Overview

Timeline

• Project start date: FY16
• Project end date: 10/2017*

*Project continuation and direction determined annually by DOE

Barriers

• H₂ molar yield (AX)
• Feedstock cost (AY)
• System engineering (AZ)

Budget

• FY16 DOE Funding: $1M
  – Include $300K to partners
• Planned FY17 DOE Funding: $700K
  – Include $150K to partner
• Total DOE funds received to date: $1.85M

Partners

• Dr. Bruce Logan
  Pennsylvania State University
• Drs. Steven Singer, Lawrence Berkeley National Lab (LBNL) and Ken Sale, Sandia National Lab (SNL)
• Dr. James Liao at UCLA (no cost)
Overall Objective: Develop direct fermentation technologies to convert renewable lignocellulosic biomass resources to $H_2$.

Directly Address Barriers
- Feedstock cost (AY): via bioreactor development using lignocellulose (Task 1), and biomass pretreatment via ionic liquid (Task 2).
- Hydrogen molar yield (AX) ($N_1$ & $N_2$: mol $H_2$/mol hexose): via genetic engineering (Task 3) and integration with Microbial Electrolysis Cell (MEC) (Task 4)

Address Key DOE Technical Targets

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Units</th>
<th>2011 Status</th>
<th>2015 Target</th>
<th>2020 Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feedstock cost$^a$</td>
<td>Cents/lb sugar</td>
<td>13.5</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Yield of $H_2$ production from glucose</td>
<td>Mol $H_2$/mol glucose</td>
<td>3.2$^b$</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>MEC production rate</td>
<td>L-$H_2$/L-reactor-day</td>
<td>-</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

$^a$ Status and target of the DOE Bioenergy Technology Office (BETO) – leverage BETO funding.

$^b$ Low carbon substrate loading (1 g/L) led to high $H_2$ molar yield.
Approach

Task 1: Bioreactor Performance

- **Approach:** Optimize bioreactor in batch and fed-batch modes by testing parameters such as corn stover lignocellulose loadings (DMR), hydraulic retention time (HRT), and liquid volume replacement and frequency, using the cellulose-degrading bacterium *Clostridium thermocellum*, one of the fastest cellulose-degraders.

Pretreatment – **De-acetylated and Mechanically Refined (DMR)**

- **Solid:** 44% cellulose, 23% xylan, 17% lignin, Ash, etc.
- **Liquid:** 79% acetate, 2.9% xylan

Ferment all the sugars to $\text{H}_2$ in one bioreactor

Engineer Cellulose Microbe to Co-metabolize C5 Sugars

- Cellulose (C6 polymer)
- Hemicellulose (C5 polymer)
- Cellobiose/glucose (C6)
- Xylose (C5)

C. *thermocellum*
Task 1 – Accomplishments/Progress

High Rate of H₂ Production from Fermenting DMR Corn Stover

DMR corn stover retains more intact biomass structure, hence more recalcitrant. Yet it can be fermented directly to H₂ by *C. thermocellum* without adding expensive enzyme cocktail.

<table>
<thead>
<tr>
<th>Carbon Concentration (g L⁻¹ d⁻¹)</th>
<th>Cycle</th>
<th>Average H₂ Production Rate</th>
<th>Average cellulose consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Avicel (mL L⁻¹ d⁻¹)</td>
<td>DMR (g L⁻¹ d⁻¹)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1119</td>
<td>738</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1140</td>
<td>1064</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1621</td>
<td>763</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1098</td>
<td>1733</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td>1244</td>
<td><strong>1075</strong></td>
</tr>
<tr>
<td><strong>stdev</strong></td>
<td></td>
<td>22%</td>
<td><strong>43%</strong></td>
</tr>
</tbody>
</table>

* High SD likely from incomplete biomass hydrolysis in cycles 1&3.

Sequencing Fed-Batch fermentation of 10 g/L/d of DMR feeding at a 48 h HRT generated an average (4-day period) H₂ production rate of **1075 mL H₂/L_{reactor}/d** at a 48 h HRT (max rate = 1.7 L H₂/L_{reactor}/d, 1-day period).

Work is ongoing to increase DMR loadings to attain an average rate of 2.5 LH₂/L/d – FY17 Q4 Milestone.

