Biological sulfide oxidation by natron-alkaliphilic bacteria

Application in gas desulfurization

Promotor
Prof. dr. ir. A.J.H. Janssen
Hoogleraar biologische gas- en waterreiniging

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Prof. dr. ir. R.H. Wijffels	Wageningen Universiteit

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Pim L.F. van den Bosch

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‘Nederigheid tegenover de bloem aan de boomgrens
opent de weg die bergopwaarts voert.’

Dag Hammarskjöld, merkstenen

ABSTRACT

Hydrogen sulfide (H_2S) is a toxic, corrosive and smelly gas that contributes to the formation of acid rain. It is present in many natural gas fields and in refinery gases that arise from oil processing. Existing physicochemical processes to remove H_2S from these 'sour' gasses are associated with some distinct disadvantages when treating gasses with a medium size H_2S load, e.g. 0.2 to 50 tons per day. In this thesis, an alternative biotechnological process is investigated to remove and convert H_2S from these sour gas streams. The process consists of an absorber, where H_2S is removed from the gas by an alkaline washing solution. The sulfide-loaded alkaline solution is sent to a bioreactor, where the sulfide is oxidized to elemental sulfur by chemoautotrophic sulfur oxidizing bacteria (SOB). Although the first full-scale installations have been taken into operation, there is significant room for improvement, thereby enabling the treatment of higher H_2S loads. The improved process operates at natron- (high sodium concentration) alkaline (high pH) conditions, making use of bacteria obtained from soda lake sediments. In the process, formation of sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) is unwanted, as it decreases the selectivity for formation of re-usable sulfur and leads to acidification of the alkaline washing solution.

The main goal of the current research was to maximize the conversion of H_2S to elemental sulfur. Bioreactor experiments show that sulfur formation takes place at highly reduced, oxygen-limiting conditions. At these conditions, sulfide reacts with sulfur particles to form polysulfide anions. The combined concentration of sulfide and polysulfides ("total sulfide") is the main parameter that determines the selectivity for biological formation of sulfate. By maintaining the total sulfide concentration above a minimum level, the undesired formation of sulfate can be completely suppressed. However, at such highly reduced, oxygen-limited conditions, always some thiosulfate is formed as a result of abiotic oxidation of polysulfides. Depending on the pH, between 5 (pH 8.5) and 20 mol% (pH 10) of the H_2S load is converted to thiosulfate. Selectivity for sulfur formation can be optimized by control of the total sulfide concentration. This can be achieved by using the measured oxidation-reduction potential (ORP) in the alkaline reactor liquid to steer the oxygen supply rate. A mathematical equation has been derived to calculate the total sulfide concentration on basis of ORP and pH.

Microbiological analysis of the bacterial population in sulfide-oxidizing bioreactors revealed a domination of obligate chemolithoautotrophic SOB belonging to the genus *Thioalkalivibrio*. The subgroup enriched from reactors operating at pH 10 and high polysulfide concentrations clustered with the extremely halo-alkaliphilic core group of *Tv. jannaschii - Tv. versutus*.

Besides H_2S, several sour gasses contain volatile organic sulfur compounds such as methanethiol (MT). When MT is absorbed by the alkaline washing liquid, it greatly inhibits the biological oxidation of sulfide and polysulfide. However, in the alkaline solution MT reacts with sulfur particles to form a complex mixture consisting of inorganic polysulfides, dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS). By this reaction, any inhibiting effect on biological sulfide oxidation is reduced. Moreover, in presence of MT, sulfate formation ceases, even when oxygen is available at excess amounts. As thiosulfate formation is absent at these conditions, full (>99%) conversion of H_2S to elemental sulfur is achieved.

The research described in this thesis increases the understanding of the underlying processes that take place in a biotechnological process for the removal of H_2S from sour gas streams at natron-alkaline conditions. Compared to conventional physicochemical technologies, this novel process is expected to lead to substantial savings on energy and chemical consumption.

CONTENTS

Chapter 1

General Introduction

1.1 INTRODUCTION

1.1.1 Anthropogenic sulfur emissions

Anthropogenic sulfur emissions play a crucial role in local air pollution and acid deposition. In particular the emission of sulfur dioxide (SO_2), which is formed from the combustion of sulfur-containing fuel sources, is an important cause for these environmental problems. In the past, the combination of fog and SO_2 emissions from burning of coal ("classical smog"), has led to severe local air pollution. In the atmosphere, sulfur dioxide is oxidized to sulfuric acid. By this process, sulfur dioxide is the main precursor for acid rain and dry acid deposition, known for their adverse effects on forests, lakes and soils.

Fortunately, since the 1970s SO_2 levels in many industrialized nations declined as a result of various emission control strategies such as the selection of fuels with a low sulfur content, specialized combustion processes and flue gas desulfurization [1]. At present, fossil fuel combustion still accounts for approximately 90% of the global man-made emission of SO_2. Other anthropogenic sources of SO_2 are the processing of sulfide ores, oil refining and sulfuric acid production. Natural sources of SO_2 are emissions from soils, plants, burning of biomass and volcanoes [2].

An important aspect of SO_2 emission control is the treatment of gas streams containing hydrogen sulfide (H_2S). This toxic, malodorous gas can be present in many hydrocarbon gas streams such as landfill gas, biogas, natural gas, refinery gases and synthesis gas. Removal of H_2S (desulfurization) from these gases is required, as upon combustion it is converted to SO_2. In the atmosphere, H_2S causes acid rain due to its reaction with ozone to form sulfuric acid. Other reasons why H_2S has to be removed from gas streams is because of its toxicity, corrosivity and bad smell ("rotten eggs"). In biogas, a mixture of methane and CO_2 that is produced from anaerobic degradation processes, H_2S concentrations are in the range of 0.2–2 vol% (2000–20000 ppmv), depending on the sulfate concentration in the influent and pH value [3]. In natural gas, H_2S concentrations vary widely, from none at all to more than 50%.

Apart from H_2S, sour gasses may also contain volatile organic sulfur compounds (VOSCs) [4]. Like H_2S, most VOSCs are toxic, malodorous and have the same adverse environmental impact. The most common VOSC in biogas and natural gas is methanethiol (CH_3SH, or MT), but also higher thiols may be present [5, 6].

1.1.2 Characteristics of hydrogen sulfide and methanethiol

At room temperature and atmospheric pressure, both H_2S and MT are gasses. H_2S is a weak acid which dissociates into bisulfide (HS^-) and sulfide (S^{2-}). The pK_a values are 6.90 and 12.92 (at 25°C), respectively [7]. The term "sulfide" is applied for all three entities. Methanethiol (also known as methylmercaptan) is a weak acid, with a pK_a of 10.30 (at 25°C). Both H_2S and MT are toxic at low concentrations. The human toxicity of H_2S is well documented (Table 1.1). The MAC-value (maximum allowable concentration in workplace conditions) for H_2S is 1.6 ppm in the Netherlands and 10 ppm TWA (Time Weighted

Average) in the United States. Hydrogen sulfide becomes progressively more dangerous at H_2S levels above 70 ppm and lethal at 600 ppm. Inhalation studies with rats indicate that MT is less toxic compared to H_2S, but more toxic compared to higher thiols [8]. However, the MAC-value of MT (0.5 ppm in the Netherlands and in the United States) is even lower than that of H_2S.

H_2S is also a corrosive gas. By a combination of stress and corrosion, pipeline steels may break as a result of exposure to sulfide ("sulfide stress cracking") [9]. Therefore, pipeline specifications for the transport of natural gas limit H_2S concentrations to low levels of typically 4 ppm(v). Due to its corrosive effects and the formation of SO_2, the use of biogas in power engines is limited to H_2S concentrations of around 100 to 200 ppm(v).

TABLE 1.1 Health effects associated with releases of H_2S.

H_2S concentration (ppm)	Health effects
1	Rotten egg smell, odor complaints
10	Occupational exposure limit for 8 hours (USA)
20	Self-contained breathing apparatus required
100	May cause headaches/nausea, sense of smell lost in 2-15 minutes
200	Rapid loss of smell, burning eyes and throat
500	Loss of reasoning and balance, respiratory distress in 200 minutes
700	Immediate unconsciousness, seizures

1.2 H_2S REMOVAL FROM HIGH-PRESSURE NATURAL GAS

Natural gas primarily consists of methane, but can also include longer hydrocarbons, such as ethane, propane, butane and pentane. In the processing of natural gas, these longer hydrocarbons are separated as a valuable by-product, known as 'natural gas liquids' (NGLs). The typical composition of natural gas before refinery treatment is shown in Table 1.2.

TABLE 1.2 Typical composition of natural gas (after ref. [11]).

Compound	Formula	Concentration
Methane	CH_4	70-90%
Ethane	C_2H_6	0-20%
Propane	C_3H_8	0-20%
Butane	C_4H_{10}	0-20%
Carbon Dioxide	CO_2	0-8%
Oxygen	O_2	0-0.2%
Nitrogen	N_2	0-5%
Hydrogen sulfide	H_2S	0-5%
Rare gasses	Ar, He, Ne, Xe	trace

The main pollutants in natural gas are carbon dioxide and H_2S, although their concentrations vary widely. Natural gas is considered "sour" if the H_2S concentration exceeds 5.7 mg m^{-3} (4 ppmv). The search for new natural gas fields focuses on deep gas formations which often contain elevated H_2S concentrations [10].

The market for processes to remove H_2S from high-pressure natural gas and other refinery gasses can be roughly divided into three niches: small-size (<0.2 tons per day (TPD) of H_2S), medium-size (0.2 - 50 TPD) and large-size (>50 TPD). For the removal of H_2S from these gas streams, various well-established physicochemical techniques are available. Most of these processes can be grouped into the categories listed in Table 1.3 [1].

TABLE 1.3 **Examples of physicochemical processes available for treatment of H_2S containing sour gasses (after ref. [13]).**

Separation technologies	**Example**	**Absorbents**	**Products**
Liquid phase chemical reaction	Aqueous methyl-diethanolamine (MDEA)	Alkanolamines	H_2S
	Potassium carbonate	Alkaline salts	H_2S
Liquid phase physical absorption	Selexol	Dimethyl ether of polyethylene glycol	H_2S
Physical and chemical absorption	Sulfinol	Sulfolane and amines	H_2S
Conversion technologies	**Example**	**Reagents**	**Products**
Dry bed adsorption	Iron sponge	Iron oxide	S^0
	Molecular sieve	Crystalline alkali-metal Aluminosilicates	S^0
Direct conversion	Stretford	Sodium carbonate, sodium vanadate, anthraquinone	S^0
	Lo-Cat and SulFerox	Iron complexes	S^0
	Claus	Air (oxygen), catalyst	S^0
	Incineration	Air (oxygen)	SO_2

For low H_2S loads (i.e. <0.2 TPD), caustic scrubbing is often applied, resulting in the formation of sulfide-rich caustic streams, often referred to as "sulfidic spent caustics". These streams are regularly fed to biological wastewater treatment installations where they stimulate the growth of poor settling filamentous bacteria [12]. Alternatively, iron sponge beds, consisting of hydrated iron oxide on wood chips as a carrier are used for H_2S adsorption and conversion to iron sulfide (FeS). However, due to the heat of reaction and the pyrophoric nature of the formed FeS, considerable safety measures have to be taken.

Besides caustic scrubbing also other liquid phase processes are used to remove H_2S from sour gas streams via absorption. The most applied method is the "amine" treating process (left part in Fig. 1.1). In an absorber, H_2S is absorbed from the sour gas, by a solvent down to levels that are typically around 4-10 ppm(v). Depending on the type of solvent CO_2 can be absorbed as well. The extracted H_2S and CO_2 are released from the amine solvent by steam stripping, resulting in a concentrated H_2S stream often referred to as "acid gas". The regenerated amine solution is reused in the absorber. Obviously, a second process step is required to further treat this concentrated acid gas stream. At low H_2S loads (e.g. 0.1 TPD), the acid gas is sometimes sent to a flare, where it is burned to SO_2. Obviously, this option is not recommended due to its negative environmental impact.

An alternative for the treatment of acid gas is re-injection into the gas field. However, this solution is expensive and not always feasible as it depends on the composition of subsurface rock formations [14]. For H_2S loads above 20 TPD, a combination of amine treatment and the Claus process is commonly applied (Fig. 1.1). In the Claus process, 1/3 of the H_2S is burned to SO_2, whereafter the remainder reacts with SO_2 to elemental sulfur and water (Eq. 1):

$$2\ H_2S + SO_2 \rightleftarrows 3\ S^\circ + 2\ H_2O \qquad (1)$$

Because this is an equilibrium reaction, it is not possible to reach full (i.e. 100%) H_2S removal efficiency. In a line-up that consists of 3 consecutive process steps, an overall efficiency of 96% is possible. In order to reach more than 99% H_2S conversion, a so called "Tail Gas Treatment" (TGT) is required, such as the SCOT or Superclaus™ process. Obviously, this leads to a significant increase in the treatment costs, both capital and operational.

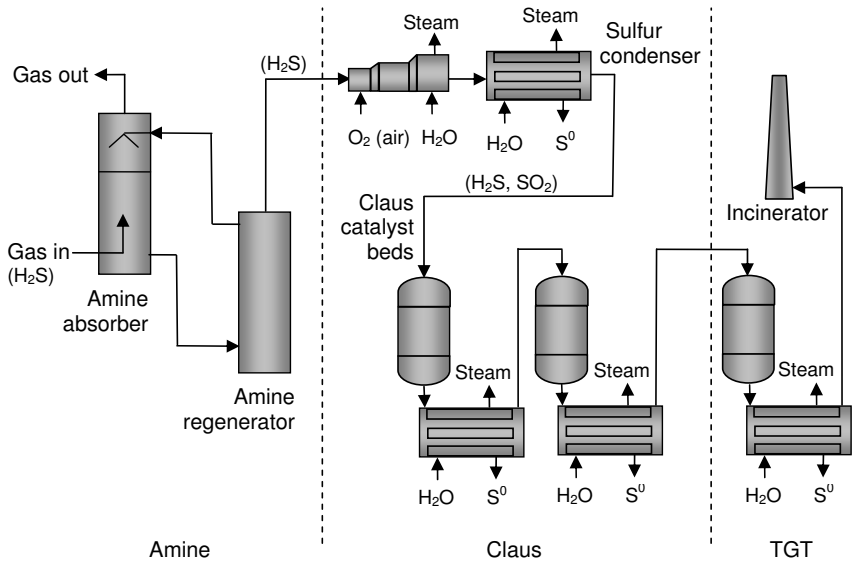

FIGURE 1.1 **Simplified scheme for the Amine/Claus/TGT process for the treatment of high-pressure sour gas (after ref. [15]).**

Treatment of natural gas in the medium-size niche of 0.2-20 TPD is challenging. The Stretford process was the first commercial liquid phase process for converting H_2S into elemental sulfur, by using vanadium salts as an oxidant. However, due to the formation of large amounts of thiosulfate as a by-product and problems related to the disposal of toxic vanadium-containing wastes generated by this process, it is no longer applied [16]. In the Lo-Cat® and SulFerox processes, conversion of H_2S to elemental sulfur is carried out in a liquid redox process. Sulfide ions are converted to elemental sulfur by the reduction of ferric iron (Fe^{3+}), which is kept in solution by a chelating agent, to ferrous iron (Fe^{2+}), which is subsequently re-oxidized Fe^{3+} by air. Due to sulfur plugging and foaming problems, many of these plants have high maintenance costs and downtime. They are primarily applied at relatively low pressures (i.e. < 35 bar) [15].

The lack of acceptable technologies for the treatment of natural gas in the medium-size niche means that often the well-proven amine/Claus/TGT combination is used. However, this combination is expensive and better applies for large-size applications. Therefore, the Gas Technology Institute (GTI, Des Plaines, United States) concluded that none of the existing technologies is suitable for treating high pressure natural gas in the medium-scale range of 0.2- 20 TPD [17]. Gas transport pipelines usually operate at high pressures (e.g. 40 bar or higher). Therefore, the equipment used for natural gas desulfurization must also operate at these high pressures. The presence of high CO_2 partial pressures complicates H_2S removal due to its negative effects on H_2S removal and sulfur recovery. The most important features that make a process suitable for the treatment of high-pressure natural gas are [15]:

- Ability to remove H_2S down to less than 4 ppmv;
- Unaffected by high CO_2 partial pressures;
- Low liquid circulation rates (i.e. low pumping costs);
- Liquids suited for high-pressure pumps;
- Low foaming tendency;
- Low plugging tendency;
- Low corrosivity;
- No formation of hazardous or difficult disposable end-products.

To overcome the problems related to gas desulfurization in the medium-size niche, microbiological processes are being considered as an alternative to existing technologies. As microbiological processes proceed around ambient temperatures and atmospheric pressure, the need for heat, cooling and pressurization power are limited and thereby cut the energy costs. Biological processes for H_2S removal involve microbial conversions that are part of the biological sulfur cycle.

1.3 APPLICATION OF THE BIOLOGICAL SULFUR CYCLE

Sulfur atoms occur in a wide range of oxidation states, from -2 to +6 (Table 1.4). Only sulfide, elemental sulfur and sulfate are thermodynamically stable under ambient conditions in the presence of water. Their particular stability depends on temperature, redox potential and pH value. Due to the large activation energy needed for the formation of sulfate, reduced sulfur compounds like polysulfides and thiosulfate can be present as metastable products in aqueous solution at ambient conditions [18]. The most stable form of sulfur is orthorhombic sulfur, which consists of eight S-atoms (S_8). Elemental sulfur of oxidation state zero is often described as S^0, which should not be mistaken for atomic sulfur.

TABLE 1.4 Oxidation state of some inorganic sulfur compounds (after ref. [19]).

-2	0	+2	+4	+6
Sulfide:	Elemental sulfur:	Sulfur monoxide:	Sulfur dioxide:	Sulfur trioxide:
H_2S	S_8	SO	SO_2	SO_3
HS^-	S^0	Sulfoxylic acid	Sulfurous acid:	Sulfuric acid:
S^{2-}		H_2SO_2	H_2SO_3	H_2SO_4
			Sulfite:	Sulfate:
			SO_3^{2-}	SO_4^{2-}
			Thiosulfate:	
	S----------		----------SO_3^{2-}	
			Thiosulfuric acid:	
H_2S----------				---------- SO_3
Polysulfide:				
S^{2-}----------	$S_{(x-1)}$			
Polysulfane:				
H_2S----------	----------$S_{(x-1)}$			

1.3.1 The biological sulfur cycle

Cyclic conversion of the element sulfur between the oxidation states of +6 and -2 plays an important role in nature. Continuous reduction and oxidation of sulfur compounds by bacteria are combined in the biological sulfur cycle (Fig. 1.2). Oxidized sulfur compounds (e.g. sulfate) are used as an electron acceptor in the metabolic pathways of a wide range of anaerobic bacteria [20], while reduced sulfur compounds (e.g. sulfide) serve as an electron donor for anaerobic phototrophic bacteria or provide energy for growth of the colorless sulfur bacteria. For the conversion of H_2S from gas streams, elemental sulfur is the preferred end-product, as it can be removed from the aqueous phase and re-used as a valuable raw material (see also section 1.4.3). Oxidation of H_2S by sulfur oxidizing bacteria (SOB) can be divided into phototrophic and chemotrophic processes. Phototrophic bacteria catalyze the photosynthetic "Van Niel" reaction under anaerobic conditions [21] (Eq.2):

$$2n\ H_2S + n\ CO_2 \xrightarrow{hv} 2n\ S^0 + (CH_2O)_n + n\ H_2O \qquad (2)$$

In this reaction, sulfide is used as an electron donor for the reduction of CO_2 to carbohydrates, using light as an energy source. Application of phototrophic bacteria on a large scale is not attractive, as extremely transparent solutions are needed for the provision of light. Moreover, the separation of sulfur and biomass is difficult, as many phototrophic SOB accumulate elemental sulfur internally.

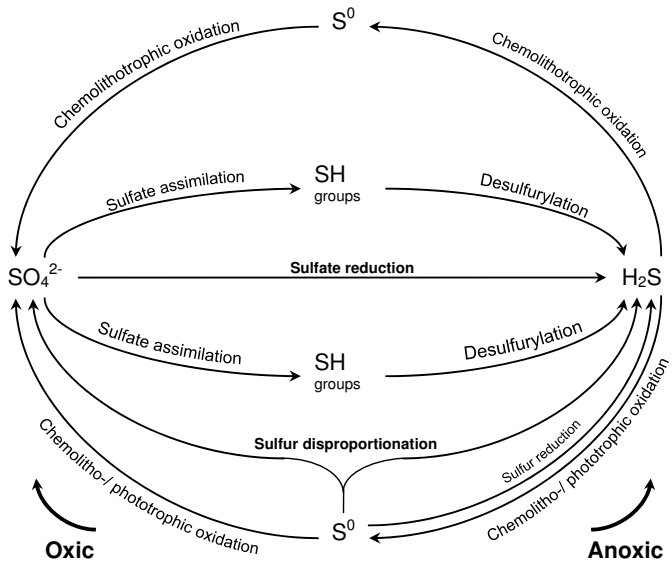

FIGURE 1.2 **The biological sulfur cycle.**

Chemotrophic ("colorless") SOB obtain their energy from the chemical aerobic oxidation of reduced sulfur compounds, with CO_2 (autotrophic) or organic compounds (heterotrophic) as the main source of carbon. With sulfide, the following two biological overall reactions can occur:

$$HS^- + ½\ O_2 \rightarrow S^0 + OH^- \qquad \Delta G^0 = -169.35\ kJ\ mol^{-1} \qquad (3)$$

$$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+ \qquad \Delta G^0 = -732.58\ kJ\ mol^{-1} \qquad (4)$$

With respect to bacterial respiration, sulfate formation (Eq. 4) is preferred over the formation of elemental sulfur (Eq. 3), as it yields the most energy. Therefore, sulfate formation will occur if there is enough oxygen available, while formation of elemental sulfur will only proceed at oxygen-limiting conditions [22, 23]. Due to their simple nutritional requirements, autotrophic bacteria are widely used for the removal of sulfide. In this respect, bacteria belonging to the genus *Thiobacillus* have been extensively studied [24-29]. The use of heterotrophic bacteria, that need organic carbon as a carbon source, has also been studied (e.g. *Xanthomonas* sp. strain DY44 [30]). However, application of heterotrophic organisms is not favorable as organic carbon compounds are in general not available in gas purification processes. Table 1.5 lists examples of chemotrophic sulfide oxidizing bacteria, together with some of their properties.

TABLE 1.5 Examples of chemotrophic sulfide oxidizing bacteria.

Species	Growth pH range	C-source	Sulfur globules	Ref.
Thiobacillus thiooxidans	2-5	autotrophic	extracellular	[31]
Thiobacillus thioparus	6-8	autotrophic	extracellular	[32]
Thiobacillus denitrificans	6-8	autotrophic	extracellular	[32]
Thiobacillus sp. W5	7-9	autotrophic	extracellular	[33]
Beggiatoa alba		mixotrophic	intracellular	[34]
Thiomicrospira pelophila	6-8	autotrophic	extracellular	[35]
Acidothiobacillus thiooxidans	2-5	autotrophic	extracellular	[32]
Acidothiobacillus ferrooxidans	2-6	mixotrophic	extracellular	[32]
Thioalkalivibrio versutus	7.5-10.5	autotrophic	extracellular	[36]
Thioalkalivibrio Jannaschii	7-11	autotrophic	extracellular	[37]
Thioalkalimicrobium cyclicum	7.5-10.5	autotrophic	extracellular	[37]
Xanthomonas	7	heterotrophic		[30]

Besides oxidative processes, also reductive processes in the biological sulfur cycle are applied in environmental biotechnology. In the presence of a suitable electron donor, such as hydrogen gas or ethanol, oxidized sulfur species such as thiosulfate and sulfate can be reduced to H_2S [20, 38-42]. It should be noted that from reductive processes, sulfide is always the end-product. Incomplete reduction of sulfate to elemental sulfur has so far not been reported [43].

Reductive processes are applied for the removal of sulfate from process streams with a too high sulfate concentration. The produced sulfide can be subsequently removed by partial oxidation to elemental sulfur. Biological sulfide production is commercially applied to remove metals from polluted groundwater or from aqueous streams in the mine and metal industry by the formation of the corresponding metal sulfide precipitates [44].

Microbial conversions involving volatile organic sulfur compounds (VOSCs) play an important role in cycling of volatile sulfur compounds between oceanic and freshwater compartments and the atmosphere. In marine ecosystems, dimethyl sulfide (DMS) and methanethiol (MT) can be formed by degradation of amino acids or dimethylsulfoniopropionate (DMSP), a common osmolyte in algae and halophilic plants in marine ecosystems. In freshwater, DMS and MT are formed by methylation of sulfide and to a lesser extent by the degradation of S-containing amino acids [45]. Microbial degradation of these VOSCs can occur under both aerobic and anaerobic conditions. Aerobically, MT can be completely oxidized to sulfate and CO_2 by chemolithotrophic SOB (e.g. *Thiobacillus* spp. [27, 31]) and methylotrophs (e.g. *Hyphomicrobium* spp.[46, 47]). Biological MT oxidation was proposed to occur after auto-oxidation of MT to DMDS and subsequent oxidation of DMDS to sulfate and carbon dioxide [48]. Anaerobic MT conversion is performed by methanogenic archaea and sulfate-reducing, phototrophic and denitrifying bacteria. Recently, methanogenic MT degradation at elevated salt concentrations and pH was studied for the development of a process for the biological conversion of MT, ethanethiol and propanethiol from LPG [49].

1.3.2 Biologically produced sulfur

From all elements, elemental sulfur has the largest number of allotropes (different chemical forms). Most sulfur allotropes consist of cyclic nonpolar molecules, while all are hydrophobic and hardly dissolve in water (5 μg L^{-1}) [50]. The build-up of elemental sulfur inclusions by *Beggiatoa* was already described in 1887, by the famous microbiologist Winogradsky [51]. Many authors have since described the formation and properties of elemental sulfur for both phototrophic [52-57] and aerobic bacteria [58-60]. Biologically produced elemental sulfur forms transparent droplets ("globules") which can be deposited inside or outside the cell and can reach diameters of up to 1 μm. While chemically produced ("inorganic") sulfur is very poorly soluble in water, biologically produced sulfur (or "biosulfur") particles are hydrophilic and dispersible in water [58]. Biosulfur has a white or pale-yellow color and has a lower density compared to inorganic sulfur. The buoyant density of elemental sulfur produced by *Chromatium* has been determined at 1.22 g cm^{-3} [52], while the average density of chemical sulfur allotropes is around 2 g cm^{-3} [61]. The different properties of biosulfur and inorganic sulfur allotropes indicate that biologically produced sulfur is not a standard sulfur form. Studies by X-ray absorption near edge spectroscopy (XANES) indicated that sulfur globules produced by chemotrophic bacteria consist of either S_8 rings or polythionates [60, 62, 63]. Because of their instability at alkaline conditions, it is however not likely that polythionates

play a role at elevated pH values. Research on sulfur globules, which can be stored intracellularly or excreted outside the cell membrane, indicate that the nature of these globules is different for different bacteria [63, 64]. As extracellular sulfur globules allow an easy separation of the sulfur, this type of biosulfur is of most interest for biotechnological application. From studies on the properties of sulfur produced by bacteria of the genus *Thiobacillus*, it was concluded that excreted colloidal biosulfur particles consist of a core of sulfur, on the surface of which polymeric organic compounds are adsorbed [65]. The relatively thick adsorbed layers of polymeric compounds are probably proteins, as indicated by charge density measurements, dynamic light scattering experiments and electrophoretic mobility experiments [66-68]. These organic polymers are assumed to play a role in the formation of larger sulfur aggregates, by sterical and electrical stabilization of the colloidal particles.

1.3.3 Application of biologically produced sulfur

Sulfur is one a fundamental feedstock for the chemical industry, mainly in the form of sulfuric acid. The vast majority (96%) of sulfur is used for fertilizer manufacturing. Other uses include production of carbon disulfide, sulfur dioxide, phosphorous pentasulfide, pulp and paper uses and rubber vulcanizing. In 2006, China imported more than 30% of the of the global trade in sulfur, corresponding to about 10 million tons [69]. In recent years, the price of sulfur on the world market has increased tremendously, mainly to the increased use of fertilizer in the United States and in China. In 2007, the price for sulfur was about 200 US$ per ton in 2007, 2.7 times the figure of that in 2006, while halfway 2008 the price increased further to up to 500 US$ per ton [70].

Dried biosulfur can be used in sulfuric acid production if its purity is at least 99%. As the sulfur purity of filtered sulfur sludge from biological desulfurization processes is around 95-98 %, additional washing steps are required to make it suitable for sulphuric acid production [71]. Alternatively, dried sulfur sludge can be sent to a smelter, where it is converted to high purity sulfur (>99.9%). At present, biologically produced sulfur is mostly sent to landfills. The increasing prices on the global sulfur market may however change this situation. Besides for sulphuric acid production, biologically produced sulfur can be used directly as a soil fertilizer e.g. for high sulfur-demanding crops or in agricultural areas with sulfur deficits [72]. The use of biosulfur as a soil fertilizer was demonstrated with canola, a rapeseed grown for the production of vegetable oil. Addition of biologically produced sulfur resulted in a 50% increase of the grain yield, even higher compared to commercially available sulfur sources used in the same study [71]. Biosulfur may also be used in agriculture as a biological fungicide, e.g. for the prevention of apple scab [73]. Another application for biosulfur is in bioleaching processes that are used in metal mining [74]. Sometimes additional sulfur is added in these processes, where low pH values are achieved by biological sulfide oxidation to solubilize metals in sulfide ores.

1.4 BIOLOGICAL PROCESS FOR H_2S REMOVAL

1.4.1 Bioscrubber process

One of the oldest and most commonly found applications of biological gas treatment involves the vent-air treatment at wastewater treatment plants. Worldwide more than 15000 of these systems are in operation of which most belong to the "biotrickling" and "biofilter" type [75, 76]. In the 1980s, laboratory research was initiated at Wageningen University into applications of the biological sulfur cycle to mitigate environmental problems caused by H_2S emissions [77]. This led to the development of a new process for the treatment of sour gasses such as biogas and landfill gas, based on the bioscrubber principle. A schematic representation of this process is given by the left-hand part of Figure 1.3. In an absorber column sulfide is absorbed from the gas into a slightly alkaline liquid (Eq. 5.A and B). The spent alkaline liquid is collected at the bottom of the absorber and directed to a second process step: the bioreactor. Here, air is dispersed at the bottom in order to enable the microbiological oxidation of dissolved sulfide to elemental sulfur (Eq. 6). By this process, hydroxyl ions (OH^-) that are consumed in the first step are produced, regenerating the alkaline liquid. The regenerated alkaline liquid is recycled over the absorber column for continuous removal of H_2S. The biologically produced sulfur particles are separated by gravity settlement whereafter dewatering of the sulfur sludge takes place, e.g. in a decanter centrifuge. The filtrate is returned to the bioreactor.

$H_2S(aq) + OH^- \rightleftarrows HS^- + H_2O$	(H_2S absorption)	(5.A)
$H_2S(aq) + CO_3^{2-} \rightleftarrows HS^- + HCO_3^-$	(H_2S absorption)	(5.B)
$HS^- + ½\ O_2 \rightarrow S^0 + OH^-$	(Biological sulfur production)	(6)
$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+$	(Biological sulfate formation)	(7)

At excess oxygen conditions, complete oxidation of H_2S to sulfate takes place (Eq. 7). Major disadvantages of sulfate formation are that:

- less re-usable elemental sulfur is formed;
- the formation of protons leads to acidification of the reaction medium;
- sulfate ions have to be removed by the addition of make-up water and the formation of a bleed stream.

To prevent the complete oxidation of H_2S to sulfate, the process is operated at oxygen-limiting conditions [23]. Advantages of this biotechnological process over the physicochemical technologies previously mentioned in section 1.2 are:

- high removal efficiency for hydrogen sulfide from sour gas;
- high biological activity, so that peak load and other variables in the production processes can be handled effectively;
- easily controlled process;

- operation at ambient temperature;
- operation at wide pressure range (0 to 80 bar);
- low operational costs;
- no production of a sulfide-containing waste stream;
- no use of chemical chelating agents;
- no hazardous bleed streams;
- beneficial use of produced elemental sulfur.

At present, the bioscrubber process is applied at moderately high salt concentrations and pH (e.g. 0.1 - 0.2 M Na^+ and pH 8). Most applications are used for the removal of H_2S from biogas, originating from anaerobic wastewater treatment facilities (UASB reactors) and landfill sites (around 80 installations worldwide, see Fig. 1.4). The first units for biogas desulfurization were applied in 1993. In the mid-nineties, the technology was further developed for the desulfurization of high pressure natural gas and refinery gas (commercialized as the *Shell-Paques* process, by Paques B.V. and Shell Global Solutions International B.V.). An overview of the development of this process is given in Figure 1.4 (Paques B.V., personal communication).

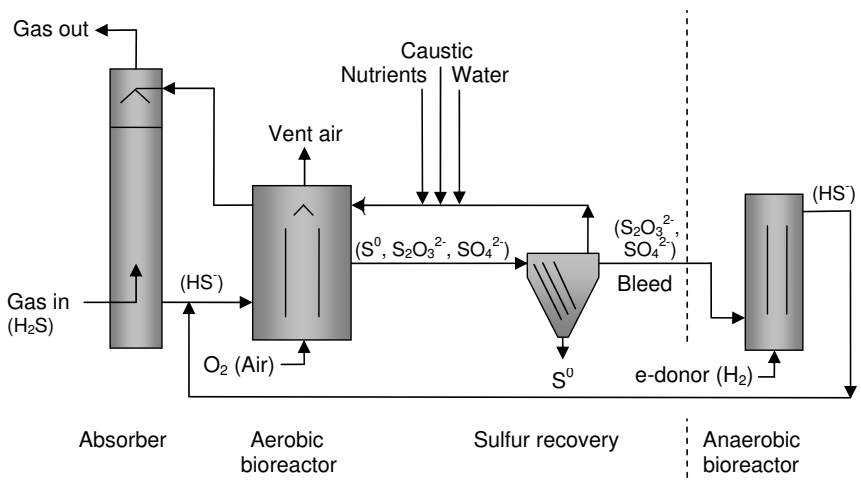

FIGURE 1.3 Biotechnological process for gas desulfurization. The part on the right of the dashed line shows the sulfate reduction step that is part of the new process innovations.

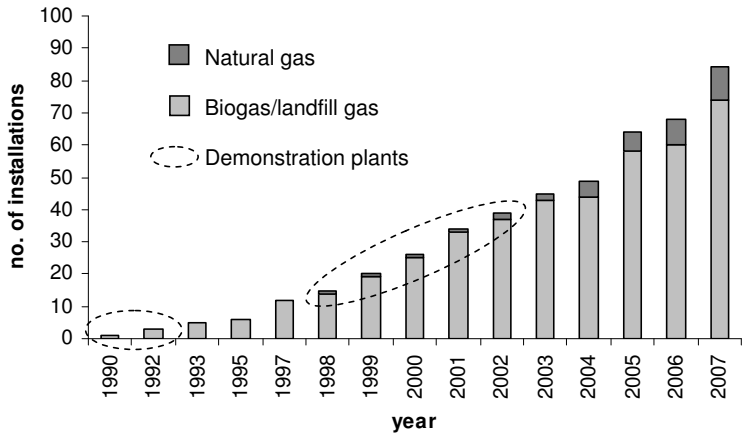

FIGURE 1.4 **Cumulative number of *Shell-Paques* and *Thiopaq* installations for biotechnological gas desulfurization.**

1.4.2 New biological process for natural gas desulfurization

The range of applicability of the current process can be extended when also treatment of gasses with high CO_2 pressures is feasible, such as high pressure (80 bar) natural gas with relatively high CO_2 concentrations (e.g. >1% v/v) . Therefore, a new biotechnological process has been developed to remove H_2S from sour gasses as produced in the oil and gas industry. In comparison to biotechnological removal of H_2S at moderately high salt and pH conditions, operating the process at high salt (halophilic) and high pH values (alkaliphilic conditions) leads to enhanced H_2S absorption and therefore to lower energy costs as less wash-water is recycled from the bioreactor to the absorber column.

Simultaneously with H_2S, also carbon dioxide (CO_2) is absorbed from the sour gas (Eq. 8.A and B). As a result, the bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) concentration will increase significantly at high pH values. Because sodium acts as the main counter ion, its concentration must also be much higher as compared to low pH values and low CO_2 partial pressures in the treated gas.

$$CO_2 + OH^- \rightleftarrows HCO_3^- \qquad (CO_2 \text{ absorption}) \qquad (8.A)$$

$$HCO_3^- + OH^- \rightleftarrows CO_3^{2-} + H_2O \qquad (CO_3^{2-} \text{ formation}) \qquad (8.B)$$

The bleed stream that is required to discharge the sulfate ions which are produced from the small fraction of H_2S that is completely oxidized can be prevented by directing the stream to a biological sulfate-reduction step. In this second -anaerobic- bioreactor, sulfate reducing bacteria (SRB) convert sulfate to sulfide with an electron donor such as ethanol or hydrogen. In a sulfate-reducing bioreactor fed with hydrogen gas, the following reaction will occur [20]:

$SO_4^{2-} + 4\ H_2 \rightarrow HS^- + OH^- + 3\ H_2O$ (Biological sulfate reduction) (9)

The produced sulfide is subsequently recycled to the first, sulfur-producing, reactor. By this, the need for make-up water and caustic can be reduced, which would be a major improvement in the feasibility and practical application of the new process. Because the sulfide-oxidizing bioreactor and the sulfate-reducing bioreactor are integrated, the sulfate-reducing bacteria are also exposed to high salt levels and pH values.

In case a sulfate reduction step is omitted, the bleed stream is preferentially kept as small as possible. This is especially important in dry areas or in case the bleed stream needs to be transported for disposal. If the process is operated at increased cation concentrations, also concentrations of sulfate can be higher. Consequently, the bleed stream and the use of make-up water can be reduced. Besides sulfate, also sodium (bi)carbonate is removed from the system with the bleed stream. Therefore, reducing the bleed stream also leads to reduced consumption of caustic (NaOH) to re-establish the (bi)carbonate equilibrium that is disturbed by addition of fresh make-up water.

The elevated sodium (bi)carbonate and -sulfate concentrations and the high pH in the new process will affect the (bio)chemistry of sulfur formation in at least two ways. First of all, the new process requires sulfide-oxidizing and sulfate-oxidizing bacteria that thrive at these double-extreme conditions. Another difference with the existing process is that due to the elevated pH values, polysulfide anions will play a more important role in the inorganic sulfur chemistry. Both issues will be discussed in more detail in the next sections.

1.5 SULFUR CHEMISTRY

1.5.1 Polysulfide chemistry

Polysulfides are unbranched chains of sulfur atoms, related to the polysulfanes (H_2S_x). Polysulfide ions in solution have a characteristic yellowish color and can be formed from the reaction of sulfide with elemental sulfur (Eq. 10):

$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+$ (abiotic polysulfide formation) (10)

Polysulfide ions of chain length x can be represented by the formula S_x^{2-}. The chain length x varies from 2 to 9 but at moderately alkaline conditions, S_4^{2-}, S_5^{2-} and S_6^{2-} ions dominate [78]. Polysulfide solutions absorb radiation in the UV region, which can be used to quantify the concentration of polysulfide ions in solution [79-81]. However, this method cannot distinguish between polysulfide ions of different chain lengths. To overcome this problem, Kamyshny et al. developed a method in which polysulfide ions are methylated and separated by chromatography [78, 82].

Polysulfide ions play an important role in alkaline biotechnological H_2S removal processes and are considered to be intermediates in the oxidation of sulfide, both biologically as well as abiotically [83, 84]. This becomes increasingly important at elevated pH values as the equilibrium (Eq. 10) shifts towards the right hand side. The reaction between sulfide and biosulfur takes place at the surface of the biologically produced sulfur particles. Polysulfides formed in this reaction exhibit an a autocatalytic effect on the reaction rate [68]. The equilibrium can be expressed by an equilibrium constant K_x, which consists of a weighted sum of constants for polysulfide ions of different chain length. For biologically produced sulfur, this value is 9.17±0.09 with an average polysulfide chain length of 4.59±0.31 (both at 35 °C) [85]. The value of K_x found for biosulfur is higher than that of inorganic sulfur. A straightforward explanation cannot be provided, but this effect is probably related to the presence of a polymeric organic layer on the surface of the biosulfur particles [60]. From lab-scale bioreactor experiments, it was found that the reaction between sulfide and elemental sulfur is close to equilibrium in the liquid coming from the gas absorber. If small hydrophilic sulfur particles are present in the absorber liquid, the formation of polysulfides enhances H_2S absorption. Conditions favoring this enhancement are low liquid side mass transfer, high polysulfide concentration, high pH, high sulfur content and small particle size [86].

1.5.2 Abiotic oxidation of sulfide and polysulfide

Besides biological oxidation, also abiotic oxidation of sulfur compounds may play a role in biotechnological processes for H_2S removal from sour gasses. Abiotic oxidation of sulfide anions is complex and several different reaction products can be formed such as sulfur, thiosulfate, sulfite and sulfate. Which product is formed depends on the experimental conditions such as pH, sulfide and dissolved oxygen concentration, type of buffer used and the presence of metal ions which may catalyze abiotic sulfide oxidation [87]. Abiotic oxidation of sulfide and polysulfide is thought to be the main cause for the formation of thiosulfate at neutral to alkaline pH conditions, following Equation 11 and 12. Apart from biological sulfur formation, also the oxidation of polysulfide ions yields elemental sulfur (Eq. 12). Abiotic oxidation of polysulfide anions proceeds faster than sulfide oxidation [88, 89]. Kleinjan et al. found that in phosphate buffers, the specific rate of oxygen decrease for polysulfide oxidation is approximately 3-5 times higher than for sulfide solutions [90]. At 30 °C and above pH 10, the same authors report formation of thiosulfate and sulfide from the disproportionation of elemental sulfur (Eq. 13). For inorganic sulfur, this reaction is reported to occur at temperatures close to 100 °C [91].

$$HS^- + O_2 \rightarrow 0.5\ S_2O_3^{2-} + 0.5\ H_2O \quad \text{(abiotic sulfide oxidation)} \qquad (11)$$

$$S_x^{2-} + 1.5\ O_2 \rightarrow S_2O_3^{2-} + (x\text{-}2)\ S^0 \quad \text{(abiotic polysulfide oxidation)} \qquad (12)$$

$$4\ S^0 + 4\ OH^- \rightarrow S_2O_3^{2-} + 2\ HS^- + H_2O \quad \text{(sulfur disproportionation)} \qquad (13)$$

1.6 NATRON-ALKALIPHILIC MICROORGANISMS

The high pH value and high sodium (bi)carbonate concentrations that are inherent to the new process for H_2S removal (Fig 1.3) require SOB that can thrive at these double-extreme conditions. Organisms living in natural alkaline environments with a high salt content, such as soda lakes, have developed strategies to cope with the high pH and salinity. Therefore suitable candidates for application in a biological desulfurization process can be found in such environments.

1.6.1 Soda lakes

Soda lakes are generally confined to arid and semi-arid regions, where evaporation rates exceed inflow rates. Well-known soda lakes are located in the East African Rift Valley (lake Bogoria), Egypt (Wadi Natrun lakes), North America (Mono lake, Soap lake) and central Asia (Kulunda steppe lakes). The concentration of salts and other minerals in soda lakes is often higher than that of seawater. Of the cations in solution, sodium is usually the most abundant, followed by calcium (Ca^+) and magnesium (Mg^+). The major anions are chloride (Cl^-), sulfate (SO_4^{2-}) and (bi)carbonate. As a result of high concentrations of (bi)carbonate, the lakes have a high buffer capacity, with pH values up to 10.5. Despite the double extreme conditions in soda lakes, most of these lakes have a large microbial diversity [92-99]. In general, microorganisms living in soda lakes are referred to as halo-alkaliphilic. The term “halophilic” refers to sodium chloride loving organisms, while the term “alkaliphilic” refers to preference for a high pH. However, in case of microorganisms living at high sodium carbonate concentrations instead of sodium chloride, it would be better to use the term “natronophiles”. For microorganisms living at high sodium concentrations and high pH, as in the new biological desulfurization process, the term “natron-alkaliphilic” is suggested.

1.6.2 Natron-alkaliphilic sulfur bacteria

The sulfur cycle is among the most active microbial processes in soda lakes [99]. Sulfate generated by microbiological oxidation processes is converted by sulfate-reducing bacteria (SRB) such as *Desulfonatronovibrio* and *Desulfonatronum* [95]. As mentioned above, aerobic chemolithoautotrophic SOB are of most interest for the application of alkaliphilic bacteria in the desulfurization process. In soda lakes, two different groups have been discovered, belonging to the genera *Thioalkalimicrobium* and *Thioalkalivibrio* [100]. In general, *Thioalkalimicrobium* strains show high growth rates, low growth yield, high maintenance and high rates of thiosulfate and sulfide oxidation. This strategy makes that *Thioalkalimicrobium* strains become dominant during short periods of excessive substrate supply, being able to outcompete other SOB (“R-strategist”). *Thioalkalivibrio* strains on the other hand are in general slow growing bacteria with a high growth yield, low maintenance and low thiosulfate and sulfide oxidation rates. As a result, this group can survive during prolonged periods of starvation (“K-strategist”). The growth pH range (7.5-10.6) and optimum (9.5-10) for both

types is more or less the same. In contrast to the similar response to pH, an important difference between both groups is their response to sodium concentrations. Whilst *Thioalkalimicrobium* strains generally are able to grow in the presence of 0.3–1.5 M Na^+, most *Thioalkalivibrio* strains show active growth and respiration within a very broad salinity range between 0.3 and 4 M Na^+, with an optimum between 0.5–2 M. This feature makes representatives of *Thioalkalivibrio* the most suitable candidates for application in the new process for gas desulfurization. The genus *Thioalkalivibrio* is found in soda lakes all over the world, with more than a hundred strains isolated from sediment samples [101].

The soda-lake sulfur bacteria that are used in the new biodesulfurization process have to withstand double extreme conditions (i.e. high salt concentration and high pH values). As the cell membrane is permeable to water, the osmotic pressure on the inside of the cell (the cytoplasm) needs to be equal to that of the external environment to prevent the loss of water. It has to be mentioned that the osmotic pressure depends on the type of salt. Because sodium carbonate is a weaker electrolyte than sodium chloride, the latter requires a higher level of bacterial adaptation [102]. Moreover, sodium carbonates are not completely dissociated at high pH, leading to lower free sodium concentrations compared to sodium chloride. Natron-alkaliphilic SOB solve the problem of osmo-adaptation by the formation of small organic molecules, referred to as "compatible solutes". In the extremely salt tolerant *Thioalkalivibrio versutus* strain ALJ 15, the compatible solute glycine betaine was found as the main compatible solute [103]. The formation of compatible solutes is energetically expensive and thereby decreases the amount of energy available for growth [104]. Also adaptations of the membrane are related to osmo-adaptation, to prevent leakage of Na^+ and H^+ ions [105]. Besides adaptations to osmotic stress, also adaptations to high pH values are required, as bacterial membranes become instable at high pH and functioning of intracellular enzymes is limited to a pH around 10 [106]. Adaptations to high pH, include cell wall modifications and trapping of protons combined with removal of sodium ions [107].

1.7 SCOPE AND OUTLINE OF THE THESIS

This PhD thesis is part of a larger project: "Application of the biological sulfur cycle under halo-alkaliphilic conditions for high-pressure natural gas desulfurization" which was funded by the Dutch Technology Foundation STW. As a part of this project, the detection and identification of halo-alkaliphilic bacteria involved in the sulfur cycle in soda lakes and bioreactors has been described in the PhD thesis of Mirjam Foti (Delft University of Technology) [99]. Physiological properties of halo-alkaliphilic sulfide-oxidizing bacteria, their interaction with sulfate-reducing bacteria and their spatio-temporal distribution in reactor systems were studied by Dimitry Sorokin (Department of Biotechnology, Delft University of Technology). Chapter 7 of this thesis is a direct result of his efforts to study the microbial population of the lab-scale bioreactors used in the current PhD research. Characterisation of alkaliphilic sulfate-reducing bacteria that may be suitable for application in the new process

for H_2S removal from sour gas streams was studied by Bart Lomans and Anne-Meint Henstra (Laboratory of Microbiology, Wageningen University).

The goal of the current thesis is to study the engineering aspects of biological sulfide oxidation at natron-alkaline conditions in lab-scale bioreactors. Particular emphasis is given to the formation of elemental sulfur and the side-products sulfate and thiosulfate. **Chapter 2** describes experiments that demonstrate the feasibility of the new process for H_2S oxidation. In this chapter it is shown that not the formation of sulfate, but the formation of thiosulfate plays a major role in the selectivity of the process for sulfur formation. In **Chapter 3,** the unwanted formation of thiosulfate is studied in more detail. A pathway is proposed by which the selectivity for thiosulfate formation is related to pH. The use of the redox potential to control product formation is explained in more detail in **Chapter 4**. In this chapter also the effect of fluctuations in the redox conditions are evaluated. In **Chapter 5** a study of the toxicity of methanethiol and the products from the reaction between methanethiol and biosulfur particles is presented. Lab-scale bioreactor experiments with methanethiol are presented in **Chapter 6**, focusing on the effect of methanethiol on product formation. The composition and characteristics of the microbial population in lab-scale bioreactors are described in **Chapter 7**. Finally, **Chapter 8** summarizes the findings presented in previous chapters and combines the results to shortly discuss the application of the new process in practice.

