Dissolution of Sulphur Particles by *Thiobacillus ferrooxidans*: Substrate for Unattached Cells

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The growth of *Thiobacillus ferrooxidans* on sulphur is known to proceed through the attachment of cells to the sulphur particles. Experiments, however, show that the cells in the liquid phase, which are not attached to the sulphur particles, also grow. It has been shown through the use of a two-compartment membrane reactor that this increase is partially due to the release of ions, corresponding to partially oxidized states of sulphur, into the solution by the attached cells. The main soluble ion has been found to be thiosulphate, but traces of sulphite have also been detected. ©1993 John Wiley & Sons, Inc.
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INTRODUCTION

The bacterium, *Thiobacillus ferrooxidans*, is the most important microorganism involved in the bioleaching of a number of sulphide minerals. In utilizing the solid substrate, the bacteria predominantly function through the direct mechanism,[5,7] which involves the attachment of the bacteria to the sulphide mineral[6] and oxidation of its sulphur part.[10] However, for minerals containing iron, dissolution of sulphide by direct attachment results in the production of Fe^{2+} in the solution, which can also be oxidized by the bacterium to Fe^{3+}. This Fe^{3+}, in turn, chemically dissolves the mineral, thereby providing an indirect mechanism of leaching of the mineral. Thus, the dissolution occurs through two mechanisms (direct and indirect), and the two cannot be investigated independently. This is true for most of the studies where the sulphide oxidation has been investigated using chalcopyrite or pyrite.[1,5,7,10] Further, under certain conditions of pulp density and particle size, this oxidation of Fe^{2+} does not take place,[10] in spite of the presence of a large number of cells in the liquid phase. The survival of the unattached cells under such conditions, though important from kinetic point of view, is still not understood. Further complications arise as Fe^{3+} has been shown to be inhibitory toward bacterial activity.[9] Even in minerals where iron is absent, generation of other metal ions such as Cu^{2+} inhibits the growth of cells.[15]

In an attempt to understand the direct mechanism, many investigators have studied the direct dissolution of elemental sulphur by the bacterium *T. ferrooxidans*,[8,18] where these complications are absent. However, apart from the bacteria growing on sulphur, considerable growth of bacteria in the liquid phase surrounding the solid is also observed, similar to that reported with chalcopyrite and pyrite.[1,5,7] An understanding of the mechanism of growth of these unattached cells is necessary to obtain a reliable kinetic expression. The objective of this article is to investigate the mechanism of growth of the unattached cells in the liquid phase. A number of mechanisms could be imagined.

Each cell could frequently attach and detach from the sulphur particles and metabolize sulphur. A cell can indeed reach the sulphur surface due to hydrodynamic and Van der Waals attractive forces. However, Gormely and co-workers[3] have reported that the rate of detachment of the cells is negligible. Thus, the mechanism involving frequent attachment and detachment from the sulphur particles appears unlikely, though new cells formed at the sulphur surface could well be released into solution and thereby contribute to the increase in cell population there.

The second possible mechanism of metabolism envisages that the attached cells produce water-soluble ions corresponding to partially oxidized states of sulphur, which are released to the liquid phase. These ions could act as substrate for the free cells. Indirect support for this hypothesis exists in literature. Silver and Lundgren[11] obtained the sulphur-oxidizing enzyme from ruptured *T. ferrooxidans*, and found that it was able to oxidize sulphur to sulphite in the presence of reduced glutathione. Similar findings were reported earlier by Suzuki and Silver,[14] who found that the sulphur-oxidizing enzyme of *T. thioparus* and *T. thiooxidans* oxidized sulphur to sulphite, which in turn chemically reacted with sulphur to form thiosulphate. Thus, within the cell, the oxidation has been proposed to take place through a number of partially oxidized states of sulphur.[15] If some of these ions are released into the liquid phase, they could indeed provide an alternate substrate for the growth of unattached cells in the liquid phase.

There could be yet another mechanism operating to provide the substrate for unattached cells. If thiosulphate is released into the liquid, as proposed in the second mechanism, it could decompose into sulphur and sulphite, under

the acidic conditions prevailing in the liquid phase. The solution would then become supersaturated with respect to sulphur, which would tend to precipitate out. The cells could act as nuclei for the deposition of sulphur from the supersaturated solution. This would be an alternate mechanism by which elemental sulphur may be supplied directly to the free cells without their coming into contact with the sulphur particles initially added.

