Sulfur Reduction at Hyperthermoacidophilic Conditions with Mesophilic Anaerobic Sludge as the Inoculum

Adrian Hidalgo-Ulloa, Irene Sánchez-Andrea, Cees Buisman, and Jan Weijma

ABSTRACT: Sulfur reduction at hyperthermoacidophilic conditions represents a promising opportunity for metal sulfide precipitation from hot acidic metallurgical streams, avoiding costly cooling down. The suitability of mesophilic anaerobic sludges as the inoculum for sulfur-reducing bioreactors operated at high temperature and low pH was explored. We examined sludges from full-scale anaerobic reactors for sulfur-reducing activity at pH 2.0–3.5 and 70 or 80 °C, with H2 as an electron donor. At pH 3.5 in batch experiments, sulfitogenesis started within 4 days, reaching up to 100–200 mg L−1 of dissolved sulfide produced after 19–24 days, depending on the origin of the sludge. Sulfitogenesis resumed after removing H2S by flushing with nitrogen gas, indicating that sulfide was limiting the conversion. The best performing sludge was used to inoculate a 4 L gas-lift reactor fed with H2 as the electron donor, CO2 as the carbon source, and elemental sulfur as the electron acceptor. The reactor was operated in semibatch mode at a pH 3.5 and 80 °C, and stable sulfide production rates of 60–80 mg L−1 day−1 were achieved for a period of 24 days, without formation of methane or acetate. Our results reveal the potential of mesophilic anaerobic sludges as seed material for sulfur-reducing bioprocesses operated at hyperthermoacidophilic conditions. The process needs further optimization of the volumetric sulfide production rate to gain relevance for practice.

1. INTRODUCTION

Hydrogen sulfide (H2S) reacts with divalent chalcophile metal ions resulting in precipitation of metal sulfides (eq 1), which can be used to remove dissolved metal ions such as Cu2+ and Zn2+ from aqueous streams. Metal sulfide precipitation has several advantages compared to the still commonly used hydroxide precipitation, such as lower residual metal concentrations and generation of denser and thus less voluminous sludge with better dewatering characteristics. Moreover, the use of sulfide allows selective precipitation when more than one metal is present.

\[
\text{H}_2\text{S}_{(aq)} + \text{Me}^{2+}_{(aq)} \rightarrow \text{MeS}_{(s)} + 2\text{H}^+_{(aq)}
\]

Sulfitogenic processes mediated by microorganisms that target sulfite as the terminal electron acceptor are already applied on an industrial scale. Sulfate-reducing bacteria (SRB) use a wide variety of electron donors, achieving conversion rates in bioreactors of 10–30 g SO4\textsuperscript{2−}/L·day\textsuperscript{−1} at circumneutral pH1−10 and temperatures from 30–65 °C. However, metallurgical streams are often acidic (2–4) and hot (65–80 °C),11 which appears incompatible with existing sulfate reduction technologies. Moreover, sulfate-reducing microorganisms that thrive at (hyper)thermophilic conditions have not been identified to the best of our knowledge, even though mesophilic—thermophilic and thermophilic—neutrophilic sulfate reducers have been isolated.

