

Systems Biology for DOE Energy and Environmental Missions

Bioenergy

Biofuels > Bioenergy Research Centers

1

Great Lakes Bioenergy Research Center

Timothy Donohue,^{1*} Kenneth Keegstra,² Richard Amasino,¹ Bruce Dale,² Robert Landick,¹ John Ohlrogge,¹ George Phillips,² Phillip Robertson,² and Michael Sussman¹

¹University of Wisconsin, Madison, Wisconsin and
²Michigan State University, East Lansing, Michigan

Project Goals: Remove the bottlenecks in converting biomass into biofuels.

The Great Lakes Bioenergy Research Center (GLBRC) is led by the University of Wisconsin-Madison, in close partnership with Michigan State University (www.great-lakesbioenergy.org).

Additional scientific partners include the Pacific Northwest National Laboratory, Oak Ridge National Laboratory, University of Florida, Illinois State University, Iowa State University, and Lucigen Corporation. Located in the world's most productive agricultural region, the GLBRC is taking scientifically diverse approaches to converting sunlight and diverse plant feedstocks (agricultural residues, wood chips, grasses) into fuels. GLBRC programs are organized in the following areas:

Improving Plant Biomass: In addition to studying how genes affect cell-wall assembly, GLBRC will be breeding plants to produce hemicellulose, starches and oils that are more easily processed into fuels. GLBRC researchers aim to increase the energy density of grasses and other non-traditional oil crops by understanding and manipulating the metabolic and genetic circuits that control accumulation of oils and other easily digestible polymers in plant tissues.

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Improving Biomass Processing: New and improved catalysts created by the GLBRC protein-production pipeline will be used with a range of plant materials and pretreatment conditions to identify the best mix of enzymes, chemicals, and physical processing for enhanced digestibility and increased fuel production from specific biomass sources. To decrease the costs of producing and using enzymes to breakdown plant cellulose, researchers are working to express biomass-degrading catalysts in the stems and leaves of plants—essentially designing crops that “self-destruct” on cue in the biofuel-production facility.

Improving Biomass Conversion to Energy Compounds: GLBRC biomass-conversion research is driven by the need to increase the quantity, diversity, and efficiency of energy products derived from plant biomass. Cellulosic ethanol is a major focus of GLBRC research, but the center will also improve biological and chemical methods for converting plant material into hydrogen, electricity, or other chemicals that can replace fossil fuels.

Fostering Sustainable Bioenergy Practices: For a bio-economy to positively impact the energy grid, complex issues in agricultural, industrial, and behavioral systems must be addressed. To create a better understanding of what will ultimately influence the direction and acceptance of new bioenergy technologies, GLBRC scientists will examine the environmental and socioeconomic dimensions of converting biomass to biofuel. To determine the best practices for biofuel production, GLBRC researchers will study how to minimize energy and chemical inputs for bioenergy crop production, reduce greenhouse gas emissions from the biofuel production life cycle, and predict environmental impacts of removing stalks, stems, and leaves from food crops. GLBRC scientists also will study the social and/or financial incentives needed to adopt these best practices.

Creating Technologies to Enable Advanced Bioenergy Research: Core GLBRC activities will provide cutting-edge, genome-based technologies to enable the innovative discoveries and creative solutions needed to advance bioenergy research. GLBRC researchers will deploy high-throughput, automated screens for genes and proteins in plants and microbes that affect biomass and

* Presenting author

biofuel production; integrate information from multiple research approaches; and develop predictive models for relevant enzymes, pathways, or networks that can guide the development of new plants, enzymes, or microbes that would enhance a biofuel-production pipeline.

Education and Outreach: With a history of excellence in the land-grant missions of education, training, and outreach, the GLBRC is committed to training the bioenergy leaders of tomorrow while removing today's bottlenecks in the biofuels pipeline. The GLBRC academic partners will offer new bioenergy-focused summer research programs, seminars, special courses and labs. By working with existing university programs, GLBRC scientists will develop workshops and educational modules for K–12 teachers on carbon chemistry, sustainability, and biodiversity issues related to biofuel production. GLBRC researchers will also develop general education materials and host public forums to raise awareness of and generate support for biofuels among farmers and communities.

2 GTL The Joint BioEnergy Institute (JBEI): Biomass Conversion to Alternative Transportation Fuels

Jay Keasling^{1,2*} (jdkeasling@lbl.gov), Harvey Blanch,^{1,2} Wolf Frommer,³ Blake Simmons,⁴ Paul Adams,¹ and Kathe Andrews-Cramer⁵

¹Lawrence Berkeley National Laboratory, Berkeley, California; ²University of California, Berkeley, California; ³Carnegie Institution for Science, Stanford, California; ⁴Sandia National Laboratory, Livermore, California; and ⁵Sandia National Laboratory, Albuquerque, New Mexico

Project Goals: This BioEnergy Research Center's program is directed at conversion of lignocellulosic biomass to transportation fuels.

Today, carbon-rich fossil fuels, primarily oil, coal and natural gas, provide 85% of the energy consumed in the United States. The release of greenhouse gases from these fuels has spurred research into alternative, non-fossil energy sources. Lignocellulosic biomass is renewable resource that is carbon-neutral, and can provide a raw material for alternative transportation fuels. Plant-derived biomass contains cellulose, which is difficult to convert to monomeric sugars for production of fuels. The development of cost-effective and energy-efficient processes to transform the cellulosic content of biomass into fuels is

hampered by significant roadblocks, including the lack of specifically developed energy crops, the difficulty in separating biomass components, the high costs of enzymatic deconstruction of biomass, and the inhibitory effect of fuels and processing byproducts on organisms responsible for producing fuels from biomass monomers.

The Joint BioEnergy Institute (JBEI) is addressing these roadblocks in biofuels production. JBEI draws on the expertise and capabilities of three national laboratories (**Lawrence Berkeley National Laboratory (LBNL)**, **Sandia National Laboratories (SNL)**, and **Lawrence Livermore National Laboratory (LLNL)**) two leading U.S. universities (**University of California** campuses at **Berkeley (UCB)** and **Davis (UCD)**), and the **Carnegie Institution for Science** at Stanford University to provide the scientific and technology underpinnings needed to convert the energy stored in cellulose into transportation fuels and other chemicals. Based in Emeryville, California, JBEI co-locates scientists and engineers from all the member organizations. JBEI's approach is based in three interrelated scientific divisions and a technologies division. The **Feedstocks Division** will create the knowledge required to develop improved plant energy crops to serve as the raw materials for biofuels. The **Deconstruction Division** will investigate the conversion of this lignocellulosic plant material to usable forms of sugars and aromatics. The **Fuels Synthesis Division** will create microbes that can efficiently convert sugar and aromatics into ethanol, butanol and advanced biofuels. JBEI's cross-cutting **Technologies Division** will develop and optimize a set of enabling technologies—including high-throughput, chip-based and 'omics platforms, tools for synthetic biology, multi-scale imaging facilities, and integrated data analysis. This division thus supports and integrates the scientific programs.

The objectives and approaches of JBEI's divisions will be described, together with the initial research accomplishments of each of JBEI's divisions.

JBEI web site: www.jbei.org

3 GTL The BioEnergy Science Center: An Overview

Martin Keller* (kellerm@ornl.gov)

Oak Ridge National Laboratory, Oak Ridge, Tennessee

Project Goals: By combining engineered plant cell walls to reduce recalcitrance with new biocatalysts

* Presenting author

to improve deconstruction, BESC within five years will revolutionize the processing of biomass. These breakthroughs will be achieved with a systems biology approach and new high-throughput analytical and computational technologies to achieve (1) targeted modification of plant cell walls to reduce their recalcitrance (using *Populus* and switchgrass as high-impact bioenergy feedstocks), thereby decreasing or eliminating the need for costly chemical pretreatment; and (2) consolidated bioprocessing, which involves the use of a single microorganism or microbial consortium to overcome biomass recalcitrance through single-step conversion of biomass to biofuels.

<http://www.bioenergycenter.org>

The challenge of converting cellulosic biomass to sugars is the dominant obstacle to cost-effective production of biofuels in sustained quantities capable of impacting U.S. consumption of fossil transportation fuels. The Bio-Energy Science Center (BESC) research program will address this challenge with an unprecedented interdisciplinary effort focused on overcoming the recalcitrance of biomass. In addition to Oak Ridge National Laboratory (ORNL), the BESC core team consists of the University of Georgia, the Georgia Institute of Technology, the University of Tennessee, the National Renewable Energy Laboratory, Dartmouth College, the Samuel Roberts Noble Foundation, and industrial partners ArborGen, Verenum, and Mascoma. Other individual PIs complete the team. The home base of BESC will be the Joint Institute for Biological Sciences building, funded by the state of Tennessee and occupied in December 2007. Located on the ORNL campus, the facility is designed specifically for interdisciplinary bioenergy research using systems biology tools. Other BESC anchor facilities include the University of Georgia's Complex Carbohydrate Research Center with extensive carbohydrate analytical and plant science expertise, the National Renewable Energy Laboratory's unique capabilities in comprehensive biomass analysis and bioprocessing, and ORNL's National Leadership Computing Facility.

By combining engineered plant cell walls to reduce recalcitrance with new biocatalysts to improve deconstruction, BESC within five years will revolutionize the processing of biomass. These breakthroughs will be achieved with a systems biology approach and new high-throughput analytical and computational technologies to achieve (1) targeted modification of plant cell walls to reduce their recalcitrance (using *Populus* and switchgrass as high-impact bioenergy feedstocks), thereby decreasing or eliminating the need for costly chemical pretreatment; and (2) consolidated bioprocessing, which involves the

use of a single microorganism or microbial consortium to overcome biomass recalcitrance through single-step conversion of biomass to biofuels.

Within five years the Center will remove biomass recalcitrance as a barrier to cost-effective biofuels production by achieving a minimum two-fold reduction in the projected cost of processing for conversion of biomass to ethanol. Through this effort we will greatly enhance our understanding of cell wall structure during synthesis and conversion. The data generated will be made available through a Web portal in order to support and catalyze the bioenergy research community. The benefits of the basic research will extend beyond the five-year program by laying the foundation for developing other biomass sources and fuel products, improving productivity of switchgrass and poplar, and ensuring sustainability of lignocellulosic biofuel production.

This talk will provide an overview of the BESC start-up activities and some initial results.

4

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BESC: Biomass Formation and Modification: *Populus*, A Case Study

G. Tuskan^{1*} (tuskanga@ornl.gov), T. Tschaplinski,¹ U. Kalluri,¹ T. Yin,¹ L. Gunter,¹ S. Jawdy,¹ X. Yang,¹ R. Priya,² X. Zhang,¹ M. Cheng,² F. Chen,³ S. DiFazio,⁴ G. Slavov,⁴ M. Hinchey,⁵ D. Mohnen,⁶ A. Darvill,⁶ and **M. Keller¹**

¹Oak Ridge National Laboratory, Oak Ridge, Tennessee; ²University of Tennessee, Knoxville, Tennessee; ³Samuel Roberts Noble Foundation, Ardmore, Oklahoma; ⁴West Virginia University, Morgantown, West Virginia; ⁵Arborgen, Inc., Summerville, South Carolina; and ⁶University of Georgia, Athens, Georgia

Project Goals: The overriding goal of the Center is to engineer wall biosynthesis pathways to generate plants with less recalcitrant walls that are deconstructed effectively by improved consolidated microbial bioprocessing, ultimately achieving optimal sugar release and conversion from the biomass with minimal or no chemical pretreatment. These efforts will be supported by the development of chemical and molecular models that predict how wall structure, recalcitrance, and biocatalyst-biomass interactions are related and by experimental and theoretical approaches to refine these models.

* Presenting author

This poster illustrates our approach to understanding Biomass formation transformation using *Populus*, which along with switchgrass are our primary feedstock targets. The goal of this area is to develop a thorough understanding of the genetics and biochemistry of plant cell wall biosynthesis so the process can be modified to reduce biomass recalcitrance. Preliminary data supporting our hypothesis that modification of cell wall structure will result in lower recalcitrance of biomass have been shown in alfalfa.

The *Populus* genome was sequenced, assembled and annotated in 2006. As a result, there have been numerous genetic and genomics tools developed for the discovery of genes and gene function in *Populus*. These tools include saturated genetic maps, whole-genome microarrays, libraries of polymorphic features within the genome, and a catalog of 45,500 predicted gene models. Moreover, there are in place extensive structured pedigrees, routine, high-throughput transformation protocols and mutated genetic lines available for study. These tools and resources will be brought to bear on the issue of the recalcitrance of plant cell walls to microbial and enzymatic deconstruction and conversion into liquid transportation fuels. Although such resources exist, we know very little about the regulation, formation and synthesis of plant cell walls.

