ARTICLE

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Engineering of a High-Throughput Screening System to Identify Cellulosic Biomass, Pretreatments, and Enzyme Formulations That Enhance Sugar Release

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ABSTRACT: The recalcitrance of cellulosic biomass, the only abundant, sustainable feedstock for making liquid fuels, is a primary obstacle to low cost biological processing, and development of more easily converted plants and more effective enzymes would be of great benefit. Because no single parameter describes recalcitrance, superior variants can only be identified by measuring sugar release from plants subjected to pretreatment and enzymatic hydrolysis. However, genetic modifications of plants coupled with molecular engineering of deconstruction proteins and definition of pretreatment conditions create a very large sample set, and previous methods for biomass pretreatment at elevated temperatures and pressures prevented use of a fully integrated high-throughput (HTP) screening pipeline. Herein, we report on the engineering of a novel HTP pretreatment system employing a 96 well-plate format that withstands extreme pretreatment conditions for rapid screening of biomass–enzyme-pretreatment combinations. This includes the development of new approaches to steam heating and water quenching the system that result in much faster heat up and cool down than previously possible and show consistent temperature histories across the multiwell plate. Coupled pretreatment and enzymatic hydrolysis performance of the well plate pretreatment system is shown to be consistent among the many wells in the device and also with performance of conventional tubular reactors.

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KEYWORDS: co-hydrolysis; enzymatic hydrolysis; high throughput; multiwell plate; pretreatment

Introduction

Cellulosic biomass in such forms as agricultural residues (e.g., corn stover), forestry wastes (e.g., forest slash), portions of municipal solid waste (e.g., yard waste and paper), and ultimately herbaceous (e.g., switchgrass) and woody (e.g., poplar wood) energy crops provides the only sustainable resource that can be converted into liquid transportation fuels on a scale sufficient to have substantial impact (Greene, 2004; Lugar and Woolsey, 1999; Perlack et al., 2005). Furthermore, the cost of cellulosic biomass is competitive with oil; for example, corn stover at $60/metric ton has about the same energy cost as petroleum at about $20/barrel (Lynd et al., 1999). Biological conversion is favorable for breakdown of the hemicellulose and cellulose that generally account for two thirds to three quarters of plant matter to fermentable sugars because of the high yields possible and the potential for biotechnology to dramatically reduce costs. Yet, high doses of very expensive enzymes are currently needed, and even then, a costly pretreatment step is essential to overcome the natural resistance to breakdown by these biocatalysts (Wyman, 2007). Consequently, biological conversion of cellulosic biomass to ethanol, butanol, alkanes, or any other liquid fuel will not be competitive until the cost of the sugars from which they are made is lowered significantly (Lynd et al., 2008).

Recently the U.S. Department of Energy selected 3 new Centers to develop novel biological approaches, and one, the BioEnergy Science Center (BESC), is focused on altering plant features to make them more amenable to conversion coupled with advancing microbial and enzymatic conversion technologies for better sugar release. The former strategy entails searching for plant phenotypes with less recalcitrance to breakdown and to understand gene variants

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and biochemical characteristics advantageous for enhanced sugar release (Lynd et al., 1991, 1999), providing a foundation for rational engineering of superior plants. Unfortunately, no single analytical parameter describes biomass recalcitrance, and promising species must be identified based on sugar release from coupled operations of pretreatment and enzymatic hydrolysis similar to those expected commercially.

In the conventional laboratory approach to evaluating pretreatment with dilute sulfuric acid or just water coupled with subsequent enzymatic hydrolysis, raw biomass is held at temperatures over about 140°C, and the solids and liquid are separated after pretreatment by filtration, the solids are washed, and their moisture and carbohydrate content are measured. Cellulase and supplementary enzymes are then added to the pretreated wet solids based on glucan plus possibly xylan content of the remaining solids. The amount of sugars in the liquids produced by both pretreatment and enzymatic hydrolysis is measured, with post hydrolysis often employed to deduce oligomer concentrations. Tedious and exacting wet chemistry methods are required to measure how much sugar is left in the solids. HPLC is generally employed to quantify sugar concentrations in all of the liquid streams that are produced by pretreatment and enzymatic hydrolysis and released from the residual solids. Finally, this data is combined with measurements of mass for each stream to calculate yields (Wyman et al., 2005).

