Bioleaching of Molybdenum by Two New Thermophilic Strains Isolated and Characterized

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ABSTRACT: *This study involves the isolation and characterization of a bacterial strain capable of bioleaching molybdenum ore. Bacterial growth was observed when rock sample was incubated in 9K at 70 ºC. The isolates were identified as extremely acidophilic, thermophilic and chemolithotroph archaebacteria. Following PCR amplification of the 16S rDNA of the isolated strain, the sequencing of this region and comparison with the Gen-Bank database identified the strains as Acidianus ambivalens and Sulfolobus solfataricus. An experimental design was carried out to optimize bioleaching of molybdenum by these bacteria. Factors of pulp density, initial pH, the concentration of Fe^{3+} and the ratio of two bacteria are the variables and molybdenum and uranium recovery were selected as responses. Bioleaching was carried out using molybdenum ore and pulp density of 4%, initial pH of 1.5, Fe^{3+} concentration of 11.5 g/L and Sulfolobus solfataricus to Acidianus ambivalens ratio of 2.0 were selected as optimum conditions. Molybdenum and uranium recoveries were 43.2% and 79.1% respectively.*

KEYWORDS: *Bioleaching, Thermophilic bacteria, Molybdenum extraction, Sulfolobus solfataricus, Acidianus ambivalens.*

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INTRODUCTION

Microbial leaching processes play an important role in the recovery of valuable metals such as copper and gold from mineral ores and in leaching out environmentally hazardous heavy metals [1, 2]. In fact, metal leaching from metal sulfides is enhanced by certain acidophilic iron and/or sulfur oxidizing bacteria [3]. Such strains have mostly been isolated from mining and ore leaching sites [4]. Isolation of microorganisms from such sites and their use in subsequent bioleaching is more advantageous than using standard strains obtained from other sources [5- 8]. Furthermore, the bio-oxidation of the sulfur to sulfate, generated as a result of sulfide dissolution, indicates that such microbes also help maintain the necessary acidic environment for optimum mineral dissolution [9]. For efficient bio-oxidation of most ores, the presence of bacteria capable of carrying out the oxidation of both iron and sulfur is necessary. *Sulfolobus solfataricus* and *Acidianus ambivalens* are capable of sulfur oxidation and are used in bioleaching processes [10, 11].

Molybdenum makes an important contribution to sustainable development as a metal, alloying element, and constituent of chemical products. Molybdenum-based alloys have a unique combination of properties, including high strength at high temperatures, high thermal and electrical conductivity, and low thermal expansion.

The dissolution of metal sulfides is controlled by their solubility product and thus, the H^+ concentration of the solution; and is further enhanced by a number of chemical mechanisms leading to a disruption of sulfide chemical bonds [12].

Molybdenite oxidation with bacteria was first experimented by *Bryner, et al.* in 1971. Tuovinen showed that the higher concentration of 5 mg/L molybdenum was lethal for *Acidithiobacillus thiooxidans.* Increasing oxidation and dissolution of molybdenite using thermophilic species, especially a variety of *Sulfolobus*, has been associated with relative success. Brierley and Murr proved the ability of thermophilic bacteria species such as *Sulfolobus* to be higher than mesophilic counterparts [13, 14]. A maximum recovery of 13.3% after 30 days with the addition of 0.02% yeast extract and 1% ferrous sulfate have been obtained; while the recovery of molybdenum in control sample was only 1.0% [15]. In one of the latest research contributions in the field of bioleaching of molybdenite concentrates, bacteria like moderate thermophilic and thermophilic were used [16,17]. Molybdenum has different valence that it can be recovered at hexavalent state. Therefore, molybdenum cannot be extracted at redox potential lower 950 mV for this reason. Molybdenum is tetravalent state in molybdenite mineral (MoS2) which should be oxidized to the hexavalent state. Molybdenum (IV) oxidizes to molybdenum (VI) at redox potential of 945 mV. The main difference between molybdenum and uranium bioleaching is minerals of these elements. Mineral of molybdenum is molybdenite that is relatively refractory but mineral of uranium is uraninite that is not refractory. The refractory minerals need hard conditions for recovery of elements therefore, molybdenum need so harder conditions for leaching or bioleaching processes [37, 38]. Mesophyll bacteria are used for bioleaching of uranium at lower temperature (25-45 °C) and ORP (478 mV) but thermophile bacteria are used for bioleaching of molybdenum at higher temperature (65-80 °C) and ORP (945 mV). Studies show that molybdenum is resistant against environmental factors and molybdenite is a refractory mineral. Thermophilic bacteria have more tolerance against molybdenum and can extract the metal from ore. Bioleaching of molybdenum sulfide minerals are carried out using thermophilic bacteria under the indirect mechanism. One of the key points in bioleaching of molybdenum is oxidation reduction potential. Molybdenum recovery increases with temperature increase and particle size decrease [18-20].

