

IV.E.4 Fermentation Approaches to Hydrogen Production

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

Objectives

- Screen and identify cellulolytic microbes and select one for pathway engineering.
- Perform pathway engineering to improve H₂ molar yield via fermentation.

Technical Barriers

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

- AI. H₂ Molar Yield
- AJ. Waste Acid Accumulation
- AK. Feedstock Cost

Technical Targets

Table 1. Progress Toward Meeting DOE Technical Targets in Dark Fermentation

Characteristics	Units	2010 Target	2005 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 H₂ from glucose: The 2010 target of H₂ molar yield of 4 uses glucose as the feedstock. Our accomplishment in fiscal year (FY) 2005 results in a molar yield of 2.1 using cellulose as the feedstock, which is much more difficult to use although less costly.
- Feedstock cost: Research to meet the 2010 target of 10 cents/lb glucose is being conducted by the DOE Biomass Program. The National Renewable Energy Laboratory's (NREL's) approach is to use cellulolytic microbes to ferment cellulose and hemicellulose directly, which will result in lower feedstock costs.

Approach

- To address the feedstock cost barrier, we will screen microbes that can directly ferment cellulose and hemicellulose, *in lieu of glucose*, to support H₂ production.
- To address the H₂ molar yield barrier, we will select the best cellulolytic microbe identified from the screening and perform metabolic engineering to divert cellular flux toward maximal H₂ production.

Accomplishments

- Screened nine strains of cellulolytic bacteria and identified the three best strains in terms of the highest rate of H₂ production from cellulose. This accomplishment meets the FY 2005 milestone and addresses technical barrier AK.
- Performed scale-up bioreactor experiments using pure cellulose (Avicel[®]) and obtained a H₂ molar yield of 2.1. This accomplishment provides a very good H₂ molar yield baseline from which to improve using a more difficult and yet less costly substrate. With a less expensive feedstock, the final cost of H₂ will become economical even at a lower H₂ molar yield.
- Demonstrated that the cellulolytic microbe can ferment the lignocellulose from corn stover biomass pretreated with a steam explosion process. The United States produces more than 1 billion tons of corn stover per year, the abundance and lesser cost of which makes corn stover a more realistic substrate in terms of meeting DOE's objectives. Moreover, the pretreatment protocols and the characterization of corn stover are well established.

Future Directions

- Perform scale-up H₂ fermentation experiments using lignocellulose derived from steam explosion of corn stover under both acidic and neutral conditions.
- Develop a genetic transformation system in *Clostridium thermocellum* for metabolic pathway engineering with the intent to improve H₂ molar yield.

Introduction

The biomass-derived glucose feedstock is a major operating cost driver for H₂ production via fermentation. One of the cost contributors is the expense of the pure cellulase enzymes needed to hydrolyze cellulose to glucose. The DOE Hydrogen, Fuel Cells and Infrastructure Technologies (HFCIT) 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 address the glucose feedstock technical barrier (AK) is to identify microbes that can use cellulose and hemicellulose directly, *in lieu of glucose*, for H₂ production. Numerous microbes are known to degrade cellulose and hemicellulose, via a suite of extracellular cellulase and hemicellulase enzymes, to support cell growth with an immense potential to produce H₂ simultaneously [1]. This project is identifying and screening cellulolytic microbes to select those that produce H₂ most efficiently.

Another technical barrier to fermentation is the relatively low molar yield of H₂ from glucose (mol H₂/mol sugar; technical barrier AI), which results from the simultaneous production of waste organic

acids and solvents. Even though 12 moles of H₂ can theoretically be produced from 1 mole of glucose (the theoretical maximum), biological pathways maximally yield only 4 moles of H₂ per 1 mole of glucose (the biological maximum) [2]. Most laboratories have reported a molar yield of 2 or less [3, 4]. Once a suitable microbe is selected, we will perform molecular engineering of the model microbe to block competing pathways. This will redirect cellular metabolic energy toward maximal hydrogen production while minimizing acid and solvent production. Blocking competing pathways via genetic engineering has been proven to improve H₂ molar yield in *Enterobacter aerogenes* [5]. Addressing technical barriers AI and AK will realize the potential of H₂ production via fermentation while overcoming technical barrier AJ (waste acid accumulation).

