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Biogenic Production of Thiosulfate from Organic and Inorganic Sulfur Substrates for Application to Gold Leaching

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Abstract: Gold mining and processing is an activity with large environmental impact due to the low concentration of gold in ore deposits and chemical resistance to most chemicals. Over 75% of gold is leached from ores using cyanide, however less toxic lixiviants have been proposed in the literature. Thiosulfate is one of these alternative reagents, but high reagent consumption has slowed acceptance in mining operations. Reducing the cost and impact of thiosulfate production is a way to reduce the cost of reagent consumption during leaching. The objective of this study was to evaluate the feasibility of leaching gold from ore with biogenic thiosulfate. Biogenic thiosulfate was produced using a marine methylotroph bacterium from three substrates: sodium sulfide, elemental sulfur, and dimethyl sulfide for application in bioleaching. The different substrates were evaluated to determine conversion efficiency from the sulfur source to biogenic thiosulfate and verified by titration and ion chromatography. Optimal conditions for conversion to thiosulfate were determined to be in the range of pH = 7–8, 25–30 °C, with sodium sulfide as a substrate in a sealed system to prevent sulfide from escaping as hydrogen sulfide gas. An oxide gold ore with a grade of 4.02 g/t was selected as a gold source for leaching experiments. The leaching of gold using the biogenic thiosulfate was compared with chemical thiosulfate solutions under experimental conditions of pH = 9.5, 50 mg/L copper, 500 RPM mixing, and 0.1 L/min air. The efficiency of gold bioleaching was measured using flame atomic absorption spectroscopy and fire assay. Gold extraction efficiencies ranging from 20–60% were achieved using the biogenic thiosulfate, and 27–77% with sodium thiosulfate solutions, respectively. It was concluded that the sodium sulfide substrate was best for producing higher biogenic thiosulfate concentrations and leaching efficiency.

Keywords: bioleaching; gold; oxide ore; sulfur; thiosulfate

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1. Introduction

Gold is a unique noble metal with high conductivity, ductility, and resistance to corrosion. It is utilized globally in the finance, jewelry, dentistry, aerospace, and electronics industries [1]. Gold extraction from primary resources is an energy and chemically intensive process that is driven by size reduction (i.e., crushing and grinding) followed by chemical leaching with a suitable lixiviant. The grade of gold in deposits has decreased significantly in the last few decades, meaning a larger volume of ore must be processed to maintain constant production [2]. With this and the advent of new technologies many new mines being developed to process 'refractory' ores, or ores with significant concentrations of sulfur and organic carbon which may impact the conventional crushing—cyanidation process. These ores must often be subjected to pre-treatment to remove or alter the chemical state of these materials. This may include fine grinding to increase the rate of the pre-treatment or expose fine gold particles within the mineral grains [3]. With these considerations combined with the demand for more environmentally sustainable processing, the mining industry has been forced to consider alternatives to the cyanidation process which has been the primary method for gold leaching since the late 1800's.

Cyanide is currently used in over 75% of global gold mining operations where strict controls and procedures make it a safe process despite the extremely high toxicity of the chemical [4]. The largest risk comes during transportation of cyanide, and as such many jurisdictions have banned its movement, making the process effectively banned for use in mining. In addition, the prevalence of lower grade and more refractory ores have contributed to higher cyanide consumption during leaching, producing hazardous tailings that must be treated further to be stored safely [5]. There have been several alternative gold lixiviants proposed as technically viable at the bench scale including thiosulfate, thiocyanate, thiourea, chlorination, and bioleaching using various biogenic lixiviants [5,6]. Thiocyanate and thiourea have not been implemented at mining sites to date despite high initial leaching rates and acidic operating conditions due to high reagent consumption when applied to many ores and significant toxicities [7,8].

Thiosulfate is favored above other alternative lixiviants like thiourea and thiocyanate due to its low toxicity, acceptable leaching rate, and stability in the presence of many components of gold ores [9]. The leaching reaction of gold by thiosulfate with oxygen as an oxidant is shown in Equation (1) [10]. Copper is often added as a catalyst to increase the leaching rate [11].

$$4Au + 8S_2O_3^{2-} + O_2 + 2H_2O \rightarrow 4Au(S_2O_3)_2^{3-} + 4OH^- \quad (1)$$

The thiosulfate leaching system is not without its shortfalls, and to date has only been applied at scale in one commercial operation, Goldstrike, operated by Nevada Gold Mines Ltd., Elko, NV, USA, where calcium thiosulfate leaching of pressure oxidized concentrate was applied [12]. Despite issues with high reagent consumption and gold recovery from solution it is the most promising of the alternatives with respect to environmental and human health concerns [5]. Aside from these concerns, in jurisdictions including Germany, Czechia, and U.S. state Montana cyanide use for mining is fully banned [13]. Therefore, in these locations any return on gold mined as a byproduct of other metals must be extracted by other means.

Biogenic lixiviants pose an interesting option when comparing lixiviants. While they consist of the same leaching agents the option to produce them using microorganisms and a suitable substrate could be a method to reduce the cost and risk of bringing large quantities to the leaching plant from other locations [14]. The largest potential benefits of applying biogenic lixiviants for gold mining are a lower cost of production at scale, less toxic chemicals, less energy intensive chemical production, and scalability for smaller e-waste recycling operations [15,16]. Biogenic cyanide, thiosulfate, iodine, organic acids, and amino acids have been reported in the literature to leach or improve the leaching rate of gold from ores [6,17,18]. There are still many knowledge gaps in the suitability of biohydrometallurgy techniques for gold mining, despite their commercially accepted application to copper extraction, recycling, and pre-treatment of gold ores [19–21].

Many microorganisms produce thiosulfate as biogenic products of metabolism or as a consequence of side reactions involving other biogenic products, such as polysulfide and sulfite [22]. *Methylophaga sulfidovorans* is a species of particular interest due to its ability to produce thiosulfate stoichiometrically as a product of chemolithoheterotrophic growth on organic and inorganic sulfur compounds [23]. Chemolithoheterotrophy is a mixed metabolic mode that some microorganisms employ to grow on organic carbon sources while deriving extra energy for cellular activities from the oxidation or reduction of inorganic compounds to conserve energy [24]. In the case of *M. sulfidovorans* the primary growth substrates are methanol, dimethyl sulfide (DMS), methylamine, and dimethylamine. These primary substrates are catabolized by *M. sulfidovorans*, and carbon atoms are assimilated into biomass by way of the ribulose monophosphate pathway [23]. The bacteria can also oxidize H_2S during growth on the organic substrates in a process that does not contribute directly to cell proliferation [18,25]. It is assumed that the oxidation of inorganic substates is for increased energy production [24]. *M. sulfidovorans* is a marine strain that was isolated from salt marsh sediments in the Netherlands. It grows near the interface of the oxic and

anoxic layers of mixed microbial mats where many reduced sulfur compounds coexist as products of other bacterial processes [23].

