Toward Biochemical Conversion of Lignocellulose On-Farm: Pretreatment and Hydrolysis of Corn Stover \textit{In Situ}

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TOWARD BIOCHEMICAL CONVERSION OF LIGNOCELLULOSE ON-FARM: PRETREATMENT AND HYDROLYSIS OF CORN STOVER IN SITU

A. A. Modenbach, S. E. Nokes, M. D. Montross, B. L. Knutson

ABSTRACT. High-solids lignocellulosic pretreatment using NaOH followed by high-solids enzymatic hydrolysis was evaluated for an on-farm biochemical conversion process. Increasing the solids loadings for these processes has the potential for increasing glucose concentrations and downstream ethanol production; however, sequential processing at high-solids loading similar to an in-situ on-farm cellulose conversion system has not been studied. This research quantified the effects of high-solids pretreatment with NaOH and subsequent high-solids enzymatic hydrolysis on cellulose conversion. As expected, conversion efficiency was reduced; however, the highest glucose concentration (40.2 g L⁻¹), and therefore the highest potential ethanol concentration, resulted from the high-solids combined pretreatment and hydrolysis. Increasing the enzyme dosage improved cellulose conversion from 9.6% to 36.8% when high-solids loadings were used in both unit operations; however, increasing NaOH loading and pretreatment time did not increase the conversion efficiency. The enzyme-to-substrate ratio had a larger impact on cellulose conversion than the NaOH pretreatment conditions studied, resulting in recommendations for an on-farm bioconversion system.

Keywords. Corn stover, Enzymatic hydrolysis, Enzyme loading, High solids, Low solids, Sodium hydroxide.

The potential for recovering energy from lignocellulose has long been recognized by the academic and industrial communities. However, developing a feasible large-scale process to achieve this recovery has proven challenging (Klein-Marcuschamer and Blanch, 2015). Lignocellulose is composed of polymers of glucose (cellulose), mixed carbohydrate polymers consisting predominately of xylose (hemicellulose), and polymers of phenyl-propanoid units (lignin). The structure of lignocellulose is highly recalcitrant to depolymerization (Isroi et al., 2011). The operations thought necessary to recover glucose and xylose from plant biomass include harvesting and storing the plant material, comminution (particle size reduction), pretreatment to improve accessibility to carbohydrates, and hydrolysis (Klein-Marcuschamer and Blanch, 2015). Fuel alcohols are then produced by fermenting the hydrolysate with alcohol-producing microorganisms (Liong et al., 2012). Historically, these operations have been studied in isolation; however, as they are all interdependent, a systematic approach must be employed to optimize the production of energy and minimize environmental impacts from the process (Klein-Marcuschamer and Blanch, 2015).

Large-scale on-farm production of biofuels is an attractive alternative to a centralized biorefinery because on-farm processing eliminates biomass transport, which is one of the higher-cost operations (Klein-Marcuschamer and Blanch, 2015). Storage, comminution, and pretreatment are the first three unit processes that must be considered when envisioning an on-farm biofuel production scheme. Ideally, on-farm processes would require low capital equipment investments and low energy inputs. One of the major energy sinks associated with biofuel production is product recovery (Jurgens et al., 2012; Singhania, et al., 2009). However, the separation costs decrease markedly with increased product concentration in the fermentation broth (Ezeji et al., 2004). One approach for increasing product concentration during fermentation is to perform the bioconversion at high solids content (Triwahyuni et al., 2015; Banerjee et al., 2012; Viamajala et al., 2010). Advantages of bioconversion at high solids content are increased sugar concentration in the saccharification step and hence higher ethanol concentration in the fermentation broth, as well as reduced capital and operating costs (Banerjee et al., 2010; Humbird et al., 2010; Hodge et al., 2008). Additionally, while interest in the use of high-solids loadings for pretreatment or enzymatic hydrolysis is increasing, few investigations into the integration of the pretreatment and hydrolysis steps at high-solids loadings are available (Rabelo et al., 2014; Larsen et al., 2008; Lau and Dale, 2009).

