

## II.E.3 Fermentation and Electrohydrogenic Approaches to Hydrogen Production

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Projected End Date: Project continuation and  
direction determined annually by DOE

### Overall Objectives

- Optimize rates and yields of hydrogen production in a sequencing fed-batch bioreactor by varying hydraulic retention time (HRT) and reactor volume replacement
- Optimize genetic tools to transform *Clostridium thermocellum* and obtain mutants lacking the targeted competing pathway to improve hydrogen molar yield
- Demonstrate hydrogen production from the NREL fermentation effluent and harness the energy in a chemical gradient to improve overall energy efficiency in hydrogen production using a microbial electrolysis cell (MEC) reactor

### Fiscal Year (FY) 2014 Objectives

- Optimize sequencing fed-batch parameters and convert corn stover lignocellulose to hydrogen by the cellulolytic bacterium *Clostridium thermocellum*, aimed to lower feedstock cost.
- Use the genetic tools developed at NREL tailored for *C. thermocellum* and delete the competing ethanol pathway, aimed to improve hydrogen molar yield via fermentation.
- Redesign a microbial reverse-electrodialysis electrolysis cell (MREC) to examine the scalability of the MREC for hydrogen production from fermentation effluent without an external energy input, aimed to improve H<sub>2</sub> molar yield.

### Technical Barriers

This project supports research and development on DOE Technical Task 6, subtasks Molecular and Systems Engineering for Dark Fermentative Hydrogen Production and Molecular and Systems Engineering for MEC and it addresses barriers AX, AY, and AZ.

(AX) H<sub>2</sub> Molar Yield

(AY) Feedstock Cost

(AZ) System Engineering

### Technical Targets

Technical targets for this project are listed in Table 1.

**TABLE 1.** Progress toward Meeting DOE Technical Targets in Dark Fermentation

Characteristics	Units	Current Status	2015 Target	2020 Target
Yield of H <sub>2</sub> from glucose	Mole H <sub>2</sub> /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	6 months
MEC cost of electrodes	\$/m <sup>2</sup>	\$2,400	\$300	\$50
MEC production rate	L-H <sub>2</sub> /L-reactor-d	1	1	4

\*Yield of H<sub>2</sub> from glucose: DOE has a 2015 target of an H<sub>2</sub> molar yield of 6 (4 from fermentation and 2 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.

### FY 2014 Accomplishments

- Conducted sequencing fed-batch reactor experiments and demonstrated that by using a HRT of 48 h and displacing 50% of the reactor liquid every 24 h, *C. thermocellum* converted corn stover lignocellulose (5 g/L loading based on cellulose content) to H<sub>2</sub> with a maximal rate of 1,102 mL H<sub>2</sub>/L<sub>reactor</sub>/d. The accumulation of up to 28 g lignin/L did not inhibit rate of H<sub>2</sub> production, a promising finding for using lignocellulosic biomass.

- Both total and specific rate of H<sub>2</sub> production was increased by nearly 1.5-fold in log-phase *C. thermocellum* culture when its formate competing pathway was deleted using the NREL proprietary genetic tools. This mutant yielded 1.6-fold more ethanol, which prompted its deletion, an ongoing effort in FY 2014 to further improve H<sub>2</sub> output.
- Obtained a volumetric current density of 78–110 A/m<sup>3</sup> by treating NREL fermentation wastewater in an MREC with an 8 h HRT and a reverse electro dialysis stack potential of 0.6–0.75 V. The maximum hydrogen production rate was 0.9 L-H<sub>2</sub> L<sub>reactor</sub><sup>-1</sup> d<sup>-1</sup> with a chemical oxygen demand (COD) removal of 60% and an H<sub>2</sub> yield of 1.0 L H<sub>2</sub>/g COD. Increased HRT to 24 h resulted in an increase in COD removal to 73%, but decreased H<sub>2</sub> production rates to 0.3 L-H<sub>2</sub> L<sub>reactor</sub><sup>-1</sup> d<sup>-1</sup> and H<sub>2</sub> yields to 0.8 L H<sub>2</sub>/g COD.



