Published In Biohydrometallurgy: Fundamentals, Technology and Sustainable Development, Part A. pages 461-470
V.S.T. Ciminelli and O. Gacia Jr. (editors)

Role of pure and mixed cultures of Gram-positive eubacteria in mineral leaching

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Research on the biooxidation of sulfidic minerals has tended to be heavily biased towards Gram-negative bacteria, such as *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans*. Currently, just three species of mineral-oxidising Gram-positive bacteria are recognised: *Sulfobacillus thermosulfidooxidans*, *Sulfobacillus acidophilus* and *Acidimicrobium (Am.) ferrooxidans*, all of which are thermotolerant prokaryotes. We have isolated and characterised a number of phylogenetically distinct Gram-positive iron-metabolising bacteria, including mesophilic and moderately thermophilic strains. Mesophilic isolates include (i) novel *Sulfobacillus* spp., some of which are the more acidophilic than all known iron-oxidising bacteria, (ii) "*Ferrimicrobium acidiphilum*", an actinobacterium most closely related to *Am. ferrooxidans*, and (iii) a group of low GC Gram-positives which appear to represent a novel genus. Moderately thermophilic isolates include a novel *Sulfobacillus* sp., an *Alicyclobacillus* spp. that, in contrast to currently recognised species, grows anaerobically by reduction of ferric iron and thrives in mineral leaching environments, and a new genus/species of iron- and sulfur-oxidising bacterium with the proposed name "*Caldibacillus ferrivorus*". These novel prokaryotes exhibited varying degrees of mineral leaching efficiencies, with the mesophilic *Sulfobacillus* spp. being particularly adept at solubilising pyrite at very low (<1) pH values. All novel Gram-positive isolates catalysed the oxidative dissolution of pyrite at lower redox potentials than Gram-negative mesophiles. Mixed cultures of Gram-positive bacteria, either with other Gram-positives or with Gram-negative bacteria, were often more effective mineral-leaching systems than corresponding pure cultures.

1. INTRODUCTION

The primary organisms responsible for the bioleaching of many sulfide minerals, such as pyrite, are those acidophiles that can oxidize ferrous iron to ferric. The best studied of these is the mesophile *Acidithiobacillus (At.) ferrooxidans*, though this bacterium is now recognised to play a minor role in mineral oxidation in many commercial bioleaching operations and in generating acid mine drainage at abandoned mines and spoils [1-3]. In contrast, *Leptospirillum ferrooxidans*, although less readily isolated by conventional enrichment methods, has been shown to be more abundant and active in these situations. Both are Gram-negative eubacteria, as is the moderately thermophilic sulfur-oxidiser *At. caldus* [4] which, although lacking the ability to oxidise ferrous iron and leach pyritic minerals in pure culture, exploits the more energy-rich reduced sulfur compounds formed chemically by ferric iron

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attack on mineral sulfides [5] and, by so doing, is often the numerically-dominant organism in bioleaching tanks that operate at *ca.*40-50ºC [6]. The first Gram-positive bacteria found in mineral leaching environments were moderately thermophilic, and originally described as "*Thiobacillus ferrooxidans*-like"; however, they were shown to be quite distinct (e.g. in their nutritional versatilities and formation of endospores) from the Gram-negative iron/sulfur-oxidisers, and were ascribed the novel genus *Sulfobacillus*. There are currently two *bona fide* species of this genus: *S. thermosulfidooxidans* [7] and *S. acidophilus* [8]. These differ in some physiological traits, GC contents etc. (Table 1). Another moderately thermophilic iron-oxidiser, *Acidimicrobium (Am.) ferrooxidans,* was later shown [9] to be unrelated to *Sulfobacillus* spp.. *Am. ferrooxidans* is also a Gram-positive eubacterium, but is positioned (from 16S rRNA gene analysis) within the class *Actinobacteria* [10], well away from the low GC Gram-positives. Besides these bacteria, obligately heterotrophic thermotolerant Gram-positive acidophiles have been described (*Alicyclobacillus (Alb.)* spp.) though none of the four recognised species has been isolated from mineral leaching environments. An ambiguous situation exists for the bacterium currently listed as *Sulfobacillus disulfidooxidans* [11], as this sulfur-oxidising mesophile is more closely related to *Alicyclobacillus* spp. than to other *Sulfobacillus* spp. (an error arising from an initial incorrect sequence being deposited for *S. thermosulfidooxidans*).

