BioHydrogen (BioH2) Consortium to Advance Fermentative Hydrogen Production

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Subcontractor(s):
- Lawrence Berkeley National Laboratory
- Pacific Northwest National Laboratory
- Argonne National Laboratory

Project Start Date: October 1, 2018
Project End Date: September 30, 2021

Overall Objectives

- Improve the rates and molar yields of hydrogen production (mol H_2/mol sugar) via metabolic engineering of the cellulose degrader *Clostridium thermocellum* (National Renewable Energy Laboratory [NREL])
- Optimize a bioreactor for the fermentation of lignocellulosic biomass at high-solid loadings to reduce reactor cost (Lawrence Berkeley National Laboratory [LBNL])
- Develop an integrated microbial electrolysis cell (MEC) system to improve hydrogen molar yield and reduce fermentation waste product (Pacific Northwest National Laboratory [PNNL])
- Conduct technoeconomic analysis (TEA) and life cycle analysis (LCA) with data generated by the above three labs to identify major cost drivers and guide integration efforts (Argonne National Laboratory [ANL]).

Fiscal Year (FY) 2019 Objectives

- Use a cellulose-degrading microbe to ferment biomass (without adding enzyme cocktail) to lower feedstock cost
- Generate an engineered biomass strain, combining the best metabolic features to improve hydrogen production rate, molar yield, and biomass utilization, the latter lowering biomass feedstock cost
- Develop bioreactor processes for high-solid loadings that will lower capital expenses
- Integrate fermentation with MEC to increase overall hydrogen molar yield
- Conduct TEA, LCA, and strategies for system integration
- Collectively advance fermentative hydrogen and increase its techno-economic feasibility for scale-up hydrogen production, leveraging the unique capabilities delivered by the four National Lab teams.

Technical Barriers

This project addresses technical barriers from the Molecular and Systems Engineering for Dark Fermentative Hydrogen Production and Molecular and Systems Engineering for MEC sections of the Fuel Cell Technology Office Multi-Year Research, Development and Demonstration Plan[1]:

- (AX) H_2 Molar Yield
- (AY) Feedstock Cost
- (AZ) System Engineering.

Technical Targets

The DOE technical targets and our current project

FY 2019 Accomplishments

- We obtained an average hydrogen production rate of 2.75 L/L-d in *Clostridium thermocellum* fermenting pretreated biomass in a batch bioreactor, a 24% improvement over

[1] https://www.energy.gov/eere/fuelcells/downloads/fuel-cell-technologies-office-multi-year-research-development-and-22

the non-engineered strain, suggesting the simultaneous utilization of hemicellulose in the biomass

- We uncovered 113 genes as highly differentially expressed genes when *C. thermocellum* was engineered to metabolize xylose. These affected genes will be subjected to more detailed analysis to correlate the changes in expression with enhanced xylose utilization
- Cultivation of *C. thermocellum* at 30 g/L was successful, with a maximum of 11 mmol/L-hr (~6.5 L H_2/L/d) H_2 production recorded to determine limiting factors in high solids cultivation
- Five exoelectrogenic microbes were identified based on their ability to donate electrons from waste organic acids (acetate, or lactate, or simulated effluent) to an insoluble electron acceptor (a proxy for electrode) in support of cell growth. These strains will be tested in the future for the current generation and hydrogen production
- We have modeled the following blocks in Aspen Plus Model: feedstock handling, feedstock pretreatment, fermentation, *C. thermocellum* bacteria production, pressure swing absorption (PSA), and MEC. The modeled blocks will be updated upon experimental updates and further communication with collaborators from other national labs for the TEA and LCA efforts.

Table 1. Progress toward Meeting DOE Technical Targets in Dark Fermentation

Characteristics	Units	Current Status	2015 Target	2020 Target
Yield of hydrogen from glucose	Mole H_2/mole glucose	2–3.2	6*	--
Feedstock cost	Cents/lb glucose	13.5	10	8
Duration of continuous production (fermentation)	Time	17 days	3 months	--
MEC cost of electrodes	$/m^2	$2,400	$300	$50
MEC production rate	L-H_2/L-reactor-d	2.8	1	---

*Yield of hydrogen from glucose: DOE has a 2015 target of a hydrogen molar yield of six (four from fermentation and two from MEC) from each mole of glucose as the feedstock, derived from cellulose.

