Enzymatic digestibility and pretreatment degradation products of AFEX-treated hardwoods (Populus nigra)

Enzymatic digestibility and pretreatment degradation products for AFEX treated hardwoods (*Populus nigra*)

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Abstract:

There is growing need to find alternatives to crude oil as the primary feed stock for the chemicals and fuel industry and ethanol has been demonstrated to be a viable alternative. Among the various feed stocks for producing ethanol, poplar (*Populus nigra X populus maximowiczii*) is considered to have a great potential for biorefineries in the US, due to their widespread availability and good productivity in several parts of the country. We have optimized AFEX pretreatment conditions (180 ^{o}C, 2:1 ammonia to biomass loading, 233% moisture, 30 min. residence time) and by adding different combinations of enzymes (commercial cellulases and xylanases) in order to achieve high glucan and xylan conversion (93 and 65%, respectively). We have also identified and quantified several important degradation products formed after AFEX using liquid chromatography

followed by mass spectrometry (LC-MS/MS). As a part of degradation product analysis we have also quantified oligosaccharides in the AFEX water wash extracts by acid hydrolysis. It is interesting to note that corn stover (C4 grass) can be pretreated effectively using mild AFEX pretreatment conditions, while on the other hand hardwood poplar needs much harsher AFEX conditions to obtain equivalent sugar yields upon enzymatic hydrolysis. Based on the oligosaccharide analysis comparison between AFEX treated stover and poplar, we conclude that pretreatment severity and enzymatic hydrolysis efficiency are dictated to a large extent by lignin carbohydrate complexes and arabinoxylan cross-linkages for the AFEX process.

Key words: Corn stover, Poplar, AFEX pretreatment, enzymatic hydrolysis, Lignocellulose

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Introduction:

The growing U.S. appetite for petroleum, fueled together with growing demand in China, India, and rest of the world, has pushed crude oil prices to a new high. The United States consumes over 20 million barrels of petroleum per day, of which over 60% is imported. Crude oil prices have risen as high as $147 per barrel (bbl) in July 2008, a remarkable 400% increase in cost over the last decade (www.wtrg.com). Hence, there is a

growing urgency to find suitable alternatives to petroleum-derived fuels. Bioethanol is one such suitable prospect that can provide a potentially low cost, environmentally-friendly way to reduce gasoline consumption while helping reduce net carbon dioxide emissions (*1*). Thus, a considerable amount of research is currently underway to economically produce ethanol from lignocellulosics, which are far more abundant in nature and cheaper to produce compared to conventional feedstocks (e.g. sugarcane, corn starch) (*2, 3*).

Cellulose, one of the major components of the plant cell wall, is a linear condensation polymer consisting of D-anhydroglucopyranose joined together by β-1,4-linkage with a degree of polymerization ranging from 100 to 20,000. Adjacent cellulose molecules are coupled by extensive hydrogen bonds and Van der Waals forces resulting in a parallel crystalline alignment, and producing a rigid and stable supramolecular structure with low accessibility to chemicals and enzymes (*4*). In addition, cellulose is embedded in a hemicellulose and lignin matrix which makes it even more recalcitrant during enzymatic hydrolysis (*5-7*). Disruption of these bonds by thermochemical pretreatment, using acid or base, increases cell wall porosity and drastically enhances the accessibility of enzymes to the sugar polymers (*8*).

A novel alkaline pretreatment method to improve lignocellulose digestibility is the ammonia fiber expansion (AFEX) process (*9*). Ammonia is added to the biomass under high pressure (200-700 psi) with varying temperatures (60-200 °C) before rapidly releasing the pressure. The AFEX process appears to be economically attractive and on-

going research has allowed further cost improvements (*10*). AFEX is thought to decrystallize cellulose, while partially hydrolyzing hemicellulose through hydrolysis of lignin-hemicellulose ester cross-linkages (*11*).

Effectiveness of pretreatment and enzymatic hydrolysis is dictated to a large extent by biomass composition, among other factors. During enzymatic hydrolysis, enzyme tends to irreversibly bind to lignin, an aromatic polymer, through hydrophobic interactions that cause loss in activity. Hence, the amount and composition of lignin critically affect the digestion of sugar polymers to soluble sugars. Lignin content in grasses (15-20%) is relatively low compared to hard and softwood (20-35%) (*12*). This could be one of the major reasons that grasses, can be more easily digested via hydrolytic enzymes compared to hardwood following AFEX pretreatment (13). In addition to lignin, the arabinoxylan content of the biomass is also thought to play a crucial role to the effectiveness of the pretreatment process. In monocots of the arabinoxylan is connected to lignin via ester and ether linkages (*5, 14-17*). These linkages are between arabinose side chains of hemicelluloses and hydroxyl/carboxyl functionalities of lignin (e.g. ferulic acid). Ammonia has the tendency to cleave these ester linkages (*18-20*) in biomass via ammonolysis. It is interesting to note that the arabinose (found mostly as arabinoxylan) content of grasses (3-6%) is significantly higher compared to hardwood (<1%). Thus the higher the arabinoxylan content and possible ester linkages to arabinoxylan, the greater its susceptibility to cleavage during the AFEX process (21). Cleavage of these linkages promotes the disruption of the cell wall complex structure in such a way that the enzymes can access the cellulose and hemicellulose more efficiently. Fundamental understanding

of how these linkages are chemically modified during the pretreatment process may help eventually design a genetically modified cell wall which can be more easily pretreated under mild conditions using fewer chemicals (*17*).

Water washing of AFEX treated corn stover was shown to remove some of the AFEX-mediated surface deposits resulting in a 13-15% weight loss (w/w) (*22*). The cleavage of the acetyl groups from hemicellulose results in the formation of acetamide and acetic acid. Lactic acid and formic acid are also formed during AFEX, likely due to alkali induced degradation of reducing sugars and lignin (*23, 24*). Moreover, phenolic compounds like p-coumaric and ferulic acid are also produced due to the hydrolysis of lignin-hemicellulose cross-linkages (*25*). Phenolic aldehydes were more likely to be produced in oxidative alkaline conditions (*24*). Some components such as 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid are thought to be monomeric lignin extractives that are easily released after the pretreatment process, unlike the other lignin cleavage products (*26, 27*). Identification and quantification of these degradation products using liquid chromatography-tandem mass spectrometry (LC-MS/MS) will provide valuable information that will help understand the kinetics and mechanisms by which these compounds are formed and enable design of optimized AFEX pretreatment conditions.

Biologically inhibitory effects of compounds from wood hydrolysates depend on the chemical structure and reactivity: e.g. terpenes > aldehydes > polyhydroxy aromatics, and formic acid > acetic acid (*28*) for yeast fermentations. Low molecular weight (MW)

compounds and salts are able to penetrate cell membranes, and are thus more toxic to fermentative microbes compared to high molecular weight compounds (*29*). Lower MW compounds influence the expression and activity of sugar and ion transporters in the cell membrane. Mechanisms for inhibition of microbial growth and ethanol production due to weak acids, furans and phenols have been reviewed recently (*30*).

Among the various sources of biomass, agricultural residues like corn stover and hardwoods like hybrid poplar (*Populus nigra x Populus maximowiczii*) are of interest. Woody biomass has several advantages compared to agricultural residues including ability to be stored "on the stump", higher mass density that reduces transport cost and increased sugar content (i.e. glucan (~40-50%) and xylan (~20-30%) content. Considerable information is available on steam explosion (*31*), organosolv pretreatment (*32*), acid pretreatment (*33, 34*) followed by enzymatic hydrolysis of poplar, but very little work has been reported on alkaline pretreatment (in particular AFEX) (*35*) of hardwoods. Corn stover can be pretreated effectively using mild AFEX pretreatment conditions, while on the other hand poplar needs much harsher AFEX conditions to obtain equivalent sugar yields upon enzymatic hydrolysis. In this paper we discuss how AFEX pretreatment severity and enzymatic hydrolysis efficiency are dictated by the plant cell wall ultra structure and composition of various components, such as lignin carbohydrate complexes (LCC) and arabinoxylan cross-linkages. Details of the AFEX pretreatment conditions, degradation products formed during pretreatment, sugar conversions for varying enzyme loadings and the mass balance for poplar and corn stover are also discussed in this article.

Materials and Methods:

Lignocellulosic substrate. Hardwood poplar was provided by National Renewable Energy Laboratory (CO, Denver), and was milled using a 50 mm sieve. The moisture content was measured using a moisture analyzer (Model MF-50, A&D). The samples had approximately 50% (total weight basis) moisture and were stored at -20 ^{o}C freezer until further experiments were carried out.

Compositional Analysis. Compositional analyses of the samples were performed according to NREL Laboratory Analytical Procedures (LAPs): "Preparation of samples for compositional analysis" and "Determination of structural carbohydrates and lignin in biomass" (*36*). Monomeric sugars were quantified using a Bio-Rad Aminex HPX-87H high performance liquid chromatography (HPLC) column.

