Original article

Investigation of some heavy metals toxicity for indigenous *Acidithiobacillus ferrooxidans* isolated from Sarcheshmeh copper mine

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Abstract

Introduction and objective: Today mining operations in the metallurgy generate secondary products which cannot be used directly in the basic technology. The mineral industry is increasingly faced with the need to process low grade ores and waste from current mining operations. Heap bioleaching of low grade sulfide is a developing technology that has been applied to the extraction of metal from secondary sulfide minerals. The microorganisms used in the bioleaching of the low grade sulfide ores are mostly different strains of *Thiobacillus*. The resistance of indigenous *Acidithiobacillus ferrooxidans* to metals such Cd^{+2}, Co^{+2} and Zn^{+2} was investigated in the process of bioleaching tailing and low grade ore.

Materials and methods: Iron-sulfur oxidizing bacterium, *At. ferrooxidans*, has been isolated from pregnant leaching solution (PLS) of the Sarcheshmeh copper mine. At. Ferrooxidans was isolated, then it's resistance to some heavy metals was investigated with shaking flasks at 150rpm and 30°C.

Results: The results indicated indigenous *At. ferrooxidans* tolerated high levels of Zn^{2+} about 30 (g/L) concentration, but it had less tolerance to Cd^{2+} and Co^{2+} (10g/L Cd^{2+} and 110ppm Co^{2+}).

Conclusion: This experiment demonstrated that Zn^{+2} toxicity is less than Cd^{+2} and Co^{+2} for isolated *At. ferrooxidans.* For this reason, isolated *At. ferrooxidans* can be used for bioleaching of tailing with high amount of zinc and waste with relative amount of cadmium.

Significance and impact of the study: Using of isolated *At. ferrooxidans* in bioleaching of low grade sulfide ore which can tolerate presence of other heavy metals in mineral concentrate.

Keywords: Biomining; Resistant bacteria; Heavy metals; *Acidithiobacillus ferrooxidans*

Introduction

Bacterial leaching processes are still applied for copper, gold, silver and uranium extraction from low grade and refractory ores. With the use of bacteria it is possible to extract valuable metals from the primary and secondary raw materials and obtain them subsequently in the metallic form by application of common hydrometallurgical methods. Many processes in metallurgy generate secondary products, which cannot be used directly in the basic technology again [1,2]. According to the regulation 383/2001 Coll metals such as Zn, Cd, Hg, As, Co, etc. are most frequent grounds of metallurgical waste enlistment into dangerous waste. For this reason using and tolerance of these metals by microorganisms are important in bioleaching process of low grade ore and waste.

A number of works have tried to find solution to the problem at present deposited metallurgical wastes. They deal with availability of bioleaching application to this sort of waste and removing heavy metals from waste by bacteria [3]. In some processes e.g. bioleaching, microorganisms play a major role as catalysts of chemical reactions, which in their absence, would be carried out at low speed, making the process inefficient. Today it is accepted that iron oxidizing microorganisms are critical for keeping a high oxidizing potential in solution [4].

Acid-leaching solution characterized by high metal concentrations that are toxic to most life and have historically been considered sterile. However, microorganisms surviving in acid leaching environments should possess the most advanced metal resistance mechanisms, making them ideal systems to study and improve understanding of metal resistance especially in leaching of tailing ore and dangerous wastes [5]. The introduction of heavy metals, in various forms, in the environment, can produce considerable modification of the microbial communities and their activities. In the naturally polluted environments, the response of microbial communities to heavy metals depends on the concentration and availability of metals and is dependent on the action of multiple factors such as the type of metal, the nature of medium and microbial species.

The presence of metals in the growth medium allowed us to maintain the tolerance of bacteria at a comparable level with that observed in naturally polluted environments [6,7]. A number of microorganisms have the capacity of solubilizing heavy metals present in aqueous solution bioleaching which are able to adsorb heavy metals through their cellular structures biosorption or can precipitate heavy metals in solution to facilitate removal of the contaminant bioprecipitation [8]. An important characteristic of the acidophilic chemolithotrophs is their general tolerance to high concentration of metallic and other ions and resistant acidophilic bacteria have been isolated from environments where heavy metal levels are elevated from mining [8,9].

