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High-throughput Pretreatment and Hydrolysis Systems for Screening Biomass Species in Aqueous Pretreatment of Plant Biomass

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22.1 Introduction: The Need for High-throughput Technologies

The primary barrier to low-cost biological conversion of lignocellulosic biomass to renewable fuels and chemicals is plant recalcitrance, that is to say, resistance of cell walls to deconstruction by enzymes or microbes [1,2]. However, the discovery and use of biomass species with reduced recalcitrance, when combined with optimized pretreatment processes and enzyme mixtures, could potentially improve the commercial viability of fuels and chemicals production from lignocellulosic biomass [3,4]. Unfortunately, the current understanding of biomass recalcitrance is limited, making it difficult to rationally select superior plant species without prior sugar release testing. As a result, there is a need to generate and screen a large variety of plants to identify those that exhibit both superior and sub-par sugar release. To this end, there are two central methodologies in generating and screening plants: (1) generation of mutants to see what effect targeted modifications have and (2) evaluation of natural variants to identify outliers for further characterization, in order to relate observed differences in behavior to structural features and biomass characteristics.

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Both methodologies require testing of thousands of samples. When considering that each sample should be screened over a range of pretreatment conditions including various times, temperatures, and chemical concentrations, as well as subsequent hydrolysis by a wide range of enzyme sources and formulations, the number of experiments easily reaches tens of thousands.

Due to the time-consuming and laborious nature of biomass analytical techniques, conventional testing of sugar release from pretreatment and enzymatic hydrolysis would be prohibitive. As a result, there has been a recent push to develop high-throughput pretreatment and hydrolysis (HTPH) systems that are capable of providing basic sugar release data in a rapid and automatable manner, while using significantly less biomass than conventional techniques. The benefits of implementing a fast and automatable procedure are clear: the throughput of data can be dramatically increased and the screening of multiple biomass-pretreatment-enzyme formulation combinations can be possible in a greatly reduced timeframe and with lower costs. In addition, high-throughput systems allow more replicate samples to be analyzed, improving estimation of measurement uncertainties.

Equally important benefits can be realized due to the low material requirements of HTPH systems. First, results can be obtained sooner. For genetically modified mutants, it can often take years for plants to adequately mature to produce sufficient amounts of material for analysis; this however is not a concern with downscaled HTPH systems for which only milligram amounts of biomass would be required. Additionally, when screening woody biomass with HTPH systems, entire trees do not have to be sacrificed for analysis; instead, analysis of a small core sample can be adequate without harming the tree. Finally, the reduced material requirement also allows analysis of individual biomass fractions that was previously not possible, in addition to making more biomass material available for other analyses.

22.2 Previous High-throughput Systems and Application to Pretreatment and Enzymatic Hydrolysis

The development and use of high-throughput (HT) technologies is relatively new. Since the 1990s, there has been a push to automate and increase sample throughput in a variety of fields, ranging from pharmaceuticals and drug discovery to genetic sequencing. In fact, efforts have also been directed at downscaling enzymatic hydrolysis of lignocellulosic biomass. Conventionally, enzymatic hydrolysis is typically performed in either 20 mL scintillation vials or 125 mL Erlenmeyer flasks, into which around 0.2 g or 3 g of wet pretreated solids are weighed, respectively [5,6]. In 2005 however, a "rapid microassay to evaluate enzymatic hydrolysis of lignocellulosic substrates" was reported that was based on a 96-well microplate for which each *c.* 300 μL well served as a reactor for enzymatic hydrolysis [7]. In 2007, the use of a standard 96-well plate was similarly reported for cellulase accessibility experiments [8], while more recently, additional reports have been made of microplate-based approaches to evaluate sugar release from the enzymatic hydrolysis of both untreated and pretreated biomass [9,10]. Other downscaled systems, such as 1.5 mL plastic Eppendorf tubes and 2 mL glass high-performance liquid chromatography (HPLC) vials, have also been used [11,12]. Although these systems provided a significant advancement in scaling down and automating biomass analyses, the development of downscaled and high-throughput pretreatment still remained a challenge. Because sugar yields are extremely low without pretreatment, a pretreatment step is required prior to enzymatic hydrolysis in order to adequately screen plant samples for their applicability for conversion to fuels and chemicals [2]. However, extending high-throughput applications to pretreatment processes presents a number of difficulties. Whereas enzymatic hydrolysis is performed at $\leq 50\,^{\circ}C$ and near-neutral pH, pretreatments typically require high temperatures, high pressures, and the addition of corrosive chemicals, virtually ruling out use of glass HPLC vials and plastic microplates and Eppendorf tubes.

Table 22.1 *Types of batch pretreatment reactors commonly employed in conventional laboratory processes, with pretreatment chemical environment and required reaction mass listed for each.*

Reactor type	Reaction mass (g)	Pretreatment type	Reference
11 mm Pyrex glass tubes	0.4	Dilute acid	[13]
4″ Hastelloy tubing with Swagelok caps	0.4–2	Water only	[14]
300 mL metal Parr stirred tank reactor	2	Dilute acid	[15]
Batch metal tube reactors: 12.5 mm OD, 0.8255 mm wall thickness, 10 cm length	6	Dilute acid	[16]
Batch metal tube reactors: 1.5 in schedule 40 pipe nipples	7.5	Calcium hydroxide	[17]
500 mL metal stirred autoclave	40	Liquid hot water	[18]
1 L Parr metal stirred tank reactor	50	Dilute acid	[19]
2 gal metal Parr stirred tank reactor	500	Dilute acid	[20]

To date, a variety of pretreatment reactors have been utilized at the laboratory bench scale. Metal tubes or stirred tank reactors are often employed for three of the most commonly used pretreatments: hydrothermal (just hot water), dilute acid, and dilute alkali. Such reactors can safely handle acid or base (usually $<2\%$ concentrations) at the elevated pressures and temperatures typical for pretreatments (90–220 °C). Table 22.1 summarizes features of a variety of batch pretreatment reactors that have been reported in the literature to demonstrate reactor types, reaction volumes, and biomass material requirements that are often employed for laboratory experiments. The table does not attempt to provide a comprehensive review of all reactor types, but instead demonstrates the range of equipment and conditions that have been used in the past. Such reactor systems are typically heated by steam, fluidized sand baths, or oil baths to achieve fairly uniform heating as well as fast heat-up, although some employ electric heating jackets that suffer from slow heat-up rates. Due to the large vessel size conventionally required for a single pretreatment reaction and the space needed for heating devices, only a limited number of pretreatments can be performed simultaneously.