A thermophile is an organism that thrives at relatively high temperatures, between 41 and 122 °C [21-23]. Thermophilic bacteria are suggested to have been among the oldest types. They survive at temperatures capable of exterminating other species of bacteria [1]. Archaea recycle elements such as carbon, nitrogen and sulfur through their various habitats. In the sulfur cycle, archaea that metabolize on oxidizing sulfur compounds release this element from rocks, making it available to other organisms. However, *Sulfolobus* archaea produce sulfuric acid in the process as byproduct, and the growth of these organisms in abandoned mines can contribute to acid mine drainage and other environmental damages [24-26]. *Sulfolobus* species grow in volcanic springs with optimal growth occurring at pH 1.5-3 and temperatures of 70-80 °C, making them acidophiles and thermophiles

respectively. *Sulfolobus* cells are spherical shaped and flagellar. Species of *Sulfolobus* are generally named after the location from which they were first isolated, e.g. *Sulfolobus solfataricus* was first isolated in the Solfatara volcano. Also in taxonomy, *Acidianus* is a genus of the Sulfolobaceae. *Acidianus* is aerobic, extremely acidophilic, thermophilic and sulfur-metabolizing [23].

In this study, microbial flora of rocks sampled from a molybdenum mine in central Iran were isolated. Two new thermophilic bacteria; *Sulfolobus solfataricus* and *Acidianus ambivalens* strains; isolated from this mine were characterized and their sulfur oxidation and bioleaching potentials were evaluated. Bioleaching tests were conducted at 70 ºC. Recovery of molybdenum and uranium from ore by bioleaching process with thermophilic bacteria was the main objective of this work.

EXPERIMENTAL SECTION

Sampling, enrichment and isolation

Solid and liquid samples were collected aseptically from various points including acid mine drainage within and around the mine. The liquid samples had an approximate pH of 8.0 and hence devoid of conditions necessary for survival of acidophilic bacteria. They were thus disregarded in the research. However, the rock samples were added to 9K medium, supplemented with sulfur (10 g/L) as energy source, in Erlenmeyer flasks. The pH of the medium was adjusted to 1.8 with sulfuric acid. Cultures were initially incubated at 70°C, shaking at 120 rpm. When growth was detected, culture samples were further enriched in 9K free iron medium containing sulfur and yeast extract at 70 ºC. Growth was indicated by sulfur oxidation, an increase in Oxidation-Reduction Potential (ORP) and decrease in the pH of the culture medium. This was indicative of bacterial activity and hence growth.

At the next step, 50 ml of mixed culture of two bacteria was added to the specific media of *Sulfolobus* and *Acidianus* strains (2015 DSMZ GmbH) separately. The *Sulfolobus* medium was dissolved ingredients (except yeast extract or other substrates) and the pH of the salt solution was adjusted at room temperature to 2.0 using 10 N H_2SO_4 and autoclave. The pH of the *Acidianus* medium was adjusted to 1.5 - 2.5 with 6 N H_2SO_4. Yeast extract (10% w/v in distilled water) was autoclaved separately. Sulfur was sterilized by steaming for 3 hours. These cultures were incubated at 70°C, shaking at 120 rpm. Some subcultures were prepared from these strains for increasing bacteria count.

Identification and characterization of the isolate

Morphological and growth analyses

Determining cell concentration is necessary in microbiology, cell culture, and other applications that require the use of cell suspension. The device used in determining the number of cells per unit volume of a suspension is called a counting chamber (Neubauer chamber). The charged counting chamber is placed on the microscope. The total count is the number of particles per mL (mL^{-1}).

Molecular biological techniques

Using a DNA based assay, one can easily detect bacterial strains directly from clinical samples or from small amounts of cultured bacterial cells, thus improving the sensitivity and decreasing the time required for bacterial identification. PCR has been particularly useful in this regard, which relies on primer sequences designed to facilitate bacterial identification at any level of specificity: strain, species or genus.

