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Development of a system for characterizing biomass quality of lignocellulosic feedstocks for biochemical conversion

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Development of a system for characterizing biomass quality of lignocellulosic feedstocks for biochemical conversion

by

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Abstract

The purpose of this research was twofold: (i) to develop a system for screening lignocellulosic biomass feedstocks for biochemical conversion to biofuels and (ii) to evaluate *brown midrib* corn stover as feedstock for ethanol production.

In the first study (Chapter 2), we investigated the potential of corn stover from *bm1-4* hybrids for increased ethanol production and reduced pretreatment intensity compared to corn stover from the isogenic *normal* hybrid. Corn stover from hybrid W64A X A619 and respective isogenic *bm* hybrids was pretreated by aqueous ammonia steeping using ammonium hydroxide concentrations from 0 to 30%, by weight, and the resulting residues underwent simultaneous saccharification and co-fermentation (SSCF) to ethanol. Dry matter (DM) digested by SSCF increased with increasing ammonium hydroxide concentration across all genotypes ($P>0.0001$) from 277 g kg^{-1} DM in the control to 439 g kg^{-1} DM in the 30% ammonium hydroxide pretreatment. The *bm* corn stover materials averaged 373 g kg^{-1} DM of DM digested by SSCF compared with 335 g kg^{-1} DM for the *normal* corn stover ($P<0.0001$). Of the *bm* mutations, *bm3* had (i) the greatest effect on cell-wall carbohydrate hydrolysis of corn stover, (ii) the lowest initial cell-wall carbohydrate concentration, (iii) the lowest dry matter remaining after pretreatment, and (iv) the highest amount of monosaccharides released during enzymatic hydrolysis. However, *bm* corn stover did not reduce the severity of aqueous ammonia steeping pretreatment needed to maximize DM hydrolysis during SSCF compared with *normal* corn stover.

In the remaining studies (Chapters 3 thru 5), a system for analyzing the quality of lignocellulosic biomass feedstocks for biochemical conversion to biofuels (i.e., pretreatment, enzymatic hydrolysis, and fermentation) was developed. To accomplish this, a carbohydrate availability model was developed to characterize feedstock quality. The model partitions carbohydrates within a feedstock material into fractions based on their availability to be converted to fermentable sugars, including non-structural carbohydrates (C_N) (monosaccharides, starches, oligosaccharides), biochemically available carbohydrates (C_B) (structural carbohydrates susceptible to enzymatic hydrolysis) with an associated 1st-order availability rate constant (k_B) and unavailable carbohydrates (C_U) (hemicellulose and cellulose in close association with lignin). The model partitions the non-carbohydrate dry matter into extractives, lignin, and ash. Quality parameters were determined using a biomass quality assay that combined established wet-chemistry analyses techniques, including total non-structural carbohydrates (TNC), alcohol insoluble residue (AIR), simultaneous saccharification and fermentation (SSCF), and Klason lignin. The assay was used to analyze four compositionally diverse biomass feedstocks: corn cobs (*Zea mays* L.), hybrid poplar (*Populus x canadensis* Moench), kenaf (*Hibiscus cannabinus* L.) and switchgrass (*Panicum virgatum* L.). In these feedstocks, C_N ranged from 27 to 127 g kg^{-1} DM, C_B ranged from 34 to 344 g kg^{-1} DM, k_B ranged from 0.071 to 0.415 h^{-1}, total available carbohydrates (C_A) ranged from 61 to 517 g kg^{-1} DM, and Klason lignin ranged from 139 to 244 g kg^{-1} DM.

The next study evaluated multiple high-throughput (HTP) modifications to the original assay methods, including (i) using filter bags with batch sample processing,

(ii) replacement of AIR with neutral detergent fiber (NDF) as a cell-wall isolation procedure, and (iii) elimination of the fermentation organism in the SSCF procedures used to determine biochemically available carbohydrates. The original and the HTP assay methods were compared using corn cobs, hybrid poplar, kenaf, and switchgrass. Biochemically available carbohydrates increased with the HTP methods in the corn cobs, hybrid poplar, and switchgrass, but remained the same in the kenaf. Total available carbohydrates increased and unavailable carbohydrates decreased with the HTP methods in the corn cobs and switchgrass and remained the same in the hybrid poplar and kenaf. There were no differences in total carbohydrates (C_T) between the two methods. The HTP methods consistently assayed less lignin than did the original method. Despite the slight differences parameter values, the HTP assay methods essentially gave a similar summary of the feedstock quality as did the original assay methods while significantly reducing the time and cost for feedstock quality analysis. The HTP assay methods was used to analyze 19 additional biomass feedstocks, including cool-season grasses, warm-season grasses, corn residues, and woody materials.

The final study evaluated the variability of biomass quality parameters in a set of corn stover samples, and developed calibration equations for determining parameter values using near infrared reflectance spectroscopy (NIRS). Fifty-two corn stover samples harvested in Iowa and Wisconsin in 2005 and 2006 were analyzed using the HTP assay for determining feedstock quality for biochemical conversion. Non-structural carbohydrates ranged from 84 to 155 g kg^{-1} DM, C_B ranged from 354 to 557 g kg^{-1} DM, k_B ranged from 0.20 to 0.33 h^{-1}, C_A ranged from

463 to 699 g kg^{-1} DM, and neutral detergent lignin (NDL) ranged from 32 to 74 g kg^{-1} DM. Significant differences ($P<0.0001$) among samples were observed for all parameters, except for the availability rate constant of C_B. Near infrared reflectance spectroscopy calibration equations were developed for C_N, C_B, C_A, C_U, C_T, and NDL. It was not possible to generate a meaningful calibration equation for k_B.

Chapter 1.

General Introduction

Dissertation organization

This dissertation is organized into six chapters. The first chapter contains a general introduction, consisting of a description of the dissertation organization and objectives and a comprehensive literature review. The second chapter contains the manuscript “Bioavailability of cell-wall carbohydrates in *brown midrib* corn hybrids during pretreatment and conversion to ethanol.” The third chapter contains the manuscript “Carbohydrate availability model for determining lignocellulosic biomass feedstock quality.” The fourth chapter contains the manuscript “High-throughput assay for screening biomass feedstocks for biochemical conversion to fuel”. The fifth chapter contains the manuscript “Rapid biomass quality determination of corn stover using near infrared reflectance spectroscopy.” All manuscripts were prepared for publication in Bioenergy Research. The sixth chapter contains general conclusion derived from the four manuscripts.

Objectives

The overall objectives of this dissertation work were twofold: (1) to evaluate *brown midrib* corn stover as feedstock for ethanol production and (2) to develop a system for characterizing lignocellulosic biomass quality that can be used to screen biomass feedstocks in the laboratory and at future biorefineries. The objective of the first study (Chapter 2) was to determine how *brown midrib* mutations *bm1-bm4* in corn and pretreatment severity influence substrate availability of corn stover for ethanol production. The objectives of the second study (Chapter 3) were to develop a series of parameters for characterizing biomass quality of feedstocks for biochemical conversion to fuel and to develop an assay to determine the quality parameters. The objectives of the third study (Chapter 4) were to evaluate multiple high throughput modifications to the biomass quality assay developed in the previous study and to validate the high throughput assay on diverse feedstock types, including cool-season grasses, warm-season grasses, corn residues, and woody materials. The objectives of the fourth study (Chapter 5) were to evaluate the variability of quality parameters for a set of corn stover samples and to determine which, if any, parameter values could be predicted using near infrared reflectance spectroscopy.

Literature Review

Production of domestic renewable fuels lessens U.S. dependence on foreign petroleum resources, decreases the net environmental impacts of liquid fuel usage when compared to petroleum-derived fuels, and provides additional demand for U.S. agricultural products [1, 2]. The Energy Independence and Security Act of 2007 mandates the production of 136 billion L of renewable fuels by 2022, with 60 billion L derived from lignocellulosic materials [3] via biochemical and/or thermochemical conversion platforms. It is expected that a considerable portion of the lignocellulose-derived fuels will be produced by biochemical conversion – that is, pretreatment followed by enzymatic hydrolysis and fermentation – due to the high commercialization potential of this conversion technology, as evidence by the current number of federal-funded demonstration projects using biochemical conversion technology [4]

Pretreatment, hydrolysis, and fermentation

Fermentation and enzymatic hydrolysis, an enzyme-catalyzed process, are thought to be the most flexible biochemical means to produce biobased industrial products [2] and transportation fuels [1] from lignocellulosic materials.

The goal of pretreatment is to improve the accessibility of cell-degrading enzymes in order to increase fermentable sugar yields from lignocellulosic materials. Increased accessibility is achieved by altering the physical and chemical association

between lignin and hemicellulose and by disrupting the crystalline structure of cellulose [4]. An effective pretreatment technology possesses the following characteristics: improves the formation of fermentable sugars during hydrolysis, avoids degradation or loss of carbohydrates, limits formation of byproducts inhibitory to hydrolysis and fermentation, such as furfural, weak acids, and phenols, and is cost effective [6]. Pretreatment constitutes approximately 33% of the cost total of processing [7], so employment of low-cost technologies is highly desirable. Pretreatment methods can be classified as physical, chemical, or biological; however some methods utilize both physical and chemical means. Some of the more promising technologies include comminution or particle size reduction, steam explosion, ammonia fiber explosion (AFEX), acid treatment, and alkali treatment, but many other methods have been studied [5, 6, 8].

The purpose of hydrolysis is to convert structural carbohydrates to fermentable sugar. In addition to enzymatic hydrolysis, hydrolysis can be carried using concentrated or dilute mineral acids. Concentrated acid hydrolysis operates at room temperature using 72% sulfuric acid and dilute acid hydrolysis occurred at elevated temperatures (100–220 ºC) using acid concentrations of about 1% by weight [9]. However, the harsh conditions can result in degradation of fermentable sugars to undesirable compounds (i.e., furfural and hydroxymethylfurfural) and collection and recycling of acid is necessary for this process to be economic viable [10]. Acid hydrolysis also requires the use of corrosion resistant equipment and the resulting hydrolyzed material must be neutralized before fermentation.

Enzymatic hydrolysis has many advantages over acid hydrolysis, including milder reaction conditions, limited degradation of pentose sugars during cellulose hydrolysis, and less need for corrosive-resistant equipment. As a result, processing costs are lower for enzymatic hydrolysis [11]. In addition, hydrolysis and microbial fermentation can occur simultaneously in the same reactor, reducing the potential for end product inhibition of hemicellulases and cellulases due to the accumulation of monomeric sugars. This process is referred to as simultaneous saccharification and fermentation (SSF). Enzymatic hydrolysis is catalyzed by hemicellulase and cellulase enzymes, which are mixtures of several enzymes that work synergistically to breakdown hemicellulose and cellulose into their component sugars. Much of the recent research in the area of enzymatic hydrolysis has focused on improvement of cellulase activity and development of more economical methods for cellulase production [12]. At present, high enzyme costs prohibit competition of cellulose-derived commodity fuels and chemicals with conventional production methods [13]. However, the U.S. Department of Energy has contracted with commercial enzyme producers to drastically lower cellulase costs [14].

Soluble carbohydrates are converted to a variety of chemical products during fermentation by a host of microorganisms, including bacteria and fungi species. Of these products, ethanol production has been studied most extensively. Ethanol from biomass, or cellulosic ethanol, is produced by hydrolyzing cellulose to glucose and fermenting the sugar to ethanol by yeast. Production of ethanol from pentose sugars (xylose and arabinose), hydrolyzed from hemicellulose, is less attractive because yeasts can not directly ferment ethanol, yields are low, and detoxification of

the hemicellulose hydrolyzate is necessary to achieve favorable conversion [15, 16, 17].

Biomass feedstocks

U.S. agricultural and forest lands have the potential to supply over 1 billion metric dry tons of lignocellulosic materials annually, for production of renewable fuels, chemicals, and other products [18]. Most of the biomass supply originates from two main sources: agricultural and forestry residues and dedicated energy crops.

Agricultural and forestry residues can be described as excess biomass resulting after the harvest of agriculture and silviculture crops. Generation of these residues can be characterized as being relatively dispersed and therefore they would require collection and transportation to a central facility for use in a biomass conversion process. Agricultural and forestry residues include materials such as corn stover, wheat straw, manure from livestock operations, tree residues from logging, and trees not merchantable for lumber production.

Dedicated energy crops refer to lignocellulosic crops grown specifically for energy production, whether for combustion to produce electricity or for conversion to fermentable carbohydrates for production of liquid transportation fuels. As with agricultural and forestry residues, production of dedicated energy crops is characterized as being dispersed. Grain may be considered a dedicated energy crop; however its primary purpose is for food and feed, rather than for production of biobased products. Dedicated biomass crops are grouped into two categories:

herbaceous energy crops and short-rotation woody crops [9]. Herbaceous crops are plant species that contain minimal or no woody tissues and are harvested one to three times per year. Herbaceous energy crops include crops such as forage sorghum, kenaf, reed canarygrass, miscanthus, switchgrass, big bluestem, and eastern gamagrass. Short-rotation woody crops contain primarily woody tissues and are harvested on a 3 to 10 year rotation. Woody crops include species such as hybrid popular, eucalyptus, willow, silver maple, sycamore, and mesquite.

The National Research Council [2] has identified cropland idled under the Conservation Reserve Program as possibly available to produce additional biomass. This federal program provides payments to land owners and farmers who remove highly erodible cropland from production for a period of 10 to 15 years. These lands could be planted to perennial cool or warm season grasses, depending on the location, and produce modest biomass yields, while continuing to control erosion.

Currently corn stover is the most abundant biomass feedstock with an estimated 75 million dry metric tons available for collection, annually [18]. Biochemical conversion of corn stover is limited by the recalcitrance of cell-wall materials in corn stover to hydrolysis is impacted by a number of structural features including crystallinity, lignification, and acetylation [19]. The close association of cell-wall carbohydrates with lignin creates a physical barrier to their accessibility by hydrolytic enzymes [20, 21]. Genetic manipulation of lignification while maintaining lignin structure-function within the plant has potential to improve ethanol yield from lignocellulosic feedstocks.

The *brown midrib* (*bm*) mutations in corn presents an avenue for altering lignin concentration and/or composition [22]. Corn *bm* genotype exhibit a reddish brown pigmentation associated with lignified tissues of the leaf midrib and stalk pith, at the four to six-leaf of growth. Since its discovery in dent corn in 1924, four *bm* mutants have been documented: *bm1* [23], *bm2* [24], *bm3* [25], and *bm4* [26]. The *bm*1 mutation results from differential expression of the cinnamyl alcohol dehydrogenase gene [27]. The *bm*3 mutation results from structural changes in the caffeic acid O-methyltransferase gene [28] and a number of deletion mutations have been identified [29]. No information is currently available on the genes affected in the *bm*2 or *bm*4 mutations.

Investigation of *bm* corn stover as feedstock for ethanol production has been limited. Vermerris et al. [30] evaluated glucose yields from the enzymatic saccharification of unpretreated corn stover from *bm* single and double mutations and a inbred non-*bm* control. They found that glucose released during enzymatic hydrolysis from *bm1* and *bm3* corn stover was higher than the non-*bm* control while the *bm2* and *bm4* mutations produced glucose yields that were similar to the control [30]. The *bm1*-*bm3* double mutation produced the highest glucose yield which was roughly twice the yield of the control [30]. When the control, *bm2*, and *bm3* materials were subjected to varying severities of pH-controlled pretreatment, there were no differences in xylose release during pretreatment across genotypes [30].

Biomass quality characterization

Evaluating the quality of lignocellulosic biomass feedstocks is important throughout the feedstock supply chain. Characterizing biomass quality is important to plant breeders, agronomists, and foresters, at the beginning of the supply chain, for evaluating genetic improvements and management practices for feedstock production. Biomass quality is equally important to biorefinery personnel at the end of the supply chain. Feedstock quality information could be used to determine the economic value of a load of biomass at the point of delivery and make process adjustment in the biorefinery based on incoming feedstock quality.

Biomass grading systems have been developed for the forage industry, but a single system has not been widely implemented because grading systems are specific to forage species and end-use [31]. Grading systems are based either on analytical values, - determined using wet chemistry methods or near infrared reflectance spectroscopy (NIRS) - organoleptic assessment, or a combination of the two methods [32]. Systems based on analytical parameters, such as crude protein and relative feed value, would not be applicable for grading cellulosic ethanol feedstock because the parameters are related to value of biomass as a feedstuff for ruminant animals, not for producing ethanol. Systems which use visual characteristics, such as dustiness, color, or maturity, are subjective and many of the characteristics are unrelated to the potential of a feedstock for conversion to ethanol.

Laboratory methods used to characterize lignocellulosic biomass properties have potential as de facto-biomass quality analyses. These methods comprise

analyses used to determine lignocellulosic composition (hemicellulose, cellulose, lignin content) including monomeric sugar composition of the lignocellulose constituents (glucose, xylose content, etc.), fermentability (ethanol or fermentable sugar yield), cellulose polymerization, cellulose crystallinity, and degree of acetylation [19, 33, 34].

Compositional analysis and enzymatic saccharification analyses are the most often employed characterization methods used by biomass researchers; however, each technique has advantages and disadvantages as a method for determining biomass quality. Compositional analysis can provide extensive data on the carbohydrate makeup of a biomass sample, but it does not indicate the availability of component sugars in carbohydrate groups, specifically hemicellulose and cellulose, for hydrolysis and fermentation. This is a result of the differences in the structural features of plant cell wall materials, including specific surface area, cellulose crystallinity, cellulose reactivity, degree of polymerization, lignin content, and degree of acetylation [33]. Fermentable sugar yields from enzymatic saccharification have been successfully correlated to these structural features for a broad range of biomass materials [19]; therefore, enzymatic saccharification serves as a simplified method to determine the impact of structural features on the availability of hemicellulose and cellulose for hydrolysis. Simultaneous saccharification and fermentation (SSF) is an often used saccharification method in biofuels research; however, direct comparison of results produced by different researchers is made difficult but there is minimal standardization in hydrolysis and fermentation conditions between researchers which make direct comparison of results difficult [35].

Enzymatic saccharification also does not provide the spectrum of carbohydrate data that compositional analysis is capable of providing. Combining compositional analysis and enzymatic saccharification into single method would produce a concise and robust assay for determining quality of lignocellulosic biomass feedstocks.

The objectives of the following studies were to evaluate *brown midrib* corn stover as feedstock for ethanol production and develop a system for characterizing lignocellulosic biomass quality that can be used to evaluate genetic improvements and management practices for producing biomass feedstocks. If successful, derivatives of this method might be deployed at future biorefineries for process control.

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Chapter 2.

Bioavailability of cell-wall carbohydrates in *brown midrib* corn hybrids during pretreatment and conversion to ethanol

A manuscript submitted to BioEnergy Research

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Abstract

Brown midrib (*bm)* mutations in corn (*Zea mays* L.) result in altered lignin structure and composition of cell walls, an important factor influencing the recalcitrance of lignocellulosic materials to biochemical conversion for biofuel production. We investigated the potential of corn stover from *bm1-4* hybrids for increased ethanol production and reduced pretreatment intensity compared to corn stover from the isogenic *normal* hybrid. Corn stover from hybrid W64A X A619 and respective

isogenic *bm* hybrids was pretreated using aqueous ammonia steeping by soaking materials for 24 h in ammonium hydroxide concentrations of 0, 3.75, 7.5, 15, and 30% (w/w) and resulting residues underwent simultaneous saccharification and co-fermentation (SSCF) to ethanol. The *bm* genotypes generally had lower hemicellulose, cellulose, and lignin concentrations than the *normal* genotype. A high correlation coefficient (R=0.88) was observed between dry matter (DM) digested during SSCF and ethanol yield, so DM digested by SSCF was used as a surrogate for ethanol production. Dry matter digested by SSCF increased with increasing ammonium hydroxide concentration across all genotypes (P>0.0001) from 277 g kg^{-1} DM in the control to 439 g kg^{-1} DM in the 30% ammonium hydroxide pretreatment. The *bm* corn stover materials averaged 373 g kg^{-1} DM of DM digested by SSCF compared with 335 g kg^{-1} DM for the *normal* corn stover (P<0.0001). Of the *bm* mutations, *bm3* had (i) the greatest effect on cell-wall carbohydrate hydrolysis of corn stover, (ii) the lowest initial cell-wall carbohydrate concentration, (iii) the lowest DM remaining after pretreatment, and (iv) the highest amount of monosaccharides released during enzymatic hydrolysis. Use of *bm* corn stover did not reduce the severity of aqueous ammonia steeping pretreatment needed to maximize DM hydrolysis during SSCF compared with *normal* corn stover.

