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## Third generation biofuels from lignocellulosic biomass materials

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# Oral Presentations

## **Oral Presentation 1-01**

### **The French initiative on renewable carbon for green chemistry and bioenergies**

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The European Union plans to cut its carbon emissions by 20% while raising renewable sources up to 20% of total energy use by 2020. Simultaneously, chemical industry aims at raising the share of renewable carbon up to 17% in the broad family of chemicals and materials by 2017.

Plant biomass has therefore a great potential to become a major alternative source to fossil carbon.

The foresight workshop « Which plants and sustainable production systems for biomass in the future? », launched in April 2008 aims at characterizing annual and perennial plants, micro-algae and biomass production systems that would meet the needs and requirements of new bioenergy and green chemistry chains. Sustainability being a key issue, the workshop also integrates environmental, social and economic dimensions.

The foresight workshop federates 20 French bodies: public research or higher education organizations, professional unions, private companies and associations which play a leading role in their respective area.

The set of experts who participate to the workshop includes specialists from many disciplines (e.g. plant physiology and genetics, biotechnologies, agronomy, ecology, economics and social sciences).

This workshop articulates 3 interrelated approaches: a reverse engineering approach that starts from the needs expressed by different industries; the exploration and optimization of production systems based on relevant plant and algal species, including biorefinery as well as green and white biotechnologies; the assessment of the environmental, territorial and economic performances of these systems.

The presentation will outline the first results before the final deliverables scheduled for Spring 2010.

## **Oral Presentation 1-02**

### **Plant growth promoting microorganisms allow for sustainable growth and increased biomass production of poplar on marginal soils**

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Looking at the drivers behind a biofuel economy it is clear that once the problem of the cost efficient decomposition of lignocellulosic biomass has been solved, the sustainable production of lignocellulosic biomass will become the major critical success factor.

Poplar is considered as a model tree species for bioenergy feedstock production. Plants live in close association with symbiotic microorganisms. We showed that specific endophytic bacteria had a beneficial effect on the development and growth of poplar on marginal soils, resulting in up to 50%-80% increase in biomass production.

Short term beneficial effects of plant growth promoting microorganisms result in improved plant establishment on marginal soils. These effects include accelerated root development resulting in better access to nutrients and water, and consequently a faster initial growth, which will allow the plants to out compete weeds for available resources, thus resulting in less need to apply herbicides. Long term beneficial effects of plant growth promoting microorganisms will result in improved plant growth, health and survival, leading to economically sustainable feedstock production. This can be obtained by counteracting stress responses caused by drought and contamination, protection against pathogens via competition for available resources, and by assisting the plant's defense response against pathogenic invasions. The genomes of four plant growth promoting endophytic bacteria were sequenced and genome annotation and "omics" approaches were used to better understand their synergistic interactions with poplar. This basic knowledge will be further exploited to improve plant establishment and sustainable bioenergy feedstock production on marginal, non-agricultural land.

## Oral Presentation 1-03

### Plants begetting plants: Lignocellulose saccharification by plant-expressed cellulases

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Significant conversion of recalcitrant cellulosic biomass to fermentable sugars requires, at a minimum, *endo*-glucanase (EC 3.2.1.4) and *exo*-glucanase (EC 3.2.1.91) activities to reduce the insoluble cellulose chains to soluble cellobiose units which are subsequently hydrolyzed to glucose by  $\beta$ -glucosidase (EC 3.2.1.21). A major obstacle to the development of a commercially viable cellulosic ethanol industry is the cost associated with vast quantity of cellulase required. At ~50mg enzyme/gram cellulose, the current level of microbially-expressed enzymes required for efficient degradation of lignocellulosic biomass makes microbial expression an economically untenable means for enzyme production. While efforts to address this problem have primarily focused upon engineering more efficient cellulases and maximizing fungal expression, utilizing plants to produce and deliver the enzymes offers a convenient and cost effective alternative. Syngenta has pioneered the concept of *in planta* expression of enzymes and traits focused on the biofuels industry. Indeed, Syngenta is the only company to date to have taken a plant-expressed biofuels trait, our corn-expressed amylase for dry grind ethanol production, into the US regulatory system. We have successfully generated transgenic crop plants, including maize and tobacco, expressing active bacterial and fungal cellulases at high levels. This paper will outline the characterization of *in planta*-expressed microbial cellobiohydrolases (CBH I and II) and *endo*-glucanases and will demonstrate the capacity of these enzymes to function in defined enzyme cocktails for the degradation of lignocellulosic biomass.

## Oral Presentation 1-04

### Transgenic Expression of Endoglucanase and Xylanase Genes Increases Tobacco Digestibility and Biomass Conversion

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The objective of this project was to test the processing performance of transgenic feedstocks expressing cell wall hydrolyzing enzymes and to identify ways to exploit the properties of these plants during biorefining to improve conversion efficiency. Transgenic tobacco lines expressing an endoglucanase, E1, from *Acidothermus cellulolyticus* and a xylanase, Xyn Z, from *Clostridium thermocellum* as single enzymes or transgenic tobacco expressing both enzymes were tested using dilute acid pretreatment and enzyme hydrolysis, as well as *in vitro* dry matter digestibility analysis, to evaluate their performance as value-added cellulosic ethanol feedstocks. Compared to wild-type tobacco, transgenic lines displayed greater digestibility and glucan conversion when biomass was digested with commercial enzyme cocktails. These properties were further enhanced by incubating the slurried biomass at moderate temperatures prior to hydrolysis with commercial enzyme cocktails. These results demonstrate that transgenic crop feedstocks have potential to improve the efficiency and lower the cost of existing bioprocessing regimes by reducing enzyme loading, and point to the possibility of further modifying bioprocessing to exploit the properties of transgenic feedstocks. This project was supported by grant 0810640 from the National Science Foundation.

## Oral Presentation 1-05

### Enhanced bioprocessing of maize cell wall mutants

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Modification of lignin subunit composition can significantly increase the yield of fermentable sugars from maize stover. The *brown midrib1* (*bm1*) and *bm3* mutations each increase the yield of glucose per gram dry stover by 50% relative to the wild-type control (inbred A619). When combined in a near-isogenic *bm1-bm3* double mutant, the two mutations act in an additive manner, resulting in a doubling of the yield of glucose. Even though there is no apparent increase in cellulose content, based on kinetic studies both the rate of hydrolysis and the overall yield of glucose increase as a result of the mutations. We are investigating the basis of the enhanced hydrolysis in these *bm* mutants by assaying the adsorbance of cellulases to stover, using recombinant proteins consisting of the cellulose binding module (CBM) isolated from *Trichoderma reesei* endoglucanases labeled with green-fluorescent protein (GFP). Because of lignin autofluorescence, this approach can not be performed *in situ*, but instead has to rely on a fluorescence subtraction assay. We have also shown that biomass from these mutants yields high levels of fermentable sugars under less severe pretreatment conditions compared to biomass from wild-type control plants. The more efficient cell wall deconstruction in these mutant can thus be viewed as genetic pretreatment. The combined data from these experiments will be of value for the design of plant cell wall composition in such a way that agronomic properties and biomass conversion are optimally balanced.

## Oral Presentation 1-06

### Impact of divergent selection on the abundance and activities of lignin biosynthetic enzymes in switchgrass, and characterization of recombinant switchgrass CAD and COMT proteins

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The relative composition of lignin monomers in cell walls provides key information on the integrated functions of the underlying biosynthetic machinery, as well as a useful window into the efficacy of broad or narrow selection criteria for improvement of plants with more optimal biomass quality. Here, we evaluated a number of switchgrass genotypes by thioacidolysis for lignin composition and content, and by biochemical characterization of protein levels and activities of select enzymes in internode extracts. The data indicated that divergent selection of switchgrass for digestibility resulted in changing the ratios of G to S lignins in plants, and impacted the relative levels of cinnamyl alcohol dehydrogenase (CAD) and caffeic-acid-O-methyl transferase (COMT) proteins, but levels of caffeoyl-CoA-O-methyl transferase (CCoAOMT) were unchanged. Enzyme activity data generally mirrored protein level data. These findings suggest that (i) enzymes required for lignin biosynthesis in switchgrass can be differentially affected by broad selection for digestibility; and (ii) discovery of the mechanisms controlling the endogenous levels of these proteins could uncover novel markers and lead to accelerated improvement of switchgrass via traditional breeding. We have also cloned and initiated biochemical characterization of recombinant switchgrass and sorghum CAD and COMT proteins. Recombinant grass CADs displayed greater substrate preference for sinapyl aldehyde and sinapyl alcohol when compared to coniferyl derivatives. There was essentially no activity against caffeoyl alcohol. Initial modeling of sorghum CAD suggested that observed changes in specific amino acid residues in monocot CADs relative to dicot CADs could account for changes in substrate specificity.

## Oral Presentation 1-06A

### Functional genomic analysis of plant biomass deconstruction by extremely thermophilic, cellulolytic bacteria in pure and co-culture

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The breakdown of lignocellulosic biomass to fermentable sugars remains a key challenge in the production of biofuels, such as hydrogen and ethanol. To this end, microbial consortia need to be considered so that natural synergistic interactions between biomass deconstruction can be used advantageously. To develop such consortia, an understanding of the relevant molecular microbial ecology of the constituent organisms is critically important. In our lab, functional genomics approaches are being used to explore interspecies interactions between extremely thermophilic bacteria that have the capacity to degrade lignocellulosic biomass. Two gram-positive, oligotrophic, fermentative anaerobes, with growth  $T_{opt}$  of ~75°C, *Caldicellulosiruptor saccharolyticus* (Csac) and *Anaerocellum thermophilum* (Athe), are being investigated as model cellulolytic extreme thermophiles. Although 16S rRNA phylogeny suggests that these two bacteria are closely related, genome sequence analysis revealed that Athe contains almost 700 ORFs not present in Csac, while Csac has over 600 ORFs missing from Athe. A key objective is to determine the physiological and ecological significance of genome sequence differences as this relates to biomass deconstruction. Using whole genome oligonucleotide microarrays, both pure and co-cultures of *C. saccharolyticus* and *A. thermophilum* were monitored at various stages of growth on monosaccharides, polysaccharides and plant biomass substrates. Operons, regulons, and key protein-encoding ORFs responsive to specific substrates, growth conditions and interspecies interactions were identified. The results illustrate how strategic use of transcriptional response analysis can be a powerful tool for examining microbial biomass deconstruction by pure and co-cultures capable of consolidated bioprocessing.

## Oral Presentation 2-01

### One-step Cellulosic Ethanol: Can We Really Do This?

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A whole-cell biocatalyst system was developed in our research laboratories to directly produce ethanol from cellulose in a single step; initially we used a model amorphous cellulose (Phosphoric acid swollen cellulose, PASC). This whole cell biocatalyst was constructed with LY01, which is one of the most developed ethanologenic *Escherichia coli* strains, as a host cell. The cellulase genes, celCCA, celCCE and  $\beta$ -glucosidase, were prepared from the mesophilic strain *Clostridium cellulolyticum*. To enhance the stability and activity of the cellulosic enzymes, these enzymes were co-displayed with the anchor protein PgsA on the surface of the host cell. For inducing the synergism of enzymes, this recombinant cellulolytic microorganism co-expressed endoglucanase, cellobiohydrolase, and  $\beta$ -glucosidase, simultaneously. The saccharification product, monosaccharides, can be uptaken immediately by the host cell and produce ethanol so that the inhibition of the catalytic activity of enzymes due to high substrate (sugars) concentration, can be effectively minimized. In this research, we also applied the whole-cell biocatalyst system in a bioethanol production process with the lignocellulosic biomass as a substrate. With the enzymatic hydrolysis of natural biomass, there are several factors, which determine the hydrolysis rate, e.g. crystallinity, degree of polymerization, particle size, pore volume, and accessible surface area. Since cellulose hydrolysis occurs on the surface of cellulose, we especially focus on the relationship between particle size of cellulose and hydrolytic rate in whole-cell biocatalyst system. The results are very promising.

## Oral Presentation 2-02

### Genetically engineering yeast for CO<sub>2</sub> capture during ethanol fermentation

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Biomass represents an abundant carbon-neutral renewable resource for the production of bioenergy and biomaterials. Bio-ethanol is one of the predominant bio-fuels, which is produced mainly by yeast (*Saccharomyces cerevisiae*) fermentation. During ethanol fermentation, more than forty percent of the carbons are released into the atmosphere as carbon dioxide. The ability to capture part of the CO<sub>2</sub> released during fermentation will provide an alternative way to improve ethanol productivity per unit of biomass used. To achieve this objective, we explored the feasibility of expressing cyanobacterial photosynthetic enzymes in fermentative yeast to capture CO<sub>2</sub>. The genes for ribulose biphosphate carboxylase and phosphoribulokinase were isolated from cyanobacterium *Synechococcus* sp. and were heterologously expressed in the yeast under the control of the yeast actin, pgk1 or adh1 promoters. RNA and protein blotting analyses confirmed that both genes were properly expressed in *S. cerevisiae*. Codon optimization of both genes significantly improved protein accumulation in the yeast. The <sup>14</sup>C labeling analysis demonstrated that ribulose biphosphate carboxylase was active in the yeast cells though the activity was low. The effects of both enzymes on yeast ethanol production were also examined in culture media supplemented with a xylose-xylose mixture. Some improvement was observed. Further improvement of this CO<sub>2</sub> capturing process is on-going.

