Bacterial Oxidation of Pyritic Materials in Coal

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ABSTRACT

SILVERMAN, MELVIN P. (Pittsburgh Coal Research Center, Pittsburgh), MARTIN H. ROGOFF, AND IRVING WENDER. Bacterial oxidation of pyritic materials in coal. Appl. Microbiol. **9**:491–496. 1961.—Applicability of the manometric method for studying the oxidation of pyritic material in the presence of bacteria has been demonstrated. Resting cells of *Ferrobacillus ferrooxidans* accelerated the oxidation of coal pyrites and coarsely crystalline marcasite, but were inactive on coarsely crystalline pyrite. Resting cells of *Thiobacillus thiooxidans* were inactive on all pyrites tested. Oxidation rates in the presence of *Ferrobacillus* were increased by reducing the particle size of pyritic samples, and, in one case, by removing the $CaCO_3$ from a calcite-containing sample.

The problem of stream pollution caused by acid mine drainage has prompted several studies (Leathen, 1952; Leathen, Braley, and McIntyre, 1953*a*, *b*; Braley, 1954; Temple and Koehler, 1954; Ashmead, 1955; Moulton, 1957; Brant and Moulton, 1960). These investigators agree that the ultimate source of the sulfuric acid and iron pollutants is the pyritic material associated with coal and coal-bearing strata. Oxidation of these iron disulfides results in the production of ferrous sulfate and sulfuric acid. Subsequent oxidation of ferrous sulfate yields additional sulfuric acid and hydrated oxides of iron. The latter form the unsightly yellow-to-red muds characteristic of streams receiving acid mine drainage.

Iron- and sulfur-oxidizing chemoautotrophs have been isolated repeatedly from acid waters, both in this country (Colmer and Hinkle, 1947; Leathen and Madison, 1949; Colmer, Temple, Hinkle, 1950; Bryner et al., 1954; Brant and Moulton, 1960) and in Europe (Ashmead, 1955; Zarubina, Lyalikova, and Shmuk, 1959), and have been shown to accelerate the rate of oxidation of pyrite and other sulfide minerals. These workers measured pyrite oxidation by titrating for increased acidity, or by determining the release of soluble iron or sulfate-S from the insoluble minerals.

Current interest in the problem of air pollution by sulfur dioxide from the combustion of sulfur-containing fuels and continued concern with the acid mine water problem have stimulated the study of the role of bacteria in the oxidation of the pyritic constituents of coal. Consequently, studies of the iron-oxidizing chemolithoautotroph *Ferrobacillus ferrooxidans* as an agent for the desulfurization of coal were started; supporting physiological studies of the organism were required to determine the bacteriological, chemical, and physical factors relating to its ability to oxidize the pyrites in coal. The use of iron-oxidizing autotrophs in the removal of pyrite from coal (Zarubina et al., 1959; Ashmead, 1955) and in the secondary recovery of copper and molybdenum from their sulfide minerals has already been reported (Bryner et al., 1954; Bryner and Anderson, 1957; Bryner and Jameson, 1958).

This study reports the use of the standard Warburg manometric method for studying the oxidation of pyrites in the presence of bacteria. Methods such as flask culture or percolation, used by earlier investigators, required several weeks for a single experiment. In contrast, the Warburg method yielded comparable data in a matter of hours. These studies include investigation of the effect of various physical factors on the rates of oxidation, the role of *F. ferrooxidans* and *Thiobacillus thiooxidans* as oxidizing agents, and the susceptibility of various forms of pyritic sulfur to oxidation by bacteria.

MATERIALS AND METHODS

Media and cultures. For growth of *F. ferrooxidans*, medium 9K (Silverman and Lundgren, 1959) was prepared and contained the following concentration of salts (per liter): $(NH_4)_2SO_4$, 3.00 g; KCl, 0.10 g; K_2HPO_4, 0.50 g; $MgSO_4 \cdot 7H_2O$, 0.50 g; $Ca(NO_3)_2$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 44.2 g. The medium was prepared with distilled water and 0.10 ml of 10 N H_2SO_4 added per liter. The pH is 3.0 to 3.6 without further adjustment. For growth of *T. thiooxidans*, $FeSO_4 \cdot 7H_2O$ was replaced by 10 to 20 g per liter of elemental sulfur (sublimed flowers), which were sterilized separately by autoclaving at 100 C for 3 hr. The pH was adjusted to 3.5 with H_2SO_4. The cultures were obtained from the Syracuse University collection and were maintained in 50-ml portions of the foregoing media in shake culture.

