Video Article

High-throughput Screening of Recalcitrance Variations in Lignocellulosic Biomass: Total Lignin, Lignin Monomers, and Enzymatic Sugar Release

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Abstract

The conversion of lignocellulosic biomass to fuels, chemicals, and other commodities has been explored as one possible pathway toward reductions in the use of non-renewable energy sources. In order to identify which plants, out of a diverse pool, have the desired chemical traits for downstream applications, attributes, such as cellulose and lignin content, or monomeric sugar release following an enzymatic saccharification, must be compared. The experimental and data analysis protocols of the standard methods of analysis can be time-consuming, thereby limiting the number of samples that can be measured. High-throughput (HTP) methods alleviate the shortcomings of the standard methods, and permit the rapid screening of available samples to isolate those possessing the desired traits. This study illustrates the HTP sugar release and pyrolysis-molecular beam mass spectrometry pipelines employed at the National Renewable Energy Lab. These pipelines have enabled the efficient assessment of thousands of plants while decreasing experimental time and costs through reductions in labor and consumables.

Video Link

The video component of this article can be found at http://www.jove.com/video/53163/

Introduction

As the global supply of non-renewable fuels and their associated products declines, scientists have been challenged to create similar fuels and chemicals from plant-derived sources[1]. A key aspect of this work is determining which species of plants may be suitable for the production of biofuels and biomaterials[2,3]. Typically, these feedstocks are evaluated for lignin, cellulose, and hemicellulose content; as well as their susceptibility to deconstruction (recalcitrance) through thermal, mechanical, and/or chemical pretreatment with or without subsequent enzyme saccharification. More detailed analyses are used to determine the specific composition of the lignin and hemicellulose fractions as well as optimal enzyme activities needed. Transgenic modifications of plants that do not intrinsically possess ideal traits for biochemical or thermochemical conversion to desired commodities have provided researchers with a greatly expanded source of potential feedstocks[4]. The standard analytical methods for quantifying the chemical traits of a plant, while quite useful for small sample sets, are unsuited for the rapid screening of hundreds or thousands of samples[5-7]. The HTP methods described herein have been developed to rapidly and efficiently evaluate large numbers of biomass variants for changes in cell wall recalcitrance to thermochemical and/or enzymatic degradation.

It is critical to understand that the HTP screening assays described herein have not been designed to maximize conversion or yield. The objective is to determine relative differences in the intrinsic recalcitrance of related biomass samples. As a result, many of the analysis steps differ from the “typical” biomass conversion assays, where the objective is to obtain maximum conversion rate or extent. For example, lower pretreatment severities and shorter enzyme hydrolysis times are used to maximize differences between samples. In most cases, relatively high enzyme loadings are used to reduce differences due to experimental variation in enzyme activity, which could skew the results significantly.

Rapid techniques for determining the composition of plant cell-walls and the monomeric sugars liberated following enzymatic saccharification include robotics, customized, thermochemically compatible 96-well plates, and modifications of standard laboratory methods[8-11] and instrumental protocols, such as vibrational spectroscopy (infrared (IR), near-infrared (NIR), or Raman) and nuclear magnetic resonance (NMR)[12-17]. These methodologies are key to isolating feedstocks with high cellulose or low lignin contents, or those expected to yield the highest glucose, xylose, ethanol, *etc.* These methods have enabled downscaled analyses that employ smaller quantities of biomass and consumables, leading to reductions in experimental expense[18]. Another feature of this methodological approach is that various experimental conditions can be rapidly, and in some cases simultaneously, evaluated. For example, a variety of different pretreatment strategies or enzyme cocktails can be tested, allowing the most optimal experimental parameters to be quickly identified and employed. Popular feedstocks, such as corn stover[9], poplar[8,10], sugarcane bagasse[8], and switchgrass[8] have been successfully evaluated using these HTP methods.

Total lignin and lignin monomeric composition are also commonly quantified biomass traits. Reductions in lignin content have been shown to increase the enzymatic digestibility of polysaccharides[19,20]. The role that the lignin monomeric ratio (often reported as syringyl/guaiacyl (S/G) content) plays in the deconstruction of the plant cell wall is still under investigation. Some reports have indicated that reductions in the S/G ratio led to increased glucose yields following hydrolysis[21], while other studies unveil the opposite trend[19,22]. High throughput methods for evaluating lignin and its monomers include vibrational spectroscopy (IR, NIR, and Raman[23-26]) coupled with multivariate analysis, and pyrolysis molecular beam mass spectrometry (pyMBMS)[27,28].

When developing HTP methods for screening biomass, several integral considerations need to be kept in mind. One key aspect is the complexity of the method. What is the required skill level for the technique? Chemometric analyses, for example, require specific skills for constructing, evaluating, and maintaining predictive models. The standard methods exhibit undesirable preparatory or data analysis steps or employ toxic reagents. Development of the models is an ongoing process where new data is incorporated into the model over time to increase the model's robustness. Another consideration is the cost-savings and decreased experimental analysis times of the proposed high-throughput methods. If the method is quite rapid, but very costly, it may not be a feasible technique for many labs to adopt. The methods illustrated in this manuscript are variants of standardized techniques, modified to amplify the throughput capabilities. These protocols quantitatively measure the biomass traits of interest without necessitating the development of predictive models. This is a key attribute of these techniques, since predictive methods, while exhibiting strong correlations with the standard analyses used to develop the models, are not as accurate as actually measuring the quantity of interest for the samples. Whereas the methods used are essentially scaled down versions of standard bench-scale analytical methods, accuracy and precision are traded for speed and throughput. Mostly, this outcome is due to higher errors in small volume pipetting and weighing; as well as increased sample heterogeneity as sample size is decreased. While large sample sets can be screened and compared, great care must be exercised when making comparisons between separate campaigns and to bench-scale results.

The most time-consuming steps involve the physical manipulation of the biomass. Grinding samples may take several min per sample, including cleaning out the mill between samples. Manually loading, unloading, and cleaning hoppers and filling and emptying tea bags and sample bags is also very labor intensive. While each step may take a minute or more, doing thousands of samples may take many hours or even days. The robots can load a typical reactor plate with biomass in about 3 to 4 hr or 6 to 8 plates day^{-1} $robot^{-1}$. This situation depends on the precision parameters used as well as the type and amount of biomass to be tested. Filling reactor plates with water, dilute acid, or enzyme is quickly done using a liquid handling robot. Pretreatment of a plate stack (1 to 20 reactor plates) takes between 1 and 3 hr when assembly, cool down, and disassembly is included. Enzyme hydrolysis takes 3 days and the sugar analysis requires about 1 hr of prep time plus 10 min per reactor plate to complete the assay and read the results. A weekly schedule of set pretreatment and analysis days accommodates a reasonable work schedule, minimizing odd-hour and weekend efforts for the human component of the assay and allows for processing ~800 to 1,000 samples per week on an ongoing basis. The maximum throughput depends on several factors, mainly how much hardware (robots, reactors plates, *etc.*) and how much "software" (*i.e.*, staffing) are available to do the manual work. The practical upper limit is 2,500 to 3,000 samples/week; however, that output requires 7 day-a-week operation and multiple student interns and technicians. In comparison, 3,000 samples by HPLC would require approximately 125 days of sample analysis plus the additional labor of manually weighing samples into reactors and filtering samples prior to analysis.

