Green Chemistry

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Green Chem.*, 2013, **15**, 2579

One-pot ionic liquid pretreatment and saccharification of switchgrass†

Jian Shi,[a,b] John M. Gladden,[a,b] Noppadon Sathitsuksanoh,[a] Pavan Kambam,[c] Lucas Sandoval,[c] Debjani Mitra,[c] Sonny Zhang,[a] Anthe George,[a,b] Steven W. Singer,[a,d] Blake A. Simmons[a,b] and Seema Singh*[a,b]

Biomass pretreatment using certain ionic liquids (ILs), such as 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]), can be highly effective at reducing the recalcitrance of lignocellulosic biomass to enzymatic degradation. However, current commercial enzyme cocktails, derived from filamentous fungi and developed for dilute acid pretreatment, are inhibited by the most effective ILs used for pretreatment and require excessive amounts of water to remove the ILs from biomass after pretreatment in order to be effective. The associated IL recycling and waste disposal costs of this process pose significant economic and process engineering challenges for the commercial scale-up of IL pretreatment-based technologies. For the first time, we have demonstrated a one-pot, wash-free process that combines IL pretreatment and saccharification into a single vessel. After treating the switchgrass with [C_2mim][OAc] and dilution with water to a final IL concentration of 10–20%, the pretreatment slurry was directly hydrolyzed using a thermostable IL tolerant enzyme cocktail previously developed at the Joint BioEnergy Institute (JBEI). This one-pot process liberated 81.2% glucose and 87.4% xylose (monomers and oligomers) at 72 h at 70 °C with an enzyme loading of 5.75 mg g^{-1} of biomass at 10% [C_2mim][OAc]. Glucose and xylose were selectively separated by liquid–liquid extraction with over 90% efficiency, thus eliminating extensive water washing as a unit operation. This study opens avenues for developing more efficient and cost effective processes for product recovery and IL recycling.

Received 22nd March 2013,
Accepted 8th July 2013

DOI: 10.1039/c3gc40545a

www.rsc.org/greenchem

Introduction

The high abundance and geographic availability of lignocellulosic biomass makes it a promising feedstock for commercial scale production of biofuels and chemicals. The recalcitrance of biomass to enzymatic hydrolysis to fermentable sugars poses a barrier to the realization of economical biochemical conversion technologies. This barrier can be overcome by implementing various physical and/or chemical pretreatment processes to reduce the recalcitrant characteristics of the biomass. Several pretreatment approaches have been investigated over the years, with dilute sulfuric acid, ammonia based pretreatment, hot water, steam explosion, and lime being the most extensively studied.[1] A more recent form of pretreatment using certain ionic liquids (ILs), such as 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]), has emerged, and compared to some pretreatment technologies, it can dramatically reduce biomass recalcitrance and enhances the enzymatic hydrolysis of fermentable sugars.[2,3] As one of the few feedstock agnostic pretreatment technologies known, IL pretreatment using [C_2mim][OAc] is capable of efficiently handling softwoods, hardwoods, herbaceous materials, and agricultural residues, both individually and in combination.[4,5] In addition, [C_2mim][OAc] pretreatment can handle densified pellets of biomass, a feature that very few pretreatment technologies can match.[5,6]

Compared with the traditional pretreatments, IL pretreatment is a relatively new field of study and has several parameters that need more thorough investigation before a commercially viable technology can be realized. The main challenges facing IL pretreatment are the cost of the ILs and the system complexity associated with IL recycling, biomass solute separation and downstream processing.[4,7] In the conventional approach to IL based bioprocessing, IL pretreatment is a separate unit operation from downstream saccharification and fermentation (Fig. 1a). This pretreatment configuration typically requires extensive washing of the biomass post-

[a]*Deconstruction Division, Joint BioEnergy Institute, Emeryville, CA, USA. E-mail: seesing@sandia.gov; Fax: +1 510-486-4252; Tel: +1 925 294 4551*
[b]*Biological and Materials Science Center, Sandia National Laboratories, Livermore, CA, USA*
[c]*The Advanced Biofuels Process Demonstration Unit (ABPDU), Lawrence Berkeley National Laboratory, Emeryville, CA, USA*
[d]*Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA*
†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3gc40545a

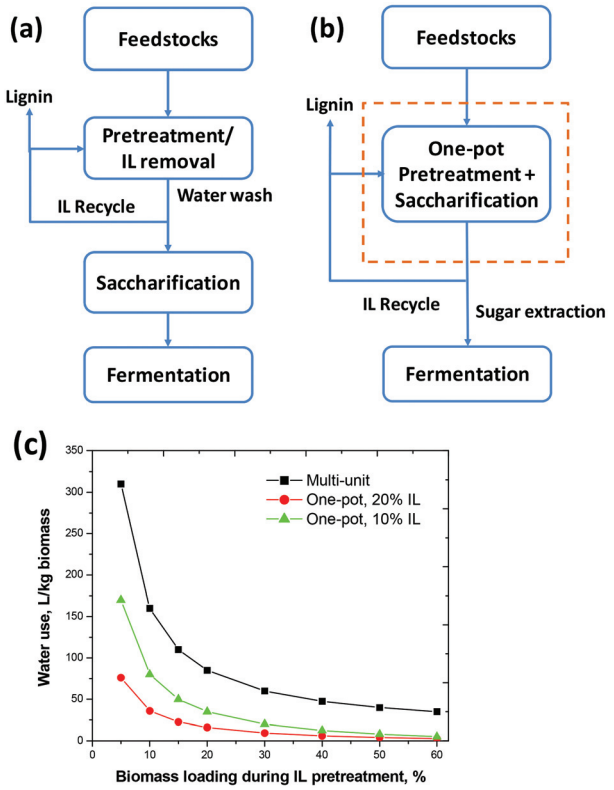

Fig. 1 One-pot pretreatment and saccharification reduces water use: (a) separated pretreatment and saccharification (multi-unit) configuration based on methods currently established in the literature; (b) process intensified one-pot ionic liquid (IL) pretreatment and saccharification configuration; (c) water use (L kg^{-1} of biomass) for multi-unit and one-pot configurations as a function of biomass loading during IL pretreatment. Assumptions: with a multi-unit configuration, approximately 15 times the reaction volume of water is used to remove IL from pretreated biomass based on a survey of the literature; and with a one-pot configuration, a smaller amount of water is added to dilute the pretreatment slurry to 10% or 20% IL.

pretreatment to remove residual amounts of ILs, which can inhibit downstream saccharification and fermentation.[8–10] This excessive use of water and waste disposal associated with washing poses a challenge for the scale-up of any IL pretreatment technology.

The configuration in Fig. 1b outlines our approach to developing a one-pot, wash-free scheme that combines IL pretreatment and saccharification, followed by direct extraction of sugars, recovery of lignin and recycling of [C_2mim][OAc] in order to minimize costs and enhance sustainability. Fig. 1c illustrates an estimated 2–15× reduction in water use with this one-pot process compared with the more conventional approach of separate pretreatment and saccharification steps. A wash-free configuration both reduces the costs associated with energy-intensive evaporation or reverse osmosis recycling of ILs and downsizes the water footprint of the biorefinery by greatly reducing grey water generation.[11] However, in order to reduce water use of the proposed one-pot process to a similar level as for dilute acid or other conventional pretreatment technologies, *i.e.*, 3–5 L water per kg biomass,[12,13] the biomass to IL ratio during pretreatment and the IL concentration during saccharification must be optimized. Less than 5 L water use per kg biomass could be achieved under such preferable conditions, *i.e.* a 1 : 1 biomass-to-IL ratio and 10% IL concentration during saccharification, respectively. Furthermore, eliminating water washing with a one-pot pretreatment and saccharification process could simplify the downstream sugar/lignin recovery and IL recycling and greatly improve the economics of IL pretreatment technology. Known sugar extraction methods, such as chromatography, molecular sieves, membrane-based separation and liquid–liquid extraction, can potentially be used for removing fermentable sugars from IL–water mixtures.[14] For example, boronate complexes can extract up to 90% of sugars from an aqueous IL solution,[15] eliminating the requirement for extensive washing and potentially excluding biomass-derived fermentation inhibitors from the downstream system altogether.[10]

