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High throughput determination of glucan and xylan fractions in lignocelluloses

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Abstract The analysis of structural glucan and xylan in lignocellulose was scaled down from original two-stage sulfuric acid hydrolysis methods (Moore WE and Johnson DB 1967 Procedures for the chemical analysis of wood and wood products. U.S. Forest Products Laboratory, U.S. Department of Agriculture., Madison, WI) and integrated into a recently-developed, high throughput pretreatment and enzymatic saccharification system. Novel 96 × 1.8 ml-well Hastelloy reactor plates (128 × 86 × 51 mm) based on previously described 96-well pretreatment reactor plates were paired with custom aluminum filler plates (128 × 86 × 18 mm) for use in Symyx Powdernium solids dispensing systems. The incorporation of glucose oxidase and xylose dehydrogenase linked assays to speed post-hydrolysis sugar analysis dramatically reduced the time for analysis of large lignocellulosic sample sets. The current system permits the determination of the glucan and xylan content of 96 replicates (per reactor plate) in under 6 h and parallel plate processing increases the analysis throughput substantially.

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Introduction

Previously, we presented a platform for high throughput pretreatment and enzymatic saccharification of lignocellulosic material utilizing a custom designed and manufactured multi-96-well plate steam reactor system (Selig et al. 2010); this system was developed in parallel with a lower throughput single-plate system described by Studer et al. (2010). Whereas these systems are useful in comparative studies within defined sample sets, conversion yields and extents cannot be assessed without concurrent high-throughput compositional analysis of the starting material. The determination of the structural carbohydrate content of lignocellulosic materials is a crucial experimental step in the study of lignocellulose recalcitrance and it's conversion to fuels and chemicals.

The determination of the structural carbohydrate content of lignocellulosic materials has long been a crucial, yet time consuming, experimental step in studying biomass conversion. Typical procedures require cumbersome equipment and mundane, time consuming steps to produce adequately accurate and precise results, yet this information is critical in order to understand the extent of conversion, yield, and rate

of the biomass conversion processes. The current standard for determining structural carbohydrates in lignocelluloses is derived from the two-stage sulfuric acid hydrolysis method originally outlined by Moore and Johnson (1967). The standard Laboratory Analytical Protocol of the National Renewable Energy Laboratory (NREL) was developed from this work and was also adopted by the American Society for Testing and Materials (ASTM) as the standard method for determining structural carbohydrate content in biomass (ASTM 2007). This method is reliable and accurate, although the complexity and tedious nature of the procedure prohibited the analysis of sample numbers much greater than 25 per week by an individual.

Past attempts to address the time-consuming nature of compositional analyses have considered spectroscopic methods, such as near-infrared (NIR), Molecular Beam Mass Spectroscopy (MBMS), and NMR with some success (Hames et al. 2002, 2003; Hames 2009; Templeton et al. 2009; Labbe et al. 2005; Ye et al. 2008; Kelley et al. 2004). The limited nature of these methods, however, requires extensive analysis of samples using more traditional (standard) methods in order to build models for determining compositional information from the various spectra. In addition, these models are often only effective on similar types of substrates, thus creating the requirement for a new model to be built for each potential substrate type. Even then, the success of such system/model combinations at predicting lignocellulose composition is limited.

Building on our earlier high-throughput pretreatment platform, we have expanded our capabilities to include a 96-well method for measuring the quantity of the key structural carbohydrates present in lignocellulosic materials. The system utilizes a deeper ($\sim$1.8 ml) 96-well Hastelloy reactor to perform a scaled down version of the most commonly cited two-stage sulfuric acid hydrolysis protocol (Sluiter et al. 2008). Rapid determination of glucose and xylose released by the process is accomplished using automated glucose oxidase and xylose dehydrogenase coupled assays. We feel the introduction of such technology will rapidly accelerate the pace of substrate-based studies in the lignocellulose recalcitrance field.

