

Enzymatic Hydrolysis of Pretreated Corn Stover at High Solids Loadings

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ABSTRACT

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Biochemically produced cellulosic ethanol holds promise for the future of renewable liquid transportation fuels. Cellulosic ethanol must demonstrate an economic and large-scale viability in order to realize its full potential, and therefore intermediate processes such as enzymatic cellulose digestion must be more fully understood. In this study, the digestibility of dilute sulfuric acid pretreated corn stover was explored with respect to pretreatment severity, particle size, and regimen of pH adjustment before enzymatic hydrolysis. A series of experiments were performed at 20% insoluble solids weight, which is considered a high solids concentration because the material has an appreciable yield stress. Conversion of cellulose to glucose and cellobiose by 20 mg protein/ g cellulose GC220 enzyme was assessed over a period of 7 days. Each sample consisted of 60 g of slurry in 125-mL cylindrical bottles rotated on mechanized rollers at 4 rpm incubated at 48.5 °C. Enzymatic hydrolysis of pretreated insoluble solids suspended in DI water and citrate buffer was performed in duplicate with and without mechanical size reduction. GC220 enzyme is most effective at a pH near 5, thus necessitating the buffer in the insoluble solids samples. Pretreated whole biomass slurries include soluble and insoluble solids and have a pH around 1.5 – 2.0. Ammonium hydroxide was mixed into the whole slurry samples to increase the pH before enzyme addition. Results showed that increasing extent of pretreatment predicts increasing digestibility of the pretreated insoluble biomass samples in both cases. Despite particle size reduction of 10-40%, there was little difference in conversion between the two different particle size distribution materials. The results indicate that pretreatment is effective and the well-established correlation between smaller particle size and high digestibility is not a simple causal relationship. In all cases, whole slurry is more difficult to break down with enzymes than insoluble solids suspended in the equivalent amount of water, but ultimately this challenge will need to be overcome to efficiently convert biomass to fuels. This series of experiments begins to show the effect that process-relevant conditions have on cellulose digestibility. Further investigation into these operations can lead to modifications and improvements that may help cellulosic ethanol become the liquid fuel of the future.

INTRODUCTION

Ethanol and other bio-fuels demonstrate significant potential for reducing our nation's dependence on foreign oil, decreasing greenhouse gas emissions, and reducing the ecological impacts of energy production [1]. Cellulosic ethanol is a promising new bio-fuel that is produced from the inedible portions of grain as well as woody and herbaceous plants [2]. Corn stover, for example, consists of leaves and stalks of the corn plant [3]. Stover comprises the majority of the crop and is thus available in large quantities as an agricultural residue [3]. The difficulty of cellulosic ethanol production lies in converting the cellulosic material into simple fermentable sugars, as it is recalcitrant to enzymatic breakdown [2]. The biochemical formation of cellulosic ethanol consists of four processes: pretreatment, enzymatic hydrolysis, fermentation, and product separation [4]. Pretreatment modifies the structure of the biomass to make the cellulose more readily available for sugar conversion [4]. Currently, leading pretreatment options involve acid, hot water, lime, ionic solids, or ammonia [4]. Hydrolysis breaks solid cellulose into simple, fermentable glucose monomers [5]. Fermentation converts sugar into alcohol and lastly, the product is separated and purified. Because of ethanol's large-scale viability, the U.S. Department of Energy aims for 30% of the petroleum transportation fuels to be replaced with biofuels by 2025 [1]. To accomplish this goal, researchers at the National Renewable Energy Laboratory (NREL) are working to develop an inexpensive and effective cellulosic ethanol production process.

Biomass consists mainly of three materials: cellulose, hemicellulose, and lignin [6]. Cellulose is often highly ordered and can be hydrolyzed into its base units: simple, monomeric glucose molecules [7]. Crystalline cellulose is contained within a matrix of hemicellulose and lignin [7]. Hemicellulose is a digestible, non-heterogeneous chain made of many sugars such as arabinose,

mannose, galactose, xylose, and glucose [8]. Lignin is a highly recalcitrant aromatic polymer that, thus far, resists breakdown [9]. Pretreatment works to expose the cellulose core for enzymatic hydrolysis and begins to hydrolyze the hemicellulose into fermentable sugars [3],[9]. Increased pretreatment severity, or higher concentrations of the dilute acid and higher reactor operating temperatures, increases the amount of cellulose available for hydrolysis [10]. However, it also leads to the production of unwanted byproducts such as furfural and hydroxymethyl furfural (HMF) which inhibit enzyme activity and fermentation [10]. Finding the optimal conditions for pretreatment and hydrolysis while avoiding unfavorable conditions is a balancing act. Additionally, enzymes operate optimally around pH 4.8-5, thus the pH of the acidic slurry must be neutralized or conditioned after employing acid pretreatment [11]. Neutralization uses a base to simply raise the pH to an acceptable range, while conditioning raises the pH to about 8.5 and then uses concentrated acid to reduce it to 5.

