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Removal of Headspace CO₂ Increases Biological Hydrogen Production

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For biological hydrogen production by fermentation to be a useful method of hydrogen generation, molar yields of hydrogen must be increased. While heat treatment of a soil inoculum increases hydrogen yields by preventing loss of hydrogen to methanogenesis, hydrogen is still lost to acetic acid generation from hydrogen and CO_2. To reduce hydrogen losses via acetogenesis, CO_2 concentrations in the headspace were substantially reduced during hydrogen production using a chemical scavenger (KOH). CO_2 in the headspace was decreased from 24.5% (control) to a maximum of 5.2% during the highest gas production phase, resulting in a hydrogen partial pressure of 87.4%. This reduction in CO_2 increased the hydrogen yield by 43% (from 1.4 to 2.0 mol of H_2/mol of glucose). The soluble byproducts in all tests consisted primarily of acetate and ethanol. Higher concentrations of ethanol (10.9 mM) remained in solution from bottles with CO_2 removal than in the control (1.2 mM), likely as a result of hydrogen inhibition of biological ethanol conversion to acetic acid. These results show that hydrogen production can be increased by removing CO_2 in the reactor vessel, likely as a result of suppression of acetogenesis.

Introduction

Hydrogen has been suggested as a promising alternative to fossil fuels as an energy carrier for transportation needs. Hydrogen gas has a high energy capacity per unit mass (118.2 kJ/g), and its reaction with oxygen does not produce greenhouse gases such as CO_2. In contrast, methane has a relatively lower energy capacity (36.3 kJ/g) and CO_2 is released during its combustion in conventional engines. However, most hydrogen is usually produced from stored methane reserves and other nonrenewable materials, resulting in net increases of CO_2 in the environment. Shifting a fossil fuel economy to a hydrogen economy offers few environmental advantages if both are based on the net consumption of fossil fuels (*1*). Thus, it is essential for reducing CO_2 emissions that hydrogen production not release a net amount of CO_2 into the atmosphere, and that the technologies and materials used to produce hydrogen are sustainable.

Anaerobic fermentation is a promising method of sustainable hydrogen production since organic matter, including waste products, can be used as a feedstock for the process (*2*, *3*). The highest yields of hydrogen have been reported with strains of clostridia in pure cultures or mixed cultures where clostridia are predominant. For example, Collet et al. obtained 2.1−3.0 mol of H_2/mol of lactose with *Clostridium thermolacticum* and Yokoi et al. achieved 2.6 mol of H_2/mol of glucose with a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* in continuous culture tests (*4*, *5*). Batch tests often produce relatively lower yields than continuous culture tests. For example, Mizuno et al. obtained a yield of 0.88 mol of H_2/mol of glucose using a mixed anaerobic culture in batch tests (*6*). A yield of 0.99 mol of H_2/mol of glucose was obtained in batch tests with a heat-treated inoculum (*7*), but the same inoculum produced a yield of 0.91−1.8 mol of H_2/mol of glucose in continuous culture (*8*). Continuous culture experiments with mixed cultures have produced yields in other studies of 1.7−2.54 mol of H_2/mol of glucose (*6*−*11*).

The reasons for lower yields in batch versus continuous culture tests using mixed cultures are not fully understood, although differences in the microbial community are certainly important (*8*). Using pure cultures helps to minimize hydrogen losses by excluding methanogens and certain homoacetogens. However, the use of pure cultures is not feasible for waste materials due to the different bacteria needed to break down the various components of the organic matter in the waste, and the prohibitive cost of sterilizing wastewater streams. Mixed cultures may contain a diverse array of bacteria, but *Clostridium* spp. and other hydrogen-producing, spore-forming bacteria can easily be obtained by heat treatment of a soil or digested sludge sample by boiling or oven drying (*7*, *12*). Heat treatment kills non-spore-forming methanogens so that hydrogen losses due to methanogenesis can be prevented in batch tests (*13*, *14*). Even with a heat-treated inoculum, however, the efficiency of hydrogen production in batch tests is relatively low.

