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Influence of Ultrasonics in Ammonia Steeped Switchgrass for Enzymatic Hydrolysis

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Abstract. *The bioconversion of lignocellulosic materials into fuels is of great environmental and economic importance, because of the large amounts of feedstock (est. over 1 billion tons per year), the potentially low cost of this feedstock, and the potentially high net energy balance the overall process. Switchgrass (Panicum virgatum L.) is a candidate dedicated lignocellulosic feedstock in the US. However, lignocellulosic materials, including switchgrass, are hampered by the recalcitrance of lignocellulose to enzymatic degradation into fermentable sugars. Various types of pretreatment have been developed to overcome this recalcitrance. In this study, we examined sequential ammonia-steeping and ultrasound pretreatment of switchgrass. The experimental variables included ultrasound energy dissipation and source amplitude, biomass concentrations, and antibacterial agents. Specifically, the 35-mL samples received either 2000 J or 5000 J, while biomass concentration was at 10% and 30% (mass basis). Antibacterial agents were employed to determine the extent to which sugars were being metabolized by naturally occurring bacteria in the unsterilized pretreated samples. Analytical glucose analysis was conducted to verify the amount of fermentable sugars released and low-vacuum SEM was used to establish the physical effect of ultrasonics on the biomass. The sequential ammonia steeping-ultrasonic pretreatment released about 10% more fermentable sugars than did ammonia steeping alone. However, the net energy balance (additional chemical in free sugars minus energy consumption of ultrasound process) was not favorable – this contrasts with Grewell's work using ultrasonics for enhancing sugar release from starches. We recommend further investigations on re-evaluating the design and conditions which could make ultrasonic work better as a lignocellulosic pretreatment.*

Keywords. Lignocellulosics, ethanol, switchgrass, ultrasonics, ammonia steeping, pretreatment, enzymatic hydrolysis

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Introduction

As world energy demand has grown and supplies of fossil fuel have become less stable, large countries such as the US have decided to shift their dependence from petroleum to alternative fuels. It has been predicted that annual global oil production will decline from the current 25 billion barrels to approximately 5 billion barrels by 2050 (Sun and Cheng, 2002). In this climate of decreasing supply and increasing uncertainty, alternative transportation fuels are receiving a great deal of attention. Currently, one of the most widely used alternative transportation fuels is ethanol, which unlike fossil fuel, a fuel produced from renewable biomass,. Biorenewable energy is defined as a sustainable natural resource as it renews itself at such a rate that it will be available for use by future generations (Brown, 2003). Additionally, ethanol, compared to gasoline is an oxygenated fuel that contains 35% O_2, which reduces particulate and NO_x emissions from combustion (Badger, 2002).

Ethanol can be made synthetically from petroleum or by microbial conversion of biomass materials through fermentation. In the US, corn grain has been widely used as the substrate for ethanol production. Ethanol production in the US is expected to grow to 7.5 billion gallons by 2012 from the current 4.5 billion gallons to meet the recently passed Energy Policy Act (EPACT 2005) (Farrell, et al., 2006). Solomon et al. (2007) commented that although corn grain ethanol has the advantage of being derived from renewable resources, its use for fuel has often been criticized as technically, economically, and environmentally undesirable. In addition, greater production of corn grain ethanol may conflict with food production needs. In contrast to corn grain-derived ethanol, lignocellulosic biomass is both more available and less food-chain critical.

Lignocellulosic materials, which are mostly comprised of lignin, hemicellulose and cellulose, can be obtained from woody parts of trees and plants, perennial grasses and crop residues. Extensive research has been conducted on converting lignocellulosics to ethanol over the last two decades (Dale, et al., 1994; Azzam, 1989; Duff and Murray, 1996). Ethanol can be produced from cellulose by enzyme hydrolysis of long chain cellulose to fermentable sugars and fermentation to alcohol by yeast or bacteria. However, lignin inhibits enzymatic conversion of cellulose and hemicellulose. Various pretreatment technologies have been developed to overcome this problem. One of the promising pretreatment methods is ammonia pretreatment in a flow-through column reactor. According to Kimet al. (2003), this method was highly effective in delignifying biomass, reducing lignin content by 70-85%. Ammonia was chosen as a pretreatment reagent because it is widely available and residual ammonia will serve as a nitrogen source for fermentative organisms..

