Appl Microbiol Biotechnol (1996) 45:169–175
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ORIGINAL PAPER

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Quantitative measurement of sulphur formation by steady-state and transient-state continuous cultures of autotrophic *Thiobacillus* species

Received: 15 May 1995/Received revision: 4 August 1995/Accepted: 22 August 1995

Abstract Sulphur formation by the obligately chemolithoautotrophic *Thiobacillus* o and *Thiobacillus neapolitanus* was studied in aerobic, substrate-limited continuous cultures. The performance of transient-state and steady-state cultures was compared using different methods for measuring sulphur production. Below a dilution rate (D) of 0.3 h^{-1} (at 50% air saturation), sulphate-producing steady states were obtained, and cultures grown with sulphide or thiosulphate (at $D = 0.06\ h^{-1}$) showed similar characteristics (e.g. cell yields, oxidation capacities and CO_2-fixation capacities). Elemental sulphur was a major product above $D = 0.3\ h^{-1}$, but steady states were difficult to achieve, because of adherence of sulphur to the fermentor surfaces and the accumulation of sulphide. These problems could be circumvented using transient-state experiments of 1 h. It was then found that elemental sulphur was formed under oxygen limitation or at high substrate load. The rates of sulphur formation obtained by sulphur analysis agreed with the values calculated from stoichiometric balances. Sulphide and thiosulphate proved to be equivalent substrates for both *Thiobacillus* species during elemental sulphur formation under the conditions tested. It is concluded that transient-state cultures of thiobacilli, pregrown as sulphate-producing steady-state cultures, provide experimental conditions for the quantitative assessment of sulphur formation from (labile) sulphide and from thiosulphate.

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Introduction

Sulphide-containing effluents are usually treated by biological oxidation to sulphate. Conversion of sulphide to elemental sulphur could be an interesting alternative treatment process, because it allows the removal of solid sulphur, resulting in a decrease in the total sulphur content of waste water. A fixed-film reactor for sulphur production has already proven successful on the pilot-plant scale (Buisman et al. 1991). Improvement of this process requires understanding of the sulphur biochemistry and the (eco)physiology of the bacteria involved. However, the complexity of the bacterial community and the inhomogeneity of the pilot-scale reactor make the in situ study of the fundamentals of sulphur production difficult. Pure cultures grown under the defined conditions of a chemostat are more appropriate for this type of research.

In this study, neutrophilic *Thiobacillus* species have been chosen as model organisms because they almost always deposit the sulphur outside the cell (Kuenen 1989; Kelly and Harrison 1989). Although these bacteria have been extensively studied in sulphate-producing chemostat cultures (for reviews see Kelly 1982, 1989; Kuenen and Beudeker 1982), little systematic research on sulphur formation by thiobacilli has been carried out. The few reports are based on batch experiments using pulses of substrate (Parker and Prisk 1953; London and Rittenberg 1964; Saxena and Aleem 1973; Hazeu et al. 1986; Chan and Suzuki 1993), or perturbations of a steady state.

Investigations with sulphide as the substrate in aerobic cultures pose several practical problems. Sulphide is oxidized spontaneously by oxygen, especially in the presence of metal ions (Chen and Morris 1972). Being a weak acid, sulphide becomes increasingly volatile at low pH. At high pH it may precipitate with metal ions. It may also react chemically with elemental sulphur to form polysulphides (Giggenbach 1972). Finally, sulphide (0.15–1.0 mM) can inhibit thiobacilli (Hirayama

and Vetter 1989; Sublette and Sylvester 1987). Most of these problems can be avoided by growing the organisms under sulphide limitation in the chemostat (Beudeker et al. 1982).

This paper describes and compares properties of thiosulphate- and sulphide-limited chemostat cultures of *Thiobacillus* o and *T. neapolitanus* during chemolithoautotrophic growth. Furthermore, a quantitative description of elemental sulphur formation will be given. Special attention is paid to the quality of growth conditions during transient-state and steady-state sulphur production.

Materials and methods

Microorganisms

Thiobacillus species, strain o (LMD 82.6; Delft culture collection) was isolated and described previously (Suylen and Kuenen 1986). *T. neapolitanus*, strain x (Kuenen and Veldkamp 1973) was also obtained from the Delft culture collection (LMD 80.58). Both bacteria are obligate chemolithoautotrophs.