The pilot-scale bioethanol Process Development Unit (PDU), funded by the US Department of Energy (DOE) and NREL, supplied the material for this study. The PDU operates at a capacity of 1 dry ton of biomass per day [12]. While pretreatment options are numerous, NREL focuses on dilute sulfuric acid (H_2SO_4). Trials conducted at the PDU thoroughly explore the potential of a high temperature, dilute sulfuric acid pretreatment at high solids fractions (20 %) [3].

Operating with higher concentrations of biomass increases the amount of fermentable sugars produced [13] and can potentially reduce economic costs [14]. However, flow properties and fluid behavior changes at higher biomass concentrations, and presents difficulties for unit operation design and control [14]. Because operating at high solids makes cellulosic ethanol production more economically viable, a better understanding of material behavior and properties needs to be developed [14]. In addition to solids concentrations, research shows that initial

particle size has an effect on conversion and process parameters. Studies demonstrate that smaller initial biomass size leads to higher digestibility [15]. Additionally, size has an effect on process relevant characteristics such as viscosity and yield stress [15].

In this study, we explore the effect of pretreatment severity, particle size, and the neutralization method on the conversion of biomass to useful sugars. First, enzymatic hydrolysis was conducted on pretreated corn stover at various pretreatment severities. Next, the size of the biomass with the same pretreatment severity was mechanically reduced. Enzymatic hydrolysis on the resulting material helped to determine whether particle size itself causes higher digestibility or if another factor causes the correlation between size and digestibility.

Additionally, pH neutralization and conditioning of the pretreated biomass before enzymatic hydrolysis was studied to determine the effect on the overall conversion. The analyzed results provide insight into how these parameters of process effect the overall conversion of cellulosic material into simple, useable sugars.

Materials and Methods

i. Fraction Insoluble Solids and Particle Volume Fraction

For quantitative analysis of biomass samples, understanding biomass structure is key. The material has a number of properties which makes it difficult to precisely define the material. Biomass is porous and contains liquid both inside and outside of the particles, and completely removing the water alters the fundamental structure of the solid. The biomass also contains both particulate and fibrous material and the nature of the material causes unpredictable liquid passage and occasional bed collapse of the solid material.

A relevant distinction arises between fraction total solids (FTS) and fraction insoluble solids (FIS). Insoluble solids represent the portion of the biomass that cannot be removed by dissolution in water, and fraction total solids includes all solid components that do not evaporate during drying. Fraction soluble solids (FSS) represents the solid portion of material that can be dissolved by water. FTS and FIS can both be measured on a bench top moisture balance (Metler Toledo, Columbus, OH). However, measuring FIS on the moisture balance can only occur after the soluble solids are removed by dissolving all soluble solids in water.

Biomass slurry density of the biomass is also an important piece of information during initial sample preparation and when performing subsequent calculations. However, measuring densities of corn stover slurries is difficult due to the porous structure of the biomass. In order to calculate volume fraction, a simple method, as described by Stickel, was used [16]. Simply, slurry was spun at differing speeds for varying amounts of time. The amount of liberated liquid was measured and plotted against the centrifugal acceleration multiplied by the time ($a * t$). Theoretically, the amount of removed liquid should reach a limit at high values of $a * t$. Thus, the limit corresponds with the total amount of free liquid in the sample. The subsequently calculated mass fraction can be converted to the particle volume fraction, as explained in [16].

Additionally, the liquor density of the material is essential for converting concentration to mass basis for overall conversion calculations. To determine the liquor density, small amounts of the biomass were centrifuged at 12500 rpm for about 5 minutes to liberate the liquid portion of the slurry. A density meter (DMA5000, Anton Paar, Ashland, VA) determined the density of a 1mL sample of the liquor.

