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Biological Hydrogen Production Measured in Batch Anaerobic Respirometers

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The biological production of hydrogen from the fermentation of different substrates was examined in batch tests using heat-shocked mixed cultures with two techniques: an intermittent pressure release method (Owen method) and a continuous gas release method using a bubble measurement device (respirometric method). Under otherwise identical conditions, the respirometric method resulted in the production of 43% more hydrogen gas from glucose than the Owen method. The lower conversion of glucose to hydrogen using the Owen protocol may have been produced by repression of hydrogenase activity from high partial pressures in the gastight bottles, but this could not be proven using a thermodynamic/rate inhibition analysis. In the respirometric method, total pressure in the headspace never exceeded ambient pressure, and hydrogen typically composed as much as 62% of the headspace gas. High conversion efficiencies were consistently obtained with heat-shocked soils taken at different times and those stored for up to a month. Hydrogen gas composition was consistently in the range of 60−64% for glucose-grown cultures during logarithmic growth but declined in stationary cultures. Overall, hydrogen conversion efficiencies for glucose cultures were 23% based on the assumption of a maximum of 4 mol of hydrogen/mol of glucose. Hydrogen conversion efficiencies were similar for sucrose (23%) and somewhat lower for molasses (15%) but were much lower for lactate (0.50%) and cellulose (0.075%).

Introduction

Hydrogen has been an unrealized "fuel of the future" for over 30 years, but there are signs that hydrogen may finally become an important component of the energy balance of a global economy (*1*, *2*). Low-cost hydrogen based fuel cells, which have been expensive or not readily available, are now entering commercial production and are finding applications in residential housing and buses. Several automobile manufacturers, including Ford and Toyota, will be introducing cars in the next few years that use fuel cells, and Shell and BP have established core hydrogen divisions in their companies. Despite the "green" nature of hydrogen as a fuel, it is still primarily produced from nonrenewable sources such as natural gas and petroleum hydrocarbons via steam reforming. In order for hydrogen to become a more sustainable and green source of energy, hydrogen must be produced either through photosynthetic or fermentative routes using waste or renewable substrates (*1*, *3*).

Many bacteria contain enzymes (hydrogenases) that can produce hydrogen during the fermentation of a variety of substrates. ATP is produced by substrate-level or electron transport phosphorylation, but the ATP yields of fermentation are quite low as compared to those of aerobic oxidation reactions. Fermentation reactions can produce many different end products such as hydrogen, acetate, ethanol, and others. The hydrogen−acetate couple produces more ATP per mole of substrate than alcohols such as ethanol and butanol and is the energetically "preferred" bacterial fermentation product for a sugar (*4*). The accumulation of hydrogen and other degradation byproducts during fermentation, however, can make the hydrogen−acetate reaction unfavorable leading to solvent production (*5*−*8*). In mixed anaerobic cultures, the accumulation of hydrogen is normally balanced by rapid hydrogen consumption by methanogens resulting in little net hydrogen accumulation in the system (*9*). If high concentrations of hydrogen are desired, a system must be designed to remove hydrogen before it can lead to repression of its production and to prevent interspecies hydrogen transfer leading to methanogenesis. Although fuel cells are being developed that can use methane, the conversion of hydrogen to methane represents a net loss of energy because, on a mass basis, methane has only 42% of the energy content of hydrogen.

The culture conditions that can adversely affect hydrogen production are only beginning to be studied and are therefore not well-understood. Batch tests using mixed cultures have demonstrated that very low pH's and high substrate concentrations can reduce biohydrogen production (*10*). Increasing the substrate loading increases relative production of volatile acids and decreases the pH, which can shift the reactions to solvent production (*8*). The overaccumulation of dissolved hydrogen in the liquid and high hydrogen partial pressures are also thought to inhibit hydrogen production, but the extent of inhibition is not known (*11*, *12*). Heat-shocking has been used to reduce the concentration of nonspore forming bacteria such as methanogens, but the effect of this procedure on the storage of the material and the differences between different batches of mixed cultures has not been tested.

