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# BACTERIAL PYRITE OXIDATION I. THE EFFECT OF PURE AND MIXED CULTURES OF *THIOBACILLUS FERROOXIDANS* AND *THIOBACILLUS THIOOXIDANS* ON RELEASE OF IRON

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Fundamental investigations were made on the oxidation of pyrite and consequent dissolution of iron by pure and mixed cultures of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. The release of iron from pyrite was remarkably enhanced with large inocula above ca. $10^9$ cells per flask (or 1% pulp density) of 2-day (active) cultures of *T. ferrooxidans*, but not with inoculum of $10^8$ cells or less. Furthermore, a phenomenon was observed that the enhanced oxidation of pyrite always proceeded with the coexistence of a $10^8$ cells or less inoculum of *T. ferrooxidans* and *T. thiooxidans* incapable of oxidizing pyrite. During the bacterial oxidation of pyrite, high iron oxidation ratios ($Fe^{3+}$/total Fe) above 90% were maintained, coincident with the enhanced release of iron from pyrite. Contrarily, in the absence of *T. ferrooxidans*, a major portion of iron was in the ferrous form and iron release was not promoted. Thus, it was thought that *T. ferrooxidans* contributes to the oxidation of pyrite through the regeneration reaction of ferric iron.

The bacterially assisted oxidation of insoluble sulfide minerals has been studied by many investigators using the acidophilic, chemolithotrophic, iron-oxidizing bacteria *Thiobacillus ferrooxidans* and related species (*1* – *4*). Two main mechanisms of bacterial attack on sulfide minerals, indirect and direct oxidation mechanisms, have been proposed (*3* – *8*). In the indirect oxidation mechanism, sulfide minerals are oxidized chemically by ferric iron serving as an effective oxidizing agent, and the bacteria are involved in the oxidation of ferrous iron formed to the ferric state (*3*, *4*). In the direct oxidation mechanism, sulfide minerals are oxidized biochemically by the close contacts between the bacterial cells and solid substrates,

independent of the action of ferric iron (*5 – 8*). The role of ferric iron in the chemical oxidation of sulfide minerals is apparent (*9 – 11*), but the contribution of the bacteria to regeneration of ferric iron and the actual mechanism of the direct bacterial attack on sulfide minerals are yet not fully resolved. In this paper we report fundamental investigations on the oxidation of pyrite and consequent release of iron by the pure and mixed cultures of *T. ferrooxidans* and *Thiobacillus thiooxidans*.

## MATERIALS AND METHODS

*Bacterial strains.* The bacteria used in this study were *Thiobacillus ferrooxidans* strain Fel and *Thiobacillus thiooxidans* strain S3, which were originally isolated from the Matsuo Sulfur and Iron Sulfide Mine in Iwate Prefecture, and *Thiobacillus ferrooxidans* strain NCIB 8455 obtained from the National Collection of Industrial Bacteria, Aberdeen, U. K.

*Media and culture conditions.* *T. ferrooxidans* and *T. thiooxidans* were grown in 500-ml flat-bottomed flasks with 50 ml of SILVERMAN 9K mineral salt medium (*12*) supplemented with $FeSO_4$ or elemental sulfur (Table 1), respectively. Cultivation was carried out at 30° for a given period on a reciprocal shaker operating at 120 rpm with a 7-cm amplitude. A large volume of the culture of *T. ferrooxidans* was made using 3 *l* of $FeSO_4$-9K medium in a 3 *l* flat-bottomed flask under forced aeration at 25° for 2 – 3 days. The stock cultures of *T. ferrooxidans* and *T. thiooxidans* were maintained on 50 ml of $FeSO_4$-9K and S°-9K media in 500-ml flat-bottomed flasks, respectively. The cultures were serially transferred into new media at 4 – 5 day intervals for *T. ferrooxidans* and monthly for *T. thiooxidans*.

