Physicochemical Changes of Cellulose and Their Influences on *Populus trichocarpa* Digestibility after Different Pretreatments

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Pretreatment is commonly used to reduce recalcitrance of the lignin-carbohydrate matrix. In this study, leading pretreatment technologies, including dilute sulfuric acid, liquid hot water, alkaline, and organosolv pretreatments, were applied to the selected *Populus trichocarpa* genotype with relatively low lignin content to elucidate cellulose physicochemical property changes and digestibility-related factors. Pretreated *Populus trichocarpa* (BESC 131) exhibited higher accessibility and glucose yield than the untreated biomass. Chemical composition and Fourier transform infrared (FTIR) analysis results revealed that hemicellulose and lignin were removed to a varying extent depending on the pretreatment techniques applied. The degree of polymerization of the cellulose was decreased to the largest extent after dilute acid pretreatment, followed by organosolv, alkaline, and liquid hot water pretreatments. Cellulose crystallinity index was slightly changed after the pretreatments; however, its differences were not remarkable between those pretreatment techniques. Among four different pretreatments, organosolv was the most effective pretreatment technology in terms of sugar release, which was three times higher than that of the untreated native biomass. Among all of the tested cell wall traits, the lignin content of *Populus trichocarpa* was the most remarkable feature associated with glucose release, though *Populus trichocarpa* recalcitrance was not solely dependent on any single factor.

Keywords: Populus trichocarpa; Dilute acid pretreatment; Liquid hot water pretreatment; Alkali pretreatment; Organosolv pretreatment; Cellulose characterization

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INTRODUCTION

The biological processing of lignocellulosic biomass continues to attract research attention due to the increasing demand for alternative energy and biobased products, as well as environmental concerns associated with traditional fuel supplies. Typically, second-generation bioethanol production involves pretreatment, enzymatic hydrolysis, fermentation, and ethanol purification (Meng *et al.* 2016). Lignocellulosic bioresources are

mainly composed of (1) cellulose, a linear polymer linked by β-1,4 glycosidic bonds with cellobiose as its repeating unit (Sun *et al.* 2014a); (2) hemicellulose, an amorphous branched heteropolymer of pentose and hexose sugars (Pu *et al.* 2008); and (3) lignin, an amorphous and three-dimensional phenolic polymer of cross-linked phenylpropane units (*i.e.*, syringyl, guaiacyl, and p-hydroxyphenyl) (Ragauskas *et al.* 2014). Due to the natural recalcitrance of lignocellulosic biomass, pretreatment is viewed as a necessity to effectively increase cellulose accessibility before cellulase hydrolysis step (Salapa *et al*. 2018). However, several pretreatment methods, due to their unique reaction mechanisms, may alter biomass properties and, subsequently, the generation of different co-products (Sun *et al.* 2014b). Pentose and furfural, released from hemicellulose during dilute acid or auto-hydrolysis pretreatment, are two of the most important co-products (Zhao *et al.* 2012). Furthermore, aromatic substrates derived from lignin, which could be removed extensively during alkaline or organosolv pretreatment, have been applied in many fields (Crestini *et al.* 2011; Sadeghifar *et al.* 2017). In general, the recovered lignin is being explored as a value-added component in composites and resins and as a resource for carbon fibers. Alternatively, lignin depolymerization protocols are sought to utilize lignin as a feedstock for biofuels and bio-derived chemicals and materials (Ragauskas *et al.* 2014; Wang *et al.* 2019). Furthermore, the efficient valorization of these co-products is crucial to make the industrialization of biorefinery cost-competitive.

Dilute acid pretreatment (DAP) is one of the most common pretreatment methods, and it can effectively remove the majority of hemicelluloses (Kim *et al.* 2014). The DAP has been applied to various plant species on an industrial scale for bioethanol production (Dien *et al.* 2006; Yao *et al.* 2010; Cao *et al.* 2012). Although cellulose accessibility increases during DAP, lignin can form droplets on the surface of cellulose. In particular, under severe conditions (Selig *et al.* 2007), lignin re-deposition is associated with an adverse effect on glucose release by 1) acting as a physical barrier and 2) binding to cellulases unproductively (Yao *et al.* 2017, 2018a,b). Changes of cellulose ultrastructure, such as an increase in the crystallinity index (CrI), cellulose crystallite size, and a reduction in the degree of polymerization (DP) after DAP, have also been reported (Sun *et al.* 2014a).

Hydrothermal pretreatment, also known as liquid hot water (LHW) pretreatment, is another promising pretreatment method as it is environmentally friendly and cost-effective (Yang *et al.* 2018). The hydrothermal conditions are known to release acetates from hemicellulose components of biomass, thus increasing the acidity of water under LHW conditions, and acidic by-products promote the reduction of the degree of polymerization of cellulose (Yang and Wyman 2008). An increase of cellulose accessibility has also been reported after LHW pretreatment with minimal inhibitor formation (Li *et al.* 2014).

Compared to DAP and LHW pretreatments, alkaline pretreatment (Alkali) is directed at the disruption of lignin structures and cleavage of acetates and ester bonds between lignin and hemicellulose (Yang *et al.* 2016). The reduction of lignin and lignin-hemicellulose cross-linkages tends to increase the accessibility of pretreated biomass to enzymes (Shahabazuddin *et al.* 2018). Numerous studies have examined the influence of alkali pretreatment on the enzymatic digestibility of various feedstocks and reported enhanced sugar release from hemicelluloses in particular (Jin *et al.* 2013; Yoo *et al.* 2013).

Organosolv (OS) has also been used to pretreat biomass, removing substantial amounts of lignin through the cleavage of β-aryl ether bonds *via* either acidolysis and/or homolytic cleavage, while solubilizing and degrading some of the hemicellulose (Nakagame 2011). The beneficial effect of organosolv pretreatment on subsequent enzymatic hydrolysis has been explored in previous studies (Guo *et al.* 2015; Santo *et al.*

2018). Organosolv is more expensive than some other pretreatment methods, but it can generate lignin-derived, value-added products, which could be applied in many fields (Sadeghifar *et al.* 2017; Moniz *et al.* 2018).

