II.H.2 Fermentation and Electrohydrogenic Approaches to Hydrogen Production

Pin-Ching Maness (Primary Contact), Katherine Chou, Lauren Magnusson, and Shiv Thammannagowda
National Renewable Energy Laboratory (NREL)
1617 Cole Blvd.
Golden, CO 80401
Phone: (303) 384-6114
E-mail: pinching.maness@nrel.gov

DOE Manager
HQ: Roxanne Garland
Phone: (202) 586-7260
E-mail: Roxanne.Garland@ee.doe.gov

Subcontractor:
Bruce Logan
Pennsylvania State University, University Park, PA

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Fiscal Year (FY) 2011 Objectives

- Perform hydrogen fermentation using cellulolytic bacteria and lignocellulosic biomass to lower feedstock cost.
- Perform metabolic pathway engineering to improve hydrogen molar yield via fermentation.
- Develop microbial electrolysis cell to improve hydrogen molar yield using waste from the fermentation of lignocellulosic biomass.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section (3.1.4) of the Hydrogen, Fuel Cells and Infrastructure Technologies Program's Multi-Year Research, Development and Demonstration Plan:

(AR) H_2 Molar Yield
(AS) Waste Acid Accumulation
(AT) Feedstock Cost

Technical Targets

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

Characteristics	Units	2013 Target	2018 Target	2011 Status
Yield of H_2 from glucose	mole H_2/mole glucose	4	6	3.2
Feedstock cost	cents/lb glucose	10	--	12

Yield of H_2 from glucose: DOE has a 2013 target of an H_2 molar yield of 4 using glucose as the feedstock. In FY 2010 we achieved a molar yield of 3.2, accomplished by *Clostridium thermocellum* fermenting avicel (commercial cellulose) via fermentation only.

Feedstock cost: The DOE Biomass Program is conducting research to meet its 2013 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 2011 Accomplishments

- Conducted sequencing fed-batch reactor experiments and determined hydraulic retention time and optimal amounts of solid feeding in bioreactor using the cellulose-degrading bacterium *C. thermocellum* fermenting avicel. We realized improved rates of H_2 production via retaining those microbes that were adapted to degrade cellulose.
- Using a custom-designed plasmid (University of Manitoba, Canada) and improved transformation protocols, we obtained two mutant lines of *C. thermocellum*, which serve as the foundation for future genetic engineering effort in this microbe.
- A prototype two-chamber microbial electrolysis cell (MEC) was designed, constructed, and tested to eliminate methane generation. The reactor was operated at three different hydraulic retention times (HRTs; 24 h, 16 h, and 10 h) and produced H_2 gas at a maximum rate of up to 191 ± 34 mL/d and a maximum volumetric current of 62 ± 1 A/m^3 at HRT 10 h. Using this new reactor design, nearly pure hydrogen was obtained.

Introduction

Biomass-derived glucose feedstock is a major operating cost driver for economic H_2 production via fermentation. The DOE Fuel Cell Technologies Program is taking advantage of the DOE Biomass Program's investment in

developing less expensive glucose from biomass to meet its cost target of 10 cents/lb by 2013. Meanwhile, one alternative and valid approach to addressing the glucose feedstock technical barrier (AT) is to use certain cellulose-degrading microbes that can ferment cellulose directly for H_2 production. One such example is the cellulose-degrading bacterium *Clostridium thermocellum* 27405 (*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 H_2 from glucose (mol H_2/mol sugar; Technical Barrier AR), which results from the simultaneous production of waste organic acids and solvents. Biological pathways maximally yield 4 mole of H_2 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 H_2 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 Bruce Logan at Pennsylvania State University (PSU). 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 H_2 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 mole of H_2 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 barriers AR and AS (waste acid accumulation) and improve the techno-economic feasibility of H_2 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 H_2 molar yield, we are selectively blocking competing metabolic pathways in this organism via developing genetic methods. Via a subcontract, PSU is testing the performance of a MEC 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 continuous feeding will eventually suffer from clogging of feed lines and over-exhaustion of the feed pump. A more feasible strategy for cellulose fermentation is via feeding the substrate at a predetermined interval in lieu of continuous feeding. This strategy can be realized via the development of a sequencing fed-batch bioreactor. This method also simultaneously retains the acclimated microbes to increase rate of H_2 production. We carried out the experiment in an Electrolab bioreactor with a working volume of 1 L. The medium was continuously sparged with N_2 at a flow rate of 16 ccm and agitated at 100 rpm. The HRT tested was 48 h with a daily carbon loading of 2.5 or 5.0 g/L of avicel. The reactor was initiated by running the fermentation using avicel at 2.5 g/L for 24 h, turning off the agitation for 1 h during which the unfermented substrate along with the attached microbes settled, followed by removing 500 ml of the clear supernatant, and adding back 500 ml fresh medium replenished with avicel (2.5 or 5.0 g/L). We completed a total of eight cycles, four cycles for each carbon loading condition (Table 2).