Protocol

1. High-throughput Determination of Glucose and Xylose Yields Following Enzymatic Saccharification[9,29]

1. Sample Preparation (Grinding, De-starching, Extraction, Pretreatment)
    1. Grind at least 300 mg of each biomass sample using a Wiley mill, such that the particles pass through a 20 mesh (850 µm) screen. Transfer to anti-static zip-top bags (typically bar-coded) and record sample information to the barcode database.
    2. Add approximately 250 mg or more of the ground biomass from anti-static bag to a numbered (with pencil, do not use ink or marker) tea-bag, carefully roll up the teabags, being sure to fold the ends over the biomass to prevent loss during de-starching and extraction.
    3. Wrap the teabag closed using tin-coated copper wire. Record the teabag number for each barcoded sample.
    4. Prepare de-starching enzyme solution from commercial enzymes (typically, 0.25% (v/v) glucoamylase (~1,600 AGU/L) and 1.5% (v/v) alpha-amylase (~2,900 KNU-S/L) in 0.1 M sodium acetate (pH 5.0)). Prepare 16 ml per g of bulk biomass or 500 ml per 120 teabag samples. Note: Loadings capable of removing starch from ground biomass should be determined empirically by testing for starch after digestion with a range of enzyme loadings and ratios.
    5. In a plastic container, add 16 ml of de-starching enzyme solution per 1 g of bulk ground biomass. For a batch de-starching protocol, add 120 teabags to 500 ml of the buffer-enzyme solution.
    6. Incubate in a shaker at 55 °C for 24 (± 4) hr, at 120 rpm to remove possible starch.
    7. After the de-starching incubation, rinse and soak the biomass in several liters of deionized water for 30 min. Repeat this process two additional times to remove buffer salts that can cause bumping (formation of gas bubbles in the solution that can result in a sudden and violent rise in the level of the solution, causing hot liquid ethanol to cascade out of the reaction vessel) during the extraction.
    8. Following the exhaustive rinsing of the de-starched biomass, place the teabags into the Soxhlet column for extraction. No thimble is required for this step.
    9. Set up the Soxhlet reflux, using 95% ethanol and extract the samples for 24 hr.
    10. Remove the teabags from the Soxhlet reactor and spread out in a single layer on a flat tray.
    11. Allow the samples to dry overnight at room temperature in a fume hood.
    12. Unroll the teabags and return the dried biomass to original barcoded anti-static bags.
        Note: The anti-static bags are efficient at reducing static electricity in the biomass, improving the handling characteristics. If other storage options are used, additional biomass may be needed due to the biomass clinging to the side of the storage container.
    13. Transfer at least 50 mg of the dried biomass to a barcoded hopper (using more material is better for accurate dispensing). Scan barcodes of anti-static bags and the receiving hopper to ensure accurate sample tracking.

14. Load hoppers onto solids weighing robot, paying close attention to the order of the samples in the racks. Load enough reactor plates to contain all the samples and select the dispensing protocol based on the number of plates used.
15. Robotically weigh 5 mg of the dried samples (± 0.3 mg) into acid-resistant stainless steel 96-well plates. Weigh out 3 replicates of each sample distributed around the plate to minimize any localized variations.
16. Include 8 control biomass samples of a well-characterized biomass standard material in order to track assay performance. For multiple plates, the use of multiple standard biomass hoppers will minimize particle size drift caused by sieving in the hoppers over repeated dispensing cycles.
    Note: In each plate, there should be 24 samples with 3 replicates, 4 blanks consisting of deionized water and enzyme, and 8 controls, using the previously characterized standard biomass. The 3 corner wells of each of the 4 corners of the plate are reserved for the sugar standards.
17. Check blank and sugar standard wells for errant biomass particles and remove if present.
18. Add 250 µl of deionized water to each well, and seal the plates with silicone adhesive-backed polytetrafluoroethylene (PTFE) tape.
19. Using a 1/8" soldering iron tip, pierce the PTFE tape at each steam port (117 total) for each plate. Use an empty plate stacked on sealed reactor plate as a guide and to restrict PTFE film movement.
20. Clamp the plates tightly with 0.031" thick glass-reinforced PTFE gaskets (pre-punched with holes for steam ports) between plates and empty plates on top and bottom of stack. Pretreat the samples using a steam reactor set to 180 °C for 17.5 min, or other temperature/time combinations based on desired severity.
21. Cool reactor plates to 50 °C by flooding with deionized water.

2. Enzymatic Saccharification
    1. Prepare an enzymatic saccharification solution consisting of 8% (v/v) enzyme solution in 1.0 M sodium citrate, pH 5.0. Prepare 5 ml per reactor plate. Note: Dilution required should be determined based on activity and protein content of the specific stock enzyme solution.
    2. When the plates are cool, centrifuge them in a swinging bucket rotor at 1,500 x g for 20 min. Remove sealing film. Note: These plates are heavy and the centrifuge specifications should be checked for compatibility.
    3. Add 40 µl of the 8% enzyme stock solution to each well (70 mg enzyme per g biomass).
    4. Reseal with new PTFE tape. Place sealed plate into magnetic plate clamp.
    5. Gently mix the samples by inversion (at least 15 times), and incubate at 50 °C for 70 hr.
    6. When the saccharification has concluded, mix by inversion and centrifuge the plates at 1,500 x g for 20 min.

3. Sugar Assay
    1. Prepare a set of 6 glucose and xylose combined calibration standards in 0.014 M citrate buffer (pH 5.0) ranging from 0 to 0.750 mg/ml (0, 0.2, 0.3, 0.45, 0.65, and 0.75 mg/ml recommended) and 0 to 0.600 mg/ml (0, 0.1, 0.2, 0.35, 0.45, and 0.6 mg/ml recommended) for glucose and xylose, respectively.
    2. Prepare the glucose oxidase/peroxidase (GOPOD) and xylose dehydrogenase (XDH) reagents according to instructions in the kit.
    3. Using a pipette, remove 200 µl of liquid from the 4 corner wells and edge wells adjacent to the corner wells (12 total wells) of the reactor plate.
    4. Using a pipette, dispense 180 µl deionized water to each well of a 96-well polystyrene flat-bottom dilution plate. Do not add water to the 12 sugar standard and corner wells (remove those tips if using a 96-channel head).
    5. Using a pipette, dispense 180 µl GOPOD reagent to each well of the glucose assay plate.
    6. Using a pipette, dispense 180 µl XDH reagent to each well of the xylose assay plate.
    7. Using a pipette, transfer 20 µl hydrolysate aliquots from the 96-well reactor plate to the dilution plate. Pipet from the upper section of the wells to avoid biomass solids and residual liquid in corner wells. Mix by trituration for at least 10 cycles.
    8. Using a pipette, transfer 110 µl of sugar standards, in duplicate, to the corner wells of the dilution plate.
    9. Using a pipette, transfer 20 µl aliquots from the dilution plate to the glucose and xylose assay plates. Mix by trituration.
    10. Incubate the glucose and xylose assay plates at room temperature for 30 min. Carefully break any surface bubbles before reading. A heat gun briefly passed over the surface of the plate works well.
    11. Using an ultraviolet/visible 96-well plate reader, set the measured wavelength to 510 nm and record the absorbance against a reagent blank. This measurement monitors the formation of quinonimine, which is proportional to glucose concentration. The glucose concentration is calculated from the calibration curve based on the calibration standards prepared in 1.3.1.
    12. Using an ultraviolet/visible 96-well plate reader, set the measured wavelength to 340 nm and record the absorbance. This measurement monitors the reduction of NAD^+ to NADH, which is proportional to xylose concentration. The xylose concentration is calculated from the calibration curve based on the calibration standards prepared in 1.3.1.