Cell crops were grown in a glass air-lift fermentor (Lundgren and Russell, 1956) containing 6 liters of medium. Cell suspensions of *F. ferrooxidans* containing a minimum of precipitated iron were obtained by the

harvesting procedure of Silverman and Lundgren (1959). *T. thiooxidans* was harvested by centrifugation, and residual sulfur was removed by filtration through Whatman no. 1 filter paper (Suzuki and Werkman, 1958). All cell crops were stored at 4 C in distilled water acidified to pH 3.5 with H_2SO_4. These conditions of harvesting and storage yielded active cell suspensions with no detectable endogenous metabolism.

Manometric. Oxygen uptake was measured in Warburg manometers in the conventional manner (Umbreit, Burris, and Stauffer, 1957). Unless otherwise indicated, each Warburg flask contained pyritic material plus 1.0 ml distilled water (acidified to pH 3.5 with H_2SO_4) in the main compartment, 0.5 ml of cell suspension in the side arm, and 0.2 ml of 20% (w/v) KOH plus a 2-cm square of Whatman no. 42 filter paper in the center well. The temperature was 30 C. The gas phase was air. Weight of sulfuritic material used in any particular experiment is indicated in the figure legends. Occasionally, to avoid the chance of insoluble substrates clinging to the vessel and side arm walls, the cells were added directly to the main compartment with the substrate.

Pyritic substrates. Description of the sulfuritic materials used for the experiments is given in Table 1. The materials were used as received (passing through 65-mesh screen) or were ground in an agate mortar to pass a 325-mesh screen. The crystalline form of the sulfuritic materials of the concretions and the concentrates was verified as pyrite by X-ray diffraction pattern analysis. Samples of pure pyrite and marcasite in the form of large, coarse crystals were ground to pass 325 mesh before use.

Analytical. Soluble iron was determined by the colorimetric American Society for Testing Materials (ASTM) *o*-phenanthroline method. A standard curve over the concentration range 20 to 240 μg of iron was prepared, reading optical density at 500 mμ in a Bausch and Lomb Spectronic 20[1] spectrophotometer-colorimeter. Reaction mixtures, after incubation, were treated with an equal volume of 2 N HCl, heated for 30 min on a steam bath, and filtered. Suitable aliquots were then taken for the iron determination. Bacterial nitrogen was determined by a micro-Kjeldahl procedure.

[1] Bausch and Lomb Optical Company, Rochester, N. Y.

TABLE 1. *Sulfuritic materials used in oxidation studies*

Sample no.	Description	Per cent pyrite
29	Pyrite concentrate obtained by beneficiation of washery waste from Tebo coal (Henry Co., Mo.)	77.0
30	Pyrite concentrate obtained by beneficiation of washery waste from Illinois #6 coal (Christian Co., Ill.)	60.0
34*	Pyrite concretion (sulfur ball) from Pittsburgh Seam coal (Monongalia Co., W. Va.)	54.4
35	Pyrite concretion (sulfur ball) from Meigs Creek #9 coal (Harrison Co., Ohio)	74.5

* Contained 18.8% $CaCO_3$ as calcite.

Experimental Results

Effect of particle size on the oxidation of pyrites. A series of experiments was carried out to determine whether the pyrite-containing samples underwent oxidation in the presence of *F. ferrooxidans*. Samples of the pyrites as received (65 mesh) were weighed into the Warburg vessels in amounts ranging from 10 to 200 mg, and oxygen uptake was measured in the presence of several concentrations of resting cells. At this particle size only pyrite concretion sample 35 showed an appreciable oxygen uptake in the presence of the test organism. Fresh samples of the above were then ground to pass a 325-mesh screen, and the experiments were repeated. Decreasing the particle size markedly enhanced the oxidation of the pyrite in all samples except pyrite concretion sample 34 (which contained 18.8% calcium carbonate). Results of these experiments are summarized in Table 2.