Systems are considered “high-solids” at solids loadings of ≥15% (w/w), where essentially no free water is available. However, at >15% (w/w) solids, several challenges emerge that are not as apparent at low or moderate solids loadings. For example, the lack of available water in the system and...
inadequate mixing of the solids can limit heat and mass transfer (Viamajala et al. 2010; Singhania et al., 2009). In pretreatment, these limitations can lead to temperature gradients that may result in non-uniform treatment of biomass (Luterbacher et al., 2010). In hydrolysis, these limitations can lead to regions of sub-optimal temperatures and pockets of increased inhibitor concentrations, both of which are detrimental to enzyme activity (Modenbach and Nokes, 2013).

Pretreatment with sodium hydroxide (NaOH) is one of the more attractive pretreatments for on-farm use because it is effective at lower temperatures and pressures than most pretreatments (Modenbach and Nokes, 2014). NaOH pretreatment results in several structural modifications of lignocellulose that are beneficial for enzymatic hydrolysis (Modenbach and Nokes, 2014; Cui et al., 2012; Cheng et al., 2010; Xu et al., 2010) by breaking bonds linking the protective lignin barrier with hemicellulose. Depending on the pretreatment conditions, lignin is more or less solubilized, and degradation of the hemicellulose fraction may occur. Sodium hydroxide pretreatment also swells the lignocellulose particles, leading to an increase in surface area and greater enzymatic accessibility to the cellulose fraction (Hendriks and Zeeman, 2009). Additionally, a decrease in the degree of polymerization and crystallinity of the cellulose is likely, which is linked to increased enzymatic digestibility of the polysaccharide (Eronen et al., 2009; Mittal et al., 2011). The true test of the efficacy of pretreatment, therefore, is the success of the subsequent enzymatic hydrolysis.

Several studies have measured the effectiveness of varying NaOH pretreatment conditions on cellulose conversion of agricultural residues (Sills and Gossett, 2012a; Cui et al., 2012; Wan et al., 2011; Cheng et al., 2010; Gupta and Lee, 2010; Duguid et al., 2009) and were summarized by Modenbach and Nokes (2014); however, most of these studies were performed at low-solids loadings (<4% w/w). NaOH pretreatment results in several structural modifications of lignocellulose that are beneficial for enzymatic hydrolysis (Modenbach and Nokes, 2014). One study reported a cellulose conversion of 99.8% from corn stover following pretreatment at 5% (w/w) solids with 50 g NaOH per 100 g solids at 60°C for 24 h. However, the temperature and NaOH loading were some of the highest reported in the literature. Another study reported a cellulose conversion of 80% from corn stover following pretreatment at 5% (w/w) solids with 20 g NaOH per 100 g solids at 25°C for 24 h (Sills and Gossett, 2012a). A study investigating high-solids (20% w/w) NaOH pretreatment of rice straw reported cellulose conversions of 35% to 40% when the pretreatment was performed with 4 g NaOH per 100 g solids at 55°C for 2 to 3 h (Cheng et al., 2010). Subsequent enzymatic hydrolysis performed in each of these studies was conducted at low-solids loadings (<4% w/w).

NaOH pretreatment is strongly alkaline, with a pH of ~13 to 14, much higher than the optimal pH (~4.8) of cellulase enzymes. Neutralization of the biomass following pretreatment is crucial for optimal performance of the enzymes during hydrolysis. Typically, large volumes of water (10 to 20 volumes) are used to rinse the pretreated biomass (Sills and Gossett, 2012a, 2012b; Cheng et al., 2010; Banerjee et al., 1995). One of the objectives of working with high-solids loadings is to reduce the amount of water consumed in the conversion process, so extensively washing biomass following pretreatment to obtain a neutral pH is counterproductive.

Enzymatic hydrolysis is often identified as the rate-limiting step in lignocellulosic conversion (Jørgensen et al., 2007a). The release rate of glucose slows over time during enzymatic hydrolysis. The use of high-solids loadings in enzymatic hydrolysis has aided in producing a more concentrated glucose product (Jørgensen et al., 2007b), but the reduction in glucose release rate over time is more pronounced, likely caused by the inhibition of enzymes by higher concentrations of glucose and other inhibitory products. Even with these limitations, the use of high-solids loadings in enzymatic hydrolysis is still regarded as a promising solution for developing a more economically feasible process (Triawahyuni et al., 2015; Banerjee et al., 2011, 2012).

