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ABSTRACT

Background: Knowledge regarding the performance of densified biomass in biochemical processes is limited. The effects of densification on biochemical conversion are explored here.

Methods: Pelleted corn stover samples were generated from bales that were milled to 6.35 mm. Low-solids acid pretreatment and simultaneous saccharification and fermentation were performed to evaluate pretreatment efficacy and ethanol yields achieved for pelleted and ground stover (6.35 mm and 2 mm) samples. Both pelleted and 6.35-mm ground stover were evaluated using a ZipperClave® reactor under high-solids, process-relevant conditions for multiple pretreatment severities (R_o), followed by enzymatic hydrolysis of the washed, pretreated solids.

Results: Monomeric xylose yields were significantly higher for pellets (~60%) than for ground formats (~38%). Pellets achieved ~84% of theoretical ethanol yield (TEY); ground stover formats had similar profiles, reaching ~68% TEY. Pelleting corn stover was not detrimental to pretreatment efficacy for both low- and high-solids conditions, and even enhanced ethanol yields.

KEY TERMS:

- *Biomass densification*—increasing the density of biomass; densification is thought to be a key process in the development of a commodity-scale, biomass feedstock supply system
- *Pelleting*—utilizes mechanical and thermal processing for the agglomeration of small particles into larger particles; a process whereby finely ground biomass can be converted into a dense, flowable, and durable product [1] through application of heat and/or moisture and extrusion of ground material through a die [2, 3]
- *Recalcitrance of biomass*—describes the complex structural and chemical nature of plant material that makes it resistant to microbial/enzymatic attack and hinders the

depolymerization of structural sugars [4]; difficulty in conversion of biomass to soluble sugars

- *Thermochemical pretreatment of biomass*—technology needed to improve the enzymatic digestibility of biomass; dilute-acid pretreatment relies on the combined effect of acid, temperature, and time to solubilize hemicellulose in order to improve enzymatic accessibility to cellulose
- *Bioconversion*—microbial-mediated conversion of organic matter into a source of energy (e.g., conversion of biomass to ethanol via fermentation)

INTRODUCTION

Global demands for energy, diminishing petroleum reserves, and growing concerns about climate change have prompted considerable interest in lignocellulosic biomass as a sustainable and renewable energy source. Under the U.S. Energy Independence and Security Act (EISA) of 2007, the Renewable Fuels Standard (RFS) mandates the use of 36 billion gallons per year (BGY) of renewable fuels by 2022, of which 16 BGY are to be derived from cellulosic ethanol [5]. Recent estimates of a baseline scenario suggest that 600 million dry tons of biomass will be available by 2022 to supply sufficient feedstock for achieving this goal, with the potential to produce more than 20 BGY of cellulosic ethanol [6]. The costs associated with acquiring such quantities of biomass are significant; with an estimated cost of $60 per dry matter ton (DMT). A consistent supply of high-quality, economical feedstock is critical to achieving national biofuels goals [7]; however, low-density biomass feedstocks pose challenges to supply chain operations and hinder the large-scale use of biomass for biofuel production. Consequently, increasing the bulk density of biomass through mechanical densification offers logistical benefits that translate into improvements in handling, storage stability, and transportation costs [8].

The development of a uniform-format, solid feedstock supply system is critical to achieve a consistent supply of feedstock with enhanced physical properties for bioenergy production [9]. The economics and physical properties of densified biomass formats produced from agricultural residues have been explored in several studies [2, 10-12]. Pelleting of biomass can increase unit density of raw biomass resources by as much as 10-fold [1], resulting in a flowable and durable product that is compatible with existing biomass supply system infrastructure. Biomass pellets are produced from raw, ground material that is conditioned with heat and/or moisture, compacted, and extruded through a die [2, 3]. It has been shown that activating or softening the natural binders in biomass, such as lignin, through a combination of moisture and temperature during the process of densification is key to the development of particle-particle bonding required for pellet durability [10]. The degree of lignification contributes strongly to biomass recalcitrance [4], and its alteration during the process of densification could impact biomass reactivity to pretreatment and enzymatic hydrolysis [13].

The process of extrusion, which is similar to pelleting, has been shown to enhance sugar recoveries for switchgrass and prairie cord grass [14]. However, there are limited studies that examine the impact of densification on biomass pretreatment and bioconversion. A recent study by Shi and colleagues demonstrated that there were no negative impacts on sugar yields following ionic liquid pretreatment of mixed feedstock pellets (equal masses of switchgrass, lodgepole pine, corn stover, and eucalyptus) relative to the mixed flour format [15]. Rijal et al. [13] reported that the enzymatic hydrolysis of ground and pelleted switchgrass increased glucose and xylose yields by 37% and 42%, respectively, relative to the original biomass following soaking in aqueous ammonia (SAA) pretreatment. The yield improvements measured for pelleted switchgrass were attributed to reduced particle size, shear development, and thermal softening during the pelletization process. Theerarattananooon et al. [3] showed that enzymatic conversion of cellulose (ECC) for pelleted biomass was equivalent to or greater than the corresponding unpelleted material for corn stover, big bluestem, wheat straw, and sorghum stalk; in particular, wheat straw pellets achieved the highest ECC of 94.1%. The improved yields were attributed to the opening of the biomass structure prior to dilute-acid pretreatment through the process of pelleting. This study also demonstrated that the conditions of the pelleting process had a significant effect on biomass composition; the glucan content of the biomass was positively affected by die thickness and negatively affected by mill screen size, while the opposite trend was observed for xylan content. Given the improved sugar yields upon enzymatic hydrolysis for pelleted biomass relative to the unpelleted source materials as described above [3, 13], it may be postulated that the process of densification causes shearing of biomass tissue that translates into increased surface area and improved accessibility for enzymatic action.

The specific surface area of pretreated biomass is thought to aid in sugar release for enzymatic hydrolysis [16]. Chen et al. [17] showed an increase in surface area of rice straw correlated to improved accessibility of cellulose to enzymatic degradation upon implementation of an integrated pretreatment process of dilute-acid and steam explosion. In addition, the diameter of pores in plant cell walls has a strong influence on enzymatic accessibility to cellulose [18]. Cellulases are reported to range in size from 4 to 13 nm, which exceeds the diameter of most pores present in plant cell walls [19]; the small pore size and reduced surface area of native plant cell walls are important factors that pose severe limitations to enzymatic accessibility and thereby contribute to the natural recalcitrance plant biomass [4].

In this study, we sought to investigate the performance implications of biomass densification on bioconversion to ethanol. Pelleted corn stover was used as a model densified feedstock. The objectives of the present study were to 1) examine whether pelleting renders corn stover more recalcitrant to low-solids, dilute-acid pretreatment; 2) determine whether pelleting is detrimental to ethanol yields in the fermentation of dilute-acid pretreated biomass; 3) examine the effects of pelleting and dilute-acid pretreatment on surface area and pore diameter; and 4) understand the impact of pelleting on combined pretreatment and enzymatic saccharification at process relevant conditions, across increasing pretreatment severities.

MATERIALS & METHODS

Materials

Corn stover was single-pass harvested and baled in Palo Alto County, Iowa, between September and November of 2010. The stover was baled and stored field-side for approximately 9 months. Bales were shipped to the Idaho National Laboratory (INL; Idaho Falls, ID) in June of 2011. The average moisture content of the bales as received was ~13% (wet basis), and bales were stored outdoors prior to grinding and densification.

Saccharomyces cerevisiae D_5A was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Accellerase® 1500 enzyme complex, (exoglucanase/endoglucanase activity 54 FPU/mL) was provided by Genencor® (Rochester, NY).

Pellet Production

Six bales were fed through a two-stage, full-scale grinding process using the Feedstock Process Demonstration Unit (PDU) at INL (Idaho Falls, ID). First, material was fed through a Vermeer BG-480 (Pella, IA), which has two horizontal grinding drums with swinging hammers powered by two 200 HP motors [20], and passed through a 25.4-mm screen. The second stage of grinding was completed by feeding the 25.4-mm screened stover through a Bliss hammer mill (Ponca City, OK) with a 6.35-mm screen. Corn stover pellets were generated from the resulting 6.35-mm screened material with a Bliss pellet mill (Pioneer 200; Bliss Industries LLC, Ponca City, OK) in June 2011. The Bliss Pioneer 200 pellet mill is rated for 5 tons/hr and operates at ~200 amps/motor (dependent upon crop type and moisture content). The die has a 12:1 length to diameter ratio, with a 6.35-mm diameter and an effective length of 7.62 cm. The optimal moisture content of feed for the pellet mill is ~10-12%. Steam injection was used to preheat the biomass to 65.6°C, and on the day the stover was pelleted, the die temperature was recorded at 98°C using an infrared thermometer.

