Comparison of laboratory delignification methods, their selectivity, and impacts on physiochemical characteristics of cellulosic biomass

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HIGHLIGHTS

▶ Delignification was performed employing sodium chlorite–acetic acid and peracetic acid.
▶ Various raw and pretreated biomass solids and pure cellulose were used.
▶ Delignification selectivity and effects on cellulose structure were determined.
▶ Peracetic acid was more selective than sodium chlorite–acetic acid.
▶ Cellulose MW, reducing ends, and CrI were affected less in delignification with PAA.

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ABSTRACT

Two established delignification methods employing sodium chlorite–acetic acid (SC/AA) and peracetic acid (PAA) are often used, and are reportedly highly selective. However, these reports are mostly for highly recalcitrant and unpretreated softwoods and hardwoods species, and information for less recalcitrant lignocellulosic feedstocks and pretreated biomass is scarce. Furthermore, the effects on cellulose structure are not documented. Thus, in this study, delignification kinetics and selectivity were evaluated when SC/AA and PAA were applied to untreated switchgrass, poplar, corn stover, and pine sawdust; poplar subjected to AFEX, controlled pH, lime, and SO_2 pretreatments; and the cellulose model compounds. Both methods proved effective in removing >90% lignin, but selectivity for lignin and carbohydrates removal was substrate and pretreatment dependent. For untreated biomass, PAA was more selective in removing lignin than SC/AA; however, both methods were less selective for pretreated solids. Cellulose characterizations revealed that PAA had less pronounced impacts on cellulose structure.

1. Introduction

Cellulose, hemicellulose, and lignin are three major components of lignocellulosic biomass, with amounts varying with biomass types (hardwood, softwood, agricultural residues, and energy crops), primary vs. secondary cell walls, ages, and locations (Chundawat et al., 2010; Wyman, 1990). Lignin is believed to surround cellulose and hemicellulose as a complex structure that makes cellulosic biomass highly recalcitrant to enzymes, pathogens and microorganisms (Lynd et al., 1991; Studer et al., 2011). To understand the complex structure of cellulosic biomass and the impact of biomass features on its enzymatic digestibility, delignification is often performed by two common laboratory methods: acidified sodium chlorite or peracetic acid (Chang and Holtzapple, 2000; Ding et al., 2012; Ishizawa et al., 2009; Naran et al., 2009). The sodium chlorite–acetic acid (SC/AA) method, originally known as the Wise method (Wise et al., 1946), is usually performed at 60–70 °C for 4–8 h with successive addition (every hour or two) of fresh sodium chlorite and acetic acid at loadings of 0.3–0.6 g sodium chlorite/g dry biomass and 0.1–0.6 ml acetic acid/g dry biomass (Ahlgren and Goring, 1971; Hubbell and Ragauskas, 2010; Timell, 1961). Whereas, peracetic acid (PAA) delignification is performed at more moderate conditions: 25 °C with PAA loadings of 4–5.5 g/g dry biomass and times of 24–48 h (Chang and Holtzapple,

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2000; Poljak, 1948; Zhu et al., 2008). The SC/AA method has become an established mostly for softwoods and proven to be highly selective at less harsh conditions (Ahlgren and Goring, 1971; Jungnikl et al., 2008). Similarly, PAA delignification of poplar wood was shown to be highly selective in terms of acetate removal (<14%) (Chang and Holtzapple, 2000). However, these two methods have not been evaluated for their selectivity and impacts on other biomass features for considerably lesser recalcitrant feedstocks such as agricultural residues and energy crops. Furthermore, delignification by these two methods and others such as alkaline peroxide is often performed on pretreated biomass to evaluate the effects of residual lignin on biomass digestibility (Ishizawa et al., 2009; Kumar et al., 2012; Selig et al., 2009; Yang et al., 2002).

It is well known that most leading thermochemical pretreatments remove, dislocate, and/or change lignin structure during pretreatment, and, therefore, the residual lignin after pretreatment is physicochemically different from that in the untreated starting material (Hu and Ragauskas, 2011; Kumar et al., 2009; Samuel et al., 2010; Sannigrahi et al., 2008). However, highly selective lignin removal is important to pinpoint its effects on biomass digestibility but has rarely been reported for application of PAA or SC/AA to pretreated biomass. Furthermore, the chemical reagents employed to delignify cellulosic biomass are known oxidizing agents and, therefore, can affect cellulose reactivity through oxidation (Xu et al., 2009) and structural changes. For example, Ishizawa et al. showed that following SC/AA delignification, Avicel cellulose reactivity was unchanged but amorphous cellulose suffered a loss in reactivity (Ishizawa et al., 2009), most possibly due to oxidation of reducing ends. Hubbell and Ragauskas (Hubbell and Ragauskas, 2010) consistent with a study by Kumar et al. (Kumar et al., 2009) showed that extensive delignification of pure cellulose via the SC/AA method can affect cellulose degree of polymerization (DP). However, such data is not available for PAA delignification.

In this study, delignification kinetics and lignin selectivity of SC/AA and PAA were evaluated for untreated switchgrass (SWG), poplar, corn stover (CS), and pine sawdust (PSD) and for poplar pretreated by leading pretreatment technologies of ammonia fiber expansion (AFEX), controlled pH (CpH), lime, and sulfur dioxide (SO$_2$). To determine the effects of delignification on cellulose structure, gel permeation chromatography, NMR, and FT-IR analysis were applied to solids delignified by these two methods. Furthermore, to evaluate the effects of the delignification methods on cellulose reducing ends and crystallinity, cellulose model compounds were also subjected to SC/AA and PAA delignification.

2. Methods

2.1. Substrates and reagents

Switchgrass (Dacotah, *P. virgatum*; ¼ inch) was generously provided by Ceres, Inc. (Thousand Oaks, CA), more information on the material can be found elsewhere (Shi et al., 2011). Corn stover (¼ inch) was kindly provided by National Renewable Energy (NREL) in Golden, Colorado. Poplar (¼ inch) was graciously provided by Dr. Venkatesh Balan at Michigan State University, MI. Poplar solids pretreated by some of the leading pretreatments were prepared by the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) partners as follows: ammonia fiber expansion (AFEX) by Dr. Bruce Dale at Michigan State University, controlled pH (CpH) by Dr. Michael Ladisch and Dr. Nathan Mosier at Purdue University, lime by Dr. Mark Holtzapple at Texas A&M University, and SO$_2$ by Dr. Jack Saddler at the University of British Columbia. The pretreatment conditions for these technologies are reported elsewhere (Kumar et al., 2009). Pine sawdust (PSD) was obtained from Dr. Joesph Norbeck's laboratory at the

Center for Environmental Research and Technology (CE-CERT), University of California, Riverside. Avicel® PH 101 cellulose (Lot No. BCBD6923V, Fluka), cotton linter (Lot No. 090M0144V), α-cellulose (Lot No. 050M0140V), and peracetic acid (32 wt.%, Batch No. 53796CM) were purchased from Sigma–Aldrich (St. Louis, MO, US). Glacial acetic acid (Acros, Lot No. B0512763) and unstablized sodium chlorite (80% purity, Acros organics, Lot No. B0130435) were from Fisher Scientific (Pittsburgh, PA).

2.2. Delignification

Prior to all experiments, Dacotah SWG was washed several times with hot DI water (80 °C) to remove free non-structural sugars that have been shown to be present in significant amounts (Garlock et al., 2011; Shi et al., 2011). The washed switchgrass was squeezed by hand to remove excess water and then dried in a 45 °C incubator (Model No. 472960, Labline, Melrose Park, IL) for several days. Other substrates were used as received.