To better understand the factors that can affect hydrogen production, we examined three factors relative to hydrogen production by mixed cultures. First, we measured biohydrogen production in two types of batch tests (Owen and respirometer) that differ by the methods used to measure gas production in the vessel headspace. Second, a mixed culture that was used as an inoculum for hydrogen production was tested over a period of several weeks to see if high levels of hydrogen production could be consistently obtained. Third, we examined whether it was possible to quantify hydrogen production either by scavenging the carbon dioxide in the off-gas or by using a mass balance assuming continuous hydrogen production at a fixed concentration of hydrogen during exponential growth in the culture. Fourth, we compared hydrogen production using several different substrates.

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Methods

Culture Conditions. Samples were taken ~10 cm deep from soils used for tomato plants from a local farming area in State College, PA. Each sample was heat-shocked by drying 1-cm thick samples at 104 °C in an aluminum pan for 2 h. Samples were then sieved using a #20 mesh (850 μm) and stored in plastic bottles in a refrigerator (4 °C). This dried sample was used as the reactor inoculum by adding 8 g to each reaction vessel.

Electron donor was added with a concentrated nutrient solution to produce a final concentration of 4 g of COD/L of the substrate in 250 mL of liquid. Electron donors tested were glucose, sucrose, lactate, and potato starch (Fisher Scientific, Pittsburgh, PA), cellulose (Sigma, St. Louis, MO), and molasses (Brer Rabbit, Roseland, NJ). The nutrient solution contained (per L of water) 2.0 g of NH_4HCO_3, 1.0 g of KH_2PO_4, 100 mg $MgSO_4 \cdot 7H_2O$, 10 mg of NaCl, 10 mg of $Na_2MoO_4 \cdot 2H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 15 mg of $MnSO_4 \cdot 7H_2O$, and 2.78 mg of $FeCl_2$. The solution was buffered with 0.05 M 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES; J.T. Baker, Phillipsburg, NJ) and adjusted to a pH = 6 using 1 M NaOH. Bottles were capped with rubber septum stoppers (Wheaton Scientific, Millville, NJ). Oxygen was removed from the headspace by nitrogen gas sparging for 15 s.

Reactors. Biological hydrogen experiments were conducted in batch tests either by periodic (Owen method) or continuous (respirometric method) release of gas from the headspace of the reaction vessels (294-mL capacity bottles; Wheaton Scientific). Tests were run in triplicate (unless indicated otherwise) and results averaged. All bottles were mixed using a stir bar on a commercial respirometer stir plate (dial setting 2−3; Challenge Environmental Systems AER-200 respirometer, Fayetteville, AR) in a constant temperature room (26 °C). For the Owen method tests, the total gas volume was measured by releasing the pressure in the bottles using a glass syringe (2, 20, or 50 mL capacity; Perfektum Syringes; Popper & Sons, Inc., New Hyde Park, NY) and measuring the volume necessary for it to equilibrate with room pressure (*13*). Gas production in the respirometer system was measured with a bubble meter calibrated according to the manufacturer's instructions. In some tests, the oil in the respirometer was replaced with 1 M NaOH in order to absorb carbon dioxide in the gas stream.

Analytical. Hydrogen in headspace of vessels was periodically measured using a gastight syringe (0.5 mL injection volume) and a gas chromatograph (GC; model 310; SRI Instruments, Torrence, CA) equipped with a thermal conductivity detector and a molecular sieve column (Alltech Molesieve 5A 80/100 6 ft × 1/8 ft × 0.085) with nitrogen as the carrier gas.

Hydrogen gas production was calculated from headspace measurements and the total volume of biogas produced for each time interval using the mass balance equation

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (1)$$

where $V_{H,i}$ and $V_{H,i+1}$ are cumulative hydrogen gas volumes at the current (i) and previous ($i-1$) time intervals, respectively, $V_{G,i}$ and $V_{G,i+1}$ are the total biogas volumes in the current and next time intervals, $C_{H,i}$ and $C_{H,i+1}$ are the fraction of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current and next time intervals, and V_H is the total volume of headspace in the reactor (44 mL).

