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BIOTECHNOLOGY and BIOENGINEERING

Application of High Throughput Pretreatment and Co-Hydrolysis System to Thermochemical Pretreatment. Part 1: Dilute Acid

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ABSTRACT: Because conventional approaches for evaluating sugar release from the coupled operations of pretreatment and enzymatic hydrolysis are extremely time and material intensive, high throughput (HT) pretreatment and enzymatic hydrolysis systems have become vital for screening large numbers of lignocellulosic biomass samples to identify feedstocks and/or processing conditions that significantly improve performance and lower costs. Because dilute acid pretreatment offers many important advantages in rendering biomass highly susceptible to subsequent enzymatic hydrolysis, a high throughput pretreatment and co-hydrolysis (HTPH) approach was extended to employ dilute acid as a tool to screen for enhanced performance. First, a single-step neutralization and buffering method was developed to allow effective enzymatic hydrolysis of the whole pretreated slurry. Switchgrass and poplar were then pretreated with 0.5% and 1% acid loadings at a 5% solids concentration, the resulting slurry conditioned with the buffering approach, and the entire mixture enzymatically hydrolyzed. The resulting sugar yields demonstrated that single-step neutralizing and buffering was capable of adjusting the pH as needed for enzymatic saccharification, as well as overcoming enzyme inhibition by compounds released in pretreatment. In addition, the effects of pretreatment conditions and biomass types on susceptibility of pretreated substrates to enzymatic conversion were clearly discernible, demonstrating the method to be a useful extension of HTPH systems.

Biotechnol. Bioeng. 2013;110: 754–762.

KEYWORDS: high throughput; dilute acid; pretreatment; co-hydrolysis; biomass; yield

Introduction

Sustainable energy sources are needed to supplement petroleum use in light of limited reserves and growing energy demands, as well as to reduce the environmental impacts associated with production and combustion of these fossil fuels (Farrell et al., 2006). Lignocellulosic biomass, such as agriculture and forestry residues (e.g., corn stover and sawdust) and woody and herbaceous crops (e.g., poplar and switchgrass; Wyman et al., 2005), is recognized as a sustainable source of sugars that can be converted to biofuels and other biomaterials by a number of routes (Himmel et al., 2007). To produce biofuels economically, a large number of factors such as feedstock quality, conversion conditions, and catalyst loadings as well as their complex interactions must be better evaluated to identify combinations that can overcome the natural recalcitrance of biomass at the lowest cost (Lynd, 1996; Wyman, 1999). However, it is impractical to apply conventional testing for such purposes because it is slow, demands considerable labor, and requires larger sample sizes than may be available. Fortunately, high throughput pretreatment and enzymatic co-hydrolysis (HTPH) systems have been developed to considerably

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Contract grant sponsor: Office of Biological and Environmental Research
Contract grant sponsor: BioEnergy Science Center (BESC)
Received 12 August 2012; Revision received 27 September 2012;
Accepted 1 October 2012
Accepted manuscript online 10 October 2012;
Article first published online 1 November 2012 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.24751/abstract)
DOI 10.1002/bit.24751

streamline these tests and allow evaluation of large number of combinations of variables effectively (Santoro et al., 2010).

Several high throughput systems have been developed and applied to screen large biomass sample sets for sugar release from pretreatment and enzymatic hydrolysis, optimization of enzyme formulations, and more rapid biomass compositional analyses (Chundawat et al., 2008; DeMartini et al., 2011; Navarro et al., 2010; Santoro et al., 2010; Studer et al., 2010). Such high throughput systems offer a number of important attributes in addition to the ability to process multiple samples quickly. For one, only milligram quantities of biomass are needed to complete a pretreatment and enzymatic hydrolysis reaction. HTPH also lends itself to being highly automated and significantly reduce labor and time requirements (Navarro et al., 2010). In addition, some of the conditions employed are in fact more similar to those expected commercially than conventional approaches typically used for such tests, making the results more commercially relevant (Studer et al., 2010).

In most high throughput systems that target identifying favorable combinations of biomass types and pretreatment and enzymatic hydrolysis conditions, a ''co-hydrolysis'' method is applied (Studer et al., 2011). In this approach, the entire pretreated slurry is directly subjected to enzymatic hydrolysis rather than separating the solid and liquid phases and washing the solids prior to enzyme addition. For application of co-hydrolysis to identification of the effects of hydrothermal pretreatment on substrate susceptibility to enzymes, low solids loadings (e.g., 1–2 wt%) and high enzyme loadings (e.g., 100 mg enzyme/g sugar in raw biomass) are generally used to minimize enzyme inhibition that could otherwise obscure differences in substrate digestibility (Kumar and Wyman, 2009; Palmqvist et al., 1996; Qing et al., 2010).

