Biological Water Gas Shift Development

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Objectives

- Improve biological water-gas shift (WGS) reaction for producing biomass-derived hydrogen, using the photosynthetic bacterium *Rubrivivax gelatinosus* CBS as the biological catalyst.
- Understand the biological machineries governing the CO-shift process in order to improve both CO-shift rates and durability.
- Understand high pressure bioreactor dynamics.
- Design, construct, and operate bioreactor capable of operating at elevated pressure.
- Elucidate CO pressure effects on biological CO-shift reaction.

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 R,D&D Plan:

- F. Feedstock Cost and Availability
- G. Efficiency of Gasification, Pyrolysis, and Reforming Technology

Approach

- Physiology study to determine energy generation during CO shift. If verified, this would assure sustained hydrogen production in darkness.
- Molecular study to clone and characterize genes involved in CO-shift reaction. This would enable us to optimize the CO-shift process.
- Integrated bioreactor engineering and operation to understand high-pressure bioreactor dynamics.

Accomplishments

- Physiological studies have confirmed that adenosine triphosphate (ATP) is generated dur-ing CO shift, which simplifies dark bioreactor operation and maintenance while improv-ing its durability.
- Molecular biology studies have identified 16 genes involved in CO shift, which enables us to manipulate them for over-expression of CO-shift activity.
- Developed a novel recirculating bubble-column reactor which provides good gas-liquid mass transfer.
- Obtained intrinsic CO-shift kinetics of Rubrivivax gelatinosus at various CO concentrations.

Future Directions

- Understand the biochemistry of the hydrogenase enzyme in the CO-shift pathway. The CO-shift hydrogenase is durable and more tolerant to oxygen, unlike most hydrogenases reported in literature. An in-depth investigation could result in insight regarding a structure-function relationship.
- Continue to understand the genetic system of the CO-shift hydrogenase. This knowledge would allow its genetic transfer to other microbial systems for fermentative and photo-lytic hydrogen production.
- Elevated-pressure bioreactor operation with gaseous substrates is a unique research area. Continued development in this area could lead to process improvement and other applica-tions.

Introduction

This project is investigating the use of the photosynthetic bacterium *Rubrivivax (Rx.) gelatinosus* CBS to quantitatively shift and condition crude synthesis gas into a hydrogen-rich stream according to the reaction: $CO + H_2O \leftrightarrow H_2 + CO_2$. In contrast to conventional high-temperature catalytic shift processes, this biologically mediated reaction can operate at ambient temperatures, making it potentially promising for large-scale H_2 production. This project has two different goals: a nearer-term goal to investigate the utility of this biological process as an alternative to conventional shift technology, and a longer-term goal to understand the enzymatic processes involved in the CO-to-H_2 reaction.

The nearer-term goal to determine the utility of this process as a "drop-in" replacement to the conventional high-temperature shift process is important because the process may represent a less-expensive option to the conventional process for certain process streams. The longer-term goal of understanding the enzymatic processes involved may allow the transfer of the hydrogen production system to another organism, which would represent a completely new approach to biological hydrogen production.

Approach

Work on this project is divided into fundamental experiments designed to understand the metabolic CO-shift pathway in *Rx. gelatinosus* CBS, and applied experiments focusing on the scale-up of the overall process.

Our approach to the fundamental experiments is to use both microbiological and molecular biology techniques to help elucidate the overall hydrogen production pathway. Based on evidence from the closely related photosynthetic bacterium Rhodospirillum rubrum, we hypothesize that CO oxidation is catalyzed via a CO dehydrogenase (CODH) enzyme generating reducing equivalents, which are then transferred via a ferredoxin-like iron-sulfur protein to the terminal hydrogenase to yield H_2. Thus, the enzyme complexes responsible for CO oxidation and hydrogen production are distinct and can be studied separately.

Our approach to the scale-up experiments is guided by an independent and very detailed techno-economic analysis of the overall process. This analysis, begun in FY 2001, was refined during FY 2002 and FY 2003. This analysis showed the need to operate the bioreactors at moderate pressures (150-200 psi). Bioreactor operation at these pressures allows significantly higher volumetric hydrogen production rates while incurring no additional reactor costs (conventional reactor materials, e.g., carbon steel, can easily withstand these modest pressures). Our overall approach is to build incrementally larger and higher-pressure bioreactors. At each stage during scale-up, reactor models are developed and compared to actual reactor data to help us fully understand the reactor dynamics.

