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ORIGINAL PAPER

Influence of ionic-liquid incubation temperature on changes in cellulose structure, biomass composition, and enzymatic digestibility

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Abstract Varying ionic liquid, 1-ethyl 3-methyl imidazolium acetate, pretreatment incubation temperature on lignocellulosic biomass substrates, corn stover, switchgrass and poplar, can have dramatic effects on the enzymatic digestibility of the resultant regenerated biomass. In order to delineate the chemical and physical changes resulting from the pretreatment process and correlate changes with enzymatic digestibility, X-ray powder and fiber diffraction, ^{13}C cross polarization/magic angle spinning nuclear magnetic resonance spectroscopy, and compositional analysis was completed on poplar, corn stover and switchgrass samples. Optimal pretreatment incubation temperatures were most closely associated with the retention of amorphous substrates upon drying of regenerated biomass. Maximal glucan to glucose conversion for 24 h enzyme hydrolysis was observed for corn stover, switchgrass and poplar at ionic liquid incubation temperatures of 100, 110 and 120 °C, respectively. We hypothesize that effective pretreatment temperatures must attain lignin redistribution and retention of xylan for optimal enzyme digestibility.

Keywords Cellulose structure · Ionic liquid pretreatment · Mercerization · Enzyme digestibility

Introduction

Lignocellulosic biomass is an abundant carbon source which can provide a renewable platform for carbon based fuels and chemicals and unlike corn, wheat or

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soy is not a food source. Lignocellulose is comprised of three major components: cellulose, a polymer of glucose monomeric units (30–55 %); hemicellulose, a highly substituted polysaccharide mixture of pentose and hexose sugars (8–25 %); and lignin (10–35 %), a macromolecule consisting of phenyl propanoid subunits (Zhao et al. 2012). The cellulose component is highly crystalline whereas the hemicellulosic and lignin components are less structured. The hydrolysis of the polysaccharide fractions, cellulose and hemicellulose, to monomeric sugars forms a sugar platform for production of fuels and value-added chemicals through fermentation or chemical conversion. Monomeric sugars can be produced through a two-step biochemical process consisting of biomass pretreatment followed by enzymatic hydrolysis. Pretreatment of the biomass produces a more readily hydrolyzed substrate while enzymatic deconstruction of polysaccharides to monomeric sugars avoids the formation of sugar decomposition products that can be inhibitory to downstream fermentation steps. Structural and chemical changes in biomass during pretreatment have a profound effect on enzyme digestibility and downstream processing.

Lignocellulosic structure with highly crystalline cellulose imbedded in a matrix of less structured hemicellulose covalently cross linked with hydrophobic lignin creates a major barrier to conversion of polysaccharides to monomeric sugars. This is due largely to the inaccessibility of cellulose to water and enzymes. Properties of biomass that affect recalcitrance to enzyme hydrolysis include cellulose crystal morphology and crystallinity (Samayam et al. 2011; Cheng et al. 2011; Zhu et al. 2008; Puri 1984), lignin and hemicellulose content (Zhu et al. 2008; Selig et al. 2009; Bura et al. 2009), and particle size (Yeh et al. 2010). A number of chemical pretreatment technologies have been developed to counteract the natural recalcitrance of biomass to deconstruction. Some pretreatment technologies (i.e. acid pretreatment) can lead to the degradation of hemicellulose (Torget et al. 1988) resulting in the substantial loss of carbon and the formation of fermentation inhibitors (Chheda et al. 2007) as well as retention or enhancement of cellulose crystallinity (Pingali et al. 2010a, b).