Cellulose ultrastructure, characterized mainly by CrI, accessibility, and cellulose DP, has been reported to remarkably impact the performance of enzymatic hydrolysis of biomass (Hall *et al.* 2010; Hallac and Ragauskas 2011). Natural *Populus* variants have been tested and applied in studying gene function and biomass recalcitrance (Meng *et al.* 2016; Yoo *et al.* 2017). It has also been demonstrated that natural variants displayed different recalcitrant properties from their control counterparts (Studer *et al.* 2011). In the past, studies on the enzymatic hydrolysis of pretreated natural *Populus trichocarpa* variants suggested that low recalcitrant variants had higher sugar yields after hydrothermal pretreatment (Meng *et al.* 2016). In other studies, it was reported that low lignin content, low cellulose DP, high cellulose accessibility, and high lignin S/G ratio improved glucose release from untreated natural *Populus trichocarpa* variants by cellulase (Yoo *et al.* 2017). A recent study demonstrated that a transgenic hybrid poplar with low lignin content showed an improvement in the efficiency of biomass conversion (Mansfield *et al.* 2012). However, the effects of different pretreatment methods on cellulose ultrastructure and sugar release of biomass have not yet been directly compared in any study before. In addition, understanding cellulose-related properties and their correlation with biomass recalcitrance are still in their infancy, and literature even reports conflicting trends on the effects of cellulose-related characteristics on the biological deconstruction of lignocellulosic biomass (Marcus *et al.* 2012).

In the present study, a *Populus trichocarpa* natural variant, BESC-131, with relatively low lignin content was selected as a substrate feedstock. Enzymatic digestibility and cellulose accessibility of differently pretreated (*i.e.*, DAP, LHW, Alkali, and OS) *Populus trichocarpa* were evaluated and compared. Attenuated total Reflection-Fourier transform infrared (ATR-FTIR) spectroscopy was employed to compare the *Populus trichocarpa* variant before and after various pretreatments. Cellulose was then isolated from each of the pretreated *Populus trichocarpa* samples, and their physicochemical characteristics were determined through gel permeation chromatography (GPC) and ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR), which provide an in-depth understanding of the roles of these cellulose-related factors in biomass recalcitrance.

EXPERIMENTAL

Materials

Four-year-old *Populus trichocarpa* (BESC-131) was harvested from a field site in Clatskanie, OR, USA. The biomass was debarked, and its size was reduced using a Wiley mill and a 0.420-mm screen. The plant growth conditions and filed establishment were described in a previous study (Meng *et al.* 2016). Cellulase from *Trichoderma reesei* ATCC 26921, β-glucosidase from *Aspergillus niger*, and antibiotics (Antibiotic Antimycotic Solution, A5955) were purchased from Sigma Aldrich (St. Louis, MO, USA). The chemical reagents were purchased from Fisher Scientific (Waltham, MA, USA) and used without further purification.

Pretreatment

Pretreatment conditions were determined according to previous research studies (Arato *et al.* 2005; Pan *et al.* 2006; Cao *et al.* 2012; Meng *et al.* 2016; Li *et al.* 2017). Dilute sulfuric acid (0.5% w/w) was employed to pretreat *Populus trichocarpa* (liquid:solid of 20) at 170 °C for 2 h for DAP. *Populus trichocarpa* (liquid:solid of 20) was also pretreated using deionized water at 180 °C for 44 min for LHW. Additionally, *Populus trichocarpa* (liquid:solid of 20) was pretreated with sodium hydroxide (1% w/w) at a temperature of 120 °C for 1 h for an alkali pretreatment. For the OS pretreatment, *P. trichocarpa* (liquid:solid of 8) was pretreated using a 65:35 ethanol:water solution (v/v) with 1.0 wt% sulfuric acid as a catalyst at 180 °C for 60 min. The pretreated *P. trichocarpa* was washed with warm (60 °C) ethanol:water solution (0.65:0.35, 3 × 200 mL) after OS pretreatment. All the pretreatments were conducted in a stirred Parr 1-L reactor (Model 4842; Parr Instrument Co., Moline, IL, USA). After being submerged in a cold water bath to halt the pretreatment, the pretreated residue was obtained by vacuum filtration and was washed with deionized water until the pH was neutral. The pretreatments were conducted in duplicates, and the average number and error bars are given in the relevant tables and figures.

Methods

Enzymatic digestibility

Populus trichocarpa samples were hydrolyzed at 2% (w/v) consistency in 0.05 M acetate buffer (pH 4.8) at 150 rpm and 50 °C for 72 h. Cellulase and β-glucosidase loading was 25 FPU/g and 50 IU per grams of glucan, respectively. Antibiotics (Sigma A5955) were added at a 10 mL/L charge to avoid microbiological contamination. Liquid samples were periodically taken (2, 4, 8, 12, 24, 48, and 72 h) from the hydrolysate, quenched by submersion in a boiling water bath for 10 min, and then immediately frozen to -20 °C prior to sugar analysis *via* a Dionex high performance liquid chromatography (HPLC) system (ICS-3000, Thermo Fisher Scientific, Sunnyvale, CA) equipped with Dionex CarboPac PA20 column, with an injection volume of 10 µL. The enzymatic hydrolysis of pretreated *P. trichocarpa* samples was performed in duplicates, and the results are represented by their mean value ± standard deviation (SD).

Chemical composition analysis

Extractive-free *Populus trichocarpa* biomass samples were treated with 72% sulfuric acid at 30 °C, and then 4% dilute acid at 121 °C according to the National Renewable Energy Laboratory protocols to determine the carbohydrate and lignin contents (Sluiter *et al.* 2008). After the two-step acid hydrolysis, the hydrolysate was diluted, filtered, and analyzed using an HPLC equipped with pulsed amperometric detection, a Dionex ISC-3000 with a conductivity detector (Thermo Fisher Scientific, Sunnyvale, CA, USA), a guard CarboPac PA1 column (Dionex, Sunnyvale, CA, USA), a CarboPac PA1 column (Dionex, Sunnyvale, CA, USA), and an AS40 automated sampler (Thermo Fisher Scientific, Sunnyvale, CA, USA). Calibration was performed with standard solutions of glucose, xylose, arabinose, mannose, and galactose, with fructose used as an internal standard. The chemical composition analysis of *P. trichocarpa* samples was completed in duplicates, and the results are presented by their average number ± SD.