## Oral Presentation 2-03

### Production of a xylose utilizing *Zymomonas mobilis* strain for ethanol production from high concentrations of mixed sugars

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Maximizing yield of ethanol from C5 utilizing micro organisms requires both high rates of sugar utilization and minimizing the production of by products that detract from carbon yield to ethanol. *Zymomonas mobilis* that has been engineered to utilize xylose by way of the pathway through xylose isomerase and xylulose kinase to the endogenous sugar phosphate pathway produces xylitol and xylitol phosphate as byproducts. Xylitol production results in loss of ethanol yield and xylitol phosphate is a general metabolic inhibitor as a dead end phosphate sink. In order to correct these deficiencies in xylose utilizing *Z. mobilis*, the pathway to xylitol and xylitol phosphate was determined and the gene for the enzyme at the head of the pathway was inactivated to produce a strain that has better fermentation properties and a higher ethanol yield. Effective means for achieving osmotic balance in high initial sugar fermentations was also established for the mutant and parent strain.

## Oral Presentation 2-04

### Construction of pentose fermenting industrial *Saccharomyces cerevisiae* strains expressing a bacterial xylose isomerase

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We have cloned and successfully expressed a prokaryotic xylose isomerase with high activity in the yeast *Saccharomyces cerevisiae*. The corresponding gene was isolated from the anaerobic bacterium *Clostridium phytofermentans*. The enzyme has only very limited sequence similarities to the xylose isomerases from *Piromyces* and *Thermus thermophilus* which up to now were the only xylose isomerases which could be expressed in yeast in a functional form. Activity and kinetics of the new enzyme are comparable to the *Piromyces* xylose isomerase. However, it is far less inhibited by xylitol, which typically is produced by yeast cells during xylose fermentations. We have expressed a codon-optimized version of the gene in industrial yeast strains. Evolutionary engineering enabled the strains to ferment xylose efficiently.

Additionally, we have also integrated genes of a bacterial arabinose pathway into the yeast strains together with an arabinose-transporter gene from *Pichia stipitis*. Codon-optimization of the heterologous genes considerably improved pentose fermentations. To this end, we have obtained industrial yeast strains able to produce ethanol from the main pentose sugars, xylose and arabinose, present in lignocellulosic biomass.

## Oral Presentation 2-05

### Development of a Robust Yeast Biocatalyst for Low pH Lactic Acid and Cellulosic Ethanol Fermentation.

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Common characteristics are required for an economically viable biocatalyst for cellulosic ethanol and lactic acid production for commodity applications like Poly Lactic Acid (PLA). These include high yield, fast fermentation, robust growth in simple media, and tolerance to organic acids at low pH. Cargill started with non-conventional yeast naturally possessing some of these characteristics and successfully developed it to efficiently produce a new end product (lactic acid) or to ferment new sugars (pentoses). Replacing yeast's ethanol pathway with lactic acid pathway was straight forward. Development of a strain capable of producing polymer grade lactic acid at commercially interesting titer, yield and productivity required concerted utilization of genome wide tools, targeted modifications, evolution and classical mutagenesis. The developed strain and low pH fermentation process offers considerable cost savings over conventional lactic acid processes.

Cargill has previously demonstrated efficient fermentation of xylose to ethanol in yeast (USPatentApp 10/554887). We are now combining our xylose fermentation technology into the acid tolerant yeast first developed for lactic acid production. Goals have been set for ethanol production from mixed sugars (dextrose, mannose, xylose, and arabinose) in the presence of 10 g/L acetate at 40°C and at a pH less than 5.0. Under these conditions Cargill host can utilize 80 g/l of dextrose and 80 g/l of mannose in less than 36 hours, producing ~ 70 g/l ethanol. A xylose utilization pathway has been engineered into this host and efficient fermentation of xylose to ethanol demonstrated both in defined medium and in hydrolyzate.

## Oral Presentation 2-06

### Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol

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#### Background

Increasing energy costs and environmental concerns have motivated engineering microbes for the production of "second generation" biofuels that have better properties than ethanol.

#### Results & Conclusions

*Saccharomyces cerevisiae* was engineered with an n-butanol biosynthetic pathway, in which isozymes from a number of different organisms (*S. cerevisiae*, *Escherichia coli*, *Clostridium beijerinckii*, and *Ralstonia eutropha*) were substituted for the Clostridial enzymes and their effect on n-butanol production was compared. By choosing the appropriate isozymes, we were able to improve production of n-butanol ten-fold to 2.5 mg/L. The most productive strains harbored the *C. beijerinckii* 3-hydroxybutyryl-CoA dehydrogenase, which uses NADH as a co-factor, rather than the *R. eutropha* isozyme, which uses NADPH, and the acetoacetyl-CoA transferase from *S. cerevisiae* or *E. coli* rather than that from *R. eutropha*. Surprisingly, expression of the genes encoding the butyryl-CoA dehydrogenase from *C. beijerinckii* (*bcd* and *etfAB*) did not improve butanol production significantly as previously reported in *E. coli*. Using metabolite analysis, we were able to determine which steps in the n-butanol biosynthetic pathway were the most problematic and ripe for future improvement.

## Oral Presentation 2-06A

### Integration of genomics and bioinformatics to identify genetic differences in an ethanol tolerant *Clostridium thermocellum* ATCC27405 strain

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*Clostridium thermocellum* is a gram-positive, anaerobic, thermophilic bacterium that can ferment cellulose at one of the highest growth rates directly to ethanol via a large extracellular enzyme complex termed the cellulosome. *C. thermocellum* is a candidate industrial biocatalyst for future lignocellulosic fuel production. The metabolic byproducts of fermentation can inhibit fermentation performance and lignocellulosic biomass pretreatment processes also produce a variety of inhibitory chemicals that can adversely affect the fermentation. Limited information is available on the mechanisms and responses of *C. thermocellum* to different inhibitors. The genetic differences between wild-type *C. thermocellum* and an ethanol tolerant mutant have been identified through microarray based comparative genome sequencing and 454-pyrosequencing. We detected more than 400 differences in the ethanol tolerant mutant compared to the *C. thermocellum* wild-type strain. The resequencing data were in agreement with published membrane proteomic data and identified new mutations in key genes such as alcohol dehydrogenase. Bioinformatics analyses identified 16 mutational hot-spots in the ethanol tolerant strain, with 7 out of 16 related to cellulose degradation and likely accounted for the strain's decreased growth on cellulose. Further work to identify and verify important loci and physiological changes conferring tolerance to inhibitors will assist in the development of industrial strains for consolidated bioprocessing (CBP) of lignocellulosic biomass and therefore reduce biofuel production costs.

## Oral Presentation 3-01

### On Size Reduction for Woody Biomass Conversion

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Physical size reduction through mechanical means is a necessary step in bioconversion of woody biomass to increase the surface accessible to enzymes to achieve satisfactory cellulose conversion efficiency. Unfortunately, reducing wood size to typical substrate scale of millimeter requires a significant amount of electric-mechanical energy. It is estimated that the energy consumed in size reduction can be 10-30% of the total biomass ethanol energy produced using current technology.

In this study, we used various fiber fractionations, different chemical pretreatments, and mechanical milling (size reduction) processes to produce wood substrates with varied physical sizes, chemical structures and physical properties. We demonstrated a wet imaging technique to determine two dimensions of these woody fibrous substrates. The measured two dimensions were used to estimate the substrate specific surface by using a cylinder model for individual fibers. The determined substrate specific surface was related to the enzymatic hydrolysis cellulose conversion of the substrate. We also compared the effectiveness of different chemical pretreatments applied directly to wood chips (~2x3x0.5 cm) on reducing size-reduction energy consumption and enhancing enzymatic saccharification, so that the efficiencies of different chemical pretreatments and size-reduction processes can be compared objectively. It was found that Chemical pretreatment affects not only cellulose conversion efficiency, but also post-pretreatment size-reduction energy consumption and liquefaction of substrates during high solids enzymatic hydrolysis. The SPORL pretreatment process that we developed is the most efficient with cellulose conversion of over 90% and post-pretreatment wood chip size-reduction energy consumption of about 30 Wh/kg.



### Oral Presentation 3-02

#### Glucose and xylose yields from switchgrass for ammonia fiber expansion, ammonia recycle percolation, dilute sulfuric acid, hot water, lime, and sulfur dioxide pretreatments followed by enzymatic hydrolysis

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Switchgrass promises to become a major resource for making fuels and chemicals by cellulose conversion technologies. However, it must be pretreated to realize reasonable yields of sugars by enzymatic hydrolysis, but pretreatment is expensive and strongly influences cost and performance of other operations. The Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) was formed in 2000 to develop the first comparative data of sugar yields from leading pretreatment operations followed by enzymatic hydrolysis of resulting solids. The CAFI Team achieved high sugar yields from corn stover for all pretreatments but found much greater variations in performance for poplar wood with changes in pretreatment technologies and biomass source. In this project, pretreatment by ammonia fiber expansion (AFEX), ammonia recycle percolation (ARP), dilute acid, hot water, lime, and sulfur dioxide steam explosion were applied to shared sources of switchgrass, and the same enzymes, experimental protocols, and material balance approaches were employed by all the members of the team. Three types of switchgrass, Alamo, Shawnee, and Dacotah, were evaluated from different locations and harvest times to determine whether these factors influence glucose and xylose yields from the combined operations of pretreatment and enzymatic hydrolysis. Comparisons will be reported for sugar yields from pretreatment alone (Stage 1), enzymatic hydrolysis (Stage 2), and the two combined over a range of enzyme mass loadings and formulations for each pretreatment approach. These results should help select pretreatment technologies for commercial operations and define new directions to improve plants, enzymes, and pretreatment technologies.

### Oral Presentation 3-03

#### Sub- and super-critical water technology for biofuels: Switchgrass to ethanol, biocrude and hydrogen fuels

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Sub- and super-critical water (critical point: 374 °C, 221 bar) provide a novel reaction medium for the efficient conversion of lignocellulosic biomass to usable liquid and gas fuels. Recently, this medium has attracted much attention as a non-toxic, environmentally benign, inexpensive and tunable reaction medium for conducting ionic/free radical reactions. Dielectric constant of water near the critical point decreases considerably, which enhances the solubility of organic compounds. Almost complete conversion of crystalline cellulose (>90%) to water-soluble products above 330 °C in a short residence time (3-5 s) was possible, and high yield (65-67%) of hydrolysis products (glucose and oligomers) was achieved in subcritical water (335 - 354 °C) (Kumar and Gupta, Ind. Eng. Chem. Res., 2008).

Subcritical water was used for the pretreatment of switchgrass in a flow through reactor in temperature range 150 to 180 °C and pressure 35 to 136 bar. The process mainly removed hemicelluloses causing structural changes, which improved the accessibility to enzymes to cellulose. This pretreatment method can be effectively used for ethanol production.

At a higher temperature, subcritical water converts biomass to biocrude, a mixture of oxygenated hydrocarbons. Liquefaction of switchgrass for biocrude production in subcritical water (230-260 °C) was studied. More than 80% of switchgrass was solubilized in only 20 minutes.

At even higher temperature, supercritical water can effectively convert carbohydrates into hydrogen fuel (Byrd, Pant, and, Chem. Res., 2007). Biocrude produced from switchgrass liquefaction was reformed in supercritical water. The gaseous products contained mainly hydrogen and CO<sub>2</sub>.

### Oral Presentation 3-04

#### The technical advantages and challenges of ionic liquid-based biomass pretreatments

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The dissolution and derivitization of cellulose in ionic liquids (ILs) has been demonstrated at laboratory scale. The use of ILs to totally dissolve lignocellulosics and to selectively dissolve lignin in biomass have also been reported. Proposed methods of IL and biomass recovery based on anti-solvent addition complete descriptions of novel closed-loop IL-based biomass pretreatments that have advantages over more conventional processes. For example, the IL-based processes are relatively rapid and are conducted at atmospheric pressure. Furthermore, cellulosic fractions recovered from IL-based pretreatments are more amenable to enzymatic hydrolysis than those recovered from other pretreatments. However, there remain several technical and economic barriers to the use of IL processes in industrial settings. The technical advantages and challenges of ionic liquid-based biomass pretreatments are described.

### Oral Presentation 3-05

#### Ultra-structural and physicochemical modifications within ammonia pretreated lignocellulosic cell walls that influence enzyme accessibility

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The development of an economically viable and environmentally sustainable bio-based chemical industry has been impeded due to the native recalcitrance of lignocellulosics to chemical and biological processing. Lower severity ammonia based pretreatments (e.g. AFEX) and minimizing enzyme usage could help reduce processing costs. However, unlike other pretreatments AFEX does not extract lignin and hemicellulose into separate liquid fractions. Instead, AFEX enhances enzymatic digestibility through certain ultra-structural and chemical modifications within the cell wall that are currently not well understood.

An important goal of this research was to identify the major ultra-structural and chemical modifications incorporated within lignocellulosic cell walls during AFEX using several microscopic, spectroscopic and spectrometric techniques. High resolution microscopic (SEM, TEM) and 3D-EM-Tomographic studies indicate an ultra-structural alteration of AFEX treated cell walls via formation of a nanoporous tunnel-like network. Closer analysis (via ESCA, AFM and confocal fluorescence microscopy) of outer cell wall surfaces shows heterogeneous deposits rich in AFEX cell wall extractives. Raman spectral data indicates conversion of cellulose I to III is intricately dependent on AFEX pretreatment conditions. More than 45 degradation products have been quantified using LC-MS/MS and GC-MS. Some of the major degradation products include organic acids, aromatics, phenolic acids and amides.

A fundamental understanding of physicochemical modifications incorporated within lignocellulosic cell walls during pretreatment and its effect on enzyme accessibility are critical to further advancements in reducing cell wall recalcitrance to bioprocessing. This understanding would be critical to re-engineer plant cell walls, hydrolytic enzymes and ethanologenic microbes amenable for cellulosic biorefineries.

