2
The effect of ball milling on birch, pine, reed, walnut shell enzymatic hydrolysis recalcitrance and the structure of the isolated residual enzyme lignin

This chapter is based on:
Wang Z, Zhu X and Deuss PJ. The effect of ball milling on birch, pine, reed, walnut shell enzymatic hydrolysis recalcitrance and the structure of the isolated residual enzyme lignin. Industrial Crops and Products. 2021 Sep 1;167:113493. https://doi.org/10.1016/j.indcrop.2021.113493
The effect of ball milling on birch, pine, reed, walnut shell enzymatic hydrolysis recalcitrance and the structure of the isolated residual enzyme lignin

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.
2.1. 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.\textsuperscript{1} It is build-up out of \textit{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.\textsuperscript{1–4} The aromatic unit and linkage distributions are variable by plant species, producing areas, plant age and even in different parts within individual plant.\textsuperscript{5} Additionally, various linkages, and different amounts of these, have been suggested to exist between lignin and carbohydrates such as phenyl glycosides, ethers and esters.\textsuperscript{6,7} These heterogenous 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.\textsuperscript{8,9} Alternatively, an extremely high molecular weight (MW, \textasciitilde100,000 g/mol) compared to lignins isolated by extraction (\textasciitilde2000 g/mol) can be obtained by enzymatic mild acidolysis.\textsuperscript{10} 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.\textsuperscript{11} 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.\textsuperscript{12–16} 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.\textsuperscript{13,17,18} 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.\textsuperscript{18–22} 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.\textsuperscript{23,24} However, the solubility of this kind residual enzyme lignin (Rel) is very low in
common organic solvents and therefore, limited characterization is typically conducted.\textsuperscript{23} In addition, contamination with protein residue is reported for Rel, since extra load of cellulosic enzyme is normally used for the Rel isolation,\textsuperscript{25} and original protein residues from biosynthesis process of constituents in plants,\textsuperscript{26} which can be eliminated by further treating the Rel with proteinase.\textsuperscript{27} Nevertheless, recent studies showed that Rel is ideal for its structural elucidation and serves as an excellent control for studying catalytic degradation of lignin.\textsuperscript{28,29}

The performance of cellulolytic enzymes for Rel isolation is enhanced by increasing the accessibility of digestible substrates.\textsuperscript{30,31} Mechanical pretreatments are typically used, especially ball milling,\textsuperscript{11,32,33} as chemical treatments are known to easily induce degradation and condensation of lignin.\textsuperscript{32,34} 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.\textsuperscript{25–37} 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\textsuperscript{10,38–40} based on different milling conditions and types of biomass.\textsuperscript{41,42} 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 shown in Scheme 2.1. 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.
2.2. Experimental section

2.2.1. General

The cellulolytic enzyme cocktail (Ctec2, mixture of cellulbiohydrolase, endoglucansase 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 were dewaxed by a Soxhlet with 1:2 (v/v) ethanol/toluene 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.

2.2.2. Ball milling and enzymatic hydrolysis

Ball milling was conducted in a planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) equipped 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

Scheme 2.1. Isolation of residual enzyme lignin.
Chapter 2

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.

2.2.3. 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_{enzyme}/g_{biomass}, 0.25 mL_{enzyme}/g_{biomass}, 0.5 mL_{enzyme}/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 2.1.

![Scheme 2.2. Schematic guide of the experiments performed for the parameter study.](image-url)
2.2.4. 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 enzyme / g biomass and a 25 liquid (mL) to solid (g) ratio. The influence of different concentration of enzyme loading were carried out by using biomass samples collected from time courses of ball milling 6 h, and the enzyme loading are 1 mL enzyme / g biomass, 0.5 mL enzyme / g biomass, 0.25 mL enzyme / g biomass, 0.125 mL enzyme / g biomass, 0.0625 mL enzyme / g biomass, and a 25 liquid (mL) to solid (g) ratio was used. A detailed illustration of the parameters study was shown in Scheme 2.2. For all the enzymatic hydrolytic treatments the total incubation is 72 h. After the hydrolysis, the hydrolyzed liquids were removed by centrifugation and solids were 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 \( y = a - b \cdot c^x \) equation for the curve of cellulose and hemicellulose. The values of \( a, b, c \) and \( R^2 \) were listed in Table S2.2 for Fig. 2.2a–d and Table S2.5 for Fig. 2.2a1–d1 to allow us to compare difference of different biomasses (See supplementary text S2.2 for more details).

2.2.5. 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 \( \mu \)m 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 poly-methylmethacrylate standards (Agilent and PSS, Mw from 550 to 1190000 g/mol). The samples were filtered over a 0.2 \( \mu \)m PTFE filter prior to injection, and a 200 \( \mu \)L 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 interna delay; the d24 delay was set to 86 ms. The total acquiring time is 3.54 h. 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 ($\delta^C_{13}$ 39.5, $\delta^H_{2}$ 2.49 ppm). The data was managed by MestReNova x64-12.04-22023.