Elemental sulfur has also been proposed as a sulfur source for biological sulfitogenesis.15 Under mesophilic conditions and at circumneutral pH, sulfur reduction rates of 0.5–3.0 g S/L·day\textsuperscript{−1} can be achieved with electron donors such as acetate, glucose, or mixed organics in bioreactors of various design.13,16–18 Although, hyperthermoacidophilic sulfur reducers are commonly isolated from natural environments where high temperature and acidic conditions are predominant (e.g., volcanic areas and thermal waters),12,19,20 it has not been investigated for its potential for high-rate sulfide generation. Sulfur reduction at hyperthermoacidophilic conditions represents an interesting microbiological opportunity, as it would allow an all-in-one process for base metal (e.g., copper) recovery from hot and acidic metallurgical streams like those generated during hydrometallurgical gold processing.15 Furthermore, operating sulfur reduction processes at low pH offers the potential for selective metal recovery from wastewater and process streams laden with multiple metals, as metal sulfide solubility is greatly affected by pH.22 This is an appealing
option for the metallurgical industry as it results in more pure metal solid products with a higher potential for recycling.\textsuperscript{19} For hydrometallurgical applications of sulfate or sulfur reduction, addition of an electron donor to the process is needed as the streams lack organic compounds.\textsuperscript{20,23,24} Sulfate requires eight electrons for reduction to sulfide (eq 2), resulting in the consumption of fairly large amounts of organic bulk chemicals or inorganic H\textsubscript{2}. This constitutes a large share of the operational costs, which limits the industrial application of sulfate reduction.\textsuperscript{25}

\[
\text{SO}_4^{2-} + 10\text{H}^+ + 8\text{e}^- \rightarrow \text{H}_2\text{S} + 4\text{H}_2\text{O}
\]

(2)

By metabolizing elemental sulfur, a stoichiometric fourfold decrease in the electron donor consumption is achieved compared the sulfate reduction (eq 3).\textsuperscript{25,26} Thus, reduction of elemental sulfur under low pH and high temperature conditions could potentially improve the economy and widen the scope of application of biological sulfide generation technologies.

\[
\text{S}^0 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{S}
\]

(3)

However, the start-up of industrial sulfur-reducing bioreactors would require large amounts of microbial inoculation material. Anaerobic granulation reactors for treatment of industrial wastewaters are widespread, for example, in the paper and food processing industries,\textsuperscript{27} and therefore granular sludge from such reactors is widely available. Granular sludge from such reactors has a high microbial diversity and has been used previously as an inoculum for successful start-up of methanogenic and sulfate-reducing laboratory-scale bioreactors at (hyper)thermophilic conditions.\textsuperscript{14,28,29} We hypothesized that anaerobic sludges might also serve as a suitable inoculation material for sulfur reduction at hyperthermophilic conditions in bioreactors at the laboratory, pilot, and full scale.

In this study, we screened three granular sludges originating from industrial bioreactors treating wastewater, for assessing elemental sulfur-reducing activity at pH 4.0 and high temperature (70–80 °C). Two of the granular sludges were further examined at pH 2.0–3.5. The most active sludge was used to inoculate a 4 L gas-lift bioreactor, which was operated in semibatch mode for 97 days at pH 3.5 and 80 °C.

2. MATERIALS AND METHODS

2.1. Origin of Sludges and Precultivation. The sludges of three industrial anaerobic full-scale reactors operated at mesophilic temperature conditions and circumneutral pH were used as the inoculum in our experiments. These sludges are referred to as Emmen, Eerbeek, and Lenzing. The Emmen sludge was granular and originated from a sulfate-reducing bioreactor with low methane production at the company Emmetc, located in Emmen, the Netherlands. This bioreactor is of the upflow anaerobic sludge blanket (UASB) type and is fed with ethanol as the sole electron donor and carbon source.\textsuperscript{30} Eerbeek granular sludge originated from a UASB bioreactor at the company Industriewater Eerbeek, located in Eerbeek, the Netherlands, treating waste water from paper mills. Sludge from this reactor has been analyzed previously for its microbial composition.\textsuperscript{31,32} Lenzing granular sludge came from a bioreactor from the company Lenzing, located in Lenzing, Austria, which is fed with a viscose factory wastewater containing complex COD and sulfate.\textsuperscript{33} The sludges were stored at 4 °C until use.