2. High-throughput Determination of Total Lignin and Lignin Monomeric Content Using pyMBMS [28]

1. Sample Preparation
    1. Grind and extract the biomass using the methods described in steps 1.1.1, and 1.1.6-1.1.8. This includes grinding and extracting of a set of standards to use as experimental controls.
       Note: The pyMBMS measurements are not particle-size dependent, so if only using the pyMBMS protocol, biomass preparation should be carried out to allow the sample to fit in the sample holder, <4 mm.
    2. Using a small spatula dispense approximately 4 mg (3 to 5 mg preferred) of the prepared biomass into an 80 µl stainless steel cup designed for the auto-sampler.
    3. Ensure that at least 10% of the samples being analyzed are control standards such as those available from the National Institute of Standards and Technology (sugarcane bagasse-8491; poplar-8492; pine-8493; or wheat straw-8494). The standard can also be any species analogous to the samples to be analyzed that have already been characterized using standard methods.

4. Randomly load the samples into the auto-sampler cups using tweezers to avoid bias due to possible spectrometer drift over time. Note: A typical randomization of the samples would include the measurement of all samples once, followed by a re-randomization of the order of the samples for a duplicate measurement. Randomization programs are available online.
5. Using a standard hole-punch, manually produce glass filter discs from type A/D glass fiber sheets, with no binder. Hold the round glass fiber filter with tweezers, center it over the sample cup, and push into the sample using a 3.5 mm Allen wrench to confine the material in each cup during the experiment.

2. Instrumental Protocol
    1. Calibrate the mass spectrometer using a known standard that has peak intensities over the entire range of compounds that may exist for the experimental samples. For typical biomass samples, use perfluorotributylamine (PFTBA).
    2. Set the helium carrier gas flow rate to 0.9 L/min using a gas flow meter.
    3. Set the auto-sampler furnace to a pyrolysis temperature of 500 °C and the interface temperature to 350 °C using the auto-sampler software. Note: A 1/8" stainless steel heated transfer line wrapped in heat tape that connects the auto-sampler to the mass spectrometer is controlled using a heat controller at 250 °C.
    4. Begin data acquisition on the mass spectrometer and wait at least 60 sec to obtain sufficient data for background spectra collection.
    5. Start the automated auto-sampler method with the specifications from 2.2.2. Note: The auto-sampler drops each sample individually into the auto-sampler furnace. The total data acquisition time is approximately 1.5 min; however, pyrolysis of a typical 4 mg sample is complete after 30 sec.
    6. Record total ion content (TIC) of each sample using mass spectrometer software at 0.5 sec scan rate. Record intensities between m/z 30 to 450.
    Note: Typical biomass compounds use soft ionization at 17 eV. The instrument can record larger intervals from m/z 1 to 1,000; however, scan rate will be limited by computer CPU power.
    7. Remove background from the spectra using the manual enhance feature in the software. Note: A 60 scan portion of the baseline at the beginning of data collection is used to calculate an average background value. This average background spectra is removed from the experimental sample spectra automatically in the mass spectrometer software.
    8. Import the single column text file created by the mass spectrometer software containing the spectral data for each sample, into a database program and combine all the samples into one database. Add any applicable metadata to the spreadsheet. Import the formatted data (spreadsheet/CSV file) into a statistical software package and mean normalize the spectra to account for variation in the pyrolyzed sample masses.
    9. Use a statistical software package to perform a principal component analysis (PCA) using the spectral data to analyze grouping of the replicated standard samples used in the measurements, as well as to evaluate which peaks are integral to the classification of chemical compounds in the loadings plot[28].
    Note: PCA groups the samples based on the similarity of their spectra, and allows a check of the standards to gauge experimental error due to instrument drift during the run.
    10. To calculate the lignin syringyl (S)/guaiacyl (G) ratio, sum the areas of the S peaks (m/z = 154, 167, 168, 182, 194, 208, and 210) and divide by the sum of G peaks at 124, 137, 138, 150, 164, and 178.
    11. To calculate the total lignin content, sum the lignin peaks with m/z = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 182, 194, and 210.
    12. Calculate a correction factor for scaling the pyMBMS measurement to a standard method for estimating total lignin, such as Klason lignin. Divide the Klason lignin value of the individual standard by the total lignin content measured for that standard sample using pyMBMS.
    13. Apply this correction factor to all like-species in the data set. Repeat for each type of biomass analyzed. Note: The correction factor can vary significantly based on the S/G of the biomass analyzed.

Representative Results

The combined effect of the thermochemical pretreatment and subsequent enzyme saccharification is measured as a function of the mass of glucose and xylose released at the end of the assay. The results are reported in terms of milligrams of glucose and xylose released per gram of biomass. This is in stark contrast to data reported from bench-scale assays, which is usually reported as percent theoretical yield based on compositional analysis of the starting material. As it is not yet practical to carry out compositional analysis on thousands of samples per week, data is best reported as conversion levels on a mass basis. This allows for reasonable sample evaluation as long as the comparisons are made between closely related samples of biomass and the composition of the samples does not vary too much.

The primary goal of the assay is to evaluate the relative differences in resistance of the plant cell wall to combined thermochemical/enzymatic saccharification across a range of individual variants. It is worth noting that the assay does not attempt to optimize any of the conditions used for saccharification. In fact, pretreatment severity conditions are significantly suboptimal in order to expand the sensitivity range of the assay, targeting a final conversion yield of 50% to 70% of theoretical. Pretreatment conditions at or near optimal would push all samples to high conversion, greatly narrowing the dynamic range of the assay. Conversely, the enzyme loading is much greater than that typically reported for bench-scale digestions. Again, the goal of the method is not to find the best enzyme or minimize the enzyme cost, but to measure the intrinsic recalcitrance of the cell walls to conversion and using very high enzyme loadings removes variability from the assay.

Lastly, it is important to understand that the variability in the overall assay is significantly higher than that reported for bench-scale work. Typical standard errors are around 8%, though the range is much wider. This is simply the nature of small-scale, HTP research. Pipetting and weighing errors are proportionally higher at the µl and mg scale, as are evaporative and condensation losses. Biomass heterogeneity becomes a very large variable when assaying several orders of magnitude fewer particles in each sample, *i.e.*, 5 mg vs. multiple g. The colorimetric assays used to determine glucose and xylose correlate well with HPLC results; however, while enzyme-linked assays for sugar analysis are quick and can be carried out in parallel, they are not as precise as analytical methods such as HPLC, introducing another layer of imprecision (**Figure 1**).

Due to all of the above considerations, results should only be interpreted and reported within certain limitations. Data should be examined in entire sets, not as individual samples. Replicates are required for meaningful results. Trends and outliers are meaningful, but single results are not. Comparisons are best made within single experimental sets. Comparisons across temporally or spatially separated campaigns require extremely tight controls and careful scrutiny to be meaningful. HTP data is not directly comparable to bench- or other-scale data. Trends track between the HTP and other scales, but direct sample comparisons do not yield identical results.