Feedstock cost: The DOE Bioenergy Technologies Office is conducting research to meet its 2015 target of 10 cents/lb biomass-derived glucose. NREL's approach is to use cellulolytic microbes to ferment cellulose and hemicellulose directly, which will result in lower feedstock costs.

INTRODUCTION

Lignocellulosic biomass is an attractive resource for hydrogen production via dark fermentation due to its abundance and high sugar content (~70%). Yet an FCTO cost analysis reveals that biomass feedstock is a major cost barrier for hydrogen production. Lowering the cost of biomass feedstock is, therefore, one of the project objectives. Biomass is comprised of cellulose (~40%; six-carbon C6-glucose polymer), hemicellulose (~30%; five-carbon C5-xylose polymer), lignin (~26%), ash, and extractives. A promising approach to lower feedstock cost is to use microbes that can degrade cellulose and hemicellulose directly, in a consolidated bioprocessing (CBP) configuration without adding the expensive cellulase and hemicellulase enzymes cocktail. One such model CBP microbe is *Clostridium thermocellum*, which is reported to have one of the highest rates in cellulose hydrolysis [1]. Genetic engineering is a valid strategy to enable cellulose and hemicellulose co-utilization to increase hydrogen output in a CBP microbe. To further lower fermentation cost, researchers must develop bioreactors that ultimately can ferment high biomass solids at 30% (w/v) loading to lower reactor cost, as identified in a cost analysis conducted by Strategic Analysis, Inc.

Despite relatively high conversion rates, the theoretical maximum of hydrogen recovery via dark fermentation cannot exceed 33% of the total stored energy, i.e., the maximum hydrogen molar yield is 4 mol H_2/mol sugar [2]. Currently, most laboratories reported a hydrogen molar yield between one and two [3, 4]. The rest of the energy is released *via* low molecular weight alcohols and organic acids. To that end, significant advancements towards improving hydrogen molar yield could be achieved through genetic engineering to redirect metabolic

pathways toward maximal hydrogen production [5] and integration of MEC to convert fermentation end-products to additional hydrogen to further improve hydrogen molar yield [6, 7].

FCTO guides the R&D of hydrogen production through TEA and environmental LCA. The TEA evaluates the cost of hydrogen production of a specific technology using the current state-of-the-art materials and systems and then identifies major cost drivers (e.g., feedstock/energy cost, equipment cost, product yield, etc.) for that technology. This TEA helps inform FCTO of R&D needs and potential pathways to achieve hydrogen production cost targets in the near- and long-term. The LCA evaluates energy and environmental metrics of the production technology such as fossil and petroleum energy use, greenhouse gas and criteria air pollutant emissions, and water consumption, which can be compared to the incumbent technology (e.g., steam methane reforming of natural gas) to evaluate the magnitude and scale of the benefits provided by emerging hydrogen production technologies. Thus, TEA and LCA are crucial to the evaluation of the economic and environmental sustainability of emerging technologies before additional resources are committed to future development and demonstration of these technologies.