The present study aims at experimental investigation of the possibility of release of intermediates into the liquid phase, which could form the alternate substrate for sustaining the growth of free cells. This has been achieved through the use of a two-compartment reactor, in which the compartments are separated through a semipermeable membrane. The membrane reactor provides a situation where the cells in one of the compartments have no access to sulphur placed in the second compartment, but have access to the soluble intermediates (generated by the attached cells) through the membrane. The possibility of sulphur directly deposited on the cells, through the decomposition of thiosulphate as the alternate substrate, has also been experimentally investigated.

MATERIALS AND METHODS

Bacteria and Culturing Conditions

The strain, SIMA 8621, of *T. ferrooxidans*,[9] was grown on powdered sulphur at 2.5% (w/v) pulp density (p.d.) in the nutrient basal salt medium (Silverman and Lundgren[12] 9K medium without $FeSO_4$). Five grams of sulphur powder were placed in a 500-mL conical flask with 180 mL of the nutrient basal salt solution and 20 mL of similarly grown culture was added as inoculum. The flask was shaken in an incubator shaker at 30°C and 240 rpm. After 8 days, the culture was filtered through Whatman no. 1 filter paper to remove the sulphur particles and centrifuged at 15,000 rpm in a refrigerated centrifuge. The cell pellet thus formed was washed with the nutrient basal salt medium and centrifuged again. Finally, the pellet was resuspended in the same volume of the nutrient basal salt medium as that of the original culture. This washed, sulphur-free culture was used in all experiments as inoculum at a 10% (v/v) basis. For obtaining the cell-free broth, the fully grown culture was filtered through Whatman no. 1 filter paper to remove sulphur. The resultant solution was filtered through a millipore membrane (0.22 μm) to filter off all the cells. The resulting solution, free from both sulphur and cells, was employed as *cell-free broth*. Commercially obtained sulphur rolls were crushed to obtain the fine powder used for harvesting the cells.

Analyses

The sulphuric acid formed by the microbial oxidation of sulphur was analyzed by titration with 0.01N sodium tetraborate solution.[17] The cell counts in the liquid were

obtained using Petroff hausser slide on a phase-contrast microscope. It was not possible to count the cells attached to the solid. Solutions were tested for elemental sulphur by forming ferric thiocyanate in acetone[17] and for thiosulphate and sulphite by iodometric titrations.[17] A Shimadtzu-2100 spectrophotometer (double beam) was also used for analyzing the cell-free broth, based on an absorbance spectrum in the range of 190 to 700 nm. Differential pulse polarography using a dropping mercury electrode and cyclic voltammetry with a platinum microelectrode were used to identify thiosulphate and sulphite in the cell-free broth. The cell-free broth for these analyses were prepared by growing the culture in the nutrient basal salt medium without $Ca(NO_3)_2$, as NO_3^- was suspected to interfere with the analysis. X-ray photoelectron spectroscopy was employed to detect elemental sulphur on dried cells.

Experimental

A two-compartment membrane reactor, shown in Figure 1, was fabricated from perspex sheets. The rectangular reactor consists of two compartments, A and B, separated by a millipore membrane (0.22 μm). Compartment A was provided with a stirrer and its corners were packed with wax to avoid dead zones. This compartment was loaded with sulphur particles (obtained by filtering a growing culture) at 2.5% p.d. and 10% (v/v) inoculum of *T. ferrooxidans*. Compartment B contained only the nutrient basal salt medium and was inoculated with *T. ferrooxidans* at 1% (v/v). Blank runs in which compartment B was not inoculated were conducted both before and after the main experiment, to ensure that there was no leakage of cells from compartment A to B. Compartment B was closed at the top, with a small hinged lid giving access to the solutions inside. The contents in this were aerated using an aquarium aerator. Arrangements were made to saturate the air with water to reduce evaporative losses from the reactor. An experiment was also conducted to observe the attachment of cells to the perspex walls by taking 10% inoculum in nutrient basal salt medium in both compartments of the reactor, and measuring the cell density as a function of time. There
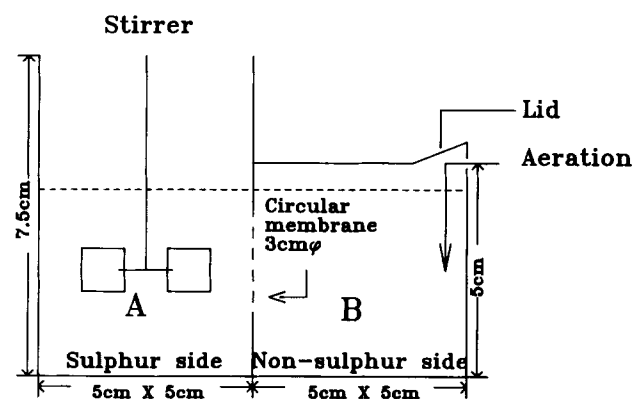