To determine whether the oxygen uptake in the presence of resting cell suspensions was a valid indication of pyrite oxidation, parallel experiments with *F. ferrooxidans* were carried out in shaken flasks, and the release of hydrochloric acid-soluble iron was measured. The experimental flasks (25 ml conical) each contained 100 mg of pyritic sample, an aliquot of a cell suspension containing 0.84 mg bacterial-N, and sufficient H_2SO_4-acidified water (pH 3.5) to provide a total volume of 4.0 ml. The flasks were incubated for 24 hr at room temperature on a shaker. After incubation the flasks were removed and the contents sampled (described under Materials and Methods) for the determination of released iron. Data obtained in typical experiments (Table 3) showed that in all cases, resting

TABLE 2. *Effect of particle size on the oxidation of pyritic materials in the presence of resting cells of Ferrobacillus ferrooxidans*

Sample no.	Microliters O_2 taken up: 1.2 mg bacterial-N: 3 hr: 20 mg pyritic material			
	Passing through 65 mesh		Passing through 325 mesh	
	No cells	Cells	No cells	Cells
29	Ev*	40	60	1,342
30	12	16	Ev	1,137
34	Ev	Ev	91	Ev
35†	Ev	336	46	2,040

* "Ev" indicates some small evolution of gas.

† Thirty microliters of O_2 would be required to completely oxidize the acid-soluble iron initially present in 20 mg of this sample.

cells of *F. ferrooxidans* accelerated the release of acid-soluble iron from the pyrite.

When comparable samples, such as the two pyrite concentrates (samples 29 and 30), were ground to the same mesh size, the rate of oxygen uptake was proportional to the pyrite content of the sample. The data in Fig. 1 show that the oxygen uptake rate with sample 29 (77% pyrite) as substrate was higher than when sample 30 (60% pyrite) was used. Factors other than pyrite content are active in determining oxidation rates, however, since the highest rates were obtained with sulfur ball sample 35 (74.5% pyrite). This sample was also the only one to show significant oxidation at a particle size of −65 mesh (Table 2).

Role of T. thiooxidans in the oxidation of pyritic material. Since *T. thiooxidans* has invariably been found associated with *F. ferroxidans* in studies of the microflora of acid mine water by ourselves and others (Leathen, 1952; Temple and Koehler, 1954; Brant and Moulton, 1960), manometric experiments were set up to determine whether this bacterium would accelerate oxidation of pyrite. The results of these studies demonstrated that no appreciable increase in the rate of oxidation of any of the pyrite samples tested occurred in the presence of resting cells of *T. thiooxidans*.

Additional manometric experiments were undertaken to determine whether mixtures of the two organisms would be more effective than *F. ferrooxidans* alone in accelerating the rate of oxidation of pyrite samples 29, 30, and 35. In this series of experiments, a resting cell suspension of *F. ferrooxidans* was tipped into the Warburg vessel from one side arm of a two-side arm flask, and oxygen uptake was measured over a 1-hr period; then *T. thiooxidans* was tipped in from the other side arm and oxygen uptake measurements were continued. The addition of resting cells of *T. thiooxidans* to the reaction mixture resulted in a slight decrease of the rate of oxidation of all the pyrite samples. Typical results, as exemplified by tests on sample 29, are shown in Fig. 2. To determine whether this effect was due to purely mechanical as opposed to physiological blocking of oxidizable sites on the pyrites, the experiments were repeated using heat-killed cells of *T. thiooxidans*. The same effect was noted when heat-killed cells were added as was observed with their viable counterparts, demonstrating that physiological blocking was not a factor.

Effect of crystalline form and presence of extraneous material on rate of oxidation of sulfide minerals. Although X-ray diffraction pattern analysis had shown that the sulfide minerals in all of the samples were pyrites, the physical state of the samples differed. Samples 34 and 35 represent a massive deposition of pyrite accompanied by other mineral material. In sample 34, 18.8% calcite had been deposited along with the pyrite. The pyrite concentrates, samples 29 and 30, are completely different material. These represent grains and bands of crystalline pyrite found in a coal matrix. Concentration, after grinding of pyrite-rich segregations, yielded these samples of high pyrite content (60 and 77%) from raw coals whose original pyrite contents were 4.5 and 8.2%, respectively. It must be recognized that the concretions (sulfur ball material) consist of pyrite with some inclusion of extraneous material, whereas the

TABLE 3. *Oxidation of pyritic materials in the presence of resting cells of Ferrobacillus ferrooxidans as measured by release of hydrochloric acid-soluble iron**

Sample no.	Cells	Iron			Pyrite oxidized
		Initial	24 hr	Released	
		μg	μg	μg	%
29	+	1,024†	4,400	3,376	9.42
	−	808	1,080	272	0.76
30	+	1,250	4,000	2,750	9.84
	−	1,034	1,376	342	1.22
35	+	3,040	7,200	4,160	11.99
	−	2,824	3,140	316	0.91

* Release of iron from 100 mg pyritic material in the presence of 0.84 mg bacterial-N.

