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Authors: D.W. Shiers, D.E. Ralph, H.R. Watling

PII: S1369-703X(11)00041-6
DOI: doi:10.1016/j.bej.2011.02.018
Reference: BEJ 5290

To appear in: *Biochemical Engineering Journal*

Received date: 16-3-2010
Revised date: 21-2-2011
Accepted date: 26-2-2011

Please cite this article as: D.W. Shiers, D.E. Ralph, H.R. Watling, Batch culture of *Acidithiobacillus caldus* on tetrathionate, *Biochemical Engineering Journal* (2010), doi:10.1016/j.bej.2011.02.018

Batch culture of *Acidithiobacillus caldus* on tetrathionate

D.W. Shiers[1,3], D.E. Ralph[2,3] and H.R. Watling[1,3]

[1]CSIRO Minerals Down Under Flagship, CSIRO Process Science and Engineering, PO Box 7229, Karawara, Western Australia 6152, Australia.

[2]School of Chemical and Mathematical Sciences, Murdoch University, South Street, Murdoch, Western Australia 6150, Australia.

[3]Parker Cooperative Research Centre for Integrated Hydrometallurgy Solutions,

Abstract

Acidithiobacillus caldus (DSM 8584) grew aerobically in minimal medium at 45 °C with potassium tetrathionate as the sole energy source. Oxidation of tetrathionate during batch culture involved the production of sulfite, thiosulfate, penta- and hexathionate which were then consumed after the tetrathionate was exhausted. Average growth yields over the batch were 3.5 g(dry wt) mol$(S_4O_6)^{-1}$, somewhat less than yields reported for continuous growth on the same substrate. Thiosulfate was unstable under sterile culture conditions and reacted spontaneously to give tetra-, penta- and hexathionate. It is suggested that the occurrence of polythionates during growth of *At. caldus* on tetrathionate is due to formation of thiosulfate as the first step in tetrathionate oxidation. Observed growth yields were compared with a thermodynamic framework which suggested a growth efficiency of *ca* 10 %. The pattern of growth yield and thermodynamic analysis suggest the formation of elemental sulfur although this was not observed.

Keywords: *Acidithiobacillus caldus*, tetrathionate oxidation, cell yields, polythionates.

1. Introduction

The oxidation of sulfur from its state in a solid mineral sulfide, to its stable soluble form as the sulfate ion, is a complex but important hydrometallurgical process. The complexity of the process arises from sulfur's unique chemistry while its importance is reflected by the amount of base metals recovered from sulfidic ores. A large volume of work has been published about this complex process that can be simply described as the oxidation of sulfur, or sulfide, to sulfate. With the purpose of understanding the aqueous bio-hydrometallurgical environment, this work is focussed on the synthesis of the chemolithotrophic bacterial cells that use sulfur as an energy source while catalysing the release of base metal cations into solution. The approach taken was to determine the yield of biomass produced autotrophically and examine the efficiency with which cells use chemical energy to produce more cells. In non-sterile biohydrometallurgical processes where sulfur is a major energy source and competition between species exists information about cell yields may assist in understanding the succession of species as time proceeds. The efficiency of energy conversion from substrate to cell mass is expected to be a major factor in the success of a particular species in an environment. The amount of chemical energy available within a system undergoing change sets a maximum thermodynamic limit to the growth of cells catalysing the change therefore an understanding of the efficiency of the conversion of chemical energy into bacterial biomass is required.

In taking this approach, an attempt has been made to combine the thermodynamic analysis of sulfur oxidation developed by Kelly [1] with a representation of carbon dioxide reduction that reflects the average composition of chemolithotrophic biomass [2-4]. Although the oxidative path from elemental sulfur to sulfate is now better understood [5-7], it is not possible to define a closed system that includes the anodic sulfur couple, the electron transport and reduction couples. The analysis attempted here is that of the system containing

a volume of liquid medium that includes chemolithotrophic cells and their required nutrients all at thermal equilibrium. The change is described by a general equation (1) and it is assumed that no gaseous or solid products, other than those determined gravimetrically as cell mass, are formed.

Substrates + cells = more cells + water (1)

1.1. Yield of biomass from generated energy

Equation (1) is the sum of three reactions describing the change in this system as the cells grow, an anodic reaction suppling electrons from the oxidation of the substrate and two cathodic reactions. The first cathodic reaction results in a net reduction of CO_2 to biomass while the second reduces oxygen and protons to water.

These three general reactions are considered in terms of their potential in a schematic energy diagram (Fig. 1) where electrons spontaneously flow 'uphill' toward higher potentials [4]. The anodic half-reaction is combined with each of the O_2 and CO_2 reduction half-reactions to produce two electrochemical cells which produce and consume energy respectively.

FIG 1 NEAR HERE

In Fig. 1 the potential of the electrochemical cells E_{ox} and E_g, the respective energy generating and consuming reactions, can be used to calculate a theoretical maximum yield (Y_{max} cell mass/substrate) by assuming no resistance to charge transfer and 100 % efficient coupling between the energy producing (E_{ox}) and consuming (E_g) reactions. The energy to drive 1.0 equivalent of charge through E_g is derived from the movement of $E_g/|E_{ox}|$ equivalents through E_{ox}. A total of $\{1 + (E_g/|E_{ox}|)\}$ equivalents would have been removed

from the anodic couple for the formation of one equivalent of cell mass. The sign change for E_{ox} in equation 2 is due to the convention of expressing the potential energy of a cell as $E_{anodic} - E_{cathodic}$. Values calculated for Y_{max} represent a theoretical maximum amount of growth and a comparison with the observed yields (Y_{obs}) gives the growth efficiency under the particular solution conditions.