**FY16 Q4 Milestone (regular) - NREL**

| Optimize the hydraulic/solid retention time with respect to H₂ production and media utilization by testing HRT between 12 and 48 h in a sequencing fed-batch reactor, and obtain a continuous average H₂ production rate of 1L/L_{reactor}/d using DMR. | 9/2016 Complete |
Task 1 – Accomplishments/Progress
Industrial Waste Reduced Growth Medium Cost by 49%

- In FY16 AMR, we reduced the medium cost by ~89% via replacing/eliminating three expensive ingredients (MOPS buffer, cysteine, and resazurin) from the rich medium without impacting H₂ production.
- The FY2017 goal is to replace yeast extract (0.45%; $201.5/kg) in the medium with an industrial waste.
- Corn steep liquor (CSL; $49.4/kg, Industrial CSL; $0.61/kg) or brewers yeast (BY; $114.5/kg) could replace yeast extract as they contain the needed vitamins and amino acids to boost growth and H₂ production.

We realized a 49% reduction ($0.95/$1.85) in medium cost when yeast extract is replaced with industrial waste corn steep liquor with increased H₂ production.

*already excluding MOPS, cysteine, and resazurin.

<table>
<thead>
<tr>
<th></th>
<th>H₂ (mM)</th>
<th>Medium* Cost ($/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rich</td>
<td>38.8</td>
<td>1.85</td>
</tr>
<tr>
<td>BY</td>
<td>48.1</td>
<td>1.38</td>
</tr>
<tr>
<td>CSL</td>
<td>45.6</td>
<td>1.39</td>
</tr>
<tr>
<td>Industrial CSL</td>
<td>47.8</td>
<td>0.95</td>
</tr>
</tbody>
</table>

We realized a 49% reduction ($0.95/$1.85) in medium cost when yeast extract is replaced with industrial waste corn steep liquor with increased H₂ production.

**FY17 Q2 Milestone (regular) - NREL**

Develop an industrially relevant growth medium for *C. thermocellum*, by evaluating and supplementing either commercially available components or industrial waste products that will maintain similar level of cell fitness and rate of H₂ production compared to the growth medium currently used with the aim to reduce medium cost (NREL).

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FY17 Q2</td>
<td>3/2017</td>
<td>Complete</td>
</tr>
</tbody>
</table>

*already excluding MOPS, cysteine, and resazurin.*
Task 1 – Accomplishments/Progress
Engineered *C. thermocellum* Can Co-utilize C6/C5 Sugar

- Up to 32% of biomass is hemicellulose, a polymer of C5 sugar (mostly xylose)
- We engineered *C. thermocellum* (*xylAB* mutant strain) to co-utilize C5 sugar while degrading cellulose – leveraging NREL LDRD success.
- C5 (xylose) and C6 sugars (glucose or cellobiose) were metabolized simultaneously *without* cross inhibition – a breakthrough finding.

**A. Xylose and Glucose Co-metabolism**

**B. Xylose and Cellobiose Co-metabolism**

C6 and C5 sugars co-metabolism improves biomass utilization and lowers H₂ selling price.
Task 1 – Accomplishments/Progress

Xylose and Cellulose Co-utilization Increases H₂ by 2.1-fold

- **C. thermocellum** mutant was cultured in 2.5 g/L cellulose (Avicel), +/- 2.5 g/L xylose.
- Engineered strain can also co-metabolize xylose with xylo-oligomer, and glucose with xylan, the latter is a modeled hemicellulose.

---

**Xylose and Cellulose Co-metabolism**

![Graph showing H₂ production and substrate consumption over time](image)

- 16.6 mM H₂ (cellulose+xylose)
- 7.9 mM H₂ (cellulose)

**Cellulose and xylose co-utilization increased H₂ output by 2.1-fold**

<table>
<thead>
<tr>
<th>FY17 Q1 Milestone (regular) - NREL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profile H₂ production, substrate consumption, and metabolite profiles in an engineered C. thermocellum mutant using xylose alone, and in co-metabolism of xylose with xylan, xylose with cellobiose, and xylose with avicel cellulose, aimed at developing microbes fermenting all the sugars in biomass to improve biomass utilization (NREL).</td>
</tr>
</tbody>
</table>
Approach/Accomplishments/Progress

Task 2. Fermentation of Pretreated Biomass using Ionic Liquid (LBNL/SNL)

- Ionic liquid (IL) pretreats biomass via electrostatic/hydrogen-bonding interactions, which compliments NREL’s approach using DMR-pretreated biomass.
- Task 2 goal is to conduct IL pretreatment and fermentation in the same reactor to save cost, hence the GNG target of tolerating 10% IL.
- *C. thermocellum* displayed a lag phase of 24 days in 10% cholinium glutamate ([Ch][Glu]), with growth yield at 23% that of the control (Go/No-go criterion is 85%).

