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What can be Learned from Silage Breeding Programs?

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Abstract Improving the quality of cellulosic ethanol feedstocks through breeding and genetic manipulation could significantly impact the economics of this industry. Attaining this will require comprehensive and rapid characterization of large numbers of samples. There are many similarities between improving corn silage quality for dairy production and improving feedstock quality for cellulosic ethanol. It was our objective to provide insight into what is needed for genetic improvement of cellulosic feedstocks by reviewing the development and operation of a corn silage breeding program. We discuss the evolving definition of silage quality and relate what we have learned about silage quality to what is needed for measuring and improving feedstock quality. In addition, repeatability estimates of corn stover traits are reported for a set of hybrids. Repeatability of theoretical ethanol potential measured by near-infrared spectroscopy is high, suggesting that this trait may be easily improved through breeding. Just as cell wall digestibility has been factored into the latest measurements of silage quality, conversion efficiency should be standardized and included in indices of feedstock quality to maximize overall, economical energy availability.

Keywords Silage breeding · Corn stover · Repeatability · Quality

Introduction

Large amounts of funding and significant advances in research are making the production of fuels from cellulosic sources a reality. At least six "biorefineries" received funding from the Department of Energy and are expected to be completed within the next 5years [1]. Corn stover is widely recognized as a low-cost feedstock for initial use on a large scale because of its current abundance and proximity to existing corn grain ethanol plants. Examples of other potential feedstocks include perennials such as switchgrass, miscanthus,

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popular, and native prairie grass mixtures. Energy for fuel production from any cellulosic source will be derived from the plant cell wall, which is primarily composed of cellulose (polysaccharide of glucose) and hemicellulose (complex polymer of both five- and six-carbon sugars). These sugars will be made available for fermentation through chemical pretreatment of the feedstock followed by enzymatic hydrolysis of the remaining cellulose [2].

Carbohydrates bound in cellulosic biomasses are also the primary energy source for ruminant animals. One example is corn silage, which is a high-yielding and high-quality feed commonly fed to dairy cows. Corn silage is produced by harvesting whole corn plants a few weeks before physiological maturity and ensiling. The most significant carbohydrate sources in corn silage are from the starch and cell wall fractions, which are made available to the animal by a complex community of anaerobic microbes that reside in the gastro-intestinal tract [3]. Both highly degradable (starch and simple sugars) and less degradable (cell wall bound) carbohydrates are converted to volatile fatty acids and absorbed through the rumen wall [4]. Metabolizable energy that exceeds maintenance requirements in a healthy, lactating dairy cow is used for milk production.

Crop varieties with greater biomass yield, carbohydrate concentration, and carbohydrate availability would be better adapted for use as cellulosic feedstocks and could significantly improve the economics of this industry by increasing the amount of energy produced from an area of land as well as reducing costs associated with pretreatment and enzyme hydrolysis [2]. When a plant breeder wants to improve a species for novel uses, three simple but fundamental questions must be answered: (1) “What are we selecting for?” (2) “How are we going to measure it?” (3) “Can we do it efficiently?” Evaluation of biomass yield and composition for selection and recombination requires logistical efficiency if significant gains are to be realized in the near future. Characterizing biomass properties that influence ethanol production, developing tools for efficient measurement of these properties, and synthesizing relevant properties into a single, accurate, and intuitive index would facilitate variety selection and breeding. Novel variation for cell wall properties has been generated through genetic engineering, and its potential for increasing biomass yield is being investigated by manipulating genes involved in photosynthesis and nitrogen metabolism [5]. However, the need for these tools still remains as such engineered variation must be entered into breeding programs for recombination with agronomically elite genetic backgrounds. The effect different genetic backgrounds have on transgene expression and phenotype should be studied in a field setting.

