Iran. J. Biotechnol. April 2023;21(2): e3278 DOI: 10.30498/ijb.2023.328969.3278

Research Article

Multi-Objective Optimization of Copper Bioleaching: Comparative Study of Pure and Co-Cultured Cultivation

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Received: 2022/03/21; **Accepted:** 2023/01/09

Background: Bioleaching is a practical method to recover metals from low-grade mineral sulfides. The most frequent bacteria involved in the bioleaching of metals from ores are *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. Experimental design is a method through which the optimum activity condition will be obtained, avoiding numerous trials and errors.

Objectives: This study aimed to optimize the bioleaching condition of two indigenous iron- and sulfur-oxidizing bacteria from the Meydouk mine, Iran, and evaluate their function in a semi-pilot operation in pure and mixed cultures.

Material and Methods: After treatment with sulfuric acid, the bacterial DNA was extracted, and further 16S rRNA was sequenced to characterize the bacterial species. The cultivation condition of these bacteria was optimized using Design-expert (6.1.1 version) software. The copper recovery rate and the differentiation in the ORP rate in the percolation columns were also investigated. These strains were isolated from the Meydouk mine for the first time.

Results: 16S rRNA analysis revealed that both bacteria belong to the *Acidithiobacillus* genus. The factors with the most significant impact on *Acidithiobacillus ferrooxidans* with their optimum level were temperature=35 °C, pH=2.5, and initial $FeSO_4$ concentration=25 $g.L^{-1}$. Also, initial sulfur concentration had the most significant impact on *Acidithiobacillus thiooxidans* with the optimum level of 35 $g.L^{-1}$. Moreover, the mixed culture determined higher bioleaching efficiency compared with the case of employing the pure cultures.

Conclusions: Utilizing a mixture of both bacteria, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* elevated the Cu recovery rate due to the synergetic function of the strains. Also, introducing an initial dosage of sulfur and pre-acidification could elevate metal recovery efficiency.

Keywords: Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Bioleaching, Optimization, Percolation column, Response surface methodology,

1. Background

Different types of copper-containing minerals within copper mining sites, such as pyrite, chalcopyrite, and chalcocite, are generally considered law-grad copper minerals (1-4). Generally, bioleaching proposes the conversion of metals into water-soluble forms by microorganisms (4-6). In terms of low-grade mineral depletion, microbial leaching is a more environmentally-friendly and cost-effective alternative to other techniques, such as hydrometallurgy, in the mining industry (4, 6).

Leaching environments are considered extreme sites because they frequently have a hyper-acidic state (pH=3) and a high concentration of heavy metals such as iron and copper (5). Iron- and sulfur-oxidizing bacteria are the most effective of all the microbiota in mines that perform leaching activities (5-7). *Acidithiobacillus ferrooxidans*, a Gram-negative chemolithoautotrophic iron-oxidizing bacteria, is one of the most active organisms capable of bioleaching sulfide ores and can survive in extreme environments. *A. ferrooxidans* can sustain going by obtaining energy from the oxidation of elemental sulfur and ferrous compounds. This bacterium can solubilize metal either directly by secreting the enzyme or indirectly by oxidizing sulfidic ore with ferric ions (1, 6, 8, 9). *Acidithiobacillus thiooxidans* is another thermophilic bacteria that may get energy by oxidizing various sulfur compounds to promote cell growth and bioleaching activities. Because it oxidizes elemental sulfur and sulfide to sulfuric acid, A. thiooxidans is essential in bioleaching metals from sulfide ores (10).

Several physicochemical and biological factors impact the operation of the bioleaching process. Only when the leaching systems are administered under optimal bacteria cultivation conditions can the optimum efficiency of metal solubilization be obtained (11). The concentration and size of solid material (12-14), pH (6, 13, 14), temperature (6, 15), pre-treatment with S and acid (7, 11, 16), as well as the impact of mixture inoculation of microorganisms (1, 2, 17) are the examples of the factors that their influence on the leaching efficiency has been evaluated.

Based on the physicochemical properties of minerals and the budget invested, there are different techniques of leaching applied, including percolation and agitation. Percolation leaching is the selective removal of metal values from a mineral by allowing a suitable solvent or leaching agent to permeate into and through a mass or pile of material comprising the mineral (18). Compared with the agitation method, percolation leaching has the advantages of not requiring significant initial investment and having a relatively low operational cost (19). Column tests closely approximate field conditions and can analyze the long-term discharge of chemical elements. The benefit of a column test over a batch test is that it enables the observation of high initial concentrations of percolates at low concentrations and time-dependent chemical release, which is necessary for predicting leaching performance under field settings (20).

