Competition for H₂ between sulfate reducers, methanogens and homoacetogens in a gas-lift reactor

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Abstract
 Reported values for growth kinetic parameters show an order in competitiveness of heterotrophic sulfate reducing bacteria > methanogens > homoacetogens for the substrate hydrogen. This order suggests that methanogens can successfully compete with consortia of heterotrophic SRB and homoacetogens when H₂/CO₂ is present as sole substrate. However, we found in experiments using gas-lift reactors inoculated with anaerobic sludge and fed with H₂/CO₂ and sulfate, that heterotrophic sulfate reduction rapidly and completely outcompeted methanogenesis, whereas a low amount of acetate was formed. Thus, in disagreement with the above competitiveness order, hydrogen is more readily consumed by homoacetogenesis than by methanogenesis, indicating that the competition is not kinetically determined. The superior settling velocity of sulfidogenic-acetogenic sludge compared to that of methanogenic sludge suggests that the former sludge is better retained, which can explain the predominance of sulfate reduction/homoacetogenesis over methanogenesis.

Keywords
Acetate; gas-lift reactor; homoacetogenesis; hydrogen; methanogenesis; sulfate reduction

Introduction
Sulfate-reducing bioreactors can be applied for desulfurization of wastewater and process water polluted with sulfate and for combined removal of heavy metals and sulfate from acid mine drainage (Ueki et al., 1988; Hammack et al., 1994). Partial oxidation of the generated sulfide by Thiobaccilli spp. under micro-aerobic conditions results in formation of reusable elemental sulfur (Janssen et al., 1997). The sulfate reduction stage requires the presence of an electron donor. When insufficient electron donors are present in the wastestream, they need to be added externally. H₂/CO₂ is a highly attractive external electron donor and carbon source for biotechnological sulfate reducing processes. However, the efficiency of the process decreases when methane and acetate formation from H₂/CO₂ occurs, according to:

\[
\text{sulfate reducing bacteria: } 4 \text{H}_2 + \text{SO}_4^{2-} + \text{H}^+ \Rightarrow \text{HS}^- + 4 \text{H}_2\text{O} \quad (1)
\]

\[
\text{methanogens: } 4 \text{H}_2 + \text{HCO}_3^- + \text{H}^+ \Rightarrow \text{CH}_4 + 3 \text{H}_2\text{O} \quad (2)
\]

\[
\text{homoacetogens: } 4\text{H}_2 + 2 \text{HCO}_3^- + \text{H}^+ \Rightarrow \text{CH}_3\text{COO}^- + 4 \text{H}_2\text{O} \quad (3)
\]

When H₂ is limiting and sulfate is in excess, sulfate reducing bacteria (SRB) compete with methanogens and homoacetogens for the available H₂. Growth kinetics, quantified by the maximum specific growth rate, substrate affinity and substrate threshold are often used to explain the outcome of bacterial competition. Reported values for these parameters reveal an order of competitiveness of heterotrophic SRB > methanogens > homoacetogens at low (limiting) H₂ concentration (Cord-Ruwisch et al., 1988; Oude-Elferink et al., 1994). When H₂/CO₂ is added as the sole substrate, heterotrophic SRB depend for an organic carbon
source on growth of homoacetogens. The competitiveness order suggests that methanogens under this condition compete successfully with heterotrophic SRB and homoacetogens. In a previous study methanogenesis did not occur in gas-lift reactors fed with \( \text{H}_2/\text{CO}_2 \) (Houten et al., 1994). However, hydrogenotrophic methanogens were possibly not present in sufficiently high numbers in the seed sludge of the reactors for development of substantial methanogenesis in the relatively short-term (40 days) experiments. Obviously in practice, reactors are in operation for long periods giving methanogenic populations ample opportunity to develop.

In this paper we report on the competitive and cooperative interaction between SRB, methanogens and homoacetogens in sulfate-reducing gas-lift reactors fed with \( \text{H}_2/\text{CO}_2 \). The role of acetate as an additional carbon source was investigated.

