A study of *Acidithiobacillus ferrooxidans* DSMZ 583 adaptation to heavy metals

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Abstract
In this study the ability of *Acidithiobacillus ferrooxidans*, with regard to the biorecovery of heavy metals in shake flask has been investigated. Adaptation experiments with the single metal ions Ni, Co, V, Mo, W and a mixture of the first four metal ions in the medium was developed through serial sub-culturing. Adaptation showed that *A. ferrooxidans* could tolerate up to 2.3 g/l Ni, 1.4 g/l Co, 1.4 g/l V, 0.045 g/l Mo and 0.005 g/l W, singly. In the presence of multi-metals considering a mixture of Ni-Co-V-Mo, the bacteria was able to tolerate up to 1.5 g/l Ni, 0.8 g/l Co, 0.8 g/l V and 0.05 g/l Mo in steps of 50-100 mg/l for Ni, Co and V, while for Mo and W with increments in concentration of 1-5 ppm, because of the high toxicity of these two metals to the bacteria. Adaptation of the bacterial strain was carried out in batch cultures by continually growing the bacteria in an environment containing increasing concentrations of the toxic metal such that a culture tolerant to the toxic metal proliferates. Effects of several variables such as pH, Eh, bacterial concentration in the solution as well as its resistance to heavy metals and ferrous and ferric iron concentration for the specific bacterial growth rate, were also investigated. This study showed that the various concentrations of Ni, Co and V had little effect on the oxidation of ferrous iron or the cell growth of *A. ferrooxidans*, whereas Mo and W ions were very inhibitory towards the Fe^{+2} oxidation ability of *A. ferrooxidans*.
Keywords**:** *Acidithiobacillus ferrooxidans*; Adaptation; Biorecovery; Heavy metals

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INTRODUCTION

Acidithiobacillus ferrooxidans is an acidophilic bacterium which can either grow on reduced sulphur compounds or on ferrous iron [Fe^{+2}] (Solisio *et al*., 2002; Nemati *et al*., 1998). It utilizes energy obtained from oxidation of inorganic sulphur compounds as well as ferrous iron dissolved in liquid medium. Ferrous iron is oxidized to ferric iron [Fe^{+3}] by *A. ferrooxidans* under acidic conditions, while its chemical oxidation by means of oxygen can be extremely low.
The reaction mechanism for the above mentioned microbial activity (Solisio *et al*., 2002) can be described as follows:

$$4FeSO_4+2H_2SO_4+O_2 \xrightarrow{Thiob.ferr.} Fe_2(SO_4)_3+2H_2O \quad (1)$$
$$Fe_2(SO_4)_3+6H_2O \rightarrow 2Fe(OH)_3+3H_2SO_4 \quad (2)$$
$$3Fe_2(SO_4)_3+12H_2O \rightarrow 2HFe_3(SO_4)_2(OH)_6+5H_2SO_4 \quad (3)$$

The oxidation of ferrous iron by *A. ferrooxidans* is responsible for the large increase in the redox potential (Eh) of Fe^{+3}/Fe^{+2}. Precipitation of ferric iron as (1) sulphate (2) iron hydroxides or (3) jarosite can explain the acidification of the solution.

The equilibrium of reactions (2) and (3) shifts to the right by precipitation of ferric compounds; which is responsible for H_2SO_4 production that can be utilized in leaching of the metals. Therefore, metals solubilization occurred by a chemical effect in the presence of H_2SO_4.

It is well known that ferrous iron oxidation by *A. ferrooxidans* rapidly decreases at pH values greater than 2.5 (Pesic *et al*., 1989; Nakamura *et al*., 1986). In

most reports of ferrous iron oxidation by acidophiles, bacterial catalysis is significant only up to pH ~ 3.5 (Pesic *et al*., 1989). According to the chemiosmotic theory, a decrease in the ferrous iron oxidation activity of *A. ferrooxidans* only occurs when there is an increase in the pH of the surrounding environment (Ingledew, 1982).

In bioleaching and biorecovery systems, metal ions, such as Ni^{+2}, Co^{+2} accumulate, and beyond certain concentrations become toxic to bacteria (Mei Li and Jun Ke, 2001). Ginn *et al*. (2006) have proposed that the toxic effects of heavy metals on the growth of microbial cultures are not just a function of the heavy metal concentration, but they are also dependent on the cumulative contact time. However, prolonged heavy metal-microorganism contact times may result in microbial acclimatization to the particular heavy metal; thus, acclimatized microorganisms can ultimately grow at significantly higher heavy metal concentrations, compared with the same non-acclimatized microbial strains (Dilek *et al*., 1998; Giller *et al*., 1998; Chang *et al*., 1986). Repeated sub-cultivation of a microorganism in the presence of increased concentrations of heavy metals may allow the isolation of heavy metal-tolerant mutants (Shehata and Whitton, 1982). The adaptation of acclimatized microorganisms to relatively high heavy metal concentrations has often been attributed to the activation of alternative biochemical pathways which allow cells to continue growing.

The adaptation of acclimatized microorganisms to relatively high heavy metal concentrations has often been attributed to the activation of alternative biochemical pathways which allow cells to continue growing (Nies, 1992). Haghshenas *et al*. (2009) confirm that another important point when adapting *A. ferrooxidans* cells to metal ion or pulp is the use of criteria for deciding when the cell can be considered as ‘‘adapted”. In previously mentioned studies on adapting *A. ferrooxidans* to metal ions, the cells were usually considered adapted when the ferrous iron oxidation rate became similar to those obtained in metal ion free media; however, in studies aimed at adapting acidophilic bacterial cultures to metal sulfide sources different criteria have been used, and in many cases the criterion employed has not been explicitly stated. This might partly explain why the adaptation period reported by various investigators encompasses such a wide range of time (Haghshenas *et al*., 2009). For example, in the study by Xia *et al*. (2008). *A. ferrooxidans* cells were considered as adapted to chalcopyrite when the cell concentration at the end of the culturing period and at a given pulp density reached 10^7 cells/ml. The adaptation criterion in the study by Astudillo and Acevedo (2008) was the appearance of significant amounts of soluble iron and copper and a Fe^{+2}/Fe^{+3} ratio lower than one, indicative of an efficient ferrous iron biooxidation. However, in the study by Mason and Rice (2002) adaptation was considered to have been achieved when the metal dissolution rate in the two consecutive subcultures of *A. ferrooxidans* on a nickel sulfide concentrate became similar.

