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INVITED REVIEW

Commercial cellulosic ethanol: The role of plant-expressed enzymes

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Abstract The use and production of biofuels has risen dramatically in recent yr. Bioethanol comprises 85% of total global biofuels production, with benefits including reduction of greenhouse gas emissions and promotion of energy independence and rural economic development. Ethanol is primarily made from corn grain in the USA and sugarcane juice in Brazil. However, ethanol production using current technologies will ultimately be limited by land availability, government policy, and alternative uses for these agricultural products. Biomass feedstocks are an enormous and renewable source of fermentable sugars that could potentially provide a significant proportion of transport fuels globally. A major technical challenge in making cellulosic ethanol economically viable is the need to lower the costs of the enzymes needed to convert biomass to fermentable sugars. The expression of cellulases and hemicellulases in crop plants and their integration with existing ethanol production systems are key technologies under development that will significantly improve the process economics of cellulosic ethanol production.

Keywords Biofuels · Cellulosic ethanol · Cellulases · Pretreatment · Biomass · Enzymes

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The Rise of Biofuels

The global production and use of biofuels has increased dramatically in recent yr, from 18.2 billion liters in 2000 to 60.6 billion liters in 2007, with about 85% of this being bioethanol (Coyle 2007). Bioethanol production from first-generation technologies is projected to increase to 113.6 billion liters by 2022 (Goldemberg and Guardabassi 2009). Several primary drivers underlie the increase in biofuels. One is the increasing uncertainty of petroleum supplies in the face of rising demand from emerging economies and the decline in known reserves. These reserves are primarily located in regions with governments that are unstable or unfriendly to Western democracies, making the long-term petroleum supply subject to political developments. A second factor is that uncertainty in petroleum supplies has led to government programs promoting biofuels and accomplishing two main policy goals: energy independence and support for rural economies. Significantly, the USA, Brazil, the European Union, and China together account for about 90% of global biofuels production, a direct result of government support in these countries (Coyle 2007). Thirdly, concerns over global warming and greenhouse gas emissions associated with fossil fuel usage have contributed to increasing interest in biofuels that reduce carbon emissions or are at least carbon-neutral. The use of bioethanol is estimated to reduce greenhouse gas emissions by approximately 30% to 85% compared to gasoline, depending on whether corn or sugarcane feedstock is used (Fulton et al. 2004). Finally, biofuels are unique among available alternative energy sources in their general compatibility with our existing liquid transport fuel infrastructure despite the potential for corrosion of existing pipelines by high concentrations of ethanol.

The USA and Brazil are currently the primary producers of fuel ethanol, producing 49.6% and 38.3% of 2007 global production, respectively (http://www.ethanolrfa.org/industry/statistics/#E, accessed 7 Jan 2008). US bioethanol production is almost entirely from maize (corn) starch, which is converted to fermentable glucose by the addition of amylase and glucoamylase enzymes. In 2007, 24.6 billion liters of ethanol was produced in the USA, yet this comprised only 3.2% of gasoline consumption on an energy-equivalent basis (Tyner 2008). Increases in agricultural commodity prices in 2007 and early 2008 were blamed by some on the increasing use of grain for biofuels. However, economic factors such as increasing demand in emerging economies and supply restrictions caused by weather, low carryover stocks, and yr of low investment in agricultural R&D have been identified as the major contributors to increases in grain prices (Gressel 2008; Anonymous 2008). Further evidence for biofuel production's minor role in driving commodity prices is that despite continued expansion in corn ethanol production, commodity prices decreased in late 2008. Nonetheless, it is estimated that restrictions on available acreage and price pressures will limit the contribution of grain-based ethanol to the US liquid transport fuel mix to less than 8% of gasoline consumption on an energy-equivalent basis (Tyner 2008).

Sugarcane juice is the preferred feedstock in Brazil, accounting for about 80% of production, with the remainder being sugarcane molasses (Sanchez and Cardona 2008). Sugarcane bioethanol production is expected to increase in Brazil with the construction of new mills and associated plantings, from 19 billion liters in 2007 to 36 billion liters in 2013, and ethanol is expected to continue providing approximately 50% of Brazil's transport fuel needs (Goldemberg and Guardabassi 2009). It is predicted that sugarcane ethanol production in Brazil will ultimately reach 79.5 billion liters in 2022, and increases of 50–100% in global sugarcane tonnage are expected in the coming decade (Kline et al. 2008). However, sugarcane ethanol production using current technologies will eventually be limited by the same agro-economic factors that restrict grain-based ethanol production: the lack of suitable land and competing demand by alternative uses, in this case sugar production.

New technologies are required if biofuels are to significantly contribute to planetary energy needs and the reduction of greenhouse gas emissions. Despite meeting about one third of the increase in global oil demand in recent yr (Lavelle and Garber 2008), in 2006, biofuels represented merely 0.8% of total global energy usage (Martinot et al. 2007), or only about 2% of our transport fuel (Koonin 2006). Fermentative production of ethanol and other alcohols from lignocellulosic materials represent the most attractive option for continued expansion of biofuel production. Some of the benefits include a high efficiency of carbohydrate recovery compared to other technologies, the possibilities for technological improvement afforded by biotechnology, and lower capital costs (Wyman et al. 2005a; Carroll and Somerville 2009).

This review addresses the role of plant-expressed enzymes and other developing technologies in enabling the commercial viability of cellulosic ethanol. The emphasis is on approaches and technologies with significant promise in reducing production costs to make cellulosic ethanol competitive with first-generation ethanol production and in identifying areas for further research to address the interrelated technical challenges of converting biomass to fermentable sugars.

Cellulosic Ethanol: Economic Aspects

The major economic barrier to viable commercial cellulosic ethanol production are high production costs, estimated to be between US$102 and 123 per barrel (Tyner 2008), or more than US$2.50 per gallon (US$0.66 per liter; Coyle 2007). Feedstock, enzymes, and processing costs, together with capital expenses associated with new plants, all combine to make cellulosic ethanol production using current technologies expensive in comparison to first-generation bioethanol. In addition, since there are no commercial-scale cellulosic ethanol production plants currently in operation, techno-economic process models rely on estimates, laboratory experiments, or, at best, pilot-scale plants (Galbe et al. 2007), increasing the investment risk profile.

Thus, it is not surprising that the first commercial cellulosic ethanol plants currently under construction take advantage of pre-collected feedstocks, proximity to existing infrastructure, and government funding. One such plant now under construction is termed Project Liberty (http://www.slideshare.net/rhapsodyingreen/project-liberty/, accessed 4 Jan 2009). It is being built by Poet, a major US corn ethanol producer, at a cost of US$200 million at the site of an existing corn ethanol plant in Emmetsburg, Iowa. The plant intends to use corn cobs and fiber for cellulosic ethanol production, with process economics improved by fractionation technologies to produce higher value co-products in addition to 94.6 million liters of cellulosic ethanol annually. The project benefits from a US$80 million grant from the US Department of Energy under the Energy Independence and Security Act of 2007, which calls for the production of 379 million liters per yr of cellulosic ethanol by 2010 (Greer 2008). More recently, Verenium announced that they intend to build the first commercial cellulosic ethanol plant in Florida

(see http://phx.corporate-ir.net/phoenix.zhtml?c = 81345&p = Rss Landing&cat = news&id = 1244987, accessed 27 Jan 2009).