Reducing the partial pressure of hydrogen in reactor vessels can increase hydrogen production. In batch tests, continuous versus intermittent release of gas pressure in batch tests increased hydrogen production by 43% (*7*). Reducing the hydrogen partial pressure using a vacuum or by sparging the reactor vessel with nitrogen gas has also increased hydrogen yields (*7*, *15*). It was assumed in these tests that this increase in hydrogen production was related to reduction in the hydrogen partial pressure. However, hydrogen can also be consumed via acetogenesis, according to (*16*)

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad (1)$$

In batch tests using mixed cultures, Oh et al. demonstrated that hydrogen yields were lowered by acetogenesis by showing that, in the absence of glucose, a heat-treated inoculum produced acetic acid from hydrogen and CO_2 (*16*). The fact that both CO_2 and H_2 are required for acetogenesis suggested to us that limiting CO_2 concentrations in the reactor might provide a viable strategy for reducing acetogenesis. To examine this hypothesis, we compared hydrogen pro-

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parameter	CO_2 scavenging	no CO_2 scavenging
pH(initial)	6.20 ± 0.01	6.20 ± 0.01
pH(final)[b]	6.14 ± 0.01	5.78 ± 0.01
time for 90% conversion of glucose, h	11.0 ± 0.5	14.0 ± 0.5
total gas production,[c,d] mL	99 ± 1	108 ± 1
total H_2 production,[c,e] mL	120 ± 5	88 ± 4
total CO_2 production,[c] mL	4.5 ± 0.2	24.5 ± 1.0
maximum H_2 concentration, %	87.4 ± 0.3	64.5 ± 0.5
hydrogen yield,[c] mol of H_2/mol of glucose	2.0 ± 0.1	1.4 ± 0.1

[a] "$\pm$" indicates the data range based on duplicate samples. [b] Measurement after 52 h. [c] Measurement after 20 h. [d] Gas production is defined as the volume of gas leaving the reactor. Note that this volume does not include hydrogen produced that remains in the headspace. [e] Hydrogen production includes gas leaving the reactor plus that remaining in the headspace of the bottles, calculated according to eq 2.

duction in batch tests using a heat-treated inoculum in the presence and absence of a chemical CO_2 scavenger.

Methods

Culture and Medium. Dewatered and thickened sludge was obtained from The Pennsylvania State University Wastewater Treatment Plant in State College, PA, and heat treated as previously described (*7*). The sample was either used within 1 day or stored in a sealed container at room temperature (24 °C). Glucose (2 g/L = 11.1 mM) and sludge (1 g/L) were added to a mineral salt medium (*11*) buffered to a pH of 6.2 using 0.07 M 2-(*N*-mopholino)ethanesulfonic acid monohydrate (MES) (Mallinckrodt Baker Inc., Phillipsburg, NJ).

Experimental Apparatus. For gas tests, four media bottles (300 mL) were used as the bioreactors. Batch tests were conducted (in duplicate) using 250 mL of media in bottles, stirred at 300 rpm in a constant temperature room (30 $\pm$ 1 °C). All bottles were initially flushed with nitrogen gas and sealed. CO_2 was removed from the headspace using 6 mL of KOH (30 wt %) placed in a small tube open only to the headspace. Biotic controls were prepared in the same manner using distilled water instead of KOH. Gas production was measured using a respirometer (Challenge Environmental Systems AER-200, Fayetteville, AR) with bio-hydrogen production calculated from headspace gas measurements and the total volume of biogas produced at each sampling interval as described below. The difference in gas production between the treatment and control was used to calculate total CO_2 gas production.

Four additional bottles were prepared in the same manner (with and without CO_2 scavenging), but these bottles were connected to 1 L polypropylene gas packs (Supelco) instead of the respirometer, and were used for analysis of liquid products. Liquid analysis was conducted to evaluate the effect of CO_2 scavenging on the change in byproduct composition. It was assumed that the reaction conditions were the same in both sets of bottles, and the small change in the volume of headspace caused by liquid sampling was neglected.

Analysis. Hydrogen gas was periodically sampled using a gastight syringe (0.25 mL injection volume) and analyzed using a gas chromatograph (model 310, SRI Instruments, Torrence, CA) equipped with a thermal conductivity detector (TCD) and a molecular sieve column (Alltech Molesieve 5A, 80/100, 6 ft $\times$ 1/8 in. $\times$ 0.085) with nitrogen as the carrier gas. Hydrogen gas production was calculated as previously described (*16*) from the gas composition of the headspace and the total volume of biogas produced using the mass balance equation

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (2)$$

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 $V_{G,i-1}$ the total biogas volumes in the current and previous

time intervals, and $C_{H,i}$ and $C_{H,i-1}$ the fractions of hydrogen gas in the current and previous intervals, respectively, and V_H is the total volume of headspace in the reactor. The hydrogen gas composition between sampling times was calculated assuming a linear change in concentration over the sampling interval (*16*).

