PRODUCTION OF HETEROLOGOUS HYDROLYSIS ENZYMES WITHIN CROP BIOMASS FOR BIOFUEL ETHANOL

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Summary

As environmental and national security issues become more urgent, production of biofuels becomes more important (see also – *Biorefineries –Concept of sustainability and human development*). An untapped resource for the production of alcohol fuels is lignocellulosic biomass (see also. – *Lignocellulose Biorefinery*). This chapter contains an overview of the process, the current status of the technology and its promise and roadblocks, and a look to the future. It begins with an overview of the plant cell wall, essential to the understanding of the process. Then, it describes hydrolysis of the cell wall by microorganisms and how scientists have utilized this knowledge to manipulate and optimize the method. Next, molecular farming of hydrolysis enzymes in crop plants is discussed as a novel idea for maximizing enzyme production. Finally, other methods useful for the optimization and maximization of biofuel output are explored.

1. Introduction

Ethanol fuel is a promising alternative to fossil fuels, which damage the environment by contributing to net carbon dioxide increase. In addition, they will eventually be depleted, and increase dependence on foreign oil imports. According to a recent report from the Natural Resources Defense Council and the Institute for the Analysis of Global Security, the dependence of the United States on foreign petroleum both undermines its economic strength and threatens its national security. The use of ethanol fuel, obtained either from grain or from cellulosic materials, can help decrease the need for petroleum fuel. Accordingly, the ethanol fuel industry has been growing significantly in many countries throughout the world. In the US, ethanol production capacity reached 3.5 billion gallons in 2004, up by 303 million gallons from 2003. Ethanol fuel is clean-burning and does not contribute to net carbon dioxide increase, is renewable, and can be produced using resources the country already possesses.

Ethanol is produced from the fermentation of sugars (usually sucrose or glucose) by yeast. The carbon (sugar) source is called the feedstock. Most feedstocks are plant materials. The most widely used feedstocks today are sugarcane (see also – *Technology and econmics of fuel ethanol production from* sugarcane) and maize grain. The sugar in sugarcane is easily extracted and used directly for fermentation, while the maize grain must be milled and its starch hydrolyzed to glucose by α-amylase (see also – *Biorefineries*). In the US, ethanol is mostly produced from the starch of maize grain with a net energy balance of 1.34; that is, for every unit of energy expended in growing corn and converting it to ethanol, 1.34 units of energy (automotive fuel) are obtained. The most efficient farming and ethanol production systems in place can achieve a balance of 2.09. Starch fermentation is thus relatively efficient. However, there is a very rich source of glucose that has so far been underutilized: cellulose.

Cellulose, composed of β-glucose units, is the most abundant polymer on earth. It is a structural component of the plant cell wall. It has traditionally not been used as a carbon source because its location inside microfibrils, which are wrapped in hemicellulose and embedded in a matrix of lignin, makes it inaccessible to hydrolysis enzymes unless the plant material goes through extensive pretreatment. However, recent advancements have made using this resource a possibility. In this chapter, we explore the problems,

challenges, and solutions to ethanol production from cellulosic materials, with a focus on utilizing plants as biofactories for hydrolysis enzyme production.

2. The Plant Cell Wall

The plant cell wall is a highly organized structural component composed of a myriad of different polysaccharides, proteins, aromatic substances and other compounds. It has several important functions: it provides structure to the cell, thus determining its shape and even function; it aids in defense against invading pathogens; and it contains signaling molecules that can alert the cell to various environmental stimuli, including pathogenic attack. It is a dynamic structure, and its configuration and composition can vary by plant species, age, tissue, cell types and even within cell wall layers (see also – *Plant Cell Culture*). The primary cell wall is formed first from the cell plate during cell division and forms the outside of the cell. Between primary cell walls of adjacent cells is the middle lamella. Secondary cell wall synthesis, if present, usually begins after the primary cell wall has stopped growing, being deposited on the interior of the primary cell wall, often in layers.

Polysaccharides are the primary constituents of the cell wall and form its main structural scaffold. They are composed of long chains of sugar molecules that are covalently linked at various positions and may have side chains. They are made up of various combinations of the 11 monosaccharide sugars commonly found in plant cell walls: glucose (from which all the others are derived), rhamnose, galactose, galacturonic acid, glucouronic acid apiose, xylose, arabinose, mannose, mannuronic acid and fucose.

2.1. Cell wall components

2.1.1. Cellulose

Cellulose is a long, unbranched polymer of up to 15,000 molecules of anhydrous glucose. The glucose molecules are arranged in β-1,4 linkages, which means that each unit is orientated 180° relative to the unit it is attached to. In other words, cellulose is composed of cellobiose units (diglucose molecules connected via β-1,4 linkages). Cellulose is an important polysaccharide found in the primary and secondary cell walls in the form of microfibrils. It makes up 15-30% of the dry mass of primary cell walls and up to 40% of secondary cell walls. The cellulose chains in microfibrils are lined up parallel to each other and consist of crystalline regions, where the cellulose molecules are tightly packed, and amorphous (also called soluble) regions, where the arrangement is less compact. The amorphous regions are staggered so that the overall structure remains strong. A microfibril has a diameter of around 30 nm and consists of around 36 cellulose chains, but the number varies with species.

2.1.2. Cross-linking glycans

Microfibrils are coated with other polysaccharides, cross-linking glycans (also called hemicelluloses), which link them together. The two major types are xyloglucans, found in dicots and around half of the monocot species, and glucuronoarabinoxylans, which are found in commelinoid monocots, including the cereals and grasses. Xyloglucans

have a backbone of glucosyl residues in 1,4-β linkages, with xylosyl units attached; glucuronoarabinoxylans have a backbone of xylosyl residues in 1,4-β linkages to which glucosyluronic acid and arabinosyl units are attached. The grasses also have a third major cross-linking glycan, called "mixed linkage" (1→3),(1→4)β-D-glucans (β-glucans), which are unbranched polymers with a 2:1 ratio of cellotriose to cellotetraose units connected by (1→3)β-D-linkages, resulting in a coiled shape. Various mannans are also present in smaller amounts. Hemicellulose accounts for 20-40% of the total dry weight of plant matter.

2.1.3. Pectins and other substances

Pectins are a mixed group of various branched, hydrated polysaccharides abundant in galacturonic acid. In dicots, they account for approximately 35% of the dry weight; in monocots they are much less abundant. They serve many functions in the cell wall: they establish wall porosity, adjust wall pH and ion balance through charged surfaces, control bonding between cells at the middle lamella, and also function as recognition molecules to alert the cell to the presence of microorganisms or insects. Pectins are mostly made up of homogalacturonan and rhamnogalacturonan I; rhamnogalacturonan II, arabinans, galactans, and arabinogalactans are also present in smaller quantities. In addition to pectins, structural proteins and aromatic substances can also be present.