Results

The goal of our microbiology experiments was to demonstrate that the photosynthetic bacterium *Rx. gelatinosus* CBS could grow solely on CO in the dark. Previous work has shown CO-supported growth in the dark in *Rx. gelatinosus* Strain 1 (Uffen,

1976), in *Rs. rubrum* (Kerby, et al., 1995), and in *Carboxydothermus hydrogenoformans* (Svetlichny et al., 1991). However, all of these experiments used a carbon-containing complex nutrient such as yeast extract or trypticase in the growth medium. Because of this, it is hard to conclude whether CO alone can generate the energy necessary to support cell growth or whether the complex nutrients played a role as well. We have performed a number of experiments to unequivocally demonstrate that bacterium *Rx. gelatinosus* CBS can grow solely on CO in the dark.

Figure 1 shows the results of an experiment in which *Rx. gelatinosus* CBS was grown in minimal medium with CO as the sole carbon substrate. This dark-grown culture has a doubling time of approximately two days in CO, as reflected both by the increase in optical density at 660nm (data in Figure 1), and by an increase in the number of colony-forming units on agar plates (data not shown).

We collected complementary evidence of the ability of *Rx. gelatinosus* CBS to grow solely on CO in darkness by measuring ATP generation during dark growth. Since ATP is a universal energy carrier in biological systems, generation of ATP is generally accepted as proof of growth on a given substrate. We cultured two identical CBS cultures in the presence of CO and light. The two cultures were then placed in darkness, without CO, to exhaust the endogenous ATP levels. After an overnight incubation, the headspace of one culture was filled with 20% CO gas while the other culture headspace was filled with argon gas as a control. At various intervals, aliquots of cell samples were withdrawn and quantified for endogenous ATP levels (Tran and Unden, 1998). Data from Figure 2 clearly indicate that the culture that received CO immediately began to produce ATP over a time period of 15 min, while no net ATP production was measured in the argon gas control. Data from both Figures 1 and 2 therefore indicate that CO supports both cell growth and ATP synthesis in darkness. ATP can be used to generate new cell mass, support nutrient uptake and cell repair, and synthesize new enzymes such as the WGS catalysts, once the existing ones are turned over. These findings therefore have significant implications in that CO shift bioreactor can operate in darkness to sustain long-term hydrogen production, which will greatly simplify the design and operation of the bioreactor.

Our work this year in molecular biology was remarkably successful. We have identified 16 putative genes involved in the pathway of H_2 production (Figure 3). Of these 16 genes, four (*hypA, cooF, cooS*, and *cooC*) reside on the same operon and are thought to be involved in the first

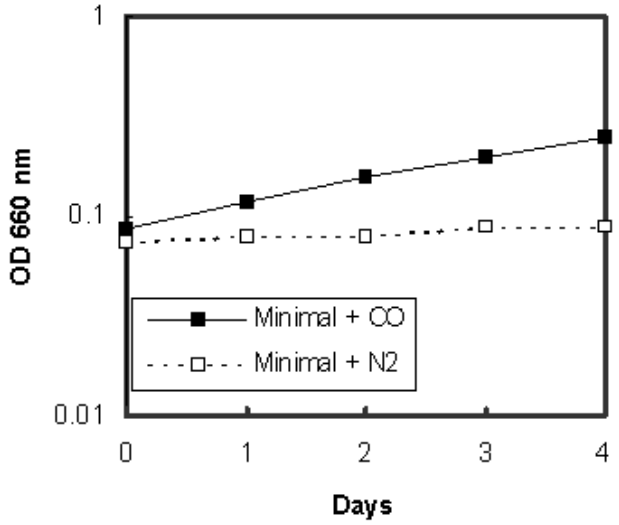

Figure 1. Growth of *Rubrivivax gelatinosus* CBS with CO as the sole carbon substrate in the dark. The culture was bubbled daily with 20% CO (solid line) while the control received N_2 gas (dashed line).