More recent studies have shown that lower IL concentrations (25–50% w/v) in water may also be effective in pretreating biomass, potentially reducing the amount of washing required prior to enzymatic saccharification.[16,17] Pretreatment with lower IL concentrations opens up the possibility to explore alternative, potentially less expensive, wash-free bioprocessing configurations. These studies indicate that, at a minimum, a combined pretreatment/saccharification approach would require biomass-deconstructing enzymes that are tolerant to 20% IL. However, at these high IL concentrations, most commercial glycoside hydrolase enzymes derived from filamentous fungi are severely inhibited, necessitating the development of a robust IL tolerant cellulase cocktail in order for this approach to be feasible.[8,18] We have previously reported the development of a thermophilic and IL tolerant cellulase cocktail, called JTherm, that has activity on IL pretreated switchgrass in the presence of 20% exogenously added [C_2mim][OAc], potentially making the one-pot configuration practical.[18,19] The objectives of this study are to (1) test the concept of one-pot ionic liquid pretreatment and saccharification of switchgrass using JTherm; (2) investigate the effect of pretreatment severity, enzyme loading, IL concentration, and xylanase and surfactant supplementation on sugar yield; (3) test the effectiveness of boronate extraction of sugars from one-pot hydrolysates; and (4) characterize lignin streams for future recovery and valorization.

Results and discussion

JTherm tolerance to [C_2mim][OAc]

ILs based on imidazolium cations, such as 1-allyl-3-methylimidazolium chloride ([C_1mim][Cl]), 1-*n*-butyl-3-methylimidazolium chloride ([C_4mim][Cl]), and [C_2mim][OAc], possess an excellent capacity for dissolving cellulose partially due to their high hydrogen-bond basicity.[4] However, these aforementioned ILs are known to deactivate cellulases at fairly low concentrations.[8,18] Enzymes sourced from extremophile organisms, *e.g.* those from high temperature and pH, and salty

environments, *etc.*, tend to be highly stable and there is growing evidence that this stability can enable these enzymes to tolerate ILs, at least in the cases of thermo- and halo-tolerance.[20] Leveraging this concept, a thermophilic and IL tolerant enzyme cocktail, called JTherm, was developed from a combination of thermophilic enzymes that can maintain activity on IL-pretreated switchgrass in the presence of 20% IL.[18,19]

To explore the capabilities of this cocktail further, it was tested on microcrystalline cellulose (MCC) and regenerated amorphous cellulose (RAC), spiked with 0–30% [C_2mim][OAc], using the CTec2 cellulase cocktail from Novozymes as a control (Fig. 2). The JTherm cocktail retained 81 and 68% of its activity on MCC at 70 °C with 10 and 20% [C_2mim][OAc], respectively, and its activity decreased to 52% in the presence of 30% [C_2mim][OAc]. In contrast, a commercial cocktail showed significantly lower activity compared with JTherm in the presence of ILs (37% and 19% activity in 10% and 20% (w/v) IL, respectively, at 50 °C) and had almost no activity in the presence of 30% IL. Similar results were observed when enzymes were applied to RAC, except that the commercial cocktail had slightly higher relative activities, likely due to the reduced recalcitrance of RAC. JTherm is more IL-tolerant than the commercial cocktail as JTherm retains most of its activity in 20% IL. The only other report that demonstrates moderate IL tolerance of cellulase enzymes is from Wang *et al.*,[21] who reported that the combination of commercial cellulases and β-glucosidase (Celluclast1.5L, from *Trichoderma reesei*, and Novozyme188, from *Aspergillus niger*, respectively) retained 77% and 65% activity after being pre-incubated in 15% and 20% (w/v) [C_2mim][OAc] solutions, respectively, at 50 °C for 3 h. However, these results were only obtained at very high enzyme loading levels (75 FPU cellulase and 80 CBU β-glucosidase per gram of cellulose), which (1) makes it difficult to infer the actual effect of ILs on enzyme activity and (2) is not an economically feasible enzyme dosage for a biorefinery.[21]

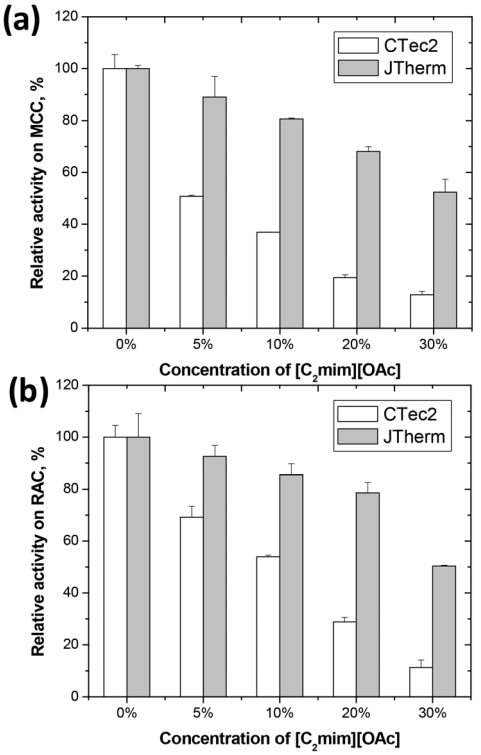

Fig. 2 Enzymatic hydrolysis of microcrystalline cellulose (MCC) and regenerated amorphous cellulose (RAC) by Novozymes Cellic CTec2 and the thermophilic IL-tolerant JTherm, in the presence of various 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) concentrations: 2% substrate in 0–30% of [C_2mim][OAc] (w/w) were incubated at 50 °C, 72 h for CTec2 at a loading of 15 mg g^{-1} starting biomass and 70 °C, 72 h for JTherm at a loading of 5.75 mg g^{-1} starting biomass. The relative enzyme activities are reported as percentages of residual activity, setting the glucose yield with IL free enzyme as 100% activity. Error bars indicate the standard deviation.

Pretreatment severity

Pretreatment conditions (*i.e.* temperature, time, and biomass loading) and biomass recovery methods greatly affect the saccharification efficiency of IL-pretreated biomass.[22] At a given biomass loading, a combination of parameters, including pretreatment temperature and time, determines the outcome of pretreatment, which affects the level of hemicellulose and lignin depolymerization, the crystallinity and morphology of cellulose, substrate reactivity, and accessibility to enzymes. Furthermore, pretreatment severity can potentially be optimized to reduce the amount of biomass-derived cellulase inhibitors generated during IL pretreatment, further increasing sugar yields. In this study, we sought to determine the optimal pretreatment conditions for using JTherm to saccharify biomass in a one-pot configuration.

Despite the abundance of existing knowledge regarding how IL-pretreatment conditions affect sugar yields from fully washed pretreated biomass, very little information is available on saccharification of pretreatment slurry that includes ILs and water. Previous findings indicate that efficient depolymerization of hemicellulose occurs after pretreatment with [C_2mim][OAc] at 120–160 °C for 1–3 h with most of the hemicellulose converted to oligosaccharides.[22] Delignification can only be significantly enhanced when biomass is heated with [C_2mim][OAc] above 150 °C, a finding that is consistent with the reported process temperatures of acid and ammonia fiber expansion pretreatment technologies.[1]

Based on this information, we selected 120 °C and 3 h as a starting point for the one-pot process and explored a range of pretreatment conditions from 70 to 160 °C for various time intervals. The biomass loading was set at 10% and glucose/xylose release was monitored over a course of 168 h with enzyme loadings fixed at 5.75 mg JTherm per g starting biomass. Fig. 3a shows the glucose and xylose yields for 72 h and Fig. S1† provides supplemental information on the hydrolysis kinetics. As shown in Fig. 3, the highest glucose yield of 65% was observed with a pretreatment temperature and time of 160 °C for 3 h, while the highest xylose yield was achieved using 140 °C for 3 h. Pretreatment at temperatures below 140 °C led to lower glucose yields, likely because the pretreatment may not be severe enough to effectively overcome the