$$Y_{max} = 1/\{1 + (E_g/|E_{ox}|)\} \quad (2)$$

1.2. Net cathodic reactions in the system

The reduction of CO_2 to form cell biomass can be expressed as a half-reaction consuming one equivalent of electrons (1 equiv) with biomass represented as normalised ratios of the elements C, H, O and N [8-10]. The mass and charge balance is given in Eq. 3 where 1 equiv of electrons is consumed and 0.243 C-mol of biomass formed [2, 9].

$$0.243CO_2 + 0.061NH_4^+ + 0.94H^+ + e^- = 0.243CH_{1.70}O_{0.42}N_{0.25}(s) + 0.384H_2O(l) \quad (3)$$

Where no phase description is given, reactants and products are assumed to be in the aqueous phase taking their appropriate forms at pH 2. Using data from the enthalpy of combustion of dried chemolithotrophic cells and the estimate of biomass formation entropy published by Blight and Ralph [4], the standard Gibbs energy for reaction 3 was calculated as $\Delta_3G°$= +5.1 kJ. This value is somewhat larger than that calculated earlier (ibid) due to the slightly different reaction used here (Ferguson and Ingledew [11]). The prevailing conditions in the bulk medium control the actual Δ_3G value, estimated using equations 3a-c [11].

$\Delta_3G = \Delta_3G° + RT\ Ln\{a_{CO2}^{-0.243}\ a_{NH4}^{-0.061}\ a_H^{-0.94}\}$ (3a)

$\Delta_3G = +21.6\ kJ\ equiv^{-1}$ (3b)

and $E_3 = -\Delta_3G/F = -0.22\ V$ (3c)

Equation (3) represents a general and potentially useful representation of the system change provided that the biomass term ($CH_{1.70}O_{0.42}N_{0.25}$) is an adequate representation of the products of the change. The second reduction reaction combines protons and oxygen and a value for $\Delta_4G° = -118.7$ kJ is given by Lide [12]. The actual potential Δ_4G can be calculated assuming that the water produced is released to the bulk medium (Eq. 4 - 4c) and the activities are those within the bulk medium [11].

$e^- + H^+ + 0.25O_2 = 0.5H_2O(l)$ (4)

$\Delta_4G = \Delta_4G° + RT\ Ln\{a_H^{-1}\ a_{O2}^{-0.25}\}$ (4a)

$\Delta_4G = -105.2\ kJ$ (4b)

$\Delta_4E = -\Delta_4G\ /F = +1.09\ kJ\ equiv^{-1}$ (4c)

1.3. Anodic reactions

The potential of the anodic reaction depends on the nature and activity of the initial and final states of the substrate oxidised. This is more complicated for growth on sulphur oxy ions compared to growth on iron because the chemical species that releases electrons into the chemiosmotic circuit is not known. Kelly [1] suggests that electrons from the oxidation of the sulfate/sulfite couple (SO_4^{2-}/SO_3^{2-}) are used in the chemiosmotic circuit (Eq. 5) and data from this reaction are used in the present analysis. The standard Gibbs energy of reaction 5

and its theoretical potential can be found from standard tables ($\Delta_5G° = -10.4$ kJ, $E_5° = +0.11$ V).

$$0.5SO_3^{2-} + 0.5H_2O(l) = 0.5SO_4^{2-} + H^+ + e^- \quad (5)$$

$$\Delta_5G = \Delta_5G° + RT\ Ln\{a_H\ a_{SO4}^{0.5}\ a_{SO3}^{-0.5}\} \quad (5a)$$

The value for E_5 depends on the activities of the components in equation 5a. The activity of sulfite (SO_3^{2-}) is difficult to define because it is an intermediate during oxidation of sulphur oxy ions. It reacts with oxygen and can be protonated to form SO_2(g) (pK_{a1} 1.9 and pK_{a2} 7.2, [12]) giving a characteristic smell. Assuming the activity of SO_3^{2-} is low but constant throughout a batch culture (0.25 mM) where the activities of the proton and SO_4^{2-} change characteristically, a potential range for this half–reaction of $0.16 < E_5 < 0.21$ V can be calculated. Lowering the estimated activity of SO_3^{2-} by a factor of 10 reduces this range to $0.13 < E_5 < 0.17$ V showing that the theoretical potential of the SO_4^{2-}/SO_3^{2-} couple is relatively insensitive to low SO_3^{2-} activity. Using a value of 0.2 V for E_5 it is possible to calculate values of Y_{max} from equation 2; $E_g = E_5 - E_3 = +0.42$ V and $|E_{ox}| = E_4 - E_5 = +0.89$ V and $Y_{max} = 0.66$.

It is also possible that a non-sulfur mediator (cytochrome c551, $E° = +0.24$ V, [13]) is the substrate that supplies electrons into the chemiosmotic circuit (reaction (6)).

$$Cyt_{red} = Cyt_{ox} + H^+ + e^- \quad (6)$$

$$\Delta_5G = \Delta_5G° + RT\ Ln\{a_H\ a_{Cox}^{+0.5}\ a_{Cred}^{-0.5}\} \quad (6a)$$

Using the same logic and equal activities of the oxidised and reduced forms of the cytochrome, a range of values $0.36 < E_6 < 0.40$ is found and $Y_{max} = 0.58$ can be calculated.

