Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2019.23723

147 (2019) 264–272
April

Characterization of heterotrophic iron and sulfur-oxidizing bacteria from acid mine drainage in Sarcheshmeh copper mine Iran

Mehdi Hassanshahian[a,*], Shiva Toorani[b], Rasoul Roghanian[b], Giti Emtiazi[b,c], Maria Genovese[d]

[a]*Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran, Tel. +989132906971; Fax: +983222032; email: mshahi@uk.ac.ir*
[b]*Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran, emails: tooranishiva@yahoo.com (S. Toorani), r.roghanian@sci.ui.ac.ir (R. Roghanian), emtiazi@yahoo.com (G. Emtiazi)*
[c]*Department of Biotechnology, Faculty of Biological Science and Technology, Shahid Ashrafi Esfahani University, Isfahan, Iran*
[d]*Institute of Biological Resources of Marine Biotechnology (IRBIM)-CNR of Messina, Sp. San Raineri 86, 98122 Messina, Italy, email: maria.genovese@cnr.it*

Received 8 August 2018; Accepted 1 January 2019

ABSTRACT

Acid mine drainage (AMD) is an environmental pollution that is generated from the oxidation of sulfide minerals that exist in the mine dumps. AMD causes pollution of surface and underground waters and lead to distribution of heavy metals into the environment. The aim of this research is isolation and identification of heterotrophic iron and sulfur-oxidizing bacteria from AMD of Sarcheshmeh copper mine. The mine samples were collected from six different locations of the mine. Iron and sulfur-oxidizing heterotrophic bacteria were quantified with serial dilution and most probable number methods. Heterotrophic oxidizing bacteria were screened by three methods: decrease in pH, sulfate ion production activity and growth rate. Most efficient heterotrophic oxidizing bacteria were identified by molecular method. The results of this research show that the maximum quantity of these bacteria in Sarcheshmeh copper mine relate to dumps number 32 and 17 with value 8 × 10 and 5.9 × 10, respectively. Thirty two strains were isolated from six different locations of this copper mine and after screening tests 15 effective strains were selected and 8 strains were identified by molecular method. The results of the molecular identification showed that eight strains belong to: *Bacillus thuringiensis* strain DP26-1, *Bacillus* sp. strain DP-15-4, *Pseudochrobactrum* sp., *Brevundimonas* sp., *Brevibacillus formosus*, *Sphingomonas ginsenosidimutans*, *Pseudomonas brassicacearum*, *Sphingomonas paucimobilis*. Most efficient heterotrophic oxidizing bacteria related to DP-17-3 and DP-30-1 strains that produce high percentage of sulfate ion (270 and 223 ppm) also decrease dramatically the pH (4.2 and 4.9). Application of these bacteria in bioleaching process can enhanced efficiency of metal extraction in this copper mine.

Keywords: Acid mine drainage; Heterotrophic bacteria; Sarcheshmeh copper mine

1. Introduction

Acid mine drainage (AMD) is a source of environmental pollution as a result of oxidation of sulfide minerals in the mining and mineral waste. Mining of certain ore deposits, including gold, copper and nickel, along with acidic drainage problem have long-term detrimental effect on the environment. In most cases, bacteria play major role in intensifying the rate of acid production. Preventing the activity of bacteria can be an obstacle to speed the formation of acid. Exposing sulfide minerals to atmosphere and water exacerbates the production of drainage [1].

Damages into the environment and pollution from mines are accompanied by decrease in pH or an increase

* Corresponding author.

in the concentration of heavy metals in adjacent soils and waters. There are many examples of increasing the concentration of metals or reducing their concentration, even when neutral pH is present. The composition of AMD creates an inadequate environment for many aquatic organisms. However, many microorganisms are able to survive under these extreme conditions [2].

High concentrations of metals, acidity and low levels of nutrients are factors limiting the growth of microorganisms in the AMD. Bacteria, fungi, yeasts and algae have been reported from conventional microorganisms that are compatible with the AMD environment. The presence of a symbiotic relationship between microorganisms increases their ability to cope with the difficult environment and survive in the wastewater. Bioleaching is one of the simple technologies for extracting metals from low-grade ore minerals and concentrates that cannot be economically exploited by conventional methods. Bioleaching has advantages over the traditional physicochemical methods as follows: (1) it does not require a high level of energy and is more economical; (2) no harmful environmental gases are produced; (3) almost, all microbial processes are more environmentally friendly; (4) in the case of low-grade ores and many of these ores cannot be recycled by conventional means [3–5].

Typically, bioleaching is carried out by chemolithotrophic iron and sulfur-oxidizing bacteria. These bacteria have been studied extensively and have a specialty and high-performance for bioleaching activity. Chemolithotrophic iron and sulfur-oxidizing acidophilic bacteria share mineral leaching environments with a range of other microorganisms, including fungi, algae, protozoans and rotifers, as well as other bacteria. Recently, a novel group of mesophilic heterotrophic acidophiles has been described. These bacteria are able to oxidize ferrous iron to ferric iron, but in contrast to chemolithotrophic acidophiles, they require organic carbon for growth. The heterotrophic iron and sulfur-oxidizing bacteria were not studied well [6].

