OPTIMIZATION OF LIQUID AMMONIA PRETREATMENT VARIABLES FOR MAXIMUM ENZYMATIC HYDROLYSIS YIELD OF ENERGY CANE BAGASSE

Abstract

This study aimed at optimizing a liquid ammonium hydroxide pretreatment for energy cane bagasse for maximum sugar yields (glucose and xylose) via response surface methodology. Optimized pretreatment parameters included temperature (160-220°C), ammonium hydroxide to biomass ratio (0-0.5:1) and residence time (30-60 min). Temperature was found to be the dominant pretreatment parameter followed by ammonium hydroxide to biomass ratio. High temperatures and long residence times had a negative effect on sugar yields. Sugar yields had the highest correlation with lignin removal. Based on our quadratic models fitted on the experimental results, optimum pretreatment conditions for maximum glucose yield were 208°C, for 36 min and at an ammonium hydroxide to biomass ratio of 0.4:1. A glucose yield of 30.77 g glucose/100 g (dry weight) untreated biomass and a xylose yield of 3.99 g xylose/100 g (dry weight) untreated biomass were predicted by the model. Optimum pretreatment conditions for maximum xylose yield were 160 °C, for 60 min and at an ammonium hydroxide to biomass ratio of 0.31:1. These conditions resulted in a predicted xylose yield of 9.10 g xylose/100 g (dry weight) untreated biomass and a glucose yield of 23.34 g glucose/100 g (dry weight) untreated biomass. The low xylose yields observed were attributed to the high amounts of xylan lost due to solubilization. The quadratic models were found to be reliable for the prediction of sugar yields within the design space. All predicted values were experimentally confirmed with 95% confidence of the predicted values.

Keywords: Process optimization; Pretreatment; Bioconversion; Energy cane.

1. Introduction

Cellulose and hemicellulose from lignocellulosic material can be utilized in the sustainable production of bio-based fuels and chemicals (Kim et al., 2003). However, the crystalline structure of cellulose and the presence of lignin tightly linked to the hemicellulose create a rigid structure that resists bioconversion (Aita et al., 2011). Therefore, a pretreatment technology is needed in order to disrupt the recalcitrant nature of lignocellulosic biomass (Haghighi et al., 2013). Unless some type of pretreatment is performed prior to enzymatic hydrolysis, no more than 20% cellulose digestibility can be achieved (Mosier et al., 2005).

There are still inherent shortcomings with each type of pretreatment available to date (Pérez et al., 2008). Among all the available technologies, ammonia-based pretreatments have some advantages in that they are non-corrosive, non-pollutant and non-toxic, remove lignin, and preserve most carbohydrates with minimal generation of by-products. These by-products (i.e., furans, carboxylic acids, phenolic compounds) can inhibit enzymes and microorganisms during enzymatic hydrolysis and downstream fermentation processes (Behera et al., 2014). Ammonia-based pretreatment technologies are versatile in terms of applied temperature, residence time and ammonia to biomass ratio. In addition, the volatility of ammonia facilitates its recovery post pretreatment. Residual amounts of ammonia can serve as a nitrogen source for microorganisms during fermentation (Kim et al., 2008; Salvi et al., 2010; Tae Hvun et al., 2006; Wyman et al., 2005). Ammonia-based pretreatments can increase the porosity and surface area of the biomass due to swelling. Furthermore, ammoniation of the methoxyl groups present in lignin prevents the lignin from non-productively binding to cellulases (Yoo et al., 2011).

Choosing the right crop for a bioconversion process is crucial since 60-75% of the total cost of biofuel production is allotted to the purchase price of the feedstock (Salassi et al., 2014). Energy cane has great potential as an energy crop and it is a cross breed between sugarcane and

its wild ancestors. It is more resistant to cold, drought and disease than sugarcane (Kim and Day, 2011). Variety Ho 02-113 (used in this study) is a non-commercial energy cane variety with a replanting period of seven years compared to three years for sugarcane (Salassi et al., 2014). This variety yields 9% Brix in pressed juice, a fiber content of 264 g/kg (dry basis) and a cane yield of 88.9 t/ha (dry basis) as compared to sugarcane commercial variety LCP 85-384 which has 13.5% Brix in pressed juice, a fiber content of 135 g/kg (dry basis) and a cane yield of 69.2 t/ha (dry basis) (Knoll et al., 2013; Kim and Day, 2011).

Response surface methodology (RSM) is a statistical modeling technique that determines a multivariate equation by utilizing quantitative data from an appropriately designed experiment (Bas and Boyacı, 2007). This modeling technique solves the equation by finding an optimal response. RSM has become a practical method for optimizing different chemical and biochemical processes (Bas and Boyacı, 2007). The main advantage of RSM is minimizing the number of experimental trials required to evaluate the effect of multiple variables as well as their interactions (Kim and Han, 2012).

This study is the first one to evaluate the interactive effect of liquid ammonia pretreatment variables including temperature, residence time, and ammonium hydroxide to biomass ratio on the glucose and xylose yields from energy cane bagasse. Subsequently, these variables were optimized for maximum sugar yield (glucose, xylose) using RSM modeling technique.

2. Material and Methods

2.1. Biomass

Energy cane non-commercial variety Ho 02-113 was bred in Houma, LA through collaboration between the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) and the Sugar Research Station at Louisiana State University Agricultural

Center in St. Gabriel, LA. Energy cane was harvested at the Sugar Research Station and the entire plant was milled three times using a roller press (Farrel Company, Ansonia, CT) to extract the juice. The remaining solids or fibrous material (bagasse) was oven dried at 45 °C overnight to a final moisture content of 10%. Partially dried energy cane bagasse was milled (Wiley Mill, Arthur Thomas Co, PA), sieved (2 mm mesh sieve) and stored at -20 °C until further use.

