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BIOLOGICAL SOLUBILIZATION OF SOME METALS BY A NEW ACIDITHIOBACILLUS SPECIES ISOLATED FROM A MODERATE SULFUR HOT SPRING

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ABSTRACT

A chemolithotrophic bacterium was isolated from sulfur hot spring. According to phenotypic traits and 16-23S rDNA intergenic spacer region analysis, the isolate was identified and named as *Acidithiobacillus* sp. MR39, which was a gram-negative, rod- shape and non-motile bacterium. The strain was able to grow in a synthetic liquid medium supplemented with the mineral ore as the source of energy. The optimum conditions were found to be within initial pH range of 2.0-2.5, at 34±1˚C and with shaking at 120 rpm. The bacterium had a remarkable potential for mineralization of 88% iron, 75% copper, 59% zinc, 59% nickel and 40% cobalt upon their growth in the liquid media. After adapting the bacterial cells to copper ions in 100 mM for 5-day incubation, biorecovery of Cu increased about 10% comparing to unadapted cells that are able to dissolve approximately 15% of total cu of mineral concentrate. Considering the finding in this study, the strain MR39 offers a great prospect for in situ extraction of metals from various ores along with other indigenous bacteria that can grow under ambient conditions.

Keywords: *Acidithiobacillus* sp. MR39, Bioleaching, Mineral ore, Chemolithotrophic

INTRODUCTION

Bioleaching refers to extraction of different minerals using environmentally adapted microorganisms as a cost-benefit and eco-friendly process in low-grade mineral ores **(Martínez-Bussenius *et al.*, 2017)**. These microorganisms have been recognized as the operators in bioreactors processing metal recovery through oxidation reactions **(Zhao *et al.*, 2013)**. Among the known microorganisms involved in bioleaching process, *Acidothiobacillus* species has been investigated by many researchers and companies **(Raheb *et al.*, 2007; Wei *et al.*, 2018)**.

In many extremely acidic environments, sulfur-oxidizing microorganisms are known as unique types of highly tolerant to extreme acidic conditions **(Wang *et al.*, 2016; Romo *et al.*, 2013)**. Such bacteria acquire their energy from the oxidation of mineral sulfides for metabolic activity **(He *et al.*, 2012; Qiu *et al.*, 2017)**. Therefore, following the bacterial activity in the presence of the metals, they might be readily solubilized in aqueous media and recovered from the mine. Herein, these bacteria could be exploited to extract precious metals like gold and copper from various mineral ores, and so the bacteria could be applied for the bioremoval of the toxic metals from agricultural lands for improving structure of the soils for production of the various crops **(Potysz *et al.*, 2018; Shin *et al.*, 2013; Li *et al.*, 2018)**. The majority of acidophilic bacteria are discovered to be responsible for extraction of several metals such as copper, iron, uranium, gold, among others suggesting their reliability in metal mining activities **(Mishra & Rhee, 2014; Pathak *et al.*, 2018; ShahrozKhan *et al.*, 2012; Wang *et al.*, 2017)**.

Bioleaching processes by living organisms could be affected by various parameters such as physiochemical and biological conditions. These parameters might play important roles in the bioleaching efficiency where the growth of microorganisms is critically affected by environmental changes (**Zhou *et al.*, 2019**). Several literatures have declared that one of the main difficulties reported on the development of biological leaching by such bacteria is the lack of a strain with desirable growth rate under in situ ambient conditions **(Ralitsa *et al.*, 2011; Wang *et al.*, 2014)**. In this research, we focused our attempts on finding native acidophilus bacteria with interested criteria, which would be suitable for industrial uses.

MATERIAL AND METHODS

Media and reagents

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO); except that elemental sulfur was obtained from Merck (Merck, Darmstadt, Germany). Molecular weight marker was purchased by Roche Company (Basel, Switzerland). A mineral ore concentrate was obtained from Sarcheshmeh copper mine, Kerman, Iran consisted of copper compositions as seen in Table 1.

