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

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http://www.bioenergycenter.org/

June 30, 2009

U.S. DEPARTMENT OF ENERGY

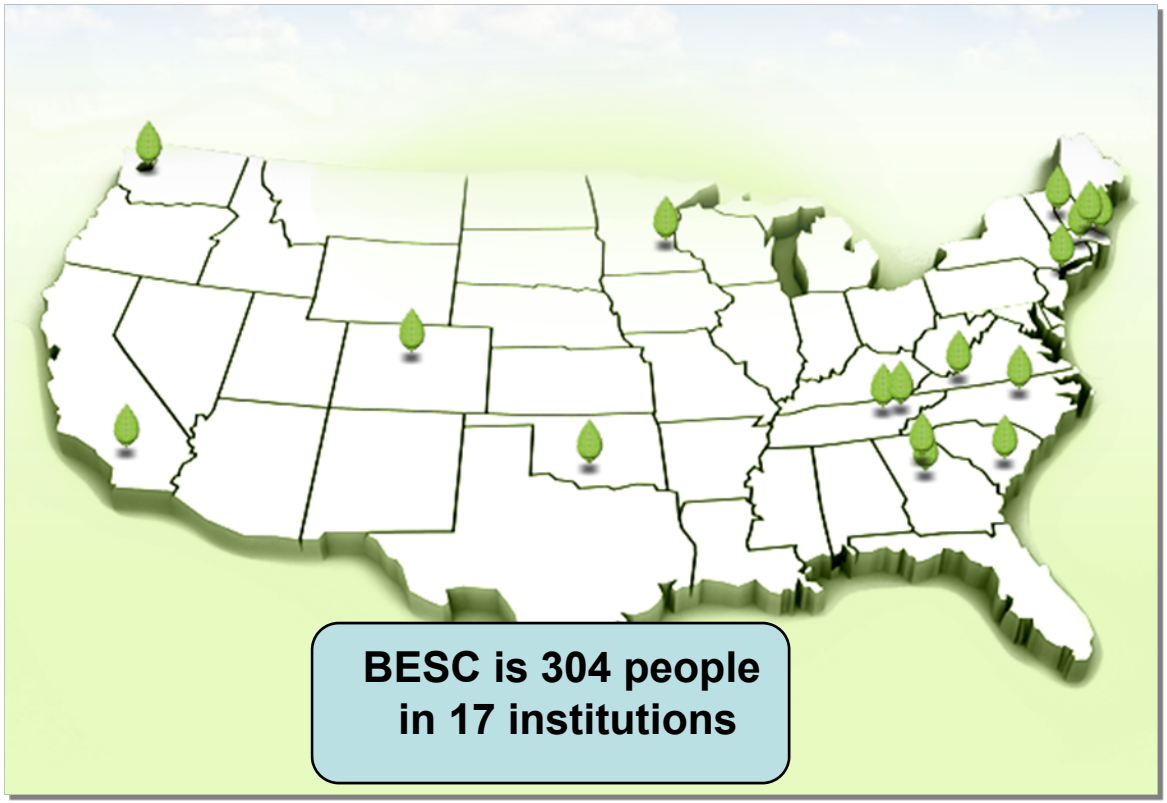
BESC BioEnergy Science Center

OAK RIDGE National Laboratory

The BioEnergy Science Center

U.S. DEPARTMENT OF ENERGY

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



- Oak Ridge National Laboratory
- University of Georgia
- University of Tennessee
- National Renewable Energy Laboratory
- Georgia Institute of Technology
- Samuel Roberts Noble Foundation
- Dartmouth College
- ArborGen, LLC
- Verenium Corporation
- Mascoma Corporation
- University of California-Riverside
- Cornell University
- Washington State University
- University of Minnesota
- North Carolina State University
- Brookhaven National Laboratory
- Virginia Polytechnic Institute

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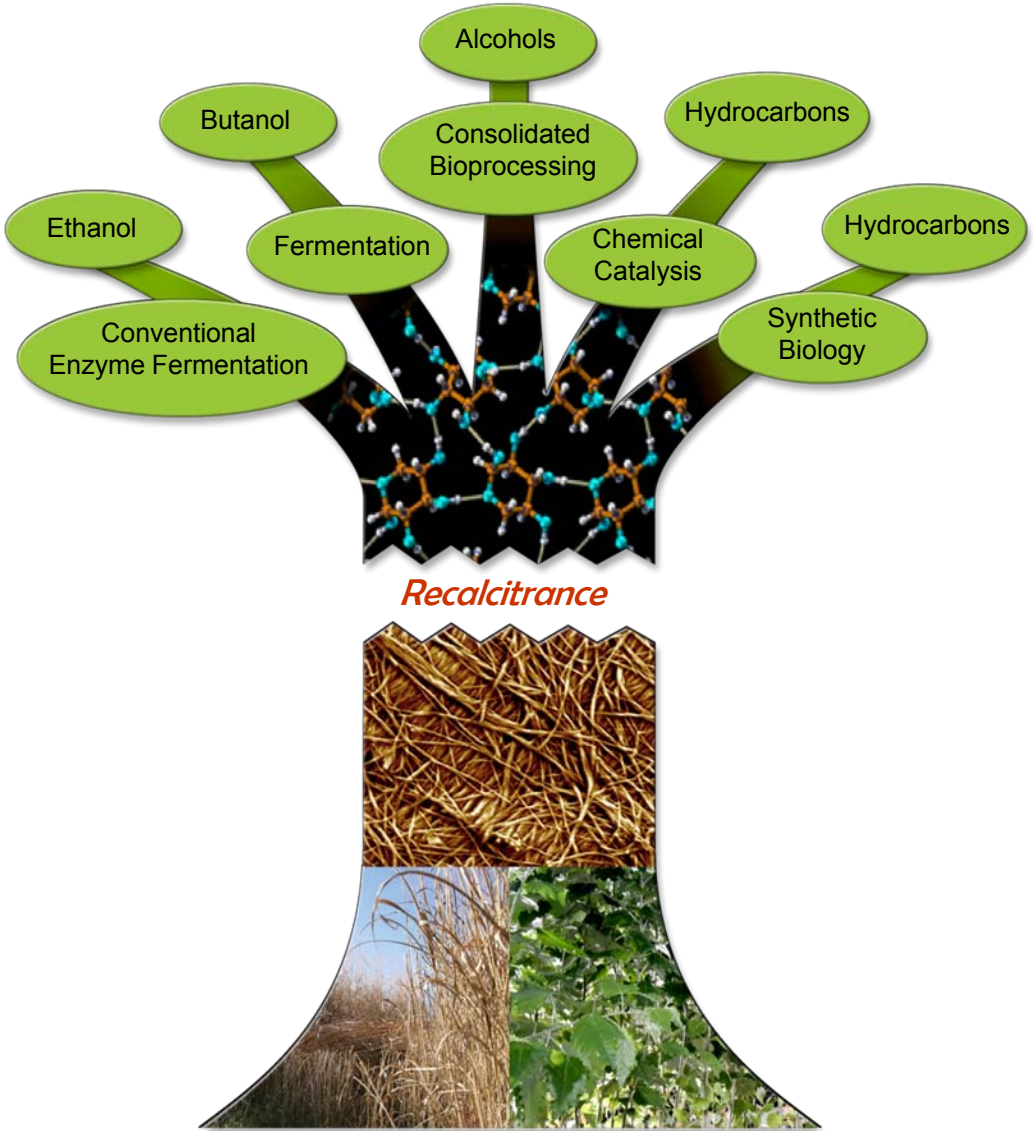
Access to the Sugars in Lignocellulosic Biomass is the Current Critical Barrier



- 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

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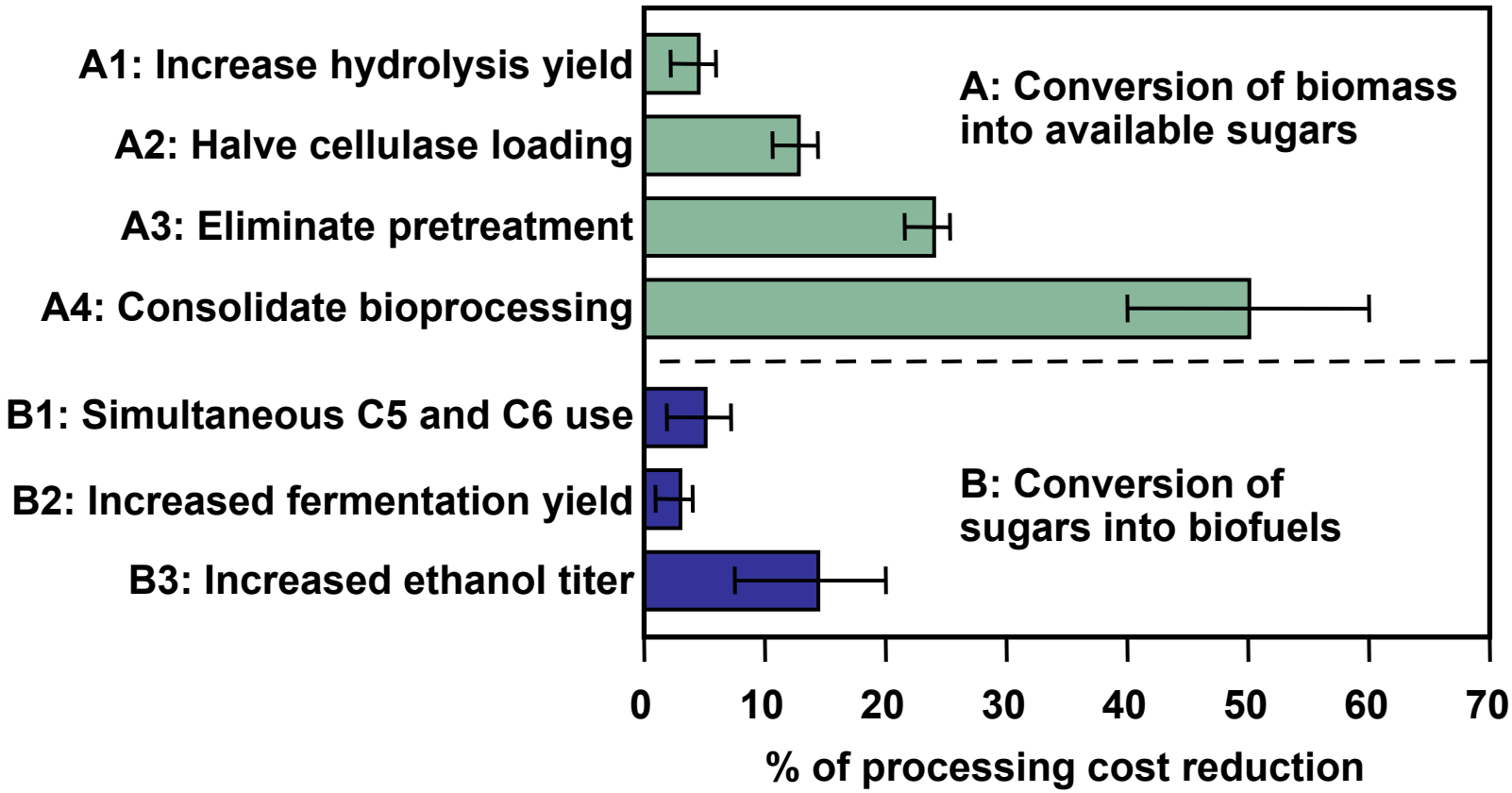
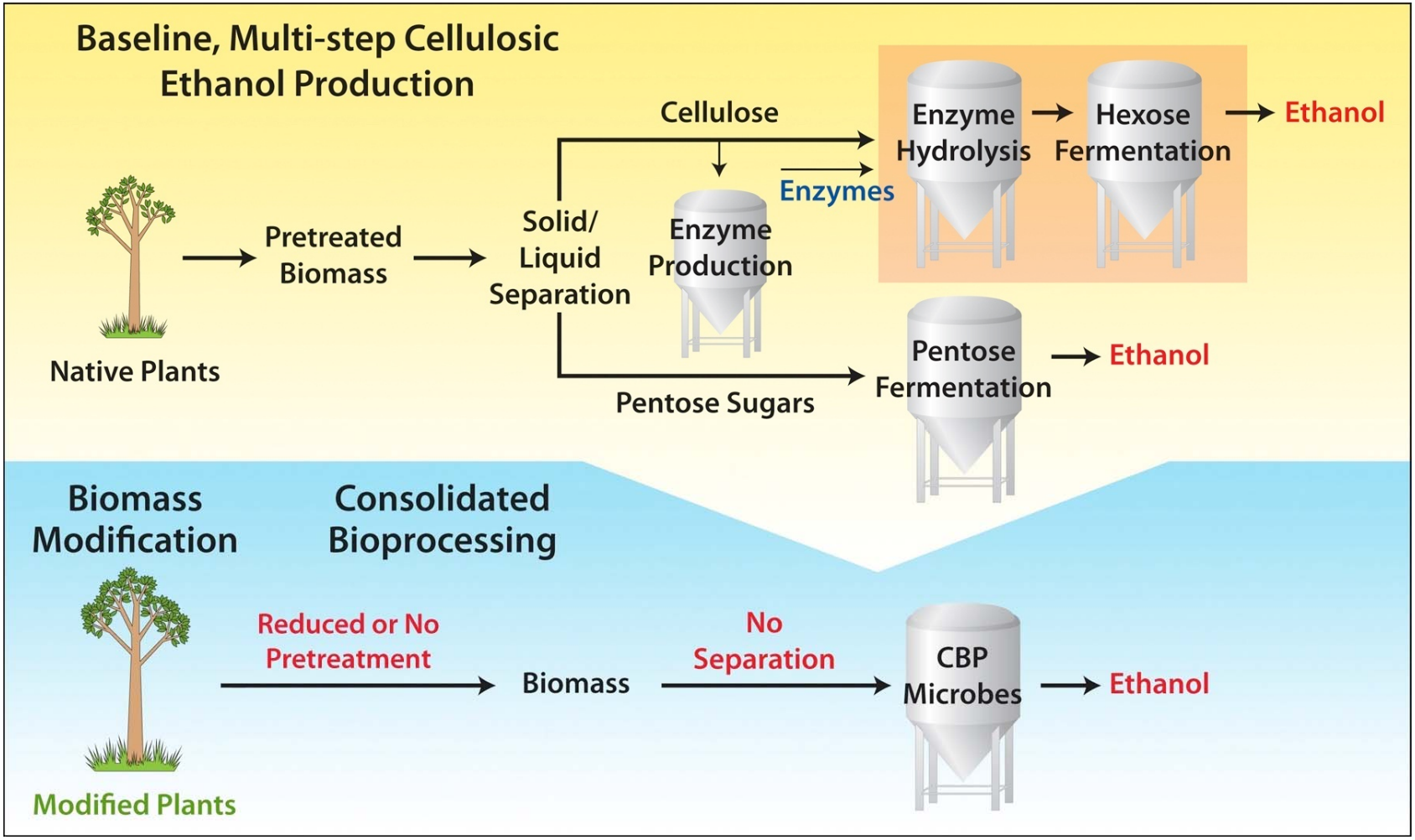
Comparative Impacts of R&D on Biomass Processing Cost



