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Comparison of Different Biomass Pretreatment Techniques and their Impact on Chemistry and Structure

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Abstract

Pretreatment of lignocellulosic biomass is a prerequisite to overcome recalcitrance and allow enzyme accessibility to cellulose and maximize product recovery for improved economics of second-generation lignocellulosic bio-refineries. Recently, the three US-DOE funded Bioenergy Research Centers (Joint Bioenergy Institute (JBEI), Great Lakes Bioenergy Research Center (GLBRC), and BioEnergy Science Center (BESC)) compared ionic liquid (IL), dilute sulfuric acid (DA), and ammonia fiber expansion (AFEXTM) pretreatments and published comparative data on mass balance, total sugar yields, substrate accessibility, and microbial fermentation (Biotechnology for Biofuels 7: 71; 72 (2014)). In this study, corn stover solids from IL, DA, and AFEX pretreatments were compared to gain comprehensive, in-depth understanding of induced morphological and chemical changes incorporated to corn stover, and how they overcome the biomass recalcitrance. These studies reveal that biomass recalcitrance is overcome by combination of structural and chemical changes to carbohydrates and lignin after pretreatment. Thermal analysis indicates that AFEX and IL pretreated corn stover showed a lower thermal stability while DA pretreated corn stover showed the opposite. The surface roughness variations measured by small angle neutron scattering (SANS) were correlated to the removal and redistribution of biomass components and was consistent with compositional analysis, atomic force microscopy (AFM) and confocal fluorescence imaging results. With AFM and confocal fluorescent microscopy, lignin was found to be re-deposited on cellulose surface with average cellulose fiber width significantly decreased for DA pretreated corn stover (one third of IL and AFEX). HSQC NMR spectra revealed a ~17.9% reduction of β -aryl ether units after AFEX, ~59.8% reduction after DA and >98% reduction after IL. Both NMR and SEC showed similar patterns of lignin depolymerization with highest degree of depolymerization observed for IL followed with DA and AFEX.

Keywords: corn stover, pretreatment, biomass characterization, recalcitrance

AFEXTM is a trademark of MBI International, Lansing, Michigan.

1 Introduction

2 Lignocellulosic biomass is considered as sustainable and renewable feedstock to produce biofuels that is alternative to petroleum derived fuels. In order to realize this 3 potential, lignocellulosic biomass must be pretreated to overcome recalcitrance(Yang and 4 Wyman, 2008: da Costa Sousa et al., 2009). There are numerous physico-chemical 5 factors that contribute to lignocellulosic recalcitrance. Substantial work is being done to 6 commercially develop scalable and economical pretreatment technologies that 7 dramatically enhance biological conversion of resulting pretreated solids and total sugar 8 vields for downstream fermentation processes (Balan et al., 2013). Pretreatment of 9 biomass is an essential processing step for producing biofuels with high yields via 10 biological routes(Haghighi Mood et al., 2013). The interactions between pretreatment and 11 12 plant cell walls determine their compositional and structural changes, which ultimately affect the extent of enzymatic hydrolysis(Ong et al., 2014). To identify various 13 recalcitrant structures present in plant cell walls, substrate-related properties such as 14 cellulose crystallinity and lattice structure, cellulose accessibility, extent of lignifications 15 have been correlated with sugar production efficiency(Chundawat et al., 2011, Yang et 16 al., 2011; Foston and Ragauskas, 2012; Zhao et al., 2012; Pu et al., 2013; Singh et al., 17 2014). Those factors are often coupled together and their relative contributions to the 18 biomass recalcitrance can vary greatly, depending on the types of biomass and enzymes 19 as well as the pretreatment conditions, etc. (Yang et al., 2011; Foston and Ragauskas, 20 2012; Zhao et al., 2012). 21

22 The three US Department of Energy (DOE) funded bioenergy research centers (BRCs), the Joint BioEnergy Institute (JBEI), BioEnergy Science Center (BESC), and 23 24 Great Lakes Bioenergy Research Center (GLBRC) are developing and optimizing ionic 25 liquid (IL), dilute acid (DA), and ammonia fiber expansion (AFEX) pretreatment technologies respectively. DA pretreatment is one of the pretreatment methods used for 26 second-generation biofuel production (Saha et al., 2005;Sun and Cheng, 2005;Foston and 27 28 Ragauskas, 2010;Cao et al., 2012;Gao et al., 2013), and has been known to effectively solubilize hemicelluloses from biomass. AFEX pretreatment has been shown to be 29 capable of breaking off ester and lignin-carbohydrate bonds and increasing cellulose 30 31 enzyme accessibility by producing nanoporous tunnel-like networks(Balan et al., 2009; Lau and Dale, 2009; Chundawat et al., 2011; Harun et al., 2013; Bals et al., 2014). IL 32 pretreatment is a relatively new technology and it can decrease lignin content and convert 33 34 microcrystalline cellulose to its amorphous form or decrease its crystallinity(Singh et al., 2009; Mora-Pale et al., 2011; Tadesse and Luque, 2011; Brandt et al., 2013). 35 CertainILs, such as 1-ethyl-3-methylimidazolium acetate (abbreviated as [C₂mim][OAc]), 36 have been found to completely dissolve the biomass at specific operating conditions, and 37 hence provide a unique platform to fractionate the three major components of biomass -38 cellulose, hemicellulose, and lignin (Verdia et al., 2014). All three pretreatments are 39 capable of decreasing biomass recalcitrance via morphological and physicochemical 40 changes. 41

