Biomass to Hydrogen (B2H2)

Pin-Ching Maness (Primary Contact), Katherine
Chou, Lauren Magnusson, Jonathan Lo, and
Wei Xiong
National Renewable Energy Laboratory
15013 Denver West Parkway
Golden, CO 80401
Phone: (303) 384-6114
Email: pinching.maness@nrel.gov

DOE Manager: Katie Randolph
Phone: (720) 356-1759
Email: Katie.Randolph@ee.doe.gov

Subcontractor:
Pennsylvania State University, State College, PA

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Project 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.

- Improve biomass utilization by engineering *C. thermocellum* to co-utilize both the six-carbon (C6) sugars (cellulose) and five-carbon (C5) sugar (hemicellulose) to lower feedstock cost.

- Optimize genetic tools to transform *C. thermocellum* and obtain mutants lacking the targeted competing pathways to improve hydrogen molar yield.

- Demonstrate hydrogen production from the National Renewable Energy Laboratory (NREL) fermentation effluent to improve overall energy efficiency in hydrogen production from cellulosic biomass using a microbial electrolysis cell (MEC) reactor.

Fiscal Year (FY) 2018 Objectives

- Optimize batch parameters and ferment corn stover lignocellulose to hydrogen with a rate of 2.5 L-H_2/L/d.

- Engineer *C. thermocellum* to co-utilize both the C6 (cellulose-derived) and C5 (hemicellulose-derived) sugars to improved biomass utilization, hence lowering feedstock cost.

- Use ^{13}C-metabolic flux analysis and identify the most important pathway leading to maximal hydrogen production from cellulose-derived sugar.

- Redesign the MEC reactor by doubling cathode area (one cathode on each side of the anode chamber) to increase hydrogen production when using a platinum-free cathode catalyst.

- Improve anode performance by replacing small brushes (0.8 cm diameter) with seven larger brush anodes (1.5 cm diameter) to increase fiber density.

- Examine using a nickel catalyst (salts on activated carbon or a Ni powder) to see if hydrogen production rate can be improved compared to stainless steel.

- Investigate the performance of a more compact MEC by also looking at a flat anode.

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 Multi-Year Research, Development, and Demonstration Plan[1]:

(AX) H_2 Molar Yield

(AY) Feedstock Cost

(AZ) System Engineering.

Technical Targets

Progress toward meeting DOE's dark fermentation technical targets is shown in Table 1.

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[1] https://www.energy.gov/eere/fuelcells/downloads/fuel-cell-technologies-office-multi-year-research-development-and-22

FY 2018 Accomplishments

- We obtained a hydrogen production rate of 2.6 L/L-d in *C. thermocellum* fermenting pretreated biomass in a batch bioreactor.

- Using laboratory adaptive evolution, we improved the rate of xylose utilization by 3.5-fold in a *C. thermocellum* mutant engineered to co-utilize both C6 sugar and C5 sugar simultaneously without cross inhibition.

- By feeding *C. thermocellum* with ^{13}C-labeled glucose, we constructed a high-resolution cellular carbon flux map and identified the most important glycolytic pathway leading to maximal hydrogen production.

- Using two cathode chambers (one on each side of the anode chamber) increased the hydrogen production from 1.3 ± 0.3 L-H_2/$L_{reactor}$-d to 2.3 ± 0.0 L-H_2/$L_{reactor}$-d (based on current density).

- The operation of the MEC with larger-diameter brush anodes (1.5 cm) and stainless steel wool cathodes further increased hydrogen production to 2.8 ± 0.3 L-H_2/$L_{reactor}$-d.

- Using a cathode made by adsorbing nickel onto activated carbon (AC) produced a higher hydrogen production rate (1.1 ± 0.1 L-H_2/$L_{reactor}$-d) than using a Ni foam cathode (1.0 ± 0.1 L-H_2/$L_{reactor}$-d), but lower than that of stainless steel wool.