Data representation typically falls into trends, outliers, or sub-population/variant comparisons. An example of trends is a survey of recalcitrance of 755 natural variants of poplar sampled over a wide range in the Pacific Northwest of the United States[19]. The recalcitrance of these samples was plotted against their lignin content and syringyl/guaiacyl ratio as determined by pyMBMS. The results indicate that as S/G rises, recalcitrance decreases until an S/G ratio of around 2, where the recalcitrance improvement levels off (**Figure 2**). Outliers can be clearly seen in **Figure 3**, where the Pt4CL1 gene was down-regulated in poplar. Several hundred cultivars were screened and several are clearly increased in recalcitrance, as evidenced by the decreased sugar release. **Figure 4** depicts a study in which several different wheat-straw varieties were grown on several sights over two years and harvest after several variations in growth conditions, resulting in 20 distinct populations based on combinations of the above variables. These sample sets are readily distinguished and indicate positive and negative variables for recalcitrance. **Figure 5** shows how pyMBMS can be used to evaluate changes in cell-wall composition. Mass spectral fragments are assigned to different lignin monomers (**Table 1**). When comparing a sample with high lignin vs. high carbohydrate content, the disparities in the spectral data are apparent. The use of pyMBMS data coupled with a principal component analysis (PCA) is illustrated in **Figure 6**. In this example, the samples are classified according to low or high nitrogen fertilization. Lignin and carbohydrate contents align inversely along PC1. The use of the principal component loadings plot can aid in identifying which chemical traits are being explained in the scores classification plot, as well as elucidating which traits are changing between the sample clusters.

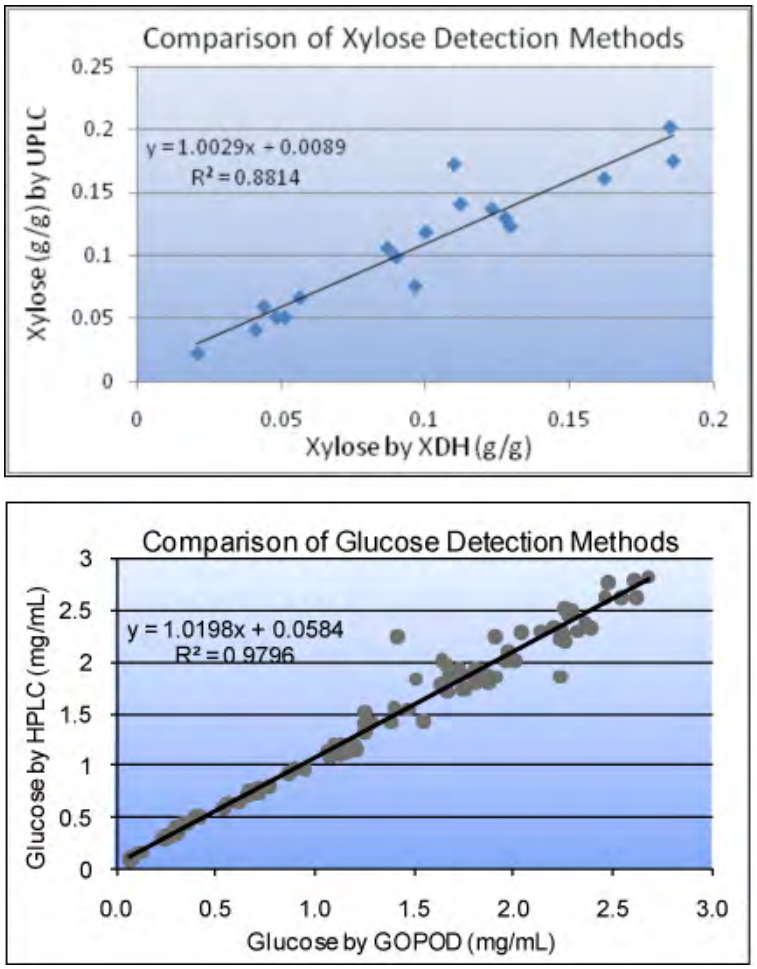

Figure 1: Comparison of glucose and xylose quantitation using high-throughput colorimetric assays vs. HPLC[9]. Comparison of glucose and xylose detection was carried out using Xylose DeHydrogenase (XDH, top panel) and Glucose Oxidase/Peroxidase (GOPOD, lower panel) high-throughput colorimetric enzyme-linked assays and high-performance liquid chromatography.

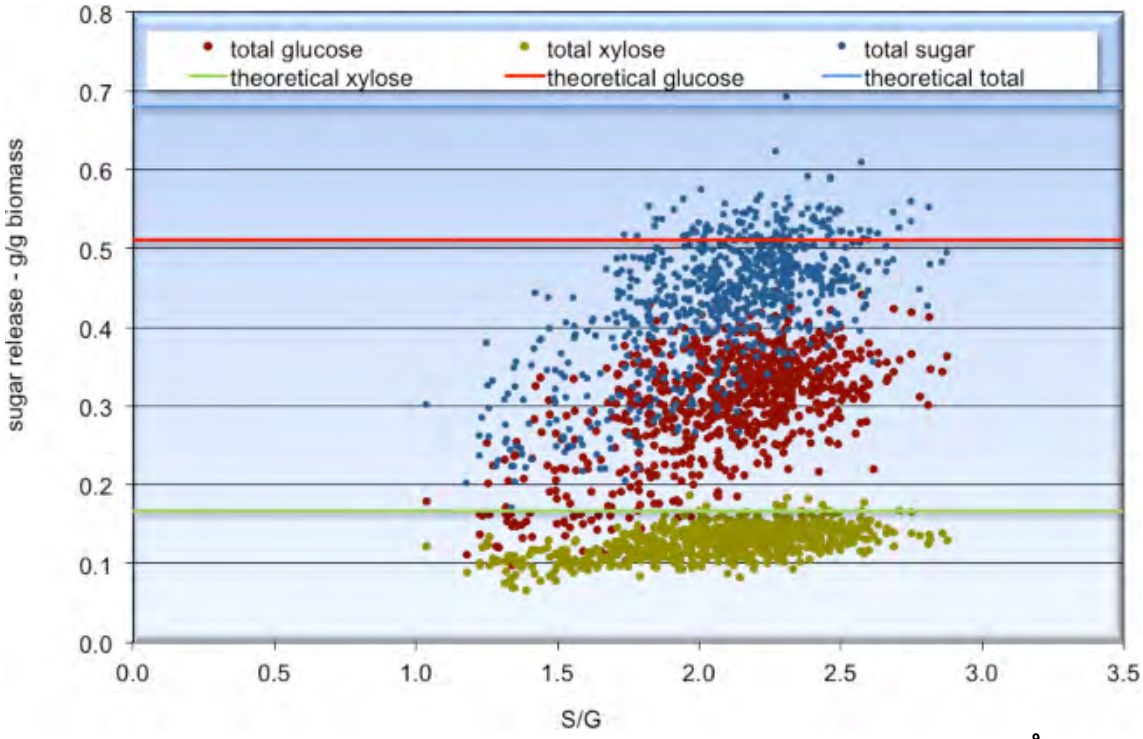

Figure 2: Recalcitrance of poplar cell walls as a function of syringyl to guaiacyl (S/G) content in lignin[9]. Increasing the recalcitrance of poplar cell walls as a function of syringyl to guaiacyl (S/G) content in the lignin fraction has been observed and reported. 755 poplar core samples of the same poplar cultivar were taken over several hundred miles of forest in the North American Pacific Northwest and screened for glucose and xylose release after thermochemical pretreatment and enzyme saccharification. Results were plotted against the S/G ratio in the cell wall lignin as determined by pyMBMS. Recalcitrance decreases with increasing S/G until about 2, where it remains constant at higher S/G. Theoretical yield values are based on the BESC "standard" poplar and are provided as a reference for comparison.

