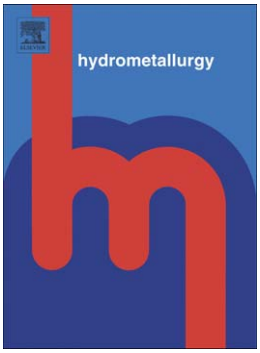

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A Comparative Study Of Substrate Utilisation By *Sulfobacillus* Species In Mixed Ferrous Ion And Tetrathionate Growth Medium

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Abstract

Concurrent ferrous ion and tetrathionate utilisation by *Sulfobacillus acidophilus*, *Sb. thermosulfidooxidans*, *Sb. thermotolerans*, and *Sb. sibiricus* grown separately in batch culture in dual substrate media containing ferrous ion and tetrathionate was investigated. For all species, tetrathionate-adapted cells oxidised both substrates concurrently, achieving at least 20 % oxidation of the second substrate before the first substrate was exhausted. Sequential substrate utilisation was observed for iron(II)-adapted cells for three of the four species and all iron(II)-adapted cell lines commenced oxidation of ferrous ions ahead of tetrathionate. Adaptation to iron(II) or tetrathionate of the test species had little impact on subsequent ferrous ion oxidation. However, tetrathionate oxidation was affected by growth history. Compared with their respective tetrathionate-adapted cell lines, cells adapted to iron(II) exhibited either significantly longer lag times and/or longer periods to complete tetrathionate oxidation once it had commenced. Polythionate intermediates measured during tetrathionate oxidation to sulfate included thiosulfate, pentathionate and hexathionate for the four species. The intermediate trithionate was only detected in *Sb. thermotolerans* cultures.

1. INTRODUCTION

While a number of organisms active in bioleaching systems have been described, little is known of their behaviour in complex environments. The behavior of bacteria when both iron(II) and sulfur substrates are available, particularly whether the organisms display any preferences for particular energy sources or whether utilisation of different energy sources occurs concurrently, is of interest. Such fundamental knowledge will assist in understanding microbial behaviour and activity in heap or tank bioreactors for the extraction of metals from sulfide ores or concentrates.

Only a few acidophiles capable of oxidizing both ferrous ions to ferric ions and sulfur oxy anions to sulfate have been described. They include *Acidithiobacillus ferrooxidans*, some *Thiomonas* species, the *Sulfobacillus* species, some *Alicyclobacillus* species and some archaea from the genera *Acidianus*, *Metallosphaera* and *Sulfolobus* [1].

Previous studies on bacterial behavior in the presence of multiple inorganic substrates mostly used *At. ferrooxidans,* providing it with elemental sulfur and ferrous ions as energy sources. The results from these studies varied. For example, Margalith et al. [2] found that sulfur-adapted *Ferrobacillus ferrooxidans* (probably *At. ferrooxidans*) cells could oxidise ferrous ions almost as rapidly as iron(II)-adapted cells without any observable lag phase. But Landesman et al. [3] and Oliver and Van Slyke [4] found that iron(II)-adapted *At. ferrooxidans* oxidised ferrous ions up to fifty times faster than sulfur-adapted cells and Kulpa et al. [5] found that sulfur-adapted cells exhibited a significant lag phase when transferred to iron(II) growth medium. Landesman et al. [3] were the first to record the simultaneous oxidation of sulfur and ferrous ion by *At. ferrooxidans,* estimating that oxidation proceeded at a rate equivalent to the sum of the maximum individual rates under the selected experimental conditions, but Sand [6] noted preferential oxidation of ferrous ion with a lag phase between the utilisation of ferrous ions and the commencement of sulfur oxidation.

Elemental sulfur is largely insoluble and the amount of cell-surface contact is an additional variable that has been shown to be rate-limiting for bacterial growth [7]. Tetrathionate was used in the present study to avoid this limitation.

The utilisation curves of ferrous ions and tetrathionate in dual-substrate media by *Sulfobacillus thermosulfidooxidans, Sb. sibiricus*, *Sb. acidophilus* and *Sb. thermotolerans* were compared. The four species are known to assist metals extraction during the bioleaching of sulfide ores [8] but no comparative studies on substrate utilisation in mixed growth media have been found. Cell growth and substrate oxidation were studied in batch culture in media initially containing (i) equimolar amounts of ferrous ions and tetrathionate ions and (ii) equal electron equivalents [9].