- Cathodes made using nickel powder blended with AC had a hydrogen production rate 36% higher than only Ni powder (AC-NiP, 0.38 ± 0.08 L-H_2/$L_{reactor}$-d; pure Ni 0.28 ± 0.02 L-H_2/$L_{reactor}$-d) in different MEC (smaller chamber, 56 mL, no recirculation).

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[a]	--
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	2.8	1	---

[a] 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.

INTRODUCTION

Biomass-derived glucose feedstock is a major operating cost driver for economic hydrogen production via fermentation. DOE's Fuel Cells Technologies Office 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 cents/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 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 (PSU). In the absence of oxygen, and by adding a slight amount of positive 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 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 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 batch and sequencing fed-batch reactor to improve longevity, yield, and rate of hydrogen production, using corn stover biomass pretreated via a de-acetylation and mechanically refined (DMR) process. We also engineer *C. thermocellum* to utilize all the sugars in biomass (both C6 and C5) aimed to lower feedstock cost. 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

Achieving High Rate of Hydrogen Production from Lignocellulose Fermentation

We conducted several batch experiments with the goal of optimizing and increasing biomass substrate loading in batch and fed-batch mode, to obtain an average hydrogen production rate of at least 2.5 L/L$_{reactor}$/day for a duration of minimally 24 h using DMR biomass as the substrate. We tested 30 g/L DMR (as cellulose) in batch mode and Figure 1 summarizes the hydrogen and carbon dioxide (CO_2) production of *C. thermocellum*. When calculating the rate over the most productive portion of the fermentation (25 hours in the linear portion of the fermentation), we achieved a rate of 2.6 L/L$_{reactor}$/day. *C. thermocellum* also exhibited a lag phase of approximately 10 hours. As we have shown in repeated fed-batch fermentation, we are able to significantly decrease, if not eliminate, the lag phase completely.
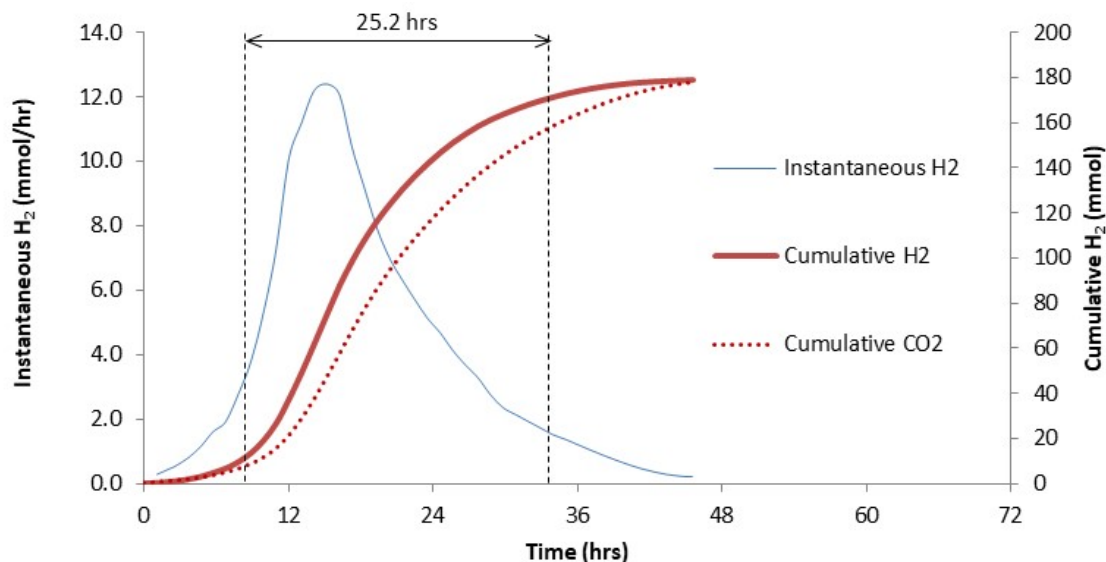
Figure 1. Hydrogen and CO₂ production by *C. thermocellum* when cultured in batch mode on 30 g/L (as cellulose) DMR substrate