Ultrasound is sound at a frequency above the normal hearing range of humans (> 15-20 kHz). When the ultrasound wave propagates in a liquid medium, it generates a repeating pattern of compressions and rarefactions in the medium where microbubbles are formed. Khanal, et al. (2007) explained that as microbubbles oscillate under the influence of variable pressure, their size begins to increase before they violently collapse. As the microbubbles collapse, a localized temperatures of up to 5000K and pressures of up to 180Mpa will be produced (Suslick, 1990). This same collapsing phenomenon, also known as cavitation, creates enough energy to mechanically shear the particles in the bulk liquid surrounding the bubbles. Another mechanism that occurs when a liquid is sonicated is acoustic streaming. Acoustic streaming has been studied since 1831 (Faraday) and occurs at a solid/liquid (horn/slurry) interface when the solid interface experiences harmonic vibrations. The main benefit of streaming is mixing of the particles (i.e., switchgrass), which facilitates uniform distribution of ultrasound energy within the feedstock mass, convection of the liquid and dissipation of any heating that occurs. Acoustic streaming was also reported in Khanal et al. (2007).

Ultrasonic technology has been employed in several studies in waste activated sludge. It was used to disrupt the recalcitrant bacterial cells so they were available for anaerobic digestion (Tiehm, et al. (2001); Chockhalingam, et al. (2004)). Khanal et al. (2007) has successfully used ultrasonics to enhance sugar yield in corn slurry for enzymatic hydrolysis. Wood et al. (1997) also studied the effects of ultrasonic treatment on ethanol fermentation from mixed office paper. Following the success of the previous studies, both in using ultrasonic technology and ammonia pretreatment, the current study is an attempt to couple the two technologies as a pretreatment in cellulosic ethanol production. The main objective of the research is to determine the potential of ammonia steeping-ultrasonics pretreatment of switchgrass in releasing glucose in enzymatic hydrolysis. The study will also evaluate the overall energy impact of the pretreatment system.

Material and Methods

Materials

The cellulosic biomass used in this study was switchgrass (*Panicum Virgatum L.*). The switchgrass was ground to approx. 5-6 mm. Then it was sent to the Agronomy laboratory of Iowa State University, Ames, IA for composition analysis. The composition of the biomass is given below. The study used a Branson 2000 series benchscale ultrasonics unit with a maximum power output of 2.2 KW and a frequency of 20 kHz. All chemicals used in this study were obtained from Fisher Scientific with the exception of cellulase enzyme (Spezyme CP; 60FPU/g cellulose) which was provided by Genencor International (Palo Alto, CA).

Table 1 Switchgrass composition

Composition	**% Concentration**
Cellulose	42
Hemicellulose	30.97
Klason Lignin	22
Ash	0.67

Methods

Switchgrass samples of 42.68 g (approximately 40 g dry weight) were weighed in a plastic container. Then 200 ml of aqueous ammonia (29.46%) was added to the sample. It was left to soak in the fume hood for 5 days. Then samples were harvested by washing the soaked switchgrass with 20L of distilled water. The first 4 L of samples were analyzed for lignin removal by the Klason method (Isci et al, 2007). Random samples were weighed and dried at 70°C for 2 d to obtain the moisture content of the wet ammonia steeped switchgrass.

All ultrasonication tests were conducted by taking 10g and 3.78 g of the wet ammonia steeped biomass samples into a 50 ml polypropylene plastic tubes where they were mixed with 15 ml of acetate buffer (to maintain a pH of 4.7 to 4.8). The volume was then completed to 35 ml before being subjected to sonication. The control samples contained the same concentrations but were not treated with ultrasonics. The samples were treated at 5000 J and 2000 J of end dissipated ultrasonic energy with an amplitude of 160 μm_{pp}. The ultrasonic horn used in the study was a standard 20 kHz half-wavelength catenoidal titanium horn with a flat 13 mm diameter face (gain = 1:8).

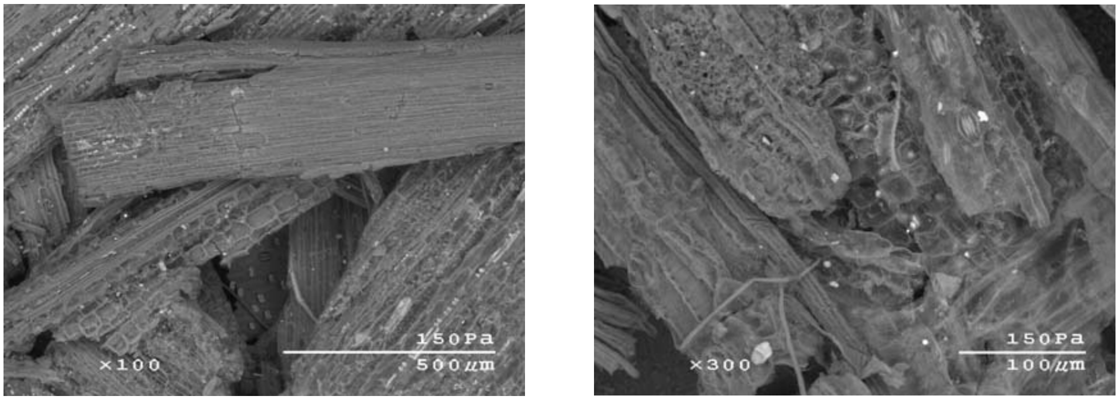
The same concentrations of samples were added with 0.21ml cycloheximide (100mg/ml) to determine the extent of possible bacterial contamination since sterilization was not employed before enzyme hydrolysis. After the samples were sonicated, Spezyme CP of 0.42 ml and 1.12 ml was added to switchgrass solutions of 10 and 30 wt percent, respectively for hydrolysis. Samples were then incubated at 37°C, shaking at 150 rpm for 24 hours. As the samples were harvested after 24 hours, 2 ml of 4M HCl tris buffer (pH 7.0) was added to terminate the enzyme activity. Samples were centrifuged at 10,000g for 20 min and then the supernatant was analyzed for glucose concentration using a modified DNS method (Miller, 1954). The solids pellet was sent to Iowa State University Materials Analysis and Research Laboratory for scanning electron microscopy (SEM) analysis using a low vacuum of 150kPa (Hitachi S2460N). All the experiments were conducted in duplicate and analytical tests were done in triplicate. The error bars in the results correspond to the standard deviation of the experimental population using the standard "n-1" method.

