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A method to evaluate corn stover biomass for fermentability to ethanol and the characterization of maize (Zea mays) brown midrib mutants

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A method to evaluate corn stover biomass for fermentability to ethanol and the characterization of maize (Zea mays) brown midrib mutants

by

Lisa Julia Haney

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
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Iowa State University

Ames, Iowa

2007

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ABSTRACT

The economic feasibility of lignocellulosic biofuels can be improved by evaluating the fermentation potential of different feedstocks. The objective of this thesis is to provide a method to evaluate corn stover biomass for fermentability to ethanol and to characterize maize brown midrib mutants. This objective was addressed by the research presented in each research chapter. The objective of the first research chapter was to develop a sugar-consuming biosensor to monitor hydrolytic reactions and to demonstrate its application in monitoring corn stover hydrolysis. We found that growth rate of the biosensor was proportional to GFP-specific fluorescence, and total growth and growth rate depend upon how much sugar is present at inoculation. We also demonstrated that stovers can be differentiated based on sugar yields in enzymatic hydrolysis reactions. This biosensor could be used in screening methods to characterize hydrolysis of feedstocks or to evaluate the performance of hydrolytic systems. The objective of the second research chapter was to determine the repeatability of the screening method used to evaluate the fermentability of corn stover biomass. We found that there was no significant effect within a technical replication, but there was a significant effect between technical replications. These results suggest how to best design experiments to use the method of simultaneous saccharification and catabolism to precisely obtain quantitative measurements of sugar availability, or fermentability, in corn stover. The objective of the third research chapter was to perform genetic complementation tests with maize lines carrying four known brown midrib mutant alleles and lines carrying unknown brown midrib mutant alleles in order to determine if unknown brown midrib mutant alleles are actually mutant alleles of brown midrib genes that
have not been characterized genetically. We found that two of the lines carrying unknown brown midrib alleles failed to complement a line carrying the $bm1$ allele, one line failed to complement a line carrying the $bm3$ allele, one line failed to complement both the line carrying the $bm1$ and the line carrying the $bm2$ allele, and three lines complemented lines carrying each of the four of the known brown midrib alleles. These new mutant alleles may encode enzymes or regulatory elements of the phenylpropanoid pathway that are either undiscovered or have not had a mutant phenotype associated with them yet. The elucidation of the phenylpropanoid pathway may be very useful in terms of biofuel production because we could use this information to alter lignin composition to optimize biofuel feedstocks.
CHAPTER 1. GENERAL INTRODUCTION

RATIONALE

Biofuels are an area of interest now for several reasons. Biofuels carry the promise of supporting our efforts of national security, helping to solve our problems associated with global climate change, and ultimately providing us with cheaper liquid fuels. Although these are all positive potential consequences of biofuels, it is important to consider the negative consequences or difficulties that lie with making fuels out of biomass as well. The specific areas to address are environmental impact, storage, harvest, and transportation technologies, and acceptance in the agricultural industry as well as details like what feedstocks and procedures to use to make the biofuels. All of these areas are currently being addressed by several groups in hopes of realizing the potential energy from biofuels. This thesis will not address the negative impacts of biofuels, but rather attempt to explain some of the current biofuels used today and present some information that may be useful to improving future biofuel feedstocks.

The objective of the research for this thesis was to provide a method to evaluate corn stover biomass for fermentability to ethanol and to characterize maize brown midrib mutants. This objective was addressed in three research chapters by: developing a sugar-consuming biosensor to monitor hydrolytic reactions and to demonstrate its application in monitoring corn stover hydrolysis, evaluating the quality of this application of the biosensor to monitoring corn stover biomass, and elucidating the genetics of maize germplasm that may be useful for developing highly fermentable corn stover biomass for ethanol production.
THESIS ORGANIZATION

This thesis consists of a general introduction, three research articles, and a general
discussion as well as cited references and acknowledgements. The general introduction
consists of the thesis objective, the organization of the thesis, the author’s role in the
research presented in this thesis as well as contributions to future publications, and a
literature review. The first research article is entitled “Development of a Real-time
Sugar-Consuming Biosensor and its Application to a Corn Stover Hydrolysis Bioassay.”
This article presents a new method for screening corn stover feedstocks and evaluating
their potential fermentability. It was demonstrated that stovers can be differentiated
based on sugar yields in enzymatic hydrolysis reactions, and it was possible to monitor
the course of enzymatic hydrolysis in real-time. This biosensor has potential application
as a screening method to characterize hydrolysis of feedstocks or to evaluate the
performance of hydrolytic systems. The second research article is entitled “Quality
Control of Simultaneous Saccharification and Catabolism.” This article discusses the
repeatability of the screening method simultaneous saccharification and catabolism. The
third research article is entitled “Complementation Testing of Maize Genetic Cooperation
Stock Center Lines Containing Unknown brown midrib Alleles.” This article describes
the complementation tests performed on lines containing unknown brown midrib alleles
with lines containing known brown midrib alleles. Three lines were shown to be allelic
to known brown midrib alleles and their new designations were reported. Following the
third research article is a general discussion including general conclusions from the
research presented in this thesis as well as insights to future directions for biosensors and
biofuels in general. References for the general introduction and each research article are reported at the end of each chapter.

AUTHOR’S ROLE

Role in Research Articles

All of the research articles were written by the primary author with the guidance and assistance of the co-authors. In the first research article (Chapter 2), each author contributed to the work in a different way: M. Paul Scott contributed by providing new approaches and guidance, D. Raj Raman and Robert Anex contributed by providing intellectual discussions and insight, James G. Coors and Aaron J. Lorenz contributed by collecting and grinding corn stover samples. Besides chopping, weighing, and grinding corn stover samples, all the methods described in the first research article were performed by the primary author with use of materials and equipment in M. Paul Scott’s laboratory.

In the second research article (Chapter 3), M. Paul Scott provided guidance and assistance while Krystal Kirkpatrick, Kendall R. Lamkey, and the primary author helped to harvest the corn stover sample used in the single batch replication experiment. Dirk Phillip provided all the corn stover samples for the technical replication experiments.

In the third research article (Chapter 4), M. Paul Scott provided guidance and assistance with the germplasm selection and experimental design. The Maize Genetic Cooperation Stock Center provided the maize seeds for evaluation. Members of the M. Paul Scott laboratory helped with planting and pollinating, and the screening was performed by the primary author.
Role in and Contribution to other efforts

In addition to the efforts of the primary author in the research articles, contributions were made by the author of this thesis that will be included in future publications. The method simultaneous saccharification and catabolism (SSC), described in the first research article (Chapter 2), was used to evaluate selected germplasm for suitability for ethanol production as part of a joint USDA-DOE grant called “Integrated Feedstock Supply Systems for Corn Stover Biomass.” The germplasm consisted of 50 maize lines selected for various traits. These entries were replicated three times and grown in two locations in Iowa and two locations in Wisconsin in 2005 and 2006 for a total of 1200 corn stover samples. These corn stover samples were analyzed by the author of this thesis by SSC. In 2006, the husks were separated from the corn stover at both Iowa locations which produced 300 husk samples from Iowa. These husk samples were also analyzed by the author of this thesis by SSC. These SSC data will be included in future publications involving plant breeding and method comparison. Also, a subset of 30 corn stover samples from Wisconsin were reevaluated by SSC in an attempt to make a near-infrared (NIR) calibration equation for corn stover fermentability.

The SSC method has also been used by the author of this thesis to evaluate biomass pretreatment methods and feedstocks for other researchers. In 2006, Dirk Phillip, a post-doctoral scientist in Kenneth J. Moore’s laboratory, produced 506 corn stover samples that had been subjected to various pretreatment methods. These were all evaluated by the author of this thesis by SSC. In 2007, Tom Richard, faculty member at Pennsylvania State University, produced 150 corn stover samples and 50 switch grass samples that were evaluated by SSC by the author of this thesis. Finally, in 2007, Bruce
Dien, a USDA-ARS scientist at the National Center for Agricultural Utilization Research, produced 50 Bermuda grass samples that were evaluated by SSC by the author of this thesis with assistance by Adrienne Lauter, a biological laboratory science technician in M. Paul Scott’s laboratory.

LITERATURE REVIEW

Introduction

This literature review contains six sections, each of which provides enough background information required to understand the following chapters thoroughly. The six sections are: 1) using biomass for energy, 2) cell wall structure and synthesis, 3) the phenylpropanoid pathway and its mutants, 4) GFP discovery and properties, 5) history of breeding for ruminant digestibility, and 6) high-throughput screening in breeding. The section on using biomass for energy describes several different types of liquid fuels made from bio-based products and focuses on cellulosic ethanol production. The sections on cell wall structure and the phenylpropanoid pathway describe each of these topics in detail in an attempt to give enough information about the way sugars are organized in the cell wall and why plants are recalcitrant to degradation. The section on GFP describes the general properties of this protein and is important because GFP is the fluorescent marker used in simultaneous saccharification and catabolism (Chapter 2) to detect sugar availability. The last two sections describe the breeding aspect and techniques for creating highly fermentable feedstocks for ethanol production.
Section 1. Using Biomass for Energy

Overview of Biomass for Energy

A common goal in the US is to reduce the cost of fuels and reduce greenhouse gas emissions (Hahn-Hagerdal et al. 2006). Fossil fuels currently provide about 95% of our global energy whereas biofuels supply only 10% of all energy used by humans (Somerville 2007). The most important reasons to use biofuels are to increase energy independence and to help solve our problems associated with climate change. Estimates show that about half of the recoverable petroleum has been used, and the countries using the most energy have only enough coal reserves to supply them with energy for the next 210 years (Somerville 2007). Both the US and the EU have set timelines for increasing the amount of biofuels used as liquid transportation fuels. The US plans to supply 30% of the 2004 gasoline demand with biofuels by 2030, and the EU plans to supply one-quarter of the transportation fuels used with biofuels by 2030 as well (Himmel et al. 2007). There are many different types of biofuels that can be used to supply our energy needs, including liquid biofuels such as ethanol and biodiesel, and other fuels such as syngas. Each of these biofuels is made from different biological resources called biomass. Ethanol is made from corn grain, sugarcane, and cellulosic material, while biodiesel is made from plant oils typically derived from oil seed crops, and syngas is made by converting biomass into carbon monoxide and hydrogen which are converted to fuel by the Fischer-Tropsch process (Somerville 2007). There is a large supply of coal that could be used for energy; however, biomass is better suited than coal for liquid fuel. Coal is better suited than biomass for combustion because it can be transported fairly easily and burns with less ash residue at a higher efficiency (Somerville 2007).
A Focus on Liquid Fuels

The two most common renewable liquid fuels used today are ethanol and biodiesel. Ethanol is a promising liquid fuel because it can be blended with gasoline or used by itself in ethanol-specific engines (Hahn-Hagerdal et al. 2006). Ethanol is currently made from two main sources of biomass: Sugarcane (*Saccharum* sp.) and corn (*Zea mays*). Cane ethanol has an output:input ratio of about 8 whereas corn grain ethanol only produces about 25% more energy than it consumes (Somerville 2007).

In sugarcane ethanol production, found mostly in Brazil, the sucrose inside the sugarcane is released by crushing the plants and then this sucrose is fermented to produce ethanol. The crushed sugarcane bagasse is then burned to provide energy for the ethanol plant (Pandey et al. 2000). In corn grain ethanol production, found in the US, the grain is finely ground in one of two processes, wet- or dry-milling, and the ground corn is then cooked so the starch gelatinizes. The cooked starch is hydrolyzed by starch-degrading enzymes, and the resulting sugars are fermented to produce ethanol. The co-product created from the dry-milling method of grain ethanol production is called distillers dried grains with solubles (DDGS). This co-product can be added to livestock diets as an additional source of protein (Bothast and Schlicher 2005; Somerville 2007).

Unfortunately, there is a major problem with corn grain ethanol in that it reduces the amount of corn grain that can be used as food for both animals and humans. This introduces competition which raises the price of corn causing increased prices of meat and potential negative environmental impacts from farmers growing more acres of corn to gain the higher profit. This is why corn grain ethanol may not be the most beneficial long-term biofuel (Hahn-Hagerdal et al. 2006; Somerville 2007).
Biodiesel is another widely used liquid biofuel. Biodiesel is made from lipids found in vegetable oils produced by plants (particularly soybeans) and can be used in diesel engines as fuel (Meher et al. 2006). Biodiesel has a net energy gain higher than that of corn grain ethanol but may not provide sufficient amounts liquid fuel due to the relatively small amount of lipid produced per acre. Also, vegetable oils are needed for food so a small change in the supply may create a large increase in the price of the feedstock and therefore an increase in the price for the biofuel (Somerville 2007).

In addition to corn grain ethanol and biodiesel, a new liquid fuel called cellulosic ethanol is being developed. Cellulosic ethanol is made from fermenting the sugars produced from lignocellulosic plant cell walls of biomass feedstocks, and the whole plant can be used so the amount of sugar produced per acre is potentially higher than with grain ethanol (Somerville 2007). There is approximately 1.3 billion tons of biomass available in the US today that could supply more than 50% of our liquid fuel consumption (Somerville 2007). 80-100 million dry tons of corn stover alone could be sustainably collected per year (Kadam and McMillan 2003). The fact that there is so much biomass and that it is rather inexpensive makes biomass an attractive feedstock for making liquid fuels (Wright et al. 1988).

Many have considered producing butanol instead of ethanol because, although it is toxic at low concentrations, it is better suited to transport in pipes, and solutions of greater than 9% butanol will separate from water, greatly reducing the high energy and monetary cost of distillation (Somerville 2007).
The Cellulosic Ethanol Conversion Process

Although it is abundant, lignocellulosic biomass is difficult to break down. The keys to increasing lignocellulosic digestibility are to increase the pore size, thereby increasing access to cellulose by large cellulase enzymes, and to remove the xylan (Wright et al. 1988). The typical lignocellulosic ethanol conversion process consists of 4 steps: pretreatment, enzymatic hydrolysis, fermentation, and distillation (Somerville 2007). A process known as simultaneous saccharification and fermentation (SSF) has been commonly used as a small scale example of the typical lignocellulosic ethanol conversion process (Himmel et al. 2007). SSF was first described in 1977 by Takagi et al. (1977) as a method to combine hydrolysis and fermentation into one step. It was later evaluated by Wright et al (1988). This method is superior to separate hydrolysis and fermentation (SHF) procedures used previously because it has lower capital costs and achieves a higher ethanol concentration in the final product which inhibits the growth of unwanted, lactic-acid producing bacteria (Ohgren et al. 2006a). Several papers describe the process of SSF with modifications (Hoskinson et al. 2007; Ohgren et al. 2006a; Ohgren et al. 2007; Ohgren et al. 2006b; Philippidis et al. 1993) but they all involve carrying out the hydrolysis and fermentation of the feedstock at the same time. Most of the differences between the methods occur in the first step of the process: the pretreatment step.