APPROACH

The Consortium's approach to addressing high feedstock cost is to endow the cellulose-degrading bacterium *C. thermocellum* the ability to metabolize C5 sugars (both monomeric and polymeric) aimed to utilize most of the sugars in biomass [8]. The simultaneous utilization also reduces the number of bioreactors leading to lower capital costs. One approach to improve hydrogen molar yield is to selectively block competing metabolic pathways in this organism via genetic methods to maximize hydrogen yield per mol of sugar consumed [9]. Improved strains will be tested in high-solid bioreactors loaded with pretreated biomass, up to 17.5% (w/v) loading (ultimate goal is 30%), in order to lower capital cost. To achieve this goal, we are optimizing the various parameters in batch and sequencing fed-batch reactors to improve longevity, yield, rate of hydrogen production, and better mixing via improved impeller design, using corn stover biomass pretreated via a de-acetylation and mechanically refined (DMR) process. We will also integrate fermentation with MEC to generate additional hydrogen via electro-fermentation to improve hydrogen molar yield. Several technical challenges associated with MEC will be addressed, including electron-transfer efficiencies at both the anode and cathode, replacing cathode with non-platinum materials, increasing ionic current through the electrolyte, improving anodic microbial consortium to increase current generation, etc. Experimental data will be input into a TEA and LCA to guide research directions.

RESULTS

Strain Development and Improvement

We previously have generated *C. thermocellum* mutant lines capable of co-metabolizing monomeric xylose (C5; hemicellulosic sugar) with cellobiose (cellulose-derived glucose dimer) with no cross-inhibition [8]. Yet xylose metabolism still displays a lag phase, and the engineered strain cannot utilize complex xylose, including xylo-oligomer and xylan. Via adaptive evolution (repeated sub-culture and transfer in xylose), we have evolved a variant that displays s 3.5-fold improvement in the rate of xylose metabolism with minimal lag phase. When the evolved strain was fed DMR biomass (38 g/L as cellulose and 21.2 g/L as xylan) in bioreactors, an average hydrogen production rate of 2.75 L H_2/L/d was obtained which is 24% higher than that of the control strain (2.2 L H_2/L/d using DSM 1313 Δ*hpt* strain). The promising outcomes suggest that although the strain was originally evolved in xylose, it has fortuitously gained the ability also to utilize the more complex hemicellulose, as evidenced with improved hydrogen production from DMR biomass.

Transcriptomic Analysis of Xylose-Utilizing Strain

Improved hydrogen production requires an in-depth knowledge of *C. thermocellum*'s physiology at utilizing C5 substrates. Since *C. thermocellum* does not naturally metabolize xylose, the introduced xylose-catabolizing pathway must result in cellular changes that help the bacteria adapt to the sensing, uptake/transport, and utilization of a substrate not naturally metabolized by the bacteria. Therefore, we set forth to compare system-wide changes in gene expression using transcriptomic analyses via RNA-sequencing (RNA-seq) of our rationally engineered, xylose-catabolizing strain (KJC335) grown in xylose (5 g/L) versus the strain cultured in

cellobiose (5 g/L) as the main carbon source. By identifying the significantly differentially expressed genes (DEGs) between the two culturing conditions, we will understand how the strain adapts to xylose and how the adaptation contributes to xylose utilization. The ultimate goals from transcriptomic analyses are to uncover and enhance mechanisms which help bacteria utilize xylose and xylan efficiently and potentially eliminate mechanisms which prevent the bacteria from efficiently co-metabolizing C5 and C6 sugar substrates with the aim of increasing hydrogen production.

The degree of differentiation between the two conditions was represented using the absolute value of $\log_2$ (fold change [FC]). Based on our data, 372 DEGs with $\log_2$ (FC) >|1| and p <0.05 were identified when comparing strain KJC335 grown in xylose to the same strain grown in cellobiose (Figure 1). When using a cut-off of $\log_2$ (FC) >|2|, 113 genes out of 372 genes were categorized as highly differentially expressed genes with 40 significantly down-regulated and 73 up-regulated genes. These affected genes will be subjected to more detailed analysis to correlate the changes in expression with enhanced xylose utilization. Their targeted manipulations will afford better biomass utilization for enhanced total hydrogen production.

