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A High-Throughput Platform for Screening Milligram Quantities of Plant Biomass for Lignocellulose Digestibility

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Abstract The development of a viable lignocellulosic ethanol industry requires multiple improvements in the process of converting biomass to ethanol. A key step is the improvement of the plants that are to be used as biomass feedstocks. To facilitate the identification and evaluation of feedstock plants, it would be useful to have a method to screen large numbers of individual plants for enhanced digestibility in response to combinations of specific pretreatments and enzymes. This paper describes a high-throughput digestibility platform (HTDP) for screening collections of germplasm for improved digestibility, which was developed under the auspices of the Department of Energy-Great Lakes Bioenergy Research Center (DOE-GLBRC). A key component of this platform is a custom-designed workstation that can grind and dispense 1–5 mg quantities of more than 250 different plant tissue samples in 16 h. The other steps in the processing (pretreatment, enzyme digestion, and sugar analysis) have also been largely automated and require 36 h. The process is adaptable to diverse acidic and basic, low-temperature pretreatments. Total throughput of the HTDP is 972 independent biomass samples per week. Validation of the platform was performed on *brown midrib* mutants of maize, which are known to have enhanced digestibility. Additional validation was performed by screening approximately 1,200 *Arabidopsis* mutant lines with T-DNA insertions in genes known or suspected to be involved in cell wall biosynthesis. Several lines showed highly significant ($p<0.01$) increases in glucose and xylose release (20–40% above the mean). The platform should be useful for screening populations of plants to identify superior germplasm for lignocellulosic ethanol applications and also for screening populations of mutant model plants to identify specific genes affecting digestibility.

Keywords Biomass conversion · Biomass pretreatment · Cellulase · Lignocellulose · High-throughput screening · Maize · Arabidopsis

Abbreviations

HTDP	High-throughput digestibility platform
OTP	One tube process
DW	Dry weight
Glu	Glucose
Xyl	Xylose
CV	Coefficient of variation
SD	Standard deviation

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Introduction

To reduce US dependency on imported petroleum and to lower greenhouse gas emissions, the US Department of Energy has made major investments in basic research to develop ethanol and other biofuels from lignocellulosic biomass [1]. Plant biomass is a highly complex and resistant meshwork of multiple polymers, which impedes extraction of the desirable free fermentable sugars such as glucose (Glu) and xylose (Xyl) [2–4]. The development of plant feedstocks with enhanced digestibility would help to remove a major obstacle in biofuel production. Plants specifically bred for ethanol production would yield higher amounts of Glu and Xyl with milder thermochemical pretreatments and lower enzyme concentrations, both of which contribute to the cost of lignocellulosic ethanol. Among the factors that are known to contribute to the recalcitrance of plant cell walls are degree of cellulose crystallinity, lignin content and structure, and heterogeneity in cell wall composition between plants and between cell types and tissues [3, 5]. Some plant genotypes, especially those with mutations in genes encoding lignin biosynthetic enzymes, are known to be more amenable to enzymatic hydrolysis [6, 7].

Progress forward on the development of better biomass crops suitable for the lignocellulosic ethanol industry will require methods to identify and evaluate germplasm for the critical trait of digestibility. Current methods of screening plant populations for ethanol yield potential have low-throughput, require relatively large amounts of plant material, and/or rely on indirect measurements of enzyme digestibility such near infrared spectroscopy or in vitro ruminal fermentation [6, 8, 9]. A particularly difficult bottleneck in evaluating hundreds to thousands of samples is to grind and weigh milligram amounts of biomass, as is the case when screening individual mutants of small plants such as *Arabidopsis* [10]. High-throughput methods for pretreatments, enzyme digestions, and sugar assays are essential corollaries to high-throughput grinding and dispensing. Here, we describe a complete high-throughput platform designed to screen small samples from a large number of plants for altered sensitivity to enzyme digestion. The system uses a custom-designed grinding and weighing robot to dispense 1–2 mg quantities of plant materials into individual tubes, followed by automated pretreatment, enzyme digestion, and colorimetric measurement of released Glu and Xyl.

Methods

Plant Material

Homozygous T-DNA lines of *Arabidopsis thaliana* with insertions in cell wall-associated genes were identified by PCR-based genotyping. The majority of the lines came from the SALK T-DNA collection, with a few supplemental lines from the SAIL and WiscDsLox collections [11–14]. Seeds were sterilized in 0.6% sodium hypochlorite plus 0.1% Triton X-100, and plated on half-strength Murashige and Skoog medium containing 0.8% agar and no sucrose [15]. After treatment at 4°C for 2–5 days, the plates were placed vertically (~80° angle) under constant light (100 $\mu E \cdot m^{-2}\ s^{-1}$). At 7 days after germination, the seedlings were transplanted to soil (Jiffy Mix #901 plus perlite, 2:1). Plants from each line were transplanted into two 4-inch (10.2 cm) pots with three plants per pot, for a total of six plants per line. Potted plants were grown in the University of Wisconsin-Madison Biotron under 16-h light/8-h dark at a fluence rate of at least 250–300 $\mu E \cdot m^{-2}\ s^{-1}$. Plants were watered four times per week, using an ebb and flow bench, with half-strength Hoagland's solution [16]. The greenhouse temperature was maintained at 18°C±1° during the dark period and 21°C±1° during the light period.

