Evaluation of Tannic and Fulvic Acids as Inhibitors of Cell Growth and Iron and Sulfur Oxidation in *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*

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The inhibitory effects of fulvic and tannic acids on the cell growth, and iron and sulfur oxidation in *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* were studied around pH 2, and compared with those of oxalic acid. Tannic acid at 500 ppm inhibited the cell growth, and iron and sulfur oxidation in 8.00 × 10⁹ cells of *T. ferrooxidans* and *T. thiooxidans*, more than two weeks, possibly due to its phenolic group, while fulvic acid inhibited them only within initial two days. Both were more effective inhibitors than oxalic acid.

**KEY WORDS**: *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, Fulvic Acids, Tannic Acids, Inhibition

**1. Introduction**

Pyrite (FeS₂) weathering in mine drainage causes environmental problems. Chemoautotrophic iron- and sulfur-oxidizing bacteria, *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, often grow actively in mine water and play an important role in pyrite weathering, especially in aerobic environments (Taylor, et al., 1984).

The organic inhibition of chemoautotrophic bacteria of genus *Thiobacillus* has been discussed by Rittenberg (1972). Anionic surfactants, such as sodium lauryl sulfate (SLS) and alkylbenzene sulfonate (ABS) function as effective inhibitors to cell growth and iron-oxidizing activity in *T. ferrooxidans* (Dugan, 1975; Kleinmann, et al., 1981; Onysko, et al., 1984). However, the extensive use of detergents is not environmentally safe. The World Health Organization recommends that stream detergent concentration be less than 1.0 ppm.

In pyrite weathering, Fe(III) ions are not only direct oxidants in indirect contact mechanism of the iron-oxidizing bacteria in aerobic environments, but also important oxidants in anaerobic environments (Luther, et al., 1991). It has been reported that the oxidation of pyrite with Fe(III) ions was suppressed by addition of oxalate in anaerobic environments (Sasaki, et al., 1995a) and by coexisting humic acid (Lalvani and Zhang, 1994). Both suppressions were explained by lowering the standard redox potential of Fe(III)/Fe(II), $E^\circ_{Fe(III)/Fe(II)}$, caused by complexation of Fe(III) to oxalic and humic acids. Though it has been reported that oxalic acids inhibited the cell growth as well as iron and sulfur oxidation in *T. ferrooxidans* (Dugan, 1975; Tuttle, et al., 1976) and *T. thiooxidans* (Iwatsuka and Mori, 1960), inhibitory effects of humic substances on chemoautotrophic bacteria is not well known.

Humic substances are widely distributed in both water and soil environments, and they are weak-acid polyelectrolytes and involved in many reactions, such as chelation, reduction, and adsorption, due to their functional groups of carboxylic, phenolic, quinolic and alcoholic groups. Fulvic acid makes up more than 50% of humic substances in natural environments, they are soluble at all pHs while humic acid is insoluble below pH 2, and fulvic acid has more functional groups than humic acid (e.g., Kuwatsuka, et al., 1992). Tannic acid is abundant in oak-leaves, is soluble at acidic pHs, and has carboxylic and phenolic groups. Due to its simpler structure, tannic acid is often used as a reference compound when evaluating humic substances. Fulvic and tannic acids are soluble and capable of reducing Fe(III) to Fe(II) ions around pH 2 (Skogebroe and Wilson, 1981).

In the present work, the inhibitory effects of fulvic and tannic acids on the cell growth, and iron and sulfur oxidation in *T. ferrooxidans* and *T. thiooxidans* were studied around pH 2, and compared with those of oxalic acid. This work has potential utility for selective control of acid production in acid mine drainage caused by *T. ferrooxidans* and *T. thiooxidans*.

**2. Experimental**

**2.1 Microorganisms**

Iron-oxidizing bacteria, *Thiobacillus ferrooxidans* (HUTFY8906), were cultured and harvested as described elsewhere (Sasaki, et al., 1995b). Sulfur-oxidizing bacteria, *Thiobacillus thiooxidans*, were supplied by American Type Culture Collection (ATCC8085), cultivated routinely in 150 cm³ of solution containing the basal salt solution of the 9K medium (Silverman and Lundgren, 1959) plus 1.50 g of elemental sulfur as an energy source, adjusted to pH 2.0 with H₂SO₄ in a silico-plugged 500-cm³ Erlenmeyer flask. Cells were harvested in the same manner as *T. ferrooxidans*. Cell
numbers were directly counted by microscopic observation (×600).

2.2 Organic acids
Tannic acid (TA, e.g., C₆H₂(OH)₃COOC₆H₂(OH)₂COOH, Koso Chemicals, analytical reagent, AR), fulvic acid (FA), and oxalic acid (OA, COOH)₂·2H₂O, Wako Chemicals, AR) were used as inhibitors. Fulvic acid was isolated from the Dando Soil according to International Humic Substances Society (IHSS) method, and provided from the Humic Substances Society of Japan (Watanabe, et al., 1994). The elemental composition of fulvic acid was 45.57% C, 4.96% H, 1.70% N, 48.09% O, and 0.68% S on ash and water free basis. Functional analysis showed 7.27 meq. COOH, 1.37 meq. phenolic OH, 1.76 meq. alcoholic OH, 5.80 meq. C=O.

