

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

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

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*	--
Feedstock cost	Cents/lb glucose	13.5	10	8
Duration of continuous production (fermentation)	Time	17 days	3 months	--
MEC cost of electrodes	\$/m ²	\$2,400	\$300	\$50
MEC production rate	L-H ₂ /L-reactor-d	1	1	---

*Yield of H₂ from glucose: DOE has a 2015 target of an H₂ 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 Biomass Program 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.

Fiscal Year (FY) 2012 Objectives

- Optimize sequencing fed-batch parameters in converting cellulose to hydrogen by the cellulolytic bacterium *Clostridium thermocellum*; aimed at lowering feedstock cost.
- Improve plasmid stability in *C. thermocellum*; aimed at metabolic pathway engineering to improve hydrogen molar yield via fermentation.
- Complete analysis of fermentation effluent as the first step in examining gas production from an actual fermentation effluent.
- Generate hydrogen without an external energy input in a microbial reverse-electrodialysis electrolysis cell (MREC).

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 microbial electrolysis cell (MEC)” and it addresses barriers AX, AY, and AZ.

(AX) H₂ Molar Yield

(AY) Feedstock Cost

(AZ) System Engineering

FY 2012 Accomplishments

- Conducted sequencing fed-batch reactor experiments and demonstrated scalability of the system, with both total hydrogen output and volumetric rate of hydrogen production proportional to the amount of cellulosic substrate added in the bioreactor containing the cellulose-degrading bacterium *C. thermocellum*. The improved rates of H₂ production were realized via retaining those microbes that were adapted to degrade cellulose.
- Plasmid stability was improved by approximately 150-fold by ensuring the compatibility of the deoxyribonucleic acid (DNA) restriction profiles between *C. thermocellum* and the plasmid used for its transformation. This finding will serve as the foundation for a future genetic engineering effort with this microbe.
- Fermentation wastewater produced from NREL (from a sequencing fed-batch fermentation reactor fed with 5 g/L cellulose) was analyzed in terms of volatile fatty acids (VFAs), alcohols, carbohydrates, and proteins. In the soluble chemical oxygen demand (COD) (10,810 ± 21 mg/L), protein (33%) was a main component, with fewer carbohydrates present (12%). Alcohols and VFAs accounted for 18% and 28% of the soluble COD, respectively. Only 9% of the soluble COD could not be identified.

- Hydrogen gas was successfully produced without an external electrical energy input in an MREC using salinity gradient energy. In order to generate salinity gradient energy, we used ammonium bicarbonate salts, which can be regenerated using low-temperature waste heat as a saline solution in the reverse electrodialysis (RED) stack. The maximum hydrogen production rate was $1.6 \text{ m}^3 \text{ H}_2/\text{m}^3\cdot\text{d}$, with a hydrogen yield of $3.4 \text{ mol H}_2/\text{mol acetate}$ at an essentially infinite salinity ratio (SR) (distilled water as the low concentration solution).



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 2015. Meanwhile, one alternative and viable approach to addressing the glucose feedstock technical barrier (AY) is to use certain cellulose-degrading microbes that can ferment biomass-derived cellulose directly for H_2 production. One such example 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 H_2 from glucose ($\text{mol H}_2/\text{mol sugar}$; technical barrier AX), which results from the simultaneous production of waste organic acids and solvents. Biological pathways maximally yield 4 moles of H_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 Prof. 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 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 [7]. Combining fermentation with MEC could therefore address technical barriers AX 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 hydrogen molar yield, we are selectively blocking competing metabolic pathways in this organism via genetic methods. Through 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

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 in lieu of 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. The hydraulic retention time (HRT) tested was 48 h with a daily carbon loading of 2.5, 5.0, or 10.0 g/L of cellulose, four cycles each. The reactor was initiated by running the fermentation using cellulose 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, then removing 1 L of the clear supernatant and adding back 1 L of fresh medium replenished with cellulose (2.5, 5.0, or 10.0 g/L). We completed a total of 12 cycles, four cycles for each carbon loading condition (Table 2).

