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Mechanism of arsenic tolerance and bioremoval of arsenic by *Acidithiobacilus ferrooxidans*

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Abstract

This paper reports the studies on mechanism of arsenic tolerance and bioremoval of arsenic ions (arsenite or arsenate) by *Acidithiobacillus ferrooxidans*. Exposure of cells to arsenic ions resulted in increased cell surface hydrophobicity, decreased electrophoretic mobility and stronger adsorption affinity towards arsenopyrite. The mechanism of tolerance to arsenic ions were specific and could be attributed to the changes in specific protein expression in the outer membrane and cytosolic membrane fractions. Biosorption studies showed decrease in solution arsenic concentration only with ferrous–grown cells indicating that presence of ferric ions in the EPS was necessary for binding or entrapment of arsenic ions in the EPS. Bacterial EPS of ferrous–grown wild cells were able to uptake arsenate ions due to the strong affinity of ferric ions towards arsenate ions. Neither cells nor the ferric ions were capable of precipitating or oxidizing arsenite ions directly. Both arsenate ions and arsenite ions were co–precipitated with ferric ions formed during the growth of the bacteria.

Key words: *Acidithiobacillus ferrooxidans*, arsenic, biosorption.

Introduction

Environments containing high levels of dissolved metals include active and abandoned mine sites, where the production of acid mine drainage (AMD) is catalyzed by the action of microorganisms (Ledin & Pedersen 1996). The generation of mine wastes causes a huge environmental problem where drainage from tailings (generated whilst processing the ore) and waste rock (produced when uncovering the ore) releases high levels of acid and toxic heavy metals. If these heavy metal–rich acidic solutions enter natural water systems they can cause devastating effects, altering river ecologies, destroying commercial and recreational fishing industries and contaminating drinking water.

Acidithiobacillus ferrooxidans has been shown to have a high tolerance to many metal ions like nickel, copper, cadmium, zinc etc (Modak and Natarajan 1995; Novo et al. 2000). Natarajan et al (1994) suggested that the bacterium is naturally tolerant to certain metals and given adequate culture time it can adapt to higher concentrations of metals. Survival of *Acidithiobacillus ferrooxidans* in arsenic–rich mine waters and the ability of this organism to oxidize ferrous to ferric indicate possible role of the organism in removal of arsenic.

Studies on mechanisms of metal ion tolerance by bacteria and its specificity has been an area of much research and has been extensively reviewed by Silver (1996). Specific studies with *Acidithiobacillus ferrooxidans* strains, particularly with respect to expression of specific membrane proteins during arsenic tolerance, and mechanism of arsenic precipitation during growth are limited. Changes in the protein profiles of outer and cytosolic membrane fractions upon exposure to arsenic and the mechanism of arsenic resistance is discussed in this paper.

There are a variety of methods currently available for removal of arsenic from contaminated water such as alum and iron precipitation, lime softening, use of membranes, colloidal flotation, adsorption by activated iron and alumina and use of ion exchange resin (Hering et al. 1997; McNeill and Edwards 1997). As against the conventional remediation techniques, biological methods are gaining prominence because of their potential in providing a cost-effective technology for heavy metal remediation. Oxidation of ferrous to ferric and subsequent precipitation of ferric ions formed in presence of ferrous-oxidizing microorganisms, such as *Acidithiobacillus ferrooxidans*, offers a promising alternative to the remediation of arsenic. Knowledge of metal toxicity and how acidophiles survive in acidic metal-rich environments may provide insights into bioremediation of AMD sites, the optimization of existing techniques and the development of novel biotechnological processes.

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Materials and Methods

Microorganism

The bacterial culture used was a strain of *Acidithiobacillus ferrooxidans* that was isolated from Hutti Gold Mines (HGML). The bacteria were cultured in sterile 9K medium developed by Silverman and Lundgren (1959).

Growth and exposure of bacteria to different metal ions

The sterilized medium was inoculated with 10% (v/v) of active inoculum in standard Erlenmeyer flasks and incubated at 30°C on a rotary shaker at 200 rpm. Bacterial count was monitored by direct counting under a Leitz phase contrast microscope (Labrolux K Wild MPS12) using a Petroff Hausser counter. The solution pH was also regularly monitored using a Systronics digital pH meter.

The grown culture was initially filtered through Whatman 42 filter paper to remove the precipitates. This was then centrifuged at 10,000 rpm for 20 min in a Sorvall RC-5B refrigerated high-speed centrifuge at 5°C. The pellet obtained was resuspended in pH 2 solution and then centrifuged as before to obtain metabolite free cells.

Exposure of the bacterium to metal ions was carried out by serial subculturing in 9K salts medium (with ferrous sulphate) in presence of externally added sodium arsenite or sodium arsenate as the sources of arsenite and arsenate ions respectively. The concentration of the metal ions were maintained at 3 g/L. Iron oxidation rate of these cultures were monitored and compared with that of the control without toxic metal ions (Unadapted strain). The bacterium was considered to be adapted to the metal ion when its iron oxidation rate matched that of the wild strain.

Hydrophobicity measurements

Hydrophobicity of the different strains was determined by bacterial adherence to hydrocarbons (BATH) technique using n-hexadecane as the hydrocarbon (Rosenberg et al. 1980). The aqueous phase after separation were carefully collected using a Pasteur pipette and air, at the rate of approximately 3 ml/s, was bubbled through them for 1 min to remove any contamination due to hexadecane. Absorbance of the aqueous phase was recorded using a Shimadzu UV-260 UV-Visible spectrophotometer at 660 nm keeping the first test tube without hexadecane as the blank control. The percentage reduction in the absorbance with increasing hexadecane concentration was evaluated.

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 1×10^{8} cells/ml. This was equilibrated for 30 mins before recording the electrophoretic mobility data.

Adsorption studies

The cell pellet of desired concentration was suspended in 100ml 10^{-3} M KCl solution at the desired pH in 250ml standard Erlenmeyer flask. 1g of the mineral sample was pulped to this 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. For experiments on adhesion kinetics, the above procedure was repeated at regular intervals and the cell data with respect to time recorded.

