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The Relative Effectiveness of pH Control and Heat Treatment for Enhancing Biohydrogen Gas Production

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Hydrogen gas can be recovered from the microbial fermentation of organic substrates at high concentrations when interspecies hydrogen transfer to methanogens is prevented. Two techniques that have been used to limit methanogenesis in mixed cultures are heat treatment, to remove nonsporeforming methanogens from an inoculum, and low pH during culture growth. We found that high hydrogen gas concentrations (57−72%) were produced in all tests and that heat treatment (HT) of the inoculum (pH 6.2 or 7.5) produced greater hydrogen yields than low pH (6.2) conditions with a nonheat-treated inoculum (NHT). Conversion efficiencies of glucose to hydrogen (based on a theoretical yield of 4 mol-H_2/mol-glucose) were as follows: 24.2% (HT, pH = 6.2), 18.5% (HT, pH = 7.5), 14.9% (NHT, pH = 6.2), and 12.1% (NHT, pH = 7.5). The main products of glucose (3 g-COD/L) utilization (≥99%) in batch tests were acetate (3.4−24.1%), butyrate (6.4−29.4%), propionate (0.3−12.8%), ethanol (15.4−28.8%), and hydrogen (4.0−8.1%), with lesser amounts of acetone, propanol, and butanol (COD basis). Hydrogen gas phase concentrations in all batch cultures reached a maximum of 57−72% after 30 h but thereafter rapidly declined to nondetectable levels within 80 h. Separate experiments showed substantial hydrogen losses could occur via acetogenesis and that heat treatment did not prevent acetogenesis. Heat treatment consistently eliminated the production of measurable concentrations of methane. The disappearance of ethanol produced during hydrogen production was likely due to acetic acid production as thermodynamic calculations show that this reaction is spontaneous once hydrogen is depleted. Overall, these results show that low pH was, without heat treatment, sufficient to control hydrogen losses to methanogens in mixed batch cultures and suggest that methods will need to be found to limit acetogenesis in order to increase hydrogen gas yields by batch cultures.

Introduction

Hydrogen is a clean fuel because it produces only water during its reaction with oxygen, and because it is not a greenhouse gas (*1*). Despite its inherently "green" nature, most hydrogen is currently produced primarily from nonrenewable sources, such as natural gas, oil, and coal (*2*). Global oil production will peak in the next 10−20 years, and so new sources of hydrogen will need to be found (*3, 4*). Hydrogen can be produced through electrolysis and thermochemical decomposition of water, but the cost of production by these routes is higher than those based on fossil fuels (*5*).

The biological generation of hydrogen from anaerobic fermentation of organic substrates promises to be an economical and sustainable technology for hydrogen production if conversion efficiencies can be increased (*6*). Anaerobic fermentation has been previously used for the production of methane and solvents, such as butanol, ethanol, and acetone, but only recently has fermentation been studied for hydrogen production. The coupling of hydrogen production to utilization of waste materials containing high concentrations of organics, such as municipal solid waste, industrial wastewater, and agricultural waste, may simultaneously provide economic and environmental benefits. Using nonsterile substrates (such as wastewaters) will likely prohibit pure culture fermentations. To recover hydrogen during mixed culture fermentations, loss of hydrogen through interspecies transfer—primarily to methanogens—must be prevented. To scale-up processes to make industrial production of hydrogen economical, inhibition of methanogen activity must be achieved economically, and in a manner that favors hydrogen production (*7, 8*). This means that chemicals often used in the laboratory to inhibit methanogenesis, such as bromoethanesulfonate, cannot be used for large scale systems (*9*).