Although there has been some success in scaling down pretreatments to use less than 1 g [16], a number of time-consuming steps still remained after pretreatment in order to prepare the materials for subsequent enzymatic hydrolysis: (1) separation of pretreated solids and liquids; (2) washing of the solids; and (3) wet chemistry compositional analysis on pretreated washed solids. Enzymatic hydrolysis was then typically performed on the washed pretreated solids, with enzyme addition based on the composition of pretreated biomass [5,6]. The amount of sugars released by both pretreatment and enzymatic hydrolysis was measured, with a post-hydrolysis procedure often applied to determine oligomeric sugar concentrations [21]. The procedures were tedious and time-consuming, required significant amounts of material, and did not easily lend themselves to automation or increased throughput. As a result, unlike the mild reaction conditions of enzymatic hydrolysis that are more readily applied at a small scale, the development of HT pretreatments lagged behind.

22.3 Current HTPH Systems

To date, descriptions of four high-throughput pretreatment and enzymatic hydrolysis systems have been published, all of which were based on the diagram shown in Figure 22.1b [12,22–25]. As opposed to conventional pretreatment and enzymatic hydrolysis (Figure 22.1a) that involved large-scale pretreatments from which pretreated solids were then distributed to multiple hydrolysis experiments, HTPH systems involved downscaled pretreatments that were then used directly for subsequent enzymatic hydrolysis. To accomplish this, three of the HTPH systems employed a process termed “co-hydrolysis” [12,24–26] or “one-tube process” [23] in which both pretreatment and enzymatic hydrolysis were performed in the same

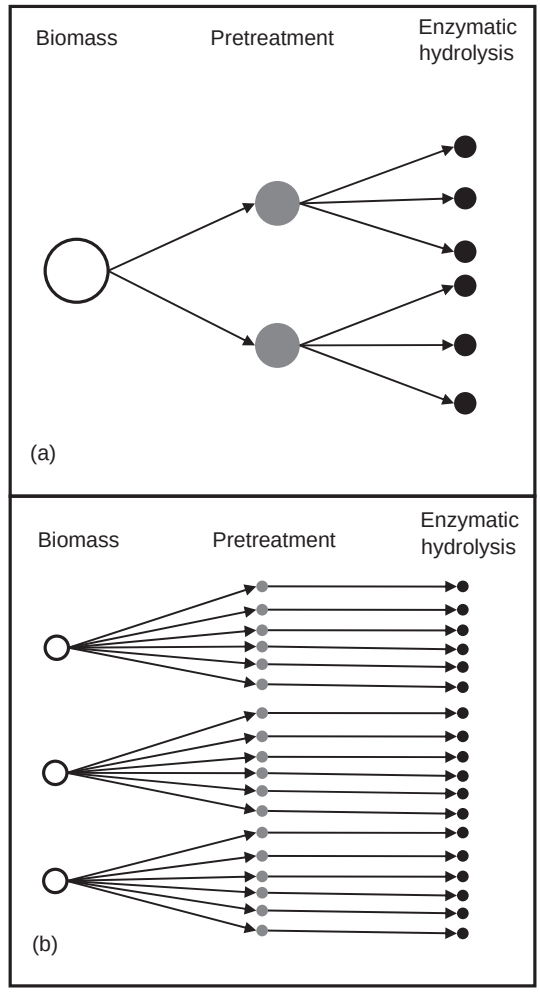

Figure 22.1 *Comparison of (a) conventional pretreatment and subsequent enzymatic hydrolysis laboratory experiments with (b) HTPH approach, in which the same reactor is employed for both pretreatment and enzymatic hydrolysis to avoid processing between the two operations. (Adapted from Studer* et al. *[26] © BioMed Central Ltd.).*

reactor without typically practiced procedures such as solid/liquid separation and solid washing between the pretreatment and hydrolysis steps.

Figure 22.2 outlines the differences between conventional pretreatment and enzymatic hydrolysis with the new co-hydrolysis or one-tube processes. Work by Santoro *et al.* [23] and Studer *et al.* [25] demonstrated that these processes achieved similar sugar yields to conventional washed solids hydrolysis. More detailed analyses showed that leaving the pretreatment liquid (hydrolyzate) with the pretreated solids could introduce inhibitors to enzymatic hydrolysis that could require lower solids concentrations or higher enzyme loadings for co-hydrolysis results to be more comparable to those from conventional processes [26]. As a result, particular attention must be paid to select conditions that best mimic the sugar yields obtained from conventional pretreatment and enzymatic hydrolysis for HTPH systems that employ co-hydrolysis or one-tube processes. To avoid this concern, one of the HTPH systems [22] took a somewhat different

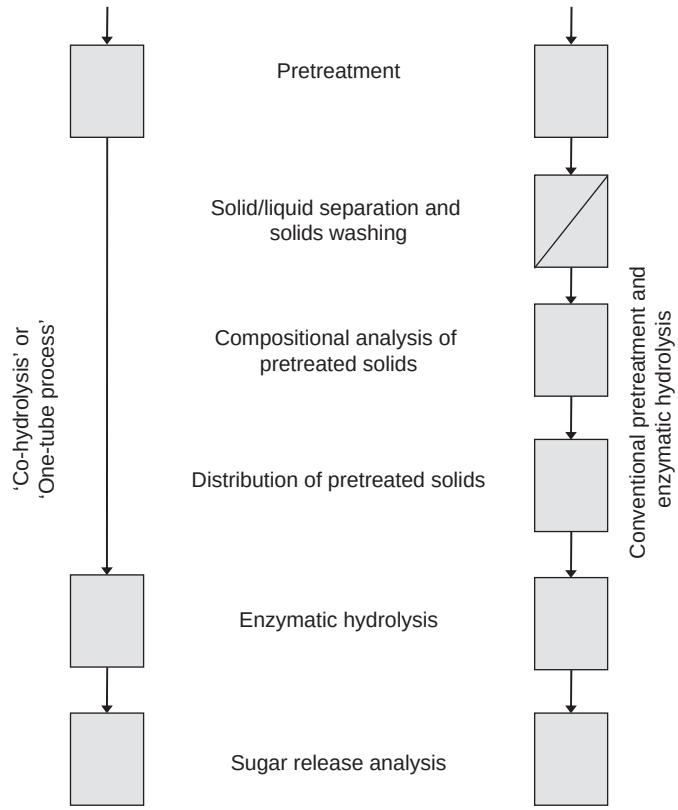

Figure 22.2 *Flow diagrams of conventional pretreatment and enzymatic hydrolysis versus an HTPH approach that employs a co-hydrolysis or one-tube process. (Adapted from Studer* et al. *[26] © BioMed Central Ltd.).*

approach from the other three: prior to enzymatic hydrolysis, pretreated solids were rinsed several times with a buffered solution to closely mimic the effects of solid/liquid separation and solid washing performed in conventional processes. In this way, the pH of the pretreated material was brought to the same value as that for enzymatic hydrolysis, and the majority of inhibitors generated during pretreatment were removed.

