The BioEnergy Science Center: An Integrated Strategy to Understand and Overcome Biomass Recalcitrance

Martin Keller, Ph.D.
Director, BioEnergy Science Center
http://www.bioenergycenter.org/

June 30, 2009
The BioEnergy Science Center

**BESC**: A multi-institutional DOE-funded center dedicated to understanding and modifying plant biomass recalcitrance

BESC is 304 people in 17 institutions

http://www.bioenergycenter.org/
Access to the Sugars in Lignocellulosic Biomass is the Current Critical Barrier

- Solving this will cut processing costs significantly and be used in most conversion processes
- This requires an integrated multidisciplinary approach
- Timeframe
  - Modified plants to field trials: Year 5
  - New or improved microbes to development: Years 4–5
  - Analysis and screening technologies: Year 3 on
Comparative Impacts of R&D on Biomass Processing Cost

Without overcoming biomass recalcitrance (A), cellulosic biofuels will be more expensive than corn biofuels. Improved sugar conversion (B) is not enough.

BESC Will Revolutionize How Biomass is Processed

Baseline, Multi-step Cellulosic Ethanol Production

Native Plants

Pretreated Biomass → Solid/Liquid Separation → Enzyme Production → Enzyme Hydrolysis → Hexose Fermentation → Ethanol

Pentose Sugars → Pentose Fermentation → Ethanol

Biomass Modification

Reduced or No Pretreatment → Biomass

Consolidated Bioprocessing

No Separation → CBP Microbes → Ethanol

Modified Plants
A Two-pronged Approach to Increase the Accessibility of Biomass Sugars

Modify the plant cell wall structure to increase accessibility

Improve combined microbial approaches that release sugars and ferment into fuels

Both utilize rapid screening for relevant traits followed by detailed analysis of selected samples
A Two-pronged Approach to Increase the Accessibility of Biomass Sugars

Modify the plant cell wall structure to increase accessibility

Improve combined microbial approaches that release sugars and ferment into fuels

Both utilize rapid screening for relevant traits followed by detailed analysis of selected samples
Strategy Part 1: Identify, Understand and Manipulate the Plant Cell Wall Genes Responsible for Recalcitrance
Functional modifications of both 1° and 2° walls may decrease recalcitrance.

Malcolm O’Neill, CCRC
Mohnen et al. (2008)
How Do We Identify Recalcitrance Genes?
• **Targeted cell wall synthesis approach:**
  * Test known putative recalcitrance genes in via *Populus* and switchgrass transgenics (TP)
  * Basic research to identify unknown genes and decipher how they effect recalcitrance

• **Discovery-based natural variation approach:**
  * Identify natural variation in recalcitrance
  * Identify gene responsible
  * Test via *Populus* and switchgrass transgenics (TP)
  * Activation tagging
What Genes Control Cell Wall Synthesis (and Access to the Sugars)?

- Native plants
- High-throughput screening (HTS) for sugar accessibility
- Phenotyping
- Key genes and cell wall structure
- Modified plant with accessible sugar
- Transformation pipeline
- Targeted putative recalcitrance genes
- “Omics”
- Detailed analysis
- Database
Targeted Plant Genes and Transformation Pipeline

• Gene transformation pipeline established and running
  – 70 *Populus* genes per set
  – 4 Switchgrass for stable transformation per set
  – 30 Switchgrass by VIGS (viral induced gene silencing) per set
  – Three sets totaling >300 genes in pipeline after three rounds of review

• *Populus*
  – Transformation: 200 genes per year
  – Activation Tagging: 1000 genes per year

• Switchgrass
  – Transformation: 20 genes Year 1; 40-60 Year 2
  – VIGS: 200 genes per year, RNAi

• Higher perennial plants have fewer genetic tools and so targets must be selected carefully

<table>
<thead>
<tr>
<th>Functional category</th>
<th># genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall biosynthesis</td>
<td>50</td>
</tr>
<tr>
<td>Cell division and expansion</td>
<td>46</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>26</td>
</tr>
<tr>
<td>Stress response</td>
<td>20</td>
</tr>
<tr>
<td>Metabolism</td>
<td>19</td>
</tr>
<tr>
<td>Intracellular traffic</td>
<td>9</td>
</tr>
<tr>
<td>Protein fate</td>
<td>9</td>
</tr>
<tr>
<td>Transcription</td>
<td>9</td>
</tr>
<tr>
<td>Plant defence</td>
<td>4</td>
</tr>
<tr>
<td>Nucleic acid or nucleotide binding</td>
<td>2</td>
</tr>
<tr>
<td>Transporters</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>196</td>
</tr>
</tbody>
</table>
Targeted Cell Wall Synthesis