The US government alone provided over US$1 billion in funding for cellulosic ethanol projects in 2007 (Waltz 2008). In addition to government funds in the USA and elsewhere, large oil and automobile companies are supporting cellulosic ethanol research by commercial and academic technology providers. Royal Dutch Shell has a 50% stake in Iogen (http://www.iogen.ca/), a company that has been running a large pilot-scale cellulosic ethanol plant in Ottawa, Canada and is planning a commercial cellulosic ethanol facility. British Petroleum has a joint venture with Verenium (http://www.verenium.com/), gaining access to their cellulosic ethanol technology, and is also providing US$500 million for biofuel research at the Energy Biosciences Institute (http://www.energybiosciencesinstitute.org/), an academic consortium including the University of California at Berkeley, the Lawrence Berkeley National Lab, and the University of Illinois. General Motors is also interested and has invested in two cellulosic ethanol technology companies, Mascoma (http://www.mascoma.com/) and Coskata (http://www.coskata.com/).

Other countries are also investing in cellulosic ethanol. China is the third largest ethanol producer globally, with 2.2 billion liters of production in 2007 (http://www.ethanolrfa.org/industry/statistics/#E, accessed 7 Jan 2008). China Alcohol Resources Corporation, the second largest ethanol producer in China, has a demonstration cellulosic ethanol plant under continuous operation, producing 6.4 million liters annually based on SunOpta (http://www.sunopta.com/bioprocess/index.aspx) technology. The Chinese government has committed to spending US$500 million on cellulosic ethanol research (Waltz 2008).

Cellulosic Ethanol: Challenges

The reason for increasing investment is that despite significant challenges, cellulosic ethanol has the potential to sustainably supply a significant proportion of our transport fuel needs. Importantly, cellulosic ethanol can significantly reduce greenhouse gas emissions compared to fossil fuels (Wang et al. 2007). Worldwide, biofuel production potential from agricultural crop residues alone are estimated at 30% of global gasoline consumption (Kim and Dale 2004; Koonin 2006). Research activity in cellulosic ethanol technology has accelerated as a result of increasing interest and funding. Below, the progress and challenges in key research areas are summarized (Table 1), with a focus on pretreatment/enzymatic hydrolysis as the most promising approach in the near term (Wyman et al. 2005a).

Feedstocks. Feedstock costs represent a major portion of first-generation ethanol production, ranging from 37% for sugarcane in Brazil to 40–50% for corn grain ethanol in the USA (Coyle 2007). The costs of feedstock for cellulosic ethanol production run from US$30 to US$90 per metric ton (Galbe et al. 2007), or about a third of production costs (Wyman 2007). A large proportion of feedstock costs (up to US$25 per ton) are attributable to the harvesting and transportation of bulky biomass feedstock (Rath 2007).

Table 1. Major research areas, progress and challenges in cellulosic ethanol development

Area	Description	Progress	Challenges
Feedstocks	Use and modification of biomass sources: agricultural, forestry or municipal wastes, or dedicated energy crops	Initial analyses of feedstock yields and collection costs; compositional analyses; research into cell wall biosynthesis and chemistry	Reducing collection/feedstock costs; determination of desired feedstock characteristics; genetic modification of feedstocks to maximize value
Pretreatment	Mechanical and chemical treatments to facilitate conversion of lignocellulosic biomass to fermentable sugars	Evaluation of effectiveness of different pretreatment processes on variety of feedstocks; characterization of inhibitors of downstream processes	Reducing capital expenses and input costs; reducing energy inputs; recycling/usage of waste streams; process integration
Enzymatic hydrolysis	Enzymatic conversion of cellulose and hemicellulose polymers to fermentable sugars	Reduction in cost of cellulase enzymes; understanding of *T. reesei* and *A. niger* cellulases	High enzyme costs; poor activity/long incubation times; optimized enzyme mixtures for specific feedstocks/processes
Fermentation	Conversion of fermentable sugars to ethanol or other fuels and bio-products	Characterization of C5/C6 sugar fermenting organisms; analysis of tolerance to inhibitors in fermentation	Organisms with rapid growth, improved tolerance to inhibitors and fermentation of multiple sugars under industrial conditions
Process engineering	Engineering designs to enable economic biomass processing at commercial scale	Process models constructed, tested and revised	Optimized process integration; incorporating best (sometimes proprietary) data into models

Process economics thus favor pre-collected feedstock such as sugarcane bagasse over agricultural residues (corn stover and grain straws) and dedicated energy crops where delivered costs to a centralized processing facility need to be considered.

The major components of plant biomass (lignocellulose) are cellulose, hemicellulose, and lignin; in dicots, pectins are also important. Cellulose is a linear homopolymer of β1,4-linked cellobiose (glucose dimer) subunits. The primary chains are organized into hydrogen-bonded layers, then into compact 4- to 5-nm elementary fibrils of about 100 chains, and finally into 7- to 30-nm micrcofibrils consisting of cellulose elementary fibrils within a hemicellulose matrix and coated with lignin (Zhang and Lynd 2004). Cellulose exists primarily in crystalline form in the plant cell wall, interspersed with more disorganized (amorphous) regions, and is insoluble in water and commonly used solvents.

Plant hemicelluloses are heterogeneous branched polymers of pentose (C5) and hexose (C6) sugars whose composition varies according to species. For example, glucuronoarabinoxylans (GAXs) are the primary hemicelluloses in grasses and are composed of C5 sugars, including a β1,4-linked xylose backbone, together with arabinose and glucuronic acid. Another major hemicellulose component in grasses are the mixed linkage or β-glucans, polymers of glucose with both β1,3 and β1,4 linkages (Vogel 2008). While GAXs fill in the space between cellulose microfibrils and provide structural rigidity, the β-glucans tightly coat the microfibril (Carpita et al. 2003).

Lignin is a complex phenylpropanoid heteropolymer, a network of coumaryl, coniferyl, and sinapyl alcohols that acts as a glue to link and strengthen the polysaccharide components (Jorgensen et al. 2007). Lignin from grasses differs from that in dicots in containing significant quantities of ρ-hydroxyphenyl acid monomers in the structure and of ferulic acid attached to GAX that may serve as nucleation sites for lignin polymerization (Vogel 2008). Unlike cellulose and hemicellulose, the complex structure and diversity of chemical bonds in lignin make enzymatic deconstruction difficult (Weng et al. 2008).

Sugarcane bagasse and corn stover are attractive feedstocks due to their availability in proximity to existing bioethanol production plants. Sugarcane bagasse in particular is already present in substantial quantities at existing sugar and ethanol mills and has a lower ash content (5.0%) compared to other agricultural residues such as corn stover (11.6%; US Department of Energy 2009). The average composition of sugarcane bagasse is approximately 39% cellulose, 23% hemicellulose (with 89% of this being xylan), and 24% lignin. By comparison, corn stover has a lower average lignin content (19%) than bagasse. Corn stover is 35% cellulose and has about the same amount of hemicellulose as sugarcane bagasse. Corn stover and grain straw feedstocks for cellulosic ethanol production will incur collection and transport costs and thus have a higher cost basis than sugarcane bagasse.

