II.F.6 Fermentation and Electrohydrogenic Approaches to Hydrogen Production

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

Objectives

- Perform hydrogen fermentation using cellulolytic bacteria and lignocellulosic biomass to lower feedstock cost.
- Perform metabolic pathway engineering to improve hydrogen molar yield via fermentation.
- Develop microbial electrolysis cell to improve hydrogen molar yield using waste from the fermentation of lignocellulosic biomass.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section (3.1.4) of the Hydrogen, Fuel Cells & Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

(AR) H_2 Molar Yield
(AS) Waste Acid Accumulation
(AT) Feedstock Cost

Technical Targets

Progress Toward Meeting DOE Technical Targets in Dark Fermentation

Characteristics	Units	2013 Target	2007 Status
Yield of H_2 from glucose	Mole H_2/mole glucose	4	2.1 (from cellulose)
Feedstock cost	Cents/lb glucose	10	13.5 (as of 2003)

- Yield of hydrogen from glucose: DOE has a 2013 target of an H_2 molar yield of 4 using glucose as the feedstock. In Fiscal Year 2006 we achieved a molar yield of 2.1 using lignocellulose from corn stover as the feedstock, which is much more difficult to use yet more abundant and a realistic substrate.
- Feedstock cost: The DOE Biomass Program is conducting research to meet the 2013 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.

Accomplishments

- Optimized growth and performed scale-up bioreactor experiments using the cellulose-degrading bacterium *Clostridium thermocellum* 27405, the genome of which is sequenced. This microbe produced hydrogen via direct fermentation of lignocellulose prepared from the acid hydrolysis of corn stover biomass.
- Demonstrated that when competing metabolic pathways were selectively blocked with inhibitors, output of hydrogen was improved by up to 81%.
- Performed microbial electrolysis cell (MEC) reaction and observed hydrogen production using synthetic solution containing typical waste products of NREL lignocellulosic fermentation.

Introduction

Biomass-derived glucose feedstock is a major operating cost driver for economic hydrogen production via fermentation. The DOE Hydrogen, Fuel Cells, and Infrastructure Technologies Program will take advantage of the DOE Biomass Program's investment in developing inexpensive glucose from biomass to meet its cost target

of 8 cents/lb by 2015. Meanwhile, one alternative and valid approach to addressing the glucose feedstock technical barrier (AT) is to use certain cellulose-degrading microbes that can ferment cellulose directly for hydrogen production. We will use the cellulose-degrading bacterium *Clostridium thermocellum* 27405 as the model organism, which was reported to exhibit the highest growth rate 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 AR), 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]. Most laboratories have however reported a molar yield of 2 or less [3,4]. To circumvent the low hydrogen molar yield, one approach is to perform molecular engineering of a model microbe to block competing pathways. This will redirect cellular metabolic energy toward maximal hydrogen production while minimizing acid and solvent production. This approach has been proven to improve H_2 molar yield in *Enterobacter aerogenes* [5]. We will begin to modify the metabolic pathways in *C. thermocellum*, the genome of which is sequenced.

A promising parallel approach to move past the biological fermentation limit has been developed by 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 (MFC) called a microbial electrolysis cell (MEC) [6]. It demonstrates for the first time a potential route for producing eight or more mole of H_2 per mole glucose when coupled to a dark fermentation process. Via a subcontract to PSU (start date 2nd Quarter, FY 2008), we will examine MEC for hydrogen production using mixed waste products generated from the biomass fermentation process conducted at NREL, lower material costs for and increase output by this new system. Addressing both Technical Barriers AR and AT will realize the potential of hydrogen production via fermentation while overcoming Technical Barrier AS (waste acid accumulation).

Approach

NREL's approach to address feedstock cost is to optimize the performance of the cellulose-degrading bacterium *C. thermocellum*. We will test various cellulosic substrates and optimize reactor parameters to improve longevity, yield, and rate of H_2 production. We will selectively block competing metabolic pathways via chemical inhibitors and test their effects on H_2 production. The outcome will serve as a proof of concept for the genetic engineering approach. PSU will test the performance of MEC initially using a synthetic mixture with composition of organic acids and solvent comparable to that of dark fermentation waste stream. They will then conduct similar experiments using the real waste stream from lignocellulosic fermentation generated at NREL.

Results

Lignocellulose Fermentation

We performed biomass fermentation in scale-up bioreactors with automated temperature (50°C) and pH (6.8), and pressure controls. The bioreactor was bubbled with nitrogen (N_2) gas (10 cc/min) to allow real-time sampling of H_2 and carbon dioxide (CO_2) via an on-line gas chromatograph. *C. thermocellum* previously cultured in crystalline avicel cellulose was inoculated into a 600 mL (working volume) bioreactor fed with 0.14% (w/v) lignocellulose from the dilute-acid hydrolysis of corn stover. Figure 1 displays the kinetics of H_2 and CO_2 production during a period of 90 hours. Determination of molar yield of H_2 and carbon mass balance is underway. A similar experiment performed with the crystalline avicel (0.5%, w/v) generated a H_2 molar yield of 1.8.

