Sulfide Oxidation at Halo-Alkaline Conditions in a Fed-Batch Bioreactor

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ABSTRACT: A biotechnological process is described to remove hydrogen sulfide (H2S) from high-pressure natural gas and sour gases produced in the petrochemical industry. The process operates at halo-alkaline conditions and combines an aerobic sulfide-oxidizing reactor with an anaerobic sulfate (SO4^2-) and thiosulfate (S2O3^2-) reducing reactor. The feasibility of biological H2S oxidation at pH around 10 and total sodium concentration of 2 mol L^-1 was studied in gaslift bioreactors, using halo-alkaliphilic sulfur-oxidizing bacteria (HA-SOB). Reactor operation at different oxygen to sulfide (O2:H2S) supply ratios resulted in a stable low redox potential that was directly related with the polysulfide (Sx^-) and total sulfide concentration in the bioreactor. Selectivity for SO4^2- formation decreased with increasing Sx^- and total sulfide concentrations. At total sulfide concentrations above 0.25 mmol L^-1, selectivity for SO4^2- formation approached zero and the end products of H2S oxidation were elemental sulfur (S0) and S2O3^2-. Maximum selectivity for S0 formation (83.3±0.7%) during stable reactor operation was obtained at a molar O2:H2S supply ratio of 0.65. Under these conditions, intermediary Sx^- plays a major role in the process. Instead of dissolved sulfide (HS^-), Sx^- seemed to be the most important electron donor for HA-SOB under S0 producing conditions. In addition, abiotic oxidation of Sx^- was the main cause of undesirable formation of S2O3^2-. The observed biomass growth yield under SO4^2- producing conditions was 0.86 g N mol^-1 H2S. When selectivity for SO4^2- formation was below 5%, almost no biomass growth was observed. Biotechnol. Bioeng. 2007;97: 1053–1063.

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KEYWORDS: gas desulfurization; sulfide oxidation; halo-alkaliphilic; polysulfides; fed-batch bioreactor

Introduction

Hydrogen sulfide (H2S) is known for its toxicity, corrosive properties and bad odor. When H2S is combusted, sulfur dioxide (SO2) is formed, which contributes to acid rain. Biotechnological removal of H2S from biogas originating from anaerobic wastewater treatment facilities and landfill sites, has been successfully applied since 1993 (Cline et al., 2003).Bulk removal of H2S from natural gas and gas streams produced in the petrochemical industry is mostly performed by a combination of physicochemical processes, for example an amine and Claus unit, which removes about 95% of the H2S. A post-treatment step is usually applied to obtain conversion efficiencies up to 99.9%. With the continuing search for new natural gas fields and the further development of gasification technologies, relatively small gas streams with high H2S concentrations are expected to be treated in the near future. This creates a need for new desulfurization technologies, such as the biotechnological removal of H2S, using micro-aerophilic bacteria (Kleinjan et al., 2006). The first full-scale plant for biotechnological desulfurization of natural gas, designed to treat 1,000 kg H2S per day, has been taken into operation in 2003 (Cline et al., 2003). For H2S loads up to 50 tons per day, a biotechnological process has several advantages compared to existing physicochemical methods (Cline et al., 2003): (1) the process is safe because all H2S is immediately absorbed; (2) there is no use of expensive chemicals; (3) the process proceeds at ambient temperatures and, apart from the absorber, also at atmospheric pressure; (4) the end product of the process is biologically produced elemental sulfur (S0). In contrast to chemically produced S0, biologically produced S0 is hydrophilic (Janssen et al., 1994) and can be used in water-based applications, such as soil fertilizer or fungicide.

Innovations in the biotechnological desulfurization process are described to treat high-pressure natural gas and sour gases produced in the petrochemical industry. The most important improvements proposed are the operation of the process under halo-alkaline conditions and the addition of an anaerobic reactor to reduce the bleed stream flow. A simplified flow scheme of the process is presented in...
In this process, a gas absorber (G) is integrated with a bioreactor (B). In the absorber, which can be operated at high pressure (e.g., 100 bar), the H$_2$S-containing gas is contacted with an alkaline solution. Hydroxide (OH$^-$) ions and carbonate (CO$_3^{2-}$) ions are consumed to absorb H$_2$S gas under the formation of hydrogen bisulfide (HS$^-$, Eqs. 1 and 2A,B). The loaded solution is fed to a bioreactor, where HS$^-$ is biologically oxidized with dissolved oxygen (O$_2$) to S$^0$, according to Equation (3). In the oxidation step, which operates at atmospheric pressure, the formation of insoluble S$^0$ is accompanied by the regeneration of OH$^-$ ions. Elemental sulfur is separated from the reactor liquid by gravity sedimentation in a settler (S), while a liquid overflow is recycled to the bioreactor.

\[
\text{H}_2\text{S}(g) \rightleftharpoons \text{H}_2\text{S(aq)} \quad (1)
\]
\[
\text{H}_2\text{S(aq)} + \text{OH}^- \rightleftharpoons \text{HS}^- + \text{H}_2\text{O} \quad (2A)
\]
\[
\text{H}_2\text{S(aq)} + \text{CO}_3^{2-} \rightleftharpoons \text{HS}^- + \text{HCO}_3^- \quad (2B)
\]
\[
\text{HS}^- + \frac{1}{2}\text{O}_2 \rightarrow \text{S}^0 + \text{OH}^- \quad (3)
\]
\[
\text{HS}^- + 2\text{O}_2 \rightarrow \text{SO}_4^{2-} + \text{H}^+ \quad (4)
\]