Sarcheshmeh copper mine is a large open pit copper mine in the Kerman province of Iran, considered to be the second largest copper deposit worldwide. Also containing substantial amounts of molybdenum, gold and other rare metals. It is also a settlement, enumerated at Iranian census. The Sarcheshmeh copper mine is located 65 km southwest of Kerman and 50 km south of Rafsanjan. The region's altitude averages about 2,600 m, the highest spot of which approximates 3,000 m. Sarcheshmeh ore bodies, situated in the central part of Zagros ranges, consist of folded and faulted early tertiary volcano sedimentary rocks [6].

The aim of this research is enumeration, isolation, identification and characterization of heterotrophic iron and sulfur-oxidizing bacteria from Sarcheshmeh copper mine. There is not any study for description of these main bacteria in this mine.

2. Material and methods

2.1. Sampling

AMD samples were collected from six different locations of Sarcheshmeh copper mine at Kerman province (58° 30, N; 29° 15, E) in two seasons (Spring and Autumn of 2017). First, 10 cm was removed from the surface of the mine soil and about 500 g of the underlying mine soil was taken into sterile containers. Mine soil samples were transported to the laboratory on ice and kept at 4°C until further study. Also, AMD waste samples (500 mL) were collected in the same condition. The sample locations with abbreviations name were presented in Table 1. Also, the map of sampling zones is illustrated in Fig. 1 [6].

2.2. Enumeration of iron and sulfur-oxidizing heterotrophic bacteria in mine samples

Iron and sulfur-oxidizing heterotrophic bacteria in each mine samples (soil and water) were enumerated by two methods: most probable number (MPN) and serial dilution. For MPN, a miniaturized MPN method was used according to Brown and Braddock [7]. Thiosulfate sodium glucose broth (TSGB) medium was used for enumeration of iron and sulfur-oxidizing heterotrophic bacteria. Mine soil and AMD waste samples were diluted in saline buffer solution that contained 0.1% sodium pyrophosphate (pH 7.5). Tenfold serial dilution was performed in microplates that were inoculated by adding 20 μL of each dilution to 1 of the 12 row wells. The first row of each plate served as a sterile control. Microplates were incubated at 20°C ± 1°C for 7 d. MPN was carried out as triplicate. MPN counts were performed with the computer program MPN Calculator version 4.1 [8].

Oxidizing-heterotrophic bacteria abundances in each mine samples were measured also by serial dilution method. Oxidizing-heterotrophic bacteria in the samples were estimated by spreading 100 μL of 10-fold diluents on plates of thiosulfate sodium glucose agar (TSGA) medium and incubating at 30°C for 3 d. The results were expressed as colony forming unit per gram (CFU g^{-1}) [8–10].

2.3. Enrichment and isolation of iron and sulfur oxidizing heterotrophic bacteria

TSGB medium was used for isolation of iron and sulfur-oxidizing heterotrophic bacteria. TSGB contained (per liter

Table 1
The abbreviation of locations that mine samples were collected

Sampling station	Abbreviation
Dump Number 11	DP-11
Dump Number 15	DP-15
Dump Number 17	DP-17
Dump Number 18	DP-18
Dump Number 19	DP-19
Dump Number 21	DP-21
Dump Number 26	DP-26
Dump Number 30	DP-30
Dump Number 31	DP-31
AMD waste of mine floor	AW
AMD waste after leaching hip	RH
Sulfuric mine soil after leaching hip	SY
AMD waste near to pound of leaching solution (PLS)	BL