As part of our research activities, we have been examining the microbial diversity of a range of anthropogenic and natural metal-rich acidic environments, and have isolated a number of novel Gram-positive eubacteria. Most of these isolates oxidise both ferrous iron and reduced sulfur, though not all are moderate thermophiles. These isolates have been charatacterised physiologically and phylogenetically, and their capacities for accelerating sulfide mineral oxidation, in pure or in mixed cultures, have been assessed.

2. NOVEL MESOPHILIC GRAM-POSITIVE ACIDOPHILES

To date, three groups of mesophilic Gram-positive iron-oxidising bacteria have been isolated and characterised at UW Bangor. Two of these cluster within the low GC Gram-positives, while the third is closely related to the actinobacterium *Am. ferrooxidans*.

2.1. Mesophilic *Sulfobacillus* spp.

During a microbiological expedition to Montserrat, W.I., (just prior to the recent major period of volcanism on the island [12]) samples were obtained from which two strains of mesophilic spore-forming, iron-oxidising mesophiles were isolated [13, 14]. The major physiological characteristics of these bacteria were (in common with other *Sulfobacillus* spp.) (i) a remarkable metabolic flexibility (facultative anaerobes able to use a range of inorganic and organic electron donors), (ii) in being extremely acidophilic (capable of growth at pH 0.7, the lowest recorded for any iron-oxidizing microorganism) and, (iii) in having temperature maxima of about 40ºC (Table 1). The two isolates (L-15 and Riv-14) share 97% 16S rRNA gene similarity, indicating that they are separate species, and both cluster within the *Sulfobacillus* clade, being more closely related to *S. thermosulfidooxidans* than to *S. acidophilus* (Fig. 1). We propose the species name "*Sulfobacillus montserratensis*" for isolate L-15 and "*Sulfobacillus ambivalens*" for Riv-14.

Table 1. Some physiological characteristics of classified and recently-isolated Gram-positive acidophilic eubacteria.

	Endospore Formation	G+C (mol %)	$T_{opt.}$ (ºC) (range)	$pH_{opt.}$ (range)	Fe^{2+} ox.	S ox.	FeS_2 ox.	Fe^{3+} red.	CO_2 fix.	Het.[a] Growth	ω-cyclic fatty acids
S. thermo-sulfidooxidans	+	48-50	45-48 (<30-60)	~ 2 (1.5-5.5)	+	+	+	+	+	(+)	ND
S. acidophilus	+	55-57	45-50 (<30-55)	~ 2 (ND)	+	+	+	+	+	+	ND
"*S. yellowstonensis*" **YTF1**	+	55.5	55 (<35->60)	2.5 (1.7->3)	+	+	+	+	+	+	-
"*S. montserratensis*" **L-15**	+	52.3	37 (<30-43)	1.5 (0.7->2)	+	+	+	+	+	+	ND
"*Al. acidiphilus*" **YTH1**	+	ND	~ 45 (ND)	~ 2 (ND)	-	-	-	+	-	+	+
"*C. ferrivorus*" **GSM**	+	50.5	45 (<35->55)	1.8 (ND)	+	+	+	+	+	+	-
Isolate **SLC1**	+	ND	37 (<10-<45)	~ 2.5 (1.7-3.7)	+	-	(+)[b]	ND	-	+	-
Am .ferrooxidans	-	67-68.5	45-50 (<30-55)	~ 2 (ND)	+	(-)	(+)[b]	+	+	+	ND
"*F.acidiphilum*" **T-23**	-	54.8	37 (<10-<45	2.5 (<1.7-5)	+	-	(+)[b]	+	-	+	ND

a = heterotrophic growth, b = pyrite oxidation only in the presence of an organic compound