In addition to the biological oxidation of HS$^-$ to S$^0$, undesirable side reactions involving HS$^-$ can take place. Two important reactions that are expected to play a role in the new process are biological sulfate (SO$_4^{2-}$) formation and abiotic polysulfide (S$_2^{2-}$) oxidation. Formation of SO$_4^{2-}$ was already described in studies involving sulfur-oxidizing bacteria (SOB) at neutrophilic conditions (Buismann et al., 1989; Kuenen, 1975). It was found that under HS$^-$ limited conditions, HS$^-$ is completely oxidized to SO$_4^{2-}$, according to Equation (4). Dissolved sulfide can also react with biologically produced S$^0$ to S$_2^{2-}$ ions, according to Equation (5) (Steudel, 1996). In presence of dissolved O$_2$, these S$_2^{2-}$ ions are abiotically oxidized to S$^0$ and S$_2$O$_5^{2-}$, according to Equation (6) (Steudel et al., 1986). Kleinjian et al. (2005b) and Chen and Morris (1972) found that the abiotic oxidation of S$_2^{2-}$ occurs more rapidly than abiotic oxidation of HS$^-$ to S$_2$O$_5^{2-}$ (Eq. 7).

Major disadvantages of SO$_4^{2-}$ and S$_2$O$_5^{2-}$ formation are that (1) less re-usable elemental sulfur is formed; (2) formation of protons (H$^+$) leads to acidification of the medium; (3) formation of SO$_4^{2-}$ and S$_2$O$_5^{2-}$ results in a higher O$_2$ demand; (4) to prevent accumulation, SO$_4^{2-}$ and S$_2$O$_5^{2-}$ ions have to be removed by means of a bleed stream and the addition of make-up water. Consequently, also (bi)carbonate is removed from the system leading to an increased chemical (caustic) demand to re-establish the (bi)carbonate equilibrium.

If the process can be operated at increased cation concentrations, also concentrations of SO$_4^{2-}$ and S$_2$O$_5^{2-}$ can be higher. Consequently, the bleed stream and the use of make-up water are much lower, provided that the selectivity for SO$_4^{2-}$ and S$_2$O$_5^{2-}$ formation is not affected. The formation of a bleed stream can be avoided when a part of the reactor content is directed to an anaerobic bioreactor (A in Fig. 1). In this second reactor, any SO$_4^{2-}$ and S$_2$O$_5^{2-}$ is biologically reduced to HS$^-$, where after the produced HS$^-$ is recycled to the S$^0$ producing reactor. For this anaerobic step, a suitable e-donor such as hydrogen, ethanol or methanol is required (Lens et al., 2002; Van Houten et al., 1994; Weijsma et al., 2000).

To reduce energy costs for pumping the liquid recycle from the bioreactor to the high pressure absorber, it is beneficial to maximize the H$_2$S loading capacity of the alkaline solution. This can be achieved by increasing the pH and the CO$_3^{2-}$ concentration by the addition of sodium hydroxide (NaOH). Simultaneously with H$_2$S, also CO$_2$ is absorbed from the sour gas. At high pH values (e.g., pH 10), this will result in increased concentrations of HCO$_3^-$ and CO$_3^{2-}$. If only NaOH is used to produce alkalinity, scaling problems due to the formation of NaHCO$_3$ precipitates may occur. This can be
prevented by using potassium hydroxide (KOH) instead, as the K⁺ salt of HCO₃⁻ has a higher solubility (3.9 mol in 1 L water, at 30°C) compared to the Na⁺ salt (1.3 mol in 1 L water, at 30°C, Perry et al., 1999). However, KOH is more expensive than NaOH and therefore a mix of NaOH and KOH is recommended.

In comparison to conventional biotechnological processes for H₂S removal from biogas, the new halo-alkaline process is developed to treat much higher H₂S and CO₂ concentrations in more compact units. Table I gives an overview of the conditions with respect to pH and salt concentration for both technologies. In the new process, specialized bacteria are needed that are capable of growth under extreme conditions. Halo-alkaliphilic chemolithoautotrophic SOB of the genera *Thioalkalimicrobium* and *Thioalkalivibrio* recently isolated from soda lakes seem to be perfectly fit for this application. These bacteria combine the capability of growth under extremely alkaline saline conditions with the formation of S⁰ under O₂ limiting conditions (Sorokin and Kuenen, 2005). *Thioalkalimicrobium* strains have relatively high specific growth rates, a low growth yield, high maintenance and high sulfide oxidation rates, while *Thioalkalivibrio* strains are in general slow-growing, have a high growth yield, low maintenance and low sulfide oxidation rates (Sorokin et al., 2003). Both genera grow optimally at pH around 10, while *Thioalkalivibrio* strains are in general more salt tolerant compared to *Thioalkalimicrobium* strains (Sorokin and Kuenen, 2005).

The aim of the present study was to determine the feasibility of biological H₂S oxidation under halo-alkaline conditions in gas-lift reactors by using new isolates, originating from soda lakes. Special attention was paid to selectivity for S⁰ formation in relation to the molar O₂:H₂S consumption ratio. In addition, biomass growth yield and the role of S₂⁻ in product formation was studied.

### Materials and Methods

#### Experimental Set-Up

Two identical gas-lift reactors were used with a wet volume of 4.8 L each (Fig. 2). The gas flow (300 L h⁻¹) was completely recycled to prevent any release of H₂S gas and to reach low O₂ concentrations. Pure H₂S gas (99.8%) and pure O₂ gas (99.995%) were supplied by mass flow controllers (Brooks Thermal Mass Flowmeter (Brooks Instruments, Veenendaal, The Netherlands), type 5850e, model 0154, 0–30 mL min⁻¹ (H₂S) and 0–150 mL min⁻¹ (O₂)). Nitrogen gas (N₂, 99.995%) was added to the gas flow when the pressure dropped below atmospheric pressure. In case of pressure build-up, excess gas was discharged via a water-lock. The reactors were operated at 35°C using a thermostat bath.