A sample size of 100 µl was removed from the supernatant, and then mixed thoroughly with 1 ml of DNS reagent. The DNS reagent consisted of 0.25 g of 3,5 dinitrosalicylic acid; 75 g sodium potassium tartrate; 50 ml of 2M NaOH; and distilled water up to 250 ml. The solution was heated to 90-95°C for 10 min and then cooled down in an ice bath. The absorbance of the sample was measured at a wavelength of 570 nm using a spectrophotometer (ThermoSpectronic Genesys 2–model W1APP11). Glucose concentrations were calculated from the calibration curve obtained using absorbance data for standard solutions of D-glucose reacted with DNS reagent prepared as above.

Results and Discussion

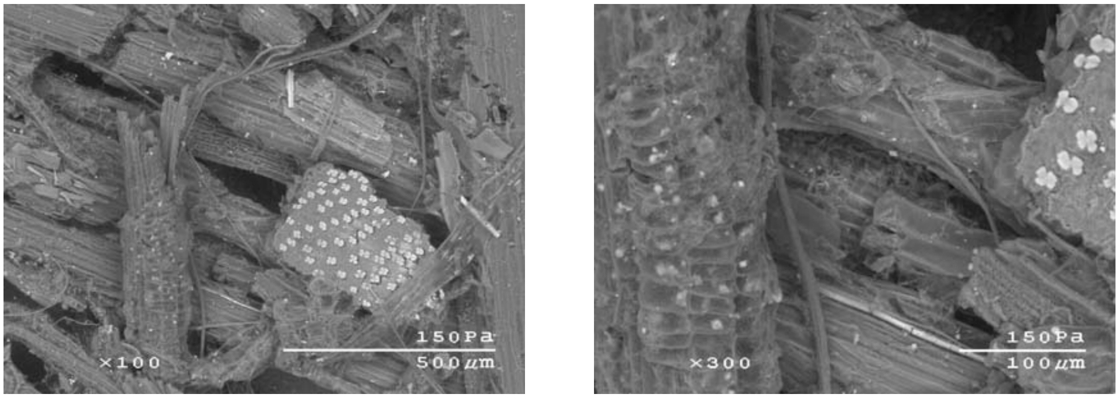
Scanning Electron Microscope Photographs

The scanning electron micrographs (SEM) of switchgrass after ammonia steeping (a-b), after ammonia steeping and ultrasonics at 2kJ (c-d), and after ammonia steeping and ultrasonics at 5kJ (e-f) are shown in Figure 1 at 100x and 300x magnification. In a and b, it was observed that the crystallinity of the samples are still intact at both 100x and 300x magnification. After subjecting the samples to 2000J of ultrasonics, the switchgrass have reduced in size as observed in Fig. 1 (c & d) in comparison with the unsonicated samples in Fig.1 (a & b). Although there are still large pieces of cellulosic material remained in the sample, it is significant to note that the bulk volume of the material has obviously decreased. The star shaped white structure found in the SEM picture was found to be silica adhering to the cellulose. In the same figure, e & f has shown that switchgrass samples sonicated at 5000J has broken down the chunks of cellulosic material initially observed into long and small strips of cellulosic material. Eventhough it was evident in the picture (Fig.1 e, f) that not all of the material in switchgrass has been broken down, the transition of the sample from a very crystalline form into a long strip material is significant to deduce that ultrasonics have changed the structure of the switchgrass.