However, most observed yields are given in units of g(biomass) per mol(substrate) and the following conversion factors are necessary; 4.11 equiv($CH_{1.7}O_{0.42}N_{0.25}$) per C-mol($CH_{1.7}O_{0.42}N_{0.25}$) and 5.83 g($CH_{1.7}O_{0.42}N_{0.25}$) per equiv($CH_{1.7}O_{0.42}N_{0.25}$)(equation 3). For Y_{max} = 0.68 values of {0.68/4.11} Y_{max} = 0.17 C-mol equiv^{-1} and {0.68×5.83=} 3.96 g($CH_{1.7}O_{0.42}N_{0.25}$) equiv^{-1} can be calculated. Correcting this value for characteristic values of ash and bound moisture gives Y_{max} = 4.5 g(dry wt.) equiv(anode)$^{-1}$ [4, 14]. In converting anodic equivalents to mole of substrate we have used the argument of Kelly [1] that only the electrons from the oxidation of sulfite are 'conserved' to the chemiosmotic circuit and therefore one mole of tetrathionate yields 4 mole of sulfite and thus 8 equiv of electrons. The Y_{max} expected for growth on tetrathionate is then 36 g(dry wt.) mol(S_4O_6)$^{-1}$. Published values for the observed yield (Y_{obs}) of chemolithotrophic organisms growing on polythionate ions vary [14-17] but Kelly [1] shows that the 'apparent efficiencies' (Y_{obs}/Y_{max}) lie in the range $0.056 < Y_{obs}/Y_{max} < 0.12$. It was considered worthwhile to examine the growth of a defined species, *Acidithiobacillus caldus*, on tetrathionate in batch cultures to determine yield of cells. *At. caldus* is a moderate thermophile and grows in minimal medium at 45 °C.

2. Materials and methods

2.1. Data acquisition

All chemicals used in this study were analytical grade reagents (AR) unless otherwise stated and all solutions were prepared with de-ionised water. A pH meter (TPS – Smartchem model) and glass membrane electrode (Ionode GL20) were used to measure pH and were calibrated using pH 1.00, 1.68, 2.00, 3.56 and 4.00 buffers [18]. For pH calibration, the buffers and probe were heated to 45 ± 0.5 ºC. Cell counts were performed using a Helber Bacteria Counting Chamber (Thoma ruling, 0.02 mm cell depth). Optical density (OD) was

determined using a 5.0 cm path-length *in-situ* photometer at a wavelength of 638 nm [19, 35]. Concentrations of thiosulphate, trithionate, tetrathionate, pentathionate and hexathionate were determined using a Waters 2695 HPLC separation module utilising an Ionpac AS16 ion exchange column. All analytes were detected using a Waters 2996 Photodiode Array Detector at 214 nm except trithionate, which was detected at 192 nm [20]. A pump flow rate of 1.5 mL min^{-1} was used and the column temperature was maintained at 25 °C. A sodium perchlorate solution (0.15 M) was used as the eluent. The software package 'Empower' was used to integrate spectra.

2.2. Growth media and inoculation

Acidithiobacillus caldus (DSM 8584) was maintained in a minimal medium consisting of $K_2S_4O_6$ as the sole energy source supplemented by macro and micro-nutrients. A macro-nutrient concentrate consisting of $(NH_4)_2SO_4$ (8.60 g), K_2HPO_4 (1.80 g), $MgSO_4.7H_2O$ (3.90 g) dissolved in 1.00 L of deionised water and adjusted to pH 2.50 ± 0.05 with concentrated H_2SO_4 was prepared. A micro-nutrient concentrate consisting of $CoSO_4.7H_2O$ (2.49 g), $CuSO_4.7H_2O$ (2.81 g), $MnSO_4.H_2O$ (1.69 g), $(NH_4)_6Mo_7O_{24}.4H_2O$ (1.77 g), $NiSO_4.6H_2O$ (2.62 g) and $ZnSO_4.7H_2O$ (2.87 g) dissolved in 1.00 L of deionised water and adjusted to pH 2.50 ± 0.05 with concentrated H_2SO_4 was prepared. The minimal medium was prepared by combining 10 mL of macro-nutrient concentrate and 0.5 mL of micro-nutrient concentrate in 990 mL of pH 2.5 H_2SO_4 solution. This solution was autoclaved at 121 °C for 30 minutes. After cooling, 0.75 g of $K_2S_4O_6$ was dissolved in 30 mL of the autoclaved medium and filter-sterilized back into the 1 L solution to prepare the final medium (2.5 mM tetrathionate). Shake-flask cultures (100 mL) were sub-cultured every 72 hours under laminar flow using sterile techniques to provide inocula for the 1.0 L reactor experiments. Cells grown on

minimal medium were collected by filtration using 0.45 μm pore-size membranes. These cells were resuspended in 20 mL of medium; 10 mL was added to the 1 L reactor and cell counts performed on the remainder. Blank experiments where the tetrathionate medium was not inoculated were conducted. Experiments where the tetrathionate was replaced by 1 mM thiosulfate in the uninoculated medium were also conducted.

2.3. Reactor experiments

A jacketed glass reactor contained a 1.0 L charge of minimal medium was aerated, stirred and maintained at 45 °C. The pH of the medium in the 1.0 L reactor was monitored but was not controlled. The batch culture reactor experiments used an initial pH ~ 2.5 which fell to *ca* pH 1.9 at the completion of the cycle. Data from the optical probe and pH were logged continuously via a data-taker (DT50) over the course of the batch culture cycle. Periodic cell counts were made to check the calibration between the optical probe readings and cell numbers. A Gilson 222 XL Liquid Handler was used to sample 4 mL from the reactor every three hours to provide samples for HPLC analysis. Bacterial activity in these samples was quenched through the addition of 1 mL of saturated NaCl solution.

2.3. Determination of cell yields

The correlation between cell numbers and cell dry mass was determined by filtering 2.0 L volumes of cell suspension through 0.45 μm pore-size membranes after counting solution cell numbers. The filters were dried under vacuum at 22 °C to constant weight before and after filtering. Bacterial cell numbers attached to the reactor walls were estimated by a total DNA extraction of planktonic and attached populations. Planktonic cells were harvested by

filtration through a 0.45 μm pore-size membrane and resuspended in a pH 1.8 sulfuric acid solution. DNA was extracted using a modified method described in Plumb et al. [21]. Cell lysis was achieved using lysozyme. DNA was quantified using a NanoDrop spectrophotometer as described in Zammit et al. [22]. Attached bacteria were detached from the reactor walls via agitation with a 1% Tween 20 detergent solution for 45 minutes. Cells were collected and DNA quantified as for the planktonic cells. Attached cell numbers were estimated to be less than 2.5 % of the planktonic cell numbers.

