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A Proposed Biochemical Protocol to Isolate and Characterize Acidophilic Bacteria from Tailings Soil

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Abstract

Indigenous acidophilic bacteria separated from mine-waste can be used in return for the addition of the reagents like sulfuric acid. Among the tailings bacteria, Acidithiobacillus ferrooxidans and Acidithiobacillus thiooxidans are of the most-studied ones for the bioleaching and bioremediation of elements. In this work, the isolation and characterization of the mentioned bacteria are studied by a proposed biochemical protocol. The sequential cultivation of the soil bacteria in a series of liquid media and solid culture medium cause the separation of bacteria. A biochemical method is used to characterize the isolated strains of the native bacteria. The changes in the Eh, pH, and culture medium color are checked in order to control the bacterial growth in a 9K liquid medium. At the first step of the sequential cultivation, the amount of nutrient broth is the main factor that affects the complete isolation of Acidithiobacillus bacteria. The trivial dosage of nutrient broth does not lead to the desired proliferation of the bacteria in the tailings soil. 8 g L-1 of the nutrient broth is suitable to increase the initial number of bacteria in the soil significantly. In the next steps, the bacteria are separated from the soil, and pure strains of A. ferrooxidans and A. thiooxidans are isolated using a 9K medium. Final pure strains are achieved during the two steps of streak cultivation of bacteria in the soil medium of nutrient agar.

1. Introduction

The soil of mine-wastes commonly consists of indigenous microorganisms [1]. A fundamental understanding of the mine tailings environments is not possible until the lack of comprehensive knowledge about microbial processes is included in the results interpretation, models, theories, etc. since microorganisms (i.e. bacteria, fungi, some algae, and archaeal organisms) can affect many reactions involved in the leaching of elements from mine-wastes [2]. Piervandi *et al*. have studied the effect of native bacteria on the bioleaching of toxic elements from mine tailings soil [3]. Usui *et al*. suspended the soil samples in sterilized water, and then the supernatants were spread onto brain–heart infusion agar plates to isolate the soil bacteria [4]. Okoye *et al*. have separated the bacteria from oil-polluted soil by culturing in Bushnell-Haas agar and sub-culturing Luria Bertani agar plates [5]. Callewaert *et al*. have purified and isolated the strongly hydrophobic bacteriocin amylovorin L471 from *Lactobacillus amylovorus* DCE 471 from a complex culture broth by a three-step protocol involving 1) ammonium sulfate precipitation, 2) extraction/precipitation using chloroform/methanol, 3) reversed-phase HPLC, in which only the chromatographic stage is performed [6].

Acidophilic bacteria are of the most used bacteria for the bio-dissolution of sulfide minerals [7]. *A. thiooxidans* is a sulfur-oxidizing bacteria that uses different sulfur compounds. *A. ferrooxidans* utilizes both sulfur compounds and Fe^{2+} ions as

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energy sources [8]. In the bioleaching of metal sulfides by *Acidithiobacillus* bacteria, a reduction in pH occurred due to the oxidization of sulfur to sulfuric acid [9]. The Eh increases during the sulfide bioleaching using *Acidithiobacillus* bacteria due to the production of ferric ions and SO_4^{2-} [3]. The indigenous acidophilic bacteria can leach valuable elements. Using inorganic materials or organic (yeast extract) nutrients, pure cultures of the bacteria separated from the tailings soil were far less efficient for the bioleaching of the tailings samples in comparison to the mixed culture [10].

Joerger and Klaenhammer have examined *L. helveticus* 481 for bacteriocin generation by the direct [11] and deferred [12] techniques [13]. Roberto *et al*. have used the methods of bacterial strains and plasmids and fatty acid analysis for the biochemical characterization of acidophilic bacteria [14]. Estepar *et al*. have determined the different biochemical parameters by standard methods for the identification of *'Peñamellera'* cheese: total solids, fat, NaCl, pH, titratable acidity, protein, total nitrogen, non-casein nitrogen, and non-protein nitrogen [15]. Lavermicocca *et al*. have characterized the fungicidal activity of *Lactobacillus plantarum* bacteria using eight fungal species or strains as indicators [16]. Sánchez *et al*. have used the API 50 CH system (API System, bioMe´rieux, Montalieu Vercie, France) in order to characterize the isolates of lactic acid bacteria. They determined the fermentation of carbohydrates using microtitre plates (Sterilin, Staffordshire, UK) [17]. Raval *et al*. have employed the circular dichroism spectroscopy for the Ve2-20-91 protease to characterize the seawater haloalkaliphilic bacteria [18]. In the most recent papers, Yu *et al*. and Sahoo *et al*. have used the analysis of polymerase chain reaction (PCR) to characterize the bacteria [19-20]. Tan *et al*. have cultivated acidophilic sulfur- and iron-oxidizing strains, moderately acidophilic iron-oxidizing bacteria, and acidophilic heterotrophs by the overlay media (FeSo, Feo, FeTo, and YEo, respectively). They utilized PCR in order to identify the bacteria [21].

In this work, for the first time, a proposed biochemical protocol based on the sequential cultivation was studied to isolate and characterize the indigenous acidophilic bacteria from sulfide mine-waste. The cultivation at four steps resulted in the isolation of the *Acidithiobacillus* bacteria. The results showed that the sequential technique was an efficient method to separate the desired acidophilic bacteria from tailings soil. At the first step, the number of bacteria was multiplied in the soil sample. Then the pure strains of acidophilic bacteria were prepared during the studied method. A biochemistry technique during the sequential cultivation was used to characterize the acidophilic bacteria.

2. Materials and methods

2.1. Preparation of samples

The deposit of the Sarcheshmeh copper complex is of the largest Oligo-Miocene porphyry copper deposits in the world. It is on a continental arc with a well-developed supergene sulfide zone, covered mainly by a hematitic gossan [22]. The plant of Sarcheshmeh smelting is considered as the biggest Cu producer of Iran that produces around 2 tons of slag per ton Cu [23]. The mining activity and mineral processing plant have generated massive amounts of low-grade wastes, which can result in many environmental issues [24]. The average precipitation in the tailings site changes annually from 300 mm to 550 mm [25]. The tailings of the Sarcheshmeh mine include sulfide minerals, commonly pyrite. The exposure of pyrite and other sulfides to water and oxygen causes the rapid oxidation of minerals, the production of acid mine drainage (AMD) [24].

In this work, the soil samples were collected from the tailings dam of the Sarcheshmeh copper complex, Iran. Sampling was performed from a depth of 20 cm. The soil of Sarcheshmeh contained several strains of native acidophilic bacteria, capable of generating AMD. The samples were dried in a suitable condition to preserve the native mesophilic bacteria and also inhibit the oxidation of sulfide minerals. According to the literature review, the optimum temperature for the growth of *Acidithiobacillus* bacteria is in the ranges of 20-40 $^{\circ}$C. Therefore, the tailings samples were dried at room temperature (≈25 $^{\circ}$C) during one week. Then the dried soil was sieved using vibrator shifter in order to obtain a homogeneous mixture and the separation of the fraction size of 75 μm [3].

2.2. Separation of native bacteria from tailings soil

The indigenous bacteria were isolated from the soil sample of the Sarcheshmeh copper tailings dam. The process of bacteria separation from soil included an initial enrichment step and then three sequential purification steps. These steps were performed as what follows.

2.2.1. Enrichment of bacteria

This step is aimed to enrich and initially increase the number of soil bacteria. Due to the high dosage of nutrients in the nutrient broth [26], this culture medium was used to increase the number of soil bacteria rapidly during 48 h. Bacterial enrichment was performed using the nutrient broth in two 250-mL Erlenmeyer flasks (Figure1). First, 0.8 g of the nutrient broth was added to 100 mL of deionized water in each flask. The dissolved nutrient broth was sterilized by autoclave in order to prevent the growth of minor bacteria of the laboratory environment. Then 2 g of tailings sample was added to each Erlenmeyer flask. Finally, the flasks were placed in an incubator shaker. The operating conditions of the tests inside the incubator included 32 °C and 150 rpm. After 48 h from the start of the experiment, the population of the soil bacteria reached several times its initial amount in the soil.