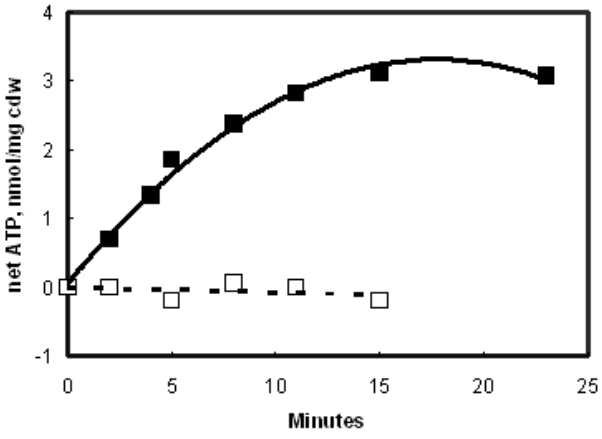

Figure 2. Kinetics of ATP production by *Rubrivivax gelatinosus* CBS in darkness with CO (solid line) or argon gas (dashed line). (cdw = cell dry weight)

enzymatic step of the pathway, the oxidation of CO to CO_2 and the release of protons and electrons from water. The remaining 12 genes are believed to play a role in the hydrogenase reaction, the reduction of protons to H_2. Unlike most other hydrogen-related genes cloned to date, these genes are clustered within the chromosome, encompassing approximately 15 kilobases. The *coo*-genes (for CO-oxidation) all show a high degree of similarity to those found in *Rhodospirillum rubrum*, which also produces hydrogen via the biological WGS (BioWGS) reaction (Fox et al., 1996). Unlike the gene organization in *Rs. rubrum*, *Rx. gelatinosus* CBS contains Hyp (hydrogenase pleiotrophy) genes clustered around the *coo* genes. Hyp genes encode hydrogenase accessory proteins involved in the insertion and co-ordination of Ni^{2+}, Fe, CO, and CN ligands found within the catalytic portion of the hydrogenase. The *hyp* genes are similar to those found in a wide variety of hydrogenase-containing bacteria, including *Chlorobium tepidum*, *Rhodobacter sphaeroides*, *Aquifex aeolicus*, and *Nostoc punctiforme*. Lastly, a putative Ni^{2+} transporter is associated with the BioWGS genes. The translation of this gene sequence predicts that the putative transporter contains an N-terminal signal peptide and six transmembrane domains. Ni^{2+} is an essential component of the *Rx. gelatinosus* CBS hydrogenase, and is also incorporated into multiple enzymes involved in the BioWGS reaction. The putative Ni^{2+} transporter is comparable to those found in *Ralstonia metallidurans*, *Rhodopseudomonas palustris*, and *Bradyrhizobium japonicum*. A *cooS* mutant (termed GV1214) was obtained and analyzed during FY 2002 and determined to be lacking carbon monoxide dehydrogenase (CODH) activity (Vanzin *et al*., 2002). This mutant was complemented by adding back to the mutant the region of the chromosome containing HypA through *cooC*. This cell line then produced approximate wild-type levels of hydrogen (data not shown).

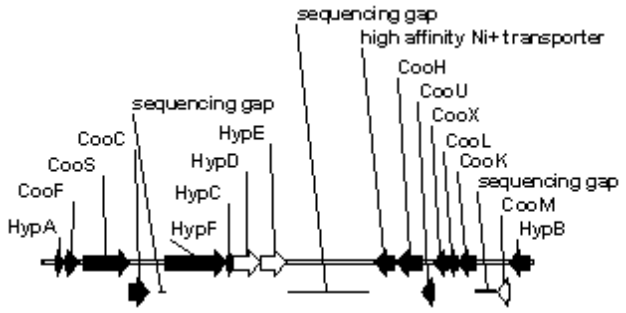

Figure 3. BioWGS gene organization. Filled arrows indicate completed genes, while open arrows indicate partially sequenced genes. The genomic information encompasses approximately 15 kilobases. Scale is approximate.

