H₂ consumption by anaerobic non-methanogenic mixed cultures

C. Dinamarca, M. Gañán, J. Liu and R. Bakke

ABSTRACT

The impact of H₂ consumption, by homoacetogens, on the overall hydrogen yield in mixed culture fermentation is the focus of this study. Batch reactors were used to test the ability of anaerobic digested sludge, heat treated to eliminate methanogens, to produce and consume molecular hydrogen. The measured average hydrogen production rate from 4.2 g glucose/L was 44 ± 4 mmol H₂/L sludge/d, while the H₂ consumption rate varied much more, in the range from 4 to 62 mmol H₂/L sludge/d. Hydrogen consumption rate depends on acetic acid concentration, headspace H₂ partial pressure and mass transfer rates. Different but relatively long lag-phases for hydrogen consumption were observed. It is concluded that homoacetogenesis can have a significant negative effect on bio-H₂ production by mixed cultures, limiting the possibilities for sustainable solutions.

Key words | fermentation, homoacetogenesis, hydrogen consumption, hydrogen production

INTRODUCTION

Hydrogen is an alternative clean energy carrier. Anaerobic fermentation has been proclaimed to be a sustainable and energy-effective method for hydrogen production (Das & Veziroglu 2001). Most studies on bio-hydrogen production focus on yields and rates of products formation from sugars oxidation, ignoring the autotrophic hydrogen consumption potential. The sustainability of hydrogen gas production by mixed culture fermentation is questioned (Dinamarca & Bakke 2009). Low net hydrogen yields, attributed to the production of reduced products, are often reported (Li & Fang 2007).

In anaerobic non-methanogenic environments, H₂ can be used as electron donor in several autotrophic pathways to generate acetate, formate, methanol and acetaldehyde with CO₂ as the electron acceptor, Equations 1–4 (Burton 1957). Sulphur (S) and, sulphate (SO₄²⁻) and sulphite (SO₃²⁻) can also act as electron acceptors (Burton 1957). Hydrogenotrophic methanogens and sulphur-reducing bacteria generally out-compete homoacetogens because these have much higher H₂ threshold levels, between 520–950 ppm (Batstone et al. 2002). The basis for the present study is the assumption that autotrophic H₂ consumption (Equations 1–4, ΔG = at pH 7, all other reactant 1 M, 25 °C) can play an important role in anaerobic hydrogen production reactors (Dinamarca & Bakke 2009).

\[
\begin{align*}
\text{HCO}_3^- + \text{H}_2 & \rightarrow \text{Formate}^- + \text{H}_2\text{O} \quad \Delta G^- = -0.84 \text{ kJ} \\
\text{HCO}_3^- + 3\text{H}_2 + \text{H}^+ & \rightarrow \text{Methanol} + 2\text{H}_2\text{O} \\
& \quad \Delta G^- = -22.60 \text{ kJ} \\
\text{HCO}_3^- + 2\text{H}_2 + \text{H}^+ & \rightarrow \text{Formaldehyde} + 2\text{H}_2\text{O} \\
& \quad \Delta G^- = +22.60 \text{ kJ} \\
2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ & \rightarrow \text{Acetate}^- + 4\text{H}_2\text{O} \\
& \quad \Delta G^- = -107.18 \text{ kJ}
\end{align*}
\]

According to Schink (1994) homoacetogens (acetogens that use the Acetyl-CoA pathway to produce acetate) are the most versatile physiological group of anaerobic microorganisms on the planet. They can catabolise different organic compounds that are energetically more favourable than get-
H₂ consumption by anaerobic non-methanogenic mixed cultures

The main aim of this study is to investigate the ability of typical H₂ producing cultures, obtained from heat treated anaerobic digested sludge, to also consume H₂. The results are discussed based on basic thermodynamic principles and stoichiometric relations from energetics of microbial growth (McCarty 1971, 1975).

MATERIALS AND METHODS

Sludge inoculum

The inoculum comes from an anaerobic digester (AD) treating primary sludge at the domestic wastewater treatment plant, Porsgrunn, Norway. The mixed reactor is operated semi-continuously (fed 4 times per day), at 40 °C and 12 days hydraulic retention time. The inoculum was collected from the AD effluent and was first sieved at 600 μm and then heat treated at 104 °C for 12 hours to kill methanogens. The inoculum was kept at the experimental temperature (35 °C) for two days in most cases and for 40 days in one case after the heat treatment procedure, prior to the start-up of the experiments.