Table 1 chemical composition of used media for bacterial cultivation

Mineral	chemical formula	Composition Percent
Elemental Copper	Cu	0.18
Chalcopyrite	$CuFeS_2$	69.44
pyrite	FeS_2	24.51
chalcocite	Cu_2S	2.11
Covelite	CuS	2.53
Molybdenite	MoS_2	0.37
bornite	Cu_5FeS_4	0.71
cuprite	Cu_2O	0.16

Analytical methods

The bacterial growth was followed by means of a cell-counting chamber under a light microscope (Olympus CK40). Total copper and iron in the culture media were analyzed by an atomic absorption spectrophotometer (German, AAS 5EA). Copper (II) generated indirectly by reduction of ferric ion was also determined by producing a blue complex by Neocuproine with absorbance at 595 nm. Ferrous ions were determined by their titration with potassium dichromate and ferric ion level was calculated by subtracting the ferrous iron values measured at each interval of incubation **(Gouda & Amin, 2010)**. The oxidation-reduction potential (ORP) value was determined by an Ag/AgCl reference electrode .

Sample preparation and bacterial enrichment

Water samples were taken from a sulfur hot spring, Fars, Iran. Bacterial isolation was conducted with inoculating the samples in 9K medium, all components of the media were prepared as stock solutions, filter-sterilized (0.22 µm pore size)

and added to sterile distilled water with the final volume of 90 mL in 250 mL Erlenmeyer flasks. The pH was adjusted with sulfuric acid to 2.5 and then, 10 mL inoculum was added to each flask containing 90 mL the media and incubated at 34±1 ºC with rotary shaking at 120 rpm for two weeks. The samples were gone through such an enrichment process to increase the chance of isolating acidophilic and autotrophic bacteria. Bacterial enrichment was studied by cultivating primary inoculums in four different culture media including 9K, TK, thiosulfate and elemental sulfur as detailed listed in Table 2. All experiments were conducted in the flasks with the following condition: pH 2.5, temperature at 34±1 ºC and shaking speed of 140 rpm for a 15-day incubation period. The growth of the bacterium in all media was measured based on total protein assay by Bradford method (**Bradford, 1976**).

Table 2 Media components prepared in sterilized distilled water for primary cultivation

Composition (g/L)	9K	TK	Sodium thiosulfate	Elemental Sulfur
$(NH_4)_2SO_4$	3	0.4	0.4	-
$MgSO_4.7H_2O$	0.5	0.4	0.5	0.25
K_2HPO_4	0.5	0.1	3	0.25
KCl	0.1	-	-	-
$Ca(NO_3)_2$	0.01	-	-	0.01
$FeSO_4.7H_2O$	33	24.6	0.01	-
$Na_2S_2O_3$	-	-	5	-
Elemental sulfur	-	-	-	10

Growth on solid media

A modified solid medium derived from 9K medium served to get colonies of the chemolithotrophic bacteria especially, *Acidithiobacillus* species. The medium was prepared according to the current method with the following modifications: I) agarose was used at the concentration of 8 g/L instead of 6 g/L and II) agarose was washed by distilled water three times. Inoculated solid medium with 10^{-2} serial dilution of the bacterial suspension was incubated at 34±1 °C in the humid atmosphere for two weeks to develop the maximum number of colonies.

Phenotypic and genotypic Characterization

Preliminary experiments were conducted to determine the biochemical activities according to Bergey's Manual of Systematic Bacteriology **(Kelly & Harrison, 1989)**. Morphological properties were analyzed based on the colony formation and bacterial gram staining. The amplification of the 16S–23S rDNA spacer regions was performed to explore its phylogenetic relationship to other bacterial species. Accordingly, the genomic DNA was first extracted by Genomic DNA Buffer Set (sucrose 20%, EDTA 50 mM, Tris HCl 50 Mm, pH 4.7), lysozyme 5 mg/mL, SDS 25% w/v, proteinase K1 mg/mL, ammonium acetate 7.5 M and cool isopropanol. Afterward, a sequence of the genome between 16S and 23s genes with the length of 500 base pair was amplified by PCR using forward primer G1-F (5'-GAAGTCGTAACAAGG-3') and reverse primer L1-R (5'-CAAGGCATCCACCGT-3') **(Pandey *et al.*, 2011)**. The thermal cycle profile used for PCR was as following: 95 °C for 5 min as initial denaturation, 35 cycles of 95 °C for 45 seconds, 58.1 °C for 1 min and 72 °C for 45 seconds as respective sequentially denaturation, annealing and extension, and a final extension step of 72 °C for 10 min **(Wu *et al.*, 2013)**. The PCR product was run on 1% agarose gel and stained with 1% ethidium bromide and 1X TAE electrophoresis buffer. Nucleotide sequencing analysis was performed with an automated sequencer 3700 ABI (Macrogene Seoul, Korea). Subsequently, sequence alignment was performed using the BLAST software in the Genbank database site (http://www.ncbi.nlm.nih.gov/BLAST) to determine similarity with nucleotide sequences deposited in Genbank database. A phylogenetic tree was also constructed by Mega4 software using neighbor-joining and distance matrix estimated with bootstrap values from 1000 replicates.

