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Electronic Supplementary Information

Additional methods and results

Corn stover

Comparisons between the EA, IL, and DA pretreatments were performed using corn stover harvested at Michigan State University (MSU, East Lansing, Michigan, USA) in September 2008. The corn hybrid used was NK 49-E3 (Syngenta, Basel, Switzerland), a corn hybrid typically used in the Great Lakes region. The corn stover was milled to a 40 mesh size using a Wiley mill and stored at 4 °C until further use. Details on the composition of this corn stover are available in Uppugundla *et al.* (2014)[1].

Other studies reported here were performed using corn stover generously provided by the Great Lakes Bioenergy Research Center (GLBRC). This stover was derived from a Pioneer 36H56 corn hybrid, harvested in September 2009 in Wisconsin (USA) and oven dried at 60 °C for approximately 2 weeks. The biomass was ground to pass through a 4 mesh size screen in a Christy hammer mill (Christison Scientific LTD, England) and stored at 4 °C in heat-sealed bags until used. The moisture content of the dried and milled corn stover was approximately 6% (wet-weight basis). The biomass compositional analysis was performed using NREL protocols NREL/TP-510-42618 and NREL/TP-510-42620. Based on this protocol, the untreated corn stover contained approximately 38% glucan, 23% xylan, 1% galactan, 3% arabinan, 14% Klason lignin, 2% acid-soluble lignin, 5% ash and 15% extractives (i.e., ethanol/water-soluble compounds), on a dry weight basis.

EA pretreatment of corn stover

For response surface analysis experiments, EA pretreatment was conducted in 33 mL in-house designed tubular reactors as described in Fig. 1a. The moisture in the corn stover was raised to 10% (total weight basis) by spraying distilled water homogeneously throughout the corn stover before transferring it to the reactor cells. The desired amount of ammonia was loaded with a syringe pump (Harvard Apparatus, model PHD 2000, Holliston, MA, USA). Immediately after loading ammonia, the reactors were heated and then maintained at the desired temperature. The top reactor fitting was connected to a nitrogen line and the bottom fitting was connected to the extractives collector. The extractives collector was pressurized with nitrogen to equalize the pressure developed in the reactor during the course of pretreatment. After reaching the desired residence time, nitrogen overpressure of 1250 psi was applied to the reactor to maintain ammonia in the liquid state, the valve between the reactor and collector was opened and the extractives were filtered and drained into the collector. The exhaust connected to the side port of the extractives collector was partially opened to slowly release the nitrogen and ammonia out of the system. The nitrogen valve in the top of the reactor was kept opened to maintain a pressure of approximately 1250 psi in the system for ~5 min. During this process, nitrogen was allowed to slowly flow through the system to help filter and drain ammonia with the extractives to the collector.

After pressurized extraction, the nitrogen inlet valve was closed and the system was depressurized slowly (~3 to 5 min). The pretreated biomass was transferred from the reactor to a tray, which was placed under the fume hood overnight to remove any residual ammonia. The extractives collector and all the system lines were cleaned with 70% (v/v) ethanol and 90% (v/v) acetone (both in water) to remove residual extractives, which were drained to the extractives collector. After drying, the total

weight and moisture content of the EA-pretreated corn stover (EA-CS) was measured with an analytical balance and moisture analyzer A&D MX-50 (A&D Engineering, Inc., San Jose, CA, USA), respectively, for determining the mass balance.

EA pretreatment for high-solid-loading enzymatic hydrolysis, as well as for lignin extractability and lignin characterization studies, was conducted using an in-house built larger scale reactor of 700 mL with a similar design as the 33 mL reactor described above. In these reactors, 40 g of biomass (dry weight basis) were used for pretreatment and the ammonia was added gravimetrically by weighing the ammonia transferred from a pre-weighed vessel to the EA pretreatment reactors. All other procedures were identical to those used in the small-scale reactions.

To evaluate the effect of lignin extraction on enzymatic hydrolysis performance, EA pretreatment was carried out with and without extraction of biomass components. The pretreatment without extraction was performed at the same operating conditions as the regular EA pretreatment, however, ammonia was evaporated from the top of the reactor and released in the gas phase at the end of stage 2. This procedure allowed biomass extracts to deposit on the surface of the biomass, instead of being extracted as it occurs during regular EA pretreatment.

AFEX, IL, and DA pretreatment of corn stover

IL pretreatment was performed at JBEI (Berkley, CA, USA) and DA pretreatment was performed at BESC (University of California, Riverside, CA, USA) as previously described[1]. AFEX pretreatment for high-solid-loading enzymatic hydrolysis experiments (industrially relevant conditions) on GLBRC corn stover was generously performed by MBI International (Lansing, MI, USA), using 1:1 ammonia-to-biomass ratio, 60% (w/w) moisture (dry weight basis), 140 °C for 15 min residence time. AFEX pretreatment on GLBRC corn stover for low-solid-loading enzymatic hydrolysis experiments was as previously described[2].

Fluorescence microscopy

Fixation and processing of pretreated and untreated stem samples were carried out as described previously by Avci *et al.* (2012)[3]. Semi-thin (250 nm thick) sections were stained with 10% (w/v) safranin solution (Sigma, Cat#94635) for 5 min or 0.05% (w/v) calcofluor for 1 minute. Images were captured at the same exposure time in an epifluorescence microscope (Nikon Eclipse 80i, DS-Ri1 camera, NIS-Elements Basic Research Software; Nikon Instruments Inc., Melville, NY). Bar = 100 µm and applies to all images.

X-ray powder diffraction (XRD)

XRD was performed on an X-ray powder diffractometer with its beam parallelized by a Gobel mirror (D8 Advance with Lynxeye detector; Bruker, Bruker AXS Inc., Madison, WI, USA). CuKα radiation (wavelength = 1.5418 Å) was generated at 40 kV and 40 mA. The detector slit was set to 2.000 mm. The sample was analyzed using a coupled 2θ/θ scan type with a continuous PSD fast scan mode. 2θ started at 8.000° and ended at 30.0277° with increments of 0.02151°, whereas θ started at 4.0000° and ended at 15.0138° with increments of 0.01075°. Step time was 1.000 s (i.e., 1025 total steps, effective total time 1157 s per run). Biomass samples (approximately 0.5 g) were placed in a specimen holder ring made of PMMA with 25 mm diameter and 8.5 mm height, rotating at 5°/min during analysis.

Preparation of cellulose standards

High purity (>95%) microcrystalline CI, purchased from Sigma-Aldrich (Avicel PH-101), served as CI standard in this study. The CIII standard was produced from Avicel PH-101 using anhydrous liquid ammonia, prepared in a high-pressure stirred batch reactor at 90 °C for 30 min residence time, using a 6:1 ammonia-to-cellulose ratio. The reactor pressure was maintained constant at 1000 psi with nitrogen overpressure. After pretreatment, ammonia was slowly evaporated from the reactor through a venting valve. During this process, the temperature of the reactor slowly decreased due to heat of vaporization of ammonia and was stabilized at 25 °C with a heating controller. After reaching atmospheric pressure, the cellulose sample was removed from the reactor, transferred to a flat container and placed overnight in the fume hood to evaporate residual ammonia present in pretreated biomass. No visual differences in color were observed after ammonia treatment of cellulose at these conditions. The CIII standard was stored at 4 °C in a zip-sealed bag prior to usage.