Culture conditions that can be used to limit growth of methanogens include low pH, short hydraulic retention times (HRT), and sludge retention times (SRT) in continuous cultures and low temperatures. Another method that can enhance biohydrogen production is a simple heat-shock treatment to remove from any inoculum nonsporeforming bacteria, such as methanogens, that consume hydrogen (*8, 10, 11, 12*). In a previous study (*12*), we demonstrated that heating ordinary soil was sufficient to remove hydrogen-consuming methanogens and leave spore forming hydrogen-producing bacteria. Heat treatment allowed production of a 60% pure hydrogen gas stream (40% CO_2) generated using carbohydrates such as glucose, sucrose, and starch. However, a low pH was simultaneously used to limit methanogen growth in batch tests, so the effects of heat treatment and pH on methane production were not separated. Van Ginkel et al. (*11*) have shown that a pH of 5.5−6.0 was optimal for biological hydrogen production using sucrose and a heat-treated inoculum, but they did not examine the effect of pH on methanogenesis for nonheat-treated samples. In addition, they used an intermittent biogas release procedure that has been shown to lower hydrogen production in comparison to continuous-release gas systems (*12*). Fang and Liu (*13*) similarly found an optimum pH of 5.5 for hydrogen production in a mixed culture chemostat using a detention time (6 h) that prohibited the growth of slower-growing methanogens.

In this study we designed an experimental matrix to study the combined effects of low pH and the heat treatment procedure on biological hydrogen production in batch, continuous-gas-release, respirometric tests. We noticed during these tests that at the end of a batch cycle (when glucose was completely consumed) that hydrogen gas remaining in the headspace was reabsorbed into the culture but at that time were unaware of the cause of this disappearance. Therefore, in the current study we monitored the production of major fermentation products in the gas and liquid phases to determine whether methanogensis was fully

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inhibited by these two treatments (heat and low pH), and the fate of the hydrogen after glucose had been consumed. We found that heat shocking was a more effective means of preventing methanogenesis than pH control and that substantial conversion of remaining alcohols to acetate occurred following the disappearance of hydrogen from the bottle at the end of the test.

Methods

Culture and Medium. Dewatered sludge from an anaerobic digestor was used in biological hydrogen production tests to ensure that methanogens were present in the test bottle inoculum. Dewatered anaerobic sludge (85% water) was collected from the municipal wastewater treatment plant in State College, PA. Heat-treated sludge samples were prepared by a baking procedure consisting of drying a 0.5-cm thick sample at 104 °C in an aluminum pan for 2 h (*12*). Samples were either used within 1 day or stored in sealed containers at ambient temperature (24 °C).

Glucose (3 g-COD/L) was used as a substrate in all tests in a nutrient solution containing (per liter of deionized water) the following: 0.5 g of NH_4Cl, 0.25 g of KH_2PO_4, 0.25 g of K_2HPO_4, 300 mg $MgCl_2 \cdot 6H_2O$, 25 mg of $FeCl_3$, 16 mg of $NiSO_4$, 25 mg of $CaCl_2$, 11.5 mg of $ZnCl_2$, 10.5 mg of $CoCl \cdot 6H_2O$, 5 mg of $CuCl_2 \cdot 2H_2O$, and 15 mg of $MnCl_2 \cdot 4H_2O$. Oxygen was removed by sparging with nitrogen gas. The solution was buffered with 0.07 M phosphate and 0.07 M 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES) (J. T. Baker) for pH 7.5 or pH 6.2, respectively, and the pHs were adjusted using 1 M NaOH. A pH of 6.2 was chosen in order to fix the pH at a value that was near to the optimum pH range for hydrogen production and at a pH that could be achieved with an inexpensive and nontoxic buffer (MES) used in previous studies (*11, 12*). The concentration of glucose used here was chosen to be low enough to minimize pH changes from acids produced during fermentation.

Hydrogen Production. Biogas production was measured using a respirometric system (Challenge Environmental Systems AER-200 Respirometer, Fayetteville, AR) and 294 mL glass bottles (Wheaton TM Scientific) as previously described (*12*). Heat-treated (HT) sludge (0.5 g) or nonheat-treated (NHT) sludge (4 g; 85% water) was added to each bottle. Media (250 mL) was added to each bottle, and the pH was adjusted to 7.5 and 6.2 using NaOH (2 M). Bottles were flushed with nitrogen gas, sealed, and connected to the respirometer in a constant temperature room (25 ± 1 °C). Two additional bottles containing media, but no glucose, and each of the inocula were used as biotic controls for gas production in the absence of added substrate. All tests (except biotic controls) were run in duplicate. Initial and final pH and alkalinity were measured in all bottles. Liquid samples were obtained from the bottles using a 3-mL syringe, filtered through a 0.2 μm (pore diameter) membrane, and stored in 2-mL GC vials at 4 °C.

