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Study of Resistance of Indigenous Bacteria Involved in the Process of Copper Bioleaching to Silver and Mercury Toxic Metals in Different Concentrations

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Bioleaching is an economic and effective procedure in which, metals like cupper can be extracted in low-grade mineral ores by proper bacteria. Acidophilic and chemolithotrophic bacteria in mines can extract metals in sulphide minerals by bioleaching process and convert metal sulphides to soluble metal sulphates. Since in these mines toxic metals are present, in the process of solution making of mineral sulphides, toxic metals in the form of soluble sulphates leak to natural waters and pollute the water. The present microorganisms in the mines specially in the species of *Acidithiobacillus* are toxic metal toxicity resistant and reduce the toxicity of these minerals in nature. In this research, the resistance of bacteria that involved in bioleaching process was determined in three concentrations of toxic ions of Ag^{1+} and Hg^{2+}. The concentration Fe^{3+} was measured by UV-VIS Spectrophotometric method and Cu^{2+} concentration was determined by Neocuprion reagent. In order to identify the local species, bacterial DNA was extracted and 16s rDNA regions genes amplified by PCR. The alignment result showed a great amount of similarity between the new bacteria and *Acidithiobacillus ferrooxidans*.

Keywords: Bioleaching- *Acidithiobacillus*- Toxic metals- Chemolithotrophic

Environmental pollution caused from toxic metals is one of the most important problems nowadays. Wastewaters and acid mine drainage (AMD) have enormous amounts of toxic metals like cupper arsenic, silver, mercury and zinc which, cause devastating in environment such as soil contamination, soil texture destruction, decreasing nutrients lifetime and natural waters[11]. Leakage of these polluted waters to the seas will endanger the life in seas, causing fish death and decrement of shore living animals. Also, prolonged effects on health of aquatic organisms and accumulation of toxic metals in their bodies will interfere in the food chain [2, 3]. Bioremediation contaminated water containing acid and toxic metals by biochemical methods are costly processes. By applying the biotechnology methods and the use of microorganisms can reduce the pollution of environment. Biological methods are inexpensive, high performance and compatible with the environment. Existant microorganisms in the mines, show high resistance to toxic metals in acidic waters and by applying different methods such as

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capacity changes, and sequestration of toxic metals, reduces toxicity in the environment[5]. Biohydrometallurgy is one of the biological methods which include bioremediation, bioleaching, biological absorption and bioaccumulation. Among the above mentioned processes, bioleaching is the most effective and efficient way in which, microorganisms can help insoluble metals from low-grade ores to be extracted into soluble form[12, 20]. The effectiveness of bioleaching process depends on physical, chemical and biological factors such as kind of contaminating materials, strains of bacteria and cell concentration[11].

Among microorganisms involved in the bioleaching process, *Acidithiobacillus* species is able to grow under acidic conditions and a pH between 1.8 to 2.5 and by stabilizing CO_2 in the air and by chemolithotrophic growth can have a good role in extraction process of metals by bioleaching [1, 6].

The purpose of this research is isolation of iron and sulfur oxidizing bacteria especially in the species of *Acidithiobacillus* that under certain concentrations of Hg^{2+} and Ag^{1+} ions could grow and achieve bioleaching process.

MATERIAL AND METHODS

The test samples are taken from the pregnant leaching solution (PLS) that was isolated from Sarcheshmeh copper mine. The samples were cultured in TK medium including K_2HPO_4, $(NH_4)_2SO_4$ and $MgSO_4$, each amount of 0.4 g/l and $FeSO_4.7H_2O$ with the amount of 33.4 g/l in 250 ml Erlenmeyer which, incubated in shaker incubator at 30 °C in 10 days. This was done in order to give this opportunity to the bacteria to be increased in numbers respect to other potential bacteria in the PLS solution. Then, to determine the growth of bacteria in the TK medium containing Ag^{1+} ions, isolated bacteria were enriched in 100, 300 and 500 ppm of silver nitrate with 10% inoculation. Also, isolated bacteria were inoculated in TK medium containing 100, 300 and 500 ppm of mercury chloride in the above mentioned condition.

