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The effect of ball milling on birch, pine, reed, walnut shell enzymatic hydrolysis recalcitrance and the structure of the isolated residual enzyme lignin

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ABSTRACT

Methodologies for the high-yield recovery of lignin with retention of its native C—O bonded structure is an essential prerequisite for many novel high-end lignin applications. Enzymatic residual lignin isolation is such a methodology that leaves the lignin untouched by using enzymatic desaccharification. Thus, a series of representative lignocellulose substrates (birch, pine, walnut shell and reed) were evaluated for effective native lignin isolation, with emphasis on the effect on the lignin structure and purity. The effect of enzyme loading and ball milling severity were studied by tracking residual saccharides and the structural integrity of the isolated lignin. Prolongation of ball milling time could achieve a higher carbohydrate removal and avoid the loading of extra enzyme. However, the application of two or more steps of enzymatic hydrolysis with higher enzyme loading and short ball milling time was shown as an alternative to long ball milling time to achieve similar carbohydrate removal and avoid extensive decrease of the lignin molecular weight (MW). This MW decrease was caused by breaking of some linkages, but not too a large enough extent to cause significant differences in the 2D HSQC NMR spectra. The recalcitrance towards increased enzyme hydrolysis activity by ball milling was different for the four representative biomasses and followed an order of walnut shell > reed ≈ pine > birch by comprehensive analysis the obtained data. Overall, the results showed a clear two-way synergy between enzymatic treatment and ball milling efficiency to isolate lignin with high yield, high native linkage content, purity and minimal MW reduction.

1. Introduction

Lignin is a highly abundant natural aromatic polymer which accounts for 18–35 % weight of lignocellulosic biomass and therefore of great interest as for the production renewable aromatic (co)polymers (Rinaldi et al., 2016). It is build-up out of *p*-hydroxyphenyl, guaiacyl, and syringyl moieties connected by different linking motifs that arise from the radical nature of the biosynthesis from the parent monolignols of which the aryl β-aryl ether (β-O-4) motif is dominant (Humphreys and Chapple, 2002; Ragauskas et al., 2014; Ralph et al., 2008; Rinaldi et al., 2016). The aromatic unit and linkage distributions are variable by plant species, producing areas, plant age and even in different parts within individual plant (Vanholme et al., 2010). Additionally, various linkages, and different amounts of these, have been suggested to exist between lignin and carbohydrates such as phenyl glycosides, ethers and esters (Giummarella et al., 2019; Nishimura et al., 2018). These heterogenous

Abbreviations: Rel, residual enzyme lignin; Srel, residual enzyme lignin obtained from one time cycle treatment; Drel, residual enzyme lignin obtained from second cycle treatment; Trel, residual enzyme lignin obtained from third cycle treatment; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum coherence spectroscopy; FT-IR, Fourier transform infrared spectroscopy; SEM, scanning electron microscopy; TGA/DTG, thermal gravimetric analysis/first derivative thermogravimetric; GPC, gel permeation chromatography; XRD, X-ray diffraction; G, guaiacyl unit; G', guaiacyl unit with oxidized α-ketone; S, syringyl unit; S', syringyl unit with oxidized α-ketone; H, *p*-hydroxylphenyl unit; β-O-4(G), aryl ether linked to G unit; β-O-4β(S), aryl ether linked to S unit; β-O-4′, aryl ether with γ-position acetylated; β-β, resinol; β-5, phenylcoumaran; D, dibenzodioxocine; 4-O-5, biphenyl ether; SP, spirodienone; X1, cinnamyl alcohol end group; X1′, acylated cinnamyl alcohol group; X2, cinnamyl aldehyde group; FA, ferulate; *p*CA, *p*-coumarate; Phe, phenylalanine; Tyr, tyrosine; NREL, National Renewable Energy Laboratory; I, Internal units; NR, non-reducing end; R, reducing end; X, xylan; C, cellulose.

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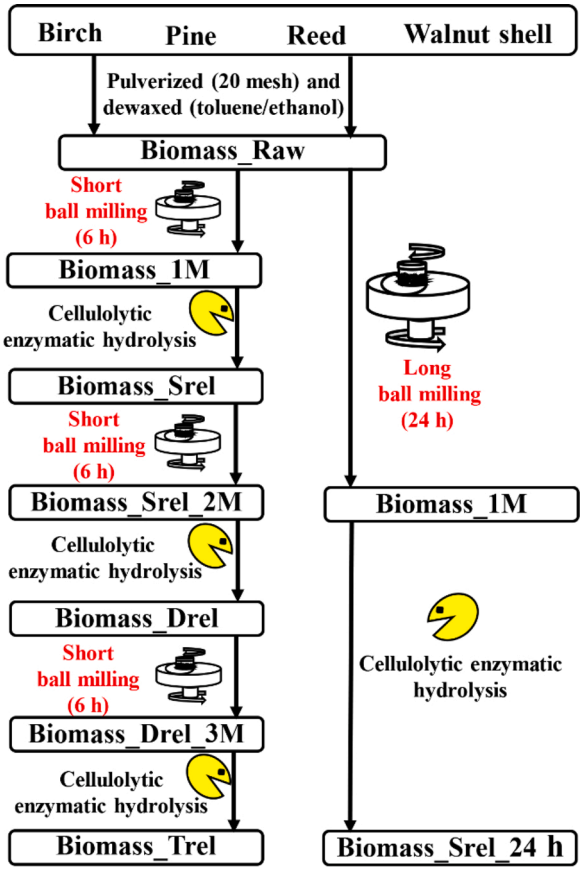
structural characters all increase the challenge of effective valorization.

The lignin isolated from biomass using mild treatments and extraction methods such as mild organic solvent extractions with carefully tuned conditions and setups such as the usage of flow-through extractor with short retention time of lignin inside the reactor, give the potential to provide lignin with a relatively highly functionalized C—O bonded backbone structure compared to typical technical lignins (Zijlstra et al., 2020, 2019). Alternatively, an extremely high molecular weight (MW, ~100,000 g/mol) compared to lignins isolated by extraction (~2000 g/mol) can be obtained by enzymatic mild acidolysis (Guerra et al., 2006). Such isolated high molecular weight lignin materials have completely different properties and potential applications compared to extracted lignins that currently available. For instance, lignins applied as dispersants and plasticizers can benefit greatly from having a higher MW (Areskogh et al., 2010). Moreover, the development of valorization strategies for lignin by chemical modification and catalytic degradation for example lignin first approaches can greatly benefit from model native-like lignins to use as substrates for fundamental studies. Thus, it is important to develop proper protocols for the isolation of high MW native-like lignins and understand how closely these resemble the native lignin that is part of the parent biomass sample.

Isolation/fractionation processes easily affect the chemical structure of lignin, which poses a serious challenge for native-like high MW lignin isolation. Nevertheless, methods used to extract native-like lignins have been developed by carefully control of extraction conditions. Original methods like the isolation of Björkman lignin (also known as milled wood lignin) rely on mild extraction conditions (Björkman, 1956; Chang et al., 1975; Luterbacher et al., 2015; Vvu and Argyropoulos, 2003; Wen et al., 2014). However, by relying on an extraction step only soluble (typically small) fragments can be extracted, and several publications have shown that such lignin only partially represents the whole structure of lignin (Chang et al., 1975; Holtman et al., 2007; T. Ikeda et al., 2002). Other alternative systems such as deep eutectic solvent (DES) and ionic liquid (IL) systems can dissolve large MW material but thus far these lignins always showed some structural breakdown and condensation (Liu et al., 2017; Shen et al., 2019; Sun et al., 2019; Tang et al., 2017; Xia et al., 2018). Isolation of lignin by enzymatic hydrolyzing the saccharides is currently the sole way to achieve almost complete separation of lignin under a milder condition with reportedly minor structural alteration (Pew, 1957; Pew and Weyna, 1962). However, the solubility of this kind residual enzyme lignin (Rel) is very low in common organic solvents and therefore, limited characterization is typically conducted (Chang et al., 1975). In addition, contamination with protein residue is reported for Rel, since extra load of cellulosic enzyme is normally used for the Rel isolation (Rencoret et al., 2015), and original protein residues from biosynthesis process of constituents in plants (Rencoret et al., 2011), which can be eliminated by further treating the Rel with proteinase (Kim et al., 2017). Nevertheless, recent studies showed that Rel is ideal for its structural elucidation and serves as an excellent control for studying catalytic degradation of lignin (Shen et al., 2017; Wang et al., 2017).