Hydrogen gas production was calculated from headspace measurements of gas composition and the total volume of biogas produced, at 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, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current and previous intervals, and V_H is the total volume of headspace in the reactor (*12*).

Hydrogen Consumption. In preliminary batch tests it was noted that after biogas production had ceased a negative gas pressure developed in the bottle, and the remaining hydrogen in the gas headspace was depleted. To further examine the fate of this hydrogen gas, separate experiments were conducted using heat-treated sludge as an inoculum, sodium bicarbonate (2.5 g/L) as a carbon source, and hydrogen as an electron donor. Serum bottles were inoculated with heat-treated sludge (0.4 g), filled with 200 mL (99 mL headspace) of the nutrient solution (no glucose), with the pH adjusted to 6.2. Bottles were flushed with pure hydrogen gas, capped with rubber and aluminum seals, and stirred resulting in an initial gas composition of 84% H_2 and 16% CO_2. Abiotic (no inoculum) and biotic (inoculum with nitrogen sparging instead of hydrogen) controls were also prepared. Hydrogen gas in the headspace and the acetate in the liquid were measured over time.

Analysis. Hydrogen in headspace of vessels was periodically measured using a gastight syringe (0.3 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′ × 1/8′ × 0.085) with nitrogen as the carrier gas. Carbon dioxide and methane were similarly analyzed except that a molecular sieve column (Alltech Porapak Q 80/100 6′ × 1/8′ × 0.085) was used with hydrogen as the carrier gas.

The concentrations of several solvent compounds (acetone, ethanol, propanol, and butanol) and organic acids (acetate, propionate, and butyrate) were determined by gas chromatography (Varian Star 3400) with injector and flame ionization detector temperatures of 250 °C. Nitrogen was used as a carrier gas with a 20 mL/min flow rate (25 psi). The oven temperature was programmed as follows: 60 °C for 2 min, increasing to 140 °C at 5 °C/min, and then constant at 140 °C for another 6 min. A 50 m × 0.32 mm internal diameter fused silica capillary column was used, coated with 0.2 μm CP-Wax 57 CB.

Glucose was analyzed after filtration through a 0.2 μm (pore diameter) membrane filter using the phenol-sulfuric acid method for reducing sugars (*14*). Alkalinity was determined according to Standard Methods 2320B (*15*) by titration using bromophenol blue to pH 4.3 with 0.05 N H_2SO_4 (expressed as mg $CaCO_3$/L). A pH probe and meter (Fisher Scientific accumet model 10 and VWR SympHony) was used for pH measurements.

Results

Hydrogen Production. Both heat treatment and the lower pH (6.2) were required to maximize biological hydrogen production in batch tests. In all cases there was a high rate of biogas production after a lag phase of ~18 h (Figure 1). This high rate of biogas production produced a maximum of 145 mL when the inoculum was heat treated and at a pH = 6.2. Based on hydrogen concentrations (Figure 2A), this is equal to 93 mL of pure hydrogen gas. In all other cases the biogas production was lower, with initial plateaus of 94.7 mL (NHT, pH = 6.2), 84.6 mL (HT, pH = 7.5), and 65.2 mL (NHT, pH = 7.5) after ~24 h. Biogas production at pH = 7.5 was consistently smaller than that at pH = 6.2 independent of heat treatment.

