Proceedings of the 1998 U.S. DOE Hydrogen Program Review

NREL/CP-570-25315

Biological H_2 from Fuel Gases and from H_2O

P. Weaver, P.-C. Maness, C. Rhodes, J. Scahill, S. Dundorf, and S. Martin
National Renewable Energy Laboratory
Golden, Colorado 80401

Summary

The two stand-alone objectives of the research are to economically produce H_2 in the near term from biomass (thermally gasified to syngas) and in the mid term from H_2O using cyanobacteria or algae with an oxygen-tolerant bacterial hydrogenase.

Photosynthetic bacteria have four different terminal enzymes that mediate their H_2 metabolisms—nitrogenase, uptake hydrogenase, fermentative hydrogenase, and carbon monoxide-linked hydrogenase. Each has been microbiologically and biochemically examined for their potential to specifically generate H_2 in large-scale processes. Based on measurements of maximal activities, stabilities, energy requirements, equilibria, and partial pressures of the H_2 producing reactions, the CO-linked hydrogenase is easily the most suited for practical applications. The enzyme mediates H_2 production from CO at rates up to 3 $mmol \cdot min^{-1} \cdot g$ cell dry $weight^{-1}$ at near ambient temperature and pressure. At biological temperatures, equilibrium for the CO shift into H_2 lies far towards H_2 production. Less than 0.1 ppm of CO remains after a 20% CO gas phase is acted upon by bacteria. The necessary contact time between CO and bacteria is approximately ten seconds. Similar biological activities are observed with thermally generated fuel gases. The product gas can be directly used in fuel cells. New bacterial isolates from nature and mutant strains are being selected to further improve the novel technology. Oxygen-resistant enzymes identified in some bacterial strains could lead to a more general, second generation technology mediating the solar production of H_2 from H_2O.

Presently, mass transfer of gaseous CO limits the bacterial production of H_2 from fuel gases. New bioreactor designs have significantly enhanced shift rates. Vapor-phase and bubble-train bioreactors employing immobilized or suspended bacteria are being scaled up. A User Facility has been established for the safe engineering scale up and validation of solar or dark technologies for the Hydrogen Program. The first system to be tested at the site will integrate fuel gas generators with biological shift reactors and a PEM fuel cell.

Introduction: An economic process for producing hydrogen, whether biologically or chemically based, would ideally be (1) H_2O derived, (2) solar driven, (3) highly efficient, (4) durable, (5) insensitive to hydrogen partial pressure, and (6) inexpensive to build and operate. A complete system fulfilling all of these goals is not currently available. Of the biological options, systems employing intact cells of photosynthetic bacteria are the most advanced. Unlike cyanobacteria or algae, however, photosynthetic bacteria do not oxidize water and therefore do not directly fulfill criterion (1). They do, however, evolve H_2 from biomass (previously photosynthetically generated from H_2O and CO_2). These bacteria employ several different enzymatic mechanisms that may have commercial potential for possible near term applications of H_2 production from biomass. One mechanism that incorporates thermal and biological processes appears particularly promising: thermally generated fuel gases derived from biomass can be sufficiently conditioned by bacterial catalysts in a one-step process that they may be directly injected into platinum-electrode, hydrogen fuel cells.

A complete system of sustained hydrogen production based on the direct photooxidation of water is more difficult to achieve. Oxygen production is inherent in the oxidation of water, and the hydrogen-evolving enzymes of cyanobacteria and algae are usually rapidly inactivated by oxygen. A number of newly isolated photosynthetic bacterial strains contain an O_2-resistant, evolving hydrogenase enzyme. This hydrogenase has been partially purified from two strains of bacteria, where it is found tightly bound to membrane fractions. The enzymes require an additional electron mediator for H_2 production. Genetic transfer and expression of the oxygen-resistant, bacterial hydrogenase enzyme and cofactor in a cyanobacterial or algal host could provide a method for the linkage of photoreduced ferredoxin to an evolving hydrogenase, even in the simultaneous presence of photoevolved oxygen. This type of genetic construct could grow naturally when CO_2 was present, but would concomitantly photoevolve H_2 and O_2 from water in the absence of CO_2. Creation of a recombinant microbe to fulfill all of the ideal criteria listed above is considered an early mid-term goal.

Approach: The two stand-alone objectives of the research are to economically produce H_2 in the near term from biomass (thermally gasified to syngas) using ambient temperature bacteria as catalysts and in the mid term from H_2O using cyanobacteria or algae with an oxygen-tolerant bacterial hydrogenase.

