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Development and characterization of a high-solids deacetylation process

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Abstract

Background: Dilute-acid pretreatment has proven to be a robust means of converting herbaceous feedstock to fermentable sugars. However, it also releases acetic acid, a known fermentation inhibitor, from acetyl groups present in the biomass. A mild, dilute alkaline extraction stage was implemented prior to acid pretreatment to separate acetic acid from the hydrolysate sugar stream. This step, termed deacetylation, improved the glucose and xylose yields from enzymatic hydrolysis and ethanol yields from fermentation of the sugars relative to the control experiments using dilute-acid pretreatment of native corn stover without deacetylation. While promising, deacetylation as it was historically practiced is conducted at low solids loadings, and at fixed conditions. Thus, many questions have been left unanswered, including the relationship between sodium hydroxide and solids loading, and acetate and xylan solubilization, as well as the impact of temperature and residence time on the process efficacy.

Results: A central composite experiment was designed to evaluate the impact of solids loading, sodium hydroxide loading, reaction time and temperature during deacetylation on the acetate and xylan solubilization of corn stover. Using the ANOVA test, it became apparent that neither of the responses was significantly impacted by the solids loading, while the reaction time was a minor factor—the responses were largely driven by reaction temperature and the sodium hydroxide loading. Based on the results, we successfully demonstrated the ability to transition the low-solids (10 % w/w) deacetylation process to a higher-solids process (30 % w/w) with minimal impact on the ability to extract acetate from biomass. Conditions were selected to minimize xylose loss during deacetylation, while also removing 70 % of acetyl groups. The impact of selected conditions on the enzymatic hydrolysis and fermentation was further investigated.

Conclusions: Evaluation of the whole-process impact demonstrated that despite the upfront reduction in carbohydrate loss during deacetylation, the overall process sugar yields were depressed by the high-solids, low alkali process relative to the historical control. Consequently, ethanol titers were reduced, though strong fermentation performance was still observed, indicating that 70 % acetate removal is sufficient to depress acetic acid concentrations to a level that does not substantially inhibit ethanol fermentation by *rZymomonas*.

Keywords: Deacetylated corn stover, Dilute acid, Bioethanol, Pretreatment, Enzymatic hydrolysis, Fermentation, Deacetylation

Background

Renewable liquid transportation fuels present a challenge distinct from other energy sources, as the tradable commodity and fuel type has yet to be defined as it is in the case of electrical power. Thus, many potential fuel products are being considered, ranging from alcohol fuels, such as ethanol and butanol, to low-oxygen hydrocarbon products that may be directly substituted for currently available fuels. Further complicating the issue, each of these potential solutions can be either blended into the current fuel supply or used as a standalone replacement, although the latter scenario would require potentially significant infrastructure changes in many cases. While this lack of a standardized product specification

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has presented challenges to the industry, many biological conversion processes utilize sugars to synthesize their respective target products, and require solutions with high concentrations of monomeric sugars and low concentrations of inhibitory compounds, including furfural, 5-hydroxymethylfurfural (HMF), and acetic acid.

Dilute-acid pretreatment has proven to be a robust means of converting herbaceous lignocellulosic feedstock to fermentable 5- and 6-carbon sugars [1]. However, it is also prone to producing potent fermentation inhibitors (i.e., furfural), and also releases acetic acid from the acetyl groups present in biomass, commingled with the desired sugars. Furfural concentrations can be reduced by implementing an effective flash system at the conclusion of the pretreatment process, which has been demonstrated to remove approximately 50 % of furfural from the hydrolysate and enable furfural concentrations less than 2 g/L (in a 20 % total solids whole slurry) [2]. Similarly, acetate, in the form of acetic acid, can be separated from the hydrolysate sugar stream through a mild, dilute alkaline extraction stage implemented prior to pretreatment [3]. This step, termed deacetylation, has been demonstrated to solubilize approximately 80 % of acetyl groups present in native corn stover, which can then be separated from the biomass prior to pretreatment though a wash process. Deacetylating corn stover prior to a typical dilute sulfuric acid pretreatment improved both glucose and xylose yields during enzymatic hydrolysis and ethanol yields relative to the control experiments using dilute-acid pretreatment of native corn stover [4].

Other processes have been explored that use neutral, ionic liquids or alkaline chemistry, including hydrothermal pretreatment [5], ionic liquid pretreatment using imidazole [6], peroxide pretreatment, oxidative lime pretreatment, and pulping-like sodium hydroxide pretreatments [7–15]. However, these processes are typically intended to function as a stand-alone pretreatment, and the severity of the process is increased to enable effective enzymatic hydrolysis with no further processing. The increased severity, while effective in achieving high cellulose conversion yields through lignin extraction, also results in the solubilization and degradation of a significant fraction of hemicellulosic sugars; in many cases, greater than 20 % is found in the alkaline liquor stream [8, 11]. Due to the low concentrations of sugars and comparatively high concentrations of undesirable components, including salts and acetic acid, this stream is typically treated as waste. Coupling deacetylation, which solubilizes less than 10 % of the hemicellulose, with dilute-acid pretreatment has proved to be a cost-effective, process-relevant means to achieve high sugar yields, while also obtaining one of the primary benefits of alkaline pretreatment (i.e., low inhibitor concentrations).

