Effect of ultrasonication of Switchgrass on fermentable sugar production and biomass physical structure

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Abstract: Pretreatment of lignocellulosic biomass for enhancing sugar yields has been studied extensively over the years. Conventional methods employed to preprocess biomass to make it conducive for enzymatic hydrolysis have been hampered by issues like poor energy efficiency and production of undesirable by-products. Ultrasonication, which involves the treatment of biomass through ultrasonic sound waves in a liquid medium without additional chemicals, is believed to have potential for biomass pretreatment. In this study the effects of ultrasonication on switchgrass, a potential feedstock for bioethanol production due to its high cellulosic content, were investigated. Results of compositional analysis and scanning electron microscopy conducted to visualize structural disintegration in sonicated samples were used to select pretreated samples for enzymatic hydrolysis at different enzyme loadings. Temperature controlled ultrasonication for 60 min at 100% amplitude in a stainless steel vessel, resulted in the highest carbohydrate conversions of 84.6% and 84.7% with Cellic® CTec2 and Alternafuel 200 L at 0.3 g enzyme protein g^{-1} dry biomass, respectively. However ultrasonication did not significantly impact composition of switchgrass and subsequent hydrolysis of untreated and treated samples was not significantly different. Based on the overall results, it is inferred that although ultrasonication alone did not provide enhanced sugar generation from switchgrass, its tendency to disrupt biomass structure could be utilized for preliminary size reduction steps and performance could be improved by further investigation of frequencies, amplitudes, enzyme loadings and process operation parameters.

Keywords: *Panicum virgatum*, amplitude, pretreatment, hydrolysis, scanning electron microscopy

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1 Introduction

The choice of feedstock for biofuel production has been central to the controversy surrounding biomass conversion today. Current technologies associated with the use of food as fuel and large-scale changes in land use have raised numerous concerns regarding feasibility and sustainability. For biofuels to have any meaningful impact on energy, biomass feedstock must be widely available at low cost and without negative environmental impact. Lignocelluloses - the non-food component of plants fit this description (Mousdale, 2008). Switchgrass is a potential lignocellulosic feedstock with high renewability and sugar content (Keshwani and Cheng, 2009). It however presents a need for pretreatment to break down the lignin and to disrupt the crystalline structure of cellulose, so that enzymes can easily access and hydrolyze it (Cadoche and Lopez, 1989; Kumar et al., 2009).

Physical pretreatments such as mechanical grinding, pyrolysis, steam explosion, and ammonia fiber explosion can be effective in mechanical disruption of the cell wall and lignin bonds but have proven to be energy intensive (Kersten and Garcia-Perez, 2013; Galbe and Zacchi, 2007; Kilzer and Broido, 1965). Chemical pretreatment agents such as acid and alkali dissolve, hydrolyze or oxidize the lignin bonds to expose the carbohydrates but may hamper

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subsequent enzyme hydrolysis by undesirable inhibitor production and require extensive washing and hence have proven to be cost ineffective (Sun and Cheng, 2002; Quesada et al., 1999).

Ultrasonication is a physical technique that uses sound waves at ultrasonic frequencies (typically > 20 kHz) to alter the molecular structure of biomass (Rehman et al., 2013). These sound waves travel in a viscous flow pattern or steady streaming in liquid medium and create pressure vibrations, which impact treatment intensity (Riley, 2001). The intensity of these waves is also dependent on temperature of the medium, and it has been observed that increase in temperature decreases intensity of sonication (Feng et al., 2011). Ultrasonication has been applied in biological processes for disruption of cell membranes and release of cellular enzymes in a process known as sonoporation. The acoustics from an ultrasound irradiated system in liquids have been shown to effect particles in the range of 0.15 to 100 mm (Suslick et al., 1986; Flint and Suslick, 1991; Suslick and Kemper, 1994). The acoustic phenomenon results in non-linear or pulsating movement of bubbles thus causing turbulence (Crum, 1985; Rehman et al., 2013). In conjunction, cavitation which is defined as the growth and implosive collapse of bubbles in a liquid irradiated by ultrasound, causes a heating effect. The implosive cavity hot spots created by the collapsing bubbles have temperatures of roughly 5300 K (5027°C), pressures of about 1720 bar, and heating and cooling rates above 10^{10} Ks^{-1} (9.9^{10} $°Cs^{-1}$) (Suslick et al., 1986; Flint and Suslick, 1991).

Sun et al. (2004) investigated the extractability of hemicelluloses from bagasse by ultrasound-assisted extraction and found that ultrasonic treatment and sequential extractions with alkali and alkaline peroxide led to release of over 90% of the original hemicelluloses and lignin. The hemicellulosic fractions obtained after ultrasonic extraction contained relatively low amounts of associated lignins (0.41% to 7.36%). While working on developing cellulose fiber for use in composites, Zhang et al. (2007) showed that cellulose nano fibers could be extracted from lignocellulose by the application of high intensity ultrasonication to produce small fibrils at nano and micro scales. Ultrasonic hydrodynamic forces produce very strong oscillating mechanical power, which may lead to the separation of cellulose microfibrils. This indicates that ultrasonic waves impact the complex lignocellulosic matrix and there is potential for more refined work in the area (Zhang et al., 2007). An added benefit of ultrasonication is the elimination of a toxic waste stream typically generated by conventional chemical methods. Hence this study was undertaken, as proof-of-concept, to determine the impact of reaction vessel construction material (glass or steel), treatment time, amplitude and temperature control during ultrasonication of switchgrass on its lignin content and sugar generation potential. The structural changes in switchgrass subjected to ultrasonication were examined by scanning electron microscopy (SEM).

