II.D.3 Fermentation and Electrohydrogenic Approaches to Hydrogen Production

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Start Date: October 1, 2004
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 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.

Fiscal Year (FY) 2013 Objectives

- Optimize sequencing fed-batch parameters in converting cellulose to hydrogen by the cellulolytic bacterium *Clostridium thermocellum*, aimed at lowering feedstock cost.
- Develop genetic tools in *C. thermocellum*, aimed at metabolic pathway engineering to improve hydrogen molar yield via fermentation.
- Examine performance of the microbial electrolysis cell (MEC) system with boosted voltages to convert the subcomponents in the NREL fermentation effluent to hydrogen.
- 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.

Technical Barriers

This project supports research and development on DOE Technical Task 6, (Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan) 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) Hydrogen Molar Yield

(AY) Feedstock Cost

(AZ) Systems Engineering

Technical Targets

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

Characteristics	Units	Current Status	2015 Target	2020 Target
Yield of H_2 from glucose	Mole H_2/mole glucose	2–3.2	6*	9
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	$/m^2$	$2,400	$300	$50
MEC production rate	L-H_2/L-reactor-d	1	1	4

*Yield of H_2 from glucose: DOE has a 2015 target of an H_2 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 2013 Accomplishments

- Conducted sequencing fed-batch reactor experiments and demonstrated that by using a hydraulic retention time (HRT) of 24 h and displacing 50% of the reactor liquid every 12 h, the rate of H_2 production and hydrogen molar

yield can be increased by 2.7 fold and 62%, respectively. The improved rates and yield of hydrogen production were realized via retaining those microbes that were adapted to degrade cellulose.

- Designed a proprietary plasmid suitable for deleting genes encoding the competing pathways in *C. thermocellum*. This capability will serve as the foundation for improved hydrogen molar yield in *C. thermocellum* by redirecting more electrons from cellulose toward hydrogen rather than other carbon byproducts.
- Obtained a volumetric current density of 44 A/m^3 with NREL fermentation wastewater in the MEC with 0.9 V applied voltage. The chemical oxygen demand (COD) removal was 73% and the maximum hydrogen production rate was 0.5 L-H_2 L^{-1} d^{-1}. Among the components in fermented wastewater, protein degradability (48% removal) was lower than alcohols and volatile fatty acids (>90%) and carbohydrate (89%). Increased applied voltages resulted in an increase in protein removal, but decreased H_2 yields.

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* (*C. 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_2/mol sugar; technical barrier AX), which results from the simultaneous production of waste organic acids and solvents. Biological pathways maximally yield 4 moles of hydrogen 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_2 molar yield. This strategy had resulted in improved hydrogen 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_2, 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. Indeed, 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*. To achieve this goal, we are optimizing the various parameters in a sequencing fed-batch reactor to improve longevity, yield, and rate of H_2 production. To improve hydrogen 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

Cellulose 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 cellulose 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 rate of hydrogen production. We carried out the experiment in a Sartorius bioreactor with a working volume of 2 L. The medium was continuously sparged with N_2 at a flow rate of 16 ccm and agitated at 100 rpm. We tested HRT of 12, 24, and 48 h, four cycles each, with a constant carbon loading of 5.0 g/L of cellulose. At each constant HRT, we also altered the feeding strategy by displacing a varying portion of the reactor liquid at different time intervals in order to uncover the most optimal parameters. The reactor was initiated by running the fermentation using cellulose at 5 g/L for 36 h. We turned off the agitation for 1 h, during which the unfermented substrate along with the attached microbes settled, then removed 1.5 L (75%) of the clear supernatant and added back 1.5 L of fresh medium

replenished with cellulose (5.0 g/L). In the subsequent four cycles, the HRT was kept at 48 h, yet only 25% of the liquid was displaced at 12-h intervals. This was then followed by four more cycles at an HRT of 24 h with displacement of 50% of the liquid every 12 h. In the end we operated another four cycles with an HRT of 12 h and displaced 50% of the liquid volume every 6 h (Table 2).

Initial results indicate that with an HRT of 48 h, more frequent displacement of a smaller amount of reactor liquid (25% vs. 75%) led to a 38% increase in both rate and yield of hydrogen. This finding strengthens the assumption that more frequent liquid displacement retains the acclimated microbes. Collectively the data suggest that an HRT of 24 h with a 12-h interval of liquid displacement generates the highest production rate and yield of hydrogen. An HRT of 12 h is deemed impractical because it would consume a large volume of liquid.