Due to the complex nature of the interactions between biomass and pretreatment,
 comparative evaluations of different pretreatment technologies on single substrate would
 offer important insights into the mechanism of the pretreatment processes since it

45 eliminates substrate variability. Literature reports on this comparative study are relatively scant, partly because it requires collaborations between different groups and institutions. 46 There have been a number of studies focused on comparing DA and AFEX pretreatment 47 48 techniques on corn stover, poplar and swichgrass(Kumar et al., 2009; Lau et al., 2009; Chundawat et al., 2010; Garlock et al., 2011; Kim et al., 2011; Pallapolu et al., 2011; Tao 49 et al., 2011; Wyman et al., 2011). In contrast, comparative studies between IL and DA(Li 50 et al., 2010; Li et al., 2013), IL and AFEX(Li et al., 2011) pretreatment are few in number, 51 due in part to the fact that IL pretreatment is a relatively new approach. To gain a better 52 understanding of IL, DA, and AFEX pretreatments and to provide a side by side 53 54 comparison of these technologies, the BRCs used the same cultivar of corn stover processed and distributed between institutions for pretreatment. Samples of the IL, DA, 55 and AFEX pretreated corn stover were then exchanged to investigate and compare 56 enzyme accessibility and sugar yields (BESC), cocktail optimization, mass balance, and 57 fermentation (GLBRC) and thermo, physico-chemical changes to corn stover upon 58 pretreatment (JBEI). 59

60 This work correlates the impact of all three different pretreatments on corn stover using a wide range of analytical approaches to provide new insights into the mechanisms 61 and impacts of each. Thermo-gravimetric analysis (TGA) was used to study thermal 62 behavior of biomass(Muhammad et al., 2012; Poletto et al., 2012; Singh et al., 2013; 63 Zhang et al., 2014). The changes in percent crystallinity and the crystalline structure of 64 the cellulose in pretreated corn stover were analyzed using x-ray diffraction (XRD). 65 66 Small-angle neutron scattering (SANS), confocalfluorescence microscopy and atomic force microscopy (AFM) were used to track the changes in surface morphologies of 67 biomass as a result of the different pretreatment methods(Singh et al., 2009; Cheng et al., 68 2011; Li et al., 2011).NMR(Kim et al., 2008; Yelle et al., 2008; Cetinkol et al., 2009; 69 Kim and Ralph, 2010; Samuel et al., 2011a; Samuel et al., 2011b; Çetinkol et al., 2012; 70 Yelle et al., 2013) and size exclusion chromatography (SEC) (Gidh et al., 2006; George 71 72 et al., 2011; Sathitsuksanoh et al., 2014) are used to chemically characterize the different linkages present in the corn stover and lignin breakdown and size distribution. These 73 results provide a new comparative insight into the effects of biomass pretreatment and 74 help explain recalcitrance factors that are important to overcome for high sugar yields. 75

76 Materials and Methods

The corn stover used in this study was obtained from Michigan State 77 University Farms (East Lansing, MI, USA). The corn hybrid used was NK 49-E3 78 (Syngenta, Basel, Switzerland) which is a typical CS hybrid used in the Great Lakes 79 Region, and was harvested in September of 2008. The harvested biomass was 80 shreaded into fine pieces followed by air drying in room temperature untill the 81 moisture content of biomass was <10% (dwb). Then it was further size reduced using 82 a Wiley mill with a 2-mm diameter sieve and then stored in refrigeratore untill further 83 use.By following the National Renewable Energy Laboratory (NREL, Denver, CO, 84 USA) protocols, the composition of untreated corn stover was 33.4% glucan, 24.9% 85 xylan, 3.7% arabinan, 2.1% acetyl, 17.2% insoluble lignin, 3.6% ash, and 10.4% 86 extractives. All compositions were given on dry biomass samples. 87

88 Pretreatment of Corn Stover

89

90 Ionic Liquid Pretreatment

Ionic liquid pretreatment of corn stover was conducted using 1-ethyl-3-91 abbreviated as [C₂mim][OAc], purchased from methylimidazolium acetate, 92 BASF($\geq 90\%$, cat #51053) and used as received. The pretreatment was conducted at 93 140°C for 3 hrs using 15% (wt/wt) loading of biomass. After the pretreatment 94 process, biomass was separated from IL and purified using a series of ethanol and 95 water washes. The composition of IL treated corn stover was 46.9% glucan, 29.8% 96 xylan, 0.3% arabinan, 1.5% acetyl, 2.7% insoluble lignin, 1.3% ash, and 13.1% 97 98 extractives.

99 Dilute Acid Pretreatment

Dilute acid pretreatment was performed at 160°C for 20min with 10% w/w solid loading and 0.5% w/w sulfuric acid using a 1 L Parr reactor with two stacked pitched blade impellers (Model 4525, Parr Instruments Company, Molline, IL). The heating system was a 4-kW model SBL-2D fluidized sand bath (Techne, Princeton, NJ). The composition of DA treated corn stover was 59.1% glucan, 6.5% xylan, 3.6% arabinan, 0% acetyl, 22.2% acid insoluble lignin, 2.5% ash, and 15.4% extractives.

