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CELF pretreatment of corn stover boosts ethanol titers and yields from high solids SSF with low enzyme loadings†

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A major challenge to economically produce ethanol from lignocellulosic biomass is to achieve industrially relevant ethanol titers (>50 g L^{-1}) to control operating and capital costs for downstream ethanol operations while maintaining high ethanol yields. However, due to reduced fermentation effectiveness at high biomass solids loadings, excessive amounts of enzymes are typically required to obtain reasonable ethanol titers, thereby trading off reduced operating and capital costs with high enzyme costs. In this study, we applied our newly developed Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) pretreatment to produce highly digestible glucan-rich solids from corn stover. Simultaneous saccharification and fermentation (SSF) was then applied to pretreated solids from CELF at 15.5 wt% solids loadings (corresponding to 11 wt% glucan loadings) in modified shake flasks to achieve an ethanol titer of 58.8 g L^{-1} at 89.2% yield with an enzyme loading of 15 mg-protein per g-glucan-in-raw-corn-stover (-RCS) in only 5 days. By comparison, SSF of corn stover solids from dilute acid pretreatment at 18.3 wt% solids loading (or 10 wt% glucan loading) only achieved an ethanol titer and a yield of 47.8 g L^{-1} and 73.0%, respectively, despite needing longer fermentation times (~20 days) and an additional 18 h of prehydrolysis at 50 °C. Remarkably, although longer fermentation times were required at more economical enzyme loadings of 5 and 2 mg-protein per g-glucan-in-RCS, high solids SSF of CELF pretreated corn stover realized final ethanol titers consistently above 50 g L^{-1} and yields over 80%.

Introduction

Ethanol is a dominant commercial biofuel in the United States, with production from lignocellulosic biomass offering significant environmental, economic, and strategic benefits.[1,2] According to the Renewable Fuels Association, 198 ethanol plants in the U.S. were in operation as of January 2015, producing about 14.58 billion gallons of ethanol.[3] Although more than 98% of this ethanol is currently made from corn starch, the Renewable Fuels Standard (RFS2) program mandates increased production of renewable fuels from lignocellulosic sources, such as corn stover, to complement the current supply of ethanol and alleviate concerns about increased corn starch production.[3] However, although full-scale biological conversion of lignocellulosic biomass to ethanol is currently being introduced in the U.S., costs for pretreatment and enzymatic digestion of lignocellulosic feedstocks impede commercial success. As a result, the US EPA recently reduced the volume of cellulosic ethanol required by RFS.[4]

Various processing strategies can effectively extract sugars from lignocellulosic biomass and ferment them to ethanol.[5] For example, in separate hydrolysis and fermentation (SHF), the solids are first hydrolyzed by enzymes followed by fermentation of the sugars released to ethanol by yeast or other microbes. The major advantage of this strategy is that enzymes and yeast can both be operated at optimal temperatures and pH. Additionally, because it is possible to send an aqueous

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†Electronic supplementary information (ESI) available: Ethanol yields from the SSF of CELF pretreated corn stover at an enzyme loading of 5 mg-protein per g-glucan-in-RCS, component mass balance from the SSF of DA pretreated corn stover at an enzyme loading of 15 mg-protein per g-glucan-in-RCS, cell viability of SSF flasks with CELF pretreated corn stover at an enzyme loading of 2 mg-protein per g-glucan-in-RCS. See DOI: 10.1039/c5gc01977j

stream to fermentation, recycling of the culture broth for subsequent fermentations is more feasible. An alternate approach is simultaneous saccharification and fermentation (SSF) in which the enzymatic hydrolysis and fermentation steps are performed together with the major advantage of an integrated process that limits the effect of enzyme inhibition by hydrolysed sugars through their immediate conversion to ethanol.[6] Other SSF advantages include lower capital costs and a reduced risk of contamination if high enough ethanol titers are achieved. For SSF, a prehydrolysis (PH) step may be employed prior to adding yeast so the enzymes have a "head start" to release sugars at a higher optimal temperature and liquefy some of the solids before lowering the temperature to accommodate the subsequent introduction of yeast.

Achieving ethanol titers of >50 g L^{-1} from fermentation of cellulosic biomass is important to reduce energy demand for downstream ethanol recovery and achieve significant savings in operating as well as capital costs.[7,8] However, realizing this goal while maintaining high ethanol yields is challenged by key technical barriers including biomass recalcitrance,[9] enzyme inhibition by sugars and oligomers,[10] surface blockage and/or unproductive binding of enzymes by lignin and pseudo-lignin,[11–13] and large enzyme demands for high solids operation.[14] Consequently, an important opportunity is to improve the biomass pretreatment technology to enable pretreated solids to be more susceptible to enzymatic digestion so that lower loadings of costly enzymes are needed.[15] Several pretreatment technologies are now being applied in newly opening commercial facilities such as Abengoa, POET/DSM, and DuPont Cellulosic Ethanol including hydrothermal, steam explosion, dilute acid (DA), and dilute ammonia pretreatments to disrupt the biomass cell wall structure.[16] Although these pretreatments reduce biomass recalcitrance, high enzyme dosages (>15 FPU per g-dry-matter) are still needed to achieve high yields, particularly at high solids loadings.[8,17,18] Consequently, achieving >50 g L^{-1} ethanol titers at low enzyme loadings for economic downstream processing has been challenging.