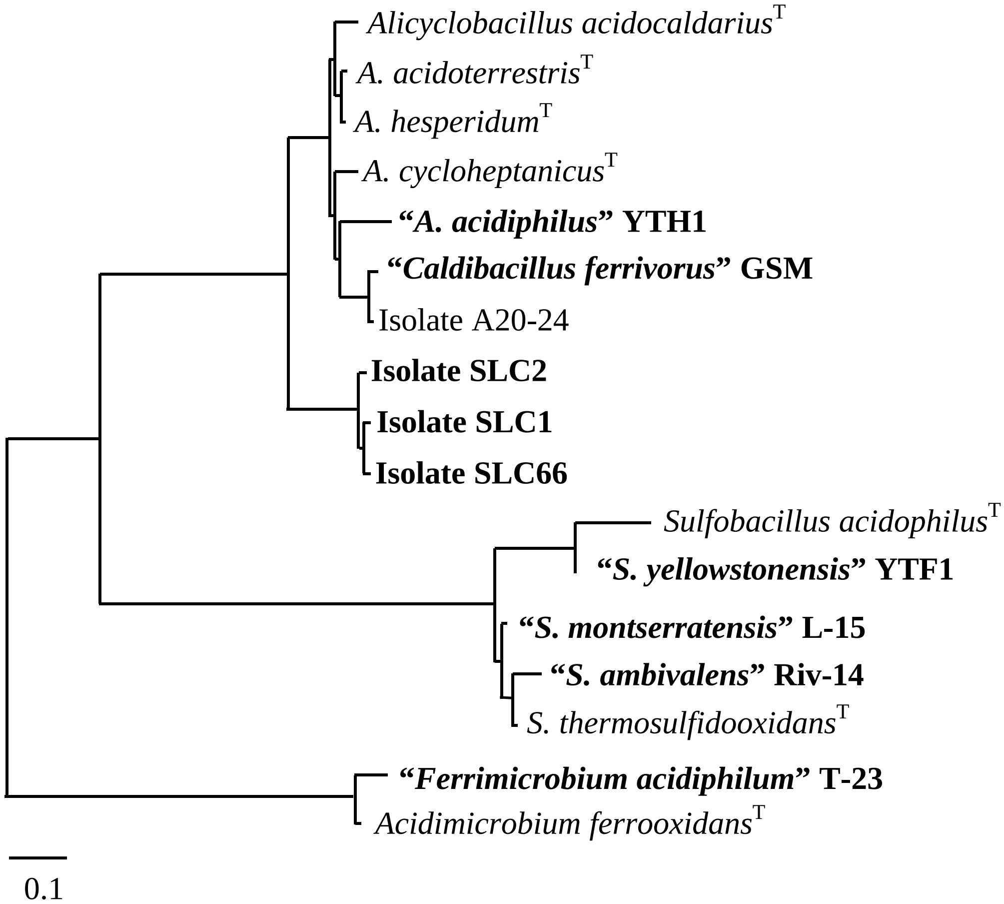

Figure 1. Dendrogram showing the phylogenetic relationships of known Gram-positive bacteria and those discussed here (in bold). The scale bar represents 0.1 inferred substitutions per site.

2.2. Other low GC Gram-positive, iron-oxidizing mesophiles

A number of apparently diverse iron-oxidising mesophilic bacteria were isolated from sulfidic regoliths subjected to accelerated oxidation in `humidity cell chambers' [15]. Some of these grew as `fried egg' colonies, which is characteristic of heterotrophic iron-oxidising acidophiles. As with *"Ferrimicrobium acidiphilum*" (described below) these isolates apparently lacked the ability to fix CO_2 and did not oxidise ferrous iron unless media were supplemented with organic materials (e.g. yeast extract). In contrast to *"F. acidiphilum*", however, these isolates formed endospores. Nearly complete (~1465 bases) 16S rRNA gene sequences have been obtained for three of these isolates ("SLC series"). As shown in Figure 1, these bacteria cluster closely together but as a distinct group within the low GC Gram-positive *Sulfobacillus/Alicyclobacillus* clade. The "SLC series" bacteria currently await more complete physiological characterisation.