Genetic modifications of plants and microbes, molecular engineering of proteins for biomass deconstruction, and definition of appropriate pretreatment conditions create a very large sample set to be tested for enhanced sugar release. For example, the BESC plans to screen up to about 4,000 biomass variants annually to identify those that promise enhanced sugar release when pretreated with just hot water or dilute acid with the goal of developing combinations of plants and processes that need little if any pretreatment to realize high yields. This load equates to around 400 samples per day if three pretreatment and three enzymatic hydrolysis conditions were run in triplicates for 5 days per week year round. In addition, limited amounts of many of these materials are available as sample size can be limited to about 50 mg in order to allow continued growth of trees being studied in the Center. Thus, previous protocols would be impractical to employ, particularly for a screening operation of this nature, and the BESC needed to streamline pretreatment and enzymatic hydrolysis testing. It was viewed as important to integrate pretreatment, which had been an obstacle to developing a fully integrated HTP pipeline, as seamlessly as possible with an existing multiwell plate method (Decker et al., 2003) that employs robots for evaluation of combinations of enzymes and feedstock. For screening to identify more easily hydrolysable biomass materials, we decided to add enzymes directly to the liquor from pretreatment and to add excess enzyme and perform pretreatment at only 1% (w/w) glucan concentrations to minimize inhibition of enzymes by sugars that could otherwise mask differentiating biomass recalcitrance from enzyme inhibition while still assuring adequate sugar concentrations for analysis (Kumar and Wyman, 2009; Palmqvist et al., 1996; Panagiotou and Olsson, 2007; Sanderso, 1965; Yourchisin and Van Walsum, 2004). We termed this approach that we have demonstrated to work successfully in conventional reactors as co-hydrolysis. This system could also be employed at higher solids concentrations with a fixed biomass material to screen multiple enzyme formulations for those that exhibit reduced inhibition by compounds released in pretreatment and enzymatic hydrolysis. The overall goal is to rapidly screen large numbers of materials, enzymes, and/or pretreatment conditions and that more detailed conventional analysis will be employed to more fully characterize those candidates that the HTP co-hydrolysis system suggests are most promising.

In this article, we focus on demonstrating that the HTP pretreatment device provides the rapid heat up and cool down of biomass needed to minimize the effects of temperature transients on results. We also show that the HTP system provides uniform temperatures among the well plate so that differences in performance are not impacted by differences in temperature histories. Furthermore, the well plates are shown to be effectively sealed to assure that loss of contents does not affect performance comparisons. Finally, the consistent sugar release measured for all 96 wells in a plate confirms that the device provides consistent data and that representative biomass can be distributed to each well despite the small sample size.

Materials and Methods

Plant Material

A genotype of *Populus trichocarpa* grown at the Oak Ridge National Laboratory was used for all experiments and termed BESC standard poplar in this article. The logs were debarked, split with an axe, chipped (Yard Machines 10HP, MTD Products Inc., Cleveland, OH), and knife milled (Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) through a 1 mm screen size. The wood was air dried in Colorado at the National Renewable Energy Laboratory for approximately one month (about 5% moisture content). All the material was then sieved to less than 20 mesh ($<$0.85 mm) and greater than 80 mesh ($>$0.180 mm) (Ro-Tap RX-29, W.S. Tyler, Mentor, OH). Particles larger than 20 mesh were reground, sieved again, and the resulting 20–80 mesh fraction was mixed with the 20–80 fraction obtained in the first place.

96 Well-Plate

As shown in Figure 1, the custom-made well-plate has the same footprint as a standard well-plate, and the inner diameter, shape and the depth of the wells are the same as for a standard 300 μL COSTAR 96 round bottom well-plate

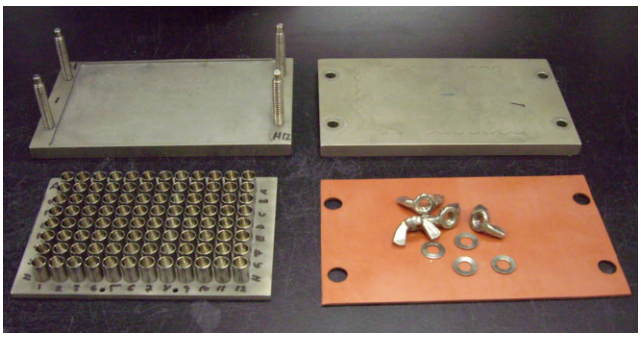

Figure 1. Custom-made 96 well-plate with free standing cups attached to a plate is sandwiched between a top and bottom plate together with a flat gasket to seal each well individually. The well-plate has standard dimensions (127.8 mm in length × 85.5 mm in width) to allow loading with biomass, liquid, and enzymes by robotic tools prior to being clamped together for pretreatment or enzymatic hydrolysis.