Keywords Aqueous ammonia pretreatment, *Brown midrib* mutation, Corn stover, Lignocellulosic feedstocks, Simultaneous saccharification and co-fermentation

Abbreviations

bm	*brown midrib*
bm1	*brown midrib*-1 genotype
bm2	*brown midrib*-2 genotype
bm3	*brown midrib*-3 genotype
bm4	*brown midrib*-4 genotype
DM	dry matter
SSCF	simultaneous saccharification and co-fermentation
SHF	separate hydrolysis and fermentation
NDF	neutral detergent fiber
ADF	acid detergent fiber
ADL	acid detergent lignin
FPU	filter paper units
CBU	cellobiose units
HPLC	high performance liquid chromatography
DML	dry matter loss
IVDMD	*in vitro* dry matter digestibility

Introduction

Production of domestic renewable fuels lessens U.S. dependence on foreign petroleum resources, decreases the net environmental impacts of liquid fuel usage when compared to petroleum-derived fuels, and provides additional demand for U.S. agricultural products [1, 2]. The Energy Independence and Security Act of 2007 mandates the production of 136 billion L of renewable fuels by 2022, with 60 billion L derived from lignocellulosic materials [3] via biochemical and/or thermochemical conversion platforms.

Corn (*Zea mays* L.) stover is the most abundant agricultural biomass resource in the U.S. [4] and has been examined extensively as a lignocellulosic feedstock for ethanol production [5]. Corn stover is the residue remaining after grain harvest, including the leaves, stalk, husks, and cobs of the corn plant. Pretreatment followed by enzymatic hydrolysis and fermentation has been studied as a means to convert cell-wall carbohydrates in corn stover to ethanol [6].

The recalcitrance of cell-wall materials in corn stover to hydrolysis is impacted by a number of structural features including crystallinity, lignification, and acetylation [7]. The negative relationship between cell-wall digestibility and lignification in both ruminant nutrition and biomass conversion is well documented [7, 8, 9]. Lignin is a complex polymer derived from monolignols – *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol – produced via the phenylpropanoid pathway. Lignin deposition occurs after cell elongation ceases, forming cross-linked structures with hemicellulose and possibly other cell-wall constituents [8, 10]. Functionally, lignin

facilitates water transport in xylem tissues, provides mechanical strength for plant stems, and defends against attack by pests and pathogens [11]. However, its close association with cell-wall carbohydrates creates a physical barrier to their accessibility by hydrolytic enzymes [8, 9]. Genetic manipulation of lignification while maintaining lignin structure-function within the plant has potential to improve ethanol yield from lignocellulosic feedstocks.

Brown midrib mutation

The *brown midrib* (*bm*) mutations in corn presents an avenue for altering lignin concentration and/or composition [12]. Corn *bm* genotype exhibit a reddish brown pigmentation associated with lignified tissues of the leaf midrib and stalk pith, at the four to six-leaf of growth. Since its discovery in dent corn in 1924, four *bm* mutants have been documented: *bm1* [13], *bm2* [14], *bm3* [15], and *bm4* [16]. The *bm*1 mutation results from differential expression of the cinnamyl alcohol dehydrogenase gene [17]. The *bm*3 mutation results from structural changes in the caffeic acid O-methyltransferase gene [18] and a number of deletion mutations have been identified [19]. No information is currently available on the genes affected in the *bm*2 or *bm*4 mutations.

Application of *bm* mutations in corn for improvement of forage quality has been investigated extensively over the past 40 years [10, 20]. Overall, lignin concentration of *bm* genotypes has been found to be lower than isogenic *normal* counterparts resulting in a increase in dry matter (DM) digestibility of forage from *bm*

corn when compared to conventional corn [21, 22]. Increases in DM intake, milk production, and body weight gain have been observed in lactating dairy cattle when fed *bm* corn silage compared to silage from *normal* corn [23, 24]. Keith et al. [25] also reported an increase in DM intake in steers and heifers when *bm* compared to *normal* corn silage was fed.

In contrast, performance of *bm* corn stover as feedstock for ethanol production has not been examined extensively. Vermerris et al. [26] evaluated glucose yields from the enzymatic saccharification of unpretreated corn stover from *bm* single and double mutations and an inbred, non-*bm* control. They found that glucose released from *bm1* and *bm3* corn stover was higher than the non-*bm* control, whereas the *bm2* and *bm4* mutations produced glucose yields that were similar to the control [26]. The *bm1-bm3* double mutation produced the highest glucose yield which was roughly twice the yield of the control [26]. When the control, *bm2*, and *bm3* materials were subjected to varying severities of pH-controlled pretreatment, there were no differences in xylose release during pretreatment across genotypes [26]. To our knowledge, no additional work has been done to investigate the potential of *bm* corn stover as a bioenergy feedstock.

The applicability of *bm* corn stover as a feedstock in a biochemical conversion platform - i.e., pretreatment followed by enzymatic hydrolysis and fermentation - is of particular interest, due to the high commercialization potential of this conversion technology. Pretreatment increases the accessibility of cell-wall carbohydrates during hydrolysis by altering the physical and chemical association between lignin and hemicellulose and disrupting the crystalline structure of cellulose [27].

Pretreatment costs are estimated to constitute approximately 20% of the total cost of producing ethanol from lignocellulosic materials [28], so reductions in pretreatment requirements are highly desirable. Pretreatment intensity is likely the most important process variable directly impacting the rate and extent of enzymatic hydrolysis. We hypothesize that *bm* genotypes will produce more ethanol than conventional genotypes, as well as reduce the pretreatment intensity necessary to maximize ethanol yield.

Pretreatment, enzymatic hydrolysis, and fermentation

A number of physical, chemical, and biological methods have been investigated as pretreatment technologies [27, 29, 30]. For this study, aqueous ammonia soaking was selected as the pretreatment method because it has been examined extensively on a range of herbaceous biomass feedstocks [31, 32, 33] and it can be implemented at room temperature and atmospheric pressure. Simultaneous saccharification and co-fermentation (SSCF) was used as the combined enzymatic hydrolysis and fermentation method in this study. Use of SSCF allows for hydrolysis of structural carbohydrates and fermentation of hexose and pentose sugars to occur in one vessel using a single fermentation organism. It also overcomes end-product inhibition of enzymes and reduces the possibility of bacterial contamination which can occur in separate hydrolysis and fermentation (SHF) systems [30].

The objective of this study was to determine how *brown midrib* mutations *bm1-bm4* in corn and pretreatment severity influence substrate availability of corn stover for ethanol production.

Materials and Methods

An experiment was conducted to compare the effects of four *bm* mutations in corn and the severity of pretreatment by aqueous ammonia steeping on the hydrolysis of carbohydrates during pretreatment and SSCF of corn stover for ethanol production. Corn stover samples containing the *bm1*, *bm2*, *bm3*, and *bm4* mutations and an isogenic *normal* sample were pretreated in solutions of 0, 3.75, 7.5, 15, and 30% (w/w) ammonium hydroxide for 24 h. Pretreatment residues underwent SSCF using a mixture of commercial cellulase and cellobiase enzymes and a hexose and pentose fermentor. Ethanol production was measured during SSCF and the fermentation residues collected by filtration. Initial substrates, pretreatment residues, and fermentation residues were digested and the neutral sugars analyzed to determine carbohydrate digestibility during pretreatment and fermentation.

Corn stover preparation

Plots of corn hybrid W64A x A619 (*normal*) and *bm*1, *bm*2, *bm*3, and *bm*4 isogenic lines of hybrid W64A X A619 were grown near Ames, Iowa in 2005 in a randomized complete block design with three replications. Corn stover (leaves, stalk, and husks)

was harvested in October 2005 using a commercial silage chopper modified for small plot research following manual harvest of the corn ear (grain and cob). Subsamples of the harvested corn stover were dried at 38°C for 4 d, ground using a Udy mill (Udy Corporation, Fort Collins, CO) to pass through a 1-mm screen, and allowed to equilibrate to laboratory conditions. Dry matter was determined for ground samples by drying 0.5 g of sample at 103°C in a forced-air oven for 4 h to correct for the moisture contained in the samples in subsequent analyses. Initial samples were analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) to determined hemicellulose, cellulose, and ADL concentration. Neutral detergent fiber and ADF were determined according to Vogel et al. [34] using the ANKOM fiber system [35, 36]. Acid detergent lignin was analyzed using the ANKOM procedure [37] and corrected for ash concentration by ashing the samples at 550°C for 4 h in a muffle furnace. Hemicellulose concentration was calculated as the difference between NDF and ADF values and cellulose concentration was calculated as the difference between ADF and the sum of ADL and ash concentration.

Pretreatment

Duplicate sets of samples were pretreated with 0, 3.75, 7.5, 15, and 30% (w/w) ammonium hydroxide solutions using an ammonia steeping procedure similar to Isci et al. [38]. Samples weighing 0.5 g were transferred into preweighed F57 filter bags (ANKOM Technology, Macedon, NY), shortened to 4.5 cm to improve bag

movement within the pretreatment reactor, and the bags were subsequently heat sealed. The filter bags are designed for sequential digestion methods [34] and simplify the sample transfer and washing procedures that normally accompany small-scale pretreatment trials. Samples receiving the same pretreatment solution within each field replicate plus two blank bags containing no sample material (12 total filter bags) were placed into 1-L polyethylene bottles with 200 mL of the respective pretreatment solution. The bottles were capped, shaken until the filter bags were thoroughly saturated with solution, and allowed to incubate for 24 h at room temperature (23°C) in a fume hood. After incubation, filter bags were manually squeezed to remove excess pretreatment solution and washed using deionized water for 15 min, with intermittent manual squeezing to remove any residual ammonium hydroxide. One set of filter bags was freeze-dried for subsequent SSCF, which is necessary because heated drying can collapse the cell wall matrix of pretreated biomass [39], limiting enzyme accessibility. The other set was dried for 4 h at 103 °C and reweighed to determined DM loss during pretreatment. Blank bags were used to adjust for weight loss in the filter bags during pretreatment. Following weighing, pretreatment residues were stored in a desiccator until further analysis.

Simultaneous Saccharification and Co-Fermentation (SSCF)

The set of pretreated and freeze-dried samples were digested by SSCF using a modification of the procedure described by Wyman et al. [6]. Speyzme CP (Genencor, Rochester, NY; Lot No. 301-05021-011) and Novozyme® 188 (Sigma-

Aldrich No. C6150; Lot No. 037K0698) were used as hydrolytic enzymes and recombinant *Escherichia coli* ATCC® 55124 (KO11) (American Type Culture Collection, Rockville, MD) was used as a fermentation organism capable of fermenting both hexose and pentose sugars to ethanol. Spezyme CP was assayed at 71 filter paper units (FPU) mL^{-1} [40] and Novozyme® 188 was assayed at 268 cellobiase units (CBU) mL^{-1} [41].

Stock enzyme, chloramphenicol, and inoculum solutions were made as follows: The enzyme solution was prepared using sterile deionized water such that the activity of the Spezyme CP was 60 FPU g^{-1} of the sample material and the activity of the Novozyme® 188 was 2 CBU FPU^{-1} in the final 10-mL fermentation volume. The chloramphenicol solution was prepared by filter sterilizing a stock solution of 400 μg mL^{-1} chloramphenicol through a 0.2 μm nylon filter. The inoculum solution was prepared using a 1-mL pellet of freeze-dried, chloramphenicol resistant *E. coli* KO11 culture. A 100 mL working volume of culture broth, containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 2% (w/v) glucose was prepared in a 250 mL DeLong fermentation flask with aluminum closure (Bellco Glass, Vineland, NJ) and autoclaved at 121°C for 20 min. The *E. coli* pellet and 2 mL of filter sterilized 2 mg mL^{-1} chloramphenicol solution were added aseptically to the fermentation flask and the flask was vortexed and incubated at 35°C for 24 h in a shaking water bath (New Brunswick Scientific, Edison, NJ) at 150 rpm.

Pretreated, freeze-dried materials weighing 0.3 g were loaded into 25 mL DeLong fermentation flasks fitted with aluminum closures (Bellco Glass, Vineland, NJ) to maintain anaerobic conditions during fermentation. To the flasks, 1 mL 1.0 M

phosphate buffer (pH 5.8), 1 mL growth media solution (0.1 g mL^{-1} tryptone, 0.05 g mL^{-1} yeast extract, 0.1 g mL^{-1} NaCl), and 5 mL deionized water was added and autoclaved at 121 °C for 20 min. After cooling, 1 mL of enzyme solution, 1 mL of chloramphenicol solution, and 1 mL of *E. coli* inoculum was added aseptically to each flask. The final 10-mL fermentation volume was buffered to pH 6.0 and contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 40 μg mL^{-1} chloramphenicol.

Samples were incubated at 35°C for 96 h in a shaking water bath at 150 rpm. Maximum ethanol production would ideally be analyzed at the endpoint of fermentation (96 h); however, previous SSCF experiments have shown that under fermentable-sugar limiting conditions, *E. coli* KO11 converts ethanol to acetic acid (unpublished data). A previous experiment using the same procedures as above and a substrate of pretreated corn stover found that ethanol concentration was maximized at 24 h of fermentation.

After 24 h of incubation, flasks were sampled aseptically for ethanol production by centrifuging a 2 mL aliquot of fermentation broth at 11,900 g for 10 min and syringe filtering the liquid fraction through a 0.2 μm cellulose acetate filter. The residue pellets were resuspended with 1.5 mL of sterile deionized water, returned to the flasks, and incubation continued. After 96 h of incubation, the fermentation residues were collected by vacuum filtering the fermentation liquids through preweighed Whatman #54 filter papers (20-25 μm particle size retention) and washing the residue with 50 mL of deionized water. Filter papers were dried for 4 h in a 103°C oven, and reweighed to determine dry matter digested during SSCF.

Blank filter papers and composite samples of the unfermented freeze-dried materials were also dried to correct for moisture in the preweighed filter papers and moisture in the initial SSCF samples, respectively. Following weighing, SSCF residues were stored in a desiccator until further analysis.

Chemical analyses

Fermentation filtrates were analyzed for ethanol by high performance liquid chromatography (HPLC) with a refractive index detector (Varian 355 RI Detector, Varian, Palo Alto, CA) using an Aminex® HPX-87H column (Bio-Rad Laboratories, Hercules, CA). Separations were made using 0.01 N sulfuric acid as the mobile phase at a flow rate of 0.6 mL min^{-1}, a column temperature of 65°C, and an injection volume of 20 μL.

Monosaccharides released during pretreatment and SSCF were determined by analyzing neutral sugar composition of the initial substrates, pretreatment residues, and SSCF residues using the 2-step hydrolysis procedure in the Uppsala Method [42]. Due to the limited amount of SSCF materials, the procedure was scaled to a sample size of 0.05 g. Samples were weighed into 50-mL test tubes and incubated with 1.25 mL of 12 M sulfuric acid for 1 h at 30 °C. The test tubes were diluted to 0.4 M by addition of 35 mL of water and autoclaved at 125°C for 1 h. After cooling, 1-ml aliquots of the hydrolysates were diluted to a volume of 100 mL in deionized water and filtered using 0.45 μm cellulose acetate syringe filters. Filtrates were analyzed for arabinose, xylose, mannose, galactose, and glucose by HPLC

with an electrochemical detector (Dionex ED 50 Electrochemical Detector, Dionex, Sunnyvale, CA) using a Carbopac™ PA200 column (Dionex, Sunnyvale, CA). Separations were made using 2 mM NaOH as the mobile phase at a flow rate of 0.48 mL min^{-1}, a column temperature of 30°C, and an injection volume of 25 μL.

Statistical analysis

The corn stover field trial was conducted using a randomized complete block design with three replications, as explained previously. All subsequent laboratory procedures were conducted using the same block structure. Statistical analysis was done using SAS software [43]. The significance of the effects of pretreatment severity (ammonium concentration), corn stover genotype, and their interaction on dry matter loss during pretreatment, ethanol production and dry matter digestibility during SSCF, and monomeric sugars released during pretreatment and SSCF was determined using the general linear model procedure. All comparisons were determined to be significant at $p < 0.05$, unless otherwise stated. Trends in data were fitted using the following monomolecular model with the NLIN procedure in SAS where appropriate:

$$Y = A\ (1 - Be^{-kx})$$

where

Y = response variable

A = asymptote value of response variable (theoretical maximum or minimum value)

B = constant associated with Y-intercept

k = rate constant for response to pretreatment severity

x = ammonium hydroxide concentration

Results

The *bm* genotypes had lower hemicellulose, cellulose, and lignin concentrations than the *normal* genotype, with the exception of *bm2* which had a similar hemicellulose concentration to its *normal* counterpart (Table 2.1). On average, *bm* genotypes had 285 g kg^{-1} DM of hemicellulose, 318 g kg^{-1} DM of cellulose, and 19 g kg^{-1} DM of ADL compared with 319 g kg^{-1} DM of hemicellulose, 369 g kg^{-1} DM of cellulose, and 27 g kg^{-1} DM of ADL for the *normal* genotype. Across genotypes, xylose accounted for, on average, 28% and glucose accounted for 64% of the total neutral sugars (Table 2.1). Differences in xylose concentration were fairly consistent with hemicellulose results, with the *normal* genotype having a higher xylose concentration than *bm1* and *bm2*. The *normal* genotype did not yield higher glucose levels than the *bm* genotypes, but glucose concentration was higher in *bm2* and *bm4* than in the *bm3* hybrid. Differences in arabinose and galactose concentration were observed; however, these differences were negligible in comparison to differences in xylose and glucose concentration.

Dry matter remaining after pretreatment ranged from 754 g kg^{-1} DM in the *normal* genotype at 0% ammonium hydroxide to 614 g kg^{-1} DM in the *bm3* genotype at 15% ammonium hydroxide (Figure 2.1). Dry matter recovered decreased as the concentration of ammonium hydroxide increased during pretreatment ($P<0.0001$).

The effect of genotype was also significant (P<0.0001), with the *normal* genotype retaining more DM after pretreatment than the *bm* mutants. The *bm3* mutant had the greatest DM loss, losing on average 32 g kg^{-1} DM more DM than the other *bm* genotypes. Changes in neutral sugar composition during pretreatment were dominated by xylose and glucose (Table 2.2). Xylose and glucose loss did not increase with increasing ammonium hydroxide concentration. Xylose release was the same across genotypes, but glucose release was higher in the *bm* hybrids than the *normal* hybrids.

Ethanol production expressed on the basis of the initial DM prior to pretreatment ranged from 137 to 192 g kg^{-1} DM at 24 h of fermentation (Figure 2.2). Ethanol yield varied across genotype (P<0.05) and ammonium hydroxide concentration (P<0.0001). However, the trends in several genotypes (*bm1*, *bm2*, and *bm4*) across the range of ammonium hydroxide treatments were inconsistent. This resulted in lack of fit when trends were modeled using a monomolecular equation. A high correlation coefficient (R=0.88) was observed between DM digested during SSCF and ethanol yields, so DM digested by SSCF was used as a surrogate for ethanol production (Figure 2.3).

