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Dilute Ammonia Pretreatment of Sorghum and Its Effectiveness on Enzyme Hydrolysis and Ethanol Fermentation

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Abstract A new pretreatment technology using dilute ammonium hydroxide was evaluated for ethanol production on sorghum. Sorghum fibers, ammonia, and water at a ratio of 1:0.14:8 were heated to 160 °C and held for 1 h under 140–160 psi pressure. Approximately, 44% lignin and 35% hemicellulose were removed during the process. Hydrolysis of untreated and dilute ammonia pretreated fibers was carried out at 10% dry solids at an enzyme concentration of 60 FPU Spezyme CP and 64 CBU Novozyme 188/g glucan. Cellulose digestibility was higher (84%) for ammonia pretreated sorghum as compared to untreated sorghum (38%). Fermentations with *Saccharomyces cerevisiae* D_5A resulted in 24 g ethanol /100 g dry biomass for dilute ammonia pretreated sorghum and 9 g ethanol /100 g dry biomass for untreated sorghum.

Keywords Dilute ammonia pretreatment · Sorghum · Ethanol · Lignocellulosic · Biomass · Hydrolysis

Introduction

The definition of biomass refers to organic non-fossil material of biological origin constituting a renewable energy source [1]. Lignocellulose is a type of biomass and refers to any plant material produced by photosynthesis. Wood, sugarcane bagasse, sorghum, corn fibers, rice straw, wheat straw, barley straw, coconut husks and pineapple leaves are just a few examples [2, 3]. The major components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. The facility to make fuels and or other value added products from lignocellulosics depends on the ability to separate and to breakdown each of these structures into their main components glucose, xylose and phenols; respectively.

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Thermochemical pre-hydrolysis of biomass, typically referred to as pretreatment, is required to break down the structure of biomass and to increase its susceptibility to subsequent enzymatic hydrolysis by cellulase enzymes. A number of pretreatment methods have been developed for improving hydrolysis of lignocellulosics including physical disruption, solvent-based, dilute acid, autohydrolysis, wet-oxidation, biological and alkali treatment [4–7]. Most of these technologies suffer from relatively low sugar yields, severe reaction conditions, large capital investment, high processing costs, and great investment risks [8].

Ammonia-based pretreatments with or without heat and or pressure have shown great success in the delignification of lignocellulosics [3, 9–16]. Ammonia pretreatments remove lignin by cleaving C–O–C bonds in lignin and other ether and ester bonds in lignin-carbohydrate complex. Delignification of the biomass results in the release of cellulose and hemicellulose which after hydrolysis can be converted into ethanol by fermentation. As lignin is inhibitory during hydrolysis and toxic to microorganisms, delignification improves both hydrolysis and fermentation yields. Ammonia being a selective reagent for lignin, non-corrosive and a relatively less expensive chemical is an appropriate choice for pretreatment [11]. Pretreatment studies on anhydrous ammonia (NH_3) or aqueous ammonia (NH_4OH) including soaking in aqueous ammonia (SAA), ammonia recycle percolation (ARP), and ammonia fiber expansion (AFEX) have mainly concentrated on low-lignin containing biomass such as corn stover [11–15, 17] and only a few on sugarcane bagasse [9, 16]. More research is needed to determine the effect of ammonia pretreatments on ethanol yields for high-lignin containing energy crops such as sugarcane bagasse, sweet sorghum, and energycane.

The objective of this study was to evaluate the effect of a new pretreatment technology using dilute ammonium hydroxide (PCT/US2009/033173) for the hydrolysis and fermentation of sorghum to ethanol.

Materials and Methods

Biomass Preparation

Sorghum (*Sorghum bicolor* (L.) Topper) was harvested from the Hill Farm Research Station (Homer, LA). Leaves, roots, and grains were removed and the stalks were crushed in a roller press (Farrel Company, Ansonia, CT) thrice to extract the juice. Sorghum fibers (1.5 kg dry weight) were pretreated with ammonium hydroxide (28% *v*/*v* solution, Fisher Scientific) and water at a ratio of 1:0.5:8, respectively, for 1 h at 160 °C under a pressure of 160 psi. Biomass (both dilute ammonia pretreated and untreated) was dried to 20% moisture at 40–45 °C. Untreated biomass was used as control. Composition analysis for untreated and pretreated biomass was determined.