ii. Pretreated Corn Stover

The initial milling and pretreatment of the corn stover was conducted in NREL's pilot scale cellulosic ethanol plant, the Process Development Unit (PDU) as detailed by Schell et al. [3]. Other material processing occurred in the horizontal reactor in the PDU (Figure 1). After pretreatment, the water soluble fraction of the biomass was separated from the corn stover with the intent of removing all soluble sugars before hydrolysis. Removal of the sugars provided accurate analysis of the sugar conversion due to hydrolysis without including the effects of pre-existing sugars and other soluble compounds produced during pretreatment. The extraction method consisted of several cycles of suspending the corn stover in deionized water in an approximate 1:5 solid to liquid ratio and allowing the solids to settle. The resulting supernatant liquid was removed through pouring or via pipet. The decanting of the material continued until the supernatant contained less than 0.005 g/L of glucose as determined by YSI 2700 Select Biochemistry Analyzer (YSI, Yellow Springs, OH). Excess water was removed by centrifugation and, in some cases, ambient evaporation until about 22% insoluble solids remained.

iii. Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in 125-mL wide-mouth polypropylene roller bottles (Thermo Fisher Scientific, Inc., Waltham, MA). Each bottle contained biomass of 20% insoluble solids and 2.61mL of 5wt% sodium citrate buffer (pH 4.8). Tetracycline (1µg/g biomass), an antimicrobial, was used to prevent contamination. The enzyme loading was set to 20 mg protein/g cellulose and the chosen enzyme was GC220 (Lot #4900756448, Genencor-Danisco, Rochester, NY). Each roller bottle contained a total of 60 g working mass, with the balance deionized water.

The hydrolysis occurred in a three deck roller apparatus (Bellco Biotechnology, Vineland, NJ) which was enclosed within an incubator designed to maintain the temperature at 48.5 °C (Figure 2). The bottles rotated horizontally on the rollers at a rate of 4 rpm. Samples were tested for sugar conversion at 8 hours after initial enzyme addition and once a day thereafter for 7 days total. The cellobiose, glucose, and xylose concentrations were determined by High Powered Liquid Chromatography (HPLC). The analysis was conducted in an Agilent 1100 series HPLC with a Shodex SP0810 Sugar Column (Kawasaki, Japan) run at 85 °C with deionized water pumped at 0.6mL/min as the eluent. HPLC sample preparation consists of collecting a small amount of biomass slurry, centrifuging to separate the free liquid, and diluting with deionized water. Typically, after the completion of hydrolysis, percent conversion is used to measure the effectiveness of hydrolysis. For this study, total cellulose conversion was described. Reported total cellulose conversion gives the amount of cellulose that was converted into glucose and cellobiose, a disaccharide composed of two glucose molecules.

iv. Experimental Variations on Enzymatic Hydrolysis

Initially, enzymatic hydrolysis was conducted at high solids loadings for various pretreatment severities. In order to investigate the effect of particle size on cellulose conversion, the same materials underwent mechanical size reduction. Each material was put separately into an industrial sized blender (Figure 3). The material was “blended” for a total of about 3 minutes in 30–45 second increments between which the sides of the blender were scraped to ensure homogeneity. The same enzymatic hydrolysis regime was conducted on the size-reduced material.

Additionally, a study was conducted on pH adjusted and conditioned material. The experiment was designed to show the difference in conversion between washed and unwashed materials. Washing pretreated slurry is a long and intensive process and in order to explore alternatives, this pretreated biomass was not washed before enzymatic hydrolysis. Thus, soluble solids such as existing sugars and salts remained in the slurry. First, the pH of the pretreated biomass was determined using an Orion 520A+ pH Meter (Thermo Electron Corporation, Waltham, Massachusetts). Due to the acidic nature of the pretreatment, the biomass had a pH of about 2. For the control material (97% xylan removal), approximately 12 mL of 30% ammonium hydroxide was added to bring the pH to approximately 5. To condition the 88% xylan removal material, it was exposed to 5 mL of ammonium hydroxide and the pH increased to 8.3 and then was brought back down to 5 using 97% (10 N) sulfuric acid. The same conditioning procedure was applied to the control material with 14 mL of ammonium hydroxide added, and then neutralized with the concentrated sulfuric acid.

RESULTS

i. Characterizing “High-Solids” Definition

Literature defines operating at high concentrations of biomass, or “high-solids,” at concentrations above anywhere from 12-15% insoluble solids. In order to further classify the point where biomass slurry becomes “high-solids,” a number of fraction insoluble solids (f_{is}) measurements were taken at differing biomass concentrations. The theory is that high solids concentration occurs where the material reaches homogeneity, thus the deviation of the f_{is} measurements should be small. Analysis indicated that a steep drop in standard deviation of f_{is}

calculations occurs around 13% solids (Figure 4), indicating that the solution was acceptably homogenous.

