The results obtained during the course of the present investigation could be briefly summarized as follows:

The present work was initiated by isolating arsenite and arsenate resistant bacteria from the arsenic contaminated and non contaminated samples procured from different environmental ecosystems. A total of 131 heterotrophic arsenite resistant and 16 arsenate resistant bacterial cultures were isolated. Ten autotrophic ferrous oxidizing isolates were also isolated from HGM reactor samples. From the isolated 131 heterotrophic cultures 8, 6, 2, 1 and 1 isolate were resistant to 140, 160, 180, 200 and 400 mM of arsenite respectively. From 16 arsenate resistant bacteria, two isolates were resistant to 100mM of arsenite. Isolate RP 15 isolated from Reliance petrochemical effluent sample was found to be most promising isolate as it was able to resist 400 mM of arsenite.

As the main aim of the studies was on detoxifying arsenic by oxidizing arsenite to arsenate further work was carried on arsenite resistant bacteria. After extensive screening of culture on the basis of their resistant towards arsenite 13 different heterotrophic isolates were selected from different ecosystems.

All these 13 isolates were thoroughly studied for their characterization and identification. These isolates were also able to use arsenate, selenate and selenite as electron donor in absence of arsenite and in presence of oxygen as an electron acceptor. However, none of the isolate was able to use nitrate or nitrate as electron acceptor.

Out of these thirteen heterotrophic isolates seven isolates and one autotrophic isolates were identified on the basis of 16S rDNA sequencing. The seven isolates HGM 1, HGM 28, HGM 32, HGM 38, RP 15, KG 36, KG 38 were identified as *Brachybacterium conglomeratum* DV1, *Microbacterium* sp. DV 2, *Exiguobacterium* sp. Jl-4, *Citricoccus* sp. SRHGAs 38, *Oceanobacillus* sp. RP 15, *Bacillus* sp. KG 36 and *Pseudomonas stutzeri* KG.
38. Where as autotrophic isolate HGM 8 was identified as *Acidithiobacillus ferrooxidans*. Isolate *Citricoccus* sp. HGM 38 and *Oceanobacillus* sp. RP 15 was found to be a novel strain on the basis of 16S r DNA sequencing.

Isolate HGM 8 was able to tolerate 200 mM of arsenite and arsenate separately. HGM 8 was also resistant to 60, 80 and 60 mM of copper, zinc and nickel respectively and 1% of NaCl. Even in the presence of 50mM of arsenite this culture showed 6% inhibition and $K_s$ was 8 and 12.5 g/L respectively. The observed optimum pH and temperature was 1.8 and 30°C respectively.

Isolates RP 15 and KG 38 were able to oxidize >99.9 % of 1mM arsenite where as isolate RP 4, RP 8, KG 36 and KG 42 were able to oxidize 20.5, 28.3, 22.8 and 20.5 % of arsenite respectively. Isolate KG 38 and RP 15 showed 80 fold better oxidation in comparison to other isolates.

Isolate RP 15 was also found to grow as chemolithoautotroph, the pattern of growth in the presence of arsenite as energy source was observed. The obtained $V_{max}$ value for KG 38 and RP 15 were 2.83 and 3.16 respectively. Where as $K_s$ was same for both the isolates of 1.2 mM. RP 15 was able to oxidize 20 % of 1 mM arsenite in minimal medium. KG 38 was not able to oxidize arsenite in minimal medium. Both the isolates were able to oxidize arsenite in present of any organic source; yeast extract was preferable choice for RP 15 and peptone for KG 38.

The optimum pH for arsenite oxidation was pH 7. The arsenite oxidation was inhibited in presence of fumarate as a carbon source, where as in presence of acetate, citrate and lactate as carbon source >99.9 % of 1 mM arsenite was oxidized within 120 h of incubation. When tested for effect of manganese 40 ppm of manganese was found to be optimum concentration for arsenite oxidation.
A yellow precipitate formed after arsenite oxidation by RP 15 was confirmed as precipitate of arsenite, by EDX analysis. EDX revealed the relative arsenic composition of the precipitated samples to be 16.93% and Na to be 75%.

EDX and ICP analysis confirmed existence of arsenic, sulphur and iron suggesting the ore to be an arsenopyrite ore. The concentration of ferrous, sulphur and arsenic was 20.1, 1.4 and 7.5% respectively. All the four isolates were able to oxidize 40 g/L of pyrite, 20 g/L refractory gold concentrate and 100 g/L of refractory gold ore. HGM 10 showed 3.1, 2.0, 3.2, 5.8 and 3.5% better arsenopyrite oxidation than other isolates studied.