AFEX Pretreatment. A bench-top reactor consisted of a 22 ml # 316 stainless steel pressure vessel (PARR Instrument Co, IL). The vessel was loaded with feedstock containing the appropriate moisture content. The vessel was clamped shut and the required amount of ammonia was injected using a pre-weighed sample cylinder. The reactor was heated by placing it inside a slotted aluminium block attached to a Vela hot plate (Cole Parmer, Inc.). The slots in the aluminium heating block were precision mille to present a tight fit around the pressure vessel for even heating and good heat transfer. The reactor was maintained at the desired temperature during the course of the pretreatment. The residence time in the reactor depended on the feedstock treated (e.g. 5

minutes for corn stover and 30 minutes for poplar). It took approximately 30-60 minutes to complete one AFEX reaction. At the end of the residence time, the pressure was explosively released by abruptly opening the half a inch NPT #316 stainless steel ball valve installed on the reactor. The biomass was promptly removed from the reactor and left in the hood overnight to allow the residual ammonia to evaporate..

Washing. Some samples were washed after AFEX treatment to remove soluble lignin and other compounds prior to enzymatic hydrolysis. AFEX treated biomass was washed using distilled (de-ionized) water with a substrate to water loading of 1:10 (w/w). The slurry was mixed for 30 minutes and the wash liquid was removed from the substrate by squeezing the slurry through a filtration cloth (Miracloth, Calbiochem, CA) with typical pore size of 22-25 μm. The filtrate was centrifuged at 10,000 RPM (24,000 g) using a Beckman Coulter Avanti J-26 XP centrifuge, with a JLA-16.500 rotor, to remove fine solid particles which were added back to the solid stream. The wash stream was used for further oligosaccharide analysis.

Enzymatic Hydrolysis. The NREL standard protocol (LAP-009) was followed for enzymatic hydrolysis. All samples were hydrolyzed in a 0.05 M citrate buffer (pH 4.8) at 1% glucan loading with the necessary commercial cellulase enzyme (Spezyme CP generously provided by Genencor, CAS 9012-528) and β-glucosidase (Novo 188, Novozyme). All the enzymes were stored at 4 ^{o}C for until further use. Certain samples were also hydrolyzed using commercial xylanases (Multifect Xylanase, Genencor). The hydrolyzed samples were boiled to denature the enzymes and filtered through a 0.2

micron nylon membrane filter at predetermined time periods (72 and 168 h). The samples were frozen for subsequent high performance liquid chromatography (HPLC) sugar analysis. Sampling was performed at two intervals (72 and 168h) to determine glucan and xylan conversions. The protein concentrations of the enzymes were determined by the BCA protein assay (Pierce, Rockford, IL). The protein concentrations of the respective enzymes were as follows; Spezyme CP (123 mg/ml; 59 FPU/ml, where FPU is filter paper units), β-glucosidase (130 mg/ml) and Multifect xylanase (42 mg/ml).

Mass Balance: A mass balance for AFEX pretreatment and enzymatic hydrolysis (1% glucan) of corn stover and poplar was performed starting with 100 grams (dry weight) of biomass. Experiments were done in duplicates, with standard deviations less than 5%. For each process step, the glucan, xylan and arabinan compositions of the solid and liquid streams were determined using the NREL LAP protocol. For poplar, either 31.3 or 125 mg protein/g of glucan of cellulase was used, while, for corn stover only 31.3 mg protein/g of glucan of cellulase was used. In both cases 33.3 mg protein/g of glucan of β-glucosidase was added to prevent cellobiose inhibition. In addition, xylanase was also supplemented for corn stover (3.1 mg protein/g of glucan) and poplar (31.3 or 125 mg protein/g of glucan). The total masses of each enzyme added (including cellulase, β-glucosidase and xylanase) are also shown in the mass balance. These experiments were run in duplicates and showed standard deviations below 5%.

LC-MS/MS Analysis of Degradation Products after AFEX: Analytical characterization of organic degradation products was carried out at Baylor University.

Details of the analytical methodology have been reported elsewhere (*37*) and are summarized below. Degraded products in the pretreated solids were initially extracted with water at 70 °C using an ASE-200 accelerated solvent extraction apparatus (Dionex Corp., Sunnyvale, CA, USA).The pH of the solution was found to be alkaline and was acidified to pH 2 before processing as given in the protocol (38). A 5-ml aliquot of each aqueous extract (hereafter referred to as the aqueous wash stream) was then extracted two times with methyl *tertiary*-butyl ether (MTBE) using the procedure reported by Chen *et. al*. (*38*). The resulting MTBE phases were combined and solvent was evaporated at 55 °C under a gentle stream of nitrogen. All samples were reconstituted in 5 ml water prior to analysis.

Instrumentation employed for analysis consisted of a Varian (Palo Alto, CA, USA) ProStar Model 210 binary pump system, Model 410 auto sampler, and Model 1200L triple-quadrapole mass analyzer. A binary solvent gradient consisting of two solvents A and B. Solvent A consists of aquous formic acid (0.025% (v/v) formic acid in water) and solvent B consists of 10% aqueous formic acid and 90% acetonitrile was used to achieve chromatographic separation on a 150 mm × 4.6 mm (S 03 µm, 99) YMC Carotenoid column (Waters, Milford, MA, USA) connected in series to a 1 mm RP C18 OPTI-Guard column (Altech, Deerfield, IL, USA). Additional chromatographic parameters were as follows: injection volume, 50 µL; column temperature, 30 ºC; flow rate, 750 µL/min. It is important to note that these chromatographic conditions are similar to those reported in previous work. The only major difference was the substitution of formic acid for phosphoric acid. This change was implemented to improve mobile phase compatibility

with MS detection. Upon exiting the column, the mobile phase was directed to both a UV-visible photodiode array (PDA) detector and the mass analyzer, which was operated exclusively in negative electro spray ionization (–ESI) mode. The majority of target analytes were assessed by monitoring an optimized MS/MS for each compound, with parent ion $[M - H]^-$ selected in the first quadrapole. A microL flow-splitter was inserted between the PDA detector and the mass spectrometer such that the volume of liquid passing through the flow-splitter was diverted 50:50 between the mass analyzer and the waste line. Mass spectrometry parameters held constant during all experiments were as follows: nebulizing gas, O_2 at 60 psi; drying gas, N_2 at 20 psi; drying gas temperature, 400 °C; needle voltage, 4500 V; collision gas, Ar at 2.0 mTorr. Exceptions include acetic acid, furfural, and 5-hydroxymethylfurfural (5-HMF) which were not amenable to mass spectral monitoring under these conditions and were instead detected via UV spectroscopy.

Accelerated solvent extractor (ASE) protocol: The extraction was done using a Dionex ASE 200 extractor at 70°C with two cycles of water. The samples used for each extraction were between 0.5-1 gm (in 11 ml cell) at 70 °C and 1500 psi with static time for 10 mins and purge time for 60 seconds.

Quantitation of target analytes by mass spectrum analysis: This was accomplished using a multipoint, internal-standard calibration curve. Calibration standards were prepared by successive dilutions of a stock solution consisting of the neat chemicals in water. Aliquots of each calibration standard were extracted with MTBE prior to analysis

(as described above). Response factors were determined for each analyte by dividing the peak area of the analyte by the peak area of the internal standard, and calibration curves were constructed by plotting a linear regression ($r^2 \geq 0.99$) of response factor versus analyte concentration. It has been previously demonstrated (*38*) that this approach to quantitation does not require independent knowledge of extraction efficiencies in order to assess analyte concentrations. However, the approach does not correct for the potential influence of co-extracted matrix components on electro spray ionization.

For this reason, data quality was assessed via analysis of a matrix spike for each analyzed sample. Calculated spike recoveries (data not shown) revealed negligible matrix influence in samples derived from untreated poplar (i.e., spike recoveries were essentially quantitative in these samples). More pronounced matrix interference was identified for some analytes in both samples derived from pretreated materials, and matrix effects were most pronounced in the sample derived from high-lignin poplar. Nevertheless, spike recoveries demonstrated that analyte concentrations derived from calibration curves were accurate within a factor of 1-3, independent of sample type, and this was deemed sufficient to support the goals of the present study.

Monomeric and Oligomeric Sugar Analysis: A high performance liquid chromatography (HPLC) system was used for sugar analysis. The HPLC system consisted of Waters (Milford, MA) Pump and Waters 410 refractive index detector. An Aminex HPX-87P carbohydrate analysis column (BioRad, Hercules, CA) equipped with a deashing guard cartridge (BioRad) was used for quantifying sugars in hydrolyzate.

Degassed HPLC grade water was used as the mobile phase at 0.6 ml/min at a column temperature of 85 ^{o}C. The injection volume was 10 µl with a run time of 20 min. Mixed sugar standards were used to quantify cellobiose and other monosaccharides (glucose, xylose, galactose, arabinose and mannose) in the samples.

Oligosaccharides in the liquid stream were quantified by acid hydrolysis based on the NREL LAP protocol (http://www.nrel.gov/biomass/pdfs/42623.pdf). The monomeric sugars produced after acid hydrolysis were quantified by high-performance liquid chromatography (HPLC) using a Bio-Rad Aminex HPX-87H ion exclusion column (60°C; 5 mM H_2SO_4; flow rate, 0.6 ml min^{-1}; injection volume, 10 µl) and differential refractive index detector.