ii. The Effect of Pretreatment on High and Low Solids Loadings

The severity of pretreatment is often characterized by the amount of xylan, a general term for polysaccharides present in plant material, removed in the process. For this experiment, three pretreatment severities were used, ranging from 0.5–1.8% H₂SO₄ and from 150–160 °C. The xylan removal ranged from 20–88%, as detailed in Table 1. Figure 5 shows the visible difference in the material due to the extent of pretreatment. The lowest severity results in material that still retains physical similarities to the original material, and the higher severity samples become more broken down and distinct from the corn stover substrate. Enzymatic hydrolysis results showed that higher pretreatment severities led to a higher total cellulose conversion (Figure 6).

iii. The Effect of Mechanical Size Reduction

After mechanical size reduction was performed on the three samples, the same enzymatic hydrolysis regime was conducted on the material. Table 2 outlines the difference in particle size before and after size reduction. The particle size was reduced by 10–40% due to blending. Additionally, the yield stress, or amount of stress at which a material permanently deforms and flows, reduces with blending. Figure 8 shows that the effect of mechanical size reduction is minimal on the total cellulose conversion.

iv. Effect of pH adjustment and conditioning

Figure 9 depicts the results of the pH adjustment and conditioning study. In previous studies, it was noted that high pretreatment severities led to the highest conversions. In this study, however,

the higher pretreated material (97% xylan removal) showed decreased digestibility. The graph also shows that the total cellulose conversions of the biomass in this study were significantly lower than the conversions in the preceding studies.

DISCUSSION AND CONCLUSION

Experiments clearly showed that the more highly pretreated materials converted more cellulose to cellobiose and glucose. As the severity of pretreatment increases, more of the lignin and hemicelluloses materials break down and make the cellulose available for hydrolysis. Thus, the results are intuitive.

Mechanical size reduction demonstrated little change in overall conversion. The results of this experiment indicate that there is not a simple causation relationship between small particle size and high digestibility due to the fact that smaller particles did not necessarily show higher digestibility. The correlations that exist between size and conversion must lie in another process factor, such as pretreatment severity. The results also show that the yield stress of the material was altered after the size reduction. For the least pretreated material (20% xylan removal), an increase in yield stress was observed. While this result is unexpected, it may be explained by the newly-formed small particles which act as pivots in the junctions between the slightly larger particles, thereby increasing the yield stress of the material. For the two higher pretreated samples, the yield stress decreased due to general breakdown of the structure of the material. An increase in homogeneity was also seen after blending. The changes observed in yield stress and material homogeneity are both process relevant results because material properties must be fully understood before full-scale cellulosic ethanol plants become realistic.

The conditioning study gives some insight into how pH adjustment affects biomass conversion. During initial conditioning, the 88% xylan removed material required only 2.8 mL ammonium hydroxide per hundred grams of slurry to reach a high pH, while the 97% xylan removed material required 6.2 mL per hundred grams of slurry. The lower digestibility of the material with higher ammonium addition suggests that ammonium inhibits the activity of the enzyme. Additionally, the total cellulose conversions of the biomass in this study were significantly lower than the conversions in the preceding studies. The decrease in digestibility is due not only to the ammonium inhibition, but also because this study was conducted at increased total solids and decreased enzyme loading.

Overall, this study infers that higher pretreatment severity of the materials studied makes high-solids materials more easily digestible. Also, mechanical size reduction has little effect on overall conversion but affects other process relevant characteristics such as material homogeneity and yield stress. More conclusions for the pH adjusted and conditioning runs.

FUTURE WORK

Additional work in high-solids enzymatic hydrolysis is necessary to tweak parameters to give optimal cellulose conversion. The results of the particle size reduction study indicate that further research into pretreatment conditions is necessary. In order to determine where the correlation between particle size and digestibility originates, further studies should be conducted by varying pretreatment parameters and investigating particle size and subsequent digestion. Additionally, further studies in the field of conditioning will be necessary to fully understand the effects of pH adjustment. For example, studies using different bases may provide some insight into exactly what inhibits the enzyme in conditioned material.