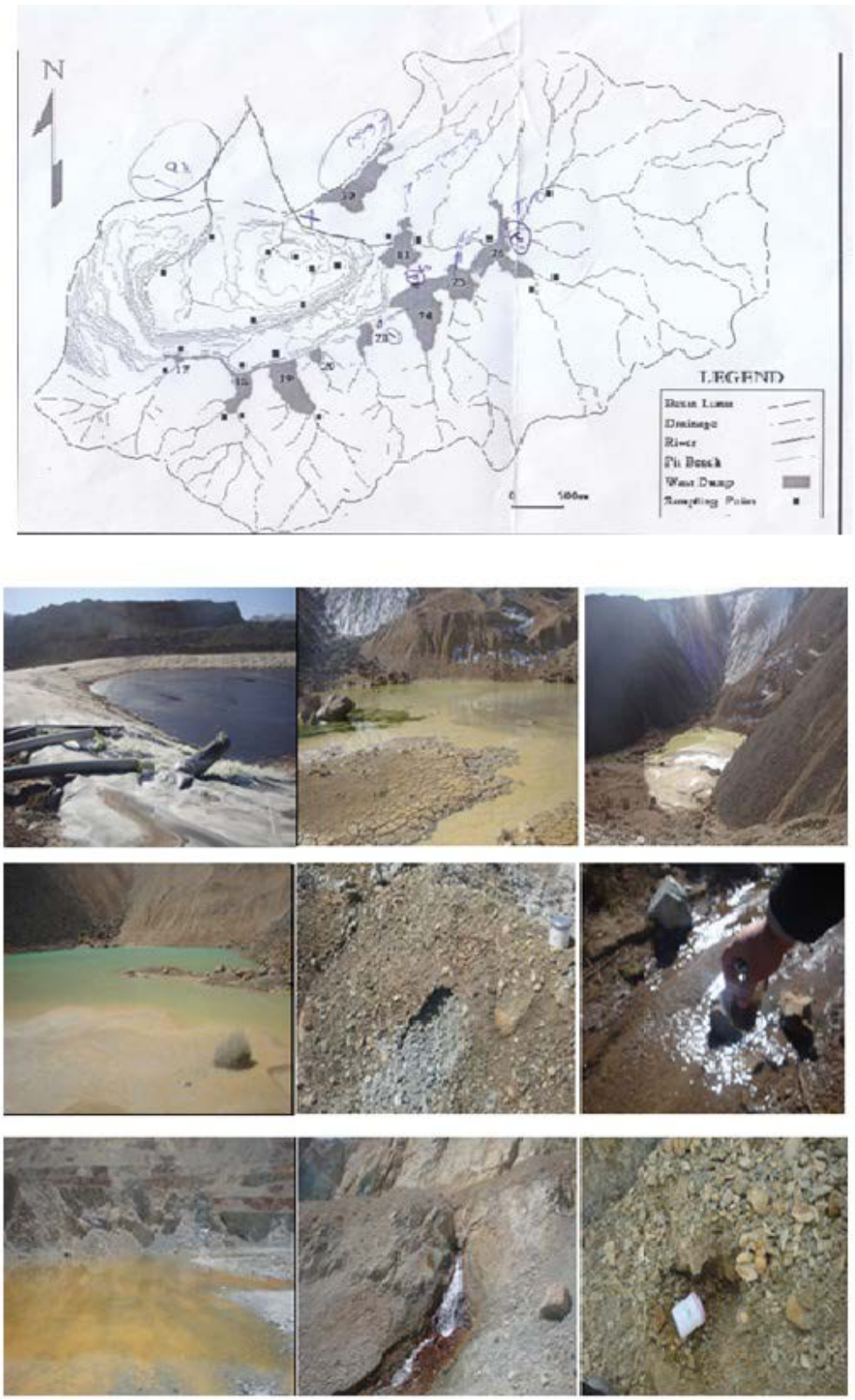

Fig. 1. Map of sampling locations at Sarcheshmeh copper mine.

of distilled water) 0.2 g K_2HPO_4, 30 g $Na_2S_2O_3$, 0.1 g $MgSO_4$, 0.1 g $CaCl_2$, 0.1 g $(NH_4)_2SO_4$, 0.02 g $FeCl_3$, 10 mL H_2SO_4 (1 N), 15 g glucose. For solid media, Bacto Agar (Difco) (15 g L) was added to the solution. Portion of mine soil (10 g) or waste of AMD (10 mL) was added to Erlenmeyer flasks containing 100 mL of medium and the flasks were incubated for 10 d at 30°C on rotary shaker (180 rpm, INFORS AG, Switzerland). Then 5 mL aliquots were removed to fresh medium. After a series of four further subcultures, inoculums from the flask were streaked out and phenotypically different colonies purified on TSGA medium. The procedure was repeated and isolates only exhibiting pronounced growth on TSGA were stored in stock media with glycerol at –20°C for further characterization [11–13].

2.4. Screening of iron and sulfur-oxidizing heterotrophic bacteria

Three tests were used for screening and selection of the iron and sulfur-oxidizing heterotrophic bacteria. These tests were described below.

2.4.1. Decrease of pH in sulfur heterotrophic medium

One of the important indicators in the screening of heterotrophic bacteria that can oxidize iron and sulfur is reduction of the medium pH by increasing the incubation time. This screening test was performed for all isolated strains. The sulfur heterotrophic (SH) medium was used for this analysis. The composition of SH medium was (per liter of distilled water): 2 g K_2HPO_4, 30 g elemental sulfur, 0.1 g $MgCl_2$, 0.1 g $CaCl_2$, 0.1 g NH_4Cl, 0.02 g $FeCl_3$, 10 mL H_2SO_4(1 N) and 15 g glucose. For solid media, Bacto Agar (Difco) (15 g L) was added to the solution. The pH of SH medium was adjusted to 7 by sulfuric acid (1 N). Then, all isolated bacteria were grown in nutrient broth medium for 24 h and 5 mL from this medium were inoculated to 100 mL SH medium in flask. The flasks were incubated in rotary shaker (160 rpm) at 30°C for 2 weeks. During this period of 14 d, four stages within 3 d, pH was measured with pH meter. Strains that had a significant reduction in pH of 2–3 were selected for the next steps [14].

2.4.2. High growth ability in SH medium

The isolated bacteria were cultured in SH medium. Glucose is the sole carbon source of SH medium also contains elemental sulfur for oxidation; the growth potential in this medium was considered as a screening index. After growth, optical density (OD) of strains was read and recorded in at 600 nm by spectrophotometer [15,16].