Results

Following a lag phase of ~20 h, there was gas production for 75 h in all test bottles inoculated with a heat-shocked soil and glucose (1 g of COD/bottle) (Figure 1). Total gas

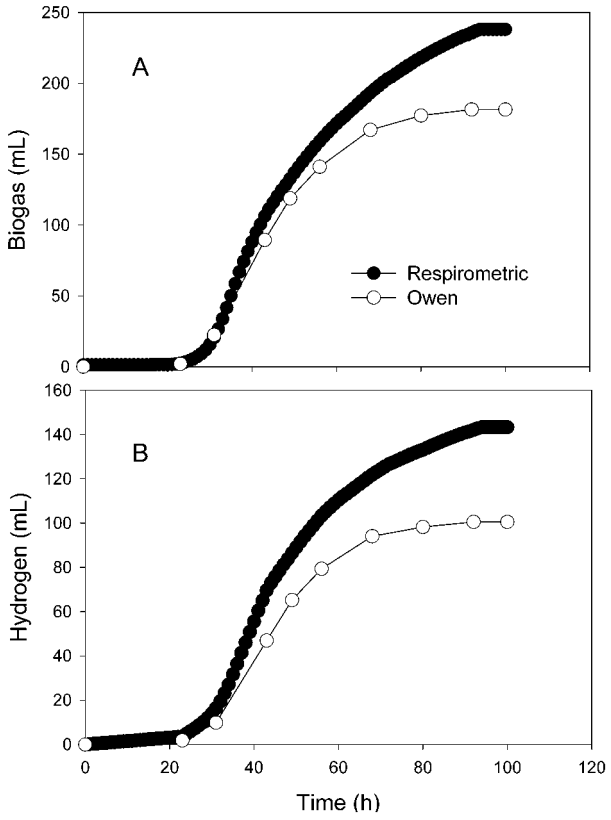

FIGURE 1. Comparison of (A) total biogas production and (B) hydrogen gas production measured using the Owen or respirometer methods. Values shown are averages of bottles run in triplicate.

production was 238 mL in the respirometer bottles when gas pressure was continuously released versus only 182 mL when the gas pressure was intermittently released in the experiment shown in Figure 1. Hydrogen production was 43% larger in the respirometer bottles than in bottles where the gas was released and measured using the Owen method or 143 versus 101 mL, respectively. Overpressure in the Owen method bottles reached a maximum of 254 kPa or a gas release of 67 mL from the headspace (44 mL). There was no measurable hydrogen production in controls in the absence of glucose addition. Assuming a theoretical maximum yield of 4 mol of hydrogen/mol of glucose, the conversion efficiency of glucose to hydrogen was 26% in this respirometer test.

The heat-shocking process produced a stable inoculum for biogas production. Experiments for biogas production repeated 5 times over a 30-day period using the same heat-shocked sample produced an average of 262 ± 24 mL of biogas with no apparent trend in gas production with the age of the stored sample (Figure 2). A second soil sample from the same site produced an average of 246 ± 25 mL of biogas, which was not significantly different than the results from the first set of samples ($p = 0.11$; t-test). Overall, there was an average production of 254 ± 13 mL for the 10 tests. On the basis of these results, we concluded that biogas production experiments conducted at different times using different inocula (from the same source) could be compared, even if the experiments were not conducted at the same time. Experiments with heat-shocked soils from other local sites have so far provided similar results in our laboratory (data not shown).