The ideal feedstock composition for production of cellulosic biofuels and other bio-based products is currently uncertain. Optimal feedstock composition depends on the economics of the processing technologies used, the value of potential co-products, waste disposal costs, and the costs associated with engineering solutions to problematic constituents such as ash. Feedstock composition can be modified by genetic modification and classical breeding approaches. For example, considerable progress has been made in understanding the lignin biosynthesis pathway in diverse plant species (Li et al. 2008), and there is evidence that changes in lignin content and/or composition can facilitate hydrolysis of cellulose and hemicellulose to fermentable sugars. Although decreases in lignin can be accompanied by increases in cellulose content (Li et al. 2003; Chen and Dixon 2007), concerns remain about possible effects on structural strength (standability), resistance to diseases and insect pests, and biomass yield (Chapple et al. 2007; Weng et al. 2008). In addition, it may be that in future biorefineries, lignin-derived products such as chemical feedstocks would have greater value than biofuels derived from fermentable sugars. Nonetheless, lignin modification of biomass feedstocks to facilitate production of biofuels represents a promising avenue for ongoing cellulosic ethanol research (Dunn-Coleman et al. 2001; Houghton et al. 2006; Buanafina et al. 2008; Carroll and Somerville 2009).

A better understanding of cell wall synthesis and the complex interactions between its different components will likely provide information on how to genetically modify additional biomass components to reduce recalcitrance to hydrolysis (Himmel et al. 2007). For example, although a number of biosynthetic enzymes are known, it is not currently possible to genetically engineer major changes in plant cell walls, such as the amount or composition of hemicellulose, as can be done for lignin (McCann and Carpita 2008). In addition, depending on the feedstock, other biomass components may be important. For example, despite being the most abundant agricultural biomass feedstock globally (Kim and Dale 2004), rice straw has a high silica content that acts to inhibit enzymatic hydrolysis and may cause other problems in biomass processing (Gressel 2008).

Pretreatment. The goals of commercial pretreatment include improving the efficiency of subsequent enzymatic hydrolysis and fermentation, maximizing the recovery of fermentable sugars, and minimizing costs associated with

energy and chemical inputs, inhibitor removal, and waste stream disposal (Galbe and Zacchi 2007; Jorgensen et al. 2007). Pretreatment is a critical step, consuming about 18% of production costs and impacting the efficiency of downstream processes as well (Yang and Wyman 2008). Capital costs are typically high for most pretreatment technologies due to the need for expensive corrosion-resistant materials or for specialized recovery and/or waste disposal systems (Eggeman and Elander 2005).

The resistance of biomass substrates to enzymatic hydrolysis is termed recalcitrance and is due to a number of factors. Lignin sterically hinders enzyme access to substrates by coating the cellulose microfibrils and in addition binds irreversibly to proteins, reducing enzymatic activity (Yang and Wyman 2006). Hemicellulose also hinders cellulose hydrolysis, and lignocellulosic modifications such as acetylation and the presence of other compounds such as ash can negatively affect enzymatic action (Himmel et al. 2007). Reduction of biomass particle size and an increase in the porosity of the material can help reduce recalcitrance to enzymatic hydrolysis by facilitating access to substrates by hydrolytic enzymes. Biomass recalcitrance can be reduced by chemical as well as physical pretreatment methods.

The mechanisms by which pretreatments alter biomass structure and composition to improve downstream enzymatic hydrolysis differ depending on the methodology employed. Among the possible mechanisms are improved substrate access by removal or modification of hemicellulose and lignin, by increasing porosity, and by changing the degree of polymerization or the crystallinity of the cellulose. Mechanical treatments such as hammer-milling to a fine particle size or extrusion of the biomass (Litzen et al. 2006) facilitate downstream chemical pretreatments and enzymatic hydrolysis, but are likely too energy- and cost-intensive to be commercially viable. Chemical pretreatments are often combined with high temperatures (typically 100–200°C) and pressures and sometimes with rapid explosive decompression. Diverse pretreatment methodologies have been researched using a variety of feedstocks, and each has advantages and disadvantages (Table 2), although all seem to obtain high sugar yields from corn stover feedstock (Wyman et al. 2005b).

Acids and alkalis are the two major pretreatment chemicals used. Acid-based pretreatments act primarily by hydrolyzing hemicellulose, which is efficiently converted first to oligomers then to monomeric pentose sugars with increasing temperature and pressure. Dilute acid hydrolysis (typically using sulfuric acid) is among the most extensively studied methods and is thought to be the closest to commercialization (Jorgensen et al. 2007). Hot water or steam pretreatments similarly rely on the generation of organic acids during the process to affect additional hydrolysis and thus can be thought of as mildly acidic auto-hydrolyses. There is a trade-off in reaction conditions, since acid-based pretreatments of increasing temperature and pressure lead to the loss of sugars and the generation of inhibitors of downstream processes, especially fermentation. These inhibitors include acetic acid and other organic acids; aldehyde lignin derivatives; and furfural and 5-hydroxymethylfurfural, furan degradation products of pentose and hexose sugars, respectively (Almeida et al. 2007).

Table 2. Features of selected pretreatment technologies

Pretreatment	Description	Advantages	Issues
Dilute acid hydrolysis	Dilute (0.5–3%) H_2SO_4 at 130–200°C/3–15 atm pressure	Low operating costs; extensively researched; highly efficient hemicellulose hydrolysis; broadly applicable to different feedstocks	Low solids loading (~5%); pH neutralization required (cost, waste disposal; formation of inhibitors of downstream processes (washing required); loss of sugars; lignin binding by cellulases slows hydrolysis
Steam explosion	160–240°C/6–34 atm pressure	High (30%) solids loading possible; efficient hemicellulose hydrolysis; broadly applicable to different feedstocks	Low xylose recovery; generation of inhibitors of downstream processes, washing required; lignin binding by cellulases slows enzymatic hydrolysis
Ammonia fiber explosion (AFEX)	Anhydrous ammonia –NH_3/biomass 1:1 at 70–90°C/15–20 atm pressure, followed by rapid decompression	Very high (60%) solids loading possible; no liquid stream; no loss of fermentable sugars; no inhibitors formed; NH_3 is recoverable; residual ammonia is N source in fermentation	Safety hazards of dealing with ammonia; need for hemicellulases to complete conversion to C5 sugars; mixed C5/C6 sugar hydrolysate; suitable only for agricultural feedstocks (not wood)
Lime	0.05–0.15 g $Ca(OH)_2$ per gram biomass at either 70–130°C/1–6 atm pressure (1–2 h), or under ambient conditions (wk)	Removes acetyl groups and ~1/3 of lignin, improving enzymatic hydrolysis; recoverable by CO_2 addition + lime kiln	Need for hemicellulases to complete conversion to C5 sugars; mixed C5/C6 sugar hydrolysate; suitable only for agricultural feedstocks

Further degradation of furans can generate formic acid, a cellulase inhibitor (Panagiotou and Olsson 2007). On the other hand, lower temperature acid-based pretreatments minimize the formation of inhibitors but can lead to poor enzymatic hydrolysis due to residual hemicellulose. Lignin is often partially melted and then redistributed under acid pretreatment conditions, improving access by cellulases, but complete removal improves subsequent enzymatic hydrolysis (Ohgren et al. 2007).