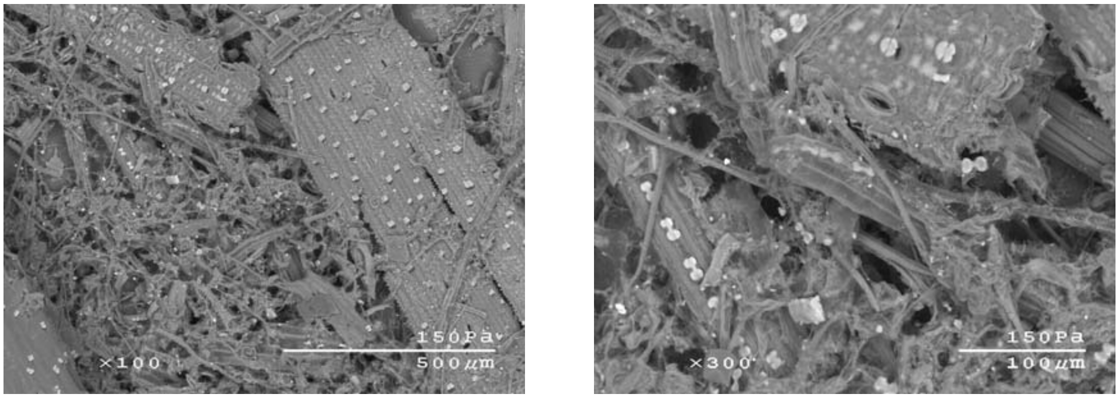
(a) Control

(b) Control



(c) Sonicated at 2000J

(d) Sonicated at 2000J



(e) Sonicated at 5000J

(f) Sonicated at 5000J

Figure 1 SEM of switchgrass samples (a & b) ammonia steeping only, (c & d) ammonia steeping with ultrasonics at 2000J, (e & f) ammonia steeping with ultrasonics at 5000J

Effect of Antibacterial in Glucose Yield

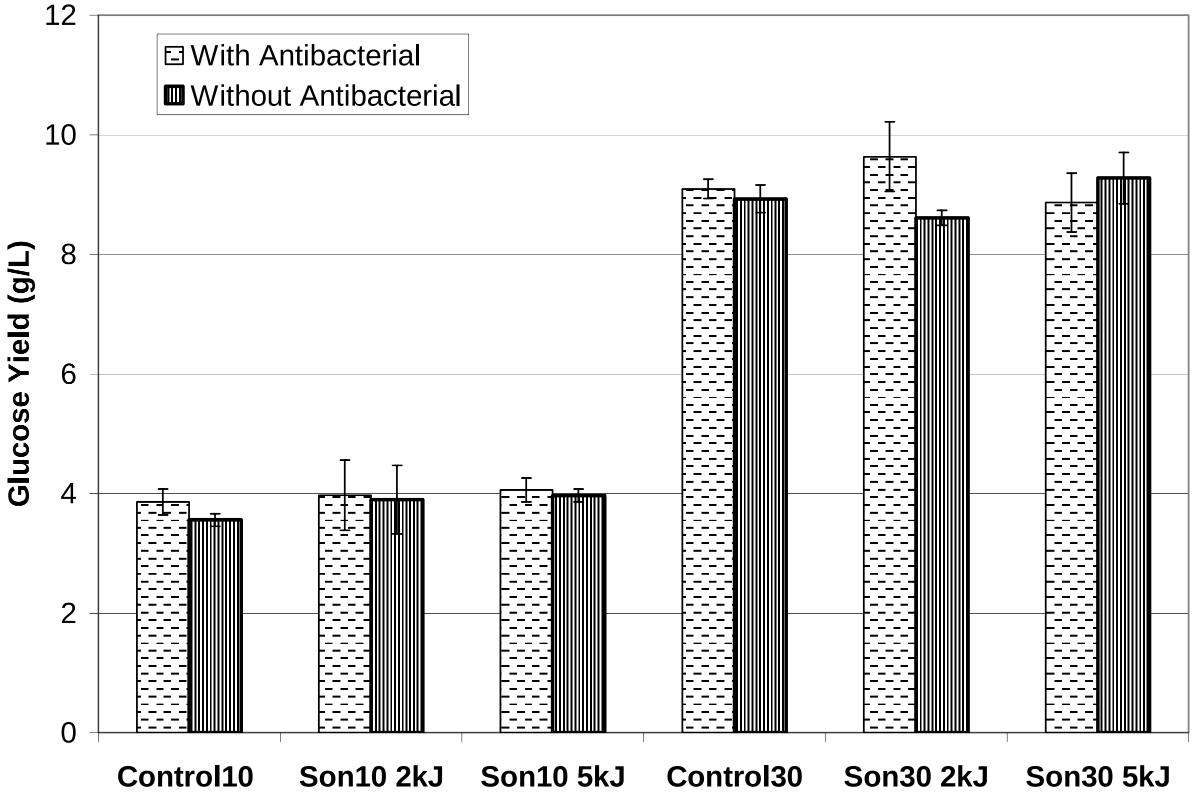


Figure 2 Effect of Antibacterial Agent (Cycloheximide) on Glucose Yield of Enzymatic Hydrolysis