The University of Wisconsin (UW) has conducted a silage breeding program for the past 15years using near-infrared spectroscopy (NIRS) based predictions of forage quality. Collaboration with nutritionists and agronomists has been instrumental in defining agronomic and quality characteristics for their improvement. The relationship between developing corn varieties for silage use and developing varieties for use as a cellulosic feedstock is obvious: In both cases, we want to maximize total available energy from plant biomass that is converted into a product through the action of microorganisms. The objective of this paper is to provide insight into what is needed for the measurement and thus genetic improvement of cellulosic feedstocks. We will accomplish this by reviewing the development and operation of a corn silage breeding program and discuss the evolving analysis of silage quality. Although yield is tremendously important, this paper will focus on quality because it is harder to measure, and the session topic under which this paper was originally presented centered on feedstock composition. We will also highlight the concept of repeatability, discuss why it is important for breeding, and report repeatability estimates of corn stover composition measurements related to ethanol production.

Measuring Energy Value of Corn Silage

Because grain makes up 40–45% of the total silage dry matter and starch, the largest constituent of grain, is a highly available energy source, it was previously believed that the best grain-producing varieties were also the best silage varieties [6]. However, we now know that this is not necessarily true because of variability in neutral detergent fiber (NDF, total cell wall concentration determined by Van Soest detergent analysis) digestibility and thus amount of energy that can be utilized from this substantial fraction [7]. Public and private breeding projects as well as university extension programs have developed and identified corn varieties for dedicated silage use. High quality is desired in a silage variety and is important for maximizing the amount of milk that can be produced from 1ton of silage; it is influenced by both total available energy content and dry matter intake potential of the silage [6]. Dry matter intake is defined as the amount of silage an animal can consume in a given amount of time. Feeding trials for determining the quality of individual varieties are practically impossible, so ranking varieties based on milk potential must rely on measuring chemical constituents and in vitro assays that are functionally and statistically associated with milk production in vivo. Constituent concentrations and their availability are entered into a model for prediction of total available energy for lactation.

The calculation of net energy of lactation (NEL) has been continually refashioned to better reflect both the amount, as well as the ruminal availability, of nutrients contributing to dietary energy used for milk production. Up until the early 1990s, the energy value of forages, including corn silage, was commonly assessed by measuring acid detergent fiber (ADF) [8]. The negative correlation of ADF, which is mostly composed of cellulose, lignin, and mineral constituents, with fiber digestibility is largely due to the fact that lignin restricts ruminal microbial degradation of plant fiber. When evaluating corn silage, nutritionists usually assumed complete digestion of starch and calculated dietary energy accordingly. This approach was used in spreadsheets for prediction of milk potential developed at the UW named MILK91 and MILK95.

It has long been recognized that the simplistic calculation of NEL based solely on amount of starch, lignin, or ADF ignores variation in the digestibility of fiber, starch, and other dietary components. Digestibility and NEL predictions based on ADF concentration alone can have high errors and empirical equations used depend upon the set of samples evaluated [6, 9]. However, initially, there were few analytical tools that accurately measured rate or extent of degradation of individual dietary constituents in the rumen. Furthermore, dry matter intake was assumed to be solely a function of NDF concentration in the diet because fiber increases bulk density in the rumen and decreases the amount of feed an animal can process over time. Results from feeding trials in which NDF was held constant and NDF digestibility (NDFD) was varied showed that digestibility of the NDF fraction also affects dry matter intake [10, 11]. These deficiencies have been addressed in the several refinements of early dietary models (MILK1991, MILK1995, MILK2000) that eventually resulted in the current system of ration evaluation, MILK2006.

The major refinement during these revisions was the development a multicomponent summative energy equation [12, 13]. The digestibility of protein, fatty acids, fiber (as measured by NDF), nonfiber carbohydrates, and starch are all used in the calculation of NEL. Because dry matter intake is not only a function of NDF concentration but also of NDFD, both factors were taken into consideration. MILK2006, therefore, now combines a robust summative energy calculation of NEL along with an estimate of intake potential to predict potential milk production per unit weight of forage.

Table 1 depicts the relative effects of NDF and starch on potential milk production as the dietary models changed over the last 15+years. It is apparent that the early nutritional models (MILK1991 and MILK 1995) did not consider effects of the ruminal availability of these constituents, and this is reflected in the lack of correlation between milk production potential and digestibility of either NDF or starch. MILK91 and MILK95 applied no weight to starch digestibility and NDFD. The small, negative correlations are due to the lack of independence between constituent concentrations and their digestibilities. This was corrected with the advent of MILK2000 and the further modifications for MILK2006. Nutritional studies have shown that varieties with higher fiber concentrations (low starch percentage) can outperform varieties with lower fiber concentrations (high starch percentage) with respect to actual milk production measured in vivo [6]. This is thought to be due to differences in fiber digestibility and the earlier models (MILK91 and MILK95) would have incorrectly ranked these varieties. Moreover, MILK2006 more accurately predicts actual production values taken from the literature than MILK2000 [13].