Thermochemical pretreatments with dilute acid or base typically realize higher yields of sugars from hemicellulose and cellulose in the combined operations of pretreatment and subsequent enzymatic hydrolysis than possible with hydrothermal pretreatment (Yang and Wyman, 2008). Thus, it is desirable to be able to screen sugar yields from these pretreatments over a wide range of feedstocks and conditions. Dilute acid (2 wt% sulfuric acid) and base (0.025 wt% NaOH) have been employed in an HTPH system (Santoro et al., 2010), with buffering and neutralization accomplished by simply adding a stoichiometric amount of NaOH or HCl to the hydrolyzate before enzymatic hydrolysis. However, this approach resulted in a decrease in hydrogen ion concentration due to the ion-exchange reaction between inorganic cations associated with the bound and free anions contained in wood and hydrogen ions in the applied solution (Springer and Harris, 1985). This drop in acid concentration from neutralization was especially apparent at low pH and with small amounts of solution (Lloyd and Wyman, 2004; Springer and Harris, 1985). Thus, neutralization capacity should be accounted for when buffering the hydrolyzate or subsequent enzymatic hydrolysis will suffer and give results that do not truly reflect differences in biomass recalcitrance. However, because titration of the hydrolyzate to adjust the pH is labor and time intensive, it is not practical for application to HTPH system. Therefore, an efficient and effective neutralizing and buffering method was needed to expand the range of applicability of HTPH to thermochemical pretreatment with dilute acid.

In this work, a novel buffering method was proven to successfully adjust the pH value of biomass slurries from dilute acid pretreatment to an appropriate range for co-hydrolysis. Then, dilute acid pretreatment followed by co-hydrolysis was applied to poplar and switchgrass in an HTPH format. Sugar release was measured for various pretreatment conditions and enzyme dosages to demonstrate that dilute acid HTPH can reproducibly screen performance over a range of conditions. Furthermore, favorable pretreatment and enzymatic hydrolysis conditions were identified to help select initial conditions for future studies. Finally, the dilute sulfuric acid HTPH system was employed to reveal differences in sugar release and recalcitrance of four Aspen samples that varied in age and composition, and the results were compared to those from hydrothermal pretreatment.

Materials and Methods

Biomass Feedstocks

Two kinds of biomass, *Panicum virgatum* and *Populus trichocarpa*, more commonly known as switchgrass and poplar wood, were the primary substrates for this study. The switchgrass was from Pierre, South Dakota. The BioEnergy Science Center (BESC) provided the poplar which was then debarked, split, and chipped (Yard Machine 10HP, MTD Products Inc., Cleveland, OH). The resulting poplar wood chips and switchgrass were both knife milled (Model 4, Wiley Mill, Thomas Scientific, Swedesboro, NJ) through a 1 mm screen. After that, both materials were air dried for approximately one month followed by sieving to collect fractions with a particle size between 20-mesh (<0.85 mm) and 80-mesh (>0.180 mm; RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20-mesh were collected and sieved again, and the resulting 20–80 mesh fraction was mixed with the previously obtained 20–80 mesh fraction. The composition was analyzed according to NREL Laboratory Analytical Procedures (Sluiter et al., 2008). As summarized in Table I,

Table I. Glucan, xylan, and lignin contents in switchgrass, poplar wood, and aspen wood.

	Switchgrass	Poplar	Aspen7[a]	Aspen11[a]	Aspen14[a]	Aspen bark[a]
Glucan	32.4	46.5	37.3	45.7	46.1	16.4
Xylan	21.2	20.3	15.3	17.4	17.8	8.8
Lignin	18.8	23.4	29.5	27.3	21.5	32.7

[a]Full dataset reported elsewhere (DeMartini and Wyman, 2011).

the resulting switchgrass contained 32.4% glucan and 21.2% xylan. The poplar contained 46.5% glucan and 20.3% xylan.

Several Aspen (*Populus tremuloides*) samples were also tested in this study. A cross-section of Trembling Aspen (*P. tremuloides*) tree classified as 20–30 years in age was obtained from Benchmark International in Alberta, Canada (DeMartini and Wyman, 2011). The wood was debarked and fractionated into its individual annual rings that were labeled as 1–26 from pith to bark, according to the relative year in which that ring was formed. All sections were milled to pass through a 20-mesh screen (<0.85 mm). The bark sample, as well as samples 7, 11, and 14, were selected to use in this study because they offer a range of glucan, xylan, and lignin contents, as shown elsewhere (DeMartini and Wyman, 2011).

Pretreatment in Conventional Tube Reactors

Biomass was soaked in 0.5% (w/w) or 1% (w/w) sulfuric acid solutions at room temperature overnight to allow full penetration. To establish baseline performance at a 5% (w/w) solids concentration, the equivalent of 0.1 g of dry biomass in the soaked slurry was then added to conventional tube reactors along with enough of the appropriate acid solution to give 2 g total weight. These reactors were made from 150 mm lengths of 12.5 mm OD Hastelloy tubing with a 0.8255 mm wall thickness and stainless steel end caps (Swaglok, San Diego, CA). Each tube reactor had an internal volume of approximately 14 mL. Teflon plugs (McMaster-Carr, Santa Fe Spring, CA) were inserted in each end to avoid acid corrosion of the stainless steel caps. The tubes were heated in a 4-kW model SBL-2D fluidized sand bath (Techne, Princeton, NJ), as described elsewhere (Lloyd and Wyman, 2005). After pretreatment at 160°C for 5, 10, 20, or 40 min, the reactions were quenched by submerging the reactors in room temperature water. The reactors were then opened, and 8 mL of deionized (DI) water was added to each reactor to dilute the hydrolyzate for subsequent enzymatic hydrolysis. All hydrolyzate was then collected in a 15 mL centrifuge tube (Corning Life Science, Fisher Scientific, Fair Lawn, NJ) and centrifuged for 10 min at 4,200*g* (Allegra X-15R, Beckman Coulter, Fullerton, CA) to separate the solid and liquid. The liquid was collected for pH measurement.