Figure1. Enrichment step to multiply the initial number of soil bacteria.

2.2.2. Purification in liquid medium

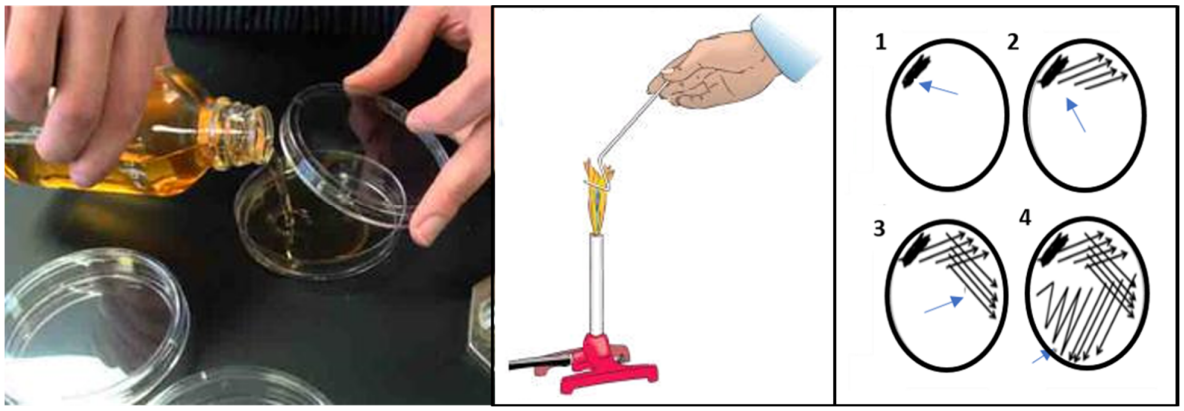
After increasing the number of soil bacteria at a primary step, it was necessary to use a specific culture medium to separate the pure acidophilic bacteria from the soil. Therefore, a 9K medium was used to isolate the iron-oxidizing bacteria and sulfur-oxidizing bacteria. The bacterial cultivation was performed in two different liquid media: 9KFe (for iron-oxidizing bacteria) and 9KS (for sulfur-oxidizing bacteria). The 9K medium contains 3 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g KCl, 0.01 g $Ca(NO_3)_2$, and 10 mL sulfuric acid (one normal) in 1 L of deionized water [8]. In order to prepare the 9K culture medium for the iron-oxidizing bacteria, 44.22 g L^{-1} of $FeSO_4.7H_2O$ was added to the 9K culture medium [27]. The culture medium for the sulfur-oxidizing bacteria was provided by the addition of 1.5% (w/w) sulfur to the 9K culture medium [28]. Finally, 2 mL of the solution containing enriched bacteria was added to 100 mL of the 9K medium.

Also two samples were prepared from the waste-water of the Sarcheshmeh tailings dam to study the pure strains of bacteria in the aquatic environment surrounding the mine tailings. The waste-water samples were cultivated in the culture media in similar cultivation conditions in comparison to the tailings soil for isolation of the iron-oxidizing bacteria and sulfur-oxidizing bacteria. Experiments were performed to regenerate pure bacteria from the nutrient broth medium and inoculate into the 9K medium.

The process of bacterial growth and proliferation in the 9K media was controlled by checking the Eh, pH, and change of medium color.

2.2.3. Purification of bacteria by solid medium

In this step, in order to achieve the pure strains of bacteria, the purified bacteria from the 9K media were sub-cultivated in a solid culture medium. Due to the high percentage of nutrients in the nutrient agar [29], it was selected as a solid culture medium. 2.8 g of the nutrient agar was dissolved in 100 mL of deionized water to prepare the solid medium. The resulting solution was sterilized using an autoclave at 121 °C for 15 min. The nutrient agar medium in a hot temperature (about more than 80 °C) was liquid. It became solid and jelly-like when the nutrient agar cooled. Cultivation was performed in a solid medium inside a biological hood. The biological hood was sterilized by turning on a UV lamp inside the hood for 20 min. It should be noted that when turning on the UV lamp, a special coating should be used on the device, and no one should be inside the laboratory. The steps were such that the hot nutrient agar would be solidified gradually by pouring the liquid nutrient agar into the sterile plates (Figure2a). Finally, the bacteria grown from the liquid culture medium were sub-cultivated in the solid medium using a flame-sterilized loop (Figs. 2b-2c). The goal of this step was to achieve a higher degree of purity for the acidophilic bacteria. The details of the streak cultivation method in a solid medium are shown in Figure2c. The streak method is done in four steps. The loop must be sterilized before each step by flame, and then cooled with the help of a corner of the sterilized solid medium.



(a) (b) (c)

Figure2. Streak method for bacterial cultivation in the solid medium using sterile loop a) pouring liquid nutrient agar into sterile plates; b) sterilizing the cultivation lop by the flame; c) streak cultivation of the bacteria from the 9K medium into the nutrient agar.

2.2.4. Production of pure strains by streak method

Due to the cultivation of bacteria from the liquid medium in the solid medium, the colorful colonies grow in the nutrient agar. Each colony belongs to a specific strain of bacteria. In this work, the colonies were re-cultivated by the streak method in the new solid medium to produce pure bacteria. The average growth time of colonies in the new medium of nutrient agar at 32 °C was 48 h. The different steps of the streak method for the cultivation of colonies in the solid medium are as follow (Figure3):

1. Preparation of sterile nutrient agar medium;
2. Sterilizing a special loop for bacterial cultivation with the flame (Figure3a);
3. Cooling the sterile loop with a corner of sterilized culture medium (Figure3b);
4. Removing the pure colony from the previous solid medium by the loop (Figure3c);
5. Four-steps streak cultivation (Figure3d).

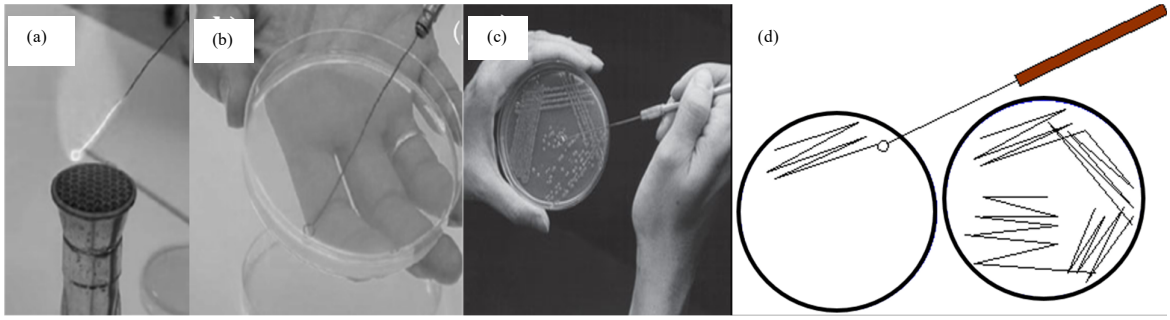


Figure3. Details of the streak technique for the colony cultivation in a solid medium: a) Sterilization of loop for bacteria cultivation; b) Cooling the sterile loop by a corner of the sterile solid medium; c) Taking colonies by loop from the previous solid medium; d) Streak cultivation in four stages.

2.3. Bacteria counting

Bacterial counting is one of the methods used to control the growth of bacteria during their cultivation. It is also a method to study the growth and activity of bacteria during the microbial experiments. Bacterial counting requires the use of special equipment as follows:

1) Thoma counting chamber (Figure4a-1);

2) Glass slide (Figure4a-2);

3) Sampler (Figure4b);

4) Biological microscope (Figure4c); OPTIKA microscope used to count the bacteria. Magnification of 40× was used to count the bacteria.