*Cell suspension.* After cultivation of *T. ferrooxidans* and *T. thiooxidans*, followed by centrifuging at 1,500×*g* for 10 min to remove iron precipitates or sulfur particles, the bacterial cells in the supernatant were harvested by centrifugation

Table 1. Culture media and their composition.

| | $FeSO_4$-9K medium | S°-9K medium | Modified 9K medium |
|---|---|---|---|
| $(NH_4)_2SO_4$ | 3.0 (g) | 3.0 (g) | 0.6 (g) |
| KCl | 0.1 | 0.1 | 0.1 |
| $K_2HPO_4$ | 0.5 | 0.5 | 0.1 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 | 0.5 | 0.2 |
| $Ca(NO_3)_2$ | 0.01 | 0.01 | 0.01 |
| $FeSO_4 \cdot 7H_2O$[a] | 80 | | |
| S°-powder[b] | | 20 | |
| Distilled water | 1,000 ml | 1,000 ml | 1,000 ml |
| pH[c] | 2.0 | 3.0 | 2.5 |

[a] Ferrous sulfate solution (pH 2.0) was separately prepared, filtered through a membrane filter (0.2 μm) (Toyo Roshi Co., Ltd), and added aseptically to basal salt solution.

[b] Powdered elemental sulfur was autoclaved at 120° for 20 min and added aseptically to basal salt solution.

[c] pH values were adjusted by the addition of $H_2SO_4$ solution.

at 12,000 × *g* for 10 min. The resulting cell pellet was washed by both resuspending and centrifuging twice with sulfuric acid solution (pH 1.5) for *T. ferrooxidans* or with distilled water for *T. thiooxidans*, and finally resuspended in a small volume of distilled water. The cell suspension was used as an inoculum the day it was prepared. The preparation procedures were all done aseptically.

*Cell count.* The total number of bacterial cells was measured with a bacterial counting chamber (Erma Optics Co., Tokyo) under a phase-contrast microscope.

*Pyrite used.* Matsuo and Yanahara pyrite ores, which were mined from the Matsuo Mine in Iwate Prefecture and the Yanahara Mine in Okayama Prefecture, respectively, were used throughout this work. Pyrite ores were preserved anaerobically in a vacuum desiccator with a drying agent. Chemical analysis showed that Matsuo pyrite contained 43.1% Fe and 49.7% S, and Yanahara pyrite, 45.8% Fe and 51.6% S.

*Preparation of ore powder.* The pyrite ore samples were prepared just before use. Small pieces of the ore were crushed into fine powder in a porcelain mortar and pestle and passed through a screen of 250-mesh (63 μm opening). The ore powder of minus 250 mesh was washed thoroughly by decantation with 1 N sulfuric acid solution and then with distilled water three times, respectively. During washing with distilled water, ore particles of 63 – 6.3 μm diameter were collected by a precipitation procedure according to the Stokes's law equation (*13*). The washed powder was dried overnight at 30° on a filter paper and used as a pyrite powder sample.

*Pyrite medium.* Pyrite medium was prepared just before use by placing Matsuo pyrite powder (1%, w/v) or Yanahara pyrite powder (4%, w/v) in a 500-ml flat-bottomed flask containing 50 ml modified 9K medium (Table 1), and autoclaved at 120° for 15 min. The modified 9K medium containing pyrite powder thus prepared was given the brief term pyrite medium.

*Pyrite oxidation experiment.* Pyrite medium was inoculated with 1 ml of the bacterial cell suspension and incubated at 30° on a reciprocal shaker at 120 rpm. All pyrite oxidation experiments were carried out in duplicate and repeated to check precision. Chemical controls containing pyrite medium, but not bacteria, were run simultaneously. When analysing, the culture flask was allowed to stand for 30 min to sediment ore particles, and the supernatant fluid of the culture was sampled quantitatively with a sterilized measuring pipette at intervals, filtered through a filter paper (Toyo-roshi No. 4), and analysed for pH, soluble iron (total and ferrous iron), and sulfate. Oxidation of pyrite was monitored mainly by measuring release of total soluble iron from the ores. Whenever samples were taken from the leached suspension in a flask, sterilized distilled water was added prior to sampling to compensate for the water loss due to evaporation during the course of experiments, and furthermore the suspension volumes removed were replaced with sterilized distilled water immediately after sampling.

*Analysis.* Iron and sulfate were determined colorimetrically by the $\alpha$, $\alpha'$-

dipyridyl method (*14*) and turbidimetrically by the barium sulfate method (*15*). The degree of iron oxidation was expressed as the ratio of ferric to total iron (%). pH was measured with a Tōa Denpa Kōgyo HM-5B pH meter. Elemental sulfur was determined as follows: A combined volume (100 ml) of cultures in two flasks was shaken with 20 ml of carbon disulfide in a 300-ml separatory funnel for 1 hr. After the layer of carbon disulfide was taken out and passed through a separatory filter (Toyo-roshi No. 2S), elemental sulfur extracted in the solvent was determined spectrophotometrically according to the method of FLIERMANS and BROCK (*16*).