2.4.3. Sulfate ion production ability

The amount of sulfate ion (SO_4) produced during growth of sulfur-oxidizing bacteria on SH medium was determined spectrophotometrically. Sulfate was measured by adding 1:1 barium chloride solution (10% w/v) with bacterial culture supernatant followed by mixing the suspensions vigorously. A resulting white turbidity due to barium sulfate formation was measured at 450 nm with a spectrophotometer (Carry 50). The value obtained was compared with the sulfate standard curve. Potassium sulfate (K_2SO_4) was used as standard to construct a sulfate calibration curve according to Holt et al. [17]. Standard sulfate solutions were made by dissolving K_2SO_4 in deionized water to known concentrations in the range 0–3 mM. The amount of turbidity formed is proportional to the sulfate concentration.

2.5. Identification of the isolates

2.5.1. Biochemical identification

The bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterization such as: oxidation/fermentation, production of acid from carbohydrates, hydrolysis of gelatin and citrate.

Table 2
Results of quantification of iron and sulfur-oxidizing heterotrophic bacteria in collected mine samples with MPN and serial dilution methods

Sample	Quantity of iron and sulfur-oxidizing bacteria (CFU g)	MPN of iron and sulfur-oxidizing heterotrophic bacteria (MPN g)
DP-11	1.5×10^7	2.18×10^8
DP-15	4.6×10^4	1.2×10^5
DP-17	1.1×10^8	5.9×10^9
DP-18	1.5×10^6	2×10^7
DP-19	2.9×10^7	3.5×10^8
DP-21	2.4×10^3	1×10^4
DP-26	2.4×10^7	5.4×10^8
DP-30	4.31×0^5	1.4×10^6
DP-31	2.3×10^8	8×10^9
RH	1.1×10^5	1×10^6
SY	2.3×10^7	9×10^8
AW	4.6×10^6	1.3×10^7
BL	1.5×10^5	3.7×10^5

These tests were carried out according to the Bergey's manual for taxonomic identification [18,19].

2.5.2. Molecular identification

Analysis of 16S rRNA was performed for the taxonomic characterization of isolated strains. Total DNA extraction of bacterial strains was performed with the CTAB method (Hassanshahian et al. [22] 2016). The bacterial 16S rRNA loci were amplified using the forward domain specific bacteria primer, Bac27_F (5′-AGAGTT TGATCCTGGCTCAG-3′) and universal reverse primer Uni_1492R (5′-TACGYTACCTTGTTACGACTT-3′). The amplification reaction was performed in the total volume of 25 μL consisting, 2 mM $MgCl_2$ (1 μL), 10X PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 μL), 2 mM each dNTP (2 μL), 0.15 mM each primer (1 μL), 1U (0.5 μL) Taq DNA polymerase (Qiagen, Hilden, Germany) and 2 μL of template DNA (50 pm). Distilled water was added for remaining of reaction (15 μL).

Amplification was performed in a thermal cycler GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was kept, 94°C for 5 min, 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, 30 cycles; then 72°C for 10 min and finally storage at 4°C (Hassanshahian 2014). The 16S rRNA amplified was sequenced with a BigDye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems, USA). Similarity rank from the Ribosomal Database Project and FASTA Nucleotide Database. Query were used to determine partial 16S rRNA sequences to estimate the degree of similarity to other 16S

Table 3
Reduction in the pH of SH medium for screening of most efficient iron and sulfur-oxidizing heterotrophic bacteria

Isolates	pH (beginning of experiment)	pH (3 d after experiment)	pH (6 d after experiment)	pH (9 d after experiment)	pH (12 d after experiment)	OD_{600} nm in SH medium
26.1	7	5.82	5.64	7.57	7.71	1.3
26.2	7	6.66	5.89	5.85	5.82	1
26.3	7	5.42	5.47	5.6	5.6	0.5
26.4	7	6.02	6.51	6.35	6.28	1.5
26.5	7	7.18	7.3	7.54	7.4	1.6
RH.1	7	6.25	6.19	6.36	6.58	0.75
BL.1	7	6.41	6.29	6.22	6.35	0.5
15.1	7	6.4	6.91	7.43	7.34	1.9
15.2	7	6.31	6.51	6.56	6.33	1.9
15.3	7	6.67	5.69	4.83	4.78	1.4
15.4	7	6.19	5.62	5.33	5.36	1.5
21.1	7	6.33	6.72	7.35	7.43	1.9
21.2	7	6.4	7.45	8.37	8.24	1.9
21.3	7	6.42	4.89	4.93	4.74	0.5
21.4	7	6.63	7.83	8.4	7.9	2
SY.1	7	6.63	6.48	4.92	4.72	0.8
SY.2	7	6.27	6.18	6.11	6.29	1.9
SY.3	7	6.23	6.24	6.16	6.11	1.9
SY.4	7	6.84	5.27	5.71	5.71	1.3
SY.5	7	6.47	6.11	5.84	5.64	0.75
30.1	7	6.8	7	4.9	4.9	1.2
30.2	7	6.8	7.6	7.12	4.67	1.8
30.3	7	4.6	4.8	4.12	4.17	1.9
30.4	7	4.88	5.31	4.8	4.8	1.9
19.2	7	4.8	5.5	4.5	4	1.1
19.3	7	4.8	5.1	4.6	4.3	1.4
17.3	7	5	5	5.5	4.3	1.9
17.4	7	6.6	8	7.9	7.7	1.9
31.1	7	5.5	5.7	5.1	5.5	1.7
31.2	7	5.8	5.3	4.6	4.5	1.9
AW.1	7	5.12	5.32	5.06	5	1.6
AW.3	7	5.19	5.3	5.18	5.14	1.7

rRNA gene sequences. Analysis and phylogenetic affiliates of sequences were performed as previously described protocols [20–23].