The formats used for comparison in this study were: (1) source material, which underwent a two-stage grinding process, was screened to less than 6.35 mm, and used as the feed material for the pellet mill; (2) source material that was subsequently ground using a laboratory knife mill (Thomas Wiley Laboratory Mill, Model 4, 1 HP; Thomas Scientific, Swedesboro, NJ) with a screen size of 2 mm (circular shaped screen opening); (3) pellets; and (4) pellet meal that was generated with a 2-mm screen on the laboratory knife mill described previously.

Bulk Density, Durability, and Calorific Value

The bulk density and durability of corn stover pellets were measured according to standard ASABE S269.4 [21]. Prior to testing, pellets were sieved through a 5.6-mm screen to remove fines. Quadruplicate 500 g samples of sieved pellets were placed in the tumbling device and then tumbled for 10 min at 50 rpm, in a dust-tight enclosure. After tumbling for 10 min, the sample was removed, sieved, and the percent of whole pellets was calculated. This procedure was performed twice for a total of eight replicates. Pellet durability is a quality parameter that represents the physical strength and the ability of a pellet to remain intact during storage, handling, and transportation [1, 22]. The calorific value of pellets was measured using a LECO AC600 bomb calorimeter according to the standard ASTM D5865-10a [23].

Particle Size Distribution (PSD)

A dynamic image analyzer, Camsizer (HORIBA Instruments, Inc., Irvine, CA), equipped with two digital cameras was used to analyze particle characteristics. This method was reported to be highly correlated with a well accepted static/quantitative technique using light microscopy ($r>0.9$ for both aspect ratio and sphericity) [24]. PSD was determined according to a standardized method developed by the International Organization for Standardization [25]. Particle distribution was calculated based on volume, which was defined using an ellipsoid model; diameter was calculated based on $X_{c\ min}$, the greatest width of a particle projection at a right angle.

Compositional Analysis

The chemical composition for corn stover grinds (6.35 mm ground to 2 mm and 2 mm) and pellets (ground to 2 mm) were measured according to the National Renewable Energy Laboratory's (NREL) Laboratory Analytical Procedures (LAPs) for standard biomass analysis [26]. Briefly, biomass was extracted using water followed by ethanol. Then a two-stage acid hydrolysis of the extracted material was performed. The resulting hydrolysis liquor was analyzed for monomeric sugars, sugar degradation products, and organic acids using high performance liquid chromatography (HPLC) with a refractive index detector (Waters, Milford, MA). The acid hydrolysis and HPLC analysis were performed as described by NREL [27]. Sugars were analyzed on an Aminex HPX-87P carbohydrate column with a mobile phase of 18 MΩ nanopure water at a flow rate of 0.6 mL/min at 85°C. Duplicate 50 µL injections were performed for each sample. Acid-soluble lignin fractions were analyzed using ultraviolet-visible spectroscopy.

Low-Solids Pretreatment

Dilute-acid pretreatment was conducted using an autoclave method as previously described [28]. The low-solids pretreatment was conducted at a solids loading of 3.3% (w/w) using dilute sulfuric acid (0.8% H_2SO_4 w/w) and an autoclave at 121°C for 30 minutes. Briefly, 3.3 ± 0.5 g (recorded to the nearest 0.0001 g) of biomass was added to a 500 mL Erlenmeyer flask, followed by the addition of 100 milliliters of 0.8% H_2SO_4 (w/w). Flasks were autoclaved at 121^{o}C, 145 kPa for 30 min. Then, flasks were cooled to 23^{o}C, and the pretreated biomass and hydrolysis liquor were filtered in a vacuum manifold through Gooch crucibles lined with Whatman™ binder-free glass microfibre type GF/D filters with 2.7-µm particle retention (Buckinghamshire, UK). Residual biomass was recovered from pretreatment flasks by rinsing with deionized water; this water was further used to rinse the biomass contained in the Gooch crucibles and collected with the pretreatment liquor in a 200 mL volumetric flask. The pretreatment liquor was brought to a final volume of 200 mL with deionized water that was used to wash the pretreated solids. The biomass was then washed with deionized water until the pH of water filtrate was between 5.5 and 7.0.

Soluble components in pretreatment liquors (monomeric, oligomeric, and total sugars) were measured using previously described techniques [29]. Total xylose and glucose are defined as the sum of their monomeric and oligomeric forms. Furfural dehydration products were not measured in pretreatment liquor for the low-severity pretreatment experiments. Liquor samples were neutralized and filtered prior to HPLC analysis. Samples of hydrolysis liquors were analyzed by an HPLC (Waters, Milford, MA) equipped with an autosampler and refractive index detector. Sugars were analyzed on an Aminex HPX-87P carbohydrate column with a mobile phase of 18 MΩ nanopure water at a flow rate of 0.6 mL/min at 85°C. Duplicate 50 µL injections were performed for each sample.

Simultaneous Saccharification and Fermentation of Low-Solids Pretreated Materials

Simultaneous saccharification and fermentation (SSF) was performed anaerobically at 30˚C and 100 rpm in 60 mL serum vials using *Saccharomyces cerevisiae* D_5A and Accellerase 1500. One day before the experiment, the moisture content of pretreated biomass was measured by drying biomass overnight at 100˚C. The following media and solutions were prepared and sterilized by either filtration with 0.2 µm filter or autoclaved at 121˚C and 20 psi: solid YPD media (10 g/L yeast extract, 20 g/L peptone 20 g/L dextrose, 20 g/L agar); liquid YPD media (10 g/L yeast extract, 20 g/L peptone, 50 g/L dextrose); 10X YP (100 g/L yeast extract, 200 g/L peptone); phosphate buffered saline (PBS, 11.8 mM phosphate buffer at pH 7); 200 mM NaCl; 27 mM KCl; and 1.0 M citric acid buffer (CAB at pH 4.5).

A yeast solution was prepared by taking a single CFU of *S. cerevisiae* D_5A, inoculating into 300 mL YPD in a 2.0 L Erlenmeyer flask, incubating at 30˚C and 175 rpm for 24 hours, and growing the culture until a 1/100 dilution of culture in YPD reached an OD_{600} of 9.5 to 11. The yeast culture was centrifuged at 4˚C and 4500 rpm for 5 min, and the supernatant was discarded. The yeast pellets were resuspended in 300 mL 1X PBS and centrifuged again at 4˚C and 4500 rpm for 5 min. Finally, the yeast pellet was suspended in 100 mL 1X PBS, and an OD_{600} on a 1:20 dilution was measured by spectrophotometer. Depending on the measured OD_{600}, the calculated volume of PBS was gradually added in order to obtain an ideal yeast solution with an OD_{600} of 15.

SSF vials contained 1 g of pretreated biomass (dry weight basis) in a fermentation volume of 30 mL. Following addition of pretreated biomass, water was added to a volume of 24 mL, which accounted for the moisture content of biomass. Serum vials were purged with ultra high purity nitrogen gas in order to remove residual oxygen and then sealed with butyl rubber stoppers and aluminum closures. The samples were autoclaved at 121˚C and 20 psi for 30 min and then placed in a walk-in cooler at 4˚C within 10 min of the autoclave run. Samples were then cooled, and the following additions were made aseptically: 5 mL enzyme cocktail—which included 3 mL 10X YP, 1.5 mL CAB , and 0.5 mL enzyme mixture (Accelerase 1500, 15 FPU/g dry weight biomass)—and 1 mL yeast solution. Vials were incubated at 30˚C and 100 rpm for ethanol production. The vials were then analyzed for ethanol by injecting 200 mL of the headspace gas for gas chromatographic analysis with a flame ionization detector (GC-FID HP 6890 equipped with autosampler; Hewlett-Packard, Palo Alto, CA). The percentages of theoretical maximum ethanol yield (% TEY) were calculated based on the fraction of glucan in pretreated biomass solids according to NREL's LAP [30].

High-Solids Pretreatment

High-solids pretreatment (25% w/w) was conducted on both pelleted and 6.35-mm ground stover using dilute sulfuric acid pretreatment under process-relevant conditions with a ZipperClave® reactor (Parker Autoclave Engineers; Erie PA) as previously described [31], followed by enzymatic hydrolysis of the washed pretreated solids. Although not directly scalable to commercial or pilot-scale [32], the ZipperClave® is a batch reactor with internal mixing, heated by direct steam injection for rapid heating. The reactor is capable of pretreating 0.3 kg of biomass at high-solids loadings (≤25% w/w). These capabilities simulate key process parameters that can provide valuable insight into the pretreatment performance of biomass under commercially-relevant processing conditions. Both pelleted corn stover and 6.35-mm ground stover were pretreated using five different pretreatment conditions, listed in Table 1. All materials were pretreated using 0.8% H_2SO_4 (w/w). To compare sugar yields between the low-solids one pretreatment severity (R_o=2.07) was used as a point for comparison between the low solids pretreatment method. Triplicate experiments were performed for both feedstock types (corn stover pellets and 6.35-mm ground stover) and each pretreatment condition. For each pretreatment condition, the following protocol was used. The corn stover, either pellets or 6.35 mm ground, was added to the canister followed by an addition of 0.8% H_2SO_4 to achieve a 25% solids loading (w/w). The resulting slurry was mixed for 5 min and then loaded into the ZipperClave® reactor. After the reactor was sealed, mixing was initiated followed by steam injection into the top and bottom of the reactor, achieving reaction temperature in 30 to 45 sec; reaction time began when the target temperature was reached. At the end of the reaction, steam is vented through the top plate and into a condenser. Following depressurization, the canister is removed and placed into an ice bucket to quench the reaction. Any remaining solids on the impellor and head plate were rinsed, collected, and analyzed for percent total solids. The hydrolyzate, rinsate, and condensate were weighed and then analyzed for sugars, organic acids, and inhibitors, as previously described [29].

