Abstract of the thesis entitled

Study of ability and role of native bacteria on the Bioleaching of Zinc and optimization of growth conditions

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By

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The term bioleaching refers to the conversion of an insoluble metal (usually a metal sulfide, e.g., CuS, NiS, and ZnS) into a soluble form (usually the metal sulfate, e.g., CuSO$_4$, NiSO$_4$, ZnSO$_4$). When this happens, the metal is extracted into water; this process is called bioleaching. These processes are oxidative in nature and when microorganisms mediate these, they are termed as biooxidation. Here the recovery of a metal is enhanced by microbial decomposition of the mineral. In this process the metal being recovered remains in insoluble form. An example is the recovery of gold from arsenopyrite ores where the gold remains in the mineral after biooxidation and is extracted by cyanide in a subsequent steps. Hence the term bioleaching is clearly inappropriate when referring to gold recovery (although arsenic, iron, and sulfur are bioleached from the mineral). Biomining is a general term that may be used to refer to both the processes. Populations of leaching bacteria are found ubiquitously and may be isolated with little difficulty wherever oxidizable ore bodies are exposed to the surface. However, newly isolated bacterial populations cannot be expected to oxidize ores at very high rates. The challenge to the biotechnologist is to improve the rate of cell growth and ore oxidation by the microbes responsible for leaching.

Different types of bacteria have been isolated from industrial leaching operations or from natural leaching sites or mines etc. These strains are capable of attacking sulfides. These are classified according to their preferred temperatures for growth Mesophilic bacteria such as *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptosprillum ferooxidans* operating at ~ 40° C are the most extensively used microorganisms for the bioleaching of sulphide minerals with commercial interest. All three of these species are highly acidophilic (optimal pH 1.5– 2), obligate autotrophs, and grow optimally at temperatures ranging from 25 to 35°C. *At. ferrooxidans* obtain energy from the oxidation of either ferrous iron or reduced sulfur compounds, and rapidly decompose mineral sulfides in pure culture.

Several toxic metals have no effect on the growth of *At.ferrooxidans*. However, strain specific difference in the level of tolerance have been reported for *At.ferrooxidans* isolated from various mine sites. Among different metals *At. ferrooxidans* shows an
unusual resistance to some metals, such as zinc, nickel, cobalt and copper, unlike most heterotrophic bacteria which exhibit high degree of sensitivity to most of these toxic metals. Some metals (e.g. mercury and silver) are highly toxic to the bacteria even at very low concentrations. It is pertinent to note that most of these metals are present at high concentrations i.e. beyond the tolerance limit of the bacterial strains at mines. Thus the bioextraction / bioleaching of metals from low-grade sulfide ores can only be effective if the bacterium is resistant to the metal or it can sustain itself at a concentration higher than the one that is prevalent in the mines.

Keeping this view in mind, we have proposed to study isolation of mesophilic bacteria form Iranian zinc and lead mine, optimization of culture conditions and optimization of its bioleaching activity by manipulation of NH4, Fe^{2+}, Mg^{2+}, pH and temperature, study of heavy metal tolerance and mechanism of resistance to lead and development of large scale column bioreactor for bioleaching study.

The objectives of the study were:

1- Isolation and identification of the mesophilic bacteria from the ore samples

2- Study the process optimization for higher recovery of Zn from the mine sample by using five different factors including pH, temperature, iron concentrations, nitrogen source concentration and magnesium concentration in four different levels.

3- Developing suitable column (bioreactor) for bioleaching capacity for large scale production

4- To study resistance to heavy metals and the mechanism of resistance to zinc and lead

In the present study Iranian Zinc and lead sulphied complex mine has been selected for bioleaching propose whose capacity is 120 million tones. The chemical and mineralogical analyses of the ore samples by XRD and XRF revealed that Pyrite, Calcite, Dolomite, Gypsum and Sphalerite are the main components of the mine and Pyrite and Sphalerite making up the main sulphide part. This mine has 3.1 % zinc and 1.27 % lead thus results indicated that this mine is low grade mine. The other properties of mine like
temperature of different site of mine, size of the mine, water availability and type of water was also detected.

A total of five acidophilic bacteria and one fungus were isolated from the mine by using three different media including TK medium Leathen and 9-K out of those three media, we find 9-K media is the best. The cultures were purified by using modified 9-K solid 2:2 medium. Among acidophilic bacteria one (MY2) was selected for further studies. The 16S ribotyping analysis of the 753 base pairs of the above mentioned isolate was done at the National Center for Biotechnology Information (NCBI) server using universal protobacterial primers. The indicated that organism is a new strain of *Acidithiobacillus ferrooxidans* and we have named it as an *Acidithiobacillus ferrooxidans*. D.F.1. *At.ferrooxidans* is a gram-negative, motile, coco-bacillus non-spore forming.