Figure 2 shows the antibacterial effect of Cycloheximide in the glucose yield of the enzymatic hydrolysis. Since the samples were not sterilized before digestion, the study has opted to add antibacterial agent and evaluated its effect on the sugar released. Fig. 2 has indicated that the 10% biomass concentration indicated about 8.5%, 2% and 2.4% difference between the sugar yield with antibacterial and without antibacterial for control, sonicated at 2kJ and 5kJ, respectively. While for 30% biomass concentration, it gave 2%, 12% and -4.6%, for control, sonicated at 2kJ and 5kJ, respectively. Overall, the antibacterial implicated an impact only on control at 10% biomass, sonicated 2kJ and sonicated 5kJ, both at 30% biomass. In the sonicated samples, it is hypothesized that the antibacterial effect could have been enhanced due to ultrasonics where possible bacterial lysis has occurred. Although it is interesting to note that the 30% switchgrass concentration in Fig. 2, which was sonicated at 5kJ had a negative value wherein without antibacterial addition even gave higher glucose yield. It is speculated that since it is at higher biomass concentration, then more cavitation could have occurred and that could have also broken down some bonds in the cycloheximide compounds which could have reduced its antibacterial effectivity.

Table 2 presents the difference expressed in percent of the glucose release in sonicated switchgrass samples relative to the control or non-sonicated samples. The data were obtained by subtracting the glucose released in control switchgrass from the sonicated switchgrass, then dividing it by the sugar value of the nonsonicated sample (multiplied by a hundred). The values presented in the table were taken from the samples without antibacterial addition. The highest increase of glucose yield was obtained from the 10% biomass samples sonicated at both 2kJ and 5kJ. However, at 30% biomass concentration sonicated at 2kJ, the glucose yield decreased by 3.5%, while at 5kJ, it only increased to approx. 3.9%, which is a relatively small amount compared to the 10% biomass. Due to the larger amount of biomass in the solution, both 2kJ and 5kJ of ultrasonic energy might have not been enough to break down large chunks of crystalline structure switchgrass.

Table 2 Percent difference of glucose release in sonicated switchgrass relative to control

Switchgrass Samples	**% Glucose Yield Difference**
Son 10 2kJ	9.6
Son 10 5kJ	11.5
Son 30 2kJ	-3.5
Son 30 5kJ	3.9