Although all four HTPH systems were based on the same principle (Figure 22.1b), each one varied its reactor configuration and processing conditions. Developed through support of the BioEnergy Science Center (BESC), the HTPH system at the University of California Riverside (UCR) pictured in Figure 22.3 was based on a custom-built 96-well plate design. However, the base plate was constructed of either aluminum or brass, and the reactor wells were made of Hastelloy. The original design [25] included an aluminum base plate into which Hastelloy wells that employed a reaction mass of 250 mg were press fit (Figure 22.3a).

More recently, an updated well plate design was developed with larger Hastelloy wells that held a reaction mass of 450 mg. These wells were also free-standing via a small pin in the well bottom to allow the wells to stand upright on the brass base plate instead of being press fit into the plate. This modification enabled grippers in a robotics platform to pick up and move the individual wells to more accurately tare and add ingredients.

For sealing both designs, the well plate was clamped between a top and bottom stainless steel plate with a flat silicone gasket positioned between the upper plate and the well openings. For pretreatment, the well

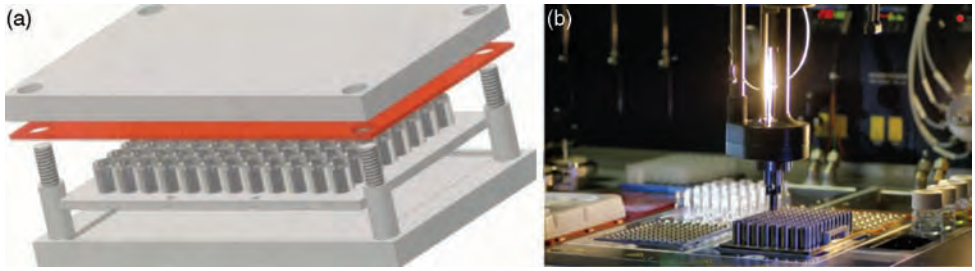

Figure 22.3 *UCR's HTPH reactor system [25] including the original reactor design in which Hastelloy wells with a 250 mg reaction mass were (a) press fit into an aluminum plate clamped between two stainless steel plates during pretreatment, and (b) the updated reactor with larger free-standing Hastelloy wells (450 mg reaction mass) being loaded by a Symyx Core Module.*

plate assembly was placed into a custom-built chamber into which steam was introduced to penetrate the space between individual wells and distribute heat evenly to all sides. At the completion of the pretreatment reaction, the steam inlet valve was closed, a valve was opened to vent steam from the chamber, and cooling water was flooded into the chamber. UCR's HTPH system was therefore capable of pretreating 96 biomass samples in one plate, with multiple plates potentially heated at once in the steam chamber.

As shown in Figure 22.4, the NREL also developed an HTPH system through support of the BESC based on custom-built gold-plated aluminum or Hastelloy stackable 96-well plates for hydrothermal or dilute acid pretreatments of c. 300 mg total reaction mass in each well [12,24]. After loading, each plate was sealed by placing an adhesive-backed aluminum foil Teflon gasket between the plates. Then up to 20 custom-made 96-well plates could be clamped on top of one another and placed in a 2-gal Parr reactor for pretreatment with indirect steam. Holes were drilled into each well plate to allow steam to penetrate into all of the plates and provide more rapid and uniform heating during pretreatment. After a target pretreatment time was reached, cooling water was forced through the channels in the plate. NREL's HTPH system was capable of pretreating up to 1920 biomass samples simultaneously.

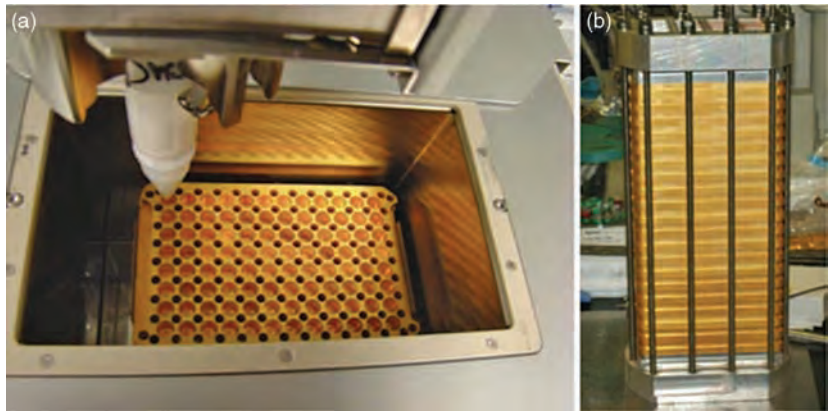

Figure 22.4 *The NREL HTPH reactor system uses the Symyx Powdernium to dispense biomass into (a) the wells of the 96-well reactor plate with a reaction mass of 255 mg per well with (b) 20 reactor plates stacked together in a modified 2-gal Parr reactor for pretreatment. (Reproduced from Decker* et al. *[12]).*