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

HRT (h)	Liquid Displacement (%)	Interval (h)	Rate of H_2 Production (mL H_2/L/d)	H_2 Molar Yield (mol H_2/mol hexose)
48	75	36	764	0.79
48	25	12	1,057	1.09
24	50	12	2,045	1.28
12	50	6	1,179	1.21

Metabolic Engineering

The ultimate goal of this approach is to develop tools to inactivate genes encoding competing metabolic pathways, thus redirecting more cellular flux to improve hydrogen molar yield. Transformation in *C. thermocellum* has been challenging, likely due to either an inefficiency of the plasmids used or an incompatibility of the deoxyribonucleic acid (DNA) restriction system between the host and the plasmid [8]. To circumvent both challenges, we have achieved the following: (1) redesigned a commercial 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 first generated a Δ*hpt* mutant of *C. thermocellum*, which was then used as the recipient strain to delete a gene of interest encoding a specific competing pathway, based on two rounds of counter-selection using two suicide substrates. The resultant double mutant (Δ*hpt*Δ*goi*) failed to produce the specific product due to the deletion of its encoding gene. The mutant produced equal amounts of total hydrogen when compared to the control, the parental strain. Yet the double mutant produced up to 60% more ethanol and exhibited a nearly 50% increase in specific rate of hydrogen production early in its growth, which warrants further investigation.

Microbial Electrolysis Cell

The prototype two-chamber MEC was designed for improved hydrogen recovery and to eliminate methane production. The reactor had a total liquid volume of 302 mL, and it was initially fed with sodium acetate (synthetic wastewater). Following that, the substrate was shifted to fermentation wastewater from NREL. The HRT was 24 h and the applied voltage was 0.9 V. Catholyte was 50 mM of phosphate buffered saline and was supplied to the cathode chamber. The volumetric current density (44 A/m^3) obtained with fermented wastewater decreased slightly compared to that obtained with the synthetic wastewater (51 A/m^3) due to the more complex substrates in the fermentation effluent (Figure 1). The use of the fermented wastewater also resulted in a slightly lower COD removal of 73%, when compared to an 87% of COD removal using synthetic waste water (acetate). The average gas volume was 159 mL with fermented wastewater, which was slightly lower than that of the synthetic wastewater (183 mL). The maximum hydrogen production rates were 0.5 L-H_2 L^{-1} d^{-1} and 0.6 L-H_2 L^{-1} d^{-1}. Among the components in fermented wastewater, protein degradability (48% removal) was lower than alcohols and volatile fatty acids (>90%) and carbohydrate (89 %).

Although more hydrogen gas was generated at increased applied voltages, H_2 yields were lower with higher applied voltage (Figure 2A). The highest H_2 yield (1.1 H_2 L/g COD) was obtained at E_{ap}= 0.9 V. Also, energy recovery (η_E=223%) was highest at E_{ap}=0.9 V, with overall energy recovery (η_{E+S}) of 64%. The increase in the applied voltages resulted in higher rates of organic matter removals; however, the amount of that organic matter going into current production (Coulombic efficiency) decreased, suggesting that this

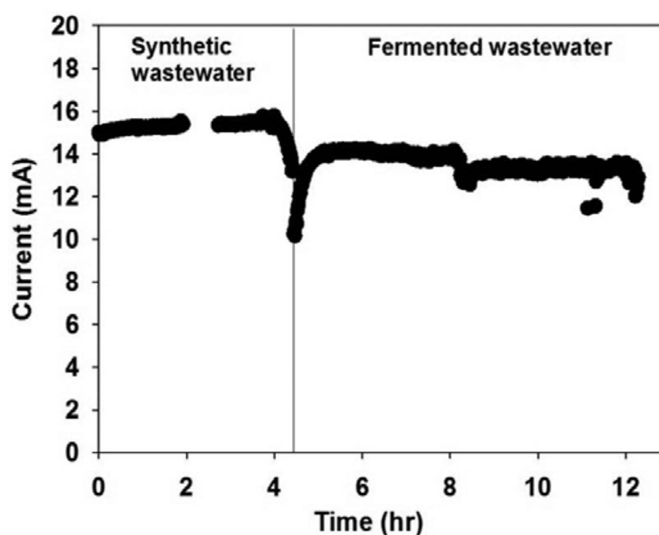

Figure 1. Current Generation from MEC using Synthetic Wastewater (Sodium Acetate) and Fermented Wastewater