2.2.1. Sodium chlorite–acetic acid (SC/AA)

Solids were delignified at 70 ± 2 °C in a water bath (Model 10 l, Cole-Parmer, Vernon Hills, IL) with an initial liquid to solids ratio of 32. Five grams of dry biomass was weighed into 250 ml Erlenmeyer flasks (Fisher Scientific, Pittsburgh, PA) in duplicates, and then 160 ml of deionized (DI) water was added followed by 0.6 g/g dry biomass of sodium chlorite (NaClO$_2$) and 0.6 ml/g dry biomass of acetic acid. The slurry was thoroughly mixed by shaking the flasks, and then a 50 ml Erlenmeyer flask was inverted in the neck of the reaction flask. The flasks were incubated in a fume hood at 70 °C with intermittent mixing, and fresh charges of sodium chlorite and acetic acid were added to the reaction every 2 h for up to 8 h. Controls with biomass and just water were also run under identical conditions. Similarly, pure cellulose model compounds were subjected to the same delignification conditions at 70 °C for 6 h. After reaction, the slurry was vacuum filtered on a glass fiber filter to separate the liquid from the solids (Cat No. 09-804-110A, Fisher Scientific, Pittsburgh, PA), and the solids were repeatedly washed with room temperature DI water until the pH of the filtrate was nearly neutral. Solids were carefully scraped off the glass fiber filter paper and collected in Ziplock bags. For mass balances, the wet weight of the delignified solids was recorded, and the solids moisture content in triplicates was analyzed using a Halogen moisture analyzer (MX5, Mettler Toledo, Columbus, OH). The amount of biomass recovered (yield) following delignification was calculated as:

Biomass recovery (yield; Y$_B$), % = 100 ∗ [Amount of wet biomass recovered after reaction (g) ∗ (100-% Avg. moisture content of the recovered solids)]/Initial amount of dry solids (g).

2.2.2. Peracetic acid (PAA)

Following the procedure of Chang and Holtzapple, peracetic delignification was performed in duplicates at 25 ± 2 °C and 5 wt.% solids loadings for 24–48 h (Chang and Holtzapple, 2000). The reaction was conducted in 250 ml Erlenmeyer flasks (Fisher Scientific, Pittsburgh, PA) heated in a temperature controlled water bath (Model 10L, Cole-Parmer, Vernon Hills, IL). PAA loadings of 0.75 g/g dry solids, 2.0 g/g dry solids, 3.5 g/g dry solids, and 5.5 g/g dry solids were used. All reactions were conducted for 24 h at all loadings except 5.5 g/g dry solids, which was run for 48 h. For cellulose model compounds, the reaction was only conducted at a PAA loading of 5.5 g/g dry solids at 25 °C for 48 h. After the reaction, the slurry was vacuum filtered, and the solids were washed repeatedly with room temperature DI water until the filtrate pH was close to neutral. Solids were collected, and the moisture content was determined as described in the previous section for SC/AA.

2.3. Compositional analysis and components yield calculations

The untreated and delignified solids were dried in a 50 °C oven (Model No. 472960, Labline, Melrose Park, IL) for several days and then milled (Mini mill, Model 3383-L20, Thomas Scientific, Swedesboro, NJ) to pass through a 20 mesh size (0.841 mm) screen. Analyses for structural carbohydrates and acid insoluble lignin (Klason-lignin) were performed on the solids according to the National Renewable Energy Laboratory (NREL, Golden, Colorado, USA) method (Sluiter et al., 2008). For analysis, the samples along with appropriate calibration standards were run on a Waters Alliance HPLC system (Model No. e2695) equipped with a 2414 RI detector and a 2998 column heater (Waters Corporation, Milford, MA). The sugars were separated with a Bio-Rad Aminex HPX-87P or Aminex HPX-87H column (Cat No. 125-0098) equipped with appropriate micro-guard cartridges (Bio-Rad, Hercules, CA). Ultrapure DI water (18 Ω, Milli-Q gradient, Millipore, Billerica, MA) and 5 mM H_2SO_4 in ultrapure DI water were used as carriers at a flow rate of 0.6 ml/min for the Bio-Rad Aminex HPX-87P and Aminex HPX-87H column, respectively. The recovery of glucan, xylan, arabinan, acetyl, and lignin after delignification was calculated as:

$$Component(i)\ recovered\ (yield, Y_i), \% = Y_B * f_{i,r} / f_{i,o}$$

where i is the biomass component (glucan, xylan, arabinan, acetyl, or lignin), Y_B is the % biomass yield after reaction, $f_{i,r}$ is the fraction of component i in the residual biomass, and $f_{i,o}$ is the fraction of component i in the original biomass.

2.4. Gel permeation chromatography (GPC) analysis

Gel permeation chromatography (GPC) was utilized to evaluate the effects of SC/AA and PAA delignification on cellulose DP. The molecular weights of the delignified solids from SWG and poplar were determined by GPC of derivatized cellulose because native cellulose is insoluble in common GPC solvents. Direct derivatization of delignified samples affords a molecular weight distribution of the remaining holocellulose by GPC. Since the solvents used for GPC cannot solubilize and derivatize cellulose completely for samples that had significant amounts of lignin, the samples were further subjected to SC/AA delignification at Georgia Tech following a procedure described elsewhere (Hubbell and Ragauskas, 2010). Furthermore, to obtain quantitative information on the cellulose chains, the cellulose fraction must first be isolated. Therefore, following delignification, an alkaline extraction was performed to remove residual hemicellulose. In brief, a holocellulose sample (1.0 g) was extracted with a 17.5% (w/w) NaOH solution (50.0 ml) at 25 °C for 30 min. Then, 50 ml of deionized filtered water was added to the NaOH solution, and the extraction was continued with the diluted NaOH solution (100 ml) at 25 °C for an additional 30 min. The isolated alpha-cellulose samples were then collected by filtration and rinsed with 50 ml of 1% acetic acid followed by excess water and then air dried.

The holocellulose and cellulose solids were derivatized according to a previously established procedure (Hubbell and Ragauskas, 2010). Briefly, dry samples were reacted with phenyl isocyanate in anhydrous pyridine at 70 °C for 48 h. The resulting solution was then precipitated in a 7:3 methanol/water mixture, and the solids were collected via filtration and dried at reduced pressure. Prior to GPC analysis, derivatized samples were dissolved in tetrahydrofuran (THF, ~1 mg/ml), filtered through a 0.45 μm membrane, and placed in a 2 ml auto-sampler vial. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA, US) equipped with Waters Styragel columns (HR1, HR2, HR4, and HR5; Waters Corporation, Milford, MA, US) and an Agilent UV detector (270 nm) using THF as the mobile phase (1.0 ml/min) with injection volumes ranging from 20–50 μl, depending on sample concentration. A calibration curve was constructed based on ten polystyrene standards, each with narrow ranges in molecular weight from 2.2 to 3600 kDa. Data collection and processing were performed using Polymer Standards Service (PSS, US) WinGPC Unity software, and the software calculated number and weight average molecular weights (M_n and M_w, respectively) relative to the universal polystyrene calibration curve. Number-average degree of polymerization (DP_n) and weight-average degree of polymerization (DP_w) were obtained by dividing M_n and M_w, respectively, by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeating unit.