106 Ammonia Fiber Expansion Pretreatment

AFEX pretreatment procedure was similar to the one described previously by 107 Balan et al.⁴⁶AFEX pretreatment conditions used in this study include: Ammonia to 108 109 dry biomass loading 1:1, biomass moisure 60% (dry biomass weight basis), temperature 140°C and a residence time 15min. Pretreatment was done in a high 110 pressure stainless steel reactor system (PARR Instrument Co, IL). Pretreated biomass 111 was dried in the hood overnight to remove residual ammonia and then packed and 112 stored in zip lock bags in a refrigerator with < 10% moisure untill further use. The 113 composition of AFEX treated corn stover was 33.5% glucan, 24.8% xylan, 3.3% 114 arabinan, 0.6% acetyl, 17.2% insoluble lignin, 4.3% ash, and 24.8% extractives. 115

116 X-Ray Diffraction (XRD) Measurement

XRD measurements were performed on a PANalytical Empyrean system 117 equipped with a PIXcel^{3D} detector. The Bragg-Brentano geometry comprises a Cu X-118 ray tube (operated at 45 kV and 40 mA; $\lambda = 1.5418$ Å), incident beam optics with a 119 1/8° fixed divergence and a 1/4° antiscatter slit, as well as a 0.04 radian soller slit and 120 receiving optics which include another 0.04 radian soller slit, a Ni K_{β} filter and the 121 PIXcel^{3D} detector in scanning mode. A reflection-transmission spinner was used as a 122 123 sample holder and the spinning rate was set at 8 rpm throughout the experiment. The patterns were collected in the 2θ range of 5 to 65°, the step size was 0.026°, and the 124 125 exposure time was 300 seconds. The crystallinity index was determined by a curve fitting procedure of the measured diffraction petterns using with the software package 126 127 HighScore Plus[®].

128 Small Angle Neutron Scattering (SANS)

129 SANS experiments were conducted at Oak Ridge National Laboratory (ORNL) 130 the CG2 (GP-SANS) instrument (http://neutrons.ornl.gov/hfir on instrument systems/CG-2.shtml) with a neutron wavelength of $\lambda = 4.8$ Å ($\Delta \lambda / \lambda \sim$ 131 132 0.14). Powder samples were sandwiched into 1mm home-made quartz cells. Two sample-detector distances were used (1.5 and 14.5 m with a 40 cm detector offset), 133 which resulted in an overall q range of 0.004 Å⁻¹ < q = $4\pi \sin\theta / \lambda < 0.5$ Å⁻¹. The data 134 were corrected for instrumental background and detector efficiency. Due to 135 uncertainty in the packing density of the powder in the cells, the absolute scattering 136 intensities are reported without normalization to sample mass or volume. 137

138 Atomic Force Microscopy (AFM)

Asylum Research MFP-3D-Bio atomic force microscopy system was used to 139 140 image pretreated corn stover. All the biomass samples are fixed on small metal plate. Since the samples are not of regular shape and do not have a flat surface for AFM 141 imaging, the pretreated corn stover samples are first spread gently and homogenously on 142 the metal plate after painting a thin layer of epoxy used to fix the samples. Then, a freshly 143 peeled mica thin layer was used to press against the biomass samples to generate a less 144 rough and flat surface. After the epoxy was dried using nitrogen gas to spray off the top 145 146 covered mica. Due to the steep surface slope we selected a high aspect ratio cantilever (MSS FMP-13 from NanoTools) as our AFM probe. For each type of biomass samples, 147 the scan size ranged from couple of micron to couple hundred nanometer with highest 148 149 resolution <1 nm. All the images are processed using the Igor software from Asylum Research AFM systems. 150

151 Confocal Fluorescent Microscope

The confocal fluorescent microscope images are taken using Zeiss LSM 710 with two laser sources (405 nm and 488 nm). The samples were directly imaged without any modification on the thin glass cover slide using 40X and 10X objectives.

155 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis consists of measuring the decrease of mass of a 156 sample with increasing temperature. Thermogravimetric analysis was conducted using 157 a TGA/DSC system (STAR^e system, Mettler – Toledo Inc, Colombus, OH) equipped 158 with a high throughput auto sampler. A 5 mg of sample was weighed into 40 µl 159 aluminum crucible and analyzed in the presence of Argon (10 ml/min) under 160 isothermal conditions. The temperature of the sample and the reference cell was 161 ramped at a rate of 10°C/min from 35°C to 500°C. The change in the mass of the 162 sample was recorded by the microbalance in the TGA instrument. The 1st differential 163 (DTG) curves of the TGA curves are easier to read and infer from when compared to 164 the TGA curves. The plot shown in this work represent dTG curves of the original 165 TGA curves obtained from the instrument. The untreated corn stover samples were 166 167 compared to pretreated samples for changes in the decomposition temperature and weight loss percent. 168