Biomass for eventual DNA extraction was prepared by growing cells as mentioned above. Following chromosomal DNA extraction of the isolate Bacterial Genomic DNA Isolation kit (Metabion), amplification by polymerase chain reaction (PCR-Master kit) of the 16S rRNA was carried out using the primers of UF 5-CCAGCAGCCGCGGTAATACG-3 and UR2 5-ATCGGCTACCTTGTTACGACTTC-3. The isolated strains as *Acidianus ambivalens* and *Sulfolobus solfataricus* (accession numbers: KM555276.1 and KM555275.1) were registered in NCBI (National Center for Biotechnology Information of the USA).

Biochemical and analytical measurements

Cultures were grown at temperatures of 70ºC during which their pH, ORP and count of bacteria were monitored. The mineral content of the rock sample was analyzed through the X-Ray Fluorescence (XRF) of the samples grounded to a particle size d80 of 74 µm (Table 1). Mineralization studies were carried out on the ore sample (Table 2). The mineral of molybdenum is molybdenite (Fig. 1).

Table 1: Analysis of ore sample by XRF.

Composition	Content	Composition	Content	Composition	Content
P_2O_5 (%)	2.31	Mo (ppm)	955	Y (ppm)	27
MnO (%)	0.137	U (ppm)	181	Zr (ppm)	208
TiO_2 (%)	0.755	Zn (ppm)	269	Sr (ppm)	60
K_2O (%)	2.63	Th (ppm)	76	Rb (ppm)	129
MgO (%)	3.26	Cl (ppm)	371	Pb (ppm)	76
Na_2O (%)	3.13	S (ppm)	7954	Ni (ppm)	73
CaO (%)	4.28	Ba (ppm)	317	Nb (ppm)	16
Fe_2O_3 (%)	4.85	Ce (ppm)	65	Cu (ppm)	175
Al_2O_3 (%)	13.92	Co (ppm)	24	Cr (ppm)	176
SiO_2 (%)	58.6	V (ppm)	124		

Table 2: Mineralization of ore sample.

Mineral Name	Formula	Mineral Name	Formula
Magnetite	Fe_3O_4	Apatite	$Ca_5(PO_4)_3(F,Cl,OH)$
Thorianite	ThO_2	Biotite	$K(Mg,Fe)_3(Al,Fe)Si_3O_{10}(OH,F)_2$
Thorite	$(Th,U)SiO_4(OH)_4$	Muscovite	$KAl_2(AlSi_3O_{10})(F,OH)_2$
Molybdenite	MoS_2	Pyrite	FeS_2
Zircon	$ZrSiO_2$	Calcite	$CaCO_3$
Arsenopyrite	FeAsS	Ankerite	$CaFe(CO_3)_2$
Cassiterite	SnO_2	Quartz	SiO_2
Amphibole	$Ca_2(Mg,Fe)_5(Si_8O_{22})(OH,F)_2$	Iron Oxide	FeO
Sphalerite	ZnS	Talc	$Mg_3Si_4O_{10}(OH)_2$

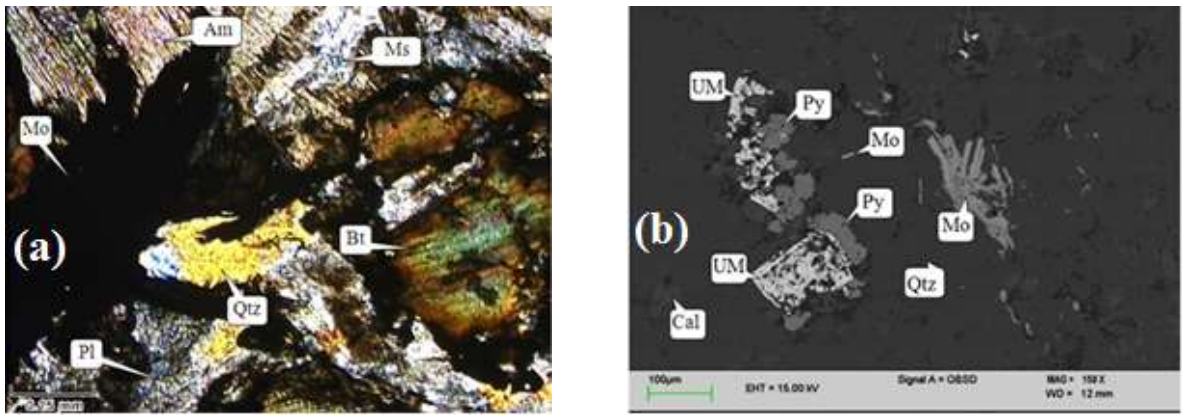

Fig. 1: Molybdenite between other minerals with (a) Electron Probe Micro-Analyzer (EPMA) and (b) Scanning Electron Microscopy (SEM).