GPC Analysis

α-Cellulose was isolated from *Populus trichocarpa* using peracetic acid and sodium hydroxide (Meng *et al.* 2016). The isolated holocellulose was mixed with anhydrous pyridine and phenyl isocyanate and warmed to 70 °C for 48 h, which generated cellulose tricarbanilate, as described in the literature (Meng *et al.* 2016). The cellulose derivative was dissolved in tetrahydrofuran (THF; 1.00 mg/mL), and the solution was filtered through a 0.45-μm polytetrafluoroethylene (PTFE) filter and placed in a 2-mL vial. The molecular weight distributions of the cellulose tricarbanilate were analyzed on an Agilent GPC SECurity 1200 system equipped with four Waters Styragel columns (HR0.5, HR2, HR4, and HR6) (Waters Corporation, Milford, MA, USA). The THF was used as the mobile phase (1.0 mL/min).

ATR-FTIR analysis

A PerkinElmer Spectrum 100 FTIR spectrometer (Perkin-Elmer, Inc., Waltham, MA, USA) was employed to analyze the structural features of *Populus trichocarpa* biomass samples. Spectra were obtained by 64 scans accumulated from 4,000 to 500 cm^{-1} with a resolution of 4 cm^{-1}.

CP/MAS ^{13}C CP/MAS-NMR analysis

A Bruker DSX-400 spectrometer (Bruker Corporation, Billerica, MA, USA) was used to perform the solid-state NMR determination at frequencies of 100.55 MHz. The CP/MAS experiments utilized a 5 μs (90°) proton pulse, 1.5 ms contact pulse, 4.0 s recycle delay, and 8 K scans.

Simons' staining

Direct Orange 15 and Direct Blue 1 (Pylam Products Co., Inc., Tempe, AZ, USA) were employed to study the cellulose accessibility of *Populus trichocarpa*. Briefly, biomass samples (100 mg) were mixed with 1 mL phosphate buffer (0.3 M, pH 6.8), 1 mL NaCl solution (1%), and 1 mL of dye mixture (Direct Orange 15: Direct Blue 1 = 1:1, with increasing concentration). After dye absorption, the absorbance of the supernatant solution was determined with a PerkinElmer ultraviolet-visible (UV-Vis) Lambda (Spectrum One FTIR system; Perkin Elmer, Wellesley, MA, USA) at 455 nm and 624 nm, representing the maximum absorbance length for Direct Orange 15 and Direct Blue 1, respectively. The Langmuir adsorption equation determined the maximum amounts of orange and blue dye adsorbed by the biomass substrates. The ratio between orange and blue dye adsorption capacities can be calculated as a measure of large-to-small pore ratio of the lignocellulosic substrates.

RESULTS AND DISCUSSION

Solid Recovery and Chemical Composition of *Populus trichocarpa* After Pretreatments

The solid recoveries after different pretreatments are shown in Fig. 1A. Organosolv pretreatment resulted in the lowest solid recovery (approximately 55%), suggesting that more biomass components such as hemicellulose and lignin were removed by this pretreatment (Fig. 1B). The solid yields of each pretreatment decreased in the following order: Alkali (89%) > LHW (78%) > DAP (68%) > OS (55%). The glucan loss was less

than 20% in all pretreatment methods; in particular, Alkali and OS pretreatment resulted in the lowest glucan loss (approximately 4 to 5%). Alkali pretreatment has been shown to remove hemicellulose and increase the efficiency of enzymatic hydrolysis (Zhang *et al.* 2012). Regarding hemicellulose removal, acid-involved methods, such as DAP, LHW, and OS pretreatments, were more effective than alkali pretreatment, which removed less than 25% of hemicellulose. Lignin can adversely affect enzymatic hydrolysis of lignocellulosic materials; thus, it is one of the major targets for many pretreatments (Yang *et al*. 2016). In this study, the four pretreatment technologies exhibited different lignin removal capabilities. Organosolv removed most of the lignin (94%), followed by Alkali (22%), LHW (5%), and DAP (4%). The enhanced ability of organosolv to dissolve and remove hemicellulose and lignin, which has been previously reported (Sannigrahi *et al*. 2010), readily explains the low solids recovery from the organosolv pretreatment.

