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The Confounding Effects of Particle Size and Substrate Bulk Density on *Phanerochaete chrysosporium* Pretreatment of *Panicum virgatum*

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Phanerochaete chrysosporium treatment is less effective as a biological pretreatment on feedstock with larger particle sizes. We hypothesized that the improved effectiveness of the pretreatment when smaller particle sizes are used may be due to the inherently higher bulk density with smaller particle sizes. The effects of substrate bulk density and particle size on the efficacy of *P. chrysosporium* pretreatment of switchgrass (*Panicum virgatum*) was tested experimentally. *Phanerochaete chrysosporium* was grown on senesced switchgrass (2 different particle sizes) with various bulk densities. In all treatments, the fungal-pretreated samples released more glucose during enzymatic saccharification than the control sample. Substrate bulk density was a statistically significant factor in explaining the variation in the amount of glucose released per gram of substrate used. However, the particle size was not found to be a significant factor. On-farm switchgrass pretreatment may not require particle size reduction if the switchgrass is supplied in high-density bales.

INTRODUCTION

Current practice for the biochemical conversion of lignocellulosic biomass into fuel includes some form of pretreatment (chemical, thermo-chemical, or biological) to deconstruct the lignin components and to allow a saccharifying agent (enzymes or acid) access to the polysaccharides. Biological pretreatment is attractive because it is generally thought to be more environmentally benign than other pretreatments, given the mild reaction conditions needed, yet still results in reasonable product yields (Moreno *et al.* 2014; Pinto *et al.* 2012). In addition, the biological pretreatment has few side reactions, decreased energy demand, reduced reactor requirements (temperature/pressure), and does not form compounds that hinder downstream reactions (Moreno *et al.* 2014). Biological pretreatment involves growing white rot, brown rot, or soft rot fungi on the lignocellulosic substrate (Shirkavand *et al.* 2016) in a high-solids environment and allowing the fungal lignolytic enzyme system to deconstruct the lignin (Couto and Sanroman 2005). The literature contains mixed conclusions about the effectiveness of biological pretreatment. However, care must be taken to differentiate between the studies' factors because the fungus used; substrate type and growth stage; length of pretreatment; substrate moisture

content; temperature; and substrate preparation method significantly affect the success of the pretreatment (Moreno *et al.* 2015).

Phanerochaete chrysosporium is the most studied white-rot fungus used for biological pretreatment and is attractive because of its effectiveness as a pretreatment in as little as seven days (switchgrass) (Mahalazmi *et al.* 2010) or 14 d (cotton stalks) (Shi *et al.* 2009). The shorter incubation time could compensate for a decrease in pretreatment effectiveness compared with other species of white-rot fungi. To date, two studies have quantified the effects of *P. chrysosporium* pretreatment on switchgrass. Air-dried, sterilized senesced switchgrass (2 mm, 66% initial water content wet basis (w.b.) was pretreated with *P. chrysosporium* for 30 d at 30 °C, and subsequent glucan digestion was approximately 12% of the theoretically estimated amount (Liu *et al.* 2015). Sterilized air-dried, green switchgrass (3 mm, 80% initial water content (w.b.)) was pretreated with *P. chrysosporium* for 7 d at 37 °C, and subsequent glucan digestion yielded approximately 5.5% of the theoretical yield (control with no *P. chrysosporium* yielded 8% of the theoretical yield) (Mahalazmi *et al.* 2010).

The lignocellulolytic enzymes produced by *P. chrysosporium* are excreted at the hyphae tip of the fungus. Fungal hyphae grow through a combination of extension and generation of new hyphae through branching. The hyphae contain a solid mycelium tip that can more easily penetrate the solid substrate (Raimbault 1998). Studies have shown that *P. chrysosporium* produces three major classes of lignolytic enzymes including laccases (E.C. 1.10.3.2), manganese peroxidases (MnP) (E.C. 1.11.1.13), and lignin peroxidases (LiP) (E.C. 1.11.1.14)) (Plácido and Capareda 2015).

