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

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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 Fuel Cell Technologies Program 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 Target in Dark Fermentation

Characteristics	Units	2013 Target	2009 Status	2010 Status
Yield of H_2 from glucose	Mole H_2/mole glucose	4	9.95 (Fermentation-MEC Integrated System)	3.2 (Fermentation alone)
Feedstock cost	Cents/lb glucose	10	12	12

MEC = microbial electrolysis cell

- 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 Fiscal Year (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.

Accomplishments

- Determined effects of substrate loadings on both rates and yields of H_2 production in scale-up bioreactor experiments using the cellulose-degrading bacterium *C. thermocellum* fermenting various amounts of avicel cellulose and lignocellulose, the latter prepared from the acid-hydrolysis of corn stover biomass.
- Tested genetic transformation protocols with a custom-designed plasmid in collaboration with University of Manitoba (Canada). Initial finding is promising which warrants further improvement.
- Designed, constructed and tested a bench-scale prototype microbial electrolysis cell of 2.5 L in volume that contained eight pairs of electrodes. The reactor produced H_2 gas at a rate of up to 1,250 mL/d, and produced a steady current of 155 to 180 mA demonstrating the usefulness of this design.

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 inexpensive glucose from biomass to meet its cost target of 8 cents/lb by 2015. 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 hydrogen production. One such example is the cellulose-degrading bacterium *Clostridium thermocellum* 27405 (*C. thermocellum*), which was reported to exhibit the highest growth rate 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 AR), which results from the simultaneous production of waste organic acids and solvents. Biological pathways maximally yield 4 mole 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 Bruce Logan at 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 (MFC) called an MEC [6]. It demonstrates for the first time a potential route for producing 8 or more moles 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. 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 testing various amounts of cellulosic substrates and optimizing reactor parameters to improve longevity, yield, and rate of H_2 production. We are selectively blocking competing metabolic pathways in this organism via developing a genetic method to accomplish this goal. Via a subcontract, PSU is testing the performance of an MEC using both a synthetic effluent and the real waste stream from lignocellulosic fermentation generated at NREL.

Results

Lignocellulose Fermentation

We tested effects of substrate loadings on rates and yields of H_2 production in *C. thermocellum*. Two types of substrates were tested: avicel cellulose and the dilute-acid pretreated corn stover lignocellulose, the latter with a composition of 59.1% cellulose, 25.3% lignin, 6.4% residual hemicellulose-derived sugars, and 3.7% ash. We performed fermentation in scale-up bioreactors with automated temperature (55°C), pH (7.0), and pressure controls. The bioreactor was bubbled with nitrogen (N_2) gas (10 cc/min) to allow real-time sampling of H_2 and carbon dioxide (CO_2) via an online gas chromatograph. *Clostridium thermocellum*, previously cultured in crystalline avicel cellulose, was inoculated into a 1.5 L (working volume) bioreactor fed with various amounts of the individual substrates described above. Table 2 summarizes rates, molar yields of H_2 production, and carbon mass balance during a period of 80 hours. Calculation of the carbon mass balance did not account for those carbon substrates assimilated into bacterial cell mass. These experiments conclude that higher carbon loading leads to faster rate of H_2 production whereas lower carbon loading results in higher H_2 molar yield. The molar yield of 3.2 obtained with 1 g/L avicel was the average of two experiments. Overall, these experiments provide the parameters (hydraulic and solid retention time) to conduct fermentation in fed-batch mode in the more realistic scale-up process. Typical compounds found in the fermentation waste are: acetic, formic, lactic, and ethanol, which are ideal substrates for the MEC reaction.

TABLE 2. Effect of Substrate Loadings on Rates, Yields of H_2 Production in Clostridium Thermocellum

Substrate	g/L	Rate (mmol H_2/L/h)	H_2 Molar Yield	Carbon Balance (%)
Avicel	1.0	0.58	3.2	74
Avicel	2.5	0.89	2.1	70
Avicel	5.0	0.98	1.6	70
Corn Stover	1.0	0.51	2.8	70
Corn Stover	2.5	1.06	2.0	94
Corn Stover	5.0	1.21	1.2	51

