Overview

Timeline

• Project Start Date: FY16 (leveraging past NREL Fermentation and Electrohydrogenic Approaches project)
• Project End Date: 10/2016*

Barriers

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

Budget

• FY16 planned DOE Funding: $1M
• Total DOE funds received to date: $1M

Partners

• Dr. Bruce Logan
  Pennsylvania State University
• Dr. Steven Singer, Lawrence Berkeley National Lab (LBNL)
• Drs. John Gladden and Ken Sale, Sandia National Lab (SNL)

*Project continuation and direction determined annually by DOE
**Overall Objective:** Develop *direct* fermentation technologies to convert renewable lignocellulosic biomass resources to $\text{H}_2$.

**Directly Address Barriers**
- Feedstock cost ($\text{AY}$): via bioreactor development using lignocellulose (Task 1), and biomass pretreatment via ionic liquid (Task 2).
- Hydrogen molar yield ($\text{AX}$) ($N_1$ & $N_2$: mol $\text{H}_2$/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 $\text{H}_2$ production from glucose</td>
<td>Mol $\text{H}_2$/mol glucose</td>
<td>$3.2^b$</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>MEC production rate</td>
<td>L-$\text{H}_2$/L-reactor-day</td>
<td>-</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

b. Low carbon substrate loading (1 g/L) led to high $\text{H}_2$ molar yield.
Task 1: Bioreactor Performance

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

**Pretreated Corn Stover – Acid Hydrolysis (PCS)**

1% H$_2$SO$_4$

160-190°C

Solid: 59% cellulose
27.5% lignin

Liquid: 90% xylan
9% glucan

More sugar loss, more inhibitors.

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

0.4% NaOH

70°C

Solid: 42% cellulose
25% xylan
16% lignin

Liquid: 79% acetate
2.9% xylan

Less sugar loss, less inhibitors.
Task 1 – Accomplishments/Progress
H₂ from DMR Corn Stover and Carbon Mass Balance

• 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.

• Mass analysis indicated (batch fermentation):
  – 94% total DMR solid was solubilized;
  – 98% cellulose solid was consumed by microbe;
  – 97% of xylan is solubilized, genetic engineering would to convert xylose to H₂ also.

<table>
<thead>
<tr>
<th>Cellulose 5 g/L</th>
<th>Dry Weight</th>
<th>Glucan (g/L)</th>
<th>Xylan (g/L)</th>
<th>Lignin (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>11.9 +/- 0.37</td>
<td>5.3 +/- 0.38</td>
<td>3.7 +/- 0.02</td>
<td>1.7 +/- 0.08</td>
</tr>
<tr>
<td>Final</td>
<td>0.6 +/- 0.28</td>
<td>0.1 +/- 0.02</td>
<td>0.1 +/- 0.02</td>
<td>0.1 +/- 0.03</td>
</tr>
<tr>
<td>Solid Hydrolyzed (%)</td>
<td>94% +/- 0.01</td>
<td>98% +/- 0.00</td>
<td>97% +/- 0.01</td>
<td>91% +/- 0.02</td>
</tr>
</tbody>
</table>

• Sequencing Fed-Batch fermentation of 5 g/L/d DMR produced an average H₂ production rate of **791 mL H₂/L_reactor/d** at a 48 h HRT (max rate = 1.6 L H₂/L_reactor/d).

• Higher substrate loading will meet toward Q4 milestone.

<table>
<thead>
<tr>
<th><strong>FY16 Milestone (regular) - NREL</strong></th>
<th><strong>9/2016</strong></th>
<th><strong>On Track</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Q4 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.</td>
<td>9/2016</td>
<td>On Track</td>
</tr>
</tbody>
</table>
Task 1 – Accomplishments/Progress
Reduce Medium Cost to Lower H₂ Selling Price

- The normal complex growth medium (CTFUD) has buffer (MOPS) and yeast extract (0.45%; w/v).
- Eliminating resazurin ($1.57/L_{medium}$; redox-sensing dye) and cysteine ($1.31/L_{medium}$; poising redox potential) have no effect on H₂ production, nor cell growth.
- MOPS buffer is costly ($10.92/L_{medium}$) yet essential for pH control and cell fitness.
- Similar H₂ output in bioreactor with pH control, with or without MOPS, the latter lowers final cost of H₂ from $0.55 to $0.18/mM H₂.