The overall goal of pretreatment is to break down the lignin barrier in the cell wall and to allow hydrolytic enzymes access to the cellulose. The pretreatment step of lignocellulosic conversion to ethanol is usually a chemical hydrolysis of the feedstock, although many different pretreatment methods have been used. Ammonia fiber/freez
explosion (AFEX), liquid hot water, steam, dilute acid, alkali, and sulfur dioxide impregnation are some of the more common methods of pretreatment. Mosier et al. (2005) carried out a thorough analysis of several pretreatment methods. In the AFEX pretreatment, liquid ammonia is added to biomass at ambient temperature and high pressure for 30 minutes. Then the pressure is released and the fibers explode because the ammonia gasifies under ambient pressure at -33.4°C (Dale 1986; Dale et al. 1996). The AFEX pretreatment method reduces the lignin content of the feedstock, hydrolyzes some of the hemicellulose, and increases the accessibility of cellulases by decrystallizing the cellulose (Mosier et al. 2005). The most commonly used acid pretreatment is dilute sulfuric acid at high temperatures for very short periods of time. The acid pretreatment hydrolyzes most of the hemicellulosic fraction of the feedstock, increasing enzymatic hydrolysis of the cellulose fraction, but it also produces inhibitory compounds such as furfural (Mosier et al. 2005). Sodium hydroxide is the most commonly used alkali for pretreatment. This pretreatment consists of adding sodium hydroxide to lignocellulose under normal temperature and pressure conditions for several hours or even days. The alkali pretreatment modifies the lignin and some hemicellulosic side-chains in order to increase availability of the cellulose and hemicellulose fractions. However, salts are produced in this process which may inhibit the pretreatment (Mosier et al. 2005). Sulfur dioxide impregnation followed by steam pretreatment has been used by Ohgren et al. in several papers (2006a; 2007; 2006b). By impregnating the lignocellulose with sulfur dioxide and then using steam pretreatment, cellulose becomes more available to enzymatic hydrolysis and the hemicellulosic sugars are recovered (Saha 2003).
After the biomass has been pretreated, the next step in the conversion process is enzymatic hydrolysis. This step is combined with fermentation in SSF. During enzymatic hydrolysis of lignocellulose, two enzyme preparations are generally used: cellulase and hemicellulase. Cellulases are commonly made by the filamentous fungus, *Trichoderma reesei*, and hemicellulose degrading enzymes can be found in microorganisms such as *Penicillium capsulatum* (Saha 2003). The cellulase preparation usually consists of three different enzymes in relative amounts that vary among different preparations. These enzymes are endoglucanase, exoglucanase, (also called celllobiohydrolase), and beta-glucosidase. The endoglucanase breaks the bonds between two glucose molecules within one cellulose polymer to create two polymers, whereas the exoglucanase breaks the bonds between two glucose molecules at the non-reducing end of the cellulose chain creating one cellobiose molecule and a shorter cellulose polymer. Beta-glucosidase breaks the bond between the two glucose molecules of cellobiose, creating two glucose monomers (Wright et al. 1988). The cellulase enzymes are inhibited by their end products, specifically cellobiose and glucose. Cellobiose inhibits the exoglucanase activity and glucose inhibits the beta-glucosidase activity. This inhibition can slow the process of enzymatic hydrolysis (Wright et al. 1988). The hemicellulase preparation may consist of many enzymes but the main enzymes are used to break down xylan, the main component of hemicellulose. The xylanases are endo-xylanase, exo-xylanase, beta-xylosidase, and other enzymes that hydrolyze side-chains of modified xylans. Endo-xylanase hydrolyzes bonds between xylose molecules in the middle of xylan polymers whereas exo-xylanase hydrolyzes bonds between xylose molecules at the ends of xylan polymers making xylobiose. The beta-xylosidase
hydrolyzes xylose monomers from short xylan polymers in addition to hydrolyzing the bond between the two xylose monomers of a xylobiose molecule (Saha 2003). Although enzyme hydrolysis may be more expensive than chemical hydrolysis, using enzymatic hydrolysis is more attractive than using chemical hydrolysis because the reactions of enzymes are specific and don’t create inhibitory side products (Wright et al. 1988).

The third step of the process in converting lignocellulosic biomass to ethanol is fermentation. As stated earlier, this is combined with enzymatic hydrolysis in SSF. Fermentation of the hydrolyzed biomass is carried out under anaerobic conditions by a fermentative organism, usually *Saccharomyces cerevisiae*, commonly referred to as baker’s yeast. This organism naturally metabolizes 6-carbon (hexoses) but not 5-carbon sugars (pentoses) (Hahn-Hagerdal et al. 2006). *Pichia stipitis* CBS 6054 naturally ferments xylose but is inhibited by pretreatment and hydrolysis (Hahn-Hagerdal et al. 2006). In order to overcome these obstacles, many different efforts have been made to engineer bacteria and yeast to best suit the industrial application of lignocellulosic ethanol production. In order to obtain organisms that will ferment both pentoses and hexoses, ethanol-producing, pentose-fermenting strains of *Escherichia coli* and *Klebsiella oxytoca* have been generated by introducing genes from *Zymomonas mobilis* (Hahn-Hagerdal et al. 2006). Two different strains of *S. cerevisiae* have been engineered to ferment pentoses by the addition of genes encoding xylose isomerase from *Thermus thermophilus* and *Piromyces sp.* (Hahn-Hagerdal et al. 2006). Many other engineered organisms to ferment both pentoses and hexoses are described in reviews (Lynd et al. 1999; Saha 2003). Also, in order to increase the tolerance of organisms to ethanol and inhibitory products, a recombinant *Z. mobilis* 8b has been engineered to withstand high
ethanol concentrations and large amounts of the inhibitory product, acetic acid (Mohagheghi et al. 2004). The best case scenario is a fermentative organism capable of producing cellulase and hemicellulase enzymes necessary for hydrolysis (Somerville 2007), fermenting both 5- and 6-carbon sugars, tolerating inhibitors produced by pretreatments, and surviving under high ethanol concentrations. This process is known as integrated bioprocessing (Lynd et al. 2005).

The final step in the process of converting lignocellulosic biomass to ethanol is distillation. This step is performed in order to recover the ethanol from fermentation and to dehydrate it so it can either be used by itself or blended with gasoline. The process of recovering the ethanol is well-characterized for the corn grain ethanol process (Bothast and Schlicher 2005). The ethanol is distilled to 95% through distillation columns, and then molecular sieves are used to dehydrate the ethanol to make 100% or anhydrous ethanol.

Section 2. Cell Wall Structure and Synthesis

Overview of Cell Wall Structure of Biomass Plants

Plants have evolved extensive systems to make them resistant to damage from both microbial and animal predators. These systems include thick, dense tissues, covered in some areas with trichomes and waxes, and cell walls consisting of crystalline and amorphous sugars interconnected and packed in water tight lignin (Himmel et al. 2007). Plant cell walls can also inhibit growth of microorganisms because many cell wall components are insoluble and therefore not conducive to enzymatic degradation. They may also contain or produce compounds that interfere with cell growth and development of the invading organisms (Himmel et al. 2007). Most of the information we have about
plant cell walls and cell wall growth comes from Arabidopsis thaliana because it is amenable to molecular and genetic characterization (Somerville 2006).

The plant cell wall is comprised of three main components. The most abundant component of plant cell walls is cellulose followed by hemicellulose and lignin. Proteins, oil, and ash also contribute to the plant cell wall in smaller quantities (Saha 2003). Cellulose, the main component of the cell wall, is comprised of beta-linked glucans that mainly provide structural support. These celluloses are embedded in and may bind to hemicelluloses to provide another layer of structural support (Cosgrove 2005). In addition to cellulose, plant cell walls contain matrix polysaccharides that can be divided into two classes: hemicellulose and pectin. This polysaccharide matrix is what causes plant cell walls to be rigid but also allows them to grow and elongate (Cosgrove 2005). Hemicellulose, a very important component of cell walls, is composed of heteropolymers of 5- and 6-carbon sugars including xylose, arabinose, galactose, mannose and glucose as well as sugar acids (Saha 2003). Lignin is the final major component of plant cell walls. It is responsible for waterproofing the cell wall as well as providing extensive structural support and a barrier to pathogens (Boerjan 2003; Humphreys and Chapple 2002; Moore and Jung 2001).

The Cell Wall Components and Their Synthesis

Cellulose

Cellulose is the most abundant biological polymer on earth. Cellulose is organized into microfibrils consisting of polymers of about 500 to 14,000 1,4-linked beta-D-glucopyranose, with 36 polymers held together by hydrogen bonds (Somerville 2006). There are two different types of cellulose: cellulose Iα and Iβ. Both of these types have
cellulose polymers that run in parallel to each other, but different crystal structures. Another type of cellulose with polymers that run anti-parallel can be created synthetically through treatment with alkali. This cellulose is called cellulose II, but has not been found to exist in nature. All cellulose polymers within a microfibril are polymerized at the same time (Somerville 2006). The glucose units that make up the cellulose microfibrils are arranged so that in the chair conformation, all of the hydroxyl groups are in equatorial positions and the hydrogen atoms are in axial positions. This configuration allows for strong hydrogen bonds to form between the hydroxyl units of the different microfibrils, forming sheets of cellulose. With hydrogen atoms in the axial positions on either side of the sheets, the sheets become hydrophobic on both surfaces. A dense layer of water molecules fills in the areas between cellulose sheets, inhibiting other molecules from coming into contact with the cellulose (Himmel et al. 2007).

Cellulose is synthesized in the cell membrane by cellulose synthase (CESA) genes. Ten genes in the CESA family have been identified in *Arabidopsis thaliana*. Three CESA genes are thought to encode six proteins that form the heterohexameric subunits of particle rosettes which form the cellulose microfibrils (Cosgrove 2005). As the cellulose is synthesized in the cell membrane, the microfibrils created are about 5-10 nm wide and wrap around the cell many times causing the matrix polysaccharides to get caught in the microfibrils, providing strength against the high turgor pressure (Cosgrove 2005). In addition to the cellulose microfibrils, there are amorphous forms of hemicellulose that intertwine with the cellulose to form more complex structures (Himmel et al. 2007).
Matrix Polysaccharides

Hemicellulose

Hemicellulose has a structure similar to cellulose in that it has beta-1,4 linked pyranosyl sugar monomers, but these monomers may not necessarily be glucose (Cosgrove 2005). The most abundant hemicelluloses are xylans. Xylan is composed of beta-D-xylopyranose units linked 1,4 in a chain to form a backbone with many different types of side groups. Common side groups are O-acetyl, alpha-L-arabinofuranosyl, or glucuronic acid, but side groups are not necessary to classify a xylose backbone as xylan. Homoxylans that are long polymers of beta-1,4 linked D-xylopyranose exist (Saha 2003). Different sources of xylan have different compositions but xylose is always the most abundant sugar monomer component of xylan (Saha 2003). Xylans with many or different types of side chains are called heteroxylans. These xylans are cross-linked by diferulic acid bridges adding another layer of support to cell walls by interacting with cellulose and reinforcing the cell wall structure (Saha 2003).

Two other common forms of hemicellulose are xyloglucan and arabinoylcan. Xyloglucan has a glucan backbone like cellulose, but the side chains are mostly comprised of xylose. Arabinoylcan has a xylan backbone with arabinose side chains as well as some acids or acid ester side chains (Cosgrove 2005).

Pectin

Pectin is the second class of matrix polysaccharides found in plant cell walls. These polysaccharides are soluble and form gels when hydrated. These gels aid in cell growth by allowing the cellulose microfibrils to move. When cell growth stops, these polysaccharides can help hold the microfibrils in place supporting the plant cell wall...
structure. Pectins are thought to be linked to each other by covalent bonds and to hemicellulloses by covalent and non-covalent bonds (Cosgrove 2005).

In contrast to cellulose, hemicellulose and the other matrix polysaccharides including pectin are created in the Golgi apparatus and transported to the plasma membrane. These polysaccharides are thought to be synthesized by cellulose synthase like (CSL) genes. The gene products are localized to the Golgi and have sequence motifs similar to beta-glycosyltransferases (Cosgrove 2005). Other proteins are required for the synthesis of polysaccharides in the cell wall but many are not fully characterized. Proteins that actually break down or ligate polysaccharides are necessary for proper cell wall synthesis, most likely in order to correct mistakes in synthesis or to create bonds between the cellulose microfibrils and the matrix polysaccharides (Cosgrove 2005).

Lignin

Lignin is the second most abundant biological polymer on earth (Boerjan 2003). It is also a structural component of plant cell walls that is composed of monolignols produced from the phenylpropanoid pathway and deposited during cell maturation. Most likely, lignin is cross-linked to cell wall polysaccharides such as hemicellulose, reinforcing the cell wall structure (Moore and Jung 2001). There are three types of lignins: p-hydroxyphenyl (H lignin), guaiacyl (G lignin), and syringyl (S lignin). These three types are mainly composed of three different hydroxycinnamyl alcohols that differ in the number of methoxy groups: p-coumaryl (found in H lignin, no methoxy groups), coniferyl (found in G lignin, methoxy group on carbon 3), and sinapyl (found in S lignin, methoxy groups on carbons 3 and 5). In addition to these alcohols, there are acetylated monolignols that also get incorporated into lignins that recently have been identified as
true lignin monomers as well (Boerjan 2003). Different plant sources have different types of lignins based on their genetic makeup as well as the environment. Of the three main types of lignin, G lignin predominates in gymnosperms, and both G and S lignins are abundant in angiosperms (Moore and Jung 2001). Monocots have a much higher amount of G lignin compared to dicots which have a higher amount of S lignin (Boerjan 2003). Many different changes in the environment can affect lignification such as temperature, moisture level, exposure to light, and soil fertility. For example, plants exposed to high temperatures generally have a higher extent of lignification (Moore and Jung 2001). Plant maturity also greatly influences the amount of lignification of plant cell walls. More mature plants have higher amounts and degrees of lignification than their less mature counterparts, especially in grasses (Moore and Jung 2001). It is important to note that lignin prevents lodging, a fundamental characteristic of agronomic quality, in many agronomic species (Humphreys and Chapple 2002).

The monolignols produced from the phenylpropanoid pathway go through a process of lignification during which they become their respective H, G, or S lignins. During lignification, monolignol units are bonded together by radical coupling reactions. These monomers are usually added at the end of the growing lignin polymer in the beta position (Boerjan 2003). After the lignin is formed, it is deposited in the cell wall, usually during secondary cell wall synthesis (Boerjan 2003). Ferulate esters aid the deposition of lignin in grass species by acting as nucleation sites (Moore and Jung 2001).

There are three layers of the secondary cell wall: the outer layer, the middle layer, and the inner layer. After the polysaccharides have been deposited into a layer of the secondary cell wall, the lignin is then deposited starting at the corners of the cell near the
middle lamella. Most of the lignin gets deposited in the inner layer of the secondary cell wall (Boerjan 2003; Moore and Jung 2001). The specific type of lignin determines when it gets deposited in terms of developmental growth. Usually, the order of deposition is H lignin followed by G lignin and finally S lignin. In addition, the polysaccharide matrix can influence where lignins get deposited. Lignin deposition is highly regulated based on genetic and environmental factors (Boerjan 2003). While lignin is being deposited into the cell wall, it can form bonds with polysaccharides such as hemicellulose, thereby excluding water from the polysaccharide matrix and improving resistance to water soluble enzymes that may try to degrade the cell wall (Boerjan 2003).

