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High-Throughput Screening Techniques for Biomass Conversion

Stephen R. Decker · Roman Brunecky · Melvin P. Tucker · Michael E. Himmel · Michael J. Selig

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Abstract High-throughput (HTP) screening of biomass or biomass-degrading enzymes, regardless of the desired outcome, is fraught with obstacles and challenges not typically faced in more traditional biotechnology. The enzyme systems are complex and synergistic and the substrate is highly heterogeneous, insoluble, and difficult to dispense. Digestions are often carried out for days at temperatures of 50°C or higher, leading to significant challenges regarding evaporation control in small well volumes. Furthermore, it is often desirable to condition or "pretreat" the biomass at extreme temperatures and/or pH to enhance enzyme digestibility. Once the substrate has been saccharified, evaluation of the extent and efficiency of conversion is made more difficult by time-consuming and tedious techniques used to measure the sugar products. Over the past decade or so, biomass researchers have creatively addressed these challenges by developing techniques to reduce biomass heterogeneity, uniformly distribute biomass samples at the small scale, pretreat the biomass at the small scale, quantitatively load these samples with enzymes, control evaporation of small reaction volumes for multiday incubations, and rapidly quantify the products. Other aspects of these measurements remain problematic and are being addressed. This review will address some of these challenges in detail, but more importantly, we will endeavor to educate the reader about the trials, tribulations, and pitfalls of carrying out HTP screening in biomass conversion research.

Keywords Biomass conversion · Biomass pretreatment · Cellulase assay · Lignocellulosic biomass · High-throughput screening

Abbreviations

BESC	BioEnergy Science Center
NREL	National Renewable Energy Laboratory
HTP	high throughput
US DOE	United States Department of Energy
MS	mass spectrometry
GC	gas chromatography
HPLC	high-performance liquid chromatography
UPLC	ultra-performance liquid chromatography
SBS	Society for Biomolecular Screening
AFEX	ammonium fiber expansion
DNS	dinitrosalicylic Acid
BCA	bicinchoninic Acid
MBTH	3-methyl-2-benzothiazolinonehydrazone
RI	refractive index
PAD	pulsed amperometric detection

S. R. Decker (✉) · R. Brunecky · M. E. Himmel · M. J. Selig
Biosciences Center, National Renewable Energy Laboratory,
1617 Cole Boulevard, MS 3323,
Golden, CO 80401, USA
e-mail: steve.decker@nrel.gov

M. P. Tucker
National Bioenergy Center,
National Renewable Energy Laboratory,
Golden, CO, USA

Introduction

Up until the early 1990s, discovering new microorganisms, improving enzyme activities, or testing novel pharmaceuticals in biotechnology was often accomplished by tedious manual effort. The advent of highly accurate, precise, and reproducible liquid-handling robots introduced a new era in biotechnology commonly referred to as laboratory automa-

tion or high-throughput (HTP) screening. The introduction and widespread acceptance of new techniques for biotechnology, such as bioinformatics, combinatorial chemistry and biochemistry, directed evolution, and genomics provided significant impetus for automation manufacturers to develop and market instruments capable of generating, manipulating, and evaluating these large sample sets. The most lucrative market was, of course, pharmaceuticals, so the great majority of these new automation products were geared toward addressing challenges in this area by focusing on systems that operated at 37°C, handled uniform powders or liquids, and evaluated results using simple assays. Unfortunately, few of these systems could handle the very different challenges faced by biomass conversion researchers, where fragments of insoluble, highly heterogeneous substrates were often subjected to temperatures in excess of 200°C and pH conditions less than pH 2 or greater than pH 12. The multiple sugar products, generated by multicomponent, synergistic enzyme systems at elevated temperatures over multiple days, were measured by complex sugar assays. Despite these and other obstacles, the lure of HTP screening was strong, and over the next decade or so, considerable progress was made in each of these challenging areas of automation.

The major bottleneck in lignocellulose enzymatic conversion research has always been the time and expense of setting up, monitoring, sampling, and analyzing an ever-increasing number of enzyme digestions and/or fermentation experiments, driving a need for automation and scale reduction. In addition to the issues of cost and effort, other key aspects of HTP methodologies applied to biomass conversion must be considered. Biomass heterogeneity is the first and foremost problem to consider when reducing the scale of the process to the microliter range. Temperature and evaporation control, precision in aliquoting biomass and enzymes, and rapid and quantitative product detection must also be addressed. Other issues include storage of pretreated biomass, analysis of biomass composition, and in the case of pretreatment, corrosion and pressure containment. While these have long been addressed at the bench scale, only recently have researchers begun to truly apply and adapt these levels of control to HTP methods.

In 2007, the Energy Independence and Security Act defined a path forward for research and development needed to meet the United States' cost and volumetric goals for liquid transportation fuels. The US DOE Office of the Biomass program has identified a 2012 process technology cost target for bioethanol production of $1.33/gal. Detailed process cost sensitivity studies recently conducted by Aden and coworkers concluded that, for a processing plant consuming 2,000 dry tons per day of corn stover, three process steps are the primary contributors to lignocellulosic ethanol cost: feedstock, thermal/chemical pretreatment, and enzymes used to produce fermentable sugars [1]. It is, therefore, critical to improve the digestibility of targeted feedstocks as well as the enzymes that hydrolyze them; however, the technology landscape is complicated by a diversity of feedstocks, enzyme systems, pretreatment technologies, as well as the inherent biomass heterogeneity issue that impacts all lignocellulose conversion research. It became readily apparent to our group at the National Renewable Energy Laboratory (NREL) that a concerted effort in HTP screening technique development was necessary to gain a deeper understanding of biomass recalcitrance.

As a partner in the BioEnergy Science Center (BESC), one of the NREL's key roles is the development and implementation of an effective HTP "biomass pipeline" for evaluating critical aspects of biomass conversion. One technical goal is the screening of thousands of genetic and environmental variants of different feedstocks to evaluate and map potential traits or factors that can enhance biomass conversion efficiencies. Another goal is the screening of new enzymes from a variety of sources for unique, enhanced, or synergistic activities that can benefit the biomass conversion industry. In this review, we will report how biomass researchers have historically addressed some of these issues. More critically, we hope the reader will come away with an understanding of the complexities and challenges involved in carrying out these types of experiments at throughput rates that are rapidly becoming the biotechnology industry standard.