For many years *At. ferrooxidans* was assumed to be the most important microorganism in the bioleaching of sulfide ore operating at temperature lower than 40°C [10,11]. Major contribution of *At. ferrooxidans* to metal extraction is its ability to attack sulfide containing minerals and convert the insoluble sulfides of metals such as copper, lead, zinc or nickel to their soluble metal sulfates [12]. *At. ferrooxidans* is an obligate chemolithoautotrophic bacterium that uses elemental sulfur and/or reduced sulfur compounds as well as ferrous iron as a source of energy and carbon dioxide as a source of carbon that grow optimally at pH<3, [13-15], so

investigation of metal resistance of this bacteria due to its important role in bioleaching of low grade ore and tailing is of interest. The purpose of this work is to determine the tolerance levels of isolated indigenous *At. ferrooxidans* to the following heavy metals ion: Cd^{2+}, Co^{2+} and Zn^{+2} for the possible application in bioleaching of waste tailing consist of each heavy metals.

Materials and methods

Microorganisms

Pregnant leaching solution (PLS) was obtained from Sarcheshmeh copper mine in the region of Kerman located in the south of Iran and used for isolating indigenous *At. ferrooxidans*.

Media

The medium used to grow and maintain *At. ferrooxidans* was 9K medium [16], containing $FeSO_4.7H_2O$ (44.0g/l), $(NH_4)_2 SO_4$ (3.0g/l), $MgSO_4$ (0.5g/l), K_2HPO_4 (0.5g/l), KCl (0.1g/l) and $Ca(NO_3)_2$ (0.01g/l). The sodium thiosulfate liquid medium was used to differentiate iron and sulfur oxidizing *At. ferrooxidans* from *Leptospirillum ferrooxidans*, [17,18], because it cannot use sulfur and its derivatives as the energy source. This medium contains $Na_2S_2O_3$ (5g/l as the energy source), $(NH_4)_2 SO_4$ (0.4g/l), $MgSO_4$ (0.5g/l), K_2HPO_4 (3g/l) and $CaCl_2$ (0.25g/l). 9K modified solid medium [19] was used to get colonies of *At. ferrooxidans* on solid medium.

This medium was prepared according to the above method with the following modifications: I) agarose was used at the concentration of 8g/l instead of 6g/l, II) agarose was washed with distilled water in 10mins for three times. Inoculated solid medium with 10^{-2} serial dilution of bacterial suspension was incubated at 30°C in humid atmosphere for two weeks to develop maximum number of colonies. 9K liquid media were inoculated with single colonies for other experiments.

Experimental conditions

Experiment was carried out in 250ml flasks containing 50ml of medium and 10 % (v/v) of inoculum. The initial pH was adjusted at 2.5 in 9K medium in order to avoid the excessive precipitation of ferric ion production and on 3.0 for sodium thiosulfate medium. Flasks were incubated at 30°C on a rotatory shaker at 150rpm [12]. The pure colonies of *At. ferrooxidans* obtained on 9K modified solid medium. Growth and morphology of pure colonies were compared to ATCC 23270 *At. ferrooxidans*.

Influence of heavy metal ions on the bacterial growth examined by adding different concentration of each of the following ions: Co^{2+}, Cd^{2+} and Zn^{2+}. The salts used in this study were $CdCl_2$, $CoCl_2$ and $ZnCl_2$ (Merck, Germany). All of the mediums supplemented with metal chloride solution to a final volume of 50ml. Sterile controls were also run by replacing the bacterial inoculum with an equal volume of medium. All of the experiments carried out in 30°C and 150rpm by performing several series of experiments with different concentration of each metal in triple with a control culture medium.

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 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 of 50µl, containing in each case 1µl

each 10µ sense/ antisense primer, 1µl dNTP (0.4mM), 5µl 10X PCR buffer, 1.2µl $MgCl_2$ 50mM, 0.2µl 1.25u/µl Taq DNA polymerase and 1µl (10-100ng) of the genomic DNA.

The amplification program was 95°C for 5mins as initial denaturation, followed by 35 cycles of 94°C for 45 seconds, 58.1°C for 1min and 72°C for 45 seconds, and finally extension was carried out at 72°C for 10mins [4]. 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.