Batch tests

Hydrogen production (HP). Four 500 mL glass bottles where used as reactors (A1-A4) to measure the hydrogen production capacity of the mixed culture, each with 250 mL inoculum. Seeded reactors were flushed with N₂ to ensure anaerobic conditions, and 5.8 mmol glucose was added to reactors A1-A3. A blank reactor (A4) without the addition of glucose was used to measure the HP of the sludge. Reactors were constantly mixed with magnetic stirrers and kept at 35 °C in a water bath.

Hydrogen consumption (HC). Reactors A1 and A2 were kept in operation without exhausting the gas produced, nor adding new gas or organic feed, after the HP test. The rate of hydrogen consumption and organic acid production was measured. Three additional series of batch experiments, series B, C and D, each in triplicate, were run to test HC at approximately constant partial pressure of ≈0.4 atm H₂ (series B) and ≈0.6 atm H₂ (series C and D). The difference between C and D was the age of the inoculums; in C it was stored the standard 2 days after heat treatment and for 40 days in D. All B, C and D reactors were initiated the same way as A4, but with 200 mL inoculum. A mixture of H₂ and CO₂ gas was added to headspace. The reactors were connected to 1500 mL gas bags containing H₂ and CO₂. The content of the gas bags were kept approximately constant through the experiments by replacing the gas when the ratio of H₂ to CO₂ had changed by 10% or when the gas volume in the bags were reduced to 600 mL. Hydrogen consumption without an initial H₂ production nor dissolved hydrogen in the liquid phase was measured in these reactors. Table 1 summarizes the experimental design.

Experiments were carried out until no more hydrogen consumption was observed. Hydrogen consumption was...
measured periodically. Organic acids concentration was measured periodically for reactor series A, C and D, and at the start and end operation of reactors serie B. Reactors were constantly mixed with magnetic stirrers and kept at 35 °C in a water bath.

Analytical methods

Filtered samples (0.45 μm) were analyzed for volatile fatty acids (VFAs), chemical oxygen demand (COD) and glucose concentration. Gas volume (mL) and composition (% v/v) was also measured. VFAs (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caprionate) were analyzed by gas chromatography (Hewlett Packard 6890) with a flame ionisation detector and a capillary column (FFAP 30 m, inner diameter 0.250 mm, film 0.25 μm). The oven was programmed to go from 80 °C, hold for one minute, to 180 °C at a rate of 30 °C/min, and then to 230 °C at a rate of 100 °C/min. The carrier gas was helium at 24 mL/min. The injector and detector temperatures were set to 200 and 250 °C, respectively. Enzymatic kit reagents Boehringer Mannheim were used for the determination of D- and L-lactate (Cat. No. 11112821035) and formate (Cat. No. 10979732035) for reactor samples A1-A3. COD was analyzed according to US standard 5220D (APHA 1995). Glucose was analyzed using the phenol-sulphuric acid method described by Dubois et al. (1956).

Gas composition (H₂, CO₂ and CH₄) was quantified by gas chromatography (Hewlett Packard 5890 A) equipped with a thermal conductivity detector and two columns connected in parallel: Column 1, CP-Molsieve 5 A (10 m × 0.32 mm) and Column 2, CP-PoraBOND Q (50 m × 0.53 mm). The gas carrier was argon at 7 bar pressure. The oven temperature was kept constant at 40 °C.

RESULTS AND DISCUSSION

Hydrogen production (HP)

More than 98% of the glucose added to reactors A1-A3 was consumed after 19 hours. The hydrogen production yield was on average 1.4 ± 0.1 mol H₂/mol glucose and the corresponding hydrogen production rate was 44 ± 4 mmol H₂/L sludge·d. The gas composition was about 40% H₂ and 60% CO₂. Hydrogen production for the sludge without glucose addition (A4) was zero. The carbon mass balance shows that 94% of the C-glucose consumed was converted to fermentation products. The fractions of the formed products, on a C-mol basis, are: 9.2, 15.8, 10.1, 1.5, 37.0 and 26.4 percent for formate, acetate, lactate, propionate, butyrate and CO₂, respectively. Measured hydrogen yield corresponds to levels found in the literature for mixed acid fermentation (Li & Fang 2007).