2. Objectives

This research aimed to assess the bioleaching capacity of copper ores from the Iranian Meydouk Mine as an alternative to the conventional methods applied. To accomplish this, we assessed the impact of several parameters on the leaching activity of two indigenous *Acidithiobacillus* strains retrieved from the mine. In addition, a comparative study was conducted on the copper recovery efficiency of the bacteria as individuals and in a mixture. These strains were isolated from this mine for the first time, and during the isolation of these strains, other bacteria were also isolated that were not further investigated.

One of the main reasons that this research first went to the native bacteria of the Meydouk copper mine for the bioleaching process was that most native bacteria are more adapted to the chemical, physical, and climatic conditions of the mine than non-native bacteria; therefore, investigations to find efficient species will be minimized leading to cost alleviation.

3. Material and Methods

3.1. Preparation of Culture Media

For the enrichment and isolation of the bacteria, we utilized 9K liquid and 2:2 solid media. 9K medium consisted of the following compounds: $(NH_4)_2SO_4$ 3.0 $g.L^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.5 $g.L^{-1}$, K_2HPO_4 0.5 $g.L^{-1}$, KCl 0.1 $g.L^{-1}$ and $Ca(NO_3)_2$ 0.01 $g.L^{-1}$ and 30 $g.L^{-1}$ $FeSO_4 \cdot 7H_2O$ as the source of energy. The initial pH was adjusted to 1.8. (21). Also, the 2:2 solid medium had these ingredients: $(NH_4)_2SO_4$ (4.5 $g.L^{-1}$), KCL (0.15 $g.L^{-1}$), $MgSO_4.7H_2O$ (0.75 $g.L^{-1}$), $Na_2S_2O_3.5H_2O$ (2 $g.L^{-1}$), $FeSO_4.7H_2O$ (2 $g.L^{-1}$) and Agar (8 $g.L^{-1}$) (22).

3.2. Sampling of Mine Ores and Microbial Adaptation

Copper ore samples were collected from several places

in the Meydouk Mine in Kerman Province, Iran. The samples were sieved to a size of 0.15 mm. The size of the columns was chosen to be 1.5 – 3 mm for the leaching experiments (percolation). Ores were deposited in big plastic containers, and 0.5 mL of regular sulfuric acid was sprayed four times daily to provide a natural bioleaching habitat for the bacteria. These samples were kept at a temperature of 30 °C. After a few weeks, the acid residue from the blue-green solution was cultivated in the 9k medium with 1% sulfur at 30 °C. This might provide the target bacteria an advantage over other potential bacteria in terms of numbers. After two weeks, 5 mL of the culture media were transferred into other Erlenmeyer flasks. The second series of Erlenmeyer flasks were sub-cultured a few days later. Simultaneously, the I and II series were cultured on the solid medium, and the process was repeated numerous times on solid and liquid media.

3.3. Identification of Isolated Bacteria

3.3.1. Morphological Characterization Method

Several serial dilutions were conducted to isolate the bacteria until turbidity was observed in the 9K medium. The bacteria were then cultured on the 2:2 solid medium for morphological analysis. To achieve a pure culture, multiple subcultures were repeated. Because acidophilic bacteria generally do not absorb gram dye, fluorescent staining was utilized instead.

3.3.2. PCR Amplification, Sequencing, and Phylogenetic Analysis of 16S rRNA

Following the isolation of pure cultures, bacterial DNA was extracted using the CTAB procedure. The forward primer 27F (5'-AGAGTTTGATCCTGGCT CAG-3') and reverse primer 1492R (5'-GGTTACC TTGTTACGACTT-3') were used to amplify the 16S rRNA gene. The overall volume of the reaction was 25 µL, which included 12.5 µL of 2X master mix, 0.5 µLof F primer, 0.5 µL of R primer, and 3 µL of DNA templates, to which 8.5 µL of distilled water was added. The amplification process includes an initial denaturation at 95 °C for 5 mins, followed by 30 cycles of 90 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 mins. The PCR products were purified by gel electrophoresis with a 1 percent agarose gel in TAE buffer solution, and the gel image was inspected using a UV trans-laminator at 260 nm. The PCR product was extracted from the gel and delivered to Takapozist Company to be sequenced using a 3730xl DNA Analyzer (Applied Biosystems co, Massachusetts, USA). The sequences were analyzed using the National Center for Biotechnology Information's BLAST software (NCBI). Ultimately, using MEGA4 software and the neighbor-joining method, the phylogenetic tree of the novel strains isolated from the Meydouk mine was plotted (23).

3.4. Experimental Design to Optimize the Bioleaching Process

The experimental design method is often used to regulate the effects of factors and process modeling. Using it minimizes the number of experiments, time, material consumption, and experimental errors. The response function in our design was the rate of Fe^{2+} oxidation to Fe^{3+} and the ORP value (mV). These two dependent variables could be attributed to the growth process of *Acidithiobacillus ferrooxidans,* and the rate of OD changes could be attributed to the growth of *Acidithiobacillus thiooxidans*. Among the factors affecting the response, eight factors were evaluated. The levels of these variables were carefully selected according to previous studies. Each variable was considered at two levels, low and high, and their average as the midpoint (**Table 1A**).