2.3. Results and discussion

2.3.1. Multiple treatments residual enzyme lignin

As intensive ball milling was reported to induce oxidation, degradation and condensation of lignin, 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) was performed (Fig. 2.1b-c) and compared to a single extended ball milling treatment (Fig. 2.1a, 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 $0.5 \text{ mL enzyme/} g \text{ biomass}$ in $25 \text{ mL buffer/} g \text{ biomass}$ was used and the saccharides removal over 2-3 cycles was monitored (Fig. 2.1b). Cellulose and hemi-cellulose 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 certain types hemicellulose constituents and possible different amounts of lignin-carbohydrate linkages. 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. 2.1c). As expected, the efficiency of the removal of saccharides decreased for all the bio-masses. 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. 2.1d). 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.
The effect of ball milling on residual enzyme lignin

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. S2.1. 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. S2.2), 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

Fig. 2.1. 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 represented by glucan, hemicellulose was represented 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 Fig. S2.3 and Table S2.1. Recovery yields are based on the total mass of recovered material after free-drying 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.

- 50 -
from the multiple treatments to those of a single treatment with longer ball-milling time (24 h), the saccharide removal was almost the same (Fig. 2.1a). 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. 2.1) and the comparison between lignin content inside the raw material and the residue yield see Fig. S2.3).

2.3.2. 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. 2.2a-d. FT-IR was also applied on tracking the change of the residual saccharide, and the result was correlated with that of saccharide analysis by comprising the peaks of C-O of cellulose and acetyl group of hemicellulose (Fig. S2.4, and detailed assignment of FT-IR was listed in the published paper45). 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 S2.2.1) 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.
Fig. 2.2. The saccharide analysis of the residues obtained from enzymatic hydrolysis of biomass (2.5 g samples from 30 g starting material) (a) 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).
The influence of added enzyme load was also evaluated, and the result was also monitored by saccharide composition analysis and FT-IR spectra (Fig. S2.5) of the samples after enzymatic hydrolysis (Fig. 2.2a1-d1, and detailed assignment of FT-IR was listed in the published paper\ref{45}). 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

Fig. 2.3. 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 grinder 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.
analysis of the curves (detailed discussion is in S2.2.2) 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. S2.6 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 the 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.

2.3.3. 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. 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 S2.2). 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. 2.3a), 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 (Fig. 2.4 and S2.7). 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. 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. 2.3b). 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.\textsuperscript{46,47} No obvious difference was observed between samples milled 0.5 h and 48 h in a 500 nm level of graphs.

2.3.4. 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.\textsuperscript{10} 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 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\textsubscript{enzyme}/g\textsubscript{biomass} enzyme load were collected for all the four biomasses (Fig. 2.5, assignment is listed in our published paper\textsuperscript{45} according to previous papers).\textsuperscript{3,41,43,48} 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. S2.8).

In comparison with raw biomass samples the samples obtained after milling were much easier swelled
in the DMSO-d$_6$/pyridine-d$_5$ 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. S2.2). 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

Table 2.1. The main units and linkage 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       | 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  | 6.8   | 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 were 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 an α-ketone structure, H, p-hydroxyphenyl unit. $^c$ β-O-4, aryl ether, β-5, phenylcoumaan β-β, resinol. $^d$ 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 around 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 (G3), 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').
the amounts of the main β-O-4 linkages. In contrast, the other linkages content such as β-5 and β-β linkages.

Fig. 2.5. 2D HSQC NMR spectra of lignin samples obtained from time course experiments after 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).
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. 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. 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. A detailed parameter overview and conclusions of cited papers are attached in the our published paper.

The MW distribution determination using GPC gave us more insight (Fig. 2.6). 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

![Fig. 2.6. 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.](image)
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 in 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.

2.4. 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 biomasses were 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.
Supplementary Material-Chapter 2

S2.1. Experiment support section
S2.1.1 Chemical composition analysis
S2.1.2. General characterization
S.2.2. Supplementary text
S2.2.1. analysis of curve fitting for Fig. 2.2 a-d
S2.2.2. analysis of curve fitting for Fig. 2.2 a1-d1
S2.2.3. Analysis of XRD spectra for samples obtained from different ball milling times
S2.2.4. Analysis thermal stability of starting material and lignin obtained from 24 h and 48 h ball milling
S2.3. Supplementary Fig. S2.1-S2.9
S2.4. Supplementary Tables S2.1-S2.2
S2.1. Experimental support section:

S2.1.1. Chemical composition analysis

The carbohydrate composition analysis was conducted according NREL\textsuperscript{49}, for the analysis of raw material with a scale of 0.3 g, and the lignin and pretreated biomass samples were in a scale of 10 mg. Samples were hydrolyzed by 72% sulfuric acid at 30 °C for 1 h and further diluted to 4% at 121 °C for another 1 h. In addition, the sugar degradation during the two steps acid hydrolysis was corrected by their corresponding monosaccharides. The amounts of monosaccharides in the resulting solution was determined by HPLC (1200 Agilent Technologies, USA) with a refractive index detector (RID). An aminex column HPX-87H (Bio-Rad, USA) was used to separate the monosaccharides at 50 °C with 5 mM sulfuric acid at a flow rate of 0.6 mL/min. Under these conditions, xylose, mannose and galactose was eluted at the same retention time which were integrated at a single peak. Since xylan is the main constituent and the calibration constant among these three monosaccharide was not very big. Thus, that overlapped peak was directly quantified by the calibration curve of xylose, which is similar to a reported method\textsuperscript{50} and the hemicellulose of the samples was represent as the total amount of xylan, mannan, galactan and arabinan.