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 unsuccessful thus far in the literature likely due to either an inefficiency of the plasmids used or an active restriction system in the host thus destroying the incoming plasmid. To ascertain any restriction endonuclease activity in *C. thermocellum*, crude cell extracts were prepared from a 20-mL culture grown to late-exponential phase, and adjusted to contain 50% (v/v) glycerol. For restriction assays pIKM1 plasmid deoxyribonucleic acid (DNA, isolated from *TOP10* cells) were incubated with a buffer (10 mM Tris/HCl, ph7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.01% bovine serum albumin) at 55°C for 2-3 hr. The products were analyzed on 1% agarose gel (Figure 1). Gel electrophoresis analysis indicated that cellular extract indeed digested the pIKM1 DNA in the cellular extract confirming our previous speculation about the *C. thermocellum* endonuclease activity on the plasmid DNA (Figure 1, lane 2). Plasmid DNA incubated without the cellular extract remained undigested (data not shown). To test the endonuclease activity of *C. thermocellum* crude extract on methylated DNA, plasmid PIKM1 was treated with GpC methylatransferase (M.CviPI) that selectively methylate all cytosine residues at the 5th carbon position within the double stranded DNA sequence. Our results indicated that the methylated pIKM1 DNA was protected from the endonuclease activity of *C. thermocellum* (Figure 1, lane 1). Methylated *C. thermocellum* DNA without cell extract or methylated pIKM1 DNA both serve as the controls (Figure 1, lanes 3 and 5, respectively). The outcomes warrant the importance of plasmid DNA methylation to improve overall transformation efficiency.

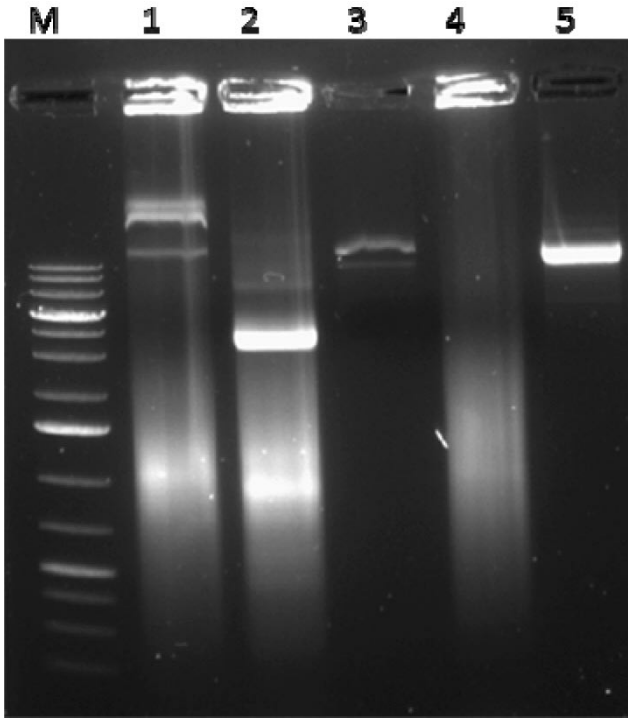

FIGURE 1. Restriction endonuclease activity of *Clostridium thermocellum* crude extract on the methylated pIKM1 plasmid DNA. M: 1kb plus DNA ladder, 1: Methylated pIKM1 DNA, 2: Unmethylated pIKM1 DNA, 3: methylated DNA without the crude extract, 4: crude extract, 5: methylated pIKM1 DNA.

Microbial Electrolysis Cell

Previously examined MECs contained a single set of electrodes and were relatively small (0.03 L) in volume. In order to examine the scalability of the existing single-chamber MEC designs, a multiple-electrode system was designed, constructed and tested for hydrogen production and current density achievable using acetate as a feedstock. The bench-scale MEC was 2.5 L in volume when empty, and contained eight separate electrode pairs made of graphite fiber brush anodes (pre-acclimated for current generation), and 304 stainless steel mesh cathodes (64 m^2/m^3) (Figure 2). Under continuous flow feeding conditions, and a one-day hydraulic retention time, a maximum current of 181 mA was produced within three days of operation. This is 1.18 A/m^2 of cathode surface area, and equivalent to 74 A/m^3. The maximum hydrogen production (day 3) was 0.53 L/L-d, reaching an energy efficiency relative to electrical energy input of η_E= 144%. Current production remained relatively steady (days 3 to 18), but the gas composition dramatically shifted over time from hydrogen to methane. After 16 days of operation, only small amounts of H_2 gas were recovered, and methane production had increased to 0.118 L/L-d. When considering the energy value of both hydrogen and methane, efficiency relative to electrical input remained above 100% until near the end of the experiment (day 17) when only methane gas was being produced. These results showed that MECs could be scaled up primarily based on cathode surface area, but that hydrogen produced in the single-chamber MEC can be completely consumed in a continuous flow system unless methanogens can be completely eliminated from the system.

Conclusions and Future Direction

- Using both avicel cellulose and corn stover lignocellulose as the substrate and a sequenced strain of *C. thermocellum*, we found that low substrate loading gives rise to higher H_2 molar yield while high substrate loading yields faster rate of H_2 production.
- We determined that plasmid DNA methylation is necessary for successful transformation in *C. thermocellum*.