Plant tissue was collected 60 days after germination. Only internodes that were at least 2 cm in length were collected. Internodes were cut from the stem starting at the base, continuing apically until the internode length became less than 2 cm. Sufficient tissue was collected to yield at least 20 mg dry weight (DW). All tissues were collected directly into 2-mL tubes (part number 72-694-007, Sarstedt, Germany), closed with a cap containing a needle hole, and placed on dry ice. Samples were stored at -80°C until they were lyophilized (Model 77545 freeze-dryer, Labconco, Kansas City, MO).

A *brown midrib* (*bm*) isogenic series homozygous recessive for three maize *bm* genes (*bm1*, *bm3*, *bm4*) in a W64A background were a generous gift of Natalia de Leon, Department of Agronomy, University of Wisconsin-Madison. The preparation of stover from these maize lines as well as their detailed compositional analysis has been previously described [6, 9]. Care was taken during the harvest and initial grinding (to 1–2 mm particle size) to insure lack of bias in the sampling [6].

Automated Grinding, Feeding, and Weighing System ("iWALL")

The automated grinding, feeding, and weighing was performed by a custom-designed robot known as iWALL (Fig. 1), designed by Labman Automation Ltd., United Kingdom. The system was built around a modified single-arm TECAN (Männedorf, Switzerland) RSP 9000 auto-sampler, which became an XYZ vial transfer system. The liquid handling capabilities were removed from the auto-sampler, and a vial gripper was installed on the arm in place of the sampling needle. The gripper is pneumatically actuated and has four fingers to grip the microtubes. The

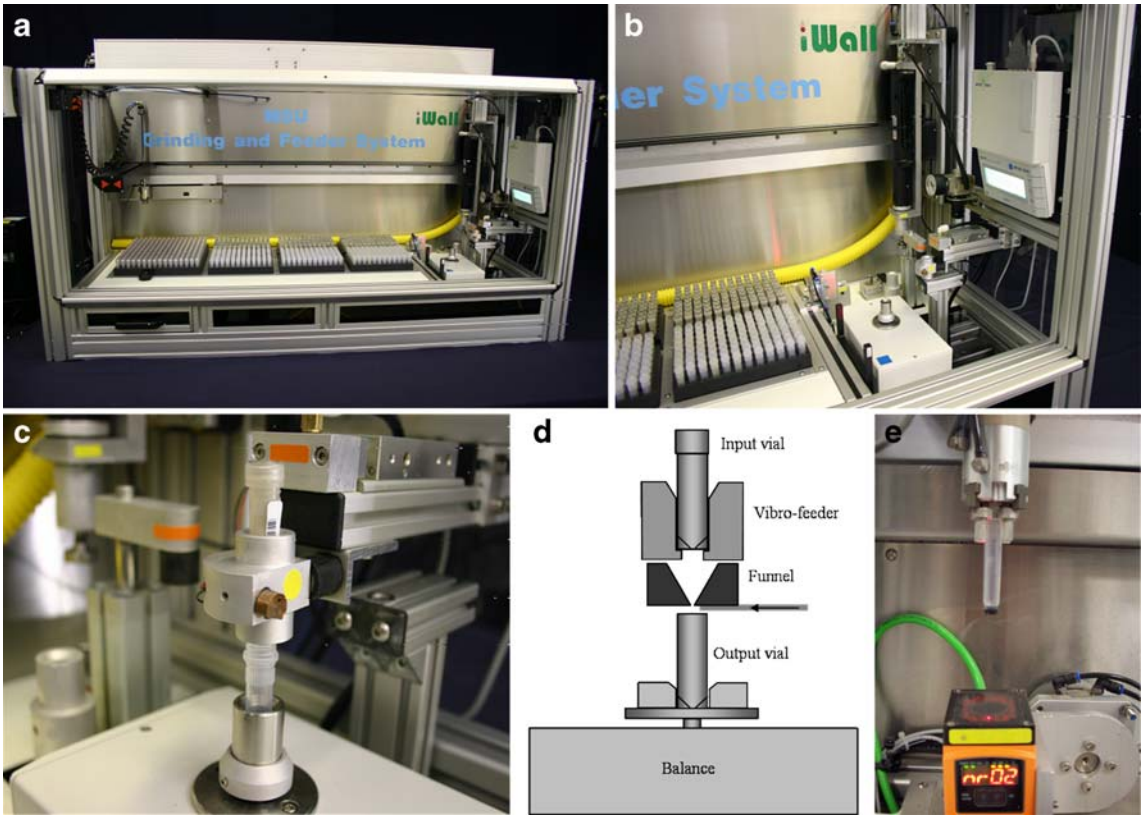

Fig. 1 iWALL, the custom-built grinder and feeder system **a** View of the entire system, except the dedicated computer. **b** Weighing sub-station. **c** Close-up view showing balance and vibro-feeder dispensing from input (*upper*) to output (*lower*) tube. **d** Diagram of weighing substation shown in (**c**). **e** Bar code scanner sub-station

system deck has the capacity for six 96-tube racks. While originally designed to hold 2-mL tubes (10 mm × 50 mm, diameter × height), additional racks were made to hold 1.4-mL sample storage tubes (7.5 mm × 44 mm). The remaining racks were filled with empty tubes that serve as output tubes.