2.1.4. Lignin

Lignin is almost nonexistent in primary cell walls but is a chief constituent in some secondary walls, and accounts for about 10-25% of the total dry weight. It is composed of aromatic compounds called phenylpropanoids arranged in complex systems. These networks are linked to the carbohydrates, including cellulose and xylose, in various bonds, including ester, ester; phenyl, phenyl; and covalent bonds. Lignin protects the cell against pathogen invasion and will often be deposited in response to attack, providing additional structure and strength.

2.2. Two major types of primary cell wall

The basic structure of primary cell walls consists of the scaffold of cellulose and cross-linking glycans, embedded in a second (and sometimes third) complex. There are two types of primary cell wall that differ in the kind of cross-linking glycan, which determines the wall type. Type I walls are found in those plants that have xyloglucans; they have approximately equal amounts of xyloglucan and cellulose. Xyloglucans coat the cellulose microfibrils and bind them together, and this complex is embedded in a matrix of pectin. Type II walls are found in plants whose major cross-linking glycans are glucuronoarabinoxylans; they lack pectin and structural proteins, instead amassing phenylpropanoids. Type II cell walls are found in cereals and grasses, and thus are of greatest interest for cellulosic ethanol research.

3. Cell wall degradation

3.1. Microorganisms

Several microorganisms (bacteria and fungi) have been studied for their ability to break down cell walls, including anaerobes (such as those present in the rumen) and aerobes (such as those that decompose dead plant matter). Most organisms that can degrade cellulose produce a number of enzymes, which form a system that hydolyzes various polysaccharides, since the enzymes first have to penetrate the hemicellulose shield before they can attack the cellulose (see also – *Cell thermodynamics and energy metabolism; – Basic Strategies of Cell Metabolism*).

Anaerobic microorganisms known for their cell-wall degrading ability include the bacteria *Butyrivibrio fibrisolvens* H17c, *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* 17, *R. albus*, *Prevotella ruminicola* B1 4, *Clostridium thermocellum*, *C. cellulovorans*, *C. cellulolyticum*, *C. stercorarium* and *Caldocellulosiruptor saccharolyticus*, and the fungus *Neocallimastix frontalis*. Aerobic microorganisms include the bacteria *Acidothermus cellulolyticus, Pseudomonas fluorescens* subsp. *cellulosa*, *Streptomyces lividans* 66, *S. reticuli*, *S. halstedii*, *Cellulomonas fimi*, *C. uda* and *Microbispora bispora*, and the fungi *Thermomonospora fusca*, *Trichoderma reesei* and *Phanerochaete chrysosporium.*

These organisms produce many different enzymes that may be grouped according to their primary activities: endoglucanases, exoglucanases (or cellobiohydrolyases), β-glucosidases, cellodextrinases, xylanases, xylosidases, lichenases, mannanases, laminarinases, arabinofuranosidases and avicelases. In order to decrystallize and hydrolyze cell walls, they must produce systems of many different enzymes (for each of the cell wall components) that act synergistically; this has been well documented. The enzymes vary in their substrate specificity: some exclusively act on a particular substrate, while others can utilize more than one; some have more activity on one substrate over another; and some can break only certain bonds, while others can cleave more than one bond type. In addition, different enzymes often produce different products from the same substrate. Therefore microorganisms may produce several different enzymes, for specific substrates or bonds or both (see also – *Enzyme production*). Some microorganisms, such as *Clostridia* spp., produce cellulosomes, complexes of multiple enzymes held together in a specific conformation by proteins that are very efficient at cell wall hydrolysis.

3.2. Hydrolysis

The major classes of enzymes needed for cell wall hydrolysis are cellulases, hemicellulases and ligninases.

3.2.1. Cellulases

Three types of cellulases are needed to obtain glucose from cellulose: endoglucanase (E1; E.C. 3.2.1.4), cellobiohydrolase (also called exoglucanase) (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21). Enymatic hydrolysis of plant cell wall polysaccharides to glucose is a three-step process. First, endoglucanase randomly cleaves the crystalline regions of cellulose, exposing chain ends. Then, cellobiohydrolase attaches to the chain end and threads it through its active site, processively cleaving off cellobiose units; it can also act on amorphous regions with exposed chain ends without prior

endoglucanase activity. Exoglucanases work from either the reducing or non-reducing end of the sugar, not both; cellulase hydrolysis is more efficient if both types are produced. Finally, β-glucosidase breaks the bonds of cellobiose to produce single glucose units.

3.2.2. Hemicellulases

For cellulases to access the cellulose, the hemicellulose surrounding it must be removed. While cellulose consists of a single monosaccharide and type of bond, hemicelluloses are amorphous and diverse. Since the major constituent of hemicellulose is β-1,4-xylan, the most abundant class of hemicellulase is xylanase, which can have both endo- and exo- activity.

3.2.3. Ligninases

Lignin degradation by microorganisms is less well understood than that of polysaccharides. The most effective lignin-degrading microbes in nature are thought to be white rot fungi, especially *Phanerochaete chrysosporium* and *Trametes versicolor*. The three major families of lignin-modifying enzymes produced by fungi are laccases, manganese-dependent peroxidases, and lignin peroxidases. They oxidate compounds by using or creating radicals.

4. Ethanol production

4.1. Maize grain ethanol production

Ethanol produced from maize grain is a mature technology. It is attractive because it benefits farmers and local communities by providing jobs, a valuable resource, and valuble coproducts (such as distillers grains and corn gluten). As of 2007, 124 biorefineries are in operation and 76 more are being constructed. Ethanol production currently stands at nearly 6.5 billion gallons a year and will reach 12.9 billion gallons per year upon the plants' completion, which could displace 4.7 and 9.3 billion gallons of gasoline respectively (if E85, a fuel blend of gasoline and up to 85% ethanol, is used). However, this only covers around 3% or 6.7% respectively of the total gasoline consumed annually in the US (137 billion gallons in 2006).

US maize growers produced 10.5 billion bushels of maize grain in 2006; 18.3% was used in ethanol production. This is the equivalent of 2.2 billion bushels, or 6.2 billion gallons of ethanol, which likely displaced 4.4 billion gallons of gasoline (3.2% total consumption). Since current ethanol plant capacity is 2.3 billion bushels, becoming 4.6 billion bushels, grain production must increase to meet capacity, or must be diverted from other uses. Currently, 50.8% of total production, or 6 billion bushels, is used for livestock feed. Much of this could be successfully diverted to ethanol fuel production as the grain could be replaced with nutritious distillers grains. To meet capacity, only 1.7% (currently) or 40% (when the plants are completed) need be diverted from grain destined for livestock feed (0.1 billion bushels and 2.4 billion bushels respectively). Therefore meeting production capacity from maize grain is an attainable goal and likely to be realized. However, if all the maize grain produced in the US were used for ethanol fuel

production, only 29.4 billion gallons would be produced, the equivalent of 21.2 billion gallons of fuel, or 15.4% of current usage. Clearly, an alternative to maize grain ethanol is needed.