### Medium Composition

The medium consisted of a carbonate buffer with 0.66 mol L⁻¹ Na⁺ and 1.34 mol L⁻¹ K⁺, as carbonates. Furthermore, the medium contained (in g per 1 L of demineralized water): K₂HPO₄, 1.0; NaNO₃, 0.83; NaCl, 6.0; MgCl₂·6 H₂O, 0.20. NO₃⁻ was used as nitrogen (N) source, because of its stability under alkaline conditions (Sorokin et al., 2000). Trace elements solution was added as described in Pfennig and Lippert (1966). After addition of all compounds, the pH of the medium was 10.2 ± 0.1 at 35°C.

### Inoculum

Reactors were inoculated with centrifuged biomass from a sulfur producing gas-lift bioreactor, operated under fed-batch conditions. The original inoculum of this reactor was a mixture of pure cultures and enrichments containing halo-alkaliphilic *Thioalkalivibrio* and *Thioalkalimicrobium* strains. Original strains were obtained from Delft University of Technology and were isolated from sediments from soda

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**Table I.** pH, salt concentration and ionic strength in the biotechnological process for H₂S removal under slightly alkaline conditions (e.g. for the treatment of biogas) compared to the halo-alkaline conditions in the new process (e.g. for the treatment of sour natural gas).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Slightly alkaline (biogas)</th>
<th>Halo-alkaline conditions (sour natural gas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8</td>
<td>9.5–10.5</td>
</tr>
<tr>
<td>[Na⁺] + [K⁺] (mol L⁻¹)</td>
<td>0.11</td>
<td>2.0</td>
</tr>
<tr>
<td>[Na⁺] : [K⁺]</td>
<td>250:1</td>
<td>1:2</td>
</tr>
<tr>
<td>Ionic strength (mol L⁻¹)</td>
<td>0.12</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*In fed-batch experiments (Janssen et al. 1995)
lakes in Mongolia, Central Asia and Kenya. A large number of these soda lake isolates were studied in detail. An overview of isolates and inoculum sources is given by Sorokin et al. (2003) and Sorokin and Kuenen (2005). Each subsequent fed-batch experiment was started with biomass from previous experiments.

Reactor Start-Up

Reactors were filled with medium and inoculated with centrifuged biomass (750–1,000 mL before centrifugation). The total liquid volume after inoculation was 4.7 L at 35°C. After temperature stabilization, addition of H₂S was started. Throughout all experiments, the H₂S load was kept constant at 10 mmol h⁻¹, unless stated otherwise. A start-up phase (0–20 h) was applied to activate the biomass and to increase the biomass concentration. During this phase, the dissolved oxygen concentration (DO) was kept between 50 and 70% saturation, by control of the O₂ supply to the gas recycle flow. After passing the start-up phase, the O₂ supply rate was set to a constant value, which was varied for different experimental runs. A typical run-time for a fed-batch experiment was 120 h. As the supply of H₂S was kept constant throughout all experiments, the O₂:H₂S supply ratio could only be varied by changing the O₂ supply. Because the medium was highly buffered, no pH control was necessary.

Analysis

Reactors were equipped with sensors for temperature, pH (Hamilton Flushtrode T200, Hamilton, Reno, NV), DO concentration (Mettler Toledo Inpro 650/120 Mettler Toledo, Greifensee, Switzerland) and oxidation-reduction potential (ORP, WTW SenTix ORP Ag/AgCl electrode, WTW, Weilheim, Germany). The DO concentration was measured as % saturation (% sat.). The ORP was measured versus a saturated KCl, Ag/AgCl reference electrode. Sulfide concentration was measured as total sulfide (\(S_{\text{total}}^2\)). At the experimental pH range of 9.5–10.1, concentrations of H₂S and S²⁻ are negligible. Polysulfides of chain lengths 2 and 3 can be present in solution in both protonated and non-protonated form (pKa of HS⁻ is 9.7, pKa of HS₂⁻ is 7.5, Schwarzenbach and Fischer, 1960). However, it was shown by Kamysny et al. (2006) that the fraction of S₂ and S₃ species is low compared to the total polysulfide concentration (around 5% at pH 9.5, less at higher pH values). Consequently, the concentrations of HS²⁻ and HS₃⁻ will be negligible compared to the total sulfide concentration (0.5% of \(S_{\text{total}}^2\) at pH 9.5). Therefore, the following equation applies:

\[
[S_{\text{total}}^2] = [\text{HS}^-] + [S_x^2^-] \tag{8}
\]

The method used was based on a modified methylene blue method as described by Trüper and Schlegel (1964). Immediately after sampling, 1 mL zinc acetate (20 g L⁻¹) per mL of sample was added to prevent oxidation of sulfide. The formed precipitate was washed and diluted with demineralized water to remove dissolved salts. \(S_{\text{total}}^2\) in the diluted zinc sulfide precipitate was measured with the Lange cuvette test LCK653 (Hach Lange, Düsseldorf, Germany). The concentration of \(S_x^2^-\) was determined spectrophotometrically as described by Kleinjan et al. (2005a), at a wavelength of 285 nm (Perkin-Elmer Norwalk, CT), Lambda 2, UV/VIS-spectrophotometer). With this method, the total concentration of zerovalent sulfur atoms in \(S_x^2^-\) was determined: \(S^0\) in \(S_{\text{total}}^2\). When the average chain length \(x\) is known, \(S_x^2^-\) can be calculated according to:

\[
(x - 1) \cdot [S_x^2^-] = [S^0] \text{ in } S_{\text{total}}^2 \tag{9}
\]