The Thoma counting chamber includes networking of small, medium, and large squares (Figure5a). There are 256 small squares inside the networking of the counting chamber. Every 16 small squares make a medium square. The length of each small square is 0.05 mm. Therefore, the

area of each small square is 25 × 10^{-6} cm^2. The height of the liquid between the counting chamber networking and the glass slide is 0.1 mm. Therefore, the liquid volume on the surface of each square is 25 × 10^{-8} cm^3. According to these calculations, when the cells are counted in 16 small squares, the number of cells per mL^2 is calculated as follows:

$$Bacteria\ number\ in\ 1\ mL = counted\ bacteria \times 2.5 \times 10^5 \times dilution\ coefficient \quad (1)$$

A medium square of the Thoma counting chamber under the microscope is shown in Figure5b. Furthermore, Figure5c illustrates the path of bacteria counting on the surface of the middle square. It should be noted that only the motile bacteria must be counted.

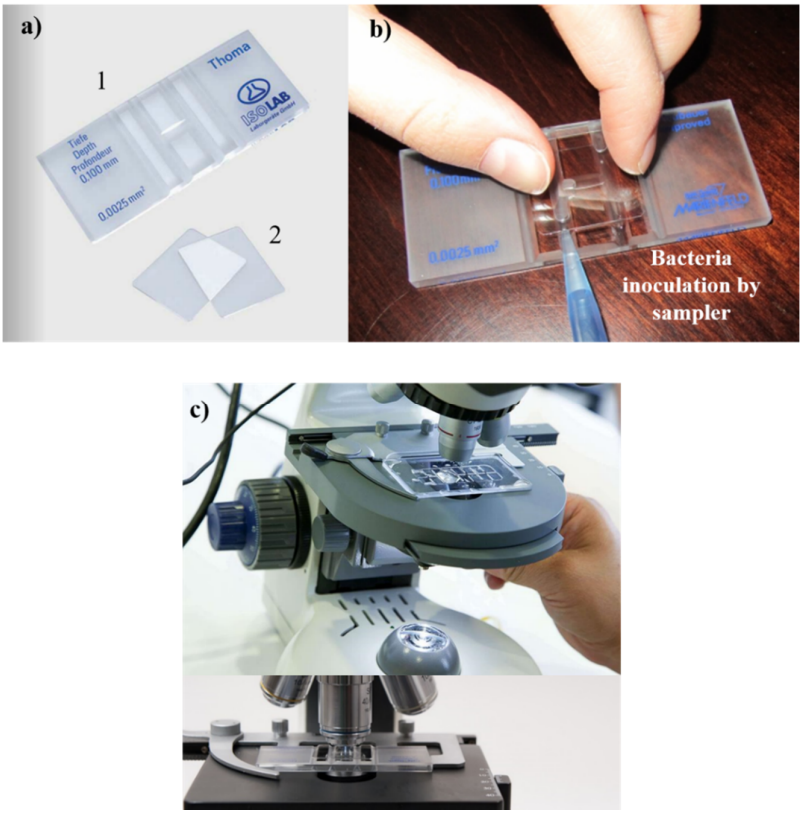


Figure4. a) Thoma counting chamber (1) and glass slide (2); b) Bacterial inoculation between the counting chamber and the glass slide for bacteria counting; c) Biological microscope (magnification of 40x is required for cell counting and 100x is useful to look at a larger grid in the hemocytometer).

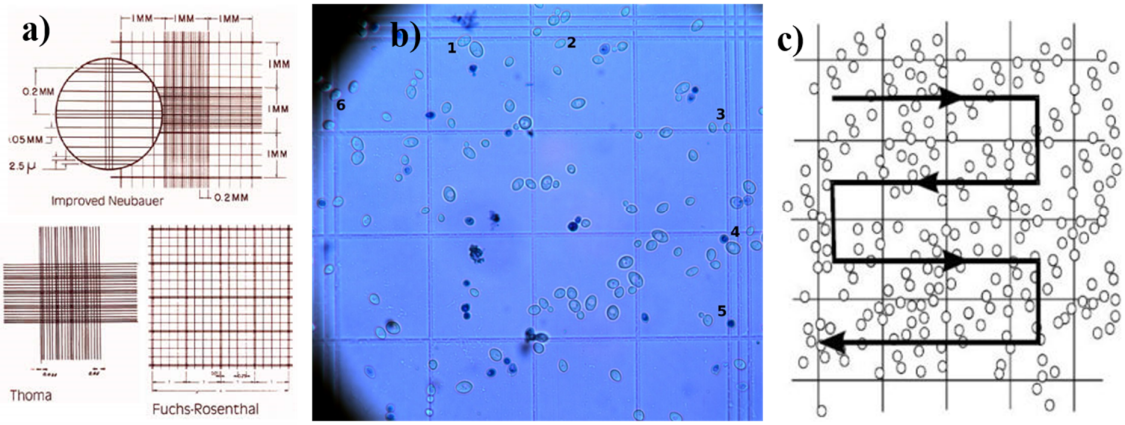


Figure5. a) Networking or hemocytometer of the Thoma counting chamber; b) The hemocytometer under the lens of the microscope; c) Route of the counting bacteria.

2.4. Preparation of a mixed culture of acidophilic bacteria

In order to prepare a mixed culture from pure strains, the fresh colonies of each strain were cultivated from the nutrient agar in 100 mL of the 9K medium. The cultivation was performed in sterile conditions near the flame and under the hood. The growth of fresh bacteria in the liquid medium lasted six days, after which, two bacteria grown in the liquid medium were added to a 500-ml Erlenmeyer flask. The flask was placed inside an incubator shaker at 32 °C for 24 h to prepare a mixed culture of thetwo bacteria namely *Acidothiobacillus ferrooxidance* and *Acidothiobacillus thiooxidation*.

2.5. Gram staining

Gram staining for the colonies of bacteria included four steps:

1. Heat-fixed cells were stained at the first step using a crystal violet stain. It colored the colonies purple.
2. Iodine solution was added. It generated a complex with the crystal violet stain.
3. Colonies were washed by ethanol solution. This step decolorized the Gram-negative cells (but not the Gram-positive bacteria).
4. Gram-negative bacteria were visualized using safranin. It was a pink-colored counterstain.

2.6. Proposed biochemical method for characterization

Our proposed protocol for characterization of the acidophilic bacteria included the study of the biochemical parameters such as pH, Eh, bacteria number, color of the culture medium, ferrous ion consumption, elemental sulfur consumption, gram staining, shape of bacterial colonies, specific culture medium for the iron-oxidizing and sulfur-oxidizing bacteria, and color of colonies in the nutrient agar. The parameters were analyzed in order to characterize the native bacteria isolated from the Sarcheshmeh mine tailings.

3. Results and Discussion

3.1. Separation of native bacteria

3.1.1. Enrichment of bacteria in liquid nutrient broth

In this step, the number of soil bacteria multiplied during the cultivation in the nutrient broth.

3.1.2. Purification in liquid medium

The bacteria grew and proliferated during the cultivation. The growth of bacteria was checked by controlling the change in Eh, pH, and the culture medium color and the bacteria counting. The 9KFe medium was initially light green due to the presence of ferrous ions. It turned red-brown through the Fe^{2+} oxidization to Fe^{3+} as the bacteria grew (Figs. 6a-6d). As the iron-oxidizing bacteria grew in this environment, the color of the medium darkened. Comparing the color of the primary 9K medium and the culture medium containing proliferating the bacteria could be a method to investigate the process of bacterial activity and growth. The 9KS culture medium was initially colorless including the elemental sulfur dispersed on the surface of the medium. When the sulfur-oxidizing bacteria grew, the solution of the 9KS culture medium became opaque (Figs. 6e-6g).