2.2. Experimental design and statistical analysis

Central Composite Design (CCD) was employed utilizing the software Design-Expert 9.0.3 (State Ease Inc., Minneapolis, MN) to assess the effect of temperature, residence time and ammonium hydroxide to biomass ratio on the glucose yield of pretreated energy cane bagasse. CCD consists of 2^k factorial points, $2k$ axial points ($\pm\alpha$), and six center points for replications, where k is the number of variables. The range and levels of independent variables are shown in Table 1. Range of independent variables (temperature, residence time and ammonium hydroxide to biomass ratio) were selected based on published literature and preliminary studies. A total of 20 experiments were performed in duplicate and shown in Table 2. Center points of the design were replicated six times to estimate the pure error sum of squares. A quadratic polynomial equation (Equation (1)) was assumed to approximate the true function.

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j + \epsilon_i \quad (1)$$

Where Y , response variable; X_i and X_j , the explanatory variables; β_0 , constant coefficient; β_{ij} , two factor interaction coefficient; ϵ , random error. Significance of each coefficient was evaluated with analysis of variance (ANOVA).

“Table 1 goes here”

Xylose yields collected from experimental data were used to statistically optimized the pretreatment conditions for maximum xylose yield (independently from glucose yield) using the software Design-Expert 9.0.3.

2.3. Liquid ammonia pretreatment

Liquid ammonia pretreatment of energy cane bagasse was carried out in a 4 L stirrer reactor (Autoclave Engineers, Erie, PA). Experiments were carried out at ammonium hydroxide (NH₄OH, 28% v/v solution, Fisher Scientific, Pittsburgh, PA) to dried biomass to water ratio of 0-0.5:1:20. Pretreatment temperatures ranged from 160 to 220 °C and residence times ranged from 30 to 60 min. Residence time was monitored once the desired temperature had been reached. At the end of each pretreatment, the bioreactor was cooled down to room temperature using cold water spray to accelerate the process. The pretreated material was passed through a stainless steel sieve (0.2 mm mesh) to separate the liquid and solid fractions. The solid fraction was used for chemical composition analysis and enzymatic hydrolysis assessments. All pretreatments were carried out in duplicate and the mean values calculated.

2.4. Chemical composition analysis

Untreated energy cane bagasse and liquid ammonia pretreated energy cane bagasse samples (solid fraction) were analyzed for glucan, xylan, lignin, arabinan, mannan, and also ash content following NREL's Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42622). NREL reference material 8491 (for sugarcane bagasse) was analyzed as an internal sample to ensure the accuracy of the procedures.

2.5. Enzymatic hydrolysis

Enzyme concentrations used were kept constant for all the treatments in order to evaluate the effect of pretreatment on biomass digestibility. Spezyme CP (Genencor, Danisco US Inc.,

Rochester, NY, USA), and Novozyme 188 (Sigma–Aldrich, Inc., St. Luis, MO, USA) were used in combination during enzymatic hydrolysis. Activity of the enzymes were measured following Ghose, (1987) method for cellulase activity and Bailey et al. (1992) method for xylanase activity. Subsequently, Spezyme CP at 30 FPU/g glucan and Novozyme 188 at 30 CBU/g glucan were used in enzymatic hydrolysis of samples following NREL LAP TP-51043629. A biomass loading of 5% (w/v) was mixed with 0.1 M sodium citrate buffer (pH 4.8) and incubated at 50 °C in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 150 rpm for 72 h. Samples were taken at 0 h (before the addition of enzymes), 24 h, 48 h, and 72 h. Samples were frozen at -20 °C until further analysis. Enzymatic hydrolysis experiments were carried out in triplicate and the mean values calculated.

2.6. Chemical composition of hydrolyzed samples

All collected samples from enzymatic hydrolysis were centrifuged at 10,000 rpm (Spectrafuge 24D, Labnet International Inc., Woodbridge, NJ) for 5 min and filtered (0.2 µm Syringe Filters, Environmental Express Inc., Mt. Pleasant, SC). Properly diluted samples were analyzed for sugars by high performance liquid chromatography (HPLC) (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), lead form, 3000 × 7.8 mm (ID), 9 µm column and a differential refractive index detector (G1362A Agilent).

Equation 2 and 3 as described in NREL’s LAP TP-510-43630 was used to calculate percent theoretical yield for cellulose and hemicellulose, respectively.

$$\% \text{ Theoretical Cellulose Digestibility} = \frac{[\text{Glucose}] + 1.053[\text{Cellobiose}]}{1.111 f [\text{Biomass}]} \times 100\% \quad (2)$$

$$\% \text{ Theoretical Hemicellulose Digestibility} = \frac{[\text{Xylose}] 0.9 + 0.9[\text{Arabinose}]}{1.136 f [\text{Biomass}]} \times 100\% \quad (3)$$

Where [Glucose], residual glucose concentration (g/L); [Cellobiose], residual cellobiose concentration (g/L); 1.053, multiplication factor that converts cellobiose to equivalent glucose;

1.111, converts cellulose to equivalent glucose; [Biomass], dry biomass concentration at the beginning of the fermentation (g/L); f , cellulose fraction in dry biomass (g/g). [Xylose], residual xylose concentration (g/L); [Arabinose], residual arabinose concentration (g/L); 1.136, converts xylan to equivalent xylose.

Glucose and xylose yields were reported as the mass of glucose or xylose released per 100 g of untreated biomass after enzymatic hydrolysis and it was calculated using equation 4 as shown below.

$$\text{Glucose (xylose) yield} = 100 \times \text{recovered solids} \times 1.111(1.136) f \times \% \text{ theoretical cellulose (hemicellulose) digestibility} \quad (4)$$

3. Results and Discussion

3.1. Effect of liquid ammonia pretreatment on the chemical composition of biomass

Results from the compositional analysis of energy cane bagasse before and after pretreatment are shown in Table 2. Untreated energy cane bagasse was composed of $40.14 \pm 0.16\%$ glucan, $24.23 \pm 0.51\%$ xylan, $2.76 \pm 0.04\%$ arabinan, $24.41 \pm 0.37\%$ lignin (5.97 ± 0.16 acid soluble, 18.44 ± 0.21 acid insoluble), 3.76 ± 0.62 extractives, and $4.7 \pm 0.04\%$ ash (dry weight). These results are in agreement with those reported by Aita et al. (2011) and others (Aita et al., 2011; Benjamin et al., 2014; Knoll et al., 2013; Qiu et al., 2012).