2 Materials and method

2.1 Biomass preparation

Alamo switchgrass was obtained through an intercropping sustainability study performed by Weyerhaeuser Inc. The study investigated cultivation of loblolly pine (*Pinus taeda L.*) silviculture for solid wood products intercropped with switchgrass (*Panicum virgatum L.*) for biofuels production near Dover, NC and plants were harvested in January 2011. The biomass was dried in cloth bags at 50°C for 48 h in a convection oven at the Metabolism Education Unit, department of Animal Science, North Carolina State University, Raleigh, NC. Dry biomass was ground to pass a 2 mm sieve in a Willey mill and stored in zip-locked bags at room temperature until used. Initial studies in a glass vessel were performed with feedstock harvested by Dr. Joe Burns from a research station near Clayton, NC in June 2008. The acid insoluble lignin (AIL) and total reducing sugar content of the two switchgrass batches was not significantly different.

2.2 Compositional analysis

Acid soluble lignin (ASL) and acid insoluble lignin (AIL) in untreated and sonicated samples was determined using Laboratory Analytical Procedures (LAP) established by National Renewable Energy Laboratory (Sluiter et al., 2008). Reducing sugar analysis for

untreated and sonicated solids, and enzyme hydrolysate samples was conducted using the 3, 5-Dinitrosalicylic acid (DNS) assay (Miller, 1959; Ghose, 1987). Filtrate from the 2 step acid hydrolysis used to determine AIL was used for determination of reducing sugars. Ash content in untreated biomass was determined by ashing the samples in a muffle furnace (Sluiter et al., 2005).

2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) of untreated and selected ultrasonicated switchgrass samples was performed for comparative visual analyses. The analysis was conducted with Hitachi S-3200 N SEM equipment at the Analytical Instrumental Facility (AIF), NC State University. Two gram each of the select samples was vacuum dried at 40°C for 48 h. Each sample was then dried to 0% moisture in a liquid nitrogen drying assembly before being sputter gold coated to be visualized through the SEM.

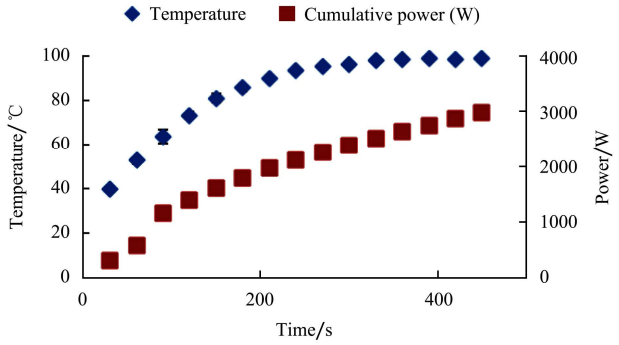
2.4 Pretreatment

Switchgrass samples were ultrasonicated by the Hielscher UIP 1000hd, which consisted of a transducer, booster, sonotorode fuel cell and amplitude control unit. The unit generates ultrasonic frequencies of 20 kHz with 1 kW power input for maximum amplitude of 150 micron (μm) at the face of the sonotrode. The sample slurry was sonicated using the sonotrode which transferred oscillations created in the transducer to the medium by converting electrical signals into mechanical oscillations. Height of the sonotrode base from the bottom of the beaker impacted biomass distribution during sonication and was adjusted to be closer (within 0.5 cm) to the bottom of the beaker by setting up a 'jiffy jack' assembly accordingly.