2.3. Mesophilic iron-oxidizing *Actinobacteria*

Obligately heterotrophic iron-oxidizing mesophilic bacteria ("T-series" isolates) have been isolated from mine sites in north Wales, the USA and elsewhere [15]. Phylogenetic analysis (16S rRNA gene sequences) shows that they are most closely related to the iron-oxidising moderate thermophile *Am. ferrooxidans* (Fig. 1). However, the relatively low 16S rRNA gene sequence similarity (95%) of “T-series” bacteria to the gene from *Am. ferrooxidans*, together with their lower GC content (a difference of about 10 mol% G+C) and significantly lower temperature optima and maxima, lead us to propose the novel genus “*Ferrimicrobium*” for the “T-series” isolates, with the type species (represented by isolate T-23) being “*F. acidiphilum*”.

2.4. Novel moderately thermophilic iron-oxidizing Gram-positive bacteria

Mine spoil materials from the Golden Sunlight mine, Montana, were analysed for indigenous acidophilic microflora by plating dispersed samples onto selective solid media [14]. One of these, originally isolated on plates incubated at 30ºC, was later shown to be a moderate thermophile with a temperature optimum of about 46ºC. This isolate (GSM) has been studied in detail and has been shown to be a facultative anaerobe, capable of oxido-reduction of iron. GSM is, however, far more “heterotrophically inclined” than *Sulfobacillus* spp. and grows readily on a range of organic substrates, producing high cell yields (>10^9/ml). It can also grow mixotrophically and autotrophically (in yeast extract-containing and yeast extract-free media, respectively), and oxidises elemental sulfur. The iron-oxidase system in GSM appears to be inducible and can be suppressed by subculturing in heterotrophic media [14]. Phylogenetically, this isolate is positioned more closely to *Alicyclobacillus* spp. (obligate heterotrophs) than to the iron-oxidising *Sulfobacillus* spp. (Fig. 1), though its low sequence similarity (88% to *S. thermosulfidooxidans* and 93% to *Alicyclobacillus cycloheptanicus*) is again sufficient to warrant a novel genus label: “*Caldibacillus ferrivorus*” is the proposed species name for isolate GSM. Interestingly, bacteria branching closely to “*C. ferrivorus*” (e.g. isolate A20-24 in Fig. 1) have been isolated from the Sydney bay area, Australia, by Holden *et al.* [16].

Other iron-oxidising Gram-positive moderately thermophilic bacteria have been isolated from the Frying Pan Hot Springs (Norris Geyser basin) and the Sylvan Springs area in Yellowstone National Park, Wyoming [17]. One of these (isolate YTF1) studied in detail, is a novel *Sulfobacillus* sp. (proposed species name “*S. yellowstonensis*”) which grows very rapidly under aerobic (doubling time of less than one hour) and anaerobic conditions [18] and is more thermotolerant than other *Sulfobacillus* spp. (Table 1).

2.5. Moderately thermophilic iron-reducing, obligately heterotrophic Gram-positive bacteria

Several obligately heterotrophic Gram-positive moderately thermophilic bacteria (“YTH series”) were also isolated from the Sylvan Springs area of Yellowstone National Park [17]. These endospore-forming rods had many physiological features in common with *Alicyclobacillus* spp., and their relatedness to this genus was later confirmed by fatty acid analysis (which confirmed the presence of diagnostic ω-fatty acids) and phylogenetic analysis (Fig. 1) for “*Al. acidiphilus*” YTH1. Although not able to oxidize ferrous iron or reduced sulfur, several YTH isolates were found to catalyze the dissimilatory reduction of ferric iron.