Initial results indicate that 5.0 g/L loading works better than 2.5 g/L, the residual avicel from the former caused retention of the acclimated *C. thermocellum*, which displayed an intense yellow color. Higher substrate also leads to faster rate of H_2 production. One of the benefits of sequencing batch fermentation is the decrease in the lag phase upon subsequent substrate feedings, once adapted. This is shown as a dramatic decrease in "time to peak H_2 production" (Table 2). The t_1 in batch one was almost 19 h, yet by the fourth cycle, it has dropped to mere 4 h, providing compelling evidence as to feasibility of the sequencing fed-batch process in fermenting solid substrates.

TABLE 2. Rate of Hydrogen and Metabolite Production in Sequencing Fed-batch Bioreactor with *Clostridium thermocellum* Fermenting Avicel Substrate

Batch	Avicel Concentration	Time to peak H_2 production	Amount of H_2 produced	Average H_2 Production Rate	Lactate	Formate	Acetate	Ethanol
	(g/L)	(t, h)	(mmoles)	(mmol L^{-1} h^{-1})	(mmol L^{-1})			
1	2.5	18:43	14.92	0.60	1.98	3.06	28.15	18.46
2-4		4:14	13.85					
5	5.0	8:09	17.57	0.92	13.07	5.02	40.79	32.12
6-8		5:27	22.11					

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 H_2 molar yield. Transformation in this organism has been challenging likely due to either an inefficiency of the plasmids used or an active restriction system in the host thus destroying the incoming plasmid. NREL established an active collaboration with the researchers from University of Manitoba, Canada. Using their proprietary plasmid along with optimized protocols, we successfully generated two mutant lines in *C. thermocellum* harboring the plasmid. The success is based on two lines of evidence: (a) growth in the antibiotic chloramphenicol (100 mg/L) (Figure 1A); and (b) polymerase chain reaction (PCR) of the chloramphenicol-encoding gene (cm^r) in the transformant (Figure 1B). For the latter, briefly, plasmid deoxyribonucleic acid (DNA) was isolated from *C. thermocellum* transformants using Qiagen mini prep kit protocol except 20 mg/ml lysozyme was added to the buffer for cells lysis. PCR was carried out using the primer for chloramphenicol-resistance gene. We obtained the anticipated 218 bp PCR product and confirmed the presence of plasmids in the two transformants tested (Figure 1B). Moreover, plasmid DNA was isolated from four *C. thermocellum* transformants and transformed to the *E. cloni* elite electro-competent cells (Lucigen, WI, USA). Plasmid DNA was then isolated from the *E. cloni* transformants and resulted in the restriction pattern with EcoRI (1.2 kb fragment) that is consistent with the presence of the correct proprietary plasmid in *C. thermocellum* (data not shown).

