Inhibition of biohydrogen production by ammonia

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\textbf{ABSTRACT}

Ammonia inhibition of biohydrogen production was investigated in batch and continuous flow reactors with glucose as a substrate. In batch tests, biohydrogen production rate was highly dependent on pH and ammonia (defined as the sum of NH\textsubscript{3} of NH\textsubscript{4}\textsuperscript{+} species) concentrations above 2 g N/L. At pH = 6.2, the maximum production decreased from 56 mL/h at 2 g N/L to 16 mL/h at 10 g N/L. At pH = 5.2, production decreased from 49 mL/h (2 g N/L) to 7 mL/h (16 g N/L). Hydrogen yield remained relatively constant in batch tests, varying from 0.96 to 1.17 mol-H2/mol-glucose. In continuous flow tests, both hydrogen production rates and yields were adversely affected by ammonia. When the reactor (2.0 L) was first acclimated under batch conditions to a low nitrogen concentration (<0.8 g N/L), H2 production and yields under continuous flow mode conditions were 170 mL/h and 1.9 mol-H2/mol-glucose, but decreased with increased ammonia concentrations up to 7.8 g N/L to 105 mL/h and 1.1 mol-H2/mol-glucose. There was no hydrogen production under continuous flow conditions if the reactor was initially operated under batch flow conditions at ammonia concentrations above 0.8 g N/L. It is concluded that the hydrogen production is possible at high concentrations (up to 7.8 g N/L) of ammonia in continuous flow systems as long as the reactor is initially acclimated to a lower ammonia concentration (<0.8 g N/L).

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1. Introduction

Bacterial hydrogen production via fermentation has the potential to treat wastewater streams while producing a clean, renewable energy carrier in the process (Benemann, 1996; Logan, 2004). Different wastewaters that have been considered for biohydrogen production via “dark” fermentation (i.e. non-photobiological-based processes) include sewage sludge (Cai et al., 2004), food wastes (Kim et al., 2004; Zhu et al., 2002; Han and Shin, 2004), and animal (Angenent et al., 2004) wastewater streams. Hydrogen can be produced using a variety of reactor and operating conditions, including batch (Logan et al., 2002; Van Ginkel et al., 2001) and continuous flow stirred tanks (Fang and Liu, 2002; Iyer et al., 2004), membrane (Oh et al., 2004), and upflow anaerobic sludge blanket (UASB) reactors (Chang and Lin, 2004).

High hydrogen yields are needed to make the process economical (Logan, 2004). Obligate anaerobes such as Clostridia species are mainly responsible for fermenting sugars to hydrogen at high yields (Iyer et al., 2004), producing acetate, butyrate, and other fermentation end products as waste products (Gottschalk, 1986). For these bacteria, the maximum theoretical yield of hydrogen by known biochemical pathways is 4 mol-H2/mol-glucose, when acetate is produced according to

\[ C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \]  

(1)

However, the need to regenerate NAD\textsuperscript{+} often results in the production of other volatile acids and solvents. For example,
bacteria produce only 2 mol-H2/mol-glucose when butyrate produced (Levin et al., 2004) via
\[
C_4H_9O_4 \rightarrow 2H_2 + 2CO_2 + C_4H_8O_2. \tag{2}
\]

Overall yields are lowered from these maximum values due to the production of cell biomass.

Several factors must be considered for maximizing hydrogen production in fermentation systems. It is important to avoid the loss of hydrogen to hydrogen-consuming anaerobes, such as methanogens (Lay, 2001). This can be done by heat-treating the inoculum to select for spore-formers, such as Clostridia, for glucose-fed reactors or even heat-treating the wastewater to kill methanogens (Levin et al., 2004; Loga. 2001; Oh et al., 2003; Ting et al., 2004). Low pH can also be used to minimize the growth of methanogens (Pelphs and Zeikus, 1984). It has been shown that longer hydraulic retention times (HRT) and lower chemical oxygen demand (COD) levels (factored together as COD loading rate) contribute to greater overall efficiency of hydrogen production in continuously fed reactors (Dabrock et al., 1992; Van Ginkel and Logan, 2005). Other factors such as inoculum, substrate, temperature, nitrogen sparging, and initial start up have all been examined by various researchers in an effort to optimize the production of hydrogen (Dabrock et al., 1992; Hawkes et al., 2002; Iyer et al., 2004).