Cellulose Conversion

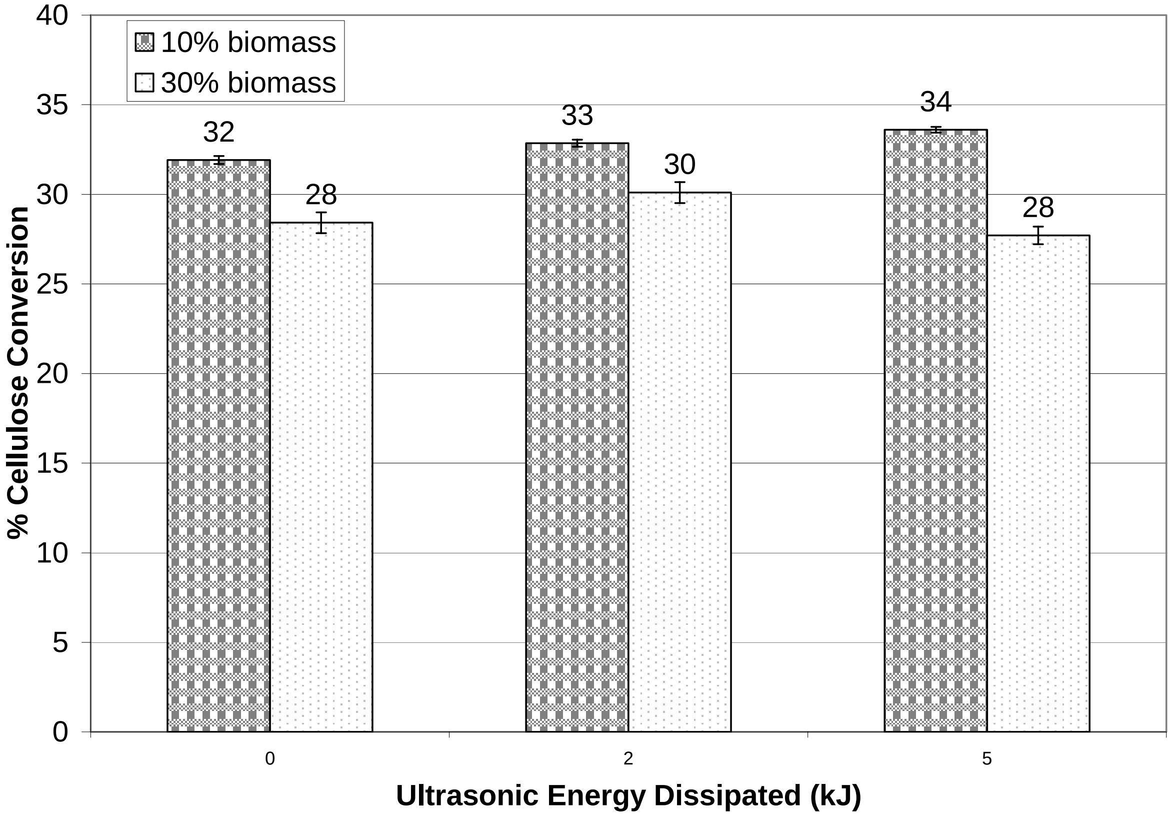


Figure 3 Percent cellulose conversion at 24 hours enzymatic hydrolysis relative to the ultrasonic energy dissipated

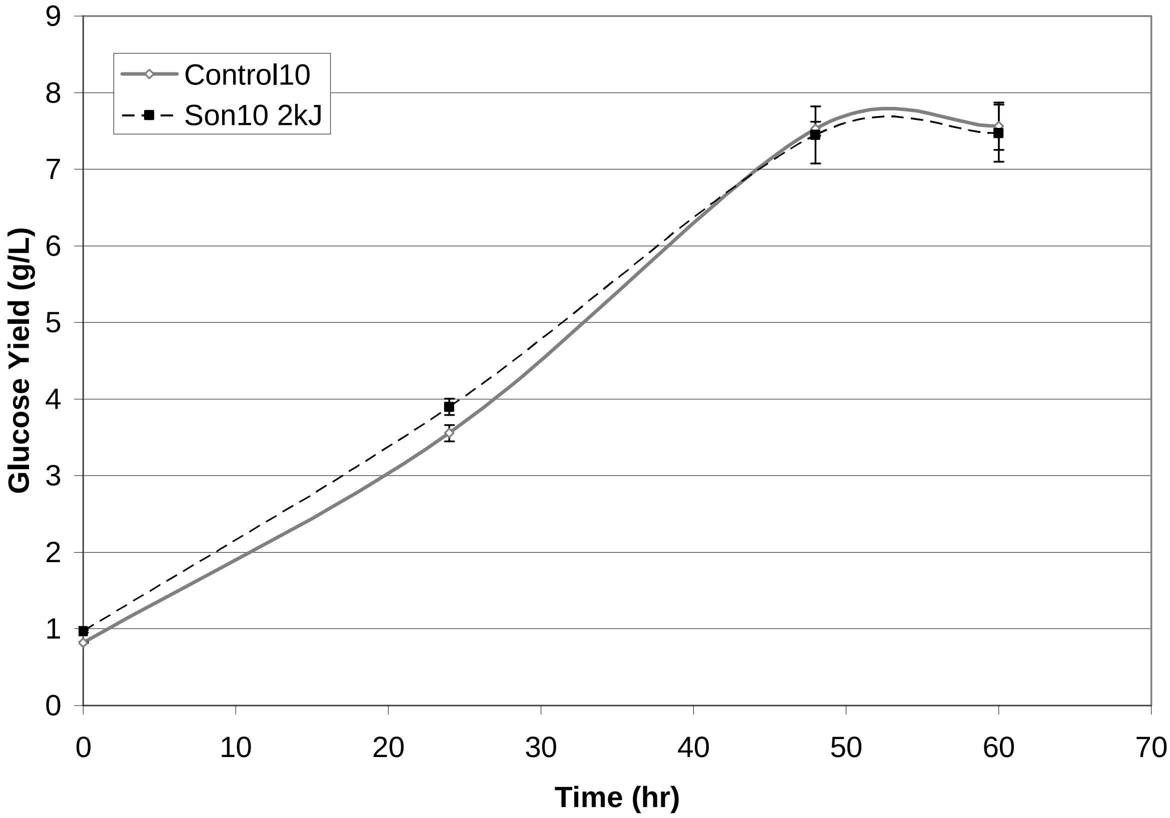


Figure 4 Glucose release with time for switchgrass at 10% concentration sonicated at 2KJ

The study has considered cellulose conversion as an indication of the extent of the pretreatment in terms of breaking down the crystallinity of the biomass structure so it is available for enzyme digestion. Figure 3 presents the percent cellulose conversion in relation to ultrasonic energy dissipation, while Figure 5 displays the percent cellulose conversion in relation to time of hydrolysis. It is apparent that the highest cellulose conversion obtained after 24 hours of digestion was at 34%, achieved with 10% biomass sonicated at 5kJ. The cellulose conversion has increased about 1% after every increase of ultrasonic treatment. On the other hand, the 30% biomass provided a 2% increase after 2kJ of ultrasonics but gave the same conversion at 5kJ. The higher biomass provides lower conversions because it has a higher amount of switchgrass in the solution, thus it will take longer time for conversion than for the 10% biomass concentration.