Biomass Handling at the Small Scale

Plant cell walls are comprised of three basic components: cellulose, hemicellulose, and lignin, with the hemicelluloses and, to a lesser extent, the lignins having highly variable composition. Both the ratio of the three components and their specific composition vary from species to species as well as across tissue and cell types in the same plant. Additionally, variability of these components can often be influenced by geographic location and other environmental factors, such as climate, soil conditions, and nutrient application. The largest practical problem, however, can be illustrated by the following example. If you plan on utilizing corn stover as a feedstock, you must evaluate the suitability of the stover in toto and not just a selected subset or surrogate feedstock. At the large scale, the overall digestibility of the corn stover can be determined by harvesting a field of stover, grinding it, mixing well, pretreating a large fraction of the entire mass, digesting it with enzymes, and measuring the product. This approach yields the overall digestibility, and although the leaves may be more digestible than stem nodes, it does not really matter at this scale because it is easy to ensure that the multikilogram sample being evaluated is compositionally equivalent

to the entire 40 acres output of corn stover. Good uniformity at this scale can be obtained with fragments as large as 1/4 in. or more. But, if you want to evaluate this same material in high throughput, where the substrate sample mass can be as low as 5 mg, then a 1/4-in. fragment might be a single piece of stem, rind, pith, or leaf. You must, therefore, reduce the size further in order to obtain representative and uniform distribution of all tissue types within that 5-mg sample. One obvious solution is to mill the feedstock down to "flour"; however, size reduction beyond 80 mesh can significantly increase the biomass susceptibility to conversion.

Once biomass heterogeneity has been addressed vis-à-vis size reduction, other practical aspects of applying automation and high throughput to biomass conversion can be considered. Distributing the sample(s) uniformly and with precision is a challenge at the small scale. To date, only a few methods have been reported for biomass allocation: dispensing from slurries, distribution of biomass "paper" disks, and automated weighing of dry biomass. Once distributed, subjecting the samples to controlled pretreatment under reproducible conditions presents an enormous chemical and mechanical engineering challenge. Arraying and aliquoting defined enzyme activities at precise levels is problematic, thus quickly and accurately determining the products of the digestion is not straightforward. The physical state of the material, whether wet, dry, or dried after wetting, also affects both the material handling and the experimental results.

Size Reduction of Biomass

Size reduction is critical because the heterogeneity of biomass dictates that studying small samples of large pieces will result in inconsistent biomass composition and subsequently widely varied pretreatability and enzymatic conversion. While fine comminution and careful mixing can mitigate this, care must be taken to avoid affecting the digestibility of the material due to size reduction alone [2–4]. The material used must be fine enough to minimize variations in dispensing due to heterogeneity, but not so fine as to affect digestibility [5]. In our experience, biomass can be milled to 20 to 80 mesh particle size in order to be distributed using automation without significantly affecting its digestibility. Most size reduction of biomass at the laboratory scale is carried out using a Wiley knife mill with sequential sieving to isolate specific size fractions. Ball milling is also used; however, it is known that ball milling can significantly increase the digestibility of the biomass, adding another variable and reducing the sensitivity of digestion assays by pushing all sample digestion yield curves closer to theoretical maximum [6, 7]. With excessive size reduction, changes in digestibility between samples are more a function of chemical and compositional differences rather than structural variations.

Physical State of the Biomass

Most pretreatment processes result in a wet product, usually a mixture of hydrolysate liquor and solids ranging from 5% to 40% (*w*/*w*) solids. A notable exception is biomass resulting from ammonium fiber expansion (AFEX) pretreatment. AFEX materials leave the reactor already dry due to the evaporation of the ammonia upon pressure release. With biomass solids from other pretreatments, vacuum oven-dried or lyophilized materials can be used to improve the handling and dispensing of the substrates [8]. Several methods have been developed for this purpose, including weighing by hand, punching disks from "biomass paper" (also called "handsheets"), and using commercial powder-dispensing robots [9]. Experimentation with wet (never-dried) pretreated material is preferred, however, as "hornification" of cellulosic materials during the drying process can negatively affect enzymatic conversion [10, 11].

Small-Scale Biomass Distribution

Weighing aliquots of a well-mixed biomass sample or pipetting a known volume of prepared slurry using wide-bore tips or positive-displacement pipettes are common distribution methods. Hand weighing is time consuming, tedious, and prone to human error, whereas slurry pipetting, although simple, is problematic for other reasons. Dilute slurries can be pipetted by hand or automated liquid handlers in either single-channel or multichannel format; however, suspensions must be continually well mixed to prevent settling and there are limits to biomass concentrations that can maintain even distribution. Wide-bore pipette tips are necessary for distributing biomass slurries and tip clogging can still present problems [12]. When pipetting into microtiter plates, care must be taken to ensure adequate distribution between wells, as small changes in concentration can result in large differences in digestion with small sample volumes. Another advantage of biomass distribution by pipetting is that most pretreatments result in a slurry of biomass and hydrolysate liquor. Although few reports have been published regarding pipetting of slurried biomass, our laboratory routinely utilizes this technique in assays of enzyme digestibility.

Positive displacement pipetting is used when the desired final solids loading in the pretreatment or enzyme digestion reaction is too high to allow consistent pipetting by standard means. Biomass solids loadings of up to 40% *w*/*w* can be pipetted by this technique; however, care must be taken to be consistent in the choice of technique employed, as small variations in dispensed volumes can result in significant variations in final biomass loadings.

Biomass Paper

Paper is a very uniform biomass product that is easy to consistently size and aliquot at a variety of scales. Filter paper has long been used as a standard substrate in cellulase and biomass conversion research and one of the earliest reports of an automated biomass assay technique used filter paper as the substrate [13]. Several groups have taken advantage of this property by developing handsheets from biomass in order to divide and evenly distribute the material in small aliquots in preparation for HTP screening. In 2006, Berlin and coworkers reported making paper handsheets from ethanol organosolv pulped yellow poplar and dispensing it into microtiter plates as disks generated using a paper punch, similar to the method by Decker and coworkers for a filter paper-based HTP assay [9, 13]. The assays conducted by Berlin and coworkers were applied to the problem of screening enzyme mixtures on a pretreated substrate and compared to more standard flask-based digestion assays. The results showed good agreement between the two techniques using two different cellulase systems. While resulting in fairly uniform material, the capability of making paper from biomass does not exist in most research laboratories and may be limited to facilities with the required expertise and equipment.