2.5. Determination of cellulose reducing ends

The amount of cellulose reducing ends (μmoles/g cellulose) was determined by the modified BCA method described elsewhere (Zhang and Lynd, 2005). BCA solution A contained 1.94 g/l of bicinchoninic acid disodium salt hydrate (Sigma, Lot No. 031M5307V, Cat No. D8284), 54.3 g/l of Na_2CO_3 (Sigma, Lot No. 049K0086), and 24.2 g/l of $NaHCO_3$ (Sigma, Batch No.118K0966) in DI water; and solution B was made with 1.25 g/l of $CuSO_4.5H_2O$ (Sigma, Lot No. 069K0099) and 1.26 g/l of L-serine (Sigma, Batch No. 117K0835) in DI water. About 2.5 ml of sample containing 1–5 g/l of cellulose in DI water was added into the test tubes run in triplicates followed by 2.5 ml of freshly made solution of equal amounts of BCA solution A and B. The test tubes along with duplicate glucose calibration standards (0–55 μM; 0–0.01 g/l) were incubated at 75 °C for 30 min in a temperature controlled circulating water bath (Fisher Scientific, Pittsburgh, PA). The tubes were shaken every five min to avoid solids settling. Following 30 min of incubation, the tubes were cooled to room temperature, and the solution was transferred into 2 ml microcentrifuge tubes that were centrifuged at 14,600 rpm for 5 min. Then about 3 ml of solid free supernatant was transferred to a cuvette, the absorbance was read at 560 nm on a UV–Vis spectrophotometer (SpectraMax M2e, Molecular Devices, Sunnyvale, CA), and the amount of cellulose reducing ends was determined from absorbance calibration plots generated for standards.

2.6. Solid-state NMR and FTIR-ATR spectroscopic analysis

2.6.1. Sample preparation
Untreated and delignified Avicel cellulose were vacuum dried overnight at 50 °C and used as received. For biomass samples, isolated cellulose was prepared from delignified samples (1.00 g) by hydrolysis in HCl (100.0 ml of 2.50 M) at 100 °C for 4 h (Sannigrahi et al., 2010a). The isolated cellulose samples were then collected by ultrafiltration, rinsed with excess DI filtered water, and air dried in a hood.

2.6.2. NMR analysis
NMR samples were prepared by packing ground isolated cellulose samples into 4-mm cylindrical ceramic MAS rotors. Each sample was repetitively pressed into the rotor to load the maximum amount of sample. Solid-state NMR measurements were carried out on a Bruker Avance-400 spectrometer operated at frequencies of 100.55 MHz for ^{13}C in a Bruker double-resonance MAS probehead at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5 μs (90°) proton pulse, 1.5 ms contact pulse, 4 s recycle delay, and 2048 scans. All spectra were recorded on wet samples (60–80% moisture content), and the line-fitting analysis of spectra was performed using NUTS NMR Data Processing software (Acorn NMR, Inc). Error analysis was conducted by two individual isolations, NMR acquisitions and line-fit data processing.

2.6.3. FTIR-ATR spectroscopic analysis

The Spectrum One FT-IR system (Perkin Elmer, Wellesley, MA) with a universal attenuated total reflection (ATR) accessory was used to characterize the isolated cellulose samples. Each sample was pressed uniformly and tightly against the diamond surface using a spring-loaded anvil. FT-IR spectra were obtained by averaging 64 scans from 4000 to 650 cm^{-1} at 4 cm^{-1} resolution. Baseline and ATR corrections for penetration depth and frequency variations were carried out using Spectrum One software supplied with the equipment. These spectra were normalized at 3350 cm^{-1} (O–H stretching peak).

3. Results and discussion

### 3.1. Delignification kinetics

Delignification kinetics and lignin selectivity can be influenced by various reaction parameters such as temperature, reagent loadings, and time. However, in this study, only the effects of reaction time for SC/AA and of reagent loadings for PAA delignification were studied, unless otherwise noted. Other reaction conditions for both methods were the same as described in Section 2 (Chang and Holtzapple, 2000; Hubbell and Ragauskas, 2010).

3.1.1. Sodium chlorite–acetic acid (SC/AA)

Switchgrass and poplar solids were delignified for up to 8 h at 70 °C with fixed doses of sodium chlorite (0.6 g/g dry solids) and acetic acid (0.6 ml/g dry biomass) added every 2 h. Fig. 1a shows the amounts of total solids (TS), carbohydrate, and lignin removed over treatment time for both SWG and poplar. The carbohydrate removal for SWG was much higher than poplar, with the amount after 6 h being about 25% and <2.5% for SWG and poplar, respectively. Although the amount of lignin removal was almost the same for all reaction times, the amount of TS removed from SWG was much higher than from poplar. For instance, after 2 h of reaction time, the residual solids yield for SWG was about 78% and dropped to about 50% after 8 h, whereas for poplar, it was ~90% at 2 h and ~72% after 8 h. For both substrates, more than 90% of the lignin was removed after 6 h of reaction time, and the reaction time beyond 6 h seemed to have little impact on further delignification. After 6 h of reaction, the delignified solids for SWG and poplar had about 2.5 and 1.7 wt.% dry basis K-lignin, respectively, as shown in Table 1. Therefore, it appears that for these reagent loadings (total 3 dosages of NaClO$_2$ and acetic 360 acid), a reaction time of 6 h was enough to remove most of the lignin. Fig. 1a also shows the amount of TS, carbohydrate, and lignin removed for corn stover (CS) and PSD for reaction time of 6 h at 70 °C with similar reagents loadings as SWG and poplar. The amounts of TS removed for these two substrates were almost the same (31–33%), with the extent of delignification being 90% and 96.7% for CS and PSD, respectively. The amount of carbohydrate removal was higher for CS (~15% of total) than PSD (~7.5%). The controls containing biomass and water showed a negligible solids loss (e.g.,<3% for SWG).The composition of untreated and delignified solids following 6 h of reaction time is shown in Table 1.