## RESULTS

*Elemental sulfur, sulfate, and iron in the leachates*

Matsuo and Yanahara pyrite media were inoculated with the cultures of *T. ferrooxidans* strain NCIB 8455 and elemental sulfur, sulfate, and iron leached were determined at the time of cell addition and after incubation for a given period (Table 2). The amount of elemental sulfur in the cell-absent flasks increased after 24 or 29 days' incubation, but rather decreased in the flasks containing the bacteria in spite of remarkable dissolution of pyrite. The ratios of total sulfate to total soluble iron leached in the flasks with the bacteria were almost equal to the values calculated from the chemical analysis of pyrite samples (Matsuo pyrite, 3.31; Yanahara pyrite, 3.37). However, in the flasks lacking the bacteria, the ratios of sulfate leached to total soluble iron leached were small, but those of total sulfate to total soluble iron leached approached the calculated values.

*Effect of T. ferrooxidans on Matsuo pyrite oxidation*

An initial series of experiments was carried out to determine whether pyrite underwent oxidation and consequent dissolution of iron in the presence of *T. ferrooxidans*. Matsuo pyrite media were inoculated with the pure cultures of *T. ferrooxidans* strains Fel and NCIB 8455, and total soluble iron, sulfate, and corresponding changes in iron oxidation ratios and pH values were successively measured.

A typical representation of pyrite oxidation curves is given in Figs. 1 and 2. The release of total soluble iron and sulfate from the pyrite was greatly enhanced by the presence of *T. ferrooxidans*, and pH values of the leached suspension shifted rapidly from an initial pH of 2.5 to 1.5. Although the iron oxidation ratios of the uninoculated controls were almost constant, averaging about 20%, those of pyrite media inoculated with *T. ferrooxidans* increased rapidly just after the addition of the bacteria and were maintained at high values of above 90% during the leaching of pyrite.

Percentage dissolution of pyrite in the presence of *T. ferrooxidans*, calculated from the concentration of both total soluble iron and sulfate leached, reached 87 – 92% (strain Fel) and 91 – 95% (strain NCIB 8455) within 576 hr, about five times higher than control flask values.

Table 2. Release of elemental sulfur, sulfate, and soluble iron in the pyrite media inoculated with and without *T. ferrooxidans* (strain NCIB 8455).

| Pyrite[a] | Cell addition | Elemental sulfur released (ppm) | | S°-sulfate[c] (ppm) | Sulfate[d] leached (ppm) | Total[e] sulfate (ppm) | Total soluble[d] iron leached (ppm) | Total sulfate[f] / Total soluble iron leached |
|---|---|---|---|---|---|---|---|---|
| | | At the time of cell addition | After cultivation | (a) | (b) | (a+b) | (c) | (a+b/c) |
| Matsuo pyrite | Absent | 332 (5)[b] | 558 (24)[b] | 678 | 1,628 | 2,306 | 756 | 3.05 (2.15)[g] |
| | NCIB 8455 | | 138 (24) | — | 12,910 | 12,910 | 3,886 | 3.32 |
| Yanahara pyrite | Absent | 122 (5) | 196 (29) | 222 | 984 | 1,206 | 343 | 3.51 (2.87) |
| | NCIB 8455 | | 102 (29) | — | 14,162 | 14,162 | 4,223 | 3.35 |

[a] 0.5 g Matsuo pyrite or 2 g Yanahara pyrite in 50 ml modified 9K medium per flask.
[b] Parentheses: period of incubation (days).
[c] Calculated amout of sulfate equivalent to elemental sulfur increment.
[d] Sulfate and iron increment: differences of analytical values between the time of cell addition and after incubation for a given period.
[e] A total of S°-sulfate and sulfate leached (a+b).
[f] Ratio of sulfate to iron calculated from the chemical analysis of pyrite sample: Matsuo pyrite, 3.31; Yanahara pyrite, 3.37.
[g] Parentheses: ratio of sulfate leached to total soluble iron leached (b/c).