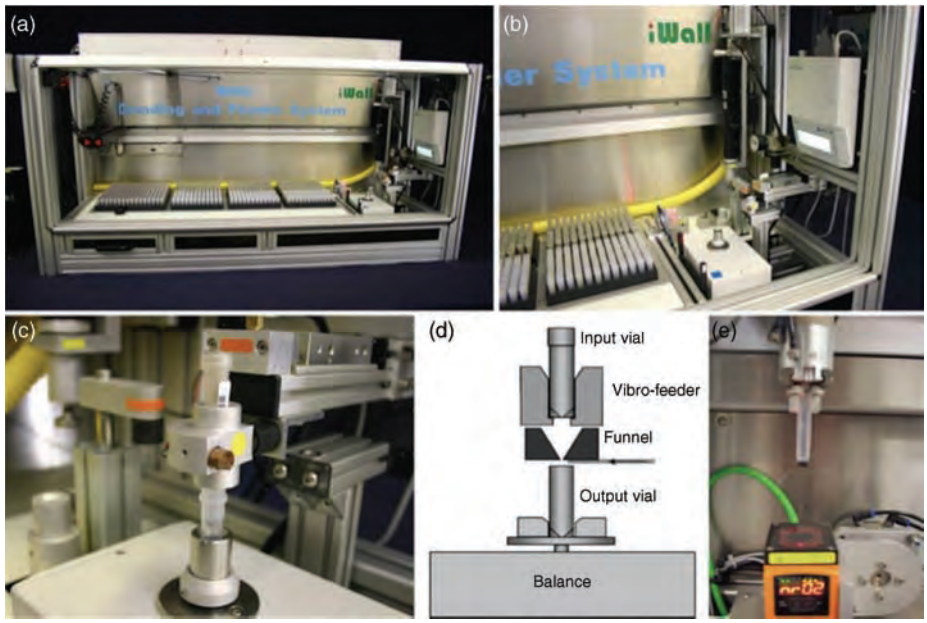

Figure 22.5 *GLBRC's HTPH system, including (a) iWall robotics platform for milling and dispensing. More detailed views of the (b) weighing substation, (c) balance and vibro-feeder dispensing from input (upper) to output (lower) tube, (d) diagram of weighing substation, and (e) bar code scanner substation are also shown. (Reproduced from Santoro* et al. *[23] with permission from Springer).*

The Great Lakes Bioenergy Research Center's (GLBRC's) HTPH system shown in Figure 22.5 was based on an off-the-shelf 96-tube Stabo-rack that held 1.4 mL polypropylene microtubes, each of which employed a reaction mass of *c.* 750 mg [23]. After loading, the tube racks were sealed with an elastopolymer seal and placed into a water bath for pretreatment. At the completion of the pretreatment reaction, the tube racks were cooled on ice. GLBRC's HTPH system could pretreat three 96-tube racks simultaneously, that is, 243 biomass samples at a time.

The fourth HTPH system shown in Figure 22.6 was developed by researchers at the University of York and the University of Dundee and was based on a standard off-the-shelf plastic 96-well plate [22]. In this system, each well employed a pretreatment reaction volume of 350 μL. After loading, the plates were sealed with a silicone cover and placed onto a heating block for pretreatment. This system could pretreat 360 biomass samples at a time. However, reaction temperatures were limited to less than 100 °C.

Although it did not measure the sugar release from combined pretreatment and enzymatic hydrolysis, a fifth high-throughput system accomplished downscaled and high-throughput pretreatment coupled with an alternative measure of biomass-pretreatment performance. In this system, a standard off-the-shelf polystyrene 96-well plate was employed (*c.* 200 mg reaction mass per well) in which various ionic liquids could be tested for their ability to dissolve the cellulose portion of biomass samples [27]. For pretreatment, a block containing heating rods through its interspaces was heated by temperature-controlled water. The block itself also acted as a seal to prevent water uptake by the ionic liquids. This HTPH system was capable of pretreating and measuring *in situ* 96 biomass samples at a time.

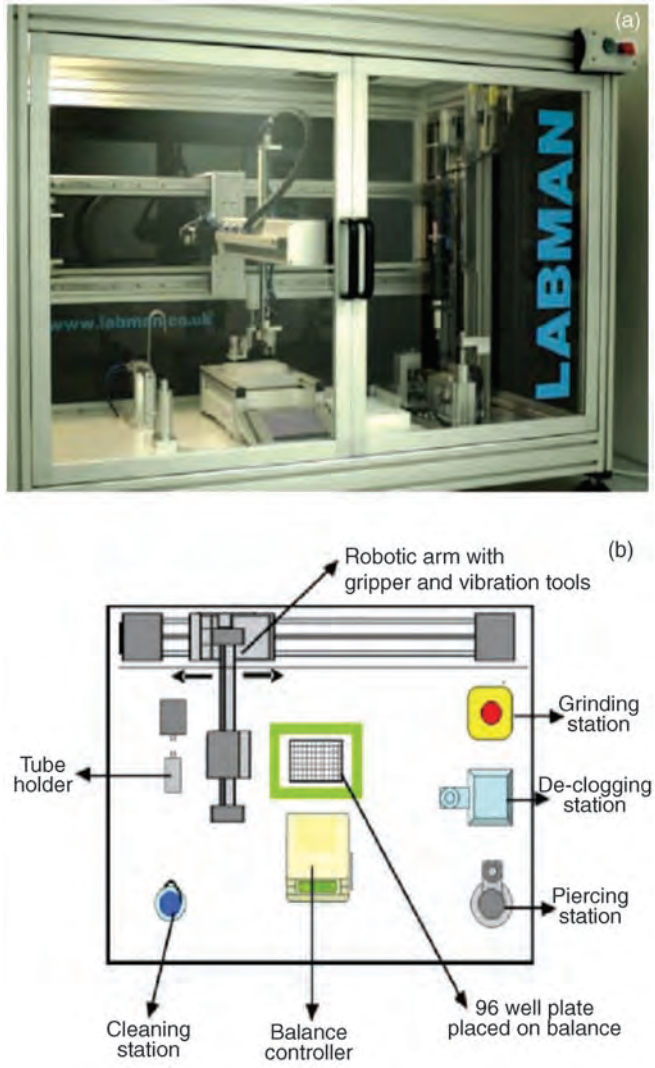

Figure 22.6 *HTPH system described by Gomez* et al. *including (a) general view of robotics platform for milling and dispensing and (b) schematic of robot's different substations. (Figure obtained from Gomez* et al. *2010 [22]).*

22.4 Key Steps in HTPH Systems

Four basic steps have to be accomplished for all HTPH systems: (1) material preparation; (2) material distribution; (3) pretreatment reaction; and (4) sample preparation and analysis. Each step is described in the following sections, and the techniques applied to accomplish these steps are summarized.

22.4.1 Material Preparation

Preparing biomass materials for high-throughput pretreatment and enzymatic hydrolysis experiments is a crucial step that impacts all downstream processes, as well as the validity of any results. The material has to

be homogeneous so that each small sample is representative of the larger mass. The three primary steps involved include sampling, particle size reduction, and any subsequent conditioning to obtain the prepared sample. In NREL's HTPH system, biomass material was knife-milled until it passed through a 1 mm screen [24]. No further conditioning was reported. Similarly, UCR's HTPH system employed knife-milled material; however, milling was typically followed by sieving to obtain a 20–80 mesh fraction (0.18–0.85 mm) [25]. Zavrel *et al.* [27] reported use of wood chips produced by sawing in their HT pretreatment system; the resulting particle sizes were in the range of 1–2 mm in length.