(Corning Inc., Acton, MA). However, the wells were made by cutting Hastelloy 276 bars to a length of 11.57 mm with the core of the bar milled to a wall thickness of 0.5 mm and with the top rim of the well tapered at an angle of 80° down to a wall thickness of 0.3 mm. Each well had a volume of 320 μL. The wells were press fit into a 3 mm thick bottom plate made of Aluminum 7075 that was 127.8 mm in length by 85.5 mm in width. Each well on the plate was sealed individually during pretreatment and enzymatic hydrolysis by clamping the well-plate between a bottom and top plate made of 304 stainless steel together with a flat Silicone gasket (thickness 1.5875 mm, durometer hardness A40). The sandwich was clamped together using four 1/4 inch-20 threaded bolts (6.35 mm-20) placed in each corner of the plate together with spring washers (flat load 1,500 N) and wing nuts to allow rapid closing and opening.

Tube Reactors

Tube reactors were made of 5.91 inch (0.150 m) long by 1/2 inch (12.7 mm) outer diameter stainless steel (316) tubes (wall thickness 0.035 inch or 0.889 mm) with both ends closed by stainless steel (316) tube fittings and caps (Swagelok, San Diego Fluids System Technologies, CA) (Lloyd and Wyman, 2005).

Heat-Up/Cool-Down of the Reactors During Pretreatment

The tubes and the well-plate were placed horizontally and lengthwise inside a custom made steam chamber for heat up to pretreatment temperatures and cool down at the end. The well-plate was placed in the chamber with column 1 noted in Figure 3 inserted first. The chamber was made of readily available steam rated (to 1 MPa steam pressure) stainless steel (316) 4 inch (0.102 m) nipples and fittings (McMaster, Santa Fe Springs, CA). The central piece of the chamber was a horizontally placed, 2 ft (0.610 m) long pipe nipple, which was closed on one end using a pipe cap and on the other end connected to a pipe cross, which was again connected to a ball valve. This configuration provided a lockable chamber that could be easily accessed by opening the ball valve. The cross was turned vertically, with a steam trap connected to the lower outlet and an air vent and release valve connected to the upper one. A steam boiler (FB-075-L, Fulton Companies, Pulaski, NY) and cooling water were connected to the chamber through the pipe cap. Steam allowed rapid heating of the chamber, while rapid cooling was accomplished by shutting the steam inlet valve to the chamber, opening the vent from the chamber, and flooding the chamber with cold water in rapid succession. A thermocouple (type K) (Wilcon Industries, Lake Elsinore, CA) connected to a portable measuring device (Fluke 51-2, McMaster) and an analog pressure gauge installed in the chamber allowed monitoring of temperature and pressure in the chamber. Heat-up and cool-down temperature profiles were measured in the tubes as well as in selected wells of the well-plate using stainless steel thermocouples (type K) inserted in the liquid phase. Thermocouples with a diameter of 1/16 inch (1.5875 mm) were installed in the cap of the tube reactors and in the closing lid of the well-plate using bored through brass screw fittings (Swagelok, San Diego Fluids System Technologies, CA). The positions on the cap of the tube and on the top plate of the well-plate were distributed such that the thermocouples stuck into the centers of the tube and the wells, respectively. The metal parts of the thermocouple, which stuck out of the screw fitting, as well as the fittings themselves were insulated using Teflon sealing tape, which was wrapped around all metal parts to a thickness of ca. 0.04 inch (1 mm). Because temperature was measured inside the steam chamber but recording was done outside the chamber (CR10X Measurement & Control Datalogger, Campbell Scientific, Inc., Logan, UT), the cables of the thermocouples were passed through and cast (J-B weld, J-B Weld Company, Sulphur Springs, TX) into a 3/8 inch (9.525 mm) stainless steel (316) pipe (McMaster), which was connected to the steam chamber using a bore through screw fitting (Swagelok).

Leak Testing

The liquid volumes in all wells were quantified before and after pretreatment by employing the fluorometric assay commonly used to calibrate multichannel pipettes. 250 μL of phosphate buffer (pH 9, for optimal fluorescein signal) was accurately measured into each well using a multichannel pipette (8 channel pipetter, 30–300 μL, Eppendorf, Hamburg, Germany). The well-plate was sealed as described above and held at the maximum possible temperature of 180°C for 55 min. After rapid cooling and opening of the well-plate assembly, 20 μL (8 channel pipetter, 10–100 μL, Eppendorf) of a fluorescein solution was added to a final

concentration of 100 nM. Then, 200 μL of the mixed solutions was transferred to a well-plate appropriate for fluorescent reading using the same multichannel pipette. The solutions were excited at 485 nm, and the fluorescence intensity was measured at 525 nm (SpectraMax M5e, Molecular Devices, Sunnyvale, CA), with the readings providing a direct function of the liquid volume in the wells after pretreatment. An analogous test was applied to the larger reaction tubes with an initial buffer volume of 9 mL.