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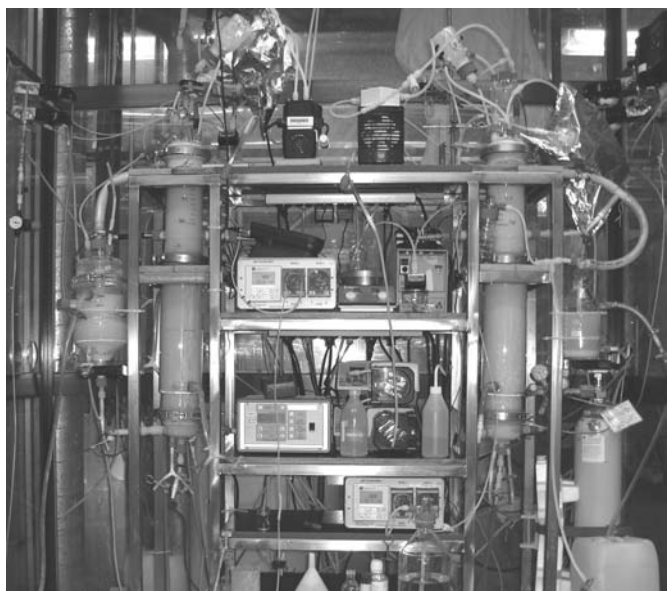

Chapter 2

Sulfide oxidation at natron-alkaline conditions in a fed-batch bioreactor

Abstract

A biotechnological process is described to remove hydrogen sulfide (H_2S) from high-pressure natural gas and sour gasses produced in the petrochemical industry. The process operates at halo-alkaline conditions and combines an aerobic sulfide-oxidizing reactor with an anaerobic sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) reducing reactor. The feasibility of biological H_2S oxidation at pH around 10 and total sodium concentration of 2 mol L^{-1} was studied in gas-lift bioreactors, using halo-alkaliphilic sulfur oxidizing bacteria (SOB). Reactor operation at different molar oxygen to sulfide (O_2/H_2S) supply ratios resulted in a stable low redox potential that was directly related with the polysulfide (S_x^{2-}) and total sulfide (S^{2-}_{tot}) concentration in the bioreactor. Selectivity for sulfate formation decreased with increasing polysulfide and total sulfide concentrations. At total sulfide concentrations above 0.25 mM, selectivity for sulfate formation approached zero and the end products of H_2S oxidation were elemental sulfur (S^0) and thiosulfate. Maximum selectivity for sulfur formation (83.3±0.7%) during stable reactor operation was obtained at a molar O_2/H_2S supply ratio of 0.65. Under these conditions, intermediary polysulfide plays a major role in the process. Instead of dissolved sulfide (HS^-), polysulfide seems to be the most important electron donor for SOB at sulfur producing conditions. In addition, abiotic oxidation of polysulfide is the main cause of undesirable formation of thiosulfate. The observed biomass growth yield under sulfate producing conditions was 0.86 g N mol^{-1} H_2S. When selectivity for sulfate formation was below 5%, almost no biomass growth was observed.

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2.1 INTRODUCTION

Hydrogen sulfide (H_2S) is known for its toxicity, corrosive properties and bad odour. When H_2S is combusted, sulfur dioxide (SO_2) is formed, which contributes to acid rain. Biotechnological removal of H_2S from biogas originating from anaerobic wastewater treatment facilities and landfill sites, has been successfully applied since 1993 [1]. Bulk removal of H_2S 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 H_2S. 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 H_2S concentrations are expected to be treated in the near future. This creates a need for new desulfurization technologies, such as the biotechnological removal of H_2S, using micro-aerophilic bacteria [2]. The first full-scale plant for biotechnological desulfurization of natural gas, designed to treat 1000 kg H_2S per day, has been taken into operation in 2003 [1]. For H_2S loads up to 50 tons per day, a biotechnological process has several advantages compared to existing physicochemical methods [1]: (1) the process is safe because all H_2S 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 (S^0). In contrast to chemically produced sulfur, biologically produced sulfur is hydrophilic [3] 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 gasses 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 Figure 2.1. 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_2S-containing gas is contacted with an alkaline solution. Hydroxide (OH^-) ions and carbonate (CO_3^{2-}) ions are consumed to absorb H_2S gas under the formation of hydrosulfide (HS^-, further referred to a sulfide, Eq. 1 and 2.A-B). The loaded solution is fed to a bioreactor, where sulfide is biologically oxidized with dissolved oxygen (O_2) to elemental sulfur, according to Eq. 3. In the oxidation step, which operates at atmospheric pressure, the formation of insoluble sulfur is accompanied by the regeneration of hydroxide 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.

$$H_2S(g) \rightleftarrows H_2S(aq) \quad (1)$$

$$H_2S(aq) + OH^- \rightleftarrows HS^- + H_2O \quad (2.A)$$

$H_2S(aq) + CO_3^{2-} \rightleftarrows HS^- + HCO_3^-$ (2.B)

$HS^- + ½\ O_2 \rightarrow S^0 + OH^-$ (3)

$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+$ (4)

$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+$ (5)

$S_x^{2-} + 1½\ O_2 \rightarrow S_2O_3^{2-} + (x\text{-}2)\ S^0$ (6)

$HS^- + O_2 \rightarrow ½\ S_2O_3^{2-} + ½\ H_2O$ (7)

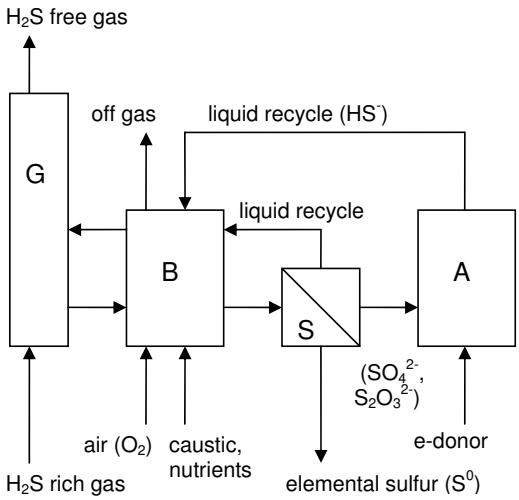

FIGURE 2.1 Simplified scheme of the process for biotechnological removal of H_2S from gas streams. G = gas absorber, B = sulfide oxidizing bioreactor, S = sulfur settler, A = anaerobic bioreactor.

In addition to the biological oxidation of sulfide to sulfur, undesirable side reactions involving sulfide 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_x^{2-}) oxidation. Formation of sulfate was already described in studies involving sulfur oxidizing bacteria (SOB) at neutrophilic conditions [4, 5]. It was found that under sulfide limited conditions, sulfide is completely oxidized to sulfate, according to Eq. 4. Dissolved sulfide can also react with biologically produced sulfur to polysulfide, according to Eq. 5 [6]. In presence of dissolved oxygen, polysulfide is abiotically oxidized to sulfur and thiosulfate, according to Eq. 6 [7]. This abiotic oxidation of polysulfide occurs more rapidly than abiotic oxidation of sulfide to thiosulfate (Eq. 7) [8, 9].

Major disadvantages of sulfate and thiosulfate 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 sulfate and thiosulfate results in a higher oxygen demand; (4) to prevent accumulation, sulfate and thiosulfate 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 sulfate and thiosulfate can be higher. Consequently, the bleed stream and the use of make-up water are much lower, provided that the selectivity for sulfate and thiosulfate 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 sulfate and thiosulfate is biologically reduced to sulfide, where after the produced sulfide is recycled to the sulfur producing bioreactor. For this anaerobic step, a suitable e-donor such as hydrogen, ethanol or methanol is required [10-12].

To reduce energy costs for pumping the liquid recycle from the bioreactor to the high pressure absorber, it is beneficial to maximize the H_2S loading capacity of the alkaline solution. This can be achieved by increasing the pH and the carbonate concentration by the addition of sodium hydroxide (NaOH). Simultaneously with H_2S, 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 HCO_3^- 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 [13]). However, KOH is more expensive than NaOH and therefore a mix of NaOH and KOH is recommended.

In comparison to conventional biotechnological processes for H_2S removal from biogas, the new halo-alkaline process is developed to treat much higher H_2S and CO_2 concentrations in more compact units. Table 2.1 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 sulfur under oxygen-limiting conditions [14]. *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 [15]. Both genera grow optimally at pH around 10, while *Thioalkalivibrio* strains are in general more salt tolerant compared to *Thioalkalimicrobium* strains [14].

The aim of the present study was to determine the feasibility of biological H_2S oxidation under halo-alkaline conditions in gas-lift reactors by using new isolates, originating from soda lakes. Special attention was paid to selectivity for S^0 formation in relation to the molar O_2/H_2S consumption ratio. In addition, biomass growth yield and the role of S_x^{2-} in product formation was studied.

TABLE 2.1 pH, salt concentration and ionic strength in the biotechnological process for H_2S removal under slightly alkaline conditions (e.g. for the treatment of biogas) compared to the natron-alkaline conditions in the new process (e.g. for the treatment of sour natural gas).

	slightly alkaline conditions (biogas)[a]	natron-alkaline conditions (sour natural gas)
pH	8 - 8.5	8.5 – 10.5
$[Na^+] + [K^+]$ (M)	0.11	2.0 - 4.0
$[Na^+] : [K^+]$	250:1	1:2
Ionic strength (M)	0.12	>2.7

[a] In fed-batch experiments [16]

2.2 MATERIALS AND METHODS

2.2.1 Experimental Set-Up

Two identical gas-lift reactors were used with a wet volume of 4.8 L each (Fig 2.2). The gas flow (300 L h^{-1}) was completely recycled to prevent any release of H_2S gas and to reach low oxygen concentrations. Pure H_2S gas (99.8%,) and oxygen (99.995%) were supplied by mass flow controllers (Brooks Thermal Mass Flowmeter, type 5850e, model 0154, 0-30 mL min^{-1} (H_2S) and 0-150 mL min^{-1} (O_2)). Nitrogen gas (N_2, 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.

2.2.2 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 demineralised water): K_2HPO_4, 1.0; $NaNO_3$, 0.83; NaCl, 6.0; $MgCl_2 \cdot 6\ H_2O$, 0.20. NO_3^- was used as nitrogen (N) source, because of its stability under alkaline conditions [17]. Trace elements solution was added as described in [18]. After addition of all compounds, the pH of the medium was 10.2±0.1 at 35°C.

2.2.3 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 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 [15] and [14]. Each subsequent fed-batch experiment was started with biomass from previous experiments.

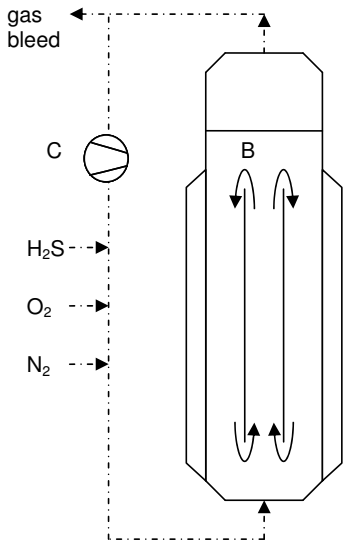

FIGURE 2.2 Flow sheet of the experimental set-up used for fed-batch experiments. C = compressor, B = bioreactor.

2.2.4 Reactor start-up

Reactors were filled with medium and inoculated with centrifuged biomass (750 – 1000 mL before centrifugation). The total liquid volume after inoculation was 4.7 L at 35°C. After temperature stabilization, addition of H_2S was started. Throughout all experiments, the H_2S 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% sat. by control of the O_2 supply to the gas recycle flow. After passing the start-up phase, the O_2 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 H_2S was kept constant throughout all experiments, the O_2/H_2S supply ratio could only be varied by changing the O_2 supply. Because the medium was highly buffered, no pH control was necessary.

2.2.5 Analysis

Reactors were equipped with sensors for temperature, pH (Hamilton Flushtrode T200), DO concentration (Mettler Toledo lnpro 650/120) and oxidation-reduction potential (ORP, WTW SenTix ORP Ag/AgCl electrode). 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^{2-}_{tot}). At the experimental pH range of 9.5 – 10.1, concentrations of H_2S and S^{2-} are negligible. Polysulfides of chain lengths 2 and 3 can be present in solution in both protonated and non-protonated form (pKa of HS_2^- is 9.7, pKa of HS_3^- is 7.5, [19]. However, it was shown by [20] that the fraction of S_2 and S_3 species is low compared to the total polysulfide concentration (around 5% at pH 9.5, less at higher pH values). Consequently, the concentrations of HS_2^{2-} and HS_3^{2-} will be negligible compared to the total sulfide concentration (0.5% of $[S^{2-}_{total}]$ at pH 9.5). Therefore, the following equation applies:

$$[S^{2-}_{tot}] = [HS^-] + [S_x^{2-}] \quad (8)$$

The method used was based on a modified methylene blue method as described by [21]. 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 demineralised water to remove dissolved salts. The S^{2-}_{tot} concentration in the diluted zinc sulfide precipitate was measured with the Lange cuvette test LCK653 (Hach Lange, Germany). The concentration of polysulfide was determined spectrophotometrically as described by Kleinjan et al., (2005), at a wavelength of 285 nm (Perkin-Elmer, Lambda 2, UV/VIS-spectrophotometer) [22]. With this method, the total concentration of zerovalent sulfur atoms in polysulfide was determined: $[S_x^{2-}\text{-}S^0]$. When the average chain length x is known, the polysulfide concentration can be calculated according to:

$$(x\text{-}1) \cdot [S_x^{2-}] = [S_x^{2-}\text{-}S^0] \quad (9)$$

Before analysis, samples were filtrated over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66). The molar extinction coefficient was determined for the high salt medium and was found to be 1300 L mol^{-1} cm^{-1}. This value is somewhat lower than reported data of 1325 L mol^{-1} cm^{-1} [23], 1350 L mol^{-1} cm^{-1} [22] and 1360 L mol^{-1} cm^{-1} [19]. Possibly, this is due to matrix effects of the high salt medium. Concentrations of sulfate and thiosulfate were determined by ion chromatography (Dionex DX-600 model 50, Salt Lake City, USA). An ionpac AS17 column was used at 30ºC 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, Salt Lake City, USA), equipped with a KOH cartridge, using deionised 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 mannitol solution to avoid

interference by carbonate ions. Presence of sulfite (SO_3^{2-}) was detected using a test-strip (Quantofix 91306). The concentration of elemental sulfur in the reactor was calculated by the mass balance on basis of the H_2S supply and measurement of dissolved sulfur products formed. Composition of the gas phase (H_2S, N_2, CO_2 and O_2) was determined with a gas chromatograph (Fisons Instruments GC 8000) equipped with two columns: 1.5 m x 1/8 inch teflon packed with Chromosorb 108 (60-80 mesh) and 1.2 m x 1/8 inch stainless steel packed with molecular sieve 5A (60-80 mesh). Both columns were connected in parallel with a split of 1:1. The carrier gas was helium, with a flow rate of 45 mL min^{-1}. Temperatures were 40ºC for the column, 110ºC for the injection port and 100º for the thermal conductivity detector. The gas flow leaving the bioreactor was also analysed incidentally by a gas detector (Dräger polytron 2 transmitter). This detector detects all volatile sulfur compounds, including organic sulfur compounds, with an electrochemical sensor. Biomass concentration was measured as the amount of total nitrogen, based on the absorbance of nitrophenol at 370 nm with the Lange cuvette test LCK238 (Hach Lange, Germany). Prior to analysis, samples were centrifuged (10 min. 10000 rpm) and washed two times with nitrogen-free medium to remove all dissolved nitrogen. 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 sulfur. Presence of biologically produced sulfur did not affect the results.

2.3 RESULTS

2.3.1 Results of a typical fed-batch experiment

Results of a typical fed-batch experiment are shown in Figure 2.3.A-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_2S supply (t=0 h), the ORP dropped to -230 mV and some thiosulfate 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_2S added. Under these conditions, some sulfide accumulated in the medium and was abiotically oxidized to thiosulfate, as was also found in several other studies at more neutral pH conditions [16, 24]. At t=5 h, the ORP increased to +90 mV, while all H_2S supplied was completely oxidized to SO_4^{2-}. In the subsequent 10 h, also the accumulated thiosulfate was oxidized to sulfate (Fig. 2.3.A, period I). The O_2/H_2S supply ratio in this period was 1.9, which is somewhat below the stoichiometrical value of 2 for sulfate formation (Eq. 4). The slightly lower value is due to assimilation of CO_2 into cellular material, for which reductive equivalents are needed [16, 25]. At t=18.5 h, the oxygen supply was manually decreased from 19 to 7 mmol h^{-1}, resulting in a molar O_2/H_2S supply ratio of 0.7. As a result of the limited oxygen addition, the DO concentration dropped rapidly below the detection limit of 0.1 mg L^{-1} (Fig. 2.3.C, period II). It can be seen that in the next 3 hours, some sulfide and polysulfide accumulated, which was accompanied by an initial rapid drop of the ORP (Fig. 2.3.B, period II). The formation of

polysulfide within 1 hour after reducing the oxygen supply indicates that sulfur formation also started within 1 hour. However, the biological sulfide oxidation rate was too low to convert all incoming H_2S. From 20 to 22 h, sulfide accumulated at an average rate of 1.6 mmol h^{-1} (data not shown) to a final concentration of 0.73 mM S^{2-}_{tot}. This means that at least 16% of the supplied H_2S was not converted.

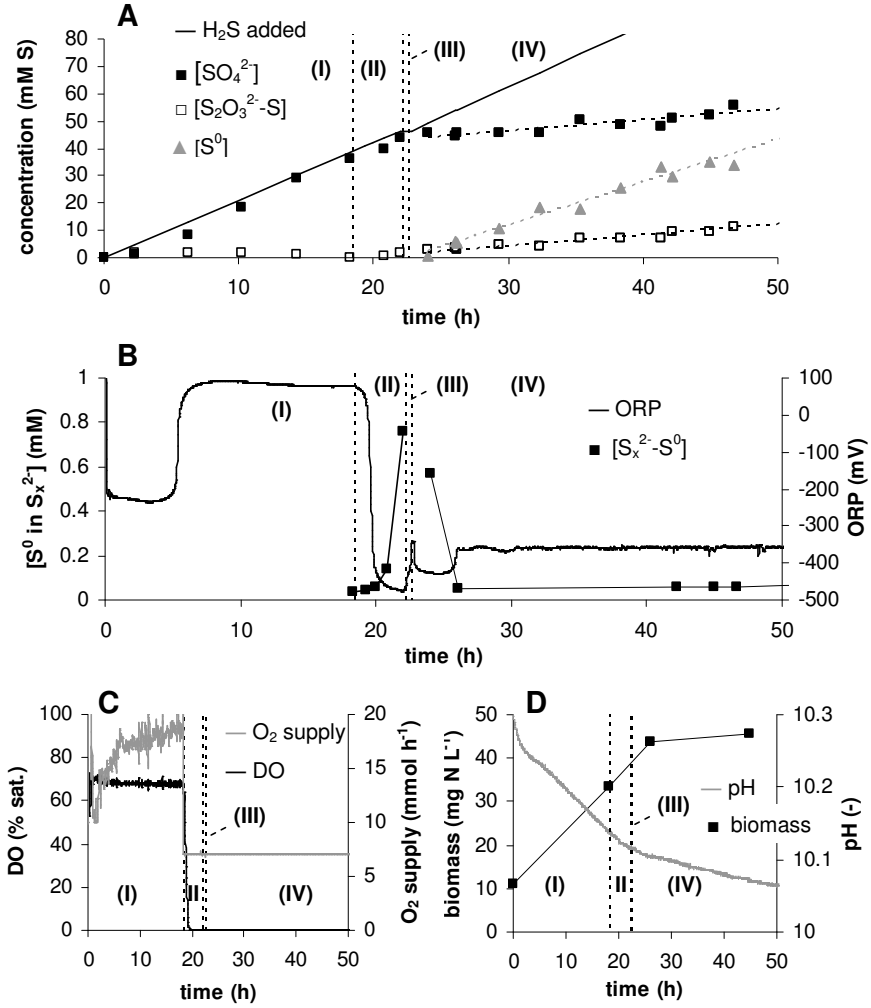

FIGURE 2.3 A-D **Results of a typical fed-batch experiment. A: Concentrations of sulfate, $S_2O_3^{2-}$ and S^0 and total H_2S added; B: Concentration of S_x^{2-}-S^0 and ORP; C: O_2 supply and DO concentration; D: Biomass concentration and pH. The vertical dashed lines border the start-up phase (I), operation with a molar O_2/H_2S supply ratio of 0.7 (II), brief stop of the H_2S supply (III) and resume of the H_2S supply at a molar O_2/H_2S supply ratio of 0.7 (IV).**

To prevent inhibition of the biomass, the H_2S supply was briefly stopped at t=22.1 h. The system responded immediately by a decrease of the polysulfide concentration and an increase of the ORP (Fig. 2.3.B, period III). When the ORP reached -340 mV, H_2S 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 polysulfide (0.059±0.001 mM S_x^{2-}-S^0) remained stable, while the S^{2-}_{tot} concentration remained below 0.02 mM.

During the period of limited oxygen supply of 7.0 mmol h^{-1} (O_2/H_2S supply ratio = 0.7), sulfate, thiosulfate and sulfur accumulated (Fig. 2.3.A, period IV). No sulfit could be detected. The oxygen concentration in the gas phase was stable at 11.1±0.4% and no H_2S was detected in the gas flow leaving the reactor, even when sulfide accumulated in the reactor medium. This demonstrates that the transfer rate of both gasses 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 2.3.A during the period of limited oxygen supply (period IV, 22.8-120 h), the formation rate of the different sulfur species can be accurately determined. For this particular experiment, H_2S was converted to sulfate (16%, r^2=0.95), thiosulfate (17%, r^2=0.99) and sulfur (67%, r^2=0.98). The pH decreased over time due to the formation of sulfate and thiosulfate (Fig. 2.3.D). The rate of acidification of the reactor medium decreased when the rate of sulfate production decreased, which is in agreement with Eq. 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 oxygen 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. 2.3.D).

2.3.2 Product selectivity in relation to the molar O_2/H_2S supply ratio

A series of experiments similar to the experiment described above was performed at a constant H_2S supply of 10 mmol h^{-1}, but at various oxygen supply rates. After passing the start-up phase of 20–30 h at DO concentrations of 50-70%, the O_2/H_2S supply ratio was varied from 1.0 to 0.8, 0.7, 0.65 and 0.6. Experiments at an O_2/H_2S supply ratio of 1, 0.8 and 0.7 were performed in duplicate. The effect of the molar O_2/H_2S supply ratio on the selectivity of sulfur species produced is shown in Figure 2.4. Selectivity was determined over a period of at least 25 h of stable reactor performance. It can be seen that selectivity for sulfate as well as thiosulfate formation decreased with decreasing O_2/H_2S supply ratio. At an O_2/H_2S supply ratio of 1.0, selectivity for sulfate formation was 36.4±0.9%, while at an O_2/H_2S supply ratio of 0.65, this value approached zero (1.4±0.6%). Selectivity for thiosulfate formation was 31.7±3.7% at an O_2/H_2S supply ratio of 1.0 and 15.3±0.1% at an O_2/H_2S supply ratio of 0.65. In all experiments, the H_2S concentration in the gas flow leaving the reactor was below the detection limit of 0.1 ppm.

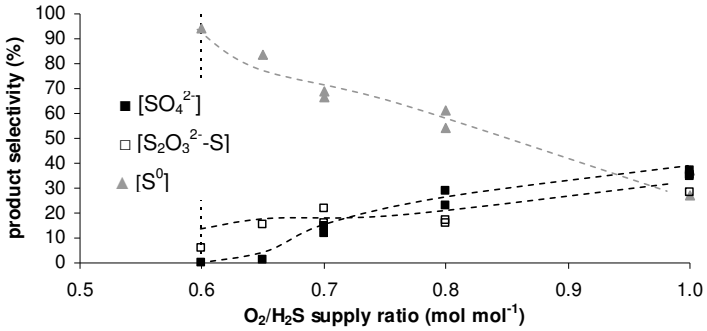

FIGURE 2.4 **Product selectivity at different molar O_2/H_2S supply ratios. The H_2S supply was 10 mM h^{-1} for all experiments. The vertical dashed line represents the boundary for stable reactor operation. At O_2/H_2S supply ratios of 0.65 and higher, no sulfide accumulated in the medium. At an O_2/H_2S supply ratio of 0.6 and below, sulfide accumulated after 20 h.**

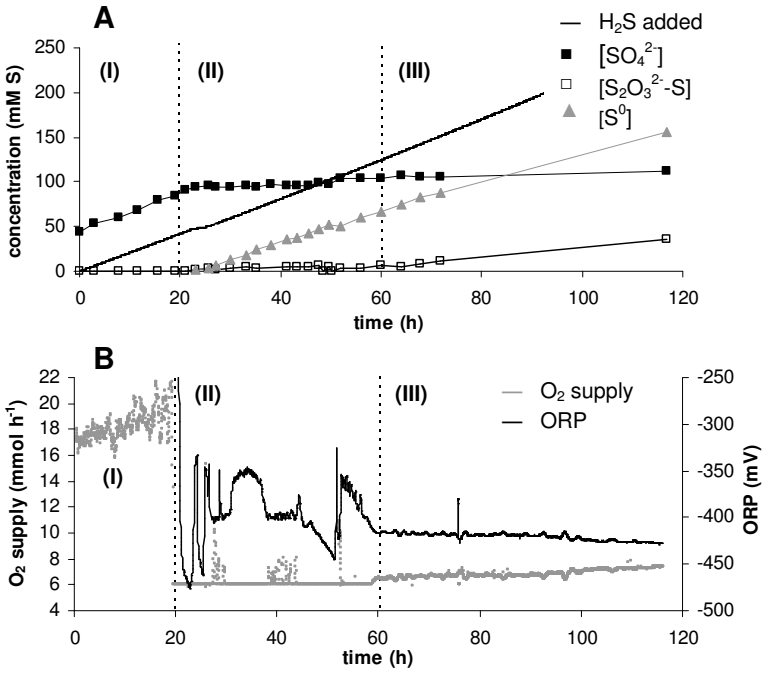

FIGURE 2.5 A-B **Results of a fed-batch experiment where the O_2 supply was decreased to 6 mmol h^{-1}. A: Concentrations of sulfate, $S_2O_3^{2-}$ and S^0 and total H_2S added; B: O_2 supply and ORP. The H_2S supply was constant at 10 mM h^{-1}. The vertical dashed lines border the start-up phase (I), operation with a molar O_2/H_2S supply ratio of 0.6 (II) and operation with the O_2 supply based on ORP (III). The arrows in Fig.5 B indicate periods in which the H_2S supply was briefly interrupted.**

Figure 2.5 A and B show that at an O_2/H_2S supply ratio of 0.60, initially only 6% of the total H_2S was converted to thiosulfate, while no sulfate formation was detected at all. However, this situation was only stable for a short period. In 30 hours following the limited oxygen supply, the ORP gradually decreased. At t=51 h, the H_2S supply was stopped to prevent toxification of the biomass due to accumulation of (poly)sulfide. In the subsequent period, the oxygen supply was controlled on the basis of ORP. The oxygen 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 oxygen supply gradually increased to 7.3 mmol h^{-1}, at an ORP of -426 mV. Under these conditions, the increase in oxygen demand was related to thiosulfate formation (selectivity for thiosulfate increased to 27%), and not to sulfate formation (selectivity for sulfate remained <2%). At O_2/H_2S supply ratios below 0.60, polysulfide rapidly accumulated and conversion of the supplied H_2S failed (data not shown). Figure 2.6 shows the stoichiometric amount of oxygen required for the formation of the sulfur products versus the actual oxygen supply for different O_2/H_2S supply ratios. From this figure it is clear that at an oxygen supply of 7 mmol h^{-1} and above, more oxidized sulfur compounds are produced than can be expected on basis of the oxygen supply. An explanation for this is the use of part of the reductive capacity for assimilation of CO_2 into cellular material.

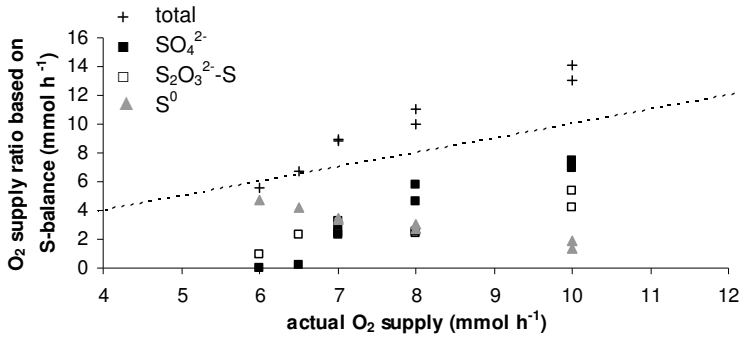

FIGURE 2.6 Electron balance, based on O_2. The stoichiometric amount of O_2 required for the formation of the different sulfur products (SO_4^{2-}, $S_2O_3^{2-}$ and S^0 and S total) is shown versus the actual O_2 supply. The dashed line represents equality between the O_2 supply based on the S-balance and the actual O_2 supply.

2.3.3 Relation between sulfide and polysulfide concentrations and redox potential

The relation between the S^{2-}_{tot} concentration and the concentration of zerovalent sulfur in polysulfide at pH 10.1±0.1 in presence of sulfur, measured during various fed-batch experiments is shown in Figure 2.7. This figure shows that the average ratio between the S^{2-}_{tot} concentration and the concentration of zerovalent sulfur in polysulfide was 1:4.4 (r^2=0.94). Combining Equation 8 and 9, the following equation can be derived:

$$\frac{[S_x^{2-} - S^0]}{[S^{2-}_{tot}] - [HS^-]} = x\text{-}1 \qquad (10)$$

With this equation, the minimum possible average polysulfide chain length (x) can be calculated when assuming that the measured S^{2-}_{tot} concentration consists of polysulfide only ($[HS^-] = 0$). With $[S^{2-}_{tot}]:[S_x^{2-}\text{-}S^0] = 1:4.4$, 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 [26], 4.59 [22], 4.79 [27], 4.93 [28], 4.97 [29], 5.11 [30], 5.33 [31] to 5.5 [32]. Applying the maximum reported average chain length of 5.5 in Eq. 10, it can be calculated that with $[S^{2-}_{tot}]:[S_x^{2-}\text{-}S^0] = 1:4.4$, at most 2.2% of the S^{2-}_{tot} concentration was present as (free) sulfide. It therefore can be concluded that in our experiments, polysulfide was the main reduced sulfur compound in the medium, and not sulfide.

The ORP in a sulfide oxidizing bioreactor is determined mainly by the S^{2-}_{tot} concentration [33]. As the polysulfide concentration was high compared to the sulfide concentration, we propose that under stable reactor operation, the ORP is directly related with the polysulfide concentration. From the various fed-batch experiments presented in this study, the relation between the negative logarithm of the concentration of zerovalent sulfur in polysulfide (in mM) and the ORP in presence of sulfur can be described as:

$$ORP = -43.2 \cdot \log [S_x^{2-}\text{-}S^0] - 411 \qquad (11.A)$$

With $[S^{2-}_{tot}] : [S_x^{2-}\text{-}S^0] = 1:4.2$, this becomes:

$$ORP = -43.2 \cdot \log [S^{2-}_{tot}] - 438 \qquad (11.B)$$

Figure 2.8 shows the ORP and $[S_x^{2-}\text{-}S^0]$ during stable reactor operation for each applied O_2/H_2S supply ratio. It is clear that at lower O_2/H_2S supply ratios, the ORP stabilizes at lower values, related with higher S_x^{2-} concentrations.

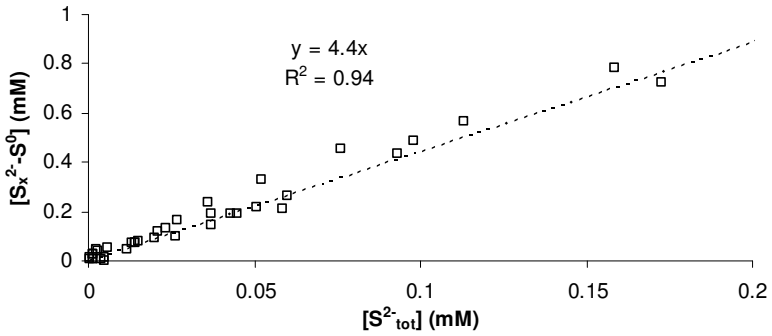

FIGURE 2.7 Relation between concentrations of total sulfide (S^{2-}_{tot}) and zerovalent sulfur in polysulfide ($S_x^{2-}\text{-}S^0$), measured in several fed-batch experiments. The pH for these measurements was between 10.0 and 10.2.

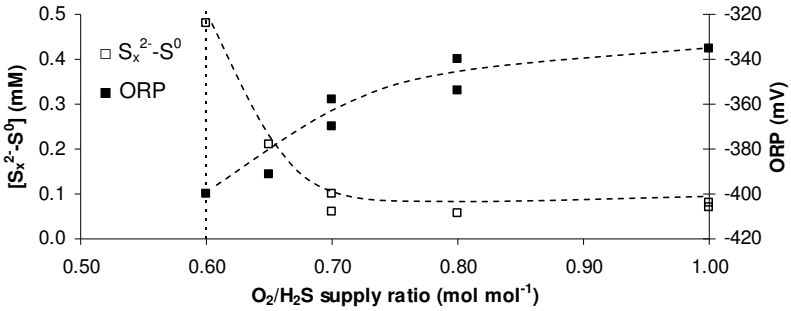

FIGURE 2.8 Polysulfide concentration (as $[S_x^{2-}$-$S^0]$) and ORP at different molar O_2/H_2S supply ratios. The H_2S supply was 10 mmol h^{-1} for all experiments. The pH for these measurements was between 10.1 and 9.6. At a molar O_2/H_2S supply ratio of 0.6 and below, sulfide accumulated after 20 h.

2.3.4 Biomass characteristics

Biomass concentration measurements were performed during the sulfate producing start-up phase as well as during sulfur producing conditions. Figure 2.9 shows biomass growth versus O_2/H_2S supply ratio. During the start-up phase, when sulfate 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_2S 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 thiosulfate [15]. Under oxygen-limiting conditions, when selectivity for sulfate 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) [34]. Obviously, this is related to the difference in the metabolically available energy from the oxidation of sulfide to either sulfate or sulfur [25]. The very low growth obtained during sulfur formation can be explained by the energy requirements for maintenance.

The relation between ORP and sulfate formation rate under oxygen-limiting conditions is shown in Figure 2.10. Below an ORP of -400 mV, selectivity for sulfate formation dropped below 2%, while at ORP values above -400 mV, the sulfate formation rate increased with increasing ORP. On basis of the relation between ORP and the S^{2-}_{tot} concentration (Eq. 11.B), it can be calculated that at a S^{2-}_{tot} concentration above 0.25 mmol L^{-1}, the sulfate formation rate was below 2%.

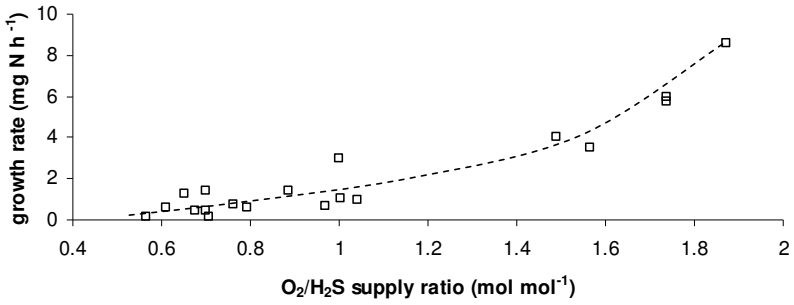

FIGURE 2.9 Biomass growth rate in mg N h^{-1} in relation to the molar O_2/H_2S supply ratio during fed-batch experiments. The H_2S load for all points was constant at 10 mM h^{-1}.

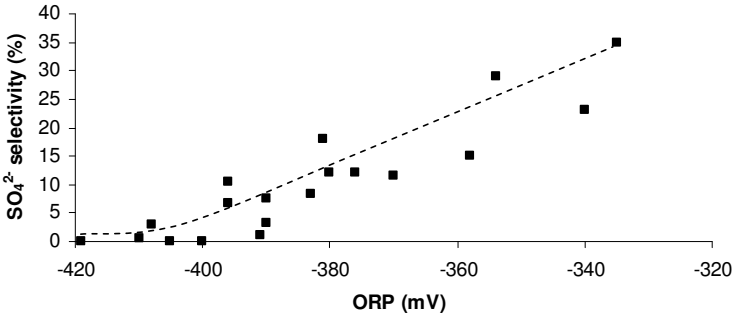

FIGURE 2.10 Selectivity for sulfate formation versus ORP during fed-batch experiments under O_2 limitation. The applied sulfide load was 10 mmol h^{-1}, T=35ºC, pH = 9.6 – 10.1, I=2.7 M.

2.4 DISCUSSION

2.4.1 Formation and speciation of polysulfide ions

For the reaction of sulfide with biologically produced sulfur particles (Eq. 5), Kleinjan et al. (2005) reported an equilibrium constant (pK_x) of 9.17±0.09 with an average polysulfide chain length of 4.59 at 35°C [22]. To exactly determine whether in our fed-batch experiments sulfide and polysulfide are in equilibrium, the activity constants of (poly)sulfide at the high salt conditions as well as the average polysulfide chain length should be known. Although this is not the case, an estimate can be made, based on the relation between the S_x^{2-}-S^0 concentration and the S^{2-}_{tot} concentration, determined at pH 10.0 – 10.2 (Figure 2.7). Applying the equilibrium constant of Kleinjan et al. (2005) [22], it can be calculated that 10.5% of S^{2-}_{tot} is present as sulfide at pH 10.1, assuming equilibrium between sulfide and polysulfide. 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 [22, 32]. Although the average polysulfide chain length in our experiments was not exactly known, it can be concluded that in excess of sulfur, the concentration of polysulfide in the medium was close to equilibrium with sulfide. This was also found in bioreactor experiments with H_2S removal under mildly alkaline conditions [2]. The formation rate of polysulfide from colloidal sulfur particles and sulfide was studied by Fossing and Jørgensen (1990), who found the halftime of isotope exchange between radiolabeled inorganic sulfur ($^{35}S^0$) and polysulfide to be 1.8 minutes [35]. In our experiments, the polysulfide formation rate was probably faster as the particle size of freshly produced sulfur particles is likely to be much smaller than the size of colloidal sulfur particles used in the study of Fossing and Jørgensen (1990). As the reaction between sulfide and sulfur takes place at the surface of sulfur particles, the formation rate of polysulfide will increase with decreasing particle size [36]. Another cause for rapid polysulfide formation in our experiments is the autocatalytic effect of polysulfide on the reaction rate, as was shown by Kleinjan et al. (2005) [36].

The minimum average chain length of polysulfide ions in our experiments was more than 4.59 as was reported by Kleinjan et al. (2005) [22]. A possible explanation for the longer average chain length in our experiments is that oxidation of short polysulfide chains occurs faster than oxidation of longer chains. Results of Banciu et al. (2004) indeed indicate that the biological oxidation rate of polysulfides of shorter chain length by the HA-SOB *Thioalkivibrio versutus*, occurs faster than biological oxidation of polysulfides of longer chain length [37]. Kamyshny et al. (2003) found the disproportionation reaction of polysulfide in aqueous solutions with excess sulfur to reach equilibrium within seconds [38]. It can be calculated that in our experiments the polysulfide oxidation rate is too low to have any effect on the average polysulfide chain length, even if all supplied H_2S is oxidized via polysulfide as an intermediate. High concentrations (up to 20 g L^{-1}) of thiosulfate and sulfate did not have a significant effect on the measurement of the polysulfide concentration (data not shown). An adequate explanation for the longer average polysulfide chain length in our experiments remains elusive.

2.4.2 Origin of thiosulfate

Our results show that at oxygen limiting conditions, thiosulfate formation plays a more important role than sulfate formation in the selectivity for sulfur formation. Previous publications on sulfide oxidation at more neutral pH conditions [4, 16, 39] suggest abiotic reactions to be the cause of thiosulfate formation. We assume that also in our experiments, thiosulfate originates from abiotic processes. As mentioned above, thiosulfate can be formed upon abiotic oxidation of polysulfide according to Eq. 6. Kinetics of polysulfide oxidation was studied by Kleinjan et al. (2005) [9]. The following empirical relation for the rate of oxygen consumption by abiotic oxidation of polysulfide was found:

$$d[O_2]\ dt^{-1} = -k\ [S_x^{2-}]\ [O_{2(aq)}]^{0.59} \quad (12)$$

At 35ºC, pH 10 and I=0.1 M, they found a value for k of 1.65. Combining this with the stoichiometry of abiotic polysulfide oxidation (Eq. 6), the following equation can be derived:

$$d[S_2O_3^{2-}]\ dt^{-1} = -1.10\ [S_x^{2-}]\ [O_{2(aq)}]^{0.59} \quad (13)$$

Combining Eq. 13 with the polysulfide concentration and thiosulfate formation rates in the fed-batch experiments, a theoretical DO concentration can be calculated, assuming that thiosulfate is formed upon abiotic oxidation of polysulfide only. With this approach, the DO concentration should be 1.6 mg L^{-1} at an O_2/H_2S supply ratio of 0.8 and 0.14 mg L^{-1} at an O_2/H_2S supply ratio of 0.65. However, during experiments with limited oxygen 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 polysulfide under halo-alkaline conditions occurs more rapidly than calculated by Eq. 13, or that besides abiotic polysulfide oxidation, another process results in the formation of thiosulfate. Hydrolysis of elemental sulfur is an eligible process explaining extra formation of thiosulfate. Hydrolysis of sulfur can be described with the following equation [32]:

$$4\ S^0 + 4\ OH^- \rightarrow S_2O_3^{2-} + 2\ HS^- + H_2O \quad (14)$$

The additional formation of sulfide leads to an increase in the sulfide load of the bioreactor and therefore to an increase in oxygen demand. According to Steudel (2000), with inorganic sulfur this reaction occurs at pH > 11.5 at 20ºC and at pH 7.6 at 80ºC [32]. Also biologically produced sulfur was found to hydrolyse at 55°C and pH 10 [40]. Kleinjan et al. (2005) concluded that at pH values above 9, colloidal sulfur formed upon abiotic oxidation of polysulfide, so called 'nascent' sulfur, is subject to hydrolysis [9]. 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 hydrolysis 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 hydrolysis of nascent sulfur, and possibly also hydrolysis of crystalline sulfur, has taken place during these experiments. The mechanisms for thiosulfate formation in the halo-alkaline sulfide oxidation process are subject of future study.

2.4.3 Biological oxidation of (poly)sulfide

It was shown that instead of sulfide, polysulfide was the main reduced sulfur species present in our experiments at sulfur producing conditions. Banciu et al. (2004) have studied the oxidation of both compounds by the HA-SOB *Thioalkalivibrio versutus*, strain ALJ 15 [37]. Their results indicated that biological oxidation of polysulfide to sulfate occurs in two phases:

a first, rapid phase of oxidation of sulfane atoms ($-S^{2-}$) and a second, slow phase of zerovalent sulfur oxidation. They also found that oxidation of sulfide alone occurs at a lower rate than the first phase of polysulfide oxidation, indicating that the oxidation of sulfane atoms is faster than the oxidation of sulfide. With polysulfide being the main reduced sulfur species in our experiments, this indicates that polysulfide was the main substrate for biological oxidation by HA-SOB under sulfur producing conditions.

As thiosulfate formation was probably partly a result of hydrolysis of (nascent) sulfur, it was not possible to determine the biological sulfur production rate. However, the relation between ORP and sulfate formation shown in Figure 2.10, indicates that also the activity of sulfur formation is ORP related. This was already suggested by Visser et al. (1997) [41]. They proposed that sulfide is oxidized to sulfate via intermediary sulfur. Under limited availability of oxygen, the degree of reduction of the cytochrome pool increases, thereby blocking the conversion of sulfur to sulfate. In our process, where polysulfide was the main reduced sulfur compound, the second, slow phase of intermediary sulfur oxidation may be influenced by the ORP in a similar way.

In all experiments, (poly)sulfide accumulated in the reactor medium when the shift to a limited oxygen supply was applied for the first time after start-up. Like in the experiment shown in Figure 2.3, the ORP and the polysulfide concentration stabilized when the H_2S supply was resumed after a brief period in which no H_2S was supplied. An explanation for this is that during the time needed for the metabolic shift from sulfate to sulfur formation, the S^{2-}_{tot} concentration increases to a level where inhibition of the bacteria results in a too low biological (poly)sulfide oxidation rate to convert all H_2S supplied. During the brief stop of the H_2S supply, the (poly)sulfide concentration decreases due to biological and abiotic oxidation. When after this stop, the H_2S supply is resumed, the biological sulfur formation rate is high enough to convert all supplied H_2S. Another possibility is that the conversion of sulfide to sulfur is too low to convert all H_2S supplied. When some sulfur is present to react with sulfide to polysulfide, the biological oxidation rate of the supplied H_2S is high enough, as polysulfide is more rapidly oxidized than sulfide.

2.5 CONCLUSIONS

Our results show that it is possible to biologically oxidize H_2S to elemental sulfur at halo-alkaline conditions (pH 10 and $[Na^+] + [K^+] = 2$ mol L^{-1}) in a fed-batch reactor. Maximum selectivity for sulfur formation during stable reactor operation (83.3±0.7%) was obtained at a molar O_2/H_2S supply ratio of 0.65. At sulfur producing conditions, intermediary polysulfide, produced by the reaction between sulfur and sulfide, plays a major role in the process. In excess of sulfur, the concentration of polysulfide in the medium was close to equilibrium with sulfide. Instead of sulfide, polysulfide seemed to be the most important e-donor for HA-SOB under sulfur producing conditions. In addition, abiotic oxidation of polysulfide was the most important cause of undesired formation of thiosulfate. Another possible cause for the

formation of thiosulfate could be the hydrolysis of (nascent) sulfur. The selectivity for sulfate formation was found to be related with concentrations of polysulfide and S^{2-}_{tot}. At a total sulfide concentration above 0.25 mM, selectivity for sulfate formation approached zero. At these conditions, the end products of H_2S oxidation were sulfur and thiosulfate. Biomass growth yield under fully sulfate producing conditions was 0.86 g N mol^{-1} H_2S. When selectivity for sulfate formation was below 5%, hardly any increase in the biomass concentration was observed. Reactor operation at set molar O_2/H_2S supply ratios of 0.65 and above resulted in a stable ORP that was directly related with the polysulfide and S^{2-}_{tot} concentration in the bioreactor. This gives good perspectives for the use of the ORP as a parameter to control the oxygen supply for optimal selectivity for sulfur formation in future research.

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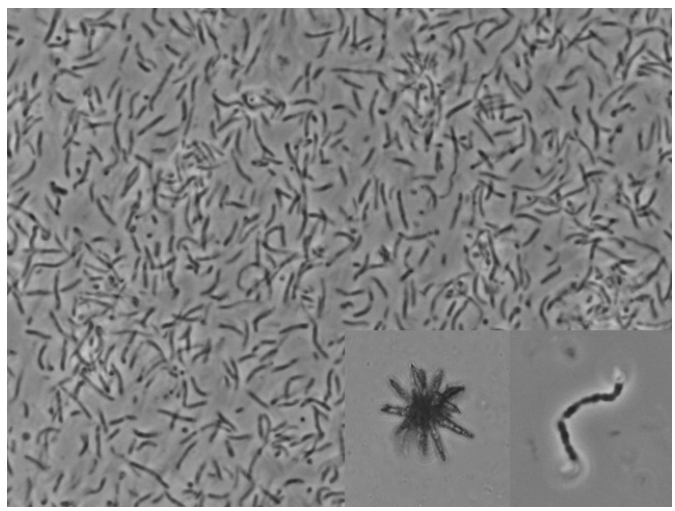

Chapter 3

The effect of pH on thiosulfate formation

Abstract

In a biotechnological process for hydrogen sulfide (H_2S) removal from gas streams at natron-alkaline conditions, formation of thiosulfate ($S_2O_3^{2-}$) is unfavourable as it leads to a reduced sulfur production. Thiosulfate formation was studied in gas-lift bioreactors, using natronophilic biomass at $[Na^+]+[K^+]$ = 2 mol L^{-1}. The results show that at sulfur producing conditions, selectivity for thiosulfate formation mainly depends on the equilibrium between sulfide (HS^-) and polysulfide ions (S_x^{2-}), which can be controlled via the pH. At pH=8.6, 21% of the total dissolved sulfide is present as polysulfide and selectivity for thiosulfate formation is 3.9 –5.5%. At pH=10, 87% of the total dissolved sulfide is present as polysulfide and 20-22% of the supplied H_2S is converted to thiosulfate, independent of the H_2S loading rate. Based on results of bioreactor experiments and biomass activity tests, a mechanistic model is proposed to describe the relation between thiosulfate formation and pH.

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3.1 INTRODUCTION

A biotechnological process is developed for hydrogen sulfide (H_2S) removal from high pressure natural gas and sour gas streams produced in the petrochemical industry. The process is based on an earlier described technology [1-3], but operates at much higher salt concentrations and alkalinity, using natronophilic sulfide-oxidizing bacteria (SOB). The high carbonate concentration makes it possible to treat gasses at high pressure (up to 100 bar) with high partial CO_2 concentrations [4]. In the process, H_2S is removed from the gas stream by an absorber using an alkaline carbonate solution (Eq. 1-2). The dissolved sulfide (HS^-) is subsequently oxidized to elemental sulfur (S^0 or "biosulfur") at oxygen-limiting conditions by natronophilic SOB in a bioreactor, thereby regenerating the hydroxyl- (OH^-, Eq. 3) and carbonate ions (CO_3^{2-}, Eq. 4) that are consumed by the absorption of H_2S. The biologically produced sulfur particles are separated from the process in a gravity settler, while the regenerated alkaline solution is recycled over the gas absorber. A more detailed description of the process was given in Chapter 2 [4].