169 **2D**¹³C-¹H HSQC NMR Analysis

170 Corn stover cell walls from different pretreatments were ball-milled, solubilized in DMSO-d₆, and then analyzed by 2D HSQC NMR. 2D ¹³C-¹H HSQC NMR of plantcell 171 wall samples were performed as previously described(Kim and Ralph, 2010a). In short, 172 173 ball-milled samples (\sim 50 mg) were then placed in NMR tubes with 600 µl DMSO-d₆. The samples were sealed and sonicated until homogenous in a Branson 2510 tabletop cleaner 174 (Branson Ultrasonic Corporation, Danburt, CT). The temperature of the bath was closely 175 monitored and maintained below 55°C. HSQC spectra were acquired at 398 K using a 176 Bruker Avance-600 MHz instrument equipped with a 5 mm inverse-gradient ${}^{1}\text{H}/{}^{13}\text{C}$ 177 cryoprobe using a q hsqcetgp pulse program (ns = 64, ds = 16, number of increments = 178 179 256, $d_1 = 1.5$ s) (Heikkinen et al., 2003). Chemical shifts were referenced to the central DMSO peak (δ_C/δ_H 39.5/2.5 ppm). Assignment of the HSQC spectra was described 180 elsewhere (Kim and Ralph, 2010a). A semi-quantitative analysis of the volume integrals 181 of the HSQC correlation peaks was performed using Bruker's Topspin 3.1 (Windows) 182 processing software. 183

184 Isolation of enzymatic mild acidolysis lignin (EMAL)

185 Ball-milling of untreated corn stover was performed using a Retsch PM 100 planetary ball mill spinning at 600 rpm with zirconium dioxide (ZrO₂)container and balls. 186 The ball milling conditions were described elsewhere (Kim and Ralph, 2010b). Briefly, 187 the ball-milled biomass samples were treated with cellulase (Cellic Ctec 2 of 188 Novozymes) and hemicellulase (Cellic Htec 2 of Novozymes) in the amount of 50 mg 189 protein/g biomass. The enzymatic hydrolysis was carried out at 50 °C for 48 h at 2% 190 191 consistency in the presence of 2% Tween 20 in 50 mM citrate buffer (pH ~4.8). The insoluble materials were washed with deionized water and a fresh batch of enzymes, in 192 the same quantity, was added for another 48 h. The insoluble materials remaining after 193 194 enzymatic hydrolysis were washed with deionized water to remove soluble sugars. 195 Residual proteins on the surface of solid pellets were then washed twice with 6M guanidine hydrochloride (GndHCl) and freeze dried. The crude lignin obtained was 196 197 further subjected to mild acid hydrolysis using an azeotrope of dioxane-water (96:4 (v/v)) containing 0.01 N HCl under nitrogen atmosphere. The resulting suspension was 198 centrifuged, and the supernatant was collected. The supernatant was neutralized with 2 M 199 200 sodium bicarbonate and then added drop-wise into 1 L acidified water (pH 2.0). The precipitated lignin was allowed to equilibrate overnight, recovered by centrifugation, 201 washed with deionized water twice, and freeze dried. 202

203 Size Exclusion Chromatography (SEC)

204 To understand changes of lignin molecular weight distribution during pretreatment, size exclusion chromatography (SEC) was performed on extracted lignin 205 from pretreated corn stover. An Agilent 1200 series binary LC system (G1312B) 206 equipped with diode-array (G1315D) and fluorescence (G1321A) detectors was used. 207 Separation was achieved with a Mixed-D column (5 µm particle size, 300 mm x 7.5 mm 208 i.d., linear molecular weight range of 200 to 400,000 u, Polymer Laboratories) at 80 °C 209 210 using a mobile phase of NMP at a flow rate of 0.5 ml per min. Absorbance of materials eluting from the column was detected at 300 nm (UVA) as well as excitation at 250 nm 211 and emission at 450 nm were used for UVF. Intensities were area normalized and 212

molecular mass estimates were determined after calibration of the system with
polystyrene standards (George et al., 2011). The enzymatic mild acidolysis lignin
(EMAL) process (Guerra et al., 2006) was used to extract lignin from corn stover and this

was used as both the process control and the initial material for the three pretreatments.