The medium composition for batch experiments was (in mM): CaCl\textsubscript{2}-2H\textsubscript{2}O, 0.75; KH\textsubscript{2}PO\textsubscript{4}, 3.01; (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2.8; NaCl, 5.13; MgSO\textsubscript{4}-7H\textsubscript{2}O, 0.49; and 0.1 g L\textsuperscript{-1} BBL yeast extract. A trace element solution (1 mL per liter of culture medium) was also added which contained (in mM): ZnSO\textsubscript{4}·7H\textsubscript{2}O, 3.48; CuSO\textsubscript{4}·5H\textsubscript{2}O, 8.01; MnSO\textsubscript{4}·H\textsubscript{2}O, 5.92; NaMoO\textsubscript{4}·2H\textsubscript{2}O, 2.28; CoCl\textsubscript{2}·6H\textsubscript{2}O, 2.47; SeO\textsubscript{2}·5H\textsubscript{2}O; H\textsubscript{2}BO\textsubscript{4}·1; and NiCl\textsubscript{2}·6H\textsubscript{2}O, 4.21. The pH of the medium was adjusted to 4.0 ± 0.1 using sulfuric acid. Prior to inoculation, bottles with the medium were flushed with nitrogen gas (O\textsubscript{2} < 0.5 ppmv, Linde Gas Benelux B.V., the Netherlands) during 20 min per liter of medium with a gas flow rate of 25 mL s\textsuperscript{-1}. The medium was then distributed over 250 mL batch bottles, with 150 mL of medium per bottle and 190 mg of elemental sulfur (1.3 g L\textsuperscript{-1}). Five grams of wet sludge were added for inoculation, the pH was corrected to the intended value for study, and the bottles were inserted in an anaerobic hood and left overnight. The subsequent day the electron donor was added and the bottles were closed with butyl stoppers. The following electron donors were tested independently: methanol (5 mM), ethanol (5 mM), acetate (5 mM), formate (5 mM), glucose (5 mM), and H\textsubscript{2} (80 vol % in headspace). After the addition of the electron donor, the headspace of the bottles was flushed with N\textsubscript{2} and CO\textsubscript{2} in a ratio 80:20 (vol %), except for the bottles containing H\textsubscript{2} as the electron donor, which replaced nitrogen. Control experiments were done with bottles following the aforementioned procedure with the corresponding modifications: without electron donor, without elemental sulfur, and with formaldehyde addition (500 mg L\textsuperscript{-1}) for microbial inhibition. The bottles were incubated at 70 and 80 °C in an orbital incubated shaker at 160 rpm. From the bottles with sulfide production, suspended solids were recovered from the whole bottle after 25 days by centrifuging at 3620 g for 30 min. The pellet was transferred to 250 mL bottles with 125 mL fresh medium with pH 3.5 ± 0.1, and the bottles were inoculated with the same electron donor and at the same temperature. For each subsequent transfer the pH was lowered by 0.5 units, down to pH 2.0 ± 0.1. Batch experiments were performed in duplicate.

Microbiological produced sulfur has shown to promote microbial growth,\textsuperscript{21} for elemental sulfur used in experiments originated from a full-scale sulfide oxidizing bioreactor treating biogas of a food waste digester. Soluble impurities in sulfur were removed through multiple 2:1 extractions with demineralized water. The sulfur–water mixture was agitated for 2 min and sulfur was recovered by centrifugation at 3620 g for 10 min. The resulting pellet was resuspended with demineralized water and the procedure repeated until the suspension reached a conductivity ≤20 μS cm\textsuperscript{-1} after which the suspension was dried for 12 h at 105 °C. The resulting solid was ground and sieved to a particle size ≤200 μm.

2.2. Electron Donor Mass Balance. To determine the electron donor conversion, two batch bottles were inoculated using hydrogen as the electron donor following the aforementioned procedure at a pH of 3.5. Once prepared and sealed, the headspace of the bottles was replaced with a combination of N\textsubscript{2} and CO\textsubscript{2} 80:20 (vol %). Hydrogen gas (5 mL) was injected into the bottles and then incubated at 80 °C. Daily liquid and gas samples were taken at normal temperature for sulfide analysis and hydrogen composition analysis,
respectively. Internal bottle pressure was determined for gas mass calculations. After all the initial hydrogen was consumed, an additional 5 mL of hydrogen was injected into the bottles and the analysis procedure repeated.