Preparation of a Buffer Solution

One molar citrate buffer was prepared by adding 1–4 mL of 37% (w/w) hydrochloric acid (Sigma–Aldrich, St-Louis, MO) to 40 mL 1 M trisodium citrate (Sigma–Aldrich) to produce a buffer solution for adjusting the pH of the hydrolyzate into the proper pH range following pretreatment. The buffering method was tested by adding 75 μL of the prepared 1 M citrate buffer into 1,350 μL sulfuric acid solutions that had pH values that mimicked the liquid resulting from dilute acid pretreatment. The final mixture corresponded to an approximately 0.05 M final buffer concentration, and the pH value was determined.

pH Measurements

All pH measurements were performed using a MI-414 Micro-combination pH electrode (Microelectrodes, Bedford, NH) coupled with a Core Module robotics platform (Freeslate, formerly Symyx Technologies, Sunnyvale, CA). The pH meter was calibrated using four standard buffer solutions with pH values of 2.0, 4.0, 7.0, and 10.0 (Fisher Scientific). A series of sulfuric acid solutions with gradient pH values from approximately 1.5–3.0 were prepared in 2 mL high recovery glass vials (Agilent Technologies Inc., Santa Clara, CA) loaded in a 6×8 rack on the robotics platform. Their pH was measured automatically by running a pre-coded program.

Dilute Sulfuric Acid Pretreatment and Enzymatic Co-Hydrolysis in HTPH System

Dilute sulfuric acid pretreatment and enzymatic co-hydrolysis were performed on all samples using a high throughput pretreatment and co-hydrolysis (HTPH) system described elsewhere (Studer et al., 2010). The HTPH system is based on a 96-well plate format, but the wells are made of Hastelloy to withstand the temperatures and pressures of thermochemical pretreatment. HTPH pretreatments were performed at a 5% (w/w) solids loading with a total reaction mass of 90 mg in each well, corresponding to 4.5 mg of dry biomass. Biomass loading was accomplished with the solid and liquid dispensing robotics platform (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance, Freeslate). Next, 85.5 μL of dilute sulfuric acid solution (either 0.5% (w/w) or 1% (w/w) concentration) was pipetted into each well with an 8-channel pipette (30–300 μL, Eppendorf North America, Hauppauge, NY). A flat pre-cut Silicone gasket (thickness 1.5875 mm, durometer hardness A40) was placed over the top of the wells to cover their openings, and the assembly was placed between bottom and top plates made of 304 stainless steel. The resulting "sandwich" was then clamped together using four 1/4 in.-20 threaded bolts (6.35 mm-20) placed in each corner of the two plates, with spring washers (flat load 1,500 N) and wing nuts to allow rapid closing and opening (Studer et al., 2010). After sitting at room temperature overnight, the plate assemblies were inserted into a custom-built steam chamber (Studer et al., 2010) for pretreatment at a temperature of 160°C at times of 5, 10, 20, or 40 min. Upon reaching the target pretreatment time, the chamber was flooded with cold water to quench the reaction.

After the well plate was removed from the chamber and opened, 360 μL (8 channel pipette, 30–300 μL, Eppendorf) of DI water was added to each well to dilute the hydrolyzate and bring the total volume to 450 μL. Then, 32–35 μL of a

mixture containing citrate buffer (1 M, pH 5.0), sodium azide, and enzymes (cellulase, xylanase, and β-glucosidase) was added to each well, depending on the enzyme loading. The final concentrations of citrate buffer and sodium azide were 0.05 M and 0.01 g/L, respectively. The total final reaction volume was 482–485 μL. The pH of the resulting hydrolyzate was adjusted to a range of 4.7–4.9 for enzymatic hydrolysis by addition of the prepared buffer mixture.

Cellulase (Spezyme CP®, protein concentration 116 mg/mL, activity 58 FPU/mL, Lot # 3016295230) and Multifect® xylanase (protein concentration 42 mg/mL, Lot# 4900667792), both from Genencor, a division of Danisco, now DuPont, Palo Alto, CA, were mixed at a protein ratio of 3:1. Three levels of enzyme loadings measured as mg cellulase + xylanase protein/g glucan+ xylan in the original raw materials were employed: 75 + 25 mg (low), 105 + 35 mg (medium), and 135 + 45 mg (high). These were supplemented with β-glucosidase (Novozyme®188, activity-665 CBU/mL) at an activity ratio of 1.5: 1 (CBU:FPU) to enhance cellobiose hydrolysis. The activity and protein numbers assumed in this study were previously reported by Dien et al. (2008). After enzyme addition, the 96-well plate assembly was clamped shut again and held in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD) at 50°C and 150 rpm for 72 h. All experiments were carried out in quadruplicate. After 72 h of enzymatic hydrolysis, the plates containing slurries in the individual wells of the well plate were centrifuged for 10 min at 4,200*g* (Allegra X-15R, Beckman Coulter) using a 96-well plate carrier adaptor (Microplate carriers SX4750, VWR International, West Chester, PA) to separate the solids and liquid. Two hundred sixty micro liter of the solids free supernatant liquid was then pipetted into 500 μL polyethylene HPLC vials for sugar analysis.