S2.1.2. General characterization

The samples after enzymatic hydrolysis were analyzed by scanning electron microscopy (SEM) with an accelerating voltage of 5.0 Kv at ZEISS EVO 18 (Carl Zeiss, Inc., Oberkochen, Germany). The surface area, the pore volume, the pore diameter, and the pore diameter distribution of the samples was determined by ASAP 2420 with N\textsubscript{2}-physisorption. Fourier transform infrared (FT-IR) were collected on a Nicolet In10 FT-IR spectrophotometer (Thermo Scientific, USA) with an MCT detector. The spectra were recorded in the region of 4000-400 cm\textsuperscript{-1} at a resolution of 16 cm\textsuperscript{-1}. Thermal gravimetric analysis (TGA) and first derivative thermogravimetric was obtained on a simultaneous thermal analyzer (TGA 4000, PerkinElmer, USA) with a 20 mL/min flow of nitrogen at a heating rate of 10 °C/min. The determination of X-ray diffraction was conducted by a D8 ADVANCE Bruhers instrument (Bruker, Germany) with Ni-filtered Cu Ka radiation source (k = 0.1542 nm) generated at 40 Kv and 30 Ma. The scattering angle (2\(\theta\)) ranged from 5 ° to 40 ° using the reflection method at a scanning speed of 2 °/min. The relative degree of crystallinity were calculated from the intensity ratio of [(22 \(\theta\))-I\textsubscript{AM}] and I (22 \(\theta\)), in which I\textsubscript{AM} is the minimum intensity between 22 ° and 16.4 °.\textsuperscript{51} 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\cdot min\textsuperscript{-1}. The columns and detectors were held at 50 °C. Data acquisition and calculations were performed using Viscotek OmniSec software version 5.0. Molecular weights were determined based on a conventional calibration curve generated from narrow dispersity polymethylmethacrylate 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. Bruker standard pulse sequence 'hsqcetgpsisp.2' was used for the \textsuperscript{13}C-\textsuperscript{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 (\textsuperscript{1}H) (acquisition time 130 ms), 160 to 0 ppm in F1 (\textsuperscript{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.\textsuperscript{43} The signal of DMSO solvent was used as internal reference (\(\delta\textsuperscript{C} 39.5, \delta\textsuperscript{H} 2.49 \text{ppm}\)). The data was managed by MestReNova x64-12.0.4-22023.

S2.2. Supplementary text

S2.2.1. Analysis of curve fitting for Fig. 2.2 a-d

In order to compared the difference of the data among four biomasses, curve fitting of saccharide remained in the residue as a function of ball milling time were conducted, and the key constants and variation degree of cellulose and hemicellulose calculated from the formula obtained from curve fitting between ball milling of 0.5h and 48h were listed in this published paper.\textsuperscript{45} More than 90% variation of cellulose was found for all the four biomasses, and the order of the variation followed walnut shell (96.5%) > birch (963 %) > pine (92.9%) > reed (92.3%), but if we evaluate the variation of cellulose between 0.5 h and 6 h by the formula, the order switched to pine (85.5%) > birch (81%) > reed (76.7%) > walnut shell (70.7%), indicating pine and birch had a higher
sensitivity towards ball milling than that of reed and walnut shell. In terms of hemicellulose reduction, the order was walnut shell (77.2%) > birch (69.3%) > reed (62.5%) > pine (46.1%) between the range of 0.5 h and 48 h, and the same order was observed for the range between 0.5 h and 6 h, indicating that removal of hemicellulose was lower than that of cellulose, which was consistent with the observation during the isolation of multiple residual enzyme lignin.

S2.2.2. Analysis of curve fitting for Fig. 2.2 a1-d1

In order to make comparison between the data in different biomasses, curve fitting analysis of the data was also performed to compare the relative hydrolytic rate among different biomasses, the key constants and variation degree of cellulose and hemicellulose calculated from the formula obtained from curve fitting between enzyme loading of 0 mL $\text{enzyme}/\text{g biomass}$ to 1 mL $\text{enzyme}/\text{g biomass}$ were listed in this published paper. For the four biomasses, a sensitivity order of cellulose towards added amount of enzyme volume was evaluated as pine (92.5%) > birch (90.5%) > reed (88.2%) > walnut shell (74.1%), but the saccharide removal between enzyme load at 0 mL $\text{enzyme}/\text{g biomass}$ and 0.125 mL $\text{enzyme}/\text{g biomass}$ switched to birch (90.2%) > pine (87.5%) > reed (86.5%) > walnut shell (71.9%). These data indicating cellulose of birch and pine showed relatively higher susceptibility than reed and walnut shell to the enzymatic digestibility with added amount of enzyme, and therefore, for the isolation of residual enzyme lignin of pine and birch, lower concentration of enzyme load were needed. The relatively lower removal of hemicellulose was also observed and the order of hemicellulose removal followed birch (62.8%) > walnut shell (50.3%) > reed (41.0%) > pine (33.2%) between enzyme load 0 mL $\text{enzyme}/\text{g biomass}$ to 1 mL $\text{enzyme}/\text{g biomass}$, and same order was also founded at the range of 0 mL $\text{enzyme}/\text{g biomass}$ to 0.125 mL $\text{enzyme}/\text{g biomass}$.

