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RESEARCH ARTICLE

Surface properties of *Thiobacillus ferrooxidans* and its adhesion to mineral surfaces

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Thiobacillus ferrooxidans **cells grown on sulphur, pyrite and chalcopyrite exhibit higher hydrophobicity than ferrous iron-grown cells. The isoelectric points of sulphur, pyrite and chalcopyrite-grown cells were at a pH higher than that for ferrous iron-grown cells The effect of duration of biotreatment under static and agitation conditions on bacterial adhesion is reported. Further, scanning electron microscopy was carried out to visualize the process of bacterial adhesion to the mineral surfaces.**

THIOBACILLUS ferrooxidans, a gram-negative chemoautotrophic acidophile, is the most important microorganism implicated in the bio-oxidation of various sulphide minerals[1]. *T. ferrooxidans* is commercially used in the extraction of copper, uranium, silver and gold from their ores. It can oxidize virtually any sulphide mineral such as that of lead, zinc, nickel, molybdenum and cobalt. Two mechanisms, indirect and direct, are known to be responsible for the biodissolution of sulphidic ores. Indirect mechanism operates by the chemical action of acidic ferric sulphate produced by bacterial metabolism. In this mechanism adhesion of the bacteria to mineral surfaces is not required. Direct mechanism, on the other hand, involves enzymatic attack of the mineral by the bacteria for which intimate contact and hence adhesion is required[2,3]. After the bacteria comes in contact with the mineral the enzymes on the outer membrane carry out the dissolution of the mineral. Previous studies in this laboratory have shown that direct attack plays an important role in the bio-

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leaching of pyrite and chalcopyrite[4,5]. Although it has been known that bacterial cells play an important role in biooxidation the actual mechanism of adhesion of *T. ferrooxidans* to the mineral has not been well understood.

Bacterial adhesion is dependent not only on the biochemical properties of the organism but also on the interfacial properties corresponding to various interfaces existing in a bioleaching system . The surface properties of bacteria that have been found to affect adhesion are the cell surface hydrophobicity and electrokinetic potential[6,7]. Ferrous iron provides a soluble substrate for growth of *T. ferrooxidans* whereas minerals such as sulphur, pyrite (FeS_2) and chalcopyrite ($CuFeS_2$) provide insoluble substrates. Adhesion is a necessary event for bacterial growth on sulphur, pyrite and chalcopyrite (solid substrates) unlike during their growth in a ferrous iron medium (soluble substrate). Thus this provides a model system to investigate the mechanisms involved in adhesion. This work was undertaken to study the role of surface properties in the adhesion of *T. ferrooxidans* to minerals. Adhesion of *T. ferrooxidans* to pyrite and chalcopyrite was quantified and visualized.

Methods

Culturing and harvesting

T. ferrooxidans MAL 4–1 used in this study was isolated from Malanjkhand copper mines and maintained on 9 K medium where ferrous iron is the energy source at pH 2.3 (ref. 8). The 9 K medium consists of the following ingredients per litre: $(NH_4)_2SO_4$ 3.0 g; K_2HPO_4 0.5 g; $MgSO_4$ 0.5g; KCl 0.1 g; $Ca(NO_3)_2$ 0.01 g; pH adjusted to 2.3 with H_2SO_4. Ferrous iron grown cells were obtained by growth on 9 K medium for 2 days at 240 rpm and 30°C. The culture was filtered through Whatman No. 1 filter paper and centrifuged at 27,000 *g* for 15 min. The pellet was washed and resuspended in pH 2.0 sulphuric acid at desired cell densities according to the experiment. Sulphur-grown cells were obtained by growth on 9 K^- mineral salts medium (without ferrous sulphate) containing 10 g sulphur powder/100 ml, adjusted to pH 2.3 and incubated at 30°C and 240 rpm for 10 days. For growth of *T. ferrooxidans* on sulphur it was found necessary to supplement the medium with trace amounts of iron (10 mg of ferric chloride/L). The culture was filtered through Whatman No. 1 with repeated washing, and centrifuged as mentioned earlier. Pyrite-grown and chalcopyrite-grown cells were obtained by growing the bacteria in 9 K^- medium supplemented with 4 g of the mineral per 100 ml of the solution. The cells were harvested as described above. The cell count was monitored using a phase contrast microscope (Laborlux K Wild MPS12) and a Petroff-Hausser counting chamber.

Minerals

Hand-picked pure samples of pyrite and chalcopyrite and analytical grade sulphur particles were used in all the experiments. They were ground to the desired size using an agate mortar and pestle.

