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Lignocellulose Recalcitrance Screening by Integrated High Throughput Hydrothermal Pretreatment and Enzymatic Saccharification

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We report a novel 96-well multi-plate reactor system for comparative analysis of lignocellulose recalcitrance via integrated hydrothermal pretreatment and enzymatic saccharification. The system utilizes stackable nickel/gold plated 96-well aluminum reactor plates, a clamping device fit to a standard Parr reactor, and robotics for efficient liquids and solids handling. A capacity of twenty plates allows up to 1920 separate hydrothermal reactions per run. Direct and rapid analysis of key end-products, glucose and xylose, is facilitated by the use of glucose oxidase/peroxidase and xylose dehydrogenase linked assays. To demonstrate efficacy, a set of 755 poplar core samples from the Department of Energy's BioEnergy Science Center was tested. Total sugar release ranged from 0.17 to 0.64 g/g of biomass and correlated strongly with the ratio of syringyl to guaiacyl lignins in the samples. Variance among sample replicates was sufficiently minimal to permit clear assignment of differences in recalcitrance throughout this large sample set.

Keywords: lignocellulose, recalcitrance, pretreatment, saccharification, high-throughput

Understanding of the fundamental nature of biomass recalcitrance is critical to the advancement of technologies used in the conversion of lignocelluloses[1]. A key objective of the DOE-funded

BioEnergy Science Center (BESC) is to identify intrinsic and environmental factors that contribute to biomass recalcitrance and to apply this knowledge to the development of less recalcitrant plant cell walls. To facilitate this work, a high-throughput pretreatment and enzymatic saccharification pipeline is required to screen environmental and genetic plant variants for differences in susceptibility to pretreatment and enzymatic saccharification. As with any high throughput methodology, the quality of the information acquired must be balanced by the realities of sample size, acquisition time, and system complexity.

In recent years, there has been an increasing demand for high throughput (HTP) screening methods to speed biocatalyst development for the nascent lignocellulose to biofuels industry. Much effort has been focused on HTP enzymatic assays, particularly those screening for or measuring cellulase activity [2-7]. Decker and coworkers were the first to automate and miniaturize the IUPAC filter paper assay for cellulase activity using the 96-well plate format[4,8]. The reproducibility and precision of this approach were later improved with the utilization of a 96-well plate temperature cycler[7]. In the development of HTP lignocellulose screening methods, the uniform distribution of small quantities of biomass into sample tubes or plates has been a consistently noted challenge[9,10]. Berlin and coworkers resolved this dilemma utilizing disks cut from paper hand-sheets formed from pretreated biomass and manually loaded into micro-titer plates[9]. Other researchers effectively dispensed biomass from liquid slurries, though settling and tip fouling were found to be problematic[10].

Whereas much of the research focused on thermochemical pretreatment of lignocelluloses has frequently utilized smaller bench-scale (10-200 mL) reaction systems, there have been very few studies aimed at further reducing the experimental scale of this work[11-15]. We have previously reported milliliter-scale hot water and dilute acid pretreatments using HPLC vials at temperatures

up to 180°C, although this work was low-throughput and relied on time consuming HPLC for sugar quantitation[16]. Zavrel and coworkers have proposed a high-throughput, micro-titer plate method for screening ionic liquid pretreatments of lignocelluloses, however this work was done in 96-well plates at temperatures below 85°C and utilization of ionic liquids as a viable pretreatment technology is still in the early investigatory research phase[17]. At this time, there are no publically available reports of HTP and high temperature pretreatment of lignocelluloses in the standard 96-well plate format.

In order to meet the high-throughput demands of the BESC, we report here the development of an integrated plate processing system for screening large sets of lignocellulosic samples for their susceptibility to combined hydrothermal pretreatment and enzymatic saccharification. The system incorporates robotics systems for dispensing solids and liquids, a novel 96-well-plate pretreatment reactor system, and enzyme-linked oxidation-reduction assays for the rapid detection of the principle sugars released by the combined processes.

METHODS

96-well reactor plate system

The novel single-piece 96-well plate reactors were machined by Aspen Machining (Lafayette, Colorado) from 0.625-inch thick 6061 T6 aluminum to dimensions consistent with the Society for Molecular Biology standards for microtiter plates (Figure 1A). The only major dimensional alteration, increasing the well diameter from 6.8 to 7.0 mm, increased the well volume from 330 to 417 µL. Individual plates are stackable, allowing more than one plate to be run

simultaneously. Steam ports (4 adjacent to each well, 117 per plate) are centered between individual wells to facilitate steam transport throughout an entire stack when heated in a 2-gallon Parr reactor (Parr Instrument Company, Moline, Illinois). The ports are also intended for water transport during post-pretreatment cooling. To add corrosion resistance, a 5 micron electroless plated nickel layer followed by an electroplated 1.3 micron layer of gold was added to the surface following machining; all surface plating was performed by Advanced Surface Technologies (Arvada, Colorado).