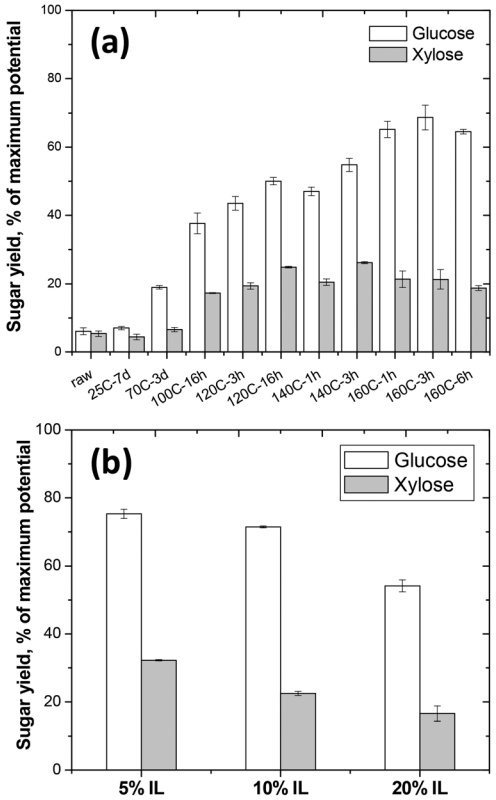

Fig. 3 (a) Effect of pretreatment severity on glucose and xylose yields; (b) effect of 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]), the IL concentration, on glucose and xylose yields under the optimal pretreatment conditions (160 °C, 3 h). JTherm loading: 5.75 mg g^{-1} starting biomass.

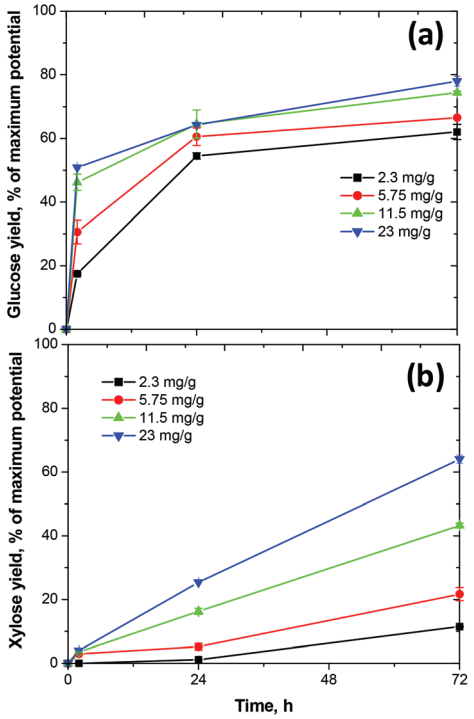

Fig. 4 Effect of JTherm loading on (a) glucose and (b) xylose yield on pretreated switchgrass.

biomass recalcitrance. Compared to pretreatment at 160 °C for 3 h, increasing the pretreatment time to 6 h actually decreased sugar yields, likely due to sugar degradation and inhibitor generation associated with the more severe pretreatment conditions. When considering time, the plateau in the glucose yield curve after 24 h indicates fast cellulose saccharification kinetics (Fig. S1†). This result agrees with previous reports on saccharification of fully washed IL-pretreated switchgrass where the saccharification was nearly complete within 24 h due to the abundance of easily digestible amorphous cellulose from the ionic liquid pretreatment.[3,25] Therefore, 160 °C for 3 hours is selected for further optimization of enzyme loading. It is worth noting that high pretreatment temperature may lead to degradation of [C_2mim][OAc]. As reported elsewhere, the onset temperature (T_{on}) for [C_2mim][OAc] is above 180 °C, whereas the T_{on} are only slightly decreased for cellulose/IL solutions compared to pure ILs.[23,24] At the temperatures tested in this study, IL degradation is considered minimal; however, temperatures may be reduced for pretreatment in agitated reactors without sacrificing sugar yield.

JTherm loading

Enzyme cost poses one of the main economic challenges in the production of lignocellulosic biofuels.[25,26] In order to achieve an economically viable biofuel technology, the enzyme loading must be reduced while maintaining a high saccharification efficiency.[27] Fig. 4 demonstrates the effect of JTherm loading on glucose and xylose yields during the one-pot IL pretreatment and saccharification process with pretreatment conditions fixed at 160 °C for 3 h. Glucose yields were improved from 60% to 80% when JTherm enzyme loading was increased from 2.3 to 23 mg g^{-1} starting biomass, while a more significant improvement (from 10% to 60%) was observed for xylose yield. In addition, higher JTherm loadings (11.5 and 23 mg g^{-1} starting biomass) resulted in a significantly accelerated initial hydrolysis rate (first 2 h glucose yield) compared with low JTherm loadings (2.3 and 5.75 mg g^{-1} starting biomass). After 24 h the overall sugar yields for the high and low enzyme loading reactions were similar, indicating that the lower enzyme dose, while slower in terms of kinetics, works well within a 24 h time frame. These saccharification kinetics results agree with those previously reported on fully washed IL-pretreated switchgrass.[3] Xylose release showed a different pattern compared to glucose: the initial hydrolysis rate was surprisingly low and the rate remained nearly constant over 72 h. It is possible that the xylan or partially hydrolyzed xylan (xylooligomers) is embedded within the regenerated cellulose and occluded from the enzymes, or perhaps xylanase activities in JTherm are inhibited by a combination of the [C_2mim][OAc] and other biomass derived compounds.

Xylanase supplementation

The observation of low xylose yields merited further investigation into the impact of hemicellulose degradation products

on saccharification. A majority of the hemicellulose that is solubilized during IL pretreatment is in the form of oligomers.[22] Previous studies showed that xylobiose and higher xylooligomers can strongly inhibit enzymatic hydrolysis, likely due to competitive inhibition of cellulose hydrolysis.[28] Furthermore, the strong inhibition of cellulose hydrolysis by xylooligomers could be attributed to the negative impact of xylooligomers on cellulase adsorption to cellulose.[29] This reinforces the hypothesis that it is critical to have sufficient xylanase loadings to ensure efficient hydrolysis of xylooligomers and to mitigate the inhibition effects. The low xylose yield (Fig. 3 and 4) and slow xylose release kinetics (Fig. 4b and Fig. S1†) from one-pot processing indicated that the JTherm xylanases were not functioning well, potentially leaving inhibitory xylooligomers in solution.

When the pretreatment slurry was pre-incubated for 24 h with a dose of HTec2 (200 mg bulk HTec2 per g starting biomass) prior to JTherm addition, a ~10% increase in xylose yield was noted in comparison with JTherm (Fig. 5a). Supplementation of the same dose of a commercial hemicellulase cocktail, HTec2 (200 mg bulk HTec2 per g starting biomass), to JTherm during saccharification liberated ~15% more xylose yield compared with the control with only JTherm. However, the overall xylose yields were below 40% and only a slight improvement was observed in glucose yield. Information is scarce about the inhibition of xylanase activity by [C_2mim][OAc], though Thomas and co-workers reported that compared with the fungal glucosidases, xylanase and arabinofurosidases are more tolerant to imidazolium-based ILs.[30] It is plausible that the xylanase from the commercial cocktail, HTec2, may have been inhibited by the presence of 10% IL, or perhaps lignin derivatives, but more specific inhibition studies will be required to sort out their individual effects on xylanases. In either case, these results indicate the need to develop IL tolerant xylanases.