The substrates chosen for this study on the potential sources for Bio-TS and the uses of methylotrophic sulfur oxidizers in the mining industry were DMS ($(CH_3)_2S$), sodium sulfide (Na_2S), and elemental sulfur (S^0). Two of these reagents were specified in an earlier study using *M. sulfidovorans* and elemental sulfur is a potential waste product encountered in gold mining operations [10,25]. The possibility of chemolithoheterotrophic growth with these sulfur substrates and methanol deserves further investigation.

When considering the sustainability of gold mining and mining operations in general, the use of fresh water for processing is of similar impact to the control of chemical contaminants in wastewater with respect to acquiring operational permits [26]. Seawater or desalinated water has been used in some mining operations in arid regions and usually comes with more difficult process and equipment design from increases to corrosion of equipment and ionic strength of process water [27]. The most common processing step to use seawater was flotation, however given that *M. sulfidovorans* is a marine bacterium the potential for partial substitution of seawater in Bio-TS production is of interest and should be investigated. The high water usage in arid mining areas has impacts to surrounding communities that could potentially be lessened by partial substitution of groundwater with seawater [28].

The purpose of this research is to investigate the potential of applying Bio-TS produced by *M. sulfidovorans* to gold leaching process options using different potential substrates. The application of biogenic thiosulfate for metallurgical purposes is a poorly understood topic, therefore comparing the feed materials and the outcome of leaching was studied to see if it holds potential for further development. Despite decades of research thiosulfate has failed to be accepted in the gold mining industry, and therefore other methods to improve it must be explored to see if they might improve the case for alternative lixiviants. Biogenic thiosulfate was applied to solubilize gold from oxide ore and gold powder. The viability of each substrate was compared with respect to biogenic thiosulfate concentration after growth, tolerance of the bacteria to increasing concentrations of the substrates, toxicity, ease of handing, and cost. Another point of novelty in the study is the mixing of seawater with bacterial growth mediums to simulate how leaching with biogenic lixiviants may reduce freshwater draws in mining areas.

2. Materials and Methods

2.1. Materials

Oxide gold ore from a Canadian gold deposit was used for leaching experiments. The ore was received in coarse form from the crusher and riffled to form representative samples. The ore was then pulverized to form a fine particle size distribution, shown in Figure S1. The pulverized samples were riffled again in an automatic micro splitter (Quantachrome Instruments, Boynton Beach, FL, USA) to produce representative samples for the leaching experiments. Gold and other metal content was quantified using fire assay and ICP-MS (Perkin Elmer, Waltham, MA, USA, NexION 300X) at an external laboratory.

Gold powder was produced by heating set amounts of gold standard solution in Erlenmeyer flasks followed on a hot plate until only solid Au remained.

2.2. Bacterial Culture

The bacteria *M. sulfidovorans* (DSMZ 11578) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). It was cultured in DSMZ951 media and synthetic seawater (Instant Ocean, Spectrum Brands) or mixtures of both solutions. The contents are listed in Table 1. All growth media were enriched with 1.0 mL/L of both trace element solution and vitamin solution (Cedarlane). Methanol 10.0 mL/L (VWR) was the carbon source added in experiments without DMS. Sulfur sources DMS, $Na_2S{\cdot}9H_2O$, and elemental sulfur were purchased from Millipore Sigma.

Table 1. Details of culture media and synthetic seawater.

DSMZ 951			Instant Ocean		
Component	**Concentration**	**Unit**	**Component**	**Concentration**	**Unit**
NaCl	15	g/L	Cl^-	17.07	g/L
$(NH_4)_2SO_4$	0.5	g/L	Na^+	9.45	g/L
$MgSO_4 \cdot 7H_2O$	1.0	g/L	$SO_{4^{2-}}$	2.37	g/L
Na_2CO_3	2.0	g/L	Mg^{2+}	1.16	g/L
$CaCl \cdot 6H_2O$	330	mg/L	Ca^{2+}	360	mg/L
KCl	200	mg/L	K^+	370	mg/L
KH_2PO_4	20	mg/L	HCO_{3^-}	180	mg/L
$FeSO_4 \cdot 7H_2O$	1	mg/L	B^{3+}	0.54	mg/L
			Sr^{2+}	0.036	mg/L

Bacteria cultures were grown at 30 °C and 150 RPM shaking in an incubator. A volume of 100 mL growth medium or synthetic seawater was measured into 250 mL screw sealed Erlenmeyer flasks that were closed after inoculation, substrate addition, or sampling to minimize the escape of volatile substrates. Mixtures were tested at 10% intervals from 100% seawater to no seawater. Growth solutions were sterilized at 121 °C for 60 min and cooled before methanol and vitamin solution were added. The prepared media was inoculated with 5 mL of a viable culture grown in DSMZ 951 at its exponential growth phase. Liquid samples were filtered using 0.45 µm syringe filters before analysis. Error bars were calculated as standard deviations from duplicate cultures.

2.3. Bio-TS Production Method

The Bio-TS production experiments were conducted with each reagent at different concentrations and in kinetic experiments. First, a set amount of reagent was administered and sampled periodically before thiosulfate determination and mass balancing. Once the time for full conversion, or time required to stop sulfur oxidation was reached the experiments were terminated and the time for each reagent was chosen for experiments with increasing concentrations to determine the effect of increased dosage on conversion and toxicity to the culture. The ranges of each reagent tested are shown in Table 2.

Table 2. Range of concentrations for each reduced sulfur substrate tested.