The majority of biological pretreatment studies have been conducted on ground lignocelluloses, typically with particle sizes between 1 mm and 2 cm (Wan and Li 2010, 2011; Liong *et al.* 2012; Ray *et al.* 2012; Liu *et al.* 2015). Decreases in particle size have been shown to correlate with increases in glucose and xylose amounts released upon saccharification when 5-, 10-, and 15-mm corn stover particles were pretreated with *Ceriporiopsis subvermispora* for 18 d at 28 °C and 75% moisture content (Wan and Li 2010) and enzymatically hydrolyzed. Glucose yields were found to be 58%, 54%, and 46%, respectively, of theoretical yield (Wan and Li 2011). Statistical analysis showed that there was no significant difference between the glucose released from the 5-mm and 10-mm corn stover. However, there was a significant decrease in glucose yield when the particle size increased from 10 to 15 mm (Wan and Li 2010). It is tempting to draw the conclusion that the decrease in glucose yield was caused by the larger particle size used. However, because the fungus grows on the lignocellulose *via* hyphal contact with the biomass, we hypothesize that the improved effectiveness of the pretreatment with smaller particle sizes may be due to the higher bulk density inherent with smaller corn stover and switchgrass particle sizes (Mani *et al.* 2006), and not strictly due to the particle size. Bulk density is determined both by particle density and porosity, and smaller particles of switchgrass and corn stover pack in a manner which reduces the porosity, thereby increasing the bulk density (Mani et al. 2006). The higher bulk density would allow the fungus hyphal tips easier access to new biomass, allowing more fungal growth. Ligninases are growth associated, therefore increased fungal growth results in increased enzyme production for lignin degradation. If this hypothesis is true, it implies that biomass would not need to be ground to be effectively pretreated, as long as the bulk density of the larger particle size material was sufficiently high.

The objective of this study was to quantify the relative magnitude of the substrate bulk density *versus* particle size and its impact on the effectiveness of biological

pretreatment of switchgrass using *P. chrysosporium*. Four bulk densities (32, 80, 120, and 180 kg/m^3) and two particles sizes (≤ 0.5 cm and 10.16 cm) were tested in a modified full factorial experiment.

EXPERIMENTAL

Inoculum Preparation

Phanerochaete chrysosporium (parent strain: ATCC 24725) culture was stored in a freezer at -40 °C and reconstituted by culturing on potato-dextrose agar (PDA) plates at 35 °C for 7 d. The PDA plugs were transferred to the center of multiple PDA plates containing eight sterilized equally spaced 1 cm by 1 cm No. 2 filter paper squares. The new plates were incubated for an additional 7 d under the same conditions. A uniform mass of fungal conidia was present on all filter paper units. The filter paper squares were used to inoculate all treatments.

Biomass Preparation

To prepare treatments of sample sizes ≤ 0.5 cm, air-dried switchgrass samples with moisture contents of 7% to 9% (w.b.) were harvested from the University of Kentucky's Spindletop farm in January 2014. The samples were ground to ≤ 0.5 cm size using a Thomas Wiley® rotary mini-mill (Arthur H. Thomas Company, USA). The 10.16-cm pieces were cut using a table saw. The prepared switchgrass samples were stored in zip-lock bags under ambient laboratory conditions.