Alkali pretreatments with commercial potential include lime and ammonia fiber explosion (AFEX) technologies. In contrast to acid-based methodologies, alkali pretreatments remove lignin rather than the hemicellulose, and thus, inhibitor formation is minimized. AFEX technology has several promising features, including high solids loading, no separate liquid output stream, low temperature/energy input, and the potential for efficient ammonia recovery (Wyman et al. 2005a). However, hemicellulases as well as cellulases need to be included in the enzymatic hydrolysis step to obtain high fermentable sugar yields, and the presence of both C5 and C6 sugars in the resultant hydrolysate requires a microorganism capable of fermenting both types of sugars efficiently. Additional work needs to be done to validate alkali-based methodologies at pilot plant scale.

Pretreatment options need to be considered in the context of the overall process, including the nature of the output streams into enzymatic hydrolysis; the enzymes used in hydrolysis; the type of fermentation, including the microorganism(s) used; and waste stream handling (Wyman et al. 2005b). Two-stage pretreatments using different process conditions or methodologies may ultimately prove to be most effective in maximizing efficient recovery of fermentable sugars and other valuable by-products (Kim and Lee 2006).

Enzymes. Cellulases and hemicellulases belong to the large glycosyl hydrolase family of enzymes. Cellulases are one to two orders of magnitude less efficient than other polysaccharidases in this family, such as amylases (Zhang and Lynd 2004), primarily due to the difficulty of hydrolyzing solid crystalline cellulose as opposed to a soluble substrate. In addition to binding irreversibly to lignin, cellulases can bind unproductively to cellulose. While this effect can be mitigated by the addition of surfactants such as Tween20™, surfactants can act as inhibitors of downstream fermentation (Sun and Cheng 2002).

Complete cellulosic hydrolysis requires at least three key enzymes: an endoglucanase, an exoglucanase, and a β-glucosidase. Endo-1,4-β-D-glucanases (EG; EC3.2.1.4) carry out the first step by hydrolyzing internal β-1,4 glucosidic bonds in the cellulose polymer. This action frees up ends that are attacked by exo-1,4-β-D-glucanases or cellobiohydrolases (CBH; EC3.2.1.91) that processively move along the cellulose chain, cleaving cellobiose units. Cellobiohydrolases come in two forms, CBH1 and CBH2, which work from the reducing and non-reducing ends of the cellulose polymer, respectively. Finally, 1,4-β-D-glucosidases (BG; EC3.2.1.21) hydrolyze cellobiose to glucose to relieve product inhibition of the cellobiohydrolases and generate fermentable sugar.

Reduction in the costs of enzymes used in lignocellulosic hydrolysis is a key issue in commercial cellulosic ethanol production. The high cost of enzymatic hydrolysis is due to the poor activity of cellulases. Although production costs are similar, 40 to 100 times more enzyme is needed to digest cellulose as compared to starch, on a mass basis (Merino and Cherry 2007). In addition to requiring about 15–25 kg of enzymes per ton of biomass (Houghton et al. 2006; Taylor et al. 2008), long incubation times add to the capital cost of vessels for enzymatic hydrolysis. Despite the significant cost reductions that have been reported by researchers from the National Renewable Energy Laboratory (NREL), Novozymes and Genencor (http://www.nrel.gov/awards/2004hrvtd.html; Anonymous 2005), the cost of cellulases for biomass hydrolysis remains high. Enzymes needed for maize grain ethanol production cost US$2.64–5.28 per cubic meter (=1,000 l) of ethanol produced (Houghton et al. 2006), whereas cellulase enzymes for a commercial process are projected to cost about US$79.25 per cubic meter of ethanol (Lynd et al. 2008), or at least 20–40 times more, depending on whether enzymes are produced on-site or purchased from commercial suppliers (Somerville 2007; Sanchez and Cardona 2008). Enzymes thus comprise an estimated 20–40% of cellulosic ethanol production costs.

Hemicellulases reflect the diversity of hemicellulose itself, but include endo-1,4-β-D-xylanases (EC3.2.1.8), which hydrolyze the internal bonds in the xylan chain, and 1,4-β-D-xylosidases (EC3.2.1.37), which attack the non-reducing end of the polymer, releasing xylose. GAX side chains are attacked by several enzymes, including α-L-arabinofuranosidases (EC3.2.1.55), α-glucuronidases (EC3.2.1.139), acetyl xylan esterases (EC3.1.1.72), and feruloyl and ρ-coumaric acid esterases (Jorgensen et al. 2007). Access to the GAX xylan backbone is facilitated by removal of ester linkages and the arabinose and glucuronic acid side groups.

A large number of fungi and bacteria produce cellulases and hemicellulases (Sun and Cheng 2002). The best-studied microbial cellulase producers are *Trichoderma* spp., particularly *Trichoderma reesei*. The composition of *T. reesei*-secreted cellulases varies according to substrate, but is typically about 60% CBH1, 20% CBH2, 12% EG2 (Zhang and Lynd 2004) and roughly 0.5% BG1 and BG2 (Merino and Cherry 2007). In addition to two cellobiohydrolases,

five endoglucanases, and two β-glucosidases (with additional putative cellulases), *T. reesei* has four endoxylanases (Jorgensen et al. 2007). Despite difficulties in production, most commercial cellulases are produced by *T. reesei* fermentation (Taherzadeh and Karimi 2007).

Cellulases act synergistically with hemicellulases and other enzymes in breaking down plant cell wall material. The addition of hemicellulases and pectinases to commercial cellulase mixture can increase the yield of fermentable sugars, in part by boosting yields of cellulose hydrolysis (Berlin et al. 2007). The optimal combination of enzymes to affect hydrolysis depends on the nature of the substrate and the interactions between the individual enzymes. As an example, the activity of standard *T. reesei* cellulase mixtures can be significantly increased by supplementation with β-glucosidases or with heterologous glycosyl hydrolases of unknown function (Merino and Cherry 2007; Taherzadeh and Karimi 2007). Merino and Cherry (2007) describe experiments to determine the optimal ratio of arabinofuridases to xylosidase to hydrolyze an arabinoxylan substrate. Intriguingly, a number of anaerobic cellulolytic bacteria feature large extracellular enzyme complexes of cellulases, hemicellulases, and pectinases called cellulosomes. Cellulosomes are up to 5–7 mDa in size and act together to digest plant cell walls (Murashima et al. 2003; Doi 2008). The composition of cellulosomes is modular and changes according to the nature of the substrate encountered.

Hydrolysis and fermentation. The rate and extent of enzymatic hydrolysis is affected by the pretreatment method, substrate concentration and accessibility, enzyme activity (loading), and reaction conditions such as pH, temperature, and mixing (Merino and Cherry 2007; Taherzadeh and Karimi 2007). Different strategies for enzymatic hydrolysis and ethanolic fermentation have been developed to address specific process engineering issues (Table 3). These strategies require different levels of additional fermentation technology development. Separate hydrolysis and fermentation (SHF) effectively addresses the current significantly different temperature optima for enzymatic hydrolysis (45–50°C) and fermentation (30–35°C) by carrying out these reactions sequentially. Although SHF offers the advantage of cell culture recycling, increasing enzymatic product inhibition during the course of hydrolysis impacts productivity. Simultaneous saccharification and fermentation (SSF) addresses this issue by having the fermenting microorganisms consume the products of hydrolysis (cellobiose and glucose), lowering their concentration and thus their inhibitory effects on cellulase activity. However, this requires a compromise on temperature optima, or the use of less efficient thermotolerant fermentation organisms. Ethanol generated in SSF can help reduce the risk of culture contamination, and the use of a single vessel helps to reduce capital costs. SSF has been extensively studied at the laboratory and pilot plant scale.