While promising, previous work on the deacetylation process was conducted at low (~10 %) total solids loadings, and at fixed conditions (0.4 % w/w NaOH, 80 °C, 120 min) [4]. Thus, many questions have been left unanswered, including the relationship between sodium hydroxide loading and acetate and xylan solubilization, as well as the impact of temperature and residence time on the process efficacy. Further, process economics favor reductions in chemical and water utilization. In particular, increasing the solids loading during deacetylation would offer significant cost savings by reducing reactor volume requirements, decreasing energy requirements for heating during deacetylation, and reducing wastewater generation. Other significant cost drivers include the process residence time and temperature.

In this work, we characterized low-severity alkaline extraction of corn stover, termed deacetylation, using response surface methodology. Experimental conditions range from sodium hydroxide loadings of 0.1–0.5 g/g of dry biomass, temperatures between 60 and 100 °C and extraction times of 45–105 min. The characterization experiments were evaluated to select a promising condition reflecting more favorable economics (e.g., decreased xylan loss, increased solids loading, decreased chemical usage) while maintaining >60 % acetyl group removal. This condition was replicated to generate more deacetylated biomass used in further experiments to evaluate the impact of the new conditions on enzymatic hydrolysis and fermentation at low and moderate pretreatment severities compared to those pretreated at control conditions.

Results and discussion

While the previous low-solids deacetylation process is effective and capable of consistently solubilizing 80 % of the acetate in corn stover, the process also is water-intensive; approximately 5 % of hemicellulosic sugars are extracted and lost during the washing process. Thus, the need has arisen to develop an improved deacetylation process that is capable of solubilizing a similar fraction of acetate as the previous process, while reducing the fraction of hemicellulose solubilized. The improved process should also be robust at high solids (>20 % w/w) and, if possible, have reduced chemical requirements. To examine the feasibility of these requirements, a central composite experiment was designed to evaluate the impact of solids loading, sodium hydroxide loading, reaction time, and process temperature on the acetate and xylan solubilization of a single corn stover feedstock during deacetylation. The parameters tested are displayed in Table 1.

In total, 30 experiments were carried out—16 factorial points, 8 star points, and 6 replicate center points. Samples of the post-extraction liquor were analyzed for acetic

Table 1 Parameters and test levels selected for development of the central composite design

Experimental variable	Axial (−2)	−1	Center point (0)	+1	Axial (+2)
Solids loading (wt%)	10	15	20	25	30
Temperature (°C)	60	70	80	90	100[a]
Time (min)	45	60	75	90	105
NaOH loading (g/g acetate)	0.5	1	1.5	2	2.5

[a] Temperature was held just under 100 °C to prevent evaporation and vessel pressurization

acid and monomeric and oligomeric xylose and extracted solids were analyzed for xylan content. The weight of extracted liquor and solids was determined and used to calculate the total remaining insoluble solids mass after deacetylation, the amount of residual xylan and acetate remaining in the solids, and xylan component mass closure. Results for xylan mass closure are shown in Fig. 1. The xylan solubilized during deacetylation was not completely depolymerized and presented in the liquor as monomeric and oligomeric sugars.

Xylan balance closure was consistently between 90 and 100 %, with only a few instances of closure outside this range, indicating that we can account for most of the xylan-derived species solubilized in this low-severity alkaline process. However, the remainder of the xylan that is unaccounted for is likely degraded due to the peeling reactions under alkaline conditions and referred to as xylan loss in this paper. The percent of xylan loss is calculated by dividing the part of unaccountable xylan after deacetylation process by the total amount xylan in the initial biomass.

In addition, the solubilization of acetate was calculated based on the acetate concentration in the liquor phase divided by the total amount of acetate groups in the initial biomass.

These results were used to generate a quadratic response surface model, with only terms that have statistical significance at a 95 % confidence level being retained in the model. The models are listed in Eqs. 1 and 2,

$$Xylan\ Loss(\%) = 17.4 - 0.193\,T - 17.0\,C + 0.188\,TC + 2.19\,C^2 \quad (1)$$

$$AcetateSolubilization\,(\%) = -28.3 + 0.162\,t + 111\,C - 27.4\,C^2 \quad (2)$$

