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Modified 9K Medium for Ore Bioleaching

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INFORMATION

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ABSTRACT

Bioleaching applications with *Acidithiobacillus ferrooxidans* (*A.f*) have shown significant progress in recent years. Before being used in bioleaching applications, bacterial isolation processes must be performed and they must be provided to reach sufficient purity, activity, and population. For this, mediums are prepared by using various nutrient mixtures in different ratios. In this study, it was tried to obtain a modified medium that could be an alternative to the 9K medium of Silverman and Lundgren. At the same time, bacterial growth rates and Fe oxidation rates were investigated. During the modification process, KH_2PO_4 was used instead of K_2HPO_4, and its effects on bacterial growth were investigated by increasing the amount of $FeSO_4.7H_2O$. The experiments were conducted in 250 mL shaken flasks for 16 days in a continuous incubator system. In the experiments, bacterial growth and Fe oxidation were investigated by analyzing ferric ion concentration in specific periods and observing the color change of the solution. At the end of the experiments, it was determined that the highest iron oxidation efficiency, the lowest acid consumption and the best bacterial growth were in Medium 6 (0.1 g/L $FeSO_47H_2O$, non-KH_2PO_4).

1. Introduction

Nowadays bioleaching occupies an increasingly important place among the available mining technologies (Acevedo, 2002; Mutch et al., 2010; Seitkamal et al., 2020; Cheng et al., 2021). The most important group of bacteria which are involved in sulfide minerals leaching are the acidophilic Thiobacilli. The elements such as nitrogen, phosphor, sulfur, and magnesium are essential for the growth of *A.f.* (Seifelnassr and Abouzeid, 2000). In order to cultivate the iron-oxidizing bacteria in a liquid medium, a lot of medium has been developed. The most commonly used medium for heterotrophic and iron-growing bacteria from acidic mine drainage is the 9K medium which was described by Silverman and Lundgren in 1959 (Silverman and Lundgren, 1959). Before being used in bioleaching, bacteria obtained from acidic mine drainage should be subjected to several isolation processes to reach sufficient purity and population.

Metal leaching from metal sulfides can be accelerated by some acidophilic iron and/or sulfur-oxidizing bacteria. These bacteria are isolated from industrial leaching operations or natural leaching and acidic mine drainage areas. In one study, three acidophilic, chemolithotrophic, and iron-oxidizing bacteria were isolated from the Agrio River using a 9K enrichment medium and then purified on a solid ferrous-agarose medium (Lavalle et al., 2005). The general understanding of bioleaching of sulfide minerals is to keep pH low. Some published articles have reported the effect of pH on bio-oxidation rates of ferrous-iron and/or sulfur by bioleaching microbes, although most of these studies were conducted at optimum or near optimum temperatures for microbial performance (Ongendangenda and Ojumu, 2011).

Studies on the effect of nutrient concentrations on bacterial leaching indicated that bacterial activity is markedly affected by variations in $(NH_4)_2SO_4$ and K_2HPO_4 concentrations of the leaching medium (Tuovinen and Kelly, 1973; Lazaroff, 1963). The absence of calcium potassium, nitrate, and chloride ions has no measurable influence on metal sulfide oxidation. These nutrients are required for bacteria in such small quantities that the impurity of their sulfur-containing substances is sufficient to meet their needs (Bryner and Andersen, 1957).

Microorganisms used for metal extraction from sulfide materials are chemolithoautotrophic bacteria and therefore

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only inorganic compounds are required for growth. In general, the mineral nutrients are obtained from the material to be leached. For optimum growth, iron and sulfur compounds may be supplemented together with ammonium, phosphate, and magnesium salts (Bosecker, 1997). The basic nutrient needs are nitrogen, potassium, phosphorus, and a variety of elements depending on the particular species (Spencer, 2001).