“Table 2 goes here”

3.1.1. Recovered solids

All pretreatments caused considerable solid losses. A quadratic model was significant ($p < 0.01$) for determining the effect of all variables on the solid recovery of pretreated samples with $R^2 = 0.97$ and adjusted $R^2 = 0.94$ (Table A.1). Recovered solids ranged from 55.50% to 74.01% (Table 2) depending on the severity of the pretreatment conditions. Similar findings for

solid recovery (75.90%) of energy cane bagasse pretreated with dilute ammonia at 160 °C for 60 min and ammonium hydroxide to biomass to water ratio of 0.5:1:8 has been reported by Aita et al. (2011). Ammonium hydroxide to biomass ratio had a significant positive effect on solid recovery. However, our model showed that temperature had the most significant effect (as confirmed by the strong negative correlation observed, -0.88) on recovered solids (Table 3). The interactive effect of temperature and time on solid recovery at an ammonium hydroxide to biomass ratio of 0.25:1 (Figure 1) showed that the amount of recovered solids decreased with the increase of pretreatment temperature. However, residence time had no significant effect on recovered solids. Qiu et al. (2014) reported the same significant effect of pretreatment temperature on the solid recovery of ionic liquid pretreated energy cane bagasse. Jiang et al. (2015) observed a linear relationship between the severity of liquid hot water pretreatment and solid recovery of cotton stalk. Solid loss in ammonia-based pretreatments can be attributed mostly to hemicellulose solubilization followed by lignin removal. In our study, this was confirmed by the high correlation (-0.81) seen between xylan loss and solid recovery followed by a correlation of -0.40 between lignin removal and solid recovery (Table 3).

“Table 3 goes here”

“Figure 1 goes here”

3.1.2. Lignin removal

Lignin is known for its underlying recalcitrant nature in biomass. Thus lignin removal is one of the main factors defining the effectiveness of a pretreatment. Lignin was significantly removed by all the pretreatment conditions evaluated in this study. Lignin removal ranged from 35.96% to 66.85% (Table 2). A 2FI model (two factor interaction model) was significant for the effect of pretreatment variables on lignin removal ($p < 0.01$) with $R^2 = 0.91$ and adjusted $R^2 = 0.87$

(Table A.2). The interaction effect of temperature and ammonium hydroxide to biomass ratio as well as the interaction effect of residence time and ammonium hydroxide to biomass ratio posed a significant effect on the lignin removal of pretreated samples (Table A.2). For example, increasing the ammonium hydroxide to biomass ratio from 0:1 to 0.5:1 at a residence time of 40 min resulted in an increase in lignin removal from 44.88% to 64.23%. Similarly, at an ammonium hydroxide to biomass ratio of 0.25:1 and a residence time of 40 min, increasing the temperature from 160°C to 220 °C increased lignin removal from 40.51% to 60.83%, respectively. Ammonia-based pretreatments are known to preferentially remove the lignin as compared to some other methods such as acid and hydrothermal pretreatments (Gírio et al., 2010). Ammonia removes the lignin from the lignocellulosic matrix through the saponification of ester bonds present between the lignin and the carbohydrates (Kim et al., 2003). However, the location of the lignin within the biomass combined with its hydrophobicity and its strong polymering bonds make the complete removal of lignin difficult to achieve (Kim et al., 2003). Qin et al. (2013) reported the effect of ammonia on the dissociation of ester linkages between lignin and hemicellulose, the disruption of the C-O bonds of guaiacyl units in lignin, and the ammonolysis of the acetyl groups present in hemicellulose in ammonia treated corn stover. In our study, lignin removal was enhanced by the addition of ammonium hydroxide at any pretreatment temperature and at 45 min residence time (Figure 2). This is due to the effect of ammonia in reducing the solubilization temperature of lignin (Aita et al., 2011). Ko et al. (2009) reported a maximum of 60.60% lignin removal for rice straw soaked in 20% w/w ammonia for 12 h at 70 °C. Chen et al. (2012) reported a 48.00% lignin removal and a 10.00% cellulose loss for microwave-assisted ammonia pretreated sorghum bagasse at 160 °C for 1 h and at a biomass, ammonium hydroxide (28% v/v solution) and water ratio of 1:0.5:8, respectively.

“Figure 2 goes here”

3.1.3. Xylan Loss

Alkaline pretreatments including ammonia-based pretreatments work non-selectively toward lignin and can result in the removal of the hemicellulose along with the lignin (Gírio et al., 2010). A positive correlation of 0.62 was observed between xylan loss and lignin removal meaning that more xylan was being solubilized as more lignin was removed. Dilute ammonia pretreatment of sorghum bagasse at 160 °C, for 60 min, and at an ammonium hydroxide to biomass ratio of 0.14:1 resulted in a considerable loss of hemicellulose (35.00%) simultaneously with 44.00% lignin removal (Salvi et al. 2010). In general, the harsh pretreatment conditions required to achieve an effective lignin removal do not overlap with the milder conditions under which hemicelluloses can be preserved (Gírio et al., 2010). In our study, a linear model was significant ($p < 0.01$) for xylan loss with $R^2 = 0.88$ and adjusted $R^2 = 0.86$. Temperature was the only variable posing a significant effect on the response at 0.01 significance level. Figure 3 shows the contour plot of the combined effect of pretreatment temperature and residence time on xylan loss. Confirmed by the strong correlation observed (0.93), increasing the temperature from 160 °C to 220 °C resulted in xylan losses ranging from 27.94% to 95.79%, respectively. Xylan solubilization starts around 180 °C and increases as temperature goes up (Hendriks and Zeeman, 2009). Bahcegul et al. (2012) reported an improvement in hemicellulose extraction from cotton stock when alkaline pretreatment temperatures were increased. Removal of hemicellulose in liquid hot water pretreated sugarcane bagasse was increased from 8.40% to 100.00% when temperatures were increased from 140 °C to 200 °C (Yu et al., 2013). Kim et al. (2009) reported retaining 80.00% of the hemicellulose and 100.00% of the cellulose in the solid fraction of liquid ammonia pretreated corn stover by using lower temperatures (30-80 °C) and longer residence

times (4-24 h). Pérez et al. (2008) reported that no hemicellulose remained in the solid fraction when the temperature of the liquid hot water pretreatment of wheat straw was increased to 220 °C.