3. Results

3.1. Batch culture experiments

In the medium containing tetrathionate, inoculation promoted significant growth of cells and an increase in the optical density was observed. During each batch culture experiment the pH fell from 2.5 to 1.9 while cell numbers increased; the tetrathionate concentration fell to zero after *ca* 60 hours (Fig. 2). The change in tetrathionate concentration in uninoculated flasks was insignificant over 60 hours (Fig. 2). This same pattern of tetrathionate utilisation was reliably reproduced by *At. caldus* in replicate experiments. At the same time, analysis of the medium filtrate revealed the concomitant production of other polythionate species (Fig. 3).

FIGURE 2 NEAR HERE

Experiment 13 was typical of the batch experiments and showed a complex pattern of polythionate occurrence (Fig. 3). No significant loss of tetrathionate or formation of polythionates was observed over 80 hours in sterile experiments but the presence of *At. caldus* resulted in the formation of thiosulfate, penta- and hexathionate from the point of inoculation. No trithionate was detected during any of the batch experiments. However,

traces of sulfite were detected at very low concentration and could not be quantified reliably (<1 μM). Cell growth during the first 20 hours was minimal, although a significant reduction in the tetrathionate concentration occurred during this period. Towards the end of the experiment, tetrathionate was exhausted first followed by pentathionate, thiosulfate and finally by hexathionate.

FIGURE 3 NEAR HERE

3.2. Yields from batch culture experiments

Four replicate experiments with 2.0 L of inoculated medium were incubated until the substrate was exhausted. The numbers of cells were counted and the media filtered using pre-weighed 0.45 μm pore-size membranes which were then dried to constant weight. These data were used to calculate the equivalence between cell number and dry weight and values of Y_{obs} for *At. caldus* cells (Table 1).

TABLE 1 NEAR HERE

The calculated value of the equivalence between cell number and cell dry weight was 4.6×10^{12} cell g(dry wt.)$^{-1}$, comparing favourably with the value derived for a mixed culture of iron-oxidising cells (6.3×10^{12} cell g^{-1}, [4]), given the uncertainty involved in cell counts. By considering the amounts of 'bound moisture' and ash present in the dry cell mass (12.4 %, [4, 17, 23]) the number of C-mole and the number of electron equivalents represented by that biomass were calculated. The conversion figures from the above references and implicit in Eq. 3 are:

1 g(dry wt.) = 0.876 g($CH_{1.7}O_{0.42}N_{0.25}$);

1 C-mol ($CH_{1.7}O_{0.42}N_{0.25}$) = 23.93 g($CH_{1.7}O_{0.42}N_{0.25}$);

1 C-mol($CH_{1.7}O_{0.42}N_{0.25}$) = 4.11 equiv($CH_{1.7}O_{0.42}N_{0.25}$);

1 equiv($CH_{1.7}O_{0.42}N_{0.25}$) = 5.83 g (dry wt.) [2, 4].

The appearance of many different intermediates during the experiment makes any energetic analysis daunting but the initial and final states of the batch culture are well defined. In the final state, it was assumed that all reduced sulfur had been converted to sulfate and that the inoculum numbers, wall growth and losses due to cell lysis were insignificant. The observed biomass yield over the period of 3.53 g(dry wt.) mol$(S_4O_6)^{-1}$ corresponds to a value for Y_{obs} = 0.53 equiv($CH_{1.7}O_{0.42}N_{0.25}$) mol$(S_4O_6)^{-1}$ (Table 1).

3.3. Acidic reactions of thiosulfate

Medium was made up with 1 mM thiosulfate substituted for the tetrathionate and incubated without *At. caldus* for 160 hours. Over that period the thiosulfate reacted to produce a mixture of higher polythionates (Fig. 4). Tetra-, penta- and hexathionate were all found in the mixture but no light scattering due to solid sulfur was observed. A mass balance around sulfur as the tetra-, penta- and hexa- forms showed that there was no significant conversion to sulfur or sulfate.

FIGURE 4 NEAR HERE

4. Discussion

4.1. Growth yields on tetrathionate

Endeavouring to understand these results, previous studies of autotrophic cells growing on polythionates were examined but no consensus between the published values of Y_{obs} emerged. Hazeu et al. [14] reported yields for *At. ferrooxidans* growing on tetrathionate in continuous culture at pH = 3 between 6 and 12 g(dry wt.) mol^{-1} depending on the dilution rate. Wood et al. [16] studied four *Sulfolobus* strains at 65 °C and pH = 3 and found yields of

7 g(dry wt.) mol$(S_4O_6)^{-1}$ for continuous growth on tetrathionate. Mason and Kelly [17] reported Y_{obs} values for *Acidiphilium acidophilum* (previously *Thiobacillus acidophilus*) of 15.6 g(dry wt.) mol$(S_4O_6)^{-1}$ during continuous growth on tetrathionate at 30 °C and pH =3. Wood and Kelly [15] reported *ca* 15 g(dry wt.) mol$(S_4O_6)^{-1}$ for *Thermithiobacillus tepidarus* (previously *Thiobacillus tepidarus)* growing at 45 ºC and pH = 7. There are no reported Y_{obs} values for growth on sulfite to our knowledge and yields for growth on elemental sulfur are problematic due to the difficulty in estimating cell numbers attached to the solid phase. The Y_{obs} from this study 3.53 g(dry wt.) mol$(S_4O_6)^{-1}$ (± 14 %) is significantly lower than these reports, possibly due to the use of batch culture and a lower pH range (1.9 < pH < 2.5).