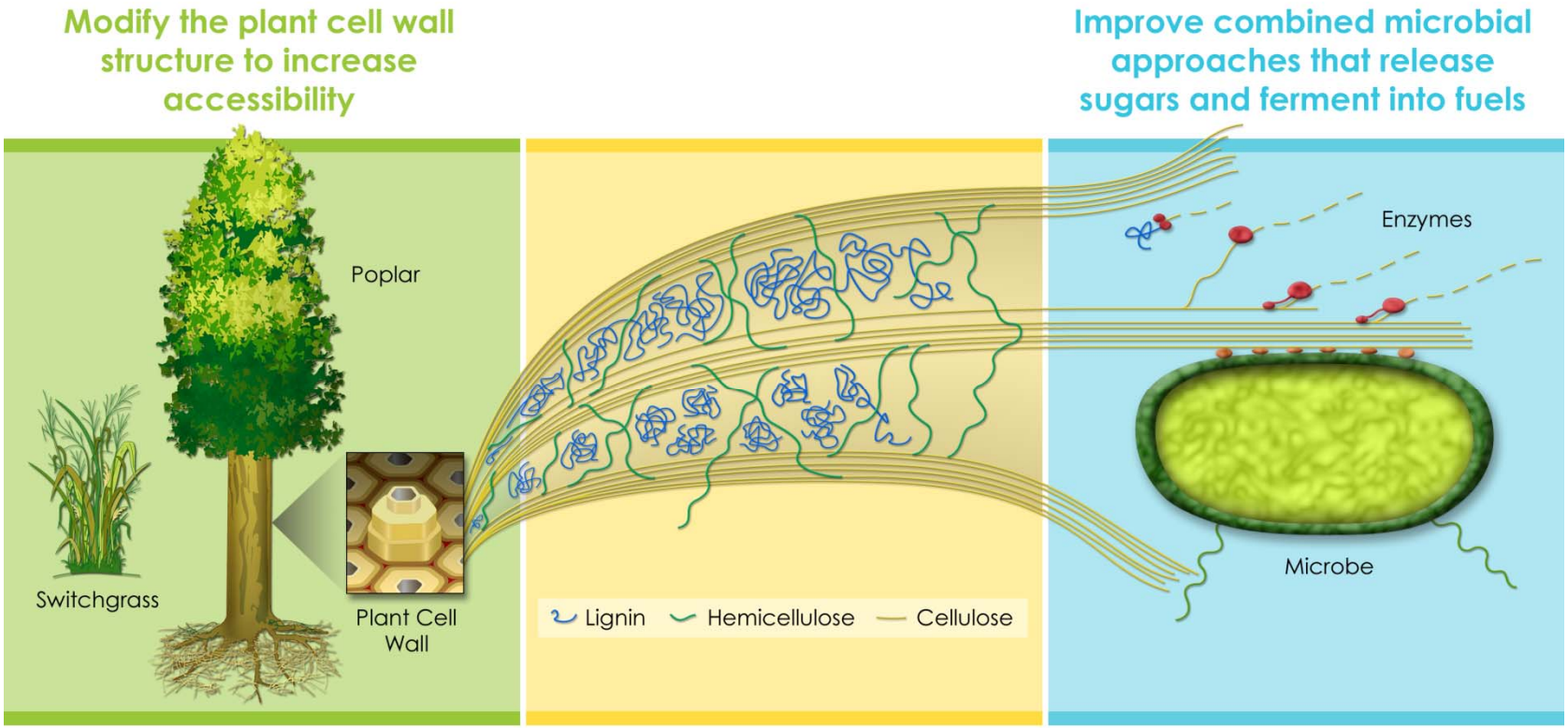
Without overcoming biomass recalcitrance (A), cellulosic biofuels will be more expensive than corn biofuels. Improved sugar conversion (B) is not enough.

Ref: Lynd, L.R., M.S. Laser, D. Bransby, B.E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J.D. McMillan, J. Sheehan, C.E. Wyman, "How Biotech can transform biofuels," *Nature Biotechnology* 26:169-172 (2008)

BESC Will Revolutionize How Biomass is Processed

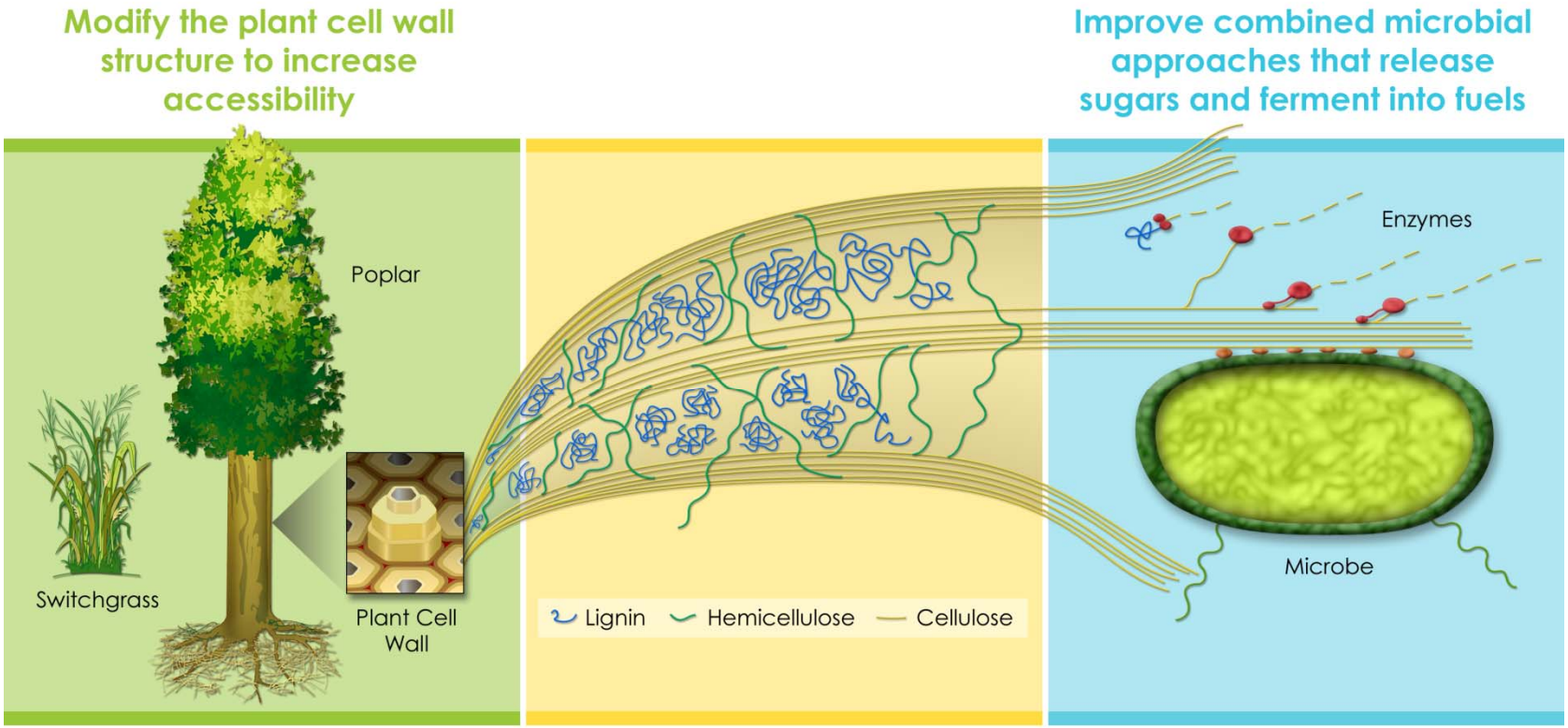


A Two-pronged Approach to Increase the Accessibility of Biomass Sugars



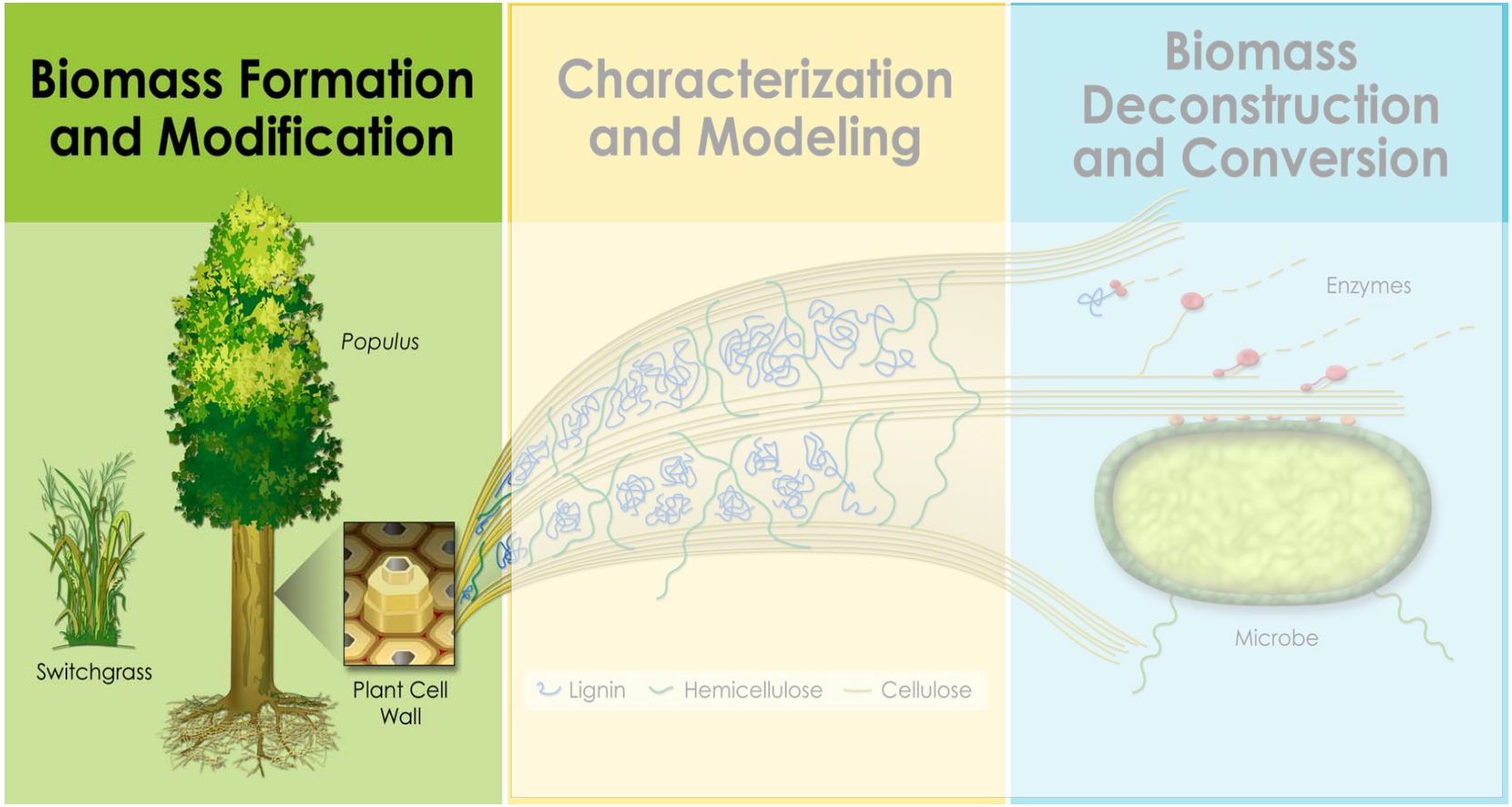
Both utilize rapid screening for relevant traits followed by detailed analysis of selected samples

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

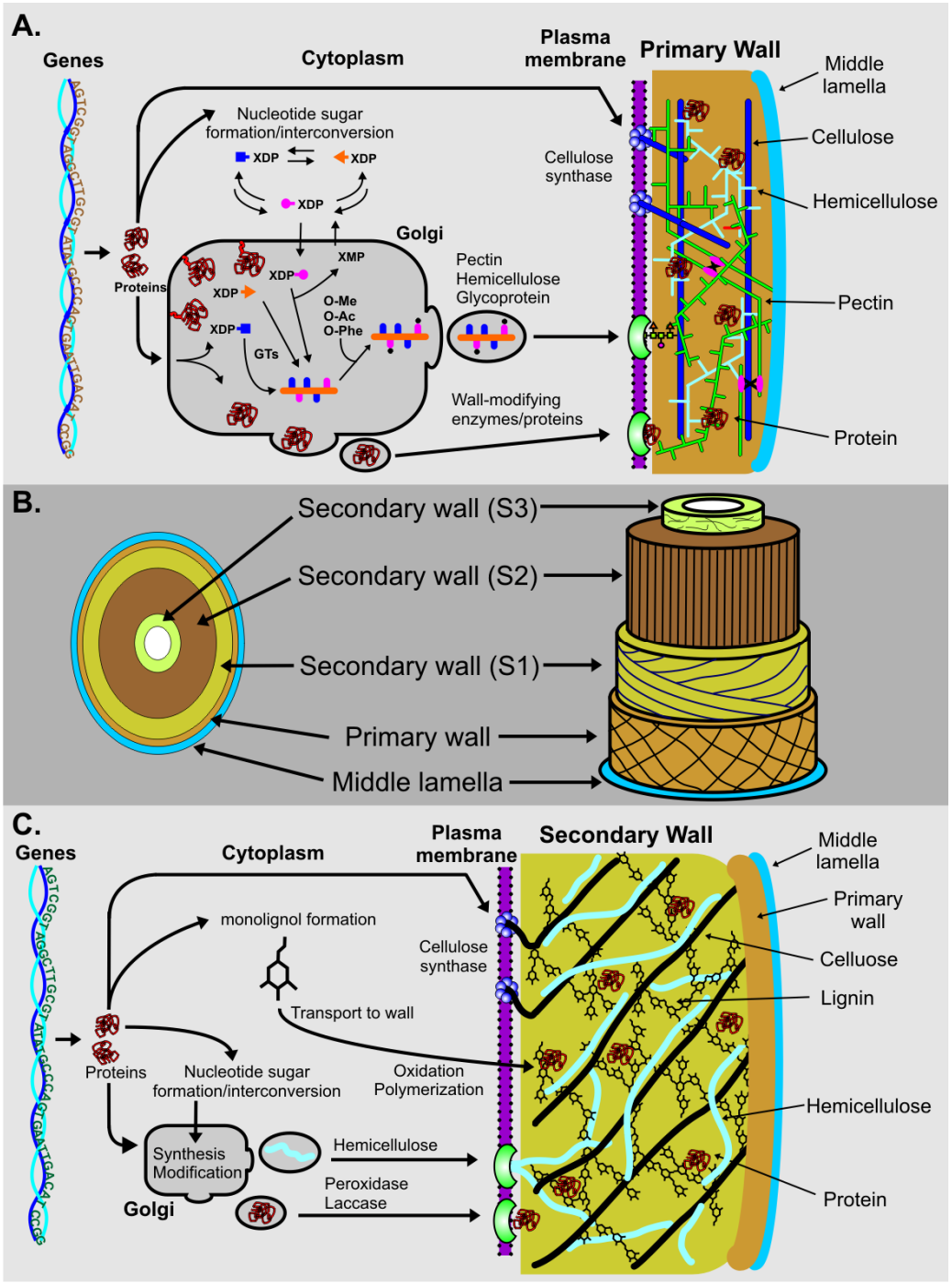


Both utilize rapid screening for relevant traits followed by detailed analysis of selected samples

