

Letter

Preserving Aryl Ether Linkages and Higher Yields of Isolated Lignin through Biomass  
Fibrillation

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## **Abstract**

Ball milling is used for size reduction of biomass for isolating lignin. However, it is known that it can lead to some structural alteration to lignin. Fibrillation using ultra-friction grinding is a newer size reduction technology which was applied to poplar for isolating cellulolytic enzyme lignin. Compositional analysis of poplar, enzymatic hydrolysis yield, water retention value, lignin yield, lignin structural characterization and lignin molecular weight analysis were carried out to compare lignin isolated from fibrillated and ball milled poplar. By using fibrillation instead of planetary ball-milling for size reduction of poplar, higher  $\beta$ -O-4' linkage content, lignin yield and molecular weight were obtained. Replacing ball milling with fibrillation for size reduction allows recovery of lignin whose structure is closer to that of lignin in biomass.

## **Keywords**

Ball milling; fibrillation; lignin; lignocellulosic biomass, poplar

## **Introduction**

Milled wood lignin (Bjorkman lignin) (MWL)<sup>1</sup> and cellulolytic enzyme lignin<sup>2-4</sup> (cellulase enzyme lignin) (CEL) are the two most popular types of isolated lignin that are used for studying chemical structure of lignin in plant material (lignocellulosic biomass). Both procedures start with extractives removal followed by ball milling. In the former, 96 v/v% p-dioxane is then used for lignin extraction<sup>5</sup>. In the latter, ball-milled material undergoes enzymatic hydrolysis to solubilize polysaccharides followed by p-dioxane in water (usually also at 96 v/v%)<sup>6</sup> as the enzymatic hydrolysis step can allow higher lignin yields compared to MWL<sup>4</sup>. The isolated lignin can then be purified by dissolving in 90% acetic acid, and further by 2:1 v/v 1,2-dichloroethane:ethanol if needed. Size reduction of biomass by ball milling increases the surface area that allows lignin recovery through organic solvents. However, ball milling can cause structural changes to lignin<sup>7</sup>. Until recently, ball milling was probably the only choice for carrying out size reduction of biomass to low micrometer scale. Ultra-friction grinding and high-pressure homogenization are some of the newer size reduction techniques that have been used to prepare nanocellulose<sup>8</sup> and can also be used to fibrillate biomass to micro to nanoscale. Therefore, this work investigated the prospect of replacing ball milling with fibrillation for the purpose of isolating lignin that has a structure which better represents the native lignin structure in biomass. Lignins were recovered from poplar through the CEL isolation procedure using either size reduction technology. Yield, chemical structure and molecular weight, and biomass compositional analysis, enzymatic hydrolysis sugar yield and water retention value testing were carried out.

## **Materials and Methods**

### Size Reduction

Knife-milled extractives-free poplar (8% moisture content) (see supporting information) was ball-milled in a planetary ball mill (PM 200, Retsch). A 5 g (8% moisture content) of poplar and 10 zirconia balls of 10 mm diameter were added to each 50 mL jar. Ball-milling was carried out at 600 rpm for 2 h with 5 min resting interval. For fibrillation, a 5 w/w% suspension of knife-milled poplar was fibrillated in ultra-friction grinder (Masuko Sangyo Supermasscollider, model MKCA6-5, grinding stone model MKGA6-80 Standard Ditch) with 20 passes at -200  $\mu\text{m}$  clearance at RT. The fibrillated poplar was never dried and kept as a suspension in water. The solids contents of ball-milled and fibrillated poplar were determined by moisture analyzer (Mettler Toledo model HG63). A small amount of sample from each type of milling was sieved through a stacked set of standard sieve no. 25, 50, 60, 80, 100, 200, 400 and receiver pan to estimate the mass distribution with particle size.