Sugar Analysis

Sugar concentrations were measured by high performance liquid chromatography (1200 series, Agilent Technologies Inc.). An Aminex HPX-87H column (Bio-Rad, Hercules, CA) heated to 65°C was used on a separation module (1200 series) equipped with a refractive index detector (G1362A, Agilent Technologies Inc.) and using 5 mM sulfuric acid as the mobile phase. For screening purposes, all sugars that fell under the xylose peak were included in the resulting xylose concentration, even though a minor amount of additional sugars such as mannose, fructose, and galactose may also have coeluted with the xylose.

Results and Discussion

Buffering Method

A major challenge for thermochemical pretreatments in HTPH systems is adjusting the pH value prior to subsequent enzymatic hydrolysis, as well as eliminating or minimizing the effect of inhibitors released during pretreatment. Unlike hydrothermal pretreatment, due to the residual acid, the buffer capacity of the citrate buffer solution used in NREL Laboratory Analytical Procedures (Selig et al., 2008) is not enough to bring the hydrolyzates from dilute acid pretreatment to an appropriate range for enzymatic hydrolysis (data not shown). In previous work that demonstrated the concept of "co-hydrolysis" on a 100 mL-scale (Studer et al., 2011), a 50 wt% NaOH solution was used to titrate slurries from pretreatment with chemicals such as dilute sulfuric acid to pH 5 prior to enzymatic hydrolysis. However, titration was tedious and would be too labor intensive and impractical for application to HTPH systems.

Herein, an alternative method was developed. First, pretreatment was performed with a 90 mg reaction weight at 5% (w/w) solids loading for sulfuric acid concentrations of 0.5% or 1% (w/w), and the pretreated slurries were then diluted by adding 360 μL DI water to produce a 1% solid (~0.5 wt% glucan) concentration. Figure 1 compares the one-step neutralization and buffering method with the previous buffer method by titration. The major benefit of the new approach is twofold. First, a higher solids loading that more closely mimics larger scale applications is used in pretreatment without increasing the actual amount of biomass required. Second, less acid was added, reducing

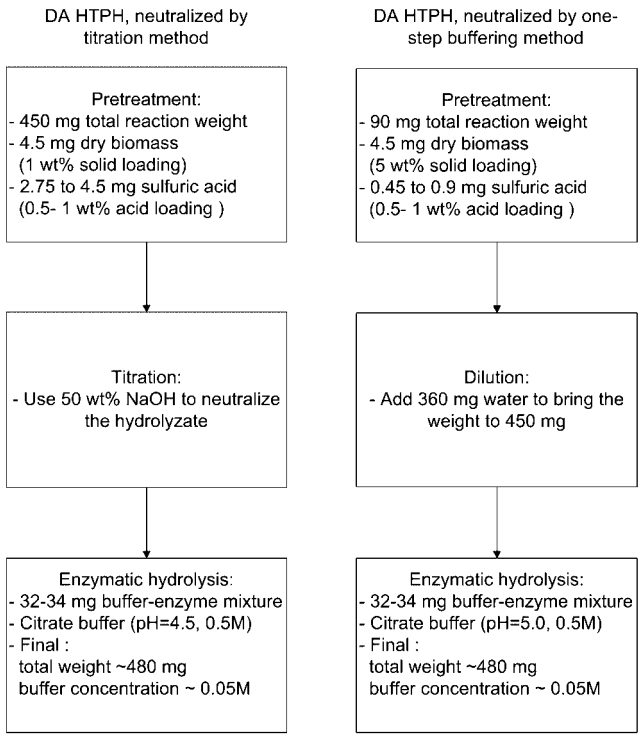

Figure 1. Flowchart of thermochemical pretreatment in HTPH system and the neutralization by titration or one-step buffer method.

the amount of buffering required to bring the hydrolyzate to an appropriate pH range. Third, dilution reduced the concentration of possible inhibitors to enzymatic hydrolysis.

Trisodium citrate was chosen for a single step neutralization and buffering approach because of its wide buffering capacity as long as the ratio and concentration of conjugated acid-base pair is well-controlled (Christian, 1994). According to the National Renewable Energy Laboratory Analytical Procedure (Selig et al., 2008), a suitable pH range for enzymatic hydrolysis of lignocellulosic biomass is 4.8–5.0, which falls in the buffering range of the conjugated pair of monosodium citrate (H_2A^-) and disodium citrate (HA^{2-}). To adjust pH in a single step, a buffer solution containing H_2A^- and HA^{2-} but with a higher pH than the final target value was added to the pretreated slurry. The excess hydrogen ions (H^+) in the pretreated hydrolyzate adjusted the ratio of the conjugated pair and led to the desired pH for enzymatic hydrolysis. In this way, neutralizing and buffering were accomplished simultaneously.