Extensive hemicellulose removal during hydrothermal pretreatment of biomass leads to structural changes in the biomass and modification of lignin distribution in the biomass (Donohoe et al., 2008). A 2D NMR spectra from aqueous ammonia pretreated miscanthus showed that ammonia pretreatment resulted in the deacetylation of the xylan backbone. Moreover, ammonia was shown to hydrolyze the carboxyl-ester linkages between the lignin units and the hemicellulose. This would leave the pretreated biomass with high amounts of cellulose (Qin et al., 2013).

“Figure 3 goes here”

3.1.4. Glucan loss

Cellulose sensitivity to temperature is less as compared to hemicellulose and lignin. However, the more severe the ammonia pretreatment the more cellulose is removed (Jung et al., 2011). As compared to the losses observed with xylan and lignin, only a small amount of glucan (4.49% to 12.95%) was lost during pretreatments with ammonium hydroxide. Aita et al. (2011) reported a 9.00% sugar loss in energy cane bagasse pretreated at 160 °C for 60 min with ammonium hydroxide (1:0.5:8 ratio of dry biomass to ammonium hydroxide to water). A quadratic model was found significant for the effect of pretreatment parameters on glucan loss ($p < 0.01$) with $R^2 = 0.99$ and adjusted $R^2 = 0.98$ (Table A.3). Ammonium hydroxide to biomass ratio was found to be the dominant variable followed by temperature. The effect of residence time was only effective at the quadratic level. Interactive effect of all variables was found to be significant for glucan loss ($p < 0.01$).

3.2. Statistical analysis and model fitting

Glucose yield was used to fit the model using Design–expert 9.0.3. The quadratic model was chosen due to the observed high adjusted R-squared and predicted R-squared values (Table 4). Credibility of the fitted quadratic model was confirmed by lack of the fit test (Table 4). Coefficients of the second order polynomial equation (Equation 1) for glucose yield were calculated by Design-expert 9.0.3 and the final equation (Equation 5) is presented below.

$$\text{Glucose yield} = +27.51 + 3.64A + 0.83B + 1.64C - 2.04AB - 1.05A^2 - 1.24B^2 \quad (5)$$

Where, A: Temperature (°C), B: Time (min), C: Ammonium hydroxide to biomass ratio.

Analysis of variance (Table 4) was performed to evaluate the significance of the developed model as well as each of the coefficients (p-value<0.01). P-value of the model was less than 0.0001 meaning that there is only 0.01% chance that the model was fit due to the noise. Additionally, all the pretreatment parameters had a significant relationship with the response while only time and temperature interaction was significantly effective at 99% confidence interval. Reliability and precision of the experiment was confirmed by a low coefficient of variation (3.60). A 20.73 signal to noise ratio was gained from our model. Adequate precision represents the signal to noise ratio with a minimum desired number of four. Additionally, a strong correlation between actual and predicted data was confirmed by the high correlation coefficient ($R^2 = 0.97$) observed, meaning the model fails to explain only 3% of the total variance. The normality of residuals was confirmed by the normal probability plot of the studentized residuals as shown in Figure 4.

“Table 4 goes here”

“Figure 4 goes here”

3.3. Cellulose and hemicellulose digestibility and sugar yield

Cellulose digestibility of our samples ranged from 41.77% to 76.11% as compared to 9.27% in untreated samples. Three-dimensional response surface graphs were plotted based on the developed quadratic model within the range of experimental design (Figure 5 and Figure 6). All the pretreatment variables had a significant effect on glucose yield ($p < 0.01$). Among the variables, temperature had the highest correlation (0.77) (Table 3). At lower temperatures, for example 160 °C, glucose yield increased from 9.14 g/L to 21.53 g/L as residence time increased from 30 to 60 min. However, at higher temperatures, extending the residence time had a negative effect on the glucose yield (Figure 5). The negative effect of residence time was more significant as temperatures increased from 190°C to 220 °C. At 220 °C, increasing the residence time from 30 to 60 min resulted in a drastic drop in glucose yield from 32.41 g/L to 17.22 g/L. Longer residence times at high temperatures favor the formation of degrading products from lignocellulosic components. Harsh pretreatment conditions degrade pentose and hexose sugars into furfural and 5-hydroxymethyl furfural, respectively (Jiang et al., 2015). Moreover, hydrolysis of acetyl groups present in the hemicellulose results in the production of organic acids, mainly acetic acid, formic acid and levulinic acid (Nichols et al., 2008). Xylo-oligomers, from the incomplete break down of hemicellulose, can compete with cellulose over the binding to hydrolyzing enzymes thus blocking their activity (Qing et al., 2010; Zhang et al., 2012). As illustrated in Figure 5, the combination of a higher temperature and a shorter residence time improved the glucose yield (larger slope of the response line) as compared to the combination of a lower temperature and a longer residence time.

Our results are in agreement with those reported by Qin et al. (2013) on ammonia pretreatment of corn stover where glucose yield was primarily affected by temperature. They

also reported the same less significant effect of residence time, at temperatures below 180 °C, as compared to other pretreatment variables (temperature, ammonia concentration and solid loading). A cellulose digestibility of 86.80% was reported in corn stover pretreated at 180 °C for 60 min with 20% w/w ammonium hydroxide and hydrolyzed with 60 FPU cellulase loading (Qin et al., 2013). A similar dominant effect of temperature as compared to acid concentration and residence time was reported in RSM optimization of dilute phosphoric acid pretreatment of corn stover (Avci et al., 2013). Increasing the ratio of ammonium hydroxide to biomass also resulted in a better cellulose digestibility (Figure 6). For example, pretreatment of energy cane bagasse at 190 °C for 45 min containing no ammonium hydroxide resulted in a glucose yield of 23.15 g glucose /100 g (dry weight) untreated biomass and 44.88% lignin removal. However, glucose yield was improved to 28.89 and 29.91 g glucose/100 (dry weight) untreated biomass when ammonium hydroxide was added to the mix at an ammonium hydroxide to biomass ratio of 0.25:1 and 0.5:1, respectively. This can be explained by the effect of ammonium hydroxide on lignin removal with observed values of 53.41% and 64.23%, respectively.

