UC Berkeley
UC Berkeley Previously Published Works

Title
Two-Step Delignification of Miscanthus To Enhance Enzymatic Hydrolysis: Aqueous Ammonia Followed by Sodium Hydroxide and Oxidants

Permalink
https://escholarship.org/uc/item/2n62t96n

Journal
Energy & Fuels, 28(1)

ISSN
0887-0624

Authors
Liu, Zhongguo
Padmanabhan, Sasisanker
Cheng, Kun
et al.

Publication Date
2014-01-16

DOI
10.1021/ef401317z

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Two-Step Delignification of Miscanthus To Enhance Enzymatic Hydrolysis: Aqueous Ammonia Followed by Sodium Hydroxide and Oxidants

Zhongguo Liu,[†,‡,‖,⊥] Sasisanker Padmanabhan,[†,‡] Kun Cheng,[†] Hongxue Xie,[†,‡] Amit Gokhale,[§] Waheed Afzal,[†,‡] Hui Na,[‖] Markus Pauly,[†] Alexis T. Bell,[†,‡] and John M. Prausnitz*[,†,‡]

[†]Energy Biosciences Institute, University of California at Berkeley, 2151 Berkeley Way, Berkeley, California 94720-1462, United States

[‡]Department of Chemical and Biomolecular Engineering, University of California at Berkeley, 201 Gilman Hall, Berkeley, California 94720-1462, United States

[§]BP America, Incorporated, 2151 Berkeley Way, Berkeley, California 94720, United States

[‖]Alan G. MacDiarmid Institute, College of Chemistry, Jilin University, Changchun 130012, People's Republic of China

[⊥]Dalian Lubricating Oil Research and Development Institute, PetroChina, Dalian 116032, People's Republic of China

ABSTRACT: Pretreatment of miscanthus is essential for enzymatic production of sugars to yield bioethanol. A two-step process using 10 wt % aqueous ammonia in the first step is followed by 1 wt % sodium hydroxide (with or without oxygen or 1 wt % hydrogen peroxide) in the second step. The first step retains about 90% of the cellulose and about 67% of the hemicellulose in the solid while removing about 62% of the lignin. Both steps together achieve 83−90% delignification. Subsequent enzymatic conversion to fermentable sugars is close to 90% after 96 h. While an oxidant does not significantly increase delignification, it has a favorable effect on saccharification of the recovered solid. Infrared spectroscopy, X-ray diffraction, and two-dimensional nuclear magnetic resonance spectroscopy provide data concerning the chemical composition of the recovered solid. Inclusion of an oxidant to the pretreatment breaks β-O-4′-linked aryl ether bonds of the remaining lignin in the recovered solid.

INTRODUCTION

Miscanthus is a promising lignocellulosic feedstock for sustainable production of a liquid fuel. Miscanthus has some significant advantages: rapid growth, low fertilizer requirement, high yield, and low mineral content. To overcome the recalcitrance of raw miscanthus to enzymatic hydrolysis of its polysaccharides, pretreatment is necessary to remove lignin.[1−4]

Several researchers have reported that a two-step pretreatment process is more effective than a one-step pretreatment process.[5] Xu et al. investigated a two-step hot-water process, where hot water was used to open the cellulose structure in eucalyptus to enhance enzymatic digestibility of cellulose.[6] Lee et al. showed that the efficiency of biomass conversion can be significantly improved by a two-step process, where hot-water treatment is followed by aqueous ammonia, achieving 75−81% lignin removal.[7] Hage et al. studied a process where the hot-water step is followed by contact with an organic solvent, resulting in better delignification.[8]

Pretreatment with alkali catalysts hydrolyzes ester bonds that cross-link lignin to hemicellulose, making the polysaccharides more accessible to enzymes for hydrolysis.[1] In comparison to other pretreatment methods, alkali pretreatment uses lower temperature and pressure.[9] Hsu showed that aqueous ammonia or sodium hydroxide can be used separately for biomass pretreatment.[10] These alkaline reagents effectively remove lignin from biomass mainly by hydrolyzing intralignin bonds. Oxidants added to alkaline reagents enhance lignin degradation.[11,12] The resulting low-lignin residue can be enzymatically hydrolyzed with high efficiency.[13]

In our previous work, we showed that aqueous ammonia is a good reagent for delignification of miscanthus but conversion by enzymatic hydrolysis was only modest; a two-step aqueous ammonia process at 180 °C achieved 60% conversion of glucose and 70% conversion to xylose.[14] Here, we investigate a two-step alkali pretreatment process, where the first step uses 10 wt % aqueous ammonia, followed by 1 wt % aqueous sodium hydroxide with or without an oxidant. The recovered solid was studied by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), and two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy to ascertain the structural changes in the recovered solid.

EXPERIMENTAL SECTION

Substrate and Reagents. *Miscanthus* × *giganteus* from the University of Illinois at Urbana−Champaign was milled to 4 mm particles using a Retsch grinder. The moisture content was 6.5 wt %. On a water-free basis, the dried miscanthus contains approximately 42% cellulose, 25% hemicellulose, 24% lignin, 8% ash, and others (pectin, lipid, and salts).[15] Aqueous ammonia was obtained from EMD Chemicals, Inc. (Gibbstown, NJ). Solid sodium hydroxide was purchased from Fisher Scientific (Fair Lawn, NJ). Aqueous hydrogen peroxide and sodium azide were obtained from Sigma-Aldrich (St. Louis, MO). Prior to pretreatment, all liquid reagents were diluted with purified water to the desired concentration.