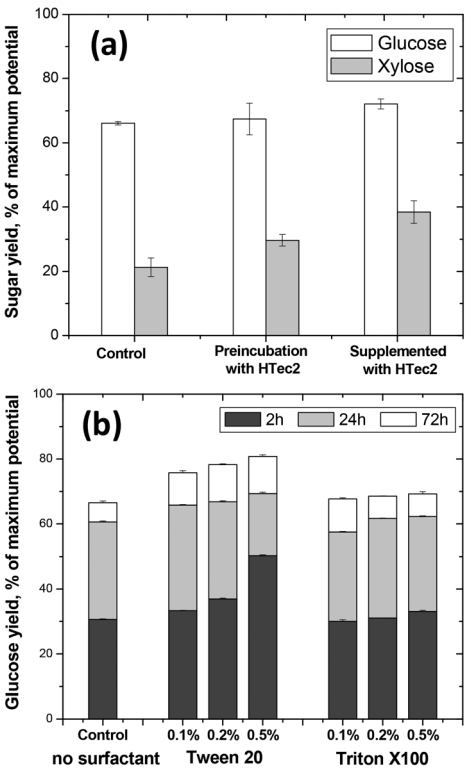

Fig. 5 Effect of (a) xylanase and (b) surfactant supplementation on enzymatic digestibility of 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]), the IL pretreated biomass. JTherm loading: 5.75 mg g^{-1} starting biomass; HTec2 loading for xylanase supplementation: 200 mg bulk HTec2 enzyme product per g starting biomass.

Surfactant addition

Surfactants, especially non-ionic surfactants, have been shown to improve enzymatic hydrolysis or reduce the amount of enzyme needed to achieve a given conversion owing to a few possible mechanisms: alteration of the substrate structure for improved accessibility to enzymes; stabilization of enzymes and relief of deactivation during hydrolysis, and reduced non-productive adsorption of enzymes to lignin.[31] Recent studies have also demonstrated that non-ionic surfactants can help enzyme recycling,[32] and if applied during dilute acid pretreatment can improve lignin solubility and cellulose digestibility by reducing unproductive enzyme binding.[33] Furthermore, enzymes can be stabilized in ionic liquid by the formation of nano/micrometer-sized water domains with addition of suitable surfactants, such as glycerin, TritonX, Tween 20 and Tween 80.[34]

We tested the effect of surfactant addition (Tween 20 and Triton X100 at 0.1, 0.2, 0.5%, v/v, respectively) on sugar yield during the one-pot process. Addition of Triton X100 did not significantly improve sugar yield; however, Tween 20 addition to a level of 0.2% v/v greatly increased glucose yield by 10–15% and reduced enzyme use by 4 fold while producing the same sugar yield (Fig. 5b). Since [C_2mim][OAc] is capable of solubilizing both polysaccharides and lignin, aromatic compounds released during IL pretreatment may inhibit the enzyme.[35] In addition to the aforementioned mechanisms, it is possible that the presence of the surfactant passivates the lignin derived aromatics thus mitigating the inhibitory effects on enzymes.

Sugar extraction

Several methods have been employed to extract sugars from aqueous IL including chromatography,[36] molecular sieves,[14] an aqueous biphasic system (ABS) using kosmotropic salts,[37] and liquid–liquid extraction.[15] Liquid–liquid extraction of sugars into organic phases through the formation of a complex with lipophilic–boronic acids has been used to recover up to 90% of sugars from aqueous solutions. For naphthalene-2-boronic acid (N2B), the most efficient removal of sugars occurs at pH levels between 11 and 12, above its pK_a of ~9. The negatively charged complex is then stabilized in the organic phase by the quaternary alkyl amine cation. The complexation reaction is reversible under acidic conditions and the sugar is recovered from the organic phase by stripping with a dilute acid solution.

Brennan *et al.*[15] have demonstrated the application of a boronic acid complex to extract sugars from a [C_2mim][OAc] and water mixture; however, its effectiveness has not been tested on the sugar streams derived from saccharification of IL pretreated cellulosic biomass. In this study, sugars produced from the one-pot process were extracted using boronic acid based liquid–liquid extraction and further stripped into a dilute HCl. Results indicate that the front-extraction efficiency is dependent on the pH of the mixture, with NaOH concentrations over 80 mM leading to >90% glucose extraction. The xylose extraction efficiency was observed to be lower and the highest percentage extraction was achieved with 240 mM NaOH. During back-extraction, glucose and xylose were selectively recovered in dilute HCl with over 90% efficiency at a NaOH concentration of 320 mM (Table 1). It was also noted that over 100% xylose extraction efficiency was achieved, probably due to the hydrolysis of xylooligomers in the dilute acid solution during extraction.

We further demonstrated preliminary results for recycling of [C_2mim][OAc] from "one-pot" hydrolyzate and reuse of the recycled IL for a next batch of "one-pot" pretreatment and saccharification (see ESI†). Briefly, after sequential filtration and vacuum evaporation, 90.8% IL was recovered from sugar-extracted hydrolyzate. Some of the IL might be lost due to wetting of the membranes we used or during transferring. ^{1}H NMR of recycled IL confirmed preservation of [C_2mim][OAc] as indicated by the persistence of the triplet and quartet proton shifts around 1.5 ppm and 4.3 ppm, respectively. The broadening of regions ~4 ppm is probably due to the trace lignin or sugar residues in recycled IL (Fig. S5†). Compared with neat IL, reuse of the recycled IL for a new batch of one-pot pretreatment and saccharification showed 93.5% and 89.2% of the original glucose and xylose yield as obtained with neat IL, respectively (Fig. S6†). The "one-pot" process eliminates the excess washing of pretreated solids, reduces water use and can potentially simplify the IL recycling and product recovery. The IL recycling process we have described would require an input of energy for vacuum evaporation and sequential filtration that is not optimized to be energy and cost effective, even with extremely optimistic assumptions about process optimization.[4,14,38] This underscores the need for developing more efficient processes of recovering IL from "one-pot" hydrolyzate other than distillation/evaporation.

Table 1 Liquid–liquid sugar extraction by the boronic acid based solvent system[a]

NaOH (μL)	NaOH (mM)	Sugar extracted to organic phase (%)		Sugar extracted to aqueous phase (%)	
		Glucose	Xylose	Glucose	Xylose
0	0	3.8 ± 0.1	6.5 ± 5.2	3.2 ± 0.4	1.0 ± 0.2
4	80	89.2 ± 0.6	61.1 ± 6.2	73.7 ± 0.5	38.1 ± 3.3
8	160	95.0 ± 0.1	76.2 ± 0.9	73.0 ± 0.7	90.8 ± 0.2
12	240	93.6 ± 1.8	87.5 ± 3.0	67.9 ± 1.9	82.4 ± 0.3
16	320	93.3 ± 0.6	75.8 ± 2.8	93.5 ± 3.6	113.5 ± 6.3

[a] Front-extraction to organic phase by the boronic acid complex (150 mM Aliquat 336™ and 70 mM boronic acid in 85 : 15 (v/v) *n*-hexane and 1-octanol; sugar stripping (back-extraction) to aqueous phase by 0.5 N dilute HCl).

Material balance

Fig. 6 summarizes the material balances for the one-pot IL pretreatment and saccharification of switchgrass for two scenarios: (a) with 23 mg JTherm per g starting biomass and (b) with 5.75 mg JTherm per g starting biomass supplemented with 0.2% v/v Tween 20. In general, a similar mass flow and allocation was observed for both scenarios. On the basis of 100 g of raw switchgrass (stream 1), the majority of the biomass is solubilized in the liquid (stream 2) during one-pot pretreatment and saccharification. At 23 mg JTherm per g starting biomass, the composition of stream 2 is 31.5 g glucose, 13.6 g xylose, 6.0 g xylose oligomers and 7.7 g lignin. Only a small fraction of the starting biomass (16.8 g) remained after pretreatment and saccharification, mainly composed of lignin and a small amount of glucan and xylan. By adding Tween 20, a similar mass flow and sugar yield can be achieved with JTherm loading reduced to 5.75 mg g^{-1}.