Extraction of outer membrane and cytosolic protein and SDS-PAGE analysis

The protocols described by Amaro et al (1991) and Booth and Curtis (1977) were used for outer membrane and cytosolic protein isolation. The general protocol for extracting both plasma (cytosolic) and membrane (microsomal) proteins from cells is given below:

(1) Washed cells were suspended in the sonication buffer and vortex mixed vigorously.
(2) Cells were lysed using a probe sonicator and the suspension was centrifuged at 2800 ×g at 4°C for 20 minutes. The supernatant obtained was decanted into a separate vial and placed in an ice bath at 4°C.
(3) The debris obtained was resuspended in 1mL of Extraction Buffer and vortex mixed again. This was again centrifuged at 2800 ×g, the supernatant decanted and pooled with the supernatant from step 3. The debris was discarded.
(4) 5mL of ice–cold aqueous 0.1 M Na_2CO_3 solution (pH >11) was added to the pooled supernatants (from Step 4) and agitated gently on ice at 4°C for 1h.
(5) The solution was ultracentrifuged at >100,000 ×g for 1 h at 4°C. The supernatant was decanted into a separate vial and placed in an ice bath at 4°C.
(6) Pellet from step 5 was resuspended in 1mL of 50mM TRIS (pH 7.3) and ultracentrifuged at >100,000 ×g for 20min at 4°C
(7) Supernatant was decanted and pooled with the supernatant from step 6. Pellet was stored at 4°C.

The pooled supernatants contain the soluble plasma (cytosolic) proteins and the pellet contains the membrane (microsomal) proteins.

The proteins were solubilized with laemmli's sample buffer and separated by SDS-PAGE (Laemmli 1970). Gels were stained with Coomassie brilliant blue R stain.

Extraction of EPS by centrifugation

The bacterial cells were removed by centrifugation at 20,000 x g for 20 min at 4°C, and the polysaccharide was precipitated from the culture supernatant. The supernatant containing extracted EPS, after filtration through 0.22 μm cellulose acetate membrane, was precipitated using chilled ethanol, the precipitates redissolved in deionized water and dialyzed against acidified double distilled water (pH 1.5) for 24 h (cut off 12000 Da) (Sklodowska and Matlakowska 1997). The dialysate was then reprecipitated and centrifuged at 3000 rpm at 4°C for 10 min, lyophilized and stored at –20°C until used for the experiments.

Metal binding by EPS

Extracted EPS solution was taken in a dialysis bag and suspended in 250 ml beakers having desired concentrations of arsenic and incubated at 30°C on a rotary shaker at 200 rpm. After incubation, the dialysis bags containing the EPS were removed and transferred to another beaker having deionised water and dialyzed overnight to remove any loosely bound metal ions. The dialyzed EPS was collected and analyzed for arsenic. Blanks in which EPS was

replaced with deionised water were incubated under similar conditions and analyzed for arsenic. For control experiments, EPS was incubated in deionised water without metal ions.

Biosorption studies

Bacterial cells from actively growing culture were separated by centrifugation and washed 2-3 times to remove ferric precipitates. Known concentration of washed cells was suspended in a beaker containing either sodium arsenite or sodium arsenate solution having a predetermined arsenic concentration at pH 2. The beaker which had a magnetic pellet was placed on a magnetic stirrer. Aliquot samples were collected at regular intervals, centrifuged to separate the cells and the supernatant obtained was analyzed for arsenite and total arsenic concentration. Arsenate concentration was determined from the difference. The binding capacity of the cells was calculated from the initial and final concentration data.

Chemical analysis

Ferrous iron concentration was determined by the ASTM standard technique with 1,10–Phenanthroline as indicator using Shimadzu UV–260 UV–Visible spectrophotometer at 512 nm wavelength.

Arsenic analysis was done either by Inductively Coupled Plasma (ICP) spectrometer for total arsenic analysis or by Flow Injection Hydride Generation Atomic Absorption Spectrometry (FI–HG–AAS) for arsenic speciation.

Results and Discussion

Surface chemical changes of adapted cells

Figure 1 shows the changes in hydrophobicity, electrophoretic mobility and adhesion behaviour towards minerals for the wild strain and strains grown in presence of arsenic.

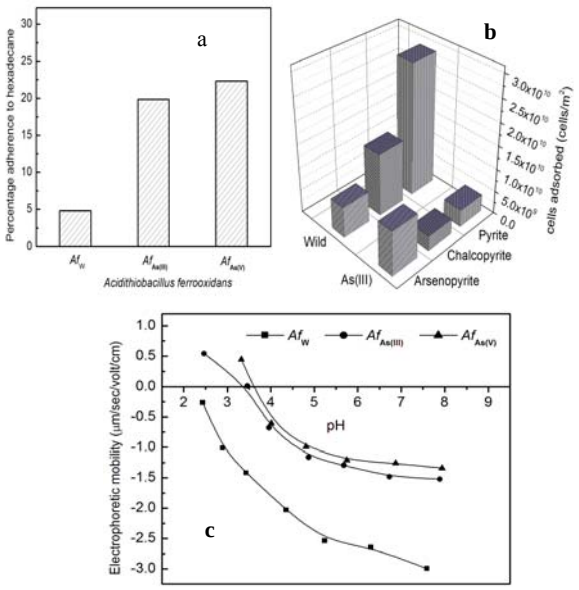

Figure 1: Hydrophobicity (a), Electrophoretic mobility (b) and Adhesion behaviour (c) of Acidithiobacillus ferrooxidans grown in presence of arsenic ions.

When grown in presence of arsenic ions the isoelectric point of the cells was shifted to a higher pH value when compared to ferrous grown wild cells (iep at pH 2.3). Since surface charge increases the likelihood of polar interactions with water molecules in the vicinity, the more charged the cell surfaces, the less hydrophobic they become. Hydrophobicity tests showed that cells grown in presence of arsenic became more hydrophobic compared to wild cells. The observed electrokinetic behaviour of the bacterial cells confirms with the hydrophobicity results. Hence, any treatment that lowers the cell negative charge would enhance its hydrophobic character. The cells of higher hydrophobicity and lower electrophoretic mobility would then be more adherent.

The strains grown in presence of arsenic had stronger adsorption affinity towards arsenopyrite. However, the affinity of these strains towards the other minerals was lower than that of the normal strain. Also, increase in surface hydrophobicity did not result in increased adsorption of adapted strains towards pyrite.

The above results indicate that hydrophobic interactions are not principally responsible for the initial adsorption of adapted strains of *Acidithiobacillus ferrooxidans.* Specific adherence of adapted strains to the respective minerals could be attributed to the expression of specific proteins in the outer membrane of the cells. Changes in the protein profiles of outer and cytosolic membrane fractions upon exposure to arsenic was analysed to understand the mechanism of arsenic resistance.

SDS–PAGE analysis of cytosolic and membrane proteins

SDS–PAGE analysis of the outer membrane and cytosolic protein fractions of ferrous–grown wild cells and cells tolerant to arsenic is shown in Figure 2. Lanes 1, 3, 5 indicate the cytosolic protein profile of wild, arsenate–adapted, arsenite–adapted cells respectively, while lanes 2, 4, 6 represent membrane protein profiles in the same order.