Here, we demonstrate the integration of a novel pretreatment called Co-solvent Enhanced Lignocellulosic Fractionation (CELF) with SSF to achieve >50 g L^{-1} ethanol production from corn stover while maintaining higher ethanol yields at reduced enzyme loadings compared to DA pretreatment. CELF has been previously introduced as a promising new pretreatment technology for hardwoods and corn stover by applying an aqueous co-solvent mixture consisting of tetrahydrofuran (THF) in combination with dilute acid to enhance biomass deconstruction through promoting extensive delignification and solubilization of biomass with minimal sugar degradation. When applied to corn stover, CELF achieved over 95% recovery of total xylose, glucose, and arabinose sugars using enzyme loadings as low as 2 mg-protein per g-glucan-in-RCS.[19,20] Furthermore, THF was selected for CELF because it is a low boiling renewable solvent that can be easily recovered, with any makeup. THF regenerated catalytically from furfural,[20] a natural pretreatment byproduct that is inhibitory to ethanol fermentation. While our previous work focused on establishing optimized CELF pretreatment conditions that achieved high sugar and ethanol yields at low enzyme loadings,[19] we extend here the application of CELF in combination with high solids enzymatic hydrolysis to (1) differentiate the performance in combination with SHF and SSF processing strategies, (2) compare maximum ethanol yields and titers achieved from corn stover solids produced by CELF and DA pretreatments, and (3) demonstrate that CELF can reduce enzyme loadings required to realize >50 g L^{-1} ethanol titers while maintaining 80–90% ethanol yields (of theoretical). We also explore whether prehydrolysis of CELF and DA pretreated corn stover solids can enhance the SSF performance as advocated by others.[21]

Experimental

Materials

Air-dried Kramer corn stover for this study was kindly provided by the National Renewable Energy Laboratory (NREL, Golden, CO). The corn stover was knife milled to pass through a 1 mm particle size interior sieve using a laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA). Compositional analysis of corn stover was performed according to the established NREL procedure (version 8-03-2012)[22] in triplicates, with a resulting mass composition of 34.2 ± 0.3% glucan, 23.7 ± 0.2% xylan, 3.8 ± 0.1% arabinan, 17.9 ± 0.9% K-lignin, and 20.2% other materials that include extractives, organic acids, inorganics, and ash. Cellulolytic enzyme cocktail Accellerase® 1500 was generously provided by DuPont Industrial Biosciences (Palo Alto, CA). The BCA protein concentration and activity were about 82 mg ml^{-1} and 50 FPU ml^{-1}, respectively, as reported by Kumar *et al.*[13] The non-xylose fermenting *Saccharomyces cerevisiae* D_5A yeast strain used for SSF was kindly supplied by NREL in plate monocultures. A frozen culture stock was prepared as previously described.[19] Before each SSF run, the seed inoculum was prepared by thawing, transferring, and growing the frozen stock using a shaker incubator at 130 rpm and 37 °C for 12 h in 250 mL baffled flasks using sterilized YPD medium. The inoculum was then centrifuged and re-suspended in sterile deionized (DI) water for washing and prepared for inoculation at a 0.5 optical density (O.D.) determined at 600 nm.

Analytical procedures

All chemical analyses were based on Laboratory Analytical Procedures (LAPs) documented by the National Renewable Energy Laboratory (NREL, Golden, CO) (http://www.nrel.gov/biomass/analytical_procedures.html). Liquid samples along with appropriate calibration standards were analysed by HPLC (Waters Alliance 2695 system equipped with a Bio-Rad Aminex® HPX-87H column and Waters 2414 RI detector) with an eluent (5 mM sulfuric acid) flow rate of 0.6 ml min^{-1}. The chromatograms were integrated by using the Empower® 2 software package (Waters Co., Milford, MA).

Corn stover pretreatment

Pretreatments were performed in a 1 L Hastelloy Parr® autoclave reactor (236HC Series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotated at 200 rpm. The reaction solutions, temperatures, and times for the CELF and DA pretreatment technologies were selected based on conditions that achieved maximum total sugar yields, as reported in previous studies.[19,23] Thus, CELF and DA reaction solutions were both loaded with 0.5 wt% (based on liquid mass) sulfuric acid (Ricca Chemical Company, Arlington, TX), while in CELF reactions THF (>99% purity, Fisher Scientific, Pittsburgh, PA) was added at a 1 : 1 (v : v) ratio to water based on adding equal volumes of each. Temperatures for CELF and DA reactions were 150 °C and 160 °C, respectively, while reaction times were 25 and 20 min, respectively. Prior to each reaction, corn stover (7.5 wt%) was added to the acid solution and soaked overnight at 4 °C. All reactions were maintained at temperature (±2 °C) by convective heating with a 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ) as previously described,[19] with the reactor temperature directly measured by an in-line K-type thermocouple (Omega Engineering, Inc., Stamford, Connecticut). After the reactions, the solids were separated from the reaction liquor by vacuum filtration at room temperature through glass fiber filter paper (Fisher Scientific, Pittsburgh, PA) and washed with room temperature DI water until the filtrate reached a neutral pH.

Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF)

Consistent with NREL standard protocols,[24] SHF and SSF were performed in triplicates in 125 mL flasks with a 50 g working mass containing 50 mM citrate buffer (pH 4.8), 10 g L^{-1} yeast extract (Becton, Dickinson and Company, Redlands, CA), 20 g L^{-1} peptone (Becton, Dickinson and Company, Redlands, CA), 40 mg L^{-1} tetracycline (Sigma Aldrich, St. Louis, MO) as an antimicrobial agent, Accellerase® 1500 cellulase (loaded at 2, 5, or 15 mg-protein per g-glucan-in-RCS), and D_5A yeast inoculum (from Materials section). Millipore water along with the washed solids was loaded into flasks with attached bubble traps to achieve 4, 8, or 11 wt% glucan loadings of CELF pretreated solids residues and 4, 8, or 10 wt% glucan loadings for DA pretreated solids. The mass of the whole flask assembly was recorded before and after autoclaving at 121 °C for 30 min. The lower glucan content of DA pretreated solids compared to CELF coupled with its moisture content after just filtering and mass of enzyme, buffer, media, and inoculum additions required, limited the highest glucan loading to 10 wt% for DA for these experiments. The flasks were then cooled, reweighed, and placed into a laminar flow hood (Baker and Baker Ruskinn, Sanford, ME) for aseptic addition of pre-sterilized Millipore water (to replenish water loss), yeast extract, citrate buffer, tetracycline, and Accellerase® 1500 cellulase. The NREL protocol was modified after this step depending on the processing strategy of interest. For SHF experiments, the flasks were placed in an orbital shaker incubator set at 150 rpm and 55 °C as determined to be optimal conditions for just hydrolysis, and another shaker was set at 130 rpm and 37 °C determined to be optimal for SSF. For SSF experiments, the yeast inoculum prepared in the Materials section was also added to flasks that were then placed in the incubator set at fermentation conditions. For those SSF experiments that were preceded by a prehydrolysis step, the flasks were initially placed in an incubator set at 150 rpm and 50 °C for 18 h followed by allowing the flasks to cool before adding yeast inoculum prior to being placed in an incubator set at fermentation conditions. Samples were drawn every day or every other day as needed to effectively track the entire time course for short and long fermentations and centrifuged at 15 000 rpm for 10 min so that the supernatant could be withdrawn for HPLC analysis for sugars, ethanol, lactic acid, and acetic acid.

Ethanol yield calculations at high solids operations

If yield calculations assume that no volume change occurs during biomass hydrolysis and the initial volume and final sugar and ethanol concentrations in solution are used in calculations, yields can be overestimated by up to 36% at high solids concentrations (10–40 wt%).[25] Consequently, the following more accurate calculation was applied to take into account the changing proportion of insoluble solids to aqueous liquid, as well as changes in the specific gravity and aqueous phase concentrations:

$$\begin{aligned}\text{Ethanol yield } (\%) &= (M_{\text{Et,G}}/M_{\text{G}}) \times 100 \\ &= ([\text{EtOH}] \times V_{\text{L}} \times 0.9)/(0.51 \times M_{\text{G}}) \end{aligned} \quad (1)$$

in which M_{G} is the mass of glucan in g, $M_{\text{Et,G}}$ is the mass of ethanol in glucan equivalents in g, [EtOH] is the ethanol concentration in the liquid in g L^{-1}, V_{L} is the volume of the liquid phase in L, 0.9 accounts for the stoichiometry of glucan conversion to glucose, and 0.51 accounts for the stoichiometry of glucose conversion to ethanol. The volume of the liquid phase can be estimated by the following equation to take into account the change during hydrolysis:

$$\begin{aligned} V_{\text{L}} &= (M_{\text{W}} + M_{\text{DS}})/\rho \\ &= (M_{\text{W}} + V_{\text{W}} \times ([\text{EtOH}] + [\text{glucose}] + [\text{glycerol}] + etc.))/\rho \end{aligned} \quad (2)$$

in which M_{w} is the mass of water initially loaded, M_{DS} is the mass of dissolved solids, ρ is the density of the solution as estimated from the weight percent ethanol in solution, and V_{w} is the volume of water initially added. The mass of the dissolved solids was estimated from the concentrations of all possible products from biomass, including ethanol, glucose, cellobiose, glycerol, acetic acid, lactic acid, xylitol, and xylose. The density at a specific weight percent of ethanol was estimated from published data.[26]

Results and discussion

Enhancing glucan content reduces solids loadings required to realize commercial desirable ethanol titers over 50 g L^{-1}

In order to realize commercially feasible production of ethanol from lignocellulosic biomass, high solids operation will be necessary to achieve high enough ethanol titers to significantly reduce energy and capital costs for distillation, waste treatment, and other downstream operations.[27,28] Based on an approximately exponential decay in energy demand for distillation *vs.* ethanol concentration, an ethanol concentration of at least 50 g L^{-1} in the product stream from fermentation is often cited as needed to realize reasonable energy and other costs.[29–32] High glucan and low moisture contents in pretreated biomass are important attributes to realizing these ethanol concentrations while keeping overall solids loadings low enough for good mixing. Furthermore, removing lignin in pretreatment can prove powerful for increasing ethanol concentrations at a given solids concentration fed to fermentation since the yeast can only convert sugar from the hemicellulose and cellulose fractions to ethanol. In addition, the action of cellulolytic, hemicellulolytic, and accessory enzymes that are responsible for cleaving the glycosidic linkages of biomass polysaccharides is generally hindered by the presence of lignin.[11,33]