Although the material balance indicated loss of some mass during pretreatment and enzymatic hydrolysis, the one-pot process liberated 81.9% glucose and 85.4% xylose (mono- and oligomers) in the liquid stream using 23 mg JTherm per g starting biomass at 70 °C for 72 h, or 81.2% glucose and 87.3% xylose (mono- and oligomers) in the liquid stream using 5.75 mg JTherm per g starting biomass with 0.2% v/v Tween 20 at 70 °C for 72 h. The overall glucan and xylan closure from both liquid and solid streams was higher than 90% for both scenarios, confirming that the one-pot IL

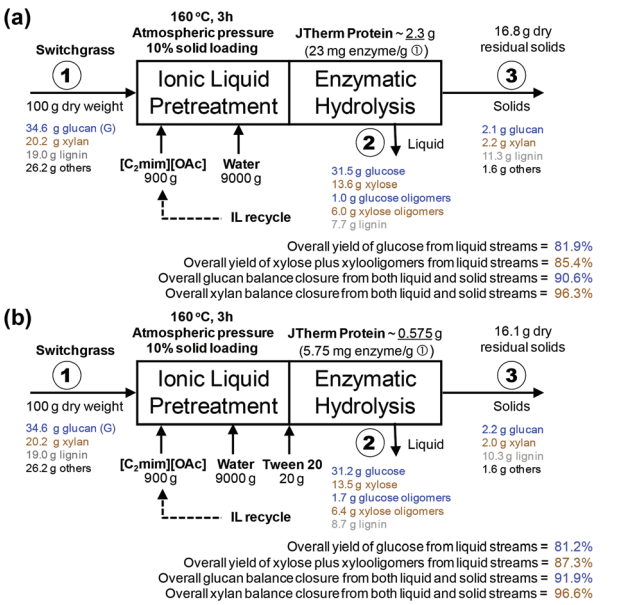

Fig. 6 Material balance on proposed one-pot pretreatment and saccharification processes: (a) with 23 mg JTherm per g starting biomass and (b) with 5.75 mg JTherm per g starting biomass supplemented with 0.2% v/v Tween 20.

pretreatment and saccharification can preserve and recover most of the sugars initially present. During pretreatment, a large portion of lignin was solubilized into the liquid stream; however, the residual solids after enzymatic hydrolysis are rich in lignin (>65%), indicating potential opportunities for lignin valorization. Compounds such as proteins, sugar degradation products and other extractives were not determined in this study, and additional study is needed to fully account for all components and reach mass closure.

Characterization of lignin in liquid and residual solids

Elution profiles of lignin before and after one-pot IL pretreatment and saccharification are shown in Fig. S2.† Relative areas of excluded and retained regions of lignin from different processing streams are determined as shown in Table 2. Enzymatic mild acidolysis lignin (EMAL) of untreated switchgrass showed a strong signal in the excluded region (t < 15.5 min) with an $A_{E/R}$ of 2.4, suggesting that EMAL of untreated switchgrass consisted mainly of large molecular mass materials. After one-pot IL pretreatment and saccharification for 72 hours, a distinct signal in the retained region (t > 15.5 min) was observed with an $A_{E/R}$ of 0.11, suggesting that lignin was solubilized and depolymerized in the liquid stream as previously observed.[39] Residual solids after one-pot IL pretreatment and saccharification for 72 hours eluted at slightly later times than that of EMAL, indicating that under the current conditions no condensation reactions occurred. This result raises the possibility of recovering deferential lignin from liquid and solid streams: small molecular weight lignin from the liquid stream and large molecular weight materials from the solids.

To better understand what happens to the lignin composition and chemical structure during IL pretreatment, 2D HSQC NMR spectra of untreated switchgrass and residual solids after one-pot IL pretreatment and saccharification after 72 h were obtained and are shown in Fig. S3.† The cross peaks were assigned and the peak assignment is shown in Table S1.† The aliphatic region of the cell wall spectrum of untreated switchgrass revealed that β-aryl ethers (substructure A) are major interunit linkages of switchgrass lignin with weak signals of phenylcoumaran (substructure B) and resinol (substructure C) at the current contour level. The aromatic region of the cell wall spectrum of untreated switchgrass showed that switchgrass lignin is an S/G type lignin containing *p*-coumarates and ferulates, which is in agreement with previous studies.[40,41] The weaker signal intensity of β-aryl ethers from residual solids suggested cleavage of β-aryl ethers, which was also revealed by an increase in elution time as determined by SEC. The absence of dibenzodioxocin (substructure D) in residual solids suggested that lignin points of branching were removed. Moreover, the absence of β-correlation of *p*-coumarates and ferulates in residual solids implied breakage of lignin–carbohydrate complex (LCC) linkages after one-pot IL pretreatment and saccharification, supporting high sugar yields from the process and trace amounts of carbohydrates in the residual solids. No condensed structures were observed in the aromatic region (Fig. S3D†), which is in agreement with the SEC profile of residual solids.

Table 2 Elution time and relative molecular mass of lignin after one-pot IL pretreatment and saccharification

Region	Elution time (min)	EMAL	Lignin in liquid phase	Lignin in solid residue
Excluded (%)	t < 15.5 (MW > 46k)	0.71	0.10	0.74
Retained (%)	t > 15.5 (MW < 46k)	0.29	0.90	0.26
$A_{Excluded/Retained}$ ($A_{E/R}$)		2.40	0.11	2.90

Conclusions

Our results indicate that IL pretreatment and saccharification can be combined into a single-unit or one-pot process by using a thermo-stable IL-tolerant enzyme cocktail, JTherm. The JTherm cocktail is capable of efficiently liberating monomeric sugars from pure cellulose and the whole slurry of IL-pretreated switchgrass at temperatures higher than that used for current commercial cellulase enzymes (70 °C *vs.* 50 °C), and can maintain sufficient activity in up to 20% (w/w) [C_2mim][OAc]. IL pretreatment conditions and JTherm loading were optimized to allow effective saccharification of the whole pretreatment slurry and potentially reduce the inhibitory factors of enzymes. Supplementation of hemicellulases to JTherm mildly improved xylose yield; however, the overall xylose yields were still low, indicating the need of developing IL-tolerant xylanases. We also found that addition of 0.2% v/v of the surfactant Tween 20 greatly increased glucose yield by 10–15% and reduced enzyme loads by 4 fold under the same process conditions. Furthermore, it has been demonstrated that following one-pot pretreatment and saccharification, sugars can be efficiently recovered from aqueous IL solutions by liquid–liquid extraction. Lignin was fractionated into a low molecular weight liquid stream and a high molecular weight solid stream after processing. This offers the possibility of integrating IL pretreatment and enzymatic saccharification with lignin recovery and valorization.

The results of this study provide the foundation for developing an economically viable IL based pretreatment technology for biofuels/chemical production based on one-pot pretreatment and saccharification. While the initial sugar yields obtained are promising, further improvements must be realized, primarily in the composition and activity of the enzyme cocktails used, in order to improve sugar yields to >95% for all fermentable sugars and eliminate the need for surfactants. Furthermore, it is of high priority to develop more efficient and cost effective processes for IL recycling and product recovery from "one-pot" hydrolyzate other than the energy-intensive distillation/evaporation methods, and higher recovery efficiencies could be achieved at low cost through further process optimization and technology development during scale-up.

Experimental

Materials

Switchgrass (*Panicum virgatum*) was provided by Dr Daniel Putnam, University of California at Davis. Switchgrass was ground by a Wiley Mill through a 2 mm screen and separated by a vibratory sieve system (Endecotts, Ponte Vedra, FL). The switchgrass fractions falling between 20 and 80 mesh were collected for use in this study. The switchgrass contains 34.6% cellulose, 20.2% xylan, 19.0% lignin and 26.2% of other compounds remaining unidentified, on dry basis. Microcrystalline cellulose (MCC, trademark name: Avicel) was purchased from Sigma-Aldrich (St. Louis, MO) and regenerated amorphous cellulose (RAC) was prepared according to Sathitsuksanoh *et al.*[42] 1-Ethyl-3-methylimidazolium acetate, abbreviated hereafter as [C_2mim][OAc], was purchased from BASF (lot no. 08-0010, purity > 95%, Basionics™ BC-01, BASF, Florham Park, NJ) and used as the IL for all pretreatments. The commercial enzyme products cellulase (Cellic® CTec2, Batch#VCN10001) and hemicellulase (Cellic® HTec2, Batch#VHN00001) were gifts from Novozymes, North America (Franklinton, NC).