† A 216-μg quantity of iron was carried over in the cell suspension.

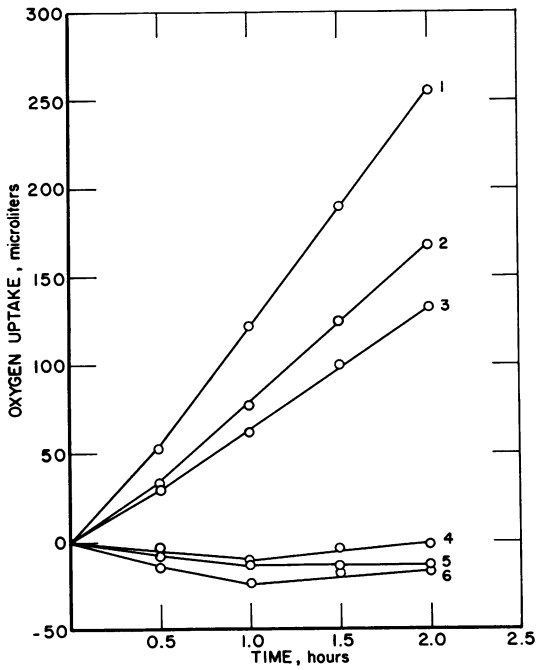

FIG. 1. *Effect of absolute pyrite content on the rate of oxidation of pyritic materials in the presence of resting cells of Ferrobacillus ferrooxidans. Each flask contained 20 mg pyritic material. Curves 1, 2, and 3 represent oxidation of samples 35 (74.5% pyrite), 29 (77.0% pyrite), and 30 (60.0% pyrite), respectively, in the presence of cells (0.21 mg bacterial-N); curves 4, 5, and 6 represent oxidation of samples 35, 30, and 29, respectively, in the absence of cells.*

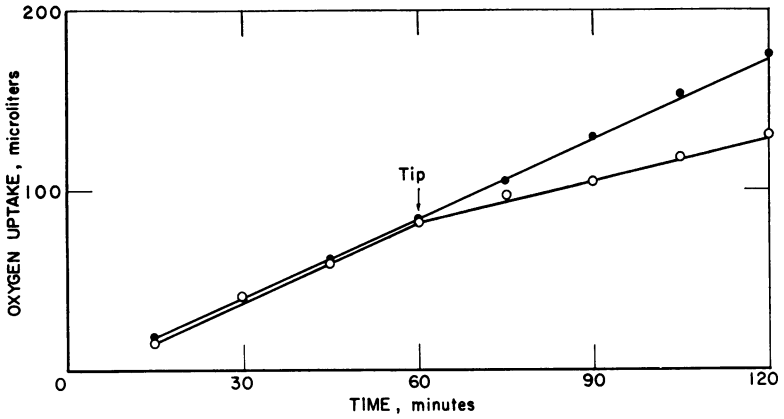

FIG. 2. *Effect of Thiobacillus thiooxidans (0.40 mg bacterial-N) on the rate of oxidation of 20 mg of pyrite concentrate sample 29 in the presence of Ferrobacillus ferrooxidans (0.21 mg bacterial-N). Closed circles, acidulated water (pH 3.5) tipped in; open circles, Thiobacillus tipped in.*

TABLE 4. *Oxidation of coarsely crystalline pyrite and marcasite (−325 mesh) in the presence of resting cells of Ferrobacillus ferrooxidans or Thiobacillus thiooxidans*

Substrate	Cells	Microliters O_2 taken up: 3 hr: mg bacterial-N: 20 mg pyritic material	
		*T. thiooxidans**	*F. ferrooxidans*†
Pyrite	−	15	38
	+	Ev‡	Ev
Marcasite	−	82	209
	+	128	734

* Bacterial-N per flask, 0.40 mg.
† Bacterial-N per flask, 0.21 mg.
‡ "Ev" indicates some small evolution of gas.

pyrite concentrates, on the other hand, consist of a variety of pyrite forms which were embedded in coal and associated clay partings, but which now have much of the pyrite surfaces exposed.

To examine the abilities of both test organisms to enhance oxidation of iron disulfides of a more homogeneous nature than the materials associated with coal, samples of large crystals of pyrite and marcasite were used as substrates in manometric experiments. Neither organism accelerated the oxidation of the coarsely crystalline pyrite. Both *Ferrobacillus* and *Thiobacillus* enhanced oxidation of the marcasite sample, although *Thiobacillus* did so only to a slight extent. Data obtained from these experiments are summarized in Table 4.