Materials and methods

Microplate deep-well hydrolysis reactor

Custom 96-deep-well microtiter plates were machined at Aspen Machining (Lafayette, Colorado) from C-276 Hastelloy alloy to handle the severely acidic conditions dictated by the two-stage sulfuric acid hydrolysis protocol (Fig. 1). These deep-well reactor plates were designed with features similar to the 96-well pretreatment reactor plates described previously (Decker et al. 2009; Selig et al. 2010), with the exception of increased plate height and well depth to increase the well volume to 1.8 ml; the dimensions are approx. 128 $\times$ 86 $\times$ 51 mm. The well diameter was reduced slightly (6.8 mm) to accommodate machining limitations; however, steam ports identical to our previously described system were included, allowing compatibility with the existing clamping and heating systems.

Robotics systems and accommodations

The automation utilized for the liquids and solids handling of this scaled-down compositional method were similar to those described for our earlier pretreatment/co-saccharification system; this includes Symyx Powdernium systems for dispensing dry biomass solids and Biomek FX systems from Beck-

Fig. 1 Deep well Hastelloy compositional analysis reactor plate with dimensions of 128 $\times$ 86 $\times$ 51 mm; well diameters are 6.8 mm. The larger openings are the samples wells (96 at 1.8 ml capacity each). The smaller openings are steam channels that are contiguous between the reactor plate clamping system and stacked reactors

mann-Coulter for dispensing all liquids. The large mass (~6 kg) of the Hastelloy deep-well plate made it impossible to load biomass directly into the plate without overloading and damaging the balances of the Symyx systems. To circumvent this, we developed two aluminum 48-well trap-door microtiter "filling" plates (approx. 128 × 86 × 18 mm; Fig. 2) for the purpose of loading the deep well plates with accurate quantities of biomass as dispensed by the Symyx systems. These plates were offset so that the wells of each plate lined up over a different set of 48 wells in the deep well plate. After filling the trapdoor plates, they were aligned on top of the deep well reactor and the trapdoor slid to one side, placing holes in the sliding door under each of the 48 wells and allowing the biomass to drop into the wells of the deep well reactor. This procedure was then repeated for the second trapdoor plate, filling all 96 wells.

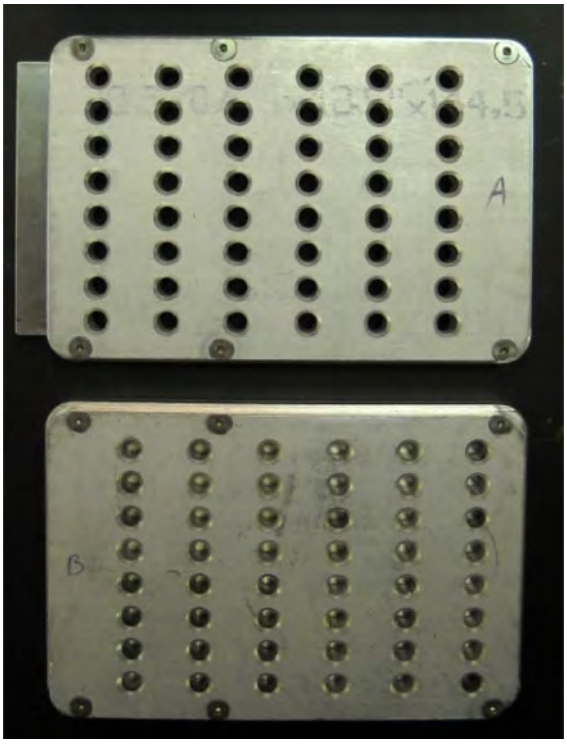

Fig. 2 Trapdoor dispensing plates for filling deep well reactors; dimensions are 128 × 86 × 18 mm. Two plates are required to dispense biomass into all 96 wells. The columns of wells are offset to allow for the trapdoor openings to slide aside for filling on the powder dispensing robot. In this figure, the sliding trapdoor is open on the top plate (wells are open and metal plate is protruding on left side) and closed on the bottom plate