Hydrogen to acetate

Hydrogen consumption occurred during the continued operation of reactors A1 and A2, where the gas produced was not removed from the head space. 7 hours after the glucose was consumed, a notable decrease in the formic acid concentration was observed followed by an increase in acetic acid (8.7 mmol C₂H₄O₂/L sludge·d) without a significant change in the amount of the head space hydrogen (Figure 1 and 2). Unintended methane production was observed after 50 hours, however it cannot be explained by hydrogenotrophic methanogenesis (Equation 5) because headspace CO₂ concentration also increased with methane at a similar rate. The observation is explained by homoacetogenesis (Equation 4) followed by aceticlastic methanogenesis (Equation 6). This explanation is supported by the observed steady

Table 1 | Experimental design

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Start pH</th>
<th>pH₂ (atm)</th>
<th>Operated (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-A4</td>
<td>8.1</td>
<td>–</td>
<td>8</td>
</tr>
<tr>
<td>A1-A2</td>
<td>6.2*</td>
<td>0.4*</td>
<td>8</td>
</tr>
<tr>
<td>B1</td>
<td>8.5</td>
<td>0.6</td>
<td>33</td>
</tr>
<tr>
<td>B2-B3</td>
<td>6.2</td>
<td>0.6</td>
<td>33</td>
</tr>
<tr>
<td>C1-C3</td>
<td>8.1</td>
<td>0.4</td>
<td>23</td>
</tr>
<tr>
<td>D1-D3</td>
<td>8.0</td>
<td>0.4</td>
<td>21</td>
</tr>
</tbody>
</table>

*Start pH for the H₂ consumption phase in reactors A1-A2.

Figure 1 | Hydrogen production and consumption batch test (A1-A2), H₂, CO₂ and CH₄ progress.
concentrations of organic acids (from 50 h of operation) when head space hydrogen consumption was established at a rate of 4.4 ± 0.5 mmol H₂/L sludge·d. These tests confirm the autotrophic metabolic capability of anaerobic sludge to consume hydrogen, first by consuming the hydrogen from formate conversion at a high rate (35 mmol H₂/L sludge·d) and then by consuming the hydrogen in head space at a lower rate (4.4 mmol H₂/L sludge·d). The lower rate in the latter case can be explained by mass transfer limitations.

\[
\begin{align*}
\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ &= \text{CH}_4 + 3\text{H}_2\text{O} \\
\Delta G &= -135.01 \text{ kJ} \\
\text{Acetate}^- + \text{H}_2\text{O} &= \text{HCO}_3^- + \text{CH}_4 \\
\Delta G &= -28.42 \text{ kJ}
\end{align*}
\]

**Hydrogen consumption test at constant H₂ pressure**

Consistent hydrogen consumption was observed in reactors B1-B3 at constant hydrogen partial pressure in the reactors’ headspace (P_H₂ ≈ 0.4 atm). Accumulated hydrogen consumption (mmol H₂/L sludge) and hydrogen consumption rate (mmol H₂/L sludge·d) are presented in Figures 3 and Figure 4, respectively. Reactors B1 and B2 consumed hydrogen until the acetic acid reached a concentration of 63.8 and 52.6 mmol/L, respectively. No change was observed in the reactor concentrations of the other measured organic acids (propionate, butyrate, Iso-butyrate, valerate, Iso-valerate and caprionate). The stop in hydrogen consumption can be explained by product (acetate) inhibition of the homoaceticogenic hydrogen consumption, suggesting complete product inhibition at about 60 mmol acetate/L. The final accumulated acetate in reactors B1 and B2 is stoichiometrically 70 ± 2% of the hydrogen consumed according to Equation 4. Other catabolic autotrophic products, not measured, such as formate (Equation 1) or methanol (Equation 2), can account for the rest of the hydrogen consumed.

Reactor B3 had the highest hydrogen consumption rate at the start, 13 mmol H₂/L sludge·d, presumably because the start acetate concentration was 2.6 and 2.0 times lower than in reactors B1 and B2, respectively (Figure 4). Reactor B3 started however to produce methane after 550 hours, while reactors B1 and B2 did not produce any methane. The methane produced in B3 before 600 hours was presumably from acetoclastic methanogenesis, because no change in the hydrogen consumption rate was observed and some extra CO₂ accumulated in the headspace, but after 600 hours a shift in the hydrogen consumption and methane production rates suggest that hydrogenotrophic methanogenic activity became significant. No adequate explanation of why hydrogen consumption stopped completely, at a final acetate concentration in reactor B3 of 7.4 mmol/L and pH of 6.2, has been found.

