PROSPECTS OF CELLULOSIC ETHANOL FROM SUGARCANE BAGASSE

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Abstract

AGRICULTURAL residues such as grain straws and corn stover are abundant, readily available biomass feedstocks for the production of next generation biofuels, with collection and transport costs being a major component of their cost. Sugarcane bagasse is thus an especially attractive biomass feedstock in that it is an agricultural residue already present in large quantities at sugar and ethanol mills. The efficient conversion of bagasse lignocellulose to fermentable sugars is the major techno-economic challenge to the commercially viable production of ethanol from this feedstock. Bagasse is first pre-treated by a combination of high temperatures and pressures in the presence of chemicals, to facilitate subsequent enzymatic hydrolysis to break down the cellulose and hemicellulose to fermentable sugars. The sheer volume (an estimated 15–25 kg/t bagasse) and costs of the enzymes required for hydrolysis make the process economically unfeasible using current fermentation-based enzyme production technologies. The expression of enzymes in crop plants represents a promising approach to reduce the costs of enzyme production, and is an area in which Syngenta has strong intellectual property and advanced proprietary technologies. Syngenta's corn amylase, currently undergoing regulatory approval in the United States, features a thermostable α-amylase expressed in the grain for dry grind ethanol production applications, the first of a series of Syngenta traits designed for the biofuels industry. To enable the *in planta* production of cell wall-degrading hydrolases for commercial next generation biofuels production requires an integrated approach within an overall engineering process model, incorporating upstream pre-treatment and downstream fermentation methodologies in addition to enzyme expression, extraction, storage and hydrolysis technologies. Progress in the development of this suite of technologies for the conversion of bagasse lignocellulose to fermentable sugars will be described.

Biofuels

Global production and use of plant-derived transport fuels tripled from 2000 to reach 76.5 billion litres in 2008 (Sims *et al*., 2008). The increase in biofuels is due to several factors, including increasing petroleum supply uncertainty and rapidly rising demand for transport fuels, primarily from emerging economies. Government policies have promoted biofuels, driven by energy security concerns and political support for rural economies. Global biofuels production is dominated by the US, Brazil, the European Union and China, all of whom have strong policies supporting biofuels.

Concerns over greenhouse gas emissions have also contributed to increasing interest in biofuels. Replacement of gasoline with ethanol 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). Importantly, biofuels are unique in their general compatibility with our existing liquid transport fuel infrastructure, despite the potential for ethanol-mediated corrosion of existing pipelines.

The United States and Brazil produce 52% and 37% of 2008 global fuel ethanol production, respectively (http://www.ethanolrfa.org/industry/statistics/#E – accessed 2 Oct 2009). US ethanol production is primarily from corn starch converted to fermentable glucose by the addition of enzymes. Limitations on available acreage and price pressures will likely restrict US grain-based ethanol to an estimated 8% of gasoline consumption on an energy-equivalent basis, or about twice current production (Tyner, 2008). Sugarcane ethanol production in Brazil was approximately 24.5 billion litres in 2008, but is estimated to increase to 79.5 billion litres by 2022 (Goldemberg and Guardabassi, 2009), consistent with projected increases of 50–100% in global sugarcane tonnage in the coming decade (Kline *et al*., 2008). However, ethanol production from sugarcane will eventually also be limited by lack of suitable land and by competing demand by alternative uses, in this case sugar production.

New technologies are needed for biofuels to significantly contribute to the global energy matrix and to greenhouse gas emission reduction. Biofuels currently represent less than 3% of our transport fuel (Koonin, 2006). The enzymatic hydrolysis of biomass to sugars that are fermented to ethanol represents the most attractive technology for continued expansion of biofuels production. Advantages of this technology include a high carbohydrate recovery efficiency, potential for continuous improvement through biotechnology, and lower capital costs (Carroll and Somerville, 2009; Wyman *et al*., 2005).

Cellulosic ethanol

Cellulosic ethanol has the potential to supply a significant portion of our transport fuel needs, while reducing greenhouse gas emissions (Wang *et al*., 2007). An estimated 30% of total gasoline consumption could be provided by biofuels from agricultural crop residues alone (Kim and Dale, 2004; Koonin, 2006). Cellulosic ethanol technology research has accelerated as a result of increasing funding and investment, but significant interrelated techno-economic challenges remain.

High production costs make commercial cellulosic ethanol production unviable at present. These are estimated at between US$102-123 per barrel (Tyner, 2008), or more than US$0.66 per litre (Coyle, 2007). Capital expenses and operating (feedstock, processing and enzyme) costs combine to make cellulosic ethanol production expensive relative to first-generation ethanol. In addition, cost estimates are based on models and pilot-scale research due to the lack of operational commercial-scale cellulosic ethanol plants, increasing the investment risk profile (Galbe *et al*., 2007).