As such, the first efforts within the *Populus* Transformation activity will be to identify all genes related to the construction of primary and secondary plant cell walls. It is estimated that as many as 5000 genes may be active during the process of cell wall formation. Genetics and genomics approaches will be used to identify 1000 genes with the largest impact on cell wall chemistry and thermochemical conversion to sugars. These 1000 genes and their effect on cell wall biosynthesis, recalcitrance and sugar release will then be tested and validated in over- and under-expression transgenic lines. An annotated and curated plant wall biosynthesis gene database will be created and the database will be used to construct cell wall biosynthesis pathways to facilitate future approaches to reduce recalcitrance of plant biomass.

The goal of the BESC *Populus* Transformation effort is to create improved plant lines that express enhanced biomass production and reduced recalcitrance, i.e. plants that are more easily deconstructed for biofuel production. Our specific goals are to: 1) identify and characterize genes involved in cell wall biosynthesis and structure and to establish which genes alter biomass recalcitrance and 2) determine how changes in cell wall composition and structure affect plant recalcitrance and to begin to develop a systems biology understanding of the genetic

basis for wall structure and recalcitrance. A detailed experimental approach will be presented.

5 BESC: Biomass Deconstruction and Conversion: A Systems Biology Analysis of Biomass Ethanol from *Clostridium thermocellum*

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Babu Raman,¹ Jonathan R. Mielenz,¹ Catherine K. McKeown,¹ Miguel Rodriguez Jr.,¹ Steven D. Brown,¹ Patricia K. Lankford,¹ Chongle Pan,¹ Gregory B. Hurst,¹ L. Lynd,² and **M. Keller**^{1*} (kellerm@ornl.gov)

¹Oak Ridge National Laboratory, Oak Ridge, Tennessee and ²Dartmouth College, Hanover, New Hampshire

Project Goals: The overriding goal of the Center is to engineer wall biosynthesis pathways to generate plants with less recalcitrant walls that are deconstructed effectively by consolidated microbial bioprocessing, ultimately achieving optimal sugar release and conversion from the biomass with minimal or no chemical pretreatment. These efforts will be supported by the development of chemical and molecular models that predict how wall structure, recalcitrance, and biocatalyst-biomass interactions are related and by experimental and theoretical approaches to refine these models.

This poster illustrates part of our approach in Biomass deconstruction and Conversion using an example of the analysis of *Clostridia thermocellum*. The conversion goal is to develop an understanding of enzymatic and microbial biomass deconstruction, characterize and mine biodiversity, and use this knowledge to develop superior biocatalysts for consolidated bioprocessing (CBP). Four biologically mediated events occur in fuel production from biomass featuring enzymatic hydrolysis—saccharolytic enzyme production, hydrolysis, fermentation of six-carbon sugars, and fermentation of five-carbon sugars. Although these events are accomplished in several process steps in near-term designs, they could in principle be combined into a single step mediated by one microbe or microbial community. Recent data suggest a microbe-enzyme-substrate synergy results from this consolidation. A CBP approach has been described as the “ultimate low-cost configuration for cellulose hydrolysis and fermentation.” Development of CBP will result in the largest projected cost reduction of all R&D-driven improvements. One of the leading candidate microorgan-

* Presenting author

isms for development of this CPB process is *Clostridium thermocellum*.

In this study, we used microarray technology to probe the genetic expression of *C. thermocellum* ATCC 27405 during cellulose and cellobiose fermentation. We also used multidimensional LC-MS/MS technology and ¹⁵N-metabolic labeling strategy to quantify changes in cellosomal proteins in response to various carbon sources (cellobiose, amorphous/crystalline cellulose (avicel) and combinations of avicel, pectin and xylan). Transcriptomic analysis involved a time-course analysis of gene expression to identify gene clusters with similar temporal patterns in expression during cellulose fermentation. Broadly, genes involved in energy production, translation, glycolysis and amino acid, nucleotide and coenzyme metabolism displayed a progressively decreasing trend in gene expression. In comparison, genes involved in cell structure and motility, chemotaxis, signal transduction, transcription and cellosomal genes showed an increasing trend in gene expression. Proteomic analysis identified over 50 dockerin- and 6 cohesin-module containing components, including 20 *new* subunits. The list included several proteins of potential interest that specifically respond to the presence of 'non-avicel' substrates in the culture medium. Quantitative proteomic results also highlighted the importance of glycoside hydrolase (GH) family 9 enzymes in crystalline cellulose hydrolysis. Overall, the transcriptomic and proteomic results suggest a well-coordinated temporal and substrate-specific regulation of cellosomal composition in *C. thermocellum*.

6 BESC: Characterization and Modeling: the Biomass HTP Characterization Pipeline for Assessing Improved Cell Walls and Enzymes

M. Davis^{1*} (mark_davis@nrel.gov), W. Adney,¹ S. Decker,¹ M. Himmel,¹ S. Ding,¹ C. Wyman,² A. Ragauskas,³ E. Uberbacher,⁴ W. York,⁵ B. Davison,⁴ and M. Keller⁴

¹National Renewable Energy Laboratory, Golden, Colorado; ²University of California, Riverside, California; ³Georgia Institute of Technology, Atlanta, Georgia; ⁴Oak Ridge National Laboratory, Oak Ridge, Tennessee; and ⁵University of Georgia, Athens, Georgia

Project Goals: The overriding goal of the Center is to engineer wall biosynthesis pathways to generate plants

with less recalcitrant walls that are deconstructed effectively by consolidated microbial bioprocessing, ultimately achieving optimal sugar release and conversion from the biomass with minimal or no chemical pretreatment. These efforts will be supported by the development of chemical and molecular models that predict how wall structure, recalcitrance, and biocatalyst-biomass interactions are related and by experimental and theoretical approaches to refine these models.

BESC will pursue the following aims in three research focus areas covered in three posters. The other focus areas will develop improved plant materials and CBP methods that facilitate cost-effective conversion of biomass to fermentable sugars. The strength of BESC is the cross-cutting integration of diverse experimental, theoretical, and computational approaches. This integration and shared analysis is the mission of the Characterization and Modeling Focus area. For example, HTP physical characterization of biomass that is being screened for decreased recalcitrance will provide the basis for subsequent data mining that can reveal previously unknown correlations between recalcitrance and biomass structure. Such correlations will invariably lead to new hypotheses, which can be tested by integrating experimental and model-building approaches.

This poster will illustrate our establishment of a high-throughput (HTP) pretreatment and characterization pipeline that enables study of the structure, composition, and deconstruction of biomass to elucidate the underlying causes of recalcitrance. We will share many samples and use data management techniques to allow knowledge exchange across the center. The HTP pretreatment and characterization pipeline will screen the structure, composition, and deconstruction of biomass (approximately 5,000 samples per month) and identify the most promising samples for more detailed characterization.

We will discuss the plans to screen multiple samples from at least 10,000 *Populus* and switchgrass plants to identify variants with modified recalcitrance. Initial tests on baseline samples and some other materials will be discussed.

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Biofuels > Analytical and Imaging Technologies for Studying Lignocellulosic Material Degradation

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Three-Dimensional Spatial Profiling of Lignocellulosic Materials by Coupling Light Scattering and Mass Spectrometry

P.W. Bohn^{1*} (pbohn@nd.edu) and J.V. Sweedler² (sweedler@scs.uiuc.edu)

¹Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, Indiana and ²Department of Chemistry, University of Illinois, Urbana, Illinois

Project Goals: The goals of the project are to:

(1) develop correlated optical and mass spectrometric imaging approaches for sub-surface imaging to address the highly scattering nature of LCMs in different states of processing; (2) adapt mass spectrometric imaging protocols to enable spatially-resolved LCM characterization; (3) create surface optical and mass spectrometric measures of lignin-hemicellulose-cellulose degradation at specific processing stages; and (4) correlate the *in situ* optical (Raman and SH-OCT) and mass spectrometric information to generate depth-resolved maps of chemical information as a function of spatial position and processing time.

The physical and chemical characteristics of lignocellulosic materials (LCMs) pose daunting challenges for imaging and molecular characterization: they are opaque and highly scattering; their chemical composition is a spatially variegated mixture of heteropolymers; and the nature of the matrix evolves in time during processing. Any approach to imaging these materials must (1) produce real-time molecular speciation information *in situ*; (2) extract sub-surface information during processing; and (3) follow the spatial and temporal characteristics of the molecular species in the matrix and correlate this complex profile with saccharification. To address these challenges we are implementing tightly integrated optical and mass spectrometric imaging approaches. Employing second harmonic optical coherence tomography (SH-OCT) and Raman microspectroscopy (RM) provides real-time *in situ* information regarding the temporal and spatial profiles of the processing species and the overall

chemical degradation state of the lignin heteropolymer; while MALDI and SIMS provide spatially-resolved information on the specific molecular species produced by pre-enzymatic processing. The goals of the approach are to: (1) develop correlated optical and mass spectrometric imaging approaches for sub-surface imaging to address the highly scattering nature of LCMs in different states of processing; (2) adapt mass spectrometric imaging protocols to enable spatially-resolved LCM characterization; (3) create surface optical and mass spectrometric measures of lignin-hemicellulose-cellulose degradation at specific processing stages; and (4) correlate the *in situ* optical (Raman and SH-OCT) and mass spectrometric information to generate depth-resolved maps of chemical information as a function of spatial position and processing time.

8

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Identify Molecular Structural Features of Biomass Recalcitrance Using Non-Destructive Microscopy and Spectroscopy

Shi-You Ding^{1*} (shi_you_ding@nrel.gov), John Baker,¹ Mike Himmel,¹ Yu-San Liu,¹ Yonghua Luo,¹ Qi Xu,¹ John Yarbrough,¹ Yining Zeng,¹ Marcel G. Friedrich,² and X. Sunney Xie²

¹National Renewable Energy Laboratory, Golden, Colorado and ²Harvard University, Cambridge, Massachusetts

Project Goals: We propose to exploit recently developed techniques to image the plant ultrastructure, map the chemistry of cell-walls, and study the time-course changes during conversion processes. These techniques are novel and developed or modified specifically for biomass characterization at the nanometer resolution. They include atomic force microscopy (AFM), solid-state NMR, small angle neutron scattering (SANS), and total internal reflection fluorescence (TIRF) microscopy. We will also use fluorescence labeling approaches to map biomass surface chemistry and to track single enzyme action *in vitro* and *in vivo*. For example, one novel technique under development is to integrate AFM with TIRF-M capability to permit us to image the cell-wall substrate at the nanometer spatial resolution and to track single cell-wall/enzyme/cellulosome-substrate interaction. The application of these advances to the study of plant cell walls, and par-

* Presenting author

ticularly, those focused on issues relevant to biomass conversion, is currently uncharted territory.

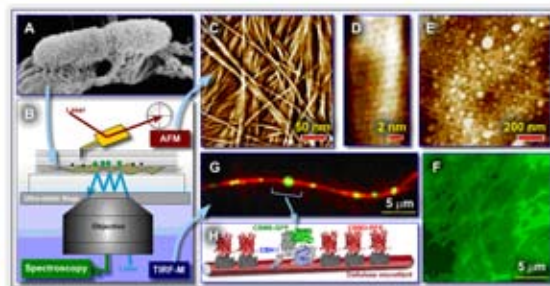
Lignocellulosic biomass has long been recognized as a potential sustainable source of mixed sugars for fermentation to fuels and other bio-based products. However, the chemical and enzymatic conversion processes developed during the past 80 years are inefficient and expensive. The inefficiency of these processes is in part due to the lack of knowledge about the structure of biomass itself; the plant cell wall is indeed a complex nano-composite material at the molecular and nanoscales. Current processing strategies have been derived empirically, with little knowledge of the nanostructure of the feedstocks, and even less information about the molecular processes involved in biomass conversion. Substantial progress towards the cost effective conversion of biomass to fuels is contingent upon fundamental breakthroughs in our current understanding of the chemical and structural properties that have evolved in the plant cell walls which prevent its disassembly, collectively known as “biomass recalcitrance.”

In nature, biomass degradation is a process of molecular interaction and reaction between plant cell wall polymers (i.e., cellulose and matrix polymers) and cellulolytic microbes and their secreted enzymes (Figure A). An integrated system (Figure B) has been set up to combine microscopic and spectroscopic modules that allow us to characterize biomass conversion processes at high spatial and chemical resolution. For example, atomic force microscopy (AFM) is used to map the surface topography of the plant cell wall and the binding of microbial cells and enzymes to the walls; total internal reflection fluorescence (TIRF) microscopy and single molecule spectroscopy is used to track the distributions and movements of labeled microbial cells and enzymes; and spectroscopy (e.g., coherent anti-stokes Raman scattering, CARS, see also Poster by *Friedrich et al.*) is used to monitor the resultant chemical changes in cell wall polymeric component during biochemical, as well as chemical, conversions of biomass.