Typical hydrogen consumption and acetate production of series C (reactors C1, C2 and C3) and D (reactors D1, D2, D3), run with a head space P_H₂ of 0.6 atm, is presented in Figure 5. The rate of hydrogen consumption for all cases, presented in Table 2, was between 18 and 62 mmol H₂/L sludge·d, which is 3 to 10 times higher than those observed in

![Figure 2](image-url)  
**Figure 2** | Volatile fatty acids progression during hydrogen production and consumption batch test (A1-A2).

![Figure 3](image-url)  
**Figure 3** | Accumulated hydrogen consumption in mmol/L at constant P_H₂ (0.4 atm).

![Figure 4](image-url)  
**Figure 4** | Hydrogen consumption rate (mmol H₂/L sludge·d) at constant P_H₂ (0.4 atm).
aceticlastic methanogenesis mainly, rather than hydrogenotrophic methanogenesis in this case also. The observed rates of hydrogen consumption are different in the series of experiments and to a lesser extent between replicates, in the range from 4 to 62 mmol H₂/L sludge · d. The rate seems to depend on several factors, such as acetic acid concentration, hydrogen partial pressure and mass transfer phenomena. Previous studies of interspecies electron transfer for methanogenic environments (Boone 1984, 1985; Conrad et al. 1985; Thiele et al. 1988; Thiele & Zeikus 1988) conclude that short distance between hydrogen producers and hydrogen consumers increase the rate of hydrogen utilization. Conrad et al. (1985) found, for example, that ~95% of the methane produced by hydrogenotrophic methanogens was derived from the hydrogen produced by juxtapositioned acetogenic bacteria and not from the common pool of dissolved hydrogen. This indicates that most of the H₂ produced do not equilibrate with the common pool of H₂, but is rather directly consumed by adjacent methanogens. It seems likely that a similar situation will evolve in non-methanogenic hydrogen producing and consuming environments. Observed lag-phases of 60 to 100 hours in homoacetogenic hydrogen consumption in batch test were used by Ryan et al. (2008) to argue that H₂ consumption is of minor importance in bio-hydrogen schemes. Such schemes can however not be based on batch reactors to be efficient so preventing homoacetogenic hydrogen consumption is a real challenge, especially in high rate reactors with high biomass densities where close associations between hydrogen producer and consumers will develop.
The hydrogen consumption rates measured in our experiments are therefore probably much lower than the maximum possible, since the hydrogen is supplied via the common pool of dissolved hydrogen in these experiments. One exception is the early stage of experiments A1-A2, where a rapid increase in acetate concentration, equivalent to a consumption rate of 35 mmol H₂/ L sludge · d⁻¹ is observed just after depletion of the organic feed (Figure 2). After that, the hydrogen consumption was much slower (4.4 mmol H₂/ L sludge · d⁻¹) even if the head space hydrogen partial pressure was maintained. This can be explained as a transition from autotrophic hydrogen consumption of hydrogen produced by juxtapositioned cell converting formic acid to H₂ and CO₂, to consumption from the common pool of dissolved hydrogen. The hydrogen consumption rates determined are, therefore, probably mass transfer limited and higher maximum rates are possible.

Alternative methods to better determine hydrogen consumption rates by autotrophic bacteria are required to determine maximum consumption rates. The technique used by Conrad and collaborators (1985), where they partially avoided gas transfer limitation, is an option. The non-methanogenic case is, however, more challenging since the homoacetogens have more diverse metabolic capabilities and can switch between autotrophic and heterotrophic metabolism depending on the availability of organic substrates.