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)

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Primary Wall

90% polysaccharide

Dividing and growing cells

Pectin
Hemicellulose
Cellulose
(proteins)

Secondary Walls

70 – 80% polysaccharide

Some cells with structural roles

↓ Pectin
Hemicellulose
Cellulose
Lignin
(proteins)

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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:***

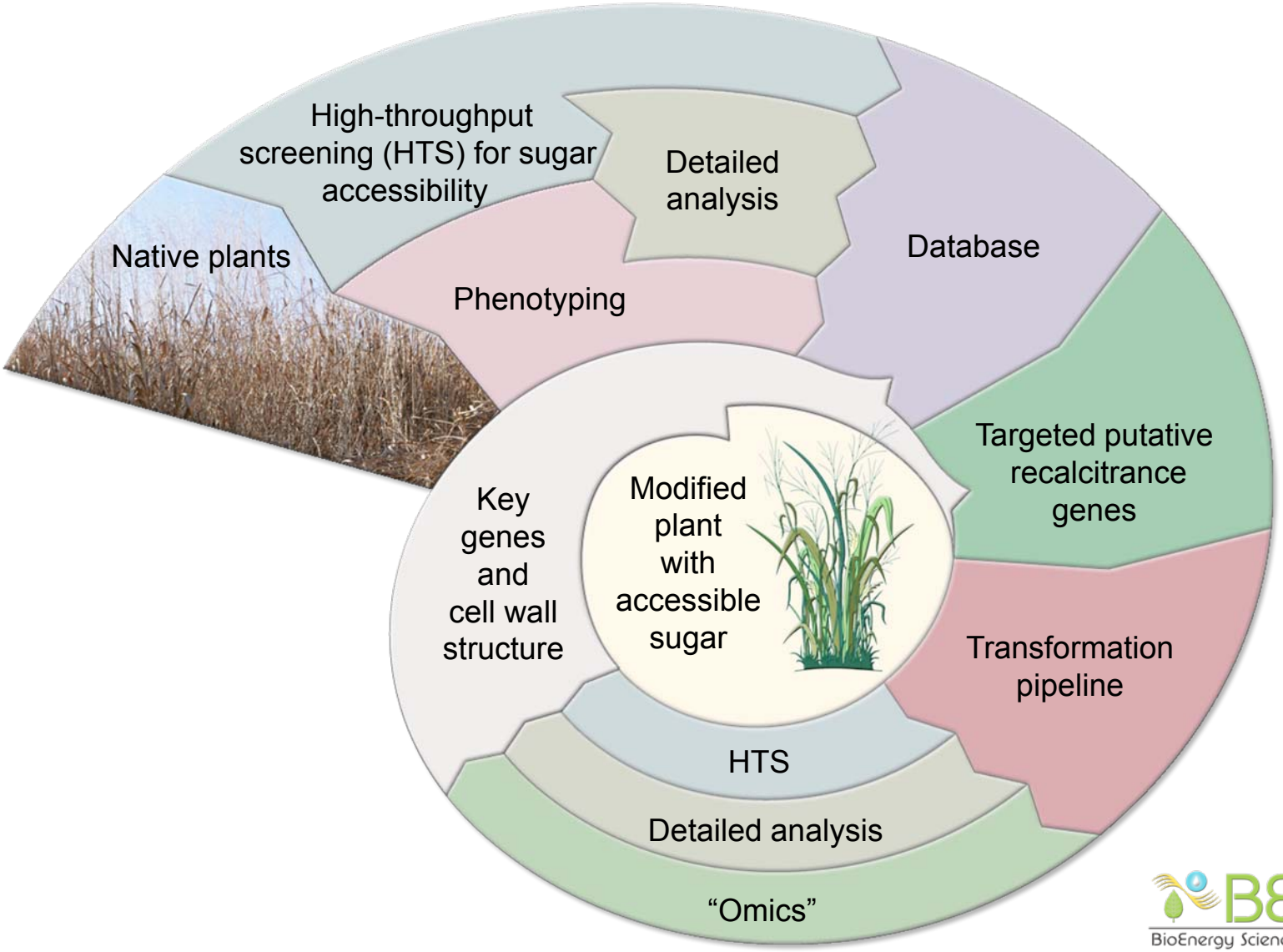
  * Identify natural variation in recalcitrance

  * Identify gene responsible

  * Test via *Populus* and switchgrass transgenics (TP)

  * Activation tagging

What Genes Control Cell Wall Synthesis (and Access to the Sugars)?



Targeted Plant Genes and Transformation Pipeline

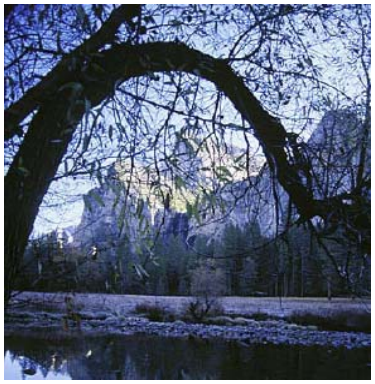
- Gene transformation pipeline established and running
  - 70 *Populus* genes per set
  - 4 Switchgrass for stable transformation per set
  - 30 Switchgrass by VIGS (viral induced gene silencing) per set
  - Three sets totaling >300 genes in pipeline after three rounds of review
- *Populus*
  - Transformation: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

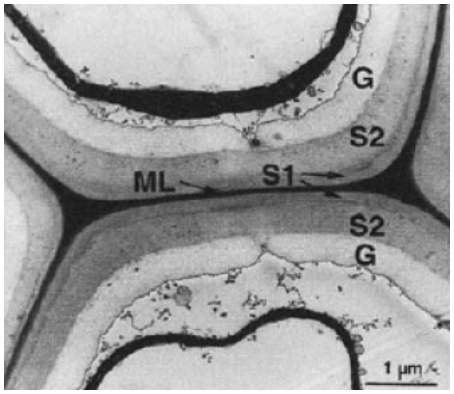
Functions of initial targets (set #1)

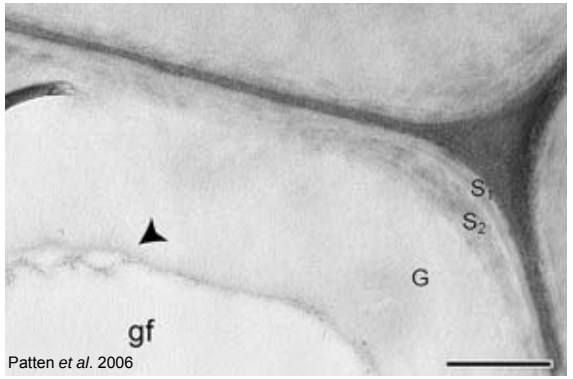
Functional category	# genes
Cell wall biosynthesis	50
Cell division and expansion	46
Signal transduction	26
Stress response	20
Metabolism	19
Intracellular traffic	9
Protein fate	9
Transcription	9
Plant defence	4
Nucleic acid or nucleotide binding	2
Transporters	2
Total	196

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

Control erect plants

Mechanically bent plants

Tension Stress Study: Characterization

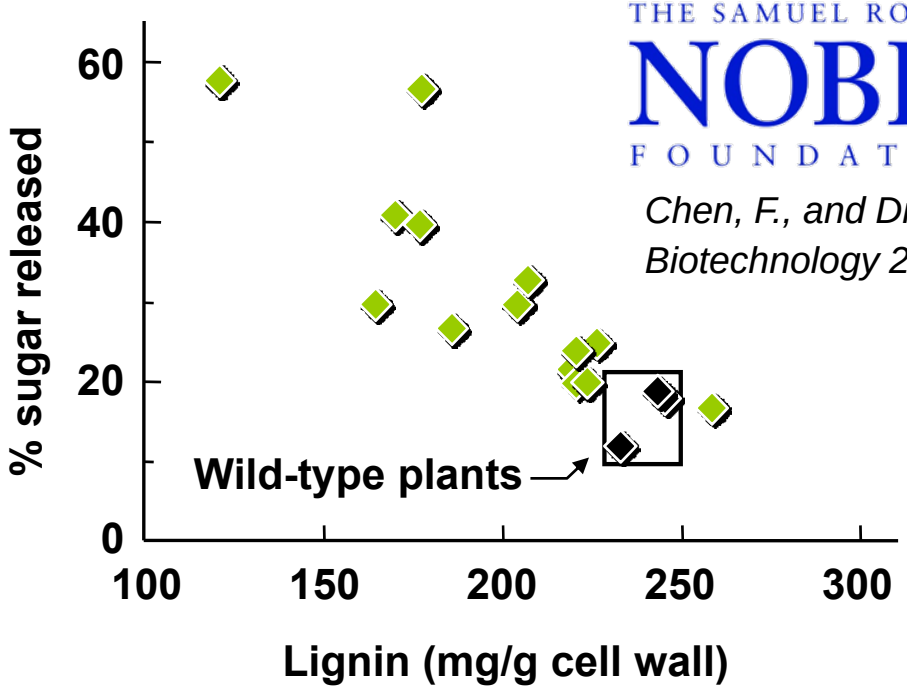
-Omics
- Metabolomics
- Proteomics
- 454 Transcriptomics
- RTPCR

LIMS
- Sample workflow
- Barcodes

Spectroscopy
- MBMS
- NMR
- FTIR
- LIBS

Imaging
- WoodCAT
- AFM
- Optical microscope

Modifying Cell Wall Composition and Structure Can Reduce Recalcitrance

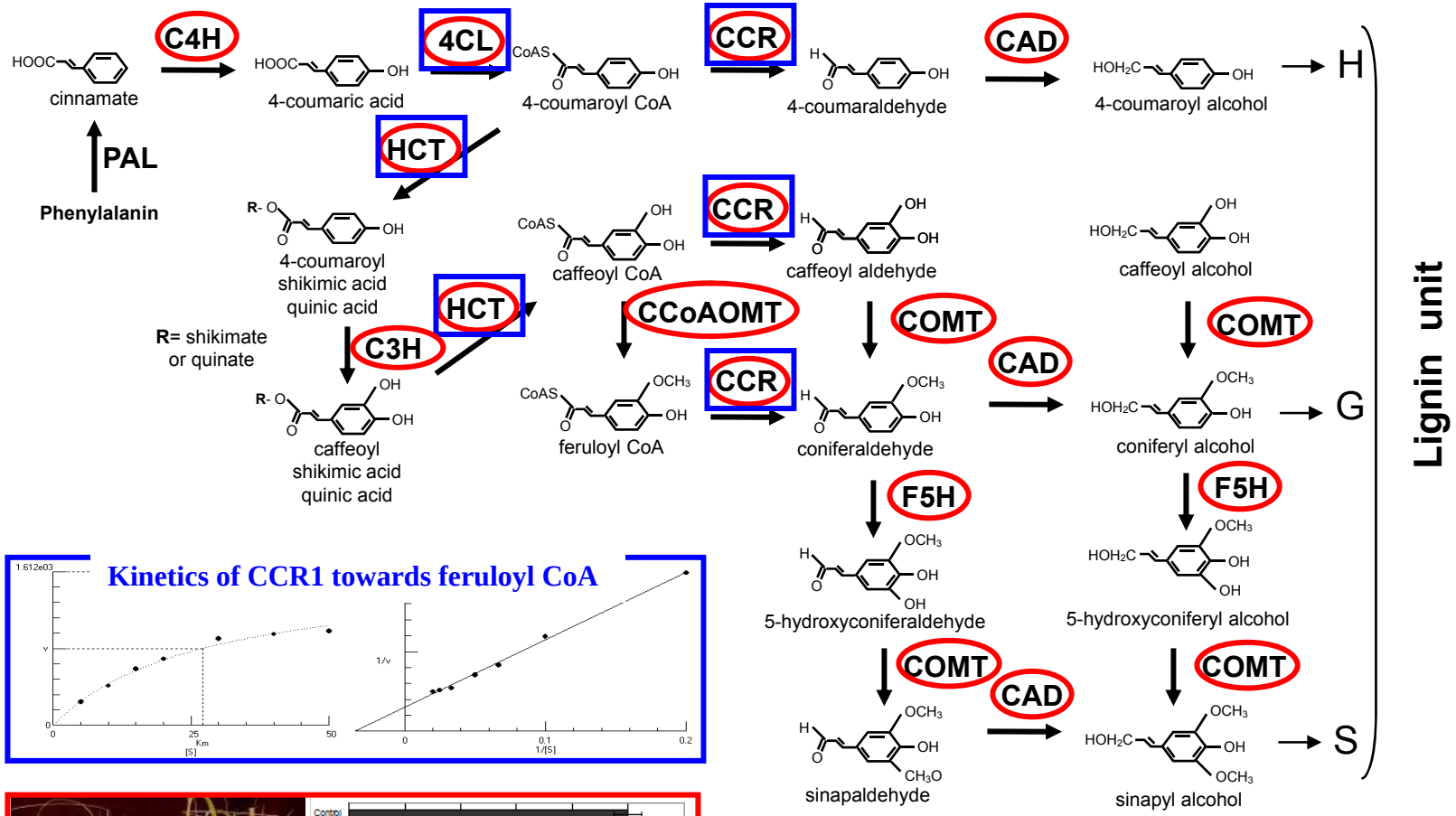


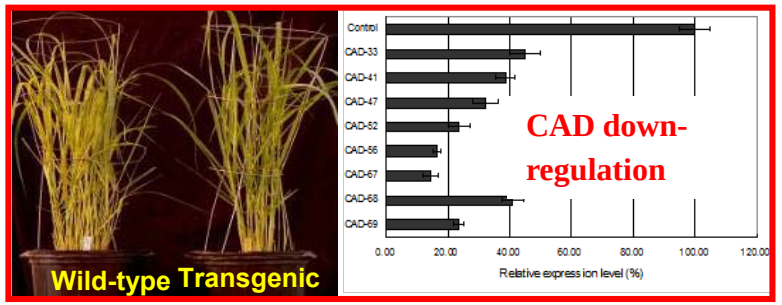
THE SAMUEL ROBERTS NOBLE FOUNDATION

Chen, F., and Dixon, R.A. (2007) Nature Biotechnology 25, 759-761.

- 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





Transgenic plants generated or in the transformation process

Recombinant enzyme expressed and purified

Dixon et al.