Microbial Electrolysis Cell

Previously, a 2.5 L-single chamber MEC equipped with eight electrode pairs was used to produce H_2 under continuous flow conditions. However, in this type of single-chamber MEC, all H_2 gas produced was converted to methane. In order to suppress methane production in single chamber MECs, anode potentials were set at different values (from –0.4 V to +0.2 V vs. Ag/AgCl) using a potentiostat. MEC performance with a potentiostat was compared to that obtained with an applied voltage of 0.6 V using a power supply. In batch tests the largest total gas production (46 ± 3 mL) and best overall energy recovery in terms of electrical energy used and substrate energy ($\eta_{E+S} = 58 \pm 6\%$) was achieved at a set anode potential of –0.2 V, and methane production was reduced at the higher set anode potentials (Figure 2). However, although the optimum set anode potential (–0.2 V) suppressed methane generation in batch tests, and stable H_2 was obtained at the beginning of the continuous flow operation, the gas composition became predominantly methane in continuous flow tests. Switching the anode to a new reactor immediately resulted in H_2 production and recovery with little methane production. This indicated that the methane originated primarily from non-anode associated microorganisms in the continuous flow tests.

A new type of tubular type MEC was designed and built for additional tests. In this new type of MEC the anode and the cathode chambers were separated by an anion exchange membrane. Although this increases costs, this greatly benefits gas purity and has minimal impact on production rates. The average volumetric current density (I_{vol}) was 59 ± 1 A/m^3 at an HRT of 24 h using acetic acid as a substrate.

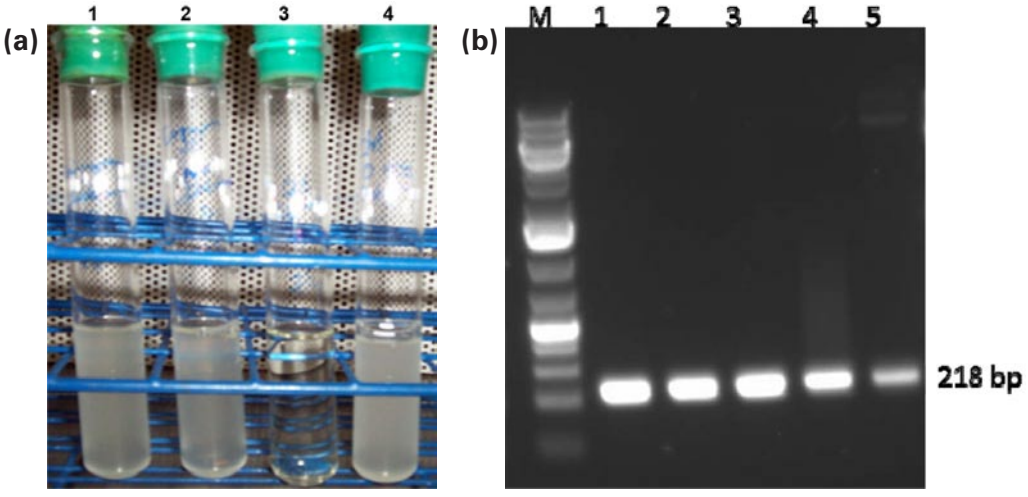

FIGURE 1. (A) Growth of *Clostridium thermocellum* transformants in liquid selection medium. 1. Transformants in 100 mg/L chloramphenicol, 2. Transformants without selection, 3. Control untransformed cells with 100mg/L chloramphenicol, 4. Control untransformed cells without antibiotic selection. (B) PCR amplification of chloramphenicl gene (cm^r) from plasmid DNA isolated directly from transformed *C. thermocellum.* Lanes 1, 2: colony # 1; lanes 3, 4: colony # 3, lane 5: plasmid positive control. M: molecular weight marker.

Shorter HRTs slightly increased the current, with average volumetric current densities of 60 ± 1 A/m^3 (HRT 16 h) and 62 ± 1 A/m^3 (HRT 10 h) (Figure 3). There were similar gas generation rates of 145 ± 34 mL/d at an HRT 16 h and 163 ± 51 mL/d at an HRT 24 h, but gas production increased to 191 ± 34 mL/d at HRT 10 h. This two-chamber MEC produced nearly pure H_2 in continuous mode as a result of the design that kept the cathode separated from the microorganisms on the anode and anode chamber.