Task 2 was closed-out in FY17/Q1, not meeting the GNG Decision.

<table>
<thead>
<tr>
<th>FY16 Q4 Go/No-go (GNG) Decision – LBNL/SNL</th>
<th>Completion Date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demonstrate biocompatibility of at least one ionic liquid with <em>C. thermocellum</em> by comparing cell growth with and without the presence of 10% selected ionic liquid in DSM 1191 growth medium with cellobiose as carbon source and achieving a similar growth rate (&gt;85%) with and without ionic liquid.</td>
<td>9/2016</td>
<td>Incomplete</td>
</tr>
</tbody>
</table>
Task 3 – Generate Metabolic Pathway Mutant in *C. thermocellum*

**Approach:** Redirect metabolic pathways to improve H$_2$ molar yield via developing genetic methods.

- Blocking the lactate and formate carbon-competing pathways led to **86% increase** in rate of H$_2$ production (2016 AMR).
- A new strategy in FY17 is to directly manipulate enzymes (Nfn and/or Rnf) to increase the pools of NADH and reduced ferredoxin (Fd$_{red}$), both are electron donors for H$_2$ production.

<table>
<thead>
<tr>
<th>FY17 Q3 Milestone (Regular) - NREL</th>
<th>Completion Date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generate a mutant lacking either ferredoxin: NAD$^+$ oxidoreductase (Rnf) and/or NADH-ferredoxin: NADP$^+$ oxidoreductase (Nfn) and profile H$_2$ and carbon metabolites production. This mutant will then serve as the host for deleting the ethanol competing pathway using H$_2$ production as the new electron sink aimed at maintaining redox balance to yield stable mutants with increased H$_2$ production</td>
<td>6/2017</td>
<td>Complete</td>
</tr>
</tbody>
</table>
Nfn: transhydrogenase; its deletion should yield more $F_{d_{red}}$ and NADH for $H_2$ production.

- PCR data confirm deletion of $nfnAB$ genes from $C.\ thermocellum$ genome: $\Delta nfn$ mutant yields a lower band (lane 1) or no band (lane 3) vs. the parental control (gDNA); P1-P4: PCR primers.

Nfn mutant: total $H_2$ production was increased by 29%, and specific rate of $H_2$ production by 55%.
Task 3 – Accomplishments/Progress
Rnf Mutant Produced 35% More H₂

- Rnf: F₅dred-NAD⁺ oxidoreductase
- The enzyme Rnf converts Fdred to NADH which yields energy.
- Its deletion should increase the pool of Fdred toward more H₂ production.
- Generated Rnf mutant that produced **35% more H₂**.
- Rnf mutant also produced ~30% less ethanol, suggesting electron redirection.

The 35% increase in H₂ production validates that redirecting electron pool is an effective strategy to boost H₂ production.
Approach/Milestone
Task 4 – Electrochemically Assisted Microbial Fermentation

Microbial Electrolysis Cell (MEC) — Conversion of Organic Waste to Hydrogen Gas

**Goal:** Achieving high rate of $H_2$ production with non-Pt based cathode using NREL fermentation effluent.

<table>
<thead>
<tr>
<th>Milestones (PSU)</th>
<th>Completion Date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY16 Design MEC cathodes with reduced width to increase $H_2$ production rate to a maximum $H_2$ rate of 1.2 L/L reactor/day based on overall reactor volume reduction</td>
<td>9/2016</td>
<td>Complete</td>
</tr>
<tr>
<td>FY17 Investigate alternative cathode materials and increase electrode loading to double reactor performance to 2.4 L/reactor-d using synthetic fermentation effluent.</td>
<td>9/2017</td>
<td>On track</td>
</tr>
</tbody>
</table>
Task 4 – Accomplishments/Progress
Cathode Chamber Optimization: replaced Pt with alternative materials

MEC with cathode flow chamber

Abiotic electrochemical tests (chronopotentiometry) at catholyte flow rates from 0 to 80 mL/min (mean ± SD, n=3)

Cathode materials
- SS mesh (SSM)
- SS fiber felt (SSFF)
- SS wool (SSW)
- Pt/C (as control)

SS: stainless steel

Kyoung-Yeol Kim

- Higher variability at low current densities or with no flow.
- Higher current produces specific trends with materials.
- Performance ranking: Pt/C > SSW>SSFF>SSM in abiotic tests.