Two variants of SSF, non-isothermal simultaneous saccharification and fermentation (NSSF) and simultaneous saccharification and co-fermentation (SSCF), are less well studied but promising (Taherzadeh and Karimi 2007). In NSSF, hydrolysis and fermentation occur in two vessels at their respective temperature optima, but simultaneously, the effluent from the hydrolysis reaction is shunted to the fermenter during the process. Despite higher capital costs, this technology has the potential to significantly reduce

Table 3. Selected hydrolysis and fermentation strategies

Name	Description	Features
SHF: Separate hydrolysis and fermentation	Enzymatic hydrolysis and fermentation done sequentially in different vessels	Hydrolysis and fermentation at respective optimal conditions; enzyme product inhibition; separate treatment of C5 and C6 sugar streams
SSF: Simultaneous saccharification and fermentation	Enzymatic hydrolysis and fermentation done simultaneously in same vessel	Compromise in conditions for optimal hydrolysis and fermentation; improved rates and yields; separate treatment of C5 and C6 sugar streams
HHF: Hybrid hydrolysis and fermentation	Enzymatic hydrolysis and fermentation done roughly sequentially in same vessel	Hydrolysis continues after shift to fermentation conditions; process optimization difficult; separate treatment of C5 and C6 sugar streams
NSSF: Non-isothermal simultaneous saccharification and fermentation	Enzymatic hydrolysis and fermentation done roughly simultaneously in different vessels	Hydrolysis and fermentation at respective optimal conditions; process optimization difficult; separate treatment of C5 and C6 sugar streams
SSCF: Simultaneous saccharification and co-fermentation	Like SSF, only both C5 and C6 sugars are fermented in same vessel	Fewer vessels, lower capital costs; requires engineered microorganism optimized for efficient C5/C6 fermentation
CBP: Consolidated bioprocessing	Enzymatic hydrolysis and fermentation carried out in single vessel by single or combination of microorganisms	Fewer vessels, lower capital costs; requires engineered microorganism optimized for enzyme production and C5/C6 fermentation

enzyme loadings and/or retention times. SSCF refers to enzymatic hydrolysis and co-fermentation of C5 and C6 sugars in a single vessel. The challenge here is identifying microorganisms that can carry out this fermentation efficiently, such as the engineered *Zymononas mobilis* strain used by the US NREL in their techno-economic process analysis (Aden et al. 2002, Aden 2008). One potential problem is that most commonly used fermentation organisms strongly prefer glucose as a carbon source, leading to inefficient or underutilization of xylose and other C5 sugars (Wyman et al. 2005b). Fermentation of xylose to ethanol, separately or by co-fermentation with C6 sugars, is crucial to cellulosic ethanol process economics (Merino and Cherry 2007).

Consolidated bioprocessing (CBP), also known as direct microbial conversion, is a technology in which one or more microorganisms carry out enzymatic hydrolysis and ethanolic fermentation simultaneously in a single vessel (Taherzadeh and Karimi 2007). Despite the potential for significant cost reductions, the technology faces challenging technical hurdles. CBP requires strains that can efficiently (in terms of yield and rates) convert glucose and C5 sugars to ethanol, effectively express multiple cellulases, exhibit robustness under industrial conditions, and for which the requisite genetic and metabolic pathway background knowledge exists (Chang 2007). No such strain has yet been reported even at lab scale, but progress is being made on CBP strain engineering (Lynd et al. 2005; Jorgensen et al. 2007).

Microorganisms are a key component of the technology used in different fermentation regimes. The potential of a number of different microorganisms has been studied, including yeasts such as the naturally pentose fermenting yeast *Pichia stipitis* (Agbogbo and Coward-Kelly 2008), mesophilic bacteria such as *Klebsiella oxytoca* (Ingram et al. 1999), and cellulolytic thermophilic anaerobes such as *Clostridium thermocellum* (Lu et al. 2006). *Saccharomyces cerevisiae* remains the gold standard in industrial ethanol production, attaining a production rate of 170 g ethanol per liter per hour on glucose under optimal laboratory conditions (Cheryan and Mehaia 1984). The ability of *S. cerevisiae* to tolerate low pH and rapidly produce ethanol helps to prevent contamination, and it has good tolerance to ethanol and other inhibitors, making it a strong candidate for further development and commercialization in cellulosic ethanol production (Almeida et al. 2007). An alternative fermentation microorganism is the mesophilic Gram-negative bacterium *Zymomonas mobilis*. Although not as hardy as industrial *S. cerevisiae* strains, the ethanol yield of *Z. mobilis* per unit of fermented glucose is 5–10% higher than that of *S. cerevisiae* due to its unique glucose metabolism (Lin and Tanaka 2006). Both species have been engineered to metabolize pentose sugars (Zhang et al. 1995; Ho et al. 1998). *Escherichia coli* has also been studied for use in lignocellulosic fermentation and has been engineered to produce and tolerate ethanol levels as high as 7.5 g/L from xylose or glucose under laboratory conditions (Yomano et al. 1998). However, ethanol concentrations produced from lignocellulosic hydrolysate fermentation are typically higher (10–35 g/L; Taherzadeh and Karimi 2007).

Expression of Enzymes in Crops

Efficient enzymatic hydrolysis of biomass substrates remains an economic and technical challenge in the development of cellulosic ethanol (Himmel et al. 2007; Wyman 2007). Reducing the costs of enzymes used in the process is crucial to favorable cellulosic ethanol process economics and commercialization (Stephanopoulos 2007). Given that loadings have been extensively optimized, improvements in enzyme performance or reduction in enzyme production costs will be required. Improved cellulases with higher specific activity, reduced allosteric inhibition, and improved tolerance to high temperatures and specific pH optima can be achieved using protein engineering methodologies. An example of a promising approach is the screening of cell-surface-tethered mutant enzyme libraries on solid lignocellulosic substrate for enhanced activity (Zhang et al. 2006). Production costs can be reduced by expressing cellulases and hemicellulases in crop plants (Sticklen 2008; Taylor et al. 2008), getting around the capital and operating costs associated with fermentation. Combining the two approaches with multiple improved enzymes expressed in crop plants would dramatically improve cellulosic ethanol process economics.

Plants provide a significantly lower cost alternative to fermentation for the production of industrial enzymes and can have additional processing benefits. Syngenta (www.syngenta.com) will soon be marketing the first commercial crop-produced enzyme product designed for corn grain ethanol production. Corn Event 3272 expresses an α-amylase gene with an improved temperature and pH profile. Amylase is used in corn grain ethanol production to convert starch to glucose for fermentation. The amylase in Event 3272 replaces commercial amylase enzymes produced by fermentation, with additional processing benefits that reduce production costs (Syngenta 2009). The product also features the environmental benefits of estimated 10% reductions in both processing water and greenhouse gas emissions. While these benefits are likely to vary depending on plant configuration, adoption of transgenic corn amylase technology has the potential to significantly improve the efficiency and environmental footprint of the US corn ethanol industry.