Glucose was completely (99.1 ± 1%; 95% C.I.) consumed in all bottles, with a maximum conversion efficiency of glucose to hydrogen (based on a theoretical yield of 4 mol-H_2/mol-glucose) of 24.2% for heat-treated samples at a pH = 6.2. This conversion rate is consistent with that previously reported (24.5%) under these conditions (*12*). Conversion efficiencies of glucose to hydrogen (based on a theoretical yield of 4 mol-H_2/mol-glucose) were as follows: 24.2% (HT, pH = 6.2), 18.5% (HT, pH = 7.5), 14.9% (NHT, pH = 6.2), and 12.1% (NHT, pH = 7.5). There was no hydrogen gas

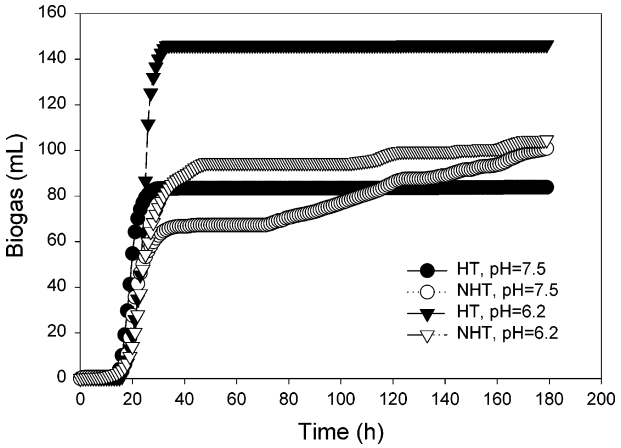

FIGURE 1. Cumulative biogas production (hydrogen, carbon dioxide, and methane) in the pH and heat shock experiments. Initial concentrations: glucose, 3 g-COD/L; anaerobic sludge, 2 g/L. (HT = heat treatment, NHT = non-heat treatment). Values shown are averages of bottles run in duplicate.

production in the biotic control (no glucose) bottles, and total biogas production was less than 0.1 mL.

The hydrogen concentration in the biogas in glucose-fed bottles reached a maximum of 57−72% after ca. 30 h (Figure 2A). The apparent initial increase in hydrogen composition prior to 30 h in all bottles was due to dilution of the nitrogen headspace with hydrogen gas (*12*). It has been previously shown that during the initial fermentation step that hydrogen gas produced during glucose fermentation had a constant composition of 60−64% (*12*), similar to the maximum concentrations observed here.

In both heat-treated samples, biogas production ceased after ca. 30 h. Following the plateau in biogas production, the remaining hydrogen gas in the headspace was reabsorbed and had completely disappeared by 80 h (Figure 2A). For the NHT samples, the disappearance of the hydrogen gas coincided with the production of methane (Figure 2B) and a lower overall concentration of CO_2 in the headspace (Figure 2C). Decreasing the pH from 7.5 to 6.2 for NHT samples decreased methane production from 50.5 to 7.7 mL. There was no methane production by HT samples. The concentration of CO_2 in bottles at a pH = 6.2 were higher than those at a pH = 7.5 due in part to changes in CO_2 solubility with pH. Note that the decrease in hydrogen gas concentration shown in Figure 2A was not reflected in total biogas production (shown in Figure 1) because the respirometers were only used to record gas production, not consumption. Because the consumption of hydrogen gas in the headspace reduced the total pressure in the vessel, gas percentages given in Figure 2 assume ambient pressure (i.e. 40% CO_2 in the absence of methane or hydrogen is equal to 100% CO_2 at a pressure 40 kPa).

Volatile Acid and Solvent Production. Seventy hours after the start of the batch test, volatile acids and solvents were measured in all bottles (Figures 3 and 4). Acetate, propionate, and butyrate were the predominant volatile fatty acids (VFAs), while ethanol was produced at the highest concentration among the measured alcohols and acetone. Overall, there was a greater percentage of conversion of glucose to VFAs than to other compounds. The degree of acidification can be expressed based on the ratio of the COD equivalent of acidogenic products (organic acids, alcohols, and hydrogen) to the initial COD (*16*). The main products of glucose (3 g-COD/L) utilization (≥99%) in batch tests were acetate (3.4−24.1%), butyrate (6.4−29.4%), propionate (0.3−12.8%), ethanol (15.4−28.8%), and hydrogen (4.0−8.1%), with lesser amounts of acetone, propanol, and butanol (COD basis). In