Few studies have focused on enzymatic hydrolysis conditions, such as solids loadings and enzyme dosages, using NaOH-pretreated corn stover (Zhang et al., 2012; Chen et al., 2009). One study reported cellulose conversions of 50% to 80% from NaOH-pretreated corn stover (pretreated at 12.5% solids) then hydrolyzed using enzyme dosages of 7 to 20 FPU g⁻¹ solids and 8% (w/w) solids loadings during hydrolysis (Chen et al., 2009). Another study conducted by Zhang et al. (2012) used a fed-batch approach to achieve a final solids loading of 30% (w/w) in the hydrolysis reaction. Cellulose conversions reached 39% and 55% for wheat straw and sugarcane bagasse, respectively. NaOH pretreatment (4 g NaOH per 100 g biomass) was conducted at 5% (w/w) solids.

Because of the large interactions between the pretreatment and enzymatic hydrolysis conditions, including substrate type, substrate preparation, and solids loadings during each processing stage, a systematic approach is needed to identify an optimal system for on-farm processing, rather than optimizing individual stages of the process. Simply recombining process stages that have been optimized independently does not reliably result in an optimal system (Singhania et al., 2009).

The objective of this study was to quantify the combined effects of NaOH pretreatment and enzymatic hydrolysis on cellulose conversion from corn stover when both unit operations are performed at high solids, such as envisioned for an on-farm biomass conversion process. Control treatments involved both high and low solids contents at each processing stage for comparison. To date, this is the first known comprehensive study on the combined effects of solids loadings for pretreating and saccharifying NaOH-pretreated corn stover.

**MATERIALS AND METHODS**

**SUBSTRATE**

Corn stover (CS) was collected directly from the field at the Woodford County Animal Research Center (38.084716° N, 84.726672° W) in Woodford County, Kentucky, in September 2010. The corn (P1253 Pioneer) had been planted using conventional tillage practices in April 2010. Stover is composed of the above-ground material other than grain (MOG). After collection, the samples were prepared for laboratory storage by drying at 45°C for 24 h in an oven and ground to ≤0.5 cm with a Thomas Wiley rotary mini-mill.
COMPOSITION OF CORN STOVER

Laboratory Analytical Procedures (LAP) established by the National Renewable Energy Laboratory (NREL) were used to determine total solids, structural carbohydrates, soluble and insoluble lignin, and ash of raw and pretreated biomass (Sluyter et al., 2005, 2008a, 2008b). HPLC was used to measure the sugars derived from cellulose and hemicellulose (glucose, xylose, arabinose, mannose, and galactose).

HPLC ANALYSIS

A Dionex U3000 HPLC system equipped with a Bio-Rad Aminex HPX-87P column and Micro-Guard de-ashing column was used and operated at 85°C with deionized water as the mobile phase at a flow rate of 0.45 mL min⁻¹. The sample components were detected with a Shodex-101 refractive index detector.

SODIUM HYDROXIDE PRETREATMENT

Sodium hydroxide pretreatment was performed according to Duguid et al. (2009) with some modifications. Weighed samples of dried, ground corn stover (5 mm) were placed in 500 mL Erlenmeyer flasks in the amount necessary to obtain a solids loading of 5% or 20% (w/v). The dry samples were autoclaved on a liquid cycle at 121°C for 30 min to ensure no loss of biomass due to microbial contamination during pretreatment. The flasks were allowed to cool to room temperature prior to equilibration at the selected pretreatment temperature. Four experiments were conducted using NaOH pretreatments. For the initial screening, 20 g NaOH per 100 g CS was used to pretreat corn stover at 5% and 20% solids content for 24 h. The second experiment examined NaOH loadings and pretreatment times using three levels of NaOH loading (4, 10, and 20 g NaOH per 100 g CS) and at 2 and 24 h pretreatment times on corn stover pretreated at 20% (w/v) solids. These loading and treatment times were selected based on appropriateness for on-farm use from a thorough literature review (Duguid et al., 2009; Sills and Gossett, 2012b). The conditions for the third experiment were selected based on the previous study’s results; corn stover at 5% (w/v) solids was pretreated with 4 g NaOH per 100 g CS for 2 h. The final experiment was conducted on corn stover pretreated at 10 g NaOH per 100 g CS for 24 h in order to mimic conditions used in a study we wished to replicate as a check on our hypothesis explaining the results seen in previous experiments. All samples were placed in a 25°C shaking incubator set to 150 rpm.