Compositional analysis of plant cell wall components

UT-CS and EA-CS solids were subjected to compositional analysis according to the standard NREL protocols NREL/TP-510-42618 and NREL/TP-510-42620, except that EA-CS was not water- and ethanol-extracted, to avoid removing water and ethanol-soluble lignin from the biomass and thereby over-predicting the percent lignin extraction. Mass balances on glucan, xylan, arabinan, and lignin were performed before and after the EA pretreatment.

For determining ester-linked ferulate content remaining in the pretreated cell walls, 40 mg of milled biomass (0.5 mm particle size) was subjected to ethanol extraction by adding 6 mL of 80% (v/v) ethanol to a capped test tube. This mixture was incubated at 50 °C for 1 h with periodic mixing (every 15 min). After incubation, the sample was centrifuged for 4 min at 3500 rpm. The supernatant was aspirated from the tube prior to a second addition of 6 mL of 80% (v/v) ethanol. The sample was gently vortexed at room temperature and centrifuged for 4 min at 3500 rpm before the liquid was aspirated from the tube. To facilitate drying, the sample was washed twice with 6 mL of 100% ethanol. The extracted biomass was left in a 50 °C oven overnight to dry before digestion. Quantification of ester-linked ferulate in the biomass was performed using a 2 M sodium hydroxide (NaOH) digestion as described in the literature[4]. Diethylether extraction of the hydroxycinnamic acids after NaOH digestion was not performed to avoid error propagation. Instead, the alkaline mixture was filtered (0.22 μm syringe filter) into a micro-centrifuge tube and diluted prior to quantification using LC-MS. LC-MS analyses were done using a QTRAP 3200 mass spectrometer (AB/Sciex) equipped with binary LC-20AD pumps (Shimadzu, Japan), a SIL-HTc auto sampler and Ascentis Express C18 column (5 cm x 2.1 mm; 2.7 μm particle size). The analytes were eluted with a reversed phase gradient using Solvent A (0.15% (v/v) aqueous formic acid) and Solvent B (methanol). Total solvent flow was maintained at 400 μL/min, and a gradient elution was performed using the following solvent compositions: initial, 5% B, held for 1 min; linear gradient to 18% B at 8 min and then to 50% B at 9 min; sudden increase to 99% B at 9.01 min and held until 10 min; and finally returned to initial condition at 10.1 min and held until 12 min. Injection volume was 5 μL, and column temperature was 50 °C. Enhanced Product-Ion (EPI) scans were generated for the $[M+H]^+$ ions of ferulic acid using electrospray ionization in positive-ion mode to identify abundant product ions. The parent ion selected for fragmentation was the protonated molecular species of ferulic acid. This analysis was performed to select suitable product ions for Multiple Reaction Monitoring (MRM) analysis. Source and gas parameters were all optimized for the standards to generate signals with highest intensity. After optimization, source parameters were as follows: curtain gas, 20 (arbitrary units); ion spray voltage,

4,500 V; temperature, 500 °C; gas 1, 25 (arbitrary units); gas 2, 25 (arbitrary units). After optimization, ferulic acid was analyzed using the 195>117 MRM transition, via a de-clustering potential of 20 V, entrance potential of 10 V and collision energy of 30 V. All mass spectrometric data were acquired and processed using Analyst v. 1.4.2 software from AB/Sciex. The quantification of ester-linked ferulates was performed in quadruplicate for each EA-CS sample as well as the untreated corn stover control, using appropriate standard curves. The results were further processed to calculate the percent of ester-linked ferulate that were depleted via EA pretreatment, with respect to the untreated corn stover.

Experimental design for EA pretreatment

A statistical design of experiments (DoE) was used in this study to evaluate the effect of EA pretreatment temperature, ammonia-to-biomass ratio (NH_3:BM), and residence time on 24 h glucan and xylan conversion. For this purpose, a Box-Behnken experimental design was created using Minitab software (Minitab Inc., State College, PA, USA), with two replicates, an alpha value of 1.15 and with high and low values for temperature of 25 °C and 115 °C, NH_3:BM ration of 3:1 and 6:1 and time of 5 min and 30 min, respectively.

A full quadratic response surface analysis was performed on the experimental results as a function of temperature, NH_3:BM, and residence time. All interaction effects between factors were considered in this analysis and parameters were included in the model based on their *P* value, as well as their influence on the predictive ability of the model. The regression equations were used to predict the responses of the various effects as a function of the pretreatment conditions within the boundaries set by the experimental design.

NMR characterization of lignin

Crude extracts generated from EA pretreatment of corn stover (~30 mg) were dissolved in DMSO-d_6/pyridine-d_5 (4:1, v/v, 600 µL) and transferred into NMR sample tubes. Enzymatic lignin (EL) from corn stover, described previously[5], was similarly prepared, and the whole corn stover material, after fine milling, was subjected to gel-NMR as previously described[5,6,7]. NMR spectra were acquired on a Bruker Biospin AVANCE 700 MHz spectrometer fitted with a cryogenically-cooled 5-mm TXI gradient probe with inverse geometry (proton coils closest to the sample). The central DMSO solvent peak was used as internal reference (δ_C, 49.5; δ_H, 3.49 ppm). Adiabatic HSQC experiments (hsqcetgpsisp2.2) were carried out using the parameters described previously[6,7]. Processing used typical matched Gaussian apodization in F2 (LB = −0.5, GB = 0.001) and squared cosine-bell apodization and one level of linear prediction (32 coefficients) in F1. Volume integration of contours in HSQC spectra (processed using no linear prediction) used Bruker's TopSpin 3.1 (Mac) software with no correction factors; i.e., the data represent volume integrals only; end groups (such as *p*-coumarate and tricin) are severely over-estimated by these methods due to their relaxation rate properties compared to the internal units of a chain. For quantitation of lignin aromatic distributions, only the carbon/proton-2 correlations from G and G' units and the carbon/proton-2/6 correlations from S and S' units were used, and the G and G' integrals were logically doubled; other aromatic integrals are reported relative to the total lignin aromatics (G + G' + S + S' = 100).

Low-solid-loading enzymatic hydrolysis

Enzymatic hydrolysis performed to evaluate the impact of EA pretreatment variables on sugar release was performed at 1% glucan loading, using 15 mg or 20 mg protein of enzyme per gram of glucan in 15 mL vials, incubated at 50 °C, with pH 4.8 for 24 h in an orbital shaking incubator (New Brunswick, USA). The enzymes utilized in this work were Cellic® CTec2 (138 mg protein/mL, batch No.VCNI0001) and Cellic® HTec2 (157 mg protein/mL, batch No.VHN00001), generously provided by Novozymes (Franklinton, NC, USA). The protein concentration for the enzymes was determined using the Kjeldahl nitrogen analysis method[8] (AOAC Method 2001.11, Dairy One Cooperative Inc., Ithaca, NY, USA). The enzyme ratios were 50% Cellic® CTec2 and 50% Cellic® HTec2 on a dry protein weight basis. These ratios were previously optimized to maximize total sugar conversion on EA-pretreated corn stover. After enzymatic hydrolysis, samples of the hydrolyzate were analyzed for glucose and xylose using an HPLC equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as previously described[9]. All the low-solid-loading enzymatic hydrolysis experiments used pretreated GLBRC corn stover.