Unlike the above three systems that required time-consuming manual material preparation steps, the HTPH systems reported by Santoro *et al.* [23] and Gomez *et al.* [22] were unique in that they performed size reduction automatically via grinding robotics platforms, namely iWALL (TECAN, Mannedorf, Switzerland) and Labman Automation (Stokesley, North Yorkshire, UK), respectively. With the former, dried plant material (20–40 mg) was manually loaded into 2 mL screw cap microtubes along with three 7/32 inch stainless steel balls. These tubes were then loaded into racks and placed in the robotics system. Pulverization of the biomass was accomplished by ball milling to a fine powder with a particle geometric mean diameter of between 0.034 and 0.055 mm (depending on biomass type); more than 90% of the particles were smaller than 0.35 mm for all plant types tested. The system described by Gomez *et al.* [22] similarly employed automated shaking at *c.* 5000 rpm with three ball bearings within biomass-filled vials, but the particle size range of the resulting material was not reported.

22.4.2 Material Distribution

Following preparation, the materials had to be distributed to the pretreatment reactors. For the small amounts of biomass that were used in HT technologies, accurate solids distribution could be a tedious and time-consuming step. Prior to the development of HTPH systems, some of the downscaled and HT enzymatic hydrolysis set-ups discussed above applied creative methods to accomplish small-scale solids distribution. For example, Berlin *et al*. [7] took advantage of the uniform nature of biomass paper to produce "handsheets" of ethanol organosolv pulped poplar, from which 6 mm disks that weighed on the order of 1–2 mg were produced by a paper punch. Each disk was then manually placed in each individual well for subsequent enzymatic hydrolysis. This method was similar to that developed by Decker *et al.* [28] in which 2.65 mg filter paper disks were produced and placed in the wells of a standard microtiter plate to automate the filter paper assay of cellulase activity. Others [9,10] distributed small amounts of biomass into 96-well plates by suspending the solids in water or buffer (1–5% w/w) and transferring the slurry into individual wells of a 96-deepwell microplate while mixing the slurry to ensure uniformity during transfer.

For HT pretreatment and enzymatic hydrolysis systems, some workers reported that the solids were manually weighed into individual wells of microtiter plates [25,27]. In the case of the initial UCR HTPH design described by Studer *et al.* [25], a small brass weighing cup that held a volume corresponding to the target mass to be dispensed (2.5 mg) facilitated distribution of biomass to the individual wells. However, weighing of milligram quantities of biomass was time-consuming and dramatically reduced the overall throughput of these systems. As a result, all four HTPH systems migrated to solids-dispensing robotics platforms. At NREL, a Symyx MTM Powdernium powder dispensing system (Symyx, Sunnyvale, CA) distributed 5 mg of biomass to each well of their 96-well plate reactor [12,24]. The deck of this robotic platform could accommodate up to 80 plastic 10 mL biomass-dispensing hoppers at a time, each of which could handle small amounts of biomass (50–100 mg). For this system, the entire reactor plate was moved to a modified Sartorius LP330 balance that recorded the final weight of biomass dispensed into each well to 0.1 mg accuracy. After solids distribution, an automated pipetting system (Biomek FX) added liquids for all subsequent steps. The total reaction mass employed in each well was 255 mg, comprising 250 μL of water or dilute acid added to 5 mg of milled biomass.

At UCR, a Standard Configuration 2 Symyx Core Module dispensed 4.5 mg of biomass into each well for the updated well plate design [29]. The robotics platform could accommodate 10 metal 25 mL biomass-dispensing hoppers, each of which could contain 5–5000 mg of biomass. The UCR system differed from NRELs in that the individual wells of the microtiter reactor were moved into a Sartorius WZA65-CW balance one at a time, allowing determination of the amount of each ingredient added to a single well to within 0.01 mg. Subsequent liquid handling steps were accomplished either with the liquid handling set-up on the same Core Module robotics platform or with multichannel pipettes (8 channel pipetter, Eppendorf, Hamburg, Germany). In this system, 445.5 μL of water was typically added to 4.5 mg of milled biomass for hydrothermal pretreatment. For dilute acid and dilute alkali pretreatments, the reaction was performed with 85.5 μL of acid or 40.5 μL of base added to 4.5 mg of biomass.

In GLBRC's HTPH system, the same iWALL robotics platform accomplished both biomass milling and solids dispensing [23]. After automated milling in a 1.4 mL tube, vials were sent to a de-clogging station to break up clumped material, followed by transfer to a piercing station where a 1 mm hole was bored into the base of each vial. Next, 1.5 mg of this milled biomass was dispensed through a funnel by the action of a vibro-feeder into an empty vial located below the original vial and placed on a Mettler Toledo SAG 205 balance. The balance recorded the amount of biomass dispensed into each tube to within 0.01 mg. A PerkinElmer (Waltham, MA) Janus workstation subsequently added 750 μL of pretreatment liquid, typically dilute NaOH, into each tube.

The system reported by Gomez *et al.* [22] was similar to that described immediately above. However, important differences included use of a different robotics platform, namely a Labman Automation platform (Stokesley, North Yorkshire, UK), that dispensed 4.0 mg of material from the original vials filled with milled biomass into the individual wells of a 96-well microplate. The amount of biomass in each plate was monitored to within 0.1 mg by placing the entire microplate on a balance during dispensing. Subsequently, 350 μL of either dilute NaOH or H_2SO_4 were added by the same robotics platform for pretreatment.

22.4.3 Pretreatment and Enzymatic Hydrolysis

After material distribution, the next key steps in any HTPH system were pretreatment and enzymatic hydrolysis. NREL performed either hydrothermal (just hot water) or dilute acid pretreatment in their system at about a 2% solids loading. As an example, they reported hydrothermal pretreatment for 40 min at 180 °C [24] and, more recently, applied dilute acid pretreatment typically with 0.3% H_2S0_4 for 30 min at 180 °C (SR Decker, personal communication, 2011).

UCR's HTPH system is also compatible with hydrothermal [25], dilute acid [30], and dilute alkali pretreatments (H. Li, personal communication, 2011). For hydrothermal pretreatments, a 1% solids loading was used, while for dilute acid and alkali, the solids loadings were 5% and 10%, respectively. Temperatures between 120 and 180 °C and times from 10 to 300 min were applied, with exact conditions determined for the biomass to be tested. For dilute acid and alkali pretreatments, concentrations of 0.5–1% H_2S0_4 and 1% NaOH, respectively, were applied.