Pretreatment and Enzymatic Hydrolysis

The co-hydrolysis experiments reported here were all performed at a solids concentration of 1% (w/w), and for co-hydrolysis in the well-plates, a wet reaction mass of 250 mg was used, corresponding to 2.63 mg of biomass, based on a biomass moisture content of about 5%. To enable manual weighing of these small amounts in a reasonable time, uniformly milled and mixed biomass was scooped with a specially made aluminum weighing beaker that held a volume corresponding to the target mass. Then, the mass in the beaker was weighed (AB135S, Mettler-Toledo Inc., Columbus, OH), thereby allowing normalization of the sugar release and later application of enzyme loadings, and the contents were poured into the individual wells. The mass distributed to the wells for the experiment presented in Figure 4 was 2.78 ± 0.23 mg. Next, 247.4 μL (8 channel pipetter, 30–300 μL, Eppendorf) of DI water was added to each well, to produce a range of solid concentrations from 0.97% to 1.14% (w/w). The biomass was then soaked in water for exactly 4 h before pretreatment. After pretreatment, 20 μL of 1 M citric acid buffer (pH 4.95), sodium azide solution (1 g/L), and enzyme were pipetted into each well (8 channel pipetter, 10–100 μL, Eppendorf) using a solution of 5 mL of buffer, 1 mL of sodium azide solution, and 1.989 mL of cellulase and xylanase at a total protein mass ratio of 3:1 to which was added DI water at a ratio of 3:1. The result corresponded to an enzyme loading of 75 mg of cellulase plus 25 mg of xylanase protein/g of total glucan plus xylan in the raw biomass (49.7 FPU/g glucan in raw biomass and 45,955.9 OSX/g xylan in raw biomass). This high enzyme loading was applied when screening for the effects of changes in biomass species and/or pretreatment conditions to be sure that inhibition of enzymes by compounds released in pretreatment and enzymatic hydrolysis did not interfere with enzyme action (Wyman CE et al. 2005a, Wyman CE et al. 2005b, Wyman CE et al. 2008); lower enzyme loadings could be applied if the goal is to screen for enzymes that are less subject to inhibition. Following enzyme addition, the plate would be re-sealed as previously described, and placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD) at 50°C for 72 h, shaking at 150 rpm.

Biomass for control experiments was pretreated in tube reactors that we and numerous others have used many times before. The total mass of the biomass slurry per tube was 8.3 g for the 1% and 5% (w/w) solid concentrations, and the contents of three tubes were needed for one enzymatic hydrolysis experiment, each of which was performed in triplicate. For co-hydrolysis, the entire pretreated material was poured directly into a 125 mL Erlenmeyer screw cap flask (FisherScientific, Pittsburgh, PA). In the case of washed solids hydrolysis, pretreated material was poured into 50 mL centrifuge tubes (Corning 50 mL PP centrifuge tubes, FisherScientific), and the mass of each tube was recorded (MXX-601, Denver Instruments, FisherScientific). The contents of these tubes were washed 3 times by spinning the pretreated biomass down (CS-6R Centrifuge, Beckman, Fullerton, CA) and re-suspending it with 50 mL of DI water each time. After the final wash step, biomass was re-suspended with DI water to meet the original weight of the tube holding the pretreated slurry. The slurry was then transferred to 125 mL Erlenmeyer flasks for enzymatic hydrolysis. Next, 1.25 mL of 1 M citric acid buffer (pH 4.95), 250 μL of a sodium azide solution (1 g/L), and 497 μL of a dilute enzyme mixture were added to all flasks. The enzyme mixture contained cellulase and xylanase at a protein mass ratio of 3:1 and was again diluted with DI water at a 1:3 ratio. After enzyme addition, flasks were placed in an incubation shaker (Multitron Infors-HT, ATR Biotech) at 50°C for 72 h, shaking at 150 rpm.