Analytical determination

To follow Bradford protein assay, heavy metal resistance was determined by the flasks containing 9K medium which were periodically analyzed for the bacterial growth [21]. The blue complex was measured by spectrophotometry at 595nm. In this method, 5ml colour reagent is used for 100µl sample volume. The preparation a calibration curve, protein standard bovine serum albumin at a concentration of 5-55µg/ml in distilled water was used as a stock solution.

Results

At. ferrooxidans resistance to different concentrations of each heavy metals

The heavy metal resistance of *At. ferrooxidans* was studied, in pure culture of 9K medium. Rate of the bacterial growth during bioleaching in 9K medium with presence of different concentration of each heavy metal was measured by Bradford protein assay. Changes in bacterial population in 9K medium contained cadmium (II) chloride, cobalt (II) chloride and zinc (II) chloride in series concentration 10, 110, 210 and 310ppm and 10, 20 and 30g/l are shown in figures 1 to 8, respectively.

The results showed that isolated *At. ferrooxidans* grew in the presence of 10, 110, 210 and 310ppm of cadmium (II) chloride and zinc (II) chloride, but grew shortly with cobalt (II) chloride with the same concentrations and cell growth decreased. Isolated *At. Ferrooxidans*, grew in 10ppm of each metal in 9K medium, however the bacteria grew better without metals in the medium. The bacteria didn't tolerate 10, 20 and 30g/l of cobalt (II) and cadmium (II) chloride but grew in the presence of zinc chloride with 10, 20 and 30g/l concentration in the medium (Fig. 8).