Figure 1. Schematic representation of the membrane reactor.

was no measurable change in cell density, indicating that the reactor walls did not interfere with the measurements.

Shake-flask experiments were conducted at 10% pulp density of sulphur particles, of 840 to 1201 μm in size, with 10% inoculum of *T. ferrooxidans*.

RESULTS AND DISCUSSION

The results of the shake-flask experiment, in which both the H_2SO_4 and the cell count in liquid phase were monitored as a function of time, are presented in Figure 2. It is seen that the cell count in the liquid phase increases as a function of time. The increase could at least partly be due to the release of newly generated cells from sulphur particles. However, the mechanism of sustenance of all the unattached cells, without coming in contact with sulphur particles, is not understood and, hence, was investigated further.

In the present work, the notion of frequent contacts was not investigated further due to both attachment being very strong[2] and the detachment rates being reported negligible.[3] However, the other two mechanisms were considered more feasible and, hence, have been investigated in some detail. If availability of elemental sulphur for bacteria by precipitation is to occur, it should be possible to detect elemental sulphur on the cells. Therefore, bacteria were filtered from a growing culture, centrifuged, and dried in a vacuum dryer. The dried pellet was tested for elemental sulphur by X-ray photoelectron spectroscopy. The spectrum did not indicate the presence of any elemental sulphur on the cell walls. This negative finding cannot be considered conclusive because of the possibility of sulphur being chemically oxidized during drying. Therefore, two identical samples of wet centrifuged cells were resuspended in the nutrient basal salt medium. One of them was sonicated to rupture the cells. The resultant liquid was then tested for elemental sulphur through the formation of iron thiocyanate in acetone. The other sample of unruptured cells was also subjected to the same test for elemental sulphur. Both these tests also

were negative, thus indicating that the cells do not have elemental sulphur on their walls. This leaves the mechanism of availability of alternate liquid-phase substrate for the bacteria to be tested.

If an alternate substrate as a soluble compound is available, then the free bacteria should be able to grow in the liquid even when they are physically separated from the sulphur particles, but have access to the solution. To verify this, the experiment using a two-compartment membrane reactor was performed. A blank run was conducted, as described earlier, to make sure that there was no leakage of cells or sulphur from compartment A to B. In this experiment, compartment A was loaded with sulphur and inoculated, as described earlier, but compartment B had the nutrient basal salt medium without any cells. The liquid in compartment B was monitored for 15 days for cells and sulphur, but neither of these could be detected, confirming that the membrane was impervious to both sulphur and cells. The next run was identical except that compartment B was now inoculated with *T. ferrooxidans* at 1% (v/v). The liquid in both the compartments was monitored for increase in number of cells. The variation of cell number densities with time for both the compartments is shown in Figure 3.

The conditions in compartment A were similar to those in shake-flask experiments and a rapid growth of cells in it was observed. It is interesting to note that the cell number density in compartment B also increased after a lag phase. There was no external substrate added to compartment B and there was no leakage of cells or sulphur from compartment A. Further, there can be no growth possible through attachment to perspex. Therefore, the growth in compartment B was the direct result of diffusion of some soluble substrate from compartment A to B. As no soluble substrate was added to either compartment in the beginning, the intermediate substrate must be produced due to some cellular activity of the cells attached to the sulphur particles in compartment A. This supply of the alternate substrate would be continuous, but with the progress of time, compartment A will also have a large number of free cells in the liquid, which would compete for this alternate
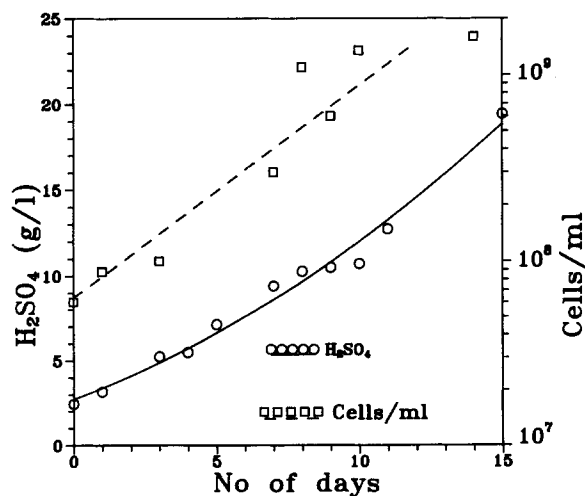