#### Cellulolytic Enzyme Lignin Isolation

The experimental strategy for lignin isolation is shown in Fig S1. Enzymatic hydrolyses on ball-milled poplar and fibrillated poplar suspensions were carried out in duplicate flasks following the NREL enzymatic hydrolysis procedure<sup>9</sup>. The conditions were: 1% biomass solids, 50 mM sodium citrate pH 5.0 buffer, 0.02% sodium azide and 150 mg total protein per g glucan+xylan in poplar at 50 °C and 150 rpm in Erlenmeyer flasks. The protein loading was 75 wt.% Accellerase 1500, 20 wt.% Accellerase XY and 5 wt.% pectinase. The protein contents of Accellerase 1500 and Accellerase XY were 82 mg/mL and 51 mg/mL, respectively, as determined previously<sup>10,11</sup>. The hydrolysis was carried out for 48 h twice. After the first 48 h, the supernatants from suspensions were removed by centrifugation at 8000 rpm for 10 min. Then a fresh solution of enzymes, buffer and antibiotic at the same concentrations were added to the centrifuge tubes, shaken, and the slurries were poured back into Erlenmeyer flasks and

hydrolysis was carried out for another 48 h. The supernatants were removed in the same manner followed by repeated washing with distilled water by centrifugation. The solids were then transferred back into Erlenmyer flasks and protease was added at 100 U/g original solids in 50 mM pH 7.0 sodium phosphate buffer at 37 °C, 150 rpm for 24 h to hydrolyze any cellulase, xylanase or pectinase enzymes that were adsorbed onto biomass solids. Then protease was deactivated at 90 °C for 15 min followed by washing of solids with water through repeated centrifugation. The solids in 50 mL polypropylene centrifuge tubes were never dried and contained a small quantity of residual water. Then 96% p-dioxane in water was poured into the centrifuge tubes to prepare ~10 w/v% slurries. The tubes were shaken and the poplar-dioxane slurries were transferred to Erlenmeyer flasks. The dioxane extractions were carried out at RT (~21 °C) at 150 rpm in an orbital shaker for 24 h. The dioxane extracts were recovered by collecting the supernatants after centrifugation. The dioxane from these extracts was removed by rotary evaporation (Buchi R-200). Then water was added to the leftover solution and transferred to 50 mL centrifuge tubes which were then frozen and freeze-dried at -80 °C to recover crude lignin. Then 90% acetic acid in water were added to the centrifuge tubes to dissolve the crude lignin followed by centrifugation. The supernatants were then transferred to beakers and excess water was added to precipitate lignin. The suspensions were then centrifuged and further washed with DI water by repeated centrifugation to completely remove acetic acid. The solids with residual water in centrifuge tubes were frozen and then freeze-dried to recover purified lignin. Average yields of crude and purified lignins were calculated based on mass of Klason lignin in knife-milled poplar. Other methods that include materials, compositional analysis<sup>12,13</sup>, molecular weight, spectroscopy, sugar quantification and water retention value<sup>14</sup> are available in supporting information.