Samples of dried plant material (20–40 mg) were loaded manually into Sarstedt 2-mL screw-cap microtubes along with three 7/32 inch (5.56 mm) stainless steel balls (Salem Specialty Ball Co, Canton, CT). These input tubes were placed into racks and positioned in the machine. A mechanical arm transferred the sample input tubes between barcode reader, grinder, piercer, and feeding funnel (Fig. 1). Pulverization of the biomass was accomplished by a grinder actuator that is a pneumatic reciprocator (modified Model SA346, Sealey Power Tools, UK) with a peak-to-peak amplitude of 25 mm and a frequency of 5,000 cycles per minute. The length of the grind time was adjusted for the particular biomass being ground, sufficient to reduce it to a fine powder (see below). After grinding, the vial was sent to a de-clogging station to break up any clumped material. The de-clogger is necessary because during grinding the ground material can become compacted in either the base or cap of the sample tube. This leads to poor powder feeding performance for some materials. The de-clogger consists of a fast-acting rotary pneumatic cylinder, an off-center tube holder, and a dead stop. The tubes were placed into the holder and were rapidly rotated through 180° and the holder was struck against the dead stop. The process was repeated in the opposite direction. The de-clog cycle was repeated two to five times depending on the material in question and the level of compaction present, as determined empirically. The vial was then transferred to the piercing station where a 1-mm hole was created in its base using a needle driven by a large bore (32 mm) pneumatic actuator.

A second, empty vial was scanned and placed on a five-decimal place analytical balance (Model SAG 105, Mettler Toledo, Columbus, OH). The balance has a modified weighing pan which locates the output vial (Fig. 1). The tube was positioned below the funnel, and a vibro-feeder dispensed the material through the pierced hole into the output vial. Experimentation during the design study established that a 1-mm hole was optimal, giving a reasonable feed rate without loss of powder during vial transfers. The precise weight of dispensed material was recorded by the balance and stored digitally. Normally, the target value was 1.5 mg per tube. The tolerance (reject limits) can be set to any value; narrower tolerances take

longer to weigh and result in more rejected tubes. Normally, the tolerance was set to ±0.2 mg. For a visual demonstration of iWALL see the video in [17].

The material was weighed into 1.4-mL 2D Tracker U-bottom tubes (1765-2011, Micronic, USA Scientific, Inc., Ocala, FL). These tubes were verified to withstand 121°C without leaking. Samples were typically weighed and dispensed in triplicate. The tubes were arranged into Stabo-Racks (Micronic North America) in sets of up to 90. This arranged the tubes into a standard 96-well microplate footprint while fixing the position of each vial. This format is defined by the Society for Biomolecular Screening (http://www.sbsonline.org) and was maintained throughout the protocol. The exposed bottom of the rack was scanned with a flatbed scanner to record the barcodes and positions of each sample.

Pretreatment and Enzyme Digestion

The rack was placed onto a PerkinElmer (Waltham, MA) Janus automated workstation and 750 µL of pretreatment solution was pipetted into each tube. The typical pretreatment solution was 0.025% (6.25 mM) NaOH [18, 19]. All subsequent liquid transfers were performed using the Janus workstation. Sample plates were sealed with a thermoplastic elastomer cap mat (# MP53000, Micronic North America), incubated at 90°C for 3 h in a water bath, and cooled on ice. An elastopolymer seal was chosen over alternatives such as ethylene vinyl acetate, which melted at temperatures above 90°C for extended times.

To each Micronic tube was added 50 µL of a solution containing 0.25 µL Accellerase 1000 (Genencor, Rochester, NY) in 30 mM citrate buffer (pH 4.5) plus 0.01% sodium azide. This was sufficient to neutralize 6.25 mM NaOH (the standard pretreatment). HCl, 5 µL or 50 µL, was used to neutralize 62.5 mM or 625 mM NaOH, respectively. When 2% (*v*/*v*) H_2SO_4 was used as the pretreatment, the solution was neutralized with 50 µL 5 M NaOH. The final total solids loading was 0.19% in a final volume of 0.8 mL. Typically, digestions were done at an enzyme concentration of 30 mg protein/g glucan.

Racks were incubated in a hybridization rotisserie oven (VWR, Model 5420) at 50°C for 20 h with end-over-end rotation. The racks were then centrifuged at 1,500×*g* for 3 min in a swinging bucket centrifuge (Eppendorf, Germany, Model 5417R) to separate the solid residue from the digested biomass. The supernatants were transferred into deep-well 96-well plates (Abgene, Fisher Scientific). The liquids were then sampled in duplicate into two 384-well plates (BD Falcon, BD Biosciences, San Jose, CA), one plate of which was used for measuring Glu and the other for Xyl.

Enzyme-Based Assays for Glu and Xyl

The Glu and Xyl content of samples was determined using enzyme-based assay kits. The assay volumes were reduced to allow the procedure to be performed in 384-well microtiter plates and to save expense. Unless stated otherwise, incubation times and temperatures followed the manufacturer's instructions. Glu was assayed with the glucose oxidase/peroxidase (GOPOD) method (K-GLUC, Megazyme, Ireland) using 4 µL of the supernatant of the digestion reaction mixture and 64 µL of the GOPOD assay reagent. Xyl was assayed enzymatically (K-XYLOSE, Megazyme) using 8 µL sample and 62 µL assay reagent. The incubation time (20 min) was twice the manufacturer's recommended time, as this gives more reproducible results with the microassay format.

Testing was carried out with various potential inhibitors of the Glu and Xyl assay enzymes, including cellobiose through celloheptose, xylobiose through xyloheptose, cellulose, xyloglucan, citrate, and various volumes of neutralized digestion mix. No interference was detected with any tested compounds, including the acid and base pretreatments or the salt concentrations resulting from neutralization.

The total monosaccharide content of the wild type corn stover was determined to be 34% Glu and 22% Xyl by the alditol acetate/gas chromatography method [20]. Starch was assayed using Megazyme kit K-TSTA [21].