FT-IR ATR analysis: A Spectrum One FTIR system (Perkin Elmer, Wellesley, MA) with a universal ATR (Attenuated Total Reflection) accessory was used to qualitatively monitor chemical changes in the AFEX treated and untreated poplar and corn stover respectively. The samples were pressed uniformly and tightly against the diamond surface using a spring-loaded anvil. Mid-IR spectra were obtained by averaging 4 or 16 scans from 4,000 to 600 cm^{-1} at 2 cm^{-1} resolution. Baseline and ATR corrections for penetration depth and frequency variations were carried out using the Spectrum One software supplied with the equipment. The region between 1,550 and 1800 cm^{-1} was selectively monitored to check the ester and amide linkage stretching frequency at 1740 and 1664 cm^{-1} respectively.

Results and Discussion:

Poplar AFEX Optimization. In order to optimize the AFEX condition for poplar, both moisture and temperature were varied for fixed ammonia to biomass loadings of 1:1 (w/w). Pretreated samples were tested at 1% glucan loading (15 ml reaction volume) using 31.3 mg of celluase and 33.3 mg of β-glucosidase per gram of glucan at 50 ^{o}C over a period of 168 hours. As we raised the pretreatment temperature from 120 ^{o}C to 200 ^{o}C we saw a steady increase in glucan conversions. Further increases in glucan conversion were also noticed when we raised the moisture from 50% to 233% (dwb, dry weight basis of feedstock) (Figure 1)

Based on preliminary AFEX optimization studies we found that higher glucan conversions were obtained for AFEX done at 180 ^{o}C and 233% (dwb) moisture. To study the effect of varying ammonia to biomass loadings the pretreatment temperature and moisture was held at 180 ^{o}C and 233% (dwb) moisture, respectively. Of the three ammonia:biomass loadings (i.e. 1:1, 2:1 and 3:1, w/w) , 2:1 loading gave the highest glucan conversion (Figure 1). Compared to acid pretreatment, AFEX is a relatively dry to dry process where both cellulose and hemicellulose are retained in the solids stream. Commercially available cellulases (e.g. Spezyme CP) do not have sufficient hemicellulase activity to adequately digest AFEX treated biomass (*39*). Therefore, we supplemented it with xylanases (using Multifect Xylanase) at varying concentrations (0-100% of the total milligrams of cellulase protein) (figure not shown). Increasing xylanase supplementation increases both glucan and xylan conversion. There is however little

improvement noticed when xylanase loadings were beyond 100% of the total cellulase protein loadings. For the sake of simplicity we have only shown enzymatic hydrolysis results for xylanase supplementation at 100% of total cellulase protein. About 15% improvement in glucan conversion was noticed for enzymatic hydrolysis done with xylanase supplementation when compared to using cellulase alone. Xylanase supplementation aids the removal of xylan polymer embedded in the cellulose matrix which in turn synergistically helps improve cellulase accessibility to cellulose (39). In addition, 125 mg per gram of glucan of cellulase loading produced about 25% higher glucan conversion when compared to 31.3 mg of cellulase per gram of glucan for AFEX treated Poplar (Figure 1).

AFEX Pretreatment for Poplar vs. Corn Stover. It is well known that grasses (monocots) and woody (dicots) species have a complex cell wall structure which are quite different from each other. Hardwoods are a good source of cellulosic fiber (higher than stover cellulose content) and their lignin and monolignol composition are very different from corn stover. Our results show that poplar needed much higher temperature, moisture and ammonia loadings during AFEX pretreatment in order to achieve significant glucan hydrolysis yields (using 31.3 mg of cellulase and 33.3 mg of β-glucosidase per gram of glucan) compared to corn stover. Both untreated and AFEX treated corn stover hydrolysis results were similar to the one we reported earlier (22). Even higher temperatures (e.g. 180 ^{o}C) could yield close to 50% glucan and 35% xylan conversion, with 1:1 ammonia to biomass loadings. (Figure 2). For corn stover, the best AFEX condition was found to be close to 90 ^{o}C, 60% moisture and 1:1 ammonia to

biomass loadings. Closer inspection of the biomass composition (Table 1), reveals that corn stover has lower glucan, lignin and higher xylan, arabinan and ash content compared to poplar. Corn stover was also found to contain higher extractives (8.5%, of which 2.2% is sucrose) compared to poplar (~3.4%). Of the various components in biomass, it has been demonstrated that the percentage of lignin in biomass will influence the enzymatic hydrolysis (40). Since ammonia cleaves the arabinoxylan and acetyl-xylan ester linkages, their composition in biomass appear to be a determining factor on ultimate sugar yields during pretreatment using ammonia (41-42).

Lignin, an aromatic polymer, is one of the major components contributing to biomass recalcitrance. Lignin binds irreversibly to enzymes and reduces available enzyme activity during hydrolysis (43). Pretreatment improves enzyme accessibility by cleaving certain lignin and lignin-hemicellulose cross-linkages (*8*). In acidic pretreatments and organosolv processing, most of the hemicellulose and some of the lignin is hydrolyzed and chemically extracted from the insoluble cellulose matrix. AFEX also produces some lignin derived products but does not physically separate them into a separate stream. Water washing the biomass prior to enzymatic hydrolysis improves glucan conversion by up to 7% for corn stover (*22*) and by up to 6% for poplar for low enzyme loading (results not shown). Multiple explanations are possible for this observation including: 1) presence of enzyme-inhibiting products produced during AFEX pretreatment, 2) partial lignin removal leading to less enzyme adsorbtion to lignin or 3) opening up of the cellulose-hemicellulose-lignin matrix thereby allowing easier penetration of enzymes.

In addition to lignin, the arabinoxylan composition of monocots is an important factor affecting digestibility of the biomass. Most of the arabinoxylan in the cell wall is connected to lignin through ether and ester linkages (*5, 14-17*). These linkages are typically between glucuronic acid and arabinose side chains of hemicelluloses and hydroxyl/carboxyl functionalities of lignin (e.g. ferulic and coumaric acid). Ammonia has tendency to cleave these ester linkages (*18, 20*) via ammonolysis. It is interesting to note that the arabinoxylan content of grasses (3-6%) is significantly higher than hardwoods (<1%, for poplar). With a higher arabinoxylan content of the cell wall, more ester linkages are likely to be cleaved during AFEX pretreatment. Arabinoxylans help form bridges between lignin and hemicellulose/cellulose that reduce enzyme accessibility. Cleavage of these linkages would help increase the cell wall pore volume, reduce the protective barrier of lignin and enhance enzyme accessibility to cellulose and hemicellulose. This is one factor that could explain why grasses are more easily digestible after AFEX pretreatment compared to hardwood poplar. Further support for this hypothesis would come from detailed quantification of oligosaccharides and other degradation products using Matrix-Assisted Laser Desorption Ionization-Time Of followed by mass spectroscopy (MALDI-TOFMS) produced during AFEX pretreatment for both corn stover and poplar.

Mass Balance. A complete mass balance for AFEX pretreatment and enzymatic hydrolysis was done for both corn stover and poplar. For poplar, enzymatic hydrolysis was performed either using 31.3 or 125 mg of cellulase per gram of glucan, while for corn stover only 31.3 mg of cellulase per gram of glucan was used. In both cases, 33.3

mg of β-glucosidase per gram of glucan was used to prevent cellobiose inhibition of the cellulases. In addition, xylanase was supplemented for both corn stover (3.1 mg/g of glucan) and poplar (either 31.3 or 125 mg/g of glucan). Both for corn stover and poplar the mass balance was done for both unwashed washed material after AFEX pretreatment and prior to enzymatic hydrolysis.All enzymatic hydrolysis experiments were done in duplicates, at a 100 ml scale with 1% glucan loading for 168 h at 50 ^{o}C (Figure 4 and 5). Close to 95% mass closure was achieved both for corn stover and poplar. For poplar, about 66 % glucan and 44% xylan conversion (31.3 mg of cellulase, 33.3 mg of β-glucosidase and 31.3 mg of xylanase per gram of glucan) was noticed for lower enzyme loading in unwashed samples. On the other hand, for higher enzyme loadings (125 mg of cellulase, 33.3 mg of β-glucosidase and 125 mg of xylanase per gram of glucan), we get 93% glucan and 65% xylan conversion respectively. When we washed poplar sample between pretreatment and enzymatic hydrolysis we see an improvement (~5%) in glucan and xylan conversion at lower enzyme loading. However, no improvement was noticed in glucan conversion when we increased the enzyme loading up to 125 mg of cellulase and xylanase per gram of glucan (Figure 4).

For corn stover (using 31.3 mg of cellulase, 33.3 mg of β-glucosidase and 1.4 mg of xylanase/gram of glucan), the glucan and xylan conversions for unwashed samples (88% and 68%) and washed samples (93% and 66%) are shown in Figure 5. Compared to unwashed corn stover, we see an increase in glucan and a slight decrease in xylan conversion for washed samples. As reported earlier *(44, 45)* washing helps reduce

removal of several degradation products like acetic acid, phenolic acids, HMF, furfural which are potentially inhibitory during enzymatic hydrolysis.