Improving the Rate of Xylose Metabolism by 3.5-Fold

DMR-pretreated biomass generates a solid fraction containing both cellulosic (C6) and hemicellulosic (C5) sugars. *C. thermocellum* naturally can ferment cellulose directly to hydrogen without needing expensive cellulase enzyme cocktail, yet the wild-type strain lacks the ability to metabolize C5 sugar. Demonstrating co-metabolism of C6/C5 sugars efficiently hence is the goal in FY 2018 to improve the economic feasibility of fermentative hydrogen production. We previously have generated *C. thermocellum* mutant lines capable of co-metabolizing monomeric xylose (C5 hemicellulosic sugar) with cellobiose (cellulose-derived glucose dimer) with no cross-inhibition. Yet xylose metabolism still displays a lag phase. Via adaptive evolution (repeated sub-culture and transfer in xylose), we have evolved a variant that displays 3.5-fold improvement in the rate of xylose metabolism. The outcomes illustrate a significant improvement in biomass conversion to hydrogen with lower biomass feedstock cost.

Probing the Most Important Hydrogen Production Pathway via ^{13}C-Metabolic Flux Analysis

Using isotope tracer (feeding ^{13}C-glucose), gas chromatography-mass spectrometry, and metabolic flux modeling, we deciphered the metabolic network of *C. thermocellum* and uncovered that the Embden–Meyerhof–Parnas (EMP) pathway is the predominant glycolytic route supporting hydrogen production whereas the Entner–Doudoroff (ED) pathway and oxidative pentose phosphate pathway are inactive. To gain a quantitative understanding, we further formulate a fluxome map to quantify the metabolic fluxes through central metabolic pathways (Figure 2). The EMP pathway yields both NADH and reduced ferredoxins (2 moles each) compared to the ED pathway (3 NADH and 1 NADPH). Both NADH and reduced ferredoxin are the putative electron mediators toward hydrogen production based on its homologs in other related microbes. Cellulose hydrolysis via the EMP pathway therefore should yield more hydrogen compared to the ED pathway, which explains the copious amount of hydrogen produced by *C. thermocellum*. This work represents the first global *in vivo* investigation of the principal carbon metabolism of *C. thermocellum*. Further manipulations of the EMP pathway should increase hydrogen production further.
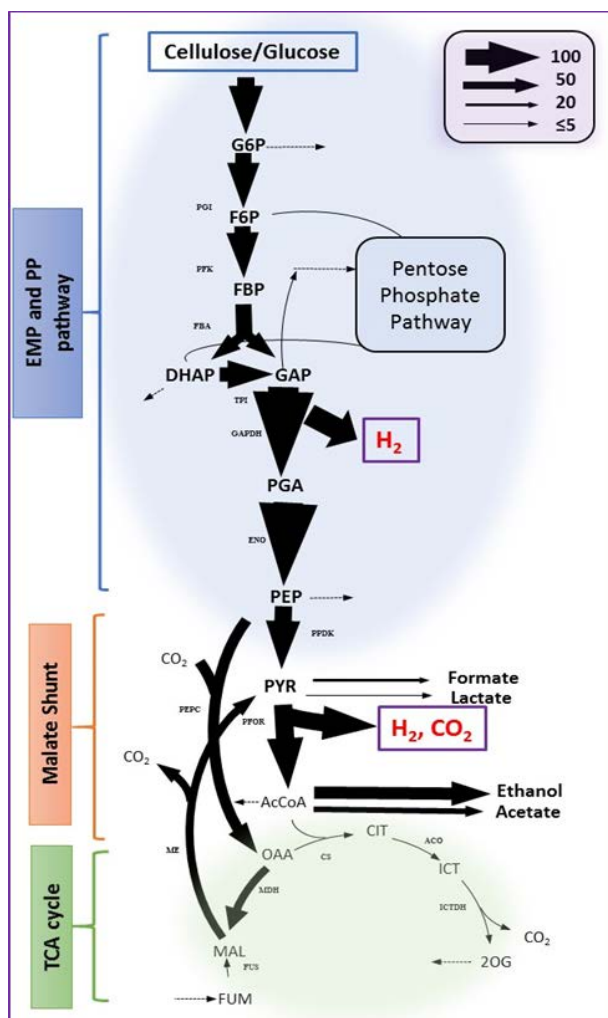
Figure 2. Carbon metabolic map of *C. thermocellum*. The growth medium was supplemented with U-13C-glucose (5.6 mM) in 22.2 mM unlabeled glucose (80%). Cells in late log phase were harvested (OD600 near 0.6) and the intracellular amino acids were extracted for gas chromatography-mass spectrometry analysis. G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; FBP: fructose 1,6-bisphosphate; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde-3-phosphate; PGA: 3-phosphate glycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; AcCoA: acetyl CoAOAA: oxaloacetate; MAL: malate; CIT: citrate; ICT: isocitrate; 2OG: 2-oxoglutarate; FUM: fumarate.