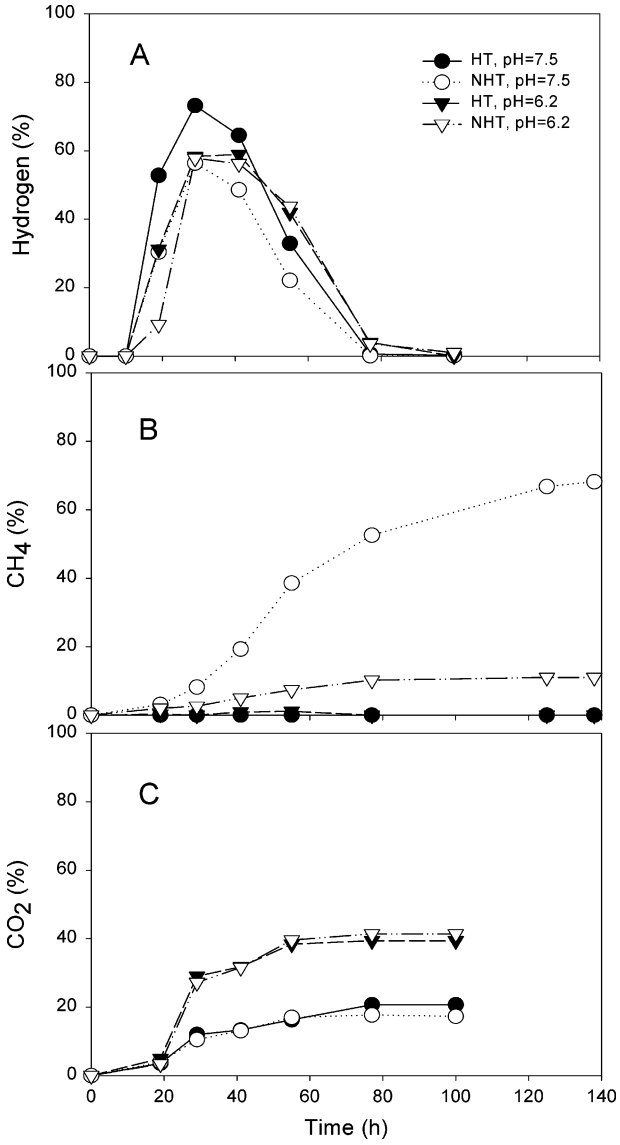

FIGURE 2. Gas concentrations (%) in the vessel headspace over time: (A) hydrogen, (B) carbon dioxide, and (C) methane. Note that the percentages are calculated on the basis of ambient pressure inside the vessel.

these experiments, glucose (3 g-COD/L) produced for VFA and alcohols: 51% and 28% for the HT samples at a pH 7.5, 37%, and 28% with NHT samples at pH 7.5; 51% and 17% with HT samples at a pH 6.2; and 33% and 15% with NHT sludge at a pH 6.2 (Figure 3). The balance of these numbers was assumed to be biomass (all samples) or methane (NHT samples), but biomass produced by glucose consumption could not be evaluated against a background of the high concentration of sludge solids used as the inoculum.

The production of high concentrations of organic acids decreased the pH of the buffered (0.05 M MES) solutions. The pH change was 8% in the case of the low pH samples (from 6.2 to 5.8) and 12% for the higher pH (from 7.5 to 6.6) (Figure 5). These pH changes corresponded to a consumption in overall alkalinity of 375 mg/L as $CaCO_3$ at an initial pH = 7.5 and 250 mg/L as $CaCO_3$ at an initial pH of 6.2 (Figure 5).

Volatile acid and solvent concentrations in the bottles were again sampled after 180 h. It was observed that ethanol, originally initially measured at high concentrations in the bottles at 70 h, was absent from all bottles at 180 h (Figure 4). It was also found that acetic acid concentrations had increased in most of the bottles. Based on these observations, it appears that ethanol was used to produce acetic acid.