S2.2.3. Analysis of XRD spectra for samples obtained from different ball milling time

Ball milling could induce some structure changes of the residues that could better enhance the enzyme cocktail performance. Except reduction of particle size, the key factor that ball milling can alter for cellulosic biomass is the crystal structure of cellulose. In order to figure out why ball milling do more contribution than recharging enzyme to the isolation of residual enzyme lignin. The XRD spectra of samples obtained from multiple residual enzyme lignin isolation of walnut shell were firstly collected and plotted in Fig. 2.4a. Three typical peaks of lattice planes of crystalline cellulose were clearly observed at 16.4 °, 22.5 ° and 34.5 ° for the raw material. For walnut shell samples collected in multiple residual enzyme lignin isolation, a broad peak around 20.5 ° appeared after one time 6 h ball milling which may ascribe to amorphous cellulose or reflections of other crystal patterns of cellulose, however, this peak disappeared after the next enzyme treatment and the peak of 22.5° became dominant again. The variation of crystalline cellulose directly revealed on the change of relative crystallinities of the residues, which decreased from 34.3% in raw material to 7.3% in walnut shell after 6h ball milling, and then increased to 19.6% as a result of removing amorphous saccharides by enzyme treatment, and a further ball milling broke the remained crystalline cellulose and enhanced enzyme treatment, and therefore a relatively lower crystallinity index (12.9%) was determined for Walnut _Dcel in contrast to Walnut _Srel (19.6%). It seems likely that the curve first shift to left by ball milling and then went back to right after enzymatic treatment, which indicated that ball milling might induce transformation of crystal patterns but it did not completely destroy crystalline cellulose with a 6 h ball milling. In order to clear observe the transformation of crystal cellulose as a function of ball milling prolongation, XRD spectra of samples collected from time course of ball milling were also collected and profiled in Fig. S2.7, and spectra of walnut shell was picked up to present at Fig. 2.4b. It was found that the peak 34.5° disappeared for all the sample after milling 1 h, and a broad peak around 13.1% gradually appeared, which may be caused by the transformation of crystalline cellulose. Whereas the peak of 22.5 ° was the most stable one among all crystalline cellulose peaks, and it became weak with increase of ball milling time and vanished after milling 24 h, and at the same time, broad peak at 20.5 ° became evident in the spectra, indicating the crystallite size gradually decreased according to Scherrer equation. This transformation is in favor for the next enzyme treatment, as cellulose I had the highest recalcitrance than the other patterns crystal cellulose and amorphous cellulose and towards hydrolysis of endoglucanases. The relative crystallinity also gradually decreased with the prolongation of ball milling and reached 0 at the ball milling time of 24 h. Similar trend was also found for birch, pine and reed (Fig. S2.7), a roughly susceptibility order of the biomass crystalline cellulose towards ball milling was found as reed > pine > birch > walnut shell by evaluating the decrease of the relatively crystallinity of cellulose. The crystalline cellulose of walnut shell showed the highest recalcitrance to ball milling which was correlate the lower performance of enzyme cocktails for removing cellulose. However, the saccharide removal of birch is easier than reed even though crystalline cellulose of reed was more easily broke by ball milling than that of birch, which indicated that some other constituents of reed may influence the
performance of enzyme, such as the linkage between lignin and polysaccharide. In addition, for Arbecol® BWW40 cellulose fibres, one hour milling with a 10 weight ratio of balls to substrate at a speed of 450 rpm could entirely break crystal structure of cellulose.\textsuperscript{35} Therefore, some other constituents of biomass had impediment in breaking of crystalline cellulose, and it was necessary to removal of amorphous cellulose and hemicellulose released from less intensive ball milling, which aided in further destroying the remained crystalline cellulose to enhance efficiency of the fresh step of enzyme treatment. This may explain why a multiple short milling time (6 h) with same total amount of enzyme load (0.5 mL enzyme/g substrate) could achieve a similar saccharides removal in contrast to single treatment with double time milling (24 h) (Fig. 2.1c and a). The detailed cellulose crystalline analysis revealed that the key factor in removing saccharides during enzyme treatment was the crystalline cellulose, and the relative better saccharide removal in single step of long time ball milling and enzyme treatment was ascribe to a higher degree damage of crystalline cellulose.