Figure 2. Typical growth of cells and acid generation in a sulphur culture.
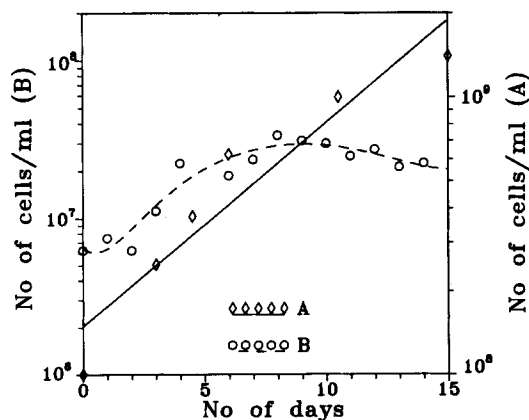

Figure 3. Cell number densities on both the sulphur and the nonsulphur sides of the membrane reactor.

substrate with the free cells in compartment B. Thus, the supply of alternate substrate to compartment B would be reduced. Such a change may cause stationary and death phase in compartment B, which is also seen in Figure 3. It can thus be concluded that although attachment of cells to solid particles is necessary, the cells in liquid can grow without coming in direct contact with the solid utilizing an alternate substrate generated by the attached cells. An effort was then made to identify the nature of the alternate substrate.

Identification of Alternate Substrate

The oxidation of sulphur is known to progress through a series of partially oxidized states inside the cell.[15] The earlier work on the reaction of enzymes (obtained from the cells through cell rupture) with sulphur indicates that sulphite and thiosulphate could be expected.[14] Iodometric titrations to detect these ions were carried out on the cell-free broth. However, appreciable amounts of these could not be detected. A UV spectrum of the cell-free broth from a shake-flask culture (against the nutrient basal salt medium as blank) was then obtained and is shown in Figure 4. The liquids from compartments A and B also showed a similar UV spectrum. It can be seen that there is a peak around 225 nm and a shoulder extending from 275 to 300 nm. To ensure that these peaks are due to bacterial activity, a fresh shake-flask experiment for culturing *T. ferrooxidans* on sulphur was started and the UV spectra of cell free broth (obtained from samples that were periodically withdrawn from the culture) was monitored. The spectra, at different times after inoculation, are presented in Figure 5. It is seen that the peaks at 225 and 275 nm increase with time, clearly indicating that they are due to bacterial activity.

If any of these peaks correspond to partially oxidized states of sulphur, it should be possible to remove it by oxidizing the concerned ions to sulphate. With this in mind, the oxidation of the solution was conducted with H_2O_2 (1 mL of 30% H_2O_2 in 10 mL of cell-free broth). The UV spectrum of the resultant solution is also shown in Figure 4. In this case, the spectrum was obtained by
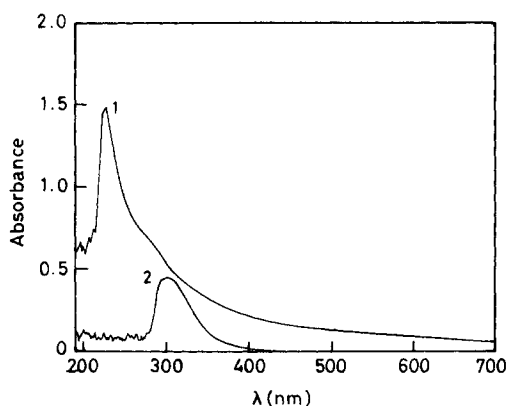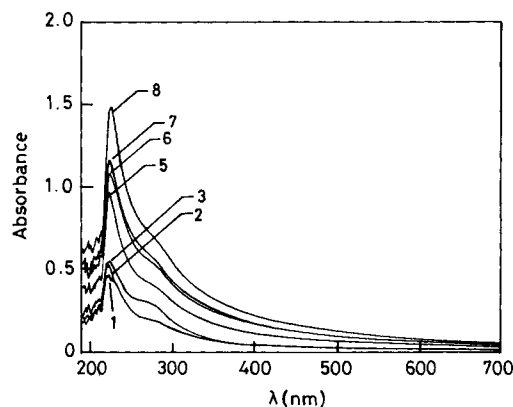
Figure 5. UV spectra of the culture medium during the growth of culture. Number for each curve indicates the time in days after inoculation, when the spectrum was analyzed.