The overall purpose of the research being undertaken in our laboratory, a small part of which is presented here, is to describe bacterial behaviour under controlled but varied conditions relevant to heap bioleaching using one or more bacterial species. It therefore complements such studies as those above and cited within and provides fundamental data from which microbial behaviour in response to (anticipated) leaching conditions can be predicted and/or confirmed.

2. MATERIALS AND METHODS

2.1. Culture Preparation and Maintenance

Sulfobacillus thermosulfidooxidans DSM 9293^T, *Sb. acidophilus* DSM 10332^T, *Sb. sibiricus* DSM 17363^T and *Sb. thermotolerans* DSM 17362^T were grown at 45 ºC in BSM-YE medium [8] supplemented with either ferrous sulfate (10 g L^{-1}) or potassium tetrathionate (0.75 g L^{-1}) as the primary energy source. Cultures were grown successively in their respective media for up to five sub-cultures before inoculation into test flasks.

2.2. Experimental

The BSM-YE medium was prepared with 35 mM ferrous sulfate ($FeSO_4.7H_2O$, 9.71 g L^{-1}) and 2.5 mM potassium tetrathionate ($K_2S_4O_6$, 0.75 g L^{-1}), filter sterilized, and 100 mL transferred into 250 mL flasks (equi-equivalent medium). The inocula contained either tetrathionate- or iron(II)-adapted cells and the cultures were incubated at 45 ºC. Aliquots for analysis and bacterial enumeration were removed periodically under aseptic conditions. Similar conditions and inocula were used in media consisting of 2.5 mM ferrous sulfate and 2.5 mM potassium tetrathionate in BSM-YE (equimolar medium).

2.3. Culture Analysis

Redox potential was measured using an E_H electrode (Ionode model IP1306) and ferrous ion concentrations were calculated as described previously [8]. E_H electrode calibration was performed on an aliquot of the medium before tetrathionate was added. With the exception of trithionate, polythionates were determined using a Waters 2695 HPLC separation module utilising an Ionpac AS16 ion exchange column. Polythionates were detected using a Waters 2996 Photodiode Array Detector at 214 nm; trithionate was detected at 192 nm [10]. A pump flow rate of 1.5 mL min^{-1} was used and the column temperature was maintained at 25 °C. A 0.15 M sodium perchlorate solution was used as the eluent. “Empower” was the software used to integrate spectra. Cell counts were performed using a Helber Bacteria Counting Chamber (Thoma ruling, 5.0 x 10^{-5} mm^3 chamber volume).

3. RESULTS

A summary of the observations is presented in Table 1. In this summary, sequential substrate utilisation describes those cases where less than 20 % of the second substrate was oxidised at the completion of primary substrate oxidation. Similarly, concurrent substrate utilisation describes cases where more than 20 % of the second substrate was oxidised at the completion of primary substrate oxidation. The term 'diauxic growth curve' refers to a two-stage growth curve where the initial stage of cell growth is separated by a lag period from a further stage of cell growth, typically indicating sequential utilisation of two substrates [11].

TABLE 1

3.1. Growth on 35 mM ferrous ions and 2.5 mM tetrathionate

Sb. thermosulfidooxidans grown in medium initially containing 35 mM ferrous ions and 2.5 mM tetrathionate ions generated similar utilisation curves regardless of the substrate to which the bacterium was previously adapted (Fig.1). Significant tetrathionate oxidation by the tetrathionate-adapted cells commenced shortly before the exhaustion of the ferrous ion substrate but there was a hiatus in cell growth between the end of ferrous ion utilisation and the start of tetrathionate utilisation. In cultures with very similar cell numbers (Fig. 1B), tetrathionate-adapted cells and iron(II)-adapted cells utilised the available ferrous ions almost identically within 40 h (Fig. 1A). However, tetrathionate-adapted cells utilised the available tetrathionate within 60 h compared with iron(II)-adapted cells which required about 100 h.

Biomass formation showed a diauxic growth curve. The first occurred during ferrous ion oxidation and was followed by a period of relatively stable cell numbers during the early period of tetrathionate oxidation. A sharp rise in biomass was seen towards the end of tetrathionate oxidation, followed by another stationary phase once both substrates had been consumed (Fig. 1B).