Surface properties

The hydrophobicity of cell suspensions was measured by liquid-liquid partition in aqueous and organic phase as described previously using *m*-xylene[9]. The zeta potential of the bacteria was determined[10] using Zeta-Meter 3.0. Zeta potential was measured on bacterial suspensions of 1.0×10^7 cells/ml which were conditioned in 10^{-3} M KCl at the required pH for 1 hour.

Determination of adhesion

To study the adhesion of *T. ferrooxidans* to the minerals 100 ml flasks containing the respective sulphidic mineral (mesh size:200 + 300) at 2% pulp density in 20 ml of 9 K^- medium were incubated with 5ml of ferrous iron-grown innoculum containing 5×10^8 cells/ml at 200 rpm and 30°C. The adhesion of *T. ferrooxidans* to pyrite and chalcopyrite was studied by determining the cell protein in the solid and liquid phases at various intervals as described previously[11]. To

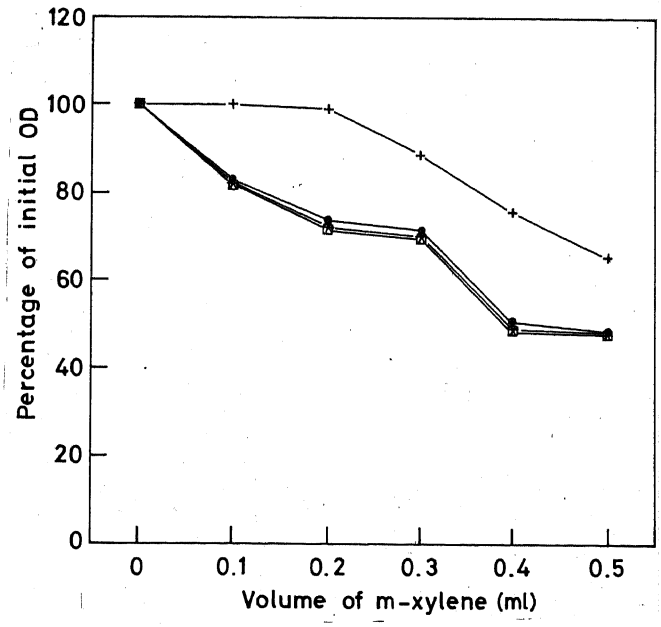

Figure 1. Hydrophobicity of *T. ferrooxidans* grown on (+) ferrous iron, (•) sulphur, (□) pyrite and (×) chalcopyrite. Varying volumes of *m*-xylene were interacted with cell suspension and the OD of the aqueous phase monitored at 400 nm. The difference from the intial OD is expressed. S. D. for each point was less than 3% and $n = 3$.

release the cells from the mineral for determination of protein the following procedure was adopted. The mineral sample was filtered through Whatman No. 1 and washed gently with 15 ml of pH 2.0 sulphuric acid solution. The mineral was then transferred into a sterile 50 ml beaker and digested with 10 ml of 0.1 N NaOH for 30 minutes to extract the protein of the attached cells. The sample was cooled and filtered. The filtrate was assayed for protein by the above mentioned method. A reference curve was constructed relating the cell number with the protein content. For scanning electron microscopy, pyrite and chalcopyrite mineral particles incubated with bacteria were collected by filtration, rinsed twice with distilled water and air-dried at room temperature. A small amount of the prepared mineral particles was mounted on specimen stubs, coated with gold in an ion coater and examined with JEOL 840 microscope operating at 20 kV.

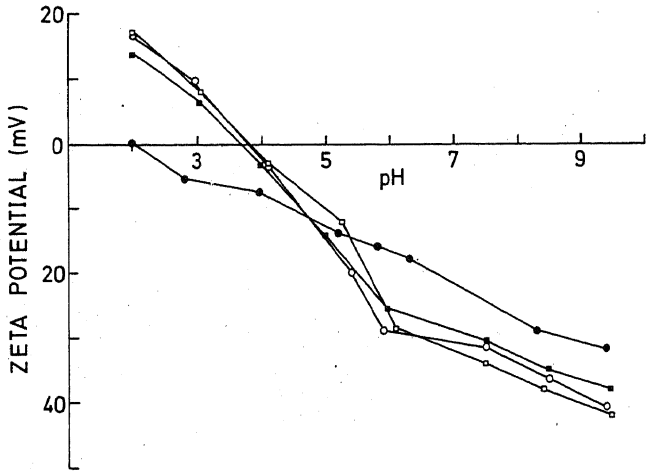

Figure 2. Zeta potential of *T. ferrooxidans* grown on (•) ferrous iron, (○) sulphur, (▪) pyrite and (□) chalcopyrite. S. D for each point was less than 10% and $n = 10$.