Formation of sulfate- (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) ions decreases the fraction of H_2S that is converted to re-usable biosulfur particles, i.e. lowers the selectivity for sulfur formation. Other disadvantages of sulfate and thiosulfate formation are: (I) no OH^- regeneration takes place, leading to a demand for caustic to control the pH; (II) the O_2 demand increases, leading to higher energy consumption; (III) sulfate and thiosulfate ions need to be removed from the process by means of a bleed stream. With this stream also (bi)carbonate ions are removed, leading to an additional caustic demand.

In previous papers on biological removal of H_2S [2, 5], a decreasing selectivity for sulfur formation was attributed to sulfate production as a result of complete biological oxidation of sulfide (Eq. 5). However, in Chapter 2 [4], it was demonstrated that also thiosulfate formation is an important cause for a decreased selectivity for sulfur formation. It was shown that at pH 10, sulfate formation gradually decreases with increasing total dissolved sulfide (S^{2-}_{tot}) concentrations and even approaches zero at S^{2-}_{tot} concentrations of 0.25 mmol L^{-1}. Thiosulfate formation, on the other hand, ranges from 15.3 to 31.7% at O_2 limiting conditions. In order to maximize selectivity for sulfur formation, more research is needed into processes that lead to thiosulfate formation. Laboratory studies show that abiotic oxidation of sulfide can result in the formation of thiosulfate (Eq. 6) [6, 7]. The rate of sulfide oxidation depends on several factors, such as sulfide and dissolved oxygen concentrations, type of buffer used and the presence of metal ions, which may act as a catalyst [6], [8] . Besides abiotic sulfide oxidation, also abiotic oxidation of polysulfide ions (S_x^{2-}) can result in thiosulfate formation (Eq. 7) [9]. Polysulfide ions are formed when biologically produced sulfur particles react with sulfide (Eq. 8) [10, 11]. For S_x^{2-} originating from biologically produced sulfur particles, an equilibrium constant of $pK_x = 9.17 \pm 0.09$ (Eq. 9) is reported at 35°C, with an average S_x^{2-} chain length of $x=4.59 \pm 0.31$ [12]. Previous research has shown that at moderate alkaline conditions (pH 8.5, 0.26 mol L^{-1} Na^+) as well as at natronophilic conditions (pH 10,

$[Na^+]+[K^+]$ = 2 mol L^{-1}), the S_x^{2-} concentration is close to equilibrium with sulfide (Chapter 2) [4, 13]. It is assumed that thiosulfate mainly originates from abiotic S_x^{2-} oxidation, because the abiotic S_x^{2-} oxidation rate is higher than that of sulfide, even at very low concentrations and in absence of a catalyst [14, 15]. In addition to sulfide and S_x^{2-} oxidation, also non-oxidative processes result in thiosulfate formation. For instance, disproportionation of sulfur particles leads to sulfide and thiosulfate formation (Eq. 10) at elevated temperatures and alkaline conditions [15, 16]. This may be especially important when colloidal sulfur particles are formed due to abiotic oxidation of S_x^{2-}. It was found that disproportionation of this, so called, 'nascent sulfur' already takes place at 30°C at pH values above 9 [14].

$$H_2S(g) \rightleftarrows H_2S(aq) \quad (1)$$

$$H_2S(aq) + OH^- \rightleftarrows HS^- + H_2O \quad (2.A)$$

$$H_2S(aq) + CO_3^{2-} \rightleftarrows HS^- + HCO_3^- \quad (2.B)$$

$$HS^- + ½\ O_2 \rightarrow S^0 + OH^- \quad (3)$$

$$OH^- + HCO_3^- \rightleftarrows CO_3^{2-} + H_2O \quad (4)$$

$$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+ \quad (5)$$

$$HS^- + O_2 \rightarrow ½\ S_2O_3^{2-} + ½\ H_2O \quad (6)$$

$$S_x^{2-} + 1½\ O_2 \rightarrow S_2O_3^{2-} + (x-2)\ S^0 \quad (7)$$

$$HS^- + (x-1)S^0 \rightleftarrows S_x^{2-} + H^+ \quad (8)$$

$$K_x = \frac{[S_x^{2-}][H^+]}{[HS^-]} \times \frac{\gamma_{S_x^{2-}}\gamma_{H^+}}{\gamma_{HS^-}} \quad \text{with } pK_x = 9.17 \quad (9)$$

$$4\ S^0 + 4\ OH^- \rightarrow S_2O_3^{2-} + 2\ HS^- + H_2O \quad (10)$$

Depending on the process conditions, all of the above-mentioned reactions can play a role in the formation of thiosulfate in a process for the removal of H_2S from gas streams at natronophilic conditions. It is however not known what the contribution of each individual reaction is. This paper therefore focuses on the effect of pH and S_x^{2-} concentration on thiosulfate formation in a H_2S oxidizing bioreactor operating at natronophilic conditions, with the overall goal to maximize the selectivity for sulfur formation.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Reactor experiments were conducted in two identical gas-lift bioreactors with a wet volume of 4.8 L each, as described elsewhere (Chapter 2) [4]. The same analytical and side equipment (pH-, redox- and dissolved oxygen (DO) electrodes, water baths, H_2S, O_2 and N_2 gas and

mass flow controllers) was used. The gas flow (300 L h^{-1}) was completely recycled to prevent any release of H_2S gas and to reach low O_2 concentrations. Nitrogen gas 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±1°C. No biomass support material was applied.

3.2.2 Medium

The mineral medium consisted of a mixture of a bicarbonate (pH 8.3) and a carbonate (pH 12.3) buffer. Both buffers contained 0.66 mol L^{-1} Na^+ and 1.34 mol L^{-1} K^+, as carbonates. The final pH of the medium ranged from 8.5 to 10.5. Furthermore, the medium contained (in g L^{-1} demineralised water): K_2HPO_4, 1.0; urea, 0.6; NaCl, 6.0; $MgCl_2 \cdot 6\ H_2O$, 0.20. Trace elements solution was added as described in [17].

3.2.3 Inoculum

Reactors were inoculated with centrifuged biomass from a sulfur producing gas-lift reactor. The original inoculum of this reactor consisted of a mixture of sediments from hypersaline soda lakes in Mongolia, south-western Siberia and Kenya, obtained from Delft University of Technology. An overview of the physiology of the SOB present in the inoculum is given elsewhere [18, 19]. Each fed-batch experiment was started with a biomass fraction taken from a previous experiment.

3.2.4 Reactor operation

Reactors were first filled with medium, whereafter biomass was added. The total reactor liquid volume after inoculation was 4.7 L at 35°C. After temperature stabilization, addition of H_2S was started at a load of 10 mmol h^{-1}, unless stated otherwise. A start-up phase (0-20 hours) was applied to allow the biomass to activate and to increase the biomass concentration. During this phase, the reactor was operated with an excess O_2 supply, i.e. the DO concentration remained above 70% sat. After completion of the start-up phase, the O_2 supply rate was reduced to obtain O_2 limiting conditions, while the H_2S supply was kept at a constant rate. Selectivity for product formation was determined over a period of at least 48 hours of stable reactor operation in fed-batch mode. Fresh medium was only supplied to make up for the sample volume (20 – 50 mL d^{-1}).

3.2.5 Oxygen supply strategy

As shown previously in Chapter 2 [4], the measured redox potential (ORP) in the reactor can be related to the logarithm of $[S^{2-}_{tot}]$ by the following equation:

$$ORP = - a \cdot \log [S^{2-}_{tot}] - b \qquad (11)$$

Values for a and b were obtained by calibration in the bioreactors at each experimental pH value. The calculated $[S^{2-}_{tot}]$ was used to control the O_2 supply rate, using a proportional control law, according to the following equation:

$$O_2 \text{ supply} = (1+P) \cdot ([S^{2-}_{tot}] - [S^{2-}_{tot}]_{set}) \cdot 0.5 \ H_2S \text{ supply} \quad (12)$$

with:
P = proportional control factor
$[S^{2-}_{tot}]$ = total sulfide concentration, based on ORP
$[S^{2-}_{tot}]_{set}$ = total sulfide concentration setpoint

For $[S^{2-}_{tot}] \leq [S^{2-}_{tot}]_{set}$, the O_2 supply was set to 0.5 times the H_2S supply. The value of P was manually set to values between 0.5 and 4. This control strategy turned the reactor into a 'sulfido-stat', maintaining constant S^{2-}_{tot} concentrations slightly above the desired setpoint.

3.2.6 Respiration measurements

Bacterial cells present in reactor samples were separated from extracellular sulfur by subsequent steps of low-speed (500 rpm) centrifugation, washed and resuspended in a pure (bi)carbonate buffer (pH 9.5, 2 mol L^{-1} Na^+), at a cell protein density of 30 g L^{-1}. The final protein content in the experiments was 0.065 g L^{-1} with an initial O_2 concentration of 0.150 mmol L^{-1}. Respiration rates with different sulfur substrates (sulfide, thiosulfate, S_x^{2-} and elemental sulfur) were measured at 30°C in a 5 mL glass chamber mounted on a magnetic stirrer and fitted with a DO electrode (Yellow Springs Instr., OH, USA). The buffers contained 2 mol L^{-1} of Na^+ and consisted of HEPES+NaCl (pH 7-8); $NaHCO_3/Na_2CO_3$ + NaCl (pH 8.5) and $NaHCO_3/Na_2CO_3$ (pH 9-11).

3.2.7 Analysis

Sulfide was measured as total sulfide (S^{2-}_{tot}), being the sum of concentrations of H_2S, HS^-, S^{2-}, H_2S_x, HS_x^- and S_x^{2-}. Of these, only HS^- and S_x^{2-} are taken into account as the sum of the other species is less than 3.7% of $[S^{2-}_{tot}]$ at pH > 8.5, calculated according to disproportionation constants of inorganic polysulfides as determined by [20]. Because HS^- and S_x^{2-} comprise at least 96.3% of the measured S^{2-}_{tot} concentration, the following equation applies:

$$[S^{2-}_{tot}] = [HS^-] + [S_x^{2-}] \quad (13)$$

The method used was based on a modified methylene blue method as described in Chapter 2 [4]. The concentration of S_x^{2-} was determined spectrophotometrically as described by [12], at a wavelength of 285 nm (Perkin-Elmer, Lambda 2, Norwalk, CT, USA). With this method,

the total concentration of zerovalent sulfur atoms in S_x^{2-} is determined: $[S_x^{2-}\text{-}S^0]$ [11, 21]. When the average chain length x is known, $[S_x^{2-}]$ can be calculated according to:

$$(x\text{-}1) \cdot [S_x^{2-}] = [S_x^{2-}\text{-}S^0] \qquad \text{with } x=4.59 \qquad (14)$$

The average chain length of S_x^{2-} produced with biological sulfur, was found to be x=4.59±0.31 at 35°C [12]. Before analysis, samples were filtrated over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66, Dassel, Germany). The molar extinction coefficient (1300 L mol^{-1} cm^{-1}) was determined as described by [12], using the high salt medium. In some occasions, the analysis of $[S_x^{2-}\text{-}S^0]$ was affected by unknown compounds with an absorbance at 285 nm. This was found by detection of a residual absorbance after removal of S_x^{2-} by respectively oxidation and acidification. These data were however not used in this study.

Sulfate and thiosulfate concentrations were determined by ion chromatography as described elsewhere (Chapter 2) [4]. Because the biosulfur particles partly attached to the reactor wall, the sulfur concentration in the reactor was not measured, but calculated based on the sulfur mass balance according to:

$$[S^0]=(H_2S \text{ supplied}/V_{liq})-[SO_4^{2-}]-[S_2O_3^{2-}\text{-}S]-[HS^-]-[S_x^{2-}\text{-}S]-[H_2S_{gas}] \qquad (15)$$

In this calculation, also the sample volume was taken into account. The gas phase composition (H_2S, N_2, CO_2 and O_2) was determined by a gas chromatograph as described in Chapter 2 [4].

Biomass concentration was measured as the amount of total N, as described in Chapter 2 [4]. In some experiments, the cell protein concentration was determined as described in [22].

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of H_2S loading rate on thiosulfate formation

To asses the contribution of abiotic sulfide and S_x^{2-} oxidation to thiosulfate formation, a bioreactor experiment was performed at two different H_2S loading rates, while $[S^{2-}_{tot}]$ was controlled at a stable value (experiment 1, Table 3.1). Figure 3.1 shows the results of a fed-batch experiment at pH 10. During the start-up phase, some thiosulfate accumulated, probably due to abiotic sulfide oxidation resulting from a low biological activity. After three days, all H_2S and thiosulfate were completely oxidized to sulfate (Period I). During this sulfate producing period, the biomass concentration gradually increased from 16 (start) to 93 mg N L^{-1} (t=5 days, results not shown). At t=6 days (Period II), the O_2 supply rate was reduced to obtain a $[S^{2-}_{tot}]$ setpoint of 0.25 mmol L^{-1}, because it is known from a previous study that at these conditions, sulfate formation approaches zero (Chapter 2) [4].

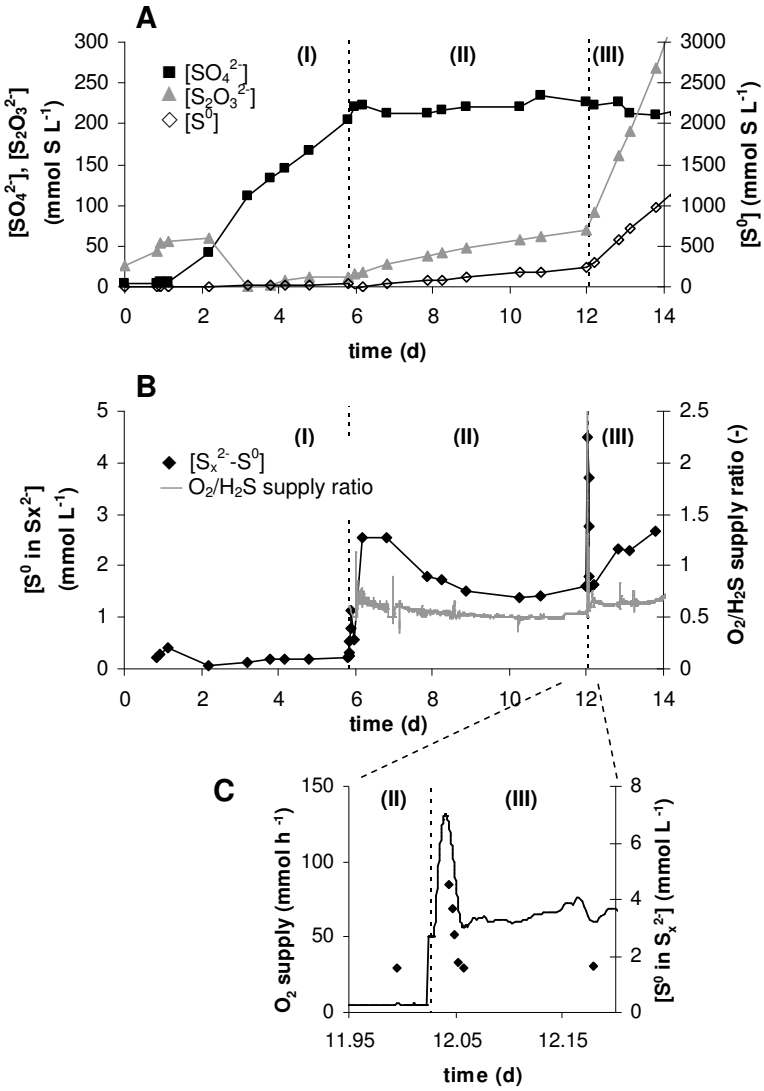

FIGURE 3.1 A-C. Results of a fed-batch experiment at pH 10.1 and 35°C (experiment 1). A: Course of sulfur compounds concentration; B: $[S_x^{2-}\text{-}S^0]$ and molar O_2/H_2S supply ratio; C: O_2 supply (line) and $[S_x^{2-}\text{-}S^0]$ (diamonds) in the period just before (II) and after (III) increase of the H_2S supply from 10 to 100 mmol h^{-1}. The dashed lines separate three periods: start-up phase (I), 10 mmol h^{-1} H_2S load (II) and 100 mmol h^{-1} H_2S load (III).

Within 3.5 hours, sulfate formation stopped completely, resulting in a shift to sulfur (79%) and thiosulfate (21%) formation (see also Table 3.1). These results correspond to earlier findings (Chapter 2) [4]. At t=12 days (Period III), the H_2S supply was increased from 10 to 100 mmol h^{-1}. As a result, concentrations of S_x^{2-} and S^{2-}_{tot} immediately increased, but then decreased to their original values within 50 minutes, as a result of the proportional increase of the O_2 supply (see inset Fig. 3.1 C). Immediately after the 10-fold increase of the H_2S supply, the rate of thiosulfate formation also increased 10-fold, so the selectivity for thiosulfate formation remained constant (22%, period III). In the following two days, the average S_x^{2-}-S^0 concentration gradually increased from 1.6 to 2.3 mmol L^{-1}, while the DO concentration remained below the detection limit of 0.1% sat. As $[S^{2-}_{tot}]$ and the DO concentration remained stable, the biological activity and substrate affinity were sufficiently high to accommodate the increased H_2S loading rate. The average O_2 supply was 5.7 mmol h^{-1} at an H_2S supply of 10 mmol h^{-1} (period II), and increased more or less proportionally to 63 mmol h^{-1} at an H_2S supply of 100 mmol h^{-1} (period III). Thus, in both cases the molar O_2/H_2S supply ratio did not deviate substantially from the calculated ratio of 0.61, as based on the electron balance for sulfur compounds. This confirms that all relevant sulfur species are included in the sulfur balance.

The rate of thiosulfate formation as a result of abiotic oxidation of sulfide and S_x^{2-} is determined by the kinetics of these abiotic processes :

$$\frac{d[S_2O_3^{2-}]}{dt} = -0.5\frac{d[HS^-]}{dt} - \frac{d[S_x^{2-}]}{dt} \qquad (16)$$

Kinetics of abiotic oxidation of sulfide and S_x^{2-} have been studied in detail by several authors [6, 7, 14]. All describe the oxidation rate by a general relation of the form:

$$\frac{d[HS^-]}{dt} = -k_1[HS^-]^{\alpha}[O_2]^{\beta} \qquad (17)$$

$$\frac{d[S_x^{2-}]}{dt} = -k_2[S_x^{2-}]^{\gamma}[O_2]^{\delta} \qquad (18)$$

With k_1 and k_2 as reaction rate constants, α and γ as reaction order with respect to the sulfide concentration and the polysulfide concentration, respectively and β and δ as reaction order with respect to the dissolved oxygen concentration. According to Eq. 17 and 18, the thiosulfate formation rate only depends on the concentrations of polysulfide, sulfide and dissolved oxygen. However, the results of experiment 1 show that at constant concentrations of polysulfide, sulfide and dissolved oxygen, the thiosulfate formation rate is directly related to the H_2S supply. Therefore, it can be concluded that at O_2 limiting conditions, direct abiotic oxidation of sulfide and S_x^{2-} is not the main process that is responsible for thiosulfate formation. However, when no O_2 limiting conditions apply, such as during the start-up phase

of experiment 1, thiosulfate formation mainly is the result of abiotic oxidation of sulfide, that accumulates due to insufficient biological activity [2].

3.3.2 The Effect of pH on thiosulfate selectivity

If S_x^{2-} plays an important role in thiosulfate formation, it is expected that also the pH defined equilibrium between sulfide and S_x^{2-} affects the selectivity for thiosulfate formation. To investigate this hypothesis, the effect of pH on thiosulfate formation was studied. Similar to the experiment described above, a series of reactor experiments were performed at a constant H_2S supply of 10 mmol h^{-1} and varying $[S^{2-}_{tot}]$ at pH values ranging from 8.5 to 10.2 (experiments 2-7, Table 3.1). In Figure 3.2, it can be seen that above pH 9.9, selectivity for thiosulfate formation was at least 20%. At lower pH values however, selectivity for thiosulfate formation decreased to 3.9% at pH 8.6. At this pH, selectivity for thiosulfate formation was also studied after doubling the H_2S supply from 10 to 20 mmol L^{-1} (experiment 2, period II-III). Similar to the results at pH 10, an increase of the H_2S supply hardly affected the selectivity for thiosulfate formation (3.9% at 10 mmol h^{-1} versus 5.5% at 20 mmol h^{-1}). At pH values of 8.5 and below, sulfide and S_x^{2-} slowly accumulated in the reactor liquor and stable reactor operation could not be achieved.

TABLE 3.1 **Selectivity for product formation during fed-batch operation at different operational conditions.**

Reactor Experiment Nr. (period)	Sulfide supply (mmol h^{-1})	pH	$[S^{2-}_{tot}]$ (mM) (±0.05)	Selectivity (%)			O_2/H_2S consumption ratio (mol mol^{-1})	
				$S_2O_3^{2-}$	SO_4^{2-}	S^0	actual	theoretical*
1, (I , SO_4^{2-})	10	10.1	<0.005	<0.1	100	0	n/d	2
1, (II)	10	10	0.40	21	<0.1	79	0.57	0.61
1, (III)	100	9.9	0.64	22	<0.1	78	0.63	0.61
2, (I)	10	8.6	0.12	3.9	3.2	93	0.59	0.57
2, (II)	20	8.6	0.11	5.5	2.2	92	0.59	0.56
3	10	8.7	0.12	5.1	11	84	0.69	0.69
4	10	8.9	0.09	7.9	5.4	87	0.63	0.62
5	10	9.0	0.60	8.7	<0.1	91	0.52	0.54
6	10	9.5	0.28	16	1.5	82	0.61	0.60
7	10	10.2	0.09	20	8.1	72	0.69	0.72

*Calculated value based on the electron- and sulfur balance, not taking into account biomass growth or cell maintenance; n/d = not determined.

Figure 3.2 also shows the relation between pH and the calculated $[S_x^{2-}]/[S^{2-}_{tot}]$ ratio, using Eq. 9 (dashed line), as determined for biologically produced sulfur at 35°C [12]. With decreasing pH, the sulfide equilibrium shifts from S_x^{2-} towards sulfide, and a smaller fraction of H_2S is converted to thiosulfate. These results imply that a linear relationship exists between sulfide speciation and the selectivity for thiosulfate formation, which runs via the pH in the bioreactor.

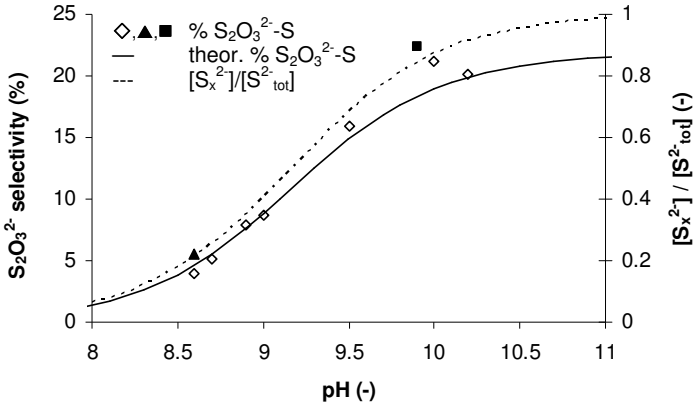

FIGURE 3.2 Selectivity for thiosulfate formation versus pH as measured in fed-batch experiments. The $[S^{2-}_{tot}]$ setpoint was always between 0.1 and 0.25 mmol L^{-1}. H_2S loads were 10 mmol h^{-1} (◇), 20 mmol h^{-1} (▲) and 100 mmol h^{-1} (■). The dashed line represents the ratio between $[S_x^{2-}]$ and $[S^{2-}_{tot}]$ (pK_x = 9.17). The solid line represents the theoretical selectivity for thisulfate formation, based on the proposed pathway (see section about reaction pathways), an average S_x^{2-} chain length of x=4.59 and pK_x=9.17.

3.3.3 Biological activity related to pH and substrate

Because the pH plays an important role in the selectivity for thiosulfate formation, the `sulfide` oxidizing activity of biomass sampled from experiment 6 (Table 3.1), was determined versus pH. With an optimum pH between 9.5 and 10 and a rapid decrease of activity above pH 10 (results not shown), the profile was typical for natronophilic SOB of the genus *Thioalkalivibrio* [19], which were present in the sediment mixture used as seed material. At pH 8.5, only 50% of the maximum activity was achieved. This indicates that the accumulation of sulfide and S_x^{2-} that occurred during reactor experiments at a pH of 8.5 and below, was a result of the obligately alkaliphilic nature of the biomass.

Figure 3.3 shows that the biomass taken from experiment 6 (pH 9.5) had a low S_x^{2-} oxidizing capacity compared to sulfide. When exposed to S_x^{2-} (pH 9.5, 2 M Na^+, total sulfur=0.3 mmol L^{-1}), visible sulfur particles appeared in the cell suspension within 5 min. Microscopic observations revealed that these particles were associated with the cells. In contrast, oxidation of S_x^{2-} without cells did not result in visible sulfur accumulation, although being almost as fast as in presence of cells. Since S_x^{2-} is very reactive, trace metals and other compounds excreted by the cells might influence its conversion [23, 24]. Therefore, actual reaction rates in the reactor matrix may be different from those obtained in experiments with washed cells in a pure buffer system. To study the effect of the reactor matrix on the oxidation rate, supernatant was obtained by centrifugation of the bulk biomass and an additional filtration step through a 0.2 µm filter. In this supernatant, S_x^{2-} (0.2 mmol L^{-1} S^{2-}_{tot}) vanished with the same O_2 consumption rate as observed with cells in pure buffer (1.8 mmol L^{-1} h^{-1}). In

the presence of cells, the S_x^{2-} oxidation rate in the supernatant did not significantly increase, indicating that in the reactor liquor, cells do not directly participate in the oxidation of S_x^{2-}.

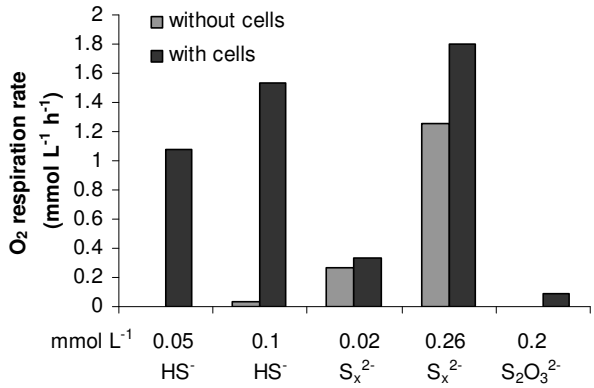

FIGURE 3.3 Respiration rates of sulfide, S_x^{2-} and thiosulfate in oxygen saturated buffers at pH 9.5, 2 mol L^{-1} Na^+, 30°C with and without presence of biomass (65 mg prot. L^{-1}, taken from reactor experiment 3 at pH 9.5). No respiration was observed with elemental sulfur dissolved in acetone.

3.3.4 Reaction pathways

The experimental results presented in this study provide grounds for possible reaction steps leading to the formation of thiosulfate and biosulfur (Fig. 3.5). The reaction sequence is initiated by the biological oxidation of sulfide to sulfur (Eq. 3), whereafter the freshly formed sulfur reacts rapidly with sulfide to form S_x^{2-} (Eq. 8). The latter was tested in batch experiments by addition of various amounts of a 2 mM sodium sulfide solution to a 2 mM solution of biologically produced sulfur, both dissolved in O_2-free acetone. Upon addition of sulfide to the dissolved biosulfur, the solution instantaneously coloured yellow as a result of S_x^{2-} formation, which was accompanied by an increase of the absorbance at 285 nm. The amount of sulfur that is transferred to S_x^{2-} in this reaction, depends on the equilibrium between sulfide and S_x^{2-} and on the S_x^{2-} chain length [11]. Most likely, this rapid formation of S_x^{2-} takes place in the cell periplasm. Indeed, cell-bound sulfur particles were observed by light microscopy, while the presence of cell-bound S_x^{2-} could be deduced from the formation of sulfide and sulfur after acidification of centrifuged biomass, as described elsewhere [25]-[26]. The excess amount of sulfur that is not consumed by the reaction with sulfide, is released from the membrane into the reactor liquor as colloidal particles. Based on the relation between the $[S_x^{2-}]/[S^{2-}_{tot}]$ ratio and the selectivity for thiosulfate formation shown in Figure 3.2, abiotic S_x^{2-} oxidation is suggested to proceed via an overall reaction with $\Delta S_2O_3^{2-}/\Delta S_x^{2-} = 0.5$ (Eq. 19). The stoichiometry of this reaction is different from the known reaction of direct

abiotic oxidation of S_x^{2-}, with $\Delta S_2O_3^{2-}/\Delta S_x^{2-} = 1$ (Eq. 7). Combining the proposed reaction sequence with the equilibrium between sulfide and S_x^{2-}, an equation can be derived that relates the selectivity for thiosulfate formation directly to pH (Eq. 20). This relation is shown in Figure 3.2 (solid line), with pK = 9.17 and x=4.59 [12]. It can be seen that the theoretical selectivity is in good agreement with the obtained experimental results. At elevated pH values, most H_2S is converted via S_x^{2-} as an intermediate to sulfur and thiosulfate according to Eq. 19. At lower pH values, a smaller fraction of H_2S is converted to S_x^{2-}. Correspondingly, a smaller fraction of the H_2S supply is converted to thiosulfate.

$$S_x^{2-} + O_2 + \tfrac{1}{2}\, H_2O \rightarrow \tfrac{1}{2}\, S_2O_3^{2-} + (x-1)\, S^0 + OH^- \quad (19)$$

$$\text{fraction } S_2O_3^{2-} = \frac{K}{x(10^{-pH} + K)} \quad (20)$$

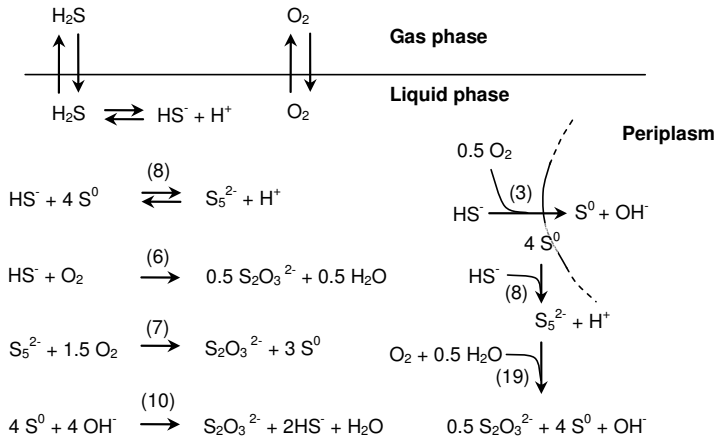

FIGURE 3.4 Reaction pathways that lead to the formation of sulfur and thiosulfate in the process H_2S removal at natronophilic conditions. On the right-hand side is the proposed new reaction pathway, based on results presented in this study. On the left-hand side are alternative routes, which may play a more important role at different conditions. For the sake of simplicity, an average S_x^{2-} chain length of x=5 is assumed. Numbers inside parenthesis refer to reaction equation numbers mentioned in the text.

It should be noted that besides this proposed pathway, other routes for thiosulfate formation may also be possible. These may become more pronounced at other process conditions. For example, abiotic oxidation of sulfide and S_x^{2-} (Eq. 6 and 7) at $[S^{2-}_{tot}]$ above 0.25 mmol L^{-1} and DO above 0.1 % sat., or disproportionation of elemental sulfur (Eq. 10) at temperatures above 35°C and pH values above 10 [14].

3.3.5 Gas treatment considerations

In the present study it is shown that at natronophilic conditions, selectivity for the conversion of H_2S to thiosulfate at oxygen limiting conditions is related to the pH of the bioreactor liquor. The results have an impact on the application of the process. The positive effect of high pH on the H_2S absorption is counteracted by a higher selectivity for thiosulfate formation and hence a higher consumption of make-up water and caustic. An optimal pH value should be low enough to minimize thiosulfate formation, but still sufficiently high to accommodate complete H_2S absorption and growth of natronophilic SOB. Given the results presented in this manuscript, this implies that the pH should be at least 8.5, as at lower values, biomass limitations occur. This study also emphasizes the importance of an accurate control of the S^{2-}_{tot} concentration. Too low [S^{2-}] (<0.25 mmol L^{-1}) will lead to the undesired formation of sulfate by complete biochemical oxidation of the reduced sulfur compounds. At too high [S^{2-}_{tot}] (> 0.25 mmol L^{-1}) however, additional formation of thiosulfate may take place due to a higher abiotic oxidation rate of sulfide and S_x^{2-}.

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Chapter 4

Application of the redox potential to control product formation

Abstract

A biotechnological process has been recently developed for the desulfurization of natural gas and refinery gas. In this process, which operates at high sodium concentrations and high pH values, maximizing selectivity for the formation elemental sulfur is one of the most important challenges. Formation of sulfate and thiosulfate is unwanted as it leads to caustic requirements for pH control and the necessity of a bleed stream. This study shows that the dissolved total sulfide (S^{2-}_{tot}) concentration is a key parameter that determines the selectivity for sulfur formation. Control of the S^{2-}_{tot} concentration can be achieved by using the measured oxidation-reduction potential (ORP) in the alkaline reactor liquid to steer the oxygen supply rate. The measured ORP depends mainly on the polysulfide concentration, which is related to the S^{2-}_{tot} concentration and pH. Therefore, an equation was derived that calculates the S^{2-}_{tot} on basis of ORP and pH, by use of electrochemical relationships and experimental results.

From investigations of the effects of ORP fluctuations on the selectivity for product formation, it follows that it takes about 25-40 min. after changing from highly reduced (ORP≤-390 mV) to less reduced (ORP>-300 mV) redox conditions, before the bacteria shift from sulfur to sulfate formation. Remarkably, at relatively oxidized redox conditions (ORP≥200 mV) and high (>130 mM) thiosulfate concentrations, all H_2S is fully converted to biosulfur, while thiosulfate is completely oxidized to sulfate.

This chapter is in preparation for submission.

4.1 INTRODUCTION

For desulfurization of biogas, natural gas, synthesis gas and other sour gasses produced in the petrochemical industry, a new biotechnological process provides an attractive alternative to existing physicochemical methods such as amine and modified Claus processes. A process for gas desulfurization, consisting of an absorber, a bioreactor and a sulfur settler, has been recently developed [1, 2]. In this process, microbiological oxidation of sulfide (H_2S) to elemental sulfur ("biosulfur", S^0) occurs under oxygen limited conditions, at DO (dissolved oxygen) concentrations below 0.1 mg L^{-1} (Eq. 1) (Chapter 2) [1, 3]. Formation of more oxidized sulfur compounds, such as sulfate (SO_4^{2-}, Eq. 2) and thiosulfate ($S_2O_3^{2-}$, Eq. 3) is unwanted as it leads to caustic requirements for pH control and the formation of a bleed stream to remove the formed sulfoxyanions (Chapter 1) [4]. Therefore, one of the most important challenges for the further development of this biotechnological process is to maximize the selectivity for biosulfur formation. As a result of high CO_2 partial pressures occurring in sour natural gas and to reduce the consumption of make-up water, biological gas desulfurization is preferably performed at elevated concentrations of the cations (i.e. Na^+ and K^+) that counter the bicarbonate, carbonate, sulfate and thiosulfate anions present in the bioreactor liquid. As a high pH is beneficial for the absorption of H_2S (Eq. 4), the process is carried out at "natron-alkaline" conditions i.e. at elevated Na^+/K^+ concentrations (>1.5 M), in combination with a high pH (>8.5).

$H_2S + ½\ O_2 \rightarrow S^0 + H_2O$	(biological reaction)	(1)
$H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2\ H^+$	(biological reaction)	(2)
$2\ H_2S + 2\ O_2 \rightarrow S_2O_3^{2-} + H_2O + 2\ H^+$	(non-biological reaction)	(3)
$H_2S + OH^- \rightleftarrows HS^- + H_2O$	(dissociation of H_2S)	(4)
$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+$	(non-biological reaction)	(5)
$S_x^{2-} + 1½\ O_2 \rightarrow S_2O_3^{2-} + (x\text{-}2)\ S^0$	(non-biological reaction)	(6)

Several studies show that the dissolved sulfide concentration is a key parameter that determines the selectivity for sulfur formation (Chapter 2) [1, 5]. At natron-alkaline conditions, the total sulfide (S^{2-}_{tot}) concentration comprises sulfide (HS^- and S^{2-}) and polysulfide anions (S_x^{2-}), which are formed from the reaction between sulfide and biosulfur particles (Eq. 5). The S^{2-}_{tot} concentration can be controlled by controlling the oxygen supply rate to the reactor [6].

Control of the S^{2-}_{tot} concentration can be achieved by using the measured oxidation-reduction potential (ORP) in the alkaline reactor liquid to steer the oxygen supply rate. The ORP of an aqueous solution is determined by its tendency to either accept or donate electrons and is a result of the proportion of the oxidized and reduced forms of all dissolved

compounds. The tendency of a redox-active molecule to donate or accept electrons can be expressed by the value of E_h, also termed the electromotive force, which is expressed relative to a standard hydrogen electrode (SHE). The measured value of E_h in a sulfide oxidizing bioreactor depends on the kinetics of reduction and oxidation reactions. In the neutral pH region, the measured ORP in a sulfide-oxidizing bioreactor is mainly determined by the sulfide concentration [6].

4.1.1 Polysulfide chemistry

Polysulfide anions of chain length x can be represented by the formula "$(x-1)S^0$-S^{2-}". Thus, a polysulfide anion comprises (x-1) zerovalent sulfur atoms (S^0) and one sulfide atom of divalent state (S^{2-}) [7]. The formation of polysulfide anions (Eq. 5) is the result of various hydrolysis and interconversion reactions and the polysulfide species have been detected with chain lengths varying from 2 to 9 ([7] and references therein). At mildly alkaline conditions and in equilibrium with excess inorganic sulfur, reported average polysulfide chain lengths vary from 4.39 to 5.5 [7-14]. Kamyshny et al. [7] show that in a solution where elemental sulfur is in excess, the fraction of polysulfide species of a specific chain length is not affected by pH or by the total polysulfide concentration. This means that the average polysulfide chain length is also not affected by pH or polysulfide concentration. Previous bioreactor experiments show that in a sulfur producing bioreactor, sulfide- and polysulfide concentrations are in equilibrium, or at least very close to equilibrium (Chapter 2) [1, 9], according to:

$$K_x = \frac{[S_x^{2-}][H^+]}{[HS^-]} \times \frac{\gamma_{S_x^{2-}}\gamma_{H^+}}{\gamma_{HS^-}} \quad \text{with } pK_x = 9.17 \qquad (7)$$

Thus, depending on the pH of the reactor liquid, the majority of the S^{2-} concentration is present as sulfide (at $pH < 9.17$), or as polysulfides ($pH > 9.17$).

Assuming that the activity coefficients (γ) for polysulfide and sulfide are identical, Eq. 7 can be simplified to:

$$K_x = \frac{[S_x^{2-}] \cdot 10^{-pH}}{[HS^-]} \qquad (8)$$

It is however quite likely that this assumption is not fully correct. Because the ionic strength of the reactor medium is around I = 2.7 M, many available models to calculate the activity coefficient cannot be applied. For example, the Debye-Hückel theory is limited to an ionic strength up to 0.5 M [15], while application of Pitzer ion-interaction models is complicated due to a lack of parameter values [16]. Investigative calculations of activity coefficients have

been performed with the Environmental Simulation Program (ESP) from OLI Systems, Inc. (Morris Plains, NJ). This simulation programme uses the revised Helgeson-Kirkham-Flowers equations of state for the calculation of standard state thermodynamic properties [17]. It was found that the activity coefficient for polysulfide is somewhat lower compared to that of bisulfide. Neglecting the effect of activity coefficients in Equation 8 may lead to small errors in the determination of the average polysulfide chain length (by combination of Eq. 8, 9 and 21, see also section 4.2.5). Moreover, the high ionic strength may also affect the value of pK_x.

In the alkaline pH range of the process (8.5-10), polysulfides with chain lengths 2 and 3 can be present in solution in both protonated and deprotonated forms (pKa of HS_2^- is 9.7, pKa of HS_3^- is 7.5, [18]). However, as the sum of the fractions of S_2 and S_3 species is low compared to the total polysulfide concentration (together 4.5% of all polysulfide species, calculated according to ref. [19]), also HS_2^{2-} and HS_3^{2-} concentrations are low as compared to the S^{2-}_{tot} concentration. In the prevailing pH range (8.5-10.1), concentrations of H_2S (1.3% of S^{2-}_{tot} at pH 8.5), HS_2^{2-} (2.1% at pH 8.5) and HS_3^{2-} (0.14 % at pH 8.5) are not taken into account. Therefore, it has been assumed that a simplified equation for the S^{2-}_{tot} concentration applies (Eq. 9):

$$[S^{2-}_{tot}] = [HS^-] + [S_x^{2-}] \quad (9)$$

4.1.2 Redox chemistry

In order to apply the measured ORP as an online control parameter to steer the oxygen supply rate, a mathematical relation is required that describes the S^{2-}_{tot} concentration as a function of ORP. Obviously, also the effect of pH needs to be taken into account. Such an equation can be derived from the electrochemical reactions involving sulfide and polysulfide species. As mentioned above, sulfide and polysulfide ions are the highest in concentration. Therefore only these species are taken into account. For each polysulfide species, the half-reaction describing the equilibrium with sulfide ions can be represented as follows [20]:

$$S_4^{2-} + 4\ H_2O + 6\ e^- \rightleftarrows 4\ HS^- + 4\ OH^- \quad (10.A)$$

$$S_5^{2-} + 5\ H_2O + 8\ e^- \rightleftarrows 5\ HS^- + 5\ OH^- \quad (10.B)$$

$$S_6^{2-} + 6\ H_2O + 10\ e^- \rightleftarrows 6\ HS^- + 6\ OH^- \quad (10.C)$$

Likewise, a generic equation for other polysulfides with of a chain length “x” can be written as follows:

$$S_x^{2-} + x\ H_2O + (2x-2)\ e^- \rightleftarrows x\ HS^- + x\ OH^- \quad (11)$$

The theoretical ORP value of a solution versus a standard H_2 electrode potential can be thermodynamically calculated using the Nernst equation:

$$E_h = E_h^0 - \frac{R \cdot T}{n \cdot F} \ln \frac{\prod_j [red]^{n_j}}{\prod_i [ox]^{n_i}} \quad (12)$$

Application of this general equilibrium equation (Eq. 11) to the Nernst equation (Eq. 12), the electrode potential (E) can be represented as:

$$E = E^0 - \frac{R \cdot T}{n \cdot F} \ln \frac{[HS^-]^x [OH^-]^x}{[S_x^{2-}]} \quad (13)$$

With n = (2x-2) (in Eq. 13) and neglecting activity coefficient corrections, Equation 13 can be combined with the pH defined equilibrium between $[HS^-]$ and $[S_x^{2-}]$ (Eq. 8). This yields an equation that shows the electrode potential in relation to the polysulfide concentration (Eq. 14):

$$ORP = E^0 - a - b \cdot \ln[S_x^{2-}] \quad (14)$$

with:

$$a = \frac{RT}{(2x-2)F} \cdot \ln\left(\frac{10^{-14}}{K_x}\right)^x \quad (15)$$

and

$$b = (x-1) \cdot \frac{RT}{(2x-2)F} \quad (16)$$

Combination of Eq. 14 and 8 yields an equation that relates the ORP to the S^{2-}_{tot} concentration and pH:

$$ORP = E^0 - a - b \cdot \ln\left(\frac{[S^{2-}_{tot}] \cdot K_x}{K_x + 10^{-pH}}\right) \quad (17)$$

or:

$$[S^{2-}_{tot}] = \left(1 + \frac{10^{-pH}}{K_x}\right) \cdot e^{-\frac{ORP - E^0 + a}{b}} \quad (18)$$

As the S^{2-}_{tot} concentration is assumed to be the decisive parameter for sulfur formation, Equation 18 can be applied to control the oxygen supply rate while optimizing the selectivity for sulfur formation.

4.1.3 Objective

The aim of the current study is (1) to assess the experimental relationship between pH, (poly)sulfide concentration and ORP and (2) to show how this relation can be applied to maximize selectivity for biosulfur formation. Finally (3), the effect of process disturbances on the selectivity for product formation is investigated.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The results described in this chapter were obtained with the same bioreactor set-up as described previously in Chapter 2. Also the same analytical and experimental equipment was applied.

4.2.2 Medium

In all reactor experiments, a liquid reactor medium was used containing 0.66 M Na^+ and 1.34 M K^+. The medium was prepared as described previously by mixing sodium- and potassium (bi)carbonate buffers. Macro nutrients (K_2HPO_4, urea, NaCl, $MgCl_2$) and trace elements were added accordingly.

4.2.3 Inoculum

The natronophilic biomass that was used to inoculate the bioreactor was collected from previous reactor experiments (Chapter 3) [2]. The original seed material to grow biomass consisted of a mixture of sediments taken from hypersaline soda lakes in Mongolia, south-western Siberia and Kenya and was kindly provided by Dr. Sorokin, Department of Biotechnology, Delft University of Technology [21].

4.2.4 Reactor operation

Data obtained by both off-line liquid analyses (e.g. polysulfide and S^{2-}_{tot} concentration) and on-line measurements (pH and ORP) from a large number of independent bioreactor runs was used in the investigation of the relation between ORP, (poly)sulfide concentration and pH (section 4.3.1). The mode of reactor operation (i.e. fed-batch or a continuously fed system, with or without pH control), H_2S and oxygen supply rates (maintained at a fixed value or controlled on basis of ORP), as well as concentrations of biomass, sulfate, thiosulfate and sulfur may vary for the various reactor runs. Results were grouped in three pH ranges, viz.8.5±0.2, 9.0±0.2, and 10±0.2.

A number of reactor experiments were selected to study the effect of the S^{2-}_{tot} concentration on product formation at two different pH ranges (section 4.3.2). For this study, only those runs were selected that provided sufficient analytical data at stable reactor operation (ORP deviation of ±5 mV around and average value). A total of 16 reactor runs were selected, of which 9 belong to the pH range 8.3-9.2 and 7 to the pH range 9.8-10.2. The H_2S supply rate

for these runs was 51.0 mmol L^{-1} d^{-1}. The oxygen supply rate was based on control of the S^{2-}_{tot} concentration at a desired setpoint value. A proportional control mechanism was applied, as schematically represented in Fig. 4.1.

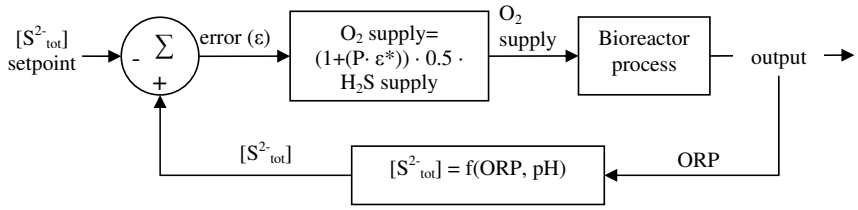

FIGURE 4.1 Schematic representation of the oxygen supply rate control strategy, applying a proportional control mechanism based on the calculated S^{2-}_{tot} concentration.

A proportional control law was implemented according to the following equation:

$$O_2 \text{ supply rate} = (1+P) \cdot \varepsilon^* \cdot 0.5 \; H_2S \text{ supply rate (mmol } h^{-1}) \qquad (19)$$

with:

H_2S supply rate	(mmol h^{-1})
P = proportional control factor	(-)
ε = error calculated by $[S^{2-}_{tot}] - [S^{2-}_{tot}]_{set}$	(mM)

ε^* = error used in control proportional control,
with $\varepsilon^* = \varepsilon$ if $\varepsilon > 0$
0 if $\varepsilon \leq 0$

As a result of this control law, the O_2 supply rate amounts to 0.5 times the H_2S supply rate for $[S^{2-}_{tot}] \leq [S^{2-}_{tot}]_{set}$. The value of P was manually set to values between 0.5 and 4. By application of this control strategy, the reactor was operated as a "sulfido-stat", maintaining S^{2-}_{tot} concentrations slightly above the desired setpoint value (0.01 - 0.05 mM). By using the S^{2-}_{tot} concentration as a control variable, pH and logarithmic ORP effects are all included in the "error" calculation. This is an improvement compared to an earlier presented control scheme in which the ORP is directly used as a control variable [6].

4.2.5 Analytical methods

The S^{2-}_{tot} concentration was measured based on a modified methylene blue method as described by ref. [22]. Before analysis, samples were filtrated over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66). To prevent abiotic sulfide oxidation, 1 mL zinc acetate (20 g L^{-1}) per mL of sample was added immediately after sampling. The formed ZnS precipitate was washed and diluted with demineralised water to remove any dissolved

salts. The S^{2-}_{tot} concentration in diluted ZnS precipitate was measured using Lange cuvette test kits (LCK653, Hach Lange, Germany).