Past Results: Photosynthetic bacteria have four different terminal enzymes that mediate their H_2 metabolisms—nitrogenase, uptake hydrogenase, fermentative hydrogenase, and carbon monoxide-linked hydrogenase. Each has been microbiologically and biochemically examined for their potential to specifically generate H_2 in large-scale processes.

Of the intact cell metabolisms of phototrophs that evolve hydrogen, the nitrogenase-mediated reactions have been the most studied. Nearly all isolates of photosynthetic bacteria have a nitrogenase enzyme complex (Weaver et al. 1975), which, in the absence of ammonium ion or dinitrogen gas and in the presence of oxidizable organic materials, functions to reduce protons and evolve hydrogen. A large variety of soluble organic acids, alcohols, and sugars can be nearly totally photoconverted into H_2 and CO_2 by this metabolism. Rates of 131 μmol H_2 evolved/min·g cell dry weight have been obtained in saturating light. Hydrogen evolution is largely light dependent (Schultz et al. 1985) and strongly exergonic. The hydrolysis of about 4 ATP (largely synthesized

in light) is required to generate each H_2 and can drive the gas production to equilibrium pressures in excess of 100 atmospheres. Radiant energy conversion efficiencies (ignoring the chemical energy of the organic substrate) are about 5.3% for the most active strains of photosynthetic bacteria. The best outdoor, solar-driven efficiency is 3.4%. Cultures grown on glutamate as the nitrogen source produced H_2 at linear rates for 7-10 days before *nif*$^-$ strains began to dominate the cultures. Experiments with weekly feedings of N_2 maintained the *nif*$^+$ wild-type genotype dominant and active H_2 production could be observed for more than 30 days. Even with the assumption that the best strains and conditions could be maintained, the maximum solar conversion efficiencies that could be expected are probably less than 10%, however, due to the large energy expenditure of the bacteria in performing this H_2-evolving metabolism. Non-sterile, solar-driven cultures (200-300 liter scale) were susceptible to contamination by sulfate-reducing and methanogenic bacteria growing on the evolved H_2 plus CO_2. Limiting the available fixed nitrogen, including that present in sedimented photosynthetic bacteria, is necessary to limit H_2S and CH_4 evolution by the contaminants. Algal growth is similarly inhibited by this method and also by the strongly reducing conditions. An in-house cost analysis of the process has been performed (Herlevich and Karpuk). First year costs for H_2 production, clean-up, and compression are estimated at \$24.40 per 10^6 Btu at 5% solar energy conversion and \$15.70 per 10^6 Btu at 10% conversion efficiency.

Many strains of photosynthetic bacteria also produce hydrogen from organic substrates by way of a fermentative hydrogenase enzyme when grown in intermittent or low, continuous light (Schultz and Weaver 1982). High, continuous light represses synthesis of the enzyme. The enzyme does not require ATP. It can mediate hydrogen production at rates more than 3-fold those of nitrogenase, or about 440 μmol H_2/min·g cells. However, it equilibrates at low partial pressures of about 0.1 atmospheres of H_2. The active rates of hydrogen production can thus only be maintained by sparging with inert gas, by vacuuming, or by scavenging with a hydrogen-consuming process. Sparging or vacuuming are considered prohibitively expensive, although closed-loop, sparged systems connected to fuel cells may be effective. Methanogenic bacteria strongly contaminate non-sterile cultures. In fact, methanogenic bacteria naturally scavenge the H_2 to such low levels that they are extremely effective in maintaining the H_2 partial pressure considerably below the equilibrium pressure, which functions to "pull" the conversion of organic materials into H_2 and CO_2 and then into CH_4. This apparently natural process is the basis for an NREL patent on solar-enhanced anaerobic digestion (Weaver, 1990).