2.2.4. Analysis thermal stability of starting material and lignin obtained from 24 h and 48 h ball milling

Thermal stability is significant for the application and chemical modification of lignin, and TGA is common method to analyze the stability of polymer with program heating under nitrogen. In this work, the raw biomasses, and their corresponding Rel obtained from samples milled 24 h and 48 h from time course after enzyme treatment with a 0.5 mL enzyme/g biomass enzyme load were selected and comparatively studied by TGA/DTG. The curves were plotted in Fig. S2.9. From the curve of the raw material, three apparently periods were shown, the first period was between 210 °C and 320 °C as a result of decomposition of hemicellulose and amorphous cellulose and side chain of lignin, and the range between 320 °C and 400 °C was mainly ascribed to the degradation of crystalline cellulose and decomposition of fragments of lignin. The main back bone of lignin was decomposed after the temperature reaching 400 °C.\textsuperscript{57} The derivatives of TGA curves was calculated and stacked with DTG, and in curves of raw material, two clear peaks were observed for birch, reed, and walnut shell, and pine showed an intensive main peak at around 350 °C with a very weak shoulder peak at 300 °C. These two peaks were attributed to the fast degradation of cellulose and hemicellulose. Both samples obtained from milling 24 h and 48 h have similar weight loss curves and almost overlapped with each other, and the degraded trend of lignins were more smooth in contrast to that of raw material, which indicated similar constituents compositions of samples obtained from different milling times and their relatively high purity. Only one main peak were observed on the DTG curves for all lignins, and this peak just between peaks for the fastest mass loss of hemicellulose and cellulose, which was correlate to the constituent composition of the residue enzyme lignin, as relatively high amount of hemicellulose and less amount of cellulose remained in the Rel.
Fig. S2.1. The carbohydrate analysis combination of samples from multiple residual enzyme isolation and residues obtained after a further enzymatic treatment without further ball milling, and enzyme treatment was conducted with a 0.5 mL enzyme/g biomass enzyme loading in 25 mL buffer/g biomass. Srel_0.5 are samples obtained from a 6 h ball milling and an enzyme treatment with a 0.5 mL enzyme/g biomass enzyme loading. Srel_De and Drel_0.5 are samples obtained from treating Srel_0.5 without and with a further 6 h ball milling before a further enzyme treatment with a 0.5 mL enzyme/g biomass enzyme loading. For walnut shell, Walnut_Drel_Te_0.5 and Walnut_Tre_0.5 are samples obtained from treating Drel_0.5 without and with a further 6 h ball milling before a further enzyme treatment with a 0.5 mL enzyme/g biomass enzyme loading.
Fig. S2.2. Graphs of samples collected from multiple residual enzyme lignin. Srel in the topside was obtained from one time enzymatic treatment of biomass after 24 h milling with a 0.5 mL \( \text{enzyme/g biomass} \) enzyme load of 1 M in 25 mL \( \text{buffer/g biomass} \). STM was the starting material obtained from smashing with normal grander with 20 mesh screen, 1M was obtained from one time 6 h ball milling of raw, Srel was obtained from one time enzymatic treatment with a 0.5 mL \( \text{enzyme/g biomass} \) enzyme load of 1 M in 25 mL \( \text{buffer/g biomass} \), Srel-2M, was obtained from treating Srel for another 6 h ball milling. Drel was obtained another enzymatic treatment of Srel_2M with a 0.5 mL \( \text{enzyme/g biomass} \) enzyme load in 2 5mL \( \text{buffer/g biomass} \). Walnut_Drel_3M was collected by treating Walnut_Drel for another 6 h ball milling. Walnut_Trel was obtained from treating Walnut_Drel_3M for another enzymatic hydrolysis with a 0.5 mL \( \text{enzyme/g biomass} \) enzyme in 25 mL \( \text{buffer/g biomass} \). Graphs outside the dash frame belongs to walnut samples collected from time course of ball milling of 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 48 h.
Fig. S2.3. Yield of the residues after enzyme treatment, and the composition of the starting material (STM). 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 25 mL buffer/g biomass. Others, no determined components, cellulose was represented by glucan, and hemicellulose was represented by the total amount of xylan and arabinan. AIL, acid insoluble lignin, ASL, acid soluble lignin, Rel*, residual enzyme lignin after removing the cellulose and hemicellulose. Srel, sample obtained from a single cycle treatment, Drel & Trel samples were obtained from double and triple cycle treatment. Detailed data about the composition of the raw biomasses see in Table S1.
Fig. S2.4. FT-IR spectra of residues obtained from enzymatic hydrolysis of samples collected from time course of ball milling 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 48 h with a 0.5 mL enzyme/g biomass enzyme loading in 25 mL buffer/g biomass. (a) birch, (b) pine, (c) reed, (d) walnut shell.

Fig. S2.5. FT-IR spectra of residues obtained from 6 h ball milling and with different enzyme loading of 0 mL enzyme/g biomass, 0.125 mL enzyme/g biomass, 0.25 mL enzyme/g biomass, 0.5 mL enzyme/g biomass, 1 mL enzyme/g biomass in 25 mL buffer/g biomass. (a) birch, (b) pine, (c) walnut shell, (d) reed.
Fig. S2.6. (a), (b), (c) and (d) are saccharide contents of birch, pine, reed and walnut obtained from 6 h ball milling of around 60 g starting material and with different enzyme loading of 0 mL enzyme/g biomass, 0.03125 mL enzyme/g biomass, 0.0625 mL enzyme/g biomass, 0.125 mL enzyme/g biomass, 0.25 mL enzyme/g biomass, 0.5 mL enzyme/g biomass, 1 mL enzyme/g biomass in 25 mL buffer/g biomass.

Fig. S2.7. X-ray diffraction curves of biomasses obtained from time course samples from the ball milling of (a) birch, (b) pine, (c) reed and (d) reed.
Fig. S2.8. 2D HSQC NMR spectra of Srel_Walnut (a), (d), Drel_Walnut (b), (e), Trel_Walnut (c), (f) obtained from multiple residual enzyme lignin with an enzyme load of 0.5 mL\textunderscore enzyme/g\textunderscore biomass in 25 mL\textunderscore buffer/g\textunderscore biomass. A aromatic region, AL aliphatic region and anomeric region.