4.2. The promise of cellulosic ethanol

Worldwide wasted crops and lignocellulosic waste crop residue could translate into 129.7 billion gallons of ethanol and replace 93.4 billion gallons of gasoline (about 32% of current consumption) if E85 is used. About 90% of this estimate comes from crop residue waste. This number could be much higher if biofuel crops were grown to supplement this amount and if the technology were in place to produce it. Worldwide availability of lignocellulosic feedstocks is estimated at over 1.7 billion tons per year, with some estimates reaching 10-50 billion tons of crop biomass annually. In addition to being inexpensive and widely available, lignocellulosic biomass has the added benefit of being renewable and thus sustainable. It is believed that with proper management, roughly 1.3 billion tons of crop and forest residues and energy crops can become available annually in the US, the majority of which could be used for conversion to alcohol fuels, yielding the equivalent of approximately 108.5 billion gallons of gasoline. A current goal for enhancing US economic security is to meet 10% of chemical feedstock demand by 2020 with plant-derived materials, or a fivefold increase over current usage level. Crops that have a high amount of lignocellulosic biomass, such as corn, rice, sugarcane and fast growing perennial grasses have been recommended for conversion to alcohol fuels.

Construction of commercial biomass ethanol facilities is currently underway in the US. These facilities will have the capactiy to collectively produce 226.4 million gallons per year. They include: Abengoa Abengoa Bioenergy, NE; Akico, Inc., FL; Bluefire Ethanol, CA; Broin Companies, IA; Iogen Biorefinery Partners, ID; and Range Fuels, GA. In Canada, Iogen Corporation has a demonstration biomass ethanol plant currently in operation that can produce about 660,000 gallons of ethanol per year.

4.2.1. Cellulosic ethanol production

To produce ethanol from biomass, several events must take place: the hydrolysis enzymes must be produced (usually in microbial fermentation tanks), the biomass must undergo a pretreatment process to disrupt the lignin and expose the cellulose, the enzymes must be added to the pretreated feedstock, and the resulting sugars must be fermented and distilled.

4.2.2. Challenges to cellulosic ethanol production

Although production of fermentable sugars for alcohol fuels from plant biomass is an exciting and attractive idea, and substantial efforts have been made toward improving ethanol yield through this technology and reducing its production costs, major roadblocks still stand in the way of widespread commercial implementation of this technology. These include prohibitive costs of pretreatment processing of the lignocellulosic matter, with estimates of up to $0.30/gallon and production of microbial cellulase enzymes used in the conversion of cellulosic matter to fermentable sugars.

Removal of lignin is the major roadblock to this process and an area of intense research because of the high cost involved. Although research is ongoing in the area of fungal ligninases (mentioned above) and reduction of lignin content (described below) in order to decrease the necessity (and thus the cost) of pretreatment, pretreatment is currently required. Several pretreatments have been developed so far, including dilute acid, acid flow-through, ammonia fiber explosion (AFEX), ammonia recycle percolation, steam water explosion, lime, and organosolv pulping.

Currently, production of hydrolysis enzymes in microbial fermentation tanks is expensive. Although decades of research have been devoted to reducing microbial production costs, resulting in significant decreases since 1980, enzyme production is still costly. The latest cost-reduction model designed by the National Renewable Energy Laboratory (NREL) and Genencor is to produce cellulases at around $0.10-$0.20 per gallon of ethanol. A possible solution to these problems is to use biomass crops as biofactories to produce these enzymes on a large scale.

5. Production of Hydrolysis Enzymes in Biomass Crops

5.1. Plants as molecular biofactories

Plants are already being used successfully for molecular farming of enzymes and other proteins, carbohydrates, lipids, polymers such as polyhydroxybutyrate and pharmaceuticals. Plant-based production of enzymes has several critical advantages compared to microbial fermentation or bioreactors. For example, plants can use the sun's energy directly, requiring fewer energy inputs. Furthermore, proteins produced in plants generally display correct folding, glycosylation, activity, reduced degradation and increased stability. In addition, the infrastructure and expertise are already available for plant genetic transformation, growing, harvesting, transporting and processing plant matter.

The US Government has recently urged the agricultural and petrochemical industries to discover and employ alternatives to fossil fuels to both decrease dependence on foreign oil and promote a cleaner environment. A specific recommendation was to develop technology that would allow production of cellulases and other hydrolysis enzymes in plants, which has the potential to reduce enzyme production costs. Extraction of plant total soluble protein (TSP) from leaves is quick and easy, and could be done at the ethanol production facilities; alternatively, the enzymes could be extracted and lyophilized for inexpensive storage and easy transport.

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Bibliography

Arioli, Tony, Liangcai Peng, Andreas S. Betzner, Joanne Burn, Werner Wittke, Werner Herth, et al. 1998. Molecular Analysis of Cellulose Biosynthesis in Arabidopsis. *Science* 279(5351):717-720. [This is one report of several on the topic of elucidating cellulose synthesis in plants.]

Baker, J.O., W.S. Adney, R.A. Nieves, S.R. Thomas, D.B. Wilson, and M.E. Himmel. 1994. A new thermostable endoglucanase, *Acidothermus cellulolyticus* E1. Synergism with *Trichoderma reese*i CBH I and comparison to *Thermomonospora fusca* E5. *Appl. Biochem. Biotech.* 45/46:245-256. [This report describes the thermostable endo-1,4-β glucanase E1 from *Acidothermus cellulolyticus*.]

Baucher, M., M.A. Bernard-vailhé, B. Chabbert, J.M. Besle, C. Opsomer, M. Van Montagu, and J. Botterman. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. *Plant Mol. Biol.* 39(3): 437-447. [This describes research on the down-regulateion of cinnamyl alcohol dehydrogenase (CAD) in alfalfa that resulted in increased in situ digestibility and modified lignin composition, although there was no overall reduction in lignin content.]

Biomass Program: Net Energy Balance for Bioethanol Production and Use. http://www1.eere.energy.gov/biomass/net_energy_balance.html (accessed August 1, 2007). [This website explains energy balances.]

Biswas, Gadab C. Ghosh, Callista Ransom, and Mariam Sticklen. 2006. Expression of biologically active *Acidothermus cellulolyticus* endoglucanase in transgenic maize plants. *Plant Science* 171(5):617-623. [This report describes the successful production of the catalytic domain of the thermostable endo-1,4-β glucanase (E1) of *A. cellulolyticus* in maize.]

Blaschke, Lothar, Michel Legrand, Carsten Mai, and Andrea Polle. 2004. Lignification and structural biomass production in tobacco with suppressed caffeic/5-hydroxy ferulic acid-*O*-methyl transferase activity under ambient and elevated CO2 concentrations. *Physiol. Plant.* 121(1): 75-83. [In this report, suppression of *O*-methyl transferase (OMT) in tobacco (*Nicotiana tabacum*) resulted in increased biomass production and a shift from the structural to non-structural fraction in biomass partitioning, but no overall decrease in lignin content.]

Bohmert, K., I. Balbo, A. Steinbuchel, G. Tischendorf, and L. Willmitzer. 2002. Constitutive Expression of the β-Ketothiolase Gene in Transgenic Plants. A Major Obstacle for Obtaining Polyhydroxybutyrate-Producing Plants. *Plant Physiol*. 128(4): 1282-1290. [This report describes the successful use of plants for molecular farming of the polymer polyhydroxybutrate.]