217

218 **Results and Discussion**

219 Effect of Pretreatment on Cellulose Crystallinity

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221 The XRD of untreated and treated corn stover samples are presented in Figure 1. There are three broad peaks at 16.3°, 21.9° and 35.0° for untreated corn stover, 222 which are consistent with cellulose I lattice. The broad peak at 16.3° is a composite 223 from several peaks. (Cheng et al., 2011) Upon pretreatment in [C₂mim][OAc], the 224 225 cellulose becomes amorphous while cellulose I lattice is preserved in the DA and AFEXsamples. Although the main peak position remains roughly the same for the DA 226 and AFEXsamples, there are noticeable shifts of the secondary peak from 16.3° to 227 15.8° upon DA pretreatment. The peak's full width at half maxim (FWHM) drops 228 from 3.5° to 3.1°, suggesting an increase of the apparent size of cellulose 229 crystallites. The increase in apparent cellulose crystal sizes due to coalescence of 230 cellulose microfibrils duirng hydrothermal pretreatment (DA, AFEX and steam 231 explosion) has been reported recently(Ibbett et al., 2013; Langan et al., 2014; 232 Nishiyama et al., 2014; Pingali et al., 2014). The fraction of crystalline cellulose in 233 the corn stover sample, defined as the biomass crystallinity index (CrI), was estimated 234 by comparing area under the XRD pattern to that of a amorphous lignin sample. There 235 is an apparent increase of biomass CrI for DA pretreated corn stover while for the 236 AFEX pretreated one, it stays roughly constant (Figure 1). The apparent increase in 237 238 CrI is caused by the removal of amorphous hemicelluloses by the DA pretreatment. However, the true impact of pretreatment on the cellulose crystalline structure is the 239 change in cellulose crystallinity index. Taking into consideration of the weight faction 240 241 of glucan in the samples (materials and methods section), the cellulose CrI in untreated, AFEX and DA pretreated samples are 1.0, 0.98 and 0.82, respectively. 242 Depending on the pretreatment conditions, AFEX may decrystallize cellulose to a 243 different extent(Chundawat et al., 2011). The decrease in cellulose CrI upon DA 244 pretreatment was perhaps due to simultaneous decrystallization and degradation(Xu et 245 al., 2011). 246

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249 Small Angle Neutron Scattering (SANS)

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SANS has been used before to reveal the surface roughness of biomass samples(Çetinkol et al., 2009;Cheng et al., 2011;Li et al., 2011).Due to the morphological inhomogeneity of the cell wall, the structural parameters averaged over

several orders of length scales are believed to resemble its true property. The SANS 254 data were collected in the q range of 0.4 to 0.002 Å⁻¹ which reveals averaged 255 structural properties on the order of 10 to 100nm. The scattering from corn stover 256 257 samples is due to the contrast (the difference in scattering length density (SLD)) between the air and biopolymer matrix. The air may exist in the pores or cracks 258 259 whose sizes vary from nanometers to microns in the cell wall(Cheng et al., 2011).As shown in Figure 2, for the untreated corn stover sample, the exponent of the fitted 260 power law function is 3.8, which corresponds to the scattering from a rough surface 261 and the roughness can be characterized by a surface fractal dimension: $D_{surface} = 6.0$ -262 3.8 = 2.2 (Cheng et al., 2011). A perfectly smooth surface has a dimension of 2.0. The 263 dimension increases with the increase of the surface roughness. After the DA and 264 AFEX pretreatment, as shown in Figure 2, the surface fractal dimension increased to 265 2.5 and 2.4, respectively. The increase in surface roughness is caused by removal 266 redistribution of cell 267 and/or wall components during pretreatment processes(Chundawat et al., 2011). SEM images showed the deposition of lignin 268 droplets on the surface of DA and AFEX pretreated samples(Selig et al., 2007; 269 270 Chundawat et al., 2011). Based on the compositional analysis, there is slight decrease in lignin content after the AFEX pretreatment; while the DA pretreatment removes 271 most of the hemicelluloses. It is believed that the in both cases the carbohydrate-272 273 lignin network was disrupted which led to the increased cellulose accessibility (Chundawat et al., 2011). On the other hand, the surface fractal dimension drops to 274 2.0 for the IL pretreated sample. This change is correlated with the removal of most 275 of the lignin from corn stover samples after IL pretreatment, and better defined 276 surfaces were formed upon regeneration. 277

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Confocal fluorescenceand Atomic Force Microscopy (AFM)

Confocal fluorescence microscopy and AFM were used to investigate the surface 281 morphologies of AFEX, DA, and IL pretreated corn stover at macro and micro scales. 282 Clear differences wereobserved by confocal fluorescence imaging in the extent of 283 physical alteration of the corn stover biomass upon different pretreatments(Figure 3a-c). 284 At macro-scale investigation using fluorescence microscopy, the AFEX and DA 285 286 pretreated corn stover appear similar to un-pretreated biomass (supplementary material). Closer observation of pretreated corn stover reveals that DA pretreated corn stover 287 288 exhibiting morphological changes to cell wall, whereas AFEX pretreatment did not significantly alter the morphology of the cell walls. Compared to DA and AFEX, IL 289 pretreatment led to significant morphological changes of the cell walldue todissolution 290 and then regeneration upon addition of water as an anti-solvent. The IL pretreated 291 292 samples do not show any resemblance to the original morphology present, and also do 293 not fluoresce, indicative of no or very low lignin in the sample. Further investigation of AFEX pretreated corn stover using confocal fluorescence imaging showed that although 294 295 there was no sign of any morphological changes in the corn stover, lignin is dissolved and displaced during AFEX (see supplementary material). 296