3. Results

3.1. Quantity of iron and sulfur-oxidizing heterotrophic bacteria in mine samples

The quantity of iron and sulfur-oxidizing heterotrophic bacteria was determined in all collected samples by two enumeration method: CFU and MPN. The results are presented in Table 2. As shown in this table the maximum quantity of these bacteria in Sarcheshmeh copper mine relate to dumps number 17 and 31. In the dumps number 15 and 21, quantity of iron and sulfur-oxidizing heterotrophic bacteria was minimum in comparison with other mine locations.

3.2. Screening of the best iron and sulfur-oxidizing heterotrophic bacteria

The most efficient iron and sulfur-oxidizing heterotrophic bacteria were screened by three tests. The results are shown in Table 3. As shown in this table after incubation period (12 d), some strains significantly reduced the pH of SH medium (two unit). These strains include: 21.3, 21.4, SY.1, SY.4, SY.5, 30.2, 30.3, 19.2, 19.3, 17.4, 31.2, AW.1 and AW.3. Some isolates such as BL.1, 15.2, SY.2, SY.3 and RH.1 had average reduction in medium pH. Also, a few strains such as 26.1, 26.5, 15.1, 21.1 and 21.2 had not any changes in the medium pH. These strains were eliminated for further studies. Table 3 also shows the optical density (OD_{600} nm) of isolated strains in SH medium. As shown in this table all isolates had sufficient growth in this medium.

3.3. Sulfate ion determination

The amount of sulfate ion produced by each isolated bacteria was calculated from the calibration curve. The results are presented in Table 4. As shown in this table the strains 17.3, 17.4 and 30.1 produced maximum amount of sulfate ion in SH medium. The minimum sulfate ion production related to strains 19.3, SY.2 and 21.2.

3.4. Identification of the most efficient iron and sulfur-oxidizing heterotrophic strains

Thirty two strains of iron and sulfur-oxidizing heterotrophic bacteria were isolated from mine samples after enrichment cultures that were incubated at 30°C for 2 weeks. Nineteen strains that show high reduction in pH at SH medium and maximum sulfate ion production were selected for further study. These strains were first identified by classical biochemical tests. The results of biochemical tests for these strains are presented in Table 5. According to this table, the strains that had similar pattern in biochemical tests were deleted and eight strains were selected for molecular identification. The most efficient strain (8 strain) was molecularly identified. This identification was done by amplifying and sequencing the 16S rRNA gene. The results of the molecular identification showed that eight isolated bacteria belong to: *Bacillus thuringiensis* strain DP26-1, *Bacillus* sp. strain DP-15-4, *Pseudochrobactrum* sp. strain DP26-4, *Brevundimonas* sp. strain DP-21-4, *Brevibacillus formosus* strain SY-5, *Sphingomonas ginsenosidimutans* strain DP-31-2, *Pseudomonas brassicacearum* strain DP-30-1, *Sphingomonas paucimobilis* strain DP-19-1.

All sequences of eight bacteria were submitted to the genetic sequence database at the National Center for Biotechnology Information (NCBI). The gene bank IDs of these strains in NCBI are LN909504 to LN909511. The phylogenetic trees of these eight isolated strains were illustrated in Fig. 2. This figure show that strain DP-26-4 and SY-5 have high similarity with other sequences that present in gene bank database, although the similarity of other strains is less in compare to these two strains.

4. Discussion

Acidic mines drainage is one of the environmental problems of open pit mines. There are several ways to manage this acid drainage. Understanding the microbial community of AMD has importance for selecting the strategies to remediation of this waste [24]. In this study, microbial population of iron and sulfur-oxidizing heterotrophic bacteria was investigated in acid drainage of Sarcheshmeh copper mine. The results of this study showed that AMD had diversity of these two bacteria groups. So far, no research has been carried out on oxidizing heterotrophs in this mine, and this study is the first report of oxidizing heterotrophs in this mine [26].

In this research, iron and sulfur-oxidizing heterotrophic bacteria in mine samples quantified by MPN and serial dilution methods. The results of enumeration confirmed that the maximum quantity of these bacteria present in the dump number 31 and 17. These two dumps produced the highest level of AMD in the Sarcheshmeh copper mine. Then, there are a direct relationship between the quantity of heterotrophic bacteria and the amount of AMD that produced by dumps. Sajjad et al. [19] studied the quantity of heterotrophic and mixotrophic acidophilic bacteria from black shale and AMD in Pakistan. Their results had shown that the microbial population is dependent on the concentration of metals and the acidity of the mine samples. So, the quantity of oxidizing-heterotrophic bacteria was minimum in the mine samples with acidity or metal concentration. However, acidophilic bacteria had maximum quantity in these samples.