Bolwell, G.P. 2000. Biosynthesis of Plant Cell Wall Polysaccharides. *Trends Glycosci. Glycotech*. 12(65): 143-160. [This is one report of several on the topic of elucidating cellulose synthesis in plants.]

Boominathan, K., and C.A. Reddy. 1992. Fungal degradation of lignin: biotechnological applications. *In Fungal Biotechnology*, 763-882. [This report defines the three major families of lignin-modifying enzymes produced by fungi as laccases, manganese-dependent peroxidases, and lignin peroxidases.]

Bordetsky, A., R. Hwang, A. Korin, D. Lovaas, and L. Tonachel. 2005. *SECURING AMERICA: Solving Our Oil Dependence Through Innovation*. Natural Resources Defense Council. [This report asserts that dependence of the United States on foreign petroleum both undermines its economic strength and threatens its national security and that the use of ethanol fuel, obtained either from grain or from cellulosic materials, can help decrease the need for petroleum fuel.]

Bothast, R.J., and M.A. Schlicher. 2005. Biotechnological processes for conversion of corn into ethanol. *Appl. Microbiol. Biotech.* 67(1): 19-25. [This report describes the cell wall as a dynamic structure that varies dramatically with plant species, age, tissue, cell type and within cell wall layers.]

Boudet, A.M., S. Kajita, J. Grima-Pettenati, and D. Goffner. 2003. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. *Trends Plant Sci.* 8(12): 576-581. [This report describes an experiment studying alteration in lignin composition in *Populus* spp; cinnamoyl CoA reductase (CCR) was down-regulated and *Clostridium cellulolyticum* was better able to digest the polysaccharides; as a result, twice as much fermentable sugar was released.]

Boudet, Alain-M. 2000. Lignins and lignification: Selected issues. *Plant Physiol. Biochem.* 38(1-2): 81-96. [This describes industries' interests in modifying plant lignin: increasing digestibility, decreasing

bleaching necessity and reducing chemical usage.]

Bruins, M.E., A.E. M. Janssen, and R.M. Boom. 2001. Thermozymes and their applications-A review of recent literature and patents. *Appl. Biochem. Biotech.* 90:155-186. [This report describes thermostable enzymes from thermophilic microbes and explains that they are usually stable at high temperature and pH.]

Carpita, N.C., and D.M. Gibeaut. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant J*. 3(1): 1-30. [This report contains a description of pectins.]

Carpita, N. and M. McCann. 2002. Chapter 2. The cell wall. In *Biochemistry & Molecular Biology of Plants*, ed. B. Buchanan, W. Gruissem, and R.L. Jones, 52-108. John Wiley & Sons. [This chapter provides a very comprehensive, detailed analysis of the plant cell wall, including structure, biochemistry and function.]

Chabannes, M., A. Barakate, C. Lapierre, J.M. Marita, J. Ralph, M. Pean, S. Danoun, C. Halpin, J. Grima-Pettenati and A.M. Boudet. 2001. Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *The Plant J.* 28(3): 257-270. [This report describes an experiment in which CCR was reduced in tobacco and resulted in a decrease in lignin and an increase in xylose and glucose associated with the wall.]

Chiang, C.M., F.S. Yeh, L.F. Huang, T.H. Tseng, M.C. Chung, C.S. Wang, H.S. Lur, J.F. Shaw, and S.M. Yu. 2005. Expression of a bi-functional and thermostable amylopullulanase in transgenic rice seeds leads to autohydrolysis and altered composition of starch. *Mol. Breeding* 15:125-143. [This report describes the successful use of plants for molecular farming of an enzyme (amylopullulanase).]

Conrad, U. and U. Fiedler. 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. *Plant Mol. Biol.* 38(1): 101-109. [This review describes antibody production in plants and its optimization via subcellular localization, with a focus on the advantages of the ER.]

D'Souza, T.M., C.S. Merritt, and C.A. Reddy. 1999. Lignin-Modifying Enzymes of the White Rot Basidiomycete *Ganoderma lucidum*. *Appl. Environ. Microbiol*. 65(12): 5307-5313. [This report describes the lignin-modifying enzymes of white rot fungi, thought to be the most effective lignin-degrading microbes in nature.]

Dai, Z., B.S. Hooker, R.D. Quesenberry, and J. Gao. 1999. Expression of *Trichoderma reesei* exo-cellobiohydrolase I in transgenic tobacco leaves and calli. *Appl. Biochem. Biotech*. 77-79:689-99. [This reports the expression of CBHI in transgenic tobacco.]

Dai, Z., B.S. Hooker, D.B. Anderson, and S.R. Thomas. 2000a. Expression of *Acidothermus cellulolyticus* endoglucanase E1 in transgenic tobacco: biochemical characteristics and physiological effects. *Trans. Res.* 9(1): 43-54. [This report describes the expression of the full-length E1 peptide in tobacco.]

———. 2000b. Improved plant-based production of E1 endoglucanase using potato: expression optimization and tissue targeting. *Mol. Breeding* 6:277-285. [This report describes the expression of the full-length E1 peptide in potato.]

Dai, Z., B.S. Hooker, R.D. Quesenberry, and S.R. Thomas. 2005. Optimization of *Acidothermus cellulolyticus* Endoglucanase (E1) Production in Transgenic Tobacco Plants by Transcriptional, Post-transcription and Post-translational Modification. *Trans. Res*. 14(5): 627-643. [This report describes the expression of the full-length E1 peptide in tobacco.]

Dean, J.F.D. 2004. Synthesis of Lignin in Transgenic and Mutant Plants. In *Biotechnology of Biopolymers: From Synthesis to Patents*, ed. A. Steinbuchel and Y. Doi, 4-21. Wiley-VCH. [This describes industries' interests in modifying plant lignin: increasing digestibility, decreasing bleaching necessity and reducing chemical usage.]

Demain, A.L., M. Newcomb, and J.H.D. Wu. 2005. Cellulase, Clostridia, and Ethanol. *Microbiol. Mol. Biol. Rev.* 69(1): 124-154. [This review describes the production of cellulosomes by microorganisms such as *Clostridia* spp.]

Ding, S.Y., and M.E. Himmel. 2006. The Maize Primary Cell Wall Microfibril: A New Model Derived

from Direct Visualization. *J. Agric. Food Chem.* 54(3): 597-606. [This report describes the cell wall as a dynamic structure that varies dramatically with plant species, age, tissue, cell type and within cell wall layers.]

Dodd, A.N., N. Salathia, A. Hall, E. Kevei, R. Toth, F. Nagy, J.M. Hibberd, A.J. Millar, and A.A.R. Webb. 2005. Plant Circadian Clocks Increase Photosynthesis, Growth, Survival, and Competitive Advantage. *Science* 309(5734): 630-633. [This describes synchronization of circadian clock and external light-dark cycles in plants, which could be potentially useful for manipulation to increase biomass production.]