The low cost stainless steel wool is the best performer to replace Pt
**Task 4 – Accomplishments/Progress**

Achieved 1.3 L H₂/L-d with low cost non-Pt Cathode

**H₂ production rates and current densities with different SS materials**

<table>
<thead>
<tr>
<th>Material</th>
<th>Flow</th>
<th>No flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSW</td>
<td>1.3±0.3</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>SSFF</td>
<td>1.9±0.2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>SSM</td>
<td>1.2±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Pt/C</td>
<td>1.5±0.2</td>
<td>0.6±0.1</td>
</tr>
</tbody>
</table>

- Hydrogen production rates ranged from 1.3±0.3 L/L-d with SSW, to 0.9±0.1 L/L-d SSM.
- Pt/C only marginally better than SSW
- 4.5 ±0.5 A/m² with SS wool (versus 5.1±0.3 A/m² with Pt/C; per cathode projected area)

**Cathodic hydrogen recoveries (CR) = percent of coulombs (electrons) converted to H₂**

<table>
<thead>
<tr>
<th>Material</th>
<th>Flow</th>
<th>No flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSW</td>
<td>105±18 %</td>
<td>71±13 %</td>
</tr>
<tr>
<td>SSFF</td>
<td>95±2 %</td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>80±10 %</td>
<td></td>
</tr>
<tr>
<td>Pt/C</td>
<td>90±10 %</td>
<td></td>
</tr>
</tbody>
</table>

- CRs with flow: 105±18 % for SSW; 95±2 % with Pt/C
- With no flow, some H₂ lost: CR=71±13 % for SSW

**H₂ production rate meets 2016 milestone (1.2 L/L-d) with low cost SS wool (a non-Pt based cathode), and with ~100% H₂ recovery from current**
Responses to Previous Year Reviewers’ Comments

Utilization of xylose by C. thermocellum is likely diauxic, so incorporation of this into the fermentation process will be an engineering challenge.

• Our data have proved to the contrary, that xylose and cellulose are co-utilized without cross inhibition (i.e., xylose usage is not inhibited by the presence of cellulose). This breakthrough finding warrants continued research to realize more complete biomass conversion to increase H₂ production.

The reasoning for using IL-derived sugars is not clear, what it adds to the project.

• The IL Task was closed out in FY17/Q1, due to not meeting the GNG Decision.

It is unclear how novel the mutations are completed...overexpression of hyd2 is good

• Mutating two carbon-competing pathways led to near 86% increase in H₂ production (end point data). This combined mutation has not been reported in literature. Work is ongoing to over-express either native or foreign hydrogenases to improve H₂ production while also maintaining redox and energy balance to maximize cell fitness.

The rate for the MEC should be normalized to biomass not reactor volume, state rate as initial, peak or average, and give the amount of energy or net energy yield.

• The biomass concentration is not known in the MEC, so that cannot be done, and it is not needed as the critical factor is current normalized to electrode area or volume (we used volume here). The rate given is an average rate (now stated), and the energy input is calculated as energy efficiency (energy in the H₂ produced relative to the energy input in the electrolyzer). As noted in our previous work, this electrolyzer energy could be substituted with a reverse electrodialysis stack that would be powered using waste heat.
Collaborations

• **Task 1 (Bioreactor)**
  Drs. Ali Mohagheghi and Melvin Tucker, National Bioenergy Center at NREL: provide DMR pretreated corn stover and their characterizations - leveraging DOE BETO funding.

• **Task 2 (Ionic Liquid)**
  Drs. Steve Singer (LBNL) and Kent Sales (SNL): conducted biomass pretreatment using ionic liquid as a complementary pretreatment approach to lower feedstock cost.

• **Task 3 (Genetic Methods)**
  Dr. James Liao of UCLA in pathway engineering of *C. thermocellum* – leveraging DOE Office of Science funding, leading to a joint publication in PNAS, 2016.

• **Task 4 (MEC)**
  Dr. Bruce Logan at Penn State University: microbial electrolysis cells to improve H₂ molar yield.
Remaining Challenges and Barriers

Task 1. Bioreactor Performance
• High solid-substrate loading (175 g/L) is needed to lower H₂ selling price, which might present a challenge to ensure sufficient mixing.
  – Impeller design with high power/low torque will address this challenge.

Task 2. Fermentation of Pretreated Biomass using Ionic Liquid (LBNL/SNL)
• This task was closed out in FY17/Q1.

Task 3. Generate Metabolic Pathway Mutant in C. thermocellum
• Deleting carbon-competing pathways to increase H₂ molar yield might cause a redox imbalance (excess NADH or reduced ferredoxin) and compromise microbial fitness.
  – Over-express hydrogenase-encoding genes to maintain redox balance.