<table>
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<th>FY16 Milestone (regular) - NREL</th>
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<tbody>
<tr>
<td>Q2 Evaluate components in the <em>C. thermocellum</em> growth medium, eliminate, reduce, or replace one to two nutrients with minimal impact to cell fitness aimed to reduce medium nutrient cost.</td>
</tr>
<tr>
<td>3/2016 Complete</td>
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$\text{$/mM H}_2$
**Approach**

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

- Ionic liquids (IL) has been proposed as a method for biomass pretreatment, driven by the combination of electrostatic and hydrogen-bonding interactions between the IL and plant polymers.
- In a parallel and complimentary approach to NREL, LBNL/SNL will test three biocompatible ILs in biomass pretreatment followed by fermentation and compare with NREL DMR pretreatment in cost and H₂ output.

**Task Leads**

Steve Singer  
LBNL

John Gladden  
Ken Sale, SNL

<table>
<thead>
<tr>
<th>FY16 Milestone (regular)</th>
<th>Completion Date</th>
<th>Status</th>
</tr>
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<tbody>
<tr>
<td><strong>Q1</strong> Develop procedures at LBNL to grow <em>C. thermocellum</em> (LBNL)</td>
<td>12/2015</td>
<td>Complete</td>
</tr>
<tr>
<td><strong>Q1</strong> Perform corn stover pretreatment with three biocompatible ionic liquids and perform compositional analysis of the pretreated biomass (SNL)</td>
<td>12/2015</td>
<td>Complete</td>
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</tbody>
</table>
Task 2 – Accomplishments/Progress

Biocompatibility of Ionic Liquid Biomass for H₂ Production (LBNL)

- *C. thermocellum* showed no growth at ≥3% of ionic liquids.
- One culture with 5% cholinium glutamate grew after 9 days; growth in 5% [Ch][Glu] was maintained in successive cultures.

- Culture grown in 5% [Ch][Glu] is being adapted to grow on 6-8% [Ch][Glu].
- Agar plating of *C. thermocellum* culture with tolerance to 6-8% [Ch][Glu] will isolate individual clones to test for tolerance to 10% [Ch][Glu] (Q2 milestone).
NREL sent untreated corn stover and SNL pretreated with three ionic liquids successfully: [Ch][Glu], [Ch][Mal], and [Ch][Su].

(A) Sugar Yields via Acid Hydrolysis

Complete FY16/Q1 milestone: [Ch][Glu] has the best sugar recovery.

(B) Sugar Yields via Cellulase Enzyme Cocktail

Complete FY16/Q2 milestone: Use cellulase enzyme cocktail to measure amounts of glucose and xylose release.

Compositional Analysis of Pretreated Cornstover

- IL, 20% (w/w)
- 140°C 3h, pH 12
- Solid: cellulose, xylan, lignin
- Liquid: lignin, ILs for recycling

Glucose and Xylose Yields from Corn Stover Pretreated using Choline-based Ionic Liquids
Approach: Redirect metabolic pathways to improve H₂ molar yield via developing genetic methods.

- NREL developed proprietary genetic tools in *C. thermocellum* that very few labs can rival (FY14 accomplishments).
- Single mutants lacking either lactate dehydrogenase (LDH, pyruvate-to-lactate step) or pyruvate-formate lyase (PFL; pyruvate-to-formate step) have been generated, but NOT in a combined strain (FY14/15 accomplishments).
- The goal in FY15/FY16 is to delete both competing pathways in the same strain and determine outcomes on H₂ production.

### FY16 Milestone – Regular

| Q3 | Measure transcriptional expression profiles of the three hydrogenases as well as H₂ production, the latter in rate and volume, at early-log, mid-log, and late-log phases of cell growth to best predict their role in either H₂ production or H₂ consumption, which will guide future genetic engineering strategy (NREL) | 6/2016 | Complete |
Task 3 – Accomplishments/Progress
Delete Both Competing pathways to Make Formate and Lactate

- Generated double mutant in *C. thermocellum* lacking both the pyruvate-to-lactate step (conserves 2 electrons) and pyruvate-to-formate step (conserves more pyruvate) aimed to produce more H₂.
- The double mutant displayed ~90% increase in specific rate of H₂ production.
- The double mutant did not produce formate and with negligible amount of lactate.