The polysulfide concentration was determined spectrophotometrically as described by ref. [9], at a wavelength of 285 nm (Perkin-Elmer, Lambda 2, UV/VIS-spectrophotometer). With this method, the total concentration of zerovalent sulfur atoms in polysulfide is measured: $[S_x^{2-}\text{-}S^0]$. the polysulfide concentration can be calculated when the average chain length x is known, according to:

$$(x\text{-}1) \cdot [S_x^{2-}] = [S_x^{2-}\text{-}S^0] \qquad (20)$$

As mentioned above, reported average for polysulfides in equilibrium with excess inorganic sulfur, chain lengths vary from 4.39 to 5.5 (section 4.1). Before analysis, samples were filtrated over a 0.2 µm cellulose acetate membrane filter (Schleicher & Schuell OE 66). The molar extinction coefficient was determined for the high salt medium and was found to be 1300 L mol^{-1} cm^{-1}.

The ORP was measured versus a saturated KCl, Ag/AgCl reference electrode (SenTix ORP, WTW, Germany). The electric potential measured by this electrode deviates from a standard H_2 reference electrode (SHE) by +200 mV at 35ºC.

Analytical methods used for measurement of the biomass concentration and sulfoxyanions concentration were described previously in Chapter 2 [1].

4.3 RESULTS AND DISCUSSION

4.3.1 Relation between ORP, (poly)sulfide concentration and pH

To apply the equation that calculates the S^{2-}_{tot} concentration on basis of the measured ORP and pH (Eq. 18), it is necessary to know the values for x (the average polysulfide chain length) and E^0. Moreover, the value of x also plays a role in the calculation of the polysulfide concentration, as it is measured as the concentration of zerovalent sulfur in polysulfide: $[S_x^{2-}\text{-}S^0]$. Hence, it also affects the sulfide concentration, which is calculated by subtraction of the polysulfide concentration from the S^{2-}_{tot} concentration (Eq. 9): $[HS^-] = [S^{2-}_{tot}] - [S_x^{2-}]$.

It was shown previously that at a pH of 10±0.1, the ratio between the zerovalent sulfur concentration in polysulfide and the S^{2-}_{tot} concentration ($[S_x^{2-}\text{-}S^0]$:$[S^{2-}_{tot}]$) is 4.4 : 1 (Chapter 2) [1]. According to Equation 8, 89.5% of S^{2-}_{tot} is present as S_x^{2-} at pH 10.1, under the assumption that sulfide and polysulfide in the bioreactor are at equilibrium. This results in a theoretical average polysulfide chain length of 6.0 (Eq. 20). This value is however higher than the equilibrium value 4.59±0.31 that was reported previously for polysulfide anions prepared from biosulfur particles at lower salt concentrations at 35ºC [9]. Possibly, the effect of ion activities plays a role in the difference between both values. As mentioned in section 4.1.4, the activity coefficient for polysulfide is somewhat lower compared to that of bisulfide (HS^-). As a result, application of Equation 8 leads to lower S_x^{2-} concentrations and therefore to

higher values for the polysulfide chain length when using Equation 20. It is assumed that the value of x = 6.0 can be used as a "lumped" value, incorporating activity coefficient effects.

In Figure 4.2, the measured S_x^{2-}-S^0 concentration is plotted versus the measured S^{2-}_{tot} concentration for results obtained from bioreactor experiments at three different pH values. For pH 9.0±0.2 and 10.0±0.2, the slope ($[S_x^{2-}\text{-}S^0]/[S^{2-}_{tot}]$) corresponds reasonably well with the theoretical relation for x=6.0 (solid lines in Fig. 4.2). For pH 8.5±0.2, the measured S_x^{2-}-S^0 concentration is somewhat higher than expected.

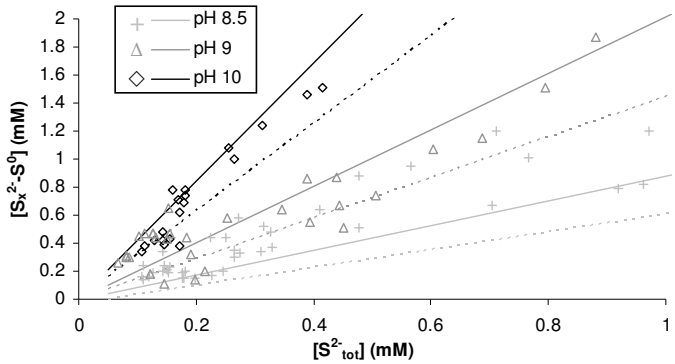

FIGURE 4.2 Relation between measured concentrations of S_x^{2-}-S^0 and S^{2-}_{tot}, at different pH values. The straight lines show the theoretical relation for pK_x = 9.17 and x = 6.0 (solid lines) and x = 4.59 (dashed lines).

Based on the Nernst equation (Eq. 13), the slope of a graph depicting ORP versus $\ln([HS^-]^x[OH^-]^x/[S_x^{2-}])$ reveals the value of -RT/(2x-2)F while the intercept with the y-axis yields the value of E^0. With a value of x=6.0, the calculated value of $\ln([HS^-]^x[OH^-]^x/[S_x^{2-}])$ obtained from bioreactor experiments at three different pH values, is plotted against the measured ORP in Figure 4.3. The theoretical value of -RT/(2x-2)F is –2.65. This slope was used in a minimum least square error linear fit to retrieve the value of E^0 (dashed line in Fig. 4.3). The intercept with the x-axis results in a value of E^0 = -702 mV, corresponding to -502 mV vs. the standard H_2 electrode potential (SHE) (not shown in graph). This value is close to -510 mV as determined for S_4^{2-} by ref. [23]. Unfortunately, the data obtained from gas-lift experiments show too much scatter to provide a reliable estimate of the slope (the fitted linear trendline has a slope of -1.3, with R^2 is 0.6, not shown)

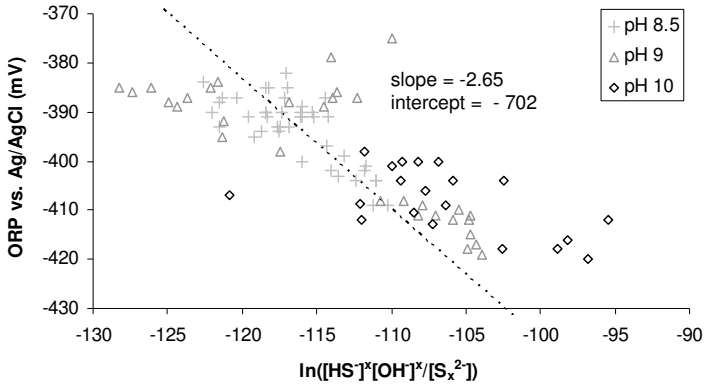

FIGURE 4.3 Measured ORP (vs. Ag/AgCl) in the bioreactor, versus $\ln([HS^-]^{6.0}[OH^-]^{6.0}/[S_x^{2-}])$ (concentrations in mol L^{-1}). The black dashed line shows the best fit with the measured data, assuming a slope of -2.65 as calculated by RT/nF with n = (2x-2), x (polysulfide chain length) = 6.0 and T = 35ºC.

With the estimated values for x (6.0) and E^0 (-702 mV) as described above, the measured ORP can be related to the polysulfide concentration (Eq. 14), or to the S^{2-}_{tot} concentration and pH value (Eq. 17). The theoretical relation between the polysulfide concentration and ORP and the S^{2-}_{tot} concentration and ORP are shown in Figure 4.4 A and B, respectively, for three different pH values, viz.: 8.5, 9.0 and 10.0 (all ±0.2). Figure 4.4 C shows the theoretical and experimental relation between the sulfide concentration and ORP, obtained by subtraction of the polysulfide concentration from the S^{2-}_{tot} concentration (Eq. 9). Although the measured concentrations match quite well with the theoretically calculated values, some points deviate substantially. The reason can, most probably, be found in an accumulation of small errors in measurements of pH, ORP and (poly)sulfide concentrations. Moreover, deviations from the theoretical slope and used values for pK_x and x may also affect the outcome of the calculations.

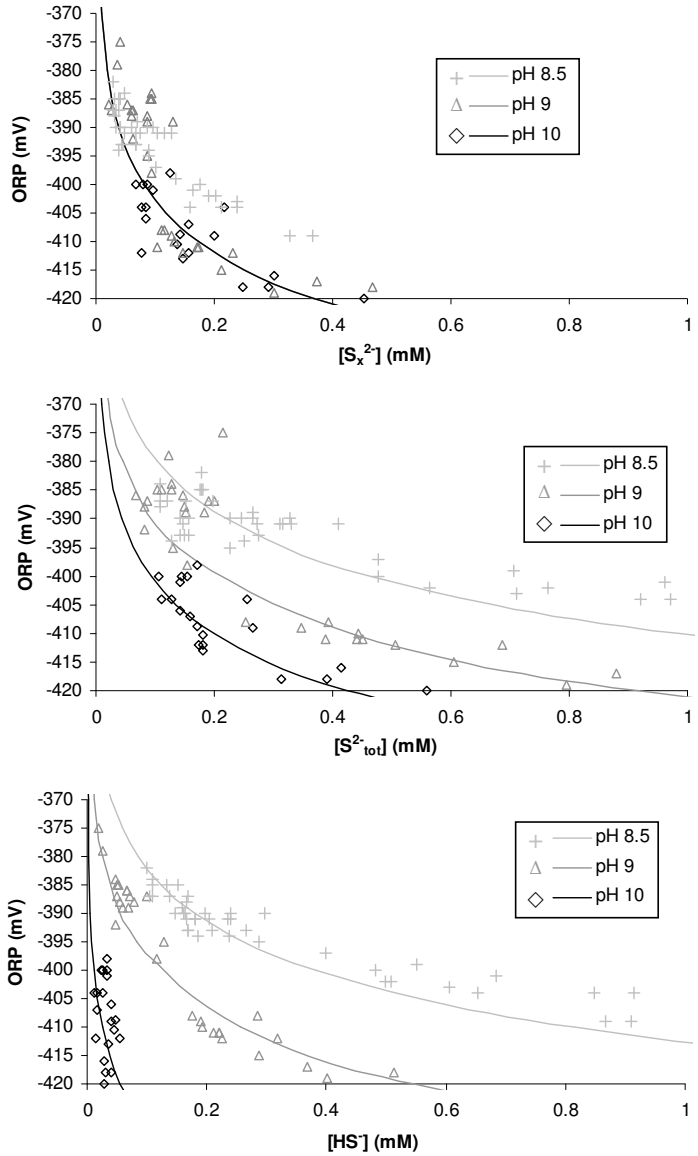

FIGURE 4.4 A-C **Relation between ORP and concentrations of polysulfide (A), S^{2-}_{tot} (B) and sulfide (C), at different pH values. The lines represent calculated concentrations based on Eq. 14 (A) and 17 (B), (pK_x = 9.17, x = 6.0 and E^0 = -0.702 V). The marks represent measurements from reactor experiments. The sulfide concentration (C) is calculated by subtraction of the polysulfide concentration from the S^{2-}_{tot} concentration: $[HS^-] = [S^{2-}_{tot}] - [S_x^{2-}]$, for both the reactor measurements and the theoretical lines.**

In general, the experimental results are in good agreement with the electrochemical calculations that are based on spectroscopic analysis of the equilibrium that exists between sulfide and polysulfide in the bioreactor. It is shown that the measured ORP mainly depends on the polysulfide concentration, which is determined by the S^{2-}_{tot} concentration and the pH. As a result, the measured ORP increases with decreasing pH values when S^{2-}_{tot} concentrations remain constant. It has to be noted that this relation is only valid for a system where biologically produced sulfur particles are present in excess amounts. Hence, concentrations of sulfide and polysulfide are close to equilibrium. The implication of these findings on process operation is dealt with in the next section.

4.3.2 Combined effects of pH and S^{2-}_{tot} concentration on product formation

Effects of pH and S^{2-}_{tot} concentration on sulfate formation.

The aim of this paragraph is to obtain more insight in the combined effects of pH, S^{2-}_{tot} concentration and ORP on product formation in a continuously operated bioreactor. The relation between S^{2-}_{tot} concentration and selectivity for sulfate and thiosulfate formation in several bioreactor experiments is grouped in two pH ranges: an “upper” pH (9.6-10.1) and a “lower” pH (8.5-9.0) range. As shown in the previous sections, pH affects the equilibrium between sulfide and polysulfide; at the lower pH range (8.5-9.0), about 18-40% of S^{2-}_{tot} is present as polysulfide, while at the upper pH range (9.6-10.1), the polysulfide content is as high as 73-89%. For both pH ranges, the selectivity for sulfate formation was very low (0-1.6 mol%) at S^{2-}_{tot} concentrations above 0.25 mM, while at S^{2-}_{tot} concentrations below 0.25 mM, sulfate formation increases with decreasing S^{2-}_{tot} concentrations (Fig 4.5 A). For all experiments the molar O_2/H_2S supply ratio was within 90% of the theoretical ratio, as calculated based on the electron balance (data not shown).

As a result of the sulfide-polysulfide equilibrium, the ORP at which sulfate formation ceases is different for both pH ranges i.e. around -390 mV at the lower pH range and around -410 mV at the upper pH range (see arrows in the inserted figure of Fig. 4.5 A). Figure 4.5 B shows that for the same S^{2-}_{tot} concentration, the ORP value is different for both pH ranges. This indicates that selectivity for sulfate formation is much stronger correlated to the S^{2-}_{tot} concentration than to the ORP. From a microbiological perspective this is understandable because the bacteria detect the S^{2-}_{tot} concentration directly, whereas the ORP is only a derivative parameter.

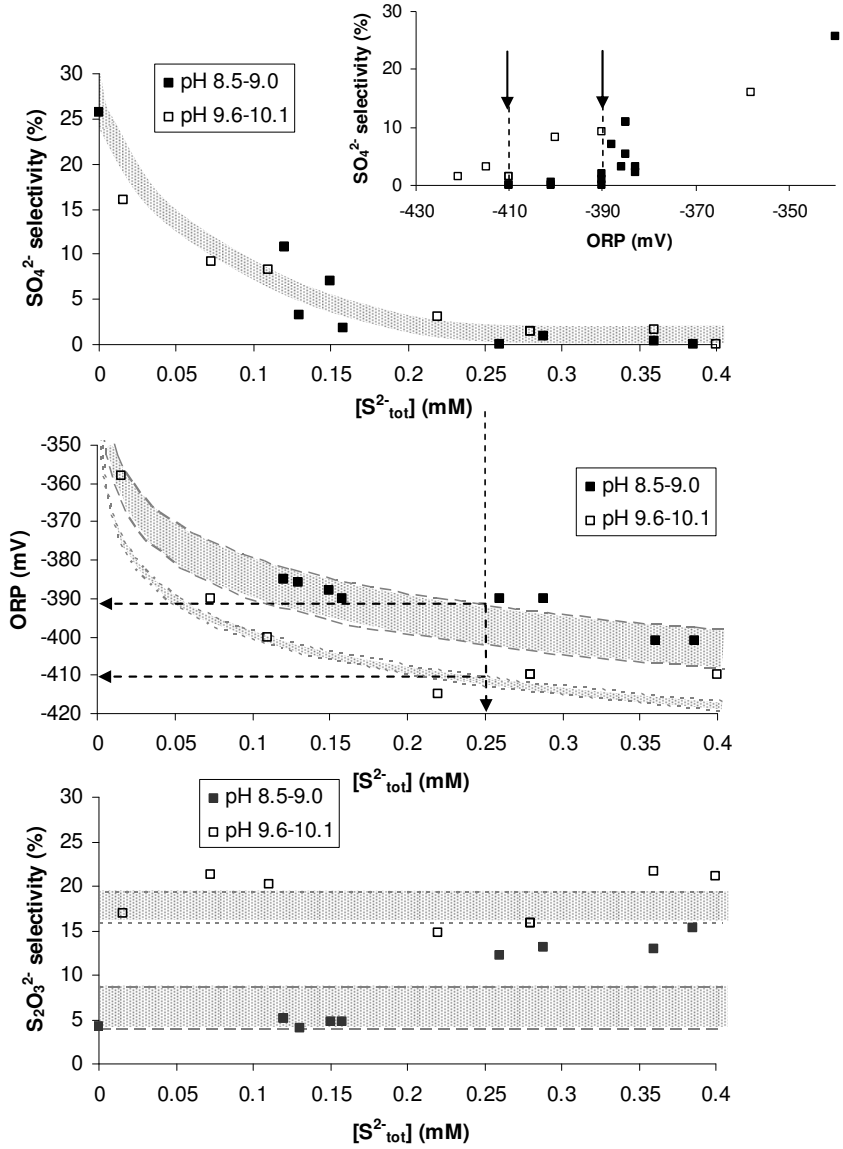

FIGURE 4.5 A-C **Effect of S^{2-}_{tot} concentration during H_2S oxidation at two different pH ranges: 8.5-9.5 ("lower" pH range) and 9.6-10.1 ("upper" pH range). A: selectivity for sulfate formation. The insert shows the relation between $\%SO_4^{2-}$ and ORP; B: ORP; C: selectivity for thiosulfate formation. The dashed lines represent: B: Calculated ORP values, based on the pH and the S^{2-}_{tot} concentration, as shown by Eq. 17, for a pH of 8.5-9.0 (···) and a pH of 9.6-10.1 (---), with pK_x = 9.17, x = 6.0 and E^0 = -0.702 V. C: calculated values of minimum thiosulfate formation, based on pH, as described by Eq. 21 for a pH of 8.5-9.0 (···) and a pH of 9.6-10.0 (---), with pK_x = 9.17 and x = 4.59. Further explanation in text.**

Effects of pH and S^{2-}_{tot} concentration on sulfate formation

The relation between the S^{2-}_{tot} concentration and selectivity for thiosulfate formation is different for both pH ranges (Fig. 4.5 C). For the upper pH range, selectivity for thiosulfate formation is not so much affected by the S^{2-}_{tot} concentration (up to 0.4 mM) and ranges from 15 to 22 mol%. For the lower pH range on the other hand, at a S^{2-}_{tot} concentration below 0.2 mM, selectivity for thiosulfate formation is around 5 mol%, while at S^{2-}_{tot} concentrations above 0.25 mM, selectivity for thiosulfate formation increases with increasing S^{2-}_{tot} concentrations, up to 15 mol% at 0.4 mM S^{2-}_{tot}.

The relation between reactor pH and selectivity for thiosulfate formation at oxygen-limited (sulfur forming) conditions was also investigated previously and reported in Chapter 3 [2]. It is shown that selectivity for thiosulfate formation is affected by pH, following the equilibrium between sulfide and polysulfide. Membrane-bound polysulfide is assumed to play a role as an intermediate. Based on a mechanistic model, it is shown that the fraction of H_2S that is converted to thiosulfate can be calculated according to:

$$\text{fraction } S_2O_3^{2-} = \frac{K_x}{x \cdot (10^{-pH} + K_x)} \qquad (21)$$

Application of Equation 21 to the two pH ranges shows that selectivity for the lower pH range (8.5-9.0) ranges from 3.8 – 8.8 mol% and for the upper pH range (9.6-10.1) from 15.9 – 19.5 mol%, assuming an average length of the polysulfide ion of 4.59, with pK_x = 9.17 (dashed lines in Fig 4.5 C). For the upper pH range, the selectivity for thiosulfate formation corresponds quite well with the calculated values and is not very much influenced by the S^{2-}_{tot} concentration. For the lower pH range on the other hand, at S^{2-}_{tot} concentrations above 0.25 mM, more thiosulfate is formed than calculated based on Equation 21. An explanation for this observation is that apart from thiosulfate formation via the proposed membrane-bound polysulfide pathway (Eq. 21), extra thiosulfate is likely formed from the abiotic oxidation of sulfide and polysulfide (Eq. 22 and 23). These reactions were not taken into account in the derivation of Eq. 21.

$$HS^- + O_2 \rightarrow ½\ S_2O_3^{2-} + ½\ H_2O \qquad (22)$$

$$S_x^{2-} + 1½\ O_2 \rightarrow S_2O_3^{2-} + (x\text{-}2)\ S^0 \qquad (23)$$

4.3.3 Effect of process disturbances on product formation

In previous studies on biological H_2S oxidation at natron-alkaline conditions, selectivity for product formation was always determined at stable redox conditions (i.e. operation at constant ORP values and (poly)sulfide concentrations; see Chapter 2 and 3 and the previous section). In a full-scale gas-lift bioreactor however, redox conditions may vary over the height of the

reactor. Close to the inlet point of the sulfide-loaded influent, (poly)sulfide concentrations will be higher than further downstream, due to mixing and conversion. Moreover, fluctuations in gas flows and/or gas H_2S concentrations can result in variations in the redox conditions. As these dynamics very likely affect the process selectivity, the aim of this section is to assess the effects of ORP fluctuations on product formation.

Mixing dynamics may play an important role in the performance of large full-scale bioreactors. In order to study this phenomenon in small scale laboratory reactors (with a liquid height of 65-70 cm), the loop circulation time and the mixing time of the bioreactor were determined by using a fluorescein tracer. At a gasflow of 5 L min^{-1}, a mixing intensity of 95% (as defined in ref. [24]) was achieved after about 25 sec, with a loop circulation time of 6.0±0.5 sec (data not shown). As will be shown later, the time scale for mixing in the lab-scale reactor is low compared to that of ORP changes in the experiments that will be discussed below. It is therefore assumed that the experimental results were not affected by mixing dynamics.

Three different experiments were performed to simulate the effect of mixing and fluctuations in the H_2S loading rate on product formation in full-scale bioreactors: (1) interruption of the H_2S addition; (2) intermittent H_2S addition and (3) long-term shift from reduced to relatively oxidized redox conditions. A detailed description of these experiments and the corresponding results is given below.

Short interruption of the H_2S addition

The molar O_2/S^{2-}_{tot} consumption ratio in the lab-scale bioreactor can be followed in time by combination of oxygen and H_2S loading rates with online calculation of the S^{2-}_{tot} concentration in the liquid phase (via the ORP) and online measurement of the oxygen concentration in both the gas and liquid phase. According to the stoichiometry of Equation 1 and 2, the O_2/S^{2-}_{tot} consumption ratio is 0.5 mol mol^{-1} for sulfur formation and 2.0 mol mol^{-1} for sulfate formation. Variations in the O_2/S^{2-}_{tot} consumption ratio after a short interruption of the H_2S addition were used to detect changes in product formation. Prior to a first 16-min interruption of H_2S addition, the reactor was operated at reduced, sulfur forming redox conditions (0.035 mmol H_2S L^{-1} min^{-1}; $[S^{2-}_{tot}]$ = 0.33 mM; O_2/H_2S consumption ratio of 0.55 mol mol^{-1}; Fig. 4.6 A-C, t= -20 to 0 min.). During the first 16-min interruption of the H_2S supply (t= 0 - 16 min.), the S^{2-}_{tot} concentration gradually decreased from 0.33 mM down to 0.07 mM. To reduce the S^{2-}_{tot} concentration down to a level that constant ORP values would lead to significant sulfate formation (i.e. $[S^{2-}_{tot}]<<0.01$, leading to >25 mol% SO_4^{2-}), the H_2S addition was interrupted for a second 16-min period (t= 26-42 min.). During this second interruption, the S^{2-}_{tot} concentration decreased to levels below the detection limit ($[S^{2-}_{tot}]<0.01$ mM) and for a period of 11 min. the ORP was above -300 mV, indicating complete depletion of (poly)sulfide. If applied for a long period (e.g. several hours), such oxidized conditions would lead to significant sulfate formation. Hence, the O_2/S^{2-}_{tot} consumption ratio would be around 2 mol mol^{-1}. However, after a short period of (poly)sulfide depletion, the O_2/S^{2-}_{tot}

consumption ratio remained around 0.55 mol mol^{-1} (Fig 4.6 A, t= 42-100 min.). This indicates that after abandoning reduced redox conditions for a short period (i.e. up to at least 10 min.), the biomass does not shift from sulfur to sulfate production.

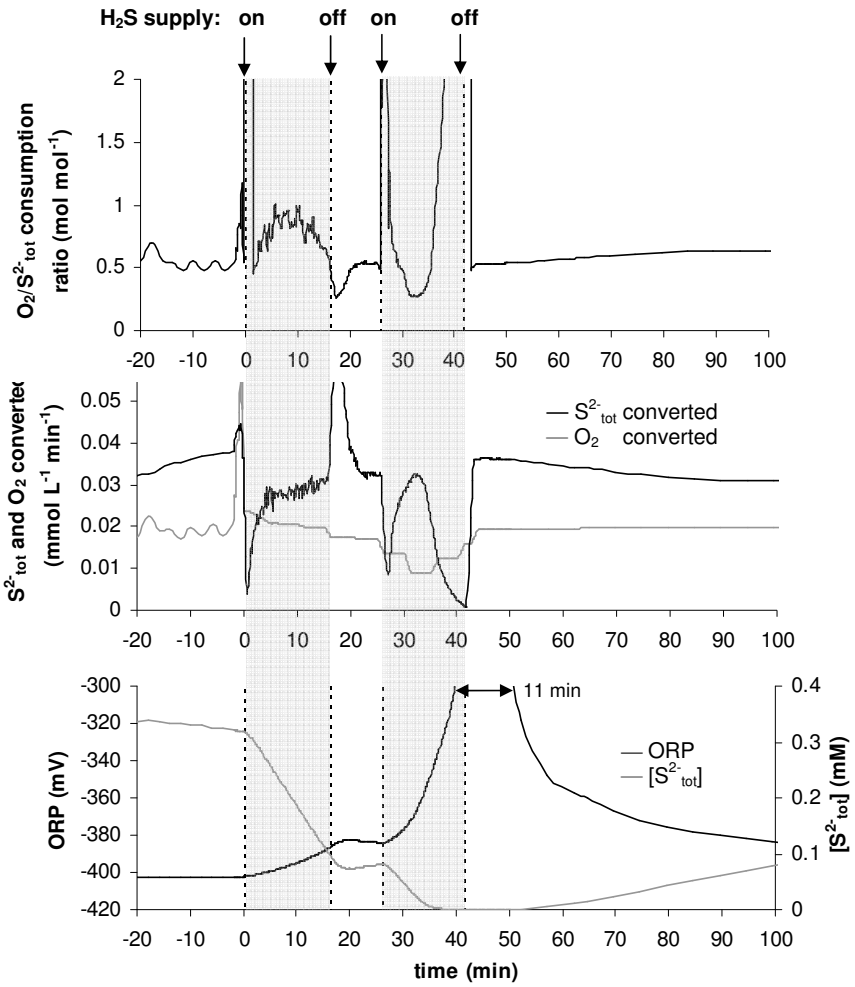

FIGURE 4.6 A-C **Results from temporary interruption of the H_2S supply. A: S^{2-}_{tot} and O_2 consumption rate; B: molar O_2/S^{2-}_{tot} consumption ratio; ORP and S^{2-}_{tot} concentration (based on ORP). The shaded areas show the periods in which the H_2S supply was interrupted. The dashed lines and the horizontal arrow indicate the period in which the ORP was above -300 mV. pH= 9.1, T= 35 °C, biomass concentration = 32 mg N L^{-1}.**

In this experiment, the biological (poly)sulfide oxidation rate of around 0.035 mM min^{-1}, (1.1 μM mg N^{-1} min^{-1}, Fig. 4.6 B) was too low to deplete the reactor from (poly)sulfide within a 16-min interruption of the H_2S addition. However, the oxidation rate was most likely limited due to a limited availability of oxygen. It was shown previously that H_2S oxidation rates up to 0.35 mM min^{-1} (3.8 μM mg N^{-1} min^{-1}) are well possible, provided that sufficient oxygen and biomass are available (Chapter 3) [2]. Therefore, in a full-scale bioreactor operating at high volumetric loading rates (e.g. 20 kg S m^{-3} d^{-1}), (poly)sulfide depletion might occur if oxygen availability is not limited. As shown above, no shift from sulfur to sulfate formation is observed after 10 min. of (poly)sulfide depletion. With an estimated circulation time of a full-scale airlift reactor to be in the order of 1-2 minutes [24], this implies that in a full-scale reactor, fluctuations in redox conditions along the height of the reactor will probably not lead to a shift in product formation.

The absence of a shift from sulfur to sulfate formation can be explained by the presence of cell-bound polysulfides. This form of sulfur behaves as polysulfide bound to the cell, which in acidic conditions liberates sulfide and hydrophobic sulfur [25]. Cell-bound polysulfides were detected as an intermediate compound during the oxidation of polysulfide by the natron-alkaliphilic chemolithoautotrophic sulfide oxidizing bacterium *Thioalkalivibrio versutus* strain ALJ 15 [26]. While the (poly)sulfide concentration in the bulk solution has dropped to low levels, these membrane-bound polysulfides may still need to be oxidized. As a result, the cytochrome pool does not become sufficiently oxidized to enable electrons from intermediary sulfur to enter the respiratory chain, which would lead to sulfate formation [5].

Intermitted H_2S supply

In a second experiment, H_2S was intermittently supplied (0 or 51.0 mM d^{-1}) for a period of 80 hours to test if selectivity for product formation is affected by continuously fluctuating redox conditions. Whenever ORP values decreased to less than -420 mV (corresponding to $[S^{2-}_{tot}]$ = 0.43 mM at pH 9.9), the H_2S supply was automatically interrupted. As a result of biological and non-biological oxidation reactions, the decrease in (poly)sulfide concentration leads to an increase in ORP values. When the ORP reached values above -370 mV (0.01 mM S^{2-}_{tot}), the H_2S supply was automatically resumed (later this was changed to -400 mV, or 0.10 mM S^{2-}_{tot}). The oxygen supply rate was kept constant at 30.6 mM d^{-1}, also during the short interruptions in H_2S supply, resulting in an average O_2/H_2S supply ratio of 0.6 mol mol^{-1}.

Due to the chosen H_2S supply strategy, this low concentration exists 5 times per day, for a short period of time (3-4 min, Fig 4.7 A). During the 80-hour period of intermitted H_2S supply, the average selectivity for sulfate formation is around 2.7 mol% and for thiosulfate 19 mol% (Fig. 4.7 B). These values correspond to the selectivity for product formation that is found at stable reduced redox conditions (see section 4.3.2), indicating that slow fluctuations in redox conditions do not disturb the biological oxidation reactions or affect the selectivity for sulfate and thiosulfate formation.

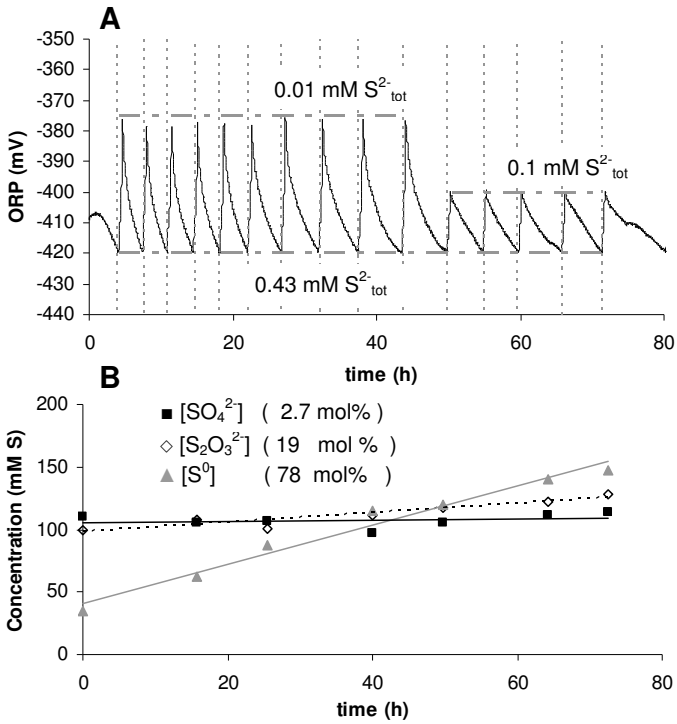

FIGURE 4.7 A-B Results of intermitted H_2S supply (0 - 51.0 mM h^{-1} H_2S; 30.6 mM h^{-1} O_2). A: ORP; B: Oxidation product concentrations. The vertical gray dashed lines in Fig. A show the periods when the H_2S supply was turned off (30-35 min). Horizontal gray lines in Fig. B indicate the minimum and maximum S^{2-}_{tot} concentrations, corresponding to the ORP boundaries. Lines in Fig. B show the linear trendlines that are used to calculate the average selectivity for product formation. During the experimental run, the pH was controlled at 9.9, T=35°C, biomass concentration = 44 mg N L^{-1}.

Long-term shift to oxidized redox conditions

In the previous two experiments, redox conditions in the bioreactor were changed by interruption of the H_2S supply. As a result, (poly)sulfide concentrations decreased till depletion. In the experiments described in this section, a step change from reduced to relatively oxidized redox conditions is achieved by an increase of the oxygen supply rate, while keeping the H_2S supply rate constant. In this way, a shift in product formation may occur without achieving a complete depletion of the (poly)sulfide substrate.

Results of preliminary experiments (data not shown) indicated that 25-40 min. after changing from highly reduced (ORP = -390 mV) to relatively oxidized (ORP >-300 mV) redox conditions, the O_2/S^{2-}_{tot} consumption ratio increases as a result of a shift in product formation. Results of these experiments also indicated that at oxidized redox conditions and

excess amounts of thiosulfate, H_2S is still fully converted to biosulfur, while thiosulfate is oxidized to sulfate. A dedicated reactor experiment was conducted (in duplicate) to further study the preference for thiosulfate over (poly)sulfide as a substrate for the formation of sulfate. In this experiment, a shift from reduced to oxidized redox conditions was made after the deliberate addition of thiosulfate. The pH was controlled at 9.5.

During the first 4.8 days from the start of the experiment, the reactor was operated at a $[S^{2-}_{tot}]$ setpoint value of 0.2 mM. In this period, 89 mol% of the H_2S supply was converted to biosulfur (45 mM d^{-1}) and 11 mol% to thiosulfate (5.5 mM d^{-1}), at a O_2/H_2S supply ratio of 0.57±0.2 mol mol^{-1} (Fig. 4.8 A and B, day 0 - 4.8). On day 4.5, sodium thiosulfate was added to reach a final concentration of 480 mM, whereafter a shift was made from reduced to oxidized redox conditions by changing the control setpoint (day 4.8). The oxygen supply rate was controlled on basis of ORP, at a setpoint value of -200 mV. The reason for applying ORP instead of the S^{2-}_{tot} concentration as a control variable, is that the latter approaches zero at oxidized redox conditions and therefore cannot be used for process control purposes. Within 30 minutes after changing the oxygen supply rate, the ORP value increased (Fig. 4.8 B). The S^{2-}_{tot} concentration dropped below the detection limit ($[S^{2-}_{tot}] < 0.01$ mM) while the DO concentration reached up to 30% sat. (data not shown). After this shift in redox conditions, sulfur formation unexpectedly continued at the same rate as during reduced redox conditions (45 mM d^{-1}). However, the thiosulfate-S concentration decreased while sulfate was produced at exactly the same rate. Figure 4.8 C shows that during the first day after the shift from reduced to oxidized redox conditions, thiosulfate is removed from the liquid at a high rate (147 mmol L^{-1} d^{-1}). In the following days, the conversion rate gradually decreases until the moment that the reactor is depleted from thiosulfate (on day 11.9).

Up to day 8, sulfate is formed at the same rate as thiosulfate-S is converted. This indicates that thiosulfate is completely oxidized to sulfate (Eq. 24). Hence, all H_2S is converted to biosulfur.

$$S_2O_3^{2-} + O_2 \rightarrow 2\ SO_4^{2-} \qquad (24)$$

After day 8, the thiosulfate conversion rate slowly decreases until all thiosulfate is depleted. Sulfate formation however continues at a rate of 46 mM d^{-1}, i.e. close to the H_2S supply rate of 51.0 mM d^{-1}, even after thiosulfate depletion on day 11.8. This phenomenon is accompanied by an increase of the O_2/H_2S supply ratio from about 1.2 mol mol^{-1} (day 11) up to value somewhat above 2 mol mol^{-1} on day 11.8 (Fig. 4.8 A), corresponding to the stoichiometry of complete oxidation of H_2S to sulfate (Eq. 2). Moreover, an increase of buffer addition used for pH control, is observed from day 11 onwards (Fig. 4.8 C). This indicates an increased consumption of alkalinity by the formation of sulfuric acid.

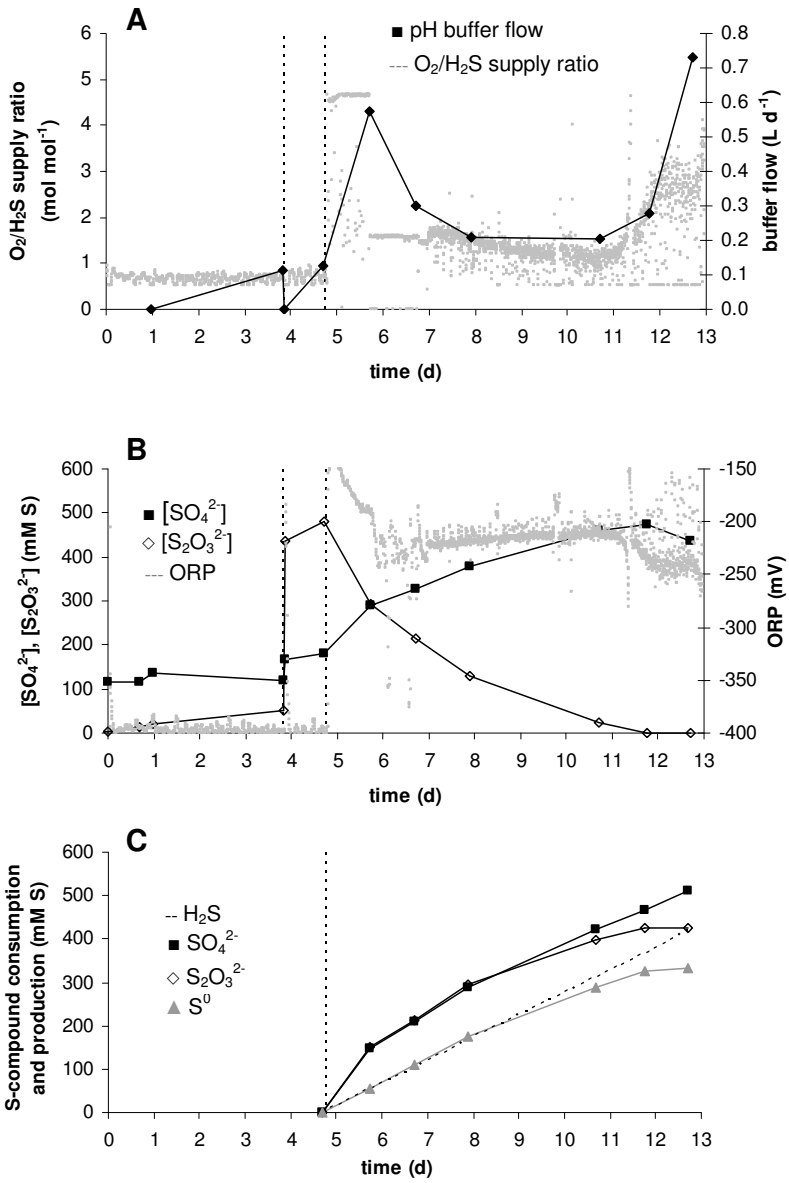

FIGURE 4.8 A-C **Effect of a shift from reduced to oxidized redox conditions on product formation. A: molar O_2/H_2S supply ratio and pH controller buffer flow; B: ORP and sulfate and thiosulfate concentrations; C: H_2S and thiosulfate consumed and sulfate and biosulfur produced after the shift in redox conditions. The H_2S supply rate was constant at 51.0 mM d^{-1}. pH = 9.5, biomass concentration = 80 (day 6) to 123 mg N L^{-1} (day 13).**

A straightforward explanation for the effect of thiosulfate on the stoichiometry of sulfide oxidation cannot be given due to the co-existence of multiple pathways for the formation of sulfate [27, 28]. However, some insight may be gained from thermodynamic considerations. Mean free-energy changes for the partial and complete aerobic oxidation of sulfur compounds as evaluated by ref. [29] are shown in Table 4.1. However, as the amount of useful energy that is obtained from substrate oxidation depends on the intermediate steps involved, these ΔG^0 values are of limited value. Oxidation of sulfide and thiosulfate to sulfate is assumed to proceed according to a number of subsequent steps [29]:

$$S^{2-} \xrightarrow{1} S^0 \xrightarrow{2} SO_3^{2-} \xrightarrow{3} SO_4^{2-} \quad (25)$$

$$S_2O_3^{2-} \xrightarrow{4} S^0 + SO_3^{2-} \xrightarrow{5} 2\ SO_3^{2-} \xrightarrow{6} 2\ SO_4^{2-} \quad (26)$$

TABLE 4.1 Mean free-energy change for the partial and complete aerobic oxidation of sulfur compounds (after ref. [29])

Reaction (step)	Mean ΔG^0 (kJ per mol substrate)
Thiosulfate to sulfate (4+5+6)	-750.1 ± 16.1
Sulfide to sulfate (1+2+3)	-701.8 ± 31.4
Sulfide to sulfur (1)	-168.6 ± 28.9
Sulfur (S^0) to sulfate (2+3)	-537.9 ± 43.7
Sulfur (S^0) to sulfite (2,5)	-280.2 ± 22.2
Sulfite to sulfate (3,6)	-230.1 ± 22.0
Thiosulfate to sulfur and sulfite (4)	+26.8[a]
Thiosulfate to sulfide and sulfate	-21.9[b]

[a]It is uncertain how this step is achieved in vivo.
[b]Disproportionation of thiosulfate, after ref. [30].

Disproportionation of thiosulfate to sulfide and sulfate [30] (Eq. 27) is assumed not to play a role, as it does not match with the observed formation rate of sulfate.

$$S_2O_3^{2-} + OH^- \rightarrow HS^- + SO_4^{2-} \quad (27)$$

If the oxidation of sulfide to sulfate (Eq. 25) involves an oxygenase for step 2, then no metabolically available energy arises from the conversion of sulfur to sulfite (step 2). The energy conserving steps 1 and 3 would then provide -398.7 kJ mol^{-1} [29]. The same applies for the oxidation of thiosulfate to sulfate (Eq. 26), yielding -460.2 kJ mol^{-1} useful energy obtained from step 6. At the experimental conditions, the sulfide concentration was always below the detection limit (<0.01 mM), while the thiosulfate concentration was very high (up to 480 mM). The huge difference between sulfide and thiosulfate concentrations may result in an increased difference in the amount of useful energy obtained from their oxidation to sulfate, leading to a preference for thiosulfate over sulfide. Sulfide oxidation to the level of elemental sulfur may then still take place e.g. to prevent sulfide toxicity. Substrate inhibition

by high thiosulfate concentrations (60 - 200 mM) on thiosulfate oxidation has been reported for *Thiobacillus ferrooxidans* [31]. Possibly, inhibition of sulfide oxidation by extremely high thiosulfate concentrations also played a role in our experiments.

4.4 CONCLUSIONS

The main results of this work may be summarized as follows:

- A good agreement exists between electrochemical calculations and spectroscopic analysis of sulfide and polysulfide species in the bioreactor. It is shown that the measured ORP mainly depends on the polysulfide concentration and not on the S^{2-}_{tot} concentration. As a result, the measured ORP increases with decreasing pH values in case the S^{2-}_{tot} concentration remains constant.
- Selectivity for sulfate formation is much stronger correlated to the S^{2-}_{tot} concentration than to the ORP. As a result it has been found that the sulfide and polysulfide concentration at which sulfate formation stops, depends on the pH.
- At S^{2-}_{tot} concentrations up to 0.25 mM, selectivity for thiosulfate formation corresponds to predicted values based on a mechanistic model described previously in Chapter 3 [2]. At S^{2-}_{tot} concentrations above this value more thiosulfate is formed, most probably due to abiotic (poly)sulfide oxidation.
- After a single interruption of the H_2S supply, it takes about 25 - 40 min. at oxidized reactor conditions (ORP>-300 mV) before the bacteria shift from sulfur to sulfate formation.
- At excessive thiosulfate concentrations (>130 mM) and fully oxidized conditions (i.e. DO = 30% sat. and ORP>-300 mV), H_2S is fully converted to elemental sulfur while thiosulfate is completely oxidized to sulfate. This implies that the incidental occurrence of oxidized redox conditions, for example after a process disturbance, does not necessarily lead to a loss in selectivity for sulfur formation.

Acknowledgements

We thank Nadine Boelee for performing part of the experimental work and Dr. Dimitry Sorokin (Department of Biotechnology, Delft University of Technology, The Netherlands) for providing sediment samples from soda lakes.

Nomenclature

ORP	Oxidation Reduction Potential
x	polysulfide chain length (x in S_x^{2-})
E_h	electrode potential versus standard H_2 electrode (electronmotive force)
E	electrode potential versus Ag/AgCl
E^0_h	standard redox potential versus standard H_2 electrode

E^0	standard redox potential versus Ag/AgCl
R	gas constant
T	temperature (K)
n	number of electrons transferred (-)
F	Faraday constant
K_x	equilibrium constant
γ_a	activity coefficient of compound "a"
R_{max}	maximum selectivity for sulfate formation (100%)
k_s	affinity constant
k_i	inhibition constant
$[S^{2-}_{tot}]_{set}$	control setpoint value of the S^{2-}_{tot} concentration
P	proportional control factor
SHE	Standard Hydrogen Electrode, indicates the reference electrode for redox potential measurements.

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Chapter 5

Inhibition of microbiological sulfide oxidation by methanethiol and dimethyl polysulfides

Abstract

To avoid problems related to the discharge of sulfidic spent caustics to a wastewater treatment plant, a three-step biotechnological process is developed for the treatment of gasses containing both H_2S and methanethiol (MT). In the process, which operates at natron-alkaline conditions (>1 M of sodium- and potassium carbonates and a pH of 8.5-10), MT reacts with biologically produced sulfur particles to form a complex mixture predominantly consisting of inorganic polysulfides, dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS). The inhibitory effect of MT, DMDS and DMTS was studied on bacterial oxidation of sulfide and polysulfide. Results show that biological oxidation of sulfide to elemental sulfur is reduced by 50% at 0.05 mM MT, while for DMDS and DMTS, 50% reduction was estimated at 1.5 and 1.0 mM, respectively. Results obtained with a reaction mixture of MT and biologically produced sulfur particles show that MT is detoxified by its reaction with biological sulfur. Adaptation of natronophilic biomass to MT was shown after prolonged exposure to (di)methyl sulfur compounds. The results indicate that application of the biotechnological process for the treatment of gasses containing both H_2S and MT is feasible as long as MT, DMDS and DMTS do not accumulate in the bioreactor.

This chapter has been submitted as:
Van den Bosch, P. L. F.; De Graaff, M.; Fortuny-Picornell, M.; Van Leerdam, R. C.; Janssen, A. J. H., Inhibition of microbiological sulfide oxidation at natronophilic conditions by methanethiol and methylated polysulfides.

5.1 INTRODUCTION

Biogas and gasses produced in the refining and petrochemical industry often contain volatile sulfur compounds, of which hydrogen sulfide (H_2S) is the most common. To prevent emission of sulfur dioxide and problems related to odour, toxicity and corrosivity, H_2S needs to be removed from the gas before combustion. For the removal of H_2S from sour gasses, caustic scrubbing is often applied. In this process, H_2S is absorbed into an aqueous alkaline solution in an absorber, under the formation of bisulfide (HS^-, referred to as "sulfide"):

$$H_2S(aq) + OH^- \rightleftarrows HS^- + H_2O \qquad (pK_a = 7.04) \qquad (1)$$

In conjunction with H_2S, sour gasses often contain carbon dioxide (CO_2). Presence of high CO_2 partial pressures leads to the formation of (bi)carbonate in the alkaline scrubber solution:

$$H_2CO_3 + OH^- \rightleftarrows HCO_3^- + H_2O \qquad (pK_a = 6.36) \qquad (2)$$

$$HCO_3^- + OH^- \rightleftarrows CO_3^{2-} + H_2O \qquad (pK_a = 10.25) \qquad (3)$$

Besides H_2S and CO_2, sour gasses may also contain volatile organic sulfur compounds (VOSCs) such as methanethiol (CH_3SH, or MT), ethanethiol (ET), propanethiol (PT), carbonyl sulfide (COS) and dimethyl disulfide (DMDS) [1]. Most of these VOSCs are even more toxic and malodorous than H_2S. Methanethiol is one of the most common VOSCs in sour gasses [2-4], and can also be adsorbed in the alkaline scrubbing liquid:

$$CH_3SH + OH^- \rightleftarrows CH_3S^- + H_2O \qquad (pK_a = 9.7) \qquad (4)$$

The sulfide-loaded alkaline solution, also referred to as "sulfidic spent caustics", is often directed to a wastewater treatment plant (WWTP). Several problems are related to the discharge of sulfide to a WWTP, including corrosion, release of odors and safety hazards [5, 6]. Negative impacts of sulfide on the performance of wastewater treatment facilities have also been observed, e.g. problems related to nitrification [7] and the formation of bulking sludge due to the growth of filamentous sulfur-oxidizing bacteria such as *Thiothrix* and *Beggiota* species [8].