Enzymes. Cellulases (Celluclast 1.5L, product C2730, 50 mL) and β-glucosidase (Novo188, product C6105, 50 mL) were purchased from Sigma-Aldrich (St. Louis, MO). The reported activity of the β-glucosidase (Novozyme 188 from Novo, Inc., Lot 11K1088) was >700 cellobiase units (CBU)/g. The activity of the Celluclast 1.5L was 100 filter paper units (FPU)/mL of solution. Enzyme loading was 20 FPU/g of glucan (cellulase and β-glucosidase), according to the procedure of Ghose et al.[16]

Experimental Procedure. Figure 1 shows a schematic diagram of our pretreatment process.

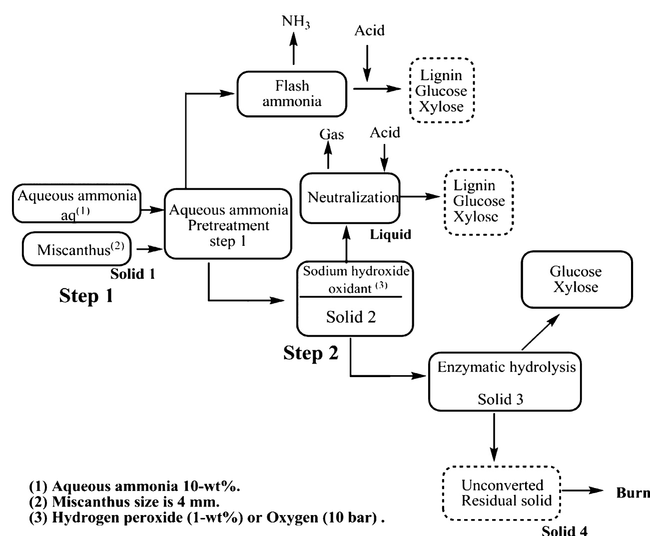


Figure 1. Schematic for a two-step pretreatment process using aqueous ammonia and sodium hydroxide with an oxidant.

First Step. A total of 2 g of miscanthus and 20 g of 10 wt % aqueous ammonia were put into a 25 mL stainless-steel reactor (4740 HP/HT pressure vessel, Moline Parr Instruments). S/L = 1:10 is found empirically to provide good pretreatment. The experiments were conducted without agitation. Kang et al. have also investigated the pretreatment of rapeseed straw by soaking in aqueous ammonia without stirring. The reactor was submerged for 1 h in a preheated silicone−oil bath at 150 °C. Reaching the desired temperature required about 15 min. The pressure inside the reactor was about 5 bar. After 1 h, the reactor was cooled to room temperature; the slurry was filtered using vacuum filtration. The solid was washed with Milli-Q pure water until it reached neutral pH. The solid was air-dried at room temperature for 48 h. We recovered 1.4 g of miscanthus; the remainder was dissolved in aqueous ammonia.

Second Step. A total of 1 g of pretreated miscanthus (first step) and 10 g of 1 wt % aqueous sodium hydroxide were placed inside a stainless-steel reactor. Both steps were kept consistent to avoid the different S/L ratio effects. In some experiments, no oxidant was used; in others, oxygen (pure, 10 bar) or aqueous hydrogen peroxide was added. The final concentration of hydrogen peroxide was 1 wt %. The experiments were conducted without agitation. The reactor was submerged in a 150 °C bath. After 1 h, the reactor was cooled to room temperature. The suspension was filtered, and the solid was washed until the wash water achieved neutral pH. The solid was dried in a 105 °C oven overnight. Each experiment was carried out in duplicate.

Composition of the Recovered Solid. Analyses of the recovered solid were performed using a standard National Renewable Energy Laboratory (NREL) analytical procedure.[17]

The liquid fraction was analyzed for the main monomeric sugars (glucose, xylose, galactose, arabinose, acetate hydroxymethylfurfural (HMF), and furfural) using high-performance liquid chromatography (HPLC, Perkin-Elmer LC200) using a refractive index detector and an Aminex HPX-87H column under the following conditions: mobile phase of 0.005 M H_2SO_4 and a flow rate of 0.60 mL/min. Sample injection was 20 μL. The refractive index detector was maintained at 50 °C for all applications.

A brief description of the procedure is given in our previous publication.[14] Lignin (acid-soluble and acid-insoluble) [(Klason) lignin] and ash contents were measured as described in the NREL analytic procedure.[17]

Solid recovery was calculated from

$$\text{percent solid recovery} = \frac{\text{weight of recovered miscanthus (g)}}{\text{weight of untreated miscanthus (g)}} \times 100\%$$

Enzymatic Hydrolysis of Recovered Miscanthus. Following the second step, 3 g of recovered solid (containing 1 g of dry miscanthus and 2 g of water) was mixed with citrate buffer (pH 4.8, 50 mM) and cellulose enzymes at 20 FPU/g of glucan in a 125 mL flask; the total solid loading was 2 wt %. Sodium azide is added to prevent the growth of organisms (20 mg/mL). Enzymatic hydrolysis was carried out at 50 °C in a shaker at 150 rpm following the NREL enzyme protocol.[18] Cellulase enzymes remain stable at 50 °C. Small samples were withdrawn at several time intervals (1, 3, 7, 24, 48, 72, and 96 h) and diluted with nanopure water to measure glucose and xylose concentrations using a Shimadzu high-performance liquid chromatograph. Each experiment was performed in triplicate; results from three measurements agreed to within ±5%.