Solids-Dispensing Robotics

The BESC at NREL has taken the approach of utilizing automation to dispense biomass into microtiter plates for automation-friendly assays. Two BESC partners, NREL and the University of California-Riverside (UCR), have adapted solids-dispensing robots to distribute biomass into custom HTP pretreatment reactor plates (see the pretreatment section below). The automation approach is very recent, and while not yet published, we have successfully dispensed corn stover, poplar, alfalfa, and wheat straw in the 3- to 5-mg range with high reproducibility and accuracy. Since these platforms are designed for dispensing very finely divided and uniform powders for the food and pharmaceutical industries, optimization of dispensing methods was required to handle the heterogeneous composition, nonsymmetrical particle size distribution, and small size of biomass samples. At NREL, we have designed and implemented a novel 96-well plate reactor, whereas UCR uses a custom 96-cup reactor (see below).

High-Throughput Biomass Pretreatment

Biomass pretreatment is complicated by factors intrinsic to biomass as well as the temperature, pH, and pressure required for various pretreatment methods. Although bench-scale and larger-scale bulk pretreatments are readily carried out under tightly controlled conditions, the resulting substrate is limited to a single feedstock per run. Tighter parameters, well-defined and highly controlled equipment, and reduced heterogeneity through large sample size minimize the effect of small process variables on the outcome of pretreatment. In contrast, small-scale pretreatments are highly subject to the vagaries of sample heterogeneity and small differences in experimental conditions can lead to significant differences in the results. Also, because heat transfer and other effects are highly scale-dependent, biomass pretreated at the 1-kg scale is not likely to be equivalent to the same biomass pretreated at the milligram scale. We conclude that, in a HTP pretreatment regimen, the results are effectively relative, not absolute, and comparison of results should be made carefully between samples subjected to identical conditions and not between experiments carried out at different scales or under other varied parameters.

In order to study the effect of pretreatment conditions and severities on a large number of disparate biomass samples, a true HTP pretreatment method is required. The physical parameters of elevated temperature and pressure, as well as the chemical consequences of extreme pH, become significantly more important and more difficult to control as the scale is reduced to the HTP level. Uniform heat transfer is critical, as slow and/or uneven heat transfer throughout the biomass can result in samples that have effectively been partially or overly pretreated under varied conditions. In general, large surface area to volume ratio reactors, such as long thin tubes, and high capacity heat transfer media, such as fluidized sand, oil, and live steam, have been used to alleviate some of these limitations. Despite these challenges, a few research groups have attempted to address some of these issues. While some of this work has been presented at various national and international meetings, no reports regarding HTP pretreatment of biomass at elevated temperature, pressure, or extreme pH have yet been published. The general trend reported has been incremental progression from simple parallel reactors, such as glass tubes, high-performance liquid chromatography (HPLC) vials, and small pipe reactors to the use of reactors based on the 96-well plates in the Society for Biomolecular Screening (SBS) footprint that fit in standard liquid and solid robotic dispensers. The custom-designed reactor systems of the BESC represent the first truly HTP pretreatment systems for biomass conversion.

Sealed Glass Reactors

The use of glass as a pretreatment containment vessel offers several advantages as well as disadvantages. Glass is inexpensive, resistant to most pretreatment chemistries, readily scaled to milliliter volumes, and can be sealed by

a variety of means, some automated. It suffers from poor heat transfer as well as limited pressure resistance and general fragility. Regardless, several methods have been developed incorporating glass-container pretreatment.

One of the earliest reports of simultaneous multiple samples pretreatment was by Saeman who reported the use of nested sealed glass tubes to study the dilute sulfuric acid degradation of cellulose at high temperature and pressure [14]. Nested copper pipes held sealed test tubes containing wood and dilute sulfuric acid and were heated by live steam in a rotating chamber. Later, Baugh and McCarty used glass ampoules as reactors to pretreat monosaccharides at high temperatures (170°C to 230°C) under various pH and times [15]. The glass ampoules were filled with 1.0 mL of sugar solution plus acid, purged with helium, flame sealed, and placed in brass pressure vessels which were filled with water and sealed. A set of reactors were heated in an oil bath at the desired reaction temperature. Plunging in ice water completed the pretreatment experiment.

In an attempt to overcome the fragility of glass ampoules and slow heat transfer through the secondary pressure vessel, Chen and coworkers filled 11.0 mm i.d. Pyrex tubes with 0.4 g of biomass and 6.0 mL of dilute sulfuric acid, flame sealed them, and immersed them in hot oil baths [16]. The first oil bath was set 50°C higher than target and after 50 s, the tubes were transferred to a second oil bath at the target temperature, held for various times, and cooled in a cold water bath. The two-stage heating allowed for rapid heating while minimizing temperature overshoot. This method is commonly used in tube-type or pipe-type pretreatment reactors.

In a study by Selig and coworkers, the pressure limitation of 2-mL HPLC vials was mitigated by enclosing the crimped-sealed vials containing feedstock and acid in water-filled Swagelok® 1-in. unions sealed with end caps [17]. Parallel pretreatments under different acid concentrations, temperatures, and reaction times were carried out. Multiple unions were lowered into dual air-fluidized bed sand baths, with the first bath set above the target temperature and the second set at the desired reaction temperature. Thermocouples in some unions were used to monitor the temperature of the reactors. The reactions were quenched by transferring the reactors to an ice water bath. Some losses were experienced, but most of the glass HPLC vials survived the pretreatment and quenching conditions.

Tube and Pipe Reactors

Metallic tube and pipe reactors are significantly more robust than glass and can contain much higher pressures. Depending on the metallurgy, they also resist both acid and alkali thermochemical pretreatment. Due to their high heat conductivity and ability to survive direct contact with the heating medium (usually fluidized sand or live steam), metal reactors are of particular benefit when rapid heating and cooling are required for limited pretreatment volumes. The ready availability of stainless steel pipe fittings and tubing allows for ready customization of the reactors, although these materials are typically unsuitable for extreme pH conditions. Various coatings, such as gold and fluoropolymer, can and have been be used to circumvent this limitation. Some parallel reactor systems are commercially available; however, most have been custom-designed and fabricated in-house.