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Discovery-based Approach to Identify Recalcitrance-Associated Genes via Analysis of Natural Variation

Mining Genetic Variation in Switchgrass

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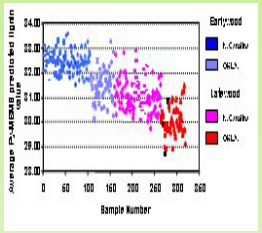
Create diverse population by cross "lowland" SG AP-13 and "upland" SG VS-16 into 385 pseudo F1 clones

Pseudo F_1 population of 385 genotypes

Clones ready for field planting

HTS Pipeline

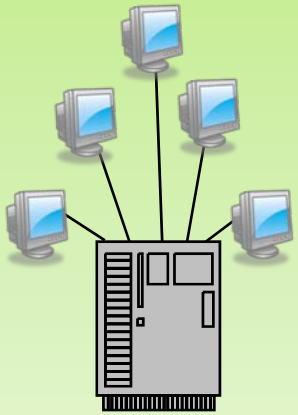
Sugar Release Assay



Analytical Pyrolysis

Create Genetic Marker Map to identify allelic variation

Identify Marker Trait Association



Cell Wall Biosynthesis Database

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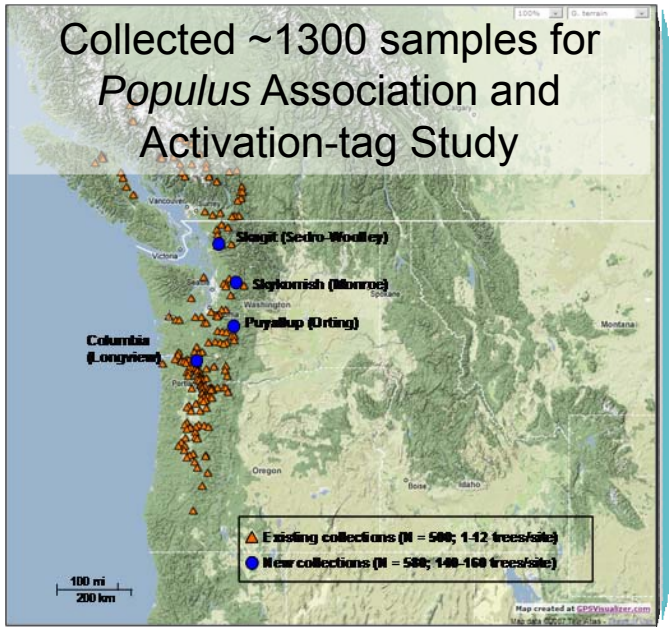
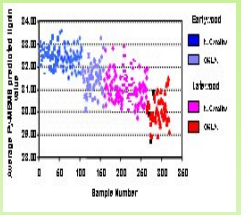
Mining Variation to Identify Key Genes in Biomass Composition and Sugar Release



HTS Pipeline

Sugar Release Assay



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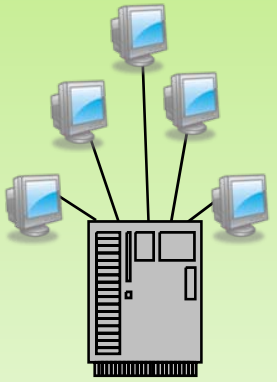

Cell Wall Biosynthesis Database

Establish common gardens for association and activation tag populations with 1000s of plants

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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

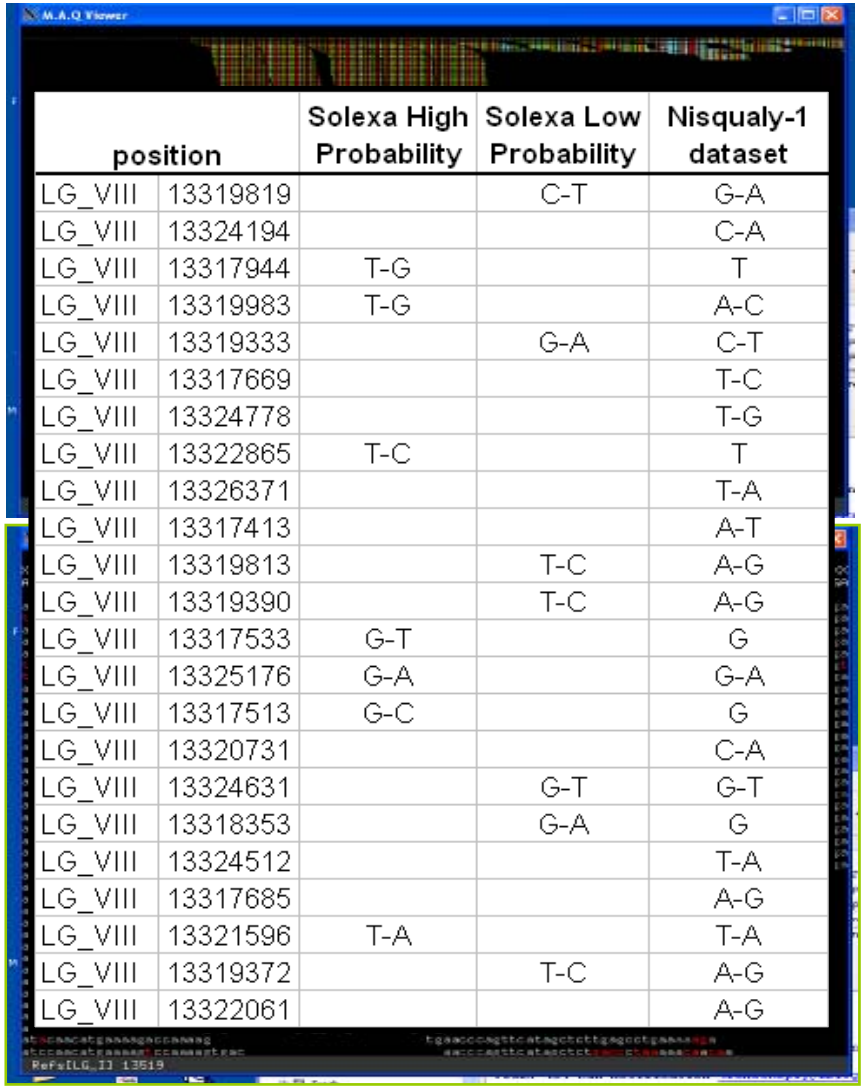
Association Genetics Study –

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

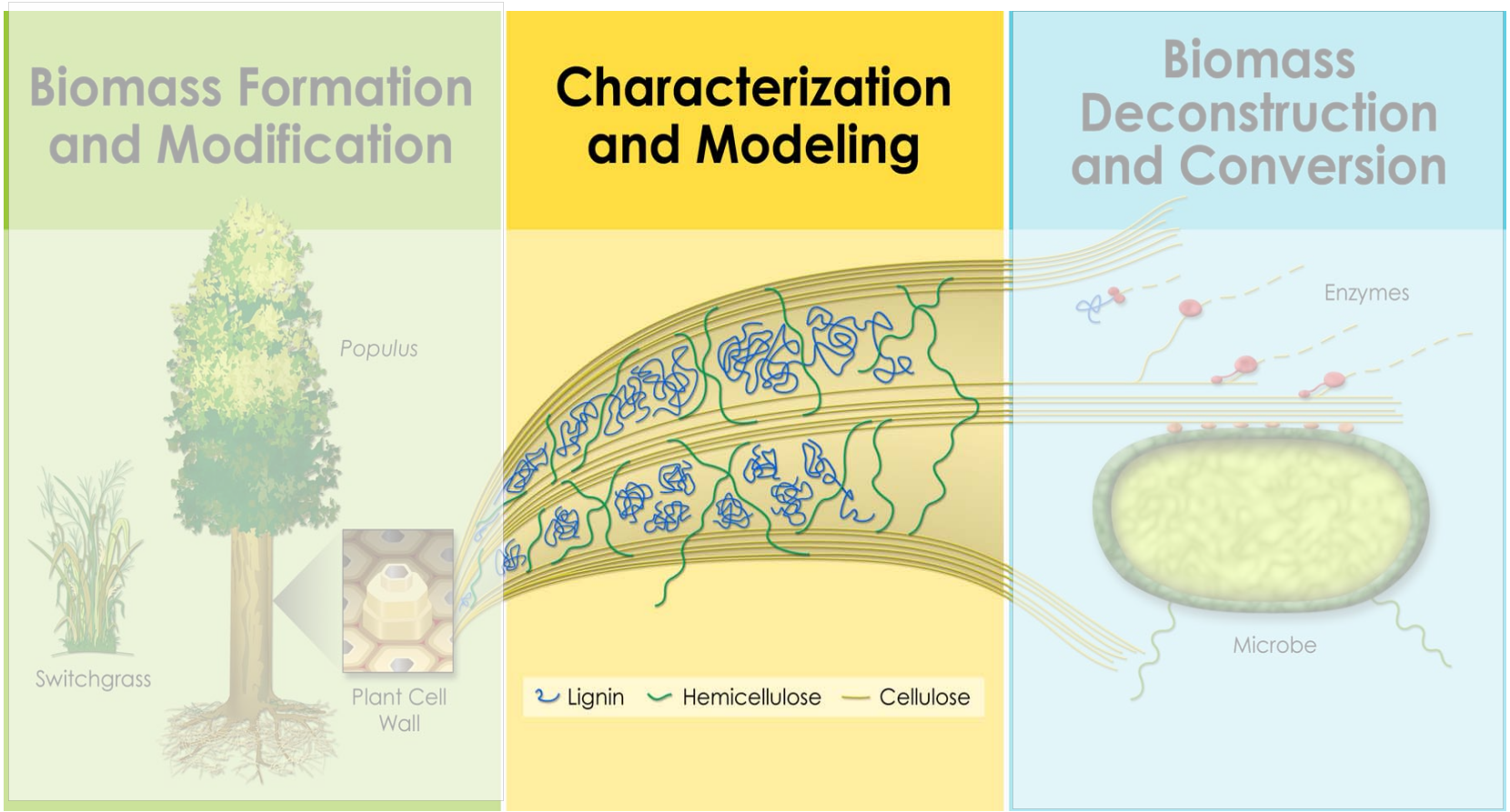
Preliminary Results

- **28x depth from 6 Solexa runs**
- **85% align to Nisqually-1**
- **843,000 SNP loci relative to reference**
- **78,000 SNP loci are heterozygous**



position		Solexa High Probability	Solexa Low Probability	Nisqualy-1 dataset
LG_VIII	13319819		C-T	G-A
LG_VIII	13324194			C-A
LG_VIII	13317944	T-G		T
LG_VIII	13319983	T-G		A-C
LG_VIII	13319333		G-A	C-T
LG_VIII	13317669			T-C
LG_VIII	13324778			T-G
LG_VIII	13322865	T-C		T
LG_VIII	13326371			T-A
LG_VIII	13317413			A-T
LG_VIII	13319813		T-C	A-G
LG_VIII	13319390		T-C	A-G
LG_VIII	13317533	G-T		G
LG_VIII	13325176	G-A		G-A
LG_VIII	13317513	G-C		G
LG_VIII	13320731			C-A
LG_VIII	13324631		G-T	G-T
LG_VIII	13318353		G-A	G
LG_VIII	13324512			T-A
LG_VIII	13317685			A-G
LG_VIII	13321596	T-A		T-A
LG_VIII	13319372		T-C	A-G
LG_VIII	13322061			A-G

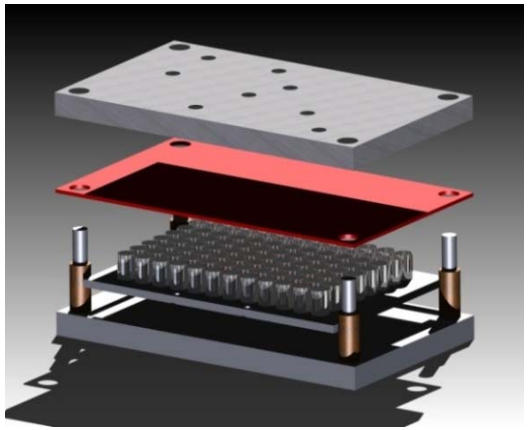
Strategy Part 2: Biomass Recalcitrance Measure, Understand, and Model



HTP Characterization Pipeline for the Recalcitrance Phenotype

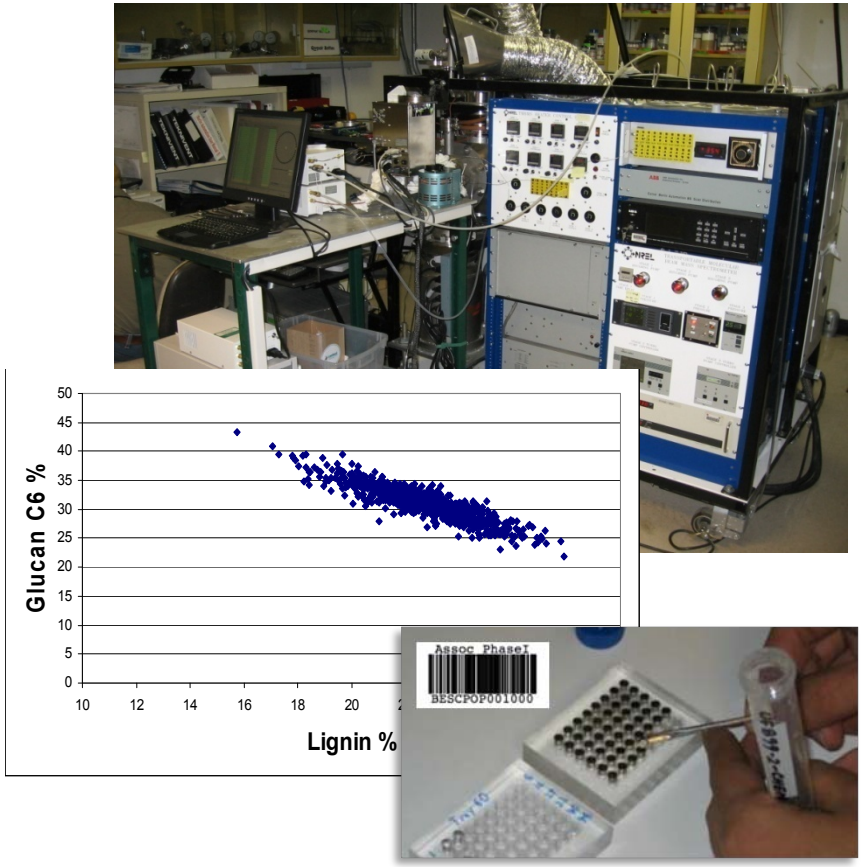
- Screening of 1000's of samples

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

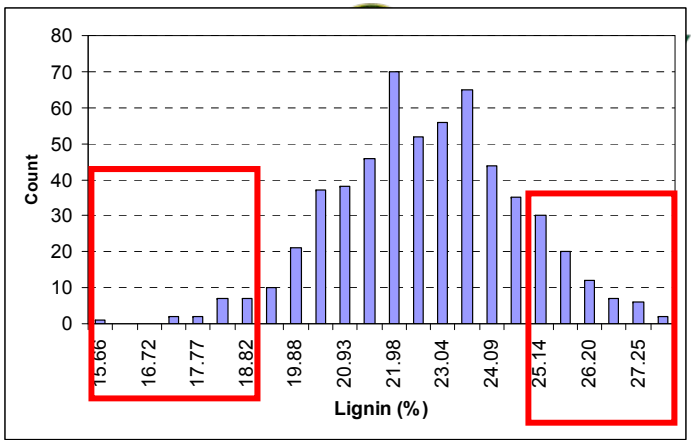


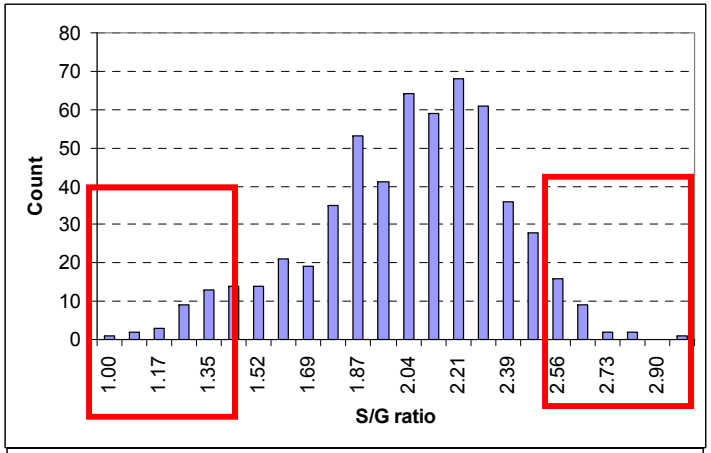
Composition data from *Populus* association study (798 samples) represents full range of known *Populus* variation