## INTRODUCTION

Biomass-derived glucose feedstock is a major operating cost driver for economic hydrogen production via fermentation. The DOE Fuel Cell Technologies Office is taking advantage of the DOE Bioenergy Technology Office's investment in developing less expensive glucose from biomass to meet its cost target of 10 cents/lb by 2015. Meanwhile, one alternative and viable approach to addressing the glucose feedstock technical barrier (AZ) is to use certain cellulose-degrading microbes that can ferment biomass-derived cellulose directly for hydrogen production. One such model microbe is the cellulose-degrading bacterium *Clostridium thermocellum*, which was reported to exhibit one of the highest growth rates using crystalline cellulose [1]. Another technical barrier to fermentation is the relatively low molar yield of hydrogen from glucose (mol H<sub>2</sub>/mol sugar; technical barrier AX), which results from the simultaneous production of waste organic acids and solvents. Biological pathways maximally yield 4 moles of H<sub>2</sub> per 1 mole of glucose (the biological maximum) [2]. However, most laboratories have reported a molar yield of 2 or less [3,4]. Molecular engineering to block competing pathways is a viable option toward improving H<sub>2</sub> molar yield. This strategy had resulted in improved H<sub>2</sub> molar yield in *Enterobacter aerogenes* [5].

A promising parallel approach to move past the biological fermentation limit has been developed by a team of scientists led by Prof. Bruce Logan at Pennsylvania State University. In the absence of O<sub>2</sub>, and by adding a slight amount of negative potential (–250 mV) to the circuit, Logan's group has produced hydrogen from acetate (a fermentation byproduct) at a molar yield of 2.9–3.8 (versus a theoretical maximum of 4) in a modified microbial fuel

cell called an MEC [6]. It demonstrates for the first time a potential route for producing eight or more moles of hydrogen per mole glucose when coupled to a dark fermentation process. In FY 2009 the team reported a combined molar yield of 9.95 when fermentation was coupled to MEC in an integrated system [7]. Combining fermentation with MEC could therefore address technical barrier AX and improve the techno-economic feasibility of hydrogen production via fermentation.

## APPROACH

NREL's approach to addressing feedstock cost is to optimize the performance of the cellulose-degrading bacterium *C. thermocellum* using corn stover lignocellulose as the feedstock. To achieve this goal, we are optimizing the various parameters in a sequencing fed-batch reactor to improve longevity, yield, and rate of H<sub>2</sub> production. To improve H<sub>2</sub> molar yield, we are selectively blocking competing metabolic pathways in this organism via genetic methods. Through a subcontract, Pennsylvania State University is testing the performance of an MEC and MREC using both a synthetic effluent and the real waste stream from lignocellulosic fermentation generated at NREL.

## RESULTS

### Lignocellulose Fermentation

Lignocellulose is a solid substrate, and, with continuous feeding, the system will eventually suffer from clogging of feed lines and over-exhaustion of the feed pump. A more feasible strategy for lignocellulose fermentation is to feed the substrate at a predetermined interval instead of using continuous feeding. This strategy can be realized via the use of a sequencing fed-batch bioreactor. This method also simultaneously retains the acclimated microbes to increase the H<sub>2</sub> production rate. We carried out the experiment in a Sartorius bioreactor with a working volume of 2 L. The medium was continuously sparged with N<sub>2</sub> at a flow rate of 16 ccm and agitated at 100 rpm. We used an HRT of 48 h, a liquid displacement of 50% working volume every 24 h, and four cycles each of carbon loadings of 2.5, 5.0, or 10 g/L of cellulose (with lignocellulose feedstock concentration adjusted based on cellulose content). The pretreated lignocellulose material contained 59% glucan, 3.9% xylan and 27.5% lignin, generated via acid hydrolysis, kindly supplied by the NREL National Bioenergy Center. In general the rate of H<sub>2</sub> increased proportionally when the cellulose substrate was increased from 2.5 g/L/d to 10 g/L/d, despite an accumulation of the undigested lignin, up to 28 g/L (Table 2). This finding is promising and suggests that lignin does not inhibit fermentation, a concern when fermenting lignocellulosic biomass. Total H<sub>2</sub> produced at 10 g/L/d feeding, however, was not proportional to that at

5 g/L/d, suggesting not all carbon substrate is consumed at this feeding.

**TABLE 2.** Rate and Yield of Hydrogen Production in Sequencing Fed-Batch Bioreactor with *Clostridium thermocellum* Fermenting Corn Stover Lignocellulose

Pretreated Corn Stover (g L <sup>-1</sup> d <sup>-1</sup> )	HRT (h)	Displacement volume (%)	Average H <sub>2</sub> Production (mL L <sup>-1</sup> D <sup>-1</sup> )	Maximum H <sub>2</sub> Production (mL L <sup>-1</sup> d <sup>-1</sup> )
2.5	48	50	239	474
5.0	48	50	550	974
10	48	50	902	1,466