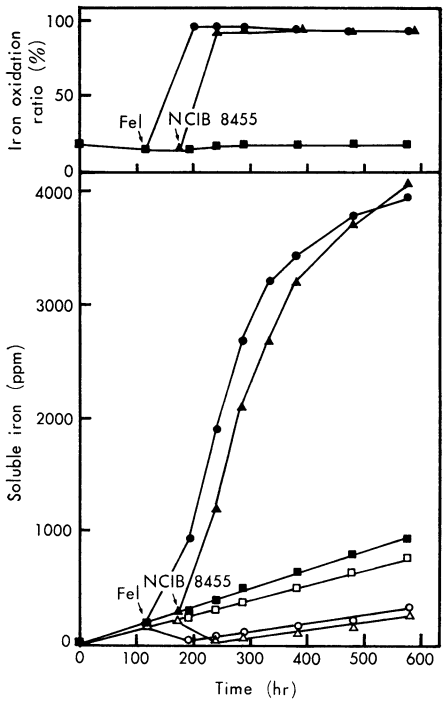


Fig. 1.

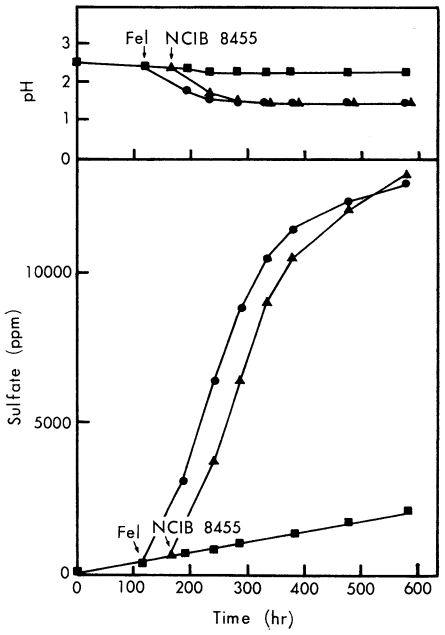


Fig. 2.

Fig. 1. Effect of *T. ferrooxidans* on oxidation of Matsuo pyrite: Release of soluble iron from pyrite and changes in iron oxidation ratios.

Matsuo pyrite media at 1% w/v pulp density (50 ml in a 500-ml flat-bottomed flask) were inoculated with *T. ferrooxidans* strains Fel and NCIB 8455 grown on $FeSO_4$-9K medium for 2 and 5 days, respectively. Inoculum size (cells/flask): Fel $7.3 \times 10^9$ (●, ○), NCIB 8455 $5.8 \times 10^9$ (▲, △), cells absent (■, □). Closed and open symbols in the lower figure denote total soluble and ferrous iron, respectively. Arrows in this and subsequent figure (s) indicate inoculation of bacteria.

Fig. 2. Effect of *T. ferrooxidans* on oxidation of Matsuo pyrite: Release of sulfate from pyrite and changes in pH values.

The experiment corresponds to that in Fig. 1. The symbols are the same as those in Fig. 1.

*Effect of T. thiooxidans on Matsuo pyrite oxidation*

As shown in Fig. 3, the addition of pure cultures of *T. thiooxidans* strain S3 to Matsuo pyrite media resulted in only a slight increase in release of total soluble iron and sulfate, and pH values of the inoculated flasks dropped to about 1.5. Iron oxidation ratios remained low in the inoculated flasks owing to the absence of iron-oxidizing activity of *T. thiooxidans*.

*Effect of T. ferrooxidans and T. thiooxidans on Yanahara pyrite oxidation*

Since Yanahara pyrite was less susceptible to chemical oxidation in air and

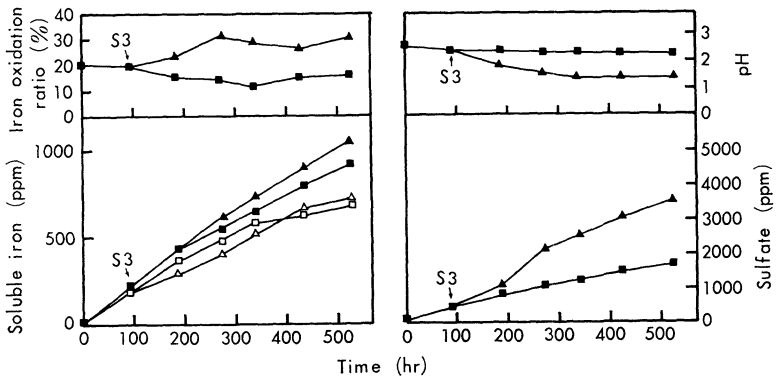


Fig. 3. Effect of *T. thiooxidans* on oxidation of Matsuo pyrite: Release of soluble iron and sulfate from pyrite and changes in iron oxidation ratios and pH values.