To achieve the best condition for growth and activity of bacteria the composition of medium (9-K) and growth conditions were optimized by varying five different factors & each one were studied in four different levels; pH (1.4, 1.6, 1.8 and 2); temperature (25, 30, 35 and 40 °C); Fe$^{+2}$ concentration (2, 4, 6, and 8 g/l), (NH$_4$)$_2$SO$_4$ (1, 2, 3 and 4 g/l) and Mg$^{2+}$ (20, 40, 60 and 80 mg/l). The concentration of free bacteria in solution is considered as an indicator of the improving of growth of bacteria that was determined by direct counting using a Thoa chamber of 0.1 mm depth and 0.0025 mm$^2$ area with an optical microscope (X1000), and ferric ion concentration was used as an indicator of bacterial activity.

Resulted suggest that:

1) In the relation of pH, acidity of the environment controls the bacterial activity within a system. The H$^+$ ion is in fact vital for acidophilic microorganisms since bacteria utilize it as a proton source for the reduction of O$_2$. The optimum pH for the growth of D.F1 bacterium was 1.4 and the optimum pH for efficiency was 1.6. Thus pH required for its growth and efficiency was different. Also an increase in the pH to 2 will decrease the efficiency of bacteria for the conversion of ferrous to ferric which maybe due to ferric precipitation on bacterial surface, which hinders the diffusion of protons.
ii) Over the optimal pH range we studied the effect of 4 different concentrations of iron on growth and activity of bacteria. Results indicated that at high (6-8 g/l) concentration of Fe$^{2+}$ bacterial cell has longer lag phase 24-28h but at low concentration of Fe$^{2+}$ the lag phase was decreased to 10-15h which showed that the extent of the lag phase depends on the initial concentration of ferrous iron. In the case of activity of bacteria (At. ferrooxidans D.F1), concentration of Fe$^{3+}$ in solutions was measured in the time interval of 2 hours. As the oxidation of Iron takes place in the exponential phase of growth in the early stage of growth the concentration of ferric iron was low. It also indicated that 4 g/l of initial Fe$^{2+}$ led to the maximum biooxidation rate (0.24g/l h) and it shows that shorter lag phase and better specific growth in compared with 6 and 8 g/l Fe$^{2+}$.

iii) We have studied the effect of temperature in the range of 25 to 40 $^0$C over the optimal pH and Iron concentration range. The bioleaching efficiency and growth of bacteria tended to increase with increasing and the optimum temperature for growth and bioleaching activity was 35$^0$C. 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.

iv) A culture medium (9-K) is mixtures of chemical compounds, which provide all the elements required for cell growth and biosynthesis. In this culture medium of Thiobacillus (9K) after iron, (NH$_4$)$_2$SO$_4$ is the main part of medium and essential for growth. Effect of different nitrogen concentrations on growth and activity of bacteria revealed that increasing the concentration of nitrogen increased cell density and maximum growth occurred at 3 g/l (NH$_4$)$_2$SO$_4$. The reason that, why the oxidation activity of bacterium decreased in 4 g/l (NH$_4$)$_2$SO$_4$ than 3 g/l; is the possible precipitation of phosphate, potassium and ammonium as jarosites due to higher concentration in the medium, which is also one of the major detractions to 9-K liquid medium.

v) The effect of Mg$^{2+}$ concentration at 20, 40, 60 and 80 mg/l, on the efficiency of the biooxidation process has been studied. The Concentration higher than 40-mg/l doses not has any effect on the biooxidation of ferrous sulfate. A higher concentration of Mg up to 80 mg/l has slightly negative effect on oxidation process.
After the standardization of growth conditions the ability of bacterium for extraction of zinc and lead has examined at two different scales including laboratory scale in flasks and large scale in column bioreactor. At laboratory scale the bioleaching experiment was carried out in conical flasks. The bioleaching ability of bacterium has examined at optimal and non optimal conditions. We have found that with the use of optimum conditions the efficiency of our isolate for extracting the zinc and lead increases more than 30-40% (from 65% to 86% for zinc and 16% to 22% for lead). Further the effect of different concentrations of ferrous iron on the ability of bacterium for zinc and lead extraction was examined. It is evidence from the results on the ability of bacterium for extraction of zinc and lead form the ore samples at 2, 4, 6, and 8 g/l Fe\(^{2+}\) that 4 g/l initial Fe\(^{2+}\) is most optimum and resulted in 88% Zn and 22% Pb recovery. The efficiency of 86% extraction of zinc is one of the advantages of the isolate for bioleaching of this metal from the mines.