The media have placed in shaker incubator with 150 rpm at 30 °C in ten days. Growth was measured by increasing in optical density at 640 nm (OD_{640}). Also, ferric iron (Fe^{3+}) concentration was measured with an ultraviolet spectrophotometric method by standard curve after 5 days in 300 nm wave length to survey the capability of bacteria in oxidizing ferrous iron (Fe^{2+}). In order to evaluate the oxidation of copper (I) and indirect bioleaching process rate, about 1g of copper (I) was added to media containing bacteria in late logarithmic phase. Then, the concentration of copper (II) was measured by using copper protocol and Neocuprion reagent. Also for identification of bacteria and superior strain, genes of 16srDNA were amplified and sequenced by PCR.

RESULTS AND DISCUSSION

Evaluation of bacterial growth in TK medium containing Ag^{1+} and Hg^{2+} ions

Isolated bacteria have a logarithmic growth in three concentrations of Ag^{1+} and Hg^{2+} in the TK medium. It can be seen in curves 1 and 2, the bacteria in TK medium containing three concentrations of 100, 300 and 500 ppm of silver nitrate and mercury chloride, reached to a maximum of logarithmic growth after 8 days and then, showed a decrease of growth. Also, the rate of bacteria growth was decreased with increase of concentration of Ag^{1+} and Hg^{2+} ions. In fact, due to toxicity of Ag^{1+} and Hg^{2+} ions, with increasing of silver nitrate and mercury chloride, growth of bacteria were prevented. By comparing the curves 1 and 2, it can be shown that the bacteria have less growth in TK medium containing Hg^{2+} than Ag^{1+}. Thus, according to results, toxicity of Hg^{2+} is much more than Ag^{1+}.

Evaluation of oxidation ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) rate in TK medium containing Ag^{1+} and Hg^{2+} ions

For evaluation of bacteria capability in oxidation process and production of ferric iron (Fe^{3+}), the concentration of Fe^{3+} was measured by using an ultraviolet spectrophotometric method in 300 nm wave lenght after 5 days. In curves 3 and 4, result of Fe^{3+} concentration measurement in TK medium with Spectrophotometry are shown. As it can be seen, the amount of Fe^{3+} iron in 500 ppm concentration is less than two other concentrations.

Measurement of Cu^{2+} concentration in TK medium containing of Ag^{1+} and Hg^{2+} ions

For evaluation of indirect bioleaching

Table 1. Amount of Cu^{2+} in TK medium containing three different concentrations of Ag^{1+}

	Measurement of copper in silver nitrate medium (microgram)	
100 ppm	104	197.5
300 ppm	66.97	180.34
500 ppm	25.5	87.44

Table 2. Amount of Cu^{2+} in TK medium containing three different concentration of Hg^{2+}

	Measurement of copper in mercury cloride medium (microgram)	
100 ppm	21.2	100.77
300 ppm	16.9	68.51
500 ppm	16.47	25.5

process in presence of Ag^{1+} and Hg^{2+} ions and oxidation of copper (I) to copper (II) by ferric iron (Fe^{3+}), concentration of copper (II) was assessed with Neocuprion reagent.

By considering the results listed in tables 1 and 2, increasing of Ag^{1+} and Hg^{2+} ions concentrations will reduce Cu^{2+} concentration. Therefore, the isolated bacteria have ability of oxidation of Fe^{2+} to Fe^{3+} in presence of toxic Ag^{1+} and Hg^{2+} ions and these bacteria are considered to oxidizing iron and also able to do indirect bioleaching process.

Thus, these bacteria are resistant to toxic metals and this feature should be found in their genetic characteristics and existence of toxic metals resistance genes can be proved in them.

