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Deciphering 'water-soluble lignocellulose' obtained by mechanocatalysis: new insights into the chemical processes leading to deep depolymerization

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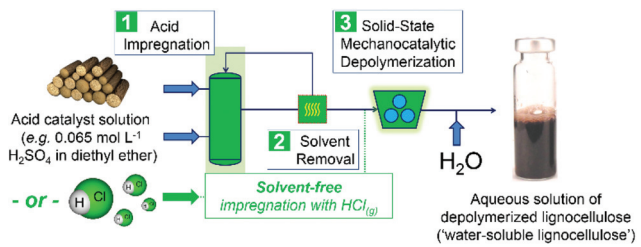
Recently, the mechanocatalytic depolymerization of lignocelluloses yielding 'water-soluble lignocelluloses' was demonstrated. Water-soluble C_5 & C_6 sugars and sulfur-free lignins are formed through the saccharification of the oligosaccharides, allowing for the fractionation of biomass by simple filtration. Herein, we present an in-depth analysis of water-soluble products obtained from beechwood. The complex nature of the 'water-soluble beechwood' is investigated by 2D HSQC NMR, HPLC and gel filtration chromatography. The detailed analysis of the 'water-soluble beechwood' lends significant insights into the chemical nature of the lignocellulose depolymerization driven by the mechanical forces.

Introduction

Mechanocatalytic depolymerization of lignocellulose constitutes a new frontier in biorefining.[1–4] Through the combination of mechanical and acid-catalyzed chemical processes, deep depolymerization of lignocellulose (*i.e.* the full conversion of lignocellulose into water-soluble oligosaccharides and lignin fragments) is easily achieved. Scheme 1 illustrates the steps involved in this emerging approach:[2]

1. Lignocellulose is impregnated with small quantities of a strong acid (*e.g.* H_2SO_4 or HCl). As demonstrated for cellulose depolymerization,[2] this process can be performed either by wet impregnation (*e.g.* in a dilute H_2SO_4 solution in diethyl ether) or solvent-free impregnation (*e.g.* by exposing the substrate fibers to gaseous HCl).[5]



Scheme 1 Schematic representation of the method for deep depolymerization of lignocellulose by mechanocatalysis.[2]

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2. In the wet impregnation, the acid is loaded on the substrate surface upon solvent removal. The energy efficiency of the wet impregnation might be debatable due the costs associated with solvent recovery and reuse. Thus, the solvent-free impregnation with gaseous HCl offers a more energy efficient and environmentally friendly alternative to the wet impregnation method.

3. In sequence, the acid-impregnated substrate is processed for relatively short milling durations (*e.g.* 2 to 3 h),[2,5,6] compared with conventional mechanical pretreatment (*e.g.* days to months).[7]

We have demonstrated the 'water-soluble lignocellulose' to serve as a platform for fractionation of biomass into C_5 & C_6 sugars in addition to sulfur-free lignins.[6] In aqueous solution, the fractionation is obtained by the saccharification of the 'water-soluble lignocellulose' at temperatures as low as 140 °C for 1 h. As a result, high yield of sugars (*e.g.* 88–92% glucose, 3.5–8% glucose dimers and 93–98% xylose relative to the glucan and xylan fractions, respectively) and sulfur-free lignins were obtained from beechwood, pinewood and sugarcane bagasse.[6]

We also demonstrated the water-soluble oligosaccharides (WSO) can be used as a unique replacement for glucose and xylose, enabling high-yield production of sugar alcohols[8] or furfurals.[9] Using a similar methodology for the mechanocatalytic depolymerization of cellulose developed by us, Beltramini *et al.* have confirmed the promising results for the production of sugar alcohols from 'water-soluble cellulose'.[3,10]

Despite the great potential of 'water-soluble lignocelluloses' as biomass feeds for catalytic valorization, so far there has been little discussion about the chemical nature of the water-

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soluble lignocelluloses. Aiming to lend detailed insights into the nature of the chemical processes involved in the deep depolymerization, we provide here an in-depth analysis of the chemical nature of 'water-soluble beechwood.' This paper is organized as follows. First, the chemical nature of the deep depolymerization (acid-catalyzed *vs.* radical reaction) is addressed. Next, the effects of the acid type (H_2SO_4 or HCl) on the mechanocatalytic depolymerization of beechwood are discussed. Surprisingly, unlike cellulose, which is fully converted into water-soluble oligomers either in the presence of H_2SO_4 or HCl,[2] the mechanocatalytic depolymerization of HCl-impregnated beechwood reaches only 74% yield of water-soluble products after milling for a duration of 2 h. The effect of the acid type on the extent of depolymerization of the carbohydrate and lignin fractions is assessed by 2D HSQC NMR, HPLC and gel filtration chromatography. Unexpectedly, the acid type exerts an effect mostly on the depolymerization of lignin. Finally, it is demonstrated by ^{13}C CP-MAS NMR that water-insoluble products obtained from the mechanocatalytic depolymerization of HCl-impregnated beechwood is mostly composed of lignin.

Results and discussion

The chemical nature of the deep depolymerization

Seminal works on the depolymerization of cellulose,[11] lignocellulose,[12] and lignin models[13–16] by mechanochemistry showed that radicals are formed through milling the substrates in absence of acid catalysts. This observation poses an important question: *What is the contribution of radicals to the mechanocatalytic depolymerization?* To answer this question one should first consider that lignin is a radical scavenger, which is in close contact with cellulose and hemicellulose. As such, if radical processes are of importance for the depolymerization extent, the yield of water-soluble products from lignocellulose will be decreased, compared to that from cellulose. To check this hypothesis, the formation of water-soluble products throughout the course of mechanocatalytic depolymerization of beechwood and α-cellulose (both impregnated with H_2SO_4 at a loading of 0.9 ± 0.1 mmol H_2SO_4 per gram of substrate) were compared.

Fig. 1 shows similar trends for the formation of water-soluble products obtained from the depolymerization of beechwood and α-cellulose. This observation tends to confirm the chemical nature of the deep depolymerization to be only acid-catalyzed. Nonetheless, one should bring awareness and attention to the fact that the susceptibility of a lignocellulosic substrate to undergo mechanochemical reactions depends also on several factors related to the receptivity of mechanical forces by the substrate, such as chemical composition (*e.g.* hemicellulose and lignin contents), mechanical properties as well as substrate microstructure. Accordingly, the need for a prolonged milling duration can arise in order to achieve full conversion of a lignocellulosic substrate into 'water-soluble lignocellulose'. For instance, we recently found that the acid-catalyzed depolymerization of pinewood and sugarcane bagasse is completed within 3 h under the same milling conditions used for the

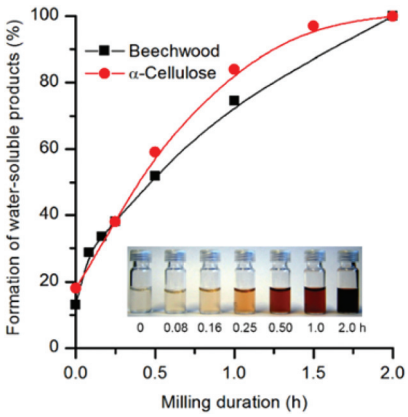

Fig. 1 Formation of water-soluble products from beechwood or α-cellulose as a function of milling time. Reaction conditions: H_2SO_4-impregnated substrate (1 g, 0.8 ± 0.1 mmol H_2SO_4 per gram of substrate) milled in a planetary mill at 800 rpm. The data was collected in separate experiments, performed at the indicated milling durations.

mechanocatalytic depolymerization of beechwood or α-cellulose, which are deep depolymerized in 2 h.