It is important to recognize why these nutritional models were first established and what use they currently serve. In well-managed dairies, rations are balanced to provide a nutritionally complete feed with maximum energy value. Feedstocks vary on a daily and seasonal basis, so a flexible and rapid nutritional assay is necessary. MILK2006 provides means for predicting energy content and intake potential of harvested forages so that these forages can be appropriately combined with other fiber and nonfiber dietary components when fed as part of a complete ration. Plant breeders also quickly realized the utility of these simple and rapid predictive assays to provide selection indices when choosing among new forage strains or cultivars for breeding purposes. In addition, MILK2006 is now commonly used in extension trials of new silage corn hybrids throughout the USA and Europe, and these evaluations are used by individual farmers to purchase hybrid seeds for planting [14].

Fortunately, NIRS can be employed to estimate most components of nutritive value important for forages, and these estimates can be used directly with MILK2006. For example, NDF, starch, protein, and NDFD of corn silage can easily be measured with NIRS, and these constituents along with MILK2006 have been routinely used by the UW corn breeding program to develop superior silage varieties [4, 15]. In a typical year, the UW silage breeding program collects forage samples from 4,000 to 6,000 field plots. Based on forage yield, approximately one half of the samples from the most productive hybrids are then analyzed for quality. This must be completed within a 4–6-week period between early

Table 1 Correlation coefficients (r) for neutral detergent fiber (NDF) and starch, as well as NDF and starch digestibilities (NDFD and StarchD) with milk per ton estimates from MILK1991 to 2006 dietary models.

r values	MILK2006[a]	MILK2000[b]	MILK1995[c]	MILK1991[d]
NDF	−0.46	−0.40	−0.94	−0.99
Starch	0.48	0.44	0.75	0.74
NDFD, % of NDF	0.49	0.70	0.16	−0.10
StarchD, % of starch	0.30	0.21	−0.25	−0.27

Data provided by J. G. Lauer (UW—Madison Agronomy Department, n=3727 treatment means for corn silage hybrids evaluated in Wisconsin). Adapted from Shaver [13].

[a] Calculated as per Shaver [13]

[b] Calculated as per Schwab et al. [12]

[c] Calculated as per Undersander et al. [8] except for in vitro dry matter digestibility adjustment

[d] Calculated as per Undersander et al. [8] using ADF and NDF.

September and mid-October so seeds of selected families can be sent to a winter nursery for generation advancement. Finally, after each season of silage evaluation, the ability to predict the nutritive value of the germplasm under development is upgraded by including new NIRS spectral samples and corresponding laboratory evaluations in the calibration database.

Although NIRS prediction equations have proven to be reliable and accurate, knowledge of nutritional requirements has increased from the time the equations were initially developed. This will always be the case because of the need to assemble a large nutritional database encompassing a large array of different forages grown in different environments for NIRS prediction to work adequately. Assembling such a database takes several years to accomplish. While data are being accumulated, the nutritional concepts validating the initial effort of NIRS prediction change somewhat requiring that altered or new nutritional characteristics be considered. The UW NIRS calibration data for NDF, NDFD, and protein were developed through compositional evaluations of a large number of corn hybrids by the UW corn breeding project beginning in 1992 and continuing to the present time (Table 2). Starch concentration was added in 1995.

Without doubt, important new nutritional characteristics will emerge as our knowledge increases and our analytical abilities improve. One potential improvement relates to the fact that current NDF digestion analyses may not provide the best representation of how fiber is utilized. The energy from the fiber component of a diet is a function of amount of total fiber and both rate and extent of fiber digestion. A single time point (e.g., 48-h) determination of NDFD represents a combination of both potential rate of digestion and total extent of fiber digestion, but it may be more efficient to deal with these factors independently. Increasing the rate of digestion would permit a greater extent of digestion before particles pass from the rumen, and many researchers have recommended that NDF digestion rate should be included, if possible, in the nutritional assessment of corn silage [16]. There is considerable variation in the rate of NDF digestion, especially apparent in hybrids carrying the *brown midrib3* (*bm3*) mutation such as F657 (Fig. 1), and perhaps this characteristic will eventually be included in the suite of traits used to evaluate silage hybrids.