Section 3. The Phenylpropanoid Pathway and its Mutants

Phenylpropanoid pathway basics

The phenylpropanoid pathway is responsible for producing plant secondary metabolites derived from phenylalanine, including lignin. These secondary metabolites perform a plethora of functions in the plant including structural support, pollen viability, disease resistance, and protection from the elements (Humphreys and Chapple 2002; Nair et al. 2004). Although many compounds are produced from this pathway, lignin captures most of the carbon cycled through it (Humphreys and Chapple 2002). The word lignin is derived from the Latin word for wood, lignum. Lignin is the insoluble material left over after the main polysaccharides are extracted from plant material, and the different monolignols were the most easily detectable parts of the lignin in wood when it was first characterized (Cherney et al. 1991; Dean 2001). Monolignols are produced in the phenylpropanoid pathway through a series of steps starting with the deamination of phenylalanine to cinnamic acid. The cinnamic acid is then led through a series of
hydroxylations and O-methylations of the aromatic ring before finally producing a series
of alcohols which are precursors to the monolignols (Boerjan 2003).

**Recent pathway changes**

The model of the phenylpropanoid pathway has undergone several changes in the
last 25 years. The original model consisted of phenylalanine being converted to cinnamic
acid and then, through a series of hydroxylation and methylation reactions, to
hydroxycinnamic acids: p-coumaric acid, caffeic acid, ferulic acid, 5-hydroxy-ferulic
acid, and sinapic acid. These hydroxycinnamic acids were thought to be the precursors to
the monolignols that make up lignin (Humphreys and Chapple 2002). Since the early
90s, this model of the phenylpropanoid pathway has undergone three significant changes.
The first change occurred in the mid-90s with the discovery of the enzyme caffeoyl CoA
3-O-methyltransferase (CCoAOMT). This enzyme was found to convert p-coumaroyl
CoA to feruloyl CoA whereas in the conventional model ferulic acid was converted to
feruloyl CoA. The second major change redefined the function of the enzyme ferulate 5-
hydroxylase (F5H). Originally it was thought that F5H converted ferulic acid to 5-
hydroxy-ferulic acid which would eventually give rise to syringyl lignin. Studies showed
that F5H functions in converting coniferaldehyde and coniferyl alcohol to their 5-
hydroxy forms which in turn give rise to syringyl lignin (Guillaumie et al. 2007;
Humphreys and Chapple 2002). This also helped to elucidate the function of caffeic
acid/5-hydroxyferulic acid O-methyltransferase (COMT) as the enzyme that converts the
5-hydroxy forms of coniferaldehyde and coniferalcohol to sinapaldehyde and sinapyl
alcohol respectively (Boerjan 2003; Humphreys and Chapple 2002). The third and most
recent change to our model of the phenylpropanoid pathway occurred just a few years
ago with the discovery that ferulic acid and sinapic acid were actually end products of the pathway produced by coniferaldehyde and sinapaldehyde respectively. The enzyme responsible for this is an aldehyde dehydrogenase encoded by the REF1 gene in *Arabidopsis thaliana* (Nair et al. 2004). There are gene products in other plant species that share amino acid sequence similarity to REF1 in *Arabidopsis*. Two of these are in *Zea mays*, RF2C and RF2D (Skibbe et al. 2002), and one is in *Oryza sativa*, OsALDH1a. All of these genes are shown to encode aldehyde dehydrogenases (Nair et al. 2004).

**Brown Midrib Mutants**

The brown midrib mutants of maize are the most studied of the lignin mutants. The brown midrib phenotype was first described in 1931 by Jorgenson (1931). Mutant alleles of four different *brown midrib* (*bm*) loci were characterized in maize by Kuc’ and Nelson (1964). These alleles result in the accumulation of a reddish brown pigmentation along the leaf midrib and are called *bm1*, *bm2*, *bm3*, and *bm4* (Dean 2001). This brown pigmentation is due to the changes in the components that make up the lignin (Marita et al. 2003; Vermerris 2002). The maize *bm1* mutation causes a reduction in lignin content by 10-20\%, the *bm2* mutation by 15-25\%, the *bm3* mutation by 25-40\%, and the *bm4* mutation by about 15\% (Guillaumie et al. 2007). Although the phenotypes of each *brown midrib* mutant have specific characteristics, the environment can impact the amount of lignification of each mutant despite the genotype (Marita et al. 2003). The maize *brown midrib* genes *bm1*, *bm2*, *bm3*, and *bm4* map to bins 5.04, 1.11, 4.05, and 9.07(/08) respectively (Guillaumie et al. 2007). All four mutant *bm* alleles are recessive and map near QTL for flowering time (Vermerris 2002).
The bm1 mutants are defective in the enzyme cinnamyl alcohol dehydrogenase (CAD) which causes the accumulation of coniferaldehyde (Dean 2001). Although the bm1 mutation affects the activity of CAD, the last enzyme in the known phenylpropanoid pathway to make monolignols, it is unclear as to whether CAD is encoded or regulated by Bm1 (Marita et al. 2003). Because the CAD protein and mRNA level in bm1 plants are not completely eliminated, it would seem that the bm1 mutation affects the regulatory region of cad and not the coding region (Guillaumie et al. 2007; Halpin et al. 1998). The bm3 mutants are defective in caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) which causes an accumulation of 5-hydroxyguaiacyl residues and a reduction in S to G lignin ratios in the plant cell wall (Dean 2001; Marita et al. 2003; Vermerris 2002). The different bm3 alleles are likely derived from a transposable element inserting into and excising from the gene encoding COMT (Guillaumie et al. 2007). The bm2 and bm4 alleles do not have known enzyme deficiencies associated with their mutant phenotypes yet (Marita et al. 2003; Vermerris 2002), although there is some evidence that bm2 correlates with the over expression of an O-methyltransferase (OMT). In addition, the bm2 allele has less lignin cross-linking (Marita et al. 2003) and causes the reduction in monolignols that make G-lignin as well as changes in the deposition of lignin (Dean 2001). There is not much known about the bm4 allele or the changes that may occur in the cell wall of this mutant (Marita et al. 2003; Vermerris 2002). Although the bm2 and bm4 alleles have not been associated with specific genes in the phenylpropanoid pathway, they could possibly encode regulatory elements that affect enzymes within the pathway or undiscovered enzymes that are part of the pathway (Guillaumie et al. 2007).
Section 4. GFP Discovery and Properties

Background Information on GFP

Jellyfish (*Aequorea victoria*) are one example of the organisms that produce fluorescent compounds. In 1962, Shimomura et al. (1962) were the first to purify the fluorescent substance that these *Aequorea* produce. They discovered that this purified, active substance was a protein and called it “Aequorin.” Shimomura et al. characterized this protein and determined that all aequorin needed to fluoresce was a Ca$^{2+}$ ion. However, the light emitted was blue light, not the green light emitted by the jellyfish *in vivo*. Shimomura et al. (1962) also concluded that oxygen was not required for the emission of the blue light by aequorin. We now know that the green fluorescence produced by jellyfish is actually caused by the interaction of two different fluorescent proteins: blue fluorescent protein (BFP) and green fluorescent protein (GFP). The GFP purified from *Aequorea victoria* absorbs blue light and emits green light maximally at 395 nm and 509 nm respectively (Chalfie et al. 1994).

What Does GFP Look Like?

In addition to being the first to purify Aequorin, Shimomura was also the first to propose a model for the structure of the fluorophore of GFP (Shimomura 1979). This structure was determined through the use of a model compound and by comparing the spectra of the model compound to the native peptide. It was proposed that this fluorophore may be similar to fluorophores in other GFPs (Shimomura 1979). Then, in 1993, Cody et al. (1993) revised Shimomura’s structure of the GFP fluorophore using HPLC and updated technologies such as cDNA sequencing. The authors agreed with Shimomura (1979) that the main functional structure of the fluorophore was the 4-(p-
hydroxybenzylidene)-5-imidazolone (Cody et al. 1993). The authors determined that the fluorophore responsible for GFP fluorescence was found within a hexapeptide of the protein and became functional by the cyclization of the Ser-dehydroTyr-Gly sequence (Cody et al. 1993). Since then, the structure of GFP has been clearly defined. GFP is a molecule of 238 amino acids and has a molecular weight of about 27 kDa (Heim et al. 1994). GFP exists as an 11-stranded $\beta$-barrel with the fluorophore on the interior. After the protein is translated, a spontaneous post-translational modification occurs in the presence of oxygen to produce the fluorophore. There are three main steps to forming GFP: cyclization, dehydration, and oxidation. The cyclization step occurs by an intrachain ring closure to make a cyclopentyl ring with the series of amino acids Ser65, Tyr66, and Gly67. Then there is a loss of water from the cyclized molecule and an oxidation by molecular oxygen to form the mature fluorophore (Wachter 2006). The GFP fluorophore formation requires oxygen. This was demonstrated by Heim et al. (1994) who grew $E.\ coli$ under anaerobic conditions and then exposed them to oxygen. Not only is oxygen required for fluorescence, availability of oxygen actually limits the rate of the fluorophore formation. The time constant for fluorophore formation is approximately 4 hours in native GFP. After these experiments, a new scheme was drawn for the mechanism of fluorophore formation (Heim et al. 1994).

**Mechanism of Green Light Production**

Jellyfish (*Aequorea sp.*) emit green light through a postulated energy transfer mechanism. The protein aequorin, in the presence of $Ca^{2+}$, becomes activated and fluoresces blue creating blue fluorescent protein (BFP). When both aequorin and GFP are present in close proximity the energy of BFP is then transferred to GFP which emits
green light. This energy transfer takes place by a Förster reaction (Chalfie et al. 1994; Morise et al. 1974).

**Using GFP as a Sensor**

Because of the unique properties of GFP, it lends itself well to use as a reporter molecule. GFP is an excellent choice when monitoring something in a biological system because it is specific, sensitive, and non-toxic. Also, it doesn’t require addition of substrate which could interfere with its measurement (Chalfie et al. 1994). GFP has been expressed in a number of organisms such as *Escherichia coli*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* (Heim et al. 1994) and is stable when expressed in *Aequorea victoria* as well as *E. coli* and *C. elegans* (Chalfie et al. 1994). Some of the first authors to suggest using GFP as a reporter in other organisms were Inouye and Tsuji (1994). They showed that the characteristics of the *E. coli* expressed GFP were nearly identical to the native *Aequorea* protein (Inouye and Tsuji 1994). GFP has also been improved from the native *Aequorea* protein. Heim et al. (1994) improved GFP by mutating Ser-65 to a Thr. This caused there to be only one excitation peak at 490 nm. Wild type GFP has two excitation peaks at 396 nm and 475 nm. Also, by reducing the excitation energy, this modified protein potentially has increased photostability (Heim et al. 1995). With the improved characteristics of this GFP mutant, S65T, we can now use this GFP as a specific sensor in many applications.

*Section 5. History of Breeding for Ruminant Digestibility*

**Overview of Silage Maize**

Corn has been developed for ensiling in order to preserve it and to improve its palatability it to animals. Corn is ensiled by drying it to a certain moisture level and
storing it under anaerobic conditions. Fermentative microorganisms consume the corn and produce lactic acid that brings the pH down to about 4.0, inhibiting unwanted microbial growth and spoiling. The ensiled corn is a type of forage fed to ruminants such as cows or sheep (Coors et al. 1994). Ruminant digestibility is a measurement of the silage quality. Good digestibility results in increased milk production from animals.

Silage maize breeders once thought that the best forage would come from modern maize hybrids with high grain yields. However, grain yield and stover yield are not necessarily correlated, making new corn hybrids inferior for silage production (Barriere et al. 1992).

**Ruminant Digestibility Measurements**

There are several ways to evaluate the ruminant digestibility of forages. Since forage maize includes both the stover and the grain, it is important to consider both when determining the digestibility of the forage (Wolf et al. 1993). The least digestible fractions of forage in ruminants are lignin and silica. The rest of the cell wall components, such as cellulose and hemicellulose, vary widely in their digestibilities (Deinum and Struik 1986). Forage maize can be bred for improved digestibility by using an *in vitro* analysis to screen forage samples. Although this method is an *in vitro* evaluation of forage quality, it is accepted by many because an *in vivo* approach would not be feasible for evaluating breeding germplasm (Deinum and Struik 1986). Tilley and Terry (1963) were the first to introduce a method that measured forage quality *in vitro* by using rumen microflora and ground forage. This method revolutionized forage breeding by allowing the rapid screening of many forage samples (Casler and Vogel 1999). The Tilley and Terry method (1963) consisted of a 2-day fermentation of the forage by rumen serum followed by a 2-day digestion with acid pepsin. Later, the Van Soest method
(1966) improved upon the *in vitro* method of Tilley and Terry to get better correlations to *in vivo* results. The authors eliminated the 48-hour acid pepsin digestion and replaced this step with a cell-wall determination step using neutral detergent. The result of the new *in vitro* process gave them digestibility values closer to those obtained from *in vivo* studies thereby improving the *in vitro* process (Van Soest et al. 1966). Using the Van Soest method (1966) to analyze forage dry matter content, the maize cell wall consists of about 44% of the total dry matter, and the cell content consists of about 56% of the total dry matter (Deinum and Struik 1986). Using these methods, *in vitro* digestibility has been determined for many silage maize hybrids making these methods valuable screening tools.

**Breeding Silage Maize for Stover Quality**

**Variability**

When breeding for digestible corn stover, it is important to have variability. Originally, all corn was treated equally, and ensiled corn was not specifically selected for its forage quality characteristics but for its grain yield. This caused breeders to speculate that there would not be much diversity in forage. However, there have been studies that show there is a lot of variation for the ruminant digestibility trait in forage maize, and much of this variation is found in plants not carrying a *brown midrib* mutation (Deinum and Struik 1986). One study involved screening maize forages by taking maize digestibility measurements to see if there was variability between different genotypes. Included in these genotypes were *brown midrib* mutants. There were differences between genotypes of different maize forages, but the differences were more significant with the *brown midrib* genotypes than without them (Barriere et al. 1992). An important
result from this study was that fiber digestibility of the forage explained more of the genetic variability among the different forage samples than the actual total fiber content (Barriere et al. 1992). This study showed that there was much forage quality diversity for which positive traits could be bred (Barriere et al. 1992). Another study found little variation in silage quality traits among most US corn hybrids, so a new maize population specific for silage breeding was developed. The Wisconsin Quality Synthetic (WQS) population was created in order to capture the diversity of silage traits (Frey et al. 2004). Relatively little selection for forage quality traits was carried out before breeders actively selected for traits such as \textit{in vitro} dry matter digestibility. This is good for lignocellulosic feedstocks used for ethanol production because it provides us with an extensive amount of variation to explore when breeding for highly digestible feedstocks (Casler and Vogel 1999).