In this study, we used three screening tests for selecting the efficient iron and sulfur-oxidizing bacteria from collected mine samples. These screening tests were also applied by other researchers for the selection of these bacteria. For example, Behera et al. [20] screened sulfur-oxidizing bacteria from mangrove soil of Mahanadi river delta, Odisha, India, and evaluate their sulfur-oxidation ability. Their results showed that in 30 sulfur-oxidizing bacteria were isolated from six different location of mangrove soil. From the qualitative screening, it was found that out of the 30 bacterial isolates, 12 isolates could reduce the pH of the medium up to 4.2 from the initial pH 8.0. Their sulfate ion production abilities were in the range of 125–245 mg mL. All researchers confirmed that between these three screening tests sulfate ion production

ability is the effective method because this method has the lowest error and can be applied in research [23].

Many research works has been done on autotrophic iron and sulfur-oxidizing in mines and AMD. While, a few significant study has been reported on heterotrophs. Some researches in this field are described as follows. Baker and Banfield [26] studied microbial communities in AMD at United States. They isolated and characterized *Acidobacterium* and *Propionibacterium* as two sulfur-oxidizing heterotrophic bacteria. Nicomrat et al. [23] screened heterotrophic oxidizing bacteria from acid coal mine drainage by molecular methods. Their isolates were identified as: *Stenotrophomas maltophilia, Bordetella* spp., *Alcaligenes* sp., *Alcaligenes faecalis*, *Alcaligenes xylosoxidans.*

In this study, iron and sulfur-oxidizing heterotrophic bacteria were isolated and identified from Sarcheshmeh copper mine. We isolated 32 strains from 6 different locations of this copper mine and after screening tests we reach to 15 strains and 8 strains were identified by molecular methods. These strains belong to seven different genus. Also, the bacterial genera described in this study are consistent with the bacterial genera reported by other researchers [25].

The heterotrophic iron and sulfur-oxidizing bacteria have potential application in biotechnology and bioleaching. Study of the bioleaching activity of these bacteria will be suggested in the future [20].

The results of this study confirmed that heterotrophic iron and sulfur-oxidizing bacteria have diversity in dumps of Sarcheshmeh copper mine. This research is the first report of these bacteria in this copper mine. Some of these bacteria decreased the pH of medium and produced sulfate ion. These results indicate oxidizing potential of these bacteria despite the heterotrophic metabolism of them. In further, by investigating the ability of metal leaching by these bacteria, the production efficiency of metals from low-grade ore can be increased.

Table 4
Sulfate ion production by most efficient iron and sulfur-oxidizing heterotrophic bacteria

Strain	Sulfate ion production (ppm)
26.1	174
26.2	171
26.3	201
26.4	186
15.1	189
15.2	162
21.1	185
21.2	156
21.4	183
SY.2	149
30.1	223
30.2	202
19.3	139
17.3	270
17.4	212

Table 5
Biochemical characterization of selected iron and sulfur-oxidizing heterotrophic bacteria

Strain name	Gram stain	Catalase	Oxidase	O	F	Nitrate	Citrate	S	I	M	TSI
30.1	–	+	+	–	+	+	+	+	–	+	Alkali/alkali
30.2	+	+	+	–	+	+	+	–	–	–	Acid/alkali
30.3	–	+	+	+	–	+	+	+	–	–	Acid/alkali
31.2	–	+	+	–	+	–	–	–	–	–	Acid/alkali
Sy.1	–	+	+	–	+	+	–	–	–	–	Alkali/alkali
Sy.4	–	+	+	–	+	–	–	–	–	–	Acid/alkali
Sy.5	–	+	–	–	+	+	–	–	–	+	Alkali/alkali
17.3	–	+	+	+	–	+	+	–	–	–	Acid/alkali
17.4	–	+	+	+	–	–	+	–	–	–	Acid/alkali
26.1	–	–	–	–	+	+	–	+	–	+	Alkali/alkali
26.2	–	+	+	+	–	+	–	–	–	–	Alkali/alkali
26.3	–	+	+	–	+	–	–	–	–	+	Alkali/alkali
26.4	–	+	+	+	–	–	+	–	–	+	Alkali/alkali
15.3	–	+	–	–	+	+	–	+	–	+	Alkali/alkali
15.4	–	+	+	–	+	+	–	+	–	+	Acid/alkali
21.2	+	+	+	–	+	+	–	+	–	–	Alkali/alkali
21.4	+	+	+	–	+	–	–	–	–	–	Acid/alkali
AW.1	+	+	+	+	+	–	+	–	–	+	Alkali/alkali
19.3	–	+	+	–	+	–	–	–	–	+	Acid/alkali