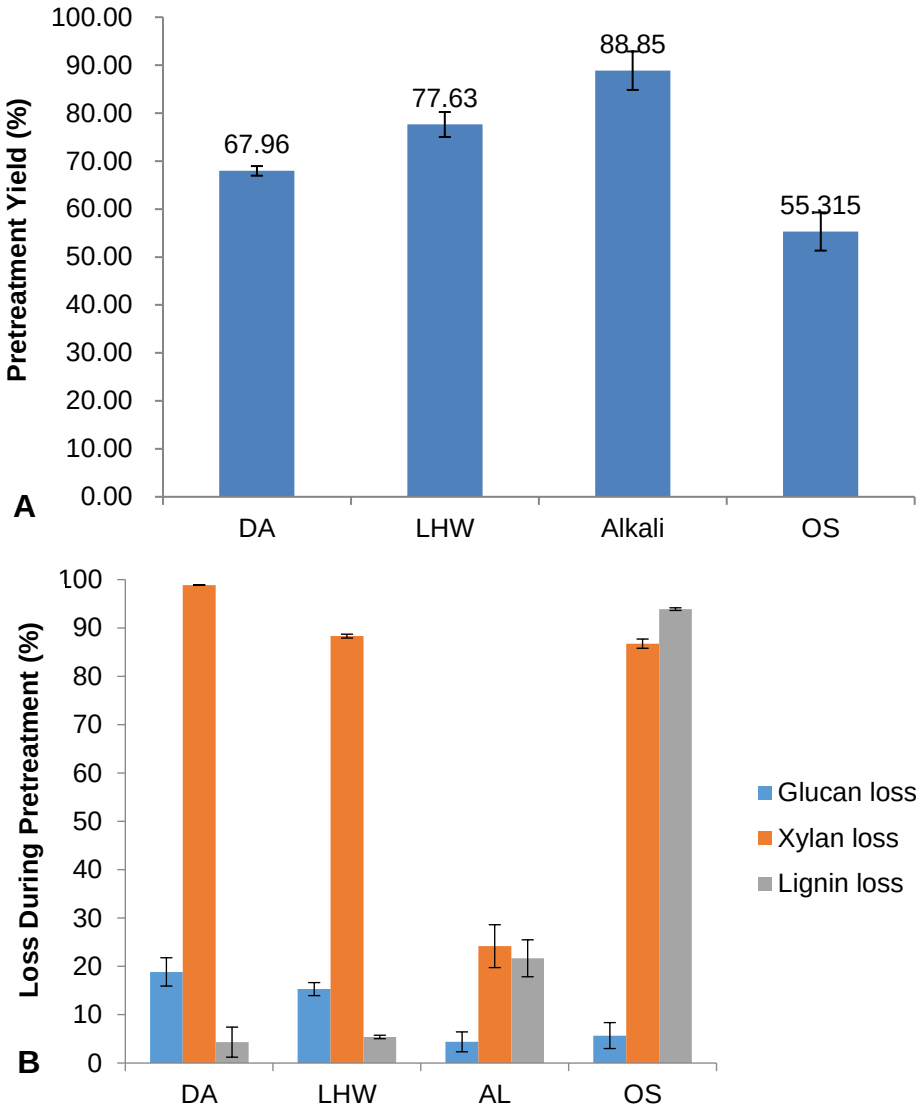

Fig. 1. Pretreatment yield (A) and loss of various components (B) during alternate pretreatment processes; DA- Dilute acid pretreatment; LHW- liquid hot water pretreatment; AL- alkaline pretreatment; OS- Organosolv pretreatment

The chemical compositions, including glucan, xylan, and Klason lignin, of the pretreated and untreated (Raw) *Populus trichocarpa* are presented in Table 1. In general, the relative glucan content in solid residues increased after pretreatments, due to variation in the removal of hemicellulose and/or lignin. Both DAP and LHW pretreatments solubilized most of the hemicelluloses, while alkali pretreatment had moderate removal of hemicellulose (*i.e.*, xylan, arabinan, and galactan) and lignin. Most of the lignin was removed during the organosolv pretreatment, resulting in the highest glucan content in the organosolv-pretreated *Populus trichocarpa*.

Table 1. Composition Analysis of Untreated and Pretreated *Populus* After Different Pretreatments (%)

	Glucan	Xylan	Klason Lignin
Raw	51.40 ± 0.37	11.76 ± 1.51	20.61 ± 0.29
DAP	61.40 ± 3.55	0.21 ± 0.01	29.03 ± 1.78
LHW	56.11 ± 1.39	181.00 ± 0.17	25.13 ± 1.10
Alkali	55.42 ± 4.09	10.82 ± 0.32	18.17 ± 0.19
OS	87.79 ± 4.45	3.34 ± 0.38	2.28 ± 0.09

Enzymatic Digestibility of *Populus trichocarpa* Before and After Pretreatments

Glucan and xylan digestibilities of untreated and pretreated *Populus trichocarpa* were tested using a mixture of cellulase and β-glucosidase with a solid loading of 2% (w/v) at 45 °C in acetate buffer for 72 h. As presented in Fig. 2, glucan digestibility was relatively high in the first 4 h, and then it diminished after 72 h.

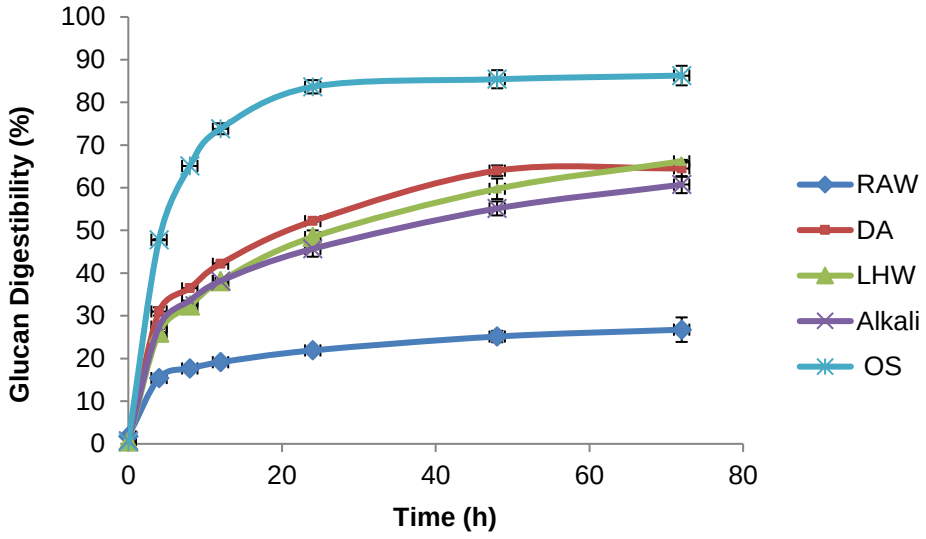

Fig. 2. Glucan digestibility of the untreated (Raw), DAP-, LHW-, OS-, and Alkali-pretreated *Populus trichocarpa*

Although it seems that the digestibility of glucan from LWH- and AL-pretreated *P. trichocarpa* was not constant at 72 h of cellulose hydrolysis, the extension of the cellulase reaction time from 72 to 96 h could not increase sugar generation further. Glucan digestibility of *Populus trichocarpa* after 72 h of enzymatic hydrolysis varied from 26.8% (untreated) to 86.3% (OS-pretreated). The OS-pretreated *Populus trichocarpa* showed the

highest glucan digestibility, while the digestibilities of the biomass in the three other pretreatments were comparable. The amount of monosaccharides obtained during the enzymatic hydrolysis process is presented in Table 2. Due to the substantial removal of xylan during DAP, LHW, and OS pretreatments (Fig. 1B), xylan digestibility was not remarkably improved after pretreatments and the xylan digestibility of DAP-pretreated *Populus trichocarpa* was even less than that of the raw biomass. In conclusion, the sugar (*i.e.*, glucose + xylose) yield was decreased in the following order: OS > Alkali > LHW > DAP > Raw.