Rate of H₂ production can be increased by deleting competing pathways.
Task 3 – Accomplishments/Progress
Hydrogenase Expression Profiles

• H₂ production (A) peaked at mid-log phase of growth (OD ~ 0.45), catalyzed by three FeFe-hydrogenases (Hyd1, 2, 3), and a NiFe-hydrogenase.
• Using real-time quantitative PCR (qRT-PCR) (B), Hyd2 expression profile coincided with peak H₂ production, suggesting that Hyd2 plays a major role in H₂ production – meeting Q3 Milestone.

(A). Cell Growth and H₂ Production

(B). Hydrogenase Expression (qRT-PCR)

Hydrogenase expression profiles suggest that over-expression of Hyd2 most likely will boost H₂ production, which guides metabolic engineering strategy.
**Approach/Milestone**

**Task 4 – Electrochemically Assisted Microbial Fermentation**

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

**Goal:**
Achieving $1.2 \text{ L-H}_2/\text{L-reactor/d}$ over 3 HRT, using NREL fermentation effluent.

<table>
<thead>
<tr>
<th>Milestones</th>
<th>Completion Date</th>
<th>Status</th>
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</thead>
<tbody>
<tr>
<td>FY15</td>
<td>9/2015</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>Optimize the design of the cathode chamber to increase the volumetric hydrogen production rate to $1.2 \text{ L H}_2/\text{L-reactor/d}$ (over 3 HRT, using synthetic effluent) in a continuous flow MEC, using Pt/C cathodes and improved configurations.</td>
<td></td>
</tr>
<tr>
<td>FY16</td>
<td>9/2016</td>
<td>On Track</td>
</tr>
<tr>
<td></td>
<td>Design MEC cathodes with reduced width to increase maximum H$_2$ production rate to $1.2 \text{ L/Lreactor/day}$ based on overall reactor volume reduction.</td>
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</table>
Hydrogen production in the MEC was increased to $1.4\pm0.2\ \text{L-}H_2/\text{L-reactor/d}$ by decreasing volume of the cathode chamber (76 mL $\rightarrow$ 28 mL). Rates comparable to MEC with a reverse electrodialysis (RED) stack. These experiments met the 2015 milestone ($1.2\ \text{L-}H_2/\text{L-reactor/d}$).

**MEC-S**
(S=small cathode chamber: 28 mL)

**MEC-M**
(M= modified cathode chamber: 76 mL)

**MREC**
(Result from previous MREC study)

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**Cathode chamber volume adjustment**

Kyoung Yeol Kim
Task 4 – Accomplishments/Progress
Cathode Chamber Optimization

**Impact of buffer/pH on H₂ production**

- Change in pH did not adversely affect H₂ production with >1.2 L-H₂/L-reactor/d.
- Can achieve compact reactor design.

**Electrochemical Performance**

- Cathodes tested:
  - PTSS: Pt/C on SS mesh
  - SSFF, non-Pt: SS Fiber Felt
  - SSM: SS mesh only

Stainless steel (SS) mesh showing good promise of matching performance of Pt/C; further tests needed.
MEC with continuous flow adjustable volume cathode chamber

- Examine electrochemical performance of cathode by improving hydrodynamics of flow through thin chambers

- Examine alternative materials and catalysts for the cathode.
  → Metal-loaded carbon fibers; and nitrogen, phosphorous, and sulfur-loaded activated carbon fibers.
Task 4 – Accomplishments/Progress
Gas Diffusion Chamber: Abiotic tests

Gas Diffusion Cathode (3 chamber-setup). **Goal is to eliminate liquid catholyte**

Aqueous Cathode - 2 Chamber-Setup (Control)

Catalyst layer:
- Carbon black (CB) 10%
- Platinum with PDMS binder

Gas diffusion layer:
- Activated carbon (AC) with PVDF binder

Preliminary test results: Cathodic efficiencies were significantly different (p<0.05) according to the type of cathode chamber.
Response to Previous Year Reviewers’ Comments

- No Reviewers’ Comments - the previous fermentation project was presented but *not reviewed* in the 2015 AMR.