Small scale two-stage sulfuric acid hydrolysis

All scaled-down methods for performing two-stage sulfuric acid hydrolysis were derived from the methods described in the NREL laboratory analytical procedure outlined by Sluiter et al. (2008). Lignocellulose samples were milled to pass a 1 mm screen and dispensed into the plates at 5 mg/well, which is around 60-fold less than what is used in the standard bench scale method. When using the single 96-well plate, typical analyses included 24 independent lignocelluloses samples run in triplicate, with the remaining 24 deep-wells reserved for internal standard curves and controls. To avoid relying on what we believe are misrepresentative sugar recovery standards (SRS) of monomeric glucose and xylose (as described for the full-scale method), we incorporated 6-point glucan and xylan standards (to generate standard curves) in the plates; these standards consist of varied loadings of the commercially available Sigmacel 50 (Sigma-Aldrich) cellulose and Oat Spelt Xylan (75% xylan w/w; Sigma-Aldrich). All replicates and standards are distributed uniformly throughout the 96-well plates to avoid bias from possible localized irregularities, such as seal failure.

Following lignocellulose loading, 50 µl 72% (w/w; ~12 M) H_2SO_4 was added to each well by the Biomek FX system using a Span-8 hydraulic pipetting head. (During this step, it is important that the acid is dispensed directly above the lignocellulose aliquots in order to most effectively wet the materials while avoiding tip immersion into the slurry; this would result in material clinging to the tips.) Prior to incubation, the plates were vibrated for 2 min on a standard vortexing apparatus in order to ensure thorough sample wetting. Incubation was at 30°C in a sonicating water bath for 1–2 h depending on substrate type. (Pretreated and some grassy lignocellulose samples typically require less time compared to hardwood and softwood samples.) After incubation, 1.4 ml of distilled water was added to each well in the reactor plate to adjust to a final sulfuric acid concentration of 4% (w/w; ~0.4 M). The plates were then sealed with a high-temperature adhesive-backed foil seal and clamped into the custom plate clamping system described previously for our original 96-well plate pretreatment system (Selig et al. 2010; Decker et al. 2009). The clamp was then fitted into a two-gallon Parr reactor, sealed, and

heated with steam to 121°C for 1 h before being flushed with cooling water.

Sampling and sugar analysis

Once the two-stage hydrolysis is complete, the plate was hand-mixed (three to five inversions) and the seal pierced with a custom-made 96-point punch. Hydrolysate, 0.2 ml, was transferred from each reactor plate well to a 96-well glass fiber filter plate and filtered under vacuum into a standard, flat-bottom polystyrene 96-well microplate. Filtered hydrolysate, 20 µl, from this plate was then diluted 1:10 into 200 mM citrate buffer (pH ~5.0) in a second polystyrene plate. After mixing on a microtiter plate shaker, aliquots of the diluted hydrolysates were transferred into assay plates to assess glucose and xylose concentration using the glucose oxidase and xylose dehydrogenase-based assays described previously (Selig et al. 2010; Decker et al. 2009). In brief, the glucose oxidase based assay results in the transfer of an electron from glucose to promote the creation of an quinoneimine dye which can be detected by measuring absorbance at 510 nm and the xylose dehydrogenase based assay transfer a H^+ from xylose molecules to NAD^+ to form NADH which can be detected by measuring absorbance at 340 nm; both reaction pathways can be purchased as kits from Megazyme International (Wicklow, Ireland).

System validation

The system described here was initially tested on four different lignocellulosic sample materials for which structural carbohydrate composition was known. The sample set included: (1) extractives-free corn stover, (2) a ground whole poplar tree, and externally validated (3) wheat straw and (4) Monterey pine samples from the National Institute of Standards and Technology. This four sample set was processed in replicates in multiple 96-well reactor runs for initial testing of the system. A second set of experimental samples was also analyzed to validate the process. Twenty-four differently pretreated corn stover samples with known compositional data (three replicates each) were analyzed. Pretreatment chemistries in this set included dilute acid, hot water, sulfite steam explosion, lime, ammonia fiber expansion, and alkaline peroxide; all samples were either prepared in-house at NREL or obtained via subcontract with the CAFI 2 pretreatment group which has been described previously (Selig et al. 2009; Wyman et al. 2005). The structural carbohydrate composition for these substrates was originally determined according to the full-scale method described by Sluiter et al. (2008).