Approach: A Few Examples
Tension Stress Study: Background

Tension wood is formed on upper side of bent stems and characterized by:

- Increased number of xylem cells
- Increased cell wall thickness
- Special layer of wall: Gelatinous G-layer
- Increased cellulose content
- Decreased lignin content
- Parallel orientation of microfibrils

Tension stress study is a cross-project effort.
Tension Stress Study: Experiment

- Two genotypes of *Populus* plants used in the bending experiment
- Two weeks of mechanical bending
- Harvested, pooled and processed (where needed) developing xylem and phloem tissues from tension, opposite and normal wood types
Tension Stress Study: Characterization

-Omics
  • Metabolomics
  • Proteomics
  • 454 Transcriptomics
  • RTPCR

Spectroscopy
  • MBMS
  • NMR
  • FTIR
  • LIBS

Imaging
  • WoodCAT
  • AFM
  • Optical microscope

LIMS
  • Sample workflow
  • Barcodes
Modifying Cell Wall Composition and Structure Can Reduce Recalcitrance

- More sugar is solubilized by cellulase when the lignin content of alfalfa cell walls is reduced
- Strategy is feasible for *Populus* and switchgrass

Biochemical and Genetic Dissection of Lignin Biosynthesis in Switchgrass

Wild-type Transgenic

Kinetics of CCR1 towards feruloyl CoA

Transgenic plants generated or in the transformation process

Recombinant enzyme expressed and purified

Dixon et al.
Discovery-based Approach to Identify Recalcitrance-Associated Genes via Analysis of Natural Variation
Mining Genetic Variation in Switchgrass

Create diverse population by cross “lowland” SG AP-13 and “upland” SG VS-16 into 385 pseudo F1 clones

Pseudo F₁ population of 385 genotypes

Clones ready for field planting

HTS Pipeline

Sugar Release Assay

Analytical Pyrolysis

Create Genetic Marker Map to identify allelic variation

Identify Marker Trait Association

Cell Wall Biosynthesis Database
Mining Variation to Identify Key Genes in Biomass Composition and Sugar Release

Collected ~1300 samples for *Populus* Association and Activation-tag Study

**HTS Pipeline**

Sugar Release Assay
Analytical Pyrolysis

*Create Genetic Marker Map to identify allelic variation*
*Identify Marker Trait Association*

Establish common gardens for association and activation tag populations with 1000s of plants
Populus Association Study
Plant Materials

- Cuttings propagated by Mt. Jefferson Farms (Salem, OR)
- Propagation successful for 100% of the genotypes
- Plants moved to Oregon State University for overwintering
Association Genetics Study – Whole-genome Resequencing in effort to discover SNPs across *Populus* genome

In collaboration with the Joint Genome Institute 10 alternate *Populus* genomes are being resequencing

Preliminary Results

- 28x depth from 6 Solexa runs
- 85% align to Nisqually-1
- 843,000 SNP loci relative to reference
- 78,000 SNP loci are heterozygous
Strategy Part 2: Biomass Recalcitrance
Measure, Understand, and Model

Biomass Formation and Modification

Characterization and Modeling

Biomass Deconstruction and Conversion
HTP Characterization Pipeline for the Recalcitrance Phenotype

• Screening of 1000’s of samples

Composition analytical pyrolysis, IR, confirmed by wet chemistry

Pre-treatment new method with dilute acid and steam

Enzyme digestibility sugar release with enzyme cocktail

Detailed chemical and structural analyses of specific samples
Composition Data from Analytic Pyrolysis (MBMS) for High-throughput Screening of Transgenic Populations

• Rapid (50/h w/ 4mg)
• Reliable
• Gives values for glucan, xylan, lignin, and details on monomers – e.g., S/G
• Complements time-consuming and more variable wet chemistry, molecular and biochemical analyses

Composition data from Populus association study (798 samples) represents full range of known Populus variation
Composition Data from *Populus* Association Study

- The association samples display extreme variation in lignin, S/G ratio, and sugar content.