Table 3: Factors and their levels in optimization experiments.

No.	Factor	Unit	-α	-1	c	+1	+α
1	A- Pulp Density	%	2.0	4.0	6.0	8.0	10.0
2	B- Initial pH	-	1.0	1.5	2.0	2.5	3.0
3	C- Fe^{3+}	g/l	1.0	4.5	8.0	11.5	15.0
4	D- *S.s./A.a.*	-	0.5	1.0	1.5	2.0	2.5

Table 4: Conditions of bioleaching tests.

Conditions	Unit	Content
Inoculation	%	10
Particle size	µm	74
	Mesh	200
Temperature	°C	70
Time	d	12
Shaking speed	rpm	120
Medium	-	9K free iron
$FeSO_4.7H_2O$	g/l	8.844
Sulfur	g/l	10
Yeast extract	%	0.01
Initial count	ML^{-1}	10^7

Bioleaching Experiments

Statistical designs of the experiments were employed to optimize the bioleaching process. Four factors, namely pulp density, initial pH, ferric concentration and the ratio of *Sulfolobus Solfataricus* to *Acidianus Ambivalens* were considered for optimization of bioleaching process through design expert software using the response surface method; and the effects of these factors were studied on molybdenum and uranium recoveries. Levels are shown in Table 3, with other conditions of bioleaching tests displayed in Table 4. The specifications of 30 runs of experiments and results of molybdenum and uranium recoveries are included in Table 5.

The ORP and pH of the culture media were measured during the 12-day period. All flasks were weighed each day, and the reductions in water level due to evaporation at 70°C were compensated for using distilled water.

RESULTA AND DISCUSSION

Isolation and Characterization of Bacteria

Acidophilic prokaryotes have been heavily utilized in bioleaching processes worldwide, and have thus become the subject of active research from a microbiological, biochemical and genetics point of view [27]. Commercially available microbial reference strains are often ineffective in the leaching of metal sulfides due to their inability to adapt and survive on sulfides and other minerals present in the ore [28]. Hence, more adaptable and robust microbes are routinely and extensively searched for in nature, which are capable of enhancing the bioleaching of such ores. The use of native bacterial strains, acclimatized to sulfide ores through many generations has been found to enhance metal extraction efficiency significantly [28]. Furthermore, the use of such bacterial strains eliminates the need for using standard reference strains, especially for large-scale studies [28]. In this study, two spherical-shaped, acidophilic and thermophilic bacteria isolated from a molybdenum mine in Iran were identified by PCR and registered in NCBI with the Gen-Bank Accession Numbers KM555276.1 and KM555275.1 for *Acidianus ambivalens* and *Sulfolobus solfataricus* respectively. In order to identify the isolated strains, sequencing of the bacterial Intergenic Spacer Region (ISR) (16S rDNA) was carried out.

Table 5: The specifications of 30 runs of experiments and results of molybdenum and uranium recovery.

Run	Pulp Density (%)	Initial pH	Fe^{3+} (g/L)	*S.s./A.a.*	Mo Recovery (%)	U Recovery (%)
1	6.0	2.0	8.0	0.5	25.1	39.4
2	8.0	1.5	4.5	1.0	28.1	65.0
3	6.0	2.0	8.0	1.5	31.9	74.1
4	6.0	2.0	8.0	1.5	35.0	72.8
5	8.0	2.5	11.5	1.0	27.5	75.1
6	6.0	2.0	8.0	1.5	30.0	62.9
7	4.0	1.5	11.5	1.0	44.3	79.3
8	8.0	2.5	11.5	2.0	31.2	63.3
9	6.0	2.0	15.0	1.5	34.0	69.5
10	6.0	2.0	8.0	2.5	34.1	71.2
11	4.0	2.5	11.5	1.0	32.7	63.2
12	10.0	2.0	8.0	1.5	29.1	68.1
13	6.0	2.0	8.0	1.5	31.2	72.4
14	2.0	2.0	8.0	1.5	34.3	70.3
15	6.0	2.0	8.0	1.5	31.7	67.4
16	8.0	1.5	11.5	2.0	37.2	71.7
17	4.0	1.5	11.5	2.0	34.4	79.3
18	4.0	2.5	11.5	2.0	38.5	74.1
19	6.0	2.0	1.0	1.5	26.8	53.2
20	8.0	1.5	4.5	2.0	27.6	79.4
21	6.0	2.0	8.0	1.5	32.7	69.5
22	4.0	1.5	4.5	2.0	41.1	72.8
23	6.0	1.0	8.0	1.5	34.3	85.1
24	8.0	2.5	4.5	2.0	22.3	69.0
25	4.0	2.5	4.5	2.0	35.7	68.3
26	6.0	3.0	8.0	1.5	24.2	61.9
27	8.0	1.5	11.5	1.0	33.2	48.3
28	8.0	2.5	4.5	1.0	19.2	65.2
29	4.0	1.5	4.5	1.0	17.9	71.1
30	4.0	2.5	4.5	1.0	21.3	57.8