“Figure 5 goes here”

“Figure 6 goes here”

A negative effect was observed between recovered solids and glucose yield. By decreasing the recovered solids from 74.01% to 55.50%, the corresponding glucose yield increased from 18.74 to 25.74 g glucose /100 g (dry weight) untreated biomass. This was expected as decreasing the recovered solids in the pretreated samples can be translated to higher lignin removal and hemicellulose solubilization. Similar observations were reported by Hongdan et al. (2013) in liquid hot water pretreatment of sugarcane bagasse. It was reported that increasing the pretreatment temperature from 160 to 200 °C resulted in a decrease in recovered solids from

86.16% to 62.60%. The decrease in solid recovery was accompanied by an increase in sugar yield as well as the generation of by-products (acetic acid, furfural and 5-hydroxymethyl furfural) in the pretreatment hydrolysate.

Glucose yield showed the highest positive correlation with lignin removal (0.81) followed by hemicellulose loss (0.72) (Table 3), indicating the importance of removing lignin to improve enzymatic digestibility (Hendriks and Zeeman, 2009; Jeoh et al., 2007). Yu et al. (2013) and Gao et al. (2013) also reported that lignin removal had a more prominent effect on the enzymatic digestibility of sugarcane bagasse regardless of pretreatment type. Ko et al. (2009) also reported a strong correlation ($R^2 = 0.93$) between lignin removal and cellulose digestibility in aqueous ammonia soaking of rice straw. Kim and Han (2012) reported a high degree of correlation ($R^2 = 0.88$) between lignin removal and enzymatic digestibility of alkaline (sodium hydroxide) pretreated rice straw. Ammonia can disrupt the linkages between lignin, cellulose and hemicellulose leaving the cellulose available to enzymes (Chen et al., 2012; Li and Kim, 2011). Furthermore, lignin can non-productively bind to cellulases or decrease the reaction rate by interfering during enzymatic hydrolysis (Zabed et al., 2016; Saini et al., 2016). Therefore, removing lignin enhances the enzymatic digestibility of cellulose.

Studies have shown that as important as the lignin removal is on improving enzyme digestibility, location and distribution of lignin plays an equally important role. Donohoe et al. (2008) reported that increasing the temperature above the lignin phase transition (120-200 °C) during thermochemical pretreatment of biomass can cause the molten lignin to merge into larger droplets and migrate to the surface of the plant cell walls. After pretreatment, these relocated lignin droplets can deposit on the surface of the biomass and provide a physical barrier for the hydrolyzing enzymes (Kaparaju and Felby, 2010). Aita et al. (2011) observed drastic

morphological changes in aqueous ammonia pretreated energy cane bagasse that included disruption of the rigid structure of the biomass, increase in the surface area of the biomass as well as the formation of micropores. All these changes favor enzyme accessibility to the pretreated biomass which translates into higher cellulose digestibility.

It was also reported that since cellulose and hemicellulose are physically connected, removal of hemicellulose can potentially enlarge the pore sizes present on the cellulose structure and increase cellulose accessibility to hydrolyzing enzymes (Kaparaju and Felby, 2010). Cao et al. (2013) reported a cellulose digestibility of 66.00% for ammonium hydroxide pretreated sugarcane bagasse at 1:20:0.5 bagasse to water to ammonium hydroxide ratio with 3% Tween 80 and hydrolyzed with 30 FPU Spezyme CP along with 30 CBU Novoxyme 188. Kim and lee (2005) reported a 53.60% solid recovery after pretreatment of corn stover with ammonia recycle percolation (15 wt% ammonia, 170 °C, 5 mL/min flow rate and 2.3 MPa), 84.70% lignin removal and 60.00% hemicellulose solubilization resulting in a 93.30% cellulose digestibility with 60 FPU cellulase enzyme activity. Despite the fact that hemicellulose provides a physical barrier for cellulases, more hemicellulose removal does not necessarily translate to better cellulose digestibility. Yang et al. (2013) studied the correlation between hemicellulose removal and cellulose digestibility on hulless oat straw and observed that the highest bioconversion was achieved while still 33.00% hemicellulose remained intact.

Hemicellulose digestibility of our samples ranged from 28.28% to 79.83% as compared to 4.40% in untreated samples (Table 2). Hemicellulose digestibility of pretreated samples was lower than those observed for cellulose. This can be attributed to the composition of the enzymes cocktail used in this study which mostly contained cellulases over xylanases. Xylose yields of pretreated samples were low and ranged from 0.71 to 7.74 g xylose/ 100g (dry weight) untreated

biomass. The low xylose yields stemmed from the low amounts of hemicellulose present in the biomass post ammonium hydroxide pretreatment. A xylose yield of 7.74 g xylose/100g (dry weight) untreated biomass was achieved when 67.04% of the xylan was preserved at 172 °C, for 36 min, and at an ammonium hydroxide to biomass ratio of 0.4:1, and resulted in a xylose digestibility of 42.55% (Table 2). However, under these pretreatment conditions only 35.96% of the lignin was removed which resulted in a poor cellulose digestibility (48.19%) and a low glucose yield (19.28 g glucose/100g (dry weight) untreated biomass). A negative correlation was observed between glucose yield and xylose yield (Table 3) as pretreatment conditions for their optimum sugar yields did not overlap.