- There is a negative correlation between sugar content and lignin content.

- All sampled genotypes are being replicated and will be established in a common garden experiment.
Enabling Technology: An HTP Pretreatment for 1000s of Small Samples

**Biomass + Water Distribution**

**Pretreatment**

**Co-Hydrolysis**

**Sugar Analysis**

Unique and Important
- *Steam*: efficient uniform heating
- *No separation*: saves time and increases accuracy
- *2-4mg sample size*: reduces material costs

HTP Enzymatic Digestion Assays

• Recalcitrance is ultimately determined by enzyme access to carbohydrates and sugar release
• HTP assays are needed to assess recalcitrant phenotypes and to screen for more effective enzymes
• 1st tier assays: >1000 samples/week
  – Evaluate base-line susceptibility of pretreated biomass as well as enzymes from natural diversity
• 2nd tier assays: ~200 samples/day
  – Hits from primary screen subjected to multi-dimensional assays using engineered enzyme cocktails for precise assessment of cell wall changes

Response surface output for multi-dimensional digestibility assays
Populus Association Study

- Tested for enhanced sugar release characteristics through pretreatment and enzymatic hydrolysis
  - Hot water pretreatments at 160 and 180°C
- HTP pretreatment and co-hydrolysis in 96 well-plates

- Preliminary observations:
  - Sugar yield increases with S/G ratio
  - Lignin content has minimal effect
  - Some outlier poplar samples exhibit very high sugar release

- Characterization pipeline works

Pretreatment conditions: 180°C, 18Min, 160°C, 68Min

Studer, Wyman et al.
Detailed Analysis of Specific Samples
Inform Cell-wall Chemistry and Structure

**Chemistry**
- NMR for cellulose crystallinity
- Fractionation and chromatography
- Mass Spectrometry for key metabolites
- 2D $^1$H-NMR sees altered bonds in polysaccharides and lignin in biomass

**Imaging**
- AFM of switchgrass showing cellulose microfibrils
- Bio-ultraCAT for 3-D density of *Populus* cell walls
- Immunolocalization using wall antibodies on *Populus* protoplasts
Analytical Pyrolysis of Low Lignin Alfalfa

36 minutes of analysis for 6 (x3) samples

Solid State $^{13}$C NMR Spectroscopy

- Whole cryo-milled cell wall residue
- Normalized for carbohydrate content

$^{13}$C NMR spectra for the control versus C3H9a and HCT30a
CARS (Coherent Anti-Stokes Raman Scattering) Imaging of Lignin in Interfascicular Fiber Cell Walls in Alfalfa

Wild-type

C3H

HCT

CML: Compound middle lamellae; SW: secondary cell walls

S-Y Ding (NREL) and X. S. Xie (Harvard)
tools under BER imaging grant; sample analysis under BESC, MS in preparation
Preliminary Conclusions from Detailed Analysis of Alfalfa Mutants

• Crosslinking between polymers is critical
• Altered localization does occur in mutants
• Crystallinity was not a major factor
• Multiple techniques on same samples add insights in the hands of experts
Strategy Part 3: Identify, Understand and Manipulate “Biological Catalysts” to Overcome Recalcitrance
Exploring Novel Environments

- Rumen endosymbionts
- Caecum endosymbionts
- Coleopteran larvae
- Biotraps
- Shipworms
- Fungi
Sequencing of 3kb and 8kb Insert Libraries (Plasmids) and 40kb Fosmid Insert Libraries - Distribution of Glycoside Hydrolases

- 220 glycoside hydrolases present on 6688 contigs (6M bp total)
- GHase families 2, 3, 31, 38 and 43 are most abundantly found

Niels van der Lelie et al.
Clone Library Activity Screening

- **Rumen** endosymbionts, **Caecum** endosymbionts
  - Animals—18
  - Microbial samples—13
  - DNA extracted—5

- **Coleoptera** larvae gut endosymbionts
  - DNA extracted—21 larvae
  - Clone libraries constructed—3

- **Biotraps**
  - DNA extracted—21 unique biotrap
  - Ribosomal diversity analysis in progress

- **Shipworm** endosymbionts
  - Specimens—>100
  - Preliminary dissection and microbial isolation complete

- **Fungal isolates**
  - DNA extracted—78 unique isolates
Microbial Hydrolysis and Enzymatic Hydrolysis: A Fundamentally Different Relationship Between Microbes and Cellulose