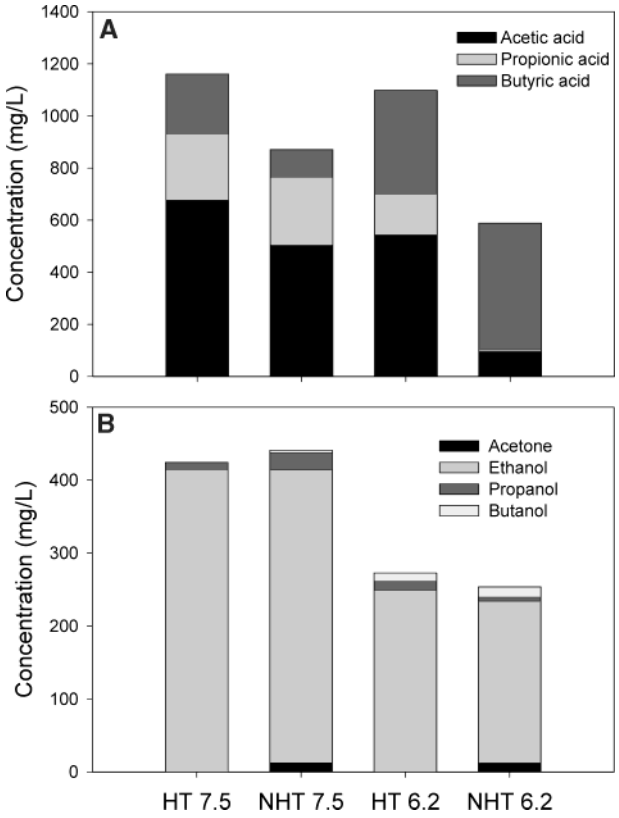

FIGURE 3. Organic acid (A) and alcohol (B) concentrations in the liquid in the bottles at 70 h.

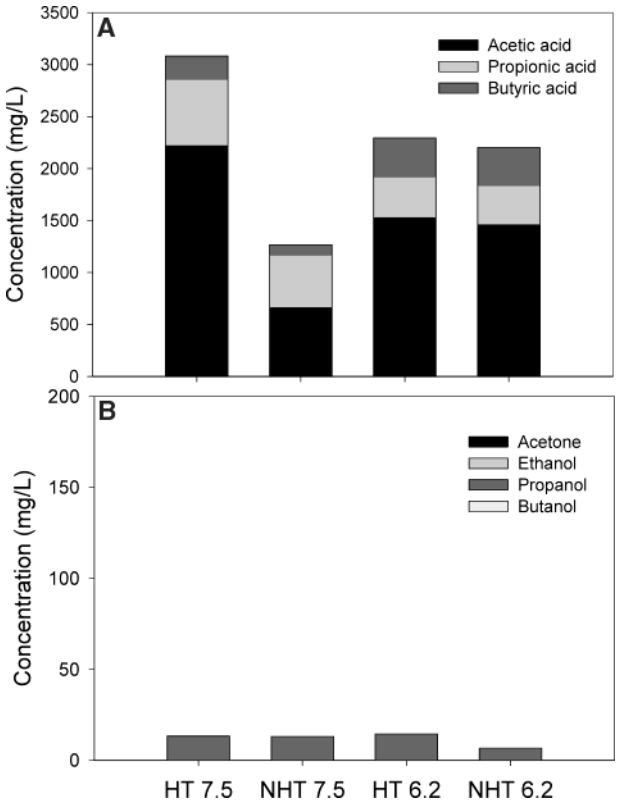

FIGURE 4. Organic acid (A) and alcohol (B) concentrations in the liquid in the bottles at 180 h. Note that acetone and ethanol were not detected in these samples.