The performance of cellulolytic enzymes for Rel isolation is enhanced by increasing the accessibility of digestible substrates (Fan et al., 1980; Himmel et al., 2007). Mechanical pretreatments are typically used, especially ball milling (Alvira et al., 2010; Chandra et al., 2007; Mittal et al., 2017), as chemical treatments are known to easily induce degradation and condensation of lignin (Mittal et al., 2017; Yao et al., 2018). Ball milling induces size reduction and shape change as well as breaking crystalline structure of cellulose, which is of great assistance to enhance the efficiency of saccharide removal and lignin recovery (Avolio et al., 2012; Baraton, 2003; Gorrasi and Sorrentino, 2015). However, several works have also insinuated that intensive ball milling and in particular the process of vibratory milling can induce partial oxidation and the cleavage of aryl ether linkage accompanied by the creation of new phenolic hydroxyls as well as some self-condensation (Fujimoto et al., 2005; Guerra et al., 2006; Hu et al., 2006; Tsutomu Ikeda et al., 2002) based on different milling conditions and types of biomass (Kim and Ralph, 2010; Polčin and Bezuch, 1978). Thus, finding a balance between ball milling treatment intensity and the quality and yield of isolated lignin is of great significance. Therefore, it is necessary to get further insight into to what extend ball-milling can affect the performance of cellulolytic enzyme and lignin structure from different plant species.

The aim of this work is to isolate lignin that should contain as much as possible the structural elements of native lignin in a high yield to serve as high MW, high β-O-4 content substrates or model lignins. In addition, we aim to carefully study how the isolation process affects the structure of lignin for different plant species and the key parameters (milling time, enzyme load, hydrolysis time) for the efficient isolation. For this purpose birch, pine, reed and walnut shell, which represent different abundant residual lignocellulosic biomass types (hard wood, soft wood, grass and non-wood) were selected to study the influence of planetary ball milling pretreatment combined with Ctec2 carbohydrate hydrolysis on the lignin structure. A detailed illustration of the whole process and how the naming of the different samples obtained from the isolation steps are showed in Scheme 1. The changes of crystalline cellulose during ball milling was tracked by X-ray diffraction (XRD), and particle size and the surface morphology of biomasses was monitored by scanning electron microscope (SEM). The influence of long time milling on structure of lignin was mainly assessed by a combination of molecular weight distribution analysis by GPC, and chemical structure analysis by gel-state heteronuclear single quantum coherence (HSQC) 2D NMR.



Scheme 1. Isolation of residual enzyme lignin.

2. Experimental section

2.1. General

The cellulolytic enzyme cocktail (Ctec2, mixture of celluobiohydrolase, endogucansase and β-glucosidase) and chemicals used in this study were all purchased from Sigma-Aldrich unless otherwise stated. Solvents were supplied by Fisher Scientific. Birch, pine, reed and walnut shell were manually cut into small sections and grounded in a grinder with a 20-mesh screen. The smashed samples was dewaxed by a Soxhlet with 1:2 (v/v) ethanol/benzene for 8 h. After leaving the solvents to evaporate in the back of the fume hood, the extractive-free sample were further dried at 65 °C for 16 h and stored in a valve bag before use.

2.2. Ball milling and enzymatic hydrolysis

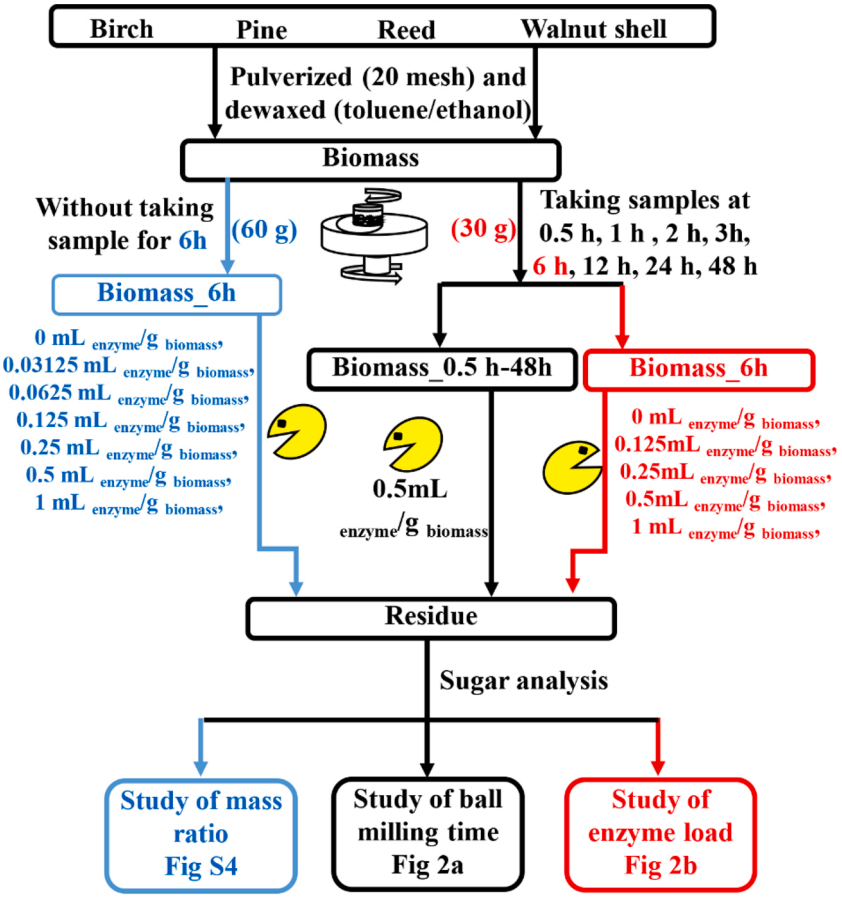
Ball milling was conducted in a planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) equiped with a 250 mL ZrO_2 jar and ZrO_2 balls (5 × 15 mm, 10 × 5 mm). A program with 450 rpm rotation, interchanging between 10 min milling followed by a 15 min pause was used for all the samples, and the time mentioned in the manuscript is all actual milling time by eliminating the pause time. The 50 mM acetate buffer was prepared by directly dissolving the pre-calculated sodium acetate in Milli-Q water and adjusting the pH to 5.5 with acetic acid. Tetracycline chloride was added into the buffer to make a concentration to 0.8 mM based on the volume of buffer to inhibit the growth of bacteria. Enzymatic hydrolysis treatments were conducted at 50 °C in a VWR incubating orbital shaker (Model 3500 L) at 250 rpm for 72 h with a typical liquid-solid (mL/g) ratio of 25 unless otherwise specified.