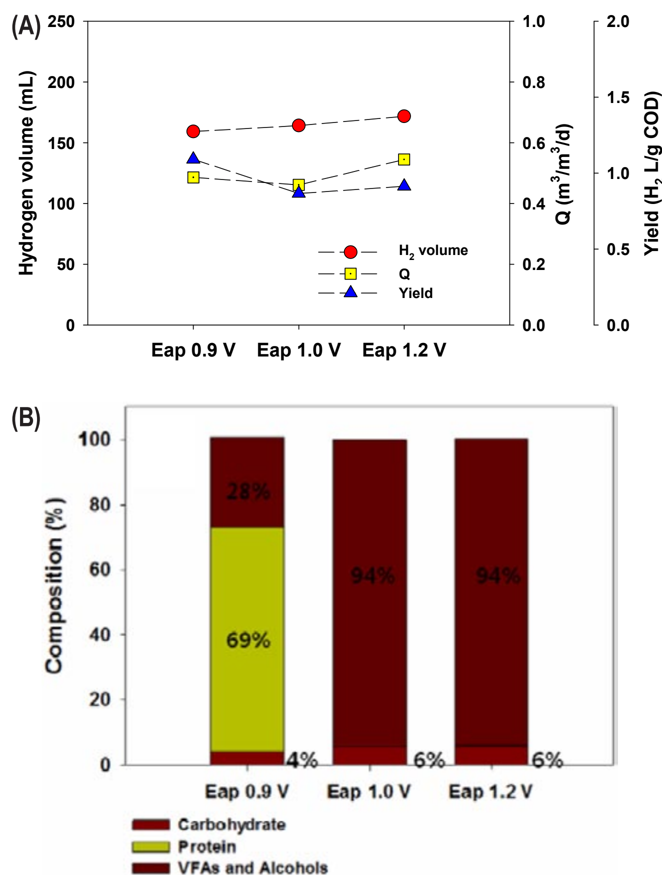

Figure 2. MEC Performance with Different Applied Voltage: (A) hydrogen generation and (B) effluent composition removal as a function of applied voltage

increase in removal did not benefit hydrogen production. Amongst the components in the MEC effluents generated at different applied voltage, protein was almost completely removed at higher applied voltage (Figure 2B). Degraded protein seemed to be used more for cell growth than electricity generation.

Microbial Reverse-Electrodialysis Electrolysis Cell

The MREC was redesigned to examine its scalability for hydrogen production from fermentation effluent without an external energy input. The MREC reactor (Figure 3) was modified to create larger anode (150 mL) and cathode (165 mL) chambers.

CONCLUSIONS AND FUTURE DIRECTION

- Using cellulose (5 g/L) as the substrate in a sequencing fed-batch reactor, we determined that using an HRT of 24 h and displacing 50% of the reactor liquid volume at 12-h intervals resulted in the highest production rate and yield of hydrogen (2.7-fold and 62% increase, respectively). Longer HRT led to retaining cultures in the inactive stationary phase of growth while shorter HRT resulted in large water consumption.
- We redesigned a commercial plasmid suited for transformation in *C. thermocellum*. We also deleted the *dcm* gene in the *E. coli* host for cloning purposes to ensure compatibility of the plasmid DNA with that in the *C. thermocellum* host. Following published protocols, we deleted the gene of interest. Yet the resultant mutant produced the same amount of total hydrogen comparing to its parental control. Nevertheless we observed a 60% increase in ethanol production as well as a 50% increase in specific rate of hydrogen production in the mutant due to the deletion of the competing pathway. The outcome should aid in future site-directed mutagenesis by deleting multiple competing pathways to improve hydrogen molar yield.
- Using an applied potential is an effective method for producing hydrogen gas. The increase in applied voltages results in higher set anode potentials, but the cathode potential does not appreciably vary. Hydrogen gas production is increased with applied potentials, as is