Bioleaching with Isolated Bacteria

The design of response surface methodology experiments, the high and low levels of each factor and molybdenum and uranium recovery responses are presented in Table 5. These results were analyzed with software using a 2FI model for molybdenum, and a reduced 2FI model for uranium recovery. The P values less than 0.05 indicate that the models are significantly within 95% confidence level. The predicted versus actual responses of molybdenum and uranium recovery are shown in Fig. 2 that is (a) for molybdenum recovery and (b) for uranium. The two bacteria of *Sulfolobus solfataricus* and *Acidianus ambivalens* are used for bioleaching of molybdenum ore and their results

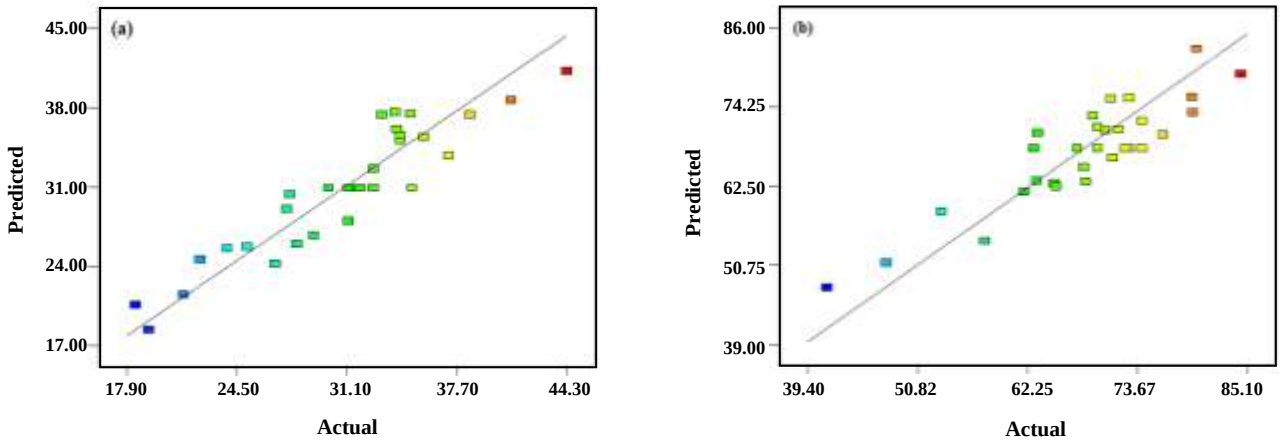

Fig. 2: Predicted versus actual plot for (a) Molybdenum recovery and (b) uranium recovery.

of molybdenum and uranium recoveries are included in Table 5. The pH, ORP and bacteria count are measured during tests. The pH was decreased to below 1 and ORP and bacteria count increased during the 12-day test period. At the end of the 12-day period, the pulp of each test was filtered and the filtrate analyzed for calculating molybdenum and uranium recoveries (Table 5). The software-analyzed results and the final equations for the models achieved as functions of factors for molybdenum and uranium follow:

The effects of the factors are displayed in Fig. 3 as: (a) interaction effect of pulp density and initial pH on molybdenum recovery, (b) interaction effect of pulp density and ratio of *Sulfolobus Solfataricus* to *Acidianus Ambivalens* on molybdenum recovery, (c) interaction effect of initial pH and ratio of *Sulfolobus Solfataricus* to *Acidianus Ambivalens* on molybdenum recovery, (d) interaction effect of Fe^{3+} concentration and ratio of *Sulfolobus solfataricus* to *Acidianus ambivalens* on molybdenum recovery, (e) interaction effect of pulp density and initial pH on uranium recovery and (f) interaction effect of pulp density and Fe^{3+} concentration on uranium recovery.