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FIGURES

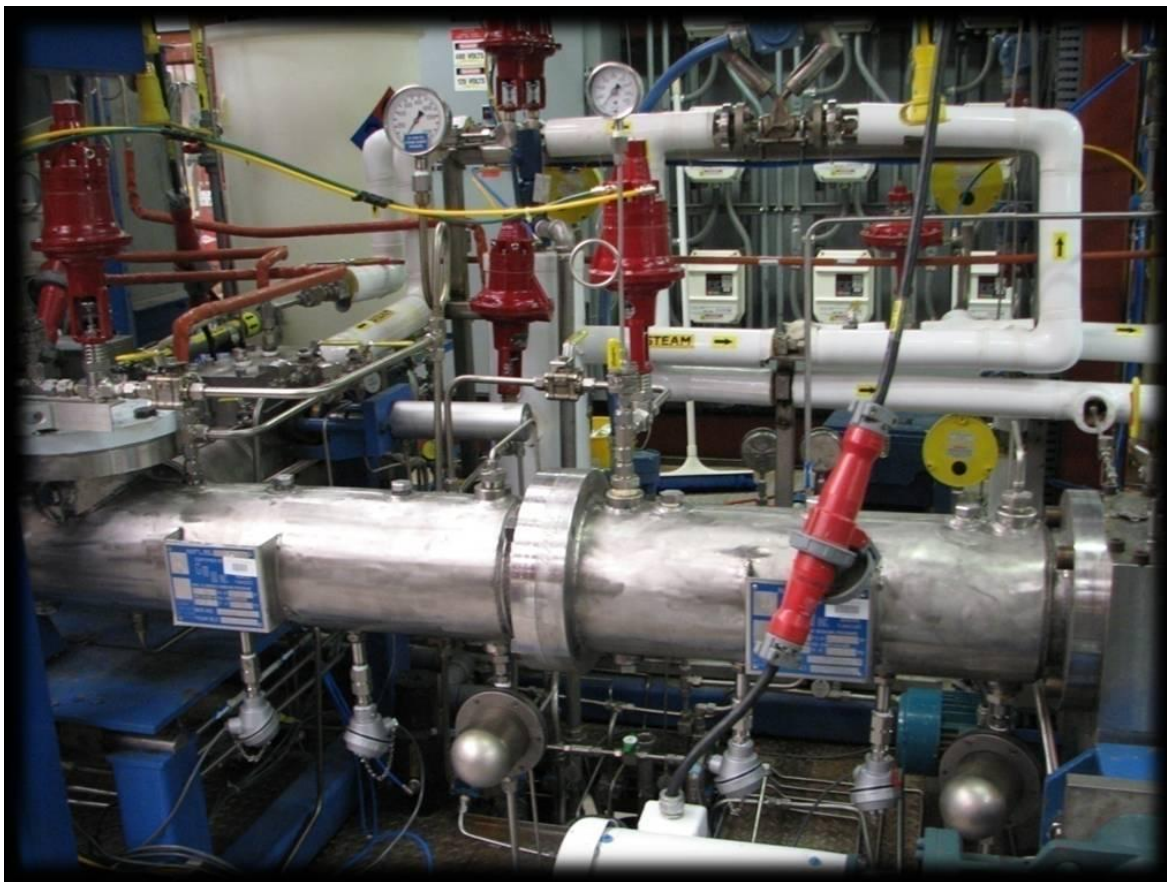


Figure 1: Horizontal Reactor located in the Process Development Unit (PDU) at NREL that produced the pretreated biomass used in this study.



Figure 2: The roller bottle apparatus used for rotating the enzymatic hydrolysis roller bottle reactors enclosed in an incubator held at 48.5 °C.



Figure 3: Industrial sized blender used to mechanically reduce the particle size of biomass samples.