Initial results indicate that when cellulose substrate loading was increased from 2.5 g/L to 5.0 g/L, both total hydrogen output and volumetric rate of hydrogen production increased proportionally. This finding demonstrates the scalability of the system between two substrate loadings, a very important criterion in a scale-up process. However, proportionally less hydrogen is produced with cellulose at 10 g/L compared to the other loadings; largely this is because not all the substrate was consumed at this loading. The operating principle of sequencing fed-batch fermentation is to have a small amount of excess cellulose substrate to retain acclimated microbes during each cycle of draining and feeding. The excess substrate hence lowers both the total hydrogen output and the hydrogen molar yield (Table 2), as the latter is calculated based on substrate added, not substrate consumed.

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

Cellulose Concentration	Amount of H ₂ produced	Max H ₂ production rate	H ₂ Yield	H ₂ /CO ₂
(g/L/day)	(mmol)	(mmol/L-h)	(mol H ₂ /mol hexose)	
2.5	18.5	1.2	1.2	1.2
5	35.3	2.6	1.1	1.3
10	51.9	3.5	0.84	1.4

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 H₂ 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 the University of Manitoba, Canada. Using their proprietary plasmid along with optimized protocols, in 2011 we successfully generated via conjugation two mutant lines in *C. thermocellum* harboring the plasmid. However, we discovered later that the plasmids were lost in the *C. thermocellum* transformants after several sub-culturings, likely attributed to an incompatibility of the DNA restriction system between the host and the plasmid [8]. To circumvent this issue, we (1) tested the effect of *dcm* gene knockout in *E. coli* S17-1; and (2) compared two recipient hosts of *C. thermocellum*: ATCC 27405 and DSM 1313. The *E. coli dcm* gene encodes a DNA methylase that specifically methylates the internal cytosine residues of DNA in the sequences of CCAGG and CCTGG at the C⁵ position. Results of colony formation in different strains are summarized in Table 3. When using DSM 1313 as the recipient, conjugating with a Δdcm *E. coli* mutant results in a more than 150-fold increase in the number of colonies growing on a chloramphenicol (Cm, 30 mg/mL) plate (comparing rows 2 and 3, Table 3). While using the same Δdcm *E. coli* strain for conjugation, DSM 1313 conjugants formed more than 450 colonies on a Cm plate, whereas wild-type ATCC 27405 conjugants formed only five colonies on the same plate (comparing rows 1 and 3, Table 2). Based on our data, we concluded that Δdcm *E. coli* and DSM 1313 render better conjugation efficiency for future metabolic engineering efforts. This finding also confirmed the observation reported by Guss et al. [8].

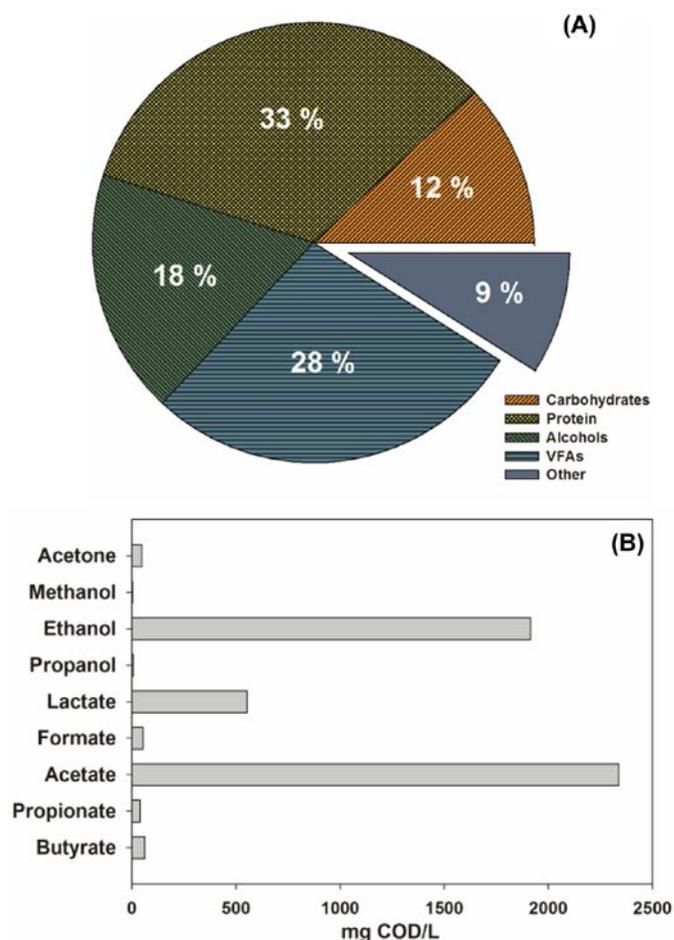
Microbial Electrolysis Cell

Fermentation wastewater produced from NREL was analyzed and found to be composed of proteins,