Particle Size Analysis

Particle size distribution of plant samples ground on iWALL was analyzed with a Partica LA-950 Laser Diffraction Particle Size Analyzer (Horiba Instruments, Irvine, CA). The refractive index for the ground plant samples was 1.50 and 0.1i, and the calculated particle size distributions were consistent with images captured by the PSA-300 Image Analysis System (Horiba Instruments).

Data Collection and Analysis

All samples and tubes were bar-coded and tracked at each stage of the process from sample submission through final data output. Every microplate contained its own standards for generating a standard curve, which was used to determine the amount of Glu or Xyl in each sample. Biomass in each input tube was dispensed in triplicate into three independent output vials, and each digestion assay was quantitated for Glu and Xyl in quadruplicate. Thus, final data points were the average of 12 measurements. The exception was when a result is stated to be for each of the triplicates, in which case the final result is the average of four measurements. The scanned input barcode, the

scanned output barcode, and dispensed weight were stored as a comma-separated value text file.

Results

An overview of the high-throughput digestibility platform (HTDP) is shown in Fig. 2. iWALL, the custom grinding and feeding system is a key component of the HTDP. The manufacturer's specifications are that iWALL can grind and dispense as little as 1 mg of dried plant material with a precision of ±0.15 mg. Accuracy is determined by the quality of the balance used to weigh the tubes plus samples. The actual weights are recorded and all values are reported "per mg DW".

A single run on iWALL usually consists of one 96-tube rack of input vials and three 96-tube racks of output vials. In this format, material from one input vial is weighed into three separate vials in the output racks. Processing of one rack of input tubes on iWALL (and three racks of output tubes) requires ~16 h. After subtracting plate controls and standards, processing three 96-tube racks on the HTDP translates into 243 samples per run. Processing the samples through the remainder of the platform takes another ~36 h. Post-iWALL processing can be interleaved with subsequent iWALL runs, and so the throughput of the entire system is four complete runs (~970 total samples) per week.

The plant material is ground with a high-speed pneumatic reciprocator in the presence of metal balls and is dispensed through a 1-mm hole in the input tube. Particle size analysis of the ground plant material indicated that the geometric mean diameter (±1 standard deviation [SD]) for pulverized corn stover were 55 µm±3 µm and for *Arabidopsis* stem were 34 µm±3 µm. More than 90% of the particles for all plant samples tested were smaller than 350 µm. Thus, more than 90% of the pulverized sample can pass unrestricted through the 1-mm hole in the bottom of the input tube. The particle size distribution curves for sequentially ground samples of the same tissue overlaid extremely well, indicating that the grinding configuration has good reproducibility (data not shown). Together, these results indicate that iWALL does not introduce bias during the sampling of the material in the input tube.

The HTDP can be adapted to various thermochemical pretreatments as long as the temperature does not exceed 121°C, which causes microtube deformation and sample leakage around the O-ring. With the goal of finding a pretreatment that could be used on different biomass materials to evaluate enzyme digestibility, a series of concentrations of both acid and base were tested. Acid pretreatment is a common industry standard, but ammonia fiber expansion and other base treatments are also effective and offer certain advantages [18, 19]. It is important that the pretreatment used for analyzing plant populations be adequate to overcome biomass recalcitrance, but not so strong that differences in digestibility are obscured; an example of the importance of choosing the right pretreatment is shown below. The effect of different NaOH concentrations on digestibility is shown in Fig. 3. Without any pretreatment, Glu yields were low, as expected [18, 19]. A NaOH concentration of 0.025% (6.25 mM) was moderately effective, and a NaOH concentration of 0.25% was saturating. On this basis, 0.025% NaOH was chosen as the standard pretreatment for corn stover. Comparisons between base and acid pretreatment are shown below.

Incorporating the pretreatment and digestion steps into a one-tube process (OTP) offers the possibility of simplifying the biomass to sugar process substantially, but it was necessary to determine whether an OTP would generate results as valid as separating the pretreatment liquor from the solid material before enzyme digestion. Removal of the pretreatment solution and washing the biomass solids before adding the enzymes generates two liquid product streams, composed of the pretreatment liquor plus washates (stage 1) and the solids digestion liquor (stage 2), both of which must be treated with enzyme and analyzed for free Glu and Xyl. A side-by-side comparison was made between the two-stream method and the OTP. We determined that the yields of Glu are similar for both methods (Fig. 4). Note that although total Glu yield was higher in the two-tube method at an enzyme loading of 30 mg/g glucan, this difference was offset by higher background (i.e., Glu released with no enzyme treatment) (Fig. 4).

A potential drawback of the OTP is that the salts generated from neutralizing the acid or base pretreatment solutions might adversely influence the activity of the digesting enzymes. This is probably not a problem with the standard pretreatment of 6.25 mM NaOH (Fig. 3), which can be neutralized by the citrate buffer, but could be a problem when using stronger base solutions (62.5 mM NaOH or 625 mM NaOH) or acid (2% H_2SO_4), which require independent neutralization. We tested for the possibility of salt interference using microcrystalline cellulose (Avicel; FMC BioPolymer, Philadelphia, PA) in the presence of a range of salt concentrations. Concentrations of salt above those present in our digestion reactions did not significantly inhibit the digestion of Avicel. Also, our conditions for dilute base pretreatment and digestion with Accellerase 1000 did not result in detectable hydrolysis of starch (data not shown). In conclusion, an OTP was deemed to give satisfactory results and therefore the best choice for economic and efficient determination of digestibility.