Eggeman, T., and R.T. Elander. 2005. Process and economic analysis of pretreatment technologies. *Biores. Tech.* 96(18): 2019-2025. [This describes several pretreatment processes used in cellulosic ethanol production.]

Eriksson, M.E., M. Israelsson, O. Olsson, and T. Moritz. 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nat. Biotech.* 18(7): 784-788. [This reports that hybrid poplar displayed improved growth and biomass when gibberellin biosynthesis was increased.]

Fischer, R., E. Stoger, S. Schillberg, P. Christou, and R.M. Twyman. 2004. Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* 7(2): 152-158. [This describes the ER as oxidizing environment with a profusion of molecular chaperones and few proteases.]

Genencor Celebrates Major Progress in the Conversion of Biomass to Ethanol - Genencor a Danisco division. http://www.genencor.com/cms/connect/genencor/media_relations/news/archive/2004/gen_211004_en.htm (accessed August 3, 2007). [This website describe the latest cost-reduction model designed by the National Renewable Energy Laboratory (NREL) and Genencor, to produce cellulases at around $0.10-$0.20 per gallon of ethanol.]

Greene, N., F.E. Celik, B. Dale, M. Jackson, K. Jayawardhana, H. Jin, E.D. Larson, M. Laser, L. Lynd, D. MacKenzie, J. Mark, J. McBride, S. McLaughlin and D. Saccardi. 2004. *Growing Energy: How Biofuels Can Help End America's Oil Dependence.* [This report estimates the worldwide availability of lignocellulosic feedstocks and describes their benefits.]

Haigler, C.H. 2006. Establishing the cellular and biophysical context of cellulose synthesis. In *The Science and Lore of the Plant Cell Wall: Biosynthesis, Structure and Function.* ed. T. Hayashi, 368. Brown Walker Press (FL). [This is one report of several on the topic of elucidating cellulose synthesis in plants.]

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiol. Rev.* 13:125-135. [This defines the three major families of lignin-modifying enzymes produced by fungi as laccases, manganese-dependent peroxidases, and lignin peroxidases.]

Herbers, K., I. Wilke, and U. Sonnewald. 1995. A Thermostable Xylanase from *Clostridium thermocellum* Expressed at High Levels in the Apoplast of Transgenic Tobacco Has No Detrimental Effects and Is Easily Purified. *Nat. Biotech.* 13(1): 63-66. [This reports the expression of a thermostable xylanase from *Clostirdium thermocellum* in the apoplast of tobacco.]

Hong, C.Y., K.J. Cheng, T.H. Tseng, C.S. Wang, L.F. Liu, and S.M. Yu. 2004. Production of two Highly Active Bacterial Phytases with Broad pH Optima in Germinated Transgenic Rice Seeds. *Trans. Res*. 13(1): 29-39. [This report describes the successful use of plants for molecular farming of enzymes (phytases).]

Horn, M.E., S.L. Woodard, and J.A. Howard. 2004. Plant molecular farming: systems and products. *Plant Cell Rep*. 22(10): 711-720. [This describes advantages of plant molecular farming.]

Houghton, J., J. Ferrel, and S. Weatherwax. 2005. From Biomass to Biofuels: A Roadmap to the Energy Future. *Proceedings of the Biomass to Biofuels Workshop*, December 7. [This describes the current ethanol production capacity.]

Howard, J.A., and E. Hood. 2005. Bioindustrial and Biopharmaceutical Products Produced in Plants. *Adv. Agron*. 85: 91-124, Elsevier Inc. [This report describes the successful use of plants for molecular farming for pharmaceuticals.]

Howard, R.L., E. Abotsi, E.L. Jansen van Rensburg, and S. Howard. 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. *Afr. J. Biotech.* 2(12): 602-619. [This describes the expense of hydrolysis enzyme production in fermentation tanks.]

Hu, W.J., S.A. Harding, J. Lung, J.L. Popko, J. Ralph, D.D. Stokke, C.J. Tsai, and V.L. Chiang. 1999. Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nat. Biotech.* 17(8): 808-812. [In this report, down-regulation of 4-courmarate CoA ligase (4CL) in *P. tremuloides* resulted in a 45% decrease in lignin and a corresponding 15% increase in cellulose.]

Hyunjong, B., D.S. Lee, and I. Hwang. 2006. Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells. *J. Exp. Bot.* 57(1): 161-169. [This report describes the success of the approach of targeting the same enzyme (xylanase) to different compartments in the same plant (choloplasts and peroxisomes) to maximize production.]

Ingledew, W.M. 1995. The Biochemistry of Ethanol Production. In *The Alcohol Textbook*, ed. T.P. Lyons, D. Kelsall, and J. Murtagh, 55-79. Nottingham, UK: Nottingham University Press. [This explains that substantial efforts have been made toward improving ethanol yield and reducing its production costs from plant biomass.]

Kabel, M.A., M.J.E.C. van der Maarel, G. Klip, A.G.J. Voragen, and H.A. Schols. 2006. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. *Biotech. Bioeng*. 93(1): 56-63. [This describes the prohibitive costs of production of microbial cellulase enzymes used in the conversion of cellulosic matter to fermentable sugars.]

Kawagoe, Y., and D.P. Delmer. 1997. Pathways and genes involved in cellulose biosynthesis. *Gen. Eng*. 19:63-87. This is one report of several on the topic of elucidating cellulose synthesis in plants.]

Kim, S., and B.E. Dale. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass Bioen.* 26(4): 361-375. [This report estimates the worldwide availability and potential ethanol output from lignocellulosic biomass.]

Kimura T., T. Mizutani, T. Tanaka, T. Koyama, K. Sakka, and K. Ohmiya. 2003. Molecular breeding of transgenic rice expressing a xylanase domain of the *xynA* gene from *Clostridium thermocellum*. *Appl. Microbiol. Biotechnol.* 62(4): 374-379. [This describes the expression of the catalytic domain of the *xynA* gene from *Clostridium thermocellum* in rice.]

Kimura, T., T. Mizutani, K. Sakka, and K. Ohmiya. 2003. Stable expression of a thermostable xylanase of *Clostridium thermocellum* in cultured tobacco cells. *J. Biosci. Bioeng*. 95(4): 397-400. [This describes the expression of the catalytic domain of the *xynA* gene from *Clostridium thermocellum* in tobacco.]

Kiran, N.S, L. Polanská, R. Fohlerová, P. Mazura, M. Válková, M. Smeral, J. Zouhar, J. Malbeck, P.I. Dobrev, I. Macháckova, and B. Brzobohaty. 2006. Ectopic over-expression of the maize β-glucosidase Zm-p60.1 perturbs cytokinin homeostasis in transgenic tobacco. *J. Exp. Bot.* 57(4): 985-96. [This report describes the expression of maize β-glucosidase in tobacco to study cytokinins.]

Kirk, T.K., and R.L. Farrell. 1987. Enzymatic "combustion": the microbial degradation of lignin. *Annu. Rev. Microbiol.* 41:465-505. [This report defines the three major families of lignin-modifying enzymes produced by fungi as laccases, manganese-dependent peroxidases, and lignin peroxidases.]