**Stover Quality**

In addition to variability in a population, it is important to breed for a trait with good agronomic characteristics. In general, forages with a higher amount of lignin, fiber, and silica will be less digestible than forages with lower amounts of these components. Based on several studies, it has been shown that lignin concentration negatively correlates with cell wall digestibility. Brown midrib mutants have been selected or induced by mutagenesis in different species in order to determine digestibility such as the presence of diferulic acid bridges (Moore and Jung 2001). However, breeding for lower concentrations of cell wall components such as these would be a mistake. In silage maize, the plants are harvested early so lodging and insect damage may not be an issue. In stover from maize used for grain, which is where the agricultural residues proposed for
use for cellulosic ethanol come from, the plants are harvested at grain maturity so damage to the plants may be more realized (Wolf et al. 1993). Barriere et al. (1997) indicate that we need to alter the composition of lignin in order to improve cell wall digestibility, not just reduce the amount of lignin. Casler et al. (2002) performed a study determining that agronomic performance was hindered, specifically by increased mortality rates, by the reduction in lignin content or increased \textit{in vitro} dry matter digestibility. They showed this in four different perennial grass species. Although this was a study done to improve livestock production, it is extremely applicable to biofuels research because much of our cellulosic feedstock for ethanol may come from perennial grasses such as these. Argillier et al. (2000) determined the digestibility of the cell wall components of the biomass was independent of the actual biomass yield in the hybrids. Studies by Allen et al. (1990) and Argillier et al. (1995) also reinforce this statement. This is good to know because it may allow breeders to select maize hybrids that make a lot of biomass and, at the same time, be very digestible. We can learn a lot about how to breed feedstocks for biofuel production from the rich history of forage quality breeding programs because similar digestibility characteristics are crucial for both types of end-products: forage and biofuel feedstocks.

\textit{Section 6. High-throughput Screening in Breeding Programs}

Agricultural crops have been bred for many different traits to increase the yield, strength, or pest resistance. It is no secret that high-throughput screening methods are essential for viable plant breeding programs. Techniques such as PCR and marker assisted selection (MAS) have been at the forefront of plant breeding since the mid-90s. Some traits are not as easy to select for by MAS because the genes that control these
traits may not be known or no markers are linked to the genes of interest. In these cases, other selection methods must be used such as multiple trait selection (Falconer and Mackay 1996). Because maize is used primarily as livestock feed, many traits are selected for the ability to improve the diets of these livestock.

One example of improving methionine in corn and soybeans is described by Wright and Orman (1995). They used a microbe to catabolize the methionine and measured the cell density of the culture to determine the amount of methionine in the substrate. This microbial method was much less expensive than the accepted chemical method, and the authors showed that the microbial method was highly correlated to the chemical method. This is an example of a bioassay because they were using a microbe instead of a chemical to quantify the substrate. This microbial assay suggests a measure of bioavailability, not chemical availability, which was important to their study when discussing the utilization of substrates in livestock. A modified version of this method was also used by Darrigues et al. (2005) to evaluate methionine in maize. The authors suggested that the methods used to do their high-throughput screening may not be as accurate as accepted chemical methods, but their methods are much less expensive, repeatable, and can produce results where the samples can be ranked. These are some of the more important features of screening methods in plant breeding programs.

Plant cell wall digestibility is not well-suited to MAS because, as of yet, it is poorly characterized genetically. Simultaneous saccharification and fermentation (SSF) is an accepted method to determine the digestibility of the cell wall in order to create ethanol, but requires a week’s time and special, expensive equipment. Other high-throughput screening methods that are precise and reliable need to be developed in order
to make progress with this trait in breeding programs. One of these methods recently
developed is described by Weimer et al. (2005). The authors describe in vitro gas
production as a high-throughput method to evaluate feedstocks for ethanol production
potential. The method basically consists of an in vitro digestion of untreated, ground
feedstock by anaerobic ruminant microorganisms in sealed serum bottles. During the
fermentation, the gas pressure is recorded and converted to the amount of gas produced
by the reaction. The amount of gas produced correlates to the amount of ethanol that
would be produced by the feedstock. This method gets around having to measure the
ethanol directly by HPLC or GC as in the SSF procedure. This method can be used to
evaluate up to 64 samples in a single replication and takes 24 or 96 hours to complete,
depending on what parameters are used. There are two main advantages of this particular
assay over SSF: 1) this method does not require any sterilization due to the high
inoculum levels added, and 2) this method is more sensitive than SSF due to the
measurement of gas production rather than ethanol directly. The main disadvantage of
this assay is that it uses rumen microflora to conduct the digestion rather than an enzyme
cocktail and yeast which are easier to store, distribute, and collect. Both SSF and the
method described by Weimer et al. (2005) are bioassays, which model the process of
producing ethanol from yeast better than chemical methods, but they still require a lot of
time, are run in rather large vessels, and each sample must be read one at a time at the
end of the reaction. The method presented in Chapter 2 called simultaneous
saccharification and catabolism (SSC) improves upon each of these weaknesses while
also increasing the number of samples that can be evaluated in one batch. SSC uses a
microbial biosensor in order to evaluate the availability of sugars in corn stover, a lignocellulosic feedstock.

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CHAPTER 2. DEVELOPMENT OF A REAL-TIME SUGAR-CONSUMING BIOSENSOR AND ITS APPLICATION TO A CORN STOVER HYDROLYSIS BIOASSAY

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ABSTRACT

Availability and low cost of lignocellulosic biomass has caused tremendous interest in the fermentation of lignocellulosic-derived sugars for the production of liquid fuels. The economic feasibility of lignocellulosic biofuels can be improved by evaluating the fermentation potential of different feedstocks. During conversion, pretreated biomass is combined with hydrolytic enzymes that convert polymeric sugars into monomers. Such hydrolysis is feedback-inhibited by sugar products, limiting the extent and rate of the reaction. This feedback inhibition can be overcome by removal of sugar products in the reaction, as is done in simultaneous saccharification and fermentation (SSF). The objective of this study was to develop a sugar-consuming biosensor to monitor hydrolytic reactions and to demonstrate its application in monitoring corn stover hydrolysis. The biosensor is based on *Escherichia coli* strain CA8404, modified to produce green fluorescent protein (GFP), and is capable of catabolizing both five- and six-carbon sugars. After several biosensor characterization experiments, we found that growth rate was proportional to GFP-fluorescence, and total growth and growth rate depend upon how much sugar is present at inoculation. This biosensor has a dynamic range of 0.100-1.600 mg glucose/mL and can accurately measure sugar mixtures where 50-80% of the
total sugar is glucose, the remainder xylose. We also demonstrated that stovers can be differentiated based on sugar yields in enzymatic hydrolysis reactions. It was possible to monitor the course of enzymatic hydrolysis in real-time. This biosensor could be used in screening methods to characterize hydrolysis of feedstocks or to evaluate the performance of hydrolytic systems.

INTRODUCTION

Fermentation reactions are important for production of many valuable products including pharmaceuticals, beverages, and biofuels. In lignocellulosic ethanol production, biomass feedstocks are chemically pretreated, hydrolyzed by cellulases and hemicellulases, and the resulting sugars are fermented by yeast or bacteria to produce ethanol (Hahn-Hagerdal et al. 2006). In large-scale production of biocommodities like ethanol, feedstocks have a large and often dominant impact on process economics and process development (Lynd et al. 1999). For example, the amount of sugar available to fermentation reactions is important because in the absence of limiting factors, substrate availability determines product yield; thus methods to measure sugar available to a fermentation reaction are desirable. There are many sugar detection and quantitation methods available including chemical reducing sugar assays and enzymatic assays (Fox and Robyt 1991; Kimura and Robyt 1995). All of these methods require sampling the fermentation reaction and measuring sugars in the sample.

The value of measuring post-hydrolysis sugar concentrations using the methods mentioned above is limited in reactions carried out by cellulases and hemicellulases since these enzymes are feedback inhibited by their sugar products. Equilibrium is reached during the hydrolysis and the feedstock is never completely hydrolyzed. Therefore, in
order to monitor cellulase and hemicellulase hydrolysis reactions that run beyond the limit imposed by product inhibition, the sugars must be removed as they are sensed. Microorganisms engineered for sugar biosensing are attractive because they can report on the sugar content in the media through the course of microbial growth while they metabolize the sugars produced by the hydrolysis reactions. An example of a sucrose biosensor is described by Jaeger III et al (1999).

One current method used to quantify ethanol production potential of lignocellulosic feedstocks is simultaneous saccharification and fermentation (SSF). In SSF, a pretreated substrate (usually a lignocellulosic feedstock) is subjected to hydrolysis by cellulases (or a crude enzyme preparation containing cellulases, hemicellulases, and other hydrolytic enzymes) and the resulting glucose is fermented to ethanol (Wright et al. 1988). Residual sugars and inhibitory products such as glucose, cellobiose, and acetic acid are often measured by HPLC, and ethanol concentration can be measured by gas chromatograph (GC) or HPLC. The whole procedure takes about 168 hours according to the protocol from the National Renewable Energy Laboratory (NREL), (Dowe and McMillan 1995). Recently, Weimer et al. (2005) have developed a more high-throughput method to predict the fermentability of cellulosic biomass to ethanol through \textit{in vitro} gas production. In this procedure, fermentations are carried out in sealed serum bottles, and the gas produced is used as measurement of the digestibility of the cellulosic biomass.

We have developed a microbial biosensor that reports sugar levels in a reaction that mimics the NREL lignocellulosic ethanol production process (Dowe and McMillan 1995). This high-throughput, sugar-consuming biosensor overcomes the feedback inhibition problems associated with enzyme hydrolytic reactions. \textit{Escherichia coli} strain
CA8404 was selected for the biosensor because it carries the crp* mutation which reduces catabolite repression and thereby allows both the 5- and 6-carbon sugars produced from corn stover hydrolysis reactions to be metabolized simultaneously (Sabourin 1975). As the *E. coli* metabolize the sugars from the hydrolysis reaction, their cell growth can be monitored by light scattering measurements of cell density. However, certain reactions, including corn stover hydrolysis, contain substances that may interfere with light scattering measurements. In order to avoid this issue, the *E. coli* strain CA8404 was modified to produce a visual marker, green fluorescent protein (GFP), (Axtell and Beattie 2002; Miller and Lindow 1997). The version of GFP used in this study (S65T) has a maximum excitation wavelength of 490 nm and a maximum emission wavelength of 510 nm (Heim et al. 1995).

The objective of this study was to develop a sugar biosensor that can be used to monitor hydrolytic reactions and demonstrate its application to monitoring corn stover hydrolysis in a process similar to that used for lignocellulosic ethanol production. Such a biosensor would be useful for rapidly screening varieties for suitability as biomass feedstocks in plant breeding programs and for evaluating different hydrolytic systems. Furthermore, this biosensor could be used to measure sugar production in a wide range of other experiments. To accomplish our objective, we performed several experiments to characterize the biosensor. In this report we characterize the growth of the biosensor in a variety of conditions and determine the repeatability of the biosensor. We demonstrate the utility of the biosensor for differentiating stover samples based on their suitability for hydrolysis to fermentable sugars.
MATERIALS AND METHODS

Site-Directed Mutagenesis and Transformation

Site-directed mutagenesis was conducted according to the Stratagene product QuikChange II® Site-Directed Mutagenesis kit in order to produce a pPNptGreen plasmid without a functional GFP fluorophore (Stratagene, La Jolla, CA). Approximately 300 bp into the gfp coding sequence, DNA encoding a glutamate residue (GAA) was changed to encode a stop codon (TAA). (Primers for mutagenesis: Forward: GATGACGGAACTACAAGACACGTGCCTAAGTCAAGTTTGAAGG; Reverse: CCTTCAAAACTTGACTTAAGCAGTGGTCTTGTAGTTCCCGTCATC.) The new plasmid was designated pPNptOchre. The original pPNptGreen plasmid and the pPNptOchre plasmid without the functional GFP fluorophore were transformed separately into E. coli strain CA8404 to produce the two strains crp*-gfp and crp*-gfp.

Preparation of Corn Stover Samples

At grain maturity, cobs were removed from the corn plants and all corn stover samples were cut at approximately 6 inches above the soil by a forage chopper. Approximately 0.8 kg of sample (wet weight) at a moisture content of about 35% was collected from each plot and samples were dried. The material from each sample was ground by a hammer mill with a 1 mm screen to obtain a uniform particle size.

Hydrolysis measured by glucose assay

To characterize the product inhibition of the enzyme preparation Multifect® A-40 (a cellulase/hemicellulase mixture from Genencor Intl.), we carried out hydrolysis reactions in the presence or absence of 10 mM D-glucose (CAS# 50-99-7, Sigma-Aldrich)
Inc., St. Louis, MO). We chose this enzyme because it is a preparation of cellulases and hemicellulases commercially used in the paper pulping industry for fiber modification. Each treatment was run with four replicates using 5 mg of a stover sample treated with a 1:20 dilution of enzyme Multifect® A-40 in citrate-phosphate buffer (21 mL 0.1 M citric acid and 29 mL 0.2 M sodium phosphate, in a final volume of 100 mL, pH 5.5).

Hydrolysis was conducted at 60°C for 90 min. Following hydrolysis, the tubes were centrifuged for 1.5 min. at 10,000 x g in a microcentrifuge (Spectrafuge, Orem, UT). An aliquot of the supernatant from the hydrolysis reaction was measured with a Hexokinase glucose assay kit (Sigma-Aldrich Inc., St. Louis, MO). The absorbance was measured at 340 nm (OD$_{340}$) using the MRXII plate reader by DYNE (Magellan Biosciences Company, Chelmsford, MA). The absorbance value was converted to percent substrate hydrolyzed with a standard curve constructed by plotting OD$_{340}$ values versus glucose concentrations following analysis of a series of solutions with known glucose concentrations.

_Growth of liquid cultures for growth characterization experiments_

Cultures of _E. coli_ crp*-gfp and crp*-gfp$^-$ paired by treatment were grown in modified 1X M9 minimal media (Sambrook and Russell 2001). The M9 media was modified by the addition of Kanamycin (50 µg/mL), thiamine (0.01% w/v), and ammonium chloride (5 mg/mL). Also, different carbon sources were provided to the cultures than the carbon source described by Sambrook et al. D-glucose (CAS# 50-99-7, Sigma-Aldrich Inc., St. Louis, MO), and D-xylose (CAS# 58-86-6, Sigma-Aldrich Inc., St. Louis, MO) solutions were made in the appropriate concentrations indicated in each experimental procedure below. All sugar solutions were filter-sterilized and frozen.
Sugar mixtures were combined from separate, sterilized glucose and xylose sugar solutions. Cultures were grown in clear, 96-well cell culture plates (Product # 92096, Techno Plastic Products, Trasadingen, Switzerland) and sealed with AirPore™ seals (Qiagen, Valencia, CA) in order to ensure that enough oxygen was available to the cultures. The plates were then securely fastened down in the Innova 4300 incubator shaker (New Brunswick Scientific, Edison, New Jersey), and allowed to incubate with shaking at 37°C and 225 rpm. When it was time to take a measurement, the AirPore™ seal was removed only from the wells to be measured, and absorbance (OD$_{595}$) measurements were taken by the MRXII plate reader (Dynex – a Magellan Biosciences Company, Chelmsford, MA). The samples from the wells to be measured were then transferred into a black, 96-well cell culture plate (Corning Incorporated Life Sciences, Lowell, MA), and fluorescence (excitation wavelength: 485 nm, emission wavelength: 535 nm) measurements were taken by the SpectraFluor Plus plate reader (Tecan US, Research Triangle Park, NC). Note that these wavelengths (595 nm for absorbance, 485 nm for excitation, and 535 nm for emission) were used consistently throughout the study. The AirPore™ seal was replaced on the clear 96-well plate and returned to the incubator.