Table 2. Monosaccharide Release from Untreated and Pretreated *Populus* During the Enzymatic Hydrolysis Process

Sample	Amount of Raw Biomass Loading (g/g biomass)		
	Glucose	Xylose	Glucose + Xylose
Raw	0.14 ± 0.01	0.02 ± 0.00	0.16 ± 0.01
DA	0.28 ± 0.01	0.01 ± 0.00	0.29 ± 0.01
LHW	0.29 ± 0.00	0.02 ± 0.00	0.31 ± 0.00
Alkali	0.34 ± 0.01	0.08 ± 0.00	0.42 ± 0.01
OS	0.45 ± 0.01	0.03 ± 0.00	0.48 ± 0.01

The OS-pretreated *Populus trichocarpa* had the highest glucan digestibility due to the remarkable removal of xylan (87%) and lignin (94%), presumably making cellulose more accessible for enzymatic hydrolysis. The results coincide with a previous study showing that the structure of lignocellulosic materials becomes relaxed after OS pretreatment due to the dissolution of lignin and hemicellulose, thus promoting the adsorption of cellulase onto the pretreated residue (Koo *et al.* 2011). Alkali-pretreated *Populus trichocarpa* had the second highest sugar release among the tested pretreatments, which was 0.42 g/g biomass. It was reported that during alkaline pretreatment, the esters linkages and glycosides could be degraded, leading to lignin modification/dissolution, cellulose swelling, cellulose de-crystallization, and hemicellulose solvation (Kumar and Sharma 2017). Lignin removal not only increased cellulose accessibility to cellulase, but it also decreased the non-productive adsorption of cellulases to lignin during enzymatic hydrolysis (Sun and Cheng 2002). Both DAP- and LHW-pretreated *Populus trichocarpa* showed comparable glucose and xylose release. Both pretreatment methods had similar interactions with biomass, including hemicellulose solubilization and lignin distribution to various extents. The DAP-pretreated *Populus trichocarpa* showed nearly 99% of xylan removal, while the LHW-pretreated sample had 87% of xylan removal. However, the amounts of the released glucose after these two pretreatments were not remarkably different, suggesting that lignin removal was probably more crucial than xylan removal for improving the enzymatic digestibility of *P. trichocarpa*. Demartini *et al.* also reported that lignin content likely plays an essential role in the recalcitrance of *Populus trichocarpa*, while hemicellulose was the critical recalcitrance-causing factor for switchgrass (Demartini *et al.* 2013).

Degree of Polymerization of Cellulose After Pretreatments

It has been reported that the molecular weight of cellulose could affect cellulase hydrolysis (Hall *et al.* 2010; Hallac and Ragauskas 2011; Meng *et al.* 2016; Yoo *et al.* 2017). A lower cellulose DP means shorter cellulose chain length, thus possessing more reducing ends. Therefore, cellulose with lower molecular weight may be readily processed

by exoglucanase (Pan *et al.* 2007). The GPC analysis was used to determine the number average molecular weight (M_n), weight average molecular weight (M_w), the DP, and polydispersity index (PDI) of celluloses from untreated and pretreated *P. trichocarpa* (Table 3). The DP of cellulose was remarkably reduced after all the pretreatments. Cellulose isolated from DAP-pretreated biomass had the lowest molecular weights (M_n and M_w), and the cellulose DPs for the pretreated samples from lowest to highest values were Organosolv, LHW, and Alkali, respectively. Therefore, the increased sugar release after these pretreatments was likely due, in part, to cellulose molecular weight reduction, which was in accordance with previous studies (Hu and Ragauskas 2012; Pu *et al.* 2013). However, DAP- and LHW-pretreated *P. trichocarpa* indicated similar cellulose conversion, while the M_w of cellulose from LHW-pretreated *Populus* was approximately three times higher than that of cellulose from DAP-pretreated *Populus trichocarpa*. Hence, as previously suggested, biomass recalcitrance is a multi-variant and multi-scale phenomenon that cannot be simply judged solely on a substrate factor, such as cellulose DP (Meng *et al.* 2016).

Table 3. Molecular Weights and Degree of Polymerization of Cellulose in *Populus*

Sample	M_n (g/mol)	M_w (g/mol)	DP_n	DP_w	PDI
Raw	343640	1858600	662	3581	5.4
DA	49689	178750	96	344	3.6
LHW	87846	553890	169	1067	6.3
Alkali	90701	495500	175	955	5.5
OS	64341	384300	124	740	6.0

PDI- polydispersity index

ATR-FTIR Analysis

The FTIR spectra of *Populus trichocarpa* are presented in Fig. 3. Relative changes of essential signals can be calculated from the ratio of various absorption bands to that of 1424 cm^{-1}, which is ascribed to cellulose (Table 4). The broad and strong signal at around 3340 cm^{-1} is from the hydroxyl group, whose spectral intensity was decreased after the pretreatments, indicating a rupture of hydrogen bonding of cellulose (He *et al.* 2008). Chemical composition and pretreatment yield also indicated that DAP and LHW pretreatments caused the most cellulose degradation. The vibration at 2900 cm^{-1} is attributed to the C-H stretching, whereas the signal at 1367 cm^{-1} corresponds to C-H bending modes (Kumar *et al.* 2009). Bands at 1745 cm^{-1} and 1720 cm^{-1} are attributed to the carbonyl vibration of lignin and carboxylic acids, respectively (Sun *et al.* 2005). The intensity of these signals in all pretreated *Populus* samples decreased most during Alkali pretreatment, followed by DAP and LHW pretreatments, suggesting the side chains of lignin were cleaved during these pretreatments. Previous studies also showed that pretreatment with alkali or base resulted in the most remarkable reduction of signals at 1745 cm^{-1} and 1720 cm^{-1} (Kumar *et al.*. 2009). The typical bands at 1595 and 1510 cm^{-1} are caused by skeletal vibrations of the aromatic ring (Yang *et al.* 2016), the lowest signal intensity was in Organosolv-pretreated *Populus*, suggesting the greatest extent of lignin removal. These results were in accordance with the solid recovery and chemical composition results of *Populus*.