**Materials and methods**

**Reactors.** For continuous experiments, two glass gas-lift reactors (Figure 1) with a working volume of 5 L were used. The effluent was led into a settler, from which settled sludge was recycled to the reactor. The operating conditions for both reactors were: \( \text{pH} = 7.0, \text{T} = 30^\circ\text{C} \), hydraulic retention time (HRT) 4 h. Reactors were fed with \( \text{H}_2/\text{CO}_2 \) (80/20 v/v) and with sulfate and a macro- and micronutrient solution. Anaerobic sulfate reducing sludge was pre-cultivated on \( \text{H}_2/\text{CO}_2 \) and sulfate for a period of 3 weeks. The resulting sludge was used to seed two reactors, giving an initial TSS-concentration of 1–2 g.L\(^{-1}\). To one reactor (R\(^+\)) 200 mg.L\(^{-1}\) acetate was added as additional carbon source (besides \( \text{CO}_2 \)) for the SRB. Reactor R\(^-\) did not receive acetate. Both reactors were initially fed with only 0.67 g.L\(^{-1}\) sulfate. After approximately 30 days, in both reactors R\(^+\) and R\(^-\) the influent sulfate concentration was increased to 3.3 g.L\(^{-1}\). After the sulfate increase, 1/15 of the reactor volume (sludge + medium) was removed daily, to facilitate relatively rapid changes in biomass composition. At the end of the continuous experiments the \( \text{H}_2 \)-load was increased and the acetate addition was switched from reactor R\(^+\) to reactor R\(^-\).

**Activity assay.** The maximum specific sulfidogenic, methanogenic and acetogenic activity of the cultivated sludge on \( \text{H}_2/\text{CO}_2 \) was determined in batch activity assays. The assays were conducted in closed 120 mL vials containing 50 mL mineral medium. Mineral medium had a similar composition as the influent of the gas-lift reactors. To sulfidogenic assays 5 g.L\(^{-1}\) \( \text{Na}_2\text{SO}_4 \) was added. When desired, addition of 200 mg.L\(^{-1}\) acetate supplied heterotrophic sulfate reducing bacteria with a carbon source. Addition of 25 mM...
2-bromoethanesulfonic acid (BES) inhibited methanogenic activity in homoacetogenic assays. The headspace consisted of H₂/CO₂ (80/20 v/v) or N₂/CO₂ (80/20 v/v), with a total pressure of 1.5–1.7 bar. After addition of fresh sludge (0.03–0.1 gTSS per vial) the vials were shaken at 175 rpm and 30°C. The specific activities were calculated from the linear increase of the amount of acetate, methane and sulfide in the vials and the amount of biomass (as N-Kjeldahl).

Settling velocity. Settling velocity of sludge was determined with the homogeneous suspension sedimentation technique (Stockman and Fochtman, 1977).

Analytical methods. The following analyses were done three times per week: sulfide (methylene blue method), sulfate (HPLC), acetate (GC), CH₄, CO₂, N₂, H₂S and O₂ in biogas (GC), TSS (standard methods), N-Kjeldahl (elemental analyzer).

Results

Competition experiments in continuous gas-lift reactors

Reactors R⁺ and R⁻ were used to study the microbial competition for H₂. Reactor R⁺ received 200 mg.L⁻¹ acetate as additional C-source (besides CO₂). The reactors first were loaded with H₂/SO₄²⁻ at a ratio of 12 (mol/mol) which means that 3 times more hydrogen is supplied than needed for complete sulfate reduction (reaction 1). Thus, methanogens and homoacetogens do not compete with the SRB for the excess of hydrogen. After approximately 30 days, increasing the influent sulfate concentration resulted in a 5 times higher sulfate load in both reactors of 6.76 gSO₄²⁻.L⁻¹.day⁻¹. This lowered the ratio H₂/ SO₄²⁻ to a value of 2.5 (mol/mol) which means that the amount of hydrogen supplied to the reactor is insufficient for complete sulfate reduction. Accordingly, methanogens and homoacetogens have to compete with SRB for the available H₂. The day of sulfate load increase is defined as day 0 in Figure 2.