A comparison between the bacterial full growth and the bacterial incomplete proliferation was done for both the 9KFe medium and the 9KS medium. The color changes of the primary culture media in the mentioned conditions are shown in Figs. 7a-b and 7c-d, respectively. The main difference between the colors of the two culture media at the end of experiments was due to the different growth rates of pure bacteria, which was according to the amount of the nutrient broth. For full growth and incomplete growth, respectively, 8 g/L and 0.8 g/L of nutritious broth were added to the medium. Therefore, the insufficient nutrition or enrichment of bacteria occurred in the presence of small amounts of the nutrient broth.

Figure 8a shows that pH changed for the growth of the iron-oxidizing bacteria in two identical 9KFe media and sulfur-oxidizing bacteria in two identical 9KS media. Figure8b represents the variations of Eh in the experiments. The activity of the acidophilic bacteria due to the production of H^+ reduced the pH of the reaction solution. The activity of these bacteria increased the amount of Eh. The enhacenment in potential could be attributed to the production of remarkable concentrations of Fe^{3+} and SO_4^{2-} in the culture medium of the iron-oxidizing bacteria and sulfur-oxidizing bacteria, respectively [3]. According to the previous studies [30], the increase in these ion concentrations enhanced the potential amount.

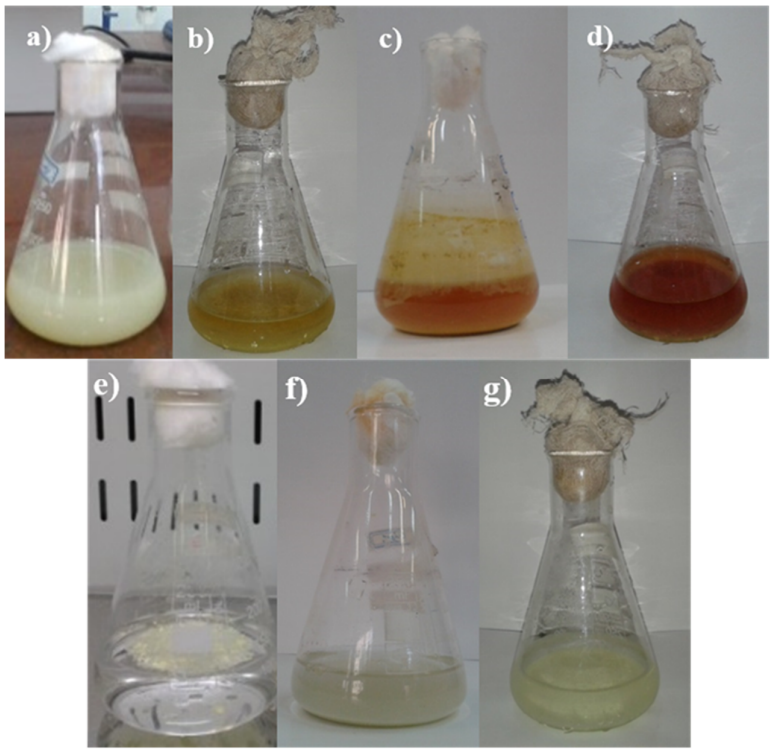


Figure6. Steps of color changes of 9K media during the growth of pure bacteria: a) Primary culture medium with a light color (green due to the presence of Fe^{2+} ions); b) Turbid solution compared to the primary culture medium (cream-brown color); c) Dark medium (reddish-brown); d) Final dark medium (dark red; rich in Fe^{3+} ions due to the bacterial activity); e) Colorless solution; f) Milky color; g) Clear cream-colored medium.

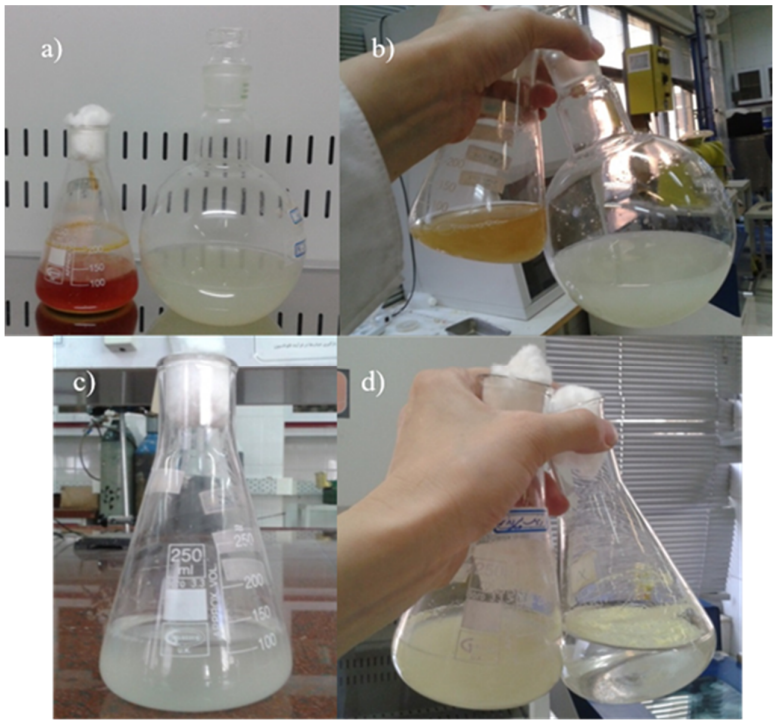


Figure7. a) Comparison between primary 9KFe medium and culture medium by incomplete growth after 30 days; b) Comparison between primary 9KFe medium and culture medium by complete proliferation after 30 days; c) Comparison between primary 9KS medium and culture medium by incomplete growth after 30 days; d) Comparison between primary 9KS medium and culture medium by complete proliferation after 30 days.

The reason for the pH increase in the 9K medium during the first days of the experiments (Figure8a) was the dissolution of the acid-consuming minerals of the soil. The amount of potential in the 9KS culture medium was associated with a continuous oscillation during the experiment. It could be attributed to the sedimentation and consumption of the elemental sulfur during the experiment.

Figure9 shows the microscope image of the bacterial grown in the 9KFe medium. The shape of these bacteria was bacilli or cocco-bacilli.

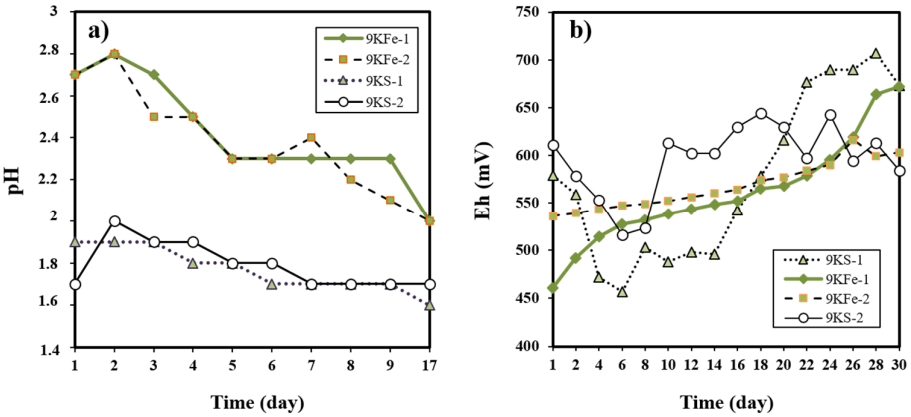


Figure8. a) Changes in pH over the time (days) for two identical 9KFe media and two identical 9KS media; b) Eh variations for bacteria growth in each 9KFe and 9KS medium.