Task 4. Electrochemically Assisted Microbial Fermentation of Acetate (PSU)
• Maximizing current generation with non-precious metal catalysts
  – Already demonstrated good performance with SS wool; no others have yet shown better and more promising MECs.
• Further reducing reactor size, which will increase overall reactor production rates; examining new anode configurations which may help.
Proposed Future Work

Task 1 (NREL)
- Optimize sequencing-fed batch reactor using DMR corn stover to obtain average rate of 2.5 L H₂/L_reactor/d (FY17 Q4 Milestone).
- Work with Argonne NL in a Life Cycle Analysis of the fermentative H₂ technology
- Optimize cellulose and hemicellulose co-fermentation to improve biomass utilization to achieve a rate of 2.5 L H₂/L_reactor/d and higher (FY17/18).

Task 2 (LBNL/SNL)
- None

Task 3 (NREL)
- Over-express native or heterologous hydrogenases to increase H₂ production and to maintain redox balance for cell fitness in either Rnf or Nfn mutant host (FY17/18).
- Profile expression pattern to identify the most important ferredoxin(s) to improve H₂ production as *C. thermocellum* contains multiple ferredoxins with unknown functions (FY17/18).

Task 4 (Penn State)
- Further improve anode and cathode chamber (increasing electrode loading) to double performance of MEC to produce 2.4 L-H₂/L_reactor/d (FY17) using synthetic effluent.
- Examine alternative materials and catalysts for the cathode (FY17).
- Design a multi-chamber MEC module (FY17/18).
Technology Transfer Activities

Technology-to-market or technology transfer plan or strategy

• Air Product and Chemicals, Inc.
  – Main interest in H₂ from biomass can be low carbon or even potentially carbon neutral; have funded the Logan lab in the past for work on MECs and RED for H₂ production from wastewaters
  – Large-scale process of greatest interest, but currently there are no larger reactors.
  – Cost needs to be near to, or lower than, making H₂ from alternative sources (natural gas).

Plans for future funding

• Network with biofuels industry to expand the use of H₂.
• Advocate the advantages of “green” H₂ rather than fossil-fuel derived H₂

Patents, licensing

• A Record of Invention (ROI-14-70) is filed for developing the proprietary genetic tools tailored for *C. thermocellum*.
• A second ROI-15-42 has been filed for generating xylose-metabolizing strain, leading to enhanced biomass utilization.
Summary

Task 1
- Achieved an average (4 days) H₂ production rate of $1075 \text{mL H}_2/\text{L}_{\text{reactor}}/\text{d}$ fermenting DMR-pretreated corn stover directly to H₂.
- Lowered growth medium cost by 49% by replacing the costly yeast extract with industrial waste corn steep liquor, without impacting H₂ production.
- Engineered *C. thermocellum* to co-metabolize C₅ sugar with cellulose, without cross inhibition, leading to 2.1-fold increase in total H₂ production, which improves biomass utilization and lowers the cost of H₂.

Task 2
- Closed out in FY17/Q1, not meeting GNG.

Task 3
- Generated a *nfn* mutant that produced 29% more H₂ and with a 55% increase in specific rate of H₂ production.
- Generated a *rnf* mutant that produced 35% more H₂. Either *nfn* or *rnf* mutant can serve as the host to over-express hydrogenases.

Task 4
- SS cathodes improved with flow, with the SS wool the best performer to replace Pt.
- H₂ production rate meets 2016 milestone ($1.2 \text{L/L-d}$) with SS wool (a non-Pt based cathode) with ~100% H₂ recoveries from current.
Technical Back-Up Slides
Task 3 – Accomplishments/Progress
Genetic Protocol to Generate the Nfn or Rnf Mutant

- The parental strain lacks *hpt* (phosphoribosyl transferase) gene, which allows counter selection with the antimetabolite 8AZH (8-azahypoxanthine) to lose the antibiotic marker thiamphenicol.
- The integration plasmid contains *tdk* (thymidine kinase) gene, which allows counter-selection with the antimetabolite FUDR (fluoro-deoxyuracil) to cure the plasmid.
- P1 – P4 are PCR primers to validate the mutagenesis steps.
Task 4 – Accomplishments/Progress

Anode Chamber Optimization: Minimizing Anode Size

Goal: Reduce anode chamber volume by using flat anodes or smaller brushes

Thinner flat anodes could produce a more compact MEC reactor and increase H₂ production rates.

- Ongoing results (no flow): only slightly reduced H₂ production rates for thinner carbon felt electrodes (0.6 cm thick) than the brush electrode (1.5 cm diameter).