Oligosaccharide analysis.. The wash streams generated from untreated/AFEX treated poplar and corn stover were analyzed for monomeric and oligomeric sugars using the Aminex 87P column giving rather interesting results. The untreated poplar wash stream had a little monomaric/polysaccharide, while untreated corn stover had approximately 26.0, 0 and 0.12 mg of glucose, xylose and arbinose per gram of biomass respectively (Table 2). We also saw an increase in glucose, xylose and arabinose concentration of 27.8, 1.22 and 0.39 mg respectively per gram of biomass after acid hydrolysis of the untreated corn stover wash stream. This confirms the presence of very low concentrations of short-chain saccharides (mostly DP 2 based on Aminex 87P chromatograms, data not shown) in untreated corn stover, unlike poplar.

The AFEX pretreated poplar wash stream also showed very low concentrations of monomeric sugars prior to acid hydrolysis. After acid hydrolysis, we observe a small increase in glucose concentration and about 54 fold increases in xylose concentration and 0.8 fold increases in arabinose concentration (Table 2). Compared to untreated corn stover, AFEX pretreated samples have decreased monomeric glucose concentration by up to 6.6 while there is not much change in xylose and arabinose concentration. The reduction in soluble sugar concentration during AFEX pretreatment could be due to alkali induced degradation of these monomeric sugars (*46*). Upon performing mild acid hydrolysis of AFEX treated corn stover wash stream, we observe about 2.9, 300 and 79

fold increase in glucose, xylose and arabinose concentrations, respectively. Our present results are in accordance with previous work (*47, 48*) in which aqueous ammonia treated corn plant fractions produced more arabinoxylans in the wash stream. Hemicellulose contains multiple side chains, rich in arabinose as the linker molecule bound to other hemicellulose side chains or phenolic acids bound to lignin, that cause steric inhibition of enzymes (*49*). AFEX cleaves these lignin-hemicellulose ester linkages, thereby releasing water extractable arabinoxylan rich oligomers along with other phenolics. The wash stream is now being further analyzed to determine the effect of biomass source and AFEX pretreatment severity on the nature of oligosaccharides obtained.

FT-IR Analysis. Further evidence confirming cleavage of ester linkages in both corn stover and poplar comes from FT-IR analysis. Both untreated and AFEX treated samples were analyzed for various stretching frequencies in the region 1550 to 1800 cm^{-1}. Some of the important peaks identified from literature that can serve to illustrate the effect of AFEX on biomass composition are the ester carbonyl peak at 1740 cm^{-1}, the aldehyde peak at 1640 cm^{-1} and amide linkages at 1664 cm^{-1} (*18, 19*) The ester-carbonyl bonds are typically present in the hemicellulose and hemicellulose–lignin complexes. A decrease in these peaks at 1740 and 1640 cm^{-1} are directly related to deesterification of hemicellulose during AFEX (*18-20*). From figure 3 we can see that the stretching ester carbonyl frequency at 1740 cm^{-1} totally disappears upon AFEX pretreatment. A 1640 cm^{-1} peak appears in both AFEX pretreated samples of corn stover and poplar. This observation further confirms the fact that hemicellulose based ester linkages are ammonolyzed, resulting in the formation of their respective amides during AFEX.

Degradation product analysis by LC-MS. ASE/water extractions of AFEX treated samples gave insight into the mechanism of AFEX, which was further explored by qualitative and quantitative analysis of the extract. The protocol employed for water washing the biomass is reported elsewhere (*22*). Approximately 6-8% and 13-15% by mass (based on initial dry weight) of the untreated and AFEX treated corn stover, respectively was lost in the washing step. These results are also consistent with previously reported water wash data (weight loss of 12%, based on dry weight of biomass) for super/sub-critical ammonia treated birch wood (*50, 51*). However, the wash extractive for ammonia treated birch wood was found to be largely acetamide (5-7% based on dry wt) formed due to the ammonolysis of the heavily acetylated hemicellulose under severe reaction conditions. Detailed compositional analysis of the wash extractives for AFEX treated poplar showed an interesting range of compounds as explained below.

Comparison of ASE extracts derived from untreated and AFEX-treated poplar demonstrated that the concentrations of certain compounds increased several folds following AFEX pretreatment. Analytical concentrations (μg/g dry weight of extracted material) of components monitored by LC-MS/MS are shown in Table 3. These data demonstrate that AFEX treatment resulted in the production of a variety of aliphatic organic acids. As compared to samples derived from untreated poplar, concentrations of lactic, malonic, methylmalonic, succinic and levulinic acids in wash streams derived from treated materials were increased between 10 and 190 fold. The highest concentrations were observed for lactic acid, which is known to be formed via alkali induced peeling and

terminal degradation of polymeric sugars (*23*). Succinic acid was also present at relatively high concentrations. Furfural and 5-HMF were not detected in AFEX treated samples..However, a noticeable increase in the concentration of 2-furoic acid was observed in both AFEX treated samples as compared to the untreated materials. It is possible that furoic acid could have been formed directly via hydrolytic/oxidative cleavage of lignin. The cleavage of the acetyl groups in hemicellulose and lignin also resulted in increased concentrations of acetic acid in the AFEX wash streams (data not shown). Sugar-derived aldehydes were not monitored as part of this work, as they are expected to undergo condensation reactions under high temperature and alkaline conditions (*52*). Some of these compounds were found to inhibit microbes during ethanol fermentation (*53, 54*).

AFEX also released measurable amounts of various aromatic acids and aldehydes, presumably due to base-catalyzed cleavage of lignin polymers. A significant increase in the phenolic content of the wash streams was observed following AFEX treatment, as indicated by increased concentrations of salicylic acid, 4-hydroxybenzaldehyde, syringic acid, vanillin, vanillic acid, homovanillic acid, 4-hydroxyacetophenone, benzoic acid and syringaldehyde. Phenolic acids such as 4-hydroxycoumaric acid and ferulic acid are expected to be produced upon hydrolysis of hemicellulose-lignin ester cross-links (*25*). However, relatively low concentrations of these compounds were noticed for AFEX-treated poplar. It is possible that these components were first formed and then further degraded via hydrolytic/oxidative cleavage at the more severe poplar pretreatment conditons, resulting in lower observed concentrations. Phenolic aldehydes are more

likely to be produced in oxidative alkaline conditions (*24*). Nevertheless, some components such as 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid are thought to be monomeric lignin extractives that are easily released after the AFEX process, unlike other lignin cleavage products (*26, 27*).

Conclusion:

Varying AFEX pretreatment conditions and enzyme combinations were tested on poplar and corn stover. Based on different AFEX conditions tested, it was found that the optimal AFEX conditions for poplar (2:1 ammonia to biomass loading, 233% moisture on dwb and 180 ^{o}C) and for corn stover (1:1, ammonia to biomass loading, 60% moisture and 90 ^{o}C) respectively. Adding xylanase enzymes along with commercial cellulase preps improved both glucan and xylan conversion both for poplar and corn stover. Complete mass balance for both pretreatment and hydrolysis has been shown for both poplar and corn stover. Based on the present results, corn stover required much less severe AFEX conditions (i.e. less ammonia and lower treatment temperatures) compared to poplar. These differences have been correlated to both lignin and arabinoxylan content of the biomass. The ester linkages connecting arabinoxylan to lignin phenolics are broken during AFEX based on evidence from FT-IR and wash stream oligosaccharides analysis. In addition to oligosaccharides, several aliphatic and aromatic organic acids were also generated from both high and low lignin poplar. These were quantified using a recently-developed LC-MS/MS methodology. Required pretreatment severity and enzyme consumption both might be significantly reduced by making alterations to several cell

wall components (e.g. lignin and arabinoxylan content). Comparison of other herbaceous and woody species will help us better understand the relationships between biomass composition, cell wall ultra-structure to effectiveness of AFEX pretreatment and enzymatic hydrolysis.