Knauf, M., and M. Moniruzzaman. 2004. Lignocellulosic biomass processing: A perspective. *Int. Sugar J.* 106:147-150. [This describes the high costs of hydrolysis enzyme production; the advances that have been made in their production, efficiency and cost; and recommends biomass crops for conversion to fermentable sugars.]

Li, L., Y. Zhou, X. Cheng, J. Sun, J.M. Marita, J. Ralph, and V.L. Chiang. 2003. Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proc. Nat. Acad. Sci. USA.* 100(8): 4939-4944. [In this report, down-regulation of 4-courmarate CoA ligase (4CL) in *P. tremuloides* resulted in a 45% decrease in lignin and a corresponding 15% increase in cellulose, which was enhanced even further (52% less lignin and 30% more cellulose) when coniferaldehyde 5-hydroxylase (CAld5H) was also present.]

Liu, H.L., W.S. Li, T. Lei, J. Zheng, Z. Zhang, X.F. Yan, Z.Z. Wang, Y.L. Wang and L.S. Si. 2005. Expression of Human Papillomavirus type 16 L1 protein in transgenic tobacco plants. *Act. Biochim. Biophys. Sin.* 37:153-158. [This report describes the successful use of plants for molecular farming of the

protein Human Papillomavirus type 16L1.]

Luo, Y., J.L. Chen, J.F. Reynolds, C.B. Field, and H.A. Mooney. 1997. Disproportional increases in photosynthesis and plant biomass in a Californian grassland exposed to elevated CO_2: a simulation analysis. *Funct. Ecol.* 11(6): 696-704. [This describes increased photosynthesis and plant biomass from elevated CO_2, which could be potentially useful for manipulation to increase biomass production.]

Lynd, L.R., W.H. van Zyl, J.E. McBride, and M. Laser. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Curr. Opin. Biotech.* 16: 577-583. [This describes efforts to improve ethanol yield and reduce its production costs through designer microbes that secrete all necessary hydrolysis enzymes and able to use the resulting sugars as a feedstock for fermentation.]

Michaels, S., and R. Amasino. 1999. *FLOWERING LOCUS C* Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering. *The Plant Cell* 11: 949-956. [This describes the *FLC* floral repressor gene of Arabidopsis.]

Mosier, N., C. Wyman, B. Dale, R. Elander, Y.Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Biores. Tech.* 96(6): 673-686. [This describes the function of lignin in the plant and describes pretreatments for its removal before processing and their costs.]

Murray C., P.W. Sutherland, M.M. Phung, M.T. Lester, R.K. Marshall and T. Christeller. 2002. Expression of Biotin-Binding Proteins, Avidin and Streptavidin, in Plant Tissues Using Plant Vacuolar Targeting Sequences. *Trans. Res.* 11(2): 199-214. [This report describes research on producing avidin in tobacco, noting that production in the cytosol is toxic to the plant, so vacuolar targeting was employed.]

Nuttall, J., N. Vine, J.L. Hadlington, P. Drake, L. Frigerio, and J.K.C. Ma. 2002. ER-resident chaperone interactions with recombinant antibodies in transgenic plants. *Eur. J. Biochem.* 269(24): 6042-6051. [This report shows that the molecular chaperone BiP interacts specifically with antibodies in transgenic plants that are targeted to the secretory pathway.]

O'Neill, M.A., T. Ishii, P. Albersheim, and A.G. Darvill. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol.* 55:109-39. [This report describes the functions of pectins in the cell wall.]

Oraby, H., B. Venkatesh, B. Dale, R. Ahmad, C. Ransom, J. Oehmke, and M. Sticklen. 2007. Enhanced conversion of plant biomass into glucose using transgenic rice-produced endoglucanase for cellulosic ethanol. *Trans. Res.* Published Online First: 20 January 2007. [This reports the successful production of the catalytic domain of the thermostable endo-1,4-β glucanase (E1) of *A. cellulolyticus* in rice.]

Pan, X., N. Gilkes, J. Kalda, K. Pye, S. Saka, D. Gregg, K. Ehara, D. Xie, D. Lam and J. Saddler. 2005. Biorefining of softwoods using ethanol organosolv pulping: Preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products. *Biotech. Bioeng.* 90(4): 473-481. [This report describes the organosolv pulping pretreatment process.]

Patel M., J.S. Johnson, R.I.S. Brettell, J. Jacobsen, and G.P. Xue. 2000. Transgenic barley expressing a fungal xylanase gene in the endosperm of the developing grains. *Mol. Breed.* 6(1): 113-124. [In this report, a modified xylanase gene (*xynA*) from the rumen fungus, *Neocallimastix patriciarum*, was successfully expressed in barley endosperm, retaining activity after desiccation and storage.]

Perlack, R.D., L.L. Wright, A.F. Turhollow, R.L. Graham, B.J. Stokes, and D.C. Erbach. 2005. *Biomass as a Feedstock for a Bioenergy and Bioproducts Industry: The Technical Feasibility of a Billion-Ton Annual Supply*. U.S. Dept. of Energy and U.S. Dept. of Agriculture. [This estimates the crop and forest residues and energy crops that could become available annually in the US for conversion to fermentable sugars and alcohol fuels.]

Persson, S., H. Wei, J. Milne, G.P. Page, and C.R. Somerville. 2005. Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *Proc. Nat. Acad. Sci. USA.* 102(24): 8633-8638. [This is one report of several on the topic of elucidating cellulose synthesis in plants.]

Pilate, G., E. Guiney, K. Holt, M. Petit-Conil, C. Lapierre, J.C. Leple, B. Pollet, I. Mila, E.A. Webster, H.G. Marsttrop, D.W. Hopkins, L. Jouanin, W. Boerjan, W. Schuch, D. Cornu, and C. Halpin. 2002. Field and pulping performances of transgenic trees with altered lignification. *Nat. Biotech.* 20(6): 607-612.

[This report describes the down-regulation of CAD, which resulted in improved lignin solubility in alkaline medium and allowed easier delignification; this could decrease costs associated with pretreatment because fewer chemicals are needed.]

Qi, B., T. Fraser, S. Mugford, G. Dobson, O. Sayanova, J. Butler, J.A. Napier, A.K. Stobart, and C.M. Lazarus. 2004. Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nat. Biotech.* 22(6): 739-745. [This report describes the successful use of plants for molecular farming of lipids.]

Ragauskas, A.J., C.K. Williams, B.H. Davison, G. Britovsek, J. Cairney, C.A. Eckert, W.J. Frederick, J.P. Hallett, D.J. Leak, C.L. Liotta, J.R. Mielenz, R. Murphy, R. Templer, and T. Tschaplinski. 2006. The Path Forward for Biofuels and Biomaterials. *Science* 311(5760): 484-489. [This reviews many aspects of lignocellulosic biofuel production: hydrolysis enzymes and their production in plants, lignin modification in plants and pretreatment processes.]

