II.E.1 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 to improve overall energy efficiency in hydrogen production from cellulosic biomass using a microbial electrolysis cell (MEC) reactor

Fiscal Year (FY) 2015 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 ethanol and lactate competing pathway; aimed to improve hydrogen molar yield via fermentation
- Optimize a two-stage MEC to increase the removal of protein during treatment of fermentation effluent while maintaining hydrogen production rates with the overall aim of improving H_2 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 from the Hydrogen Production section of the Fuel Cell Technologies Office (FCTO) Multi-Year Research, Development, and Demonstration Plan.

(AX) Hydrogen Molar Yield

(AY) Feedstock Cost

(AZ) System 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	mol H_2/mol glucose	2–3.2	4	6
Feedstock cost	¢/lb sugar	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

Feedstock cost: The DOE Bioenergy Technologies Office is conducting research to meet its 2015 target of 10¢/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 2015 Accomplishments

- Sequencing fed-batch reactor experiments were conducted and demonstrated that by using a hydraulic retention time (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_2 with a maximal rate of 1,373 mL $H_2/L_{reactor}$/d. The lignocellulose has undergone a new and milder pretreatment process via alkaline de-acetylation. *C. thermocellum* can hydrolyze the more recalcitrant de-acetylated substrate for H_2 production using its innate hydrolytic enzyme cocktails, a novel finding.

- A *C. thermocellum* mutant lacking the pyruvate-to-lactate electron-competing pathway has been generated yet with no change in total H_2 production albeit 24% more ethanol, suggesting the importance of deleting the ethanol-competing pathway, which is ongoing.
- A two-stage MEC treatment process was developed to increase the removal of protein from the fermentation effluent. Using a combined continuous flow (first stage) and batch-fed (second stage), the process achieved 93±3% chemical oxygen demand (COD) removal and 84±3% protein removal, respectively. The total hydrogen production rate of this combined treatment process was 0.3±0.1 L $H_2/L_{reactor}$/d. The first stage of the process achieved a hydrogen production rate of 2.1±0.4 L $H_2/L_{reactor}$/d.

INTRODUCTION

Biomass-derived glucose feedstock is a major operating cost driver for economic hydrogen production via fermentation. DOE FCTO is taking advantage of the DOE Bioenergy Technology Office's (BETO) investment in developing less expensive glucose from biomass to meet its cost target of 10¢/lb by 2015. One alternative and viable approach to addressing the glucose feedstock technical barrier (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_2/mol sugar; Technical Barrier AX) using existing metabolic pathways in the cells. Biological pathways maximally yield 4 mol hydrogen per 1 mol 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 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 (MFC) called an MEC [6]. It demonstrated for the first time a potential route for producing up to 8 moles of hydrogen per mole of acetate or potentially up to 12 moles of hydrogen per mole of 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 an MEC in an integrated system [7]. Combining fermentation with MECs 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_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

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 rate of H_2 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 used HRT of 48 h, a liquid displacement of 50% working volume every 24 h, and seven cycles of carbon loadings at 5.0 g/L of lignocellulose (based on cellulose content). Corn stover was pretreated with a de-acetylation and mechanically refined (DMR) process that has less severity (using mild alkaline solution) hence better sugar recovery and less inhibitors generation. Biomass pretreated with the DMR process could therefore lower the biomass feedstock cost. The pretreated DMR lignocellulose material, kindly supplied by the NREL National Bioenergy Center, contained 42% glucan, 25% xylan and 16% lignin. Data from Figure 1 shows that a longer length of time is required to adapt the microbes to hydrolyze the more recalcitrant DMR biomass, which reached a maximal rate of H_2 production at the fifth cycle (fourth cycle upon DMR substrate loadings). The average H_2 production rate is 757 mL $H_2/L_{reactor}$/d (average of cycles 2–8), with a maximal rate at 1,373 mL/$L_{reactor}$/d (Cycle 5). This is the first report that