2.3. Gas-Lift Reactor Inoculation and Operation. A glass gas-lift reactor with a working volume of 4 L (Figure 1) was used for a larger scale experiment on hyperthermoacidophilic sulfur reduction with H2 as the electron donor and CO2 as the carbon source. The temperature of the reactor was maintained at 80 °C with a water jacket heated with a Julabo F32-HL heater (Julabo, Seelbach, Germany), and the pH in the reactor was controlled at 3.5 using 1 M sulfuric acid. The reactor was inoculated with 15 g·L⁻¹ of wet Emmen sludge, and elemental sulfur (41 grams, dry weight) was suspended in 50 mL anaerobic demineralized water and supplied to the reactor through a feed port. The reactor was operated in a semibatch mode by replacing 1 L of reactor solution by fresh medium every 8 days to ensure sufficient supply of nutrients. Additional sulfur was supplied regularly, aiming to maintain a sulfur concentration of at least 10 g·L⁻¹. Sulfur has a tendency to accumulate as a foam layer above the water–gas interface on the top of the reactor. The accumulated sulfur was regularly manually pushed back to the reactor solution with a piece of tubing. Hydrogen and carbon dioxide were supplied as the electron donor and as the carbon source, using two mass flow controllers (type El-FLOW, model FG-201CV-RAD-22-V-DA, Bronkhorst) (Veenendaal, the Netherlands) at a rate of 1.5 and 0.5 L·h⁻¹ (volume at standard conditions), respectively. Enhanced gas transfer and mixing was achieved by recirculating the gas at a rate between 50–80 L·h⁻¹ using a vacuum pump KNF Neuberger type N820.3FT.18 (Freiburg, Germany). Gas and liquid flow tubing and connections were made from PTFE (Schott A.G, Mainz, Germany and Serto A.G, Fuldabrück, Germany). The redox potential and pH were continuously measured with a Prosense QP181X-TJ/Ag/AgCl/12 × 250 mm glass electrode.

During the first 67 days of operation, a H2S absorption solution was installed in the recycle gas line. However, this led to inefficient scrubbing and an underestimation of sulfide production. Therefore, on day 67 the configuration was changed as shown in Figure 1, with absorption of H2S from the effluent gas in a 2 M NaOH solution, after which sulfide production could be monitored effectively.

2.4. Chemical Analyses. Samples for sulfide analysis were diluted in a solution containing 1 mM NaOH and 0.1 mM zinc acetate for sulfide fixation. Dissolved sulfide was analyzed using a Hach Lange kit LCK-653 (Hach, Germany) which is based on the reaction of sulfide with dimethyl-p-phenylenediamine and ferric chloride. The absorption was measured in a spectrophotometer DR3900 (Hach, Germany). Sulfate was measured by ion chromatography on a Dionex ICS 2100 equipped with an IonPac AS19 column (4 × 2550 mm) (Dionex, USA) eluted at 30 °C with potassium hydroxide (5 mM, 1 mL·min⁻¹).

The headspace composition of the batch bottles was analyzed with gas chromatography (Shimadzu GC-2010 series) equipped with two columns in parallel, a Porabond Q (50 m × 0.53 mm) and Molsieve 5A (25 m × 0.53 mm) for nitrogen, oxygen, and methane. Hydrogen analysis was carried out with a Hewlett Packard GC-5890 series equipped with a CP-Molsieve 5A column (30 m × 0.32 mm). Acetate, propionate, butyrate, methanol, and ethanol were measured with an HPLC system Ultimate 3000 (Thermo Fisher, USA) equipped with an Alltech OA-1000 column (300 mm × 6.5 mm) (Hichrom, UK) eluted at 60 °C with sulfuric acid (1.25 mM, 0.6 mL·min⁻¹).