3.1.2. Peracetic acid (PAA)

PAA delignification was performed following the Chang and Holtzapple method originally developed by Poljack, 1948 on SWG and poplar at 25 °C with PAA loadings of 0.75, 2.0, and 3.5 g/g dry biomass for 24 h, whereas for PAA loading of 5.5 g/g dry biomass, the reaction time was 48 h (Chang and Holtzapple, 2000). Fig. 1b shows the effect of PAA loading on TS, carbohydrate, and lignin removal for poplar and SWG. The biomass yield at each loading was similar for both poplar and SWG; however, the extent of delignification for poplar was much higher than for SWG, except
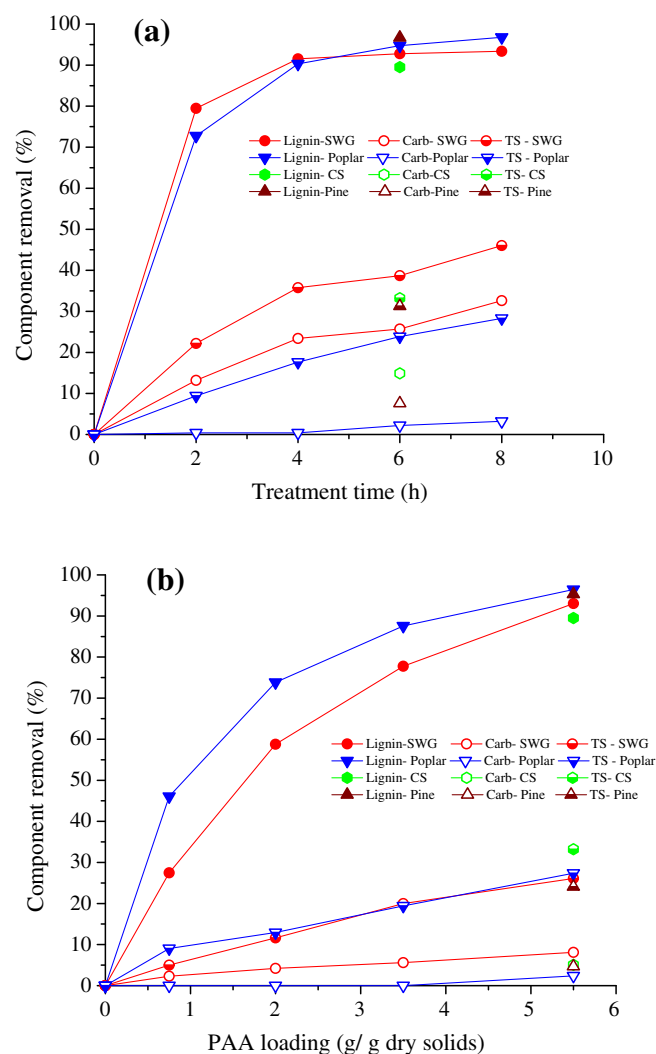
Fig. 1. Effect of reaction time for (a) sodium chlorite–acetic acid (SC/AA), and (b) reagent loadings for peracetic acid (PAA) delignification of switchgrass (SWG) and poplar lignin, carbohydrate (carb), and total solids (TS) removal. Also shown are the TS, carb, and lignin removal for corn stover (CS) and pine sawdust (PSD) for SC/AA delignification performed at 70 °C for 6 h and PAA delignification performed at 25 °C and 5.5 g PAA/g dry solids for 48 h. Note: For PAA delignification, the reaction time for all loadings was 24 h except at a loading of 5.5 g/g dry solids (48 h), unless noted otherwise.

the highest loading of 5.5 g PAA/g biomass resulted in the same amount of lignin removal for both feedstocks. Although the causes for such differences in delignification kinetics at low PAA loadings is unclear, it appears that different lignin structure and the higher ash content in SWG may contribute to such behavior (Sannigrahi et al., 2010b). A PAA loading of 5.5 g/g dry biomass and delignification time of 48 h appeared to be necessary to achieve >92% delignification for both feedstocks at 25°C. As shown in Table 1, solids following delignification of poplar and SWG at the highest PAA loading had about 1.2 wt.% and 2.1 wt.% K-lignin, respectively. For a PAA loading of 5.5 g/g dry biomass, shorter reaction times than 48 h were also run to evaluate the effect on delignification but resulted in less lignin removal. For example, SWG delignification at a PAA loading of 5.5 g/g dry biomass at 25 °C for 6 h and 24 h of reaction time resulted in only 53 wt.% and 88% lignin removal, respectively, compared to 94% achieved for 48 h reaction time (data not shown). The effects of other variables such as time, temperature, and PAA loadings, which could possibly affect kinetics, and their combinations were not explored in this study.

Table 1
Biomass yields following SC/AA and PAA delignification and composition of untreated and delignified switchgrass (SWG), poplar, corn stover (CS), and pine sawdust (PSD).

Substrate	Delig. method	Biomass yield$^\chi$, %	Components, avg. (%, dry basis)					Total mass, %
			Glucan	Xylan	Arab/Man*	Acetyl	K-lignin**	
Poplar	None	na	42.4	14.2	2.0	2.7	26.7	88.0
	SC/AA	76.2	55.6	17.1	2.5	3.1	1.7	80.0
	PAA	72.6	58.5	17.4	2.9	2.9	1.2	83.0
SWG	None	na	35.5	21.7	1.8	2.2	22.8	84.0
	SC/AA	61.3	46.9	22.5	2.3	1.9	2.5	76.1
	PAA	73.9	46.6	25.4	1.9	2.6	2.1	78.6
CS	None	na	37.4	22.8	3.2	nd	14.5	77.9
	SC/AA	66.8	51.5	25.4	3.8		2.3	82.9
	PAA	76.8	46.2	28.3	3.8		1.6	79.9
PSD	None	na	45.1	19.4$^\psi$	1.3		28.1	93.9
	SC/AA	68.7	65.0	22.3$^\psi$	1.1		1.3	89.7
	PAA	75.9	56.8	22.3$^\psi$	1.7		1.7	82.5

$^\chi$ Biomass yield = amount of dry biomass left after delignification (g)/initial dry biomass amount (g).
* For SWG, CS, and PSD, it represents arabinan, whereas for poplar-mannan; SC/AA-sodium chlorite–acetic acid delignification at 70 °C for 6 h with successive addition of 0.6 g sodium chlorite/g dry biomass and 0.6 ml acetic acid/g dry biomass every 2 h; PAA-peracetic acid delignification performed at 25 °C for 48 h at loading of 5.5 g PAA/g dry biomass.
** ash free-acid insoluble lignin; ψ-xylan + mannan + galactan; Delig., delignification; na, not applicable; nd, not determined.

Fig. 1b also shows that delignification performed at 25 °C with a PAA loading of 5.5 g/g dry biomass for 48 h on CS and PSD also resulted in >90% of lignin removal and similar amounts of total solids removal as SWG and poplar. The amount of carbohydrate lost for all substrates, even at the highest loading of 5.5 g/g dry solids, was <9%. The composition of the delignified solids is shown in Table 1. Although the amount of lignin removed by the PAA method at the highest PAA loading was more or less the same as for SC/AA, the amount of total solids, at least for SWG and CS, and carbohydrate removal were higher for SC/AA.

3.2. Lignin selectivity of SC/AA and PAA delignification methods

Lignin delignification selectivity was evaluated for untreated biomass and poplar pretreated by AFEX, CpH, lime, and SO$_2$ pretreatments. Pretreatments at lower pH such as SO$_2$ and CpH mostly remove hemicellulose and some lignin, whereas high pH pretreatments such as lime mostly remove lignin with some hemicellulose. However, AFEX is an exception in that it does not physically remove much of any components (Kumar et al., 2009; Mosier et al., 2005). For untreated poplar and SWG, selectivity was evaluated over reaction time for SC/AA and for different reagent loadings for PAA. However, for untreated CS, PSD, and pretreated poplar solids, selectivity was only determined for 6 h reaction time for SC/AA and 5.5 g PAA/g dry solids loading for PAA method.

3.2.1. For untreated (UT) biomass

Yields of biomass components including glucan, xylan, arabinan, mannan, acetyl groups, and lignin are shown in Fig. 2a and Fig. 2b for both poplar and SWG employing SC/AA and PAA delignification, respectively. SWG and poplar contained negligible amounts of mannan and arabinan, respectively; therefore, their yields were not determined.

Although lignin yields for both SWG and poplar over reaction time were similar for SC/AA delignification, Fig. 2a shows that yields of other SWG components including total solids were much lower than for poplar, as shown previously in Fig. 1. For example, after 2 h of reaction, the glucan, xylan, arabinan, acetyl, and lignin yields for SWG were 86.4%, 86.5%, 98.4%, 79.9%, and 20.5%, respectively, whereas for poplar, the corresponding yields were about 99.2% for glucan, 101% for xylan, 97.3% for mannan, 95.0% for acetyl, and 27.2% for lignin. As can be seen in Fig. 2a, component yields decreased with reaction time but were much lower for SWG than poplar. Component yields over a range of PAA loadings are shown

in Fig. 2b for SWG and poplar for reactions performed at 25 °C. It can be observed that PAA, irrespective of feedstock type, was more selective for lignin. Yields for the PAA method were greater than 90% for all components except acetyl even at the highest PAA loading of 5.5 g/g dry biomass, in agreement with data by Chang and Holtzapple for poplar (Chang and Holtzapple, 2000).