The enzymatic release of Glu from corn stover using the HTDP as a function of pretreatment, time, and enzyme concentration is shown in Fig. 5. The results indicate that

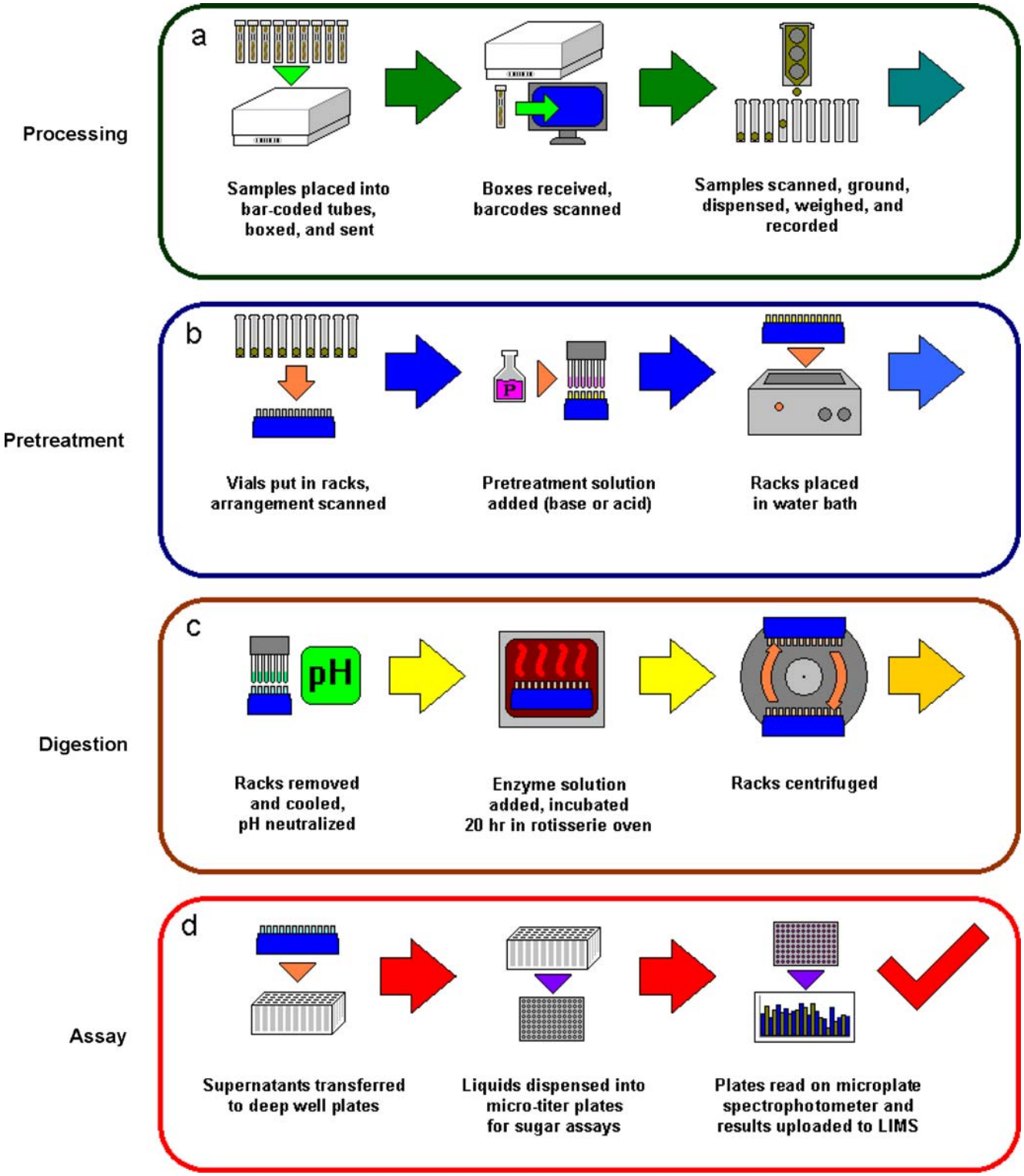

Fig. 2 The high-throughput digestibility platform pipeline. **a** Processing includes tracking incoming samples, automated grinding, and gravimetric dispensing. **b** Pretreatment includes adding an acidic or basic solution and incubation. **c** Digestion involves neutralization, addition of digestive enzymes, and incubation. **d** Assay involves sampling and measurement of Glu and Xyl

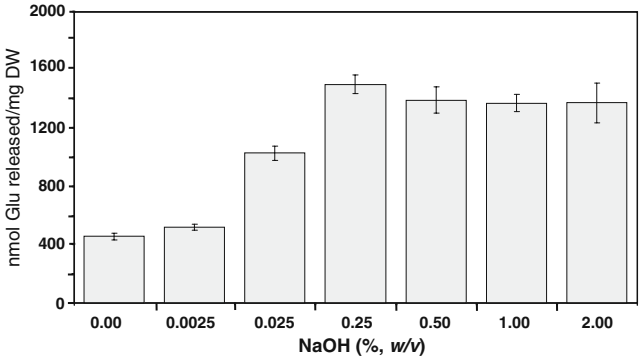

Fig. 3 Effect of the concentration of NaOH as a pretreatment on subsequent release of Glu by Accellerase 1000 (30 mg/g glucan). All pretreatments were for 3 h at 90°C

the level of digestibility can be finely controlled by changing these variables. For example, similar amounts of Glu were released using five-fold less enzyme and digesting approximately six times longer. This flexibility in controlling total digestion yields allows conditions to be adapted for different sample populations in order to detect both higher and lower digestible phenotypes. Maximum Glu yields from maize stover were in the range of 1,400 to 1,600 nmol Glu/mg DW (Figs. 4, 5). These levels correspond to 74% to 85% of maximum possible Glu release. These values are within the range seen by others using other digestibility methods [e.g., 9].