2.5. Statistical Analysis. The sulfide data here presented was analyzed with a chi-square test for normality followed by an analysis of variance (ANOVA) for the establishment of significant differences. The ANOVA single factor was calculated using an α-value of 0.05 for the data clustered in...
three groups: (1) the sludge origin and temperature; (2) the sludge origin and pH; and (3) pH and temperature. The differences in data sets discussed here lie within the framework of this analysis.

3. RESULTS AND DISCUSSION

3.1. Screening of Anaerobic Sludges for Sulfur-Reducing Activity at Initial pH 4.0 and 70 and 80 °C

Anaerobic sludges from three full-scale bioreactors, at mesophilic conditions, treating industrial wastewaters were screened for elemental sulfur-reducing activity at pH 4.0 and temperatures of 70 and 80 °C. Less than 2 mg L⁻¹ sulfide was found after 60 days incubation with methanol, ethanol, acetate, formate, and glucose with the three sludges, showing that these organic compounds did not support sulfur reduction in our experimental setup. Microbial activity controls with the three sludges at 30 °C yielded sulfide using methanol, ethanol, and hydrogen (Supporting Information, Table S1). However, mesophilic conditions were not the focus of this study and were therefore not further investigated. In bottles with Emmen, Eerbeek, and Lenzing sludges incubated with H₂ (80 vol %) as the electron donor and CO₂ (20 vol %) as the carbon source, >50 mg L⁻¹ sulfide was produced within 18 days (data not shown). Chemical controls without addition of sulfide but with added elemental sulfur, biological controls with sludge but without added elemental sulfur, and biological controls with added elemental sulfur and inactivated sludge (with 500 mg L⁻¹ of formaldehyde) all showed <1 mg L⁻¹ sulfide accumulation, after 60 days of incubation.

The biological control experiment without sulfur confirmed that sulfide was not formed through the reduction of sulfate, which was present in the medium at a concentration of around 450 mg L⁻¹ due to addition of sulfuric acid for pH adjustment. The sulfate concentration remained constant (±20 mg L⁻¹) during incubation, providing further evidence that sulfate reduction did not account for sulfidogenesis. Biological disproportionation of sulfur to sulfide and sulfate could also be excluded as the sulfide and sulfate concentrations did not increase without H₂ but with sulfur and sludge (results not shown). Furthermore, in the biological controls where formaldehyde (500 mg L⁻¹) for microbial inactivation was added, sulfide remained below 1 mg L⁻¹ for 80 days, revealing the biological nature of sulfidogenesis. Additionally, in the incubations generating up to 100 mg of dissolved and gaseous sulfide per L of medium (calculated using eq.4), an increase of 100 mg L⁻¹ of sulfate would be expected based on biological sulfur disproportionation. For the same reason, chemical sulfur disproportionation (also called sulfur hydrolysis), reported to occur at temperatures above 80 °C and elevated pressures, could therefore be excluded. Formate, acetate, propionate, and butyrate concentrations remained below 5 mg L⁻¹ in incubations with H₂/CO₂.

Thus, sulfide formation in the bottles with Emmen, Lenzing, and Eerbeek sludge could be unambiguously attributed to sulfur reduction with H₂ as the electron donor at hyperthermophilic conditions. The substrates, besides sulfate, on which these sludges were cultivated in their respective full-scale reactors, were ethanol (Emmen), paper mill wastewater (Eerbeek), and viscose factory wastewater (Lenzing). The finding that microorganisms from anaerobic sludges grown on a wide range of substrates show sulfur-reducing activity at hyperthermophilic conditions suggests that this characteristic is fairly widespread among such sludges.