Approach

In FY 2005, our main goal has been to address technical barrier AK by screening cellulolytic microbes capable of using cellulose directly, in lieu of glucose, for fast rates of H₂ production. Once suitable strains are identified, we will select one for metabolic pathway engineering in FY 2006 and beyond. Most microbes that degrade cellulose also

excrete a suite of hemicellulase enzymes to hydrolyze hemicellulose [6]. Together, cellulose and hemicellulose constitute up to 90% of the biomass and contain nearly 100% of the carbohydrate fractions [7]. Our strategy will simplify biomass pretreatment processes and thus significantly lower feedstock cost. Moreover, it will result in a near complete utilization of most of the carbohydrate fractions of biomass for H₂ production, instead of using only the glucose component of biomass (mostly from cellulose), which is only 40%-60% for most lignocellulosic biomass.

Results

Screening of Cellulolytic Microbes

We have established collaboration with Lee Lynd (Dartmouth College) and Ed Bayer (Weizmann Institute of Sciences, Israel). Both individuals kindly supplied us with a total of eight strains of *Clostridium thermocellum* isolated from anaerobic composts and the hot springs of Yellowstone National Park. *C. thermocellum* is a thermophile, with optimal growth temperature between 55°C and 60°C. Upon exposure to cellulosic substrate, this microbe is known to excrete a suite of extracellular cellulase enzymes to hydrolyze cellulose to cellobiose (a glucose dimer). The cellobiose is then transported inside the cells to support microbial growth. We tested eight strains along with an American Type Culture Collection (ATCC) culture for H₂ production using a commercial grade of pure cellulose (Avicel) as the substrate. Five strains were found to produce H₂ almost immediately from Avicel [0.5%, weight by volume (w/v)] and leveled off between 8 to 9 μmol H₂/ml culture gas phase (Figure 1A and 1B). Table 2 summarizes the rate of H₂ production from kinetic studies. Although the ATCC strain displays the highest rate of H₂ production, it consistently grew slower in Avicel with an initial lag phase. Cell growth rate is an important parameter in culture selection along with fast rates of H₂ production. We will continue to optimize growth parameters for the ATCC strain. Meanwhile, we performed scale-up experiments using *C. thermocellum* strain 1.1.1, which grows very fast in Avicel.

Table 2. Rates of Hydrogen Production from Avicel Cellulose

Clostridium thermocellum Strains	nmol H ₂ /hr/ml gas
ATCC	1018
1.1.1	595
YS	477
7.10.4	477
7.12.1	407
7.7.10	35
7.8.3	Trace
6.3.2	Trace
7.9.4	Trace

Our strategy of screening using Avicel cellulose meets our objective. Commercial Avicel is prepared by removing most of the amorphous regions of cellulose with mild acid, which yields a product that contains the most crystalline fraction of cellulose [6]. Finding microbes capable of hydrolyzing Avicel almost guarantees that these microbes will degrade lignocellulose from biomass at a faster rate because lignocellulose is much more amorphous. The identification of best cellulolytic strains meets the FY 2005 milestone for this research.

H₂ Molar Yield and Carbon Mass Balance Determination

To determine H₂ molar yield and carbon mass balance more accurately, we must perform fermentation in scale-up bioreactors with automated controls for temperature (50°C) and pH (6.8). To facilitate real-time measurement of product gas, we bubbled the reactor with nitrogen (N₂) gas (10 cc/min) to allow continuous sampling of H₂ and carbon dioxide (CO₂) via an on-line gas chromatograph. *Clostridium thermocellum* 1.1.1 was chosen as the model strain for feeding with .5% (w/v) Avicel. Data from Figure 2 display kinetics of H₂ and CO₂ production during a 150-hr period. Both H₂ and CO₂ production reach a stationary phase at around 75 hr because of the complete depletion of the cellulosic substrate. Carbon mass balance is approximately 95% without