### Oral Presentation 3-06

#### A novel biochemical platform for fuels and chemicals production from cellulosic biomass

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One significant obstacle impeding the large scale production of fuels and chemicals from cellulosic biomass is the lack of a low cost processing technology. The conventional biochemical platform for biorefinery involves four distinct steps: pretreatment, enzymatic hydrolysis, fermentation, and product recovery. Sugars are produced as the reactive intermediate for the subsequent fermentation. Steps involved with overcoming the recalcitrance of cellulosic biomass (pretreatment and enzymatic hydrolysis) are the two most costly steps in the whole process. Here we propose a novel biochemical platform for fuels and chemical production that will replace the two most costly steps in the conventional platform with a single biological step. Cellulolytic microorganism(s) that can secrete all the enzymes needed to hydrolyze cellulose and hemicellulose in spite of the presence of lignin will be modified to convert most of the carbohydrate contained in the cellulosic biomass to sugar aldonates. In a second step, sugar aldonates will be utilized as the carbon source to produce ethanol and other products. The new platform can potentially lower the cost of cellulosic bioprocessing substantially. Factors contributed to the cost reduction include elimination of the high capital cost and high operating cost associated with thermo-chemical pretreatment process, consolidation of the process, and reduction of the product recovery cost due to higher ethanol concentrations produced in the fermentation step. Feasibility study has demonstrated that sugar aldonates can be produced from modified *Neurospora crassa* under simulated conditions; and sugar aldonates can be converted to ethanol at high efficiency and at high yields by fermentation.

### Oral Presentation 4-01

#### Genetic dissection of bioenergy traits in sorghum

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Sorghum has a number of characteristics that make it a very attractive biomass crop for ethanol production: low water and fertilizer requirements, tolerance to heat and drought, high biomass yield, and great genetic diversity. Two traits of particular interest are the sweet sorghum trait, which results in the accumulation of fermentable sugars in the juice of the stems, and the *brown midrib* (*bmr*) trait, which changes the color and the chemical composition of the vascular tissue, and results in higher yields of fermentable sugars obtained after enzymatic saccharification of the lignocellulosic biomass. The genetic basis of these traits, however, is poorly understood and impedes the full exploitation of sorghum as a bioenergy crop. High throughput expression profiling using 454-sequencing is being applied to identify the gene(s) underlying a recently mapped quantitative trait locus (QTL) for stem sugar concentration. In addition, we are developing a population of recombinant inbred lines to map QTL for juice volume. To identify novel genes affecting cell wall composition, we are using a sorghum TILLING population to identify mutants, including *bmr* mutants, with improved saccharification properties. The *Brown midrib* genes from the most promising mutants are being cloned using a candidate-gene approach. This approach recently resulted in the identification of *Bmr6* as the gene encoding cinnamyl alcohol dehydrogenase2. These combined approaches will enable the development of sorghums that offer maximum flexibility for the production of food, feed, fiber and fuel. Funding from the US Department of Energy for this project (DE-FG02-07ER64458) is gratefully acknowledged.

### Oral Presentation 4-02

#### *Acidothermus cellulolyticus*: From genome sequence to plant cell wall deconstruction

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The 2.4 Mb genome of the cellulose-degrading thermophile, *Acidothermus cellulolyticus* 11B, was completely sequenced by the Joint Genome Institute (DOE) in 2006. The genome offers useful insights into the lignocellulose deconstruction capabilities of the organism. The *A. cellulolyticus* genome revealed several new secreted glycoside hydrolases and carbohydrate esterases, indicating a diverse biomass-degrading enzyme repertoire, and significantly elevating the industrial value of this organism. A sizeable fraction of these hydrolytic enzymes break down plant and fungal cell walls. Findings from functional enzyme studies and insights into the evolutionary and functional implications of the *A. cellulolyticus* cellulolytic and xylanolytic capabilities will be presented.

### Oral Presentation 4-03

#### Community structure and functional diversity of thermophilic cellulolytic microbial consortia

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In nature, cellulose degradation is performed by complex microbial communities in synergistic fashion. The enrichment in mixed cultures from nature of new microbial consortia with high cellulolytic activity is essential in the identification of novel organisms, novel metabolic capabilities and novel functions that will enhance our fundamental understanding of how the potential benefits of CBP (consolidated bioprocessing) can be realized at the industrial scale. In this work we identify the key players in cellulolytic enrichment cultures with thermophilic compost as an inoculum. Community structure and functional diversity have been characterized with clone libraries targeting both the 16S rRNA gene and glycosyl hydrolase gene fragments from mixed microbial cultures. Our studies have revealed varying levels of diversity and community composition, with a narrower range of novel and very specific clostridial cellulose degraders playing the main functional role in these cultures.

### Oral Presentation 4-04

#### Discovery of genes that mediate and regulate hemicellulose biosynthesis

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One attractive strategy for identifying the required genes is to perform expression profiling during periods of rapid hemicellulose deposition. Many plants produce large quantities of specific polysaccharides as storage polymers in developing seeds. Earlier studies of these seed systems has been used to identify proteins involved in mannan and xyloglucan biosynthesis. We are extending these earlier studies by using 454 sequencing technology to perform deep EST sequencing at various stages of seed development during and just before rapid synthesis of mannan (*Fenugreek-Trigonella foenum-graecum*), xyloglucan (*Nasturtium-Tropaeolum majus*), or arabinoxylan (*Psyllium-Plantago ovata*). Analysis of the sequences obtained has confirmed the expression of genes known to be involved in the biosynthesis of these polysaccharides. In addition, a number of other genes have emerged as strong candidates for involvement in the production of these polysaccharides or in regulation of these pathways. Among the candidates that have been identified are putative sugar nucleotide biosynthetic enzymes, putative sugar nucleotide transporters, putative and known glycosyltransferases and glycan synthases, proteins of unknown function, and transcription factors. Many of the genes have homologs that are expressed in developing wood or in other plant tissues where secondary wall synthesis is occurring rapidly, providing support for the hypothesis that the same genes are involved in depositing these polymers in secondary cell walls. Promising examples from each class of candidate genes have been selected for detailed functional analysis. Selected examples from each polysaccharide will be presented on the poster.

## Oral Presentation 4-05

### Mining the metatranscriptome of the rumen microbiota for feedstock-targeted glycosyl hydrolases

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Stable and highly active cellulolytic enzymes are essential for the efficient conversion of lignocellulosic biomass into fermentable sugars. Natural cellulolytic systems such as the bovine rumen are known to harbor fibrolytic microbes and represent promising sources of enzymes for biomass degradation. In the project presented here, pyrosequencing has been employed to identify feedstock-targeted enzymes within the transcriptome of rumen microbial communities.

Switchgrass and alfalfa were incubated for 72 hr in the bovine rumen and nucleic acids were extracted from the fiber-associated microbial communities. Based on 16S rRNA sequencing, the microbial community tightly associated with switchgrass differed significantly from that associated with alfalfa, suggesting that distinct sets of organisms are involved in degrading each of these two feedstocks.

Expression profile of the switchgrass-associated organisms was determined by 454-pyrosequencing. We identified 85 highly expressed putative glycosyl hydrolases and 201 unique glycosyl hydrolase transcripts. ~4,000 genes without assigned function were highly expressed and some of them might encode truly novel proteins involved in biomass degradation. We will expand our analysis to expression profiles of rumen microbial communities associated with other biofuel crops.

The results obtained in the course of our project indicate that the fiber-bound microbes are indeed a rich source of putative cellulolytic enzymes that might be useful for large-scale biofuel production. Currently we are developing techniques to capture the full-length sequence of selected transcripts from rumen community DNA. Expressing the recombinant proteins and subjecting them to detailed physicochemical characterization will allow us to verify the sequence-based annotation of the transcript tags.

## Oral Presentation 4-06

### Discovery of Switchgrass Genes through Genomics to Improve Biomass Composition and Conversion

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The ability to manipulate the relative proportions of cell wall components is critical to improving a bioenergy crop like switchgrass. An essential step towards this goal is to understand the genetic components involved in cell wall biosynthesis. To address this problem, we are using genomics tools to discover and select genes from switchgrass. We are using two parallel approaches in the selection process: (1) identification of differentially expressed genes through gene expression analysis utilizing GRASS chip technology, and (2) misexpression analysis in Arabidopsis to evaluate the potential effects of identified genes on cell wall composition & conversion and on biomass accumulation. In this poster, we will present preliminary data to demonstrate the effectiveness of our selection efforts to identify candidate genes that can be used to transform and manipulate switchgrass to improve conversion efficiency to biofuels.

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## Oral Presentation 5-01

### Thermostable fungal lignocellulosic biomass saccharification enzyme cocktail

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Lignocellulosic biomass is the most abundant, least expensive renewable natural biological resource for the production of biobased products and bioenergy is important for the sustainable development of human civilization in 21st century.

For making the fermentable sugars from lignocellulosic biomass, a reduction in cellulase production cost, an improvement in cellulase performance, and an increase in sugar yields are all vital to reduce the processing costs of biorefineries. Improvements in specific cellulase activities for non-complexed cellulase mixtures can be implemented through cellulase engineering based on rational design or directed evolution for each cellulase component enzyme, as well as on the reconstitution of cellulase components. In this presentation, we will update on DSM efforts on developing thermostable enzyme cocktail for saccharification of lignocellulosic biomass.

## Oral Presentation 5-02

### Impact of solids loading on the economics of a lignocellulosic biomass to ethanol conversion process

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Technoeconomic modeling describes the impact of performance tradeoffs on process economics and helps guide research efforts. A rigorous model in Aspen Plus was used to compute material and energy balances for a biomass-to-ethanol conversion process using dilute acid pretreatment of corn stover, separate enzymatic cellulose hydrolysis and fermentation, and ethanol distillation. Subsequent economic analysis determined the minimum ethanol selling price (MESP) for the process, assuming nth-plant equipment and operating costs. To understand the cost impact of solids loading in the hydrolysis step, a correlation of cellulose conversion as a function of enzyme and solids loading was developed from bench-scale enzymatic hydrolysis experiments on pretreated corn stover slurries. Higher solids processing should show more favorable economics, since stream volumes are reduced and less energy is required to separate the product from water. However, using the correlation it was found that the MESP rises at high solids loading due to a drop in cellulose conversion yields. For assumed enzyme costs of \$10-\$15/kg protein, the minimum MESP was between 15-20% total solids. For projected costs of \$2.50-\$5/kg, the minimum MESP occurred at 25% total solids, and was relatively flat from 15-25%. Only in the ideal case where conversion was independent of solids loading did the MESP decrease monotonically from 5 to 30% total solids. These results indicate the economic benefit of processing at higher solids loading, but highlight the need to develop enzymes that maintain conversion yields at high solids.

## Oral Presentation 5-03

### Substrate-based Limitations in the Enzymatic Hydrolysis of Cellulose: Crystallinity, Reactivity and Adsorption

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The enzymatic hydrolysis of crystalline cellulose encounters various limitations that are both substrate- and enzyme-related. They directly impact the rate of the reaction and lead to its dramatic slowdown, observed especially at high degrees of conversion. Although the initial crystallinity of cellulose plays a major role in determining the rate of hydrolysis, it was shown not to evolve over the time of conversion by cellulases, implying other reasons for the decrease in rate. Using increasing concentration of phosphoric acid to generate acid-swollen Avicel, samples with intermediate crystallinity indexes were obtained and their subsequent enzymatic hydrolysis gave a clearer overview of the relevance of the initial degree of crystallinity on reaction rate. Change in adsorption capacity and decrease in reactivity along conversion were also confirmed to be involved. Reactivity (measured as of glucose production rate from restart experiments) experienced a serious drop already after 5% conversion, supporting the hypothesis that the cellulose surface has been modified by the action of the enzymes. Both X-ray diffraction and solid state CP/MAS 13C-NMR were employed and gave insight into molecular changes occurring with cellulose along the conversion. The (021) face was shown to be converted first by pure cellobiohydrolase. Strategies to improve the overall reaction rate will be presented.



## Oral Presentation 5-04

### Characterization of novel bacterial expansins that promote enzymatic hydrolysis of plant cell wall polymers

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Plant cell wall polymers, which are considered to be the most abundant renewable resource on earth, are mainly composed of cellulose, hemicellulose and lignin. Due to the recalcitrance of cellulose itself and the protective barriers of lignin, the enzymatic hydrolysis of cellulose and hemicellulose to obtain sugars has long been a challenge. In the process of cell growth, a plant cell wall protein, expansin, is found to be involved in inducing extension of cell wall without hydrolysis. In our study, the functions of novel expansins from bacterial sources, which are the structural homologs of maize expansin, EXPB1, were targeted and elucidated for the first time in this area (Kim, E. S. et al., J. Biotechnol. 136S: S426, 2008; H. J. Lee et al., J. Biotechnol. 136S: S343, 2008; Kim, E. S. et al., Biotechnol. Bioeng. In press) since expansins only from eukaryotic sources such as plants, animals or fungi were so far functionally characterized. Many eucaryotic expansins were found, but none of their overexpression in microorganisms has been successful yet. The bacterial expansins expressed in a soluble form in the present work showed binding and weakening activities towards cellulose or xylan and also exhibited significant synergistic activity with enzymes in the hydrolysis of cellulose or xylan. These findings might have opened a door to the possible applications of bacterial expansins in effective enzymatic conversion of lignocellulosic biomass into sugars.