DNA extraction and Sequencing

DNA was extracted by set buffer (sucrose 20%, EDTA 50mM, TrisHCl 50Mm, pH: 4.7), lysozyme 5mg/mL, SDS 25%, proteinase- k 1mg/mL, ammonium acetate 7.5M and cool isopropanol. Fragment of 16SrDNA was amplified by PCR using

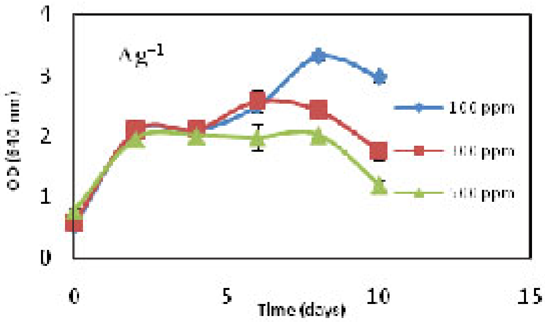

Fig. 1. Curve of bacterial growth in TK medium of containing Ag^{1+}

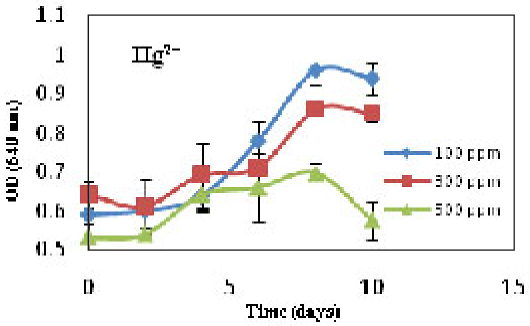

Fig. 2. Curve of bacterial growth in TK medium of containing of Hg^{2+}

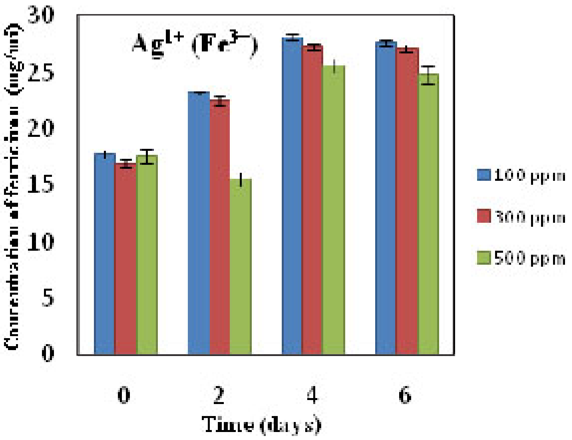

Fig. 3. Curve of measurement of ferric iron (Fe^{3+}) concentration in TK medium containing of Ag^{1+}

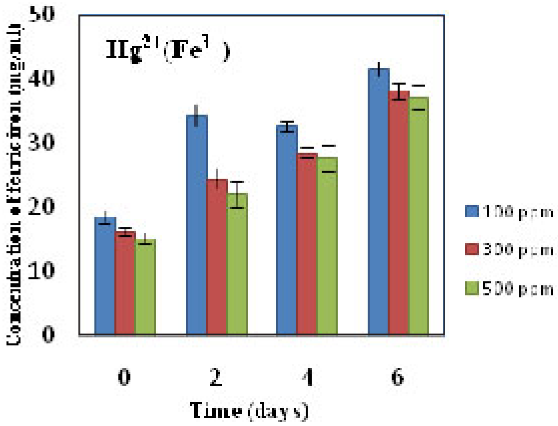

Fig. 4. Curve of measurement of ferric iron (Fe^{3+}) concentration in TK medium containing of Hg^{2+}