Substrate	Concentration	Unit
$Na_2S \cdot 9H_2O$	1.91–9.55	g/L
$(CH_3)_2S$	10–72	mg/L
S^0	1–5	g/L

Batch Bio-TS production experiments were performed at the conditions listed in Section 2.2. In growth experiments with DMS, the methanol was replaced with DMS given it is also a primary growth substrate, in the case of other sulfur substrates methanol was present at 1 mL/L. Due to the vapor pressure of DMS it was administered with a syringe through Erlenmeyer flasks plugged with rubber stoppers, and samples for kinetic experiments were taken the same way. After substrates were added pH was readjusted to 7.5 using 0.1 mol/L HCl. All liquid samples were filtered with 0.45 µm syringe filters before Bio-TS quantification using IC/UV-Vis or iodometric titration. In the experiments with reduced sulfur reagents an H_2S monitor (Honeywell, Charlotte, NC, USA; BWC2-C) and 3M organic acid P100 filters (60923) were used for personal protective equipment. All open samples were handled within a fume hood.

2.4. Leaching Method

The Bio-TS solutions from all sources were used to leach oxide ore and gold powder. The leaching experiments were conducted using the method proposed in previous work [18]. Standard leaching conditions for both materials are shown in Table 3.

Table 3. Reactor experimental parameters for gold extractions.

Parameter	Ore Leach	Au Powder Leach
Cu^{2+} conc. (mg/L)	50	50
Temp. (°C)	50	50
Pulp density (%)	10	1 mg per 100 mL
Mixing speed (RPM)	500	350
Air flow (L/min)	0.1	0
Initial pH	9.5	9.5
Duration (h)	24	8

Prior to leaching, the bacteria incubated with methanol for 48 h in DSMZ 951 or mixed seawater cultures. After the initial growth, the flasks were supplemented with the sulfur substrate and grown for longer based on the results of the production experiments. Prior to leaching, the Bio-TS solutions were centrifuged, decanted, and passed through 45 µm syringe filters to remove the biomass. The pH was adjusted with 0.5 N NaOH and copper added as $CuSO_4$ prior to addition of the gold source. Water washed residues were dried for analysis. Error bars were calculated in the same way as for Bio-TS production.

2.5. Analysis

Gold and copper from liquid samples was analyzed by atomic absorption spectroscopy (AAS; Thermo Scientific iCE 3000 Series, Waltham, MA, USA). Mineral components of the ore were determined by X-ray diffraction (XRD; Philips, Amsterdam, The Netherlands) with PANalytical X'Pert HighScore 5.1 software and the International Centre for Diffraction Data (ICDD) database. Carbon and sulfur in solid samples were quantified with a carbon sulfur analyser (Eltra CS 200). Particle size distributions were determined using laser particle size analysis (Malvern Panalytical, Malvernn UK; Mastersizer 3000).

The growth of bacteria at different conditions was compared using a UV-Vis spectrophotometer (Thermo Scientific; GENESYS 10S) against a bacterial media blank at 430 nm [23]. Turbidity based growth data was validated using microscopic counts as in Fey et al. [29]. In cultures where elemental sulfur was added, the flasks were removed from the incubator 20 min prior to sampling to minimize the error associated with increased turbidity from sulfur particles. Dissolved oxygen (D.O.) was measured using an optical dissolved oxygen sensor (METTLER TOLEDO InLab OptiOx, Fisher Scientific, Waltham, MA, USA). Oxidation reduction potential (ORP) and pH were measured using a multi-channel probe (VWR, Radnor, PA, USA; Symphony H30PCO).

Bio-TS was quantified using an ion chromatograph equipped with ultraviolet-visible spectrometer (IC/UV-Vis, Methrohm 930 Compact IC Flex/947 Professional UV/Vis Detector Vario SW) equipped with a Metrosep A Supp 5-250/4.0 separation column. The eluent was composed of 10 mmol/L $NaClO_4$ and 1 mmol/L NaOH in deionized water. Standards were prepared using a 1000 µg/mL thiosulfate standard (Agilent) and made at concentrations of 10, 20, 50, and 100 mg/L prior to each analysis. Samples form Bio-TS solutions were drawn no earlier than 1 h before analysis and diluted from the same deionized water used to make the standards and eluent.

Gold extraction efficiency of aqueous samples was calculated using fire assay data from the feed ore and AAS determinations of gold in solution after Bio-TS leaching, Equation (2). The final residue was subjected to fire assay and ICP-MS at an external laboratory to confirm the mass balance.

$$\text{Gold Extraction Efficeny } (\%) = \left(\frac{C_s \cdot V_s}{g_o \cdot M_o}\right) \times 100\% \quad (2)$$

where C_s, V_s, g_o, and M_o are the concentration of extracted metal in solution (mg/L), volume of solution (L), grade of metal in ore sample before leaching (mg/kg), and mass of ore leached (kg), respectively. In the experiments on gold leaching from ore the liquid assay was balanced against the fire assay of the residue after water washing.

2.6. Sample Characterization

The ore was assayed to a gold content of 4.02 ± 0.12 g/t. Concentrations of other metals, carbon, and sulfur are shown in Table 4. The XRD pattern of the ore is shown in Figure 1. In the XRD analysis the main phase was determined to be silicate. There was also significant amounts of goethite and magnetite, as well as some chlorite. Goethite and magnetite are iron oxyhydroxide and oxide minerals, respectively. The chlorite mineral was an aluminum silicate with calcium and magnesium in the structure.

Table 4. Composition of elements in oxide gold ore.

Analyte:	Au	Cu	Fe	Zn	Mn	Pb	As	C	S
Unit:	mg/kg	mg/kg	%	mg/kg	mg/kg	mg/kg	mg/kg	%	%
Concentration	4.02	70	6.82	57	1100	13	26	3.21	0.32

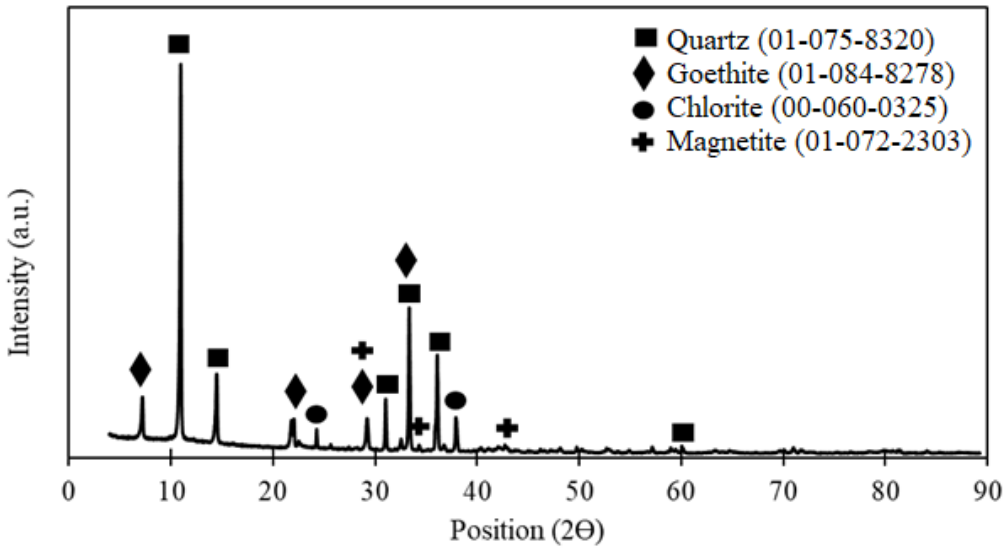

Figure 1. XRD pattern of oxide gold ore.