Characterization of Raw and Recovered Miscanthus with 2D NMR. The two-dimensional $^{13}C-^{1}H$ heteronuclear single-quantum coherence nuclear magnetic resonance (2D HSQC NMR) spectrum of miscanthus material was obtained after each pretreatment according to the method described in our previous reports.[14,19]

In brief, biomass (300 mg) was ball-milled for 7 h with alternating 5 min grinding with 5 min breaks using a Retsch PM 100 (Retsch, Germany). Milled material (25 mg) was dissolved in DMSO-d_6/EmimOAc-d_{14} (0.75 mL/10 mg). Two-dimensional HSQC NMR spectra were acquired using a Bruker standard pulse sequence "hsqcetgpsisp.2" on a Bruker AVANCE 600 MHz NMR spectrometer equipped with an inverse gradient 5 mm TXI $^{1}H/^{13}C/^{15}N$ cryoprobe.

All spectra were calibrated using the central DMSO-d_6 solvent peak (δ_C, 39.9 ppm; δ_H, 2.49 ppm). NMR data processing and analysis were performed using Bruker's Topspin 3.1 software.

FTIR Spectra. Attenuated total reflection-infrared (ATR-IR) characterization of raw and recovered miscanthus was performed at room temperature using a Nicole 6700 FTIR spectrometer (Thermo Scientific). Samples were pressed against the crystal surface of a spring-loaded diamond anvil to secure uniform pressure. All spectra were adjusted to the same background spectra and were measured using 128 scans with 4 cm^{-1} resolution to achieve a good signal-to-noise ratio.

XRD Analysis and Crystallinity Index (CI). Pretreated miscanthus and raw miscanthus were studied by XRD using a Bruker diffractometer at 40 kV and 20 mA with Cu Kα radiation (λ = 0.154 nm). The scanning rate was 0.02°/s with 2θ ranging from 3° to 60°.

The cellulose CI is defined as the ratio of the mass of crystalline cellulose (cellulose I_b) to the total mass of dry material that includes crystalline and amorphous cellulose, lignin, hemicellulose, pectin, etc. CI is obtained using the height of the space 200 (I_{200}; 2θ = 22.7°) and the minimum between the space 200 and 110 peaks (I_{AM}; 2θ = 18°). I_{200} represents both crystalline and amorphous material, while I_{AM} represents amorphous material only.

$$\text{percent CI} = \frac{I_{200} - I_{AM}}{I_{200}} \times 100\% \tag{1}$$

As discussed by Segal et al,[20] eq 1 assumes that the intensity of the amorphous material at 18° is the same as that at 22.7° and that the crystalline cellulose does not contribute to the intensity at 18°.

RESULTS AND DISCUSSION

Miscanthus biomass was processed with two sequential steps; the first used 10 wt % aqueous ammonia, followed by a second step of 1 wt % aqueous sodium hydroxide with or without an

oxidant, such as oxygen or hydrogen peroxide (Figure 1). Figure 2 and Table 1 show the cellulose and hemicellulose contents in the recovered solid after the first and second processing steps. After the first step, we achieve 92.9% recovery of cellulose and 66.6% recovery of hemicellulose. Additional treatment with aqueous sodium hydroxide solubilizes about 10% cellulose, resulting in a total cellulose recovery of 80% after the second step. Using the two-step procedure, the hemicellulose content was further reduced to 40%, independent of oxidant addition. The larger loss of hemicellulose is likely due its amorphous structure that provides high accessibility of the alkaline reagent to its active sites.[12] As a result of the two-step procedure, solid recovery is 53−55%, independent of the addition of an oxidant. The two-step process studied here is not fully optimized; there is still scope for further improvement.

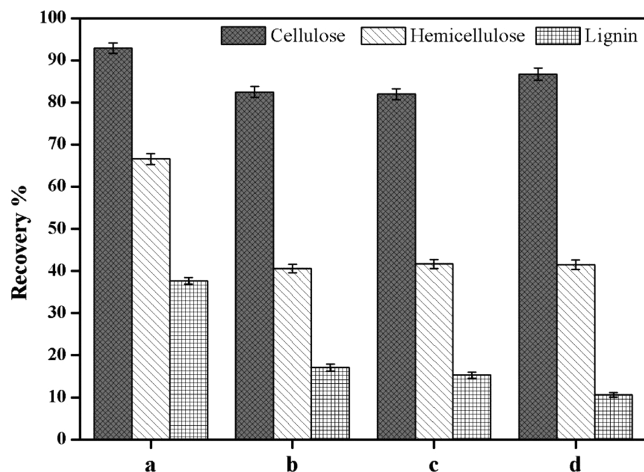


Figure 2. Effect of two-step pretreatment on the composition of recovered miscanthus: (a) 10 wt % aqueous ammonia (step 1), (b) step 1 followed by 1 wt % aqueous sodium hydroxide (step 2), (c) step 1 followed by 1 wt % aqueous sodium hydroxide + oxygen (step 2), and (d) step 1 followed by 1 wt % aqueous sodium hydroxide + hydrogen peroxide (step 2).