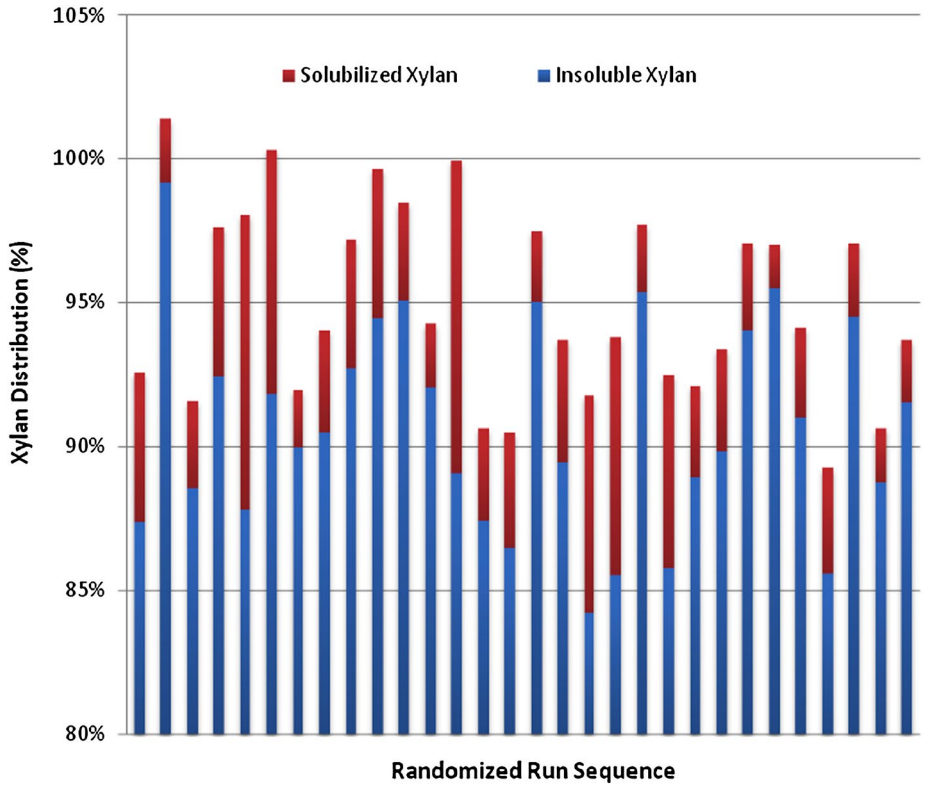

Fig. 1 Xylan component closure for 30 low-severity alkaline extraction experiments

where T is temperature (°C), C is NaOH loading (g/g), and t is time (min).

Upon analyzing the results from a one-way ANOVA test (available in the Additional file 1), it became apparent that neither response was significantly impacted by the solids loading during deacetylation, while the reaction time was a minor factor—the responses were largely driven by reaction temperature and the sodium hydroxide loading. We also found that in experiments conducted at sodium hydroxide loadings of less than 1.5 g/g acetate, the pH of the post-extraction liquor was consistently in a neutral range (6–8). This indicates that the sodium hydroxide is actually consumed in the reaction, rather than simply acting as a catalyst. Thus, the extent of acetate removal can be controlled by limiting the alkaline reagent loading during deacetylation, which could also reduce the extent of xylan solubilization (Fig. 2).

These data suggest that solids concentration is not a significant factor within the range of conditions tested, and also provides more confidence in the scalability and ultimate economic viability of the process. The CAPEX and OPEX of the unit operation will be potentially reduced through decreased process volumes at the higher solids operation. The effluent stream from the deacetylation process has the potential for producing valuable side products because high titers are achieved from high-solids operation.

Based on the results of these experiments, a set of conditions was selected that minimized the loss of xylan to the aqueous effluent stream after deacetylation, while also extracting a sufficient fraction of acetate to enable much improved fermentation performance. The selected conditions (10–25 % total solids, 1 g NaOH per g acetate, 70 °C, 90 min) solubilized approximately 65 % of acetyl groups and 2 % of the xylan present in the corn stover. These conditions were used to produce larger quantities of deacetylated corn stover for use in downstream dilute-acid pretreatment, enzymatic hydrolysis, and ethanol fermentation experiments to assess the impact of the modified deacetylation process. The measured component solubilization of acetate and xylan produced in these experiments are shown in Fig. 3.

Relative to previous low-solids deacetylation work (control), less xylose was measured in the post-extraction liquor, indicating a significant reduction in xylan loss. The amount of acetate removed from the corn stover was also decreased, with 70 % recovered in the post-extraction liquor in the high-solids process, and 80 % solubilized in the low-solids control process.

The carbohydrate composition of the solids was greatly enriched relative to the original feedstock, due to the removal of water extractible compounds and the removal of some lignin, as shown in Fig. 4. The solids compositional analysis also reveals that the low-solids deacetylation process has lower lignin content (14 %) and higher glucan and xylan content (34 and 23 %, respectively), while the high-solids deacetylation has more lignin (18 %) and less glucan and xylan (39 and 26 %, respectively). This is mainly caused by lower sodium hydroxide loading (0.24 vs. 0.4 w/w%) in the high-solids deacetylation, resulting in less severe delignification compared to the low-solids deacetylation process.