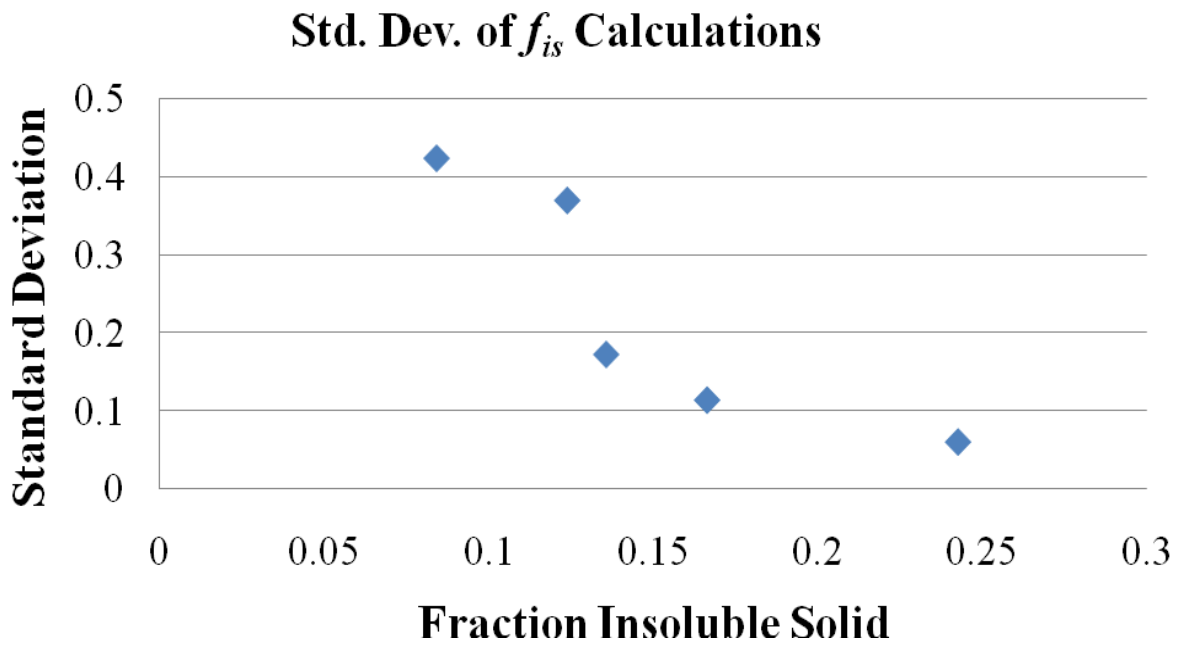


Figure 4: Standard Deviation of f_{is} which shows a smaller deviation at high solids and thus an increased homogeneity of the samples collected from each material.

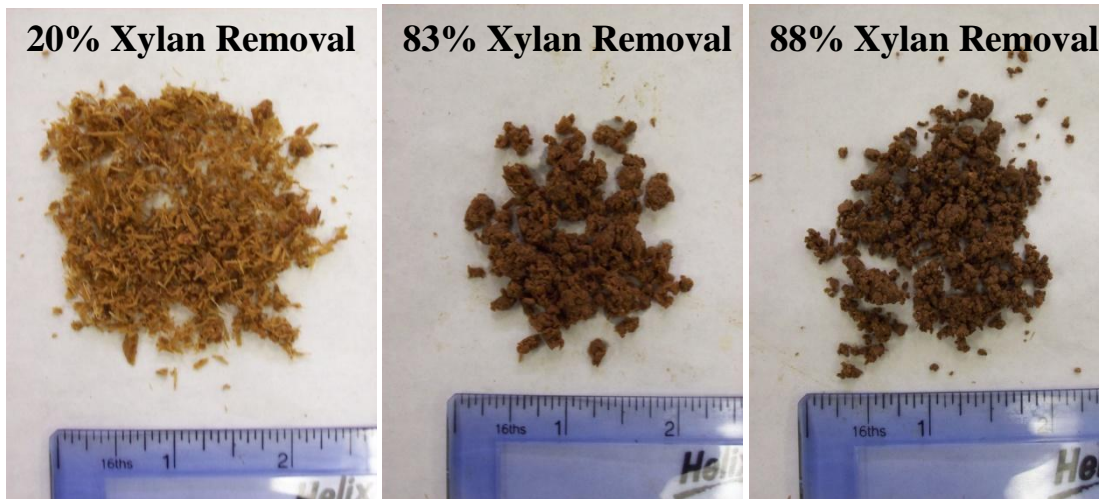


Figure 5: Visible differences in the biomass result due to differences in pretreatment severity.