The DM digested by SSCF is expressed on the basis of the initial DM prior to pretreatment and is displayed in Figure 2.4. Dry matter digested increased with ammonium hydroxide concentration across all genotypes (P<0.0001) from 277 g kg^{-1} DM in the control pretreatment to 439 g kg^{-1} DM in the 30% ammonium hydroxide pretreatment. On average, 38 g kg^{-1} DM more DM was digested in the *bm* genotypes compared with the *normal* genotype (P<0.0001). No differences in DM

digested was observed between the *bm* genotypes (P=0.38). Responses to ammonium hydroxide concentration were fitted appropriately using a monomolecular model for each genotype. The interaction of genotype and ammonium hydroxide concentration was not significant (P=0.99). Neutral sugars released during SSCF were dominated by xylose and glucose (Table 2.3). Xylose and glucose released increased as the ammonium hydroxide concentration in pretreatment increased. Xylose hydrolysis was higher in the *bm3* genotype at 100 g kg^{-1} DM compared to all other genotypes which averaged 85 g kg^{-1} DM. Glucose hydrolysis was higher in the *bm* genotypes, averaging 200 g kg^{-1} DM, compared the *normal* genotype at 171 g kg^{-1} DM. Interactions between genotype and ammonium hydroxide concentration on xylose and glucose released during SSCF were also not significant.

Discussion

The lower lignin concentration of the *bm* materials is consistent with past studies [10, 20]. The order of the genotypes in decreasing lignin concentration (*normal*, *bm4*, *bm1*, *bm2*, *bm3*) was the same as observed by Lechtenberg et al. [22] with materials in the Tr inbred background. Cell wall (hemicellulose, cellulose, and lignin), hemicellulose, and cellulose concentration were all lower in the *bm* genotypes in this study. Differences in cellulose and hemicellulose concentrations of *normal* and *bm* corn stover have not been documented across studies, but it is generally accepted that *bm* materials have lower cell wall concentrations than their *normal* counterparts due, in part, to lower lignin concentrations [10].

Carbohydrate availability in corn stover from *bm* genotypes was increased compared with isogenic *normal* corn stover, both in terms of carbohydrates converted to ethanol during enzymatic hydrolysis and fermentation and carbohydrates solubilized during pretreatment. These results are fairly consistent with the work of Vermerris et al. [26]. However, increased levels of glucose were observed in all *bm* materials compared with the *normal* corn stover, whereas Vermerris et al. [26] only reported improvements with *bm1* and *bm3* materials.

Of the *bm* mutations, the *bm3* mutation had the greatest effect on cell-wall carbohydrate digestibility of the corn stover. The *bm3* corn stover had the lowest initial cell-wall carbohydrate concentration, the lowest dry matter remaining after pretreatment, and the highest amount of monosaccharides released during enzymatic hydrolysis. Barnes et al. [21] examined *in vitro* dry matter digestibility (IVDMD) of *bm1*, *bm3*, and *normal* corn stover across a series of harvests and found the *bm3* material to be superior in IVDMD compared with the *normal* and *bm1* materials. Follow up studies with *bm1-bm4* genotypes, further confirm the improvement in IVDMD of corn stover with the *bm3* mutation compared with the other *bm* genotypes and the *normal* genotype [10, 20]. *In vitro* dry matter digestibility is analyzed using a two-step digestion process with rumen microorganisms and acid-pepsin to simulate the action of the ruminant digestion system. There are clear differences in ruminant digestion and pretreatment, enzymatic hydrolysis, and ethanol fermentation processes; however, the enzymatic hydrolysis mechanisms of rumen microorganisms are similar to hydrolysis with commercial cellulase enzymes. Based on this, much of our knowledge about cell-

wall digestibility of *bm* materials as feedstocks for ruminants would be applicable to evaluating *bm* materials as feedstocks for ethanol production.

Values for DM digested by SSCF at the 30% ammonium hydroxide concentration are all within 3% of the maximum DM digested values predicted by the monomolecular models, which suggests that the range of ammonium hydroxide concentrations used in this study adequately bracket the response of the genotypes to pretreatment severity in the aqueous ammonia soaking pretreatment technology. Admittedly, the 0% ammonium hydroxide concentration does not truly present a control treatment due to the need to sterilize (121°C for 20 min) the corn stover prior to SSCF. If these materials were not sterilized and microbial contamination was not to occur during SSCF, it is expected that DM digested in the 0% ammonium hydroxide treated materials would be much lower than what was observed.

Lack of a significant genotype X ammonium hydroxide concentration interaction term with DM digestion during SSCF indicates that the various genotypes responded similarly to increasing pretreatment severity. Pretreatment technologies using aqueous ammonia have been known to reduce lignin concentration in lignocellulosic materials with increasing pretreatment intensity [44]. Dry matter digested during SSCF was 38 g kg^{-1} DM higher in the *bm* genotypes and 50 g kg^{-1} DM higher in the *bm3* genotype and than *normal* genotype. These values are much lower than the differences in IVDMD observed between *bm* and *normal* corn stover materials. Barnes et al. [21] found an average increase of 100 g kg^{-1} DM in IVDMD with corn stover having the *bm3* mutation and *bm1-bm3* double mutation versus *normal* corn stover. Increases in digestibility of 92 and 129 g kg^{-1} DM were observed

in *bm3* corn stems compared than *normal* corn stems [45, 46]. It is likely that the aqueous ammonia soaking pretreatment is so effective at delignification that it effectively reduces the differences in digestibility between *bm* and *normal* genotypes. However, this is an opposite result than reported by Hartley and Jones [46], who found that the digestibility of cell walls treated with NaOH increased more in *bm* than *normal* corn stover. The difference is likely because alkaline pretreatment can result in significant delignification [27], but oxidative conditions are required [47].

It is unclear how *bm* and *normal* materials would perform with pretreatment technologies, where limited or no delignification occurs, such as liquid hot water and dilute acid pretreatment [27]. Future comparisons of isogenic *normal* corn stover and *bm* corn stover, particularly *bm3* materials, as feedstocks for ethanol production should be accomplished using a range of pretreatment technologies to understand how the performance of pretreatment processes – alkaline hydrolysis, acid hydrolysis, oxidation, etc. – are affected by feedstock lignin concentration and structure and which technologies have the greatest potential for pretreating *bm* materials. Additionally, the extent of digestion was only investigated in this study, but the rate of digestion is also an important process variable which affects the size and quantity of SSCF vessels and ultimately the economic feasibility of an ethanol biorefinery. Future investigations with *bm* and *normal* corn stover materials for biofuel production should consider both the extent and rate of digestion during SSCF.

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Table 2.1. Fiber and neutral sugar composition of corn stover from hybrid W64 X A619 (*normal*) and isogenic brown midrib (*bm*) hybrids grown near Ames, Iowa in 2005.*

	Hybrid					
Constituent, g kg^{-1} dry matter	W64A X A619 *normal*	W64A X A619 *bm1*	W64A X A619 *bm2*	W64A X A619 *bm3*	W64A X A619 *bm4*	SE†
Extractives‡	276^{b}	376^{a}	392^{a}	358^{a}	355^{a}	22.0
Hemicellulose	319^{a}	277^{b}	278^{b}	297^{ab}	286^{b}	9.1
Cellulose	369^{a}	317^{b}	302^{b}	322^{b}	330^{b}	12.0
Lignin§	27^{a}	20^{bc}	19^{cd}	16^{d}	23^{b}	1.2
Arabinose	25.1^{b}	24.2^{b}	24.8^{b}	28.0^{a}	24.2^{b}	0.55
Xylose	200^{a}	176^{b}	178^{b}	189^{ab}	189^{ab}	5.4
Mannose	7.6	7.1	7.0	7.8	7.0	0.42
Galactose	15.3^{bc}	16.3^{b}	16.0^{b}	18.3^{a}	14.7^{c}	0.41
Glucose	407^{ab}	413^{ab}	441^{a}	390^{b}	442^{a}	15.2

*Data are means of triplicate samples. Values in the same row with unlike superscript letters are significantly different ($P < 0.05$).

†Standard error of the mean.

‡Neutral detergent extractives.

§Acid detergent lignin.

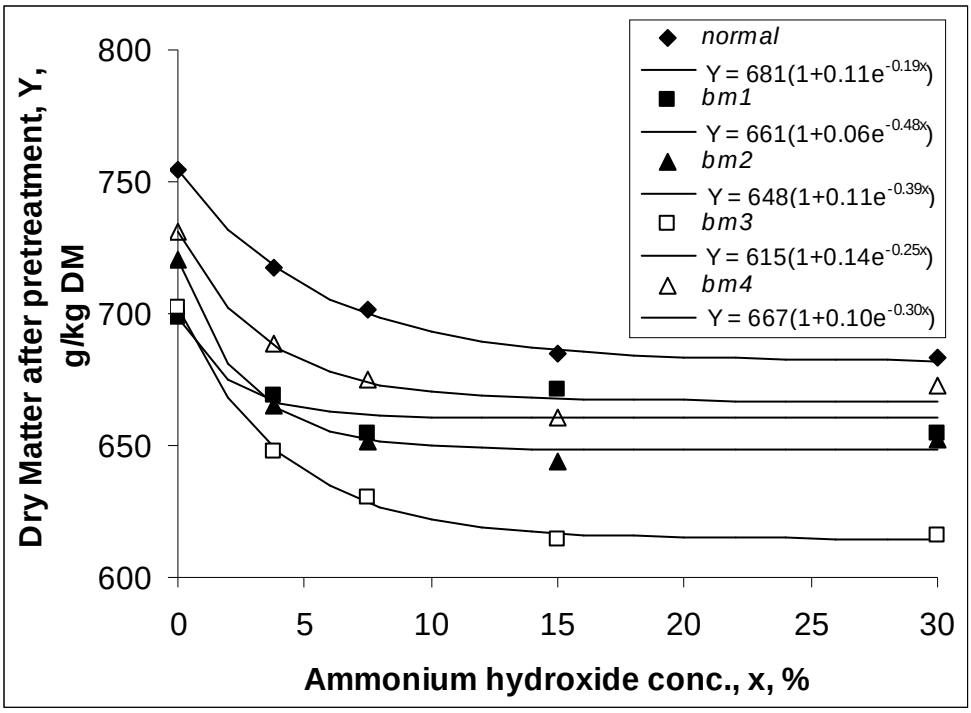

Figure 2.1. Dry matter remaining after pretreatment with ammonium hydroxide from corn stover of W64 X A619 hybrid (*normal*) and isogenic brown midrib (*bm*) hybrids. SE=11 (n=3).

Table 2.2. Neutral sugars lost during pretreatment with ammonium hydroxide from corn stover of hybrid W64 X A619 (*normal*) and isogenic brown midrib (*bm*) hybrids.

	Arabinose	Xylose	Mannose	Galactose	Glucose
	g kg^{-1} dry matter				
Genotype					
normal	5.3	28	3.0	5.8	76
bm1	5.2	24	2.4	6.4	84
bm2	6.2	33	2.8	6.7	123
bm3	7.5	33	3.4	7.7	98
bm4	5.1	30	2.5	5.7	99
SE†	0.43	2.1	0.28	0.17	7.2
Ammonium hydroxide conc., %					
0	5.4	28	2.7	5.6	87
3.75	5.3	25	2.8	6.1	97
7.5	5.7	28	2.7	6.5	99
15	6.7	32	3.0	7.0	103
30	6.2	34	2.9	7.0	93
SE	0.43	2.1	0.28	0.17	7.2

ANOVA

Source	df					
Genotype (G)	4	0.0009	0.0158	NS	<0.0001	0.0005
normal vs *bm*	1	NS‡	NS	NS	0.0002	0.0036
within *bm* types	3	0.0007	0.0086	NS	<0.0001	0.0045
Conc. (C)	4	NS	NS	NS	<0.0001	NS
G X C	16	NS	NS	NS	NS	NS

†Standard error of the mean.

‡NS = Not significant (P>0.05).

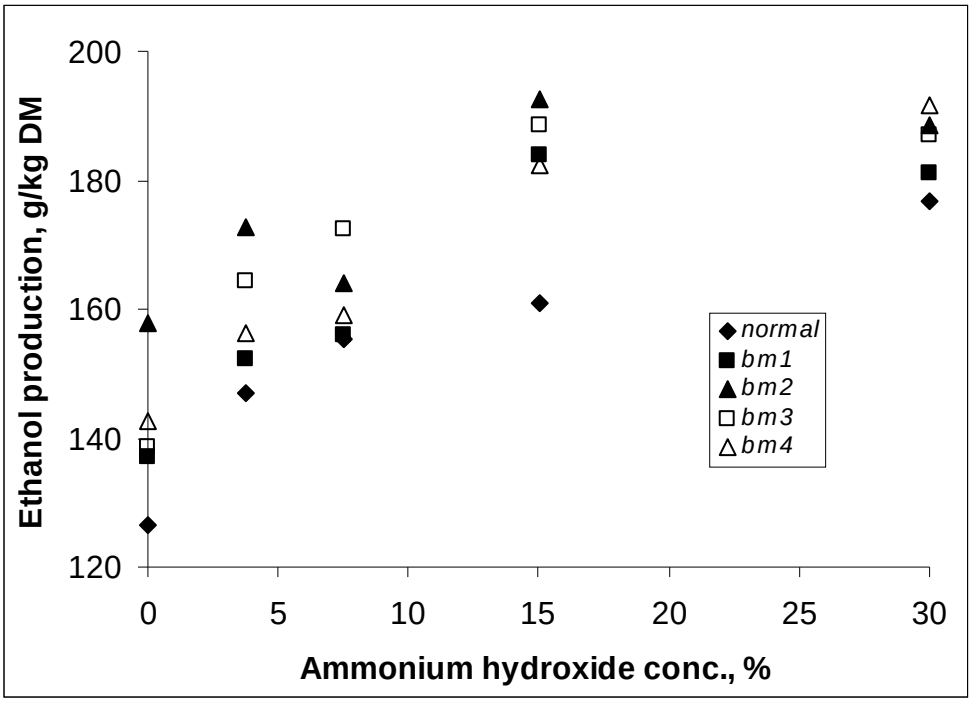

Figure 2.2. Ethanol production from corn stover of hybrid W64 X A619 (*normal*) and isogenic brown midrib (*bm*) hybrids pretreated with ammonium hydroxide. Ethanol production is expressed on a dry matter (DM) basis of the initial material prior to pretreatment. SE=11 (n=3).

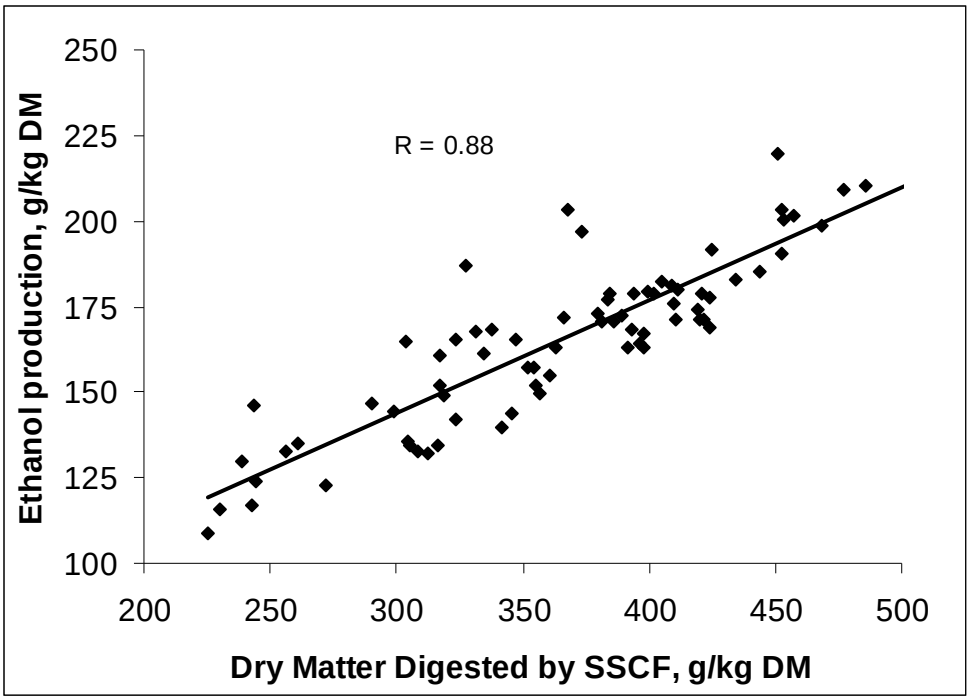

Figure 2.3. Correlation between dry matter (DM) digested during simultaneous saccharification and co-fermentation (SSCF) and ethanol production from corn stover of hybrid W64 X A619 (*normal*) and isogenic brown midrib (*bm*) hybrids pretreated with ammonium hydroxide. Dry matter digested during SSF and ethanol production are expressed on a dry matter basis of the initial material prior to pretreatment.

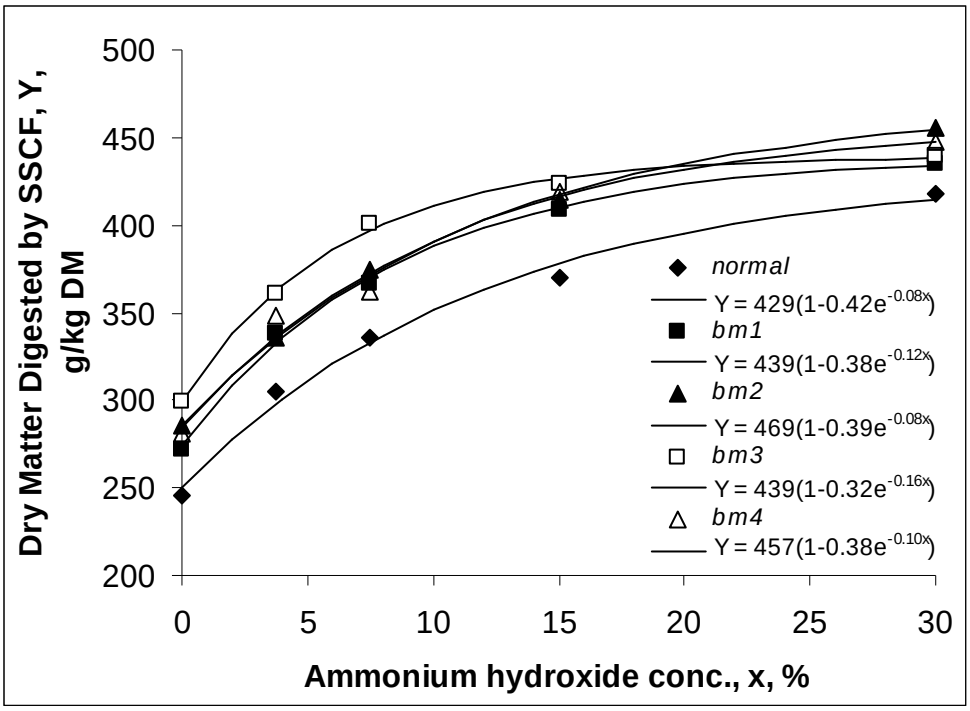

Figure 2.4. Dry matter (DM) digested during simultaneous saccharification and co-fermentation (SSCF) from corn stover of hybrid W64 X A619 (*normal*) and isogenic brown midrib (*bm*) hybrids pretreated with 0 to 30% (w/w) ammonium hydroxide. Dry matter digested is expressed on a dry matter basis of the initial material prior to pretreatment. SE=19 (n=3).

Table 2.3. Neutral sugars hydrolyzed during simultaneous saccharification and co-fermentation (SSCF) from corn stover of hybrid W64 X A619 (*normal*) and isogenic brown midrib (*bm*) hybrids.

	Arabinose	Xylose	Mannose	Galactose	Glucose
	g kg^{-1} dry matter				
Genotype					
normal	14.3	87	3.0	7.1	171
bm1	14.0	86	3.2	7.3	199
bm2	13.1	80	2.8	6.6	200
bm3	16.1	101	3.4	8.2	191
bm4	13.6	86	2.9	6.4	209
SE†	0.46	2.8	0.16	0.18	10.1
Ammonium hydroxide conc., %					
0	9.9	53	2.4	5.3	160
3.75	14.0	78	3.0	7.1	175
7.5	15.0	90	3.2	7.6	191
15	15.5	103	3.3	7.8	208
30	16.7	117	3.4	7.9	235
SE	0.46	2.8	0.16	0.18	10.1

ANOVA

Source	df					
Genotype (G)	4	0.0005	0.0002	NS	<0.0001	NS
normal vs *bm*	1	NS‡	NS	NS	NS	0.0141
within *bm* types	3	0.0002	<0.0001	NS	<0.0001	NS
Conc. (C)	4	<0.0001	<0.0001	0.0005	<0.0001	<0.0001
G X C	16	NS	NS	NS	NS	NS

†Standard error of the mean.