To overcome these problems, a three-step biotechnological process has been recently developed for the removal of H_2S from sour gasses with high CO_2 partial pressures [9]. The first step consists of absorption of H_2S and, if present also MT, into an alkaline scrubbing solution as described above. In the second step, the sulfide-loaded alkaline solution is sent to a bioreactor where it is biologically oxidized to elemental sulfur (S^0, referred to as "biosulfur"). The overall reaction for the conversion of H_2S to biosulfur is shown in Equation

5. To maximize the recovery of biosulfur and to reduce the consumption of caustic and make-up water, complete oxidation of H_2S to sulfate (SO_4^{2-}, Eq. 6) is unwanted. Sulfate formation can be controlled by operating the bioreactor at oxygen limiting conditions (Chapter 4) [10].

$$H_2S + ½\ O_2 \rightarrow S^0 + H_2O \quad (5)$$

$$H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2\ H^+ \quad (6)$$

In a third step, biosulfur particles are separated from the reactor liquid by sedimentation. To treat sour gasses with a high partial CO_2 pressure and to maximize the H_2S loading capacity of the alkaline solution, the process has to be operated at high concentrations of (bi)carbonate (Chapter 1) [10]. With sodium (Na^+) and potassium (K^+) as counter ions of the (bi)carbonate, the process operates at natron-alkaline conditions (e.g. 2 M Na^+/K^+, pH 9). Therefore, specialized natron-alkaliphilic sulfur oxidizing bacteria (SOB) are applied in the process [11].

To avoid the discharge of sulfidic spent caustics in a WWTP, the abovementioned biotechnological process further developed for the treatment of gasses containing both H_2S and MT. Previous research on sulfidic spent caustics suggests that biological oxidation of MT to sulfate at neutral pH conditions occurs via intermediary DMDS [12]. The effect of MT on the biological conversion of H_2S to elemental sulfur at natron-alkaline conditions is however not yet known. Being a strong nucleophile, MT can react with sulfur particles in an aqueous solution, initially forming methyl polysulfide by opening the sulfur ring (Eq. 7) [13]. In subsequent spontaneous reaction steps, shorter dimethyl polysulfides are formed along with inorganic (poly)sulfides (Eq. 8).

$$CH_3SH + S_8 \rightarrow CH_3S_9^- + H^+ \quad (7)$$

$$CH_3SH + CH_3S_9^- \rightarrow CH_3S_nCH_3 + S_x^{2-} + H^+ \qquad (n + x = 10) \quad (8)$$

In previous research we have shown that these reactions indeed take place between MT and biosulfur particles [14]. The main end-products when MT is added to an excess of biosulfur at pH 8.7 are polysulfide, sulfide and (di)methyl polysulfides, consisting predominantly of DMDS and DMTS. In the presence of oxygen, DMDS also can be formed by rapid auto-oxidation of MT (Eq. 9) [15].

$$2\ CH_3SH\ + ½\ O_2 \rightarrow CH_3SSCH_3 + H_2O \quad (9)$$

This paper focuses on the inhibitory effects of MT and the formed reaction products, on oxidation of sulfide and polysulfide by natronophilic SOB. Knowledge on the potential toxic effects of these compounds is required to further develop a biotechnological process for

removal of H_2S and MT from sour gasses as alternative to treatment of sulfidic spent caustics in a WWTP.

5.2 MATERIALS AND METHODS

5.2.1 Respiration tests

Respiration tests were performed in a thermostated 7.5 mL glass chamber mounted on a magnetic stirrer and fitted with a piston holding a dissolved oxygen (DO) electrode (Yellow Springs Instr., OH, USA). This piston contained a small opening to allow air to be removed from the chamber and to add reactants. A schematic representation of the setup is shown elsewhere [16]. Cell suspensions (70-100 µL) were added to a carbonate buffer solution (pH 9.0), to a final concentration of 14-21 mg N L^{-1}, while the solution was saturated with oxygen by bubbling with air for at least 5 minutes. Experiments were started by injection of 20 – 320 µL of sulfur substrate stock solutions (sulfide, polysulfide, a mixture of MT and biosulfur, MT, DMDS and/or DMTS). The decrease of the DO concentration was measured in time and the initial slope $(d[O_2]/dt)_0$ was used as a measure of the oxidation rate. Oxidation rates were measured in the absence (chemical controls) and presence of cells. Biological oxidation rates were calculated by subtracting the rates in the absence of cells (chemical oxidation) from the rates in the presence of cells (combined chemical and biological oxidation). At the end of each respiration experiment, samples were taken for analysis of the residual total sulfide (S^{2-}_{tot}) concentration, to enable the calculation of the molar O_2/S^{2-}_{tot} consumption ratio. All experiments were performed in duplicate. Controls were performed with autoclaved cells (20 min, 121°C).

5.2.2 Biomass source

Natronophilic SOB were obtained from a lab-scale gas-lift bioreactor inoculated with a mixture of hypersaline soda lake sediments from Mongolia, south-western Siberia and Kenya [17], operating at natron-alkaline, sulfur producing conditions (2M Na^+/K^+, pH 9.0±0.2, $[S^{2-}_{tot}]$ = 0.2-0.3 mM, DO <0.1% sat., 2.2 mM h^{-1} H_2S supply, 35°C). A detailed description of the reactor setup and a composition of the microbiological population is given in Chapter 2 [10, 11]. Bacterial cells were separated from extracellular sulfur by several successive steps of low-speed centrifugation (500 rpm), washed and resuspended in a (bi)carbonate buffer (pH 9.0, 1.67 M K^+, 0.33 M Na^+, see section *Chemicals used*), resulting in cell suspensions with a final concentration of 1100-1700 mg N L^{-1}.

In a dedicated experiment, MT (1.5-3.3 µM h^{-1}) and H_2S (2.2 mM h^{-1}) were continuously supplied to the bioreactor (pH 9.1±0.1). This way, natronophilic biomass was exposed to (di)methyl sulfur compounds for a prolonged period. The DO concentration was controlled at >5% sat. and the S^{2-}_{tot} concentration was kept below 0.01 mM, to prevent limitation. After 27 days of operation, cells were harvested from the bioreactor as described above and used in respiration tests.

5.2.3 Analytical procedures

The maximum oxygen solubility of the alkaline buffer solution (see section *Chemicals used*) was determined by placing buffer (pH 9.0) in an air-tight thermostated vessel (400 mL). After oxygen saturation by bubbling with air (DO = 100% sat.), the vessel was equipped with a DO electrode. A 50 mM sodium sulfite solution ($NaSO_3$, Merck, Darmstadt, Germany) was stepwise added to the vessel in the presence of copper sulfate to act as a catalyst. Based on the stoichiometry of the oxidation of sulfite to sulfate, combined with the decrease of the DO concentration, the maximum oxygen solubility of the buffer was found to be 0.15 mmol L^{-1} at 35°C (data not shown).

Total sulfide (S^{2-}_{tot}) concentrations were measured on the basis of a modified methylene blue method as described previously in Chapter 2 [10]. At the experimental pH of 9.0, the main sulfide species are HS^- and S_x^{2-}. Therefore, the total sulfide concentration can be described as:

$$[S^{2-}_{tot}] = [HS^-] + [S_x^{2-}] \qquad (10)$$

Polysulfide anion concentrations were determined spectrophotometrically as described elsewhere (Chapter 2) [10, 18], at a wavelength of 285nm (Perkin-Elmer, Lambda 2, Norwalk, CT, USA). With this method, which can only be used in the absence of VOSCs, the total concentration of zerovalent sulfur atoms in polysulfide (S_x^{2-}-S^0) is determined. Biomass concentrations were measured as the amount of total nitrogen, as described in Chapter 2 [10].

5.2.4 Chemicals used

Carbonate buffer was prepared by mixing a bicarbonate (pH 8.3) and carbonate (pH 12.3) buffer to a final pH of 9.0. Both buffers contained 0.67 M Na^+ and 1.33 M K^+ as (bi)carbonate (Merck, Darmstadt, Germany).

Sodium sulfide stock solutions (20-30 mM) were freshly prepared by dissolution of $Na_2S{\cdot}9H_2O$ crystals (Merck, Darmstadt, Germany) in de-aerated Milli-Q water. Before dissolution, the oxidized surface of the crystals was removed by flushing with de-aerated water. The exact sulfide concentration of the stock solutions was determined afterwards.

Polysulfide stock solutions were prepared by reaction of excess biosulfur (250 mM) with a 30 mM sulfide solution at 50°C, as described elsewhere [16] (Eq. 11). Polysulfide solutions as prepared in this way consist of a mixture of polysulfide (S_x^{2-}, with an average value of x = 4.9 [18]) and sulfide (HS^-), of which the equilibrium is defined by Equation 12 [18, 19]. The pH of the polysulfide stock solutions was 10.3. According to the equilibrium (Eq. 8) at this pH, 93% of the S^{2-}_{tot} concentration is present as polysulfide. This was confirmed by analysis of S^{2-}_{tot} and polysulfide concentrations.

$$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+ \qquad \text{with } x = 4.9 \text{ (average)} \qquad (11)$$

$$K_x = \frac{[S_x^{2-}][H^+]}{[HS^-]} \qquad \text{with } pK_x = 9.17 \qquad (12)$$

A sodium methylmercaptide ($NaCH_3S$) solution (2.5 M) was supplied by Arkema Group (Rotterdam, the Netherlands). Stock solutions of 20 mM MT were prepared by dilution with oxygen-free ultrapure (MilliQ) water and kept at a slight nitrogen gas overpressure to prevent oxidation by air.

Biosulfur was obtained from a full-scale biogas treatment facility (Eerbeek, the Netherlands) and dialyzed in demineralised water to remove salts to a conductivity below 40 μS cm^{-1}. The mixture of MT and biosulfur (further referred to as "MT-S^0 mixture") was prepared by addition of 20 mM MT and 400 mM biosulfur to oxygen-free carbonate buffer (pH 9.0) as described above. The MT-S^0 mixture was incubated overnight at 30°C. After incubation, the S^{2-}_{tot} concentration in the MT-S^0 mixture was 9.6 mM, which is close to the expected value of 10 mM, based on the reaction stoichiometry according to Equations 7 and 8. According to this stoichiometry, the total concentration of dimethyl polysulfides should also be 10 mM. The exact composition of the MT-S^0 mixtures was not determined, but a more detailed description of the composition of similar MT-S^0 mixtures (30°C, pH 8.7) is given elsewhere [14]. Before use, remaining biosulfur particles were allowed to settle so that no biosulfur was introduced to the respiration chamber.

Stock solutions of DMDS and DMTS (20 mM) were prepared from pure solutions (Merck, Darmstadt, Germany) by dilution in water (DMDS) or HPLC-grade methanol (DMTS).

5.3 RESULTS

5.3.1 Sulfide and polysulfide oxidation rates in the absence of methyl sulfur compounds

Oxidation rates of sulfide and polysulfide ions were initially determined in the absence of (di)methyl sulfur compounds. Upon addition of biomass (15 mg N L^{-1}) to oxygen saturated buffer, the DO concentration decreased up to a rate of 4.8 mM O_2 h^{-1} (data not shown), even without addition of substrate. This phenomenon was also previously observed and is assumed to be attributed to the oxidation of membrane-bound polysulfur compounds, such as polysulfide [20, 21]. After re-aeration for at least 5 minutes, only endogenic oxygen consumption was observed and experiments were started. Oxidation rates were determined with sulfide (Fig. 5.1 A) and polysulfide (Fig. 5.1 B) as substrates (0.05 to 0.5 mM S^{2-}_{tot}), in the absence and presence of cells. In the absence of cells (chemical controls), oxidation rates increased slightly with increasing sulfide concentrations. For polysulfide, chemical oxidation rates increased proportionally to the concentration, indicating first-order reaction kinetics, as was also reported by Kleinjan et al. [16]. The rate of chemical polysulfide oxidation in the buffer was approximately twice as high as that of sulfide.

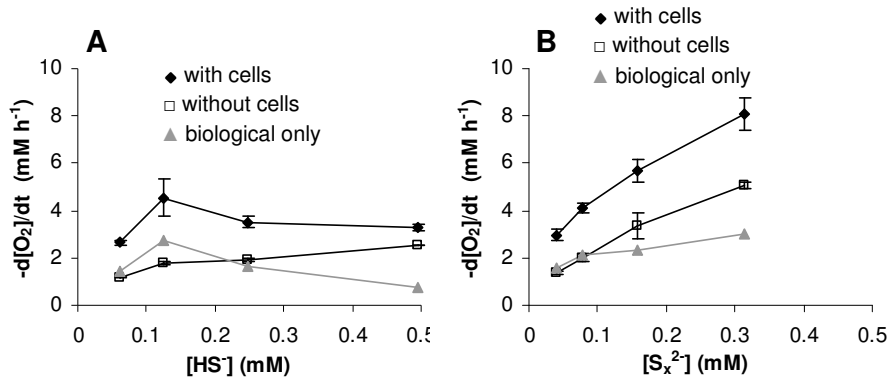

FIGURE 5.1 A-B **Oxidation rates with different concentrations of HS^- (A) and S_x^{2-} (B). Both Figures show rates in the presence of cells, in the absence of cells and rates as a result of biological oxidation only. Biomass concentration = 15 mg N L^{-1}; pH=9.0; total salt = 2 M Na^+/K^+ as carbonates.**

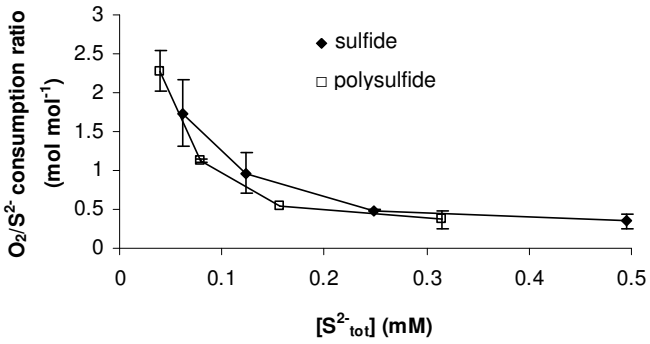

FIGURE 5.2 **Molar O_2/S^{2-}_{tot} consumption ratio as a result of biological oxidation of sulfide and polysulfide. Biomass concentration = 15 mg N L^{-1}; pH=9.0; total salt = 2 M Na^+/K^+ as carbonates.**

The biological oxidation of sulfide showed a maximum rate of 2.7±0.3 mM O_2 h^{-1} (0.18±0.2 mM O_2 mg N^{-1} h^{-1}) at a sulfide concentration of 0.12 mM, whereas at higher sulfide concentrations, the oxidation rate decreased. For biological polysulfide oxidation, a similar maximum oxidation rate of 3.0±0.1 mM O_2 h^{-1} (0.20±0.1 mM O_2 mg N^{-1} h^{-1}) was observed at the highest polysulfide concentration tested (0.31 mM S^{2-}_{tot}). By measurement of the molar O_2/S^{2-}_{tot} consumption ratio both in the absence and presence of cells, the stoichiometry of biological substrate oxidation could be calculated (Fig. 5.2). For both the sulfide and polysulfide bio-oxidation, the molar O_2/S^{2-}_{tot} consumption ratio was around 2 at a S^{2-}_{tot} concentration of around 0.05 mM. At increasing S^{2-}_{tot} concentrations, the molar O_2/S^{2-}_{tot}

consumption ratio gradually decreased to a final value of around 0.5 at a S^{2-}_{tot} concentration of 0.20-0.25 mM and above.

5.3.2 Inhibition by methanethiol and (di)methyl polysulfides

The effect of MT, DMDS and DMTS on sulfide oxidation was studied at various concentrations of these VOSCs. As elemental sulfur is the preferred end-product (Chapter 1) [10, 22], a S^{2-}_{tot} concentration of 0.25 mM was applied. At this S^{2-}_{tot} concentration, sulfide is oxidized to elemental sulfur, as described above. Oxygen consumption rates with only MT, DMDS and DMTS were negligible both in the absence and presence of cells (<0.1 mM h^{-1} at 0.4 mM). Chemical oxidation of sulfide was only slightly affected by these (di)methyl sulfur compounds (data not shown). Biological sulfide oxidation rates were strongly affected by MT (Fig. 5.3). A 50% decrease of the oxidation rate (IC_{50}) was already observed at a MT concentration of 0.05 mM, while at concentrations above 0.65 mM, biological oxidation was completely inhibited. Inhibition of biological sulfide oxidation by DMDS and DMTS was less severe. At the highest concentration applied (0.85 mM), the oxidation rate of sulfide in the presence of DMDS or DMTS decreased to 55-60%. Based on a log-linear fit, it was estimated that for DMDS and DMTS, the IC_{50} value was 1.5 and 1.0 mM, respectively.

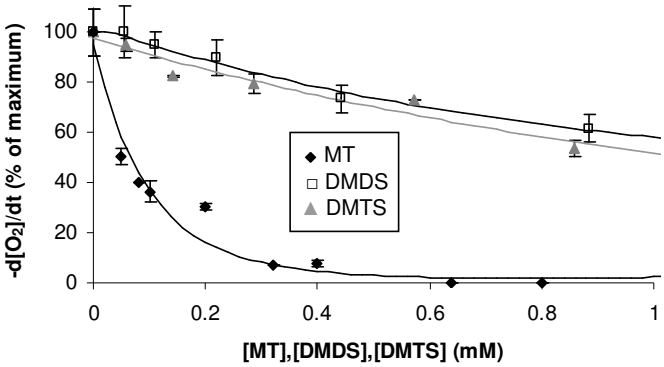

FIGURE 5.3 Relative biological oxidation rates during sulfide oxidation (0.25 mM HS^-) in the presence of varying concentrations of MT, DMDS and DMTS. Biomass concentration: 20.5 mg N L^{-1}, specific biological oxidation rate with sulfide only (100%): 0.18±0.02 mmol O_2 mg N^{-1} h^{-1}. pH=9.0; total salt = 2 M Na^+/K^+ as carbonates. Solid lines represent the results of a log-linear regression model fitted to the results.

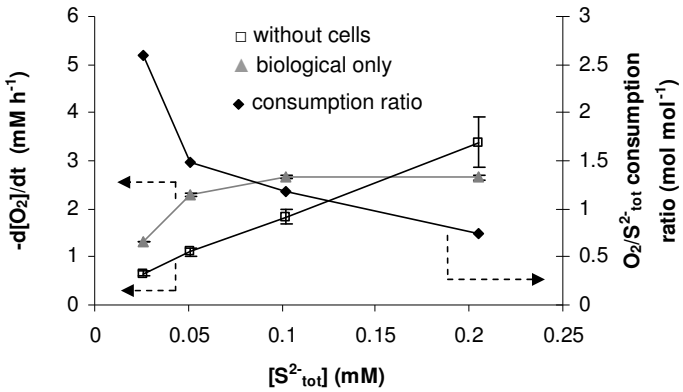

FIGURE 5.4 **Oxidation rates with the MT-S^0 mixture. The x-axis shows the initial S^{2-}_{tot} concentration after addition of the mixture, being equal to the sum of concentrations of (di)methyl polysulfides, according to the stoichiometry of the reaction between MT and biosulfur (Eq. 3 and 4). The primary y-axis shows oxidation rates in the absence of cells and as a result of biological oxidation only. The secondary y-axis shows the molar O_2/S^{2-}_{tot} consumption ratio as a result of biological oxidation only. Biomass concentration = 18 mg N L^{-1}; pH=9.0; total salt = 2 M Na^+/K^+ as carbonates.**

Oxidation rates of the MT-S^0 mixture were also tested in the absence and presence of cells. Chemical oxidation rates increased proportionally to the amount of MT-S^0 mixture added (Fig. 5.4) and the relation between oxidation rate and S^{2-}_{tot} concentration was comparable to that of polysulfide (Fig. 5.1 B). Biological oxidation rates obtained with the MT-S^0 mixture increased with increasing S^{2-}_{tot} concentrations, thereby reaching a maximum rate of 2.7±0.3 mM O_2 h^{-1} (0.18±0.2 mM O_2 mg N^{-1} h^{-1}) at 0.1 mM S^{2-}_{tot}. The measured rates were comparable to those of biological polysulfide oxidation (Fig. 5.1B). The relation between the S^{2-}_{tot} concentration and the molar O_2/S^{2-}_{tot} consumption ratio as a result of biological oxidation of the MT-S^0 mixture shows a similar pattern as observed with only sulfide and polysulfide as substrates (Fig. 5.2). According to the stoichiometry of the reaction between MT and biosulfur (Eq. 7 and 8), the formed dimethyl polysulfide concentration is equal to the formed inorganic polysulfide concentration. As the maximum (inorganic) polysulfide concentration applied in the respiration tests was 0.2 mM, this implies that at least up to the same concentration of 0.2 mM, dimethyl polysulfides (DMDS and DMTS) do not inhibit biological sulfide oxidation.

5.3.3 Respiration rates after exposure to MT

In the respiration experiments discussed so far, the biomass had not been exposed to MT, DMDS or DMTS prior to the respiration experiments. To test whether adaptation of biological sulfide oxidation to MT occurs after long-term exposure to (di)methyl sulfur

compounds, biomass was pre-exposed to MT and the products formed from its reaction with biosulfur in a H_2S-oxidizing bioreactor operating without O_2 limitation. At a continuous MT supply of 1.5-3.3 μM h^{-1}, all H_2S supplied to the reactor (2.2 mM h^{-1}) was converted to sulfur, whilst little, if any, accumulation of sulfate, sulfide or thiosulfate was found. This indicates that the biological activity was not severely inhibited by MT. After 27 days of adaptation, a comparison was made between pre-exposed and not pre-exposed biomass. For both type of cells, comparable sulfide oxidation rates were found at a sulfide concentration of 0.16 mM (Fig. 5.5).

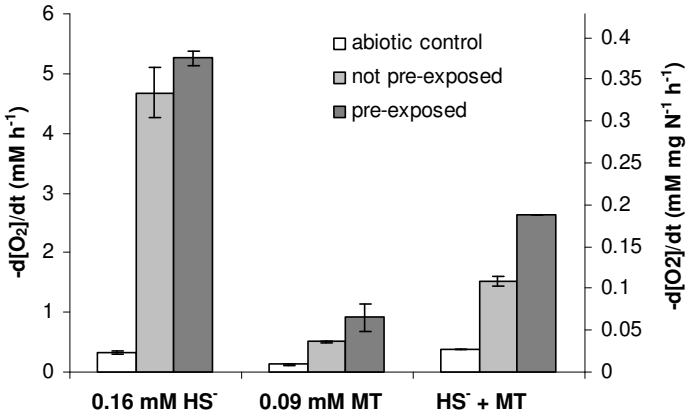

FIGURE 5.5 Oxidation rates with HS^- (0.16 mM), MT (0.09 mM) and a mixture of HS^- (0.16 mM) and MT (0.09 mM), for respiration experiments without biomass (abiotic control), biomass that has not been pre-exposed to MT (light gray) and biomass that was pre-exposed to MT (dark gray) in a H_2S oxidizing bioreactor.

The primary y-axis shows volumetric oxygen consumption rates. The secondary y-axis shows the specific biological oxygen consumption rates, corrected for abiotic oxidation. Biomass concentration = 14 mg N L^{-1}; pH=9.0; total salt = 2 M Na^+/K^+ as carbonates.

Oxidation rates with only MT (0.09 mM) were slightly higher for pre-exposed cells (0.07±0.02 mM mg N^{-1} h^{-1}) compared to the not pre-exposed cells (0.04±0.00) mM h^{-1}). Although the oxidation rate with MT was somewhat higher in the presence of cells compared to abiotic control experiments, it was not clear if any biological MT degradation occurred. The reason for this is that concentrations of MT and DMDS could not be measured. When both sulfide (0.16 mM) and MT (0.09 mM) were added, respiration rates with pre-exposed cells (0.19±0.01 mM mg N^{-1} h^{-1}) were significantly higher (73±6 %) compared to rates with not pre-exposed cells (0.11±0.01 mM mg N^{-1} h^{-1}).

5.4 DISCUSSION

5.4.1 Inhibition by methylated sulfur compounds

This study shows that sulfide oxidation to elemental sulfur by natron-alkaliphilic SOB is severely inhibited in the presence of MT, already at low concentrations (IC_{50} = 0.05 mM MT). As an intermediate in the methionine metabolism, MT was reported to be responsible for inhibition of cytochrome *c* oxidase activity [23]. It was suggested that inhibition by MT is caused by steric hindrance and ionic or hydrophobic interactions. As alkaliphilic SOB have a high cytochrome *c* content and a very high cytochrome *c* oxidase activity [24, 25], it is likely that inhibition of the oxidation rate by MT is caused by inhibition of cytochrome *c* oxidase activity. Being a strong nucleophile, MT can also break S-S bonds in proteins and thereby rendering enzymes inactive ([26]. If the nucleophilicity of MT indeed plays a role in the inhibiting effect, a high pH is expected to result in more severe inhibition compared to low pH values, as the deprotonated form of MT (CH_3S^-) is a stronger nucleophile than molecular MT. At the experimental pH of 9.0, 2% of MT is present in the deprotonated form (pK = 10.4). Most information about the inhibiting effects of MT on microorganisms originates from experiments in anaerobic environments. Reported IC_{50} values for methanogenic granular sludge are 6-8 mM (with acetate), 10 mM (with methanol) and 7 mM (with hydrogen) [27, 28]. Under aerobic conditions, much lower substrate inhibition values for MT are reported: 8 µM for *Thiobacillus thioparus* [29] and 14 µM for *Hyphomicrobium* species [30]. The IC_{50} values for MT on sulfide oxidation at natron-alkaline conditions found in our tests are thus comparable to values reported for aerobic neutrophilic SOB.

It was shown that compared to MT, DMDS and DMTS exhibit a less severe inhibiting effect on sulfide oxidation by natron-alkaliphilic SOB. This may be explained by the lower nucleophilicy of DMDS and DMTS compared to MT. Apparently, hydrophobic interactions do not play a major role in the inhibition of sulfide oxidation by VOSCs, as DMDS and DMTS are more hydrophobic compared to MT. As polysulfide, DMDS and DMTS are the main products from the reaction between MT and biosulfur, this reaction effectively results in a partial detoxification of MT. This was confirmed by bio-oxidation experiments performed with MT-S^0 mixtures. It was found that the results of these tests were similar to those using only polysulfide as a substrate (Fig. 5.1 B and Fig. 5.4). Formation of DMDS was also proposed to play a role in detoxification of MT in the aerobic treatment of sulfidic spent caustics [12]. It was hypothesized that biological oxidation of MT proceeds in 2 steps. First, MT is chemically oxidized to DMDS, where-after DMDS is biologically oxidized to sulfate. Any biological oxidation of DMDS was not observed during the short duration of our respiration experiments (max. 15 min.), but may take place after prolonged incubation periods. Although DMDS seems to play an important role in detoxification of MT, it can also be toxic to microorganisms [31]. Kiene et al. [32] for example, showed that while DMDS and MT could stimulate methanogenesis in different sediments when added at low concentrations (26-56 µM DMDS and 20-52 µM MT), strong inhibition on methanogenesis occurred at

concentrations above 0.26 mM DMDS. Little is known about the effect of DMTS on microorganisms. Rappert et al. [33] found that *Pseudonocardia asacharolytica* was able to oxidize 0.5 mM DMTS as sole carbon and energy source. It was hypothesized that DMTS was first converted into DMDS and subsequently to sulfate and CO_2. Our results indicate that adaptation of the natronophilic biomass to MT takes place after prolonged exposure to low concentrations of MT. It is not known if this was the result of adaptation of the cells, or a change in the composition of the mixed bacterial population.

5.4.2 Influence of the S^{2-}_{tot} concentration

This study shows that the biological molar O_2/S^{2-}_{tot} consumption ratio varies with the S^{2-}_{tot} concentration. At S^{2-}_{tot} concentrations around 0.05 mM, the molar O_2/S^{2-}_{tot} consumption ratio was around 2, while at $[S^{2-}_{tot}]$ >0.2-0.25 mM, the molar O_2/S^{2-}_{tot} consumption ratio was around 0.5 (Fig. 5.2). The explanation for these results is that at S^{2-}_{tot} concentrations around 0.05 mM, (poly)sulfide is completely oxidized to sulfate, with a theoretical molar O_2/S^{2-}_{tot} consumption ratio of 2 (Eq. 6). At $[S^{2-}_{tot}]$ >0.2-0.25 mM, (poly)sulfide is biologically converted to elemental sulfur, with a theoretical molar O_2/S^{2-}_{tot} consumption ratio of 0.5 (Eq. 5). Intermediate molar O_2/S^{2-}_{tot} consumption ratios can be explained by a combined sulfate and sulfur formation, with the selectivity shifting towards sulfur formation with increasing S^{2-}_{tot} concentrations. This relation was found for all substrates used (sulfide, polysulfide and the MT-S^0 mixture, Fig. 5.4). The reaction stoichiometry could not be confirmed by analysis of the oxidation products (sulfate, thiosulfate and biosulfur), as the concentrations were too low to be detected in the carbonate buffer. The same relation between S^{2-}_{tot} concentration and the molar O_2/S^{2-}_{tot} consumption ratio was also observed in bioreactor studies described in Chapter 2 [10]. In these bioreactor studies also the products of (poly)sulfide oxidation could be analyzed, confirming the stoichiometry of sulfate and sulfur formation according to Equations 5 and 6. While the DO concentration in the bioreactor study was always below the detection limit of 0.1% sat., the respiration experiments were performed at saturated DO conditions. This indicates that the S^{2-}_{tot} concentration and not the DO concentration determines the selectivity for the various products (i.e. sulfur or sulfate) from biological oxidation of (poly)sulfide.

Although chemical and biological oxidation rates of sulfide and polysulfide observed in the respiration experiments were comparable (Fig. 5.1 A and B and Fig. 5.4), biological oxidation of these substrates can outcompete chemical oxidation in a bioreactor. Chemical oxidation rates of sulfide and polysulfide are higher at increased DO concentrations [16, 34]. Consequently, at the low DO concentration (<0.1% sat.) prevailing in a sulfur-producing bioreactor [10, 22], chemical oxidation rates are much lower compared to the rates found in the respiration experiments, which were performed at saturated DO concentrations.

5.4.3 Gas treatment considerations

Application of the newly developed process for treatment of gasses containing both H_2S and MT at natron-alkaline conditions will mainly depend on the concentrations of MT, DMDS and DMTS prevailing in the bioreactor. Usually, the MT concentration in sour gasses is much lower than the H_2S concentration [2, 3]. Moreover, as a result of the reaction between MT and biosulfur particles, MT is converted in the absorber column into the far less toxic DMDS and DMTS. Also auto-oxidation of MT to DMDS may contribute to this apparent detoxification of MT. The rate of these reactions determines if the MT concentration in the bioreactor remains below values that severely inhibit biological sulfide oxidation (<0.05 mM). Another prerequisite for biotechnological treatment of H_2S and MT containing gasses, is the degradation of the dimethyl polysulfides (mainly DMDS and DMTS) that are produced from the reaction between MT and biosulfur. If these compounds are degraded at a sufficient rate, no accumulation will occur, preventing inhibitory concentrations (1-1.5 mM). Degradation of DMDS and DMTS may proceed by biological oxidation, although this was not observed in the respiration tests presented in this study. Further study on MT, DMDS and DMTS in a H_2S oxidizing bioreactor operating at natron-alkaline conditions is therefore essential to give more insight in the feasibility of treatment of gasses containing both H_2S and MT.

In our experiments, only MT and its derived compounds produced from the reaction with biosulfur particles were studied for their inhibitory effects. However, besides MT also higher organic sulfur compounds like ethanethiol and propanethiol may be present in sour gasses [1]. Like MT, also these higher thiols can react with biosulfur particles [14]. Possibly, this reaction has the same detoxifying effect as observed with MT, so that treatment of sour gasses containing these higher thiols may also be feasible.

5.5 CONCLUSIONS

The main results of this work may be summarized as follows:

- Microbiological oxidation of sulfide to elemental sulfur by natron-alkaliphilic SOB is severely inhibited in the presence of MT. A 50% decrease of the oxidation rate (IC_{50}) occurs at a concentration of 0.05 mM MT.
- Compared to MT, DMDS and DMTS exhibit a less severe inhibiting effect on sulfide oxidation at sulfur-forming conditions ($[S^{2-}_{tot}]$ = 0.25 mM). For these the IC_{50} value was estimated to be 1.5 (DMDS) and 1.0 mM (DMTS).
- As DMDS and DMTS are products from the reaction between MT and biosulfur particles, this reaction effectively results in a partial detoxification of MT in a sulfur-producing bioreactor.
- At S^{2-}_{tot} concentrations of 0.20-0.25 mM and above, the product of microbiological (poly)sulfide oxidation is elemental sulfur, while at S^{2-}_{tot} concentrations around 0.05 mM and below, (poly)sulfide is completely oxidized to sulfate. At intermediary S^{2-}_{tot} concentrations, both biosulfur and sulfate are formed. This relation is found for sulfide

and polysulfide, as well as for a reaction mixture of MT and biosulfur, containing polysulfide, sulfide, DMDS and DMTS.

- Adaptation of natronophilic biomass to MT takes place after prolonged exposure to (di)methyl sulfur compounds in a H_2S oxidizing bioreactor.
- Application of a biotechnological process for the treatment of gasses containing both H_2S and MT at natron-alkaline conditions is feasible as long as MT, DMDS and DMTS do not accumulate in the bioreactor. Accumulation of MT can be prevented by auto-oxidation or the reaction between MT and biosulfur particles. Further research is required to study microbiological degradation of MT, DMDS and DMTS at natron-alkaline conditions.

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pH Controller

Chapter 6

The effect of methanethiol on product formation

Abstract

The effect of methanethiol (MT) on biological sulfide oxidation was studied in a continuously operated bioreactor, in which chemolithoautotrophic bacteria belonging to the genus *Thioalkalivibrio* convert hydrogen sulfide (H_2S) to elemental sulfur particles at natron-alkaline conditions. In the process, part of the MT reacts with the freshly produced sulfur particles under the formation of dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS). The organic sulfur compounds also partly adsorb onto the biosulfur particles. Previous bioreactor experiments have shown that a fraction of the supplied H_2S is oxidized to sulfate or thiosulfate. This is unwanted, as it leads to caustic requirements for pH control and the formation of a bleed stream. The current research shows that the addition of MT prevents sulfate formation, even at fully oxidized redox conditions (i.e. dissolved oxygen concentration ≥10% sat.). As a result, all supplied H_2S is completely converted into elemental sulfur (≥99mol%). However, presence of dissolved MT, DMDS and DMTS results in biomass decay at concentrations above 10 μM. Treatment of a continuous supply of 51.0 mM d^{-1} H_2S and 79 μM d^{-1} MT was feasible for a prolonged period (>20 days), with 99 mol% selectivity for sulfur formation.

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6.1 INTRODUCTION

Biological oxidation of sulfide at natron-alkaline conditions (i.e. more than 1 M Na^+ and pH above 8) provides an attractive alternative to physicochemical methods for the removal of hydrogen sulfide (H_2S) from high pressure natural gas and sour gas streams produced in the petrochemical industry (Chapter 1) [1, 2]. The process consists of a gas absorber, where H_2S is removed from the sour gas stream by an alkaline carbonate solution and dissociates to form bisulfide (HS^-, further referred to as "sulfide"). In a bioreactor, the sulfide is biologically oxidized to elemental sulfur (S^0 or "biosulfur", Eq. 1), regenerating the alkaline carbonate solution. The bulk amount of the formed biosulfur is separated from the alkaline solution in a gravity settler, while the remaining biosulfur is fed back into the gas absorber with a liquid recycle stream. A detailed description of the process is given in Chapter 2 [1]. Recent microbiological analysis of lab-scale bioreactors studying this process has shown a domination of chemolithoautotrophic, alkaliphilic sulfur-oxidizing bacteria belonging to the genus *Thioalkalivibrio* [3]. Previous research has also shown that depending on the process conditions, a fraction of the H_2S is converted into sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) (Chapter 2 and 3) [1, 4]. At oxidized redox conditions (i.e. a redox potential (ORP) above -250 mV vs. Ag/AgCl), sulfate formation (Eq. 2) is favoured, while at reduced redox conditions (ORP < -350 mV), selectivity for sulfate formation decreases. However, at reduced redox conditions, also thiosulfate formation occurs due to the rapid abiotic oxidation of intermediary polysulfide anions (S_x^{2-}) formed from the reaction between sulfide and biosulfur (Eq. 3 and 4). Formation of sulfate and thiosulfate is unwanted, as it leads to caustic requirements for pH control and the formation of a bleed stream to remove the formed sulfoxyanions [2]. The maximum fraction of H_2S that is converted to biosulfur (selectivity for sulfur formation) is achieved at a S^{2-}_{tot} concentration of 0.20 - 0.25 mM, and a moderately alkaline pH of 8.5 (95 mol%) (Chapter 3) [4]. At these conditions, almost no sulfate formation takes place and selectivity for thiosulfate formation is limited to 5 mol%.

$HS^- + ½\ O_2 \rightarrow S^0 + OH^-$	(biological reaction)	(1)
$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+$	(biological reaction)	(2)
$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+$	(non-biological reaction)	(3)
$S_x^{2-} + 1½\ O_2 \rightarrow S_2O_3^{2-} + (x\text{-}2)\ S^0$	(non-biological reaction)	(4)

Besides H_2S, natural gas and sour gasses produced in the petrochemical industry may also contain volatile organic sulfur compounds (VOSCs) [5]. Of these compounds, methanethiol (MT) is the most abundant [6]. Therefore, the application range of the biotechnological process can be further expanded when also the treatment of gasses containing MT is feasible. Previous research shows that MT reacts with biologically produced sulfur particles to form a mixture of (poly)sulfur compounds [7]. From this reaction, sulfide, polysulfide, dimethyl

disulfide (DMDS) and dimethyl trisulfide (DMTS) are the main products. Longer-chain dimethyl polysulfides are formed at trace levels and possibly also monomethyl disulfide is formed. In literature, no evidence is reported for the existence of monomethyl polysulfides with more than two sulfur atoms [8]. The effect of MT on the biological oxidation of sulfide and polysulfide by a mixed culture of natronophilic sulfide oxidizing bacteria (SOB) has been studied in Chapter 5. The MT concentration at which biological oxidation of sulfide to biosulfur at natron-alkaline conditions was decreased by 50% (IC_{50}), was found to be 0.05 mM. Moreover, it was shown that the products of the reaction between MT and biosulfur (mainly DMDS and DMTS) are less inhibitory than MT. The 50% inhibitory concentrations of DMDS and DMTS on biological (poly)sulfide oxidation were found to be 1.5 and 1.0 mM, respectively.

This chapter describes the effect of MT and dimethyl polysulfides on biological sulfide oxidation in a bioreactor operating at natron-alkaline conditions. Focus is on the effect of MT on the selectivity for product formation.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Reactor experiments were conducted in a gas-lift bioreactor with a wet volume of 4.7±0.1 L. A detailed description of the reactor and analytical and side equipment (pH-, redox- and dissolved oxygen (DO) electrodes, water bath, H_2S and oxygen mass flow controllers) is given in Chapter 2 [1]. No biomass support material was used.

6.2.2 Medium

Liquid reactor medium was prepared by mixing a bicarbonate (pH 8.3) and a carbonate (pH 12.3) buffer, both containing 0.66 M Na^+ and 1.34 M K^+ as carbonates, to a final pH of 9.0±0.1. Furthermore, the medium contained the following macro-nutrients (in g L^{-1}): K_2HPO_4, 1.0; urea, 0.6; NaCl, 1.0; $MgCl_2 \cdot 6\ H_2O$, 1.0. Trace elements were added as described in ref. [9].

6.2.3 Inoculum

The natronophilic biomass that was used for the start-up of each experiment was collected from previous reactor experiments (Chapter 3) [4]. The original seed material consisted of a mixture of sediments from hypersaline soda lakes in Mongolia, south-western Siberia and Kenya and was obtained from Delft University of Technology [10]. Biomass from the sediment mixture was grown at natron-alkaline, sulfide-oxidizing conditions (2M Na^+/K^+, pH 9.2±0.5) at 35°C. The biomass was not exposed to VOSCs prior to the experiments described in this study.

6.2.4 Reactor operation

The reactor was filled with liquid medium and biomass was added to a final concentration of 10-40 mg N L^{-1}. After temperature stabilization at 35±1 °C, a start-up phase of 1-2 days was applied during which H_2S was added at a low loading rate (25.5 mM d^{-1}) and the reactor was operated without oxygen limitation (DO≥10% sat.), to allow biomass activation and growth. After the start-up phase, the H_2S loading rate was increased to 51.0 mM d^{-1}. The oxygen loading rate was based on control of the S^{2-}_{tot} concentration, using a proportional control law as described in Chapter 4. The S^{2-}_{tot} concentration was calculated based on the redox potential (ORP) as described in Chapter 4. When the S^{2-}_{tot} concentration exceeded the control setpoint (resulting a decrease in ORP), the oxygen loading rate was automatically increased. When the S^{2-}_{tot} concentration was below the control setpoint, the O_2/H_2S supply ratio was limited to 0.5 mol mol^{-1}. VOSCs were added after at least 5 days (pulse additions) or 1 day (continuous MT supply) of operation at stable ORP values. Stock solutions of MT, DMDS/DMTS and a mixture of MT and biosulfur ("MT-S^0 mixture") were added to the bioreactor using a diaphragm pump (STEPDOS 03, KNF Neuberger, Freiburg, Germany), either as a "pulse" with a duration of 1-1.5 hours, or continuously. When a pulse of the MT-S^0 mixture was added, the H_2S load was decreased to keep the total sulfide load constant. A pH controller (custom made at the electronic workshop WUR, Wageningen, the Netherlands) was used to maintain the pH at the desired setpoint. Fresh medium as described above was only added to make up for the sample volume and for pH control.

Six reactor runs were performed, at either "reduced" or "oxidized" redox conditions (Table 6.1). When operating at "reduced" redox conditions, the reactor was controlled at a S^{2-}_{tot} concentration of 0.15-0.25 mM, corresponding to an ORP of -395 to -405 mV (vs. Ag/AgCl). When operating at "oxidized" redox conditions, the reactor was controlled at a DO concentration of at least 10% sat. and an ORP above -250 mV, which corresponds to a S^{2-}_{tot} concentration below 1.0 µM. In run 1, a pulse of the MT-S^0 mixture was dosed while the reactor operated at reduced, sulfur forming redox conditions (run 1A). The VOSC concentration in the reactor after addition of the pulse was 3.0 mM as dimethyl polysulfides, assuming that all MT added to the mixture reacted with biosulfur. In the same run, a pulse of the MT-S^0 mixture was dosed after the bioreactor was switched to oxidized, sulfate forming redox conditions (run 1B). In run 2, again a pulse of the MT-S^0 mixture was added at oxidized redox conditions, but now at a lower concentration (1.5 mM as dimethyl polysulfides). In run 3 (at reduced redox conditions) and 4 (at oxidized redox conditions), pulse additions of MT (0.87 mM) were given to the bioreactor. A mixture of DMDS and DMTS (both 1.5 mM) was added as a pulse in run 5. Finally, in run 6, MT was continuously added to the bioreactor, at two different loading rates (35.0 and 79.3 µM d^{-1}).

TABLE 6.1 Overview of reactor runs: operating conditions

Run Nr.	1A	1B	2	3	4	5	6
Redox conditions	Red.	Ox.	Ox.	Red.	Ox.	Ox.	Ox.
$[S^{2-}_{tot}]$ setpoint (mM)	0.15	< 0.001	< 0.001	0.25	< 0.001	< 0.001	< 0.001
ORP setpoint (mV)	-395	> -250	> -250	-405	> -250	> -250	> -250
DO setpoint (% sat.)	-	10	10	-	10	10	10
Type of VOSC added	MT-S^0 mixture	MT-S^0 mixture	MT-S^0 mixture	MT	MT	DMDS/ DMTS	MT
Pulse/ Continuous	Pulse	Pulse	Pulse	Pulse	Pulse	Pulse	Cont.
Initial VOSC concentration (mM)	3.0[a]	3.0[a]	1.5[a]	0.87	0.87	1.5/ 1.5	35-79 µM d^{-1}

[a] Based on dimethyl polysulfide (mainly DMDS and DMTS) concentration in the MT-S^0 mixture.

6.2.5 Total sulfide concentration

The total sulfide (S^{2-}_{tot}) concentration, being the sum of concentrations of dissolved sulfide (HS^-) and polysulfides (HS_x^- and S_x^{2-}), was measured after filtration of samples (0.2 µm, Schleicher & Schuell OE 66, Dassel, Germany) and 1:1 dilution with a 20 g L^{-1} zinc acetate solution. The formed zinc sulfide precipitate was washed to remove salts, and resuspended in demineralised water. The S^{2-}_{tot} concentration was measured with the Lange cuvette test LCK653 (Hach Lange, Düsseldorf, Germany), which is based on a modified methylene blue method as described by ref. [11]. The relation between the ORP and the S^{2-}_{tot} concentration was determined for each reactor run, as described in Chapter 4.

6.2.6 Concentrations of sulfate, thiosulfate, sulfur and biomass

Sulfate and thiosulfate concentrations were measured by ion chromatography (IC) as described in Chapter 2 [1]. The sulfur concentration in the reactor was calculated based on the sulfur mass balance according to:

$$[S^0] = (H_2S \text{ supplied} / V_{liq}) - [SO_4^{2-}] - 2 \cdot [S_2O_3^{2-}] \qquad (16)$$

with V_{liq} for the bioreactor wet volume. Concentrations of sulfide, polysulfides and VOSCs were not taken into account, as their combined contribution to the total concentration of sulfur species was negligible.

The biomass concentration was measured as the amount of organically bound nitrogen, as described in Chapter 2 [1].

6.2.7 Dissolved VOSC concentration and gas composition

Concentrations of (di)methyl disulfide and dimethyl trisulfide in the liquid phase were measured by High Pressure Liquid Chromatography (HPLC) as described elsewhere [12], but with a modified eluent consisting of 70% methanol and 30% water. The concentration of MT could not be determined with this method. No distinction between mono- and dimethyl

disulfide could be made, probably due to the use of methanol in the eluent, resulting in methylation of monomethyl disulfide. This method was only used for the first experimental run (run 1, see Table 6.1), since the presence of high sulfate concentrations lead to the system clogging. For subsequent runs, 1.0 µL of liquid sample was injected into the gas chromatograph used for the detection of VOSCs, as described below. Both filtrated (0.2 µm, Schleicher & Schuell OE 66, Dassel, Germany) and non-filtrated samples were analyzed, to distinguish between dissolved VOSCs and VOSCs adsorbed to sulfur particles or biomass.

Volatile sulfur compounds (H_2S, MT, DMDS and DMTS) were analyzed using a Hewlett Packard (Palo Alto, CA) 6890 gas chromatograph equipped with a Supelco (Bellefonte, PA) sulfur SPB-1 column (length 30m, inner diameter 0.32 mm, film thickness 4 µm) and an Antek (Houston, TX) 704E sulfur chemoluminescence detector (SCLD). Helium was used as the carrier gas, at a flow rate of 5.5 mL min^{-1}. The injection port temperature was 250°C, the oven temperature was 105°C and the interface heater temperature for the transfer interface between the GC and the SCLD was 250°C. A sample injection volume of 100 µL was used for gas samples, and 1.0 µL for liquid phase samples. Combustion furnace temperature was 950°C. Rotameter settings were 5 for O_2 and 3.5 for O_3 and H_2.

6.2.8 Chemicals used

A 2.5 M sodium methylmercaptide ($NaCH_3S$) solution was supplied by Arkema Group (Rotterdam, the Netherlands). Stock solutions of 20 mM MT were prepared by dilution of the $NaCH_3S$ solution with oxygen-free demineralised water. A stock solution containing DMDS and DMTS (both 70 mM) was prepared from pure solutions (Merck, Darmstadt, Germany) by dilution in oxygen-free demineralised water. The solution was continuously mixed by a magnetic stirrer to prevent coalescence. Reaction mixtures of MT and biosulfur ("MT-S^0 mixtures") were prepared by addition of 215 mM MT to excess biosulfur in oxygen-free medium without nutrients or trace elements, as described above. MT-S^0 mixtures were incubated overnight at 30°C to achieve equilibrium between the reactants (MT and biosulfur) and the products, i.e. (di)methyl polysulfides and inorganic polysulfide. Before use, any remaining biosulfur particles were allowed to settle so that almost no biosulfur was introduced into the bioreactor. Fresh biosulfur was obtained from a full-scale biogas treatment facility (Eerbeek, the Netherlands). Before use, dissolved salts (e.g. sodium bicarbonate and sodium sulfate) were removed from the fresh biosulfur suspension by dialysis in demineralised water to a specific conductivity below 40 µS cm^{-1}.

6.3 RESULTS

An overview of the results of reactor runs at different operating redox conditions is given in Table 6.2. The results of these runs are described in more detail in the following sections.