3. LEACHING OF SULFIDE MINERALS BY GRAM-POSITIVE BACTERIA

All of the iron-oxidizing Gram-positive acidophiles have been found to accelerate the oxidative dissolution of pyrite in laboratory cultures, though some isolates only do so (or are much superior) when grown either in mixed culture with Gram-negative acidophiles, or in media containing organic materials, such as yeast extract (e.g. [19, 20]). For example, “*F. acidiphilum*” T-23 leaches pyrite in yeast extract-containing but not in purely “inorganic” media in pure culture, though mixed cultures of this obligate heterotroph and *At. thiooxidans* or *Acidiphilium (A.) acidophilum* (sulfur-oxidising acidophiles that are unable to oxidise pyrite in pure culture) can accelerate the oxidative dissolution of this mineral (Fig. 2).

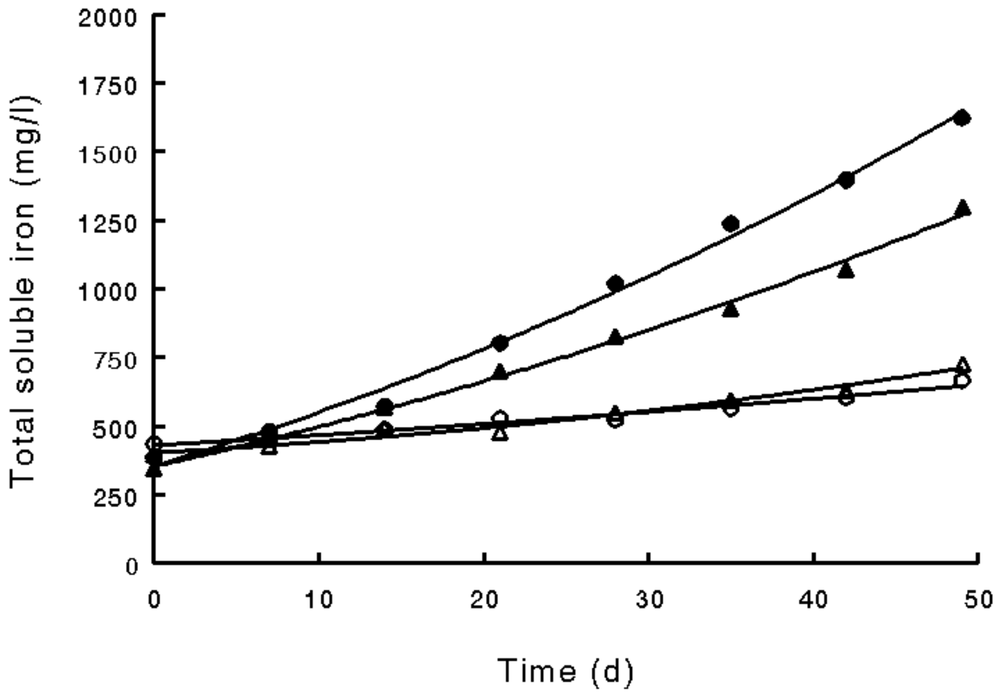

Figure 2. Leaching of pyrite by mixed cultures of "*F.acidiphilum*" and sulfur-oxidising Gram-negative bacteria. Key:) , *At. thiooxidans*; # , *At. thiooxidans* + "*F.acidiphilum*" T23; + , *A. acidophilum*; %, *A. acidophilum* + "*F.acidiphilum*" T23. Rates of iron solubilisation in pure cultures of "*F.acidiphilum*" T23 and in uninoculated controls were similar to those in pure cultures of the sulfur-oxidisers.

Of the other novel mesophilic Gram-positive isolates, the "SLC series" bacteria have been found to oxidize pyrite very slowly, even in organic-amended media, while both of the Montserrat *Sulfobacillus* isolates L-15 and Riv-14 can leach this mineral very effectively, particularly at very low pH (<1) where they are superior to the Gram-negative acidophiles ([14]; Fig. 3). Another characteristic of these bacteria (and many other Gram-positive acidophiles) is that they maintain significantly lower redox potentials (100-200 mV) during mineral leaching than both *L. ferrooxidans* and *At. ferrooxidans* (data not shown).