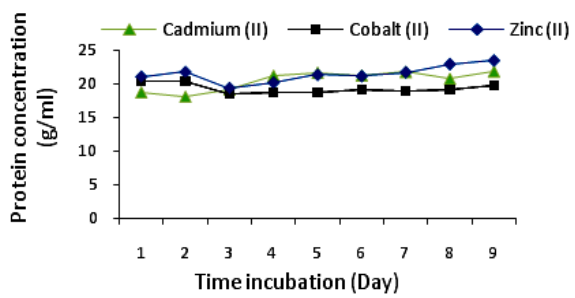

Fig. 1: Bacterial growth in the presence of 10ppm of each heavy metal

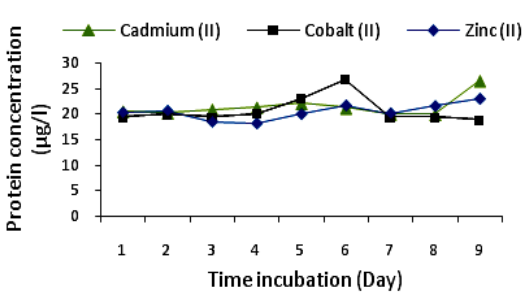

Fig. 2: Bacterial growth in the presence of 110ppm of each heavy metal

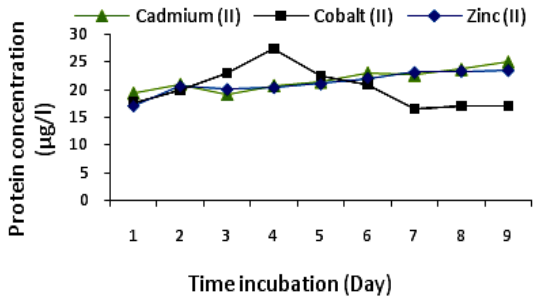

Fig. 3: Bacterial growth in the presence of 210ppm of each heavy metals

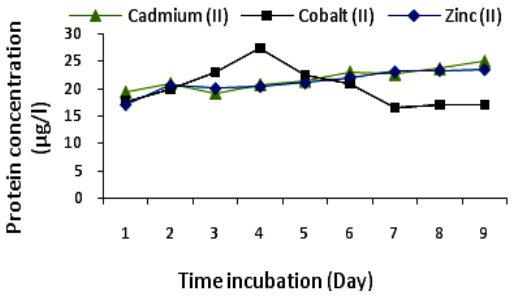

Fig. 4: Bacterial growth in the presence of 310ppm of each heavy metal

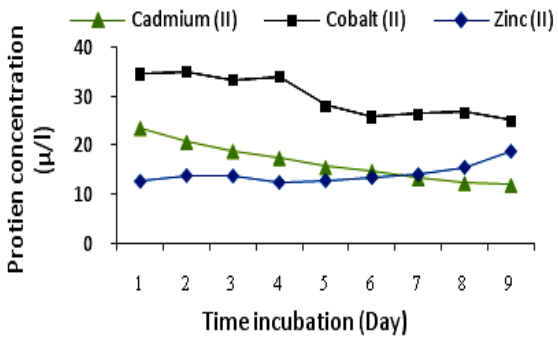

Fig. 5: Bacterial growth in the presence of 10g/l of each heavy metal

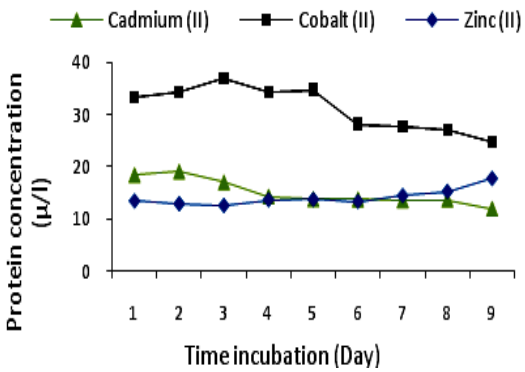

Fig. 6: Bacterial growth in the presence of 20g/l of each heavy metal

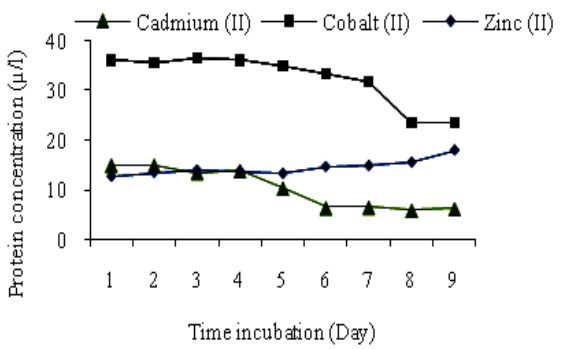

Fig. 7: Bacterial growth in the presence of 30g/l of each heavy metals

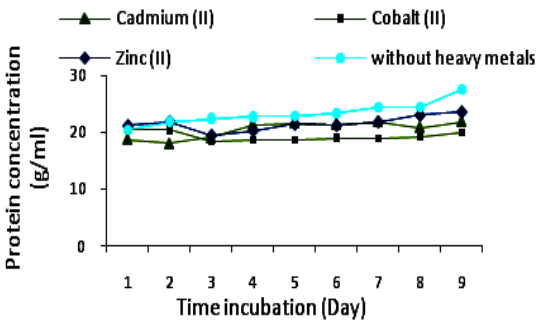

Fig. 8: Comparison bacterial growth in the presence and absence 10ppm of each heavy metals

Strain determination by genetic analysis

PCR products run on electrophoresis gel showed sequences of PCR product had 500bp nucleotides (Fig. 9). The comparison between sequences obtained from this strain with sequences presented in NCBI showed that this bacterium had 100% similarity with *AT. ferrooxidans* ATCC 23270. The results of genetic analysis confirmed the biochemical tests of isolating and identifying the bacterium.

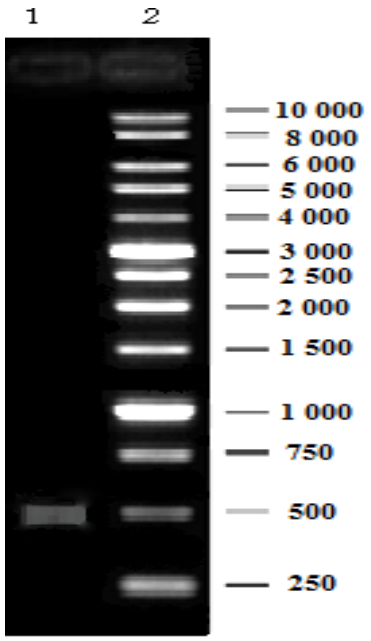

Fig. 9: PCR product of 16SrRNA fragment of indigenous bacterium 2. DNA ladder

Discussion

It is well known that bacterial heavy metal resistance depends on the nature of strain. The level of resistance of several acidophilic bacterium and archaea to As^{3+}, Cu^{2+}, Cd^{2+}, Zn^{+2} and Ni^{2+} have been reviewed and will not be covered here in detail [8,9]. The results presented here indicated that indigenous *At. ferrooxidans* growth is affected by the level and type of heavy metal in the medium, which is consistent with some previously reported findings [9].

