CHAPTER FOUR:

DISCUSSION
At the beginning of the XXI century, bioleaching occupies an increasingly important place among the available mining technologies. The situation is now quite different than what it was 25 years ago, when the first international meeting on the subject took place in Socorro, New Mexico (Murr et al. 1978), initiating the now traditional International Biohydrometallurgy Symposia. Today bioleaching is no longer a promising technology but an actual economical alternative for treating specific mineral ores.

Examination of the current large-scale bioleaching operations reveals that a large number of them are located in developing countries. This is not purely accidental but the necessary result of two important factors: for one thing, many developing countries have significant mineral reserves and mining constitutes one of their main sources of income; on the other hand, bioleaching is a technique especially suitable for developing countries like Iran and India because of its simplicity and low capital cost requirement (Acevedo, 2002). For example by the year 2000 more than 50% of the world copper production was recovering from this phenomena (Table 4.1)

In light of the above it can be seen the importance of bioleaching application for extracting low grade mines in developing countries. One such developing country which has the huge source of metal ores is Iran. The main metal ores located in Iran are copper and iron and after these two metals zinc and lead mines are having large reserves in the country. Currently the bioleaching process is applied for extraction of copper from Sarcheshmeh mine in Kerman but till this day there is no report of bioleaching application
for zinc and lead recovery in Iran at industrial scale. Among the zinc and lead mines there is the Angoran mine in Zanjan, northwest of Iran, and Koshk and Mehdibad in Yazd, central Iran. According to Iranian Ministry of Mining and Industry, reservoirs of Angoran mine is about 17 million tonne and it is in the form of metal oxide. However oxide stones do not make any problem during extraction by non biological methods hence this mine is not considered in this study. But as we said before Koshk and Mehdibad mines are having 120 and 180 million tone capacity respectively and most of these reservoirs is in the form of metal sulfides. And we have selected Koshk mine for our study to find out technology to apply bioleaching process for this mine to have highest efficiency.
Table 4.1 World copper production (thousand of tones)\(^a\)

<table>
<thead>
<tr>
<th>Country</th>
<th>1998</th>
<th>1999</th>
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<tbody>
<tr>
<td>Australia</td>
<td>607.0</td>
<td>711.0</td>
<td>829.0</td>
</tr>
<tr>
<td>Canada</td>
<td>705.8</td>
<td>620.1</td>
<td>634.2</td>
</tr>
<tr>
<td>Chile</td>
<td>3,686.9</td>
<td>4,391.6</td>
<td>4,602.0</td>
</tr>
<tr>
<td>China</td>
<td>486.0</td>
<td>520.0</td>
<td>588.5</td>
</tr>
<tr>
<td>Indonesia</td>
<td>809.1</td>
<td>790.3</td>
<td>1,005.5</td>
</tr>
<tr>
<td>Mexico</td>
<td>384.3</td>
<td>381.2</td>
<td>344.6</td>
</tr>
<tr>
<td>Peru</td>
<td>483.3</td>
<td>563.3</td>
<td>553.9</td>
</tr>
<tr>
<td>Poland</td>
<td>436.2</td>
<td>463.6</td>
<td>463.2</td>
</tr>
<tr>
<td>Russia</td>
<td>518.0</td>
<td>510.0</td>
<td>510.0</td>
</tr>
<tr>
<td>United States</td>
<td>1,860.0</td>
<td>1,601.0</td>
<td>1,480.0</td>
</tr>
<tr>
<td>Zambia</td>
<td>378.8</td>
<td>271.0</td>
<td>320.1</td>
</tr>
<tr>
<td>Others</td>
<td>1,932.7</td>
<td>1,915.9</td>
<td>1,912.7</td>
</tr>
<tr>
<td>World</td>
<td>12,288.1</td>
<td>12,712.0</td>
<td>13,243.7</td>
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Oxides and silicates are the most frequently encountered minerals but only few of them bear valuable metals that can or need to be extracted by bioleaching techniques. However, metal sulfides are a very important class of compounds because some of the base metals occur mainly in sulfide ore deposits.
It is pertinent to note that producing concentrate is economical if the mineral is high grade but in zinc and lead mines sometime there are locations that have mineral of very low grade and producing concentrate from such an area is not economical and has to be considered as waste. But the biological process can solve this problem because the bacterial cell can easily ignore the rigors of metals and convert the low grade metals to the soluble form and extract them. This is one of the main advantages of bioleaching and is the reason why we have selected this method for extracting zinc and lead from this mine. In this study we have also the same problem, because the concentration of zinc and lead in this mine is 3.1 and 1.2 % respectively from which we could extract more than 90% of zinc by using of isolated bacterium (D.F.1). This is therefore the evidence that microorganisms can ignore the wastes which surround the metals and easily can attack the target metal. The second reason of selection the bioleaching method for extracting zinc and lead was the composition of mine which is sulfide base and one of the main problems of such kind of mines is to contaminate environment while using other methods, so by using bioleaching we can avoid the environmental contamination. The third advantage that established the good ability of application of bioleaching for this mine is the presence of indigenous At. ferrooxidans strain which is one of the best bacteria in bioleaching process. The good range of temperature at the mine location during the year (30-37 °C) can provide the good habitat for bacterial activity whereas in the Angoran mine the average temperature is 10-18 °C during the year.
Physicochemical properties of the minerals subjected to dissolution and of the reaction products and reaction kinetics are of great importance for the economy of a process. Hence we have studied the physicochemical and composition of mine sample at the first stage of our study for better planning of further study by XRD and XRF analysis.