Biogas production appeared to be growth-associated in batch tests. The rate of biogas production reached a maximum of 10 mL/h at 42 h (Figure 3A), and this maximum rate corresponded to the end of the log phase in biogas production (Figure 3B). During the period following the

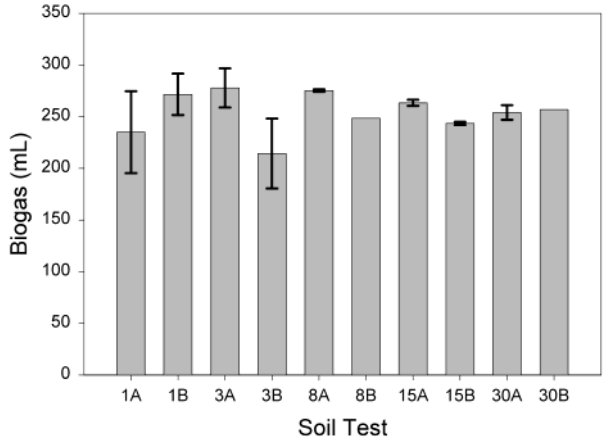

FIGURE 2. Biogas production from two different heat-shocked soils (A and B) collected on different days from the same site. Error bars indicate total biogas production ±SD for experiments conducted in triplicate (days 1, 3, 8, and 15) or duplicate (day 30).

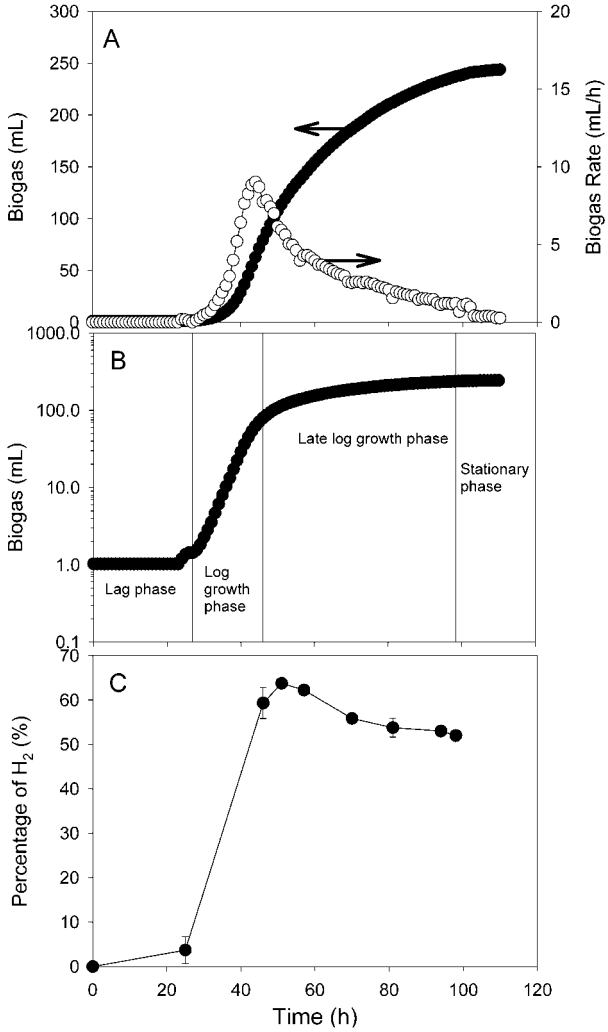

FIGURE 3. Examination of hydrogen production in a respirometer test with glucose. (A) Cumulative hydrogen production (●) and the rate of hydrogen production (○) in the reactors. (B) Biogas production plotted on log axes to show growth phases. (C) Hydrogen gas concentrations (%) in the vessel headspace.

maximum biogas rate, biogas was still produced but at a decreasing rate until the end of the test at 100 h. The percentage of hydrogen gas reached a maximum of 64% at 44 h, but it then declined to 52% at 92 h (Figure 3B). Hydrogen