To further investigate the glucose yield trend of the sonicated switchgrass relative to its control, the samples were harvested at different times. Figure 4 and 5 has shown the behavior of the glucose released and cellulose conversion in terms of hydrolysis time, respectively. After 48 hours, the glucose released has remained almost constant indicating that the enzyme was not able to hydrolyze the remaining cellulose available. It is interesting to note that at 48 hours, the glucose difference between sonicated and nonsonicated samples was almost negligible, while there was a 10% increase after 24 hours. It could only imply that sonication sped up the enzyme hydrolysis at 24 hours, however, the control was able to catch up at 48 hours. The result may entail that the amount of crystalline structure of the switchgrass that was broken down by ultrasonics was insufficient to make an impact in the enzymatic hydrolysis. The highest cellulose conversion (Fig. 5) obtained by pretreating switchgrass with ammonia steeping and ultrasonics was up to 63% at 60 hours of digestion.

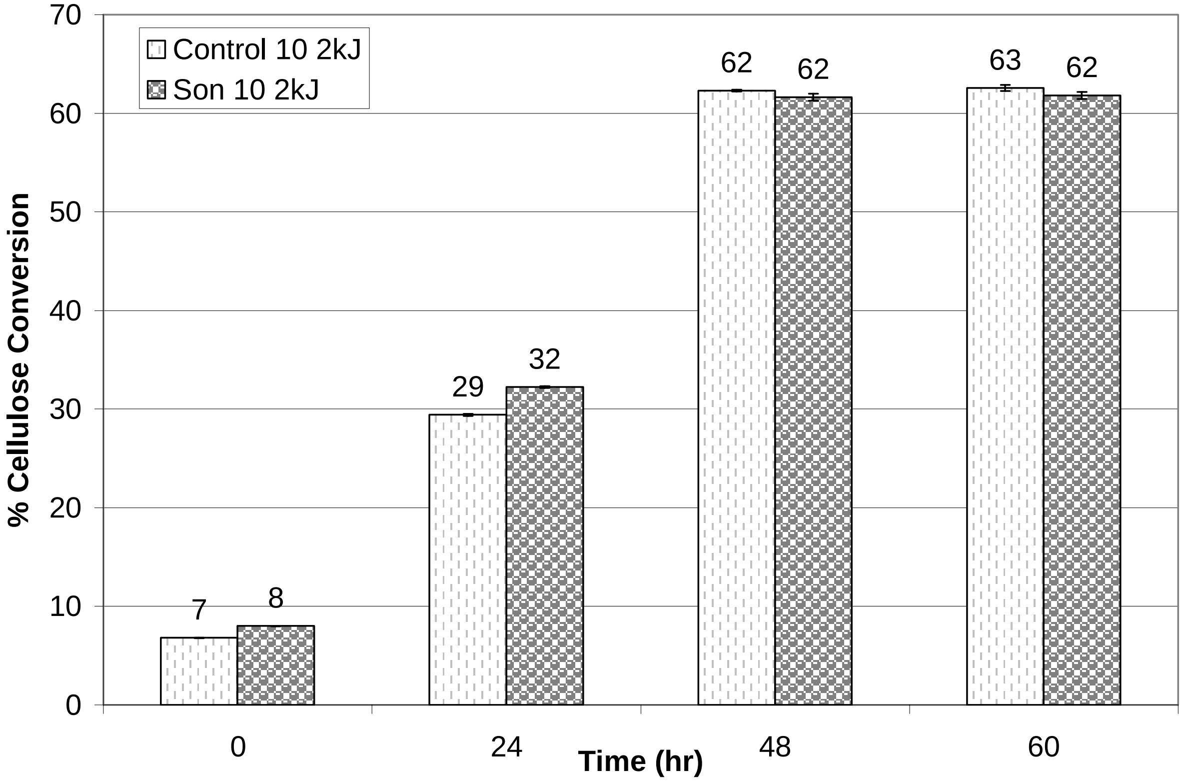


Figure 5 Percent cellulose conversion for varying enzyme hydrolysis periods with 10% switchgrass

Energy Balance

One of the major concerns of any biorenewable technology is the balance of input and output energy, which aims to measure the overall efficiency of any given system. In this study, the efficiency of the ultrasonics treatment in terms of the different experimental conditions was taken into consideration, as shown in Figure 6. The energy balance was based upon the ultrasonic efficiency which is the difference of the ultrasonic energy in and the glucose energy out due to ultrasonics, then divided by the amount of energy in, multiplied by a hundred. In order to convert the glucose difference in terms of energy units, a conversion factor of 16,000 kJ/ kg glucose was used. According to Figure 6, there were negative net energy balances in all the sonicated biomass conditions. This implies that the glucose difference released due to ultrasonics was low compared to the amount of ultrasonic energy dissipated into the system. It is rather unfortunate that the coupling of ultrasonics with ammonia steeping did not show significant advantage, especially in the energy balance.