Sugar Analysis

Sugar concentrations were analyzed using high performance liquid chromatography. An Aminex HPX-87H column (BioRad, Hercules, CA) heated to 65°C was used on a separation module (Alliance 2695, Waters, Milford, MA) equipped with a refractive index detector (2414, Waters) and using 0.005 M sulfuric acid as the eluent in an isocratic mode. 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 been included. Additionally, enzyme blanks were injected on the HPX-87H column to demonstrate that soluble sugars in the enzyme solutions did not interfere with glucose and xylose peaks.

Statistical Analysis

In order to test whether pretreatment and the enzymatic hydrolysis in tubes and in the well-plate yielded the same sugar recovery, the probability of equal means of sugar recovery was calculated using a two-tailed, heteroscedastic (unequal variance) Student's *t*-test. The three different experimental designs–washed solids hydrolysis in tubes, co-hydrolysis in tubes, and co-hydrolysis in the well-plate–were tested against each other. The number of necessary replicates in the well-plate required to obtain the same accuracy as in pretreatment in tubular reactors and co-hydrolysis in 125 mL Erlenmeyer flasks, was calculated

following the approach that the standard deviation of the mean,

$$\sigma_{\bar{x}} = \frac{\sigma_x}{\sqrt{N}} \quad (1)$$

must be equal for both methods. Therefore the number of replicates in the well-plate can be calculated following Equation (2):

$$\frac{\sigma_{\text{well}}}{\sqrt{n}} = \frac{\sigma_{\text{tube}}}{\sqrt{3}} \Rightarrow n = 3 \times \frac{\sigma^2_{\text{well}}}{\sigma^2_{\text{tube}}} \quad (2)$$

Results

The following questions had to be addressed before we could be certain that the design could meet our needs: (1) would co-hydrolysis after pretreatment tell us whether some plant materials were more easily broken down to sugars than others, that is, would co-hydrolysis yield the same sugar recovery as conventional washed solids hydrolysis, (2) is the seal of the well plates effective in preventing loss of solids or liquid, (3) do all the well plates heat up rapidly and experience similar temperature histories, (4) would sugar release by co-hydrolysis be similar for a material regardless of which well it occupied, (5) could the multiwell plate produce similar results to larger scale tube runs, and (6) how many replicates in the well-plate were required to achieve the same accuracy as in the tubular reactors? We addressed the first question by comparing sugar yields from the conventional approach based on hydrolysis of washed solids to co-hydrolysis using BESC standard poplar wood, both in the larger tubular reactor systems that we have employed many times in the past and pretreated in the steam chamber. A pretreatment optimization curve for 1% slurries at 180°C in just water for five different times was developed (Fig. 2). A portion of the pretreated material from the tubular reactors was washed prior to adding enzymes, but enzymes were also added to the entire slurry without separation for the remaining pretreated portion in the co-hydrolysis approach. Total sugar yields from pretreatment in tubular reactors plus co-hydrolysis and washed solids hydrolysis, respectively, using an enzyme loading of 75 mg of cellulase plus 25 mg of xylanase/g of total glucan and xylan in the raw biomass, were the same, and the trends were clearly similar, in that the curves overlapped especially for longer pretreatment times and the sugar yields peaked at a value of 88% for the same times. This observation is also supported by the statistical analysis using a Student's *t*-test, in which the discrepancies between the means of the sugar recoveries for co-hydrolysis and washed solids hydrolysis in tubular reactors are not significant at the 5% level for pretreatment times longer than 28 min.

It was vital to be sure the plates did not leak from the point they were loaded with biomass, water, and catalysts

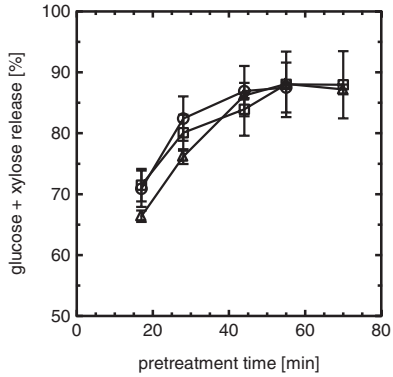

Figure 2. Total yields of glucose plus xylose as a function of pretreatment time from the combined operations of pretreatment and enzymatic hydrolysis for pretreatment at 180°C in just water for different reactors and enzymatic hydrolysis regimes. Co-hydrolysis, well-plate; Co-hydrolysis, tube reactors; Washed solids hydrolysis, tube reactors. An enzyme loading of 75 plus 25 mg respectively of cellulase and xylanase protein/g of total glucan plus xylan in the raw biomass was used for all experiments. The total sugar yields from pretreatment and enzymatic hydrolysis were a maximum at a pretreatment time of 55 min for all cases. The pretreatment of the washed solids hydrolyses were done at a solids loading of 5% (w/w), the co-hydrolyses at 1% (w/w) solids loading. The total sugar yields achieved in the 96 well-plate are identical to the yields found in the tube reactors for both hydrolysis regimes (plotted are the means and the standard deviations of three replicates for the experiments pretreated in tube reactors, and of 96 replicates in the well-plate).