High-solid-loading enzymatic hydrolysis

Enzymatic hydrolysis was performed in 250 mL Erlenmeyer flasks with 100 mL reaction volume, incubated at 50 °C, with pH adjusted to 4.8, in an orbital shaking incubator at 250 rpm (New Brunswick, USA). Biomass was added in fed-batch mode to improve mixing. The enzymes used in these experiments were Cellic® CTec2, Cellic® HTec2, from Novozymes, and Multifect Pectinase (MP) (72 mg protein/mL, batch No. 4861295753) a gift from Dupont Industrial Biosciences (Palo Alto, CA, USA). The protein concentration for the enzymes was determined using the Kjeldahl nitrogen analysis method (AOAC Method 2001.11, Dairy One Cooperative Inc., Ithaca, NY, USA). The enzyme ratios were 50% Cellic® CTec2, 25% Cellic® HTec2, and 25% MP on a dry protein weight basis for EA-pretreated corn stover. This commercial enzyme mixture was previously optimized to maximize total sugar conversion (data not shown). The optimal enzyme ratios for AFEX, IL, and DA pretreated corn stover were determined and used as described previously[1]. High-solid-loading enzymatic hydrolysis for comparing EA with AFEX was performed on GLBRC corn stover. MBI corn stover was used for comparing EA performance with IL and DA pretreatments, where two enzymatic hydrolysis conditions were used: Condition 1 used 7.5 mg protein/g glucan enzyme loading, at 8% glucan loading for 96 h; Condition 2 used 30 mg protein/g glucan enzyme loading, at 6% glucan loading for 72 h.

Hydrolysate fermentation

Saccharomyces cerevisiae 424A(LNH-ST), which is a genetically modified yeast strain that ferments xylose[10], was kindly provided by Prof. Nancy W. Y. Ho, Purdue University and was used for fermentation of hydrolysates. A yeast extract-tryptone medium with 100 g/L glucose, 25 g/L xylose, 10 g/L yeast extract, and 20 g/L tryptone was used as seed culture medium. Seed culture was prepared in a 250 mL Erlenmeyer flask with 100 mL medium inoculated with a frozen glycerol stock. After inoculation, the seed culture had an initial optical density at 600 nm (OD_{600}) of 0.1. The flask was capped using a rubber stopper with a needle pierced through and was incubated at 30 °C and 150 rpm for 22 h. The seed culture OD_{600} reached 14 at 22 h and was centrifuged at 4000 rpm for 5 min. The resulting yeast cell pellets were used for inoculation of hydrolysate fermentation. Hydrolysate fermentation was performed in a 125 mL Erlenmeyer flask with working volume of 50 mL at pH 5.5, 30 °C and 150 rpm for 120 h. The initial OD_{600} for fermentation was 2.0. A 0.5 mL sample was taken at different time points during fermentation for HPLC analysis.

Ammonia recycling and thermodynamic simulations for EA

To determine the energy required to recycle ammonia after EA pretreatment, process simulations were performed using ASPEN Plus V.8.6 software. Ammonia, water and nitrogen were the components included in the simulation. The Peng Robinson thermodynamic model was used to predict the thermodynamic properties of ammonia-water mixtures. Vapor-Liquid equilibrium (VLE) predictions for ammonia-water mixtures using the Peng Robinson method in ASPEN Plus fit reasonably well to the corrected experimental data from Wucherer, J. *et al.* (1932),[11] which has been also published by Tillner-Roth, R. *et al.* (1998).[12] The ammonia loss was experimentally calculated by performing EA pretreatment without the extraction process. The pretreated and untreated biomass were further analyzed for nitrogen content using a nitrogen analyzer (Skalar Primacs^{SN}, The Netherlands). Nitrogen mass balance was performed and converted to ammonia equivalents.

Ammonia recycling data for AFEX

The data used to estimate the energy cost for ammonia recycling and respective ammonia recovery yields was experimentally obtained by MBI International (Lansing, MI) in their pilot scale unit. The process design and ammonia recycling method during AFEX pretreatment was previously reported by Campbell, T. *et al.* (2014)[13].

Fig. S1 Effect of ammonia loading on (a), 24 h enzymatic digestibility of corn stover at 1% glucan loading and 15 mg protein/g glucan enzyme loading and (b), CIII formation during EA pretreatment. Pretreatment was performed at 120 °C for 1 h, varying the NH_3:BM ratio from 1:1 to 6:1. CIII was not completely formed at 1:1 NH_3:BM ratio. However, for ammonia loadings higher than or equal to 2:1, XRD data shows the pattern associated with CIII and no evidence of the presence of CI. Glucan conversion data shows a considerably lower sugar release for 1:1 NH_3:BM ratio, whereas for 2:1 there is a slightly lower sugar release compared to the remaining samples. These differences may be connected to the lower degree of lignin solubilization and extraction. For samples prepared at ammonia loadings higher than 2:1, no major differences were detected in sugar release, possibly because enzymatic hydrolysis is approaching maximal conversion at 24 h.

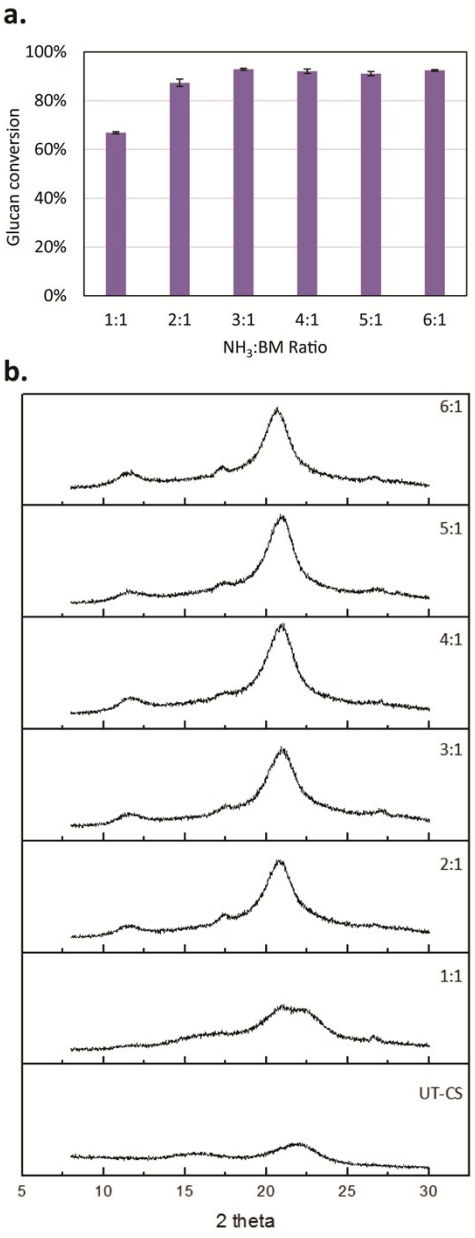


Fig. S2 EA pretreatment temperature effect on the depletion of mono-ferulate ester residues from corn stover. Pretreatment was performed at 6:1 NH_3:BM ratio and 30 min residence time. Coumaroyl and feruloyl amides have been detected by NMR, proving evidence that ammonolsis reactions are taking place during cleavage of coumarate and ferulate linkages. The sugar conversion relationship with pretreatment temperature is also shown.