A quadratic model was fitted ($p < 0.01$) based on the xylose yield of our samples with $R^2 = 0.95$ and adjusted $R^2 = 0.91$ (Table A.4). The model showed that temperature and the ratio of ammonium hydroxide to biomass were the most significant variables ($p < 0.05$). Figure 7 shows the contour plot effect of temperature and ammonium hydroxide to biomass ratio on xylose yield. Pretreatment temperature had a negative and close to linear relationship with xylose yield (correlation of -0.79) (Table 3). The opposite was observed for pretreatment temperature and glucose yield having a strong positive correlation (0.77) (Table 3). An indication that while increasing the pretreatment temperature improved the glucose yield, it caused a decrease in xylose yield. Yu et al. (2013) reported an increase in total xylose yield (including monomeric sugars and xylo-oligomers) by increasing the temperature of liquid hot water pretreatment of sugarcane bagasse up to 160 °C. However, increasing pretreatment temperature above 180 °C resulted in a significant decrease in the xylose yield while xylan loss was also increased. They also observed that the same increase in pretreatment temperature (from 160°C to 180 °C) resulted in a drastic increase in the generation of degrading products from hemicellulose.

391 Additionally, by the same increase in temperature, the concentration of xylo-oligomers in the
392 hydrolysate was decreased in favor of an increase in the concentration of xylose; meaning that no
393 oligomeric sugars were found in the pre-hydrolysate as the pretreatment temperature approached
394 200 °C. The drop observed in the xylose yield can be justified by the inhibitory effect of these
395 compounds on the hydrolyzing enzymes. There are other strategies suggested to preserve both
396 pentose and hexose sugars for maximum total sugar yield. Kim and Lee (2005) investigated the
397 effect of a two-stage pretreatment process (hot water followed by ammonia percolation) in corn
398 stover. Liquid hot water pretreatment (190 °C, 30 min) removed 84.00% of the xylan which can
399 be recovered from the liquid fraction. The lignin-rich solid fraction was further treated with
400 ammonia recycle percolation (170 °C, 60 min, at 2.3 MPa and 15% NH₃ at 5 mL/min of flow
401 rate) resulting in a 75.00% lignin removal. A 93.60% cellulose digestibility was observed with
402 the two-stage pretreatment process as compared to 94.40% cellulose digestibility for the one-
403 stage ammonia recycle percolation pretreatment.

404 The highest combined total amount of fermentable sugars (glucose and xylose) was achieved
405 at 190 °C, for 45 min, and at an ammonium hydroxide to biomass ratio of 0.25:1, where 28.89 g
406 of glucose along with 7.22 g of xylose were obtained for every 100 g (dry weight) of untreated
407 biomass (Table 2). Under these conditions, about 50.22% lignin was removed and 54.50% of
408 xylan was solubilized.

409 “Figure 7 goes here”

410 3.4. Optimization of process conditions and experimental validation

411 Optimal pretreatment conditions for maximum glucose yield (as our foremost sugar) were
412 predicted using Design-expert 9.0.3. Data related to the predicted points as well as the results
413 from the experimental evaluation of these points are summarized in Table 5. Based on these

defined conditions, the optimum pretreatment parameters for maximum glucose yield were 208 °C, for 36 min and at an ammonium hydroxide to biomass ratio of 0.4:1. The predicted value for glucose yield was 30.77 ± 0.93 g glucose/100 g (dry weight) untreated biomass.

Based on our model for xylose yield, the maximum xylose yield within our experiment design range was predicted as 9.10 g xylose/100 g (dry weight) untreated biomass at 160 °C, for 60 min and at an ammonium hydroxide to biomass ratio of 0.31:1. However, the glucose yield obtained under these conditions was 23.34 g glucose/100g (dry weight) untreated biomass. Experimental results fell within the 95% confidence interval of the predicted value which supported the reliability and robustness of our model (Table 5).

“Table 5 goes here”

4. Conclusions

Pretreatment parameters including temperature, residence time and ammonium hydroxide to biomass ratio were optimized for maximum sugar yields from energy cane bagasse. Temperature was found to be the most effective variable having the greatest impact on the response followed by ammonium hydroxide to biomass ratio. A quadratic model was fitted (P value < 0.01) with the experimental values and the generated equation was used to find the optimum value for each variable. The highest glucose yield was predicted to be 30.77 g glucose/ 100 g (dry weight) untreated biomass with a xylose yield of 3.99 g xylose/100 g (dry weight) untreated biomass obtained at 208°C for 36 min and at an ammonium hydroxide to biomass ratio of 0.4:1. A separate quadratic model (P value < 0.01) was fitted for the experimental values of xylose yield. Based on the model, the maximum xylose yield was predicted to be 9.10 g xylose/100 g (dry weight) untreated biomass obtained at 160°C for 60 min and at an ammonium hydroxide to biomass ratio of 0.31:1. However, these pretreatment conditions resulted only in a predicted

437 glucose yield of 23.34 g glucose/ 100 g (dry weight) untreated biomass. Results of actual
438 experiments carried out at the optimal points from our models fell within 95% confidence
439 interval of the predicted value. The low xylose yields observed in this study can be attributed to
440 the substantial amounts of xylan being lost during pretreatment due to solubilization.

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Table 1

Variables	Unit	Coded level					
		Coding	$-\alpha^a$	-1	0	+1	$+\alpha^a$
Temperature	°C	A	160	172	190	208	220
Residence Time	min	B	30	36	45	54	60
Ammonium Hydroxide ^b to Biomass Ratio	v/w	C	0	0.10	0.25	0.40	0.50

^a Axial distance = $\sqrt[4]{N}$, where N is the number of experiments of the factorial design.

^b H₄OH, 28% v/v solution

Table 2

Table 2. Composition analysis and sugar yields of energy cane bagasse.