until they were opened after pretreatment as losses could alter the ratios of biomass to water and result in misleading sugar concentrations. Because differential pressures between the wells and surrounding atmosphere can reach 1 MPa at the start of heat up and when steam pressure is released prior to cool-down (the saturation pressure of steam at the maximum used temperature of 180°C is 1 MPa), fluorometric readings of a well plate, cooked for 55 min were compared to those of a control plate, which underwent identical steps except that the control plate was never sealed or cooked. The mean of the ratios of the fluorescent readings of the cooked versus the control was 1.00 ± 0.04, and the standard deviation of the liquid volumes in the 96 wells was 4.2% after pretreatment, compared to 2.3% measured in the control experiment.

Figure 3 presents temperature profiles measured by thermocouples inserted into selected cups of the multiwell plate system, with their locations shown in this illustration. The plate was placed lengthwise into the steam chamber described earlier, and steam and cooling water was introduced from the side of column 1. After holding at 180°C for about 2 min, the inlet steam valve was closed, the chamber vent opened to release steam pressure, and the chamber flooded with cold water to cool down the HTP device. The data in Figure 3 show that the temperature in the cups rose from room temperature to 180°C in about 40 s and that less than 30 s was needed to increase the temperature from 120 to 180°C. The temperature dropped back to 120°C in only about 10 s after cold water was introduced. Just as

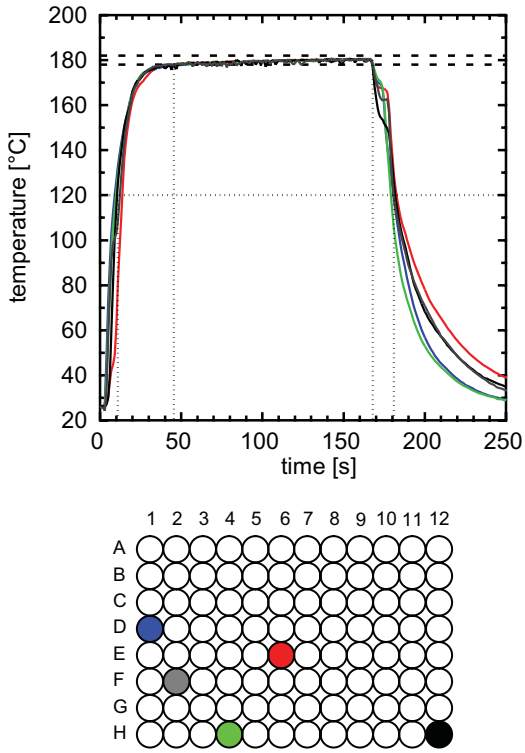

Figure 3. Heat-up and cool-down temperature profiles for water in the wells of the custom-made 96 well-plate heated in a chamber with condensing steam and cooled by flashing the steam and flooding with cold water. The color of the lines encode for the position of the thermocouples in the well-plate, as shown in the 96-well plate schematic. The heat-up and cool-down from 120 to 180°C and vice-versa are on the order of 30 and 10 s, respectively, a very short period compared to the reaction time. Wells located in the interior of the well plate lag only a few seconds behind the ones located at the periphery, and divergence of temperatures for the different wells only becomes important for temperatures below 120°C where further reaction is very slow (the two dashed horizontal lines represent the error of thermocouples Type K).

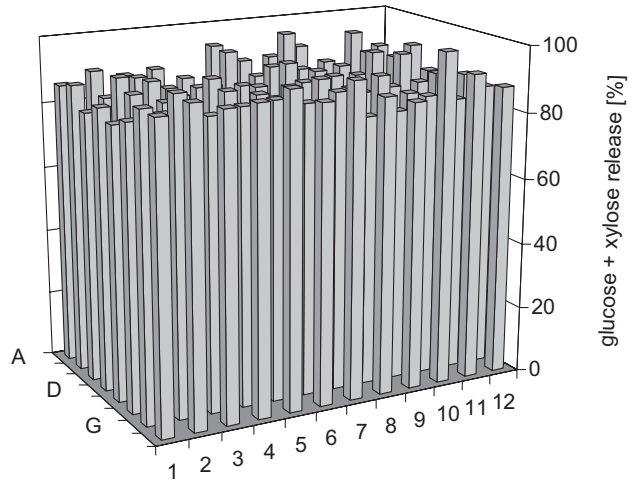

Figure 4. Total sugar release from pretreatment and enzymatic hydrolysis for the 96 wells. The biomass slurries were pretreated at 180°C for 55 min, and enzymatic hydrolysis was done at an enzyme loading of 75 plus 25 mg respectively of cellulase and xylanase protein/g of total glucan plus xylan in the raw biomass. The small variation between the individual wells is random and not correlated to the position of the well on the plate. The standard deviation of the total sugar yield across all 96 wells is 4.1%.