Further comparison of Y_{obs} with Y_{max} calculated from the energy frame-work of Fig. 1 requires a value for the anodic couple (reactions (5) and (6)). The thermodynamic maximum yields (Y_{max}) predicted from these two possible anodic reactions are 0.66 (sulfite/sulfate) and 0.58 (cytochrome couple) equiv$(CH_{1.7}O_{0.42}N_{0.25})$ equiv(anode)$^{-1}$. Comparing this to the observed value, Y_{obs} = 0.53 equiv$(CH_{1.7}O_{0.42}N_{0.25})$ mol$(S_4O_6)^{-1}$ (Table 1), still presents at least two problems, the number of equivalents from tetrathionate coupled to growth via the chemiosmotic circuit and the efficiency of the coupling between energy generating and energy consuming reactions. Following Kelly's argument [1] of 8 equiv(anodic) mol$(S_4O_6)^{-1}$, a value for Y_{obs} of 0.066 equiv(biomass) equiv(anode)$^{-1}$ can be calculated giving 'apparent efficiencies' of *ca* 10 % for anodic reactions 5 and 6. These coupling efficiencies are similar to the value estimated by Kelly [1] for growth on tetrathionate (9.4 %) using the published framework based on Gibbs energy calculations. However, they are somewhat lower than the 20 - 40 % range most often quoted for microbial growth efficiency [10, 24-27]. This could be because growth on sulfur-oxy species is inherently less efficient due to the unique chemistry of the species and environment or that the number of electrons conserved to the

chemiosmotic circuit is less than the 8 potentially available from the oxidation of each mole of $S_4O_6^{2-}$ when metabolised via sulfite.

4.2. Production of polythionates during batch experiments.

The appearance and subsequent oxidation of polythionates during growth of *At. caldus* on tetrathionate has been reported by other workers [23, 28, 29]. Given the stability of tetrathionate in uninoculated experiments, these polythionate species must arise from some aspect of the metabolic activities of *At. caldus*. A tetrathionate hydrolase enzyme was isolated from *At. caldus* DSM8584 [30] in the periplasm fraction and the tentative stoichiometry proposed resulted in the disproportionation of tetrathionate (reaction 7). Our thermodynamic analysis of this stoichiometry gives a large positive $\Delta G°$ suggesting it is unlikely.

$$2S_4O_6^{2-} + H_2O = S_2O_3^{2-} + S_5O_6^{2-} + SO_4^{2-} + 2H^+ \qquad \Delta_7G° = +103.7\ (kJ) \qquad (7)$$

Other reports about this enzyme [31-33] proposed stoichiometry to account for the formation of higher polythionates but only that reported by Meulenberg et al. [34], reaction 8 gave a $\Delta G°$ value less than +30 kJ $mol(S_4O_6)^{-1}$.

$$S_4O_6^{2-} + H_2O(l) = S_2O_3^{2-} + S(s) + SO_4^{2-} + 2H^+ \quad \Delta_8G° = +4.2\ (kJ) \qquad (8)$$

The relatively low value for $\Delta_8G°$ means that $\Delta_8G < 0$ under the concentrations found in these experiments, making reaction 8 spontaneous while reaction 7 is not, unless the sum of the $S_2O_3^{2-}$ and $S_5O_6^{2-}$ activities is less than ca 1×10^{-16} M. Although the stoichiometry is still

debated, a common feature is the production of thiosulfate which, under acidic conditions and the presence of oxygen, can abiotically react to form polythionates (Fig. 4). A cycling of the $S_2O_3^{2-}/S_4O_6^{2-}$ couple is proposed in the sulfur metabolic pathways reviewed by Rohwerder et al. [6].

The appearance of these higher polythionates can be explained most simply by *At. caldus* producing thiosulfate from the tetrathionate as a first step in its metabolic pathway. Thiosulfate is unstable in acidic conditions and as well as being metabolised, could disproportionate abiotically, forming tetra- penta- and hexathionate as the major products (>95 %, Fig. 4). The polythionates observed during these batch culture experiments can all arise from the spontaneous decomposition of thiosulfate produced as the first step in tetrathionate metabolism. Furthermore, the lower pH range used in these experiments compared to other reports [14, 15, 16, 17] would destabilise thiosulfate, favouring its abiotic conversion to polythionates and reducing the amount immediately available for oxidation.

4.3 Growth yields during the batch culture

The complex mixture of polythionate species makes description of intermediate states in the batch experiment very difficult. This was attempted by constructing a mole balance around sulfur found from analyses (sulfite, thiosulfate, trithionate, tetra-, penta- and hexathionate) and assuming that any other sulfur was in the form of sulfate. Data of cell numbers collected during two experiments was used to estimate the number of equivalents represented by that biomass and plotted against the estimated amount of sulfate produced (Fig. 5). The data is scattered but a linear trend is evident, giving a Y_{obs} of *ca* 0.037 equiv($CH_{1.7}O_{0.42}N_{0.25}$) mol(SO_4)$^{-1}$.

FIGURE 5 NEAR HERE

The pattern in Fig. 5 shows instantaneous Y_{obs} values over the batch culture follow a sigmoid pattern with initial values lower than the average and final values higher than the average. Since sulfate was not measured directly but assumed to include all sulfur not found as sulfite, thiosulfate, tetrathionate, pentathionate and hexathionate, a number of hypotheses to explain this pattern of Y_{obs} over time, are possible. Although elemental sulfur was not observed during the abiotic oxidation of thiosulfate (Fig. 4), a colloidal form of elemental sulfur, invisible under light microscopy, could have been produced via reaction 8 (Meulenberg et al. [34]) during inoculated experiments. Net production of colloidal sulfur during the early half, and net metabolism during the latter part of the batch culture, is a possible explanation for the observed pattern of Y_{obs} values. This pattern also shows as a 20 hour 'lag' period where 25 % of the tetrathionate is removed for only a small increase in cell numbers (Fig. 2). Approximately 60 % of the tetrathionate removed by 20 hours can be found as pentathionate and less than 5 % as thiosulfate or hexathionate (Fig. 3). The initial activity for the inoculum cells appeared to involve transformation of the tetrathionate rather than growth. After 20 hours, the cell concentration increased rapidly while the concentrations of pentathionate, hexathionate and thiosulfate remained relatively steady until after the tetrathionate was exhausted at 60 hours. The initial production of components necessary for the transformations, at the expense cell mass, could also explain the Y_{obs} pattern observed. It might be expected from earlier work that cell yields would be reduced as the pH of the medium was reduced (Dopson et al. 2002). This may explain the lower Y_{obs} value observed during this study compared with yields reported earlier [14, 15, 16 and 17]. This decreasing efficiency with increasing a_{H+} does not explain the Y_{obs} pattern observed within each batch culture because the pH decreased over the course of the batch while the Y_{obs} value rose.