Conclusive evidence on the acid-catalyzed nature of the process is provided by the fact that the reaction takes place at appreciable rates only when a strong acid (*i.e.* $pK_a \leq -3$) is used as a catalyst.[2,3,5] This observation clearly indicates that the protonation of the glycosidic O site is also not easy even on the acid-impregnated substrate.[5,17] In light of the current results, it is apparent that the contribution of radicals to the overall extent of the mechanocatalytic depolymerization is small.

Interestingly, it is clear from Fig. 1 that directly after impregnation and prior to milling, 13 and 18% of water-soluble products, from beechwood and α-cellulose, were already formed. However, an increase in impregnation duration does not yield improved substrate solubilization.[5] This observation agrees with findings by Nevell and Upton.[18] They demonstrated the initial depolymerization of cotton cellulose by HCl in benzene as a rapid and random process taking place at the amorphous domains, and contrastingly as a slow and specific process at the chains ends in the crystalline domains. The depolymerization is thus limited to a rather low extent. Hence, partial hydrolysis of the amorphous domains of cellulose and hemicellulose most likely results in *ca.* 10–20% of the substrate solubilization shortly after acid impregnation.

Finally, Fig. 1 also shows that the color of the solutions containing the water-soluble products from beechwood changed from pale yellow to dark reddish brown with the increase in the milling duration. In contrast, the solution of water-soluble oligosaccharides from α-cellulose is a colorless, clear solution.[2] This result is visual evidence that not only the carbohydrates but also lignin undergoes depolymerization rendering water-soluble products.[2]

Effect of the acid catalyst on the product composition

Since the energy efficiency of the wet impregnation of acid might be debatable due the solvent recovery and reuse, we

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examined the dry impregnation with gaseous HCl as an alternative to the wet impregnation with H_2SO_4 solutions. In this section, we will focus our analysis on the depolymerization of H_2SO_4- and HCl-impregnated beechwood milled for 2 h.

By exposing beechwood (6 g) to gaseous HCl for 15 min (1 bar, 25 °C), a loading of 1.0 mmol of HCl per gram of substrate was achieved after evacuation for 1 h (10^{-3} mmHg). As previously demonstrated by us, this acid loading provides comparable results to those of the depolymerization of a commercial cellulose (α-cellulose) impregnated with 0.9 mmol H_2SO_4 per gram of substrate. The mechanocatalytic depolymerization of HCl-impregnated α-cellulose yields 100% of water-soluble products within a milling duration of 2 h.[2]

The H_2SO_4-processed beechwood is fully soluble in both water and DMSO. In contrast, the HCl-processed beechwood is only partially soluble in water (74%), but still fully soluble in DMSO. To shed light on the effect of acid catalyst on the general composition of chemical entities in the samples, the depolymerized beechwood samples were analyzed in detail by 2D NMR HSQC experiments (Fig. 2 and 3). Since both depolymerized beechwood samples are fully soluble in DMSO-d_6, the HSQC NMR analysis thus provides the best way for the characterization of the entities present in the depolymerized beechwood samples.

Nonetheless, HSQC spectrum data must be interpreted with caution, since the $^1J_{CH}$ dependence of polarization transfer in HSQC experiments is not suppressed in regular HSQC pulse sequences.[19] As a result, the absolute intensity of cross peaks is not fully quantitative in the entire spectral range, although considerable progress has been achieved in the field of HSQC NMR that is giving rise to quantitative analysis protocols.[19–21] Nevertheless, regular HSQC NMR experiments still offer extremely valuable (direct) semiquantitative information for characterization and comparison of lignins as well as whole plant cell compositions.[22–25] Semiquantitative determination of volume integral ratios is possible for ^1H–^{13}C pairs in a similar chemical environment (*e.g.* as for the correlations involving the anomeric H signals, the C_α–H_α signals for the side-chain of lignin units, or the C_2–H_2 and C_6–H_6 signals for lignin aromatic units), due to the fact that the $^1J_{CH}$ values for the specific entities are reasonably similar.[22,23] Accordingly, for the different regions of the HSQC spectra, semiquantitative analysis was performed separately by integration of ^1H–^{13}C pairs of interest.[22,23]

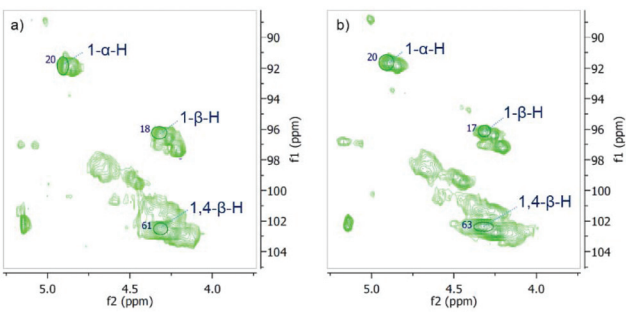

Fig. 2 Partial HSQC spectra of depolymerized beechwood samples dissolved in DMSO-d_6. Spectra "a" (H_2SO_4-processed beechwood) and "b" (HCl-processed beechwood) display the anomeric region showing the signals for α- and β-reducing end units and that for the 1,4-β internal anomeric H.