Fig. S1 in the ESI† compares the individual fate of each major biomass component (xylose, glucose, arabinose, lignin, and other) during DA and CELF pretreatment under optimal conditions used to produce pretreated solids for fermentation in this study. The conditions used in this study were based on a previous study that determined that CELF pretreatment achieved the highest sugar recovery at 150 °C, 10 °C lower than the optimal temperature for DA pretreatment.[19] Fig. S1† shows that both DA and CELF pretreatments were effective in solubilizing and recovering sugars from hemicellulose, as evidenced by reduced xylan and arabinan content in the solids, but CELF pretreatment removed 57.4% more lignin than DA pretreatment after accounting for total solids yields following both pretreatments. Thus, CELF pretreatment produced a solids comprised of 71.2% glucan, 10% lignin, and 4.4% xylan, compared to solids following DA pretreatment that contain 54.9% glucan, 25.3% lignin, and 3.9% xylan, thereby resulting in a 30% advantage for CELF in glucan content.

The pretreated corn stover in this study was not allowed to air dry, as other studies have shown that drying of pretreated solids can reduce enzymatic digestibility due to lower enzyme binding on the substrate.[34,35] Consequently, differences in the moisture content of solids following filtration and washing of solids from each pretreatment limited the maximum solids loadings. Additionally, the maximum solids loadings are further limited by the need for vital hydrolysis and fermentation components, *i.e.*, enzymes, yeast, nutrients, and buffer, which account for up to 9–10 g of the 50 g working weight in the flasks. Accordingly, the moisture content of CELF pretreated corn stover was 80.6% resulting in a maximum solids loading of 15.5 wt% that translated into an effective glucan loading of 11 wt%. The moisture content of DA pretreated corn stover was 77.2% resulting in a maximum solids loading of 18.3 wt% that translated into an effective glucan loading of 10 wt%.

SSF of CELF pretreated corn stover solids achieved higher yields than SHF in less time

SHF and SSF strategies have disparate features for biological conversion of cellulosic biomass into ethanol. For example, while SSF can enhance hydrolysis rates by reducing glucose inhibition of enzymes, SHF can be operated at higher temperatures optimal for the highest enzyme activity. Additionally, if liquid and solids are separated following enzymatic hydrolysis, SHF offers the possibility of cell recycling to enhance subsequent fermentation rates and cut costs.[36] Of relevance to this study, the variability in final ethanol yields for SSF *vs.* SHF when applied to different substrates and loadings reported in the literature[37–39] underlines the importance of characterizing the performance of each with CELF pretreated corn stover at different solids loadings. For example, a study by Alfani *et al.* showed that SHF achieved 13% higher overall ethanol yields compared to SSF (81% *vs.* 68% yield)[37] reports that SSF achieved 13% higher overall ethanol yields than SHF (72% *vs.* 59% yield) for steam pretreated corn stover.[38] SSF achieved higher ethanol yields than SHF (76% *vs.* 65%, respectively) at a 9 wt% loading of olive pruning solids produced by liquid hot water pretreatment. On the other hand, when the solids loading was increased to 17 wt%, SHF achieved ethanol yields of 63% while SSF yields were only 46%.[39]

To facilitate comparisons between the amount of ethanol produced in SSF and sugars produced by SHF, SHF glucose concentrations were translated into expected ethanol concentrations based on the assumption that glucose could be fermented to ethanol at a yield of 90% of theoretical. The ethanol concentrations and yields shown in Fig. 1A and 1B for SSF of CELF pretreated solids clearly show that SSF achieved better performance than SHF at both 5.6 and 15.5 wt% solids loadings that correspond to glucan loadings of 4.0 and 11.0 wt%, respectively. SHF was carried out at 37 °C and 55 °C to understand how the lower temperature required to accommodate SSF yeast impacted rates and yields compared to operation at the higher temperature that is optimal for enzymatic hydrolysis. SSF at 37 °C realized higher ethanol concentrations and yields than SHF at the same temperature, especially at the highest solids loading of 15.5 wt%, as summarized in Fig. 1. More specifically, Fig. 1A shows that day 2 ethanol concentrations of 19.4 g L^{-1} for SSF were more than double the value of 8.14 g L^{-1} for SHF at 37 °C. Moreover, at the higher glucan loadings presented in Fig. 1B, ethanol concentrations reached 58.8 g L^{-1} in 5 days for SSF, compared to only 22.5 g L^{-1} in 7 days for SHF. Consistent with previous findings, the higher yields and faster rates for SSF are likely due to removal of sugars that would otherwise inhibit the enzymes.[40,41]

Although one would expect that operation at 37 °C would favor SSF due to removal of inhibitory sugars, this advantage could be lost if improved enzyme activity at the higher temp-