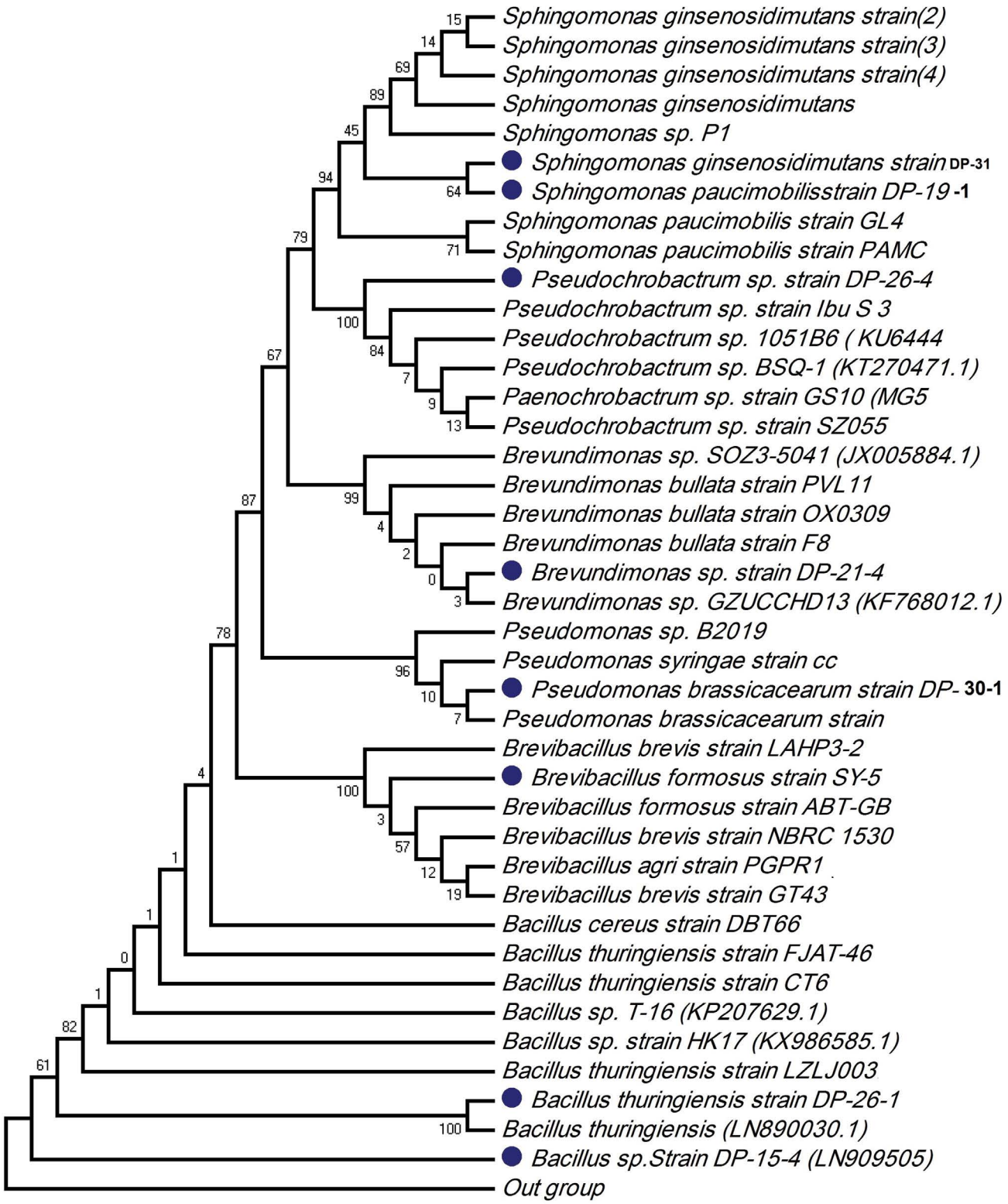

Fig. 2. Phylogenetic tree showing the inter-relationships of isolated strains with the most closely related type strains inferred from sequences of 16S rRNA gene. *Vibrio anguillarum* was used as an out group. The tree was generated using the neighbor-joining method. Bootstrap values expressed as a percentage of 1,000 replications, are given at the branching points.

5. Conclusion

Acidic mines drainage is one of the environmental problems of open pit mines. In this research, three screening tests were used for selection of the most efficient iron and sulfur-oxidizing bacteria. The results of this study confirmed that sufficient diversity of iron and sulfur-oxidizing bacteria was exist in Sarcheshmeh copper mine. Use of these bacteria in bioleaching process can enhance the efficiency of metal extraction in this copper mine.

Acknowledgments

This research was financially supported by Shahid Bahonar University of Kerman and research and development center of Sarchshmeh copper mine. Also authors would like to thank the University of Isfahan for financial help.

Conflict of interest

There are not any conflict of interest between authors.