To obtain a value for GFP-specific fluorescence for each culture pair, the fluorescence reading of the crp*-gfp$^-$ strain was subtracted from the fluorescence reading of the crp*-gfp strain.

Characterization of the Biosensor

Growth Curves with Different Glucose Concentrations

To establish whether it was possible to use GFP to detect differences in cell growth in response to sugars, cultures of *E. coli* crp*-gfp and crp*-gfp$^-$ were grown in
modified 1X M9 minimal media with 2, 4, or 8% (v/v) D-glucose solution. Absorbance and fluorescence were measured every two hours for 22 h, and the GFP-specific fluorescence was determined.

**Sensitivity and Dynamic Range**

To determine the sensitivity and dynamic range of the sugar biosensor, cultures of *E. coli crp*-gfp and crp*-gfp were grown in modified 1X M9 minimal media with D-glucose concentrations ranging from 0.025 mg/mL to 6.0 mg/mL. Absorbance and fluorescence measurements were taken 20 h after inoculation, and the GFP-specific fluorescence was determined.

**Standard Curve and Repeatability**

Because the ratio of 5 to 6 carbon sugars may vary with different sources of biomass, we wanted to determine the accuracy of the biosensor with different sugar mixtures and concentrations. Six different sugar mixtures of D-glucose and D-xylose in different ratios were made as indicated in Table 1. These mixtures were diluted to make sugar standard solutions containing six different total sugar concentrations for each mixture. These sugar standard concentrations are indicated in Table 2. Eight replications of each sugar standard concentration within each sugar mixture were examined. Within a 96-well plate, two adjacent wells were assigned the same sugar mixture and concentration treatment at random and *E. coli* cultures crp*-gfp and crp*-gfp were randomly assigned to one of the two wells. Cultures were inoculated and grown in modified 1X M9 minimal media. Absorbance and fluorescence measurements were taken after 20 h of incubation, and the GFP-specific fluorescence was determined. One-way analysis of variance (ANOVA) was performed on the GFP-specific fluorescence
values to determine if variation in the experiment was significant. A line was fit to the
data from each sugar ratio and the slope and R-squared were calculated for each line.
Repeatability was evaluated by computing the coefficient of variation for each sugar
concentration within each sugar ratio.

**Glucose Spiking**

To determine the response time of the biosensor, glucose was added to the
reaction when the culture reached stationary phase. Two sets of three replications of both
*E. coli* strains crp*-gfp* and crp*-gfp- were grown in modified 1X M9 minimal media
with 2% (v/v) D-glucose solution for 20 h. After 20 h, half of the cultures (one set) were
randomly selected to receive an addition of 8% (v/v) D-glucose solution for a total of
three replications each of spiked cultures and unspiked cultures. Absorbance and
fluorescence were measured every two hours, and the GFP-specific fluorescence was
determined.

**Stopping Protein Production**

Another way we examined the response time of the biosensor was by stopping
protein production when the culture was in mid-log phase. Six replications of both *E.
coli* strains crp*-gfp* and crp*-gfp- were grown in modified 1X M9 minimal media,
containing 20% D-glucose (v/v). Chloramphenicol was added to half of the cultures at
random after 13 h for a total of three replications each of cultures with chloramphenicol
and without chloramphenicol. Absorbance and fluorescence were measured every hour,
and the GFP-specific fluorescence was determined.
Application of the Biosensor

Post-hydrolysis Monitoring

A bioassay, utilizing the biosensor described here, that we call simultaneous saccharification and catabolism (SSC) was used to analyze stover samples. For each sample to be analyzed, 25.0 ± 0.2 mg of dried and ground corn stover was weighed into two separate 14-mL sterile test tubes (BD Biosciences, San Jose, CA). Two tubes were used to control for variations in fluorescence of the corn stover samples: an experimental tube to be inoculated with crp*-gfp and a control tube to be inoculated with crp*-gfp. The difference in the fluorescence of these two tubes was used to determine the GFP-specific fluorescence. Then, 1150 µL of 0.5% (v/v) sulfuric acid were added to each tube, and the tubes were incubated at 100°C for 1 h (Mohagheghi et al. 2004). The tubes were allowed to cool for 15 min. after incubation, after which 3850 µL of bacterial media inoculum (essentially 2X M9 media inoculated with the appropriate bacterial culture) were added to each tube. 1 L of bacterial media inoculum contained 620 mL sterile water, 330 mL 5X M9 salts, 6.6 mL 1M MgSO₄, 164.2 µL 1M CaCl₂, 1.7 mL thiamin at 10%, 8.3 mL Kanamycin at 10 mg/mL, and 33 mL crp*-gfp or crp*-gfp⁻ liquid culture (grown overnight at 37°C in 1X M9 media to an OD₅₉₅ of ~0.6). In addition, 25 µL of 1:1 GC220: Multifect® Xylanase (Genencor Intl.) were added to each tube. The tubes were allowed to incubate with shaking at 37°C and 225 rpm. Samples containing 100 µL of 0, 2, 4, 6, 8, 10, 12, 14, 16, or 18% sugar at ratios of 37.5 xylose: 62.5 glucose in place of corn stover were included to as positive controls. Absorbance and fluorescence were measured after 20 h of incubation.
The GFP-specific fluorescence values were computed by subtracting the fluorescence from crp*-gfp cultures from crp*-gfp cultures. These values were then analyzed by ANOVA in order to determine variability in the experiment. When variation was significant, a student’s t-test was performed on each pair to compare means of the samples. The coefficient of variance (CV) was also computed in order to determine the variation in measurements for each genotype.

**Real-time-hydrolysis Monitoring**

The SSC method described above was used to analyze stover samples. Absorbance and fluorescence were measured every two hours for 24 h and once at 36 h.

**RESULTS**

*Feedback inhibition of a preparation of cellulases and hemicellulases*

We conducted our first experiment in order to determine if the enzyme Multifect® A-40 was feedback inhibited by the products of enzymatic hydrolysis. Feedback inhibition would interfere with this measurement because hydrolysis would not proceed to completion. We therefore set out to determine the extent of feedback inhibition in an enzymatic hydrolysis reaction. Two sample types were used: stover with glucose added to 10 mM and stover without added glucose. Enzyme Multifect® A-40 was strongly inhibited by this relatively low level of glucose. Specifically, we observed 10-fold or greater reductions in glucose yield when hydrolyzing corn stover in the presence of added glucose (data not shown). From this, we concluded that enzyme Multifect® A-40 was inhibited by glucose, the product of the hydrolysis reaction. To overcome this difficulty, we needed an assay in which the hydrolysis products were removed from the hydrolysis reaction as they were produced. A whole-cell biosensor involving a microbe that
constitutively produces a reporter gene should fulfill this need. By growing a microbe on carbon limited media with sugars supplied by the experimental treatment, cell growth, which can easily be monitored by the reporter, will be limited by the level of sugars in the experimental treatment.

**Characterization of the Biosensor**

The next objective was to establish whether it was possible to detect differences in cell growth in response to sugars using GFP as a reporter and to determine the relationship between cell growth and GFP fluorescence. Our hypothesis was that because GFP is expressed from a constitutive promoter, GFP fluorescence will track culture density. Three different concentrations of glucose were added to culture media and the absorbance and fluorescence were measured over time. The absorbance data were fit to the Gompertz equation (Gompertz 1825; Zwietering et al. 1990) which describes the normal growth of bacteria over time (Figure 1a). The fluorescence data, however, fit a Gompertz equation poorly because fluorescence decreased late in the experiment (Figure 1b). These data were better fit to the first derivative of the Gompertz equation which describes the rate of change of bacterial growth over time (Lavrencic et al. 1997). The culture density and rate of growth were proportional to the sugar concentration at time points between 16 and 22 h after inoculating the *E. coli* (Figures 1a and 1b). The rate of growth was also directly proportional to the level of fluorescence throughout growth (Figure 1c). To further characterize the relationship between sugar concentration and GFP-specific fluorescence, we plotted the glucose concentration against the fluorescence units from either GFP-specific fluorescence or integrated fluorescence at 20 h and fit a linear equation to each of these data sets. The respective R-squared values for these fits
were 0.91 and 0.98 (data not shown). The integrated fluorescence correlated best to the sugar concentration, so it would be useful to use this value when it is feasible to take measurements throughout the course of the reaction. The GFP-specific fluorescence at 20 h predicted sugar concentration fairly well, (R-squared of 0.91), so this value would be useful in a high-throughput screening method where it may not be realistic to take measurements throughout the reaction. Based on these observations, we conducted several experiments by taking end-point measurements at 20 h after bacterial inoculation.

It was also important to ascertain the sensitivity and dynamic range of our biosensor in order to determine whether we could use the biosensor for a corn stover hydrolysis application. Several different concentrations of glucose were added to culture media, and the absorbance and fluorescence were measured 20 h after inoculation. As shown in Figure 2, the sensitivity of the biosensor was 0.100 mg glucose/mL solution, and the dynamic range was from 0.100 mg to 1.600 mg glucose/mL solution, which should be sufficient for monitoring stover hydrolysis.

Glucose/xylose mixtures at different concentrations were tested to determine the precision of our biosensor with different concentrations of sugar mixtures. Also, we wanted to know what effect different ratios of glucose and xylose would have on the biosensor because this ratio may vary with different sources of biomass. Several different glucose/xylose mixtures at different concentrations were added to culture media and the absorbance and fluorescence were measured 20 hours after inoculation. An analysis of variance (ANOVA) indicated that there were significant differences between the sugar concentrations within each sugar mixture except the 0 glucose: 100 xylose sugar mixture (data not shown). Sugar concentration was plotted against GFP
fluorescence for each sugar mixture and a linear equation was fit to the data. The slopes of these curves differed with differing sugar ratios (Table 1). Standard curves with larger slopes yield higher resolution for differentiating glucose concentrations. The standard curve with the highest slope was the 62.5 glucose: 37.5 xylose sugar mixture and the standard curve with the lowest slope was the 0 glucose: 100 xylose sugar mixture. We concluded that we can best resolve sugar concentrations in glucose/xylose mixtures containing between 50% and 80% glucose based on the slope of the linear trend computed for each sugar mixture standard curve.

To characterize repeatability of the assay, we computed the coefficient of variation (CV) of the GFP-specific fluorescence for each concentration within each sugar mixture. There was no significant linear trend from low to high sugar concentration for the CV% for any of the sugar mixtures. The average CV% was around 24% for all sugar mixtures except the 0 glucose: 100 xylose sugar mixture which had the highest amount of variation at 54.8% (Table 2).

We conducted two experiments to determine the response time of the biosensor to either a flux in sugar concentration or a sudden limitation in sugar during a hydrolysis reaction. The first objective was to determine how quickly the biosensor responded to adding glucose to a reaction where glucose was limiting. To test this, after growing several cultures on 2% glucose to stationary phase, we spiked half the cultures with 8% sugar then monitored the response of the biosensor. This spiking caused the absorbance and fluorescence to increase (Figure 3). As before, the fluorescence level changed in proportion to the change in the growth rate. Within two hours the effect of glucose addition was evident and fluorescence reached a maximum after four hours. Because we
conducted a time-course experiment, we were also able to calculate the integrated fluorescence. By plotting the glucose concentrations (2% and 10%) against the fluorescence units from either GFP-specific fluorescence or integrated fluorescence at 18 h, the R-squared values were 0.86 and 0.99 respectively (data not shown). The integrated fluorescence values were therefore the most accurate predictors of sugar concentration even when glucose was being metered over time.

In addition to determining how quickly the biosensor can respond to an increase in sugar concentration, it is important to establish the response time to a sudden limitation in sugar. We reasoned that the response to a sudden limitation in sugar would be limited by the rate of decay of existing GFP, so we sought to determine this parameter by halting protein production in mid-log phase, and determining the effect on cell density and GFP-specific fluorescence. This was accomplished by the addition of Chloramphenicol (a bacterial translation inhibitor) to the bacterial cultures and measuring absorbance and fluorescence over time. Absorbance measurements showed that the bacteria entered stationary phase one hour after addition of Chloramphenicol, and the fluorescence decreased proportionally to the decrease in growth rate (Figure 4). Within an hour, GFP-specific fluorescence decreased dramatically, reaching a minimum after four hours.

Application of the Biosensor

Post-hydrolysis monitoring is important to show the applicability of the biosensor to screening corn stover samples for their suitability for hydrolysis. The SSC bioassay, described here, is a high-throughput screening method that has been shown to differentiate corn stover samples based on their sugar yield from hydrolysis. The corn stover samples chosen for this experiment were five near-isogenic lines, four of which
were near-isogenic for a different brown midrib allele: W64A X A619 (wild type), W64A X A619 bm1, W64A X A619 bm2, W64A X A619 bm3, and W64A X A619 bm4. The brown midrib mutations either alter the composition or reduce the amount of lignin in the corn stover, making the stover more conducive to hydrolysis (Marita et al. 2003). We hypothesized that the brown midrib lines would yield more sugar upon hydrolysis than the line without the brown midrib mutation when performing endpoint hydrolysis on this set of corn stover samples. There was a significant difference in the mean GFP-specific fluorescence values for all four brown midrib mutants when compared to the near-isogenic line without the brown midrib mutation (Table 3). The brown midrib lines containing alleles bm1, bm2, and bm3 had significantly higher mean GFP-specific fluorescence values than the line containing the bm4 allele. It is important to note that none of the stover samples were completely hydrolyzed to available sugars. This was done intentionally in order to allow us to differentiate samples.

For some applications, it may be important to monitor the products of a hydrolysis reaction with our biosensor in real-time. For example, this may be useful for optimizing mixtures of hydrolytic enzymes. The near-isogenic hybrids W64A X A619 and W64A X A619 bm1 were analyzed using SSC and the biosensor growth and fluorescence were measured over time. Our hypotheses, based on the previous growth curve observations, were that 1) instantaneous GFP-specific fluorescence would be proportional to the sugar catabolism rate (bacterial growth rate) and 2) the integral of fluorescence would be proportional to the amount of sugar consumed by the biosensor. It was expected that the integrated fluorescence values would be the most accurate predictors of sugar concentrations during SSC because they are not based on a specific growth model but on
the total sugar catabolized by the biosensor (as shown earlier by the glucose-spiking experiment). Under this assumption, there was no reason to fit a Gompertz equation or the first derivative of the Gompertz equation to the data because neither would be expected to accurately model culture growth when sugars are being produced during culture growth. The absorbance and fluorescence data are shown in Figures 5a and 5b, respectively. The integral of fluorescence was the best indicator of sugar concentration in previous experiments and these values are presented in Figure 5c. For single time-point measurements, the difference between the corn stover with more available sugars and the corn stover with less available sugars is best determined during the time period of steady state fluorescence (16 to 24 h) whether the GFP-specific fluorescence value or the integrated fluorescence value is used. However, it is likely that the integrated fluorescence value more accurately predicts the total amount of sugar catabolized by the biosensor up to a specific time point.