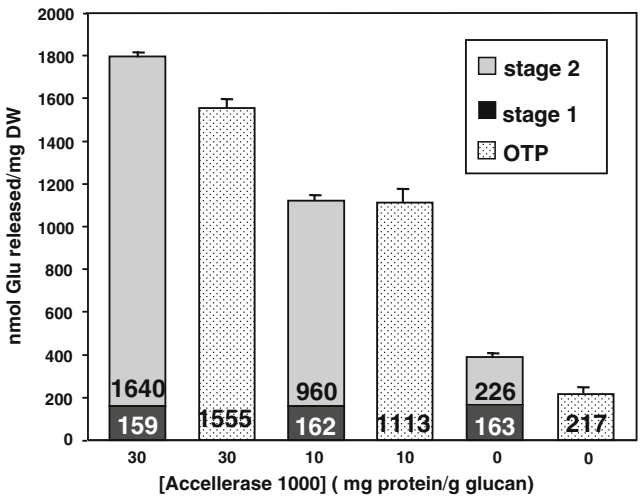

Fig. 4 Comparison of Glu release from corn stover using either separation and independent processing of liquid and solid phases after pretreatment (the two-step method) or the one-tube process (OTP). In the two-step method, stage 1 was the post-pretreatment liquid phase and stage 2 was the post-pretreatment solid phase; both were treated with Accellerase 1000. In the OTP, the liquid and solid were not separated, and the entire reaction mix was neutralized prior to addition of enzyme. The *error bars* indicate ±1 SD of three independent assays for each sample. Digestion conditions were 20 h at 50°C with 0.19% glucan loading. Comparisons were made at three concentrations of Accellerase 1000

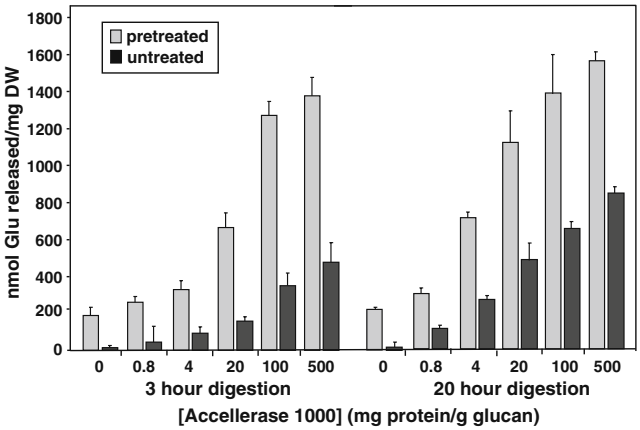

Fig. 5 Glu release from corn stover as a function of pretreatment, enzyme concentration, and time of digestion. The pretreatment was dilute base (6.25 mM NaOH, 90°C, 3 h). The error bars indicate ±1 SD of three independent assays for each sample

The utility of a system such as the HTDP depends on its reproducibility and experimental error. The system is comprised of several different processes that collectively contribute to the experimental variability reflected in the final quantitation. In order to insure statistical robustness, the method was implemented using triplicate independent determinations for every sample. A typical screening run on the HTDP consists of 90 sample tubes, leaving six empty rack positions for standards. Figure 6a shows a measure of the variability observed between 12 portions of a single biomass (W64A maize stover) ground and dispensed on iWALL into eight independent replicate tubes (i.e., showing variation between output tubes from a single source as well as variation between input tubes sampled from an identical source). The coefficient of variation (CV) for the entire high-throughput digestibility process was 7% (Fig. 6a). This CV represents the entire process from grinding/dispensing through digestion as well as the monosaccharide assays. The within subset Glu mean yield and variation was approximately equal to the Glu mean and variation observed over different subsets of the same material. Thus, iWALL contributes little to the experimental error.

The ultimate use of this platform is to screen a number of samples from different plant species. To test the platform for another plant, we used *Arabidopsis* (Columbia wild type) stem tissue harvested from 30 different plants. The CV for an entire rack of 96 samples was 7% (Fig. 6b).

To determine whether plant samples with differences in digestibility could be identified using the HTDP, we compared wild type and the *brown midrib* (*bm*) maize genotypes *bm1*, *bm3*, and *bm4*. Some of the *bm* mutants have been shown to have enhanced rumen digestibility and enzyme digestibility [6, 7]. Using acid pretreatment, *bm1* and *bm3* had a statistically significant ($p<0.0001$) increase (20%) in Glu release compared with the parental W64