5. Conclusions

Chemolithotrophic growth of *At. caldus* on tetrathionate involved a complex series of reactions during which tetrathionate appeared to disproportionate to form polythionates and sulfate. Batch experiments consistently resulted in the formation of polythionates during the consumption of tetrathionate in the first half of a batch culture followed by consumption of all the polythionates in the latter stages of the batch. The growth yield observed over the entire batch culture was 3.5 g (dry wt.) $mol(S_4O_6)^{-1}$, somewhat lower than other reports using similar chemolithotrophic organisms in continuous culture. Interpreting these observations against the derived theoretical framework shows results of thermodynamic efficiency consistent with the analysis of Kelly [1] reporting *ca* 10 %. The formation of polythionates was observed but whether created by the enzymic process described by Bugaytsova et al. [30] acting on tetrathionate or spontaneous abiotic reaction of thiosulfate could not be resolved. The sinusoidal pattern of Y_{obs} values can be explained by the undetected production of elemental sulfur in colloidal form.

Acknowledgements

Grateful thanks are extended to D. Hewitt for valued technical assistance in maintenance and method development in respect of HPLC analyses. The financial assistance of the Australian Government through CSIRO Minerals Down Under Flagship and the Parker Cooperative Research Centre for Integrated Hydrometallurgy Solutions is gratefully acknowledged.

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FIGURE CAPTIONS

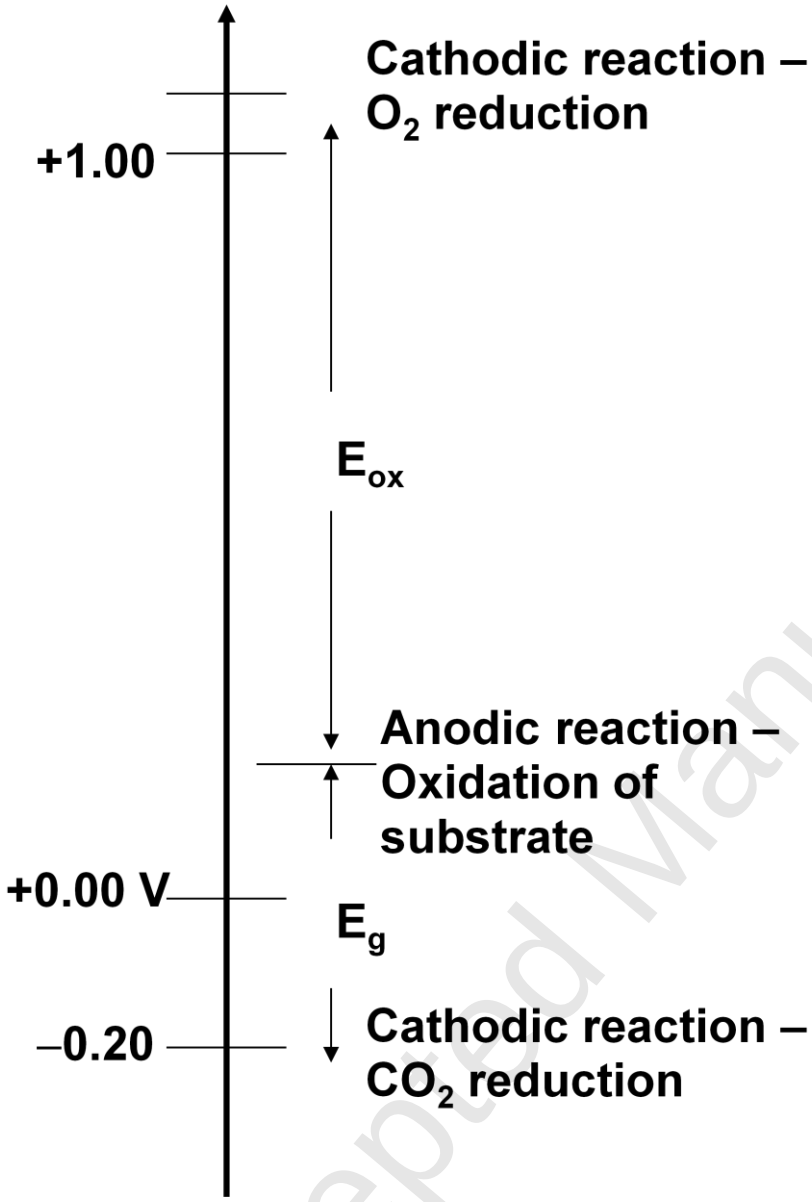
Figure 1. A schematic view of the chemiosmotic circuit in chemolithotrophic bacteria. E_{ox} is the spontaneous electrochemical cell generating energy. E_g is the non-spontaneous cell resulting in CO_2 reduction and biomass formation.

Fig. 2. Cell number (dotted and solid lines) and tetrathionate concentration (□ and ◊) changes during two replicate batch culture experiments.

Fig. 3. Thiosulfate (●), tetrathionate (■), pentathionate (◊) and hexathionate (Δ) changes during batch a experiment.

Fig. 4. Formation of tetrathionate (□), pentathionate (Δ) and hexathionate (○) from thiosulfate (♦). TS (---) is the calculated total sulfur expressed as mM thiosulfate.