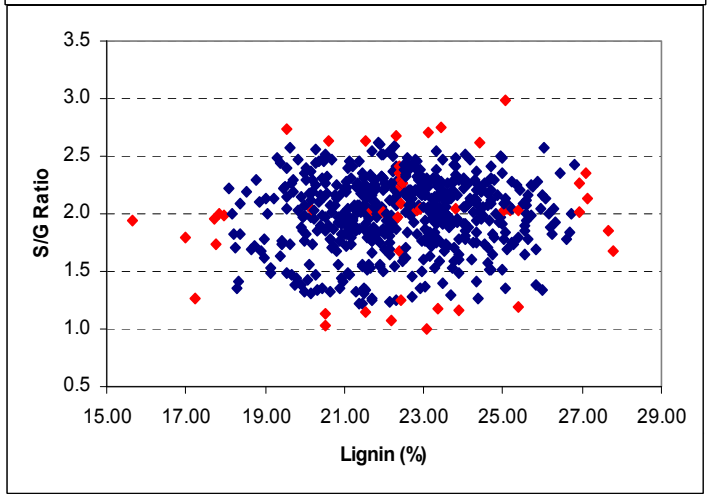
- Rapid (50/h w/ 4mg)
- Reliable
- Gives values for glucan, xylan, lignin, and details on monomers – e.g., S/G
- Complements time-consuming and more variable wet chemistry, molecular and biochemical analyses

Composition Data from *Populus* Association Study

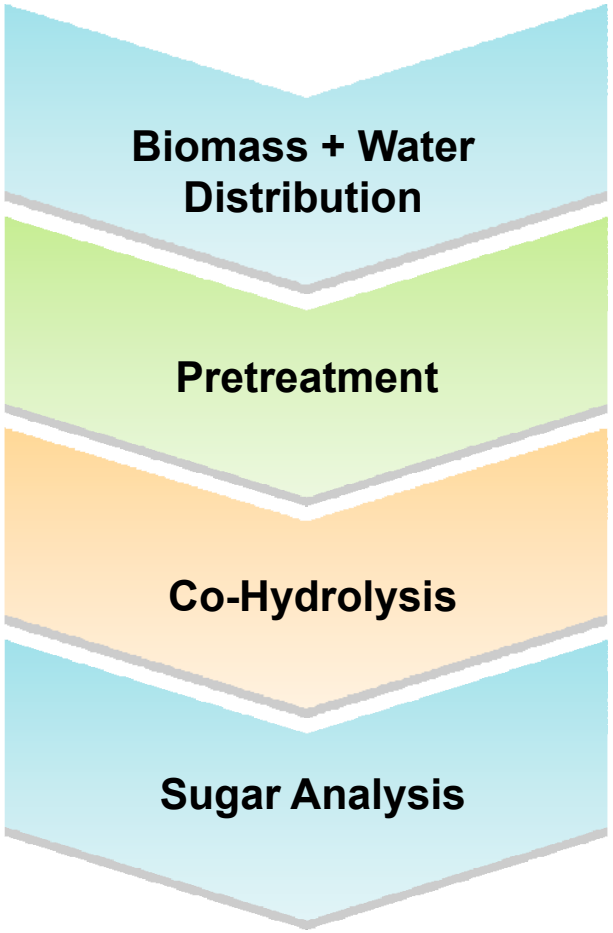
- The association samples display extreme variation in lignin, S/G ratio, and sugar content
- There is a negative correlation between sugar content and lignin content
- All sampled genotypes are being replicated and will be established in a common garden experiment







Enabling Technology: An HTP Pretreatment for 1000s of Small Samples



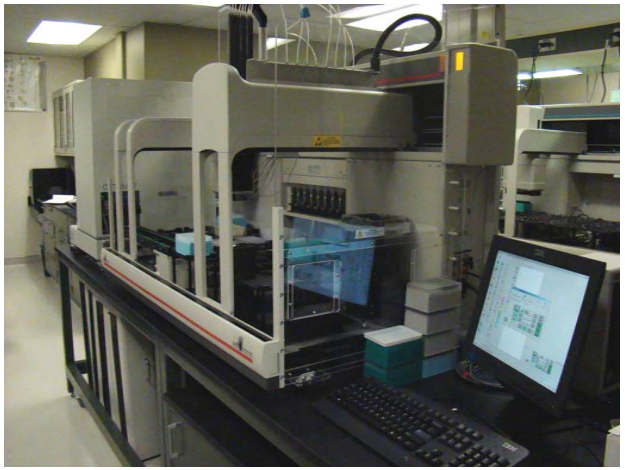
Studer *et al*., presented at the 30th *Symposium on Biotechnology for Fuels and Chemicals*, May 2008

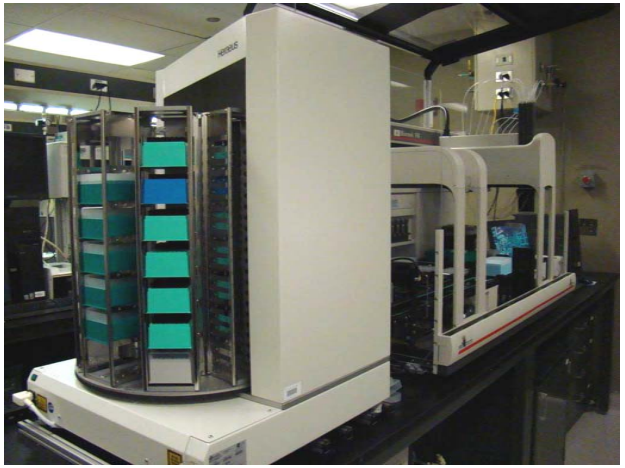
Unique and Important

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

HTP Enzymatic Digestion Assays

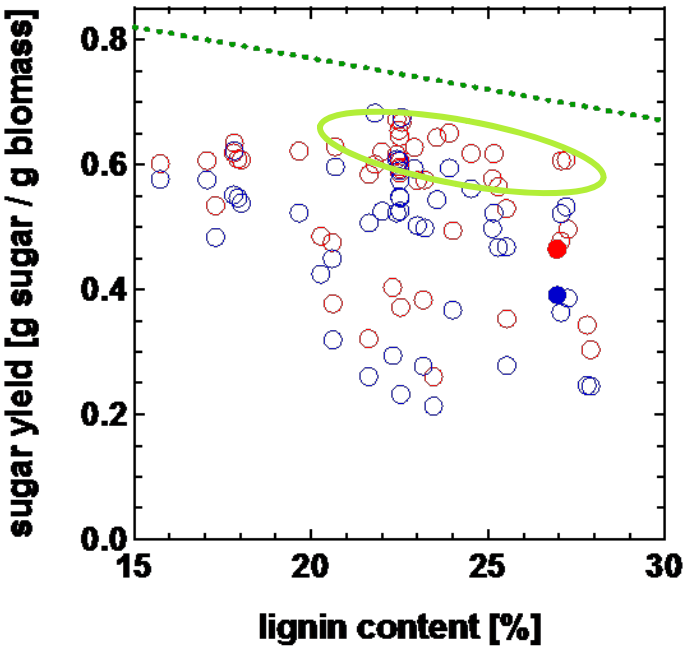
- Recalcitrance is ultimately determined by enzyme access to carbohydrates and sugar release
- HTP assays are needed to assess recalcitrant phenotypes and to screen for more effective enzymes
- 1st tier assays: >1000 samples/week
  - Evaluate base-line susceptibility of pretreated biomass as well as 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

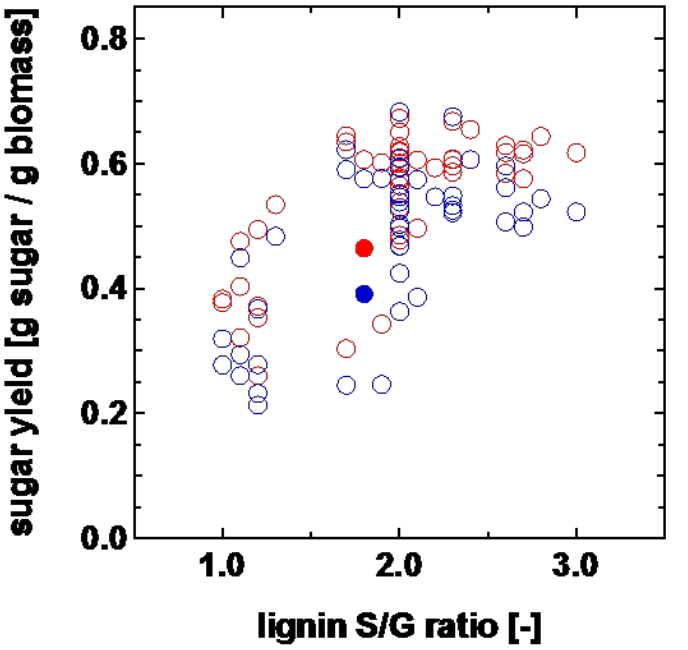




Response surface output for multi-dimensional digestibility assays

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**

●● **Standard BESC poplar**
···· **Theoretical sugar yield**

Studer, Wyman *et al.*

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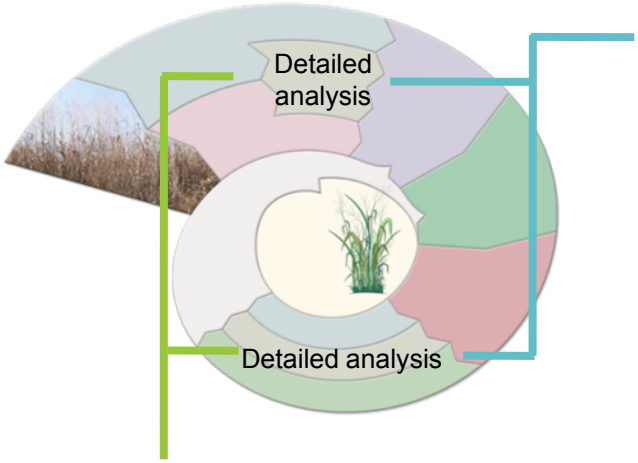
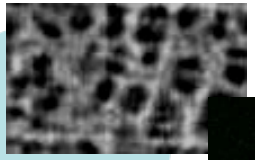
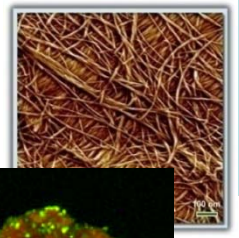
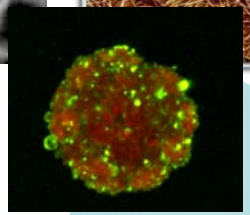
Detailed Analysis of Specific Samples Inform Cell-wall Chemistry and Structure



Imaging





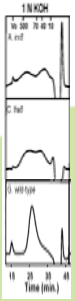


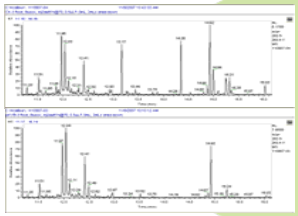
AFM of switchgrass showing cellulose microfibrils

Bio-ultraCAT for 3-D density of *Populus* cell walls

Immunolocalization using wall antibodies on *Populus* protoplasts

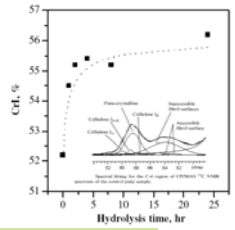
Chemistry



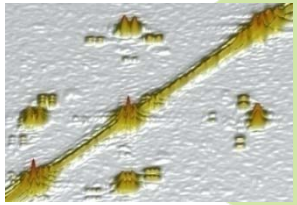


Mass Spectrometry for key metabolites

Fractionation and chromatography



NMR for cellulose crystallinity

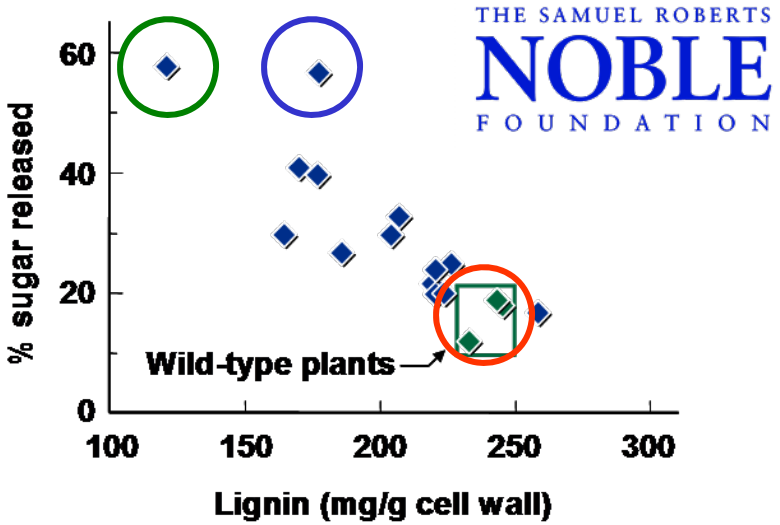


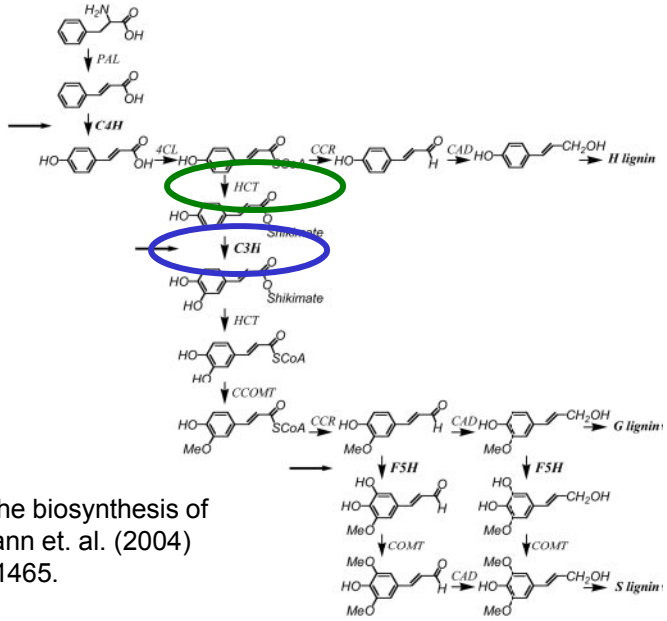
2D ^{1}H-NMR sees altered bonds in polysaccharides and lignin in biomass