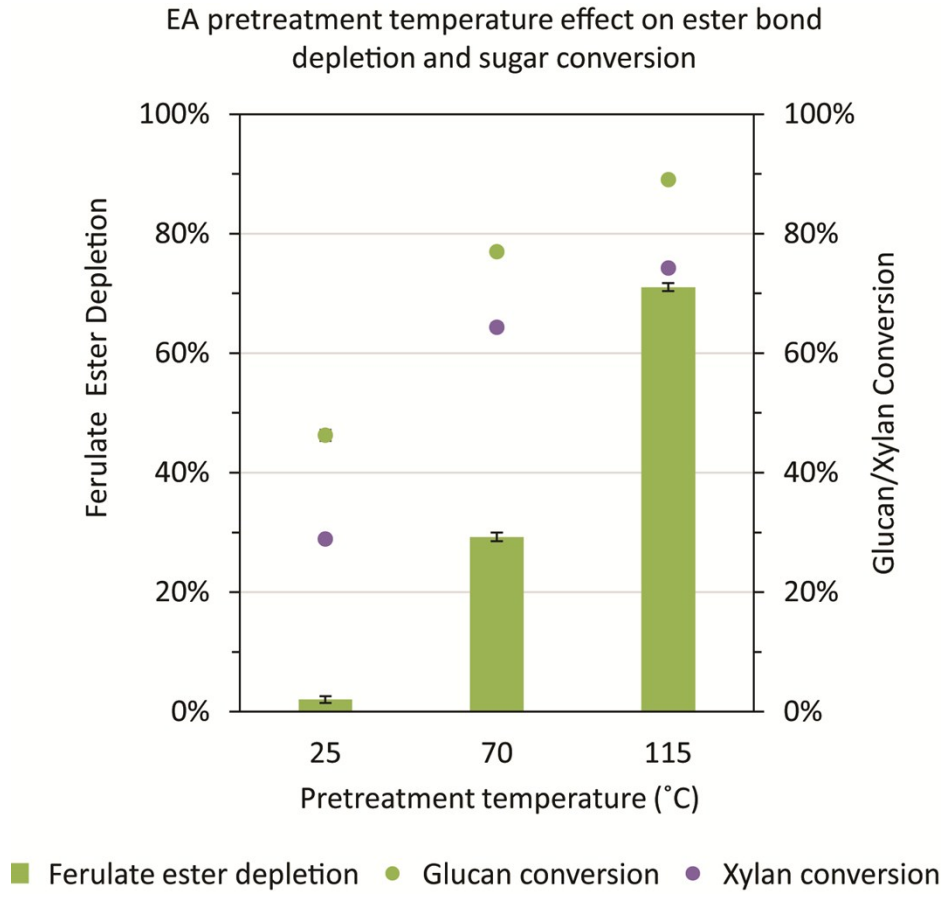
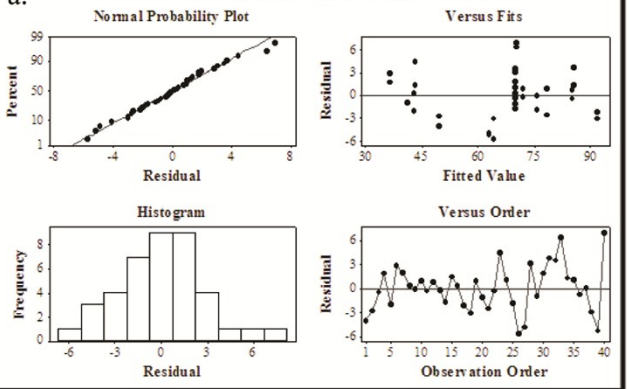


Fig. S3 Residual plots for regression of (a), 24 h glucan conversion and respective (b), regression coefficients. Residual plots for regression of (c), 24 h xylan conversion and respective (d), regression coefficients. Regression equations for glucan and xylan conversions are also presented.

a.

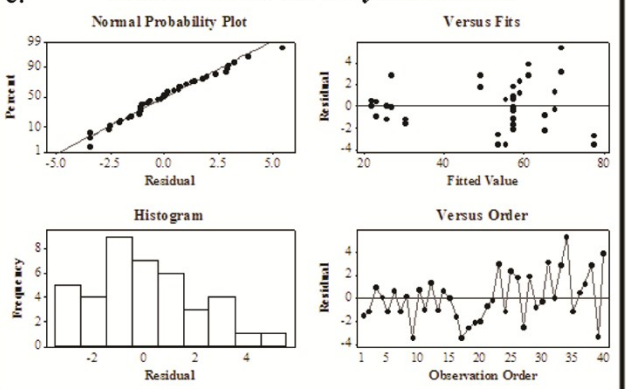


b. Regression Coefficients for 24h % Glucan Conversion

Term	Coef	SE Coef	T	P
Constant	-8.4167	8.4671	-0.994	0.327
Temperature	0.7285	0.07479	9.741	0
NH_3:BM	12.3293	4.02309	3.065	0.004
time	0.2021	0.11141	1.814	0.079
Temperature*Temperature	-0.0025	0.00049	-5.028	0
NH_3:BM*NH_3:BM	-1.1312	0.44418	-2.547	0.016
time*time				
Temperature*NH_3:BM	-	-	-	-
Temperature*time	0.0028	0.00139	2.047	0.049
NH_3:BM*time	-	-	-	-

S = 3.128 **PRESS** = 506.812
R-Sq = 96.64% **R-Sq (pred)** = 94.73% **R-Sq (adj)** = 96.03%

c.



d. Regression Coefficients for 24h % Xylan Conversion

Term	Coef	SE Coef	T	P
Constant	-19.8537	6.83575	-2.904	0.007
Temperature	0.9504	0.06154	15.443	0
NH_3:BM	6.6245	3.32887	1.99	0.055
time	0.6163	0.20533	3.001	0.005
Temperature*Temperature	-0.004	0.00041	-9.796	0
NH_3:BM*NH_3:BM	-0.4878	0.36772	-1.327	0.194
time*time	-0.0144	0.0053	-2.717	0.011
Temperature*NH_3:BM	-	-	-	-
Temperature*time	0.0033	0.0011	2.978	0.005
NH_3:BM*time	-	-	-	-

S = 2.48153 **PRESS** = 342.335
R-Sq = 98.15% **R-Sq (pred)** = 96.78% **R-Sq (adj)** = 97.74%

24h % Glucan Conversion $= (-8.4167) + (0.7285 X_1) + (12.3293 X_2) + (0.2021 X_3) - (0.0025 X_1X_1) - (1.1312 X_2X_2) + (0.0028 X_1X_3)$

24h % Xylan Conversion $= (-19.8537) + (0.9504 X_1) + (6.6245 X_2) + (0.6163 X_3) - (0.004 X_1X_1) - (0.4878 X_2X_2) - (0.0144 X_3X_3) + (0.0033 X_1X_3)$

X_1 = Temperature (°C) [18.25 to 121.75 °C]; X_2 = NH_3:BM [2.78 to 6.23]; X_3 = Time (min) [3.13 to 31.88 min].

Fig. S4 Effect of pretreatment conditions on corn stover crystallinity by X-ray diffraction (II) and impact on 24 h glucan and xylan conversion (I); enzymatic hydrolysis was performed at 1% glucan loading and 15 mg protein/g glucan enzyme loading. As temperature and residence time increased, corn stover became more crystalline due to improvements in lignin extractability. Samples pretreated with short residence times at lower temperature contained a mixture of cellulose I and cellulose III. The samples pretreated at higher temperatures and residence times had a higher crystallinity and were fully converted cellulose I to III, achieving the best enzymatic hydrolysis results. At lower temperatures, even samples that do not contain cellulose I could not achieve high sugar yields. AFEX glucan conversion is presented in green as a control under the same enzymatic hydrolysis conditions. Control CI is Avicel PH-101 and CIII is ammonia treated Avicel PH-101.