Results and discussion

The time required to perform 96 separate analyses in the deep-well plate format was considerably reduced from the bench-scale method. This time ranged from 5–6 h depending on the number of different samples that were analyzed within a single plate. Much of the time reduction observed is associated with the change from HPLC sugar analysis methods to enzyme-linked assays for the quantitation of glucose and xylose. Use of these redox-enzyme based assays allows for analysis of all samples simultaneously instead of requiring 30 min per well for HPLC analysis (i.e., 48 h for 96 samples). Eliminating the HPLC analysis also eliminated the need to neutralize the hydrolysates (usually through $CaCO_3$ addition), saving additional preparation time during the post-hydrolysis analysis.

Initial tests of the system were performed on native lignocellulose samples and results compared well with those obtained by the "full-scale" determination (Fig. 3). Incubation time with 72% (v/v) sulfuric acid was the most influential component related to achieving "complete hydrolysis" of the structural carbohydrates. Incubation times between 90 and 120 min were suitable for most substrates. Whereas a shorter (e.g., 60 min) incubation left the poplar and wheat straw replicates only partially hydrolyzed (data not shown), this period was found to be sufficient for most pretreated materials tested.

Analysis of 24 different pretreated corn stover samples (each in triplicate) within a single plate proved successful and results matched adequately with those obtained by the standard bench-scale method (Fig. 4). An exception was one of the hot water pretreated substrates, which appeared to only reach partial hydrolysis with respect to the glucan fraction; this result was observed in repeated runs of an identical sample set. In this case, the sample was pretreated under fairly mild conditions, which suggests that a longer incubation period may be

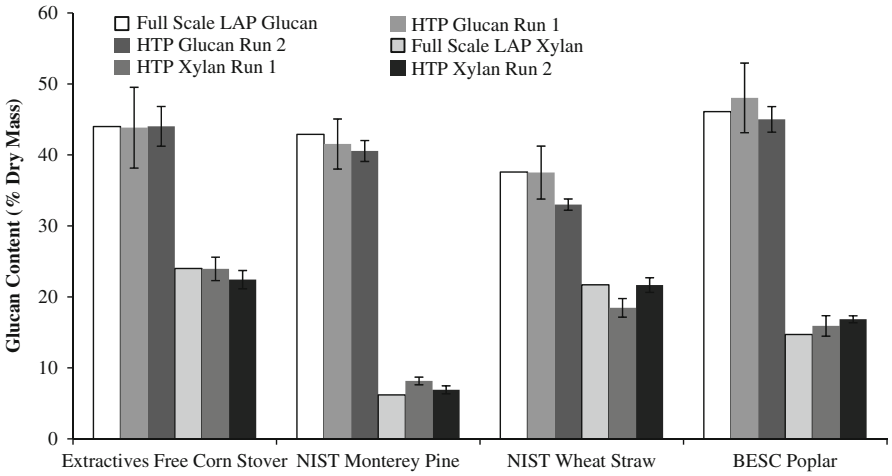

Fig. 3 Comparison of two separate high throughput compositional analysis runs of standard reference biomass to results from the standard NREL compositional analysis LAP

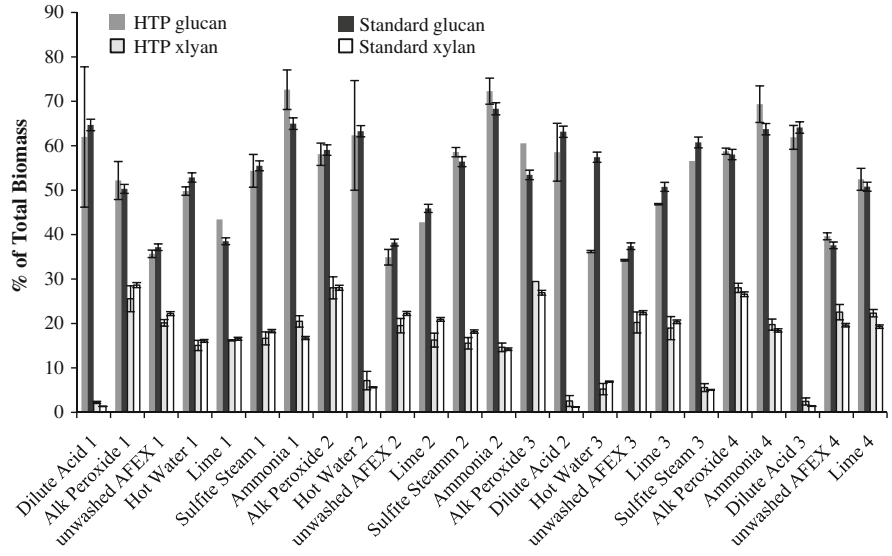

Fig. 4 Comparison of high throughput and standard compositional analysis of corn stover after various pretreatment chemistries and severities. Conditions for each pretreatment are given in Table 1. Pretreated samples were obtained via in house production at NREL or from members of the CAFI 2 pretreatment group