## Results and Discussion

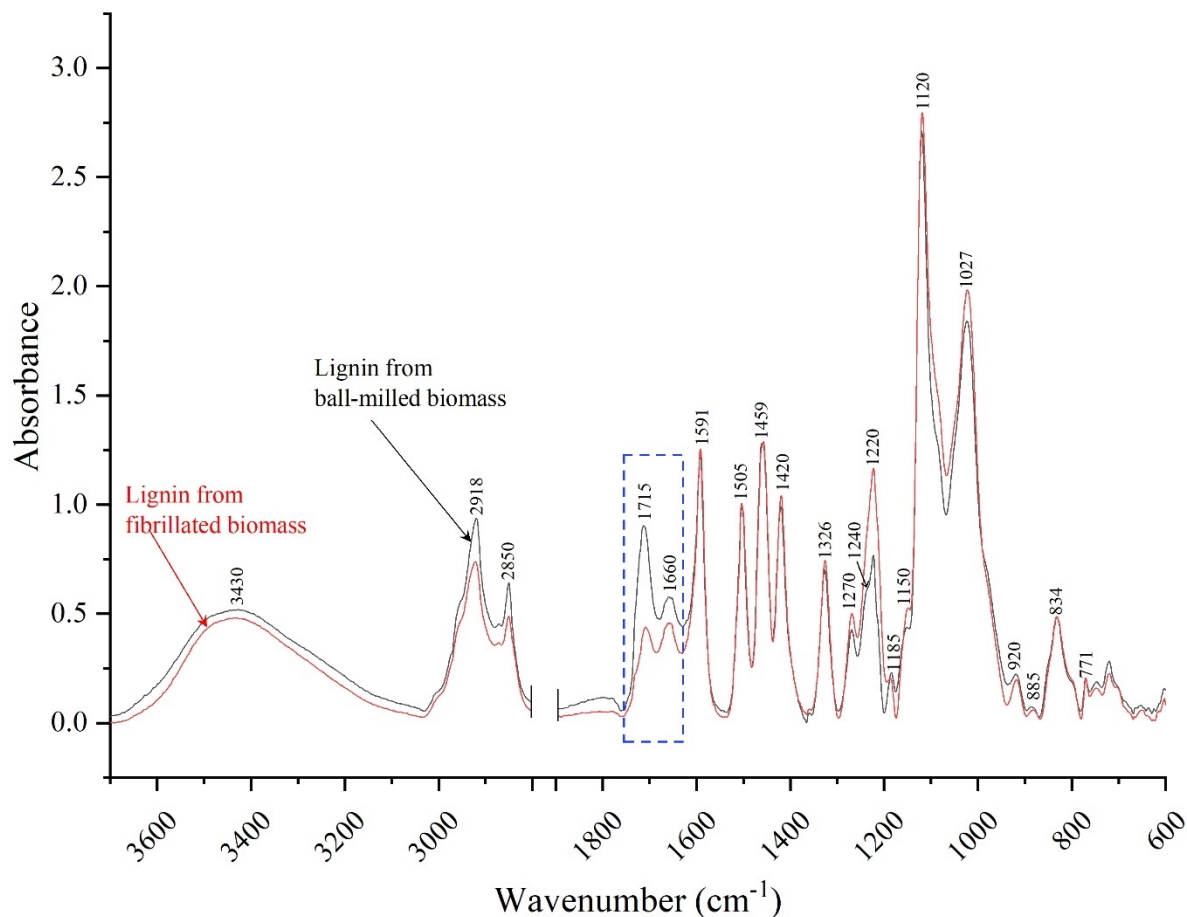
The experimental path followed for isolation of cellulolytic enzyme lignin (CEL) is shown in Fig S1 (see supporting information). Soxhlet extraction of knife-milled poplar was carried out so that any wood aromatic extractives do not interfere with the lignin signal in spectroscopy. The poplar was composed of  $42.1 \pm 0.8\%$  glucan,  $19.7 \pm 1.0\%$  xylan, and  $20 \pm 0.7\%$  acid insoluble lignin (Klason lignin) and  $4.1 \pm 0.1\%$  acid soluble lignin. The planetary ball milling conditions and fibrillation conditions were chosen based on laboratory routines as they allowed efficient size reduction. For ball milled samples, for the particle size range of 707-297, 297-250, 250-177, 177-149, 149-74, 74-37 and  $<37 \mu\text{m}$ , the weight fractions were roughly 3, 3, 6, 3, 12, 11 and 63 wt.% respectively. For fibrillated poplar, for 74-37 and  $<37 \mu\text{m}$  they were roughly 7 and 93%, respectively. Fibrillated biomass fibers can aggregate irreversibly and be paper-like if dried completely, which otherwise would have considerably reduced the surface area. Therefore, it is important to use fibrillated never-dried biomass in suspension form for enzymatic hydrolysis. Since fibrillated suspensions are usually dilute, the only requirement is that the solids% of suspension be higher than solids% in enzymatic hydrolysis to allow for buffer, antibiotic, enzyme and water to make up to total volume; otherwise a decantation step can be performed. For better enzymatic hydrolysis, a mixture of 75% cellulase, 20% hemicellulase and 5% pectinase preparations instead of just cellulase was used as untreated biomass contains high hemicellulose content<sup>15</sup> and pectin is also found in middle lamella where lignin is at higher concentration than primary and secondary cell walls in plants<sup>16</sup>. Enzymatic hydrolysis was carried out 2 times for 2 days each. This is done because the enzymes deactivate over the course of reaction<sup>10</sup>. Replacing the enzyme containing liquid at the mid-point of total reaction time of 4 days assures that excessive active enzyme is present for hydrolysis for maximizing polysaccharide solubilization. The glucan, xylan and glucan+xylan yields were quantified after

