BioEnergy Science Center: An Integrated Strategy to Understand Biomass Recalcitrance

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The challenges: Lignocellulosic biomass is complex and heterogeneous

Fig. II.2. Biosynthesis of primary and secondary walls: from genes to polymers. A.
Switchgrass –
Atomic force microscopy (AFM)

Crystalline cellulose microfibrils

NREL, Ding, et al, unpublished results
Access to the sugars in lignocellulosic biomass is the current critical barrier for cellulosic biofuels

- Overcoming this recalcitrance barrier will cut processing costs significantly and be used in most conversion processes.

- This requires an integrated, multi-disciplinary approach.

- **BESC believes biotechnology-intensive solutions offer greatest potential.**
BioEnergy Science Center (BESC)
A multi-institutional, DOE-funded center performing basic and applied science dedicated to understanding biomass recalcitrance and improving yields of biofuels from cellulosic biomass.

300+ People in 17 Institutions

Oak Ridge National Laboratory
National Renewable Energy Laboratory
Samuel Roberts Noble Foundation
ArborGen, LLD
Ceres, Incorporated
Mascoma Corporation
DuPont
GreenWood Resources

University of Georgia
University of Tennessee
Cornell University
Dartmouth College
West Virginia University
Georgia Institute of Technology
University of California--Riverside
North Carolina State University
University of California—Los Angeles

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BESC is organized into three focus areas to understand biomass recalcitrance

Better Plants

Better Microbes

Better Tools and Combinations
Systems Biology:

• Philosophical approach to consider biology as integrated complex microbial and metazoan systems including:
  – Molecular complexes & interactions
  – Molecular networks including cell signaling and gene regulation

• Experimental approach to generate and analyze HTP data

Recombinant engineering is the intentional manipulation of organisms via altered genes or gene expression. Synthetic biology accelerates the process by manipulating entire pathways not just single of several genes.
ORNL (and others) has “omic” capabilities for Systems Biology

Single component
- Gene
- Transcript (mRNA)
- Protein
- Metabolite

“All” components
- Genomics
- Transcriptomics
- Proteomics
- Metabolomics

Together these data can provide a deeper picture of how an organism is functioning. This can help identify where improvements need to be made.
Do I need a why feedstock – switchgrass and poplar slide
Genetic Block in Lignin Biosynthesis in Switchgrass Increases Ethanol Yields

Phenylalanine → PAL

Agrobacterium-mediated transformation of switchgrass

Wild-type (L) and 3 transgenic switchgrass plants (R)

X. Fu and Z. Wang (Noble), J. Mielenz (ORNL), support from USDA/DOE

Ethanol Yield per Weight of Biomass (g/g)

25% more ethanol

The Samuel Roberts NOBLE Foundation
Top performing transgenic greenhouse plants must be evaluated in the field

- Greenhouse plants have minimal stresses
- The stresses in a field may result in plants responding differently
- First year field-grown data is qualitatively consistent and second-year field grown data is better

Mining variation to identify key genes in biomass composition and sugar release

Collected ~1300 samples for *Populus* association and activation-tag study

High-throughput screening pipeline

Sugar release assay

Establish common gardens for association and activation-tag populations with thousands of plants

Cell wall biosynthesis database
- Create genetic marker map to identify allelic variation
- Identify marker trait association

- Skagit (Sedro Woolley)
- Skykomish (Monroe)
- Puyallup (Orting)
- Columbia (Longview)

Existing collections (N = 500; 1–2 trees/site)
New collections (N = 580; 140–160 trees/site)
Start with Nature’s Best: Feedstocks

Some feedstocks are less recalcitrant than others
Even in the same species!

*Populus trichocarpa*
No pretreatment other than autoclaving

Variation in diameter from different 3-year-old individuals from a common garden

Sequential hydrolysis & fermentation, fungal cellulase (15 mg Ctec2/g solids), yeast
Discovering and utilizing natural variants: *Populus* association genetics study

- Combines common gardens, phenotyping, GWAS and sequencing of ~1000 lines by JGI
  - Sugar release analysis shows up to 1.4x lower lignin, 2.7x increase in sugar release, and up to 2.4x higher ethanol yield compared to wild-type genotypes.
  - We have identified natural variants from the genome-wide association population with:
    - increased levels of sugar release,
    - high productivity in field trials across diverse sites, and
    - causal alleles.
- These genotypes can be clonally replicated and planted on a large scale in near-term bioenergy plantations.
Transcriptional regulation of flavonoid and phenylpropanoid pathways in *Populus*