Fig S2.9. DTG/TGA curves of raw materials and samples obtained from time course of ball milling of 24 h and 48 h after enzyme treatment with a 0.5mL\textunderscore enzyme/g\textunderscore biomass in 25 mL\textunderscore buffer/g\text underscore biomass. (a), birch, (b), pine, (c), reed, (d), walnut.
S2.4. Supplementary Tables

Table S2.1. Carbohydrate analysis whole cell wall (WCW), residual enzyme lignin (DREL) and milled wood lignin (MWL) from four biomasses.

| Sample         | Cellulose | Hemicellulose | Acid soluble lignin | Acid insoluble lignin | Ash |
|----------------|-----------|---------------|---------------------|-----------------------|-----|
| Birch_WCW      | 39.9      | 24.4          | 6.5                 | 18.4                  | 0.5 |
| Pine_WCW       | 41.8      | 22.2          | 4.0                 | 25.8                  | 0.2 |
| Reed_WCW       | 38.3      | 24.1          | 1.4                 | 20.1                  | 2.5 |
| Walnut_WCW     | 26.7      | 24.9          | 5.5                 | 29.3                  | 0.3 |

\(^a\) Data was duplicated collected and the error less than 3%. \(^b\) The cellulose was represented by glucan. \(^c\) Hemicellulose was represented by the total amount of xylan and arabinan. \(^d\) Acid soluble lignin was calculated by absorbance of UV light at recommended wavelength. \(^e\) Acid insoluble lignin was calculated by the mass difference after acidic hydrolysis. \(^h\) Ash content was calculated by the mass difference after calcination. Nd, not determination.

Table S2.2. Surface area, pore volume, pore size of sample obtained from isolation of multiple residual enzyme lignin with a 0.5 mL enzyme/g substrate enzyme load.

| Samples        | Surface Area (m²/g) | Pore Volume (m³/g) | Pore Size (nm) |
|----------------|---------------------|--------------------|----------------|
|                | A\(^a\)  | B\(^b\)  | C\(^c\) | D\(^d\)  | E\(^e\)  | F\(^f\)  | G\(^g\)  |
| Birch_1M       | 2.97    | 3.09    | 2.78   | 0.02    | 0.02    | 20.49   | 22.35   |
| Birch_Scel     | 30.05   | 30.07   | 34.32  | 0.20    | 0.22    | 26.49   | 25.82   |
| Birch_Scel_2M  | 2.23    | 2.12    | 2.50   | 0.02    | 0.02    | 31.14   | 26.09   |
| Birch_Dcel     | 73.22   | 85.34   | 235.27 | 0.36    | 0.40    | 16.76   | 6.84    |
| Pine_1M        | 3.47    | 3.11    | 3.21   | 0.02    | 0.02    | 28.43   | 27.36   |
| Pine_Scel      | 14.52   | 14.38   | 16.45  | 0.07    | 0.07    | 19.43   | 16.97   |
| Pine_Scel_2M   | 3.68    | 3.67    | 3.31   | 0.03    | 0.03    | 30.72   | 33.83   |
| Pine_Dcel      | 26.32   | 28.63   | 39.83  | 0.08    | 0.08    | 11.85   | 8.48    |
| Reed_1M        | 3.45    | 3.25    | 3.15   | 0.02    | 0.02    | 25.49   | 26.17   |
| Reed_Scel      | 34.20   | 32.52   | 36.31  | 0.19    | 0.23    | 23.62   | 24.94   |
| Reed_Scel_2M   | 3.64    | 3.75    | 3.43   | 0.02    | 0.02    | 20.11   | 21.82   |
| Reed_Dcel      | 6.46    | 7.13    | 6.83   | 0.03    | 0.03    | 17.04   | 17.30   |
| Walnut_1M      | 0.68    | 2.14    | 3.91   | 0.03    | 0.03    | 54.24   | 26.02   |
| Walnut_Scel    | 3.35    | 2.84    | 2.64   | 0.02    | 0.02    | 23.06   | 24.79   |
| Walnut_Scel_2M | 2.88    | 3.01    | 2.30   | 0.01    | 0.01    | 11.87   | 15.03   |
| Walnut_Dcel    | 9.42    | 8.66    | 10.56  | 0.10    | 0.10    | 46.02   | 37.72   |
| Walnut_Dcel_3M | 3.27    | 3.41    | 2.61   | 0.01    | 0.01    | 11.52   | 14.80   |
| Walnut_Tcel    | 53.73   | 57.23   | 98.69  | 0.20    | 0.20    | 13.72   | 7.98    |

\(^a\) BET Surface Area; \(^b\) BJH Adsorption cumulative surface area of pores between 1.70 nm and 300.00 nm width; \(^c\) BJH Desorption cumulative surface area of pores between 1.70 nm and 300.00 nm width; \(^d\) BJH Adsorption cumulative volume of pores between 1.70 nm and 300.00 nm width; \(^e\) BJH Desorption volume of pores between 1.70 nm and 300.00 nm width; \(^f\) BJH Adsorption average pore width (4V/A); \(^g\) BJH Desorption average pore width (4V/A); \(^h\) 1M sample after one time ball milling treatment, 2M sample after two times ball milling, Scel, sample obtained from one time enzymatic treatment, Dcel, sample obtained after two time enzymatic treatment.
References

1. Rinaldi, R. et al. Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. Angew. Chemie - Int. Ed. 2016, 55, 8164–8215.