We further evaluate the relative impact that homoacetogenic hydrogen consumption can have on bio-H₂ fermentors by using the approach of McCarty (1971, 1975) to calculate the stoichiometry of hydrogen production (Equation 8) and consumption (Equation 9), together with the optimal growth rate of the mixotrophic Clostridium acetium reported by Braun et al. (1981) as 0.125/h and 0.04/h for heterotrophic and autotrophic metabolism, respectively. We use this information to calculate the theoretical percentage of hydrogen consumed (of the total hydrogen produced) as a response of different biomass ratio between producers and consumers, for example as two metabolic expressions of the same microorganism (Figure 6).

\[
\begin{align*}
C_6H_{12}O_6 + 1.85HCO_3^- + 0.22NH_4^+ &\rightarrow 1.63CH_3COO^- + 5.48CO_2 + 3.26H_2 + 0.22C_3H_7O_2N + 0.89H_2O \quad (8) \\
4H_2 + 1.09CO_2 + 0.91HCO_3^- + 0.06NH_4^+ &\rightarrow 0.52CH_3COO^- + 0.059C_3H_7O_2N + 5.09H_2O \quad (9)
\end{align*}
\]

Figure 6 shows that a ratio of 1:5, between hydrogen consumers and producers, could result in a 30% of H₂ loss by homoacetogenic metabolism but a complete loss of net production is observed at a ratio of 4:5. Previously Dinamarca and Bakke (2009) reported steady state hydrogen gas composition of about 4% (zero methane), confirming the almost complete loss of net production according to this theoretical analysis. A more realistic equation for hydrogen production with multiple fermentation products and hydrogen yields close to 1.5, as observed (average values reactors A1-A3; Equation 10), implies that the activity of homoacetogens is especially relevant.

\[
\begin{align*}
C_8H_{12}O_6 + 0.076NH_3^+ \\
+ 1.182H_2O &\rightarrow 0.560CH_3OH + 0.52NO_2^- + 0.21N_2 \\
+ 0.552CHO_3^- + 0.474C_2H_5O_2^- + 0.202C_3H_7O_3^- \\
+ 0.035C_5H_7O_2^- + 0.555C_2H_7O_2^- + 1.584CO_2 \\
+ 1.52H_2 + 5.679H^+ 
\end{align*}
\]

The Gibbs free energy for the hydrogen consuming reaction to acetate is markedly exergonic at standard conditions (Equation 4). Figure 7 shows the viability of this reaction under different P₂H₂ and acetate concentrations. From the figure we observe an exothermic reaction, for acetate = 64 mmol/L, when P₂H₂ is more than 0.2. This is consistent with the observations that there was no inhibition of hydrogen consumption at 17 mmol/L, while total free energy is only about –10 to –20 kJ at 64 mmol/L which may not be enough useful energy to promote growth. This can explain why hydrogen consumption stopped completely at about 60 mmol/L, as a case of product inhibition.

Implications for modelling product distribution

Several studies have been devoted to syntrophic cooperation between acetogens (hydrogen producer from VFAs
Simultaneous hydrogen production and consumption occur in non-methanogenic fermentation, since anaerobic sludge holds both metabolic capabilities, leading to low net observed hydrogen yield. H₂ consumption between 6 and 62 mmol H₂/L sludge d are measured in a series of batch tests. The large observed differences in hydrogen gas consumption rates under relatively similar conditions are not well explained, but it is probably influenced by a variety of factors, including mass transfer limitations, acetic acid concentration, history of the inoculums and hydrogen partial pressure. Acetate production accounted for a large portion of the H₂ consumed, between 24% and 70%. A significant portion must have been converted to not measured products (such as formate, methanol or ethanol) to close the mass balance. This was also the case in experiments where some methane unintentionally was produced. CO₂ production associated with the observed methane production implies that it occurred by aceticlastic methanogenesis.

A lag-phase (1 to 13 days) was observed before significant H₂ consumption occurred in all experiments. The lag-phase length was influenced by inoculum history. A longer lag-phase led to higher H₂ consumption rates once it started.

It appears that hydrogen supplied through the headspace is more slowly consumed than hydrogen produced in the reactors. This is explained by mass transfer limitations and suggests that H₂ production and consumption by juxtaposition organisms can be efficient, as suggested by the cited literature, and play a major role in such processes. Close associations between hydrogen producers and consumers can make homoacetogenesis a relevant process in bio-hydrogen production reactors. Avoiding such is an obstacle for the development of sustainable hydrogen production by dark fermentation.

The homoacetogenic hydrogen consumption is product inhibited and stops completely at product (acetate) concentration close to 60 mmol/L. This is explained by bioenergetics.

Acknowledgments

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References