Media and culture conditions

Continuous culture medium contained ($g\,l^{-1}$): KH_2PO_4 0.5, K_2HPO_4 0.5, NH_4Cl 0.4, $MgSO_4 \cdot 7H_2O$ 0.8, and 2.0 $ml\,l^{-1}$ trace element solution described by Vishniac and Santer (1957), except that it contained 2.2 $g\,l^{-1}$ $ZnSO_4 \cdot 7H_2O$ instead of the originally reported 22 $g\,l^{-1}$. Thiosulphate was added after sterilization (12.5 g $Na_2S_2O_3 \cdot 5H_2O$ l^{-1}). Continuous cultures on sulphide received equal flows of mineral medium and anoxic sulphide solution (100 mM Na_2S). Sulphide was pumped into the culture (below surface level) through oxygen-impermeable tubing (butyl rubber, Teflon and Tygon). The mineral medium was double strength and additionally contained 5.5 ml $H_2SO_4\,l^{-1}$. When low-sulphate medium was required, H_2SO_4 was replaced by HCl (21.5 $ml\,l^{-1}$) and $MgSO_4 \cdot 7H_2O$ by $MgCl_2 \cdot 6H_2O$ (1.32 $g\,l^{-1}$). Chemostat cultures were grown at 30°C in 2-l fermentors (Applikon, the Netherlands). The pH was maintained at 6.8 by automatic titration with 1 M Na_2CO_3, which served as the carbon source for autotrophic growth. Batch culture medium (pH 7) contained ($g\,l^{-1}$): KH_2PO_4 4.0, K_2HPO_4 4.0, NH_4Cl 0.4, $MgSO_4 \cdot 7H_2O$ 0.8, $Na_2S_2O_3 \cdot 5H_2O$ 7.5, and 2.0 $ml\,l^{-1}$ of trace elements solution described above. Solid media were made by adding 1.6% (w/v) Difco Bacto agar. Culture purity was checked by microscopy and by streaking on thiosulphate/agar or inoculation in a rich medium (Suylen et al. 1986).

Transient-state chemostat cultures

Transient-state experiments were performed in a 1.3-l fermentor (Harder et al. 1974). The fermentor was equipped with a polarographic oxygen electrode (Ingold; detection limit 0.1 μM O_2), a redox/pH electrode (Phoenix) and a culture-recycling system (residence time: 10 s) with an optical sensor. Sulphur formation was detected by the increase in culture turbidity at 540 nm. The sulphide inlet was located above the culture surface. Each experiment was started with the effluent of a sulphate-producing, steady-state culture (dilution rate, $D = 0.06\,h^{-1}$; biomass concentration: 110–120 mg protein l^{-1}) which had been collected overnight at 0–4°C. After equilibration at 30°C, the culture was run for 10–15 min under the original steady-state conditions. The growth conditions were then changed, as described in the text. A typical experiment consisted of two or three transient-state periods of 20–30 min. During each period, the culture conditions were kept constant and four or five successive samples were taken for the analysis of sulphur compounds. When the effect of the substrate load was tested (at constant dissolved oxygen concentration), the medium supply rate was increased, rather than using a higher reservoir concentration of substrate. Oxygen-limiting conditions were obtained by vortex aeration (i.e. air supply above surface level; 1 l air/min) and different stirring rates ranging from 400 rpm to 500 rpm. When measurement of sulphate accumulation was required, steady-state cells were centrifuged and resuspended in mineral medium (with 8–10 mM sulphate), and low-sulphate medium was used for continuous cultivation. For experiments at different pH values, cultures were titrated with sodium carbonate or hydrochloric acid by changing the set-point of the pH control of the fermentor.

Sampling procedures for sulphur analysis

Samples from steady-state cultures containing high concentrations of sulphur were centrifuged (20 min, 38 000 *g*, 4°C) and washed twice with 50 mM phosphate buffer (pH 7.0). A faster inactivation method was required for the measurement of low concentrations of sulphur (e.g. in transient-state cultures). Such samples were filtered through nitrocellulose membranes with a pore size of 0.45 μm (Schleicher &, Schüll, type RC55) and then washed with ice-cold water. By taking identical intervals of 30 s between sampling and inactivation, the change in S concentration caused by the metabolic activity of the cells was apparently similar for each sample. Sulphur formation rates were thus intrinsically corrected for these errors.

Analysis of sulphur compounds

The sulphur/biomass residues obtained by filtration or centrifugation were extracted with acetone overnight. Extracts were analysed for elemental sulphur by cyanolysis (Bartlett and Skoog 1954), using NaSCN in acetone as a standard and unused filters as blanks. Elemental sulphur was also determined by HPLC (Möckel 1984) with a Waters HPLC using a Nucleosil-C18 column (Macherey-Nagel), with 97% methanol as eluent (flow rate: 1 $ml\,min^{-1}$) and detection by UV at 217 nm (detection limit: 5 μM S^0).