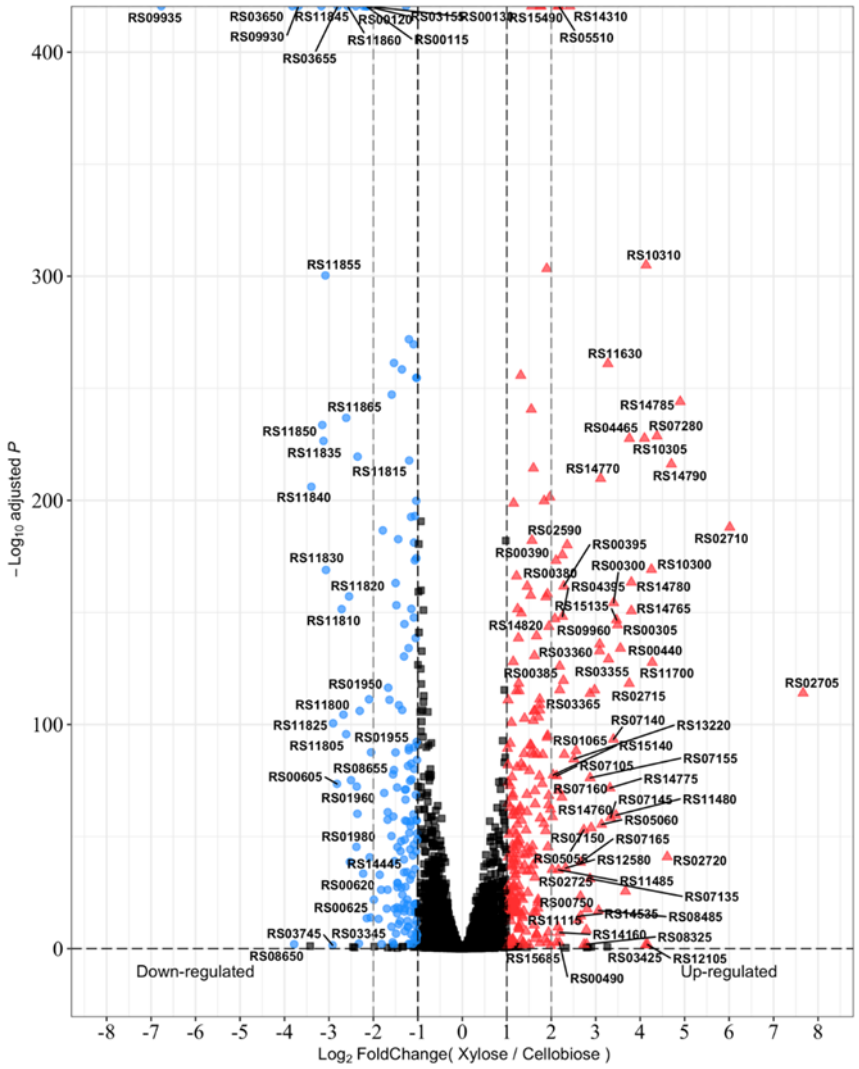

Figure 1. A volcano plot representing differentially expressed genes in the engineered C. thermocellum strain (KJC335) capable of growing on xylose as the sole carbon source. DEGs were obtained from comparisons made between cultures grown in D-xylose vs. D-cellobiose. Down-regulated genes are shown in blue and up-regulated genes are shown in red. Numbers in bold correspond to the numerical suffix of gene locus tags in C. thermocellum DSM 1313 (e.g., RS10310 = CLO1313_RS10310

High-solid Bioreactor Development.

Following receipt of cultures and protocols from NREL, LBNL completed technology transfer of serum bottle cultivation, culture storage techniques, and setup of the bioreactor system, including online gas analysis and required safety approvals. The completed setup was then tested for biohydrogen production with *C. thermocellum* using 1.5% (15 g/L) Avicel as the fermentation feedstock. This 1L-scale fermentation campaign was successful, achieving >15 mmol/L-hr hydrogen production at peak productivity (data not shown). This is followed by culturing *C. thermocellum* in 30 g/L Avicel to determine limiting factors in high solids cultivation. Cultivation at 30 g/L was successful, with a maximum of 11 mmol/L-hr hydrogen production recorded (6.5 L H_2/L/d) (Figure 2). Replicate cultivations were performed at 30 g/L, indicating that the process was reproducible. Metabolite profiles vary significantly between the LBNL campaigns and experiments conducted at NREL under similar conditions, indicating that metabolite distribution can be influenced by site-specific or process-specific factors. The reduction in gas production at higher solids loading indicates potential process bottlenecks, including rheology, osmotic stress, or metabolite formation–these process bottlenecks will be interrogated in future quarters. We have also commissioned a new Thermo Prima benchtop off-gas mass spectrometer, which will enable improved gas production resolution. In addition, the new system will enable the use of the full fermentation suite at the pilot plant in LBNL, improving throughput and enabling additional customization of reactor geometries, reactor scales, and impeller geometries in upcoming campaigns.