PH ADJUSTMENT

Two methods were compared for neutralizing the alkaline pH resulting from NaOH pretreatment. The first method was to wash the pretreated corn stover (PCS) with DI water (10 to 20 volumes) while vacuum filtering until a neutral pH was achieved in the biomass. The pH was quantified using pH paper applied to the biomass. The second method used the volume of concentrated glacial acetic acid calculated to neutralize the pH based on the volume of NaOH used during pretreatment. The acetic acid was added to a reduced amount of wash water (3 to 5 volumes). Following both methods, the samples were dried in a 45°C oven for 24 h. The pretreated corn stover was stored at 4°C until further use, typically 24 h or less.

ENZYME LOAD IN ENZYMATIC HYDROLYSIS

Two separate experiments were performed to investigate enzyme loading effects as a function of solids loading. First, a low and high enzyme dosage (5.2 and 60 FPU g⁻¹ solids, respectively) was applied to pretreated corn stover. To further investigate results from the high and low enzyme loading experiments, an additional experiment was conducted using enzyme loadings of 15, 30, 45, and 60 FPU g⁻¹ solids.

Calculation of Cellulose Conversion

Cellulose conversion is typically calculated as the ratio between the amount of glucose (and sometimes cellobiose) released during hydrolysis to the theoretical amount of glucose in the substrate. This calculation is based on several assumptions: (1) the specific gravity is the same (1.0 g mL⁻¹) for all components in the reaction, (2) the volume of the liquid is equivalent to the volume of the hydrolysis slurry, and (3) the volume of the liquid remains constant throughout the entire reaction. However, these assumptions do not necessarily hold true at high-solids loadings. By diluting the sample 10-fold following enzymatic hydrolysis at high solids, cellulose conversions can be calculated according to Kristensen et al. (2009a, 2009b) with equation 1:

$$\text{Cellulose conversion (\%)} = \frac{[\text{Glu}]+1.0526\times[\text{Cel}]}{1.111\times F_c \times DM} \times 100$$

(1)

where [Glu] and [Cel] are the glucose and cellobiose concentrations (g L⁻¹), respectively, 1.0526 and 1.111 are conversion factors accounting for the water molecule required.
to hydrolyze glucose and cellobiose from cellulose, $F_c$ is the fraction of cellulose in the corn stover, and $DM$ is the initial dry matter solids loading (g L$^{-1}$).

**RESULTS AND DISCUSSION**

**EFFECTS OF SOLIDS LOADING IN PRETREATMENT AND ENZYMATIC HYDROLYSIS**

Table 1 presents the corn stover composition when raw, pretreated at low solids (5% w/v), and pretreated at high solids (20% w/v). The highest reduction in lignin occurred in the low solids treatment, decreasing from 18.9% in the raw feedstock to 12.8% in the low-solids pretreated feedstock. The percent lignin in the high-solids pretreatment appears to have increased, but this is likely an artifact of the increased variability seen when working with high solids. What can be safely assumed is that pretreatment in high solids did not result in a measureable reduction in lignin content in absolute amount. It is important to note that although the percent glucose content increased, the amount of actual glucose was constant (numerator remained constant; Modenbach and Nokes, 2014) because when compared to samples that were not pretreated or were pretreated under other conditions, the total amount of available material decreased by removal of components from the original sample (smaller denominator).