In the aforementioned HPLC vial study by Selig and coworkers, larger-volume parallel array pretreatments were carried out using gold-plated Swagelok® unions as the pretreatment reactors. Feedstock and 15 mL of acid were loaded into the unions and sealed. Pretreatment and quenching operations were carried out as described above using 2-mL HPLC vials with multiple reactors being heated simultaneously [17]. Montane and coworkers used 1-in. pipe reactors (100 mL) for parallel hot water pretreatments from 180°C to 240°C at solids concentration of 7% (*w*/*w*) to pretreat almond shells [18]. Up to four reactors could be used for pretreatment at constant temperature. Although not truly high throughput, these small-scale reactors offer higher sample throughput than traditional pretreatment such as a Parr reactor.

Parallel arrays of pipe reactors have also been used for pretreatments. Custom-fabricated pipe reactors (12.7 mm× 102 mm) placed in a custom heating block were used in the pretreatment of a number of different biomass samples under the same conditions [19]. The reactors were constructed of Carpenter 20 Cb-3 stainless steel for corrosion resistance and were later gold plated to increase the corrosion resistance. The heating block fit six reactors side-by-side to ensure rapid and uniform heating of all reactors, after which the tubes were removed from the swing open split heating block and quenched in an ice water bath. This parallel array of reactors could test three different biomass samples in duplicate under the same conditions or test a single biomass feedstock under different acid-loading conditions, keeping the temperature and time at temperature constant for the set of reactors.

In 2003, Lloyd and Wyman utilized small pipe reactors (12.5 mm×10 cm) constructed of acid-resistant Hastelloy C-276 in the dilute acid pretreatment of milled corn stover over a wide range of acid concentrations, temperatures, and residence times for modeling the kinetics of dilute acid pretreatment [20]. A large number of these reactors could be fit into an air-fluidized bed sand bath for parallel pretreatment with replicates. In 2006, Michel and coworkers used a commercially available Multiclave® reactor (Autoclave Engineers, Erie, PA, USA) to pretreat up to ten samples simultaneously [21]. The reactor system was

constructed of Hastelloy C-276 to resist the corrosion in dilute acid pretreatments and consisted of ten pipe reactors in a ring that were loaded with biomass and acid and sealed with a special top via O-ring seals. The entire ring of reactors was placed in a large air-fluidized bed sand bath preheated to 220°C to rapidly bring the reactors to near pretreatment temperatures, followed by immersion in a second sand bath at reaction temperature for pretreatment. An ice water plunge was used to stop the pretreatment. In 2009, Ximenes and coworkers also used small pipe reactors for pretreatment coupled with rapid enzymatic hydrolysis and monitoring of sugar production [22]. The small stainless pipe reactors in this study were loaded with 50 mg of biomass and water, sealed, and immersed in an air-fluidized bed sand bath for hot compressed water pretreatment. A throughput of nine parallel reactors per 10 min reaction time was claimed for pretreatment, followed by rapid enzymatic saccharification and enzymatic detection of released sugars.

Microtiter Plate Format Pretreatment

Pretreatment in microtiter plates has the obvious advantage of high sample density and throughput, but faces challenges beyond those above. The key obstacles include uniform distribution of a heterogeneous substrate and pressure and evaporation control of very small pretreatment volumes. True HTP pretreatment depends on the use of robotics and pretreatment reactors designed to the SBS microtiter plate footprint that are essential to the success of the HTP technique. The allure of microtiter plate format pretreatment is quite obvious; massively parallel pretreatment allows for a greatly expanded range of variables to be explored with high statistical validation and the use of SBS standard dimensions facilitates integration with robot plate handlers, automated microtiter plate incubators, pipetting platforms, and plate readers. Some pretreatment chemistries, such as ionic liquid, alkaline peroxide, or lime, may be suitable for standard, commercially available plastic microtiter plates operated at temperatures less than 100°C; however, those chemistries employing elevated temperature and pressure require special consideration.

Few actual pretreatments have been carried out in the microtiter plate format. Zavrel and coworkers utilized plastic 96-well microtiter plates to screen ionic liquids for efficient solvation of lignocellulosics [23]. In this case, solubilization of biomass was determined directly using either scattered or transmitted light measurements in a plate reader. The reader was equipped with interwell metal "fingers" that could control the temperature of the plate from 4°C to 85°C and act as a sealing surface to prevent water uptake by the ionic liquids. Ionic liquids are only just beginning to be considered as a biomass pretreatment method, and in this case, enzymatic digestibility was not determined.

The BESC has invested significant research effort into developing HTP pretreatment and enzyme digestion screening platforms. An initial design by Studer and coworkers [24] at developing a high-temperature, HTP pretreatment system employed a custom-fabricated reactor consisting of 96 individual Hastelloy cups, each individually loaded robotically with 2 to 5 mg of biomass plus water or dilute acid using a Symyx Core Module 1123 robot and inserted into a carrier base plate. The design allowed for each cup to be robotically moved to a high-precision balance where biomass was added and then returned to the base plate, eliminating significant potential for error (Fig. 1a). A gasket placed on top of the array was used to clamp seal the cups using a top and bottom plate (Fig. 1b). The assembled reactor was placed in a custom-fabricated steam chamber, heated to reaction temperature with steam, and cooled in place with water. Both heating and cooling rates were greatly enhanced

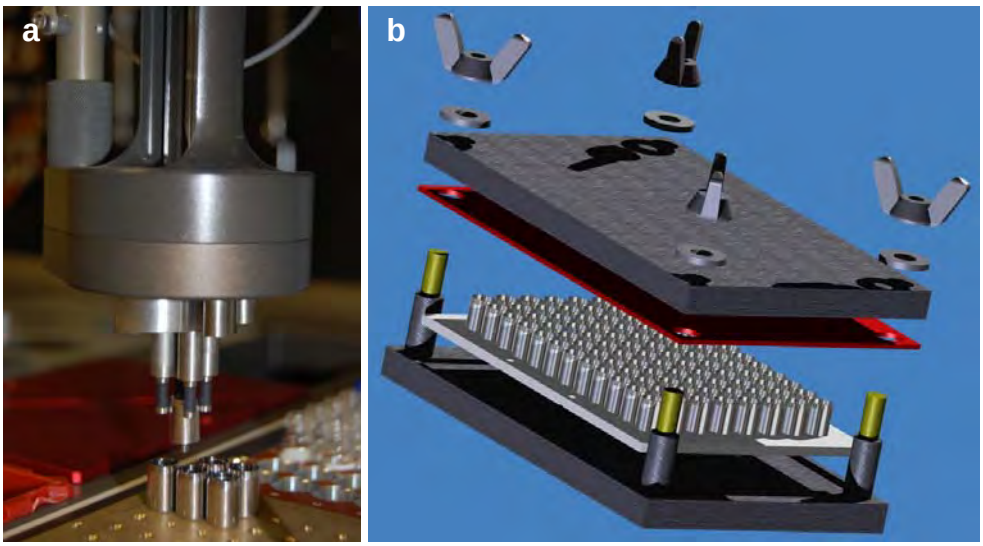

Fig. 1 The UCR HTP pretreatment reactor system: **a** individual cup being moved by robot for automated biomass dispensing and weighing and **b** design drawing of reactor configuration showing clamp-sealing system. The assembled reactor with cups loaded with biomass and catalyst are pretreated in a custom steam chamber

by the 360° access of heat transfer medium (live steam for heating or cold water for cooling) to the wells.