The prices of metals such as Mo, W, Ni, Co and V in the world market determine the economic viability of metal recovery from the environment and especially from spent catalysts (Marafi and Stanislaus, 2008). Fresh catalysts consist of molybdenum oxide mixed mainly with oxides of V, Co or Ni on an alumina (Al_2O_3) carrier. During the operation of the catalysts, trace amounts of V and Ni impurities in the crude oil gradually deposit on-to the catalysts. The operating conditions are favorable for the formation of metal sulfides, such as those of Mo, Co, Ni and V (Biswas, 1985)

The inhibition of *A. ferrooxidans* growth and activity by Ni has been more widely studied with respect to the other inhibitory metals, such as Co, V, Mo and W. The results indicate a greater tolerance to Ni but, at the same time, huge variations between strains, ranging from 0.1 mM (6 mg/l) (Tuovinen *et al*., 1971) to 850 mM (50 g/l) Ni^{+2} (Natarajan and Iwasaki, 1983). *A. ferrooxidans* has been reported to tolerate Ni^{+2} up to a concentration of 160 mM (Leduc and ferroni, 1994). But adapted strains of *A. ferrooxidans* can tolerate Ni^{+2} and Co^{+2} up to concentrations of 1M and 500 mM, respectively (Dew *et al*., 1999). Metal resistance of bacteria can be strengthened by adapting the culture to an environment containing the various metal cations (Sampson and Phillips, 2001). In comparison to the results of this study, a Ni tolerant strain (from a mining environment) was reported to also resist Ni concentrations as high as 160 mM (Leduc and Ferroni, 1994). However, they also reported that the growth of one strain of *A. ferrooxidans* was completely inhibited by Ni at a concentration of 1mM, and partially inhibited at 0.1 mM. In addition, they reported another strain of *A. ferrooxidans*, which was able to resist 170 mM of Ni, although it was unknown if the strain was adapted to a Ni environment (Sampson and Phillips, 2001). It was also reported that in the case of the mesophiles, during the oxidation of the ferrous substrate, the bacterium’s ability to oxidize Fe^{+2} was found to be unaffected at 8 mM of each of Ni and Co individually and

as the concentration of each metal increased, the oxidizing ability of the mixed culture became inhibited. Kai *et al*. (1995) was also able to adapt a strain of *A*. *ferrooxidans* to a modest Ni concentration of 17 mM (1 g/l) by carrying out 20 sub-cultures of the strain in ferrous iron growth medium. They showed that the growth response of the strain after 20 transfers in Ni-rich medium was similar to that of the unadapted strain in Ni free medium. Similarly, other researchers were able to adapt *A*. *ferrooxidans* to 21 mM (1.2 g/l) Ni^{+2}, which was capable of oxidizing 95% of the available ferrous ion under the conditions of the experiments (Dave and Pandhi, 1995). By contrast Li and Ke (2001) succeeded in adapting *A*. *ferrooxidans* to 510 mM (30 g/l) Ni^{+2} by repeated sub culturing at 30°C and pH 2 for a year.

Zeng and Cheng (2009) recently reviewed applications of the bioleaching process to recovering metal values from spent petroleum catalyst. In another study, the treatment of the spent nickel catalyst generated during the hydrogenation of vegetable oil was conducted using *A*. *thiooxidans* cultures (Bosio *et al*., 2008). More recently, some reports described bioleaching procedures applied to the recovery of metals from spent refinery catalysts by means of iron/sulfur oxidizing bacteria (Beolchini *et al*., 2010).

Although the adaptation process of *A*. *ferrooxidans* to heavy metal ions looks promising, however there is a lack of studies about the adaptation concerning a mixture of such heavy metals. Therefore, the objective of the present work was to investigate the influence of Ni, Co, V, Mo and W ions on the ability of *A*. *ferrooxidans* to oxidize Fe^{+2} ions, and thus determine the degree of resistance of *A*. *ferrooxidans* to such metals.

MATERIALS AND METHODS

Microorganism and bacterial culture preparation: A pure strain of *A*. *ferrooxidans* DSMZ 583 (PTCC 1646) obtained from Iranian Research Organization for Science and Technology (*IROST*) was used in this study. The cultures were maintained in shake flasks before being used for the sub-culturing experiments. The medium used for growing the *A*. *ferrooxidans* strain was composed of: KH_2PO_4 (0.4 g/l), $MgSO_4.7H_2O$ (0.4 g/l), $(NH_4)_2SO_4$ (0.4 g/l), and $FeSO_4.7H_2O$ (33.3 g/l, Merck). The experiment was performed in 500 ml Erlenmeyer flasks with 100 ml of the medium and 10% (v/v) inoculums with an initial density of approximately 4×10^7 cells/ml, on a rotary shaker at 180 rpm and 32°C. Deionized water was added to the flasks to compensate for evaporation losses. The pH of the solution was initially basic; therefore it was necessary to decrease the pH by using 0.1 N H_2SO_4 in order to create a favorable environment for the employed microbial strain. All of the experiments were conducted at least in duplicate.