‡NS = Not significant (P>0.05).

Chapter 3.

Carbohydrate availability model for determining lignocellulosic biomass feedstock quality

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Abstract

Development of a system for characterizing lignocellulosic biomass quality is necessary for evaluating genetic improvements and management practices for producing biomass feedstocks for biochemical conversion (i.e., pretreatment, enzymatic hydrolysis, and fermentation). This project was conducted to develop a series of parameters for determining feedstock quality and to develop a laboratory assay to determine the quality parameters. A carbohydrate availability model was

developed, which partitions feedstock carbohydrates within a feedstock material into fractions based on their availability to be converted to fermentable sugars, including non-structural carbohydrates (C_N) – monosaccharides, starches, oligosaccharides –, biochemically available carbohydrates (C_B) – structural carbohydrates susceptible to enzymatic hydrolysis – with an associated 1^{st}-order availability rate constant (k_B), and unavailable carbohydrates (C_U). The model partitions the non-carbohydrate dry matter into extractives, lignin, and ash. The assay combines established compositional analysis and digestibility techniques, including total non-structural carbohydrates (TNC), alcohol insoluble residue (AIR), simultaneous saccharification and fermentation (SSCF), and Klason lignin, to determine model parameters. The assay was used to analyze four compositionally diverse biomass feedstocks: corn cobs (*Zea mays* L.), hybrid poplar (*Populus x canadensis* Moench), kenaf (*Hibiscus cannabinus* L.) and switchgrass (*Panicum virgatum* L.). In this group of feedstocks, C_N ranged from 27 to 127 g kg^{-1} DM, C_B ranged from 34 to 344 g kg^{-1} DM, k_B ranged from 0.071 to 0.415 h^{-1}, total available carbohydrates (C_A) ranged from 61 to 517 g kg^{-1} DM, and lignin ranged from 139 to 244 g kg^{-1} DM. Based on the parameter values, corn cobs would be most amenable to biochemical conversion to ethanol of the feedstocks tested.

Keywords Biomass quality, Lignocellulosic feedstocks, Biochemical conversion, Simultaneous saccharification and co-fermentation

Abbreviations

HTP	high throughput
NIRS	near infrared reflectance spectroscopy
SSF	simultaneous saccharification and fermentation
TD	true digestibility
DM	dry matter
TNC	total non-structural carbohydrates
AIR	alcohol insoluble residue
SSCF	simultaneous saccharification and co-fermentation
CV	coefficient of variation
NDF	neutral detergent fiber

Introduction

U.S. agricultural and forest lands have the potential to supply over 1 billion metric dry tons of lignocellulosic materials annually, for production of renewable fuels, chemicals, and other products [1]. Processing even a small portion of these feedstock materials into biofuels will require a framework for quality determination, analogous to the grading and standardization system for grains established under the United States Grains Standard Act [2]. Because a significant portion of the lignocellulose-derived fuels will be produced by biochemical conversion – that is, pretreatment followed enzymatic hydrolysis and fermentation – due to the high commercialization potential of this conversion technology, we posit that development of a biomass quality system requires: (i) quality parameters that can be correlated to process data and (ii) high throughput (HTP) methods to precisely determine quality parameter values.

De facto biomass quality measures

Biomass grading systems have been developed for the forage industry, but a single system has not been widely implemented because of differences in forage species and animal type [3]. Grading systems are based either on analytical values, – determined using wet chemistry methods or near infrared reflectance spectroscopy (NIRS) – organoleptic assessment, or a combination of the two methods [4]. Systems based on analytical parameters, such as crude protein and relative feed

value, are not directly applicable for grading cellulosic ethanol feedstock because the parameters are related to value of biomass as a feedstuff for ruminant animals, not for producing ethanol. Systems which use visual characteristics, such as dustiness, color, or maturity, are subjective and many of the characteristics are unrelated to the potential of a feedstock for conversion to ethanol.

Laboratory methods used to characterize lignocellulosic biomass properties have potential as de facto-biomass quality analyses. These methods comprise analyses used to determine lignocellulosic composition (hemicellulose, cellulose, lignin content) including monomeric sugar composition of the lignocellulose constituents (glucose, xylose content, etc.), enzymatic saccharification potential (ethanol or fermentable sugar yield), cellulose polymerization, cellulose crystallinity, and the degree of acetylation [5,6,7].

Compositional analysis and enzymatic saccharification tests are the most often employed characterization methods used by biomass researchers; however, each technique has advantages and disadvantages as a method for determining biomass quality. Compositional analysis can provide extensive data on the carbohydrate makeup of a biomass sample, but it does not indicate the availability of these component sugars in carbohydrate groups (e.g., hemicellulose and cellulose), for hydrolysis and fermentation. Differences in availability result from the differences in the structural features of plant cell wall materials, including specific surface area, cellulose crystallinity, cellulose reactivity, degree of polymerization, lignin content, and degree of acetylation [6]. Fermentable sugar yields from enzymatic saccharification have been successfully correlated to these structural features for a

broad range of biomass materials [5]; therefore, enzymatic saccharification serves as a simplified method to determine the impact of structural features on the availability of hemicellulose and cellulose for hydrolysis. Simultaneous saccharification and fermentation (SSF) is an often used saccharification method in biofuels research; but, direct comparison of SFF results produced by different researchers is complicated by a lack of standardization in hydrolysis and fermentation conditions between researchers [8]. Enzymatic saccharification also does not provide the spectrum of carbohydrate data that compositional analysis is capable of providing. Combining compositional analysis and enzymatic saccharification into single method would produce a concise and robust assay for determining quality of lignocellulosic biomass feedstocks.

The objective of this paper is to address the first requirement of developing a biomass quality system, to develop quality parameters that are process-related, in the context of a biochemical conversion platform.

Theory

Model Development

We have developed a model which provides the conceptual framework for determining lignocellulosic biomass quality using a combined compositional analysis and enzymatic saccharification assay. This model is based on a true digestibility (TD) model for ruminant feedstuffs (Figure 3.1) [9]. The TD model partitions plant

dry matter based on its potential for ruminal digestion into cell solubles (C_S), digested fiber (C_D), and undigested fiber (C_I) with a 1st-order rate constant (k), lag time (L), and fermentation time (t) to describe digestion kinetics. Cell solubles include soluble sugars, starches, pectins, and proteins. Digested fiber comprises cellulose and hemicellulose and undigested fiber consist of lignin and the remaining cellulose and hemicellulose. True digestibility is calculated according to Equation 1.

$$TD = C_S + C_D (1 - e^{-k(t-L)}) \quad [1]$$

The carbohydrate availability model we have developed partitions plant dry matter constituents based on their availability for biological conversion – that is, enzymatic hydrolysis and fermentation – as opposed to availability for ruminant digestion (Figure 3.2). Carbohydrates are separated into three groups based on the relative severity of conditions necessary to hydrolyze them to fermentable sugars. These groups include: non-structural carbohydrates (C_N), biochemically available carbohydrates (C_B), and unavailable carbohydrates (C_U). The C_N fraction includes monomeric sugars, oligosaccharides, starches, and fructans and requires none or minimal processing to be converted to fermentable sugars. These sugars can be potentially lost during processing at a biorefinery if washing is needed to remove inhibitory compounds contained in the biomass. The C_B fraction includes structural carbohydrates, hemicellulose and cellulose, which are susceptible to enzymatic hydrolysis. This group is comparable to digested fiber in the TD model, with its availability with time described using a first-order constant, k_B. The C_U group consists of carbohydrates which are unavailable for biological conversion, mainly hemicellulose and cellulose in close association with lignin and inaccessible to

enzymes. Total available carbohydrates, C_A, and total carbohydrates, C_T, are calculated according to Equations 2 and 3.

$$C_A = C_N + C_B \quad [2]$$

$$C_T = C_N + C_B + C_U \quad [3]$$

The remaining constituents are lignin, ash, and extractives, which from the standpoint of biological conversion, generally represent anti-quality factors. Lignin concentration has been found to be the most important factor governing enzymatic saccharification of lignocellulosic biomass to fermentable sugars [10]. Ash content is of interest because high levels can cause excessive wear on material handling equipment, increase energy requirements to grind biomass, and cause fouling in combustors and gasifiers if fermentation residues are converted thermochemically for heat and power [11]. Fermentation inhibitors, such as furfural, weak acids, and phenols [12], are contained in the extractives faction. Proteins are also a constituent of extractives, but do not constitute an anti-quality factor.

Carbohydrate and lignin are most likely to be of most interest to biorefineries. Differing levels of these parameters can affect process operation parameters such as residence time during pretreatment, enzyme loading rates, loading rates for the fermentation organisms, and solids loading rates in reactors. This information can be employed to determine the premium or dockage level for each load of biomass delivered by a producer and to establish a regime for blending biomass to produce a uniform substrate or to make on-line process adjustments based on the quality of the incoming material.

Materials and Methods

Model parameters were determined using a composite gravimetric and spectroscopic assay based on a combination of forage quality, cell wall isolation, enzymatic hydrolysis, and fermentation procedures (Figure 3.3). Parameter values were calculated on the basis of grams of constituent per kg dry matter (DM) of the initial sample. Total-non structural carbohydrates (TNC) were used to determine C_N. Alcohol insoluble residue (AIR) was prepared by removing cell-soluble materials from the initial sample to produce a cell wall isolate suitable for subsequent digestion. Extractives concentration was calculated by subtracting the sum of the AIR and TNC values from 1000.

Biochemically available carbohydrates and the rate constant k_B were determined by digesting the AIR by simultaneous saccharification and co-fermentation (SSCF) using a co-fermenting organism for periods of 4, 8, 24, 48, 72, and 96 hours. Lignin concentration was determined by analyzing the AIR for Klason lignin. Ash concentration was determined by ashing the AIR sample. Unavailable carbohydrates concentration was calculated as the difference between the AIR value and the sum of C_B, lignin, and ash values.

Feedstocks

Switchgrass ‘Cave-in-Rock’ (*Panicum virgatum* L.) and kenaf ‘Tainung 2’ (*Hibiscus cannabinus* L.) were harvested from a field plots near Ames, Iowa in November

2006. Whole corn ‘Pioneer hybrid 34A20’ (*Zea mays* L.) plants were harvested from a field plots near Ames, Iowa in October 2007 and the corn cobs were separated from all other corn grain and stover components by hand. Hybrid poplar ‘Eugenei’ (*Populus x canadensis* Moench) was harvested from Iowa State University poplar breeding plots located in Ames, Iowa in December 2006 and chipped to an average particle size of approximately 2 cm. All samples were dried at 60°C in a forced air oven for 72 h [13] and ground using a Wiley mill (Thomas Scientific Inc., Swedesboro, NJ) fitted with a 1-mm sieve. Dry matter was determined for ground samples by drying 1 g of sample at 103°C in a forced air oven for 4 h to make moisture corrections for all laboratory analyses [13].

Laboratory Procedures

Total non-structural carbohydrates were determined according to a modified procedure of Guiragossian et al. [14], in which, 0.125 g of sample was refluxed in 25 mL of 0.2 N sulfuric acid for 1 h followed by filtering through Whatman No. 42 filter paper. To a 1-mL aliquot of the filtrate diluted by a factor of 20, 1 mL of 5% phenol solution and 5 mL of 18 M sulfuric acid were added, and the solution’s absorbance was measured at 490 nm. TNC values were determined using a glucose reference calibration and calculated on the basis of grams of glucose per kg sample DM.

Alcohol insoluble residue samples were prepared by sequentially digesting 6 g of the initial sample with α-amylase, protease, and amyloglucosidase [15]. Following enzyme treatment, AIR materials were precipitated and washed using

three volumes of 80% (v/v) ethanol. After precipitation with ethanol, the method [15] calls for two washes of acetone followed by oven-drying the sample. We believed that this could irreversibly collapse the cell wall matrix of the materials, limiting subsequent hydrolysis with cellulase enzymes [16]. To remove excess ethanol, samples were washed with two volumes of deionized water and then freeze-dried. Alcohol insoluble residue samples were hydrolyzed and fermented using SSCF procedures described by Murphy et al. [17] using enzyme loading rates of 60 FPU g-AIR^{-1} Spezyme CP and 2 CBU FPU^{-1} Novozyme 188 [8]. Alcohol insoluble residue samples weighing 0.5 g were loaded into 25 mL DeLong fermentation flasks fitted with aluminum closures (Bellco Glass, Vineland, NJ) to maintain anaerobic conditions during fermentation. To the flasks, 1 mL 1.0 M phosphate buffer (pH 5.8), 1 mL growth media solution (0.1 g mL^{-1} tryptone, 0.05 g mL^{-1} yeast extract, 0.1 g mL^{-1} NaCl), and 5 mL deionized water was added and autoclaved at 121°C for 20 min. After cooling, 1 mL of enzyme solution, 1 mL of chloramphenicol solution, and 1 mL of *E. coli* inoculum was added aseptically to each flask. The final 10-mL fermentation volume was buffered to pH 6.0 and contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 40 μg mL^{-1} chloramphenicol. Flasks were incubated at 35°C for 4, 8, 24, 48, 72, or 96 h in a shaking water bath at 150 rpm. Saccharification residues were recovered by vacuum-filtration using pre-weighed Whatman No. 42 filter paper, washed with deionized water, and dried for 4 h at 103°C.

Klason lignin was determined on AIR samples according to the two-step sulfuric acid digestion described by Theander et al. [18]. Alcohol insoluble residue

subsamples weighing 0.1 g were loaded into 50-mL test tubes and incubated with 1.25 mL of 12 M sulfuric acid for 1 h at 30°C in a water bath. Solutions were diluted to 0.4 M by addition of 35 mL of water and autoclaved at 125°C for 1 h. Klason lignin residues were recovered by vacuum filtration using pre-weighed glass filter paper, washed with deionized water, and dried for 4 h at 103°C. Klason lignin concentrations were corrected for residual mineral concentration by ashing the filters at 550°C for 4 h. Ash concentrations were determined by ashing a 0.5-g subsample of AIR at 550°C for 4 h in pre-weighed crucibles.

Statistical analysis

Feedstock samples were analyzed using a randomized complete block design with three replications. Statistical analysis was done using SAS software [19]. The significance of the effect of feedstock was analyzed for all model parameters. SSCF data was fitted using a monomolecular model (Equation 4) with the NLIN procedure to estimate C_B and k_B:

$$C(t) = C_B (1- e^{- k_B t}) \quad [4]$$

where

C(t) = dry matter digested by SSCF with time (g AIR kg^{-1} initial DM)

C_B = biochemically available carbohydrates (g AIR kg^{-1} initial DM)

k_B = rate constant for C_B (h^{-1})

t = digestion time (h)

Results and Discussion

Simultaneous saccharification and co-fermentation data was fit using Eq. 4 to determine C_B and k_B values (Figure 3.4). Parameter values for the four feedstocks analyzed are presented in Table 3.1. Large ranges in values were observed across the feedstock species for most of the parameters. Non-structural carbohydrates ranged from 27 to 127 g kg^{-1} DM, C_B ranged from 34 to 344 g kg^{-1} DM, k_B ranged from 0.071 to 0.415 h^{-1}, C_A ranged from 61 to 517 g kg^{-1} DM, C_U ranged from 419 to 641 g kg^{-1} DM, C_T ranged from 701 to 935 g kg^{-1} DM, and lignin ranged from 139 to 244 g kg^{-1} DM.

The coefficient of variation (CV) values ranged from 0.9 to 26.1 for the model parameters. Coefficient of variation values provide a general estimate of how precisely a value is determined. All parameters had CV values less than 10, with exception of the rate constant, k_B, which had a CV of 26.1. Based on these values, all dry matter constituent values were measured with relatively high precision. The high CV value for k_B is a product of the high variation in SSCF data from the 4 and 8 h samples. Using kinetics that approximate the rate constant for the 1^{st}-order saccharification period [20], rather than using a series of time points, should improve the precision in determining k_B.

The negative extractives value for corn cobs is not theoretically possible. However, because extractives are measured indirectly as the difference between 1000 and the sum of the AIR and TNC values, slight overestimations in AIR and TNC can cause some feedstocks to have negative extractives values in the model.

As indicated earlier, TNC is determined on the basis of glucose per mass of dry matter. Total non-structural carbohydrates are overestimated in feedstocks having significant concentrations of non-structural polysaccharides, such as starch, due to the mass increase through the hydrolysis of polysaccharides to glucose. This in combination with feedstocks having low extractives concentrations and the general tendency for the acid digestion TNC procedure used in the assay to overestimate TNC, are the likely cause of negative extractives values as quantified by the model. Such negative extractives values are a minor concern because the extractives parameter is likely one of the least important parameters in determining the overall quality of biomass feedstocks.

Of the model parameters biochemically available carbohydrates, C_B, is the single most important parameter because in lignocellulosic biomass feedstocks it represents the largest source of fermentable carbohydrates, i.e., those available for biofuel production. The assay produced C_B values from 34 to 344 g kg^{-1} DM. If the feedstocks were to be subjected to a potential commercially viable pretreatment technology (e.g., aqueous ammonia soaking, pH-controlled pretreatment, ammonia fiber explosion), it is anticipated that the concentration of carbohydrates made available for enzymatic hydrolysis would be higher than predicted by the C_B values in all feedstocks. This is because these methods have more severe reaction conditions (i.e., higher temperatures, longer reaction times, higher pressures, more caustic reagents) than the AIR method used in the assay. Total sugar yields during enzymatic hydrolysis of corn stover pretreated using leading pretreatment technologies have been reported in the range of 800 – 950 g kg^{-1} initial cell-wall

carbohydrate [21]. By comparison, total sugar yields during enzymatic hydrolysis of the four feedstocks analyzed with the assay ranged from 50 to 450 g kg^{-1} initial cell-wall carbohydrate.

It is reasonable to argue for the use of a leading pretreatment technology in place of the AIR method in light of the relatively limited cell-wall hydrolysis which occurred. Most pretreatment technologies hydrolyze hemicellulose to varying extents [22], resulting in a loss of carbohydrates from C_B. To that end, using a pretreatment technology and reaction conditions suited for highly recalcitrant feedstocks (i.e., woody materials) on a highly bioavailable feedstock (i.e., corn cobs) would likely result in significant hemicellulose hydrolysis during pretreatment and underestimation of C_B. As such, the pretreatment method would need to be adjusted for a particular feedstock. The intent of this assay is to produce parameter estimates related to the pretreatment, enzymatic hydrolysis, and fermentation processes which can be correlated to performance of a range of biomass feedstocks; the lower intensity nature of AIR is consistent with this intent.

Although the primary intent of this validation exercise was not to compare these feedstocks to one another, there are clearly differences in the amount and availability of carbohydrates between feedstocks. Corn cobs are superior to the other feedstocks because of its higher total carbohydrate concentration and biochemically available carbohydrates, and lower lignin and ash concentrations. Hybrid poplar performed the worst in the assay, due to its low carbohydrate availability and high lignin concentration. Kenaf and switchgrass values were intermediate between corn cobs and hybrid poplar. It is clear that the model and

assay are applicable to a wide range of biomass feedstocks; however, determination of model values for a larger population of feedstocks would better indicate how parameter values compare within and across various feedstock types (e.g., warm season grasses, woody species, high-fiber dicots, etc.).

During completion of the laboratory procedures for this assay, several challenges were identified which need to be addressed before this method can be adopted for routine biomass quality analysis. The multiple solvent washing steps in the AIR procedure and the filtration procedure used to recover SSCF and Klason residues are extremely time-consuming and limit sample throughput. Use of filter bags and batch processing of samples, both commonly used in forage quality research [23], can be used to address both these problems. The AIR procedure is not compatible with filter bags, so use of an alternative method to produce a cell-wall isolate, such as neutral detergent fiber, would be necessary.

