II.E.1 Biomass to Hydrogen (B2H2)

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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.
- 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 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) 2017 Objectives

- Optimize sequencing fed-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 the genetic tools developed at NREL tailored for *C. thermocellum* and delete enzymes involved in the interconversion of the various cellular redox cofactors, aimed to improve hydrogen molar yield via fermentation.
- Examine alternative materials and catalysts for the cathode and achieve H_2 production rate to 1.2 $L/L_{reactor}$/d without platinum catalyst.
- Design smaller volume of MEC reactor to further enhance the H_2 production rate to a goal of 2.4 L-H_2/L/d.

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.

(AX) H_2 Molar Yield

(AY) Feedstock Cost

(AZ) System Engineering

Technical Targets

See Table 1.

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.

FY 2017 Accomplishments

- Identified and replaced the yeast extract nutrient component with industrial waste product in the *C. thermocellum* growth media with no impact on cell fitness or H_2 production. The outcome reduced relative cost of the medium by up to 49% when the costly yeast extract was replaced with industrial corn steep liquor without impacting H_2 production.
- A *C. thermocellum* mutant was generated which can co-utilize both C6 sugar and C5 sugar simultaneously without cross inhibition. We detected twice as much hydrogen production when both sugars were present as that in either sugar alone.
- Two *C. thermocellum* mutants were generated; one lacks the enzyme catalyzing the interconversion of nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH) (Mutant 1) and another enzyme in the interconversion of NADH and ferredoxin (Mutant 2). Mutant 1 exhibited 29% increase in total H_2 production and 55% increase in specific rate of H_2 production. Mutant 2 produced 35% more total H_2. Findings from both mutants validate the effectiveness of metabolic engineering.
- H_2 production rate with stainless steel (SS) wool (a non-Pt based cathode) reached to 1.3±0.3 L-H_2/L/d which is comparable to the rates with Pt cathode using synthetic fermentation effluent.

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 H_2 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 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 called an 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 H_2 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

Lower Medium Cost for Lignocellulose Fermentation

C. thermocellum is normally cultured in a growth medium containing buffer, mineral salts, and vitamins. The addition of hydrolyzed yeast extract (0.45%, w/v at $201.5/kg) boosts cell growth due to the presence of amino acids (also serve as nitrogen nutrient), vitamins, and a few inorganic compounds. We evaluated and tested several sources of yeast extract, including Brewers yeast ($95.7/kg), corn steep liquor (CSL; $49.4/kg) (both costs cited from the Sigma Aldrich Chemical Co.), and the industrial waste corn steep liquor from Solulys ($0.61/kg). Hydrogen production with these supplements were compared with two controls where autohydrolyzed yeast extract was added (Rich) and where the cells were cultured in the absence of both yeast extract and

vitamins (Base). Data from Table 2 show that higher amount of H_2 was produced in all cases where various forms of yeast extract were added, and the outputs were comparable with that from the "Rich" medium. Industrial CSL show the most promise due to its low cost. Table 2 summarizes the amounts of H_2 produced and the medium cost per liter, with industrial CSL from Solulys realized a 49% saving in the growth medium cost, without compromising H_2 production.

TABLE 2. Amount of H2 Produced, and Overall Medium Cost when Supplemented with Comparable Concentration (0.45%, w/v) of Brewer's Yeast, Corn Steep Liquor, and Solulys Industrial CSL

	H_2 (mM)	Medium* Cost ($/L)
Rich	38.8	1.85 (1X)
BY	48.1	1.38
CSL	45.6	1.39
Industrial CSL	47.8	0.95 (0.51X)

Co-metabolism of six-carbon (cellulose-derived) and five-carbon (hemicellulose-derived) sugars to improve biomass utilization

DMR-pretreated biomass generates a solid fraction containing both cellulosic (C6) and hemicellulosic (C5) sugars. *C. thermocellum* naturally can ferment cellulose directly to H_2 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 hence is the goal in FY 2017 to improve the economic feasibility of fermentative H_2 production. We generated *C. thermocellum* mutant lines capable of co-metabolizing xylose (C5 hemicellulosic sugar) with either glucose or cellobiose (cellulose-derived glucose dimer) with no cross-inhibition, a seminal observation in a cellulose-degrading microbe. The mutant line yielded twice as much H_2 when both avicel (model cellulose) and xylose are present, when compared to either substrate alone. The outcomes illustrate a significant improvement in biomass conversion to H_2 with lower biomass feedstock cost.

Metabolic Engineering

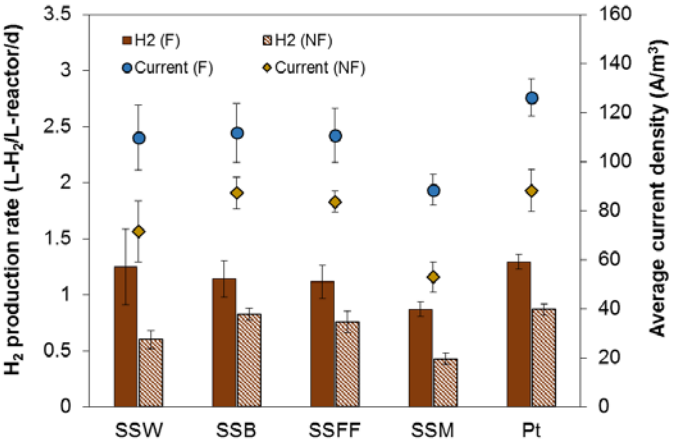
The goal of this approach is to use genetic tools to inactivate genes encoding competing metabolic pathways, thus redirecting more cellular flux (i.e., electrons) to improve H_2 molar yield. We have designed a plasmid suited for genetic transformation in *C. thermocellum* strain DSM 1313 as the model cellulose-degrader. Following the protocols developed by Argyros et al. [10], we have created mutants lacking either the enzyme catalyzing the interconversion of NADPH and NADH (Mutant 1), or the interconversion of reduced ferredoxin and NADH (Mutant 2). The aim is to conserve more reduced ferredoxin and/or NADH. Mutant 1 exhibited a 29% increase in total H_2 production and 55% increase in specific rate of H_2 production. Mutant 2 produced 35% more total H_2 with a concomitant decrease in both ethanol and lactate production. Findings from both mutants validate the effectiveness of metabolic engineering in increasing H_2 output without compromising cell growth or fitness.