Table 1. Pretreatment conditions for high-solids pretreatment experiments using the ZipperClave®.

Severity (Log R_o)	Temperature (°C)	Reaction Time (min)
1.51	125	6
2.07	150	4
2.47	150	10
2.81	175	4
3.29	175	12

Enzymatic Digestion of High-Solids Dilute Acid Pretreated Materials

Both ground (stover screened to < 6.35 mm) and pelleted corn stover, pretreated in the ZipperClave®, were subjected to enzymatic hydrolysis, as previously described [30]. The pretreated materials were washed prior to enzymatic hydrolysis, and the solids content of the wet solids was determined prior to saccharification. Non-pretreated pellets and 6.35-mm ground stover were used as controls to compare with the pretreated materials. The enzymatic hydrolysis was conducted at 20% solids loading, using CTec2® (15 FPU/mL; Novozymes; Franklinton, NC) at an enzyme loading of 20 mg of cellulase/g dry wt. of pretreated washed solids. All enzymatic hydrolysis studies were carried out for 5 days at 50°C. At the end of the assay, the samples were filtered and the liquors analyzed for soluble sugars using standard methods [29]. To calculate glucose release in the washed solids the concentration of glucose released (g/g dry wt.) during enzymatic hydrolysis was multiplied by the mass of insoluble solids remaining after pretreatment, determined by the fraction of insoluble solids (FIS) analysis. Glucose yield was determined by dividing glucose release by the mass of initial glucose in the biomass sample.

Scanning Election Microscopy

Scanning electron microscopy (SEM) was performed with a JEOL JCM-5000 NeoScope in high vacuum mode (JEOL, Arvada, CO). Micrographs were taken of raw biomass samples (7.6-cm source material and pellets) in order to identify morphological differences among biomass formats that occurred as a result of densification.

Gas Adsorption

The Brunauer-Emmett-Teller (BET) surface area, pore volume, and pore size for source, pelleted, and pretreated corn stover was determined by nitrogen adsorption at 77K (Autosorb iQ, Quantachrome Instruments, Boynton Beach, FL). Corn stover samples were dried at 80˚C under vacuum for a minimum of 24 hours and a maximum of 113 hours. Before vacuum drying, source material, pellets, and pellets ground to 2 mm were dried in an oven at 80˚C for a minimum of 24 hours and a maximum of 310 hours. Drying times varied because of sample form and nature, and samples were considered dry if the sample was isolated and the pressure rise was less than 21.00 μm/min. Pellets were broken to approximately 1.27 cm in length before drying, and five pellets were placed into the Quantachrome sample cells for each replicate (1.8-2.0 g). Sample cell bulbs were filled approximately half-way with source material, ground pellets, and acid-pretreated samples (0.2-1.7 g). Autosorb iQ software (ASiQwin V2.0, Quantachrome Instruments, Boynton

Beach, FL) was used to obtain BET surface area, total pore volume, and average pore size data from nitrogen adsorption curves. BET suface area was determined using 11 adsorption points in the relative pressure range from 0.05-0.30.

Statistical Analysis

Data presented are the mean of at least three replicates unless otherwise noted, and error bars represent one standard deviation. All statistical analyses were performed in the open-source language R (version 2.15.2) [33] or StatPlus®: mac 2009 (AnalystSoft Inc. 2010). One-way analysis of variance (ANOVA) was performed to identify significant differences, and Tukey's Honest Significant Difference (HSD) test was performed for multiple-level comparisons of statistical equivalency. Two-way ANOVA was used to identify significance in feedstock reactivity experiments.

For BET gas adsorption studies, one-way ANOVA was used to determine how surface area, pore volume, and pore size were affected by pelleting. A Bonferroni adjustment was used to control for experiment-wise error rate, and differences were considered significant at a level of $p \leq 0.05$. Total pore volume data was $\log_{10}$ transformed to meet the assumptions related to normality and homogeneity of variance.

RESULTS & DISCUSSION

Physical Properties, Size Distribution, and Composition of Corn Stover

The physical and thermal properties of the corn stover pellets produced with the Feedstock PDU were measured. The moisture content of the pellets was 11.3%. High-quality corn stover pellets were produced with the Feedstock Process Demonstration Unit (PDU) and had a measured durability of 98.5% ± 0.6%; this complies with the standard durability requirement for pellets during handling and transportation that establishes a minimum limit of

97.5% [22]. Pelleting the 6.35-mm ground source material improved bulk density by 4.3 times (from 149 to 645 kg/m^3). The bulk density and energy density of the corn stover pellets produced with the Feedstock PDU (645 kg/m^3 and 16.55 MJ/kg) were about half that for coal (1346 kg/m^3 and 31.75 MJ/kg).

The average particle size distribution (PSD) parameters for each of the ground corn stover formats are presented in Table 2. There was no significant difference between the geometric mean particle size, D_{50}, of the 6.35-mm source material or the pellet meal ($p > 0.05$), while the geometric mean particle size for the 2-mm ground source material was about 33% less than the other two formats ($p < 0.001$; Table 2). The particle diameters for the 84th and 16th percentiles of particle size distributions were significantly different for all three stover formats ($p < 0.001$). The 6.35-mm source material had the widest range of variation (D_{84}=2.27 mm, D_{16}=0.30 mm) and least uniform particle size, as calculated by the size range variation coefficient (%) [34].

Table 2. PSD parameters (mean ± 1 SD; n=3) of corn stover formats; all dimensions are in mm. The source material (6.35 mm) was generated from a 2-stage grinding process with the Feedstock PDU. The 2-mm format was generated from the source material using a lab-scale knife mill. Pellet meal represents size-reduced pellets generated with a 2-mm screen on a lab-scale knife mill.

Treatment	D_{84}	D_{50}	D_{16}
Source	2.27 ± 0.02	0.77 ± 0.01	0.30 ± 0.004
2 mm	1.22 ± 0.02	0.52 ± 0.01	0.18 ± 0.002
Pellet meal	1.35 ± 0.003	0.75 ± 0.01	0.24 ± 0.01

Compositional data suggest that neither grinding nor pelleting had a significant effect on the chemical composition of the corn stover formats examined here. Glucan and xylan contents were reduced slightly for pelleted corn stover (31.3% ± 0.5% and 20.8% ± 0.6%, respectively) compared with the ground formats (33.2% ± 0.6% and 25.1% ± 1.8% for 6.35 mm; 32.1% ± 1.4% and 21.5% ± 1.2% for 2 mm), although these differences were not significant ($p > 0.05$). There were no significant differences in lignin content among the three corn stover formats tested ($p > 0.05$). Similar glucan, xylan, and lignin compositional data for corn stover have been reported previously [32, 35]. In addition, results from this study are consistent with recent reports [3, 13] that examined the effect of pelleting on composition and sugar yields for a variety of biomass feedstocks, including corn stover, wheat straw, big bluestem, sorghum stalks, and switchgrass.

Low-Solids Dilute-Acid Pretreatment

Monomeric and total xylose yields, and ethanol yields were measured following dilute-acid pretreatment and SSF, respectively. Figure 1 provides the average monomeric and total xylose yields following dilute-acid pretreatment. Monomeric xylose yields are a critical performance parameter used to assess the efficacy of dilute-acid pretreatment [32]. Under the low-severity pretreatment conditions examined here, monomeric xylose yields were significantly higher for the pellet meal (Tukey's HSD $p < 0.01$) than for the other formats tested with a monomeric xylose yield of 59.3% ± 3.4% (mean ± SD; Figure 1). Average monomeric xylose yields were similar (~38%) for both the 6.35-mm and 2-mm formats. There were no significant differences in average total xylose yield among the three stover formats ($p > 0.05$; Figure 1). The 2-mm corn stover format exhibited high variability in both monomeric and total xylose yields (SD 7.1% and 15.8%, respectively). The average total xylose yield achieved with pellet meal

(81%) under the low-severity conditions examined here (R_o=2.07) was similar to what has been achieved under high-severity, dilute sulfuric acid pretreatment conditions using a continuous, pilot-scale reactor with corn stover ground to less than 1.9-cm at a 30% solids loading [32]. Findings by Rijal et al. [13] demonstrated that pelleting of switchgrass was not detrimental to the efficacy of dilute-acid pretreatment. The improved monomeric xylose yields achieved with the corn stover pellets, and total xylose yields that are comparable to what has been achieved under high-severity, commercially-relevant pretreatment conditions, may suggest that densification, and more specifically, pelleting, has the potential to reduce the severity of pretreatment conditions that are required for bioconversion.