BioEnergy Science Center

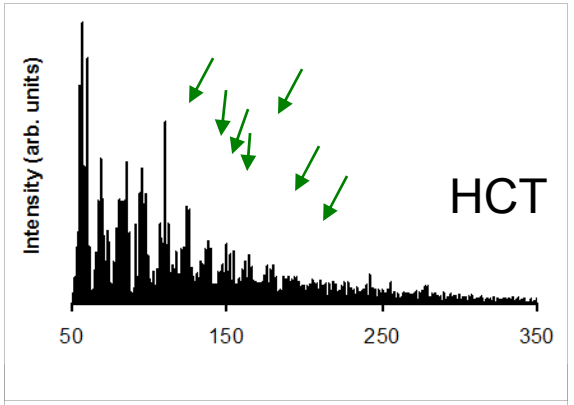
Analytical Pyrolysis of Low Lignin Alfalfa

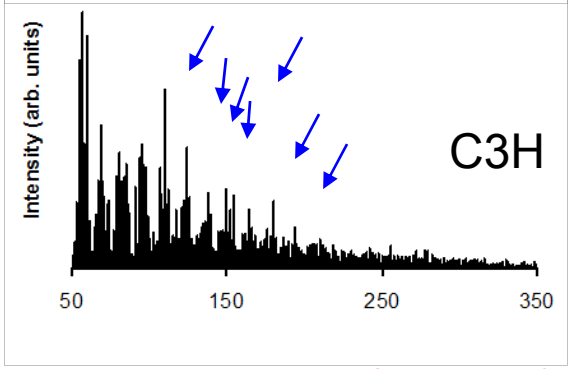
36 minutes of analysis for 6 (x3) samples

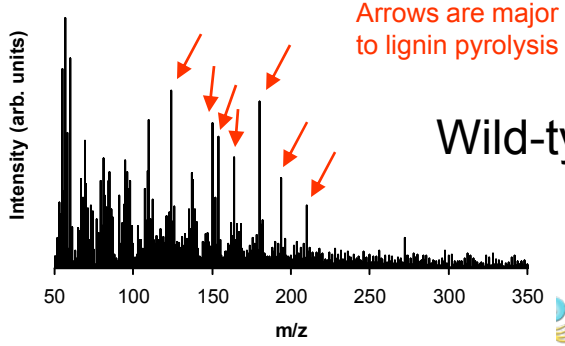




Current scheme for the biosynthesis of monolignols. (Hoffmann et. al. (2004) Plant Cell 16, 1446–1465.



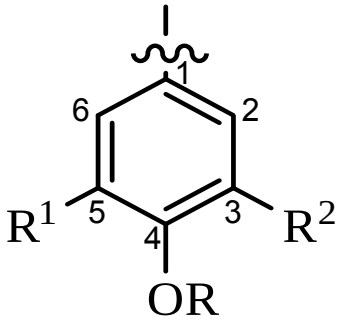




Arrows are major peaks assigned to lignin pyrolysis fragments

Solid State 13CNMR Spectroscopy

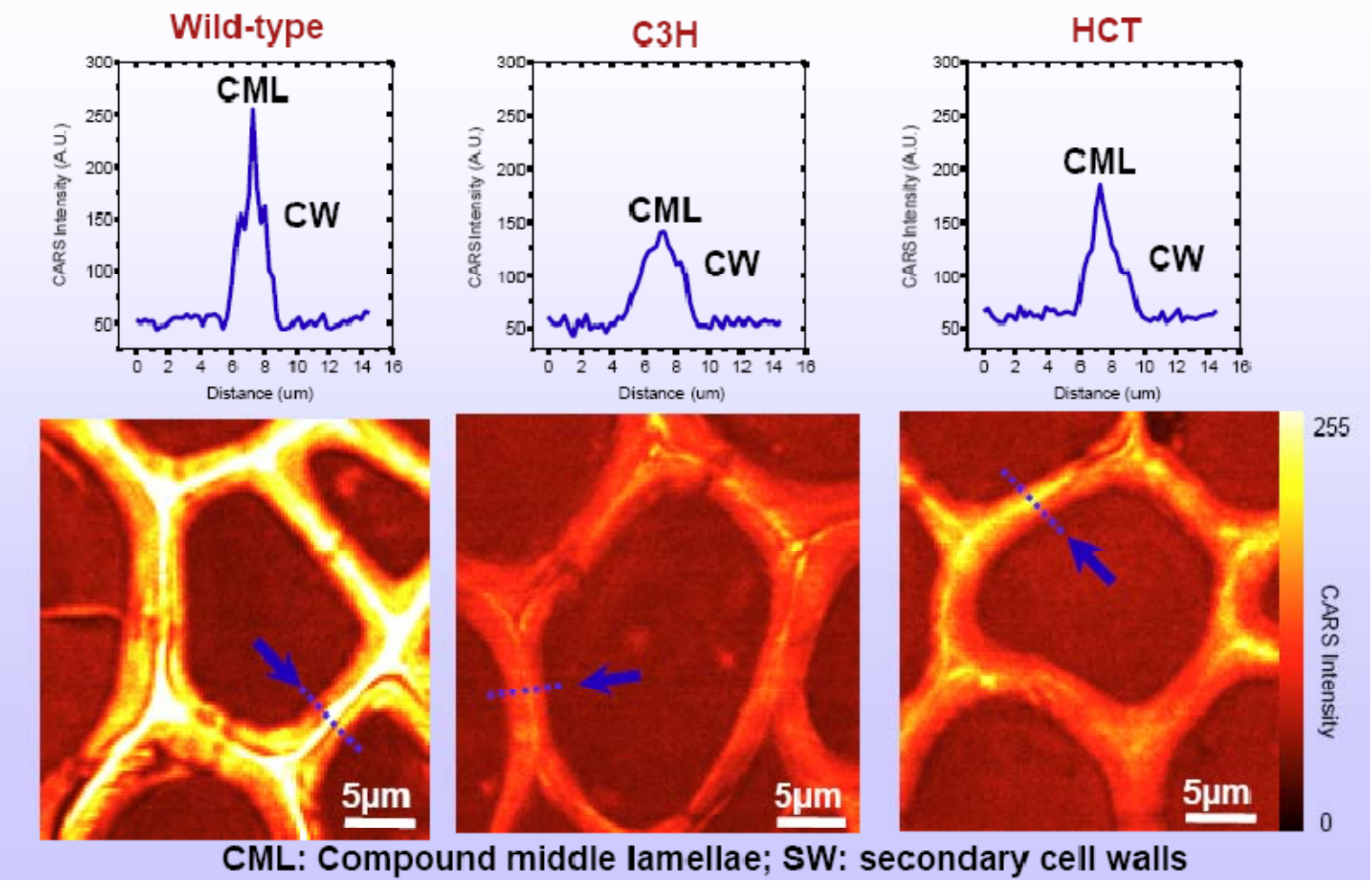
- **Whole cryo-milled cell wall residue**
- **Normalized for carbohydrate content**



^{13}CNMR spectra for the control versus C3H9a and HCT30a

BESC BioEnergy Science Center

CARS (Coherent Anti-Stokes Raman Scattering) Imaging of Lignin in Interfascicular Fiber Cell Walls in Alfalfa

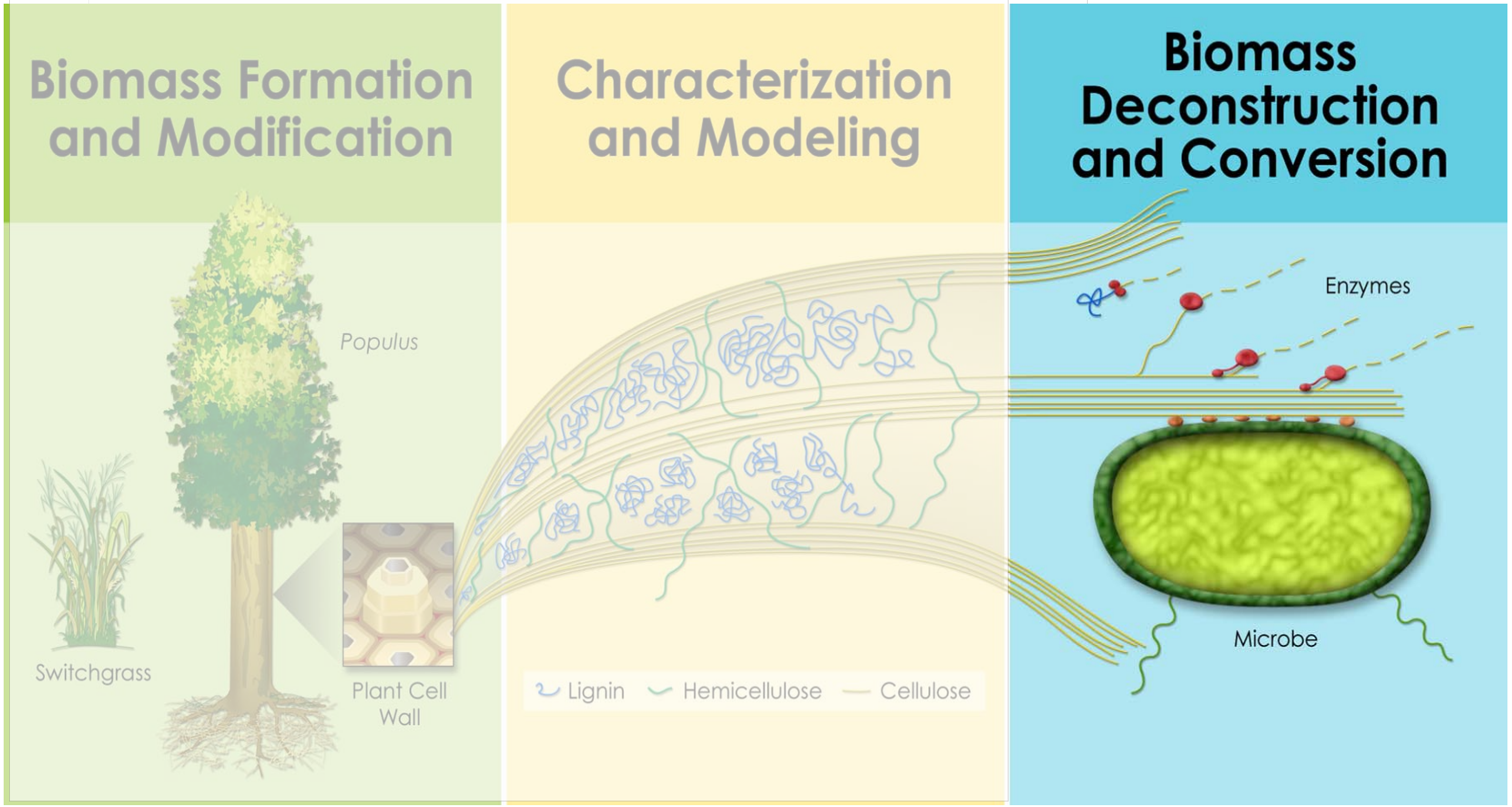


S-Y Ding (NREL) and X. S. Xie (Harvard)
tools under BER imaging grant; sample analysis under BESC, MS in preparation

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

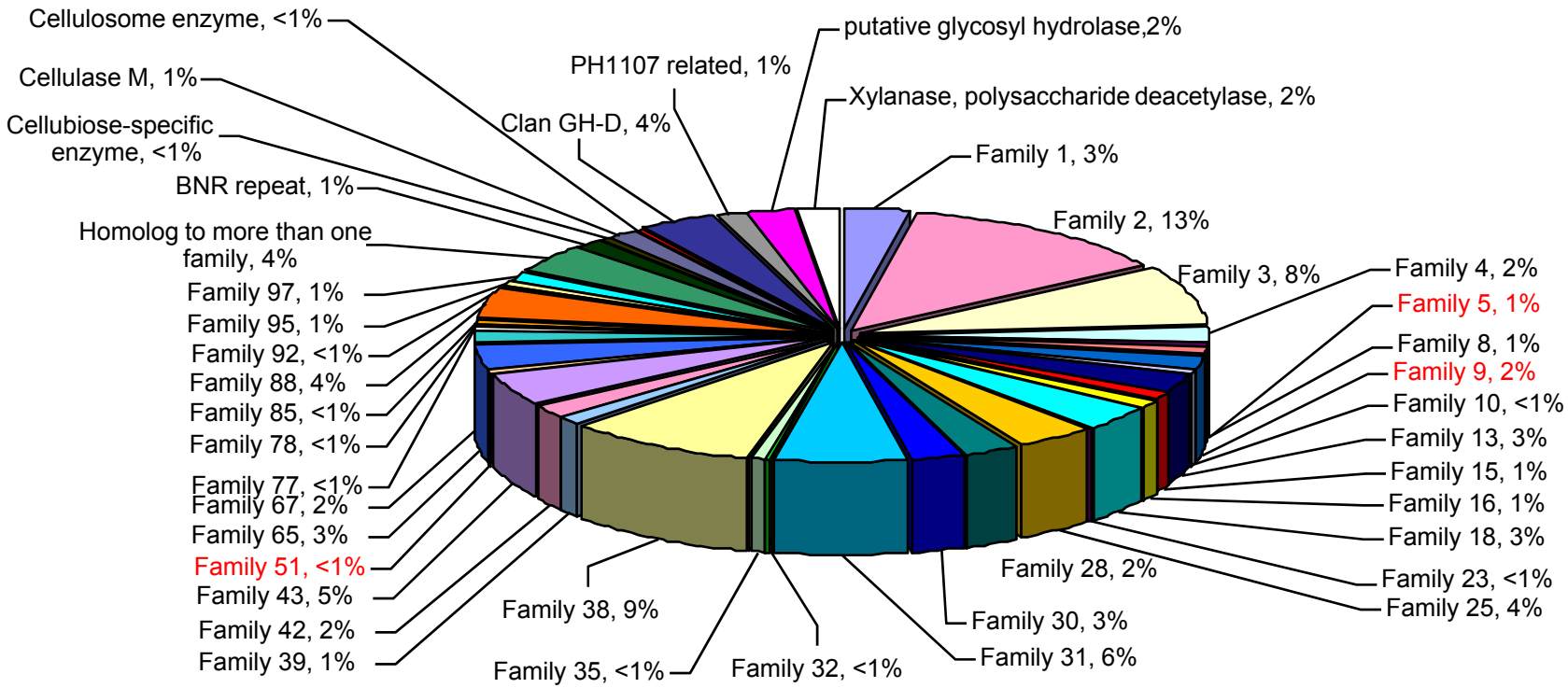
Strategy Part 3: Identify, Understand and Manipulate "Biological Catalysts" to Overcome Recalcitrance



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

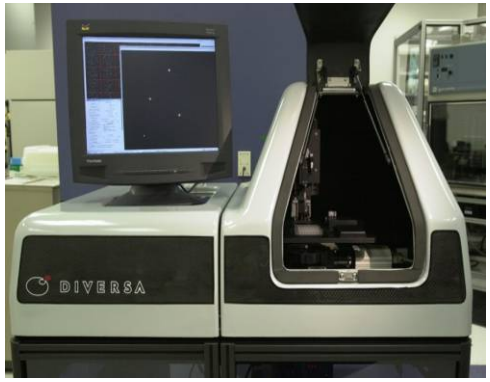


- **220 glycoside hydrolases present on 6688 contigs (6M bp total)**
- **GHase families 2, 3, 31, 38 and 43 are most abundantly found**

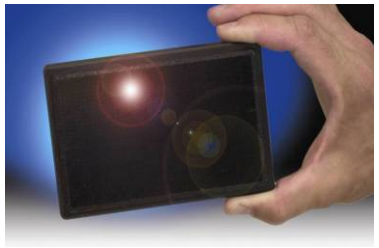
Niels van der Lelie *et al*.