Delignification. Delignification is arguably the most important requirement to increase enzymatic digestibility of lignocellulosic biomass.[21] Table 1 shows the second step effect of 1 wt % aqueous sodium hydroxide (with or without oxidant) on the delignification of miscanthus. After the first treatment, 62.3% of lignin was already removed; together with the second step, 82.9% delignification is achieved. Panagiotopoulos et al. reported that, rather than hindering subsequent organosolv delignification, the prior steam treatment enhanced lignin solubilization with more than 66% of the original lignin removed after the two-step pretreatment.[22] Delignification by aqueous sodium hydroxide with oxygen (10 bar) is increased slightly to 84.7%. Nearly 90% of delignification of miscanthus is achieved when hydrogen peroxide is added to the aqueous sodium hydroxide in the second step. Phenolic groups in lignin react with oxygen to result in phenoxyl radicals that undergo further reactions with superoxide radicals to form hydroperoxide intermediates.[23] An oxidant may open the aromatic rings and/or produce lignin epoxidation.[24] Lai et al. reported that, upon adding hydrogen peroxide to sodium hydroxide pretreatment, oxidative delignification occurred because of cleavage of C−C bonds.[25,26]

Spectroscopic Characterization of the Residues. The effect of biomass pretreatments on the biomass was monitored using several spectroscopic techniques. Figure 3 shows a 2D HSQC NMR spectrum for untreated and treated miscanthus biomass. Interpretation of the spectra is based on a previous report.[19]

As reported previously,[14] ammonia pretreatment leads to deacetylation of the hemicellulose xylan ($^{13}C/^{1}H$ chemical shifts at δ = 75.0/4.79 ppm and δ = 73.7/4.48 ppm; Figure 3b). In addition, signals representing 2-α-L-arabinofuranoside and other non-terminal arabinofuranoside residues disappear after the ammonia pretreatment. Terminal arabinofuranoside residues increase 3-fold compared to the untreated sample, as calculated by the volume integral of its corresponding peak (107.4/5.30 ppm; Figure 4). The loss of substituents from arabinosyl residues is likely due to the cleavage of ferulic acid ester bonds accounting for most of the hemicellulose−lignin linkages.[27] Moreover, one-step ammonia pretreatment results in the removal of *p*-hydroxybenzoate (PB), *p*-hydroxyphenyl (H), and ferulate (FA) and a significant decrease (∼80%) of *p*-coumarate (pCA). In addition, one-step ammonia pretreatment cleaves many lignin side-chain linkages, including the β-5′-linked phenylcoumaran (B), β-β′-linked resinol (C), and 5-5′/4-O-β′-linked dibenzodioxocin (D).

However, after the first step, more than 70% of the β-O-4′-linked aryl ether (A) remained in the pretreated solid miscanthus. Delignification is improved by the second step using alkali with an oxidant (panels c and d of Figure 3). The β-O-4′-linked aryl ether bonds are almost completely cleaved. Syringyl (S) lignin decreases from about 50% for one-step ammonia pretreatment to about 5% for two-step pretreatment under the conditions used in the present study. The guaiacyl units (G) decrease from about 60% for one-step ammonia pretreatment to less than 15% for two-step pretreatment, as shown in Figure 4.

Figure 5 shows FTIR spectra for solid samples. After the first step, the absorption bands at 1730 cm^{-1} disappeared; these bands are assigned to unconjugated C═O stretching in the acetyl group of hemicellulose. The disappearance of this band

Table 1. Chemical Composition of Miscanthus after Pretreatment[a]

pretreatment condition	solid recovery (wt %)[b]	recovery of components of miscanthus (wt %)			CI (%)	delignification (wt %)
		cellulose	hemicellulose	lignin		
raw miscanthus	100	42	25	26	43	
aqueous ammonia[c]	70 ± 0.2	92.9 ± 0.7	66.6 ± 0.5	37.7 ± 1.2	66	62.3
aqueous sodium hydroxide[d]	55 ± 0.5	82.5 ± 0.5	40.6 ± 0.3	17.1 ± 0.8	64	82.9
aqueous sodium hydroxide + oxygen[e]	53 ± 0.4	82.0 ± 0.4	41.7 ± 0.2	15.3 ± 0.5	62	84.7
aqueous sodium hydroxide + hydrogen peroxide[f]	53 ± 0.2	86.7 ± 0.3	41.5 ± 0.3	10.6 ± 0.8	44	89.4

[a]All recoveries are for the recovered solid only. [b]Solid recovery is based on the raw miscanthus; recovery of components is based on the biopolymer of the raw miscanthus. [c]Aqueous ammonia is 10 wt %. [d]Aqueous sodium hydroxide is 1 wt %. [e]Oxygen is10 bar. [f]Hydrogen peroxide is 1 wt %.