Metal Tolerance assessment

This study was conducted in standard 9K liquid medium, supplemented with heavy metals in the sulfate forms. These metals were prepared as sterile stock solutions including aluminum, cobalt, nickel, zinc, cadmium, and copper. A series of triplicate experiments were performed in 250 mL flasks containing 90 mL 9K basal nutrients supplemented with 100, 200, 300 and 400 mM of each metal, inoculated with 10 mL of 5 day-incubated bacterial suspensions (cell number of 1.5×10^8 cells/mL) in 9K culture media. The pH of the media was first adjusted to 2.0 with 1N H_2SO_4 and incubated in a shaker at 34±1 ºC with 140 rpm for 15 days. Tolerance study was carried out by enumerating bacterial cells into the culture media. A stress response indicator with an appellation of tolerance index (Ti) value was defined based on the growth ability of the bacterial cells in the presence of different metal concentrations. This value was calculated from the growth rate in metal-treated cultures divided by untreated control cultures.

Bioleaching experiment

As aforementioned, a mineral sulfide ore provided from Sarcheshmeh copper mine was powdered, passed through a mesh sieve with 100-300 µm pore size and then used for bioleaching experiment. Bioleaching was investigated by the addition of 10 g the mineral ore into 250 mL flasks containing 90 mL of 9K liquid medium (with initial pH adjusted to 2.0) that $FeSO_4$ was removed from the medium. After that, the flasks were inoculated by the bacteria (10 mL inoculums with cell number of 1.5×10^8 cells/mL) and incubated in a shaker incubator (140 rpm) at 34±1 ºC for 24 days. Two set of experiments were conducted for evaluating copper mineralization by adapted bacterial cells in the presence of 100 mM Cu and unadapted cells without heavy metal treatment. A control flask was also remained uninoculated and maintained under the same condition as the experimental ones. During the incubation, bacterial growth was monitored by sampling at regular intervals for bacterial cell counting **(Xiao *et al.*, 2016)**. Moreover, the amount of dissolved metals in the samples was measured as described previously in "analytical techniques" section indicating the metal oxidizing activity. Prior to any measurement, the volume of flasks was adjusted to the original volume (100 mL) by adding sterilized distilled water to compensate for the decreased volume of the media subjected to evaporation.

RESULTS AND DISCUSSION

Bacterial identification revealed the isolate is a strain of *Acidithiobacillus* genus. Hence, the isolate was named *Acidithiobacillus* sp. MR39 based on morphology and metabolism studies along with 16S rRNA phylogeny analysis. The morphology of colonies appeared on agarose-based medium was circular with a light brown center and a yellow halo with 1-3 mm in diameter (Figure 1A) and upon microscopic observation (Figure 1B). Regarding *Acidithiobacillus* sp. MR39, the physiochemical properties were compared with those of five different Acidithiobacillus species (Table 3); indicating some similarities with these bacteria. However, this strain showed some differences between our strain and its closest relative, as unlike this strain, our strain did not demonstrate motility and nitrogen-fixing capability.

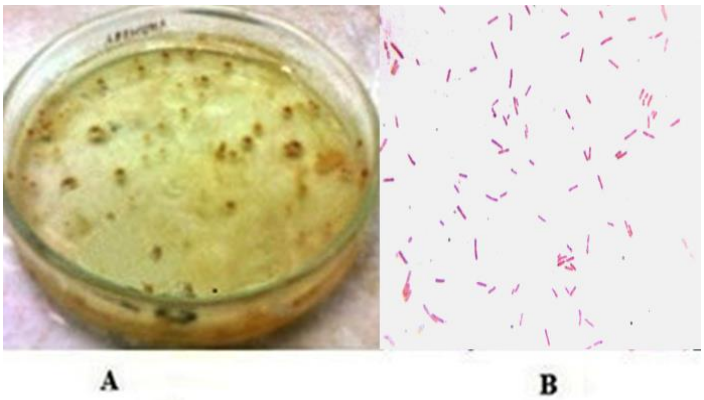

Figure 1 Colony morphology of the bacterial isolate. (a) Colony appearance on 9K medium (b) light microscopic scheme of gram stained cells