## Oral Presentation 5-05

### Industrial level production of enzymes by *Trichoderma reesei* for cellulosic bioethanol

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Efficient expression is the key for economically-viable bulk production of enzymes. Filamentous fungi naturally secrete large amounts of proteins into the growth medium and are commonly used for the large-scale manufacture of proteins, particularly industrial enzymes (1). However, although other fungal proteins are efficiently expressed, expression of gene products from other organisms such as thermophilic bacteria, is subject to a number of bottlenecks that reduce yield. Following a proteomic analysis of the *Trichoderma reesei* secretome, we constructed four expression vectors that utilise either the *cbh2* or *egl2* promoters. Thermophilic xylanases are of particular interest for efficient hydrolysis of the hemicellulosic component of woody materials into fermentable pentose sugars (2). The *xynB* gene encoding xylanase B from the thermophilic bacterium *Dictyoglomus thermophilum* has been inserted into the vectors for heterologous expression as a model system. This enzyme is particularly effective in the hydrolysis of both soluble and insoluble xylan in hemicellulose. The codon usage of the *xynB* gene has been modified for expression in *T. reesei*. Expression of *xynB* from the pEG2-cbmlin and pCBH2-sigpro vectors was found to be greater than that using the EG2-sigpro and CBH2-cbmlin constructions, based on zymogram analysis and liquid enzyme activity assays. We will discuss the ability of the new promoter constructions to drive xylanase production as influenced by the structural differences in the expression cassette motifs and their contribution to improved yields under fermentor conditions (1) Nevalainen et al (2005), Trends Biotechnol. 23: 468 (2) Viikari et al (2007), Adv. Biochem. Eng. Biotech. 108: 121

## Oral Presentation 5-06

### Use of palm kernel press cake for production of bioethanol and feed

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Palm kernel press cake (PKC) is a residue from palm oil extraction, composed mainly of polysaccharides from cell-wall material, of which the carbohydrate mannan is the most predominant. Due to increased demand for plant oils globally there has been a tremendous increase in palm oil production in recent years. In Indonesia and Malaysia large amounts of palm kernels are processed at centralised plants, which make PKC an ideal feedstock for further processing into e.g. bioethanol or other biorefinery processes. At present the PKC is only used for feeding purpose, but due to the high content of mannan, it cannot be included in high amounts in broiler diets.

Mannose can readily be fermented to ethanol by normal yeast *Saccharomyces cerevisiae*. The first step in the conversion of PKC is then an efficient hydrolysis of polymers, mainly mannan, into fermentable monomers. The present study examines the pretreatment requirements and especially the enzymatic hydrolysis of polysaccharides from the cell-wall material present in PKC by various enzyme preparations in order to achieve high amounts of free monosaccharides. The process has been tested at high solids concentrations and it was possible to operate at up to 50% DM. The resulting hydrolysates were easily fermented by *Saccharomyces cerevisiae* with high ethanol yields. Various process configurations (SHF and SSF) were also tested. In addition, the processing of PKC resulted in a new feed product with increased protein content, which could be beneficial for the feeding value of this new residue.

## Oral Presentation 6-01

### Metagenomics for mining new deconstructive enzymes, exploring enzyme diversity and screening cellulolytic activities

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Plant biomass is the most abundant biopolymer on earth and has long been recognized as a potential source of mixed sugars for bioenergy production. Our goals are to understand the diversity and metabolic capabilities of the complex microbial communities, and to exploit their dynamics for converting plant biomass into feedstock for biofuels production. Metagenomics allows the discovery of new enzymes from microbial communities, especially from organisms that are unknown or have never been cultivated. From an anaerobic microbial community actively decaying poplar biomass, metagenomic DNA was isolated and microbial species distribution was investigated via 16S and 18S rRNA sequencing. *Saccharomycetes* composed the major group among the Eukaryotes, and *Clostridiales* composed the major group among the Bacteria. No major population of Archaea was found in this microbial community. Using the 454-GS-FLX Titanium pyrosequencing, approximately 580Mbp metagenomic DNA was sequenced. Preliminary blastx searches identified approximately 4,000 glycosyl hydrolase homologues. Five candidates were selected for further investigation based on homology to enzyme families of interest (families 5, 9, 48, and 51 representing cellulase, hemicellulase, and xylanase activities) and quality of sequences. Full-length open reading frames were obtained using inverse PCR and DNA walking, and gene cloning is presently in process. A lambda-based expression library of one isolated strain from the community was also constructed and enzyme activity screening is in process. Our metagenomic studies successfully provided insight into the microbial community composition as well as a resource of diverse, community-encoded glycosyl hydrolases, for mining new deconstructive enzymes and screening cellulolytic activities.

## Oral Presentation 6-02

### Cellulolytic Extreme Thermophiles and Hyperthermophiles from Terrestrial Hot Springs

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Extremely thermophilic microorganisms are good candidates to provide highly stable, active enzymes for cellulose deconstruction. Elevated operating temperatures may also be beneficial in fermentations producing biofuels. To that end, cellulose-degrading microorganisms from hot springs in Nevada and Northern California were enriched on anaerobic medium containing ground (80 mm diameter particles) *Miscanthus sinensis* as the sole carbon source. Enrichments were performed at temperatures between 70°C and 90°C. Organisms growing in the enrichments were identified by 16S RNA clone libraries generated from PCR amplification of 16S coding sequences from DNA extracted from the enrichments, or 454 sequencing with bar-coded primers from the same DNA. Enrichments at 70°C contained a variety of thermophilic bacteria related to strains known to be cellulolytic as well as some that were not closely related to any characterized strain and may represent new genera of cellulolytic organisms. In order to verify that cellulolytic organisms were present in *Miscanthus* enrichments, secondary enrichments were made using Whatman #1 and #3 filter paper as the carbon source and a portion of the primary enrichments as the inoculum. At 90°C the dominant microorganisms present in *Miscanthus* or filter paper enrichments were archaeal, establishing a role for archaea as cellulose degrading organisms and as potential targets for the identification of new cellulases. Cellulase assays were performed to determine the activity of free and bound cellulases in each of the enrichment cultures. The media constituents were refined to promote accelerated cellulose degradation, and pure cultures, including archaeal species, were isolated from the enrichments.

## Oral Presentation 6-03

### Mining *Clostridium thermocellum* for Enzymatically Active Carbohydrases

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The efficient hydrolysis of biomass to 5 carbon and 6 carbon sugars is limited by the lack of affordable, high specific activity enzymes. Screening of genomic and metagenomic libraries for new biomass-degrading enzymes has had only limited success. We examined a number of screening strategies using *Clostridium thermocellum* (Cth) as a target-rich model organism to validate the efficiency of capturing carbohydrases that may prove useful for biomass degradation. The Cth genome has been sequenced and is predicted to have genes for over 60 potential biomass-degrading enzymes associated with the cellulosome, and another 18 enzymes that are noncellulosomal. Two different cloning systems were used for gene expression and two different screening methods were utilized for identification of positive clones. A comparison of the methods showed large differences both in the total number of positive clones identified as well as large differences in the total number of different enzymes captured. This poster also describes the gene products that were captured by the individual screens.

## Oral Presentation 6-04

### Genome shuffling of *Penicillium decumbens* to improve its cellulase production

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Genome shuffling is an efficient approach for the rapid improvement of important industrial microorganisms. The cellulase production of *P. decumbens* was improved by genome shuffling of an industrial catabolite-repression-resistant strain JU-A10 with its mutants. The mutants were obtained by UV-EMS mutation or N<sup>+</sup> ions implanting of JU-A10, and prepared for protoplasts fusion. Six improved fusant strains were selected as parents for the second genome shuffling. Three fusants, GS2-15, GS2-21 and GS2-22, were selected based on their capacity to show clear hydrolysis halo on the two-layer plate containing 2% glucose and 5% ball-milled microcrystalline cellulose. The fusants showed 100%, 109% and 94% increase in filter paper activity, respectively. The cellulase production of the fusants on various substrates, such as corn stover, wheat straw, bagasse and the corncob residue from xylitol production, were studied. It was obvious that the three fusants could produce abundant cellulase much earlier than the parental strain JU-A10, the maximum volumetric productivity of GS2-15, GS2-21 and GS2-22 was 92.15, 102.63, and 92.35 FPU/L/h respectively when fermented with the corncob residue at 44 h, which was 117%, 142%, 118% higher than that of JU-A10 (42.44 FPU/L/h at 90 h). Higher glucose yield from the corncob residue were also observed by using the fermented broth of the fusants as crude cellulase. The improved cellulase production of the fusants was proposed to be mainly due to their increased growth rates and enhanced secretion of extracellular proteins.

## Oral Presentation 6-05

### Development and characterization of xylose-fermenting strains of *Saccharomyces cerevisiae* based on structure-based engineering of key metabolic enzymes

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Metabolic engineering of *Saccharomyces cerevisiae* for xylose fermentation has often relied on insertion of a heterologous pathway consisting of NAD(P)H-dependent xylose reductase (XR) and NAD-dependent xylitol dehydrogenase (XDH). Low ethanol yield and formation of fermentation by-products such as xylitol and glycerol seen for many of the strains constructed in this way have been ascribed to incomplete coenzyme recycling in the steps catalyzed by XR and XDH. We have used structure-guided engineering of *Candida tenuis* XR and *Galactocandida mastotermitis* XDH to obtain enzyme pairs that display well matched utilization of NAD(H) and NADP(H). Yeast strains producing XR and XDH variants that show altered coenzyme selectivity exhibit notably improved fermentation capabilities as compared to the reference strain expressing the genes for the wild-type enzymes.

## Oral Presentation 6-06

### Understanding the Relationship of Toxic Compounds in Corn Stover Hydrolysates and Their Inhibitory Effects on Ethanologen Growth and Fermentation

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Overcoming the effects of hydrolysate toxicity after pretreatment and/or enzymatic hydrolysis is a key technical barrier in the biochemical conversion process for biomass feedstocks to ethanol. Yet, the complexity of the hydrolysate toxicity phenomena and the lack of systematic studies and tools surrounding this issue has prevented us from fully understanding relationships involving toxic compounds in hydrolysates, their relative inhibitory effects on ethanologen growth and fermentation, and the impact of various conditioning approaches to effectively mitigate these inhibitory effects. We conducted systematic studies to analyze chemical composition of the hydrolysates and developed quantitative, high throughput biological growth assays to obtain the inhibitory kinetics for individual compounds, along with correlation of growth and fermentation performance in conditioned diluted acid corn stover hydrolysates and hydrolysate fractions. These key findings provide important insights for understanding hydrolysate toxicity and provide guidance for potential process development in both pretreatment and hydrolysate conditioning operations, along with potential future strain improvement and tolerance strategies. The tools that have been developed can also be more broadly applied to other feedstock and pretreatment process situations as well as other ethanologens.

## Oral Presentation 7-01

### Commercialization of Second Generation Biofuels: An Independent Engineer's View

Doug Dudgeon, Harris Group, Seattle, Washington

The renewable fuels industry, which experienced steady progress through first generation biofuels such as corn based ethanol and oil seed based biodiesel has slowed significantly in the commercialization of second generation technologies including cellulosic ethanol and algae based biodiesel. While the base technologies are promising, there are structural issues that go beyond technology development which impede implementation. The purpose of this presentation is to provide a detailed background on the way large scale renewable energy technology is financed and implemented, outlining a roadmap for stakeholders bringing these technologies to market.

A brief overview of the factors that allowed first generation biofuels to grow rapidly will be covered and contrasted with the factors that are limiting next generation technologies. A review of the capital cost of emerging biofuel technologies will be provided, along with a summary of the engineering approach and construction contracting options, and how they can impact the ability to gain financing. Common forms of equity and project financing will be reviewed. Finally, the topics will be summarized with a roadmap of how large, commercial scale emerging biofuel projects can be moved forward. This presentation comes from direct experience by the author in taking an emerging technology from pilot scale to a full size commercial facility.

## Oral Presentation 7-02

### Successful commercialization of second generation biorefineries

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Successful commercialization of second generation biorefineries will require navigating significant technical, commercial and supply chain challenges. This presentation will review the technical challenges and report on progress to date on the integration and optimization of technologies from parent companies and others. It will also report on the innovations in business models and collaborations that will mitigate the commercial risk of pioneer deployment.

## Oral Presentation 7-03

### Development and Deployment of Consolidated Bioprocessing for Production of Ethanol

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Consolidated Bioprocessing ("CBP") employs single microorganism that simultaneously generates sugars and produces ethanol from wood and other forms of pretreated lignocellulosic biomass. Pretreatment opens up the structure of wood by disrupting the lignin seal and exposing cellulosic plant cell wall components. This enables CBP microorganisms to access the cellulosic constituents, hydrolyze them, and produce ethanol. The microorganisms not only ferment sugars to ethanol, but also generate the biocatalysts – enzymes – that are needed to break down cellulose into fermentable sugars. Mascoma's research is combining naturally occurring metabolic activities into a single microorganism by modifying the fermentative pathways of nature's most efficient processors of cellulose, including the thermophilic anaerobic bacterium, *Cl. thermocellum*, to produce high yields of ethanol from hardwoods and biomass feedstocks. In addition, the ability to modify the fermentative pathways of a thermophilic anaerobe to achieve high ethanol yield from sugars was previously demonstrated through metabolic engineering of *T. saccharolyticum*. The practical application of Consolidated Bioprocessing is based on combining new biotechnology and unique but established process engineering. We discuss the four basic steps convert wood to ethanol; (1) feedstock preparation (chipping); (2) simple pretreatment of wood to make it accessible to microbial action; (3) fermentation to ethanol; and (4) product separations for recovery of fuel-grade ethanol and lignin. The design of a commercial facility is being informed by pilot and demonstration scale validation of fermentation parameters and designs that have evolved from work of NREL, DOE and USDA sponsored programs in both the public and private sectors.

## Oral Presentation 7-04

### Demonstration plant scale production of lignocellulosic ethanol

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The Energy Independence and Security Act (EISA) of 2007 calls for the blending of at least 36 billion gallons of biofuels in 2022. The Act mandates that cellulosic biofuels are to contribute 16 of the 36 billion gallons by 2022 with 100 million gallons by 2010. A number of companies have announced plans to build and operate pilot and demonstration facilities in the United States to validate proprietary cellulosic technologies at scale. Verenium completed construction of a 1.4 MM gal/yr facility in mid 2008 and has been in the process of commissioning the plant. This talk will describe and discuss progress towards completion of the commissioning of Verenium's demonstration plant and commercialization efforts in order to achieve the targets set forth in the EISA of 2007.