2. Humphreys, J. M. & Chapple, C. Rewriting the lignin roadmap. Curr. Opin. Plant Biol. 2002, 5, 224–229.

3. Ragauskas, A. J. et al. Lignin valorization: Improving lignin processing in the biorefinery. Science. 2014, 344, 6185.

4. Ralph, J. et al. Lignification: are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template replication. Recent Adv. Polyphen. Res. 2008, 1, 36–66.

5. Vanholme, R., Demedts, B., Morreel, K., Ralph, J. & Boerjan, W. Lignin biosynthesis and structure. Plant Physiol. 2010, 153, 895–905.

6. Giummarella, N., Pu, Y., Ragauskas, A. J. & Lawoko, M. A critical review on the analysis of lignin carbohydrate bonds. Green Chem. 2019, 21, 1573–1595.

7. Nishimura, H., Kamiya, A., Nagata, T., Katahira, M. & Watanabe, T. Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls. Sci. Rep. 2018, 8, 1–11.

8. Zijlstra, D. S. et al. Mild Organosolv lignin extraction with alcohols: the importance of benzylic alkoxylation. ACS Sustain. Chem. Eng. 2020, 8, 5119–5131.

9. Zijlstra, D. S., Analbers, C. A., de Korte, J., Wilbers, E. & Deuss, P. J. Efficient mild organosolv lignin extraction in a flow-through setup yielding lignin with high β-O-4 content. Polymers (Basel). 2019, 11, 1913.

10. Guerra, A. et al. Toward a better understanding of the lignin isolation process from wood. J. Agric. Food Chem. 2006, 54, 5939–5947.

11. Alvira, P., Tomás-Pejó, E., Ballesteros, M. & Negro, M. J. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour. Technol. 2010, 101, 4851–4861.

12. Luterbacher, J. S. et al. Lignin monomer production integrated into the γ-valerolactone sugar platform. Energy Environ. Sci. 2015, 8, 2657–2663.

13. Chang, H., Cowling, E. B. & Brown, W. Comparative studies on cellulolytic enzyme lignin and milled wood lignin of sweetgum and spruce. Holzforschung-International J. Biol. Chem. Phys. Technol. Wood 1975, 29, 153–159.

14. Wen, J.-L., Yuan, T.-Q., Sun, S.-L., Xu, F. & Sun, R.-C. Understanding the chemical transformations of lignin during ionic liquid pretreatment. Green Chem. 2014, 16, 181–190.

15. Vvu, S. & Argyropoulos, D. An improved method for Isolating lignin in high yield and purity. J. pulp Pap. Sci. 2003, 29, 235–240.

16. Björkman, A. Studies of finely divided wood. Part 1. Extraction of lignin with neutral solvents. Sven. Papperstidn 1956, 59, 477–485.

17. Holtman, K. M., Chang, H. & Kadla, J. F. An NMR comparison of the whole lignin from milled wood, MWL, and REL dissolved by the DMSO/NMI procedure. J. wood Chem. Technol. 2007, 27, 179–200.

18. Liu, Y. et al. Efficient cleavage of lignin–carbohydrate complexes and ultrafast extraction of lignin oligomers from wood biomass by microwave-assisted treatment with deep eutectic solvent. ChemSusChem 2017, 10, 1692–1700.

19. Sun, Y.-C., Liu, X.-N., Wang, T.-T., Xue, B.-L. & Sun, R.-C. Green process for extraction of lignin by the microwave-assisted ionic liquid approach: Toward biomass biorefinery and lignin characterization. ACS Sustain. Chem. Eng. 2019, 7, 13062–13072.

20. Xia, Q. et al. Multiple hydrogen bond coordination in three-constituent deep eutectic solvents enhances lignin fractionation from biomass. Green Chem. 2018, 20, 2711–2721.

21. Shen, X.-J. et al. Facile fractionation of lignocelluloses by biomass-derived deep eutectic solvent (DES) pretreatment for cellulose enzymatic hydrolysis and lignin valorization. Green Chem. 2019, 21, 275–283.

22. Tang, X. et al. Green processing of lignocellulosic biomass and its derivatives in deep eutectic solvents. ChemSusChem 2017, 10, 2696–2706.

23. Pew, J. C. Properties of powdered wood and isolation of lignin by cellulolytic enzymes. Tappi 1957, 40, 553–558.

24. Pew, J. C. & Weyna, P. Fine grinding, enzyme digestion, and the lignin-cellulose bond in wood. Tappi 1962, 45, 247–256.