When previously adapted cultures were tested the maximum iron leached was 49.5, 51.6, 52.0, 53.8 and 58.6% with 20, 40, 60, 80, and 100 g/L pulp density by isolate HGM 10 respectively. Whereas isolate HGM 5 was slow in comparison to HGM 10 with 42.8, 44.1, 44.2, 45.2 and 46.4% with 20, 40, 60, 80, and 100 g/L pulp density respectively.

Culture HGM 10 was found to be most promising for refractory gold ore leaching as the isolate was fastest of all the four studied isolates also isolate HGM 8 was able to resist 6000 mg/L of arsenic liberated during the leaching operations from 100 g/L pulp density.

To remediate arsenic contaminated waste water, typical contaminant water was formulated in the laboratory for these studies. Arsenic was reported to be absorbed on iron precipitates, keeping this theory in mind a packed bed column was inoculated with *Pseudomonas stutzeri* KG 38, as KG 38 was able to precipitate iron and biofilm was developed to treat the waste water. The treated water complied with the statutory limits prescribed by pollution control board. It was found that within 90, 120 and 180 minutes of contact 30, 60 and 120 μg/L of arsenic was removed.
The process developed in the presence investigation has distinct advantages such as, no chemical additives are required, production of low volume of sludge, easy to operate and maintain. Thus, the process has the potential of becoming an economical and reliable alternative for treatment of arsenic contaminated water.

*Acidithiobacillus ferrooxidans* HGM 8 which was able to grow between pH range 1.5-2.2 and 10 mg/L arsenite was removed with oxidation of 20g/L of ferrous iron up to 15th cycle and 10 mg/L of arsenite was removed with oxidation of 40 g/L of ferrous iron after 29th cycle. The present work shows that the biotic co precipitation of As (III) and Fe (III) efficiently removes As(III) from heavily contaminated waters.

The importance of the investigations carried out could be described as follows:

- Bacterial diversity of fourteen ecosystems has been studied, which could be further studied to know the relevance of each genus separately and role of these isolates on arsenic transformation in the ecosystem.

- Two novel isolates were found during the course of study which could be further explored. One isolate was able to grow in presence on 400mM of arsenite which is the first ever report in World, the isolate could be further explore to understand the arsenic resistant genes.

- *Acidithiobacillus ferrooxidans* HGM 10 strain was developed for biooxidation of refractory gold ore which could be useful for remediation of arsenic contamination in environment due to cyanidation process generally used for pre oxidation of ore.

- A laboratory scale microbiological process for the treatment of arsenic containing groundwater developed using *Pseudomonas stutzeri* KG 38.
The process was found to be highly efficient and may provide a useful approach for remediating arsenic contamination in ground water.

- The bioremediation technique explored in the present study for treatment of arsenic containing acid mine water has the process efficiency of > 99.9% with arsenic concentration of 10 mg/L making it an ideal alternative for treatment of heavily contaminated water.

In the light of the above facts the present work tends to the advancement of knowledge and it is hoped that these studies would pave a way for future research. The studies highlight the utility of microbial technology for environment pollution.
Interactions of Acidithiobacillus ferrooxidans with heavy metals, various forms of arsenic and pyrite

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Abstract. An arsenic resistant ferrous iron oxidizing bacterium Acidithiobacillus ferrooxidans (GenBank no. EF010878) was isolated from reactor leachate. The reactor leachate showed extreme environmental parameters. Ferrous iron concentrations of more than 60 g/L were found to be inhibitory in the presence and absence of arsenite. Ks values of 12.5 and 8.0 g/L ferrous sulphate and Vmax of 0.124 and 0.117 g/L/h/0.8 mg of protein were found in the presence and absence of arsenite respectively. At 14.9 g/L of arsenite and arsenate the culture showed 26.8 and 59.7 % ferrous iron oxidizing activity respectively. Amongst the metals studied, copper was found to be more toxic as compared to nickel and zinc. In the presence of 3.51 g/L nickel or 4.68 g/L zinc, about 30 % biooxidation activity was registered. In the pyrite oxidation study 87, 67 and 64 % of pyrite oxidation was found and 2.02, 3.19 and 5.96 g/L total iron was solubilized with 5, 10 and 20 g/L of pyrite respectively. The isolate was also able to oxidize refractory arsenopyrite gold ore and 0.531 g/L of arsenic was solubilized along with 0.872 g/L of soluble total iron. During this period the numbers of planktonic bacteria increased from 2.4 x 10^6 to 1.0 x 10^8 cells/mL.