To enhance sealing of the wells and assure alignment of the ports, a clamping system was developed to hold stacked plates together (Figure 1B and 1C). The aluminum base and top plates (Figure 1B) were designed for rigidity while maintaining continuity with the steam/water distribution ports of the plates. The clamping device was designed to hold up to 20 plates, allowing for up to 1920 individual reactions to be run simultaneously. Pressure is placed on the stack by 12 threaded studs around the plate perimeter and one center stud tightened evenly with a torque wrench (Figure 1C). Well liquid volume changes from evaporative losses, condensation, or water incursion were minimized by sealing individual reactor plates with high temperature aluminum foil tape. This seal kept the wells sealed during disassembly and enabled the plates to be centrifuged after mixing. The aluminum foil seal was reinforced by dual polytetrafluoroethene (PTFE) gaskets (0.01 inch thickness each). The PTFE gaskets were perforated along the ports with a custom-built 117-hole-punch die. Two PTFE gaskets were placed on top of each sealed plate and the stacked plates were tightened into the clamp. The seals were perforated along the steam channels with a metal scribe.

The entire reactor stack and clamp were fit to a modified 2-gallon Parr reactor (Parr Instrument, Moline, IL), model 4554, constructed of Hastelloy C-276 (Figure 1B). The Parr

reactor head plate was fitted with a supporting disk connected by four stay rods to the Parr reactor head plate. This disk supported the clamped plate stack. Steam or cooling water (selected by a T-valve), was introduced underneath the base plate through a 3/8" tube and was channeled through the ports in the stack. A clearance of 0.025" between the sides of the top and bottom plates and the Parr reactor wall allowed steam and water to also move along the outside of the stack. The Parr reactor head plate was vented through a valve to allow air to be displaced by steam and the bottom port on the vessel was equipped with a steam trap to allow drainage of condensate during pretreatment.

Solids and liquids handling systems

A Powdernium powder dispensing system (Symyx, Geneva, Switzerland) was used to dispense milled lignocellulosic biomass into the pretreatment reactor plates. The system's deck configuration allows for up to 80 plastic 10 mL dispense heads to be stored for a single run; these dispensing heads easily handle small powder quantities in the 50-100 mg range. In addition, the system utilizes a modified Sartorius LP330 balance (Goettingen, Germany), which accurately records the final weight dispensed into each well to 0.1 mg. All liquids handling for the recalcitrance screenings was performed on a Biomek FX automated pipetting system equipped with Span-8 and ninety-six channel pipetting heads (Beckman-Coulter, Fullerton, CA).

Pretreatment processing

For all HTP pretreatments, control and experimental biomass samples were milled to pass a 20 mesh screen and dispensed into the reactor plates in 5.0 ± 0.3 mg/well aliquots using the Symyx system. The BESC standard poplar was utilized in all wells for reactor testing and validation. The standard poplar was obtained by milling a whole *Populus trichocarpa* tree to pass a 1 mm screen size; the tree harvested from forest land in the Northwestern United States. For biomass variant recalcitrance screening, reactor plates were loaded with 24 samples, each in triplicate. An additional twenty wells were loaded with BESC standard poplar; twelve for use in generating "with-biomass" glucose and xylose standard curves and eight as normalization control samples for inter-plate comparisons. Four empty wells in each reactor plate served as enzyme-only controls. The samples and standards were distributed evenly across the plate to minimize any localization bias that may occur in the event of an occasional broken seal. Prior to pretreatment, deionized water was added to the plates at a rate of 300 µL per well.

The loaded reactor plates were each sealed with an aluminum foil seal. Two PTFE gaskets were placed on top of each sealed plate and the stacked plates were tightened into the clamp; after tightening the clamp all plate wells are independently sealed and able to become pressurized during heating. The Parr reactor was electrically preheated to 180^{o}C and the entire reactor plate stack and clamp assembly was fitted to the hot Parr reactor head plate carrier and bolted to the Parr reactor chamber. Steam was introduced for 15 seconds with the vent open to displace air and then the vent was closed. The reactor was held at 180^{o}C (as determined by a thermocouple in the reactor vessel) for 40 minutes. Afterwards, the steam was vented and cooling water was allowed to flow directly into the Parr reactor and through the steam ports of the clamped stack. When the reactor temperature dropped below 50^{o}C, the water was drained and the plate stack was removed. After pretreatment, the plates were separated from the stack and

centrifuged for ten minutes at 1000 rpm in Jouan KR4i swinging-bucket centrifuge (Thermo Scientific, Winchester, VA).

Enzymatic saccharification

Enzymatic saccharification of the resultant pretreated lignocellulosic solids and hydrolysate was carried out in the 96-well reactor plates without solid/liquid separation. Following pretreatment and centrifugation, the aluminum seals were pierced with a 96-point punch to permit the addition of buffer and enzymes. The pH of the pretreatment slurry was buffered at pH 5.0 by direct addition of 1 M sodium citrate buffer in combination with the desired enzymes to ensure an optimal environment for enzymatic hydrolysis. A total volume of 40 μL of buffer and enzyme was added to each well. All saccharifications included an excess loading (70 mg/g initial biomass) of a commercial *Trichoderma reesei* cellulase preparation (SpezymeCP, Danisco, Copenhagen, Denmark) augmented with a 2.5 mg/g initial biomass loading of a commercial *Aspergillus niger* β-glucosidase preparation (Novozyme 188, Novozymes A/S, Bagsværd, Denmark). This over-optimal enzyme loading was intended to ensure that enzyme activity was maximal and not the limiting factor regardless of sample variability, allowing for a more clear interpretation of sugar release data with respect to variable characteristics of the biomass samples. Following enzyme and buffer addition, the pierced reactor plates were resealed with a second aluminum foil seal, inverted by hand five times to ensure sufficient initial mixing, and statically incubated at 40^{o}C for 72 h. After incubation, the plates were remixed by several hand inversions, centrifuged, and the wells pierced for sampling and analysis of released sugars.