Before analysis, samples were filtered over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Schleicher & Schuell, Dassel, Germany). The molar extinction coefficient was determined for the high salt medium and was found to be 1,300 L mol⁻¹ cm⁻¹. This value is somewhat lower than reported data of 1,325 L mol⁻¹ cm⁻¹ (Teder, 1967), 1,350 L mol⁻¹ cm⁻¹ (Kleinjan et al., 2005a), and 1,360 L mol⁻¹ cm⁻¹ (Schwarzenbach and Fischer, 1960). Possibly, this is due to matrix effects of the high salt medium. Concentrations of SO₃²⁻ and S₂O₂⁻ were determined by ion chromatography ( Dionex DX-600 model 50, Dionex, Sunnyvale, CA). An ionpac AS17 column was used at 30°C and a flow rate of 1.5 mL min⁻¹. The injection volume was 25 µL. The eluent was made by an eluent generator (EG40, Dionex), equipped with a KOH cartridge, using deionized water as a carrier. Detection was based on conductivity. An ASRS-ULTRA suppressor was used to suppress eluent conductivity. Samples were diluted (1:500) with a 30 mM mannit solution to avoid interference by carbonate ions. Presence of sulfite (SO₃²⁻) was detected using a test-strip (Quantofox 91306, Merck, Darmstadt, Germany). The concentration of S⁰ in the reactor was calculated by the mass balance on basis of the H₂S supply and measurement of dissolved sulfur products formed. Composition of the gas phase (H₂S, N₂, CO₂, and O₂) was measured as the amount of total N, based on the absorbance of nitrophenol at 370 nm with the Lange cuvette test LCK238 (Hach Lange, Düsseldorf, Germany). Prior to analysis, samples were centrifuged (10 min, 10,000 rpm) and washed
two times with N-free medium to remove all dissolved N. This method was tested by standard addition of ureum and nitrate to reactor samples as well as fresh medium, with and without the presence of biologically produced $S^0$. Presence of biologically produced $S^0$ did not affect the results.

Results

Results of a Typical Fed-Batch Experiment

Results of a typical fed-batch experiment are shown in Figure 3A–D. The reactor was inoculated with biomass to a final concentration of 11.0 mg N L$^{-1}$. During the start-up phase, the O$_2$ supply to the gas recycle flow was based on the measured DO concentration, which was kept at 70% sat. Immediately after starting the H$_2$S supply ($t = 0$ h), the ORP dropped to $-230$ mV and some S$_2$O$_3^{2-}$ was produced until $t = 5$ h. These results indicate that in the first hours after start-up, the biological activity was too low to treat all H$_2$S added. Under these conditions, some HS$^-$ accumulated in the medium and was abiotically oxidized to S$_2$O$_3^{2-}$, as was also found in several other studies at more neutral pH conditions (Janssen et al., 1995; Krishnakumar et al., 2005). At $t = 5$ h, the ORP increased to $+90$ mV, while all H$_2$S supplied was completely oxidized to SO$_4^{2-}$. In the subsequent 10 h, also the accumulated S$_2$O$_3^{2-}$ was oxidized to SO$_4^{2-}$ (Fig. 3A, period 1). The O$_2$:H$_2$S supply ratio in this period was 1.9, which is somewhat below the stoichiometrical value of 2 for SO$_4^{2-}$ formation (Eq. 4). The slightly lower value is due to assimilation of CO$_2$ into cellular material, for which reductive equivalents are needed (Janssen et al., 1995; Kelly, 1999).

At $t = 18.5$ h, the O$_2$ supply was manually decreased from 19 to 7 mmol h$^{-1}$, resulting in a molar O$_2$:H$_2$S supply ratio of 0.7. As a result of the limited O$_2$ supply, the DO concentration dropped rapidly below the detection limit of 0.1 mg L$^{-1}$ (Fig. 3B, period 2). It can be seen that in the next 3 h, some HS$^-$ and S$_2^-$ accumulated, which was accompanied by an initial rapid drop of the ORP (Fig. 3C, period 2). The formation of S$_2^-$ within 1 h after reducing the O$_2$ supply indicates that S$_0$ formation also started within 1 h. However, the biological HS$^-$ oxidation rate was too low to convert all incoming H$_2$S. From 20 to 22 h, [S$_2$O$_3^{2-}$] accumulated at an average rate of 1.6 mmol h$^{-1}$ (results not shown) to a final concentration of 0.73 mmol L$^{-1}$. This means that at least 16% of the supplied H$_2$S was not converted. To prevent toxification of the biomass, the H$_2$S supply was briefly stopped at $t = 22.1$ h. The system responded immediately by a decrease of the S$_2^-$ concentration, and an increase of the

![Figure 3](https://example.com/figure3.png)

Figure 3. A–D: Results of a typical fed-batch experiment. Concentrations of SO$_4^{2-}$, S$_2$O$_3^{2-}$, and S$^0$ and total H$_2$S added (A); O$_2$ supply and DO concentration (B); Concentration of S$^0$ in S$_2^-$ and ORP (C); Biomass concentration and pH (D). The vertical dashed lines border the start-up phase (1), operation with a molar O$_2$:H$_2$S supply ratio of 0.7 (2), brief stop of the H$_2$S supply (3), and resume of the H$_2$S supply at a molar O$_2$:H$_2$S supply ratio of 0.7 (4).
ORP (Fig. 3C, period 3). When the ORP reached $-340$ mV, H$_2$S addition was resumed ($t = 22.8$ h). After an initial drop of the ORP, it stabilized around $-360$ mV and remained stable until the end of the experiment ($t = 120$ h). In this period also the concentration of $S^{2-}$ ($0.059 \pm 0.001$ mmol S$^0$ in $S^{2-}$) remained stable, while the $S^{2-}$ concentration remained below $0.02$ mmol L$^{-1}$.

