II.E.1 Biomass to Hydrogen

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Start Date: July 2015
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.
- Demonstrate hydrogen production by *Clostridium thermocellum* from biomass pretreated either with ionic-liquid or alkaline de-acetylation aimed at lowering feedstock cost.
- Optimize genetic tools to transform *C. 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) 2016 Objectives

- Optimize sequencing fed-batch parameters and ferment corn stover lignocellulose to hydrogen and replace or reduce medium components aimed to lower hydrogen cost.
- Demonstrate growth of *C. thermocellum* in the presence of up to 10% of cholinium-based ionic liquids; determine most appropriate ionic liquid for future experiments based on *C. thermocellum* tolerance.
- Determine the highest saccharification efficiency for corn stover pretreated with several cholinium-based ionic liquids and correlate with hydrogen production by *C. thermocellum*.
- Identify the most important hydrogenase in *C. thermocellum* for over-expression aimed to increase hydrogen molar yield via fermentation.
- Use the genetic tools developed at NREL tailored for *C. thermocellum* and delete both the formate and lactate competing pathways; aimed to improve hydrogen molar yield via fermentation.
- Design MEC cathodes with reduced volume to increase maximum hydrogen production rate to 1.2 $L/L_{reactor}/d$ based on overall reactor volume reduction using Pt/C cathodes.
- Examine alternative materials and catalysts for the cathode and achieve hydrogen production rate to 1.2 $L/L_{reactor}/d$ without platinum catalyst.

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 in the Hydrogen Production section of the Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan.

(AX) Hydrogen Molar Yield

(AY) Feedstock Cost

(AZ) System Engineering

Technical Targets

See Table 1.

FY 2016 Accomplishments

- Identified, reduced, or eliminated three nutrient components (resazurin, cysteine, and a buffer reagent) in the *C. thermocellum* growth media with no impact on cell fitness or hydrogen production. The outcome reduced relative cost of the medium by up to 66% when the costly buffer chemical was replaced with KOH in a bioreactor with pH control.

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 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.

- Initial experiments showed that growth of *C. thermocellum* was inhibited at ≤3% of the cholinium-based ionic liquids. Adaptation experiments identified cholinium glutamate ([Ch][Glu]) as the most promising ionic liquid for tolerance experiments. Cultures of *C. thermocellum* were adapted to be tolerant to up to 9% [Ch][Glu].
- Pretreatment and saccharification with cholinium-based ionic liquids ([Ch][Glu], cholinium succinate, cholinium malate) demonstrated that corn stover pretreated with [Ch][Clu] yielded the highest amount of glucose. The saccharification results correlated with hydrogen production results, which indicated that [Ch][Glu]-pretreated biomass was a good substrate for release of hydrogen by *C. thermocellum*.
- A *C. thermocellum* mutant was generated which lacks both the pyruvate-to-formate and pyruvate-to-lactate electron-competing pathways. An increase in specific rate of hydrogen production was detected, yet with a concomitant increase in ethanol production, highlighting the importance of deleting the latter competing pathway.
- Hydrogen production in the MEC was increased to 1.4±0.2 L-H_2/L-reactor/d using hydraulic flow control past the electrode, in a reduced cathode chamber volume to improve performance. It was determined that increased pH did not adversely impact current generation.

INTRODUCTION

Biomass-derived glucose feedstock is a major operating cost driver for economic hydrogen production via fermentation. DOE's FCTO is taking advantage of the DOE's Bioenergy Technology Office's 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 (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*, 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 hydrogen molar yield. This strategy has 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 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 (MFC) called an microbial electrolysis cell (MEC) [6]. It demonstrated for the first time a potential route for producing up to eight 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 high 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 hydrogen production. Two types of biomass pretreatment technologies were tested, one via alkaline de-acetylation at NREL, and a second approach using ionic liquid, conducted at Lawrence Berkeley National Laboratory and Sandia National Laboratories. 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 using both a synthetic effluent and the real waste stream from lignocellulosic fermentation generated at NREL.