Metabolic Engineering

The ultimate goal of this approach is to develop tools to inactivate genes encoding competing metabolic pathways thus providing more cellular flux to improve H_2 molar yield. To test our hypothesis, we determined effects of metabolic pathway inhibitors on H_2 production. We chose two inhibitors: (1) hypophosphite

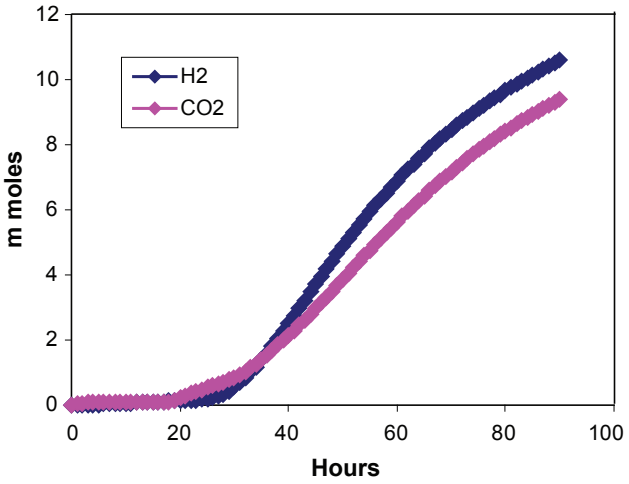

FIGURE 1. Kinetic H_2 and CO_2 Production from Corn Stover Lignocellulose (0.14%, w/v) in a Bioreactor Inoculated with *Clostridium thermocellum* 27405

(HPP), and (2) disulfiram; the former selectively blocks formate synthesis from pyruvate [7], whereas the latter blocks ethanol synthesis from acetyl-CoA [8]. The metabolic pathways of *C. thermocellum* clearly suggest that blocking formate production could channel more pyruvate toward hydrogen production whereas blocking ethanol production conserves more reduced nicotinamide adenine dinucleotide (NADH), a potential substrate for hydrogen production via the NADH-linked hydrogenase. When 0.5 mM to 2 mM HPP was added to *C. thermocellum* cultured in cellobiose, we indeed observed a 31% to 58% increase of H_2 in the culture gas phase (Figure 2a). Moreover we detected a 61% to 74% increase in the hydrogenase activity measured by H_2 production coupling to reduced methyl viologen, suggesting a combination of elevated pyruvate level and new hydrogenase protein synthesis are responsible for the overall increase in H_2 output. The disulfiram (50 μM) similarly enhanced H_2 production by 81%, yet *in vitro* hydrogenase activity remained the same, suggesting a higher NADH pool or a yet unidentified factor may be responsible for the increase in total H_2 output (Figure 2b). These biochemical and physiological studies consequently provide the strategy to guide the most efficient genetic engineering effort while serving as proof of concept that our strategy is scientifically sound.

Microbial Electrolysis Cell

In order to increase overall yields, we also examined H_2 production from the lignocellulose fermentation end products using a synthetic solution having the same composition of main volatile acids and solvents (acetic acid, 26 mM; succinic acid, 5.6 mM; lactic acid, 1.8 mM; formic acid, 0.6 mM; ethanol, 14 mM). The reactor being used is a recently-developed 4-cm long, single-chamber MEC containing a brush anode and flat cathode (Pt catalyst on the cathode only) (Figure 3). Initially with a mixed culture and synthetic substrates, we obtained a maximum yield of H_2 of 30 mL H_2/g-COD (chemical oxygen demand). The gas was produced over a cycle as shown in Figure 4. The high concentration of substrate (compared to other tests) resulted in a long time for the batch cycle, and we found appreciable methane gas production. In successive cycles, methane gas concentration increased and H_2 gas production decreased. In order to improve the process performance, we are looking into an alternative strategy for inoculation by acclimating mixed cultures to the individual substrates in MFC mode before testing them in MEC mode. All the acclimated cultures are starting up as expected except for formate, where the power does not appear be increasing. Maximum voltages are:

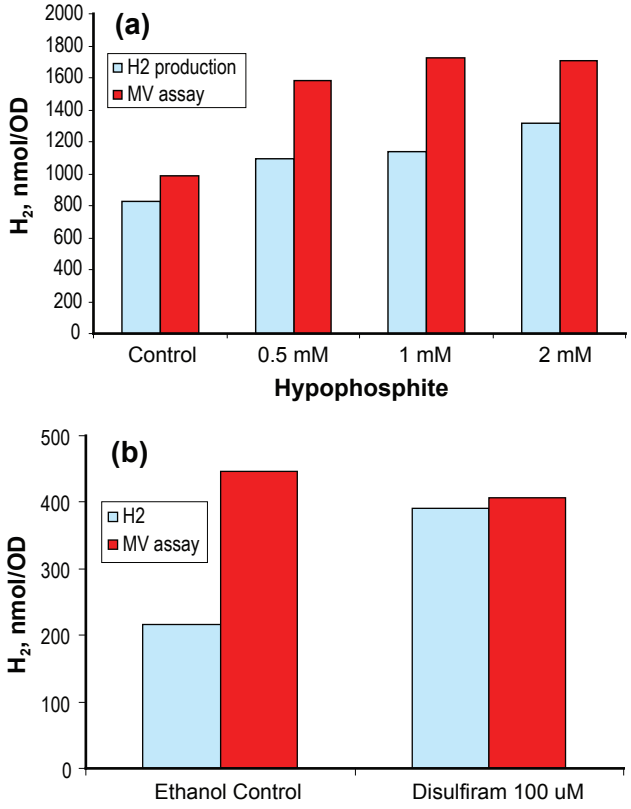

FIGURE 2. Total H_2 Production and Hydrogenase Activity upon the Addition of (a) Hypophosphite and (b) Disulfiram. MV: Methyl Viologen, OD: Optical Density

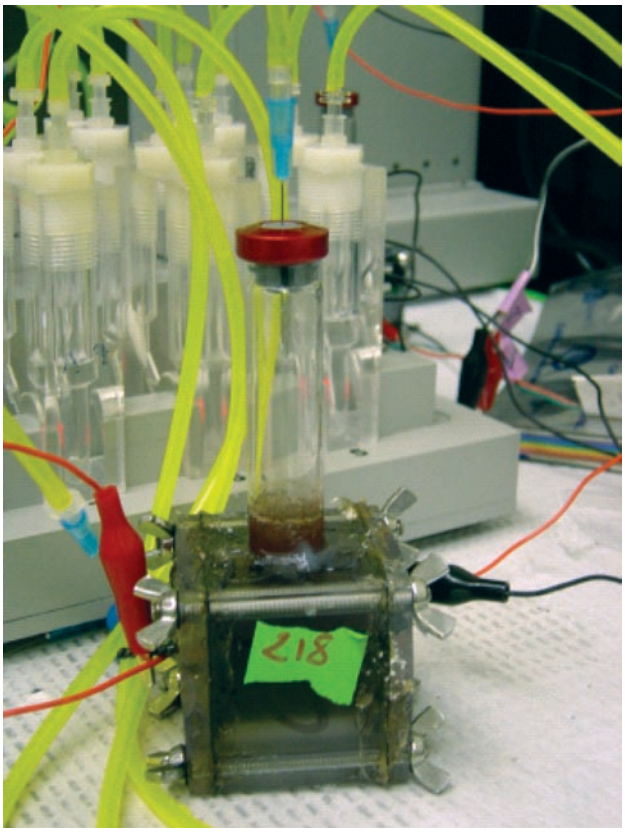

FIGURE 3. MEC Reactor used at Pennsylvania State University for Tests

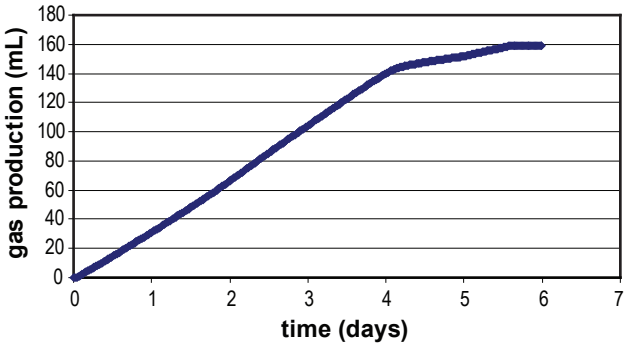

FIGURE 4. Gas Production by a Mixed Culture Using Synthetic Fermentation End Product Solution

acetate, 556 mV; lactate, 543 mV; ethanol, 523 mV; succinate, 412 mV; and formate, 228 mV.

Conclusions and Future Directions

- We demonstrated H_2 production via the fermentation of lignocellulose derived from dilute acid hydrolysis of corn stover, using a sequenced strain of *C. thermocellum*;
- Blocking competing pathways indeed improved yields of H_2 production; and
- The MEC process can produce H_2 from a synthetic solution having the same composition of organic acids and solvent as the typical end products of lignocellulose fermentation.

As for future directions, we will optimize bioreactor performance, and determine carbon mass balance and molar yield of lignocellulose fermentation. We will develop tools for molecular engineering in *C. thermocellum*. In the MEC area, we will test different methods of reducing methane production in MEC while testing real fermentation effluent from NREL.

FY 2008 Publications/Presentations

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3. Huang, L. and B.E. Logan. 2008. Electricity generation and treatment of paper recycling wastewater using a microbial fuel cell. *Appl. Microbiol. Biotechnol*. In press.

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