Significant advances in the expression of cellulases and hemicellulases in crop plants have occurred over the past 15 yr (Table 4). Some of the earliest work was the

Table 4. Expression of cellulases and hemicellulases in plants

Enzyme	Enzyme source	Host	Targeting	Reference	Comments
1,3-1,4-β-Glucanase	*Bacillus* spp.; chimeric	Barley aleurone protoplasts[z]	Apoplast; cytoplasm	Phillipson 1993	Thermostable mixed-linkage glucanase
EG1 endoglucanase	*Trichoderma reesei*	Barley cultured cells[y]	Apoplast	Aspegren et al. 1995	Secreted into culture medium; could not transform tobacco
XynZ xylanase	*Clostridium thermocellum*	*N. tabacum* cv Samsum NN	Apoplast	Herbers et al. 1995	Thermostable; normal plant growth
XYLD-A xylanase	*Ruminococcus flavefaciens*	*N. tabacum* cv Samsum NN	Apoplast	Herbers et al. 1996	*xynD*-encoded protein domain; xylanase
XYLD-C 1,3-1,4-β-glucanase	*Ruminococcus flavefaciens*	*N.tabacum* cv Samsum NN	Apoplast	Herbers et al. 1996	*xynD*-encoded protein domain; mixed-linkage glucanase
1,3-1,4-β-Glucanase	*Baccillus* spp.; chimeric	*H. vulgare* cv Golden Promise	Seed aleurone	Jensen et al. 1996	Thermostable codon-optimized mixed-linkage glucanase
EG1 endoglucanase	*Ruminococcus albus* F-40	*N. tabacum* BY2 cultured cells	Cytoplasm	Kawazu et al. 1996	Also has xylosidase activity; no effect on cell growth
E2 cellobiohydrolase	*Thermonospora fusca*	Tobacco, maize and wheat[x]	Various[w]	Lebel et al. 2008[v]	Provisional patent applications filed in 1996 and 1997
E1 endoglucanase	*Thermonospora fusca*	Tobacco, maize and wheat[x]	Various[w]	Lebel et al. 2008[v]	Provisional patent applications filed in 1996 and 1997
E5 endoglucanase	*Thermonospora fusca*	Tobacco, maize and wheat[3]	Various[w]	Lebelx et al. 2008[v]	Provisional patent applications filed in 1996 and 1997
XynC xylanase	*Neocallimastix patriciarum*	*B. napus*	Seed oil body	Liu et al. 1997	Oleosin fusion protein; enzyme works immobilized
XynB xylanase	*Clostridium stercorarium*	*N. tabacum* BY2 suspension cells		Sun et al. 1997	Intracellular and culture supernatant enzyme activity
CBH1 cellobiohydrolase	*Trichoderma reesei*	Tobacco[u]		Dai et al. 1999	Normal growth
EG1 endoglucanase	*Ruminococcus albus* F-40	*N. tabacum* cv Xanthi nc	Cytoplasm	Kawazu et al. 1999	Apoplast targeting deletion needed to get transformants
EG1 endoglucanase	*Trichoderma reesei*	Barley[t]	Seed	Nuutila et al. 1999	No enzymatic assay data; catalytic domain only
E2 endoglucanase	*Thermonospora fusca*	Alfalfa, tobacco and potato[s]		Ziegelhoffer et al. 1999	Thermostable; activity in dried leaf material
E3 cellobiohydrolase	*Thermonospora fusca*	Alfalfa, tobacco and potato[s]		Ziegelhoffer et al. 1999	Thermostable; activity in dried leaf material
E1 endoglucanase	*Acidothermus cellulolyticus*	Tobacco[u]	Chloroplast	Dai et al. 2000a	Normal growth/photosynthesis; truncated; dry leaf activity
E1 endoglucanase	*Acidothermus cellulolyticus*	Potato and tobacco[r]	Various[q]	Dai et al. 2000b	Cellulose binding domain (CBD) deletion retained activity
1,3-1,4-β-Glucanase	*Baccillus* spp.; chimeric	*H. vulgare* cv Golden Promise	Endosperm Vacuole	Horvath et al. 2000	Codon-optimized; targeted to protein bodies
XynA xylanase	*Neocallimastix patriciarum*	*H. vulgare* cv Golden Promise	Seed endosperm	Patel et al. 2000	Modified xylanase; stable activity in storage
E1 endoglucanase	*Acidothermus cellulolyticus*	Arabidopsis and BY2 cells[p]	Apoplast	Zeigler et al. 2000	Thermostable; high expression and activity in Arabidopsis
E1 endoglucanase	*Acidothermus cellulolyticus*	Potato and tobacco[o]	Various[n]	Hooker et al. 2001	Activity in dried leaf material
E1 and CD endoglucanases	*Acidothermus cellulolyticus*	*N. tabacum* cv W38	Various[m]	Ziegelhoffer et al. 2001	Higher activity from catalytic domain (CD) version
CelE endoglucanase	*Clostridium thermocellum*	*N. tabacum* cv Samsum NN	Cytoplasm; apoplast	Abdeev et al. 2003	Pleiotropic phenotypic alterations observed
E1 and CD endoglucanases	*Acidothermus cellulolyticus*	*N. tabacum* cv W38	Chloroplast	Jin et al. 2003	Spacer length impacts chloroplast import
XynA xylanase	*Clostridium thermocellum*	*O. sativa* cv Notohikari		Kimura et al. 2003	Activity in rice leaves and dessicated grain
Cel-Hyb1 endoglucanase	Chimeric[l]	*H. vulgare* cv Golden Promise	Seed endosperm	Xue et al. 2003	Codon-optimized; heritable; mixed linkage glucanase
CD endoglucanase	*Acidothermus cellulolyticus*	*N. tabacum* cv W38	Apoplast	Teymouri et al. 2004	AFEX pretreatment causes substantial loss of activity
E1 endoglucanase	*Acidothermus cellulolyticus*	Tobacco[u]	Various[k]	Dai et al. 2005	Apoplast best, vacuole worst targeting for E1 activity

Table 4 (continued)

Enzyme	Enzyme source	Host	Targeting	Reference	Comments
CD endoglucanase	*Acidothermus cellulolyticus*	*Z. mays* Hi II	Apoplast	Biswas et al. 2006	
XYL2 xylanase	*Trichoderma reesei*	*A. thaliana* Columbia	Various[j]	Hyunjong et al. 2006	Higher expression and activity with dual targeting
E1 endoglucanase	*Acidothermus cellulolyticus*	*Z. mays* Hi II	Various[i]	Hood et al. 2007	Codon-optimized; high seed ER, vacuole activity; heritable
CBH1 cellobiohydrolase	*Trichoderma koningii*	*Z. mays* Hi II	Various[i]	Hood et al. 2007	Codon-optimized; high seed ER, cell wall activity; heritable
CD endoglucanase	*Acidothermus cellulolyticus*	*O. sativa* cv Taipei 309	Apoplast	Oraby et al. 2007	Activity on pretreated corn stover and rice straw
CD endoglucanase	*Acidothermus cellulolyticus*	*Zea mays* Hi II	Apoplast	Ransom et al. 2007	Activity on pretreated corn stover
E1 endoglucanase	*Acidothermus cellulolyticus*	Duckweed[h]		Sun et al. 2007	Low expression of proteolytically derived CD
Cel6A endoglucanase	*Thermobifida fusca*	Tobacco[g]	Chloroplast	Yu et al. 2007a, b	Chloroplast transformation; homoplastomic events
Cel6B cellobiohydrolase	*Thermobifida fusca*	Tobacco[g]	Chloroplast	Yu et al. 2007a, b	Chloroplast transformation; homoplastomic events
CD endoglucanase	*Acidothermus cellulolyticus*	*Z. mays* Hi II	Various[f]	Mei et al. 2009	