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References:

1. Ohara, H. Biorefinery, *Appl. Microbiol. Biotechnol.* **2003**, *62*, 474–477.
2. Ragauskas, A. J.; Williams, C. K.; Davison, B. H.; Britovsek,G.; Cairney, J.; Eckert, C. A.; Frederick Jr, W. J.; Hallett, J. P.; Leak, D. J.,; Liotta, C. L.; Mielenz,J. R.; Murphy, R.; Templer, R.; Tschaplinski, T. The Path Forward for Biofuels and Biomaterials. *Science* **2006**, *311*, 484-489.
3. Gray, K. A.; Zhao, L.; Emptage, M. Bioethanol. *Current Opinion in Chemical Biology* **2006**, *10*, 141–146.
4. Mantanis, G. I.; Young, R. A.; Rowell, R. M. Swelling of compressed cellulose fiber webs in organic liquids. *Cellulose* **1995**, *2*, 1-22.
5. Bidlack, J.; Malone, M.; Benson R. Molecular Structure and Component Integration of Secondary Cell Walls in Plants. *Proc. Okla. Acad. Sci.* **1992**, *72*, 51-56.
6. Somerville, C.; Bauer, S.; Brininstool, G.; Facette, M.; Hamann, T.; Milne, J.; Osborne, E.; Paredez, A.; Persson, S.; Raab, T.; Vorwerk, S.; Youngs, H. Toward a Systems Approach to Understanding Plant Cell Walls. *Science* **2004**, *306*, 2206 – 2211.
7. Grabber, J. H. How Do Lignin Composition, Structure, and Cross-Linking Affect Degradability? A Review of Cell Wall Model Studies. *Crop Sci.* **2005**, *45*, 820–831.
8. Mosier, N.; Wyman, C.; Dale, B. E.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96*, 673-686

9. Dale, B. E, Method for increasing the reactivity and digestibility of cellulose with ammonia. **1986**, US patent No. 4600590.

10. Eggeman, T.; Elander, R. T. Process and economic analysis of pretreatment technologies. *Bioresource Technol.* **2005**, *96*, 2019–2025.

11. Teymouri, F. Laureano-Perez, L.; Alizadeh, H.; Dale, B. E. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresource Technol.* **2005***, 96*, 2014–2018.

12. Chang, M. C. Harnessing energy from plant biomass. *Curr Opin Chem Biol.* **2007**, *11*, 677-684.

13. Chen, F.; Dixon, R.A. Lignin modification improves fermentable sugar yields for biofuel production. Nature Biotechnology 2007, 25, 759 – 761.

14. Jeffries, T. W. Biodegradation of lignin and hemicelluloses. *Biochemistry of Microbial Degradation, C. Ratledge (ed.), Kluwer Academic Publisher, Netherlands,* **1997***, p*233–277.

15. Hatfield, R. D.; Ralph, J.; Grabber, J. H. Cell wall cross-linking by ferulates and diferulates in grasses. *J. Sci. Food Agric.* **1999**, *79*, 403-407.

16. Cosgrove, G. J. Growth of the plant cell wall. Nature reviews **2005**, 6, 850 - 861.

17. Ralph J. What Makes a Good Monolignol Substitute? In The science and Lore of the Plant cell wall: Biosynthesis, structure and function, Hayashi T (eds.) Brown walker press, Boca Raton. **2006**, *p* 285-293.

18. Buettner, M. R.; Lechtenberg, V.L.; Hendrix, K. S.; Hertel, J. M. Composition and Digestion of ammoniated tall fescue Hay. *J. Animal Science* **1982**, *54*, 173-178.

19. Pawlak, Z.; Pawlak, A.S. A Review of Infrared Spectra from Wood and Wood Components Following Treatment with Liquid Ammonia and Solvated Electrons in Liquid Ammonia. *Applied Spectroscopy Reviews* **1997**, *32*, 349–383.

20. Rosca, L.; Puhringer, R.; Schmidt, H.; Tanczos, I. New aspects in studying and application of ammonia treatment of softwood. Kudela J, Kurjatko S (edt), Wood Structure and Properties Arbora publishers. **2002**, *p* 127-129.

21. Grabber JH, Hatfield RD, Lu F, Ralph J. Coniferyl ferulate incorporation into lignin enhances the alkaline delignification and enzymatic degradation of cell walls. Biomacromolecules. **2008**, 9, 2510-2516.

22. Chundawat, P.S.; Venkatesh, B.; Dale, B. E. Effect of Particle Size Based Separation of Milled Corn Stover on AFEX pretreatment and Enzymatic Digestibility. *Biotechnol. Bioeng.* **2007**, *96*, 219-231.

23. Sjöström, E. Carbohydrate degradation products from alkaline pretreatment of biomass. *Biomass Bioenergy* **1991**, *1*, 61–64.

24. Klinke, H.B.; Ahring, B.K.; Schmidt, A.S.; Thomsen, A. B. Characterization of degradation products from alkaline wet oxidation of wheat straw. *Bioresour. Technol.* **2002**, *82*, 15–26.

25. Lawther, J. M.; Sun, R. The fractional characterisation of polysaccharides and lignin components in alkaline treated and atmospheric refined wheat straw. *Ind. Crops Prod.* **1996**, *5*, 87–95.

26. Baeza, J.; Freer, J. Chemical characterization of wood and its components. In: Hon DNS, Shirashi N (eds) Wood and cellulosic chemistry. Dekker, New York, **1997**, p 275–374.

27. Jönsson, L. J.; Palmqvist, E.; Nilvebrant, N. O.; Hahn-Hägerdal, B. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 691–697

28. Leonard, R. H.; Hajny, G. J. Fermentation of wood sugars to ethyl alcohol. *Ind. Eng. Chem.* **1945**, *37*, 390–395.

29. Sierra-Alvarez, R.; Lettinga, G. The methanogenic toxicity of wastewater lignins and lignin related compounds. *J. Chem. Tech. Biotechnol*. **1991**, *50*, 443–455.

30. Palmqvist, E.; Hahn-Hägerdal, B. Fermentation of lignocellulosic hydrolysates. I. Inhibition and detoxification. *Bioresour. Technol.* **2000**, *74*, 17–24.

31. Cantarella, M,; Cantarella, L.; Gallifuoco, A.; Spera, A.; Alfani, F.; Effect of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF. *Biotechnol Prog*. **2004**, *20*, 200-206.

32. Pan, X.; Gilkes, N.; Kadla, J.; Pye, K.; Saka, S.; Gregg, D.; Ehara, K.; Xie, D.; Lam, D.; Saddler, J. Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: optimization of process yields. *Biotechnol. Bioeng.* **2006**, *94*, 851-61.

33. Esteghlalian, A.; Hashimoto, A, G.; Fenske, J. J.; Penner, M. H. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresource Technology.* **1997**, *59*, 129-136.

34. Davison, B.H.; Drescher, S. R.; Tuskan, G. A.; Davis, M. F.; Nghiem, N. P. Variation of S/G ratio and lignin content in a Populus family influences the release of xylose by dilute acid hydrolysis. *Appl. Biochem. Biotechnol.* **2006**, *129-132*, 427-435.

35. Chang, V. S.; Nagwani, M.; Kim, C. H.; Holtzapple, M. T. Oxidative lime pretreatment of high-lignin biomass: poplar wood and newspaper. *Appl. Biochem. Biotechnol.* **2001**, *94*, 1-28.

36. LAP protocol available at National Renewable Energy Lab website *(http://www.nrel.gov/biomass/analytical_procedures.html).*

37. Sharma, L. N.; Becker, C.; Chambliss, C. K. Analytical characterization of fermentation inhibitors in biomass pretreatment samples using liquid chromatography, UV-visible spectroscopy, and tandem mass spectrometry," *Methods in Molecular Biology* **2008** (in press).

38. Chen, S-F.; Mowery, R. A. Castleberry VA, van Walsum GP, and Chambliss CK High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates. *J. Chromatogr. A*, **2006**, *1104*, 54-61.

39. Dien BS, Ximenes EA, O'Bryan PJ, Moniruzzaman M, Li XL, Balan V, Dale B, Cotta MA Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol. *Bioresour. Technol.* **2007** (ahead of print, doi:10.1016/j.biortech.2007.09.030).

40. Vanholme, R.; Morreel, K.; Ralph, J. And Boerjan, W. Lignin engineering. Current opin. plant Biol. 2008, 11, 278-285.

41. Lizbeth L-P, Teymouri, F.; Alizadeh, H.; Bruce E Dale, B. E. Understanding factors that limit enzymatic hydrolysis of biomass: characterization of pretreated corn stover. Appl Biochem Biotechnol. **2005**,121-124,1081-1099.

42. Laureano-Perez L, Dale BE, Zhu L, O'Dwyer JP, Holtzapple M. Statistical correlation of spectroscopic analysis and enzymatic hydrolysis of poplar samples.Biotechnol Prog. **2006**, 22, 835-841.

43. Sutcliffe, R. and Saddler, J.N. The role of lignin in the adsorption of cellulases during enzymatic treatment of lignocellulosic material. *Biotechnol. Bioeng. Symp.* 1986, **17**, 749–762.

44. Maria Cantarella, M.; Cantarella, L.; Gallifuoco, A.; Spera, A.; and Alfani, F. Effect of Inhibitors Released during Steam-Explosion Treatment of Poplar Wood on Subsequent Enzymatic Hydrolysis and SSF. *Biotechnol. Prog.* **2004**, 20, 200-206.

45. Sakai S, Tsuchida Y, Nakamoto H, Okino S, Ichihashi O, Kawaguchi H, Watanabe T, Inui M, Yukawa H. Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested Corynebacterium glutamicum R. *Appl Environ Microbiol.* **2007**, 73, 2349-53.

46. Agyei-Aye K, Chian MX, Lauterbach JH, Moldoveanu SC. The role of the anion in the reaction of reducing sugars with ammonium salts. **2002**, *Carbohydrate Research*, 337, 2273-2277.