the first hydrolysis 2 days and repeat 2 days hydrolysis with fresh enzyme solution. Table S1 shows that sugar yields were higher for fibrillated biomass than ball-milled biomass. However, even for fibrillated biomass, at best 60% of glucan+xylan could be solubilized in total 4 days of total hydrolysis. The repeat 48 h of hydrolysis only yielded 7% and 13% glucan+xylan for ball-milled and fibrillated biomass which suggests that only one round of 48 h of hydrolysis at high enzyme loading is probably sufficient to remove ~40-50% of the polysaccharides from biomass. Multilayer nitrogen adsorption can give a good estimate of surface area but cannot be applied to fibrillated biomass as it would require drying. Instead, water can be used in this case to compare ball-milled and fibrillated biomass through the water retention value (WRV) test. Since the samples are the same chemically and only differ in size and morphology, a higher WRV can indicate higher surface area. The WRV of knife-milled, ball milled and fibrillated poplar were  $2.59 \pm 0.09$ ,  $3.68 \pm 0.04$  and  $7.55 \pm 0.16$  g/g, respectively. Thus, very high surface area of fibrillated biomass likely increased the saccharification efficiency.

Lignin yields from ball-milled and fibrillated poplar were 19% and 31% based on Klason lignin content, respectively. After purification, about 35% purified lignin based on crude lignin weight was recovered from either type. Mid-infrared spectra (FTIR) of the purified lignins were baseline-corrected and normalized using  $1505\text{ cm}^{-1}$  peak height as it arises from vibration of aromatic skeleton in lignin<sup>17</sup> to get relative quantitation of lignin substructures. The processed FTIR spectra are shown in Fig. 1 and band assignments are shown in Table S2. The only discernible difference appears to be at  $1660$  and  $1715\text{ cm}^{-1}$  bands that correspond to vibrations of carbonyl groups in ketones, aldehydes and carboxylic acids. The spectra suggest that CEL from ball-milled poplar is more oxidized than CEL from fibrillated poplar. Presence of bands at  $1240$  and  $1185\text{ cm}^{-1}$  shows presence of polysaccharides even in purified lignins. It is well known that

some carbohydrates linkages are covalently linked to lignin and are present in CEL and MWL, which has led to development of an acid hydrolysis procedure (enzymatic mild acidolysis lignin or EMAL) to cleave the linkages between carbohydrate and lignin during dioxane extraction step (0.1 M HCl in 85 v/v% dioxane)<sup>18</sup> for applications that require lignin of highest purity. Table 1 shows quantitative data on lignin substructures, Fig. S2 shows the lignin chemical structures, Fig. S3 and S4 shows the NMR spectra and Table S3 shows the signal assignments from <sup>13</sup>C-<sup>1</sup>H heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy. The only difference in lignin structure between ball-milled and fibrillated poplar CEL appears to be in  $\beta$ -O-4' linkage content: ~64% through planetary ball milling and ~70% through fibrillation. The standard deviation of lignin structures quantified through NMR using CEL procedure on 6 ball-milled poplars in a previous study has been shown to be 1.4% on an average<sup>19</sup>. Therefore, such a difference in aryl ether linkage content was likely caused by fiber size reduction technology rather than deviations in CEL procedure. The lignin and carbohydrate signals were assigned based on prior literature<sup>19-22</sup>. Other signals that likely originate from carbohydrates can be seen in the NMR spectra. Due to higher enzymatic hydrolysis efficiency of fibrillated poplar, the NMR signals from xylan, arabinan, galactan and glucan at 69-76/3.0-3.8 ppm appear less intense in CEL isolated from fibrillated poplar than ball-milled poplar. C<sub>1</sub>-H<sub>1</sub> signals of hemicellulose and pectin appear in the region between 90-105/4.2-5.3 ppm. Due to higher surface area, enzymes may have had better access cellulose, hemicellulose and pectin in fibrillated poplar. Thus, the differences between CEL from ball-milled and fibrillated poplar in this region are likely due to the differences in type and quantity of remaining hemicellulose and pectin in enzymatically hydrolyzed biomass.