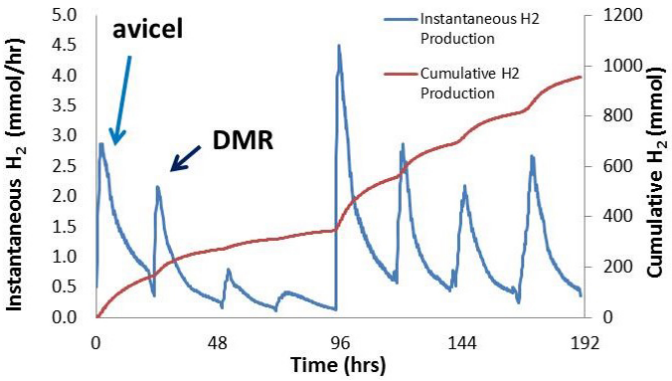

FIGURE 1. H_2 production from corn stover pretreated with a DMR process. Pure cellulose (avicel) was fed at Cycle 1 followed by seven cycles of DMR-biomass in a sequencing fed-batch bioreactor.

C. thermocellum or a cellulose-degrader can hydrolyze the more recalcitrant DMR biomass without adding expensive enzyme cocktail. Future work will devise strategy to shorten the lag time of *C. thermocellum* to start hydrolyzing the more recalcitrant DMR biomass.

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 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 DNA restriction system between the host and the plasmid [8]. To circumvent both challenges, we have redesigned a plasmid suited for genetic transformation in *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 pyruvate-to-lactate pathway encoded by lactate dehydrogenase with the aim to redirecting more electrons toward H_2 production. The lactate pathway mutant yielded 24% more ethanol and with no change in total H_2 output, highlighting the importance of deleting the ethanol pathway. An effort to delete the ethanol pathway was initiated. We have obtained single colony on agar plate growing in the presence of the antibiotic marker, suggesting the deletion of the ethanol pathway-encoding gene. However, the single colony cannot be revived, likely due to metabolic or redox imbalance. Work is ongoing to devise new strategy for stable deletion of the ethanol pathway.

Two-Stage MEC Process for Increased Protein Removal

Previous research showed unexpected high concentrations of proteins in the fermentation effluent. To convert both acetate and protein to H_2, MEC anodes were conditioned separately to degrade acetate and protein (bovine serum albumin [BSA]) to achieve a goal of 80%) protein

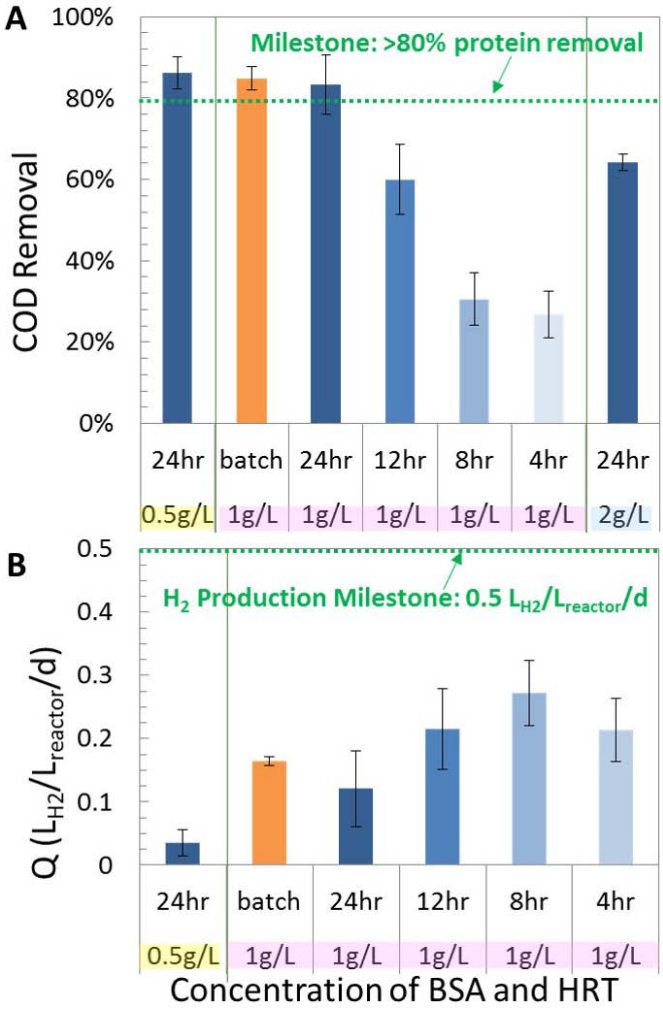

FIGURE 2. (A) Percent removal of protein (BSA) and (B) hydrogen production in MECs fed only BSA