Liquid samples (triplicate) were prepared for analysis by adding 20 μL of $HgCl_2$ (16 g/L) into 1 mL of sample in a 1.5 mL centrifuge tube, centrifuging the tubes at 6610g for 15 min, and then removing the supernatant for analysis. Samples were stored at 4 °C prior to analysis. Glucose was analyzed using the phenol/sulfuric acid method (*17*). All other liquid components were analyzed with a gas chromatograph (Agilent 6890N) equipped with an FID detector and a DB-FFAP capillary column (30 m $\times$ 0.32 mm, with 0.5 μm thick film) with helium as the carrier gas.

Results

Enhancement in Hydrogen Production and Glucose Conversion Rate. Hydrogen production was enhanced 43% by reducing the CO_2 concentration in the headspace. Overall, hydrogen yields increased from 1.4 mol of H_2/mol of glucose (175 mL of H_2/g of glucose) to 2.0 mol of H_2/mol of glucose (241 mL of H_2/g of glucose) with CO_2 removal (Table 1). CO_2 concentrations in the headspace could not be completely depleted, with CO_2 concentrations reduced from 24.1% (control) to 5.2% (maximum percent) using the KOH trap during the period of the greatest rate of hydrogen production (from 12 to 17 h). As indicated in Table 1, trapping the CO_2 reduced the measured gas production (compared to that of the control), but increased overall hydrogen production. Note in Table 1 that the gas production (the total volume of gas leaving the reactor) is less than the total volume of hydrogen gas production. This is because the hydrogen gas that is produced includes both gas leaving the reactor and gas produced in the reactor. When CO_2 and H_2 are both produced, but only the CO_2 is scavenged from the headspace, it is possible for H_2 to accumulate, and thus, less gas leaves and is recorded as "gas production" rather than "hydrogen gas production". Thus, hydrogen accumulates in the reactor and overall can exceed total gas production up to a value equal to the headspace of the reactor.

In the absence of CO_2 removal, the pH decrease (from pH 6.2 to pH 5.78) was larger than that of the control (final pH 6.14) due to the higher concentration of CO_2 and volatile acids. This slight difference in final pH should not have substantially affected hydrogen production as maximum hydrogen yields in batch tests have been reported to be in the pH range of 5.5−6.0 (*18*). The rate of glucose degradation was also increased slightly when CO_2 was removed (Figure 1). With CO_2 removal, 99% of the glucose was removed within 15 h, whereas only 92% of the glucose was removed after 15 h in the absence of CO_2 removal.
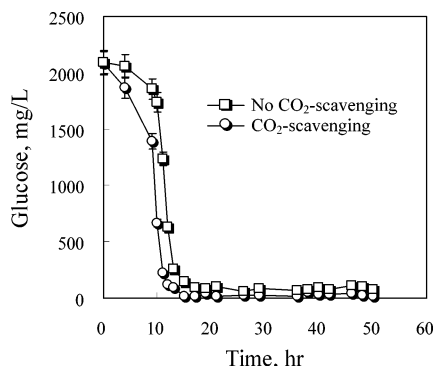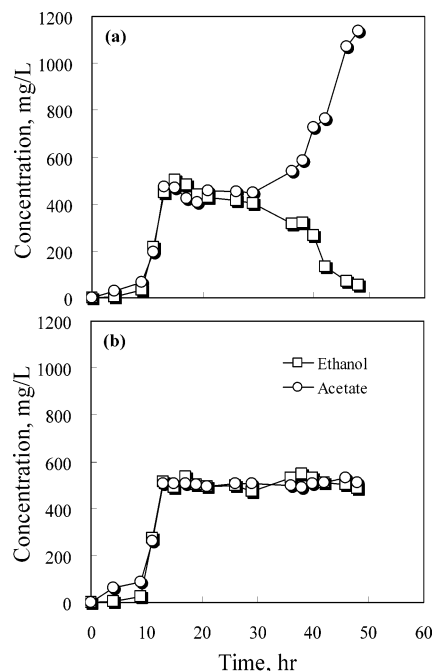
FIGURE 1. Glucose concentrations in the presence and absence of CO_2 removal (error bars indicate the data range for duplicate samples).
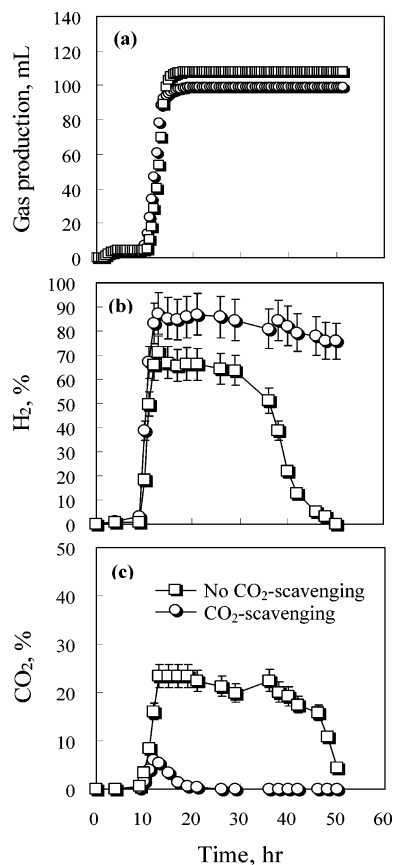
FIGURE 2. Total gas production over time (a) and concentrations in the headspace of (b) hydrogen and (c) carbon dioxide (error bars indicate the data range for duplicate samples).