Oxidation-reduction assays for sugar analysis

Analysis of glucose and xylose release from the combined pretreatment and enzymatic saccharification processes was performed using standard 96-well flat-bottomed polystyrene plates and enzyme-linked oxidation reduction assays. Internal standard curves for glucose and xylose were generated in each plate by the direct addition of glucose and xylose standards to twelve of the BESC standard poplar control wells prior to analysis.

Glucose was detected using a modified glucose oxidase/peroxidase (GOPOD) assay (Megazyme Intl., Wicklow, Ireland) based on the following reaction pathway:

(1) D-Glucose + O_2 + H_2O ➔ D-gluconate + H_2O_2

(2) H_2O_2 + p-hydroxybenzoic acid + 4-aminoantipyrine ➔ quinoneimine (A_{510})

Increased absorbance at 510 nm due to the formation of quinoneimine is the basis of glucose quantification.

Xylose was detected using a xylose dehydrogenase assay (Megazyme Intl., Wicklow, Ireland) based on the following reaction pathway:

(3) D-Xylose + NAD^+ ➔ D-xylonic acid + H^+ + NADH (A_{340})

Absorbance measured at 340 nm due to the production of NADH was the basis for xylose quantification.

The sensitivities of the assays were optimized to be linear between 0 and 1 mg/mL for each sugar. Samples from the reactor plates were diluted 1:10 prior to assaying and 20 μL of each diluted sample was pipetted into 200 μL of reagent for each assay. Assays for both sugars were run at 40°C for 45 minutes prior to measuring absorbance. All components for performing both assays were obtained from Megazyme International Ireland Ltd. (Bray, Co.Wicklow, Ireland).

The specificity of the enzyme-linked detection assays was tested on glucose, cellobiose, xylose, xylobiose, galactose, arabinose and mannose. Each sugar was assayed with each method and the response assessed on a per milligram basis. In addition, select enzymatic hydrolysis data sets were analyzed for glucose and xylose via the enzyme-linked methods as well as by HPLC. Sample analysis by HPLC was carried out using a lead-based Shodex (Kawasaki, Japan) Sugar Column (SP 0810) on a Jasco Inc. (Easton, MD) HPLC system at 80°C using purified water as an eluent flowing at 0.6 mL; sugar peak detection was performed on a Jasco refractive index detector.

System performance

A heating and cooling rate study was performed with a thermocouple-fitted reactor plate. Type-K thermocouples were inserted into wells G2, D6, and C11 through holes drilled into the reactor plate from one long side. The plate was filled with 300 μL water per well, sealed, placed in the center of an 8 plate stack, clamped, and pretreated at 180°C for 10 minutes. The temperature of each thermocouple was recorded every 0.25 sec.

The well-to-well variation in the reactor plates was heavily investigated during system development. Three tests were used; (1) a water-dye test to measure volume variation and

identify poorly sealed regions, (2) a 1.0 mg/mL glucose-only run to observe variations in the pipetting, end-product analysis, and sugar degradation during pretreatment, and (3) full-plate pretreatments of the BESC poplar standard to test the variation across the entire system using lignocellulosic material.

For the water-dye runs, 300 µL of water was added to each well. The plates were sealed, stacked, clamped, and pretreated at 180°C for 40 minutes. After pretreatment, 50 µL of green food-dye solution was added to the wells; the plates were resealed and thoroughly mixed. Two hundred microliters from each well was sampled into clear polystyrene plates and the absorbance was read at 425 nm. Similarly, the glucose-only test was carried out using 300 µL of 1.0 mg/mL glucose in each well, the same pretreatment conditions, and glucose quantification by GOPOD assay.

As a final test, a full twenty-plate stack was run with the poplar standard in every well in order to observe the consistency of the analysis throughout the system when run at maximum capacity. Including the plate filling process, the 72 hour enzymatic hydrolysis, and subsequent analyses, this run took approximately 10 days to complete; the analysis of sugar release in all 1920 wells was completed in 5.5 hours. Each well was loaded with 5 mg of standard BESC poplar and 300 µL of water, pretreated at 180°C for 40 minutes, and glucose and xylose measured as described above.

BESC poplar association study

The first large-scale experiment was run on a sample set of 755 milled poplar cores sampled from several forest regions throughout the northwest United States and southwest Canada; they

are part of an ongoing natural variation association study within the DOE-BESC (G. Tuskan, personal communication). The samples were milled to pass a 20-mesh screen and stored in desiccators to allow equilibration to uniform moisture content. Partial compositional analysis of each sample was carried out by molecular beam mass spectroscopy, providing total lignin and syringyl:guaiacyl lignin ratios for each for the 755 samples[18,19]. Triplicate 5.0 mg samples were dispensed into the reactor plates and pretreated in 300 uL of water at 180°C for 40 minutes as described. Data between plates was normalized to the BESC poplar standard controls present in individual plates.

RESULTS

Heat-up/thermal distribution tests

Thermal profiles for thermocouples placed in the modified reactor plate (Figure 2) show heating to be consistent across the reactor. Heating is minimal while the reactor is being bolted to the Parr vessel and ramps up during steam introduction and venting. Heat-up to 180°C was estimated to occur 52 seconds after vent closure. The temperature in the reactor plate was constant throughout the course of the 40 minute pretreatment. Additional profiles at multiple points in the reactor stack showed similarly consistency results (data not shown).