## Oral Presentation 7-05

### Ethanol from wheat straw – A reality in Denmark from November 2009

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In Denmark, DONG Energy subsidiary Inbicon is constructing a plant to demonstrate their proprietary process for conversion of wheat straw to ethanol to be ready for the Copenhagen Climate Summit late 2009. From 30,000 tonnes of wheat straw annually, the plant will produce 1.43 mill. gallon ethanol, 8,250 tonnes of biopellets and 11,100 tonnes of cattle feed. The investment is \$56 mill. of which \$14 mill. is funded by the Danish government.

In the 1990's, Danish power companies started using biomass for power production. In 2002, a R&D project ("Co-production biofuels") partly funded by the European Commission was initiated to extract more value of the straw. Several technological breakthroughs were achieved and a 1 ton/hr straw pilot plant was inaugurated in 2005. Based on the success of the project, the subsidiary Inbicon was formed to commercialize the technology.

The core technology, a hydrothermal pretreatment and enzymatic hydrolysis, works at high dry matter content, enabling efficient liquefaction with low enzyme doses, a robust fermentation and resulting high ethanol concentration. In addition to ethanol, the process produces a supreme dry biofuel suitable for bio pellets and a C5-molasses, which can be used for animal feed or ethanol production with suitable organisms.

The demonstration plant is the first stage of the Inbicon Biomass Technology Campus. Additional technologies for production of high value products from biomass will be developed, tested and added to the ethanol facility, making the plant an industrial scale biorefinery.

## Oral Presentation 7-06

### Third generation biofuels from lignocellulosic biomass materials

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In the presentation we will present our integrated concept for production of biofuels and bioproducts using both bio-chemical as well as thermo-chemical reactions. Wheat straw will be our base raw material. The initial step will consist of a pretreatment constructed to make the carbohydrates ready for enzymatic hydrolysis. This pentose fraction can be collected by separation from this stream and used for bioethanol production or as a feedstock for production of new chemicals using microbial catalysts producing chemicals such as succinic acid, acetoin, threonine. In the presentation we will show data from bioethanol production in pilot scale using *Thermoanaerobacter* BGL1. Furthermore, pentoses can be converted using chemical catalysis into other chemical products, for example, furfural, furfural alcohol, and levulinic acid and its derivatives. The remaining stream contains the solids including the polymers of carbohydrates which can be hydrolyzed by cellulases into mainly glucose and minority portion of other sugars. Lignin-containing residues from biorefineries have previously received little attention except as a fuel for producing the power for fuelling the biorefinery. In the presentation we will show potential alternative ways to thermochemically convert lignin-containing residues into 1) advanced biofuels (hydrocarbons), for example via pyrolysis and/or hydrothermal liquefaction and downstream processing, 2) methane, for example via wet gasification, or 3) chemical products. We will further show our initial economical assessment of the different options and how these different steps will affect the overall economics of the biorefinery.

## Oral Presentation 8-01

### An up-to-date overview and comparison of sustainability certification schemes for biofuels

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Recently, the use of biofuels is heavily criticised, doubting its GHG impact and potential to reduce emissions. Other objections relate to the possible negative impacts on water, biodiversity and (direct and indirect) land-use changes due to biomass production for biofuels. While the main concerns currently are focussed on especially first generation feedstocks, they are also highly relevant for second generation lignocellulosic feedstocks. With the strong growing increase in biofuels demand, the need to secure the sustainability of biofuels is acknowledged by various stakeholder groups. Developing principles and establishing certification schemes are recognized as possible strategies that help to ensure the sustainable production of biofuels. This paper presents an up-to-date overview of the wide range of efforts undertaken towards the development of sustainability principles, criteria, indicators and biomass certification systems. The stakeholder groups included are governments, international bodies, NGOs and companies. The paper focuses on which key differences are found between the criteria formulated, the methodologies developed and the certification systems envisioned by the various initiatives. Their feasibility, cost effectiveness, and contribution to the removal of trade barriers are evaluated. Special attention is given to (partially) conflicting methodologies, e.g. differing GHG emission calculation methodologies, which can cause major differences in total avoided emissions. The paper analyses these developments and provides recommendations on possible harmonization efforts, with a specific focus on the current developments in the European Union. Within this context, comments and possible solutions to overcome different approaches are obtained from different stakeholder groups as industry and policy makers.

## Oral Presentation 8-02

### International trade in lignocellulose biomass

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The future use of bioenergy will mostly focus on solid biofuels such as woodpellets and agri- products, as well as liquids for power generation (vegetable oils). Looking at the future developments and intertwining, the raw materials market will also be the source market for the second generation bio liquids such as bio ethanol and biodiesel. In the following graph the interfacing between those markets is highlighted. The distinction between upstream, midstream and downstream is made to clearly split market activities and areas (see fig. 1)

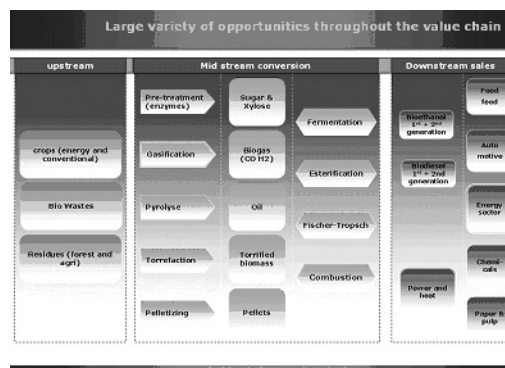


Fig 1.

This presentation will discuss the expected future developments in the international trade of lignocellulosic feedstocks for energy purposes, including the required logistics and the potential impact of the advent of large-scale second generation biofuels production. Moreover, the importance of sustainable sourcing will be highlighting, presenting the Green Gold Label for Solid biomass as an example of a track-and-trace certification system for sustainable biomass, including production, processing, transport and final energy transformation.

On the author:

Peter - Paul (P.J.W.G.) Schouwenberg, Director Biofuels and Development at Duferco Energy International and Task leader of IEA Bioenergy Task40, has long-term experience in the international sourcing of large quantities of solid and liquid biomass for electricity production. In his former position at essent, he was directly involved in the development of the Green Gold Label.



### Oral Presentation 8-03

#### Food and fuel: Investigation into integrating animal feed production with cellulosic ethanol from switchgrass

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As commercial cellulosic ethanol becomes a reality, concerns regarding land use are becoming increasingly prevalent. Critics claim a large cellulosic ethanol industry will result in decreased land available for food production, leading to increased food prices as well as increased carbon emissions due to indirect land use change. However, these concerns assume a "business as usual" approach to farm management. If bioenergy is to become a significant portion of agriculture, then it is likely that the animal feed market will be adapted to reflect these changes. Dedicated energy crops such as switchgrass have the potential to produce more carbohydrates and protein per acre than corn and soy, respectively, thus leading to the possibility that these crops could be integrated into animal feed rations. These feed co-products could be produced on-site at the biorefinery, or more likely at a regional biomass processing center (RBPCs).

We propose several methods for integrating animal feed production with cellulosic ethanol from switchgrass using ammonia fiber expansion (AFEX) pretreatment technology. AFEX-pretreated fiber can significantly increase the digestibility and feed quality of grasses, and thus potentially compete with more traditional energy feeds. Extracting proteins from grasses prior to pretreatment as well as recovering proteins after hydrolysis and fermentation will also be considered. Of particular importance is the quality and digestibility of the proteins, particularly for the essential amino acids. The potential for producing animal feed co-products is considered for both on-site production as well as at RBPCs, and the consequences of each taken into account.

### Oral Presentation 8-04

#### Sourcing Stover: Results from the ISU Integrated Corn Stover Feedstock Supply Systems Project

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Corn stover is widely recognized as the most promising high volume, low cost lignocellulosic feedstock on which to base second generation biofuel production. However, several significant challenges confront this vision. This talk will summarize the results of a four year effort aimed at: (1) developing innovative harvesting and storage technologies to efficiently and economically move corn stover from the field to the factory gate with physical and chemical properties optimal for the conversion processes; (2) identifying genetic varieties of corn with specific properties attractive for biobased industries to enable a breeding program to enhance those properties; and (3) evaluating and optimizing systems of production, harvest and storage for efficiency, and economic and environmental sustainability.

### Oral Presentation 8-05

#### A Life Cycle Impact Assessment Framework for Characterizing Human Health Benefits and Impacts from Emerging Biofuels

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Life Cycle Impact Assessment (LCIA) provides an assessment framework that addresses both benefits and impacts from emerging biofuels on human health from changes in chemical emissions. LCIA addresses the links among emissions, transport, human exposure, and health damage. We have adapted LCIA to compare the impacts of emerging biofuels, such as cellulosic ethanol and butanol, to California reformulated gasoline. This presentation describes the development of characterization factors (CFs), which yield important information on the human health effects and human damage related to the emissions-fate-exposure pathway of chemicals associated with these fuels. CFs track chemical releases to the environment at various fuel life stages including: 1) from drilling and/or harvesting to a refinery; 2) conversion and processing of fuel at a refinery; 3) storage, transport and distribution of the fuel; and 4) fuel combustion. CFs incorporate both an environmental fate factor and the human intake fraction (iF). Systems models that track the exchange of chemicals among air, water, soil, and plant compartments provide fate factors. The iF, which is the ratio of the chemical mass taken in by a population to the mass released, is used to express cumulative inhalation and ingestion exposures for a defined geographic region.

We present the CFs we use to compare emissions at various life cycle stages. Important in this analysis is the consideration of the variation and uncertainty of human exposures related to temporal and spatial scope of a fuel's life cycle, including local (e.g., refinery) and regional (e.g., agricultural systems and storage of fuel).

### Oral Presentation 8-06

#### Process technology and integration options for a sustainable biorefinery

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This presentation will illustrate how advances in process development bring the economically attractive and environmentally friendly production of cellulosic ethanol within reach.

Life Cycle Analysis (LCA), as a holistic approach to evaluate the environmental profile of a biorefinery value chain, has been used side by side with techno-economic evaluations to guide researchers to the most sustainable design alternatives. LCA scenario analyses explain how technology choices, research progress and synergies with co-located facilities affect the cradle to refinery gate footprint of a cellulosic biorefinery.

## Oral Presentation ST1-01

### One Million Tons of Biomass Per Year-Feedstock Management for Large Scale BTL Plants

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In the next plant generation, synthetic biofuels from Fischer-Tropsch synthesis (BTL) will be produced in large scale facilities of 200,000 – 300,000 tons per year BTL output. Therefore, an amount of around 1 million tons of dry biomass is needed as chipped or pelletized wood or straw for CHOREN's Carbo-V® process. To supply this huge stream of matter, a detailed supply chain management concept has to be established starting from forestry and short rotation coppice plantations to logistics and processing (chipping, drying) of wood up to storage management. CHOREN's Carbo-V® gasification concept is optimized for biomass feedstock with a water content of around 15 % w/w. By these circumstances, the following topics have to be dealt with:

- Sources: all kinds of wood can be used technologically (short rotation coppice, round wood, fresh wood, forest energy wood, waste wood, wood chips) and to a certain extent straw
- Transport and storage of untreated material (rail, road, ship)
- Elimination of contaminants
- Chipping of wood and pelletizing of straw and small wood particles
- Drying of chipped biomass particles
- Biomass storage and storage policy
- On-site biomass transport

We will present a biomass sourcing concept and the results of a supply chain management analysis and engineering study performed to gain insight into designing a specific site to manage a flow of 1 million tons of dry biomass per year from sustainable sources. This first of its kind sourcing and logistic study for large scale biomass gasification in Germany/Europe is proving to be extremely helpful to further understanding of supply concepts.

## Oral Presentation ST1-02

### POET Update on Project LIBERTY

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As the largest ethanol producer in the United States and in the world, POET is committed to meeting the ambitious targets of the Renewable Fuels Standard through the commercialization of cellulosic ethanol. Project LIBERTY will transform POET Biorefining – Emmetsburg from a strictly grain-to-ethanol plant to include ethanol from cellulose or biomass. Once complete, the facility will produce 125 million gallons of ethanol per year.

Following a successful start-up in the fourth quarter of 2008, POET Research Center in Scotland, S.D. is now producing cellulosic ethanol at a pilot scale, completing a crucial step toward development of commercially viable cellulosic ethanol. The Scotland plant is producing ethanol at a rate of 20,000 gallons per year using corn cobs as feedstock. The \$8 million endeavor is a precursor to the \$200 million Project LIBERTY that will begin production in 2011.

## Oral Presentation ST1-03

### Commercialization of Biomass Ethanol at Abengoa Bioenergy

Quang Nguyen, Abengoa Bioenergy, Chesterfield, MO

Abengoa Bioenergy is a world leader in bioethanol production with facilities in operation and under construction in Europe, USA, and Brazil. As part of the diverse portfolio of biofuels, Abengoa is advancing lignocellulosic biomass ethanol commercialization through pilot plant process development (York, NE, USA), commercial demonstration (Salamanca, Spain) and upcoming commercial operation (Hugoton, KS, USA).

This presentation provides an overview of Abengoa Bioenergy's multi-pronged approach on biofuel development and progress on biomass ethanol commercialization.

## Oral Presentation ST1-04

### Lignocellulosic Ethanol-Breaking Through the Barriers

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Novozymes is, as an independent enzyme technology provider, focusing on enabling the industry in a wider context to commercialize lignocellulosic ethanol. Through an unprecedented research effort with more than 150 scientists, Novozymes is breaking through the barriers for commercialization. Novozymes is developing highly cost effective novel enzyme systems, and has recently launched two new state-of-the-art products. With leading ethanol producers in US, China, Brazil and in Europe, processes are developed and integrated leading to total cost scenarios that could make sustainable 2G ethanol production competitive with gasoline in the near term. Novozymes is working in China with COFCO and Sinopec, two large state owned companies, and together the three parties represent all parts of the value chain. Progress from the joint development project on corn stover to ethanol will be outlined.