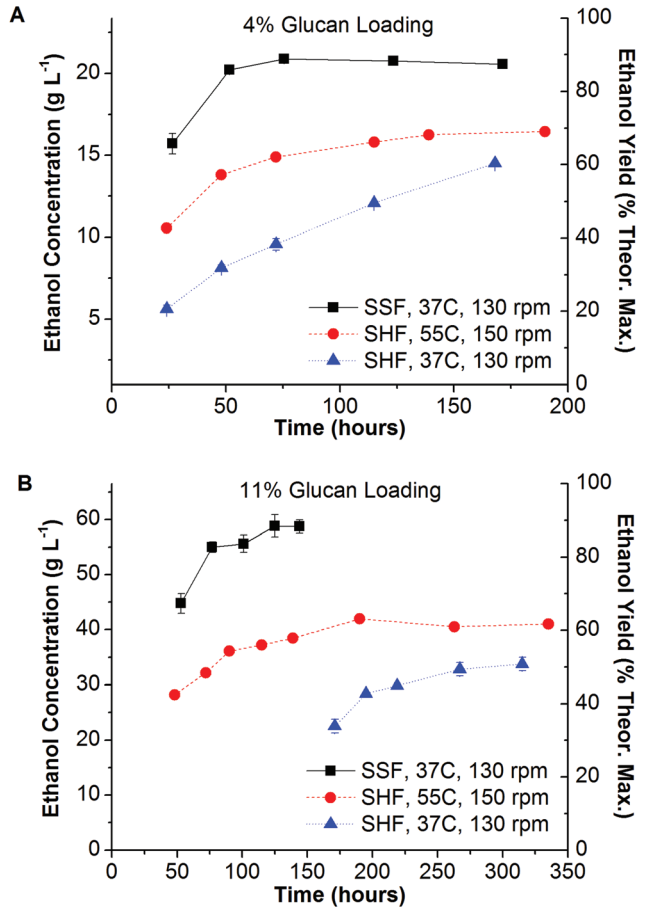

Fig. 1 Comparison of percent ethanol yields of theoretical maximum and concentrations (g L^{-1}) for SSF and SHF at 37 °C and 55 °C for CELF pretreated solids at (A) 4 wt% and (B) 11 wt% glucan loadings and a cellulase loading of 15 mg-protein per g-glucan-in-RCS. For SHF, ethanol yields and concentrations are calculated values based on 90% of theoretical maximum ethanol possible.

eratures optimal for SHF is more than compensated for sugar inhibition. However, Fig. 1 shows that SSF at 37 °C still achieved higher ethanol production rates and yields than SHF at 55 °C, supporting earlier findings that sugar inhibition has a greater impact on performance than temperature.[42] SSF also achieved higher ethanol concentrations in a shorter reaction time, especially at higher glucan loadings, further showing that reducing enzyme inhibition by sugars has a greater effect on performance than increasing temperatures.

Enhanced substrate digestibility by CELF increased yields at higher solids loadings

Although high solids operations are needed to achieve >50 g L^{-1} ethanol titers that improve the economics of downstream operations, increasing solids loadings can reduce yields, making it vital to determine trade-offs among solids loadings, enzyme loadings, ethanol titers, and resulting yields. Mohagheghi *et al.* simultaneously saccharified and fermented solids from dilute acid pretreatment of wheat straw over a range of solids loadings and observed that although an ethanol titer of 57 g L^{-1} was achieved for a 20 wt% solids loading, a high enzyme loading of 20 FPU per g-cellulose (roughly equal to 24 mg-protein per g-cellulose)[43] was required to reach that mark. It is also vital to note that ethanol yields dropped substantially from 82.0% to 68.5% when the solids loading was increased from 12.1 to 20.0 wt%.[44] Similarly, Zhang *et al.* observed that the ethanol yields dropped from 76.5% to 52.1% when the loading of solids produced by steam pretreatment of corn stover was increased from 15 to 30 wt%.[8] In addition, the maximum ethanol titer was 40.6 g L^{-1}, well under the 50 g L^{-1} commercial goal.

Because low ethanol yields resulting from solids loadings greater than 15 wt% are frequently blamed on impeded heat and mass transfer and high local inhibitor concentrations as a result of poor mixing in these very viscous slurries, a prehydrolysis step in which enzymes are employed initially alone has been applied to reduce viscosity and enhance sugar concentrations before adding yeast to complete hydrolysis and fermentation in the SSF mode.[8,21,45–47] However, this additional prehydrolysis step is not always reported to be beneficial. For example, Hoyer *et al.* demonstrated an overall yield increase from 3.9% to 62.1% for a 22 h prehydrolysis of steam pretreated spruce slurry prior to adding yeast for SSF,[21] while Zhao and Xia reported a 12 h prehydrolysis reduced overall SSF yields from solids produced by alkaline pretreatment of corn stover.[46] One could hypothesize that these differences in the impact of prehydrolysis could result from differences in substrate digestibility due to such factors as reduced substrate accessibility, reduced enzyme availability and effectiveness due to inhibition by lignin, glucose, or cellobiose, or lower water availability for enzymatic hydrolysis.[47,48] Consequently, comparing the effects of increasing solids loadings with and without prehydrolysis of solids produced by CELF and DA pretreatments of corn stover could shed some light on whether the lower lignin content and greater digestibility of solids from CELF pretreatment would enhance the performance at higher solids concentrations or whether other factors control yields. All results for this portion of our study are based on loading the same amounts of glucan in the solids from both CELF and DA pretreatments so that comparisons could be made based on the same potential ethanol titer.