FIGURE 3. Concentrations of ethanol and acetate, the two main soluble products during hydrogen production tests, in tests (a) without CO_2 removal and (b) with CO_2 removal.

The maximum hydrogen concentration in the headspace was increased from 64.5% (control) to 87.4% as a result of CO_2 removal (Table 1, Figure 2). A hydrogen concentration of 100% could not be achieved due to incomplete CO_2 removal (Figure 2c) and some remaining nitrogen gas. During the period of the greatest rate of hydrogen production (from 12 to 17 h), CO_2 reached a maximum concentration of 5.2% before being completely removed from the system (after 19 h). On the basis of previous results (*19–22*), we would expect that increased hydrogen partial pressures would have inhibited bio-hydrogen production, and not resulted in an increase of hydrogen production as observed here. Therefore, the increase in hydrogen production must result from an aspect related to the reduced CO_2 concentration in the system. The most likely explanation is that a reduction in CO_2 concentrations reduced hydrogen losses via acetogenesis, which could not be completely inhibited. While there was an overall increase in hydrogen production (from 1.4 to 2.0 mol of H_2/mol of glucose) with CO_2 removal, CO_2 could not be completely removed from the headspace. CO_2 concentrations may have been disproportionately higher in the liquid phase (relative to equilibrium) where the CO_2 was generated. Thus, it was not possible to completely inhibit acetogenesis due to the remaining CO_2 concentration in the liquid phase.

The reduction of acetogenesis can be seen by examination of hydrogen concentrations in the headspace following glucose removal. In the absence of CO_2 scavenging, hydrogen was completely removed from the headspace between 15 and 50 h (from 64.5% to 0.0%, Figure 2b). During the same time interval, acetic acid concentrations increased from 425 mg/L (7.1 mM) to 1140 mg/L (19.0 mM) (Figure 3a). Glucose could not have been a source of acetic acid as glucose concentrations were insufficient after 15 h to account for this increase in acetic acid (715 mg/L = 11.9 mM), and glucose was completely consumed after 26 h. This increase in acetic acid concentration, coupled with hydrogen reduction, demonstrated acetogenesis in the system. However, when CO_2 was scavenged from the headspace, hydrogen concentrations decreased only slightly from a maximum concentration of 87.4% following glucose removal during the same period (Figure 2b). In addition, the concentration of acetic acid was also nearly constant, demonstrating the lack of acetogenesis with CO_2 scavenging (Figure 3b).

In the absence of CO_2 scavenging the increase in the acetic acid concentration is greater than the increase that can be attributed to acetogenesis alone. On the basis of the amount of hydrogen consumed between 15 and 50 h, 59 mg (1.0 mmol) of acetate should have been produced according to eq 1. This would have resulted in an increase in acetate concentration of 236 mg/L (3.9 mM), which is significantly lower than the observed increase of 715 mg/L (11.9 mM). This observed increase in acetic acid can be attributed to ethanol degradation. Ethanol is converted to acetic acid and hydrogen according to (*19, 23*)

$$C_2H_5OH + H_2O \rightarrow CH_3COOH + 2H_2 \qquad (3)$$
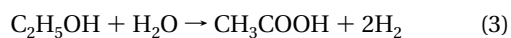
The reaction proceeds in the forward direction for most bacteria only if the concentration of hydrogen is below 10^{-3} atm for most bacteria, although it has been noted that this reaction goes forward at 1 atm of hydrogen for *Clostridium kluyveri* (*19*, *24*). In tests where CO_2 was removed, hydrogen concentration remained high (above 0.8 atm), preventing ethanol conversion to acetic acid (Figure 3b).