Supernatant and filtrate were used for the analysis of soluble sulphur compounds. Sulphate and thiosulphate were measured by HPLC (Gommers and Kuenen 1988). Thiosulphate, trithionate and tetrathionate were determined by cyanolysis (Kelly et al. 1969). Since polythionates and polysulphides would also be cyanolysed, negative results were interpreted as also being negative for polysulphides and polythionates. Sulphite was estimated with commercially available dipsticks (Merck, detection limit: 0.25 mM). Sulphide in the medium reservoir was measured by iodometric titration (Rand et al. 1985). H_2S in the off-gas of the fermentor was trapped in a solution of 2 M KOH and determined by the methylene blue method (Trüper and Schlegel 1964). Residual sulphide was determined as methylene blue, after rapid sampling (less than 2 s) of culture fluid into an equal volume of 0.2% (w/v) zinc acetate solution. Being unaware of the interference by sulphur, samples from *Thiobacillus* o cultures were analysed as such, which could have given underestimates of the sulphide concentration. In order to avoid this interference, sample treatment with *T. neapolitanus* was extended by anaerobic stripping of the sulphide from an acidified mixture to a second zinc acetate solution, using N_2 as the carrier gas.

Biomass determinations

Protein was determined by the microbiuret method (Goa 1953), using bovine serum albumin as a standard. Sulphur-containing

Table 1 Sulphur balances in steady-state chemostat cultures of *Thiobacillus* o. Cultures were grown as described in the text with Na_2S (sulphide influent concentration, $[S]_R$ = 49 mM). $[SO_4^{2-}]$ is the difference between the effluent and influent concentration of sulphate. Sulfur concentrations were obtained after membrane filtration ($[S^0]_{fil}$), centrifugation ($[S^0]_{cen}$), or by calculation from the carbonate titration ($[S^0]_{acid}$; see Materials and methods). The sulphur balance ($\Delta[S]$) was calculated using $[S^0]_{cen}$. *NM* not measured, *D* dilution rate

D (h^{-1})	$[S^{2-}]_{res}$ (mM)	H_2S loss (mM)	$[S^0]_{fil}$ (mM)	$[S^0]_{cen}$ (mM)	$[S^0]_{acid}$ (mM)	$[SO_4^{2-}]_{out}$ (mM)	$\Delta[S]$ (mM)	Biomass (mg/l)
0.06	0.008	0.6	0	0	0	48	0	116
0.18	NM	0.9	0	0	0.4	48	0	133
0.27	0.004[d]	0.8	< 0.6[a]	NM	0	48	0	124
0.31[b]	0.03[d]	2	4	29	32	21	−3	54
0.31[c]	0.04[d]	4	2	30	28	19	−4	46

[a]Value slowly decreasing
[b]Data obtained after 5 volume changes of sulphur formation
[c]Data obtained after 10 volume changes of sulphur formation
[d]Underestimated, because of the interference by sulphur

culture samples were pretreated by extraction with acetone and washing with 50 mM phosphate buffer. The correction made for protein loss by acetone extraction was 8% (determined with sulphate-producing chemostat cultures). The dry weight and biomass carbon of sulphate-producing cultures was measured as described previously (Suylen et al. 1986).

Activity measurements

Substrate-dependent oxygen uptake by whole cells was measured polarographically at 30°C with a biological oxygen monitor (Yellow Springs Instruments). The substrate concentrations used gave maximum biological oxidation rates. CO_2-fixing capacities by whole cells and ribulose bisphosphate carboxylase activity in cell-free extracts were measured according to Beudeker and co-workers (Beudeker et al. 1980).

Miscellaneous methods

The carbonate concentration in the titration solution and the acid concentration in mineral medium were determined by titration (Rand et al. 1985). The redox electrode was calibrated with saturated solutions of quinhydrone. The presence of elemental sulphur inside the cells was checked by electron microscopy after staining with $AgNO_3$, as used for *Thiobacillus ferrooxidans* (Hazeu et al. 1988). The oxygen consumption in the fermentor was monitored via analysis of the effluent gas (Van Urk et al. 1988). Prior to the analysis, H_2S and CO_2 were removed from the effluent gas by absorption in a solution of 2 M NaOH.