*T. thiooxidans* strain S3 was cultured on S°-9K medium for 5 days. Inoculum size (cells/flask): $7.8 \times 10^9$ (▲, △), cells absent (■, □). Closed and open symbols in the left lower figure denote total soluble and ferrous iron, respectively.

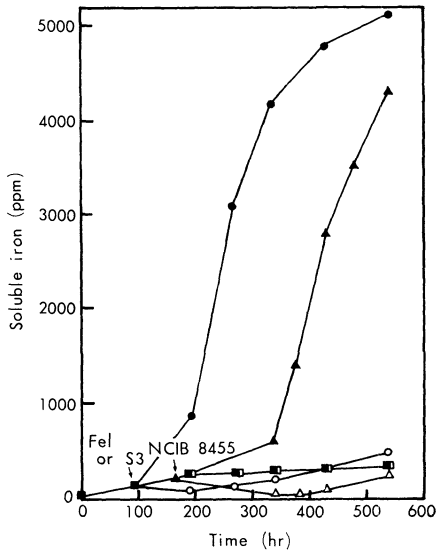


Fig. 4. Effect of *T. ferrooxidans* and *T. thiooxidans* on oxidation of Yanahara pyrite: Release of soluble iron from pyrite.

Yanahara pyrite media at 4% w/v pulp density (50 ml in a 500-ml flat-bottomed flask) were inoculated with *T. ferrooxidans* strains Fel and NCIB 8455 cultured for 2 days and *T. thiooxidans* strain S3 cultured for 5 days. Inoculum size (cells/flask): Fel $5.2 \times 10^9$ (●, ○), NCIB 8455 $5.8 \times 10^9$ (▲, △), S3 $4.6 \times 10^9$ (■, □), cells absent (■, □). Closed and open symbols denote total soluble and ferrous iron, respectively.

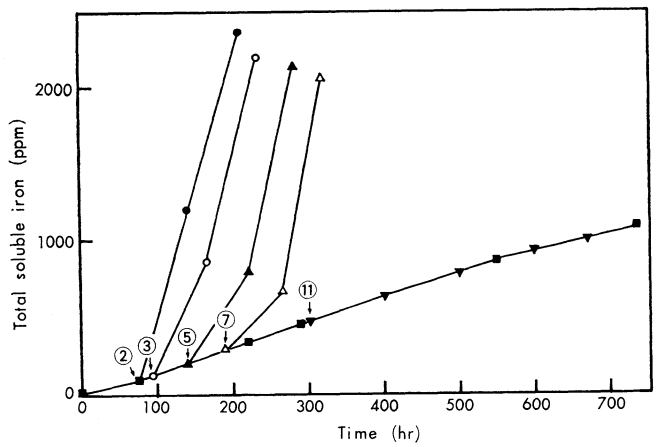


Fig. 5. Effect of varying the cell age of *T. ferrooxidans* on pyrite oxidation.

Matsuo pyrite media. *T. ferrooxidans* strain Fel was cultured on 3 *l* of $FeSO_4$ (2%)-9K medium prepared in a 3 *l* flat-bottomed flask under forced aeration, and parts of the culture (ca. 300 ml) were sampled from the flask successively on 2, 3, 5, 7, and 11 days. Cell suspensions prepared were inoculated at the time indicated by arrows into the pyrite media which had been shaken before inoculation. Numbers in circles denote bacterial cell ages. Inoculum size (cells/flask): $7.4\times10^9$ (●), $6.2\times10^9$ (○), $6.0\times10^9$ (▲), $7.1\times10^9$ (△), $5.5\times10^9$ (▼), cells absent (■).

water than Matsuo pyrite, a further experiment was carried out to demonstrate how Yanahara pyrite was oxidized by both thiobacilli. Figure 4 represents the iron release curves and indicates that oxidation of Yanahara pyrite was also remarkably enhanced by *T. ferrooxidans* and not at all by *T. thiooxidans*. The iron oxidation ratios increased swiftly even up to 90% with increasing release of iron from pyrite. The percentage dissolution of the pyrite, as determined by total soluble iron leached, was approximately 30% and 38% for strains NCIB 8455 and Fel, respectively, 15 – 18 times larger than that for the control.