High-resolution surface morphologies of the pretreated corn stover were examined using high resolution AFM imaging that can further resolve the fine surface 299 features down to the cellulose fibers (Figure 3d-f). For the AFEX pretreated corn stover, 300 we can clearly see that although some fraction of lignin is dissolved and re-deposited on the surface as evident by fluorescence imaging, the cellulose fibers, however, appear 301 unaltered in size and shape. The AFM images also show lignin deposited on the surface 302 of the cellulose fibers (confirmed with force mapping images). The detailed AFM images 303 show very different size of the cellulose fibers before and after DA pretreatment. The 304 cellulose fibers seem to be separated and piled together. The average width of the 305 cellulose fibers for the DA treated samples (209±34 nm), which is much smaller than the 306 AFEX treated sample (685±119 nm). Another interesting feature observed in these high-307 308 resolution AFM images wasthat these cellulose fibers appear to be composed of many cellulose nano-crystals. It appears that DA attacks the amorphous regions and breaks 309 down the cellulose to smaller cellulose crystals(Moon et al., 2011). This effect could be 310 generated by the combined impact of hemicellulose removal, cellulose fiber 311 dehydration(Langan et al., 2014), and breakage of fibers at the presumed weakest point 312 (amorphous regions in cellulose fiber). AFM images of IL pretreated corn stover show 313 lignin-free fibers (confirmed with fluorescent imaging) that appear to be similar in width 314 as corn stover but more randomly organized. The width of the fibers is dependent on the 315 processing conditions(choice of anti-solvent, temperature and stirring) during cellulose 316 regeneration (data not shown). These results are consistent with XRD data that shows 317 amorphous structure. 318

319

320 Effect of pretreatments on thermochemical properties of cornstover

321 The thermogravimetric curves of biomass before and after pretreatment, as shown in Figure 4, can be divided into three regions: the hemicelluloses zone (245-290°C), the 322 cellulose zone (290-350°C) and the lignin zone (350-500°C)(Singh et al., 2013;Zhang et 323 324 al., 2014). We observe from the differential thermogravimetric (DTG) curves that for untreated corn stover, the first weight loss peak occurs at 278°C and the second weight 325 loss peak occurs at 336°C. Also, the calculated weight loss for untreated corn stover in 326 hemicelluloses region is 28% and in cellulose region is 32%. For comparison, the 327 untreated corn stover contains 21.7% hemicelluloses and 34.9% cellulose. Interestingly, 328 DA treated corn stover only shows a weight loss of 61% in the cellulose region (348°C). 329 The weight loss only in the cellulose region indicates absence of hemicellulose in the DA 330 treated material, which is consistent with the compositional analysis (materials and 331 methods section). In AFEX pretreated corn stover, we noticed an increase in 332 decomposition temperature (from 278 to 285°C) in hemicellulose region and a decrease 333 in decomposition temperature in the cellulose zone (336 to 325°C). Meanwhile it shows a 334 higher weight loss of 44% in the hemicelluloses region, a lower weight loss of 15% in the 335 cellulose region. Considering similar composition and crystallinity of untreated and 336 AFEX pretreated corn stover, the DTG data suggest partial conversion of cellulose to 337 lower molecular weight cellulose and the decomposition temperature of the 338 depolymerized cellulose falls within the decomposition temperature range of the 339 hemicellulose.In the case ofIL pretreated corn stover, the first decomposition peak 340 increases to 292°C and the weight loss in this region increases to 40%. The second 341 weight loss peak (decomposition temperature) of IL pretreated sample does not change 342

when compared to untreated corn stover while weight loss in this region decreases to
27%. Considering increased cellulose and hemicellulose content of IL treated corn stover,
we hypothesize that higher the weight loss in the hemicellulose region after IL
pretreatmentis due to conversion of crystalline celluloseinto the amorphous form.

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Characterization of chemical structure of carbohydrates and lignin from pretreated corn stover

2D HSQC NMR was used to elucidate structural changes of carbohydrates and 350 lignin due to chemistry that governs different pretreatments. HSQC NMR spectra of 351 untreated and pretreated corn stover samples from aliphatic, anomerics, and aromatic 352 regions are shown in Figure 5-7, respectively. Assignments of the lignin ¹³C-¹H 353 correlation peaks in the 2D HSQC spectra of corn stover cell wall are given in Table S1 354 355 (supplementary material). Color-coded polymer structures correspond to colored crossed peaks in the spectra (right side, Figure 5 and 7). Aliphatic region of untreated corn stover 356 357 showed lignin side chain correlations found in corn stover: β-aryl ether units (A), resinol units (C), dibenxodioxocin units (D), as well as cinnamyl alcohol end-groups (I) and 358 359 methoxyls (MeO). Prominent peaks of acetylated xylopyranosides (2-acetylated xylan (2-O-Ac- β -D-Xylp, \dot{X}_{2}) and 3-acetylated xylan (3-O-Ac- β -D-Xylp, \dot{X}_{3})) were well 360 resolved and observed at δ_C/δ_H 73.5/4.5 and 75.0/4.8 ppm, respectively(Figure 6). 361 Anomeric region correlation of untreated corn stover shows some important 362 polysaccharide anomerics including $(1 \rightarrow 4)$ -linked β -D-glucopyranosyl units (β -D-Glcp, 363 cellulose), $(1 \rightarrow 4)$ -linked β -D-xylopyranosyl units (β -D-Xlyp, xylan), $(1 \rightarrow 3)$ -linked α -L-364 arabinofuranosyl units (α -L-Arap, arabinan). Aromatic region of untreated corn stover is 365 shown in Figure 7. Corn stover lignin is a typical syringyl/guajacyl (S/G) lignin with low 366 levels of p-hydroxyphenyl (H) units(Figure 7) with S/G ratio of 1.42, which is in 367 agreement with a value previously reported (Li et al., 2012). Ferulate (FA) and p-368 369 coumarates (pCA) are observed in untreated corn stover(Figure7).