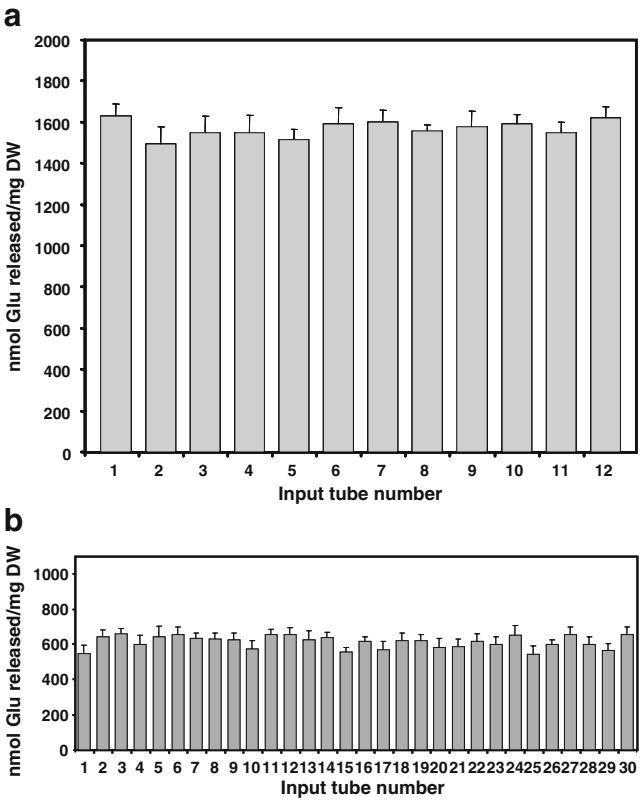

Fig. 6 Reproducibility of the platform. **a** Corn stover. Twelve input tubes containing wild-type (inbred W64A) corn stover were processed on iWALL and 1.5 mg (target weight) was dispensed into eight output tubes for each input tube. *Error bars* represent ±1 SD of the mean for each set of eight tubes. **b** *Arabidopsis*. Stem samples (1.5 mg target weight) from 30 individual wild-type *Arabidopsis* plants were processed and dispensed by the high throughput digestibility platform into triplicate output tubes. *Error bars* represent ±1 SD of the mean of the triplicates for each input sample

inbred (Fig. 7). With alkaline pretreatment, *bm3* showed a highly significant ($p<0.0001$) increase of 40% in Glu digestibility. These differences are not due to increased Glu content in the *bm* mutants, because all of the *bm* mutants have lower stover Glu levels than wild type [6]. Digestibility of *bm1* after alkaline pretreatment was not statistically different ($p<0.5$) from the wild type. At 2.5% (*w/v*) NaOH, no difference in digestibility was observed between the wild type and *bm* plants (data not shown). Thus, the HTDP can detect known differences in enzyme digestibility, but the type and severity of pretreatment can be critical and must be taken into account when screening.

In order to test the HTDP on a real plant population, we screened a set of approximately 1,200 *Arabidopsis* T-DNA insertion lines. These lines contain T-DNA insertions in genes with known or possible roles in cell wall metabolism and are thus more likely than random mutants to show an altered digestibility phenotype. Glu and Xyl yields for stem tissue taken from 81 T-DNA lines and processed in a single run on the HTDP are shown in Fig. 8. The mean Glu digestibility yield for this run was 711±82 nmol Glu/mg DW. Generally, the CV observed for genetically identical samples is 5% to 7%, but for this set it was 11.5%. This might be because although most of the mutants were the Columbia (Col) ecotype, some of them were in the Wassilewskija (Ws) ecotype. The average CV of the technical replicates ($n=3$) for each of the 81 individual samples was 5%.

Mutant line number 390 (indicated by a circle in Fig. 8a) released 1010 nmol Glu/mg DW, which is 42% (3 SD) higher than the mean. This difference was significant at $p<0.0001$. Mutant line 390 also showed an increased Xyl release almost 100% (4 SD) higher than the mean (circle in Fig. 8b). This difference was significant at $p<0.0001$. This particular mutant was regrown and verified to have enhanced digestibility. Three additional lines, indicated by asterisks in Fig. 8b, also had significantly higher Xyl release but no difference in Glu release. Another line, indicated by the asterisk in Fig. 8a, had enhanced Glu but not enhanced Xyl release. All of these differences were significant at $p<0.0001$. Thus, the HTDP can identify mutant lines with enhanced digestibility of Glu, Xyl, or both.

Discussion

Some steps in the lignocellulose to ethanol pipeline have previously been automated, e.g., pretreatments, enzyme digestions, or sugar analyses [22–27], but the system described here automates all steps from tissue grinding through sugar analysis. Human intervention is necessary to load the input tubes and add the metal grinding balls; to load the racks with output tubes; and to transfer the plates three times, first, to the liquid-handling robot for addition of pretreatment solutions and enzymes, second, to the incubating oven, and, third, back to the robot for the sugar assays.

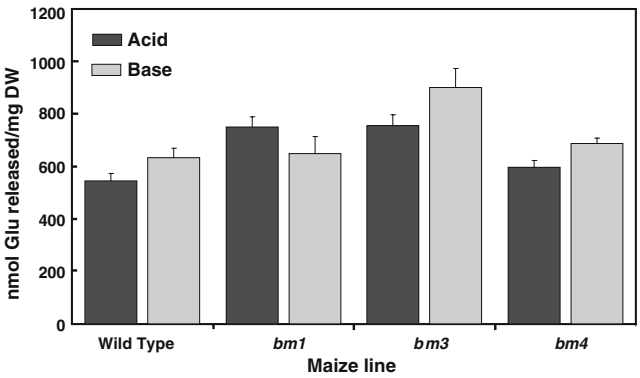

Fig. 7 Enzymatic Glu release from wild type (inbred W64A) and near-isogenic *bm* mutant corn stover lines (*bm1*, *bm3*, and *bm4*) after acid or base pretreatment. Acid pre-treatment was 2% (*v/v*) H_2SO_4 at 120°C for 45 min and base pretreatment was 6.25 mM NaOH at 90°C for 3 h. *Error bars* represent ±1 SD of the mean of three independent samples