2.3. Multiple treatments residual enzyme lignin isolation

The biomass sample was milled 6 h with a 30 g starting material, and 20 g milled biomass sample was used for the first enzymatic hydrolysis, after the first treatment, the solid product was separated by centrifugation and washed by 10 times volume of Milli-Q water for 3 time. The first cycle of treatment was finished after freeze-drying (ALPHA 2–4 LD, Appropriate Technical Resources) of the residues. The second cycle treatment started milling the residues obtained from first cycle treatment for another 6 h, and then a second enzymatic hydrolysis was conducted for the remilled samples. The solid was separated by centrifugation and washed by 10 times volume Milli-Q water for three times. After freeze-drying the washed solid, the second cycle treatment was finished. For walnut shell, ball milling was performed a third time and followed by enzymatic hydrolysis and post treatments described above. The enzyme load for the hydrolysis are 0.125 mL $_{enyzme}$ /g $_{biomass}$, 0.25 mL $_{enyzme}$ /g $_{biomass}$, 0.5 mL $_{enyzme}$ /g $_{biomass}$. Accordingly, the solids obtained were noted as A_B, which the A is the type of biomass, and B can be Srel, Drel, and Trel representing biomass that is obtained after single, double and triple cycle treatment. The whole process was depicted in the Scheme 1.

2.4. Parameter study

Time courses of the ball milling were conducted, and 30 g dewaxed biomasses sample was used for the milling, and 2.5 g sample was taken after different milling times (0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 48 h). After collecting these ball milled samples, the enzymatic hydrolysis was conducted by submerging 0.3 g sample in 7.5 mL buffer with an enzyme loading of 0.5 mL_{enyzme} /$g_{biomass}$ and a 25 liquid (mL) to solid (g) ratio. The influence of different concentration of enzyme loading were carried



Scheme 2. Schematic guide of the experiments performed for the parameter study.

out by using biomass samples collected from time courses of ball milling 6 h, and the enzyme loading are1 mL $_{enyzme}$ /g $_{biomass}$, 0.5 mL $_{enyzme}$ /g $_{biomass}$, 0.25 mL $_{enyzme}$ /g $_{biomass}$, 0.125 mL $_{enyzme}$ /g $_{biomass}$, 0.0625 mL $_{enyzme}$ /g $_{biomass}$, and a 25 liquid (mL) to solid (g) ratio was used. A detailed illustration of the parameters study was showed in Scheme 2. For all the enzymatic hydrolytic treatmentsthe total incubation is 72 h. After the hydrolysis, the hydrolyzed liquids were deserted and thoroughly washed by 10 times volume Milli-Q water for three times. The washed solids were freeze-dried. Original fitting analysis was performed, and exponential fit with a function of 'Asymptotic1′ was selected to simulate a y = a-b*c^x equation for the curve of cellulose and hemicellulose. The values of a, b, c and R^2 were listed in Table S2 for Fig. 2a–d and Table S5 for Fig. 2a1–d1 to allow us to compare difference of different biomasses (See supplementary text S2 for more details).

2.5. Lignin characterization

GPC was performed in DMF (containing 0.01 M LiBr) on a Viscotek GPC max equipped with model 302 TDA detectors, two columns (Agilent Technologies-PolarGel-L and M, 8 μm 30 cm) at a flow rate of 1.0 mL min^{-1}. The columns and detectors were held at 50 °C. Data acquisition and calculations were performed using Viscotek OmniSec software version 5.0. Molecular weight was determined based on a conventional calibration curve generated from narrow dispersity polymetylmethacrylate standards (Agilent and PSS, Mw from 550 to 1.190.000 g/mol). The samples were filtered over a 0.2 μm PTFE filter prior to injection, and a 200 μL sample with a concentration of 2.0 mg/mL was injected. 2D HSQC NMR of residual enzyme lignin were collected on a 600 MHz Bruker Biospin (Rheinstetten, Germany, BASIC PROBHD) instrument using a 4:1 DMSO-d_6:pyridine-d_5. Bruker standard pulse sequence 'hsqcetgpsisp.2′ was used for the ^{13}C-^{1}H correlation experiment. A reported parameters with minor modification was used for the analysis: spectra use 2048 data points from 11 to 0 ppm in F2 (1 H) (acquisition time 130 ms), 160 to 0 ppm in F1 (^{13}C) with 256 increments (acquisition time 6 ms) of 64 scans with 500 ms internal delay; the d24 delay was set to 86 ms. The total acquiring time is 3.54 h (Mansfield et al., 2012). 40 mg sample was swelled in 0.6 mL mixture of DMSO-d_6 and pyridine-d_5 (4:1). The signal of DMSO-d_6 was used as internal reference (Δc 39.5, Δh 2.49 ppm). The data was managed by MestReNova x64−12.0.4-22023.

3. Results and discussion

3.1. Multiple treatments residual enzyme lignin

As intensive ball milling was reported to induce oxidation, degradation and condensation of lignin (Chang et al., 1975; Fujimoto et al., 2005; Pew, 1957), we were interested in what extent that multiple short milling and enzyme treatment could reduce the influence of ball milling and increase the saccharide removal at same time. For this purpose, a modified multiple extraction sequence on a 20 g scale for four different lignocellulosic biomass resources (birch, pine, reed and walnut shell) (Pew, 1957) was performed (Fig. 1b-c) and compared to a single extended ball milling treatment (Fig. 1a, which also shows the initial biomass saccharide composition). For each the multi-step separation, a 6 h ball milling was conducted before each enzymatic treatment, while 24 h milling was performed for the single step sequence with a 10 g scale. The latter clearly showed effective carbohydrate removal, based on the residual saccharide of the four biomass sample: pine (11.2 %) > reed (8.9 %) > walnut shell (8.0 %) > birch (6.8 %).

For the multistep process, a similar high enzyme load of 0.5 $mL_{enzyme}/g_{biomass}$ in 25 $mL_{buffer}/g_{biomass}$ was trialed and the saccharides removal over 2–3 cycles was monitored (Fig. 1b). Cellulose and hemicellulose amounts were significantly reduced in the first cycle but a high percentage of saccharide was still retained in the residues of the four biomass samples: pine (32.4 %) > reed (28.4 %) > walnut shell (25.9 %) > birch (19.7 %), in which hemicellulose content was relatively harder to be removed than cellulose. The different efficiencies in hemicellulose removal for the four biomass sources were likely due to the nature of the enzyme cocktail being known to be more specific for hydrolysis of