Samples	Experimental Variables			Recovered Solids (% w/w)	Glucan (% w/w)	Xylan (% w/w)	Lignin (% w/w)	Cellulose Digestibility (% w/w)	Hemicellulose Digestibility (% w/w)	Glucose Yield (g/ 100 g of untreated biomass)	Xylose Yield (g/ 100 g of untreated biomass)	Glucan Loss (% w/w)	Xylan Loss (% w/w)	Lignin removal (% w/w)
	Temperature (°C)	Time (min)	^a Ratio											
Untreated	^b N/A	N/A	N/A	100	40.14	24.23	24.41	9.27	4.36	4.13	1.22	0	0	0
1	208	54	0.3:1:20	62.74	61.10	2.20	23.52	69.36	57.43	29.54	0.91	4.49	94.18	66.85
2	190	45	0.5:1:20	63.50	58.41	8.69	22.32	72.54	68.51	29.91	4.29	7.59	76.77	64.23
3	190	30	0.25:1:20	67.09	54.57	14.89	13.98	55.66	49.30	22.65	5.63	8.77	57.93	42.85
4	208	54	0.1:1:20	55.50	63.19	2.06	16.86	66.05	52.86	25.74	0.71	12.62	95.19	42.74
5	190	45	0:1:20	59.22	59.08	18.39	16.61	59.56	28.28	23.12	3.50	12.95	56.13	44.88
6	208	36	0.1:1:20	56.81	62.07	6.57	18.11	76.11	79.83	29.82	3.40	12.14	84.29	46.86
7	190	45	0.25:1:20	64.34	56.33	16.79	17.14	71.75	58.67	28.89	7.22	9.71	54.50	50.22
8	190	45	0.25:1:20	62.90	57.62	15.57	17.85	67.45	61.00	27.16	6.81	9.71	58.77	51.11
9	220	45	0.25:1:20	56.42	64.70	1.77	23.77	74.34	52.02	30.15	0.65	9.06	95.79	60.83
10	190	45	0.25:1:20	62.34	58.11	12.60	17.09	66.21	59.43	26.65	5.31	9.75	66.92	48.54
11	172	54	0.4:1:20	69.11	52.33	20.74	15.79	64.73	46.15	26.02	7.48	9.89	39.63	49.69
12	208	36	0.4:1:20	62.91	58.20	7.81	19.90	75.20	75.35	30.59	4.23	8.78	87.26	56.86
13	172	36	0.1:1:20	68.63	54.00	17.78	12.54	41.77	36.76	17.20	5.11	7.67	48.59	39.40
14	190	60	0.25:1:20	67.65	54.02	14.06	18.56	62.01	53.56	25.18	5.78	8.95	59.95	57.01
15	172	54	0.1:120	72.88	48.01	14.90	12.38	57.13	51.86	22.21	6.42	12.83	54.26	41.27
16	190	45	0.25:1:20	65.86	55.25	14.70	16.45	65.08	54.52	26.31	6.04	9.34	59.24	49.35
17	160	45	0.25:1:20	74.01	48.60	23.12	11.97	46.89	37.07	18.74	7.19	10.37	27.94	40.51
18	172	36	0.4:1:20	71.09	50.65	22.39	11.02	48.19	42.55	19.28	7.74	10.30	32.96	35.96
19	190	45	0.25:1:20	63.29	57.03	15.93	17.86	67.75	56.85	27.17	6.46	10.08	57.55	51.43
20	190	45	0.25:1:20	63.67	56.99	15.25	18.45	71.78	58.71	28.94	6.53	9.60	59.12	53.41

Note: Data shown are the mean value (n=2 for pretreatments and n=3 for enzymatic hydrolysis; standard error < 5%)

^a Ammonium hydroxide (NH₄OH, 28% v/v solution): bagasse (dry weight): water

^b Not applicable

Table 3

Table 3. Correlation coefficients between variables and responses.									
	Temperature	Time	Ammonium Hydroxide to Biomass Ratio	Glucose Yield	Lignin Removal	Xylan Loss	Glucan Loss	Recovered Solids	Xylose Yield
Temperature	1	0	0	0.77	0.60	0.93	-0.15	-0.88	-0.79
Time	0	1	0	0.17	0.34	0.10	0.04	0.02	-0.12
Ammonium Hydroxide to Biomass Ratio	0	0	1	0.34	0.53	0	-0.65	0.23	0.17
Glucose Yield	0.77	0.17	0.34	1	0.80	0.72	-0.22	-0.66	-0.39
Lignin Removal	0.60	0.34	0.53	0.80	1	0.62	-0.62	-0.40	-0.42
Xylan Loss	0.93	0.10	0.02	0.72	0.62	1	-0.20	-0.81	-0.88
Glucan Loss	-0.15	0.04	-0.65	-0.22	-0.62	-0.20	1	-0.11	0.14
Recovered Solids	-0.88	0.02	0.23	-0.66	-0.40	-0.81	-0.11	1	0.75
Xylose Yield	-0.79	-0.12	0.17	-0.39	-0.42	-0.88	0.14	0.75	1

Table 4

Table 4. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on glucose yield g/ 100 g (dry weight) of untreated biomass.						
Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	297.45	9	33.05	38.52	< 0.0001	significant
A-Temperature	180.79	1	180.79	210.69	< 0.0001	
B-Time	9.50	1	9.50	11.07	0.0077	
C-Ammonium Hydroxide	36.62	1	36.62	42.67	< 0.0001	
AB	33.46	1	33.46	38.99	< 0.0001	
AC	0.084	1	0.084	0.098	0.7607	
BC	2.27	1	2.27	2.64	0.135	
A ²	15.82	1	15.82	18.43	0.0016	
B ²	22.11	1	22.11	25.76	0.0005	
C ²	1.45	1	1.45	1.69	0.2223	
Residual	8.58	10	0.86			
Lack of Fit	2.21	5	0.44	0.35	0.8644	not significant
Pure Error	6.37	5	1.27			
Std. dev.	0.93			R-squared	0.972	
Mean	25.74			Adj R-squared	0.947	
Coefficient of Variation%	3.60			Pred R-squared	0.915	
PRESS	26.1			Adeq precision	20.73	

Table 5

Table 5. Predicted versus experimental results.						
Response	Predicted mean	Observed	Std. dev.	SE Pred.	95% PI low	95% PI high
Glucose Yield (g/100 g (dry weight) untreated biomass)	30.77	30.62	0.96	1.20	28.10	33.43
Xylose Yield (g/100 g (dry weight) untreated biomass)	3.99	4.20	0.66	0.84	2.12	5.87
Recovered Solid (%)	63.71	62.51	1.23	1.59	60.17	67.24
Glucan Loss (%)	8.76	9.01	0.24	0.32	8.12	9.45
Xylan Loss (%)	83.27	84.14	7.48	8.25	65.54	100.28
Lignin Removal (%)	59.18	60.26	3.05	3.91	50.73	67.63

Note: Data shown represent the mean values (n=2; Standard error < 5%).