Ionic liquid (IL) pretreatment is an alternative process that can largely retain hemicellulose and convert the native cellulose I allomorph to cellulose II or an amorphous substrate without the production of inhibitors, enabling greater access of polysaccharides for enzymatic deconstruction (Dadi et al. 2006; Li et al. 2010; Wang et al. 2011; Samayam and Schall 2010; Samayam et al. 2011). The ease of cellulose deconstruction is dependent on its structure. Amorphous cellulose is the most easily hydrolyzed followed by cellulose II (mercerized cellulose) then cellulose I (Wada et al. 2010; Samayam and Schall 2010). The transition from cellulose I to cellulose II has been found to be dependent upon IL treatment temperature and appears to vary with specific biomass feedstocks (Samayam et al. 2011; Cheng et al. 2011). It has been suggested that mercerization is furthered when lignin is redistributed as its glass transition temperature is approached (Wormald et al. 1996; Samayam et al. 2011). Lignin glass transition temperature is feedstock dependent and has been reported to range between 100 and 160 °C (Arora et al. 2010; Hatakeyama et al. 1982; Irvine 1985; Selig et al. 2007).

In order to systematically explore the dependecy of biomass structural changes with IL treatment temperature, a hardwood (poplar) and herbaceous (corn stover and switchgrass) substrates were pretreated at incubation temperatures ranging from 75 to 140 °C with the IL 1-ethyl-3-methyl imdizaolium acetate (EMIM-OAc) followed by IL displacement with water. Characteristics of the IL treated biomass were analyzed including cellulose crystallinity and morphology, composition, and enzymatic digestibility. Structural changes in cellulose were assessed using X-ray powder diffraction (XRD), X-ray fiber diffraction, and solid state cross polarization—magic angle spin ^{13}C (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy (Isogai et al. 1989; Park et al. 2009; Samayam et al. 2011). The enzyme digestibility of the pre-treated substrates was also assessed in order to identify optimal pretreatment temperatures for each substrate.

Materials and methods

Materials

1-ethyl 3-methyl imidazolium acetate (EMIM-OAc) was purchased from Sigma Aldrich (St. Louis, MO). Three different biomass sources were investigated: poplar, corn stover and switchgrass. Poplar samples were provided by the National Renewable Energy

Laboratory (Golden, CO). Corn stover and switchgrass samples were provided by the United States Department of Agriculture (USDA, NCAUR Laboratory, Peoria, IL). Commercial enzyme mixture, Cellic CTec2, was obtained from Novozymes North America, Inc. (Franklinton, NC).

Methods

X-ray powder diffraction

Biomass samples were milled, sieved (−80/+20), and dried at ~45 °C in a vacuum oven. Biomass was added to EMIM-OAc at a biomass to ionic liquid ratio of 5 % (wt. biomass/wt. IL). The biomass samples were incubated at constant temperature ranging from 75 to 140 °C for 4 h with mixing. After the 4 h incubation, the biomass samples were washed with deionized water and centrifuged in an Eppendorf centrifuge (model 5810R) with a swinging bucket rotor at 4,000 rpm (3,220×*g*) for 10 min. The supernatant was removed and the washing process was repeated until the supernatant appeared visually colorless. Pretreated biomass was vacuum-filtered and dried at ~45 °C in a vacuum oven. Before XRD or CP/MAS NMR, biomass samples were ground to powder in a mortar and pestle. The resulting powder was analyzed on an X'pert powder diffractometer PAN 188 analytical with Cu Kα radiation and X'celerator detector with a range of 5°–38° and step size of 0.0334°.

X-ray fiber diffraction

Biomass samples cut into very fine splinters were fully immersed in EMIM-OAc in a glass vial and capped with a septum lid. The biomass samples were incubated followed by IL displacement with water as described above. The samples were then pressed between two microscope slides and placed into a desiccator to dry. A sample of dried biomass was mounted and X-ray diffraction data were collected at room temperature on a Rigaku FR-E diffractometer with copper Kα radiation and an R-Axis IV++ detector. Data were also collected at the Advanced Photon Source at Argonne National Laboratory, BioCARS BM-14-C, with an ADSC Q316 detector with a wavelength of 0.979 Å. Diffraction data from native and mercerized ramie fibers, cellulose I and cellulose II, respectively, were used to guide interpretation of diffraction data. Preparation of cellulose II is described in a previous publication (Samayam et al. 2011).