Holtman et al.<sup>7</sup> have previously reported that planetary ball milling can cause oxidation of lignin side chains and decrease  $\beta$ -O-4' content in MWL from Loblolly pine. The two most popular ball milling designs are planetary ball milling and vibratory ball milling. Their effects on lignin structure are not always the same. Guerra et al.<sup>18</sup> found that lignin was oxidized and depolymerized in vibratory ball milling as carboxylic acid content of lignin increased and  $\beta$ -O-4' content decreased over 0 to 100 h of vibratory ball milling but was constant over 25 days of planetary ball milling. The 6% points higher  $\beta$ -O-4' content achieved through fibrillation in this study shows that planetary ball milling causes some decrease in  $\beta$ -O-4' content. The depolymerization caused by ball milling is further supported by molecular weight data (Fig. S5). While weight-average ( $M_w$ ) and number-average molecular weights ( $M_n$ ) for acetylated CEL isolated from ball milling were 3800 and 2300 g/mol, they were 4600 and 2900 for acetylated CEL isolated from fibrillation.



**Fig 1.** Mid-infrared spectra of cellulolytic enzyme lignin (CEL) isolated from ball-milled and fibrillated biomass (poplar)

**Table 1:** Lignin structural composition by quantitative  $^{13}\text{C}$ - $^1\text{H}$  HSQC NMR

Lignin structure	Ball-milled	Fibrillated
Syringyl (S) units, %	77.42	77.53
Guaiacyl (G) units, %	21.91	21.62
<i>p</i> -hydroxyphenyl (H) units, %	0.67	0.85
<i>p</i> -hydroxybenzoate (PB) units, %	6.78	8
$\beta$ -aryl ether ( $\beta$ -O-4') linkages, %	<b>63.64</b>	<b>69.86</b>
Resinol ( $\beta$ - $\beta'$ ) linkages, %	6.89	7.37
Phenylcoumaran ( $\beta$ -5') linkages, %	2.94	2.17
Syringyl:guaiacyl (S/G) ratio	3.53	3.59

Note: Two independent purified lignin samples from ball-milled or fibrillated poplar were combined for average value

Ball milling reduces size through impact-based fracturing by transferring momentum from heavy balls to the sample causing a rupture of covalent bonds that may generate free radicals which may initiate oxidation reactions. It appears that ultra-fine friction grinding does not cause the unwanted changes seen in ball milling as it seems to have a different mode of size reduction that entails separation of the fibers through shearing and pressure forces. Also, since fibrillation is generally carried out as a dilute suspension in water, heat generated due to friction may be better transferred to water that may prevent increase in local temperature. Thus, this newer technology can replace the ball milling step for reducing biomass size. While CEL procedure can directly use the dilute fibrillated suspension, a dewatering step (like centrifugation) is necessary to allow lignin extraction at high dioxane concentration in MWL procedure. In the future, more widespread availability of fibrillation machines would increase its application in isolation of lignin at high yields and low structural alterations. Moreover, the process is much faster than ball milling. Fibrillation operation needs at most half an hour to grind and clean the machine in comparison to ball milling that can take anywhere from 1 h to several days. Currently, a minimum of about 5-10 g of sample per liter of water is needed in the benchtop fibrillation machine. The machines have been built for larger scale grinding applications but could be miniaturized if there is a demand for grinding of limited sample quantities.