FIGURE 1

Sb. thermotolerans (data not shown; Table 1) exhibited a remarkably similar curve to *Sb. thermosulfidooxidans* for substrate utilisation in the equi-equivalent medium. The main difference was an extended lag time of about 30 h before the commencement of ferrous ion oxidation for both iron(II)- and tetrathionate-adapted cells, compared with about 20 h for *Sb. thermosulfidooxidans*. For the tetrathionate-adapted *Sb. thermotolerans* cells, significant tetrathionate oxidation commenced just prior to the complete utilisation of ferrous ions. The second obvious difference for *Sb. thermotolerans* was that, at the end of the growth cycle, the cell concentration of iron(II)-adapted cells was significantly lower than the cell concentration of tetrathionate-adapted cells (Fig. 2), even though both substrates had been completely oxidised.

FIGURE 2

Both *Sb. acidophilus* and *Sb. sibiricus* exhibited different substrate utilisation curves (Fig. 3; Table 1) compared with *Sb*. *thermosulfidooxidans* (Fig. 1A) and *Sb. thermotolerans*. *Sb. acidophilus* exhibited longer lag times than *Sb. sibiricus* before substrate utilisation commenced (Fig. 3A cf. 3B). Ferrous ion oxidation by *Sb. acidophilus* commenced at about 35 h and was completed by about 45 h for both iron(II)- and tetrathionate-adapted cells (Fig. 3A), even though twice as many of the tetrathionate-adapted cells had developed at the end of the growth cycle than of the iron(II)-adapted cells (Fig. 4), a consequence of concurrent tetrathionate utilisation. Growth history impacted on tetrathionate oxidation; iron(II)-adapted cells of *Sb. acidophilus* exhibited an extended lag time before significant tetrathionate utilisation was detected (Fig. 3A).

For *Sb. sibiricus*, concurrent substrate utilisation was observed for both iron(II)-adapted and tetrathionate-adapted cell lines (Fig. 3B). Tetrathionate-adapted cells commenced tetrathionate oxidation ahead of ferrous ion oxidation but, overall, required longer (about 40 h) for complete utilisation of both substrates compared with the iron(II)-adapted cell line (about 35 h).

FIGURE 3

Tetrathionate-adapted cells exhibited concurrent substrate utilisation for all species (Table 1), indicating that growth history had affected tetrathionate oxidation.

The cell growth curves varied considerably between species but can be correlated with the respective utilisation curves. For example, for *Sb. acidophilus* (Fig. 4), the tetrathionate-adapted cells showed a single exponential growth phase reflecting the concurrent utilisation of both substrates (Fig. 3A) whereas the growth curve of the iron(II)-adapted cells reflects the initial period of ferrous-ion utilisation followed by tetrathionate utilisation.

FIGURE 4

For all species, iron(II)-adapted cells grown in equi-equivalent medium generated about 4 x 10^7 cells mL^{-1} from total ferrous ion oxidation, except in the case of *Sb. sibiricus*, where significant concurrent tetrathionate oxidation yielded 7 x 10^7 cells mL^{-1}. For the four species grown in the equi-equivalent medium, final cell concentrations for tetrathionate-adapted cell lines were between 7 x 10^7 and 1 x 10^8 cells mL^{-1} and in all cases, cell concentrations for iron(II)-adapted cells were less than or equal to those for tetrathionate-adapted cells at the time when both substrates were utilised.

3.2. Growth on 2.5 mM ferrous ions and 2.5 mM tetrathionate

Sb. thermosulfidooxidans grown in equimolar medium initially containing 2.5 mM ferrous ions and 2.5 mM tetrathionate ions initiated growth utilising ferrous ions, only oxidising the tetrathionate once the ferrous ions were exhausted (Fig. 5A). No significant difference was seen between iron(II)-adapted and tetrathionate-adapted cells other than that the tetrathionate-adapted cells exhibited a shorter lag time than iron(II)-adapted cells before commencing tetrathionate oxidation.

Biomass formation (planktonic cell numbers) was observed during the ferrous ion oxidation phase and then again during tetrathionate oxidation phase with a stable period of cell density between and after the exponential phases (diauxic growth) (Fig. 5B).