RESULTS

Lower Medium Cost for Lignocellulose Fermentation

C. thermocellum is normally cultured in a growth medium containing buffer, mineral salts, and vitamins. The top three most expensive components per liter medium are: 3-morpholinoproprane-1-sulfonic acid (MOPS) buffer ($10.92), resazurin ($1.57), and cysteine ($1.31). Resazurin is used as a redox or O_2 indicator to ensure anaerobicity of the growth medium. Cysteine is normally added to poise lower redox potential and also scavenges O_2. The removal of either resazurin or cysteine from growth medium has no impact on cell growth (data not shown), or hydrogen production (Figure 1A). MOPS is added to maintain pH at 7.0, optimal for *C. thermocellum* cell growth. With no external pH control, feeding MOPS at 50% level has little impact on either growth or hydrogen production, yet its complete removal severely impacted both parameters (Figure 1A), suggesting the importance of proper pH maintenance. To circumvent pH effect, we carried out hydrogen production in bioreactor with pH control (maintaining at pH 7 throughout) by adding the less costly KOH. Under this condition a complete removal of MOPS has no impact on hydrogen production (Figure 1B), lowering the medium cost from $0.55/mM H_2 to $0.18/mM. The outcomes identified the cost saving components with their removal or replacement lowering medium cost without compromising final hydrogen productivity.

Fermentation of Pretreated Biomass using Ionic Liquid (Lawrence Berkeley National Laboratory and Sandia National Laboratories)

The goal of this work is to integrate a novel pretreatment process, ionic liquid pretreatment, into the production of hydrogen from biomass using *C. thermocellum*. Ionic liquids are a remarkably effective pretreatment for biomass, and biologically derived cholinium-based ionic liquids offer the possibility of performing biomass pretreatment and saccharification in the same bioreactor, reducing the cost of the process [9]. To establish whether a combined pretreatment/saccharification process is feasible, the ability

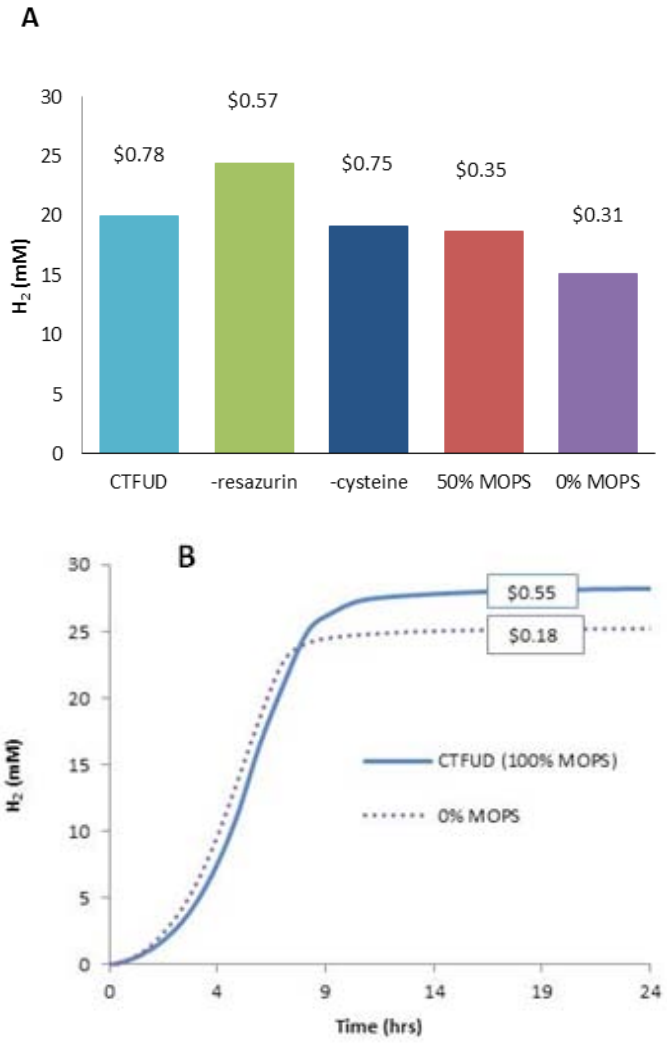

FIGURE 1. (A) Hydrogen production on CTFUD medium with removal of a single ingredient. Values above each bar represent $ per mmoles H_2 and are based on the costs of each medium after removal of the corresponding ingredient; (B) hydrogen production in pH-controlled bioreactor with and without MOPS buffer. When MOPS was removed from the medium, the cost of hydrogen decreased from $0.31/mM H_2 (1A) to $0.18/mM H_2 (1B) due to more hydrogen being produced in the latter. The nutrient cost was calculated based on catalog price of Sigma Chemical Co. It is anticipated the nutrient cost will be greatly reduced when purchase in bulk in scale-up applications.