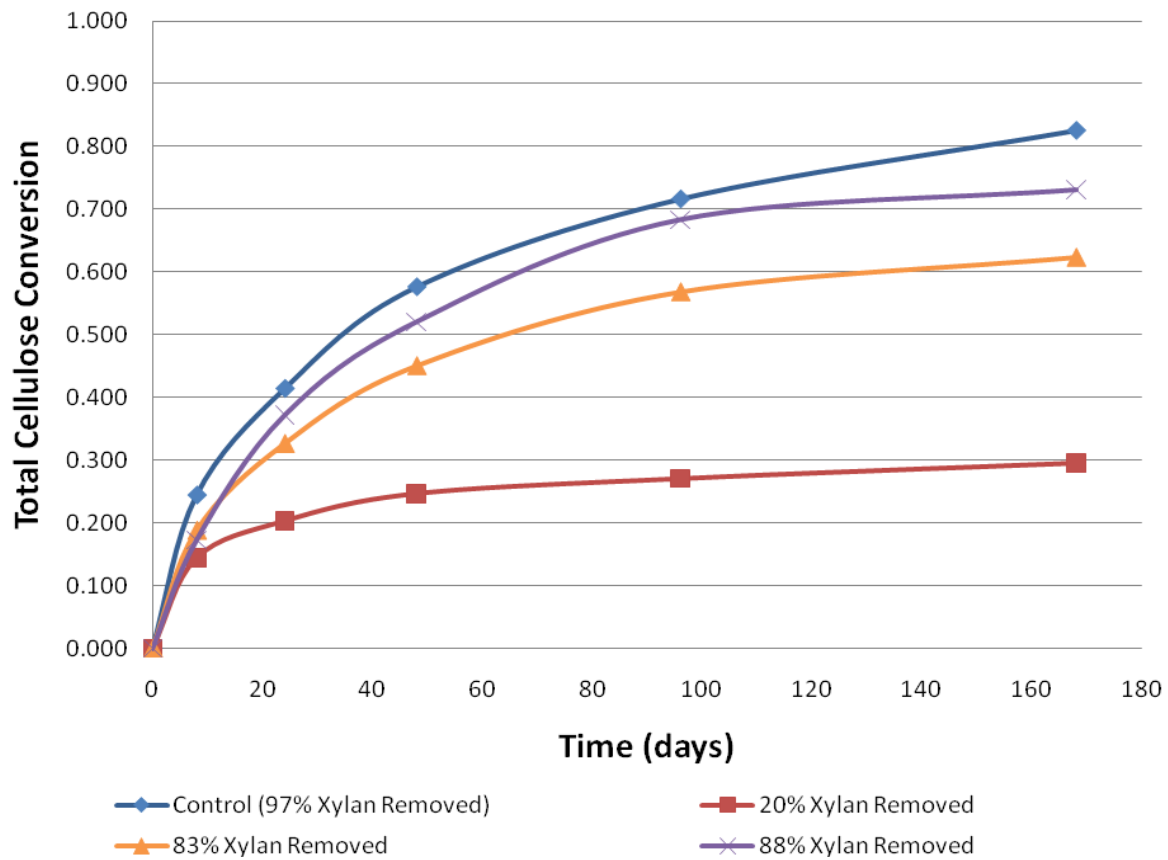


Figure 6: Total cellulose conversion decreases with decreasing pretreatment severities.