Enzymatic Hydrolysis

A combination of two commercially available enzymes, Spezyme CP (Genencor, Danisco US Inc., Rochester, NY) and Novozyme 188 (Sigma-Aldrich, Inc., St. Luis, MO), was used for the hydrolysis of dilute ammonia pretreated and untreated sorghum fibers. The enzyme mixture consisted of 60 FPU of Spezyme CP/ g of glucan and 64 CBU of Novozyme 188/g of glucan. One Liter Erlenmeyer flasks were each loaded with 50 g (dry weight) of biomass (dilute ammonia pretreated or untreated), 0.5 g yeast

extract, 1 g peptone, 25 g citrate buffer (1 M stock solution, pH 4.8), and water to bring the final weight to 500 g. The pH for each mixture was adjusted to 4.8 with concentrated hydrochloric acid. Flasks were autoclaved for 30 min at 121 °C and then cooled. Samples (5 ml) were taken prior to the addition of enzymes and labeled time 0. The enzyme mixture was added and all flasks were incubated at 55 °C in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 200 rpm for 24 h. Samples (5 ml) were withdrawn from each flask post enzyme hydrolysis (time 24 h). All samples were analyzed for sugars (glucose, cellobiose, arabinose, xylose), ethanol, HMF (hydroxy-methyl-furfural) and furfurals. Experiments were run in triplicates. The percent theoretical cellulose yield was calculated using the equation shown below as described in NREL's laboratory procedures (LAP#42630).

$$\%\,Theoretical\,Cellulose\,Yield = \frac{[Glucose] + 1.053[Cellobiose]}{1.111f[Biomass]} \times 100\%$$

where:

$[Glucose]$	Residual glucose concentration (g/L)
$[Cellobiose]$	Residual cellobiose concentration (g/L)
1.053	Multiplication factor that converts cellobiose to equivalent glucose
$[Biomass]$	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose or hemicellulose fraction in dry biomass (g/g)

Simultaneous Scarification and Fermentation (SSF)

All flasks were cooled down to 30 °C post enzyme hydrolysis. Approximately, 1 ml yeast cells (*Saccharomyces cerevisiae* D_5A, ATCC, Manassas, VA, USA) was withdrawn from a stock solution and added to each flask. The stock solution (1×10^9 CFU/ml) was prepared according to NREL's laboratory procedures (LAP#42630). Flasks were incubated at 30 °C in a shaker incubator at 200 rpm for an additional 24 h. Samples (15 ml) were withdrawn at 48 h and analyzed for sugars (glucose, cellobiose, arabinose, xylose), ethanol, HMF and furfurals. Composition analysis and total solids were determined. Percentage theoretical ethanol yield was calculated using the following equation provided by NREL's laboratory procedures (LAP#42630).

$$\%\,Theoretical\,Ethanol\,Yield = \frac{[EtOH]_f - [EtOH]_o}{0.51(f[Biomass]1.111)} \times 100\%$$

where:

$[EtOH]_f$	Ethanol concentration at the end of the fermentation (g/L) minus any ethanol produced from the enzyme and medium
$[EtOH]_o$	Ethanol concentration at the beginning of the fermentation (g/L) which should be zero
$[Biomass]$	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose fraction of dry biomass (g/g)
0.51	Conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast
1.111	Converts cellulose to equivalent glucose

Analytical Procedures

Composition Analysis

Composition analysis was determined following NREL's Laboratory Analytical Procedures (LAPs # 42618, 42619, 42620, 42621, 42622). NREL reference material (8491 sugarcane bagasse) was analyzed as an internal sample to ensure the accuracy of the procedures.

Sugar Analysis

Cellobiose, glucose, xylose and arabinose were analyzed by HPLC (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), lead form, 300 mm×7.8 mm (ID), 9 μm column and a DRI detector (G1362A Agilent). Water was used as the eluent at a flow rate of 1 ml/min with a sample volume of 20 μl.

Ethanol Analysis

Ethanol was analyzed by Gas Chromatography (Hewlett Packard 5890 Series II GC) with a wax column (Zebron ZB Wax Plus, 60 m×0.32 nm×0.50 μm) and GC-FID (flame ionization detector, 280 °C). The operating conditions were injector at 250 °C, split flow rate at 10.4 ml/min and column flow at 1.0 ml/min. Sample volume was 1 μl. Initially, the sample was held at 75 °C for 5 min and then the temperature was increased (at the rate of 10 °C/minute) to 200 °C and held for 1 min throughout the total run time of 18.5 min.

HMF and Furfural Analysis

HMF and 2-furfural were analyzed using HPLC in reverse-phase (Agilent 1100) with a C18 column, 150 mm×4 mm×5 μm (Agilent Eclipse). A diode array detector was configured to collect the absorbance at 280 and 330 nm. Methanol and water were used as eluents at a flow rate of 1 ml/min for a total run of 15 min.