Determining pH in Pretreated Hydrolyzates

As previously stated, a buffer solution containing monosodium citrate (H_2A^-) and disodium citrate (HA^{2-}) with a slightly higher pH than the final target pH was capable of neutralizing and buffering the slurries from dilute acid pretreatment. However, due to the complexity of the buffer system, the exact pH value required for the buffer solution must be determined empirically. Furthermore, the anions associated with inorganic cations in biomass can neutralize part of the mineral acid, further necessitating pH measurements rather than simply calculating the pH based on the original acid loading (Lloyd and Wyman, 2004; Springer and Harris, 1985). To accomplish this, poplar and switchgrass were pretreated in tube reactors at various conditions, and the diluted hydrolyzate was then collected to measure the resulting pH values, as displayed in Table I. For poplar pretreated at 160°C for 5–40 min at a 0.5% (w/w) sulfuric acid concentration, the pH value of the diluted hydrolyzate ranged from 1.88 to 1.93, while at 1% (w/w) sulfuric acid loading, pH values varied from 1.66 to 1.69, depending on the pretreatment time. For switchgrass pretreated at the same conditions, the pH ranged from 2.00 to 2.06 and 1.66 to 1.69 for 0.5% (w/w) and 1% (w/w) acid concentrations, respectively. As demonstrated in Table II, the pH value of the pretreated hydrolyzate was primarily determined by the original sulfuric acid loading. The type of biomass and pretreatment time had minor impacts on the final pH value. The diluted hydrolyzate from pretreatment with 0.5% (w/w) sulfuric acid tended to have a pH value between 1.8 and 2.1, while that from pretreatment with 1% (w/w) sulfuric acid had pH values that usually fell between 1.6 and 1.7. These results provided a reference on how to prepare the proper buffer solution.

Table II. pH value of diluted hydrolyzates produced by dilute sulfuric acid pretreatment of switchgrass and poplar.

Biomass	Acid loading (% w/w)	Pretreatment time (min)	pH of diluted hydrolyzate	Buffer	pH after neutralization
Switchgrass	0.5	5	2.00	C	4.60
		10	2.00		4.60
		20	2.04		4.83
		40	2.06		4.90
	1	5	1.64	B	4.58
		10	1.66		4.60
		20	1.66		4.98
		40	1.68		5.01
Poplar	0.5	5	1.92	C	4.80
		10	1.93		4.82
		20	1.97		4.83
		40	2.09		4.90
	1	5	1.62	B	4.85
		10	1.63		4.88
		20	1.65		4.92
		40	1.66		4.92

Testing and Confirmation of the Proposed Buffering Method

To prove that the proposed buffering method properly prepared the pretreated slurry for subsequent enzymatic hydrolysis, sulfuric acid solutions with pH values ranging from 1.5 to 3.0 were prepared to mimic the acidity of diluted hydrolyzate from pretreatment. All of the concentrations and volumes used were proportional to the actual conditions in the HTPH system but scaled up by factor of three for easy operation and testing. As such, 75 μL of 1 M citrate buffer was well mixed with 1350 μL acid solution for pH measurement.

Table III shows the pH of buffer solutions A through E, sulfuric acid solutions, and the final mixture. In this table, mixtures with a pH value between 4.5 and 5.0 are highlighted in bold to indicate their suitability for enzymatic hydrolysis. The results suggest that for pretreated hydrolyzate solutions with a pH value higher than 2.15, a generic buffer (1 M, pH 4.5) had enough capacity to adjust the pH to the desired value when the final buffer concentration was 0.05 M. For solutions with pH values ranging between 1.8 and 2.1, corresponding to the 0.5% (w/w) sulfuric acid concentration in pretreatment, both buffer C (1 M, pH 4.85) and buffer D (1 M, pH 4.55) effectively brought the final pH to a range appropriate for enzymatic hydrolysis. Although the pH value was slightly off, buffer C with acid solutions at a pH of 2.13 and buffer B (1 M, pH 5.09) with acid solutions at a pH of 1.66 were also effective. For solutions with a lower pH range of 1.5–1.7, such as for hydrolyzates resulting from pretreatment with 1% (w/w) sulfuric acid, buffer B achieved a final pH between 4.7 and 4.9.

These results confirmed the neutralizing and buffering method developed in this study was effective for diluted pretreatment hydrolyzates at 160°C for 5–40 min with either

Table III. pH values of buffer, dilute acid solutions, and mixtures of the two[a].

	Acid	1	2	3	4	5	6
Buffer	pH	2.41	2.13	1.83	1.66	1.55	1.47
A	5.65	—	—	5.56	5.31	5.07	**4.76**
B	5.09	—	5.41	5.12	**4.88**	**4.63**	4.35
C	4.85	5.31	5.17	**4.89**	**4.65**	4.40	—
D	4.55	5.10	**4.96**	**4.72**	4.50	—	—
E	4.24	**4.66**	**4.57**	—	—	—	—

[a]Mixture, 75 μL of 1 M citrate buffer was mixed with 1,350 μL sulfuric acid solution.