3.2. Sulfur-Reducing Activity of Pre-Exposed Emmen and Eerbeek Sludge at Initial pH 3.5 and 70 and 80 °C

The Emmen and Eerbeek sludges yielded the highest sulfide production rate and maximum sulfide concentration in the screening at pH 4.0, and therefore these were used for further study. The Emmen and Eerbeek sludge from the pH 4.0 incubations with H₂/CO₂ at 70 and 80 °C was transferred to fresh medium with pH 3.5 and then incubated at the same temperature. Control incubations at pH 3.5 yielded less than 1 mg L⁻¹ of sulfide.

Sulfide production at pH 3.5 followed a typical concave pattern in all incubations, leveling off with increasing sulfide concentration (Figure 2A,B). However, the obtained sulfide production rate and the maximum sulfide concentration were clearly different for the two sludges. For the Emmen sludge incubations, 100 mg L⁻¹ of sulfide was generated within 6 and 10 days at 70 and 80 °C, respectively. At both 70 and 80 °C, the Eerbeek sludge incubations reached 100 mg L⁻¹ of sulfide. Also, the maximum sulfide concentration reached in the incubations differed for both sludges. While Emmen sludges reached 150–200 mg L⁻¹ after 17–24 days at both temperatures, Eerbeek sludge reached 100–120 mg L⁻¹ at both temperatures after 21–31 days.

Temperature did not affect sulfidogenesis with the Eerbeek sludge, while with Emmen sludge the sulfide formation rate was approximately 1/3 less at 70 °C compared to that at 80
concentrations of 100 mg·L⁻¹. Control incubations yielded less activity at 70 °C. This procedure was consequently transferred to medium with pH 3.0, and incubated at the purpose, sludge from the incubations at pH 3.5 was recovered, reduction at pH below 3.5 was further explored. To this end, sludge from the incubations at pH 3.5 was recovered, and incubated at the corresponding temperature. This procedure was consequently repeated to pH 2.5 and 2.0. Control incubations yielded less than 1 mg·L⁻¹ of sulfdide once again.

At pH 3.0 and lower, sulfdogenesis still proceeded, although at lower rates than at pH 3.5 (Figure 3), for both sludges and at both temperatures. The Emmen sludge had a 5.8-fold reduction in the sulfdide producing rates at pH 2.0–3.0 relative to that at pH 3.5. Sulfdogenesis rates of the Eerbeek sludge had a nine and threefold decrease at 80 and 70 °C, respectively, compared to the ones at pH 3.5. Also, the maximum sulfdide concentrations were much lower, but these concentrations were still inhibitory, as revealed by the resumption of sulfdogenesis after flushing. In addition to the differences between the results obtained at pH 3.5 and pH 2.0/2.5/3.0, there were still clear differences in the sulfdogenesis rate and maximum sulfdide concentration for the two sludges in the pH range 2.0–3.0. For the incubations with the Emmen sludge, the maximum sulfdide concentrations were 40–60 mg·L⁻¹ at pH 2.0–3.0 and 80 °C, while at 70 °C the maximum sulfdide concentrations were 30–35 mg·L⁻¹. Incubations with the Eerbeek sludge reached sulfdide levels of around 30 mg·L⁻¹ at pH 2.0–3.0 and 70 °C, while at 80 °C the maximum level was 10–14 mg·L⁻¹.

Acidophilic organisms reportedly have an internal pH between 4.6 and 7. The results reveal that both sludges have sulfd-reducing activity at 70–80 °C and pH 3.5.