importantly, Figure 3 shows that the temperature is very uniform across the plate, and the profile for the center cup closely follows those at the perimeter, with only a small difference near the end of the cool down period where little reaction would occur.

Next we wanted to verify that results would be the same for all the cups in the well plate system. Thus, known masses of BESC standard poplar (1% solid slurries) were loaded into each cup, and all 96 cups containing these samples were pretreated with just water at 180°C for 55 min, followed by enzymatic hydrolysis at an enzyme loading of 75 mg of cellulase plus 25 mg of xylanase/g of total glucan and xylan in the raw biomass to be sure enzyme inhibition does not mask differences in biomass susceptibility to hydrolysis. Pretreatment and enzymatic hydrolysis via the co-hydrolysis approach but using conventional equipment established that these conditions resulted in high sugar yields (Fig. 2). Total glucose and xylose release from all 96 wells were then measured at the completion of enzymatic hydrolysis using HPLC. As shown in Figure 4, variations among sugar yields from the individual wells were not correlated to the position of the well on the plate. In addition, the standard deviation of the total sugar yield across all 96 wells was only 4.1%.

Total sugar yields from pretreatment and co-hydrolysis measured in the well-plate were compared to yields achieved in standard tubular pretreatment reactors using conventional washed solids hydrolysis as well as co-hydrolysis, using the BESC standard poplar as the feedstock. All pretreatments were conducted in the steam chamber at 180°C over the same range of times pictured in Figure 2, and enzyme loadings of 75 mg of cellulase plus 25 mg of xylanase/g of total glucan plus xylan in the untreated wood were applied once again. The sugar yields achieved in the HTP system were compared to biomass pretreated in larger tubular reactors used as control. These results show that the performance of pretreatment and co-hydrolysis in the well-plate were the same as those in pretreatment in tubular reactors and followed by enzymatic hydrolysis in Erlenmeyer flasks, a result also confirmed by application of the Student's *t*-test at the 5% significance level. Finally, the number of replicates in the well-plate needed to achieve the same accuracy as co-hydrolysis in the tubular reactors was calculated at each pretreatment time, with the mean and standard deviation determined to be 1.8 ± 0.6.

Discussion

An effective device was developed to allow rapid screening of large numbers of plant phenotypes to identify those with reduced recalcitrance and to understand the genotypic and

biochemical characteristics advantageous for enhanced sugar release, providing a foundation for rationally engineering plants. The well-plate format widely used in microbiology was customized to streamline and parallelize high temperature pretreatment followed by enzymatic hydrolysis in the same metal vessels. Because rapid and uniform heat-up without overheating (which can easily occur in electrically heated instruments or baths set to a higher temperature to speed up the heating) as well as rapid quenching of the reactions are crucial to a successful and reproducible pretreatment action, we chose to use saturated steam to take advantage of the more uniform temperature, greater penetration ability, and high heat transfer rates. Individual cylindrical cups were employed to provide spaces between the wells so that steam could penetrate to the inner wells and condense, and water could pass through these spaces for rapid cooling when the chamber is flooded with cold water at the end of the pretreatment time. The result was that rapid heat up and cool down times were realized and the temperature history was virtually the same for all wells measured.

In order to reduce the effects of heating and cooling on the pretreatment performance, the ratio of temperature transient periods to the actual pretreatment time needs to be minimized. In fact, heat up and cool down were shown to be about two orders of magnitude faster than seen for electrical heaters and air cool down approaches often sold with high pressure reactors and about an order of magnitude faster than sand baths often employed to heat up small scale pretreatment reactors. The temperatures in different wells only deviated towards the end of the cooling cycle at temperatures below 140°C, because water of its higher viscosity and density than steam can not as easily penetrate the spaces between the individual wells. However, because hemicellulose hydrolysis kinetics closely follow the heuristic that the rate of reaction doubles for every 10°C change in temperature (Abatzoglou et al., 1992), limited reaction is expected to occur below 120°C.