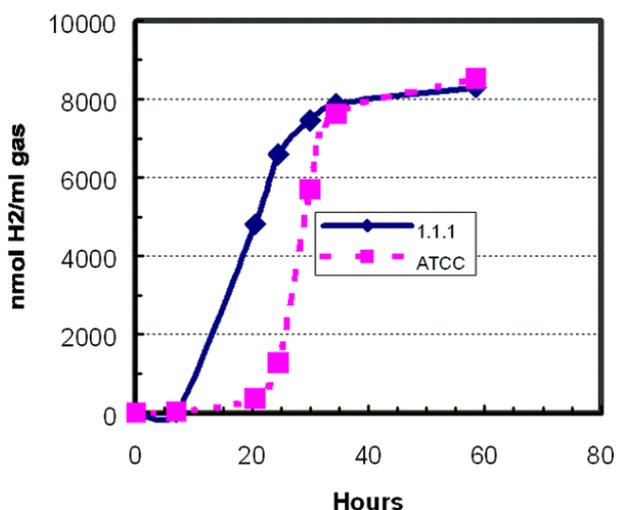
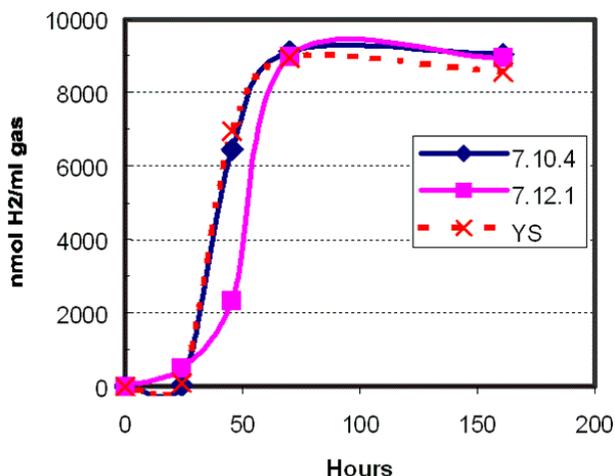


Figure 1. Hydrogen Production from Various Strains of *Clostridium Thermocellum* at 55°C Using Avicel Cellulose (0.5%) as the Substrate

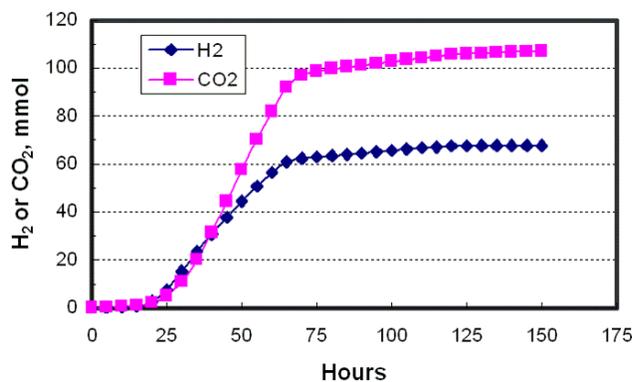


Figure 2. Kinetic H₂ and CO₂ Production from Avicel Cellulose (0.5%) in a Bioreactor Inoculated with *Clostridium thermocellum* 1.1.1

accounting for those carbon materials assimilated into new cell mass. As measured by high-performance liquid chromatography, ethanol and acetic acid are the dominant waste products, with minor amounts of formic and lactic acids also observed. Molar yield of H₂ from cellulose is 2.1, and this is the first demonstration of such a yield using Avicel cellulose as the substrate (not yet reported in the literature). Our H₂ molar yield data compare very favorably with those obtained in other laboratories, most of which reported a value around 2 or less using the more favorable and costly glucose as the feedstock. Cellulose degradation is much more challenging. Consequently, our research findings give us a very good baseline on which to improve.