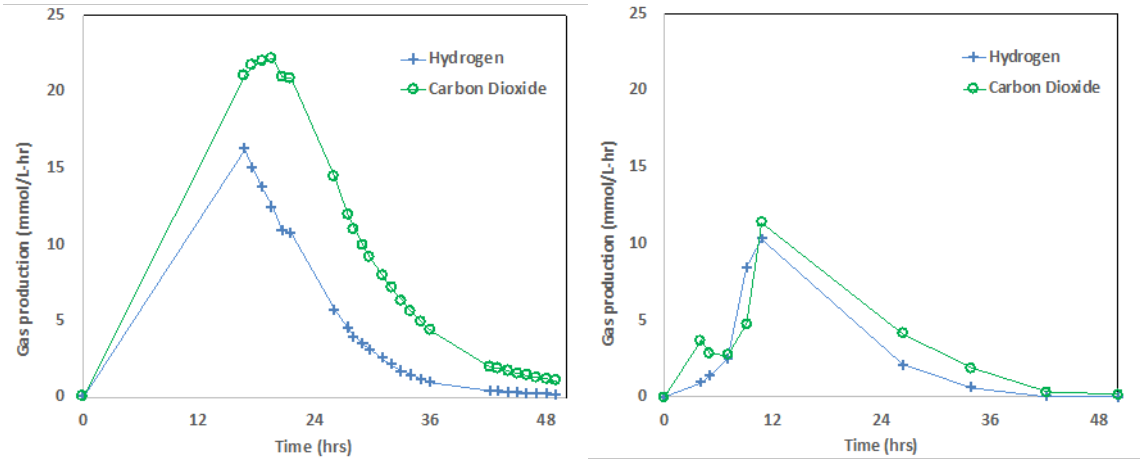

Figure 2. Gas production (left) and organic acid production (right) over 48 hours of C. thermocellum biohydrogen fermentation with 15% Avicel feedstock.

Microbial Electrolysis.

Improvements in MEC-driven hydrogen production depend on the efficient recovery of reductant from the byproducts of the lignocellulosic fermentation. To achieve this, we are employing exoelectrogenic microorganisms (grown either as pure or mixed cultures) that are capable of simultaneously oxidizing the main components in the fermentation effluent and transferring electrons to an anode for electricity generation. The PNNL group characterized the extracellular electron transfer rates of 10 exoelectrogenic microorganisms to evaluate their ability to utilize main components of a synthetic fermentation effluent (containing acetate, ethanol, lactate, format, and tryptone) for the reduction of hydrous ferric oxide (HFO), an insoluble electron acceptor that requires extracellular electron transfer to support growth of exoelectrogenic bacteria under anaerobic conditions. This insoluble electron sink was chosen as a proxy to assess current production capacity. The outcomes of the extracellular electron transfer activity measurements are provided in Figure 3. As a result, we were able to down-select five promising strains, specifically: *Shewanella* sp. W3-18-1, *S. loihica* PV-4, *Geobacter metallireducens* GS-15, *G. anodireducens* SD-1 and *Anaeromyxobacter dehalogenans* 2CP-C. Notably, the rates of HFO reduction by *Geobacter* and *Anaeromyxobater* spp. was three- to five-fold higher than these of *Shewanella*. The fastest rate of reduction using acetate as the electron donor (which is the main component of the fermentation effluent) was demonstrated by *G. metallireducens* GS-15, while *A. dehalogenans* 2CP-C had the highest overall activity on the simulated effluent indicating its ability to utilize

different electron donors simultaneously. These strains will be further investigated for current production and hydrogen evolution on simulated and actual fermentation effluent using a high-throughput bioelectrochemical system developed and constructed at PNNL.