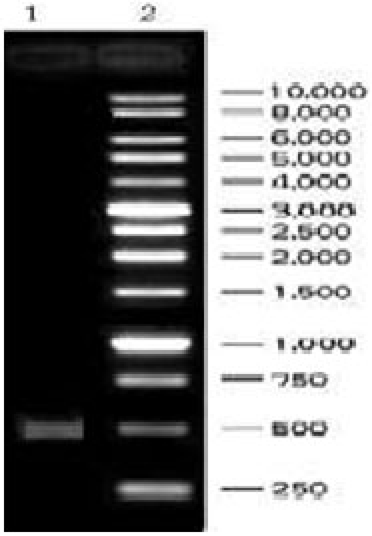

Fig. 5. 16s rRNA fragment amplified from the genomic DNA isolate d from *At. ferrooxidans AL*

forward primer G1-F (5'-GAAGTCGTAACAAGG-3') and reverse primer L_1-R (5'-CAAGGCATCCACCGT-3')[20]. PCR was carried out at a final volume 50µl, containing in each case 1µl each 10µ sense/ antisense primer, 1µl dNTP (0.4mM), 5µl 10X PCR buffer, 1.2µl $MgCl_2$ 50mM, 0.2 µl 1.25 u/µl Taq DNA polymerase and 1µl (10-100ng) of the genomic DNA. The amplification program was 95°C for 5 minutes as initial denaturation, followed by 35 cycles of 94°C for 45 second, 58.1°C for 1 minute and 72ÚC for 45 second, and finally extension was carried out at 72°C for 10 minutes. PCR product of the expected size (approximately 500bp), were checked by %1 agarose gel electrophoresis stained with 1% ethidium bromide and 1X TAE electrophoresis buffer. PCR product was directly sequenced by automated sequencing 3700 ABI (Gene fanavaran, Macrogene Seoul, Korea). Result of sequencing showed 16SrDNA partial sequence has 100% similarity with 16SrDNA of *At. ferrooxidans* strain ATCC23270.

Acidithiobacillus spp. is classified as chemolithotrophic and acidophilic bacterium. It can grow autotrophically by using ferrous iron and elemental sulfur as sole energy sources. *Acidithiobacillus ferrooxidans* is a gram-negative, mesophilic acidophilic bacterium and oxidizes Fe^{2+} to Fe^{3+} [6, 15,21].

Underground mines have large amounts of insoluble toxic metals. Oxidizing iron bacteria present in the mines, oxidize sulfide minerals by ferric iron (Fe^{3+}), followed by production of ferrous iron (Fe^{2+}) and reduction of sulfur compounds. Reduced sulfur compounds are oxidized to sulphuric acid and convert ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). Ag^{1+} and Hg^{2+} are released during activity of mineral oxidation and because of