FIGURE 5

For each of the four species there was strong similarity between ferrous ion utilisation curves for both iron- and tetrathionate-adapted cells, but *Sb. thermotolerans* (data not shown; Table 1) and *Sb. acidophilus* (Fig. 6B) exhibited extended lag times, compared with *Sb. thermosulfidooxidans* (Fig. 5A) and *Sb. sibiricus* (Fig. 6A). Ferrous ion oxidation was substantially complete for *Sb. thermotolerans* before tetrathionate oxidation commenced (data similar to *Sb. thermosulfidooxidans*; Fig. 5A). For the tetrathionate-adapted cell lines, concurrent utilisation of ferrous ions and tetrathionate was observed for *Sb. acidophilus* (Fig. 6B) and *Sb. sibiricus* (Fig. 6A). Tetrathionate-adapted cells commenced tetrathionate oxidation concurrently with or ahead of iron(II)-adapted cells for all species, confirming the impact of cell growth history on tetrathionate oxidation. Only *Sb. acidophilus* commenced tetrathionate oxidation ahead of ferrous ion oxidation and *Sb. sibiricus* completed tetrathionate oxidation in the shortest time (about 25 h).

FIGURE 6

In respect of planktonic cell counts, similar variation in cell concentrations at the end of the growth cycle was observed as with equi-equivalent growth media and, with the exception of *Sb. thermosulfidooxidans*, significantly lower numbers of iron(II)-adapted cells than tetrathionate-adapted cells developed during the experiments. For all species, iron(II)-adapted cells grown in equimolar medium commenced ferrous ion oxidation before tetrathionate oxidation and generated about 2×10^7 cells mL^{-1} from ferrous ion oxidation.

3.3. Soluble Sulfur Speciation

Sulfur speciation showed few qualitative differences among the four *Sulfobacillus* species, regardless of adaptive history and experimental conditions. During the oxidation of tetrathionate, pentathionate formed in the greatest amounts with small quantities of hexathionate and thiosulfate also being detected (Figure 7A). Trithionate was observed to form in small quantities in cultures inoculated with *Sb. thermotolerans* under all experimental conditions tested (Figure 7B). In an abiotic test, under the temperature and pH conditions tested, tetrathionate was stable in both media in the time frame of the experiment. In the presence of 35 mM ferric ion, as

would be present at the end of experiments involving the equi-equivalent medium, any polythionates generated were below the detection limit (about 10^{-5} M).

FIGURE 7

4. DISCUSSION

4.1. Cell generation and energetics

Energy generation during cell growth can be understood as an electrochemical phenomenon where electrons are transferred from the oxidised substrate, iron and tetrathionate in this study, to oxygen ([9] and references therein). This electron transfer generates chemical energy which is used to create more cells and for cellular processes, often described as 'cell maintenance' [11]. In the case of the obligate chemolithotrophic species the energy is also used to 'fix' CO_2 and to drive the synthesis of all the carbon compounds used in the cell. These four *Sulfobacillus* species are mixotrophs, requiring the inclusion of a suitable carbon compound such as yeast extract [12-13] for continued growth, indicating that carbon for the cell components is not derived solely from CO_2.

The two substrates providing electrons for growth in these experiments are ferrous ions and tetrathionate ions. The anodic reaction of ferrous (1) is a single electron transfer step while that of tetrathionate involves 14 electrons. Equation (2) represents the sum of a number of reactions that occur during tetrathionate conversion to sulfate. There is strong evidence to suggest that not all of the reactions comprising equation (2) furnish electrons that can be used by the organisms for growth [14]. Organism yields calculated during another study [15] conducted with only tetrathionate as an energy substrate suggest this is the case for *At. caldus*.

$$Fe^{+2} \rightarrow Fe^{+3} + e^{-} \quad (1)$$

$$S_4O_6^{-2} + 10H_2O \rightarrow 4SO_4^{-2} + 20H^{+} + 14e^{-} \quad (2)$$

The two media used in this study provided the same concentration of tetrathionate (2.5 mM) but different concentrations of ferrous ions. The 'equi-equivalent' medium with 35 mM ferrous and 2.5 mM tetrathionate ion offered nearly twice the theoretical number of substrate electrons available in the 'equimolar' medium (2.5 mM each of

ferrous and tetrathionate ion). It was expected that increased cell numbers would result from the greater concentration of ferrous, but more complex behavior was observed.