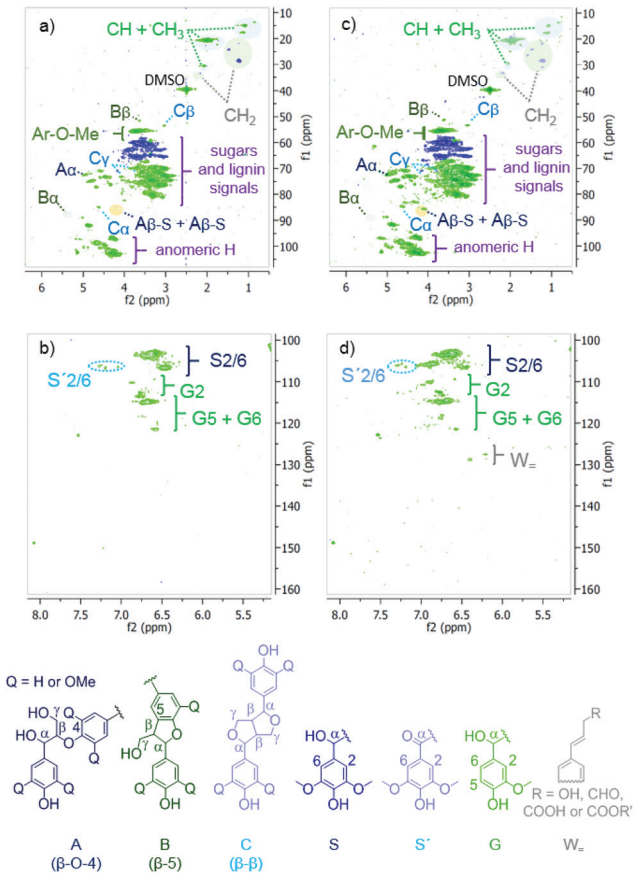

Fig. 3 Partial HSQC spectra showing the region of the C_3 side-chains of the lignin units ("a" and "b") and aromatic region of lignin ("b" and "d") for depolymerized beechwood samples dissolved in DMSO-d_6. The spectra "a" and "b" refer to the samples prepared by milling the H_2SO_4-impregnated substrate, while "c" and "d" the samples obtained by milling the HCl-impregnated substrate. The correlation signals in *green* correspond to CH and CH_3 groups, while those in *blue* to CH_2 groups (Table 1).

Oligosaccharide products. The HSQC spectra in the anomeric H region give a great deal of information concerning the configuration and connectivity of glycosidic linkages.[21,22,26] Part of these signals are certainly also associated with the oligosaccharides derived from hemicellulose in which the sugar units are not necessarily connected only by 1,4-β-linkages. However, the combination of acid catalysis and milling leads to an unexpected result, which also contributes to additional cross signals in the anomeric region. Glucans with stereochemistries differing from that of cellulose are also produced by concurrent unspecific oligomerization of the sugar units, as previously reported by us.[2] In contrast, glucose or even cellulose do not undergo oligomerization by conven-

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tional mechanical pretreatments (*i.e.* in the absence of acid catalyst).[2]

Fig. 2 shows the HSQC spectra of the depolymerized beechwood in the region of the correlation signals of the pair ^{1}H–^{13}C associated with anomeric H. Through the direct comparison of the spectra from the depolymerized beechwood samples (Fig. 2a and 2b) with that of cellobiose (not shown here), we assigned the correlation signals associated with 1,4-β-glucans: (1) α reducing end units (1-α-H, δ_C/δ_H, 91.8/4.9 ppm); (2) β reducing end units (1-β-H, δ_C/δ_H, 96.3/4.3 ppm); and (3) 1,4-β internal anomeric H (1,4-β-H, δ_C/δ_H, 102.5/4.3).

By using the volume integral of the signals 1-α-H (V_α), 1-β-H (V_β) and 1,4-β-H (V_i), the degree of polymerization (DP) in anhydroglucose units (AGU) can be estimated by eqn (1):

$$DP = \frac{\sum V_i + V_\alpha + V_\beta}{\sum V_\alpha + V_\beta} \quad (1)$$

Both HCl- and H_2SO_4-processed beechwood show the same value for the estimated DP (3 AGU). In addition, the DP values by HSQC are in good agreement with the DP values by gel filtration chromatography, which also indicates similar DP values for both samples (DP ~ 5–6 AGU, as it will be discussed later). We can thus conclude that both H_2SO_4 and HCl catalysts lead to a similar depolymerization extent in the mechanocatalytic experiments.

This conclusion may seem surprising, considering the total H^+ content present in the acid-impregnated samples. The actual H^+ content in the H_2SO_4-impregnated beechwood is about twice as high as that in the HCl-impregnated beechwood, since the acid catalyst loadings were 0.9 mmol H_2SO_4 and 1.0 mmol HCl per gram of beechwood. Nonetheless, the hydrolysis of the 1,4-β-glycosidic bonds in cellulose requires strong acids ($pK_a \leq -3$) for taking place at appreciable rates at temperatures lower than 100 °C.[2,3,27–29] In effect, for cellulose depolymerization, only half of the H^+ content of H_2SO_4 catalyzes the reaction, since the weakly basic glycosidic O site can selectively distinguish the acidity of the H^+ species in H_2SO_4 (the values of pK_{a1} and pK_{a2} are −3 and 1.99, respectively). Thereby, this fact supports the same extent of cellulose depolymerization reached by the processing of the H_2SO_4- and HCl-impregnated beechwood samples, since the relevant acid content ($pK_{a1} = -3$) is similar.

For the purpose of this study, the simple comparison of Fig. 2a and 2b reveals that both samples have identical correlation signals, indicating that both samples show rather similar types of glycosidic linkages in their oligosaccharides. This observation has an important implication for product solubility in water, since the presence of branched oligosaccharides could account for the different solubilities of the HCl and H_2SO_4 processed beechwood samples, as recently suggested for the water-soluble oligosaccharides obtained from mechanocatalytic depolymerization of cellulose.[3]

The similar DP of the oligosaccharides in the depolymerized beechwood samples, in addition to their identical patterns of correlation signals in the anomeric region of the HSQC spectra, constitute indirect evidence that the different solubility of the depolymerized beechwood samples are more likely associated with their lignin fractions rather than related to the oligosaccharide fractions formed by mechanocatalytic depolymerization.

Lignin-derived products. In conventional processes (*e.g.* Organosolv process and Kraft process), lignin undergoes depolymerization preferentially by the cleavage of ether linkages rather than C–C bonds.[30–32] In an insightful theoretical study by Gnanakaran *et al.*, a thorough examination of the bond dissociation enthalpy (BDE) of C–C and C–O linkages in representative lignin subunits was recently given.[33] This study shows that the β-5 and β–β linkages rank among the strongest C–C linkages occurring in lignin ($BDE_{\beta\text{-}5} \sim 125$ kcal mol^{-1} and $BDE_{\beta-\beta} \sim 117$ kcal mol^{-1}).[33,34] Indeed, their BDE values are even higher than 4-O-5 linkages (Ar–O–Ar), which are the strongest ether linkages occurring in lignin ($BDE_{4\text{-}O\text{-}5} \sim 80$ kcal mol^{-1}).[33,34] In turn, the ether linkages β-O-4 ($BDE_{\beta\text{-}O\text{-}4} \sim 65$ kcal mol^{-1}) and α-O-4 ($BDE_{\alpha\text{-}O\text{-}4} \sim 55$ kcal mol^{-1}) rank among the weakest linkages connecting lignin units.[33,34] Considering this and the fact that α-O-4 and β-O-4 are the most abundant linkages occurring in lignin,[35] it is natural to assume that lignin should undergo depolymerization or acidolysis primarily through the cleavage of weak ether linkages also by the mechanocatalytic method.

To assess the relative distribution of β-O-4, β-5 and β–β linkages still present in the depolymerized beechwood samples, the HSQC spectra were analyzed in the region of C_3 side-chains of the lignin units. Fig. 3 displays cross signals of the C_3 side-chains of the lignin units (Fig. 3a and 3b) and those of the aromatic region showing the color-coded corresponding structures.