Issues Related to Measuring Energy Value of Corn Stover as an Ethanol Feedstock

Similar to the silage quality situation, carrying out complete fermentations for determining the relative energy value of different crop varieties for breeding is impractical considering the large number of samples requiring analysis. High-throughput methods for determining

Table 2 NIRS calibration statistics for corn silage used at the University of Wisconsin for broad-based prediction equations used to estimate neutral detergent fiber (NDF), in vitro true digestibility (IVTD), protein, and starch of corn silage.

Trait	Number	Mean	R^2	SEC	SEV(C)	# PLS terms	Math trt.
NDF (%)	838	47.0	0.93	1.53	1.60	13	1,4,4,1
IVTD (%)	642	80.0	0.82	1.47	1.56	11	1,4,4,1
Protein (%)	844	7.5	0.92	0.32	0.34	13	1,4,4,1
Starch (%)	293	28.4	0.94	1.83	2.02	9	1,4,4,1

NDF digestibility is calculated from NDF and IVTD.

SE Standard error, *R* coefficient of determination, *SEC* standard error of calibration, *SEV(C)* standard error of cross-validation, *#PLS terms* number of terms used for modified partial least squares regression.

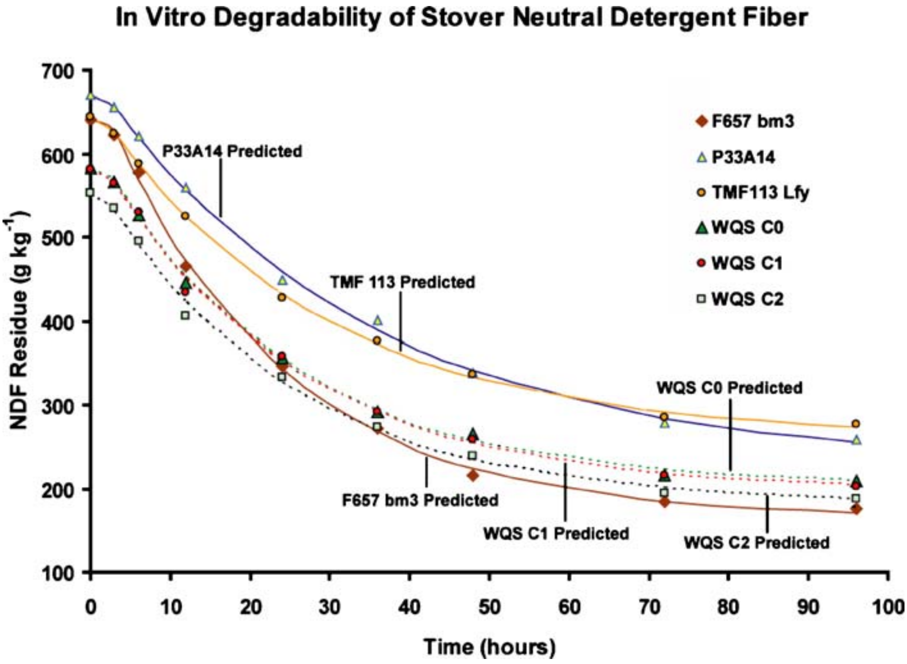

Fig. 1 In vitro neutral detergent fiber digestion for stover collected from six corn varieties over six time periods of incubation with rumen microbes. The hybrid designated P33A14 is a conventional high-yielding grain hybrid. Hybrid TMF113 carries the leafy gene that increases the number of leaves above the ear. The brown-midrib hybrid F657 carries the *bm3* gene that reduces lignin concentration of the stover. The germplasm designated *WQS C0*, *C1*, *C2* are breeding populations produced by two cycles of selection for silage yield and quality [14]. Data from Justen [20]