Calculation of sulphur production rates

Sulphur analysis showed that the accumulation of sulphur ($d[S]/dt$) in a transient state proceeded linearly in time. The net rate of sulphur formation (rS^0) could therefore be approximated by: $rS^0 = d[S]/dt + D[\bar{S}]$. Where, $[\bar{S}]$ is defined as the sulphur concentration half way through the transient-state period and D is the dilution rate (h^{-1}). Sulphur production rates were also calculated from oxygen consumption and carbonate titration rates using standard methods for stoichiometric balancing (Roels 1983). In these calculations, the sulphide consumption rate was taken as the sulphide load corrected for the H_2S loss via the off-gas and the sulphide accumulation in the culture.

Results

Comparison of sulphide- and thiosulphate-grown chemostat cultures

Steady-state chemostat cultures of *Thiobacillus* o and *T. neapolitanus*, grown with either sulphide or thiosulphate at $D = 0.06\ h^{-1}$ and 50% air saturation, completely oxidized the substrates to sulphate and gave comparable yields of dry weight (4.2–4.4 g/mol) and biomass protein (2.1–2.4 g/mol). Moreover, substrate-dependent oxygen uptake by these cultures was similar (tested with Na_2S, S^0, $Na_2S_2O_3$, $Na_2S_2O_3$ and Na_2SO_3). In agreement with earlier studies (Beudeker et al. 1982; Timmer-ten Hoor 1981), sulphide and thiosulphate thus proved to be interchangeable substrates for chemolithoautotrophic bacteria producing sulphate. The two strains were similar in many respects, except for the oxidation of exogeneously added elemental sulphur, which was eight times faster in *T. neapolitanus*.

Sulphur production by steady-state chemostat cultures

Sulphur-producing steady-state cultures, at 50% air saturation, could be obtained only at dilution rates (D) near the critical value, D_{crit}. This was accomplished by small ($0.04\ h^{-1}$) increments in D. Up to $D = 0.26\ h^{-1}$, these changes sometimes resulted in transient sulphur formation (less than 1 mM), especially in *Thiobacillus* o cultures, but the sulphur disappeared within a few hours and was normally not detected at steady state. Stable sulphur formation was observed with *Thiobacillus* o at $D = 0.31\ h^{-1}$ ($\mu_{max} = 0.33$–$0.35\ h^{-1}$). The sulphur-producing steady-state culture formed less biomass, the concentration of residual sulphide was higher, and almost 10% of the sulphide was lost as H_2S in the off-gas (see Table 1). Steady-state sulphur formation

Table 2 Sulphur balances in steady-state chemostat cultures of *T. neapolitanus*. Cultures were grown with Na_2S ($[S]_R = 48$ mM). Details are given in the legend of Table 1

D (h^{-1})	$[S^{2-}]_{res}$ (mM)	H_2S loss (mM)	$[S^0]_{fil}$ (mM)	$[S^0]_{cen}$ (mM)	$[S^0]_{acid}$ (mM)	$[SO_4^{2-}]_{out}$ (mM)	Δ[S] (mM)	Biomass (mg/l)
0.06	0.001	0.02	0	0	0	48	0	116
0.31	0.003	0.1	0	0	0	48	0	134
0.33[a]	0.04	0.3	6	6	15	23	19	34
0.33[b]	0.06	0.4	8	9	14	21	18	36

[a]Data obtained after 5 volume changes of sulphur formation
[b]Data obtained after 19 volume changes of sulphur formation

with *T. neapolitanus* was found at $D = 0.33\ h^{-1}$ (Table 2; $\mu_{max} = 0.35\ h^{-1}$). In both cultures, no oxidation products other than sulphur and sulphate were detected.

Phase-contrast microscopy and electron microscopy both proved that the elemental sulphur was deposited outside the cells. The quantification of the sulphur was strongly dependent on the method of sample preparation. Membrane filtration of *Thiobacillus* o cultures gave low amounts of sulphur, when compared to centrifugation (see Table 1). Sulphur concentrations obtained by centrifuging were correct, because they agreed both with S balances and with calculations made from acid balances (Table 1). After 30 h of sulphur formation by *Thiobacillus* o, the electrodes were visibly covered with sulphur and the control of dissolved oxygen and pH was lost. In *T. neapolitanus* cultures, centrifugation and filtration gave the same amount of sulphur, but the sulphur balances were incomplete (Table 2). Calculation of sulphur concentrations from acid balances was more in agreement with the sulphur balance (Table 2). This could be explained by the massive deposition of sulphur on fermentor surfaces and electrodes. The sulphur layer also contained biomass that contributed to sulphide oxidation. At $D = 0.35\ h^{-1}$ (not shown), the suspended cells almost washed out completely (biomass concentration: 2–3 mg C/l), and the bulk of sulphide (85%) was oxidized by the attached cells. Since the requirement for defined conditions and reproducible results was not satisfied in these long-term steady-state cultures, short-term transient-state experiments were developed to study sulphur formation from sulphide and thiosulphate.