blanking the solution against the nutrient basal salt medium containing the same amount of H_2O_2. It is seen that the peak corresponding to 225 nm vanishes, whereas the residual peak at 280 nm remains. This peak could not be removed, either by addition of H_2O_2 or by contacting the cell-free broth with organic solvents (cyclohexane, benzene, and chloroform) or activated carbon. UV spectra of several sodium salts (thiosulphate, sulphite, tetrathionate, and metabisulphite) in the nutrient basal salt medium were obtained, to identify the peak at 225 nm. The solutions of sodium salts in the nutrient basal salt medium were acidified to contain about 15 g/L of acid to simulate conditions of a fully grown culture, allowed to stand for 2 days, and then filtered through millipore membrane (0.22 μm) before obtaining their UV spectra against the nutrient basal salt medium as blank (sulphuric acid does not have any absorbances in this range).

Absorbance peaks were found at the following wavelengths (nm), with corresponding ions given in parentheses: 222 and 270 (sulphite), 230 (thiosulphate), 221 (metabisulphite), and 227 (tetrathionate). Sulphite, thiosulphate, and tetrathionate salts have been successfully used to harvest *T. ferrooxidans*.[14] Thus, the peak at 225 nm is due to one of these ions. For the identification of the ion, its oxidation and reduction were followed using the techniques of differential pulse polarography and cyclic voltammetry. Typical results obtained with these techniques indicate the presence of thiosulphate ion.

The differential pulse polarograph indicated a reduction potential at −0.170 V (with DME vs. SCE) and cyclic voltammetry was used to detect the oxidation potential of 1.18 V (with Pt microelectrode) both correspond to thiosulphate. The concentration was obtained from current and was found to be 4 to 10 ppm. The differential pulse polarograph also showed a small current peak at −0.55 to −0.65 V, corresponding to the reduction of sulphite ion (0.03 ppm). Therefore, the peak at 225 nm was mainly the result of thiosulphate. Because of the extremely small concentrations, the decomposition was insignificant, which accounts for the absence of sulphur on

Figure 4. UV spectrum of cell-free broth before (1) and after (2) addition of H_2O_2.

cells. The residual peak may be either due to cytochromes,[13] some nucleic acids,[16] or some surface-active substances including polypeptides and amino acids,[4] all of which have absorbances in the range of 260 to 280 nm and are known to be produced by *T. ferrooxidans*.[4,16] As these do not act as substrates, this peak was not investigated further.

Based on the above experimental results, the following mechanism for the growth of *T. ferrooxidans* on sulphur is proposed. Initially, the bacteria attach to the sulphur particles and start oxidizing it. The number of attached bacteria increase with time, resulting in an increase in the rate of generation of the partially oxidized sulphur compounds, important among these being thiosulphate and sulphite. These sulphur compounds are then consumed by the bacteria in the liquid phase itself, resulting in their growth. Thus, there can be an increase in the number of bacteria in the liquid due to release of newly grown cells from the solids as well as the growth in the liquid using the intermediate as the alternate substrate. Depending upon the rate of generation and the rate of consumption of intermediates the concentrations of these compounds may either increase, remain constant, or decrease in the liquid.

CONCLUSIONS

During the bioleaching of elemental sulphur by *T. ferro-oxidans*, a part of the bacteria from the inoculum first gets attached to the solid. These bacteria oxidize sulphur to its partially oxidized states, which are water-soluble and form the substrate for the bacterial activity in the liquid phase. The partially oxidized sulphur is mainly in the form of thiosulphate, though traces of sulphite are also discernible.

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