Figure 2 shows that the sulfide formation rate in reactor R⁺ attained a stable level of 2.5 gCOD.L⁻¹.day⁻¹ before day 0, whereas it varied from 1.5–2.5 gCOD.L⁻¹.day⁻¹ in reactor R⁻. In both reactors no sulfate was detected from day –20 to day 0. Methanogenesis prevailed in reactor R⁺ before day 0. However, the methane formation rate in reactor R⁻ remained low until day –10. Most likely, this was due to inhibition of methanogens by oxygen, present in the recycle gas due to leakage of air into the gas circuit. When the air leak was repaired, the methane production rose to the same level of 3–4 gCOD.L⁻¹.day⁻¹ as in reactor R⁺. Homoacetogenesis benefited from the inhibition of methanogens in reactor R⁻ between day –20 and day –5 resulting in a high acetate production rate.

From day 0 onwards the volumetric sulfide production fluctuated between 4 to 8 gCOD.L⁻¹.day⁻¹ in both reactors. Sulfate removal was incomplete resulting in a effluent sulfate concentration of 1.5–3 gSO₄²⁻.L⁻¹. Increasing the hydrogen load at day 28 (reactor R⁺) and day 23 (reactor R⁻) did not give a higher sulfide formation rate suggesting that hydrogen transfer did not limit sulfate reduction in the reactors. The level of the toxic unionized hydrogen sulfide was still relatively low (maximally 350 mgS.L⁻¹). Probably the low amount of biomass was limiting the volumetric sulfide formation rate. The increase in the sulfate load at day 0 resulted in an immediate, sharp decrease of the methane formation in both reactors. On day 5, 80% less methane was formed compared to day 0, whereas on day 20 the decrease amounted to 98%. This result indicates that SRB outcompete methanogens for hydrogen when sulfate is not limiting, irrespectively whether or not acetate is added. A net decrease of acetate in reactor R⁺ indicated consumption of this added C-source after the sulfate load increase. However, acetate was produced within 3 days after terminating the acetate supply on day 32. By contrast, reactor R⁻ produced acetate during the whole experiment. Addition of acetate from day 27 onwards decreased...
the net production, but no net consumption was observed. In both reactors the acetate concentration never dropped below 50 mg.L\(^{-1}\).

**Activity assays**

Table 1 shows results from activity assays conducted with freshly sampled sludge from the reactors. In both reactors, the specific sulfidogenic activity increased about 2–3 times after the sulfate load increase at day 0. Concomitantly, the specific methanogenic activity nearly completely disappeared within 3–4 weeks after the sulfate load increase. The specific homoacetogenic activity of sludge from reactor R\(^-\) was about 6 times higher than that of sludge from reactor R\(^+\), both before and after the sulfate load increase.

**Table 1** Maximum specific sulfidogenic, methanogenic and acetogenic activity on H\(_2)/\text{CO}_2\) of sludge taken from reactors R\(^+\) and R\(^-\) at day –6 and 28 and day –4 and 23, respectively. Before day 0, sludge was cultivated under sulfate limitation, after day 0 sludge was cultivated at an excess sulfate. H\(_2)/\text{CO}_2\) was added in excess, when desired acetate and sulfate were added to concentrations of 200 mg.L\(^{-1}\) and 3.4 g.L\(^{-1}\), respectively. Acetate was not degraded when added as the sole electron donor (data not shown).

<table>
<thead>
<tr>
<th>Type of activity</th>
<th>Maximum specific activities of reactor sludge (day of assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gCOD.gbiomass(^{-1}).day(^{-1})</td>
</tr>
<tr>
<td></td>
<td>R(^+) (–6)</td>
</tr>
<tr>
<td>sulfidogenic on H(_2)/\text{CO}_2), acetate and sulfate</td>
<td>2.3 (0.7)</td>
</tr>
<tr>
<td>sulfidogenic on H(_2)/\text{CO}_2) and sulfate</td>
<td>1.6 (0.3)</td>
</tr>
<tr>
<td>methanogenic on H(_2)/\text{CO}_2)</td>
<td>6.9 (0.8)</td>
</tr>
<tr>
<td>homoacetogenic on H(_2)/\text{CO}_2) (with 25 mM BES)</td>
<td>0.5 (0.0)</td>
</tr>
</tbody>
</table>
Settling velocity
Settling velocity was determined of sludge taken from reactor R– at days –4 (mainly methanogenic sludge) and day 36 (sulfidogenic–homoacetogenic sludge). Figure 3 shows that 50% of the mainly methanogenic sludge has a settling velocity less than 2 m.h–1, whereas for the sulfidogenic-homoacetogenic sludge this value amounts to 12 m.h–1.

