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# Role of outer membrane exopolymers of *Acidithiobacillus ferrooxidans* in adsorption of cells onto pyrite and chalcopyrite

M.N. Chandraprabha [a,*], K.A. Natarajan [b]

[a] Department of Biotechnology, M S Ramaiah Institute of Technology, Bangalore 560054, India
[b] Department of Materials Engineering, Indian Institute of Science, Bangalore 560012

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ABSTRACT

Bacterial surface polymers play a major role in the adhesion of bacterial cells to solid surfaces. Lipopolysaccharides (LPS) are essential constituents of the cell walls of almost all Gram-negative bacteria. This paper reports the results of the investigations on the role of outer membrane exopolymers (LPS) of the chemolithotroph, *Acidithiobacillus ferrooxidans*, in adsorption of the cells onto pyrite and chalcopyrite. Optimization of EDTA treatment for removal of LPS from cell surface and the surface characterization of EDTA-treated cells are outlined. There was no change in cell morphology or loss in cell motility upon treatment with upto 0.04 mM EDTA for 1 h. Partial removal of LPS by EDTA treatment resulted in reduced adsorption of the cells on both pyrite and chalcopyrite. The protein profile of the EDTA-extractable fraction showed presence of certain outer membrane proteins indicating that EDTA treatment results in temporary gaps in the outer membrane. Also, specificity towards pyrite compared to chalcopyrite that was exhibited by untreated cells was lost when their exopolymer layers were stripped off, which could be attributed to the role of outer membrane proteins in the mineral-specificity exhibited by the bacteria.



## 1. Introduction

Gram-negative bacteria, especially those important in metal reduction and bioremediation, form an extensive outer layer of lipopolysaccharides (LPS) that can account for a significant fraction (~40%) of the mass of the outer membrane, and can extend outward 5–10 nm from the cell surface. Together with the LPS, proteins are also exposed at the surface of the outer membrane (Chatterjee and Chaudhuri, 2011).

The LPS has a highly reactive electronegative surface (Peterson et al., 1985) and most of this charge is anionic because of exposed phosphoryl and carboxyl groups which can be readily ionized depending on the environmental pH. It is the molecular interactions of these bacterial surface functional groups that control metal binding and microbial attachment to surfaces (Peterson et al., 1985). Because of their physical location on the outside of the microorganisms and their negatively charged groups, LPS are believed to be a key factor in the attachment of microbes to mineral surfaces, the uptake of metal ions, and microbially induced precipitation/dissolution reactions.

*Acidithiobacillus ferrooxidans* is an important Gram-negative microorganism used in biomining operations for metal recovery. Utility of *Acidithiobacillus ferrooxidans* in selective separation of pyrite, chalcopyrite and arsenopyrite by biomodulation has been previously reported (Chandraprabha et al., 2003, 2004a, 2004b). The effect of surface lipopolysaccharides (LPS) on the electrophoretic softness of *Acidithiobacillus ferrooxidans* cells grown in presence of copper or arsenic ions have also been elucidated (Chandraprabha et al., 2009).

Contradictory results have been reported on the role of exopolymers in adsorption of *Acidithiobacillus ferrooxidans* onto mineral substrates. Arredondo et al. (1994) have reported that removal of LPS increases the adsorption of bacteria onto minerals. On the other hand, while LPS-deficient strains of ferrous-grown cells lost their ability to adhere to covellite, similar LPS-deficient strains of sulphur-grown cells showed greater adsorption compared to untreated cells (Pogliani and Donati, 1999).

EDTA treatment has shown to induce losses of lipopolysaccharides (LPS) (upto 40%), outer membrane proteins (OmpA, OmpF/C and lipoprotein), periplasmic proteins and phosphatidylethanolamine (Leive, 1965). EDTA-induced losses of up to 40% LPS from whole cells of wild-type *Escherichia coli* and several lipopolysaccharide (LPS) mutants derived from *E. coil* have been reported by Hans et al. (1989). In their studies, the material released from *E. coli* showed a constant ratio of lipoprotein, OmpA, and phosphatidylethanolamine at all EDTA concentrations tested indicating that the material was lost as specific outer membrane patches. The envelope alterations caused by EDTA did not result in cell lysis.

In this work we aim at EDTA induced removal of LPS from the cell wall of *Acidithiobacillus ferrooxidans* and understanding the role of these exopolymers in bacterial adsorption onto pyrite and chalcopyrite minerals.

* Corresponding author. Tel.: +91 9845785174.
*E-mail address:* chandraprabhamn@yahoo.com (M.N. Chandraprabha).

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## 2. Experimental

### 2.1. Microorganism

This strain of *Acidithiobacillus ferrooxidans* used was isolated from Hutti Gold Mines, India. Bacterial cells were cultured in Silverman and Lungdren 9 K salts medium (Silverman and Lundgren, 1959). The purity of the strain was ascertained by the procedure outlined by Karavaiko et al. (1988).

### 2.2. Minerals

Pure handpicked mineral samples of pyrite were obtained from Alminrock Indser Fabricks, India and chalcopyrite from Gregory Botley and Lloyd's, UK. The mineral samples were dry-ground with a porcelain ball mill and dry-sieved to obtain different size fractions. The −37 μm fraction was further ground in a Retsch mortar grinder. The particle size analysis of this sample was done using a Malvern Mastersizer 3000-model and the mean size was found to be ≈5 μm. Purity of the samples was ascertained by mineralogical, chemical analysis and XRD. Minerological analysis of pyrite indicated that the mineral contained 98–99% of pyrite with 1–2% of silicates as impurity. Trace amounts of sphalerite and carbonate were also detected. X-ray diffraction pattern showed major peaks for pyrite, and absence of other peaks confirmed that pyrite was the predominant mineral present. Mineralogical analysis of chalcopyrite sample indicated 97–98% of chalcopyrite with presence of quartz and inclusions of pyrite in trace amounts. X-ray diffraction pattern showed major peaks of chalcopyrite with other peaks being negligible, indicating that the mineral used was sufficiently pure. Surface area of the mineral samples was determined by BET nitrogen specific technique using Quantachrome surface area analyzer and was found to be 1.26 and 1.31 $m^2/g$ respectively for pyrite and chalcopyrite.