TABLE 6.2 Overview of reactor runs: results

Run Nr.	1A	1B	2	3	4	5	6
ORP (mV)[a]	-395 ±5	-150 ±50	-110 ±50	-405 ±5	-210 ±30	-50 ±20	-180 ±30
DO (% sat.)[a]	<0.1	10 - 30	30 - 35	<0.1	5 - 10	25 - 35	5 - 20
Before VOSC addition							
SO_4^{2-} (%)	2	na	96	8	100	na	na
$S_2O_3^{2-}$ (%)	17	na	<0.5	9	<0.5	na	na
S^0 (%)	81	na	4	83	<0.5	na	na
O_2/H_2S ratio (theoretical) (mol mol^{-1})	0.59 (0.54)	1.76 (1.27)	1.71 (1.95)	0.69 (0.66)	1.98 (2.00)	na	na
growth rate (mg N L^{-1} d^{-1})	0.94	11	45	na	na	na	na
After VOSC addition[a]							
SO_4^{2-} (%)	1	<0.5	<0.5	<0.5	24	na	1
$S_2O_3^{2-}$ (%)	25	1	1	15	<0.5	na	<0.5
S^0 (%)	74	99	99	85	76	na	99
O_2/H_2S theoretical) (mol mol^{-1})	0.67 (0.64)	0.53 (0.51)	0.55 (0.51)	Na (0.58)	0.89 (0.86)	na	0.52 (0.51)
decay rate (mg N L^{-1} d^{-1})	5.1	5.4	9.7	4.9	na	203	na
process deterioration	yes	yes	na[c]	yes	no	yes	no
Biomass conc. at deterioration (mg N L^{-1})	26	37	<45	42	-	109[d]	-

[a]Before deterioration of the process.
[b]Based on product formation according to the stoichiometry of Eq. 1-4.
[c]Run was stopped while biomass concentration still decreased, process failure might have occurred after prolonged operation.
[d] Immediately after addition of DMDS/DMTS, H_2S conversion failed.
na: not available

6.3.1 Pulse addition of the MT-S^0 mixture at reduced redox conditions (run 1A)

In run 1A, H_2S was supplied to the bioreactor while it was controlled at oxygen limiting, reduced redox conditions (S^{2-}_{tot} setpoint = 0.15 mM). The reason for applying reduced redox conditions is that at these conditions sulfur formation is favored (Chapter 2 and 4) [1]. Throughout a period of 26 days prior to addition of the MT-S^0 mixture, H_2S was supplied at a rate of 51.0 mM d^{-1} and oxidized to elemental sulfur (81 mol%), thiosulfate (17 mol%) and sulfate (2 mol%) (Fig. 6.1 A, day 2-28 and Table 6.2). The measured O_2/H_2S consumption ratio of 0.67 mol mol^{-1} is close to the calculated value of 0.64 mol mol^{-1}, based on product

formation according to the stoichiometry given in Eq. 1-4. The reactor biomass concentration increased slowly (Fig. 6.1 B) at a rate of 0.94 mg N L^{-1} d^{-1}.

As soon as the MT-S^0 mixture was added (reactor concentration of 3.0 mM dimethyl polysulfides, day 28), the biomass concentration decreased at an average rate of 5.1 mg N L^{-1} d^{-1}, indicating a toxic effect of the VOSCs that are added together with the MT-S^0 mixture. Four days after addition of the mixture (day 32), the sulfide concentration gradually increased, which was accompanied by a significant drop in the ORP. In spite of the increasing oxygen supply rate, sulfide ions continued to accumulate, indicating that biological sulfide oxidation was severely inhibited (Fig. 6.1 B, day 32-35). In this period, the reactor thiosulfate concentration also increased (Fig. 6.1 A), probably as a result of abiotic oxidation of inorganic polysulfides, which increases at increasing polysulfide concentrations [13]. To prevent excessive accumulation of toxic (poly)sulfides, the H_2S addition was automatically interrupted when the ORP dropped to values below -420 mV, corresponding to a S^{2-}_{tot} concentration of 1.0 mM. When the H_2S addition was interrupted, the (poly)sulfide concentration decreased as a result of abiotic oxidation, which, in turn, resulted in increasing ORP values. When the ORP was above -350 mV (0.005 mM S^{2-}_{tot}), the H_2S addition was automatically resumed. These control settings caused an intermitted supply of H_2S to the reactor, leading to an oscillating behavior of the recorded ORP values (Fig. 6.1 C).

To restore the biological activity, three mitigating actions were taken: (1) additional biosulfur (32 g) was supplied to the reactor (day 34.5); (2) the O_2/H_2S supply ratio was manually increased to 1.76 mol mol^{-1} (day 35) and (3) the H_2S loading rate was decreased from 51.0 to 25.5 mmol L^{-1} d^{-1} (day 35). As a result of these actions, biological sulfide oxidation recovered and also biomass growth was observed. The supplied H_2S and also thiosulfate that accumulated in the previous period were oxidized to elemental sulfur and sulfate (Fig. 6.1 A). Immediately after the supply of additional biosulfur, a significant drop of (di)methyl disulfide ((D)MDS) and dimethyl trisulfide (DMTS) concentrations was measured in the filtrated samples (Fig. 6.1 C). Unfortunately, no MT data were obtained due to limitations of the used HPLC method. It is believed that VOSCs adsorb onto the surface of the biosulfur particles and thus reduce their toxic effects on the micro-organisms. This hypothesis is supported by the fact that increased levels of MT, DMDS and DMTS were found after biosulfur particles taken from the reactor were dissolved in methanol. Obviously, the VOSCs were liberated from the sulfur particles.

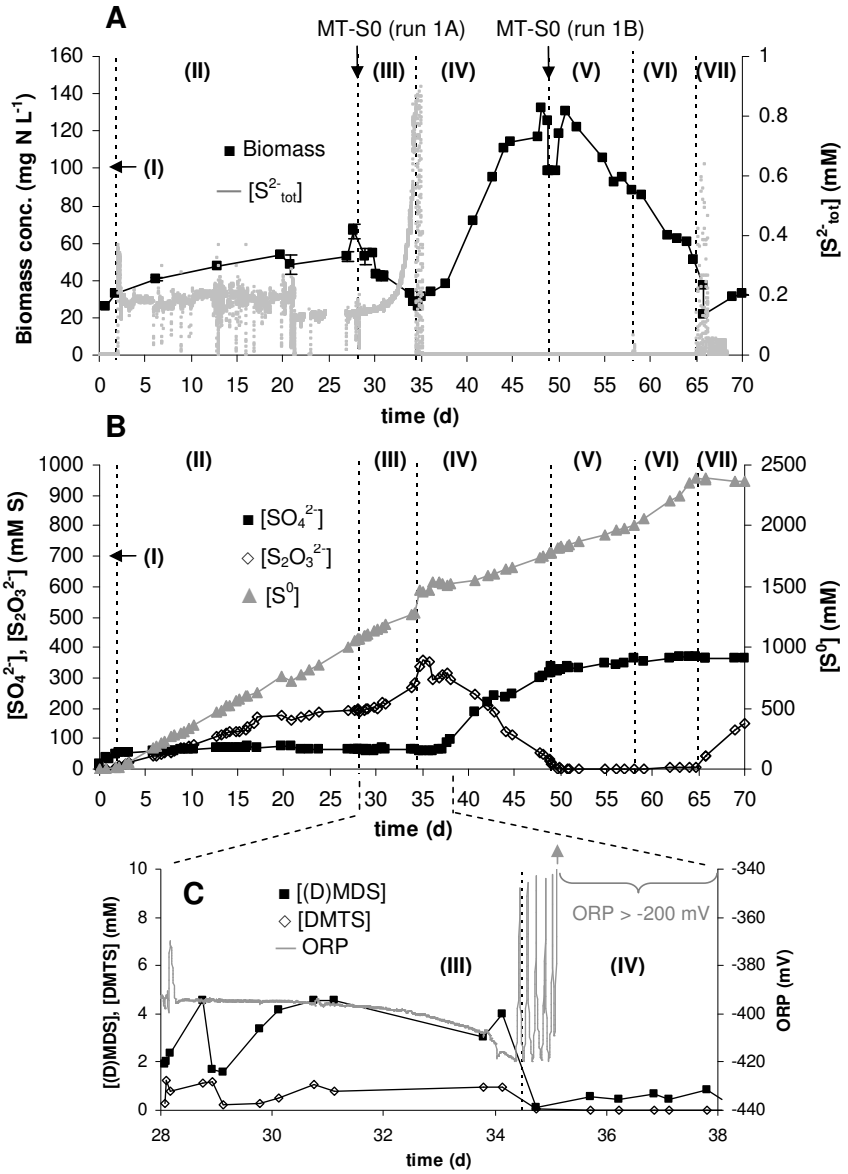

FIGURE 6.1 A-C **Performance of the H_2S-oxidizing bioreactor during run 1A and 1B. A: sulfur product speciation; B: biomass and total sulfide concentration; C: (di)methyl disulfide ((D)MDS) and dimethyl trisulfide (DMTS) concentrations in filtrated samples, and redox potential (ORP) (day 28-38). The vertical dashed lines separate different periods: (I) start-up; (II) switch to reduced conditions (H_2S supply = 51.0 mM d^{-1}) ; (III) addition of MT-S^0 mixture, run 1A; (IV) switch to oxidized conditions at reduced H_2S supply (25.5 mM d^{-1}); (V) addition of MT-S^0 mixture, run 1B; (VI) increase of the H_2S supply to 51.0 mM d^{-1}; (VII) process deterioration as a result of a too low biological activity. The arrows indicate pulse additions of the MT-S^0 mixture. pH = 9.0±0.1, Temp. = 35 °C. Additional data is shown in Table 6.2.**

6.3.2 Pulse addition of the MT-S^0 mixture at oxidized redox conditions (runs 1B and 2)

In a continuation of run 1A, on day 49 (start of run 1B), another pulse of the MT-S^0 mixture was added to the reactor, while it was still controlled at oxidized redox conditions (DO≥10% sat., ORP setpoint ≥-250 mV, reactor concentration of 1.5 mM dimethyl polysulfides). After addition of the MT-S^0 mixture, the O_2/H_2S consumption ratio dropped from 1.76 mol mol^{-1} to an average value of 0.53 mol mol^{-1}, which was accompanied by an almost complete absence of sulfate formation.

In contrast to earlier observations, where oxidized redox conditions would favour sulfate formation (Chapter 2) [1], H_2S was almost exclusively converted to elemental sulfur. After doubling the H_2S supply from 25.5 to 51.0 mM d^{-1} (day 58), sulfur formation continued, i.e. 99 mol% of the added H_2S was converted to biosulfur and only 1 mol% to sulfate. Such a high selectivity for sulfur formation has never been observed before at natron-alkaline conditions. However, upon addition of the MT-S^0 mixture, the biomass concentration decreased at a comparable rate as observed earlier at reduced redox conditions (5.4 mg N L^{-1} d^{-1}, run 1A). On day 65, the reactor performance deteriorated at a biomass concentration of around 37 mg N L^{-1}. All added H_2S accumulated in the bioreactor suspension and was converted to thiosulfate.

A pulse addition of the MT-S^0 mixture at oxidized redox conditions was applied a second time, after start-up with fresh medium and biomass (run 2, Fig. 6.2). This time only half the amount of the MT-S^0 mixture was applied as compared to runs 1A and 1B (reactor concentration of 1.5 mM dimethyl polysulfides).

Before addition of the MT-S^0 mixture, the biomass concentration increased while the supplied H_2S was mainly converted to sulfate. The O_2/H_2S consumption ratio was 1.71 mol mol^{-1} (Table 6.2). Like in run 1B, addition of the MT-S^0 mixture resulted in a steep drop of the O_2/H_2S consumption ratio, leading to an average ratio of 0.55 mol mol^{-1} (Table 6.2). Almost all H_2S was oxidized to biosulfur (99 mol%), confirming the shift in product formation that was already observed previously in run 1B. Biosulfur formation continued during the subsequent eight days while the reactor was controlled at oxidized redox conditions (DO≥10% sat., ORP≥-250 mV), until the end of the run (day 14). Addition of half the amount of the MT-S^0 mixture as compared to run 1B unfortunately did not prevent biomass decay. The absence of a complete process failure is most likely related to the high initial biomass concentration (120 mg N L^{-1}) i.e. sufficient availability of biological oxidation capacity. Prolonged reactor operation may however have resulted in a further decrease of the biomass concentration, which would eventually lead to a complete process failure.

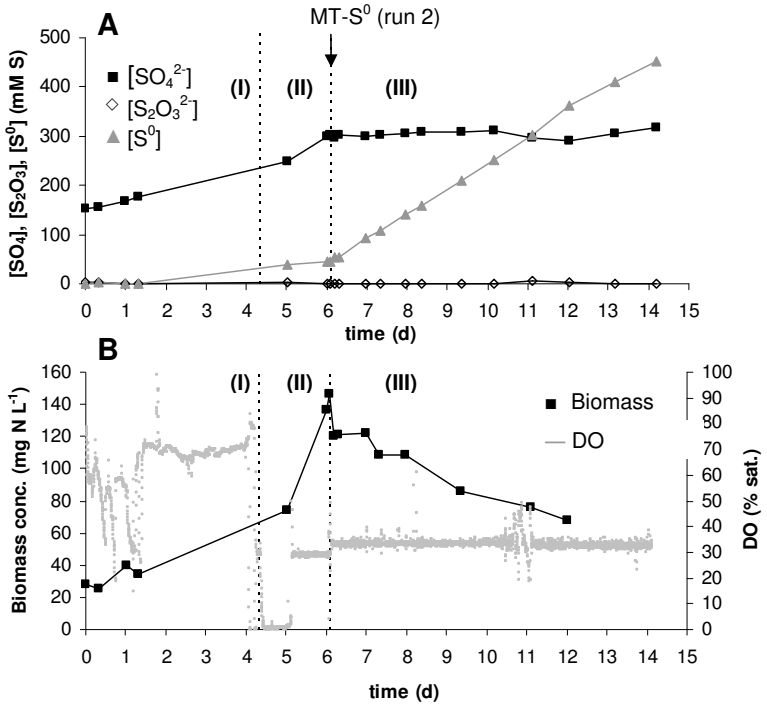

FIGURE 6.2 **Sulfur product speciation (A) and biomass and DO concentration (B) during run 2. The vertical dashed lines separate different periods: start-up at oxidized conditions (I); increase of H_2S supply to 51.0 mM d^{-1} (II); addition of MT-S^0 mixture (III). The arrow indicates pulse addition of the MT-S^0 mixture. pH = 9.0±0.1, Temp. = 35 °C.**

6.3.3 Pulse addition of MT and DMDS/DMTS (runs 3-5)

While in previous runs, MT had reacted with biosulfur before its addition to the reactor, in runs 3 (reduced redox conditions) and 4 (oxidized redox conditions), a pulse addition of MT was applied directly to the reactor. Before addition of MT (reactor concentration of 0.87 mM MT), the reactor operated at stable redox conditions for 9 days.

After addition of MT at reduced redox conditions (run 3), the biomass concentration gradually decreased and 7.2 days after addition of MT, the process failed to convert H_2S at a biomass concentration of 42 mg N L^{-1} (Fig. 6.3, Table 6.2). Immediately after MT addition at oxidized redox conditions, sulfate formation decreased to 24 mol% and H_2S was mainly converted to biosulfur (76 mol%, Table 6.2). No accumulation of sulfide or a drop in ORP was observed, indicating that the process functioned properly (data not shown).

MT concentrations were measured in both filtrated and non-filtrated bioreactor samples (Fig. 6.3). For both runs, more MT was detected in non-filtrated as compared to filtrated

samples, indicating that part of the added MT was associated to the biosulfur particles. The same was observed with DMDS and DMTS (data not shown). At reduced redox conditions, higher concentrations of MT (and DMDS and DMTS) were present in the filtrated samples, as compared to oxidized redox conditions. After deterioration of the process at reduced conditions (day 7.2), high concentrations (>1 mM) of MT were detected in both filtrated and non-filtrated samples. Most probably, presence of VOSCs affected biomass growth and decay. While at reduced redox conditions, the biomass concentration decreased at an average rate of 4.7 mg N L^{-1} d^{-1}, at oxidized redox conditions, the biomass concentration increased (3.0 mg N L^{-1} d^{-1}) when the MT concentration had decreased from 0.03 mM (immediately after addition of MT on day 0) to 0.01 mM (day 2.7, Fig. 6.3).

Addition of a pulse of a mixture of DMDS and DMTS (both 1.5 mM, run 5) at oxidized redox conditions resulted in biomass decay at a high rate (203 mg N L^{-1} d^{-1}). Immediately after addition of DMDS/DMTS, (poly)sulfide started to accumulate in the reactor and thiosulfate accumulated (data not shown), indicating inhibition of biological activity. During the following days, the biomass concentration decreased (9.4 mg N L^{-1} d^{-1}), but after three days growth was observed (1.1 mg N L^{-1} d^{-1}) until the end of the experiment (day 21). During this period, no additional sulfate formation occurred and selectivity for sulfur formation was 99 mol%.

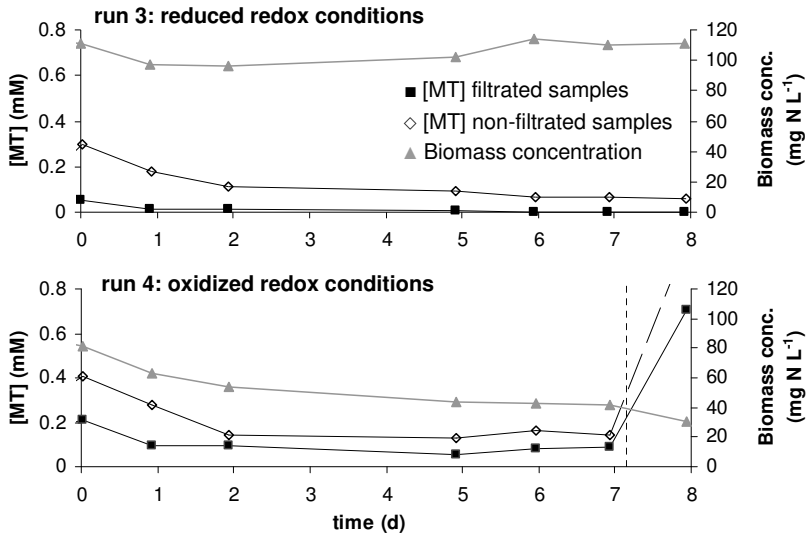

FIGURE 6.3 **MT concentrations in filtrated and non-filtrated reactor samples and biomass concentration, after a pulse addition of MT at reduced (run 3, upper graph) and oxidized redox conditions (run 4, lower graph). Addition of the MT pulse is shown as day 0. On day 7.2 (dashed line) (poly)sulfide accumulated at reduced redox conditions (run 3), while at oxidized redox conditions (run 4) the reactor remained functioning properly.**

6.3.4 Continuous addition of MT at oxidized redox conditions (run 6)

In run 6, MT was continuously supplied to the reactor at a loading rate of 35.0 to 79.3 μM d^{-1}, while the H_2S supply remained constant (51.0 mM d^{-1}). As previous results indicate that in the presence of VOSCs and at reduced redox conditions, sulfide oxidation is not feasible, the reactor was controlled at oxidized redox conditions. During the first 4 days of continuous MT addition (35.0 μM d^{-1}), the biomass concentration slightly increased (3.4 mg N L^{-1} d^{-1}), while 99 mol% of the added H_2S was converted to biosulfur (day 0-4, Fig. 6.4 A and Table 6.2).

After day 7, VOSC concentrations in the gas phase were below the detection limit (Fig. 6.4 B). After day 7, thiosulfate that was already present from the start of the experiment, was converted to sulfate. On day 14, the supply of MT was increased to 79.3 μM d^{-1}, resulting in an increase of DMDS and DMTS concentrations in the gas phase.

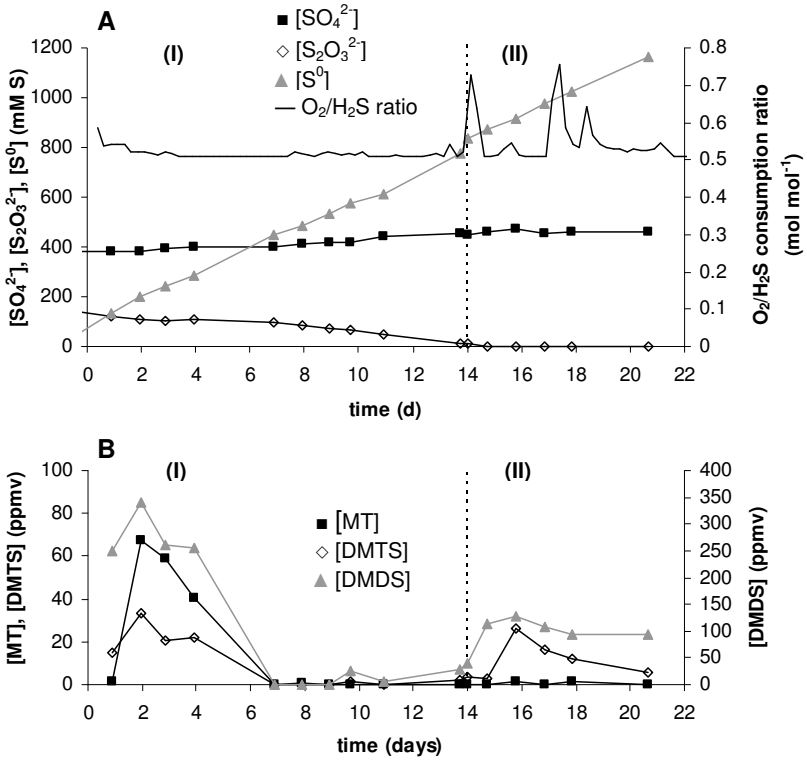

FIGURE 6.4 A-B Results of continuous supply of MT (35.0-79.3 μM d^{-1}) and H_2S (51.0 mM d^{-1}) during run 6. A: concentrations of sulfate, thiosulfate and sulfur and molar O_2/H_2S consumption ratio; B: concentrations of MT, DMDS and DMTS in the gas phase of non-filtrated reactor samples. The dashed lines separate two periods; continuous MT supply of 35.0 μM d^{-1} (I) and 79.3 μM d^{-1} (II).

6.4 DISCUSSION

6.4.1 Adsorption of VOSCs to biosulfur particles

Our results show that DMDS, DMTS and MT adsorb to the surface of biosulfur particles in the bioreactor. A significant drop in the DMDS and DMTS concentration was observed after the supply of additional biosulfur (run 1A, Fig. 6.1 C). Moreover, after dissolution in methanol, a significant amount of VOSCs was released from biosulfur taken from the reactor, indicating that VOSCs were adsorbed to biosulfur particles. It is known that gaseous VOSCs can adsorb to surfaces and the use of activated carbon, zeolites and metal surfaces for the removal of VOSCs from gas streams is well studied [14-17]. However, adsorption of VOSCs in the liquid phase becomes more complex due to the presence of water as a polar solvent [18]. To the best of our knowledge, adsorption of VOSCs to sulfur particles is not mentioned in literature. Polymeric organic substances (e.g. proteins), that are also responsible for the hydrophilicity of biosulfur particles [19] may play a role in adsorption of MT to the particles.

Adsorption of VOSCs to biosulfur particles was observed at both oxidized and reduced redox conditions. However, higher concentrations of VOSCs were present in the reactor liquid at highly reduced redox conditions (i.e. ORP <-350 mV) compared to relatively oxidized redox conditions (i.e. ORP >-250 mV, runs 2 and 3, respectively, Fig. 6.3). When the sulfide concentration in the reactor increased due to process deterioration, MT was released from the sulfur particles (run 2, Fig 6.3). Possibly this is related to the partial "dissolution" of sulfur under the formation of polysulfides, from its reaction with sulfide (Eq. 3). The apparent adsorption of MT to biosulfur particles seems to contradict DMDS and DMTS formation as a result of the reaction between MT and biosulfur particles, which was studied previously by Van Leerdam et al. [7]. In non-biological experiments, it was shown that after a reaction time of about 45 minutes, MT completely reacts away due to the reaction with biosulfur particles. Possibly, the fate of MT in presence of biosulfur particles is related to properties of the biosulfur particles. In the non-biological experiments by Van Leerdam et al., biosulfur was dialyzed in demineralised water before use, while in the bioreactor experiments described in this chapter, biosulfur was formed indigenously. The adsorption process needs to be further investigated to understand its role in the bioreactor.

6.4.2 The effect of MT on product formation

When the reactor was operated at oxidized redox conditions (DO >10% sat. and ORP ≥-250 mV), addition of the MT-S^0 mixture or MT prevented sulfate formation. A decrease of the MT concentration (e.g. after the supply of additional biosulfur or shifting from highly reduced to oxidized redox conditions) resulted in a recovery of sulfate formation (runs 1 and 4). Apparently, the concentration of dissolved MT (the fraction that is not adsorbed to biosulfur particles) determines whether or not complete inhibition of sulfate formation occurs. Our results indicate that sulfate formation is completely inhibited at MT concentrations above 0.01 mM, while at lower MT concentrations, sulfate formation is recovered (Fig. 6.3, Table 6.2).

Inhibition of sulfate formation may be caused by an effect of MT on the metabolic pathway for sulfate formation. It is known that MT inhibits cytochrome *c* oxidase activity [20-22]. When oxygen is not limiting, the sulfide:cytochrome *c* oxidoreductase (FCC) system, which delivers electrons from H_2S respiration to oxygen, is not limited [23]. As a result, H_2S can be completely oxidized all the way to sulfate, as was shown for the neutrophilic chemolithoautotrophic SOB *Thiobacillus neapolitanus* [24]. In presence of MT, cytochrome *c* oxidase activity may be inhibited, which forces electrons to be delivered to oxygen by the sulfide:quinine oxidoreductase (SQR) system, which has a less positive midpoint potential as compared to the FCC system [23]. Both enzyme systems have been shown to be present in natronophilic SOB isolated from soda lake sediments [10]. Possibly, the SQR system is not inhibited by MT, but does not sustain complete oxidation of H_2S to sulfate, resulting in the accumulation of elemental sulfur as the final product.

Like complete oxidation of H_2S to sulfate, also oxidation of thiosulfate to sulfate did not occur in presence of MT (run 6, Fig 6.4 A, day 0-7). This dismisses the possibility that sulfate formation is blocked as a result of binding of MT to biosulfur particles, as oxidation of thiosulfate to sulfate should then not be affected by MT. At low concentrations of (dissolved) MT (<10 µM), sulfate formation from both thiosulfate (run 6, day 7-14, Fig 6.4 A and run 1, day 35-50, Fig 6.1 A) and H_2S (run 4, day 2.7 – 8, Fig. 6.3) was recovered.

6.4.3 The effect of VOSCs on biomass decay

Presence of dissolved VOSCs (MT, (D)MDS and DMTS) resulted in a gradual decrease of the biomass concentration, at both reduced and oxidized redox conditions. A decrease of the dissolved VOSC concentration as a result of adsorption to biosulfur particles resulted in a recovery of growth when the bioreactor was operated at oxidized redox conditions (runs 1, 4 and 6). Our results indicate that biomass growth was recovered at MT concentrations below 10 µM. In general, biomass growth was accompanied by sulfate formation, except for the rapid increase of the biomass concentration observed in run 6 (day 4-6), for which no explanation was found.

A limitation in biological oxidation capacity resulted in thiosulfate formation as a result of chemical (poly)sulfide oxidation (runs 1A-B, 3 and 5). Such a deterioration of the process stability occurred when the biomass concentration dropped below approximately 40 mg N L^{-1}. If no VOSCs are present, a biomass concentration of 40 mg N L^{-1} should be sufficient for the oxidation of the applied H_2S load of 51.0 mM d^{-1} (start-up of run 1, see also Chapter 2 [1]). This indicates that not only a decrease in biomass concentration, but also a decrease in specific biological activity contributes to the deterioration of the H_2S conversion. When a mixture of DMDS/DMTS was added (both 1.5 mM, run 5), process deterioration occurred already at a biomass concentration of 96 mg N L^{-1}, but in this experiment, the biomass concentration dropped at a much higher rate after addition of the VOSCs, as compared to the other experiments (decay rate of about 200 mg N L^{-1} d^{-1} with DMDS/DMTS compared to 5-10 mg N L^{-1} d^{-1} with MT or the MT-S^0 mixture, see Table 6.2). Apparently, addition of

DMDS and/or DMTS as pure compounds induces biomass decay at a higher rate than when these compounds are added as part of the MT-S^0 mixture.

6.4.4 Gas treatment considerations

In a previous study it was suggested that the reaction between MT and biosulfur particles enhances the absorption of MT from sour gasses [7]. The current study shows however, that in an H_2S oxidizing bioreactor the reaction between MT and biosulfur particles does not proceed to completion. Adsorption of MT to biosulfur particles as observed in the current study is however likely to have a similar MT-scavenging effect and may also lead to an enhanced absorption of MT from the gas phase. Moreover, the present research shows that MT adsorption onto biosulfur particles leads to a reduced toxicity on the sulfide oxidizing biomass.

Already at concentrations above 10 µM, dissolved MT (i.e. not adsorbed to biosulfur particles), prevented the formation of sulfate. Hence, elemental sulfur is the sole reaction product even at oxidized redox conditions. At these conditions, thiosulfate formation by abiotic oxidation is absent, because the (poly)sulfide concentration is extremely low (below detection limit). The effect of MT on product formation can be used to optimize the selectivity for sulfur formation, either by the presence of MT in the sour gas, or by deliberate addition of MT to the reactor liquid. However, at too high MT levels, biomass decay will occur.

Acknowledgements

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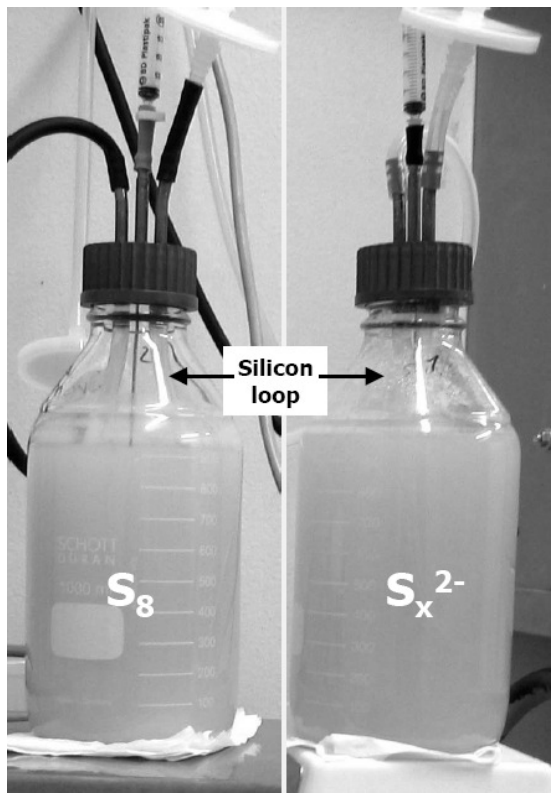
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Chapter 7

Microbiological analysis of the population of extremely halo-alkaliphilic sulfur-oxidizing bacteria dominating in lab-scale bioreactors

Abstract

Thiopaq™ biotechnology for partial sulfide oxidation to elemental sulfur is an efficient way to remove H_2S from biogasses. However, its application for high-pressure natural gas desulfurization needs upgrading. Particularly, an increase in alkalinity of the scrubbing liquid is required. Therefore, the feasibility of sulfide oxidation into elemental sulfur under oxygen limitation was tested at extremely halo-alkaline conditions in lab-scale bioreactors using mix sediments from hypersaline soda lakes as inoculum. The microbiological analysis, both culture dependent and independent, of the successfully operating bioreactors revealed a domination of obligately chemolithoautotrophic and extremely halo-alkaliphilic sulfur-oxidizing bacteria belonging to the genus *Thioalkalivibrio*. Two subgroups were recognized among the isolates. The subgroup enriched from the reactors operating at pH 10 clustered with *Tv. jannaschii-Tv. versutus* core group of the genus *Thioalkalivibrio*. Another subgroup, obtained mostly with sulfide as substrate and at lower pH, belonged to the cluster of facultatively alkaliphilic *Tv. halophilus*. Overall, the results clearly indicate a large potential of the genus *Thiolalkalivibrio* to efficiently oxidize sulfide at extremely halo-alkaline conditions, which makes it suitable for application in the natural gas desulfurization.

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Sorokin, D. Y.; Van den Bosch, P. L. F.; Janssen, A. J. H.; Muyzer, G., Microbiological analysis of the population of extremely haloalkaliphilic sulfur-oxidizing bacteria dominating in lab-scale sulfide-removing bioreactors *Applied Microbiology and Biotechnology*, in press.

7.1 INTRODUCTION

The presence of H_2S in fuel gasses causes many environmental and technical problems demanding its removal before combustion. Usually, this is done by a catalytic oxidation. An alternative bioprocess based on lithoautotrophic sulfide-oxidizing bacteria (SOB) as a catalyst has been developed and successfully applied at full scale in the Netherlands for biogas desulfurization [1-3]. The major principle of the *Thiopaq* technology is regulation of sulfide oxidation at the level of elemental sulfur by low redox potential (ORP), which provides two advantages over the complete oxidation to sulfate: (1) the oxidation does not generate protons but regenerates hydroxyl ions, thereby allowing to save on caustic absorbent; (2) formation of elemental sulfur allows easy separation of the final oxidation product and recirculation of the liquid phase. In case of biogas, the bioprocess is performed at relatively low salt concentrations and pH, i.e. at 0.5 M total Na^+ and pH 8.2-8.5, with bicarbonate as the dominant anion in solution. At these conditions marine-type SOB, such as *Halothiobacillus neapolitanus* W5 [4], function very well. However, for the removal of H_2S from high pressure natural gas and sour gas streams produced in the petrochemical industry, the total alkalinity must be substantially increased to make the scrubbing process more efficient. This dictates a shift in a type of biocatalyst from neutrophilic marine SOB to natronophilic (soda-philic), highly salt tolerant SOB [5-7]. Such SOB have recently been discovered in the sediments of hypersaline soda lakes [8, 9]. One out of three genera of haloalkaliphilic SOB described so far, the genus *Thioalkalivibrio*, is characterized by the ability to grow in saturated soda brines containing up to 4 M total Na^+ and pH from 7 to 10.5. All but one out of nine described species of this genus are obligatory alkaliphilic and soda-philic, i.e. they can only grow in carbonate brines and at a pH above 8. A single species, *Tv. halophilus,* is a facultative halophile capable of growth at neutral pH in NaCl brines as well as in soda brines at pH 10 [10]. All previously described high salt-tolerant *Thioalkalivibrio* strains were obtained from the enrichments with thiosulfate as substrate and at high redox potential, since batch cultivation at low redox potential with sulfide as an electron donor is much more complicated. Therefore, such organisms might be unsuitable for application in the *Thiopaq* process module, where the redox potential is as low as -350 mV and sulfide/polysulfide is the actual electron donor. This paper describes results of a microbiological investigation of lab-scale bioreactors oxidizing sulfide/polysulfide at pH 8.5-10 and a salt content of 2-3 M Na^+/K^+. It is shown that the populations were dominated by extremely salt-tolerant alkaliphilic SOB, represented by two subgroups of the genus *Thioalkalivibrio*.

7.2 MATERIALS AND METHODS

7.2.1 Inoculum for the bioreactors

Surface sediment samples (0-10 cm) were obtained from hypersaline soda lakes in northeastern Mongolia, southeastern Siberia and Wadi al Natrun in Egypt. 8-12 samples from

individual lakes in each region were combined into a single pool. The pH of the brines varied from 9.2 to 10.6, total salt concentration from 60 to 400 g L^{-1} and total soluble alkalinity from 0.05 to 3 M.

7.2.2 Bioreactors

Two types of lab-scale bioreactors were used for oxidation of sulfide at oxygen-limited conditions. One was a 5 L gas-lift column (FBR) fed by H_2S gas, where the oxygen supply was controlled on basis of the redox potential (Chapter 2) [6, 7]. Another type was a 1 L stirred tank reactor (SL-BR) where sodium sulfide was fed in sequential fed-batch mode (1 mM shots) and oxygen was supplied by limited diffusion through a loop from silicon tubing (Fig. 7.1). The major difference between these two types of bioreactors was the concentration of polysulfide. In the FBR it was generally maintained at a relatively low level of 100 μM sulfane using redox control. The fraction of free sulfide as compared to polysulfide was minimal at pH 10 and increased toward pH decrease. In the SL-BR the concentration of polysulfide reached 1 mM right after addition of sulfide due to a rapid spontaneous reaction with sulfur formed during the previous stage, decreasing gradually to zero due to biological activity of the SOB. Therefore the latter conditions selected for high polysulfide/sulfide resistance and polysulfide as a substrate.

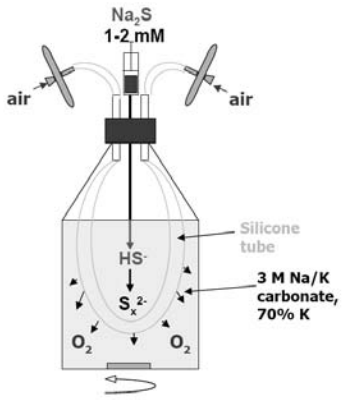

FIGURE 7.1 **Schematic presentation of the silicon loop stirred-tank reactor (SL-BR).**

7.2.3 Operating conditions

The duplicate FBR were run during 2 years at variable modes at pH starting from 10.1 and ending at 8.8 (Table 7.1). The redox potential was maintained most of the time below -390 mV (vs. Ag/AgCl). The mineral medium was based on bicarbonate/carbonate buffer containing 70% K^+ and 30% Na^+, 2 M in total. The presence of so much potassium is certainly unusual and never occurs in natural soda lakes. The reason behind employing such

an unusual buffer is the much higher solubility of potassium carbonates as compared to sodium carbonates, especially when the pH is decreased below 9 and bicarbonate is the dominant anion. Our preliminary tests with extremely haloalkaliphilic cultures of the genus *Thioalkalivibrio* demonstrated that many strains can withstand up to 50% replacement of Na^+ by K^+ at 2 M total cation in the form of carbonates, and a few strains even tolerated 90% replacement. This is in contrast to the neutrophilic halophilic SOB, which cannot grow already at 30% replacement of NaCl by KCl [11]. The N-source in the bioreactors was urea. A more detailed description of these bioreactors is given in Chapter 2 [6, 7]. The duplicate 1 L SL-BR were run within 2 months at 1.2-3.0 M of K^+/Na^+ carbonates, 70% K^+ and pH 10.1. The N-source was ammonia.

7.2.4 Isolation and cultivation of pure cultures of extremely halo-alkaliphilic SOB

Two approaches were used for the enrichment and isolation of pure cultures of halo-alkaliphilic SOB. One was based on using thiosulfate as substrate (20 mM), which is stable at aerobic conditions. Alternatively, sulfide (2 mM) was used as a substrate. The use of sulfide is much more difficult because of its volatility, toxicity and spontaneous oxidation. The former effects however, are reduced at highly alkaline pH, while the spontaneous oxidation was reduced by a 10 times decrease in trace metal content and the use of only 1% oxygen in the gas phase. To standardize the enrichment procedure, the same conditions were also used in the thiosulfate enrichments. The liquid mineral medium used for enrichment and isolation contained carbonate buffer with 2 M K^+/Na^+ (70% K^+), pH 10, 1 g L^{-1} of K_2HPO4 and 4 mM of either NH_4Cl or urea. After sterilization the medium was supplemented with trace metals [12], 1 mM $MgCl_2$ and a sulfur substrate. Serial dilutions were incubated at 30°C in 20 mL Hungate tubes with 2 mL medium and a headspace containing 1% O_2 in argon. When turbidity appeared in a highest dilution, it was plated into a solid agar medium of the same composition. The plates were incubated in closed jars (3 L) under the atmosphere of argon containing 1% O_2. In case of sulfide enrichments the plates did not contain any substrates. Instead, 4 mL of 1 M sodium sulfide solution was placed into the jar in a 10 mL vial. During the prolonged incubation (2-4 weeks), H_2S entered the gas phase and was absorbed by the alkaline agar creating optimal conditions for sulfide-utilizing SOB. Dominating colony types were picked under the binocular and placed into the liquid medium. The purity of the culture was checked by repeated plating.

The activity and the stoichiometry of oxidation of various sulfur compounds was studied with an oxygen electrode using washed cells as described previously [10]. The pH dependence was examined at 2 M total Na^+, using the following buffers: for pH 6-8, 0.1 M HEPES and NaCl; for pH 8-11, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. To study the influence of salt concentration, sodium carbonate buffer with pH 10 containing 0.1 and 4.0 M of total Na^+ was applied. All buffers contained 50 mM K^+ and 1 mM Mg^{2+}. Cell membranes were obtained by ultracentrifugation of the sonified cells at 144,000 x g for 2 h (Beckman). The membranes were resuspended in soda buffer at pH 9

containing 0.5 M total Na^+ and used to measure several enzyme activities. Cytochrome *c*-oxidase was measured by the rate of oxidation of 1 mM TMPD spectrophotometrically at 610 nm. Sulfide-quinone reductase (SQR) activity was determined in discontinuous assay of anaerobic decyl-ubiquinone-dependent oxidation of sulfide (0.2 mM each). Cytochrome *c* content in the membranes was estimated from spectroscopic measurements of dithionite-reduced minus air-oxidized preparations using a UV-visible diode-array spectrophotometer (Vectra 8453, HP, Amsterdam) and a molar absorbance coefficient of E_{550} = 20 mM cm^{-1}.

7.2.5 Analytical procedures

Chemical analysis of sulfur (sulfide, sulfur, thiosulfate and sulfite) and nitrogen (nitrite and ammonium) compounds and cell protein were performed as described previously [10]. Sulfane sulfur atoms of polysulfide were analyzed in the same way as free sulfide, i.e. after precipitation as ZnS. Zero-valent sulfur in polysulfide was analyzed in the same way as free sulfur (i.e. by cyanolysis of acetone extract) after decomposition of polysulfide molecules by acid treatment. Total protein comparison of the isolates and the reactor PCR fingerprinting comparison of the SOB isolates was performed with the 1 GTG5 primer set as described previously [13].

7.2.6 Genetic and phylogenetic analysis

Genomic DNA was extracted from the cell pellet using the UltraClean Soil DNA Extraction Kit (MoBio Laboratories, USA), following the manufacturer's instructions. For the pure cultures, the nearly complete 16S rRNA gene was obtained using general bacterial primers GM3f (5'-AGAGTTTGATCCTGGCTCAG-3') and GM4r (5'TACGGTTAC-CTTGTTACGACTT-3'). For the DGGE analysis, partial amplification with a primer pair 341F+GC/907R was employed [14]. DGGE was performed as described by ref. [15], using a denaturing gradient of 20-35 to 60-70% denaturants in 8% polyacrylamide gel. Individual bands were excised, re-amplified, and run again on a denaturing gradient gel to check their purity. PCR products for sequencing were purified using the Qiaquick PCR purification kit (QIAGEN, The Netherlands). The sequences were first compared with sequences stored in GenBank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the sequences were imported into the ARB software program [16], automatically aligned, and added to a phylogenetic tree using the Quick-add tool. Sub-trees were then built using the neighbour-joining algorithm with automatic selected correction settings.

7.3 RESULTS

7.3.1 Catalytic properties of the bioreactor biomass

Respiration profiles for different sulfur compounds were determined with washed cells directly from the bioreactors and used to characterize the activity and pH/salt response of the dominant SOB populations. A general trend could be seen that sulfide and, in case of 1 the

SL-BR reactors, polysulfide were much better respiratory substrates for the mixed SOB population in the reactors than thiosulfate (Fig. 7.2 A). A characteristic for an active respiratory chain in aerobic chemolithoautotrophs is the activity of cytochrome *c* oxidase together with the presence of a high-potential cytochrome *c* pool. This pair is responsible for the terminal delivery of the electrons obtained during oxidation of electron donor to oxygen. Comparison of the three reactor samples demonstrated that only in FBR2 (pH 8.8, 0.1±0.05 mM (poly)sulfide, ORP=-390±5 mV) it was at a level normal for aerobic haloalkaliphilic SOB (personal data on pure cultures), while in SL-BR2 (pH 10) and FBR1 (pH 9.5; 0.3±0.05 mM (poly)sulfide; ORP=-410±5 mV) the cytochrome *c* oxidase had a low specific activity (Fig. 7.2 B). This might be connected to the condition of extremely low redox potential in these reactors, especially in SL-BR2, due to the presence of polysulfide at a high concentration.

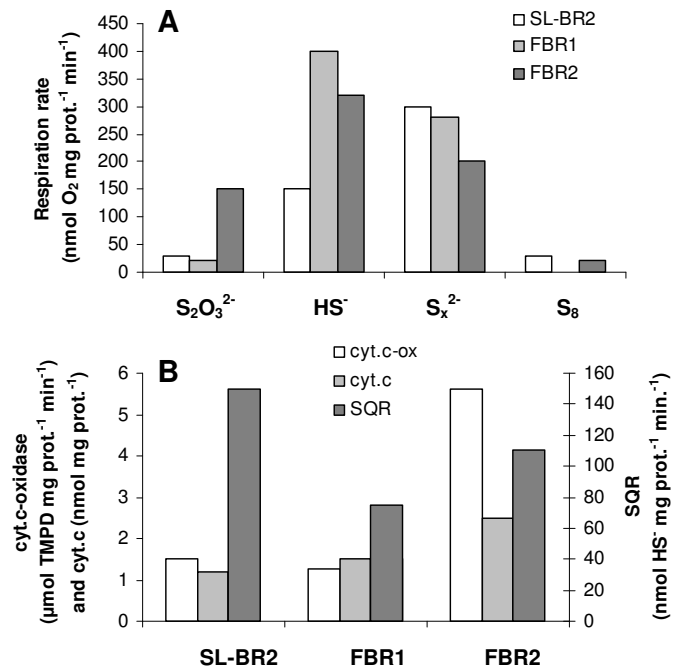

FIGURE 7.2 A-B Metabolic activity of SOB biomass from sulfide-oxidizing bioreactors (see Table 7.1). A: respiratory activity of whole cells with different sulfur substrates at pH 10 and 2 M total Na^+/K^+ (as carbonates); B: respiratory enzyme parameters in cell membranes: cytochrome *c*-oxidase, µmol TMPD (mg protein min)$^{-1}$ at pH 9; cytochrome *c* content, nmol (mg protein min)$^{-1}$ and SQR (sulfide-quionone reductase activity, nmol HS^- (mg protein min)$^{-1}$ at pH 10.

In contrast to cytochrome *c* oxidase, the activity of sulfide quinone reductase (SQR) – a flavin-containing enzyme oxidizing sulfide to elemental sulfur with quinones as electron acceptors [17] - was relatively high (Fig. 7.2 B).

One of the very important parameters of the SOB biomass activity in the reactors is its pH response. Comparison of the two FBR reactors, run at different pH (9.5 for FBR1 and 8.8 for FBR2), demonstrated a different pH response (Fig. 7.3). There was a clear difference in the neutral part of the pH profiles for sulfide between the two reactors. This indicates either the presence of a separate pH-neutral population in FBR2 (pH 8.8) or a dominance of facultatively alkaliphilic sulfide-oxidizing species in this reactor. The profile for polysulfide in FBR2 was similar to that of sulfide. In contrast, the profile for thiosulfate oxidation by FBR2 cells was typical for obligate alkaliphilic SOB. The latter might be due to the presence of different SOB populations specialized either in sulfide/polysulfide or thiosulfate, or due to a different pH response of the same population with different substrates. Our pure culture data favor the first suggestion. But, in general, the respiratory data clearly indicated domination of halo-alkaliphilic SOB in the reactors.

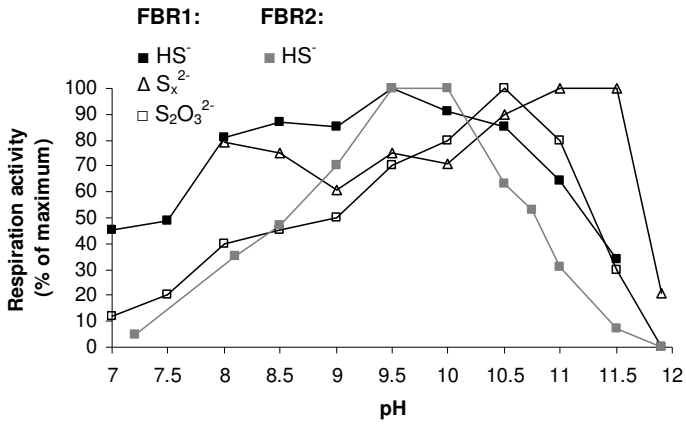

FIGURE 7.3 **Influence of pH at 2 M K^+/Na^+ on the respiratory activity of the washed cells from the reactors FBR1 and FBR2 with different sulfur substrates.**

A direct comparison of the membrane proteins, which are dominated by the respiratory enzymes, showed major similarity between the two reactors FBR1 and FBR2 (Fig. 7.4 A-B), suggesting a presence of the same dominant SOB population.

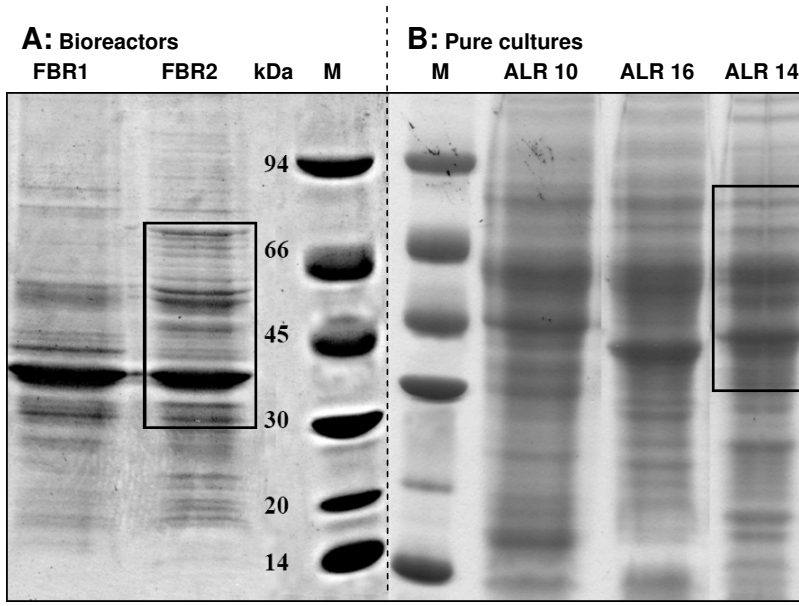

FIGURE 7.4 A-B Comparison of the membrane proteins from biomass in reactors FBR1 and FBR2, using SDS-PAGE (5-15% gradient). Boxes shows highly similar regions. Total protein concentration was 2 mg mL^{-1}.