Due to the variables and their levels, 2^8 experiments were required by complete factorial design (CFD). The fractional factorial design was used because of the limitation of the test materials and the cost of analyses. Fractional factorial designs (FFD) are experimental designs consisting of a carefully chosen subset (fraction) of the experimental runs of a complete factorial design. Since only the main effect of factors on the response was desired and their interactions were not considered in this step, the FFD with resolution IV was employed. Therefore, a fraction of 1/16 was selected that required 16 ($2^8/2^4$) experiments for *Acidithiobacillus ferrooxidans*. Two experiments were performed at midpoints of all factors to analyze the curvature, resulting in 18 experiments. All experiments were carried out in random order, and the means of two or more independent data were compared by One-Way Analysis of Variance (ANOVA) to determine whether there is statistical evidence that the associated population means are significantly different. The software Design Expert version 6.0.10 was used to design the experiments and statistical analysis. According to the results obtained

Table 1. Levels of the studied variables. The variables considered for the optimization of **A)** *A. ferrooxidans* and **B)** *A. thiooxidans.*

A)

Factors		The lowest level (-1)	The midpoint (0)	The highest level (1)
A	$(NH_4)_2SO_4$ g.L^{-1}	1.5	2.75	4
B	K_2HPO_4 g.L^{-1}	0.25	0.5	0.75
C	$MgSO_4$ g.L^{-1}	0.25	0.5	0.75
D	KCl g.L^{-1}	0.2	0.1	0
E	$Ca(NO_3)$ g.L^{-1}	0.02	0.01	0
F	$FeSO_4$ g.L^{-1}	24.7	34.7	44.7
G	pH	1.5	2	2.5
H	Temperature ^{o}C	25	30	35

B)

Factors		The lowest level (-1)	The midpoint (0)	The highest level (1)
A	pH	2	3	4
B	Temerature ^{o}C	25	30	35
C	Sulfur con g.L^{-1}	5	12.5	20

from the experiment design for *Acidithiobacillus ferrooxidans*, three significant factors were determined, and the growth of *Acidithiobacillus thiooxidans* (TTM) was investigated (**Table 1B**). At two levels, the effect of these factors was studied using CFD (23 experiments) and two other experiments for midpoints. Therefore, ten experiments were conducted according to the three factors.

3.5. Leaching Experiments

In three series, 10, 15, and 20 g of sample soil (soil particle diameter = 1 µm) (10, 15, and 20% W/V pulp density) were put into 250 mL flasks containing 95 mL of 9k medium. The first flask received 5 mL of *Acidithiobacillus ferrooxidans* medium, the second received 5 mL of *Acidithiobacillus thiooxidans* medium, and the third (control) received no bacterium inoculation. With an initial pH of 1.8, all flasks were put in a shaker incubator at 35 °C for seven days. Every day, the acidity was tested and, if necessary, adjusted with sulfuric acid. Finally, samples were transported to the Atomic Energy Agency to be analyzed using inductively coupled plasma (ICP).

3.6. Percolation Column Test

The percolation column leaching test was carried out in Plexiglas columns with a height of 0.7 m and an internal diameter of 7 cm. **Figure 1** illustrates the real setup and a shematic diagram of the bioleaching system. The column was filled with 2.4 kg of 1.5-3 mm ore samples. The top of the columns was left open to load the sample and supply the H_2SO_4 aqueous solution. Before loading the ore samples, a filter was put at the bottom of the columns, allowing only the percolate to pass through. 5 L of leach liquor was irrigated and made to circulate continually through the ore sample by gravity and cyclically re-circulation. Aeration, pH, and temperature were set at 60 mL.min^{-1}, 1.8, and 35 °C, respectively. The procedure was set to run in continuous mode for 40 days. A representative sample of leach liquid was collected from each leach tank at the end of each day and evaluated for Cu recovery and oxidation-reduction potential.

Figure 1. Percolation column. A) Real setup. **B**) Schematic diagram.

4. Results

4.1. PCR Amplification, Sequencing, and Phylogenetic Analysis of 16S rRNA

Following morphological identification, genomic DNA was extracted from the two bacteria, and PCR was performed to replicate the 16s rRNA, followed by electrophoresis on a 1% agarose gel, resulting in a band at 1500bp compared to the 1kb marker for each, indicating that the PCR reaction was performed correctly. A 1434 nucleotide fragment for the iron-oxidizing acid bacteria and a 1432 nucleotide fragment for the sulfur-oxidizing bacteria were obtained by sequencing the PCR result. The homology found using NCBI's BLAST program demonstrated that the iron- and sulfur-oxidizing bacteria isolated from the Meydouk mine were 100% compatible and 99% similar to *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, respectively. Other methods of identifying the isolated bacteria were used, such as examining the morphology and characteristics of gram staining and light and electron microscope photos. Due to the limitation of presenting figures, their data is not shown.