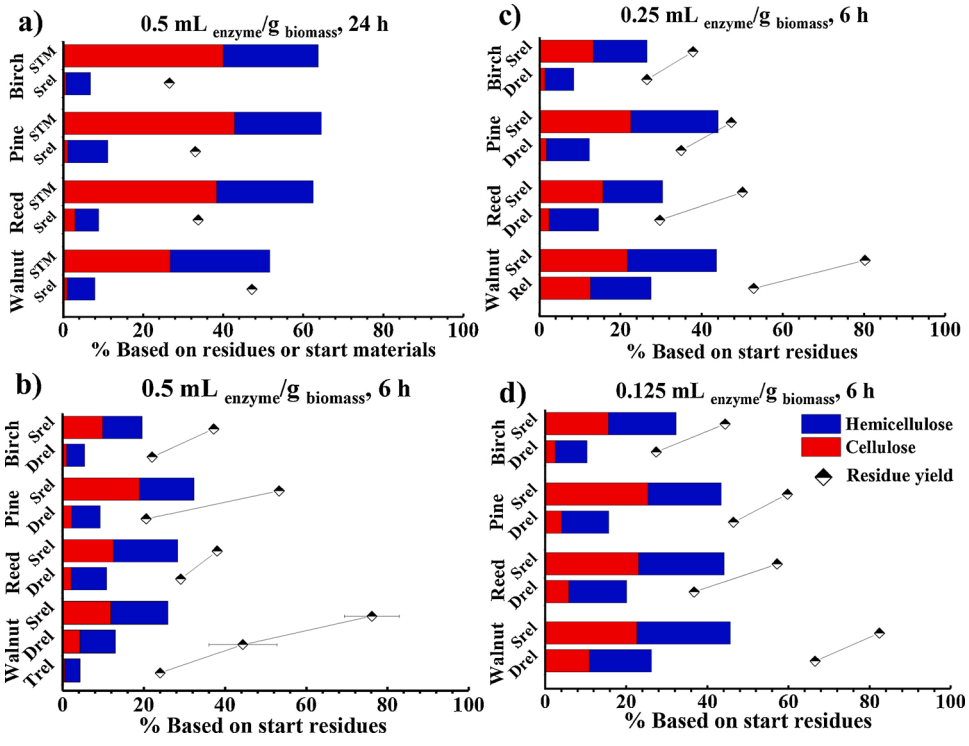


Fig. 1. Saccharide analysis of different biomass samples before and after enzyme treatment. Starting material (STM) was obtained from smashing with normal grinder with a 20 mesh screen and dewaxing with toluene/ethanol. a) One step single cycle treatment (24 h ball-milling and a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load in 25 $mL_{buffer}/g_{biomass}$). b), c) and d) are the results from multiple steps treatments. b) 6 h ball-milling and a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load hydrolysis in 12.5 mL $_{buffer}/g_{biomass}$, c) 6 h ball-milling and a 0.25 $mL_{enzyme}/g_{biomass}$ enzyme load hydrolysis in 12.5 mL $_{buffer}/g_{biomass}$, and d) 6 h ball-milling and a 0.125 $mL_{enzyme}/g_{biomass}$ enzyme load hydrolysis in 12.5 $mL_{buffer}/g_{biomass}$. Srel, sample obtained from a single cycle treatment, Drel & Trel samples were obtained from double and triple cycle treatment. Cellulose was represent by glucan, hemicellulose was represent by the total amount of xylan and arabinan. The data was duplicated collected and the error less than 3%. The percentage of the cellulose and hemicellulose was calculated by the start materials or residues before acid hydrolysis. Detailed data about the composition of the raw biomasses see in Figure S3 and Table S1. Recovery yields are based on the total mass of recovered material after freedrying and error bars at Srel and Drel from walnut shell with 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load and 6 h ball-milling were from triplicate experiments.

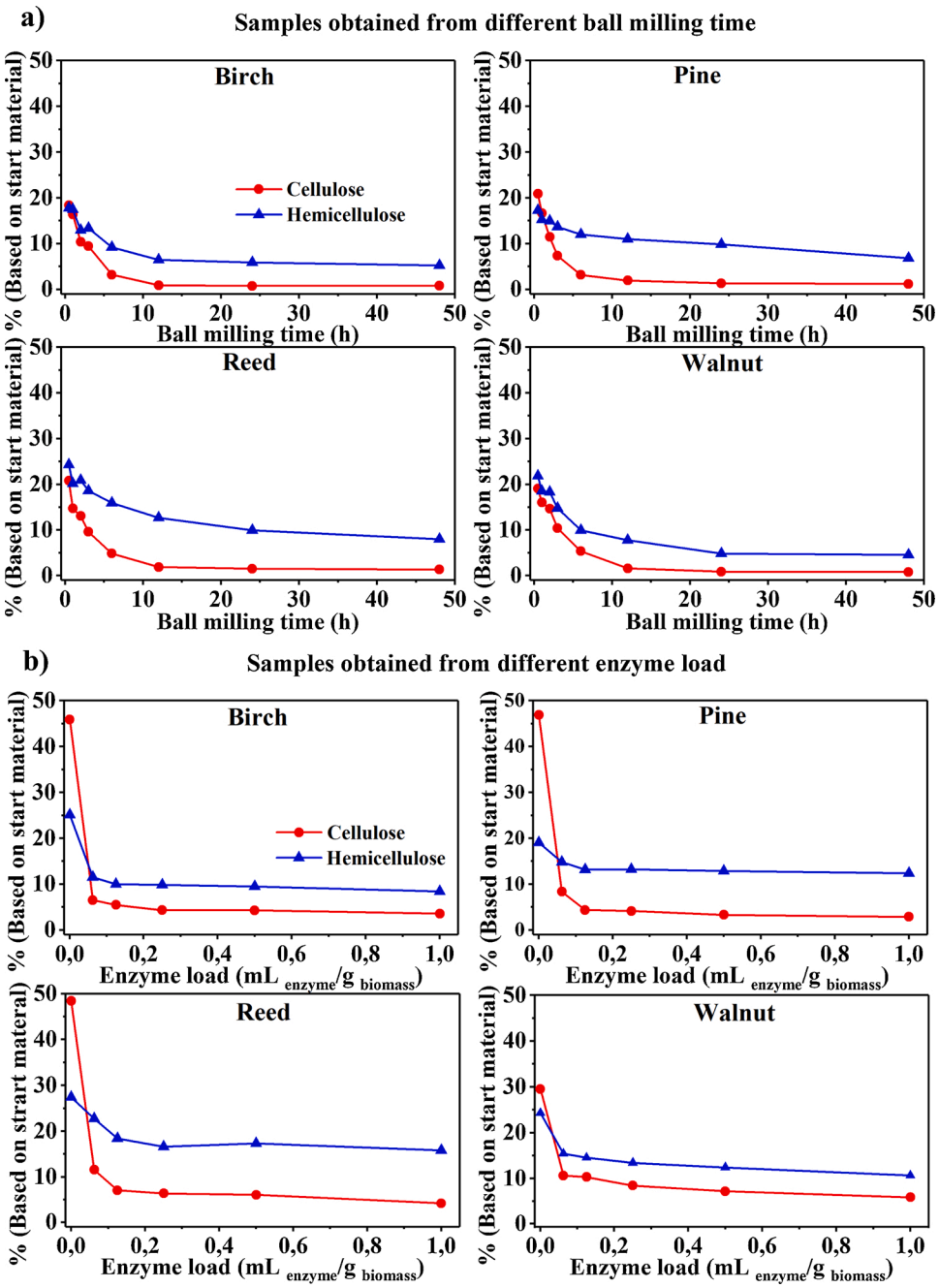


Fig. 2. The saccharide analysis of the residues obtained from enzymatic hydrolysis of biomass (2.5 g samples from 30 g starting material) after different ball milling times (0.5 $mL_{enzyme}/g_{biomass}$ in 25 $mL_{buffer}/g_{biomass}$) and b) with different enzyme loadings (6 h ball-milling).

certain types hemicellulose constituents and possible different amounts of lignin-carbohydrate linkages (Sheet, 2010). After another 6 h ball milling treatment of the freeze-dried residues, a second enzyme treatment, the saccharide content in the residue was further decreased to contents similar to those of the single enzyme treatment with long milling treatment. However, Drel from walnut shell contained relatively high saccharide content (13.0 %), which could be reduced by a third cycle treatment to around 4.3 % saccharide (Trel).

To assess possible cost reduction associated with the enzymes, we halved the enzyme load (0.25 $mL_{enzyme}/g_{biomass}$, Fig. 1c). As expected, the efficiency of the removal of saccharides decreased for all the biomasses. However, the double cycle treatment still could remove most of the saccharide content, in particular for birch. We further halved the enzyme load to 0.125 $mL_{buffer}/g_{biomass}$ and kept the same liquid to solid ratio (Fig. 1d). A similar saccharide removal was still observed for birch, and the residual saccharide of the Rel provided the following order of recalcitrance: walnut shell > reed ≈ pine > birch. The results show that the enzyme loading requirement for the isolation of Rel should be carefully tuned for different biomasses.