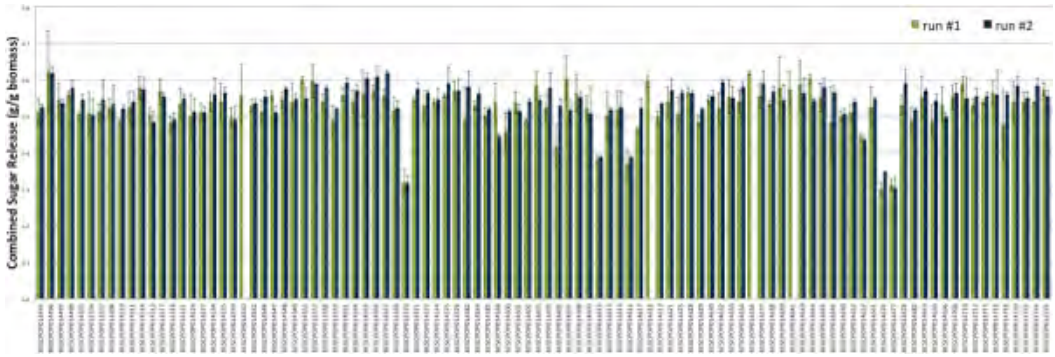

Figure 3: Recalcitrance in down-regulated Pt4CL1 expression in poplar. Down-regulation of the Pt4CL1 gene in poplar resulted in little variation in cell wall recalcitrance between clones, however several drastically increased recalcitrance variants are easily identified. Please click here to view a larger version of this figure.

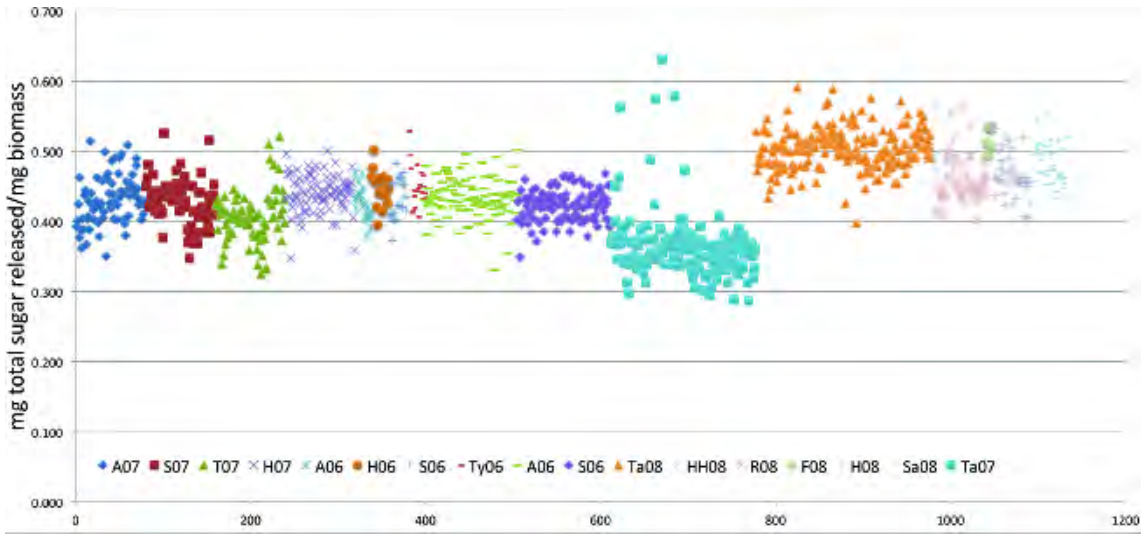

Figure 4: Recalcitrance in various wheatstraw populations. The effects of cultivar type, site of cultivation, fertilizer application, and watering rate result in clearly distinguishable recalcitrance levels. Different symbols represent different experimental growth conditions. Please click here to view a larger version of this figure.

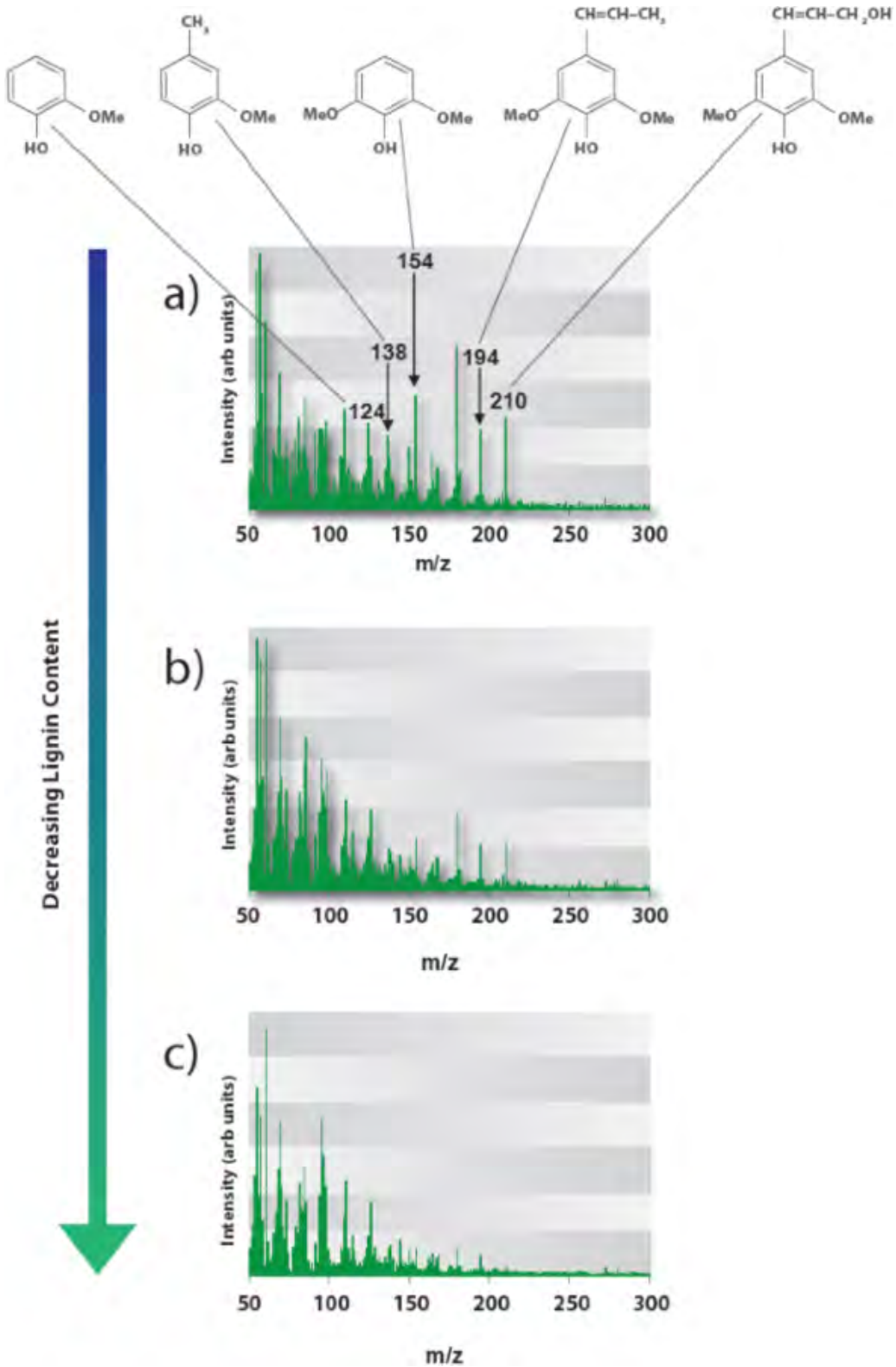

Figure 5: Application of pyMBMS for the analysis of chemical changes in plant cell walls[28]. The peaks denoted with arrows are lignin fragments, and were used to evaluate (**a**) high lignin (**b**) medium lignin or (**c**) low lignin content.

