CHAPTER FIVE:

SUMMARY

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CONCLUSION
Mining operations using bacteria are becoming popular; as the conventional methods are being costly, involve lots of energy and release toxic gases. Thus in this process, having the microorganisms with some properties like tolerance to the low pH and high concentrations of heavy metals, rapid growth, less requirement for growth and ability to extract the target metal by maximum efficiency is the best alternative. In the present investigation, we have successfully isolated the newly acidophilic bacterium from complex zinc and lead sulfide mine, located at central Iran which has the specific properties. The target mine which was studied in this work was low grade sulfide mine in central of Iran which has not been used for any leaching operation (biological and non biological). The XRD and XRF analysis of mine indicated that the mine has good reserves of metals like iron, zinc and lead. The total capacity of mine is 120 million tones.

The bacterium which was isolated in this study was a novel strain of \textit{At. ferrooxidans} and we have named it as DF.1. We have optimized the growth conditions of the bacterium to enhance the efficiency of bacterium for oxidation of iron and ultimately more metals extraction from the mine. The factors which were selected for optimization process were pH, ferrous iron concentration, temperature, ammonium concentration and magnesium concentration. The optimization of growth conditions showed that the strain can grow in the pH range of 1.2 to 3 with optimum pH of 1.4 -1.6 and this is one of the advantages of this isolate that grows in extremely low pH which
ultimately increase the bioleaching efficiency by decreasing the chance of jarosite precipitation. One of the surprising results which have obtained in this study was the difference between the optimal pH for growth (1.4) and activity (1.6) which showed that having more cell density is not the only factor for having more oxidation activity. The results also showed that the rate of growth and activity of bacteria directly affect the rate of metal dissolution. Longer lag phase in case of higher concentration of Fe$^{2+}$ was the expected results. The maximum biooxidation rate of 0.24 g/l h has obtained in initial Fe$^{2+}$ concentration of 4 g/l which is one of the highest biooxidation rates with this much concentration of initial iron. Second advantage of our isolate is that it has high rate of biooxidation with lower concentration of initial Fe$^{2+}$ (4 g/l) and having less requirement of iron for maximum growth and efficiency (4 g/l). It grows well with temperature range from 10 to 40 °C with optimum of 35 °C. The best concentration of ammonium and magnesium required for optimum growth and activity of bacterium was 3 g/l and 40 mg/l respectively.

Further, we were studied the ability of bacterium for extraction of zinc and lead at laboratory scale as well as large scale (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 increased more than 30-40 % (from 65% to 86% for zinc and 16% to 22% for
lead). The efficiency of 86% extraction of zinc is also another advantage of the isolate. Even 22% recovery of lead was one of the highest amounts of extraction of lead which has been reported before. The toxicity of lead to bacterial cell avoids more extraction of this metal. The effect of different iron concentrations on the amount of zinc and lead extraction was studied and the results showed that 4 g/l Fe$^{2+}$ is the best concentration for zinc extraction and other concentrations above and below this concentration have the adverse effect on bioleaching efficiency of the bacterium. The lead extraction did not affect by changing the initial iron concentration.

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. The column was one of the biggest column bioreactor has been ever made for study of bioleaching of zinc and lead. The bioleaching ability of bacterium was studied at basic and optimum conditions. Further the effect of different pH and iron concentrations 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 conditions 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 confirmed our optimization results as well as bioleaching experiment at laboratory scale. This showed that this bacterium can be used use in larger scales.
The present study describes response of bacterial strain to heavy metal toxicity. The isolate was studied for its tolerance to nine heavy metals, i.e., zinc, manganese, nickel, cobalt, copper, arsenate, chromium, lead and mercury by growing them in 9-K medium. 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 was quite high with respect to previous reports (Nickel; 150 mM, Cobalt; 80 mM and Copper 50 mM). The growth response of the isolate to metals was carried out by growing the bacterium in seventeen different concentrations of those metals. 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.