Results and Discussion

Effect of Pretreatment on Biomass Composition

Chemical composition of untreated and dilute ammonia pretreated sorghum is summarized in Fig. 1. The untreated sorghum consisted of 45% cellulose (glucan), 28% hemicellulose (xylan) and 22% lignin. This chemical composition was found to be within the range of published results. Mamma et al. [18] and Gnansounou et al. [19] reported values of 48–49%, 22–26% and 19–20%, respectively. It was observed that 24% of the total mass was lost during pretreatment (Fig. 1) mostly attributed to lignin removal. Approximately, 44% of the initial lignin was removed during pretreatment. Considerable amount of hemicellulose (35%) was also lost as compared to cellulose (10%). Loss of hemicellulose was expected as ammonia has been reported to remove hemicellulose along with lignin [20]. Data available on ammonia pretreatments (i.e., AFEX, ARP, SAA) with corn stover has shown delignification values of 53–85% [11–14]. Higher delignification from the above mentioned studies on corn stover can be attributed to its low lignin

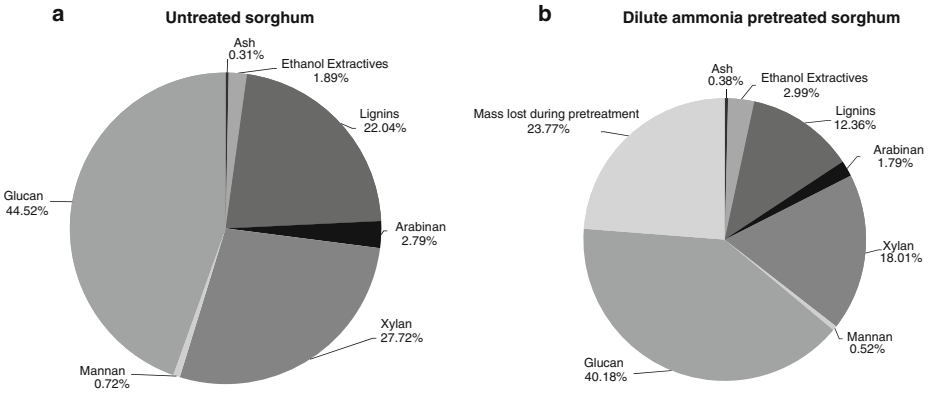

Fig. 1 Composition analysis of **a** untreated sorghum and **b** dilute ammonia pretreated sorghum. %=g/100 g dry biomass

content (17%) [11–14, 17] as compared to higher-lignin containing sorghum (22%) and to pretreatment conditions such as higher ammonia concentrations (1–15 parts ammonia per part biomass), high pressure (atmospheric to 334 psi), temperature (20 °C to 210 °C) and reaction time (1–60 days). Pretreatments such as AFEX at 1.5 parts of ammonia per part of dry sugarcane bagasse and at 473 psi resulted in 90% hydrolysis yield (for cellulose and hemicellulose) [9]. ARP pretreatment (with overnight aqueous ammonia soaked corn stover) resulted in 70–85% lignin removal at 334 psi [11]. SAA for corn stover resulted in 55–74% delignification at 1.8–3.6 parts of ammonia per part of dry biomass, at room temperature, atmospheric pressure, and at a reaction time of 10–60 days [12]. The dilute ammonia pretreatment evaluated in this study resulted in 44% delignification at lower ammonia concentrations (0.14 parts of ammonia per part of dry biomass), low pressure (160 psi) and for less reaction time (1 h) as compared to the above mentioned pretreatments. Toxic inhibitors such as 2-furfural and HMF were not formed during pretreatment and were not present during enzyme hydrolysis or fermentation as indicated by HPLC analysis. Pretreatment technologies involving the use of hot water, sulfuric acid, or organosolv have been reported to enhance the formation of these inhibitory compounds [21, 22].

Enzymatic Hydrolysis and Fermentation of Sorghum

Hydrolysis was carried out with an enzyme mixture of 60 FPU of Spezyme CP/g of glucan and 64 CBU of Novozyme 188/g of glucan for 24 h followed by an additional 24 h for yeast fermentation. Sugars and ethanol concentrations post saccharification and fermentation are presented in Fig. 2. At the end of saccharification (24 h) glucose concentration of dilute ammonia pretreated biomass was 47 g/L as compared to 17 g/L for untreated biomass (Fig. 2).

Similarly, xylose concentrations were higher in dilute ammonia pretreated biomass (17 g/L) than untreated biomass (3 g/L). Cellobiose and arabinose concentrations were also higher for dilute ammonia pretreated biomass. *Saccharomyces cerevisiae* D_5A ferments only glucose hence the concentrations of xylose and arabinose remained constant throughout yeast fermentation. Glucose yield (470 g/Kg dry sorghum) and xylose yield (170 g/Kg dry sorghum) post hydrolysis of dilute ammonia pretreated fibers were higher than those reported