47. Kurakake. M.; Kisaka, W.; Ouchi, K.; Komaki, T. Pretreatment with ammonia water for enzymatic hydrolysis of corn husk, bagasse and switchgrass. *Appl. Biochem. Biotech.* **1999**, *90*, 251-259.

48. Sewalt, V. J. H.; Fontenot, J. P.; Allen, V.G.; Glasser, W.G. Fiber composition and in Vitro digestibility of corn stover fractions in response to ammonia treatment. *J. Agric. Food Chem.* **1996**, *44*, 3136-3142.

49. Saulnier, L.; Thibault, J. F. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.* **1999**, *79*, 396–402.

50. Chou, Y. C. T. Supercritical ammonia pretreatment of lignocellulosic materials. *Biotechnol. Bioeng. Symp.* **1986**, *17*, 19-32.

51. Weimer, P.J.; Chou, Y. C. T. Weston. W.M., Chase, D.B. Effect of supercritical ammonia on the physical and chemical structure of ground wood. *Biotechnol. Bioeng. Symp.* **1986**, *17*, 5-18

52. March, J. Advanced organic chemistry reactions mechanisms and structure, 3rd ed. Wiley, New York, **1985**, *p*1346.

53. Caidian Luo, David L. Brink and Harvey W. Blanch, Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. *Biomass and Bioenergy* **2002**, *22*, 125-138.

54. Oliva, J. M.; Saez, F.; Ballesteros, I.; Gonzalez, A.; Negro, M. J.; Manzanares, P.; Ballesteros, M. Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast Kluyveromyces marxianus. *Appl. Biochem. Biotechnol.* **2003**, *105 -108*, 141-53.

Figure Captions:

Figure 1a: Enzymatic hydrolysis for AFEX treated poplar under varying conditions. Here, I) Glucan conversions for poplar as a function of AFEX conditions. All experiments were performed at 1:1 ammonia to biomass ratio and 30 minutes of residence time. In A) the effect of temperature was studied, fixing the moisture content at 233% (dwb). In B) the effect of moisture content on glucose conversions was studied using 180°C as a fixed temperature. All experiments used enzyme loadings of 31.3 mg of cellulase protein and 33.3 mg of β-glucosidase protein per gram of glucan. II.) Glucan conversions for poplar as a function of varying AFEX conditions and enzyme loadings. Here, experiments were done using two different enzyme loadings; (A) low enzyme loading (31.3 mg of cellulase protein, 33.3 mg of β-glucosidase protein per gram of glucan) and (B) high enzyme loading (125 mg of cellulase protein and 33.3 mg of β-glucosidase protein per gram of glucan). In some of experiments either 31.3 mg (low) or 125 mg (high) per gram of glucan of xylanase enzyme was also supplemented. The AFEX pretreatment conditions are at a fixed temperature (180 ^{o}C) and moisture (233%, dwb) and varying ammonia to biomass loadings (1:1, 2:1 and 3:1, w/w). All the hydrolysis experiments were done in duplicates.

Figure 2: Comparison of glucan (in dark bars) and xylan (in white bars) conversions for untreated, AFEX treated and washed AFEX treated samples prior to hydrolysis for both corn stover and poplar, respectively. Pretreatment temperatures during the AFEX process

are shown in the brackets. AFEX pretreatment was done using 1:1 ammonia to biomass loading for both the corn stover and poplar, with 60% moisture (corn stover) and 233% moisture (poplar) (% dry weight basis of substrate). Enzymatic hydrolysis was done using 31 mg/g of glucan of cellulase and 33 mg/g of glucan of β-glucosidase for both corn stover and poplar at 50 ^{o}C, for 168 h. All the hydrolysis experiments were done in duplicates.

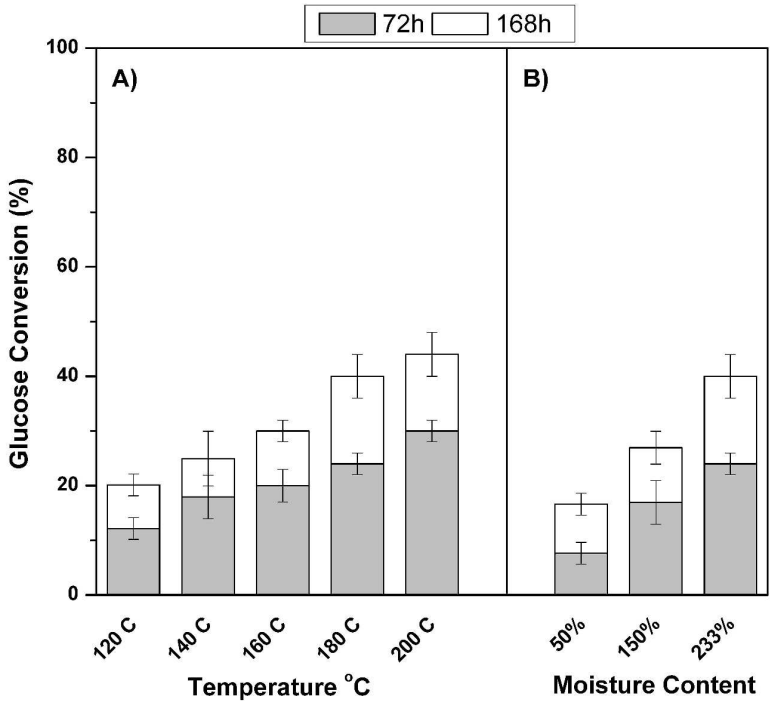
Figure 3: FT-IR ATR spectra for untreated/AFEX treated corn stover and poplar, respectively. Here, (i) Untreated corn stover (CS-UT), (ii) AFEX treated corn stover (CS-AFEX), (iii) Untreated poplar (Poplar-UT) and (iv) AFEX treated poplar (Poplar-AFEX). Stretching frequencies at 1664 cm^{-1} and 1740 cm^{-1} correspond to amide and ester linkages, respectively, as denoted by corresponding dotted lines.

Figure 4: Mass balance for AFEX treated poplar during pretreatment and enzymatic hydrolysis after 168h is shown. AFEX was performed at 180 °C and 700 psi for 30 minutes, using 2:1 ammonia to biomass ratio and 233% of moisture (dry biomass basis). Here, (A) unwashed poplar and (B) washed poplar after pretreatment and before enzymatic hydrolysis are represented. Enzymatic hydrolysis was done using 31.3 or 125 of cellulase protein and 33 mg of β-glucosidase protein per gram of glucan were used (results for the latter case of higher enzyme loading are shown in brackets for poplar). Multifect xylanase was supplemented using 31 or 125 mg protein/g of glucan.

Figure 5: Mass balance for AFEX treated corn stover during pretreatment and enzymatic hydrolysis after 168h is shown. AFEX was performed at 90°C for 5 minutes, using 1:1 ammonia to biomass ratio and 60% of moisture (dry biomass basis). Here, (A) unwashed corn stover and (B) washed corn stover after pretreatment and before enzymatic hydrolysis are represented. Enzymatic hydrolysis was done using 31.3 mg of cellulase protein and 33.3 mg of β-glucosidase protein per gram of glucan. Multifect xylanase was supplemented using 3.1mg /g of glucan in all experiments.

Figure 1

I


72h
168h
A)
B)
Glucose Conversion (%)
0
20
40
60
80
100
120 C
140 C
160 C
180 C
200 C
Temperature °C
50%
150%
233%
Moisture Content

II

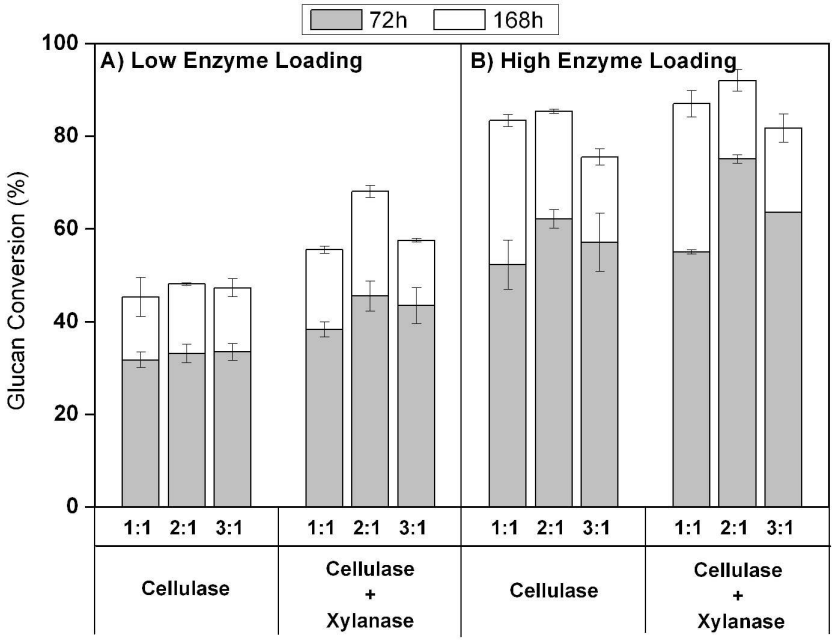

72h
168h
A) Low Enzyme Loading
B) High Enzyme Loading
Glucan Conversion (%)
0
20
40
60
80
100
1:1
2:1
3:1
Cellulase
Cellulase + Xylanase