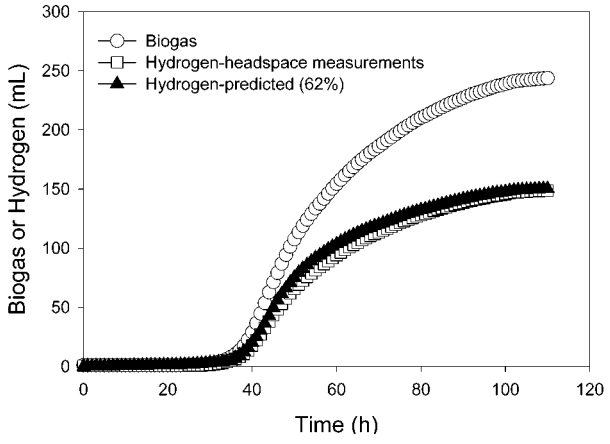

FIGURE 4. Comparison of the hydrogen gas produced in the reactors based on a mass balance and hydrogen gas measurements versus the assumption of a constant hydrogen gas concentration of 62% for all biogas production.

gas concentrations further decreased to 11.5% and 0.15% at times of 169 and 216 h after the start of the tests (data not shown). The initial decreases in hydrogen gas concentrations could reflect an increased proportion of carbon dioxide in the gas from non-hydrogen producing fermentative routes. Because of these large decreases in hydrogen gas concentrations to very low levels much later, however, it was likely that there was hydrogen consumption by the mixed culture.

The apparent increase in the concentration of hydrogen in the gas (Figure 3C) leaving the respirometer during the first 44 h was actually produced by the dilution of the hydrogen gas by nitrogen gas initially in the headspace during the first 44 h. Hydrogen gas concentrations only decreased later during the late log growth phase. Support for this assertion of constant concentration of hydrogen gas can be shown by conducting our hydrogen gas calculations assuming a constant 62% hydrogen gas concentration of all biogas produced during the experiment. This mass balance based on 62% hydrogen shows a nearly identical hydrogen production profile and final hydrogen gas production (149 mL) to that calculated from a mass balance of using actual hydrogen gas concentrations and biogas leaving the reactor (total hydrogen gas volume of 150 mL) (Figure 4). These similar profiles demonstrate that the low initial hydrogen concentrations leaving the reactor are a direct result of dilution of the biogas produced with the nitrogen atmosphere initially in the respirometer bottle headspace.

The main components of the biogas are carbon dioxide and hydrogen. It is therefore possible to measure hydrogen as total biogas production by stripping out the CO_2 in the effluent gas stream. To do this, a 1 M NaOH solution was placed in the bubble meter. When the gas reached the bubble meter, the CO_2 was absorbed by the NaOH solution leaving a bubble that was essentially only hydrogen gas. Shown in Figure 5 is a comparison of two tests, using the same inoculum and run in parallel, with hydrogen removal based on CO_2 absorption into NaOH compared to the regular test with hydrogen calculated from a mass balance. It is evident that hydrogen production is similar in both cases calculated for the same volume of hydrogen gas produced in the bottles in each test (150 mL).

Hydrogen Production Using Different Substrates. The hydrogen produced using glucose was compared to that obtained with several different substrates. All substrates except molasses (1.3 g of COD/bottle) were added on an equivalent basis of 1 g of COD/bottle (Figure 6). Lag times were similar (19−27 h) for the high sugar substrates (glucose, sucrose, and molasses). The potato starch bottles produced

TABLE 1. Summary of Total Biogas and Hydrogen Gas Production Measured Using Substrates (S)[a]

substrate (formula)	g of COD (mmol)	biogas (mL)	H_2 produced		maximum H_2 production (mol/mol of S)	conversion (%)
			mL	mol/mol of S		
glucose ($C_6H_{12}O_6$)	1.0 (5.56)	244	125	0.92	4	23
sucrose ($C_{12}H_{22}O_{11}$)	1.0 (2.92)	217	131	1.8	8	23
molasses	1.3 (−)[b]	233	134	*b*	*b*	*b*
potato starch [$(C_6H_{10}O_5)_n$]	1.0 (6.17)	134	90	0.59	4	15
lactate ($C_3H_5O_3Na$)	1.0 (8.92)	7.2	2.3	0.01	2	0.50
cellulose [$(C_6H_{10}O_5)_n$]	1.0 (6.17)	0.96	0.6	0.003	4	0.075

[a] Substrate conversion efficiencies assume production of acetate as the organic carbon end product in order to calculate the indicated maximum production of H_2. [b] Cannot be calculated.