While animal wastewaters have been considered as potential wastewater sources for biohydrogen production, these wastewaters contain high (~2 g/L) concentrations of ammonia (Ceccherini et al., 1998; Cruz et al., 2000). Ammonia is known to inhibit methanogenesis (Lay et al., 1998; Borja et al., 1996; Bhattacharya and Parkein, 1988; Koster and Koomen, 1988), but the effect of ammonia on hydrogen production has not been well examined. Zhu et al. (2001) found that NH3 concentrations of <0.14 g/L did not adversely affect hydrogen production by Clostridium butyricum. Lay et al. (1998) determined that it was the NH3 species, as opposed to the NH4+ ion, that was responsible for increased lag times in methanogenesis, but that NH4+ ions had a larger effect than NH3 on the overall methanogenic activity.

To investigate the inhibition to hydrogen-producing bacteria, various concentrations of ammonium chloride were added to a feed solution containing glucose as the energy source under both batch and continuous flow conditions. The effect of ammonia was examined in terms of hydrogen production rates and overall hydrogen yields. Ammonia is defined here as the sum of both NH3+NH4+ forms, with specific chemical formulas used to denote specific ammonia species.

2. Methods
2.1. Batch tests

Dewatered and thickened sludge was obtained from the Pennsylvania State University Wastewater Treatment Plant in State College, PA, and heat treated as previously described (Oh et al., 2003). The sample was either used within one day or stored in a sealed container at room temperature (24 °C) for no more than 1 month. The reactors were inoculated with the heat-treated sludge (4 g/L) and grown on glucose (3.76 g/L = 4 g/L COD) in a mineral salt medium (MSM) (Oh et al., 2003), and buffered to a pH of 5.2 or 6.2, using 0.07 M of 2-(N-morpholino) ethanesulfonic acid monohydrate (MES) (J.T. Baker, Phillipsburg, NJ). The MSM consisted of the following nutrients (listed per liter of feed) for all experiments: 360 mg MgSO4·7H2O, 50 mg FeCl3, 48 mg NiCl2·6H2O, 66 mg CaCl2·2H2O, 23 mg ZnCl2, 21 g CoCl2·6H2O, 10 mg CuCl2·2H2O, 30 mg MnCl2·4H2O, 175 mg K2HPO4, and 175 mg KH2PO4. NH4Cl was separately added to each reactor at concentrations of 0.5, 2, 5, 7, and 10 g N/L.

Batch tests were conducted in duplicate using 250 mL of liquid in 300 mL bottles stirred at 300 rpm in a constant temperature room (30 ± 1 °C). All bottles were initially flushed with nitrogen gas before being sealed with rubber stoppers. Gas production was measured continuously using a respirometer system (Challenge Environmental Systems AER-200, Fayetteville, Arkansas, USA), while the composition of headspace gas was periodically sampled (10–20 sample points per experiment). The biohydrogen production was calculated from the composition and the volume of biogas produced, as previously described (Logan et al., 2002).

2.2. Continuous-flow reactor experiments

Continuous flow tests were conducted using a fermentor (New Brunswick Scientific BioFlo 110) filled to 2.0 L at 30 °C. The reactor was sparged with nitrogen gas, and inoculated with heat-treated agricultural soil (Logan et al., 2002). MSM and glucose (4 g/L COD) were added to the reactor. L-cysteine (0.5 g/L) was added to scavenge dissolved oxygen and lower the initial redox potential. Bottles of feed (10 L, autoclaved) were sparged with N2 for at least 30 min before use, and then continuously sparged and mixed with a magnetic stirrer during tests. NH4Cl was added in concentrations of 0.8, 1.6, 3.9, 5.3, and 7.8 g N/L.