**Selecting TOP Lines**
- Novel isoform
- EPSP ancestral isoform

**Recalcitrance Mechanisms**
- Isoform has DNA-binding motif
- Isoform is expressed in xylem instead of chloroplasts

**Q3: Scientific achievements.**
- Association mapping study of 1,100 *Populus* sequenced natural variants
- Identified natural variants with low lignin
- EPSP variants have >2.5X more sugar release than controls
Forage Genetics plans to commercialize BESC invention in lignin regulation

- The invention provides a genetic mechanism for the reduction of lignin biosynthesis while increasing concentration of desirable flavonoids.
- Reduced lignin content increases digestibility and nutritional value of animal feedstocks such as alfalfa, corn and sorghum.
- Forage Genetics plans to evaluate commercial viability of this technology in alfalfa, corn and sorghum forage crops as animal feedstocks.

Forage Genetics International is the union of industry-leading forage companies whose history of alfalfa innovations dates back to the 1950s. Brought together in 1991, we’ve leveraged our collective strength to advance the forage industry and meet the needs of a diverse and growing world.

Our breeding expertise combined with our proprietary germplasm base and global reach allows us to develop unique seed varieties for diverse growing conditions, making us the world leader in value-added genetics. We’re proud to provide not only the seed in the bag, but the expertise, research and technology that help growers succeed.

foragegenetics.com
Biomass Fermentation Options: Reduction of Process Steps by Using CBP

Baseline, Multi-step Cellulosic Ethanol Production

Native Plants → Pretreated Biomass → Solid/Liquid Separation → Cellulose → Enzymes → Enzyme Hydrolysis → Hexose Fermentation → Ethanol

Pentose Fermentation → Pentose Sugars → Ethanol

Biomass Modification

Modified Plants

Consolidated Bioprocessing

Reduced or No Pretreatment → Biomass → No Separation → CBP Microbes → Ethanol
Snapshot of the metabolic pathways involved in central glycolysis and fermentation from BMBP pathway map

http://ca.expasy.org/cgi-bin/show_thumbnails.pl
Access biodiversity for new microbes

- State-of-the-art cultivation techniques to isolate novel high-temperature microbes with powerful lignocellulolytic enzymes
  - Collect samples from thermal biotopes
  - Establish primary enrichment cultures at relevant temperatures and conditions

Sampling at Yellowstone National Park, October 2007 and July 2008
**C. thermocellum** is effective at CBP

- *Clostridium thermocellum*, a thermophilic lignocellulose degrading anaerobe, is the most effective cellulolytic microbe.
- **C. thermocellum** uses multiple glucosyl hydrolase mechanisms:
  - Cellulosomes
  - Free cellulases
  - Free cellulolytic enzyme complexes
- High solubilization with minimal pretreatment is possible using **C. thermocellum** and other bacterial systems compared to industry standard SSF using fungal cellulases.
- Microbial solubilization of biomass selectively targets the carbohydrates

Solubilization of washed mid-season switchgrass by various biocatalysts. Xylan (white) and glucan (black) solubilization from washed mid-season switchgrass by various bacteria or SSF with yeast and fungal cellulase after 5 days. (Paye et al.)

C. thermocellum ethanol yield is poor due to side reactions

Biswa et al., "Elimination of hydrogenase active site assembly blocks H2 production and increases ethanol yield in Clostridium thermocellum" Biotechnol. Biofuels, 2015
**Highest* C. thermocellum* ethanol yield to date: “quad mutant” (pfl− hydG− ldh− pta-ack−)**

- **Cellobiose**
  - L-Lactic Acid
  - Formate
  - Acetyl-CoA
  - Pi
  - ADP
  - ATP
  - Acetic Acid

- **Pyruvate**
  - NAD(P)
  - NAD(P)H
  - 2H+

- **Acetyl-CoA**
  - Acetaldehyde
  - NADH
  - NAD+

- **Ethanol**
  - NADH
  - NAD+

- Ethanol yield 81% of theoretical

However strain grows very slowly – 10 days to consume 5 g/L cellobiose (lean medium) - secretes pyruvate, valine, alanine

Biswas et al., "Elimination of hydrogenase active site assembly blocks H2 production and increases ethanol yield in Clostridium thermocellum" Biotechnol. Biofuels, 2015
A mutated alcohol dehydrogenase (AdhE) with altered co-factor specificity was shown to enhance ethanol tolerance in *Clostridium thermocellum*, a candidate consolidated bioprocessing microbe.