The carbohydrate availability model provides a conceptual framework for determining quality of lignocellulosic biomass feedstocks for biochemical conversion. The assay used to estimate model parameters employs a combination of compositional analysis and enzymatic saccharification techniques and determined the values of model parameters for compositionally-diverse biomass feedstocks with high precision. In comparison to compositional analysis as stand-alone method to measure biomass quality, the carbohydrate availability model and assay provide vital compositional analysis data for carbohydrates and lignin concentrations. And in comparison to enzymatic saccharification tests, the model and assay partition carbohydrates based on their availability for fermentation to ethanol via enzymatic

hydrolysis, whereby indirectly measuring the net effects of cell-wall structural features. Moreover, the assay can be used for a range of feedstock species without the requirement for process optimization for specific feedstock types. However, further refinement of the assay methods is needed to improve the throughput of the assay for large sample populations.

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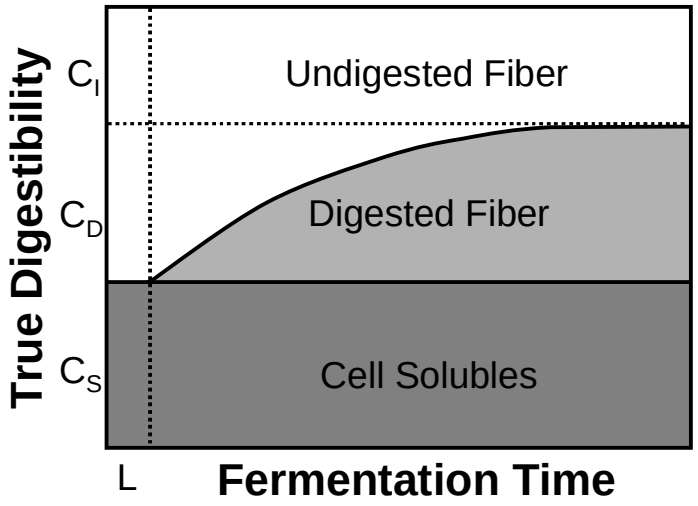

Figure 3.1. True digestibility (TD) model for ruminant livestock.

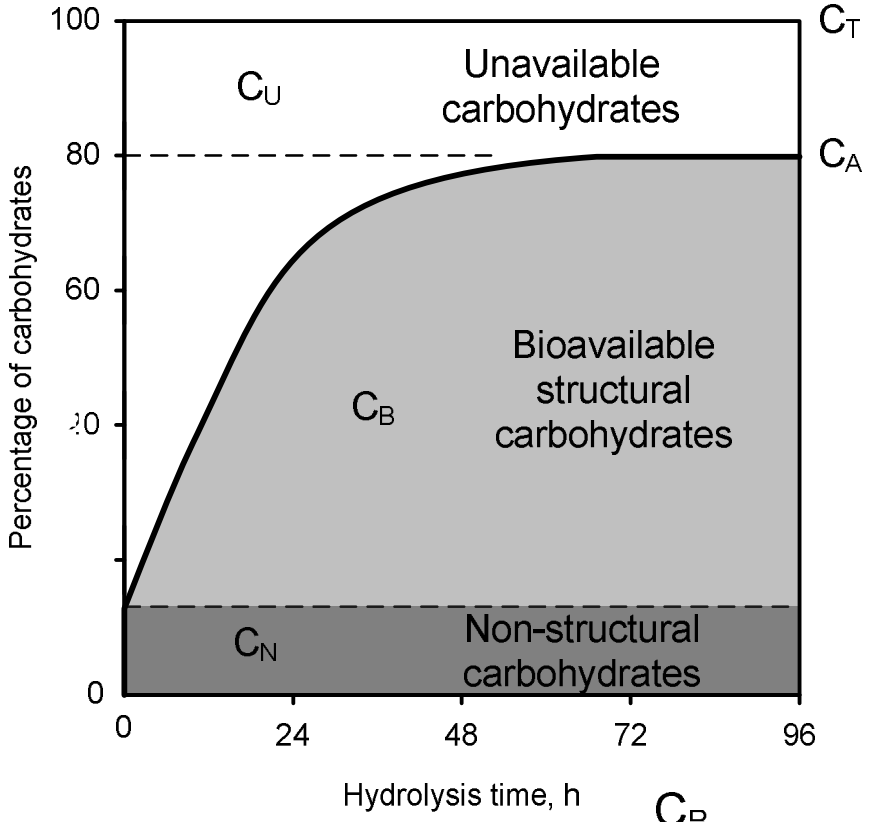

Figure 3.2. Carbohydrate availability model for lignocellulosic biomass feedstocks.

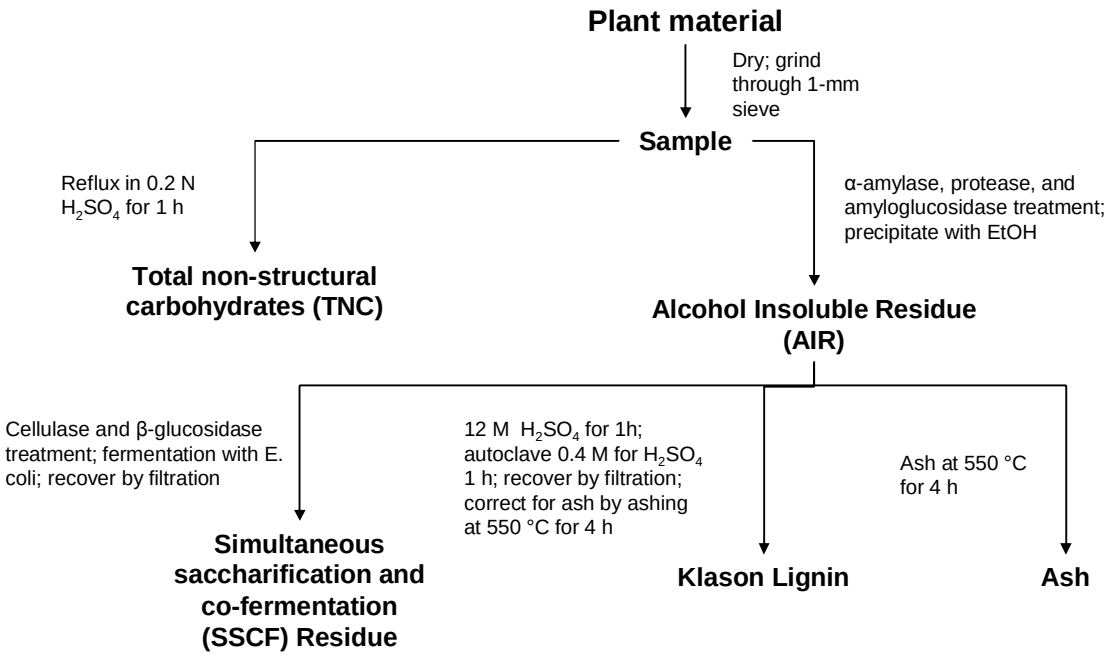

Figure 3.3. Overview of laboratory procedures for carbohydrate availability assay.

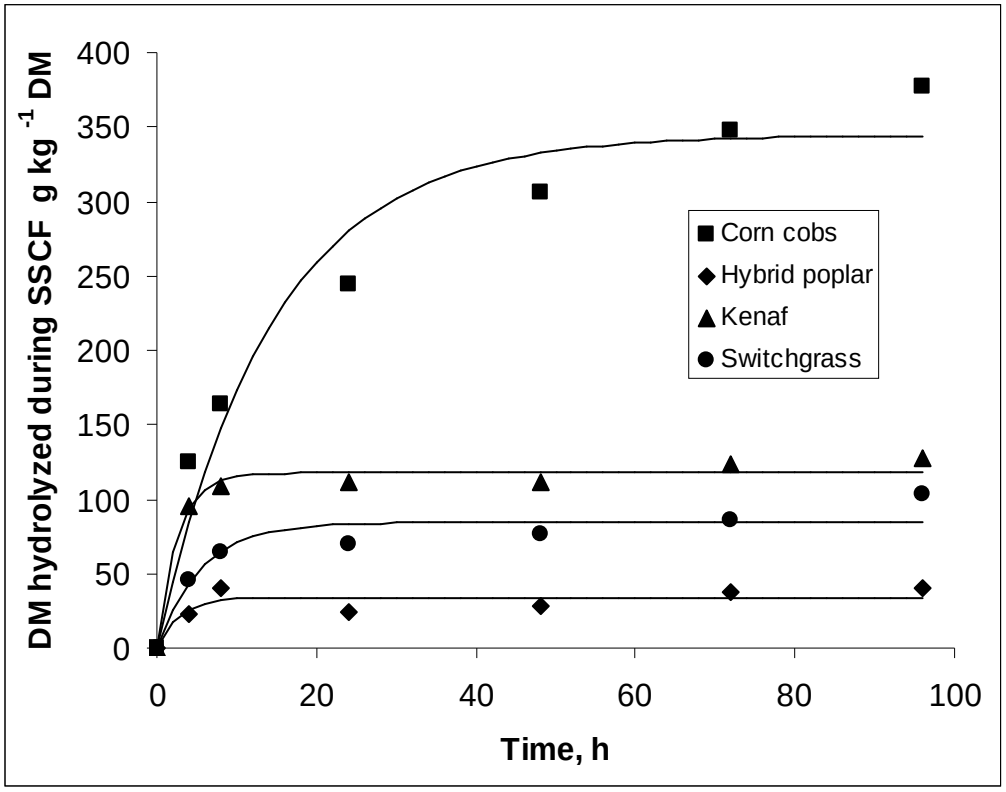

Figure 3.4. Dry matter (DM) hydrolyzed during simultaneous saccharification and co-fermentation (SSCF) of alcohol insoluble residues from biomass feedstocks.

Table 3.1. Carbohydrate availability model parameter values for diverse biomass feedstocks.

Feedstock	Extractives[a]	C_N	C_B	k_B	C_A	C_U	C_T	Lignin	Ash
	——— g kg^{-1} DM ———			h^{-1}	——————— g kg-1 DM ———————				
Corn cobs	-81	172	344	0.07	517	419	935	139	6
Hybrid poplar	46	27	34	0.41	61	641	701	244	9
Kenaf	88	37	118	0.42	154	578	732	167	13
Switchgrass	29	102	85	0.20	187	561	748	191	32
SE[b]	3.0	3.7	2.2	0.041	3.6	2.9	3.9	3.3	0.4
CV[c]	8.4	7.6	2.6	26.1	2.7	0.9	0.9	3.0	4.5

[a] Extractives = Alcohol soluble extractives C_N = Non-structural carbohydrates, C_B = Biochemically available carbohydrates, k_B = Rate constant for C_B, C_A = Total available carbohydrates, C_U = Unavailable carbohydrates, C_T = Total carbohydrates, Lignin = Klason lignin, Ash = cell-wall ash

[b] Standard error of the mean

[c] Coefficient of variation

Chapter 4

High-throughput assay for screening biomass feedstocks for biochemical conversion to fuels

A manuscript for submission to Bioenergy Research

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Abstract

Development of a method for analyzing the quality of biomass feedstocks for biochemical conversion to fuel (i.e., pretreatment followed by enzymatic hydrolysis and fermentation) requires high-throughput assay methods. We evaluated multiple modifications to a an existing biomass quality assay that partitions feedstock carbohydrates into three fractions based on their availability to be converted to fermentable sugars. Modifications included (i) using filter bags with batch sample processing, (ii) replacement of alcohol insoluble residue (AIR) with neutral detergent

fiber (NDF) as a cell-wall isolation procedure, and (iii) elimination of the fermentation organism in the simultaneous saccharification and fermentation procedures used to determine biochemically available carbohydrates. The original and the high throughput (HTP) assay methods were compared using corn cobs (*Zea mays* L.), hybrid poplar (*Populus x canadensis* Moench), kenaf (*Hibiscus cannabinus* L.) and switchgrass (*Panicum virgatum* L.). Biochemically available carbohydrates (C_B) increased with the HTP methods in the corn cobs, hybrid poplar, and switchgrass, but remained the same in the kenaf. Total available carbohydrates (C_A) increased and unavailable carbohydrates (C_U) decreased with the HTP methods in the corn cobs and switchgrass and remained the same in the hybrid poplar and kenaf. There were no differences in total carbohydrates (C_T) between the two methods. The HTP methods consistently assayed less lignin than did the original method. Despite the slight differences parameter values, the HTP assay methods essentially gave a similar summary of feedstock quality as did the original assay methods while significantly reducing the time and cost for feedstock quality analysis. Quality parameter values for 23 biomass feedstocks analyzed using the HTP assay are also included in the article.

Keywords Biomass quality, Lignocellulosic feedstocks, Biochemical conversion, Simultaneous saccharification and co-fermentation

Abbreviations

HTP high throughput

DM	dry matter
TNC	total non-structural carbohydrates
AIR	alcohol insoluble residue
SSCF	simultaneous saccharification and co-fermentation
KL	Klason lignin
NDF	neutral detergent fiber
NDL	neutral detergent

Introduction

Development of a system for determining quality of lignocellulosic biomass for biochemical conversion (i.e., pretreatment followed enzymatic hydrolysis and fermentation) requires: (i) quality parameters that can be correlated to process data and (ii) high throughput (HTP) methods to precisely determine quality parameter values. We have developed a carbohydrate availability model for determining feedstock quality that partitions plant dry matter (DM) constituents based on their availability for biochemical conversion to fuels [1] (Figure 4.1). The carbohydrate availability model separates carbohydrates into three groups based on their potential to be converted to fermentable sugars. These groups include: non-structural carbohydrates (C_N) (monosaccharides, oligosaccharides, and starches) biochemically available carbohydrates (C_B) (hemicellulose and cellulose hydrolyzed by cellulolytic enzymes) with an associated 1^{st}-order availability rate constant (k_B), and unavailable carbohydrates (C_U) (hemicellulose and cellulose in close association with lignin). Non-carbohydrate constituents are separated into extractives, lignin, and ash. Additional carbohydrate parameters, total available carbohydrates, C_A, and total carbohydrates, C_T, are calculated according to Equations 1 and 2.

$$C_A = C_N + C_B \quad [1]$$

$$C_T = C_N + C_B + C_U \quad [2]$$

Model parameters were determined using a gravimetric and spectroscopic assay based on a composite of forage quality, cell wall isolation, enzymatic

hydrolysis, and fermentation procedures [1]. Total-non structural carbohydrates (TNC) were used to determine C_N. Alcohol insoluble residue (AIR) was prepared by removing cell-soluble materials from the initial sample to produce a cell wall isolate suitable for subsequent analytical procedures. Biochemically available carbohydrates and k_B were determined by digesting the AIR using simultaneous saccharification and co-fermentation (SSCF) techniques for 4, 8, 24, 48, 72, and 96 h and fitting the data using Equation 3. Lignin concentration was determined by analyzing the AIR for Klason lignin (KL) and ash concentration was determined by ashing the AIR sample.

$$C(t) = C_B (1- e^{-k_B t}) \quad [3]$$

where

$C(t)$ = dry matter digested by SSCF with time (g AIR kg^{-1} initial DM)

C_B = biochemically available carbohydrates (g AIR kg^{-1} initial DM)

k_B = rate constant for C_B (h^{-1})

t = digestion time (h)

We identified several issues with the analytical methods used in the assay that limited sample throughput and thereby could hinder acceptance of the assay as a routine method for biomass feedstock quality [1]. The AIR procedure requires five solvent washing steps. Between each washing the sample is allowed to settle for a 24 h period and the supernatant removed from the sample. This 5-day washing period combined with the initial starch and protein digestion time and ensuing freeze-drying period results in a total of 7 days to generate AIR materials for the additional analyses. The AIR sample is then divided into subsamples for each of six

SSCF times, KL, and ash. After SSCF and KL procedures are complete sample residues are recovered individually by vacuum filtration. All these individual steps result in an assay that is time-consuming and expensive. Filter bags and batch processing of samples which are commonly used in forage quality analyses [2] could be implemented in this assay to significantly reduce time and labor requirements of the wet-chemistry procedures. The AIR procedure requires residue precipitation with ethanol [3], which is not compatible with use of filter bags, so use an alternative cell-wall isolation method is necessary. Neutral detergent fiber (NDF) has been used since the 1960's as a cell-wall isolation method for ruminant feedstuffs [4]. The NDF procedure is completed in a significantly shorter period of time (~90 min.) than the AIR procedure and the widespread use of the NDF procedure has largely been the motivating factor for inclusion of filter bag technology into routine forage quality analysis [2].

Additionally, the SSCF procedure uses six time points to estimate k and requires a hexose and pentose co-fermenting organism to prevent potential end-product inhibition of the enzymes from increases in fermentable sugar concentrations in the saccharification vessel. Because the monomolecular model in Equation 3 adequately describes the digestion curve for C_B [1], k_B and C_B can be determined using three time points [5] with a linearized logarithmic equation [6]. Moreover, sufficient dilution of the saccharification vessel would alleviate the need for a fermentation organism. Both of these changes would further reduce time and labor requirements of the assay.

Finally, the assay procedures have only been validated on four feedstocks: corn cobs (*Zea mays* L.), hybrid poplar (*Populus x canadensis* Moench), kenaf (*Hibiscus cannabinus* L.), and switchgrass (*Panicum virgatum* L.). Application of the assay on a more extensive population of feedstocks sample would improve its validity as standard method for determining biomass feedstock quality.

The objectives of the study were to evaluate the high throughput modifications to the wet-chemistry methods of the carbohydrate availability assay, including (i) filter bags with batch sample processing, (ii) neutral detergent fiber for cell-wall isolation, (iii) estimation of the digestibility rate constant for the biochemically available carbohydrate fraction using natural logarithm linearization with three time points, and (iv) elimination of the fermentation organism for cell-wall digestion. With these changes, we sought out to evaluate the high throughput assay on diverse feedstock types, including cool-season grasses, warm-season grasses, corn residues, and woody materials.

Materials and Methods

Feedstocks

Corn cobs (*Zea mays* L.), hybrid poplar (*Populus x canadensis* Moench), kenaf (*Hibiscus cannabinus* L.), and switchgrass (*Panicum virgatum* L.) were used as substrates for comparison of the original and HTP assay methods. Nineteen additional feedstocks were analyzed using the HTP assay. Species, cultivar, and

collection location and date information for all feedstocks in included in Table 4.1. All samples were dried at 60°C in a forced air oven for 72 h [7] and ground using a Wiley mill (Thomas Scientific Inc., Swedesboro, NJ) fitted with a 1-mm sieve. Dry matter was determined for ground samples by drying 1 g of sample at 103°C in a forced air oven for 4 h to make moisture corrections for all laboratory analyses [7].

Original assay procedures

Cellulase (Spezyme CP, Genencor, Rochester, NY; Lot No. 301-05021-011) and (Novozyme188, Sigma-Aldrich, St. Louis, MO; Lot No. 037K0698) β-glucosidase were used as hydrolytic enzymes and co-fermentator *Escherichia coli* ATCC 55124 (KO11) was used as a fermentation organism. Spezyme CP was assayed at 71 FPU mL^{-1} [8] and Novozyme 188 was assayed at 268 CBU mL^{-1} [9].

Total-non structural carbohydrates were used to determine C_N. Alcohol insoluble residue was prepared by removing cell-soluble materials from the initial sample to produce a cell wall isolate suitable for subsequent digestion. Biochemically available carbohydrates and k_B were determined by digesting the AIR using SSCF techniques for periods of 4, 8, 24, 48, 72, and 96 h. SSCF data was fit using the NLIN procedure in SAS [10] to estimate C_B and k_B using the monomolecular model in Equation 3. Lignin concentration was determined by analyzing the AIR for KL with correction for residual ash. Ash concentration was determined by ashing the AIR sample. C_U, C_A, and C_T were calculated as

$$C_U = AIR - C_{KL} - Ash$$

$C_A = TNC + C_B$

$C_T = C_A + C_U$

where

AIR = alcohol insoluble residue (g AIR kg^{-1} initial DM)

TNC = total non-structural carbohydrates (g glucose kg^{-1} initial DM)

C_{KL} = ash-free, residual AIR concentration after acid digestion (g AIR kg^{-1} initial DM)

C_{Ash} = residual AIR after ashing (g AIR kg^{-1} initial DM)

The original assay methods are described in Murphy et al. [1]. Total non-structural carbohydrates were determined according to a modified Guiragossian et al. [11] procedure, in which, 0.25 g of sample was refluxed in 0.2 N sulfuric acid for 1 h. TNC values were calculated on the basis of grams of glucose per kg sample DM.