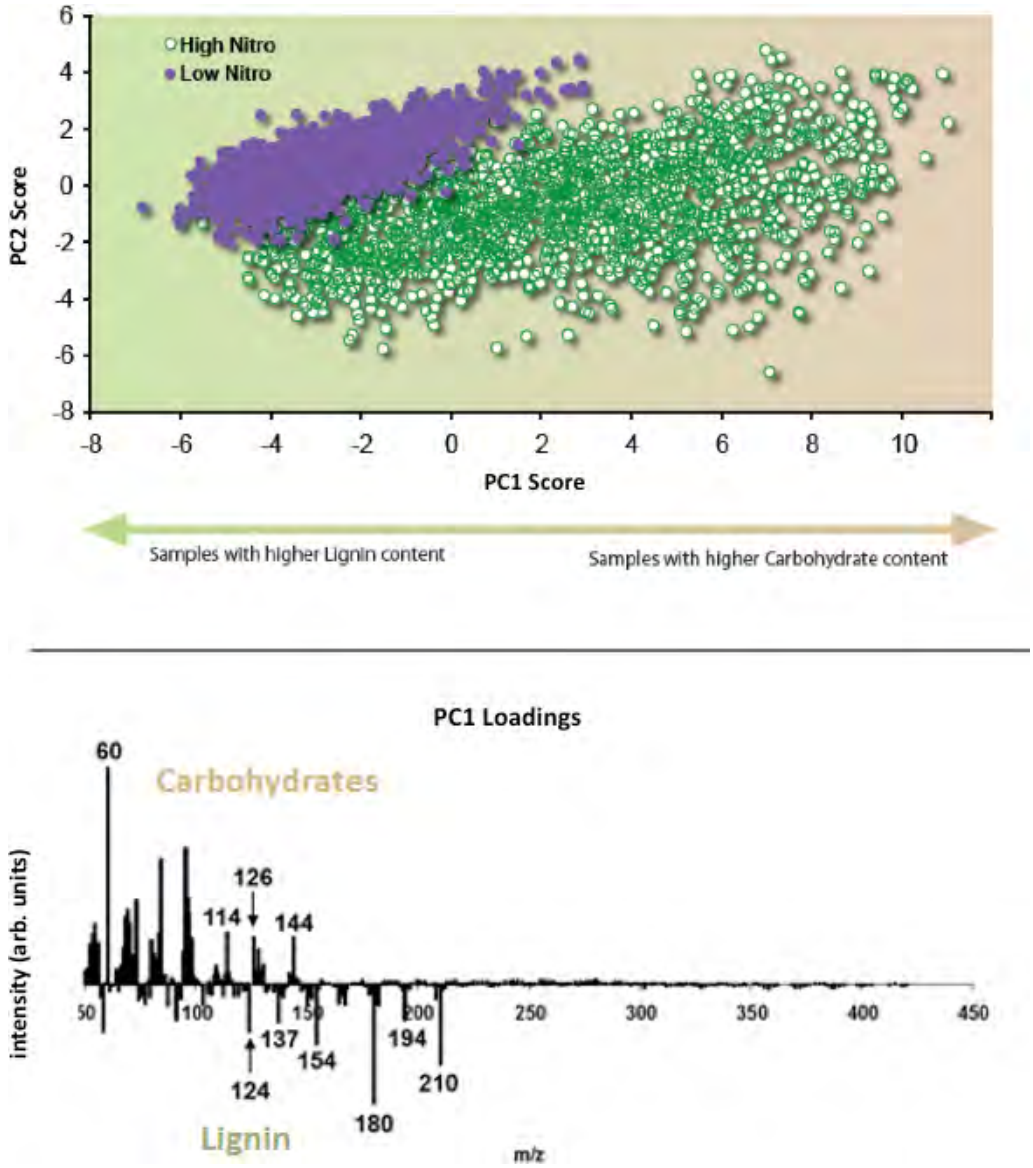

Figure 6: Principal component analysis scores plot of differences in the cell wall chemistry of populous trees grown with higher and lower fertilization rates[28]. (Top) Principal component analysis scores plot illustrating a distinct classification, and therefore, differences in the cell wall chemistry of populous trees grown with higher and lower fertilization rates. Principal component 1 partitioned the samples based on higher lignin (negative) or higher carbohydrate (positive) contents. (Bottom) The principal component loadings elucidate which chemical components change between the two clusters of samples in the scores plot. The positive loadings correlate with the positive scores, which are representative of higher carbohydrate contents, while negatively correlated loadings align with negative scores and, therefore, higher lignin content.

m/z	Molecular assignment[30]	S/G/H assignment
94	phenol	S/G/H
120	Vinylphenol	H
124	Guaiacol	G
137	Ethylguaiacol, homovanillin, coniferyl alcohol	G
138	Methylguaiacol	G
150	Vinylguaiacol	G
154	Syringol	S
164	Allyl- + propenyl guaiacol	G
167	Ethylsyringol, syringylacetone, propiosyringone	S

168	4-Methyl-2,6-dimethoxyphenol	S
178	Coniferyl aldehyde	G
180	Coniferyl alcohol, syringylethene	S, G
182	Syringaldehyde	S
194	4-Propenylsyringol	S
208	Sinapylaldehyde	S
210	Sinapylalcohol	S

Table 1: Peak *m/z* and assignments for lignin-derived pyrolysis fragments as detected by mass spectrometry. List of typical compounds detected by pyMBMS. Molecular assignments based on Evans, R. J. and T. A. Milne (1987). Molecular characterization of the pyrolysis of biomass. *Energy and Fuels* **1**(2):123–137.

Discussion

The key sample preparation steps for obtaining accurate and reproducible data when conducting high-throughput screening experiments are as follows:

Sugar Release Assay:

In general, samples are prepared in lots ranging from a few dozen to several thousand at a time. Each major step is typically carried out for all samples prior to moving forward in order to minimize variations in preparation between samples. De-starching was not originally part of the protocol and can be omitted in certain cases. Wood core samples and senesced herbaceous materials are consistently low in starch and, therefore do not require de-starching to reduce variability. Starch is a major concern; however, in sample sets of green tissue plants, especially for large sample sets where harvest occurs over several hours. As starch levels rise during daylight hours and are depleted during darkness, starch levels can vary significantly over several h and glucose from starch is indistinguishable from glucose from cellulose, therefore, high starch levels can lead to distortedly low recalcitrance measurements. The actual enzyme loading in the destarching step is of lesser concern, as long as there is enough excess activity to remove all accessible starch within the time frame of the starch hydrolysis step. Variations in the specific enzymes used, time, temperature, volume, and agitation rate of the starch-removal step can certainly lead to differences in starch removal levels and rates and should be determined empirically when possible. Lastly, when wrapping the teabags for extraction, use tin-coated copper wire. Bare copper wire can cause unwanted chemical reactions with the enzyme solution, giving false sugar values. Tin-coated copper resolved this issue while stainless steel wire was not malleable enough to keep the teabags tightly closed.