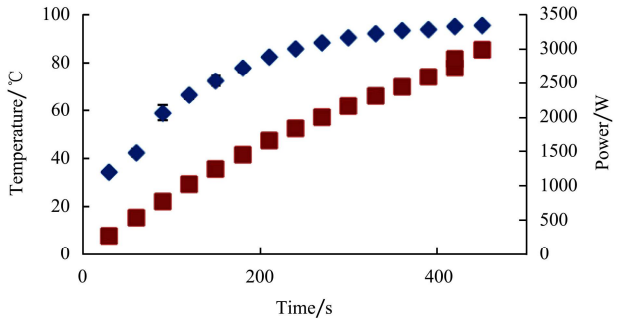
Initial studies on ultrasonication of ground switchgrass without temperature control or stirring were conducted in a 150 mL glass beaker. Treatments were performed with 10% (w/v) biomass loading in 100 mL deionized water for 5, 7.5, and 10 min at 50%, 75% and 100% amplitude (corresponding with 75, 112.5, and 150 μm at the face of the sonotrode). Treatment time was limited to 10 min to avoid possible overheating and breakage of the glass beaker due to rapid ramping of the slurry temperature to 100°C during ultrasonication (Figure 1). During treatment it was observed that a thick layer of biomass accumulated around the sonotrode. This was believed to have a non-uniform effect of ultrasonic irradiation on the biomass. Hence, stirring of the biomass slurry during sonication to maintain a uniform effect was introduced in all ensuing experiments. It has been reported that with increase in temperature in the biomass containing medium, intensity of ultrasonication waves decreases (Feng, 2011). Treatment with temperature control was therefore included in the study and subsequent sonication experiments at 10% solid loading in 100 mL deionized water were performed in a 150 ml stainless steel beaker whose shape was similar to that of the glass beaker. A magnetic stir bar was placed in the beaker and the beaker was placed over a magnetic stirrer which was then placed on the "jiffy jack" such that it was exactly below the sonotrode of the ultrasonicator. Constant stirring at 150 r min^{-1} was maintained to ensure homogeneity during various treatments. Sonication treatments were carried out for 5, 10 and 60 min at 50%, 75% and 100% amplitude. The effect of cooling the reaction vessel was investigated by placing the stainless steel beaker in an ice bath during sonication. The average temperature during the course of sonication was thus maintained at 50°C.

After treatment (in glass or stainless steel beaker) the 'jiffy jack" was lowered and beaker with treated sample was removed. Since the sonotrode was in direct physical contact with the biomass slurry, some biomass particles got stuck to the sonotrode though this amount was significantly lower after stirred treatments than after unstirred sonication. Biomass stuck to the sontrode was recovered in a clean beaker by spray washing with 100 mL deionized water. The washing water and recovered biomass were filtered in a Buchner funnel and flask assembly by vacuum filtration. Moisture content of the solids recovered after ultrasonication was determined by drying a sub-sample (approximately 2 g wet) of the recovered solids at 105°C. Five gram of wet recovered biomass per replicate was dried at 40°C in a vacuum oven for compositional analyses. Table 1 presents the experimental design of the study which was conducted to determine the impact of reaction vessel

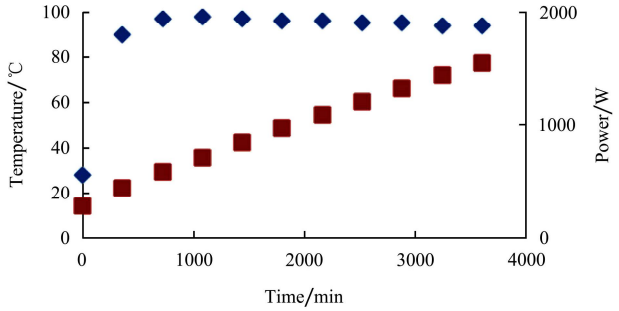
construction material (glass or steel), treatment time, amplitude and temperature control during sonication on biomass composition and structure.



a. 7.5 min at 100% amplitude in a glass beaker without stirring



b. 7.5 min at 100% amplitude in a glass beaker with stirring



c. 60 min at 100% amplitude in a stainless steel beaker with stirring

Figure 1 Temperature and power dissipation profiles during ultrasonication of switchgrass with no temperature control

Table 1 Treatment conditions investigated during ultrasonication

Treatment condition	Amplitude/%	Treatment time/min
Glass vessel, unstirred, no temperature control	50, 75, 100	5, 7.5, 10
Stainless steel vessel, no temperature control	50, 75, 100	5, 10, 60
Stainless steel vessel, temperature control	50, 75, 100	5, 10, 60

2.5 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out on select pretreated biomass to determine the effect (if any) of structural changes in ultrasonicated switchgrass on generation of fermentable sugars. Samples from two treatments, a) highest amplitude and longest time period and b) lowest amplitude and shortest time period were selected for hydrolysis. Wet sample, equivalent to 8% solid loading (1.6 g dry basis) was drawn from the pretreated biomass recovered and hydrolysis was conducted at 20 ml solution volume made up (apart from enzyme volume) by sodium citrate buffer (pH 4.9) and tetracycline hydrochloride solution equivalent to 40 μg mL^{-1} to prevent possible contamination. Hydrolysis was conducted in 50 mL centrifuge tubes for 72 h in a shaking water bath maintained at 50°C. Cellic® CTec2 provided by Novozymes NA, Franklinton, NC and AlternaFuel 200L from Dyadic International Inc., Jupiter, FL were used for hydrolysis. Both enzyme cocktails are mixtures of cellulase, hemicellulase and β-glucosidase. The CTec2 enzyme complex is reported to have an activity of 108.3 - 168.8 FPU/ml (Eckard et al., 2011; Kodaganti, 2011) and protein content 117 - 185.2 mg protein/ml (Eckard et al., 2011; Eylen et al., 2011). The samples were treated with 2 enzyme loadings each to establish the range of sugar production. A high loading of 0.3 g enzyme protein g^{-1} dry biomass for Cellic® CTec2 and AlternaFuel 200 L and low enzyme loadings of 0.1 g enzyme protein g^{-1} dry biomass for Cellic® CTec2 and 0.05 g enzyme protein g^{-1} dry biomass for AlternaFuel 200 L were investigated. The low loadings were based on the suggestions in the manufacturer's application data sheets. Pretreated samples with no enzyme addition (0 g enzyme protein g^{-1} dry biomass) and untreated samples with the low and high enzyme loadings were hydrolyzed as controls.