Fig. 3 summarizes percentage component removals for poplar, SWG, CS, and PSD following SC/AA and PAA delignification at conditions found to be optimum for maximum lignin removal in Section 3.2: (1) SC/AA at 70 °C for 6 h at NaClO$_2$ and glacial acetic acid loadings of 0.6 g/g dry biomass and 0.6 ml/g dry biomass, respectively (fresh dose every 2 h; total 3 dosages) and (2) PAA at 25 °C for 48 h with PAA loading of 5.5 g/g dry biomass. Biomass yields and compositions of untreated and delignified solids for SWG, poplar, CS, and PSD are shown in Table 1. Xylan is anhydrous xylose for SWG, poplar, and CS in Table 1 and Fig. 3; however, for PSD it represents xylan together with anhydrous mannose (mannan) plus anhydrous galactose (galactan) due to the inability of the Bio-Rad Aminex HPX-87H column to cleanly separate these three components. Both methods, irrespective of feedstock type, removed ⩾90% of the lignin, however, by comparing Fig. 3a and b, it can be seen that PAA removed less carbohydrates than SC/AA. For example, the SC/AA delignification method resulted in about 18% loss of glucan, 38% loss of xylan, and 30% loss of arabinan for SWG, whereas the PAA method lost less than 5%, 15%, and 20% of glucan, xylan, and arabinan, respectively. Similar trends were observed for other feedstocks as well, except poplar, which was more or less equally responsive to both delignification methods. Furthermore, SC/AA removed higher amounts of acetate groups than PAA (45% vs. 15%) for SWG, while for poplar, PAA removed more acetate groups than SC/AA (22% vs. 12%). Overall, it can be concluded that PAA delignification was more selective for lignin than SC/AA.

3.2.2. For pretreated solids

To evaluate the selectivity of lignin removal from pretreated solids, poplar solids prepared by the leading pretreatments of AFEX, CpH, Lime, and SO$_2$ were delignified by SC/AA and PAA methods at optimum conditions discussed previously. The percent removal of total solids, carbohydrate, and lignin for pretreated solids were compared to results with raw untreated (UT) poplar delignified at similar conditions. The biomass yields (the fraction of biomass recovered) and the composition of raw poplar and poplar pretreated by various pretreatments before and after delignification are summarized in Table 2. Following delignification,
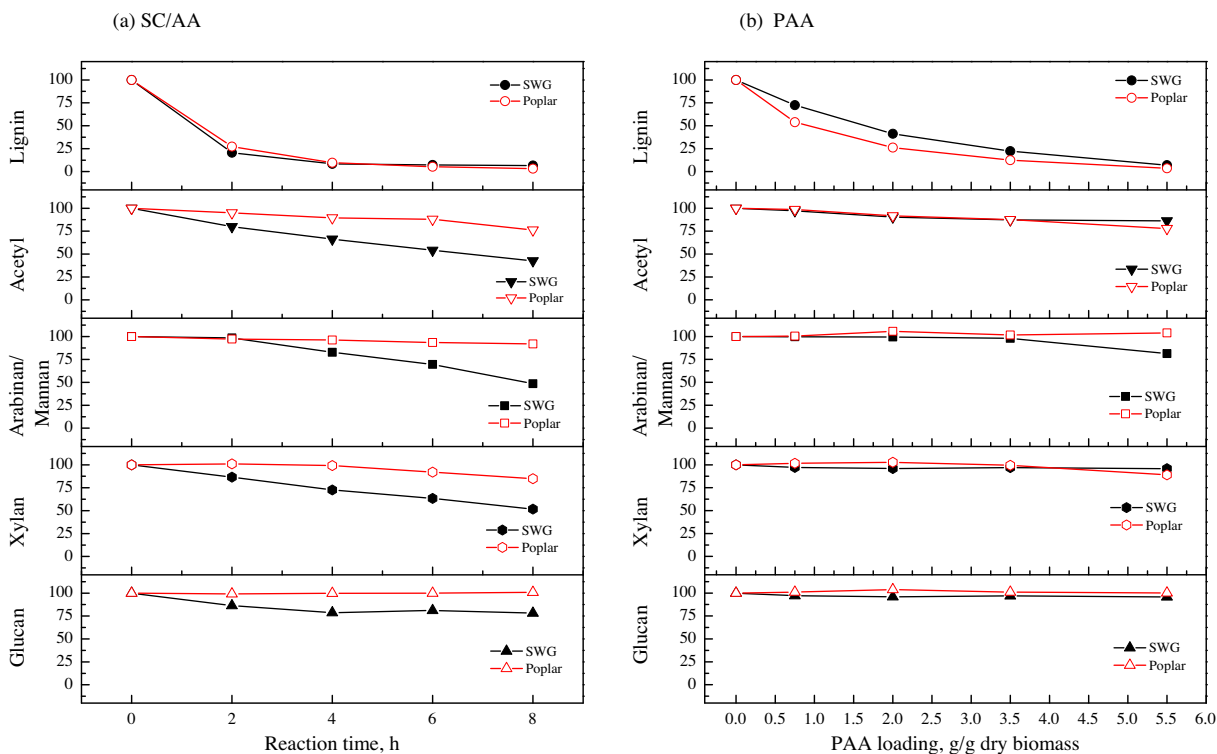
Fig. 2. Component yields (a) over reaction time for sodium chlorite–acetic acid (SC/AA) delignification, and (b) against PAA loadings for PAA delignification. Solid and open symbols are for SWG and poplar, respectively. Because the amounts of mannan in switchgrass (SWG) and arabinan in poplar were negligible, arabinan yield is for SWG and mannan yield is for poplar on the ordinate for third layer. For PAA delignification, the reaction time for all loadings was 24 h except at loading of 5.5 g/g dry solids (48 h), unless otherwise noted.
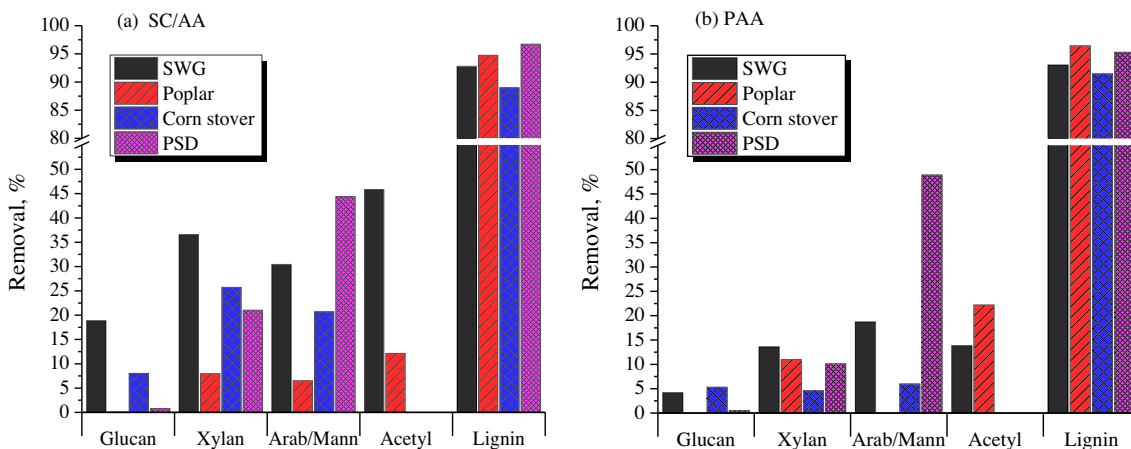
Fig. 3. Summary of biomass component removal (%) for switchgrass (SWG), poplar, corn stover (CS), and pine sawdust (PSD) following (a) sodium chlorite–acetic acid (SC/AA) delignification at 70 °C for 6 h, and (b) peracetic acid (PAA) delignification performed at 25 °C for 48 h. The effects on acetyl groups were not determined for CS and PSD. In the figures for SWG, poplar, and CS, xylan represents anhydrous xylose, whereas for PSD it represents xylan + mannan + galactan. Arab/Mann – represents arabinan for SWG, CS, and PSD, and mannan for poplar only. For SC/AA, a fresh charge of NaClO$_2$ (0.6 g/g dry biomass) and acetic acid (0.6 ml/g dry biomass) was added every 2 h. PAA delignification was performed at loading of 5.5 g/ g dry biomass. Arab, arabinan; Mann, mannan.