Chemical analysis showed that sample 34 contained 18.8% $CaCO_3$ in the form of the mineral calcite (identified by its X-ray diffraction pattern). This sample was completely resistant to oxidation (Table 2). When the calcite was removed by refluxing with 2 N HCl followed by extensive washing with distilled water, the sample became susceptible to oxidation in the presence of *Ferrobacillus* (Fig. 3).

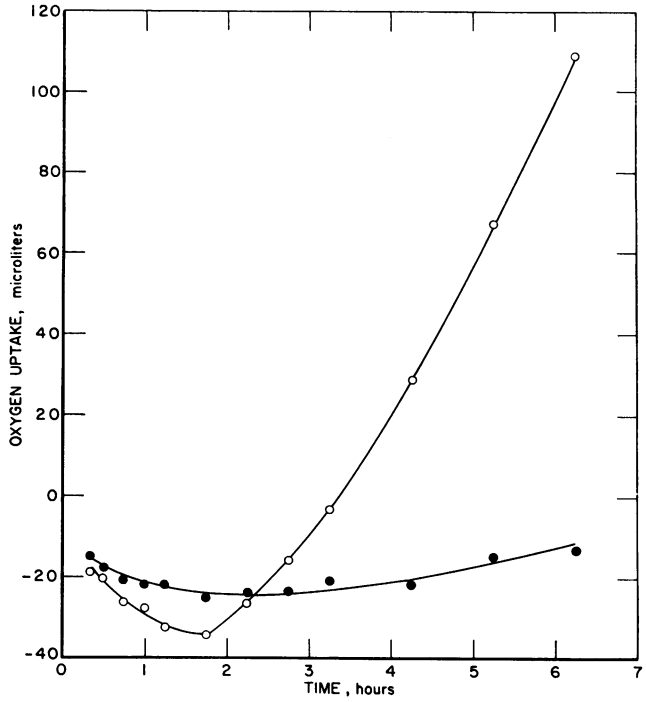

FIG. 3. *Oxidation of 20 mg of pyrite concretion sample 34 (after removal of calcium carbonate), in the presence of resting cells of Ferrobacillus ferrooxidans. Open circles, cells present (0.42 mg bacterial-N); closed circles, cells absent.*

DISCUSSION

The dependence on particle size of the rate of oxidation of the pyrite-containing samples (Table 2) was to be expected. It can be estimated that the reduction in particle size from −65 to −325 mesh increased the surface area by a factor of at least 50, so that more pyrite surface was exposed, resulting in an increase in the oxidation rates.

Both *Thiobacillus* and *Ferrobacillus* were able to accelerate the oxidation of coarsely crystalline marcasite but not the oxidation of coarsely crystalline pyrite

(Table 4). In this case the crystal structure of these minerals seems to be a factor in determining their susceptibility to oxidation. The ability of the test cultures to accelerate oxidation of marcasite has little bearing on practical considerations of microbial coal desulfurization, as this mineral is present in only minor amounts in most American and European coals and is rarely a factor in acid-producing mine areas.

The inability of *Ferrobacillus* to oxidize coarsely crystalline pyrite (Table 4), while it is able to accelerate oxidation of sulfur ball and coal pyrites (Fig. 1, Tables 2, 3), is difficult to explain. Those pyrite-containing samples responding to oxidation, including sample 34 after the calcite had been removed (Fig. 3), have X-ray diffraction patterns identical to that of the coarsely crystalline sample. Investigations concerned with variations in the properties of pyrites (Smith, 1942) have not received the support of more recent researchers (Stanton, 1957; Kullerud and Yoder, 1959). Nevertheless, the fact that some kinds of pyrite differ from others with respect to their susceptibility to oxidation may be taken as a priori evidence that there are physical and chemical differences among the different kinds of pyrites. Imperfections or impurities in the crystal lattice may be necessary for initiation of the oxidative process.

The behavior of sample 34 in the presence of *Ferrobacillus* was most interesting. The organism was unable to enhance the oxidation of this sample when calcite was present (Table 2); on removal of the carbonates by refluxing with acid, oxidation proceeded after a lag period of approximately 2 hr (Fig. 3). The resistance of this sample to oxidation can best be attributed to the neutralizing capacity of the carbonates. The quantity of $CaCO_3$ present (18.8%) in the untreated sample was sufficient to neutralize the reaction mixture so that the pH exceeded the upper limit for the activity of this acidophilic bacterium. Upon its removal, the reaction mixture remained acid and *Ferrobacillus* could exert its effect.