Figure 1 presents the results comparing low (5%) and high (20%) solids loadings in NaOH pretreatment (20 g NaOH per 100 g CS at 25°C for 24 h) and enzymatic hydrolysis (5.2 FPU g$^{-1}$ solids at 50°C for 72 h) in units of g glucose g$^{-1}$ biomass expressed as a percent and in g L$^{-1}$ as hydrolyzed. The raw corn stover data exemplify the advantages of hydrolyzing in high-solids systems compared to low-solids systems at the on-farm scale: a higher concentration of glucose in the hydrolysate (13.7 g L$^{-1}$ at 20% solids versus 4.4 g L$^{-1}$ for the 5% solids hydrolysis). The trade-off is in conversion inefficiencies that occur as the solids loading increases (14.1% cellulose conversion in the 20% solids system versus 20.5% in the 5% solids system).

Similar trends occurred in the other treatments. Considering only the g glucose g$^{-1}$ biomass conversion efficiency, the high-solids pretreatment followed by low-solids hydrolysis would be the system selected (57.4% conversion for water-washed biomass following NaOH pretreatment or 63.5% conversion for acid-neutralized pretreated corn stover). However, there are at least two problems with this selection when considering on-farm conversion strategies: (1) multiple reaction vessels would be needed for processing if the solids content is lowered between pretreatment and hydrolysis, and (2) the final glucose concentration is too low to produce a feasible concentration of alcohol to allow economical alcohol recovery. By contrast, the water-washed high-solids treatment for both the pretreatment and hydrolysis requires only one vessel for processing and results in a higher concentration of glucose (40.2 g L$^{-1}$).

Figure 1 also shows that cellulose conversion was impacted in some cases by the change in neutralization methods from all water washing to acetic acid neutralization with reduced water washing. The only treatment that was unaffected was the high-solids pretreatment and low-solids hydrolysis. One hypothesis for this observed decrease in cellulose conversion is that when acids react with bases, the main products that form are water and salts, which in this case

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**Table 1. Composition of biomass in the raw state following NaOH pretreatment (20 g NaOH per 100 g corn stover) at low solids (5% w/w), and high solids (20% w/w). Values are means (with standard deviations in parentheses).**

<table>
<thead>
<tr>
<th>Component$^{[a]}$</th>
<th>Raw (%)</th>
<th>Low Solids (%)</th>
<th>High Solids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu (%)</td>
<td>37.9 (0.5)</td>
<td>48.4 (0.1)</td>
<td>44.4 (1.1)</td>
</tr>
<tr>
<td>Xyl (%)</td>
<td>17.8 (0.4)</td>
<td>22.7 (1.2)</td>
<td>17.0 (0.3)</td>
</tr>
<tr>
<td>Ara (%)</td>
<td>2.6 (0.1)</td>
<td>4.4 (0.3)</td>
<td>1.7 (0.5)</td>
</tr>
<tr>
<td>Man (%)</td>
<td>0.4 (0.0)</td>
<td>0.0 (0.0)</td>
<td>2.3 (0.3)</td>
</tr>
<tr>
<td>Gal (%)</td>
<td>0.8 (0.1)</td>
<td>0.5 (0.0)</td>
<td>0.3 (0.1)</td>
</tr>
<tr>
<td>AIL (%)</td>
<td>18.9 (0.3)</td>
<td>12.8 (0.3)</td>
<td>22.5 (5.4)</td>
</tr>
<tr>
<td>ASL (%)</td>
<td>2.2 (0.0)</td>
<td>1.1 (0.1)</td>
<td>1.2 (0.0)</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>4.8 (0.2)</td>
<td>3.3 (0.1)</td>
<td>10.1 (1.2)</td>
</tr>
<tr>
<td>Other (%)</td>
<td>14.6</td>
<td>6.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$^{[a]}$ Values are noted as weight of component per weight of total biomass in percent: Glu = glucose, Xyl = xylose, Ara = arabinose, Man = mannose, Gal = galactose, AIL = acid-insoluble lignin, and ASL = acid-soluble lignin.