Solid state ^{13}C CP/MAS NMR

Solid state ^{13}C CP/MAS NMR spectra were collected on a Varian Unity Inova 600 MHz NMR with a magnetic field of 600 MHz and a ^{13}C frequency of 150.84 Hz with a CP/MAS unit at room temperature. Data were collected at a spinning rate of 10 kHz and a contact time of 10 ms. The spectrum was collected for 215 transients.

Commercial enzyme characterization

The protein concentration of Cellic CTec2 was determined using the colorimetric Bradford protein assay with bovine serum albumin standards (Bradford 1976). The rate of hydrolysis of a filter paper substrate was measured via a 2,5-dinitrosalicylic acid assay and expressed as filter paper units, FPU (Miller 1959). The enzyme activity of Cellic CTec2 was also determined using other specific cellulosic and xylan substrates with measurement of release of soluble reducing sugars via a Nelson-Somogyi assay (Nelson 1944). Endoglucanase activity was measured using a carboxymethyl cellulose (CMC) substrate. Cellobiohydrolase activity was measured using crystalline cellulose (Avicel) and endoxylanase activity was measured using xylan (birchwood xylan). One unit of activity, IU, is the release one μmol of soluble sugar reducing ends per minute. β-glucosidase and β-xylosidase activity were measured by the release of 4-nitrophenol from p-nitrophenyl-glucopyranoside and p-nitrophenyl-xylopyranoside, respectively (Gilead and Shoham 1995). For the PNP assay, one unit of activity, IU, is the release one μmol of p-nitrophenol per minute.

Enzymatic hydrolysis

Cellic CTec2 (Novozymes) was added to the substrate at loadings of 5 FPU/g glucan (4 mg/g glucan) for poplar and 7 FPU/g glucan (5 mg/g glucan) for corn stover and switchgrass based on untreated substrate composition. These loadings correspond to an endoglucanase activity loading of 150–200 IU/g glucan. FPU and specific activities of Cellic CTec2 was used to hydrolyze well defined substrates as described

previously and are summarized in Supplemental Table 1 (Samayam and Schall 2010; Barr et al. 2012).

Biomass was hydrolyzed in 50 mM citric acid buffer, pH 4.8, at 50 °C with a substrate loading of 1 % (w/v) with orbital shaking for 24 h. A supernatant sample was incubated at 100 °C for 5 min to stop the enzymatic hydrolysis. The released sugars were analyzed by high performance liquid chromatography (HPLC) with refractive index detection. Sugars were separated using a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis column with an isocratic mobile phase of HPLC-grade water at a flow rate of 0.6 mL/min and a column temperature of 80 °C. Mixed sugar standards of known concentrations were used to generate standard curves to calculate the concentration of released sugars. Glucan and xylan converted to glucose and xylose, respectively, were reported as a percentage of theoretical yields of monomeric sugars based on glucan and xylan composition and recovery of pretreated substrates.

Compositional analysis

Carbohydrate and lignin content in the untreated and pretreated biomass were determined in triplicate using concentrated acid hydrolysis followed by dilute acid hydrolysis according to the standard laboratory analytical procedures developed by the National Renewable Energy Laboratory (NREL) standard LAP 002 protocol (Sluiter et al. 2008). Monomeric sugars were quantified by HPLC as described above.

Results and discussions

Crystallinity and crystal polymorph

Cellulose crystallinity can be estimated via a crystallinity index, CrI, from X-ray powder diffraction (XRD) data (Segal et al. 1959) (Table 1). All IL treated biomass substrates exhibit reduced crystallinity compared to the native substrates (~70 % reduction for poplar and 58 and 44 % reductions for corn stover and switchgrass, respectively). As the incubation temperature increases, the crystallinity decreases to minimum then increases at higher temperatures. The CrI temperature minimum varies from substrate to substrate and occurs at ~100 °C for poplar, 75 to 100 °C for corn stover and 110 °C for switchgrass.