4.2. Phylogenetic Tree Based on 16S rRNA

The phylogenetic tree was constructed using Mega software version 4 after identifying the nucleotide sequences of *Acidithiobacillus ferrooxidans* (TFM) and *Acidithiobacillus thiooxidans* (TTM) and comparing them to neighboring species. By examining the phylogenetic tree, it is possible to deduce that these bacteria were closely related to the *A. ferrooxidans* and *A. thiooxidans* species (**Fig. S1**). The proximity of these bacteria to the desired species suggests that they have a similar origin.

4.3. Experimental Design to Optimize the Bioleaching Process

The quantity of pH, $FeSO_4$, and temperature had the most significance on iron oxidation, and the most substantial interaction was between A and F. To analyze the data and simplify the proposed model by the software, the factors that had a negligible effect on the response were eliminated, and the analysis of the variance of the remaining factors was obtained (**Table S1**). The Fvalue=19.17 in **Table S1** illustrates the relevance of the suggested equation with eight selected components. Prob>F less than 0.05 suggests that the participants in the equation or model are significant. The proposed equation had an R^2=0.96 and a standard deviation of 8.57. The proposed model for iron oxidation based on the coded values of the factors in **Table S1** is as follows:

Y= (51.16) + (2.38) A – (3.88) C + (3.18) D – (4)E – (7.97) F + (21.32) G + (8.03) H – (5.80) AE + (11.37) AF

The data from these 18 experiments likewise had a normal distribution, indicating that the error in the findings was not substantial, as demonstrated by the standard distribution curves (**Fig. S2A**). **Figure S2B** presents a comparison of the anticipated values with the experimental results. Because the actual values

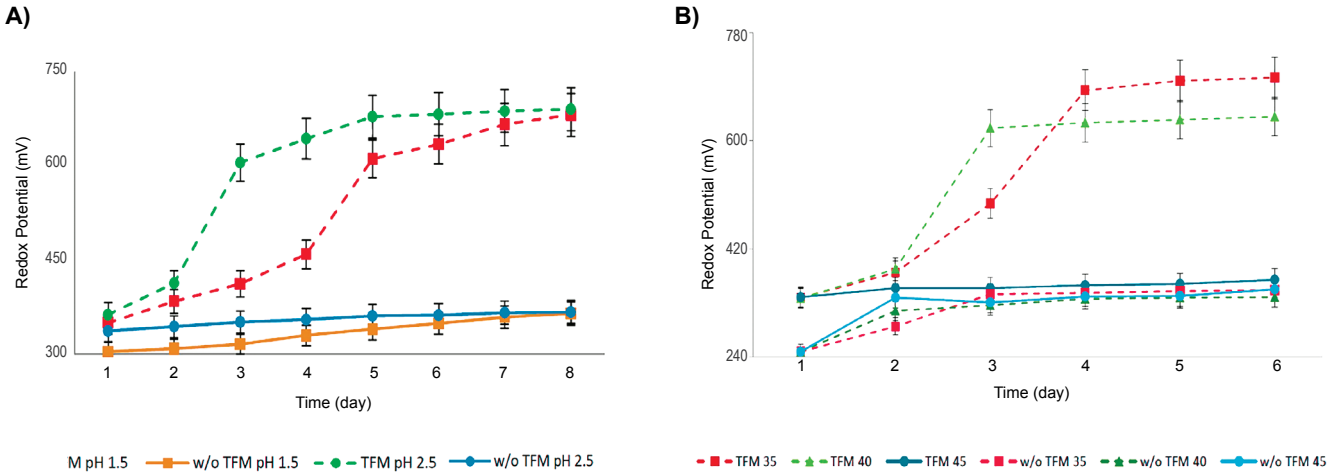

Figure 2. Evolution of redox potential. It was evaluated with the presence and absence of TFM in different **A)** pH and **B)** temperature

are around a straight line, the diagram implies that the relationship recommended by the program adequately processed the test points. **Figure S2C-D** display the interaction of the four factors when the other factors are set to zero.

Based on the results obtained and after assessing the results by the Design Expert Software, the optimal culture condition provided by the program for TFM was as follows: $(NH)_2SO_4$ 1.5 $g.L^{-1}$, $MgSO_4$ 0.39 $g.L^{-1}$, KCl 0.1 $g.L^{-1}$, $Ca(NO_3)_2$ 0.02 $g.L^{-1}$, $FeSO_4$ 25 $g.L^{-1}$, pH 2.5 and temperature 35 °C.

4.4. Leaching Experiments (pH optimization)

This test aimed to determine if the difference in iron oxidation was due to bacterial growth or if an increase in pH caused it. It was discovered that changes in the presence of bacteria in both pHs were more significant than when no bacteria were present. Furthermore, the bacteria grew faster at a pH of 2.5 than at a pH of 1.5; hence the optimal pH was 2.5. (**Fig. 2A**).