The simplicity of the genetic basis for this ethanol-tolerant phenotype informs rational engineering of mutant microbial strains for cellulosic ethanol production.

Illustrates systems biology approach including molecular modeling, ‘omics, physiological measurements and leadership class computing facilities.
Resequencing an ethanol tolerant *C. thermocellum* mutant


![Graph showing optical density and growth rate vs. ethanol concentration](image)

**Fig. 1** Effect of ethanol on growth of wild-type (circles) and ethanol-adapted (triangles) cultures. Ethanol-sensitive cells (wild-type) and cells adapted to 5% ethanol were incubated in media containing varying concentrations of ethanol. Optical density (a) and growth rate (b) were measured.

Carbon and electron flow partition differently in AdhE mutant strain

**Specific Activity**

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<tr>
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<th>NADH</th>
<th>NADPH</th>
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<tbody>
<tr>
<td>WT</td>
<td>2.7 (0.18)</td>
<td>0.025 (0.005)</td>
</tr>
<tr>
<td>EA</td>
<td>&lt;0.005$^b$</td>
<td>0.052 (0.007)</td>
</tr>
<tr>
<td>adhE*(EA)</td>
<td>&lt;0.005</td>
<td>0.12 (0.03)</td>
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$^a$ µg NAD(P)H oxidized.mg crude extract protein-1.min-1

$^b$ Below assay detection limit

**Carbon flow also effected in C. thermocellum containing mutant ADH**

Studies underway to further optimize carbon and electron flow for productivity advances

Combining modified switchgrass with engineered *C. thermocellum* improves yield

Significance

- First report of use of a microbe engineered to produce increased amounts of a biofuel on a bioenergy feedstock modified for the same purpose. Results demonstrate the potential additive advantages from combining a modified feedstock with an engineered consolidated bioprocessing microorganism.

Outcome

- Fermentation of the modified COMT switchgrass by *C. thermocellum* mutant M1570 had superior conversion relative to the wild-type control switchgrass line with an increase in conversion of approximately 20%.

  - Ethanol was the primary product, accounting for 90% of the total metabolites with conversion of 0.19 g ethanol/g glucan loaded and 0.27 g liberated.

Conversion (mg/g glucan loaded) for *C. thermocellum* mutant M1570 and wild-type DSM 1313 strains on both transgenic (T1-3-TG) and wild-type (T1-3-WT) switchgrass, which were pretreated with dilute acid. The standard deviation is from the average of triplicate buffered serum bottle fermentations.

Yee et al., *Biotechnology for Biofuels* 7:75, 2014.
Community outreach in bioenergy science education is becoming self-sustaining

Farming for Fuels lessons reach thousands of students through hands-on science activities

- BESC in collaboration with the Creative Discovery Museum (CDM) in Chattanooga, Tennessee, developed hands-on lesson plans for students in 4th, 5th and 6th grades.
- *Farming for Fuel* lessons educate students about the carbon cycle, lignocellulosic biomass as substrate for the production of biofuels and the technical and economic obstacles to a bio-based fuel economy.

Science Night events reach thousands of families

- In the last 2 years, >100 Science Nights were presented nation-wide reaching more than 25,000 students, parents and teachers.

“Hub and Spoke” model allows economical outreach national outreach using partnering with regional science centers and museums. Over six years, the outreach program has steadily expanded from Chattanooga across Tennessee to currently active hubs in Georgia, Texas, Michigan, Illinois, Florida, Oklahoma, Idaho, Montana, Washington, Oregon and Utah.

➤ A marker of self-sustaining success is that now 75% of the support for the hands-on activities now come from the schools, hubs, and other sources.

➤ This approach has allowed BESC to steadily increase hands-on science contacts to over 25,000 in the last year and over 145,000 students, parents, and teachers in the past six years.
Recalcitrance of biomass can be overcome

Better Plants

Better Microbes

Better Tools and Combinations
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