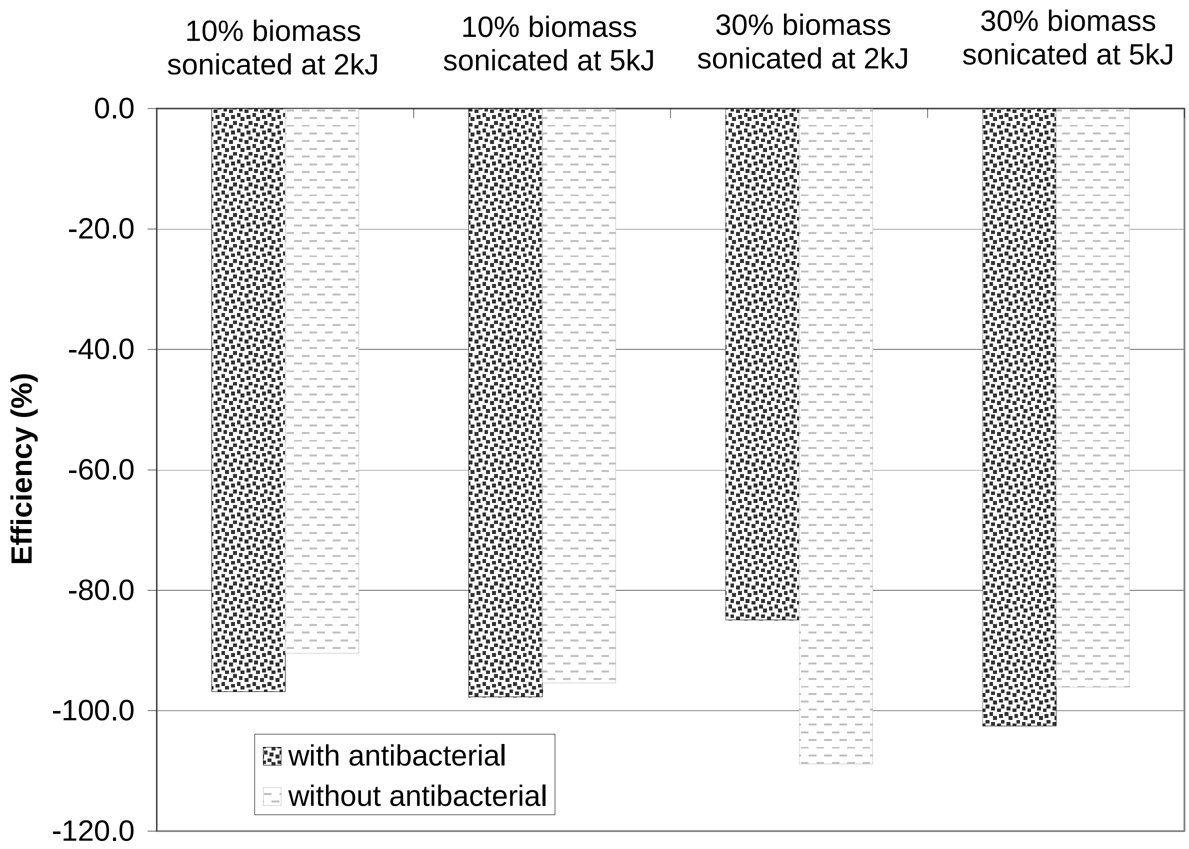


Figure 6 Energy efficiency of ultrasonics relative to glucose release

Conclusion

The study reviews the potential of ultrasonics as an additional pretreatment in the ammonia steeping of switchgrass for enzymatic hydrolysis. The experimental conditions included varying biomass concentrations, time of hydrolysis and ultrasonic energy dissipation. The SEM results showed that at 5kJ, the crystalline structure of switchgrass was disrupted and broken into shorter strands. When enzymatic digestion was conducted for 60 hours with 10% biomass concentration and sonicated at 2kJ, the highest cellulose conversion obtained was after approximately at 48-60 hours at 63% cellulos conversion. However, there was no significant difference in the sugar yield observed from sonicated and non-sonicated switchgrass samples.. The highest sugar increase achieved by sonication relative to the control was about 10%. Despite a promising concept, ultrasonics has failed to produce an increase in net energy from cellulosic ethanol production using ammonia steeping pretreatment. It is recommended that further investigation is undertaken to evaluate other pretreatment processes and conditions..

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