Cellulose I (native substrates) XRD data exhibit an intense peak at $2\theta \sim 22.6°$ corresponding to Miller index of 200 and a broad peak at ~15.5° comprised of

Table 1 Sugar losses, composition, and crystallinity indices of native and recrystallized substrates (N/A = not applicable)

Substrate	Temperature (°C)	Crystallinity Index (CrI)	Solid losses (% w/w)	Percent glucan	Percent xylan	Percent lignin	Glucan losses	Xylan losses	Lignin losses
Poplar	Native	52	N/A	43 ± 1	13 ± 0	26 ± 1	N/A	N/A	N/A
	75	16	4 ± 1	41	12	26	7	11	4
	100	14	6 ± 0	43	13	26	6	8	6
	120	17	13 ± 2	46	12	27	7	22	10
	140	20	24 ± 5	55	5	25	2	72	27
Corn Stover	Native	33	N/A	34	18	15	N/A	N/A	N/A
	75	14	18 ± 1	37 ± 2	20 ± 1	15	11	4	18
	85	12	17 ± 2	38	21	16	7	−3	11
	100	13	18 ± 5	38	19	15	8	8	18
	120	21	36 ± 6	43 ± 4	16 ± 3	18	19	40	23
Switchgrass	Native	36	N/A	32 ± 0	19 ± 1	20 ± 1	N/A	N/A	N/A
	75	20	17 ± 1	36 ± 2	20 ± 1	19	7	13	21
	110	10	24 ± 5	40	18	18	5	28	32
	120	25	36 ± 3	36 ± 8	15 ± 1	26 ± 7	28	49	17
	140	26	45 ± 8	48	6	22	18	83	40

Weight percent solid losses are the difference in solids weight before pretreatment and after recrystallization. Samples with standard deviation were completed in triplicate while those without standard deviation were completed in duplicates with <13 % deviation

a doublet peaks corresponding to Miller indices of 1–10 and 110, at 14.8° and 16.3° respectively. This doublet is more clearly seen near the beam center in fiber diffraction of untreated substrates and ramie controls (Supplemental Figure 1). In XRD data, the appearance of cellulose II is characterized by a broad XRD peak at ~20° formed by a doublet of peaks at 19.8° and 22.6° for Miller indices 110 and 020, respectively, and a singlet at a lower 2θ of ~12.1° for 1–10 (Samayam et al. 2011; Isogai et al. 1989). These diffraction features are again more clearly seen in fiber data with a singlet near the beam center and a doublet measured further from the beam center for mercerized ramie controls. A shift of XRD peaks from 2θ ~ 22° to ~20°, and 2θ from 15 to 12° in native and IL treated substrates is more apparent with increasing IL incubation temperature (Fig. 1).

X-ray fiber data can often reveal finer structural details from diffraction. (Supplemental Figures 2–4). Poplar and corn stover fiber samples retain diffraction features consistent with cellulose I at IL incubation temperatures up to 75 °C particularly in the low angle reflections (near the beam center). However at an IL pretreatment temperature of 85 °C, corn stover loses much of the native diffraction features although a broad band of diffraction intensity corresponds more closely with cellulose I than cellulose II. In all samples, as temperatures increase the biomass appears largely amorphous with weak diffraction features. Overall, the fiber diffraction features appear weakest at an incubation temperature of 100 °C for poplar, at 85 °C for corn stover and 110 °C for switchgrass. However, once pretreatment temperatures are 120 °C and higher, diffraction features reappear, indicating increasing order. This is seen in the increase in intensity in the reflections closer to the beam center. We interpret these features as corresponding most closely with cellulose II for all substrates.

While weaker diffraction features are more apparent in fiber data compared to XRD, it is still difficult to identify transitions from cellulose I to cellulose II in the limited crystalline and largely amorphous IL treated substrates. Solid-state ^{13}C CP/MAS NMR was used to further probe for the presence of cellulose I and cellulose II polymorphs. There are differences in the chemical shifts of the C4 and C6 carbons in the transition from cellulose I and cellulose II from 89.1–89.8 ppm to 88.7–88.8 ppm and from 65.5–66.2 ppm to 63.5–64.1 ppm, respectively (Isogai et al. 1989). Additionally, a small peak near 98 ppm