To overcome this hurdle, the US and other governments and companies are providing research funding and subsidies to give incentives for the development of next generation biofuels, including subsidies for biomass crop production, transport and storage.

Sugarcane bagasse

Plant biomass (lignocelluloses) is composed primarily of cellulose, hemicellulose and lignin linked by a complex web of covalent and hydrogen bonds. Cellulose primary structure consists of a linear homopolymeric chain of β1,4 linked cellobiose (glucose dimer) subunits. Primary chains are organised into higher level structures, finally comprising a 7–30 nm cellulose microfibril of hundreds of primary chains within a hemicellulose matrix, and coated with lignin (Zhang and Lynd, 2004). Importantly, cellulose exists mostly in crystalline form in plant cell walls, and is thus insoluble in water and commonly used solvents.

Hemicellulose is a heterogeneous branched polymer of pentose (C5) and hexose (C6) sugars whose composition varies according to species. In grasses, glucuronoarabinoxylans are the primary hemicelluloses, comprising a β1,4 linked xylose backbone and branching arabinose and glucuronic acid side chains. Grass hemicellulose also contains mixed (β1,3– and β1,4) linkage glucose polymers (β-glucans (Vogel, 2008). Hemicellulose acts as the filler between cellulose microfibrils and provides structural rigidity.

Lignin is a complex phenlypropanoid heteropolymeric network of coumaryl, coniferyl and sinapyl alcohols that acts as the glue linking and strengthening the polysaccharide components (Jorgensen *et al*., 2007). Unlike cellulose and hemicellulose, the complex structure and diversity of chemical bonds in lignin make enzymatic deconstruction difficult (Weng *et al*., 2008).

Sugarcane bagasse is an economically attractive biomass feedstock for cellulosic ethanol production, already present in substantial quantities at sugar and ethanol mills, and commonly burned to provide energy for the mill and, increasingly, for electricity cogeneration. The average composition of sugarcane bagasse (dry) is approximately 39% cellulose, 23% hemicellulose (with 89% of this being xylan), 24% lignin and 5% ash content (US Department of Energy, 2009). By comparison, corn stover and grain straw used for cellulosic ethanol production will incur collection and transport costs and will thus have a higher cost basis than sugarcane bagasse feedstock.

Pre-treatment

Biomass must be pre-treated to reduce the recalcitrance of lignocellulose to enzymatic hydrolysis. In untreated material, lignin and hemicellulose reduce enzymatic activity by steric hindrance, and crystalline cellulose slows cellulase action. In addition, lignin, ash and other components can irreversibly bind to and inactivate enzymes, or reduce their activity (Himmel *et al*., 2007).

Biomass recalcitrance can be reduced by chemical as well as physical pre-treatment methods. Reducing biomass particle size and increasing porosity can reduce recalcitrance by facilitating substrate access by hydrolytic enzymes, but is likely too energy-intensive to be cost-effective. Chemical pre-treatments also act to improve substrate access, by removing or altering deposition of hemicellulose and/or lignin, or by changing the cellulose characteristics such as crystallinity. Pre-treatment typically involves treatment with either acid or alkalis at high temperatures (100–200°C) and pressure. Acid-based pre-treatments act primarily by converting hemicelluloses to component pentose sugars, with reaction conditions being a trade-off between improvement of subsequent enzymatic hydrolysis, and loss of sugars and generation of inhibitors of downstream processes caused by increasing pre-treatment severity. Alkali pre-treatments remove lignin rather than hemicellulose, minimising inhibitor formation but requiring the use of hemicellulases and of microbial strains capable of mixed C5/C6 sugar fermentation.

Commercially viable pre-treatments minimise the costs associated with energy and chemical inputs, removal of inhibitors, water usage and waste disposal, while maximising the recovery of fermentable sugars (Galbe and Zacchi, 2007; Jorgensen *et al*., 2007). Pre-treatment typically represents about 18% of total production costs and also impacts the efficiency of downstream processes (Yang and Wyman, 2008). Most pre-treatment methodologies involve high capital expenses due to the materials and engineering designs required (Eggeman and Elander, 2005).