Bulk density was calculated for each treatment using the radius of the container, height of switchgrass biomass in the container, and weight (g) of switchgrass used. Substrate bulk density for the 10.16 cm particle size treatments (adjusted to 120 kg/m^3) was increased using weight to compress the substrate to the desired height. The weight was placed on a platform that rested on four legs, which held down a mesh screen (McMaster Part # 85385T62; 304 stainless steel, 15x15 mesh size, 73% open area, 0.01" wire size, opening size 0.057"), as shown in Fig. 1, to compress the biomass yet allow for sufficient oxygen exchange.
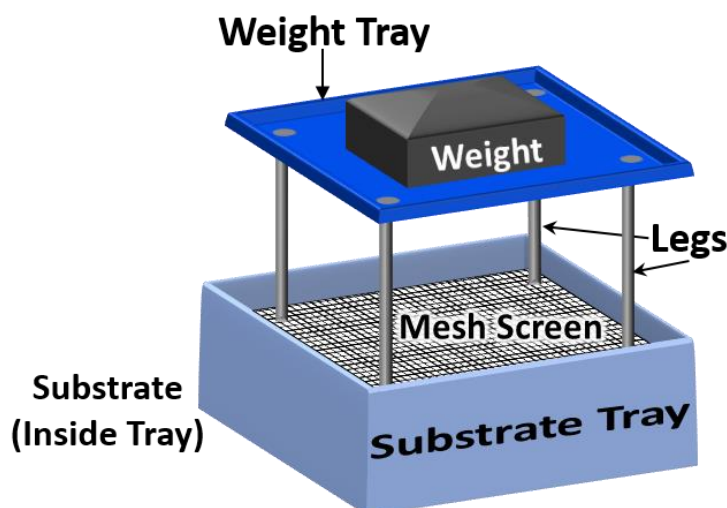

Fig. 1. Schematic details of the apparatus used for obtaining higher substrate bulk density with loose feedstock

Substrate bulk density was lowered by incorporating inert glass beads (1 mm diameter) into the ≤ 0.5 cm particle size switchgrass to achieve the desired bulk density. Glass beads were chosen because the fungal culture cannot use the glass beads as a source of carbon; their sole purpose was to increase the space (and therefore decrease the bulk density) between the ground switchgrass pieces. No fungal growth was observed on the glass beads. To achieve the correct substrate height (in 400 mL (3.66 cm radius) glass cylinders) required for the 32 kg/m^3 tests, 360 g of glass beads was added to 10 g of switchgrass and mixed thoroughly with a stir rod. The 80 kg/m^3 tests required 100 g of glass beads to be added to 10 g of switchgrass and mixed thoroughly. The 120 kg/m^3 tests did not require glass beads, as the substrate itself was already at the desired density after moisture content adjustment.

The desired bulk density in the miniature bales (10.16 cm switchgrass) was achieved by weighing the switchgrass (126, 189, and 280 g) needed, then compressing this weight into 10.16 cm x10.16 cm x 15.24 cm (4"x4"x6") bales using a Dake Arbor Press (Grand Haven, MI). Once compressed to the desired thickness, the bales were fastened with wire.

Moisture contents were determined with Ohaus MB35 Halogen Moisture Analyzer (Ohaus Company, Switzerland) and verified gravimetrically. The amount of distilled water required to bring each treatment (10 g dry matter (d.m.) of loose substrate up to 75% moisture content (w.b.) was calculated (Shi *et al*. 2008). Treatments were then autoclaved for 60 min at 121 °C at 15 Pa. An overview of the treatments with the particle sizes and density combinations is shown in Table 1. The method by which each pretreatment density was achieved is also shown in the table for clarification.

The miniature bales were autoclaved before adjusting the moisture content. The autoclaved bales were soaked in deionized water (DI) water for 10 min and then allowed to gravity drain. The bales were then inoculated with *P. chrysosporium* and each placed into separate containers in controlled temperature and relative humidity chambers. Control treatments were prepared identically (in triplicate) but were not inoculated with *P. chrysosporium*. All treatments and controls were placed in a 35 °C environment for 15 d (Bak *et al.* 2009; Shi *et al.* 2009).

Table 1. Experimental Treatments (Particle Size x Bulk Density)

Bulk Density	Particle Size	
	≤ 0.5 cm	10.16 cm
32 kg/m^3	X$_{Glass\ beads}$	
80 kg/m^3	X$_{Glass\ beads}$	X$_{bale}$
120 kg/m^3	X$_{None}$	X$_{weight}$; X$_{bale}$
180 kg/m^3		X$_{bale}$

Tested treatments are represented by the table cells containing an X. The method by which the bulk densities were achieved is also given in the table cell as a subscript to the X. All substrates were senesced switchgrass samples that had been air dried in the field. Each treatment was replicated three times.