0.5% or 1% (w/w) sulfuric acid concentrations on both switchgrass and poplar wood. Test results demonstrated the feasibility of the method, as well as the preferred buffer solution. Buffer C (1 M, pH 4.85) and buffer B (1 M, pH 5.09) were selected due to their ability to achieve a final pH close to 4.8 when 75 μL of buffer was added to 1,350 μL of pretreated hydrolyzate over a range of pretreatment conditions. Table III can also serve as a reference when preparing buffer solutions, since 0.5% and 1% (w/w) are two of the most commonly used sulfuric acid loadings for biomass pretreatment (Esteghlalian et al., 1997). For lower acid loadings, such as 0.2% (w/w), a generic buffer solution (1 M, pH 4.5) would be sufficient.

Results From Dilute Acid HTPH of Switchgrass and Poplar

After showing the neutralization and buffering concept worked effectively in adjusting and controlling pH for enzymatic hydrolysis, the performance of HTPH and larger scale conventional reactors were compared for application of dilute acid pretreatment and hydrolysis to poplar and switchgrass. In this case, both materials were pretreated at 160°C with 0.5% and 1% (w/w) sulfuric acid concentrations for 5, 10, 20, and 40 min, followed by co-hydrolysis at three enzyme loadings of 75 + 25 mg (low—L), 105 + 35 mg (medium—M), and 135 + 45 mg (high—H) of cellulase + xylanase/g glucan + xylan in the raw material (Studer et al., 2011). The hydrolyzate was then diluted and buffered as described previously.

Figure 2a shows the glucan, xylan, and total sugar yields from combined pretreatment and co-hydrolysis for switchgrass using 0.5% (w/w) acid for pretreatment. The highest glucan yield (99 ± 2.0%) was achieved for pretreatment at 160°C for 20 min with 0.5% acid, while the highest xylan yield (99 ± 1.6%) and total sugar yield (98 ± 1.9%) were for pretreatment at 160°C for 10 min at enzyme loading of 135 + 45 mg of cellulase + xylanase/g glucan + xylan in the raw material.

Figure 3a shows similar results for poplar wood. In this case, the highest glucan yield (84 ± 1.5%) was for pretreatment at 160°C for 40 min with 0.5% sulfuric acid,

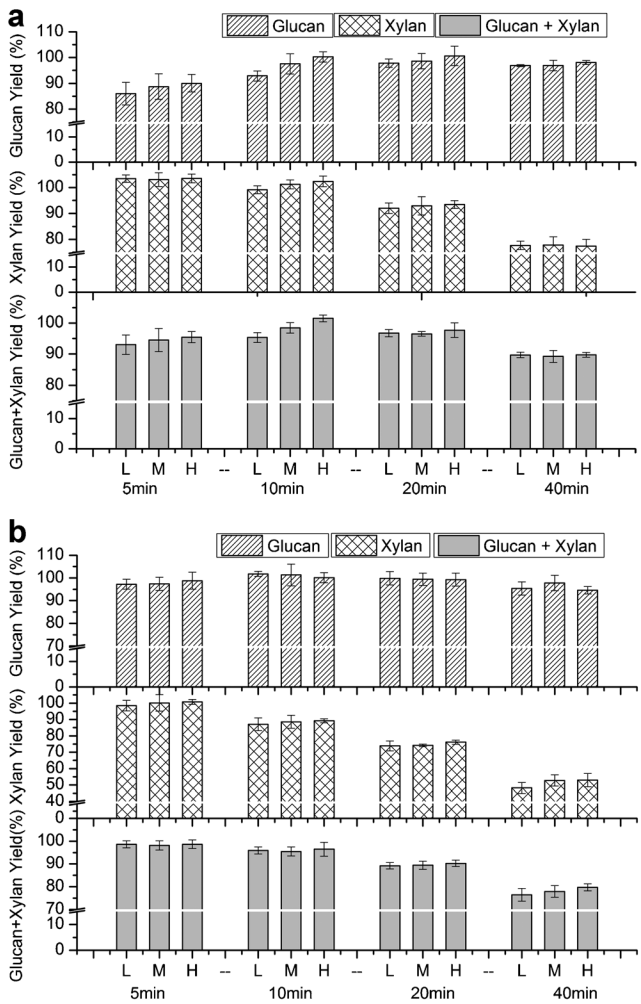

Figure 2. Glucan, xylan, and total sugar (glucan + xylan) yields at 5, 10, 20, and 40 min pretreatment from switchgrass at 160°C with (**a**) 0.5% and (**b**) 1.0% (w/w) acid loading. L, M, and H represent the following enzyme loadings: Low—75 + 25 mg, Medium—105 + 35 mg, and High—145 + 35 mg of cellulase + xylanase/g glucan + xylan in raw material. The error bars represent the standard deviation of three replicates for the multi well-plate experiments.

while the highest xylan yield (78 ± 2.1%) and total sugar yield (72 ± 1.0%) were from pretreatment at 160°C for 10 min with 0.5% acid. In Figures 2b and 3b, it can be seen that increasing the acid loading to 1% (w/w) increased the glucan yield compared to the results obtained with 0.5% (w/w) acid. However, with 1% (w/w) acid loading at the same pretreatment temperature and time, xylan degradation began noticeable at 10 min and got progressively worse with increasing pretreatment time for both poplar and switchgrass.