Results

The results of phase partitioning with *T. ferrooxidans* are illustrated in Figure 1. It can be seen that *T. ferrooxidans* cells grown on mineral substrates are increasingly transferred to the xylene organic phase unlike the cells grown in liquid ferrous iron medium. The results indicate that mineral-grown cells exhibit a higher hydrophobic nature which helps in adhesion.

Figure 2 illustrates the zeta potential of *T. ferrooxidans* grown on different substrates as a function of pH. It is observed that *T. ferrooxidans* grown on ferrous iron exhibited an isoelectric point (IEP) at about pH 2.0. This implies that the bacterial cells are negatively charged over an extended pH range below 2.0, the cells becoming increasingly more negative as the pH is increased. If the zeta potential of the iron-grown cells is compared with those grown on the mineral substrates, one could observe a significant difference. The cells grown on sulphur, pyrite and chalcopyrite exhibited IEP corresponding to a pH of about 3.8, implying that the cells are positively charged below pH 3.8. Thus one could observe that the surface chemical (electrokinetic) nature of the ferrous iron- and mineral-grown bacteria is significantly different. It is noteworthy that all the mineral-grown bacteria show similar surface chemical properties. This is of significance with respect to microbe-mineral interactions in bioleaching. From a leaching point of view, the optimum pH used in many sulphide leaching processes is between 2.0 and 2.5.Thus there is the possibility of electrostatic interactions,

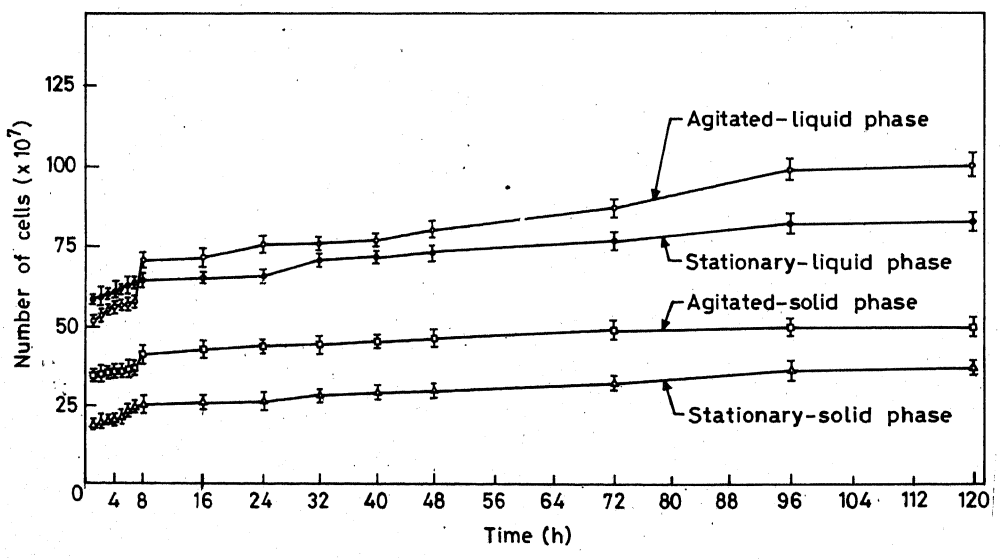

Figure 3. Interaction of *T. ferrooxidans* with pyrite. Bars indicate S. D, $n = 3$.