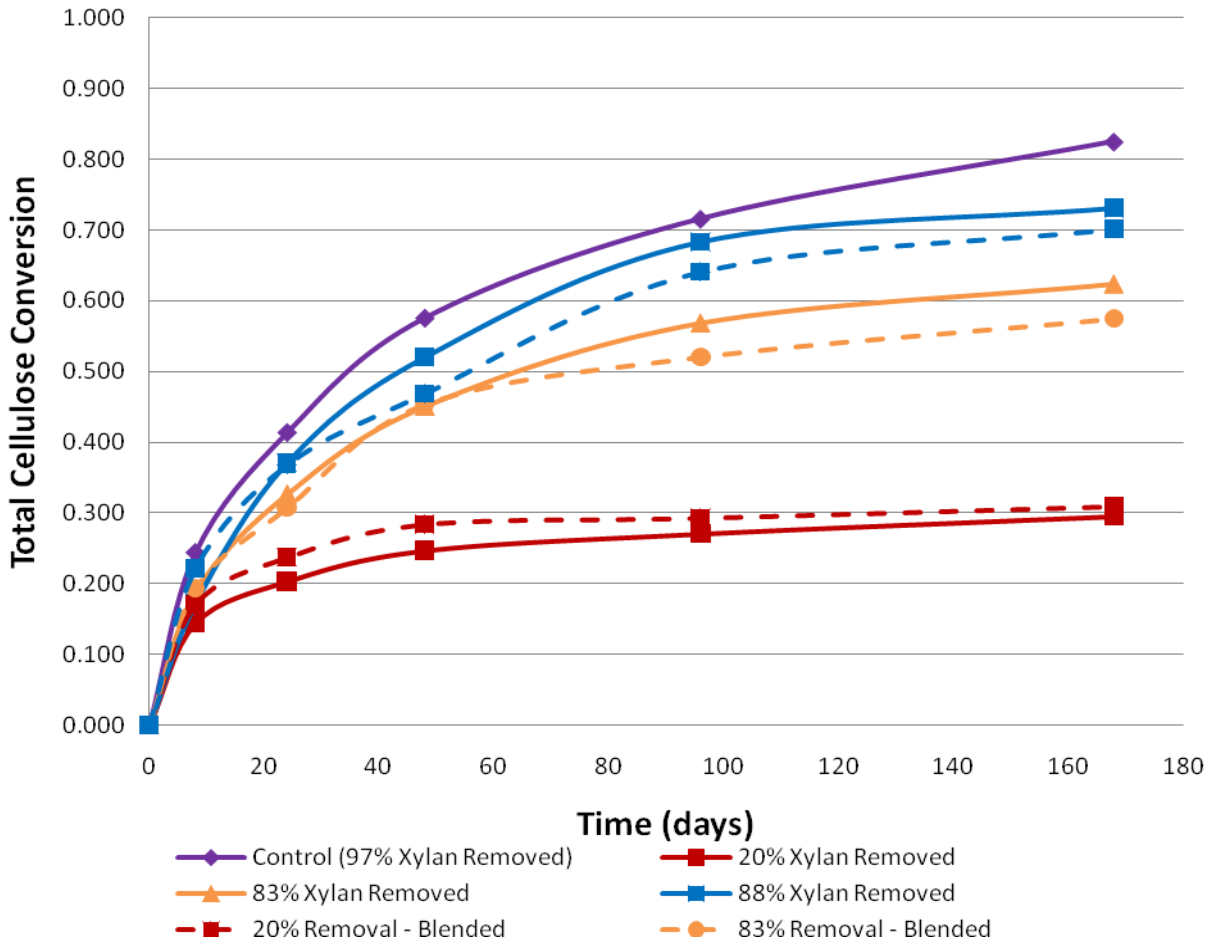


Figure 7: The conversion after mechanical size reduction shows little difference to the conversion before size reduction.

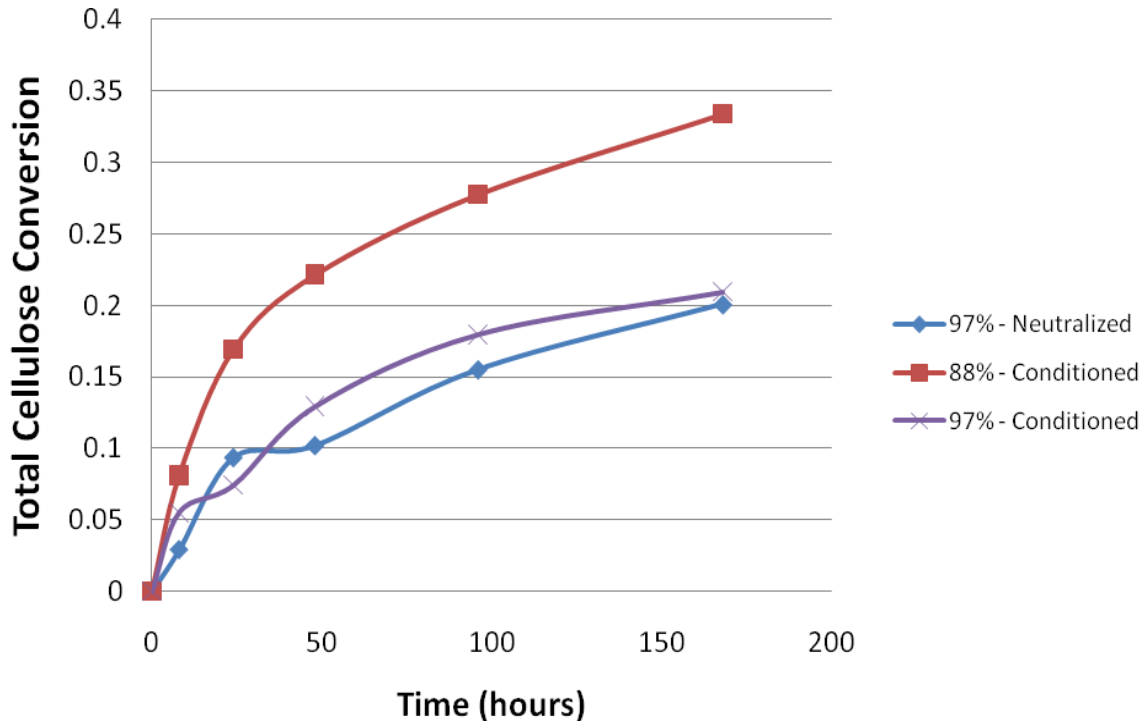


Figure 8: Experimental results for enzymatic hydrolysis completed on pH adjusted and conditioned materials.

TABLES

Name	Xylan Removed	Acid Severity	Temperature
XT090330E	20%	0.5% H ₂ SO ₄	150 °C
XT090401D	83%	1.5% H ₂ SO ₄	150 °C
XT090307D	88%	1.8% H ₂ SO ₄	160 °C

Table 1: The difference in pretreatment severity for each of the tested materials, as well as the corresponding percent xylan removed.

	Pretreated		Pretreated and Blended	
Sample:	Particle Size (mm)	Yield Stress (Pa)	Particle Size	Yield Stress
Xylan Removal				
20%	773	527	482	1019
83%	667	500	411	257
88%	414	182	385	66
97% - Control	55	126	-	-

Table 2: The difference in pretreated particle properties before and after performing mechanical size reduction (blending) of the material.