Analytical methods: Analysis of ferrous and ferric iron concentrations of culture samples taken at different time intervals was carried out using a precise quantitative method, which is not affected by the presence of iron or *A*. *ferrooxidans* in solution (Mousavi *et al*., 2007). A 0.1 ml sample of the culture was mixed with 3 ml of 10% w/v 5-sulfosalicylic acid (SSA), followed by the addition of deionized water (97 ml). The absorbance of the resulting red-colored ferric sulfosalicylate complex was measured at 500 nm using a UV-VIS spectrophotometer (Digital model: SPECTRONIC $20D^+$, USA). Subsequently, 3 ml NH_4OH (25% v/v) was added to the above mixture, causing the SSA to form a yellow complex containing iron ions. Total iron concentrations was estimated by measuring the absorbance of the yellow SSA-iron complex at 425 nm (Karamanev, *et al*., 2002). Ferrous iron concentrations were then measured by calculating the difference between the concentrations of total iron and ferric iron. Bacterial population (cell density/ml) was estimated by the visual count method using an optical microscope (Olympus, BH-2, USA), and a Thoma chamber with a depth of 0.1 mm and an area of 0.0025. The pH and Eh of the culture samples were monitored at room temperature using a digital pH/Temp/Eh meter (Metrohm, model: 827 pH lab, Switzerland) calibrated with a low pH buffer.

Adaptation technique and analysis: The adaptation of the wild strain to heavy metal ions, in the medium, was developed through serial subculturing of the strain over a 6 months period. All the experiments were performed in a 500 ml Erlenmeyer flask containing 100 ml of medium. Experiments were carried out on a rotary shaker at 180 rpm and 32°C. Throughout the experiments, a 10% (v/v) sample of the metal-adapted bacterial culture giving an initial cell density of approximately 4×10^7 cells/ml, was used as inoculums. Culture samples were withdrawn at appropriate time intervals, and used for the analysis of Fe^{+2} concentrations. The pH and Eh values of the culture medium were also measured when each flask was sampled.

To adapt *A*. *ferrooxidans* to Ni, Co, V, Mo and W,

of the following salts; $NiSO_4.(6H_2O)$, $CoSO_4$, $NaVO_3$, $Na_2MoO_4.2H_2O$ and $Na_2WO_4.2H_2O$ were used for this purpose. Increasing concentrations of each of the salts were added to the culture media. In the case of Mo and W, which are highly toxic to *A. ferrooxidans,* the procedure was repeated many more times with addition of only 1-5 ppm of the mentioned ions, while for the other metals in each step, 50-100 ppm of ions were used for the adaptation procedure, and at each stage samples from the previous culture were transferred to a fresh medium containing a higher concentration of the ion.

Finally to investigate the effect and toxicity of a mixture of heavy metals on *A. ferrooxidans,* a quaternary combination of metal ions with specified concentrations of (Ni, Co, V and Mo) was also studied. Hence, the concentration of metals varied upon the adaptability of *A. ferrooxidans* to such heavy metals.

All growth experiments were continued until a ferric iron oxidation of approximately 80-90% (corresponding to a Fe^{+2}/Fe^{+3} ratio lower than one), was achieved (Astudillo and Acevedo, 2008)).

All experiments were conducted in duplicate. The pH and Eh (redox potential in mV) values, ferrous and ferric concentrations (mmol/l) were measured twice for each. pH and Eh accuracies are ± 0.003 and ± 0.2 mV, respectively. Data points in the Figures represent means with error bars shown (±S.D.).

RESULTS

In this study, the adaptation of *A. ferrooxidans* up to 2300 ppm Ni, 1400 ppm Co, 1400 ppm V, 45 ppm Mo and 5 ppm W was conducted through several sub-cultures over a 6 month period. Following the adaptation period, the *A. ferrooxidans* culture easily tolerated Ni, Co and V; but in presence of Mo and W its lag phase of growth increased. The influence of the Ni, Co, V, Mo and W ions at different concentrations on the Fe^{+2} oxidizing ability of an adapted culture was determined during the course of growth and oxidation of ferrous iron, which are demonstrated in Figures 1A, 1B, 1C, 1D, 1E, and 1F. The *A. ferrooxidans* of this study was able to adapt to the mentioned heavy metal ions and could oxidize 81-92% of the available ferrous ions under the conditions of the experiments which were all carried out at an initial pH of 1.4, the optimum pH value of this type of microorganism, a rotation speed of 180 rpm and a temperature of 32°C.

The bacterial population (cell density/ml), ferrous iron oxidation and iron conversion percentage measurements with regard to the influence of a combination of metals (Ni, Co, V and Mo), are shown in Table 1. The maximum tolerance of *A. ferrooxidans* to a combination of heavy metals in the culture medium was achieved at 1500 ppm Ni, 800 ppm Co, 800 ppm V and 50 ppm Mo. As it is obvious, increasing the metal concentration resulted in increasing of the tolerance of *A. ferrooxidans*. Heavy metals are able to exert harmful effects due to their strong coordinating capabilities. These toxic effects include blocking of biologically important functional groups and the denaturation of enzymes (Valix and Loon, 2003). Therefore, the time of the ferrous iron oxidation also increased, because of the mentioned reason. According to the Table 1. The order of the toxicity of the metals in a mixture of Ni, Co, V and Mo is as follows:

Mo > V ~ Co > Ni

DISCUSSION

Ferrous and ferric variations: The oxidation of the ferrous iron substrate under the influence of maximum concentrations achieved by these metals with the culture shows that the ferrous oxidizing ability of the culture was unaffected in the presence of heavy metal mixtures at high concentrations, with almost complete substrate oxidation being achieved over the experimental time period. However, when the metal concentrations were at a maximum, the rate of oxidation was found to be reduced and thus the time needed to be adapted increased (Table 1).

The change in Fe^{+2} and Fe^{+3} concentrations with respect to time by *A. ferrooxidans* cultures in the presence of Co (1400 ppm), V (1400 ppm), Mo (45 ppm), W (5 ppm) and Mixed metals (#7) are demonstrated in Figures 2A, 2B, 2C, 2D, and 2E. As can be seen at the early stage of the reaction, which coincided with the lag phase of bacterial growth, the oxidation of the ferrous iron was slow. The extent of the lag phase was dependent on the initial concentration of ferrous iron. It is also evident that, for all of the experiments, increasing of the ferric ions is in concordance with decreasing of the ferrous ions.

pH variations: Some studies have shown that the adaptation of the acidophilic *A. ferrooxidans* to the heavy metals and their subsequent recovery is maximum at a pH range of 1.4-1.5 (Mohapatra *et al*. 2006; Fukuta *et al*., 2006).