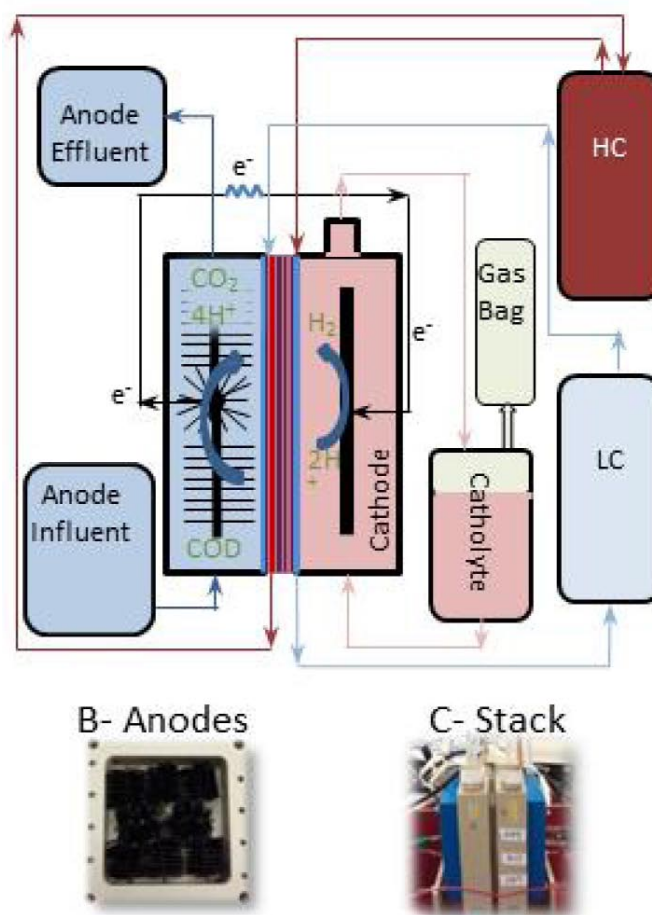
### Metabolic Engineering

The ultimate goal of this approach is to develop tools to inactivate genes encoding competing metabolic pathways, thus redirecting more cellular flux, i.e., electrons, to improve H<sub>2</sub> molar yield. Transformation in *C. thermocellum* has been challenging, likely due to either an inefficiency of the plasmids used or an incompatibility of the DNA restriction system between the host and the plasmid [8]. To circumvent both challenges, we have achieved the following: (1) redesigned an in-house plasmid with a gram-positive origin of replication suitable for *C. thermocellum*; (2) deleted the *dcm* gene in the *E. coli* host used for cloning purpose; and (3) used *C. thermocellum* strain DSM 1313 as the model cellulose-degrader. Following the protocols developed by Argyros et al. [9], we have created a mutant lacking the gene of interest encoding a specific competing pathway. The mutant yielded nearly 60% more ethanol. We have since used this strain as the recipient to delete the bifunctional acetaldehyde/ethanol dehydrogenase encoded by the *adhE* gene. We have undergone one round of selection based on resistance to the thiamphenicol antibiotic (indicating introduction of the plasmid), followed by two rounds of counter-selections using two suicide substrates (leading to gene knockout and loss of the plasmid). Work is ongoing to confirm the genotype via polymerase chain reaction, followed by phenotyping to quantify both ethanol and H<sub>2</sub> in the triple mutant lines.

### Microbial Reverse-Electrodialysis Electrolysis Cell

The larger, redesigned MREC (Figure 1) had a total (anode and cathode chamber) volume of 315 mL. The reactor was initially fed and tested using a sodium acetate (0.8 g COD/L) amended 100 mM sodium bicarbonate buffer solution, followed by a synthetic fermentation effluent (1.2 g COD/L) containing glucose, lactate, ethanol, and bovine serum albumin in addition to the acetate. Following that, the substrate was shifted to diluted fermentation wastewater (1.2 g COD/L) provided by NREL. The catholyte was 1 M sodium bicarbonate (55 mS/cm) which was recycled at a rate of 8 mL/min. The reverse electrodialysis stack (10 cell pairs)

### A- MREC Design



B- Anodes



C- Stack



**FIGURE 1.** MREC Flow Diagram

was operated with 10 L of 1.4 M ammonium bicarbonate (90 mS/cm) as the high concentrate solution and distilled water (1 μS/cm) as the low concentrate solution, which were fed through the stack and recycled at a flow rate of 300 mL/min until the stack potential dropped to a point where there was minimal H<sub>2</sub> production. The anolyte HRT was varied between 8, 12 and 24 h.