A unique type of hydrogen producing activity was found in a strain of photosynthetic bacteria by Uffen (1976) that functioned only in darkness to shift CO (and H_2O) into H_2 (and CO_2). We have isolated more than 450 strains of photosynthetic bacteria from local sites that perform this shift reaction in darkness, as does the Uffen strain, but will also quantitatively assimilate CO into new cell mass in light, unlike the Uffen strain, which makes them easy to grow. We have tested many of our strains for CO shift activity and growth with crude (water-scrubbed only) synthesis gas (primarily CO and H_2) generated from thermally gasified wood chips. In light, the novel photosynthetic bacteria assimilate the CO and H_2 components and a portion of the trace gases. In darkness, all of the isolates respond similarly by shifting the CO component of synthesis gas into additional H_2, thereby leaving a product gas highly enriched in H_2 (with CO_2 and trace pyrolysis gases). No inhibitory effects of synthesis gas on long-term photosynthetic growth were noted. At ambient temperature and pressure conditions and starting from 200,000 ppm of CO in the gas phase, less than 0.1 ppm of CO remained at equilibrium. The product gas could be fed directly into a phosphoric

acid fuel cell with generation of electrical power. No harmful effects to the fuel cell were noted.

Bacterial catalyzed shift rates as high as 1.5 mmol H_2 produced from CO per min per g cell dry weight were obtained from vigorously agitated cultures at low cell density. Less actively stirred cells at more normal cell densities (2-6 g cells per liter) exhibit H_2 production rates of 10-100 μmol per min per g cells, reflecting a limiting mass transfer of CO into solution. Gas pressures (10% CO) of more than 12 atmospheres strongly increased shift rates but were still limiting.

A variety of bioreactor designs that show enhanced mass transport of CO (at near ambient pressure) were built and tested (Markov et al. 1996; Markov et al. 1997). Hollow-fiber reactors (0.5 m^2 surface area) with bacterial cells immobilized on the outer fiber surfaces evolved H_2 at rates of about 0.3-0.7 $mmol \cdot min^{-1} \cdot g\ cdw^{-1}$. One such reactor produced H_2 from CO (10% in N_2) continuously over a 15 month period with only occasional changes of medium. No detectable levels of CO remained in the effluent gas stream.

Bacterial cultures grown in light with 20% CO in the gas phase are probably not maximally induced for CO shift activity. The specific activity for CO shift activity increased by a factor of six when light-grown cultures were incubated in darkness in a Paar cell at seven atmospheres of 20% CO gas pressure. This indicates that increased availability of CO to the cells causes a further derepression of shift activity enzymes. The capability of running syngas shift reactors at a few atmospheres of pressure is being incorporated into new reactor designs.

Due to limiting mass transfer of CO during assay conditions, it has been difficult to compare bacterial shift activities between the wild-type and mutant strains that we have isolated to-date. A new assay was developed that is independent of mass transfer. Bubbles of CO were added to suspensions of the bacterial strains in syringes and agitated vigorously. At time zero the bubble was expelled from the syringe and liquid samples of the bacterial suspension were injected at precise time intervals into an anaerobic solution of reduced hemoglobin. The hemoglobin rapidly quenches further shift activity by binding the remaining CO in solution to form carboxyhemoglobin, which has a distinctive spectral absorbance that can be readily quantitated from difference spectra. All of the bacterial strains exhibit a linear uptake of CO from solution until the dissolved CO approaches the K_s value (the CO concentration at which shift rates are one-half maximal). Different strains tested have K_s values of 3-8 μM. At normal suspended cell densities, CO concentrations in solution would drop to the K_s value in 3-5 seconds. CO concentrations would drop to 0.2μM (the assay limit of resolution) in 6-8 seconds. This value of CO in solution is equivalent to about 200 ppm of CO in an equilibrated gas phase. Kinetic rates for the further shift of remaining CO below 200 ppm and the time necessary to generate a conditioned gas with less than 10 ppm residual CO are currently unknown; however, FTIR analysis after long-term contact with a bacterial suspension at 30°C indicate the conditioned gas contained less than 0.1 ppm CO.

A mutant strain of photosynthetic bacteria was selected by growing a culture on CO in light in the presence of 5% O_2. Strain CBS-2 responded to the imposed culturing conditions by synthesizing 340% of the hydrogenase activity of its parent. Of particular importance, the O_2-resistant hydrogenase exhibited no oxy-hydrogenase activity in the presence of H_2 and O_2. This is a necessary property if the bacterial hydrogenase is to be integrated into an oxygenic host.

Current Results: The equilibrium constant of the CO shift reaction is strongly dependent upon temperature. At 400°C the calculated K_{eq} is 12, meaning that even with a chemical shift catalyst the CO concentrations in thermally generated fuel gases remains far too high for fuel cell use. At 35°C, where most of the bacterial shift strains function the K_{eq} is about 60,000; at 50°C the K_{eq} is still about 28,500, providing sufficient driving force to ensure the product gas contains less than 1 ppm residual CO.