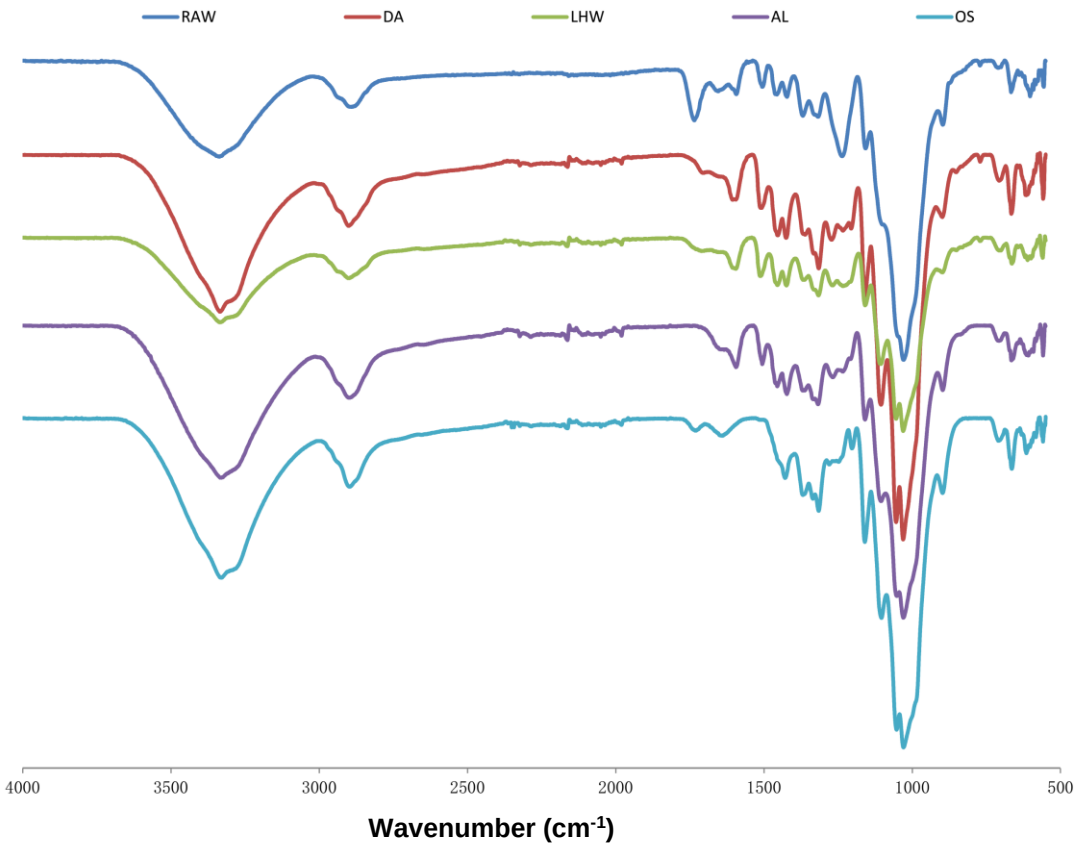

Fig. 3. FTIR spectra of *Populus*

Table 4. Signal Assignments and Relative Changes in *Populus* Solids After Leading Pretreatments

Wavenumber (cm^{-1})	Assignment	Pretreatment				
		Raw	DA	LHW	Alkali	OS
3340	O–H stretching (indicates rupture of cellulose hydrogen bonds)	2.7	1.4	1.4	1.9	2.5
2900	C–H stretching	1.3	0.7	0.7	0.9	1.1
1745	Carbonyl bonds	1.5	0.04	0.1	0.01	0.2
1720	Carboxylic acids/ester groups	1.4	0.1	0.2	0.02	0.2
1595	Aromatic ring stretch	0.9	0.4	0.5	0.5	0.1
1510	Lignin aromatic ring stretch	0.7	0.5	0.4	0.6	0.03
1423	CH_2 scissor motion in cellulose	1.0	1.0	1.0	1.0	1.0
1367	Aliphatic C-H stretch in CH_3	1.5	0.7	0.7	0.8	1.2
1265	Ester absorbance (related to removal of uronic acid)	1.2	1.0	1.0	0.8	0.8
1245	C–O adsorption (resulting from acetyl groups cleavage)	1.9	0.8	0.8	0.7	0.7
1059	C-O stretching on secondary alcohol	7.2	3.3	3.0	3.3	4.8
900	Amorphous cellulose	1.7	0.6	0.6	0.8	1.2

The decreased spectral intensity at 1245 cm^{-1} in all pretreated *Populus* was due to the cleavage of acetyl groups. The reduction of relative intensity at 900 cm^{-1} suggested the degradation of amorphous cellulose and/or possible transformation of amorphous cellulose into crystalline cellulose (Laureano-Perez *et al.* 2005; Sun *et al.* 2014a,b).

^{13}C CP/MAS-NMR Analysis

Cellulose crystallinity index analysis

Cellulose is composed of crystalline and amorphous regions. The two regions exhibit entirely different reaction rates during the enzymatic hydrolysis process. Generally, the crystalline Cellulose I region is more difficult to hydrolyze with cellulase than the amorphous region (Liu *et al.* 2017). Figure 4A shows each of the six carbon atoms of cellulose in the *Populus* and labeled accordingly in the spectra (Pu *et al.* 2006). The C4 region extends over a chemical shift range of 80 to 92 ppm. Signals assigned to cellulose amorphous domains appear broad, while those of crystalline domains are sharper (Pu *et al.* 2006; Foston 2014). The crystalline region accounted for more than half of the C4 absorption in native *Populus* (Fig. 4B). Earlier studies revealed that pretreatments under high pressure could disrupt inter- and/or intra-hydrogen bonding of cellulose and resulted in a change of crystalline structure (Mosier *et al.* 2005). As shown in Fig. 4B, CrI was slightly increased after all the pretreatments, indicating that part of the amorphous region was degraded and/or transformed during pretreatments. Both OS- and Alkali-pretreated *Populus* showed the lowest CrI followed by LHW and DAP. The OS- and Alkali-pretreated *Populus* showed similar cellulose CrI (60%), while there were remarkable differences in their sugar release. Although cellulose CrI has been proposed as an indicator of biomass recalcitrance, it did not show any correlation with glucose release from pretreated biomass (Brienzo *et al.* 2014; Meng *et al.* 2016). In recent studies, it was suggested that *Populus* variants with lower cellulose DP usually had higher CrI (Yoo *et al.* 2017), which was also found in the present study (Fig. 4C).