In the present study we have used different media for isolation and maintenance of acidophilic microorganisms. The results showed that 9-K medium is the best medium among the other media for cultivation and maintenance of acidophilic microorganism and specially *At. ferrooxidans*. These results are comparable with the report of Gomez et al. (1999), where they have investigated the influence of growth media using five different media formulations [9K (Silverman and Lundgren, 1959), T&K (Tuovinen and Kelly, 1973), ES (Norris and Barr, 1985), Leathen (Leathen et al., 1956) and Norris (Gomez et al. 1999)], with varying the concentrations of salts and they have observed significantly higher extraction of metals (Zn, Cu and Fe) within 9K medium than others.

The 16S ribotyping RNA results was indicated that the isolate is the new strain of *At. ferrooxidans*. *Acidithobacillus* is a genus of gram negative, obligately or facultatively chemolithoautorophic (or mixotrophic) bacteria which occur e.g. in soil, marine muds, mine drainage and hot springs. Cells are typically polar flagellated rods, ca. 0.5 X 1.0-3.0 μm.
Biomining bacteria belonging to this genus were previously included in the genus *Thiobacillus*. As a result of 16S rRNA sequence analysis, it became clear that the genus *Thiobacillus* (as described prior to 2000) included sulfur-oxidizing bacteria that belonged to α-, β-, and γ-divisions of the Proteobacteria. To solve this anomaly, the genus *Thiobacillus* was subdivided (Kelly 2000) and a new genus, *Acidithiobacillus*, was created to accommodate the highly acidophilic members of the former genus. These members include *At. ferrooxidans* (previously *T. ferrooxidans*), *At. thiooxidans* (previously *T. thiooxidans*), and *At. caldus* (previously *T. caldus*). Phylogenetically, the genus *Acidithiobacillus* is situated very close to the branch point between the β-, and γ-subdivisions of the Proteobacteria (Lane et al. 1992, Rawlings. 2001). These bacteria appear to be ubiquitous and have been isolated from sites that provide a suitable environment for their growth (such as sulfur springs and acid mine drainage) from many regions throughout the world.

*At. ferrooxidans* was the first bacterium discovered that was capable of oxidizing minerals (Colmer and Hinkel, 1947). Although typical *At. ferrooxidans* isolates have a genomic GCC mole ratio of 57%–59%, they have been found to belong to at least four DNA-DNA hybridization similarity subgroups (Harrison et al. 1982). The percentage DNA-DNA similarity between some of these subgroups is sufficiently low (10%–50%) for them to be considered separate species. In addition, a strain called *At. ferrooxidans* m1 has a GCC of 65%, is a β-proteobacterium, and belongs to a different, as yet unnamed, genus. Nutritionally, a typical *At. ferrooxidans* isolate are considered to be obligate autotrophs.
They are also able to grow on formic acid, provided it is added in small quantities (Pronk et al. 1991). Because formic acid is a C₁ compound, this property is not inconsistent with autotrophy. *At. ferrooxidans* is able to use either ferrous iron or a variety of reduced inorganic sulfur compounds as an electron donor. It is preferentially aerobic but also able to grow using ferric iron as an electron acceptor, provided that it has a reduced inorganic sulfur compound as an electron donor (Pronk et al. 1991).

For many years *At. ferrooxidans* was considered to be the most important microorganism in biomining processes that operate at 40°C or less. However, in some of the older studies techniques were used that were less sophisticated than those available since the early to mid-1990s. It is currently understood that *At. ferrooxidans* is not favored in situations in which the ferric iron content is much higher than the ferrous iron (high redox potential), such as is found in continuously operating stirred tank reactors operating under steady-state conditions (Rawlings et al. 1999). Nevertheless, *At. ferrooxidans* may be the dominant bacterium in some dump and heap leaching environments including those involved in uranium and copper oxide/sulfide leaching, especially if the ferrous iron concentration in solution is high (>5 g/liter) (Pizarro et al. 1996). *At. ferrooxidans* is capable of rapid growth relative to many biomining bacteria and is generally favored within the temperature range 20°–35°C and pH 1.8–2.0, providing the ratio of soluble ferrous iron to ferric iron is high. It is also possible to adapt *At. ferrooxidans* so that they are able to grow at pH values outside their optimal range (Vian, et al. 1986). As may be expected from bacteria growing in a mineral-rich environment, these bacteria are
remarkably tolerant to a wide range of soluble metal ions [reviewed in (Leduc and Ferroni. 1994). *At. ferrooxidans* is recognized as being responsible for the oxidation of iron and inorganic sulfur compounds in areas such as mine tailings and coal deposits where these compounds are abundant. *At. ferrooxidans* is acidophilic and has a physiology which is well suited for growth in an inorganic mining environment.

