II.I.4 Fermentation and Electrohydrogenic Approaches to Hydrogen Production

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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 (HFCIT) 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	2009 Status
Yield of H_2 from Glucose	mole H_2/mole glucose	4	9.95
Feedstock Cost	cents/lb glucose	10	12

- Yield of H_2 from glucose: DOE has a 2013 target of an H_2 molar yield of 4 using glucose as the feedstock. In Fiscal Year 2009 we achieved a molar yield of 9.95 from glucose (derived from cellobiose) by integrating fermentation (H_2 molar yield 1.64) with microbial electrolysis cell (MEC) (H_2 molar yield 8.31) reactor.
- Feedstock cost: The DOE Biomass Program is conducting research to meet its 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

- Performed scale-up bioreactor experiments using the cellulose-degrading bacterium *Clostridium thermocellum* 27405 fermenting various amounts of lignocellulose prepared from the acid hydrolysis of corn stover biomass.
- Demonstrated that when competing metabolic pathways were selectively blocked with the inhibitor 4-methyl pyrozole, output of hydrogen was improved by up to 28%.
- Observed hydrogen production from an MEC reaction using real waste effluent from NREL's lignocellulosic fermentation. When integrated, the two-stage process combining fermentation and electrogenesis produced an overall molar yield of 9.95 mol H_2 per mol hexose, using cellobiose as the substrate.

Introduction

Biomass-derived glucose feedstock is a major operating cost driver for economic hydrogen production via fermentation. The DOE HFCIT Program is taking 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. One such example is the cellulose-degrading bacterium *Clostridium thermocellum* 27405 (*C. thermocellum*), 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 mole of H_2 per 1 mole of 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 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 an MEC [6]. It demonstrates for the first time a potential route for producing 8 or more mole of H_2 per mole glucose when coupled to a dark fermentation process. Combining fermentation with MEC could therefore address Technical Barriers AR and AS (waste acid accumulation) 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*. To achieve this goal, we are testing various amounts of cellulosic substrates and optimizing reactor parameters to improve longevity, yield, and rate of H_2 production. We are selectively blocking competing metabolic pathways in this organism via chemical inhibitor and testing its effects on H_2 production. The outcome will serve as a proof of concept for the genetic engineering approach. Via 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

We performed biomass fermentation in scale-up bioreactors with automated temperature (50°C or 55°C), pH (7.0), 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 online gas chromatograph. *C. thermocellum,* previously cultured in crystalline avicel cellulose, was inoculated into a 1.5 L (working volume) bioreactor fed with various amounts of lignocellulose from the dilute-acid hydrolysis of corn stover. Table 1 summarizes rates and molar yields of H_2 production during a period of 90 hours, using corn stover lignocellulose, avicel cellulose and cellobiose as the substrates. Carbon mass balance for cellobiose (14.6 mM glucose) and lignocellulose (0.56% (w/v); or 20.4 mM glucose) is 86% and 74.5%, respectively. Calculation of the carbon mass balance did not account for those carbon substrates assimilated into bacterial cell mass. Typical compounds found in the fermentation waste are: acetic, formic, lactic, succinic acids, and ethanol, which are ideal substrates for the MEC reaction.

TABLE 1. Effect of substrate loading and temperature on rates and yields of hydrogen in *Clostridium thermocellum.*

Substrate	Hexose, mM	Temperature (°C)	L H_2/L/ Day	H_2 Molar Yield
Cellobiose (0.25%)	14.6	55	2.94	1.1
Cellobiose (0.25%)	14.6	50	1.65	1.64
Avicel (0.5%)	30.9	50	1.44	1.51
Corn Stover (0.25%)	9.1	50	0.25	1.67
Corn Stover (0.56%)	20.4	55	0.55	1.33
Corn Stover (0.83%)	30.9	55	1.21	Not determined

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 inhibitor on H_2 production. We chose the inhibitor 4-methyl pyrozole, which blocks two reactions: the synthesis of lactate from pyruvate and the synthesis of ethanol from acetaldehyde [7]. The metabolic pathways of *C. thermocellum* clearly suggest that blocking both pathways could potentially conserve more (reduced) nicotinamide adenine dinucleotide (NADH), an ideal substrate for H_2 production (Figure 1). When 3 mM of 4-methyl pyrozole was added to *C. thermocellum* cultured in cellobiose, we detected

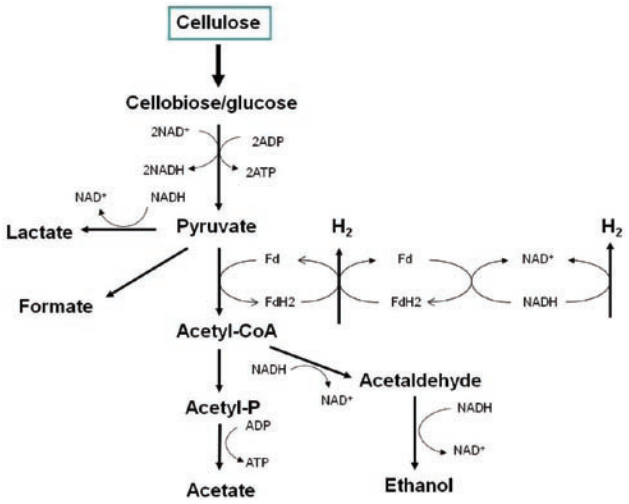

FIGURE 1. Cellulose metabolic pathway in *Clostridium thermocellum.* NAD(H): (reduced) nicotinamide adenine dinucleotide; ATP: adenosine triphosphate; ADP: adenosine diphosphate; Fd(H2): (reduced) ferredoxin