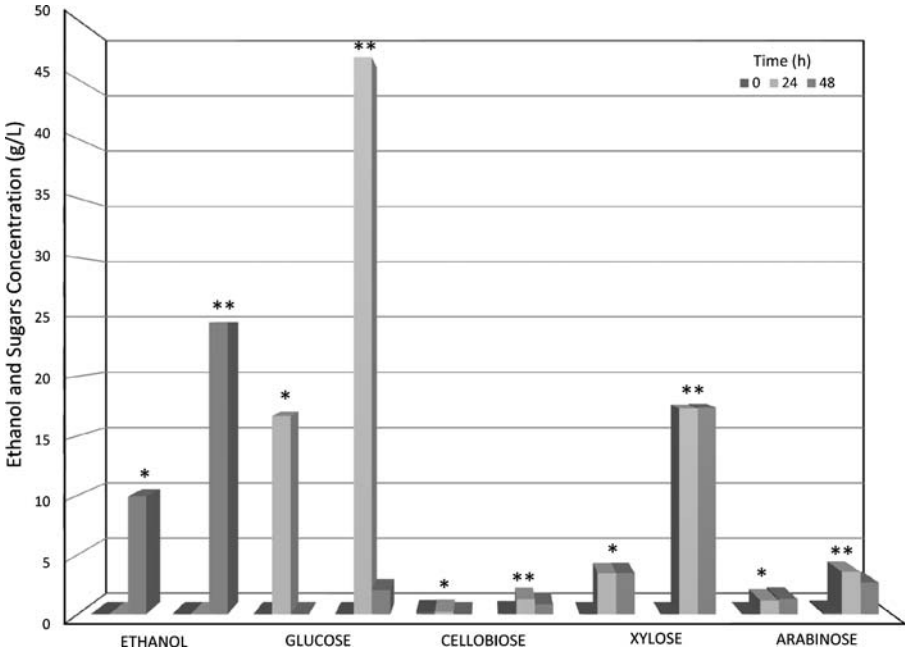

Fig. 2 Enzyme hydrolysis and fermentation of untreated (*) and dilute ammonia pretreated (**) sorghum fibers

by Kurakake et al. [16] on ammonia-water treated sugarcane bagasse (glucose-255 g /Kg dry biomass and xylose- 62 g /Kg biomass). This comparison is possible because sugarcane bagasse like sorghum is a grass and has similar chemical composition (42% cellulose, 22% hemicellulose and 24% lignin) [23].

Cellulose digestibility for dilute ammonia pretreated sorghum was 84% and 38% for untreated sorghum. The high percent digestibility in the pretreated material can be attributed to lignin removal due to the cleaving of lignin-carbohydrate complex by ammonia. This can result in pore formation and swelling of biomass thus increasing surface area and subsequently improving enzyme accessibility [11, 21].

Ethanol concentrations at the end of fermentation were 24 g/L with a theoretical ethanol yield of 84% for dilute ammonia pretreated sorghum as compared to 9 g/L with a theoretical ethanol yield of 44% for untreated sorghum. The ethanol yield obtained from dilute ammonia pretreated sorghum (240 g/kg dry sorghum) is comparable [24] or better than [18, 25, 26] those reported in literature. Gibbons et al. [26] reported 141 g ethanol/kg dry sorghum using sulfuric acid pretreated sorghum in solid phase fermentation. Ban et al. [25] used phosphoric acid treated sorghum and the fermentation of both cellulose and hemicellulose resulted in 145 g ethanol /kg dry sorghum. Mamma et al. [18] reported 115 g/Kg dry sorghum ethanol yield from sodium hydroxide treated sorghum fibers using a mixed culture of *Fusarium oxysporum* and *Saccharomyces cerevisiae*. In another study by Mamma et al. [24], ethanol yields of 160–258 g/kg dry sorghum were reported post fermentation of soluble (glucose and sucrose) as well as insoluble (cellulose) sugars from sorghum juice and fiber; respectively. A higher ethanol yield (316 g ethanol/kg dry sorghum) has been reported by Yu et al. [27] after fermenting both acid treated sorghum (30% sulfuric acid) and sorghum juice. However, unlike in our study, both sorghum juice and fibers were converted to ethanol using a mutant strain of baker's yeast.

Overall, dilute ammonia pretreated sorghum resulted in glucose and ethanol yields comparable if not better than pre-existing technologies. Studies on structural changes of sorghum post pretreatment with dilute ammonia hydroxide and the effect of lower enzyme concentrations on hydrolysis are being investigated.

Conclusions

Dilute ammonia pretreatment was successful in removing 44% of the original lignin from sorghum fibers at comparatively lower ammonia concentration, lower pressure, and with relatively less reaction time than other ammonia pretreatments. A glucan digestibility of 84% was observed in pretreated biomass due to increased surface area and porosity as compared to 38% in untreated sorghum fibers. Percent theoretical ethanol yield was 84% as compared to 44% for untreated sorghum.

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