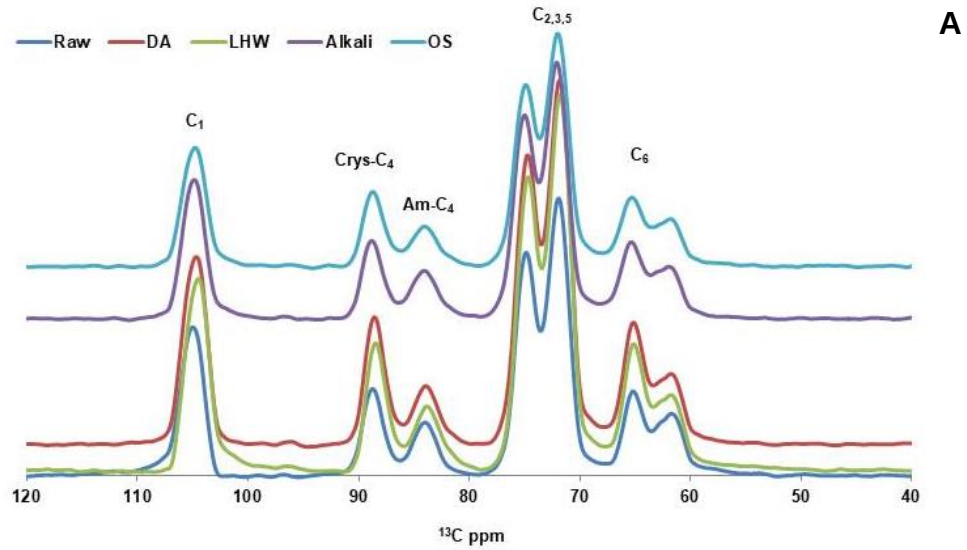

Fig. 4A. The ^{13}C CP/MAS-NMR spectra (A) and cellulose crystallinity index of cellulose

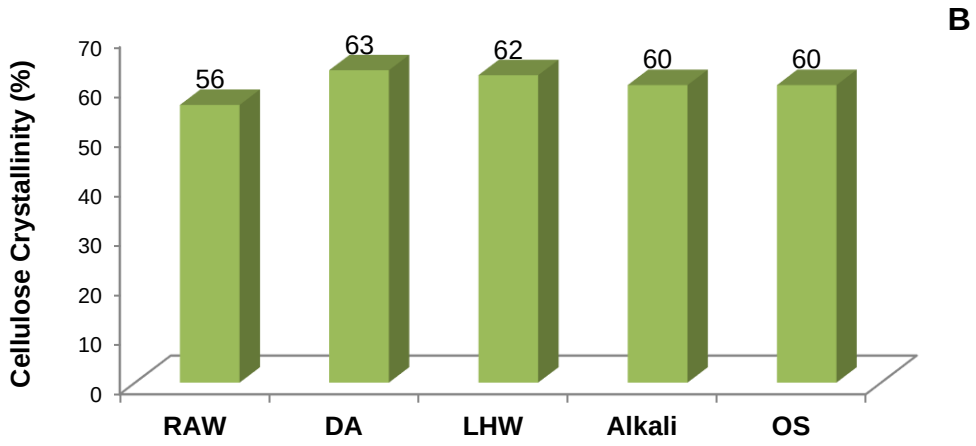

Fig. 4B. The ^{13}C CP/MAS-NMR spectra (B) from *Populus*

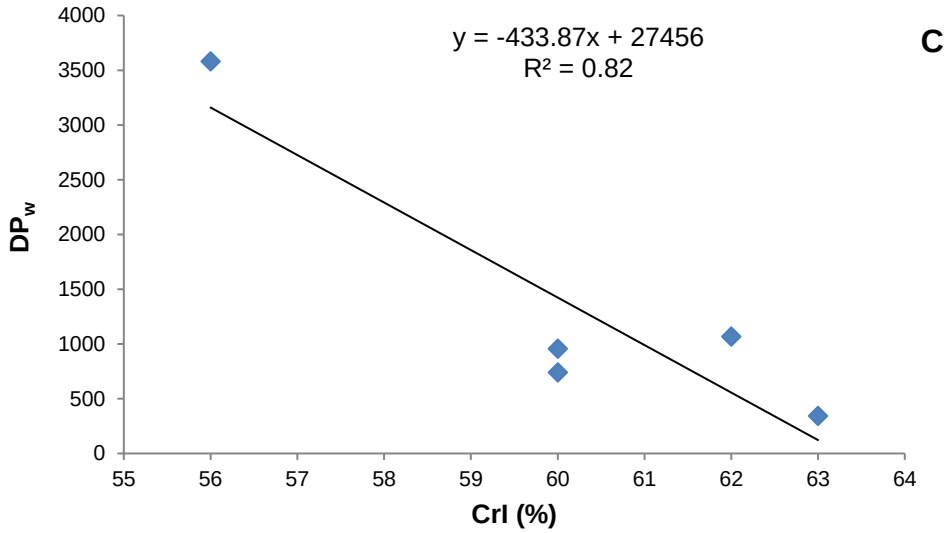

Fig. 4C. The ^{13}C CP/MAS-NMR spectra: their relationship with DPw (C)

Cellulose ultrastructure analysis by NMR

The relative intensity of the cellulosic ultrastructural components, including cellulose crystalline allomorphs, para-crystalline cellulose, and cellulose fibril surface, and their changes after each pretreatment are shown in Fig. 5. A seven-peak model and a least-squared non-linear fit of the C4-carbon region of the ^{13}C CP/MAS-NMR spectra were used (Sun *et al*. 2014a). After the pretreatments, the content of para-crystalline, which is a form of cellulose that has a degree of order between crystalline and amorphous cellulose, was increased to a varying extent (Ioelovich *et al.* 2010). In addition, cellulose Iα content was decreased, accompanied by an increase of I (α + β) content. This result was in accordance with previous studies, which suggested preferential degradation and/or transformation of cellulose Iα into cellulose Iβ during the pretreatment (Sun *et al.* 2014a,b). Furthermore, the relative proportion of amorphous (*i.e.*, accessible and inaccessible fibril surfaces) cellulose was decreased, indicating the favored hydrolysis of amorphous cellulose over that of crystalline cellulose during the pretreatment process. These results were also confirmed by the increased crystallinity index after pretreatment. The DA-pretreated *Populus* contained

the most para-crystalline cellulose, which was presumably due to the preferential degradation/removal of amorphous cellulose under acidic conditions (Foston and Ragauskas 2010). *Populus* pretreated by Alkali showed the highest content of I_β, suggesting that more cellulose I_α was converted into cellulose I_β under alkali condition.