Detection assay specificity and reliability

Sugar specificity tests indicated that each enzyme-linked assay is specific for the targeted sugars for each respective reaction pathway (data not shown). An exception was the xylose dehydrogenase (XDH) assay's sensitivity to xylobiose (about half that of xylose); this indicates a potential sensitivity to higher xylooligomers (not tested). As xylooligomers are products of the overall conversion, this sensitivity to them does not affect the comparative nature of the results, indicating that the XDH assay is a useful tool for evaluating xylan degradation within a sample set. The glucose oxidase/peroxidase (GOPOD) assay had a nearly 1:1 correlation with glucose detection by HPLC analysis, while the XDH assay slightly overestimated the amount of free xylose when compared to HPLC analysis, likely due to xylooligomer sensitivity (Figure 3).

Well-to-well variation

Water-dye tests evaluating volumetric variability demonstrated high levels of consistency across each plate. The most optimum tests showed standard deviations of ~2.6% of the mean absorbance across all plate wells; about half of this is attributable to pipetting error (~1.3 %, data not shown). Following pretreatment of 1.0 mg/mL glucose plates, standard deviations of the GOPOD assay were ~4.0 % of the mean absorbance. Poplar control plates resulted in standard deviations of 6.0 to 8.5 % of the mean absorbance after pretreatment and enzymatic saccharification. While this is a test of the consistency of the entire system, we acknowledge that this value is highly dependent on the variability of the sample substrate used. Analysis of the BESC standard poplar in all 1920 wells of a twenty-plate run showed similar consistency throughout the reactor stack (Figure 4); less 10 "blow-out" wells (where the seal ruptured) and fewer than 30 extreme outlier wells (>3 SD). The average standard deviations in absorbance

across each plate were 7.6 and 6.6 % of the average absorbance for the respective GOPOD and XDH assays. In addition, well-specific averages throughout the twenty-plate stack showed no specific plate regions to be more susceptible to the treatments than others.

BESC poplar association study

A large set of poplar cores was evaluated using this system. This 755 sample set (association study) was effective in demonstrating the utility of the system for comparing differences in glucose and xylose release following sequential pretreatment and enzymatic saccharification of large sample sets. Within this data set, glucose, xylose, and the combined sugar release respectively ranged from 0.10 to 0.45, 0.07 to 0.19, and 0.17 to 0.64 grams per gram of starting biomass (Figure 5). The average standard deviations amongst triplicates for each sample were 8.4, 8.3 and 7.2% for glucose, xylose and combined sugar release respectively. Median standard deviations were respectively 5.9, 5.7 and 5.2%. In addition, a handful of samples in this set were repeated in multiple reactor plates; these samples produced similar results in all repeat runs (data not shown).

Partial compositional data from molecular beam mass spectrometry (MBMS) analysis of the sample set was utilized to identify trends in the recalcitrance screening data associated with specific compositional factors[18,19]. Sugar release was shown to be independent of overall lignin content, but strongly correlated to the syringyl:guaiacyl ratio of the biomass (Figure 6). Both glucose and xylose release increased proportionally with increasing S/G ratio, although this trend appears reduced at S/G ratios >2.

DISCUSSION

We report a new integrated parallel-plate processing system as perhaps the first truly high throughput system for assessing the susceptibility of lignocellulosic materials to hydrothermal pretreatment and subsequent enzymatic saccharification. The key elements in this system include: A) stackable 96-well pretreatment reactor plates with pressure and heat resistive sealing systems, B) automated solids and liquids handling, C) enzyme-linked oxidation-reduction assays, and D) recognition that the slurries resulting from hydrothermal pretreatments can be taken directly to enzyme saccharification without separation or washing. We expect these features will effectively apply to more corrosive thermo chemical pretreatments with the implementation of alternative plate coatings and metallurgy. We are currently testing several additional coatings including industrial Teflon and Teflon-impregnated nickel. The inert nature of the Teflon should allow dilute acid and alkaline pretreatments such as aqueous ammonia to be carried out. In addition, we have tested reactors made from Hastelloy with dilute sulfuric acid at 180^{o}C and they show no corrosion effects, suggesting they will be suitable for high temperature dilute acid pretreatment.

To a large degree, sequential processing of the pretreatment slurries for recalcitrance screening was not an obvious decision. For example, to reduce the possibility that inhibitory effects from the hydrolysate on enzymes used for saccharification and analysis later on could occur, the following measures were taken: A) solids loading was kept below 2% (w/v), B) cellulase preparations were loaded in significant excess, and C) numerous controls for the reporter enzyme assay were included in each plate. One must consider that the desired result from such a high level screen is the identification of superior performing biomass samples from a

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very large sample set, yet done in such a manner as to be as inclusive as possible. More detailed, albeit lower-throughput, screens will be employed to conduct more extensive testing of promising samples identified through the assay described here.