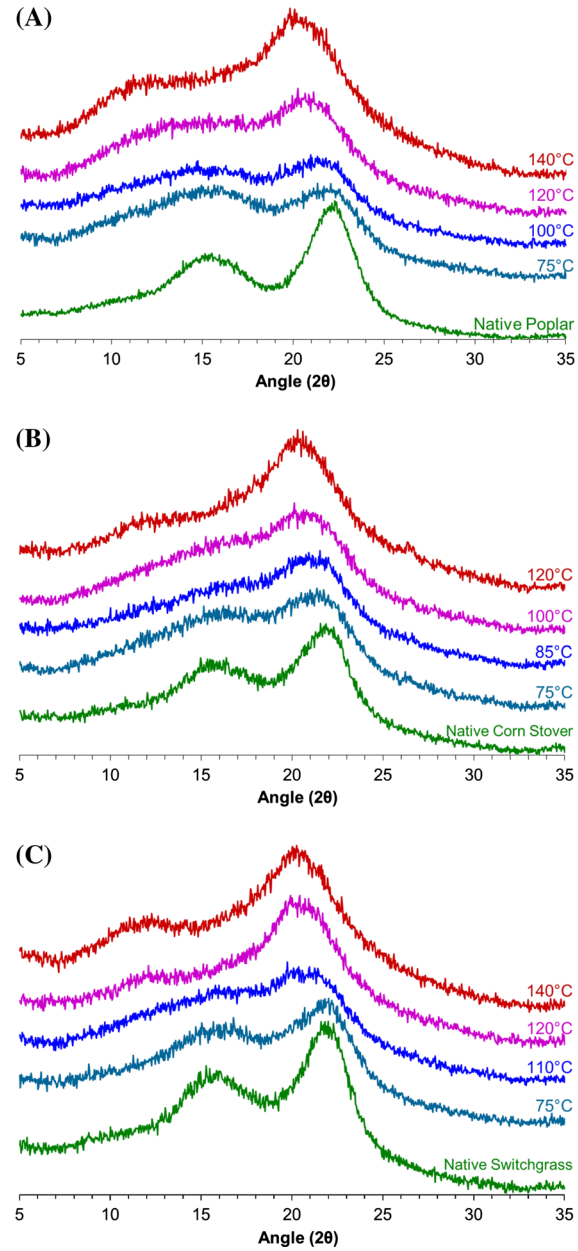

Fig. 1 X-ray powder diffraction patterns of native and IL-treated **a** poplar, **b** corn stover, and **c** switchgrass incubated in IL at the noted temperatures. Samples were dried before measurement of diffraction. XRD patterns indicate a conversion from cellulose I (native substrate) on the bottom progressing to cellulose II at the top (increasing IL treatment temperatures)

appears in cellulose II, attributed to "the α-type anomeric C1 carbon of the reducing end glucose residue in the cellulose oligomers" (Isogai et al. 1989). These chemical shifts can be observed in cellulose I and cellulose II standards (Supplemental Figure 5).

Native biomass NMR spectra are shown with positions of the aforementioned key chemical shifts for cellulose I and II (Fig. 2). For all IL incubation temperatures the resulting biomass spectra appears to display a reduction in cellulose I features and a gradual increase in cellulose II features with increasing temperature. Chemical shifts associated with cellulose II appear to become dominant between 100 and 120 °C for poplar, between 75 and 100 °C for corn stover, and between 110 and 120 °C for switchgrass.

Composition

The overall biomass losses were determined gravimetrically by measuring the weight of the dry biomass before and after pretreatment (Table 1). Biomass loss to the IL or wash solutions increases with increasing incubation temperature for all substrates, as has been observed in other studies (Arora et al. 2010). Overall mass losses of ~20 % were higher for the herbaceous substrates than ~10 % for the hardwood poplar at pretreatment temperatures of 120 °C or less.