The use of transient-state chemostat cultures

Instead of sacrificing a steady-state culture for each experiment, the overnight effluent from a sulphate-producing steady-state culture was used (see Materials and methods). The characteristics of the cells in this effluent (such as yield and oxidation capacities) were identical to those of a steady-state culture obtained by direct sampling. Transient-state sulphur formation, induced by a shift to oxygen limitation or high substrate loads, was also very similar. The transient-state sulphur formation rates were dependent on the growth history. Higher dilution (= growth) rates in steady state resulted in lower sulphur formation rates. All experiments were performed, therefore, with samples taken from steady-state cultures grown at a fixed *D* of $0.06\ h^{-1}$. Furthermore, a transient-state period was limited to 30 min, in order to diminish possible adaptation to higher growth rates, for example.

Transient-state sulphur production under oxygen limitation and different pH values

Transient-state cultures of *Thiobacillus* o supplied with sulphide or thiosulphate at a load of $3\ mmol\ l^{-1}\ h^{-1}$ at different pH values (pH 4–9) only formed sulphur under oxygen limitation (below 0.1% air saturation). Sulphur accumulated at a constant rate, provided that the oxygen transfer rate was not changed. Sulphur was not formed above pH 7.2. Instead, the substrate (sulphide or thiosulphate) gradually accumulated once oxygen became limiting. The cultures supplied with thiosulphate then also accumulated sulphite (up to 0.5–1.0 mM). The change in culture turbidity was used to estimate sulphur production rates under oxygen limitation semi-quantitatively. Both turbidity measurements and sulphur analyses indicated that sulphur production was similar with sulphide or thiosulphate as substrates. Sulphur formation under oxygen limitation was dependent on the oxygen transfer rate (controlled by the stirring rate; see Fig. 1). In order to maximize sulphur production, a control parameter for the oxygen supply was required. As turbidity measurements could only be used qualitatively to detect the onset of sulphur production, and oxygen gas analysis was not accurate enough at low oxygen uptake rates, the redox potential was tested. However, the rate of response recorded by the electrode was too slow (minutes) to use as a control parameter. Therefore, the maximum rate of sulphur formation was found by decreasing the stirring rate empirically, by hand. The redox potential was used as an indicator for sulphide accumulation. When this was done with *Thiobacillus* o at pH 6.5 and a sulphide load of $3\ mmol\ l^{-1}\ h^{-1}$, a maximum of 50% conversion to

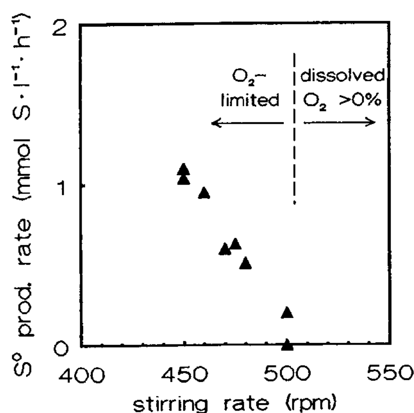

Fig. 1 Sulphur production by oxygen-limited, transient-state cultures of *Thiobacillus* o. Cultures were supplied with Na_2S (3 mmol l^{-1} h^{-1}). Oxygen limitation (less than 0.1% air saturation) was achieved by vortex aeration at different stirring rates

elemental sulphur was found. *T. neapolitanus* was able to convert a maximum of 68% of the sulphide to elemental sulphur under the same conditions.

Transient-state sulphur production at various sulphide loads and 50% air saturation

Transient-state cultures of *Thiobacillus* o also were tested at various sulphide loads and 50% air saturation. Sulphur was formed only at and above a load of 10.8 mmol S^{2-} l^{-1} h^{-1} (Table 3), while sulphide remained undetectable (below 1 μM). Once the maximum oxidative capacity of the culture was exceeded (at 13.8 mmol S^{2-} l^{-1} h^{-1}), sulphide accumulated up to 0.19 mmol l^{-1} and large amounts of H_2S were lost in the off-gas (Table 3). At this sulphide concentration, 5%–10% of the oxygen consumption in the fermentor can be attributed to chemical oxidation of sulphide