For SSF of solids from DA pretreatment at 15 mg-protein per g-glucan-in-RCS enzyme loading, Fig. 2A shows that increasing glucan loadings in SSF from 4 to 8 to 10 wt% resulted in final ethanol yields dropping from 83.3% to 78.2% and then down to 61.4%, respectively, corresponding to a maximum ethanol titer of 40.19 g L^{-1}. Although ethanol yields levelled off at about 61% for 10 wt% glucan loadings without prehydrolysis, glucose concentrations increased during the 15–22 day period from 9.7 to 19.3 g l^{-1}, suggesting that yeast had lost viability but the enzymes were still hydrolyzing glucan. When an 18 h prehydrolysis step was introduced prior to SSF at a 10 wt% glucan loading, Fig. 2A shows that the maximum ethanol concentration and yield increased to 47.8 g L^{-1} and 73.0% by day 21, respectively, demonstrating the

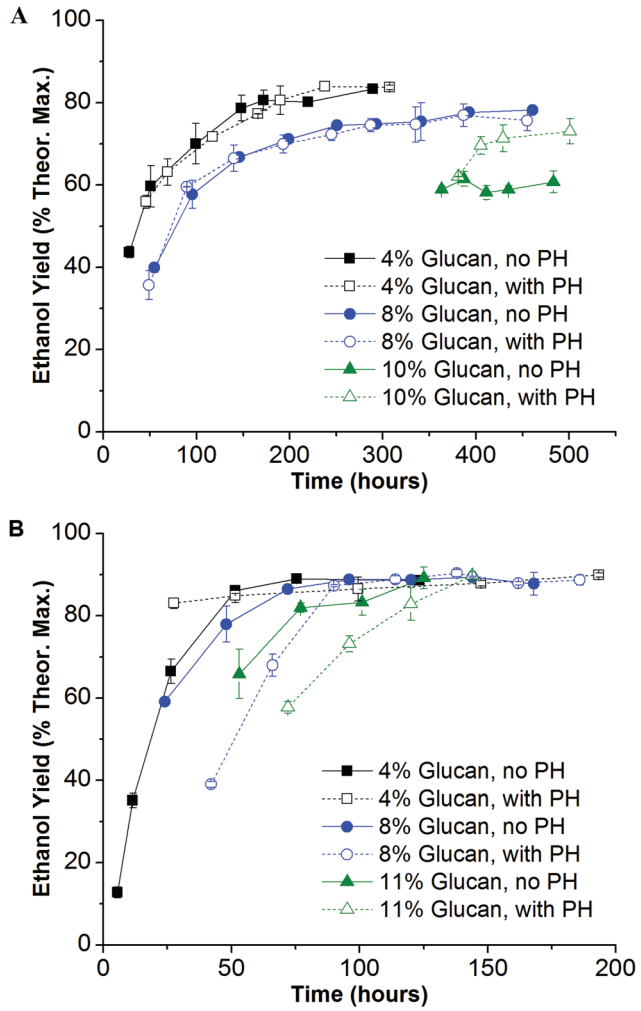

Fig. 2 Effects of glucan loading and 18 h prehydrolysis (PH) at 50 °C on percent ethanol yields of theoretical maximum for SSF of solids from (A) DA and (B) CELF pretreatments of corn stover. Accellerase® 1500 enzyme loading of 15 mg-protein per g-glucan-in-RCS. Fermentations without PH are shown as solids lines, while fermentations with PH are shown as dashed lines. The times include the time for the PH step when appropriate.

benefits of prehydrolysis at 10 wt% glucan loading for DA pretreated corn stover. However, the overlap in yields over time in Fig. 2A shows that prehydrolysis was not beneficial at lower glucan loadings of 4 and 8 wt%, likely because mixing was much better.

Previous studies have reported that increasing the initial substrate loadings from 5 to 8 wt% can reduce production costs by 19%, assuming similar yields can be achieved,[49] but maintaining yields while achieving >50 g L^{-1} ethanol titers at such high solids loadings has presented a major challenge.[8,39,44] However, as shown in Fig. 2B, the long term SSF yields for solids produced by CELF pretreatment of corn stover were substantially better at higher glucan loadings than for solids resulting from DA pretreatment in Fig. 2A. In fact, long-term yields were about 89–90% for all CELF solids loadings without prehydrolysis. The only significant difference was that yields rose more slowly at the 11 wt% glucan loadings due to poorer initial mixing (the mixture did not fully liquefy until after 2 days) so that the time required for yields to peak was about 120 h instead of only about 72 h for loadings of 4 and 8 wt%. Furthermore, although introducing prehydrolysis at 4 wt% glucan loadings of CELF solids achieved higher initial ethanol yields at 24 hours, the extra prehydrolysis step also prolonged the fermentation times needed to realize maximum yields for the higher loadings of 8 and 11 wt%. The better performance for SSF alone could be explained as resulting from higher hydrolysis rates due to yeast consuming sugars that would otherwise accumulate in high concentrations at higher solids loading and inhibit enzyme action during prehydrolysis. Although as mentioned previously, lower yields at high solids loadings are often attributed to poor mixing, the SSF results in Fig. 2B suggest that highly digestible CELF pretreated corn stover can achieve so much better results at high solids loadings that prehydrolysis has limited if any benefits. In summary, the data in Fig. 2 show that higher titers can be achieved at higher solids loadings while maintaining high yields for CELF pretreated solids than possible for solids from DA pretreatment at an enzyme loading of 15 mg-protein per g-glucan-in-RCS.