One of the Major parts of our work was the engineering part where we have designed and made up the high capacity column bioreactor (450 l) to understand the behavior of our bacterium for large scale bioleaching. On trial and error basis we have designed bioreactor. Reactor design was based on a glass column with inlet for air and outlet for effluent at the bottom. The bioreactor size was 50 cm in diameter and 3 M length. Total operation volume of bioreactor was about 450 L. Air was supplied from bottom and fresh medium from the top, also at every 30 cm of bioreactor a sampling port and temperature indicator were provided. The column is one of the biggest column bioreactor has been ever made for study bioleaching of zinc and lead. During the experiment factors like pH, Temperature and etc continually were monitored and for avoiding jarosite precipitation 1 N NaOH was added at appropriate time. Extraction of zinc and lead were monitored in the period of 100 days. Here also the bioleaching ability of bacterium was studied at basic and optimum conditions. Further the effect of different pH and iron concentrations also was studied on the bioleaching of zinc and lead. The results confirmed our results on laboratory scale and 72% extraction of zinc during 87 days was obtained within column bioreactor at optimal condition whereas it was 54% at basic condition. The best pH and iron concentration for bioleaching of zinc and lead was 1.6 and 4 g/l respectively which
can confirm our optimization results as well as bioleaching experiment at laboratory scale. This showed that this bacterium can be used in larger scales even for in situ bioleaching at the mine site or large heap bioleaching.

As the most of heavy metals are present at high concentration at mines environment thus bioextraction of metals from low-grade sulfide ores can only be effective if the bacterium is resistance to the metal recovered as well as to other in the environment. Hence in the present study we have described response of bacterial strain to heavy metal toxicity. Determination of minimum inhibitory concentration (MIC) of different metals, growth profile of strain in response of metals and proteomic approach to find out the mechanism of resistance to lead and zinc at protein level are described in this work. we have investigated the effect of ten different heavy metals on growth and activity of our bacterium. The metals include zinc, lead, copper, arsenite, arsenate, nickel, manganese, chromium, mercury and cobalt. The minimum inhibitory concentration (MIC) of each metal was determined by microdilution method. The isolate was showed high resistance to zinc and manganese (650 and 700mM). The resistance to other metals also were quite high with compare to previous reports (Nickel ; 150 mM, Cobalt; 80 mM and Copper 50 mM). These results could be expected; because the mine where this bacterium was isolated from, contain most of these metals at high different concentration, so the organisms from this mine should be adapted to high concentration of such metals. Thus we can conclude that this strain is one the most resistant strains of *At. ferrooxidans* to different heavy metals when compare to other reports available in literatures.

The growth profile of the organism was studied in 9-K medium in presence of two concentrations of all metals mentioned above. The basic toxic effect of the metals tested was to cause an increase in the lag phase of isolates growth and also more than 90 hour increment was observed in the time required for maximum oxidation of ferrous iron. Under the control condition it took 45 hour for complete oxidation of ferrous iron whereas in presence of 700 mM Zn and 10 mM Pb oxidation of ferrous iron was completed in 160 hour. Similar results were also obtained for manganese, nickel and
copper where more than 140 hours were required for oxidation of ferrous iron. For other heavy metals the same was 70 – 90 hours.

We have studied the mechanism of resistance of bacterium to these metals. Bacterial cell were exposed to 500 mM and 5 mM zinc and lead respectively. The intracellular proteins were extracted and resolved on two dimensional gel electrophoresis gels. IEF was performed using both 7 cm IPG strips and tube gel of pH range 3-10 using the BioRad IEF cell. Differentially expressed proteins were detected. Spots were excised and digested with proteolytic enzymes. Peptide mass fingerprints were created and analyzed with MADLI-TOF. Proteins were identified by different bioinformatics softwares. The results of 2D PAGE have indicated that under the influence of metals, there was a differential regulation of proteins to cope-up with the metal toxicity. More than 10 proteins have been differentially expressed. The most over expressed protein was the protein with 30-40 KDa molecular weights and with pI 4-5 which was over expressed in presence of both the metals (lead and zinc). This protein was identified as major Outer Membrane Protein of *Acidithiobacillus ferrooxidans* (OMP40) with significant ProFound score (2.25). It seems that this protein has the significant role in resistance to metals toxicity as it was over expressed in response to both the metals. Molecular characterization of this protein suggests that OMP40 is a porin. The over expression of this protein is reported in other organisms also by many workers. The second most over expressed protein has 60 KD molecular weight with pI of 6-7 which is over expressed in presence of zinc and lead. This protein was showed highest significant score (1.64) to Putative DNA restriction methylase (*Salmonella typhi*). These enzyme are believed have a role in control proteins function. In case of lead we could find four more over expressed proteins but there are many proteins which have been down regulated as compared to control. As compared to lead the number of over expressed proteins in presence of zinc was higher and most of these proteins are in the pI range of 5 -7 with different molecular weight.