AFEX. Going from untreated to AFEX, β -arvl ether units appeared to be slightly 370 371 lower than those of untreated corn stover (Figure 5). Cross peak integral of β -aryl ether units revealed a ~17.9% reduction of β -aryl ether units compared to that of untreated corn 372 stover. This result suggested that AFEX pretreatment did not have a strong effect onβ-373 aryl ether linkage cleavages. Disappearance of X'_2 and X'_3 in AFEX-pretreated corn 374 stover indicated a removal of acetylated xylopyranosides, reducing steric hindrance and 375 increasing cellulose accessibility and enhancing carbohydrate digestibility. The depletion 376 377 of dibenzodioxocin units during AFEX indicated that lignin points of branching were removed. 378

Polysaccharide anomerics of AFEX sample (Figure 6) showed a slightly weaker signal of 2-*O*-Ac- β -D-xylp(R). The depletion of β -correlation of FA and pCA was observed. The AFEX lignin aromatic region displays depletion of β -correlation of FA and pCA. No significant reduction in S_{2,6}, G₂, and $pCA_{2,6}$ cross peak intensities were observed from AFEX. No significant reduction in lignin sidechain units and peaks in aromatic regions indicated that lignin was not depolymerized to a great extent during AFEX. However, significant reduction of dibenzodioxocin units, acetylated xylopyranosides, and ferulate suggest that any lignin branching points via these bonds, and lignin-carbohydratelinkages broke off during AFEX.

DA.HSQC spectrum of DA (Figure 5) indicated ~59.8% reduction in β -aryl ether 388 units compared to that of untreated corn stover. Although cross peaks attributed to 389 polysaccharides and some lignin sidechain units are overlapped in the region δ_C/δ_H 60-390 85/2.8-4.2 ppm, a significant decrease in intensities of xylan correlations (X₂, X₃, and X₄) 391 indicated significant removal of hemicelluloses during DA. Interestingly, X_2 and X_3 392 393 cross peaks were observed on DA sample, suggesting that residual hemicelluloses after DA pretreatment contained acetyl groups. Weaker signals of 2-O-Ac-β-D-xylp(R) and 3-394 395 O-Ac- β -D-xylp(R) in anomeric region of DA were observed, which were due to removal of hemicelluloses during DA pretreatment. Similar to AFEX, aromatic region of lignin 396 397 from DA did not show a significant change in cross peak intensities. This result is in agreement with composition of DA-pretreated corn stover that DA pretreatment mainly 398 hydrolyzes labile hemicelluloses, leaving majority of lignin in the DA-pretreated corn 399 stover. β-aryl ether units of lignin from DA were lower than that from AFEX, suggesting 400 a greater degree of lignin depolymerization. 401

402 **IL**. Disappearance of β -aryl ether units from lignin from IL indicated the 403 depolymerization of β -aryl ether linkages during IL pretreatment. The absence of X²₂ and 404 X³₃ cross peaks confirmed deacetylation of hemicelluloses during IL pretreatment. Weak 405 intensities of S_{2,6}, G₅, and FA₂were observed in aromatic region of IL-pretreated corn 406 stover, supporting a low lignin content in IL-pretreated corn stover. The depletion of β -407 aryl ether units and lignin aromatic units in IL-pretreated corn stover suggested that 408 lignin was depolymerized during IL pretreatment.

409 Depolymerization of Lignin from Different Pretreatments

EMAL of corn stover was used to represent the intact lignin (lignin before 410 pretreatment). Area-normalized elution profiles of lignin before and after pretreatments 411 areshown inFigure 8a-b.UVA and UVF detections elucidate different aspects of the same 412 lignin molecule. While UVA gives information of the bulk larger molecules, it is not as 413 414 effective at detecting smaller molecular weight components that exhibit fluorescence, and vice versa. For a more complete insight into the polydispersity of the lignin both 415 detection modes are used with a focus on UVA for larger molecules and UVF for smaller. 416 In addition, the chemistry of the different pretreatments yields lignin of different 417 structures, as indicated by the NMR data in this study. Therefore, it is possible that the 418 lignin extraction method may affect the samples differently in terms of their chemical 419 420 structure and this will affect SEC data interpretation. It is also important to note that for the DA and IL samples, if depolymerization occurs the smallest molecules maybe lost to 421 the liquid phase, thereby increasing the average molecular weight of the samples. The 422 compositional analysis of AFEX-pretreated corn stover showed that AFEX retained most 423 of lignin after pretreatment, while IL pretreatment showed an almost complete removal of 424 lignin, which was confirmed by very weak signals of lignin aromatic units (Figure 7). 425 With these caveats, the relative changes between the samples can be interpreted. 426 Fractions were defined in the SEC chromatograms (Figure 8) to highlight changes in four 427 key molecular weight regions (F1-4). Table 1 shows the proportion of the sample in each 428

fraction, and also indicates the Mw range in these regions, as obtained by polystyrene calibration which is thought to be more reliable for only the smallest molecules due to the difference in lignin versus polystyrene structure.