feedstock constituents, their energy value, and conversion efficiency will be required and must be combined into an index that accurately ranks varieties for cellulosic ethanol quality at the industrial scale. The National Renewable Energy Laboratory (NREL) has developed an NIRS equation for predicting the concentration of individual cell wall carbohydrates and theoretical ethanol potential of corn stover [17]. However, the theoretical ethanol potential assumes the cell wall carbohydrates to be completely available for fermentation and does not reflect variability in stover digestibility among varieties. Moreover, hydrolysis of cellulose carried out by costly cellulases and chemical pretreatment—to make the cellulose more accessible to enzymatic hydrolysis—are currently among the most expensive steps in the process [2]. Identifying and breeding varieties with stover that is more conducive to chemical and enzymatic digestion could be of more value than those varieties that contain higher carbohydrate concentrations but are more recalcitrant. Because cellulosic ethanol production practices are under development and will likely not be uniform anyway, determining a standard method for assessing the economically available energy through combining composition and degradability will allow for breeders to make progress in improving the overall ethanol quality of corn stover, just as silage breeding and quality analysis has added additional variables for a more comprehensive model of quality.

As discussed above, the rate of digestion impacts the energy value of corn silage, and hopefully, future tools allow ruminant nutritionists and breeders to include this characteristic in quality evaluations. Likewise, genetic variability for rate of chemical degradation or conversion into ethanol may impact the economics of cellulosic ethanol plants by influencing the rate of biofeedstock throughput that can be achieved. Determining the relative importance of conversion rate, how to measure this trait, and how to incorporate it into a comprehensive model would be essential to developing a variety that is completely converted in substantially less time. While more immediate hurdles have priority (total carbohydrate content and conversion efficiency), genetic improvement of these characteristics will reach limits, making conversion rate one of the next feedstock properties that could contribute to overall process efficiency in the future.

Accurate prediction of NEL, dry matter intake, and thus milk production potential provides correct ranking of varieties for silage use, and the use of NIRS for prediction of silage constituents has made large-scale breeding and variety evaluation possible. The current status of cellulosic biofeedstock development for corn is directly analogous to that of silage breeding before the development of a dedicated breeding effort. For biofeedstock development, what now remains is to determine the logistical requirements for large-scale field evaluations. To this end, we need information on the appropriate number of unique field environments (locations and years of evaluation) as well as the amount of replication within each environment that are necessary to correctly rank potential germplasm sources. Plant breeders use the concept of “repeatability” to address these requirements for accurate assessment at the field level.

Repeatability of Corn Stover Traits Related to Cellulosic Ethanol Production

Measurement precision combined with genetic variation of a characteristic is vital to its improvement. Genetic gain for a trait made each cycle of selection by plant breeders directly relates to the heritability (h^2) of a trait and its selection differential (S: difference between mean of selected individuals and population mean).

$$\text{Genetic gain per cycle} = h^2 S$$

The selection differential is directly related to the selection intensity. The selection intensity can be increased by either selecting a smaller number of individuals for recombination or increasing the total number of individuals evaluated. The latter approach is preferred because a reduction in genetic diversity through selecting too few individuals may compromise future gains. Therefore, rapid methods of measurement are necessary to evaluate large numbers of families with adequate replication over different environments. The h^2 of a trait represents the proportion of S expected to be inherited by the next generation and is determined by the ratio of heritable genetic variance to total phenotypic variance. Repeatability sets an upper limit to h^2 and is a statistical measure used to quantify the importance of variation among individuals or families under evaluation (usually referred to as genotypes) relative to variation within genotypes [18]. Repeatabilities are dependent upon the genotypes evaluated as well as the environments in which they were evaluated. When genotypes are randomly sampled from a defined reference population, repeatability is termed broad-sense heritability or coefficient of genetic determination. Plant breeders typically calculate repeatability (R) on a family mean basis with:

$$R = \frac{\sigma^2_{\mathrm{G}}}{\sigma^2_{\mathrm{G}} + \left(\sigma^2_{\mathrm{GE}}/e\right) + \left(\sigma^2_e/re\right)}$$

where σ^2_G is the variation among genotypes, σ^2_{GE} is the variation because of genotype by environment interaction, σ^2_e is the variation because of error, e is the number of environments individuals were evaluated in, and r is the number of replications within environments. Essentially, the presence of genotype by environment interaction and random environmental noise (measurement error, within environment variation) reduces repeatability and thus reduces efficiency of selection. Increasing replication and/or the number of environments used for evaluation increases repeatability at the expense of additional resources.