[z] *Hordeum vulgare* cv Himalaya

[y] *Hordeum vulgare* cv Pokko suspension cells

[x] *Nicotiana tabacum* cv Xanthi nc and nahG transgenic derivative

[w] Constitutive: cytoplasm, vacuole; inducible: cytoplasm, vacuole; chloroplast transformed/nuclear inducible

[v] First publication date 23 May 2002 as US2002/0062502 A1

[u] *Nicotiana.tabacum* cv Petit Havana SR1

[t] *Hordeum vulgare* cv Kymppi and cv Golden Promise

[s] *Mendicago sativa* RSY27; *Nicotiana tabacum* W38; *Solanum tuberosum* PI 203900

[r] *Nicotiana tabacum* cv Petit Havana SR1; *Solanum tuberosum* cv Desiree and cv FL1607

[q] Constitutive: apoplast; green tissue: chloroplast and vacuole

[p] *Arabidopsis thaliana* Columbia; *Nicotiana tabacum* Bright Yellow 2 (BY2) suspension cells

[o] *Nicotiana tabacum* cv Petit Havana SR1; *Solanum tuberosum* cv Desiree

[n] Constitutive: cytoplasm and apoplast; green tissue: chloroplast and vacuole

[m] Constitutive: apoplast, chloroplast and cytoplasm

[l] *Neocallimastix patriciarum* CelA (activity) + *Piromyces* sp. Cel6G (thermotolerance) by gene shuffling

[k] Constitutive: apoplast; chloroplast, vacuole and endoplasmic reticulum; green tissue: chloroplast

[j] Green tissue: cytoplasm, chloroplast, peroxisome, chloroplast + peroxisome

[i] Seed embryo-preferred: apoplast, endoplasmic reticulum and vacuole

[h] *Lemna minor* 8627

[g] *Nicotiana tabacum* cv Samsun and cv 22X-1 (cv K327 non-nicotine derivative)

[f] Green tissue: cytoplasm, endoplasmic reticulum and mitochondria

expression of mixed-linkage glucanase and endoglucanase in cultured barley cells, with the objective of reducing viscosity in beer brewing (Phillipson 1993; Aspegren et al. 1995). Expression of a chimeric, codon-optimized mixed linkage glucanase in barley demonstrated the ability to express the enzyme in grain for brewing and animal feed applications (Jensen et al. 1996).

In the mid-1990s, work done at Syngenta provided the first example of the expression of active cellulases in plants. Two EG and a CBH sourced from *Thermonopora fusca* (since renamed *Thermobifida fusca*) were expressed from both constitutive and inducible promoters and were targeted to either the cytoplasm, the vacuole (using an appropriate targeting sequence), or to the chloroplast using direct transformation of the organelle (Lebel et al. 2008). Nuclear transformants of tobacco, corn, and wheat were generated that exhibited chemically induced cellulase expression. Interestingly, tobacco transgenics with a T7 promoter driving EG expression in the chloroplast were crossed with nuclear transformants that had the chemically inducible tobacco PR-1a promoter driving the T7 RNA polymerase gene fused to a chloroplast targeting sequence. The doubly transgenic lines exhibited chemical induction of chloroplast-expressed EG. The Lebel et al. (2008) patent thus demonstrates cellulase expression in plants and highlights the use of targeting sequences and inducible promoters among the tools available to do so.

The stability of plant-expressed enzymes is important to their use in industrial processes. Seeds represent one attractive storage option for enzymes. Horvath et al. (2000) demonstrated expression of codon-optimized hemicellulases fused to a signal peptide mediating secretion. Additional research in barley (Patel et al. 2000; Xue et al. 2003) and in rice (Kimura et al. 2003) provided evidence that hemicellulases were stable in stored grain. Hydrolytic enzyme activity can also be stable upon drying and when frozen as a crude extract. Stable *Acidothermus cellulolyticus* E1 endoglucanase activity was demonstrated in dried leaf material from tobacco (Ziegelhoffer et al. 1999; Dai et al. 2000a; Teymouri et al. 2004) and alfalfa (Ziegelhoffer et al. 1999) and in frozen crude extracts (Sticklen 2006). Stability, extraction, and storage are important considerations for the effective use of plant-expressed enzymes in industrial processing. Directed research and economic analyses in this area could help determine the optimal crops and tissues to target for enzyme expression and whether this varies depending on the biomass feedstock in question.

A thermostable, apoplast-targeted xylanase was the first hemicellulase to be expressed in whole tobacco plants, with normal growth being observed (Herbers et al. 1995). The strategy of expressing thermostable cellulases and hemicellulases continues to be used today to get around the problem that enzyme activity at physiological temperatures could pose for the plant. The use of thermostable enzymes in cellulosic ethanol production is a promising approach for other reasons, including improved enzyme activity and stability, lower viscosity, ease in extraction, and enhanced flexibility in process configurations (Viikari et al. 2007; Taylor et al. 2008). However, it is currently unclear how thermostable cellulases and hemicellulases would be integrated into an overall biomass processing scheme, suggesting that additional research is needed.

Cellulases and hemicellulases can be expressed in plants as truncated fully active proteins. The *xynD* gene of a ruminant microorganism was successfully expressed as separate, apoplast-targeted xylanase and mixed-linkage glucanase domains in tobacco (Herbers et al. 1996). Subsequently, *A. cellulolyticus* endoglucanase E1 was found in truncated but active form in tobacco and in duckweed, consistent with proteolytic removal of the cellulose binding domain (CBD), leaving just the catalytic domain (CD; Dai et al. 2000a; Sun et al. 2007). Expression of *A. cellulolyticus* endoglucanase CD had equal or higher activity than the full-length protein when expressed in potato or tobacco (Dai et al. 2000b; Ziegelhoffer et al. 2001), and apoplast-targeted CD has also been successfully expressed in maize (Biswas et al. 2006; Ransom et al. 2007). Importantly, although the enzyme is not able to survive a mild AFEX pretreatment (Teymouri et al. 2004), rice- and maize-expressed *A. cellulolyticus* CD has activity on pretreated corn stover and rice straw (Oraby et al. 2007; Ransom et al. 2007).

Most experiments on the expression of cellulases in plants have focused on endoglucanases, with a predominance of experiments with thermostable enzymes such as *A. cellulolyticus* E1 and the CD derivative of this enzyme. Kawazu et al. (1996) demonstrated expression of an EG from a ruminant microorganism in tobacco suspension cells, followed by expression in whole tobacco plants (Kawazu et al. 1999). *T. reesei* cellobiohydrolase (CBH1) expression in plants has been reported in tobacco (Dai et al. 1999) and in maize seed (Hood et al. 2007); in addition, expression of thermostable cellobiohydrolases have been reported in tobacco (Ziegelhoffer et al. 1999; Yu et al. 2007a), alfalfa, and potato (Ziegelhoffer et al. 1999). Given the need for relatively large amounts of CBH activity required to hydrolyze pretreated biomass cellulose, high-level expression of these enzymes in plants is needed.