Saccharification

Upon completion of pretreatment, saccharification was performed using a modified procedure from the National Renewable Energy Laboratory (NREL) standard biomass analytical procedures for enzymatic saccharification of lignocellulosic biomass,

NREL/TP-510-42629 (Selig *et al.* 2008). The standard protocol requires the substrate to be ground to a uniform particle size; however, in this study, the samples were hydrolyzed without further particle size reduction (*i.e.*, ≤ 0.5 cm and 10.16 cm). Commercial cellulase (activity 3.00 FPU/mg protein) and β-glucosidase (activity 9.75 IU/mg protein) (American Labs Inc., Omaha, Nebraska) obtained from *Trichoderma longibrachiatum* were applied (60 FPU/g cellulose and 195 pNPGU/g cellulose, respectively) to the biomass. Glucose concentrations were quantified using a YSI analyzer (YSI 2900D; YSI, Inc.; Yellow Springs, Ohio). The glucose samples were randomized, and calibration standards and duplicates were interspersed within the samples to monitor for any shift in instrument precision. Sodium azide (0.10 mL of 20 mg/mL) was used as an antimicrobial agent during saccharification. Theoretical glucose yields were calculated as shown in Eq. 1. The ratio of cellulose to dry biomass was calculated using compositional data which quantified the switchgrass as 37.5% cellulose.

$$Percent\ Theoretical\ Glucose = \frac{Measured\ Glucose\ (g/L)}{\frac{Dry\ Biomass\ (g)}{Volume\ (L)}*(ratio\ of\ cellulose\ to\ dry\ biomass)*\frac{180\frac{g}{mole}}{162\frac{g}{mole}}} * 100 \qquad (1)$$

Statistical Analysis

An analysis of variance was performed in SAS (version 9.3) to test for significant treatment effects due to; pretreatment (with and without *P. chrysosporium*), density (32, 80, 120, and 180 kg/m^3), and particle size (≤0.5 cm and 10.14 cm). The response variable tested was glucose concentration after enzymatic hydrolysis.

If the pretreatment effect was statistically significant, an additional analysis of variance was performed using data from only the treatments that were pretreated, to test for significant treatment effects due to density (32, 80, 120, and 180 kg/m^3), and particle size (≤0.5 cm and 10.14 cm).

RESULTS AND DISCUSSION

Unmodified Bulk Densities

The bulk densities of air-dried switchgrass samples as a function of their particle sizes were 113 and 41 kg/m^3 for the ≤ 0.5 cm and 10.16 cm particle sizes, respectively. When prepared to 75% water content (w.b.), the bulk densities were 120 and 67 kg/m^3, respectively. The unmodified bulk densities of the two particle sizes are statistically different, with the 10.16 cm pieces having a bulk density approximately 40% greater than that of the ≤0.5 cm pieces of switchgrass.