Enzymes

Cellulases and hemicellulases belong to the large glycosyl hydrolase (GH) family of enzymes. Cellulose hydrolysis requires at least three enzymes: an endo-glucanase, an exo-glucanase and a β-glucosidase. Endo-glucanases (EG) act by hydrolysing internal glucosidic bonds, freeing up ends that are attacked by exo-glucanases or cellobiohydrolases (CBH) that move progressively along the cellulose chain, cleaving cellobiose units. Cellobiohydrolases come in two forms, CBH1 and CBH2, that work from the reducing and non-reducing ends of the cellulose polymer,

respectively. Finally, β-glucosidases (BG) hydrolyse cellobiose to glucose to produce fermentable sugar and relieve product inhibition of CBH by cellobiose.

Enzyme cost reduction is a key issue in the commercialisation of cellulosic ethanol. On a mass basis, enzyme production costs are similar, yet it takes an estimated 40–100 times more enzyme to digest cellulose compared to starch (Merino and Cherry, 2007). Low activity requires high loadings of approximately 15–25 kg of hydrolytic enzymes per tonne of biomass (Houghton *et al*., 2006; Taylor *et al*., 2008). Long reaction times require large vessel sizes and hence high capital costs. Enzyme costs for corn dry grind ethanol production range from US$2.64–5.28 per m^3 (= 1000 litres) of ethanol produced (Houghton *et al*., 2006), compared to about US$79.25 per m^3 of cellulosic ethanol (Lynd *et al*., 2008), or at least 20–40 times more (Somerville, 2007). Enzymes thus comprise an estimated 20–40% of cellulosic ethanol production costs.

Expression of enzymes in crops

Efficient enzymatic hydrolysis of biomass substrates remains the major economic and technical challenge in the development of cellulosic ethanol (Himmel *et al*., 2007; Wyman, 2007). Enzyme cost reduction is critical to favourable cellulosic ethanol process economics; since enzyme loadings have been extensively optimised, improvements in enzyme activity and/or reduction in production costs are required. Cellulases can be improved by protein engineering to have higher specific activity, reduced allosteric inhibition, high temperature tolerance and altered pH optima. Expression of cellulases and hemicellulases in crop plants has the potential to significantly reduce production costs, by getting around the capital and operating costs associated with fermentation (Sainz, 2009; Sticklen, 2008; Taylor *et al*., 2008). In combination, the expression of multiple improved enzymes in crop plants would dramatically improve cellulosic ethanol process economics.

Plants can produce enzymes at much lower cost than fermentation methodologies, and can also provide additional benefits. Syngenta (www.syngenta.com) is currently seeking deregulation from the United States Department of Agriculture for the first crop-produced enzyme product, designed for corn grain ethanol production. Amylase is used in the conversion of starch to fermentable glucose in corn grain ethanol production. Syngenta corn event 3272 expresses an α-amylase gene with an improved temperature and pH profile, replacing commercial amylase enzymes produced by fermentation, with additional benefits that reduce production costs (Syngenta, 2009).

The environmental benefits of Syngenta Corn Amylase (Enogen™) include estimated reductions of 8% in processing water and 6–11% in greenhouse gas emissions (Urbanchuk *et al*., 2009). While actual benefits are likely to depend 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.

The technology for crop-based expression of cellulases and hemicellulases has significantly progressed over the past 15 years. A potential problem with the expression of hydrolytic enzymes in plants is that of impaired structural integrity of plant cell walls and, consequently, on crop standability. Strategies to effectively address this problem have included the use of appropriate expression tools, such as suitable promoters and subcellular targeting of enzymes.

Work done at Syngenta in the mid-1990s provided the first example of the expression of active cellulases in plants. Two endoglucanases (EG) and a cellobiohydrolase (CBH) sourced from *Thermonospora fusca* (since renamed *Thermobifida fusca*) were expressed from constitutive and inducible promoters and targeted to either the cytoplasm, the vacuole by use of 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. The Lebel *et al*. (2008) patent thus demonstrates cellulase expression in plants, and highlights thermostable enzymes, subcellular targeting sequences and inducible promoters as among the tools available to do so.

The importance of subcellular targeting in the expression of cell wall hydrolysing enzymes in plants has been confirmed in numerous reports. Successful targeting of cellulases and hemicellulases has been reported to chloroplasts, vacuoles, peroxisomes, mitochondria, endoplasmic reticulum, apoplast and cytoplasm (Sainz, 2009 and references therein). More recently, EG and CBH1 expressed in corn seed were targeted to different subcellular compartments (Hood *et al*., 2007).