A more standard microtiter plate format pretreatment reactor was developed in the BESC in parallel with the work above. Utilizing custom-fabricated aluminum or Hastelloy® C-22 96-well reactor plates based on the SBS footprint, researchers at the NREL developed a stackable plate format capable of simultaneously pretreating 1,920 biomass samples [25]. As in some of the tube reactors above, a gold coating was used on the aluminum reactors to assist in corrosion resistance. Other coatings used included polytetrafluroethylene-impregnated nickel and Teflon® PFA (a perfluoroalkoxy copolymer resin). Though significantly more time consuming and expensive to manufacture, Hastelloy® plates were subsequently designed for use in dilute acid pretreatments due to their inherent acid resistance.

The overall format of the NREL reactor plate design was held constant, consisting of 96 wells in a standard SBS format, each surrounded by four steam channels bored through the plate (Fig. 2a). The steam channels are contiguous through stacked reactors and allow for even and rapid introduction of live steam or cooling water to all wells in the plates through an upflow configuration. The SBS standard format allowed for the reactor plates to be readily integrated into both solid-handling and liquid-handling robotic platforms, while the steam channels allowed stacked plates to be uniformly heated and cooled during pretreatment. The plates were loaded with 5 mg of milled biomass feedstock using a Symyx MTM Powdernium® solids-dispensing robot. Water or acid is added to each well with a Beckman FX robot, and the plates are sealed using adhesive-backed aluminum foil. Teflon® gaskets are inserted between the plates, and up to 20 plates are stacked and clamped using a custom-fabricated clamping system. A specially fabricated holder is used to hold the stacked plates in a 2-gal Parr reactor (model 4550, Parr Instrument Company, Moline, IL, USA). Steam is directed via a pipe to the bottom of the Parr reactor and up through the channels machined in the plate stack and clamping system, with air vented from the top of the Parr reactor head plate during the initial steaming of the reactor. At the end of pretreatment, cooling water is forced through the channels in the plates and clamping system. Although the plate reactors are custom-built to an in-house design, the liquid handling, incubation, and measurement are carried out in standard, commercially available robotic platforms. The use of the aluminum foil seal provides significant advantages in evaporation control, liquid exchange with the heating/cooling media, and condensation handling through centrifugation, although clean up of the reactors necessitated by the high-temperature effects on the acrylic adhesive of the seal is not trivial.

By design, both the NREL and UCR HTP pretreatment reactors were also used for subsequent enzyme digestion, as the SBS well spacing allowed for ease of pipetting with standard equipment. To circumvent the issues of low pH affecting enzyme activity, the reaction volumes of each well were neutralized with buffer prior to enzyme digestion. This "cohydrolysis" concept is critical to the success of these reactors, as washing the biomass or doing a liquid/solid separation at this level creates significant problems in maintaining mass balance.

Enzymatic Saccharification at the Small Scale

Enzymatic conversion of biomass to free sugars and other components is a complex and only partially understood system. Substrate heterogeneity and recalcitrance, the complex and sensitive enzyme synergies, biomass alterations induced by varied pretreatment chemistries and severities, and complications of handling these materials

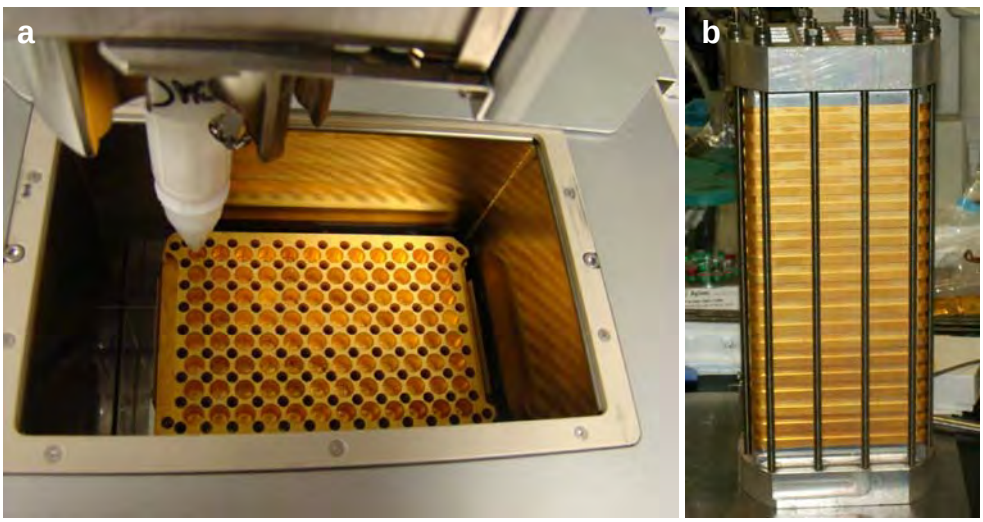

Fig. 2 The NREL HTP pretreatment reactor system: **a** single reactor plate showing wells and steam ports and **b** assembled stack of 20 reactor plates (1,920 samples). The stack is held by a custom clamping system and pretreated in a modified 2-gal Parr reactor with steam and cooling water introduced through the reactor plate ports in an upflow configuration

are all factors which must be considered. As one can surmise from the highly factorial variables given above, the possible permutations in any given biomass conversion experiment can be enormous and meeting this challenge requires automation and scale reduction.