Preliminary results have demonstrated that integrated analysis of the same cell wall samples by diverse microscopic and spectroscopic approaches is critical for characterizing the degradation processes. In the examples illustrated, correlative imaging of AFM (Figure C-E) and TIRF-M (Figure F and G) provides molecular resolution of surface structure and chemistry of cell walls.

AFM has been shown to be a powerful tool for imaging biomolecules because of its potential atomic level resolution and its ability to image surfaces under appropriately-

buffered liquids (1). Precise measurement of cellulose microfibrils (Figure C) has been reported (1, 2) and individual cellulose chains (Figure D, Ding, unpublished) can be visualized. Using a flow-cell, the same cell wall sample can be thermo-chemically treated and imaged to monitor the structural changes that occur during pretreatment. Figure E shows particles precipitated on the wall surface after dilute acid pretreatment at 140°C. These particles are probably lignin-carbohydrate-complexes (LCC) generated by partial hydrolysis with acid (Ding, unpublished). The cell wall can also be specifically labeled by a fluorescently-tagged carbohydrate-binding module (CBM) and imaged by TIRF-M. Figure F shows a CBM3-GFP (green fluorescence protein) labeled cell wall, in this case, the family 3 CBM specifically recognizes the planar face of cellulose (3). This image therefore reveals cellulose distribution in the cell wall. Using similar technique, individual cellulase species can be labeled with fluorescent protein and imaged using TIRF-M at the single molecule level. Figure G shows GFP-labeled *Trichoderma* cellobiohydrolase-I (CBH I) selectively bound on areas of an individual cellulose microfibril also labeled with CBM3-RFP (red fluorescence protein) (4).



In summary, we have demonstrated non-destructive approaches to characterize the biomolecules involved in biomass conversion processes using integrated microscopy and spectroscopy. The methods are initially developed using corn stover biomass and maize plant. As a next step, we intend to employ this imaging system to characterize more energy plants, such as switchgrass and poplar wood, and their chemical and biological conversion processes to biofuels.

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9 Dynamic Visualization of Lignocellulose Degradation by Integration of Neutron Scattering Imaging and Computer Simulation

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Barbara R. Evans^{1*} (evansb@ornl.gov), Jeremy C. Smith,^{2,3} Volker Urban,^{1,4} Dean A. Myles,^{1,4} William T. Heller,^{1,4} Hugh M. O'Neill,¹ Art Ragauskas,⁵ and Ida Lee^{1,6}

¹Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee; ²Center for Molecular Biophysics, Oak Ridge National Laboratory, Oak Ridge, Tennessee; ³Governor's Chair, University of Tennessee, Knoxville, Tennessee; ⁴Center for Structural Molecular Biology, Oak Ridge National Laboratory, Oak Ridge, Tennessee; ⁵Institute of Paper Science and Technology, School of Chemistry and Biochemistry, Georgia Institute of Technology; and ⁶Department of Electrical and Computer Engineering, University of Tennessee, Knoxville, Tennessee

Project Goals: Lignocellulosic biomass is a complex material with a molecular-level morphology and laminate structure that result in its recalcitrance to deconstruction and subsequent enzymatic hydrolysis to fermentable sugars. The objective of this research project is the development of reliable real-time in situ imaging technology integrating the various neutron and simulation techniques to obtain detailed information on the relationship of atomic and mesoscale structure to resistance to hydrolysis in lignocellulosic

materials. This structural complexity will be elucidated by applying scattering techniques over a wide range of length scales (Ångströms to micrometers using wide-, small- and ultra-small angle scattering) and using several complementary approaches to contrast variation, including x-rays with sensitivity to electron density and neutrons with sensitivity to isotope composition in combination with neutron contrast variation by using appropriate blends of hydrogenated and deuterated solvents as well as by specific isotope labeling of individual biomass components. This comprehensive and multi-scale approach is necessary to address the complexity of the multi-component, multi-length-scale and multi-phase structure of lignocellulosic biomass.

Lignocellulosic biomass is composed of plant cell walls. It is a complex material composed of crystalline cellulose microfibrils laminated with hemicellulose, pectin, and lignin polymers. Understanding the physical and chemical properties of this biomass is crucial for overcoming the major technological challenge in the development of viable cellulosic bioethanol, the minimization of biomass recalcitrance to hydrolysis via the improvement of pretreatment and the design of improved feedstock plants.

To address this problem, we propose to integrate neutron science, surface force recognition imaging, and computer simulation technology to provide in situ real-time multi-scale visualization of lignocellulose deconstruction. A full arsenal of neutron scattering techniques will be employed, ranging from diffraction through small-angle scattering and dynamic neutron scattering in combination with state-of-the-art high-performance computer simulation to achieve an understanding of the physico-chemical mechanism of biomass recalcitrance in unprecedented detail. A key part is the design and employment of multipurpose neutron imaging chamber specifically designed for in situ dynamic observation under biomass pretreatment conditions. This visualization of biomass morphological degradation during heat, pressure, chemical or enzymatic treatment will provide fundamental molecular level information that will address the following basic questions:

- Visualization of regional transitions of cellulose between crystalline and amorphous.
- Investigation of lignin degradation and re-polymerization in response to biomass pretreatment.
- Quantitative characterization of lignin associations with cellulose before, during and following heat, pressure, chemical or enzymatic treatment.
- Analysis of accessibility of bio-mass components to solvent molecules, chemical agents or enzymes

* Presenting author

- Visualization of structural changes of cellulose and lignocellulose during disruption and degradation by cellulases and cellulosomes.

We will present a summary of current progress on the research tasks that we have initiated to achieve these objectives.

This work is funded by the U. S. Department of Energy, Office of Biological and Environmental Research Genomics:GTL Program under FWP ERKP704, “Dynamic Visualization of Lignocellulose Degradation by Integration of Neutron Scattering Imaging and Computer Simulation.”

10 A New Solution-State NMR Approach to Elucidate Fungal and Enzyme/Mediator Delignification Pathways

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Kenneth E. Hammel^{1*} (kehammel@wisc.edu), John Ralph,² Daniel J. Yelle,¹ Fachuang Lu,² Semarjit Shary,¹ and Sally Ralph¹

¹USDA Forest Products Laboratory, Madison, Wisconsin and ²U.S. Dairy Forage Research Center, Madison, Wisconsin

Project Goals: Our goal is to use new NMR approaches to characterize the changes fungi cause in lignin when they biodegrade lignocellulose. This work will likely identify reactions that are pivotal in the removal of lignin.

The removal of lignin is a key step in lignocellulose biodegradation by fungi, and the mechanisms responsible may find practical applications in lignocellulose processing. However, the characterization of the chemical changes in lignin that fungi introduce to promote its biodegradation remains a challenge. We are using new NMR and isotope enrichment approaches to address this problem. One improvement is that lignocellulose samples undergoing biodegradation can now be completely solubilized and analyzed by two-dimensional ¹H-¹³C correlation (HSQC) solution-state NMR spectroscopy. For example, by using this method we have now shown that intermonomer lignin sidechains were markedly depleted in spruce wood undergoing decay by the brown rot basidiomycete *Gloeophyllum trabeum*. Additional work is needed to characterize the products, but it is already clear, contrary to the general view, that the aromatic polymer remaining after extensive brown rot is no longer

recognizable as lignin. A second improvement is that the ligninolytic reactions of some poorly understood fungi can now be characterized by ¹³C NMR spectroscopy if the lignin in the growth substrate is enriched with ¹³C. To test this approach, we spiked aspen wood with an α -¹³C-labeled synthetic lignin, inoculated the wood with the soft rot ascomycete *Daldinia concentrica* to decay it, extracted the residual lignin afterwards, and analyzed this sample by solution-state ¹³C NMR spectroscopy. The results showed that *D. concentrica* cleaved the lignin between C_α and C_β to give lignin fragments terminated by benzaldehyde and benzoic acid moieties.

11 Development of Modular Platforms for in Vivo Mapping of Local Metabolite Concentrations Important to Cell Wall Degradation by Microorganisms

GTL

Christopher G. Hunt^{1*} (cghunt@fs.fed.us) and Kenneth Hammel^{1,2} (kehammel@wisc.edu)

¹USDA Forest Products Laboratory, Madison, Wisconsin and ²Department of Bacteriology, University of Wisconsin, Madison, Wisconsin

Project Goals: Develop sensors which can be used to produce micron scale maps of metabolite activities in regions where fungi are actively degrading wood.

Background

Lignin is a major barrier to the conversion of plant cell walls into fuels or other valuable products. A complex polymer of randomly coupled phenylpropane units, lignin resists chemical degradation. It coats the cellulose and hemicelluloses of vascular plants, thus preventing enzymes from hydrolyzing these potentially valuable polysaccharides. Lignin accounts for about 25% of woody biomass and about 15% of the total biomass of agricultural feedstocks such as switchgrass. The presence of lignin is a significant hindrance to effective biomass utilization, as evidenced by the fact that fermentability of plant material is inversely related to lignin content. Cost-effective lignin removal is a significant barrier to effective fermentation of biomass.

One approach to solving the problem of lignin removal is to apply the mechanisms developed by organisms that have evolved to metabolize lignocellulose. So far, the only organisms clearly shown to biodegrade lignified tissue

* Presenting author

are certain filamentous fungi. One reason that vascular plant cell walls are so difficult to digest is that their lignin prevents even the smallest enzymes from penetrating. Filamentous fungi solve this problem by making lignocellulose more hydrophilic and porous, with the result that hydrolytic enzymes can eventually penetrate the substrate.

To make lignocellulosics permeable to enzymes, filamentous fungi use a variety of small, diffusible reactive oxygen species (ROS) such as hydroxyl radicals, peroxy radicals, and possibly phenoxy radicals. These radicals diffuse into the cell walls and initiate biodegradative radical reactions. When lignin is the target, radical attack results in various extents of oxidation and depolymerization.

These small diffusible oxidative species are important tools used by filamentous fungi to make the cell wall accessible to enzymes. Despite this, we have a poor knowledge of how these oxidants are spatially distributed in biodegrading lignocellulose relative to the fungal hypha that produce them. The goal of this project is to remedy this deficit through fluorescence microscopy of newly designed sensors that will serve as in situ reporters of biodegradative radical production. While developing these sensors, we will test the specificity of reaction of a variety of promising fluorophores to increase the arsenal of ROS specific probes available for work at low pH. We will also demonstrate how binding these fluorescent probes to beads improves fluorescent imaging by preventing dye diffusion, limiting interferences, and allowing the use of almost all dyes (lipo- or hydrophilic, cell permeant or not) to be used in extracellular environments. Finally, we will use these sensors to produce oxidative maps that will help us to understand how fungi generate ROS and how they use these ROS to make cell walls more accessible to enzymes.

Method

We are attaching fluorescent dyes to silica beads. Our first bead has BODIPY 581/591® on a 3µm porous HPLC bead. This dye's emission changes irreversibly from red to green upon oxidation by ROS. The ratio of red to green emission provides a quantitative measure of the cumulative oxidation at that point in space. Dyes with reactivity to specific ROS, pH, or other metabolites of interest are envisioned.

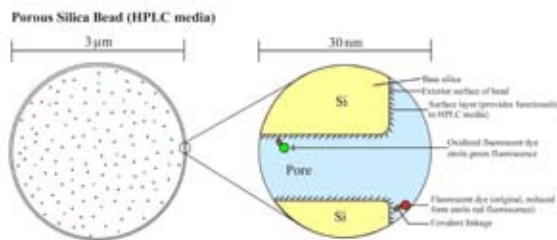


Figure. Fluorescent dye attached to silica bead

There are many advantages gained by fixing the dye to bead. We design the bead to emit two fluorescent signals, so that the ratio of the two signal intensities provides quantitative information. Immobilized dyes are prevented from moving after reaction, so partitioning is impossible, they cannot be ingested, and the fluorescence from the dye is clearly distinguishable from background.

Beads are placed on wood samples and imaged with a confocal microscope during fungal colonization. Images can be analyzed to provide the analyte concentration maps as well as an overlay of the location of fungal hypha.