JTherm production

To obtain sufficient quantities of the JTherm cocktail for this study, the basic cocktail formulation found in Park *et al.*[19] was scaled up at the Advanced Biofuels Process Demonstration Unit (ABPDU, Lawrence Berkeley National Laboratory, Emeryville, CA). The endoglucanase/xylanase component of JTherm was scaled to a 15 L culture and the cultures of the recombinant β-glucosidase and cellobiohydrolase components of JTherm expressed in *E. coli* were scaled to 50 L.

The endoglucanase/xylanase component (secretome). The endoglucanase/xylanase component of JTherm was produced by cultivating 15 L of a thermophilic bacterial community on microcrystalline cellulose (Sigma-Aldrich, St. Louis, MO). To scale to 15 L, the cultures were first scaled from 50 ml to 2 × 200 ml by inoculating 2 ml of the bacterial community culture described in Park *et al.*[19] into 200 ml of M9TE media containing 2 g of microcrystalline cellulase in a 2 L shake flask and incubating at 60 °C for 2 weeks, shaking at 200 RPM. The two 200 ml cultures were combined and 375 ml of the culture was used to inoculate the 15 L culture. Medium was prepared from M9 minimal salts powder (BD Difco) in a 19 L stirred tank reactor (STR) (NLF 22, BioEngineering, Wald, Switzerland) and sterilized by steam in place (SIP). Separately autoclaved microcrystalline cellulose and filter sterilized trace element solution,[43] $MgSO_4$, and $CaCl_2$ were then added aseptically. The reactor was inoculated with 2.5% (v/v) (375 ml) of the inoculum. The fermentation was maintained at a temperature of 60 °C, agitation at 150 RPM, an air sparging rate of 0.5 volume of gas per volume of liquid per minute (VVM), and without pH control. After two days of cultivation, the air sparging rate was decreased from 0.5 VVM to 0.07 VVM to mitigate the observed evaporative water losses. To replace the water lost due to evaporation, 2 L and 1.5 L of sterilized water was added back to the reactor at a rate of 2 ml min^{-1} on day 12 and day 17 respectively. The fermentation was carried out for 20 days.

To recover the endoglucanase/xylanase rich supernatant, the thermophilic community cultivation broth was harvested and centrifuged at 3220*g* and 22 °C for 20 min (5810R Centrifuge, Eppendorf, Hamburg, Germany). The supernatant containing the secretome was concentrated 10 fold by tangential flow filtration (Cogent M1, Millipore, Billerica, MA, USA) using a 10 kDa filter membrane with a surface area of 0.1 m^2, operated at a feed pressure of 42 psi. The concentrated enzyme solution was then filter sterilized through 0.45 μm syringe filters, aliquoted, lyophilized and stored at −80 °C. The total protein concentration in the endoglucanase/xylanase-rich supernatant, estimated by the Bradford assay, was 0.5 mg ml^{-1}.

The recombinant β-glucosidase and cellobiohydrolase component. The recombinant β-glucosidase (BG) from *Thermotoga petrophia* (UniProt ID: A5IL97) and the recombinant cellobiohydrolase (CBH, a truncated construct of CelB containing only the CBM3 and GH5 domains) from *Caldicellulosiruptor saccharolyticus* were expressed in *E. coli* with a C-terminal His(66)-tag in the pDEST42 expression vector (Invitrogen, Carlsbad, CA). The inoculums of the 50 L cultures were scaled in two steps: first 20 ml and then 500 ml using 5 ml of the first culture. All inoculum flasks were grown for 18–20 h in shaker incubators (MaxQ 8000 Incubator Shaker, Thermo Fisher Scientific, Waltham, MA, USA) at a temperature of 37 °C and shaking at 200 RPM. The 50 L culture was run in a 70 L STR (ABEC, Bethlehem, PA). Filter sterilized carbenicillin stock solution was added to the sterilized LB medium to a final concentration of 50 μg L^{-1}. The fermentation medium was inoculated with 1% (v/v) of the second stage inoculum. The fermentation was maintained with pH of 6.8 (using 2 N H_2SO_4 and 2 N NaOH), a temperature of 37 °C, agitation at 100 RPM and an air sparging rate of 0.5 VVM. Samples were taken hourly to monitor $OD_{600\ nm}$ using a DU 730 UV/Vis Spectrophotometer (Beckman Coulter, Brea, CA). Once the culture reached $OD_{600\ nm}$ between 1.2 and 1.4, temperature control was set to 30 °C and enzyme expression was induced by the addition of filter sterilized IPTG (Sigma-Aldrich) to a final concentration of 0.4 mM. The fermentation was maintained for 20 h after induction before harvesting. The CBH and BG 50 L fermentation broths were harvested by continuous centrifugation. The continuous centrifuge (MBPX 404, Alfa-Laval, Lund, Sweden) was operated with a 50 LPH flow of fermentation broth at 100 kPa of back pressure and 9000 RPM bowl speed. The resulting biomass slurry was further concentrated by centrifugation at 3220*g* and 22 °C for 15 min and disposal of the supernatant, and then frozen at −80 °C. The cell pellets (161 g for the BG and 183 g for the CBH) were each thawed and resuspended in 100 mM Tris-HCl, 100 mM NaCl pH 8.0 buffer containing 2 mg ml^{-1} lysozyme, 0.33 mM phenylmethanesulfonyl fluoride, and Benzonase (Sigma-Aldrich, St. Louis, MO) (1 : 3000 dilution) at 5 ml lysis buffer per gram of cell pellet. The samples were warmed up at 37 °C for 15 min and then shaken at 200 RPM at 30 °C for 45 min. *E. coli* proteins were shear crashed by

incubating at 70 °C for 45 min and insoluble material was removed by centrifuging at 15 000*g* for 15 min. The samples were then filtered through a 0.45 μm syringe filter, aliquoted, lyophilized and stored at −80 °C. The concentration of each enzyme was determined by comparing the BG or CBH activity in these crude lysates with purified enzymes and final yields were determined to be 251 mg of the BG and 1330 mg of the CBH. The JTherm enzyme cocktail contains 4 : 1 : 0.75 mass of CBH, BG and secretome, respectively.

One-pot pretreatment and saccharification

Switchgrass (100 mg, dry weight) was mixed with [C_2mim][OAc] at a 10% biomass loading in a 5 ml capped glass vial and pretreated in a convection oven at different temperature/time combinations including 70 °C for 72 h, 100 °C for 16 h, 120 °C for 3 and 16 h, 140 °C for 1 and 3 h, and 160 °C for 1, 3, and 6 h. Pretreatment was also conducted at 25 °C for 7 d for comparison and untreated raw switchgrass was used as a control.

After pretreatment, the pretreatment slurry was diluted with water to obtain a final IL concentration of 10 or 20%. The JTherm enzyme cocktail was then directly applied to the diluted pretreatment slurry at an enzyme loading of 5.75 mg enzyme product (EP) per g starting biomass. For comparison, a CTec2 + HTec2 (10 : 1 v/v) mixture at 15 mg EP per g starting biomass was added to the pretreatment slurry along with citrate buffer (50 mM pH 4.8). Three glass beads were added to each vial to facilitate mixing during enzymatic hydrolysis. Enzymatic hydrolysis using the JTherm or CTec2 + HTec2 cocktail was conducted at 70 or 50 °C, respectively, with constant agitation on an Enviro Genie SI-1200 rotator platform (Scientific Industries, Inc., Bohemia, NY).

For comparison, the whole pretreatment slurry was washed 6 times with hot water to remove residual ILs and soluble sugars. Washed IL-pretreated solids were dried by lyophilization, weighed and resuspended with water or buffer solution before adding the JTherm or CTec2 + HTec2 enzyme cocktail. In addition, microcrystalline cellulose (MCC) and regenerated amorphous cellulose (RAC) were hydrolyzed by 5.75 mg g^{-1} starting biomass and 15 mg g^{-1} starting biomass for JTherm and CTec2 + HTec2, respectively, spiked with 0–30% IL.