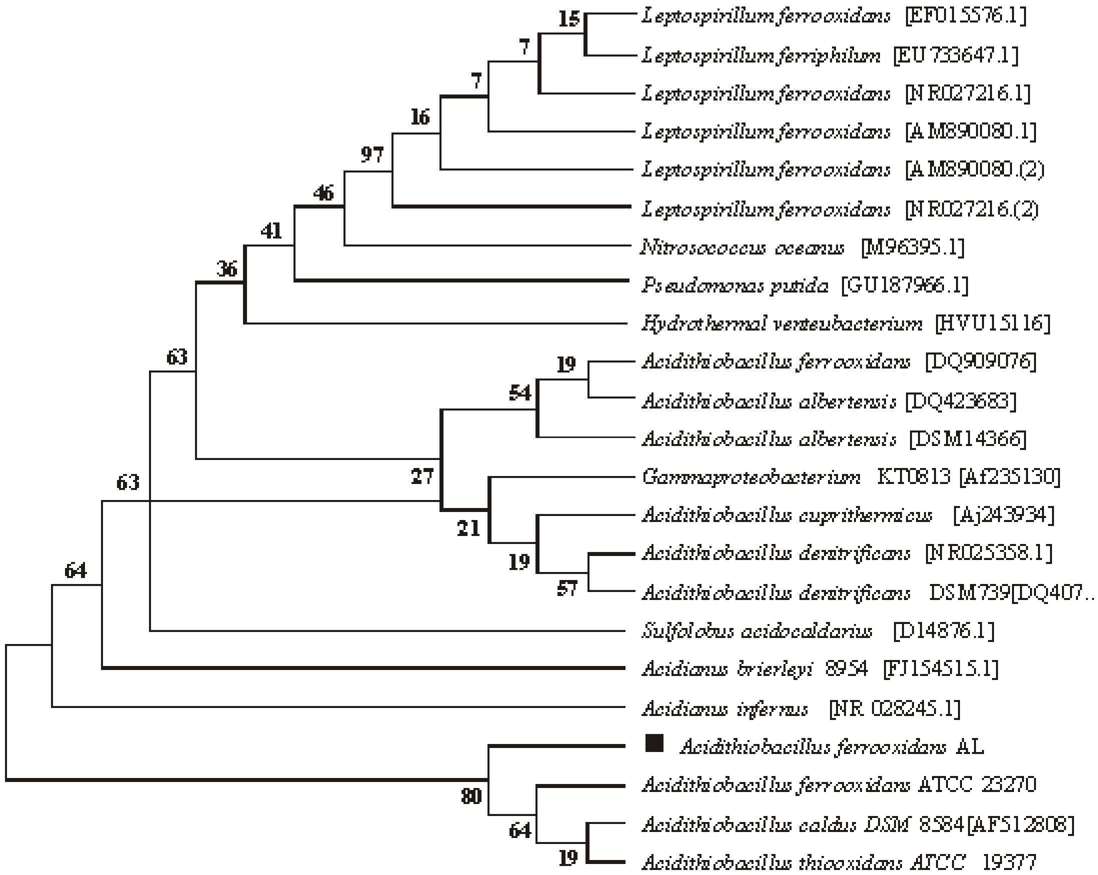

Fig. 6. Phylogenetic tree derived from 16s rRNA sequence of strain AL

presence of acidophilic bacteria such as *At. ferrooxidans* and *At. thiooxidans* in this mines, bioaccumulation in this mines are observed[5].

In general, oxidation of sulfide minerals is done in the following

$$MS + 2Fe^{3+} \longrightarrow M^{2+} + S + 2Fe^{2+}$$

Bacteria

$$2Fe^{2+} + 2H^{+} + 1/2O_2 \longrightarrow 2Fe^{3+} + H_2O$$

And

Bacteria

$$MS + 1/2O_2 + 2H^{+} \longrightarrow M^{2+} + S + H_2O$$

Acidophilic bacteria have a resistance mechanism to toxic metals that are concluded the following steps:

1. Efflux of the toxic metal to out of the cell.
2. Enzymic modification and conversion.
3. Banding specific protein to toxic metal in intracellular.
4. Exclusion toxic metal by a permeability barrier.
5. Reduction in sensitivity of cellular targets[4].

It is reported that, resistance mechanisms to Hg^{2+} in strains of *At. ferrooxidans* is based on reduction of Hg^{2+} to Hg° by flavoprotein and transferring outside of cell[16].

Also, several different mercury resistance operons have been characterized from strains of *At. ferrooxidans*. The mercury resistance operon from *At. ferrooxidans* T3.2 and *A. ferrooxidans* Tn5037 consists of: merR which, encodes a unique positive regulatory protein that twists the operator DNA to allow mRNA formation and merP encodes a proteins that binds to Hg^{2+} in periplasmic and gene expression product by merA, Hg^{2+} ion is converted to Hg° [10].

Therefore, those strains can be used for bioremediation of mercury-polluted acidic sites[19].

The toxicity of Ag^{1+} ions to *At. ferrooxidans* and *Leptospirillum ferrooxidans* growth and oxidation of Fe^{2+} has been recorded[9]. In fact, the reason of reduction of oxidation activity in acidophilic bacteria in mines is substitution of toxic ions with Fe^{2+} in active site of cytochrome-c oxidase[4].

The result of this study showed that, high concentrations of Ag^{1+} and Hg^{2+} ions will reduce rate of bacterial growth[13]. Therefore, we can conclude that resistance of bacteria to toxic metals is applicable in certain concentration and is not unlimited and the bacteria are able to grow in specified concentration of toxic metals.

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