Alcohol insoluble residue samples were prepared by sequentially digesting a 6-g initial sample with α-amylase, protease, and amyloglucosidase [3]. Following enzyme treatment, AIR materials were precipitated and washed using three volumes of 80% (v/v) ethanol. To remove excess ethanol, samples were washed with two volumes of deionized water and then freeze-dried. Between washes, residue was allowed to settle for 24 h and the supernatant removed by vacuum suction. Alcohol insoluble residue samples were hydrolyzed and fermented using SSCF procedures described by Murphy et al. [12] using enzyme loading rates of 60 FPU g-AIR^{-1} Spezyme CP and 2 CBU FPU^{-1} Novozyme 188 [13]. Alcohol insoluble residue samples weighing 0.5 g were loaded into 25 mL DeLong fermentation flasks fitted with aluminum closures (Bellco Glass, Vineland, NJ) to maintain anaerobic conditions during fermentation. To the flasks, 1 mL 1.0 M phosphate buffer (pH 5.8),

1 mL growth media solution (0.1 g mL^{-1} tryptone, 0.05 g mL^{-1} yeast extract, 0.1 g mL^{-1} NaCl), and 5 mL deionized water was added and autoclaved at 121°C for 20 min. After cooling, 1 mL of enzyme solution, 1 mL of chloramphenicol solution, and 1 mL of *E. coli* inoculum was added aseptically to each flask. The final 10-mL fermentation volume was buffered to pH 6.0 and contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 40 μg mL^{-1} chloramphenicol. Flasks were incubated at 35°C for 4, 8, 24, 48, 72, or 96 h in a shaking water bath at 150 rpm.

Klason lignin was determined on AIR samples according to the two-step sulfuric acid digestion procedure [14]. Alcohol insoluble residue subsamples weighing 0.1 g were loaded into 50-mL test tubes and incubated with 1.25 mL of 12 M sulfuric acid for 1 h at 30°C. Solutions were diluted to 0.4 M by addition of 35 mL of water and autoclaved at 125°C for 1 h. Klason lignin residues were recovered by filtration using pre-weighed glass filter paper, washed with deionized water, and dried for 4 h at 103°C. Klason lignin concentrations were corrected for residual mineral concentration by ashing the filters at 550°C for 4 h. Ash concentrations were determined by ashing a 0.5-g subsample of AIR at 550°C for 4 h in pre-weighed crucibles.

High throughput laboratory assay

A mixture of cellulase (Spezyme CP, Genencor, Rochester, NY; Lot No. 301-05021-011), xylanase (Multifect xylanase, Genencor, Rochester, NY; Lot No. 301-05357-223), and β-glucosidase (Novozyme 188, Sigma-Aldrich, St. Louis, MO; Lot No.

037K0698) enzymes were used as saccharification enzymes. All enzymes were applied in excess [15] in the high throughput assay, so that enzyme availability did not limit cell-wall digestibility in the samples.

As with the original methods, C_N was determined using the TNC procedure. Neutral detergent fiber was used as the cell-wall isolation procedure. Preliminary experiments were conducted to compare enzymatic hydrolysis of NDF samples of the four comparison feedstocks with and without a fermentation organism (*E. coli* KO11) using the following procedures. We found that at a solids loading rate of 1.5% (w/v) enzyme inhibition did not occur and that the availability rate constant for C_B, k_B, was most accurately estimated for the four feedstocks in the incubation period between 0 and 4 h (unpublished data). Consequently, NDF samples were hydrolyzed using a mixture of cell-wall degrading enzymes for 4 or 96 h to estimate C_B and k_B:

$$C_B = C_0 - C_{96}$$

$$k_B = [\ln (C_0 - C_{96}) - \ln (C_4 - C_{96})] / (4 - 0)$$

where

C_o = NDF concentration (g NDF kg^{-1} initial DM)

C_4 = residual NDF concentration following 4 h of enzymatic digestion (g NDF kg^{-1} initial DM)

C_{96} = residual NDF concentration following 96 h of enzymatic digestion (g NDF kg^{-1} initial DM)

Neutral detergent fiber samples were also digested with concentrated sulfuric acid to determine neutral detergent lignin (NDL) concentration following by ashing to

adjust for the residual cell-wall ash concentration. Neutral detergent lignin, total available carbohydrates (C_A), unavailable carbohydrates (C_U), and total carbohydrates (C_T) were calculated as

$$NDL = C_{NDL} - C_{Ash}$$

$$C_A = C_N + C_B$$

$$C_U = C_0 - C_{NDL}$$

$$C_T = C_N + C_B + C_U$$

where

C_{NDL} = residual NDF concentration after acid digestion (g NDF kg^{-1} initial DM)

C_{Ash} = residual NDF concentration after acid digestion and ashing (g NDF kg^{-1} initial DM)

TNC = total non-structural carbohydrates (g glucose kg^{-1} initial DM)

All sample analyses were done in duplicate following the laboratory procedures in Figure 4.2. Total non-structural carbohydrates were determined using the same procedure as in the original assay methods. Neutral detergent fiber was completed using the filter bag method [2] with the ANKOM fiber system [16]. After the final hot water wash, NDF samples intended for lignin and ash analysis were dehydrated with acetone and dried at 103°C for 4 h before being reweighed. The NDF samples for enzymatic hydrolysis were allowed to cool in the fiber analyzer for 5 min and aseptically transferred into a saccharification flask. Saccharification flasks consisted of 1-L DeLong flasks fitted with aluminum closures (Bellco Glass, Vineland, NJ). To the flasks, 80 mL of 1.0 M phosphate buffer (pH 5.8) and 680 mL of deionized water were added and autoclaved at 121°C for 20 min. Prior to addition

of the NDF samples, flasks were warmed to 35°C in a hot water bath. After addition of the NDF samples, 2 mL of Spezyme CP, 1 mL of Multifect Xylanase, and 0.1 mL of Novozyme 188 per sample were added to flasks and the flasks swirled to mix the samples and enzyme solutions. The total working volume of the saccharification flask was approximately 800 ml buffered to pH 6.0. Flasks were incubated at 35°C in a shaking water bath for 4 or 96 h at 100 rpm. After digestion, filter bags were washed with deionized water to removed solublized materials, dehydrated with acetone, and dried at 103°C for 4 h before being reweighed.

Neutral detergent lignin was completed on the NDF residues according to the ANKOM method [17]. Each batch of filter bags was incubated in 500 mL of 72% sulfuric acid (12 M) for 3 h at room temperature. After digestion, filter bags were washed with deionized water to removed solublized materials, dehydrated with acetone, and dried at 103°C for 4 h before being reweighed. Ash concentration was determined by ashing the NDL residues at 550°C for 4 h in a muffle furnace and used to correct NDL values for residual ash.

Statistical analysis

Statistical analysis was done using SAS software [10]. The significance of the effects of assay procedures (original versus high throughput) and feedstock type was analyzed for all model parameters using the general linear procedure. Contrast statements were used to further analyze differences between assay procedures

within each feedstock. All comparisons were determined to be significant at $p < 0.05$.

Results and Discussion

Comparison of assay methods

Parameter values for corn cobs, hybrid poplar, kenaf, and switchgrass analyzed using the original and throughput assay methods are displayed in Table 4.2. Non-structural carbohydrate values were not different between the assays. Because both methods used the same TNC procedure to determine C_N, this result was entirely expected. Biochemically available carbohydrates increased from the original to the HTP methods for the corn cobs, hybrid poplar, and switchgrass, but remained the same for the kenaf. Biochemically available carbohydrates increased from 344 to 702 g kg^{-1} DM in the corn cobs, from 34 to 57 g kg^{-1} DM in the hybrid poplar, and from 85 to 273 g kg^{-1} DM in the switchgrass. The availability rate constant for C_B, k_B, ranged from 0.071 to 0.415 h^{-1} with the original methods and from 0.131 to 0.256 h^{-1} with the HTP methods. Though k_B values changed considerably between to the two methods, there were no significant differences in k_B between the methods among the four feedstocks. The coefficient of variation (CV) of k_B in the original methods, estimated by model fitting a series of digestion values, was 26.1. The natural logarithm linearization used in the HTP methods reduced the CV to 11.9. Total available carbohydrates increased and C_U decreased with the HTP methods in the

corn cobs and switchgrass and remained the same in the hybrid poplar and kenaf. There was no difference in C_T and lignin concentrations decreased in all feedstocks between the two methods.

Both methods quantified total carbohydrate and total non-carbohydrate pools similarly; however, there was shift in dry matter partitioning within both pools between the two assay methods. The proportion of total carbohydrates that is biochemically available increased with the HTP method, presumably due to the pretreatment effects of the cell wall isolation methods used. The NDF and AIR procedures function as low-intensity biomass pretreatment methods. Because of the combination of longer treatment times at higher temperatures and the presence of stronger hydrolytic reagents in the NDF procedure, pretreatment intensity is greater in the NDF procedure than the AIR procedure. As a result, it would be expected that the C_B concentration would be greater in the HTP methods – using NDF – than the original methods – using AIR – and our results largely support this reasoning.

The fraction of cell-wall carbohydrates susceptible to enzymatic hydrolysis in the kenaf was unchanged with increasing pretreatment intensity, suggesting the kenaf cell-wall is less responsive to increased pretreatment intensity than other feedstock species. Absence of a change in C_B concentration with kenaf is possibly related to the bast and core fiber composition of the kenaf canes [18] and the high syringl to guaicyl lignin ratio of the bast fiber [19], which has been suggested to limit hydrolysis of cell wall polysaccharides [20]. This leads to kenaf being a high-quality fiber crop [21], but perhaps a poor model feedstock for biomass quality methods development.

In the non-carbohydrate pool, there was a shift in dry matter from lignin to extractives (non-carbohydrate, non-lignin constituents) between the original and HTP methods. Neutral detergent lignin values were lower that KL values most likely a result of the solublilization of lignin components [22] due to the use of sodium sulfite in the NDF method [23].

The HTP assay methods required considerably less time to analyze the 23 feedstock samples than it took to analyze the four initial samples with the original assay methods. Complete analysis of the four feedstocks, in triplicate, using the original methods took approximately 3 months, whereas analysis of all 23 feedstocks, in duplicate, using the HTP methods took 7 days. Admittedly, the HTP assay requires a fiber analyzer at cost of $5000, but this investment can easily be recovered through a reduction in analysis time and savings in labor.

The feedstocks were characterized similarly in both procedures, but with considerably time savings using HTP methods. As such, the HTP assay is the preferred methodology to analyze the quality of biomass feedstocks for biochemical conversion according the carbohydrate availability model [1].

Comparison of feedstock types

A total of 23 feedstocks, grouped into corn residues, cool-season grasses, warm-season grasses, sugar-crop, fibrous dicots, legume, and woody materials, were analyzed using the HTP assay methods (Table 4.3). Box plots for C_N, C_B, k_B, C_A, C_U, and lignin for the corn residues, cool-season grasses, warm-season grasses and

wood materials are displayed in Figure 4.3. Of these four feedstock groups, variability was generally the highest in the corn residues and the lowest in the woody materials. The corn residues, cool-season grasses, and warm-season grasses had higher C_N, C_B, and C_A concentrations and lower C_U concentrations than the woody materials. There was limited variability in k_B across feedstocks types. Neutral detergent lignin was higher in the woody materials; however, NDL concentrations in the miscanthus and switchgrass fell within the range of the woody materials. The miscanthus was harvested in the early spring, following overwintering, which causes soluble plant constituents to be leached and indigestible cell-wall components, such as lignin, to be concentrated. The switchgrass was harvested in November 2006 from a similar location as the big bluestem, eastern gamagrass, and indiangrass harvested in September 2007. The higher lignin concentration in the switchgrass is likely linked to anatomical and morphological differences between the samples [20], as a result of the later harvest date.

Though the feedstocks analyzed represent a single sample, most of which were grown in different environments and harvested at slightly different maturities, the corn cobs and husks appear to be the most suitable feedstocks for biochemically conversion, due to their comparatively high C_B concentrations.

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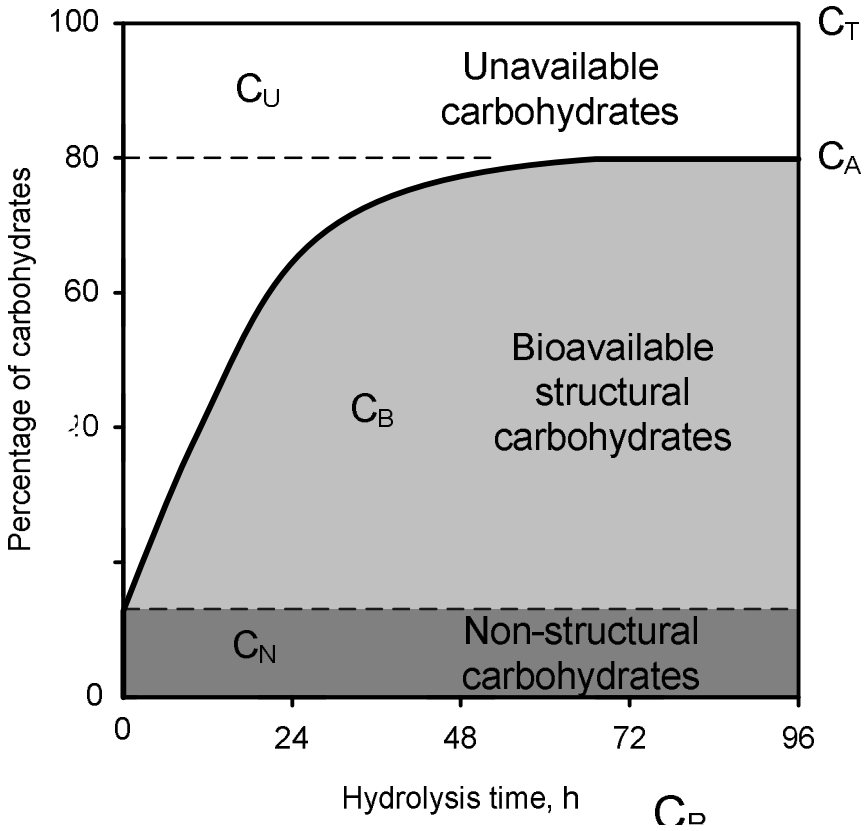

Figure 4.1. Carbohydrate availability model for lignocellulosic biomass feedstocks.

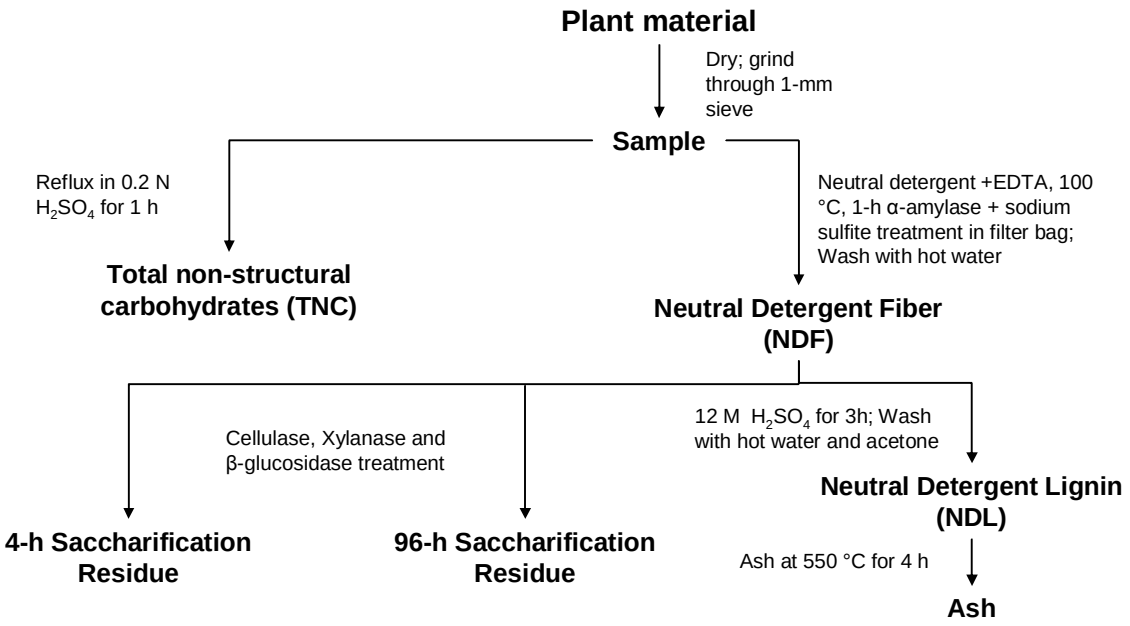

Figure 4.2. Overview of laboratory procedures for carbohydrate availability assay

Table 4.1. Description of lignocellulosic biomass feedstocks analyzed using carbohydrate availability assay.

Feedstock	Scientific name	Location	Harvest/collection date
Corn residues			
Corn cobs	*Zea mays* L. 'Pioneer 34A20'	Ames, IA	Sept. 2007
Corn fiber	*Zea mays* L.	ADM, Decatur, IL	Mar. 2007
Corn husks	*Zea mays* L. 'Pioneer 34A20'	Ames, IA	Sept. 2007
Corn leaves	*Zea mays* L. 'Pioneer 34A20'	Ames, IA	Sept. 2007
Corn stalks	*Zea mays* L. 'Pioneer 34A20'	Ames, IA	Sept. 2007
Corn stover	*Zea mays* L. 'Pioneer 34A20'	Ames, IA	Sept. 2007
Cool-season grasses			
Reed canarygrass	*Phalaris arundinacea* L.	Ames, IA	Oct. 2007
Tall fescue	*Festuca arundinacea* Schreb.	Ames, IA	Oct. 2004
Warm-season grasses			
Big bluestem	*Andropogon gerardii* Vitman 'Rountree'	Ames, IA	Sept. 2007
Eastern gamagrass	*Tripsacum dactyloides* (L.) L. 'Pete'	Ames, IA	Sept. 2007
Indiangrass	*Sorghmastrum nutans* (L.) Nash 'Rumsey 54'	Ames, IA	Sept. 2007
Miscanthus	*Miscanthus x giganteus*	Monticello, IL	Apr. 2007
Switchgrass	*Panicum virgatum* L. 'Cave-in-Rock'	Ames, IA	Nov. 2006
Sugar-crop			
Sweet sorghum	*Sorghum bicolor* (L.) Moench 'Topper 76'	Ames, IA	Sept. 2007
Fibrous dicots			
Amaranth	*Amaranthus cruentus* L.	Ames, IA	Sept. 1998
Kenaf	*Hibiscus Cannabinus* L. 'Tainung 2'	Ames, IA	Nov. 2006
Legume			
Forage soybean	*Glycine max* (L.) Merr.	Ames, IA	Sept. 2001
Woody materials			
Black alder	*Alnus glutinosa (*L.) Gaertn	Ames, IA	Mar. 2008
Eastern cottonwood	*Populus deltoides* Marshall 'ISU 91x04-03'	Ames, IA	Dec. 2006
Hybrid poplar	*Populus x canadensis* Moench 'Eugenei'	Ames, IA	Dec. 2006
Hybrid willow	*Salix matsudana x alba* 'Austree'	Ames, IA	Mar. 2008
Silver maple	*Acer saccharinum* L.	Ames, IA	Mar. 2008
White aspen	*Populus alba* L. 'ISU 2106'	Ames, IA	Mar. 2008

Table 4.2. Comparison of carbohydrate availability model parameter values for original and high throughput biomass assay methods.