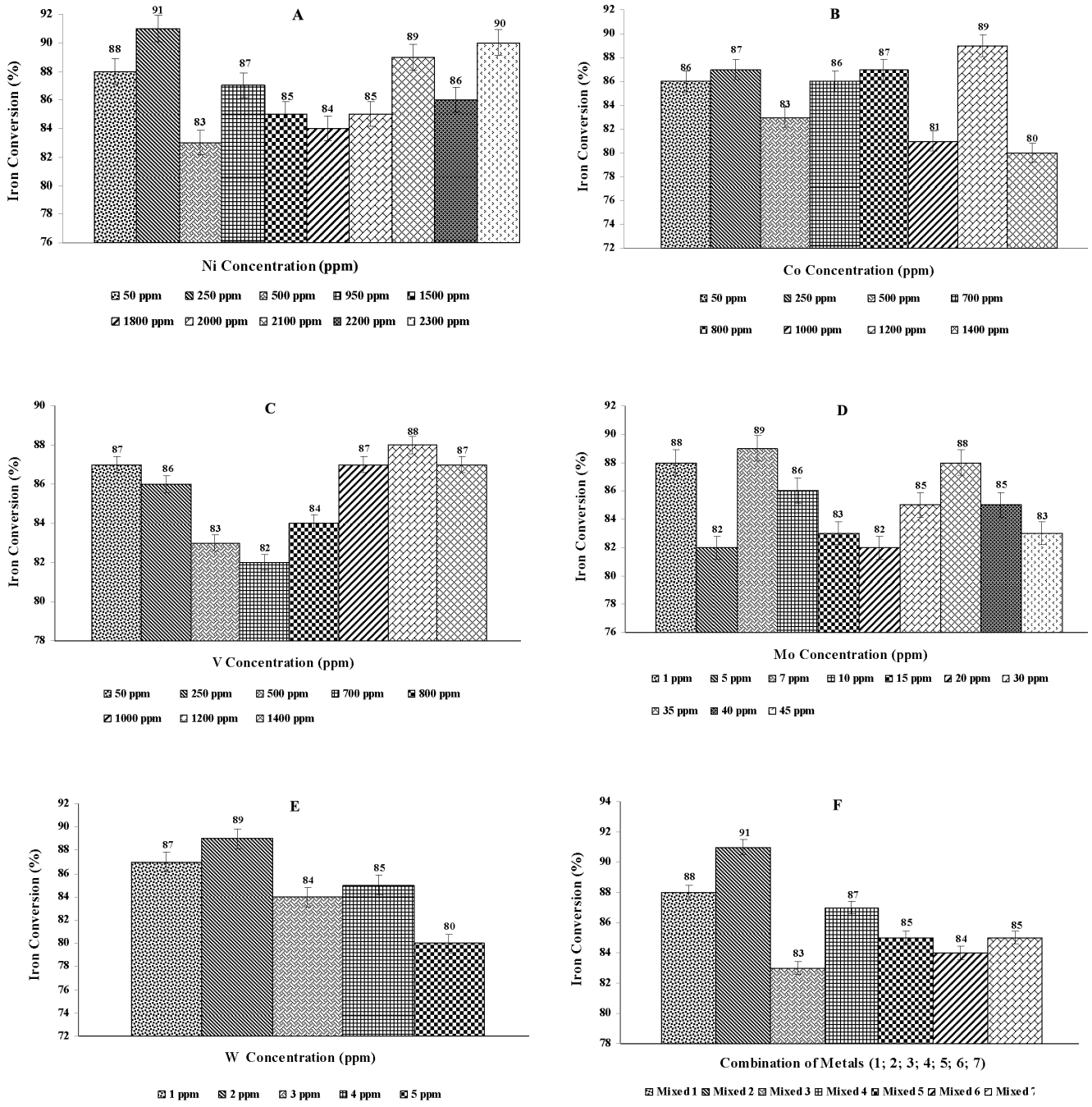

Figure 1. The influence of the metal ions at different concentrations on the Fe^{+2} oxidizing ability of an adapted *A. ferrooxidans* culture, in the presence of (A) Ni, (B) Co, (C) V, (D) Mo, (E) W and (F) combination of Ni, Co, V and Mo.

Since oxidation of ferrous iron is accompanied by the removal of acid, and the pH is ascending, the ferric iron thus formed is hydrolyzed in the aqueous solution (Mousavi *et al*., 2006). Hydrolysis of ferric iron produces acid, thus reducing the pH, to approximately 1.4.

Figures 3A, and 3B illustrate the changes in the pH and Eh of the culture medium respect to time during biooxidation of Fe^{+2} at low concentrations of Ni (200, 400, 950 and 1800 ppm), while Figure 3C shows those variations at Ni concentrations of 2300 ppm (maximum concentration tolerated). Thus from the Figure 3A, 3B, and 3C, it could be concluded that at low concentrations of Ni, at the beginning of the biooxidation,

Table 1. Cell density, ferrous iron oxidation and iron conversion percentage measurements with regard to the influence of a combination of metals (Ni, Co, V and Mo).