References

[1] R.R. Auld, M. Myre, N.C. Mykytczuk, L.G. Leduc, T.J. Merritt, Characterization of the microbial acid mine drainage microbial community using culturing and direct sequencing techniques, J. Microbiol. Methods, 93 (2013) 108–115.
[2] A. Schippers, A. Breuker, A. Blazejak, K. Bosecker, K. Kock, D. Wright, The biogeochemistry and microbiology of sulfidic mine waste and bioleaching dumps and heaps, and novel Fe(II)-oxidizing bacteria, Hydrometallurgy,104 (2010) 342–350.
[3] M. Ryan, A. Schippers, W. Sand, Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation, Appl. Microbiol. Biotechnol., 97 (2013) 74.
[4] G.M. Gadd, Metals, minerals and microbes, Geomicrobiol. Biorem. Microbiol., 156 (2010) 609–643.
[5] K.B. Hallberg, New perspectives in acid mine drainage microbiology, Hydrometallurgy, 104 (2010) 448–453.
[6] M. Hassanshahian, S. Ghorbani, Isolation and characterization of iron and sulfur-oxidizing bacteria from Maiduk Copper Mine at Shahrbabk province in Iran, Geomicrobiology, 35 (2017) 27–37.
[7] E.J. Brown, F. Braddock, Sheen screen, a miniaturized most-probable-number method for enumeration of oil-degrading microorganisms, Appl. Environ. Microbiol., 56 (1990) 3895–3896.
[8] D. Leduc, L.G. Leduc, G.D. Ferroni, Quantification of bacterial populations indigenous to acidic drainage streams, Water Air Soil Pollut., 6 (2002) 1–21.
[9] M. Hasanshahian, G. Emtiazi, Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation, Int. Biodeterior. Biodegrad., 62 (2008) 170–178.
[10] M. Hassanshahian, G. Emtiazi, R. Kermanshahi, S. Cappello, Comparison of oil degrading microbial communities in sediments from the Persian Gulf and Caspian Sea, Soil Sediment Contam., 19 (2010) 277–291.
[11] I. Ñancucheo, D.B. Johnson, Production of glycolic acid by chemolithotrophic iron-and sulfur-oxidizing bacteria and its role in delineating and sustaining acidophilic sulfide mineral-oxidizing consortia, J. Appl. Environ. Microbiol., 76 (2010) 461–467.
[12] M. Hassanshahian, H. Tebyanian, S. Cappello, Isolation and characterization of two crude-oil degrading yeast strains, Yarrowia lipolytica PG-20 and PG-32 from Persian Gulf, Mar. Pollut. Bull., 64 (2012) 1386–1391.
[13] M. Hassanshahian, G. Emtiazi, S. Cappello, Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea, Mar. Pollut. Bull., 64 (2012) 7–12.
[14] M. Hassanshahian, M. Ahmadinejad, H. Tebyanian, A. Kariminik, Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances), Mar. Pollut. Bull., 73 (2013) 300–305.
[15] A. Romanenko, A. Grassellino, O. Melnychuk, D.A. Sergatskov, Dependence of the residual surface resistance of superconducting radio frequency cavities on the cooling dynamics around, J. Appl. Phys., 115 (2014) 184903.
[16] Å. Kolmert, P. Wikström, K.B. Hallberg, A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures, J. Microbiol. Methods, 41 (2000) 179–184.
[17] S.G. Holt, N.R. Kriey, P.H.A. Sneath, J.T. Staley, S.T. Williams, Bergy's Manual of Determinative for Bacteriology, Williams and Wilkins, New York, 1998.
[18] H.M. Amminiblöthe, A. Schippers, Microbial diversity at the moderate acidic stage in three different sulfidic mine tailings dumps generating acid mine drainage, Res. Microbiol., 165 (2013) 713–718.
[19] W. Sajjad, T.M. Bhatti, F. Hasan, A.A. Shah, Characterization of heterotrophic and mixotrophic acidophilic bacteria isolated from black shale and acid mine drainage, Khala Chatta, Haripur. Pakistan, Int. J. Biosci., 6 (2015) 62–70.
[20] B.C. Behera, M. Patra, S.K. Dutta, H.N. Thatoi, Isolation and characterisation of sulphur oxidising bacteria from mangrove soil of Mahanadi River delta and their sulphur oxidising ability, J. Appl. Environ. Microbiol., 2 (2014) 1–5.
[21] F. Anjum, M. Shahid, A. Akcil, Biohydrometallurgy techniques of low grade ores: a review on black shale, Hydrometallurgy, 117 (2012) 1–12.
[22] M. Hassanshahian, Z. Bayat, S. Cappello, F. Smedile, M.M. Yakimov, Comparison the effects of bioaugmentation versus biostimulation on marine microbial community by PCR–DGGE: A mesocosm scale, J. Environ. Sci., 43 (2016) 136–146.
[23] D. Nicomrat, W.A. Dick, M. Dopsonc, O.H. Tuovinen, Bacterial phylogenetic diversity in a constructed wetland system treatingacid coal mine drainage, Soil Biol. Biochem., 40 (2008) 312–321.
[24] N.N. Perreault, C.W. Greer, D.T. Andersen, S. Tille, G. Lacrampe-Couloume, B.S. Lollar, L.G. Whyte, Heterotrophic and autotrophic microbial populations in cold perennial springs of the high Arctic, Appl. Environ. Microbiol., 74 (2008) 6898–6907.
[25] V. Ulmann, A. Kracalikova, R. Dziedznsika, Mycobacteria in water used for personal hygiene in heavy industry and collieries: a potential risk for employees, Int. J. Environ. Res., 12 (2015) 2870–2877.
[26] J.B. Baker, F.J. Banfield, Microbial communities in acid mine drainage, FEMS Microbiol. Ecol., 44 (2003) 139–152.