Dilute acid pretreatment

Three batches of deacetylated corn stover were prepared using the high-solids method in addition to a single batch of deacetylated corn stover prepared using the low-solids

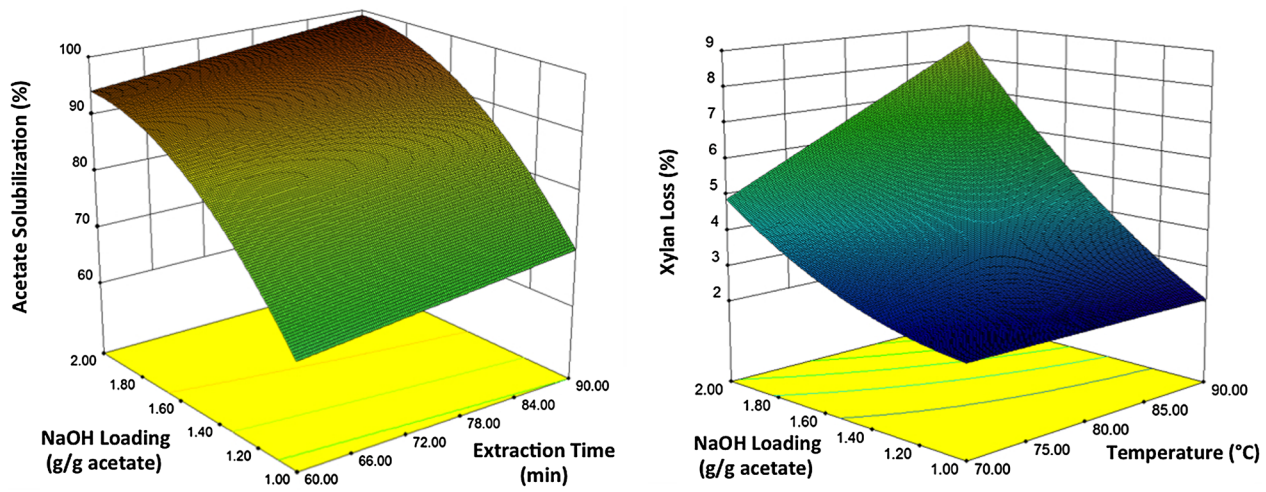

Fig. 2 Response surface characterizing the solubilization of xylan and acetate during low-severity alkaline extraction

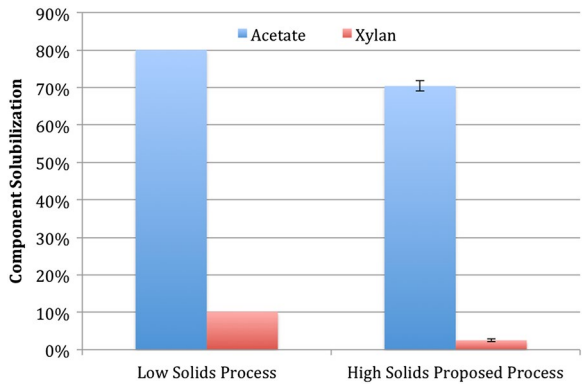

Fig. 3 Solubilization of acetate and xylan during deacetylation experiments. *Low solids process* 10 % total solids, 80 °C, 120 min, 1.7 g NaOH/g acetate (equivalent to 0.4 % w/w). *High solids proposed process* 30 % total solids, 70 °C, 90 min, 1.0 g NaOH/g acetate

control method at two different pretreatment severities. After deacetylation, each batch of material was exhaustively washed and acid impregnated with 0.5 % (w/w) sulfuric acid at 10 % total solids prior to dewatering to approximately 50 % total solids. The samples were then pretreated in a 4-L batch steam explosion reactor using pretreatment conditions of 165 °C for 10 min and 150 °C for 20 min, with triplicate experiments conducted at each condition for each feedstock batch produced. The results of these experiments can be found in Figs. 5 and 6.

As shown in Figs. 5 and 6, monomeric xylose yield was improved by roughly 10 yield points in the three high-solids deacetylation samples in both sets of pretreatment experiments. This is likely due to the decreased xylan loss in deacetylation, and thus the preservation of easily-extractible xylose in the feedstock that was solubilized during acid hydrolysis. Interestingly, the soluble xylo-oligosaccharides yield from xylan was comparable across all samples at a given pretreatment condition, despite the difference in total and monomeric xylose yield. Conversion to furfural was minimal (1–2 %), as expected, given the relatively mild pretreatment conditions and modest acid loadings (0.5 % w/w during pre-impregnation). Xylan component balance closure ranged between 97 and 100 % for all samples.

Concentrations of acetic acid were modestly higher in pretreated hydrolysates generated in the high-solids process than those from the control. In low-severity pretreatment experiments, they were 2–3 g/L in the high-solids process and <1 g/L in the low-solids process, respectively. At moderate severity, they were 3–4 g/L in the high-solids process and <1 g/L in the low-solids process. The higher concentrations of acetic acid resulting from the moderate severity pretreatment were reflective of the more severe pretreatment in which more acetic acid will be released from residue acetyl groups in the biomass.

The solids compositional analysis on the acid-pretreated corn stover substrates reveals that the low-solids