### **Conflicts of Interests**

The authors declare that they do not have any conflict of interests.

### **Acknowledgments**

We thank the Center for Renewable Carbon (CRC) and Science & Engineering Research Facility (SERF) at UTK and Joint Institute of Biological Sciences (JIBS) at ORNL for providing the facilities that made this work possible. We also thank Dr. Siqun Wang at UTK for his assistance on proper use of fibrillation equipment.

This research was funded by the U. S. Department of Energy Office of Science through the Genomic Science Program, Office of Biological and Environmental Research, under contract FWP ERKP752 and the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U. S. Department of Energy under Contract DE-AC05-00OR22725.

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### **Data Availability**

All data are contained in this article.

## References

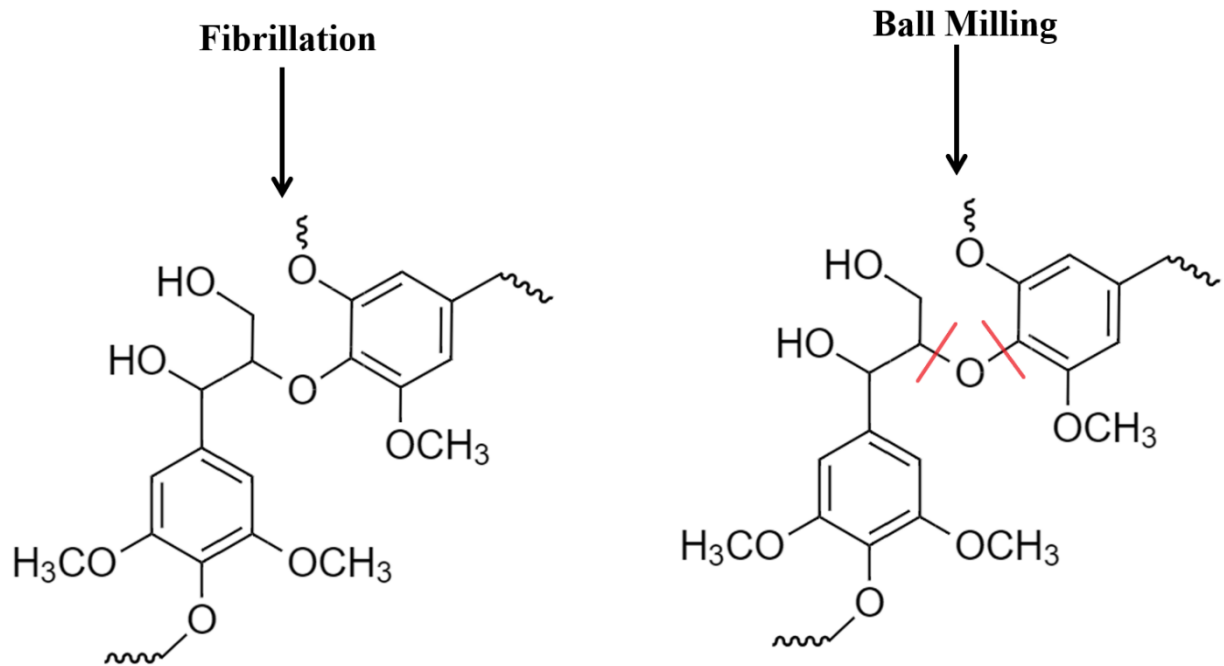
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## **Supporting Information**

Description: Experimental methods, enzymatic hydrolysis sugar yields, spectra and assignments, and chromatograms

**For Table of Contents Use Only**



Replacing ball milling with fibrillation allows better preservation of lignin structure of biomass