Fungal Pretreatment

The results of biological pretreatment using *Phanerochaete chrysosporium* on the miniature bales are shown in Fig. 2. Dense white growth and a bleaching effect were observed. Substrate bleaching studies have displayed a good relationship between lignin degradation and substrate brightening (Kondo *et al.* 1994). A positive correlation was shown between MnP activity and brightening of the substrate during pretreatment with white-rot fungi (*Phanerochaete chrysosporium*, *Phanerochaete sordida*, and *Coriolus versicolor*) of kraft pulp (Kondo *et al.* 1994).
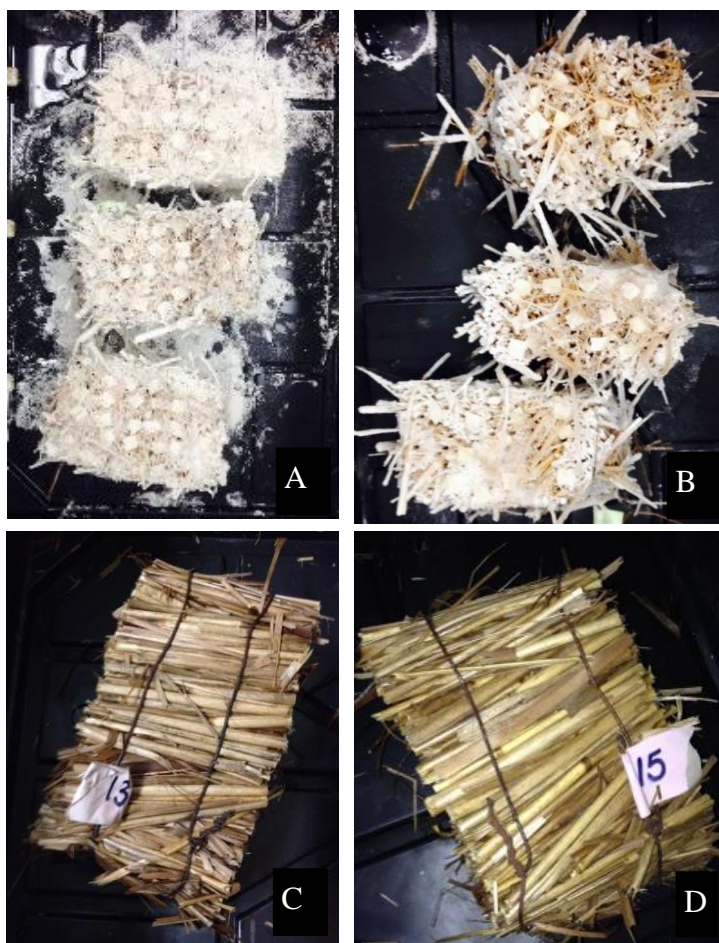
Fig. 2. *P. chrysosporium* growth on miniature bales after 14 d (A & B) and control references respectively (C & D) after 14 d: A) 180 kg/m^3 pretreated bales; B) 80 kg/m^3 pretreated bales; C) 180 kg/m^3 control bale; and D) 80 kg/m^3 control bale

When hydrolyzed, the glucose yields from the *P. chrysosporium*-pretreated biomass were statistically higher than the glucose yields released from the control sample (with no *P. chrysosporium*) with a probability of a greater F-value less than 0.0001 (Pr > F < 0.0001). The average glucose released from the pretreated samples, averaged over all particle sizes and densities, was 0.05 g of glucose per gram bone-dry switchgrass (11% of theoretical), and the un-pretreated average was 0.02 g of glucose per gram bone-dry switchgrass (5% of theoretical). The analysis of variance also revealed that there was no evidence of a difference between the tests run in flasks (loose substrate) and the tests run in bales (data not shown). Table 2 presents the full analysis of variance, including the main effects of fungus, density, and particle size, and their respective interactions. The model was significant, as were fungus (present or not), density, and particle size main effects. However, none of the interactions were significant.

Table 2. Analysis of Variance for Switchgrass

Source	DF[*]	Sum of Squares	Mean Square	F value[*]	Pr>F[*]
Model	10	0.0108	0.0011	6.42	<0.0001
Error	27	0.0045	0.0002		
Corrected Total	37	0.0153			
Source	**DF**	**Pr>F**			
Fungus[a] (Y/N)	1	<0.0001			
Density[b]	3	0.01			
Particle Size[c]	1	0.05			
Fungus*Density	3	0.43			
Fungus*Particle	1	0.70			
Density*Particle	1	0.14			
R^2=0.70	RMSE=0.013	Mean = 0.04 g/g			

a) pretreatment (with *P. chrysosporium* or without *P. chrysosporium*) b) at four densities (32, 80, 120, and 180 kg/m^3) and c) at two particle sizes (≤ 0.5 cm and 10.14 cm).
*DF referring to the degrees of freedom, F value is by definition the treatment mean square as a multiple of the error mean square, (Steel and Torrie 1980), and Pr quantifies the probability (in decimal format) of obtaining a greater F value, essentially representing the area under the normal curve from the F value to 100% certainty.