Consistent size-reduction of biomass is critical to accurate data for two reasons: 1) Finely milled biomass, while more easily and consistently dispensed, is also easier to digest than coarser material. 2) Biomass that is too coarse can clog the hoppers, resulting in insufficient material or, if the clog is only partially blocking the opening, it can act as a sieve and only allow fine particles through, decreasing the observed recalcitrance of the sample. If too little biomass is contained in the hoppers, it may coat the sides of the hopper, causing the robot to assume the hopper is empty and the sample will be skipped in the dispensing process. Additionally, low levels of biomass can contribute to exaggerated sample heterogeneity, especially as denser particles (such as rind) tend to sieve to the bottom of the cone and get dispensed first. When less than 50 mg of material is available, the assay can still be carried out; however, the samples should be hand-weighed. However, the results of hand-weighing are more variable than those from robotic weighing.

The actual amount of biomass required for the preparatory steps is not well-defined. We have suggested 250 mg as a reasonable starting point, however, each biomass sample exhibits different characteristics regarding losses due to handling, grinding, and extracting as well as being held up in the hoppers or being dispensed reproducibly. The heterogeneity of biomass across types and even within the same type precludes a more defined protocol. Those wishing to implement these assay methods will simply need to determine many of these variables for themselves, though experience does make it easier.

Another step that can introduce error is mixing in microtiter plates, which is notoriously difficult and is critical in these assays for several reasons. The enzyme solution used is concentrated and can be stratified upon addition to the wells, limiting enzyme accessibility to the biomass. As saccharification proceeds, the sugars released may also stratify and concentrate near the biomass and depending on the sampling depth, sugar assays can be artificially high or low. This is why it vital to mix samples after enzyme addition and enzyme saccharification, allowing thorough mixing of the enzymes with the biomass for even saccharification and uniform distribution of the resultant sugars to allow for consistent sugar analysis. In plates that do not contain biomass, such as dilution or assay plates, mixing by repeated pipetting (trituration) has proven to be far superior than shaking. However, as solutions in this protocol have variable densities and particulate levels, pipetting speed and vertical tip location in the liquid column is critical. If the speed is too fast, poor accuracy due to cavitation (aspiration) and liquid retention in the tip (dispensing) can occur. If the tip is too deep in the well, biomass may clog the tip or the tip may be pressed to the bottom, preventing accurate aspiration, and there is more outer surface area for liquid to cling to and carryover to the next step. The latter issue can be somewhat mitigated by control the tip withdrawal speed, as slower tip removal allows liquid to be removed to the bulk solution by surface tension. If tip withdrawal is too slow, pipetted liquid may creep back into the bulk solution as well. Biomass particles also have a tendency to cling to the tips and become transferred to the dilution plate, where they can clog the tip during mixing or transfer to the assay plates, creating inaccuracies in sample volumes being analyzed. These particles may also interfere with the spectroscopic analysis of the assay plates if they occlude the light beam during the read.

Consistent color development timing is critical for GOPOD and XDH assays. The color-development must be allowed to go to completion; however, the GOPOD assay color tends to continue rising after completion and the NADH generated by the XDH assay slowly decreases due to spontaneous oxidation of the NADH. As a result, timing variability can lead to inconsistent comparisons of data between separate plates. The

assay steps should be monitored carefully, as stoppages due to hardware or software issues can lead to large differences in assay times. The use of sugar standards and standard biomass control wells in each plate can help mitigate this to some extent.

PyMBMS:

Standards must be carefully considered for pyMBMS experiments for comparison to standard wet chemical methods. Due to the differing lignin composition (S/G ratios) of biomass species, the correction factor must be determined using a representative standard that is the same species as the experimental sample. If there are no appropriate standard available, differences in lignin concentration can be compared using the intensities of the lignin precursors directly. High S/G ratios (over ~3.5) can lead to overestimation of lignin content by pyMBMS. This is caused by the tendency for S lignin to be preferentially released under the standard conditions used in this method. In addition, the amount of sample required for pyMBMS measurements depends on the type of biomass used. Isolated lignin and lignin model compounds require less material (0.1 mg), as using larger aliquots of sample can saturate the detector.

Another critical step in the PyMBMS process is careful tuning of instrument to a known standard such as perfluorotributylamine (PFTBA) before each experiment. PFTBA contains molecular peaks across the entire spectrum of typical biomass and, therefore, allows uniform instrument tuning between experimental runs. The middle region of the PFTBA spectra is tuned so that m/z 131 and m/z 219 are approximately 50% of the intensity of m/z 69. This tuning is used to emphasize typical peaks seen under soft ionization (17 eV) used to minimize fragmentation of ions to lower masses that cannot be easily identified.

It should be noted that these methods are not analytical methods for the exact quantification of analytes of interest. Rather, these high-throughput techniques are for the screening of large biomass sets to identify those possessing the desired chemical traits. These methods should only be used for screening, ranking, and correlation of samples within a data set. Data sets cannot be directly compared when collected on different days due to sources of variation such as instrumental drift, changes in enzyme activity, ambient moisture content in the biomass due to changes in humidity, fluctuations in temperature, and differences in tips and absorbance plate lots. Additionally, the pretreatment used in the sample preparation protocol is not an optimized pretreatment strategy, but rather has been specifically designed to be sub-optimal. This enables subtle differences between the plants to be more efficiently elucidated.

The main benefit of adopting high-throughput methods is that many more samples can be measured in the same time period[18]. For example, a common method for analyzing carbohydrates produced during an acid or enzymatic saccharification is the use of high-performance liquid chromatography (HPLC). Selig *et al.*, state that the ubiquitous two-step acid hydrolysis, although quite useful and paramount to the quantification of structural carbohydrates, limits researchers to being able to evaluate approximately 25 samples per week[9]. While the analytical instrumentation employed in high-throughput methodologies may not provide the sensitivity of a standard technique like HPLC, the rapid analysis affords researchers the luxury of being able to assess large quantities of samples. In addition, the high-throughput methods often have downscaled experimental protocols, reducing the use of consumables, and therefore, decreasing experimental costs and waste.