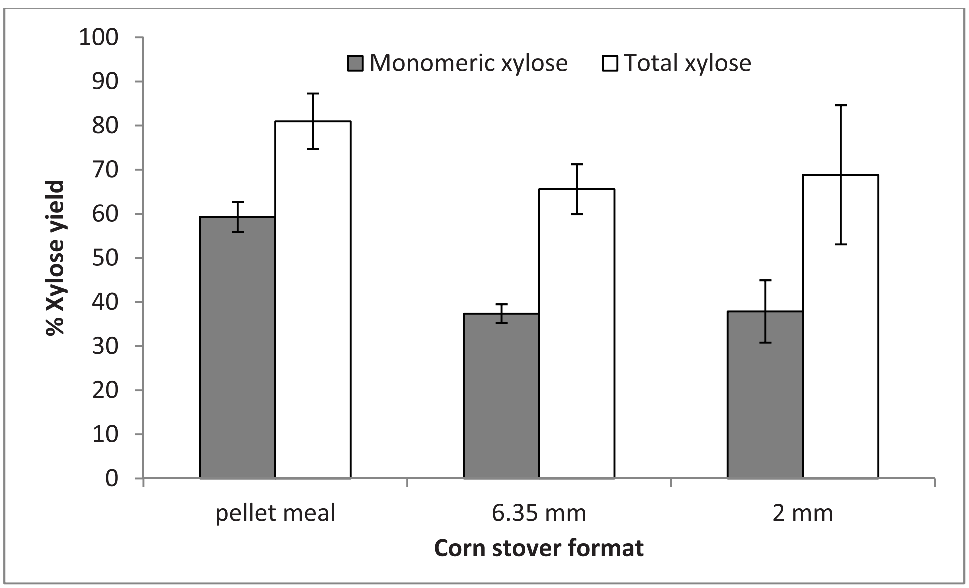

Figure 1. Monomeric and total xylose yields for corn stover formats following low-solids, dilute-acid pretreatment (mean ± 1 SD; n=4 for 6.35-mm and 2-mm formats; n=3 for pellet meal due to a problem with HPLC injection volume in one replicate).

The fraction of initial glucan solubilized during low-solids, dilute-acid pretreatment for corn stover formats is shown in Figure 2. There were no significant differences in either monomeric or total glucose solubilized upon dilute-acid pretreatment among the formats tested ($p > 0.05$); glucan hydrolysis yields ranged from 7.2% for the 2-mm grind to 8.8% for pellet meal, with solubilized glucose present in predominantly oligomeric form (Figure 2). These results are consistent with earlier studies that have demonstrated cellulose hydrolysis upon dilute-acid pretreatment yields only a small fraction of the total glucose available in the native material [31, 37, 38]. Previous reports have suggested that the presence of oligomeric glucose in pretreatment hydrolyzates corresponds to the depolymerization of amorphous glucan contained in the structure of cellulose microfibrils [37, 39].

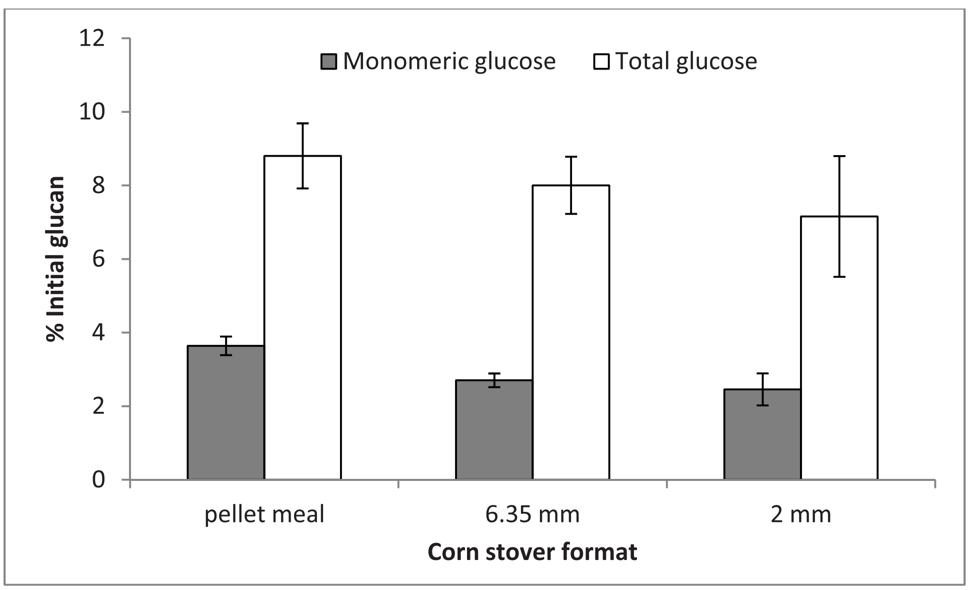

Figure 2. Glucan hydrolyzed during dilute-acid pretreatment for three corn stover formats (mean ± 1 SD; n=4 for 6.35-mm and 2-mm formats; n=3 for pellet meal due to problem with HPLC injection volume in one replicate).

Simultaneous Saccharification and Fermentation of Low-Solids Pretreated Materials

Beyond maximizing xylose yields during pretreatment, the key measure for assessing the efficacy of dilute-acid pretreatment is achieving maximum sugar release from the enzymatic hydrolysis of cellulose in pretreated biomass for fermentation [32]. Results from laboratory-scale simultaneous saccharification and fermentation (SSF) demonstrated that the pellet meal achieved the highest average percentage of theoretical maximum ethanol yield (% TEY) of the formats examined (Tukey's HSD $p < 0.001$), reaching nearly 84% TEY from glucan by the end of the experiment (Figure 3). The conventional ground formats, 6.35-mm and 2-mm grinds, had similar profiles achieving nearly 68% of TEY by day 7. Actual ethanol yields on day 5 of the fermentation were also compared for each format, with pellet meal, 6.35-mm, and 2-mm formats producing 0.15 ± 0.006, 0.12 ± 0.009, and 0.12 ± 0.004 g ethanol / g pretreated biomass, respectively. The pellet meal had higher yields compared to the other formats tested (ANOVA $p < 0.001$; Tukey's HSD $p < 0.001$). No differences were seen in ethanol yield between 6.35-mm and 2-mm stover formats ($p > 0.05$). It is important to note that, although pellet composition was slightly reduced in glucan content relative to the other formats tested, pelleted corn stover still achieved significantly higher actual ethanol yields, in addition to increased % TEY. The improved ethanol yields measured in SSF for pretreated corn stover pellets relative to the 6.35-mm and 2-mm grinds suggest that pelleting enhances saccharification of structural glucan for fermentation, which has the potential to enhance ethanol production from biomass feedstocks.

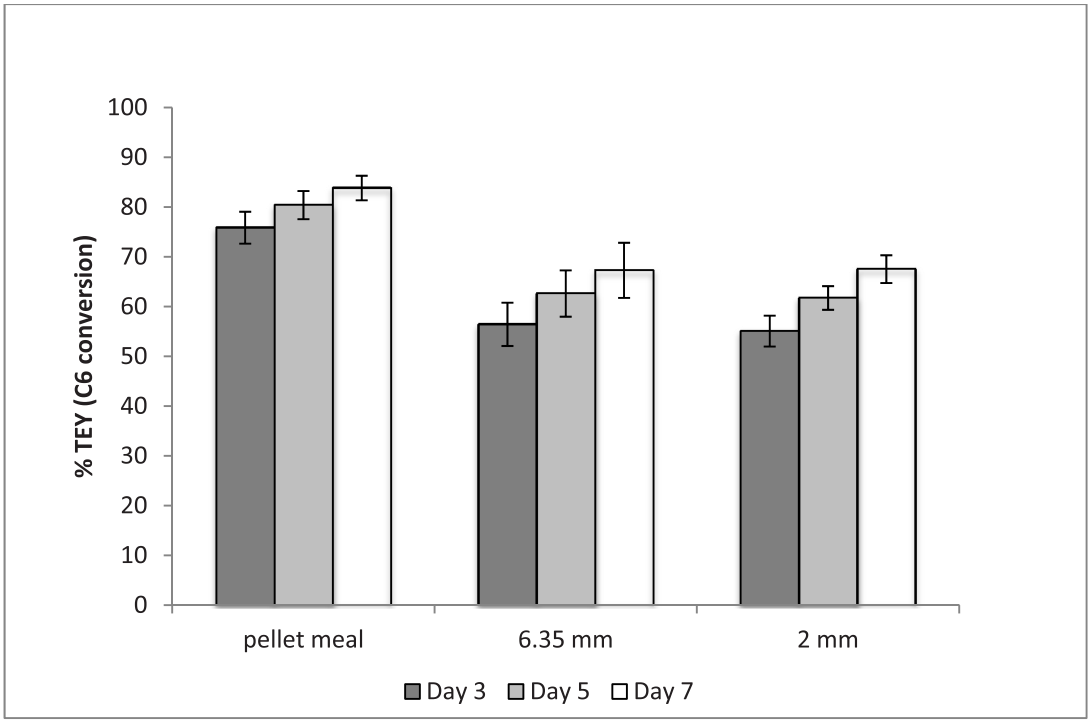

Figure 3. The percentage of theoretical maximum ethanol yield (% TEY) for three-corn stover formats on days 3, 5, and 7 of SSF (mean ± 1 SD; n=4).