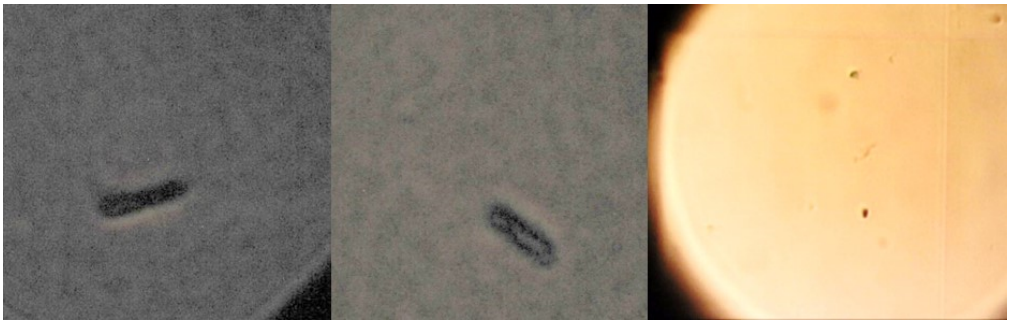


Figure9. Images of bacterial grown in the 9KFe medium under the lens of biological microscope.

3.1.3. Purification in solid medium

During the purification step in the solid medium, colonies with different colors appeared. The colonies often consisted of white ones (Figure10) but other colonies were generated with different colors as well (Figure11).

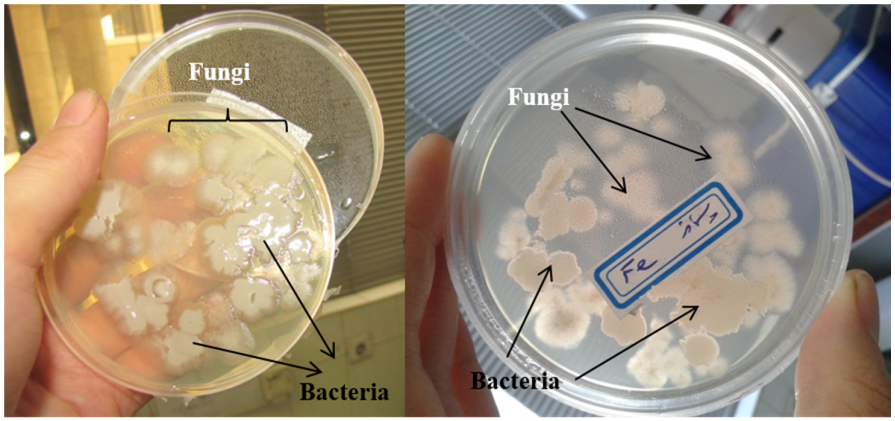


Figure10. Cultivation of bacteria from the 9KFe medium in the solid nutrient agar.

In the liquid culture medium, the conditions were desirable for the growth of biomass along with bacteria. Therefore, the growth of fungi also occurred in the solid culture medium (Figure12). Under the conditions for incomplete growth of bacteria in the liquid culture medium, the growth of a large amount of fungi was also observed (Figure12); these fungi were *Penicillium* and *Aspergillus*. Generally, the *Penicillium* and *Aspergillus* fungi were mainly able to grow in the nutrient agar medium [31].

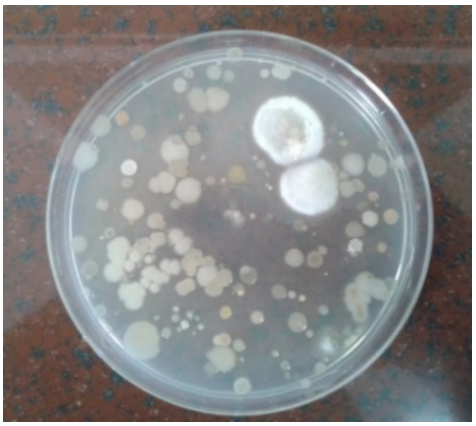


Figure11. Growth of bacteria in the nutrient agar after cultivation from the 9K medium.

3.1.4. Bacteria streak cultivation

As a result of the initial bacteria cultivation from the liquid medium to the solid medium, the colonies of different colors grew (Figure11). For the final purification and isolation of the colonies, the iron-oxidizing bacteria (*A.f*) and the sulfur-oxidizing bacteria (*A.t*) were cultivated by the streak method in the solid medium (Figure13). The time required for the growth of colonies in the new medium of nutrient agar at 32 ℃ was 48 h.

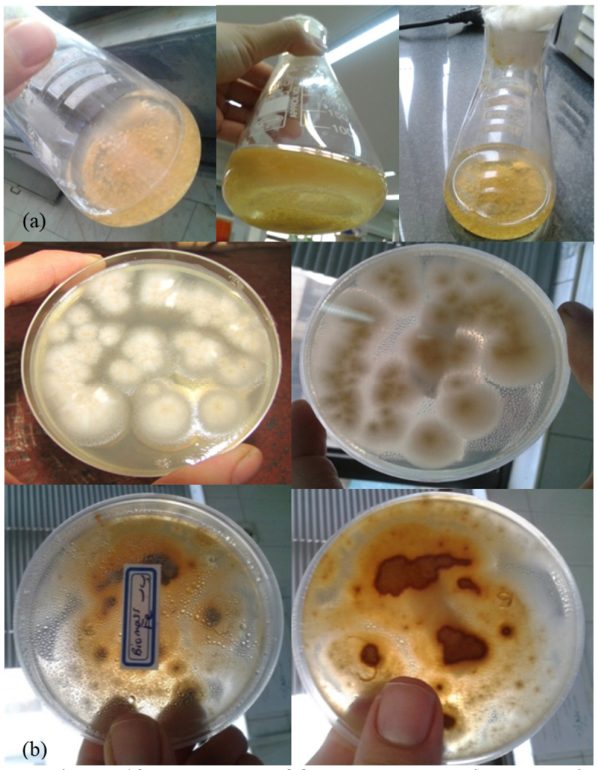


Figure 12. Examples of fungal growth with bacteria in the solid medium; *Penicillium* (white fungi) and *Aspergillus* (red fungi).

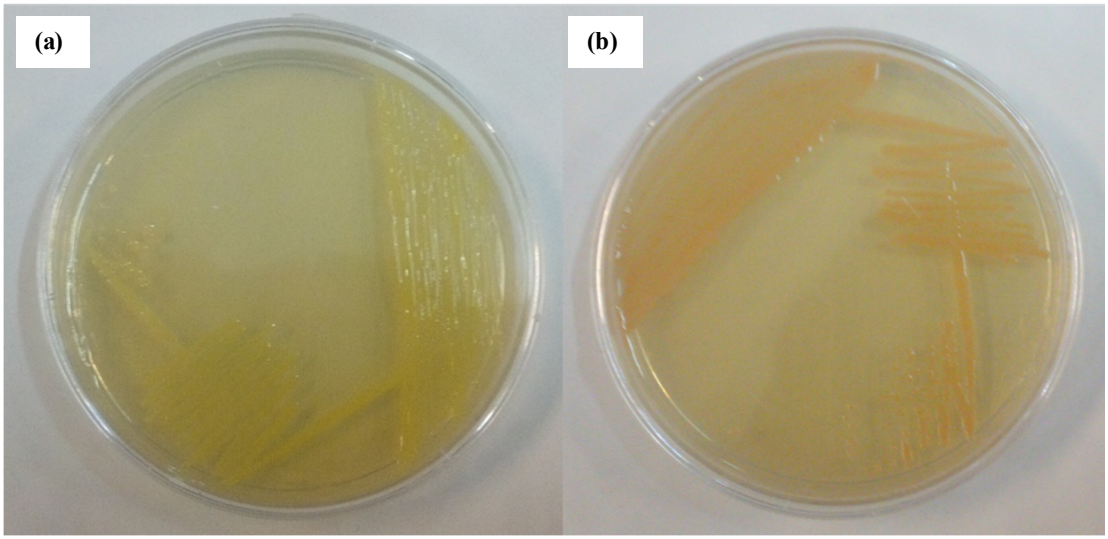


Figure 13. Streak sub-cultivation for the pure colonies of the *A.f* bacteria and *A.t* bacteria from the primary solid medium in a new solid medium after 24 h.