The sugar release data produced by this system provides a general idea regarding the variation of recalcitrance within a sample set. In combination with accurate compositional data, this system will be an extremely powerful tool that would allow for true differences in recalcitrance to be assessed. Consequently, trends in recalcitrance could be identified with respect to a number of measurable characteristics. For example, we can now confirm, for a very large dataset, the previously observed trend between xylan and glucan conversion in such combined processes (see Figure 4)[20,21]. While current high-throughput lignocellulose compositional analysis is limited to lignin measurements by molecular beam mass spectrometry, the correlation of this data with sugar release data as determined by our high-throughput pretreatment and enzyme hydrolysis system has already proven the utility of the system in providing insight into factors affecting biomass recalcitrance**.** The development of new high-throughput techniques for measuring other compositional elements, such as cell wall polysaccharide content, will significantly expand the utility of this assay. Comparisons may also include information regarding plant genetics, growth conditions, sample phenotype, and harvest parameters. With the increasing growth in sample variability and availability, such analyses will be central to the identification of key trends with respect to biomass characteristics and the mapping of factors crucial to overcoming the barriers of recalcitrance posed by lignocellulosic substrates.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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Figure 1

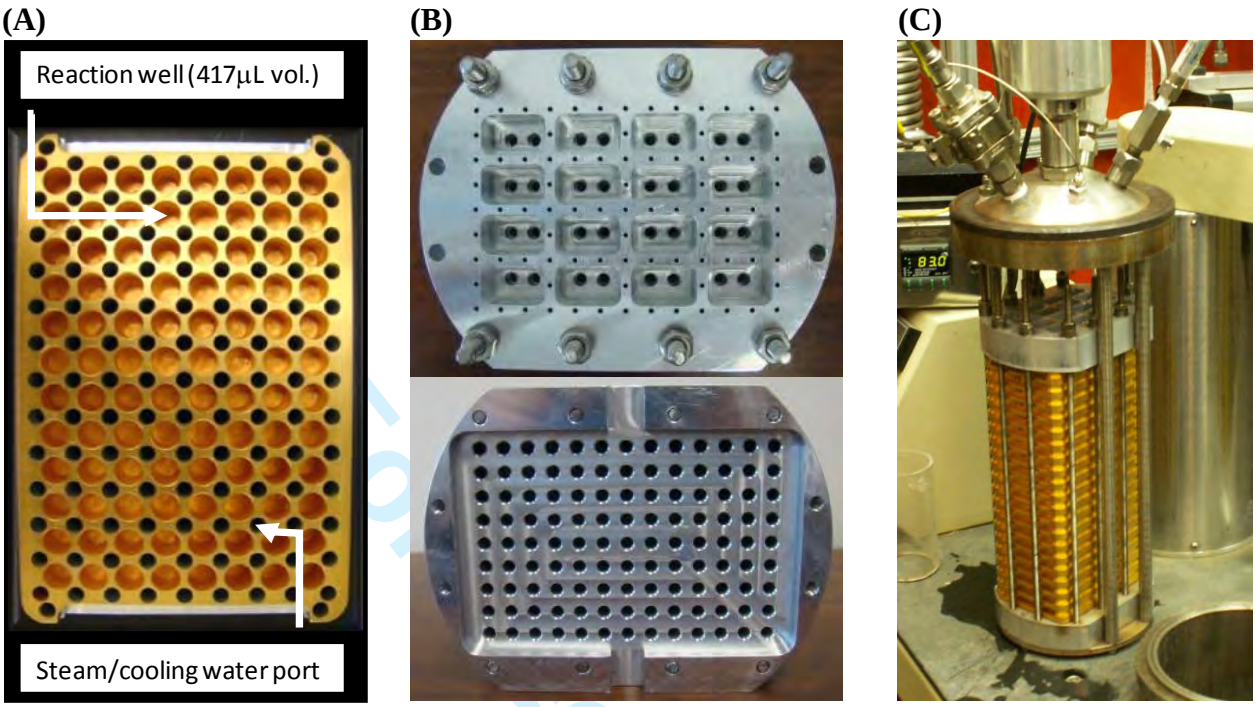

Figure 2

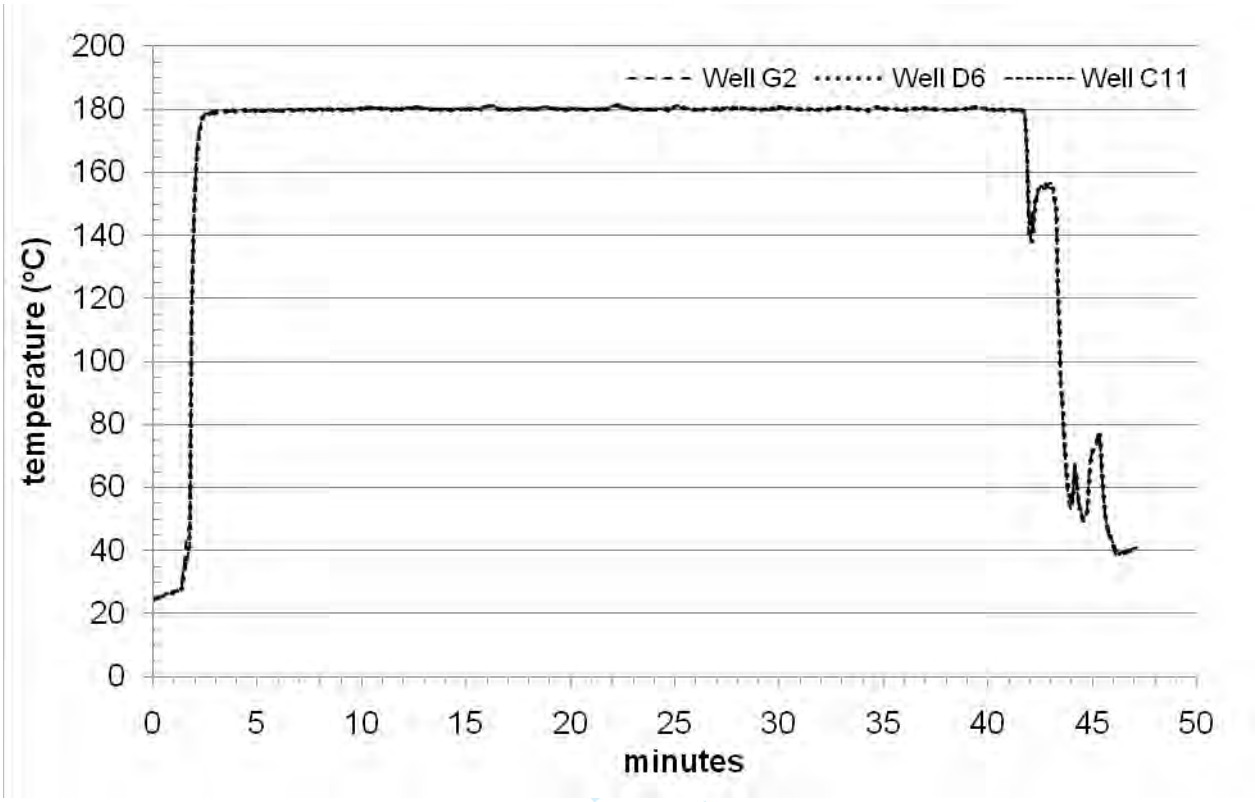

Figure 3

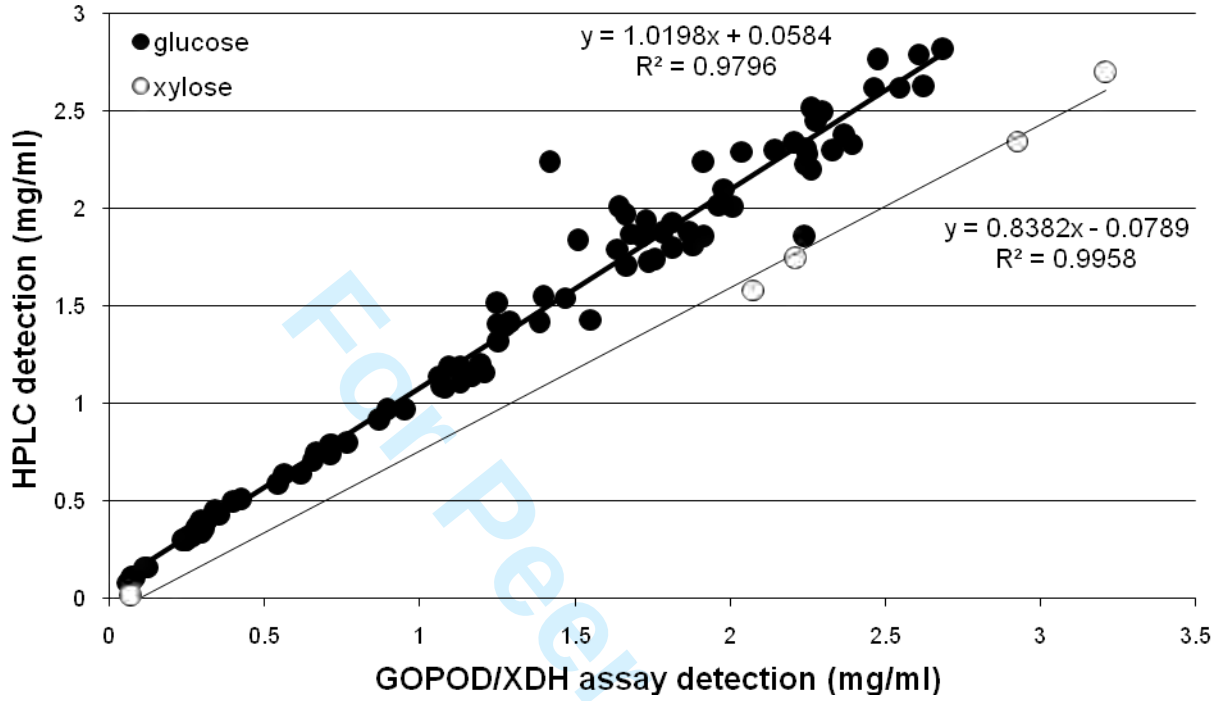

Figure 4

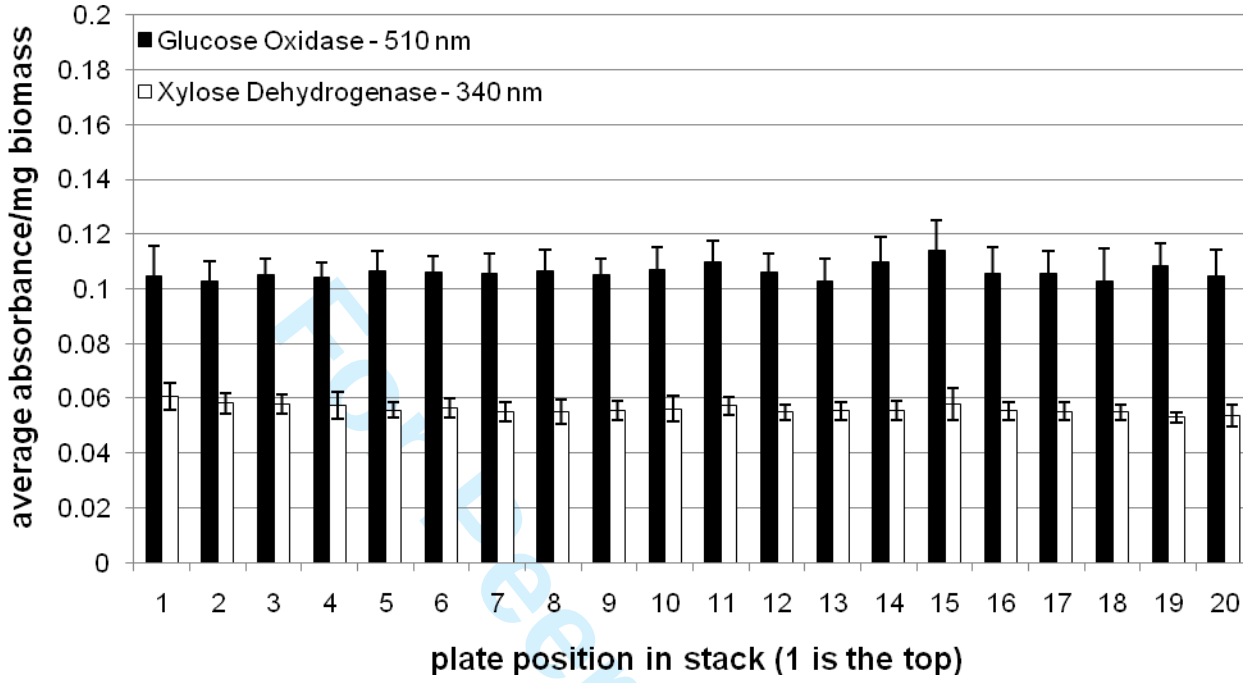

Figure 5

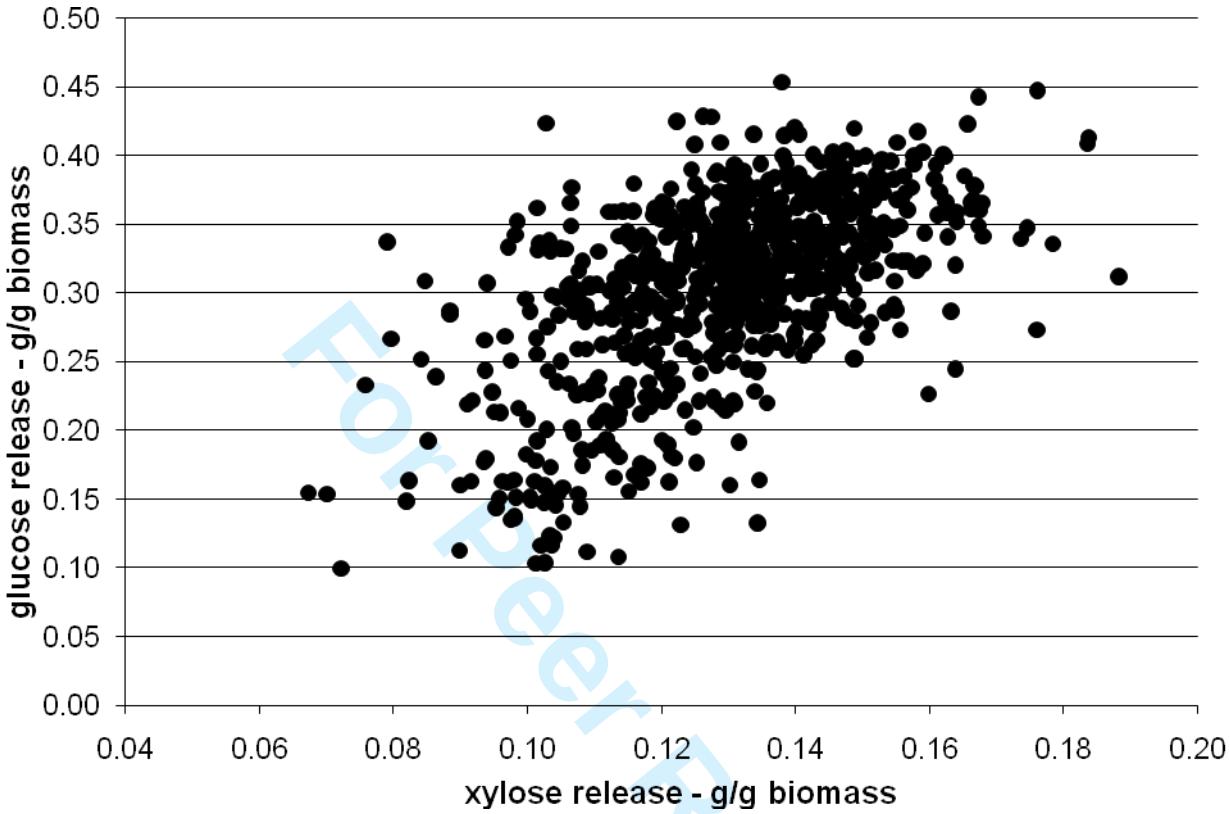

Figure 6

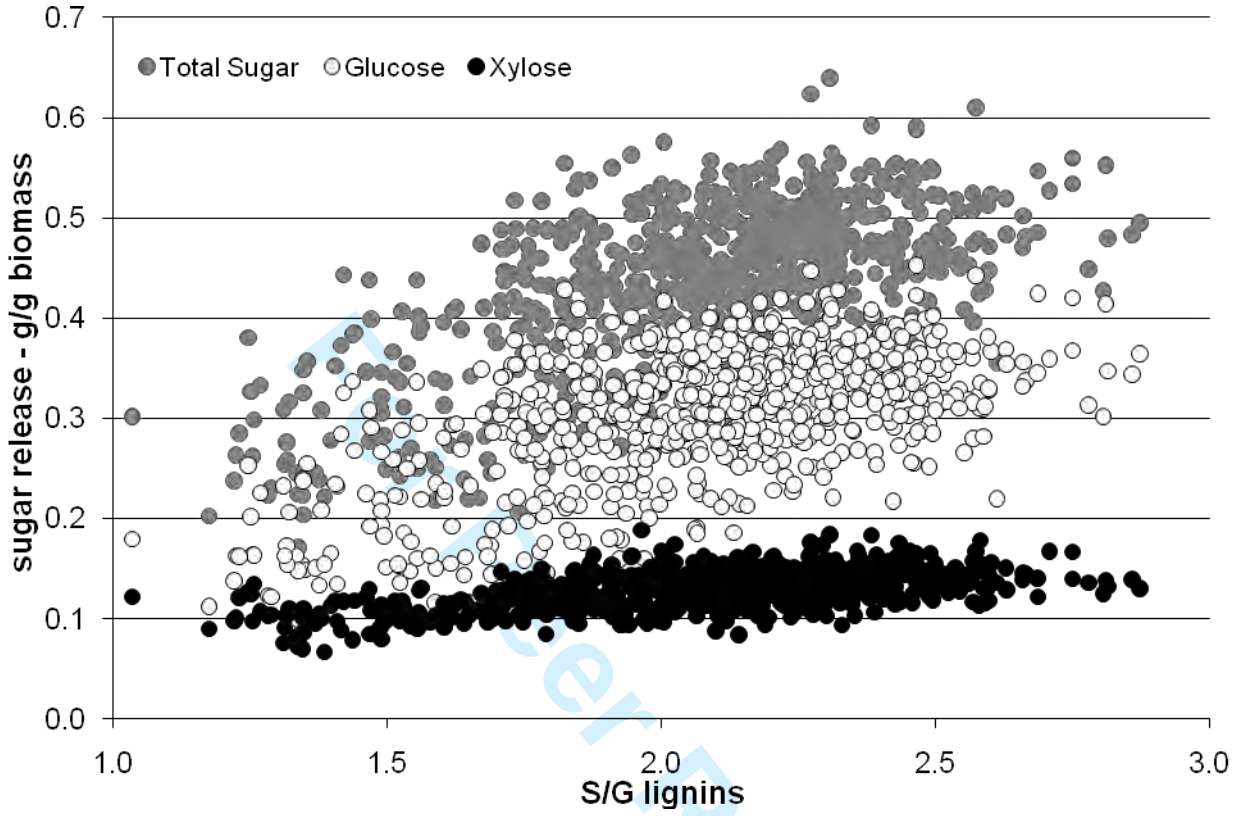

Figure Captions