Bioleaching of sulfide minerals is naturally a complex process since chemical and microbiological reactions occur concomitantly within the system. The strains of bacteria used as the mediator of oxidative reactions themselves establish optimum conditions under which they optimally grow. The optimum growth conditions could be adjusted to maximize the rate and extent of metal dissolution from sulfide ores/concentrates (Bosecker, 1997). There are a number of factors controlling the activity of bacteria with the resultant oxidation of substrate environment in order to optimize bioleaching performance. In this study the parameters including pH, initial iron concentration, ammonium concentration, temperature and magnesium concentration have been studied

pH of the growth medium significantly affects the growth and activity of acidophilic microorganisms. Amaro *et al* in 1991 reported that *At. ferrooxidans* responds to external pH changes by regulating the synthesis of several of its cellular components. The H\(^+\) ion is in fact vital for acidophilic microorganisms since bacteria utilize it as a proton source for the reduction of O\(_2\) (Apel & Dugan, 1978). In general, the acidophilic bacteria, *At. ferrooxidans*, are unable to initiate growth on Fe(II) at a pH greater than 3.0. The growth of
the bacteria is usually initiated at a very low pH range and as the growth continues the pH of the medium also increases without affecting the bacterial activity (Apel & Dugan 1978). The bioleaching efficiency by mesophilic and moderate thermophiles tended to increase with decreasing acidity (pH 1.0-2.0) (Deveci et al. 2004). In this study also we have found the pH range of 1.4 - 1.6 for optimum growth and activity of bacterium. Similarly, an optimum pH value of 1.6 has also been cited in literature for growth of *At. ferrooxidans* (Brierly et al., 1978 and Malhotra et al., 2002). These results also were consistent with the optimum pH 1.5 to 2.3 for bacterial leaching/oxidation of most sulphide minerals/ferrous iron reported (Torma, 1977). Several authors have reported that the bacterial activity is enhanced many folds by adaptation of the bacterial strain to the growth medium maintained at a suitable pH (Elzeky & Altia 1995, Menon & Dave 1995, Barr & Jordan 1992). In another similar study by Deveci et al. (2003) have studied the effect of pH at range of 1-2 on growth of *At. ferrooxidans* and they found pH 1.6-1.8 as the optima for bacterial growth.

In our study we have observed that increase in the pH to 2 decreased the efficiency of bacterium for the conversion of ferrous to ferric which maybe due to ferric precipitation on bacterial surface, which hinders the diffusion of protons (Meruane and Vargas 2003). The optimum pH range may be identified as that the optimum growth of bacteria and the most efficient oxidation of minerals are attained (Deveci, et al. 2004) but in our study results indicated that optimum pH for growth (1.4) is different from the same for activity (1.6), this can be demonstrated that although at pH 1.4 we have more number of cells due to the
low pH there is a prolonged lag phase at pH 1.4 in comparison to 1.6 and ultimately the oxidation of iron could be started faster at a better rate.

Another factor that we have studied in this project was the effect of initial iron concentration. Fe (II) is one of the main energy sources for bacterial growth so finding the best required concentration of iron for growth and activity of bacteria is important. A low Fe(III) concentration enhances the oxygen uptake by the acidophilic microorganism. At a higher concentration, Fe(III) competitively inhibited ferrous iron oxidation by *At. ferrooxidans*, (Nyavor et al., 1996). Curtchet et al. (1992) have reported that in the absence of other sources of energy the presence of soluble Fe(III) inhibits the growth of *At. ferrooxidans*.

It has been observed that at high concentration of Fe\(^{2+}\) there is a prolong lag phase (24-28 h) but at a low concentration of Fe\(^{2+}\) the lag phase was reduced to 10-15 hours which showed the duration of the lag phase depends on the initial concentration of ferrous iron. The same results were reported by Mousavi *et al.* in 2006. The maximum biooxidation rate of 0.24 g/l h was obtained at initial Fe\(^{2+}\) of 4 g/l and is comparable with previous reports; Nemati and Harrison (2000) and Mousavi *et al.* (2006), which have reported 0.2 g/l h and 0.088 g/l h respectively in same concentration of initial iron. The reason of decrease in efficiency of iron oxidation at concentrations above 4 g/l is because of the precipitation of jarosites, that the formation of it increases in higher concentration of iron and affect the biooxidation rate of bacteria by covering their surface (Hiroyoshi et al
1999). We have also observed that the biooxidation of ferrous iron proceeded with the same rate of growth rate of bacteria and this result was expected (Mousavi et al. 2006). Hence, we can see that the formation of precipitated iron compounds depends on pH, Eh and concentration of Fe(III). Therefore, by carrying out the leaching experiment under control conditions, the extent of precipitation can be minimized (Canterford et al. 1985).

One of the important features of hydrometallurgical operations is the temperature dependency of dissolution process such that the rate and extent of dissolution of sulfides increase with temperature. In effect, these processes establish a certain temperature range beyond which the rise in the rate of dissolution with temperature is not commensurate with the decrease in the oxidation activity of bacteria. In this respect, the optimum temperature for the bioleaching operations may well be defined as the temperature at which the rate of biooxidation of desired minerals is maximized. Deveci et al. (2004). Here we have studied the effect of temperature at the range of 25 to 40 °C on growth and activity of bacterium. The optimum temperature for growth of this bacterium was found 35 °C, which is similar to other reports available in literatures (Okereke and Stevens 1991; Smith et al. 1988; Lacey and Lawson 1970; Malhotra et al. 2002). Also these results can be compared with the reports of Gomez in 1999, who have reported 31°C as optimum temperature for *At. ferrooxidans*.