Figures 2 and 3 also demonstrate that all three enzyme loadings resulted in very similar sugar yields for a given biomass and pretreatment condition. Furthermore, all three loadings allowed differentiation of performance between

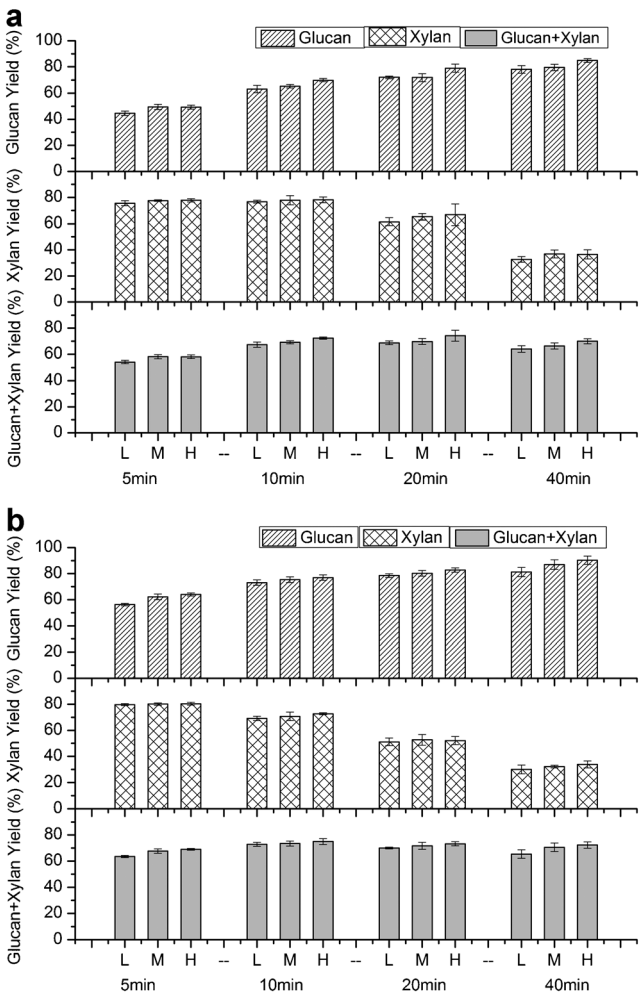

Figure 3. Glucan, xylan, and total sugar (glucan + xylan) yields at 5, 10, 20, and 40 min pretreatment times from poplar at 160°C with (**a**) 0.5% and (**b**) 1% (w/w) acid concentration. L, M, and H represent enzyme loadings: Low—75 + 25 mg, Medium—105 + 35 mg, and High—145 + 35 mg of cellulase + xylanase/g glucan + xylan in raw material. The error bars represent the standard deviation of three replicates for the multi well-plate experiments.

different pretreatment conditions, as well as between poplar and switchgrass.

Selection of Pretreatment and Enzymatic Hydrolysis Conditions for Screening Studies

The primary HTPH goal is to provide a rapid screening tool for initial indications of sugar release from different biomass-pretreatment-enzyme combinations in order to identify viable strategies to overcome biomass recalcitrance and improve sugar yields. For screening purposes, identifying biomass samples with high sugar yields at a sub-optimal pretreatment condition is usually favored because it not only minimizes sugar degradation and inhibitor production but also allows for differentiation between biomass samples with variable recalcitrance. Therefore, pretreatment at 160°C for 5–10 min with 0.5% sulfuric acid was selected as a suitable screening condition. Acid loadings higher than 1% (w/w) are not recommended because hemicelluloses degradation can be quite high even at short pretreatment times, and very precise residence time control would be needed to maximize yields. Although only one pretreatment temperature, 160°C, was tested in this study, the time required to achieve similar yields at different temperatures can be estimated from the combined severity parameter (Nicolas Abatzoglou, 1992). For example, times of 20–40 and 1.3–2.5 min would be estimated to give similar yields at 140 and 180°C, respectively, as the highest yield identified at 160°C. However, the time required for pretreatment at 140°C would not allow processing of large numbers of samples in a short period of time, while pretreatment at 180°C must be performed with equipment capable of very rapid heating and cooling and tightly controlled residence times to avoid degradation of sugars. Considering all of these factors, pretreatment at160°C for 5–10 min with 0.5% (w/w) acid was selected.

In selecting the enzyme loading for screening purposes, it is important to keep in mind that enzymatic hydrolysis in this study was performed in the same reactor as pretreatment, and dilute acid pretreatment generates inhibitors that hamper enzymatic hydrolysis (Kim et al., 2011; Kothari and Lee, 2011). Due to this, a significantly higher enzyme loading was employed compared to conventional washed solids hydrolysis to offset the effects of potential inhibitors in the pretreated slurry as a result of not separating the solids and liquids following pretreatment (Selig et al., 2010; Studer et al., 2011). The higher loading will help ensure that enzyme activity is not the limiting factor in screening studies, regardless of sample variability, and allow a clearer interpretation of sugar release data with respect to characteristics of the biomass samples (Selig et al., 2010). As demonstrated by Figures 1 and 2, all three enzyme loadings, 75 + 25, 105 + 35, and 135 + 45 mg cellulase + xylanase/g glucan + xylan in raw material gave similar trends in sugar yields, and all met the requirement of effectively converting cellulosic biomass to sugars without obscuring differences between performance with different biomass materials. Given that the three levels of enzyme loadings tested here gave similar trends in sugar yields at various pretreatment conditions, the low level enzyme loading was selected.