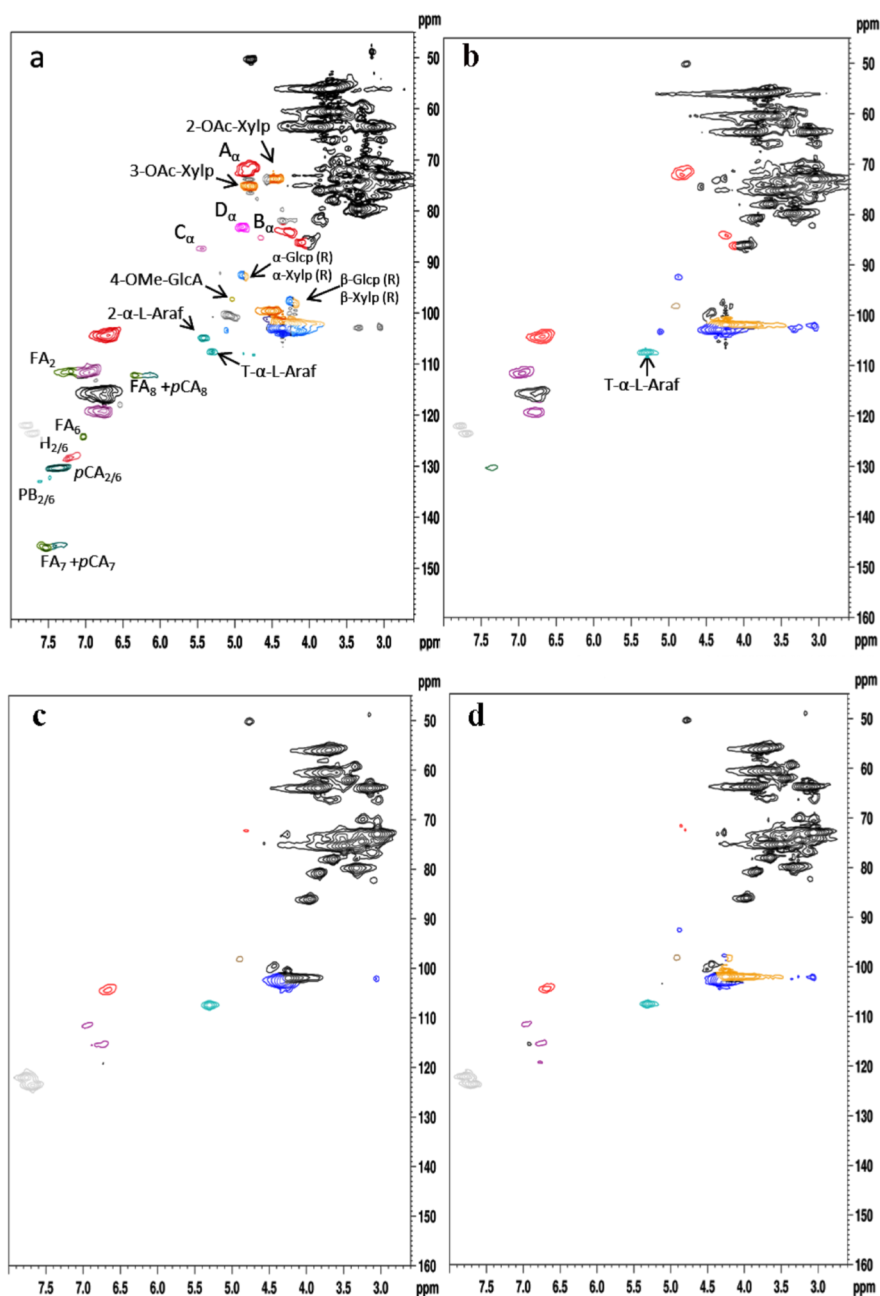


Figure 3. Two-dimensional HSQC NMR spectra for raw and pretreated miscanthus: (a) raw miscanthus, (b) pretreated miscanthus after 10 wt % aqueous ammonia pretreatment, (c) miscanthus after second step 1 wt % aqueous sodium hydroxide + oxygen pretreatment, and (d) miscanthus after second step 1 wt % aqueous sodium hydroxide + hydrogen peroxide pretreatment.

indicates deacetylation, confirming the results described above.[14] Bands at 896, 1035, and 1155 cm^{-1} were strengthened after pretreatment, indicating an increase in the abundance of cellulose after pretreatment; this increase was due to delignification and removal of some ash components. However, there is no significant difference between the spectra of the sample after the first step and those of the sample going through the two-step procedure. The intensities of the bands at 3400 and 2890 cm^{-1} (assigned to O−H and C−H stretching, respectively) became stronger after pretreatment,[28] probably because of the higher concentration of carbohydrates in the recovered solid.

Table 1 shows that the CI changes after the first step and again after the second step. Following pretreatment with aqueous ammonia, aqueous sodium hydroxide, or aqueous sodium hydroxide with O_2, the index (66, 64 and 62%, respectively) was higher than that for raw miscanthus, most likely because of the removal of lignin and some of the hemicelluloses. The cellulose content strongly affects the CI. However, XRD results following second-step pretreatment with aqueous sodium hydroxide and hydrogen peroxide show that pretreated miscanthus has a low crystallinity (44%), likely as a result of damaged cellulosic chain linkages broken during the second step because an alkaline hydrogen peroxide treatment has previously been shown not only to produce delignification but also to decrease cellulose crystallinity.[29,30] Low crystallinity should provide better access to cellulose for enzymatic saccharification.

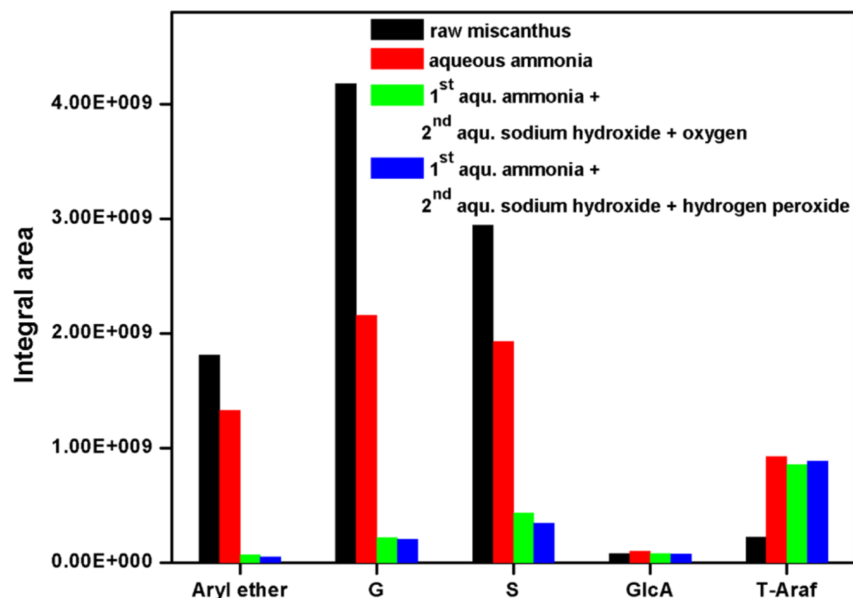


Figure 4. Absolute integral volume of aryl ether (β-O-4′), guaiacyl (G), syringyl (S), glucuronic acid (GlcA), terminal arabinofuranoside (T-Araf) in raw and pretreated miscanthus.