Hemicellulase and surfactant addition

To test the effect of HTec2 addition or pre-incubation on sugar yields, the pretreatment slurry was either pre-incubated with hemicellulase (HTec2) at a loading of 200 mg HTec2 EP per g raw SG for 24 h prior to adding JTherm at 5.75 mg g^{-1} raw SG, or HTec2 (200 mg HTec2 EP per g raw SG) was added to JTherm (5.75 mg g^{-1} raw SG) during saccharification. A control with only HTec2 was used to blank out any interfering sugar peaks. To test their effect on sugar yields, the surfactants Tween 20 and Triton X100 were added to saccharification reactions at loadings of 0.1, 0.2 and 0.5% v/v (JTherm loading of 5.75 mg g^{-1} raw SG). Aliquots for measuring sugar yields were taken at 2, 24, and 72 h.

Sugar extraction

Boronic-acid-based liquid–liquid extraction was applied to extract sugars out of the hydrolysate from the one-pot process.[15] Prior to extraction, the pH of the hydrolysate (0.5 ml) was adjusted to 11–12 through the addition of 10 M NaOH. The concentration of NaOH was varied from 80 to 320 mM (4–16 μl) to examine its effect on extraction efficiency. The pH-adjusted aqueous-IL solution was then contacted with an equal volume (0.5 ml) of an *n*-hexane–octanol (85 : 15 v/v) organic phase, containing 150 mM Aliquat 336™ and 70 mM naphthalene-2-boronic acid, in 1.7 ml Eppendorf vials. The mixture was shaken at 1000 RPM for 2 hours at 70 °C in a Thermomixer® (Eppendorf North America, Hauppauge, NY). The final mixture (1 ml) was then centrifuged (13 000*g* for 30 s) to facilitate phase separation, and the concentration of sugar remaining in the aqueous-IL phase was measured by HPLC. Percentage of sugar extracted to the boronic acid complex (front-extraction) was then calculated.[15]

The recovery of sugars from the organic phase into an aqueous dilute acid phase (back-extraction) was conducted as published.[15] In brief, 0.5 ml of the sugar-containing organic phase was contacted with 0.5 ml of 0.5 N hydrochloric acid (HCl) and mixed at 1400 RPM at 70 °C for 0.5 h in a Thermomixer® (Eppendorf North America, Hauppauge, NY). The final mixture was then centrifuged (13 000*g* for 30 s) to facilitate phase separation, and the sugar concentration on the lower-aqueous phase was measured using HPLC. The percentage recovery from back-extraction is defined as the amount of sugar in the stripping phase relative to the initial amount in the loaded hydrolyzate.

Analytical methods

The saccharification hydrolyzate was separated by centrifugation at 14 000*g* for 10 min followed by syringe filtration. The amount of cellobiose, glucose, xylose, and arabinose released in the hydrolyzate was measured by an Agilent 1100 series HPLC equipped with a Bio-Rad Aminex HPX-87H ion exchange column and a refractive index detector, using 4 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1} and a column temperature of 60 °C. Furthermore, for oligomers determination, an aliquot of hydrolyzate was mixed with an equal volume aliquot of 72% H_2SO_4, incubated at 30 °C for 1 h, diluted to 4% sulfuric acid concentration with DI water and autoclaved at 121 °C for 1 hour (post-hydrolysis) according to the NREL LAP "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples".[44] Differences between the amount of sugars following post-hydrolysis and the monomer content before post-hydrolysis were defined as the oligomeric sugar content. Enzymatic digestibility was defined as the glucose yield based on the maximum potential glucose from glucan in biomass. Sugar yields were also converted to g sugar based on 100 g starting switchgrass. After 72 h of saccharification, the remaining solids were collected by centrifugation and washed with a large volume of DI water to remove residual sugars. The solids

were then lyophilized and analyzed for acid-insoluble lignin, glucan, and xylan compositions.

Characterization of lignin in liquid and residual solids

To understand changes in lignin molecular weight distribution during the one-pot IL pretreatment and saccharification, size exclusion chromatography (SEC) was performed on the lignin in both liquid stream and residual solids after one-pot IL pretreatment and saccharification for 72 h. An Agilent 1200 series binary LC system (G1312B) equipped with a DA (G1315D) detector was used. Separation was achieved with a PL-Gel™ 5 μm Mixed-D column (300 mm L × 7.5 mm i.d., a linear molecular weight range of 200–400 000 u, Polymer Laboratories, Amherst, MA) at 80 °C using a mobile phase of NMP at a flow rate of 0.5 ml per min. The absorbance of materials eluting from the column was detected at 300 nm (UV-A). Intensities were area normalized and molecular mass estimates were determined after calibration of the system with polystyrene standards.[39] The enzymatic mild acidolysis lignin (EMAL) process[45] was used to extract lignin from switchgrass and it was used as a control.

Switchgrass cell wall and residual solids from one-pot IL pretreatment and saccharification were ball-milled, solubilized in DMSO-d_6, and then analyzed by two-dimensional (2D) ^{13}C–^{1}H heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) as previously described.[46] In short, ball-milled samples (~50 mg) were placed in NMR tubes with 600 μl DMSO-d_6. The samples were sealed and sonicated until homogeneous in a Branson 2510 table-top cleaner (Branson Ultrasonic Corporation, Danburt, CT). The temperature of the bath was closely monitored and maintained below 55 °C. HSQC spectra were acquired at 398 K using a Bruker Avance-600 MHz instrument equipped with a 5 mm inverse-gradient $^{1}H/^{13}C$ cryoprobe using the q_hsqcetgp pulse program (ns = 64, ds = 16, number of increments = 256, d_1 = 1.5 s).[47] Chemical shifts were referenced to the central DMSO peak (δ_C/δ_H 39.5/2.5 ppm). Assignment of the HSQC spectra is described elsewhere.[46] A semi-quantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin 3.1 processing software.

Acknowledgements

This work conducted by the Joint BioEnergy Institute was supported by the Office of Science, Office of Biological and Environmental Research of the U.S. Department of Energy under contract no. DE-AC02-05CH11231.