Hydrogen Consumption by Homoacetogens. The disappearance of hydrogen from the heat-treated bottles after 30 h could not have been due to methanogenesis, as no methane

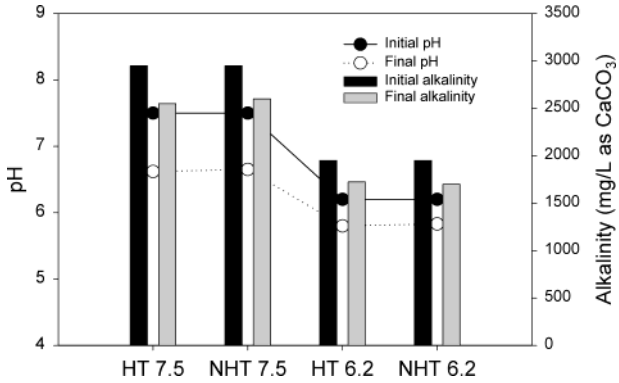

FIGURE 5. Initial and final pH of the solutions and total alkalinity consumed in the bottles, after 70 h.

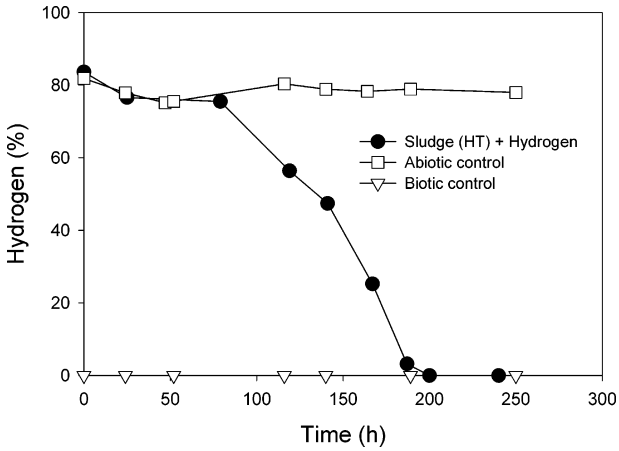

FIGURE 6. Hydrogen gas concentration (%) in samples with a high concentration of bicarbonate as a carbon source and without glucose amendment. (Abiotic control = no inoculum with hydrogen in the headspace; biotic control = inoculum and nitrogen gas in the headspace.)

was detected in these samples. We examined whether acetogenesis from H_2 and CO_2 was possible for the HT inoculum by conducting tests using a bicarbonate-rich media, no glucose, and bottles flushed with hydrogen gas. Following a lag phase of ~70 h, hydrogen gas was completely removed over the next 130 h (Figure 6). Respiking the bottle with additional hydrogen gas at 250 h produced the same hydrogen consumption pattern but no lag phase (data not shown). Acetate accumulation in the bottle reached 540 mg/L after 250 h. When adjusted for acetate production by the control (240 mg/L; due to organics in the soil inoculum), this amount of acetate production accounts for 108% of the hydrogen predicted via acetogenesis. These results therefore indicated that while heat treatment prevented methanogensis, it was unable to prevent hydrogen losses via acetogenesis.

Discussion

Heat treatment of the inoculum was essential to eliminate methanogenesis in batch tests. Lowering the pH from 7.5 to 6.2 for nonheat-treated samples reduced methane production but did not prevent it. For heat-treated samples, biogas production at a pH 6.2 was larger than that at pH 7.5. Higher hydrogen production at the lower pH was expected based on earlier tests (using a different test methodology) that showed the maximum hydrogen production occurred over a pH range of 5.5−6.0 (*11*). Lay et al. (*8*) suggested that a pH = 5.6 is optimum because a lower pH produces a transition from acid to alcohol production for mixed cultures. Hydrogen evolution by clostridia has been shown to be completely inhibited below a pH range of 4−5 (*17*−*19*).

Hydrogen Consumption by Homoacetogens. While heat treatment was sufficient to prevent methanogenesis, it did not prevent losses of hydrogen from the headspace (Figure 2A). Hydrogen losses could be coupled to microbial reduction of a variety of compounds, such as sulfate (some are sporeformers (*20*)), nitrate, and iron, but the nonspore-forming bacteria (*20*) that mediate these processes should have been killed by heat treatment. In addition, only trace amounts of these compounds were present in the medium.