12 GTL Dynamic Molecular Imaging of Lignocellulose Processing in Single Cells

Amy Hiddessen, Alex Malkin, and **Michael Thelen*** (mthelen@llnl.gov)

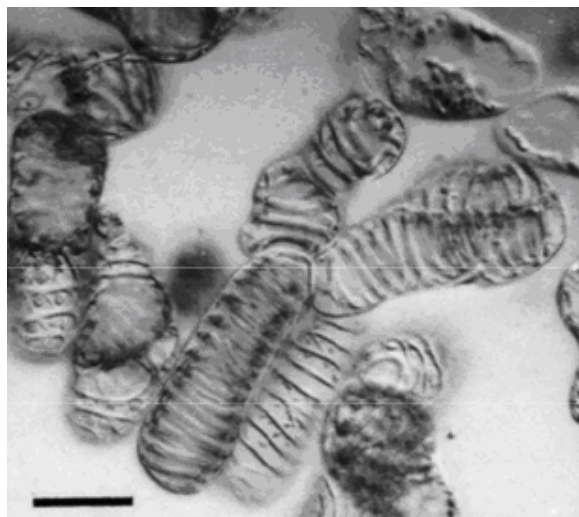
Chemistry Directorate, Lawrence Livermore National Laboratory, Livermore, California

Of central importance to our nation's energy resources is the pursuit of alternative fuels directly from plant material rich in lignin and cellulose. The abundant and intractable nature of these biopolymers limits their practical use to produce biofuel precursors, and so presents an extraordinary scientific challenge. A comprehensive understanding of the natural or engineered breakdown of plant cell wall materials must be addressed on several levels, including the examination of detailed ultra-structural changes that occur in real time. To accelerate research on the cellular and molecular details of the cell wall deconstruction process, we are developing sophisticated analytical tools specifically to visualize changes in surfaces, polysaccharides and proteins. Molecular surface characterization can be directly linked with high

* Presenting author

resolution, three-dimensional images of cellular structure by combining atomic force microscopy (AFM) with laser scanning confocal microscopy (LSCM) in a single instrument.

Highly oriented assemblies of lignocellulose are found in the water-transporting xylem tissue. To observe these thick secondary wall depositions in single cells, we are using the *Zinnia elegans* culture system in which leaf mesophyll cells are induced to differentiate in synchrony to tracheary elements (single xylem cells; see Figure). In these cultures, a high proportion of cells undergo programmed autolysis which is accompanied by a high rate of secondary wall polysaccharide synthesis and lignification. When isolated at the same stage of wall formation, cells are treated with enzyme preparations or wood-degrading microbes to stimulate specific decomposition of wall materials, and these are examined under physiological conditions using AFM for topological, near-molecular scale imaging. Corresponding processing of polymers, protein composition, and protein co-localization will be detected and visualized using gold-conjugated antibodies by AFM-based immunolabeling. Specific dyes and antibodies also will be used as cellular probes in LSCM, where live cell samples will be observed in real time in a precisely controlled environment. Methods will be developed to couple AFM and LSCM imaging techniques for monitoring natural and induced changes in wall ultrastructure, and rendered as 3D reconstructions.



Cultured cells of *Zinnia elegans*. Helically oriented secondary cell wall thickenings are characteristic of xylem cells in culture as in plant vasculature. bar = 25 μm .

13 Study of Lignocellulosic Material Degradation with CARS Microscopy

Marcel G. Friedrich^{1*} (friedric@fas.harvard.edu),
Conor L. Evans,¹ Gary Holtom,¹ John Yarbrough,² **Shi-You Ding**² (shi_you_ding@nrel.gov), and X. Sunney Xie¹ (Xie@chemistry.harvard.edu)

¹Harvard University, Cambridge, Massachusetts and
²National Renewable Energy Laboratory, Golden, Colorado

Project Goals: Finding alternative sources of energy required to maintain today's living standards is a compelling challenge. A promising substitute for fossil fuel is bioethanol produced from biomass conversion. The key step of this process is the degradation of plant cell wall polysaccharides into fermentable sugars. Lignin is the hydrophobic polymeric component of the plant cell wall. It has been proposed that lignin may play a role in preventing plant cell walls from chemical and biological hydrolysis during biomass conversion processes. The improvement of conversion efficiency is key to biofuel production and requires imaging techniques with contrast based on their chemical composition for a real-time monitoring. This project is aimed on applying coherent anti-Stokes Raman Scattering (CARS) microscopy to visualize the chemical/structural changes of the cell wall polymers during the degradation process.

Finding alternative sources of energy required to maintain today's living standards is a compelling challenge. A promising substitute for fossil fuel is bioethanol produced from biomass conversion. The key step of this process is the degradation of plant cell wall polysaccharides into fermentable sugars. Lignin is the hydrophobic polymeric component of the plant cell wall. It has been proposed that lignin may play a role in preventing plant cell walls from chemical and biological hydrolysis during biomass conversion processes. The improvement of conversion efficiency is key to biofuel production and requires imaging techniques with contrast based on their chemical composition for a real-time monitoring. This project is aimed at visualizing the chemical/structural changes of the cell wall polymers during the degradation process.

Confocal and multiphoton fluorescence microscopy have become powerful techniques for three-dimensional imaging of biological systems. However, for biochemical species or cellular components that either do not fluoresce or cannot tolerate labeling, other contrast mechanisms with molecular specificity are needed. Vibrational microscopy

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based on infrared absorption and spontaneous Raman scattering have been used for chemically selective imaging. However, IR microscopy is limited to low spatial resolution because of the long wavelength of light used. Furthermore, the absorption of water in the infrared region makes it difficult to image biological materials. Raman microscopy can overcome these limitations and has been successfully used to characterize plant cell walls. By tuning into different vibrational frequencies, lignin and carbohydrates could be imaged selectively. However, the intrinsically weak Raman signal can require high laser powers and long integration times, often hours. This poor time resolution prevents any kind of real time monitoring.

Coherent anti-Stokes Raman scattering (CARS) microscopy has matured as a powerful nonlinear vibrational imaging technique that overcomes these limitations of conventional Raman microscopy [3,4]. CARS microscopy provides a contrast mechanism based on molecular vibrations, which are intrinsic to the samples. It does not require natural or artificial fluorescent probes. CARS microscopy is orders of magnitude more sensitive than spontaneous Raman microscopy. Therefore, CARS microscopy permits fast vibrational imaging at moderate average excitation powers (i.e. up to ~10 mW) tolerable by most biological samples. Furthermore, CARS microscopy has a three-dimensional sectioning capability, useful for imaging thick tissues or cell structures. This is because the nonlinear CARS signal is only generated at the focus where the laser intensities are the highest. Finally, the anti-Stokes signal is blue-shifted from the two excitation frequencies, and can thus be easily detected in the presence of one-photon fluorescence background. We demonstrate that CARS can visualize the chemical and structural changes of the cell walls during this conversion process.

The Raman spectrum of a cell wall region in corn stover is shown in Fig. A (inset). The prominent band at 1600 cm^{-1} is the aryl symmetric ring stretching vibration which serves as a sensitive marker for the presence of lignin. The two bands at around 1090 and 1110 cm^{-1} are due to C-O and C-C stretching vibrations from cellulosic polymers at the cell wall. The structures of lignin and cellulose are shown in Fig. B. Figure a shows the CARS image tuned into the lignin band at 1600 cm^{-1} . A high density of the lignin is detected in the secondary cell walls. The integration time of a CARS image (Fig. d) is with 20 sec tremendously faster than compared to the Raman picture that requires about 55 min (Fig. c), while providing a comparable contrast. The fast integration time is critical of monitoring chemical changes during biomass pretreatment that usually occurs within minutes.

Currently, we are working on improving the sensitivity of the CARS technique to image cellulose, as well as spatial resolution. These initial results demonstrate the feasibility of the proposed method.

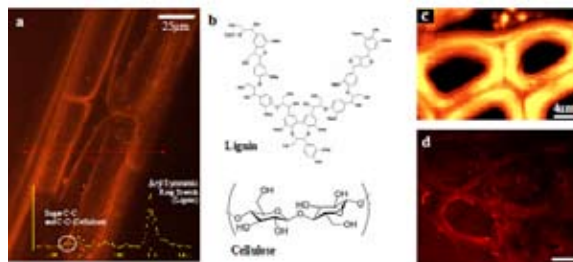


Figure. a) CARS images of cross section of corn stove cell walls by integrating over the aromatic lignin band wavelength ($1,550\text{--}1,640\text{ cm}^{-1}$). b) Chemical Structures of lignin and cellulose. c) Cross section of poplar wood imaged by Raman microscopy (integration time of 55 min) and d) CARS microscopy (integration time of 20sec).

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* Presenting author

14

Single-Molecule Studies of Cellulose Degradation by Cellulosomes

Haw Yang^{1,2*} (hyang@lbl.gov), Jamie Cate,^{1,2} Padma Gunda,¹ L. Meadow Anderson,¹ and Hu Cang¹

¹Lawrence Berkeley National Laboratory, Berkeley, California and ²University of California, Berkeley, California

A cellulosome is a large extracellular supramolecular complex that is produced by anaerobic microbes to enzymatically decompose crystalline cellulosic polymers and plant cell walls. It consists of a scaffolding protein that accommodates other essential protein and enzyme components for cellulose degradation. They include carbohydrate-binding modules for attachment to the solid cellulose substrate, various glycoside hydrolases to efficiently hydrolyze a heterogeneous substrate, and in some cases, anchoring proteins to attach cellulosomes to the bacterial cell surface. The mechanism of its function is poorly understood due to the complexity of the cellulosome itself and the natural environment in which it functions. Outstanding issues include the location and the manner in which the cellulosome is assembled, the distribution in the cellulosomal composition, and the dynamic interactions between cellulases and the insoluble substrate, to name a few.

These questions are very difficult to address quantitatively using conventional, ensemble-based methods due to the multiple layers of complexity involved. The convoluted spatio-temporal dynamics in cell and cellulosome interaction with insoluble substrates make it very hard to quantitatively study the various molecular dynamics of a functioning cellulosome. We anticipate that the single-molecule approach, due to its capability of directly monitoring the individual processes from a distribution, will prove invaluable in efforts to unravel how microscopic, molecular interactions impact macroscopic behavior in plant cell wall degradation.

In order to study the various processes involved in ligno-cellulosic degradation by cellulosomes, we are developing a single-molecule spectrometer with the capability to track individual fluorescent particles in three dimensions. We plan to follow the assembly and disassembly as well as the export of cellulosome complexes both *in vitro* and *in vivo*. We are developing quantitative single-molecule and single-particle assays for cellulosome activity. We are studying the cellulosome's formation and processivity for the degradation of lignocellulose. These experiments will exploit the genetic tools that we are developing to

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incorporate fluorescent or nanoparticle tags into the cellulosome to enable tracking. The mechanistic insights are expected to have a direct impact on the improvement and engineering of tailored biomass depolymerization systems.

Biofuels > Metabolic Engineering for Biofuels Production

15

Non-Fermentative Pathways for Synthesis of Branched-Chain Higher Alcohols as Biofuels

Shota Atsumi, Taizo Hanai, and James C. Liao* (liaoj@seas.ucla.edu)

Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California

Project Goals: The goal of this project is to develop novel pathways for production of higher alcohols.

Global energy and environmental problems have stimulated increased efforts in synthesizing biofuels from renewable resources. Compared to the traditional biofuel, ethanol, higher alcohols offer advantages as gasoline substitutes because of their higher energy density and lower hygroscopicity. In addition, branched-chain alcohols have higher octane numbers compared to their straight-chain counterparts. However, these alcohols cannot be synthesized economically using native organisms. Here we present a metabolic engineering approach using *Escherichia coli* to produce higher alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol from a renewable carbon source, glucose. This strategy leverages the host's highly active amino acid biosynthetic pathway and diverts its 2-keto acid intermediates for alcohol synthesis. In particular, we have achieved high yield, high specificity production of isobutanol from glucose. The strategy enables the exploration of biofuels beyond those naturally accumulated to high quantities in microbial fermentation.

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16

Anaerobic Expression of Pyruvate Dehydrogenase for Producing Biofuels in Fermentative Pathways

Thomas Buelter* (tbuelter@gevo.com), Renny Feldman, and Peter Meinhold

Gevo, Inc., Pasadena, California

Project Goals: Aims at determining whether a balanced butanol fermentation pathway can be expressed in a heterologous host, such as *E. coli*. The successful implementation of a metabolic pathway for the production of butanol in a heterologous host depends upon generating the necessary reducing equivalent NADH during the conversion of pyruvate to acetyl-CoA. Therefore, a heterologous host will be engineered so that it expresses pyruvate dehydrogenase, and not pyruvate formate lyase under anaerobic conditions.

Advanced biofuels, which include butanol, will deliver the performance of gasoline without the environmental impact and these biofuels will reduce our dependency on foreign oil. Butanol has a higher energy content per gallon than many first generation biofuels, it does not absorb water and can be transported through the existing oil and gas distribution infrastructure. Butanol can be used in gas-powered vehicles without modification or blending.

In order to make butanol competitive on the fuels market it has to be produced with a higher yield than currently achievable using bacteria from the genus *Clostridium*, which produces many byproducts. We are working on recombinant microorganisms that are engineered to convert biomass into butanol without byproducts. A key problem to be solved is the redox balance of the cell during butanol production from sugar. Additional reducing equivalents in the form of NADH have to be generated to produce butanol from sugar.

To accomplish this we are working on three approaches: We are using in vivo evolution and selection to select for a strain that has the ability to produce sufficient reducing power to generate butanol at high yield. The native pathway for anaerobic generation of Acetyl CoA and fermentative pathways were deleted from the strain before selection pressure was applied to only allow strains to grow that have increased NADH production. The second approach we are pursuing is the directed evolution of pyruvate dehydrogenase subunits with the aim to increase the activity of this enzyme under the conditions of butanol production. The third approach uses the engineering of the cells' regulation of pdh expression to

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increase its expression under butanol production conditions.