	Original methods using Alcohol Insoluble Residue						
Feedstock	C_N[a]	C_B	k_B	C_A	C_U	C_T	KL
	—— g kg^{-1} DM ——		h^{-1}	———— g kg^{-1} DM ————			
Corn cobs	172	344	0.07	517	419	935	139
Hybrid poplar	27	34	0.41	61	641	701	244
Kenaf	37	118	0.42	154	578	732	167
Switchgrass	102	85	0.20	187	561	748	191
SE[b]	3.7	2.2	0.041	3.6	2.9	3.9	3.3
CV[c]	7.6	2.6	26.1	2.7	0.9	0.9	3.0
	High throughput methods using Neutral Detergent Fiber						
	C_N	C_B	k_B	C_A	C_U	C_T	NDL[d]
	—— g kg^{-1} DM ——		h^{-1}	———— g kg^{-1} DM ————			
Corn cobs	169	702	0.23	871	77	948	72
Hybrid poplar	20	57	0.23	77	631	709	174
Kenaf	32	121	0.26	152	590	743	76
Switchgrass	97	273	0.13	371	397	768	105
SE	10.2	5.7	0.018	11.1	8.1	10.9	3.6
CV	18.1	2.8	11.9	4.3	2.7	1.9	4.8
	% Change in parameter values from original methods to high throughout methods						
Corn cobs	-2	+104***	+220	+69***	-82***	+1	-49***
Hybrid poplar	-24	+68**	-45	+27	-1	+1	-29***
Kenaf	-14	+3	-38	-1	+2	+1	-54***
Switchgrass	-4	+222***	-33	+99***	-29***	+3	-45***

[a] C_N = Non-structural carbohydrates, C_B = Biochemically available carbohydrates, k_B = Rate constant for C_B, C_A = Total available carbohydrates, C_U = Unavailable carbohydrates, C_T = Total carbohydrates, KL = Klason lignin

[b] Standard error of the mean

[c] Coefficient of variation

[d] NDL = Neutral detergent lignin

*,**,*** Significant at the 0.05, 0.01, and 0.001 probability level, respectively

Table 4.3. Carbohydrate availability model parameter values for various feedstocks.

Feedstock	C_N[a]	C_B	k_B	C_A	C_U	C_T	NDL
	—— g kg^{-1} DM ——		h^{-1}	———— g kg^{-1} DM ————			
Corn residues							
Corn cobs	169	702	0.23	871	77	948	72
Corn fiber	274	264	0.17	538	292	830	21
Corn husks	162	729	0.24	891	66	957	36
Corn leaves	102	521	0.23	623	125	748	42
Corn stalks	58	332	0.22	390	394	784	95
Corn stover	102	509	0.22	610	209	819	59
Cool-season grasses							
Reed canarygrass	90	327	0.17	416	155	571	87
Tall fescue	124	459	0.16	583	48	631	41
Warm-season grasses							
Big bluestem	92	401	0.16	492	301	793	91
Eastern gamagrass	93	454	0.17	547	184	731	88
Indiangrass	109	385	0.20	494	287	781	67
Miscanthus	59	259	0.15	318	478	796	129
Switchgrass	97	273	0.13	371	397	768	105
Sugar-crop							
Sweet sorghum	207	203	0.23	410	181	591	57
Fibrous dicots							
Amaranth	107	154	0.29	261	259	520	72
Kenaf	32	121	0.26	152	590	743	76
Legume							
Forage soybean	70	218	0.30	289	129	417	99
Woody materials							
Black alder	49	64	0.21	113	626	739	129
Eastern cottonwood	28	50	0.18	78	675	753	136
Hybrid poplar	20	57	0.23	77	631	709	174
Hybrid willow	33	72	0.21	105	651	756	130
Silver maple	33	72	0.24	105	722	827	110
White aspen	33	83	0.19	116	659	775	96
SE[b]	5.7	6.4	0.032	8.1	6.7	6.8	3.1

[a] C_N = Non-structural carbohydrates, C_B = Biochemically available carbohydrates, k_B = Rate constant for C_B, C_A = Total available carbohydrates, C_U = Unavailable carbohydrates, C_T = Total carbohydrates, NDL = Neutral detergent lignin

[b] Standard error of the mean

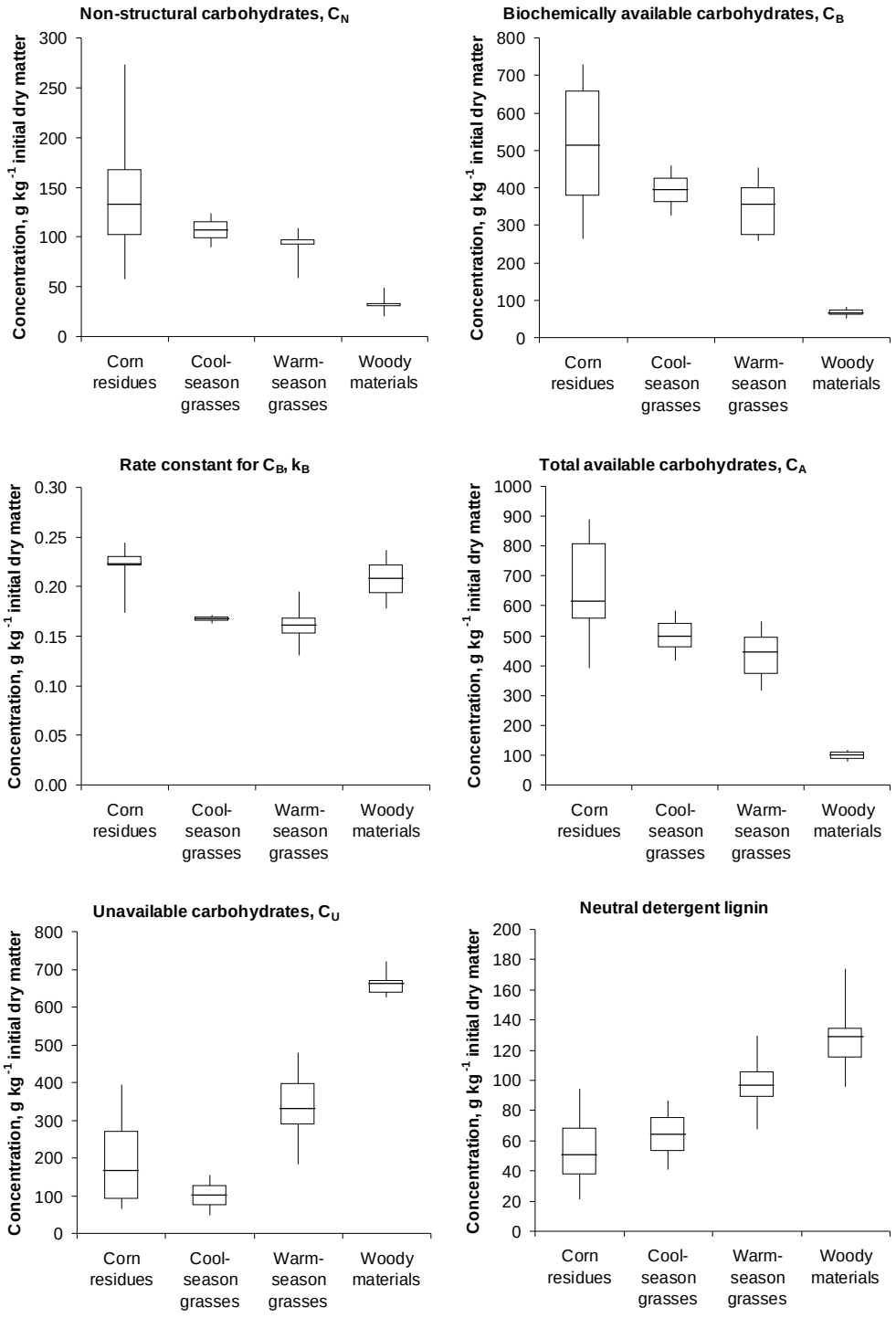

Figure 4.3. Comparison of quality parameters for corn residues, cool-season grasses, warm-season grasses and woody materials.

Chapter 5

Rapid biomass quality determination of corn stover using near infrared reflectance spectroscopy

A manuscript for submission to Bioenergy Research

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Abstract

Near infrared reflectance spectroscopy (NIRS) has been used extensively in the forage industry for rapid measurement of forage constituents and could be useful for determining quality of biomass feedstocks at the point of delivery. We evaluated the variability of biomass quality parameters in a set of corn stover samples and developed calibration equations for determining parameter values using NIRS. Fifty-two corn stover samples harvested in Iowa and Wisconsin in 2005 and 2006 were

analyzed using a high throughput assay for determining feedstock quality for biochemical conversion. Non-structural carbohydrates ranged from 84 to 155 g kg^{-1} dry matter (DM), biochemically available carbohydrates (C_B) ranged from 354 to 557 g kg^{-1} DM, availability rate constant of C_B ranged from 0.199 to 0.330 h^{-1}, total available carbohydrates ranged from 463 to 699 g kg^{-1} DM, and neutral detergent lignin ranged from 32 to 74 g kg^{-1} DM. Significant differences ($P<0.0001$) among samples were observed for all parameters, except for the availability rate constant of C_B. Near infrared reflectance spectroscopy calibration equations were developed for cell-soluble carbohydrates, biochemically available carbohydrates, total available carbohydrates, unavailable carbohydrates, total carbohydrates, and neutral detergent lignin. It was not possible to generate a meaningful calibration equation for the availability rate constant of C_B. There is significant variability within the corn stover population for several key quality-related carbohydrate and lignin constituents which can be predicted reliably using NIRS.

Keywords Biomass, Lignocellulosic feedstock, Feedstock quality, Near infrared reflectance spectroscopy

Abbreviations

HTP high throughput

NIRS near infrared reflectance spectroscopy

DM dry matter

TNC total non-structural carbohydrates

NDF neutral detergent fiber

NCNDE non-carbohydrate neutral detergent extractives

MPLS modified partial least squares

bm3 *brown midrib*-3

SEC standard error of calibration

Introduction

Development of a system for determining quality of lignocellulosic biomass for biochemical conversion to fuels –i.e., pretreatment followed by enzymatic hydrolysis and fermentation– requires: (i) quality parameters that can be correlated to process data and (ii) high throughput (HTP) methods to precisely determine quality parameter values.

The carbohydrate availability model (Figure 5.1) developed by Murphy et al. [1] provides a conceptual framework for evaluating the quality of feedstocks for biochemical conversion. An assay was developed by Murphy et al. [1] to determine the model quality parameters and further refined for high throughput screening of feedstock samples [2]. These assay methods are applicable for screening large sample sets in the research environment where determination of quality parameters in a period in days or weeks is acceptable. However, determining quality of feedstocks at delivery to a biorefinery would require that quality parameters be measured in a matter of minutes. This would allow incoming lots (e.g., truckloads, bales, modules) of materials to be sorted and blended into a consistent quality product stream as it is conveyed into the biorefinery for processing [3].

Near infrared reflectance spectroscopy (NIRS) has been applied extensively for rapid measurement of constituents in a range of biological products, including cereal and oilseed grains, forage and feedstuffs, fruits and vegetables, dairy products, meats, timber and paper, wool, and soils [4]. NIRS has been successfully used to measure moisture, nitrogenous compounds, carbohydrates, fiber

constituents, lignin, ash, and digestibility on a range of forage and feedstuffs, including several proposed biomass feedstocks –corn (*Zea mays* L.) stover and switchgrass (*Panicum virgatum* L.) [5]. Real-time knowledge of feedstuffs composition and digestibility using NIRS allows for continual adjustment of livestock rations to maximize production, analogous to blending biomass lots to maintain a consistent process feedstock.

Significant variability in quality parameter values from the carbohydrate availability model has been documented across a range of biomass feedstock types [2]. Sufficient variability in constituent values within samples of a single feedstock type is necessary for development of robust NIRS calibration equations. The objectives of this study were to evaluate the variability of quality parameters for a set of corn stover samples and to determine which, if any, parameter values could be predicted using NIRS.

Materials and Methods

Feedstocks

Corn stover samples were obtained from a larger collaborative field experiment between Iowa State University and the University of Wisconsin – Madison conducted in 2005 and 2006. A total of 28 genetically diverse hybrids, breeding populations, and population crosses, all originating from elite corn germplasm, were selected for this study. Iowa samples were grown at Ames and Ankeny, IA in 2005 and at Ames

and Belmond, IA in 2006. Wisconsin samples were grown at Arlington and Madison, WI in 2005 and 2006. All corn entries were grown in triplicate at each location in each year. After manual removal of corn ears, plots were harvested with a modified silage chopper, subsampled, and dried at 38°C for 96 h. Samples were ground using a cyclone mill (UDY Corporation, Fort Collins, CO) to pass a 1-mm sieve. Composite samples of the ground material were made by combining 2 g of each of the six samples from each location (Iowa and Wisconsin) in each year (2005 and 2006) for a total of 52 samples. Dry matter (DM) was determined for ground samples by drying 1 g of sample at 103°C in a forced air oven for 4 h to make moisture corrections for all laboratory analyses [6].

Quality Analysis

Quality parameters were determined using a high throughput assay developed by Murphy et al. [2]. Non-structural carbohydrates (C_N) were determined using the total non-carbohydrates (TNC) procedure. Neutral detergent fiber (NDF) was used as a cell-wall isolation procedure. NDF samples were subjected to enzymatic hydrolysis using a mixture of cell-wall degrading enzymes for 4 or 96 h to estimate biochemically available carbohydrates (C_B) and its rate availability rate constant, (k_B):

$$C_B = C_0 - C_{96}$$

$$k_B = [\ln (C_0 - C_{96}) - \ln (C_4 - C_{96})] / (4 - 0)$$

where

C_o = NDF concentration (g NDF kg^{-1} initial DM)

C_4 = residual NDF concentration following 4 h of enzymatic digestion (g NDF kg^{-1} initial DM)

C_{96} = residual NDF concentration following 96 h of enzymatic digestion (g NDF kg^{-1} initial DM)

NDF samples were also digested with sulfuric acid to determine neutral detergent lignin (NDL) concentration following by ashing to adjust for the residual cell-wall ash concentration. Neutral detergent lignin, total available carbohydrates (C_A), unavailable carbohydrates (C_U), and total carbohydrates (C_T) were calculated as

$$NDL = C_{NDL} - C_{Ash}$$

$$C_A = C_N + C_B$$

$$C_U = C_0 - C_{NDL}$$

$$C_T = C_N + C_B + C_U$$

where

C_{NDL} = residual NDF concentration after acid digestion (g NDF kg^{-1} initial DM)

C_{Ash} = residual NDF concentration after acid digestion and ashing (g NDF kg^{-1} initial DM)

Laboratory Procedures

Samples were analyzed using a randomized complete block design with two replications. The NDF procedure we used is limited to a total of 24 samples per

batch, so the 52 samples were split randomly into three batches for each type of analysis (NDF, NDF + 4h saccharification, and NDF+ 96 h saccharification) and replication. Two samples were run in duplicate so that each batch had an equal number of samples. High-digestibility *brown midrib* and lower-digestibility wild-type corn stover samples [7] were added to each batch for quality control and a blank bag was added to correct for changes in bag weight during analysis procedures for a total of 21 bags per batch.

Total non-structural carbohydrates were determined according to a modified procedure of Guiragossian et al. [8], in which, 0.125 g of sample was refluxed in 25 mL of 0.2 N sulfuric acid for 1 h followed by filtering through Whatman No. 42 filter paper. To a 1-mL aliquot of the filtrate diluted by a factor of 20, 1 mL of 5% phenol solution and 5 mL of 18 M sulfuric acid were added, and the solution's absorbance was measured at 490 nm. TNC values were determined using a glucose reference calibration and calculated on the basis of grams of glucose per kg sample DM.

Neutral detergent fiber was completed using the filter bag method [9] with the ANKOM fiber system [10]. After the final hot water wash, NDF samples intended for lignin and ash analysis were dehydrated with acetone and dried at 103°C for 4 h before being reweighed. NDF samples for enzymatic hydrolysis were allowed to cool in the fiber analyzer for 5 min and aseptically transferred into a saccharification flask. Saccharification flasks consisted of 1-L DeLong flasks fitted with aluminum closures (Bellco Glass, Vineland, NJ). To the flasks, 70 mL of 1.0 M phosphate buffer (pH 5.8) and 595 mL of deionized water were added and autoclaved at 121°C for 20 min. Prior to addition of the NDF samples, flasks were warmed to the

temperature for saccharification of 35°C in a hot water bath. All enzymes were applied in excess, so that enzyme availability did not limit cell-wall hydrolysis in the samples. After addition of the NDF samples, 2 mL of Spezyme CP (Genencor, Rochester, NY; Lot No. 301-05021-011), 1 mL of Multifect Xylanase (Genencor, Rochester, NY; Lot No. 301-05357-223), and 0.1 mL of Novozyme 188 (Sigma-Aldrich, St. Louis, MO; Lot No. 037K0698) per sample were added to flasks and the flasks swirled to mix the samples and enzyme solutions. All enzymes were applied in excess, so that enzyme availability did not limit cell-wall hydrolysis in the samples. The total working volume of saccharification was approximately 700 ml buffered to pH 6.0. Flasks were incubated at 35°C in shaking water bath for 4 or 96 h at 100 rpm. After digestion, filter bags were washed with deionized water to removed solublized materials, dehydrated with acetone, and dried at 103 °C for 4 h before being reweighed.

Neutral detergent lignin was completed on NDF residues according to the ANKOM method [11]. Each batch of filter bags was incubated in 500 mL of 72% sulfuric acid (12 M) for 3 h at room temperature. After digestion, filter bags were washed with deionized water to remove solublized materials, dehydrated with acetone, and dried at 103°C for 4 h before being reweighed. Ash concentration was determined by ashing the NDL residues at 550°C for 4 h in a muffle furnace and used to correct NDL values for residual ash.

Near infrared reflectance spectroscopy

Ground samples were scanned in triplicate using a Foss NIRSystems 6500 Spectrophotometer (NIRSystems, Silver Springs, MD) from 400 to 2500 nm at 2 nm intervals. Scans were averaged and calibration equations developed by modified partial least squares (MPLS) using reference wet-chemistry values for all quality parameters. Calibration equations were evaluated and validated using six-sample subsets with cross validation to prevent over fitting. Calibration equations were selected according to procedures described by Windham et al. [12].

Statistical analysis

Statistical analysis was done using SAS software [13]. Differences among corn stover samples within each quality parameter were assessed using the general linear procedure. All comparisons were determined to be significant at $p < 0.05$.

Results

Model parameter values for all corn stover samples are presented in Table 5.1. Differences among corn stover samples were observed in all parameters ($P<0.0001$), with exception of k_B ($P=0.17$). Non-structural carbohydrates ranged from 84 to 155 g kg^{-1} DM, C_B ranged from 354 to 557 g kg^{-1} DM, k_B ranged from 0.199 to 0.330 h^{-1}, C_A ranged from 463 to 699 g kg^{-1} DM, and NDL ranged from 32 to

74 g kg^{-1} DM. The sample set contains eight *brown midrib*-3 (*bm*3) samples, which purport to have lower lignin concentrations and a higher carbohydrate bioavailability [7]. The *bm3* samples generally had higher C_B and C_A concentrations and lower NDL concentrations than the non-*bm* samples across the four environments in this study.

Near infrared reflectance spectroscopy calibration equations were developed for all parameters. Calibration statistics for each quality parameter are presented in Table 5.2 and regressions of NIRS predicted and actual parameter values are displayed in Figure 5.2. Performance of the calibration equations was evaluated using the coefficient of determination (r^2), bias, and slope of the regression. Control limits for evaluation criteria are: (i) the absolute value of the bias can not exceed 0.6 times the standard error of calibration (SEC), (ii) the r^2 value is greater than 0.8, and (iii) the slope of regression line is between 0.95 and 1.05 [12,14]. Equations for C_N, C_B, C_A, C_U, C_T, and NDL met all criteria. The equation for k_B did not satisfy the control limits for any evaluation criteria.