From Fig. 3a and 3c, it is possible to identify the presence of lignin subunit structures A, B and C and thus the corresponding interunit linkage types – β-O-4 (by the presence of the correlation signals, A_α and A_β), β-5 (B_α and B_β) and β–β (C_α, C_β and C_γ). Due to the prominent and overlapping carbohydrate contours, it is very challenging to resolve the correlation signals for the ^{1}H–^{13}C pairs corresponding to A_γ (δ_C/δ_H, 59.7/3.6 ppm and 59.4/3.7 ppm) and B_γ (δ_C/δ_H, 62.8/3.7 ppm). Worth mentioning, the correlation signal for B_α shows a very low absolute intensity, and is thus hardly seen in the magnification applied to Fig. 3a and 3c. Table 1 lists the ratio of β-O-4 : β-5 : β–β, which were estimated from the volume integrals of the contours for A_α, B_α and C_α, respectively. The β–β linkage-

Table 1 Relative composition of lignin subunits linkages β-O-4 (as A_α), β-5 (as B_α) and β–β (as C_α) estimated by HSQC NMR for the depolymerized beechwood samples dissolved in DMSO-d_6

Sample	β-O-4	β-5	β–β
H_2SO_4-processed beechwood[a]	2.0	0.3	1.0
HCl-processed beechwood[b]	2.6	0.2	1.0
Organosolv beechwood lignin	2.5	0.5	1.0

Acid loading per gram of substrate: [a] 0.9 mmol H_2SO_4. [b] 1.1 mmol HCl.

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type was chosen as a "reference" for the comparison of lignins because it represents a class of strong C–C linkages[33] and shows a more intense cross signal in the HSQC compared with B_α (β-5).

Table 1 indicates that the β-O-4 linkage is still the most abundant in depolymerized wood samples as well as in beechwood lignin extracted by the organosolv process. This result is very interesting since the organosolv pulping is a fairly mild process and, consequently, the isolated lignin retains much of its native β-O-4 interunit linkages, as reported by other studies.[36,37] The results from Table 1 suggest that H_2SO_4-processed beechwood might indeed contain a lignin depolymerized to a slightly higher extent than that in HCl-processed beechwood, as revealed by a lower proportion of β-O-4 linkages found for the H_2SO_4-processed beechwood (β-O-4 : β-5 : β–β, 2.0 : 0.3 : 1.0) compared with that for HCl-processed beechwood (β-O-4 : β-5 : β–β, 2.6 : 0.2 : 1.0).

Unlike the hydrolysis of cellulose, which requires strong acids ($pK_a \leq -3$) to proceed at appreciable reaction rates,[2,3,27–29] the β-O-4 linkages undergo acidolysis even in the presence of weak acids (*e.g.*, acetic acid).[30] This fact may lead to the (false) conclusion that the β-O-4 linkages undergo acidolysis to a much greater extent in the experiments in the presence of H_2SO_4. However, the problem is not so straightforward because the conjugated base of the acid catalyst markedly affects the hydrolysis rate and the reaction pathway of the acidolysis of β-O-4 linkages, as reported by several studies.[38–46]

Recently, Yokoyama *et al.* revisited the mechanism of β-O-4 bond cleavage in the acidolysis of a lignin model.[40] They found that the rate of disappearance of veratrylglycerol-β-guaiacyl ether shows rate constants of 3.61×10^{-2} and 8.08×10^{-3} h^{-1} in the presence of HCl and H_2SO_4, respectively, when the whole disappearance process in each experiment was approximated as a pseudo-first-order reaction.[40] Based on these rate constants, the acidolysis of β-O-4 bond in the processing of H_2SO_4-impregnated beechwood would be expected to be slower than for the HCl-impregnated substrate. In contrast, the experimental evidence shows that the H_2SO_4-processed beechwood contains a lignin depolymerized to a slightly higher extent than that in HCl-processed beechwood.

Among the lignin linkages still detectable by HSQC NMR, the β-O-4 is the most thermolabile one, as indicated by its low BDE value and thermolysis experiments.[33,34] We propose that the proportion of this linkage in the sample can serve as an internal, natural probe for distinguishing the internal processes driven by mechanical forces from those thermally driven (due to microscopic hot spots formed by the impact of balls in the mill). The slightly lower fraction of β-O-4 found for the processed H_2SO_4-beechwood sample, in comparison to that found for lignin extracted by the organosolv process at 180 °C, strongly suggests that the mechanocatalytic depolymerization is as mild as the Organosolv process is to the lignin fraction. Accordingly, it is evident that the mechanocatalytic process is driven by the action of mechanical forces on the internal molecular structures of the biopolymers, rather than by the presence of hot spots. If the latter process were the predominant one, the fraction of the β-O-4 linkages would most likely be so low that it could not be detected in the HSQC spectrum – because the β-O-4 ether linkage would be then cleaved to a much greater extent relative to the other, more thermostable linkages β-5 and β–β.

The presence of β-O-4 linkages in the depolymerized beechwood samples thus strongly suggests that the action of mechanical forces is driving the acid-catalyzed depolymerization of the carbohydrate fraction and the acidolysis of the lignin linkages. This notion agrees with a very recent report on the hydrolysis of cellulose by DFT calculations, predicting that both protonation of the glycosidic O site and conformational changes are the rate limiting steps in the mechanism.[27] The action of mechanical forces in the lignocellulosic matrix is proposed to facilitate the conformational changes needed for the glycosidic bond activation towards hydrolysis.[5,27] In addition, the acid impregnation guarantees high local-concentration of H^+-species on the lignocellulosic surface, alleviating the protonation problems.[5,27] The one-pot combination of acid catalysis with mechanical forces offers great advantages over the conventional two-step approach, in which the substrate is first milled, to reduce crystallinity only, and next hydrolyzed in an acidic solution at temperatures between 150 and 210 °C, forming moderate yields of fermentable sugars.[47]

The last important information that can be extracted from the HSQC spectra is the relative composition of lignin in terms of coumaryl (H), coniferyl or guaiacyl (G) and sinapyl (S) units. To obtain the composition of H, G and S units, the aromatic region of the HSQC spectra (Fig. 3b and 3d) was analyzed. The ^1H–^{13}C pairs considered in this estimation are $H_{2/6}$ (δ_C/δ_H, 128.0/7.2 ppm, not detected in the beechwood samples studied here), $G_{2/6}$ and S_6 because of their similar chemical environment.[22] Hence, by using the half-value of the volume integral of the correlation signals $G_{2/6}$ (note that they correspond to two ^1H–^{13}C pairs), in addition to the entire integral value for S_6, the lignin composition can be estimated in terms of S and G units as given by eqn (2) and (3), respectively.