A subsequent analysis of variance was conducted using only the pretreated samples to more accurately compare the overall effects of density and particle size on *P. chrysosporium* pretreatment. The results are presented in Table 3. There was significant evidence that density impacts the pretreatment effectiveness (Pr > F = 0.006). The density means are plotted in Fig. 3. The effects of particle size however were not found to be statistically significant (Pr > F = 0.127). Therefore, there was insufficient evidence to conclude that the particle size affected pretreatment effectiveness after accounting for the effects of bulk density differences.
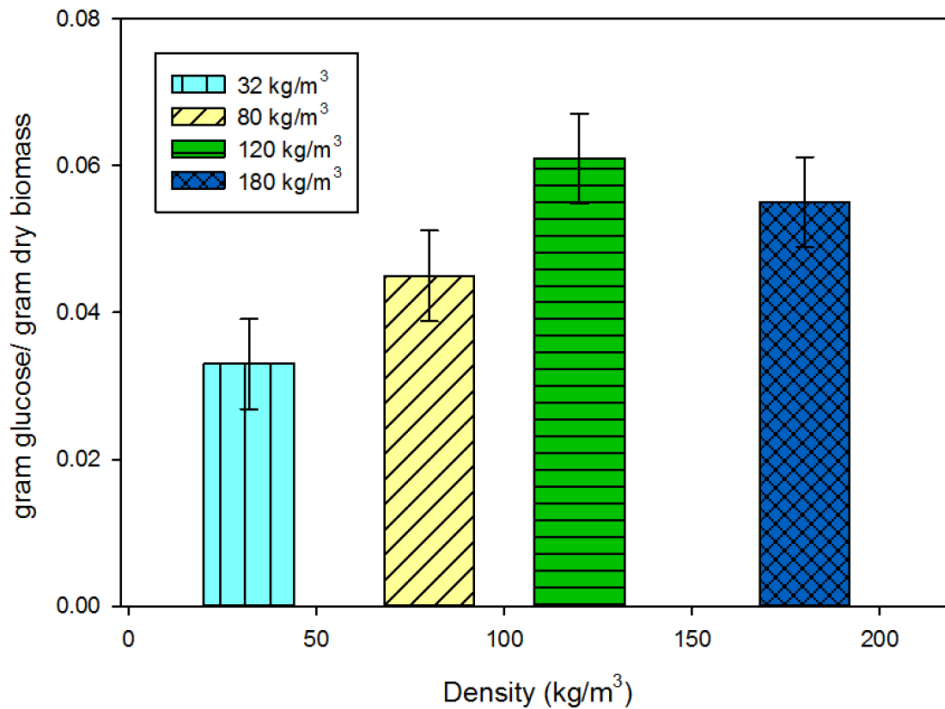
Fig. 3. Average glucose yield released per gram of bone-dry switchgrass, averaged over particle size (≤ 0.5 and 10.16 cm) for the four bulk densities tested (32, 80, 120, and 180 kg/m³). Error bars represent the root mean square error (RMSE = .0123 g glucose per gram of dry biomass).

Table 3. Analysis of Variance for Switchgrass Samples Pretreated with *P. chrysosporium* for 14 days

Source	DF	Sum of Squares	Mean Square	F value	Pr>F
Model	4	0.003	0.00074	4.89	0.007
Error	19	0.003	0.00002		
Corrected Total	23	0.006			
Source	**DF**	**Pr>F**			
Density[a]	3	0.006			
Particle Size[b]	1	0.127			
R^2=0.51	RMSE=0.0123	Mean = 0.05 g/g			

a) At four densities (32, 80, 120, and 180 kg/m³) and b) at two particle sizes (≤ 0.5 cm and 10.14 cm)