Fig. 5. Intermediate Y_{obs} values observed in an experiment (Δ) and its replicate (□).



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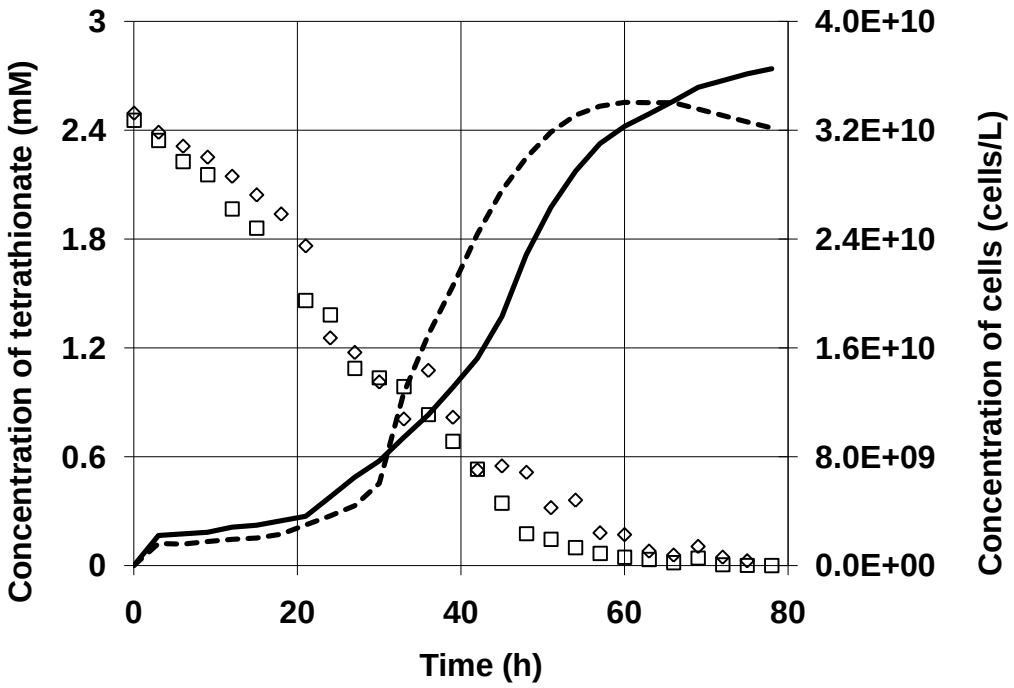

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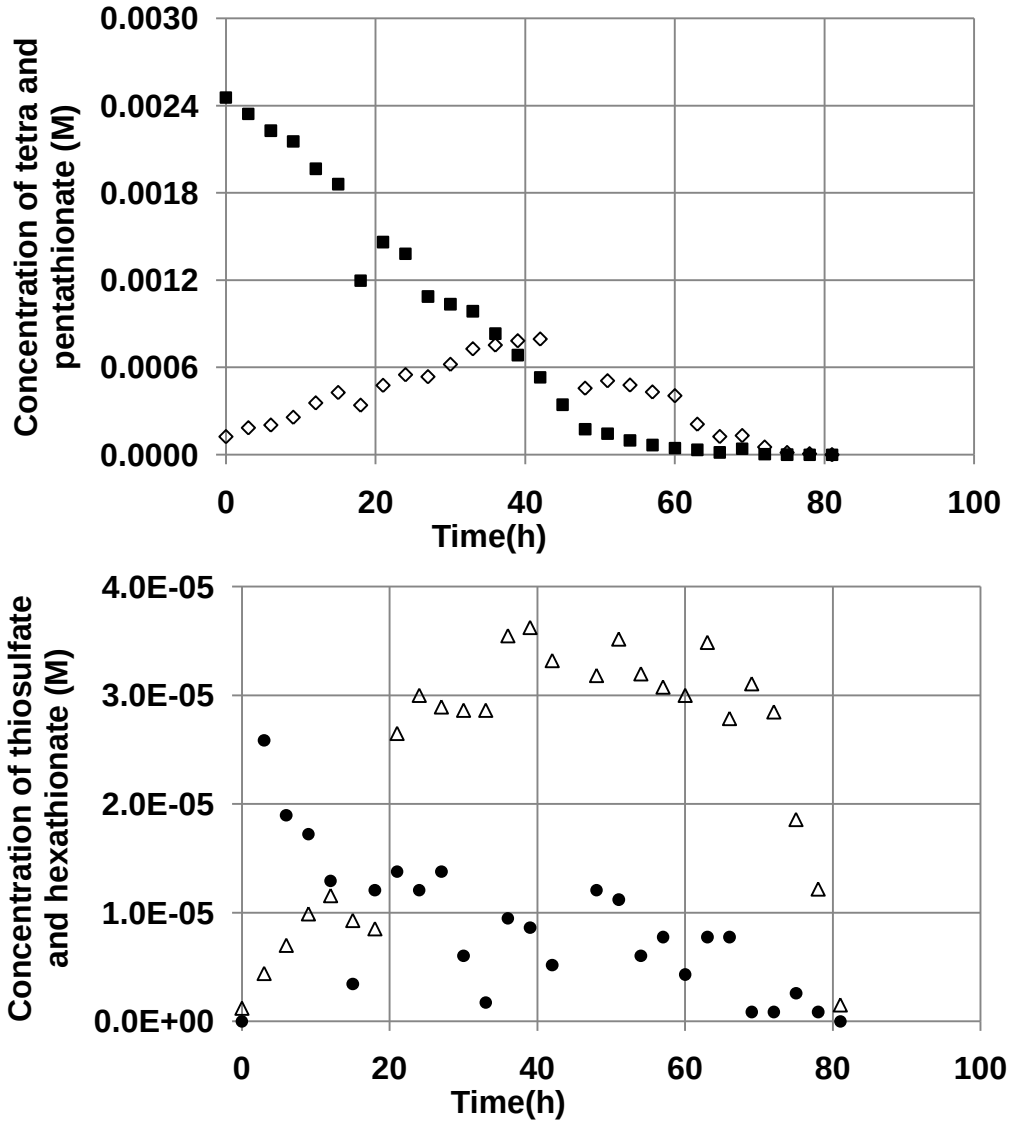