$$S(\%) = \frac{S_6}{(0.5 \times H_{2,6}) + (0.5 \times G_{2,6}) + S_6} \quad (2)$$

$$G(\%) = \frac{0.5 \times G_{2,6}}{(0.5 \times H_{2,6}) + (0.5 \times G_{2,6}) + S_6} \quad (3)$$

As expected for a hardwood, the beechwood samples contain no appreciable amount of coumaryl (H) units, and show a higher content of S units relative to G units. Again, regarding the contents of S and G units, the lignins in H_2SO_4- and HCl-processed beechwood are very similar. However, the content of S-units found for H_2SO_4- and HCl-processed beechwood (S/G ratios 1.56 and 1.63, respectively) is lower than that found here for Organosolv beechwood lignin (S/G ratio 1.94). This finding agrees with the results reported by Ragauskas *et al.* on the structural characterization of ball-milled switchgrass lignins obtained before and after dilute H_2SO_4 pretreatment.[48] They also found a decrease in S/G ratio (from 0.80 to 0.53).[48] However, the correlation of this finding with the

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chemical phenomena occurring in the acid-catalyzed depolymerization of lignocellulose remains unclear.

The data from Table 2 also reveals an important feature of the mechanocatalytic depolymerization of beechwood. The similar values for the relative volume integrals for the $^{1}H–^{13}C$ pairs – $S_{2/6}$, G_2, and $G_5 + G_6$ – suggest that the aromatic rings of lignin in the H_2SO_4-processed do not undergo sulfonation, which could account for the full water-solubility of the H_2SO_4-processed substrate in contrast to HCl-processed beechwood. Furthermore, no evidence supporting sulfonation or sulfatation of the processed substrate was detected by capillary electrophoresis experiments on the solutions of 'water-soluble beechwood' at different pH values.

Gel filtration chromatography of the water-soluble products

To further assess the composition of the water-soluble products from H_2SO_4- and HCl-processed beechwood samples, the water-soluble products from depolymerized beechwood were analyzed by gel filtration chromatography (Fig. 4). Table 3 summarizes the yields of glucose dimers, glucose, xylose, HMF and furfural. The yields of glucose dimers, glucose and 5-hydroxymethylfurfural (HMF) were calculated relative to the glucan fraction, while those of xylose and furfural are relative to the xylan fraction in the original sample of beechwood.[2]

The similar product contents listed in Table 3 indicate that the depolymerization of the carbohydrate fraction occurs similarly, irrespective of whether HCl or H_2SO_4 is used. The chromatogram (refractive index detector) shows the presence of saccharides with DP from 1 to 10 AGU in both samples. The oligosaccharide distributions are both centered around 5–6 AGU. A more intense "lump" background signal is, however, seen in the chromatogram for the HCl-processed sample (Fig. 4c). The chromatogram measured with the UV-Vis diode array detector (Fig. 4b and 4d) indicates the presence of components, which absorb UV light at about 275 nm and co-elute with the oligosaccharides (which do not absorb above 210 nm). Since both, phenols and furfural, show absorption bands at around 275 nm, to distinguish the phenolic species from the furanic ones, the pH of the post-column flow was adjusted to 12 with the co-flow of a 0.1 mol L^{-1} NaOH solution. At this pH value, the phenolic compounds are fully ionized to phenolates. As a result, bathochromic and hyperchromic shifts occur at the maxima of phenol absorption

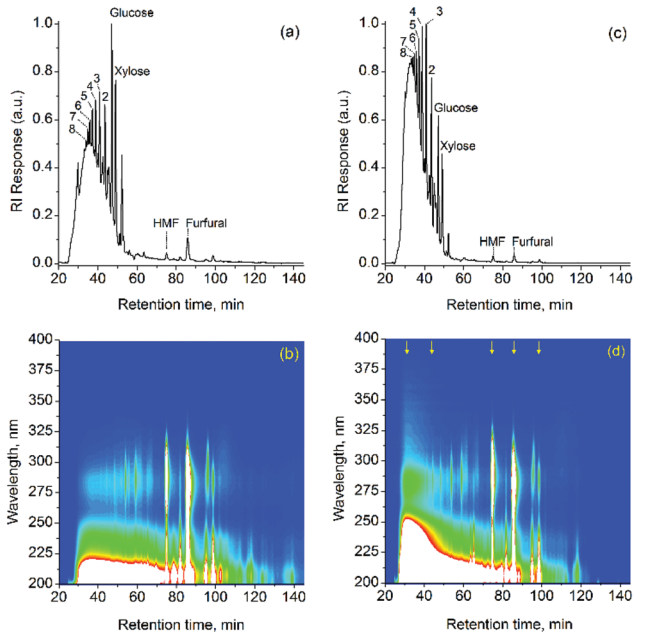

Fig. 4 Gel-filtration chromatograms of the water-soluble beechwood samples obtained from milling of H_2SO_4-impregnated beechwood (chromatograms "a" and "b") and HCl-impregnated beechwood (chromatograms "c" and "d"). The chromatograms "a" and "c" show the refractive index detector response, "b" and "d" display the UV-Vis DAD response.

Table 2 Relative composition of sinapyl (S) and guaiacyl (G) units in lignin as estimated by HSQC NMR for the depolymerized beechwood samples and organosolv beech wood lignin dissolved in DMSO-d_6

	Volume integrals			Content[a] (%)	
Sample	$S_{2/6}$	G_2	$G_5 + G_6$	S	G
H_2SO_4-processed beechwood	57	18	25	61	39
HCl-processed beechwood	56	17	27	62	38
Organosolv beechwood lignin	58	15	27	66	34

[a] The cross signal for $H_{2/6}$ entities was not detected in the beechwood samples.

spectra. In turn, furfural and HMF are not ionized. Thus, no considerable alteration in the spectra of this compound class is expected.

Fig. 5 displays the UV-Vis trace spectra extracted from the DAD chromatogram of HCl-processed beechwood at the retention times 31.3, 43.7, 74.4, 85.5, and 98.7 min, as indicated by yellow-colored arrows in Fig. 4d. It is clear from the bathochromic shifts in the UV-Vis spectra (Fig. 5a) that the co-eluting species sampled at 31.3, 43.7 and 98.7 min correspond to phenolic compounds (lignin fragments), whereas the unchanged UV-Vis spectra collected at 74.4 and 85.5 min confirm the presence of HMF and furfural, respectively. Hence, the intense "lump" background signal shown in Fig. 4a and 4c is most likely due to a response of the refractive index detector to lignin fragments co-eluting with the oligosaccharides. In the chromatogram of the HCl-processed beechwood, the intense absorption band at 280 nm between 20 and 40 min (Fig. 4d) is additional evidence of the presence of large lignin fragments ($M_w \sim 1$ to 2 kDa as estimated relative to the eluted saccharides). For comparison, in the chromatogram of the H_2SO_4-processed beechwood sample, the absorption band at 280 nm shows a less continuous profile, which at higher intensities between 50 and 70 min (Fig. 4c) indicates the presence of lignin fragments with lower molecular weight (~0.2 to 0.5 kDa). One may thus conclude that a larger quantity of water-soluble lignin fragments with high molecular weight is still present in the solution of HCl-processed beechwood. This is also supported by the trace UV-Vis spectrum at 31.3 min (Fig. 5a). The bathochromic and hyperchromic shifts produced

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Table 3 Contents of cellobiose, glucose, xylose, HMF and furfural in the depolymerized beechwood samples. Both samples were milled for 2 h

		Contents of products after mechanocatalytic depolymerization (%)				
Acid	Water soluble products (%)	Glucose dimers	Glucose	Xylose	HMF	Furfural
H_2SO_4[a]	100	0.8	2.5	4.8	<0.1	0.5
HCl[b]	74	3.6	2.8	6.3	0.1	0.7

Acid loading (per gram of substrate). [a] 0.9 mmol H_2SO_4. [b] 1.0 mmol HCl.