The amplified product of the spacer intergenic region was observed as a single band of approximately 600 bp in relation to the DNA marker .The sequence was edited with Bioedit software (version 1.5) and deposited in NCBI Genbank named *Acidithiobacillus* sp. MR39 with an accession number of KX817172. Local alignment of 16S-32S rDNA spacer intergenic related to our isolate with other bacterial strain deposited in NCBI Genbank showed the closest similarity to *Acidithiobacillus* species. Such as similarity was also highlighted by generating the phylogenetic tree based on the spacer sequences. Taken together, biochemical features and phylogenetic analysis outputs revealed our indigenous isolate' identity which was named *Acidithiobacillus* sp. MR39 (Figure 2).

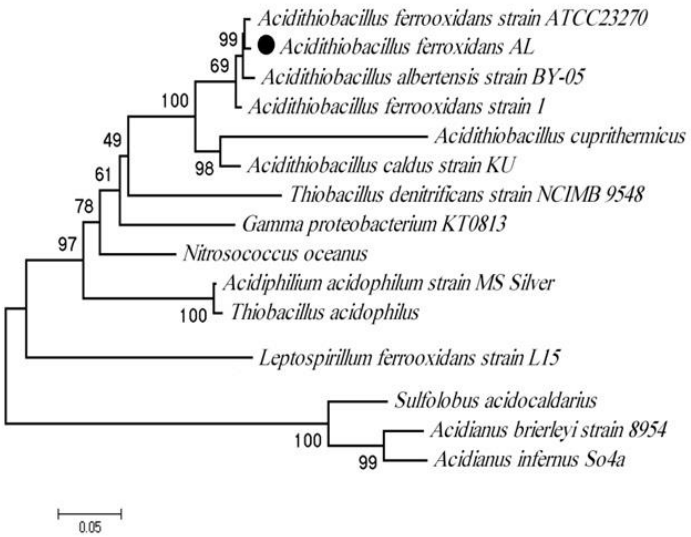

Figure 2 Phylogenetic relationship tree constructed from 16-23S rRNA sequences comparing with other chemolithotroph bacteria by MEGA 4 using neighbor-joining method with bootstrap value (1000 replicates).

Additionally, some important properties of strain MR39 were compared with four different chemolithotrophic bacteria. These metabolically comparisons indicated that strain MR39 had a great similitude to *A. ferrooxidans* ATCC 23270 (Table 3).

Table 3 Biochemical and morphology properties of isolated bacterium and its comparison with other closest strains

Characteristics	***Acidithiobacillus* sp. MR39**	***A. ferroxidans***	***A. thiooxidans***	***A. albertensis***	***A. caldus***
optimal PH	2.5	2-2.5	2-3	2-4.5	2-2.5
optimal temperature	30	30-35	28-30	25-35	40-45
G+C	57	58-59	52-57.9	63	63-64
oxidase test	+	+	-	-	+
catalase test	+	+	+	+	+
Amylase	-	-	+	+	-
Indole production	-	-	-	-	-
Urease test	-	-	-	+	-
Citrate utilization	-	-	+	+	-
Methyl red	-	-	+	+	-
Voges proskauer	-	-	-	-	-
Glucose oxidation	-	-	+	+	+
Glucose fermentation	-	-	-	-	-
Sulfur oxidation	+	+	+	+	+
Thiosulfate oxidation	+	+	+	+	+
Triple sugar iron	-	-	-	+	+
chemolithoautotrophic	+	+	+	-	+

The optimal growth condition of our isolate was found at pH 2.5, 34±1 ºC and shaking at 140 rpm in a 9K medium containing ammonium sulfate as nitrogen source, which is consistent with the report of Das in 1989 **(Das *et al.*, 1989)**. The highest bacterial growth and oxidation activity were achieved in 9K media followed by TK, elemental sulfur and thiosulfate, respectively (Figure 2). Many reports on *Acidithiobacillus* species indicate most of the bacteria are hardly able to grow on different solid media, which can be attributed to their chemolithotrophic and dilatory metabolism **(Deng *et al.*, 2017; Zhang *et al.*, 2012)**. Since detailed studies on most of the microorganisms necessitate having a pure culture, our first attempt was limited to getting a single colony of the bacterium on solid media and noticed that such a strain was very sensitive to agar and some other organic compounds resulted in bacterial growth inhibition **(Das *et al.*, 1989)**. For instance, washed agarose was used instead of agar for solidification of media as previously reported in the literature for successful enriching obligate chemolithotrophic bacteria **(Das *et al.*, 1989; Kondratyeva *et al.*, 1999; Lantican *et al.*, 2011; Mohseni *et al.*, 2011b)**.