"Shake flask" experiments have been the standard enzymatic research saccharification scale for decades; however, this method is materially expensive, organizationally complex, and often criticized for not accurately representing saccharification on an industrial process scale [26]. The latter issue undoubtedly applies to any HTP techniques, although conservation of materials (biomass and enzymes), efficient time and space utilization, and numerous error-propagating technical barriers associated with manipulating insoluble substrates has driven the need for higher throughput and more accurate bench-scale saccharification methods. This problem has been further emphasized by the recent increased interest in lignocelluloses as a viable renewable fuel feedstock elevating the demand for greater flexibility and complexity in experimental design.

Enzyme Digestion

While much of the enzymatic saccharification work is still carried out using a single, overoptimal loading of commercial *T. reesei* cellulase [27], there has been a growing interest in how xylanases and other noncellulolytic hemicellulose degrading "accessory" enzymes affect the type of pretreatment needed and the synergy between pretreatment chemistry, severity, and enzyme cocktail composition [8, 28, 29]. To better understand the complexity of these substrates and the enzymatic systems that degrade them, experiments involving hundreds of saccharifications on identical substrates are needed. From multiple enzyme loading curves to matrices of commercial and purified enzyme activities, this growing expanse of experimentation has necessitated scaled-down protocols to facilitate orders of magnitude increases in the number of conditions that can be run with limited materials [9].

Scaling the enzyme digestion work to milliliter or smaller volumes allows for several advantages. Enzymes and pretreated biomass, both expensive or time consuming to produce, can be greatly reduced. The other advantages include minimizing bench and incubator space, the application of automation for increased throughput, and the general increase in the number of experiments carried out. The scaled-down "scintillation vial" method originally developed by Brown and Torget at the NREL and later modified by Selig, Weiss, and Ji at NREL was an early step in minimizing materials usage (e.g., 100 mg cellulose loaded per saccharification in 10 mL) and laboratory space needed to conduct experiments on lignocellulosic materials [30].

Many studies over the past decade have made tenfold or even 100-fold reductions in further scaling down this methodology into HPLC vials, Eppendorf tubes, and 96-well microtiter plates [9, 12, 31, 32]. Whereas many of these improvements reduced the materials and time needed to conduct complex experiments, the scale down in size presents its own challenges by often magnifying the attention to detail that must be made to reduce error related to a number of critical factors associated with the need for accurate and homogeneous distribution of cellulosic/lignocellulosic materials into small reaction vessels [12].

To date, the smallest scale and most systematic format utilized for saccharification work has been the 96-well microtiter plate (~330 μL per well). The first successful attempt at applying this scale to biomass hydrolysis was the adaptation of the IUPAC standard filter paper assay for measuring cellulase activity to a 96-well plate format [13, 33]. This posed challenges with respect to evaporative losses during the method's incubation period which were later resolved by Xiao and coworkers by the use of a polymerase chain reaction (PCR) temperature cycler [34]. While work to apply this format to lignocellulosic saccharifications had been met with some success at a number of commercial enzyme companies as well as at NREL, the first publication of such work came from Berlin and coworkers who overcame issues associated with accurate and efficient loading of lignocelluloses into plates by punching uniform disks of biomass from handsheets made of the same material [9]. A more widely usable method was later reported by Chundawat and coworkers who used liquid-dispensing robotics to transfer slurries of pretreated biomass into plate wells [12]. With all of these methods, analysis of sugars released during saccharification were usually assessed by chemical assays which, although much higher throughput, were less specific and accurate than HPLC determinations. Methods enabling HTP sugar analysis are discussed in the following section.

Solids Loading in Enzyme Digestion

To date, most laboratory-scale saccharification work has been performed at dry solids loadings below 5% (*w*/*w*) and more typically around 1% (*w*/*w*). At the same time, those focused on bringing laboratory-tested lignocellulosic conversion processes to the industrial scale have continuously pointed to the economic necessity for running saccharification processes at solids loadings above 20% (*w*/*w*) [35–37]. Mohagheghi and coworkers first understood the necessity for high-solids saccharification research, although there are to date only a handful groups which have addressed this issue. Furthermore, no one has scaled this work to levels which would accommodate the complex enzyme arrays facilitated by the above-mentioned HTP methods [38].

Currently, at NREL, we have successfully deployed free-fall mixing strategies utilized at the 250-mL scale by Jorgensen and coworkers to development of an HPLC vial-scale high-solids saccharification system [36]. Although much of the current saccharification research may continue for some time at low solids in microplate or small-vial formats, there will be an inevitable shift towards high-solids work to accommodate industries needs.

High-Throughput Sugar Analysis

Considerations in choosing a system for carbohydrate analysis for HTP methods include the overall throughput requirement of the assay, scale of the reaction, ease of sample preparation, and the required specificity of the analysis. The difficulty in detecting the sugar of interest among other components of the mixture may also need to be considered. This is particularly critical when the sugar is at a lower concentration compared to other components present in complex mixtures. Depending on which criteria are critical, many different methods of sugar detection are available, such as chemical-reducing sugar, enzyme-linked, and instrumentation-based methods. The advantages and disadvantages of these methods are detailed below and summarized in Table 1.

Chemical Methods

Generally, chemical methods are easily applied to HTP applications. They are usually quick, inexpensive, require low-user intervention, and scale relatively well to microtiter plate-type formats. There are, however, several drawbacks that must be addressed, primarily lack of specificity and elevated assay temperatures. The various methods applicable to HTP sugar analysis are reviewed below.

Dinitrosalicylic acid (DNS) is widely used for the quantitative determination of reducing sugars [39]. This method tests for the presence of the free carbonyl group (C=O), the so-called reducing sugars. Upon oxidation of the sugar, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions [40]. It is suspected that there are many side reactions and the actual reaction stoichiometry is more complicated than that previously described. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. The DNS method is inexpensive and simple; however, due to low sugar specificity, careful controls and replicate samples are required [41]. An additional complication of the DNS assay is the high temperature and time dependency of color development; 5 min at 100°C. Small volumes typical of HTP assays are particularly subject to increased error due to substantial evaporation during this step. Slight variations in time can lead to significant variation in color development.