Lignin, xylan and glucan composition in biomass was measured allowing estimation of these component losses. Over 90 % of the glucan was recovered at all incubation temperatures explored for poplar and at temperatures <120 °C for corn stover and switchgrass. Xylan losses were higher than those of glucan for all substrates resulting in enrichment of the IL treated feedstock with glucan as incubation temperature increases. Cellulose II recrystallization appears more evident at higher incubation temperatures and is closely associated with higher xylan losses during IL pretreatment. It has been previously reported that increasing fractions of xylan and mannan components in biomass impede mercerization of cellulose I (Serkov et al. 1986; Serkov et al. 1983), consistent with our observed decreases in xylan losses and mercerization at lower ionic liquid incubation temperatures.

Losses of xylan and lignin were greater for the herbaceous substrates than the hardwood poplar for most incubation temperatures. Poplar and corn stover xylan losses were <10 % at incubation temperatures of 100 °C or under whereas switchgrass exhibited higher losses of ~30 % at an incubation temperature of 110 °C. For incubation temperatures of 120 °C or

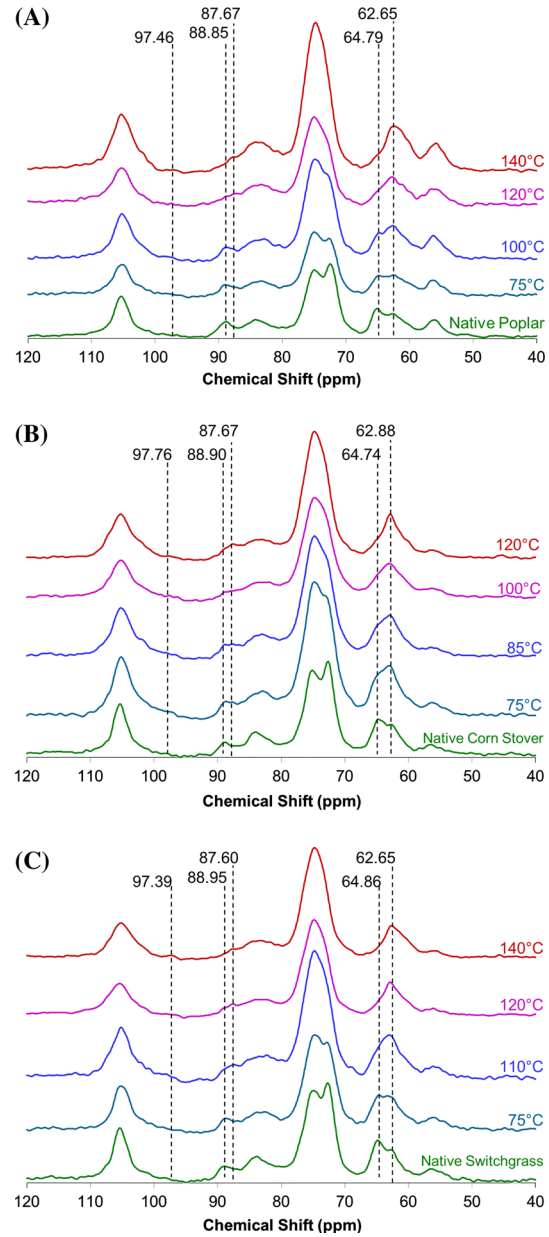

Fig. 2 Cellulose crystal structural characterization using solid state ^{13}C CP/MAS NMR of **a** native and recrystallized poplar, **b** native and recrystallized corn stover, and **c** native and recrystallized switchgrass. Spectra are offset to facilitate visualization. NMR spectra indicate a conversion from cellulose I (native substrate) on the *bottom* to cellulose II at the top. The C4 (89.1–89.8 ppm to 88.7–88.8 ppm) and C6 (65.5–66.2 ppm to 63.5–64.1 ppm) peaks indicative of the transition from cellulose I and cellulose II as well as a characteristic cellulose II peak at ~97 ppm are marked. The NMR spectrum for native biomass contains a single peak at ~88.9 ppm corresponding to the C4 peak for cellulose I

less below, poplar exhibited <10 % lignin losses, whereas corn stover and switchgrass lignin losses were approximately 20 and 30 %, respectively. At an IL treatment temperature of 120 °C, poplar xylan losses increased to ~20 %.