## Oral Presentation ST1-05

### Why Green House Gas Balances are Good for Business

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The British Sugar Group (BSG) is amongst the leading sugar producers in the world. It has 39 factories, in 8 countries, at the last count, processing sugar beet and cane to produce sugar, and a wide range of co-products, including bioethanol. When the UK Government decided to encourage biofuel production it also insisted on carbon and sustainability reporting of all biofuel in the UK. Because of this, BSG designed and integrated their bioethanol plant into existing sugar process to minimise overall fuel consumption. BSG has also worked with its beet growers to optimise agricultural inputs and this has reduced fertiliser consumption significantly over the years. Not only does this reduce green house gas emissions, it also means lower cost. Another area which has improved the cost base, improved profitability and reduced overall GHG emissions has been to diversify into other products from the same inputs to the existing process, like producing tomatoes from the waste heat and CO2 from our power plant and to cogenerate electricity for export to the local grid. It doesn't stop there. We have done a piece of work to benchmark what we do with the worlds best, this has shown that there is great scope to improve in many areas, and also shows, when you look at the world as a whole there is tremendous scope for us all to improve. If we all improve the basics and add on the satellites we will not only improve GHG balances, but also improve profitability.

## Oral Presentation ST1-06

### Deployment at Scale: A Grand Challenge for Advanced Biofuels

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Perhaps the single most distinguishing feature of the transportation fuel market is its size. To have a meaningful impact in the fuels market, deployment at scale is a key challenge to be addressed. Because even the smallest of changes are difficult to execute at mega-scale, the question of scaling new technologies or manufacturing regimes is arguably best approached by making the most conservative changes to existing infrastructure. One solution to scaling a hydrocarbon-based, advanced biofuel involves retrofitting sugar cane ethanol facilities.

By volume, the largest-scale production of biofuel is ethanol. Sugar cane ethanol facilities in Brazil deploy more than 10 million acres of sugar cane to produce 350 million tons of cane crush which is fermented into 8 billion gallons of fuel. To leverage the infrastructure built around the mills for advanced biofuel production, Amyris employs a "capital light" model, replacing the ethanol-producing yeast with a hydrocarbon-producing yeast. Because a hydrocarbon is produced, rather than a water-miscible alcohol, this advanced biofuel can be adopted into the existing fuel infrastructure. This model presents obvious advantages for rapid scaling and deployment and reveals some of the grand challenges presented to any scale up operation.

## Oral Presentation ST1-07

### Global Feasibility of Large-scale Biofuel Production

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There is currently great confusion and uncertainty regarding the role biofuels should play in the world's energy future. In response, we have initiated a project to test the hypothesis that the welfare of both humanity and the environment can be better with large-scale production of biofuels than without it. The project is structured in three stages:

- 1) Hold public meetings at five locations around the world during the second half of 2009 and first half of 2010, to develop a project plan, form a team, and recruit support for stage 2.
- 2) Answer the question: Is it possible for biofuels to meet a substantial fraction of future world mobility demand without compromising other vital needs: feeding humanity, providing fiber, maintaining and where possible improving soil fertility, air and water quality, biodiversity and wildlife habitat, and achieving large greenhouse gas emission reductions that are not substantially negated by land use changes.
- 3) Given an affirmative answer to this question, broaden the analysis and team as necessary to address desirable transition paths and policies, ethical and equity issues, impacts of climate change, and local-scale analysis including rural economic development.

Our proposed approach is distinct from prior studies of biomass resource availability and is likely necessary if a low-carbon transportation future is to be realized. Project results will provide critical guidance, both toward the overall feasibility of a biofuel-intensive future, and toward defining the policy and land use trajectories that foster this outcome.

## Oral Presentation ST2-01

### Overview of Algal Biofuels: From Cell Biology to Biotechnology

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Microalgae are typically aquatic, photosynthetic, oxygenic autotrophs that are smaller and less structurally complex than land plants. Many microalgae have the ability to produce substantial amounts (e.g. 20–50% dry cell weight) of storage neutral lipids/oils mainly in a form of triacylglycerols (TAG). Since TAG can be converted to biofuels (such as surrogates of gasoline, kerosene and diesel), microalgae have been considered a promising alternative, renewable source of feedstock for biofuels. The advantages of algae over oil crop plants for biofuels are that algae have a considerably higher TAG production potential, are a non-food source, can utilize marginal lands and wastestreams (e.g., wastewater and CO<sub>2</sub>), thereby providing additional environmental benefits. Since the concept of microalgae-based biofuels has been explored to only a limited extent over the past few decades, a scalable, commercially viable production system and process has yet to emerge. In this presentation, an overview of the current status of research on selection of high TAG-producing microalgal strains, the synthesis and regulation of TAG and the effects of environmental and biological factors on cellular TAG accumulation will be provided. An engineered system and process to produce TAG-derived biofuels and the technical limitations associated with existing algal TAG production technologies will be described. Finally, the path forward for microalgae-based biofuels with respect to both challenges and opportunities will be discussed.

## Oral Presentation ST2-02

### Biofuels from microalgae: Biochemistry and regulation of triacylglycerol accumulation in the model *Chlamydomonas reinhardtii*

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Many microalgae, including *Chlamydomonas*, accumulate triacylglycerols when cultures encounter certain environmental stresses such as nutrient limitation. However, the regulatory factors and enzymes that govern triacylglycerol biosynthesis in microalgae have not been studied at the molecular level. *Chlamydomonas* is used as a microalgal model to identify genes and regulatory mechanisms required for triacylglycerol biosynthesis following nutrient deprivation. Multiple global and focused approaches are pursued towards this goal: 1. A gene disruption mutant screen of 32,000 lines yielded 80 putative mutants, some of which are disrupted in genes central to the regulation of triacylglycerol biosynthesis. 2. Global expression analysis identified putative transcription factor genes induced under oil accumulation conditions. New expression vectors were produced for their analysis in *Chlamydomonas*. 3. Following proteomics analysis of isolated lipid droplets, the expression of a major lipid droplet associated protein was repressed by RNAi in *Chlamydomonas* resulting in increased oil body size. Betaine lipid biosynthetic enzymes were found associated with lipid droplets and are presumed to be critical to oil biosynthesis. 4. Of five putative genes present in the genome encoding diacylglycerol acyltransferases that catalyze the last reaction of triacylglycerol biosynthesis, two were confirmed to encode enzymes with oil biosynthetic activity following expression in a respective yeast mutant. These newly identified genes will provide novel targets for future engineering approaches towards optimizing microalgae oil production strains.

Funding for this work is provided by the US Air Force Office of Scientific Research and the Michigan Agriculture Experiment Station.

## Oral Presentation ST2-03

### Strain development of non-model photosynthetic microbes for biofuel production

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Aurora Biofuels has been successful in isolating photosynthetic micro-organisms which meet certain criteria important for the feedstock production process we are designing. These organisms must produce the right amount and types of oil; they must be harvestable; they must be amenable to our specialized pond mixing and oil extraction technologies; and they must grow outdoors stably. However, we have been unbiased in regards to the availability of molecular genetic tools for the organisms that we isolate; and as a result, we are working mostly with non-model organisms. As strain improvement via molecular genetics is essential to our business model, we have had to establish the requisite tools such as transformation technology and whole-genome sequences. We have also stumbled upon some interesting (and very useful) surprises.

## Oral Presentation ST2-04

### Low-cost photobioreactor technology: Promise, progress, and challenges

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Closed photobioreactors (PBRs) facilitate the cultivation of specific strains of algae and allow optimal growth conditions to be maintained. To date, however, the high capital and operating costs of photobioreactors have prohibited their use for "low-valued" products such as biofuels. Since its founding in 2006, Solix Biofuels has maintained a sustained effort to develop low-cost, high-productivity photobioreactors. Three generations of PBRs have been developed, each with successively higher productivity, lower capital cost, lower operating cost, and lower energy utilization. Key design details and a performance summary of generation is presented. Extensive process modeling and economic projections have been performed. Increasing efforts are now being devoted to reducing the costs of downstream processing: harvesting, dewatering, oil extraction, and co-product processing. The driving biological factors, design rationale, modeling results, and projected product costs will be discussed. Solix has now begun construction of a large-scale production facility in southwest Colorado, on the Southern Ute Indian Reservation; relevant technology details of this expansion facility will be presented.

## Oral Presentation ST2-05

### Feedstock-flexible renewable oil production from heterotrophic algae using a proven, scalable system

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Solazyme is a renewable oil production company. The company harnesses the power of microalgae to renewably produce clean and scalable high performance biofuels and renewable industrial chemicals. Solazyme's microbial conversion technology process allows algae to produce oil in standard fermentation facilities quickly, efficiently and at large scale. These oils are tailored not only for biofuel production, but also as replacements for fossil petroleum and plant oils in a range of products running from household cleaning supplies to cosmetics and foods. Solazyme's oils provide compelling solutions to complex issues of fuel scarcity, energy security and environmental impact while fitting into the pre-existing multi-trillion dollar fuel infrastructure. A defined pathway to high production volumes is critical for a new technology to penetrate an industry producing million of barrels a day of products. Solazyme's industrial fermentation has already been demonstrated at the commercial scale.

The company is currently producing thousands of gallons of oil at scale and has refined that oil into the world's first algal based fuels that meet ASTM standards for biodiesel (ASTM D6751), renewable diesel (ASTM D975), and jet fuel (ASTM D1655). Solazyme has road tested these fuels for thousands of miles in unmodified diesel engines.

Solazyme has pioneered the use of cellulosic feedstocks for microbial production of oil. The company has used a combination of genetic engineering and high throughput screening technology to establish proof of concept through high efficiency fermentation on a range of feedstocks, including corn stover, bagasse, sugar cane, switchgrass, and beet pulp.

## Oral Presentation 9-01

### Assessment of Potential Fermentation Inhibitors in Wet Cake Cellulosic Hydrolysate

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The cellulose-rich fiber byproduct stream of corn dry milling (wet cake), is a potential feedstock for hybrid corn-and-cellulosic ethanol production. To simulate process we prepared a high solids (30% w/w) slurry from wet cake in light stillage from a dry grind ethanol plant using laboratory-scale reactors. The slurry was pretreated under liquid hot water conditions and hydrolyzed with a mixture of cellulases and hemicellulases resulting in a hydrolysate containing 115 g/L glucose, 55 g/L xylose and 46 g/L arabinose. This hydrolysate was subsequently fermented with the Purdue glucose/xylose co-fermenting *Saccharomyces cerevisiae* 424A (LNH-ST). Results of the fermentation showed very strong inhibition effecting utilization of both xylose and glucose. Potential sources include: concentration of secreted yeast metabolic products, osmotic stress or lack of free water from the high total dissolved solids, compounds liberated during the enzymatic deconstruction of the hemicelluloses and concentrated dissolved solids (non-protein) in the enzyme solutions. In order to study the combined effects, artificial hydrolysates were prepared YEP media or corn steep liquor in water with the addition of potential fermentation inhibitors. In comparison fermentations, potential inhibitors were selectively removed from artificial hydrolysates by a bed of adsorbent resins.

## Oral Presentation 9-02

### Conversion of municipal solid waste into bioenergy

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The amount of municipal solid waste (MSW) is increasing in the developed part of the world. This is apparently an environmental problem but it also holds a large potential for energy production and recycling. However, the major challenge of utilizing MSW is the heterogeneous composition of plastics, biomass and metals.

Here we present a solid-liquid technology for separation of biomass from other waste components followed by possible gasification or fermentation. The philosophy behind this concept is sustainability, no pre-sorting of MSW, full recovery and improved usage of the different waste components. This technology is based on an initial thermal treatment of the entire MSW material which opens up cardboard based packaging and pulp & paper fractions and makes the other organic parts more accessible. Secondly an enzymatic liquefaction is initiated of the biomass fraction which turns this fraction into a pumpable slurry and ease the washing and sorting of non-biomass substances. The third and finishing step is the separation by simple filtration which may include washing for extracting the bound biomass and for cleaning recyclable non-organics.

Different commercial enzymes have been screened for their effect on liquefaction of municipal waste after thermal treatment of the material.

The overall concept of this process will be shown as well as results obtained so far from small batch experiments and a pilot plant capable of processing 100 kg/hr continuously. Also required characteristics of the slurry product in order to fulfil demanding properties of different energy systems affecting this project will be discussed.

## Oral Presentation 9-03

### Evaluation of Target Efficiencies for Solid-Liquid Separation Steps in Biofuels Production

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Development of liquid biofuels has entered a new phase of large scale pilot demonstration. A number of plants that are in operation or under construction face the task of addressing the engineering challenges of creating a viable plant design, scaling up and optimizing various unit operations. It is well-known that separation technologies account for 50-70 % of both capital and operating cost. Additionally, reduction of environmental impact creates technological challenges that increase project cost without adding to the bottom line. Different technologies vary in terms of selection of unit operations; however, solid-liquid separations are likely to be a major contributor to the overall project cost. Despite the differences in pretreatment approaches, similar challenges arise for solid-liquid separation unit operations. A typical process for ethanol production from biomass includes several solid-liquid separation steps, depending on which particular stream is targeted for downstream processing. The nature of biomass derived materials makes it either difficult or uneconomical to accomplish complete separation in a single step. Therefore, setting realistic efficiency targets for solid-liquid separations is an important task that influences overall process recovery and economics. Experimental data will be presented showing typical characteristics for pretreated cane bagasse at various stages of processing into cellulosic ethanol. Results of a generic material balance calculations will be presented to illustrate the influence of separation target efficiencies on overall process recoveries and characteristics of waste streams.