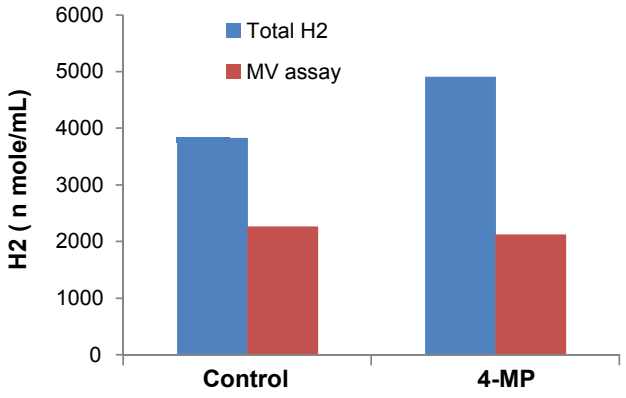

FIGURE 2. Total H_2 production and hydrogenase activity upon the addition of 3 mM 4-methyl pyrazole (4-MP) to a *C. thermocellum* culture fermenting cellobiose. MV: methyl viologen. Rates for MV assay should be divided by 10 to reflect real values.

approximately a 28% increase of H_2 in the culture gas phase (Figure 2). However, the inhibitor did not change the hydrogenase activity measured by H_2 production coupled to reduced methyl viologen. Consequently, the inhibitor study provides the strategy to guide the most efficient genetic engineering effort while serving as proof of concept that the metabolic engineering strategy is scientifically sound.

One criterion for metabolic engineering is to optimize colony formation protocol of *C. thermocellum* on solid agar plate. By lowering the agar concentration from 1.5% (w/v) to 0.7% (w/v), we improved the colony formation by more than 1,000-fold, a significant accomplishment. Using the pIKM1 plasmid (provided by Dr. Wiegel, University of Georgia) which harbors a heat-resistant antibiotic marker (lincomycin), work is

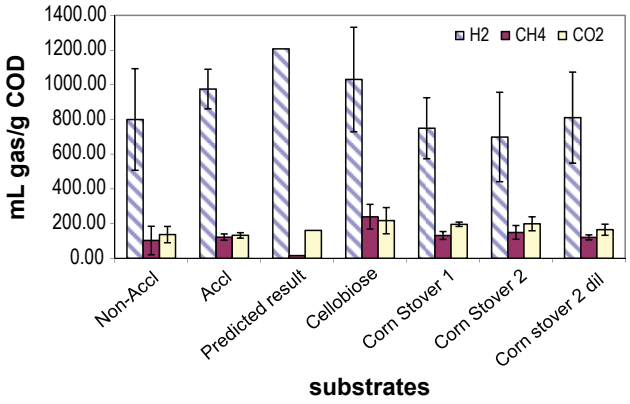

FIGURE 3. Effects of different compositions of selected sources and acclimation procedures on product gas production and composition (hydrogen, methane, and carbon dioxide) for synthetic wastewater (non-accl = not acclimated to individual substrates; accl = acclimated to individual substrates) and fermentation effluents based on cellobiose, two corn stover effluents, and a diluted corn stover.

underway to develop a genetic transformation protocol in this organism.

Microbial Electrolysis Cell

The lignocellulose and cellobiose fermentation effluents provided by NREL consisted primarily of: acetic, lactic, succinic, formic acids, and ethanol. Using just a synthetic fermentation effluent (a mixture of these five compounds) in an MEC enriched to this mixture, the MEC produced an additional 800 ± 290 mL H_2/g chemical oxygen demand (COD) above that produced by the fermentation reactor (mixed inocula tests, or MI). In order to increase hydrogen yields from the fermentation effluent, we devised a procedure to acclimate the bacteria used in the MEC to individual substrates. Using this mixture of bacteria pre-acclimated to the individual compounds, we increased the hydrogen production in the MECs to 980 ± 110 mL H_2/g COD (single substrate inocula tests, or SSI) (Figure 3). Hydrogen yields and production rates with SSI and the synthetic and actual fermentation effluents were 980 ±110 mL/g-COD and 1.11 ± 0.13 L/L-d (synthetic); 900 ± 140 mL/g-COD and 0.96 ± 0.16 L/L-d (cellobiose); and 750 ± 180 mL/g-COD and 1.00 ± 0.19 L/L-d (lignocellulose). A maximum hydrogen production rate of 1.11 ± 0.13 L H_2/L reactor/d was produced with the synthetic effluent. Energy efficiencies based on electricity needed for the MEC using SSI were 270 ± 20% for the synthetic effluent, 230 ± 50% for lignocellulose effluent and 220 ± 30% for the cellobiose effluent. COD removals were ~90% for the synthetic effluents, and 70% to 85% based on volatile fatty acid removal (65% COD removal) with the cellobiose and lignocellulose effluent. The overall hydrogen yield combining MEC with fermentation was 9.95 mol-H_2/mol-glucose for the cellobiose. These results

show that preacclimation of MFCs to single substrates improved performance, and that we could obtain very high hydrogen yields with this integrated system.

Conclusions and Future Direction

- Using lignocellulose as the substrate and a sequenced strain of *C. thermocellum*, low substrate loading gives rise to higher H_2 molar yield while high substrate loading yields faster rate of H_2 production.
- Blocking competing pathways improves yields of H_2 production.
- The MEC process can produce H_2 both from a real waste effluent from lignocellulose fermentation and a synthetic solution having the same composition as the former.

In the future, we will continue to vary substrate loading to determine its effect on rates and yields of H_2. 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, operating in continuous mode, using fermentation effluent from NREL.

FY 2009 Publications/Presentations

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