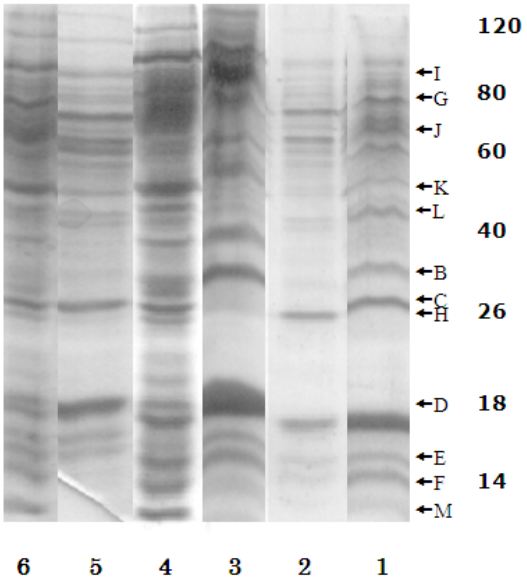

Figure 2: SDS–PAGE analysis of the proteins expressed by *Acidithiobacillus ferrooxidans* grown in absence (lanes 1 & 2) and presence of arsenate (lanes 3 & 4), arsenite ions (lanes 5 & 6). Odd numbered lanes represent cytosolic protein profiles and even numbered lanes represent outer membrane protein profiles.

Synthesis of certain cytosolic and outer membrane proteins were either induced or repressed when the cells were grown in presence of arsenic ions. Changes observed was more in the membrane

fraction compared to the cytosolic fraction indicating that the cell membrane plays a vital role in the resistance mechanism.

Cytosolic protein profile

Cytosolic protein profile of the arsenite–adapted cells (lane 5) did not show much variation when compared to similar profile of ferrous–grown wild cells (lane 1). Synthesis of most of the cytosolic proteins was induced in case of arsenate–adapted cells (lane 3). Significant over expression of 18 kDa (spot D) and 90 kDa proteins (spot I) and repression of a 28 kDa (spot C) and 44 kDa proteins (spot L) was observed in these cells.

Outer–membrane protein profile

Protein profile of outer membrane fractions of arsenate–adapted (lane 4) cells showed significant differences compared to ferrous–grown wild cell membrane profile (lane 2). Most of the membrane proteins of arsenate–adapted cells showed enhanced synthesis. Protein of apparent molecular weight 49 kDa (spot A), 28 kDa, 30 kDa, 24 KDa (spot H), 15 kDa, 14 kDa and 13 kDa (spot M) were induced in these cells. Outer membrane protein profile of arsenite–adapted cells (lane 6) was similar to that of arsenate–adapted cells. However comparatively fewer proteins were induced in these cells

Arsenic resistance mechanism

Plasmid–associated arsenic efflux resistance mechanisms have been known for many years and have been extensively reviewed (Cervantes et al. 1994; Silver, 1996; Suzuki et al. 1998; Tsai et al. 1997). Resistance to arsenic in certain bacteria is explained to be via energy–dependent efflux encoded by the *ars* operon, containing the genes *arsRBC* (Carlin et al. 1995). Arsenate enters the bacterial cell wall via the fast, unspecific and constitutive uptake systems for phosphate. Arsenate detoxification involves reduction to arsenite prior to its efflux (Ji and Silver 1992; Ji et al. 1994). As(V) is reduced to As(III) by arsenate reductase (ArsC) prior to its efflux via a membrane potential driven pump (ArsB) controlled by *trans*–acting repressor (ArsR). Butcher and Rawlings (2002) have reported that *ArsRC* genes were induced in *Acidithiobacillus ferrooxidans* irrespective of the form of arsenic added. Induction of protein of apparent molecular weight 18 kDa in the cytosolic fraction of only arsenate–adapted cells indicates expression of ArsC protein (≈ 18.1 kDa) (Butcher et al. 2000). Induction of 13.5 kDa protein in the membrane fraction of both arsenate and arsenite–adapted cells indicates ArsR protein (≈ 13.1 kDa) (Butcher et al. 2000). The regulatory protein of the operon, ArsR, has been shown to be a *trans*–acting repressor that senses environmental As(III) (Xu et al. 1996). The ArsR protein contains a very specific binding site towards As(III) and can discriminate effectively against phosphate, sulfate, cobalt, and cadmium (Scott et al. 1997). ArsR has a strikingly high affinity, as even 10-15 M As(III) could induce the *ars* promoter (Ramanathan et al. 1997). Although ArsR is known to be specific for As(III), removal of As(V) may occur through the initial conversion of As(V) into As(III) by the arsenate reductase and the subsequent sequestration by ArsR. Induction of protein of apparent molecular weight 49 KDa can be identified as ArsB (≈ 48.5 kDa) outer membrane protein. This protein was induced more for arsenate–adapted cells compared to arsenite–adapted cells. Induction of this protein in the outer membrane fraction indicates that the resistance to arsenic was by active efflux system. Induction of proteins in the outer membrane fraction indicates that the mechanism of resistance was due to active efflux in both arsenite and arsenate–adapted cells. Li et al (1997) suggested that resistance to Ag by *E. coli* was due to active efflux enhanced synergistically by decreased membrane permeability. However, in our study, repression of outer membrane porins was not observed for arsenic–adapted cells. Resistance to arsenic by this strain was mainly due to active efflux system and not supported by decreased membrane permeability as a support mechanism. The resistance mechanism for arsenic can be schematically represented as shown in Figure 3.

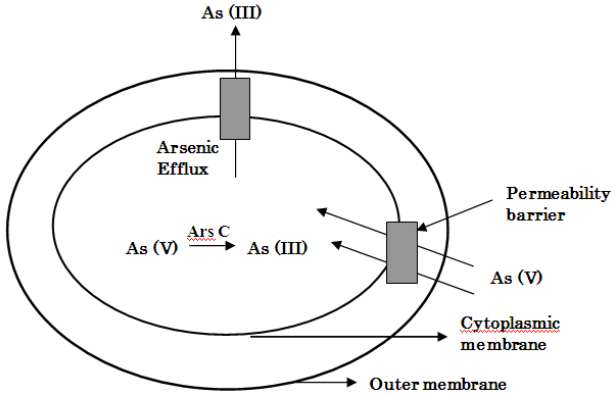

Figure 3: Schematic representation of arsenic ion tolerance by *Acidithiobacillus ferrooxidans.*

Mechanism of biological arsenic removal

Survival of *Acidithiobacillus ferrooxidans* in arsenic–rich mine waters and the ability of this organism to oxidize ferrous to ferric indicate possible role of the organism in removal of arsenic. Various experiments were carried out to understand the mechanism of biological arsenic removal and the results obtained are discussed.