Theerarattananoon *et al*. 2012 [3] recently reported that enzymatic conversion of cellulose from pelleted biomass was either equivalent to or higher than conversion yields that were achieved with the corresponding unpelleted samples following dilute-acid pretreatment; these results implied that under optimized conditions pretreated, pelleted biomass may enhance glucose recovery from cellulose. In a study by Rijal and colleagues [13], the effect of pelleting on sugar yields upon enzymatic hydrolysis for untreated and pretreated biomass was examined; the untreated pellets had higher glucose yields upon enzymatic hydrolysis than the untreated, original biomass (D_{50} ~ 7.7 mm). For dilute-acid pretreated biomass, pellets had significantly higher glucose yields than the original biomass at 24 hours, while soaking in aqueous ammonia resulted in glucose yields that were 36% greater for pellets than for original biomass at 24 hours.

Extrusion has been shown to increase the ethanol yield and fermentation efficiency of sorghum [40]; the improvements in bioconversion were attributed to depolymerization of starch, increased free sugars, and destruction of native biomass structures as a result of the extrusion process.

High-Solids Dilute Acid Pretreatment and Enzymatic Hydrolysis of Source and Pelleted Materials

Differences between the 6.35-mm source and pelleted corn stover were evident prior to pretreatment. After adding acid and mixing for 5 min, the pelleted material did not dissolve into the bulk liquor and remained as distinct pellets in the aqueous phase prior to pretreatment. The source corn stover was very hygroscopic: after mixing for 5 min, no free liquid was observed. However after pretreatment, the slurries from both the pelleted and source corn stover (R_o=2.47) were similar, both in color and volume of pretreated solids. Material recovery after pretreatment of the source and pelleted stover was 95.3% ± 5.2% and 102.98% ± 2.2%, respectively. Final sugar yields were normalized to 100% of material recovery.

In the high-solids pretreatment experiments, the corn stover pellets had glucan and xylan contents of 36.1% and 27.2%, respectively. The composition of the source material was similar in glucan content, 36.8%, with an increase in measured xylan, 32.3%. The increased xylan content of the 6.35-mm ground material may be attributed to the heterogeneous nature of this material and high cob content of the stover bales that were used in this study rather than as a result of pelleting. Previous work has shown that corn cobs are enriched in xylan relative to other fractions of corn stover [36]. Additionally, sampling and splitting techniques may have contributed to the compositional variations that were observed here.

Xylose yields from pretreatment alone and pretreatment plus enzymatic hydrolysis of washed solids is shown in Figures 4 and 5. Maximum xylose release was achieved at a severity of R_o=3.29 for both pretreated (80.8%, 78.6%) and enzymatic hydrolysis of washed, pretreated stover (86.5%, 86.1%) for the 6.35-mm source and pellets, respectively. Soluble xylose yield (monomeric plus oligomeric) for both feedstock formats increased with increasing pretreatment severity (R_o=1.51 to 3.29) for both pretreatment configurations (Figures 4 and 5). Enzymatic hydrolysis of the washed pretreated solids increased xylose yield for all samples compared to pretreatment alone.

Comparison of both feedstock formats revealed no significant differences in xylose yields for either pretreatment alone or pretreatment plus enzymatic hydrolysis, with the exception of R_o=2.81; xylose yield was ~10% higher for pelleted stover compared with source material at this severity based on one-way ANOVA ($p<0.05$). The percentage of monomeric xylose of the total xylose yield was higher for the 6.35-mm source relative to the pelleted stover for all severities tested. At the highest pretreatment severity (R_o=3.29) the percentage of monomeric xylose was 74% and 95% of the total xylose, for the pelleted and source stover, respectively. This is in contrast to the results of the low-solids pretreatment data, which showed higher monomeric xylose yield from the pelleted material. A higher fraction of oligomeric xylose from dilute-acid pretreatment would suggest a less optimal pretreatment condition. For pretreatment alone, xylose yields for pellets were greater in the low-solids (56%; R_o=2.1) than the high-solids (31%; R_o=2.07) pretreatment, but no difference was present for the source material (38%).

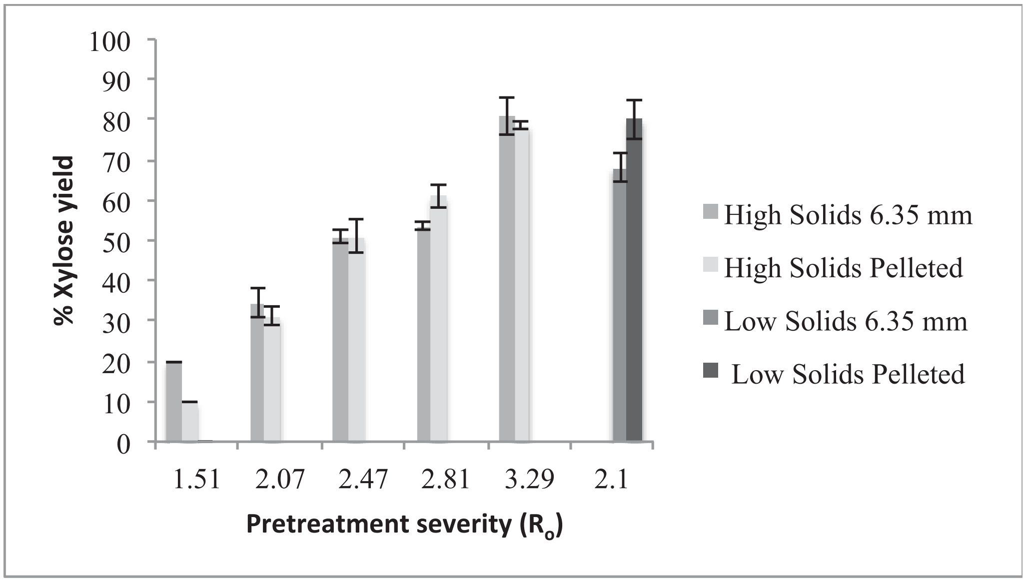

Figure 4. Xylose yield (monomeric and oligomeric) from high-solids pretreatment of pelleted and 6.35 mm ground stover using the ZipperClave® reactor (mean ± 1 SD; n=3).

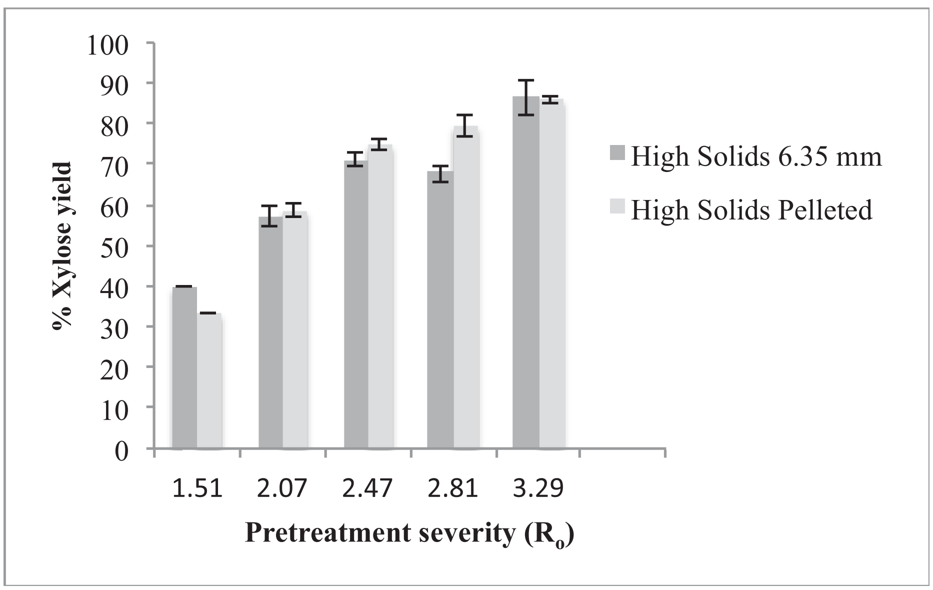

Figure 5. Xylose yield from combined high-solids pretreatment and enzymatic hydrolysis of the pelleted and 6.35 mm ground stover (mean ± 1 SD; n=3).