4.5. Leaching Experiments and Temperature Effect on TFM in Optimized Culture Medium

Because the optimal temperature (35 °C) was the highest proposed, redox potential was measured at temperatures of 35, 40, and 45 °C to evaluate the correctness of the experiment design. Control studies were also carried out at these temperatures to demonstrate the impact of bacterial activity on the redox potential (**Fig. 2B**). According to the diagram, the redox potential approached the maximum rate of around 35 °C.

4.6. Optimization of the Culture Medium for TTM by Design Expert 6.0.10

Design-Expert software was used to optimize the bioleaching conditions of *Acidithiobacillus thiooxidans* extracted from the Meydouk mine using a complete factorial method. According to **Table S2**, the amount of sulfur had the most significant impact on sulfur oxidation, and the accuracy of the model used could be assessed using a level of significance less than 0.05. As indicated in the error distribution diagram, the data from the experiments likewise had a normal distribution, indicating that the error in the results acquired from the studies was minimal (**Fig. S3A**). **Figure S3B** depicts the interaction of factors A and B when all other parameters are set to zero.

The effects of temperature and pH on sulfur oxidation are depicted in **Figure 3A-3B** (OD 600nm). According to the analyses, the highest sulfur oxidation occurred at 25 °C and pH 2.

4.7. Initial S Concentration Effect on TTM Growth in the Optimized Culture Medium

To determine the optimal S concentration for *A. thiooxidans* optimum activity, the growth of bacteria in the optimal cultivation medium of TFM with the sulfur concentrations of 25, 30, 35,45, 45, and 50 $g.L^{-1}$ was evaluated. The temperature was set at 25 °C, as suggested by the software analysis. Based on the result,