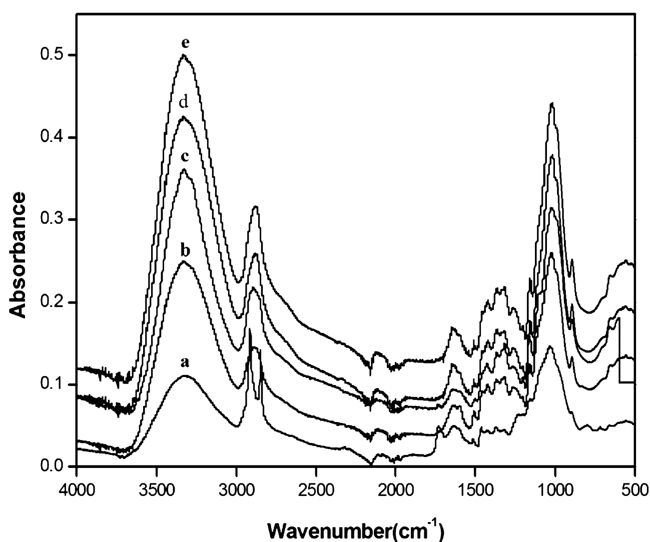
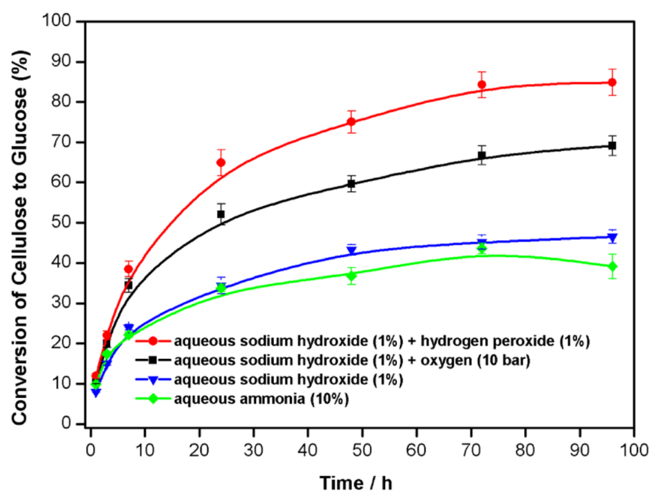


Figure 5. FTIR spectra of recovered miscanthus: (a) raw miscanthus, (b) 10 wt % aqueous ammonia (step 1), (c) step 1 followed by 1 wt % aqueous sodium hydroxide (step 2), (d) step 1 followed by 1 wt % aqueous sodium hydroxide + oxygen (step 2), and (e) step 1 followed by 1 wt % aqueous sodium hydroxide + hydrogen peroxide (step 2).



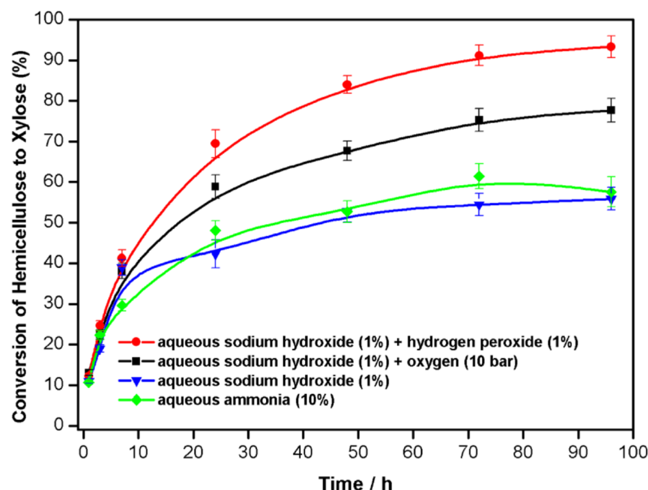


Figure 6. Glucose and xylose production by enzymatic hydrolysis following two-step pretreatment.

Enzymatic Hydrolysis of Recovered Solid. As expected,[31] an oxidant in the second process step improves conversion to sugar by enzymatic hydrolysis of the recovered solid (Figure 6). When an oxidant was included in the second step, the maximum conversion to glucose and xylose were 84.8 and 93.3% after 96 h, respectively, based on the recovered solid only. Luterbacher et al. reported a two-step, two-temperature pretreatment; a short-time high-temperature step at 210 °C (16 min for hardwood and 1 min for switchgrass) was followed by a long-time low-temperature stage at 160 °C for 60 min. Glucan−glucose conversion yields were 83% for mixed hardwood and 80% for switchgrass, obtained at 15 FPU/g of glucan after 72 h.[32]

Yu et al. reported 90.2% conversion of cellulose to glucose and 73.4% conversion of hemicellulose to xylose after 96 h.