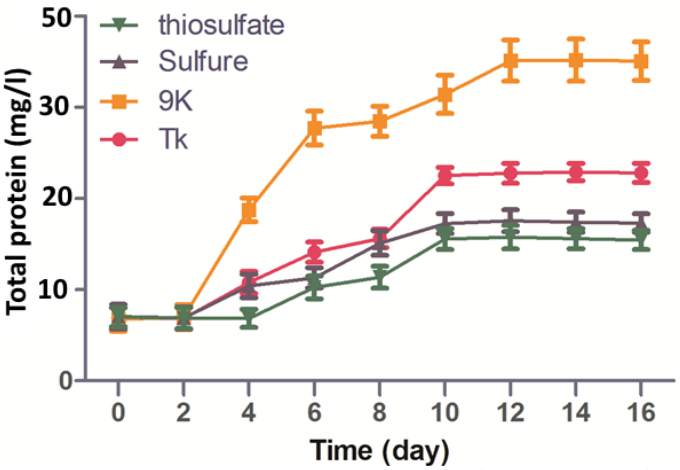

Figure 3 *Acidithiobacillus* sp. MR39 growth pattern in four culture media for 16 days incubation

Metal tolerance assessment

Figure 3A, B, C and D Show tolerance of the bacterial isolate for a 15-day cultivation course. The growth and Fe (II) oxidation was defined as tolerance index (Ti) in the presence of different metals. The results show the highest tolerance to Zn and then Cu, followed by a decreasing order of Ni, Al, Co, and Cd. A dramatic decrease in bacterial growth occurred when Cu concentration increased up to 300 mM. However, the tolerance to Cd was in the lowest level for all treatments. As seen in the experiments, the bacterium shows a favorable tolerance to Zn and Ni, even if the concentration of both metals increased. Since

bioleaching process often occurs in the presence of some heavy metals, therefore resistance to toxic metals for used strains of bacteria is of absolutely vital subject matter **(Ye *et al.*, 2017)**. Considering the mineral construction, management of bacterial types for enhancing mineralization of the metals via adaptation of bacteria at current condition such as frustration with heavy metals can be an efficient strategy **(Heydarian *et al.*, 2018)**. Many practical approaches are the serving several strains with different adaptability to harsh conditions. As the main leaching practices take place in situ, implementing all the optimum conditions achieved in the laboratory are often cost-intensive **(Tavakoli *et al.*, 2017)**. Environmental factors can affect bacterial growth and shift their leaching abilities out of the optimal conditions, which is not intended for high metal extraction. For instance, on releasing metals from the ore, which is rich in toxic metals when reaching high levels in the solution, the bio-oxidizing activity is hampered in chemolithotrophic bacteria **(Mohseni *et al.*, 2011a; Mohseni *et al.*, 2011b)**. Therefore, the more bacteria can tolerate elevated levels of metals, to higher limits their oxidizing and leaching capabilities can be exploited that makes them valuable for the purpose of the bioleaching process **(Heydarian *et al.*, 2018; Li *et al.*, 2011; Wei *et al.*, 2018)**.