The bicinchoninic acid (BCA) assay is more sensitive than the DNS method, often requiring higher dilutions to measure sugar release from biomass. The BCA method uses the reducing ends of the sugar to reduce Cu^{2+} to Cu^{1+} at elevated temperature. The assay chemistry involves two molecules of BCA chelating each Cu^{1+} ion, forming a complex that absorbs strongly at 562 nm [42]. Proteins also react with the BCA solution and reduce Cu^{2+} to Cu^{1+}. If the sugar solution also contains any protein or other reducing compounds, proper controls must be included to account for the protein content of the samples [43]. Though the temperature of the assay is 80°C, not the 100°C of the DNS assay, the incubation time is longer (up to 30 min). As this assay is more sensitive than the DNS method, higher dilution rates and/or smaller sample aliquots are often used to measure sugar release from biomass and this can result in higher errors of measurement.

Table 1 Chemical methods for quantifying reducing sugars in HTP screening of lignocellulosics conversion

Assay chemistry	Temp (°C)	Time (min)	Sugar sensitivity	Reactions with protein	Other
DNS	100	5	Low	Low	Side reactions are numerous and must be accounted for
					Hazardous waste (phenol, caustic) generated
					High temperature/time-dependent color development
BCA	80	20–30	High	High	Sensitive to other Cu reducing compounds
					Short shelf life of reagents
					Very sensitive, high dilutions needed
Nelson–Somogyi	100	15–20	High	High	Sensitive to other Cu reducing compounds
					Stable color development
MBTH	80	15	High	Very Low	Oligomers give a higher response than monomers

The Nelson–Somogyi reducing sugar detection method uses copper and arsenomolybdate reagents. Absorbance at 660 nm is proportional to the reducing sugar and is stable over long periods of time [44]. However, since it primarily relies on the reduction of copper, it shares similar problems with the BCA method.

A more recent spectrophotometric method for quantifying reducing sugars with high sensitivity uses 3-methyl-2-benzothiazolinonehydrazone (MBTH). One advantage of this method is that is largely free from interference by protein. Generally, in this reaction, an aldehyde combines with two molecules of MBTH in a two-step process. The first step, which occurs at neutral pH, has the aldehyde condensing with a single MBTH molecule to form an adduct. During the second step, which occurs under acid and oxidizing conditions, this adduct reacts with a second MBTH to form a highly colored final product which absorbs at 620 nm [45]. Similar to the BCA assay, temperatures of 80°C are used for the color development, though for a shorter time (15 min). The sensitivity of the assay may require dilution of the samples. Oligomers give a higher response than monomers and samples containing high levels of oligomeric carbohydrates, such as low-severity pretreated biomass hydrolysates or enzyme digestions low in β-glucosidase/xylosidase activity, may overestimate the extent of conversion.

For all of the chemical methods, at the test tube scale, equal heating and cooling rates can be easily achieved in water and ice baths. When using microtiter plates, however, the heating method is critical and uneven heating and edge effects can lead to dramatic differences in color development across the plate. Inconsistent or improper sealing of wells will also lead to variable evaporation across the plate. As the assay solutions must often be diluted (typically 5- to 20-fold) for accurate readings, these effects can be magnified significantly. Evaporation losses can be mitigated through proper sealing. The best seals we have found are heat seals incorporating an aluminum foil layer, which minimizes evaporation, but can be difficult to pierce for sampling. The use of a PCR machine to heat and cool the plate can also mitigate even heating as well as evaporation issues. The advantages of the PCR machine are that evaporation and heating rate and uniformity are tightly controlled and the plate can be rapidly cooled to stop the color development; however, they are expensive and integration into an automated platform requires substantial hardware and software changes.

Enzyme-Linked Assays

Compared to reducing end methods, enzyme-linked assays are more specific for a single sugar such as glucose or xylose. Some cross-reactivity can occur, specifically with oligomers of the target sugar, though with decreasing sensitivity with increased chain length. Glucose is often assayed by the glucose oxidase/peroxidase (GOPOD) assay. Glucose oxidase converts glucose to gluconic acid and H_2O_2 and peroxidase uses the H_2O_2 to oxidize *O*-dianisidine which is then detected colorimetrically at 510 nm [46]. Xylose quantitation utilizes xylose dehydrogenase which oxidizes xylose to xylonic acid with the concurrent reduction of NAD+ to NADH. The increase in NADH can be measured directly by absorbance at 340 nm or indirectly after the NAD-dependent reduction of phenazine methosulfate monitored by absorbance increase at 585 nm [47]. Similar assays can be carried out for galactose and cellobiose using galactose dehydrogenase and cellobiose dehydrogenase.

Alternative methods for enzyme-linked assays involve using hexokinase and glucose-6-phosphate dehydrogenase methods. In this method, glucose is phosphorylated by hexokinase to produce glucose-6-phosphate which then reduces NAD+ to NADH which is detected at 340 nm [48, 49]. Hexokinase also reacts with other sugars such as fructose, mannose, and glucosamine. Subsequent conversion of the phosphorylated products to glucose-6-phosphate through isomerase and/or deamidase activity can be used to quantify these sugars. Enzyme-linked assays have advantages over chemical-reducing sugar assays including single sugar specificity and a general lack of interference from other compounds. With the notable exception of cellobiose, most are also available commercially in kit form. Their primary disadvantages are that they are readily available for only a few sugars, the high cost of the enzymes and reagents, and their relative instability.

Enzyme Electrodes

Amperometric methods have existed for some time for rapid detection of glucose levels in a clinical setting. These assays utilize similar chemistry to the enzyme-linked assays; however, detection is through electron transfer to an electrode (amperometric) instead of colorimetric detection. Amperometric glucose electrodes based on glucose oxidase undergo several chemical or electrochemical steps which produce a measurable current that is related to the glucose concentration. Many methods have been developed to immobilize glucose oxidase on various electrodes including adsorption, gel–polymer entrapment, and covalent attachment. Direct physical adsorption of the enzyme on the electrode requires no reagents and has a fast response due to minimal diffusional limitations, but the electrode is susceptible to a change of environment which may damage or degrade the adsorbed enzyme. Gel–polymer entrapment uses mild reaction conditions and a wide variety of electron transfer-mediating polymers to entrap the

enzyme in a thin, three-dimensional matrix. However, gel–polymers suffer from several disadvantages. Many experimental factors must be controlled, possible deactivation of enzyme occurs during polymer formation, and enzyme size is limited by the size and structure of the polymer. There is also an increase in electrode response time due to a large diffusional barrier created by the gel–polymer [50]. To obtain a fast response, one method is to form an ultrathin polymer film at the electrode surface, while the other is to use a mediator [51]. Alternatively, the peroxide formed by glucose oxidase can be detected by immobilized peroxidase [52]. For biomass conversion, the most relevant sugars are glucose, cellobiose, and xylose. Smolander and coworkers have reported using a covalently immobilized aldose dehydrogenase electrode for xylose and glucose detection [53]. Several groups have reported the immobilization of cellobiohydrolase on electrodes for the quantitation of cellobiose [54–56]. Reports of xylose quantitation via amperometric detection are very limited, though the commercial availability of a xylose dehydrogenase-based xylose quantitation kit suggests that it should be possible. One commercial analyzer from Yellow Springs Instruments uses a membrane kit for measuring xylose.