## Oral Presentation 9-04

### Reactive separations for esterification and purification of individual organic acids from mixed solutions

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Many fermentation systems yield a mixture of products depending on the intended metabolic pathways and conditions within the bioreactor. The recovery and purification of individual species from fermenter effluents can be costly, contributing more than 50% of the overall production cost in some cases. We present here the results of reactive separation studies, building on prior work involving lactate and citrate esters, to simultaneously esterify and recover individual products from aqueous solutions of mixed organic acids. General concepts and limitations of applying simultaneous reaction and separation to mixed acid systems are discussed, and specific results from mixed succinic acid and acetic acid as a prototypical system are presented. Experiments in an elevated pressure, pilot-scale reactive distillation column in our laboratories demonstrate succinic acid conversions greater than 99%, with recovery of diethyl succinate as a pure product stream. Bench studies have focused on characterization of physical properties and phase equilibria of key species in the acetate/succinate system, and a non-ideal kinetic model for simultaneous esterification of the acids has been developed. These physical properties and reaction kinetics have been incorporated into a rigorous simulation of the reactive distillation system using AspenPlus simulation software, facilitating characterization of the pilot-scale results and scale-up to a commercial esterification facility. Reactive separations are thus emerging as versatile, economical, and "green" technologies for the biorefinery, providing opportunity for lower capital costs and greater energy efficiencies than traditional reaction and separation approaches.

## Oral Presentation 9-05

### Process development of integrated cellulose- and starch-based ethanol production

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The aim of this study is to develop processes for production of ethanol from cellulose-based raw materials, mainly agricultural residues such as wheat straw, barley straw and corn stover (second-generation bioethanol), integrated with starch-based ethanol production (first-generation bioethanol). In this manner higher ethanol yield, lower energy demand and lower production cost can be reached.

The study comprises both experimental studies and techno-economic evaluation of the results using commercial flow sheeting- and cost-estimation programs. Several process configurations for the integration are possible, from integration already at the front end, i.e., after pretreatment of the raw materials, to integration only of the downstream processes, i.e., distillation and evaporation.

Integration of the steam-pretreated wheat straw with liquefied and pre-hydrolyzed starch from wheat (5%+0.5-3% WIS (Water Insoluble Solids) respectively) in SSF (Simultaneous Saccharification and Fermentation) configuration has been investigated, using baker's yeast. Ethanol yields above 80% of the theoretical (from the hexose sugars) and ethanol concentrations around 6 wt-% have been reached. This results in lower total energy demand in comparison with separate production of ethanol in a starch- or in a cellulose-based process.

The study is now continued using genetically modified yeast to also ferment the pentose sugars and further increase the WIS content of the pretreated wheat straw in the SSF step thereby increasing both the yield and the concentration. Furthermore, integration of the steam-pretreated wheat straw with only liquefied starch in order to reduce the availability of glucose in the SSF step is also under investigation.

## Oral Presentation 9-06

### Fluorescence resonance energy transfer sensors for quantitative monitoring of pentose and disaccharide accumulation in bacteria

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Engineering microorganisms to improve metabolite flux requires detailed knowledge of the concentrations and flux rates of metabolites and metabolic intermediates *in vivo*. Fluorescence resonance energy transfer (FRET) sensors represent a promising technology for measuring metabolite levels and corresponding rate changes in live cells. Sensors for hexose and pentose carbohydrates could help in the development of fermentative microorganisms, for example, for biofuels applications. Arabinose is one of the carbohydrates to be monitored during biofuels production from lignocellulose, while maltose is an important degradation product of starch that is relevant for starch-derived biofuels production. An *Escherichia coli* expression vector compatible with phage  $\lambda$  recombination technology was constructed to facilitate sensor construction and a novel FRET sensor for arabinose was generated. In parallel, a strategy for improving the sensor signal was applied to construct an improved maltose sensor. Both sensors were expressed in the cytosol of *E. coli* and sugar accumulation was monitored using a simple fluorimetric assay of *E. coli* cultures in microtiter plates. The addition of the respective ligand led to concentration-dependent fluorescence resonance energy transfer responses allowing quantitative analysis of the intracellular sugar levels at given extracellular supply levels as well as accumulation rates. The new carbohydrate FRET sensors can be used for *in vivo* monitoring of sugar levels in prokaryotes, demonstrating the potential of such sensors as reporter tools in the development of metabolically engineered microbial strains or for real-time monitoring of intracellular metabolite during fermentation.

## Oral Presentation 10-01

### Enzymatic synergy examined using an engineered complex of cellulosomal enzymes from *Clostridium thermocellum*

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The bacterium *Clostridium thermocellum* produces a formidable array of enzymes to break down lignocellulosic biomass. Many of these enzymes are organized into structures anchored to the cell surface known as cellulosomes. Organization of cellulosomal enzymes into complexes increases their efficacy both in the cellulosome and in engineered two- and three-enzyme complexes. We have created an assembly based on a chaperonin from the hyperthermophilic organism *Sulfolobus shibatae* that binds up to eighteen cellulosomal enzymes on the ends of a nine-member double ring. We have characterized the activity of combinations of two, three or four cellulytic enzymes attached to this structure and begun to explore activity on natural biomass substrates using combinations of enzymes including those that degrade hemicellulose. These experiments have yielded new insights into the synergy between cellulosomal enzymes that act on distinct sites on a single substrate and those that act on distinct substrates in within biomass.

## Oral Presentation 10-02

### The three-dimensional structure of an intact glucoamylase gives insight on how substrate is directed towards the active site

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We present the three-dimensional structure of *Hypocrea jecorina* glucoamylase at 1.8 Å resolution. The structure model includes both the catalytic domain and the starch binding domain as well as the glycosylated linker segment between these domains. This is the first intact structure of a glucoamylase. Previously, structure models of only catalytic or starch-binding domains have been available and these have been of other fungal and yeast glucoamylases. That the protein is intact in the model allows visualization of the juxtaposition of the starch-binding domain relative to the catalytic domain. One of the proposed starch binding regions on the starch-binding domain are in close proximity of the active site on the catalytic domain. This supports the hypothesis that the starch-binding domain serves to target the glucoamylase at sites where the starch granular matrix is disrupted and where the enzyme might most effectively function. The detailed interactions between the catalytic and the starch-binding domains are confirmed by two independent structure determinations of the enzyme in two different crystal forms. The two structure models exhibit an identical conformation and show the same positioning of the starch-binding domain relative to the CD. This, in turn, suggests that the *H. jecorina* glucoamylase structure we present not only is independent of crystal lattice contacts but that it also represents the three-dimensional structure found in solution.

## Oral Presentation 10-03

### Computational Estimates of Free Energy Profiles of Cellodextrin Motion in Cel7A Reaction Tunnel

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The motion of cellodextrin through the tunnel of processive exo-cellobiohydrolases is a fundamental step in the catalytic process by which these enzymes degrade cellulose to cellobiose and other small polysaccharides. We have studied the movement of cellobiose, cellononose, and celloheptose in the reaction tunnel of Cel7A from *Hypocrea jecorina* (*Trichoderma reesei*) using molecular modeling and simulation to determine the mechanism of movement and the free energy profile of the movement. Using path sampling methods, we generated reaction paths with associated potential of mean force and have identified the mechanistic contributions to the barriers to motion and explore possible mechanisms for the leaving of cellobiose product and repositioning of cellodextrin chain for subsequent reaction.

## Oral Presentation 10-04

### A Family of Thermostable Fungal Cellulases Created by Structure-Guided Recombination

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SCHEMA structure-guided recombination of fungal cellulases has yielded a collection of novel, highly thermostable CBH2 chimeras. An appreciable fraction of a sample set of cellulase chimeras were secreted by a heterologous host in catalytically active form. Many of these chimeras have half-lives of thermal inactivation that are greater than the most stable parent cellulase. We predict that the collection of cellulase chimeras contains hundreds of highly stable cellulases. All of the active sequences chosen from the chimeras predicted to be thermostable based on the sample set sequence-stability data retained more activity than the most stable parent upon incubation at elevated temperature. These validated thermostable cellulases have high sequence diversity, differing from their closest natural homologs at up to 63 amino acid positions. Selected thermostable chimeras hydrolyzed phosphoric acid swollen cellulose at temperatures between 7 and 15°C higher than the parent enzymes. These chimeras also hydrolyzed as much or more cellulose than the parent cellulases in long-time cellulose hydrolysis assays and had pH/activity profiles as broad, or broader than, the parent enzymes. Generating this group of diverse, thermostable fungal cellulases is the first step in building an inventory

of thermostable cellulases from which optimized enzyme mixtures for biomass conversion can be formulated.

## Oral Presentation 10-05

### Engineering cellulases on their natural substrates by directed evolution

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Engineering costly cellulases on natural cellulosic substrates is of importance for emerging biomass-based biorefineries. Complicated relationship among heterogeneous cellulose and different action mode cellulase components results in great challenges for cellulase engineering. Most times, cellulase activities on cellulose analog substrates (e.g., soluble substrate or chromogenic substrates) have no relationship with their activities on natural substrates. Directed enzyme evolution is becoming a popular tool, but identification of the desired mutants from a large mutant library remains challenging sometimes.

For beta-glucosidase, we have designed a novel combinatorial selection/screening approach for fast identification of thermostable beta-glucosidase mutants on cellobiose. Several thermostable mutants were identified from a random mutant library of the *Paenibacillus polymyxa* beta-glucosidase. The most thermostable mutant A17S had an 11-fold increase in thermostability at 50°C. In addition, we also attempted to improve the family 48 exoglucanase and the family 5 endoglucanase through directed evolution. The mutant libraries are cell-surface displayed in *E. coli* or secretory across membrane in *Bacillus*. Our preliminary results clearly supported the technical feasibility of cellulase engineering through directed evolution.

## Oral Presentation 10-06

### Developing Improved Thermostable Cellulases: High-Throughput Cellulolytic Assays and Protein Engineering Strategies

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DNA family shuffling has been employed to improve the thermostability and activity of moderately thermophilic exocellulases from fungal sources. Selection of genes for shuffling was carried out using constrained homology clustering and validated *in silico* using eShuffle. Because cellulases are highly modular, clusters for the catalytic and binding domains were formed separately and recombined to create novel cellulases as the parents for DNA family shuffling.

Conventional cell-based cellulase expression methods are time and labor intensive. Previous efforts to express cellulases in *E. coli* or yeast have often failed to produce active forms. A cell-free protein expression system, on the other hand, can be used as an alternative protein expression tool to address these problems. We have developed a high-throughput cellulase expression and screening platform to generate libraries of four thermophilic archaeal endocellulases, with the aim of improving their properties for industrial application. In addition, carbohydrate binding module (CBM) domains from bacterial cellulases are being added to the archaeal enzymes. The generation of CBM fusions is directed toward improving the catalytic activity of the extremely thermophilic archaeal cellulases toward crystalline substrates.

Finally, successful implementation of directed evolution to improve cellulase activity depends on the screening method used in enzyme selection. The poor correlation between cellulase activity on soluble and insoluble cellulosic substrates requires high-throughput methods for screening cellulase activity on relevant insoluble substrates. Our protein engineering efforts have thus employed high-throughput assays that are compatible with insoluble cellulosic substrates and constraints imposed by directed evolution strategies.

## Oral Presentation 11-01

### Hydrocarbon Fuels from Plant Biomass

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In this paper we'll discuss the conversion of renewable plant carbohydrates to hydrocarbon fuels, by fermentation of carbohydrates to isobutanol followed by catalytic conversion of the isobutanol to fuel blend stock, gasoline, jet & diesel fuel components. Bioethanol has provided an excellent start to sustainable, domestically produced renewable fuels entering the fuels market. Additionally ethanol has provided an infrastructure of expertise that is extremely valuable to support the development and commercialization of advanced biofuels. One of the remaining challenges is completing the conversion of plant matter into fuels and blend stocks that fit, without special rules, exactly into the existing infrastructure for crude oil derived transportation fuels and vehicles. Ultimately, this means producing sustainable hydrocarbons from biomass. A number of companies are pursuing the production of hydrocarbons from plant biomass utilizing a variety of thermochemical and biochemical approaches. The approaches will be compared and contrasted to the approach proposed in this paper. Data using our approach for the conversion of sugars to isobutanol and conversion of isobutanol to a high value gasoline blend stock, jet fuel and terephthalic acid, will be shown.

## Oral Presentation 11-02

### Renewable Petroelum™ Products and Technologies: Engineering Microbial Fatty Acid Metabolism for Fuel and Chemical Production

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The challenge to the biofuel industry is to quickly develop products and technologies that enable the rapid and widespread adoption of renewable and sustainable substitutes for existing fossil fuels and chemicals. Success will require products to be "drop in" replacements that leverage existing consumer and distribution infrastructure and for processes to be "feedstock agnostic," scale without competing with food supplies, result in significant reduction in green house gas emissions, and most importantly be cost competitive with existing products. LS9, Inc. has developed a core technology to convert fermentable sugar to a diversity of fuel and chemical products in one-step fermentation processes. Our lead fuel product, UltraClean™ Diesel, is a secreted immiscible product that is recovered by simple centrifugation and is vehicle ready without further chemical processing, such as hydrogenation, cracking, or transesterification. Leveraging the efficient and productive fatty acid biosynthetic pathway in combination with an engineering strategy that places all chemical conversions in a single whole cell catalyst, LS9's Microrefinery™ catalysts enable a specific and efficient route to a high-performing diesel substitute that is competitive with recent oil prices without subsidy. This talk shall discuss the fundamental technology and its application to a diversity of products, but shall focus on the quality, underlying economics, GREET analysis, pilot demonstration, and commercial development plan of UltraClean™ Diesel.