In order to confirm the necessity of the second ball milling step before next further enzymatic hydrolysis, Rel obtained from one time 6 h ball milling and one time enzymatic hydrolysis with 0.5 mL $_{enzyme}/g_{biomass}$ load were directly treated with fresh enzyme cocktails without second ball milling step, and saccharide data of these samples was shown in the Fig. S1. It was found that for Srel samples with a higher proportion of residual saccharide still had a sharp decrease of saccharides after the second hydrolysis step, such as Srel of walnut shell, pine and reed, though the remaining saccharide content was still higher than

their corresponding Drel with milling. These difference might due to the activity of the enzyme decreasing with a longer incubation time and thus a fresh batch of enzyme could further hydrolyze the exposed sites left by the first treatment (*vide infra*). Going from Srel to Srel_2 M and Drel the colour also changed to become darker (Fig. S2), which did not directly correlate to impurity contents and thus could not be used as indication of the presence of carbohydrate residues and thus might hint towards changes in lignin structure.

Comparing the results from the multiple treatments to those of a single treatment with longer ball-milling time (24 h), the saccharide removal was almost same (Fig. 1a). Among them, in term of saccharide removal walnut shell is in particular suitable to use long time ball milling to isolate residual enzyme lignin, as the saccharide removal of Srel of walnut shell obtained with 24 h milling was close to Trel of walnut shell. Moreover, residue yields obtained from single ball milling and enzyme treatment resulted a relative higher lignin yield than that from multiple steps, since too much material was easily lost in the multiple steps treatment (Fig. 1 and the comparison between lignin content inside the raw material and the residue yield see Fig. S3).

3.2. Parameters study

From the above observation, it was found that both enzyme load and ball milling time played important roles in enhancing saccharide removal for the isolation of residual enzyme lignin. Therefore, we evaluated the influence of enzyme load and ball milling time on the extend of saccharide removal more carefully. A time course of balling time was conducted, and 2.5 g samples after milling 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 48 h were continually collected from a 30 g starting material. A 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load in 25 $mL_{buffer}/g_{biomass}$ was used for the subsequent enzymatic treatment. The saccharide analysis of the samples after ball milling and enzymatic hydrolysis are shown in Fig. 2a–d. FT-IR was also applied on tracking the change of the residual saccharide, and the result was correlate with that of saccharide analysis by comprising the peaks of C—O of cellulose and acetyl group of hemicellulose (Fig. S4 and Table S3 and S4). In total, it was found that with the increase of the ball milling time, saccharides of residues after enzymatic hydrolysis decreased, and the saccharide reduction level off for samples milled for more than 24 h. Cellulose removal was more than 90 % for all samples and significantly higher than the removal of hemicellulose, consistent with the results discussed in the previous section. Additionally, the enhancement of hemicellulose removal with prolongation of ball milling for pine and reed was lower than that of walnut shell and birch. The analysis of curves obtained (detailed discussion is in S2.1) showed that enhancement of prolongation of ball milling on the efficiency of enzyme treatment, and the order was pine > birch > reed > walnut shell, which correlated with our previous observation about the high saccharide removal of pine at the second cycle treatment in the isolation of Drel. This data confirmed that long time ball milling with single enzyme treatment could isolate Rel with satisfying saccharide removal, and it was also consistent with our observation that long time ball milling was suitable for the isolation of residual enzyme lignin from walnut shell and reed, while a relatively short ball milling was enough for isolation of residual enzyme lignin of pine and birch.

The influence of added enzyme load was also evaluated, and the result was also monitored by saccharide composition analysis and FT-IR spectra (Fig. S5 and Table S6 and S7) of the samples after enzymatic hydrolysis (Fig. 2a1-d1). In all the biomasses, the cellulose and hemicellulose content in the residues gradually decreased with enzyme load below 0.25 $mL_{enzyme}/g_{biomass}$, corresponding to the increased initial hydrolysis rates. The analysis of the curves (detailed discussion is in S2.2) showed that the carbohydrate content reduction then levelled off above 0.25 $mL_{enzyme}/g_{biomass}$ enzyme load with a lower saccharide removal at the final stage in contrast of the removal achieved by prolongation of ball milling time, confirming limitation of extra enzyme addition for saccharide removal. Among them, the saccharide removal of birch showed the level off at the lowest enzyme load, which was consistent with data of our Rel isolation that a relative lower enzyme was required for the birch Rel isolation. Both birch and pine had relatively higher susceptibility than reed and walnut shell with increase of enzyme load.

The milling setup and operation parameters can dramatically influence the results of saccharide removal. Fig. S6 shows the result of screening of enzyme load with material of 6 h milling directly started from around 60 g samples without taking samples during milling process. The final saccharide removal was much lower compared to their corresponding material obtained from time course of ball milling under the same enzymatic treatment. However, sensitivity orders of these samples cellulose and hemicellulose towards the enzymatic hydrolysis were roughly same with data obtained from hydrolyzing sample collected from time course of ball milling by evaluating the formula between the same range of enzyme loading (Table S8).

3.3. Characterization of the samples collected from multiple residual enzyme lignin isolation and time course of ball milling

With the clear influence of ball-milling prolongation and increase of enzyme load on the efficiency of carbohydrate removal, it is necessary to explore their effects in more detail. The morphological surface changes of the sample obtained from multiple ball milling and enzyme treatments were tracked by SEM graphs to observe morphological changes (Figs. 3 and S7 to S14). The birch, pine and reed starting samples displayed typical biomass morphological features, and smooth surface, sharp edges with lamellar debris were found for these biomasses. As expected, some porous structure appeared in the residues after each enzymatic treatment, in particular for biomasses after first time enzymatic treatment such as Birch_Srel, which was due to high extent saccharide removal. The SEM graphs showed that ball milling could significantly decrease the particle size of biomass and altered their morphological surface, and porous structure appeared on the residual after the enzyme treatment, which was confirmed by BET analysis (Table S9). Nevertheless, walnut shell showed significant differences to the other biomass samples. The walnut shell surface showed bulges and cracks prior to ball milling (A comparison with birch is shown in Fig. 3a), which could be a reason for the higher recalcitrance of the walnut shell material discussed above. Furthermore, XRD analysis clearly showed that ball milling caused the least loss of crystallinity of the cellulose in walnut shell compared to the other biomass samples (Figs. 4 and S15). Looking further with SEM at samples that were milled for a longer time showed that the lamellar debris disappeared, the surface of the milled material switched into a rough surface with cracks and corrugated sheets (compare samples of birch and reed in Fig. S7, S9 to Fig. S11, S13). The particle size of walnut shell kept decreasing from milling 0.5 h–2 h but no obvious difference was observed for samples collected after 3 h ball milling (Fig. 3b). Even a slight increase of the particle size was found after more than 6 h milling (for example compare the sample of Walnut_2 h and Walnut_24 h). This was also observed in other works where this was explained by particles aggregating and being squeezed in to thin sheets (Podgorbunskikh et al., 2018; Yuan et al., 2016). No obvious difference was observed between samples milled 0.5 h and 48 h in a 500 nm level of graphs (Fig. S11 to S14).

