MATERIALS AND METHODS

All chemicals used were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. 1-ethyl-3-methylimidazolium acetate, [C$_2$C$_1$im][OAc], was purchased from Sigma-Aldrich and used as received. Switchgrass (Panicum virgatum) was provided by Dr Daniel Putnam, University of California at Davis. The air-dried biomass was milled by a Thomas-Wiley Mini Mill fitted with a 40-mesh screen (Model 3383-L10 Arthur H. Thomas Co., Philadelphia, PA, USA) and sieved to the nominal sizes of 40-60 mesh (250-400 μm). All feedstocks were further dried in a vacuum oven at 40 °C overnight prior to pretreatment to eliminate the variability of moisture content.

IL synthesis: The synthesis of the protic ionic liquids was achieved by combining an acid and a base in stoichiometrically equal amounts, similar to techniques described elsewhere.$^{17}$ Each amine/sulfuric acid adduct was prepared by the dropwise addition of H$_2$SO$_4$ (95%) in water (3 ml of water per every 1 ml of H$_2$SO$_4$) to a solution of the amine in water (1 ml of water per every 1 ml of amine). The purity of the starting materials was considered when calculating the quantities to be combined. The mixtures were stirred at room temperature for several hours. Water was then removed in vacuo at 50 °C for 48 h. We have endeavored to control the acid:base ratio at 1:1 by careful dosing of sulfuric acid and amine based on the purity stated by the manufacturer. However, since purity of both acid and base was <100%, we cannot guarantee that the mixtures used in this study had the exact compositions we aimed for (up to 4% deviation possible, within the limits of elemental analysis).
Pretreatment: All pretreatments were conducted in the presence of 20% water (w/w IL) and data compared to [C_2C_1im][OAc]:[H_2O] (4:1 w/w) by a methodology described elsewhere. Briefly, 10% (w/w) switchgrass in IL:H_2O (4:1 w/w), 20g total reaction mass, was loaded in a Syrris globe reactor at 120 °C for 180 min, unless otherwise noted. The solution was allowed to cool to 50 °C, and then soaked in 3 parts (w/w) ethanol to retain lignin in solution and precipitate dissolved biomass, if necessary. Further to this, the solution was filtered and the solids washed twice with 3 parts (w/w) deionized water to remove residual IL and the recovered biomass was then lyophilized.

Carbohydrate and lignin assays: The carbohydrate composition of biomass and residual biomass after hydrolysis was determined with a modified quantitative saccharification (QS) procedure. In the modified QS, secondary hydrolysis was conducted in the presence of 1% (w/w) sulfuric acid at 121 °C for 1 h to more accurately determine the quantities of sugars susceptible to acid degradation (e.g., xylan). After CaCO_3 neutralization and centrifugation, monomeric sugars in the supernatant were measured with an Agilent HPLC equipped with a Bio-Rad Aminex HPX-87P column (Richmond, CA) at a rate of 0.6 mL of deionized water per min at 60 °C. Glucose yield after hydrolysis was calculated as follows, where glucan in recovered biomass was converted to glucose:

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\text{Glucose Yield \ [%] =} \frac{\text{mass glucose in hydrolysate}}{\text{mass glucose in recovered biomass}} \times 100
\]

The standard NREL biomass protocol was used to measure lignin and ash. Briefly, solids remaining after two-stage acid hydrolysis were held at 105 °C overnight. The mass of the dried solids corresponds to the amount of acid-insoluble lignin and ash in the sample. The mass of the ash-only fraction was then determined by heating the solids to 575 °C for 24 h. The acid-soluble lignin content of the sample was determined by measuring the UV absorption of the acid hydrolysis supernatant at 320 nm wavelength and an absorptivity of 25. Total lignin was calculated as the sum of acid soluble and acid insoluble lignin.

The acid soluble lignin (ASL) on an extractives free basis was calculated as follows:

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\% \text{ASL} = \frac{UV_{\text{Abs}} \times Volume_{\text{filtrate}} \times Dilution}{\epsilon \times ODW_{\text{sample}} \times Pathlength} \times 100
\]

UV_{\text{Abs}} = \text{average UV-Vis absorbance for the sample at appropriate wavelength}

\(\epsilon\) = Absorptivity of biomass at specific wavelength

ODW_{\text{sample}} = \text{weight of sample in milligrams}

Pathlength = pathlength of UV-Vis cell in cm

All carbohydrate and lignin assays were conducted in triplicate.

Enzymatic hydrolysis assay: The pretreated samples were diluted to 10 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.1% (w/v) NaN_3, to prevent growth of microorganisms. All enzymatic hydrolysis experiments were conducted in
triplicate. Pretreated samples were completely suspended in a rotary shaker at 250 rpm at 50 °C. The enzyme loadings were 20 mg protein (Novozymes Cellic® CTec 2) per gram of glucan. Eight hundred microliters of well-mixed hydrolysate were removed, followed by immediate centrifugation at 13,000 rpm for 5 min. Exactly 500 µL of the supernatant was transferred to another micro-centrifuge tube and kept at room temperature for 30 min, to allow the conversion of all cellobiose to glucose. The supernatant was then acidified by adding 30 µL of 10 % (w/w) sulfuric acid, followed by freezing overnight. The frozen samples were thawed, mixed well, and then centrifuged at 13,000 rpm for 5 min, to remove any precipitated solid sediments. The soluble glucose in the enzymatic hydrolysate was measured by HPLC equipped with a Bio-Rad Aminex HPX-87H column at a rate of 0.6 mL of 0.1 % (v/v) sulfuric acid per min at 60 °C. After all remaining hydrolysate was decanted, the pellets were re-suspended in 20 mL of water and centrifuged to remove residual soluble sugars from the pellets. The sugar content of the washed pellets was determined by modified QS as described above. Enzymatic glucan digestibility after 48 h was calculated using the ratio of soluble glucose yield in the supernatant after enzymatic hydrolysis to the sum of this soluble glucose and the soluble glucose obtained from the pellet.

X-ray diffraction assay: The XRD experiments were performed on a PANalytical Empyrean X-ray diffractometer equipped with a PIXcel3D detector and operated at 45 kV and 40 mA using Cu Kα radiation (λ= 1.5418 Å). The patterns were collected in the 2θ range from 5 to 55° with the step size of 0.026° and the exposure time of 300 seconds. A reflection-transmission spinner was used as a sample holder and the spinning rate was set at 8 rpm throughout the experiment. All spectra were subjected to baseline correction using PeakFit1 4.12 software (Systat Software Inc., Chicago, IL) assuming Gaussian distribution function as the shape of the resolved peaks and Saviszky-Golay smoothing. The crystallinity index (CrI) was determined by Segal method.

TGA assay: Thermogravimetric analysis (TGA) was performed using a Mettler Toledo model TGA/DSC 1. 1-3 mg of each specimen was placed in a 40 µL platinum crucible, and heated under argon from 30-105 °C at 5 °C min⁻¹ and the temperature held for 5 minutes. A 90-105 °C cycle with a five minute hold was repeated 5 times to remove moisture and the temperature was then increased to 350 °C at a heating rate of 10 °C min⁻¹. TONSET was determined by the standard method. Briefly this was calculated by tangentially extrapolating the pretransition mass loss curve and determining its intercept with the tangent of the point of steepest slope in the transition region. TPEAK was calculated by the peak of the first derivative of the TGA weight loss curve.