TABLE 3. *E. coli* and *Clostridium thermocellum* Conjugation Efficiencies

Conjugation Pair	Cm (μ g/mL)	Growth (Colonies)
1. <i>E. coli (dcm-)</i> /ATCC 27405	30	5
2. <i>E. coli (dcm+)</i> /DSM 1313	30	3
3. <i>E. coli (dcm-)</i> /DSM1313	30	>450

carbohydrates, alcohols, VFAs, and some organic particulates. The total COD of the fermentation effluent was $11,035 \pm 5$ mg/L and the soluble COD was $10,810 \pm 21$ mg/L, which means that 98% of the effluent consists of soluble organic matter. The soluble COD portion was analyzed in terms of VFAs, alcohols, carbohydrates, and proteins (Figure 1A). Soluble COD contained high concentrations of protein (3,600 mg/L, 33% of the soluble COD) and carbohydrates (1,250 mg/L, 12%). Alcohols and VFAs accounted for 46% of the soluble COD (alcohols 1,974 mg/L, VFAs 3,047 mg/L). Alcohols and VFAs were mainly ethanol (1,915 mg/L) and acetate (2,338 mg/L) (Figure 1B). Only 9%

**FIGURE 1.** (A) Composition of the soluble COD ($10,810 \pm 21$ mg/L) and (B) each alcohol and VFA concentration

of the soluble COD was not identified by this analysis. Due to the various techniques used and measurement errors in each technique, it is possible that this value could be smaller.

Salinity-gradient energy from ammonium bicarbonate (NH_4HCO_3) salts was used as the voltage source for making hydrogen gas in an MEC. A RED stack of alternating ion exchange membranes was placed between the anode and the cathode chambers, producing an MREC. A high concentration (HC) NH_4HCO_3 solution was added into the RED stack, with alternating membrane chambers containing a low concentration (LC) solution of this chemical. The HC solution contained 1.4 M NH_4HCO_3 , and the LC solution of NH_4HCO_3 was adjusted to produce different salinity ratios (SRs) of 100, 200, 400, 800, and infinite (distilled water in the LC solution). Current and hydrogen gas were successfully generated in the MREC using only the NH_4HCO_3 solution and no external power supply. The MREC performance was relatively insensitive to the SRs. The peak volumetric current densities varied over a small range of $137 \pm 8 \text{ A/m}^3$ to $152 \pm 8 \text{ A/m}^3$ for the different SRs. Total hydrogen generation ranged from 27 mL H_2 ($Y = 2.8 \text{ mol H}_2/\text{mol acetate}$, $\text{SR} = 200$) to 30 mL H_2 ($Y = 3.4 \text{ mol H}_2/\text{mol acetate}$, $\text{SR} = \text{infinite}$) over each fed-batch cycle. Energy recovery was 10% based on total energy applied, with an energy efficiency of 22% based on the consumed energy in the reactor. A reduction in the HC solution (1.4 M to 0.1 M) with a fixed LC solution ($\text{SR} = \text{infinite}$) decreased current generation and increased the time needed to complete a fed-batch cycle (Figure 2A). The coulombic efficiency substantially decreased with HC solution, from $\eta_{\text{CE}} = 72\%$ using the 1.4 M HC solution to $\eta_{\text{CE}} = 50\%$ using the 0.1 M HC solution. The reduction in current decreased the recovery of hydrogen gas from 30 mL H_2 to 17 mL H_2 (93%–94% H_2 , 6%–7% CO_2) and decreased the gas production rate from $1.6 \text{ m}^3 \text{ H}_2/\text{m}^3 \cdot \text{d}$ to $0.5 \text{ m}^3 \text{ H}_2/\text{m}^3 \cdot \text{d}$ (Figure 2B).