Most bacterial strains exhibit enhanced shift rates with increasing temperature, up to a sharp maximum temperature. Strain CBS-2 exhibits a maximal shift temperature of 50°C where the shift rate is about 1.3 mmol CO shifted $\cdot min^{-1} \cdot g\ cdw^{-1}$. In at least one instance rates have exceeded 3 $mmol \cdot min^{-1} \cdot g\ cdw^{-1}$. The higher temperature for shift activity may be of additional value in requiring less temperature quenching of the fuel gases. Twenty-four hot springs sites with temperatures between 45-75°C were sampled. Twelve of the cultures gave biological shift activity. A number of strains exhibiting shift activity have been isolated in pure culture, and all appear to be clostridial species, although photosynthetic bacteria and cyanobacteria dominate the hot springs biomass.

The oxygen tolerance of the evolving hydrogenase in strain CBS-2 was examined in considerable detail. This strain was isolated by forcing it to grow photosynthetically with CO as the only carbon source while in the presence of 5% O_2. It could only survive and grow if it could evolve H_2 from CO even in the presence of O_2. This strain retained 50% of its evolving hydrogenase activity after seventeen hours stirring in air (21% O_2). The membrane-bound enzyme was extracted and highly purified. It was found to have a molecular weight of about 56,000 daltons by gel exclusion chromatography. The half-life of the purified hydrogenase stirred in air was about five hours. This compares very favorably with other evolving hydrogenases which have half-lives of about two minutes in air.

Although the hydrogenase appears to be relatively oxygen resistant, it is possible that the enzyme became inactivated but protected during the time it was aerated. In order to determine whether the enzyme could functionally evolve H_2 while O_2 was simultaneously present, different assays had to be employed. The purified enzyme was placed in a 50/50 D_2O/H_2O solution under an H_2 atmosphere with varying amounts of O_2, and the rate of appearance of HD in the gas phase was measured using mass spectrometry. A. Dillon and M. Heben of the Basic Sciences Center at NREL kindly supplied their expertise and apparatus for these measurements. With 3.3% O_2 in the gas phase, the hydrogenase enzyme retained 82% of its deuterium exchange activity. At 13% O_2, the enzyme was about 55% active. Better than 90% activity was regained with subsequent removal of O_2. These data surprisingly indicate that the hydrogenase is partially active under aerobic conditions and partially reversibly inactivated.

A second assay was developed which placed the purified hydrogenase with methyl viologen in a chamber with a clark hydrogen electrode. The enzyme is slightly reversible (at about 2.5% of the evolution rate). With the addition of 4.3% O_2, the kinetic rate of H_2 consumption was instantaneously reduced to about 35% of the anaerobic rate, but remained constant thereafter for the duration of the assay. Once again, better than 90% activity was regained upon return of the solution to anaerobic conditions.

Continuous-flow bioreactors were established with 10% CO in N_2 as the sole carbon source for the

bacteria. Cultures of strain EB21 were typical in that after 7-10 days the pH dropped and after 20 days the pH was below 6.0 and became inhibitory to shift activity. The acidity could not be attributed to carbonic acid and could not be gas stripped. Analysis of spent culture medium indicated there was only 0.7 mm phosphate remaining from the 20 mm added. It is surmised, though not identified, that medium phosphate is converted into intracellular polyphosphate under these conditions. Carr and Sandhu (1966) previously demonstrated that polyphosphate is often synthesized in the absence of fixed carbon substrates. When we added 5g/6 sodium malate, a non-fermentable substrate, to the anaerobic, dark cultures, the pH and CO shift activity of the culture remained constant.

Bubble-tower bioreactors 90 cm tall were inoculated with strain CBS-2 in a malate medium with 10% CO bubbled through a glass frit in the bottom of the columns. Bubble contact time with the bacterial suspension was 6-8 seconds. In a ¾ inch diameter, 90 cm column at flow rates of 14 ml per minute, about 90% of the CO was shifted over periods of a few weeks with no drop in pH. With the addition of Tween 80, a surfactant, 100% of the CO became shifted but the effect lasted only for a day or so. Presumably, the Tween partioned into the cell fraction and the stimulation of mass transfer was lost. A 3" diameter, 90 cm column performed similarly at 140 ml per minute gas flow provided that gas bubbles do not exceed 3-4 mm in diameter.