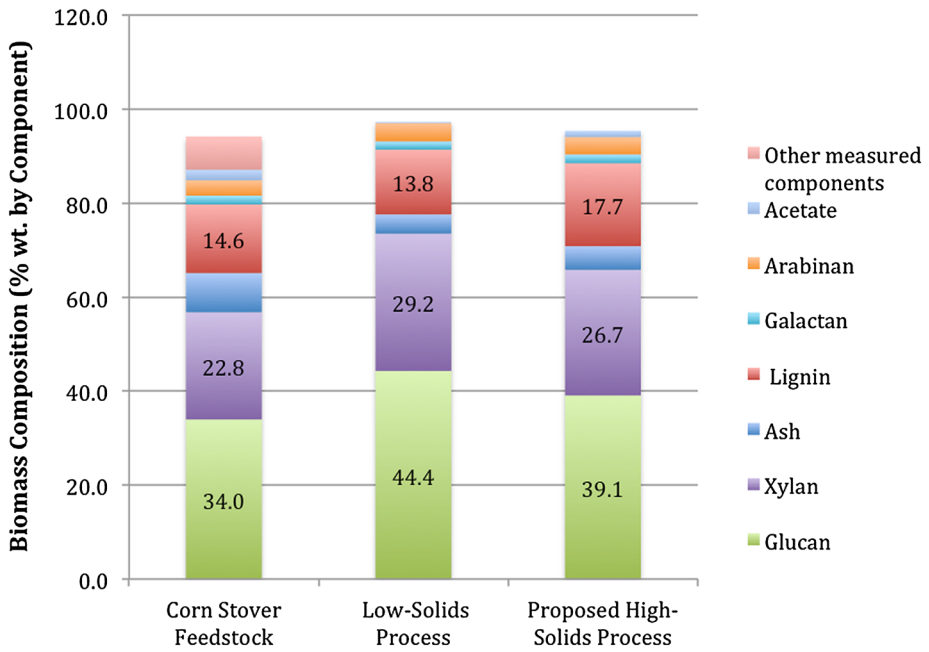

Fig. 4 Solid compositions of corn stover solids before and after deacetylation

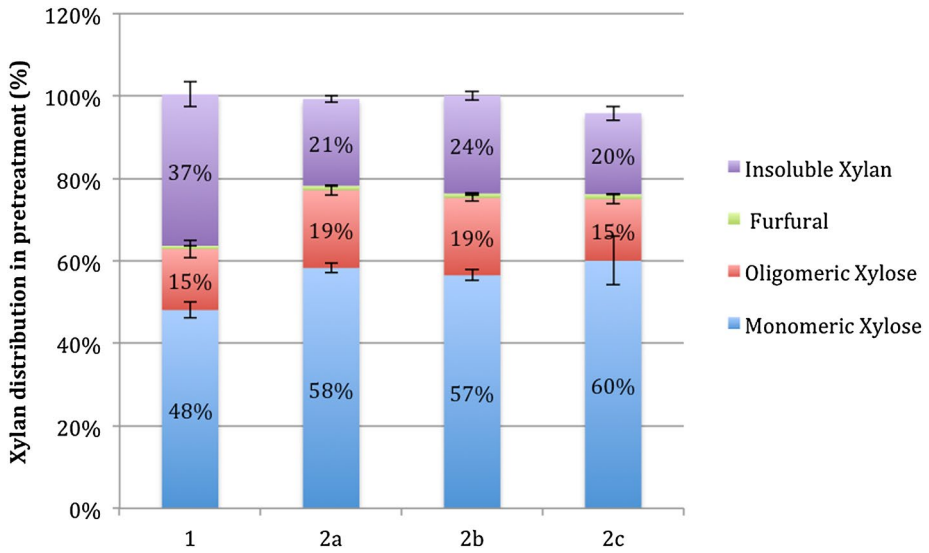

Fig. 5 Xylan component yields from pretreatment at 150 °C, 20 min, 0.5 % sulfuric acid (w/w biomass). *1* Historical deacetylation process performed at 10 % total solids, 80 °C, 120 min, 1.7 g NaOH/g acetate (equivalent to 0.4 % w/w of biomass); *2* (*a–c*) proposed high solids deacetylation process performed at 30 % total solids, 70 °C, 90 min, 1.0 g NaOH/g acetate (equivalent to 0.24 % w/w of biomass); *a–c* represent triplicate deacetylation experiment; *error bars* represent the standard deviation of sugar yields from triplicate runs of acid pretreatment using each batch of the deacetylated corn stover