Introduction

Bioleaching of minerals is a very complex process, which involves biological, mineralogical, electrochemical and engineering factors [1]. Amongst all these, the microbiology of this process is an very important aspect [2]. Several bacterial species are known to be able to grow by oxidation of sulphidic ores [3]. Acidithiobacillus ferrooxidans, Acidithiobacillus caldus, Leptospirillum ferrooxidans, Leptospirillum ferrithiophilum and Acidiphillum sp. etc. are few important microorganisms involved in the biological oxidation and solubilization of reduced mineral species and are still in the center of research in bioleaching research [4,5]. These bacteria are able to grow in a simple defined mineral medium, using CO₂ as carbon source and ferrous ion, sulphur or reduced sulfur compounds as energy sources [6,7].

The objective of this work is to examine the kinetics of ferrous iron oxidation and the effect of 1.49 g/L of arsenite on the kinetics. Interactions of heavy metals like copper, nickel and zinc on the ferrous iron oxidation rate is also examined. The effect of pyrite and arsenopyrite is also studied.

Materials and Methods

Bacterium and growth conditions. A strain of Acidithiobacillus ferrooxidans was isolated from leachate of refractory gold concentrate reactor established at Hutti Gold Mine situated at Karnataka, India. The cells were incubated at 30°C in 9K liquid medium with 20 g/L ferrous sulphate instead of 44.4 g/L in the original medium adjusted to pH 1.8 with 10% sulphuric acid [8]. Culture was grown in 200 mL medium in 500 mL Erlenmeyer flasks and kept on rotary shaker and mixed at 150 rpm.

Identification of the culture. The bacterium was identified by 16S rDNA sequencing by standard techniques. The 902-nucleotide sequence was submitted to DDBJ/EMBL/GenBank database under accession no EF 010878.
Iron oxidation measurement. Iron oxidation rate was studied by measuring the conversion of ferrous iron to ferric iron. For the kinetics studies FeSO₄ was added in different concentrations: 1.25, 2.5, 5, 10, 20, 40, 60, 80 and 120 g/L with and without arsenite.

Resistance to heavy metals. Heavy metal resistance of the bacterium was studied using 9K medium supplemented with 20 g/L of ferrous sulphate as source of energy and sodium salt of arsenite (NaAsO₂) and arsenate (NaHAsO₄), copper sulphate (CuSO₄), nickel sulphate (NiSO₄) and zinc sulphate (ZnSO₄) was added as a source of metals. Growth of the isolated strain was measured in terms of ferrous iron biooxidation.

Bioleaching studies. Arsenopyrite leaching experiments were carried out in 500 mL Erlenmeyer flasks containing 200 mL of 9K medium pH 1.5 ± 0.2 where ferrous sulphate was replaced with 40 g/L (w/v) refractory gold ore and 5, 10 and 20 g/L of pyrite. The reaction flasks were incubated at 30 ± 0.2 °C on rotary shaker at 150 rpm.

Chemical Analysis
Iron analysis. Ferrous iron was measured at regular intervals of time by potassium dichromate titrimetric method. Total soluble iron was measured in the leaching solution by Systronics UV-Vis 119, spectrophotometrically using 1,10 phenanthroline method [9].

Total arsenic analysis. The progress of bacterial oxidation of arsenopyrite was determined by measuring the concentration of total soluble arsenic in the leaching medium by double beam Atomic Absorption Spectrophotometer (Elico SL 194).

pH and redox potential. pH and mV were studied using a standard pH meter by combined glass and platinum-SCE couple electrode respectively (Systronics pH system 361).

Cell count and growth rate. Bacterial cell numbers in the leaching medium was determined by direct microscopic count using Peteroff-Houser chamber.

Results and Discussion
An arsenic resistant strain of *Acidithiobacillus ferrooxidans* (GenBank accession no. EF010878, 902 nucleotide sequence) was isolated from Hutti gold mine reactor leachate of pH 1.5, redox potential 520 mV, ferrous iron, total iron, arsenic and total dissolved solid of 0.1144, 8.242, 2.17 and 2.3 g/L respectively. The influence of arsenite and arsenate on ferrous iron oxidation by this organism is shown in Fig. 1. As can be seen from the data As (V) was found to be less toxic as compared to As (III). Iron oxidation was not inhibited even in the presence of 7.45 g/L of arsenate whereas 4.48 g/L of arsenate showed 3.3 % inhibition. When 14.9 g/L of arsenate was supplemented, 40 % of inhibition was observed in comparison to 73 % inhibition in 14.9 g/L arsenite containing medium.