2.6 Statistical analysis

All experiments in this study were conducted in triplicate. Statistical analyses using PROC GLM procedure for a balanced factorial design were conducted with SAS 9.2© (Cary, NC) to determine the significance of results for the two response variables (acid insoluble lignin and reducing sugar content in the treated and untreated samples) over two independent variables (amplitude and treatment time). Results from treatments

in the three reaction vessels- unstirred glass batch and stainless steel stirred batch with and without temperature control were also compared. The enzymatic hydrolysis data for percent carbohydrate conversion (represented by reducing sugars) in the pretreated or untreated biomass to fermentable sugars in the hydrolyzate was also statistically analyzed using one way ANOVA, with percent conversion as the response variable.

3 Results and discussion

Untreated switchgrass, from Clayton, NC, used for initial testing in a glass vessel contained 23.5±1.8% AIL, 4.7±0.02% ASL, 64.5±1.3% total reducing sugars and 1.6±0.2% ash. The switchgrass obtained from Dover, NC contained 24.3±0.6% AIL, 1.9±0.04% ASL, 69.7±1.5% total reducing sugars and 2.0±0.1% ash. Results reported here for the various experiments are not distinguished on the basis of batch.

3.1 Effect of ultrasonication on switchgrass composition

The effect of ultrasonication on switchgrass composition was characterized by three parameters, namely solid recovery, AIL, and total reducing sugars, for the various pretreatment conditions. Temperature of the slurry and power dissipated during sonication were monitored for representative samples using a thermocouple and power gauge, respectively (Figure 1). While power dissipated in the glass beaker for a 7.5 min ultrasonication pretreatment at 100% amplitude without stirring was not very different from when stirring was introduced, cumulative dissipated power after 60 min of treatment in a stainless steel beaker was much lower at 1,542 W. Temperature increase for all treatments was however similar. It appears that mechanical oscillations and movement of biomass particles were similar during sonication in both types of vessels, but material of construction (glass vs metal) significantly impacted power dissipation.

3.1.1 Solid Recovery

Since there was no chemical degradation during ultrasonication, high solid recoveries were observed in pretreated samples. The highest solid recovery of 94% was observed for the 10 min treatment at 75% amplitude and the lowest was observed to be 76% for the 7.5 min, 75% amplitude combination, both in a glass beaker with no temperature control (Table 2). Average solid recovery across all pretreatments was estimated to be 88.3%. Although ultrasonication potentially solubilized some biomass components, statistical analysis of solid recovery data showed that time, amplitude and vessel material (glass vs. steel) had no significant effect ($p > 0.05$) on solid loss. Recovery was partly impacted by how efficiently the biomass particles sticking to the sonotrode could be recovered after treatment. This resulted in potentially higher standard deviations for some samples.

Table 2 Solid recoveries for ultrasonicated switchgrass samples

Treatment	Time /min	Amplitude/%		
		50	75	100
Glass, unstirrred without temperature control	5	84.9±1.2	78.4±8.5	88.5±3.0
	7.5	87.7±1.7	76.0±9.0	89.7±1.8
	10	87.10±2.7	94.0±2.8	83.1±0.9
Stainless steel, without temperature control	5	89.7±1.1	90.5±6.0	88.7±1.9
	10	93.7±1.3	90.3±4.2	91.2±4.1
	60	91.2±2.8	90.9±2.5	90.2±1.5
Stainless steel, with temperature control	5	90.0±2.3	88.4±1.0	88.0±2.8
	10	89.4±4.9	89.7±0.9	88.6±4.0
	60	91.0±0.3	90.0±2.1	88.0±4.1

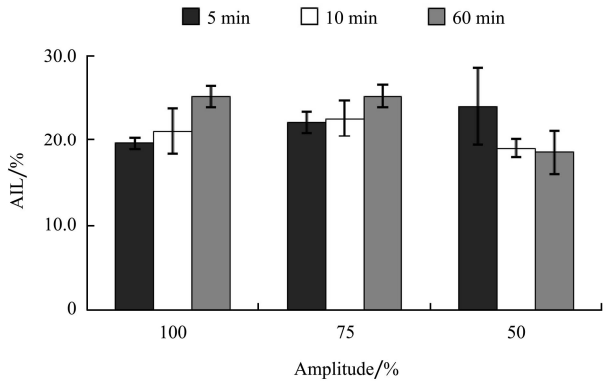
A partial material balance for samples recovered from pretreatment at 100% amplitude for 60 min in the stainless steel reactor with temperature control is presented in Equation (1).