7.3.2 DGGE analysis of the reactor biomass

Molecular analysis of the biomass from two SL-BR and two FBR bioreactors based on 16S-rRNA gene DGGE showed low genetic diversity, typical for autotrophic mix cultures with domination of 1-2 SOB genotypes and some side heterotrophic populations (Fig. 7.5). Since analysis of the SL-BR and FBR biomasses was performed on different gels, the profiles could not be directly compared. However, a general similarity of the FBR1 and FBR2 (pH difference 0.7 unit) profiles were quite obvious, while the profiles of two SL-BR (difference in salinity is equivalent to 2 M Na^+/K^+) looked different. This might be explained by the fact that while pH 9.5 and 8.8 are still within alkaliphilic range, a salt content of 1.5 and 3 M total Na^+/K^+ usually selects for different groups of halo-alkaliphilic SOB [9].

The phylogenetic analysis of the DGGE band sequences demonstrated that in all samples lithoautotrophic SOB belonging to the Gammaproteobacteria were dominant (Fig. 7.6), although its affiliation differed at different reactor conditions. At moderate halo-alkaline conditions (reactor SL-BR1) the dominant band belonged to a distant relative of the genus *Halothiobacillus,* while at higher salt (2-3 M Na^+/K^+), both in SL-BR2 and in FBR, the representatives of the genus *Thioalkalivibrio* were identified. Interestingly, in all 3 high salt reactors, despite the difference in pH, the SOB sequence was most closely related to the

facultatively alkaliphilic and extremely salt-tolerant *Thioalkalivibrio halophilus* [10]. Other identified sequences either belonged to nonsulfur oxidizing alkaliphilic heterotrophs, such as *Marinospirillum* (SL-BR2) or *Bacillus* (FBR2) or to halo-alkaliphilic facultative sulfur oxidizers, for which sulfide oxidation might provide additional energy [8, 18], such as *Alkalispirillum* 1 (FBR1) and *Rhobacteraceae* (FBR2).

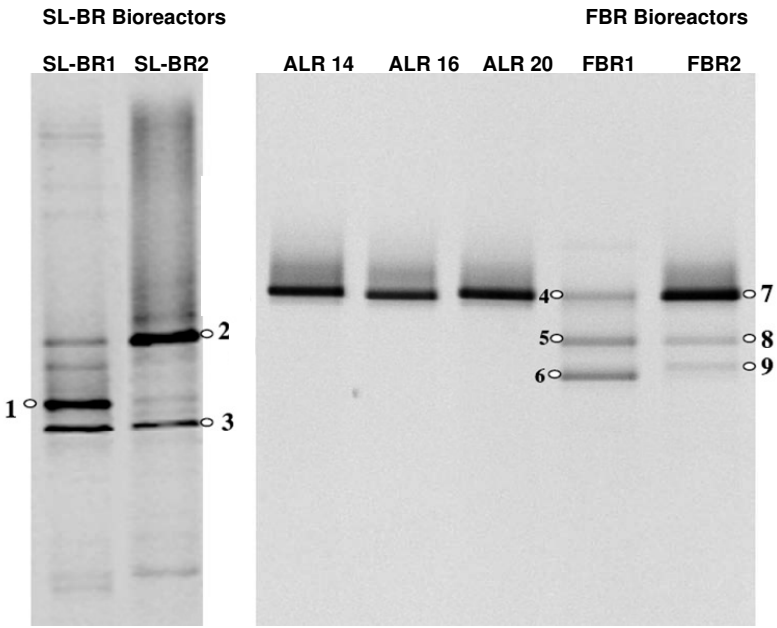

FIGURE 7.5 DGGE analysis of the biomass from SL-BR (35-70% gradient) and FBR (20-17 70% gradient) bioreactors. For comparison, three SOB isolates (ALR) from the FBR2 were also analyzed. Bands identification (by Blast): 1: 94% similarity to *Halothiobacillus kellyi*; 2 and 7: 99% similarity to *Thioalkalivibrio halophilus*; 3, 97% similarity to *Marinospirillum alkaliphilum*; 5 and 8: 97% similarity to *Bacillus agaradhaerans*; 6: 99% similarity to*Alkalispirillum mobilis*; 9: 99% to *Paracoccus denitrificans.*

7.3.3 Isolation and identification of the dominant halalkaliphilic SOB from the bioreactors

The cultivation approach demonstrated presence of culturable SOB in the reactor biomass, mostly at a very high density (Table 7.1). From the highest positive dilution more than 20 pure cultures were obtained either with thiosulfate or with sulfide as the sulfur substrate. All but one of them (strain ALR 15) were obligately chemolithoautotrophic SOB identified as the representatives of three subclusters within the genus *Thioalkalivibrio* by 16S-rRNA gene sequencing (Fig. 7.6).

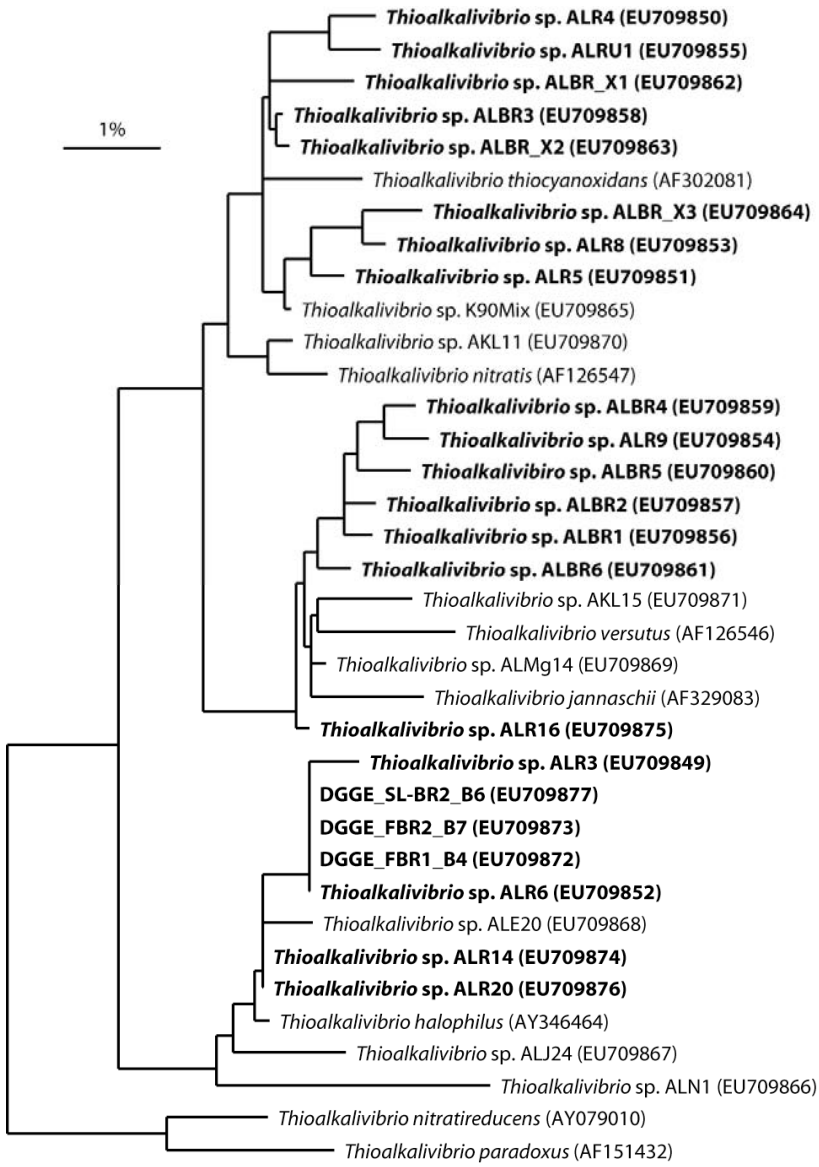

FIGURE 7.6 **Phylogenetic tree based on 16S rRNA gene sequencing, showing the position of the SOB identified by DGGE in the reactor biomass and of the pure cultures of halo-alkaliphilic SOB isolated from the bioreactors. Sequences obtained in this work are in bold. Other undescribed strains presented in the tree were isolated from various soda lake sediments: AKL11, AKL15, and Tvmix90, from Kulunda Steppe (Siberia, Russia); ALMg14, from Mongolia; ALE20 and ALN 1, from Wadi Natrun (Egypt); ALJ24, from Lake Magadi (Kenya). Scale bar represent 1% sequence divergence.**

Most of the isolates from the SL-BR reactors (pH 10) and from the FBR run at pH 10 were clustering around the core group of the genus which includes the type species *Tv. versutus* and its close relatives *Tv. thiocyanoxidans*, *Tv. jannaschii* and *Tv. nitratis*, all obligate halo-alkaliphiles. Only 2 isolates, ALR 3 and ALR 6, obtained from the high-pH FBR on sulfide, belonged to the cluster of facultative alkaliphilic halophile *Tv. halophilus*. On the other hand, all the isolates obtained with sulfide as substrate from the reactor with the lowest pH (FBR2, pH 8.8) belonged to the *Tv. halophilus* subcluster and only those enriched with thiosulfate clustered with the core group of obligate alkaliphiles. The latter results were consistent with the DGGE analysis (Fig. 7.5 and 7.6) of the biomass from FBR2 and with the pH profile of respiration (Fig. 7.3). On the other hand, the cultivation-based approach failed to produce the same results for SL-BR2, where molecular data also identified a dominance of a *Tv. halophilus*-like population, while only representatives of the core group of the genus *Thioalkalivibrio* were obtained in culture. This might be explained by the different culture conditions: in batch enrichment cultivation at pH 10 from the SL-BR2 might have provided better conditions for the secondary population of obligate alkaliphiles.

A pH-salt response of one of the *Tv. halophilus*-like isolates, strain 1 ALR 14, was studied in more detail, since this type seemed to be an important player in both types of bioreactors. Growth experiments with thiosulfate demonstrated that, despite being phylogenetically closely related to facultatively alkaliphilic *Tv. halophilus*, strain ALR 14 can be regarded as an obligate alkaliphile (Fig. 7.7 A). On the other hand, its respiratory activity (Fig. 7.7 B), especially with sulfide, was quite high already at subalkaline pH values (8.5-9.0), indicating good fitness to the reactor conditions (pH 8.8). Salt profiles corresponded to those of extremely salt-tolerant moderate halophiles (Fig. 7.7 C) and also fit very well to the reactor conditions (2 M total Na^+/K^+). An interesting pH effect was observed in case of oxidation of sulfide and polysulfide by ALR 14 (Fig. 7.7 D): the oxidation of sulfane atoms (S-) proceeded mostly to elemental sulfur at near neutral pH, while its further oxidation to sulfate was increasing at an alkaline pH range.

A similar effect of pH on product formation was found in the FBR bioreactors [7], with one important difference: in case of washed cells of a pure culture, the final oxidation product was sulfate, while in the bioreactor thiosulfate accumulated at high pH. This difference can be accounted to the nature of sulfane atom oxidation in the two systems. In the bioreactors, enzymatic oxidation of polysulfide sulfane atoms was apparently inhibited by very low redox potentials, resulting in spontaneous oxidation to thiosulfate. In contrast, the washed cells experiment was conducted at a high initial oxygen concentration, allowing enzymatic conversion of sulfane with sulfate as the final product. A single heterotrophic isolate was obtained from FBR2 from the special colonies formed on alkaline plates incubated under the H_2S-containing gas phase.

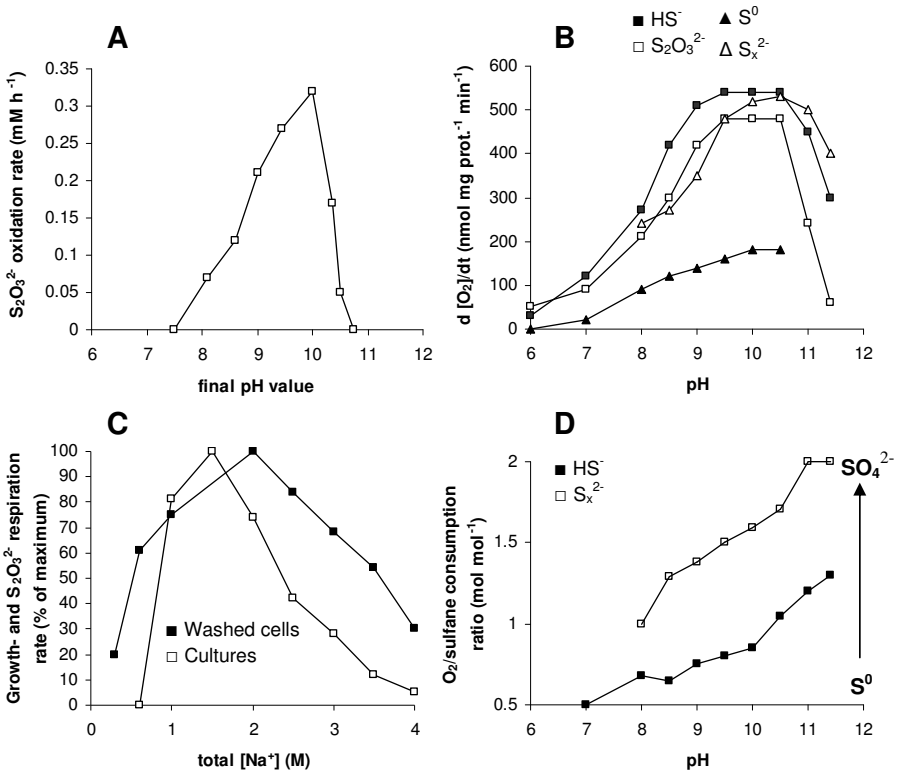

FIGURE 7.7 A-D pH-salt response of *Thioalkalivibrio* strain ALR 14 isolated from the sulfide-oxidizing bioreactor. A: pH influence on growth with thiosulfate; B: pH profile for oxidation of different sulfur compounds by washed cells grown at 2 M total Na^+, pH 10 with thiosulfate; C: Salt profile for thiosulfate oxidation at pH 10 in cultures, and by washed cells pre-grown at 2 M total Na^+; D: Influence of pH on product formation during oxidation of sulfide and polysulfide by washed cells at 2 M Na^+. The arrow indicates a shift in the product formation from sulfur to sulfate with increasing pH.

Since also DGGE analysis indicated a presence of heterotrophs in the reactor there must be a source of organic carbon in the system. Such carbon could be provided by a dominant population of *Thioalkalivibrio* either by excretion or after lysis of dead cells. Usually, sulfide-oxidizing bacteria are forming sulfur inside the colonies. In contrast, strain ALR 15 produced large sulfur halos around the colonies (Fig. 7.8 A). Further investigation showed that the bacterium, which was identified as a member of the genus *Halomonas*, was an obligate heterotroph unable to actually use sulfide or thiosulfate as energy source. However, during heterotrophic growth, it oxidized thiosulfate to tetrathionate, which is a well-known property of this group of Gammaproteobacteria [19].

Our scenario to explain this extra-colonial sulfur formation is the following (Fig. 7.8 B): H_2S was being absorbed into the alkaline agar from the gas phase and partially converted to elemental sulfur. The latter reacted with sulfide to form polysulfide (indeed a yellowish coloration was observed after prolonged incubation of the plates). Spontaneous reaction of polysulfide with oxygen resulted in the formation of thiosulfate. Here the active role of the bacterium starts, in providing tetrathionate - a powerful oxidant for sulfide. The reaction of sulfide with tetrathionate produces sulfur and regenerates the substrate (thiosulfate) for the heterotroph. So, only traces of thiosulfate are necessary to catalyze the oxidation of sulfide to sulfur in the presence of tetrathionate-forming heterotrophs. Such a microbio-chemical catalysis has been demonstrated for a marine heterotrophic bacterium *Catenococcus* [20]. The difference with *Halomonas* ALR 15 is in the internal generation of thiosulfate from polysulfide, which is stable only at highly alkaline conditions. In the case of *Catenococcus*, thiosulfate had to be supplied externally. Although the colonies with external sulfur accumulation were quite common in our enrichments, this organism was not found in the reactor biomass by molecular analysis, which makes its role in sulfide oxidation in situ in comparison with the obligate autotrophic *Thioalkalivibrio* questionable.

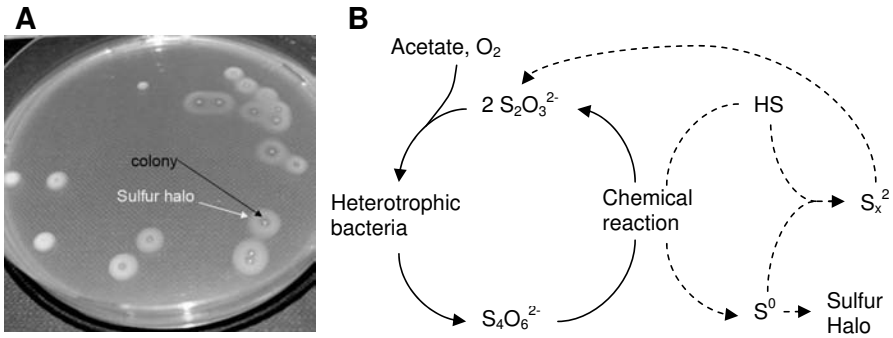

FIGURE 7.8 A-B Formation of sulfur halos on solid agar medium by heterotrophic SOB. A: Colonies with sulfur halos on solid agar medium; B: Scenario for the formation of sulfur halos via tetrathionate.

7.4 DISCUSSION

Our previous microbiology investigation of various soda lakes demonstrated a presence of highly diverse population of the obligately lithoautotrophic SOB of the genus *Thioalkalivibrio* which was dominating especially at extreme salinity [8, 9, 13]. So, it is not surprising, that in the bioreactors inoculated with the sediments from hypersaline soda lakes these halo-alkaliphilic SOB species were dominating. The reactor isolates were very closely related to the *Thioalkalivibrio* species obtained from the natural sediments isolated at fully oxic

conditions with thiosulfate as substrate. This might mean that both low-potential oxidation of sulfide/polysulfide and high potential thiosulfate oxidation pathway may coexist in the same SOB species. Indeed, that's what usually can be seen in the respiratory profiles of the halo-alkaliphilic SOB grown either with thiosulfate or sulfide at fully aerobic conditions, i.e., equal rates of sulfide- and thiosulfate-dependent respiration. On the other hand, the respiratory tests with the SOB biomass from the low redox potential bioreactors clearly indicated sulfide/polysulfide preference (see Fig. 7.2), suggesting a possibility of a different pathway for oxidation of these highly reduced electron donors as compared to thiosulfate oxidation. One of the specialized components of such a pathway might be sulfide quinone reductase (SQR). A relatively high activity of this enzyme - which oxidizes sulfide to elemental sulfur with quinones as electron acceptors [17] - was found in the biomass of the analyzed bioreactors (Fig. 7.2 B). Furthermore, the profound "sulfide/polysulfide specialization" of the biomass in SL-BR2 and FBR1 correlated with a relatively low cytochrome *c* oxidase activity. Taken together, this might indicate a substantial change in the electron flow pathway and needs further, more detailed investigation.

Another interesting result of this study is a diversity of a single culturable halo-alkaliphilic SOB taxon found in the bioreactors at triple extreme conditions (high alkalinity, high total salt, high K^+). Although all of them belonged to a single genus, most of the isolates were genetically different from each other. The exact meaning of such difference is not completely clear, although some phenotypic difference, such as correlation with the pH in the reactor and with the substrate, used for isolation, can be seen in different subgroups. Apparently, the genus *Thioalkalivibrio*, despite being a relatively narrow specialized ecotype, possess extraordinary potential for microadaptation. On the other hand, the molecular analysis (DGGE) of the reactor populations indicated a presence of a single *Thioalkalivibrio* phylotype closely related to a facultatively alkaliphilic species *Tv.halophilus*. It is not easy to explain such a big difference between the results from two approaches. We can offer two. First, this could be a result of cultivation bias, since batch enrichment/isolation from the SL-BR reactors was performed at pH 10 and resulted in isolation of numerous strains of obligate alkaliphiles related to the core group of the genus *Thioalkalivibrio*. Perhaps, if lower pH values were used, isolates related to *Tv. halophilus* would be obtained at least with sulfide as substrate, similar to the results with FBR reactors. A second explanation might be that the DNA from the obligately alkaliphilic types of *Thioalkalivibrio*, for one or another reason, was less accessible for PCR – a usual bias of the DGGE method. Judging from the previous results of microbiological study of natural alkaline lakes, the major factor determining a domination of these two types of *Thioalkalivibrio* is pH in combination with high chloride content. For example, *Tv. halophilus* –like SOB dominated among the isolates from the halo-alkaline Wadi Natrun lakes in Egypt with NaCl as a dominant salt and pH around 9, while natrono (soda)philic *Thioalkalivibrio* species dominated in true soda lakes with pH around 10 where sodium carbonates were present at molar concentration [9]. The genus *Thioalkalivibrio* has a great potential to thrive at extremely halo-alkaline conditions and is a sure candidate for

application in biological sulfide removal directly from spent sulfide caustics. In the natural soda lake sediments often another genus of low salt-tolerant alkaliphilic SOB (*Thioalkalimicrobium*) can be found, which is characterized by extremely high rates of sulfide oxidation [8, 9]. The fact that it apparently was not present in the described bioreactors could probably be explained by high salt concentration which is above its capacity to grow.

7.5 REFERENCES

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Chapter 8

Summary and general discussion

8.1 INTRODUCTION

This thesis focuses on the further development of a biotechnological process for the conversion of hydrogen sulfide (H_2S) from hydrocarbon gas streams. The new process operates at natron- (high sodium) alkaline (high pH) conditions and is mainly developed to desulfurize high pressure natural gas, refinery gasses and synthesis gas (a mixture of carbon monoxide (CO) and hydrogen gas (H_2), produced by gasification of carbonaceous fuel such as coal and biomass). Prior to combustion of these gas streams, H_2S needs to be removed for environmental reasons and because it is malodorous, toxic and corrosive. For the treatment of natural gas in the medium-size niche (0.2 - 50 tons of sulfur per day), existing processes based on iron chelates, such as the Sulferox and the Lo-Cat® process, have important disadvantages: high operational costs due to chemicals consumption and the treatment of produced waste, and the occurrence of plugging problems, leading to high maintenance costs and downtime. Large-scale desulfurization processes such as the Claus process involve high investment costs, mainly because an expensive post treatment method is needed for more than 99% H_2S removal efficiencies **(Chapter 1)**.

The basic technology of the new process was developed at the Section Environmental Technology of Wageningen University in conjunction with the Department of Biotechnology of Delft University of Technology. Originally, the biological oxidation of sulfide was studied for the removal of sulfides from anaerobic effluents [1, 2]. Hereafter, Wageningen University and Paques B.V. developed the process further to remove H_2S from biogas that is produced in anaerobic wastewater treatment plants and landfills [3]. Together with Shell Global Solutions International B.V. the process was further developed for the desulfurization of natural gas, refinery gasses and synthesis gas [4, 5]. A schematic representation of this process is given by the left-hand part of Fig. 8.1.

H_2S-containing gas is contacted with an alkaline washing solution and H_2S is absorbed under the formation of (bi)sulfide (HS^- and S^{2-}, further referred to as "sulfide", Eq. 1). The sulfide-loaded alkaline solution is pumped to a bioreactor, where the sulfide is biologically oxidized to elemental sulfur ("biosulfur", S^0) by chemoautotrophic sulfur compound oxidizing bacteria (SOB, Eq. 2). The insoluble sulfur particles are removed from the water stream and can be re-used, for instance as a soil fertilizer **(Chapter 1)**. Biological oxidation of sulfide to biosulfur occurs at oxygen-limiting conditions, whereas sulfate (SO_4^{2-}) is formed at excess amounts of oxygen (Eq. 3).

$$H_2S + OH^- \rightarrow HS^- + H_2O \quad \text{(biological reaction)} \quad (1)$$

$$HS^- + ½\ O_2 \rightarrow S^0 + OH^- \quad \text{(biological reaction)} \quad (2)$$

$$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+ \quad \text{(biological reaction)} \quad (3)$$

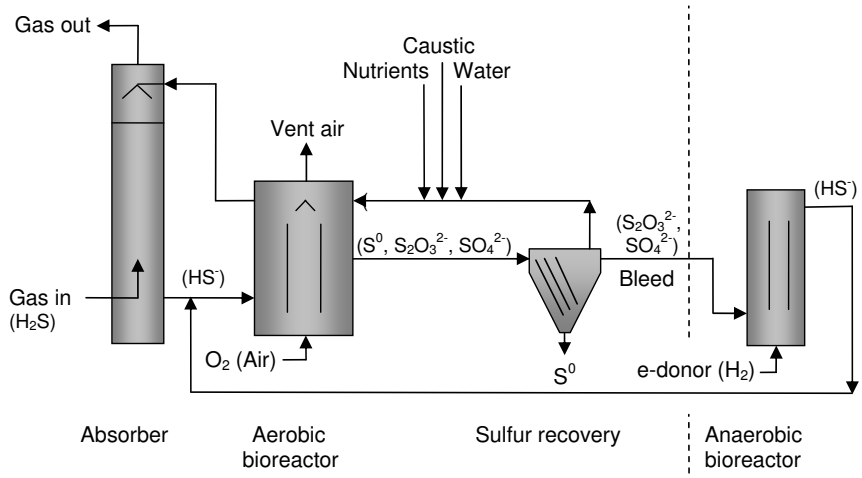

FIGURE 8.1 Biotechnological process for H_2S removal. The part on the right-hand side of the dashed line shows the (thio)sulfate reduction step that is part of the new process innovations.

At oxygen-limiting conditions thiosulfate ($S_2O_3^{2-}$) is formed, as a result of abiotic oxidation of reduced sulfur compounds. Major disadvantages of sulfate and thiosulfate formation are:

- Less re-usable elemental sulfur is formed.
- The formation of protons (H^+) leads to acidification of the alkaline solution. As a result, caustic is needed to control the pH.
- To prevent accumulation of sulfate and thiosulfate, these ions have to be removed by a bleed stream. With this stream, also (bi)carbonate ions are lost and make-up water and caustics are needed to compensate for this.

The current process operates at moderate alkaline pH (i.e. pH 8.2-8.5) and salt concentrations up to 0.5 M sodium (Na^+). However, operating at higher pH values and sodium concentrations would lead to a number of important advantages:

- Treatment of gasses with high CO_2 levels such as high pressure natural gas becomes feasible. To prevent acidification, Na^+ serves as a counter-ion for high concentrations of bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions.
- The sulfide loading capacity of the alkaline solution is considerably higher at elevated pH and high (bi)carbonate concentrations. This leads to lower energy costs for recirculation of the solution over the (high pressure) absorber column.
- The discharge costs for the bleed stream and the need for make-up water and caustics are reduced because the reactor solution contains higher concentrations of sulfate and thiosulfate ions.

If only sodium would be used as the counter-ion for (bi)carbonate, sulfate and thiosulfate, scaling problems may occur due to the formation of sodium bicarbonate ($NaHCO_3$) precipitates (max. solubility is 1.3 mol L^{-1} water at 30°C [6]). As the solubility of the

potassium (K^+) salt of bicarbonate is about 3 times higher compared to the solubility of the sodium salt, a mixture of sodium hydroxide (NaOH) and potassium hydroxide (KOH) is used to prevent acidification. The bleed stream that is required to discharge sulfate and thiosulfate can be prevented by directing the stream to a biological sulfate-reduction step (right-hand side of Fig. 8.1). In this second -anaerobic- bioreactor, sulfate reducing bacteria (SRB) convert sulfate and thiosulfate to sulfide, which can be redirected to the aerobic, sulfur-producing bioreactor. As a result, the water cycle of the process can be closed, leading to a drastic decrease in the consumption of water and caustic. For the formation of sulfide in the (thio)sulfate reducing step, an electron donor is needed, such as ethanol or hydrogen.
At the double extreme conditions in the process (high pH and high sodium and potassium concentrations), only natron-alkaliphilic bacteria can survive. In the past decade, a large number of such microorganisms has been isolated from soda-lakes around the world [7]. Within the current research there has been cooperation with the Department of Biotechnology of Delft University of Technology, where researchers collect, isolate and characterize SOB from soda-lakes. The joint project was titled: "Application of the biological sulfur cycle at halo-alkaliphilic conditions for high-pressure natural gas desulfurization" and was funded by the Dutch Technology Foundation STW, Shell Global Solutions International B.V. and Paques B.V. The research described in this thesis was aimed at the study of biological sulfide oxidation at natron-alkaline conditions. Particular emphasis was given to the formation of elemental sulfur and the side-products sulfate and thiosulfate.

8.2 BIOLOGICAL (POLY)SULFIDE OXIDATION

8.2.1 Substrate for biological oxidation

Most of the results described in this thesis were obtained with lab-scale bioreactor experiments. Oxygen and H_2S gas were simultaneously supplied to a reactor setup, which was inoculated with natron-alkaliphilic biomass obtained from soda lake sediments. It was already shown previously that at oxygen-limited conditions, biological sulfur formation prevails over the unwanted formation of (thio)sulfate [3]. Operating the bioreactor at oxygen-limited conditions results in the presence of reduced sulfur compounds such as sulfides, and thereby to highly reduced conditions (i.e. an oxidation/reduction -or "redox"- potential below -300 mV, measured against an Ag/AgCl reference electrode). The term "oxygen-limited conditions" is applied for the highly reduced conditions that lead to the conversion of H_2S to biosulfur.

At moderately alkaline conditions (pH 7.5-8.5), dissolved sulfide is the main substrate for biological oxidation processes. At elevated pH values however, sulfide reacts with biologically produced sulfur particles to form polysulfide anions (S_x^{2-}) [8]:

$$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+ \quad pK_x = 9.17 \quad \text{(non-biological reaction)} \qquad (4)$$

It was shown that at natron-alkaline conditions, sulfide, sulfur and polysulfide in the bioreactor are in equilibrium or very close to it, according to Eq. 4 **(Chapter 2 - 4).** As a result, a large fraction of the reduced sulfur species is present as polysulfide anions at high pH values, while at lower pH, this fraction decreases (e.g. 87% at pH 10 and 18% at pH 8.5). Banciu et al. (2004) showed that polysulfide oxidation by the halo-alkaliphilic SOB *Thioalkalivibrio versutus*, strain ALJ 15 occurs in two steps: first, a rapid oxidation of sulfane atoms ($-S^-$) takes place, followed by slower second step involving zerovalent sulfur oxidation [9]. They also found that oxidation of sulfide proceeds at a lower rate than the first step in polysulfide oxidation, indicating that at elevated pH, polysulfide is the preferred substrate. In **Chapter 7** it is however shown that oxidation rates at pH 10 of cells obtained from the gas-lift bioreactors are about 1.5 times higher for sulfide as compared to polysulfide. Cells obtained from stirred tank bioreactor experiments on the other hand show about two times higher oxidation rates in the presence of polysulfide compared to sulfide. In general, it can be concluded that at oxygen-limiting conditions, both sulfide and polysulfide are used as a substrate by natron-alkaliphilic biomass.

8.2.2 Influence of the total sulfide concentration on the conversion of H_2S to biosulfur

As biosulfur is the preferred end-product of the biological oxidation of H_2S, it is of paramount importance to assess which factors determine the selectivity for sulfur formation. In three different types of experiments it was shown that the sum of the sulfide and polysulfide concentration, defined as the total sulfide (S^{2-}_{tot}) concentration, is the main parameter that determines to what extend the supplied H_2S is converted to sulfur or sulfate.

First, in fed-batch bioreactor experiments, product formation was studied at fixed molar O_2/H_2S supply ratios, at pH values ranging from 9.6-10.2 **(Chapter 2)**. Without oxygen limitation (i.e. dissolved oxygen concentration > 10% sat.), the S^{2-}_{tot} concentration remains below the detection limit (<0.01 mM) and all supplied H_2S is completely oxidized to sulfate at an O_2/H_2S consumption ratio of 1.9 mol mol^{-1}. This ratio is somewhat lower than the theoretical ratio of 2.0 mol mol^{-1} that follows from the stoichiometry of Eq. 3, because part of the reducing equivalents is used to incorporate CO_2 into cell mass. When the O_2/H_2S supply ratio is fixed at a value below 1.9 mol mol^{-1}, the S^{2-}_{tot} concentration reaches stable values above the detection limit. It was found that with decreasing molar O_2/H_2S supply ratios, the S^{2-}_{tot} concentration stabilizes at a higher value. At oxygen-limiting conditions, a certain fraction of the H_2S supply rate is converted to sulfate ("selectivity for sulfate formation"). This fraction decreases with increasing S^{2-}_{tot} concentrations and approaches zero at S^{2-}_{tot} concentrations around 0.20-0.25 mM. The main end-products at these conditions are elemental sulfur and thiosulfate, while sulfate formation ceases. Due to thiosulfate formation, the maximum selectivity for sulfur formation at pH 10 is limited to 83%. The unwanted formation of thiosulfate is the result of abiotic oxidation processes, as will be further discussed in section 8.3.

In a second type of reactor experiment, the S^{2-}_{tot} concentration was controlled at a desired value by control of the oxygen supply rate **(Chapter 3 and 4)**. Results confirmed that the S^{2-}_{tot} concentration is the governing parameter to control sulfate formation in the pH range of 8.5-10.1. As with increasing pH values a larger fraction of the S^{2-}_{tot} concentration is present as polysulfides, it can be concluded that the speciation of total sulfide (i.e. sulfide or polysulfide ions) does not play an important role in the inhibition of sulfate formation.

The effect of the S^{2-}_{tot} concentration on product formation was also investigated in respiration experiments with washed cells taken from bioreactor experiments **(Chapter 5)**. Results show that at S^{2-}_{tot} concentrations of 0.25 mM and above, only sulfur is produced, at an O_2/S^{2-}_{tot} consumption ratio of 0.5 mol mol^{-1} as a result of biological activity. The response to the S^{2-}_{tot} concentration was similar to what was found in the bioreactor experiments, independently of whether sulfide or polysulfide was used as the substrate. In the respiration experiments, dissolved oxygen (DO) was present at saturation levels (i.e. 100% sat.), while in the bioreactor studies the DO concentration was below the detection limit of 0.1% sat. This indicates that the DO concentration does not directly affect the selectivity for biological product formation (i.e. sulfur or sulfate).

A mechanism for biological sulfur formation by *Thiobacillus* species was suggested by Visser et al. (1997) [10]. They proposed that sulfide is oxidized to sulfate via elemental sulfur as an intermediate. Under limited availability of oxygen, the degree of reduction of the cytochrome pool that is responsible for the delivery of electrons to oxygen increases, thereby blocking the conversion of sulfur to sulfate. Results presented in **Chapter 7** indeed show that cells obtained from the bioreactor at 0.34 mM S^{2-}_{tot} exhibit a low specific cytochrome *c* oxidase activity, while cells obtained at 0.14 mM S^{2-}_{tot} exhibit a higher specific cytochrome *c* oxidase activity. Besides the cytochrome *c* enzyme system, a different pathway, involving quinones as electron acceptors, may be involved in the biological oxidation of (poly)sulfides. This is shown by a high activity of the enzyme sulfide quinone reductase (SQR). However, its exact role in the oxidation pathway needs to be further investigated.

In full-scale gas-lift bioreactors, redox conditions may vary throughout the height of the reactor. Moreover, fluctuations in the redox conditions may also be caused by fluctuations in gas flows and/or gas H_2S concentrations. It is shown that it is not likely that these dynamics affect the selectivity for product formation **(Chapter 4)**. After a one-time interruption of the H_2S supply, it takes a period of about 25 to 40 minutes before the bacteria shift from sulfur to sulfate formation. Most probably this delay is caused by the presence of membrane-bound polysulfides [9] that need to be oxidized before the cytochrome pool is sufficiently oxidized to enable sulfate formation.

A remarkable result was obtained at relatively oxidized redox conditions (ORP>-200 mV) and high thiosulfate concentrations (>130 mM). At these conditions, all H_2S fed to the bioreactor is fully converted to biosulfur. In addition, thiosulfate is completely oxidized to sulfate. This implies that the incidental occurrence of oxidized redox conditions, for example after a process disturbance, does not directly lead to a loss in selectivity for sulfur formation.

8.3 THIOSULFATE FORMATION

While little if any sulfate is produced at S^{2-}_{tot} concentrations of 0.25 mM and above, thiosulfate formation always occurs whenever the reactor is operated at oxygen-limited conditions **(Chapter 2)**. Thiosulfate can be produced as result of abiotic oxidation of sulfide and polysulfide, or by disproportionation of biologically produced sulfur particles [11]. In bioreactor experiments, increased thiosulfate formation rates were observed whenever sulfide and polysulfide accumulated, for example as a result of a too low biological activity directly after start-up, as a result of oxygen-limiting conditions due to too low oxygen dosing rates **(Chapter 2)**, or as a result of inhibition of the biological oxidation capacity by toxic compounds such as methanethiol or (di)methyl polysulfides **(Chapter 6)**. As polysulfide is known to abiotically oxidize faster than sulfide [11, 12], it is not surprising that polysulfide plays an important role in the formation of thiosulfate.

Because it is the bottleneck for obtaining a high selectivity for biosulfur formation, thiosulfate formation was studied in more detail in **Chapter 3**. It was shown that at oxygen-limiting conditions, selectivity for thiosulfate formation is affected by pH, and is independent of the H_2S supply rate. A model was proposed to describe thiosulfate formation in the process, involving the equilibrium between sulfide and polysulfide (Eq. 4) and a hypothetical oxidation pathway involving membrane-bound polysulfide as an intermediate. Based on this model, the minimum fraction of H_2S that is converted to thiosulfate can be calculated according to the following equation:

$$\text{fraction } S_2O_3^{2-} = \frac{K_x}{x \cdot (10^{-pH} + K_x)} \qquad (5)$$

with x = average polysulfide chain length and K_x = equilibrium constant for the reaction between sulfide and polysulfide (pK_x = 9.17, Eq. 4). By assuming a value of x = 4.59, as determined by Kleinjan et al. [8] for polysulfide ions in equilibrium with excess biosulfur, this equation quite well fits experimental results. Maximum selectivity for sulfur formation is achieved in reactor experiments at pH 8.5. At this pH and a S^{2-}_{tot} concentration of 0.25 mM, 95±1 mol% of the H_2S load is converted to elemental sulfur and 5 mol% to thiosulfate. At pH values below pH 8.5, the biological activity decreases rapidly. As a result, not all H_2S is converted. When the S^{2-}_{tot} concentration increases above 0.25 mM (at pH 8.5-9), more thiosulfate is formed than calculated according to Eq. 5. Clearly not all reactions that lead to thiosulfate formation are covered in the mechanistic model that leads to this equation **(Chapter 4)**.

8.4 CONTROL OF OXYGEN DOSING BASED ON REDOX POTENTIAL

The importance of an accurate control of the S^{2-}_{tot} concentration becomes clear from the previous two sections. To reach optimal selectivity for sulfur formation, the S^{2-}_{tot} concentration should not decrease to values below 0.20-0.25 mM for a prolonged period, while at too high S^{2-}_{tot} concentrations, excess thiosulfate formation occurs and biological activity may be severely inhibited. At a constant pH, the S^{2-}_{tot} concentration in the bioreactor is logarithmically related to the redox potential (ORP) of the reactor liquid **(Chapter 2)**. This relation can be applied to control the S^{2-}_{tot} concentration at a set value, by adjustment of the oxygen supply on basis of a simple proportional feedback control strategy **(Chapter 2-4)**. However, the relation between the S^{2-}_{tot} concentration and ORP is affected by the pH. Therefore, a general applicable equation to calculate the S^{2-}_{tot} concentration should include the effect of pH. Such an equation can be obtained by combination of the Nernst equation for the reduction of polysulfide to sulfide with the pH defined equilibrium between polysulfide and sulfide ions (Eq. 4). Based on this principle, a theoretical relation has been derived that calculates the S^{2-}_{tot} concentration as a function of the variables ORP and pH **(Chapter 4)**.

8.5 THE EFFECT OF METHANETHIOL ON BIOLOGICAL SULFIDE OXIDATION

Sour gasses produced in the petrochemical industry often not only contain H_2S, but also volatile organic sulfur compounds (VOSCs). The most common VOSC found in natural gas is methanethiol (CH_3SH, or MT). Being a strong nucleophile, MT can react with sulfur particles in aqueous solution. In the PhD. thesis of Van Leerdam (2007), it was shown that the main end-products of the reaction of MT with excess amounts of biosulfur particles are polysulfide, sulfide and (di)methyl polysulfides [14]. The latter mainly consist of dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS).

The effect of MT, DMDS and DMTS on biological oxidation of sulfide and polysulfide ions was tested in respiration experiments **(Chapter 5)**. It was shown that the biological oxidation rate of sulfide to elemental sulfur is reduced by 50% (IC_{50}) at MT concentrations as low as 0.05 mM. DMDS and DMTS exhibit a less severe effect on sulfide oxidation as compared to MT, with estimated IC_{50} values of 1.5 and 1.0 mM, respectively. As polysulfide, DMDS and DMTS are the main end-products from the reaction between MT and biosulfur particles, this reaction effectively results in a partial detoxification of MT. This was confirmed by respiration experiments with a stabilized reaction mixture of MT and biosulfur, containing polysulfide, DMDS and DMTS. Biological oxidation rates with this mixture were in the same range as those of polysulfide, indicating that the presence of DMDS and DMTS did not significantly inhibit the biological oxidation of polysulfide.

The effect of MT on product formation was tested by addition of a sodium methylmercaptide ($NaCH_3S$) solution to a bioreactor that was continuously supplied with H_2S (**Chapter 6**). It was shown that the reaction between MT and biosulfur particles does not

proceed to completion i.e. not all MT reacts with biosulfur to form DMDS and DMTS. Instead, partial adsorption of MT to biosulfur particles was observed. This adsorption of MT to biosulfur particles is likely to have a MT-scavenging effect and may lead to an enhanced absorption of MT from the gas phase, as well as a reduced toxicity towards the sulfide oxidizing biomass. A remarkable result was that addition of MT prevents sulfate formation, even at fully oxidizing conditions (i.e. $[S^{2-}_{tot}]<<0.01$ mM; DO>10% sat.). Because (poly)sulfide concentrations are extremely low at these conditions, no thiosulfate formation occurs. As a result, all supplied H_2S is completely (≥ 99 mol%) converted into elemental sulfur. This is an outstanding result compared to the maximum selectivity for sulfur formation of 95 mol%, which can be achieved by control of the S^{2-}_{tot} concentration (0.20-0.25 mM) at a pH of 8.5 **(Chapter 3)**. Continuous treatment of 51.0 mM d^{-1} H_2S and 79 µM d^{-1} MT, with more than 99 mol% selectivity for sulfur formation, was feasible for a prolonged period (>20 days). The fact that MT, DMDS or DMTS did not accumulate in the bioreactor indicates that conversion of these compounds occurred. Deliberate addition of MT may be considered to obtain a high selectivity for sulfur formation. However, a drawback of the presence of dissolved MT, DMDS and DMTS is that they partly adsorb to the biosulfur, rendering it less suitable for reuse due to odor problems.

8.6 ANALYSIS OF THE MICROBIOLOGICAL POPULATION

At the double-extreme conditions, natron-alkaliphilic SOB are used for the conversion of (poly)sulfide. Analysis of the microbiological population in our gas-lift bioreactors was performed in cooperation with the Department of Biotechnology of Delft University of Technology **(Chapter 7)**. A comparison of the membrane proteins of cells harvested from bioreactors operated at pH 9.5 and 8.8 showed major similarities, suggesting presence of the same dominant population. This is not very surprising, as pH 8.8 and 9.5 are still within the same alkaline range. Within the alkaline pH range, an important factor that selects for a certain population is the dissolved salt content [15]. In this thesis, this is shown by domination of the genus *Thioalkalivibrio* (i.e. *Tv. jannnaschii*, *Tv. versutus* and *Tv. halophilus*) in reactors operated at Na^+/K^+ concentrations of 2-3 mol L^{-1}, while at 1 mol L^{-1} Na^+/K^+, a distant relative of the genus *Halothiobacillus* dominated. It can be concluded that the conditions that are applied in the new process select specifically for the genus *Thioalkalivibrio*, a group of obligately chemolithoautotrophilic and extremely halo-alkaliphilic SOB. The group of *Thioalkalivibrio* is very diverse, with high genetic and physiological heterogeneity. Within the larger framework of the project, researchers at Delft University of Technology studied *Thioalkalivibrio* strains from different locations with molecular techniques [16]. A method that was developed specifically for this purpose, on basis of Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the Internal Transcribed Spacer (ITS) region of ribosomal RNA, can be used to detect and quantify bacteria belonging to the genus *Thioalkalivibrio* in sediments and bioreactors. Unfortunately, this method was not suitable to

discriminate between individual *Thioalkalivibrio* strains in samples taken from the bioreactors described in the current thesis.

8.7 APPLICATION OF BIOLOGICAL H_2S REMOVAL AT NATRON-ALKALINE CONDITIONS

8.7.1 Specific and volumetric H_2S loading rates

The new process for biological H_2S removal at natron-alkaline conditions is primarily developed for sulfur recovery from natural gas. In this thesis, the maximum applied bioreactor H_2S loading rate was 21.7 mmol L^{-1} h^{-1}, with a sulfur formation rate of 17 mmol L^{-1} h^{-1} at a biomass concentration of 93 mg N L^{-1} and pH 10.1. This corresponds to a specific conversion rate of 0.19 mmol H_2S mg N^{-1} h^{-1}. As the bioreactor could convert this load without any problems, the maximum applicable H_2S loading rate is assumed to be substantially higher. In respiration experiments with reactor biomass, indeed a maximum specific sulfur formation rate of 0.30 mmol S mg N^{-1} h^{-1} was achieved, with both sulfide and polysulfide as substrate. Therefore, in a bioreactor containing for example 150 mg N L^{-1}, a volumetric H_2S conversion rate of 35 kg m^{-3} d^{-1} appears to be very well feasible. For the treatment of 10 tons of sulfur per day, a total bioreactor volume of only 300 m^3 would then be needed. Application of such high volumetric H_2S loading rates is however not only dependant on sufficient biological capacity, but also on sufficient oxygen transfer to achieve this capacity.

8.7.2 Biomass concentration

By minimization of the bleed flow, the overall hydraulic retention time (HRT) in the process can be very high (e.g. 20 days, based on 300 m^3 reactor volume and an average bleed flow of 15 m^{-3} d^{-1}). Therefore, wash-out of biomass does not seem to be an issue. Upon complete oxidation of H_2S to sulfate, we found a biomass growth yield of 0.86 g N per mol H_2S (**Chapter 2**). In contrast, the biomass growth yield on sulfur formation is considerably lower. Based on measured growth rates during simultaneous sulfur and sulfate formation, the growth yield on sulfur formation can be estimated at 0.015 g N per mol of sulfur produced. With this growth yield it can be calculated that biomass wash-out will not occur, even when no sulfate formation takes place. Based on an example calculation (H_2S load = 10 tons S d^{-1}, HRT = 20 days, 0 mol% sulfate-, 10% thiosulfate-, 90% sulfur formation), the steady state biomass concentration in the reactor will be more than 300 mg N L^{-1}. This is more than enough for the treatment of 10 tons of H_2S per day in a 300 m^3 reactor.

8.7.3 Application of a biological step for (thio)sulfate reduction

As mentioned in section 8.3, a certain fraction of the H_2S load will always be converted to thiosulfate. As a result, a bleed stream is required to remove thiosulfate (and -if produced- also sulfate) from the process. One of the most important advantages of an elevated Na^+/K^+ concentration is that the bleed flow can contain high concentrations of sulfate and thiosulfate

ions. As a result, a small bleed flow suffices, leading to a decreased consumption of water and caustic. The formation of a bleed stream can be prevented when thiosulfate and sulfate are biologically converted to sulfide in a subsequent reductive step (right-hand side of Fig. 8.1). As the conditions with respect to salt concentration and pH in the (thio)sulfate reducing bioreactor are similar to those of the aerobic bioreactor, the thiosulfate and sulfate reducing bacteria (SRB) should also be capable to grow at natron-alkaline conditions. Moreover, the SRB should thrive at elevated sulfide levels. For neutrophilic SRB, it is known that sulfate reduction is severely inhibited at sulfide concentrations of around 30 mM [17]. As the inhibitory effect of sulfide is presumed to be caused by undissociated H_2S [18], this concentration may be considerably higher at elevated pH values. To prevent inhibition of SRB, the recycle flow from the oxidizing to the reducing bioreactor can be adjusted in such a way that sulfide concentrations in the reduction step do not reach inhibiting levels.

Within the larger framework of the research, research was conducted at the Laboratory for Microbiology at Wageningen University on biological (thio)sulfate reduction at natron-alkaline conditions. One of the conclusions was that natron-alkaliphilic SRB reduce thiosulfate more rapidly than sulfate. Because the bleed-stream from the sulfide oxidation step contains mainly thiosulfate and not so much sulfate, this is beneficial for application of the reduction step.

Before the bleed-stream from the oxidizing reactor enters the reducing reactor, sulfur is removed in a sulfur recovery step (Fig. 8.1). If the removal of sulfur particles is not complete, colloidal sulfur particles will end up in the reducing bioreactor. This is undesirable, as the sulfur particles will (partly) be reduced to sulfide. This leads to an increased consumption of electron donor and a higher sulfide concentration.