biomass yields from raw poplar were higher than yields from pretreated solids, and among pretreated solids, SO$_2$, irrespective of delignification method employed, had the lowest biomass yield followed by CpH.

Fig. 4 shows the component removal data for both delignification methods. Both delignification methods, irrespective of pretreatment, removed close to 95% of available lignin, which was comparable to that from raw poplar. However, removal of other components varied with pretreatment and was different from

raw poplar. The amount of total solids removed was higher for pretreated solids than raw biomass, which, most probably, was due to pretreatment loosening the biomass structure (Mosier et al., 2005; Yang and Wyman, 2008) and higher amounts of lignin in pretreated solids compared to raw biomass such as following SO$_2$ pretreatment. The amount of glucan removal following SC/AA delignification of all pretreated poplar solids was very low (< 4%) and similar to that from raw poplar. However, the amount of xylan and mannan removal increased and was pretreatment dependent.

Table 2
Biomass yields following SC/AA and PAA delignification and composition of untreated (UT) and variously pretreated poplar before and after SC/AA and PAA delignification.

Substrate	Delig. method	Biomass yield$^{\chi}$,%	Components, avg. (%, dry basis)				Total mass, %
			Glucan	Xylan	Mannan	K-lignin[*]	
UT poplar	None	na	42.4	14.2	2.0	26.7	85.3
	SC/AA	76.2	55.6	17.1	2.5	1.7	76.9
	PAA	72.6	58.5	17.4	2.9	1.2	80.0
AFEX poplar	None	na	50.0	14.8	2.8	20.5	88.0
	SC/AA	67.7	74.8	19.9	3.4	1.1	99.2
	PAA	66.5	73.0	18.6	3.1	0.2	95.0
CpH poplar	None	na	56.7	8.7	2.3	31.2	98.9
	SC/AA	61.4	89.0	8.7	3.0	0.2	101.0
	PAA	62.5	84.5	7.2	2.7	1.1	95.6
Lime poplar	None	na	52.0	15.2	1.5	22.3	91.0
	SC/AA	69.0	74.9	20.8	2.0	0.7	98.4
	PAA	69.3	72.5	18.9	1.8	0.4	93.6
SO2 poplar	None	na	54.0	9.3	1.7	28.2	93.3
	SC/AA	57.2	96.4	5.2	1.1	3.7	106.4
	PAA	56.0	91.6	4.1	1.0	0.5	97.2

$^{\chi}$ Biomass yield = amount of dry biomass left after delignification (g)/ initial dry biomass amount (g); SC/AA-sodium chlorite–acetic acid delignification performed at 70 °C for 6 h with successive addition of 0.6 g sodium chlorite/g dry biomass and 0.6 ml acetic acid/g dry biomass every 2 h; PAA-peracetic acid delignification performed at 25 °C for 48 h at loading of 5.5 g PAA/g dry biomass.
[*] ash free-acid insoluble lignin; Delig., delignification; na, not applicable; AFEX, ammonia fiber expansion; CpH, controlled pH; SO$_2$, sulfur dioxide.
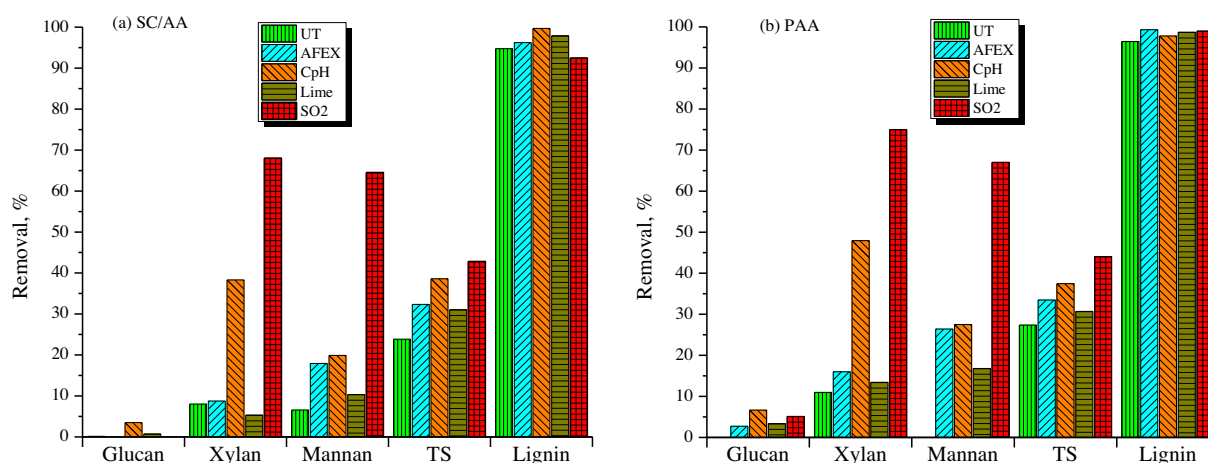
Fig. 4. Summary of biomass component removal (%) for AFEX, CpH, lime, and SO$_2$ pretreated poplar, and comparison with UT poplar following (a) sodium chlorite–acetic acid (SC/AA) delignification at 70 °C for 6 h, and (b) peracetic acid (PAA) delignification at 25 °C for 48 h. The effect on acetyl groups was not determined. For SC/AA, a fresh charge of NaClO$_2$ (0.6 g/g dry biomass) and acetic acid (0.6 ml/g dry biomass) was added every 2 h. PAA delignification was performed at 25 °C at a loading of 5.5 g/g dry biomass for 48 h. UT, untreated; AFEX, ammonia fiber expansion; CpH, controlled pH; SO$_2$, sulfur dioxide; TS, total solids.

For example, as shown in Fig. 4a following SC/AA delignification, raw poplar and AFEX pretreated solids had similar amounts of xylan removal (~9%), but CpH and SO$_2$ pretreated solids lost about 39% and 68% of xylan, respectively, and about 20% and 64.5% of mannan, respectively, that was initially available in the pretreated solids. A possible hypothesis to explain this result, other than attributing it to the disturbed structure of pretreated biomass, could be that during thermochemical pretreatments, such as uncatalyzed steam explosion, low-severity dilute acid pretreatment, and CpH (also known as liquid hot water and hydrothermal) hemicelluloses partly solubilize in the solution and precipitate back on the cellulose micro-fibrils upon cooling, which is different than the structural hemicellulose, and can be easily removed after treatments such as SC/AA delignification.