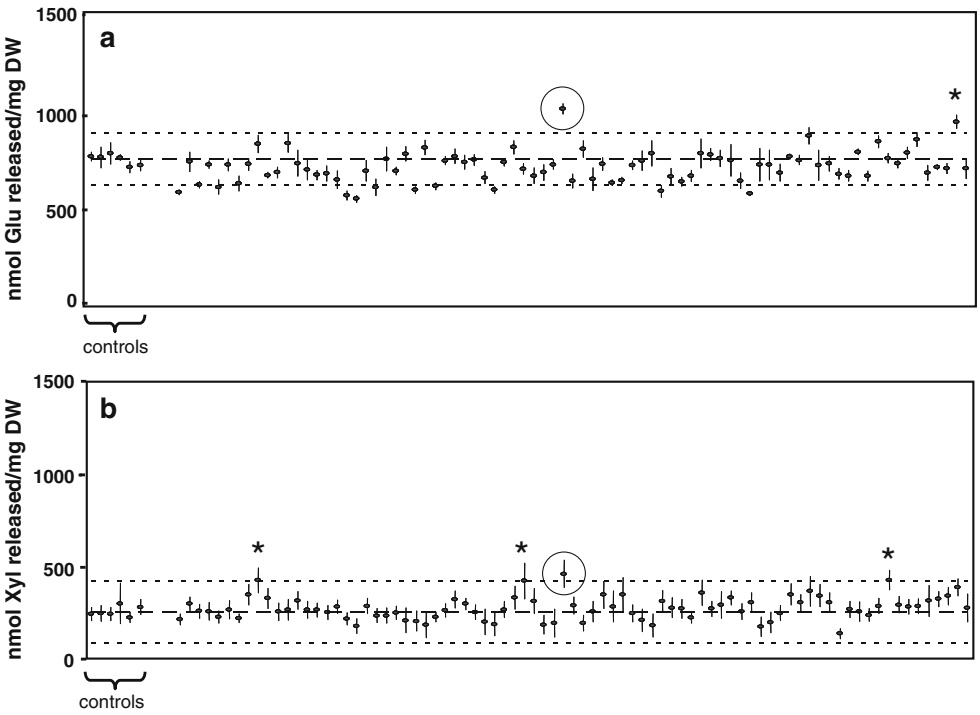

Fig. 8 Glu release from stem tissue of 81 *Arabidopsis* T-DNA insertion mutant lines. Each *line* was assayed in triplicate. **a** Glu release. **b** Xyl release. The *center dashed line* represents the mean of Glu or Xyl release value for all 81 samples plus six controls. The *upper* and *lower dashed lines* indicate ±3 SD from the mean. Data points represent the means and the *error bars* represent ±1 SD (n=3). Results from six wild-type control *Arabidopsis* stem samples are indicated on the far left of each graph. The results for mutant line 390 are *circled*. *Asterisks* indicate four additional outliers, one for Glu (**a**) and three for Xyl (**b**)

A grinder/dispenser similar to the one used in this study has been described [28]. This system dispenses ~20 mg of ground plant material with ±4 mg precision (a precision of ±0.5 mg can be obtained; Labman Automation Ltd., personal communication). Our system differs in being able to handle smaller quantities and in not being cryogenic, which was deemed not essential for dispensing dried plant materials. Since our goal is to screen large plant collections using a minimum of plant material, we developed a system that would accurately and precisely dispense a target weight that was in the range of 1–3 mg, i.e., ~10-fold lower than the machine described by Bieniawska et al. [28]. Decker et al. [10] mention a solids-dispensing robot to distribute milled biomass feedstock but do not provide any details.

The platform described here solves several technical challenges of evaluating large numbers of plant samples in an automated method [10]. The use of iWALL avoids the need to hand-weigh mg quantities of materials or to pipet slurries. The OTP avoids the need to process multiple sample streams. Colorimetric assays to measure Glu and Xyl are easily automated and allow much higher throughput than other sugar analysis methods [20].

Because our system is based on plasticware, temperatures higher than 121°C cannot be used. A metal reactor system in a 96-well microplate format that is capable of withstanding much higher temperatures and pressures has been recently described [10]. However, fixed multiwell formats have the disadvantage that a single weighing or pipetting error can invalidate an entire plate, whereas in our system the samples are in individual replaceable tubes. iWALL automatically rejects any misweighed tubes before they enter the 96-well format. Furthermore, mild pretreatments (in terms pH, pressure, and temperature), that are within the constraints of a plasticware-based platform, are not only effective (Figs. 3, 5), but for economic reasons are also more likely be representative of pretreatments ultimately adopted at industrial scales.

Our system uses enzyme-based colorimetric assays of Glu and Xyl. The advantages of this method are its higher specificity than reducing sugar assays [10, 25] and its amenability to automation. Its main disadvantage is the inability to measure the less abundant biomass sugars, such as arabinose, galactose, and mannose, because specific assays for these sugars have not been developed.

This platform should be useful for several purposes. For example, as we show, it can be used to identify genes that affect digestibility in mutant populations of model plants such as *Arabidopsis*. It could also be used to screen accessions and collections of diverse plants (maize, switchgrass, Miscanthus, etc.) in order to identify promising germplasm for breeding programs to produce superior bioenergy feedstock plants. It could also be used to evaluate biomass plants grown under different agronomic conditions (fertilizer, irrigation, etc.) that might affect digestibility.

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