However, Yu et al. use 30 wt % aqueous ammonia for 2 h in the first step and 5 wt % hydrogen peroxide for 2 h in the second step at 130 °C.[33] A two-step hot-water pretreatment process was applied to *Eucalyptus grandis* at 180 °C for 20 min, followed by 200 °C for 20 min. Enzymatic digestion reached 96.6% after 72 h at 40 FPU/g of glucan.[6] Kim et al. developed a two-step pretreatment process using aqueous ammonia and dilute sulfuric acid. Enzymatic hydrolysis yields were 96.9% with enzyme loadings of 60 FPU/g of glucan.[34] However, these pretreatment conditions and enzyme loadings were more severe than the ones used in the present study.

The addition of hydrogen peroxide in the second step increases lignin removal but also causes some carbohydrate degradation because hydrogen peroxide decomposes to free radicals that promote degradation reactions in carbohydrates and lignin at high temperatures.[35,36] The addition of oxygen to the second step improves conversion to monosacharides to 69.1% for glucose and 77.7% for xylose. Without oxidant, conversion was limited to 46.5% for glucose and 55.6% for xylose under the conditions used.

CONCLUSION

Pretreatment of miscanthus using a two-step process at 150 °C significantly reduces the lignin content and enhances enzymatic production of fermentable sugars. Pretreatment with 10 wt % aqueous ammonia, followed by further pretreatment with 1 wt % aqueous sodium hydroxide and 1 wt % hydrogen peroxide, improved delignification and sugar yield: delignification was 89.4% and enzymatic hydrolysis for 96 h achieved 84.8% conversion to glucose and 93.3% conversion to xylose. Because the two-step process described here uses inexpensive dilute aqueous ammonia and highly dilute sodium hydroxide and only a very small quantity of hydrogen peroxide (or 10 bar oxygen), solvent recycle might be circumvented. The results obtained here may provide the basis for a possible large-scale process to produce bioethanol or other sugar-based commodity chemicals from miscanthus biomass.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +1-510-642-3592. Fax: +1-510-642-4778. E-mail: prausni@berkeley.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful to the Energy Biosciences Institute (University of California at Berkeley) for financial support. Additional support was provided by a stipend from the China Scholarship Council to Zhongguo Liu.

REFERENCES

(1) Geng, X. L.; Henderson, W. A. Pretreatment of corn stover by combining ionic liquid dissolution with alkali extraction. *Biotechnol. Bioeng.* **2012**, *109*, 84−91.

(2) Zhang, X. M.; Xu, J. L.; Cheng, J. Pretreatment of corn stover for sugar production with combined alkaline reagents. *Energy Fuels* **2011**, *25*, 4796−4802.

(3) Wang, Z. Y.; Xu, J. L.; Pandey, P.; Cheng, J.; Li, R. Y.; Qu, R. D. Improvement of sugar production form transgenic switchgrass with low-temperature alkali pretreatment. *Energy Fuels* **2012**, *26*, 3054−3061.

(4) Xu, J. L.; Cheng, J. J.; Sharma-Shivappa, R. R.; Burns, J. C. Sodium hydroxide pretreatment of switchgrass for ethanol production. *Energy Fuels* **2010**, *24*, 2113−2119.

(5) Zheng, Y.; Pan, Z. L.; Zhang, R. H. Overview of biomass pretreatment for cellulosic ethanol production. *Int. J. Agric. Biol. Eng.* **2009**, *2*, 51.

(6) Yu, Q.; Zhuang, X Z.; Yuan, Z. H.; Wang, Q.; Qi, W.; Wang, W.; Zhang, Y.; Xu, J. L.; Xu, H. J. Two-step liquid hot water pretreatment of *Eucalyptus grandis* to enhance sugar recovery and enzymatic digestibility of cellulose. *Bioresour. Technol.* **2010**, *101*, 4895−4899.

(7) Kim, T. H.; Lee, Y. Y. Fraction of corn stover by hot-water and aqueous ammonia treatment. *Bioresour. Technol.* **2006**, *97*, 224−232.

(8) Hage, R. E.; Chrusciel, L.; Desharnais, L.; Brosse, N. Effect of autohydrolysis of *Miscanthus × giganteus* on lignin structure and organosolv delignification. *Bioresour. Technol.* **2010**, *101*, 9321−9329.

(9) Han, M.; Choi, G. W.; Kim, Y.; Koo, B. C. Bioethanol production by *Miscanthus* as a lignocellulosic biomass: Focus on high efficiency conversion to glucose and ethanol. *BioResources* **2011**, *6*, 1939−1953.

(10) Hsu, T. Pretreatment of biomass. In *Handbook on Bioethanol: Production and Utilization*; Wyman, C. E., Ed.; CRC Press (Taylor and Francis Group): Boca Raton, FL, 1996; pp 179−212.

(11) Xiang, Q.; Lee, Y. Y. Oxidative cracking of precipitated hardwood lignin by hydrogen peroxide. *Appl. Biochem. Biotechnol.* **2000**, *84−86*, 153−162.

(12) Banerjee, G.; Car, S.; Scott-Craig, J. S.; Hodge, D. B.; Walton, J. D. Alkaline peroxide pretreatment of corn stover: Effects of biomass, peroxide, and enzyme loading and composition on yields of glucose and xylose. *Biotechnol. Biofuels* **2011**, *4*, 16.

(13) Kim, T. H.; Kim, J. S.; Sunwoo, C. S.; Lee, Y. Y. Pretreatment of corn stover by aqueous ammonia. *Bioresour. Technol.* **2003**, *90*, 39−47.