Similar to the SC/AA method, Fig. 4b shows that the lignin selectivity by the PAA method was also pretreatment dependent. However, the PAA delignification method surprisingly removed higher amounts of carbohydrate from pretreated solids than SC/AA, quite contrary to results seen earlier for raw UT biomass. For example, the PAA method essentially doubled xylan removal from AFEX

pretreated solids compared to SC/AA (8% vs. 16%) and was about 48% for CpH pretreated solids (39% for SC/AA). Similar increases in carbohydrate removal were observed for other pretreated solids as well.

Although the clear reason for such behavior is not yet known, it can be hypothesized that the lower reaction pH for PAA delignification (pH ~1 for PAA compared to 3–4 for SC/AA) combined with disturbed biomass structure could promote higher carbohydrates removal from pretreated solids. Although this study only developed data for poplar prepared by few leading pretreatments, it is believed that the behavior of these delignification methods would change with feedstock type and pretreatment. Therefore, caution should be taken in interpreting (structural and/or enzymatic hydrolysis) data from delignification by these methods.

3.3. Effects of delignification on cellulose molecular structure

3.3.1. Cellulose molecular weight and degree of polymerization

The effect of SC/AA and PAA delignification on cellulose molecular structure for SWG and poplar was determined by GPC, as

Table 3
Number-average (M_n) and weight-average (M_W) molecular weight, degree of polymerization (DP), and polydispersity index (PDI) of cellulose in raw and SC/AA[¥] and PAA[§] delignified switchgrass and poplar.

Substrate	Delig. method	M_n (g/mol)	DP_n	M_W (g/mol)	DP_w	PDI (M_W/M_n)
Switchgrass	None	224,000	432	1650,000	3180	7.4
	PAA	195,000	376	1410,000	2720	7.2
	SC/AA	135,000	260	993,000	1910	7.4
Poplar	None	153,000	295	1240,000	2390	8.1
	PAA	116,000	224	998,000	1920	8.6
	SC/AA	114,000	220	894,000	1720	7.8

SC/AA, sodium chlorite–acetic acid; PAA, peracetic acid; Delig., delignification; DP_n, number-average degree of polymerization; DP_w, weight-average degree of polymerization.
[¥] SC/AA delignification was performed at 70 °C for 6 h with fresh charges of sodium chlorite (0.6 g/g dry biomass) and acetic acid (0.6 ml/g dry biomass) added every 2 h.
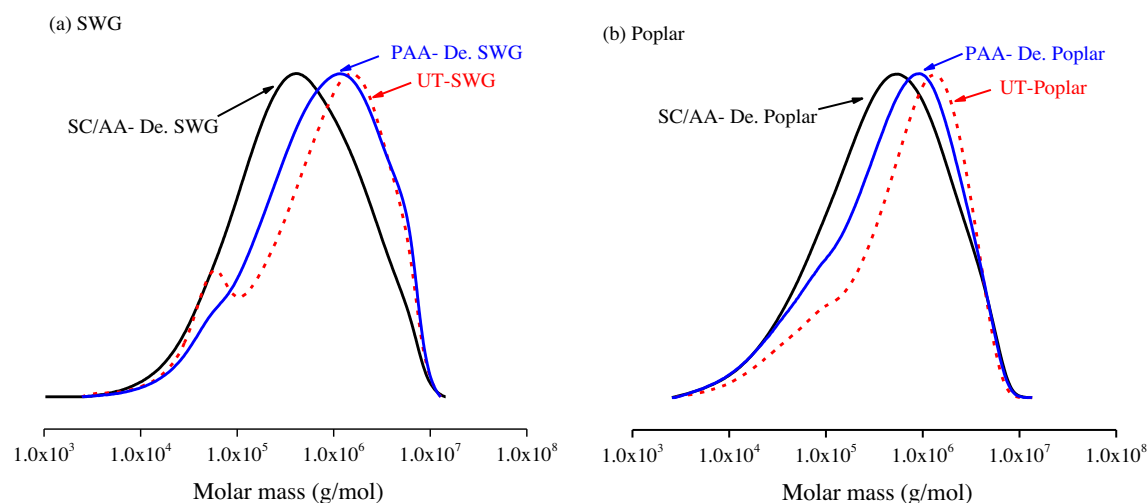[§] PAA delignification was performed at 25 °C for 48 h with peracetic acid at a loading of 5.5 g/g biomass.

described in the Materials and Methods. The number-average and weight-average molecular weight, degree of polymerization, and polydispersity index values for cellulose isolated from untreated and delignified SWG and poplar are presented in Table 3. Data for delignified samples is only shown at conditions shown to be optimum earlier, i.e., delignification at 70 °C for 6 h for SC/AA and at 25 °C for 48 h for PAA at loading of 5.5 g PAA/g solids. Table 3 shows that cellulose in SWG had a higher molecular weight than in poplar. However, as shown in Fig. 5a for poplar and Fig. 5b for SWG, cellulose molecular weight decreased following delignification by both methods. Delignification employing peracetic acid appeared to degrade the cellulose chains less than the SC/AA method. Table 3, for instance, shows a decrease in cellulose number average molecular weight (M_w) following SC/AA delignification of about 40% (from 1.65×10^6 g/mol to 0.99×10^6 g/mol) for SWG and 28% (from 1.24×10^6 g/mol to 0.89×10^6 g/mol) for poplar. Whereas, employing PAA delignification, the decrease in cellulose molecular weight was about 15% (from 1.65×10^6 g/mol to 1.4×10^6 g/mol) and 20% (from 1.24×10^6 g/mol to 0.99×10^6 g/mol) for SWG and poplar, respectively. Hubbell and Ragauskas, employing the SC/AA method at similar conditions to this study, reported similar results for cellulose model compounds such as microcrystalline cellulose and filter paper. In their study, lignin in varying amounts was deposited on these model compounds to simulate real biomass, and then SC/AA delignification was performed. The effect on cellulose molecular weight, however, varied with substrate and lignin amount (Hubbell and Ragauskas, 2010)

in that filter paper had a significantly greater MW reduction upon SC/AA delignification than microcrystalline Avicel cellulose, and samples that had significant amounts of lignin were less prone to cellulose MW change. In another study from the same lab, such changes in cotton cellulose molecular weight upon SC/AA delignification, although at a bit different chemical loadings, were also reported (Foston et al., 2011). However, these studies concluded that SC/AA delignification would have minimal impact on cellulose MW in the presence of lignin. Although it appears that the amorphous cellulose content and level-off degree of polymerization (LODP) might play a role in determining the impacts of delignification on cellulose MW, this study indicated that SC/AA delignification had a significant impact on cellulose MW.

3.3.2. Cellulose reducing ends

Cellobiohydrolase I (CBH I; Cel7A) makes a major fraction of *T. reesie* derived cellulase preparations and hydrolyzes cellulose in a processive manner from its reducing ends (Teeri et al., 1995). Therefore, reducing ends are presumed to play a vital role in enzymatic hydrolysis of cellulose. Since sodium chlorite and peracetic acid both are strong oxidants and can affect cellulose reducing ends, the effect of SC/AA and PAA treatment on cellulose reducing ends was evaluated for the three cellulose model compounds: Avicel, cotton linter, and α-cellulose. As determined by the NREL standard protocol, Avicel and cotton linter both were composed entirely of glucan, whereas, α-cellulose contained about 82.7 wt.% glucan and the rest xylan. These substrates were

Fig. 5. Effect of sodium chlorite–acetic acid (SC/AA) and peracetic acid (PAA) delignification on cellulose molecular weight distributions for (a) SWG, and (b) poplar. Delignification employing SC/AA and PAA methods was performed at 70 °C for 6 h and at 25 °C for 48 h, respectively. For SC/AA, a fresh charge of NaClO₂ (0.6 g/g dry biomass) and acetic acid (0.6 ml/g dry biomass) was added every 2 h. PAA delignification was performed at 25 °C with loading of 5.5 g/g dry biomass for 48 h. SWG, switchgrass; De, delignified.