Growth in presence of arsenic

Concentrations of arsenite and arsenate ions in the solution were monitored during the growth of the organism in 9K ferrous medium in presence of either sodium arsenite or sodium arsenate as sources of arsenite and arsenate ions respectively. When grown in presence of arsenate ions (initial concentration 2500 ppm), the solution arsenate concentration started decreasing during the exponential phase of growth (Figure 4a). During the log phase of growth, concentration of arsenate decreased from 2500 ppm to 1000 ppm. Arsenite was not detected in the solution during the course of growth. Ferric is known to precipitate arsenate as ferric arsenate over a wide pH range (Kilty et al. 2001; Leblanc et al. 1996). This explains the decrease in arsenate concentration with the oxidation of ferrous iron by cells.

When grown in presence of arsenite ions, similar behaviour was observed and the solution arsenite concentration decreased to about 1000 ppm during the log phase of growth (Figure 4b). Solution arsenate concentration was found to be below detectable limits. Thus, the decrease in arsenite concentration represents decrease in total arsenic concentration of the solution. Various experiments were carried out to understand the mechanism of arsenite precipitation and are discussed in the following sections.

Effect of cell-free metabolite on arsenic removal

The cell–free metabolite was tested for its ability to precipitate arsenite and it was interesting to note that there was no significant decrease in the concentration of arsenite in solution. This indicates that ferric ions were not able to directly precipitate arsenite. The obtained results also indicate that extracellular biopolymers secreted during the bacterial growth were unable to either oxidize or

precipitate arsenite. Thus, removal of arsenite from solution is a phenomenon happening only during the growth of bacteria and presence of cells becomes necessary for this.

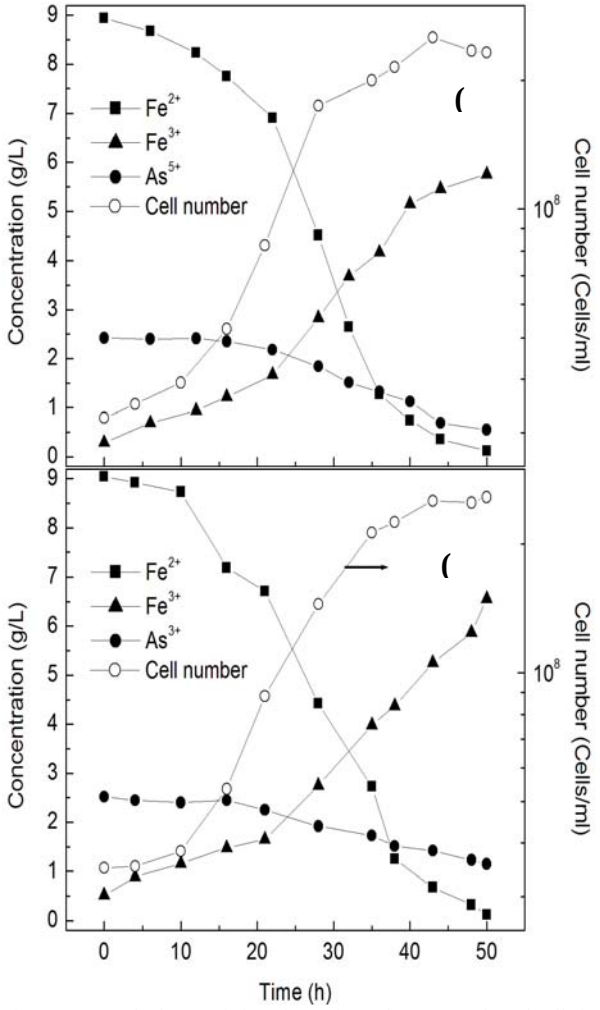

Figure 4: Growth characteristics of arsenite and arsenate adapted cells in 9K ferrous media and in presence of arsenite ions.

Growth of adapted cells in ferrous-free media supplemented with arsenite ions

The growth characteristics of arsenite and arsenate adapted cells in 9K$^-$ media devoid of ferrous iron and in presence of arsenite ions is shown in Figure 5. In case of both the strains, no growth was observed in presence of arsenite ions. Cell number for both the strains started decreasing after 6 days. The solution arsenite concentration remained almost constant indicating that the organism does not utilize arsenic for metabolic energy generation and thus does not oxidize arsenite to arsenate.

Growth of sulphur-grown arsenite-adapted cells

Growth characteristics of sulphur–grown arsenite adapted strain in presence of 2500 ppm arsenite is shown in Figure 6. Contrary to that observed with ferrous–grown cells, there was no significant decrease in solution arsenite concentration during the log phase of cell growth. Slight decrease in arsenite concentration could be attributed to its oxidation by oxygen. The results obtained indicate that the cells were not able to either oxidize or precipitate arsenite directly and presence of iron is essential for arsenite precipitation.

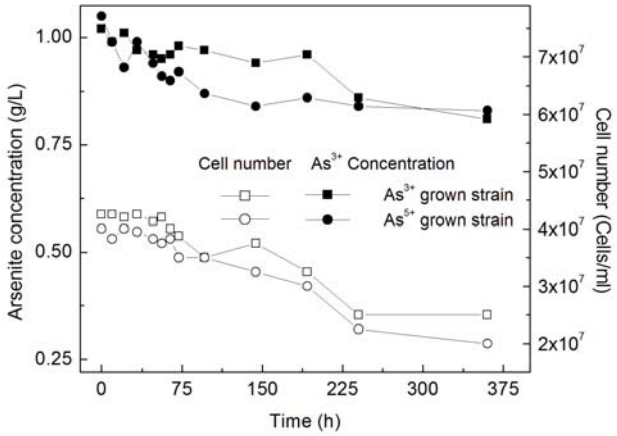

Figure 5: Growth characteristics of arsenite and arsenate adapted cells in 9K- media devoid of ferrous iron and in presence of 1000 ppm arsenite ions

It is evident from the obtained results that cells cannot oxidize arsenite directly or indirectly. Decrease in arsenite or arsenate concentration during growth could also be attributed to the biosorption of arsenite or arsenate ions on the cell surface.

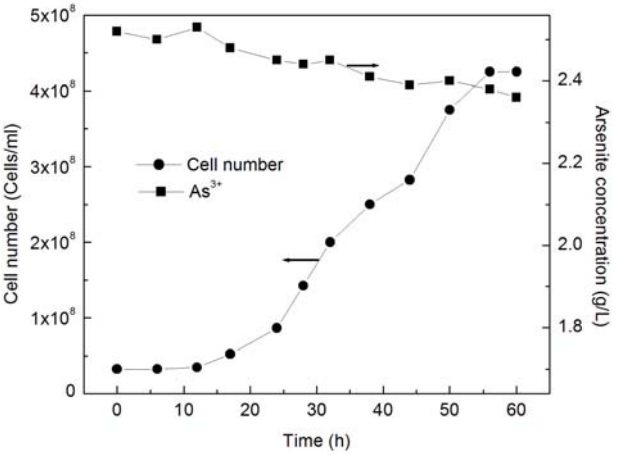

Figure 6: Growth characteristics of sulphur–grown arsenite adapted strain in presence of 2500 ppm arsenite.