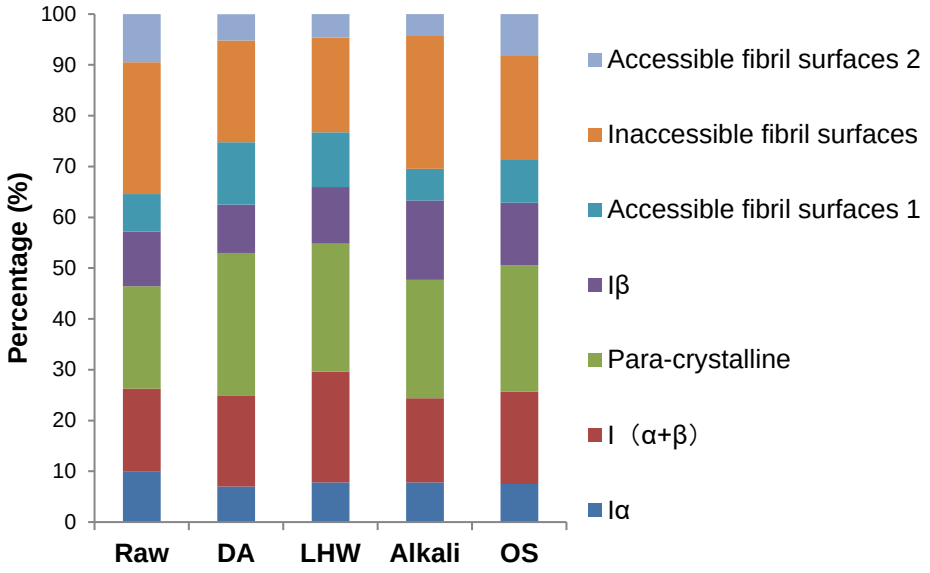

Fig. 5. Relative percentage of cellulose crystalline allomorphs, para-crystalline cellulose, and cellulose fibril surface in the pretreated *Populus*

Cellulose Accessibility Test by Simons' Stain

The influence of the four different pretreatments on cellulose accessibility was evaluated by Simons' staining (SS) method, which has proven to be a promising technique to test the accessible surface area of cellulose before and after pretreatment (Meng *et al.* 2016). The SS measures both interior and exterior accessible surface area of lignocellulosic substrates by applying two direct dyes: Direct Blue 1 (DB) and Direct Orange 15 (DO). These two dyes show different molecular size and maximum UV absorption wavelengths and are known to exhibit different absorption properties with cellulosic fibrous materials. The DO dyes have a molecular diameter of approximately 5 to 36 nm, which is similar to the nominal size of 5.1 nm and representative of the diameter of a typical enzyme, while DB dye only has a molecular diameter of approximately 1 nm (Meng *et al.* 2016). Because the DO dye has a much higher binding affinity to the hydroxyl group on the cellulose surface, the maximum adsorbed DO dye is a reliable indicator of the ease of attack by cellulases (Chandra *et al.* 2008). As shown in Table 5, the pretreated *Populus* exhibited an increase in the accessible surface area of cellulose compared with the raw material. Alkali-pretreated *Populus* showed the highest cellulose accessibility, followed by OS, LHW, and DAP pretreatments. Generally speaking, the pretreated *Populus* with higher DO dye adsorption exhibited higher sugar release during the enzymatic hydrolysis. Previous studies have also indicated a positive correlation between cellulose accessibility and digestibility of biomass (Hall *et al.* 2010; Meng *et al.* 2016). However, Alkali-pretreated biomass has the highest cellulose accessibility but releases a lower amount of glucose compared to OS-pretreated biomass, which could be a result of the relatively higher lignin content and higher cellulose DP, compared to OS-pretreated sample that negatively affects its sugar

release. This also indicates that biomass recalcitrance cannot be simply judged based on a single substrate attribute.

Table 5. The Maximum Amount of Direct Orange and Blue Dye Adsorbed by *Populus* During Simons' Stain

Substrate (*Populus*)	Maximum Adsorbed Orange Dye (mg/g Sample)	Maximum Adsorbed Blue Dye (mg/g Sample)
Raw	17.1	24.3
DA	23.5	29.4
LHW	27.9	32.2
Alkali	34.4	37.5
OS	29.0	51.5

CONCLUSIONS

1. Organosolv pretreatment was the most effective method to increase the digestibility of *Populus*, which was three times higher than that of the untreated biomass. Hemicellulose and lignin were removed to various extents during the four pretreatment processes. Dilute acid (DA) pretreatment removed the majority of the hemicellulose, while organosolv (OS) pretreatment solubilized most of the hemicellulose and lignin.
2. The gel permeation chromatography (GPC) analysis showed that the degree of polymerization (DP) of cellulose was decreased during the four pretreatment processes and it was decreased in the following order: liquid hot water (LHW) > alkaline (AL) > OS > DA. The crystallinity index (CrI) of cellulose was slightly increased after pretreatment, but the differences between different methods were negligible. Cellulose ultrastructure analysis indicated that DA-pretreated *Populus* contained the most para-crystalline cellulose and AL-pretreated *Populus* contained the highest content of I_β.
3. Among the tested properties of cell wall, including molecular weight of cellulose, cellulose crystallinity index, ultrastructure features, and cellulose accessibility, digestibility could not be judged based on any of the studied single factors by itself. Rather, digestibility can be described as a multi-scale phenomenon.

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