of *C. thermocellum* to produce hydrogen from [CH][Glu]-pretreated corn stover at 75% of the comparable production from cellobiose was demonstrated (Table 2). Preliminary cultivations of cellobiose-grown *C. thermocellum* demonstrated that growth was inhibited at ~3% [Ch][Glu] and at lower levels of [Ch][Mal] and [Ch][Suc]. Adaptation of *C. thermocellum* to grow on up to 9% [Ch][Glu] was demonstrated and hydrogen production from cellobiose at 7% [Ch][Glu] was observed that was ~74% that of the control. The growth of the cellobiose culture containing 7% [Ch][Glu]

was 50% of the control, suggesting that inhibiting growth may contribute to decreased total hydrogen production. Efforts to obtain a stable culture growing at 10% [Ch][Glu] are currently being pursued.

TABLE 2. Tolerance and Hydrogen Production in Ionic Liquids

Ionic liquid	Hydrogen production from pretreated corn stover (mL)[1]	Maximum tolerance of *C. thermocellum* (w/v)[2]
[Ch][Glu]	24 ± 2	9%
[Ch][Suc]	11 ± 1	3%
[Ch][Mal]	ND	1%

[1]Based on production in cultures containing 0.5% pretreated corn stover incubated at 55°C for 60 h
[2]Percentage of ionic liquid at which *C. thermocellum* can achieve >50% growth relative to an unamended control. Hydrogen production in 0.5% cellobiose (without ionic liquid) was 31±4 mL.

Metabolic Engineering

The goal of this approach is to use genetic tools to inactivate genes encoding competing metabolic pathways, thus redirecting more cellular flux (i.e., electrons) to improve hydrogen molar yield. We have designed 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. [10], we have created mutants lacking the pyruvate-to-lactate pathway encoded by lactate dehydrogenase (LDH) either in the wild type background or in a host lacking also the pyruvate-to-formate pathway catalyzed by pyruvate formate lyase (PFL), as verified by PCR (Figure 2). The double mutant indeed exhibited higher specific rate of hydrogen production based on cell dry weight. To further increase hydrogen production, an effort is ongoing to delete the competing ethanol pathway in the double mutant to conserve more electrons for hydrogen production.

Cathode Chamber Design

The cathode chamber electrolyte was recirculated past the cathode to improve operation and allow a reduction in volume (76 mL→28 mL) using a chamber width of 0.7 cm. The anode chamber was operated using synthetic fermentation effluent (1.2 g COD/L, HRT=8 h) comprised of acetate, BSA, dextrose, ethanol, and lactate in 50 mM phosphate buffer solution (PBS), and a catholyte with 50 mM PBS. Theoretical calculations suggested that the pH increase that would result from this operation would impair performance, but no impact was found relative to an increase in pH even with the buffered solution. Due to the smaller