Discussion

Lack of an observed difference in k_B for this group of corn stover samples is a result of the relatively narrow range (0.199-0.330 h^{-1}) of values and the comparatively high error associated with measuring k_B. Murphy et al. [2] observed a range of k_B values from 0.131 to 0.301 h^{-1} for a group of 23 biomass feedstocks using the same assay procedures. The range in k_B values for the corn stover samples was only slightly

less than the range for the group of biomass feedstocks, suggesting the methods used to determine k_B are only precise enough to detect large differences between biomass feedstocks samples.

Suitable calibration equations were developed for all parameters directly related to organic constituents: extractives, C_N, C_B, C_A, C_U, C_T, and NDL. The availability rate constant of C_B is based on temporal changes in an organic constituent group and could, presumably, be measured using NIRS just as digestibility kinetics are measured in forages [5].

Prior NIRS studies of biomass feedstocks have focused on compositional analysis [3,15]. Compositional analysis provides extensive data on the carbohydrate makeup of a biomass sample; it does not indicate the availability of structural carbohydrate, specifically glucan and xylan, for hydrolysis and fermentation. This is a result of the differences in the structural features of plant cell wall materials, including specific surface area, cellulose crystallinity, cellulose reactivity, degree of polymerization, lignin content, and degree of acetylation [16,17,18]. The constituent groups quantified by our biomass quality assay [2] partition sample dry matter both on the basis of compositional group – carbohydrates and lignin – and availability for biochemical conversion – non-structural carbohydrates, biochemically available carbohydrates, total available carbohydrates, and unavailable carbohydrates.

The sample set used in this study represents a finite population; however, because of the inclusion of highly digestible genotypes, including several *bm* hybrids, this population represents a large portion of the population of corn stover samples. The calibration equations developed are not applicable over the full range

of corn stover samples, but it has been demonstrated that NIRS calibration equations could be developed to rapidly measure quality parameters of corn stover, as well as other feedstock types. Expansion of the calibration sample set to include corn stover samples from diverse environments would improve greatly the applicability and robustness of the calibration equations.

These results indicate that there is significant variability within the corn stover population for several quality-related carbohydrate and lignin constituents, and that the concentration of these constituents can be predicted reliably using NIRS.

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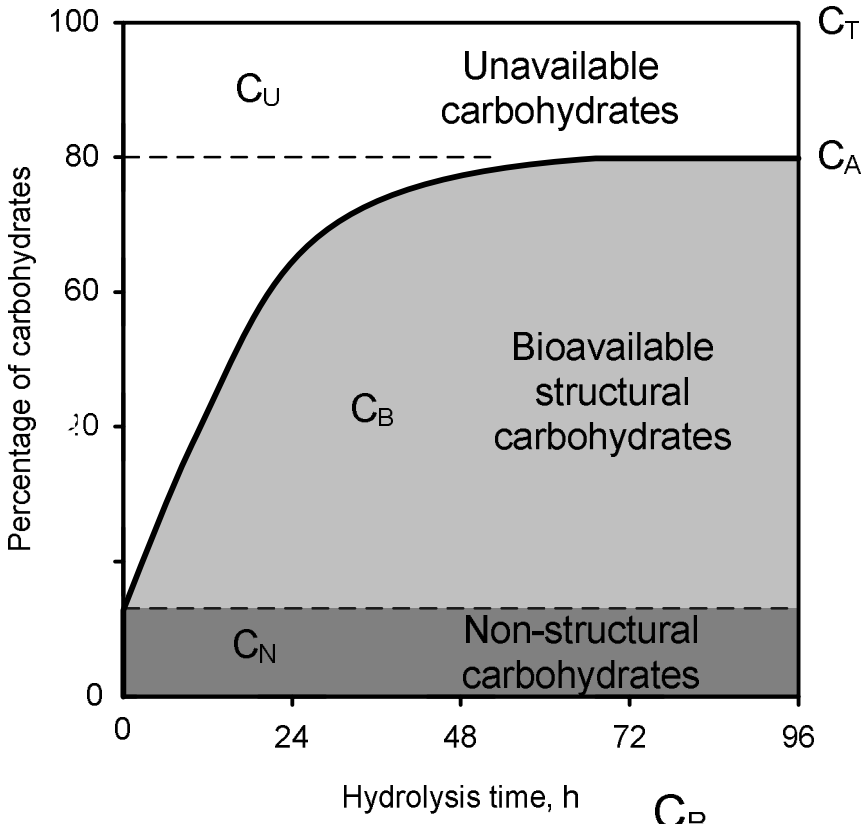

Figure 5.1. Carbohydrate availability model for lignocellulosic biomass feedstocks.

Table 5.1. Carbohydrate availability model parameter values for corn stover from corn genotypes grown in Wisconsin and Iowa in 2005 and 2006.

Entry	Location	Year	C_N [a]	C_B	k_B	C_A	C_U	C_T	NDL
			— g kg^{-1} DM —		h^{-1}	———— g kg^{-1} DM ————			
W64A X A619	WI	2005	109	466	0.22	575	175	750	52
W64A X A619 bm3	WI	2005	155	544	0.29	699	54	753	32
WQS C3 Syn2	WI	2005	140	476	0.24	617	146	763	41
WQS C3 X HC33	WI	2005	121	476	0.25	597	188	785	51
W601S X LH244	WI	2005	91	501	0.23	592	192	785	47
W602S X LH198	WI	2005	120	503	0.26	623	187	810	51
W603S X LH227	WI	2005	106	487	0.28	593	218	811	58
W604S X TR7245	WI	2005	111	490	0.20	602	193	794	50
W605S X HC33	WI	2005	109	490	0.24	599	208	807	56
LH227 X LH279	WI	2005	110	502	0.25	612	209	821	58
DK5143	WI	2005	109	493	0.23	603	201	804	54
Mycogen F697 (bm3)	WI	2005	118	557	0.27	675	71	745	37
W64A X A619	WI	2006	126	369	0.29	495	168	663	54
W64A X A619 bm3	WI	2006	132	520	0.30	653	62	714	40
WQS C3 Syn2	WI	2006	131	399	0.33	530	179	709	52
WQS C3 X HC33	WI	2006	119	406	0.24	524	196	720	59
W601S X LH244	WI	2006	103	423	0.23	526	232	758	59
W602S X LH198	WI	2006	113	439	0.24	552	208	760	63
W603S X LH227	WI	2006	86	427	0.24	513	250	763	71
W604S X TR7245	WI	2006	121	405	0.28	526	212	737	53
W605S X HC33	WI	2006	120	384	0.27	503	201	705	59
LH227 X LH279	WI	2006	96	411	0.28	507	254	762	68
DK5143	WI	2006	84	436	0.23	519	242	761	62
Mycogen F697 (bm3)	WI	2006	118	519	0.26	637	69	706	43
W64A X A619	IA	2005	108	404	0.23	512	252	765	66
W64A X A619 bm3	IA	2005	128	489	0.29	617	120	737	46
WQS C3 Syn2	IA	2005	113	433	0.30	546	213	758	60
WQS C3 X HC33	IA	2005	106	381	0.24	487	269	755	68
W601S X B126	IA	2005	124	442	0.31	567	221	788	54
W602S X SGI912	IA	2005	113	411	0.23	523	230	753	62
W603S X B129	IA	2005	104	410	0.23	514	234	748	66
W604S X TR7245	IA	2005	126	396	0.25	522	247	769	59
Renk 232	IA	2005	123	404	0.27	527	226	753	61
B73 X Mo17	IA	2005	98	377	0.29	475	284	759	74
B129 X TR7322	IA	2005	98	394	0.24	492	244	736	73
BS31(R)C0 X B116	IA	2005	113	376	0.26	488	241	730	72

BS31(R)C2 X B116	IA	2005	98	400	0.26	497	259	757	72
Mycogen F697 (bm3)	IA	2005	108	524	0.28	631	128	760	46
W64A X A619	IA	2006	119	370	0.29	489	228	717	56
W64A X A619 bm3	IA	2006	115	503	0.25	618	96	714	40
WQS C3 Syn2	IA	2006	134	377	0.27	510	206	716	48
WQS C3 X HC33	IA	2006	115	377	0.32	493	234	727	54
W601S X B126	IA	2006	94	369	0.27	463	270	733	58
W602S X SGI912	IA	2006	126	385	0.28	511	211	722	54
W603S X B129	IA	2006	131	393	0.28	524	179	702	65
W604S X TR7245	IA	2006	121	355	0.22	476	214	690	53
Renk 232	IA	2006	101	435	0.29	536	214	749	70
B73 X Mo17	IA	2006	108	373	0.28	481	250	732	64
B129 X TR7322	IA	2006	110	364	0.24	474	210	684	70
BS31(R)C0 X B116	IA	2006	113	357	0.25	470	230	700	62
BS31(R)C2 X B116	IA	2006	123	354	0.26	477	211	688	57
Mycogen F697 (bm3)	IA	2006	133	485	0.30	618	113	731	41
		SE[b]	6.0	6.3	0.03	8.9	5.8	8.4	3.2

[a] C_N = Non-structural carbohydrates, C_B = Biochemically available carbohydrates, k_B = Rate constant for C_B, C_A = Total available carbohydrates, C_U = Unavailable carbohydrates, C_T = Total carbohydrates, NDL = Neutral detergent lignin

[b] Standard error of the mean

Table 5.2. Near infrared reflectance spectroscopy (NIRS) calibration results for corn stover from genotypes grown in Wisconsin and Iowa in 2005 and 2006.

Parameter	C_N [a]	C_B	k_B	C_A	C_U	C_T	NDL
	g kg^{-1} DM		h^{-1}	g kg^{-1} DM			
Math treatment	1, 4, 4, 1						
Mean	115	432	0.265	546	197	745	6
Range	87 - 155	354 - 557	0.20 - 0.30	463 - 699	54 - 284	663 - 821	32 - 74
Stdev[b]	13.9	57.2	0.048	60.4	55.8	35.3	10.0
SEL[c]	6.0	6.3	0.025	8.9	5.8	8.4	3.2
SEC[d]	4.8	13.9	0.044	11.7	12.2	8.4	2.3
SECV[e]	5.5	16.6	0.049	18.1	15.2	11.4	3.2
R^2	0.880	0.941	0.146	0.963	0.952	0.944	0.948
F value	11.0	5.2	9.2	16.6	7.5	17.6	8.9
1-VR[f]	0.862	0.917	0.006	0.913	0.929	0.895	0.903

[a] C_N = Non-structural carbohydrates, C_B = Biochemically available carbohydrates, k_B = Rate constant for C_B, C_A = Total available carbohydrates, C_U = Unavailable carbohydrates, C_T = Total carbohydrates, NDL = Neutral detergent lignin

[b] Standard deviation

[c] Standard error of laboratory results

[d] Standard error of calibration

[e] Standard error of cross-validation

[f] Ratio of unexplained variance to total variance

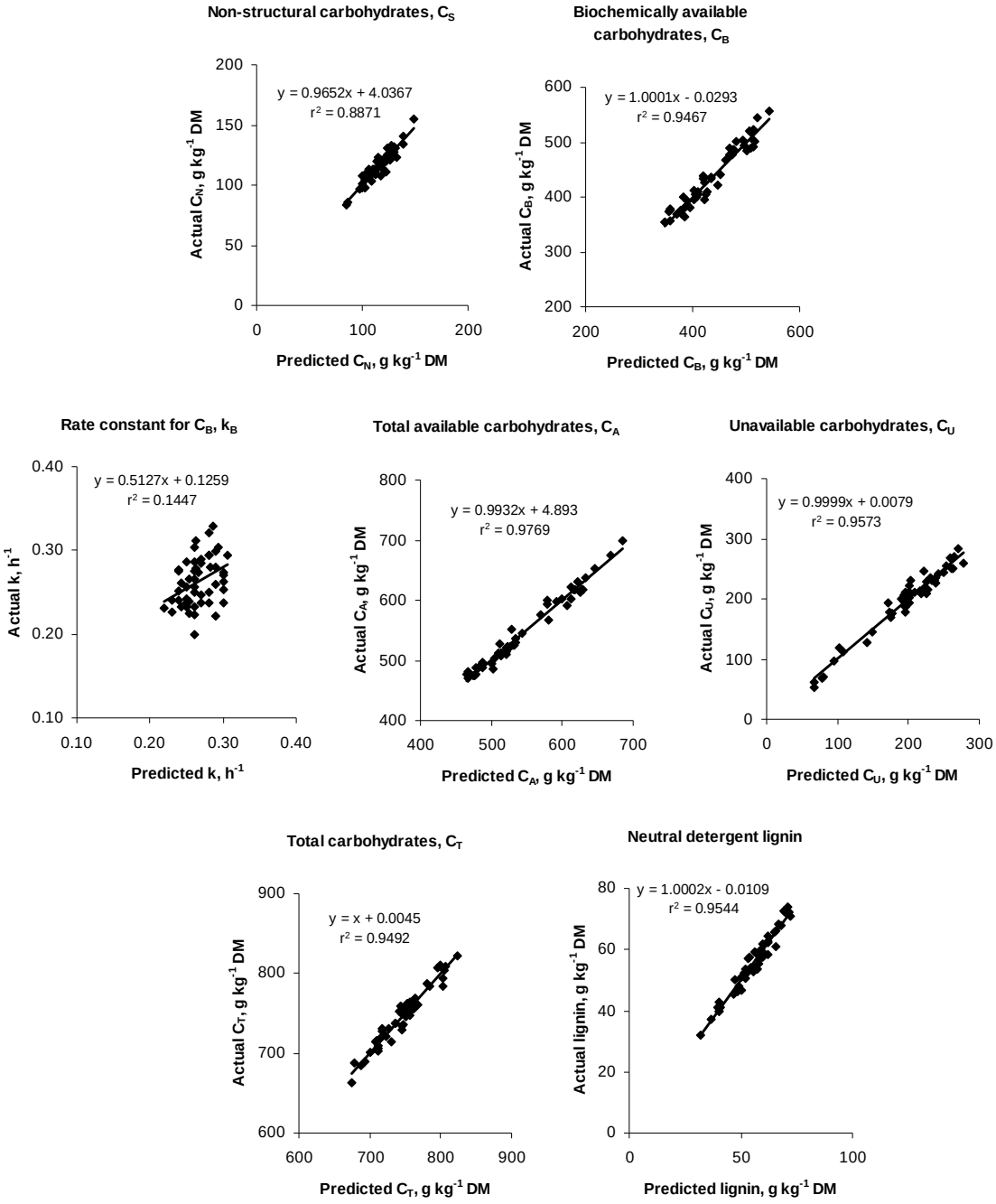

Figure 5.2. Correlations between actual values from the carbohydrate availability assay and predicted values from near infrared reflectance spectroscopy (NIRS) calibration equations for corn stover samples grown in Iowa and Wisconsin in 2005 and 2006.

Chapter 6.

General Conclusions

The primary focus of my research was to develop a system for screening lignocellulosic biomass feedstocks for biochemical conversion to biofuels. Development of this screening system was completed sequentially: initially, a series of parameters were developed for characterizing the quality of biomass feedstocks for biochemically conversion based on the true digestibility model for ruminant feedstuffs [1]. These parameters were synthesized into a carbohydrate availability model which partitions the carbohydrate portion of feedstock dry matter (DM) in into three groups based on their availability to undergo biochemical conversion: cell-soluble carbohydrates, C_N, biochemically available carbohydrates, C_B, and unavailable carbohydrates, C_U. Next, a wet-chemistry assay was developed and refined for quantifying these three quality parameters, as well as lignin concentration. The wet-chemistry assay was used to analyze a diverse group of biomass feedstocks, including cool-season grasses, warm-season grasses, corn residues, and woody materials. Finally, we demonstrated that near infrared reflectance spectroscopy (NIRS) could be used to rapidly determine quality parameter values. Such a high-throughput system would be necessary for implementation of the screening system at a biorefinery.

The biomass quality assay worked well for herbaceous feedstocks. We observed large variations in C_N, C_B, and neutral detergent lignin (NDL) across feedstocks types and within the feedstock types, particularly the corn residues.

In contrast, there was little variation within the woody species and C_B values were very low (50-83 g kg^{-1} DM) and C_U values very high (626-722 g kg^{-1} DM). This suggests the assay is likely not as applicable for woody species as herbaceous materials. Using a more disruptive cell-wall isolation method than the neutral detergent fiber (NDF) procedure for woody material would be necessary to improve partitioning of cell-wall carbohydrates from C_U to C_B. This should provide more stratification of C_B and C_U values across woody species and the improve differentiation of quality for these materials.

The end-goal of this research was to develop a standard, easily deployed methodology for analyzing end-use value of biomass feedstocks and, in doing so, to lay the groundwork for grading and commoditizing biomass feedstocks. This goal is within reach, but additional work needs to be done before it can be realized.

The chemical analysis method having the most parallelisms, in terms of development, standardization, and acceptance, to the feedstock quality methods we developed is the detergent fiber system for analyzing forage and other ruminant feedstuffs. Development of the detergent fiber system was initiated by Peter Van Soest in the early 1960's to identify a suitable replacement for the antiquated proximate analysis system. His work produced the NDF procedure and the acid detergent fiber (ADF) procedure, which made it possible to describe the nutritional value of feedstuffs in the terms of intake and digestibility. The ADF method was first published in 1963 [2] and was accepted, thereafter, as a standard method by the Association of Official Analytical Chemists (AOAC) with

little resistance [3]. The NDF was first published in 1970 [4] and finally gained AOAC approval in 2002 [5] thanks to the efforts of David Mertens over the prior 20-year period. The delay in acceptance of the NDF procedure were mainly a result of the numerous variants of the procedure developed over time and used with different feedstuff types [6], although there were likely was some political issues that caused additional delays.

The obstacles the biomass quality assay will face during the standardization process will be similar to that faced by the NDF procedure. In particular, a handful of key techniques used in this assay that will likely delay securing an AOAC-approved method, including the types of cell-degrading enzymes used, their loading rates, and the use of the NDL procedure to quantify lignin. An enzyme loading rate experiment(s) could be done to evaluate how the cocktail of commercial enzymes used in our assay and different enzyme products and loading rates affects quantification of C_B. Ben Goff at Iowa State University and I are currently in the process of comparing the NDL method (with and without the of use sodium sulfite in the NDF procedure) to other procedures for quantifying lignin, including Klason lignin, permanganate lignin, and acid detergent lignin, and their relationship to carbohydrate availability in biomass feedstocks and digestibility in forages. Regardless whether the goal is AOAC approval or not, both of issues need to be addressed to understand their effect on quantifying the quality parameters.

Development of an AOAC method for evaluating the quality of biomass feedstocks for biochemical conversion would be a career accomplishment;

however, it is ultimately more important that we developed a system that works and is implemented in the cellulosic ethanol industry. Although the work completed in this dissertation to develop this biomass quality system is concrete, with exception of some minor changes to the procedures in the assay, we have yet to demonstrate how changes in feedstock quality affect key process variables in the pretreatment, hydrolysis, and fermentation train. The most immediate future work with this biomass quality system should focus on understanding how changes in the magnitude of parameters, particularly C_N, C_B, C_U, and NDL, influence process variables, such as optimal pretreatment technology, pretreatment intensity, solids loading rates, and enzyme loading rates. Successful demonstration of the value of our biomass quality system for process control and optimization within a cellulosic ethanol plant would open the door to collaboration with cellulosic ethanol companies to develop NIRS calibrations for measuring quality parameters at the point of delivery to the plant and equations to relate quality parameter values to important process variables in their process. Hopefully this would lead to more industry-wide acceptance of our biomass quality system and a government-approved method for commoditizing biomass feedstocks.

The effort required to gain AOAC-approval for the biomass quality assay and gain industry acceptance of our biomass quality system will be non-trivial. Nonetheless, development and implementation of a feedstock quality system is paramount to the widespread success of the cellulosic ethanol industry in the twenty-first century, as was the Grain Standards Act of 1916 to the growth of the

grain marketing and export industry in the United States in the twentieth century [7].

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