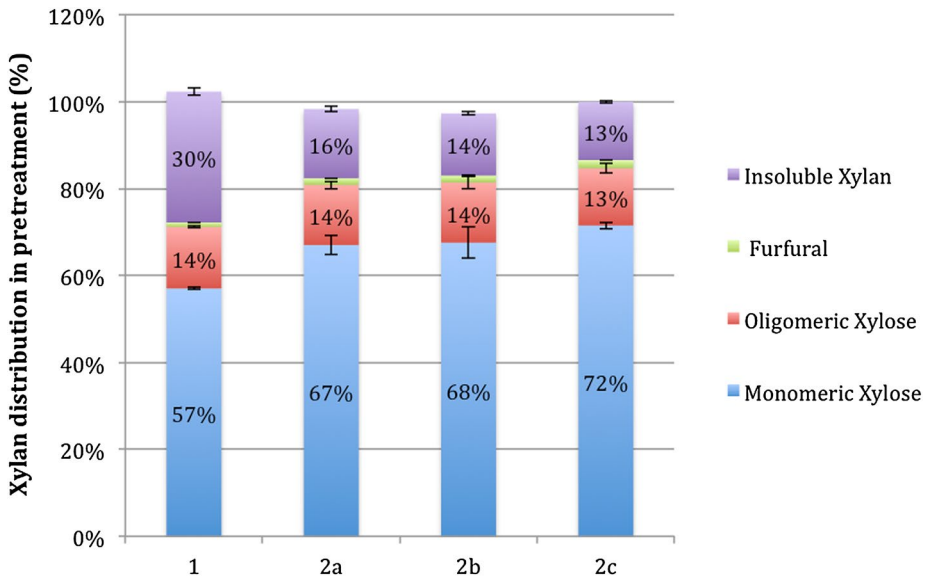

Fig. 6 Xylan component yields from pretreatment at 165 °C, 10 min, 0.5 % sulfuric acid (w/w biomass). *1* Historical deacetylation process performed at 10 % total solids, 80 °C, 120 min, 1.7 g NaOH/g acetate (equivalent to 0.4 % w/w of biomass); *2* (*a–c*) proposed high solids deacetylation process performed at 30 % total solids, 70 °C, 90 min, 1.0 g NaOH/g acetate (equivalent to 0.24 % w/w of biomass); *a–c* represent triplicate deacetylation experiment; *error bars* represent the standard deviation of sugar yields from triplicate runs of acid pretreatment using each batch of the deacetylated corn stover

deacetylation process has lower lignin content (14 %) and higher glucan and xylan content (44 and 29 %, respectively), while the high-solids deacetylation has more lignin (18 %) and less glucan and xylan (39 and 26 %, respectively). This is mainly caused by lower sodium hydroxide loading (0.24 w/w%) in the high-solids deacetylation, resulting in less severe delignification compared to the low-solids deacetylation process.

Enzymatic hydrolysis

Following dilute-acid pretreatment, the triplicate slurries produced from each experimental condition were pooled to produce a larger, homogenous batch of hydrolysate representing each deacetylated feedstock (low-solids control and high-solids process) and pretreatment condition combination (four samples total). These slurries were then neutralized with 28 % ammonium hydroxide to pH 5.1 and enzymatically digested using Novozymes Cellic CTec2 at 20 % total solids and an enzyme loading of 40 mg protein per gram of cellulose. Hydrolysis was conducted in duplicate in 1-L bottles and held at 50 °C for 5 days. Glucan-to-glucose yields during enzymatic hydrolysis (Fig. 7) were both lower in high-solids deacetylation process samples than in the control process, showing 82 % (high solids) vs. 92 % (low solids) of glucose yield at low severity acid pretreatment, and 85 % (high solids) vs. 90 % (low solids) at moderate severity acid pretreatment. The fact that less lignin and acetate were removed in the high-solids deacetylation process but more xylan was removed in the following acid pretreatment, indicates that lignin and acetate removal have more impact on sugar yields in enzymatic hydrolysis, resulting in lower sugar yields in the high-solids, reduced alkali process. Low solids deacetylation removed less xylan but more lignin and acetate; nevertheless, enzymatic hydrolysis sugar yields were higher for the low solids deacetylation process solids compared to the high solids process solids. This finding suggests that acetate and lignin removal is more impactful for improving enzymatic hydrolysis sugar yields.

Ethanol fermentation

The fermentability of each hydrolysate was tested using the glucose-xylose fermenting microorganism *Zymomonas mobilis* 8b. Fermentation was run for 72 h, though glucose was consumed (<2 g/L) in all samples within the first 24 h. The utilization of xylose was slightly higher in the low-solids control process samples, though all were within the same range (83–88 %). Ethanol titers, though, were significantly higher (70 vs 60 g/L) in the control-process-derived samples, largely due to the increased glucose yield from enzymatic hydrolysis making a greater quantity of fermentable sugars available.

Conclusions

We successfully demonstrated the ability to transition the low-solids (10 % w/w) deacetylation process to a higher solids process with minimal impact on the ability to extract acetate from biomass. Conditions were selected to minimize xylan loss during deacetylation, while also removing 70 % of the acetyl groups. Evaluation of the impacts on enzymatic hydrolysis and fermentation demonstrated that, despite the upfront reduction in carbohydrate loss in deacetylation, the overall process sugar yields were depressed by the high-solids, low-alkali process relative to the low solids control. Consequently, ethanol titers were reduced, though strong fermentation