Biosorption studies with Acidithiobacillus ferrooxidans cells

Biosorption studies of arsenite and arsenate were carried out with wild, arsenite–adapted and arsenate–adapted strains of *Acidithiobacillus ferrooxidans* (Figure 7). Cells were washed 2-3 times to ensure that there is no precipitation of arsenic due to ferric ions in cell suspension. Control experiments (without cells) under similar experimental conditions were maintained for comparison.

When the wild strains were interacted with 10 ppm arsenite solution (Figure 7a), it was observed that there was no significant change in the solution arsenic concentration even after 2 h. When interacted with 10 ppm arsenate (Figure 7b), instantaneous decrease in solution arsenic concentration of about 0.3-0.4 ppm was observed. Beyond this, arsenic concentration in solution remained almost constant. Interaction with 100 ppm of arsenate resulted in instantaneous decrease in solution arsenic concentration by 1.5-2 ppm (Data not shown).

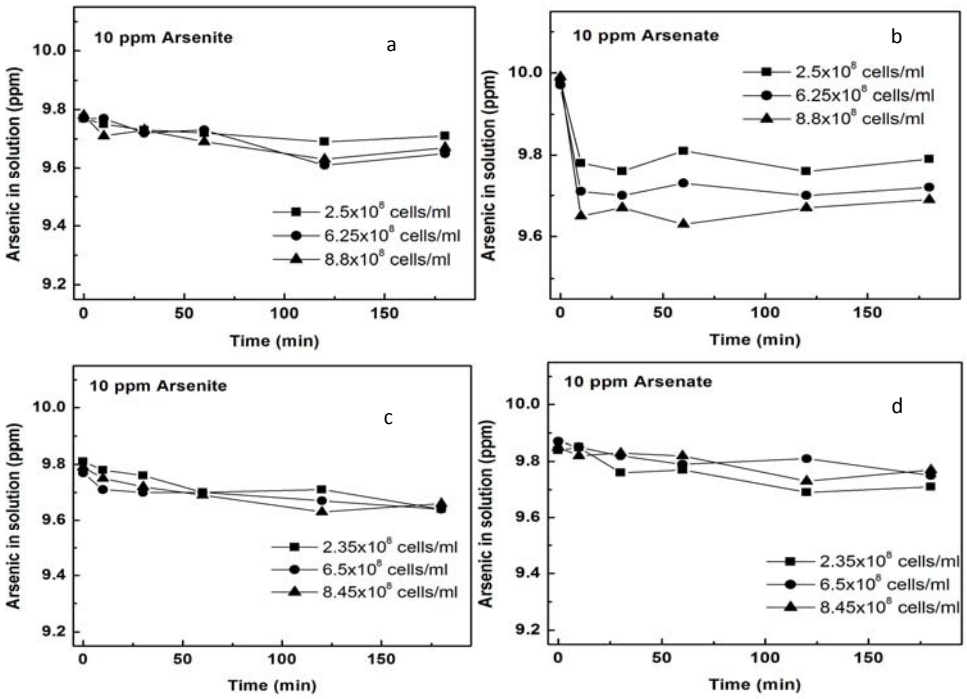

Figure 7: Effect of ferrous–grown (a, b) and sulphur-grown (c, d) wild cells of Acidithiobacillus ferrooxidans on solution arsenic concentration during biosorption studies with arsenite and arsenate ions.

Interaction of sulphur–grown cells with 10 ppm arsenite (Figure 7c) had no significant effect on the solution arsenic concentration as observed with ferrous–grown cells. However, contrary to that observed with ferrous–grown cells, interaction of sulphur–grown cells with 10 ppm arsenate (Figure 7d) did not result in any significant decrease in solution arsenic concentration.

Interaction of either ferrous–grown or sulphur–grown adapted cells with 10 ppm arsenite (Figure 8a & 8b) did not result in any significant change in the solution arsenic concentration. Arsenic in solution was not detected or below detectable limits in the control flasks. Contrary to this, the solution arsenic concentration increased during the first hour of incubation with arsenate–adapted strains (Figure 8c & 8d). With 10 ppm arsenate solution, the solution arsenic concentration increased from 9.87 ppm to 10.16 ppm with 2.25×10^8 cells/ml, 9.97 ppm to 10.31 ppm with 6.5×10^8 cells/ml and 9.78 ppm to 10.45 ppm with 8.25×10^8 cells/ml respectively. Beyond 1 hr, the solution arsenic concentration remained constant. Control experiments without arsenate also showed an increase in solution arsenic concentration (Data not shown). Increase in solution arsenic concentration was lower for sulphur–grown arsenate adapted cells when compared to ferrous–grown arsenate adapted cells.

Increase in solution arsenic concentration could be attributed to the release of arsenic from cells. Geesey et al (1988) have reported that the polysaccharides of bacterial EPS are capable of complexation of metal ions. However, in our case decrease in arsenic concentration was observed only with ferrous–grown cells indicating that the polysaccharides do not contribute towards the observed decrease in arsenic concentration. EPS of *Acidithiobacillus ferrooxidans* has been shown to contain tightly bound Fe(III) (Gherke et al. 2001). The sudden decrease in solution arsenic concentration, when interacted with ferrous–grown cells, could therefore be attributed to the precipitation of arsenate ions by the ferric ions present in the extracellular polysaccharide layer of the bacteria. Decrease in solution arsenic concentration observed only in presence of arsenate indicates the strong affinity Fe(III) has for Arsenate. By contrast, lower affinity of Fe(III) to As(III) results in insignificant changes in the solution arsenic concentration.

Role of EPS in arsenite precipitation

EPS was extracted from wild and adapted strains and chemical analysis was done to detect iron and arsenic. The extracted EPS samples were dissolved in HNO_3 and analyzed for iron and arsenic species. Results obtained for wild and adapted cells are tabulated in Table 1. The chemical species detected are indicated by tick mark. Ferric ions were detected only in the EPS of ferrous–grown cells and not of sulphur–grown cells. Arsenic species were detected in the EPS of only ferrous–grown adapted cells. Both arsenite–adapted and arsenate–adapted ferrous–grown cells had arsenic present in their EPS. Presence of arsenic in the extracted EPS indicates that the net charge on the bacterial surface was partly neutralized. EPS of bacterial cells is known to render the cell surface electropositive by complexing metal ions (Arredondo et al. 1994; Blake et al. 2001). Decrease in electrophoretic mobility of the arsenite and arsenate–adapted strains observed earlier (Figure 1) is thus the result of binding of arsenic ions to the EPS.

Table 1: Detection of ferric and arsenic species from extracted EPS of wild and arsenic–adapted strains of Acidithiobacillus ferrooxidans.