The rate of pyrite dissolution by "*C. ferrivorus*" GSM was found to be similar in "inorganic" medium (i.e. autotrophic growth) or in medium supplemented with 5 mM fructose + 0.001% yeast extract (mixotrophic/heterotrophic growth) (Fig. 4). However, the ratio of ferrous:ferric iron in these cultures (and calculated redox potentials) were very different in the different culture conditions (Fig. 4).

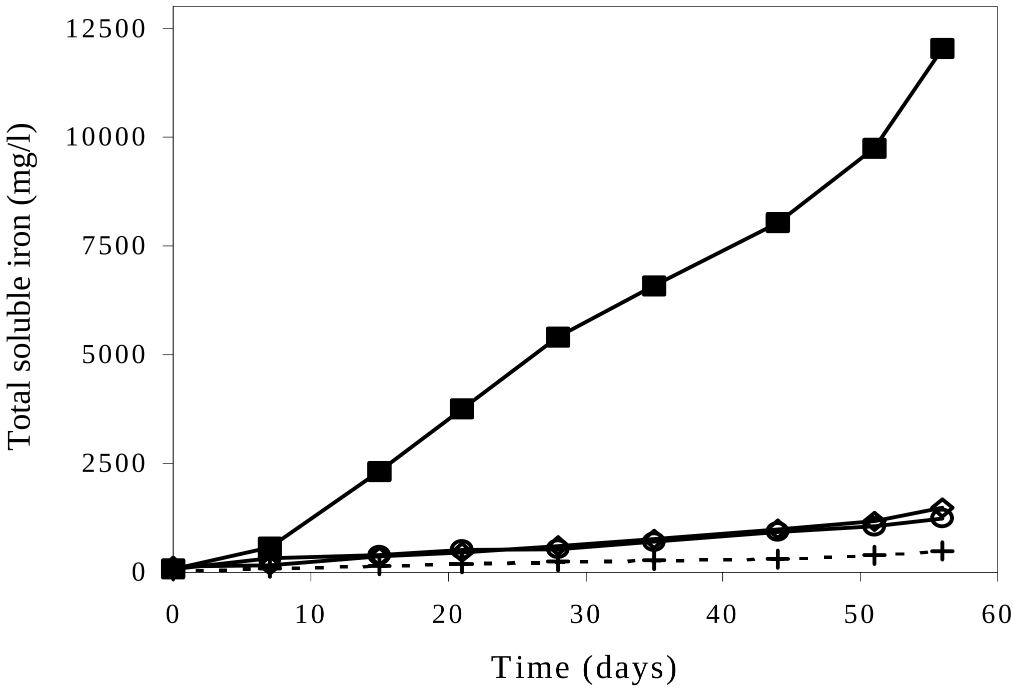

Figure 3. Oxidation of pyrite by (!) "*S. montserratensis"* L-15 in liquid medium poised initally at pH 1.2, and comparison with the type strains of *At. ferrooxidans* ( )) and *L. ferrooxidans* (( ). An uninoculated control is also shown (+).