Table 4
Effect of SC/AA and PAA delignification on Avicel, cotton linter, and α-cellulose reducing ends.

Substrate	Delignification method	Reducing ends (μmoles/g solids)	$DP_{N,r}$
Avicel	None	$26.67 \pm 1.06^{\Omega}$	231 ± 9
	PAA	27.60 ± 1.65	224 ± 13
	SC/AA	11.07 ± 1.94	566 ± 98
Cotton linter	None	17.55 ± 0.93	352 ± 18
	PAA	15.74 ± 0.62	392 ± 15
	SC/AA	6.31 ± 0.16	978 ± 24
α-cellulose	None	5.72 ± 0.21	1079 ± 39
	PAA	8.35 ± 0.45	740 ± 39
	SC/AA	9.33 ± 1.05	665 ± 73

SC/AA-sodium chlorite–acetic acid delignification was performed at 70 °C for 6 h with fresh charges of sodium chlorite (0.6 g/g dry biomass) and acetic acid (0.6 ml/g dry biomass) added every 2 h; PAA-peracetic acid delignification was performed at 25 °C for 48 h with PAA loading of 5.5 g/g dry solids; $DP_{N,r}$-number average degree of polymerization calculated based on number of reducing ends and glucose moles (6.17 mmoles) per g cellulose (Zhang and Lynd, 2005).
$^{\Omega}$ Standard deviation of quadruplet samples.

Table 5
The cellulose crystallinity index (CrI) of various samples determined by NMR and FT-IR.

Substrate	Delig. Method	CrI (NMR)	CrI (FT-IR)
Avicel	None	52	52
	SC/AA	55	55
	PAA	57	55
Switchgrass	SC/AA	53	50
	PAA	52	41
Pine Saw dust	SC/AA	61	52
	PAA	48	50

SC/AA-sodium chlorite–acetic acid delignification was performed at 70 °C for 6 h with fresh charges of sodium chlorite (0.6 g/g dry biomass) and acetic acid (0.6 ml/g dry biomass) added every 2 h; PAA-peracetic acid delignification was performed at 25 °C for 48 h with PAA loading of 5.5 g/g dry solids.

subjected to delignification by SC/AA (70 °C for 6 h with 0.6 g/g $NaClO_2$ and 0.6 ml acetic acid/g solids every 2 h; total three doses) and PAA (5.5 g PAA/g dry solids for 48 h at 25 °C). Then, the total number of reducing ends was determined by employing the modified BCA method (Zhang and Lynd, 2005). The reducing ends and reducing ends based cellulose degree of polymerization ($DP_{N,r}$), calculated by dividing the number of glycosyl residues per g cellulose (6.17 mmoles/g cellulose) by the total number of reducing ends, are presented in Table 4 for untreated and treated samples. Avicel cellulose had the highest number of reducing ends per unit weight (26.67 μmoles) followed by cotton linters (17.55 μmoles) and α-cellulose (5.72 μmoles). Consequently, the Avicel cellulose chain length was the shortest of the three cellulose compounds ($DP_{N,r}$; 231 ± 9) followed by cotton linters (352 ± 18) and α-cellulose (1079 ± 39). As shown in Table 4, peracetic acid delignification had no impact on cellulose reducing ends for cotton linters and Avicel; however, SC/AA oxidized more than 50% of the reducing ends available in both substrates. α-cellulose, on the other hand, had a higher number of reducing ends per unit weight after delignification by both methods than before. This result could be due to interference in the BCA reducing ends measurement by xylan present in α-cellulose and by cellulose chains fragmentation. The latter agrees with results in the previous section that these delignification methods alter cellulose molecular structure by fragmenting cellulose chains, with PAA again having a milder impact than SC/AA. The data here suggest that applying these delignification methods, reducing ends generation through chain scission and their oxidation, especially for SC/AA, simultaneously take place. However, it seems that reducing end generation through cellulose fragmentation was limited for Avicel and cotton linters due to their DP close to LODP (Håkansson and Ahlgren, 2005).

3.3.3. Crystallinity index by CP/MAS ^{13}C NMR and FT-IR analysis

In order to evaluate the impact of SC/AA and PAA delignification on cellulose crystallinity index (CrI), ^{13}C CP/MAS NMR analysis were conducted to determine the relative area of the cellulose ultra-structural components (i.e., cellulose I_α and I_β, para-crystalline cellulose and cellulose at accessible and inaccessible surface). Fig. S1 (Supplementary material) shows the representative NMR spectrum for untreated (Fig. S1a), SC/AA treated (Fig. S1b), and PAA treated (Fig. S1c) Avicel cellulose. Each of the six carbon atoms in the monomeric unit of the cellulose backbone are denoted by C_1 through C_6 labeled accordingly to the corresponding carbon signal in the spectrum, Fig. S1a. The C_4 region is commonly used to probe crystalline forms of cellulose (cellulose $I_{\alpha+\beta}$, I_α, and I_β) and

para-crystalline domains in the δ 86.0–92.0 ppm region; and less ordered or non-crystalline domains between δ 80.0 and 86.0 ppm (Atalla and VanderHart, 1984). Lorentzian line-shapes were applied to the carbon signals attributed to domains of cellulose $I_{\alpha+\beta}$, I_α, I_β, and para-crystalline cellulose, while Gaussian lines were used to describe the signals from inaccessible and accessible fibril surfaces comprising the amorphous domains. The ratio of the area in the δ 86.0–92.0 ppm region to the total peak area from δ 80.0–92.0 ppm is designated as the crystallinity index (CrI) (Sannigrahi et al., 2010a). CrI values for various samples following SC/AA and PAA delignification are shown in Table 5. For pure Avicel cellulose, both treatment methods had a negligible impact on CrI. However, for biomass samples, cellulose crystallinity appeared to increase with glucan removal by the SC/AA treatment. Although these values are not absolute as the hemicellulose removal procedure may have different impacts on delignified samples prepared by SC/AA and PAA, these values show the comparative impact of SC/AA and PAA delignification on cellulose CrI. To further verify the impact of delignification on cellulose CrI, FT-IR characterization was performed on pure cellulose and delignified biomass samples. The peak heights ratios at 1372 and 2900 cm^{-1} (H1372/H2900, C–H bending/C–H stretching) in the FT-IR spectra were used to determine cellulose crystallinity (Monrroy et al., 2011), with data shown in Table 5. The CrI values determined by FT-IR agree fairly well with NMR CrI values and show that SC/AA delignification may enhance cellulose crystallinity, however, again dependent on the biomass type.

4. Conclusions

SC/AA and PAA delignification applied to a range cellulosic biomass types removed >90% of lignin, but selectivity varied. For UT biomass, PAA was more selective than SC/AA. However, neither was selective for pretreated solids, and selectivity varied with pretreatment type. Characterization data showed that the SC/AA had a more pronounced impact on cellulose molecular structure than PAA. Thus, it can be concluded that PAA delignification is more selective than SC/AA and had less severe impacts on cellulose structure. However, for pretreated solids, the data obtained must be interpreted carefully as both methods removed significant amounts of hemicellulose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.12.028.

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