3. Results and Discussion

3.1. Biogenic Thiosulfate Production Using M. sulfidovorans

3.1.1. Kinetic Growth Experiment

In previous studies *M. sulfidovorans* was shown to be a slow growing bacterium with a relatively long stable phase of approximately 7 days after a 36 h exponential phase when grown on methanol or DMS [18,23]. To determine the incubation time for each sulfur substrate periodic samples were taken to determine the best time for biomass removal and spent medium bioleaching with Bio-TS. Low concentrations of each substrate were added to prevent any errors from sulfur toxicity in these experiments. The used concentrations were 624, 150, and 2500 mg/L for Na_2S, DMS, and S^0, respectively.

The growth conditions for cultures grown on each substrate are shown in Figure 2. DMS cultures were grown with no other sulfur additive, while the other cultures were grown for 48 h on methanol before the aseptic addition of the desired sulfur substrate. Figure 2a,b shows the OD_{430} and D.O. and indicated that the methanol containing cultures continued to grow steadily for 7 days after addition of the substrates with a corresponding oxygen removal. All cultures began to decrease in cellular density after this point. The DMS grown cultures had a similar oxygen removal rate to the others but resulted in lower cellular density in the flasks, this was likely due to the higher oxygen required for the bacterium to oxidize 1 mole of DMS (4 mole of O_2) compared to methanol (1.5 mole of O_2).

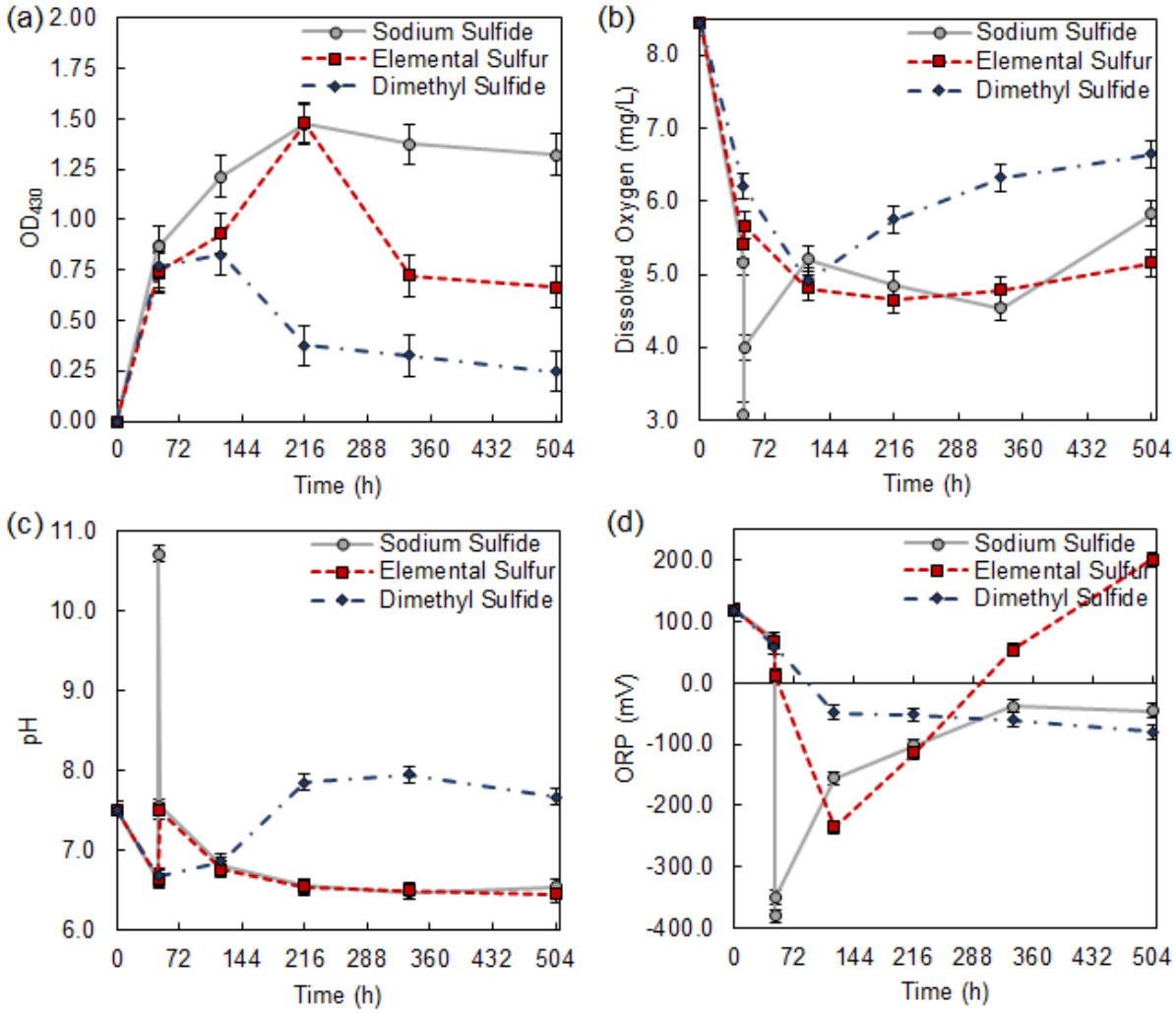

Figure 2. Growth conditions of *M. sulfidovorans* with three sulfur substrates. (**a**) optical density; (**b**) D.O.; (**c**) pH; and (**d**) oxidation reduction potential vs. staurated Ag/AgCl.