Conclusions and Future Direction

- Using cellulose as the substrate, we successfully demonstrated scalability of cellulose fermentation in the sequencing fed-batch mode. We determined that both hydrogen output and volumetric rate of hydrogen production doubled when the cellulose substrate loading was increased from 2.5 g/L to 5.0 g/L with a HRT of 48 h.
- We knocked out the *dcm* gene in *E. coli* S17-1 (the conjugation host) to have a more compatible DNA methylation system with *C. thermocellum* (both strains become *dcm*). This improved plasmid transfer and maintenance in *C. thermocellum* by at least 150-fold. The outcome should aid in future site-directed mutagenesis of competing pathways to improve hydrogen molar yield.
- The fermentation wastewater was mostly composed of soluble organic matter including proteins, carbohydrates, alcohols, and VFAs. The fact that most of the material is

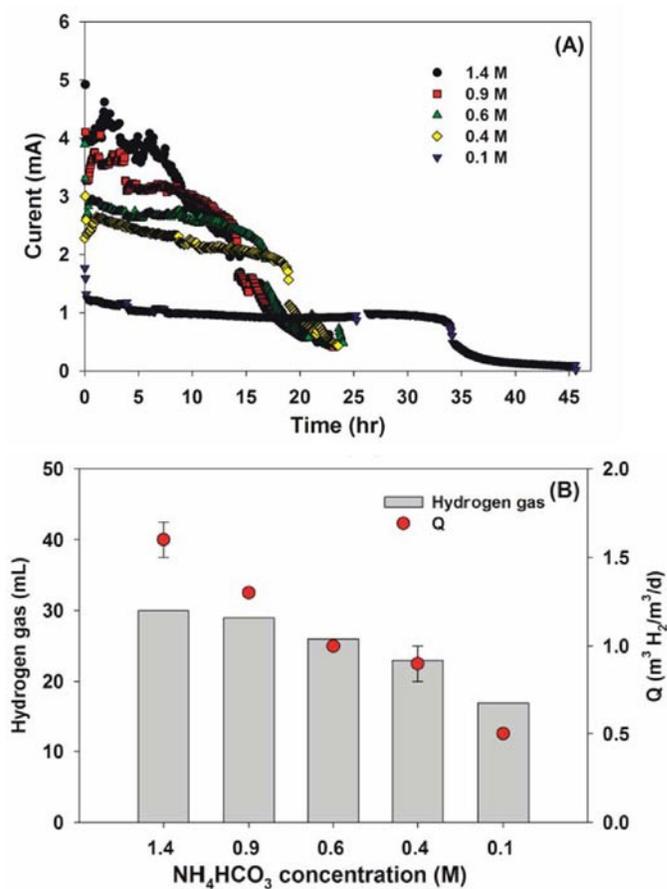


FIGURE 2. MREC performance with different HC concentration: (A) current generation and (B) gas production

soluble will help in converting this material into current and hydrogen gas in the MEC.

- Hydrogen gas was successfully produced without an external energy input in an MREC using salinity gradient energy.

In the future, we will continue to optimize sequencing fed-batch fermentation by testing HRTs of 4 h, 8 h, 12 h, and 24 h. Depending on the HRT, various volumes of liquid replacement will be tested (up to 50% of working volume) aimed at improving both rates and output of hydrogen production as well as cellulose consumption. We will continue to develop genetic tools for molecular engineering in *C. thermocellum* to alter its metabolic pathway to improve H_2 molar yield. We will redesign the plasmid by reducing its size (removing non-essential features) and replacing origin of replication suitable for an electroporation protocol to improve transformation efficiency. In future MEC tests, fermentation wastewater will be supplied to the prototype MEC in order to examine hydrogen production from an actual fermentation effluent. The MEC will be operated in continuous flow mode with the optimum operation conditions that were obtained

with synthetic wastewater (sodium acetate). Also, increased power (more positive anode potentials) will be evaluated in order to enhance treatment efficiency of fermented wastewater. The MREC will be redesigned to examine the scalability of the MREC without an external energy input, and operating conditions such as hydraulic retention times, salinity ratios, and concentrations of the saline solution will be optimized.

FY 2012 Publications/Presentations

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