Instrument Methods

Unlike the methods discussed above, instrument-based sugar quantitation offers high-precision measurement of multiple sugar species, however, at the cost of speed and ease of assaying. Some methods, such as gas chromatography, require substantial sample processing and derivatization, which can add time and error to the measurement. The key problems with liquid chromatography methods for sugar analysis include complex sample preparation, such as filtering and neutralization and run times on the order of 20 min or more per sample [57]. For example, under these conditions, a single microtiter plate would take 2 days to analyze. These drawbacks make instrument methods less than ideal for high throughput; however, they can be invaluable as more detailed second-tier analysis of interesting samples. Also, as modern methods and materials push the pressure limits of HPLC to 15,000 psi and higher, chromatography run times can be reduced to a few minutes or seconds, holding out the promise of highly specific and rapid product quantitation.

Several chromatography column configurations are available for analysis of sugars on HPLC systems, offering high resolution and sensitive detection of multiple sugars. Detection is usually via refractive index (RI) or pulsed amperometric detection (PAD) [57] under aqueous mobile phase conditions. The RI detectors provide good peak resolution and chromatographic stability, but have higher detection limits compared to PAD that, while having superior low-level detection performance, suffer from poor chromatographic peak resolution [58, 59]. Often lead-, calcium-, or hydrogen-form ion exchange columns (ion moderated partition chromatography) are used for sugar analysis. Whereas early versions of these systems may have suffered from poor stability or insufficient resolution, the use of modern column-packing resins has generally solved these problems, resulting in highly stable and robust columns [59]. There are also examples of amine-based columns available for sugar analysis; however, these columns require an organic-phase gradient elution which necessitates an alternative detection method, typically evaporative light scattering [60]. Although these systems are faster than traditional methods, with run times of 10–15 min, this time requirement is still too long to attain true HTP sample numbers. Modern ultrapressure liquid chromatography (UPLC) systems, which utilize smaller packing resins and pressures upwards of 15,000 psi, have the potential to reduce these run times to a few minutes or less; however, no commercial UPLC columns for sugar separations exist today.

Gas chromatography–mass spectrometry (GC-MS) has long been the method of choice for detecting trace amounts of carbohydrates. As sugars are nonvolatile, monosaccharide analysis by GC requires derivatization of the sugar to increase their volatility and decrease interaction with the analytical system. There are several well-established derivatization methods; the most common methods are the alditol acetate [61] and trimethylsilyl procedures [62]. The aldononitrile acetate and trifluoroacetate procedures are also commonly utilized [63].

Basic gas chromatographic analysis of sugars is commonly performed with a universal flame ionization detector while standard LC methods often employ RI or PAD. These detection methods, while sensitive, lack the specificity of MS or tandem mass spectrometry (MS-MS) [64]. More recently, the increasing popularity and availability of tandem mass spectroscopy (MS-MS) has encouraged the use of GC-MS-MS for improved specificity in trace analysis. There have also been ongoing developments in high-performance liquid chromatography–mass spectrometry (LC-MS) and MS-MS of underivatized carbohydrates. These LC-based methods have the advantage of dramatically simplified sample preparation without the derivatization required by GC. It should be recognized that LC-MS and MS-MS profiling of carbohydrate monomers in complex mixtures is still nontrivial to perform, and LC-MS and MS-MS analyses exhibit poor sensitivity compared to GC-MS or GC-MS-MS, especially in complex samples [64].

Capillary electrophoresis (CE), when combined with electrochemical detection at copper electrodes, has been shown to provide a simple and sensitive method for the direct analysis of samples containing a wide range of carbohydrate compounds including simple sugars, sugar acids, and alditols.

Typically, both the separation and the detection require the use of a strongly alkaline medium whose hydroxide content can be varied to optimize the migration times of samples and the level of resolution obtained. Detection using copper electrodes consists of direct oxidation that requires no derivatization of the sugars and yields detection limits at or below the femtomole level for most of the carbohydrate species [65]. Alternatively, fluorescence CE can used to detect and quantify fluorotagged sugars from a variety of biomass conversion processes. In at least one case, this technique was applied by modifying a 96-channel DNA sequencer in order to elevate the throughput to handle thousands of samples per day. Although very fast and, when utilized in a 96-channel instrument, very high throughput, detection is still limited to fluorescence tagging and separation of all sugar components is not ideal [66, 67].

Summary

Whereas HTP methods have rapidly expanded in the pharmaceutical, enzyme discovery, and combinatorial chemistry fields, biomass conversion research faces unique and difficult challenges in adopting these powerful methods. Only recently have researchers begun to truly scale down the pretreatment process to a format that is readily integrated into standard HTP robotics platforms. These new technologies, while still in development, hold great promise for rapidly accelerating research progress in biomass conversion, specifically in the areas of feedstock susceptibility to pretreatment and enzyme digestion combinations as well as screening huge libraries of environmental and genetic feedstock variants for relative digestibility traits.

As these developments are expanded and refined, great care must be taken in both the design of the experiments and the interpretation of the results. Nowhere is the HTP axiom "You get what you screen for" more applicable than in biomass conversion. The sample heterogeneity, necessary size reduction, distribution, and physical treatment all contribute to variability in the assay and may limit the usefulness of assay results. The array of pretreatment chemistries and severities, as well as the variety and synergy of enzyme activities available are also critical. Elevated reaction temperatures and long incubation times will affect the quality of the results as well. Product detection is a balance of sensitivity, selectivity, and speed. The final consideration to keep in mind is that the results of HTP assays are always relative within a tightly defined experimental space and are not always directly comparable to similar experiments run at larger scale. These complications present some daunting obstacles to the biomass researcher and perhaps even greater challenges to the research director, process engineer, or program manager trying to understand and apply the results of such studies.

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