BioEnergy Science Center (BESC)

Director’s Presentation to BERAC

Paul Gilna
February 27, 2015
The BioEnergy Science Center
A multi-institutional, DOE-funded center performing basic and applied science dedicated to improving yields of biofuels from cellulosic biomass

300+ People in 18 Institutions

- Oak Ridge National Laboratory
- National Renewable Energy Laboratory
- Samuel Roberts Noble Foundation
- ArborGen, Inc.
- Ceres, Incorporated
- Mascoma Corporation
- DuPont
- GreenWood Resources
- University of North Texas
- 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

2012-2014
To enable the emergence of a sustainable cellulosic biofuels industry by leading advances in science and science-based innovation resulting in removal of recalcitrance as an economic barrier to cost-effective production of biofuels.
Access to the sugars in lignocellulosic biomass is the current critical barrier

- Overcoming this 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.**
Scientific challenges as of 2006

• With a deeper understanding of:
  ➢ The resistance of lignocellulosic biomass to deconstruction
  ➢ The genetic controls of plant composition and ultrastructure
  ➢ Bioenergy crop domestication and sustainability
  ➢ The structure and function of cellulases and other plant cell wall depolymerizing enzymes
  ➢ The microbial cell’s mechanisms for toxicity response

• We could envision:
  ➢ Dedicated bioenergy crops
  ➢ Consolidated bioprocessing – cellulase production and ethanol fermentation combined
  ➢ Beyond “ethanol” to advanced biofuels
  ➢ Improved pretreatments

DOE Biomass to Biofuels Roadmap (7/2006)
http://doegenomestolife.org/biofuels/b2bworkshop.shtml
Cellulosic industry is emerging

Syngenta and Cellulosic
Ethanol Technologies team
up for battle in Iowa
July 14, 2011
In Minnesota, a successful ethanol process—one million barrels per day (bpd) in 2015.
Read More

M&G Chemicals, Anhui Guozhong ink pact to build world’s first biofuel plant in China
July 29, 2011
970,000 tons of corn ethanol per year. Why is China interested in biofuels?
Read More

Walking The Talk: A fast-rising DuPont nears completion of its cellulosic biofuel project
Celebrating completion of its cellulosic biofuel project in China.
Editor’s Sketchbook: Beta Renewables cellulosic ethanol project
Crescentino, Italy
September 25, 2014
The first commercial-scale cellulosic ethanol plant in Europe. The plant is expected to produce 5 million gallons per year.
Read More

Beta Renewables, Biochemtex ink deal for commercial-scale biofuels project in South America
October 10, 2014
Cellulosic leaders sign with 2nd Generation Ethanol pla Biochemtex and Beta Renew Energochemica SE for the construction of a commercial-scale biofuels plant.
Read More

World’s largest cellulosic biofuels plant opens: Beta Renewables, in pictures
October 8, 2014
Beta Renewables plant in Crescentino, Italy to produce 75 million liters of cellulosic ethanol per year from agricultural waste. What does a commercial-scale plant look like? In Italy, Beta Renewables and Novozymes marked the official opening in Northern Italy of the world’s largest cellulosic biofuels facility. Situated in fields outside the city of Crescentino, it is the largest plant of its kind.
Read More

BioEnergy Science Center
Key strategic goals for BESC through Year 10

• Develop a fundamental understanding of the molecular basis of recalcitrance and cell wall formation.

• Identify and characterize a high-performing set of *Populus* and switchgrass TOP reduced recalcitrance lines.

• Understand microbial cellulose utilization at all levels, including enzyme components, enzyme systems, pure cultures, and mixed cultures.