A colorimetric leakage test showed that a combination of several measures was effective in virtually eliminating the leakage encountered in an initial device we made by drilling holes in a solid aluminum block. First, the top rims of the cups were tapered to reduce the gasket compression area. In addition, an elastic gasket was employed to seal the wells, and spring washers were inserted between the top plate and the wing nuts to maintain the force necessary to seal the wells even at elevated temperatures and pressures. Thus, any differences in performance among the well plates are expected to result from differences in the substrate and reaction ingredients during pretreatment and enzyme doses and formulations during biological conversion and not differences in thermal history or leakage.

Because solids and liquid could not be accurately separated in the HTP pretreatment screening process, it was important to show that the HTP system could give similar performance to conventional approaches. For very dilute biomass slurries of only 1% (w/w), combined with rather high enzyme dosages of 75 plus 25 mg respectively of cellulase and xylanase protein/g of total glucan plus xylan in the raw biomass to avoid inhibition of enzymes masking comparisons, virtually identical yields were achieved from co-hydrolysis as for conventional washed solids hydrolysis performed with 5% (w/w) slurries (Fig. 2). Statistical analysis demonstrated that discrepancies only exist at shorter pretreatment times. Consequently, any inhibition due to substances possibly produced or released from biomass during pretreatment, such as HMF, furfural, acetic acid (Palmqvist et al., 1996), lignin degradation products (Panagiotou and Olsson, 2007; Sanderso, 1965) or glucose and xylose oligomers (Kumar and Wyman, 2009), were overcome. This key finding proves that different experimental conditions such as solids loading can produce the same hydrolysis results, and that co-hydrolysis is effective in the HTP system.

The standard deviation of total sugar release when the same biomass was used in all 96 wells was 4.1%, which is directly in the range of the 4.2% measurement for the leakage test. Thus, this result is further evidence of the consistency in performance of the multiwell plate and also shows that the biomass milling and distribution approach is acceptable. It also suggests that the multiwell plate approach should be capable of detecting differences in yields of over 10% targeted by the BESC. In addition, plots of the total sugar yield from pretreatment and enzymatic hydrolysis in the well-plate and in tubular reactors versus the pretreatment time nicely overlap, which is also confirmed by a Student's *t*-test using a 5% significance level, and all curves peak at the same time of 55 min. Thus, reaction results are essentially identical, demonstrating that the multiwell plate performance was consistent with that obtained using much more labor intensive standard methods, that is, tubular reactors and washing the substrate after pretreatment and prior to enzymatic hydrolysis.

A statistical analysis of the number of replicates in the well-plate required to reach the same accuracy as in pretreatment in tubular reactors followed by co-hydrolysis in Erlenmeyer flasks showed that triplicates are sufficient. This small number, especially when considering the limited amount of biomass used per well, can be explained by the fact that for pretreatment in tubular reactors and enzymatic hydrolysis in Erlenmeyer flasks, an additional quantifiable error is introduced due to transfer of the slurry, a process step which is omitted in the well-plate approach. In a single 96 well-plate, up to 32 samples could theoretically be tested simultaneously—in reality it is desirable to run controls, which would decrease the number of samples—proving its value as a true HTP screening device.

Conclusions

As reported above, a coupled HTP pretreatment and enzymatic hydrolysis device has been developed to support the BESC in the rapid screening of thousands of biomass

materials annually for improved sugar release characteristics. It will also be invaluable in identifying enzyme formulations that better integrate with choice of feedstocks and in identifying pretreatment conditions that work best with different enzymes and feedstocks. The HTP device is simple and can be readily employed with standard robotic systems to allow rapid performance evaluations over a large range of variables. An approach was developed to effectively seal the individual wells in the multiwell plate and prevent losses of solids or liquids that would lead to potentially large errors. Employing individual cups as wells coupled with heating with saturated steam and flooding with cold water was very effective in providing rapid and uniform heat up and cool down, respectively, thereby assuring all wells have very similar thermal histories. Hot water pretreatment followed by enzymatic hydrolysis of *Populus* with this novel device gave very similar results for all the cups in the multiwell plate and were statistically identical to those from application of standard methods. Thus, combined pretreatment and co-hydrolysis in the multiwell plate can be an effective tool for accelerating progress in developing approaches to overcome the recalcitrance of cellulosic biomass, the primary obstacle to achieving very low costs. Additionally this development could enable multi well-plate applications to fields where high pressures and temperatures are used, such as heterogeneous reactions with insoluble catalysts.

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