## Oral Presentation 11-03

### High-Titer Production of Hydroxyvalerates and 4-Valerolactone from Levulinic acid in *Pseudomonas putida*

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Hydroxyacids and lactones are versatile, chiral compounds that can readily be modified into several useful derivatives. Specifically, these compounds see use in the synthesis of antibiotics,  $\beta$ - and  $\gamma$ -aminoacids and peptides, in fragrances, and as chiral synthetic building blocks. These compounds can also be used directly as nutritional supplements and can be polymerized into biodegradable polyesters with interesting physical properties.

In this work, an economical, high-titer method for the production of 4-hydroxyvalerate (4HV), 3-hydroxyvalerate (3HV), and 4-valerolactone (4VL) from the inexpensive and renewable carbon source levulinic acid was developed. Titrers of 4HV and 3HV in shake flask cultures both reached multi-gram-per-liter scale in both minimal and rich media. To achieve these titers, we tested two strains of *P. putida* and examined two enzyme systems for removing CoA acyl carriers from intracellular hydroxyvaleryl-CoA intermediates: the *ptb/buk* system and *tesB*. Once a suitable strain and enzyme system was found, the process was optimized at the shake flask scale in minimal and rich media for the high-titer production of both 4HV and 3HV. To produce 4VL from 4HV, a pH-dependent equilibrium between 4HV and 4VL had to be overcome. Because intracellular pH was found to be too high for the appreciable production of 4VL, we employed a membrane-bound, extracytosolic lactonase to perform the lactonization reaction in acidic culture medium and achieved multi-gram-per-liter production of 4VL. To our knowledge, this work represents the first time that these hydroxyvalerates and 4-valerolactone have been produced from a feasible feedstock in shake flasks at the multi-gram-per-liter scale.

## Oral Presentation 11-04

### Sustainable Chemicals through Biotechnology: 1,4-Butanediol

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The relentless rise and volatility of petroleum prices is stimulating the chemical industry to diversify its raw material base. Genomatica has established an integrated suite of computational and experimental technologies to design, engineer, and optimize novel organisms and bioprocesses for cost-advantaged and sustainable manufacture of chemicals.

*In silico* metabolic modeling and simulation technologies greatly accelerate the pace of industrial bioprocess development by providing optimum strain designs and engineering strategies, by facilitating data interpretation, and by guiding experimental activities throughout the entire development cycle. Unique pathways to a chemical are identified and verified as superior through genome-scale metabolic models that allow prioritization in terms of parameters such as yield, energy balance and redox balance. Subsequently a strain is designed via the proprietary OptKnock algorithm, which identifies sets of genes that must be deleted in order to tightly couple product formation to growth of the organism. Following introduction of the most attractive biosynthetic pathways and designated deletions, strains are subjected to adaptive evolution methods which use controlled selection pressure to optimize strain performance following genetic manipulations. In addition to achieving superior product yield and productivities, evolved strains are genetically stable and thus ideally suitable for cell recycle or continuous bioprocessing with consistent high-level production.

The presentation will highlight successful implementation of this combined computational and experimental approach for engineering a microorganism that produces the industrial chemical 1,4-butanediol (BDO) directly from glucose and sucrose.

## Oral Presentation 11-05

### Rapid optimization of microorganisms for the cost superior production of chemicals and fuels

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The growing field of bio-refining relies upon the use of microorganisms to convert renewable carbon sources such as sugar into higher value products. Traditional bio-refining processes have taken advantage of the unique abilities of specific micro-organisms to produce desired product, or the genetic engineering of micro-organisms to produce non-natural products. Bio-processes have then been designed around these organisms. These bioprocesses are often very costly in large part due to the complex requirements of the microorganisms themselves, which can necessitate expensive growth conditions as well as separations and processing steps both before and after the micro-organisms' conversion step. OPX has developed several new high-resolution and comprehensive genomics tools that can be used to optimize industrial organisms. We have employed these generalizable methods to very rapidly construct and optimize commercially relevant microorganisms. We are able to optimize micro-organisms that enable both variable and capital cost savings across the entire bioprocess.

In particular, OPX has been able to construct and optimize micro-organisms for the production for several bioprocesses including the biorefining of 3-hydroxypropionic acid. 3-hydroxypropionic acid is a bio-product with several market applications. The most notable being the \$7 Billion acrylic acid market, as 3-hydroxypropionic acid is readily converted by conventional methodologies to acrylic acid. Our platform technology has enabled the construction of microbial strains capable of producing commercially relevant titers of 3-hydroxypropionic acid at commercial productivities in inexpensive growth conditions, a strain that will enable a cost competitive bio-processing route to acrylic acid.

## Oral Presentation 11-06

### Production of terpene based biofuels in *S. cerevisiae*

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The generation of microorganisms that can produce biofuels similar to petroleum-based transportation fuels would allow the use of existing engines and fuel transportation infrastructure. The corrosivity and high hygroscopicity of ethanol, today's preferred biofuel, are incompatible with existing technologies. "Second generation" biofuels should have better physical properties than ethanol and a higher energy content per unit. Here, a *Saccharomyces cerevisiae* platform previously engineered to overproduce farnesyl pyrophosphate has been adapted to produce a variety of terpenoid base biofuels. First, a series of terpene synthases were tested for the production of monocyclic, bicyclic, and linear sesquiterpenes in yeast. Next, the production levels of the different sesquiterpenes were optimized and the molecules classified based on their structure and yield as potential biofuel candidates. Finally, the terpene synthases leading to the most promising biofuel candidates were introduced in *Escheria coli* able to overproduce farnesyl pyrophosphate and tested for biofuel production. This is the first time that such a large array of terpene synthases has been tested for the production of sesquiterpenes in *S. cerevisiae*. Importantly, some of the successfully produced sesquiterpenes have structures similar to gasoline and jet fuel and may be useful second generation biofuels.

## Oral Presentation 12-01

### Identification of Desirable Traits in *Miscanthus* to Enhance Total Sugar Yields in Biological Conversion

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*Miscanthus*, which has a very high productivity, was selected by DOE as one of the potential biomass crops to support large scale production of fuels. Improving our understanding of features that control enzymatic hydrolysis and how pretreatment alters these features for this prolific crop would be a significant step to identifying better strategies and opportunities to genetically alter the susceptibility of biomass to deconstruction and achieve very high total sugar yields at low costs. In this study, a number of *Miscanthus* species were screened based on chemical composition and structure to identify the most promising species for more detailed characterization by pretreatment and enzymatic hydrolysis. Then, water-only and dilute acid batch and flowthrough pretreatments were applied to those varieties to define how sugar yields and release profiles vary among species and to develop meaningful cause-and-effect relationships of factors that control deconstruction of hemicellulose, cellulose, lignin, and other subcomponents. Profiles of glucose, xylose, and total sugar release from selected *Miscanthus* species were determined for various combinations of time, acid concentration, flow rate, and temperature followed by enzymatic hydrolysis of the pretreated solids. The resulting data and models provide a new perspective on how sugar yields vary with species of *Miscanthus* during pretreatment and biological processes and key features that could govern sugar yields.

## Oral Presentation 12-02

### Elucidation of Alfalfa Lignin Structures on Gene Down-Regulation

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Independently down-regulation of genes encoding 4-coumarate 3-hydroxylase (C3H) and hydroxycinnamoyl transferase (HCT) has shown to reduce the recalcitrance of alfalfa and thereby improving the yield of simple sugars after pretreatment. One-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) techniques were utilized to identify structural elements of importance to the recalcitrance of genetically engineered alfalfa. After C3H and HCT gene down-regulation, significant structural changes had occurred to the alfalfa lignin. A significant increase of p-hydroxylphenyl unit content was observed in the transgenic alfalfa lignins as well as a concomitant decrease of up to ~70% of the guaiacyl and syringyl units. Quantitative <sup>13</sup>C NMR measurement also showed a significant decrease of carboxylic group, methoxyl group and β-O-4 linkage contents in the alfalfa lignins after genetic engineering. <sup>13</sup>C-<sup>1</sup>H HSQC 2D correlation NMR demonstrated an increase of interunit phenylcoumaran and resinol contents for C3H and HCT transgenic alfalfa. In addition, <sup>31</sup>P NMR measurement revealed that phenyl hydroxyl group in p-hydroxylphenyl unit was dramatically increased for the transgenic lignins, as well as by over ~50% decrease of guaiacyl hydroxyl group content. The results of these changes in lignin structure and their relationship to recalcitrance will be examined with a perspective to future improvements in plant cell wall design for enhanced sugar production for biofuels.



## Oral Presentation 12-03

### Small-scale Enzymatic Conversion Screens to Assist in the Development of Improved Energy Crop Varieties

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In the development of improved energy crop varieties, obtaining accurate chemical composition and conversion performance characteristics will be critical. Ceres has previously developed small-scale conversion assays for the assessment of switchgrass biomass. Here we display information on small laboratory-scale, high-throughput assays that can be used to assess the conversion efficiencies of Arabidopsis and sorghum. Both acidic (APT) and basic (BPT) pretreatment methods have been employed. Ceres has generated a collection of thousands of transgenic Arabidopsis lines, each overexpressing a single full length cDNA. A selection of transgenic Arabidopsis lines bearing misexpressed genes relevant to cell wall biosynthesis have been grown to maturity and assessed for conversion efficiency. Additionally, Ceres has available hundreds of genetically diverse sorghum samples, and the biomass collected from a subset of these lines has been assessed for relative digestibility in Ceres' small-scale conversion assays. For both plant species, lines with distinct differences in glucose released per gram dry biomass and/or in percent of theoretical maximum glucose yield have been identified. In the case of the Arabidopsis lines, these conversion assays provide a direct means to identify genes that can influence conversion processing performance, leading to higher rates of conversion and higher final sugar yields. The sorghum assays assist in the identification of sorghum lines with superior conversion characteristics. This information will be invaluable to breeders and genetic engineers in the design of improved energy crop varieties that give higher conversion product yield per ton of biomass input to a conversion process.

## Oral Presentation 12-04

### Effects of Chemical Pretreatment on Enzymatic Hydrolysis of Lignocellulose Observed by AFM

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Lignocellulose has natural resistance to microbial and enzyme destruction often called "recalcitrance". The recalcitrance of lignocellulose is a major barrier to the economical development of bio-based fuels and chemicals. Chemical pretreatment is often applied to biomass feedstock to remove lignocellulose recalcitrance for efficient subsequent enzymatic saccharification. The performances of different chemical pretreatments vary significantly. Understanding of the fundamentals of nano-scale phenomena on lignocellulose substrate during enzyme actions can provide insight about why some pretreatment is more effective than others. This understanding can lead to developing efficient pretreatment processes and enzyme systems to improve lignocellulose bioconversion. This presentation will provide some observations of the effects of chemical pretreatment on enzymatic hydrolysis of lignocellulose substrate using AFM. Dilute acid, hot water, and SPORL pretreatment were applied to lodgepole pine and eucalyptus wood chips to produce substrates. Enzymatic hydrolysis of the pretreated substrates was conducted at 50°C with enzyme loading of 15 FPU/g substrate on solid. AFM imaging was applied to substrates after enzyme actions for various incubation duration times. The AFM images clearly showed the enzymes attached to the substrate and the destruction of cellulose microfibrils over time after enzyme actions. Furthermore, the effect of CBD on enzyme attachment can be clearly seen from the AFM images. The effects of pretreatment process on the dynamics of nano-scale enzyme destruction of lignocellulose can be clearly seen. When correlating the AFM imaging information with time-dependent quantitative enzymatic cellulose conversion data, the effects of nano-scale enzyme process on macro-scale cellulose conversion can be easily understood.

## Oral Presentation 12-05

### Understanding Ionic Liquid Pretreatment of Lignocellulosic Biomasses

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Pretreatment of biomass is essential for breaking apart highly ordered and crystalline plant cell walls and loosening the lignin and hemicellulose conjugation to cellulose microfibrils, thereby facilitating enzyme accessibility and adsorption and reducing costs of downstream saccharification processes. Recent reports<sup>1, 2</sup> have shown very high yields at very low enzyme loadings. However, pretreatment still remains one of the most costly steps in lignocellulosic biofuel production. Ionic liquids are novel solvents showing great promise for lignin and cellulose solubilization. Instant rejection of dissolved polysaccharides upon addition of anti-solvent shows promise for recyclability in addition to other desired attributes like low volatility, non-flammability and thermal stability. Although ionic liquids have been shown to be very effective in cellulose solubilization<sup>3,4</sup>, the disposition of hemicellulose and lignin are not fully understood. The aim of our research is to develop a fundamental understanding of ionic liquid pretreatment by monitoring and analyzing process streams. To that end, we have employed HPAEC, XRD, FTIR, NIR, and SEM to study the impact of ionic liquid pretreatment on switchgrass and corn stover. We will present the results from these measurements in the context of developing and selecting optimized ionic liquid pretreatment conditions for selective depolymerization of either cellulose or lignin, whereby fractionation of different cellulose and lignin components could be realized.

## Oral Presentation 12-06

### Single Molecule Tracking of Carbohydrate-Binding Modules Bound to Cellulose Crystals

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To develop more cost-effective approaches to liberate fermentable sugars from recalcitrant biomass, the enzyme cocktail used for saccharification must be improved. We have developed a single-molecule technique based on fluorescence imaging to track the motion of cellulase components with spatial resolution at several nanometers. We used single molecule spectroscopy to study the behavior of carbohydrate-binding modules (CBMs) labeled with quantum dots (QDs) while bound to cellulose crystals. These bio-assemblies were subjected to total internal reflection fluorescence (TIRF) microscopy. The concentrations of the CBMs and QDs were optimized to achieve single molecule resolution. This technique revealed a confined nanometer-scale movement of the CBMs bound to cellulose. Although the mechanism of CBM motion is still unknown, the single molecule approach used here offers new opportunities to guide us toward a fundamental understanding of cellulase function, especially the mechanism of the "processivity" of exoglucanase.