Figure 2c,d shows the pH and ORP changes that are associated with the oxidation of sulfur compounds. DMS grown cultures had gradual increases in pH and decreases in ORP, which is similar to methanol grown cultures. Upon addition of the inorganic substrates larger changes were observed. Na_2S addition causes a large pH spike that is countered by addition of more HCl, which then resumes the slight decrease associated with growth on methanol. This is coupled with an increase in ORP due to the oxidation of H_2S to form Bio-TS. The elemental sulfur oxidation mechanism is less understood, however the ORP steadily rose as Bio-TS became measurable in the solution. Elemental sulfur can be converted to hydrogen sulfide via a trans sulfurization reaction with NADP to produce hydrogen sulfide [30]. Another pathway may be reactions with some medium constituents, or products of cellular production other than NADP. However, the first option was deemed likely due to the qualitative measurement of H_2S gas by detector in cultures where only methanol and S^0 were added.

Figure 3 shows the Bio-TS concentrations measured using IC/UV-Vis over these experiments. Regardless of the substrate the maximum measurement was taken at 336 h or 15 days of growth, however these were within the error margin of the previous samples. The Bio-TS was stable over the entire 21 day test period and therefore was not being consumed in further biochemical reactions, conforming to previous studies [25].

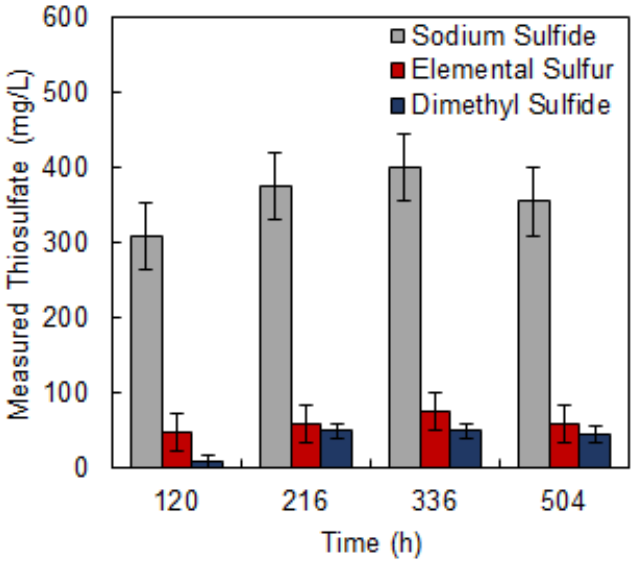

Figure 3. Bio-TS production over time by *M. sulfidovorans* with Na_2S, DMS, and S^0.

3.1.2. Bio-TS Yield at Different Substrate Concentration

After determining that most of the Bio-TS production was complete after 7 days, the concentration of each substrate was increased to determine at which dosage a reduction on grow, toxicity to sulfur, or other negative effects occurred. In these tests conversion of each substrate to Bio-TS was calculated on a molar basis using the stoichiometry of Equations (3)–(5).

$$H_2S + O_2 \rightarrow 0.5\, H_2S_2O_3 + 0.5H_2O \quad (3)$$

$$S + 0.5O_2 + 0.5H_2O \rightarrow 0.5\, S_2O_3^{2-} + H^+ \quad (4)$$

$$(CH_3)_2S + 4O_2 \rightarrow 2CO_2 + H^+ + 0.5\, S_2O_3^{2-} + 2.5H_2O \quad (5)$$

Figure 4 Shows the results of increasing concentration on Bio-TS concentration and conversion efficiency. When sodium sulfide was added to produce H_2S for metabolism at low concentrations almost 90% of the molar concentration added was converted to thiosulfate. It is likely that the remaining fraction was consumed in side reactions with dissolved oxygen upon the initial addition and pH adjustment stage and the rest was available to bacteria, while being mediated by Equation (6).

$$HS^- + H^+ \leftrightarrow H_2S \quad (6)$$

This reaction has a pKa of 7.04 and is expected to remain at approximately 50% of the aqueous sulfur by molar ratio during the oxidation by *M. sulfidovorans* [31]. The measured Bio-TS concentrations were the highest in cultures where sodium sulfide was the sulfur substrate, with reasonable high conversion up the ~1500 mg/L level. Above this concentration, OD and D.O. measurements would not recover to pre-addition levels indicating that the limiting concentration of sulfide was exceeded or shock for pH change during addition was harmful to the culture.

Elemental sulfur addition and conversion to Bio-TS was much less efficient and had larger variance than the other two substrates (Figure 4b). This could be due to a slow intermediate chemical reaction that is required to convert S^0 to another species that the bacterium can oxidize. It is also produced as a by-product in many hydrometallurgical processes and could be a cheap or free input for Bio-TS production [32].

Dimethyl sulfide grown cultures were the lowest in terms of Bio-TS concentrations measured after 7 days, with DMS concentrations above 300 mg/L (4.8 mmol/L) producing negligible cell growth. This toxicity to the bacterium considered in conjunction with the high vapor pressure make it the least ideal substrate from the perspective of biogenic

thiosulfate production. The phenomenon may be of interest to industries where DMS is produced in industrial activities and must be captured and remediated, such as pulp and paper [33]. It is also regarded as a significant chemical in the atmospheric sulfur cycle affecting climate, ecology, and global temperatures so the processing by marine bacteria may be significant to research in other fields [34].