DISCUSSION

*Feedback inhibition of a preparation of cellulases and hemicellulases*

An attractive method to characterize biomass hydrolysis reactions would be to run the reaction to completion and measure the amount of sugar released. We have shown that Multifect® A-40, a commercially available hydrolytic enzyme preparation, is feedback inhibited which means assays involving quantitation of hydrolysis products at the end of a reaction may not be very useful for evaluating sources of biomass. The sugar biosensor described here can overcome this feedback inhibition problem by removing the sugars from solution as they are sensed. This allows the enzymes to hydrolyze the biomass further and gives us the ability to differentiate samples based on their suitability.
for hydrolysis. Because the biosensor expresses GFP constitutively, we can use fluorescence as a measure of sugar concentration when the solution is too turbid to measure absorbance accurately or when other compounds interfere with this culture density measurement.

*Characterization of the Biosensor*

Because the GFP encoded by the pPNptGreen plasmid is a long-lived GFP and should not lose its fluorescence over time, we thought the fluorescence of GFP would be directly proportional to the turbidity, measured by absorbance, over time. This was not the case as shown in Figure 1 by the growth curves with different glucose concentrations. GFP-specific fluorescence can be limited by carbon as well as by nitrogen and oxygen (Cubitt et al. 1995). Because we have done experiments to determine that nitrogen and oxygen are not limiting (data not shown), we suspect that GFP is being actively degraded for use as a carbon source. We used the Gompertz equation to describe the trend of cell growth over time and the first derivative of the Gompertz equation to describe the trend of fluorescence over time. This showed that the rate of growth (equal to the first derivative of the Gompertz equation) was proportional to fluorescence at any point in time. We also showed that a higher amount of sugar in solution will cause the cells to stay in log phase, and therefore have higher fluorescence, for a longer period of time than a lower amount of sugar. (This is assuming these sugar solutions are within the dynamic range of the sugar biosensor.) These data support our new hypothesis that instantaneous fluorescence is proportional to the sugar catabolism rate. Based upon the growth curves with different glucose concentrations, it is best to measure fluorescence between 16 and 22 h of growth when the *E. coli* are limited in carbon and the rate of growth is most
different for each concentration of glucose. Because we observed a high correlation (R-squared = 0.98) by plotting the sugar concentrations against the integrated fluorescence at 20 h, we formulated the hypothesis that the integral of fluorescence is proportional to the amount of sugar consumed by the biosensor. We have evidence to support this hypothesis in the glucose-spiking experiment as well.

The sensitivity and dynamic range of the biosensor allows for the detection of sugar in the hydrolysis reactions performed here. We were able to observe significant differences between the amounts of sugar produced in the corn stover hydrolysis reactions with only 25 mg of sample. The amount of sugar produced by the hydrolysis reaction is within the dynamic range of the biosensor.

In the standard curve and repeatability experiment, different sugar mixtures fit linear equations with different slopes. Since these slopes are different, it would be difficult to compare samples containing different ratios of sugars. In lignocellulosic hydrolysis reactions, it is unlikely that feedstocks would produce a range of sugar ratios near the extremes of the range used in the standard curve and repeatability experiment. A survey of potential biomass feedstocks shows that all fall near a 60:40 ratio of 6:5 carbon sugars, making this biosensor well-suited for measuring sugar produced from hydrolysis reactions of any of these feedstocks (Lynd et al. 1999; Mosier et al. 2005; Saha 2003). Thus, the sugar ratios found in nature are close to the ratio at which the biosensor functions best, and the variation found in nature is narrow enough that this sugar ratio variation will have a relatively small impact on the biosensor function.
Application of the Biosensor

Our data suggest that post hydrolysis monitoring (i.e. measuring fluorescence levels at the end of the hydrolysis reaction) is feasible. Our evaluation of low lignin brown midrib mutants using this approach showed that they contain significantly more available sugars than a genotype with wild-type lignin levels. This result is consistent with the large body of data suggesting the low lignin genotypes of both corn and sorghum are more readily hydrolyzed and digestible than their normal lignin counterparts (Barnes et al. 1971; Bucholtz et al. 1980; Fritz et al. 1981; Lechtenberg et al. 1972; Muller et al. 1971; Porter et al. 1978). This post hydrolysis monitoring could be used as a high-throughput screening method to determine available sugars and, in turn, ethanol production potential in different feedstocks.

In the real-time corn stover hydrolysis, shown in Figures 5a and 5b, during the first 16 h, the fluorescence appears to be proportional to the bacterial growth rate. After 16 h, the fluorescence appears to be proportional to the cell density. If the values of the absorbance and fluorescence are predicted between 24 and 36 h and the integral of the fluorescence vs. absorbance is plotted, there is a clear deviation from a linear trend at an OD\textsubscript{595} of ~0.5 where the predicted values cause the slope to increase dramatically (data not shown). This deviation is most likely due to a new and steady supply of sugar to the bacteria provided by the hydrolytic enzymes. Based on the data shown in Figure 5, we would predict that this constant supply of sugar is due to the equilibrium between sugar consumption by the bacteria and feedback inhibition of the hydrolytic enzymes in high sugar concentrations. This constant supply of sugar leads to a constant growth rate,
which in turn leads to constant levels of GFP fluorescence. This explains why these data do not fit a Gompertz equation, which assumes required nutrients are in excess.

From the data presented here, we have formulated a new hypothesis to explain the behavior of the biosensor during the real-time hydrolysis of corn stover. During growth of the biosensor on sugar solutions added at time 0, the level of sugars starts high and decreases during the course of the reaction until the sugar levels limit growth. In contrast, when the sugar required for the growth of the biosensor is provided by a hydrolysis reaction such as in the SSC method, we hypothesize that a certain amount of sugar is present in solution thereby inhibiting enzymatic hydrolysis of the corn stover until the microbes can deplete the sugars enough for the enzymes to regain function. From the point in time when the enzymes regain function, the hydrolysis proceeds in equilibrium with bacterial growth until the feedstock becomes depleted and the hydrolysis reaction slows down. For example, a stover sample more conducive to hydrolysis would allow for sugar to be released by the enzyme into solution more rapidly, therefore supporting a higher steady state level of GFP. This would give a higher amount of steady-state fluorescence. A sample less conducive to hydrolysis would release the sugars more slowly causing the steady-state GFP levels to be lower. Different hydrolytic enzymes could be compared using the real-time SSC method described here by using the same feedstock and monitoring the release of sugars by measuring fluorescence during the course of the reaction.

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REFERENCES


FIGURES AND TABLES

Growth Curves with Different Glucose Concentrations

(a) Data was fit to curves described by the Gompertz equation. R-squared values: 2%, 0.85; 4%, 0.91; 8%, 0.97. (b) Each replication consisted of the fluorescence of a crp*-gfp culture subtracted from the fluorescence of a crp*-gfp culture. Data was fit to first derivative Gompertz equation. R-squared values: 2%, 0.80; 4%, 0.82; 8%, 0.82. (c) Mean fluorescence values from panel b were integrated and plotted against mean crp*-gfp absorbance values from panel a. Linear fit R-squared values: 2%, 0.90; 4%, 0.93; 8%, 0.98. (n=4)

Figure 1

Growth curves of E. coli crp*-gfp grown in modified 1X M9 minimal media with 2, 4, or 8% glucose solution. All points are the mean of four replications with error bars indicating the standard error. (a) Data was fit to curves described by the Gompertz equation. R-squared values: 2%, 0.85; 4%, 0.91; 8%, 0.97. (b) Each replication consisted of the fluorescence of a crp*-gfp culture subtracted from the fluorescence of a crp*-gfp culture. Data was fit to first derivative Gompertz equation. R-squared values: 2%, 0.80; 4%, 0.82; 8%, 0.82. (c) Mean fluorescence values from panel b were integrated and plotted against mean crp*-gfp absorbance values from panel a. Linear fit R-squared values: 2%, 0.90; 4%, 0.93; 8%, 0.98. (n=4)
Sensitivity and Dynamic Range

(a) Absorbance of crp*-gfp @ 20h
(b) Absorbance of crp*-gfp @ 20h

E. coli strain crp*-gfp grown in modified 1X M9 minimal media for 20 h with increasing amounts of glucose. (a) Each time point is the average absorbance of four replications of each E. coli strain. (b) Each time point is the average fluorescence of four replications of crp*-gfp cultures minus the average fluorescence of four replications of crp*-gfp cultures. All points are the mean ± s.e.
Biosensor Response to the Addition of Glucose

Figure 3

*E. coli* strains crp*-gfp and crp*-gfp* grown in modified 1X M9 minimal media with 2% glucose solution for 20 h. 8% glucose solution was added after 20 h to the indicated cultures. (a) Each time point is the average absorbance of three replications. (b) Each time point is the average fluorescence of three replications of crp*-gfp cultures minus the average fluorescence of three replications of crp*-gfp* cultures. All points are the mean ± s.e.
Biosensor Response to the Addition of Chloramphenicol

Figure 4

*E. coli* strains crp*-gfp and crp*-gfp– grown in modified 1X M9 minimal media, containing 20% D-glucose, with or without chloramphenicol. Chloramphenicol added at 13 h. (a) Each time point for hours 0 – 12 is the average absorbance of six replications. Each time point for hours 13 – 23 is the average absorbance of three replications. (b) Each time point for hours 0 – 12 is the average fluorescence of six replications of crp*-gfp cultures minus the average fluorescence of six replications of crp*-gfp– cultures. Each time point for hours 13 – 23 is the average fluorescence of three replications of crp*-gfp cultures minus the average fluorescence of three replications of crp*-gfp– cultures. All points are the mean ± s.e.
Figure 5

SSC method used to analyze corn stover samples W64A X A619 and W64A X A619 bm1. (a) Absorbance measured every two hours for 24 h. Each point is the mean ± s.e. (n=2). (b) Fluorescence measured every two hours for 24 h. Each point is the mean ± s.e. (n=2). (c) Mean fluorescence values integrated and plotted over time. Each point is the mean ± s.e. (n=2).
Table 1

Linear Fits of Standard Curves for Different Sugar Mixtures

<table>
<thead>
<tr>
<th>Sugar Mixture</th>
<th>R-squared $^a$</th>
<th>Slope $^b$</th>
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<tr>
<td>100 glucose: 0 xylose</td>
<td>0.88</td>
<td>4.50</td>
</tr>
<tr>
<td>80 glucose: 20 xylose</td>
<td>0.87</td>
<td>5.85</td>
</tr>
<tr>
<td>62.5 glucose: 37.5 xylose</td>
<td>0.98</td>
<td>6.86</td>
</tr>
<tr>
<td>50 glucose: 50 xylose</td>
<td>0.93</td>
<td>5.95</td>
</tr>
<tr>
<td>20 glucose: 80 xylose</td>
<td>0.92</td>
<td>4.00</td>
</tr>
<tr>
<td>0 glucose: 100 xylose</td>
<td>0.76</td>
<td>0.40</td>
</tr>
</tbody>
</table>

$^a$ R-squared for linear fit of means for each standard curve

$^b$ Slope of linear fit for each standard curve (Fluorescence (U*10^-4)/ mg sugar mixture)
Table 2

Coefficients of Variation for Different Sugar Mixtures

<table>
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<th>Sugar Mixture</th>
<th>Standard</th>
<th>CV%</th>
<th>Average CV%</th>
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<td>0 glucose: 100 xylose</td>
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<td>2</td>
<td>73.99</td>
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<td>3</td>
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<td>4</td>
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<td></td>
<td>5</td>
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<td>6</td>
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<td>54.84</td>
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<td>20 glucose: 80 xylose</td>
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a Standards 1 through 6 are 2, 4, 6, 8, 10, and 12% solution mixtures respectively.

b CV% = (Standard deviation/mean)*100. (n=8)

c Average CV% = (Sum of CV%/6)
Table 3

Endpoint Hydrolysis of Corn Stover

<table>
<thead>
<tr>
<th>Corn Stover</th>
<th>Mean GFP-specific Fluorescence (U*10^-4)</th>
<th>CV%</th>
<th>Grouping</th>
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<tr>
<td>W64A X A619</td>
<td>7.298</td>
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<td>W64A X A619 bm1</td>
<td>19.758</td>
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<td>A</td>
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<tr>
<td>W64A X A619 bm2</td>
<td>19.603</td>
<td>14.42</td>
<td>A</td>
</tr>
<tr>
<td>W64A X A619 bm3</td>
<td>21.747</td>
<td>13.84</td>
<td>A</td>
</tr>
<tr>
<td>W64A X A619 bm4</td>
<td>15.078</td>
<td>20.58</td>
<td>B</td>
</tr>
</tbody>
</table>

\(^a\) Mean GFP-specific fluorescence is the mean of the fluorescence from crp*-gfp subtracted from crp*+gfp. (n=6)

\(^b\) CV% is the standard deviation divided by the mean *100. (n=6)

\(^c\) Levels not connected by same letter are significantly different, p<0.05.
CHAPTER 3. QUALITY CONTROL OF SIMULTANEOUS SACCHARIFICATION AND CATABOLISM

ABSTRACT

The high-throughput screening method of simultaneous saccharification and catabolism (SSC), described in Chapter 2, was evaluated for its repeatability within a technical replication. A technical replication is a repeated measurement of the same sample using the same method. In an analysis of variance, the significance of laboratory parameters were evaluated as main effects in a linear model in order to determine the extent to which different laboratory procedures contribute to the variance of the experiment. None of the laboratory parameters contributed significant variation in the model when applied to experiments involving evaluation of corn stover samples from a breeding program. The SSC method was also evaluated for its precision by evaluating the sample of corn stover in several technical replications referred to as batches. Using a similar statistical model, the significance of the effects of laboratory parameters was determined. Only one of the laboratory parameters, the batch effect, was significant in the model. This difference between batches, or technical replications, means that in order to compare samples from different technical replications with the SSC method, experiments should be planned so that samples are balanced across technical replications and different technical replications should be normalized based on their means. These results suggest how to best design experiments to use the SSC method to precisely obtain quantitative measurements of sugar availability in corn stover and to identify differences
between different corn stover samples within a technical replication as well as differences between means of corn stover samples from many technical replications.

INTRODUCTION

When developing a new screening method to evaluate samples in a plant breeding program, it is important for the method to have high-throughput and be precise. Precision is a measure of the repeatability of the method. In an effort to increase the precision of a multi-step laboratory method, it is helpful to understand how much variation is derived from each step in the method since variation is the antagonist of precision. Simultaneous saccharification and catabolism (SSC) is a new procedure used to evaluate lignocellulosic feedstocks for available sugars in an effort to increase the ethanol potential of feedstocks (see Chapter 2). This method has advantages over other methods used to evaluate ethanol potential of feedstocks. The conventional method to evaluate feedstocks is simultaneous saccharification and fermentation (SSF) (Dowe and McMillan 1995). This method requires anaerobic conditions, large reaction volumes, and is limited in the number of samples that can be evaluated at one time. Another method to evaluate feedstocks uses an in vitro ruminant digestion method and measures the gas produced as a surrogate for ethanol production (Weimer et al. 2005). Although this method has higher-throughput than SSF, it requires a large volume as well as more time than SSC. The SSC method has high-throughput, but must produce repeatable results in order to be valid. This makes precision crucial to the usefulness of this method. There are several steps in the laboratory procedure of SSC that may introduce bias in the data. Measured values may be affected by the row or column position in the rack of the oven or the shaking incubator or in the well in a 96-well plate due to pipetting error. There may also
be bias introduced by a temperature gradient in a shaking incubator or by a gradient
effect of the fluorescence measurement in a 96-well plate fluorometer. All of these
biases may introduce variability in the final SSC data.