Using Tukey's least significant difference test (in which a critical value was calculated based on the variances between replications, and applied to all pair comparisons of means to determine statistical significance (Steel and Torrie 1980)), there was no evidence that the density treatment of 120 kg/m³ differed from the 180 kg/m³ treatment (120 kg/m³ = 180 kg/m³); however, there was significant evidence that the glucose released from the 120 kg/m³ pretreatment was greater than that released from either the 80 or 32 kg/m³ pretreatment. There was no evidence that 32 or 80 kg/m³ pretreatments differed from each other (120 kg/m³ > 80 = 32 kg/m³). This suggests that our hypothesis is correct: higher feedstock densities (120 or 180 kg/m³) result in better pretreatment, regardless of

particle size, than lower bulk densities (32 or 80 kg/m^3). The higher bulk density likely allows the fungal hyphal tips easier access to new biomass, allowing more fungal growth. Ligninases are growth associated, therefore increased fungal growth results in increased enzyme production for lignin degradation.

To our knowledge, 10.16 cm particle sizes have not been tested in biological pretreatment prior to this study (Wan and Li 2010, 2011; Liong *et al.* 2012; Ray *et al.* 2012; Liu *et al.* 2015). We selected this particle size because this represents a lower limit in particle size practical for large-scale baling. This research suggests that this larger particle size is a viable option for lignocellulose pretreatment, and further testing should be conducted on larger pieces such as those typically found in bales.

Higher-density square bales transport more material in a smaller amount of space; therefore, a higher-density bale is a more economical way to transport switchgrass for processing. Typically, large square bales of switchgrass have densities of 125 to 145 kg/m^3 (Montross (2015), data not shown); however, reed canary grass bales have been reported to be between 130 to 180 kg/m^3 (Lötjönen and Paappanen 2013). Dried senesced switchgrass has a loose density of approximately 70 kg/m^3. Leaving the substrate in bale form would decrease the energy input used in preparing the substrate for conversion. Size reduction is an energy intensive process and could consume up to one-third of the energy input in the entire biofuel conversion process (Bitra *et al.* 2009). Bitra *et al.* (2009) tested the total specific energy input calculations to grind switchgrass as a function of screen size, mass feed rate, and rotor speed. Increasing grinding speed from 250 to 500 rpm increased energy inputs 33% for all screen sizes tested. Total specific energy decreased by 20% and effective specific energy consumption decreased by 55% with an increase in screen size from 12.7 to 50.8 mm for switchgrass. As screen size increased, specific energy decreased; therefore, larger particle sizes resulted in less energy input for the process. Optimizing the system resulted in a knife mill screen size of 25.40 mm, rotor speed of 250 rpm, feed rate of 7.6 kg/min, and corresponding total specific energy of 7.57 MJ/mg for switchgrass (Bitra *et al.* 2009).

Avoiding grinding altogether could save energy, costs, and processing time. Previous studies have focused on particle size as a mandatory initial means of pretreatment for optimal glucose yields (Richard 2010). However, this study suggests that densifying the feedstock and pretreating biologically may be as effective as reducing particle size prior to pretreatment. Substrate densification would also improve the transportation logistics from where the crop is harvested and baled to where it will be processed and also decrease the energy input that would have gone to grinding the substrate and subsequent handling of the ground biomass.

CONCLUSIONS

1. Higher bulk density during biological pretreatment resulted in a higher glucose yields (120 kg/m^3 > 32 and 80 kg/m^3) upon saccharification.

2. There was no evidence that substrate particle size affected the efficacy of biological pretreatment once the variation from the bulk density effects was removed.

3. It was as effective to leave the substrate baled (at ≥ 120 kg/m^3) as to grind the substrate prior to biological pretreatment.

4. Biological pretreatment of high-density bales has the potential to save energy and labor.

5. Further studies are needed to determine how to most effectively hydrolyze biologically-pretreated bales.

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