In general AFEX shows the most similar profile to that of the EMAL (enzymatic 432 mild acidolysis lignin) lignin control. IL, followed by DA, has undergone the most 433 significant changes in terms of profile and size, and has a greater proportion of smaller 434 Mw components. DA shows a higher proportion of the smaller Mw components that are 435 also present in the EMAL. A new category of material is present in the IL in the smallest 436 molecular weight region in great abundance, (or with chemical functionality that exhibits 437 strong florescence), indicating even further depolymerization. Considering the fractions 438 in more detail, F1 depicts the largest molecules. EMAL, AFEX, and DA contain (19-439 26%) of material in this range, while IL contains only 9%, indicating a greater degree of 440 depolymerization in the IL of these largest molecules. This is also corroborated by UVF, 441 which indicates very small quantities of material in this region. For IL, it appears that 442 small molecular weight fragments have been cleaved from F1, to shift the sample into the 443 444 F2 region (UVA). The smaller cleaved fragments are likely making a contribution to the new peak F4 (UVF). DA has the largest proportion of material in F3 by UVF analysis. 445 This increase in abundance could either be due to de-polymerization of larger fragments 446 in the F3 range, or an increase in relative abundance of F3 due to removal of cleaved 447 448 molecules from the largest fractions (F1&F2) to the liquid phase of the DA treatment.

	Time range (min)	MW range (Polystrene calibration)		EMAL	AFEX	DA	IL
UVA							
F1	8.0-11.7	2.02E+07	1.35E+06	19.2	26.1	25.2	9.4
F2	11.7-15.5	1.35E+06	8.17E+04	46.0	50.1	56.1	65.4
F3	15.5-21.1	8.17E+04	1333.15	26.3	18.8	14.3	12.1
F4	21.1-26.0	1333.15	36.37	8.4	4.9	4.5	12.8
UVF							
F1	8.0-11.7	2.02E+07	1.35E+06	12.7	3.0	4.8	0.9
F2	11.7-15.5	1.35E+06	8.17E+04	39.2	53.9	28.0	14.4
F3	15.5-21.1	8.17E+04	1333.15	32.1	32.8	36.2	8.8
F4	21.1-22.6	1333.15	36.37	16.0	10.3	31.0	75.9

449	Table 1.Elution	time and re	elativeabund	dance of lig	nin in	different SI	EC fractions

450

451 **Conclusions**

A side-by-side comparison of the physicochemical and thermal changes to biomass rendered by IL, DA and AFEX pretreatment methods is necessary to better understand the origins of biomass recalcitrance and mechanism and impact of these pretreatments for further optimization. The separate hemicellulose and cellulose peaks in the TGA data allowedus to evaluate the impact of pretreatment on biomass components. The higher weight loss in the hemicelluloses region after TGA analysis for IL and AFEX pretreated corn stover indicates the ease by which thermal breakdown of these pretreated 459 materials occurs. Although just based on the standard compositional analysis of AFEX 460 pretreated solid that shows minimum change to the corn stover, and it has been difficult to explain the mechanism and the causes for improved digestibility of AFEX pretreated 461 materials in the past, our results indicate that disruption of lignin-carbohydrate linkages 462 of mainly polymeric lignincontributeto the efficiency of AFEX pretreatment. DA 463 pretreatment appears to start with significant lignin de-polymerization, with 50% of the 464 lignin re-condensed in the final pretreated corn stover. DA pretreated corn stover was 465 found to be thermally more stable, however, fiber width wasmeasured to be significantly 466 smaller than IL and AFEX pretreated corn stover. The small fiber width and the presence 467 of re-condensed lignin for DA may explain the slow initial hydrolysis kinetics but high 468 sugar yields at higher enzyme loadings that were reported but difficult to understand 469 These comparative results will aid in the further development and 470 previously. optimization of each pretreatment technology, and provides the biofuel community a 471 detailed knowledgebase of the impact of pretreatment as a function of type and process. 472

473 **Conflict of Interest Statement**

The authors declare that they have no competing interests.

475

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- Figure 1. Powder XRD patterns of corn stover samples before and after pretreatment.The CrI refers to biomass crystallinity index.
- Figure 2. SANS spectrum of corn stover samples before and after pretreatment. Dsrefers to surface fractal dimension.
- Figure 3.Confocal fluorescence (Top) and AFM (Bottom) images of AFEX, DA and IL pretreated corn stover. In a) and b) the inset are low magnification images. In d), e) and f) the inset plots are the cross profile of the pretreated cellulose fiber.
- Figure 4. Differentialthermogravimetric curves of untreated and pretreated corn stoversamples
- Figure 5.Aliphaticregion of HSQC NMR spectra of untreated and pretreated corn stoversamples
- Figure 6.Anomeric region of HSQC NMR spectra of untreated and pretreated corn stoversamples
- Figure 7.Aromaticregion of HSQC NMR spectra of untreated and pretreated corn stoversamples
- Figure 8.Area-normalized SEC elution profiles of lignin before and after pretreatments.(a) UVA; (b)UVF.

750





AFEX









400

DA

600

800 nm



(c)



0 2 3 5 µm 1 4

IL

0

200

Figure 3.TIFF