Mixed Metals	Composition of heavy metal mixture (ppm)	Cell density 4 x10^6 (Cell/ml)	[Fe^{+3}] (mmol/l)	[Fe^{tot}] (mmol/l)	Iron Conversion (%)	[Fe^{+2}] (mmol/l)	Time of oxidation (days)
#1	Mo: 5; V: 250 Ni: 950; Co:250	11.54	75.83	90.96	83	15.13	6
#2	Mo: 10; V: 300 Ni: 1000; Co:300	11.23	75.28	91.43	82	16.15	6
#3	Mo: 15; V: 400 Ni: 1100; Co:400	12.65	74.46	84.77	87	10.31	6
#4	Mo: 20; V: 500 Ni: 1200; Co:500	13.40	80.21	90.96	88	10.75	12
#5	Mo: 30; V: 600 Ni: 1300; Co:600	12.97	76.38	89.53	85	13.15	13
#6	Mo: 40; V: 700 Ni: 1400; Co:700	12.56	81.03	95.24	85	14.21	14
#7	Mo: 50; V: 800 Ni: 1500; Co:800	9.03	97.73	113.10	86	15.37	50

pH increases and decreases afterwards to a level lower than the initial pH of 1.4, while reverse trend is observed for the Eh (Fig. 3). These types of variations are also demonstrated for the other metals in the investigation which are illustrated in Figures 5, 6, 7, 8, and 9. For all metals at low concentrations the trends of pH and Eh were ordinary, while for higher concentrations, the pH and Eh varied sequentially. The reason for these unusual variations at higher concentrations could be probably due to jarosite formation, complex formations of iron with other metals present in the solution or sequential oxidation-reduction-oxidation of the ferrous ions in the solution.

It was observed that an initial increase in pH to a maximum was as a result of Fe^{+2} oxidation, and a latter decrease in pH, accompanied by a falling off in Fe^{+3} concentrations; since the oxidation of ferrous iron is acid consuming and the hydrolysis of ferric iron produces acid, it is therefore obvious that the pH of the system influences the oxidation and hydrolysis of reactions. Furthermore, there is a reaction which competes with the hydrolysis reaction, giving products of basic ferric hydroxysulphates with the formula $MFe_3(SO_4)_2(OH)_6$, where M can be K^+, Na^+, NH_4^+, Ag^+, or H_3O^+. These hydroxysulphate precipitates are known as jarosites, which could be inhibitory towards the mixed culture. Jarosite precipitation is also an acid-producing reaction, which is dependent on the pH, temperature and on the ionic composition and concentration of the medium (Daoud and Karamanev, 2006; Jensen and Webb). The following is the formula for jarosite precipitation:

$$3Fe^{+3} + M^+ + 2HSO^-_4 + 6H_2O \rightarrow MFe_3(SO_4)_2(OH)_6 + 8H^+ \quad (4)$$

The pH and Eh measurements during the oxidation of ferrous iron in the presence of different concentrations of Ni ions, cell density and conversion percentage at the end of each step were measured which are shown in Table 2. In all cases Eh and pH of the culture media were in the range of 290-330 mV and 1.49-1.86, respectively, following the initial addition of H_2SO_4.

Cell growth and bacterial population measurement: Growth rate depends on the medium, genotype of the strain, temperature and the degree of aeration. As the density of the culture increases, the rate of division decreases until the bacteria reach a concentration

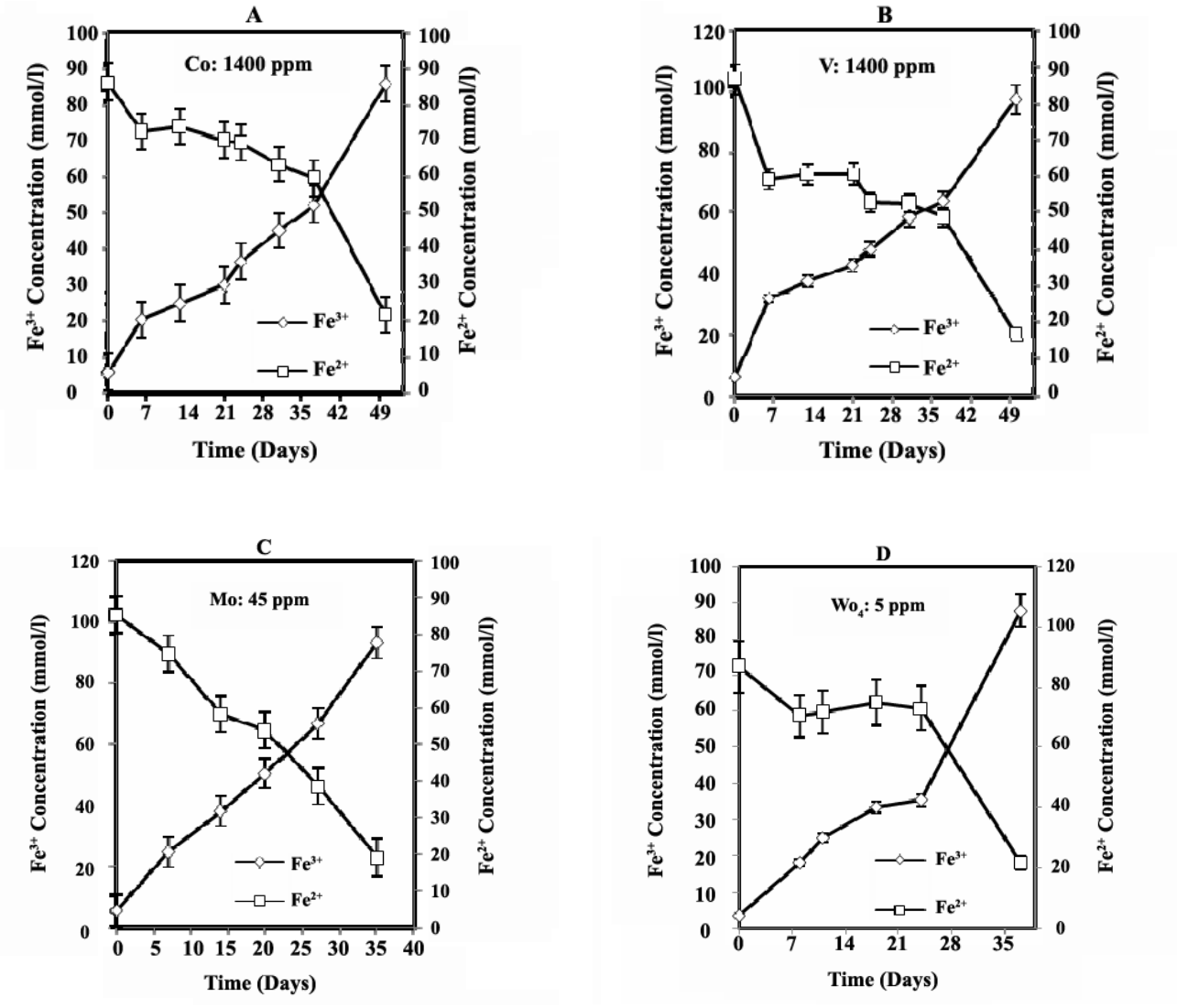

Figure 2. Changes in Fe^{+2} and Fe^{+3} concentrations with respect to time by *A. ferrooxidans*; effect of: A: Co (1400 ppm), B: V (1400 ppm), C: Mo (45 ppm), D: W (5 ppm) and E: Mixed metals (#7).