Decrease in the oxidation activity of the bacterium at temperature beyond the optimum may be attributed to the likely denaturation of proteins involved in oxidation system of
bacteria (Torma, 1977). Therefore we can conclude that for a specific bacterial strain there is a specific optimum growth temperature and a specific temperature at which the oxidation efficiency is maximum and accordingly, bioleaching systems require intimate control of operating temperature to maintain optimum range for the activity of bacteria.

A minimum concentration of salts in the liquid medium is essential to maintain the desired level of bacterial activity. A typical nutrient solution is mainly composed of nitrogen introduced as an ammonium salt, phosphorus as a potassium salt of phosphoric acid, magnesium as magnesium sulfate and other salts. In our investigation the effect of ammonium and magnesium salts were studied. Increase in the concentration of nitrogen resulted in increase of cell density and maximum growth was observed at 3 g/l (NH$_4$)$_2$SO$_4$. These results are on expected lines (Gomez et al., 1999). In another study also Deveci et al. (2003) have been indicated that 9k medium with 3 g/l ammonium sulfate was the best media for growth and activity of _At. ferrooxidans_ in comparison by other media with less concentration of ammonium sulfate. The possible reason for lower oxidation activity of bacterium at 4 g/l (NH$_4$)$_2$SO$_4$ than 3 g/l could be the possible precipitation of phosphate, potassium and ammonium as a jarosites complex due to excess concentration of salt in the medium. This problem is prominently encountered while working with 9-k liquid medium (Sand et al., 2001).

With relation to the effect of Mg$^{2+}$ concentration, the highest growth and activity of bacterium was occurred at 20 mg/l Mg$^{2+}$. This less requirement for Mg$^{2+}$ is comparable
with the reports of Mousavi et al., (2006) and Malhotra et al., (2002) where they have been reported 20 and 3 mg/l Mg\textsuperscript{2+} for optimum growth of \textit{At. ferrooxidans} respectively. Such a low magnesium ion requirement can be attributed to the slow growth of the culture (Malhotra et al., 2002). In one study, the effect of using tap water (TW) and double distilled water (DDW) has been studied on bioleaching process and it was observed that the better bioleaching activity of microorganism in TW than in DDW and is most likely due to the presence of anions and cations at relatively high concentration in TW i.e. Mg\textsuperscript{2+} and PO\textsubscript{3}\textsuperscript{3-} (Deveci et al., 2004). Hence, it showed the essential of Mg\textsuperscript{2+} for better biooxidation.

One of the main objectives of the present investigation was to evaluate the ability of isolate for bioleaching of zinc and lead from ore sample. For this propose we have studied the ability of bacterium DF.1 at two different scales. One of these scales was laboratory scale where we were studied the ability of bacterium in conical flasks at two different conditions (basic condition of 9K medium and optimal conditions which we were obtained before). Also we have examined the effect of different concentrations of initial iron on bioleaching ability of bacterium. Zinc sulphide bioleaching investigations had been carried out by some researchers (Pani, et al. 2003, Shi & Fang 2004, Shi et al. 2006, Deveci et al. 2004). Though the leaching effects reported were different in each study due to the distinct properties of the mineral specimens used in the various experiment (Shi et al. 2005). Most nature sphalerite contains traces of iron in solid solution with the temperature and chemistry of the environment (Lusk et al. 1993). High iron, zinc sulphide is often called
marmatite. The activation energies for leaching decreased with increasing Fe content of the sphalerite (Weisener et al. 2004).

In the present investigation at basic condition we had only 65% extraction of zinc while it was more than 86% at optimum conditions. Hence, these results confirmed, our results of the optimization process. These results also showed the importance of ferric ion on bioleaching process, as we had maximum extraction of zinc at optimum condition which we had maximum Fe(III) concentration.

4 g/l Fe(II) concentration was the optimum concentration for zinc and lead extraction. The reason that we had less extraction at higher concentrations of Fe(II) could be related to the composition of the mine. As this mine has more than 25% iron, it can affect the efficiency of zinc extraction at high concentration of initial iron which was added in the medium, because bacterial cells have an upper limit for tolerating iron and above that concentration is toxic. The results of the effect of initial iron concentration on zinc and lead extraction are in contrast to Deveci et al. (2004) report, where they have been reported the same amount of zinc extraction in the presence of 1-6 g/l Fe$^{2+}$ externally added but we used different amount and rate of extraction at different concentrations of Fe$^{2+}$.