removal with a H_2 production goal of 0.5 L $H_2/L_{reactor}$/d. COD removal decreased as the HRT in the anode chamber was decreased (Figure 2A). The MECs fed acetate achieved ≥80% COD removal at HRTs ≤12 h for all concentrations tested, and a H_2 production rate ≥0.5 L $H_2/L_{reactor}$/d was achieved at all HRTs tested in continuous flow mode. The highest H_2 production rate for the acetate-fed MECs was 2.5±0.3 L $H_2/L_{reactor}$/d at a 4 h HRT, but under these conditions there was <40% COD removal. In MECs treating acetate, a shorter HRT or higher substrate concentration led to increased H_2 production rates, but less COD removal. In BSA-fed MECs, increased protein removal did not result in improved current production (Figure 2B). Only MECs fed ≤1 g/L of BSA, and operated at a 24 h HRT or in fed-batch mode, achieved >80% COD removal, and none of the conditions tested achieved the target H_2 production rate, with the highest rate in the BSA fed MEC of 0.27±0.06 L $H_2/L_{reactor}$/d. When connected hydraulically in series, with the first MEC (acclimated to acetate) at an

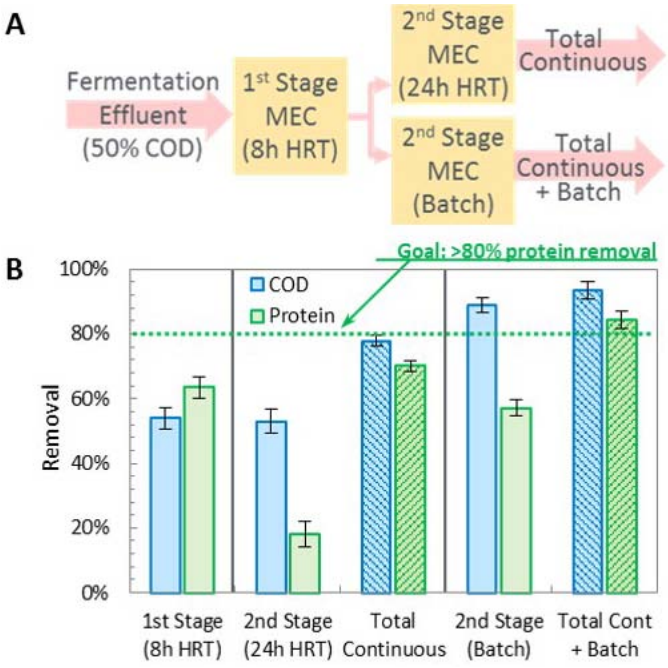

FIGURE 3. (A) Process diagram of effluent treatment stages and (B) COD and protein removal in each stage and for the combined process

8 h HRT to maximize H_2 production rates , and the second MEC (acclimated to BSA) operated at either a 24 h HRT or in batch mode, the two-stage process met the milestone of >80% protein removal (Figure 3). However, this process did not meet the goal of a H_2 production rate of 0.5 $L_{H2}/L_{reactor}$/d. The total H_2 production rate of the reactors combined in series was much lower than that of the first stage of the process (2.1±0.4 $L_{H2}/L_{reactor}$/d). The main reason for the limited H_2 production in the second stage MEC was a low COD concentration. With the effluent COD only 20% of the influent COD, there was insufficient organic matter to sustain high current densities by the anode biofilm. Going forward, we will focus on maximizing H_2 production rates.