During the period of limited O$_2$ supply of 7.0 mmol h$^{-1}$ (O$_2$:H$_2$S supply ratio $= 0.7$), SO$_4^{2-}$, S$_2$O$_3^{2-}$ and S$^{0}$ accumulated (Fig. 3A, period 4). No SO$_3^{2-}$ could be detected. The O$_2$ concentration in the gas phase was stable at $11.1 \pm 0.4\%$ and no H$_2$S was detected in the gas flow leaving the reactor, even when HS$^-$ accumulated in the reactor medium. This demonstrates that the transfer rate of both gases from the gas to the liquid phase was equal to the supply rate to the gas recycle flow. From the slope of the curves of Figure 3A during the period of limited O$_2$ supply (period 4, 22.8–120 h), the formation rate of the different sulfur species can be accurately determined. For this particular experiment, H$_2$S was converted to SO$_4^{2-}$ (16%, $r^2 = 0.95$), S$_2$O$_3^{2-}$ (17%, $r^2 = 0.99$) and S$^0$ (67%, $r^2 = 0.98$). The pH decreased over time due to the formation of SO$_4^{2-}$ and S$_2$O$_3^{2-}$ (Fig. 3D). The rate of acidification of the reactor medium was reduced when the rate of SO$_4^{2-}$ production decreased, which is in agreement with Equation (4). During the start-up phase, the biomass concentration increased from 11.0 to 43.9 mg N L$^{-1}$ at a rate of 1.3 mg N L$^{-1}$ h$^{-1}$. Under O$_2$ limited conditions, the biomass concentration increased to 45.7 mg N L$^{-1}$ at a rate of only 0.075 mg N L$^{-1}$ h$^{-1}$ (Fig. 3D).

### Product Selectivity Related With Molar O$_2$:H$_2$S Supply Ratio

A series of experiments similar to the experiment described above was performed at a constant H$_2$S supply of 10 mmol h$^{-1}$, but at various O$_2$ supply rates. After passing the start-up phase of 20–30 h at DO concentrations of 50–70%, the O$_2$:H$_2$S supply ratio was varied from 1.0 to 0.8, 0.7, 0.65, and 0.6. Experiments at an O$_2$:H$_2$S supply ratio of 1, 0.8, and 0.7 were performed in duplicate. The effect of the molar O$_2$:H$_2$S supply ratio on the selectivity of sulfur species produced is shown in Figure 4. Selectivity was determined over a period of at least 25 h of stable reactor performance. It can be seen that selectivity for SO$_4^{2-}$ as well as S$_2$O$_3^{2-}$ formation decreased with decreasing O$_2$:H$_2$S supply ratio. At an O$_2$:H$_2$S supply ratio of 1.0, selectivity for SO$_4^{2-}$ formation was $36.4 \pm 0.9\%$, while at an O$_2$:H$_2$S supply ratio of 0.65, this value approached zero ($1.4 \pm 0.6\%$). Selectivity for S$_2$O$_3^{2-}$ formation was $31.7 \pm 3.7\%$ at an O$_2$:H$_2$S supply ratio of 1.0 and $15.3 \pm 0.1\%$ at an O$_2$:H$_2$S supply ratio of 0.65. In all experiments, the H$_2$S concentration in the gas flow leaving the reactor was below the detection limit of 0.1 ppm.

Figure 5A,B shows that at an O$_2$:H$_2$S supply ratio of 0.60, initially only 6% of the total H$_2$S was converted to S$_2$O$_3^{2-}$, while no SO$_4^{2-}$ formation was detected at all. However, this situation was only stable for a short period. In 30 h following the limited supply of O$_2$, the ORP gradually decreased. At $t = 51$ h, the H$_2$S supply was stopped to prevent toxification of the biomass due to accumulation of HS$^-$ and S$_2^{2-}$. In the

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**Figure 4.** Product selectivity at different molar O$_2$:H$_2$S supply ratios. The H$_2$S supply was 10 mmol h$^{-1}$ for all experiments. The vertical dashed line represents the boundary for stable reactor operation. At O$_2$:H$_2$S supply ratios of 0.85 and higher, no sulfide accumulated in the medium. At an O$_2$:H$_2$S supply ratio of 0.8 and below, sulfide accumulated after 20 h.

**Figure 5.** A, B: Results of a fed-batch experiment where the O$_2$ supply was decreased to 6 mmol h$^{-1}$. Concentrations of SO$_4^{2-}$, S$_2$O$_3^{2-}$ and S$^0$ and total H$_2$S added (A); O$_2$ supply and ORP (B). The H$_2$S supply was constant at 10 mmol h$^{-1}$. The vertical dashed lines border the start-up phase (1), operation with a molar O$_2$:H$_2$S supply ratio of 0.8 (2), and operation with the O$_2$ supply based on ORP (3). The arrows in (B) indicate periods in which the H$_2$S supply was briefly interrupted.
subsequent period, the O2 supply was controlled on the basis of ORP. The O2 supply of 6 mmol h⁻¹ was increased with 0.08 mmol h⁻¹ per mV negative departure from the setpoint of −410 mV. During this period, the O2 supply gradually increased to 7.3 mmol h⁻¹, at an ORP of −426 mV. Under these conditions, the increase in O2 demand was related to S₂O₂⁻ formation (selectivity for S₂O₂⁻ increased to 27%), and not to SO₂⁻ formation (selectivity for SO₂⁻ remained <2%). At O2:H2S supply ratios below 0.60, S₂⁻ rapidly accumulated and conversion of the supplied H2S failed (data not shown). Figure 6 shows the stoichiometric amount of O2 required for the formation of the sulfur products versus the actual O2 supply for different O2:H2S supply ratios. From this figure it is clear that at an O2 supply of 7 mmol h⁻¹ and above, more oxidized sulfur compounds are produced than can be expected on basis of the O2 supply. An explanation for this is the use of part of the reductive capacity for assimilation of CO₂ into cellular material.