Enzymatic digestibility

Ultimately, our aim is identify maxima in enzyme digestibility of biomass polysaccharides with respect to IL incubation temperature and explore linkages between biomass structural and compositional changes and digestibility. Enzyme digestibility was evaluated by measurement of conversion of glucan and xylan to monomeric sugars after 24 h of hydrolysis (Fig. 3). Enzyme loadings were selected to reach incomplete 24 h monomeric sugar conversions to discern potential maxima. The percent conversion to monomeric sugars was based on composition and mass of IL treated substrates to account for polysaccharide losses during pretreatment (Table 1). Consistent with prior reports (Samayam and Schall 2010; Samayam et al. 2011; Barr et al. 2012), ionic liquid pretreatment greatly improves polysaccharide hydrolysis when compared to hydrolysis of native biomass (Fig. 3). This improvement is observed even at the lowest incubation temperatures.

Maxima in the glucan and xylan conversion to monomeric sugars with respect to pretreatment temperatures are observed for all substrates (Fig. 3). For poplar, a maximum conversion of glucan (60 %) and xylan (70 %) to monomers occurs at 120 °C. The optimal pretreatment temperatures appear to be lower for the herbaceous crops than poplar. At the incubation temperatures explored, corn stover has a maximum in glucan (60 %) and xylan conversion (55 %) at 100 °C. Switchgrass has a maximum glucan conversion (45 %) at 110 °C. Glucan conversion assessed at incubation temperatures of 110 °C for poplar and corn stover and 100 °C for switchgrass yielded 24 h conversions of 55, 46 and 45 %, respectively, lower than the maxima (data not shown in Fig. 3).

The maximum for xylan conversion to xylose for switchgrass was at an IL incubation temperature of 140 °C. This is likely a result of the larger xylanase loading per gram of xylan. Losses of xylan during pretreatment at this temperature were ~80 % leading