Application of Dilute Acid HTPH to Aspen Wood Rings

The primary application of HTPH systems is for screening large numbers of samples in order to select those with desired properties, such as lower recalcitrance and higher sugar yields. To evaluate the ability of dilute sulfuric acid HTPH systems to differentiate performance differences among samples, experiments were performed on four Aspen samples that differed in maturity and composition

(DeMartini and Wyman, 2011). Pretreatment in the multi-well plate was performed with a 0.5% sulfuric acid concentration at 160°C for 5 min, and subsequent co-hydrolysis was carried out for 72 h at 50°C with an enzyme loading of 75 + 25 mg cellulase + xylanase/g glucan + xylan in the raw material, as established previously. The results in Figure 4 show that sample 14, which was from the mature section of the tree and contained the most glucan and least lignin, gave the highest glucan yield of 82.6 ± 3.3%. Sample 7 (juvenile wood) displayed the highest xylan yield of 97.2 ± 5.0% of the four samples. When considering total glucan + xylan yields, samples 11 (90.0 ± 2%) and 14 (88.3 ± 1.9%) performed very similarly and slightly better than sample 7 (82.6 ± 2%). The bark sample showed both the lowest glucan and xylan yields at 57.0 ± 4.2% and 73.0 ± 3.7%, respectively.

Figure 4 also compares results from dilute acid pretreatment at 160°C with a 0.5% (w/w) sulfuric acid concentration for 5 min with previous work by our group for hydrothermal (water-only) pretreatment at 160°C for 70 min (DeMartini and Wyman, 2011). The enzyme loading for both co-hydrolysis experiments was 75 + 25 mg cellulase + xylanase/g glucan + xylan in the raw material. Overall, the sugar yield trends are in good agreement. Furthermore, the HTPH results showed that dilute acid could reduce the pretreatment time from about 70 min for hydrothermal processing to about 5 min without sacrificing accuracy or obscuring differences between biomass samples. Sugar yields from the four Aspen samples demonstrated that dilute acid HTPH was capable of discerning differences in recalcitrance among samples.

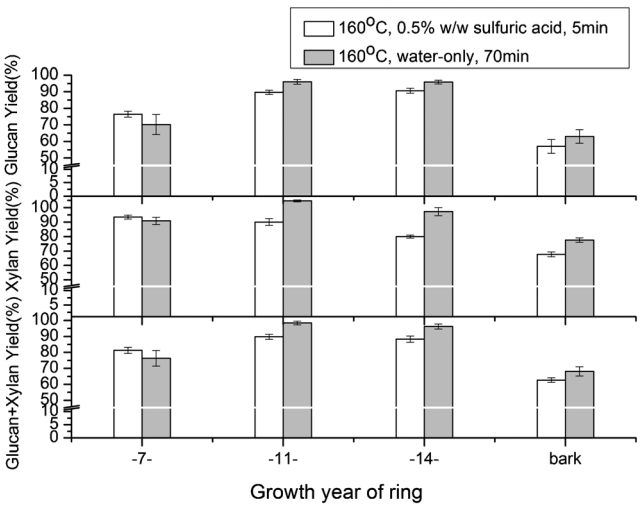

Figure 4. Glucan, xylan, and total sugar (glucan + xylan) yields of aspen wood samples 7, 11, 14, and bark pretreated at 160°C with 0.5% (w/w) H_2SO_4 for 5 min or 160°C with water only for 70 min. The enzyme loading for co-hydrolysis for both was 75 + 25 mg of cellulase + xylanase/g glucan + xylan in raw material. The error bars represent the standard deviation of three replicates for the multi well-plate experiments.

Conclusions

A novel one step buffering and neutralizing method was developed and proven to simultaneously neutralize and adjust the pH value of slurries resulting from pretreatment prior to whole slurry enzymatic co-hydrolysis. By (1) concentrating the pretreatment slurry to a 5% (w/w) solids loading, (2) diluting it to 1% (w/w) solids for co-hydrolysis, and (3) adding a buffer solution with the appropriate pH, buffering, and neutralization were possible within the limited volume of the HTPH reaction vials. This method allowed us to extend the HTPH concept to dilute acid pretreatment, thereby providing an additional tool to screen large numbers of biomass candidates and processing conditions to identify combinations that better overcome biomass recalcitrance and improve the economics of ethanol production from cellulosic biomass.

We gratefully acknowledge support for this research by the Office of Biological and Environmental Research in the DOE Office of Science through the BioEnergy Science Center (BESC). The author is also grateful to the Center for Environmental Research and Technology of the Bourns College of Engineering (CE-CERT) at the University of California, Riverside for providing key equipment and facilities. Gratitude is also extended to the Ford Motor Company for funding the Chair in Environmental Engineering at the Center for Environmental Research and Technology of the Bourns College of Engineering at UCR, which augments support for many projects such as this one.

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