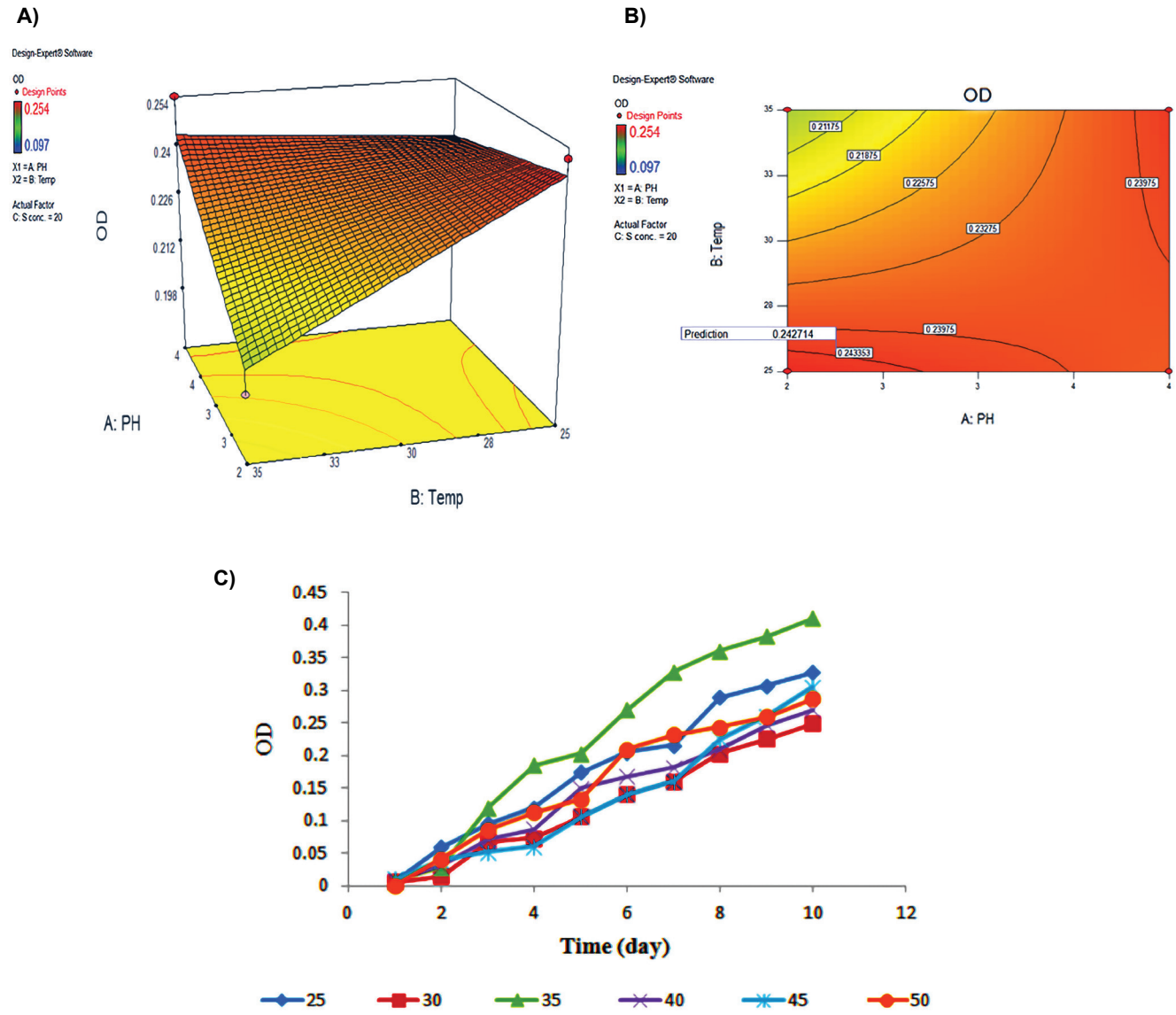

Figure 3. Impact of different parameters on the sulfur oxidation by TTM. A) Three-dimensional diagram of pH and temperature. **B**) Contour curves of pH and temperature and **C**) Initial sulfur concentration.

the highest sulfur oxidation was achieved by the 35 $g.L^{-1}$ of initial sulfur concentration (**Fig. 3C**).

4.8. The Results of the Release of Copper Through the ICP Device

Preliminary bioleaching studies were carried out to determine the copper release rate of each bacterium in the optimum culture medium at various pulp percentages (**Fig. 4A-4B**). According to the graphs, the higher the pulp concentration, the lower the Cu recovery, with the maximum copper recovery in 10% pulp concentration for both strains.

4.9. Percolation Column Tests Results

The ORP and copper recovery rate increased in the co-culture percolation columns, compared to the single-culture columns (**Fig. 5A-5B**).

5. Discussion

The experimental design makes it feasible to establish the optimal conditions for microorganism growth and activity, minimizing the need for numerous trials and

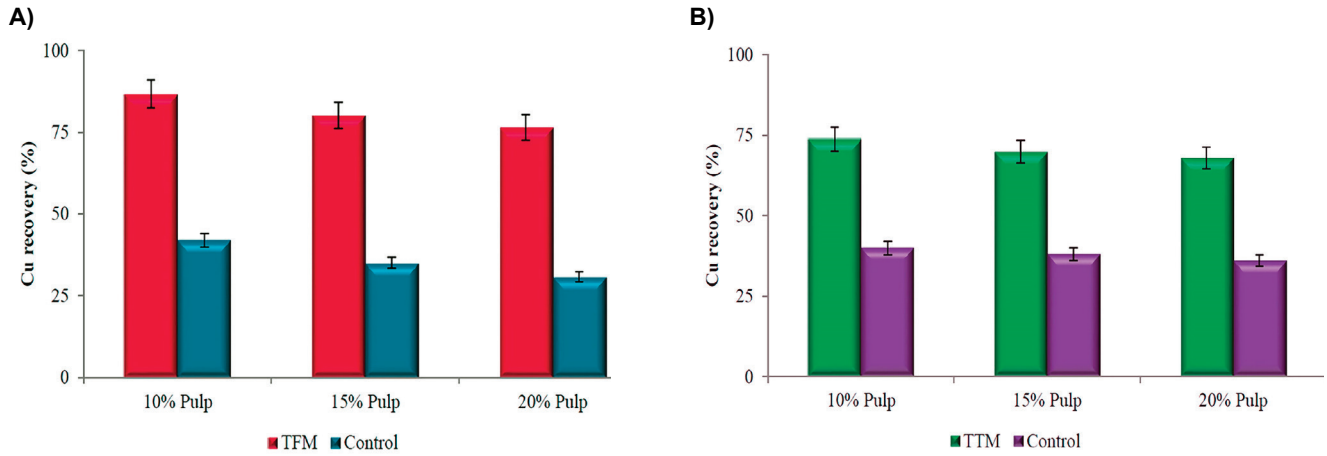

Figure 4. Copper release rate. The evaluation was done in the optimized culture medium with different pulp percentage while **A)** TFM and **B)** TTM were operating.