The growth of *Sb. thermosulfidooxidans* in the different media exhibited similar growth curves, indicated by planktonic cell numbers. Regardless of the 14-fold increase in concentration of ferrous ion, the increase in cell numbers at the higher concentration is only just significant. Cell numbers after utilisation of both ferrous ion and tetrathionate were not significantly different in either medium (Figures 1B and 5B, 8×10^7 cells mL^{-1} ±15 %). Cell numbers in flasks containing *Sb. acidophilus* and *Sb. thermotolerans* exhibited more 'concurrent' substrate utilization and more complex patterns in cell concentration. However the cell concentrations at the exhaustion of both substrates were similar in either medium, for either adaptation, with the exception of iron-adapted *Sb. thermotolerans* which produced only half of the average quoted above.

The 14-fold increase in energy available as ferrous did not result in the expected increase in cell numbers during the sequential oxidation of ferrous. However, the number of cells produced during the oxidation of only 2.5 mM ferrous appears rather high compared to published values (2.3×10^{11} cells g^{-1} compared with 2.5 – 3.8×10^{10} cells g^{-1} in data from [16]). By contrast, the yield of *Sb. thermosulfidooxidans* from the 35 mM concentration of ferrous was 2×10^{10} cells g^{-1}, much closer to the values reviewed by Nemati et al. [16]. However, the data from Nemati et al. [16], derived from obligate chemolithotrophic cells, may not provide a reasonable comparison given the availability of yeast extract in the media of the present study. Preliminary data from this study indicates that growth on dual substrates is more complicated than anticipated. While a 'preference' for ferrous oxidation appears to be common in this genus, understanding the way cells convert the chemical energy to biomass will require significant further study.

4.2. Substrate utilisation

We know of three papers in which observed results are attributed to the inhibition of sulfur oxidation by the presence of ferrous ions.

Sugio et al. [17] interpreted their results as showing that >0.1M ferrous ion in iron(II)-elemental sulfur growth medium (0.108 M Fe^{2+} and 1 % elemental sulfur) inhibited *At. ferrooxidans* utilisation of elemental sulfur as an energy source. They

proposed that two enzymes, sulfur:ferric ion oxidoreductase and sulfite:ferric ion oxidoreductase were the specific sites of inhibition and that the resultant build up of sulfite could damage cells. Das et al. [18] reported concurrent utilisation of ferrous ions and elemental sulfur by *At. ferrooxidans* but that cells only exhibited thiosulfate / tetrathionate oxidation enzyme activity after the ferrous ion concentration was depleted. They concluded that ferrous ions, but not ferric ions, inhibited thiosulfate / tetrathionate oxidation by *At. ferrooxidans*. Similarly, Cwalina et al. [19] interpreted their results as showing that ferrous ions (>20 mg L^{-1}) inhibited thiosulfate biooxidation by a *Sulfobacillus* species.

While the current results for *Sb. thermosulfidooxidans* are consistent with the hypothesis that ferrous ions inhibit tetrathionate oxidation, the data for *Sb. acidophilus, Sb. thermotolerans* and *Sb. sibiricus* showing that some tetrathionate oxidation occurred before the complete utilisation of ferrous ions under certain conditions, are inconsistent with that hypothesis. In the case of tetrathionate-adapted *Sb. sibiricus,* tetrathionate oxidation occurred while the predominant soluble iron species was ferrous ion (Figure 3B). These experimental results do not allow us to conclude that ferrous ions inhibit the metabolic pathway for tetrathionate oxidation for the *Sulfobacillus* species.

A recent study by Sugio et al. [20] found that ferric ions were associated with the growth of *At. ferrooxidans* when grown on elemental sulfur or tetrathionate. The requirement of ferric ions for growth on tetrathionate would offer one explanation why the *Sulfobacillus* species oxidise ferrous ions almost completely before beginning tetrathionate oxidation due to its physiological necessity. The observation that both substrates were oxidised simultaneously may be explained by individual *Sulfobacillus* species having lower requirements for ferric ions in solution to begin sulfur oxidation processes.

4.3. Sulfur speciation

Significant amounts of polythionates were detected during the sulfur oxidation phases of growth and the pattern of this accumulation and removal was similar for all species despite differences in cell history and growth conditions. Pentathionate was formed in the greatest amount with hexathionate and thiosulfate making up minor contributions to the total. Trithionate was only observed in cultures inoculated with *Sb. thermotolerans*, irrespective of growth conditions or adaptive histories. The

similar pattern suggests that the four *Sulfobacillus* species are utilising similar mechanisms of tetrathionate oxidation.