- This new fermentation project was started in FY2016 responding to a FCTO Lab Call via collaboration with Penn State, LBNL, and SNL.
Collaborations

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

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

• Task 3 (Genetic Methods)
  Drs. David Levin and Richard Sparling at the University of Manitoba, Canada: NREL is an international collaborator of the Genome Canada Grant award to pathway engineering in *C. thermocellum* - leveraging Canadian funding.

• 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 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)
- Overcoming potential toxicity of high concentrations of ionic liquid on microbes.
  - Acclimate microbes to tolerate high levels of ionic liquid.

Task 3. Generate Metabolic Pathway Mutant in *C. thermocellum*
- Deleting competing pathways to increase H₂ molar yield might cause a redox imbalance (excess NADH) and hinder mutant generation.
  - Over-express hydrogenase-encoding genes to maintain redox balance.

Task 4. Electrochemically Assisted Microbial Fermentation of Acetate (PSU)
- Current designs use precious metal catalysts, which must be avoided to make the process economical, but further improvements in performance are needed.
  - Demonstrate equal or improved performance using non-precious metal alternatives to Pt.
  - Further reduce reactor size by improving hydrodynamics or eliminating aqueous flow.
Proposed Future Work

Task 1 (NREL)
- Optimize sequencing-fed batch reactor using DMR corn stover to obtain average rate of 1 L H₂/L reactor/d (FY16 Q4 Milestone).
- Test “pretreatment” of de-acetylated biomass with *C. thermocellum’s* cellulosomes (cellulase enzyme cocktail) to accelerate the initial fermentation kinetics (FY16/17).

Task 2 (LBNL/SNL)
- Adapt *C. thermocellum* to grow robustly in 10% cholinium glutamate and test H₂ production (FY16).

Task 3 (NREL)
- Repeat hydrogenase expression profile experiment and verify the role of hydrogenase 2 in catalyzing H₂ production, aimed to meet Q3 milestone (FY16).
- Over-express hydrogenase 2 to balance electrons and increase H₂ production (FY16/17).

Task 4 (Penn State)
- Improve electrochemical performance of cathode by achieving better hydrodynamics of flow through thin cathode chambers (FY16).
- Examine alternative materials and catalysts for the cathode (FY16/17).
- Further examine gas diffusion cathode for improving reactor operation (FY16/17).
Technology Transfer Activities

Technology-to-market or technology transfer plan or strategy

- Air Product and Chemicals, Inc.
  - Main interest in \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) from alternative sources (natural gas).

Plans for future funding

- Network with biofuels industry to expand the use of \( \text{H}_2 \).
- Advocate the advantages of “green” \( \text{H}_2 \) rather than fossil-fuel derived \( \text{H}_2 \)

Patents, licensing

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

Task 1
• Eliminate MOPS as a costly nutrient and lower overall cost of \( \text{H}_2 \) production in bioreactor.
• Achieved an average \( \text{H}_2 \) production rate of 791 mL/L/d fermenting DMR cellulose directly to \( \text{H}_2 \). 97% of the xylan was also solubilized, genetic engineering will convert xylose to \( \text{H}_2 \) also.

Task 2
• Pretreated corn stover with three ionic liquids. Obtained highest glucose and xylose yields with the \([\text{Ch}][\text{Glu}]\) after saccharification using cellulase enzyme cocktail.
• Tested three ionic liquid and improved tolerance of \( \text{C. thermocellum} \) to 5% of \([\text{Ch}][\text{Glu}]\) ionic liquid via adaptation strategy.

Task 3
• Generated double mutant lacking both lactate- and formate-competing pathways leading to \( \sim 90\% \) increase in specific rate of \( \text{H}_2 \) production.
• Hydrogenase expression profiles reveal Hyd2 is most active in \( \text{H}_2 \) production.

Task 4
• A more compact reactor design was successful since pH changes did not adversely affect \( \text{H}_2 \) production. PSU achieved the 2015 milestone of > 1.2 L-\( \text{H}_2 \)/L-reactor/d.
• SS fiber felt (SSFF) cathode showed good promise of matching performance of cathodes containing Pt in electrochemical tests. Further MEC tests are ongoing.
• Preliminary tests show aqueous cathode still better than gas diffusion cathode.