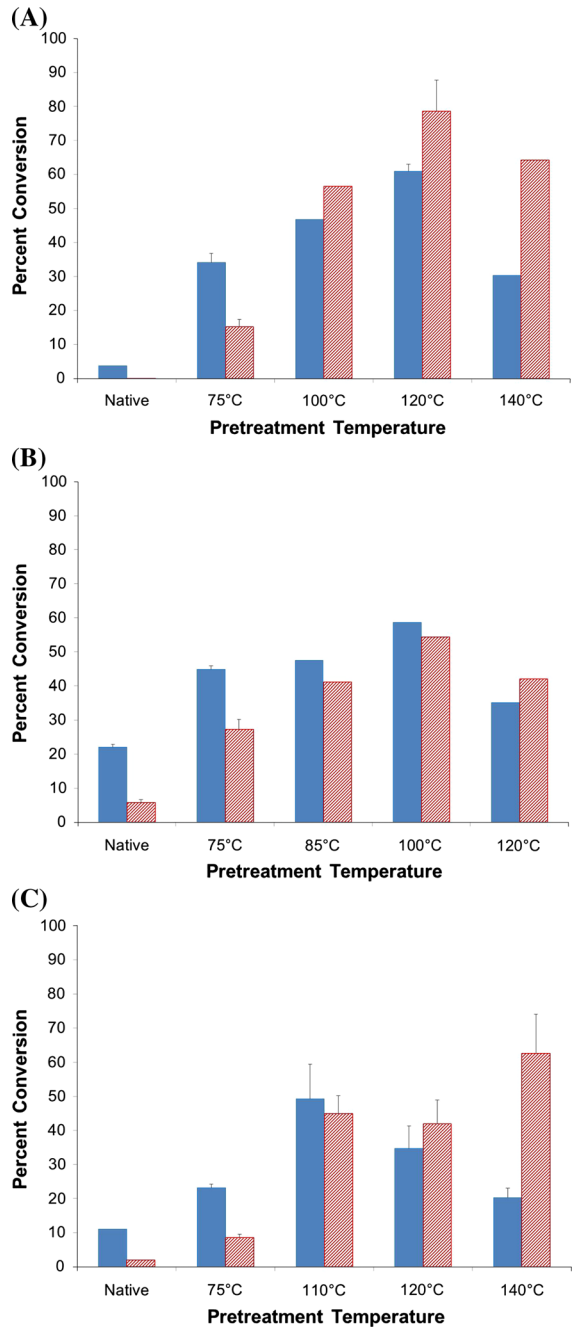

Fig. 3 24 h hydrolysis of native and recrystallized **a** poplar, **b** corn stover, and **c** switchgrass. *Solid/blue bars* denote percent glucan to glucose conversion while *hashed/red bars* denote percent xylan to xylose conversion. Percent conversion is based on the recrystallized biomass composition. Samples without error bars are duplicates with deviations <10 % while those with error bars are triplicates. (Color figure online)

to a concomitant increase in loading of xylanase per gram of xylan. Similar xylan losses with EMIM-OAc treatment of switchgrass have been reported previously (Arora et al. 2010). Since unmodified CTec2 enzyme mixture was used, xylanase loadings were not adjusted independently of the cellulase loadings.

The temperatures of the glucan 24 h hydrolysis maxima appear to be consistent with low CrI substrates. Substrates are largely amorphous with evidence of some cellulose recrystallization and little to no residual cellulose I features. At incubation temperatures higher than the enzyme hydrolysis maxima, the polymorph exhibits exclusively cellulose II features as observed in both the NMR and diffraction data. This result was true for all three substrates examined. This is consistent with an expectation of relative ease in digestion of amorphous cellulose followed by cellulose II then cellulose I. Therefore, one of the strongest factors associated with IL pretreatment enhancement of biomass hydrolysis appears associated with increasing cellulose chain mobility, affecting a transformation of cellulose I to cellulose II at incubation temperatures above the hydrolysis maxima.

Deacetylation of hemicellulose has been reported in EMIM-OAc and other cellulose dissolving ILs at temperatures ranging from 60 to 120 °C (Çetinkol et al. 2010) (Labbé et al. 2012). Acetylation of lignin has also been reported in EMIM-OAc at 120 °C (Çetinkol et al. 2010). The deacetylation of xylan in conjunction with the acetylation of lignin may indicate reactions that sever hemicellulose-lignin covalent linkages. Interruptions of theses linkages combined with temperatures nearing the glass transition temperature of lignin may lead to redistribution of lignin and increased mobility of cellulose. Lignin coalescence outside the secondary cell wall in EMIM-OAc treated biomass has been reported previously in imaging studies (Singh et al. 2009).

Interruptions in the hemicellulose-lignin linkages may facilitate increased xylan solubilization during IL pretreatment. Increasing xylan losses are observed at increasing incubation temperatures (Table 1). At higher incubation temperature the xylan removal may lead to enhancement of cellulose recrystallization upon sample drying. Previous studies have shown that xylan can impede the mercerization of cellulose (Serkov et al. 1983, 1986). In order to retain a primarily amorphous cellulosic substrate even upon drying, retention of xylan during ionic liquid pretreatment may be desirable.

Conclusions

Based on examination of multiple biomass substrates, the optimal IL incubation temperature for maximizing 24 h enzyme hydrolysis ranges from 100 to 120 °C dependent upon substrate. Optimal glucan to glucose conversion occurred at 100 °C for corn stover, 110 °C for switchgrass, and 120 °C for poplar. While these temperatures vary among biomass sources, the properties of the dried regenerated biomass at each the optimal temperatures are similar. At the optimal incubation temperatures, X-ray powder and fiber diffraction techniques show minima in CrI or primarily amorphous material. Examination of NMR spectra indicated that the residual crystalline portion of the biomass at the optimal temperatures may be predominantly cellulose II with some cellulose I. Above these optima, xylan losses greatly increase, leading increasingly to crystalline cellulose II features upon drying. Up to the optimal temperatures, lignin losses are low. The necessity of elevated incubation temperatures may be associated with the need to reach temperatures where lignin coalescence and redistribution is possible. The maxima in IL incubation temperatures appear to be a balance between minimizing xylan losses and increasing cellulose fibril mobility and associated structural transitions.

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