Figure 2

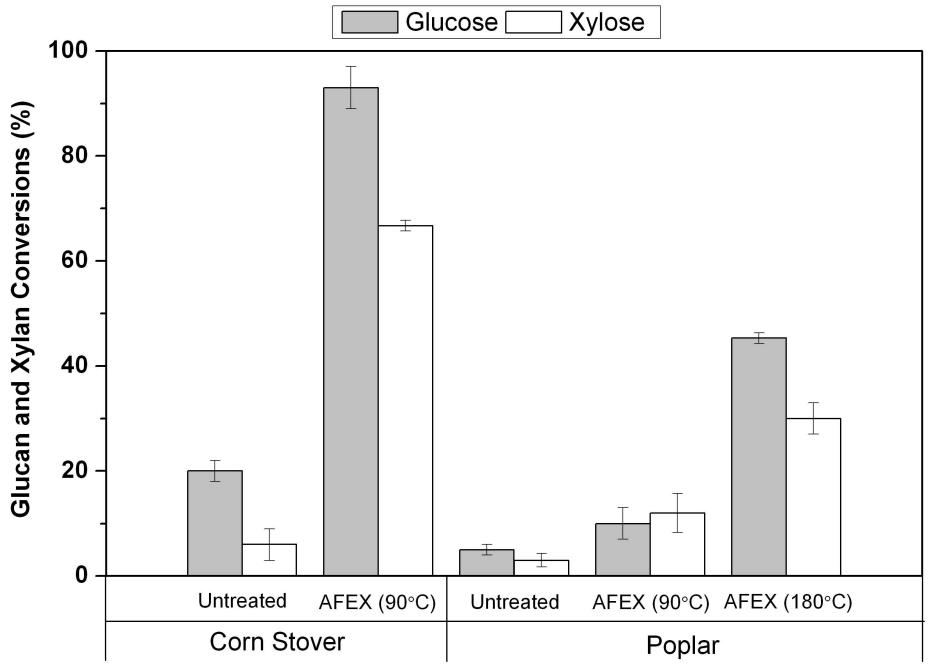

Glucose
Xylose
Glucan and Xylan Conversions (%)
100
80
60
40
20
0
Untreated
AFEX (90°C)
Untreated
AFEX (90°C)
AFEX (180°C)
Corn Stover
Poplar

Figure 3

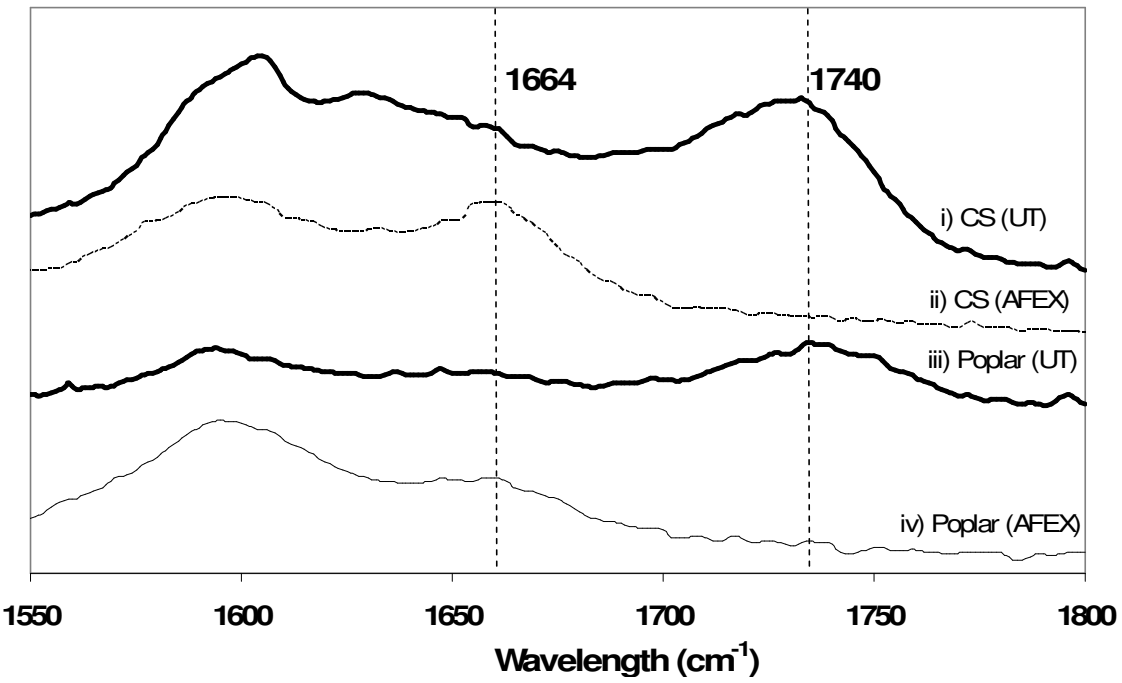

1664
1740
i) CS (UT)
ii) CS (AFEX)
iii) Poplar (UT)
iv) Poplar (AFEX)
1550
1600
1650
1700
1750
1800
Wavelength (cm-1)