17

Kinetic Modeling of Metabolic Pathways in *Zymomonas mobilis* to Optimize Pentose Fermentation

MEWG

Dhinakar S. Kompala* (kompala@colorado.edu)

University of Colorado, Boulder, Colorado

Project Goals: To investigate the complex network of metabolic pathways in engineered bacterium *Zymomonas mobilis*. To develop a quantitative understanding of the metabolic fluxes along the newly engineered pathways for pentose fermentation.

Zymomonas mobilis has been engineered with four new enzymes to ferment xylose along with glucose. A network of pentose pathway enzymatic reactions interacting with the native glycolytic Entner Doudoroff pathway has been hypothesized. We have analyzed the complex interactions between the pentose phosphate and glycolytic pathways in this network by developing a large-scale kinetic model for all the enzymatic reactions. Based on the experimental literature on *in vitro* characterization of each of the 20 enzymatic reactions, the large-scale kinetic model is numerically simulated to predict the dynamics of all the intracellular metabolites along the network of interacting metabolic pathways. This kinetic model takes into account all the feedback and allosteric regulations on the enzymatic reaction rates and is better suited to the systems level analysis of interacting metabolic pathways compared to the standard linearized methods of metabolic flux analysis and metabolic control theory.

This nonlinear kinetic model is simulated to perform numerous *in silico* experiments by varying different enzyme concentrations and predicting their effects on all the intercellular metabolic concentrations and the ethanol production rates in continuous fermentors. Among the five enzymes whose concentrations were varied and given as input to the model, the ethanol concentration in the continuous fermentor was optimized with xylose isomerase was needed at the highest level, followed by the transaldolase. Predictions of the model, that interconnecting enzyme phosphoglucose isomerase, does not need to be overexpressed, were recently confirmed through experimental investigations. Through this kinetic modeling approach, we can develop efficient ways of maximizing the fermentation of both glucose and xylose,

* Presenting author

while minimizing the expression of the heterologous enzymes.

18 Foundational Advances in RNA Engineering Applied to Control Biosynthesis

MEWG

Christina D. Smolke* (smolke@cheme.caltech.edu)

California Institute of Technology, Pasadena, California

Project Goals: The goals of this project are to: (i) generate RNA-based molecular sensors to key metabolites along the BIA pathway; (ii) engineer *Saccharomyces cerevisiae* to synthesize early BIAs; (iii) integrate the molecular sensors into the transgenic yeast strain for the noninvasive, real-time detection of key metabolite levels along this heterologous pathway; (iv) expand the utility of the engineered molecular sensors to the dynamic regulation of enzyme levels in response to metabolite accumulation; and (v) solidify a strong base in biomolecular and metabolic engineering and encourage its advancement by training and educating scientists through cutting-edge, integrated research and educational plans.

Recent progress in developing frameworks for the construction of RNA devices is enabling rapid advances in cellular engineering applications. These devices provide scalable platforms for the construction of molecular communication and control systems for reporting on, responding to, and controlling intracellular components in living systems. Research that has demonstrated the modularity, portability, and specificity inherent in these molecules for cellular control will be highlighted and its implications for synthetic and systems biology research will be discussed. In addition, tools that translate sequence information to device function to enable the forward design and optimization of new devices will be discussed. The flexibility of the specified framework enables these molecules to be integrated as systems that perform higher-level signal processing based on molecular computation strategies. The application of these molecular devices to studying cellular systems through non-invasive *in vivo* monitoring of biomolecule levels and to regulating cellular behavior will be discussed, in particular in the control and optimization of the biosynthesis of alkaloids in *Saccharomyces cerevisiae*.

19 Development of Tolerant and Other Complex Phenotypes for Biofuel Production

MEWG

E. Terry Papoutsakis* (epaps@udel.edu)

Department of Chemical Engineering and the Delaware Biotechnology Institute, University of Delaware, Newark, Delaware

Project Goals: Develop solvent tolerant phenotypes in prokaryotes for biofuel production.

In bioprocessing, in addition to maximizing the flux for a desirable product, the robustness and prolonged productivity of the biocatalyst (the cells) under realistic bioprocessing conditions is an equally important issue. Thus, the ability of cells to withstand “stressful” bioprocessing conditions without loss of productivity is a most significant goal. Such conditions include: toxic substrates, accumulation of toxic products and byproducts, high or low pH, or high salt concentrations as encountered in most applications for the production of chemicals and biofuels as well as in bioremediation applications. The difficulty—but also the intellectual and biotechnological challenge—is that the desirable phenotypic trait is determined by several genes or a complex regulatory circuit. Complex phenotypes are also encountered when one desires to develop a *de novo* capability or pathway in a particular cell type. For example, how do cells put together the regulatory elements of a sequence of genes to make a pathway or program possible? Yes, it is an evolutionary process, but if we are to “imitate” the process, what would we do? What tools could one possibly use and strategies to facilitate the development of complex phenotypes in microbial cells? From omics-based analysis to synthesis, all selection based, or hybrid? Knowledge-based and mechanistic or not? This will be the focus of this presentation, together with some data from early efforts to demonstrate some key concepts that we explore in my laboratory.

* Presenting author

20

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Thermophilic Electricity Generating Bacteria as Catalysts for the Consolidated Bioprocessing of Cellulose

T. Shimotori,¹ C. Marshall,² R. Makkar,² M. Nelson,¹ and H. May^{1,2*} (hmay@mfctech.net)

¹MFC Technologies, LLC, Mount Pleasant, South Carolina and ²Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina

Project Goals: Discover thermophilic electrode reducing bacteria that may be used with cellulolytic and ethanologenic bacteria to convert cellulose into biofuels. Apply aforementioned bacteria in modified fuel cells for the production of ethanol, electricity and hydrogen (or combination thereof) from cellulose.

The biological mediation of the conversion of lignocellulose to biofuels holds much promise, particularly when regarding the supply constraints and contribution to global warming associated with fossil fuels. Consolidated bioprocessing of cellulosic biomass leverages the catalytic activity of cellulolytic and ethanologenic bacteria to produce ethanol^{1,2}. Due to their rapid and effective ability to metabolize cellulose, the bacteria utilized in this process are thermophilic, e.g. *Clostridium thermocellum* and *Thermoanaerobacter thermosaccharolyticum*. However, the formation of acetate and other by-products during the processing and fermentation of plant biomass is common, which may inhibit the overall fermentation. Therefore, it would be advantageous to use bacteria capable of consuming acetate in combination with the cellulolytic bacteria. One clever way to consume acetate under anaerobic conditions is to use electricity generating bacteria and microbial fuel cells (MFCs), where acetate is converted to carbon dioxide and electricity is generated. A thorough description of MFCs is given in a new book authored by Bruce Logan³. We set out to discover thermophilic electricity generating bacteria that would be compatible with the thermophilic ethanologenic bacteria with the idea that ethanol and electricity could then be generated from cellulose.

To find thermophilic electricity generating bacteria we enriched for acetate-consumers in MFCs beginning with sediment fuel cells from marine and freshwater sources incubated at 60°C. This population was further enriched in sediment-free single chamber fuel cells equipped with air cathodes. Following several exchanges of the media

and several transfers to additional fuel cells with only acetate as an energy source, the enriched community from Charleston Harbor was scraped from the surface of an anode and the 16S rRNA genes of the community were cloned and sequenced. A mixed community that included bacteria most closely related to *Deferribacter* spp. but dominated by Gram positive *Firmicutes*, particularly of *Thermincola* spp., was discovered. The *Deferribacter* spp. have been isolated from deep wells and deep-sea hydrothermal vents and some species are known to reduce metals external to the cells^{4,6}. Two *Thermincola* spp. have been isolated from volcanic hot springs^{7,8}. Interestingly, our community came from a mesobiotic estuarine harbor.

Spores from the *Thermincola* are resistant to autoclaving, and our enriched community would still grow and generate electricity in a fuel cell supplied with acetate even after the inoculum was autoclaved for 30 minutes. The 16S rRNA genes of the autoclaved community are now under analysis. In addition, *T. ferriacetica*, which is capable of reducing insoluble iron oxides while consuming acetate, is in pure culture and we are now testing it for the ability to generate electricity in a fuel cell. Both the enriched community and *T. ferriacetica* are also being tested in combination with cellulolytic bacteria, e.g. *C. thermocellum*, in a consolidated bioprocess to produce ethanol and electricity from cellulose.

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BioHydrogen > Quantitative Microbial Biochemistry and Metabolic Engineering for Biological Hydrogen Production

21

Metabolic Modeling for Maximizing Photobiological H₂ Production in Cyanobacteria

GTL

Alexander S. Beliaev^{1*} (alex.beliaev@pnl.gov), Grigoriy E. Pinchuk,¹ Oleg Geydebekht,¹ Michael H. Huesemann,² Jennifer L. Reed,³ Andrei L. Osterman,⁴ John R. Benemann,⁵ and Jim K. Fredrickson¹

¹Pacific Northwest National Laboratory, Richland, Washington; ²Pacific Northwest National Laboratory Marine Science Laboratory, Sequim, Washington; ³University of California, San Diego, California; ⁴Burnham Institute for Medical Research, La Jolla, California; and ⁵Benemann Associates, Walnut Creek, California

Project Goals: In this study, we seek to improve our understanding of H₂ production by a diazotrophic unicellular cyanobacterium *Cyanothece* sp. strain ATCCC 51142 using a metabolic modeling approach for simulating the fundamental metabolism of indirect biophotolysis, as well as identifying the main metabolic and regulatory controls in this organism. From this, the potential for H₂ production by indirect biophotolysis in this organism will be assessed based on imposing new constraints to redirect the low redox potential electron transport pathways from normal metabolism towards H₂ production from accumulated carbohydrates. As a result, it will provide an *in silico* tool for manipulating such microorganisms to act as catalysts for solar energy

conversion to H₂ and potentially allow for a development of a highly efficient H₂ production process.

Advances in microbial genome sequencing and functional genomics are greatly improving the ability to construct accurate systems-level models of microbial metabolism and to use such models for metabolic engineering. With the increasing concerns over the reliance on fossil fuels, there is a revitalized interest in using biological systems for producing renewable fuels. Genomics and metabolic engineering hold great promise for the rational design and manipulation of biological systems to make such systems efficient and economically attractive.

Although there is a relatively rich body of scientific information on the biochemistry, physiology, and genetics of photosynthetic H₂ production, a systems-level understanding of this process is lacking. In this proposal, we seek to improve our understanding of H₂ production by a diazotrophic unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142 using a metabolic modeling approach for simulating the fundamental metabolism of indirect biophotolysis as well as identifying the main metabolic and regulatory controls in this organism. The genus *Cyanothece* has several attractive properties; they are aerobic, unicellular, diazotrophic bacteria that separate in time the process of light-dependent autotrophic growth and glycogen accumulation from N₂ fixation at night. The process of N₂ fixation and concomitant cyanophycin (nitrogen storage compound) accumulation is accompanied by a decrease in total cellular glycogen content.

In this project, we intend to construct a base metabolic model from genome sequences and other sources of information and integrate new data from physiology experiments, functional genomics, and comparative sequence analysis to develop high-quality, comprehensive models suitable for predictive analysis of *Cyanothece* metabolism. From this, we will assess the potential for H₂ production by indirect biophotolysis in this organism based on imposing new regulatory constraints that would redirect the low-redox-potential electron transport pathways from normal metabolism toward H₂ production from accumulated carbohydrates. We will also extend our studies to other cyanobacterial species including *Synechocystis* PCC 6803, which will allow us to develop a more robust cyanobacterial metabolic model and conduct a comparative metabolic analysis between the two organisms. The result will be an *in silico* tool for manipulating such microorganisms to act as catalysts for solar energy conversion to H₂ and will potentially allow development of a highly efficient H₂ production process.

* Presenting author

22

GTL

Engineering Microbes for Enhanced Hydrogen Production: Parameter Estimation for Transcriptional Control of Metabolism

Alex Fichtenholtz* (alex@bu.edu), Diogo Camacho, and James J. Collins

Department of Biomedical Engineering and Center for BioDynamics, Boston University, Boston, Massachusetts

Project Goals: As part of this proposed project, we plan to create a novel systems biology technology platform and resources that can be used to reverse engineer and analyze gene regulatory networks and metabolic pathways that influence and control microbial hydrogen production. We will use this novel platform to achieve the following specific objectives: (1) construct genome-scale transcriptional regulatory and metabolic models using automated model reconstruction methods, and subsequently integrate the regulatory and metabolic models for computational analysis; (2) use the integrated metabolic and regulatory models to identify relevant metabolic processes and pathways involved in hydrogen production as well as the regulatory processes that inhibit hydrogen overproduction; and (3) use the integrated metabolic and regulatory models to make computational predictions of optimal metabolic pathway modifications, which will enable enhanced biological hydrogen production. As part of this project, we plan to use our systems biology platform to study the cyanobacterium *Cyanothece*, a microbe with metabolic capabilities to produce hydrogen. We will collaborate with a team from the Pacific Northwest National Laboratory (PNNL), headed by Alexander Beliaev and involving Jim Fredrickson.