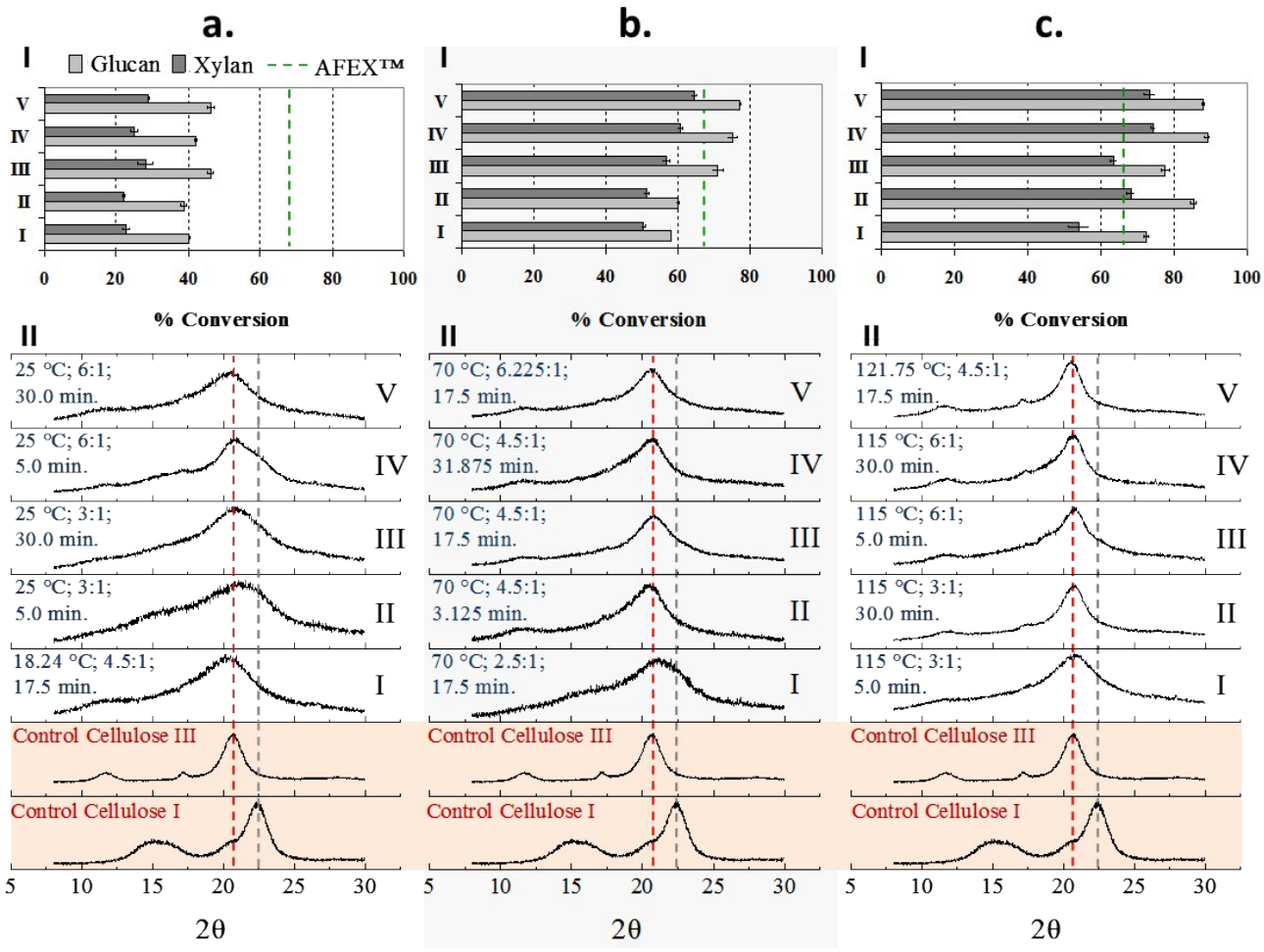


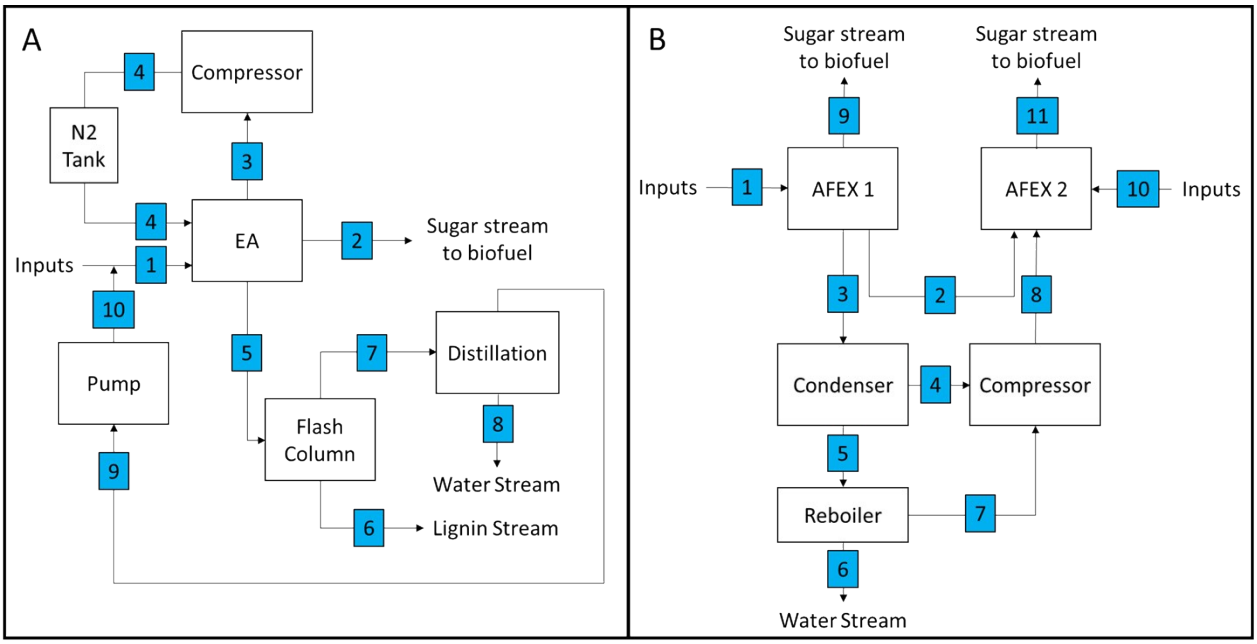
Table S1 HSQC NMR assignments and annotations[5,6,7].

	Annotation	^{1}H	^{13}C
β-Aryl Ether, (A)	C α	5.04	71.7
	C β-G	4.50	83.5
	C β-S	4.22	86.0
	C β-S	4.08	87.1
	C γ	3.64	59.7
Tricin, (T)	C 2/6	7.34	103.7
	C 3 attached	7.09	104.7
	C 3 free	7.03	103.5
	C 6	6.31	98.7
	C 8	6.63	94.1
Syringyl, (S)	C 2/6	6.79	103.6
	C 2/6 (oxidized)	7.36	105.8
	C 5/6	7.10	115.6
	C 5/6	6.83	119.0
	C 2	7.09	110.8
Feruloyl amide, (FAM)	C 2	7.09	110.8
	C 6	7.00	121.3
	C α	7.42	139.0
	C β	6.51	118.7
p-Coumaryl ester, (*p*CA)	C 2/6	7.49	129.9
	C 3/5	6.87	115.4
	C α	7.47	144.4
	C β	6.28	113.7
p-Coumaroyl amide, (*p*CAM)	C 2/6	7.41	129.1
	C 3/5	6.87	115.4
	C α	7.42	139.0
	C β	6.51	118.7
Phenylcoumaran, (B)	C α	5.57	87.1
	Methoxyl group	3.65	55.2

Table S2 *Saccharomyces cerevisiae* 424A (LNH-ST) fermentation performance in hydrolyzate derived from corn stover subjected to various pretreatments. The hydrolysate was produced using "Condition 1" on MSU corn stover, i.e., at 8% glucan loading, 7.5 mg protein/g glucan for 96 h residence time. The hydrolysate was not supplemented with nutrients, nor detoxified prior to fermentation. Standard deviations are presented in parentheses.