There were three objectives of this research. The first objective was to determine
if specific laboratory procedures biased the SSC measurement such that the procedures
had a significant effect on the measured value. The second objective was to determine if
there were significant differences between technical replications of SSC. The
combination of these two objectives will help address the third objective of determining
the repeatability of the SSC method. Since there are several laboratory parameters
involved in the method of SSC, each of these parameters was evaluated individually in
order to determine how much variation was attributed to that parameter as well as to
determine if that variation was significant.

MATERIALS AND METHODS

Preparation of Corn Stover Samples

Single Batch Replication test

Plants consisting of 50 different genotypes were produced in field plots replicated
three times and planted at two locations in Iowa and two locations in Wisconsin in 2005.
At grain maturity, ears were removed from the corn plants and the remaining parts were
cut at approximately 6 inches above the soil and chopped by a forage chopper.
Approximately 0.8 kg of chopped plant material (wet weight) at a moisture content of
about 35% was collected from each plot and these samples were dried using warm air.
The material from each sample was ground by a hammer mill with a 1 mm screen to
obtain a uniform particle size. From the many corn stover samples collected, the W64A
X A619 sample produced in Ames, IA was selected for evaluation for the single batch replication test.

**Technical Replication test**

The corn stover samples used in the technical replication test were obtained from Dr. Dirk Phillip as part of a set of corn stover samples subjected to different pretreatment methods. The samples used in this analysis were the control samples in each of the pretreatment experiments and therefore were not subjected to any pretreatment method themselves. The samples were of the same genotype and were produced in the same environment, and dried material from each sample was ground by a hammer mill with a 1 mm screen.

**Simultaneous Saccharification and Catabolism Procedures**

**Single Batch Replication test**

One objective was to assess the effects from the laboratory parameters in order to determine if corn stover samples could be differentiated. If the variation introduced by one or more of the laboratory parameters was too high, it may have caused errors in the ranking of samples. One corn stover sample was selected to determine the effects of each laboratory parameter. The sample selected was hybrid W64A X A619 produced in Ames, IA in 2005. This sample was analyzed for available sugars using the method of simultaneous saccharification and catabolism described in Chapter 2. For each step in the process of SSC, the test tubes containing the corn stover were randomly assigned to the positions shown in Figure 1a-c, and a split-plot design was used so that each position contained two locations. The left location contained the strain crp*-gfp with the non-fluorescing green fluorescent protein (GFP), and the right location contained the strain
crp*-gfp with the fluorescing GFP. The GFP-specific fluorescence was determined for each position by subtracting the fluorescence from the liquid culture containing crp*-gfp from the fluorescence from the liquid culture containing crp*-gfp. In this experiment, the corn stover sample W64A X A619 was analyzed 35 times.

Technical Replication test

The technical replication test was crucial to determining if there was significant variability between technical replications of our SSC method. This is important to know before comparing samples with different technical replications. A technical replication is a repeated measurement of the same sample using the same method (SSC). This is different than measuring many samples of the same type as previously described in the single batch replication test. In a single batch replication test, one sample is measured many times within one technical replication (one batch) whereas in a technical replication test, one sample is measured many times in several technical replications (many batches). A set of corn stover samples was provided by Dirk Phillip to evaluate the variation within each technical replication and between technical replications. Each technical replication will heretofore be referred to as a batch. The SSC method was used to determine available sugars for each corn stover sample. Each of the corn stover samples was randomly assigned to one of the positions shown in Figure 1a-c, and a split-plot design was used so that each position contained two sub-plots. The strain with the non-fluorescing GFP or the fluorescing GFP were each randomly assigned to one of the sub-plots within each plot. The GFP-specific fluorescence was determined for each position. Each control corn stover sample from each pretreatment method was replicated three times in 5 or 6 batches, depending on which pretreatment method the control sample was
associated with. In total, the number of control samples of the same genotype in each batch was 18 except for batch 6 which contained 15 total control samples. The pretreatment methods from which the untreated, control corn stover samples came are listed in Table 1 along with their number of technical replications.

Statistical Analyses

Single Batch Replication test

All statistical analyses of the data were performed using JMP statistical software (SAS Institute, Cary, NC). A preliminary analysis of the data was performed by constructing a linear model including each of the laboratory parameters and their interactions as terms in the model. None of the interaction terms contributed significant variation so they were excluded from the final model, and their variances should be captured in the residual. A linear model for analysis of variance was created for the data set with each of these categorical laboratory parameters included as fixed main effects: acid row, acid column, shelf in oven, SSC row, SSC column, plate row, and plate column. The GFP-specific fluorescence was the dependant variable. The model was fit using the standard least squares method. The model consisted of the following terms:

\[ y_{ijklmnp} = \mu + r_i + c_j + s_k + t_l + u_m + v_n + w_p + e_{ijklmnp} \]

where

\[ y_{ijklmnp} = \text{the observed value in the } ijklmnp^{th} \text{ position during SSC} \]

\[ \mu = \text{overall mean of observed values} \]

\[ r_i = \text{the effect of the } i^{th} \text{ row of the rack in the oven during acid pretreatment} \]

\[ c_j = \text{the effect of the } j^{th} \text{ column of the rack in the oven during acid pretreatment} \]

\[ s_k = \text{the effect of the } k^{th} \text{ shelf in the oven during acid pretreatment} \]
\( t_l = \) the effect of the \( l^{th} \) row in the incubator during bacterial growth

\( u_m = \) the effect of the \( m^{th} \) column in the incubator during bacterial growth

\( v_n = \) the effect of the \( n^{th} \) row of the plate during fluorescence measurement

\( w_p = \) the effect of the \( p^{th} \) column of the plate during fluorescence measurement

\( \varepsilon_{ijklmp} = \) the error associated with the \( ijklmp^{th} \) position during SSC

After the model was fit to the data, the residuals were computed and a normal quantile plot was used to identify outliers in which the residuals deviated significantly from a normal distribution.

**Technical Replication test**

Using the JMP statistical software (SAS Institute, Cary, NC), a preliminary analysis of the data was performed by constructing a linear model including each of the laboratory parameters and their interactions as terms in the model. None of the interaction terms contributed significant variation so they were excluded from the final model, and their variances should be captured in the residual. A linear model for analysis of variance was created for the data set with each of these categorical laboratory parameters included as model main effects: pretreatment experiment, sample replicate within each experiment, batch, plate, row within plate, and column within plate. The GFP-specific fluorescence was included as the dependant variable. The model was fit using the standard least squares method. The model consisted of the following terms:

\[
y_{ijklmn} = \mu + e_i + q_{ji} + b_k + p_l + d_{mn(i)} + f_{n(l)} + \varepsilon_{ijklmn}
\]

where

\( y_{ijklmn} = \) the observed value in the \( ijklmn^{th} \) position during SSC

\( \mu = \) overall mean of observed values
\( e_i = \) the effect of the \( i^{th} \) experiment on the control corn stover sample

\( q_{j(i)} = \) the effect of the \( j^{th} \) replication nested in the \( i^{th} \) experiment on the control corn stover sample

\( b_k = \) the effect of the \( k^{th} \) batch of SSC

\( p_l = \) the effect of the \( l^{th} \) plate during fluorescence measurement

\( d_{m(l)} = \) the effect of the \( m^{th} \) row nested in the \( l^{th} \) plate during fluorescence measurement

\( f_{n(l)} = \) the effect of the \( n^{th} \) column nested in the \( l^{th} \) plate during fluorescence measurement

\( \epsilon_{ijklmn} = \) the error associated with the \( ijklmn^{th} \) position during SSC

After the model was fit to the data, the residuals were computed and a normal quantile plot was used to identify outliers in which the residuals deviated significantly from a normal distribution.

RESULTS AND DISCUSSION

Single Batch Replication test

In order to determine if a laboratory step in the SSC method produced a significant effect in the model, the bioassay was conducted with one stover sample allocated to each position and the samples were re-randomized between every step of the laboratory procedure. The effect tests of the linear model fit to the GFP-specific fluorescence data, including effects representing each of the laboratory steps, showed the significance of the laboratory steps in the model. The R-squared of a fit of actual to predicted values was 0.86 which indicates that the majority of the variation observed could be explained by non-error terms in the model. The significance probabilities (p-
values) for each of the effects were calculated and are shown in Table 2. Using a threshold significance level of \( \alpha = 0.05 \), none of the laboratory parameters were significant. Since none of the laboratory parameters were significant, there was repeatability within a batch defined as the ability to place a sample in any position and still get the same result.

The repeatability of the measurement was also calculated with the coefficient of variation for the 35 corn stover samples which was 5.29% for the liquid cultures containing the strain crp*-gfp, 6.43% for the liquid cultures containing the strain crp*-gfp, and 22.93% for the GFP-specific fluorescence. The raw fluorescence data collected from the liquid cultures containing the bacterial strain crp*-gfp had a normal distribution, but the raw fluorescence data collected from the liquid cultures containing the bacterial strain crp*-gfp had a left skewed distribution. This caused the GFP-specific fluorescence distribution to also be left skewed. The skewness is likely due to fluorescence quenching at high levels. However, the CV% for the raw fluorescence data for the liquid cultures containing each bacterial strain was small. Since the fluorescence from the liquid cultures containing the strain crp*-gfp are not highly correlated to the fluorescence from the liquid cultures containing the strain crp*-gfp (R-squared = 0.23), then error is being introduced into the GFP-specific fluorescence value when the fluorescence correction is made between the two strains. This suggests that the position of the sample throughout the experimental procedure is not crucial because the liquid cultures containing the strain crp*-gfp were not correlated to the specific liquid cultures containing the strain crp*-gfp with which they were paired. In a situation where many different samples are being compared, determining the GFP-specific fluorescence will be
important because the intrinsic fluorescence of each sample may vary. Based on the normal quantile plot in Figure 2a, the data contained no outliers.

Technical Replication test

One corn stover variety was used as a control in different pretreatment experiments by Dr. Dirk Phillip and this untreated control sample was analyzed by SSC with the samples from various treatments in different batches. A linear model fit to the GFP-specific fluorescence data, including effects representing each step in the laboratory procedure, showed the significance of the laboratory parameters in the model. The technical replication model was fairly good because the R-squared was 0.70, meaning the non-error terms of the model explained 70% of the variance in the experiment. An analysis of variance (ANOVA) was conducted to determine the probability that the observed variation in means was due to chance. The p-value for the F-test was < 0.01. This significant p-value for the F-test suggests that differences in values observed are most likely due to one or more model parameters rather than error. The effect tests for the linear model yielded p-values for each of the laboratory steps and are shown in Table 3. Using a threshold significance level of $\alpha = 0.05$, the only significant effect was the batch effect. The significance in the batch effect indicated that results can be improved by correcting for a batch effect prior to comparing measurements from different batches. This result suggests that the best experimental design would include all samples to be compared in the same batch which should be treated as a complete statistical block. Based on the normal quantile plot in Figure 2b, these data also contained no outliers. With any screening method, it is important to understand which parameters will contribute to significant variation. It is also important when planning an experiment to
know what needs to be compared in order to be able to identify the significant differences in the samples rather than the significant differences in the method parameters.

REFERENCES


FIGURES AND TABLES

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Figure 1

Positions within Each Step of SSC. (a) acid pretreatment positions, (b) SSC incubator positions, and (c) 96-well plate positions for fluorescence measurement. Each position contains two locations: left location indicated by a 0, right location indicated by a 1.
Figure 2

Normal Quantile Plots of Residuals. (a) Single batch replication test and (b) technical replication test.
Table 1

Experiments from Which Control Corn Stover Samples Came and Their Number of Technical Replications

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Table 2

The Effect Tests for Each of the Laboratory Parameters Used in the Fit Model for the Single Batch Replication Test

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<tr>
<th>Parameter</th>
<th>Degrees of Freedom</th>
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<td>3</td>
<td>30.54</td>
<td>1.59</td>
<td>0.33</td>
</tr>
<tr>
<td>Acid Column</td>
<td>4</td>
<td>14.71</td>
<td>0.57</td>
<td>0.70</td>
</tr>
<tr>
<td>Shelf in oven</td>
<td>1</td>
<td>0.36</td>
<td>0.06</td>
<td>0.82</td>
</tr>
<tr>
<td>SSC Row</td>
<td>7</td>
<td>33.83</td>
<td>0.75</td>
<td>0.65</td>
</tr>
<tr>
<td>SSC Column</td>
<td>4</td>
<td>29.75</td>
<td>1.16</td>
<td>0.45</td>
</tr>
<tr>
<td>Plate Row</td>
<td>7</td>
<td>28.25</td>
<td>0.63</td>
<td>0.72</td>
</tr>
<tr>
<td>Plate Column</td>
<td>4</td>
<td>16.32</td>
<td>0.64</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Table 3

The Effect Tests for Each of the Laboratory Parameters Used in the Fit Model for the Technical Replication Test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares ($10^6$)</th>
<th>F-ratio</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Pretreatment Experiment</td>
<td>5</td>
<td>25.75</td>
<td>1.49</td>
<td>0.21</td>
</tr>
<tr>
<td>Sample Replicate [Experiment]</td>
<td>12</td>
<td>28.08</td>
<td>0.69</td>
<td>0.77</td>
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<tr>
<td>Batch</td>
<td>5</td>
<td>282.58</td>
<td>16.38</td>
<td>&lt; 0.01</td>
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<tr>
<td>Plate</td>
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<td>0.07</td>
<td>0.02</td>
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<tr>
<td>Row[Plate]</td>
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<td>0.52</td>
<td>0.91</td>
</tr>
<tr>
<td>Column[Plate]</td>
<td>8</td>
<td>17.38</td>
<td>0.63</td>
<td>0.75</td>
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CHAPTER 4. COMPLEMENTATION TESTING OF MAIZE
GENETIC COOPERATION STOCK CENTER LINES CONTAINING
UNKNOWN BROWN MIDRIB ALLELES

ABSTRACT

The phenylpropanoid pathway starts with phenylalanine and forms many different compounds, including lignin, through a series of reactions. The brown midrib mutants in maize (Zea mays) produce plants that have low or altered amounts of lignin. There are currently four known brown midrib (bm) mutant alleles: bm1, bm2, bm3, and bm4, and two of these alleles have been shown to disrupt enzymes in the phenylpropanoid pathway. The Maize Genetics Cooperation Stock Center has collected mutants with the brown midrib phenotype that are uncharacterized. The objective of this study was to perform genetic complementation tests with maize lines carrying known brown midrib alleles and lines carrying unknown brown midrib alleles in order to determine which unknown brown midrib alleles are alleles of known brown midrib genes and which are new brown midrib genes. Two of the lines carrying unknown brown midrib alleles failed to complement a line carrying the bm1 allele, one line failed to complement a line carrying the bm3 allele, one line failed to complement the line carrying the bm1 and the bm2 allele, and three lines complemented lines carrying each of the four known brown midrib alleles.
INTRODUCTION

In *Zea mays*, there are four different *brown midrib* genes that when mutated cause lower levels or altered composition of lignin. The brown midrib phenotype was first discovered in St. Paul, Minnesota in 1924. Since then, four different *brown midrib* alleles have been identified: *bm1* (Jorgenson 1931), *bm2* (Burnham and Brink 1932), *bm3* (Emerson et al. 1935), and *bm4* (Burnham 1947). All four of these known *brown midrib* alleles are recessive. The brown pigment produced in these mutants occurs in the leaves, stem, roots, tassel, and cob of the maize plant but not in the kernels or pollen grains (Jorgenson 1931).