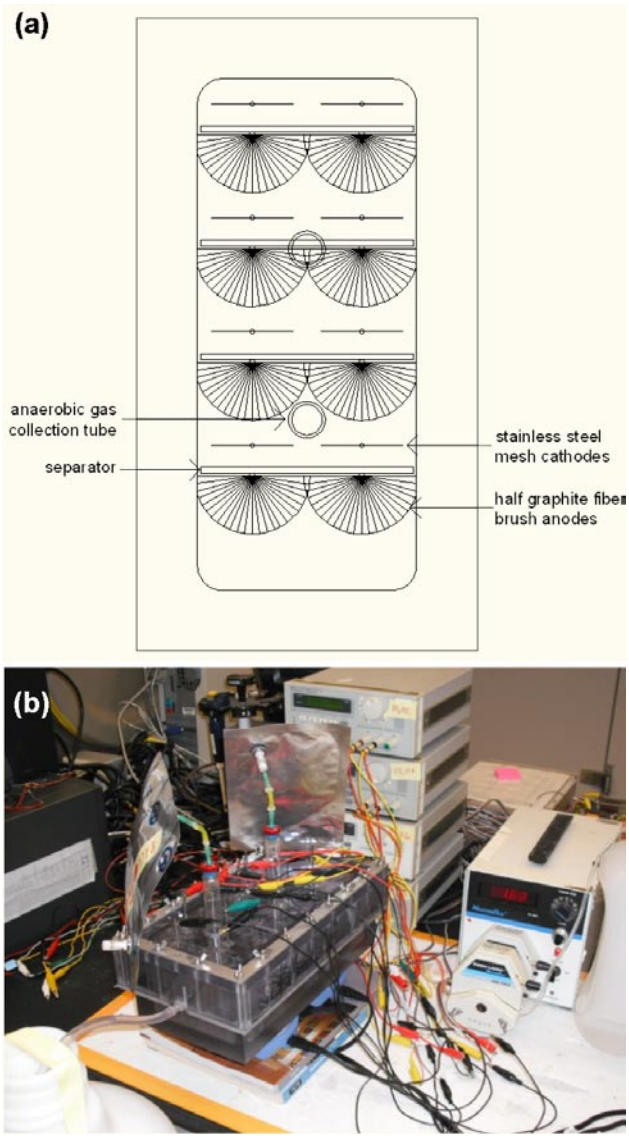

FIGURE 2. Microbial electrolysis cell (a) schematic, and (b) reactor with associated liquid feed pump, wiring and power sources for adding voltage, and gas bags for collection of gas produced.

- Hydrogen production was successful in a larger-scale (2.5 L) continuous flow MEC, but eventually all hydrogen production was converted to methane.

In the future, we will conduct fed-batch fermentation to optimize solid and hydraulic retention time for more realistic scale-up application. We will continue to develop tools for molecular engineering in *C. thermocellum*. In the MEC area, a new reactor design is now in progress to better capture hydrogen gas produced in these systems to avoid its loss to methanogens.

FY 2010 Publications/Presentations

1. Thammannagowda, S; Magnusson, L; Jo, J.H.; Maness, P.C., Seibert, M. 2010. Renewable hydrogen production from biomass. Accepted for publication in *Enclyclo. Biol. Chem*.

2. Rader, G.K. and B.E. Logan. 2010. Multi-electrode continuous flow microbial electrolysis cell for biogas production from acetate. *Int. J. Hydrogen Energy.* In press.

3. Maness, P.C. "Hydrogen from lignocellulose fermentation", Oral presentation at the MGCB^2 Grant kickoff meeting. Winnipeg, Canada. October 23, 2009.

4. Thammannagowda, S. Hydrogen production systems in cellulose degrader *C. thermocellum*, poster presentation at the 1st Annual Review meeting of the MGCB^2 Program. Hamilton, Ontario, Canada, June 12–13, 2010.

5. Maness, P.C. and Logan, B. DOE Fuel Cell Technology Program Review – Washington, D.C. – June 2010, Presentation PD#038.

6. Rader, G. 2010. Effect of long-term operation on MFC performance and the performance of a scale-up continuous flow MEC with an examination of methods to decrease CH_4 production. M.S. Thesis, Department of Civil & Environmental Engineering, Penn State University.

7. Logan, B.E. Development of microbial fuel cell technologies for renewable energy production. *Invited plenary*, Institute of Biological Engineering Annual Conference, Boston, MA, March 4–6, 2010.

8. Logan, B.E. Bioenergy production using microbial fuel cell technologies. *Invited seminar*. MIT Energy Initiative (MITEI) Seminar, Massachusetts Institute of Technology, Cambridge, MA, March 2, 2010.

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6. Cheng, S.; Logan, B. E. (2007). Sustainable and efficient biohydrogen production via electrogenesis. *Proc. Natl. Acad. Sci. USA*. 104: 18871-18873.