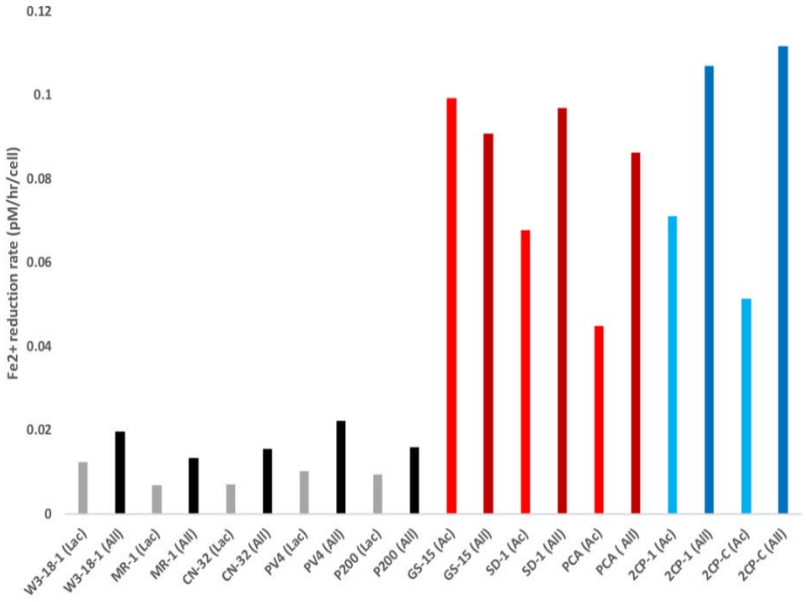

Figure 3. Rates of HFO reduction by 10 exoelectrogenic strains using lactate (lac), acetate (ac), or simulated effluent (all) as electron donor.

System Integration, Techno-economic Analysis, and Life Cycle Analysis.

We completed the preliminary direct fermentation (DF)-MEC integrated system design, divided by the following blocks: feedstock handling, feedstock pretreatment (via DMR), fermentation, MEC, microbe bacteria production (for *C. thermocellum* and for MEC bacteria of *Geobacter/ Shewanella,* respectively), wastewater treatment plant, storage, pressure swing adsorption (PSA) for hydrogen purification and collection, and utility generation. Process modeling work is underway using Aspen Plus to present the system design and obtain process mass and energy balance that are essential for both TEA and LCA. Some preliminary cost information has been incorporated in an H2A model as placeholders, which will be updated for equipment addition/removal and resizing, based on experimental and subsequent Aspen modeling results to provide yield and flow information. Similarly, for LCA, the data placeholders of hydrogen production from the integrated DF-MEC system are also in place in the Greenhouse Gases, Regulated Emissions, and Energy Use in Transportation (GREET) model.

The current research effort produces hydrogen from both DF and MEC, which reduces the amount of corn stover feedstock to about 800 MT/day. This is expected to have a significant impact on reducing overall cost and environmental release. We have modeled the following blocks in Aspen Plus Model: feedstock handling, feedstock pretreatment, fermentation, *C. thermocellum* bacteria production, PSA, and MEC. The modeled blocks will be updated upon experimental updates and further communication with collaborators from other national labs (Figure 4). In the A200 pretreatment area, the corn stover pretreatment is updated to DMR technology. In the current design, the black liquor (containing acetate, cellulose, xylan, arabinan, lignin, and ash) produced from deacetylation (without mechanical refining) is routed to the fermenter. This is to improve the carbon utilization, given the enhanced capability of *C. thermocellum* to convert both C6 and C5 sugars simultaneously (Figure 5). For the dark fermentation stage, the fermenter is designed/sized based on the current research target of 175 g substrate /L by utilizing both C5 and C6 components. Upon separation by a flash tank and liquid-solid separator, the gaseous products (mostly CO_2, H_2, and water vapor) are sent to PSA, the liquid portion is sent to MEC, and the solid portion (mainly lignin residue), is sent to the combustor for utility generation.