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**Figure 1.** Cellulose conversion of corn stover pretreated at 5% and 20% solids loadings and subsequently hydrolyzed at 5% and 20% solids loadings: (a) cellulose conversion and (b) glucose values in g L$^{-1}$ as hydrolyzed. Pretreatment was performed using 20 g NaOH per 100 g CS at 25°C for 24 h. Enzymatic hydrolysis was performed using 5.2 FPU g$^{-1}$ solids at 50°C for 72 h. Error bars indicate standard deviations.
would be sodium acetate. Actual sodium acetate concentrations were not measured in this study, but based on the amount of acetic acid added during the washing step, as much as 43 to 130 mmol sodium acetate L⁻¹ may have been present. Additionally, the pH was very high initially (~13 to 14). When pH is higher than 5.5, sodium acetate tends to dissociate into its two ionic constituents.

Residual salts (and their ionic constituents) are possible causes for the reduced glucose release in enzymatic hydrolysis for the acid-neutralized treatments. One study found that the production of cellulase by Bacillus coagulans and the subsequent hydrolysis of cellulose slowed as the acetate ion concentration increased (Romsaiyud et al., 2009). Cellulase production (4 U L⁻¹) and cellulose hydrolysis were still measurable at an acetate concentration of 10 mmol L⁻¹, but cellulase production slowed by as much as 75% to ~1 U L⁻¹ at 30 mmol L⁻¹ acetate and was completely eliminated at 60 mmol L⁻¹ acetate.

The highest cellulose conversions observed in these experiments were 45% to 65%, which are quite low when compared to cellulose conversions following other intensive pretreatments (i.e., dilute acid and steam explosion) that can reach conversions of >90%. However, these results are consistent with other studies that used NaOH for pretreating biomass at high solids (Cheng et al., 2010; Cui et al., 2012). We intentionally selected a low-input pretreatment that would be feasible on-farm, knowing that conversion efficiency would be sacrificed to gain practicality in the pretreatment.

A reduction in percent cellulose conversion to glucose as solids loadings are increased in enzymatic hydrolysis was seen in this study (fig. 1a) and has been seen in other studies (Kristensen et al., 2009a, 2009b; Cara et al., 2007; Jørgensen et al., 2007b). This decrease in conversion yields with increasing solids loadings is referred to as the solids effect (Kristensen et al., 2009a, 2009b). However, we contend that, for on-farm conversion, the increase in hydrolysate glucose concentration in high solids (fig. 1b) outweighs the negative aspects of the solids effect.

**EFFECTS OF NaOH LOADING IN PRETREATMENT**

Figure 2 presents the effects of NaOH concentration in high-solids pretreatment of corn stover when followed by acid-neutralization and enzymatic hydrolysis at both low and high solids. There is a marked difference between hydrolysis at low and high solids, which is consistent with the lower conversion in high solids when using acid neutralization (fig. 1). Among the low-solids hydrolysis treatments, 10 g NaOH per 100 g CS was statistically equal to 20 g NaOH per 100 g CS when treated for 24 h, and they were both higher than the 4 g NaOH per 100 g CS. For 2 h pretreatment, 4 g NaOH per 100 g CS resulted in the highest percent conversion.

Figure 2. Cellulose conversion of corn stover pretreated at 20% solids loadings at 25°C, acid neutralized, and subsequently hydrolyzed at 5% and 20% solids loadings. Pretreatment was performed using 4, 10, or 20 g NaOH per 100 g CS at 25°C for 2 or 24 h. Enzymatic hydrolysis was performed using 5.2 FPU g⁻¹ solids at 50°C for 72 h. Error bars indicate standard deviations.