Disclosures

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References

1. Department of Energy. *U.S. Billion-Ton Update: Biomass Supply for a Bioenergy and Bioproducts Industry.* R.D. Perlack and B.J. Stokes (Leads), ORNL/TM-2011/224, Oak Ridge National Laboratory, Oak Ridge, TN, (2011).
2. Henry, R. in *Plant Resources for Food, Fuel and Conservation.* Ch. 5, 53-80, Earthscan, (2009).
3. Henry, R. J. Evaluation of plant biomass resources available for replacement of fossil oil. *Plant Biotechnol. J.* **8** (3), 288-293, doi: 10.1111/j.1467-7652.2009.00482.x (2010).
4. Furtado, A. *et al.* Modifying plants for biofuel and biomaterial production. *Plant Biotechnol. J.* **12** (9), 1246-1258, doi: 10.1111/pbi.12300 (2014).
5. Sluiter, J. B., Ruiz, R. O., Scarlata, C. J., Sluiter, A. D., & Templeton, D. W. Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods. *J. Agric. Food Chem.* **58** (16), 9043-9053, doi: 10.1021/jf1008023, (2010).
6. Lupoi, J. S., Singh, S., Simmons, B. A., & Henry, R. J. Assessment of Lignocellulosic Biomass Using Analytical Spectroscopy: an Evolution to High-Throughput Techniques. *Bioenerg. Res.* **7** (1), 1-23, doi: 10.1007/s12155-013-9352-1, (2014).
7. Lapierre, C., Monties, B., & Rolando, C. Thioacidolysis of lignin: comparison with acidolysis. *J. Wood Chem. Technol.* **5** (2), 277-292, doi: 10.1080/02773818508085193 (1985).
8. DeMartini, J. D., Studer, M. H., & Wyman, C. E. Small-scale and automatable high-throughput compositional analysis of biomass. *Biotechnol. Bioeng.* **108** (2), 306-312, doi: 10.1002/bit.22937 (2010).
9. Selig, M. J. *et al.* High throughput determination of glucan and xylan fractions in lignocelluloses. *Biotechnol. Lett.* **33** (5), 961-967, doi: 10.1007/s10529-011-0526-7 (2011).

10. Selig, M. J. *et al.* Lignocellulose recalcitrance screening by integrated high-throughput hydrothermal pretreatment and enzymatic saccharification. *Ind. Biotechnol.* **6** (2), 104-111, doi: 10.1089/ind.2010.0009, (2010).
11. Studer, M. H., De Martini, J. D., Brethauer, S., McKenzie, H. L., & Wyman, C. E. Engineering of a high-throughput screening system to identify cellulosic biomass, pretreatments, and enzyme formulations that enhance sugar release. *Biotechnol. Bioeng.* **105** (2), 231-238, doi: 10.1002/bit.22527 (2009).
12. Gjersing, E., Happs, R. M., Sykes, R. W., Doeppke, C., & Davis, M. F. Rapid determination of sugar content in biomass hydrolysates using nuclear magnetic resonance spectroscopy. *Biotechnol. Bioeng.* **110** (3), 721-728, doi: 10.1002/bit.24741 (2013).
13. Templeton, D. W., Sluiter, A. D., Hayward, T. K., Hames, B. R., & Thomas, S. R. Assessing corn stover composition and sources of variability via NIRS. *Cellulose (Dordrecht, Netherlands)*. **16** (4), 621-639, doi: 10.1007/s10570-009-9325-x (2009).
14. Tucker, M. P. *et al.* Fourier transform infrared quantification of sugars in pretreated biomass liquors. *Appl. Biochem. Biotechnol.* **84-86**, 39-50, doi: 10.1007/978-1-4612-1392-5_2 (2000).
15. Wolfrum, E. J., & Sluiter, A. D. Improved multivariate calibration models for corn stover feedstock and dilute-acid pretreated corn stover. *Cellulose (Dordrecht, Netherlands)*. **16** (4), 567-576, doi: 10.1007/s10570-009-9320-2, (2009).
16. Ona, T. *et al.* Non-destructive determination of wood constituents by Fourier-transform Raman spectroscopy. *J. Wood Chem. Technol.* **17** (4), 399-417, doi: 10.1080/02773819708003141 (1997).
17. Ona, T., Sonoda, T., Ohshima, J., Yokota, S., & Yoshizawa, N. A rapid quantitative method to assess eucalyptus wood properties for kraft pulp production by FT-Raman spectroscopy. *J. Pulp Pap. Sci.* **29** (1), 6-10, 10.1186/1754-6834-7-93, (2003).
18. Hames, B. R., Thomas, S. R., Sluiter, A. D., Roth, C. J., & Templeton, D. W. Rapid biomass analysis. New tools for compositional analysis of corn stover feedstocks and process intermediates from ethanol production. *Appl. Biochem. Biotechnol.* **105-108**, 5-16, doi: 10.1385/abab:105:1-3:5, (2003).
19. Studer, M. H. *et al.* Lignin content in natural *Populus* variants affects sugar release. *Proc. Natl. Acad. Sci. U. S. A.* **108** (15), 6300-6305, S6300/6301-S6300/6306, doi: 10.1073/pnas.1009252108, (2011).
20. Chen, M., Zhao, J., & Xia, L. Comparison of four different chemical pretreatments of corn stover for enhancing enzymatic digestibility. *Biomass Bioenergy.* **33** (10), 1381-1385, doi: 10.1016/j.biombioe.2009.05.025, (2009).
21. Davison, B. H., Drescher, S. R., Tuskan, G. A., Davis, M. F., & Nghiem, N. P. Variation of S/G ratio and lignin content in a *Populus.* family influences the release of xylose by dilute acid hydrolysis. *Appl. Biochem. Biotechnol.* **129-132**, 427-435, doi: 10.1385/abab:130:1:427, (2006).
22. Li, X. *et al.* Lignin monomer composition affects *Arabidopsis* cell-wall degradability after liquid hot water pretreatment. *Biotechnol. Biofuels.* **3**, 27-33, doi: 10.1186/1754-6834-3-27, (2010).
23. Lupoi, J. S. *et al.* High-throughput prediction of eucalypt lignin syringyl/guaiacyl content using multivariate analysis: a comparison between mid-infrared, near-infrared, and Raman spectroscopies for model development. *Biotechnol. Biofuels* **7**, 93, doi: 10.1186/1754-6834-7-93, (2014).
24. Lupoi, J. S., & Smith, E. A. Characterization of woody and herbaceous biomasses lignin composition with 1064 nm dispersive multichannel Raman spectroscopy. *Appl. Spectro.* **66** (8), 903-910, doi: 10.1366/12-06621 (2012).
25. Sun, L. *et al.* Rapid determination of syringyl:guaiacyl ratios using FT-Raman spectroscopy. *Biotechnol. Bioeng.* **109** (3), 647-656, doi: 10.1002/bit.24348 (2012).
26. Lupoi, J. S. *et al.* High-throughput prediction of *Acacia* and eucalypt lignin syringyl/guaiacyl content using FT-Raman spectroscopy and partial least squares modeling. *Bioenerg. Res.* in press, doi: 10.1007/s12155-015-9578-1, (2015).
27. Sykes, R., Kodrzycki, B., Tuskan, G., Foutz, K., & Davis, M. Within tree variability of lignin composition in Populus. *Wood Sci. Technol.* **42** (8), 649-661, doi: 10.1007/s00226-008-0199-0 (2008).
28. Sykes, R. *et al.* High-Throughput Screening of Plant Cell-Wall Composition Using Pyrolysis Molecular Beam Mass Spectroscopy. *in Biofuels: Methods and Protocols*. Vol. 581 *Methods in Molecular Biology* (ed Jonathan R. Mielenz) Ch. 12, 169-183, doi: 10.1007/978-1-60761-214-8_12, (2009).
29. Decker, S. *et al.* Reducing the effect of variable starch levels in biomass recalcitrance screening. in *Biomass Conversion.* Vol. 908 *Methods in Molecular Biology* (ed Michael E. Himmel) Ch. 17, 181-195, Humana Press, doi: 10.1007/978-1-61779-956-3_17, (2012).
30. Evans, R. J. and Milne, T. A. Molecular characterization of the pyrolysis of biomass. *Energy Fuels.* **1**(2), 123–137, doi: 10.1021/ef00002a001 (1987).