• Achieve proof of concept for CBP with *C. thermocellum*, yeast, and *Caldicellulosiruptor spp.*

• Develop and apply chemical, immunological, physical, and imaging methods to characterize biomass; use the resulting data to build models to predict the relationships between biomass structure and recalcitrance.
Organized around our strategic goals

Better Plants

Better Microbes

Better Tools and Combinations
Feedstocks: then and now

2007

• Lignin and cellulose accessibility believed to be the primary roots of recalcitrance
• Low transformation efficiencies for switchgrass
• Range of natural variation and genetic control of recalcitrance within a species not established
• Viability of low lignin transgenic perennials not confirmed in field

2015

• Proved core concept that multiple genes – many outside of the lignin pathway, control plant cell wall recalcitrance
• Used BESC transformation pipeline to target ~900 candidate recalcitrance gene constructs to date
• Verified multiple reduced recalcitrance lines in *Populus* and switchgrass and ~20 TOP Lines selected for deeper study
• Data now returning from field trials of initial transgenic lines
Early results: high-throughput Agrobacterium-mediated switchgrass transformation

- A transformation efficiency of more than 90% has been achieved, vastly improving prior 5% efficiency.
- The system has been effectively used for producing large numbers of transgenic switchgrass plants.
Early results: COMT modification improves biofuel yield

Phenylalanine → PAL

Agrobacterium-mediated transformation of switchgrass

<table>
<thead>
<tr>
<th>Phenylalanine</th>
<th>PAL</th>
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<tbody>
<tr>
<td>HOOC-</td>
<td>cinnamate</td>
</tr>
<tr>
<td>C4H</td>
<td>4-coumaric acid</td>
</tr>
<tr>
<td>4CL</td>
<td>4-coumaroyl CoA</td>
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<tr>
<td>CCR</td>
<td>4-coumaraldehyde</td>
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<tr>
<td>CAD</td>
<td>4-coumaroyl alcohol</td>
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<tr>
<td>HCT</td>
<td>H lignin</td>
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<tr>
<td>CCoAOMT</td>
<td>feruloyl CoA</td>
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<tr>
<td>CCR</td>
<td>coniferaldehyde</td>
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<tr>
<td>CAD</td>
<td>coniferyl alcohol</td>
</tr>
<tr>
<td>F5H</td>
<td>5-hydroxyconiferyl alcohol</td>
</tr>
<tr>
<td>COMT</td>
<td>5-hydroxyconiferaldehyde</td>
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</tbody>
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Lignin pathway

Impact of enzyme levels and pretreatment conditions on biofuel production

Noble Foundation transgenic switchgrass

Fu and Wang (Noble), Mielenz (ORNL), support from USDA/DOE
Fu et al. PNAS 2011
Field study of COMT transgenic switchgrass

Significance

This is the first reported field study evaluating biofuel potential of transgenic switchgrass with reduced cell wall recalcitrance and shows that conversion phenotypes can be maintained in the field.

Outcome

• Lignin-modified transgenic switchgrass had similar gains in sugar release (up to 34% higher) and biofuel production (up to 28% higher) as observed in the greenhouse.

• Results extrapolate to 50% more liters of ethanol per hectare compared to wild-type.

BESC Synergy

• Culmination of several years and many hands;

• Prior BESC work: creation of the transgenic switchgrass (Noble), greenhouse experiments (Noble), composition analyses (Noble, NREL);

• This work: APHIS field permits and cultivation (UTK), compositional analyses (NREL, ORNL), saccharification tests (NREL), SHF fermentation tests (ORNL, UTK).
Genome-wide adaptive variation in *Populus* revealed by population genomic analysis

**Significance**
- Study is the first to explore the genomic legacy of selection across an entire tree genome and highlight both the wide range of selection pressures as well as the climatic influence on phenological systems and growth.
- Provides new insights into mechanisms of adaptive evolution, and provides potential targets for genetic improvement for biofuels production as well as the management of natural populations in the face of environmental change.

**Outcome**
- 17.9 million single nucleotide polymorphisms (mostly rare) were identified representing naturally segregating variants found in wild populations which can be utilized to track key traits.
- 379 genomic regions showed evidence of recent positive and/or divergent selection.
- These selected regions had stronger gene-trait association signals at all three test sites than expected, indicating that these outliers were driven by adaptive selection and represent natural, functional variation.