The enzyme tetrathionate hydrolase is thought to be the first step in the conversion of tetrathionate during its metabolism. Several pathways and stoichiometries have been proposed for the conversion [21-23]. All authors reported a variety of products such as sulfate, pentathionate, sulfur and protons arising from the reaction but a common feature is the formation of thiosulfate. The enzyme, which is located in the periplasm of *At. caldus* [21], has a pH optimum of 3 reflecting the acidic conditions under which these organisms thrive. Our studies have shown that thiosulfate, under acidic conditions (pH < 3), decomposes spontaneously to form a complex mixture of higher polythionates including tetra, penta and hexathionate [15]. Since thiosulfate is reported as a product in all studies of tetrathionate dehydrogenase it is possible that the mixture of reported products arises from a combination of enzyme action and abiotic reaction of thiosulfate initially produced by the tetrathionate hydrolase. The contributions of abiotic and enzymatic pathways to the pattern could be related and potentially difficult to unravel.

4.4. This study in the context of heap leaching

Not surprisingly, plant metallurgists with responsibility for metal production from heap operations are reluctant to 'interfere' with production for the purposes of research – the construction of additional test cribs or heaps or the collection of periodic solution and solids samples – unless they relate to a specific operational problem. Thus, comprehensive studies on heap mineralogy, chemistry and microbiology are rare. In addition, those microbiological data that are in the public domain tend to be sparse and selective, generally focusing on species present with only cursory attention to their relationship with prevailing leaching conditions and temporal, spatial or physico-chemically induced changes in them.

For example, bacteriological studies of a dump/heap leach at Bingham Canyon, Utah [24] revealed mesophiles (*At. ferrooxidans, At. thiooxidans, Leptospirillum ferrooxidans* and an unidentified iron-oxidising heterotroph) and moderate thermophiles, the latter being more prevalent at depth in higher temperature regions of the heap. Cell counts of around 8 x 10^6 cells g^{-1} were found at the surface but declined by up to two orders of magnitude at greater depths but there was no correlation between bacterial counts and copper extraction. Hawkes et al. [25]

described the microbial diversity of a commercial copper bioleaching heap operation in Myanmar, reporting cell counts for 15 samples collected from different parts of the circuit and identifying species using culture-based and culture-independent methods. They found higher numbers of mesophilic and moderately thermophilic organisms capable of iron(II) and sulfur oxy anion oxidation than had been reported for a similarly constructed chalcocite heap [26].

The exceptions to the above generalisation are the data arising from recent studies which together described the microbial diversity of the Escondida run-of-mine heap leaching operation and the microbial assemblages as a function of time (microbial succession in response to changed environmental pressures and/or fluctuating conditions) [27-28]. In addition, global gene expression was analysed to elucidate the roles and key metabolic functions of the active microbial community with the purpose of relating metabolic changes to the leaching cycle [29]. Most recently, Demergasso [30] presented data to suggest that bacterial iron(II) oxidation processes occur ahead of sulfur oxidation processes, which were activated once the aqueous environment reached a high redox potential. This behaviour was thought to be governed by mineralogical characteristics of the ore and microbial succession as populations changed with substrate availability in the heap.

In heap leaching operations, sulfur will be present initially as sulfide minerals and subsequently as elemental sulfur and low concentrations of sulfur oxy anions generated via oxidative and proton attack mechanisms [31]. The substantial body of work in mixed substrate media comprising elemental sulfur (largely insoluble) and ferrous ions (soluble) could lead to the conclusion that the slow kinetics of solids dissolution played a role, resulting in apparently faster oxidation of ferrous ions. However, the general finding that, in most cases, *Sulfobacillus* species preferentially oxidise iron(II) relative to soluble tetrathionate is consistent with the observation that sulfur biooxidation in the Escondida heap follows iron(II) oxidation [30] but that the phenomenon has a physiological basis rather than being determined by sulfur oxy anion availability.

5. CONCLUSIONS

A greater understanding of the behaviour of bioleaching bacteria with the ability to oxidise both iron(II) and sulfur oxy anions in bioleaching environments has been gained from this study. Substrate utilisation observed in batch cultures varied between *Sulfobacillus* species. Iron(II)-adapted cells always commenced oxidation of ferrous ions ahead of tetrathionate oxidation. For all species, tetrathionate-adapted cells oxidised both substrates concurrently, achieving at least 20 % oxidation of the second substrate before the first substrate was exhausted.