H₂ Fermentation Using Corn Stover Pretreated with Steam Explosion

The ultimate goal of this research is to use waste biomass as the feedstock. Using corn stover as the model biomass, we chose steam explosion as the pretreatment technology to evaluate its suitability for H₂ fermentation. Steam explosion is conducted by subjecting steam-saturated corn stover [200 pounds per square inch (psi)], with or without acid [1.2% sulfuric acid (H₂SO₄)], to a sudden release of pressure while forcing through a small orifice, during which the flash evaporation of water causes the biomass to rupture [8]. This thermo-mechanical force breaks open the ultrastructure of (corn stover) biomass into an aqueous hemicellulose (hydrolyzate) and a solid lignocellulose fraction, both of which are then tested for fermentation. In this work, we are focusing on the fermentation of the lignocellulose solid because H₂ fermentation of the hydrolyzate is much easier and was demonstrated earlier in our laboratory [9]. Using lignocellulose solids (0.5%, w/v) from either neutral or acid steam explosion of corn stover, we observed H₂ production almost immediately from *C. thermocellum* 1.1.1 (Figure 3). Visual inspection of the test tubes before and after fermentation indicates that the lignocellulose solids almost disappeared completely at the end of fermentation. Work is underway to scale up this fermentation process to better measure carbon mass balance and H₂ molar yield.

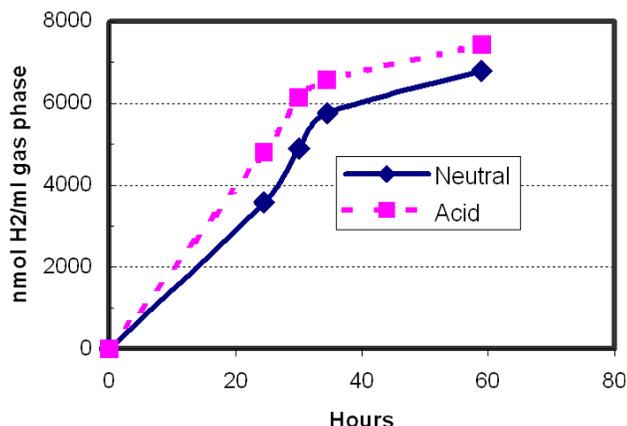


Figure 3. Hydrogen Production from *Clostridium thermocellum* 1.1.1 Using Lignocellulose Solids from Steam Explosion of Corn Stover under either Neutral or Acidic Conditions

Metabolic Engineering

We have screened and identified three best strains of *C. thermocellum*, namely ATCC, 1.1.1 and YS, with the highest rates of H₂ production from cellulose. We will choose the ATCC strain as the model microbe for genetic engineering to improve its H₂ molar yield in FY 2006. The main reason is that the genome of this particular strain has been sequenced. This accomplishment has met the FY 2005 milestone. We will begin work soon to conduct a literature search and to evaluate the genetic system of the ATCC strain for transformation.

Conclusions

- We have screened and identified several strains of *C. thermocellum* capable of producing H₂ with high rates from Avicel. This accomplishment meets the FY 2005 milestone (to identify up to three suitable strains of fermentative microbes and select one for genetic engineering).
- We will continue to optimize growth of the ATCC strain in Avicel because it demonstrates the highest rate of H₂ production seen to date.
- Using Avicel as the substrate and strain 1.1.1 in scale-up bioreactors, we obtained a H₂ molar yield of 2.1, which compares very favorably with those reported in the literature using glucose as the substrate.

- *C. thermocellum* was capable of metabolizing the lignocellulose fraction from corn stover biomass pretreated with a steam explosion process. Using waste biomass is an ultimate goal of this research. Our accomplishments in FY 2005 meet the DOE technical target of developing an inexpensive feedstock in support of fermentative H₂ production.

Special Recognitions & Awards/Patents Issued

1. Maness mentored a high-school student (Michelle Gahagan) with her Science Fair Project in H₂ production via fermentation. The student won first prize in her school and second prize in the Louisiana Regional Fair along with a special award from the U.S. Army.

FY 2005 Publications/Presentations

1. Datar, R., Huang, J., Maness, P. C., Mohagheghi, A., Czernik, S., and Chornet, E. "Hydrogen production from the fermentation of corn stover pretreated with a steam explosion process," Submitted to *Environ. Sci. Technol.*
2. Presentation to the Minnesota Delegation (Maness, October 2004).
3. Presentation to the DOE Hydrogen Production Technical Team (Maness, January 2005).
4. Oral presentation at the 10th Institute of Biological Engineering Meeting (Maness, March 2005).
5. Invited seminar at Penn State University (Maness, April 2005).
6. Presentation to the Brazilian Delegation of their USAID/DOE/MME Bilateral Project (Maness, May 2005).
7. Presentation at the International Hydrogen Energy Congress (Maness, July 2005).

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