Ethanol conversion to acetic acid was only possible in tests without CO_2 scavenging because hydrogen concentrations were decreased (Figure 3a). It appears from data in Figure 3b that the conversion of ethanol occurred after the hydrogen fraction was decreased to <53% (0.53 atm). In tests where CO_2 was removed, hydrogen concentration remained high (above 0.8 atm), preventing ethanol conversion to acetic acid (Figure 3b). Ethanol conversion to acetic acid was only possible in tests without CO_2 scavenging because hydrogen concentrations were decreased (Figure 3a). The conversion of ethanol to acetic acid is supported by a mass balance using eq 2. According to this equation, 1.0 mmol of acetate is produced from 1.0 mmol of ethanol. On the basis of a decrease in ethanol concentration from 500 mg/L (10.9 mM) at 15 h to 55 mg/L (1.2 mM) after 50 h, we estimate that 579 mg/L (9.7 mM) acetic acid could be produced. When combined with 236 mg/L (3.9 mM) acetate from acetogenesis, this would predict a total change in acetate concentration of 815 mg/L (13.6 mM) from 425 mg/L (7.1 mM) at $t = 15$ h to 1240 mg/L (20.7 mM) at $t = 50$ h. This estimated result compares well to the observed increase of 715 mg/L (11.9 mM) of acetic acid (final concentration of 1140 mg/L = 19.0 mM).

CO_2 scavenging did not substantially affect the concentrations of the other volatile acids and solvents. In the absence of CO_2 scavenging, the final concentrations (after 48 h) of the other measured byproducts were (propionate) 58 mg/L, (1-propanol) 33 mg/L, and (butyrate) 15 mg/L. With CO_2 scavenging, the concentrations of these chemicals were (propionate) 65.7 mg/L, (1-propanol) 26 mg/L, and (butyrate) 48.3 mg/L. In addition, there was a small concentration of acetate (13.5 mg/L) measured in solution with CO_2 scavenging.

Discussion

Several studies have shown that gases produced during biohydrogen production can affect hydrogen production. While this increase in production has typically been attributed to a reduced percent of hydrogen in the gas phase, our results demonstrate that changes in CO_2 concentration can also be a factor. Overall hydrogen production increased here by 43% with chemical scavenging of the CO_2 despite an increase in the hydrogen concentration in the headspace. Previous studies have shown that an increase in gas pressure in bottles during these tests can reduce hydrogen production. It was shown by Logan et al. that continuous release of gases increased H_2 production by 43% versus tests where there was an intermittent release of gas pressure in the bottles (*7*). Their analysis on the effect of hydrogen gas concentration based on thermodynamic data suggested that the hydrogen gas concentration would not be inhibitory in their tests. Results from the current study suggest that the increased pressure of CO_2 could also have been a factor in their study. It has also been shown that reducing the pressures of the evolved gases, either by applying a vacuum or by gas sparging, can increase hydrogen production. When a vacuum was applied to a reactor operated in chemostat mode, hydrogen production increased by 10%, resulting in an overall yield of 2.2 mol of H_2/mol of glucose (*26*). An alternate approach to decrease the dissolved hydrogen concentration in a reactor used by Tanisho et al. was to sparge the reactor with argon gas, which resulted in an increase in hydrogen yield from 0.52 to 1.58 mol of H_2/mol of glucose (*15*). While increased hydrogen production was attributed to a reduction in the dissolved hydrogen concentration in both of these studies, the concentration of dissolved CO_2 would also have been reduced, contributing to increases in hydrogen production.

While applying a vacuum, gas sparging, or CO_2 scavenging may all be effective methods of increasing hydrogen production, CO_2 scavenging may provide the most direct and cost-effective method of reducing CO_2. Gas sparging may also have the undesirable potential to produce a loss of volatile fatty acids in the reactor off gas. In the current study, we only examined the effect of CO_2 scavenging in batch tests. Even in these tests, it was not possible to completely scavenge CO_2 from the headspace during high rates of hydrogen production (Figure 2). If hydrogen is produce in continuous culture, it is not known how effectively we could scavenge CO_2 under these conditions. Thus, additional research is needed to extend the batch test results from this test to continuous culture tests, particularly under conditions that affect the hydrogen production rate, such as under different organic loadings and reactor detention times.

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