Figure 4

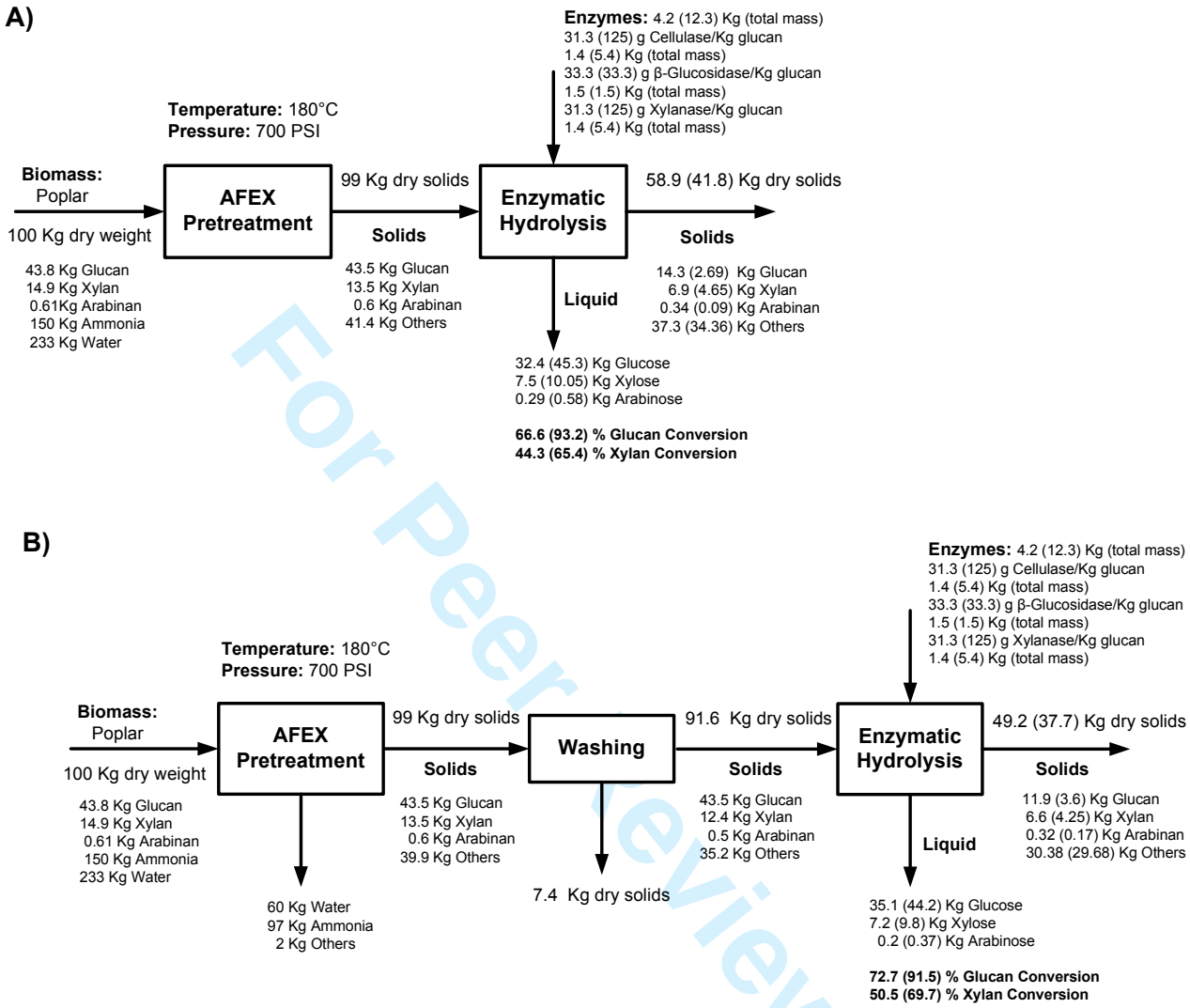


Figure 5

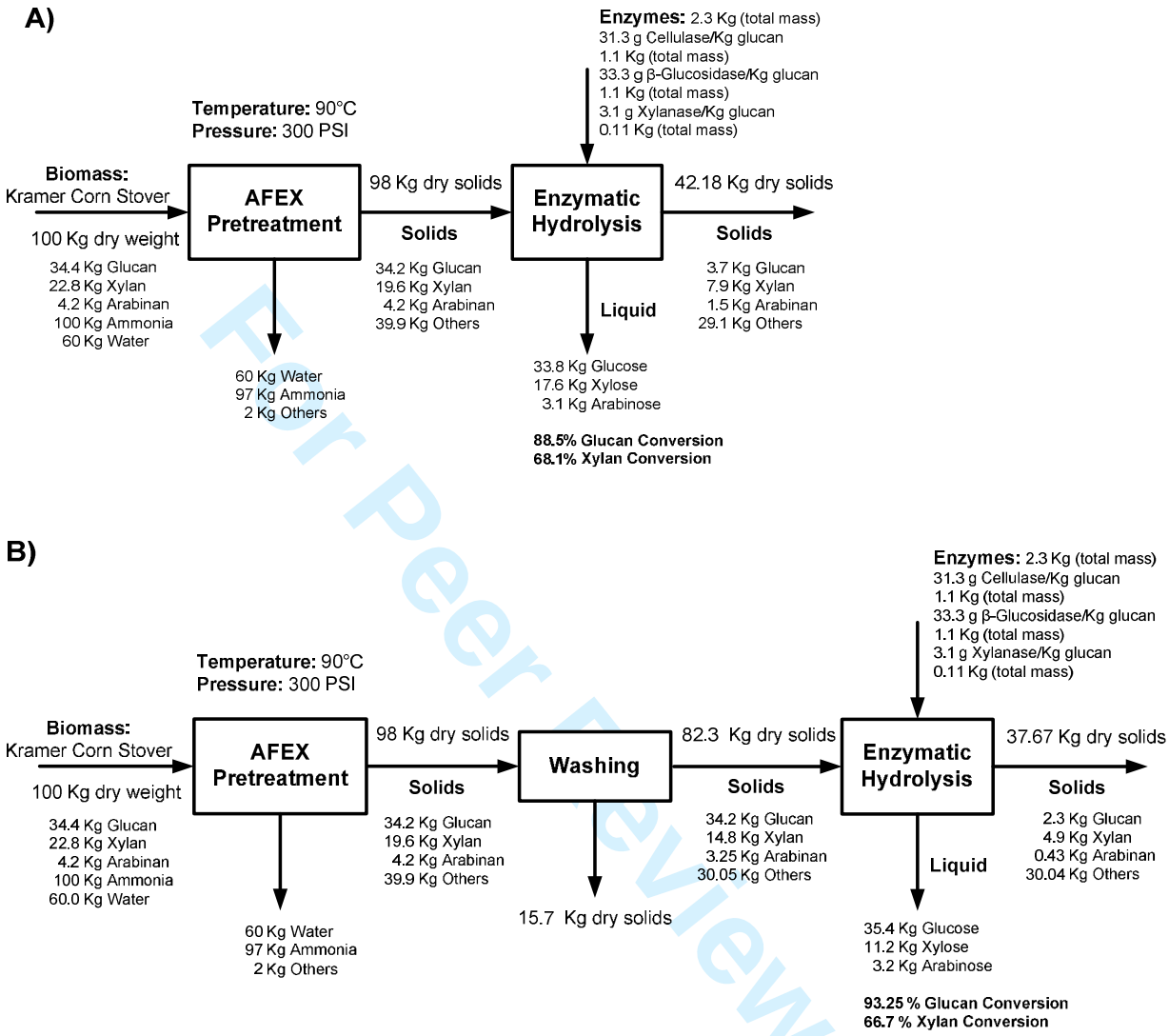


Table 1: Compositional analysis (% wt/wt, dry basis) for Kramer Corn Stover and High-lignin Poplar

Contents	Corn Stover	Poplar
Glucan	34.4	43.8
Xylan	22.8	14.9
Arabinan	4.2	0.61
Mannan	0.6	3.9
Galactan	1.4	1.0
Lignin	11.0	29.1
Protein	2.3	*Nd
Acetyl	5.6	3.6
Ash	6.1	1.1
**Extractives	8.5	3.6

* Nd- Not determined; ** Water extractives

Table 2: Water extractable carbohydrates produced during AFEX pretreatment of corn stover and poplar (mg per gm of original dry biomass). All experiments were done in triplicates.

	Glucose	**Xylose**	**Arabinose**	**Glucose**	**Xylose**	**Arabinose**
	Average (mg/gm of dry biomass)			Stdev (mg/gm of dry biomass)		
Poplar						
Untreated	0.17	0.14	0.12	0.09	0.17	0.10
Untreated (after acid hydrolysis)	2.63	0.24	0.16	0.39	0.13	0.10
AFEX treated	0.19	0.21	0.06	0.11	0.09	0.05
AFEX treated (after acid hydrolysis)	2.00	11.36	0.49	1.06	1.82	0.12
Corn stover						
Untreated	26.00	0	0.12	2.50	0	0.07
Untreated (after acid hydrolysis)	27.86	1.22	0.39	3.10	0.10	0.10
AFEX treated	6.57	0.15	0.12	2.66	0.21	0
AFEX treated (after acid hydrolysis)	17.91	48.98	9.53	1.40	12.30	2.88

Table 3: Small organics present in ASE water extracts of untreated (UT) and AFEX treated poplar (μg per gm of dry weight of substrate).

Analytes	Molecular formula	Poplar (UT) μg/g	Poplar (AFEX) μg/g	Fold Increase
Aliphatic Acids				
Malonic acid	$CH_2(COOH)_2$	23.2	11.4	--
Lactic acid	$CH_3CH(OH)COOH$	27.6	1411.9	51
Maleic acid	HOOC-CH=CH-COOH (Cis)	0.6	4.4	7
Cis -aconitic acid	HOOC-(CH_2-COOH)C=CH-COOH	0.9	1.3	1
Methylmalonic acid	HOOC-$CH(CH_3)$-COOH	0.4	74.0	74
Succinic acid	HOOC-CH_2-CH_2-COOH	2.0	196.0	97
Fumaric acid	HOOC-CH=CH-COOH (Trans)	0.5	8.2	8
Trans -aconitic acid	HOOC-(CH_2-COOH)C=CH-COOH	BDL	BDL	--
Levulinic acid	$CH_3COCH_2CH_2COOH$	BDL	53.7	54
Glutaric acid	$HOOC(CH_2)_3COOH$	30.4	21.4	1
Itaconic acid	CH_2=C(COOH)CH_2COOH	BDL	1.0	1
2-Hydroxy-2-methylbutyric acid	$C_2H_5C(OCH_3)(OH)COOH$	0.3	0.1	--
Adipic acid	$HOOC(CH_2)_4COOH$	0.5	7.2	15
Furans				
2-Furoic acid	$C_5H_4O_3$	0.3	8.6	25
Aromatic acids				
Gallic acid	$C_6H_2(OH)_3COOH$	BDL	0.2	0.2
3,4-Dihydroxybenzoic acid	$C_6H_3(OH)_2COOH$	2.5	4.5	2
3,4-Dihydroxybenzaldehyde	$C_6H_3(OH)_2CHO$	0.5	15.1	15
Salicylic acid	OHC_6H_4COOH	56.0	115.7	2
4-Hydroxybenzeldehyde	$C_6H_4(OH)CHO$	0.7	60.2	90
Vanillic acid	$OHC_6H_3OCH_3COOH$	0.8	31.3	39
Homovanillic acid	$OHC_6H_3OCH_3CH_2COOH$	BDL	25.6	26
4-Hydroxyacetophenone	$OHC_6H_4COCH_3$	0.1	10.6	11
Caffeic acid	$C_6H_3(OH)_3CH$=CHCOOH	0.2	0.4	2
Syringic acid	$OHC_6H_3(OCH_3)_2COOH$	2.4	71.9	30
Vanillin	$OHC_6H_3OCH_3CHO$	4.9	429.2	88
4-Hydroxybenzoic acid	$C_6H_4(OH)COOH$	2.0	11.2	11
Benzoic acid	C_6H_5COOH	4.1	304.7	305
Syringaldehyde	$OHC_6H_3(OCH_3)CHO$	6.0	949.3	159
4-Hydroxycoumaric acid	OHC_6H_4CH=CH-COOH	1.8	4.6	3
Ferulic acid	$OHC_6H_3(OCH_3)CH$=CHCOOH	4.7	5.4	1.1
Sinapic acid	$OHC_6H_2(OCH_3)_2CH$=CHCOOH	0.2	0.9	5
Para -toluic acid	$C_6H_4(CH_3)COOH$	9.3	9.9	1.1

BDL = below detection limit