Acidithiobacillus ferrooxidans strain EPS	Fe^{3+}	As
Ferrous–grown wild	✓	×
Sulphur–grown wild	×	×
Ferrous–grown arsenite–adapted	✓	✓
Sulphur–grown arsenite–adapted	×	×
Ferrous–grown arsenate–adapted	✓	✓
Sulphur–grown arsenate–adapted	×	×

It is interesting to note that arsenic species were not detected in the

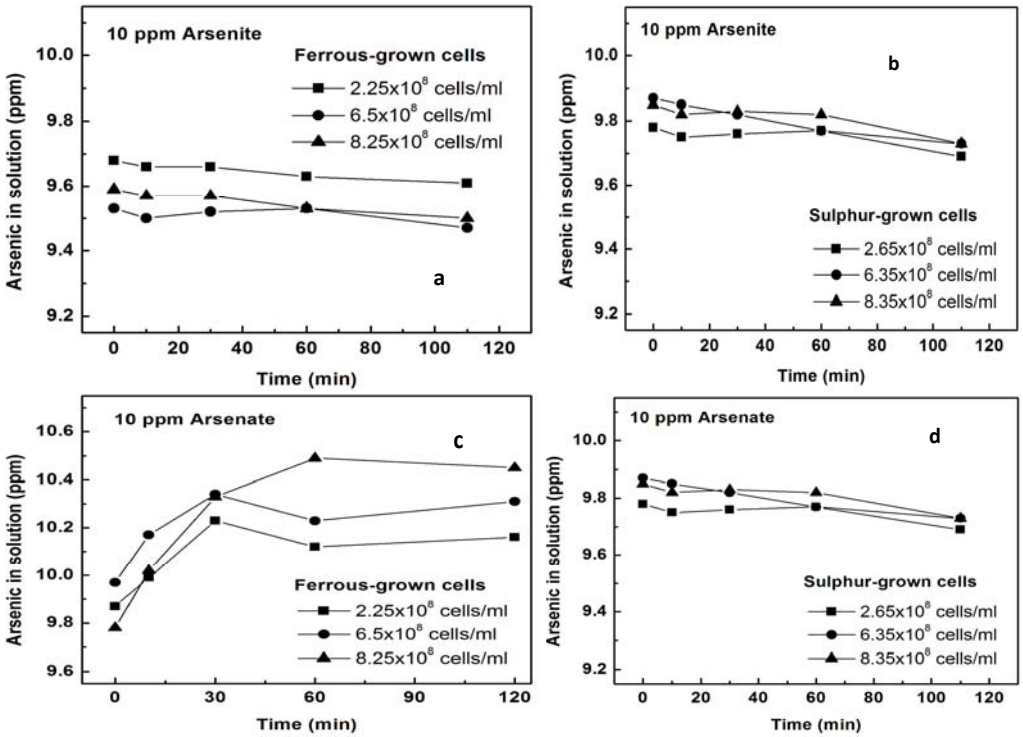

Figure 8: Effect of arsenite adapted ferrous–grown (a), arsenite adapted sulphur-grown (b) cells and arsenate adapted ferrous–grown (c), arsenate adapted sulphur-grown (d) cells of *Acidithiobacillus ferrooxidans* on solution arsenic concentration during biosorption studies with arsenite and arsenate ions.

extracted EPS of sulphur–grown adapted strains. The obtained results suggest that the presence of ferric iron is necessary for complexation of arsenic species in the EPS. It has been reported that arsenate can be readily precipitated with ferric under both acidic and alkaline conditions (Cadena and Kirk 1995). Strong affinity of ferric for arsenate species supports this observation. Presence of arsenic in EPS of ferrous–grown arsenic–adapted strains could be attributed to precipitation of arsenic with ferric ions present in the EPS.

The ability of extracted EPS of wild and adapted strains to bind arsenite and arsenate ions was tested and the results obtained are shown in Table 2. The EPS of wild and adapted strains, after interaction with metal ion solution, were dialyzed against distilled water to remove loosely bound metal ions and then analyzed for arsenic. It is interesting to note that the EPS of both wild and arsenic–adapted strains were unable to uptake arsenite ions. EPS of both ferrous–grown and sulphur–grown cells exhibited the same behaviour. Contrary to this, when interacted with arsenate ions, arsenic was detected in the EPS of both wild and arsenic–adapted ferrous–grown cells. However, when interacted with arsenate ions, no arsenic was detected in the EPS of both sulphur–grown wild and adapted cells. The present observation suggests that the binding of arsenic to EPS happens only when ferric ions were present in the EPS. This could therefore be attributed to precipitation or complexation of arsenate by ferric ions in the EPS. Binding of only arsenate ions by the EPS of ferrous–grown cells indicates the strong affinity of ferric ions towards arsenate ions. This observation explains the instantaneous decrease in the solution arsenate concentration during the biosorption studies of ferrous–grown cells with arsenate (Figure 7). Instantaneous decrease in solution arsenate concentration was not observed with arsenate–adapted ferrous–grown cells. This could be a result of saturation of EPS with arsenic ions during the growth of organism in presence of arsenic. Our earlier studies demonstrated that arsenic was present in the extracted EPS of arsenate–adapted ferrous–grown cells, which supports this explanation.

However, these observations cannot explain the initial increase in arsenic concentration during the biosorption studies with ferrous–grown and sulphur–grown arsenate–adapted cells. Although ferric ions were absent in the EPS of sulphur–grown arsenate–adapted cells, initial increase in solution arsenic concentration was observed during the incubation of these cells for biosorption studies. This behaviour of arsenate–adapted cells could be attributed to the active efflux system of arsenic resistance mechanism, which involves reduction of arsenate to arsenite prior to its efflux (Figure 3).

Table 2: Arsenite and arsenate binding capacity of extracted EPS of wild and arsenic–adapted strains of *Acidithiobacillus ferrooxidans*.

As uptake / Strain	As^{3+}	As^{5+}
Ferrous–grown wild	×	✓
Sulphur–grown wild	×	×
Ferrous–grown arsenite–adapted	×	✓
Sulphur–grown arsenite–adapted	×	×
Ferrous–grown arsenate–adapted	×	✓
Sulphur–grown arsenate–adapted	×	×

Co–precipitation with ferric ions

The results obtained so far show that both cells and their extracellular products and the ferric ions were not able to either oxidize or precipitate arsenite directly. From the growth studies of adapted cells in presence of arsenic, it was observed that the disappearance of arsenic coincides with the formation of ferric ions

by ferrous oxidation. Under acidic conditions arsenite is shown to be co–precipitated with chemical ferric ions (Kirk 1993; Wilkie and Hering 1996). XRD studies on precipitates produced by *Acidithiobacillus ferrooxidans* strains in presence of acid mine water containing arsenite ions (Morin et al. 2003) showed presence of tooeliete in the precipitate of some strains. However, this phenomenon was not observed with all the *Acidithiobacillus ferrooxidans* strains. By contrast, Duquesne et al. (2003) demonstrated that co–precipitation of arsenite in mine waters formed schwertmannite.