25. Rencoret, J., Prinsen, P., Gutiérrez, A., Martínez, A. T. & del Río, J. C. Isolation and structural characterization of the milled wood lignin, dioxane lignin, and cellulolytic lignin preparations from
brewer’s spent grain. J. Agric. Food Chem. 2015, 63, 603–613.
26. Rencoret, J. et al. Lignin composition and structure in young versus adult eucalyptus globulus plants. Plant Physiol. 2011, 155, 667–682.
27. Kim, H. et al. Characterization and elimination of undesirable protein residues in plant cell wall materials for enhancing lignin analysis by solution-state nuclear magnetic resonance spectroscopy. Biomacromolecules 2017, 18, 4184–4195.
28. Shen, X.-J. et al. Efficient and product-controlled depolymerization of lignin oriented by raney Ni cooperated with Cs$_2$H$_3$O$_5$–xPW$_3$O$_{14}$. BioEnergy Res. 2017, 10, 1155–1162.
29. Wang, H.-M., Wang, B., Wen, J.-L., Yuan, T.-Q. & Sun, R.-C. Structural characteristics of lignin macromolecules from different Eucalyptus species. ACS Sustain. Chem. Eng. 2017, 5, 11618–11627.
30. Himmel, M. E. et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 2007, 315, 804–807.
31. Mittal, A. et al. Ammonia pretreatment of corn stover enables facile lignin extraction. ACS Sustain. Chem. Eng. 2017, 5, 2544–2561.
32. Avolio, R. et al. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics? in Biofuels 2007, 67–93 (Springer, 2007).
33. Yoshida, T. & Kremling, K. Disordered biomass recalcitrance: engineering plants and enzymes for biofuels production. ACS Sustain. Chem. Eng. 2018, 6, 14767–14773.
34. Avolio, R. et al. A multitechnique approach to assess the effect of ball milling on cellulose. Carbohydr. Polym. 2012, 87, 265–273.
35. Mansfield, S. D., Kim, H., Lu, F. & Ralph, J. Whole plant cell wall characterization using solution-state 2D NMR. ACS Sustain. Chem. Eng. 2012, 7, 576–591.
36. Kim, H. & Ralph, J. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d6/pyridine-d5. Org. Biomol. Chem. 2010, 8, 576–591.
37. Fujimoto, A., Matsumoto, Y., Chang, H.-M. & Meshitsuka, G. Quantitative evaluation of milling effects on lignin structure during the isolation process of milled wood lignin. J. Wood Sci. 2005, 51, 89–91.
38. Ikeda, T., Holtman, K., Kadla, J. F., Chang, H. & Jameel, H. Studies on the effect of ball milling on lignin structure using a modified DFRC method. J. Agric. Food Chem. 2002, 50, 129–135.
39. Wang, Z., Zhu, X. & Deuss, P. J. The effect of ball milling on birch, pine, reed, walnut shell enzymatic hydrolysis recalcitrance and the structure of the isolated residual enzyme lignin. Ind. Crops Prod. 2021, 167, 113493.
40. Podgornbuskikh, E. M., Bychkov, A. L., Bulina, N. V & Lomovskii, O. I. Disordering of the crystal structure of cellulose under mechanical activation. J. Struct. Chem. 2018, 59, 201–208.
41. Yuan, X. et al. Effects of ball milling on structural changes and hydrolysis of lignocellulosic biomass in liquid hot-water compressed carbon dioxide. Korean J. Chem. Eng. 2016, 33, 2134–2141.
42. Ralph, S. A., Ralph, J., Landucci, L. L., & Landucci, L. L. NMR database of lignin and cell wall model compounds. NMR database lignin cell wall Model Compd. US For. Prod. Lab., Madison, WI (http://ars.usda.gov/Services/docs.htm. 2004).
43. Sluiter, A. et al. Determination of structural carbohydrates and lignin in biomass. Lab. Anal. Proced. 2008, 1617, 1–16.
44. Ferraz, A., Baeza, J., Rodriguez, J. & Freer, J. Estimating the chemical composition of biodegraded pine and eucalyptus wood by DRIFT spectroscopy and multivariate analysis. Bioresour. Technol. 2000, 74, 201–212.
45. Segal, L., Creely, J. J., Martin Jr, A. E. & Conrad, C. M. An empirical method for estimating the degree of
crystallinity of native cellulose using the X-ray diffractometer. *Text. Res. J.* **1959**, *29*, 786–794.

52. Cheng, G. *et al.* Impact of ionic liquid pretreatment conditions on cellulose crystalline structure using 1-ethyl-3-methylimidazolium acetate. *J. Phys. Chem. B* **2012**, *116*, 10049–10054.

53. Nelson, M. L. & O’Connor, R. T. Relation of certain infrared bands to cellulose crystallinity and crystal lattice type. Part II. A new infrared ratio for estimation of crystallinity in celluloses I and II. *J. Appl. Polym. Sci.* **1964**, *8*, 1325–1341.

54. Scherrer, P. Bestimmung der inneren struktur und der größe von kolloidteilchen mittels röntgenstrahlen. In *Kolloidchemie Ein Lehrbuch* **1912**, *387–409* (Springer, 1912).

55. Igarashi, K., Wada, M. & Samejima, M. Activation of crystalline cellulose to cellulose IIII results in efficient hydrolysis by cellobiohydrolase. *FEBS J.* **2007**, *274*, 1785–1792.

56. Amano, Y. *et al.* Reactivities of cellulases from fungi towards ribbon-type bacterial cellulose and band-shaped bacterial cellulose. *Cellulose* **2001**, *8*, 267–274.

57. Yang, H., Yan, R., Chen, H., Lee, D. H. & Zheng, C. Characteristics of hemicellulose, cellulose and lignin pyrolysis. *Fuel* **2007**, *86*, 1781–1788.

58. Ago, M., Endo, T. & Hirotsu, T. Crystalline transformation of native cellulose from cellulose I to cellulose ID polymorph by a ball-milling method with a specific amount of water. *Cellulose* **2004**, *11*, 163–167.