Maximum tolerance concentrations determined by other authors for several strains of *At. ferrooxidans* are shown in table 1. From these data, it is possible to make a comparison between MTCs found in the literature (Table 1) and the level of resistance obtained in this work. This comparison reveals a high degree of variability in the resistance to some heavy metals for different strains of the same bacteria. Zn^{+2} ion gives rise to the lowest toxic effect in pure culture of isolated *At. ferrooxidans*. Results of this work showed indigenous *At. ferrooxidans* tolerated 30g/l of Zn^{+2} ion. The same tolerance concentration of Zn^{+2} ion were prepared by other authors [8], but the concentration obtained in this research was lower than the level of tolerance concentration Zn^{+2} 70 g/l [5]. It is necessary to do further test to clarify whether this bacterium is resistant to this concentration.

Results of this work agree with Brahmaprakash *et al*. [23] in the resistance of the strains of *At. ferrooxidans* to 600mM zinc. Zinc occurs as the divalent ion Zn^{2+} which is present in a number of enzymes. Zn^{2+} toxicity is based on complex formation with various cellular components, and it may be due to competitive inhibiting of Fe^{2+} oxidation. Toxicity of Zn^{2+} for *At. ferrooxidans* depends on the growth substrate [22].

Table 1: Level tolerance some heavy metals for *At. ferrooxidans* obtained by other authors

Heavy metal	MTC (g/l)	References
Cd (II)	5.6, 1.12,.01	5,9,18
Zn (II)	6.9, 120	9,18
Co (II)	30	9

In this work zinc tolerance of *At. ferrooxidans* done in the presence of ferrous ion as the source of energy. Cd^{2+} is toxic to microorganisms through a variety of mechanisms, including binding to thiol groups, protein denaturation and interaction with potassium and zinc metabolism. Toxicity of Cd^{2+} for *At. ferrooxidans* was found to be low in comparison to Hg^{2+}, inhibiting Fe^{2+} oxidation by 2% at 10mg/l. However, Cd^{2+} toxicity is greater than zinc and copper to *At. ferrooxidans* [5].

The comparison between the results in table 1 and the results in this experiment, showed that level of tolerance concentration of Cd (II) and Co (II) for indigenous *At. ferrooxidans* was lower than results obtained by other authors for 5.6 and

1.12g/l Cd (II) tolerance concentration [9,18]. Results of Bradford protein assay showed the isolated bacteria didn't tolerate Co^{2+} and Cd^{2+} in 10, 20 and 30g/l. Indigenous *At. ferrooxidans* has high sensitivity to cobalt (II) as it couldn't grow in the presence of 10, 20 and 30g/l Co(II) as well as grew shortly with 110, 210 and 310ppm in the medium.

Acidophilic microorganism must be resistant to heavy metals due to the selective pressures that metal-rich acidic environments pose. As may be predicted, levels of resistance to different heavy metal on one hand and bacterial strain resistance on the other hand show considerable strain variation. On the basis of the obtained results, it is possible to establish the same order of toxicity for pure culture of indigenous *At. ferrooxidans*: Co^{+2}> Cd^{+2}> Zn^{+2}.

Conclusion

The studied strain, which was isolated from copper mine environment, has a particular behaviour pattern, similar tolerance level for Zn^{+2} or lower tolerance levels for Cd^{+2} and Co^{+2} in comparison to the concentrations found in contaminated media for heavy metal ions in other studies. This trend could be due to the presence of certain metallic species in the mine environment which makes the bacteria resistant to high levels of heavy metal ions in other research. Further studies should be carried out to find the tolerance levels of indigenous *At. ferrooxidans* to Zn^{+2} ion in indigenous.

Conflict of interest statement: All authors declare that they have no conflict of interest.

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