Fermentation Stage					
Pretreatment type	**Sugar Consumption (%)**		**Final Ethanol Concentration (g/L)**	**Final OD_{600}**	**Ethanol Yield (kg/100 kg Biomass)**
	glucose	**xylose**			
Dilute Acid	100 (0.0)	86.6 (0.7)	29.5 (0.2)	5.8 (0.0)	9.3 (0.3)
Extractive Ammonia	99.3 (0.3)	93.7 (0.5)	56.8 (0.2)	14.5 (1.1)	18.2 (0.2)
Ionic Liquid	99.3 (0.2)	19.4 (4.2)	49.4 (1.9)	5.5 (0.3)	17.9 (0.9)

Fig. S5 A) Process flow diagram for EA pretreatment including ammonia recycling unit operations. B) Process flow diagram for AFEX pretreatment including ammonia recycling operations.



List of key assumptions and basis for energy and mass balances:

A) Yields and consumable prices
   1) Ethanol yield of 0.18 Kg ethanol/Kg biomass, experimentally verified at the following enzyme loadings:
      a) Enzyme loading for EA pretreated biomass is 7.5 mg protein/g glucan (2.29 Kg enzyme/ tonne untreated biomass).
      b) Enzyme loading for AFEX pretreated biomass is 18.5 mg protein/g glucan (6.18 Kg enzyme/ tonne untreated biomass)
   2) 2.2 Kg of ammonia reacted with 100 Kg of untreated biomass during EA pretreatment and 2.0 Kg of ammonia reacted with 100 Kg of untreated biomass during AFEX pretreatment (value provided by MBI International)
   3) Ammonia price of $700.00 per tonne.
   4) Enzyme price of $4.24 per Kg or protein[14].
   5) Natural gas price of $2.06 per MMBTU.
   6) Electricity price of $0.08 per kWh.

B) Ammonia recycling process for EA pretreatment
   1) Ammonia-soluble biomass components (mostly lignin) extracted to the flash column were not considered for heat duty calculations.
   2) Biomass moisture was assumed to be completely extracted from the biomass during EA pretreatment. Only non-reacted ammonia and moisture inputs were both considered for ammonia recycling simulations.

3) Nitrogen is used for maintaining the pressure at 87 bar during liquid ammonia extraction from the bottom of the EA reactor. Nitrogen is further recompressed to the original vessel after pretreatment, so the biomass can be removed from the reactor at atmospheric pressure.
4) High pressure condensation of vapor ammonia during distillation was considered as a possible ammonia purification protocol. Air cooling at atmospheric conditions was used for heat exchange, based on the rectification column condenser used by NREL[14]. According to this reference, a 300 HP fan is required for a heat duty of 92.2 MMBTU/h.
5) Ammonia recycling was designed for maximum ammonia recovery with a composition of 100.0 wt% ammonia.

C) Ammonia recycling process for AFEX pretreatment
1) The ammonia recycling process flow diagram for AFEX pretreatment is based on the design developed by MBI International, Lansing, MI.
2) Heat and power requirements for ammonia recycling during AFEX pretreatment were experimentally obtained by MBI International during pilot scale runs.

Description of EA and AFEX pretreatments and ammonia recycling systems

Fig. S5A shows a preliminary process flow diagram for EA pretreatment in batch mode. In this process, liquid ammonia is added to the biomass with 10% moisture (dry biomass basis) in the EA reactor, temperature is then raised to 120˚C and maintained for 30 min residence time. Pressure is built in the EA reactor as temperature increases. For 6:1 NH_3:BM ratio, pressure will raise up to 1250 psi. Vapor-liquid equilibrium (VLE) calculations for ammonia-water mixture at an NH_3:BM of 6:1 (ignoring the fluid interactions with solid biomass) under the conditions mentioned above, show that 99.8 wt% of the vapor-phase is composed by ammonia and 97.8 wt% of the liquid-phase is composed by ammonia. The vapor-phase contains about 19 wt% of the total fluid mass and the liquid-phase contains the remaining 81%. Under these specific conditions, about 53% of the available volume in the reactor is occupied by the vapor-phase and 47% is occupied by the liquid phase. After reaction, the ammonia and extractives are filtered to a flash column. Nitrogen is used to maintain pressure at 87 bar in the reactor, so ammonia can be always in the liquid-phase during extraction. Once filtration is complete, nitrogen is compressed back to the storage tank, the extractives are collected and ammonia is recycled. Our preliminary ammonia recycling system for EA (Fig. S5A) is composed by a flash column operating at 36 bar and 137˚C, where liquid ammonia and residual water is completely evaporated from the extractives, which are condensed and recovered in the bottom of the tank. The gaseous ammonia-water mixture is further distilled at 36 bar. After distillation, gaseous anhydrous ammonia is condensed from the top of the distillation column to liquid anhydrous ammonia, which can then be pumped back to the reactor. Condensation of ammonia at 36 bar can be performed using cooling water or cooling air at room temperature. For the purpose of energy balances, we have assumed a condenser working with cooling air, similarly to what NREL proposed for ethanol condensation after rectification[14]. Make-up ammonia is required to be added, as 0.022 g of ammonia are lost for every 100 g of untreated biomass processed due to ammonia reactions with the biomass. Stream tables projecting flow rates and operating conditions for EA pretreatment performed at different ammonia loadings are presented in Tables S3 to S6.

During AFEX, a moisture content of 60% and a NH_3:BM ratio of 1:1 is used at 140˚C. Under these conditions, the pressure raises up to about 400 psi. Vapor-liquid equilibrium calculations for ammonia-water mixture using Peng-Robinson model in ASPEN Plus show that about 50% of the weight of the ammonia-water mixture is in the liquid phase, occupying only about 2.3% of the available volume. The gas-phase is composed of 91 wt% ammonia and the liquid phase is composed of 35 wt% ammonia, while the remaining is composed by water. These calculations were performed ignoring the effect of the solid biomass and extractives on the vapor-liquid equilibrium of the ammonia-water mixture. However, they provide useful insight about how ammonia-water mixtures behave under AFEX pretreatment conditions. For example, water is the dominant component in the liquid-phase, contributing with 65 wt% of the mixture under these conditions. Also, most of the volume of ammonia is in the gas phase. This is why it is important to have water adsorbed to the biomass before ammonia is loaded, so ammonia gas can be dissolved in the water and react uniformly with the biomass through the liquid film generated on the surface of the biomass. If we apply nitrogen overpressure up to 1200 psi to mimic EA pretreatment-like conditions, all the ammonia and water will be in the liquid-phase. Thus, the concentration of ammonia in the liquid layer contacting the biomass will also be higher. This may improve pretreatment effectiveness, however, water will always prevent cellulose III to be formed during AFEX pretreatment, unlike what is observed for EA pretreatment.