Impact of MEC Configuration and Operation on Hydrogen Production Rates

The goal of this project is to optimize the configuration of the MEC to maximize the hydrogen production rate. Electrically connecting two cathodes to the anode (Figure 3) doubled the hydrogen production from 1.3 ± 0.3 L-H_2/$L_{reactor}$/d to 2.3 ± 0.0 L-H_2/$L_{reactor}$-d (based on current production) using a stainless steel wool cathode. However, this increased current production could not be sustained, likely due to insufficient biofilm on the small (0.8 cm diameter) anodes. Therefore, thicker brushes (1.5 cm diameter, seven brushes) were used, which enabled stable current generation and a hydrogen production rate as high as 4.7 L-H_2/$L_{reactor}$-d over the first 7 h of recirculation of the anolyte, and 2.8 ± 0.3 L-H_2/$L_{reactor}$-d averaged over a 17 h cycle. The electrode potential was monitored with a reference electrode (Ag/AgCl) showing that the anode was primarily limiting performance, likely due to the low concentration of acetate in the synthetic fermentation effluent. Based on preliminary tests it is possible that performance can be further improved by replacing the seven 1.5-cm

diameter brushes with a single 4.5-cm diameter brush, pressed between the two anion exchange membranes, as this will provide for flow through the brush fibers rather than around the brushes.
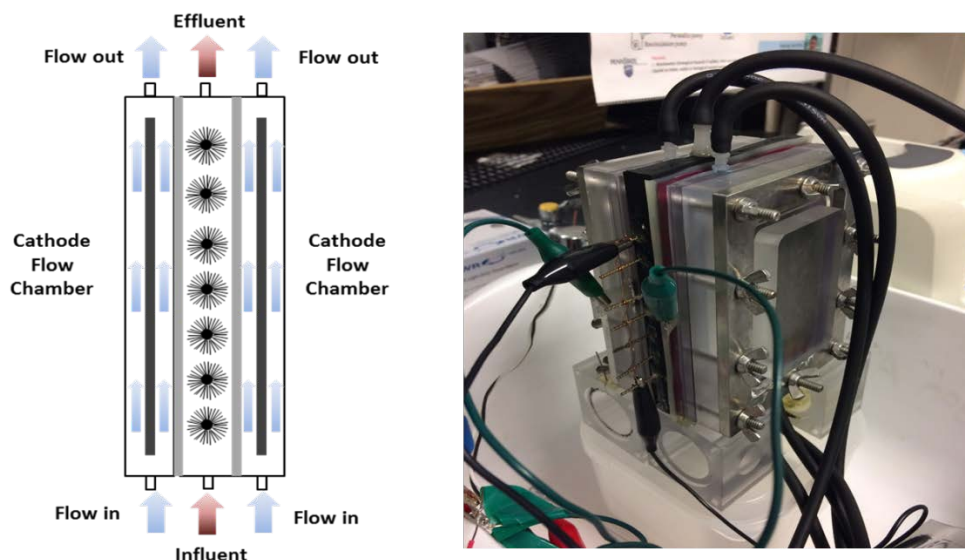