Two of the *Brown midrib* (*Bm*) genes, *Bm1* and *Bm3*, are associated with specific enzymes that are involved in the phenylpropanoid pathway. *Bm1* is involved either in regulating or encoding cinnamyl alcohol dehydrogenase (CAD), an enzyme that dehydrogenates coniferaldehyde and 5-hydroxyconiferaldehyde to their respective alcohols, and *Bm3* encodes caffeic acid O-methyltransferase (COMT), an enzyme that converts 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol to sinapaldehyde and sinapyl alcohol respectively by adding a methyl group (Boerjan 2003). Other genes in the phenylpropanoid pathway have been identified by EST sequencing in Arabidopsis, and mutants in these genes have not yet been identified in maize which may produce a brown midrib phenotype. The Maize Genetics Cooperation Stock Center has collected several recessive brown midrib mutants that are uncharacterized. The objective of this study was to perform genetic complementation tests with maize lines carrying four known *brown midrib* mutant alleles and lines carrying unknown *brown midrib* mutant
alleles in order to determine if unknown *brown midrib* mutant alleles are actually mutant alleles of *brown midrib* genes that have not been characterized genetically.

**MATERIALS AND METHODS**

The germplasm used in these experiments was obtained from the Maize Genetics Cooperation Stock Center (MGCSC), (Urbana, IL) and is listed in Table 1. Maize seeds obtained from the MGCSC were planted in the summer nursery of 2006 at the Agronomy Farms of Iowa State University (Boone County, IA). Each of the lines containing a homozygous recessive unknown *brown midrib* allele was crossed to each of the lines containing a homozygous recessive known *brown midrib* allele. The successful pollinations from the summer nursery of 2006 are shown in Table 1. The F1 seeds from these crosses were harvested at maturity and 25 seeds from each cross were planted in the summer nursery of 2007 at the Agronomy Farms of Iowa State University. Crosses were also performed with the homozygous recessive known *brown midrib* alleles and the homozygous recessive unknown *brown midrib* alleles in the summer nursery of 2007. The successful pollinations from the summer nursery of 2007 are shown in Table 2. The F1 seeds from these crosses were harvested at maturity and 10 seeds from each cross were planted in the USDA Greenhouse in 2007 (Ames, IA). The plants were screened for reddish-brown pigmentation in the leaf midrib that is characteristic of the brown midrib mutant phenotype at V8 stage of growth.

**RESULTS**

Lines containing unknown *brown midrib* alleles were crossed to lines containing the known alleles *bm1*, *bm2*, *bm3*, and *bm4*. If the progeny from a cross between a plant
with a homozygous recessive known brown midrib allele and a homozygous recessive unknown brown midrib allele show a wild-type phenotype, then the two alleles are said to complement and are most likely not derived from the same genetic locus. If the progeny from the same cross show a brown midrib phenotype, then the two alleles do not complement and are most likely at the same genetic locus. It is possible for two non-allelic loci to fail to complement, so non-complementation results must be interpreted with caution (Benzer 1955; Rine and Herskowitz 1987). Since all the plants used in crosses between lines carrying known and unknown brown midrib alleles were of the brown midrib phenotype, all of the progeny should produce one of two phenotypic classes: wild-type or brown midrib.

The number of plants scored in each class from each cross made in 2006 and 2007 are shown in Tables 1 and 2 respectively, and the new designations for the lines carrying the unknown brown midrib alleles are shown in Table 3. Two of the lines carrying unknown brown midrib alleles failed to complement the line carrying the bm1 allele and one line failed to complement the line carrying the bm3 allele. One line carrying an unknown brown midrib allele, 5803I bm*-PI267186, failed to complement both the line carrying the bm1 allele and the line carrying the bm2 allele. Three lines carrying an unknown brown midrib allele complemented all of the lines carrying the known brown midrib alleles (Table 3).

DISCUSSION

There are many enzymes involved in the phenylpropanoid pathway which may produce brown midrib phenotypes when genetically disrupted, and a study of this nature may help to characterize these enzymes. A brown midrib phenotype may be caused by a
mutation in a known or unknown enzyme in the pathway or in a regulator of the pathway, therefore, brown midrib mutants may be useful in helping to elucidate the phenylpropanoid pathway. Most likely, the two lines that did not complement the line containing the $bm1$ mutant allele contain mutations in the gene associated with the $Bm1$ allele (Table 3). Similarly, the line that did not complement the line containing the $bm3$ mutant allele most likely contains a mutation in the gene associated with the $Bm3$ allele (Table 3).

However, it is possible that these specific lines carrying unknown recessive brown midrib alleles exhibit non-allelic non-complementation and are actually mutations in gene products of the phenylpropanoid pathway that interact with the known enzymes CAD and COMT (Benzer 1955; Rine and Herskowitz 1987). Although all of the progeny scored from these crosses had a brown midrib phenotype, it would be important to self-pollinate the F1 plants and score the progeny in order to determine the occurrence of phenotypic segregation, suggesting non-allelic non-complementation.

There are two main genetic explanations for line carrying the unknown brown midrib allele that did not complement both the line carrying the $bm1$ allele and the line carrying the $bm2$ allele. The first is that the line with the unknown phenotype was a double mutant of $bm1$-$bm2$. Crossing this line to lines homozygous for the $bm1$ allele or the $bm2$ allele would only produce progeny with brown midrib phenotypes. The second is that the line with the unknown bm phenotype likely contains a mutation in a gene product that interacts in trans with the gene products encoded by $Bm1$ and $Bm2$ (Table 3). The study of this interaction may help determine the product encoded by $Bm2$ and may also help to improve the model of the phenylpropanoid pathway in producing lignin in maize.
Finally, there were three lines containing unknown brown midrib alleles that complemented all of the lines containing the four known brown midrib alleles (Table 3). These lines, however, were not crossed to each other so it is unknown whether these lines complement each other. These new mutant alleles likely encode enzymes or regulatory elements of the phenylpropanoid pathway that are either undiscovered or have not had a mutant phenotype associated with them yet. The elucidation of the phenylpropanoid pathway may be very useful in terms of biofuel production because we could use this information to alter lignin composition to optimize biofuel feedstocks.

REFERENCES


FIGURES AND TABLES

Table 1

Crosses Made in Summer Nursery in 2006

<table>
<thead>
<tr>
<th>Pedigrees of Crosses</th>
<th>Phenotypic Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female Parent</td>
<td>Male Parent</td>
</tr>
<tr>
<td>515D bm1</td>
<td>5803D bm*-PI251009</td>
</tr>
<tr>
<td>515D bm1</td>
<td>5803K bm*2001PR-1</td>
</tr>
<tr>
<td>5803F bm*-PI251930</td>
<td>515D bm1</td>
</tr>
<tr>
<td>5803G bm*-PI262480</td>
<td>515D bm1</td>
</tr>
<tr>
<td>5803H bm*-PI262485</td>
<td>515D bm1</td>
</tr>
<tr>
<td>119F bm2</td>
<td>5803J bm*-86-87-8875-6</td>
</tr>
<tr>
<td>5803F bm*-PI251930</td>
<td>119F bm2</td>
</tr>
<tr>
<td>5803G bm*-PI262480</td>
<td>119F bm2</td>
</tr>
<tr>
<td>5803I bm*-PI267186</td>
<td>119F bm2</td>
</tr>
<tr>
<td>5803K bm*-2001PR-1</td>
<td>119F bm2</td>
</tr>
<tr>
<td>5803C bm*-PI228174</td>
<td>408E bm3-91598-3</td>
</tr>
<tr>
<td>5803D bm*-PI251009</td>
<td>408E bm3-91598-3</td>
</tr>
<tr>
<td>5803G bm*-PI262480</td>
<td>408E bm3-91598-3</td>
</tr>
<tr>
<td>5803H bm*-PI262485</td>
<td>408E bm3-91598-3</td>
</tr>
<tr>
<td>5803I bm*-PI267186</td>
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<td>5803K bm*2001PR-1</td>
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<td>919A bm4</td>
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<tr>
<td>5803K bm*2001PR-1</td>
<td>919A bm4</td>
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</table>

a The pedigree of each parent of the complementation test cross according to the Maize Cooperation Genetic Stock Center

b The observed phenotype of the plants. bm = brown midrib and WT = wild type.
Table 2

Crosses Made in Summer Nursery in 2007

<table>
<thead>
<tr>
<th>Pedigrees of Crosses&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phenotypic Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female Parent</strong></td>
<td><strong>Male Parent</strong></td>
</tr>
<tr>
<td>515D bm1</td>
<td>5803J bm*-86-87-8875-6</td>
</tr>
<tr>
<td>515D bm1</td>
<td>5803L bm*-N2331B</td>
</tr>
<tr>
<td>5803I bm*-PI267186</td>
<td>515D bm1</td>
</tr>
<tr>
<td>5803I bm*-PI267186</td>
<td>515D bm1</td>
</tr>
<tr>
<td>119F bm2</td>
<td>5803C bm*-PI228174</td>
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<td>119F bm2</td>
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<tr>
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<tr>
<td>5803I bm*-PI267186</td>
<td>919A bm4</td>
</tr>
<tr>
<td>5803L bm*-N2331B</td>
<td>919A bm4</td>
</tr>
</tbody>
</table>

<sup>a</sup> The pedigree of each parent of the complementation test cross according to the Maize Cooperation Genetic Stock Center

<sup>b</sup> The observed phenotype of the plants. bm = brown midrib and WT = wild type.

* Only 6 seeds were planted.
Table 3

Complementation Test Results

<table>
<thead>
<tr>
<th>Previous Designation (^a)</th>
<th>New Designation (^b)</th>
<th>MGCSC Source Number (^c)</th>
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<tr>
<td>5803D bm*-PI251009</td>
<td>5803D bm1-PI251009</td>
<td>MGSC94-4844-9 ((M14 x W23) x bm*)self</td>
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<tr>
<td>5803I bm*-PI267186</td>
<td>5803I bm*-PI267186(^s)</td>
<td>MGSC96-6357-8 x 6357-5 (bm* x bm*)</td>
</tr>
<tr>
<td>5803L bm*-N2331B</td>
<td>5803L bm1-N2331B</td>
<td>MGSC2001-2659-4 ((B73 x Mo17) x bm*)self</td>
</tr>
<tr>
<td>5803K bm*-2001PR-1</td>
<td>5803K bm3-2001PR-1</td>
<td>MGSC2002-335-3 ((M14 x W23) x bm*)self</td>
</tr>
<tr>
<td>5803F bm*-PI251930</td>
<td>5803F bm*-PI251930(^t)</td>
<td>MGSC94-4829-2 (bm*)self</td>
</tr>
<tr>
<td>5803H bm*-PI262485</td>
<td>5803H bm*-PI262485(^t)</td>
<td>MGSC94-4827-1 (bm*)self</td>
</tr>
<tr>
<td>5803J bm*-86-87-8875-6</td>
<td>5803J bm*-86-87-8875-6(^t)</td>
<td>MGSC2002P-22-2 ((W23 x M14) x bm*)self</td>
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</table>

\(^a\) The pedigree of the maize line carrying the unknown *brown midrib* allele according to the Maize Cooperation Genetic Stock Center

\(^b\) The new pedigree of the maize line carrying the unknown *brown midrib* allele after complementation tests to all four of the known *brown midrib* alleles.

\(^c\) The Maize Cooperation Genetic Stock Center source number for each of the lines carrying unknown *brown midrib* alleles.

\(^s\) This line failed to complement both 515D bm1 and 119F bm2.

\(^t\) These lines complemented lines 515D bm1, 119F bm2, 408E bm3-91598-3, and 919A bm4.
CHAPTER 5. GENERAL DISCUSSION

GENERAL DISCUSSION

The most important reasons to use biofuels are to increase energy independence and to help solve problems associated with climate change. Biofuels made from lignocellulosic feedstocks such as corn stover may help to specifically address these issues because they are renewable resources.

There are several conclusions that can be made from the three research articles presented in this thesis. In Chapter 2, “Development of a Real-time Sugar-Consuming Biosensor and its Application to a Corn Stover Hydrolysis Bioassay,” a strain of *E. coli* was engineered to express green fluorescent protein (GFP) and grown on different varieties of corn stover. This strain was used in a high-throughput screening method called simultaneous saccharification and catabolism and can be applied to breeding programs to report differences between corn stover samples based on the amount available sugars. In Chapter 3, “Quality Control of Simultaneous Saccharification and Catabolism,” the SSC bioassay was evaluated for its repeatability within a technical replication and between technical replications. It was found that none of the steps in the laboratory procedure significantly biased the resulting mean fluorescence level for the corn stover samples, but technical replication did significantly affect the mean fluorescence level. In Chapter 4, “Complementation testing of Maize Genetic Cooperation Stock Center Lines Containing Unknown *brown midrib* Alleles,” complementation tests were performed with maize plants carrying known or unknown *brown midrib* alleles. Two of the lines carrying unknown *brown midrib* alleles failed to
complement a line carrying the $bm1$ allele, one line failed to complement a line carrying the $bm3$ allele, one line failed to complement the line carrying the $bm1$ and the line carrying the $bm2$ allele, and three lines complemented lines carrying each of the four of the known brown midrib alleles. The brown midrib phenotypes are more digestible with acid or enzymatic hydrolysis and may be good feedstock candidates for lignocellulosic ethanol production.

**FUTURE WORK**

The method of SSC has high-throughput and has been used to screen several lignocellulosic feedstocks such as corn stover, switchgrass, and bermudagrass, however, the SSC method needs to be validated with another method such as SSF.

The *E. coli* biosensor used in the method of SSC carries a plasmid that constitutively expresses GFP, but it is not the most optimal biosensor. Biosensors that are sugar specific would be better than the biosensor currently used in SSC because they would be more informative and not specifically dependent on culture density. The sugars released in the hydrolytic reactions of hemicellulases and cellulases with lignocellulosic feedstocks could then be monitored in one solution simultaneously by the different sugar-specific biosensors. This would be useful in experiments aimed at optimizing hydrolysis reactions. The sugar-specific biosensors could be used to monitor sugars produced in a mixed solution in a method like SSC to screen feedstocks for sugar production or to monitor sugars released during yeast fermentation reactions.

Based on our consumption rate of fossil fuels, we will eventually have to obtain energy from another source. Lignocellulosic feedstocks are a promising renewable resource that can be used to make biofuels. Corn stover may not be the cellulosic
biomass of the future, but because it is a readily available feedstock, it is a good transitional feedstock to develop cellulosic ethanol technologies.

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