3.2. Growth of pure strains in liquid culture medium

After separation of *A.f* and *A.t* from the soil, the colonies of these bacteria were cultivated in a 9K medium. The diagrams of pH and Eh during the growth and proliferation of pure bacteria in the 9K media are shown in Figs. 14a-b. The changes in the bacterial population during the time are shown in Figs. 14c-d. The pH diagram showed a decrease in the pH value due to the production of acid by the two acidophilic bacteria (Figure14a). The activity of these bacteria caused an increase in Eh during the first six days of bacterial cultivation. The increase in potential was due to the oxidation of ferrous ions and sulfur and the production of the Fe^{3+} and SO_4^{2-} ions. According to Figs. 14c-d, the increase in the bacteria number during the time occurred with an oscillation. It was due to the proliferation of some bacteria and the death of the other ones.

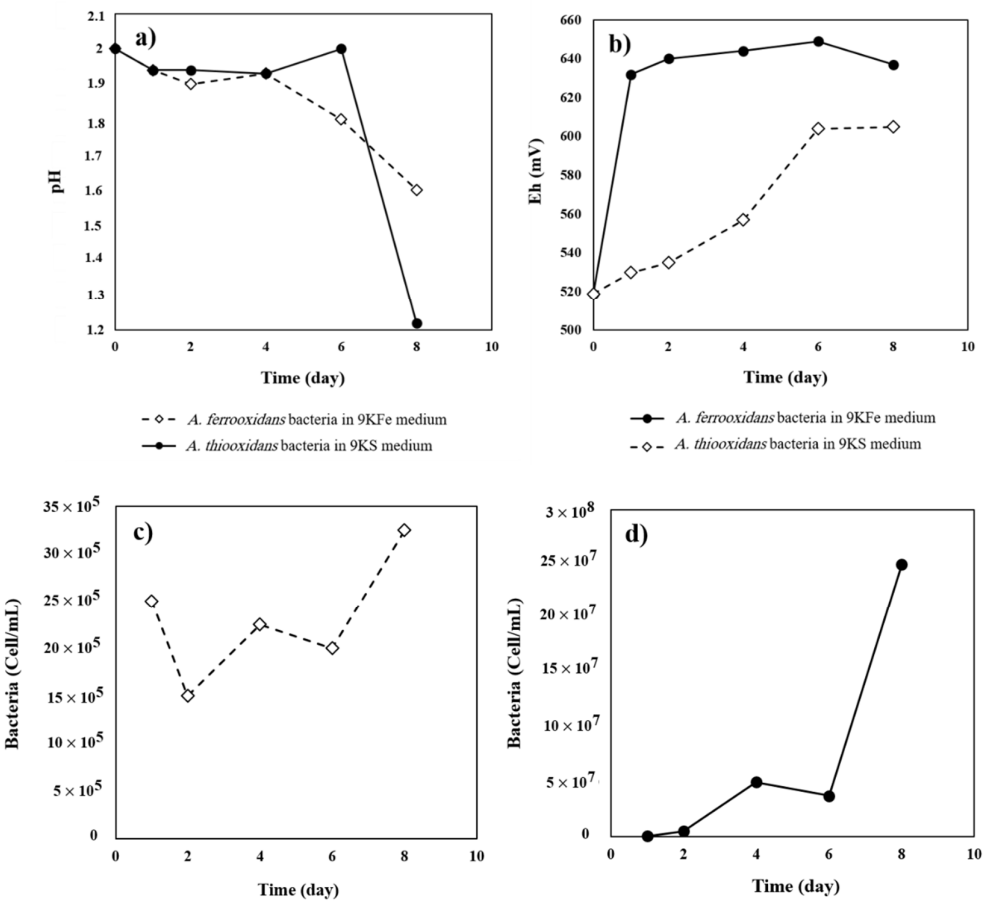


Figure 14. a) pH changes during the time; b) Eh changes over the time during the growth and proliferation of pure bacteria in the 9K medium; c) Changes in the number of *A.f* in the 9KFe medium during the time; d) variations in the *A.t* population over the time in the 9KS medium.

The results of this work showed that *A.f* and *A.t* could consume both ferrous ions and elemental sulfur during the experiments in the 9K media. The consumption rate of elemental sulfur and ferrous ions is higher for the iron-oxidizing bacteria and sulfur-oxidizing bacteria, respectively.

3.3. Bacterial process for acid production

A schematic diagram for the process of acid generation by the acidophilic bacteria is shown in Figure15. The production of sulfuric acid by the *Acidithiobacillus* bacteria can take place through two different mechanisms, contact process or indirect procedure. In the contact mechanism, the bacteria attach directly to the surface of sulfide mineral (Eq. 2) [3] or elemental sulfur (Eq. 3) and generate sulfuric acid. However, during the indirect mechanism, the iron-oxidizing bacteria oxidize Fe^{2+} to Fe^{3+} (Eq. 4) [32]. Then ferric ions attack the surface of metal sulfides and produce the mentioned acid via the sequential reactions (Eqs. 5-7) [33].

$$MS + H_2O + 1.5O_2 \xrightarrow{acidophilic\ bacteria} M^{2+} + SO_4^{2-} + 2H^+ \quad (2)$$

$$2S + 3O_2 + 2H_2O \xrightarrow{acidophilic\ bacteria} 2SO_4^{2-} + 4H^+ \quad (3)$$

$$4Fe^{2+} + O_2 + 4H^+ \xrightarrow{A.\ ferrooxidans} 4Fe^{3+} + 2H_2O \quad (4)$$

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5H_2S_n + Fe^{2+}\ (n \geq 2) \quad (5)$$

$$0.5H_2S_n + Fe^{3+} \rightarrow 0.125S_8 + Fe^{2+} + H^+ \quad (6)$$

$$0.125S_8 + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+ \quad (7)$$

Finally, the generation of sulfuric acid caused a significant reduction in the culture media (Figure14a). On the other hand, the production of the Fe^{3+} and SO_4^{2-} ions caused the enhancement of Eh (Figure14b). The lower pH and medium to high Eh provide the oxidizing condition for the leaching of sulfide minerals in the presence of the acidophilic bacteria [3].

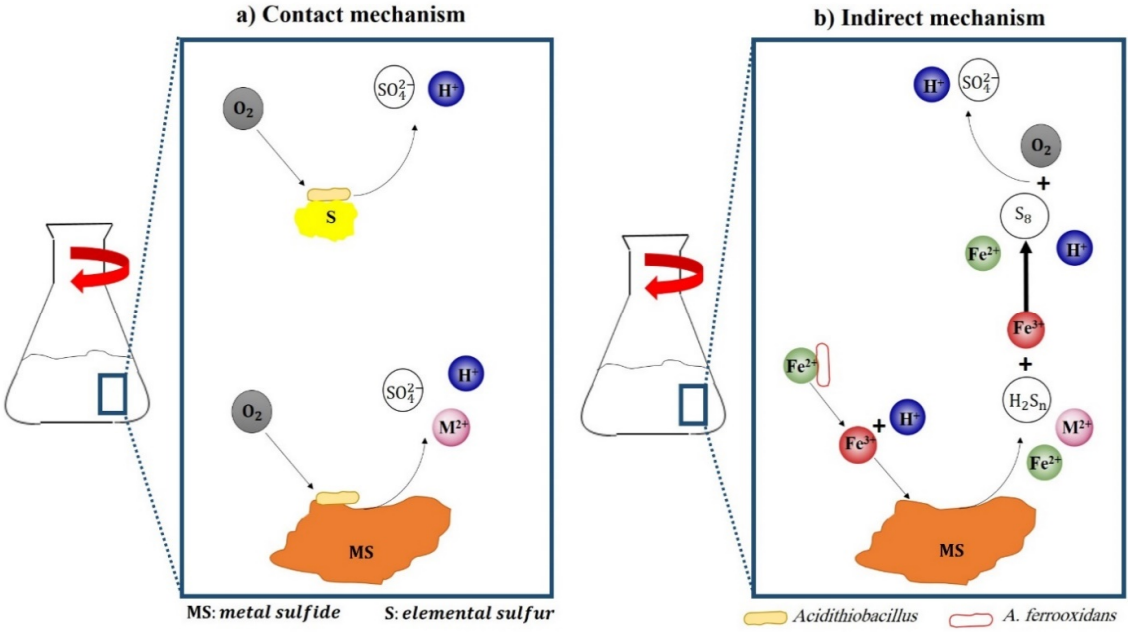


Figure 15. A schematic diagram for the mechanisms involved in acid production by the acidophilic bacteria a) contact mechanism; b) indirect mechanism.