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 biotraps
  - Ribosomal diversity analysis in progress
- **Shipworm** endosymbionts
  - Specimens— >100
  - Preliminary dissection and microbial isolation complete
- **Fungal isolates**
  - DNA extracted—78 unique isolates



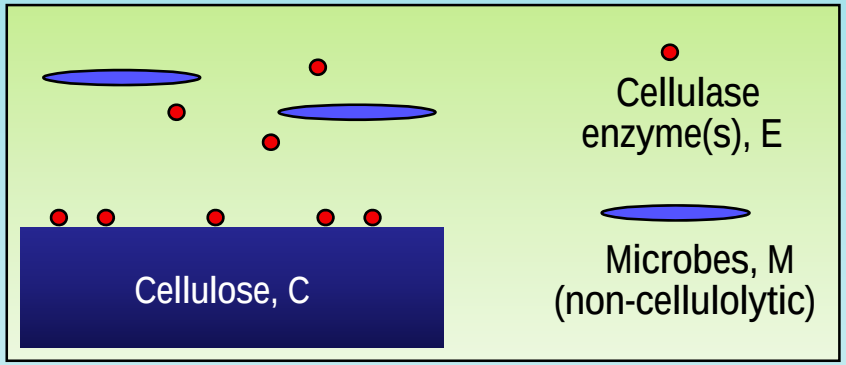
GigaMatrix® plate
400K wells, 50nL/well
200K clones/plate



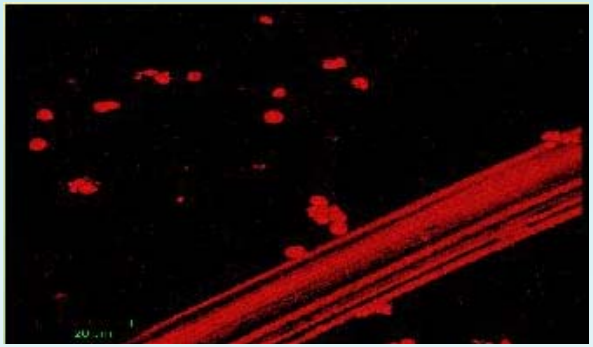
Microbial Hydrolysis and Enzymatic Hydrolysis: A Fundamentally Different Relationship Between Microbes and Cellulose

U.S. DEPARTMENT OF ENERGY

Enzymatic hydrolysis (classical approach)

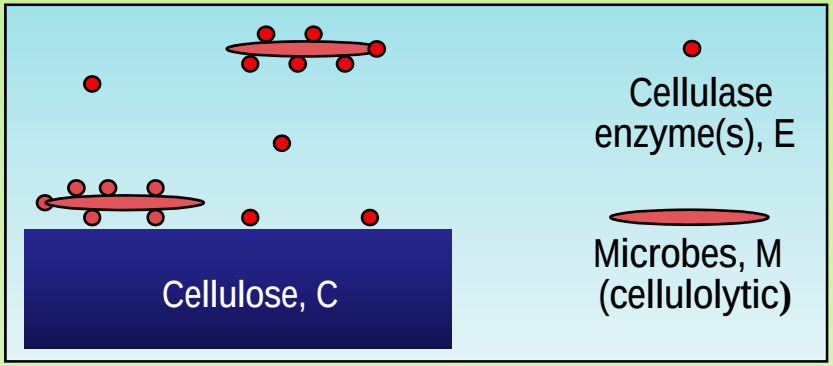


- Hydrolysis mediated by CE complexes
- Enzymes (several) both bound and free
- Cells may or may not be present

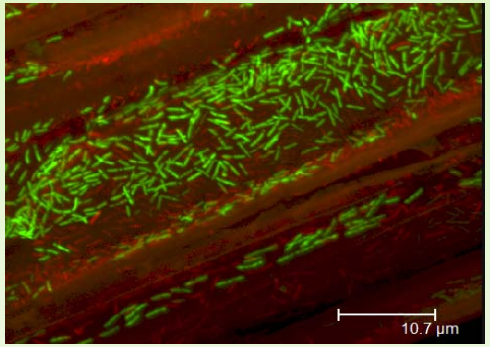


Yeast, enzymes with biomass, Dumitrache and Wolfaardt

Microbial hydrolysis (CBP)



- Hydrolysis mediated mainly by CEM complexes
- Enzymes both bound and free
- Cells both bound and free



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

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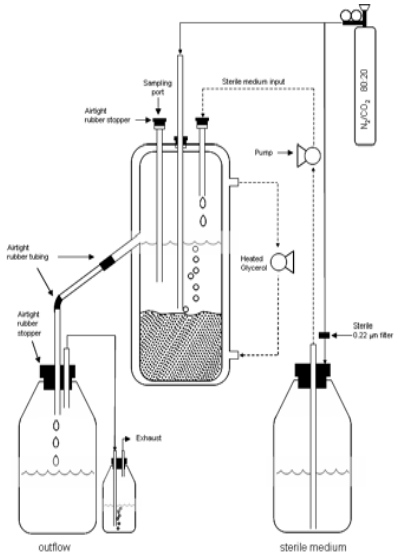
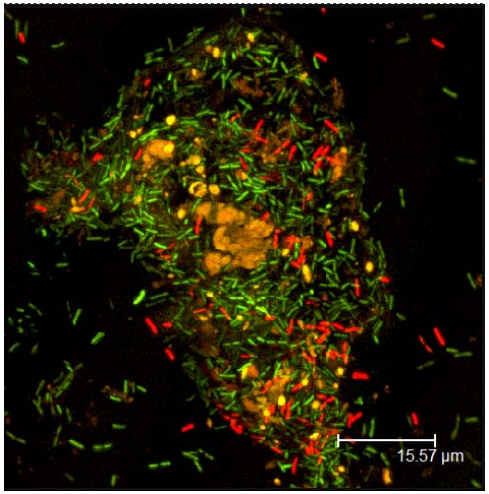
High-Throughput Isolation Using Flow Cytometry

U.S. DEPARTMENT OF ENERGY

Establish consortium

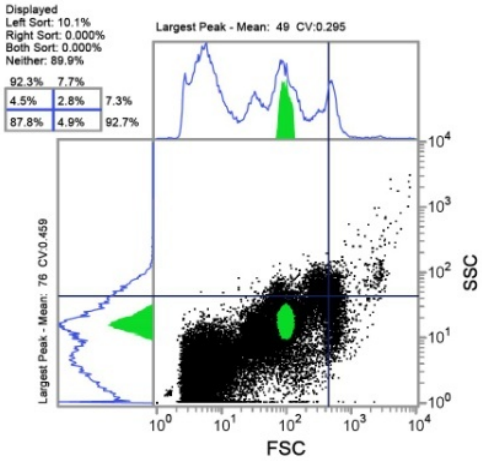


Identify members



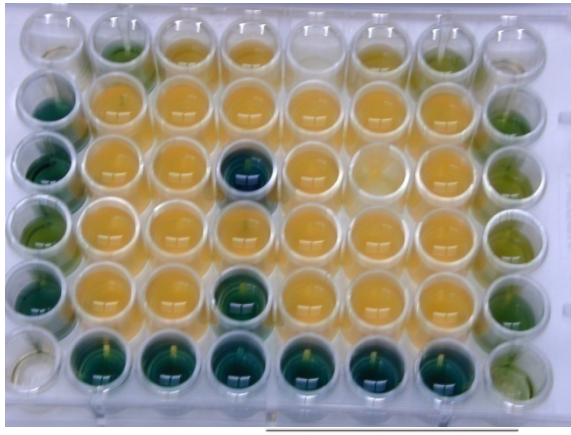
Flow-cytometer

Select different gates

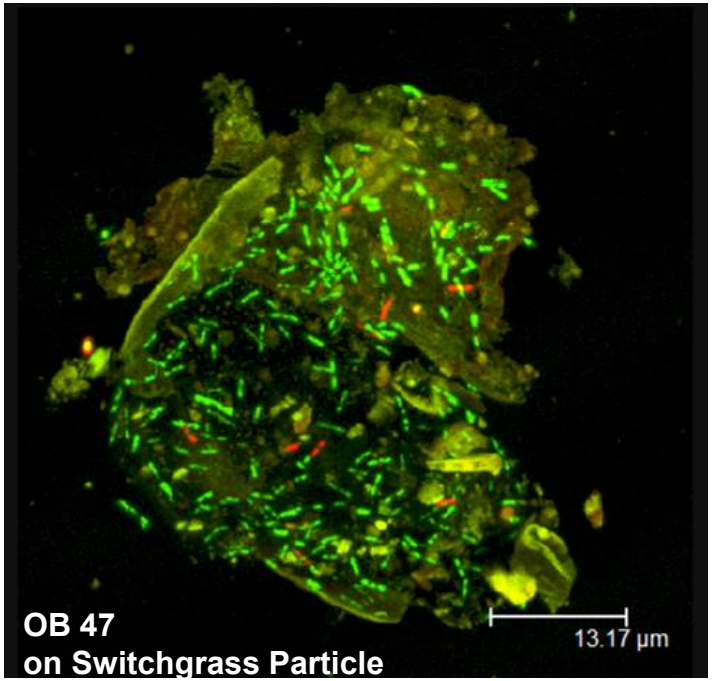


Anaerobic inc. @ 75 °C

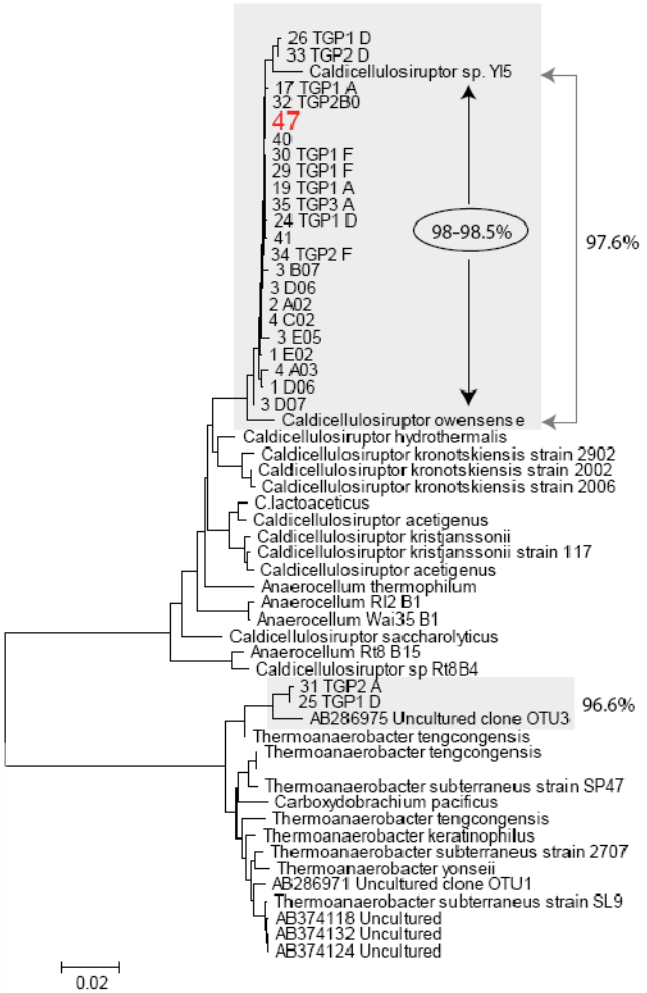
ΔpH indicates growth



Caldicellulosiruptor sp. OB47



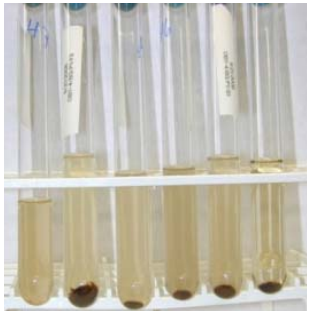
Life-Dead Stain & Confocal Microscopy Image by Jennifer Morell-Falvey



- **Gram-positive bacterium**
- **Rod Shaped ~0.5 x 1-2µm**
- **T_{opt} 75-78°C**
- **Fermentative heterotroph**
- **Produces H_2, Acetate, Lactate, CO_2 and Ethanol**