After AFEX is performed, ammonia is released in the gas phase from reactor 1 to reactor 2 until pressures equalize (Fig. S5). At this point, steam is used to strip the remaining gaseous ammonia from reactor 1 to reactor 2. Due to the pressure differential, the steam stripped ammonia needs to be compressed to reactor 2, before passing by a condenser to remove any residual moisture. The ammonia trapped in the steam condensate is further evaporated using a reboiler before being compressed to reactor 2. During AFEX pretreatment, about 0.02 g ammonia reacts with 100 g biomass. To compensate this loss, ammonia needs to be replenished in the beginning of every pretreatment cycle.

Energy requirements and consumable costs for ammonia recycling during EA and AFEX

The energy requirements for the different unit operations during EA and AFEX (including ammonia recycling) are presented in Tables S7 and S8, respectively. Energy duties for each EA pretreatment unit operation were estimated using ASPEN Plus software for various ammonia loading conditions ranging from 3:1 to 6:1 NH_3:BM. To simplify ammonia recycling calculations, the presence of ligin-rich components dissolved in ammonia-water mixtures were not considered. The condenser heat duty was converted to electrical power duty for the fan in an air cooled condenser. A linear conversion was performed using the values of fan power vs condenser heat duty proposed by NREL for ethanol condensation after rectification. This correlation may not be the most accurate, as heat transfer coefficients may vary between the two systems. However, this assumption was used as a first estimation of the power duty for ammonia condensation. Energy duties for AFEX pretreatment unit operations were experimentally obtained by MBI International during pilot scale operations.

Assuming the heat and power requirements estimated in this model, the energy costs for ammonia recycling during EA pretreatment vary significantly with NH_3:BM ratio (Table S7). While energy costs reached $0.22 per gallon ethanol for 6:1 ammonia loading, a lower ammonia loading of 3:1 reduced energy costs down to $0.13 per gallon ethanol. As expected, an even lower energy cost of $0.11 per gallon of ethanol is estimated for AFEX pretreatment (Table S7). These estimations assumed that the

price of heat is comparable to the price of natural gas ($2.06 per MMBTU) and the price of electricity was assumed to be $0.08 per kWh. Interestingly, most of the energy cost for ammonia recycling during EA pretreatment is associated to heat requirements, while during AFEX pretreatment the heat and power requirements are not very different. From this point of view, EA pretreatment would benefit more from heat integration with industries that produce medium-high temperature waste heat (e.g. steel mills, cement kilns, boiler exhausts or gas turbine exhausts from power plants, etc.) and mitigate some of the heat cost for ammonia recycling. However, it is still important to continue engineering the EA process to further reduce ammonia loading and operating pressure, so it can be more sustainable in economic and environmental terms.

To evaluate the impact of spending extra energy on ammonia recycling during EA, compared to AFEX pretreatment, the costs of enzyme and make-up ammonia must be also determined. Assuming that ethanol yields do not change significantly between 6:1 and 3:1 NH3:BM ratio during EA pretreatment, we have estimated that the total consumable costs for ammonia recycling, make-up ammonia and enzymes vary from $0.64 to $0.55 per gallon ethanol (Table S8). For AFEX pretreatment the sum of these costs raise up to $0.77 per gallon ethanol, based on our experimental ethanol yields. The high enzyme loading during AFEX pretreatment (18.5 mg protein/g glucan to achieve similar ethanol yields as EA pretreated biomass with 7.5 mg protein/g glucan) is the primary responsible for such high difference in these consumable costs. Based on this information, it is clear that AFEX-based biorefineries will be more sensitive to enzyme cost fluctuations, while EA-based biorefineries will be more sensitive to energy cost fluctuations (especially heat costs). The cost of energy and enzyme per gallon of ethanol can be further reduced if ethanol can be produced at higher yields that the ones reported in this study. A recent report shows that AFEX pretreated biomass hydrolyzed after pelletization in a pilot scale can generate higher ethanol yields that the ones reported in this study, using similar enzyme loadings[15]. The effect of scale and pelletization need to be address to understand the reasons for such ethanol yield enhancement. Biomass pretreated with other methods, such as EA pretreatment, could potentially benefit from the same processing conditions.

IL recycle and recovery compared to ammonia pretreatment

EA and IL pretreatments show very high performance at low enzyme loading (7.5 mg protein/g glucan) and high solid loading (8% glucan loading) (Fig. 5d). Also, both pretreatments allow comparable ethanol yields from corn stover under such conditions. Like EA pretreatment, IL-based pretreatments present operational challenges that need to be addressed in order to become industrially viable. One major bottleneck is the price of ILs. The production cost of some of the cheapest ILs in the literature is approximately $1.24 per Kg,[16] which is significantly higher than the price of ammonia (about $0.7 per Kg) used for EA pretreatment. Due to these considerable chemical price differences, IL-based pretreatments are significantly more vulnerable to marginal IL losses than ammonia-based processes. Thus, IL recovery and reuse are critical aspects to address in IL-based pretreatment research.

The most common approaches to recover ILs often require substantial washing steps to remove ILs adsorbed onto the surface of the solid biomass. Washing steps, often generate a large volume of solvent (usually water, but also ethanol or acetone)[17,18,19] that must be evaporated to recover the IL, which turns to be quite energy intensive. For example, in order to achieve a comparable energy duty with EA pretreatment performed at 6:1 NH_3:BM ratio, it is only possible to evaporate ~2 Kg water or ~8

Kg acetone for every 1 Kg input of untreated biomass at atmospheric pressure. Also, IL decomposition may be an issue when applying distillation processes, leading to unrecoverable losses[20]. The one-pot IL pretreatment and saccharification technology was initially proposed by Jian Shi, *et al.* (2013)[22] to avoid extensive washing steps and reduce recovery costs. IL recovery using solvent extraction was only 90.8%.[22] This low IL recovery combined with high IL loading of 9:1, represented a cost of about $17 per gallon ethanol for make-up IL, assuming an ethanol yield of 0.18 g/g biomass (as obtained in this study at Condition 1 (Fig. 5d)) and an IL price of $1.24 per Kg.[16] Efforts are being made to improve IL recyclability and recovery, as well as reduce IL production costs. Recently, Feng Xu *et al.* (2015)[21] assumed 99.9% recovery using pervaporation technology for recycling of IL in a one-pot pretreatment and saccharification process with low IL loading (0.29 IL:BM ratio). Also, the introduction of bionic liquids can potentially reduce their production cost to as low as $0.75 per Kg. If these targets are achieved, the cost of make-up ILs will be practically negligible for the biorefinery. In this case, energy costs will become the dominant factor for IL recycle.

Table S3 – Stream table showing the properties of each ammonia recycling stream for EA pretreatment using 6:1 NH_3:BM ratio.

6:1 NH_3:BM Ratio										
Stream	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
State	Liquid	Solid*	Vapor	Vapor	Liquid	Liquid	Vapor	Liquid	Liquid	Liquid
Mass Flow kg/hr										
AMMONIA	500000	1833	0	0	498167	0	498167	50	498117	498117
WATER	9250	0	0	0	9250	0	9250	9249	1	1
NITROGEN	0	0	80366	80366	0	0	0	0	0	0
Mass Fraction										
AMMONIA	1		0	0	1		1	0	1	1
WATER	0		0	0	0		0	1	0	0
NITROGEN	0		1	1	0		0	0	0	0
Total Flow kg/hr	509250		80366	80366	507417	0	507417	9299	498118	498118
Total Flow l/min	16392		13418	9291	29094	0	411701	194	16086	16171
Temperature, ˚C	76		25	113	120		137	244	74	75
Pressure, bar	87		87	173	87	36	36	36	36	87
Vapor Fraction	0	0	1	1	0		1	0	0	0
Liquid Fraction	1	0	0	0	1		0	1	1	1

Table S4 - Stream table showing the properties of each ammonia recycling stream for EA pretreatment using 5:1 NH_3:BM ratio.