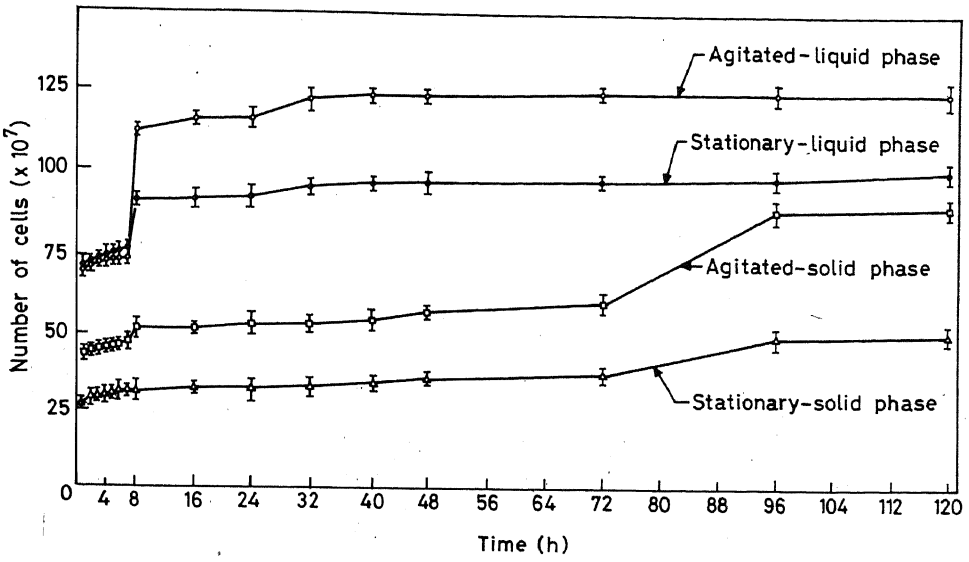

Figure 4. Interaction of *T. ferrooxidans* with chalcopyrite. Bar indicates S. D, $n = 3$.

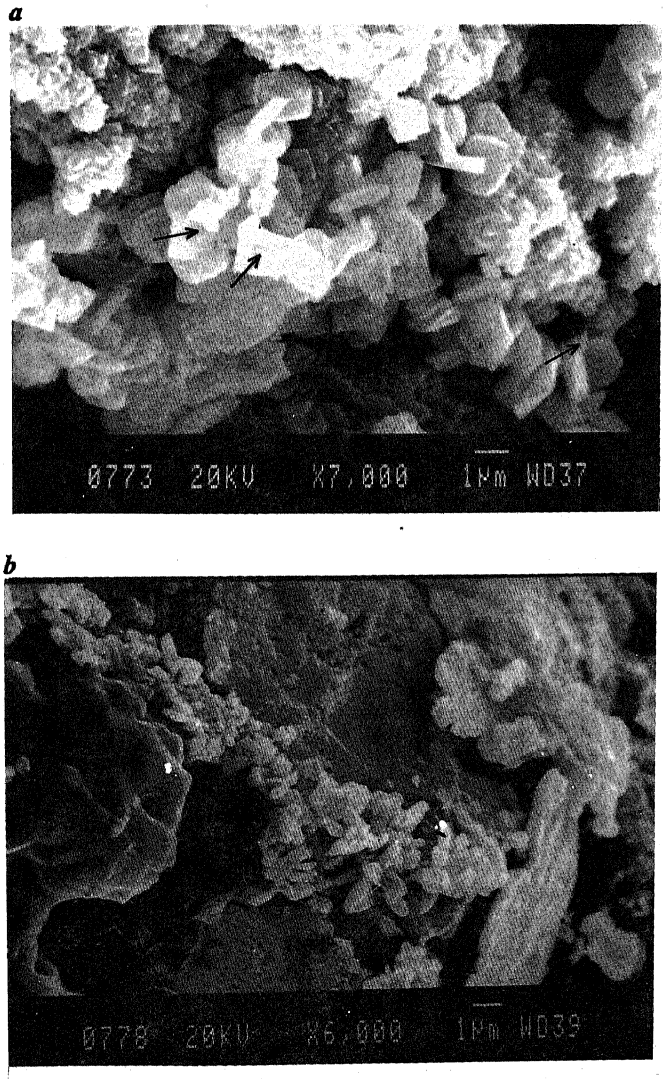

Figure 5. Scanning electron micrograph of (*a*) *T. ferrooxidans* adhering to pyrite, (*b*) leached out surface of pyrite. Arrows represent cells.

apart from hydrophobic interactions, playing a role in bringing about adhesion for leaching.

Studies were carried out to find the adhesion of *T. ferrooxidans* to the mineral surface. Cell protein estimated from solid and liquid phases of pyrite and chalcopyrite at different periods of biotreatment is shown in Figures 3 and 4 respectively. It is very difficult to directly enumerate the number of cells adhering to solid surfaces. Thus the method of estimating the cell protein was used to enable the indirect enumeration of cells. A definite lag period was observed before the onset of significant bacterial adhesion to the sulphide minerals. This may be due to the fact that the surface of the ferrous iron-grown *T. ferrooxidans* has to undergo modification for efficient adhesion. It may involve the synthesis of an organic substance reported earlier based on electron spectroscopic and X-ray fluorescence observations[12]. This is supported by the fact that ferrous iron-grown cells show a significant difference in surface properties from the mineral-grown cells. It may be noted that only in mineral-grown conditions adhesion is a necessary event.