In our study we had extraction of zinc and lead together but in another study, Deveci et al. (2004) has been reported more than 95% extraction of zinc while majority of lead (>98%) remains in the residue. Also the optimum pH of 1.6 for extraction of zinc and lead.
is one of the lowest optimum pH reported for zinc and lead extraction by *At. ferrooxidans*
and can compare with other reports (Deveci et al. 2004, Bayat et al. 2008). Results of the
present investigation revealed that maximum zinc extraction was at 4. g/l of Fe$^{2+}$ (over
86%). These results are similar to the results obtained by Shi et al. (2006), Deveci et al
(2004) and Liao and Deng (2004). But the amount of extraction of zinc in our study was
much more in comparison with the reports of Hossain et al. (2004) and Bayat et al. (2008)
where they have reported 74% and 35% zinc extraction respectively.

Data presented previously (Choi et al. 1993, Konishi et al.1992, Torma et al. 1970)
showed that bacterial leaching results in a much higher level of extraction of metal than a
sterile control (chemical leaching) under the same initial solution conditions and we have
obtained similar results. On the basis of these results, most of the researchers concluded
that the bacteria act as a catalyst for the leaching reaction by the direct mechanism.

After studying the zinc and lead extraction at laboratory scale, we have studied the
ability of the isolate at large scale inside the column bioreactor. The principal bioleaching
techniques in use for the treatment of sulfide minerals include stirred tank leaching
(Kinnunen et al., 2006), heap leaching and dump leaching (Renman et al., 2006), and
concentrate heap leaching (Sampson et al., 2005). Heap leaching provides both operation
and capital cost advantages. However, the use of heap leaching is limited to cases in which
the temperature can be maintained within the heap at a suitable temperature without
external heating (Sampson et al., 2005). From a process engineering standpoint, the
complex network of biochemical reactions encompassed in bioleaching would best be performed in reactors. The use of reactors would allow a good control of the pertinent variables, resulting in a better performance. Parameters such as volumetric productivity and degree of extraction can be significantly increased (Acevedo and Gentina, 1989; Acevedo 2000). The main limitation in the use of reactors in biomining is the very large amounts of run-of-mine ore that in most cases is to be treated. Recently, zinc sulfide investigations have been carried out by some researchers in column or in shake flasks (Mousavi et al., 2007, 2006; Deveci et al., 2004; Pani et al., 2003; Rodriguez et al., 2003). Leaching in column, with or without the recirculation of the leaching liquid, simulates percolation leaching because the conditions are very similar to those in the heap. Since, results obtained in the laboratory can be extrapolated, with slight correction, to the real situation they will help show whether bacterial leaching is possible under acceptable conditions (Lizama and Suzuki, 1989). In this sense, we might consider the columns as the heart of the heap, with the same degree of access for the leaching solution and the circulating gases in both. In other words, a column experiment simulates the flow or, at least, one of the possible paths of a liquid percolating through a mass of material by gravity.

In our investigation 450 l column bioreactor was used to study the bioleaching ability of our isolate in large scale. The size of the column used in this study was one of the largest in the world in case of zinc and lead bioleaching which has been reported earlier (Mousavi et al., 2007, 2006; Deveci et al., 2004; Pani et al., 2003; Rodriguez et al., 2003).
More than 40% increase in zinc and lead extraction was observed at optimal conditions which were obtained at laboratory scale experiments, in comparison with basic condition (non optimal). Hence, it showed the importance of process optimization of bioleaching condition even at this large scale. Another objective of this study was to investigate the effects of initial iron concentration of Fe(II) ion as well as initial pH of feed solution on the leaching of zinc and lead. The optimum pH of 1.6 and iron concentration of 4 g/l was confirmed our laboratory results and it showed that the results obtained in this study are trustable even for use at larger scale.

The reduction of zinc extraction at higher pH than 1.6 can be credited to Fe(III) precipitation which probably took place under the jarosite form that results to reduction of mass transfer on the cell surface by the formation of ferric iron precipitates (Pina et al., 2005; Malhotra et al., 2002; Mousavi et al., 2007). The possible reason of less extraction of zinc and lead at pH 1.4 was due to the slowing effect of low pH on the metabolism of microorganism and limitation of minimum pH for growth. It has been observed (Falco et al., 2003) that in bioleaching of Zn from concentrates, pH sharply decreases with time which results in suppression of bacterial activity, to avoid this abstruse during the experiment continuously the pH was maintained at initial pH by adding 1 N NaOH or 10 N H₂SO₄. It is known that Fe(II) oxidation by *At. ferrooxidans* decrease at a pH higher than 2. This is particularly due to the reduction of the mass transfer on the cell surface by the formation of ferric iron precipitations. However, in the case of the Fe(II) oxidation by *At ferrooxidans*, the proton is one of the reactants. Therefore, the decrease in the growth rate
with an increase in pH cannot be avoided (Mousavi et al., 2007). Higher bacterial growth rate were observed at the lower pH ranges (1.4 and 1.6). This fact is in keeping with the dynamic of bioleaching, whereby active bacteria account for the formation of oxidized chemical species in the solution; the oxidation being coupled to growth and thus to cell density. At the higher pH the free bacterial population started to decrease due to the attachment to the deposits (jarosite precipitation) (Pogliani and Donati, 2000).