5:1 NH_3:BM Ratio										
Stream	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
State	Liquid	Solid*	Vapor	Vapor	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Mass Flow kg/hr										
AMMONIA	416667	1833	0	0	414834	0	414834	41	414792	414792
WATER	9250	0	0	0	9250	0	9250	9249	1	1
NITROGEN	0	0	69762	69762	0	0	0	0	0	0
Mass Fraction										
AMMONIA	1		0	0	1		1	0	1	1
WATER	0		0	0	0		0	1	0	0
NITROGEN	0		1	1	0		0	0	0	0
Total Flow kg/hr	425917		69762	69762	424084	0	424084	9291	414793	414793
Total Flow l/min	13687		11647	8065	18003	0	343883	194	13395	13467
Temperature, ˚C	76		25	113	120		137	244	74	75
Pressure, bar	87		87	173	87	36	36	36	36	87
Vapor Fraction	0	0	1	1	0		1	0	0	0
Liquid Fraction	1	0	0	0	1		0	1	1	1

Table S5 - Stream table showing the properties of each ammonia recycling stream for EA pretreatment using 4:1 NH_3:BM ratio.

4:1 NH_3:BM Ratio										
Stream	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
State	Liquid	Solid*	Vapor	Vapor	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Mass Flow kg/hr										
AMMONIA	333333	1833	0	0	331500	0	331500	33	331467	331467
WATER	9250	0	0	0	9250	0	9250	9249	1	1
NITROGEN	0	0	55809.55	55810	0	0	0	0	0	0
Mass Fraction										
AMMONIA	1		0	0	1		1	0	1	1
WATER	0		0	0	0		0	1	0	0
NITROGEN	0		1	1	0		0	0	0	0
Total Flow kg/hr	342583		55809.55	55810	340750	0	340750	9282	331468	331468
Total Flow l/min	10981		9317.848	6452	14419	0	276065	193	10704	10762
Temperature, ˚C	76		25	113	120		137	244	74	75
Pressure, bar	87		87.19772	173	87	36	36	36	36	87
Vapor Fraction	0	0	1	1	0		1	0	0	0
Liquid Fraction	1	0	0	0	1		0	1	1	1

Table S6 - Stream table showing the properties of each ammonia recycling stream for EA pretreatment using 3:1 NH_3:BM ratio.

	3:1 NH_3:BM Ratio									
Stream	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
State	Liquid	Solid*	Vapor	Vapor	Liquid	Liquid	Vapor	Liquid	Liquid	Liquid
Mass Flow kg/hr										
AMMONIA	250000	1833	0	0	248167	0	248167	25	248142	248142
WATER	9250	0	0	0	9250	0	9250	9249	1	1
NITROGEN	0	0	41857	41857	0	0	0	0	0	0
Mass Fraction										
AMMONIA	1		0	0	1		1	0	1	1
WATER	0		0	0	0		0	1	0	0
NITROGEN	0		1	1	0		0	0	0	0
Total Flow kg/hr	259250		41857	41857	257417	0	257417	9274	248143	248143
Total Flow l/min	8275		6988	4839	10835	0	208246	193	8013	8058
Temperature, ˚C	76		25	113	120		137	245	74	76
Pressure, bar	87		87	173	87	36	36	36	36	87
Vapor Fraction	0	0	1	1	0		1	0	0	0
Liquid Fraction	1	0	0	0	1		0	1	1	1

Table S7 – Energy requirements for each unit operation involved in ammonia recycling during EA pretreatment for a 2000 ton biomass/day biorefinery.

	Input Streams for EA Pretreatment		Energy Requirements for Ammonia Recycling in EA Pretreatment					
NH_3:BM Ratio	**Liquid Ammonia Loading (Tonne/day)**	**Water (Tonne/day)**	**NH_3 Flash (MMBTU/day)**	**Distillation Reboiler (MMBTU/day)**	**Distillation Condenser (MMBTU/day)**	**Fan Duty (Condenser) (KW)**	**Ammonia Pump (kW)**	**Nitrogen Compressor (kW)**
6:1	12000	222	8188.6	198.6	12977.5	1319.6	1599.0	2114.0
5:1	10000	222	7837.5	273.43	10751.2	1093.2	1343.7	1835.1
4:1	8000	222	6707.9	269.1	8604.1	874.9	1087.3	1468.1
3:1	6000	222	5230.6	255.14	6466.6	657.5	829.2	1101.0

Table S8 - Energy requirements for each unit operation involved in ammonia recycling during AFEX pretreatment for a 2000 ton biomass/day biorefinery. These are experimental results from pilot scale tests provided by MBI International.

	Input Streams For AFEX Pretreatment		Energy Requirements for Ammonia Recycling in AFEX Pretreatment			
NH_3:BM Ratio	**Liquid Ammonia Loading (Tonne/day)**	**Water (Tonnne/day)**	**NH_3 Stripping (MMBTU/day)**	**Condenser (MMBTU/day)**	**Reboiler (MMBTU/day)**	**Compressor (KW)**
1:1	2000	1200	2317.7	1100	415.3	3750

Table S1 – Total energy cost for recycling ammonia during EA and AFEX pretreatments per gallon of ethanol produced.

		Energy Consumption for NH_3 Recovery per Gallon of Ethanol		Energy Cost for NH_3 Recycling Per Gallon of Ethanol		
Pretreatment	**NH_3:BM Ratio**	**Heat (MMBTU)**	**Power (KWh)**	**Natural Gas**	**Electricity**	**Total**
EA	**6:1**	0.07	0.99	**$ 0.14**	**$ 0.08**	**$ 0.22**
	5:1	0.07	0.84	**$ 0.14**	**$ 0.07**	**$ 0.20**
	4:1	0.06	0.68	**$ 0.12**	**$ 0.05**	**$ 0.17**
	3:1	0.05	0.51	**$ 0.09**	**$ 0.04**	**$ 0.13**
AFEX	**1:1**	0.02	0.74	**$ 0.05**	**$ 0.06**	**$ 0.11**

Table S2 – Total costs for make-up ammonia, ammonia recycling energy and enzyme costs for EA pretreatment at various ammonia loadings and AFEX pretreatment.

				Key Consumable Costs Per Gallon of Ethanol			
Pretreatment	**NH_3:BM Ratio**	**NH_3 Recovery**	**Recycled NH_3 Concentration**	**Make-up NH_3**	**NH3 Recycling**	**Enzyme**	**Total**
EA	**6:1**	99.62%	100.0 wt%	$ 0.26	$ 0.22	$ 0.16	**$ 0.64**
	5:1	99.55%	100.0 wt%	$ 0.26	$ 0.20	$ 0.16	**$ 0.62**
	4:1	99.44%	100.0 wt%	$ 0.26	$ 0.17	$ 0.16	**$ 0.59**
	3:1	99.26%	100.0 wt%	$ 0.26	$ 0.13	$ 0.16	**$ 0.55**
AFEX	**1:1**	98.00%	100.0 wt%	$ 0.23	$ 0.11	$ 0.43	**$ 0.77**

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