References

1 C. E. Wyman, V. Balan, B. E. Dale, R. T. Elander, M. Falls, B. Hames, M. T. Holtzapple, M. R. Ladisch, Y. Y. Lee, N. Mosier, V. R. Pallapolu, J. Shi, S. R. Thomas and R. E. Warner, *Bioresour. Technol.*, 2011, **102**, 11052–11062.
2 S. Singh, B. A. Simmons and K. P. Vogel, *Biotechnol. Bioeng.*, 2009, **104**, 68–75.
3 C. L. Li, B. Knierim, C. Manisseri, R. Arora, H. V. Scheller, M. Auer, K. P. Vogel, B. A. Simmons and S. Singh, *Bioresour. Technol.*, 2010, **101**, 4900–4906.
4 S. Singh and B. A. Simmons, in *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*, ed. C. E. Wyman, 2013.
5 C. Li, L. Sun, B. A. Simmons and S. Singh, *BioEnergy Res.*, 2013, **6**, 14–23.
6 J. Shi, V. S. Thompson, N. A. Yancey, V. Stavila, B. A. Simmons and S. Singh, *Biofuels*, 2013, **4**, 63–72.
7 D. Klein-Marcuschamer, B. A. Simmons and H. W. Blanch, *Biofuels, Bioprod. Biorefin.*, 2011, **5**, 562–569.
8 M. B. Turner, S. K. Spear, J. G. Huddleston, J. D. Holbrey and R. D. Rogers, *Green Chem.*, 2003, **5**, 443–447.
9 F. Ganske and U. T. Bornscheuer, *Biotechnol. Lett.*, 2006, **28**, 465–469.
10 M. Ouellet, S. Datta, D. C. Dibble, P. R. Tamrakar, P. I. Benke, C. L. Li, S. Singh, K. L. Sale, P. D. Adams, J. D. Keasling, B. A. Simmons, B. M. Holmes and A. Mukhopadhyay, *Green Chem.*, 2011, **13**, 2743–2749.
11 Y. W. Chiu and M. Wu, *Environ. Sci. Technol.*, 2012, **46**, 9155–9162.
12 R. Rinaldi and F. Schuth, *Energy Environ. Sci.*, 2009, **2**, 610–626.
13 R. J. Garlock, V. Balan, B. E. Dale, V. R. Pallapolu, Y. Y. Lee, Y. Kim, N. S. Mosier, M. R. Ladisch, M. T. Holtzapple, M. Falls, R. Sierra-Ramirez, J. Shi, M. A. Ebrik, T. Redmond, B. Yang, C. E. Wyman, B. S. Donohoe, T. B. Vinzant, R. T. Elander, B. Hames, S. Thomas and R. E. Warner, *Bioresour. Technol.*, 2011, **102**, 11063–11071.
14 M. Francisco, A. N. Mlinar, B. Yoo, A. T. Bell and J. M. Prausnitz, *Chem. Eng. J.*, 2011, **172**, 184–190.
15 T. C. R. Brennan, S. Datta, H. W. Blanch, B. A. Simmons and B. M. Holmes, *BioEnergy Res.*, 2010, **3**, 123–133.
16 D. B. Fu and G. Mazza, *Bioresour. Technol.*, 2011, **102**, 8003–8010.
17 A. Brandt, M. J. Ray, T. Q. To, D. J. Leak, R. J. Murphy and T. Welton, *Green Chem.*, 2011, **13**, 2489–2499.
18 J. M. Gladden, M. Allgaier, C. S. Miller, T. C. Hazen, J. S. VanderGheynst, P. Hugenholtz, B. A. Simmons and S. W. Singer, *Appl. Environ. Microbiol.*, 2011, **77**, 5804–5812.
19 J. I. Park, E. J. Steen, H. Burd, S. S. Evans, A. M. Redding-Johnson, T. Batth, P. I. Benke, P. D'haeseleer, N. Sun, K. L. Sale, J. D. Keasling, T. S. Lee, C. J. Petzold, A. Mukhopadhyay, S. W. Singer, B. A. Simmons and J. M. Gladden, *PloS One*, 2012, **7**.
20 S. Datta, B. Holmes, J. I. Park, Z. W. Chen, D. C. Dibble, M. Hadi, H. W. Blanch, B. A. Simmons and R. Sapra, *Green Chem.*, 2010, **12**, 338–345.
21 Y. Wang, M. Radosevich, D. Hayes and N. Labbé, *Biotechnol. Bioeng.*, 2011, **108**, 1042–1048.
22 R. Arora, C. Manisseri, C. L. Li, M. D. Ong, H. V. Scheller, K. Vogel, B. A. Simmons and S. Singh, *BioEnergy Res.*, 2010, **3**, 134–145.

23 F. Liebner, I. Patel, G. Ebner, E. Becker, M. Horix, A. Potthast and T. Rosenau, *Holzforschung*, 2010, **64**, 161–166.
24 F. Wendler, L. N. Todi and F. Meister, *Thermochim. Acta*, 2012, **528**, 76–84.
25 L. Tao, A. Aden, R. T. Elander, V. R. Pallapolu, Y. Y. Lee, R. J. Garlock, V. Balan, B. E. Dale, Y. Kim, N. S. Mosier, M. R. Ladisch, M. Falls, M. T. Holtzapple, R. Sierra, J. Shi, M. A. Ebrik, T. Redmond, B. Yang, C. E. Wyman, B. Hames, S. Thomas and R. E. Warner, *Bioresour. Technol.*, 2011, **102**, 11105–11114.
26 A. Aden and T. Foust, *Cellulose*, 2009, **16**, 535–545.
27 D. Klein-Marcuschamer, P. Oleskowicz-Popiel, B. A. Simmons and H. W. Blanch, *Biotechnol. Bioeng.*, 2012, **109**, 1083–1087.
28 Q. Qing, B. Yang and C. E. Wyman, *Bioresour. Technol.*, 2010, **101**, 9624–9630.
29 J. Shi, M. A. Ebrik, B. Yang, R. J. Garlock, V. Balan, B. E. Dale, V. R. Pallapolu, Y. Y. Lee, Y. Kim, N. S. Mosier, M. R. Ladisch, M. T. Holtzapple, M. Falls, R. Sierra-Ramirez, B. S. Donohoe, T. B. Vinzant, R. T. Elander, B. Hames, S. Thomas, R. E. Warner and C. E. Wyman, *Bioresour. Technol.*, 2011, **102**, 11080–11088.
30 M. F. Thomas, L. L. Li, J. M. Handley-Pendleton, D. van der Lelie, J. J. Dunn and J. F. Wishart, *Bioresour. Technol.*, 2011, **102**, 11200–11203.
31 W. E. Kaar and M. T. Holtzapple, *Biotechnol. Bioeng.*, 1998, **59**, 419–427.
32 M. Tu and J. N. Saddler, *Appl. Biochem. Biotechnol.*, 2010, **161**, 274–287.
33 Q. Qing, B. Yang and C. E. Wyman, *Bioresour. Technol.*, 2010, **101**, 5941–5951.
34 J. Pottkamper, P. Barthen, N. Ilmberger, U. Schwaneberg, A. Schenk, M. Schulte, N. Ignatiev and W. R. Streit, *Green Chem.*, 2009, **11**, 957–965.
35 E. Ximenes, Y. Kim, N. Mosier, B. Dien and M. Ladisch, *Enzyme Microb. Technol.*, 2011, **48**, 54–60.
36 D. X. Feng, L. Z. Li, F. Yang, W. Q. Tan, G. M. Zhao, H. B. Zou, M. Xian and Y. W. Zhang, *Appl. Microbiol. Biotechnol.*, 2011, **91**, 399–405.
37 N. Sun, H. Liu, N. Sathitsuksanoh, V. Stavila, M. Sawant, A. Bonito, K. Tran, A. George, K. L. Sale, S. Singh, B. A. Simmons and B. M. Holmes, *Biotech. Biofuel.*, 2013, **6**, 39.
38 D. C. Dibble, C. L. Li, L. Sun, A. George, A. R. L. Cheng, O. P. Cetinkol, P. Benke, B. M. Holmes, S. Singh and B. A. Simmons, *Green Chem.*, 2011, **13**, 3255–3264.
39 A. George, K. Tran, T. J. Morgan, P. I. Benke, C. Berrueco, E. Lorente, B. C. Wu, J. D. Keasling, B. A. Simmons and B. M. Holmes, *Green Chem.*, 2011, **13**, 3375–3385.
40 R. Samuel, M. Foston, N. Jiang, L. Allison and A. J. Ragauskas, *Polym. Degrad. Stab.*, 2011, **96**, 2002–2009.
41 R. Samuel, Y. Pu, B. Raman and A. Ragauskas, *Appl. Biochem. Biotechnol.*, 2010, **162**, 62–74.
42 N. Sathitsuksanoh, Z. Zhu and Y. H. P. Zhang, *Cellulose*, 2012, **19**, 1161–1172.
43 K. M. DeAngelis, J. M. Gladden, M. Allgaier, P. D'haeseleer, J. L. Fortney, A. Reddy, P. Hugenholtz, S. W. Singer, J. S. Vander Gheynst, W. L. Silver, B. A. Simmons and T. C. Hazen, *BioEnergy Res.*, 2010, **3**, 146–158.
44 A. Sluiter, B. Hames, R. Ruiz, J. Sluiter, D. Templeton and D. Crocker, National Renewable Energy Laboratory Technical Report, 2011.
45 A. Guerra, I. Filpponen, L. A. Lucia, C. Saquing, S. Baumberger and D. S. Argyropoulos, *J. Agric. Food Chem.*, 2006, **54**, 5939–5947.
46 H. Kim and J. Ralph, *Org. Biomol. Chem.*, 2010, **8**, 576–591.
47 S. Heikkinen, M. M. Toikka, P. T. Karhunen and I. A. Kilpeläinen, *J. Am. Chem. Soc.*, 2003, **125**, 4362–4367.