**Relation Between Sulfide, Polysulfide and Redox Potential**

The relation between [S²⁻] and [S⁰] at pH 10.1 ± 0.1 in presence of S⁰, measured during various fed-batch experiments is given in Figure 7. This figure shows that the average ratio between [S²⁻] and [S⁰] was 1:4.4 ($r^2 = 0.94$). Combining Equations (8) and (9), the following equation can be derived:

$$\log \frac{[S^{2-}]}{[S^{0}]} = \log \left( \frac{[S^{2-}]}{[S^{0}]} \right) = x - 1 \quad (10)$$

With this equation, the minimum possible average S²⁻:chain length (x) can be calculated when assuming that the measured [S²⁻] consists of S²⁻ only ([HS⁻] = 0). With [S²⁻]:[S⁰] = 1:4.4, this results in a minimum average chain length of 5.4 in our experiments. Reported average chain lengths of polysulfide in equilibrium with sulfur vary from x = 4.39 (Giggenbach, 1972), 4.59 (Kleinjan et al., 2005a), 4.79 (Maromny, 1959), 4.93 (Boulegue and Michard, 1978), 4.97 (Cloke, 1963), 5.11 (Kamyshny et al., 2004), 5.33 (Teder, 1971) to 5.5 (Steudel, 2000). Applying the maximum reported average length of 5.5 in Equation 10, it can be calculated that with [S²⁻] = 1:4.4, at most 2.2% of the total sulfide was present as HS⁻. It therefore can be concluded that in our experiments, S²⁻ was the main reduced sulfur compound in the medium, and not HS⁻.

The ORP in a sulfide oxidizing bioreactor is determined mainly by the total sulfide concentration (Janssen et al., 1998). As [S²⁻] was high compared to [HS⁻], we propose that under stable reactor operation, the ORP is directly related with [S²⁻]. From the various fed-batch experiments presented in this study, the relation between the negative logarithm of [S⁰] in S²⁻ and the ORP in presence of S⁰ can be described as:

$$\text{ORP} = -43.2 \cdot \log \left( \frac{[S^{0}]}{[S^{2-}]} \right) - 411 \quad (11A)$$

with [S²⁻]:[S⁰] = 1:4.2, this becomes:

$$\text{ORP} = -43.2 \cdot \log \left( \frac{[S^{2-}]}{[S^{0}]} \right) - 438 \quad (11B)$$

Figure 8 shows the ORP and [S⁰] during stable reactor operation for each applied O2:H₂S supply ratio. It is clear that at lower O2:H₂S supply ratios, the ORP stabilizes at lower values, related with higher S²⁻ concentrations.

**Biomass Characteristics**

Biomass concentration measurements were performed during the SO₂⁻ producing start-up phase as well as during S⁰ producing conditions. Figure 9 shows biomass growth versus O₂:H₂S supply ratio. During the start-up phase, when SO₂⁻ was the only product, the biomass concentration increased with 8.6 mg N h⁻¹, corresponding...
to a yield of 0.86 g N mol$^{-1}$ H$_2$S converted. Assuming a dry weight N content of 14% (w/w), this is comparable to the yields found with pure cultures of HA-SOB, growing on S$_2$O$_5^-$ (Sorokin et al., 2003). Under O$_2$ limiting conditions, when selectivity for SO$_4^{2-}$ formation was below 5%, almost no increase in biomass concentration was observed (0.01–0.13 mg N h$^{-1}$). A similar relation between the growth yield and product formation was found with SOB grown at more neutral pH conditions by Buisman et al. (1991). Obviously, this is related to the difference in the metabolically available energy from the oxidation of HS$^{-}$ to either SO$_4^{2-}$ or S$^{0}$ (Kelly, 1999). The very low growth obtained during S$^0$ formation can be explained by the energy requirements for maintenance.

The relation between ORP and selectivity for SO$_4^{2-}$ formation under O$_2$ limiting conditions is shown in Figure 10. Below an ORP of $-400$ mV, selectivity for SO$_4^{2-}$ formation dropped below 2%, while at ORP values above $-400$ mV, the SO$_4^{2-}$ formation rate increased with increasing ORP. On basis of the relation between ORP and $[[S^0_{\text{total}}]]$ (Eq. 11B), it can be calculated that at $[[S^0_{\text{total}}]]$ above 0.25 mmol L$^{-1}$, the SO$_4^{2-}$ formation rate was below 2%.

### Discussion

#### Formation and Speciation of Polysulfide Ions

For the reaction of HS$^{-}$ with biologically produced sulfur particles (Eq. 5), Kleinjan et al. (2005a) reported an equilibrium constant ($pK_a$) of 9.17$\pm$0.09 with an average S$_2$ chain length of 4.59 at 35°C. To exactly determine whether in our fed-batch experiments HS$^{-}$ and S$_2$ are in equilibrium, the activity constants of HS$^{-}$ and S$_2$ at the high salt conditions as well as the average S$_2$ chain length should be known. Although this is not the case, an estimate can be made based on the relation between $[S^0_{\text{in S}_2}]$ and total sulfide, determined at pH 10.0–10.2 (Fig. 7). Applying the equilibrium constant of Kleinjan et al. (2005a), it can be calculated that 10.5% of the total sulfide is present as HS$^{-}$ at pH 10.1, assuming equilibrium between HS$^{-}$ and S$_2$. Based on this value, the average polysulfide chain length in our experiments should be 5.9. It was already found that the polysulfide chain length was at least 5.4, while the maximum reported average chain length is 5.5 (Kleinjan et al., 2005a; Steudel, 2000). Although the average S$_2$ chain length in our experiments was not exactly known, it can be concluded that in excess of S$^0$, the concentration of S$_2$ in the medium was close to equilibrium with HS$^{-}$. This was also found in bioreactor experiments with H$_2$S removal under mildly alkaline conditions (Kleinjan et al., 2006). The formation rate of S$_2$ from colloidal S$^0$ and HS$^{-}$ was studied by Fossing and Jørgensen (1990), who found the halftime of isotope exchange between $^{35}$S$^0$ and S$_2$ to be 1.8 min. In our experiments, the S$_2$ formation rate was probably faster as the particle size of...
freshly produced S\(^0\) is likely to be much smaller than the size of colloidal S\(^0\) particles used in the study of Fossing and Jørgensen (1990). As the reaction between HS\(^-\) and S\(^0\) takes place at the surface of S\(^0\) particles, the formation rate of S\(_2^+\) will increase with decreasing particle size (Kleinjan et al., 2005c). Another cause for rapid S\(_2^+\) formation in our experiments is the autocatalytic effect of S\(_2^+\) on the reaction rate, shown by Kleinjan et al. (2005c).