Both dilute acid and dilute alkali pretreatments could also be performed in GLBRC's HTPH system. For example, they reported pretreatment at 90 °C in 0.025% NaOH for 3 hours with a solids loading of 0.2% [23]. Similarly, Gomez *et al.* [22] employed both dilute acid and alkali pretreatments but at lower temperatures due to pressure constraints for their system, for example, 1% H_2S0_4 or 0.5N NaOH at 90 °C for 30 min at a solids loading of about 1.1%. Finally, Zavrel *et al.* [27] added a total of 200 μL of various imidazolium-based ionic liquids to between 4 and 12 mg of biomass per well to give a solids loading of 2–6%. In this case, pretreatments were performed at 50 °C for 8–24 hours.

Following pretreatment, enzymatic hydrolysis was performed in the same reactor in a co-hydrolysis or one-tube processing approach for three of the HTPH systems [23–25]. In general, a higher enzyme loading

was employed for these methods compared to conventional washed solids hydrolysis to offset the effects of inhibitors possibly present in the pretreated biomass slurry as a result of not removing the pretreated liquids from the solids. For hydrothermal pretreatment with the NREL system, an enzyme loading of 70 mg cellulase per g initial biomass supplemented with 2.5 mg/g β-glucosidase was applied for a 72 hr static incubation at 40 °C [24]. The enzyme addition was made to the entire pretreated biomass slurry along with 1 M sodium citrate buffer to bring the pH of the pretreatment slurry to *c.* 5.0. UCR employed a similarly high enzyme loading, typically 75 mg cellulase per g glucan + xylan in the initial biomass, supplemented with 25 mg/g of xylanase for a 72 hr incubation at 50 °C and with shaking at 150 rpm [25]. Diluted enzyme was added to the pretreated biomass slurry in combination with 1 M citrate buffer and 1 g/L of the biocide sodium azide. For dilute acid and dilute alkali pretreatments, the pretreated slurry was diluted with water 5 or 10 times, respectively, prior to enzyme addition to achieve a total reaction mass of 450 mg. GLBRC's HTPH system employed an enzyme loading of 30 mg cellulase per g glucan in the initial biomass, which was added to the pretreated biomass slurry in each vial as a solution that also contained 30 mM citrate buffer and 0.01% sodium azide. Hydrolysis was performed at 50 °C with end-over-end rotation for a 20 hr incubation time [23].

As mentioned previously, the HTPH system developed at the University of York and University of Dundee [22] more closely mimicked conventional solid/liquid separation and solid washing by applying several rinses with a buffered solution prior to enzymatic hydrolysis. As a result, a lower enzyme loading of 6.3 FPU/g of material (*c.* 14 mg cellulase/g biomass [31]) was typically employed using a mixture of cellulase (Celluclast, Novozymes, Bagsvaerd, Denmark) supplemented with β-glucosidase (Novozyme 188, Novozymes, Bagsvaerd, Denmark) at a 4 : 1 ratio, respectively. Enzymatic hydrolysis was reported to be carried out in 25 mM sodium acetate buffer at 50 °C for 8 hours with constant shaking at 120 rpm.

22.4.4 Sample Analysis

The four HTPH systems reported here all measured the amount of sugar released from combined pretreatment and enzymatic hydrolysis. To accomplish this, UCR employed an HPLC equipped with a refractive index detector [25], using hydrolyzates transferred to HPLC-compatible vials or microplates following the completion of enzymatic hydrolysis. Benefits of this approach included that it was both well established and enabled measurement of sugars in addition to glucose and xylose. However, HPLC was time-consuming and could require between 15 and 30 min per sample, equating to 24–48 hours for analysis of all 96 samples from a single well plate. To avoid this HPLC load, both the NREL and GLBRC systems employed enzyme-based assays [23,24]. For the NREL system, hydrolyzates following enzymatic hydrolysis were diluted and transferred to 96-well flat-bottomed polystyrene plates in which glucose was detected via a modified glucose oxidase/peroxidase (GOPOD) assay and xylose was detected with a xylose dehydrogenase assay (Megazyme International Ireland, Wicklow, Ireland) by measuring their absorbance at 510 and 340 nm, respectively [24]. GLBRC's HTPH system similarly employed GOPOD and xylose dehydrogenase assays following transfer to 384-well microtiter plates [23]. The benefit of sugar analysis by enzyme-based assays was the speed at which they could be completed: it was estimated that a plate of 96 samples could be analyzed in 20 min [12]. However, it has also been reported that, due to the specificity of the assays, certain sugars such as xylooligomers could interfere with xylose measurements [24].

Colorimetric assays, which could easily be applied in high-throughput set-ups, have also been employed for sugar release measurements. In HT enzymatic hydrolysis systems [10,32], a standard dinitrosalicylic acid (DNS) assay was used to measure reducing sugars released during enzymatic hydrolysis. Alternatively, the HTPH system reported by Gomez *et al.* [22] measured glucose equivalents released by enzyme action

Table 22.2 *Summary of key characteristics for each of the four HTPH systems including the reaction volumes utilized in pretreatment and enzymatic hydrolysis, solids loading, chemical type and concentration, temperature, and heat source.*

Institution		Reaction volume (μL) (pretreatment/EH[a])	Solids loading (%[b]/mg)	Pretreatment type	Temp/acid concentration	Heating medium	Ref
NREL[d]	A	300/300	1.7/5.0	Hydrothermal	180 °C/0%	Steam	[24]
	B	250/250	2.0/5.0	Dilute acid/H_2SO_4	180 °C/0.3%		
UCR[d]	A	450/450	1.0/4.5	Hydrothermal	120–180 °C/0%	Steam	[25]
	B	85.5/450	5.0/4.5	Dilute acid/ H_2SO_4	120–180 °C/0.5–1%		
	C	40.5/450	10.0/4.5	Dilute alkali/ NaOH	120–180 °C/1%		
GLBRC[e]	A	750/750	0.2/1.5	Dilute alkali/ NaOH	90 °C/0.025%	Water bath	[23]
	B			Dilute acid/ H_2SO_4	90 °C/2.0%		
UY[c, e]	A	350/750	1.1/4.0	Dilute acid/ H_2SO_4	90 °C/1.0%	Heating block	[22]
	B			Dilute alkali/ NaOH	90 °C/0.5N		

[a] The reaction volume for enzymatic hydrolysis is the total slurry volume (water, dilute acid, or dilute alkali) prior to enzyme, buffer, biocide addition.

[b] Solids loading is described as % biomass weight per reaction volume.