Finally, it can be concluded that heap leaching is without question the largest scale application of microbial mineral leaching now practiced. The use of heap leaching, in which finely ground ore is agglomerated and placed on highly engineered heap, is now being considered for the biooxidation of refractory gold ores in which millions of tones of ore are treated. In the light of the above we can say that study the bioleaching of metals at large column reactor can be the primary study of heap leaching at larger scale as it gives us the real data near to the real situations. The future of bioreactors in mining appears promising. Gold biooxidation operations tend to increase in number and size in several countries the world over. The use of reactors will most probably extend to the bioleaching of other metals, such as copper. Currently studies are being carried on for the development of processes for the bioleaching of copper concentrates. The experience gained in the heap leaching of copper and in the biooxidation of gold concentrates is being used in these studies. The bioleaching of chalcopyritic copper concentrates in the next few years will constitute a big breakthrough in biomining. The application of these technologies to the processing of nickel, zinc, and other heavy metals may also become a reality in near future.
Micro-organisms require the presence of a number of metals that play essential biochemical roles such as catalysts, enzyme co-factors, activity in redox processes and stabilizing protein structures (Bruins et al., 2000). Metals may accumulate above normal physiological concentrations by the action of unspecific, constitutively expressed transport systems, whereby they become toxic. Intracellular metals can exert a toxic effect by forming coordinate bonds with anions blocking functional groups of enzymes, inhibiting transport systems, displacing essential metals from their native binding sites and disrupting cellular membrane integrity (Nies, 1999). There are five basic mechanisms that convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell; (2) enzymic conversion; (3) intra- or extracellular sequestration; (4) exclusion by a permeability barrier; and (5) reduction in sensitivity of cellular targets. In the last part of this investigation we have studied the level of tolerance to heavy metals among the bioleaching related organisms. Also we have studied the mechanism of bacterium to zinc and lead toxicity at biochemical level (proteomics). Studies on *At. ferrooxidans* from different mines, showed that this bacterium generally tolerate wide range of heavy metals (Touvinen 1971, Leduc 1994). Results of the present investigation are on similar lines. In case of some metals like Zn and Mn our isolates were highly resistant.

The resistance to 650 mM zinc and 700 mM manganese was observed, also high resistance to other metals like Ni, Co and Cu were obtained in the range of 50 – 200 mM. However, low resistance was observed with lead, chromium and mercury (10, 10 and 0.005 mM).
respectively). A similar study by Tuovinen et al. (1971) have shown that *At. ferroxidans* able to oxidize ferrous iron in the presence of high concentrations (10 g/l) of Zn, Ni, Cu, Co Mn and Al whilst Ag and anions of Te, As and Se were proved to have an inhibitory effect on the iron oxidation activity of bacteria at concentration of 50 – 100 mg/l. Resistance to 650 mM of Zn was one of the highest concentrations which has been reported earlier (Renata et al., 2005; Novo et al., 2000 and Tuovinen et al., 1971). It has been shown that the toxicity of Zn(II) to *At. ferrooxidans* depends on the growth substrate. One strain resistant to 153 mM Zn(II) while growing on Fe(II) was sensitive to 92 mM (0.6 mg l\(^{-1}\)) when growing on thiosulfate (Trevors et al., 1985). Kondratyeva et al. (1995) suggested zinc resistance is chromosomally encoded. Adaptation of the strain to increased levels of Zn(II) resulted in an increase in genome fragment size, suggesting increased copy numbers of the operon encoding the putative Zn(II) resistance genes.

The resistance of our bacterium to Ni and Cu can be compared with results obtained by Chisholm et al. (1998) indicating average of 160 mM Cu\(^{2+}\) and Ni\(^{2+}\) resistance among different strains of *At. ferrooxidans*. In another study Novo et al (2000) had reported resistance of *At. ferrooxidans* to 200 and 600 mM Cu and Ni respectively. For the mechanism of growth inhibition by Ni(II), it has been proposed that Ni(II) binds to the cell surface, where it inhibits the RISC enzymes sulfur dioxygenase and sulfite oxidase, and ultimately growth (Maeda et al., 1996; Nogami et al., 1997). Also differential protein expression of bacteria on expose to Ni(II) has been analyzed by Novo et al. (2000) but no resistance mechanisms have been characterized. Results of present investigation on Cu, Co
and Hg are matching with the results of Garcia et al. (1991) where it has been reported 50 mM and 0.005 mM MIC for Cu and Hg. Although *At. ferrooxidans* was shown to be the most resistant to Cu(II), virtually all of the acidophiles were found to be more resistant than *E. coli*. Das et al. (1997) found that *At. ferrooxidans* copper resistance was inducible and that the resistant strain extracts Cu(II) more rapidly compared to an unadapted strain. Cu(II) inhibition of growth and Fe(II) oxidation has also been demonstrated in *Sulfobacillus thermosulfidooxidans* subsp. asporogenes via competitive inhibition of Fe(II) oxidation (Vartanyan et al., 1990).

In regard to arsenate toxicity, it has been suggested that the toxicity of As(V) to microorganisms is due to replacement of phosphate in cellular processes, inhibiting a plethora of biological reactions. In metal-leaching biooxidation vessels, As(V) inhibition can be alleviated by increasing phosphate concentrations, likely counteracting the toxic action of As(V).