Figure 3. A new scalable MEC reactor with two cathode flow chambers: a schematic diagram (left), and a photo of the reactor (right)

Developing Non-Precious-Metal-Based Cathode Catalysts for MECs

Nickel is known to be a good, non-precious catalyst for the hydrogen evolution reaction. To examine its performance in MECs, Ni was adsorbed using a salt ($NiCl_2 \cdot 6H_2O$) onto high-surface-area AC to produce AC-Ni cathodes. The AC-Ni cathode (8.8 mg/cm^2-electrode surface area) was tested in a 168 mL MEC (100 mL anode chamber, with 68 mL using a single cathode chamber). Synthetic fermentation effluent (1.2 g-chemical oxygen demand/L) was continuously provided to the anode chamber and 50 mM phosphate buffer solution (pH 7) was recirculated through the cathode chamber at a rate of 40 mL/min. The MEC with the AC-Ni cathode produced 1.1 ± 0.1 L-H_2/L-d of hydrogen over 7 days of operation, which was significantly better (p <0.05) than that obtained using Ni foam (1.0 ± 0.1 L-H_2/L-d), a material that has been used by others as an alternative to Pt. The better performance of the AC-Ni cathode was assumed to be due to the high specific surface of AC, which made more Ni available as a catalyst per projected area of cathode than the foam. Because Ni could be lost over time, we tested performance of a regenerated cathode. We removed the Ni using an acid solution and re-adsorbed additional Ni and found that the regenerated cathodes performed as well as or better than the original cathodes.

A cathode was also tested using Ni powder blended with the AC, rather than having the Ni adsorbed, in MECs fed with synthetic fermentation effluent in a different MEC (smaller chamber, 56 mL, no recirculation). The AC cathodes blended with Ni powder (4.4 mg/cm^2 loading) produced 0.38 ± 0.08 L-H_2/L$_{reactor}$-d, which was higher than that obtained by Ni powder cathodes (no AC, 0.28 ± 0.02 L-H_2/L$_{reactor}$-d). These current densities were lower than that of the AC-Ni cathodes, thus this catalyst was not further tested in larger MECs.

CONCLUSIONS AND UPCOMING ACTIVITIES

- We measured an average hydrogen production rate of 2.6 L/L-d in *C. thermocellum* fermenting pretreated corn stover over a period of 25 hours.

- We generated and adapted *C. thermocellum* mutant lines and improved rate of xylose consumption by 3.5-fold.

- Via ^{13}C-metabolic flux analysis, we generated a high-resolution flux map and identified the EMP pathway is important in *C. thermocellum* for cellulose conversion to hydrogen.

- We doubled the hydrogen production rate by connecting two cathode chambers to one anode to reach 2.8 $\pm$ 0.3 L-H$_2$/L$_{reactor}$-d.

- Increasing the diameter of the brushes from 0.8 cm to 1.5 cm helped to stabilize reactor performance in terms of current production.

In the future, we will operate the batch and sequencing fed-batch bioreactors fermenting DMR-pretreated corn stover lignocellulose with increased solid loading, using *C. thermocellum* strains engineered and adapted to convert xylose (along with cellulose) at a faster rate. The aim is to increase rate of hydrogen production while lowering feedstock cost. We have generated a series of *C. thermocellum* mutants lacking carbon-competing pathways. We will conduct ^{13}C-metabolic flux analysis to gain insight as to how pathway redirections influence carbon and electron flux toward maximizing hydrogen production. The task using ionic liquid pretreatment was closed out in Q1, FY 2017. We will continue to examine alternative materials and catalysts for the cathode to improve reactor operation aimed at increasing hydrogen production and lowering MEC cost. A new larger brush will be tested in the two-cathode MEC to further improve the anode performance and biofilm development. The acetate concentration in the feed will be increased to stabilize the anode performance in both flat and brush anode MECs.