For each experiment, the reactor was initially operated for 3–4 days in batch mode (with L-cysteine), and then switched to continuous-flow conditions at a HRT of 10 h (no L-cysteine). Redox potential and pH were monitored during start up to ensure that the redox potential remained negative and that pH was constant at 5.5. Small (~2 mL total per reactor) additions of 2 M KOH were needed to maintain the pH during the initial batch mode. During continuous flow operation the pH was automatically maintained at a pH of 5.5 using KOH (2 M). The reactor was determined to be operating under steady state conditions on the basis of gas production rate, which typically required 6–8 days of continuous flow mode operation. Total gas production was measured using a bubble trap system consisting of a graduated pipette with a side inlet. The time for 2–10 mL of gas production, depending on flow rate, was recorded in triplicate.

2.3. Analysis

Hydrogen gas was measured using a GasTight™ syringe (SGE, 0.5 mL injection volume for continuous tests, 0.25 mL for batch tests) and analyzed using a gas chromatograph (GC; Model 310, SRI Instruments, Torrence, CA) equipped with a thermal conductivity detector and a molecular sieve column (Alltech Moleseies 5A 80/100) with nitrogen as the carrier gas.
Nitrogen and carbon dioxide were sampled in the same manner and analyzed using a second GC of the same model but containing a different column (Alltech Porapak Q 80/100) with helium as the carrier gas. Pure gases were used as calibration standards.

Organic acids, solvents, and alcohols were measured in the aqueous phase using a GC (Agilent GC-FID). Liquid samples were filtered using 0.2 \( \mu \)m pore-diameter filters (Whatman) and diluted before analysis with 50% formic acid. Samples were stored sealed in a refrigerator (4 °C) prior to analysis. Residual glucose was measured (in duplicate) with the phenol and sulfuric acid assay using standards in a range of 10–60 mg/L.

3. Results and discussion

3.1. Batch reactors

The rate of total biogas production in batch tests decreased, and the apparent lag increased, with increasing ammonia concentrations (Fig. 1). However, total hydrogen gas production was not significantly affected \((p > 0.18, \text{ANOVA})\), and averaged 129 ± 5 mL (± 95% confidence interval; Fig. 2) for both pH values tested (5.2 and 6.2). The change in the pH during a batch cycle was affected by organic loading, but decreased only by 3.6–9.6% and therefore was not expected to effect hydrogen production. The hydrogen yield was also not affected by ammonia concentration in batch tests \((p > 0.32, \text{ANOVA})\), and averaged 1.0 ± 0.04 mol-H₂/mol-glucose at ammonia concentrations of up to 10 g/L (Fig. 2). The bacteria continued to produce hydrogen until all the glucose in the system was consumed, which resulted in approximately the same amount of total hydrogen being produced despite the different levels of ammonia. The lag time, defined arbitrarily here as the time for 100 mL of gas production, increased with ammonia concentration (Fig. 3) from ~12 h (0.5 g N/L) to ~35 h (10 g N/L), and the maximum gas production rate decreased (Fig. 4). The maximum production rate (the highest rate of gas production) decreased as the ammonia concentration increased.
production, as determined by differentiating the gas production data) at pH 6.2 decreased from 56 mL/h (at 2 g N/L) to 16 mL/h (at 10 g N/L). At pH 5.2, it decreased from 49 to 7 mL/h, at 2 and 10 g N/L, respectively.

As the specific form of the ammonia (NH₃ or NH₄⁺) is highly dependent on pH, it is important to note the difference in reactor performance under different pH values in terms of ammonia species. At the conditions tested here, most of the ammonia is present as NH₄⁺ as the NH₃/NH₄⁺ ratio varied from 1.3 \times 10^{-4} to 1.3 \times 10^{-3} mol/mol. At a given total ammonia/ammonium concentration, the concentration of NH₃ molecules is an order of magnitude greater at a pH of 6.2 than at 5.2. For example, in tests at an ammonia concentration of 10 g/L, the NH₃ concentration was 13 mg N/L at pH = 6.2, and 1.3 mg/L at a pH of 5.2. The reactors showed longer lag times and slower production rates at a pH of 5.2, suggesting that the inhibitory mechanism is different than that expected on the basis of just NH₃ concentrations.