The minimum average chain length of S\(_2^+\) ions in our experiments was more than 4.59 as was reported by Kleinjan et al. (2005a). A possible explanation for the longer average chain length in our experiments is that oxidation of short S\(_2^+\) chains occurs faster than oxidation of longer S\(_2^+\) chains. Results of Banciu et al. (2004) indeed indicate that the biological oxidation rate of S\(_2^+\) of shorter chain length by HA-SOB *Thioalkalivibrio versutus* occurs faster than biological oxidation of S\(_2^+\) of longer chain length. Kamysny et al. (2003) found the disproportionation reaction of S\(_2^+\) in aqueous solutions with excess S\(^0\) to reach equilibrium within seconds. It can be calculated that in our experiments the S\(_2^+\) oxidation rate is too low to have any effect on the average S\(_2^+\) chain length, even if all supplied H\(_2\)S is oxidized via S\(_2^+\) as an intermediate. High concentrations (up to 20 g L\(^{-1}\)) of S\(_2\)O\(_2^-\) and SO\(_2^-\) did not have a significant effect on the measurement of [S\(^0\) in S\(_2^-\)] (results not shown). An adequate explanation for the longer average S\(_2^+\) chain length in our experiments remains elusive.

### Origin of Thiosulfate

Our results show that under O\(_2\) limiting conditions, S\(_2\)O\(_3^-\)-formation plays a more important role than SO\(_2^-\)-formation in the selectivity for S\(^0\) formation. Previous publications on HS\(^-\) oxidation under more neutral pH conditions (Janssen et al., 1995; Kuenen, 1975; Plas et al., 1992) suggest abiotic reactions to be the cause of S\(_2\)O\(_3^-\)-formation. We assume that also in our experiments, S\(_2\)O\(_3^-\)-originates from abiotic processes. As mentioned above, S\(_2\)O\(_3^-\)-can be formed upon abiotic oxidation of S\(_2^+\)-according to Equation (6). Kinetics of S\(_2^-\)-oxidation was studied by Kleinjan et al. (2005b). The following empirical relation for the rate of O\(_2\) consumption by abiotic oxidation of S\(_2^-\) was found:

\[
d[O_2]d\tau^{-1} = -k[S_2^-][O_2]_{(aq)}^{0.59} \tag{12}
\]

At 35°C, pH 10 and I = 0.1 mol L\(^{-1}\), they found a value for k of 1.65. Combining this with the stoichiometry of abiotic S\(_2^-\)-oxidation (Eq. 6), the following equation can be derived:

\[
d[S_2O_3^-]d\tau^{-1} = -1.10[S_2^-][O_2]_{(aq)}^{0.59} \tag{13}
\]

Combining Equation (13) with the S\(_2^-\)-concentration and S\(_2\)O\(_3^-\)-formation rates in the fed-batch experiments, a theoretical DO concentration can be calculated, assuming that S\(_2\)O\(_3^-\)-is formed upon abiotic oxidation of S\(_2^-\)-only.

With this approach, the DO concentration should be 1.6 mg L\(^{-1}\) at an O\(_2\):H\(_2\)S supply ratio of 0.8 and 0.14 mg L\(^{-1}\) at an O\(_2\):H\(_2\)S supply ratio of 0.65. However, during experiments with limited O\(_2\) supply, the DO concentration was never above the detection limit of 0.1 mg L\(^{-1}\). This implies that either the abiotic oxidation rate of S\(_2^-\)-under halo-alkaline conditions occurs more rapidly than calculated by Equation (13), or that besides abiotic S\(_2^-\)-oxidation, another process results in the formation of S\(_2\)O\(_3^-\)-. Hydrolysis of S\(^0\) is an eligible process explaining extra formation of S\(_2\)O\(_3^-\). Hydrolysis of S\(^0\)-can be described with the following equation (Steudel, 2000):

\[
4S^0 + 4OH^- \rightarrow S_2O_3^- + 2HS^- + H_2O \tag{14}
\]

The additional formation of HS\(^-\) leads to an increase in the HS\(^-\)load on the bioreactor and therefore to an increase in O\(_2\)-demand. According to Steudel (2000), with inorganic sulfur this reaction occurs at pH > 11.5 at 20°C and at pH 7.6 at 80°C. Also biologically produced sulfur was found to hydroylyse at 55°C and pH 10 (Buisman et al., 2000). Kleinjan et al. (2005b) concluded that at pH values above 9, colloidal S\(^0\)-formed upon abiotic oxidation of S\(_2^-\), so called “nascent” sulfur, is subject to hydrolysis. They proposed that small clusters of sulfur rings, having a high specific surface area, or small chains of sulfur atoms, having a low chemical stability, are subject to faster hydroylysis compared to biologically produced sulfur. The pH of the experiments described in the current study was between 9.6 and 10.2. It is therefore very likely that hydroylysis of nascent sulfur, and possibly also hydroylysis of crystalline sulfur, has taken place during these experiments. The mechanisms for S\(_2\)O\(_3^-\)-formation in the halo-alkaline sulfide oxidation process are subject of future study.