Flux distribution is a major problem in metabolic engineering, in that, while one can insert genes for a novel pathway of reactions for a metabolite into the cell, basic chemical kinetics dictates that much of the flux will flow through pre-existing reactions for this metabolite, and only some through this foreign pathway. While this approach is occasionally successful, it harbors a host of problems: (i) it is inherently a local approach, as the gene knockouts always focus on genes that surround a metabolite node of interest and (ii) such knockouts often significantly slow down the growth and nutrient uptake rates of the cell. The gene knockout strategy fails to be an acceptable solution on account of it consistently falling short of optimum efficiency and rates of production.

The methodology proposed here is to approach the problem by controlling the transcriptional network regulating the expression of metabolic genes. This network of transcription factors, which has considerable connectivity and feedback, has evolved as a response mechanism to control the cell's metabolism in a variety of conditions. It controls the distribution of flux throughout the cell on a global scale, from rate nutrient uptake, to ratio of reaction distributions at metabolite nodes, all the way down to the rate at which waste exits the cell. Several of these transcription factors, such as CRP, Fnr, and FruR, modulate the expression of dozens of metabolic genes. Optimizing the expression of these transcription factors for the best efficiency of production is a more global approach than knocking out individual metabolic genes, and thus much more likely to yield consistent results.

With our approach, the parameter that describes the strength of connection is determined and the network reconstructed through a parameter estimation of a generalized differential equation for the levels of expression for each gene. This methodology was applied to a two-reaction system composed of 19 differential equations and 60 parameters. Using synthetic time course data from both wild-type and knockout experiments, we were able to estimate an average of 75% of all parameters to within 5% precision.

Considering the success of our parameter estimation approach, an expansion of the model to the central metabolic network of *Escherichia coli* and the cyanobacterium *Cyanothece*, respectively, will be made and used in future studies to maximize hydrogen production for modified strains. Once the parameters for these gene regulatory networks are estimated, modifications to the networks can be attempted. These modifications will include a host of genetic engineering elements, such as dynamic toggle switches and riboregulators. Our reconstructed networks will allow us to quantitatively predict the effect of any modifications to the network topology, and thus to predict flux distribution throughout the central metabolic network with a high degree of accuracy.

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* Presenting author

23

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High-Throughput Screening Assay for Biological Hydrogen Production

Roger L. Ely^{1,2*} (ely@engr.orst.edu), Paul S. Schrader,^{2,3} and Elizabeth H. Burrows¹

¹Department of Biological and Ecological Engineering, Oregon State University, Corvallis, Oregon;

²Department of Chemical Engineering, Yale University, New Haven, Connecticut; and ³Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon

Project Goals (Abstracts 23-27): The objectives of the research are addressed in the following four tasks: **1. Evaluate the effects of various culture conditions (N, S, or P limitation; light/dark; pH; exogenous organic carbon) on H₂ production profiles of WT cells and an NDH-1 mutant; 2. Conduct metabolic flux analyses for enhanced H₂ production profiles using selected culture conditions and inhibitors of specific pathways in WT cells and an NDH-1 mutant; 3. Create PCC 6803 mutant strains with modified H₂ases exhibiting increased O₂ tolerance and greater H₂ production; and 4. Integrate enhanced H₂ase mutants and culture and metabolic factor studies to maximize 24-hour H₂ production.**

This poster describes a screening assay, compatible with high-throughput bioprospecting or molecular biology methods, for assessing biological hydrogen (H₂) production. While the assay is adaptable to various physical configurations, we describe its use in a 96-well, microtiter plate format with a lower plate containing H₂-producing cyanobacteria strains and controls, and an upper, membrane-bottom plate containing a color indicator and a catalyst. H₂ produced by cells in the lower plate diffuses through the membrane into the upper plate, causing a color change that can be quantified with a microplate reader. We used response surface methodology to optimize the concentrations of the components in the upper plate. The assay is reproducible, semi-quantitative, sensitive to 20 nmol of H₂ or less, and largely unaffected by oxygen, carbon dioxide, or volatile fatty acids at levels appropriate to biological systems.

24

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Hydrogen Production by PSS1, A *boxH* Mutant of *Synechocystis* sp. PCC 6803

Hatem Mohamed,^{1*} Paul S. Schrader,^{2,3} Elizabeth H. Burrows,¹ and Roger L. Ely^{1,2} (ely@engr.orst.edu)

¹Department of Biological and Ecological Engineering, Oregon State University, Corvallis, Oregon;

²Department of Chemical Engineering, Yale University, New Haven, Connecticut; and ³Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon

Project Goals: See goals for abstract 23.

In this research we created a *boxH* mutant of *Synechocystis* sp. PCC 6803, identified as PSS1, by drastically modifying a portion of the enzyme predicted to be adjacent to the active site. Sequence analyses of the genomes of *Ralstonia eutropha*, *Desulfovibrio gigas*, and *Synechocystis* sp. PCC 6803 show five conserved regions in the *boxH* gene, designated as L1 through L5. By deleting 300 base pairs of the gene, we removed all of region L5 and part of L4. This poster describes hydrogen (H₂) production performance of the mutant, with and without inhibitors of specific metabolic pathways, in high-throughput screening assay studies, GC vial tests, photobioreactor studies, and membrane inlet mass spectrometer measurements. In some ways, even with two of the five conserved regions eliminated, PSS1 behaves quite similarly to wild-type (WT) PCC 6803. However, some measurement methods show greater H₂ production from the mutant than from WT cells, while others show no H₂ production at all.

25

MEWG

Metabolic Flux Analysis of Metabolism in *Synechocystis* sp. PCC 6803 for Improving Hydrogen Production

Frank W.R. Chaplen,* Elizabeth H. Burrows, and Roger L. Ely (ely@engr.orst.edu)

Department of Biological and Ecological Engineering, Oregon State University, Corvallis, Oregon

Project Goals: See goals for abstract 23.

* Presenting author

Metabolic engineering of *Synechocystis* sp. PCC 6803 strains with the capability of consistent, high-yield, biosolar hydrogen (H_2) production requires continued development of comprehensive mathematical models describing the metabolism underlying H_2 production and linking genomic, proteomic, and metabolomic information. Such models help to organize disparate hierarchical information, discover new strategies, and understand the essential qualitative features of components and interactions in a complex system¹. Metabolic flux analysis is an analytical approach used to estimate the fluxes through a biochemical reaction network operating at steady state based on measured inflows and outflows. Analysis of both over- and underdetermined networks is possible, the latter with linear programming. Here we use metabolic flux analysis to examine the effect of different network parameters and constraints on photoautotrophic H_2 production by wild type (WT) *Synechocystis* sp. PCC 6803 and by a high H_2 -producing mutant (M55) with impaired Type I NADH-dehydrogenase (NDH-1) function. Two different networks are used with both WT and M55 mutant strains under chemostat growth: 1) an overdetermined network with 24 metabolites and 20 constraints, requiring at least 4 measurements of fermentation parameters for solution; and 2) an underdetermined network with increased detail for gene knockout simulations, requiring constraints-based approaches for solution. The inflows and outflows measured for both networks are H_2 , O_2 , CO_2 , glucose, glycogen, ammonium, and biomass production/consumption. The behavior of both model networks is consistent with WT and M55 mutant phenotypes, thus validating the general approach. The models are then used to provide insights into the possible effects of different mutant phenotypes on H_2 production.

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* Presenting author

26 Optimization of Media Nutrient Composition for Increased Photofermentative Hydrogen Production by *Synechocystis* sp. PCC 6803

GTL

Elizabeth H. Burrows,* Frank W.R. Chaplen, and Roger L. Ely (ely@engr.orst.edu)

Department of Biological and Ecological Engineering, Oregon State University, Corvallis, Oregon

Project Goals: See goals for abstract 23.

By optimizing concentrations of key components in nutrient media, we achieved over 60-fold greater photofermentative hydrogen (H_2) production by *Synechocystis* sp. PCC 6803 than was achieved by analogous, sulfur-deprived cultures, which produce more H_2 than cultures grown on complete media. We used response surface methodology to determine optimum conditions and found that, instead of completely starving cells of sulfur or nitrogen, the highest H_2 production occurred with low concentrations of S and N. H_2 profiling experiments provided initial screening of NH_4^+ , HCO_3^- , SO_4^{2-} , and PO_4^{3-} concentrations and identified the significant variables for H_2 production to be NH_4^+ , SO_4^{2-} , and the interactions of both NH_4^+ and SO_4^{2-} with HCO_3^- . A central composite design was implemented and subsequent response surface analysis of the data resulted in a saddle point. Ridge analysis was then conducted to identify high points within the region of interest, and those concentrations were tested and compared with sulfur-deprived cells. Our results indicate that optimized amounts of nitrogen and sulfur in the nutrient media are superior to total deprivation of these nutrients for H_2 production.

27 Performance of REHX, A *Synechocystis* sp. PCC 6803 Mutant with an Oxygen-Tolerant hoxH Subunit from *Ralstonia eutropha*

GTL

Hatem Mohamed,*¹ Paul S. Schrader,^{2,3} Elizabeth H. Burrows,¹ and Roger L. Ely^{1,2} (ely@engr.orst.edu)

¹Department of Biological and Ecological Engineering, Oregon State University, Corvallis, Oregon;

²Department of Chemical Engineering, Yale University,

New Haven, Connecticut; and ³Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon

Project Goals: See goals for abstract 23.

This poster describes hydrogen (H₂) production behavior of a *Synechocystis* sp. PCC 6803 mutant strain, designated as REHX. In REHX, we replaced the gene encoding the wild-type (WT) PCC 6803 *hoxH* subunit with the *hoxH* gene from the soluble hydrogenase of *Ralstonia eutropha*, which has been well characterized, is oxygen tolerant, and shares significant sequence homology with the PCC 6803 *hoxH*. We also inserted *hypX*, a gene that encodes an accessory protein essential for oxygen tolerance in *R. eutropha*. The *R. eutropha hoxH* and *hypX* genes were both transcribed into mRNA and hydrogen production was observed, implying that the foreign *hoxH* gene was translated and that the protein functioned with the unaltered subunits of the WT hydrogenase. H₂ production and oxygen tolerance of REHX were evaluated, with and without inhibitors of specific metabolic pathways, in high-throughput screening assay studies and in gas chromatograph and membrane inlet mass spectrometer measurements.

28

Understanding and Engineering Electron Flow to Nitrogenase to Improve Hydrogen Production by Photosynthetic *Rhodospseudomonas palustris*

James 'Jake' McKinlay* (mckinla1@u.washington.edu), Erin Heiniger, Jean Huang, and **Caroline S. Harwood** (csh5@u.washington.edu)

Department of Microbiology, University of Washington, Seattle, Washington

Project Goals: To develop and apply techniques in metabolic engineering to improve the biocatalytic potential of the bacterium *Rhodospseudomonas palustris* for nitrogenase-catalyzed hydrogen production. *R. palustris*, is an ideal platform to develop as a biocatalyst for hydrogen gas production because it is an extremely versatile microbe that produces copious amounts of hydrogen by drawing on abundant natural resources of sunlight and biomass. Anoxygenic photosynthetic bacteria, such as *R. palustris*, generate hydrogen and ammonia during a process known as biological nitrogen fixation. This reaction is catalyzed by the

enzyme nitrogenase and normally consumes nitrogen gas, ATP and electrons. The applied use of nitrogenase for hydrogen production is attractive because hydrogen is an obligatory product of this enzyme and is formed as the only product when nitrogen gas is not supplied. Our challenge is to understand the systems biology of *R. palustris* sufficiently well to be able to engineer cells to produce hydrogen continuously, as fast as possible and with as high a conversion efficiency as possible of light and electron donating substrates.

Rising energy demands and the imperative to reduce carbon dioxide emissions are stimulating research on the development of bio-based fuels. Hydrogen gas is one of the most promising biofuels, having about three times the energy content of gasoline. The purple bacterium *Rhodospseudomonas palustris* naturally uses energy from sunlight and electrons from organic waste to produce H₂ and ammonia using any of its three nitrogenases (1). We have also described *R. palustris* mutants that express nitrogenase at all times and use this enzyme to produce pure H₂ without accompanying ammonia production (2). Although we achieved higher specific H₂ productivities using these mutants, H₂ yield and rate can still be improved further. In order to effectively engineer *R. palustris* for improved H₂ production, we need to better understand the pathways and proteins that are used by cells to transfer electrons from electron donating substrates (e.g., acetate) to nitrogenase; the site of H₂ production.