Discussion
The results show that SRB rapidly outcompete methanogens for H₂ in gas-lift reactors when sulfate is in excess, irrespective of the addition of 200 mg.L⁻¹ acetate. Activity assays revealed that hardly any active hydrogenotrophic methanogens were left in the sludge when it had been cultivated at hydrogen-limiting conditions and at an excess sulfate for 3–4 weeks. To keep a low level of methanogenesis from H₂/CO₂, H₂ but not sulfate must be maintained as the growth limiting factor for SRB. When CO₂ is the only added C-source, the sludge produces sufficient acetate for growth of heterotrophic SRB, making external addition of acetate redundant. To rapidly remove methanogens applying a low SRT, e.g. by daily withdrawal of part of the sludge, seems a proper method.

Addition of 200 mg.L⁻¹ acetate, however, did affect the competition between SRB and homoacetogens for H₂. The reactor that received CO₂ as sole carbon source (R–) showed a net production of acetate from H₂/CO₂. By contrast, the reactor fed with CO₂ and acetate as carbon sources (R⁺) net consumed acetate. The actual values of net acetate consumption and production were only low resulting in a low but very similar acetate concentration (50–100 mg.L⁻¹) in both reactors.

Comparing the results with the kinetic based competitiveness order of heterotrophic SRB > methanogens > homoacetogens it appears that the competition for H₂ in the gas-lift reactor is not entirely kinetically determined. Microbiological analysis showed that the dominant sulfate reducers from both reactors were heterotrophic (data not shown). This indicates that co-cultures of heterotrophic SRB + homoacetogens in the acetate-producing reactor R– outcompete the methanogens for H₂. On the other hand, results from reactor R– before day 0 showed that methanogens outcompete homoacetogens when both H₂ and sulfate are limiting. Thus, methanogens appear to outcompete homoacetogens for H₂ when sulfate is limiting but not when sulfate is in excess. This difference cannot solely be explained from growth kinetics, as from this it must be concluded that methanogens outcompete homoacetogens irrespective of the activity of SRB. Apparently, some factor which is not of a kinetic nature gives consortia of homoacetogens and SRB a competitive advantage over methanogens.

Figure 3  Settling velocity of sludge taken from reactor R– at days –4 (○) when methanogenesis was predominant (see also Figure 2) and day 36 (○) when sulfate reduction was predominant with some homoacetogenesis.
This competitive advantage might be a higher residence time of sludge that contains consortia of homoacetogens and heterotrophic SRB compared to that of sludge mainly containing methanogens. The much higher settling velocity of sulfide + acetate producing sludge compared to that of methanogenic sludge (Figure 3) probably relates to the larger particle sizes and more densely packed structure of the former sludge, as observed microscopically. Thus, consortia of heterotrophic SRB and homoacetogens appear to adhere or aggregate better than methanogens. The difference in settling velocities may affect the competition because the used method for biomass retention selects for well settleable sludge. Thus, the retention time of methanogens in the reactors may be much lower than that of co-cultures of homoacetogens and heterotrophic SRB resulting in selective wash-out of methanogens.

The results show that acetate addition to sulfate-reducing bioreactors does neither increase the selectivity nor the rate of sulfate reduction with H₂/CO₂. The addition of acetate theoretically slightly increases the amount of H₂ available for sulfate reduction, but this will not outweigh the costs of the added acetate in large-scale applications. Although addition of acetate leads to an increased specific sulfidogenic activity of sludge this does not result in increased volumetric conversion rates because such sludge is poorly retained in the reactor. Thus, the process performance does not benefit from the addition of acetate.

Conclusions
1. Applying hydrogen limitation is a practical method to suppress methanogenesis in sulfate reducing gas-lift reactors fed with hydrogen.
2. Homoacetogens are not completely outcompeted by SRB for hydrogen when no acetate is supplied. Presumably, heterotrophic SRB then depend on the homoacetogens for supply of acetate as their carbon source when no carbon source is added.
3. The competition between methanogens and SRB seems not to be determined by kinetics. Possibly, the better retention of sulfidogenic biomass in the reactor compared to methanogenic biomass plays an important role in the competition.

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