High yields achieved with low enzyme fermentations at high solids loadings of CELF corn stover

Enzymes have recently been estimated to contribute similar operating costs to that of the lignocellulosic biomass feedstock for biological processing, with the lowest estimated enzyme cost of $0.68 per gal ethanol being for maximum theoretical yields at 20 mg-protein per g cellulose and a higher cost of $1.47 per gal for yields and loadings reported in the literature.[50] Because catalysts must cost much less than substrates for fuels to be economical, various studies have sought approaches to reduce enzyme amounts, but the sacrifice in sugar yields and titers that results hurts process economics.[8,51] On the other hand, solids produced by CELF pretreatment have been shown to realize high sugar yields at enzyme loadings as low as 2 mg-protein per g-glucan-in-RCS,[19] and the data above show that high ethanol yields are maintained at glucan loadings of 11% for an enzyme loading of 15 mg-protein per g-glucan-in-RCS. The question remained as to whether high yields can be achieved at low enzyme loadings for high enough solids loadings to achieve ethanol titers above 50 g L^{-1}.

In response, Fig. 3 shows that an enzyme loading of 5 mg-protein per g-glucan-in-RCS realized ethanol yields of 89.0%, 90.9%, and 90.7% at 4, 8, and 11 wt% glucan loadings, respectively, for SSF, resulting in a maximum ethanol titer of 56.4 g L^{-1} after 14 days of fermentation. Thus, SSF of CELF pretreated corn stover achieved overall ethanol yields over 89% at a more commercially viable enzyme loading of 5 mg-protein per g-glucan-in-RCS, producing an average of 56.4 g L^{-1} ethanol after 14 days of fermentation, while the ethanol yield for SSF of DA pretreated corn stover solids was just 73% with

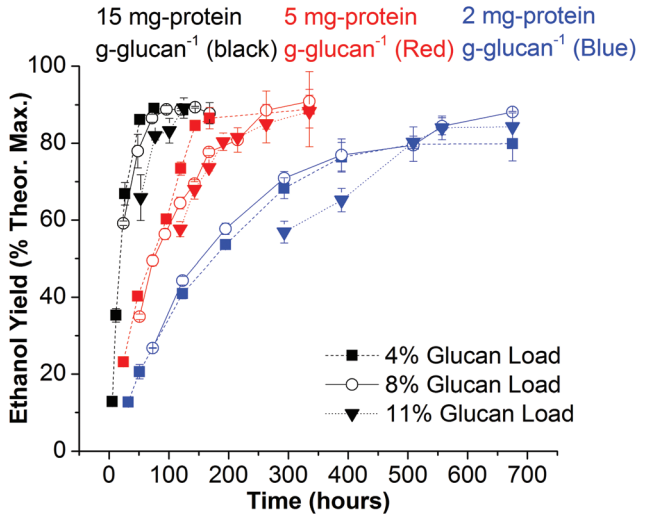

Fig. 3 The effect of enzyme loadings at 2, 5, and 15 mg-protein per g-glucan-in-RCS on SSF fermentation times and ethanol yields for CELF solids at glucan loadings of 4, 8, and 11 wt%.

47.8 g l^{-1} ethanol titer after 21 days of fermentation. It is noteworthy that the time course of SSF ethanol yields with CELF pretreated solids at an enzyme loading of 5 mg-protein per g-glucan-in-RCS (shown in Fig. S2 in the ESI†) was very similar with or without prehydrolysis, although a lag was still observed for the 11 wt% glucan loading case with prehydrolysis. Thus, although fermentation times increased as enzyme loadings were dropped and solids loadings increased, long-term ethanol yields were not sacrificed. Based on enzyme costs projected by Klein-Marcuschamer *et al.*, the near theoretical yields achieved at this enzyme loading with CELF pretreated corn stover solids could translate into reducing the enzyme cost to $0.17 per gallon ethanol, a savings of about $1.30 per gallon.[50] Additional cost savings would be realized by the ability to achieve ethanol titers >50 g L^{-1} at the higher solids loadings.

Fig. 3 also shows results from cutting enzyme loadings further to 2 mg-protein per g-glucan-in-RCS for CELF pretreated corn stover solids. Ethanol yields dropped from 88.1% to 84.3% as solids loadings were increased from 8 to 11 wt% at this low enzyme loading, with the result that the maximum ethanol titer was 53.4 g L^{-1} after 23 days of fermentation. Although these times were longer, the overall fermentation time needed to achieve the maximum yield of 11 wt% glucan was similar to those for only 4 wt% DA pretreated corn stover at an enzyme loading of 15 mg-protein per g-glucan-in-RCS. Even then, the maximum yields from DA pretreated solids were lower and the times to reach these yields much longer at higher solids loadings. Also similar to results with DA pretreated corn stover at a 10 wt% glucan loading, the increase in glucose concentration to 7.1 g L^{-1} by day 28 for CELF pretreated corn stover at 11 wt% glucan loadings suggested that yeast cell viability suffered. Yeast viability tests confirmed that cells were no longer alive when the glucose concentrations began to increase after about 22 days. Mohagheghi *et al.* also observed a loss of yeast viability with increasing solids loading, with colony forming units dropping to 0 at 20 wt% solids loading, resulting in a reduced ethanol yield of 68% and a glucose concentration of 16 g L^{-1}.[44] These results coupled with ours suggest that high solids operations are limited by yeast viability. Remarkably, despite the similarities in fermentation rates and trends, CELF pretreated corn stover still achieved higher ethanol yields and titers with a low enzyme loading of 2 mg-protein per g-glucan-in-RCS than the DA pretreated corn stover could with 7.5 times more enzyme.