Enzymatic hydrolysis (classical approach)
- Hydrolysis mediated by CE complexes
- Enzymes (several) both bound and free
- Cells may or may not be present

Cellulose, C
Cellulase enzyme(s), E
Microbes, M (non-cellulolytic)

Microbial hydrolysis (CBP)
- Hydrolysis mediated mainly by CEM complexes
- Enzymes both bound and free
- Cells both bound and free

Cellulose, C
Cellulase enzyme(s), E
Microbes, M (cellulolytic)

Yeast, enzymes with biomass, Dumitrache and Wolfaardt

C. thermocellum on poplar, Morrell-Falvey and Raman, ORNL
Biodiversity Access for New Biocatalysts

• What is the upper temperature for cellulose degradation?
• How do is it done?
• Can we make it better?

Sampling at Yellowstone National Park, October 2007 and July 2008
High-Throughput Isolation Using Flow Cytometry

Establish consortium

Identify members

Flow-cytometer

Select different gates

Anaerobic inc. @ 75 °C

$\Delta$\text{pH}$ $ indicates growth

BioEnergy Science Center
Caldicellulosiruptor sp. OB47

• Gram-positive bacterium
• Rod Shaped ~0.5 x 1-2µm
• $T_{\text{opt}}$ 75-78°C
• Fermentative heterotroph
• Produces $\text{H}_2$, Acetate, Lactate, $\text{CO}_2$ and Ethanol
Caldicellulosiruptor sp. OB47

Growth of *Caldicellulosiruptor sp. OB47* on insoluble substrates

![Image of growth cultures](image_url)
Schematic of Cellulosome

(adapted from Carlos Fontes, 2007 Gordon Research Conference on “Cellulases and Cellulosomes”)
C. thermocellum Cellulosome was Analyzed under Several Conditions

- Grown on cellubiose, Avicel, and pretreated switchgrass at ~1L
- Cellulosome is released when growth slows
- Cellulosome isolation via affinity digestion method
- In-solution trypsin digestion, following by shot-gun proteomics (LC-MS/MS)
- Quantitative proteomics with $^{15}$N labeled substrates
Characterization of a \textit{C. thermocellum} Mutant that Utilizes Cellulose Rapidly

- 454 Resequencing identified 78 mutated loci in Speedy mutant
- 25 mutant loci common with ORNL wild-type strain
- Transcriptomics and further analysis underway
Integrating Expression, Proteomics, SNPs, Metabolites on Cellular Systems

*Locus Tag:* SO_1631  
*Gene Symbol:* pyrH  
*Gene Description:* uridylate kinase, PyrH  
*Organism:* Clostridium thermocellum  
*Location:* 1713187..1713915  
*Function:* This protein, also called UMP kinase, converts UMP to UDP by adding a phosphate from ATP. It is the first step in pyrimidine biosynthesis.  
*Notes:*  
*Citation:*  
*Annotation Date:* 2007-03-24  
*Domain(s):* COG | MIST  
*INTERPRO:*  
*Pathway(s):* BeoCyc | KEGG  
*Information:* GenBank | IMG | SEED | ORNL | SEED | COG | MIST

SNP mapping  
Expression on pathways  
Metabolite tracing  
Pathway Component Details
Computational Microscope – Assay and Analysis Framework
Highlights 2008 until March 2009

• **185+** Scientific presentations at meetings and conferences worldwide
• **43** Scientific publications
• **17** Workshops and seminars for BESC researchers and graduate students
• **13** Inventions disclosed which are under evaluation by the BESC Commercialization Council and 2 additional in-preparation
• Scientific collaboration with the University of British Columbia has contributed over 250 additional *Populus* samples at no cost to BESC
• **80+** Presentations to Stakeholders (Secretary, Under Secretaries, Congressmen and Staff Members, Businessmen, etc.)
• **70+** Television, Print, and Radio Interviews
• Education program with the Creative Discovery Museum in Chattanooga, Tennessee to develop a Biofuels Outreach Lesson
• Co-sponsored Global Venture Challenge 2008 in April at ORNL
BESC Website

http://bioenergycenter.org/

• New website has been deployed
• Elements include
  – General information
  – Educational and professional level components
  – area for controlled access by BESC members
  – Inventions
Retreat February 2008
Thank you
Retreat December 2008

BESC is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science