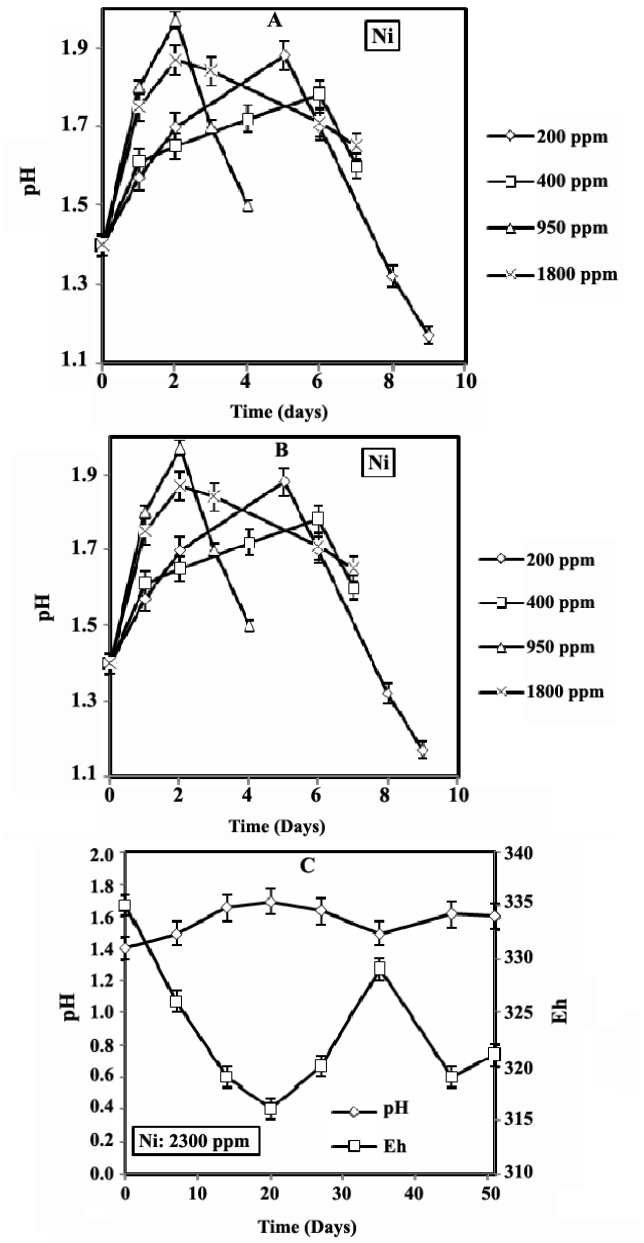

Figure 3. A: pH variations of the solution with time at different concentrations of Ni. B: Eh variations of the solution with time at different concentrations of Ni. C: The effect of Ni ions at a concentration of 2300 ppm on the pH and Eh of the culture medium.

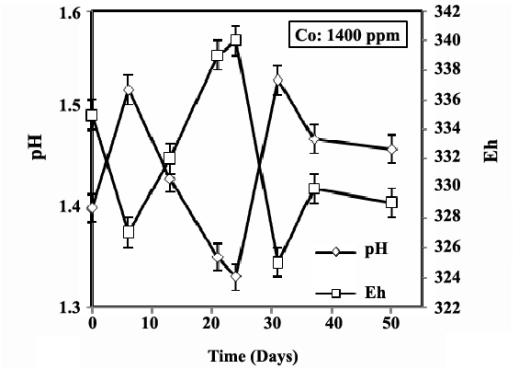

Figure 4. The effect of Co ions at a concentration of 1400 ppm on the pH and Eh of the culture medium.

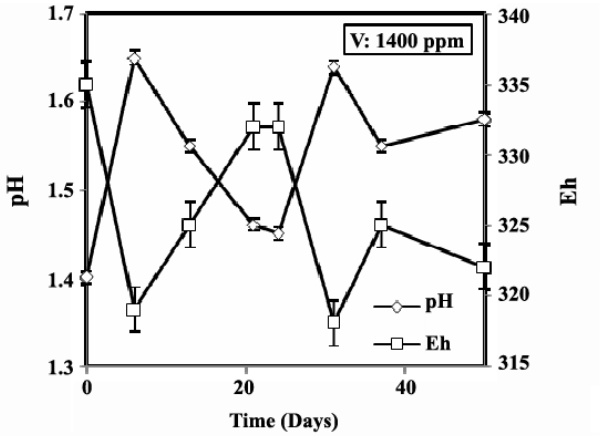

Figure 5. The effect of V ions at a concentration of 1400 ppm on the pH and Eh of the culture medium.

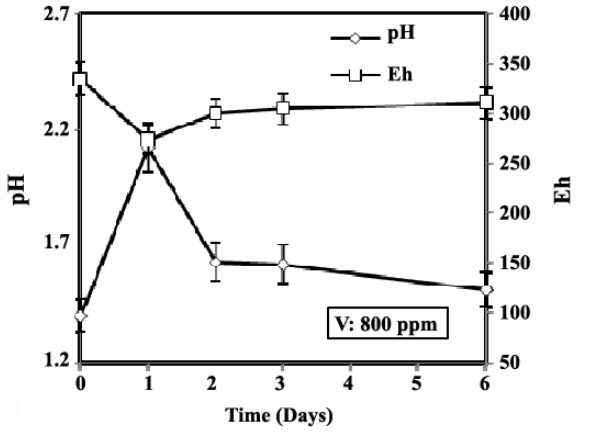

Figure 6. The effect of V ions at a concentration of 800 ppm on the pH and Eh of the culture medium.