Cathode Chamber Design

To increase the volumetric H_2 production rate, we investigated reducing the cathode chamber volume by decreasing the width to 0.7 cm. The volume was reduced to 28 mL (vs. 76 mL previously), for a total reactor volume reduction of 25% (final total volume of 144 mL). The catholyte used in these tests was 0.05 M phosphate buffer (5.6 min HRT), but more saline catholytes will be tested in the future as this can reduce reactor internal resistance. The resulting whole cell and anode potentials were similar to those measured in previous experiments with larger cathode chambers. Therefore, volumetric current density was greater than that of previous MECs with larger (76 – 163 mL) cathode chambers. The improved (reduced volume) MEC produced 1.4±0.2 L $H_2/L_{reactor}$/d over more than three anode HRT cycles. Optimization will continue as higher salinity catholytes and various catholyte HRTs will be investigated.

CONCLUSIONS AND FUTURE DIRECTIONS

- Using corn stover lignocellulose pretreated via a DMR process (5 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, we obtained an average rate of H_2 production at 757 mL/$L_{reactor}$/d, exceeding the benchmark value of the FY 2015 milestone using more recalcitrant substrate. We will optimize the feeding strategy aimed to decrease the H_2 production lag time.
- Following published protocols and using the NREL proprietary plasmid, we deleted the lactate dehydrogenase gene encoding the pyruvate-to-lactate pathway. Its phenotype of increasing ethanol production guided the design to delete the ethanol pathway. Work is ongoing toward generating mutants lacking both ethanol and lactate pathways and quantifying ethanol and H_2 production. The outcome should aid in future site-directed mutagenesis effort by deleting multiple competing pathways to improve hydrogen molar yield.
- There is a trade-off between maximum H_2 production rate and maximum COD and protein removals using a series of MECs for fermentation effluent treatment. The solution to this situation is to focus on maximizing H_2 production rate, and using a secondary process (no hydrogen gas production) to achieve overall COD removal..

In the future, we will operate the fed-batch bioreactor fermenting DMR-pretreated corn stover lignocellulose generated from a de-acetylated process. Due to its recalcitrance nature, we will feed DMR biomass in Cycle 1 to acclimate the microbes hence shortening the H_2 production lag phase. We will also test more frequent re-inoculations to decrease lag time. We will test a new mutagenesis approach via gene replacement to generate the ethanol-competing pathway mutant, determine ethanol and H_2 production profiles in the triple mutants with the outcomes guiding future mutagenesis effort to delete multiple competing pathways, aimed to improve hydrogen molar yield. We will continue to optimize the design of the cathode chamber with the aim of increasing the hydrogen production rate. In addition, we will evaluate combined aqueous/gas diffusion cathodes using electrochemical tests to determine if higher hydrogen production rates can be obtained by using a gas diffusion layer rather than an aqueous catholyte. As the cathode cost is impacted by the choice of the cathode catalyst, we will explore the use of non-precious metal cathode catalysts.

FY 2015 PUBLICATIONS/PRESENTATIONS

1. "Hydrogen metabolic network in *Clostridium thermocellum*." Oral presentation at the XIII International Clostridium Conference, September 19–21, 2014, Shanghai, China (Maness).

2. "Engineering *Clostridium thermocellum* for H_2 production." Oral presentation at the 249th American Chemical Society National Meeting & Exposition, March 22–24, 2015 (Chou).

3. Maness, P.C., and Logan, B. 2015. DOE Fuel Cell Technologies Office Annual Merit Review, June 11, 2015, Washington, DC. Presentation PD038.

4. Watson, V.J., and Logan, B. 2015. "Hydrogen production from continuous flow bioelectrochemical systems treating fermentation wastewater." DOE Fuel Cell Technologies Office Annual Merit Review Poster Session, June 9, 2015, Washington, DC. Poster Presentation PD122.

5. Watson, V.J., M.C. Hatzell, and B.E. Logan. 2015. "Hydrogen production from continuous flow, microbial reverse–electrodialysis electrolysis cells treating fermentation wastewater." *Biores. Technol.* 195: 51–56.

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