*Effect of cell age on pyrite oxidation*

In an attempt to ascertain the effect of inoculum cell age on the oxidation of pyrite by *T. ferrooxidans*, Matsuo pyrite media were inoculated with cells of *T. ferrooxidans* cultured for various periods ranging from 2 to 11 days. Figure 5 represents the iron release curves of the respective inoculum cell age, indicating that the lag times of iron release after cell addition became longer as cells aged. However, with 11-day cultured cells, no appreciable increase in dissolution of iron occurred within the time of testing. It was apparent that an active cell inoculum taken from the 2-day culture was the more effective on the leaching of pyrite when compared with the inocula from the cultures grown for a greater number of days.

*Effect of inoculum size on pyrite oxidation*

Since cell inoculum size would be an important factor for bacterial pyrite

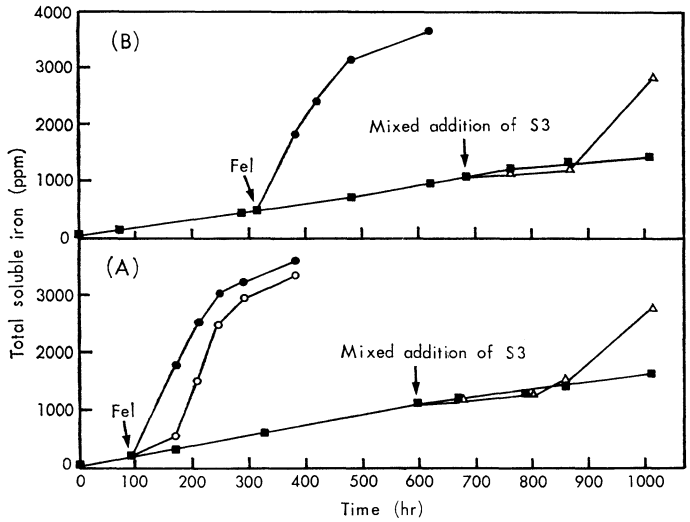


Fig. 6. Effect of varying the inoculum size of 2-day (A) and 11-day (B) cultured *T. ferrooxidans* and mixed addition of *T. thiooxidans* on pyrite oxidation.

Matsuo pyrite media. *T. ferrooxidans* strain Fel was cultured for 2 or 11 days. *T. thiooxidans* strain S3 was cultured for 5 days. (A) Inoculum size (cells/flask) of 2-day (active) culture: Fel $3.9\times10^{10}$ (●), Fel $3.9\times10^{9}$ (○), Fel $3.9\times10^{8}$ (■), Fel $3.9\times10^{8}$+S3 $7.5\times10^{9}$ (△), cells absent (■). (B) Inoculum size (cells/flask) of 11-day (inactive) culture: Fel $9.0\times10^{10}$ (●), Fel $4.5\times10^{9}$ (■), Fel $4.5\times10^{9}$+S3 $6.9\times10^{9}$ (△), cells absent (■). *T. thiooxidans* was inoculated into the flasks with $10^{8}$ active cells or with $10^{9}$ inactive cells of *T. ferrooxidans* at the time indicated by arrow.

oxidation, cell inocula of 2-day (active) and 11-day (inactive) cultures of *T. ferrooxidans*, varying from $10^{4}$ to $10^{10}$ cells per flask, were added to Matsuo pyrite media. Figure 6 represents the iron release curves for active and inactive cell inocula. In the case of active cell inocula (A), release of iron was promoted with inocula of $10^{9}$ and $10^{10}$ cells per flask and not with inocula of $10^{8}$ cells per flask or less. Furthermore, an increase of iron release was observed after a long lag time when *T. thiooxidans* was inoculated into the flask containing $10^{8}$ active cells of *T. ferrooxidans*. On the other hand, in the case of inactive cell inocula (B), iron release was promoted only in the flask with $10^{10}$ cells. When *T. thiooxidans* was mixed with $10^{9}$ inactive cells of *T. ferrooxidans*, dissolution of iron was also enhanced.