Table 3 Transient-state sulphur production by *Thiobacillus* o at different sulphide loads and 50% air saturation. The sulphate concentration in the culture was reduced to 10 mM prior to the experiments (see Materials and methods). rH_2S comprises the sulphide accumulation in the culture and the loss of H_2S via the off-gas. Δr is the difference between in and outcoming S flows (with a maximum error of 0.5–1.0 mmol S l^{-1} h^{-1}). Oxygen consumption rates (rO_2) determined by off-gas analysis were used to estimate sulphur production rates (rS^0_{O2}). (*NM* not measured)

Rates (mmol l^{-1} h^{-1})						
rS^{2-}	rS^0	rSO_4^{2-}	rH_2S	Δr	rO_2	rS^0_{O2}
2.8	0	NM	0	NM	5.1	0.06
5.5	0	5.4	0	0.1	10.2	< 0
8.2	0	7.5	< 0.1	0.7	14.6	0.2
10.8	1.7	8.9	0.1	0.1	17.3	1.7
13.8	2.8	9.5	1.7	− 0.2	19.2	2.2

(determined by oxygen uptake measurements in medium without cells). Just before sulphide accumulated in the transient state, approximately 20% of the sulphide supply was converted to elemental sulphur. The observed differences in the sulphur balance were within the limits of the experimental error, indicating that no sulphur compounds other than sulphate and elemental sulphur were formed. This was confirmed by the negative results of assays for other S compounds. Sulphur production rates calculated from oxygen consumption rates agreed with the experimental values for sulphur production (Table 3).

Transient-state cultures of *T. neapolitanus* (at 50% air saturation) also formed sulphur at and above a sulphide load of 10 mmol l^{-1} h^{-1} (not shown). Again, the highest sulphur formation rate was found when the sulphide-oxidizing capacity of the culture was exceeded at a load of 18 mmol Na_2S l^{-1} h^{-1}. At this point, 42% of the sulphide was converted to elemental sulphur.

Discussion

The experiments presented here show that transient-state continuous cultures are well suited for studying the oxidation of sulphide to elemental sulphur under defined environmental conditions. Attempts to achieve defined sulphur production in steady-state cultures failed because of the adherence of sulphur and biomass to fermentor surfaces. Although the overall sulphur formation by these cultures could always be quantified by calculation from stoichiometric balances (Table 2), culture conditions could not be adequately controlled. The agglomeration of sulphur has been reported earlier (e.g. Cadenhead and Sublette 1990), and is probably caused by the aging of bacterial (hydrophilic) sulphur to hydrophobic sulphur (Steudel et al. 1988). Also, the total quantity of sulphur formed in these experiments may have played a role. In the steady-state cultures, up to 30 mM S^0 could be formed, whereas a maximum of only 4 mM S^0 had accumulated at the end of a transient-state experiment. By using low sulphide influent concentrations (5–7 mM), steady-state, sulphur-producing *Thiobacillus thioparus* cultures have previously been obtained under oxygen limitation (Van den Ende and Van Gemerden 1993). Low influent sulphide concentrations (thus low S^0-concentrations) therefore seem advantageous for maintaining homogeneous sulphur-producing cultures. However, sulphide oxidation may occur biologically and non-biologically. Since low influent sulphide concentrations give less biomass, the relative contribution of chemical oxidation to the overall oxidation of sulphide may become high, especially when oxygen and sulphide are simultaneously present. Our interest was the biological formation of sulphur under various environmental conditions, including high dissolved oxygen concentrations. Hence, transient-state

experiments were more attractive, because they combined high biological activities and low sulphur concentrations.

Biomass-specific sulphide loads in transient-state experiments could be controlled by the rate of medium supply, because the biomass concentration did not change significantly during short-term experiments. Thus, sulphur formation could be studied at different loads under defined, sulphide-limiting conditions. During long-term experiments in the chemostat at a fixed dilution rate near D_{crit}, sulphur formation parallelled the wash-out of biomass, resulting in an (uncontrollable) increase of the biomass-specific sulphide load. Long-term sulphur formation was therefore always accompanied by accumulation of sulphide. This might have resulted in different side-effects, such as the chemical oxidation of sulphide (Chen and Morris 1972), loss of H_2S by the aeration, inhibition of bacterial respiration (Hirayama and Vetter 1989) and chemical reaction of sulphide with S^0 to form polysulphides (Giggenbach 1974; Steudel 1988). The latter might explain the low sulphur yields obtained by membrane filtration of samples from steady-state cultures of *Thiobacillus* o (see Table 1). Evidently, transient-state experiments were less prone to such phenomena.