$$\begin{array}{l} \text{Initial sample (10 g)} \xrightarrow{\text{ultrasonication}} \text{Solids recovered (8.8 g)} \\ \text{AIL - 24.3\%} \qquad\qquad\qquad\qquad \text{AIL - 20.7\%} \\ \text{ASL - 1.9\%} \\ \text{Reducing sugars - 69.7\%} \qquad\qquad \text{Reducing sugars - 66.5\%} \\ \text{Ash - 2.0\%} \end{array} \tag{1}$$

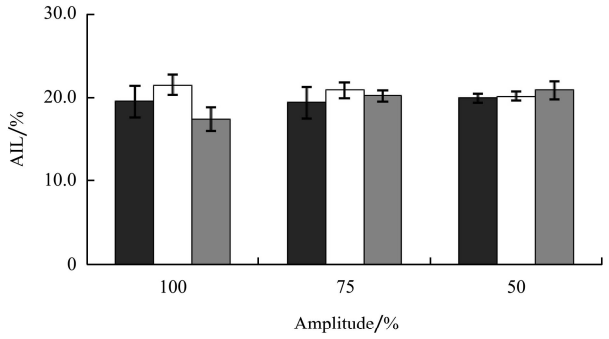
3.1.2 Acid insoluble lignin

The highest average acid insoluble lignin content of 25.2% for samples sonicated in a glass beaker with no temperature control was observed for the 10 min, 100 and 75% amplitude treatments and the lowest acid insoluble lignin content was observed for the 10 min, 50% amplitude treatment resulting in 18.6% AIL (Figure 2). When sonication was performed in a stainless steel beaker with temperature control, the highest and lowest

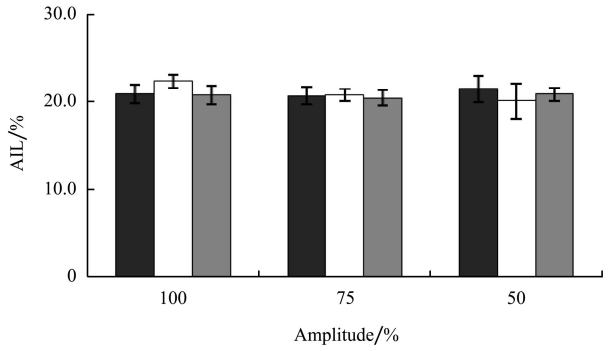
acid insoluble lignin contents were 22.4% and 20.0% observed, respectively, for the 10 min 100% amplitude treatment combination and 10 min, 50% amplitude.



a. Glass unstirred beaker with no temperature control



b. Stainless steel beaker with no temperature control



c. Stainless steel beaker with temperature control

Figure 2 Percent acid insoluble lignin in switchgrass samples ultrasonicated

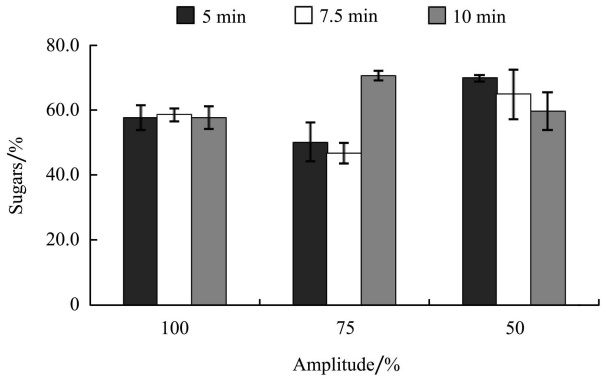
Statistical analysis of the data from the 3 ultrasonication strategies namely glass with no temperature control, stainless steel without temperature control and stainless steel with temperature control did not show a statistically significant ($p > 0.05$) difference in lignin content among the pretreated samples. In a study conducted by Gadhe et al. (2006) a sonochemical reactor at an ultrasonic frequency of 600 kHz coupled with 4 drops of 1 N NaOH to generate free radicals was utilized (Gadhe et al., 2006). An increase in non-conjugated carbonyl groups and a decrease in conjugated carbonyl groups occurred indicating degradation of the lignin polymer. Thus it may be inferred that extremely high ultrasonic frequencies and long pretreatment times coupled with a chemical aid to generate free radicals that can enhance lignin depolymerization (Rehman et al., 2013) need to be investigated to develop more effective ultrasonication pretreatment methods. Hence ultrasonication of switchgrass in this study, which utilized a maximum ultrasonic frequency of 20 kHz with no chemical additives, did not show significant change ($p > 0.05$) in the composition of samples after pretreatment.