4. LEACHING OF PYRITE BY MIXED CULTURES CONTAINING GRAM-POSITIVE ACIDOPHILES

Rates of leaching of pyrite by pure cultures of Gram-positive bacteria tend to be lower than those by Gram-negative bacteria except, as noted, in certain situations (e.g. at extremely low (<1) pH where "*S. montserratensis"* is much more effective than either *L. ferrooxidans* or *At. ferrooxidans*). Even at more elevated temperatures (~45ºC), moderately thermophilic *Sulfobacillus* spp. and "*C. ferrivorus"* GSM were found to be far less efficicient at leaching pyrite than a thermotolerant *Leptospirillum* strain [21]. However, mixed cultures of Gram-negative and Gram-positive acidophiles may be more effective mineral leaching systems than pure cultures of either. This is most notably the case for the mesophile *L. ferrooxidans*, which has been noted to form very stable mixed cultures with "*F. acidiphilum*"-like isolates that are both more robust and effective mineral leaching systems than corresponding pure cultures [22]. More recently, it has been shown that a thermotolerant *Leptospirillum* strain was unable to oxidise pyrite concentrate in pure culture, but was highly effective at so doing when grown in mixed culture with a variety of Gram-positive moderate thermophiles [21]. This phenomenon is quite possibly due to the particular sensitivity of *Leptospirillum* isolates to soluble organic materials (e.g. cell lysates, floatation chemicals) which might be metabolised by organotrophic Gram-positive acidophiles, though this hypothesis requires further testing.

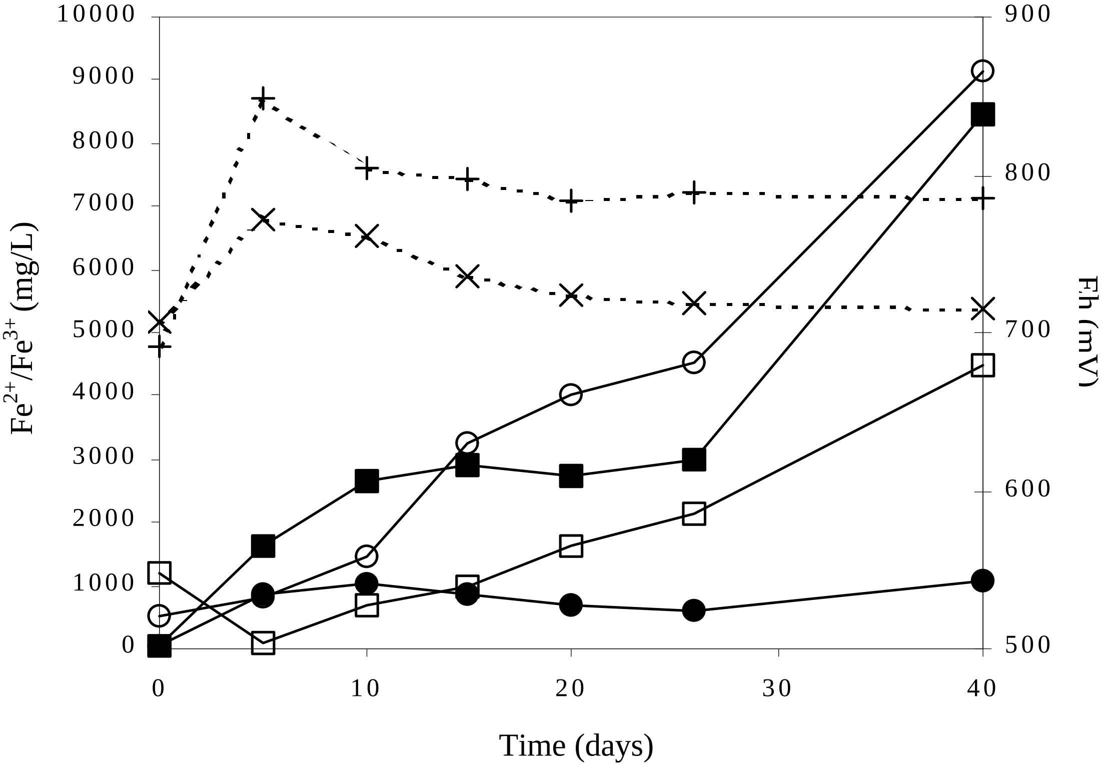

Figure 4. Speciation of soluble iron and calculated redox potentials during oxidation of pyrite by "*C. ferrovorus*" GSM in inorganic liquid medium and the medium amended with 5 mM fructose + 0.001% (w/v) yeast extract. Key:) ferrous iron, # ferric iron, X redox potential (-fructose/yeast extract); ' ferrous iron, ! ferric iron, 9 redox potential (+fructose/yeast extract).

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