Furfural production from the dilute-acid pretreatment of the pelleted and source stover was low for all samples (Figure 6); concentrations ranged from 0.1 g/L to 4.63 g/L in the condensed flash vapors. The highest furfural concentrations translated to 1.17 g and 0.71 g furfural per 100 g of dry wt. Furfural production was higher for the 6.35-mm ground compared with the pelleted stover (Figure 6).

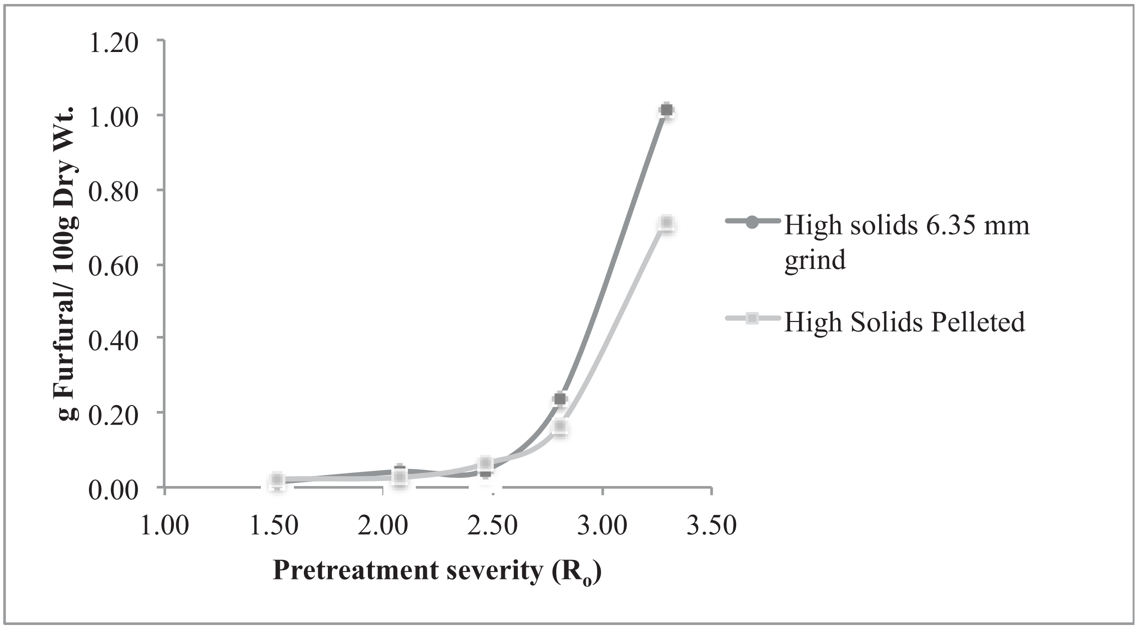

Figure 6. Furfural release from high-solids pretreated pelleted and 6.35-mm ground material (mean ± 1 SD; n=3).

Results for cellulose hydrolysis from combined pretreatment and enzymatic hydrolysis is shown in Figure 7. Glucose yield increased with increased pretreatment severity, although pretreatment contributed less than 10% to the final glucose yield, primarily monomeric glucose. The majority of glucose was released from enzymatic hydrolysis of the washed solids. Glucose yields were higher in the low-solids (58%, 83%; R_o=2.1) than the high-solids (49%, 61%; R_o=2.07) pretreatment for the pretreated source and pelleted materials, respectively. The highest glucose yield was achieved at R_o=3.29 in the high-solids pretreatment (103%, 98%) for source and pelleted materials, respectively. Glucose yields at this severity over estimated glucose release given that the accuracy decreases as 100% yield of glucan is approached. Enzymatic hydrolysis of the non-pretreated source and pelleted stover were 11.2% and 19.5%, respectively. Comparing glucose yield across feedstock format using two-way ANOVA, the only statistically significant ($p < 0.05$) differences occurred at R_o=2.07 with a ~12% increase in glucose yield for the pelleted versus the source stover. For all other severities, there was no difference between the two materials.

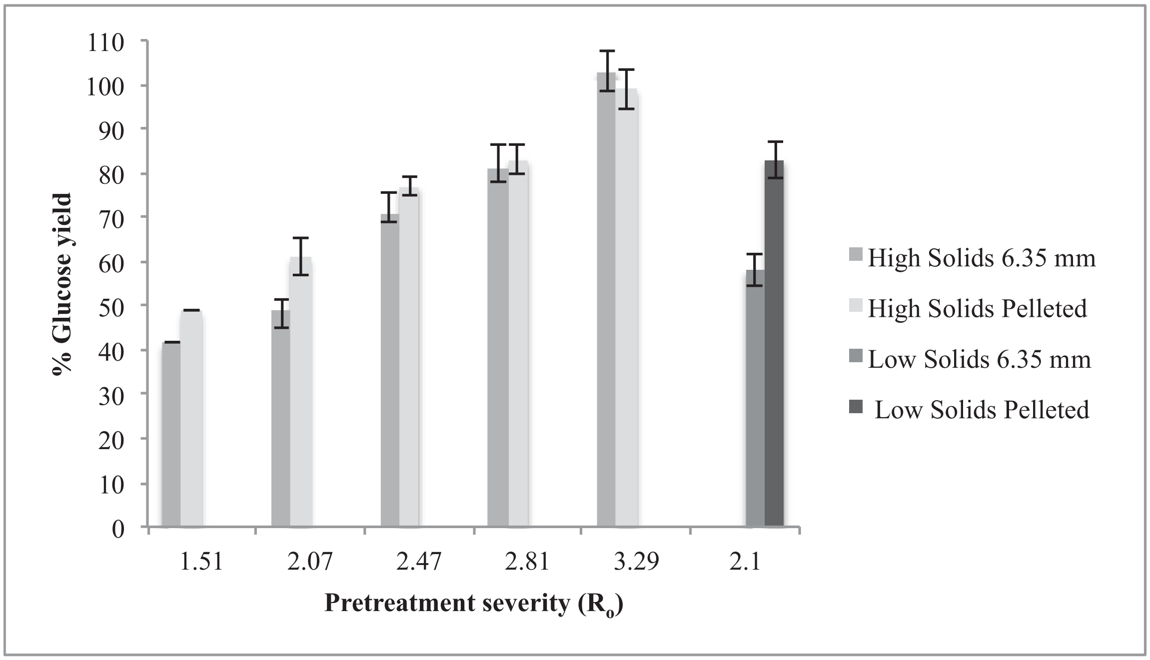

Figure 7. Glucose yield from combined high-solids pretreatment and enzymatic saccharification of pelleted and 6.35-mm source stover (mean ± 1 SD; n=3).

Feedstock reactivity is useful for comparing biomass performance across varying pretreatment severities and providing a measure of biomass recalcitrance to a given conversion process and set of conditions. Reactivity is defined as the fraction of the glucose and xylose released from pretreatment and enzymatic hydrolysis divided by the initial mass of glucose and xylose in the native feedstock. Figure 8 shows the response of the two-feedstock formats as a function of combined pretreatment and enzymatic hydrolysis. Results from a two-way ANOVA identified a small but significant increase from pelleting stover at R_o of 2.07, 2.47, and 2.81, with p-levels of 0.00095, 0.01893 and 0.0205, respectively. No difference in reactivity was observed at the lowest severity (Figure 8), and at the highest severity, pellets had slightly lower feedstock reactivity.