As mentioned above, the independent techno-economic evaluation of the overall biological water-gas shift process indicated that the bioreactor must be operated at elevated pressures to achieve favorable process economics. Our previous work with pressurized bioreactors has indicated that elevated pressures reversibly inhibit the CO-shift activity of the microorganism. Our current results strongly indicate that the presence of dissolved CO is the major cause of this inhibition.

To verify this, we performed a number of CO conversion rate experiments in agitated shake flasks to determine the effect of dissolved CO concentration on the rate of CO uptake and subsequent H_2 production by the bacterium *Rx. gelatinosus* CBS. While gas-liquid mass transfer often complicates the interpretation of reactor conversion data, we verified that these experimental results were not influenced by mass transfer limitations. The results of these experiments are shown in Figure 4. There is

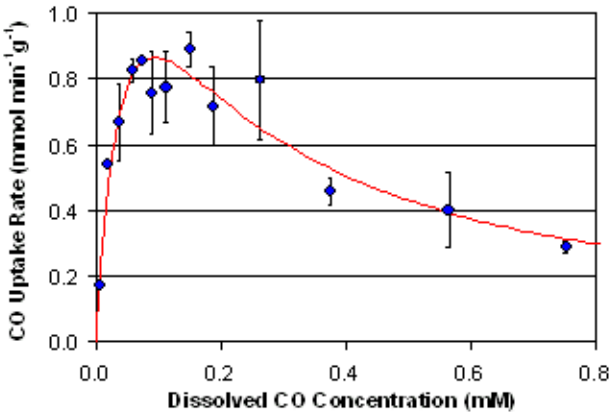

Figure 4. Effect of dissolved CO concentration on the rate of CO conversion by *Rubrivivax gelatinosus* CBS. At concentrations above approximately 0.15 mM, the conversion rate decreases due to substrate inhibition by CO.

considerable scatter in the data, but the overall trend is clear: the rate of CO conversion to H_2 passes through a maximum at approximately 0.15 mM dissolved CO, which corresponds to a gas phase concentration of CO of approximately 20%. These results are consistent with the phenomenon of "substrate inhibition", in which excessive concentrations of a reactant can interfere with the enzyme catalyzing conversion to product. The curve in Figure 4 shows a fit to a substrate inhibition kinetic model.

How does the behavior shown in Figure 4 cause reactor inhibition at elevated pressures? At elevated pressure, the solubility of CO in solution increases, and above a critical pressure (which depends on several factors), the dissolved CO concentration begins to exceed 0.15 mM, which causes a reversible inhibition of reaction rate. Thus, the inhibition can be avoided simply by keeping the dissolved CO concentration below 0.15 mM in the bioreactor. Our preliminary experimental and modeling results (not shown) indicate that elevated cell mass loadings in the bioreactor appear to be effective in keeping the dissolved CO concentration below 0.15 mM, since higher cell mass loadings increase the CO uptake rate per unit bioreactor volume. We are currently performing additional experiments to verify these results.

NREL performed a techno-economic analysis to investigate the relative economics of biological vs. conventional water-gas shift (WGS). Reactor data from our work were used to estimate the size (and therefore cost) of the biological WGS reactor. These results were then compared to the conventional process in the context of an overall system model. The thermodynamics of the biological WGS process are more favorable than the conventional WGS process, but the kinetics are less favorable. Thus, a biological WGS reactor will always be larger (and therefore more expensive) than the corresponding conventional reactor. This difference in reactor cost is balanced by the cost for low-pressure steam production and delivery for the conventional process (the biological process uses liquid water). Thus, the overall cost savings associated with the biological process result from these "balance of plant" savings, and the overall system must be considered when estimating the relative costs of the two different shift technologies. For many biomass-derived synthesis gas streams, the presence of unreacted hydrocarbons (principally methane, CH_4) requires the use of a secondary reformer downstream of the gasifier to convert the unreacted hydrocarbons to additional synthesis gas. Since this reformer requires steam, if the reformer is used, the incremental cost of steam generation for the conventional WGS reactor is minimal, and the lower cost of the conventional WGS reactor makes this technology the preferred option. Thus, it is the amount of methane present in the biomass-derived synthesis gas stream (and not the amount of CO in the stream) that controls which WGS technology is preferred. At lower methane concentrations, no reformer is needed, and the biological WGS process is preferred. At higher methane concentrations, the reformer is necessary, and the conventional WGS process is preferred. The exact methane concentration where this "crossover" occurs depends on a number of factors, but for the assumptions we made in the techno-economic analysis, we believe that the biological WGS process is a promising alternative to conventional process for CH_4 concentrations less than about 3%-5%. The results of this analysis are shown in Figure 5.