3.4. The influence of ball milling on the structure of lignin

Intensive ball milling treatment can alter the structure of lignin, such as cleavage of β-O-4 linkages and decreasing the molecular weight (Guerra et al., 2006). In order to isolate a high quality of Rel, it is important to understand the balance between efficient desaccharification and minimization of lignin structure alteration. Therefore, we tracked the structure alteration of lignin during the isolation of Rel. Some extent of ball-milling is always required for effective

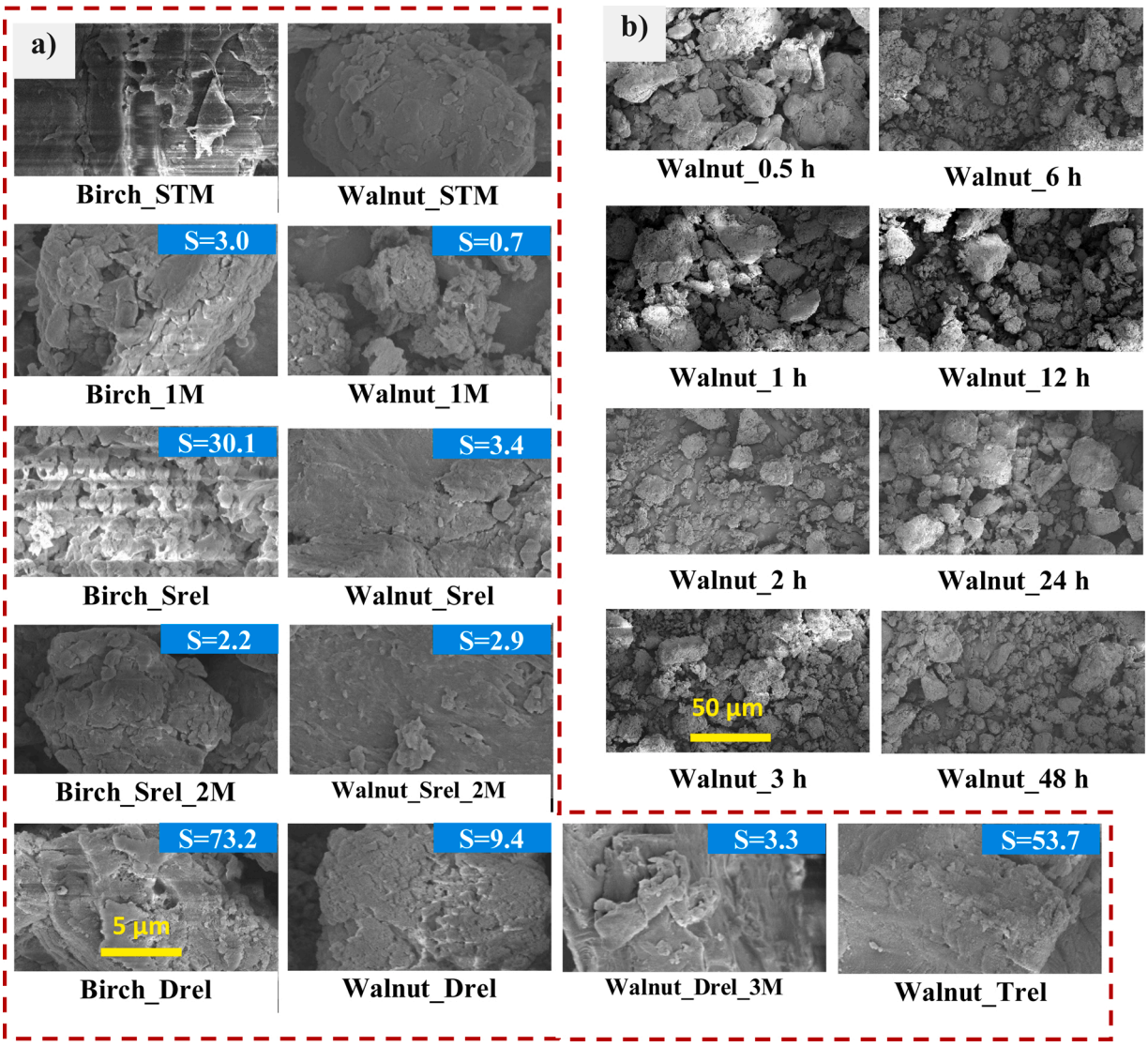


Fig. 3. SEM graphs of samples collected from multiple residual enzyme lignin isolation and time course of ball milling of walnut shell. (a) is graphs inside the frame with dashes lines. They are residual enzyme lignin of birch and walnut shell. STM was the start material obtained from smashing with normal grander with 20 mesh screen, 1 M was obtained from one time 6 h ball milling of raw, Srel was obtained from one time enzymatic treatment with a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load of 1 M in 25 $mL_{buffer}/g_{biomass}$, Srel-2 M, was obtained from treating Srel for another 6 h ball milling. Drel was obtained another enzymatic treatment of Srel_2 M with a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load 25 $mL_{buffer}/g_{biomass}$. Walnut_Drel_3 M was collected by treating Walnut_Drel for another 6 h ball milling. Walnut_Trel was obtained from treating Walnut_Drel_3 M for another enzymatic hydrolysis with a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load 25 $mL_{buffer}/g_{biomass}$. (b)is graphs outside the dash frame. These images belong to walnut samples collected from time course experiments after 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 48 h of ball milling. Data inside the frame with blue background is the surface area of the corresponding sample determined by BET. Samples zoomed for scale of 5 μm for Rel and 50 μm was showed in the figure, the pictures with 100 50, 10 μm and other sites of the samples were attached in Figure S5-12.

desaccharification, thus to gain more detailed insight into the alteration to lignin structure induced by significantly extended ball milling times the 2D HSQC NMR spectra of samples collected from time course of ball milling 24 h and 48 h after enzyme hydrolysis with a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load were collected for all the four biomasses (Fig. 5, assignment is listed in Table S10 according to published papers)(Kim and Ralph, 2014, 2010; Mansfield et al., 2012; Ralph et al., 2004). Furthermore, samples collected from multiple residual enzyme treatment of walnut shell were selected for tracking structure variation of lignin during the multiple steps isolation process (Fig. S16).

In comparison with raw biomass samples the samples obtained after milling were much easier swelled in the DMSO-*d6*/pyrdine-*d5* mixture and the 48 h sample even more so, which let to slightly better quality spectra. Also, the intensity of spectra gradually increased going from Srel to Trel, indicating that longer milling time and less carbohydrate impurities resulted in a better sample swelling and higher quality spectra. Poorly swelled samples resulted in relatively weaker carbohydrate signals and lower ratio of S/G. Based on semi-quantification (Table 1), a higher proportion of oxidized S units was found for samples obtained from longer milling time. It is hard to draw conclusions whether this is due to the morphological changes induced by the milling or by more effective oxidation by certain enzymes in the cocktail. Oxidation can also lead to darker samples due to extension of conjugated π-systems within the lignin structure. As the color clearly darkened even after a sequential enzyme treatment, this might be an indication of more extensive oxidation by enzyme activity (Fig. S2). No significant differences were observed in terms of proportion of main linkage content and S/G ratios between the different samples for all the four biomasses, indicating the limited influence of ball milling on the structure of lignin, at least following an initial round of milling. A slight of decrease of β-O-4 linkages was found for birch (<3%), indicating that doubling the ball milling time indeed might have a slight negative effect on the amounts of