Gas phase bioreactors have been constructed that immobilize bacteria on high-surface-area solid supports. Most promising is a low cost, low energy input bioreactor made by immobilizing bacteria on the 30 μm diameter fibers of an upside-down nylon carpet that was glued to the inside surface of a 3.2 cm diameter, 115 cm long clear plastic tubing. The carpet forms a semicircle within the upper portion of the horizontal tubing with the nylon extending downward and contacting an aqueous reservoir of medium filling the lower half of the tubing. The fibers wick water and minerals to the immobilized bacteria in the upper half of the tubing volume. A 10% CO gas stream at 40 ml per minute passes through the carpet nap above the reservoir and is shifted into H_2 within the first 60 cm. Most of the product CO_2 enters the reservoir as bicarbonate ion, which should be gas strippable through an external reservoir loop, thereby leaving the reactor effluent gas highly enriched in H_2. Scanning electron micrographs of the fiber surfaces indicate that bacteria colonize only a small portion of the fiber surface, which leaves opportunity for considerable increases in activity.

The Outdoor Photobiology Test Area has been expanded and soon will be considerably enhanced in capability. Both a steam reformer and a biomass gasifier are being constructed to thermally generate authentic fuel gases on a continuous basis for use as feedstock for the bacterial shift reactor. A 50W PEM fuel cell will complete the technology validation. The system will initially run at 300 ml per minute.

Future Work: Further mutant selections should result in significantly increased specific activities in the CO shift reaction. Thermophiles will also be selected. Strains with a lower K_s for CO will be sought. Necessary co-factors for the O_2-tolerant hydrogenase will be characterized. The Outdoor Photobiology Test Area will be re-established with both a propane steam reformer and a small downdraft biomass gasifier to generate fuel gases at the rate of 0.3-14 liters per minute. Scaled-up shift bioreactors will operate continuously on the fuel gases and long-term operation will be automated and monitored. Production of polyhydroxyalkanoates or single-cell protein as side products will be maximized, quantified and incorporated into economic analyses.

Economic Evaluation/Systems Analysis: A FY96 analysis was performed on a separate task in the Hydrogen Program. The bacterial shift reaction is being examined as a possible low cost, single-step gas conditioning process for crude syngas. The projected cost of hydrogen is $17/GJ using a base case of purchased biomass at $46/ton and thermal gasification with biological shift (15% ROI). If biomass is purchased at $22/ton, the price of H_2 drops to $13.5/GJ, well within current market prices. A 10-fold increase in specific shift activity (from 1.5 $mmol \cdot min^{-1} \cdot g\ cdw^{-1}$) will decrease the cost by $4.70/GJ. If further gas purification is not needed to remove the remaining trace gases CO_2, the selling price is further reduced another $3.80/GJ. For many gas uses, such as in PEM or phosphoric acid fuel cells, it is only necessary that the gas stream be free of CO and H_2S, while not necessarily totally free of CO_2. In these applications the cost of further gas purification can be automatically discounted from the base case.

Projected Goals: The equilibrium constant of the CO shift reaction at room temperature is sufficiently large to generate a bacterially conditioned syngas stream with sub-ppm concentrations of CO remaining. Assuming there is no appreciable sulfide present as well, the gas stream is clean enough (with or without CO_2 diminishment) to be directly injected into phosphoric acid or PEM fuel cells. This would effectively eliminate the costs (see Economics) for pressure-swing absorption (or similar gas clean-up process) to yield H_2 for fuel cell use. Our near-term goal will be for this type of fuel cell application.

A genetic construct containing an O_2-tolerant hydrogenase would create an inexpensive photobiological catalyst that would switch in the absence of CO_2 from a growth mode to a H_2-producing mode at nearly maximal solar conversion efficiency. The successful construction and testing of such an oxygenic phototroph is our mid-term goal to photobiologically produce H_2 from H_2O in a single-stage bioreactor.

Major Barriers: The long-term effects on bacteria of trace components in syngas have not been examined and may necessitate more frequent change-out of bacteria. Mass transfer of CO to the bacteria remains rate limiting and needs to be enhanced. Genetic enhancement can easily match any large increase in mass transfer rates. There are a few more unknowns in the photobiological process to produce H_2 from H_2O. The O_2-tolerant hydrogenase must be genetically transferred and expressed in a biophotolytic host and shown to be active in linking to photoreductants. This has been demonstrated in mixed extracts, however, where spinach chloroplasts and bacterial hydrogenase photoproduced H_2 from H_2O in the presence of the redox mediator, methyl viologen.

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