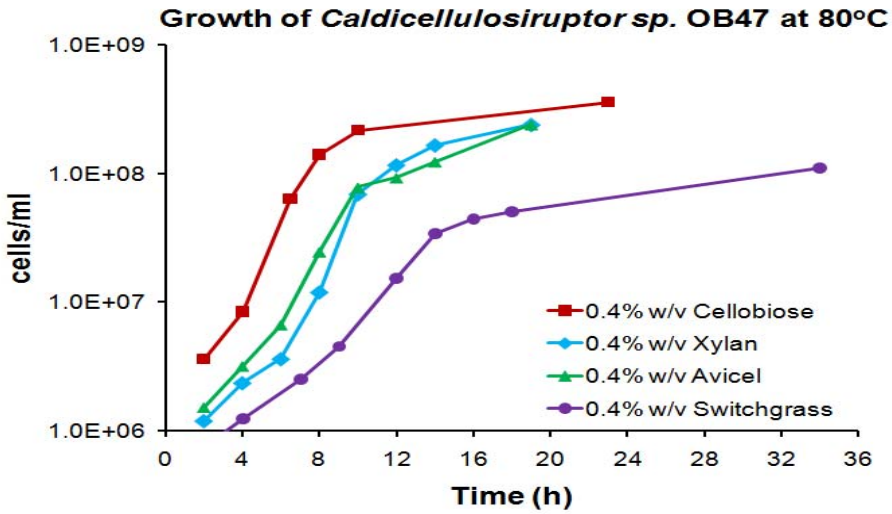
Caldicellulosiruptor sp. OB47

Growth of *Caldicellulosiruptor sp.* OB47 on insoluble substrates

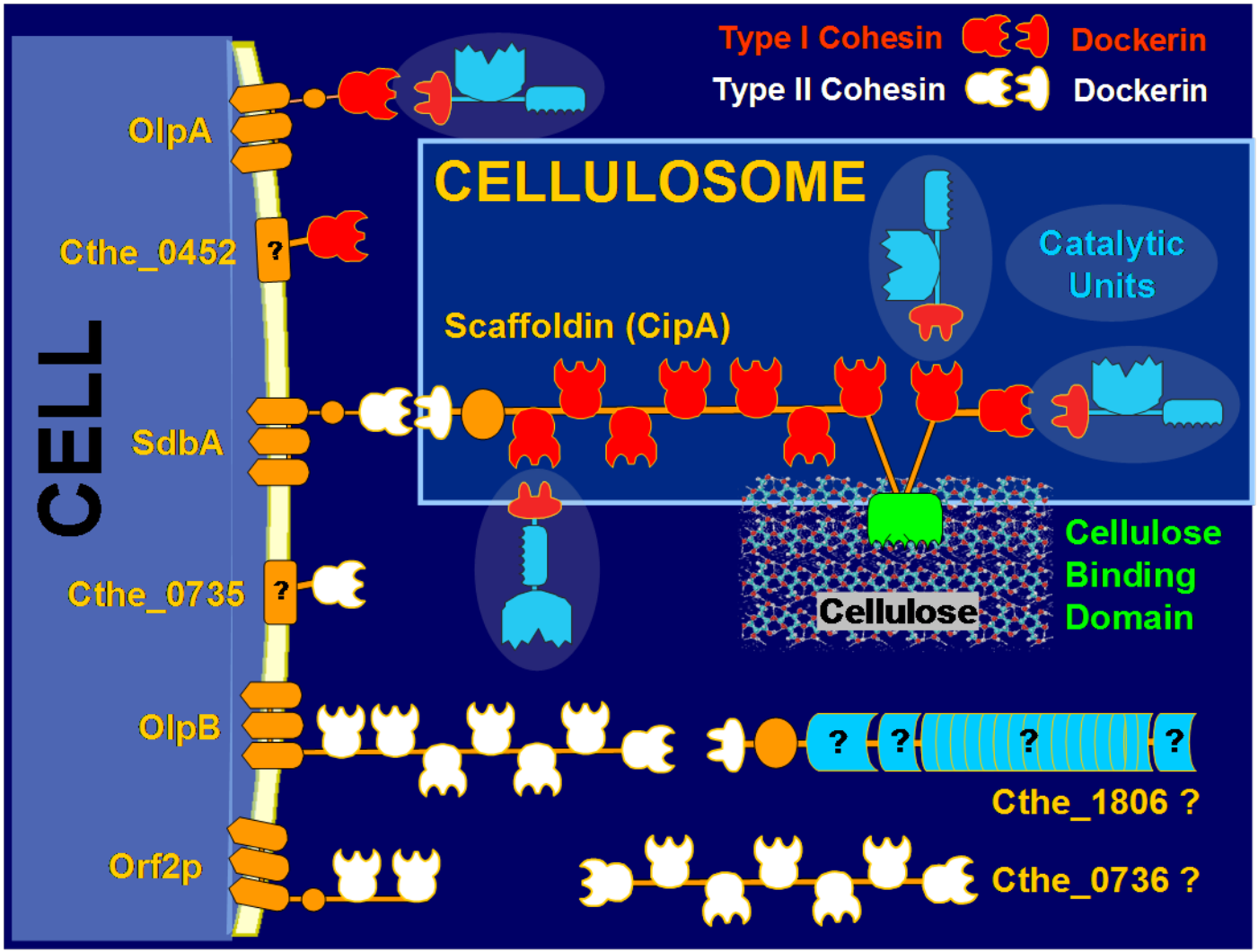


Isolate #47 - Control

control 24 h 3 days



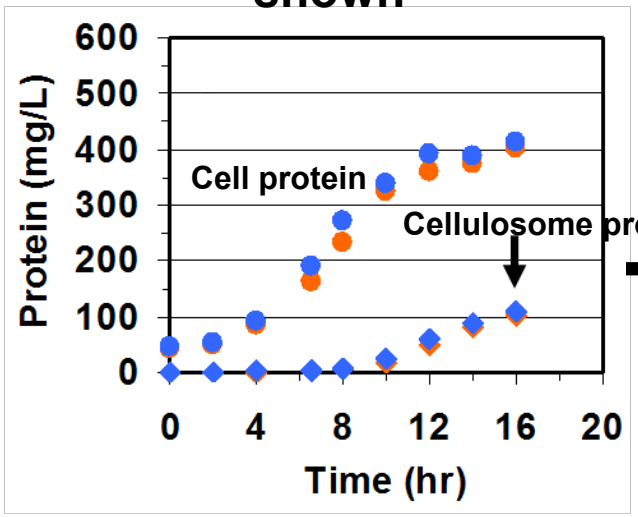
Schematic of Cellulosome

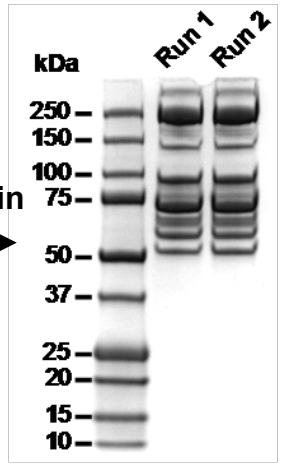


(adapted from Carlos Fontes, 2007 Gordon Research Conference on "Cellulases and Cellulosomes")

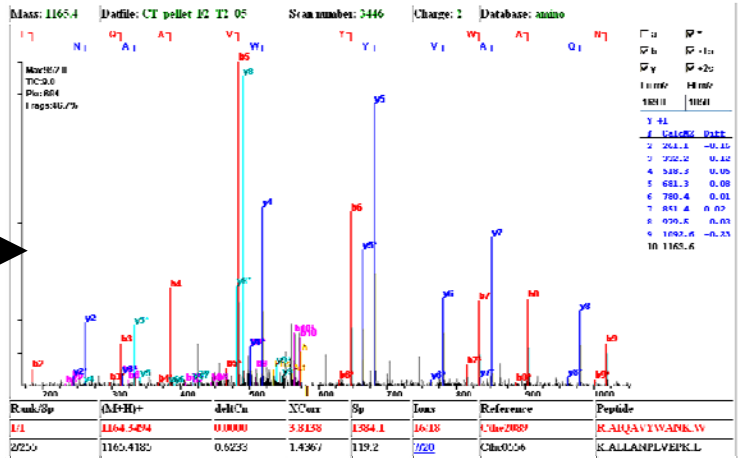
C. thermocellum Cellulosome was Analyzed under Several Conditions

Cultivation on Avicel shown





Quantitative Proteomics: Shot-gun



- 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 *C. thermocellum* Mutant that Utilizes Cellulose Rapidly

Genome position

Speedy 454 contigs

Speedy SNPs

CDS

ORNL wild-type 454 contigs

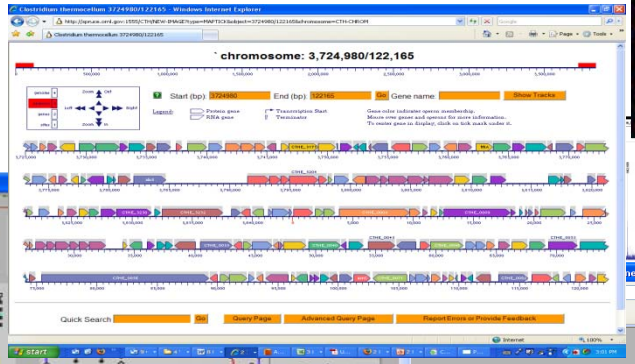
ORNL wild-type SNPs

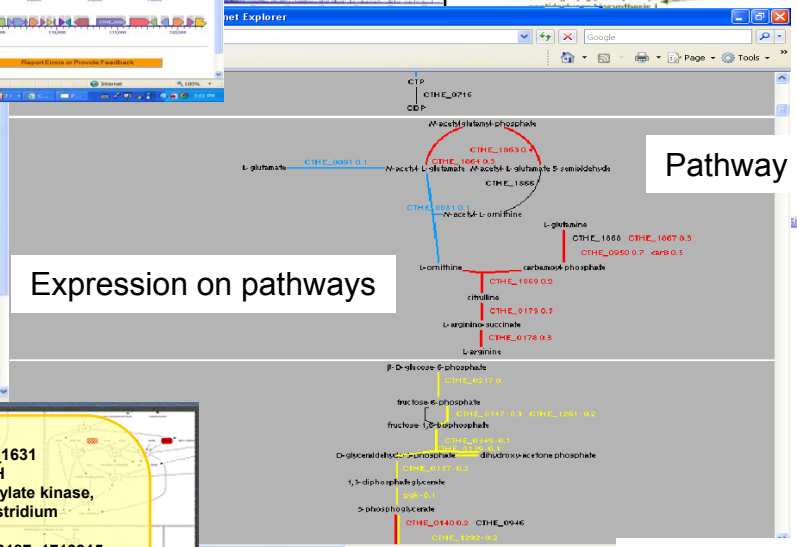
- 454 Resequencing identified 78 mutated loci in Speedy mutant
- 25 mutant loci common with ORNL wild-type strain
- Transcriptomics and further analysis underway

Integrating Expression, Proteomics, SNPs, Metabolites on Cellular Systems

Clostridium thermocellum

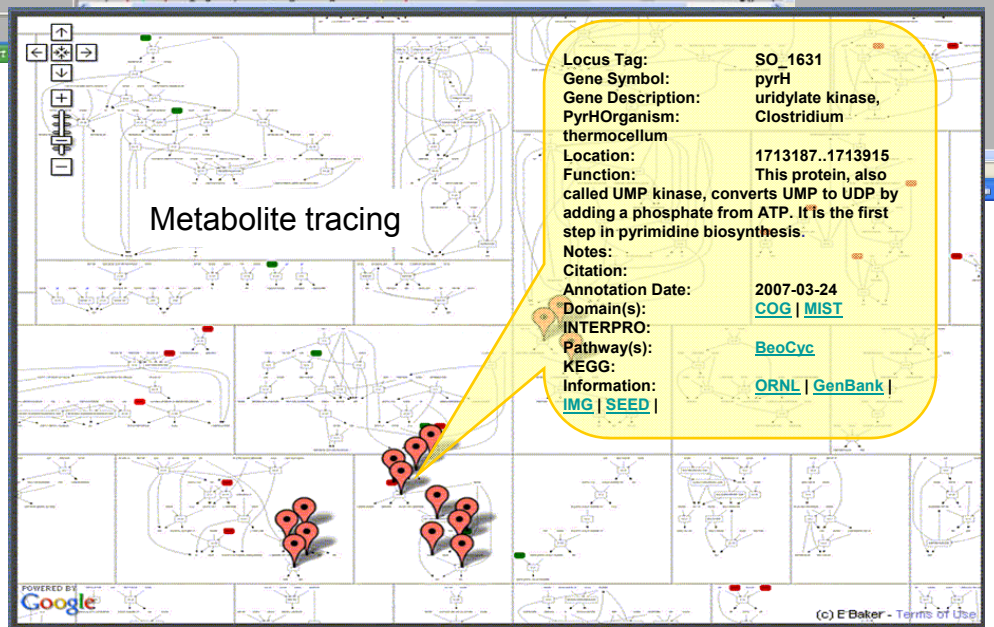
SNP mapping





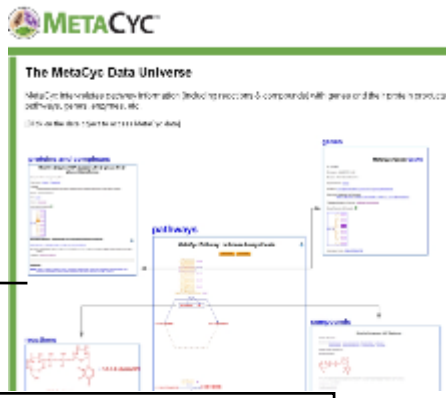
Pathway Component Details

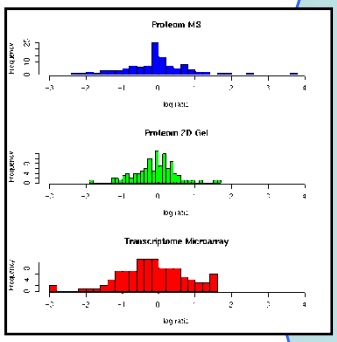
Expression on pathways

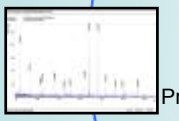


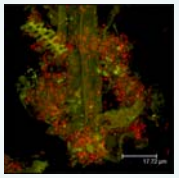
Expression on genome map

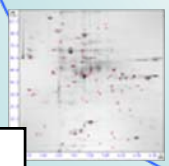
Computational Microscope – Assay and Analysis Framework

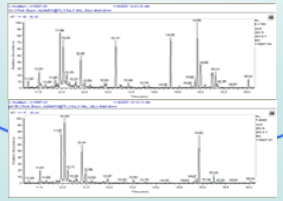


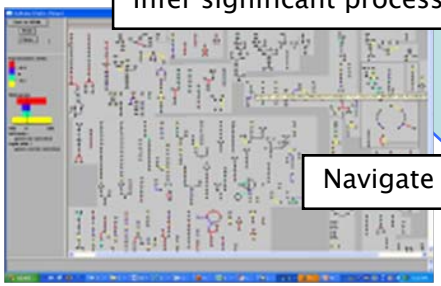












Knowledgebase

LIMS

Assays

Metabolic pathways

Ontologies

Integrate experimental data with external reference sets

Phenotypes

Microarray

Experimental conditions

Gene expression

Treatments

Mass spec

Imaging

Protein interaction

Genotypes

SNPs

Protein expression

Mutants

2D gels

DNA sequence

Orthologs

Proteins

Metabolites & concentrations

Genome sequence

Gene structure

Infer significant processes

Operons

Pathways

Navigate pathways

BESC BioEnergy Science Center

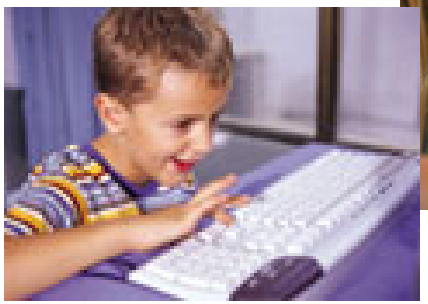
Highlights 2008 until March 2009

- ***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

DOE Bioenergy Research Centers

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OFFICE OF SCIENCE U.S. DEPARTMENT OF ENERGY

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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

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