3.3. Inhibition by H₂S at pH 3.5. Both Emmen and Eerbeek sludges showed a progressive decrease in the sulfdide production until the sulfdide concentration reached a maximum (Figure 2A,B). To assess if sulphate toxicity was the cause of the inhibition, H₂S was removed by flushing the headspace of the bottles with H₂/CO₂ (80:20 vol %) on day 24 for Emmen sludge and day 31 for Eerbeek sludge. Sulfide production resumed immediately after, indicating that sulfide at a concentration of 150–200 mg·L⁻¹ (Emmen) and 100–120 mg·L⁻¹ (Eerbeek) was indeed inhibitory. At pH 3.5 and lower, >99.9% of sulfide is present as the undissociated H₂S species. It has been suggested that the H₂S species is the most toxic for microbial life as this uncharged molecule presumably easily permeates the lipid cell membrane. The inhibitory H₂S concentrations of 100–200 mg·L⁻¹ found here for the sulfd-reducing microorganisms in the sludges lie within the range found for other anaerobic microorganisms. Also, in the period after flushing, a progressive decrease of sulfdogenesis was found with both sludges. However, with the Emmen sludge, sulfide accumulated to a higher concentration of 200–240 mg·L⁻¹ than the maximum level found previously. Presumably, the microbial community was able to adapt to a certain extent to sulfide, or the slight increase of the pH from 3.4 to 3.5–3.6 during the experiment (Supporting Information, Figure S1) favored the activity of the microorganisms. After flushing, the Eerbeek sludge reached a higher sulfide concentration only at 70 °C, with a pH increase from 3.5 to 3.7 from the start until the end of the experiment. Inhibition by hydrogen depletion was discarded because >33 mM hydrogen remained in the headspace of the bottles at the end of each cycle.

3.4. Sulfur-Reducing Activity of Emmen and Eerbeek Sludges at Initial pH 2.0–3.0 and 70 and 80 °C. The potential of the sludges for hyperthermoacidophilic sulfur reduction at pH below 3.5 was further explored. To this purpose, sludge from the incubations at pH 3.5 was recovered, transferred to medium with pH 3.0, and incubated at the corresponding temperature. This procedure was consequently repeated to pH 2.5 and 2.0. Control incubations yielded less than 1 mg·L⁻¹ of sulfide once again.

At pH 3.0 and lower, sulfdogenesis still proceeded, although at lower rates than at pH 3.5 (Figure 3), for both sludges and at both temperatures. The Emmen sludge had a 5.8-fold
The gas-lift reactor was operated at 80 °C and pH 3.5, conditions at which relatively high sulfide production and production rates were observed with the Emmen sludge in the batch experiments. During the first 67 days of the experiment, sulfide production was monitored by regular analysis of the sulfide accumulation in the scrubber solution installed in the recycle gas line. This gave an underestimate of the sulfide production, as the scrubbing efficiency rapidly declined due to CO₂ absorption (data not shown). After day 67, the scrubber solution was placed in the outlet gas line (as shown in Figure 1) after which H₂S scrubbing was effective, indicated by the absence of sulfide in the effluent gas. Sulfide production in the gas-lift reactor was measured within 1 day after start-up. From day 0 to 67, H₂S was absorbed from the large recycle gas, resulting in increased sulfide concentrations in the reactor of around 5 mg L⁻¹. In order not to limit sulfur reduction by substrate limitation, the reactor was fed with an excess of H₂/CO₂. Due to stripping of H₂S from the reactor medium low at 0.68–1.53 mg L⁻¹. After day 67, sulfide was no longer absorbed from the recycle gas, resulting in increased sulfide concentrations in the reactor of around 5 mg L⁻¹. In order not to limit sulfur reduction by substrate limitation, the reactor was fed with an excess of H₂/CO₂. Due to stripping of H₂S from the reactor medium, this also kept the sulfide concentration far below the inhibitory level of ≥200 mg L⁻¹ observed in the batch experiments. The oxidation–reduction potential (ORP) of the reactor solution after day 67 normally varied between −153 and −185 mV (Figure 4). The granules of the inoculum gradually disintegrated during the experiment. Although the results clearly reveal that stable sulfur reduction is possible under the hyperthermoacidophilic conditions tested, the volumetric rate was still fairly low compared to other microbial sulfidoxygen processes at milder conditions. For sulfur and sulfate reduction processes at moderate conditions (pH 5–7, temperature 30 °C), volumetric sulfide production rates up to 1.3 g L⁻¹ d⁻¹ (gas-lift)⁴⁻¹ and 10 g L⁻¹ d⁻¹ (CSRT)⁴⁻¹ have been reported. Thus, to become relevant for application, the sulfide production rate under hyperthermoacidophilic conditions (60–80 mg L⁻¹ d⁻¹) needs to be increased by one to two orders of magnitude. Hydrogen transfer can be ruled out as the limiting factor as this will not occur in gas-lift reactors up to conversion rates of at least 10 g L⁻¹ d⁻¹.⁴¹