### Biological Oxidation of (poly)Sulfide

It was shown that instead of HS\(^-\), S\(_2^-\)-was the main reduced sulfur species present in our experiments at S\(^0\)-producing conditions. Banciu et al. (2004) have studied the oxidation of both compounds by the HA-SOB *Thioalkalivibrio versutus*, strain ALJ15. Their results indicated that biological oxidation of S\(_2^-\)-to SO\(_2^-\)-occurs in two phases: a first, rapid phase of oxidation of sulfate atoms (−S\(_2^-\)) and a second, slow phase of S\(^0\)-oxidation. They also found that oxidation of HS\(^-\)-alone occurs at a lower rate than the first phase of S\(_2^-\)-oxidation, indicating that the oxidation of sulfane atoms is faster than the oxidation of HS\(^-\). With S\(_2^-\)-being the main reduced sulfur species in our experiments, this indicates that S\(_2^-\)-was the main substrate for biological oxidation by HA-SOB under S\(^0\)-producing conditions.

As S\(_2\)O\(_3^-\)-formation was probably partly a result of hydroylysis of (nascent) sulfur, it was not possible to determine the biological production rate of S\(^0\). However, the relation between ORP and SO\(_2^-\)-formation shown in Figure 10, indicates that also the activity of S\(^0\)-formation is
ORP related. This was already suggested by Visser et al. (1997). They proposed that HS\(^{-}\) is oxidized to SO\(_2\)\(^{-}\) via intermediary S\(_0\). Under limited availability of O\(_2\), the degree of reduction of the cytochrome pool increases, thereby blocking the conversion of S\(_0\) to SO\(_2\)\(^{-}\). In our process, where S\(_2\)\(^{-}\) was the main reduced sulfur compound, the second, slow phase of intermediary S\(_0\) oxidation may be influenced by the ORP in a similar way.

In all experiments, total sulfide and S\(_2\)\(^{-}\) accumulated in the reactor medium when the shift to a limited O\(_2\) supply was applied for the first time after start-up. Like in the experiment shown in Figure 3, the ORP and the S\(_2\)\(^{-}\) concentration stabilized when the H\(_2\)S supply was resumed after a brief period in which no H\(_2\)S was supplied. An explanation for this is that during the time needed for the metabolic shift from SO\(_2\)\(^{-}\) to S\(_0\) formation, the total sulfide concentration increases to a level where inhibition of the bacteria results in a too low biological S\(_2\)\(^{-}\) oxidation rate to convert all H\(_2\)S supplied. During the brief stop of the H\(_2\)S supply, the S\(_2\)\(^{-}\) concentration decreases due to biological and abiotic oxidation. When after this stop, the H\(_2\)S supply is resumed, the biological S\(_0\) formation rate is high enough to convert all supplied H\(_2\)S. Another possibility is that the conversion of HS\(^{-}\) to S\(_0\) is too low to convert all H\(_2\)S supplied. When some S\(_0\) is present to react with HS\(^{-}\) to S\(_2\)\(^{-}\), the biological oxidation rate of the supplied H\(_2\)S is high enough, as S\(_2\)\(^{-}\) is more rapidly oxidized than HS\(^{-}\).

### Conclusions

Our results show that it is possible to biologically oxidize H\(_2\)S to S\(_0\) under haloalkaline conditions (pH 10 and [Na\(^{+}\)] + [K\(^{+}\)] = 2 mol L\(^{-1}\)) in a fed-batch reactor. Maximum selectivity for S\(_0\) formation during stable reactor operation (83.3 ± 0.7%) was obtained at a molar O\(_2\):H\(_2\)S supply ratio of 0.65. Under S\(_0\) producing conditions, intermediary S\(_2\)\(^{-}\) produced by the dissolution of S\(_0\) by HS\(^{-}\), plays a major role in the process. In excess of S\(_0\), the concentration of S\(_2\)\(^{-}\) - in the medium was close to equilibrium with HS\(^{-}\). Instead of HS\(^{-}\), S\(_2\)\(^{-}\) seemed to be the most important e-donor for HA-SOB under S\(_0\) producing conditions. In addition, abiotic oxidation of S\(_2\)\(^{-}\) was the most important cause of undesired formation of S\(_0\)O\(_3\)\(^{-}\). Another possible cause for the formation of S\(_2\)O\(_3\)\(^{-}\) could be the hydrolysis of (nascent) S\(_0\). Selectivity for SO\(_3\)\(^{-}\) formation was found to be related with the S\(_2\)\(^{-}\) and the total sulfide concentration. At [S\(_{\text{total}}\)] above 0.25 mmol L\(^{-1}\), selectivity for SO\(_3\)\(^{-}\) formation approached zero. Under these conditions, the end products of H\(_2\)S oxidation were S\(_0\) and S\(_2\)O\(_3\)\(^{-}\). Biomass growth yield under fully SO\(_3\)\(^{-}\) producing conditions was 0.86 g N mol\(^{-1}\) H\(_2\)S. When selectivity for SO\(_3\)\(^{-}\) was below 5%, hardly any increase in the biomass concentration was observed. Reactor operation at set molar O\(_2\):H\(_2\)S supply ratios of 0.65 and above resulted in a stable ORP that was directly related with the S\(_2\)\(^{-}\) and S\(_{\text{total}}\) concentration in the bioreactor. This gives good perspectives for the use of the ORP as a parameter to control the O\(_2\) supply for optimal S\(_0\) selectivity in future research.

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### References


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