Activity of ER-targeted versions was high for both enzymes, but EG also had activity when targeted to the vacuole but not the apoplast, whereas the reverse was true for CBH1. These results suggest that expression optimisation through targeting will likely depend on enzyme type. In other experiments, the highest activity was observed in plants with dual targeting of a xylanase to both the chloroplast and the peroxisome, compared to either compartment alone, suggesting a multiple targeting strategy to maximise expression (Hyunjong *et al*., 2006). Additional research on targeting multiple enzymes to subcellular compartments would help identify potential limitations to this strategy, e.g. whether localisation in certain compartments can interfere with enzyme accumulation in others.

Hydrolytic enzymes active at physiological temperatures have the potential to negatively impact plant structural characteristics. To limit this potential damage, the strategy of using cellulases and hemicellulases with high temperature optima and low activity at physiological temperatures has also been validated. Most reported experiments on cellulase expression in plants have used endoglucanases, principally using thermostable enzymes such as the *A. cellulolyticus* E1 EG and derivatives. Expression of thermostable CBH have been reported in several plant species (Sainz, 2009 and references therein), an important result given the need for relatively large amounts of CBH activity in biomass conversion.

Besides reducing the risk of unintended effects on plant structure, the use of thermostable enzymes has other potential advantages, including improved enzyme extraction, activity and stability, and lower process flow viscosities (Taylor *et al*., 2008; Viikari *et al*., 2007). Stable *A. cellulolyticus* E1 EG activity was demonstrated in dried leaf material from tobacco and alfalfa (Dai *et al*., 2000; Teymouri *et al*., 2004; 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 processing.

High-level enzyme expression in an agricultural setting is the main challenge in delivering 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), although an estimated 10% of 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), or about 0.05% of dry grain weight. It is estimated that approximately 9 times this enzyme yield would be required to use the grain produced to process the corn stover from the same hectarage (Sainz, 2009). Target yields for different plant-expressed hydrolytic enzymes will depend on various factors, including the type of pretreated feedstock and the activity, loading and optimal ratios of the enzymes needed for efficient biomass hydrolysis, together with consideration of any losses incurred during extraction and storage prior to use.

Significant levels of cellulase expression in corn leaves (7–10% of TSP) have been obtained in experiments conducted at Syngenta Biotechnology, Inc. (Warner *et al*., unpublished data). Importantly, the performance of the corn leaf-expressed exoglucanase on pre-treated sugarcane bagasse was roughly comparable to the activity of microbially produced enzyme when assayed in defined composition enzyme cocktails. Similar studies on cellulase expression in sugarcane are being conducted at the Queensland University of Technology (QUT) in the Syngenta Centre for Sugar Cane Biofuels Development, a unique public-private research collaboration between

Syngenta and QUT (Sainz and Dale, 2009). Preliminary experiments using the constructs shown to be functional in corn have generated events with cellulase activity, mirroring the results observed in corn (Sainz *et al*., unpublished data). Taken together, these results are important in demonstrating the viability of crop-expressed hydrolytic enzyme product concepts for cellulosic ethanol production from both corn stover and sugarcane bagasse.

Conclusions

Increasing global energy demand will drive the development of technologies for environmentally sustainable transport fuels. Cellulosic ethanol and other advanced biofuels can help to meet future energy needs with a more favourable environmental profile than first-generation biofuels (Woods *et al*., 2009). Given existing infrastructure, enzymatic hydrolysis represents the most attractive near-term approach for biomass to biofuels conversion, with ongoing improvements through biotechnology (Wyman *et al*., 2005). Process models identify conversion of lignocellulosic biomass into fermentable sugars as the key challenge in reducing cellulosic ethanol production costs (Lynd *et al*., 2008). Longer-term, biomass feedstock modification to improve yield and processing characteristics, the development of improved organisms for fermentation, and improved engineering designs will increase process efficiencies, reducing capital costs per litre of cellulosic ethanol produced, costs that are currently about three times higher than equivalent costs for a corn ethanol plant (Galbe *et al*., 2007).

Sugarcane bagasse is the most cost-effective feedstock for cellulosic ethanol production, but commercial viability will depend on being able to lower the cost and improve the effectiveness of cellulases, and to develop pre-treatment technologies compatible with an optimised, integrated process, especially downstream enzymatic hydrolysis and fermentation. 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 modelling will be important in guiding research, by identifying the most promising areas for improvements and cost reductions in making cellulosic ethanol production economically viable. In addition, standardised life cycle analysis methodologies will need to be developed to account for environmental costs and benefits in effectively comparing different options towards meeting our future energy needs.

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PERSPECTIVES DE PRODUCTION DE L'ÉTHANOL CELLULOSIQUE À PARTIR DE LA BAGASSE DE LA CANNE À SUCRE

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MOTS-CLÉS: Biomasse, Biocarburants, Éthanol Cellulosique, Cellulases, Enzymes.