The effect of Fe^{3+} concentration is more than other factors on molybdenum recovery due to the latter's higher extraction potential. Some jarosite formed on the surface of the ore at higher Fe^{3+} concentration. It is therefore not possible to increase of pulp potential through ferric iron addition.

Variance analysis of response surface 2FI model for molybdenum recovery and that of response surface reduced 2FI model for uranium recovery are given in Table 6 and Table 7.

Optimized Conditions of bioleaching

The optimized conditions given by the software are 4% for pulp density, 1.5 for pH, 11.5 g/L for Fe^{3+} concentration and 2.0 for *S.s./A.a.* ratio. Software molybdenum and uranium recoveries were 39.0% and 84.4% respectively. Two confirmation tests were carried out at these optimum conditions proving molybdenum and uranium recoveries to be 43.2% and 79.1% respectively, close to software results. Two control tests were carried out in optimum conditions without bacteria. The molybdenum recovery (9.2%) and uranium recovery (20.7%) were low, proving *Sulfolobus Solfataricus* and *Acidianus Ambivalens* to be effective in the recovery of molybdenum and uranium. These results show that the bioleaching of molybdenum and uranium by native bacteria isolated from molybdenum ore is possible but it is necessary to increase ORP above 950 mV in order to increase molybdenum recovery. It is not possible to increase the ORP by conventional methods such as adding ferric iron due to jarosite formations. Jarosite is a basic hydrous sulfate of potassium and iron with a chemical formula of $KFe^{3+}{}_3(OH)_6(SO_4)_2$. This sulfate is formed in ore deposits by the oxidation of iron sulfides and forms an elimination layer on mineral surfaces. The pH is optimized at 1.5. It is the most preferable pH for these bacteria because of lower pH shocked bacteria at the beginning of the process and higher pH effect on bioleaching speed.

Table 6: ANOVA (Analysis of variance) for Response Surface 2FI Model for molybdenum.

Source	Sum of Squares	Df	Mean Square	F Value	p-value
Model	801.60	10	80.16	5.25	0.0010
A- Pulp Density	104.17	1	104.17	6.83	0.0171
B- Initial pH	128.81	1	128.81	8.44	0.0091
C- Fe^{3+}	268.00	1	268.00	17.57	0.0005
D- *S.s./A.a.*	159.14	1	159.14	10.43	0.0044
AB	16.81	1	16.81	1.10	0.3070
AC	0.25	1	0.25	0.02	0.8995
AD	33.64	1	33.64	2.21	0.1539
BC	0.56	1	0.56	0.04	0.8497
BD	6.50	1	6.50	0.43	0.5216
CD	83.72	1	83.72	5.49	0.0302
Residual	289.80	19	15.25		
Lack of Fit	275.61	14	19.69	6.94	0.0214

Table 7: ANOVA (Analysis of variance) for Response Surface Reduced 2FI Model for uranium recovery.

Source	Sum of Squares	Df	Mean Square	F Value	p-value
Model	1197.27	7	171.04	2.747	0.0328
A- Pulp Density	46.20	1	46.20	0.74	0.3983
B- Initial pH	248.97	1	248.97	4.00	0.0580
C- Fe^{3+}	61.12	1	61.12	0.98	0.3326
D- *S.s./A.a.*	565.51	1	565.51	9.08	0.0064
AB	139.83	1	139.83	2.25	0.1482
AC	132.83	1	132.83	2.13	0.1583
AD	2.81	1	2.81	0.04	0.8339
Residual	1369.85	22	62.27		
Lack of Fit	1282.15	17	75.42	4.30	0.0471

Sulfolobus solfataricus is stronger than *Acidianus ambivalens*, reproduces more rapidly and its ability to acidify solutions is more than *Acidianus ambivalens.* Some bioleaching tests were carried out at the optimum conditions with *Sulfolobus solfataricus*, *Acidianus ambivalens* and mixed culture of these bacteria and changing ORP, pH and bacteria count of were determined at the end of the 12 days. These results are shown in Fig. 4. Higher pulp density resulted in lower metal extraction but higher density in more economic leach solutions will give higher grade metals with lower chemical consumption.