Table A.1. Appendix

Table A.1. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on recovered solids.						
Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	495.11	9	55.01	36.42	< 0.0001	significant
A-Temperature	393.77	1	393.77	260.67	< 0.0001	
B-Time	0.22	1	0.22	0.15	0.7110	
C-Ammonium Hydroxide	27.07	1	27.07	17.92	< 0.0017	
AB	1.76	1	1.76	1.16	0.3060	
AC	26.83	1	26.83	17.76	0.0018	
BC	3.24	1	3.24	2.14	0.1739	
A ²	4.81	1	4.81	3.18	0.1047	
B ²	25.86	1	25.86	17.12	0.0020	
C ²	8.89	1	8.89	5.88	0.0357	
Residual	15.11	10	1.51			
Lack of Fit	7.38	5	1.48	0.95	0.5195	not significant
Pure Error	7.73	5	1.55			
Std. dev.	1.23			R-squared	0.9704	
Mean	64.50			Adj R-squared	0.9437	
Coefficient of Variation%	1.91			Pred R-squared	0.8474	
PRESS	77.85			Adeq precision	21.213	

Table A.2. Analysis of variance (ANOVA) for response surface 2FI model for the effect of pretreatment variables on lignin removal.						
Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	1210.73	6	201.79	21.77	< 0.0001	significant
A-Temperature	482.08	1	482.08	52.00	< 0.0001	
B-Time	150.35	1	150.35	16.22	0.0014	
C-Ammonium Hydroxide	373.90	1	373.90	40.33	< 0.0001	
AB	12.00	1	12.00	1.30	0.2757	
AC	106.58	1	106.58	11.50	0.0048	
BC	85.81	1	85.81	9.26	0.0094	
Residual	120.51	13	9.27			
Lack of Fit	105.54	8	13.19	4.40	0.0596	not significant
Pure Error	14.97	5	2.99			
Std. dev.	3.04		R-squared	0.9095		
Mean	49.69		Adj R-squared	0.8677		
Coefficient of Variation%	6.13		Pred R-squared	0.6405		
PRESS	478.64		Adeq precision	17.970		

Table A.3. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on glucan loss.						
Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	73.74	9	8.19	138.09	< 0.0001	significant
A-Temperature	1.76	1	1.76	29.59	0.0003	
B-Time	0.11	1	0.11	1.8	0.2089	
C-Ammonium Hydroxide	31.72	1	31.72	534.65	< 0.0001	
AB	9.16	1	9.16	154.37	< 0.0001	
AC	15.62	1	15.62	263.32	< 0.0001	
BC	13.36	1	13.36	225.24	< 0.0001	
A ²	0.048	1	0.048	0.81	0.3903	
B ²	0.86	1	0.86	14.54	0.0034	
C ²	0.90	1	0.90	15.22	0.0030	
Residual	0.59	10	0.059			
Lack of Fit	0.31	5	0.063	1.13	0.4494	not significant
Pure Error	0.28	5	0.063			
Std. dev.	0.24		R-squared	0.9920		
Mean	9.37		Adj R-squared	0.9848		
Coefficient of Variation%	2.50		Pred R-squared	0.9609		
PRESS	2.91		Adeq precision	50.871		

Table A.4. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on xylose yield g/ 100 g (dry weight) of untreated biomass.						
Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	92.20	9	10.24	23.49	< 0.0001	significant
A-Temperature	60.73	1	60.73	139.28	< 0.0001	
B-Time	1.36	1	1.39	3.20	0.1040	
C-Ammonium Hydroxide	2.86	1	2.86	6.55	0.0284	
AB	6.66	1	6.66	15.28	0.0029	
AC	1.05	1	1.05	2.41	0.1516	
BC	0.45	1	0.45	1.03	0.3330	
A ²	10.44	1	10.44	23.95	0.0006	
B ²	0.67	1	0.67	1.53	0.2450	
C ²	10.44	1	10.44	23.95	0.0006	
Residual	4.36	10	0.44			
Lack of Fit	2.17	5	0.43	0.99	0.5031	not significant
Pure Error	2.19	5	0.44			
Std. dev.	0.66		R-squared	0.9548		
Mean	5.07		Adj R-squared	0.9142		
Coefficient of Variation%	13.02		Pred R-squared	0.7955		
PRESS	19.75		Adeq precision	15.679		

Figure 1

Figure 1. Contour plot of the combined effect of temperature and time on percent recovered solids at an ammonium hydroxide to biomass ratio of 0.25:1.

Figure 2

Figure 2. Contour plot of the combined effect of temperature and ammonium hydroxide to biomass ratio on lignin removal at 45 min residence time.

Figure 3

Figure 3. Contour plot of the combined effect of pretreatment temperature and residence time on xylan loss at 5% (w/w) solid loading with ammonium hydroxide to biomass ratio of 0.25:1.

Figure 4

Figure 4. Predicted versus actual values.

Figure 5

Figure 5. Three-dimensional response surface plot of the effects of pretreatment temperature and residence time on glucose yield (g glucose/ 100 g (dry weight) untreated biomass) at ammonium hydroxide to biomass ratio of 0.25:1.

Figure 6

Figure 6. Three-dimensional response surface plot of the effects of ammonium hydroxide to biomass ratio and pretreatment temperature at 45 min residence time.

Figure 7

Figure 7. Contour plot of the combined effect of pretreatment temperature and ammonium hydroxide to biomass ratio on xylose yield (g xylose/100 g (dry weight) untreated biomass at 45 min residence time.