warranted when samples have been pretreated under low severities. Aside from this lone sample, combining data on all samples tested with the new system and modified method we have shown good correlation with standard structural carbohydrate determination methods for both glucan and xylan determination (Fig. 5). We feel that this new reduced scale, rapid throughput technique will be of significant value for studies where large numbers and limited quantities of samples must analyzed.

Whereas the equipment described here is specific to a particular system developed at NREL, we feel the concepts and alternative methods presented could easily be incorporated into any system designed for use in a standard autoclave. In addition, the highly corrosion resistant nature of the 96-well hastelloy reactor could easily prove useful for applications beyond the two-stage sulfuric acid hydrolysis. Compared to our previously described 416 µl well 96-well pretreatment reactor, the larger 1.8 ml wells and could allow for a broader range of biomass pretreatment strategies to be tested in addition to a number of different small scale and high temperature chemical catalyses. In conclusion, the modified methods and systems described here significantly enhance the high throughput screening methods developed for lignocelluloses and will be of great benefit to those studying lignocellulose composition and variance in

Table 1 Pretreated corn stover sample preparation information

Sample #	Sample ID	Time	Temp (°C)	Catalyst loaded
1	Dilute acid 1	12 min	160	H_2SO_4—0.5% (w/w)
1	Alkaline peroxide 1	3 h	50	H_2O_2—1.0% (w/w)
3	Unwashed AFEX 1	5 min	90	NH_3—1 g/g dry mass
4	Hot water 1	10 min	190	None
5	Lime 1	3 weeks	55	Lime—0.07 g/g dry mass
6	Sulfite steam 1	9 min	170	SO_2—3% in solution
7	Ammonia 1	30 min	170	NH_3—3.33 g/g dry mass
8	Alk peroxide 2	60 min	65	H_2O_2—2.5% (w/w)
9	Hot water 2	12 min	200	None
10	Unwashed AFEX 2	5 min	90	NH_3—0.7 g/g dry mass
11	Lime 2	2 weeks	55	Lime—0.7 g/g dry mass
12	Sulfite steam 2	5 min	190	SO_2—0% in solution
13	Ammonia 2	30 min	175	NH_3—6.66 g/g dry mass
14	Alk peroxide 3	60 min	25	H_2O_2—2.5% (w/w)
15	Dilute acid 2	16 min	160	H_2SO_4—4.8% (w/w)
16	Hot water 3	20 min	190	None
17	Unwashed AFEX 3	5 min	90	NH_3—0.5 g/g dry mass
18	Lime 3	3 days	55	Lime—0.07 g/g dry mass
19	Sulfite steam 3	7.8 min	210	SO_2—3% in solution
20	Alk peroxide 4	30 min	65	H_2SO_4—6.4% (w/w)
21	Ammonia 3	30 min	160	NH_3—3.33 g/g dry mass
22	Dilute acid 3	20 min	160	H_2SO_4—0.5% (w/w)
23	Unwashed AFEX 4	5 min	90	NH_3—1.0 g/g dry mass
24	Lime 4	1 week	55	Lime—0.07 g/g dry mass

Samples were either prepared by the authors in-house at NREL of obtained via sub-contract with members of the CAFI 2 pretreatment group. All samples except the AFEX pretreated materials were washed with distilled water and lyophilized following receipt or in-house preparation