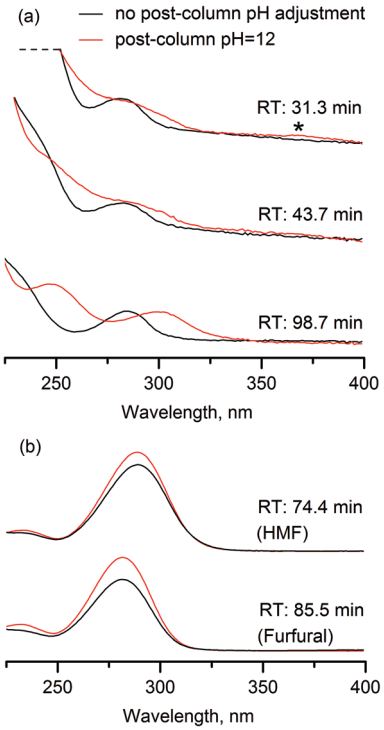
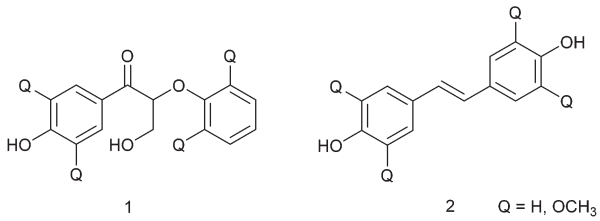


Fig. 5 UV-Vis spectra extracted from the DAD images of the eluates with no post-column pH adjustment and with post-column pH adjusted to 12. (a) Phenolic and (b) furanic components. In Fig. 5a, the absorption band at 370 nm (indicated by '*') is characteristic for lignin-dimeric or larger structures with an α-carbonyl group **1** or even *p*,*p*′-stilbene structures **2** (Scheme 2).

by the post-column adjustment of the pH to 12 causes the appearance of an absorption band at 370 nm (indicated by '*' in Fig. 5a), which is characteristic for lignin-dimeric or larger structures with an α-carbonyl group **1** or even *p*,*p*′-stilbene structures **2**,[49] as depicted in Scheme 2. The presence of **1** was confirmed by HSQC NMR (structure S′, Fig. 3), whereas **2** could not be detected in the spectrum Fig. 3d).

The chemical nature of the insoluble products

In this section, the chemical nature of the insoluble fraction obtained by milling HCl-impregnated beechwood is presented. In this endeavor, two methods are of importance to elucidate the chemical nature of the insoluble products: saccharification of 'water-soluble lignocelluloses' and CP-MAS ^{13}C NMR spectroscopy.



Scheme 2 Dimers with an α-carbonyl group **1** and *p*,*p*′-stilbene moiety **2**.

Heating a 10% aqueous solution of depolymerized beechwood (pH 1, note that no 'extra' acid is required since the mechanocatalytic method does not consume the acid content) the oligosaccharides undergo saccharification.[2,6] Simultaneously, a brown precipitate is formed, which shows similar CP-MAS ^{13}C NMR spectra (Fig. 6a and 6b) to that of the lignin extracted by the Organosolv process (Fig. 6d), as it will be discussed next in this section. Upon lignin precipitation, the solution color changes from dark reddish brown to pale yellowish (after removal of the precipitate).[6] Table 4 summarizes the results obtained from the saccharification experiments with H_2SO_4- and HCl-processed beechwood.

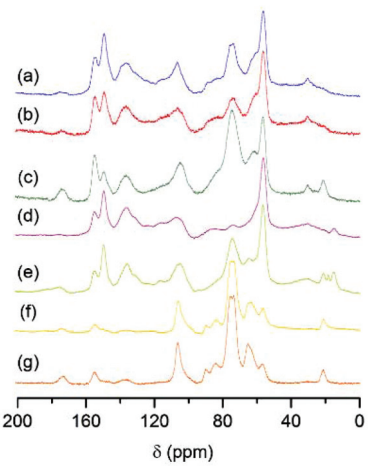


Fig. 6 CP-MAS ^{13}C NMR spectra of the precipitate obtained from the saccharification of (a) H_2SO_4- and (b) HCl-processed beechwood samples, from (c) the non-soluble fraction of the HCl-processed beechwood, from ('d' and 'e', respectively) the organosolv beechwood lignin with and without hemicellulose impurities, (f) α-cellulose and (g) unprocessed beechwood.

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Table 4 HPLC-yields of glucose dimers, glucose, xylose, HMF and furfural obtained from saccharification of depolymerized beechwood samples

		Yields relative to the corresponding glucan and xylan fractions (%)				
Acid	Sample fraction	Glucose dimers	Glucose	HMF	Xylose	Furfural
H_2SO_4	Whole	3.9	84	1.3	89	5.3
HCl	Whole	7.2	75	0.9	88	3.6
HCl	Soluble products (74%)	4.4	71	0.9	71	3.6

The best sugar yields were obtained by the hydrolysis of the H_2SO_4-processed beechwood, resulting in 84% glucose, 3.9% cellobiose and 1.3% HMF relative to the glucan fraction in addition to 89% xylose and 5.3% furfural relative to the xylan fraction. The saccharification of the whole sample of HCl-processed beechwood (*i.e.* insoluble and soluble fractions) led to slightly lower yields compared with that from H_2SO_4 water-soluble beechwood, *i.e.* 75% glucose, 7.2% cellobiose, and 0.9% HMF relative to the glucan fraction in addition to 88% xylose and 3.6% furfural relative to the xylan fraction.

Most importantly, the saccharification of the water-soluble fraction of the HCl-processed beechwood released rather similar quantities of glucose (71% *vs.* 75%) and cellobiose (4.4% *vs.* 7.1%) in comparison to the saccharification of the whole sample of HCl-processed beechwood. However, the release of xylose distinguishes these samples. While the saccharification of the whole sample produced 88% xylose, the hydrolysis of the water-soluble products led to 71%. These findings indicate that the insoluble part of the HCl-processed sample is composed of lignin associated with a small fraction of hemicellulose fragments.