**BESC Synergy**
- This work builds on several years of groundwork:
  - establishing the common gardens
  - JGI sequencing the thousand individuals
  - multiyear phenotyping team(s)
- This work represents multiple skill sets: field and population design (WVU, ORNL), field maintenance (GreenWood and Mt. Jefferson Farms), plant genome sequencing (JGI, ORNL), and GWAS bioinformatic analytics (ORNL, WVU, UTK)

Field testing of improved feedstocks

40+ *Populus* constructs in stool beds (South Carolina)

1000+ *Populus* genotypes in 4 common gardens (Pacific Northwest)

Field assessment of genetically improved switchgrass (Texas)

Field assessment of genetically improved switchgrass (Tennessee)
Reduced recalcitrance TOP Lines already selected for deeper study

Year 6
- *PvGAUT4*-KD (pectin synthesis)
- *PvFPGS1*-KD (carbon metabolism)
- *PvCCR1*-KD (lignin synthesis)
- *PvMYB4*-OE (transcription factor, lignin)
- *PvCOMT*-KD (lignin synthesis)
- *PvmicroRNA156*-OE (growth and development)

Year 7
- *PvSGAMP15_14* (natural variant)
- *PvSGAMP15_05* (natural variant)
- *PvEFR/SHINE TF*-OE (transcription factor)
- *PvGAUT1*-KD (pectin synthesis)

+18 Populus lines added

Switchgrass control and *FPGS*-KD lines at R1 stage.
Reduced recalcitrance TOP Lines already selected for deeper study

Year 6

- PvGAUT1-KD (pectin synthesis)
- PvFPGS1-KD (carbon metabolism)
- PvCCR1-KD (lignin synthesis)
- PvMYB4-OE (transcription factor)
- PvCOMT-KD (lignin synthesis)
- PvmicroRNA156-OE (growth and development)
- PvSGAMP15_14 (natural variant)
- PvSGAMP15_05 (natural variant)

BESC Synergy

- Each of the TOP Lines combine insights from multiple institutions and simple to complex analytics.
- Common shared protocols and metrics allow stronger comparisons.
- Shared improvements in genetics, transformation and field tests accelerate efforts and avoid errors.

Switchgrass control and FPGS-KD lines at R1 stage.
CBP microbes: then and now

2007

• Few cellulases expressed in yeast
• Functional genetic systems for cellulolytic thermophiles not available
• Modes of ethanol inhibition or tolerance in *C. thermocellum* not understood
• *Caldicellulosiruptor* not well characterized
• Few models and mechanisms for multifunctional cellulolytic enzymes available

2015

• Solubilization of plant cell walls more effective when mediated by cellulolytic microbes
• Multiple new structures and models for cellulosomes along with chimeric cellulosomal enzymes
• New genetic tools for thermophilic cellulolytic microbes
• Improved ethanol yields in *C. thermocellum* and demonstrated ethanol production in *Caldicellulosiruptor*
• Production of advanced biofuels in thermophilic microbes
Early results: microbial solubilization of plant cell walls

- Larger than expected differences in solubilization effectiveness as a function of the choice of both feedstock and conversion system
- Ability of several anaerobic bacteria to solubilize cellulosic biomass without (or with minimal) conventional pretreatment
- *C. bescii* grows on high solids loadings (up to 200 g/L) of crystalline cellulose and non-pretreated switchgrass
- *C. bescii* solubilizes all components of non-pretreated switchgrass (cellulose/hemicellulose/lignin) at 78°C
- Better than 74% cellulose conversion of five grassy feedstocks by *C. thermocellum* with minimal pretreatment
Cellulase architecture and mechanisms

Significance
The different modes of action of CelA and Cel7A offer new possibilities for creating synergistic mixtures of biomass-attacking enzymes drawn from different organisms.

Outcome
- The activity of CelA on Avicel is sevenfold higher than the common exo/endo cellulase standard mixture, Cel7A and Cel5A.
- CelA appears to be both surface active and active in excavating into the substrate creating cavities and additional surface area for attack, whereas Cel7A is apparently strictly surface active and creates tapered ends on Avicel.

BESC Synergy
- Draws on deep NREL expertise in fungal cellulases, hydrolysis, and imaging.
- CelA identified as a key enzyme by Caldi groups at UGA and NCSU based on sequencing from JGI and transcriptomics and proteomics at NCSU and ORNL.

Early results: genetic systems for thermophiles

BESC reported successful DNA transformation of both *C. thermocellum* and *C. bescii*:

- Used methylation with an endogenous unique α-class N4-Cytosine methyltransferase to overcome major barrier to DNA transformation in *C. bescii*.
- Strategy for *C. thermocellum* employed counter selections developed from the native *C. thermocellum hpt* gene and the *T. saccharolyticum tdk* gene; was used to delete the genes for both lactate dehydrogenase (Ldh) and phosphotransacetylase (Pta).