A technical process for sulphur production will consist of (partly) immobilized biomass. Hence, the abovementioned wash-out of biomass due to (long-term) sulphur production, coupled to the problems associated with the accumulation of sulphide, is probably less important in these systems. Furthermore, immobilized systems are less vulnerable to a fluctuating supply of sulphide. In fact, fluctuating conditions might favour the efficiency of the process, as transient-state experiments showed that sulphur production was faster at high sulphide loads, with cells previously grown at low rates. Oxygen-limiting conditions gave the highest sulphur production rates during transient state experiments (see Table 4). In a technical process, oxygen gradients inside biolayers will probably result in oxygen-limiting conditions. Oxygen-limited conversion of sulphide will therefore contribute to sulphur production as well, but this process is difficult to control. It may be influenced by many factors, including the biofilm thickness and activity, the sulphide load, and the oxygen transfer rate. It is evident from our studies that high sulphide loading rates should be the preferred method to optimize sulphur production, as it is relatively easy to control.

This study showed that transient-state experiments can be used to study sulphur formation in homogeneously suspended, substrate-limited, continuous cultures. These experiments are especially suited for the establishment of the biological factors that play a role in elemental sulphur formation. This includes the influence of the type of organism and growth conditions (for summary see Table 4), and the growth history (e.g. the type of growth limitation in the steady state). Such results can be useful for the optimization and control of biological processes (e.g. waste-water treatment) designed for sulphur formation.

Table 4 Overview of sulphur-producing capacities of transient-state cultures of *Thiobacillus* o and *T. neapolitanus* under different environmental conditions. Under oxygen limitation, the oxygen supply was optimized for sulphur production. At 50% air saturation, the sulphide load was equal to the sulphide-oxidizing capacity, *DO* dissolved O_2

Organism	rS^{2-} (mmol Na_2S $l^{-1}h^{-1}$)	DO (% air saturation	Na_2S conversion to S^0 (%)
Thiobacillus o	3	0	50
T. neapolitanus	2.7–10	0	60
Thiobacillus o	18	50	20
T. neapolitanus	18	50	42

Acknowledgements The authors are grateful to S. Roukema for excellent experimental assistance. This research was supported by the Netherlands Technology Foundation (STW).

References

Bartlett JK, Skoog DA (1954) Colorimetric determination of elemental sulfur in hydrocarbons. Anal Chem 26:1008–1011

Beudeker RF, Cannon GC, Kuenen JG, Shively JM (1980) Relations between D-ribulose-1,5-bisphosphate carboxylase, carboxysomes and CO_2-fixing capacity in the obligate chemolithotroph *Thiobacillus neapolitanus* grown under different limitations in the chemostat. Arch Microbiol 124:185–189

Beudeker RF, Gottschal JC, Kuenen JG (1982) Reactivity versus flexibility in thiobacilli. Antonie van Leeuwenhoek 48:39–51

Buisman CJN, Lettinga G, Paasschens CWM, Habets LHA (1991) Biotechnological sulphide removal from effluents. Water Sci Technol 24:347–356

Cadenhead P, Sublette KL (1990) Oxidation of hydrogen sulphide by thiobacilli. Biotechnol Bioeng 35:1150–1154

Chan CW, Suzuki I (1993) Quantitative extraction and determination of elemental sulfur and stoichiometric oxidation of sulfide to elemental sulfur by *Thiobacillus thiooxidans*. Can J Microbiol 39:1166–1168

Chen KY, Morris JC (1972) Oxidation of aqueous sulfide by O_2: 1 General characteristics and catalytic influences. In: Jenkins SH (ed) Advances in water pollution research III-32, Pergamon Press, New York, pp 1–17

Giggenbach W (1974) Optical spectra and equilibrium distribution of polysulfide ions in aqueous solution at 20°C. Inorg Chem 11:1201–1207

Goa J (1953) A microbiuret method for protein determination; determination of total protein in cerebrospinal fluid. Scand J Clin Lab Invest 5:218

Gommers PJF, Kuenen JG (1988) *Thiobacillus* strain Q, a chemolithoheterotrophic sulphur bacterium. Arch Microbiol 150:117–125

Harder W, Visser K, Kuenen JG (1974) Laboratory fermentor with an improved magnetic drive. Lab Pract 23:241–249

Hazeu W, Bijleveld W, Grotenhuis JTC, Kakes E, Kuenen JG (1986) Kinetics and energetics of reduced sulphur oxidation by chemostat cultures of *Thiobacillus ferrooxidans*. Antonie van Leeuwenhoek 52:507–518