CONCLUSIONS

In this study, two native spherical-shaped acidophilic and thermophilic bacteria, isolated from a molybdenum mine in Iran. These bacteria were identified by PCR method and registered at the NCBI. Gen-Bank Accession Numbers were KM555276.1 and KM555275.1 for *Acidianus ambivalens* and *Sulfolobus solfataricus* respectively. The bioleaching potential of these bacteria was studied proving more effective in the bioleaching of molybdenum and uranium. The bioleaching tests were carried out in 12 days at temperature of 70°C, inoculation

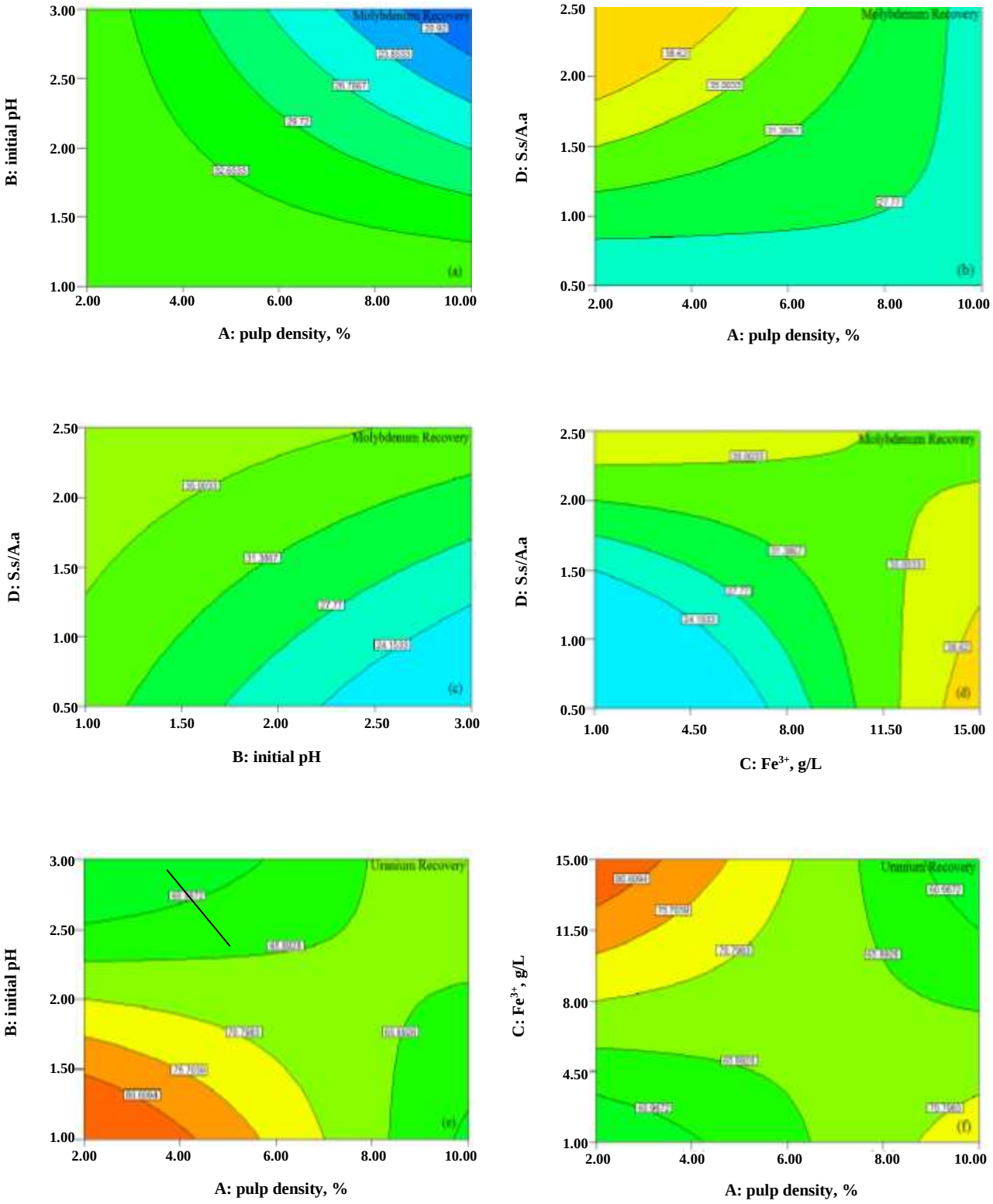

Fig. 3: Interaction effect of factors, including pulp density, initial pH, of Sulfolobus solfataricus to Acidianus ambivalens and Fe^{3+} concentration on responses (Molybdenum recovery and uranium recovery) at temperature of 70 ºC and inoculation of 10%.