The growth profiles of the organism in the presence of various concentration of the metals were investigated in this investigation. The lag phase of isolate in the presence of different heavy metals was 40 to 50 h as compared to 15 h in the control. These were expectable results because Garcia et al (1991), Touvinen et al. (1985) and Renata et al. (2005) have been reported increase in the lag phase of *At. ferrooxidans* and *At. thiooxidans* growth when exposed to different heavy metals like cobalt, copper, zinc and mercury.
At the control conditions, 45 h of incubation was necessary for complete oxidation of ferrous iron by isolate whereas in the presence of heavy metals tended to increase between 90 h to 160 h. This prolonged growth was highest in the case of 650 mM Zn and 150 mM Ni and 50 mM Cu. These results are comparable with the report of Novo et al. (2000) where 100 h incubation time was reported for completion of ferrous iron oxidation in the presence of 200 mM Cu whereas at the control condition it was 48 h.

By deduction of results from MIC of different heavy metals and growth behavior of isolates to those concentrations, we can conclude that this isolate is highly resistant strain to of toxicity of wide range of metal ions. This property could select it as an appropriate isolate for metal bioleaching investigations that could be used in the industrial leaching processes of different mines.

We have studied the mechanism of resistance of our isolate to zinc and lead toxicity by proteomics methods (2D gel electrophoresis and MALDI-TOF MS). Proteomics provide direct information of the dynamic protein expression in tissue or whole cells, giving us a global analysis. Together with the significant accomplishments of genomics and bioinformatics, systematic analysis of all expressed cellular components has become a reality in the post genomic era, and attempts to grasp a comprehensive picture of biology have become possible. One important aspect of proteomics is to characterize proteins differentially expressed by dissimilar cell types or cells imposed to different environmental conditions. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) in
combination with mass spectrometry is currently the most widely used technology for comparative bacterial proteomics analysis (Gygi et al., 2000). The high reproducibility of 2D PAGE is particularly valuable for multiple sample comparisons. In addition, it directly correlates the changes observed at the peptide level to individual protein isoforms. Several studies have been used 2D PAGE to study changes in protein expression of *At. ferrooxidans* under different growth conditions. Proteins induced under heat shock (Varela and Jerez, 1992), pH stress (Amaro et al., 1991), phosphate limitation (Seeger and Jerez, 1993; Vera et al., 2003) or presence of heavy metals like copper (Novo et al., 2003) have been reported. A set of proteins that changed their levels of synthesis during growth of *At. ferrooxidans* ATCC 19859 in metal sulfides, thiosulfate, elemental sulfur and ferrous iron was characterized by using 2D PAGE (Ramirez et al., 2004).

Changes in the protein expression pattern when the organism is grown in different conditions have been recorded with 2-D electrophoresis. The results clearly indicated that there were proteins that specifically up regulated or down regulated under specific grown conditions. There are also proteins which are specifically expressed in the presence of both the zinc and lead. This is also a confirmation of presence of some common regulatory systems for the treatment of different metals. Over all the protein in the control is found to be more than that of zinc and lead which showed that due to high toxicity of metals some proteins were completely disappeared.
The result of 2D PAGE indicated that more than 13 proteins are showing differential expression in response to a metal treatment. All these proteins have not been identified. So far only four proteins are identified. One of those proteins which were identified and seems to have central role in bacterial tolerance to zinc and lead was a major outer membrane protein or OMP40. Molecular characterization of this protein suggests that OMP40 is a porin (Guiliani and Jerez, 2000). Rodriguze et al (1986) also described a porin-like protein in the outer membrane of *At. ferrooxidans*. This protein has been reported to respond to external pH and phosphate starvation (Jerez et al. 1992) and 600 mM of copper, zinc, nickel, or cadmium sulfate (Novo et al., 2000, 2003). Hence, we have to consider this protein as the most responsible protein for resistance to lead and zinc. An outer membrane of gram-negative bacteria is a structure exposed to environmental changes by external stimuli. The bacteria make use to specialized pore-forming proteins (porins) to facilitate the passage of small hydrophilic molecules through their outer membrane. A major outer membrane protein having an apparent molecular mass of 40 KDa (OMP40) in *At. ferrooxidans* has been known to be organized in a trimetric structure and to form a slightly anionic channel (Jerez et al., 1992; Guiliani and Jerez 2000). The structure of OMP40 protein is similar to the *E. coli* OMPC involved in response to osmotic pressure (Guiliani and Jerez, 2000). The relative content of OMP40 in the outer membrane has been known to be increased when cells grown at pH 3.5 were shifted to pH 1.5 or cells were grown under phosphate starved condition. Since OMP40 is a major outer membrane protein and has a similar structure to the *E. coli* OMPC, OMP40 is expected to be involved in response to osmotic pressure. However, the detailed information on effects of increasing concentration
of salts on the synthesis of OMP40 is not available. The characterization of OMP40 identified a large external L3 loop that could control the size of the entrance to the pore and ion selectivity at the entrance (Guiliani and Jerez, 2000). The calculated charge of this loop is postulated to control influx of proton across the outer membrane. Compared to that in the cells grown on ferrous iron, a relative content of OMP40 in *At. ferrooxidans* CCM4253 cells has been recently reported to be increased in sulfur grown cells (Bouchal et al., 2006). Since pH in the medium become acidic due to the production of sulfate during sulfur oxidation, the increased amount of OMP40 in sulfur-grown cells has implied that OMP40 is involved in the adaptation of the cell to acidic conditions. Since an increased concentration of metal ions (Zn and Pb) has an inhibitory effect on growth of DF1 cells, the cell must develop the mechanism allowing it to control the free passage of metal ions from the outside. The results obtained in this study implied that OMP40 is involved in the adaptation of cell to the increased concentration of toxic metals.