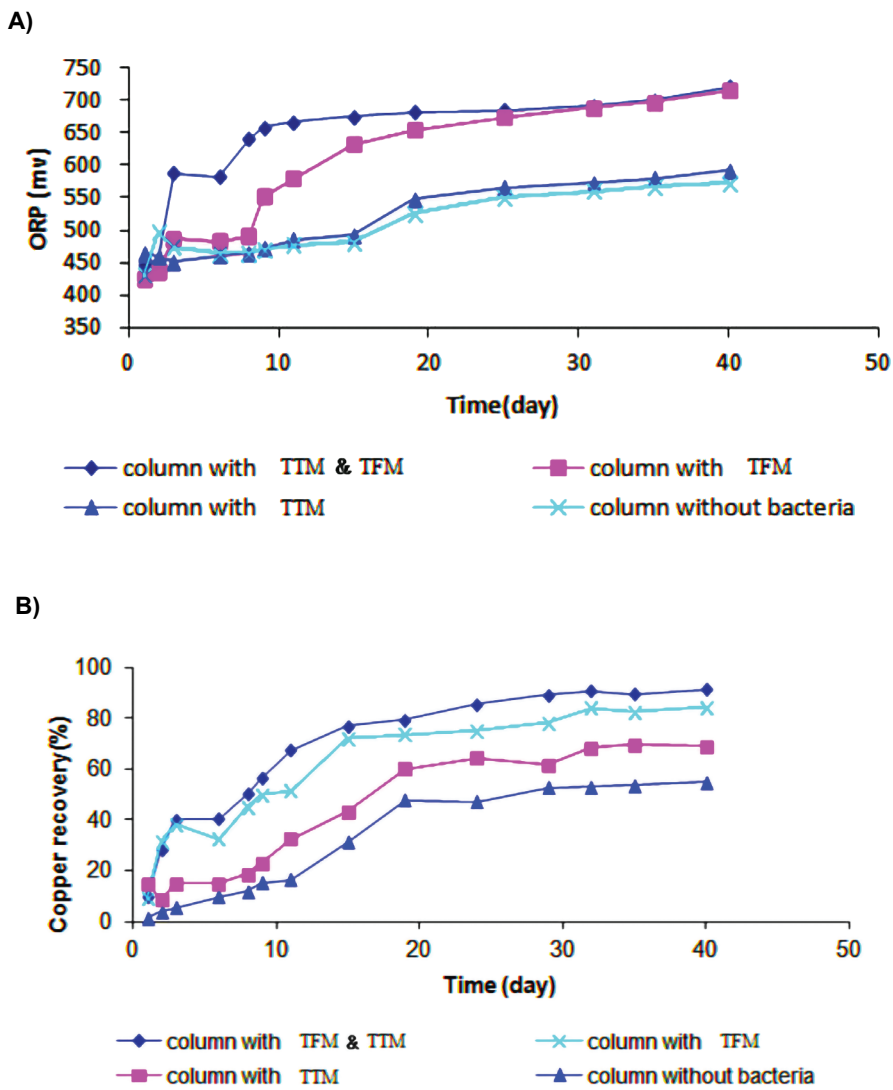

Figure 5. Activity comparison between pure and co-culture cultivation. The comparison was based on **A)** ORP rate and **B)** Cu recovery rate.

errors and eliminating extraneous factors.

The conversion of Fe^{2+} to Fe^{3+} by *Acidithiobacillus ferrooxidans* not only facilitates the removal of metals from ores but also provides energy for the bacteria to thrive (9, 24). In fact, it was expected to have iron concentration as one of the factors that significantly impact the growth rate of *A. ferrooxidans* (24), similar to what we discovered through the experimental design. Our investigation demonstrated that high $FeSO_4$ concentrations (>25 $g.L^{-1}$) had a preventive effect on bacterial iron oxidation. Fe^{2+} and Fe^{3+} have certain levels of inhibition on *A. ferrooxidans* (9, 13). Also, we observed that the influence of K^+ and Ca^{2+} ions on iron oxidation was minimal at the tested levels. Since the effect of KH_2PO_4 was negligible, we concluded that it is possible to have it eliminated from the components of the K9 culture medium.

According to the experimental design findings, pH is one of the three factors with the most significant effect on iron oxidation. In short terms, following **Figure 2A**, differentiation in initial pH demonstrated significant differences. A similar trend was observed in the study conducted by Arshadi and Mousavi in 2015. They similarly stated that the Cu recovery rate elevated as the initial pH increased from 1 to 2. They also indicated that a shift from a pH of 2 to 3 lowered the recovery of copper (13). Both sides of their study prove the accuracy of our result of 2.5 being the optimum pH. In another study where Fe oxidation by an iron-oxidizing bacteria was examined, it was discovered that as the pH decreased below 2, the bacteria's iron-oxidizing potential declined (6). Also, According to **Figure 2A** of the current work, the difference in iron oxidation was caused by bacterial action, not due to the shift in pH.

Iron-oxidizing bacteria, particularly *Acidithiobacillus ferrooxidans*, can adapt to temperatures found in deep mines. In 1989, Ahonen and Tuovinen investigated the growth of acidophilic iron-oxidizing bacteria at temperatures ranging from 4 to 37 and 46 °C and discovered that bacteria grew at all temperatures except 46 °C. (15). Kim *et al*. (2008) explored the effect of temperature on the log phase of *A.ferrooxidans* growth, discovering that 303 °K (about 30 °C) was the optimal temperature for the bacterium to thrive. The rate decreased above this temperature due to the denaturation of enzymes and proteins and ferric ion behavior (6). Although we observed bacterial function at temperatures below and above 30 °C, *A.ferrooxidans* demonstrated the most significant activity at 35 °C as the optimal temperature, which is roughly consistent with the findings of the cited works. Furthermore, at 45 °C, the redox potential exhibits a dramatic drop (**Fig. 2B**). This **Figure** further illustrates that the changes in redox potential were due to the presence of bacteria rather than a temperature rise.