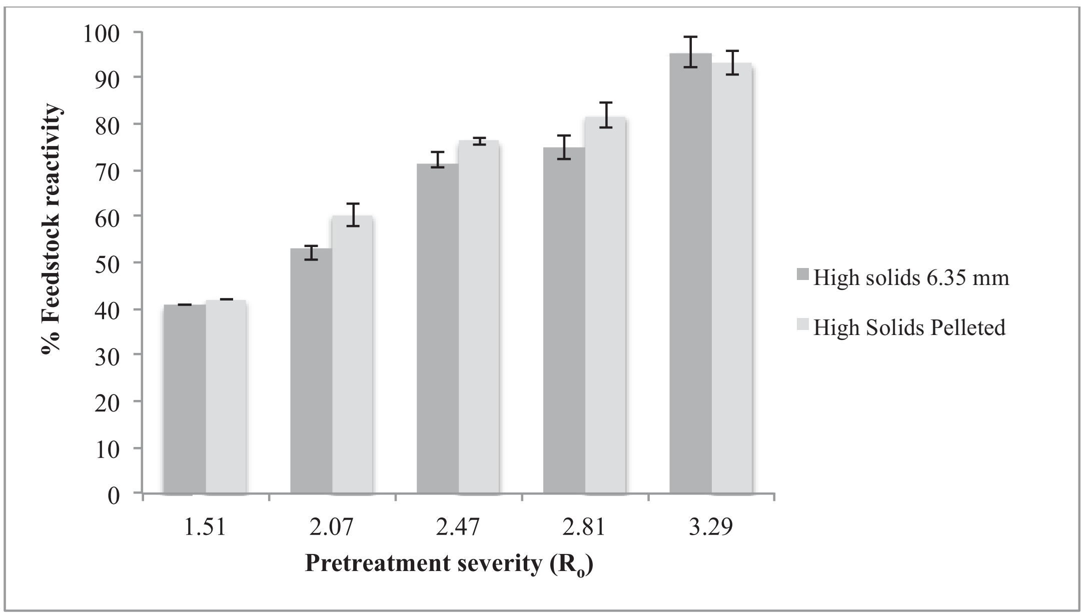

Figure 8. Feedstock reactivity for high-solids pretreated 6.35 mm grind and pelleted stover (mean ± 1 SD). Reactivity is defined as the fraction of the glucose and xylose released from pretreatment and enzymatic hydrolysis divided by the initial mass of glucose and xylose in the native feedstock.

Comparison between the low-solids and high-solids pretreatment at similar severity (R_o=2.1 vs. 2.07, respectively) and identical acid concentration showed different results. The pelleted stover at low-solids pretreatment showed a significant increase in both xylose and glucose yield compared with the high-solids pretreatment. While the severity was similar, different combinations of time and temperature can produce similar, if not identical, severity having different effects on sugar release. While 0.8% H_2SO_4 (w/w) was used for pretreatment in both the low- and high-solids pretreatment conditions, the increased solids loading (3.3% vs. 25% w/w) reduced the acid loading on a per mass basis (mg acid/g dry wt. of biomass) for the high-solids pretreatment; the mass of acid per mass of solids was significantly higher in the low-solids pretreatment. These factors would contribute to the yield differences between these two pretreatment regimes (see Figures 4 & 7).

Xylose and glucose yields obtained from both low-solids and high-solids pretreatment experiments suggest that pelletizing of corn stover did not increase biomass recalcitrance. Consistent with previous work [3, 13] increased sugar yields were observed in the pelleted stover, relative to the 6.35-mm source stover, from low-solids pretreatment and at a few conditions for under high-solids pretreatment and high solids-pretreatment followed by enzymatic hydrolysis. However no clear trend for glucose and xylose release was identified. Feedstock reactivity increased slightly for pelleted stover, relative to the 6.35-mm source material, for three of five pretreatment severities.

Scanning Election Microscopy

Scanning electron micrographs of native (a,b) and pelleted (c,d) corn stover are shown in Figure 9. These images reveal significant morphological differences between native corn stover source material and pellets; although, no specific structural or chemical alterations were identified with scanning election microscopy (SEM) that could be attributed to the improved sugar and ethanol yields that were measured during dilute-acid pretreatment and SSF (see Figures 1 and 3). A recent report examining the effect of pelleting on sugar yields in enzymatic hydrolysis of switchgrass attributed the increased yields to the grinding and heating of biomass prior to pelleting [13], although no specific mechanisms have been identified. Other factors that may have a role in the improved yields measured here include tissue shearing, mixing, and heating that occur during pelleting, which disturb biomass structure and may increase enzyme access to cellulose [3].

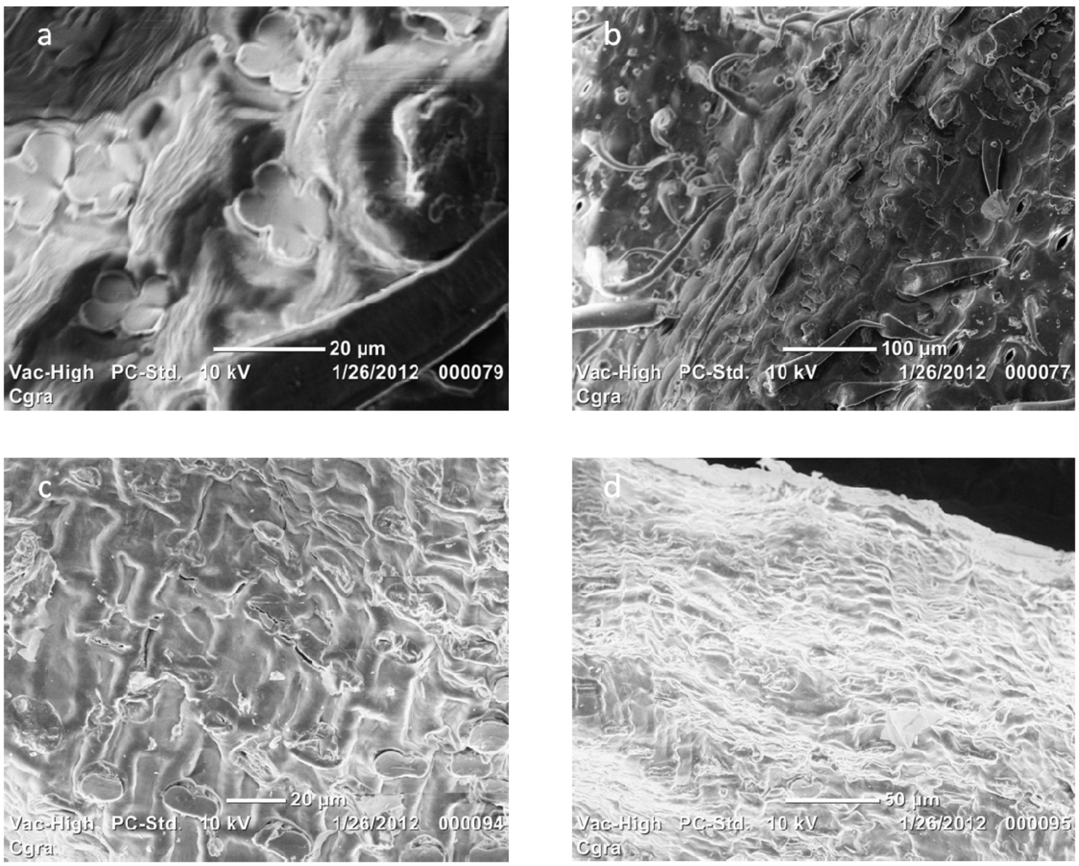

Figure 9. SEM images of (a) and (b) native corn stover; (c) and (d) pelleted corn stover.

Gas Adsorption Studies

Brunauer-Emmett-Teller (BET) theory of nitrogen adsorption was employed in order to elucidate differences in the surface area, pore volume, and pore diameter of the pelleted corn stover and the corresponding unpelleted source material. Surface area and pore volume of corn stover were lower following pelleting even if the material was subsequently ground to a 2-mm pellet meal (Bonnferroni $p < 0.01$; Table 3; Figure 10). Acid pretreatment of source material and pellets increased surface area and pore volume compared with untreated source material and pellets (Bonnferroni $p < 0.001$; Table 3; Figure 10); however, there was no difference between acid-pretreated source material and acid-pretreated pellets (Bonnferroni $p > 0.05$; Table 3; Figure 10). Average pore size increased when corn stover was pelleted (Figure 10) and was greatest following acid pretreatment of source corn stover and pellets (Bonnferroni $p < 0.01$), but there was no significant difference in pore size between acid-pretreated source material and acid-pretreated pellets (Bonnferroni $p > 0.05$). The increased pore size of the untreated pellet meal (3.59 nm) relative to the 6.35-mm source material (2.90 nm) may provide some basis for the improved TEY's achieved with pellet meal. The measured pore sizes for the pretreated pellets (4.77 nm) and pretreated source material (4.84 nm) are consistent with mesopore sizes required for cellulose accessibility by enzymes [19]. Mesopores larger than 4-5 nm were reported to be associated with the surface area available for enzymatic attack, because smaller pores hinder enzymatic access [41].

Some criticisms to the use of BET gas adsorption for measuring surface area and mesoporosity of biomass and pretreated materials have been reported. The sample preparation and oven-drying required for BET analysis may result in decreased surface area and pore volume due to collapse of pore structures during cell wall drying [19, 42], thereby reducing the overall nitrogen accessible porosity [41]. For some AFEX pretreatment conditions, BET analysis has been shown to underestimate surface area by 10-15 fold [43, 44]. Previous studies have demonstrated that increased specific surface area of pretreated biomass correlates with improved sugar release upon enzymatic hydrolysis [16]. Considering the drawbacks associated with gas adsorption techniques for measuring surface area and pore diameter, along with the demonstrated associations of increased surface area and pore diameter with enhanced sugar yields, it may be possible that this technique was not suitable for the examination of pelleted biomass performed here. Further work is needed to understand the relationship between surface area and pore diameter in pelleted biomass, enhanced xylan removal upon dilute-acid pretreatment, and improved ethanol yields in fermentation.