To identify which metabolic reactions provide electrons for H₂ production we are conducting ¹³C-labelling experiments to compare carbon flux distributions between non-H₂-producing wild type and constitutively H₂-producing mutant strains. We are also using genetic and biochemical approaches to identify electron carriers that operate between central metabolism and nitrogenase. Mutational analysis has indicated that the FixABCX complex transports electrons to nitrogenase but also that it is not the sole electron carrier involved. We are therefore also investigating novel candidate electron carriers that were up-regulated in mutants with constitutive nitrogenase activity (2).

Once potential bottlenecks in electron transfer are identified, various genetic tools will be used to engineer *R. palustris* for improved H₂ production. Towards this end we have been working to develop a plasmid vector for controlled gene expression. Recently, members of our group identified a new signaling compound from *R. palustris* that acts with the transcriptional regulator, RpaR, to control expression of a gene named *rpaI* (Schaefer et al. submitted). We have developed an expres-

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sion vector based on this novel signaling system whereby varying the amount of signaling molecule provided as the inducer can control gene expression levels over a dynamic range. We anticipate that we will be able to combine new information about mechanisms of electron flow to nitrogenase with the use of our new gene expression system and other tools to improve rates of H₂ production by *R. palustris*.

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29 Modeling Electron Flow in *Rhodobacter sphaeroides* to Quantitatively Identify Approaches to Maximize Hydrogen Production

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L. Safak Yilmaz^{1*} (yilmaz@cae.wisc.edu), Yi-Kai Su,¹ Wayne S. Kontur,² Timothy J. Donohue² (tdonohue@bact.wisc.edu), and Daniel R. Noguera¹ (noguera@engr.wisc.edu)

¹Department of Civil and Environmental Engineering and ²Department of Bacteriology, University of Wisconsin, Madison, Wisconsin

Project Goals: To determine the impact of metabolic and regulatory networks on light-powered hydrogen production by *Rhodobacter sphaeroides*. Our experiments will determine the quantitative contribution of networks that are known or predicted to impact solar-powered hydrogen production. We will use genetic, genomic, and modeling approaches to quantify the role of previously uncharacterized networks, ultimately using this information to design microbial bioreactors to efficiently produce hydrogen from solar energy.

This is a new project aimed at quantifying electron flow within networks that impact solar-powered H₂ production by the photosynthetic bacterium *Rhodobacter sphaeroides*. We plan to use genetic, genomic, and modeling approaches to quantify the role of known and previously uncharacterized networks in solar-powered H₂ production by this organism. Ultimately, this information can

be used to design microbial bioreactors that efficiently generate H₂ or other alternative fuels from solar energy.

The initial work has centered on the development of a mathematical model to represent the electron flow from an organic substrate to the different and competing electron accepting reactions, during anaerobic photosynthetic growth. As a preliminary step, the model describes the quantitative apportionment of electrons derived from the oxidation of an organic substrate to pathways related to cell mass synthesis, polyhydroxyalkanoate (PHA) formation, hydrogen production, and accumulation of soluble organic products. Experimental measurements of substrate, biomass accumulation, nitrogen and phosphorus consumption, and pH are used to calculate best-fit estimates of the fraction of electrons consumed in each pathway.

When the model is fit to data from batch experiments with exponentially growing *R. sphaeroides* 2.4.1. and succinate as the sole organic substrate, it predicts that 47% of the electron flow is related to biomass formation, 41% to PHA accumulation, and 12% to soluble microbial products. In experiments with stationary phase cultures with nitrogen limitation, 26% of the electrons are predicted to be used in PHA synthesis, 26% in hydrogen production, and 48% in the formation of reduced soluble organic substrates. When propionate is used as the sole organic substrate, the predicted apportionment of electrons during exponential growth is similar to that seen with succinate, but under nitrogen limiting conditions, the model predicts lower PHA formation, higher hydrogen production, and a higher formation of soluble organic substrates.

These preliminary evaluations are being used to inform genetic strategies to optimize hydrogen production. Finally, as more complete experimental methods are developed, we expect to be able to experimentally validate the model predictions regarding PHA formation and hydrogen production, as well as to identify the type of soluble organic substrates produced by *R. sphaeroides*.

* Presenting author

30

Towards Experimental Verification of Protein Coding Transcripts of *Chlamydomonas reinhardtii* and Comprehensive Modeling of its Metabolic Network

Ryan Murray,¹ Chenwei Lin,¹ Arvind Chavali,² Erik F.Y. Hom,³ Xinping Yang,¹ David E. Hill,¹ Marc Vidal,¹ Jason Papin,² and **Kourosh Salehi-Ashtiani**^{1*} (Kourosh_Salehi-ashtiani@DFCI.harvard.edu)

¹Center for Cancer Systems Biology (CCSB), Department of Cancer Biology, Dana-Farber Cancer Institute, and Department of Genetics, Harvard Medical School, Boston, Massachusetts; ²Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia; and ³Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts

Project Goals: Our objectives for this project are to experimentally verify, define, and validate metabolic protein-coding genes of *Chlamydomonas reinhardtii* and model a comprehensive metabolic network for this organism. The proposed experiments utilize a technology platform that can be adapted to virtually any organism, and hence serve as a prototype that can be used for gene validation in any species. *Chlamydomonas reinhardtii* is an ideal organism for this project because 1) it is an important “bio-energy” organism, and 2) a draft of its genome sequence is currently available. The obtained results will be used to build a more complete model of the metabolic circuitry of this organism. The generation of a metabolic network will in turn help validate examined genes by defining a biological role for them. From our obtained results, we should be able to formulate testable hypotheses as to how to optimize bio-fuel (including hydrogen gas) production in this organism. To achieve these objectives, we will be carrying out experiments to define and verify transcript structures of metabolic genes in *Chlamydomonas reinhardtii* by RT-PCR and RACE, functionally validate the transcripts by yeast two-hybrid experiments, and build and interpret predictive metabolic network models based on the obtained results.

Chlamydomonas reinhardtii is a promising “bio-energy” organism capable of producing hydrogen gas and other “bio-fuel” resources. Although a draft of its genome is available, annotations of most of its protein coding genes have not been experimentally verified. Furthermore, a

comprehensive metabolic map has not been generated for this organism.

To experimentally verify, define, and validate metabolically related protein-coding genes of *C. reinhardtii*, and to produce a comprehensive metabolic map, focusing on hydrogen production and other bio-energy aspects, we plan to: *i*) experimentally verify and define the transcript structures of ~2,000 metabolically related genes and clone their open reading frames (ORF) for downstream protein-based validation studies; *ii*) identify protein-protein interactions among the metabolic gene products; and *iii*) build protein interaction maps and metabolic networks that will ultimately allow the development of testable predictions of *C. reinhardtii* physiology, including gene lethality and rates of growth under defined environmental conditions.

Here we present our computational and experimental results towards completing the aforementioned goals. We have computationally examined the current JGI *C. reinhardtii* annotated transcripts (frozen version 3.1 release) for the presence of full length ORFs. Our computational analyses indicate that of the ~14,500 annotated transcripts, ~9,500 appear to be “complete”, *i.e.* they each appear to contain a complete ORF, while the remaining ~5,000 appear to be “incomplete”. Starting from KEGG and KOG databases, we selected a list of ~470 transcripts for experimental verification by RT-PCR and RACE. Our RT-PCR experiments on a large subset of these showed that the majority of transcripts with a predicted full length ORF could indeed be amplified by PCR. Cloning and sequencing of these transcripts are in progress to further verify these transcript models.

Using currently available resources, we have generated a draft for the intracellular central metabolic network of *Chlamydomonas reinhardtii*. Our *in silico* model accounts for key pathways involved in carbohydrate and energy metabolism, including glycolysis, TCA cycle, pentose phosphate pathway and oxidative phosphorylation. The current network accounts for 224 *gene products* (214 nuclearly- and 7 mitochondrially-encoded), 96 reactions, and 140 metabolites, partitioned into 6 sub-compartments: chloroplast, mitochondria, flagella, cytosol, extracellular space, and glyoxysome. Approximately 82% of the network reactions were based on gene predictions/ annotations, while the remainder were inferred to ensure model completeness, mainly to account for metabolite exchange between compartments. Our metabolic model suggests a number of adjustments are needed in the current *C. reinhardtii* gene annotations.

* Presenting author

Our iterative approach of integrating experimental data with model-building enables us to refine genome annotations and metabolic network maps progressively. We are currently in the process of completing the first of such cycles. Concurrent with carrying on these iterations, we will develop testable hypotheses to accelerate future biological discoveries relevant to our goals and those of the *C. reinhardtii* scientific community at large.

31 Addressing Unknown Constants and Metabolic Network Behaviors through Petascale Computing: Understanding H₂ Production in Green Algae

GTL

Christopher H. Chang,^{1*} David Alber,¹ Peter Graf,¹ Kwiseon Kim,¹ Arthur R. Grossman,² Glenn Murray,³ Matthew C. Posewitz,³ and Michael Seibert¹ (mike_seibert@nrel.gov)

¹National Renewable Energy Laboratory, Golden, Colorado; ²Carnegie Institution of Washington, Stanford, California; and ³Colorado School of Mines, Golden, Colorado

Project Goals: Development of photobiological H₂-production processes, a key component in pursuit of DOE's renewable energy mission, would be substantially accelerated by increasing our understanding of the extremely complex underlying biology. To address biological complexity at the molecular level, systems biology has evolved rapidly in recent years. However, the lack of comprehensive experimental data for a given organism prevents reliable predictive modeling. We will therefore employ petascale computing to address this issue by computational parameter estimation to delimit the space of stable solutions for experimentally constrained metabolic models. The response will be characterized at the level of enzyme kinetic differential equation parameters. Through development of scalable software tools, iterative model building, and incorporation of experimental constraints generated by high-throughput "omics" technologies, a model of metabolism linked to H₂ production in the green alga, *Chlamydomonas reinhardtii*, will be constructed. Once a set of acceptable kinetic parameters have been computed, the model will then be used for high performance optimization of H₂ output in the space of enzyme expression levels, subject to limitations on cell viability. Integration into the popular Systems Biology Workbench will make our tools accessible to the

general user community. The work is envisioned as an important contribution toward long-term development of a complete in silico cell.

The Genomics revolution has resulted in a massive and growing quantity of whole-genome DNA sequences, which encode the metabolic catalysts and ribonucleic acids necessary for life. However, gene annotations can rarely be complete, and measurement of the kinetic constants associated with the encoded enzymes can not possibly keep pace, necessitating the use of careful modeling to explore plausible network behaviors. Key challenges are (a) the quantitative formulation of the kinetic laws governing each transformation in a fixed model network; (b) characterizing the stable solutions of the associated ordinary differential equations; (c) fitting the latter to metabolomics data as it becomes available; and (d) optimizing a model output against the possible space of kinetic parameters, with respect to properties such as robustness of network response or maximum consumption/production. This project addresses this large-scale uncertainty in the genome-scale metabolic network of the water-splitting, H₂-producing green alga, *Chlamydomonas reinhardtii* [1,2]. Each metabolic transformation is formulated as a steady-state process in such manner that the vast literature on known enzyme mechanisms may be incorporated directly. We have encoded glycolysis, the tricarboxylic acid cycle, and basic fermentation pathways in Systems Biology Markup Language (SBML) with careful annotation and consistency with the KEGG database, yielding a preliminary model with 4 compartments, 85 species, 35 reactions, and 89 kinetic constants.

The SemanticSBML toolkit is first used to combine, validate, and annotate hand-coded SBML models corresponding to metabolic modules. In this manner, a genome-scale kinetic model may be constructed from a library of simple component pathways, making modification and maintenance plausible. The use of a standard, XML-based language and preservation of semantics in the naming convention for variables and parameters means that customization of the library to any organism may be accomplished easily. From an instance of a model, use of libSBML and SOSLib allows automatic production of a C program that when executed, optimizes the model's kinetic parameters according arbitrary test criteria. The generation of this optimizer from the model consists of several steps. First, the unified SBML model is parsed and converted to a system of ordinary differential equations (ODEs), including Jacobian and sensitivity matrices. These are then translated to C functions and embedded in code utilizing the ODE solver package CVODES, resulting in a library that can efficiently simulate the model, including calculating derivatives with

* Presenting author

respect to parameters (in our case enzyme kinetic constants). This library is in turn incorporated into code that calculates the objective functions implied by challenges (2) – (4) above, as well as the objective function derivatives. Finally these routines are built into code using the optimization package TAO to optimize the model with respect to the kinetic parameters. We illustrate the system and present numerical results.