Based on trends typically observed for theoretical glucose yields from enzymatic hydrolysis, an increase in yields was expected as the pretreatment conditions became more severe (i.e., longer time and higher NaOH loading). However, in this study, the effects of pretreatment severity were time-dependent (fig. 2). For samples pretreated for 2 h, the glucose yields decreased or remained relatively constant with increasing NaOH loading, while samples pretreated for 24 h resulted in increased glucose yields with increasing NaOH loading. The amount of time and NaOH applied during pretreatment directly affects a number of factors, including cellulose availability (the amount of theoretical glucose present), cellulose accessibility (the amount of glucose actually accessible to the enzymes for hydrolysis), and optimum processing conditions. Time and NaOH loading interact and impact the structure by breaking lignin-carbohydrate bonds and causing the lignocellulose particles to expand (Modenbach and Nokes, 2014). It is also important to note that NaOH is degraded during the pretreatment process from the resulting ester-bond cleavage between the lignin and carbohydrate fractions. Unreacted NaOH can impact the operating conditions of the subsequent downstream processing steps if the lignocellulose material is not handled appropriately. According to Sills and Gossett (2012a), the amount of NaOH reacted increased with NaOH loading, with as much as 7 g NaOH per 100 g TS reacted when corn stover was pretreated with a NaOH loading of 20 g NaOH per 100 g TS for 24 h. Unreacted NaOH and NaOH ions could then be transported into the enzymatic hydrolysis reaction, which could shift conditions away from the optimum for the enzymes, thus negatively impacting the glucose yield.

Composition data from this study do not support an increased degradation of lignin or cellulose for corn stover pretreated at all NaOH loadings examined. Similar lignin (22%) and cellulose (40%) contents were observed for samples pretreated with 4 g NaOH per 100 g TS as pretreatment time increased from 2 to 24 h (data not shown). Cellulose is very stable at low NaOH concentrations, and this result is consistent with findings reported in the literature (Cui et al., 2012; Sills and Gossett, 2012a). However, for the 4 g NaOH per 100 g TS NaOH pretreatment, 50% less glucose was released during low-solids hydrolysis (quantified as percent cellulose conversion) for 24 h pretreatment (20% cellulose conversion) as compared to 2 h pretreatment (40% cellulose conversion) as compared to 2 h pretreatment (40% cellulose conversion).
conversion), which indicates that cellulose accessibility was impacted with increased time, since cellulose availability did not change based on feedstock composition (data not shown).

Lastly, compositional data support an apparent decrease in cellulose content from 49% to 44% for corn stover pretreated with 20 g NaOH per 100 g TS as time increased from 2 to 24 h. Again, this reduction in percent cellulose available at higher NaOH loadings and/or increased pretreatment times is consistent with findings reported in the literature, and higher cellulose availability has been shown to translate to reduced glucose yields upon saccharification (Cui et al., 2012; Sills and Gossett, 2012a).

**EFFECTS OF ENZYME LOADING IN HYDROLYSIS**

The effects of enzyme loading in hydrolysis were examined to determine whether higher enzyme applications could overcome the low conversion of cellulose observed in high-solids hydrolysis systems. Only corn stover pretreated at high-solids loadings was used for this investigation. Figure 3 shows conversion of cellulose for low and high enzyme applications to hydrolysis reactions loaded with 5% and 20% solids (pretreatment at 20% solids). Cellulose conversion decreased significantly with increasing solids loadings (40.6% to 9.6%) for the low-dose enzyme application, which was further investigated and is discussed later (fig. 4). However, the opposite was observed for the high-dose enzyme application (28.9% in 5% solids to 36.8% in 20% solids), which is hypothesized to be a result of the higher enzyme-to-substrate ratio negating the diffusion limitations typically observed with high-solids systems, allowing hydrolysis to progress further.