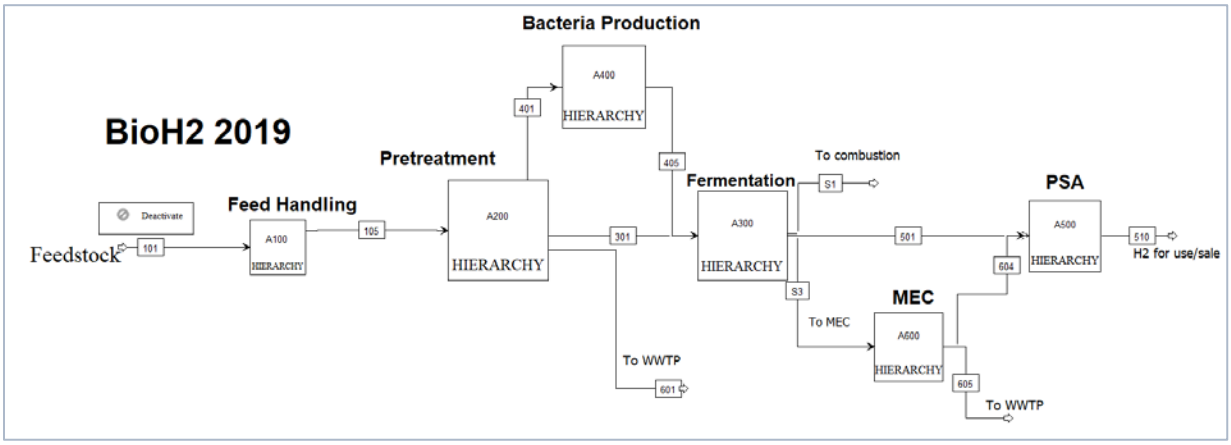

Figure 4. The flow scheme for overall direct fermentation-microbial electrolysis cell process.