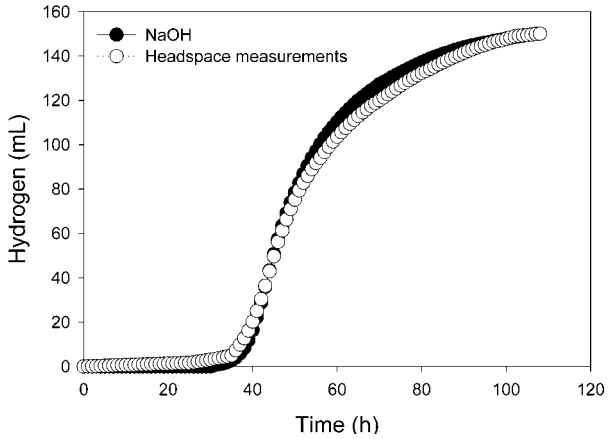

FIGURE 5. Hydrogen gas produced in glucose-fed reactors calculated from total biogas production (●) versus that measured in tests using NaOH to absorb CO_2 and assuming only hydrogen as the component of the biogas (○).

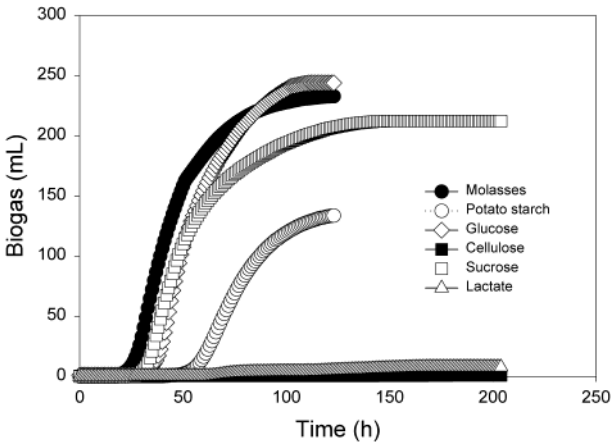

FIGURE 6. Biogas production in reactors using different substrates (average of duplicate bottles). The hydrogen gas produced in each experiment is listed in Table 1.

gas slowly, starting at about 40 h, and did not produce more than 10 mL of hydrogen gas for the next 58 h. The lactate and cellulose bottles produced little hydrogen gas (7.2 and 0.96 mL, respectively).

The conversion efficiency of these substrates was compared assuming a maximum stoichiometric conversion of the substrate to hydrogen and acetate as shown in Table 1. The highest conversion of substrate to hydrogen was obtained for glucose and sucrose (23%) based on the assumption of a maximum of 4 mol of H_2/mol of glucose and 8 mol of H_2/mol of sucrose. Hydrogen conversion efficiencies were somewhat lower for molasses (15%) but were much lower for lactate (0.50%) and cellulose (0.075%).

Discussion

The use of heat-shocked soil samples and a low pH provided a method to obtain consistent results for fermentation of glucose to produce high concentrations of hydrogen gas. Fermentation tests with two sugars (glucose and sucrose) consistently produced high hydrogen gas concentrations of 62% during log growth. The balance of the gas was mainly CO_2, and it was shown that this component could be easily removed using a NaOH solution in the bubble meter, allowing hydrogen production to be measured as total biogas production. Hydrogen gas does not ordinarily accumulate to such high concentrations in mixed culture anaerobic reactors due primarily to the consumption of hydrogen by methanogens. To avoid losses of hydrogen to methane, the reactors here were kept at a pH = 6 that is below the range of many methanogens, and a heat-shock process was used to exclude nonspore forming bacteria such as methanogens originally present in the soil. While pH control alone can increase hydrogen production by mixed cultures (*10*, *14*), the effects of pH control and heat-shocking the inoculum were not separately examined here.