3.1.3 Total reducing sugars

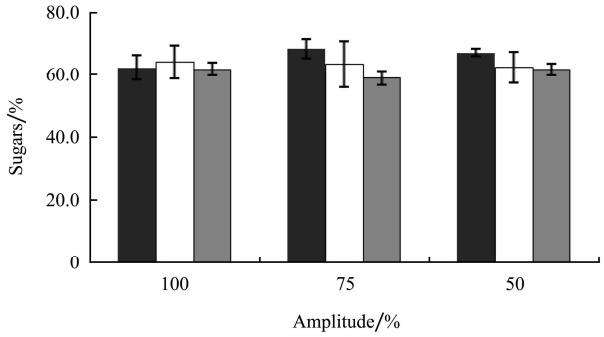
Total reducing sugars in the solids recovered after sonication were used to represent carbohydrate availability in the samples. The lowest reducing sugar value for the no temperature control, unstirred glass beaker combination, was observed to be 46.6% with the 7.5 min 75% amplitude treatment and the highest reducing sugar value of 70.6% was observed for 10 min 75% amplitude treatment (Figure 3). Interestingly, the lowest total reducing sugar value (59%) for treatments with no temperature control in a stainless steel beaker was higher than that in the glass beaker. This value was comparable to the lowest obtained (60.7%) with temperature controlled sonication in the stainless steel beaker for 10 min at 75% amplitude. Of all the stainless steel beaker treatments, the highest total reducing sugar content was 68.6%, obtained for samples treated for 5 min at 75% amplitude without temperature control. It is speculated that material of construction of the treatment vessel impacted the mechanism of sonication potentially due to different levels of power dissipation (Figure 1), although this needs further investigation to improve the overall performance of the technique.

Analysis of data from the 3 ultrasonication strategies did not show a statistically significant ($p>0.05$) difference in total reducing sugars within the pretreatments but showed a significant drop ($p<0.05$) in sugar content compared to untreated samples. Loss of sugars could be explained by the dissolution effect due to disruption of the lignocellulosic matrix. It can thus

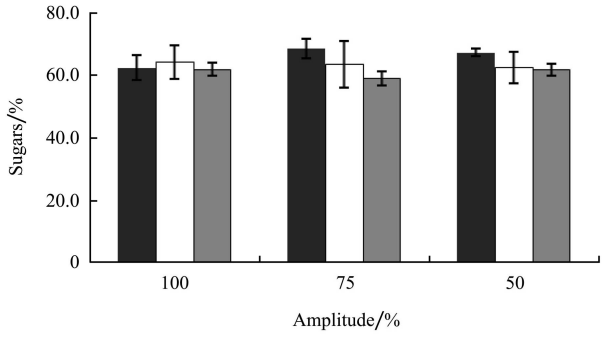
be inferred that solid loss, which ranged between 6% and 24% after sonication, was primarily due to loss of reducing sugars.



a. Glass unstirred beaker with no temperature control



b. Stainless steel beaker with no temperature control



c. Stainless steel beaker with temperature control

Figure 3 Percent reducing sugars in switchgrass solids ultrasonicated

3.2 Scanning electron microscopy

To better understand and comprehend the structural changes that occurred during ultrasonication, select switchgrass samples were observed by scanning electron microscopy (SEM). Figure 4a depicts untreated switchgrass which shows integrity and robustness of particles. It shows a clear and intact outer core. In Figure 4b, which represents a sample from temperature controlled sonication for 10 min in the metallic beaker, it was observed that slight peeling away and disruption of the outer core occurred possibly due to collapsing of bubbles and pressure variations. When ultrasonication was conducted for a longer time interval of 60 min in the stainless steel beaker without temperature control, a tendency of disruption of the outer sheath was observed but rupturing and penetrating impact on the inner core was not seen. (Figure 4c and Figure 4d). Controlling temperature during the 60 min treatment interval resulted in disruption of outer sheath and inner core (Figure 4e and Figure 4f). These samples also show a tendency of crack formation on the outer layer on some of the particles visualized, which suggests mechanical disruption through the force exerted on the particles.

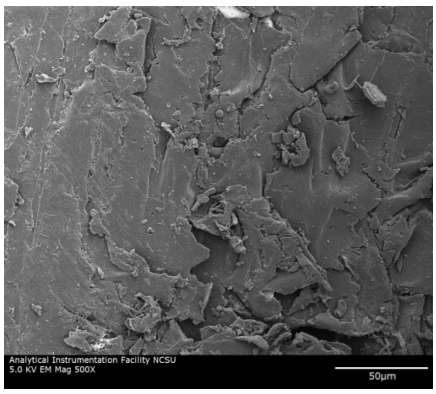
These trends of mechanical disruption were in accordance with earlier studies conducted on SEM analyses of physically pretreated lignocellulosic materials (Behera et al., 1996). There was no evidence of pore formation or any solubilization effect on the biomass structure in the ultrasonicated samples confirming the compositional analysis trends of insignificant lignin degradation.