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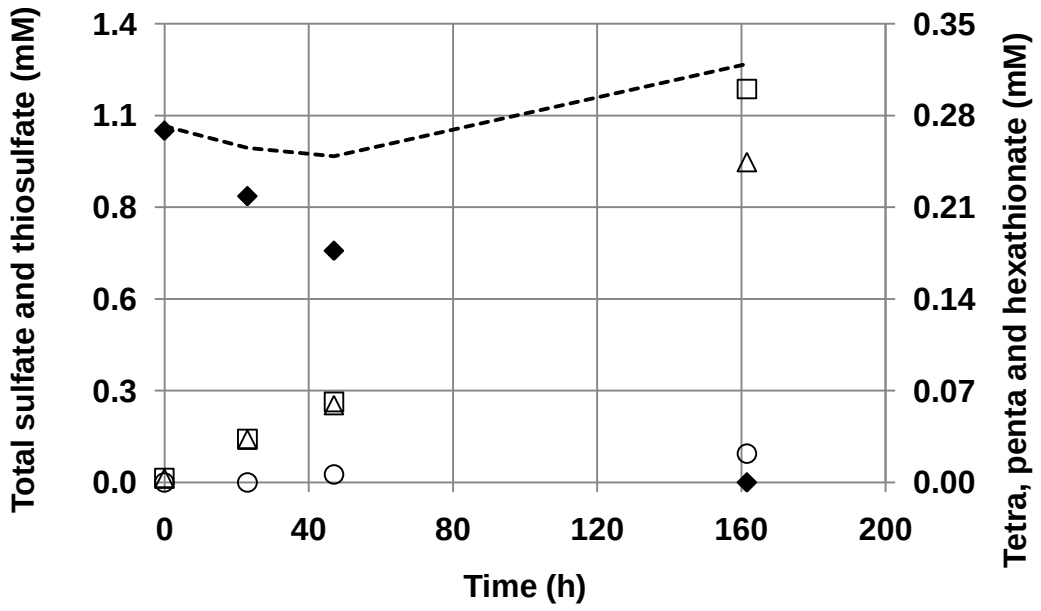

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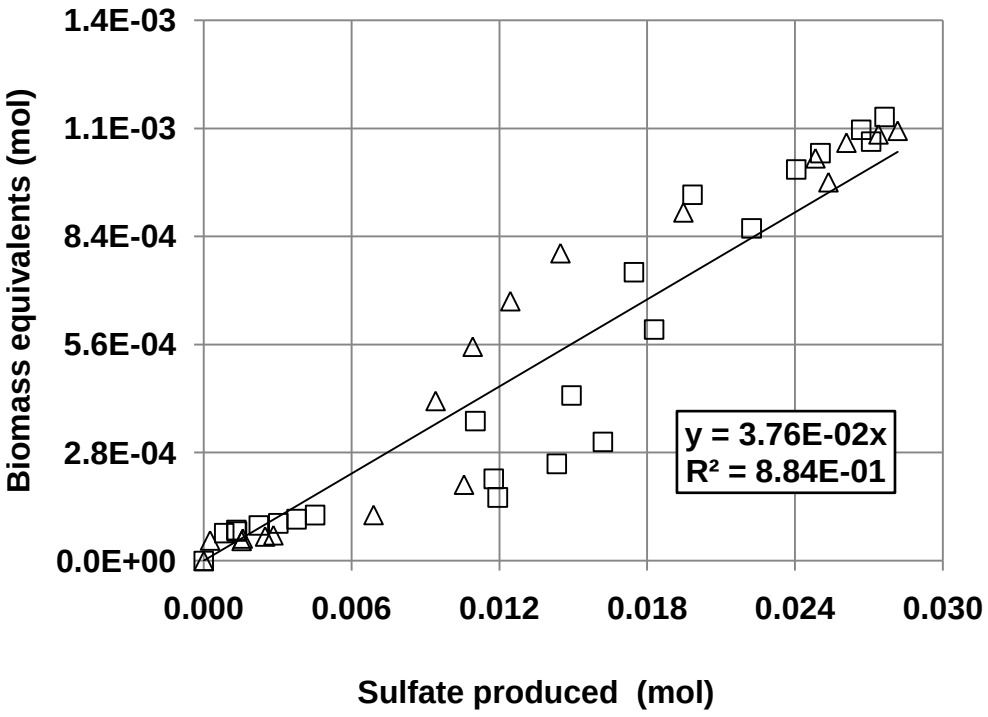

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Table 1. Observed yields (Y_{obs}) and cell number-dry weight correlation for *At. caldus*. Average and standard deviation values from four replicate experiments (n = 4).

Quantity	**average**	**standard deviation**
N (cell L^{-1})	4.00×10^{10}	6.48×10^{9}
Dry wt. (g L^{-1})	8.35×10^{-3}	1.3×10^{-3}
Number/mass (cell g^{-1})	4.59×10^{12}	9.01×10^{11}
Y_{obs} (g(dry wt.) mol$(S_4O_6)^{-1}$)	3.53	0.517
Y_{obs} (g$(CH_{1.7}O_{0.42}N_{0.25})$ mol$(S_4O_6)^{-1}$)	3.09	0.453
Y_{obs} (C-mol$(CH_{1.7}O_{0.42}N_{0.25})$ mol$(S_4O_6)^{-1}$)	0.129	0.0189
Y_{obs} (equiv$(CH_{1.7}O_{0.42}N_{0.25})$ mol$(S_4O_6)^{-1}$)	0.531	0.0779