Figure 1. (A) Reactor plate: Each plate is machined from aluminum and then Ni/Au coated. Wells are 416 mL in volume (7.0mm dia x 10.8mm depth). Steam/cooling water ports are 1/8" and contiguous between plates when stacked. (B/C) Reactor stack and clamping system: The head and base plates (B), with contiguous steam/cooling water ports, are machined from aluminum. The head plate is ribbed to reduce weight while maintaining rigidity for even clamping pressure. Threaded rods are used to tightly hold the stack between the head and base plate. Pressure is maintained during heating and cooling through using 2 sets of inversely faced bevel washers under each tightening nut and tightening all nuts to the same torque.

Figure 2. Temperature profile of reactor plate heating and cooling. Three lines indicate temperatures monitored in three well locations (inset) using Type-K thermocouples. There is an approximately 1.5 minutes slow heating while the reactor is assembled, followed by a 15 second rise while air is being purged during steam introduction. The final temperature of 180^{o}C is achieved in approximately 52 seconds by condensing steam once the purge valve is closed.

Figure 3. Comparison of redox enzyme-mediated glucose and xylose detection to HPLC analysis. Glucose is equally quantitated by HPLC or GOPOD assay. Xylose is slightly over-estimated by XDH assay compared to HPLC, likely due to sensitivity of XDH to xylooligomers not detected by HPLC.

Figure 4. Glucose and xylose detection by redox-enzyme assays in a 20 plate stack subjected to pretreatment and enzyme digestion. Plate averages of absorbance values from each assay are given for each plate position in the stack.

Figure 5 Glucose released plotted against xylose released from 755 poplar variants after pretreatment and enzymatic saccharification. Sugars were quantified by redox enzyme-mediated assays.

Figure 6. Glucose, xylose, and total sugar released from 755 poplar variants after pretreatment and enzyme digestion plotted against the ratio (S/G) of syringyl and guaiacyl (S and G) lignins. Lignin contents were determined by molecular beam mass spectrometry.