As compared to control five proteins have been found to down regulated or completed in strain exposed to zinc and lead. One of these proteins has been identified as CBBL (Ribulose bisphosphate carboxylase large subunite) of *Acidithiobacillus ferrooxidans*
with the high Profound score of 2.25 and Mascot 115. This is an enzyme that plays a role in Calvin cycle to catalyze the first major step of carbon fixation. RuBisCo is very important in term of biological impact and it’s very vital and important in chemolithotrophic bacteria for carbon fixation. There are other 2-3 proteins which have been down regulated in metal treated cells have similar molecular weigh in the pI range of 5-6. One of these proteins showed more similarity to Hypothetical protein SO-408 of *Shewanella oneidensis* with the marching of 11 of 35 peptides. Other is Putative glutamines with 9 out of 54 peptides matching.

Thus in short, work can be summaries as follow:

1- We have been studied the potential application of bioleaching for extraction of zinc and lead from Iranian zinc and lead mine for the first time.
2- Bacterial strains have been recovered from the zinc and lead mines. Characterization and identification of this strain has been undertaken.
3- Growth conditions have been standardized to increase the efficiency of the isolate and ultimately having more extraction of metals. All the factors were studied separately for growth as well as activity of bacteria (iron oxidation) and found out the optimum conditions as ; pH 1.4-1.6, ferrous iron concentration 4 g/l, ammonium sulfate concentration 3 g/l, temperature 35 °C and magnesium concentration 20 mg/l
4- Developed the modified method for DNA isolation from acidophilic bacteria that could increase the efficiency of DNA extraction. Because of the interference of iron precipitation in DNA extraction it has been suggested to use more concentration of EDTA to remove the iron and also different ratio of chloroform/ isoamayl alcohol was used which increased the efficiency of DNA extraction.
5- It is very difficult to solidify agar at low pH. We have developed a modified method for solidifying agar at a very low pH (< 3 pH)
6- The maximum biooxidation rate of 0.24 g/l h has obtained at initial ferrous concentration of 4 g/l, this is the highest biooxidation rate for this concentration ever reported.
7- Studied the heavy metal tolerance with use of all expected heavy metals that can be found at mines and found out the MIC of nine different heavy metals. It has been established that this isolate is highly resistance bacterium to most of the studied metals and especially to Zn, Mn, and Ni.

8- Analyzed the growth behavior of bacterium in response to those metals by measuring the iron oxidation versus time and established that the increase in the lag phase of bacterial growth is a basic toxic effect of heavy metals.

9- Proteomics approach has been adapted to understand the mechanism of resistance to heavy metal stress at protein level. Proteins having the role in resistance process have been identified and their functional characterization is carried out.

10- To the best our knowledge ours is the first study related to understanding the mechanism of resistance to lead for *At. ferrooxidans* at protein level by proteomics approach.

11- Studied the extraction of zinc and lead at two different scales by evaluation the effect of different factors. The large scale was the 450 l column bioreactor which is the biggest column ever used for study the zinc bioleaching.

12- Optimized the bacterial efficiency for zinc and lead extraction at laboratory scale by changing the concentration of initial iron concentration and establishment the 4 g/l Fe$^{2+}$ for maximum extraction of zinc and lead (~90%)

13- Optimized the efficiency of bacterium for zinc and lead extraction at column bioreactor where we were found; pH 1.6 and 4 g/l ferrous iron concentration for the best growth and activity of bacterium.

As a conclusion it is important to know that population of leaching bacteria are found ubiquitously and may isolate with little difficulty wherever oxidizable ore bodies are exposed to the surface. However, newly isolated bacteria population can not be expected to oxidize ores at maximum rates. Natural populations of bacteria are selected for their ability to survive under a wide range of often adverse condition, rather than for their ability to rapidly oxidize ores under the ideal condition of an industrial bioleach process. The challenge to the biotechnologist is to improve the rate of cell growth and ore oxidation by the microbes responsible for leaching. Hence it would be interesting to
isolate the strain with ability of rapid growth and oxidation as well as tolerance the adverse conditions of mine habitat like low pH and high concentration of heavy metals. And then try to improve its abilities to enhance the efficiency of oxidation process and metals extraction. Because these improved bacteria can be good source to be applying in different mines for extracting different metals. In this study also we could isolate the new strain of *At. ferrooxidans* which itself had good ability for applying in bioleaching process and by changing the growth condition we could increase its efficiency more than 35%. The other properties of this strain like high tolerance to wide range of heavy metals and ability to extract metals even at very large scale make it as ideal bacterial strain for applying in bioleaching industries for recovery of different metals especially for iron, zinc and lead.