In order to further assess the chemical nature of the insoluble product obtained by milling HCl-impregnated beechwood, CP-MAS ^{13}C NMR spectra were collected of lignin precipitates, the insoluble fraction of the HCl-processed beechwood, Organosolv beechwood lignin with and without hemicellulose impurities, α-cellulose, and unprocessed beechwood (Fig. 6).

Fig. 6 shows that CP-MAS ^{13}C NMR spectra of the precipitate obtained from the saccharification of H_2SO_4- and HCl-processed beechwood samples (Fig. 6a and 6b, respectively) and organosolv beechwood lignin (without hemicellulose impurities, Fig. 6d) are similar. The resonances characteristic for lignin are present (Table 5), in addition to a band centered at about 75 ppm, which can be assigned to $C_{2,3,5}$ of cellulose or $C_{2,3,4}$ of xylans (Fig. 6f, α-cellulose that comprises 76% glucans and 16% xylans). The existence of this band indicates the presence of residual glucan and xylan in the lignin precipitate, and agrees well with the overall yields of glucan-based products close to 90% and of xylan-based products close to 95%, as obtained by the saccharification at 140 °C for 1 h (Table 4). In addition to the strong signal at about 75 ppm, attributable to the residual content of carbohydrates, both the water-insoluble product of HCl-processed beechwood (Fig. 6c) and the unprocessed beechwood sample (Fig. 6g) show a strong band at around 170 ppm. This signal characterizes several oxidized carboxyl structures, and suggests that galacturonic units be present as carboxylic acid, carboxylate and esters. As the oligosaccharides are hydrolyzed, the intensity of this signal decreases (Fig. 6a and 6b). Altogether, this observation is indicative of a covalent bond through ester linkages between residual hemicellulose and lignin in the water-insoluble residue of HCl-processed beechwood (Fig. 6c).

Table 5 Assignment of the CP-MAS ^{13}C NMR chemical shifts for Fig. 6 according to ref. 50 and 51

^{13}C shift (ppm)	Assignment
200–160	C═O in several oxidized structures (*e.g.* galacturonic units)
153	C_3 and C_5 in etherified syringyl units
148	C_3 and C_4 in etherified guaiacyl units
135	C_1 in etherified syringyl and guaiacyl units
122	C_6 in guaiacyl units
113	C_2 in guaiacyl units
105–103	C_1 in carbohydrate and C_3 and C_6 in syringyl units
86–81	C_β in β-O-4 linked units
90–70	$C_{2,3,4,5}$ in cellulose and $C_{2,3,4}$ in xylans and C_α in β-O-4 linked units
62–60	C_6 in cellulose, C_5 in xylans and C_γ in β-O-4 linked units
56	Ar–O–CH_3
44	C_α methyne with aliphatic substitution
35	C_α in aryl propanol
31	Alkyl CH_2
20–15	CH_3 group in acetylated xylan and terminal CH_3 group

Conclusion

The in-depth analysis of the 'water-soluble beechwood' lends important insights into the chemistry of the mechanocatalytic depolymerization of lignocellulose, as follows:

1. The depolymerization is an acid-catalyzed process. The contribution of radical chemistry to the depolymerization rate appears to be negligible. Otherwise, the depolymerization rates found for beechwood would starkly contrast with that of α-cellulose, since lignin is a radical scavenger.

2. The presence of β-O-4 lignin linkages in the depolymerized beechwood samples strongly indicates that the action of mechanical forces, and not the eventual formation of hot spots, is driving the acid-catalyzed depolymerization of the carbohydrate fraction in addition to the acidolysis of the lignin linkages.

3. Either H_2SO_4 or HCl enables similar process performance with respect to the depolymerization extent of cellulose. Surprisingly, by milling HCl-impregnated beechwood, a 74% yield of water-soluble is achieved. Under similar conditions,

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the conversion H_2SO_4-impregnated beechwood leads to a 100% yield of 'water-soluble beechwood.' Again, the combination of results from 2D HSQC NMR, HPLC and gel filtration chromatography indicates that lignin is depolymerized to a lesser extent in the presence of HCl.

4. Despite the differences in the extent of lignin depolymerization, the aqueous phase saccharification of the depolymerized samples leads to quite similar yields of glucose and xylose products at 140 °C for 1 h. We found that the insoluble product, obtained by milling HCl-impregnated beechwood for 2 h, is composed mostly of lignin.

5. The solvent-free impregnation of lignocellulose with gaseous hydrogen chloride is showed to be as effective as that with H_2SO_4 performed in liquid phase. Therefore, the procedure for deep depolymerization can be simplified by contacting lignocellulose with gaseous HCl prior to milling the acid-impregnated substrate, thus eliminating the need of a solvent for the impregnation procedure.

Although the use of gaseous HCl is associated with corrosion problems, it is important to keep in mind that the acid impregnation is performed under ambient conditions (*i.e.* 25 °C, 1 bar). Under such low-severity conditions is fully enabled the utilization of inexpensive polymeric materials (*e.g.* PVC) for the construction of the impregnation reactor. With regard to corrosion problems, both the balls and the mill vial (stainless steel) have proven very resistant to corrosion. In fact, *no change* in the weight of the balls and the mill vial, even after 600 experiments, was detected.

Experimental

General

Sulfuric acid (95–97%, J. T. Baker), hydrogen chloride (99.8%, Air Liquide), α-cellulose (76% glucans, 16% xylans, 6% humidity, 0.1% ash, and 1.9% others, Aldrich) and diethyl ether (99%, Aldrich) were used as received. Pellets from beechwood (41% glucans, 21% xylans, 24% lignin, 5% humidity, 0.4% ash, and 8.4% others) were comminuted with a blender. The sawdust was sieved. Powders with a particle size smaller than 250 μm were collected and used for the mechanocatalytic experiments.

Wet impregnation with an H_2SO_4 solution in diethyl ether

The beechwood or α-cellulose (10 g) was suspended in a diluted H_2SO_4 solution in diethyl ether (150 mL). *Note*: To avoid degradation of the substrates upon the prolonged contact with the acidic solution, we chose to use a 0.065 mol L^{-1} H_2SO_4 solution for the acid impregnation. The suspension was shaken for 1 h (IKA shaker, KS 130 control, 350 rpm). The organic solvent was removed under reduced pressure at 40 °C. A fine powder with loose particles was obtained. This procedure led to an acid-loading of 0.8 ± 0.1 mmol H_2SO_4 per gram of substrate. The powder was immediately processed in a ball mill or stored in a closed vial and kept in a freezer (−10 °C) to prevent substrate decomposition that would normally occur to form grayish to black powder after several days of storage at room temperature.[9]

Solvent-free impregnation with gaseous HCl

Beechwood sawdust (5 g) was exposed to a flow of gaseous HCl for 15 min. Next, the substrate was evacuated for 1 h (10^{-3} mmHg). Again, a fine powder with loose particles was obtained. The powder was immediately processed in a ball mill or stored in a closed vial and kept in a freezer (−10 °C).