An interesting observation from SSF of CELF pretreated corn stover at 2 mg-protein per g-glucan-in-RCS was that the lowest yield of 79.2% occurred for the lowest glucan loading of 4 wt%. Because enzyme loadings were based on glucan content, consistent with accepted practice, lower enzyme cocktail loadings resulted in lower concentrations of β-glucosidase in solution (80 mg-protein per L for 4 wt% glucan compared to 160 and 220 mg-protein per L for 8 and 11 wt%, respectively) that in turn would reduce the rate of breakdown of soluble cellobiose to glucose. Thus, the lower concentration of β-glucosidase could be the limiting factor at this lower glucan loading, as evidenced by no apparent activity detected by the end of the fermentation.

Applying total mass balance to confirm component yields

To confirm the component yields reported here, a mass balance was performed on all components that could be derived from the glucan in pretreated corn stover. Although *Saccharomyces cerevisiae* is a robust organism for industrial ethanol production that is able to realize ethanol yields as high as 90–93% of theoretical, some glucan losses are attributable to supporting yeast growth and maintenance and formation of by-products such as glycerol, acetic acid, and lactic acid.[52] Fig. 4 shows that most of the glucan added to fermentation flasks initially could be accounted for since the sum of

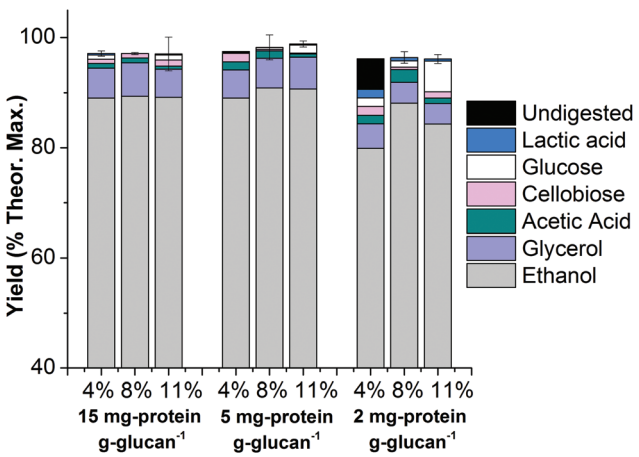

Fig. 4 Theoretical yields of ethanol, glucose, and glucose derived by-products from SSF of CELF pretreated corn stover solids over the range of glucan and enzyme loadings (*i.e.*, mg-protein per g-glucan-in-RCS).

the component yields of ethanol, by-products, unfermented glucose/cellobiose, and undigested glucan added up to about 97 ± 3%. One would expect the total to fall short of 100% due to use of glucan for maintenance and cell growth. As expected, the major fermentation by-product from SSF of CELF pretreated corn stover was glycerol (3.7–6.0%, 0.5–4.1 g L^{-1}), with minimal losses to acetic acid (0.5–2.3%, <2 g L^{-1}), and lactic acid (0–1%). The by-product yield range for glycerol production increased with enzyme loadings from 3.7% to 4.4% glycerol at 2 mg-protein per g-glucan-in-RCS, from 5.0% to 5.7% glycerol at 5 mg-protein per g-glucan-in-RCS, and from 5.2% to 6.0% glycerol at 15 mg-protein per g-glucan-in-RCS, likely due to increased osmotic pressure and lower fluxes of pyruvate from glycolytic intermediate utilization.[52] A similar mass balance was also applied to verify yields from SSF of DA pretreated corn stover (Fig. S3 in the ESI†).

Conclusions

The high cost of enzymes and low ethanol titers due to difficulties in handling high solids loadings and end-product inhibition of enzymes have presented two of the most important barriers to realizing the low cost potential of converting low cost and abundant lignocellulosic biomass to ethanol. In this paper, we have shown that CELF pretreatment technology is able to reduce both of these obstacles by removing much of the lignin and hemicellulose to leave highly glucan enriched solids that are much more easily broken down by enzymes than possible with solids from dilute sulfuric acid pretreatment at enzyme loadings of 15 mg-protein per g-glucan-in-RCS in SSF fermentations. Higher yields with shorter fermentation times were achieved for application of SSF to CELF pretreated corn stover than possible by SHF at 11 wt% glucan, the solids loadings needed to realize economically attractive ethanol concentrations: SSF at 37 °C achieved a 89.2% yield after 5 days, SHF was ran at 55 °C achieved a 61.9% yield after 8 days, and SHF at 37 °C achieved a 52.3% yield after 11 days. Thus, removing glucose and cellobiose that are strong inhibitors of enzymes proved to more than compensate for the need to run at lower temperatures to maintain yeast viability in SSF. Furthermore, the remarkably high digestibility of CELF pretreated corn stover solids resulted in ethanol yields >80% and titers >50 g L^{-1} for SSF of 11 wt% loadings of glucan even as enzyme loadings were dropped from 15 mg-protein per g-glucan-in-RCS to more economically viable levels of 5 and 2 mg-protein per g-glucan-in-RCS, although fermentation times had to be extended from 5 to 14 and 23 days, respectively. Finally, unlike the advantage of applying enzymes alone for 18 h prior to adding yeast for dilute acid pretreatment, we found that this prehydrolysis step did not improve overall SSF yields from solids produced by CELF pretreatment, suggesting that prehydrolysis is more beneficial if the pretreated solids are not highly digestible.

Conflict of interest

The authors declare no conflict of interest.

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