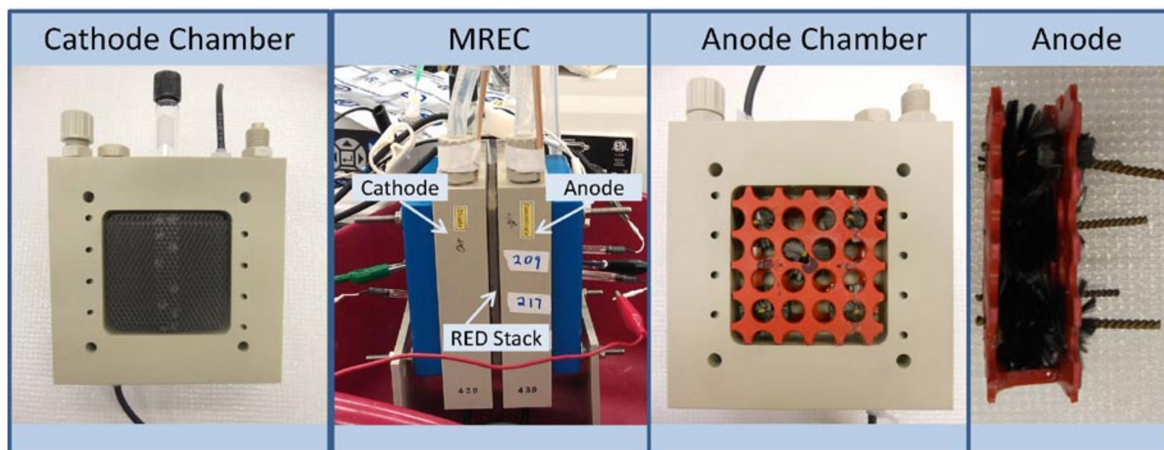

FIGURE 3. Modified MREC Reactor

COD removal. More of the protein in the fermentation effluent can be removed at the higher applied voltages.

In the future, we will operate the sequencing fed-batch bioreactor fermenting lignocellulose derived from corn stover (vs. pure cellulose) and test an HRT of 24 h with liquid volume displacement at 12-h intervals. We will test other bioreactor parameters (HRT and percent displacement) just to ensure these parameters are not substrate-dependent. We will continue to generate pathway mutants by deleting multiple competing pathways to realize an increase in hydrogen molar yield. One such candidate pathway is the ethanol pathway, since the production of one mole of ethanol consumes two moles of nicotinamide adenine dinucleotide, which could be redirected toward hydrogen production. In the future, the performance of the newly redesigned MREC will be examined and operating conditions such as HRT, salinity ratios, and concentrations of the saline solution will be optimized.

FY 2013 PUBLICATIONS/PRESENTATIONS

1. Levin, D., J. Jo, and P.C. Maness. 2012. "Biohydrogen production from cellulosic biomass." Book chapter in Integrated Forest BioRefineries, Royal Society of Chemistry, Cambridge, UK.

2. Thammannagowda, S., L. Magnusson, J.H. Jo, P.C. Maness, and M. Seibert. 2013. Renewable hydrogen from biomass. In Encyclopedia of Biol. Chem. 4: 72-75.

3. Nam, J.-Y., R.D. Cusick, Y. Kim, and B.E. Logan. 2012. Hydrogen generation in microbial reverse-electrodialysis electrolysis cells using a heat-regenerated salt solution. *Environ. Sci. Technol.* 46(9): 5240-5246.

4. Nam, J-Y., and B.E. Logan. 2012. Optimization of catholyte concentration and anolyte pHs in two chamber microbial electrolysis cells. *Int. J. Hydrogen Energy.* 37(24):18622-18628.

5. "Hydrogen production from cellulose in *Clostridium thermocellum*", poster presentation at the Clostridium XII - International Conference on the Genetics, Physiology and Biotechnology of Solvent- and Acid-forming Clostridia, Sept. 10–12, 2012, Nottingham, UK (Chou).

6. "Metabolic engineering in *Clostridium thermocellum* for hydrogen production", invited presentation at the Annual MGCB2 Review. November 5–6, 2012, Winnipeg, Canada (Maness) – paid for by Genome Canada Program.

7. "Hydrogen production via the fermentation of lignocellulosic biomass in *Clostridium thermocellum,"* poster presentation at the MGCB2 Annual Review. November 5–6, 2012, Winnipeg, Canada (Chou) – paid for by Genome Canada Program.

8. Logan, B.E. Microbial fuel cells meet salinity gradient energy. *Invited talk*, Science Writers Workshop, CASW New Horizons in Science, Raleigh, North Carolina, October 29, 2012.

9. Maness, P.C. and Logan, B. 2013. DOE Fuel Cell Technology Program Review, May 2013, Washington, DC. Presentation PD038.

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9. Argyros, D.; Tripathi, S.A.; Barrett, T.F.; Rogers, S.R.; Feinberg, L.F.; Olson, D.G.; Foden, J.M.; Miller, B.B.; Lynd, L.R.; Hogsett, D.A.; Caiazza, N.C. (2011). High ethanol titers from cellulose by using metabolically engineered thermophilic anaerobic microbes. *Appl. Environ. Microbiol.* **77**, 8288–8294.