(14) Liu, Z. G.; Padmanabhan, S.; Cheng, K.; Schwyter, P.; Pauly, M.; Bell, A. T.; Prausnitz, J. M. Aqueous-ammonia delignification of miscanthus followed by enzymatic hydrolysis to sugars. *Bioresour. Technol.* **2013**, *135*, 23−29.

(15) Dee, S.; Bell, A. T. Effects of reaction conditions on the acid-catalyzed hydrolysis of miscanthus dissolved in an ionic liquid. *Green Chem.* **2011**, *13*, 1467−1475.

(16) Ghose, T. K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257−268.

(17) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of Structural Carbohydrates and Lignin in Biomass*; National Renewable Energy Laboratory (NREL): Golden, CO, revised June 2010.

(18) Selig, M.; Weiss, N.; Ji, Y. *Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure*; National Renewable Energy Laboratory (NREL): Golden, CO, March 2008.

(19) Cheng, K.; Sorek, H.; Zimmermann, H.; Wemmer, D. E.; Pauly, M. Solution-state 2D NMR spectroscopy of plant cell walls enabled by a dimethylsulfoxide-d_6/1-ethyl-3-methylimidazolium acetate solvent. *Anal. Chem.* **2013**, *85*, 3213−3221.

(20) Segal, L.; Creely, J. J.; Martin, A. E.; Conrad, C. M. An empirical method for estimating the degree of crystalliity of native cellulose using the X-ray diffractometer. *Textile Res.* **1959**, *29*, 786−794.

(21) Zhao, X. B.; Wang, L.; Liu, D. H. Effect of several factors on peracetic acid pretreatment of sugarcane bagasse for enzymatic hydrolysis. *J. Chem. Technol. Biotechnol.* **2007**, *82*, 1115−1121.

(22) Panagiotopoulos, I. A.; Chandra, R. P.; Saddler, J. N. A two-stage pretreatment approach to maximise sugar yield and enhance reactive lignin recovery from poplar wood chips. *Bioresour. Technol.* **2013**, *130*, 570−577.

(23) Bajpai, P. *Environmentally Benign Approaches for Pulp Bleaching*; Elsevier: Amsterdam, Netherlands, 2005; pp 23−175.

(24) Gille, S.; de Souza, A.; Xiong, G.; Benz, M.; Cheng, K.; Schultink, A.; Reca, I. B.; Pauly, M. O-Acetylation of *Arabidopsis* hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* **2011**, *23*, 4041−4053.

(25) Lai, Y.-Z. Chemical degredation. In *Wood and Cellulosic Chemistry*; Hon, D. N.-S.; Shiraishi, N., Eds.; Marcel Dekker: New York, 2001; pp 443−512

(26) Suchy, M.; Argyropoulos, D. S. Catalysis and activiation of oxygen and peroxide delignification of chemical pulps: A review. In *Oxidative Delignification Chemistry*; Argyropoulos, D. S., Ed.; American Chemical Society (ACS): Washington, D.C., 2001; ACS Symposium Series, Vol. 785, Chapter 1, pp 2–43.

(27) Subramaniyan, S.; Prema, P. *Crit. Rev. Biotechnol.* **2002**, *22*, 33–46.

(28) Zhao, X. B.; Zhang, L. H.; Liu, D. H. Pretreatment of Siam weed stem by several chemical methods for increasing the enzymatic digestibility. *Biotechnology* **2010**, *5*, 493–504.

(29) Kerley, M. S.; Fahey, G. C.; Berger, L. L.; Gould, J. M.; Baker, F. L. Alkaline hydrogen peroxide treatment unlocks energy in agricultural by-products. *Science* **1985**, *230*, 820–822.

(30) Gould, J. M. Studies on the mechanism of alkaline peroxide delignification of agricultural residues. *Bioengineering* **1985**, *27*, 225–231.

(31) Yamashita, Y.; Shono, M.; Sasaki, C.; Nakamura, Y. Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo. *Carbohydr. Polym.* **2010**, *79*, 914–920.

(32) Luterbacher, J. S.; Tester, J. W.; Walker, L. P. Two-temperature stage biphasic CO_2–H_2O pretreatment of lignocellulosic biomass at high solid loadings. *Biotechnol. Bioeng.* **2012**, *109*, 1499–1507.

(33) Yu, G. R.; Afzal, W.; Yang, F. X.; Padmanabhan, S.; Liu, Z. G.; Xie, H. X.; Shafy, M. A.; Bell, A. T.; Prausnitz, J. M. Pretreatment of *Miscanthus* × *giganteus* using aqueous ammonia with hydrogen peroxide to increase enzymatic hydrolysis to sugars. *J. Chem. Technol. Biotechnol.* **2013**, DOI: 10.1002/jctb.4172.

(34) Kim, J. W.; Kim, K. S.; Lee, J. S.; Park, S. M.; Cho, H. Y.; Park, J. C.; Kim, J. S. Two-stage pretreatment of rice straw using aqueous ammonia and dilute acid. *Bioresour. Technol.* **2011**, *102*, 8992–8999.

(35) Xiang, Q.; Lee, Y. Y. Oxidative cracking of precipitated hardwood lignin by hydrogen peroxide. *Appl. Biochem. Biotechnol.* **2000**, *84–86*, 153–162.

(36) Gupta, R.; Lee, Y. Y. Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide. *Bioresour. Technol.* **2010**, *101*, 8185–9191.