Because sulfur is a solid with a low solubility of 122 μg S L⁻¹ at 80 °C,²⁵ substrate bioavailability or the rate of dissolution may also represent a limitation to the process. Strong indications exist that dissolved polysulfides represent the actual terminal electron acceptors in sulfur-reducing processes at mesophilic/neutrophilic conditions.¹³,¹⁶ However, under acidophilic conditions polysulfides are unstable and split in H₂S and elemental sulfur, resulting in extremely low polysulfide concentrations in the order of 10⁻¹¹ mole L⁻¹ at pH 3.0, 1 mM H₂S and an excess of elemental sulfur.⁴³–⁴⁵ It has been suggested that nanosized sulfur particles formed from this reaction represent the terminal electron acceptor. However, also other mechanisms for sulfur bioavailability have been proposed, yet it is not elucidated which mechanism is prevailing.⁴⁷ In any case, the mechanism of sulfur bioavailability in the reactor may have constituted a limitation for the process rate. For instance, diffusion of sulfur nanoparticles could also represent a rate-limiting step.

### 3.6. No Methanogenesis and Acetogenesis during Sulfur Reduction at Hyperthermophilic Conditions

Methane was not detected (<1%) in the head space of the batch incubations at pH 2.0–4.0, and also in the reactor methane was not detected in the outlet gas. In additional batch experiments with sludge and H₂/CO₂, but no added elemental sulfur, also no methane was found. This indicated that methanogens present in the inoculum sludge were inhibited under hyperthermoacidophilic conditions. Although the methanogenic sludge from mesophilic origin has been used successfully to inoculate methanogenic reactors operated at (hyper)thermophilic conditions,²⁸,²⁹ the pH of the medium in these reactors was circumneutral. Also, no hydrogenotrophic methanogens have been found in mixed or pure cultures to date that can grow under the hyperthermoacidophilic conditions applied in our experiments. Moreover, no acetate, propionate, or butyrate was detected (<1 mg L⁻¹) in the incubations and the reactor. Thus, sulfur reduction at thermoacidophilic conditions with H₂ in the reactor took place with little or no formation of side products, suggesting that a high selectivity is possible with the process.

Another potential metabolism which could have occurred at the operating conditions could have been acetogenesis. Acetogenic microorganisms can convert H₂ and CO₂ via the acetyl-coA pathway mostly generating acetate. Nonetheless, no acetate (nor propionate or butyrate) was detected in the incubations. With over 100 acetogenic species isolated to date, acetogens present high taxonomic and generic diversity.³⁸ Microorganisms able to perform acetogenesis at high temperature are well known such as Clostridium thermoacetium or...
Moorella thermoacetica. However, no descriptions under low pH or hyperthermoacidophile microorganisms have been reported to the best of our knowledge.

A microbial community analysis would be of interest to clarify the discussion presented here and increase certainty. We performed several trials for DNA extraction for such analysis, yet interferences with the minerals originated from the sludge, and the low biomass concentration in the system yielded low DNA concentrations necessary for this analysis. We are currently addressing these issues and intend to provide this information in future work.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02557.

Dissolved sulfide accumulation in the screening stage, change in pH during the incubation, hydrogen and sulfide mass balance in a batch system inoculated with Emmen sludge, total sulfide calculations, and electron donor mass balance (PDF)

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**Notes**
The authors declare no competing financial interest.

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