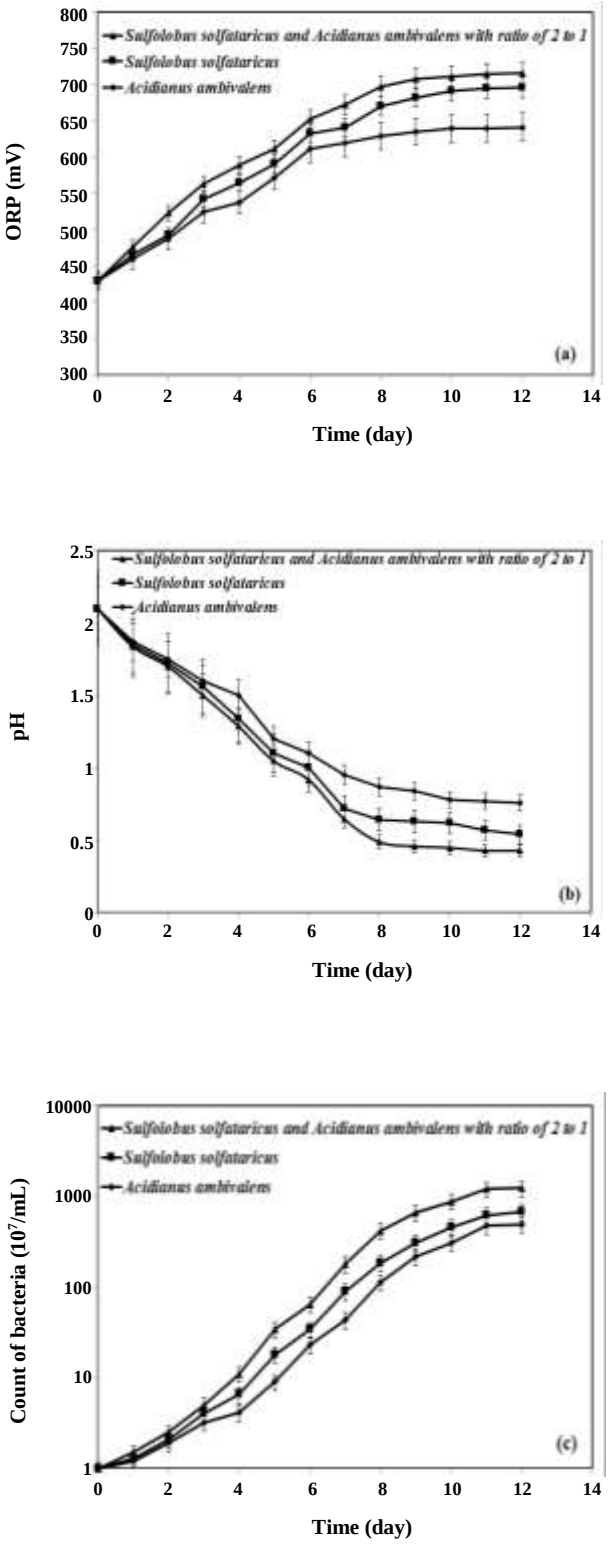

Fig. 4: Variation of (a) ORP, (b) pH, and (c) count of bacteria during 12 days of 18 bioleaching experiments were carried out in which pulp density was 4% and Fe^{3+} concentration was 11.5 g/L.

od 10%, particle size of 74 µm, and initial count of 10^7 mL^{-1}. Pulp density of 4%, initial pH of 1.5, Fe^{3+} concentration of 11.5 g/l and *Sulfolobus solfataricus* to *Acidianus ambivalens* ratio of 2.0 were selected as optimum conditions after carrying out 30 tests. It was shown that increasing of ORP above 950 mV by adding ferric iron is not possible due to jarosite formations. Molybdenum and uranium recoveries were 43.2% and 79.1% respectively; whereas molybdenum and uranium recoveries of control solutions were 9.2% and 20.7% respectively. Results prove *Sulfolobus solfataricus* and *Acidianus ambivalens* isolated from the indicated ore, effective in the recovery of molybdenum and uranium.

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Abbreviations

A.a.	*Acidianus ambivalens*
Am	Amphibole
Bt	Biotite
Cal	Calcite
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EPMA	Electron probe micro-analyzer
Mo	Molybdenite (in Fig. 1)
Ms	Muscovite
NCBI	National Center for Biotechnology Information of USA
PCR	Polymerase chain reaction
Pl	Plagioclase
Py	Pyrite
Qtz	Quartz
SEM	Scanning Electron Microscopy
S.s.	*Sulfolobus solfataricus*
UM	Uranium minerals
XRD	X-ray diffraction
XRF	X-ray fluorescence

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