**BESC Synergy**

- Significant lessons learned were applied from prior work in *C. thermocellum* and *T. saccharolyticum* (Dartmouth, Mascoma, ORNL).
- The methods are being transferred into BESC partner labs at ORNL and NCSU for metabolic engineering for the direct conversion of lignocellulose to ethanol or butanol.
Building on efforts to increase biofuel yield in BESC CBP thermophiles

Outcome

• *C. thermocellum*:
  ➢ Several engineering strategies have improved ethanol yield.
  ➢ HydG was the best targeted mutation.
  ➢ Several of the evolved strains share a point mutation in the *adhE* alcohol dehydrogenase.

• *C. bescii*:
  ➢ Also based on incorporation of *adhE* gene;
  ➢ 15 mM ethanol levels;
  ➢ Acetate, lactate and ethanol production when grown on cellobiose, Avicel and switchgrass.

BESC Synergy

Shared strategies among Dartmouth, ORNL, UGA
Highest *C. thermocellum* ethanol yield to date: “quad mutant" (*pfl*− *hydG*− *ldh*− *pta-ack*−)

**Cellbiose**

- **L-Lactic Acid**
- Formate
- Acetate
- Ethanol
- Acetyl-P
- Pi
- ATP
- ADP
- Acetyl-CoA
- NAD+ NADH
- NAD+ NADH
- Pyruvate
- Fd_{oxidized}
- Fd_{reduced}
- NAD(P)
- NADH
- 2 H+
- Ethanol

**Ethanol yield 81% of theoretical**

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

*Biswa et al.*, submitted
Conversion of switchgrass to biofuel using engineered *Caldicellulosiruptor bescii*

**Significance**
- Direct conversion of plant biomass to a fuel without pretreatment.
- First demonstration of metabolic engineering of an extreme thermophilic organism for conversion of lignocellulosic biomass to a liquid fuel.

**Outcome**
- The Idh⁻, AdhE⁺ strain redirected most of the carbon flux to ethanol, with 12.8 mM ethanol produced from non-pretreated switchgrass (compared to 14.8 mM from cellobiose).
- Switchgrass was neither chemically nor thermally pretreated prior to digestion.

**BESC Synergy**
- Led by UGA with genetic engineering and fermentation, fermentation advice and HPLC at ORNL
- Draws on shared standard switchgrass (Noble) previous characterization (NREL) and ORNL fermentation protocols using pretreated materials

Overview of *C. bescii* fermentative pathways for bioconversion of hexose sugars. Pathway 3 (red) is a new pathway resulting from heterologous expression of the *C. thermocellum adhE* gene to synthesize 2 moles of ethanol per mole of glucose.

Lactate, acetate and ethanol production by wild-type (I), Idh⁻ (J), and Idh-AdhE⁺ (K) strains of *C bescii* grown on non-pretreated switchgrass

Isobutanol production from cellulose in *C. thermocellum*

120 plasmids with different promoters constructed
40 verified and tested in *C. thermocellum*

Isobutanol production in LC medium and CTFuD medium during 96 h

Isobutanol production in CTFuD medium (0.45% YE) during 24 h

Achieved 2 g/L of isobutanol in minimal medium (LC medium)
Including cellulase expression and glycerol reduction in the C56X yeast

8-10% Yield Improvement or 2.5 mg/g TS cellulase displacement already demonstrated in an SSF process; transferable to HHF process

Given 250K Tonnes/yr scale:
Value = 625,000 Kg enzyme displaced:
$12/Kg = $7.5M/yr = $0.38/gal savings
Enabling technology: then and now

2007

- Gold standard for biomass composition was NREL method requiring ~5 g per sample and over two weeks’ effort
- Submicron analyses for catalytic imaging and chemical signatures of biomass were not established
- Limited polysaccharide epitope antibodies were just beginning to be used for imaging
- Very large biological datasets were genomic and primarily used in medical screening fields, not in bioenergy