Determination of acid loading

Typically, 1 g of the acid impregnated substrate was suspended in 40 mL water. Subsequently, titration with a 0.0100 mol L^{-1} NaOH solution was performed on a Metrohm Titrino Plus 848 automated titrator.

Mechanocatalytic depolymerization

The mechanocatalytic depolymerization of lignocellulose was performed in a stainless steel vial (12 mL; 5 stainless steel balls of 4 g each) using a planetary ball mill (Fritsch, Pulverisette P7). The acid-impregnated substrate (1 g) was processed at 800 rpm. The data displayed in Fig. 1 was collected in separate experiments performed at the indicated milling durations. Full conversion of beechwood was achieved at a milling duration of 2 h; pinewood and sugarcane bagasse required a duration of 3 h for full conversion. Under working conditions, the temperature inside the mill did not exceeded 42 °C after milling for 0.5 h. In experiments of longer duration, the mill was switched off every 0.5 h for 10 min to avoid overheating and thermal decomposition of the sample. Note that the experiment duration refers exclusively to the total milling time applied to the sample. The product was then collected and kept in an air-tight vial at −10 °C prior to analysis or saccharification experiments.

Extraction of lignin from beechwood by the organosolv process for NMR comparison purposes

Beechwood (16–17 g) was suspended in a 140 mL solution of ethanol–water (1 : 1, v/v) in a 250 mL autoclave equipped with a mechanical stirrer. The suspension was processed at 180 °C for 3 h. In sequence, the mixture was left to cool down to room temperature. A reddish-brown solution was obtained after filtering off the lignocellulose fibers (pulp). Ethanol was partially evaporated at 60 °C using a rotoevaporator. This procedure leads to lignin precipitation. The solid was collected by filtration and, in sequence, resuspended in hot water in order to remove hemicellulose sugars. Next, the suspension was filtered and the solid washed several times with hot water. Finally, the organosolv beechwood lignin was dried in oven at 40 °C for 1 d.

Determination of the water-soluble products

The processed substrate (0.500 g) was suspended in water (25 mL) and shaken for 5 min. The suspension was centrifuged for 10 min. The residue was washed one more time with water (25 mL), centrifuged and finally dried overnight at 90 °C.

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The weight of the solid residue was recorded. The solubility was then determined using the difference in weight, as described elsewhere.[2]

Determination of the solubility in DMSO

The depolymerized beechwood sample (50 mg) was dispersed in 1 mL of DMSO and sonicated for 20 min. In sequence, the sample was filtered through a 1.0 μm PTEE membrane (Omnipore membrane filter, JAWP04700, Merck Millipore) and washed with water. The membrane was dried overnight at 60 °C in an oven. The solubility was determined by weight difference.

Saccharification of 'water-soluble beechwood'

The depolymerized beechwood samples (*i.e.* HCl- and H_2SO_4-impregnated beechwood samples milled for 2 h) were solubilized in water forming a 10% solution with a pH value of 1. In a closed glass vial, the beechwood solution (9 mL) was heated at the indicated temperatures for 1 h. Upon heating, a solid residue was formed, which was isolated from the sugar solution by centrifugation. The precipitate was washed with 20 mL water six times. The aqueous solutions were combined and set aside for HPLC analysis. In turn, the solid residue was dried in an oven at 60 °C for 24 h. The solid residue was weighed and stored at −10 °C.

Sugar and furfural quantification

HPLC analysis was performed on a Shimadzu LC-20 equipped with a column switcher combining two organic acid resin columns (100 and 300 mm in length and 8 mm inner diameter). An aqueous solution of trifluoroacetic acid (2 mmol L^{-1}) was used as the eluent (1 mL min^{-1}). Glucose, glucose-dimers and xylose were analyzed using an RI detector; furfural and HMF, with a UV-Vis detector operating at 280 nm. The yields of glucose, cellobiose and 5-hydroxymethylfurfural are given relative to the glucan content of the unprocessed substrate; the yields of xylose and furfural are given relative to the xylan content.[2]

Gel filtration chromatography

The analyses were performed on a Perkin-Elmer HPLC 200 using a series of four TSKgel G-Oligo-PW columns (6 μm, 7.8 mm I.D. × 30 cm L, Tosoh) and milli-Q water as eluent (0.8 mL min^{-1}) at 80 °C. For detection of the oligosaccharides, a refractive index detector was used. For detection of the lignin fragments, a diode-array detector (DAD) was employed. To differentiate the DAD response of furanic from phenolic eluates, the pH of the post-column flow (0.8 mL min^{-1}) was adjusted to 12 by a co-flow (0.1 mL min^{-1}) of a 0.1 mol L^{-1} NaOH solution through a secondary HPLC pump (Shimadzu).

Solution NMR experiments

The NMR spectra of the samples of depolymerized beechwood (HCl- and H_2SO_4-impregnated beechwood samples milled for 2 h) were acquired at 25 °C with a Bruker AV spectrometer (400 or 500 MHz ^1H frequency) equipped with a BBFO probe head with *z*-gradient. Spectral widths of 20 ppm were used for the 1D ^1H spectrum. The relaxation delay for 1D ^1H spectrum was 5.0 s following a 30-degree excitation pulse. For 1D inverse-gated ^{13}C spectrum, the relaxation delay was set to 1.0 s following a 30-degree excitation pulse. ^1H-decoupling with the Waltz-16 sequence was applied during acquisition. The number of collected points was 64k for ^1H and for ^{13}C. The 1D ^1H spectra were processed using an exponential weighting function (lb 0.2 Hz) prior to Fourier transform. The 2D HSQC NMR (Bruker standard pulse sequence "*hsqcetgpsi*" with delay optimized for $^1J_{CH}$ of 145 Hz) were set up with spectral widths of 20 ppm and 180 ppm for ^1H- and ^{13}C-dimensions, respectively. The number of collected complex points was 2048 for ^1H-dimension with a recycle delay of 3.13 s (3.0 s relaxation delay and 0.13 s acquisition time). The number of transients for the HSQC spectra was between 12 and 24, and 512 time increments were recorded in ^{13}C-dimension resulting for in an overall experiment time of 6 to 12 h. For HSQC experiments, a squared cosine-bell apodization function was applied in both dimensions, followed by zero-filling to 1024 points in the ^{13}C-dimension prior to Fourier transform. The 1D ^1H NMR and 2D HSQC NMR spectra were processed using MestReNova 8.1.1 software.

Solid-state ^{13}C CP-MAS NMR experiments

The ^{13}C CP-MAS NMR spectra were measured on a Bruker Avance 500WB spectrometer with a double-bearing standard MAS probe (DVT BL4) at a resonance frequency of 125.8 MHz using 4 mm MAS probe spinning at 10 kHz. The experimental conditions were 2 s recycle delay, between 8000 and 28 000 scans, 1 ms contact time, and 4.3 μs ^1H π/2 pulse.

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