FY 2018 PUBLICATIONS/PRESENTATIONS

1. W. Xiong, J. Lo, K.J. Chou, C. Chao, L. Magnusson, T. Dong, and P.C. Maness, "Isotope-assisted metabolite analysis sheds light on central carbon metabolism of a model cellulolytic bacterium *Clostridium thermocellum*," *Frontier in Microbiol.* 9 (2018): Article 1947, DOI: 10.3389/fmicb.2018.01947.

2. W. Xiong, L.H. Reyes, W.E. Michener, P.C. Maness, and K.J. Chou, "Engineering cellulolytic bacterium *Clostridium thermocellum* to co-ferment cellulose- and hemicellulose-derived sugars simultaneously," *Biotechnol. Bioeng.* 115 (2018): 1755–1763, DOI: 10.1002/bit.26590.

3. K.J. Chou, "Metabolic Engineering of a cellulose-degrading bacterium *Clostridium thermocellum* for biofuels production," Invited talk at Colorado State Univ., March 22, 2018.

4. P.C. Maness, Poster Presentation PD038, DOE Hydrogen and Fuel Cells Program Annual Merit Review, June 14, 2018, Washington, D.C.

5. K.-Y. Kim and B.E. Logan, "Dynamic flow and the use of inexpensive nickel-added activated carbon cathodes to achieve cost-effective hydrogen production in microbial electrolysis cells," Abstract Proceedings of the 6th International Meeting of International Society for Microbial Electrochemistry and Technology (ISMET6), Universidade NOVA de Lisboa, Lisbon, Portugal, Oct. 3–6, 2017, poster presentation.

6. K.-Y. Kim and B.E. Logan, "Evaluation of alternative cathode materials for hydrogen production in Microbial electrolysis cells (MECs)," Abstract Proceedings of the Association of Environmental Engineering and Science Professors (AEESP) 2017 Conference, University of Michigan, Ann Arbor, June 20–22, 2017, poster presentation.

7. E. Zikmund, K.-Y. Kim, and B.E. Logan, "Hydrogen production rates with closely-spaced felt anodes and cathodes compared to brush anodes in two-chamber microbial electrolysis cells." *International Journal of Hydrogen Energy* 43, no. 20 (2018): 9599–9606.

8. K.-Y. Kim, W. Yang, and B.E. Logan, "Regenerable nickel-functionalized activated carbon cathodes enhanced by metal adsorption to improve hydrogen production in microbial electrolysis cells." *Environmental Science & Technology* 52, no. 12 (2018): 7131–7137.

9. K.-Y. Kim, E. Zikmund, and B.E. Logan, "Impact of catholyte recirculation on different 3-dimensional stainless steel cathodes in microbial electrolysis cells." *International Journal of Hydrogen Energy* 42 (2017): 29708–29715.

10. B.E. Logan, E. Zikmund, W. Yang, R. Rossi, K.-Y. Kim, P.E. Saikaly, and F. Zhang, "Impact of ohmic resistance on measured electrode potentials and maximum power production in microbial fuel cells," *Environmental Science and Technology* 52 (2018): 8977–8985.

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4. S. Van Ginkel and S. Sung, "Biohydrogen production as a function of pH and substrate concentration," *Environ. Sci. Technol.* 35 (2001): 4726–4730.

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10. D. Argyros, S.A. Tripathi, T.F. Barrett, S.R. Rogers, L.F. Feinberg, D.G. Olson, J.M. Foden, B.B. Miller, L.R. Lynd, D.A. Hogsett, and N.C. Caiazza, "High ethanol titers from cellulose by using metabolically engineered thermophilic anaerobic microbes," *Appl. Environ. Microbiol.* 77 (2011): 8288–8294.