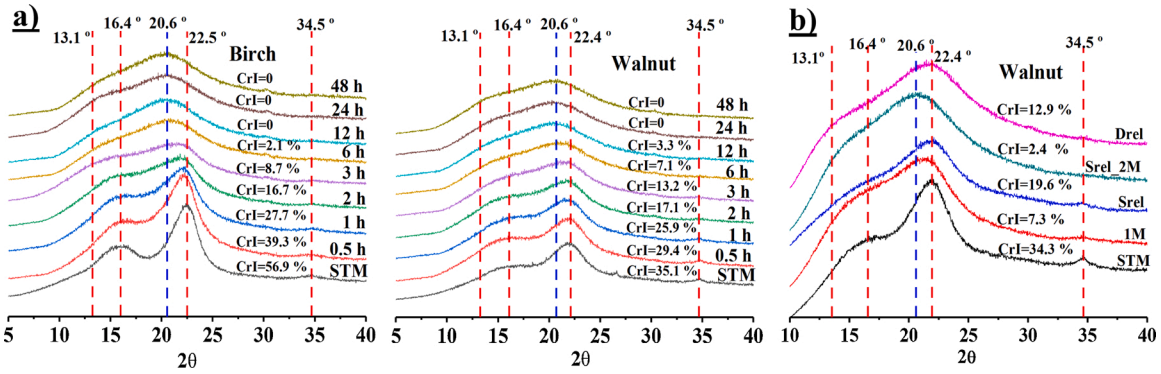


Fig. 4. XRD curves collected from samples biomass obtained from a) time course experiments from the ball milling of birch and walnut b) multiple cycles of residual enzyme walnut lignin isolation 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load in 25 $mL_{buffer}/g_{biomass}$. Crystallinity indices of the samples are shown in the Figure. Some contamination from ZrO_2 was observed as a weak peak at 30° that gradually appeared and became more evident after more intensive milling, which also observed in previous work (Ago et al., 2004).

the main β-O-4 linkages. In contrast, the other linkages content such as β-5 and β-β relatively increased for samples of birch and pine, indicating that these are more recalcitrant. This increase, which was also observed for pine and reed, might correspond to a slight improvement of the quality of the spectra and the fact that the β-O-4 signal did not increase accordingly, might indeed indicate some degradation at extended milling times. Nevertheless, it was overall found that planetary ball milling did not seriously affected total linkage of lignin within 48 h milling in our isolation, which was different with other similar works in which extensive vibratory mill seriously cleaved β-O-4 linkages (Guerra et al., 2006). While here planetary ball milling only slightly influenced the linkage contents, around 25% of β-O-4 linkages was estimated to be degraded in earlier reports (Fujimoto et al., 2005). However, it is hard to have an effective comparison as result of variable parameters, such as different types of ball milling machines, program for the milling, biomass, size and material of the jar and balls, weight ratio between balls and biomass as well as extraction or isolation methods of the lignin used for the study (Fujimoto et al., 2005; Guerra et al., 2006; Hu et al., 2006; T. Ikeda et al., 2002). A detailed parameter overview and conclusions of cited papers are attached in the Table S11.

The MW distribution determination using GPC gave us more insight (Fig. 6). In comparison with samples obtained from 24 h milling, double the milling time to 48 h dramatically decreased the MW of the recovered lignin, in particular for the reed sample. Additionally, as the multiple steps enzyme treatment (Drel) was performed with an increased weight ratio of the balls to residue before the second ball milling treatment, the efficiency of the ball milling relatively increased. This might cause more damage to the lignin upon extended ball milling and might explain why the molecular weight of lignin isolated from multiple cycle treatments (Drel) was close or even lower than that of lignin obtained from single long time milling (24 h and 48 h) and enzyme treatment. This observation further indicated the importance of regulating the isolation conditions for improving the quality of the recovered lignin. As the amount of main linkage of the lignin between the samples milling 24 h and 48 h and after multiple milling/enzyme cycles were almost the same with each other, this indicated that the polymer is cleaved to produce small fragments during the milling process. But the total linkage of the lignin almost kept constant in these samples. As these are high molecular weight polymers, the cleavage of a fraction of the linkages can already lead to a significant molecular weight decrease. Furthermore, this decrease in MW could also be related to the color changes as small amounts of cleavage could release reactive fragments that are susceptible to oxidation.

Thus, overall, these analyses revealed that ball milling used in this study could cleave part of the lignin resulting a lower MW and the cleaving of some β-O-4 linkages but overall did not lead to a significant decrease in the total linkages. This can be important when targeting applications for which high molecular weight lignin is required and shows that at these high molecular weights the relative β-O-4 linkage content is not an adequate measure of degradation.

4. Conclusion

It was shown that from four different types of biomass samples (birch, pine, reed and walnut shell) relatively pure Rel could be isolated by either a single enzymatic hydrolysis with a much higher enzyme load and intensive balling, or by multiple steps of lower enzyme treatment and short ball milling time. Intensive ball milling and different weight ratio of balls to biomass only slightly impacted the total linkage content of lignin but did significantly decrease the MW, and played a more important role compared to enzyme loading in enhancing the efficiency of the enzyme for removing polysaccharides. The enhancement of ball milling and enzyme addition for hemicellulose removal depended on the biomass type, while the relative enzymatic recalcitrance of the four biomass was different with each other and followed a roughly the order of walnut shell > reed ≈ pine > birch, which made the optimized conditions for Rel isolation of the four biomasses different. Among them, for Srel isolation with 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load, 12 h milling is enough to isolate Rel with less than 10 % saccharide impurity for birch and walnut shell, while pine and reed need more than 24 h milling. In terms of Rel from multiple steps at the given 6 h ball milling time, birch requires the lowest concentration of enzyme load (0.25 $mL_{enzyme}/g_{biomass}$), and pine needs 0.5 $mL_{enzyme}/g_{biomass}$, while walnut shell and reed need an extra third cycle treatment with 0.5 $mL_{enzyme}/g_{biomass}$ load to obtain Rel with less than 10% saccharide impurities. This shows that for obtaining lignin with high purity and very high MW systematic optimization is needed based on the biomass feed. The porous structure created by cellulolytic enzyme treatment and reduction of the amount of crystalline cellulose as well as decrease of particle size from ball milling synergistically enhanced the efficiency of the enzyme for further hydrolysis of the residual polysaccharides, in particular for biomass with higher recalcitrance such as walnut shell. This work shows that the isolation of Rel with high purity requires tuning the conditions to the respective biomass to find a good balance between minimal structural lignin degradation in terms of molecular weight and effective saccharide removal. It will depend on eventual effects of either of these for specifically applications that will determine the direction to take.

Authorship contribution statement

Z.W. contributed to the overall idea, designed and conducted all the experiments and wrote the first drafts. X.T. contributed to the collection of SEM graphs P.J.D. contributed to the data analysis supervised experimental designed, wrote parts of the manuscript and