In the case of pyrite and chalcopyrite more of the cells were found in liquid phase where solubilized ferrous iron serves as an energy source. When ferrous iron in liquid phase and the sulphide mineral is available as the solid phase the bacteria may completely utilize the ferrous iron in preference to the mineral. Solubilization of the solid substrate and the consequent growth of *T. ferrooxidans* in the liquid phase have been reported when sulphur is used as the solid substrate[13]. Another possibility is that the sites available to bacteria for adhesion on the mineral may have been saturated forcing a majority of the population to remain in the liquid phase.

Substantially increased adhesion was observed on chalcopyrite compared to pyrite. Reports indicate that pyrite surfaces are highly heterogeneous with respect to

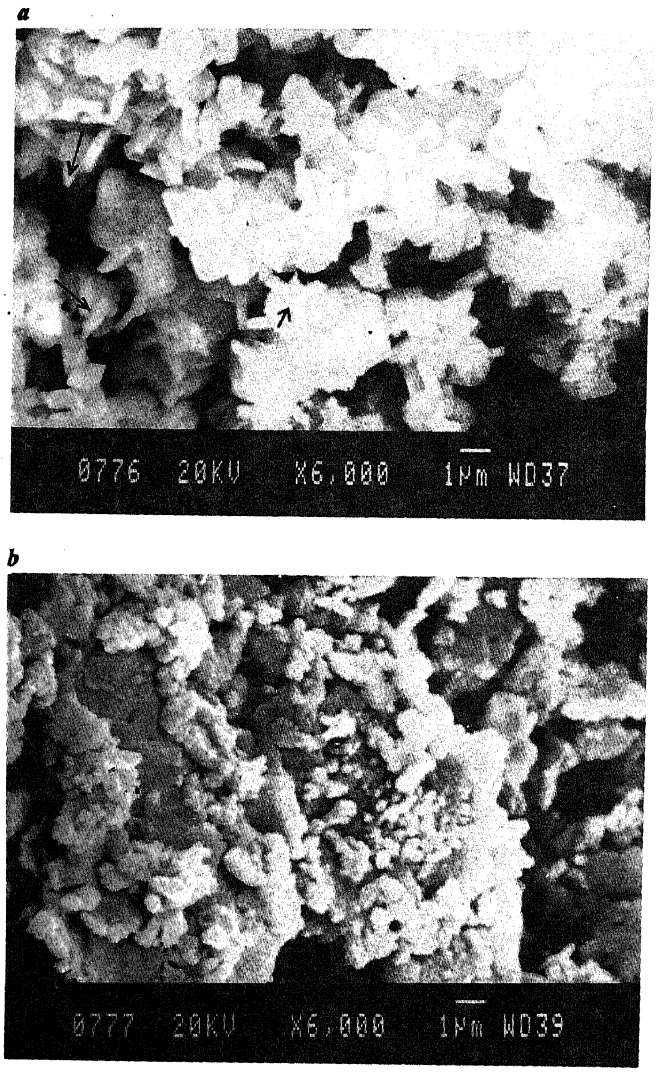

Figure 6. Scanning electron micrograph of (*a*) *T. ferrooxidans* adhering to chalcopyrite, (*b*) leached out surface of chalcopyrite. Arrows represent cells.

their affinity for *T. ferrooxidans*[14]. In the time intervals involved in these experiments, the stationary flasks consistently showed lower adhesion than agitated flasks. Slow diffusion of oxygen into the solution was probably responsible for decreased adhesion and growth. The rotary shaker for agitation of the flasks was used only at 200 rpm and the motion it imparts to the system aids bacterial contact with the mineral surface. Hence the agitated conditions show increased bacterial adhesion.

The scanning electron micrographs shown in Figures 5*a* and *b* indicate adhesion of *T. ferrooxidans* to pyrite and the leached out surface as a consequence of bacterial attack respectively. Figures 6*a* and *b* show the same events respectively for chalcopyrite mineral. Clustering of the bacteria and colonization of the mineral are noteworthy. The pitting of the surface is due to the direct attack of the bacteria.

Conclusion

This study provides a characterization of the hydrophobicity and electrophoretic mobility of *T. ferrooxidans* as important for its interaction with minerals. A time course of adhesion of *T. ferrooxidans* to pyrite and chalcopyrite for stationary and agitated conditions has been studied. Adhesion to the minerals has been visualized and the consequences of direct attack demonstrated.

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