Disagreement exists as to the ability of *T. thiooxidans* to oxidize pyritic materials. The oxidation of coarsely crystalline marcasite and certain pyrite-containing concretions by this bacterium has been reported (Temple and Delchamps, 1953; Temple and Koehler, 1954). On the other hand, Leathen et al. (1953*a*, *b*) reported that *T. thiooxidans* was unable to enhance the oxidation of any iron disulfide with the exception of museum-grade marcasite. These discrepancies have been explained as being due to differences in the susceptibility to oxidation of the particular samples used as test substrates. The present findings, however, would seem to eliminate *T. thiooxidans* from any significant role in pyrite oxidation. We propose this in view of the fact that, under our test conditions, resting cells of *T. thiooxidans* did not enhance the oxidation of a number of different samples of pyritic materials.

Sato (1959) presented evidence for the release of elemental sulfur during the oxidation of pyrite in accord with the following two-step mechanism:

$$FeS_2 + H_2SO_4 + \tfrac{1}{2}O_2 \rightarrow FeSO_4 + 2S + H_2O$$
$$2S + 2H_2O + 3O_2 \rightarrow 2H_2SO_4$$
$$\text{Net: } FeS_2 + H_2O + 3\tfrac{1}{2}O_2 \rightarrow FeSO_4 + H_2SO_4$$

Elemental sulfur produced in the first step could be utilized by *T. thiooxidans* through the second step, accounting for the organism's presence in acid mine waters. The experiments on the combined activities of *F. ferrooxidans* and *T. thiooxidans* (Fig. 2) would appear, at first glance, to have eliminated this mechanism from consideration. The preliminary oxidation of pyrite in the presence of *F. ferrooxidans* should produce elemental sulfur, and one would have expected the subsequent addition of *T. thiooxidans* to the reaction mixture to have increased the rate of oxygen uptake. However, the strain of *F. ferrooxidans* used in these experiments can oxidize elemental sulfur as well as ferrous iron. Bryner and Jameson (1958) and Beck (1960) have reported sulfur oxidation by similar strains of iron-oxidizing autotrophs. A comparison of the Q_{O_2} (N) values of the strains of *F. ferrooxidans* and *T. thiooxidans* used in these experiments is given in Table 5. Thus, it is conceivable that all the available sulfur was oxidized by *F. ferrooxidans* prior to the addition of *T. thiooxidans*.

A second consideration concerns the stability of the sulfur produced in Sato's first oxidation step. This sulfur may be in a very active form that will immediately oxidize in air and therefore be unavailable for use as a source of energy by either organism. Thus, unless a stable form of sulfur is produced, the presence of *Thiobacillus* in mine waters and the ability of *Ferrobacillus* to oxidize elemental sulfur would both appear to be fortuitous.

From the data presented in this paper the following conclusions may be drawn:

1) The rate of oxidation of pyrite increases with the exposed, i.e., available, pyrite surface (Table 2). As a corollary to this, regarding coal desulfurization, the

TABLE 5. *Oxidation of iron and elemental sulfur by Ferrobacillus ferrooxidans and Thiobacillus thiooxidans*

	Q_{O_2} (N)*	
	S (1,000 μmoles)	Fe^{++} (500 μmoles)
T. thiooxidans†	557	0
F. ferrooxidans‡	148	3672

* Microliters O_2 taken up per mg bacterial-N per hr.
† Bacterial-N per flask, 0.40 mg.
‡ Bacterial-N per flask, 0.21 mg.

coal must be in a finely divided state to expose a maximum of the embedded pyrite.

2) *F. ferrooxidans* can accelerate the oxidation of diverse pyritic materials and coarsely crystalline marcasite, but not the oxidation of coarsely crystalline pyrite.

3) *T. thiooxidans* does not enhance the oxidation of the experimental materials, with the possible exception of marcasite. This organism appears to play no role in pyrite oxidation.

4) Some unknown factor, possibly lattice imperfections or the presence of some impurity in the mineral, affects the ability of the test organisms to accelerate oxidation. Coarsely crystalline pyrite proved resistant to oxidation, whereas coarsely crystalline marcasite, and pyrite concretions and concentrates from coal were oxidizable.

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