The conversion efficiency of the two simple sugars to hydrogen was 23% based on the assumed stoichiometry of 4 (glucose) and 8 (sucrose) mol of hydrogen/mol of sugar. Nearly complete conversion efficiency of glucose (97%) was obtained by Woodward et al. (*15*) using an enzyme solution, but no natural microbiological route is known to result in more than 4 mol of hydrogen/mol of glucose. The conversion efficiency of 23% obtained here is higher than the 18% found by Roychowdhury et al. (*16*) using glucose and air-dried landfill soil, but it is lower than the maximum observed in recent batch tests with sucrose (58%) (*10*), although these other studies used much higher temperature (37 °C) than those used here (26 °C). Higher conversion efficiencies have also been found using acclimated mixed cultures or pure cultures. In batch tests, Brosseau and Zajic (*17*) measured a glucose conversion efficiency of 25−38% using *Clostridium intermedius*. In continuous culture tests, using an acclimated culture at 35 °C and a pH = 6.7, Chen et al. (*18*) found a maximum conversion efficiency of 43% (3.47 mol of H_2/mol of sucrose) for a sucrose feed concentration of 20 g of COD/L. Kataoka et al. (*19*) measured a range of 32−55% in chemostat tests with *Clostridium butyicum* stain SC-E1 and Taguchi et al. (*20*) achieved a conversion efficiency of 36−59% using *Clostridium* sp. strain No. 2 with xylose. The higher conversion efficiencies measured in these other tests suggest that the conversion efficiencies measured here could be increased through higher temperatures, culture selection, and acclimation using continuous culture conditions.

Hydrogen gas production was lower with lactate, cellulose, molasses, and potato starch than with glucose and sucrose. Part of the reason for lower gas production with substrates other than glucose or sucrose could simply be due to the degradative capabilities of the microbial inoculum relative to these different substrates. For example, there was substantially less hydrogen production from cellulose (β-linked

TABLE 2. Estimation of the Maximum Hydrogen Concentrations Based on Thermodynamic Calculations[a]

substrate	ΔG° (kJ/mol)	H_2 (atm) $\Delta G = 0$	H_2 (atm) $\Phi = 0.86$
sucrose	−458	1.2×10^8	3.1×10^{10}
glucose	−206	7.4×10^9	6.1×10^8
lactate	−4.2	2.8	0.046
butyrate	48.1	1.1×10^{-4}	7.5×10^{-7}

[a] Assumptions: 80% conversion of substrate initially present at 4 g of COD/L, $\Delta G_{critical} = 20$ kJ/mol, and 26°C.

sugars) than starch (α-linked sugars). In the case of lactate, however, it is likely that the situation was more complex. Lactate is a readily fermentable substrate, yet hydrogen concentrations in the gas phase reached only a maximum of 4.6% during the fermentation versus the 64% measured using glucose. This suggested that hydrogen gas buildup inhibited the forward reaction to hydrogen and acetate. Our results showing lower hydrogen production with the Owen method than with the respirometer method also suggest that the buildup of hydrogen gas inhibited hydrogen production when using glucose.

Fennell and Gossett (*9*) have shown using various substrates, such as lactic acid, propionic acid, and ethanol, that hydrogen production rates in anaerobic mixed cultures can be related to the overall thermodynamic potential for hydrogen production in mixed cultures. They demonstrated that the Michaelis−Menten rate constant could be reduced by a thermodynamic-defined coefficient, Φ, defined as

$$\Phi = 1 - \exp\left(\frac{\Delta G - \Delta G_{\text{critical}}}{RT}\right) \qquad (2)$$

where $0 \leq \Phi \leq 1$, ΔG is the Gibbs free energy available from the fermentation reaction at any time, $\Delta G_{critical}$ a constant reflecting some marginal free energy that microorganisms need to live and grow, R the gas constant, and T the absolute temperature. Their results supported the use of $\Delta G_{critical} = -19$ kJ/mol of donor, and they reported that many other studies supported a similar value of $\Delta G_{critical} = -20$ kJ/mol of donor. Using this model, they could explain greater percent accumulations of hydrogen that were achieved using lactic acid than those with butyric acid or ethanol for reactions under the nonstandard conditions in their cultures.