Ralph, J. 2006. What makes a good monoligol substitute? In *The Science And Lore of the Plant Cell Wall: Biosynthesis, Structure And Function*, ed. T. Hayashi, 367. Brown Walker Press (FL). [This describes industries' interests in modifying plant lignin: increasing digestibility, decreasing bleaching necessity and reducing chemical usage.]

Ralph, J., T. Akiyama, H. Kim, F. Lu, P.F. Schatz, J.M. Marita, S.A. Ralph, M.S.S. Reddy, F. Chen, and R.A. Dixon. 2006. Effects of Coumarate 3-Hydroxylase Down-regulation on Lignin Structure. *J. Biol. Chem.* 281(13): 8843-8853. [This reports a dramatic shift in the lignin profile and consequent altered lignin structure that were speculated to explain an earlier study (Reddy et al., 2005, below) reporting improved digestibility of C3H-deficient alfalfa lines in ruminants.]

Ransom, C.B. 2007. *Production and Analysis of Biologically-Active Cellulases for Ethanol Fuel in Maize Biomass.* Michigan State University. [This dissertation contains the first report of a heterologous microbial β-glucosidase expressed in a plant for the purpose of obtaining biologically-active enzyme for use in the production of ethanol fuel.]

Ransom, C.B., V. Balan, G.C.G. Biswas, B.E. Dale, E. Crockett, and M.B. Sticklen. 2007. Heterologous *Acidothermus cellulolyticus* 1,4-β-Endoglucanase E1 Produced Within the Corn Biomass Converts Corn Stover Into Glucose. *Appl. Biochem. Biotech.* 36-140:207-220. [This describes the successful production of the catalytic domain of the thermostable endo-1,4-β glucanase (E1) of A. cellulolyticus in maize and its ability to convert biomass to glucose.]

Reddy, M.S.S., F. Chen, G. Shadle, L. Jackson, H. Aljoe, and R.A. Dixon. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). *Proc. Nat. Acad. Sci. USA.* 102(46): 16573-16578. [The results of this study were speculated to be explained by the report by Ralph et al., 2006, above.]

Reggi S., S. Marchetti, T. Patti, F. De Amicis, R. Cariati, B. Bembi, and C. Fogher. 2005. Recombinant human acid β-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. *Plant Mol. Biol.* 57(1): 101-113. [In this report, human acid β-glucosidase was successfully expressed in transgenic tobacco seeds for medical purposes.]

Renewable Fuels Association. 2007. *Building New Horizons: Ethanol Industry Outlook 2007*. [This describes ethanol production capacity in the US for 2003 and 2004.]

RFA - The Industry - Plant Locations. http://www.ethanolrfa.org/industry/locations/ (accessed August 5, 2007). [This website describes the biorefineries in operation and under construction and their production.]

Richards, R.A. 2000. Selectable traits to increase crop photosynthesis and yield of grain crops. *J. Exp. Bot.* 51(1): 447-458. [This describes research to increase photosynthesis, which could be used to increase biomass for ethanol production.]

Ridley, B.L., M.A. O'Neill, and D. Mohnen. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochem.* 57(6): 929-967. [This describes the functions of pectins.]

Sahrawy, M., C. Avila, A. Chueca, F.M. Canovas, and J. Lopez-Gorge. 2004. Increased sucrose level and altered nitrogen metabolism in *Arabidopsis thaliana* transgenic plants expressing antisense chloroplastic fructose-1,6-bisphosphatase. *J. Exp. Bot.* 55(408): 2495-2503. [This report describes the successful use of

plants for molecular farming of carbohydrates.]

Salehi, H., C.B. Ransom, H.F. Oraby, Z. Seddighi, and M.B. Sticklen. 2005. Delay in flowering and increase in biomass of transgenic tobacco expressing the *Arabidopsis* floral repressor gene *FLOWERING LOCUS C. J. Plant Phys.* 162, no. 6: 711-717. [This reports the successful expression of *FLC* in tobacco, which resulted in later flowering and increased biomass.]

Saruul, P., F. Srienc, D.A. Somers, and D.A. Samac. 2002. Production of a Biodegradable Plastic Polymer, Poly-β-Hydroxybutyrate, in Transgenic Alfalfa. *Crop. Sci.* 42(3): 919-927. [This report describes the successful use of plants for molecular farming of the polymer polyhydroxybutrate.]

Schillberg, Fischer, and Emans. 2003. Molecular farming of recombinant antibodies in plants. *Cell. Mol. Life Sci*. 60(3): 433-445. [This report indicates that proteins are less stable when they are secreted rather than retained in the lumen of the ER; therefore higher accumulation is possible, generally 2-10 fold greater.]

Schillberg S., S. Zimmermann, A. Voss, and R. Fischer. 1999. Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotiana tabacum. Trans. Res.* 8(4): 255-263. [In this report, targeting of antibiodies in tobacco to either the secretory pathway or the cytosol was compared.]

Schulman, A.H. 2002. Transgenic plants as producers of modified starch and other carbohydrates. In *Plant Biotechnology and Transgenic Plants*, ed. K.M. Oksman-Caldenetey and W.H. Barz, 255-282. New York: Basel. [This report describes the successful use of plants for molecular farming of carbohydrates.]

Shapouri, H., J.A. Duffield, and M. Wang. 2002. *The Energy Balance of Corn Ethanol: An Update*. U. S. Dept. of Agriculture. [This report describes the energy balance of ethanol produced from maize grain.]

Sheldon, C.C., J.E. Burn, P.P. Perez, J. Metzger, J.A. Edwards, W.J. Peacock, and E.S. Dennis. The *FLF* MADS Box Gene: A Repressor of Flowering in *Arabidopsis* Regulated by Vernalization and Methylation. *Plant Cell* 11(3): 445-458. [This describes the floral repressor *FLC* gene of *Arabidopsis*.]

Singh, S.P., E. Ekanem, T. Wakefield Jr., and S. Comer. 2003. Emerging Importance of Bio-Based Products and Bio-Energy in the U.S. Economy: Information Dissemination and Training of Students. *Int. Food Agribus. Mgmt. Rev.* 5(3): 1-15. [This describes the goal of meeting chemical feedstock demand with plant-derived materials.]

Smidansky E.D., J.M. Martin, C.L. Hannah, A.M. Fischer, and M.J. Giroux. 2003. Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. *Planta* 216(4): 656-664. [In this report, rice was transformed to increase the activity of ADP-glucose pyrophosphorylase (AGP), a key enzyme in starch biosynthesis, and an unexpected 20% increase in plant biomass was observed.]

Sticklen, M.B. 2004. Production of microbial hydrolysis enzymes in biomass crops via genetic engineering. In: *Proceedings of the 2nd International Ukrainian Conference on Biomass for Energy.* Sept. 20-22, 2004. Kyiv, Ukraine. Ukraine Natl. Acad. Sci. Press. pp. 133-136. [This contains recommendations for biomass crops and development of technology that would allow production of cellulases and other hydrolysis enzymes in plants.]