*Effect of mixed addition of T. ferrooxidans and T. thiooxidans on pyrite oxidation*

To ascertain the promoting effect of mixed cultures of *T. ferrooxidnas* and *T. thiooxidans* on pyrite oxidation, cultures of both thiobacilli were simultaneously inoculated into Matsuo pyrite media at the start of the leaching experiment. Cell inocula of *T. ferrooxidans* were prepared from an active culture grown for 2 days.

As shown in Fig. 7, it was confirmed that release of iron was unaffected by the

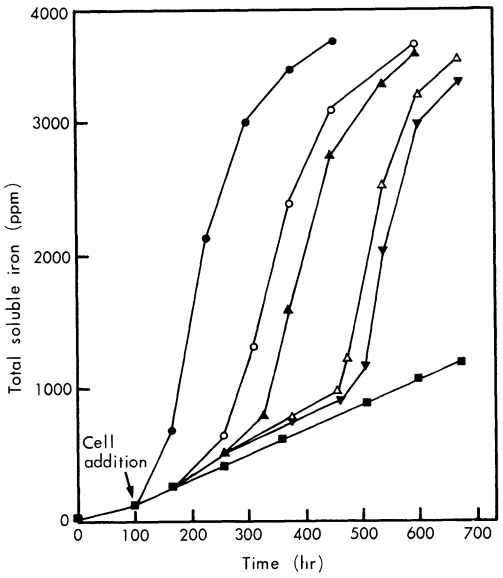


Fig. 7. Effect of mixed addition of *T. ferrooxidans* and *T. thiooxidans* on pyrite oxidation.

Matsuo pyrite media. *T. ferrooxidans* strain Fel and *T. thiooxidans* strain S3 were cultured for 2 and 5 days, respectively. Single addition of *T. ferrooxidans* (cells/flask): $4.3\times10^{9}$ (●), $4.3\times10^{8}$–$4.3\times10^{4}$ (■), cells absent (■). Mixed addition of both thiobacilli (cells/flask): Fel $4.3\times10^{9}$ (●), Fel $4.3\times10^{8}$ (○), Fel $4.3\times10^{6}$ (▲), Fel $4.3\times10^{5}$ (△), Fel $4.3\times10^{4}$ (▼). Inoculum size (cells/flask) of *T. thiooxidans* was $7.8\times10^{9}$.

addition of *T. ferrooxidans* ranging from $10^{8}$ to $10^{4}$ cells per flask. However, iron release in the flask with $10^{8}$ cells or less of *T. ferrooxidans* was considerably accelerated by the mixed addition of *T. thiooxidans*, although the lag times of iron release increased gradually as the inoculum size of *T. ferrooxidans* diminished. Thus, these results indicate that varying the inoculum size of *T. ferrooxidans* in the coexistence of *T. thiooxidans* produces a large difference in the lag phases of the iron release curves, but that release of iron from pyrite after the lag phases eventually proceeded at a similar rate and to a similar extent irrespective of the inoculum size.

## DISCUSSION

The reactions for the indirect oxidation of pyrite are as follows (*4*): At first, pyrite is oxidized chemically to ferrous sulfate and sulfuric acid. Ferrous iron is concurrently oxidized by *T. ferrooxidans* to yield ferric sulfate (Eq. 1).

$$2FeSO_4+{}^{1}/_{2}O_2+H_2SO_4 \xrightarrow{\text{Bacteria}} Fe_2(SO_4)_3+H_2O \qquad \text{(Eq. 1)}$$

The oxidation of pyrite is accelerated in the presence of ferric iron (Eq. 2).

$$FeS_2 + Fe_2(SO_4)_3 \longrightarrow 3FeSO_4 + 2S \qquad \text{(Eq. 2)}$$

The elemental sulfur is oxidized to sulfuric acid by the bacteria (Eq. 3).

$$S + 1^1/_2O_2 + H_2O \xrightarrow{\text{Bacteria}} H_2SO_4 \qquad \text{(Eq. 3)}$$

The ferrous iron formed in Eq. 2 is reoxidized biologically to ferric iron by *T. ferrooxidans* according to Eq. 1 and then the iron redox cycle is repeated successively.