The importance of subcellular targeting in the expression of cell wall hydrolyzing enzymes in plants has been confirmed in numerous reports. Early reports targeted cell wall hydrolases to the cytoplasm, vacuole, and/or the apoplast. More recently, experiments with transgenic plants bearing different constructs targeting the same protein to different subcellular compartments have been used to compare expression levels. Cellulases and hemicellulases have been successfully targeted to chloroplasts (Dai et al.

2000a, b, 2005; Hooker et al. 2001; Ziegelhoffer et al. 2001; Jin et al. 2003; Hyunjong et al. 2006), vacuoles (Dai et al. 2000b, 2005; Hooker et al. 2001; Lebel et al. 2008), peroxisomes (Hyunjong et al. 2006), mitochondria (Mei et al. 2009), and the endoplasmic reticulum (ER; Dai et al. 2005; Mei et al. 2009). In addition, direct chloroplast transformation can be used to obtain very high level expression of cellulases in homoplastomic tobacco (Yu et al. 2007a, b; Lebel et al. 2008).

In seeds, codon-optimized *A. cellulolyticus* E1 EG and *Trichoderma* spp. CBH1 were each targeted to either the apoplast, the vacuole, or the ER using an embryo-preferred promoter, and the expression and activity of the expressed enzymes was assayed (Hood et al. 2007). While the activity of both enzymes was high for the ER-targeted versions, they exhibited differences; EG had activity when targeted to the vacuole but not when targeted to the apoplast (cell wall), while the reverse was true for CBH1. These results suggest that expression optimization through targeting will likely depend on the enzyme class and individual characteristics of the protein in question. In other experiments, the highest xylanase activity was observed in plants with an enzyme with dual targeting to both the chloroplast and the peroxisome, relative to those targeted to either compartment individually, suggesting multiple targeting as a strategy to maximize expression (Hyunjong et al. 2006). Additional research on targeting multiple cellulases and hemicellulases to subcellular compartments would help resolve if there are limitations to this strategy, such as whether localization in certain compartments can interfere with enzyme accumulation in others.

High level enzyme expression in an agricultural setting is the primary challenge to delivering on the promise of inexpensive, plant-produced cellulases, and hemicellulases for cellulosic ethanol production. *A. cellulolyticus* EG has been expressed as high as 2% total soluble protein (TSP) in corn stover (Biswas et al. 2006; Mei et al. 2009), but it is estimated that 10% TSP is needed for complete hydrolysis (Sticklen 2008). In contrast, both EG and CBH1 constituted about 16–18% TSP in maize seed in the best expressing lines (Hood et al. 2007), but these represented only around 0.05% of dry grain weight. A yield of 150 bu/A of 15.5% moisture corn is equivalent to 7.97 t/ha dry weight (Graham et al. 2007). Taking the expression levels reported by Hood et al. (2007), the enzyme yield would be 7.97 t×0.05%≈ 4 kg enzyme per hectare. Assuming a 1:1 grain/stover harvest index and that 30% of the corn stover (2.4 t/ha) could be sustainably harvested (Graham et al. 2007), and a conservative hydrolytic enzyme requirement of 15 kg per ton biomass (Houghton et al. 2006), a minimum estimated enzyme yield of ~36 kg/ha would enable the grain to provide the enzymes required to process the stover from the same hectarage.

The actual target yields for different plant-expressed enzymes will depend on the amount and proportions of different enzymes needed for efficient biomass hydrolysis, which in turn depends on the nature of the pretreated feedstock and the individual activities of the chosen enzymes, taken together with any losses incurred during extraction and storage prior to use. Much progress has been made in demonstrating the utility of subcellular targeting for high level expression of cell wall hydrolyzing enzymes in plants, but more remains to be done in plant gene expression tools, including the use of strong, tissue-specific, or inducible promoters; transcriptional, translational, and intronic enhancers; and codon optimization (Streatfield 2007). In addition, developing technologies such as monocot chloroplast transformation (Bock 2007), mini-chromosomes (Carlson et al. 2007; Yu et al. 2007b; *see* also Chromatin Inc. website, http://www.chromatininc.com/), and INPACT (Dale et al. 2001, 2004; *see* also Farmacule Bioindustries website, http://www.farmacule.com/) could potentially provide additional options for boosting plant-expressed enzyme production to target levels.

Conclusions

Cellulosic ethanol can make a substantial contribution to our future energy needs and is projected to be even more environmentally friendly than first-generation biofuels, with the potential to reduce greenhouse gas emissions by an estimated 85% compared to gasoline (Fulton et al. 2004). Enzymatic hydrolysis represents the most attractive approach for biomass to biofuels conversion in the near term, with the promise of continuing improvements through biotechnology (Wyman et al. 2005a). Analysis of cellulosic ethanol process economics identifies the conversion of lignocellulosic biomass into fermentable sugars as the key technical challenge in reducing the costs of cellulosic ethanol production (Lynd et al. 2008). Over the long term, the problem of biomass recalcitrance will be addressed by improving biomass feedstock yield and processing characteristics and developing improved organisms for fermentation. The new commercial-scale cellulosic ethanol plants coming online will almost certainly give rise to improved engineering designs, driving down investments that are currently about three times higher per unit of ethanol produced than an equivalent maize grain ethanol plant (Galbe et al. 2007). However, to make cellulosic ethanol economically viable, our immediate research priorities need to be to lower the cost and improve the effectiveness of cellulases and to develop pretreatment technologies compatible with an optimized, integrated process, especially downstream enzymatic hydrolysis and fermentation.

A major theme in the research and development of cellulosic ethanol is that the individual steps in turning biomass to biofuels are inextricably linked, and research improvements need to be undertaken in the context of an integrated process. Because so much of the technology is under development, many interdependent variables need to be considered before focusing on a set of research goals. Towards this end, the Syngenta Centre for Sugarcane Biofuels Develoment (SCSBD) was formed to develop technologies enabling economically viable production of cellulosic ethanol from sugarcane bagasse (Sainz and Dale 2009). The SCSBD is a public partnership between Syngenta and the Queensland University of Technology (QUT) in Brisbane, Australia, with support from the Queensland state government. The approach is to express improved cellulases at high levels and in a controlled fashion in transgenic sugarcane and to use these enzymes in optimized processes designed to be integrated into existing sugar mills. The SCSBD project team consists of QUT and Syngenta scientists and engineers working together on solutions along a process stream that includes bagasse pretreatment and plant-expressed enzyme production to enzymatic hydrolysis and fermentation. The project plans to make use of a sugarcane bagasse cellulosic ethanol pilot plant being built in Mackay, Queensland with support from the Australian federal government.

The SCSBD example is relevant because in the future, success in improving the cost basis of cellulosic ethanol production will likely require increasingly integrated technical solutions drawing from diverse disciplines, including agronomy, plant breeding, and microbiology, in addition to biotechnology, enzymology, and engineering. Process modeling will be important in guiding research by identifying the most promising areas for improvement in reducing costs to make cellulosic ethanol production economically viable.

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