Another important protein which has been identified and showed over expression at the control conditions was Ribulose bisphosphate carboxylase. This protein is an enzyme that is used in the Calvin cycle to catalyze the first major step of carbon fixation. RuBisCo is very important in terms of biological impact and it is very vital and important in chemolithotrophic bacteria for carbon fixation. The results also showed that the level of enzyme decreased in presence of heavy metals (zinc and lead) and these are expectable results because by the toxicity result of heavy metals the growth and activity of bacteria is
decreasing and ultimately the activity and level of this enzyme also decreased in metal exposed bacteria.

Ribulose-1,5-bisphosphate carboxylase/oxygenase, most commonly known by the shorter name RuBisCO, is an enzyme (EC 4.1.1.39) that is used in the Calvin cycle to catalyze the first major step of carbon fixation, a process by which the atoms of atmospheric carbon dioxide are made available to organisms in the form of energy-rich molecules such as sucrose. RuBisCO catalyzes either the carboxylation or oxygenation of ribulose-1,5-bisphosphate (also known as RuBP) with carbon dioxide or oxygen.

RuBisCO catalyzes the most commonly used chemical reaction by which inorganic carbon enters the biosphere. RuBisCO is also the most abundant protein in leaves, and it is the most abundant protein on Earth. Given its important role in the biosphere, there are currently efforts to genetically engineer crop plants so as to contain more efficient RuBisCO. In plants, algae, cyanobacteria, and phototropic and chemoautotrophic proteobacteria, the enzyme usually consists of two types of protein subunit, called the large chain (L, about 55,000 Da) and the small chain (S, about 13,000 Da). The enzymatically active substrate (ribulose 1,5-bisphosphate) binding sites are located in the large chains that form dimers in which amino acids from each large chain contribute to the binding sites. A total of eight large chain dimers and eight small chains assemble into a larger complex of about 540,000 Da. In some proteobacteria and dinoflagellates, enzymes consisting of only large subunits have been found. In *At. ferrooxidans* the cbbL and cbbS genes encoding form I ribulose-1,5-bisphosphate carboxylase/oxygenase. *At. ferrooxidans*
obtains its cellular carbon by the fixation of carbon dioxide via the Calvin cycle. The RuBPCase from *T. ferrooxidans* has been reported to be of the form I type, a hexadecamer composed of eight large subunits (LSU) and eight small subunits (SSU) similar to those of higher plants and most photosynthetic bacteria.

The third identified protein was putative DNA restriction methylase (*Salmonella typhi*). To protect the cell from exogenous DNA, most species have DNA modification methylase but the actual role of this enzyme in metal resistance is still unclear and there is no report till date. DNA methylases catalyze the transfer of a methyl group to DNA from S-adenosyl-L-methionine, which is consequently converted to S-adenosyl-L-homocysteine (Morita et al., 2008). The later two proteins (RuBisCo and putative DNA restriction methylase) which are differentially expressed have not been reported earlier under heavy metal stress.

The Holo-(acyl carrier protein) synthase (AcpS) is another enzyme which showed threshold score with MASCOT search engine. This enzyme belongs to the family of transferees, specifically those transferring non-standard substituted phosphate groups. It is the central coenzyme of fatty acid biosynthesis and also has previously been identified and shown to be essential for *E. coli* growth. Over expression of this protein in the presence of metals suggests that the organism tried to adapt to the high concentrations of metals by over expressing this protein which can helps in enhancing the biosynthesis of fatty acids for adapt to the toxic metals.
CoA-(4'-phosphopantetheine) + apo-[acyl-carrier-protein] → adenosine 3',5'-bisphosphate + holo-[acyl-carrier-protein].

Acyl carrier protein interacts with enzymes which synthesize fatty acids (Vagelos et al., 1966, Butterworth et al., 1967) and with enzymes involved in complex lipid biosynthesis. In addition to interaction with enzymes involved in fatty acid metabolism, ACP interacts with ACP hydrolase (Vagelos et al., 1976) and holo-ACP synthetase (Elovson et al., 1968). Studies with certain acyl peptides derived from ACP have demonstrated that few chemical modifications of ACP are tolerated by enzymes in fatty acid biosynthesis. ACP hydrolase has also been shown to be quite specific (Vagelos et al., 1976).

Thus, the functional analysis of the identified proteins, though very few, explained the tolerance of heavy metals to a certain concentrations, by this bacterium. The mechanism of lead tolerance has been studied for the first time in this study and we could add more details to the zinc resistance mechanism which was studied before (Novo et al., 2003). These results have shown that, almost there is a same mechanism for zinc and lead resistance with very minor difference.