The present results decisively demonstrate that the oxidation of both Matsuo and Yanahara pyrite is accelerated remarkably by the presence of *T. ferrooxidans*. During the bacterial oxidation of pyrite, high iron oxidation ratios above 90% were maintained in the leachates and were coincident with the enhanced dissolution of iron from pyrite. Contrarily, in the absence of the bacteria, a major portion of the iron was still in the ferrous form. The reaction of Eq. 1 occurs chemically but at an extremely slow rate in a low pH environment. Furthermore, *T. ferrooxidans* are able to oxidize ferrous iron at a rate $5 \times 10^5$ times as fast as would occur in their absence (*17*). Therefore, it is confirmed from these data that *T. ferrooxidans* plays an active role in the reaction of ferrous iron oxidation (Eq. 1) and that ferric iron chemically attacks pyrite, serving as an oxidizing agent (Eq. 2).

SATO (*18*, *19*) has reported that the direct oxidation of a simple sulfide mineral is a process in which the metal atoms move into the surrounding solution to become aqueous cations, thus enriching the remaining solid phase with sulfur atoms. The remaining sulfur is oxidized to sulfate. With pyrite and marcasite, the sulfur released from the crystal structure may be in an unstable form (Eq. 4), and will be oxidized in the presence of oxidizing agent (Eq. 3).

$$FeS_2 + H_2SO_4 + {}^1/_2O_2 \longrightarrow FeSO_4 + 2S + H_2O \qquad \text{(Eq. 4)}$$

It was found that elemental sulfur accumulated in the pyrite medium without *T. ferrooxidans*, but that in the pyrite medium with the bacteria, elemental sulfur decreased and the ratios of total sulfate to total soluble iron leached became equal to the calculated values from chemical analysis of pyrite. Thus, it is conceivable that pyrite is chemically dissolved to yield elemental sulfur according to Eq. 2 and/or Eq. 4, and that elemental sulfur produced is further oxidized to sulfuric acid through Eq. 3 chemically and/or biologically in the presence of *T. ferrooxidans*.

As indicated by our and other published data, it is indisputable that *T. ferrooxidans* contributes to the leaching of pyrite by regeneration of ferric iron. However, disagreement exists as to the ability of *T. thiooxidans* to oxidize pyritic materials. The oxidation of marcasite and pyrite-containing concretions (*20*), pyrite (*21*), and also zinc sulfide (*22*) by this bacterium has been reported. On the other hand, it has been reported that *T. thiooxidans* is unable to enhance the oxidation of pyrite (*23*, *24*). The present results also indicate that *T. thiooxidans* cannot oxidize pyrite under our test conditions. Therefore, we think that *T. thiooxidans* is not concerned directly with the dissolution of pyrite.

It is recognized that iron-oxidizing bacteria must be present in sufficient

numbers for the enhancement of pyrite oxidation in aqueous solution. Judging from the present study, the critical cell number for the enhancement appears to be about $10^9$ active cells per flask (or 1 % pulp density), under which the oxidation of pyrite ceases. However, a phenomenon was observed that the enhanced oxidation of pyrite always proceeded with mixtures of *T. ferrooxidans* and *T. thiooxidans* even if the inoculum cell size of *T. ferrooxidans* was below the critical cell number. The enhancement of pyrite oxidation by the mixed inoculation of both thiobacilli is an interesting point which has important implications for the processes occurring. It has been shown that mixed cultures of iron-oxidizing bacteria, *Leptospirillum ferrooxidans* as well as *T. ferrooxidans*, with the other acidophilic sulfur-oxidizing bacteria including *T. thiooxidans*, *T. organoparus* and *T. acidophilus* are more effective than the pure cultures of iron-oxidizing bacteria for the dissolution of pyrite (*21*, *25*). It has also been reported that the leaching of a Cu-Ni sulfide concentrate is accelerated by the mutualistic association of *T. ferrooxidans* with heterotrophic bacteria *Beijerinckia lactinocogenes* (*26*). The problems of how mixed populations of bacteria effect mineral dissolution are important in natural leaching systems but have not yet been fully solved. At present, it is difficult to define the inability of *T. ferrooxidans* to oxidize pyrite below a critical cell number and the contributing role of *T. thiooxidans* in the enhancement of pyrite oxidation by the mixed flora of *T. ferrooxidans* and *T. thiooxidans*.

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