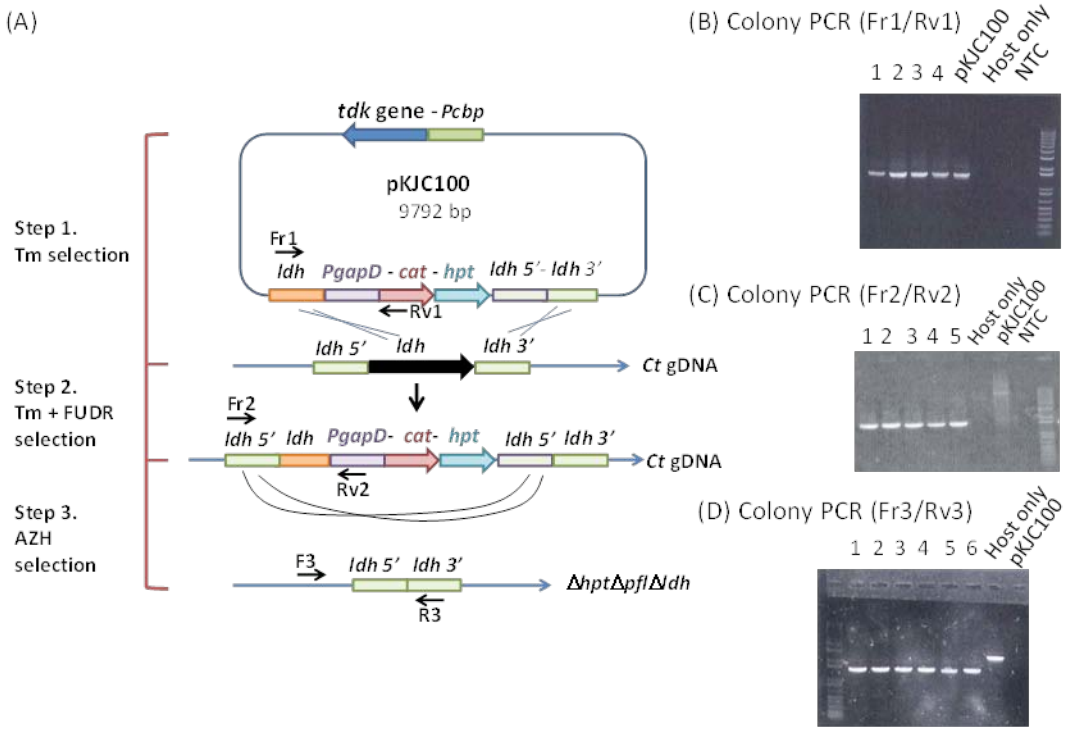

FIGURE 2. Creating the LDH pathway mutant in the Δ*hpt*Δ*pfl* strain yielding Δ*hpt*Δ*pfl*Δ*ldh*. (A) Schematic illustration of the three steps to generate the knockout; (B to D) colony PCR data validating each of the three steps and the final step yielding Δ*hpt*Δ*ldh*.

reactor volume, current density was greater than that of the previous MEC 76 mL cathode chamber, as well as a pervious MREC (microbial reverse electrodialysis electrolysis cell) that had a 163 mL cathode chamber (Figure 3). The improved (reduced volume) MEC produced 1.4±0.2 L-H_2/L-reactor/d over more than three anode HRT cycles, meeting the milestone for this part of the project. An MEC with a reduced volume cathode chamber was further tested using both 50 mM phosphate buffer (PBS, 5.6 mS/cm, initial pH 7) and 200 mM PBS (17.5 mS/cm, initial pH 7) as the catholyte. Hydrogen production using 200 mM PBS was not significantly different than gas production with the 50 mM buffer.

Alternative Cathode Materials

The cathode chamber was redesigned (63 mL) to position the electrode more centrally in the cathode chamber, in order to improve flow across the cathode surface. Three different cathode materials (stainless steel mesh, SSM; SS fiber felt, SSFF; SS wool, SSW) were tested as alternative cathode materials to platinum and a Pt/C (0.5 mg/cm^2) cathode was also tested as a control. Chronopotentiometry results at recirculation rates of 40 mL/min showed that for the alternative cathode materials, the better performances were obtained in order of SSW>SSFF>SSM, with the Pt/C cathode performing better than all three (Figure 4). The SSW cathode is a 3-dimensional material which has a high specific surface area, and this might be the reason for showing higher performance than SSM and SSFF cathodes.

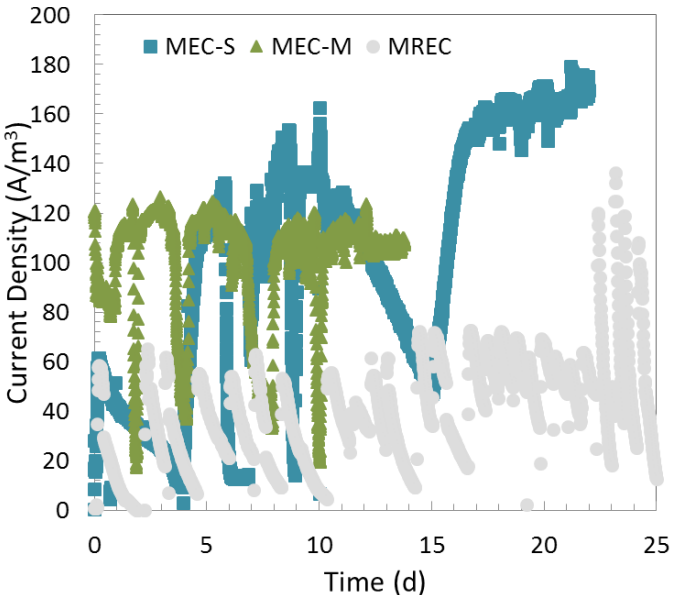

FIGURE 3. Current density of the MEC with a modular, continuous flow cathode chamber (MEC-S: 28 mL, 5.6 min HRT, 50 mM phosphate buffer; MEC-M: 76 mL, 3–15 min HRT, 50 mM phosphate buffer) compared to the previous MREC study (MREC: 163 mL, 8 min HRT, 1 M sodium bicarbonate buffer).