3.2. Continuous-flow reactor experiments

Acclimation to a low nitrogen concentration prior to the introduction of a high concentration of NH₃/NH₄⁺ (>0.8 g N/L) was critical to successful production of hydrogen. When the initial concentration of NH₃/NH₄⁺ in the reactor operated under batch conditions was 1.6 g N/L or higher, total gas production and hydrogen production were all negligible, and the reactor inoculum washed out when the reactor was switched to continuous mode operation (data not shown). Therefore, in subsequent tests the reactor was first run in batch mode at 0.8 g N/L, and then switched to a feed containing the same or higher concentration of NH₃/NH₄⁺. Ammonia toxicity has been shown for methanogens in batch experiments (Lay et al., 1998), where concentrations above 4 g N/L showed reduced methane production and increased lag times. Toxicity has also been shown in UASB reactors (Borja et al., 1996), where digestion was inhibited above 5 g N/L.

Hydrogen production in a 2 L chemostat decreased by 15% (from 170 to 145 mL/h) when the ammonia concentration was increased from 0.8 to 3.9 g N/L in the feed after reactor acclimation to 0.8 g N/L (Fig. 5). The hydrogen yield decreased from 1.9 to 1.6 mol-H₂/mol-glucose when the ammonia concentration was increased from 0.8 to 3.9 g N/L. During this feed transition, the total gas production rate decreased (259–230 mL/h) and hydrogen production decreased (170–145 mL/h). The glucose removed increased from 86% to 91% (this change was not statistically significant, p > 0.1). Hydrogen production was further inhibited, while yield was...
decreased, when the reactor was transitioned to a feed containing 5.3 or 7.8 g N/L.

The hydrogen yield consistently decreased with ammonia concentration from 1.9 mol-H₂/mol-glucose at 0.8 g N/L, to 1.1 mol/mol at 7.8 g N/L (Fig. 6). The concentration of hydrogen gas remained constant at 65 ± 1.6% for the different ammonia feed concentrations (Table 1). Thus, the decrease in hydrogen yield was due to reduced gas production, with the hydrogen production rate decreasing from 170 mL/h at 0.8 g N/L, to 105 mL/h at 7.8 g N/L. The glucose removal rate increased slightly, from 86% to 92%, as the ammonia concentration from 1.9 mol-H₂/mol-glucose via the acetate pathway, we would predict 13% less hydrogen production from the high ammonia reactor than the low ammonia reactor. This is very close to the measured decrease in hydrogen production rate of 15%. A similar analysis performed for the 7.8 g N/L conditions would predict a 37% decrease in hydrogen production, which is similar to the 38% observed decrease.

4. Conclusions

These results show that hydrogen production can be inhibited by high concentrations of ammonia, such as those present in animal wastewaters. Under fed batch conditions, the overall yield of hydrogen is not affected but the rate of hydrogen production is decreased. When a continuous flow reactor was initially operated (in batch mode) at a high (>1.6 g N/L) concentration of ammonia, hydrogen production failed. However, if the reactor was first started up at a lower ammonia concentration (0.8 g N/L), hydrogen could be produced but the overall yield was reduced from 1.9 to 1.1 mol-H₂/mol-glucose for ammonia concentrations of 0.8 to 7.8 g N/L. The concentration of hydrogen in the gas remained constant under all continuous flow conditions (65 ± 1.6%), and
thus the decrease in hydrogen yields was due to reduced gas production rates. It is therefore possible to produce hydrogen under high (up to 7.8 g N/L) concentrations of ammonia but the ammonia can reduce the overall rate or conversion of glucose to hydrogen.

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REFERENCES