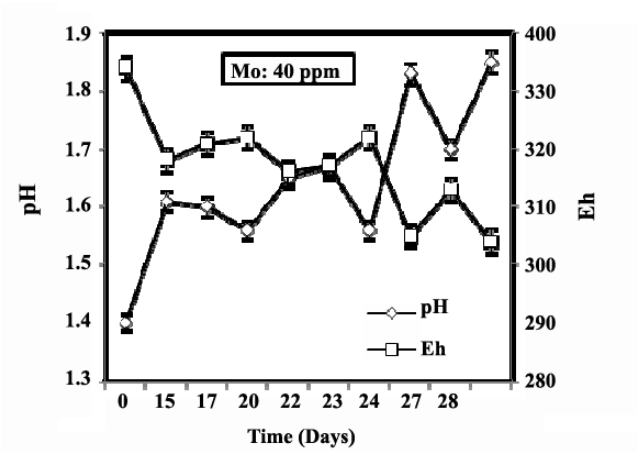

Figure 7. The effect of Mo ions at a concentration of 40 ppm on the pH and Eh of the culture medium.

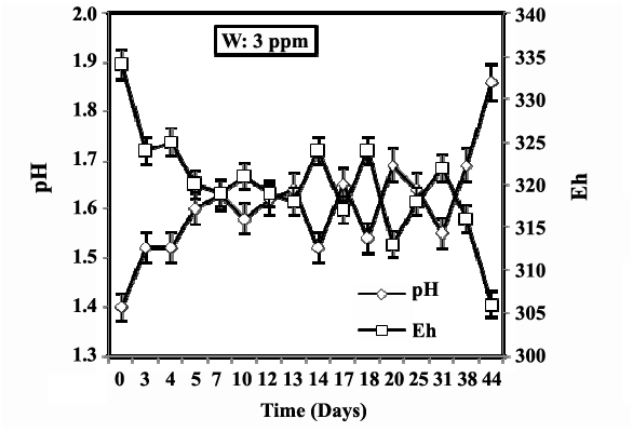

Figure 8. The effect of W ions at a concentration of 3 ppm on the pH and Eh of the culture medium.

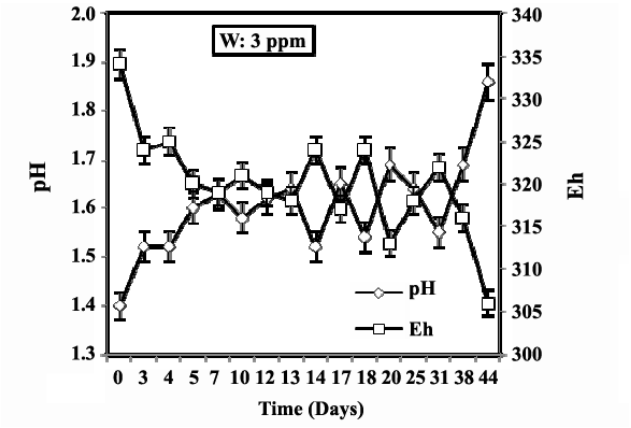

Figure 9. The effect of metals combination (#7) on the pH and Eh of the culture medium.

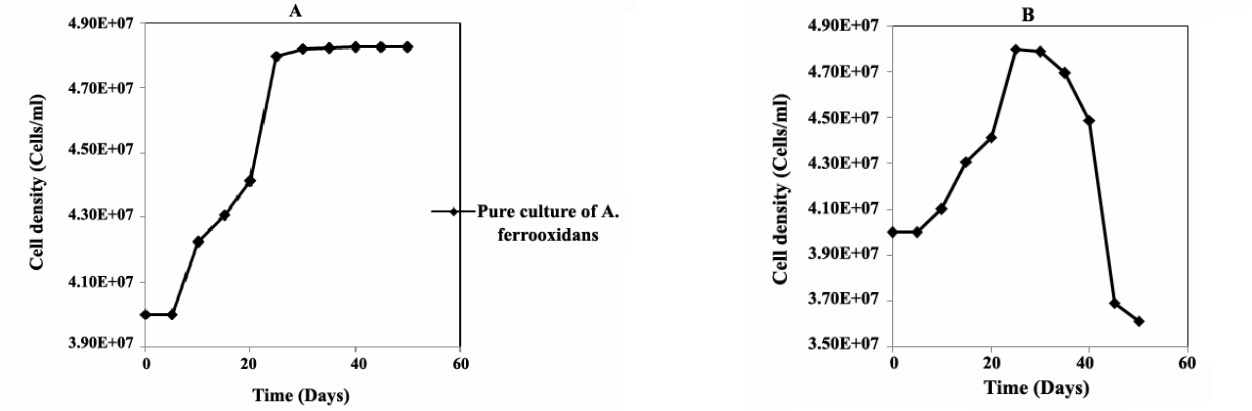

Figure10. A: Bacterial growth curve of a pure culture of *A. ferrooxidans*. B: Bacterial growth curve of *A. ferrooxidans* in a mixed metals solution.

at which they no longer divide but are viable (Mason and Rice, 2002). Direct counting by microscope was used to estimate cell numbers. A culture aliquot was taken every 5 days, in order to calculate the concentration of cells. Figures 10A and 10B represent bacterial growth curve of a pure culture of *A. ferrooxidans* and its relative growth curve in the presence of mixed metals (Ni, Co, Mo and V), respectively. For microorganisms reproducing by binary fission a typical plot of logarithm (cell number) vs. incubation time shows four distinct phases (Prescott *et al*., 1990), viz. (i) lag phase; (ii) exponential (or log) phase; (iii) stationary phase; (iv) death phase. Figures 10A and 10B follow this pattern. The exponential phase of bacterial growth started at day 5 after the lag phase and continued until day 25 when it reached the stationary phase. During the exponential phase, each microorganism is dividing at constant intervals. Thus, the population will double in number during a specific length of time. It can be concluded that the stationary phase in the culture of only A. ferrooxidans lasts more than a culture containing a solution of mixed heavy metals too, in which the death phase is also observable because of the toxicity of the mentioned metals and the reduction of nutrient source (Fe^{+2}) in the medium. Therefore, the bacterial population reaches a value of 3.6×10^7 (cells/ml) at day 50, while in the pure culture it maximizes to the value of 4.8×10^7 (cells/ml).