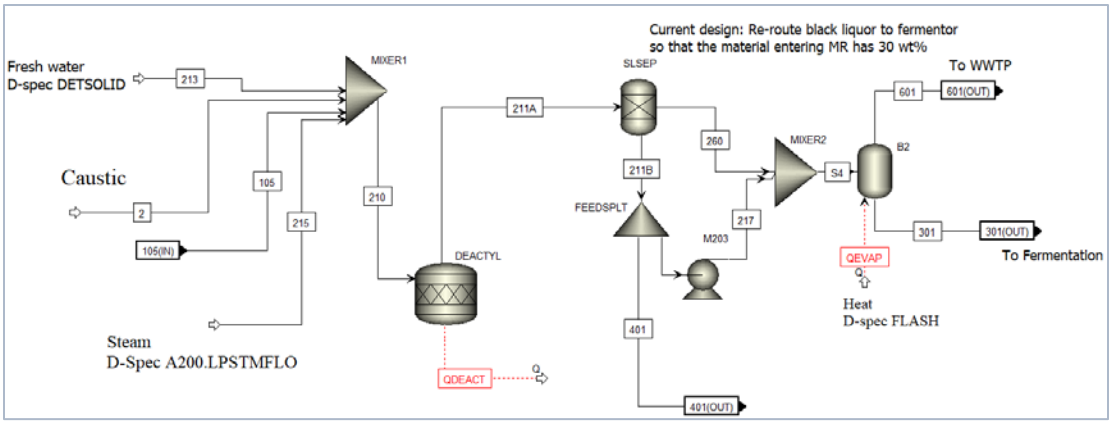

Figure 5. The flow scheme for corn stover pretreatment

CONCLUSIONS AND UPCOMING ACTIVITIES

- We measured an average hydrogen production rate of 2.75 L/L-d in *C. thermocellum* engineered and evolved to metabolize C5 sugars, suggesting its ability to co-utilize hemicellulose in the pretreated corn stover over a period of 24 hours.
- We identified 113 genes that are differentially expressed to afford C5 sugar utilization. Probing these genes in more detail will reveal the mechanisms to improve biomass conversion to additional hydrogen.
- The LBNL team obtained a high rate of hydrogen production (11 mmol/L-hr, i.e., 6.5 L H_2/L/d) recorded in bioreactors feeding with 30 g/L Avicel to determine limiting factors in high solids cultivation.
- In the MEC area, five promising exoelectrogenic strains were identified, which can extract electrons from components of synthetic fermentation effluent to potentially produce more hydrogen when integrating with fermentation.
- We have modeled the following blocks in Aspen Plus Model: feedstock handling, feedstock pretreatment, fermentation, *C. thermocellum* bacteria production, pressure swing absorption, and MEC. The modeled blocks will be updated upon experimental updates and further communication with collaborators from other national labs for the TEA and LCA efforts.

In the future, we will analyze in more detail the differentially expressed genes upon xylose utilization to identify key mechanisms to improve C5 sugar utilization to lower biomass cost and improve total hydrogen production. We will also express heterologously additional xylanase into the C5-metabolizing microbes to expedite hemicellulose utilization. We will improve hemicellulose analytical protocols to better quantify its utilization in order to validate strain improvement. A tracer-based metabolic flux analysis will be conducted to reveal metabolic changes upon C5 sugar utilization. These strains will be tested along with the higher loading of Avicel to determine limiting parameters under high solids loading conditions to reduce the capital cost of fermenters. Five promising exoelectrogenic strains were identified, which will be subjected to MEC testing for current generation and hydrogen production using both synthetic and real fermentation effluent by integrating fermentation with MEC. The Aspen Plus modeled blocks will be updated upon experimental updates and further communication with collaborators from other national labs for the TEA and LCA efforts to guide future research directions.

FY 2019 PUBLICATIONS/PRESENTATIONS

1. J.G. Marcano, J. Lo, A. Nag, P.C. Maness, K.C. Chou. "Developing Riboswitch-Mediated gene Regulatory Controls in Thermophilic Bacteria." *ACS Synthetic Biology*. DOI: 10. (2019) 1021/acssynbio.8b00487.
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3. K. Chou, Invited, Oral Presentation at International Conference of Biomolecular Engineering (ICBE Conference) on Jan 17, 2010. Titled, "Engineering Cellulolytic Bacterium Clostridium Thermocellum to Co-Ferment Cellulose- and Hemicellulose-Derived Sugars Simultaneously" in Metabolic engineering session at Newport Beach, CA, USA.
4. P.C. Maness, "Biohydrogen (BioH2) Consortium to Advance Fermentative Hydrogen Production", Poster presentation at the FCTO Annual Merit Review, April 30, 2019, Arlington VA.
5. K. Chou, Poster Presentation at the Society of Industrial Microbiology and Biotechnology (SIMB) 2019 Annual meeting, July 22, 2019. Titled, "Developing riboswitch-mediated gene regulatory controls in cellulose-degrading bacteria Clostridium thermocellum" at Washington, D.C., USA.

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8. W. Xiong, L.H. Reyes, W.E. Michener, P.C. Maness, K.J. Chou, "Engineering Cellulolytic Bacterium *Clostridium thermocellum* to Co-Ferment Cellulose- and Hemicellulose-Derived Sugars Simultaneously." *Biotechnol. Bioeng.* **115 (**2018): 1755–1763; DOI: 10.1002/bit.26590.

9. W. Xiong, P.P. Lin, L. Magnusson, L. Warner, J.C. Liao, P.C. Maness, K.J. Chou "CO2-Fixing One-Carbon Metabolism in a Cellulose-Degrading Bacterium *Clostridium thermocellum*." *Proc. Nat. Acad. Sci.* USA. **113** (2016): 13180–13185.