3.4. Gram staining

Gram staining acts due to the different effects of ethanol on the wall of the bacterial cell:

- Gram-positive cells have a thick peptidoglycan layer. It is dehydrated by ethanol, *catching* the crystal violet stain.
- Gram-negative bacteria have an outer lipid membrane. It is dissolved by ethanol, releasing the crystal violet stain.

Therefore, gram staining of isolated bacteria from mine-waste soil showed that these bacteria were gram-negative (Figure16).

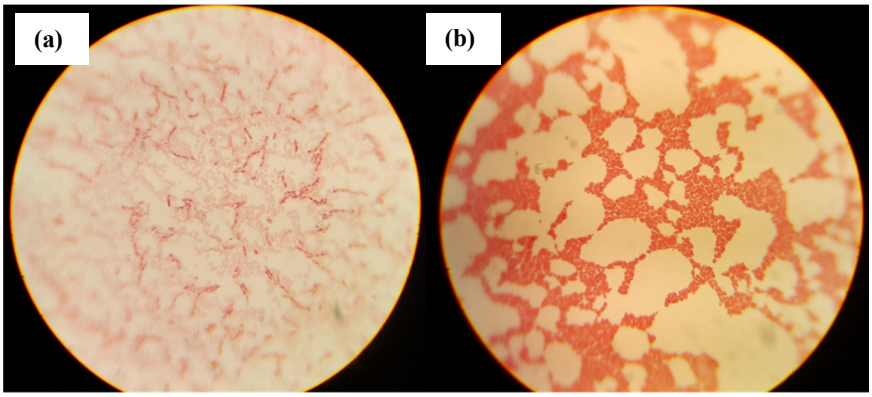


Figure 16. Gram straining of native bacteria a) iron-oxidizing bacteria; b) sulfur-oxidizing bacteria.

3.5. Characterization of indigenous bacteria

The following properties are the particular characteristics of the acidophilic bacteria; these properties were also observed for the native bacteria isolated in this work.

1. 9K medium is a specific culture medium for the cultivation and growth of *A. ferrooxidans* and *A. thiooxidans* [3].
2. These bacteria color in the nutrient agar medium, and are red and yellow due to the adsorption of Fe^{3+} and elemental sulfur, respectively.
3. The colonies of the mentioned bacteria are cocco-bacilli shaped.
4. *A. ferrooxidans* [34] and *A. thiooxidans* [35] are gram-negative.
5. The proliferation of *A. ferrooxidans* and *A. thiooxidans* in the 9K media changes the color of the culture medium from green to red and from colorless to opaque, respectively.
6. The pH reduces and the Eh increases due to the growth of the *Acidithiobacillus* bacteria.
7. *A. ferrooxidans* and *A. thiooxidans* in the 9K media can consume both ferrous ions and elemental sulfur during the experiments. The consumption rate of ferrous ions and sulfur is faster for *A. ferrooxidans* and *A. thiooxidans*, respectively.

According to the results obtained from this work, it could be concluded that the isolated bacteria were *A. ferrooxidans* and *A. thiooxidans*.

4. Conclusions

The soil of mine tailings includes a variety of indigenous bacteria. In this work, the separation of the *Acidithiobacillus* bacteria generating sulfuric acid was studied. The acid-producing bacteria can be applied to increase or inhibit the extraction of elements according to the economic or environmental target, respectively. Our proposed method to isolate pure strains of the bacteria was the sequential cultivation technique using liquid media and solid media. At the first step, the number of soil bacteria increased. In the next steps, the purification of fresh strains took place by the culture of bacteria in a 9K medium and then in a solid nutrient agar. The isolated iron-oxidizing and sulfur-oxidizing bacteria were characterized, respectively, as *A. ferrooxidans* and *A. thiooxidans* due to: 1) the growth of bacteria in a 9K specific culture medium for the *Acidithiobacillus* bacteria; 2) the change of the 9K medium from green to red for *A. ferrooxidans* and from colorless to opaque for *A. thiooxidans* as a result of the proliferation of iron-oxidizing bacteria and sulfur-oxidizing bacteria, respectively; 3) the cocco-bacilli shape of colonies of the isolated bacteria; 4) being gram-negative bacteria; 5) the pH reduction and Eh enhancement during the growth of bacteria; 6) the consumption of Fe^{2+} ions and elemental sulfur, respectively, during cultivation in the 9K media; these bacteria will be used in the future bioleaching studies.

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Submission declaration and verification

The authors confirm that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint), it is not under consideration for publication elsewhere, its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form in English or in any other language including electronically without the written consent of the copyright holder.

References

[1]. Piervandi, Z., Khodadadi Darban, A., Mousavi, S.M., Abdollahy, M., Asadollahfardi, G., Funari, V., Dinelli, E., 2019. Minimization of metal sulfides bioleaching from mine wastes into the aquatic environment. Ecotox. Environ. Safe. 182, 109443.

[2]. Ledin, M., Pedersen, K., 1996. The environmental impact of mine wastes-Roles of microorganisms and their significance in treatment of mine wastes. Earth-Sci. Rev. 41, 67-108.

[3]. Piervandi, Z., Khodadadi Darban, A., Mousavi, S.M., Abdollahy, M., Asadollahfardi, G., Funari, V., Dinelli, E., Webster, R.D., Sillanpää, M. 2020. Effect of biogenic jarosite on the bio-immobilization of toxic elements from sulfide tailings. Chemosphere 258, 127288.

[4]. Usui, K., Miyazaki, S., Kaito, C., Sekimizu, K. 2009. Purification of a soil bacteria exotoxin using silkworm toxicity to measure specific activity. Microb. Pathoge. 46, 59–62.

[5]. Okoye, A.U., Chikere, C.B., Okpokwasili, G.C., 2020., Isolation and Characterization of Hexadecane Degrading Bacteria from Oil- polluted soil in Gio Community, Niger Delta, Nigeria. Scientific African 8, e00340.

[6]. Callewaert, R., Holo, H., Devreese, B., van Beeumen, J., Nes, I.F., de Vuyst, L., 1999. Characterization and production of amylovorin L471, a bacteriocin purified from Lactobacillus amylovorus DCE 471 by a novel three-step method. Microbiology 145, 2559– 2568.

[7]. Falco, L., Pogliani, C., Curutchet, G., Donati, E., 2003. A comparison of bioleaching of covellite using pure cultures of *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* or a mixed culture of *Leptospirillum ferrooxidans* and *Acidithiobacillus thiooxidans*. Hydrometallurgy 71, 31–36.

[8]. Diao, M., Nguyen, T.A.H., Taran, E., Mahler, S., Nguyen, A.V., 2014. Differences in adhesion of A. thiooxidans and A. ferrooxidans on chalcopyrite as revealed by atomic force microscopy with bacterial probes. Miner. Eng. 61, 9–15.

[9]. Zhang, R., Hedrich, S., Römer, F., Goldmann, D., Schippers, A., 2020. Bioleaching of cobalt from Cu/Co-rich sulfidic mine tailings from the polymetallic Rammelsberg mine, Germany. Hydrometallurgy 197, 105443.

[10]. Bryan, C.G., Hallberg, K.B., Johnson, D.B., 2006. Mobilisation of metals in mineral tailings at the abandoned São Domingos copper mine (Portugal) by indigenous acidophilic bacteria. Hydrometallurgy 83, 184–194.

[11]. Tagg, J. R., Dajani, A. S., Wannamaker, L.W., 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40, 722-756.