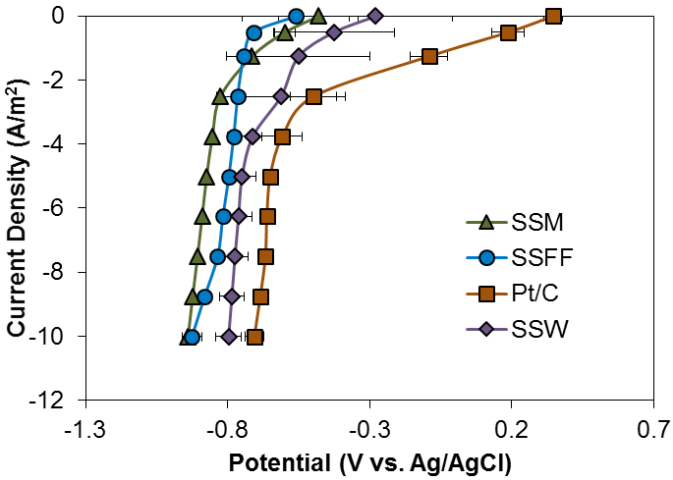

FIGURE 4. Chronopotentiometry tests with different cathode materials (SSM, SSFF, SSW, and Pt/C, recirculation rate: 20 mL/min) in the cathode chamber (set current 20 min/step, 40 cm^2 projected surface area).

CONCLUSIONS AND FUTURE DIRECTIONS

- We determined that resazurin and cysteine can be completely eliminated from growth medium for culturing *C. thermocellum* and hydrogen production. Moreover the costly MOPS can be replaced by KOH for pH maintenance, hence lowering the cost of the medium from \$0.55/mM H_2 to \$0.18/mM H_2, lowering the cost of hydrogen production from biomass.
- Following published protocols and using the NREL proprietary plasmid, we deleted both the pyruvate-to-formate pathway and the pyruvate-to-lactate pathway in a double mutant. The mutant indeed displayed higher specific rate of hydrogen production but with an increased level of ethanol production, suggesting deleting the latter is a priority for future research to increase hydrogen molar yield.
- Stable cultures of *C. thermocellum* growing on high levels of [Ch][Glu] that produce hydrogen have been established and will be tested in combined pretreatment/hydrogen production scenarios with the data applied toward a techno-economic analysis.
- Saccharification and hydrogen production experiments have demonstrated that [Ch]Glu] pretreatment of corn stover is a promising pretreatment for hydrogen production. Optimization of pretreatment and integration with *C. thermocellum* growth experiments will be continued.

- Hydrogen production in the MEC was increased to 1.4±0.2 L-H_2/L-reactor/d, and a higher catholyte pH did not adversely impact performance.

In the future, we will operate the sequencing fed-batch bioreactor fermenting DMR-pretreated corn stover lignocellulose generated from either a de-acetylated process or via ionic liquid pretreatment. We will increase solid loading to achieve higher rates of hydrogen production. We will further replace the commercial yeast extract with brewery yeast waste to decrease medium cost aimed at lowering hydrogen selling price. Deleting the ethanol-competing pathway is deemed essential to redirect more electrons toward hydrogen production and will continue to be a part of this effort. Past efforts had led to unstable isolates likely due to redox imbalance. To circumvent, we will delete the ethanol pathway in a host with higher levels of hydrogen production, hence using proton reduction as the new sink. This new host strain will be generated via replacing the native promoter with a stronger promoter to drive hydrogenase overexpression. The team will continue to adapt *C. thermocellum* to tolerate higher levels of ionic liquid (10% [Chl][Glu]) so that pretreatment and fermentation can occur in the same reactor to save reactor cost. hydrogen production and substrate utilization will be profiled to measure fermentation efficiency. The data will be input into a techno-economic model to determine the rate-limiting steps and cost drivers to guide future research direction. We will further investigate high surface area cathode materials by designing and constructing macroporous stainless steel material (brushes and fiber felt) for cathodes using the newly re-designed cathode chamber. We will also examine alternative materials and catalysts for the cathode with improved gas diffusion properties to improve reactor operation aimed at increasing hydrogen production and lowering MEC cost.

FY 2016 PUBLICATIONS/PRESENTATIONS

1. Maness, P.C., and Logan, B. 2016. DOE Fuel Cell Technologies Office Annual Merit Review, June 7, 2016, Washington, D.C. Presentation PD038.

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