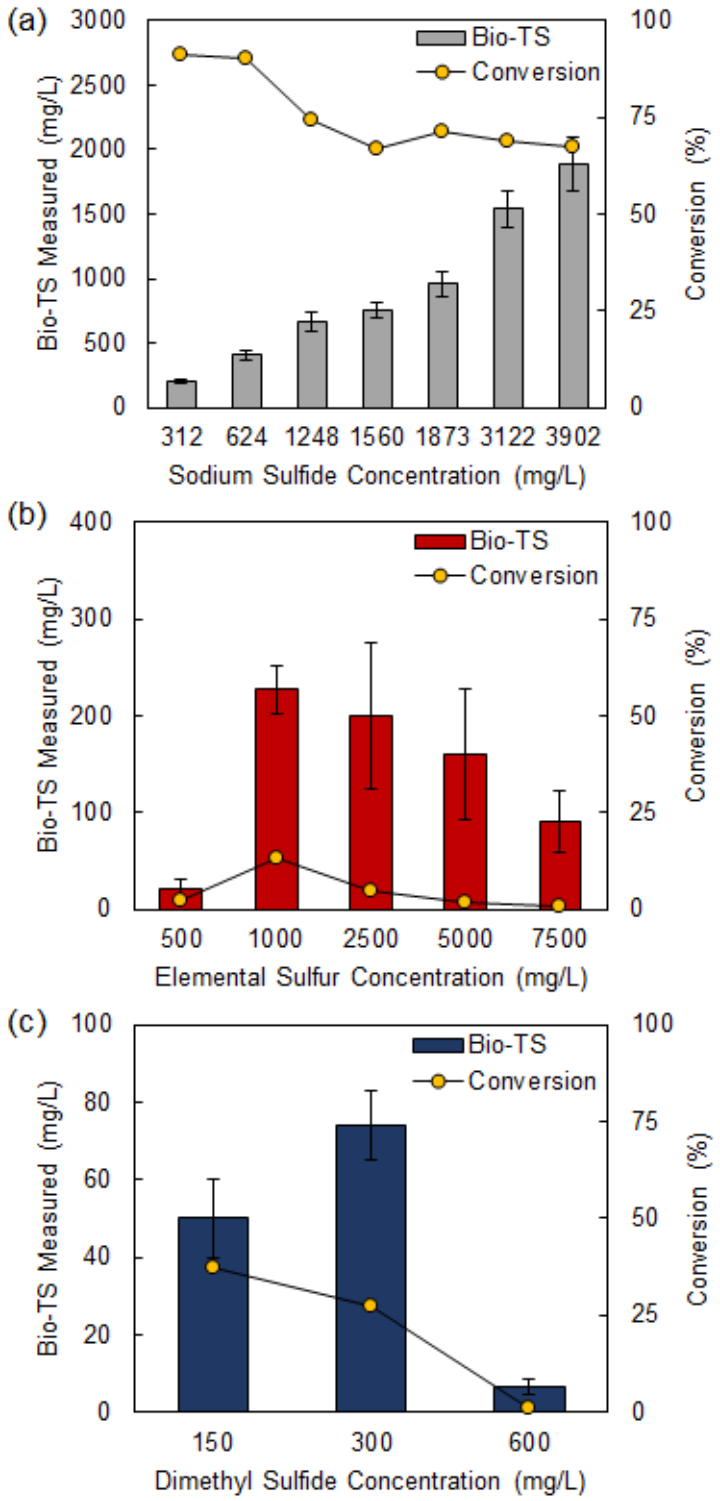

Figure 4. Bio-TS production and conversion (%) at different concentrations of (**a**) Na_2S; (**b**) S^0; and (**c**) DMS.

3.2. Bio-TS Gold Leaching

3.2.1. Gold Powder Bio-TS Leaching

After determining the substrate dosage and contact time needed to avoid negative effects to the bacterial culture the procedure was applied to solubilize pure gold powder at conditions similar to thiosulfate ore leaching. Sulfur substrates were added to cultures growing on methanol after 48 h at concentrations of 1873 and 2500 mg/L for Na_2S and S^0, respectively. DMS cultures were grown on 300 mg/L DMS. All cultures were filtered to remove biomass after 168 h of growth on the respective sulfur substrate and immediately prepared for leaching. The temperature of 50 °C and copper concentration of 50 mg/L added prior to the start of the experiments are widely reported to be effective in many reports [11,35]. No air was sparged but mixing rate was maintained at 250 RPM to facilitate adequate dissolved oxygen levels to facilitate the gold oxidation reaction. D.O. was never observed below 6.5 mg/L during any of the sample periods. The leaching pH was maintained at 9.5 throughout the experiments using 0.5 mol/L NaOH solutions.

Figure 5 shows the gold concentration measured in solution during the leaching period. In all cases the leaching rate was fastest in the first 3 h of the experiment and gold solubilized was higher in the Bio-TS solutions produced when adding Na_2S to methanol grown cultures. These cultures had Bio-TS concentrations between 770–960 mg/L and continued to increase in gold concentration until the end of the experiments. This was similar to the result obtained when applying a synthetic solution of 10 mmol/L (1120 mg/L) sodium thiosulfate. Elemental sulfur and DMS grown cultures generated 120 and 45 mg/L Bio-TS, respectively, and did not increase in gold concentration after the first sample period. This suggests that Bio-TS is as effective as sodium thiosulfate salt as a gold lixiviant. The low concentration of gold powder dissolved is characteristic of low thiosulfate concentrations when applied to pure gold in the absence of additives to prevent passivation of the surfaces [36]. This was likely exacerbated by copper precipitation during the gold leaching, which over the 24 h period was higher at lower levels of Bio-TS and most likely reduced on the gold surface, Table 5. In previous studies it has been noted that copper can be reduced to several precipitate species upon reactions with aqueous thiosulfate or due to reduction during gold oxidation [11]. This experiment further confirmed the higher potential of Na_2S provided by dissolving sulfide salts as the most promising Bio-TS substrate for producing the reagent using *M. sulfidovorans*.

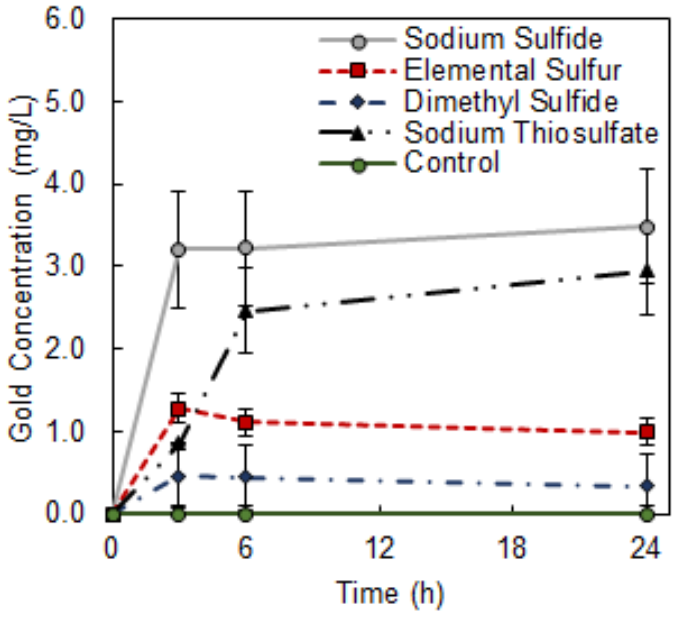

Figure 5. Gold concentration over time when leaching gold powder into Bio-TS solution produced from different sulfur substrates.

Table 5. Copper loss during gold powder leaching.