[c] UY: University of York/University of Dundee at Scottish Crops Research Institute.

[d] Employs custom-built reactor.

[e] Employs off-the-shelf reactor.

using a modified 3-methyl-2-benzothiazolinonehydrozone (MTBH) assay. In this approach, a mixture of hydrolzate, NaOH, MBTH, and dithiothreitol (DTT) with a final volume of 250 μL was incubated at 60 °C for 20 min, after which an oxidizing reagent was added and optical measurements were taken at 620 nm in an optical well plate. As with enzyme-based assays, colorimetric measurements such as the modified MTBH assay employed by Gomez *et al.* [22] had the benefit of fast detection; however, possible interference among sugars and differing response for various sugars could be concerns [12,22].

Although also testing for sugar release from combined pretreatment and enzymatic hydrolysis, Zavrel *et al.* [27] applied a different approach to evaluating pretreatment-biomass combinations by monitoring *in situ* dissolution of cellulose by ionic liquids. In their HT pretreatment system, scattered and transmitted light was measured continuously during pretreatment to follow the size and number of cellulose particles as a function of pretreatment time. Although this system was unable to quantitatively measure sugar release, greater solubilization of crystalline cellulose was associated with enhanced sugar release in subsequent enzymatic hydrolysis, providing an alternative measure of cellulose digestibility.

Table 22.2 summarizes key characteristics for each of the four HTPH approaches.

22.5 HTPH Philosophy, Difficulties, and Limitations

HTPH technologies provided a significant step for screening large numbers of plant samples for their recalcitrance to sugar release. However, it was important to recognize the distinctions between conventional and HT pretreatment and enzymatic hydrolysis testing, particularly that the latter was primarily a screening tool that provided a platform from which sugar release trends, as well as superior and sub-par outliers, could be identified for further analysis. Along these lines, HTPH processes typically measure only total monomeric sugar release from combined pretreatment and enzymatic hydrolysis. On the other hand, conventional methods track sugar release from these two steps individually by separately analyzing

the solid and liquid phase from pretreatment and also from enzymatic hydrolysis, thereby revealing more details about the sources and fates of sugars, including those in both monomers and oligomers [33], and facilitating mass balance closure.

However, it is important to note that HTPH could be extended to measuring total monomeric plus oligomeric sugars from pretreatment and enzymatic hydrolysis or easily adapted to measure sugar release from individual stages if such details are needed. It should also be kept in mind that solid/liquid separation is not likely to be desirable commercially due to higher capital and operating costs, as well as introducing additional opportunities for contamination and sugar loss. Although HTPH configurations based on co-hydrolysis or one-tube processes may differ from conventional methods, they may more closely simulate commercial practice.

The lack of solid/liquid separation following pretreatment in HTPH systems also has strong implications on enzyme loadings used in hydrolysis of the pretreated biomass slurry. Since the purpose of HTPH systems was primarily to screen multiple plants for reduced recalcitrance, this required the selection of conditions that best highlighted differences in substrate features and changes in substrate with pretreatment. High enzyme loadings were therefore applied to allow determination of differences in substrate digestibility, as opposed to enzyme inhibition or activity. Another key point in ensuring that differences in substrate digestibility were revealed was selection of proper pretreatment conditions. When the goal was to identify less recalcitrant plants, lower pretreatment severities than those identified for maximum sugar release from baseline substrates were typically applied to facilitate identification of biomass samples that were amiable to high sugar release from enzymatic hydrolysis at conditions that sacrificed less sugar to degradation during pretreatment.

Although the development of HTPH systems is a major step to accelerating screening of large combinations of biomass materials, pretreatment conditions, and enzyme loadings and formulations, important challenges remain. One such limitation, as with any downscaled system, is that errors can be significantly amplified at this small scale. In line with this, the introduction of a single air bubble during pipetting will result in inaccurate sugar concentrations and erroneous sugar release results. Furthermore, glucose released from starch in plants cannot be differentiated from glucose released from cellulose for the HTPH analysis methods discussed previously; as a result, differences in the amount of glucose release observed between samples could be due to varying levels of endogenous sugars in native biomass. Likewise, the fate of extractives and particularly free sugars during pretreatment has not yet been established; as a result, the mass of sugar released per mass of biomass could be influenced.

Unfortunately, the removal of starch and extractives is conventionally performed with 0.1 g and 5–20 g of material, respectively, with equipment and techniques that are not currently designed for large numbers of samples and high-throughput applications [34,35]. A simple way to address possible starch interference in HTPH systems is to test the enzymes used for their ability to hydrolyze starch into glucose. In this regard, both Santoro *et al.* [23] and Studer *et al.* [25] reported non-detectable or minimal levels of starch hydrolysis, respectively. Alternatively, researchers at NREL have developed downscaled and higher-throughput methods to remove starch and extractives prior to HTPH testing to ensure that endogenous sugars do not interfere with sugar release results (SR Decker, personal communication, 2011).

Perhaps the biggest concern with scaling down pretreatment and enzymatic hydrolysis processes is obtaining homogeneous biomass samples, which could be influenced by a range of steps including sampling, biomass inhomogeneity, milling, and sieving. For example, an HTPH system was applied to measure the ring-by-ring composition and sugar release from combined pretreatment and enzymatic hydrolysis across an aspen wood cross-section to show that both varied significantly across the radial direction [36]. Additionally, Garlock *et al.* [37] reported that corn stover composition and performance in ammonia fiber expansion (AFEX) pretreatment varied considerably with anatomical fraction. These findings demonstrate that when only a small portion of a plant was sampled for analysis, such as the case in HTPH testing, the location from which that sample is taken could impact sugar release results.

It has also been noted that although milling and sieving are often necessary to achieve homogeneous samples for biomass analyses, the material preparation process itself could complicate obtaining representative samples [38]. This issue has not been found to be as significant for well-mixed woody samples that are generally quite homogeneous when milled since they contain fewer cell types that segregate by particle size. On the other hand, herbaceous materials are typically composed of diverse cell types that can segregate into distinct particle size fractions. For example, larger particle size fractions of milled corn stover have been reported to be richer in cob and stalk portions that were more recalcitrant to hydrolysis than smaller size fractions, which contained higher amounts of leaves and husk [39]. Furthermore, the fine fractions of herbaceous materials in the size range of <80 mesh or <0.180 mm can contain a large fraction of inorganics. Removal of this fraction could affect composition and sugar release performance and may not provide results that are representative of the entire plant since certain anatomical fractions segregate [38]. Together, these studies all stress the importance of taking great care in sampling and material preparation for HTPH analyses to ensure that the small amounts of materials employed are representative of the entire plant sample being tested. Conversely, due to the downscaled and high-throughput nature of HTPH systems, this limitation can be easily tested by running many replicates to check for biomass inhomogeneity, an approach not taken as easily for previous larger-scale conventional pretreatment and enzymatic hydrolysis methods.