Given this information, it seems reasonable that our observation of greater volumes of hydrogen gas production when the gas pressure was continuously released in respirometer tests than when gas pressure was only periodically released (Figure 1) could reflect conditions where hydrogen accumulation inhibited the forward reaction for hydrogen production. To examine whether our results could be related to Gibbs free energies, we calculated ΔG values for our culture conditions as described by Fennel and Gossett (*9*) for the conditions 4 g of COD/L of substrate (1 g of COD/bottle), 80% stoichiometric conversion of the substrate to hydrogen and acetate, and $\Delta G_{critical} = -20$ kJ/mol of donor. We assumed that stirring maintained equilibrium between the gas and liquid phases. Our results are shown in Table 2, along with the free energy under standard conditions (ΔG°). Sugar substrates are highly exothermic under standard and culture conditions, while the value for ΔG° for lactate is much smaller but still favorable. In contrast, the forward reaction for butyrate to hydrogen is not favorable under standard conditions.

Under the conditions modeled for our system, the maximum hydrogen gas concentration that could accumulate can be calculated by finding the partial pressure of hydrogen when $\Delta G = 0$. For lactate, this is 2.8 atm, while for the sugar substrates, the values are extremely large ($>10^8$ atm). For our observed maximum headspace concentration of 4.6% hydrogen with lactate, and assuming 80% conversion of lactate to products, we find $\Phi = 0.86$. This suggests that only 14% of the maximum hydrogen concentration inhibited hydrogen production from lactate.

These thermodynamic calculations do not, however, explain the higher hydrogen production rates in the respirometer tests using glucose than in bottle tests using the Owen method to periodically release hydrogen pressure. Glucose partial pressures would need to be 6.1×10^8 atm in order to reduce the rate of hydrogen production, assuming the same value of $\Phi = 0.86$ from the lactate tests. Obviously, these high hydrogen partial pressures could not be expected to occur in our bottle tests. It is still possible that the higher concentrations of hydrogen in the Owen test did inhibit hydrogen production but in a manner not included in this thermodynamic/rate type of calculation. It may be that hydrogen inhibited just one of the individual steps or enzymes in the process. This inhibition could be incorporated into the Φ-based calculation by choosing a higher value of $\Delta G_{critical}$, but we did not make that calculation as our experiments were not designed to produce a reliable estimate of this constant. There may be other explanations for the differences in the Owen and respirometric tests as well. For example, it has been found with even high rates of mixing that dissolved hydrogen concentrations can be supersaturated with respect to gas concentrations (*11*, *12*). Therefore, it may be possible that the enzymes are exposed to a much higher hydrogen concentration than those implied by the gas-phase measurements.

Implications for Hydrogen Production During Wastewater Treatment. It has been shown here that the fermentation of organic matter and, in particular, sugars such as glucose and sucrose can provide high partial pressures of hydrogen. Our research demonstrates that even unacclimated samples can be used to consistently produce large volumes of hydrogen gas from waste products. Anaerobic wastewater treatment has been used for many years to treat wastewaters and bioreactor sludge but, so far, not to produce hydrogen or electricity from hydrogen. With the emergence of new fuel cell technologies, this hydrogen gas could be used for on-site energy production.

The recapture of energy in the form of hydrogen is a promising alternate strategy for wastewater treatment for making anaerobic wastewater treatment a more economical and sustainable technology than aerobic processes. The greatest savings may be related to reducing costs for aerators. Aeration is the major operational expense at a wastewater treatment plant with an activated sludge reactor. The conversion of sugars in wastewaters could remove about one-third of this chemical oxygen demand in the form of hydrogen gas. The remainder of the chemical oxygen demand, if converted to methane, would also provide an additional source of clean energy recovery if methane were used in a fuel cell. Both of these processes could save money spent on aeration while at the same time making a wastewater treatment plant into a local power plant.

Acknowledgments

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