Table 3. Degrees of freedom, *F* values, and p values from a one-way analysis of variance for Brunauer-Emmett-Teller (BET) surface area, total pore volume, and average pore size.

Effect	df	*F*	p
BET surface area	4,10	99.477	5.21E-08
Total pore volume	4,10	280.12	3.23E-10
Average pore size	4,10	93.937	6.87E-08

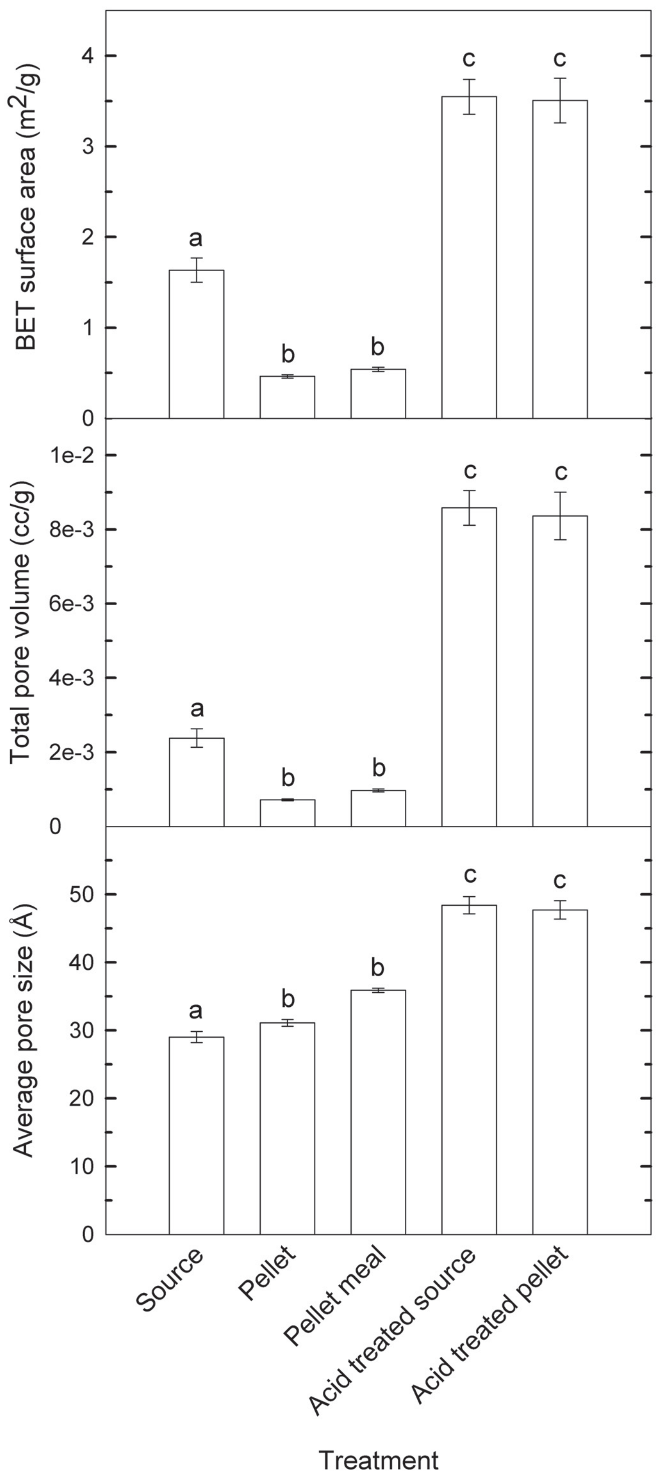

Figure 10. Brunauer-Emmett-Teller (BET) surface area, total pore volume, and average pore size (mean ± SE; n=3) for source material (6.35 mm), pellets, pellets ground to 2 mm, acid-treated

source material, and acid-treated pellets. There is a significant difference between treatments ($p \leq 0.05$) if the letters above the bars are different (Bonferroni post-hoc adjustments).

CONCLUSIONS

Pelleting corn stover had no adverse impact on pretreatment efficacy, as compared to unpelleted source material for both low-solids pretreatment and SSF and high-solids pretreatment and enzymatic hydrolysis. Corn stover pellet meal had significantly higher monomeric xylose yields upon dilute-acid pretreatment, and total xylose yields were similar for pellet meal, 6.35-mm, and 2-mm ground material. Solubilized glucan during low-solids pretreatment was similar for all formats examined and consistent with findings from the literature. Pellet meal achieved the highest ethanol yields of the formats tested here. Micrographs from SEM revealed obvious morphological differences among pelleted and unpelleted corn stover. Results from BET gas adsorption studies demonstrated that, although corn stover pellets and pellet meal initially had significantly larger pore diameter than the source material, these differences were not apparent following dilute-acid pretreatment. The combined results suggest that pelleting corn stover does not have a negative impact on pretreatment efficacy. The improved monomeric xylose yields achieved with pellet meal under the low-solids pretreatment condition indicates the potential for a reduction in required pretreatment severity for pelleted biomass; however, further studies will be required in order to elucidate whether the improved monomeric xylose yields are scalable to higher solids loading.

The conditions used for the high-solids pretreatment were representative of process-relevant conditions. Incorporation of mixing, increased severity, and higher solids and acid loading for larger-scale pretreatment systems resulted in a slight increase in overall sugar yields, as seen in 3 of the 5 pretreatment severities. Future work includes pretreatment of pelleted and non-pelleted feedstocks under continuous conditions to further assess the impact of densification on biomass recalcitrance. The enhanced ethanol yield upon fermentation and slightly increased sugar yield upon enzymatic hydrolysis highlight the potential for pelleted biomass to play an important role in the production of biomass-derived fuels beyond the densification-related benefits for storage, handling, and transportation.

FUTURE PERSPECTIVE

Densification will play a critical role in the development of the cellulosic bioenergy industry through its impact on transportation, handling, and long-term storage of biomass feedstock materials. These systems will be highly location dependent with significant variability in feedstock type, the quantity of available feedstock, the timeframe for harvesting and collecting the feedstock, weather considerations relating to storage options, and the infrastructure restrictions that govern the quantities of biomass that can be transported on the roadways [9]. The monumental task of displacing 30% of the 2004 gasoline use with biofuels by 2030 (60 BGY) will require more than 700 million dry matter tons of lignocellulosic biomass materials from a diverse variety of resources. Densification provides a uniform size, density, and moisture content that will facilitate transport using conventional logistical systems, including the nation's commodity-scale grain handling systems and storage structures. The increased bulk density of densified materials will allow highly efficient large capacity transport of commodity-formatted materials. The improved long-term storage characteristic of densified materials is of greatest importance. Bales of biomass material and feedstock resources will be more resilient to unpredictable environmental conditions. This will also minimize the variability of the commodity-scale feedstock resource, promote greater quality assurance of feedstock materials, and provide the opportunity to import materials from large supply areas.

EXECUTIVE SUMMARY

Results & discussion

- Pelleting corn stover did not have a negative impact on the efficacy of dilute-acid pretreatment. In this study, pellet meal produced significantly higher monomeric xylose yields during pretreatment than conventionally ground source material. The improved monomeric xylose yields achieved with pellet meal may offer potential for a reduction in pretreatment severity for pelleted biomass.
- The highest ethanol yields were achieved with pelleted corn stover during simultaneous saccharification and fermentation, which may suggest that the pelleting process increases enzymatic access to cellulose.
- Micrographs from SEM revealed obvious morphological differences among pelleted and unpelleted corn stover.
- Results from gas adsorption studies demonstrated that, although corn stover pellets and pellet meal initially had significantly larger pore diameter than the source material, these differences were not apparent following dilute-acid pretreatment.
- Both pretreatment regimes, low and high solids, demonstrated that biomass recalcitrance did not increase as a result of pelleting. In addition, examination of several pretreatment severities at high-solids loading revealed that overall sugar release was higher in the pelleted versus 6.35-mm ground material; however, with increased severity, the two materials showed little difference in overall sugar release.

Conclusions

- These results suggest that pelleting corn stover does not have a negative impact on pretreatment efficacy, and the improved monomeric xylose yields achieved with pellet meal indicate the potential for a reduction in required pretreatment severity for pelleted biomass. The enhanced ethanol yields upon fermentation demonstrate that pelleted biomass holds promise as an important feedstock for the production of ethanol fuels beyond the densification-related benefits for storage, handling, and transportation.
- The conditions used for the high-solids pretreatment represent some process-relevant conditions for larger-scale pretreatment systems, suggesting that these results may scale to larger continuous pretreatment regimes.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

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