When the experimental design for *A. thiooxidans* was completed, the results indicated that the initial concentration of sulfur had the most significant impact on the growth rate of TTM, possibly because this strain is dependent on sulfur to receive electrons essential for thriving. Using the Response Surface Method, Liu *et al*. (2004) optimized the acidic culture conditions of native *Acidithiobacillus thiooxidans*. They determined the optimal composition of this strain by evaluating four components in the culture medium: $(NH_4)_2SO_4$=4.9 $g.L^{-1}$, H_2PO_4=3.5 $g.L^{-1}$, $MgSO_4$=0.7 $g.L^{-1}$, and S=23.7 $g.L^{-1}$. By studying these four parameters, they concluded that the concentration of sulfur in the culture medium was the most crucial chemical impacting the growth of the bacteria and that the influence of the other three factors was minimal compared to the effect of sulfur concentration (10).

After establishing optimal cultivation conditions for these two isolated bacteria, bioleaching experiments were carried out on both strains. The results demonstrated a higher copper recovery of 10% in the pulp density. The greater the particle size in the column, the lesser the electrochemical connection was between sulfide minerals. The Cu ratio is often high in low-grade ore, where mineral interaction is expected to be less vigorous. As a result, the bacteria were more active in the thinner pulp, resulting in higher copper recovery (12).

As mentioned before, *Acidithiobacillus ferrooxidans* convert Fe^{2+} to Fe^{3+} to provide the required energy to thrive. The more Fe^{2+} is transformed to Fe^{3+}, the higher the pH will become, and, as a result, the Cu recovery rate will drop (9, 24). Also, *Acidithiobacillus thiooxidans* obtains the energy it needs by oxidizing sulfur to sulfate. Complementary to *A. ferrooxidans* activity, *A. thiooxidans* mechanism results in more sulfuric acid produced that enhances metal recovery through lowering the increased pH that was the consequence of the conversion of Fe^{2+} to Fe^{3+} by *A. ferrooxidans*. Furthermore, *A. thiooxidans* can prevent the formation of jarosites and allow for greater copper solubilization

through the action of ferric ions (1). Accordingly, in our investigation, a greater rate of Cu recovery was observed in the percolation column of the mixture of these two bacteria than in the situations where *A. ferrooxidans* and *A. thiooxidans* were used separately. Qiu MQ *et al*. 2005 and Liu H *et al*. 2011 evaluated pyrite and chalcopyrite bioleaching processes in vitro using pure cultivation vs. mixed cultivation of *Acidithiobacillus* strains, demonstrating that the mixes in vitro had more significant impacts on copper recovery (1, 2).

Typically, a pre-treatment stage is planned in industrial operations to minimize the initial stationary period. Hereupon, pre-leaching acidification and sulfur addition can enhance microorganism leaching activity by facilitating metal mobility by lowering pH, as demonstrated by Kamizela *et al*., 2021, who investigated the recovery of heavy metals from landfill leachates using *A. ferrooxidans* and *A. thiooxidans* (7). Zheng *et al*. in 2012 demonstrated that acid rain, considered an acid treatment, could significantly elevate the leaching activity of micro-organisms by declining the pH level (16). Due to the proven positive effect of pre-acidification, we decided to have this pre-treatment after sampling and before the bioleaching experiments. Moreover, adding sulfur at the beginning of the leaching can play a double-sword role, increasing in case of the optimum dose and preventing if it overdoses (**Fig. 3C**).

Although multiple studies have been conducted on the bioleaching of copper in mines located in Iran, to the best of our knowledge, this is the first report on the optimized copper bioleaching by a mixture of different *Acidithiobacillus* strains extracted from Iran's mines, and it indicates the importance of employing this method besides optimization of cultivation and bioleaching conditions.

6. Conclusions

Two mesophilic iron-oxidizing and sulfur-oxidizing acidophilic strains isolated from the Meydouk mine in Kerman Province, Iran, were identified as *Acidithiobacillus* based on morphological testing and 16S rRNA gene sequence analysis. The experimental design was performed through Response Surface Method to optimize the bioleaching condition. Initial iron concentration, temperature, and pH were the influential factors for *A. ferrooxidans*, and initial sulfur concentration had the most significant impact on *A. thiooxidans*.

When percolation columns were set up, it was discovered that using a mixture of both bacteria resulted in a higher ratio of copper recovery than individually inoculating each strain.

Acknowledgement

This work was supported by the National Iranian Copper Industries Corporation and the National Institute of Genetic Engineering and Biotechnology (Grant Number 497).

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