### 2.3. Adsorption studies

Culture containing cells in the exponential growth phase was centrifuged at 10,000 rpm for 20 min to obtain the cell pellet. The cell pellet of desired concentration (about $3.75 \times 10^8$ cells/ml) was suspended in $10^{-3}$ M KCl solution at the desired pH to obtain the mother solution. 1 g of the mineral sample was pulped to 100 ml of the mother solution and the slurry was agitated on a rotary shaker at 200 rpm for 30 min for equilibration. After equilibration, the slurry was vortex mixed for 1 min to remove loosely held cells, centrifuged at 2000 rpm for 5 min and the supernatant cell number was recorded. The bacterial count was monitored by direct counting under a Leitz phase contrast microscope (Labrolux K Wild MPS12) using a Petroff Hausser counter. For experiments on adhesion kinetics, the above procedure was repeated and the cell number with respect to time recorded. All the experiments were conducted in triplets and the average value is considered.

### 2.4. Electrokinetic studies

The electrophoretic mobilities of the bacterial cells was determined using a Malvern Zetasizer 3000 instrument. Measurements were carried out in $10^{-3}$ M KCl solutions. Immediately prior to each measurement, an aliquot of the bacterial suspension was added to the electrolyte solution such that the cell density in the final suspension is about $1 \times 10^8$ cells/ml. This was equilibrated for 30 min before recording the electrophoretic mobility data.

### 2.5. LPS-deficient cells by EDTA treatment

EDTA was used to remove the lipopolysaccharides (LPS) of bacterial cell wall (Leive et al., 1968). The cells were harvested from the culture after reaching late exponential growth phase by centrifugation at 10,000 rpm for 20 min. Cell pellets were washed with deionised water and suspended in 100 mM EDTA solution. The solution was incubated in a rotary incubated shaker at 100 rpm and 37 °C for 60 min. The solution was centrifuged as before and the bacterial pellets obtained were washed twice with distilled water. The final cell pellet obtained was termed LPS-deficient cells.

### 2.6. Ruthenium red adsorption

Cell surface polysaccharide quantification was done by Figueroa and Silverstein (1989) procedure using the polysaccharide specific stain ruthenium red dye. Solution of ruthenium red dye was prepared by dissolving 0.5 g of ruthenium red crystals in 100 ml of distilled water. This was heated slowly to 60 °C, cooled and stored in a dark bottle at 4 °C in a refrigerator. Washed cell pellet of known concentration were suspended in 10 ml of known concentration of ruthenium red dye at a desired pH. The suspension was incubated in an Orbitek shaking incubator at 37 °C for 2 h while shaking gently at 200 rpm. Interacted suspension was centrifuged at 10,000 rpm for 20 min to remove the cell pellet. The clear supernatant obtained was analyzed for residual ruthenium red concentration by UV-visible spectrophotometer at a wavelength of 535 nm. Amount of ruthenium red dye adsorbed onto the cell surface was determined and expressed as mg/g of cell.

## 3. Results and discussion

### 3.1. Effect of EDTA concentration on LPS removal

EDTA treatment induces removal of LPS from the cell surface and this would result in decrease of polysaccharide content of the bacterial surface. This was confirmed by decrease in ruthenium red dye adsorption of cells and the results obtained are shown in Fig. 1. With increase in EDTA concentration from 0.01 mM to 0.05 mM, there is gradual decrease in the adsorption of dye by the cells. Beyond 0.05 mM concentration, the decrease in dye adsorption is not significant.

The effect of EDTA concentration on LPS removal was also assessed by its sugar content and the results obtained are shown in Fig. 2. With increase in EDTA dosage there was a gradual increase in the sugar content of the extracted LPS. With increase in EDTA concentration from 0.01 mM to 0.05 mM, the sugar content of extracted LPS increased to about 55 mg/$10^{12}$ cells. Beyond 0.05 mM, there was no significant increase in the sugar content with further increase in EDTA dosage. Limited availability of divalent ions in LPS would lead to the saturation observed.

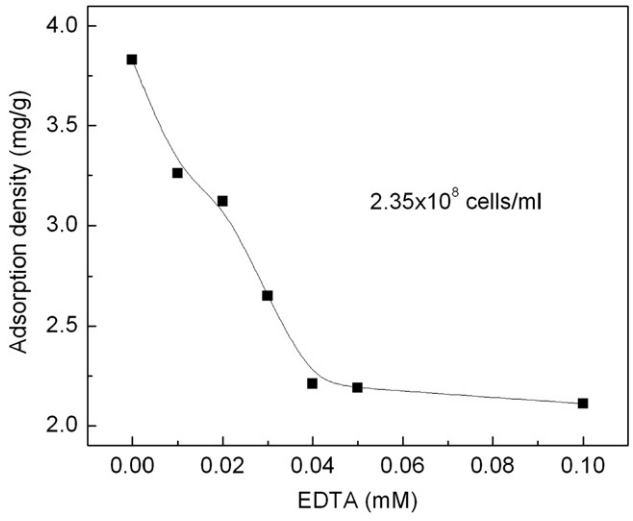


**Fig. 1.** Effect of EDTA concentration on adsorption density of ruthenium red for cells of *Acidithiobacillus ferrooxidans*.