Conclusions and Future Direction

- Using avicel cellulose as the substrate, we successfully conducted fermentation in the sequencing fed-batch mode. We determined that 5.0 g/L feeding was capable of retaining more acclimated *C. thermocellum* which attached to the residual unfermented cellulose. The outcomes lead to higher rates and yield of H_2. Retention of more acclimated microbes also significantly decreased lag time and led to a faster rate of H_2 production.
- We successfully developed genetic tools and produced two mutants in *C. thermocellum*. These tools will aid in the construction of targeted pathway mutants to improve yield of H_2.
- In single chamber MECs, methanogenesis was suppressed by using higher anode potentials set by a potentiostat. However, it was revealed that methanogen proliferation could not be eliminated in this reactor because the methanogens were not primarily anode-associated.
- By separating the cathode from biological conditions in a new reactor design, nearly pure H_2 production was successful in a two-chamber continuous flow MEC with reasonable H_2 gas flow rates.

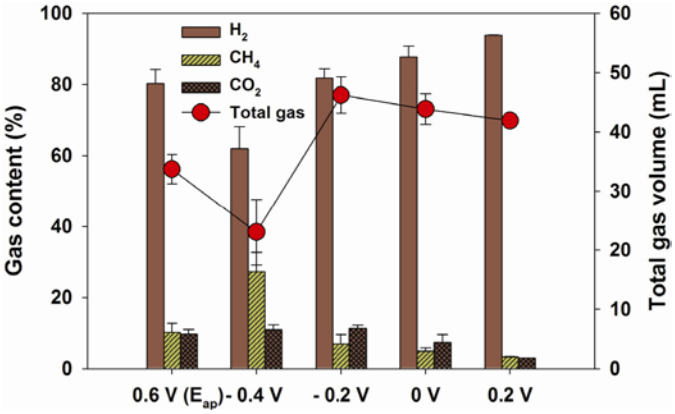

FIGURE 2. Hydrogen, methane, carbon dioxide contents and total gas volume at different set anode potentials and an added voltage of 0.6 V in batch tests.

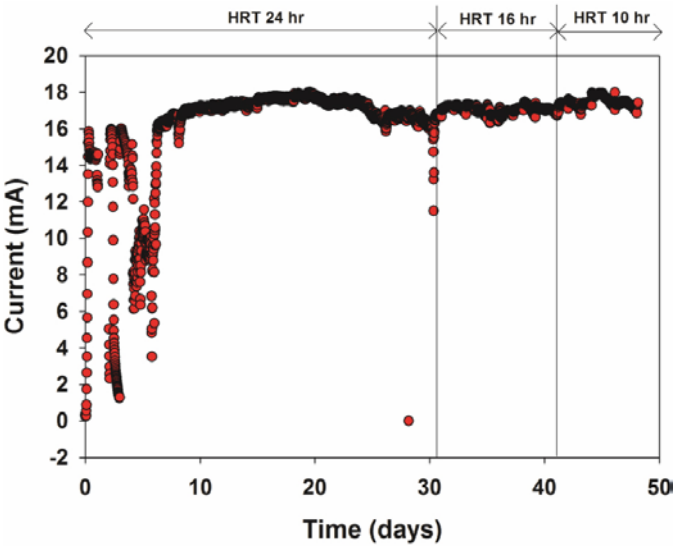

FIGURE 3. Current Generation at Different HRTs in a Two-Chamber MEC

In the future, we will conduct sequencing fed-batch fermentation to further optimize hydraulic retention time, carbon substrate loading, and the volume of liquid replacement to improve rate and yield of H_2 production. We will continue to develop genetic tools for molecular engineering in *C. thermocellum* to alter its metabolic pathway to improve H_2 molar yield. In future MEC tests, following complete analysis of fermentation effluent, fermentation wastewater will be supplied to a two-chamber MEC in order to examine H_2 production from an actual fermentation effluent.

FY 2011 Publications/Presentations

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2. Levin, D.; Jo, J.; and Maness, P.C. 2011. Biohydrogen production from cellulosic biomass. Accepted for publication in "Integrated Forest BioRefineries" (ed. L. Christopher). Royal Society of Chemistry, Cambridge, UK.

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4. Maness, P.C.; Logan, B. DOE Fuel Cell Technology Program Review – Washington, DC – May 2011, Presentation PD#038.

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