3.3 Sugar yield after enzymatic hydrolysis

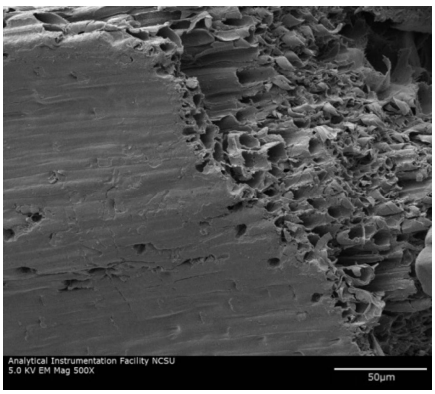
As there were no significant (p>0.05) differences in the composition of samples from various sonication conditions, samples for hydrolysis were selected on the basis of treatment intensity and its impact on structure which could be deduced from visual analyses through SEM. Two samples, each from the temperature controlled and no temperature controlled stainless steel beaker pretreatments, with one being the least intense, i.e. 50% amplitude, 5 min, stirred and the other most intense, i.e. 100% amplitude, 60 min were chosen for enzymatic hydrolysis.

Reducing sugars in hydrolysates from various treatment combinations and enzyme loadings (Table 3 and Table 4) ranged between 467 and 511 mg g^{-1} dry untreated biomass for hydrolysis with 0.3 g g^{-1} Cellic® CTec2 and between 455 and 511 mg g^{-1} for 0.30 g g^{-1} AlternaFuel 200L. A carbohydrate conversion (based on reducing sugar estimates) of 84.6% upon hydrolysis of samples drawn from the temperature controlled 60 min, 100% amplitude, stainless steel beaker sonication was the

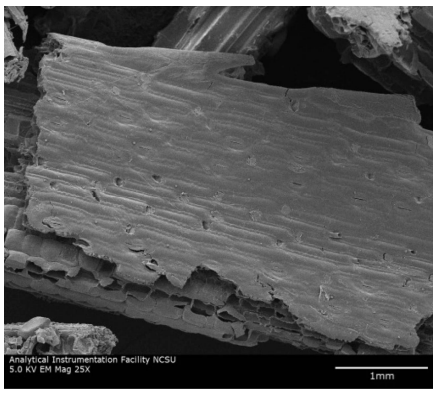
highest observed with CTec2. One way ANOVA indicated that this conversion value was significantly (p<0.05) higher than that for untreated samples and samples from other treatment combinations. The higher AlternaFuel 200L loading led to the highest (p<0.05) sugar conversion of 84.7% in the temperature controlled 60 min, 100% amplitude, stainless steel beaker while the corresponding conversion of untreated switchgrass was 81.2%. There was no significant difference (p>0.05) between the highest and untreated conversion, but these values were significantly higher than the rest of the combinations.



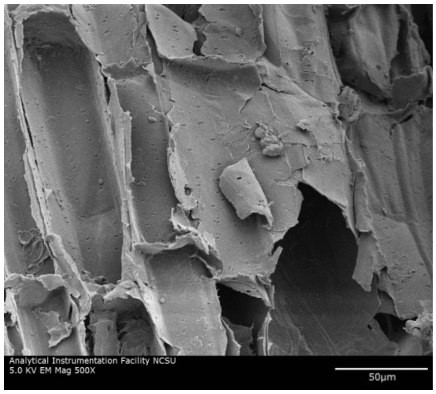
a. Untreated switchgrass (500X magnification)



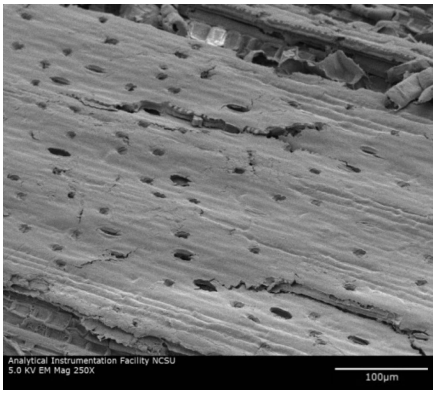
b. Switchgrass pretreated at 100% amplitude for 10 min in a stainless steel beaker with temperature control (500X magnification)



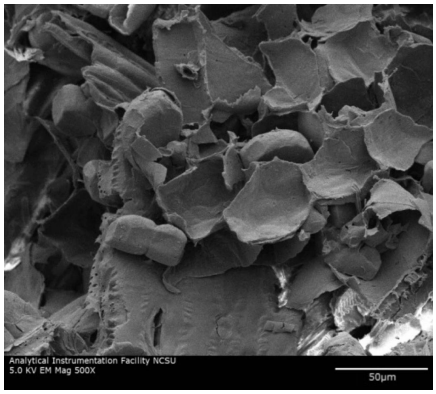
c. Switchgrass pretreated at 100% amplitude for 60 min in a stainless steel beaker without temperature control at location 1 (250X magnification)



d. Switchgrass pretreated at 100% amplitude for 60 min in a stainless steel beaker without temperature control at location 2 (500X magnification)



e. Switchgrass pretreated at 100% amplitude for 60 min in a stainless steel beaker with temperature control (250X magnification)



f. Switchgrass pretreated at 100% amplitude for 60 min in a stainless steel beaker with temperature control (500X magnification)