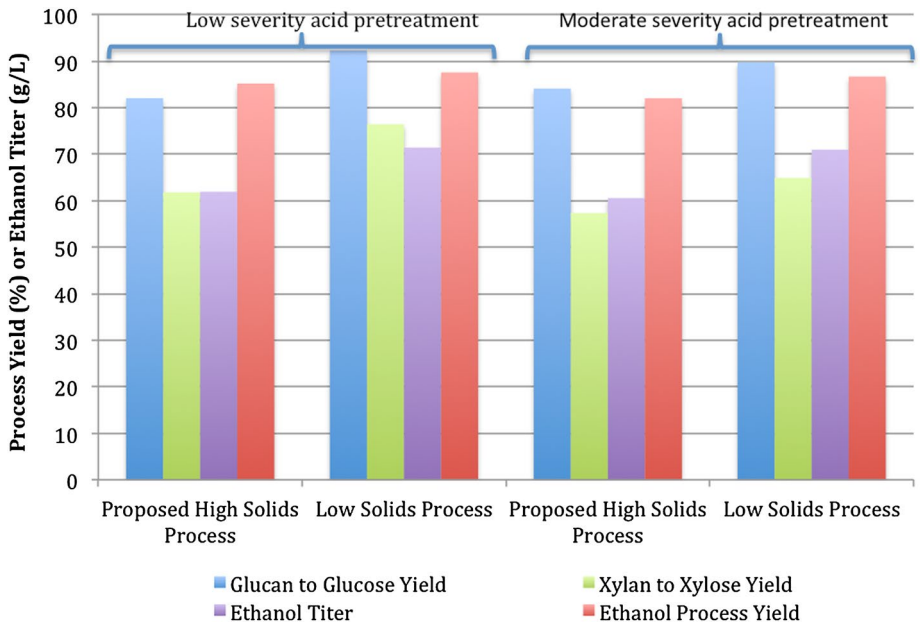

Fig. 7 Sugar conversion and ethanol fermentation yields obtained from integrated deacetylation, pretreatment, enzymatic hydrolysis, and fermentation experiments. Fermentation performed with *rZymomonas mobilis* 8b (NREL). Enzymatic hydrolysis performed with Novozymes Cellic CTec2

performance was still observed, indicating that 70 % acetate removal is sufficient to depress acetic acid concentrations to a level that does not substantially inhibit ethanol fermentation by *Z. mobilis*.

Methods

Feedstock

The corn stover used in this study was harvested in Emmetsburg, IA, USA in October 2010 and shipped to Idaho National Laboratory (INL, Idaho Falls, ID, USA). There, it was processed using the pilot-scale feedstock-handling unit operated by INL as described by Yancey et al. [16] and hammer milled to pass through a 1.9 cm (0.75 in.) round rejection screen. The raw feedstock contains approximately 14.6 % lignin, 34.0 % glucan, 22.8 % xylan, 1.9 % galactan, 3.3 % arabinan, 2.3 % acetate, and 8.4 % ash.

Deacetylation

Deacetylation was performed in a jacketed 90-L paddle mixing reactor. Dry corn stover (3–5 kg) was first loaded into the vessel and the reactor sealed. With the agitator mixing at moderate speed (approximately 100 rpm), water and sodium hydroxide were transferred into the reactor to achieve the desired sodium hydroxide and solids loading. The water and sodium hydroxide were previously mixed in a 60-L stainless steel pressurized dispensing tank. After adding the liquids, a low pressure steam jacket (35 psig supply) was used to heat the contents of the reactor to the target temperature. After a 15-min heat-up period, the steam flow was controlled by throttling the globe and ball valves upstream of the tank and adjusting periodically to maintain the headspace temperature at the target set point. The corn stover was mixed and maintained at temperature for the target amount of time before being discharged from the vessel.

Samples were taken of the deacetylated biomass and the resultant liquor for subsequent compositional analysis and characterization.

Washing and acid impregnation

If the deacetylated biomass was to be carried forward into pretreatment and enzymatic hydrolysis, the solids from deacetylation were placed in a Hastelloy C-276 20-mesh wire basket and exhaustively washed with water to achieve a wash-water pH of 8.0. A sample of the washed solids was taken for moisture analysis.

The deacetylated solids were then loaded back into the 90-L paddle reactor, and the required amount of water to achieve a 10 % total solids loading and 93 % sulfuric acid to achieve the target acid concentration were added to the pressurized transfer tank. After the reactor was sealed, the agitator was started and set to approximately 100 rpm, and the contents of the pressurized transfer tank were added to the reactor. The contents were allowed to mix at room temperature for 2 h and then discharged.

The solids were dewatered to 50 % total solids using a 25.4-cm (10-in.) hydraulic press operated at pressures of up to 440 psig (3 MPa). Samples of the impregnated and dewatered solids were taken for solids compositional analysis and moisture analysis. The prepared feedstock was stored at 4 °C for no longer than 1 week prior to use in pretreatment experiments.

Pretreatment

Dilute-acid pretreatment experiments were carried out in a 4-L steam explosion reactor, the construction and operation of which has been previously described [4]. Prepared deacetylated and acid-impregnated corn stover feedstock were loaded into the reactor (500 g) and heated to the target temperature by direct steam injection. The internal temperature and pressure of the reactor were monitored and recorded. After the desired time elapsed, the contents were discharged from the vessel through a fast-actuating ball valve and collected in a 55-gal drum liner (CDF Corporation, Plymouth, MA, USA).

Pretreatment was performed at two different conditions—150 °C for 20 min and 165 °C for 10 min. All experiments were performed in triplicate. Samples from each experiment were taken for complete compositional analysis. The remaining portions of the triplicate slurries were then pooled to create eight batches prior to enzymatic hydrolysis (three high-solids deacetylation and one low-solids deacetylation, for each pretreatment condition).

Enzymatic hydrolysis

Each of these eight hydrolysates was neutralized with 28 % ammonium hydroxide to a pH of 5.2–5.4 and tested in duplicate in enzymatic hydrolysis and fermentation. Enzymatic hydrolysis was performed in 1-L bottles using a total mass of 450 g at 20 % total solids using CTec2 at a loading of 40 mg of protein per gram of cellulose. The bottles were placed in a shaker box incubator (150 rpm) and held at 48 °C for 120 h, after which samples were taken for analysis and the remainder of the hydrolysate carried forth to fermentation.