Hydrogen Production by MECs with 3-D SS materials (non-Pt based catalyst)

The goal of this project is to avoid using precious metal catalysts while maintaining or improving hydrogen production rates. We evaluated several different SS cathodes with different 3-D architectures (SS mesh, felt, wool and brush) in a larger-scale MEC (anode volume: 100 mL, cathode volume: 68 mL) in the absence and present of catholyte recirculation (50 mM phosphate buffer solution, 250 mL). Synthetic fermentation wastewater composed of acetate, glucose, ethanol, lactate and protein was continuously provided into the anode chamber at a hydraulic retention time of 8 h. The highest H_2 production rate (1.3±0.3 L-H_2/L/d) was observed with SS wool, and the lowest with SS mesh (0.9±0.1 L-H_2/L/d). The H_2 production rate with SS wool was comparable to the rate with Pt cathode (1.3±0.1 L-H_2/L/d) (Figure 1). The good H_2 production rates with SS wool, fiber felt and brushes compared to the SS mesh was likely due to the higher specific surface areas of these materials compared to the SS mesh which was flat and relatively non-porous. Catholyte recirculation was important for improving H_2 production rates (and current densities) as the rates decreased in MECs that did not have recirculation.

Anode Chamber Design

The goal of this project is to see if the use of flat anodes, or smaller brush anodes, could further reduce the anode chamber volume and thus result in greater hydrogen



SSW – SS wool; SSM – SS mesh; SSFF – SS fiber felt; SSB – SS brushes

FIGURE 1. Hydrogen production rates (L-H_2/L-d) and current densities (A/m^3) of MECs with SS materials with a catholyte recirculation (F, 40 mL/min flow rate) or without recirculation flow (NF, 0 mL/min) (applied voltage of 0.9 V; error bars indicate ±SD, with n > 5).

production rates per volume of reactor. Two chamber reactors (14 mL anode, 28 mL cathode) with anion exchange membranes were used to examine H_2 production rates using flat carbon felt or carbon brush anodes in MECs using fed batch mode. MECs were fed sodium acetate (2 g/L) in 50 mM phosphate buffer at an applied voltage of 0.9 V. Brushes performed better, as the average current density was greater for brush anodes (3.8±0.4 A/m^2) than felt anodes (2.9±0.1 A/m^2). The average chemical oxygen demand (COD) removals were 89±2% for the brush anode and 71±2% for the felt anode. The higher current densities and greater COD removals were consistent with the higher hydrogen production rates for the brush anodes (0.38±0.06 L-H_2/L/d) compared to the felt anodes (0.32±0.02 L-H_2/L/d). The shape of the felt anode current profile (a sharp initial peak) suggested that mass transfer of acetate to the anode was limiting performance. When the anolyte was stirred, the current density and COD removals increased to 4.7±0.4 A/m^2 and 92±1%, with a hydrogen production increased to 0.41±0.04 L-H_2/L/d, confirming the mass transfer limited operation with the felt anode.

CONCLUSIONS AND UPCOMING ACTIVITIES

- We determined that the expensive yeast extract nutrient can be replaced with a commercial waste product, the CSL from Solulys, without compromising H_2 production. The replacement lowers the medium cost by 49% and is promising in scale-up H_2 production.
- We generated *C. thermocellum* mutant lines capable of using all the sugars in biomass leading to improved H_2 production with lower biomass feedstock cost.
- We deleted enzymes involved in the interconversion of NADH, NADPH, and ferredoxin, leading to conserving more reduced ferredoxin. The mutants indeed displayed higher specific rate and total H_2 production. These mutants will serve as the hosts for additional genetic engineering in future research to increase H_2 production and molar yield.
- Expensive Pt catalyst can be replaced with non-precious catalyst (e.g,. stainless steel) with larger surface area and we could obtain higher H_2 production rates by using catholyte recirculation.
- Anode volume could be further reduced using a felt electrode, but mass transfer needs to be improved to maintain performances.

In the future, we will operate the sequencing fed-batch bioreactor fermenting DMR-pretreated corn stover lignocellulose with increased solid loading to achieve a H_2 production rate of 2.5 L-H_2/L/d. We will determine what essential genes are needed to enable *C. thermocellum* mutant line to utilize xylan or hemicellulose (complex C5 sugars) directly to enhance its utilization. Deleting the ethanol-competing pathway is deemed essential to redirect more electrons toward H_2 production and will continue to be a part of this effort. Past efforts had led to unstable isolates likely due to redox imbalance. We will delete the ethanol pathway in the mutant hosts with altered balance/pools of NADH/NADPH/ferredoxin to achieve redox stability. 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 H_2 production and lowering MEC cost. We will also examine a smaller reactor volume to further enhance the H_2 production rate to a goal of 2.4 L-H_2/L/d.

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