Figure 4 SEM images

Table 3 Sugar yields and carbohydrate conversions for switchgrass samples hydrolyzed with Novozyme Cellic® CTec2 at various enzyme loading (g enzyme protein g^{-1} dry biomass)

Pretreatment	Sugar yield /mg sugar/g biomass			Carbohydrate conversions %*		
	No enzyme	0.10	0.30	No enzyme	0.10	0.30
UNTREATED	18.5±2.0	194.0±6.0	509.4±4.1	2.7±0.3	28.0±0.5	73.4±2.1
US NTC 100% 1 h	15.4±3.8	158.8±3.5	466.8±12.3	2.6±0.6	26.0±1.0	78.2±1.8
US NTC 50% 5 min	20.5±0.9	178.5±28.2	491.9±32.4	3.1±0.2	27.2±3.5	75.2±2.1
US TC 100% 1 h	22.2±2.0	189.0±7.9	497.9±12.9	3.8±0.3	32.1±0.9	84.6±2.1
US TC 50% 5 min	17.4±0.9	167.7±16.5	510.6±22.7	2.7±0.2	25.9±3.5	78.6±5.1

Note: * Calculated as g total reducing sugars in hydrolysate/ g total reducing sugars in untreated, un-hydrolyzed solids.

Table 4 Sugar yields and carbohydrate conversion for switchgrass samples hydrolyzed with Dyadic Alternafuel 200L at various enzyme loading (g enzyme protein g^{-1} dry biomass)

Pretreatment	Sugar yield /mg sugar/g biomass		Carbohydrate conversions /%*	
	0.05	0.30	0.05	0.30
Untreated	77.5±8.0	563.7±23.1	11.2±1.0	81.2±4.3
US NTC 100% 1 h	25.5±7.1	454.6±11.6	4.3±1.2	76.1±1.6
US NTC 50% 5 min	62.5±5.1	500.1±25.4	9.6±1.0	76.5±3.1
US TC 100% 1 h	61.6±5.1	498.5±23.1	10.5±1.0	84.7±3.2
US TC 50% 5 min	58.2±3.1	510.5±10.8	8.9±0.4	78.5±5.1

Note: * Calculated as g total reducing sugars in hydrolysate/ g total reducing sugars in untreated, un-hydrolyzed solids.

When temperature was controlled, carbohydrate conversion in samples from higher intensity sonication was 32.1% and 10.5%, respectively, at enzyme loadings of 0.1 g g^{-1} Cellic® CTec2 and 0.05 g g^{-1} AlternaFuel 200 L. The corresponding conversions for samples from lower intensity sonication were 25.9% and 8.9%, respectively. Hydrolysis with no enzyme yielded negligible amount of sugars in the range of 15-22 mg g^{-1} of dry untreated biomass when hydrolyzed with CTec2. Conversions at lower enzyme loadings had similar patterns as higher loadings and the highest carbohydrate conversion (p<0.05) was observed when ultrasonication was conducted under controlled temperature for 60 min at 100% amplitude. Pretreatment effectiveness of temperature controlled ultrasonication at longer residence time and high amplitude showed that temperature of the liquid medium was a key factor during mechanical disruption of the lignocellulosic matrix. It was apparent that when increase in temperature during treatment was controlled by using the ice bath, less energy dissipation took place thus allowing intensity of the irradiation to be maintained at a higher level. This confirms results of earlier studies describing the inverse relationship between intensity of ultrasonication and temperature of medium (Feng, 2011). This enhanced mechanical disruption seemed to have increased the hydrolysis efficiency of the treatment combination.

The total sugar yields from representative sonicated samples observed in this study were less than those from untreated biomass. This was potentially due to loss of some sugars during ultrasonication and the observation that ultrasonication alone did not alter the structure enough to produce significantly (p< 0.5) higher yields of reducing sugars. This finding suggests that additives such as those that reduce viscosity and increase bubble nucleation (Mazzoccoli, 2010) may be required to assist ultrasonication for high yields and effect of ultrasonication on sugar production varies based on the biomass material being treated (Montalbo-Lomboy et al., 2010).

4 Conclusions

Ultrasonication of switchgrass at 150 μm (100%) amplitude, for 60 min in a stainless steel vessel with temperature control was the most effective pretreatment strategy among the various conditions tested in this study. SEM images highlighted the treatments tendency to physically disrupt significant portions of the treated biomass due to mechanical action. The trend of considerably high sugar generation from untreated switchgrass hydrolyzed at higher enzymatic loadings however presents a new investigational direction relative to economic viability of pretreatment and hydrolysis processes and needs further scrutiny. It can be inferred from the results of this study that although at the conditions tested ultrasonication alone provided some structural alteration in lignocellulosic biomass, chemical aids may be needed for it be an effective pretreatment technique. Besides, the reported range of particle size that can be affected by ultrasonication (0.15–100 mm) is large and higher particle sizes need to be studied for determining the true potential of this technique. Although ultrasonication did not provide significant change in chemical composition of biomass, results of

this research indicate that sonication may be employed as a preliminary size reduction step to decrease the severity of primary pretreatment methods.

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Disclaimer

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