At higher HRT (24 h), the anode potential was unstable, but at the shorter HRT (8 h), the anode potential stabilized and the MREC produced higher current densities, suggesting that there is a level where low COD along the length of the anode chamber severely limits current density (Figure 2). The maximum volumetric current densities obtained with fermentation wastewater (78–110 A/m<sup>3</sup>, 8 h HRT, 0.6–0.75 V stack potential) decreased slightly compared to that obtained with the synthetic wastewater (100–130 A/m<sup>3</sup>). H<sub>2</sub> production rate increased from 0.3 to 0.9 L-H<sub>2</sub> L<sub>reactor</sub><sup>-1</sup> d<sup>-1</sup> and H<sub>2</sub> yields increased from 0.8 to 1.0 L/g COD when HRT was decreased from 24 h to 8 h (Figure 3). The composition of gas collected

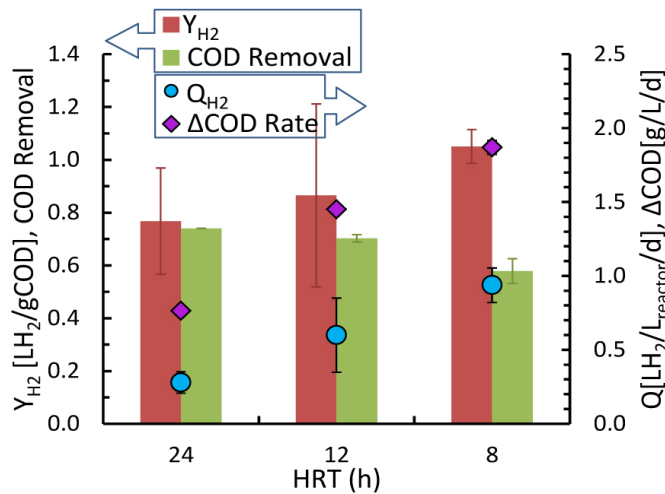


FIGURE 2. Current Generation from Continuous flow MREC using Fermentation Wastewater at HRTs of 8, 12 and 24 h

was 93–96% H<sub>2</sub> with the balance as CO<sub>2</sub>. COD removal decreased from 73% to 60% with the decrease in HRT, but the rate at which COD was removed increased from 0.8 g/L/d to 1.9 g/L/d (Figure 3).

**Protein Removal**

MEC anodes were conditioned separately to degrade acetate or protein in order to increase the protein removal while maximizing H<sub>2</sub> production by utilizing a system with

two reactors in series. Acetate fed anodes would not produce appreciable current, and current levels in protein fed reactors were limited on startup, in 100 mM sodium bicarbonate at pH 8.2 to 9. At a buffer pH of 7.6, all anodes started to acclimate rapidly to produce current. Optimal HRT is being tested for both acetate and protein removal and then the reactors will be tested in series.

**CONCLUSIONS AND FUTURE DIRECTION**

- Using corn stover lignocellulose (2.5-10 g/L based on cellulose content) as the substrate in a sequencing fed-batch reactor, an HRT of 48 h, and displacing 50% of the reactor liquid volume at 24 h intervals, rate of H<sub>2</sub> was proportional to the amount of substrate feeding. The outcomes also indicate that the accumulation of up to 28 g lignin/L does not inhibit rate of H<sub>2</sub> production, a promising finding for using lignocellulose as the feedstock.
- Following published protocols and using the NREL proprietary plasmid, we deleted the gene of interest. Its phenotype of increased ethanol production guided the design to delete the ethanol competing pathway. Work is ongoing toward making this triple mutant and quantifying ethanol and H<sub>2</sub> production. The outcome should aid in future site-directed mutagenesis effort by deleting multiple competing pathways to improve hydrogen molar yield.