In the present study, precipitates from the growth flasks were examined by XRD to find out if arsenite ions were able to

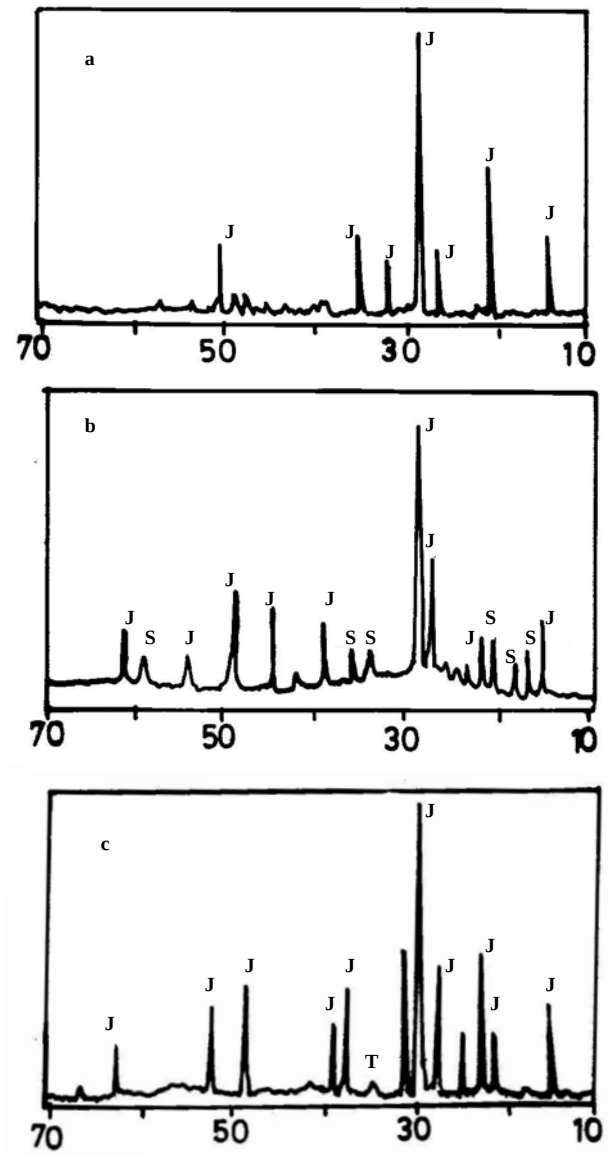

Figure 9: XRD of precipitate from (a) wild, (b) arsenate–grown and (c) arsenite–grown cells of *Acidithiobacillus ferrooxidans* (J: Jarosite, S: Scorodite; T: Tooeleite)

coprecipitate with biologically produced ferric ions during the growth of the organism. Figure 9 shows the XRD pattern of precipitates from the growth flasks of ferrous–grown wild, arsenate and arsenite–adapted strains. The major peaks obtained are identified and labeled as follows: Jarosite (J), Tooeliete (T) and Scorodite (S).

Oxidation of $FeSO_4$ by the cells produced crystalline jarosite and amorphous basic ferric–sulphates as shown by the XRD pattern of precipitate generated by wild ferrous–grown cells. The major diffraction peaks obtained can be attributed to crystalline jarosite (Carlson et al. 1992).

The XRD pattern of precipitate from arsenate–adapted cells indicates an amorphous phase represented by weak, broad bands around 2 Å to 4 Å. These broad bands can be attributed to amorphous ferric arsenate formed during the growth (Tuovinen et al. 1994). Growth in presence of arsenate also led to concurrent precipitation of crystalline jarosite. The peaks observed around 4 Å to 5 Å is identical to that of scorodite ($FeAsO_4.\ 2H_2O$) (Carlson et al. 1992). Arsenic oxyanions readily sorb to ferric hydroxide phases, which are often present as colloidal precipitates (Pierce and Moore, 1982). Scorodite is shown to be a common weathering product of arsenopyrite (Carlson et al. 1992; Ehrlich, 1995, Tuovinen et al. 1994).

XRD pattern of precipitate from arsenite–adapted strain shows presence of crystalline jarosite. Major peak at 3.1 Å can be attributed to arsenic–bearing mineral, similar to tooeleite (Morin et al. 2003, Webmineral data). No other As–bearing crystalline phase was present. Tooeleite is an iron arsenite sulphate oxy–hydroxide mineral with an approximate structural formula $Fe_6(AsO_3)_4\ (SO_4)\ (OH)_4.\ 4H_2O$ (Morin et al. 2003). Crystalline jarosites and tooeleite were found associated with an XRD–amorphous phase. Absence of amorphous ferric arsenate peaks indicates that the organism is not able to oxidize arsenite to arsenate.

The obtained results indicate that both arsenate and arsenite ions were getting co–precipitated with biologically formed ferric ions under acidic conditions.

Conclusions

Exposure of *Acidithiobacillus ferrooxidans* cells to arsenic ions resulted in increased cell surface hydrophobicity and decreased electrophoretic mobility. However, strains grown in presence of arsenic had stronger adsorption affinity towards arsenopyrite indicating that hydrophobic interactions are not principally responsible for the initial adsorption of adapted strains of *Acidithiobacillus ferrooxidans.*

Resistance to arsenic by this strain was mainly due to active efflux system and not supported by decreased membrane permeability as a support mechanism. Neither the cells nor the ferric ions were capable of precipitating or oxidizing arsenite ions directly. Presence of ferric ions in the EPS was necessary for binding or entrapment of arsenic ions in the EPS during the growth of bacteria. Bacterial EPS of ferrous–grown wild cells were able to uptake arsenate ions due to the strong affinity of ferric ions towards arsenate ions.

Both arsenate ions and arsenite ions were co–precipitated with ferric ions, formed during the growth of the bacteria, resulting in the formation of crystalline jarosite, scorodite and amorphous ferric arsenate or jarosite, tooeliete and amorphous ferric arsenite phase respectively. Co–precipitation of arsenic with the ferric hydroxide precipitate formed during growth of *Acidithiobacillus ferrooxidans* can be made use of as a process to remediate arsenic in arsenic contaminated tailings.