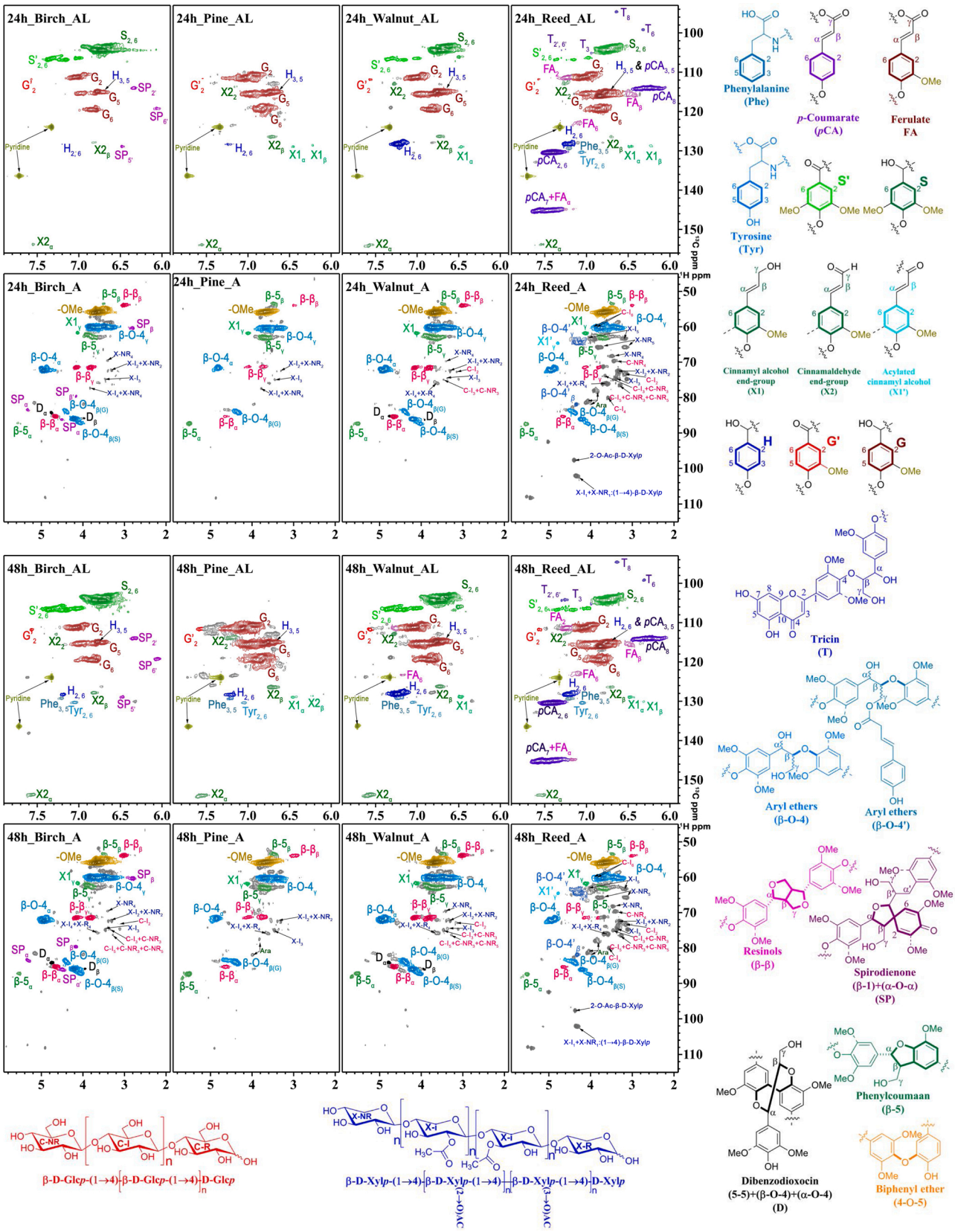


Fig. 5. 2D HSQC NMR spectra of lignin samples obtained from time course experiments ater 24 h and 48 h of ball milling and enzyme treatment with an enzyme load of 0.5 $mL_{enzyme}/g_{biomass}$ in 25 $mL_{buffer}/g_{biomass}$. A: Aromatic region, AL: Aliphatic region and anomeric region.The common polysaccharide and lignin label system is used and the signals were colored for convenience. Spectra were recorded by swelling lignin samples in a mixture of DMSO-d_6 and pyridine-d_5 (v/v, 4:1).

Table 1
The main units and linkages in samples obtained from milling 24 h and 48 h for four biomasses and residual enzyme lignin of walnut shell obtained from multiple cycle treatments.

Sample[a]	S[b]	S'[b]	G[b]	G'[b]	H[c]	β-O-4[d]	β-5[d]	β-β[d]	S/G[e]
Birch_24h	69.1	9.7	20.2	0.9	0.2	57.6	2.1	8.4	3.7
Birch_48h	67.2	11.1	20.5	0.6	0.6	56.0	3.1	9.2	3.7
Pine_24h	0.0	0.0	96.1	2.0	1.9	39.9	15.9	5.8	–
Pine_48h	0.0	0.0	95.5	1.7	2.8	39.1	17.1	6.5	–
Reed_24h	36.1	3.8	52.9	1.0	6.2	47.4	7.9	3.6	0.7
Reed_48h	37.3	5.2	50.2	1.3	6.1	47.5	7.8	4.4	0.8
Walnut_24h	50.1	6.8	31.4	1.0	10.6	51.4	7.8	12.0	1.8
Walnut_48h	50.1	8.2	31.2	1.1	9.4	51.0	7.1	11.1	1.8
Walnut_Srel	45.5	4.2	38.2	0.5	11.6	51.2	8.7	9.7	1.3
Walnut_Drel	50.1	4.1	34.1	0.6	11.0	50.2	6.9	10.1	1.6
Walnut_Trel	48.3	5.9	33.2	2.3	10.3	51.9	6.6	9.8	1.5

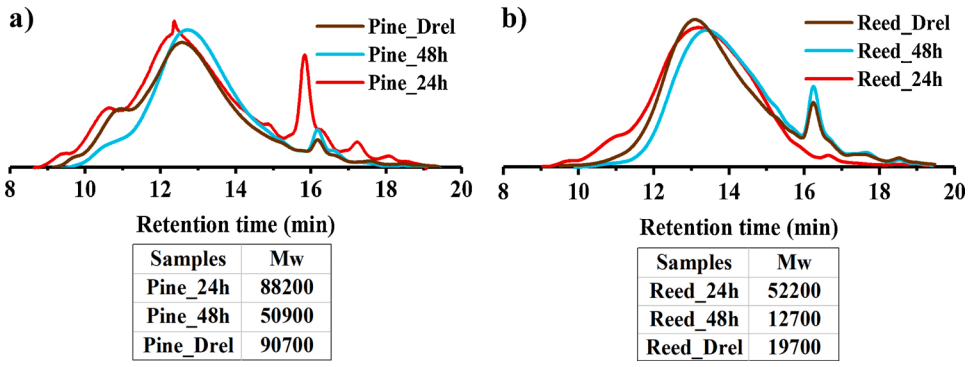
[a] Samples was selected from time course of ball milling of 24 h and 48 h after enzyme treatment with a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load in 25 $mL_{buffer}/g_{biomass}$ and isolation of multiple residual enzyme lignin with a 0.5 $mL_{enzyme}/g_{biomass}$ enzyme load in 25 $mL_{buffer}/g_{biomass}$.

[b] S, syringyl unit, S', syringyl unit with a α-ketone structure, G, guaiacyl unit, G', guaiacyl unit with a α-ketone structure, H, *p*-hydroxylphenyl unit. β-O-4, aryl ether, β-5, phenylcoumaan β-β, resinol.

[c] As Rel were contaminated during enzyme treatment, and the signals of $H_{2,6}$ units were overlapped with Phe from residue protein, so the integration of H units was based on the overlapped signals aroumd $H_{2,6}$.

[d] The data was calculated by semi-quantitative method and based on 100 Ar, and molar percentages was calculated by integration of the signal corresponding to the α position of the linkage and divide it by the total integration of H (1/2 $H_{2,6}$), G (G_2), S (1/2 $S_{2,6}$), G' (G'_2), S' (1/2 $S'_{2,6}$).

[e] S/G ratio obtained by (S + S')/(G + G').



Samples	Mw
Pine_24h	88200
Pine_48h	50900
Pine_Drel	90700

Samples	Mw
Reed_24h	52200
Reed_48h	12700
Reed_Drel	19700

Fig. 6. Molecular weight distribution of lignin samples obtained from time course experiments after 24 h and 48 h of ball milling and double cycles treatments after enzyme treatment with an enzyme load of 0.5 $mL_{enzyme}/g_{biomass}$ at a 25 liquid (mL) to solid (g) ratio. a) lignin samples of pine, b) lignin samples of reed.

conceptualized the project. All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Zhiwen Wang: Conceptualization, Methodology, Investigation, Writing - original draft. **Xiaotian Zhu:** Investigation. **Peter. J. Deuss:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113493.

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