Proteomics approach was used to identify the differentially expressed protein under heavy metal stress for this strain. The result of 2D PAGE has indicated that under the influence of metals, there was a differential regulation of proteins to cope-up with the metal toxicity. Four of the differentially expressed proteins were identified to be; major outer membrane protein of *At. ferrooxidans*, Ribulose bisphosphate carboxylase large subunit (RuBisCo) of *At. ferrooxidans*, putative DNA restriction methylase and Holo synthase (*Syntrophus aciditrophicus*).
OMP40 is a porin protein in the outer membrane of *At. ferrooxidans*. Since an increased concentration of metal ions (Zn and Pb) has an inhibitory effect on growth of bacterial 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 down regulation in metals treated cells was Ribulose bisphosphate carboxylase (RuBisCo). This protein is an enzyme that is used in the Calvin cycle to catalyze the first major step of carbon fixation. The reason of down regulation of this enzyme in metal treated cells is the decrease in growth and activity of bacteria due to the toxic effect of heavy metals and ultimately the activity and level of this enzyme also down regulated in metal exposed cells.

Over expression of Holo-(acyl carrier protein) synthase (AcpS) protein in the presence of metals suggested that the organism tried to adapt to the high concentration of metals by over expressing this protein which can helps in enhancing the biosynthesis of fatty acids to adapt to the toxic metals.

Another 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.
The later three proteins which are differentially expressed have not been reported earlier under heavy metal stress. To the best of our knowledge this is the first study related to understanding the mechanism of resistance to lead in *At. ferrooxidans* at protein level by proteomics approach. Thus, the functional analysis of the identified proteins, though very few, explained the tolerance of heavy metals to a certain concentration, by this bacterium.

Thus in short, work can be summaries as follow:

1- We have been investigated 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, magnesium concentration 20 mg/l and temperature 35 °C.

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- 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. which 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 of 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 evaluating 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 and founded that 4 g/l Fe$^{2+}$ is the best concentration for maximum extraction of zinc and lead (~90%)
13- Optimized the efficiency of bacterium for zinc and lead extraction at column bioreactor where we found; pH 1.6 and 4 g/l ferrous iron concentration for the best growth and activity of bacterium.

Hence, finally we can conclude that the current panorama of bioleaching in developing countries is encouraging. It is expected that in the coming years several new commercial-size bioleaching plants will be installed. It is likely that heap leaching will continue to be the choice for low-grade ores and tailing, while tank bioleaching technology will probably increase its application for gold, copper and other base-metal concentrates. The use of thermophilic bacteria and archea will be a major contribution, increasing the leaching rates and metal recoveries and allowing for the treatment of recalcitrant ores such as chalcopyrite. Developing countries should increase their efforts in research and development in bioleaching technology, as they have comparative and competitive advantages in this area. International cooperation should also be considered in the establishment of new operations that can significantly contribute to the economic and social development of these countries.

Bioleaching process is a widely accepted commercial technology to recover metal values from low/off grade sulfide ores which cannot be exploited commercially by usual techniques. So far the application of bioleaching technique to recover metal values from high grade ore concentrate has very limited application due to slow
kinetics. With the possible use of this technique to enhance the recovery of precious metals from refractory ores, much emphasis is now being given to modifying the kinetics. In-depth knowledge regarding the reaction mechanisms including the characterization of microorganism and the enzyme system is required in order to enhance the kinetics by finding out the rate determining steps.

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 and according to Modak & Ntarajan (1995), it is necessary to develop *At. ferrooxidans* strains more tolerant to high metals concentration and temperature fluctuations to improve bioleaching. 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.

From the literature, it is apparent that much has been learned about microbial leaching and the potential of the organisms for leaching. Regardless of the present limited applications of microbes for leaching, the future appears promising. We can look forward to a new modifications of the leaching process including: (a) redesigning of heap dumps to include finger dumps to exercise natural aeration and artificial aeration with compressed air; (b) genetic manipulation of chemolithotrophic bacteria to fit the optimum conditions needed for successful leaching or the application of naturally selected higher-yielding organisms: (c) vat leaching to allow more controls for optimizing leaching and improving pollution control and (d) the use of ore concentrates for leaching.