References

Amaro AM, Chamorro D, Seeger et al (1991) Effect of external pH perturbations on in vivo protein synthesis by the acidophilic bacteria *Thiobacillus ferrooxidans*. J Bacteriol 173:910-915

Arredondo R, Garcia A, Jerez CA (1994) Partial removal of lipopolysaccharide from *Thiobacillus ferrooxidans* affects its adhesion to solids. Appl Env Microbiol 60:2846-2851

Blake II RC, Sasaki K, Ohmura N (2001) Does aporusticyanin mediate the adhesion of *Thiobacillus ferrooxidans* to pyrite? Hydrometallurgy 59:357-372

Booth BR, Curtis NAC (1977) Separation of the cytoplasmic and outer membrane of *Psedomonas aeruginosa* PAO1. Biochem Biophy Res Comm 74:1168-1176

Butcher BG, Deane SM, Rawlings DE (2000) The chromosomal arsenic resistance genes of *Thiobacillus ferrooxidans* have an unusual arrangement and confer increased arsenic and antimony resistance to Escherichia coli Appl Environ Microbiol 66:1826-1833

Butcher BG and Rawlings DE (2002) The divergent chromosomal ars operon of *Acidithiobacillus ferrooxidans* is regulated by an atypical ArsR protein. Microbiol 148:3983-3992

Cadena R and Kirk T (1995) New Mexico Water Resource Research Institute Technical Completion Report, New Mexico State University, pp 293 - 298

Carlin A, Shi W, Dey S, Rosen BP (1995) The ars operon of *Escherichia coli* confers arsenical and antimonial resistance. J Bacteriol 177:981-986

Carlson L, Lindstrom EB, Hallberg KB, Tuovinen OH (1992) Solid-phase products of bacterial oxidation of arsenical pyrite. Appl Env Microbiol 58:1046–1049

Cervantes C, Ji G, Ramirez JL, Silver S (1994) Resistance to arsenic compounds in microorganisms. FEMS Microbiol Rev 15:355-367

Duquesne K, Lebrun S, Casiot C et al (2003) Immobilization of arsenite and ferric iron by *Acidithiobacillus ferrooxidans* and its relevance to acid mine drainage. App Environ Microbiology 69:6165-6173

Ehrlich HE (1995) Geomicrobiology, Marcel Dekker, New York

Geesey GG, Jang L, Jolley JG et al (1988) Binding of metal ions by extracellular polymers of biofilm bacteria. Water Sci Tech 11:161-165

Gherke T, Hallmann R, Kinzler K, Sand W (2001) The EPS of *Acidithiobacillus ferrooxidans* – a model for structure - function relationships of attached bacteria and their physiology. Water Sci Technol 37:159-167

Hering JG, Chen PY, Wilkie JA (1997) Arsenic removal from drinking water during coagulation. J Env Engg 123:800-807

Ji GY, Silver S (1992) Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid pI258. Proc Natl Acad Sci USA 89:9474-9478

Ji GY, Garber EAE, Armes LG et al (1994) Arsenate reductase of *Staphylococcus aureus* Plasmid pI258. Biochem 33:7294-7299

Kilty H, Moran D, Roussy J et al (2001) Acid mine water treatment: a laboratory scale study, In: VST Ciminelli and O Garcia (ed) Biohydrometallurgy: Fundamentals, Technology and Sustainable development. Elsevier, Amsterdam, pp 365-371

Kirk TL (1993) Masters Thesis, Department of Civil Engineering, New Mexico State University, Las Cruces, NM

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Leblanc M, Achard B, Ben Othman D et al (1996) Accumulation of arsenic from acidic mine waters by ferruginous bacterial accretions (stromatolites). Appl Geochem 11:541-554

Ledin M, Pedersen K (1996) The environmental impact of mine wastes - Roles of microorganisms and their significance in treatment of mine wastes. Earth-Sci Rev 41:67–108

Li XZ, Nikaido H, Williams KE (1997) Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. J Bacteriol 179:6127-6132

McNeill LS, Edwards M (1997) Arsenic removal during precipitative softening. J Env Engg 123:453-460

Modak JM, Natarajan KA (1995) Development of special strains of *Thiobacillus ferrooxidans* for enhanced bioleaching of sulphide minerals In: T Vargas, CA Jerez, JV Wiertz and H Toledo (ed) Biohydrometallurgical processing, University of Chile, Santiago, pp 33-46

Morin G, Juillot F, Casiot C et al (2003) Bacterial formation of tooeleite and mixed As(III)/(V)-Fe(III) gels in the Carnoulès acid mine drainage of France - A XANES, XRD and SEM study. Env Sci Tech 37:1705-1712

Natarajan KA, Sudeesha K, Rao GR (1994) Stability of copper tolerance in *Thiobacillus ferrooxidans*. Antonie van Leeuwenhoek 66:303-306

Novo MTM et al. (2000) *Thiobacillus ferrooxidans* response to copper and other heavy metals: growth, protein synthesis and protein phosphorylation. Antonie van Leeuwenhoek 77:187-195

Pierce M, Moore CB (1982) Adsorption of As(III) and As(V) on amorphous iron hydroxide. Water Resources Res 16:1247–1253

Ramanathan S, Shi W, Rosen BP, Daunert S (1997) Sensing antimonite and arsenite at the subattomole level with genetically engineered bioluminescent bacteria. Anal Chem 69:3380–3384

Rosenberg M, Gutnick D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring hydrophobicity. FEMS Microbiology Letters 9:29-33

Scott DL, Ramanathan S, Shi W et al (1997) Genetically engineered bacteria: electrochemical sensing systems for antimonite and arsenite. Anal Chem 69:16–20

Silver S (1996) Bacterial resistances to toxic metals - a review. Gene 179:9-19

Silverman MP, Lundgren DG (1959) Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*: An improved medium and a harvesting procedure for securing high cell yields. J Bacteriol 77:642-646

Sklodowska, Matlakowska R (1997) Influence of exopolymers produced by bacterial cells on hydrophobicity of substrate surface. Biotechnol Technol 11:837-840

Suzuki K, Wakao N, Kimura T et al (1998) Expression and regulation of the arsenic resistance operon of *Acidiphilium multivorum* AIU 301 plasmid pK301 in *Escherichia coli.* Appl Environ Microbiol 64:411-418

Tsai KJ, Hsu CM, Rosen BP (1997) Efflux mechanisms of resistance to cadmium, arsenic and antimony in prokaryotes and eukaryotes. Zool Stud 36:1-16

Tuovinen OH, Bhatti TM, Bigham JM et al (1994) Oxidative dissolution of arsenopyrite by mesophilic and moderately thermophilic acidophiles. Appl Environ Microbiol 60:3268-3274

Wilkie JA, Hering JG (1996) Adsorption of arsenic onto hydrous ferric oxide: effects of adsorbate/adsorbent ratios and co-occurring solutes. Colloids and Surfaces A: Phys EngAspects 107:97-124

Xu C, Shi W, Rosen BP (1996) The chromosomal arsR gene of *Escherichia coli* encodes a trans-acting metalloregulatory protein. J Biol Chem 271:2427–2432