2.3 Culture tests
The 500-cm³ brown Erlenmeyer flasks were filled with 150 cm³ of the 9K medium for the culture of T. ferrooxidans, or the 9K basal solution plus 1.50 g of sterilized elemental sulfur for the culture of T. thiooxidans, the initial pHs were adjusted to 2.2–2.3 and 0–75.0 mg fulvic, tannic, or oxalic acids was added. The additives are entirely dissolved in the medium. The control culture was set triply without inhibitors. All these flasks were inoculated with 8.00×10⁹ cells of T. ferrooxidans or T. thiooxidans. A sterilized experiment was also performed without microorganisms and inhibitors.

The flasks were installed in a rotary shaking culture-apparatus (Takasaki Kagaku Co., Ltd., TB-16) at 30±2°C and incubation was carried out for 14 days, under light shielding to avoid decomposition of carboxylic compounds. At intervals, cell numbers were counted, and 1 cm³ of supernatant was pipetted off and filtered with a 0.2 μm pore size membrane filter for solution analysis.

2.4 Solution analysis
The solution analysis was carried out as follows. For experiments with T. ferrooxidans, the redox potential of the medium (E vs NHE) and the concentration of Fe(II) ions were measured. The Fe(II) ions were determined by the 1, 10-phenanthroline method. The microorganisms oxidize Fe(II) to Fe(III) ions so E increases rapidly with the increase in iron-oxidizing activity of T. ferrooxidans (Sasaki, et al., 1993).
this system, increases in $E$ are correlated to iron oxidation and
to growth of the microorganism (Dugan and Lundgren, 1964).
For experiments with \textit{T. thiooxidans}, the pH and concentra-
tions of dissolved S species were measured. Concentrations of
soluble S species were determined by ICP-AES (SEIKO Co.
Ltd., SPS 1200). The microorganisms oxidize elemental S to
soluble S species and produce H$^+$ ions during the culture.

3. Results

3.1 Effect of organic acids on cell growth and
iron-oxidizing activity in \textit{Thiobacillus ferrooxidans}

The effects of fulvic, tannic, and oxalic acids on the cell
growth, $E$, and consumption of Fe(II) ions in the culture of \textit{T.
ferrooxidans} are shown in Fig. 1, where the control data with-
out inhibitors are shown with solid circles and are averages of
three experiments. The sterilized data are shown with $\times$.
With 50 ppm of fulvic acid cell growth was suppressed a little
and the iron oxidation was delayed about 12 hours. With 500
ppm of fulvic acid there was an extended lag in cell growth
and iron oxidation by \textit{T. ferrooxidans} was delayed about 48
hours, cell numbers and $E$ recovered after 5 days and were
then not inhibited.

Tannic acid prevented cell growth and iron oxidation in
\textit{T. ferrooxidans}, in longer periods than fulvic acid. With 500
ppm tannic acid inhibited cell growth completely and inhib-
ited the iron oxidation of $8 \times 10^9$ cells of \textit{T. ferrooxidans} for
more than 14 days.

Oxalic acid had little effect on the cell growth of \textit{T.
ferrooxidans}, $E$, and the oxidation of Fe(II) ions even at 500
ppm (3.97 mmol dm$^{-3}$), similar to the control experiment. It is
considered that there is no lowering of the standard redox poten-
tial, $E_0^0$, in the presence of 500 ppm oxalic acid, because the
concentration of oxalic acid was too small to reduce $E_0^0$ by
ligand effects (Sasaki, et al., 1995a).

3.2 Effect of organic acids on cell growth and
sulfur-oxidizing activity in \textit{Thiobacillus thiooxidans}

The effects of fulvic, tannic, and oxalic acids on the cell
growth, pH, and concentration of dissolved S species in the

![Graphs showing the effect of fulvic acid (FA), tannic acid (TA), and oxalic acid (OA) on cell growth, pH, and dissolved S species concentration.](image-url)
culture of *T. thiooxidans* are shown in Fig. 2, where the symbols are the same as those in Fig. 1. The 50~500 ppm of fulvic acid had a small effect on the cell growth of *T. thiooxidans*, but enhanced the sulfur oxidation.

Tannic acid dramatically inhibited cell growth, decrease in pH, and sulfur oxidation in 8.00×10^9 cells of *T. thiooxidans*, in longer periods than fulvic acid, and 500 ppm of tannic acid suppressed them completely for more than 14 days, to the same level as in the sterilized experiments.

Oxalic acid did not affect clearly the cell growth, changes in pH, and sulfur oxidation in *T. thiooxidans* even at 500 ppm.