We calculated repeatability values for a set of silage traits and a set of stover traits measured in a nearly identical set of corn hybrids. Forty hybrids were evaluated for silage traits at 30–40% dry matter, and 44 hybrids were evaluated for stover traits at physiological maturity (approximately 50% dry matter) during 2005 and 2006. Hybrids were evaluated in a randomized complete block design with three replications within each of four environments (480 silage plots, 528 stover plots). Plots consisted of two rows (0.76m apart and 6.08m long) planted to a density of 79,000 plants per hectare. A single sample was obtained from each plot and ground to pass a 1-mm screen for compositional analysis. The global NIRS equations discussed above were used to predict NDF, NDFD, and starch concentrations of each sample. These constituents were then entered into MILK2006 for prediction of milk per ton. The stover constituents glucan, xylan, galactan, mannan, arabinan, and lignin were predicted by Stover9, a NIRS equation developed at the NREL in Golden, CO [17]. Polysaccharide concentrations were used to calculate theoretical ethanol potential according to NREL's website (http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html). Silage and stover yield values were on a dry-matter basis.

Repeatability estimates of the silage traits were in the expected range and indicate that the majority of total variation observed was due to variation among hybrids (Table 3). The repeatability estimates for the stover characteristics predicted by Stover9 were excellent and mostly exceeded those of the silage traits. Stover9 predictions of individual hybrids were reliable across environments and replications within environments and suggest that ethanol potential and its components may be highly heritable. Both silage and stover yield repeatabilities were lower than those of the composition type traits, which is not surprising given the complex nature of yield and difficulty in obtaining reliable measurements in a field setting. Nevertheless, significant gains have been made for grain yield of corn through persistent breeding and selection [19].

Table 3 Repeatability estimates for silage and stover traits. Silage traits were predicted with the global NIRS equations discussed in text and MILK2000 was used to calculate milk per ton.

	R
Silage trait	
Milk per ton	0.72
NDF	0.65
NDFD	0.84
Starch	0.74
Silage yield	0.58
Stover trait	
Ethanol potential[a]	0.82
Glucan	0.81
Xylan	0.83
Galactan	0.87
Arabinan	0.95
Mannan	0.74
Lignin	0.85
Stover yield	0.75

Stover composition was predicted with Stover9 [17].

[a] Theoretical ethanol potential calculated with NREL's ethanol calculator (http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html).

Conclusions

Lessons from measuring and ranking silage varieties for potential milk production can be applied to the analogous situation of determining how to rank varieties for use as cellulosic feedstocks. Rapid methods of measurement capable of analyzing the large number of samples generated by breeding and variety evaluation programs will be required. In addition, a simple method of assigning an intuitive quality value to different varieties would be advantageous, and such an index should include total carbohydrate concentration and conversion efficiency. Commercial procedures such as pretreatment type and severity as well as specifics on hydrolysis reactions are not yet established. However, inclusion of conversion efficiency standardized by pretreatment severity, enzyme loading, and cost would better rank varieties based on economically available energy given the variation for cell wall digestibility among varieties. As more is learned about energy sources and inhibitors in cellulosic biomasses and how they relate to ethanol production, models for energy prediction will change. However, selection and improvement of feedstocks should still proceed, even when knowledge of the energy value is known to be incomplete. The rate of digestion is a silage property known to vary and impact NEL. Because increased rate of digestion could affect the economics of ethanol plants by increasing feedstock throughput in the future, this property may deserve attention, and simple measurement techniques will need to be developed that are statistically and functionally associated.

Repeatability of ethanol potential and corn stover composition as determined by NIRS prediction is high and suggests that these traits may be highly heritable. Improvements in feedstock composition through breeding could be realized with sufficient funding and effort. A comprehensive model for predicting economically available energy for ethanol production should be developed so the net amount of energy per ton of feedstock and per dollar can be maximized.

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