The effect of Cu, Zn and Ni was carried out on ferrous iron biooxidation and the results are shown in Table 1. When 1.23 ± 0.07 g/L of Zn, Cu and Ni were studied, copper was found to be most inhibitory whereas Zn was least inhibitory. When results of 3.7 ± 0.2 g/L of Cu, Zn and Ni were compared, copper was again found to be the most toxic metal, inhibiting 94 % activity where as Zn showed minimum toxicity with only 42 % inhibition.

<table>
<thead>
<tr>
<th>Concentration of metal [g/L]</th>
<th>Biooxidation activity [%]</th>
<th>Inhibition [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu</td>
<td>Zn</td>
</tr>
<tr>
<td>0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>1.23 ± 0.07</td>
<td>93.6</td>
<td>99.3</td>
</tr>
<tr>
<td>2.45 ± 0.15</td>
<td>70.5</td>
<td>72.9</td>
</tr>
<tr>
<td>3.70± 0.20</td>
<td>5.8</td>
<td>57.8</td>
</tr>
<tr>
<td>4.90 ± 0.25</td>
<td>0.0</td>
<td>29.8</td>
</tr>
</tbody>
</table>
All the metals showed higher toxicity in comparison to arsenic, which is definitely due to the presence of arsenic in the habitat from where the bacterium is isolated. Although the isolate was not exposed to copper, nickel and zinc it showed resistance towards these metals. The organism showed 6 h of lag phase in the presence of all the metals studied in all the concentrations being studied whereas the lag phase is just 4 h in the absence of these metals. In the absence of metals 0.40 ± 0.5 g/L ferrous iron was oxidized in 6 h. The organism showed iron oxidation in the presence of as high as 14.9 g/L of arsenite and arsenate and no inhibition up to 3.0 g/L of arsenic in the medium [Fig. 1].

Ferrous iron oxidation in the presence and absence of 1.49 g/L of arsenite is shown in Fig. 2. In the presence of arsenite, the iron oxidation rate was 18.5, 10, 9.5 and 3 mg/L/h higher in the medium containing 2.5, 5, 10 and 20 g/L of ferrous sulphate, respectively, than in the absence of arsenite. However, beyond 50 g/L of ferrous sulphate in the medium there was a decrease in the iron oxidation rate in the presence and absence of arsenite, which is due to the inhibitory effect of substrate concentration. The ferrous iron biooxidation kinetic data are shown in Table 2. The Ks value was decreased from 12.5 g/L to 8 g/L ferrous sulphate when 1.49 g/L of arsenite was added to the medium which may indicate that the presence of 1.49 g/L of arsenite increased the affinity of Acidithiobacillus ferrooxidans towards ferrous sulphate.

<table>
<thead>
<tr>
<th>Medium</th>
<th>( V_{\text{max}} ) [g/L/h/0.8 mg protein]</th>
<th>( \frac{1}{2} V_{\text{max}} ) [g/L/h/0.8 mg protein]</th>
<th>Ks [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>With 1.49 g/L arsenite</td>
<td>0.117</td>
<td>59</td>
<td>8</td>
</tr>
<tr>
<td>Without arsenite</td>
<td>0.124</td>
<td>62</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Oxidation of pyrite was also studied up to 20 g/L concentration and results are shown in Fig. 3a and 3b. The oxidation started from the very first day and the oxidation rate remained almost constant till 19th day of incubation. Thereafter, there was a decrease in the iron oxidation rate at all concentrations of pyrite, which indicated the beginning of the stationary phase. Pyrite oxidation of 87, 67 and 64% and 2.02, 3.19 and 5.96 g/L iron solubilization was found in medium from 5, 10 and 20 g/L of pyrite respectively.
Fig. 3a. Biosolubilisation of ferrous iron from pyrite. Pulp density is given in percent

Fig. 3b. Biosolubilisation of total iron from pyrite. Pulp density is given in percent

The isolate was also studied for its interactions with refractory arsenopyrite gold ore and it was found to solubilize 0.531 and 0.87 g/L of total iron and arsenic respectively. This was correlated with an increase in the planktonic cell count from $2.4 \times 10^6$ to $1.0 \times 10^8$ cells/mL.

Acknowledgment

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References