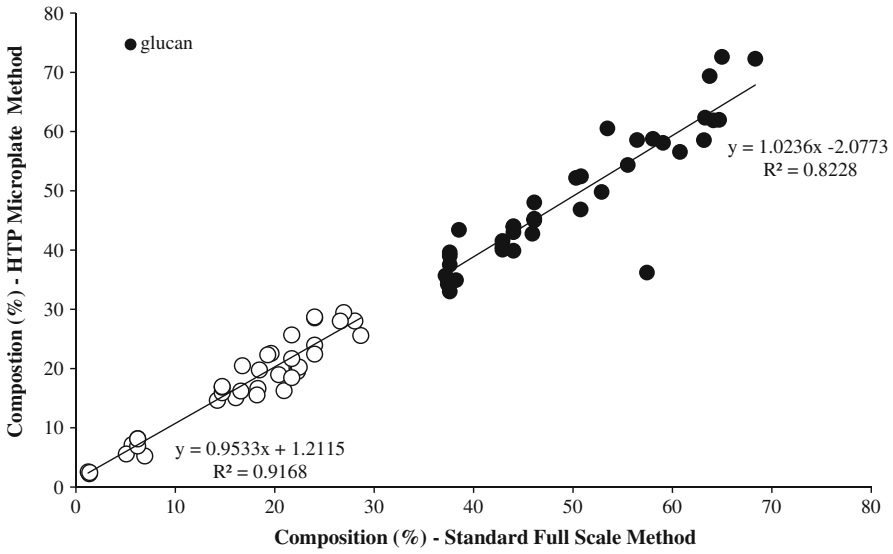

Fig. 5 Compiled comparison of all materials and components analyzed by the presented high throughput and standard two-stage sulfuric acid hydrolysis methodology

large sample populations, as well as those looking for robust 96-well chemical reaction vessels.

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References

ASTM (2007) Standard test method for determination of carbohydrates in biomass by high performance liquid chromatography. ASTM, West Conshohocken

Decker S, Brunecky R, Tucker M, Himmel M, Selig M (2009) High-throughput screening techniques for biomass conversion. BioEnergy Res 2(4):179–192

Hames BR (2009) Biomass compositional analysis for energy applications. Methods Mol Biol 581:145–167

Hames BR, Sluiter AD, Meglen RR (2002) Rapid biomass analysis: new analytical methods supporting fuels and chemical production from biomass. Abstr Pap Am Chem Soc 223:U140

Hames BR, Thomas SR, Sluiter AD, Roth CJ, Templeton DW (2003) 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

Kelley S, Rials TG, Snell R, Groom LH, Sluiter A (2004) Use of near infrared spectroscopy to measure the chemical and mechanical properties of solid wood. Wood Sci Technol 38(4):257–276

Labbe N, Rials TG, Kelley SS, Cheng ZM, Kim JY, Li Y (2005) Ft-ir imaging and pyrolysis-molecular beam mass spectrometry: new tools to investigate wood tissues. Wood Sci Technol 39(1):61–77

Moore WE, Johnson DB (1967) Procedures for the chemical analysis of wood and wood products. U.S. Forest Products Laboratory, U.S. Department of Agriculture, Madison

Selig MJ, Adney WS, Himmel ME, Decker SR (2009) The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. Cellulose 16: 711–722

Selig MJ, Tucker MP, Sykes RW, Reichel KL, Brunecky R, Himmel ME, Davis MF, Decker SR (2010) Lignocellulose recalcitrance screening by integrated high-throughput hydrothermal pretreatment and enzymatic saccharification. Ind Biotechnol 6(2):104–111

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass—laboratory analytical procedure. NREL/TP-510-42618:1–17

Studer MH, DeMartini JD, Brethauer S, McKenzie HL, Wyman CE (2010) 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

Templeton DW, Sluiter AD, Hayward TK, Hames BR, Thomas SR (2009) Assessing corn stover composition and sources of variability via nirs. Cellulose 16(4):621–639

Wyman CE, Dale BE, Elander RT, Holtsapple M, Ladisch MR, Lee YY (2005) Coordinated development of leading biomass pretreatment technologies. Bioresour Technol 96:1959–1966

Ye XP, Liu L, Hayes D, Womac A, Hong K, Sokhansanj S (2008) Fast classification and compositional analysis of cornstover fractions using Fourier transform near-infrared techniques. Bioresour Technol 99(15):7323–7332