Sulfur Substrate	Na_2S	S^0	DMS	$Na_2S_2O_3.5H_2O$
Initial Cu (mg/L)	50	50	50	50
Final Cu (mg/L)	8.5	8.2	1.6	11.2

3.2.2. Gold Ore Bio-TS Leaching

Bio-TS produced by *M. sulfidovorans* was also used in the same method to leach gold from oxide gold ore. The procedure was the same as that followed in the gold powder experiment with the exception of air sparging at 0.1 L/min and a higher mixing rate of 500 RPM. These changes were chosen based on an analysis of the literature [11]. The air sparging is intended to regenerate Cu(II) that is reduced during leaching and to facilitate the gold solubilizations following Equation (1).

The same trend was realized with oxide ore leaching experiments as that of gold powder leaching. Bio-TS produced using Na_2S had the largest concentration and the highest gold leaching, Figure 6, with an average of 60.5% gold extraction efficiency. Thiosulfate consumption was higher at the lower concentrations as well, where it increased on average from 42.5, 43.5, to 96.5% in leaching experiments with Bio-TS from Na_2S, S^0, and DMS grown cultures, respectively.

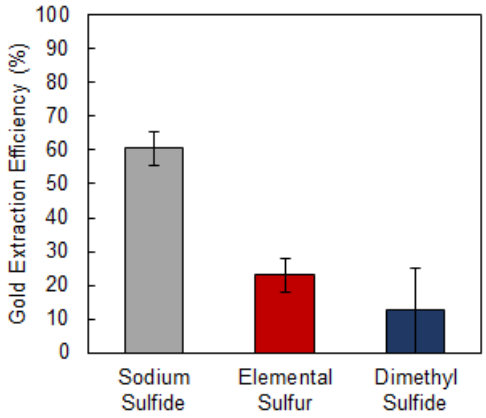

Figure 6. Gold extraction efficiency from oxide ore after 24 h leaching with Bio-TS from different sulfur substrates.

Ultimately this experiment showed that DMS and S^0 are inferior sulfur substrates to Bio-TS from the perspective of leaching gold from ore. An analysis of other factors was conducted in the discussion based on the cheap availability of S^0 in mining environments.

3.3. Biogenic Thiosulfate Production in Mixed Seawater Media

To examine the potential for more sustainable metal production, Bio-TS production in seawater cultures was determined. The increase in ionic strength and removal of specific nutrients when substituting seawater for bacterial media make it a more challenging environment for the bacteria to grow. Similarly, in most industrial applications of seawater it is used after costly desalination processes or added as a fraction of the total process water [27]. Therefore, cultures of *M. sulfidovorans* were grown in mixtures of DSMZ 951 media and synthetic seawater formulated to mimic subtropical oceans at increasing ratios from 10% to 100% seawater to gauge the effect on the growth potential of the culture.

Figure 7 shows the results of these growth experiments over the first 168 h of growth. There were very minute differences in growth rate and cell concentration of the cultures until the seawater content exceeded 70% of the solution volume. This was also confirmed by a lower D.O. consumption in cultures of 70% seawater and higher, indicating the bacteria consumed less oxygen for growth on methanol. Above 70% seawater the cultures grew slower and reached lower final cell concentration, while also increasing in pH to lower

levels than others. This is likely due to the decrease in sodium carbonate from the DSMZ 951 fractions, which slightly buffers the pH as the growth progressed in lower seawater fraction cultures. It was concluded that up to 70% seawater could be mixed with the bacterial medium without large impact on growth.

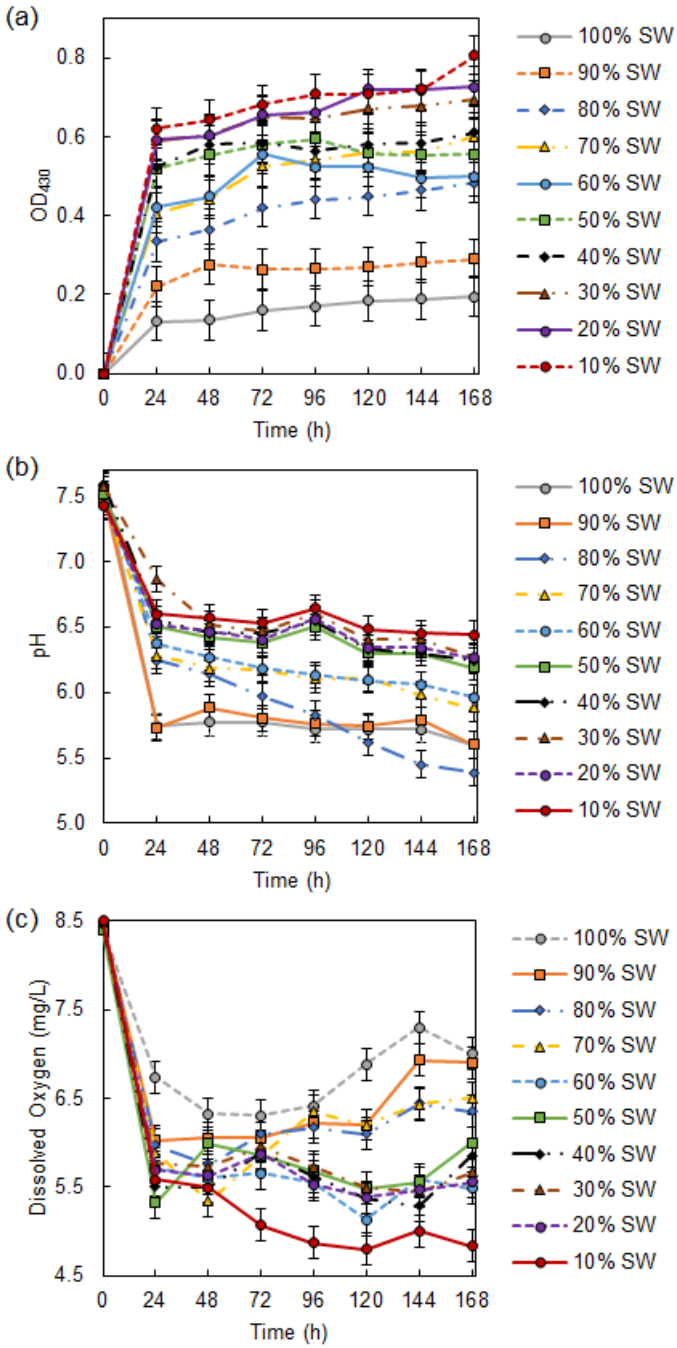

Figure 7. Growth conditions of *M. sulfidovorans* in mixtures of DSMZ 951 and synthetic seawater (SW). (**a**) optical density; (**b**) pH; (**c**) D.O.