2015

- High-throughput pipeline for analysis of composition and sugar release for thousands of samples per year
- Glycome profiling now medium throughput and validated for analyses of cell wall structure and automated
- Establishment of experimental and computational protocols for high-performance proteomics
- Use of computational modeling to increase understanding of lignin biosynthesis and polymerization
- Integration of modified feedstocks with engineered microbes
- Metabolomics analysis coupled with genetic information to identify metabolite-gene associations
Development and utilization or sample analysis pipelines

• High-throughput (1000s per month):
  ➢ lignin composition
  ➢ S/G ratio
  ➢ glucose/xylose release

• Medium-throughput (100s):
  ➢ sugar composition assay (new)
  ➢ glycome profiling
  ➢ bioconversion assay

• Low-throughput (10s)
  ➢ high resolution image analysis
  ➢ NMR (multiple modalities)
  ➢ cellulose crystallinity
  ➢ accessibility

• Over 65 techniques available
  ➢ see BESC characterization handbook
Support for identification and characterization of TOP Lines

• High-throughput cell wall chemistry and recalcitrance screening (NREL)
  - Assay lignin content, S/G ratio and sugar release identifying low recalcitrance lines and prioritizing deeper characterization work.
  - ~ 30,000 samples planned for BESC Years 6-8.

• Biomass analytical workflow (UGA, NREL, UCR)
  - Use chemical, immunological, enzymatic, and spectroscopic methods to identify changes in biomass structure (TOP selection).
  - Provide predictive knowledge to relate recalcitrance and structure.

• Whole-cell wall NMR with emphasis on lignin structure (UTK/ORNL, UGA)
  - Whole-cell ionic liquid 1D and 2D NMR to characterize the structure of lignin and hemicelluloses and correlate changes with lowered recalcitrance (TOP selection).

• Small-scale cell wall compositional assays (NREL, ORNL)
  - Analyze major and minor sugars as well as other hydrolyzate products (e.g., acetate, furfural, and HMF).
  - Screen TOP candidates for fermentation inhibitors.

• Screening for cellulose crystallinity, accessibility, and degree of polymerization (DP) (UTK/ORNL)
  - Monitor and understand changes to cellulose in transgenic lines and correlate changes to reductions in recalcitrance (TOP selection).
Capabilities of ’omics

Outcome

• Metabolomic profiling identifies growth inhibitors, fuel production pathways, and microbial intracellular redox metabolites:
  ➢ Characterized and quantified aromatic metabolites and known microbial inhibitors released and generated by *Thermoanaerobacter saccharolyticum* degrading corn cob biomass

• Metabolomic profiling of transgenic plants versus controls:
  ➢ Completed the GC-MS analysis of >60 transgenic lines of *Populus deltoides*, *Panicum virgatum*, and *Arabidopsis thaliana*.

• A proteomics-based assay measuring *C. thermocellum* cell density grown on biomass substrates:
  ➢ Applied to real-world samples (right), tMIT specifically and accurately tracks cell density across cultures that utilize both simple (cellobiose) and complex (switchgrass) carbon sources.

BESC Synergy

• ORNL ’omics expertise applied to Dartmouth, Noble, UGA and ORNL samples

• Drivers for improved methods in microbial and redox measurements came from FA2
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 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.

**BESC Synergy**

- Combines elements from across BESC: COMT switchgrass (Noble), *C. thermocellum* (Mascoma) with ORNL expertise.

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 now 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, 170 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 36,000 in the last year and over 116,000 students, parents, and teachers in the past six years.
BESC Active “Alumni”
Elements of BESC’s value system

- Focus on impactful recalcitrance science
- A high-functioning team of world-class scientists
- Emphasis on integration and collaboration
- Close connection to industry increasing potential of impact
- Acceleration of research and technology outcomes
- Growing core of well-trained young research staff
Thank you!
One more thing...
Reduced distortion with reinforced materials.

15% CF-ABS
C-lignin shows promise as a potential co-product for biorefining

May be easier to extract from plant cell walls. Replacing G/S lignin with C-lignin might lead to reduced recalcitrance of biomass. **Linear nature** makes this molecule better suited than classical lignin for manufacture of **carbon fibers** and bioplastics.

*C-lignin*  
*Kraft lignin*

Nar et al., unpublished results