[12]. Kekessy, D.A., Piguet, J.D., 1970. New method for detecting bacteriocin production. Appl. Microbiol. 20, 282-283.

[13]. Joerger, M.C., Klaenhammer, T.R., 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by Lactobacillus helveticus 481. J. Bacteriol. 167, 439–446.

[14]. Roberto, F.F., Bruhn, D.F., Wilhite, A.M., Ward, T.E., 1993. Phylogenetic and biochemical characterization of acidophilic bacteria. FEMS Microbiology Reviews 11, 31-36.

[15]. Estepar, J., Sánchez, M.D.M., Alonso, L., Mayo, B. 1999. Biochemical and microbiological characterization of artisanal '*Peñamellera*' cheese: analysis of its indigenous lactic acid bacteria. International Dairy Journal 9, 737-746.

[16]. Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., Gobbetti, M., 2000. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol. 66, 4084–4090.

[17]. Sánchez, I., Palop, L., Ballesteros, C., 2000. Biochemical characterization of lactic acid bacteria isolated from spontaneous fermentation of 'Almagro' eggplants. International Journal of Food Microbiology 59, 9–17.

[18]. Raval, V.H., Pillai, S., Rawal, C.M., Singh, S.P., 2014. Biochemical and structural characterization of a detergent-stableserine alkaline protease from seawater haloalkaliphilic bacteria. Process Biochemistry 49, 955–962.

[19]. Yu, P., Sun, Y., Huang, Z., Zhu, F., Sun, Y., Jiang, L., 2020. The effects of ectomycorrhizal fungi on heavy metals' transport in Pinus massoniana and bacteria community in rhizosphere soil in mine tailing area. Journal of Hazardous Materials 381, 121203.

[20]. Sahoo, H., Senapati, D., Thakur, I.S., Naik, U.C., 2020. Integrated bacteria-algal bioreactor for removal of toxic metals in acid mine drainage from iron ore mines. Bioresource Technology Reports 11, 100422.

[21]. Tan, G.L., Shu, W.S., Hallberg, K.B., Li, F., Lan, C.Y., Huang, L.N. 2007. Cultivation-dependent and cultivation-independent characterization of the microbial community in acid mine drainage associated with acidic Pb/Zn mine tailings at Lechang, Guangdong, China. FEMS Microbiol Ecol 59, 118–126.

[22]. Atapour, H., Aftabi, A., 2007. The geochemistry of gossans associated with Sarcheshmeh porphyry copper deposit, Rafsanjan, Kerman, Iran: Implications for exploration and the environment. J. Geochem. Explor. 93, 47–65.

[23]. Khorasanipour, M., Esmaeilzadeh, E. 2016. Environmental characterization of Sarcheshmeh Cu-smelting slag, Kerman, Iran: Application of geochemistry, mineralogy and single extraction methods. Journal of Geochemical Exploration 166, 1–17.

[24]. Jannesar Malakooti, S., Shafaei Tonkaboni, S.Z., Noaparast, M., Doulati Ardejani, F., Naseh. R., 2014. Characterisation of the Sarcheshmeh copper mine tailings, Kerman province, Southeast of Iran. Environ. Earth. Sci. 71, 2267–2291.

[25]. Khorasanipour, M., 2015. Environmental mineralogy of Cu-porphyry mine tailings, a case study of semi-arid climate conditions, Sarcheshmeh mine, SE Iran. Journal of Geochemical Exploration 153, 40–52.

[26]. Lusa, M., Knuutinen, J., Lindgren, M., Virkanen, J., Bomberg, M., 2019. Microbial communities in a former pilot-scale uranium mine in Eastern Finland – Association with radium immobilization. Sci. Total Environ. 686, 619-640.

[27]. Pourhossein, F., Mousavi, S.M., 2018. Enhancement of copper, nickel, and gallium recovery

from LED waste by adaptation of *Acidithiobacillus ferrooxidans*. Waste Manag. 79, 98–108.

[28]. Mafi Gholami, R., Borghei, S. M., Mousavi, S. M., 2011. Bacterial leaching of a spent Mo–Co–Ni refinery catalyst using *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. Hydrometallurgy 106, 26–31.

[29]. Obase, K., 2019. Extending the hyphal area of the ectomycorrhizal fungus *Laccaria parva* co-cultured with ectomycorrhizosphere bacteria on nutrient agar plate. Mycoscience 60, 95-101.

[30]. Burkin, A. R. 2001. Chemical Hydrometallurgy: Theory and Principles. ISBN: 978-1-86094-184-9.

[31]. Sheik, G.B., Abd Al Rheam, A.I.A., Al Shehri, Z.S., Al Otaibi, O.B.M., 2015. Assessment of Bacteria and Fungi in air from College of Applied Medical Sciences (Male) at AD-Dawadmi, Saudi Arabia. Int. Res. J. Biol. Sci. 4, 48-53.

[32]. Tao, H., Dongwei, L., 2014. Presentation on mechanisms and applications of chalcopyrite and pyrite bioleaching in biohydrometallurgy–a presentation. Biotechnology Reports 4, 107–119.

[33]. Pattanaik, A., Sukla, L.B., Pradhan, D., Samal, D.P.K., 2020. Microbial mechanism of metal sulfide dissolution. Materials Today: Proceedings 30, 326-331.

[34]. Quatrini, R., Johnson D.B., 2019. *Acidithiobacillus ferrooxidans*. Trends in Microbiology 27, 282-283.

[35]. Yang, L., Zhao, D., Yang, J., Wang, W., Chen, P., Zhang, S., Yan, L., 2019. Acidithiobacillus thiooxidans and its potential application. Applied Microbiology and Biotechnology 103 (3).

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چکیده

خاک سد باطله معادن سولفیدی عموما، حاوی باکتری‌های بومی و اسیددوست است. به جای اضافه کردن اسید سولفوریک در فرایند استحصال فلزات، می‌توان باکتری‌های اسیددوست را به کار برد. این باکتری‌ها، از طریق تولید اسید سولفوریک و کاهش مقدار pH، به انحلال عناصر کمک می‌کنند. از میان باکتری‌های مختلف، باکتری‌های *اسیدیتیوباسیلوس فرواکسیدانس* و *اسیدیتیوباسیلوس تیواکسیدانس*، بیشتر از سایرین برای انجام آزمایش‌های فروشویی زیستی موردمطالعه قرار گرفته‌اند. در این تحقیق، جداسازی و شناسایی این باکتری‌ها، به کمک یک پروتکل بیوشیمیایی پیشنهادی جدید، صورت گرفت. با استفاده از روش کشت متوالی باکتری‌ها در محیط کشت‌های مایع و محیط کشت جامد، خالص‌سازی باکتری انجام شد. باکتری‌ها از طریق یک شیوه‌ی بیوشیمیایی و طی مراحل کشت متوالی شناسایی گردید. کشت و رشد باکتری‌ها با بررسی تغییرات مقدار pH، Eh و تغییر رنگ محیط کشت کنترل شد. در مرحله‌ی اولیه از روش کشت متوالی، مقدار آبگوشت مغذی، تاثیر بسزایی بر روی کشت کامل باکتری در گام‌های بعدی داشت. میزان جزیی آبگوشت مغذی به رشد و تکثیر مطلوب باکتری منجر نشد. با این حال، استفاده از ۸ گرم بر لیتر آبگوشت مغذی، به افزایش قابل‌توجه تعداد باکتری خاک در مرحله‌ی ابتدایی منتهی گردید. در گام‌های بعدی، باکتری‌های *ا. فرواکسیدانس* و *ا. تیواکسیدانس* خالص‌سازی و جداسازی شدند. در نهایت، کلونی‌های خالص، طی دو مرحله کشت خطی در محیط جامد آگار مغذی بدست آمد.

کلمات کلیدی: باکتری بومی، جداسازی، باطله معدن، *ا. فرواکسیدانس* ، *ا. تیواکسیدانس*.