While some suggestions have been made in the literature that ferrous ions inhibit sulfur oxidation, these experimental results do not allow us to conclude that ferrous ions inhibit the metabolic pathway for tetrathionate oxidation for the *Sulfobacillus* species.

Biomass generation (planktonic cell numbers) under different conditions indicate that the system is not as simple as was initially thought from an analysis of the total theoretical energy for growth presented to the species. A future study to elucidate the number of electrons that might be directed into the sulfur electron transport chain from tetrathionate oxidation is planned.

Analysis of possible mechanisms of polythionate formation showed that the observed sulfur oxy anions could all be accounted for by previously described enzymatic and chemical mechanisms under the experimental conditions.

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Table 1. Comparison of substrate utilisation by four *Sulfobacillus* species with two growth histories (iron(II) adaptation and tetrathionate adaptation) in equi-equivalent and equimolar dual-substrate media.

	Sb. acidophilus	*Sb. sibiricus*	*Sb. thermosulfidooxidans*	*Sb. Thermotolerans*	*Sb. acidophilus*	*Sb. sibiricus*	*Sb. thermosulfidooxidans*	*Sb. thermotolerans*
	Iron-adapted				Tetrathionate-adapted			
Equi-equivalent medium								
Iron oxidation commenced before tetrathionate oxidation	●	●	●	●			●	○
Tetrathionate oxidation commenced before iron oxidation					●	●		
Sequential substrate utilisation (<20% 2nd substrate oxidation)	●		●	●				
Concurrent substrate utilisation (>20% 2nd substrate oxidation)		●			●	●	●	●
Diauxic growth (two stage growth pattern)	▲		▲			△	▲	
Ideal growth (one-stage growth pattern)		△		▲	▲			▲
Equimolar medium								
Iron oxidation commenced before tetrathionate oxidation	●	●	●	●			●	●
Tetrathionate oxidation commenced before iron oxidation					●	○		
Sequential substrate utilisation (<20% 2nd substrate oxidation)	●		●	●				
Concurrent substrate utilisation (>20% 2nd substrate oxidation)		○			●	●	●	●
Diauxic growth (two stage growth pattern)	△		▲	▲			▲	△
Ideal growth (one stage growth pattern)		△			△	▲		

●, ▲ positive result

○ unclear result

△ growth curve modified by partial concurrent substrate utilisation and/or utilisation that did not result in cell replication

Figure 1. Substrate utilisation (A) and cell growth (B) by *Sb. thermosulfidooxidans* in the presence of equal substrate equivalents for cultures adapted to ferrous ion (solid symbols) and tetrathionate (open symbols). ●,○ ferrous ion; ▲,△ tetrathionate; ■,□ cell concentration.

Figure 2. Cell growth by *Sb. thermotolerans* in the presence of equal substrate equivalents for cultures adapted to ferrous ion (solid symbols) and tetrathionate (open symbols).

Figure 3. Substrate utilisation by *Sb. acidophilus* (A) *and Sb. sibiricus* (B) in the presence of equal substrate equivalents for cultures adapted to ferrous ion (solid symbols) and tetrathionate (open symbols). ●,○ ferrous ion; ▲,△ tetrathionate.

Figure 4. Cell growth by *Sb. acidophilus* in the presence of equal substrate equivalents for cultures adapted to ferrous ion (solid symbols) and tetrathionate (open symbols).

Figure 5. Substrate utilisation (A) and planktonic cell numbers (B) by *Sb. thermosulfidooxidans* in the presence of equimolar substrates for cultures adapted to ferrous ion (solid symbols) and tetrathionate (open symbols). ●,○ ferrous ion; ▲,△ tetrathionate

Figure 6. Substrate utilisation by *Sb. sibiricus* (A) and *Sb. acidophilus* (B) in the presence of equimolar substrates for cultures adapted to ferrous ion (solid symbols) and tetrathionate (open symbols). ●,○ ferrous ion; ▲,△ tetrathionate.

Figure 7. Polythionate formation by *Sb. thermosulfidooxidans* (A) and *Sb. thermotolerans* (B) under equi-equivalent substrate conditions using cultures adapted to ferrous ion; △ thiosulfate; □ pentathionate; ◇ hexathionate; X trithionate.