Conclusions

- CO can serve both as the carbon and energy substrate to sustain long-term hydrogen production in darkness.

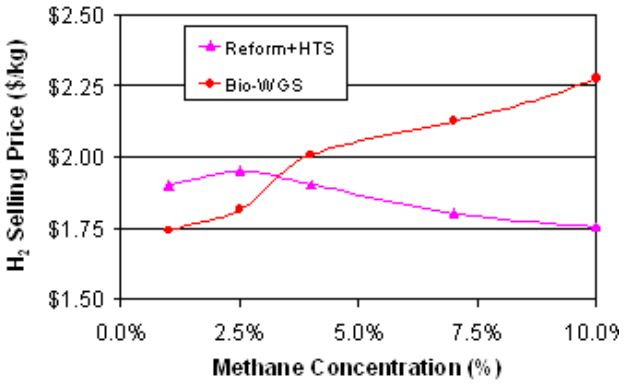

Figure 5. Effect of methane concentration on the cost of hydrogen produced for both conventional and biological WGS processes. Below approximately 3-5%, the biological WGS process appears to be the less expensive option.

- At least 16 genes are required for the CO-to-H_2 reaction in *Rx. gelatinosus* CBS. These genes encode proteins not only involved in catalysis, but also in the transport and assembly of hydrogen-producing enzymes.
- A number of *Rx. gelatinosus* WGS genes are closely related to those known in *Rs. rubrum*, which also produces hydrogen from CO.
- Unlike other hydrogenase-containing microorganisms, *Rx. gelatinosus* CBS contains a large cluster of genes related to hydrogen production. This grouping of genes should facilitate their over-production and/or heterologous expression.
- By restoring wild-type levels of hydrogen production in a CODH mutant, we have demonstrated the ability to make H_2-related proteins of our choosing in CBS. This is a critical step toward the overproduction of microbial catalysts aimed at the production of hydrogen.
- Elevated pressure bioreactor operation is complicated by the inactivation of the WGS pathway by elevated concentrations of dissolved CO. This problem can be avoided by proper bioreactor design.
- The biological WGS reaction appears to be a promising alternative to conventional technology for synthesis gas streams containing low concentrations (<5%) of unreacted hydrocarbons.

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FY 2003 Publications/Presentations

1. Maness, P.C. and P.F. Weaver. 2002. Hydrogen production from a carbon-monoxide oxidation pathway in *Rubrivivax gelatinosus*. Intl. J. Hydrogen Energy. 27: 1407-1411.

2. Merida, W., P.C. Maness, R.C. Brown, and D.B. Levin. Enhanced hydrogen production from indirectly heated, gasified biomass, and removal of carbon gas emission using a novel biological gas reformer. Accepted for publication in Intl. J. Hydrogen Energy.

3. Maness, P.C., G.V. Vanzin, J. Huang, and S. Smolinski. "The Hydrogen Production Pathway Linked to Carbon Monoxide Oxidation in Rubrivivax Gelatinosus" (Oral Presentation). 12th Western Photosynthesis Conference, Pacific Grove, CO (January 2003).

4. Huang, J., S. Smolinski, G.V. Vanzin, V. Tek, and P.C. Maness. "The Water-Gas Shift Reaction Generates Energy in Darkness" (Poster Presentation). 25th Symposium on Biotechnology for Fuels and Chemicals, Breckenridge, CO (May 2003).

5. Amos, W., Wolfrum, E., Watt, A., "Biological H_2 Production from Synthesis Gas: Preliminary Techno-Economics & Reactor Design Issues" (Poster Presentation). 25th Symposium on Biotechnology for Fuels and Chemicals, Breckenridge, CO (May 2003).