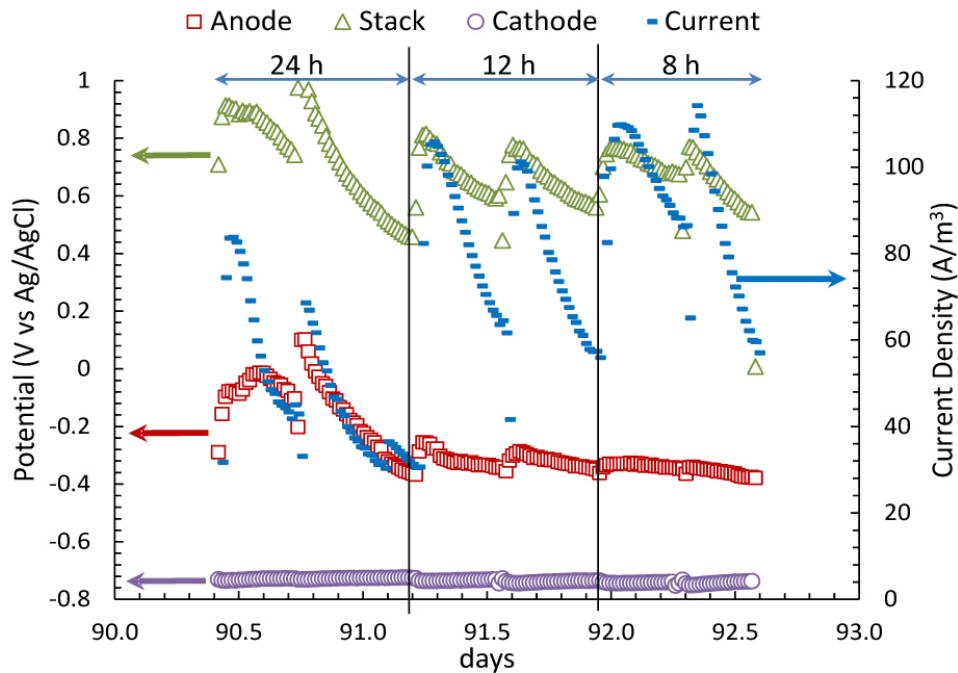


FIGURE 3. Hydrogen Generation and COD Removal from Continuous Flow MREC Treating Fermentation Wastewater at HRTs of 8, 12 and 24 h

- Larger scale MRECs can be used to produce H<sub>2</sub> without net use of electrical grid energy. HRT has a significant impact on the anode potential and the current produced in the MREC. There is a level where the COD drop within the anode chamber negatively impacts the anode potential and severely limits current density and hydrogen production, suggesting the use of a two stage system for increased COD removal with maximum H<sub>2</sub> production rates.

In the future, we will operate the fed-batch bioreactor fermenting corn stover lignocellulose generated from a de-acetylated process vs. the acid-hydrolysis pretreatment used at present. De-acetylation reaction is a milder pretreatment process and hence eliminates the non-specific binding of lignin to cellulose. The latter has rendered the lignocellulose less fermentable. We will determine ethanol and H<sub>2</sub> production profiles in the triple mutants with the outcomes guiding future mutagenesis efforts to delete multiple competing pathways, aimed to improve H<sub>2</sub> molar yield. In the future, we will continue to evaluate the performance of reactors in series, operated at different HRTs and applied potentials, for COD and protein removal efficiency as well as H<sub>2</sub> production rates. We will also be looking at maximizing H<sub>2</sub> production rates while decreasing the electrode cost by exploring the use of non-precious metal cathodes.

## FY 2014 PUBLICATIONS/PRESENTATIONS

1. Wei, H., Y. Fu, L. Magnusson, J.O. Baker, P.C. Maness, Q. Xu, S. Yang, A. Bowersox, I. Bogorad, W. Wang, M.P. Tucker, M.E. Himmel, and S-Y. Ding. 2014. Comparison of transcriptional profiles of *Clostridium thermocellum* grown on cellobiose and pretreated yellow poplar using RNA-Seq. *Frontiers in Microbiology* 5: Article 142. (doi: 10.3389/fmicb.2014.00142).
2. Nam, J.-Y., M. Yates, Z. Zaybak, and B.E. Logan. Factors affecting protein degradability in a continuous microbial electrolysis cell treating fermentation wastewater. *Bioresour. Technol.* Submitted July 2014.
3. Watson, V.J. and B.E. Logan. Effect of hydraulic retention time on hydrogen production and organics removal from fermentation effluent using a continuous flow microbial reverse-electrodialysis electrolysis cell. Kappe Environmental Engineering Seminar, Penn State University, March 6, 2014.
4. “Metabolic engineering of *Clostridium thermocellum* for H<sub>2</sub> production”, oral presentation at the MGCB2 annual review (collaboration with and paid for by the Genome Canada Program). March 18, 2014, Winnipeg, Canada (Chou).
5. Watson, V.J., M. Hatzell, and B.E. Logan. Hydrogen production from fermentation effluent using a continuous flow microbial reverse-electrodialysis electrolysis cell. Poster presentation at NA-ISMET Conference, Penn State University, May 14–15, 2014.
6. “Hydrogen Production from Cellulose in *Clostridium thermocellum*”, poster presentation at the 114<sup>th</sup> General Meeting of the American Society for Microbiology, May 17–20, 2014, Boston (Chou).
7. Maness, P.C. and Logan, B. 2014. DOE Fuel Cell Technology Office Annual Merit Review, June 19 2014, Washington, DC. Presentation PD038.

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