22.6 Examples of Research Enabled by HTPH Systems

To date, HTPH systems have enabled a number of research projects that were previously not possible, including methodologies in both plant development and screening presented in Section 22.1. One of the first reports of a large-scale project to screen sugar release from combined pretreatment and enzymatic hydrolysis of hundreds of plants was by Santoro *et al.* [23] in which 1200 *Arabidopsis* samples were tested. These samples were knock-out and knock-down mutants via T-DNA insertions to genes that were believed or known to play a role in cell-wall metabolism and possibly digestibility. In this case, HTPH successfully identified several *Arabidopsis* lines with significantly higher glucose and xylose release than most others.

Voelker *et al.* [40] also applied an HTPH system [12,24] to screen 4–7 field-grown poplar trees per transgenic event (14 events, 100 samples in total) that had undergone transgenic down-regulation of the Pt4CL1 gene based on previous evidence that this would reduce lignin content in cell walls. However, in contrast to previous studies, they found that trees with reduced lignin contents did not yield substantially higher saccharification potential; instead, very little difference was found in the sugar release from combined hydrothermal pretreatment and enzymatic hydrolysis among all trees tested.

Selig *et al.* [24] similarly employed an HTPH system to screen 755 natural poplar variants from the Pacific Northwest of North America, a feat that would not have been otherwise practical in light of the sheer number of samples. In this study, sugar release from combined hydrothermal pretreatment and enzymatic hydrolysis was independent of total lignin content but strongly related to the lignin syringyl to guaiacyl (S/G) ratio. In an extension of that study, a much smaller subsample of natural poplar variants (47 samples $\times$ 3 pretreatment conditions) from the same population was tested under a variety of hydrothermal pretreatment conditions using HTPH technology [41]. In this case, glucose release only had a strong negative correlation to lignin content for trees with low S/G (<2) ratio, while xylose release was dependent on the S/G ratio alone and not lignin content. Furthermore, certain trees featuring average lignin contents and S/G ratios exhibited exceptionally high sugar release, demonstrating that factors beyond lignin content and S/G ratio influenced recalcitrance.

HTPH systems are not only useful for screening large numbers of samples but also for their ability to provide sugar release results from very minimal amounts of biomass material. Along these lines, DeMartini and Wyman [36] applied HTPH technology to test sugar release from combined pretreatment and enzymatic hydrolysis of the individual annual rings of 26- and 8-year-old aspen trees. Although only about 35 samples

in total were tested at a single pretreatment condition, downscaled technology was essential to process such small sample amounts (<100 mg). In this case, sugar release (grams of sugar released per gram biomass) varied significantly across the radial direction of the tree, but sugar yields (grams of sugar released per gram available sugar) did not, suggesting that wood maturity impacted composition much more than recalcitrance. A similar study was also undertaken with mixed prairie species in which HTPH technology was utilized to test sugar release from combined pretreatment and enzymatic hydrolysis of grasses and legumes [42]. DeMartini and Wyman [42] found significant differences among these two types of species, as well as among individual anatomical components that influenced the recalcitrance of mixed prairie species.

22.7 Future Applications

The development of HTPH technology has opened the door for reducing sample size and increasing the throughput for a variety of biomass applications. HTPH systems can generate large amounts of sugar release data to better understand factors influencing biomass recalcitrance. However, results from these screening studies typically reported the amount of sugar released per amount of total biomass; at the time that most of the studies were performed, there was no method to accurately determine the carbohydrate content of the large numbers and small amounts of samples available. However, to gain a better sense of a plant's recalcitrance, sugar yields should be determined as the amount of sugar released per amount of sugar available. To meet this need, downscaled and high-throughput compositional analysis approaches have recently been developed [29,43]. Both are based directly on conventional two-stage acid hydrolysis compositional analysis [44] but use significantly less material (between 60 and 100 times less material, equivalent to 3 or 5 mg biomass per test). NREL applied a 96-well plate format similar to their HTPH procedure for downscaled compositional analysis [43], while an array of 48 1.5 mL glass HPLC vials with the same plate-clamping mechanism was used to support the UCR HTPH system [29]. The former method was capable of measuring glucan and xylan contents of much larger sample sets than reported for the latter; however, the latter could also estimate whole ash and Klason lignin contents through measurement of acid insoluble residue (AcIR).

Development of HTPH technologies also enable large-scale pretreatment kinetic studies that require much less time and labor compared to use of conventional reactors. Along these lines, recent work by T. Zhang (personal communication, 2011) employed UCR's HTPH system to study the kinetics of hemicellulose and cellulose conversion to sugar degradation products such as furfural, 5-hydroxymethyl-2-furaldehyde (5-HMF), and levulinic acid, by evaluating a number of different pretreatment times, temperatures, acids, and acid concentrations. Approximately 4000 samples were tested in about 1 month.

In addition to employing HTPH technology to screen sugar release from combined pretreatment and hydrolysis or developing pretreatment degradation kinetics, there are opportunities to expand it to microbial screening. For example, Cianchetta *et al.* [45] reported development of a miniaturized cultivation system based on flat-bottom 24-well plates to quickly and easily identify superior and sub-par cellulase producers on cellulose powder from over 300 *Trichoderma* strains. Extension of this technology to real biomass substrates, including pretreated materials, presents an important opportunity.

22.8 Conclusions and Recommendations

The development of HTPH systems represents a major advancement in biofuels research through providing a platform for rapid screening of large sets of plant samples in order to identify biomass outliers and trends in recalcitrance. Testing sugar release of thousands of samples in combination with various pretreatment and enzymatic hydrolysis conditions is no longer an imposing feat. Downscaling of biomass pretreatment to microplate- or small tube-based formats, in addition to development of co-hydrolysis or one-tube

processes, enables automation and increased throughput, as well as greatly reduced material requirements for recalcitrance assays. Although some difficulties arise with scaling down biomass pretreatment and hydrolysis, HTPH systems can be powerful tools if careful attention is paid to biomass sampling and distribution. With the continued application of and improvements in these systems, new insights can be gained into biomass recalcitrance that will aid in identification of superior feedstock candidates, better pretreatment conditions, and improved enzyme formulations.

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