Fermentation

Fermentations were performed in batch mode in Biostat-Q plus (Sartorius, Goettingen, Germany) fermenters with a 400 mL working volume. Fermentation conditions were: temperature 33 °C, pH 5.8 controlled using 4 N KOH, and 300 rpm. The microorganism used was the NREL strain, *Zymomonas mobilis* 8b. The fermentations were completed in 72 h.

The media used in the fermentation was rich media (RM) with the composition of 10 g/L yeast extract and 2 g/L KH_2PO_4 supplemented with desired sugar level. RM was prepared as 10× stock solution and sugars, glucose, and xylose were prepared as 50× stock solution; all were filter sterilized. To start the inoculum, first one frozen vial of strain 8b was thawed and revived in 9 mL RMGX (8:2 %) in a 15 mL Falcon tube and incubated for 8 h at 33 °C. The revived culture was used to start the main seed fermenter. The seed fermenter was a Biostat-Q plus fermenter with 400 mL media RMGX (15:2 %) plus 1 g/L sorbitol under similar conditions to those mentioned above. When the optical density (OD @600 nm) of about 10 was reached, usually in 18 h, the remaining glucose was usually between 40–50 g/L. Then the seed culture was used to inoculate the main fermenters at a 10 % (v/v) ratio to achieve an initial OD of 1 (@600 nm).

Analytical

Sugar concentrations were measured using an Agilent 1100 series high performance liquid chromatograph (HPLC) (Santa Clara, CA, USA) with a Shodex SP0810 carbohydrate column (Shawa Denko K.K., Kawasaki, Japan) and a de-ashing guard cartridge (BioRad Laboratories, Hercules, CA, USA). The HPLC was operated with a column temperature of 85 °C and 0.6 mL/min flow rate of the mobile phase (ultra-pure water). Furfural, HMF, and acetic acid concentrations were measured by HPLC using a Phenomenex Rezex RFQ Fast Fruit H + organic acid column and Cation H + guard cartridge (BioRad Laboratories) at 55 °C. The mobile phase was dilute sulfuric acid (0.01 N) at a flow rate of 0.6 mL/min. A refractive index detector was used for compound detection for both columns. Mixed component standards were periodically run with the HPLC samples to verify calibration accuracy. The density of liquid samples was measured using an Anton-Paar model DMA-500 density meter (Anton Paar USA, Inc., Ashland, VA, USA).

The composition of pretreated solids was determined using a two-stage acid digestion procedure [35]. Concentrations of total soluble sugars in pretreated liquor samples were determined using a mild dilute-acid hydrolysis procedure and HPLC analysis [36]. The oligomeric sugar concentration was the difference between total and monomeric sugar concentration. Slurry TS concentrations were determined by drying samples at 45 °C in a vacuum oven (0.6 bar) until repeated weight measurements were constant. Slurry insoluble solid concentrations were determined by a six-step washing and centrifugation procedure [7]. Triplicate measurements were performed on each sample.

The enzyme was desalted prior to measuring the protein content. Desalting was performed on a HiPrep 26/10 desalting column (GE Healthcare, Uppsala, Sweden) with a Sephadex G-25 column matrix using a 2-mL sample. The mobile phase was a 50 mM Tris, 150 mM NaCl pH 5 buffer at a flow rate of 10 mL/min. Protein concentration was measured using the Pierce BCA (BCA Protein Assay Kit, Pierce, IL, USA) assay following the manufacturer's protocols with bovine serum albumin as the protein standard. The measurement was performed in triplicate.

Response surface methodology

A four-factor central composite design was used to characterize the reaction space for deacetylation as a function of temperature, reaction time, sodium hydroxide loading on a gram per gram of acetate basis, and total solids loading. The design used in this experiment was a five-level, four-factor factorial with the center point replicated six times and six axial points with the axial points specified to maintain full rotatability. The calculation of the axial points, specification of the design, and randomization of the experimental design was done using Design-Expert 8.0 (Stat-Ease, Minneapolis, MN, USA), but was consistent with the methodology described by Meyers, Montgomery and Cook [17]. The selection of experimental conditions was based on previously published results [3, 11]. The range of conditions tested is displayed in Table 1.

Regression analysis was completed using Design-Expert 8.0. The responses modeled included the overall solubilization of acetic acid and xylose during the deacetylation stage, with the objective of maximizing the former while minimizing the latter. The software was used to determine which factors from a linear, quadratic, or two-factor interaction model were significant beyond a p value of 0.05 using one-way ANOVA analysis. Factors with the largest p values were progressively removed from each respective model until only significant ($p < 0.05$) terms remained.

Additional file

Additional file 1. ANOVA results.

Authors' contributions

JS executed the deacetylation experiments, analyzed data, and prepared the manuscript along with XC. HS designed and conducted the enzymatic hydrolysis and fermentation experiments. MPT, XC and JS conceived the work and developed the experimental design. All authors read and revised. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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