It is more likely that hydrogen losses in batch tests were due to homoacetogenesis. Spore-forming clostridia, such *C. aceticum* and *C. thermoautotrophicum*, are known to produce acetic acid from H_2 and CO_2 (*21*) via

$$2HCO_3^- + 4H_2 + H^+ = CH_3COO^- + 4H_2O \qquad \Delta G^{\circ\prime} = -105 \text{ kJ} \quad (2)$$

Although it was not possible to separately measure hydrogen production and consumption in batch bottles fed glucose, separate experiments conducted using a sludge or soil inoculum in the absence of glucose demonstrated that acetogensis from H_2 and CO_2 was not inhibited by heat treatment of the samples (Figure 6). It is not known if the same bacteria switched from hydrogen to acetic acid production when glucose was depleted from the culture, or if there were constant losses of hydrogen during the whole batch cycle. However, hydrogen gas conversion efficiencies are typically greater in continuous culture (~32−59%) (*9*, *22*, *23*) using pure cultures than in mixed batch cultures used here (23−24%), suggesting that there are constant hydrogen losses in batch tests due to the activity of the non-hydrogen-producing bacteria. The hydrogen lost via acetogenesis can be roughly estimated using data in Figure 6 as 35 mL for HT samples at a pH = 6.2. Including this hydrogen in as a part of the total production changes the conversion efficiency to 33%, a value more consistent with pure culture results under these pH and temperature conditions.

Ethanol, produced during the initial fermentation of glucose, was not stable in solution after hydrogen production ceased. Ethanol accumulated in solution during glucose utilization to concentrations of 221−414 mg/L after 70 h in all batch tests (Figure 3), but ethanol was absent in solutions by 180 h. The production of ethanol from acetic acid and hydrogen is thermodynamically favorable under conditions present in the heat-treated batch bottles (pH = 6.2) only for high H_2 partial pressures. Assuming ethanol production by the reaction

$$CH_3COOH + H_2 = C_2H_5OH + 4H_2O \quad (3)$$

$\Delta G^\circ = -49.51$ kJ/mol is calculated based on standard free energies (kcal/mol) of −369.41 for acetic acid, −181.75 for ethanol, and −237.17 for water at pH = 7 (*24*). After 70 h we infer from the data that hydrogen consumption exceeded hydrogen production, so that hydrogen was eventually removed to very low levels in the headspace at 77 h and thereafter. Based on concentrations of chemicals in the bottles at 77 h (0.0058 atm, H_2; 11.29 M, acetic acid; and 9.01 M, ethanol), ethanol production was still thermodynamically favorable ($\Delta G^{\circ\prime} = -24.5$ kcal/mol).

After 180 h, it was observed that ethanol had been depleted in solution and that acetic acid concentrations had increased in the bottles. Equation 4 is a reversible reaction, and at a hydrogen partial pressure of 4 Pa, acetic acid production from ethanol becomes thermodynamically possible. In addition, a biochemical route for microbial conversion of ethanol to acetic acid is known (*25*−*27*). Thus, it appears likely that ethanol consumption resulted in additional acetic acid production after approximately 100 h. Any hydrogen produced during this period (72−180 h) would probably be consumed via interspecies hydrogen transfer for acetogenesis, resulting in no further accumulation of hydrogen in the gas headspace.

These experiments demonstrate that methanogenesis can be prevented by heat treatment of an inoculum and that high concentrations of hydrogen can be produced by fermention of glucose in batch tests. While the conversion efficiency of hydrogen production from glucose is increased by maintaining a pH at around a pH = 6 (compared to a near neutral pH), interspecies transfer of hydrogen will occur, and hydrogen lost to acetogenesis will not be able to be recovered. Such losses will need to be better controlled to maximize biological hydrogen under batch reactor conditions.

Acknowledgments

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