Future software development will include a parallel global optimization algorithm, enhanced integration with the Systems Biology Workbench, and development of distributed data analytics and visualization tools, a graphical interface, and interactive visualization for high-dimensional datasets. The free availability of these tools will allow the broader biological community to sample and/or optimize genome-scale metabolic networks in the space of thousands of parameters, and permit study of, for example, implications of enzymatic co-evolution and system-wide metabolic engineering. The software design is specifically targeting the combined goals of no-cost availability, open standards, and wide accessibility to researchers from all sectors. The size of the addressable problems will steadily increase with the availability of parallel computing platforms ranging over now-common desktop multi-core/multi-chip workstations, massive distributed grids, workgroup clusters, and cutting-edge petascale architectures.

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32 Flexibility of Algal Anaerobic Metabolism is Revealed in a Mutant of *Chlamydomonas reinhardtii* Lacking Hydrogenase Activity

Alexandra Dubini,^{1,3} Florence Mus,² Maria L. Ghirardi,¹ Arthur R. Grossman,² Matthew C. Posewitz,³ and **Michael Seibert**^{1*} (mike_seibert@nrel.gov)

¹National Renewable Energy Laboratory, Golden, Colorado; ²Carnegie Institution of Washington, Stanford, California; and ³Colorado School of Mines, Golden, Colorado

Project Goals: Past research has shown that photosynthesis, respiration, and fermentation are all required to sustain H₂ photoproduction from water in algae. These microbes utilize [FeFe]-hydrogenases, which are the most efficient H₂-generating biocatalysts known. The long-term objective of our project is to identify the suite of genes facilitating and/or limiting H₂ photoproduction in the alga, *Chlamydomonas reinhardtii*, by conducting global gene expression and cell metabolism studies using algal cells acclimated to conditions known to induce H₂-production activity. A detailed understanding of the influences of metabolism and other environmental factors on the coordinated expression of genes and biochemical pathways associated with H₂-production activity will ultimately be required to increase the yields of renewable H₂ production for potential future applications. To accomplish this we will examine WT cells and a number of NRELS H₂-production mutants under a number of experimental conditions using *Chlamydomonas* gene microarrays along with extensive biochemical assays. Algal H₂ production requires the synergies of multiple redox proteins, sensors, biochemical pathways and regulatory processes. Knowledge gained by deconvoluting these interactions will help us identify specific targets for future strain engineering aimed at enhancing H₂ production in *C. reinhardtii*.

The green alga, *Chlamydomonas reinhardtii*, has an extensive network of fermentation pathways that are activated when the cells acclimate to anoxia, and hydrogenase activity is an important component of this metabolism. *Chlamydomonas* uses fermentative pathways for ATP production during anoxia in the dark, catabolising starch into the predominant fermentation products formate, acetate, ethanol, CO₂, and H₂ in what is classified as heterofermentation. Previous microarray studies as well as RT-PCR analysis identified increases in specific gene

* Presenting author

transcripts under these conditions [1]. Indeed, anoxia leads to the up-regulation of genes encoding proteins involved in fermentation and more specifically those associated with pyruvate catabolism, such as pyruvate formate lyase (PFL1) and pyruvate:ferredoxin oxidoreductase (PRF1). Moreover, increased levels of transcripts encoding several regulatory elements suggest that activation of specific signalling pathways and the need to control translational and post-translational processes occur in the cells as the environment becomes anoxic.

In this study we have compared metabolic and regulatory acclimation responses that accompany anaerobiosis in wild-type cells and in a mutant defective for H₂ production as a consequence of a null mutation in the [FeFe]-hydrogenase maturation protein, HYDEF [2]. The mutant exhibits both elevated accumulation of succinate and diminished production of CO₂, relative to the parental strain, after four hours of dark, anaerobic acclimation. These results are consistent with increased activity of enzymes required for anoxic succinate production, and a decreased metabolic flux through the PRF1 pathway. In the absence of hydrogenase activity, an increase in succinate suggests the need to activate alternative pathways to metabolize pyruvate and re-oxidize NAD(P)H, which allows continued glycolysis and fermentation in the absence of O₂. Activities required for succinate production include pyruvate carboxylation and/or oxaloacetate reduction, which generate malate. Malate can then be further metabolized to fumarate and finally to succinate. Enzymes that can potentially catalyze the carboxylation of pyruvate to malate *via* independent pathways are the pyruvate carboxylase and malic enzymes. Marked increases in the abundance of mRNAs encoding both of these enzymes are observed in the mutant relative to the parental strain. *Chlamydomonas* has a single gene encoding pyruvate carboxylase and six genes encoding putative malic enzymes. Only one of the malic enzyme genes, *MME4*, shows a dramatic increase in expression (mRNA abundance) in the *hydEF-1* mutant during anaerobiosis. Furthermore, there are also large increases in transcripts encoding fumarase and fumarate reductase, the enzymes required for the conversion of malate to succinate. To further identify potential metabolic and regulatory features of the *hydEF-1* mutant relative to the parental strain, we used a high density, oligonucleotide (70-mer)-based microarray to compare genome-wide transcript patterns of *hydEF-1* and parental strains after transferring cultures from aerobic to anaerobic conditions. Several transcripts encoding proteins associated with cellular redox functions and other aspects of anaerobic metabolism were observed to be differentially regulated in the mutant under anaerobic conditions.

* Presenting author

In summary, these experiments illustrate the marked metabolic flexibility of *Chlamydomonas* and also provide insights into how mutants, altered in normal H₂ metabolism, acclimate to H₂-producing conditions. Moreover the information obtained will provide the foundation for metabolic engineering and accurate *in silico* modelling of *Chlamydomonas* metabolism. The availability of the *Chlamydomonas* genome sequence, combined with high-throughput-'omics'-based approaches is critical in this effort. Moreover, the use of specific mutant strains can help establish the foundation for a more comprehensive understanding of metabolic networks, how cells adjust metabolite fluxes when specific metabolic reactions are blocked, and how to improve H₂-production yields.

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33 GTL Development of Biologically Based Assays to Study Rate-Limiting Factors in Algal Hydrogen Photoproduction

Alexandra Dubini, Matt Wecker, and **Maria L. Ghirardi*** (maria_ghirardi@nrel.gov)

National Renewable Energy Laboratory, Golden, Colorado

Photobiological H₂ production from water is a clean, non-polluting and renewable technology. Although the potential light conversion efficiency to H₂ by biological organisms is theoretically high (about 10%), the system is currently limited by biochemical and engineering constraints. These limitations include (but are not restricted to) the extreme O₂ sensitivity of the biological hydrogenases and the low availability of reductants to the hydrogenase due to the existence of competing metabolic pathways. Our research addresses the O₂ sensitivity issue by developing a new, biologically-based assay to screen large microbial populations for improved H₂-production properties. This novel assay is based on the H₂-sensing properties of systems found in nitrogenase-containing

photosynthetic bacteria. We will validate the new assay by using it to screen mutants generated through directed-evolution techniques for O₂ tolerant [FeFe]-hydrogenases. To address the issue of competitive metabolic pathways with H₂ production, we will adapt the yeast two-hybrid assay to measure the interactions between different ferredoxin isoforms present in *Chlamydomonas reinhardtii* with different proteins known to accept electrons from ferredoxin in most photosynthetic and fermentative organisms. An expanded approach will include the use of ferredoxin probes to identify unknown target genes out of a *C. reinhardtii* expression library. Identified protein-protein interactions above will be quantified by isothermal titration calorimetry. The information obtained will guide future protein and metabolic engineering efforts to divert most of the electron flux from ferredoxin to the hydrogenase. This work will develop techniques that will drive a deeper understanding of algal H₂ metabolism and accelerate the development of future photobiological H₂-production catalysts and organisms.

34 Quantitative Tools for Dissection of Hydrogen-Producing Metabolic Networks

Joshua Rabinowitz* (josh@genomics.princeton.edu), G. Charles Dismukes, and Herschel Rabitz

Department of Chemistry and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, New Jersey

Project Goals: With an eye towards eventually enabling rational optimization of microbial hydrogen production, here we aim to develop novel tools for quantitative dissection of hydrogen-producing metabolic networks. These tools will bring innovations both in high-throughput experimental metabolomics and in algorithms for predictive modeling/analysis of experiments. We will first develop quantitative experimental tools for simultaneous measurements of multiple metabolite concentrations and fluxes. These will include liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays and complementary electrochemical, fluorescence, or NMR assays. We will then apply these tools to map the dynamic metabolic responses of *Clostridium acetobutylicum* and *Synechococcus* species to changing environmental conditions. Finally, we will use the data to guide the development of predictive models of hydrogen metabolism. The

development of predictive metabolic models will pave the way to computationally-guided optimization of microbial hydrogen yields.

Overview: With an eye towards eventually enabling rational optimization of microbial H₂ production, we have recently initiated a project that aims to develop novel tools for quantitative dissection of H₂-producing metabolic networks. We hope that these tools will bring innovations both in high-throughput experimental metabolomics and in algorithms for predictive modeling/analysis of experiments. The project has three major objectives:

Aim 1: Develop quantitative experimental tools for simultaneous measurements of multiple metabolite concentrations and fluxes. We aim to create and apply a comprehensive set of experimental tools for measuring multiple (200+) intermediate metabolites and gases (including H₂) implicated in energy metabolism. Metabolomic data will be generated by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We have developed methods that enable quantitation of several hundred metabolites of general interest by LC-MS/MS, and are working to expand to include also compounds of specific importance to hydrogen metabolism. For compounds of particular importance that cannot be reliably measured by LC-MS/MS, complementary electrochemical, fluorescence, or NMR assays will be employed. A particular interest of our group is development also of highly sensitive devices for electrochemical monitoring of dissolved O₂ or H₂ that enable simultaneous measurement intracellular levels of NAD(P)H. Exemplary LC-MS/MS data will be presented, as will be data regarding monitoring of dissolved H₂ and intracellular NAD(P)H.

Aim 2: Map the organisms' dynamic metabolic responses to changing environmental conditions. Metabolic network activity, including H₂ production, is highly sensitive to the availability of a broad range of nutrients and environmental stresses. To elucidate the interactions of these inputs, we plan to modulate the cellular environment and apply the measurement techniques developed in Aim 1 to acquire high quality dynamic data describing the full network response. Currently, we are working on optimizing cell handling for *Clostridium* and *Synechococcus* species, the H₂-producing organisms of greatest interest to us. Given the preliminary status of our work on these organisms, we tentatively plan to present here exemplary data from *E. coli* to highlight the potential of the approach.

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* Presenting author

Aim 3: Develop predictive models of H₂-metabolism.

The emerging ability to generate high quality, dynamic metabolomic data raises a new challenge: how to apply these data to (a) better understand metabolic regulation, and (b) develop predictive, quantitative metabolic models. We plan to achieve these objectives via integrated computational-experimental approaches, which use experimental data to drive computational modeling, while also using computational results to guide experimental design. An example of such an effort related to nitrogen metabolism in *E. coli*, although not directly relevant to hydrogen production, will be presented to highlight the concepts involved. We are also eager to discuss our computational plans in the hydrogen arena with groups conducting related research.

Organisms: The foci of the present newly initiated project are *Clostridium acetobutylicum* (possessing the fastest and highest yielding hexose fermentation pathway to H₂ of any microbe yet reported), and a new class of thermophilic cyanobacteria that lack hydrogenase genes and produce H₂ at 62°C via a nitrogenase-dependent pathway (*Synechococcus* species). We are eager to build contacts with experts in these organisms.

35 Quantitative Tools for Characterization of H₂-Producing Cyanobacteria

Gennady Ananyev, Nicholas Skizim, and **Charles Dismukes*** (dismukes@Princeton.edu)

Department of Chemistry and Environmental Institute, Princeton University, Princeton, New Jersey

This subproject of DE-FG02-07ER64488 focuses on quantitative tools for biohydrogen detection and applications to N₂-fixing cyanobacteria. We have built a cell for simultaneous real-time detection of extracellular dissolved H₂ and intracellular reduced pyridine nucleotide levels. Ultrasensitive H₂ detection (>1 nanomolar) is accomplished with a homebuilt Clark-type electrochemical cell using live cells (3 - 5 μl volume at 1 - 8 μg dry wt. equiv.). NADPH+NADH concentrations are assayed using wavelength selective UV light-emitting diode (360 nm) excitation and fluorescence emission (450 +/- 20 nm) detection. These tools have revealed for the first time two major temporal phases of H₂ production in anaerobically poised cyanobacteria arising from reductant pools that equilibrate with [NiFe]-hydrogenase. The first phase correlates with the availability of residual NADPH produced in prior photosynthetic stage, while phase 2 arises from NADH produced by anaerobic fermentation of intracellular glycogen. Experiments with N₂-fixing cyanobacteria reveal additional kinetic phases of H₂ production presumed due to interactions with N₂-fixation pathways and which correlate with the cells' circadian rhythms. We shall describe how these tools have been used to identify more O₂-tolerant H₂-producing cyanobacteria isolated during bioprospecting studies.

* Presenting author