Résumé

LES RÉSIDUS agricoles comme la paille et les résidus du maïs sont abondants et facilement disponibles comme matières premières pour la production de la deuxième génération de biocarburants. Cependant, la collecte et le transport demeurent les composantes principales au coût. La bagasse de canne représente une matière première intéressante dans le sens que c'est un résidu disponible en grande quantité dans les sucreries et usines d'éthanol. La conversion efficiente de la bagasse ligno-cellulosique en sucres fermentables est un défi technico-économique majeur pour une production industrielle viable d'éthanol. La bagasse est d'abord prétraitée par une combinaison de haute température et de pression en présence de produits chimiques, afin de faciliter l'hydrolyse enzymatique subséquente pour la décomposition de la cellulose et de l'hemicellulose en sucres fermentables. Le volume conséquent (estimé à 15–25 kg/t bagasse) et le coût des enzymes nécessaires pour l'hydrolyse font que le processus n'est pas économiquement envisageable avec la présente technologie de fermentation enzymatique. L'expression des enzymes dans des plantes représente une approche prometteuse pour une réduction du coût de l'enzyme, et c'est dans ce domaine que Syngenta a une propriété intellectuelle solide et une technologie propriétaire avancée. L'amylase de maïs de Syngenta qui se caractérise par un α-amylase thermostable avec expression dans le grain pour le broyage à sec dans les applications de production d'éthanol, fait actuellement l'objet de la recherche d'approbation réglementaire aux États Unis. C'est le premier d'une série de caractères que Syngenta à l'intention de destiner à l'industrie des biocarburants. La production *in planta* des hydrolases pour dégrader la paroi cellulaire requiert une approche intégrée à l'intérieure du modèle de l'ensemble du processus d'ingénierie. Elle incorpore des méthodologies de fermentations de pré-traitements en amont et en aval en sus des technologies ayant trait à l'expression d'enzyme, d'extraction, de préservation et d'hydrolyse. Le progrès dans le développement de cette suite de technologies pour la conversion de la bagasse ligno-cellulosique en sucres fermentables est décrit.

PROSPECTOS PARA EL ETANOL CELULÓSICO A PARTIR DEL BAGAZO DE LA CAÑA DE AZÚCAR

Por

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PALABRAS CLAVE: Biomasa, Biocombustibles, Etabol Celulósico, Celulasas, Enzimos.

Resumen

RESÍDUOS agrícolas tales como la paja derivada de cereales y el rastrojo del maíz son abundantes materias primas, las cuales se encuentran fácilmente disponibles para producir biocombustibles de siguiente generación; sin embargo, los costos de colecta y de transporte son grandes componentes en su costo. El bagazo de caña de azúcar es, por tanto, una materia prima especialmente atractiva puesto que se encuentra presente en grandes cantidades en molinos de azúcar y etanol. La conversión eficiente de la lignocelulosa del bagazo en azúcar fermentable es el mayor reto tecnológico y económico para una viable producción comercial del etanol a partir de tal materia prima. El bagazo cursa un pretratamiento con una combinación de altas temperaturas y presiones en presencia de reactivos químicos, a fin de facilitar subsecuentes hidrólisis enzimáticas, degradar la celulosa y hemicelulosa y convertirles en azúcares fermentables. El volumen puro (estimado en 15-25 kg/tn de bagazo) y los costos de producción de los enzimos requeridos para la hidrólisis hacen económicamente inviable al proceso de usarse las actuales tecnologías de producción de enzimo para la fermentación. La expresión de enzimos en cultivos vegetales representa un enfoque promisorio para reducir los costos de la producción de enzimos, y es una área en la que Syngenta posee un gran propiedad intelectual y avanzadas tecnologías propietarias. La amilasa de maíz de Syngenta, actualmente bajo aprobación regulatoria en los Estados Unidos, presenta características de una α-amilasa termoestable expresada en el grano para aplicaciones en la producción de etanol a partir del molido seco, siendo la primera de una serie de caracteres de Syngenta diseñados para la industria de biocombustibles. Para facilitar la producción *in planta* de hidrolasas degradantes de pared celular para la producción comercial de siguiente generación de biocombustibels, se requiere un enfoque integrado dentro de un modelo completo de ingeniería de procesos, incorporando pretratamientos en fases superiores del proceso y metodologías de fermentación en fases resultantes del proceso, adicionales a aquellas de expresión enzimática, extracción, almacenaje e hidrólisis. El avance en el desarrollo de esta serie tecnológica para la conversión de lignocelulosa del bagazo en azúcares fermentables será descrito.