Hazeu W, Batenburg WH, Bos P, Plas RK van der, Kuenen JG (1988) The production and utilization of intermediairy elemental sulfur during the oxidation of reduced sulfur compounds by *Thiobacillus ferrooxidans*. Arch Microbiol 150:574–579

Hirayama A, Vetter RD (1989) Kinetics of sulfide and thiosulfate oxidation by the hydrothermal vent bacterium *Thiomicrospira crunogina* and comparison with *Thiobacillus neapolitanus*. In: Abstracts of the Annual Meeting of the American Society of Microbiology, Washington, I-43

Kelly DP (1982) Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. Philos. Trans R Soc Lond [Biol] 298:499–528

Kelly DP (1989) Physiology and biochemistry of unicellular sulphur bacteria. In: Schlegel HG, Bowien B (eds) Autotrophic bacteria, Springer Verlag, Berlin, pp 193–213

Kelly DP, Harrison AP (1989) The genus *Thiobacillus*. In: Staley JT, Pfennig N, Bryant MP, Holdt JG (eds) Bergey's manual of determinative bacteriology, vol 3. Williams & Wilkins, Baltimore, pp 1842–1858

Kelly DP, Chambers LA, Trudinger PA (1969) Cyanolysis and spectrofotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal Chem 41:899–901

Kuenen JG (1989) Colorless sulfur bacteria. In: Staley JT, Pfennig N, Bryant MP, Holdt JG (eds) Bergey's manual of determinative bacteriology, vol 3. Williams & Wilkins, Baltimore, pp 1834–1837

Kuenen JG, Beudeker RF (1982) Microbiology of thiobacilli and other sulphur-oxidizing autotrophs, mixotrophs and heterotrophs. Philos Trans R Soc Lond [Biol] 298:473–497

Kuenen JG, Veldkamp H (1973) Effects of organic compounds on growth of chemostat cultures of *Thiomicrospira pelophila*, *Thiobacillus thioparus* and *Thiobacillus neapolitanus*. Arch Mikrobiol 94:173–190

London J, Rittenberg SC (1964) Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. Proc Natl Acad Sci USA 52: 1183–1190

Möckel HJ (1984) Retention of sulphur and sulphur organics in reversed phase liquid chromatography. J Chromatogr 317:589–614

Parker CD, Prisk J (1953) The oxidation of inorganic compounds of sulphur by various sulphur bacteria. J Gen Microbiol 8:344–364

Rand MC, Greenberg AE, Taras MJ (1985) Standard methods for the examination of water and waste-water, 16th edn. APHA, AWWA, WPCF, New York

Roels JA (1983) Energetics and kinetics in biotechnology, Elsevier Biomedical Press, Amsterdam

Saxena J, Aleem MIH (1973) Oxidation of sulfur compounds and coupled phosphorylation in the chemoautotroph *Thiobacillus neapolitanus*. Can J Biochem 51:560–568

Steudel R (1988) On the nature of the "elemental sulfur" (S^0) produced by sulfur-oxidizing bacteria-a model for S^0 globules In: Schlegel HG, Bowien B (eds), Autotrophic bacteria, Springer Berlin Heidelberg New York, pp 289–303

Steudel R, Göbel T, Holdt G (1988) The molecular composition of hydrophilic sulfur sols prepared by acid decomposition of thiosulfate [1]. Z Naturforsch 43b:203–218

Sublette KL and Sylvester ND (1987) Oxidation of hydrogen sulfide by *Thiobacillus denitrificans*: desulfurization of natural gas. Biotechnol Bioeng 29:249–257

Suylen GMH, Kuenen JG (1986) Chemostat enrichment and isolation of *Hyphomicrobium* EG, a dimethyl sulphide oxidizing methylotroph and reevaluation of *Thiobacillus* MS1. Antonie van Leeuwenhoek 52:281–293

Suylen GMH, Stefess GC, Kuenen JG (1986) Chemolithotrophic potential of a *Hyphomicrobium* species, capable of growth on methylated sulphur compounds. Arch Microbiol 146:192–198

Timmer-Ten Hoor A (1981) Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. Antonie van Leeuwenhoek 47:231–243

Trüper HG, Schlegel HG (1964) Sulphur metabolism in *Thiorhodaceae*. 1. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek, 30:225–238

Van den Ende FP and Van Gemerden H (1993) Sulfide oxidation under oxygen limitation by a *Thiobacillus thioparus* isolated from a marine microbial mat. FEMS Microbiol Ecol 13:69–78

Van Urk H, Mak PR, Scheffers WA, Van Dijken JP (1988) Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. Yeast 4:283–291

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195–213