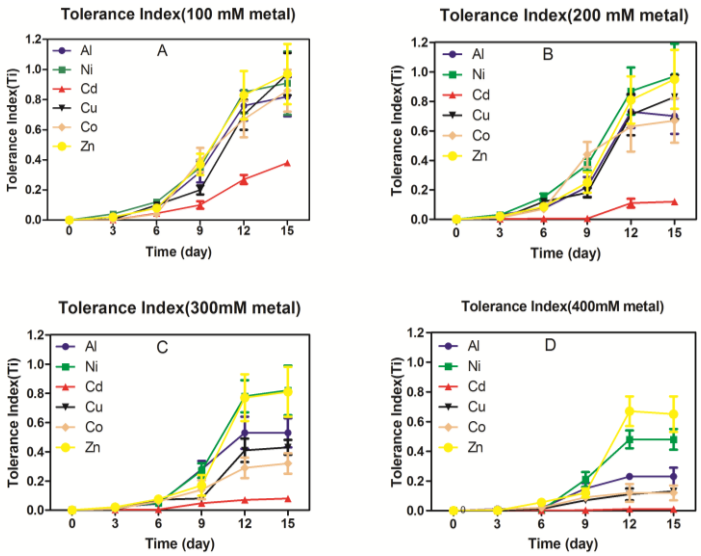

Figure 4 bacterial growth in the presence of 6 metals including (a) Tolerance index of bacterium at 100 nm of each metals for 15 days incubation. (b) Ti in the presence of 200 mM. (c) Ti in the presence of 300 mM and D: Ti in the presence of 400 mM

Bioleaching experiment

Bioleaching experiment was performed for copper oxidation from a low-grade mineral ore. In the initial stage, metal adapted bacterial cells were used as the inoculum. During a 24-day bioleaching experiment, the bacterial cells were able to oxidize about 28% of copper ions in the culture medium, comparing unadapted cells that increased about 10 % in the same conditions exerted on adapted cells. Considering our aims in this work that focused on copper recovery by *At. ferrooxidans* MR39, we observed the isolate was able to leach Cu ions in the presence of low-grade concentrate copper ore. Besides, the isolate could solubilize other metals in which it showed high tolerance like Zn and Ni.

CONCLUSION

The research revealed that our isolate could solubilize some valuable heavy metals such as appreciable degree copper, existing in mineral ores. This isolate was found to be a strain of *Acidithiobacillus* species with well-established chemolithotrophic metabolism. In our case, the bacterium was able to tolerate different levels of some heavy metals. In addition, the bacterium showed a great ability to solubilize copper from a mineral ore, which is promising especially for copper extraction from lower grade ores .

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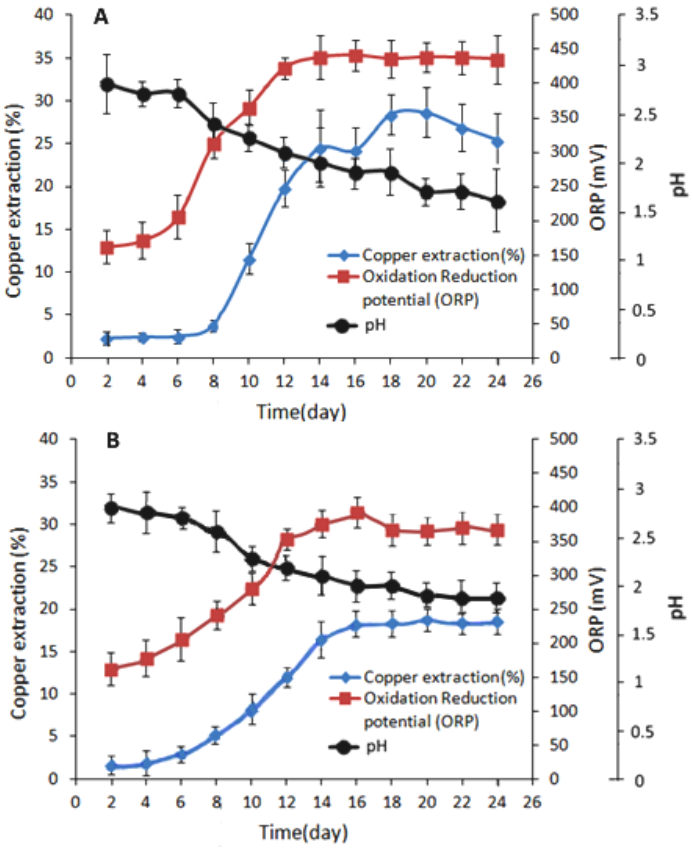

Figure 5 bioleaching experiment by adapted and unadapted inoculum obtained from fresh culture bacterial cells. (a) copper extraction by adapted bacterial cells with 100 mm of Cu for 5 days and oxidation-reduction along with pH changes. (b) Copper extraction obtained by unadapted bacterial cells and ORD along with pH changes has been shown for a 24 day process

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