The lower cellulose conversion at a loading of 60 FPU g⁻¹ solids compared to 5.2 FPU g⁻¹ solids on the 5% solids loading was initially inexplicable. Selig et al. (2012) reported similar results, and they demonstrated that soluble species found in enzyme preparations can significantly reduce the availability of water, which can negatively impact enzymatic hydrolysis, resulting in lower cellulose conversion. The soluble species include the soluble enzyme systems and other components found in commercial cellulase preparations, such as fermentation by-products, stabilizers, and preservatives. Selig et al. (2012) also determined that increasing the non-hydrolyzable insoluble solids in the enzyme preparation did not appear to affect the overall cellulose conversion. Selig et al. (2012) confirmed these results by purifying commercial cellulase preparations and applying different enzyme loadings (1 to 50 mg protein g⁻¹ cellulose) to 5% and 20% initial dry solids loadings (Sigmacel 50 cellulose). Cellulose conversion for the two solids loadings increased with increasing purified enzyme loadings when the inactive soluble components of the enzyme systems were removed. Furthermore, upon addition of increasingly concentrated non-enzymatic components that were previously removed during purification of the commercial enzyme preparation, a significant decline in cellulose conversion
was observed. Selig et al. (2012) also investigated some common compounds used as preservatives in commercial enzyme preparations (glycerol and sorbitol). These preservatives negatively impacted cellulose conversion (5% solids loading), reducing conversion of pure cellulose by 15% when sorbitol was present at 25 mg mL⁻¹ of hydrolyzate liquid (500 mg sorbitol g⁻¹ cellulose) compared to when no sorbitol was present. Conversion of pure cellulose was reduced by as much as 40% when sorbitol was present at 160 mg mL⁻¹ hydrolyzate liquid (3,200 mg g⁻¹ cellulose, or approximately 30 FPU g⁻¹ solids). This sorbitol concentration would be found in an enzyme loading ~6 times higher than the highest enzyme loading examined in the present study at the same solids loading.

According to another study that characterized multiple commercial enzyme preparations (Nieves et al., 1997), the cellulase system produced from \textit{T. reesei} and marketed as Celluclast 1.5L (the enzyme preparation used in this current work) contained ~280 mg sorbitol mL⁻¹ enzyme preparation. The protein content and specific activity of the commercial cellulase preparation surveyed by Nieves et al. (1997) were very similar to the measurements obtained in the present study (data not shown).

Enzymatic hydrolysis experiments were repeated at 5% and 20% solids loading using increasing amounts of commercial enzyme (15, 30, 45, and 60 FPU g⁻¹ solids) to determine if a systematic reduction in cellulose conversion would result. Assuming that a similar sorbitol concentration as reported by Nieves et al. (1997) was present in the enzyme preparation used in the present study translated to an application of ~120 to 500 mg sorbitol g⁻¹ cellulose for enzyme applications of 15 to 60 FPU g⁻¹ solids (82 to 328 mg protein g⁻¹ cellulose) on 5% solids loadings (fig. 4). Increasing the sorbitol concentration from 120 to 500 mg g⁻¹ cellulose in combination with the enzyme (protein) concentration from 82 to 328 mg g⁻¹ cellulose reduced the conversion of cellulose in PCS by nearly 70% and 30% at 5% and 20% solids loadings, respectively. The concurrent application of high sorbitol concentrations with increasing enzyme activities would appear to explain the low cellulose conversions observed in the hydrolysis studies presented here.

Sorbitol is used as a stabilizer in commercial enzyme preparations, which suggests the need for on-farm enzyme production and immediate use for hydrolysis, or finding a different stabilizer for use in commercial enzyme preparations that does not interfere with biomass hydrolysis.

**CONCLUSIONS**

The use of high-solids loadings in pretreatment followed by high-solids enzymatic hydrolysis has been proposed for on-farm use as a solution to increase the glucose concentration in the hydrolysate and the subsequent ethanol production. However, the impact of solids loadings of the two unit operations in succession (pretreatment and hydrolysis) on cellulose conversion has not been investigated until now. This study found that high-solids loadings in pretreatment followed by high-solids hydrolysis reduced cellulose conversion efficiency but resulted in the highest glucose concentration in the hydrolysate, which translates to higher alcohol concentrations in the fermentation and therefore lower product recovery costs. Increasing the NaOH loading and pretreatment time did not improve cellulose conversion efficiency when high-solids loadings were used in both unit operations. However, increasing enzyme dosage improved cellulose conversion from 9.6% to 36.8% when high-solids loadings were used in both unit operations. The higher enzyme-to-substrate ratio had a larger impact on cellulose conversion than the pretreatment conditions investigated. Using higher concentrations of commercial cellulase preparations introduces soluble inhibitors that can have a significant impact on high-solids hydrolysis. These results could have implications for on-farm processing, such as necessitating the use of enzymes produced on-site without soluble inhibitors.

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