8.7.4 pH and salt range

The feasibility of the process to is intimately related to the operational boundary conditions with respect to pH and Na^+/K^+ concentrations. Biological H_2S oxidation by the natron-alkaliphilic biomass applied in the process was proven to be feasible at a pH range of 8.5 - 10.1. At pH values below 8.5, biomass activity decreases significantly **(Chapter 3)**. Hence, H_2S conversion at high salt concentrations is not feasible at near neutral pH conditions. This is however not a problem, as for sufficient H_2S absorption the pH of the caustic absorber solution should at least be above pH 8 [19]. At pH values around 10.1-10.3, the biomass activity is still sufficiently high to convert a reasonable H_2S load of 51 mmol L^{-1} d^{-1}. However, significant biosulfur formation (>5 mol%) was never observed in the gas-lift bioreactor at pH values of 10.2 and above, even at severely oxygen-limited conditions. At these conditions, only sulfate and thiosulfate were detected as the products of H_2S oxidation (data not shown).

Almost all gas-lift bioreactor experiments presented in this thesis were performed at a total cation concentration of 2 mol L^{-1}, with 30% Na^+ and 70% K^+. As a result, maximum activity of bioreactor cells is also found at a Na^+ concentration of 2 mol L^{-1}, while at 4 mol L^{-1} Na^+,

still 30% of the maximum respiration rate is achieved **(Chapter 7)**. Based on previous studies of the genus *Thioalkalivibrio*, it is expected that H_2S oxidation to elemental sulfur is feasible for concentrations up to 4 mol L^{-1} Na^+/K^+ [20, 21].

As mentioned before, maximum selectivity for sulfur formation is achieved at a pH of 8.5. At this pH and 1.5 mol L^{-1} Na^+/K^+ as counter-ions for (bi)carbonate alkalinity, the equilibrium gas phase CO_2 partial pressure is around 35 kPa, which corresponds to 0.44 vol% at 80 bar. At higher CO_2 partial pressures and constant pH, the (bi)carbonate concentration will increase. Because then also more Na^+/K^+ is needed as a counter-ion for (bi)carbonate, this is at the expense of the maximum allowable (thio)sulfate concentration. At 4 mol L^{-1} Na^+/K^+ as counter-ions for (bi)carbonate, the equilibrium partial pressure of CO_2 in the gas phase is about 88 kPa, or 1.1 vol% at a pressure of 80 bar. Treatment of gasses with a higher partial CO_2 concentration (e.g. 2-3% CO_2 at 80 bar) may be feasible by the use of methods to selectively absorb H_2S and less CO_2, such as low gas residence times and high gas flow rates [19].

8.7.5 Alternative applications

Apart from the treatment of natural gasses, biological sulfide oxidation at natron-alkaline conditions may also be applied for the treatment of other sulfidic gas- or wastewater streams such as biogas, landfill gas and sour gasses as produced by the pulp and paper industry, breweries and food processing industry. For the treatment of once-through liquid streams that are discharged after desulfurization, formation of elemental sulfur may not be feasible as a result of the low biomass growth yield on sulfur formation. Without specific measures, biomass wash-out will occur. Only if sulfide in such streams is completely oxidized to sulfate, sufficient biomass growth may prevent biomass wash-out.

Biological sulfide oxidation at natron-alkaline conditions can also be applied as a process step in a recently developed process for the biological desulfurization of Liquefied Petroleum Gas (LPG) [22]. In this process, sulfide produced in an anaerobic MT degradation step is converted to elemental sulfur. The effect of MT described in the current thesis can be applied for further development of this combined process.

Another application in the petrochemical industry is H_2S removal from synthesis gas. Although the applicability of the process for synthesis gas has been established, further research is needed on the long-term effects of CO and trace impurities in synthesis gas on the process.

8.8 CONCLUDING REMARKS

Biotechnological conversion of sulfide can be applied at natron-alkaline conditions for the removal of H_2S from sour gas streams. Compared to conventional physicochemical technologies, application of the new process is expected to lead to substantial economic and energy savings for H_2S loading rates of up to at least 50 tons of sulfur per day [4]. The main

advantages over existing technologies are: (1) low energy consumption for heating and cooling; (2) low consumption of expensive chemicals; (3) simple process operation and (4) it is safe, as no free H_2S is present downstream of the absorber.

The process can be operated with a high stability and flexibility towards H_2S loading rates. However, full (100%) conversion of H_2S to elemental sulfur cannot be achieved by control of the oxygen (air) supply. Depending on the pH, a certain fraction will always be converted to thiosulfate. An additional anaerobic step is needed to convert the formed thiosulfate and sulfate ions to sulfide, thereby preventing the formation of a bleed stream.

Further research is required to investigate the role of mass transfer processes and chemical and biological processes in the dynamic behavior of full-scale reactors. Another area for further research is the properties of biosulfur particles, especially related to the formation of large sulfur aggregates and the application of biologically produced sulfur, e.g. in agriculture or in sulfur concrete.

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Chapter 8’

Samenvatting en algemene discussie

8.1 INTRODUCTIE

Dit proefschrift beschrijft de ontwikkeling van een biotechnologisch proces voor de omzetting van waterstofsulfide (H_2S) uit koolwaterstofgassen, bij natron- (hoge natrium concentratie) alkalifiele (hoge pH) condities. Dit nieuwe proces is voornamelijk geschikt voor de verwijdering van H_2S uit aardgas, raffinaderijgassen en synthesegas (een mengsel van koolstofmonoxide (CO) en waterstofgas (H_2), geproduceerd door middel van het vergassen van koolstofhoudende brandstoffen zoals steenkool en biomassa). Vóór verbranding moet H_2S uit deze gassen worden verwijderd vanwege de schadelijke gevolgen voor het milieu, omdat het stinkt en omdat het toxisch en corrosief is. Voor de behandeling van aardgas met een middelgrote zwavelvracht (0.2 tot 50 ton zwavel per dag), beschikken bestaande ontzwavelingsprocessen gebaseerd op de werking van ijzerchelaten, zoals het Sulferox en Lo-Cat® proces, over een aantal nadelen. De belangrijkste zijn de hoge operationele kosten vanwege chemicaliënverbruik en verwerking van geproduceerd afval, en het voorkomen van verstoppingsproblemen, wat kan leiden tot uitval van het proces. Grootschalige ontzwavelingsprocessen, zoals het Claus proces, gaan gepaard met hoge investeringskosten, met name omdat een kostbare nabehandelingstap is vereist voor het behalen van een H_2S verwijderingefficiëntie van meer dan 99% **(Hoofdstuk 1)**.

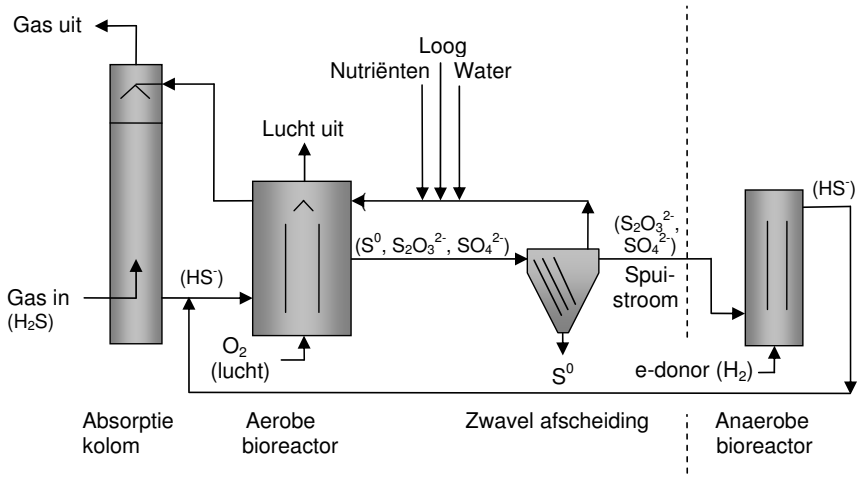
De basistechnologie van het nieuwe proces is ontwikkeld op de sectie Milieutechnologie van Wageningen Universiteit in samenwerking met de afdeling voor Biotechnologie van de Technische Universiteit Delft. Biologische sulfide oxidatie werd in eerste instantie bestudeerd voor de verwijdering van sulfiden uit anaeroob behandeld effluent [1, 2]. Wageningen Universiteit en Paques B.V. hebben dit proces vervolgens verder ontwikkeld voor de verwijdering van H_2S uit biogas, dat wordt geproduceerd bij de anaerobe behandeling van afvalwater en bij vuilstortplaatsen [3]. Samen met Shell Global Solutions International B.V. is het proces verder ontwikkeld voor de ontzwaveling van aardgas, raffinaderijgas en synthesegas [4, 5]. Een schematische weergave van het bestaande proces is weergegeven aan de linkerzijde van Fig. 8.1.

Met H_2S verontreinigd gas wordt in contact gebracht met een alkalische wasvloeistof waarin H_2S wordt geabsorbeerd onder de vorming van (bi)sulfide (HS^- en S^{2-}, hierna "sulfide" genoemd, Vgl. 1). De alkalische wasvloeistof met opgelost sulfide wordt vervolgens naar een bioreactor gepompt. Hier wordt het sulfide biologisch geoxideerd tot elementair zwavel ("biozwavel", S^0), door chemoautotrofe zwavel-oxiderende bacteriën (SOB, Vgl. 2). De onoplosbare zwaveldeeltjes worden afgescheiden van de waterstroom waarna hergebruik mogelijk is, bijvoorbeeld als meststof of als fungicide **(Hoofdstuk 1)**. Biologische oxidatie van sulfide naar biozwavel vindt plaats onder zuurstoflimiterende condities, terwijl sulfaat (SO_4^{2-}) wordt gevormd in aanwezigheid van een overmaat zuurstof (Vgl. 3).

$H_2S + OH^- \rightarrow HS^- + H_2O$ (biologische reactie) (1)

$HS^- + ½\ O_2 \rightarrow S^0 + OH^-$ (biologische reactie) (2)

$HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+$ (biologische reactie) (3)



FIGUUR 8.1 Biotechnologisch proces voor H_2S verwijdering. Het deel rechts van de streeplijn geeft de (thio)sulfaatreductie stap weer die deel uitmaakt van het nieuwe proces.

Onder zuurstoflimiterende condities wordt thiosulfaat ($S_2O_3^{2-}$) gevormd, als gevolg van abiotische oxidatie van gereduceerde zwavelverbindingen. De belangrijkste nadelen van sulfaat- en thiosulfaatvorming zijn:

- Er wordt minder herbruikbaar elementair zwavel gevormd;
- De vorming van protonen (H^+) leidt tot verzuring van de wasvloeistof. Hierdoor is dosering van loog benodigd voor pH controle;
- Om ophoping van sulfaat en thiosulfaat te voorkomen, moeten deze ionen worden verwijderd middels een spuistroom. Met deze spuistroom worden ook (bi)carbonaat ionen uit het proces verwijderd. Om hiervoor te compenseren moeten water en loog worden toegevoegd.

Het huidige proces vindt plaats bij matig alkalische pH condities (pH 8.2 - 8.5) en zoutconcentraties tot 0.5 M natrium (Na^+). Wanneer het proces bij hogere pH waarden en Na^+ concentraties kan worden bedreven, leidt dit tot een aantal belangrijke voordelen:

- Behandeling van gassen met een hoge CO_2 partiaalspanning, zoals bijvoorbeeld aardgas, wordt mogelijk. Om verzuring te voorkomen, dient Na^+ als tegen-ion voor hoge concentraties van bicarbonaat (HCO_3^-) en carbonaat (CO_3^{2-}) ionen.

- De sulfide belading van de alkalische wasvloeistof is aanmerkelijk hoger bij verhoogde pH en (bi)carbonaat concentraties. Dit leidt tot lagere energiekosten voor het recirculeren van de vloeistof over de (hoge druk) absorptiekolom.
- De lozingskosten voor de spuistroom en de consumptie van water en loog worden lager omdat de reactorvloeistof hogere concentraties aan sulfaat en thiosulfaat ionen kan bevatten.

Wanneer alleen natrium zou worden gebruikt als tegen-ion voor (bi)carbonaat, sulfaat en thiosulfaat ionen, leidt dit tot problemen ten gevolge van de vorming van natriumbicarbonaat ($NaHCO_3$) precipitaten (de maximale oplosbaarheid van $NaHCO_3$ is 1.3 mol per liter water bij 30°C [6]). Omdat de oplosbaarheid van het kalium (K^+) zout van bicarbonaat ongeveer drie maal hoger is dan van het natriumzout, wordt een mix van natriumhydroxide (NaOH) en kaliumhydroxide (KOH) gebruikt om verzuring tegen te gaan. De spuistroom waarmee sulfaat en thiosulfaat uit de zwavel vormende bioreactor wordt verwijderd, kan worden behandeld in een biologische sulfaatreductie stap (rechterzijde van Fig. 8.1). In deze anaerobe bioreactor zetten sulfaat reducerende bacteriën (SRB) sulfaat en thiosulfaat om naar sulfide, wat weer kan worden behandeld in de zwavel vormende bioreactor. Hierdoor wordt de waterkringloop van het proces gesloten, waardoor het gebruik van water en loog drastisch afneemt. Voor de vorming van sulfide in de (thio)sulfaatreductie stap is een elektronendonor nodig, zoals bijvoorbeeld ethanol of waterstofgas.

Onder de dubbel extreme condities die voor dit proces gelden (hoge pH en hoge natrium- en kalium concentraties), kunnen alleen natron-alkalifiele bacteriën overleven. In de afgelopen tien jaar is wereldwijd een groot aantal van deze micro-organismen geïsoleerd uit sodameren [7]. In het kader van het onderhavige onderzoek is samengewerkt met de afdeling Biotechnologie van de Technische Universiteit Delft, waar onderzoekers SOB uit sodameren verzamelen, isoleren en karakteriseren. Het gezamenlijke project was getiteld: "Toepassing van de biologische zwavelkringloop onder halo-alkalische condities voor de ontzwaveling van aardgas", en gefinancierd door de Technologiestichting STW, Shell Global Solutions International B.V. en Paques B.V. Het onderzoek dat in dit proefschrift staat beschreven, was gericht op de studie van biologische sulfide oxidatie onder natron-alkalifiele condities. Er is met name gekeken naar de vorming van elementair zwavel en de bijproducten sulfaat en thiosulfaat.

8.2 BIOLOGISCHE (POLY)SULFIDE OXIDATIE

8.2.1 Het substraat voor biologische oxidatie

Het merendeel van de resultaten beschreven in dit proefschrift is verkregen uit labschaal bioreactor experimenten. Bij deze experimenten werden zuurstof en H_2S gas toegevoegd aan reactoren die zijn aangeënt met natron-alkalifiele biomassa uit sodameer sedimenten. Het is bekend dat onder zuurstoflimiterende condities de vorming van elementair zwavel de overhand heeft over de ongewenste vorming van (thio)sulfaat [3]. Het bedrijven van de

bioreactor onder zulke condities resulteert in de aanwezigheid van gereduceerde zwavelcomponenten (sulfides), en leidt daarmee tot sterk gereduceerde condities (een oxidatie/reductie -of "redox"- potentiaal lager dan -300 mV, gemeten tegen een Ag/AgCl referentie elektrode). De term "zuurstoflimiterende condities" wordt gebruikt voor de sterk gereduceerde condities waarbij H_2S wordt omgezet naar biozwavel.

Bij matig hoge pH waarden (pH 7.5-8.5) is opgelost sulfide belangrijkste substraat voor biologische oxidatieprocessen. Bij hogere pH waarden reageert sulfide in de wasvloeistof echter met biologisch gevormde zwaveldeeltjes tot polysulfide anionen (S_x^{2-}) [8]:

$$HS^- + (x\text{-}1)\ S^0 \rightleftarrows S_x^{2-} + H^+ \quad pK_x = 9.17 \quad \text{(chemische reactie)} \qquad (4)$$

Onder natron-alkalische condities zijn sulfide, zwavel en polysulfide in de bioreactor met elkaar in evenwicht of hier zeer dichtbij, volgens Vgl. 4 **(Hoofdstuk 2-4)**. Als gevolg van deze reactie is bij een hoge pH een grotere fractie van de gereduceerde zwavelverbindingen aanwezig als polysulfide anionen dan bij een lagere pH (bijv. 87% bij pH 10 en 18% bij pH 8.5). Banciu et al. (2004) heeft aangetoond dat polysulfide oxidatie door de halo-alkalifiele SOB *Thioalkalivibrio versutus*, stam ALJ 15 plaatsvindt in twee stappen: eerst vindt een snelle oxidatie plaats van sulfaan atomen ($-S^-$), gevolgd door een tweede, langzamere stap waarbij ongeladen zwavel wordt geoxideerd [9]. Zij vonden tevens dat sulfide oxidatie langzamer gaat dan de eerste stap van polysulfide oxidatie, wat impliceert dat bij een verhoogde pH polysulfide oxidatie de voorkeur heeft boven sulfide oxidatie. In **Hoofdstuk 7** blijkt echter dat oxidatiesnelheden bij pH 10 met bacteriecellen uit de gaslift bioreactoren ongeveer 1.5 keer zo hoog zijn voor sulfide dan voor polysulfide. Cellen die zijn verkregen uit geroerde tank reactor experimenten hadden echter een ongeveer tweemaal zo hoge oxidatiesnelheid met polysulfide dan met sulfide. Over het algemeen kan worden geconcludeerd dat onder zuurstoflimiterende condities zowel sulfide als polysulfide ionen kunnen worden gebruikt als substraat door natron-alkalifiele biomassa.

8.2.2 Invloed van de totale sulfide concentratie op de omzetting van H_2S naar biozwavel

Omdat "biozwavel" het gewenste eindproduct is van de biologische oxidatie van H_2S, is het van cruciaal belang te bepalen welke factoren de selectiviteit voor zwavelvorming bepalen. In drie verschillende soorten experimenten is aangetoond dat de som van de sulfide- en polysulfide concentratie, die is gedefinieerd als de "totaal sulfide concentratie" (S^{2-}_{tot}), de meest belangrijke parameter is die bepaalt in welke mate H_2S biologisch wordt omgezet naar zwavel of naar sulfaat.

In eerste instantie is in "fed-batch" reactor experimenten productvorming bestudeerd bij vaste O_2/H_2S doseringsverhoudingen en pH waarden van 9.6 tot 10.2 **(Hoofdstuk 2)**. Zonder zuurstoflimitatie (opgeloste zuurstofconcentratie >10% verzadiging) blijft de S^{2-}_{tot} concentratie beneden de detectielimiet (<0.01 mM) en wordt alle H_2S volledig geoxideerd

naar sulfaat bij een O_2/H_2S consumptie verhouding van 1.9 mol mol^{-1}. Deze verhouding is enigszins lager dan de theoretische verhouding van 2.0 mol mol^{-1}, welke volgt uit de stoichiometrie van Vgl. 3. De oorzaak hiervan is dat een deel van de reductie equivalenten wordt gebruikt voor de assimilatie van CO_2 in nieuwe biomassa. Als de O_2/H_2S doseringsverhouding wordt verlaagd naar waarden lager dan 1.9 mol mol^{-1}, dan bereikt de S^{2-}_{tot} concentratie stabiele waarden die boven de detectielimiet liggen. Hoe lager de O_2/H_2S doseringsverhouding, des te hoger is de waarde waarop de S^{2-}_{tot} concentratie stabiliseert. Onder zuurstoflimiterende condities wordt een bepaalde fractie van de H_2S belasting omgezet naar sulfaat ("selectiviteit voor sulfaatvorming"). Deze fractie neemt af bij toenemende S^{2-}_{tot} concentraties, tot een S^{2-}_{tot} concentratie van 0.20-0.25 mM, waarbij nauwelijks of geen sulfaatvorming meer plaatsvindt. De eindproducten bij deze condities zijn biozwavel en thiosulfaat. Door de vorming van thiosulfaat is de maximale selectiviteit voor zwavelvorming bij pH 10 gelimiteerd tot ca. 83%. De ongewenste vorming van thiosulfaat is het gevolg van abiotische oxidatie processen. In sectie 8.3 wordt hier verder op ingegaan.

In een tweede type reactor experiment werd de S^{2-}_{tot} concentratie gecontroleerd door sturing van de zuurstofdosering (**Hoofdstuk 3 en 4**). De resultaten bevestigen dat de S^{2-}_{tot} concentratie de belangrijkste regelvariabele is voor sulfaatvorming binnen de experimentele pH bereik van 8.5 tot 10.1. Omdat bij een toenemende pH waarde een grotere fractie van de S^{2-}_{tot} concentratie aanwezig is als polysulfides, kan worden geconcludeerd dat de speciatie van het totaal sulfide over sulfide en polysulfide ionen geen belangrijke rol speelt bij de remming van sulfaatvorming.

Het effect van de S^{2-}_{tot} concentratie op productvorming is ook onderzocht in respiratie experimenten met cellen afkomstig van bioreactor experimenten (**Hoofdstuk 5**). Uit de resultaten blijkt dat vanaf een S^{2-}_{tot} concentratie van 0.25 mM alleen zwavel wordt geproduceerd, bij een O_2/S^{2-}_{tot} consumptie verhouding van 0.5 mol mol^{-1} als gevolg van biologische activiteit. De gevonden relatie tussen de S^{2-}_{tot} concentratie en productvorming is vergelijkbaar met de relatie die is gevonden bij de bioreactor experimenten, onafhankelijk van het gebruik van polysulfide of sulfide als substraat. De respiratie experimenten zijn uitgevoerd bij een verzadigde opgeloste zuurstof (DO) concentratie (100%). Bij de bioreactor experimenten was de DO concentratie echter beneden de detectielimiet van 0.1%. Dit impliceert dat de DO concentratie geen directe invloed heeft op de selectiviteit voor productvorming als gevolg van biologische omzettingen (zwavel- of sulfaatvorming).

Visser et al. (1997) [10] geeft een mechanisme voor de biologische vorming van zwavel door *Thiobacillus* soorten. Zij stellen dat sulfide naar sulfaat wordt geoxideerd via elementair zwavel als intermediair. Onder zuurstoflimiterende condities neemt de mate van reductie toe van de cytochroom enzymsystemen die verantwoordelijk zijn voor de overdracht van elektronen naar zuurstof. Hiermee wordt de oxidatie van zwavel naar sulfaat geblokkeerd. Resultaten in **Hoofdstuk 7** laten zien dat cellen uit de bioreactor inderdaad een lage specifieke cytochroom *c* oxidase activiteit hebben bij 0.34 mM S^{2-}_{tot}, terwijl bij 0.14 mM S^{2-}_{tot} een hogere activiteit wordt gemeten. Naast het cytochroom *c* enzymsysteem speelt

mogelijk ook een andere oxidatieroute, waarbij quinonen betrokken zijn, een rol bij de biologische oxidatie van (poly)sulfide in de bioreactoren. Dit blijkt uit een hoge activiteit van het enzym sulfide quinon reductase (SQR). Voor het bepalen van de exacte rol van SQR in de oxidatieroute is verder onderzoek noodzakelijk.

In “full-scale” gaslift bioreactoren kunnen redoxcondities variëren over de hoogte van de reactor. Daarnaast kunnen fluctuaties in redoxcondities ook worden veroorzaakt door veranderingen in het gasdebiet en/of de H_2S concentratie. Het is echter niet waarschijnlijk dat deze fluctuaties de selectiviteit voor productvorming beïnvloeden. Na een eenmalige onderbreking van de H_2S dosering duurt het ongeveer 25 tot 40 minuten voordat de bacteriën overgaan van zwavel- naar sulfaatvorming **(Hoofdstuk 4)**. Deze vertraagde reactie wordt waarschijnlijk veroorzaakt door de aanwezigheid van membraangebonden polysulfides [9] die geoxideerd worden alvorens het cytochroom systeem voldoende is geoxideerd om sulfaatvorming mogelijk te maken.

Een opmerkelijk resultaat is verkregen bij relatief geoxideerde redox condities (ORP> -200 mV) en hoge thiosulfaat concentraties (>130 mM). Onder deze condities wordt alle H_2S omgezet naar biozwavel, terwijl thiosulfaat wordt geoxideerd naar sulfaat. Als gevolg hiervan leidt een kortstondig voorkomen van geoxideerde condities, bijvoorbeeld na een procesverstoring, waarschijnlijk niet direct tot een afname van de selectiviteit voor zwavelvorming.

8.3 VORMING VAN THIOSULFAAT

Terwijl er nauwelijks of geen sulfaatvorming optreedt bij S^{2-}_{tot} concentraties hoger dan 0.25 mM, vindt er altijd thiosulfaatvorming plaats wanneer de reactor wordt bedreven onder zuurstoflimiterende condities **(Hoofdstuk 2)**. Thiosulfaat kan worden gevormd als gevolg van abiotische oxidatie van sulfide en polysulfide of door disproportionering van biologisch gevormde zwaveldeeltjes [11]. Wanneer sulfide en polysulfide accumuleren in de bioreactor, gaat dit gepaard met een toename van de vorming van thiosulfaat. Ophoping van (poly)sulfide vindt onder andere plaats als gevolg van een te lage biologische activiteit direct na opstart van de reactor, als gevolg van zuurstoflimiterende condities **(Hoofdstuk 2)**, of door remming van de biologische oxidatie capaciteit door toxische componenten zoals methaanthiol of (di)methyl polysulfides **(Hoofdstuk 6)**. Omdat polysulfide sneller oxideert dan sulfide [11, 12] ligt het voor de hand dat polysulfide een belangrijke rol speelt in de vorming van thiosulfaat.

Omdat thiosulfaatvorming een beletsel vormt voor het behalen van een hoge selectiviteit voor zwavelvorming, is het mechanisme hiervan in meer detail bestudeerd in **Hoofdstuk 3**. Het blijkt dat onder zuurstoflimiterende condities de selectiviteit voor thiosulfaatvorming wordt beïnvloedt door de pH en onafhankelijk is van de H_2S belasting. Op basis van het evenwicht tussen sulfide en polysulfide (Vgl. 4) en een hypothetische oxidatie route waarin membraangebonden polysulfide een rol speelt als intermediair, is een model opgesteld voor

thiosulfaatvorming in het proces. Op basis van dit model kan de minimale fractie van H_2S die wordt omgezet naar thiosulfaat worden berekend met de volgende vergelijking:

$$\text{fractie}\, S_2O_3^{2-} = \frac{K_x}{x \cdot (10^{-pH} + K_x)} \qquad (5)$$

waarin x = gemiddelde polysulfide ketenlengte en K_x = evenwichtsconstante voor de reactie tussen sulfide en polysulfide (pK_x = 9.17, Vgl. 4). Indien een waarde van x = 4.59 wordt aangenomen, zoals beschreven door Kleinjan et al. [8] voor polysulfide ionen in evenwicht met een overmaat biozwavel, dan komt deze vergelijking goed overeen met de experimentele resultaten. De maximale selectiviteit voor zwavelvorming is behaald in reactor experimenten bij een pH van 8.5. Bij deze pH en een S^{2-}_{tot} concentratie van 0.25 mM, wordt 95±1 mol% van de H_2S belasting omgezet naar zwavel en 5 mol% naar thiosulfaat. Bij pH waarden lager dan 8.5 neemt de biologische activiteit sterk af, waardoor niet alle H_2S wordt omgezet. Indien de S^{2-}_{tot} concentratie hoger wordt dan 0.25 mM (bij pH 8.5-9), dan wordt er meer thiosulfaat gevormd dan berekend volgens Vgl. 5. Hieruit volgt dat niet alle reacties die mogelijkerwijs leiden tot thiosulfaatvorming deel uitmaken van het mechanistische model dat de basis vormt voor deze vergelijking **(Hoofdstuk 4)**.

8.4 CONTROLE VAN DE ZUURSTOFDOSERING OP BASIS VAN REDOX POTENTIAAL

Uit de voorgaande secties volgt het belang van een effectieve controle van de S^{2-}_{tot} concentratie: om een optimale selectiviteit voor zwavelvorming te bereiken, mag de S^{2-}_{tot} concentratie niet langdurig zakken tot waarden beneden 0.20-0.25 mM, terwijl een te hoge S^{2-}_{tot} concentratie leidt tot een overmaat aan thiosulfaatvorming en mogelijke remming van de biologische activiteit. Bij een constante pH is de S^{2-}_{tot} concentratie logaritmisch gerelateerd aan de redox potentiaal (ORP), gemeten in de reactorvloeistof **(Hoofdstuk 2)**. Deze relatie kan worden gebruikt voor controle van de S^{2-}_{tot} concentratie op een gewenste waarde, door aanpassing van de zuurstofdosering op basis van een simpele proportionele feedback controlestrategie **(Hoofdstuk 2-4)**. De relatie tussen de S^{2-}_{tot} concentratie en ORP wordt beïnvloedt door de pH. Hierdoor moet de pH deel uitmaken van een algemeen toepasbare vergelijking om de S^{2-}_{tot} concentratie te berekenen. Een dergelijke vergelijking kan worden verkregen door combinatie van de Nernst vergelijking voor de reductie van polysulfide naar sulfide, met het door de pH bepaalde evenwicht tussen polysulfide en sulfide ionen (Vgl. 4). Op basis hiervan is een theoretische relatie afgeleid die de S^{2-}_{tot} concentratie berekent als functie van de variabelen ORP en pH **(Hoofdstuk 4)**.

8.5 HET EFFECT VAN METHAANTHIOL OP BIOLOGISCHE SULFIDE OXIDATIE

Zure gassen geproduceerd in de petrochemische industrie bevatten vaak niet alleen H_2S, maar ook vluchtige organische zwavelverbindingen (VOSCs). De meest voorkomende VOSC in aardgas is methaanthiol (CH_3SH, of MT). Omdat MT een sterk nucleofiel is, kan het reageren met gesuspendeerde zwaveldeeltjes. In het proefschrift van Van Leerdam (2007) [14] is aangetoond dat polysulfide, sulfide en (di)methyl polysulfides de belangrijkste eindproducten zijn van de reactie van MT met een overmaat aan biozwavel. De (di)methyl polysulfides bestaan voornamelijk uit dimethyl disulfide (DMDS) en dimethyl trisulfide (DMTS).

Het effect van MT, DMDS en DMTS op de biologische oxidatie van sulfide en polysulfide ionen is getest met behulp van respiratie experimenten **(Hoofdstuk 5)**. Hierbij is aangetoond dat reeds bij een MT concentratie van 0.05 mM de biologische oxidatie snelheid van sulfide naar elementair zwavel met 50% wordt geremd (IC_{50}). In vergelijking met MT hebben DMDS en DMTS een minder groot effect op de sulfide oxidatiesnelheid, met IC_{50} waarden van respectievelijk 1.5 en 1.0 mM. Omdat polysulfide, DMDS en DMTS de voornaamste eindproducten zijn van de reactie tussen MT en biozwavel, resulteert deze reactie in een gedeeltelijke detoxificatie van MT. Dit is bevestigd met respiratie experimenten uitgevoerd met een gestabiliseerd reactiemengsel van MT en biozwavel. Biologische oxidatiesnelheden met dit mengsel waren vergelijkbaar met die van polysulfide, wat impliceert dat de aanwezigheid van DMDS en DMTS de biologische oxidatie van polysulfide niet remt.

Het effect van MT op productvorming is ook bestudeerd in een bioreactor, door toevoeging van natriummethylmercaptide ($NaCH_3S$) samen met een continue dosering van H_2S **(Hoofdstuk 6)**. Hierbij is aangetoond dat de reactie tussen MT en biozwavel in de reactor niet volledig is. In tegenstelling tot een volledige reactie van MT tot DMDS en DMTS, adsorbeert een deel van het toegevoegde MT aan de zwaveldeeltjes. Dit leidt tot het wegvangen van MT uit de vloeistof. Mogelijk leidt dit tot een verhoogde absorptie van MT uit de gasfase en een vermindering van de toxiciteit van MT voor de sulfide oxiderende biomassa. Een opmerkelijk effect van toevoeging van MT aan de reactor is de inhibitie van sulfaatvorming, zelfs bij volledig oxiderende condities ($[S^{2-}_{tot}] << 0.01$ mM; DO>10%). Omdat onder deze condities de (poly)sulfide concentratie extreem laag is, vindt er geen thiosulfaatvorming plaats. Als gevolg hiervan wordt alle gedoseerde H_2S volledig ($\geq$ 99 mol%) omgezet naar elementair zwavel. Dit is een zeer opmerkelijk resultaat in vergelijking met de maximale selectiviteit van 95% die wordt behaald door middel van controle van de S^{2-}_{tot} concentratie (20-0.25 mM) bij een pH van 8.5 **(Hoofdstuk 3)**. Continue behandeling van 51.0 mM d^{-1} H_2S en 79 µM d^{-1} MT, met meer dan 99 mol% selectiviteit voor zwavelvorming is aangetoond gedurende een periode van meer dan 20 dagen. Het feit dat geen accumulatie van MT, DMDS en DMTS plaatsvond, duidt erop dat deze componenten in de bioreactor worden omgezet. Om een hoge selectiviteit voor zwavelvorming te bereiken, zou toevoeging van MT aan de bioreactor overwogen

kunnen worden. Een nadeel van de aanwezigheid van opgelost MT, DMDS en DMTS is echter dat deze deels aan het biozwavel adsorberen, waardoor dit minder geschikt is voor hergebruik vanwege stankoverlast.

8.6 ANALYSE VAN DE MICROBIOLOGISCHE POPULATIE

Vanwege de toegepaste dubbel-extreme condities, worden natron-alkalifiele SOB gebruikt voor de omzetting van (poly)sulfide. Microbiologische analyse van de bacteriepopulatie in de gaslift reactoren is uitgevoerd in samenwerking met de afdeling Biotechnologie van de Technische Universiteit Delft **(Hoofdstuk 7)**. De membraaneiwitten van cellen gegroeid bij pH 8.8 en 9.5 vertoonden een grote overeenkomst, wat impliceert dat bij beide pH waarden dezelfde dominante populatie aanwezig is. Dit is geen verrassing, aangezien beide pH waarden binnen het alkalische gebied liggen. Binnen dit alkalische gebied is de zoutconcentratie een belangrijke selectiefactor voor de groei van een bepaalde populatie [15]. Dit blijkt uit de dominantie van het genus *Thioalkalivibrio* (*Tv. jannnaschii*, *Tv. versutus* en *Tv. halophilus*) in reactoren die zijn bedreven bij Na^+/K^+ concentraties van 2-3 mol L^{-1}, terwijl bij 1 mol L^{-1} Na^+/K^+, een verre afstammeling van het genus *Halothiobacillus* domineerde. De condities die worden toegepast in het nieuwe proces selecteren dus specifiek voor het genus *Thioalkalivibrio*, een groep van obligaat chemolithoautotrofe en extreem halo-alkalifiele SOB. De groep van *Thioalkalivibrio* is zeer divers en wordt gekenmerkt door een hoge genetische en fysiologische heterogeniteit. Binnen het bredere kader van het onderzoeksproject hebben onderzoekers van de Technische Universiteit Delft *Thioalkalivibrio* stammen, afkomstig van verschillende locaties, onderzocht met behulp van moleculaire technieken [16]. Eén van de methodes die hiervoor speciaal is ontwikkeld op basis van "Denaturing Gradient Gel Electrophoresis" (DGGE) analyse van het "Internal Transcribed Spacer" (ITS) gebied van het ribosomaal RNA, kan worden gebruikt om bacteriën die behoren tot het genus *Thioalkalivibrio* te detecteren en te kwantificeren in sedimenten en bioreactoren. Helaas was het met deze methode niet mogelijk om onderscheid te maken tussen onderlinge *Thioalkalivibrio* stammen uit de bioreactoren beschreven in dit proefschrift.

8.7 TOEPASSING VAN BIOLOGISCHE H_2S VERWIJDERING ONDER NATRON-ALKALISCHE CONDITIES

8.7.1 Specifieke en volumetrische H_2S belasting

Het nieuwe proces voor biologische H_2S verwijdering onder natron-alkalische condities is voornamelijk ontwikkeld voor het ontzwavelen van aardgas. De hoogste H_2S belasting die in dit onderzoek is toegepast, bedraagt 21.7 mmol L^{-1} uur^{-1}. Hiervan werd 17 mmol L^{-1} uur^{-1} in zwavel werd omgezet bij een pH van 10.1. De biomassa concentratie bedroeg 93 mg N L^{-1}, wat overeenkomt met een specifieke omzettingssnelheid van 0.19 mmol H_2S mg N^{-1} uur^{-1}. Omdat bij deze belasting alle sulfide probleemloos omgezet kan worden, is het aannemelijk

dat de maximaal toepasbare H_2S belasting aanmerkelijk hoger is. In respiratie experimenten met biomassa uit de reactor is inderdaad een hogere specifieke zwavelvormingssnelheid gemeten van 0.30 mmol S mg N^{-1} uur^{-1}, met zowel sulfide als polysulfide als substraat. Hieruit volgt dat bij een biomassa concentratie van bijvoorbeeld 150 mg N L^{-1}, een volumetrische H_2S belasting van 35 kg m^{-3} d^{-1} zonder meer haalbaar moet zijn. Voor de behandeling van 10 ton H_2S per dag zou dan een totaal bioreactor volume van slechts 300 m^3 benodigd zijn. Toepassing van een dergelijk hoge volumetrische H_2S belasting is echter niet alleen afhankelijk van voldoende biologische capaciteit, maar tevens van voldoende zuurstofoverdracht om deze capaciteit te behalen.

8.7.2 Biomassa concentratie

Doordat het debiet van de spuistroom zo laag mogelijk wordt gehouden, is de hydraulische verblijftijd (HRT) in het proces erg hoog (bijv. 20 dagen, gebaseerd op een reactor volume van 300 m^3 en een spuistroom van 15 m^3 d^{-1}). Bij een dergelijk hoge verblijftijd lijkt uitspoeling van biomassa geen belangrijke rol te spelen. Bij volledige oxidatie van H_2S naar sulfaat is een biomassa groeiopbrengst van 0.86 g N per mol H_2S gevonden **(Hoofdstuk 2)**. Onder zwavelvormende condities is deze opbrengst echter een stuk lager. Aan de hand van gemeten groeisnelheden bij gelijktijdige zwavel- en sulfaatvorming, kan de groeiopbrengst op enkel zwavelvorming worden geschat op 0.015 g N per mol geproduceerd zwavel. Met dit getal kan worden berekend dat geen uitspoeling van biomassa zal plaatsvinden, zelfs niet wanneer er helemaal geen sulfaatvorming plaatsvindt. Gebaseerd op een H_2S belasting van 10 ton S d^{-1}, een HRT van 20 dagen, 0 mol% sulfaat-, 10% thiosulfaat- en 90% zwavelvorming, kan worden berekend dat de biomassaconcentratie in de reactor hoger zal zijn dan 300 mg N L^{-1}. Dit is meer dan voldoende voor de behandeling van 10 ton H_2S per dag in een reactor van 300 m^3.

8.7.3 Toepassing van een biologische stap voor de reductie van (thio)sulfaat

Zoals vermeld in sectie 8.3, zal altijd een bepaalde fractie van de H_2S belasting worden omgezet naar thiosulfaat. Als gevolg hiervan is een spuistroom benodigd om thiosulfaat (en mogelijk gevormd sulfaat) uit het proces te verwijderen. Eén van de belangrijkste voordelen van het toepassen van een hoge Na^+/K^+ concentratie is dat de reactorvloeistof hoge concentraties aan sulfaat en thiosulfaat ionen kan bevatten. Hierdoor kan worden volstaan met een kleine spuistroom, wat resulteert in een laag verbruik van water en loog. De vorming van een spuistroom kan echter grotendeels worden voorkomen wanneer thiosulfaat en sulfaat biologisch worden omgezet naar sulfide in een reductiestap (zie rechterzijde van Fig. 8.1). Omdat de zoutconcentratie en pH waarde in deze (thio)sulfaat reducerende bioreactor hetzelfde zijn als die in de aerobe reactor, moeten de (thio)sulfaat reducerende bacteriën (SRB) in deze stap ook kunnen groeien onder natron-alkalische condities. Daarnaast moeten deze bacteriën ook bestand zijn tegen hoge sulfide concentraties. Van neutrofiele SRB is het bekend dat sulfaatreductie ernstig wordt geremd bij een sulfide concentratie van ongeveer 30

mM [17]. Omdat het remmende effect van sulfide waarschijnlijk wordt veroorzaakt door ongedissocieerde H_2S, valt te verwachten dat deze concentratie een stuk hoger kan zijn bij hogere pH waarden [18]. Om remming van SRB te voorkomen, kan het recycle debiet van de sulfide oxiderende naar de (thiosulfaat) reducerende bioreactor zo worden aangepast, dat de sulfide concentratie in de reducerende stap beneden de waarde blijft waarbij remming optreedt.

Binnen het bredere kader van het onderzoeksproject is bij het Laboratorium voor Microbiologie van Wageningen Universiteit onderzoek verricht naar biologische (thio)sulfaat reductie onder natron-alkalische condities. Eén van de conclusies was dat natron-alkalifiele SRB sneller thiosulfaat reduceren dan sulfaat. Omdat de spuistroom uit de sulfide oxidatie stap voornamelijk thiosulfaat en weinig sulfaat bevat, komt dit de toepassing van de reductiestap ten goede.

Voordat de spuistroom van de oxiderende reactor in de reducerende reactor terechtkomt, wordt eerst het zwavel uit de waterstroom verwijderd in een zwavelscheiding stap (Fig. 8.1). Indien de verwijdering van vaste zwaveldeeltjes niet volledig is, komen colloïdale zwaveldeeltjes terecht in de reducerende reactor. Dit is ongewenst, omdat de zwaveldeeltjes (deels) zullen worden omgezet naar sulfide, wat leidt tot een verhoogde consumptie van elektronendonor en een hogere sulfideconcentratie.

8.7.4 pH en zoutconcentratie

De toepasbaarheid van het proces is nauw verbonden met de pH en zoutconcentratie waarbij het proces nog kan worden bedreven. Dit proefschrift laat zien dat biologische oxidatie van H_2S door natron-alkalifiele biomassa kan worden toegepast binnen een pH bereik van 8.5 tot 10.1. Bij een pH lager dan 8.5 vindt een sterke afname plaats van de biomassa activiteit **(Hoofdstuk 3)**. Hierdoor is H_2S oxidatie bij een hoge zoutconcentratie en neutrale pH waarden niet mogelijk. Dit is echter geen probleem, omdat voor toereikende H_2S absorptie de pH van de wasvloeistof ten minste hoger dan pH 8 moet zijn [19]. Bij pH waarden van 10.1 tot 10.3 is de biologische activiteit nog hoog genoeg om een H_2S belasting van 51 mmol L^{-1} d^{-1} om te zetten. Bij een pH van 10.2 en hoger kan echter geen hoge selectiviteit voor zwavelvorming (>5 mol%) bereikt worden, zelfs niet onder zuurstoflimiterende condities (S^{2-}_{tot} concentraties van 0.1 tot 0.8 mM). Onder deze omstandigheden worden alleen sulfaat en thiosulfaat gevonden als eindproducten van H_2S oxidatie (data niet getoond).

Vrijwel alle bioreactor experimenten beschreven in dit proefschrift zijn uitgevoerd bij een totale kation concentratie van 2 mol L^{-1}, met 30% Na^+ en 70% K^+. Als gevolg hiervan wordt de maximale biologische activiteit ook gevonden bij een Na^+ concentratie van 2 mol L^{-1}, terwijl bij 4 mol L^{-1} Na^+ nog 30% van de maximale oxidatiesnelheid wordt gevonden **(Hoofdstuk 7)**. Gebaseerd op eerdere studies van het genus *Thioalkalivibrio* kan worden aangenomen dat de oxidatie van H_2S naar elementair zwavel mogelijk is bij zoutconcentraties tot 4 mol L^{-1} Na^+/K^+ [20, 21].

Zoals eerder vermeld, vindt maximale selectiviteit voor zwavelvorming plaats bij een pH van 8.5. Bij deze pH en 1.5 mol L^{-1} Na^+/K^+ als tegenion voor (bi)carbonaat alkaliniteit, is de evenwichts-partiaalspanning van CO_2 in de gasfase ongeveer 35 kPa (bij 35°C), wat bij een druk van 80 bar overeenkomt met 0.44 vol%. Bij een hogere CO_2 partiaalspanning en constante pH, zal de (bi)carbonaat concentratie toenemen. Omdat dan ook meer Na^+/K^+ nodig is als tegenion voor (bi)carbonaat, gaat dit ten koste van de maximaal toelaatbare (thio)sulfaat concentratie. Bij 4 mol L^{-1} Na^+/K^+ als tegenion voor (bi)carbonaat, is de evenwichts-partiaalspanning van CO_2 in de gasfase ongeveer 88 kPa, ofwel 1.1 vol% bij een druk van 80 bar. Behandeling van gassen met een hogere CO_2 partiaalspanning (bijv. 2-3% CO_2 bij 80 bar) is wellicht mogelijk door het gebruik van methodes waarbij selectief H_2S en minder CO_2 wordt geabsorbeerd, zoals het toepassen van lage gas verblijftijden en hoge gas debieten [19].

8.7.5 Alternatieve toepassingen

Naast de behandeling van aardgas, kan biologische sulfide oxidatie onder natron-alkalische condities ook worden toegepast voor de behandeling van andere sulfide bevattende gas- of waterstromen, zoals biogas, stortgas en gassen uit de pulp- en papierindustrie, brouwerijen en de voedingsindustrie. Voor afvalwater dat direct na behandeling wordt geloosd is de vorming van elementair zwavel wellicht niet haalbaar. Door de lage groeiopbrengst bij zwavelvorming zal uitspoeling van biomassa plaatsvinden wanneer er geen specifieke maatregelen worden genomen om dit te voorkomen. Bij volledige oxidatie van sulfide naar sulfaat wordt uitspoeling van biomassa mogelijk voorkomen door de hogere groeiopbrengst bij sulfaatvorming.

Biologische sulfide oxidatie onder natron-alkalische condities kan eveneens worden toegepast als processtap in een recentelijk ontwikkeld proces voor de biologische ontzwaveling van “Liquefied Petroleum Gas” (LPG) [22]. In dit proces wordt MT omgezet naar sulfide in een anaerobe reactor. Dit sulfide kan vervolgens worden omgezet naar elementair zwavel. Het effect van MT op sulfide oxidatie zoals beschreven in dit proefschrift, kan worden toegepast bij de verdere ontwikkeling van dit gecombineerd proces.

Een andere toepassing in de petrochemische industrie is de ontzwaveling van synthesegas. De toepasbaarheid van het proces voor de ontzwaveling van synthesegas is reeds aangetoond, maar voor verdere ontwikkeling is vervolgonderzoek nodig naar de lange-termijn effecten van CO en onzuiverheden die aanwezig zijn in synthesegas.

8.8 TOT BESLUIT

Biotechnologische omzetting van sulfide kan worden toegepast onder natron- alkalische condities voor de verwijdering van H_2S uit zure gasstromen. In vergelijking tot conventionele fysisch-chemische technologieën kan worden verwacht dat het nieuwe proces leidt tot een aanzienlijke energie- en kostenbesparing voor de behandeling van H_2S vrachten tot tenminste 50 ton zwavel per dag [4]. De belangrijkste voordelen ten opzichte van bestaande

technologieën zijn: (1) verminderd energiegebruik voor opwarming en afkoeling; (2) lagere consumptie van dure chemicaliën; (3) de procesvoering is eenvoudig; (4) het is veilig, omdat er geen vrij H_2S gas voorkomt benedenstrooms van de absorptiekolom.

Het proces kan worden bedreven met een hoge stabiliteit en flexibiliteit bij wisselende H_2S belastingen. Volledige (100%) omzetting van H_2S naar elementair zwavel kan echter niet worden bereikt door aanpassing van de zuurstofdosering. Afhankelijk van de pH zal er altijd een bepaalde fractie worden omgezet naar thiosulfaat. Een extra anaerobe stap is benodigd om het gevormde thiosulfaat en eventueel ook sulfaat om te zetten naar sulfide. Op deze manier kan de vorming van een spuistroom worden voorkomen.

Verder onderzoek is nodig om de rol van massaoverdracht processen en chemische en biologische processen in "full-scale" reactoren vast te stellen. Daarnaast is vervolgonderzoek naar de eigenschappen van de zwaveldeeltjes gewenst, voornamelijk met betrekking tot de vorming van aggregaten en hergebruik van het biozwavel.

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Pim van den Bosch
Wageningen, September 2008

ABOUT THE AUTHOR

Pim Lambert Franz van den Bosch was born on November 30, 1978 in Oss (The Netherlands). Living in the village of Oijen, he finished his high-school education (VWO) in 1997 at "Het Maaslandcollege" in Oss. In the same year he started his study of environmental hygiene at Wageningen University (The Netherlands). The specialization of environmental technology combined his interest in technology with a desire to work on environmental problems. For his thesis he worked at the Laboratory of Microbiology on the biological production of hydrogen gas by thermophilic fermentative microorganisms. A second thesis research was performed at the Sub-department of Environmental Technology, on a combined physicochemical and biological process for the removal of NO_x from flue gasses.

The first contact with halo-alkaliphilic *Thioalkalivibrio* 'bugs' was brought about during an internship at Paques B.V. (Balk, The Netherlands), with a study on the biological treatment of sulfidic slags. In June 2003 he obtained his MSc. degree (with honors). In the same year he started the PhD. research described in this thesis, at the Sub-department of Environmental Technology of Wageningen University. From August 2008, he works at the same sub-department on the treatment of oily sludges.

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CERTIFICATE

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Place: *Wageningen* Date: *31 October 2008*

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SENSE board

Prof. dr. R. Leemans

the SENSE Director
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