4. Discussion

It is known that carboxylic acids are inhibitory to *T. ferrooxidans* (Tuttle et al., 1976), and *T. thiooxidans* (Iwatsuka and Mori, 1960). Tuttle et al. (1976) reported that the inhibitory ability of oxalic acid on cell growth and iron oxidation in *T. ferrooxidans* was greater than those of other monocarboxylic acids and α-keto acids, and that 100 % of 3.2×10^10 cells cm^{-3} of *T. ferrooxidans* were inhibited by 10^{-2} mol dm^{-3} of oxalic acid and 10 % by 10^{-3} mol dm^{-3} of oxalic acid. Iwatsuka and Mori (1960) used manometric measurements to establish that the inhibition of oxalic acid on cell growth and sulfur oxidation in *T. thiooxidans* was 15 % at 10^{-2} mol dm^{-3} and zero at 10^{-3} mol dm^{-3} around pH 3.8 (below pK2), but cell numbers were not given. In the present work, even 500 ppm of oxalic acid did not show clear inhibition of iron and sulfur oxidation in 5.33×10^6 cells cm^{-3} of *T. ferrooxidans* and *T. thiooxidans*, though there was some inhibition of cell growth in *T. ferrooxidans* and *T. thiooxidans* in the earlier periods.

Natural organic acids, tannic and fulvic acids, were more effective inhibitors than oxalic acid. The inhibitory effect on cell growth and iron and sulfur oxidation in *T. ferrooxidans* and *T. thiooxidans* was greater in the order of tannic acid > fulvic acid > oxalic acid. Both tannic acid and fulvic acid are reductants due to their phenolic groups, and abiotically they would reduce Fe(III) to Fe(II) ions in cultures of *T. ferrooxidans* (Skogerboe and Wilson, 1981). However, it was observed that both tannic acid and fulvic acid inhibited cell growth of *T. ferrooxidans* and *T. thiooxidans* at the initial period, and that tannic acid was a stronger inhibitor than fulvic acid, as shown in Figs. 1 and 2 (top). On the basis of electron microscopic observations Tuttle et al. (1977) demonstrated that carboxylic acids nonselectively disrupted the cell envelope and cytoplasmic membrane of *T. ferrooxidans* by reaction with cations which contribute to their structural integrity, leading to inhibition of iron oxidation in the microorganisms. The above results also suggest that inhibition of cell growth is correlated to iron or sulfur oxidation with *T. ferrooxidans* or *T. thiooxidans*, except for the inhibition of sulfur oxidation in *T. thiooxidans* by fulvic acid shown in Fig. 2 (left). Fulvic acid enhanced sulfur oxidation. Direct contact between microorganisms and elemental sulfur is necessary to oxidize elemental sulfur by *T. thiooxidans* (Takakawa, et al., 1979). Fulvic acid acts as a surfactant, and when it is adsorbed on particles of elemental sulfur the physicochemical affinity between microorganisms and elemental sulfur would be enhanced. Tannic acid with phenolic and carboxylic groups was more strongly inhibitory to *T. ferrooxidans* and *T. thiooxidans* than oxalic acid which has no phenolic groups, suggesting that the cell envelope and membrane are mainly disrupted by the phenolic groups rather than by carboxylic groups. Though the molecular weight of fulvic acid was not obtained in the present work, it is generally reported to be 500~800. The inhibitory ability of fulvic acid is rather weaker than tannic acid due to fewer phenolic and carboxylic groups per molecular unit. The present work indicates that the natural organic acids, tannic and fulvic acids, would function both as reductants and ligands to Fe(III) ions and also as inhibitors to *T. ferrooxidans* and *T. thiooxidans* in the environment.

5. Conclusion

Tannic acid at 500 ppm inhibited the cell growth, and iron- and sulfur- oxidation in 8.00×10^9 cells of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, more than two weeks, possibly due to its phenolic group, while fulvic acid inhibited them only within initial two days. Both were more effective inhibitors than oxalic acid.

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References


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Thiobacillus ferrooxidans and Thiobacillus thiooxidans の細胞増殖と
鉄・硫黄酸化に対する阻害剤としてのタンニン酸およびフルポ酸の評価

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フルポ酸、タンニン酸、シュウ酸を用いて、腐食物質が
Thiobacillus ferrooxidans および Thiobacillus thiooxidans の細胞増殖と鉄ならびに硫黄酸化能に及ぼす影響を pH 2 付近で調べ
た。

タンニン酸は、500 ppm の添加濃度で Thiobacillus
ferrooxidans (8.0 × 10⁶ cells) および Thiobacillus thiooxidans
(8.0 × 10⁶ cells) の細胞増殖および鉄、硫黄酸化能を 2 週間以
上にわたって阻害した。この阻害は、タンニン酸のフェノール基
に基づくものと考えられる。これに対し、フルポ酸ははじめの 2
日間しかそれらを阻害しなかった。しかし、この 2つの酸は、す
でに阻害効果があると報告されているシュウ酸よりも効果的な阻
害剤であった。

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