Although bacteria do not react well to sudden and significant changes in heavy metal ion concentrations, they can be adapted in the laboratory to incrementally-increased concentrations over a period of time, in order to increase their tolerance to such metals. The highest tolerance achieved by *A. ferrooxidans*, over a 6 months period, was towards 2.3 g/l (~40 mM) Ni, 1.4 g/l (~24 mM) Co, 1.4 g/l (~ 28 mM) V, 45ppm (~ 0.47

Table 2. The effect of Ni ions (50-2300 ppm) on the pH, Eh, bacterial population and iron conversion percentage of the culture medium.

Ni (ppm)	Eh (mV)	pH	Cell density x4x10^6 (Cell/ml)	[Fe^{+3}] (mmol/l)	[Fe^{tot}] (mmol/l)	Iron Conversion (%)
50	312	1.51	9.33	77.74	88.09	88
100	307	1.60	10.47	73.09	86.90	84
150	293	1.84	9.27	78.83	92.85	84
200	313	1.52	9.32	81.57	90.47	90
250	315	1.43	9.52	80.48	88.09	91
300	293	1.84	9.32	76.64	94.63	80
350	292	1.85	11.6	75.82	88.09	86
400	294	1.78	12.012	76.1	89.04	85
450	300	1.50	10.38	78.02	89.28	87
500	298	1.75	9.078	71.17	85.70	83
550	297	1.78	9.212	75.28	88.68	84
600	306	1.70	9.88	72.27	87.97	82
650	300	1.67	10.21	73.14	88.68	82
700	305	1.65	11.56	66.79	77.61	86
750	302	1.74	10.43	68.43	79.75	85
800	290	1.85	10.23	70.21	81.78	85
900	293	1.84	11.87	71.87	82.13	87
950	290	1.86	10.47	70.55	80.70	87
1500	310	1.43	9.26	78.02	91.54	85
1600	313	1.49	10.88	76.92	91.66	83
1700	296	1.75	10.4	75.82	91.66	82
1800	302	1.70	9.7	72.27	85.70	84
1900	312	1.51	9.23	77.2	91.90	84
2000	330	1.49	11.32	73.64	86.90	84
2100	306	1.83	10.62	84.3	94.75	88
2200	320	1.60	8.73	75.28	87.14	86
2300	320	1.60	6.00	88.15	98.21	89

mM) Mo, 5 ppm W. Furthermore it could tolerate a combination of following heavy metal ions; Ni, Co, V and Mo, up-to the concentration of 1500, 800, 800 and 50ppm, respectively. However it was observed that Mo and W are toxic even at relatively low concentrations, thus indicating that a process of bacterial adaptation is necessary. Furthermore, adaptation of bacteria to high levels of metal ions would allow for the dissolution of high metal levels without a loss in bacterial performance, such as in an industrial bioreactor.

It was evident that the various concentrations of Ni, Co and V had little effect on the oxidation of ferrous iron or the cell growth of *A. ferrooxidans*, whereas Mo and W ions were very inhibitory towards the Fe^{+2} oxidation ability of *A. ferrooxidans*. W was found to cause the largest inhibition followed by Mo, V, Co and Ni. However, during Fe^{+2} oxidation, in the presence of a combination of Ni, Co, V and Mo, it was shown that the presence of Mo as an inhibitory metal toxic to *A. ferrooxidans*, has not much of a negative influence on the oxidation of ferrous iron. In fact the bacterium could tolerate up-to 50ppm of Mo in combination with other metals. In comparison to other strains of *A. ferrooxidans*, the DSMZ 583 strain, could not tolerate high concentrations of Ni and Co, but in a combination of metals in the presence of V and Mo, it could tolerate much more than expected. The effect of metals on the oxidizing ability of the culture in the presence of a combination of four metals indicates the importance of adapting bacteria to environments containing high concentrations of valuable metal ions. However the time needed to oxidize Fe^{+2} in the last sub-cultures increased intensely. It was obvious because of the increasing inhibitory effects of these metals on *A. ferrooxidans*.

The adaptation should be performed by increasing

the concentration of heavy metals in small increments, in order to reduce the lag phase of growth of the microorganism and consequently reduce the total adaptation time.

In almost every experiment, except for those with high concentrations of heavy metals, a continuous decrease in pH and increase in Eh was observed. Shifting the Eh towards more positive values is directly related to the net increase of the Fe^{+3}/Fe^{+2} ratios in the solution. The shifting of the pH towards acidic values results from the balance between the simultaneous consumption of protons during the oxidation of ferrous iron and the release of protons during iron precipitation (Meruane and Vargas, 2003). The maximum pH achieved during the adaptation period was in the approximate range of 1.86-1.9, which must be carefully controlled throughout the experiments, since the optimum pH for the mentioned microorganism is between 1.4-1.5. In fact, when the pH of the culture medium was allowed to increase from 1.4 to 1.86, during the production of ferric iron, the oxidizing ability of the culture containing a combination of metals was inhibited and incomplete oxidation of Fe^{+2} was observed. The incomplete oxidation was suggested by Johnson *et al*., 2000 to be due to inhibition of the culture by a ferric iron complex.

CONCLUSIONS

The current study has shown how different concentrations of the commercially important heavy metals (Ni, Co, V, Mo, W), have different effects on the oxidizing ability of the culture, during the adaptation period of *A. ferrooxidans* DSMZ 583 to these metals.

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