Following on the growth experiments, cultures were grown on methanol under the standard conditions with increasing seawater consumption and administered 1783 mg/L Na_2S after 48 h of incubation with methanol. After another 168 h of growth the media were sampled and filtered before analysis for Bio-TS using IC/UV-Vis. Figure 8 shows the resulting Bio-TS concentrations and indicated that there was no negative impact on conversion of H_2S by *M. sulfidovorans* in the seawater mixed medium cultures. Conversion of sodium sulfide ranged between 65–71% on average, consistent with cultures grown on pure

bacterial media. This is a promising result from the perspective of sustainable development, where less fresh water could potentially be used in future gold leaching operations.

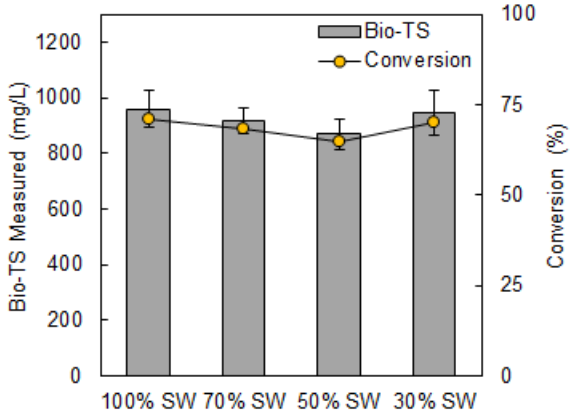

Figure 8. Bio-TS concentration measured after 144 h of growth in mixed SW DSMZ 951 media.

3.4. Discussion of Substrate Viability

After determining that the bacterium *M. sulfidovorans* has the capacity to convert organic and inorganic sulfur compounds to Bio-TS through chemolithoheterotrophic growth the next factor to consider is the practicality of each substrate. Table 6 summarizes some of the attributes of each reagent that would be relevant to application in a larger scale biohydrometallurgical process. Safety data sheets for pure compounds of each substrate from Sigma Aldrich were also used to source safety attributes.

Table 6. Attributes of different sulfur substrates used in experiments [37–39].

Attribute	Sodium Sulfide	Elemental Sulfur	Dimethyl Sulfide
Conversion to Bio-TS	High	Low	Moderate
Toxicity (LD50 Oral)	1122	>2000	>2000
Safety	Skin and eye irritant	Skin irritant	Flammable
2022 Cost (USD/t)	500	198	10,000
Volatility	Off gas as H_2S (Moderate)	Inert (Low)	High vapor pressure (High)
Availability	High	High	Low
Source	Chemical, mining, metallurgy industry	Petrochemical, mining, coal industry	Petrochemical industry

The cost of cyanide can range from 2000–3000 USD/t without considering the cost of safe handling, transportation, and disposal [40]. The recycling of cyanide is a critical step in economic gold extraction but is more difficult in the organic carbon or high copper ores where thiosulfate is often considered [5]. When considering this and the values of the table, DMS would not be a reasonable substrate for Bio-TS manufacture due to the high volatility and cost compared with the other reagents, along with the low measured Bio-TS measurements in DMS cultures.

Elemental sulfur is the most attractive reagent in terms of safety, volatility, and cost. In fact, in many hydrometallurgical processes S^0 could be produced and used on the same site. The downside of the S^0 as a substrate for *M. sulfidovorans* is the low conversion rates measured in laboratory experiments. This would be more applicable for very low grade or long-term gold extraction processes, such as leaching from gold tailings or extremely low-grade heap leaching activities. This scheme would be recommended for further study.

Sodium sulfide is in the middle of the other reagents in terms of cost and toxicity, making it only a marginal improvement over cyanide. It could in some cases be produced by metal processing activities which could make it cheaper for a diversified mining company but would be subject to many of the same safety precautions as cyanide, while being less toxic [39]. The high conversion rates in Bio-TS experiments make it a much more effective substrate than the other options and therefore demand further study of the system to search for a cost-effective solution.

However, due to the purpose of using sodium sulfide as a means of generating aqueous H_2S the potential of a mixed system is the most promising. Several sulfate reducing bacteria can reduce SO_4 and S^0 to H_2S and could be applied directly before Bio-TS production by *M. sulfidovorans* [22]. Bacteria including *Geobacter sulfurreducens* PCA and *Desulfomicrobium baculatum* have been applied to produce biogenic H_2S for application in sulfide precipitation of metals in process waters [41]. Similar processes have been applied for metal recovery in the gold mining industry previously [42]. It is most likely that new biotechnology implementation in the mining industry will be gradual due to the economic risks, so study of Bio-TS production from existing biogenic H_2S circuits poses the most likely path to success for this method.

4. Conclusions

Bio-TS was produced by *Methylophaga sulfidovorans* during chemoheterotrophic growth on methanol and Na_2S or S^0, or direct growth on dimethyl sulfide. The highest concentrations of Bio-TS produced were 1891, 200, and 91 mg/L when the sulfur substrate was Na_2S, S^0, and DMS, respectively. The highest conversion on a molar basis was when using Na_2S at 90% when low concentrations were added to methanol grown cultures. The highest rates of conversion were reached after 7 days of growth for all substrates. The Bio-TS was used to solubilize gold powder up to concentrations of 3.5, 1.0, and 0.34 mg/L when the sulfur substrate was Na_2S, S^0, and DMS, respectively. Ore leaching experiments with an oxide gold ore assayed at 4.02 g/t followed the same trend with average gold extraction efficiency of 60.5, 23, and 12% when the sulfur substrate was Na_2S, S^0, and DMS, respectively.

Sodium sulfide in bacterial medium was determined to be the most effective method of producing Bio-TS for gold leaching. The system was determined to be effective when up to 70% seawater was used as growth medium, presenting potential for decreased water use in a process that is freshwater intensive in the current form. Elemental sulfur was determined to be the most environmentally safe and cost-effective reagent but low conversion to Bio-TS makes it technically inferior to Na_2S. Intermediate bioprocessing using sulfate reducing bacteria is recommended for further study to combine the high technical efficiency of H_2S as a substrate with the low cost and easy handing of elemental sulfur. However, before biogenic thiosulfate can become competitive on an economic basis a method to concentrate the aqueous Bio-TS must be developed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142416666/s1, Figure S1: Particle size distribution of oxide ore used in leaching experiments.

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