Sticklen, M.B. 2006. Plant genetic engineering to improve biomass characteristics for biofuels. *Curr. Opin. Biotech.* 17(3): 315-319. [This report contains a recommendation to develop technology that would allow production of cellulases and other hydrolysis enzymes in plants.]

Sticklen, M.B. 2007a. Feedstock Crop Genetic Engineering for Alcohol Fuels. *Crop Sci*.: in review. [This report contains a recommendation to develop technology that would allow production of cellulases and other hydrolysis enzymes in plants.]

———. 2007b. Role of Transgenic Biomass Crops in Ethanol Refineries. *J. Biobased Mater. Bioenergy*: in review. [This report contains a recommendation to develop technology that would allow production of cellulases and other hydrolysis enzymes in plants.]

Teymouri, F., H. Alizadeh, L. Laureano-Perez, B.E. Dale, and M.B. Sticklen. 2004. Effects of Ammonia Fiber Explosion Treatment on Activity of Endoglucanase from *Acidothermus cellulolyticus* in Transgenic Plant. *Appl. Biochem. Biotech.* 116:1183-1192. [In this study, it was found that one of the mildest pretreatments available, Ammonia Fiber Explosion (AFEX), reduced the activity of E1 by about two-thirds.]

Thurston, C.F. 1994. The structure and function of fungal laccases. *Microbiol.* 140:19-26. [This report defines three major families of lignin-modifying enzymes produced by fungi as laccases, manganese-dependent peroxidases, and lignin peroxidases.]

Tucker, M.P., A. Mohagheghi, K. Grohmann, and M.E. Himmel. 1989. Ultra-Thermostable Cellulases From *Acidothermus cellulolyticus*: Comparison of Temperature Optima with Previously Reported Cellulases. *Bio/Technol*. 7(8): 817-820. [This report describes the thermostable endo-1,4-β glucanase (E1) of *A. cellulolyticus*.]

U.S. Prime Supplier Sales Volumes of Petroleum Products. http://tonto.eia.doe.gov/dnav/pet/pet_cons_prim_dcu_nus_a.htm (accessed August 5, 2007). [This website describes the total annual gasoline consumption in the US.]

Warren, R.A.J. 1996. Microbial Hydrolysis of Polysaccharides. *Annu. Rev. Microbiol.* 50(1): 183-212. [This contains information on organisms that can degrade cellulose, cellulosomes, enzymatic hydrolysis, hemicellulose and hemicellulases.]

World of Corn 2007. http://www.ncga.com/WorldOfCorn/main/consumption1.asp (accessed August 5, 2007). [This website describes the usage of maize grain in the US.]

World of Corn 2007. http://www.ncga.com/WorldOfCorn/main/production1.asp (accessed August 5, 2007). [This website describes the production of maize in the US.]

Wyman, C.E. 1999. Biomass ethanol: Technical progress, opportunities, and commercial challenges. *Annu. Rev. Energy Environ.* 24:189-226. [This describes various pretreatment processes and the reduction in enzyme production costs.]

Wyman, C.E., B.E. Dale, R.T. Elander, M. Holtzapple, M.R. Ladisch, and Y.Y. Lee. 2005a. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Biores. Tech*. 96(18): 2026-2032. [This describes various pretreatment processes.]

Yang P., Y. Wang, Y. Bai, K. Meng, H. Luo, T. Yuan, Y. Fan, and B. Yao. 2007. Expression of xylanase with high specific activity from *Streptomyces olivaceoviridis* A1 in transgenic potato plants (*Solanum tuberosum* L.). *Biotech. Lett.* 29(4): 659-667. [In this report, the *xynB* gene of *Streptomyces olivaceoviridis* A1 was expressed in potato; its enzyme activity was retained over several generations. The purpose in this case was to produce xylanase as an additive for animal feed.]

Yao, J.Q. 2004. *Genetic transformation of tobacco with a beta-glucosidase gene to induce constitutive systemic acquired resistance against tobacco mosaic virus*. Western Michigan University, Kalamazoo, MI. [This dissertation describes the expression of *Butyrivibrio fibrisolvens* H17c β-glucosidase in tobacco to study whether it could effect enhanced immune response through systemic acquired resistance (SAR).]

Zhong, H., F. Teymouri, B. Chapman, S.B. Maqbool, R. Sabzikar, Y. El-Maghraby, B.E. Dale, and M.B. Sticklen. 2003. The pea (*Pisum sativum* L.) rbcS transit peptide directs the *Alcaligenes eutrophus* polyhydroxybutyrate enzymes into the maize (*Zea mays* L.) chloroplasts. *Plant Sci*. 165(3): 455-462. [This report describes the successful use of plants for molecular farming of the polymer polyhydroxybutrate.]

Ziegelhoffer, T., J.A. Raasch, and S. Austin-Phillips. 2001. Dramatic effects of truncation and sub-cellular targeting on the accumulation of recombinant microbial cellulase in tobacco. *Mol. Breeding* 8(2): 147-158. [This report describes the expression of the catalytic domain and full-length peptide of the thermostable endo-1,4-β glucanase (E1) of *A. cellulolyticus* in tobacco; expression of the catalytic domain yielded more activity than the full-length enzyme.]

Ziegelhoffer T., J. Will, and S. Austin-Phillips. 1999. Expression of bacterial cellulase genes in transgenic alfalfa (*Medicago sativa* L.), potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.). *Mol. Breeding* 5(4): 309-318. [In this report, the thermostable endoglucanase E2 and cellobiohydrolase E3 of *Thermomonospora fusca* was expressed in tobacco, potato and alfalfa.]

Ziegler, M.T., S.R. Thomas, and K.J. Danna. 2000. Accumulation of a thermostable endo-1,4-β-D-glucanase in the apoplast of *Arabidopsis thaliana* leaves. *Mol. Breeding* 6(1): 37-46. [In this report, the catalytic domain of the thermostable endo-1,4-β glucanase (E1) of *A. cellulolyticus* was successfully produced in *Arabidopsis*.]

Biographical Sketches

Mariam Sticklen is a Professor in the Department of Crop and Soil Science at Michigan State University. She received her Ph.D. in Horticulture from The Ohio State University in 1981. After teaching at both Clemson Uinveristy in South Carolina and The Ohio State University, she came to Michigan State University. She has served as an advisor to the National Academy of Science and on the Board of Trustees of International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). She is a leading expert in the field of genetic engineering of cereals and turfgrasses. Recently, her research has focused on molecular farming of hydrolysis enzymes and other biobased industrial products and pharmaceuticals in maize, and genetic improvement of crop plants for use as feedstock biomass. She currently owns seven patents and has published three books and many peer-reviewed articles.

Callista Ransom is a doctoral student in the Plant Breeding and Genetics Program and the Department of Crop and Soil Science at Michigan State University. She expects to complete her degree in December, 2007. She received her B.S. in Agriculture and Natural Resources and her M.S. in Crop and Soil Sciences, both also from Michigan State University. Ms. Ransom's research focuses on production of hydrolysis enzymes in the biomass crop plant maize.