Leathen et al. (1956) developed an inorganic medium by using silica gel with inorganic salts in isolating Ferrobacillus ferrooxidans. These and similar studies have supported the development of iron-oxidizing bacteria. Manning, in his work in 1975, found a new solid medium for isolating acidophilic bacteria from acidic mine drainages (Manning, 1975).

The samples taken from AMD were used as 10% inoculums into Silverman and Lundgren's 9K medium. In another study by Kawabe et al. (1999), T. ferrooxidans-type bacterium was used. This bacterium was obtained in the laboratory by semi-continuous enrichment culturing of iron-oxidizing bacteria. Its chemical composition was purified on agar consisting of $(NH_4)_2SO_4$, KCl, K_2HPO_4, $MgSO_4.7H_2O$, $Ca(NO_3)_2$, $FeSO_4.7H_2O$ and $Na_2S_2O_3.5H_2O$ and in 10:10 solid medium (pH=2.2).

In this study, the modification of Silverman and Lundgren's 9K medium was carried out. It was observed that there were differences in initial pH between K_2HPO_4 and KH_2PO_4 used in Silverman and Lundgren's 9K medium. In addition, it was determined that $FeSO_4.7H_2O$ and K_2HPO_4 added to the medium caused precipitation in the solution. At the end of the experiments, the medium providing the fastest oxidation and the best bacterial growth was determined.

2. Materials and Methods

AMD samples were taken from the acidic mine drainage of the copper mine in Yomra, Trabzon, Turkey. Solutions containing $Ca(NO_3)_2$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, KCl, K_2HPO_4, KH_2PO_4, $FeSO_4.7H_2O$, distilled water, and *Af* were used for 9K medium. H_2SO_4 was used for pH adjustment. 250 ml flasks, Schott Instrument Handy Lab Multi 12 pH meter, 8 well ROSI-1000 Thermolyne Orbital Shaking Incubator shaker were used in the experiments. Fe(II) analyses were performed with a Shimadzu UV160A spectrophotometer.

The solutions prepared by inoculating 100 ml of 9K medium with 1 ml of AMD sample were put into 250 ml flasks. The inoculation process in these mixtures was cultured several times, and the necessary microscopic and analytical examinations were made and made ready for the experiments.

Then, modification processes were carried out with these obtained bacteria, and the medium mixtures were planned to be modified. Six different mediums were prepared for the modification process. These mediums were prepared in the 1.5 mL bacterial solution to 150 mL medium in 250 mL flasks. The mixtures used in the 6 mediums are given in Table 1. Experiments were started by adjusting the pH values in all mediums to 2.00 with 10% H_2SO_4.

Table 1. Contents of 9K mediums

Nutrient (g/L)	The number of 9K mediums					
	1	2	3	4	5	6
$Ca(NO_3)_2$	0.01	0.01	0.01	0.01	0.01	0.01
$MgSO_4.7H_2O$	0.5	0.5	0.5	0.5	0.5	0.5
$(NH_4)_2SO_4$	3.0	3.0	3.0	3.0	3.0	3.0
KCl	0.1	0.1	0.1	0.1	0.1	0.1
K_2HPO_4	0.5	0	0	0	0.5	0
KH_2PO_4	0	0.1	0.5	0.5	0	0.1
$FeSO_47H_2O$	14.7	14.7	14.7	33.0	33.0	33.0
Distillated water (mL)	150.0	150.0	150.0	150.0	150.0	150.0
Bacteria solution (mL)	1.5	1.5	1.5	1.5	1.5	1.5

Table 2. pH of modification experiments

9K medium number	pH values						
	Solution preparation stage	At the beginning of experiment	4th day	7th day	10th day	14th day	16th day
1	3.63	2.00	2.33	2.45	2.47	2.17	2.10
2	3.11	2.00	2.21	2.30	2.36	2.10	2.01
3	3.06	2.00	2.16	2.30	2.37	2.10	2.01
4	3.08	2.00	2.27	2.30	2.40	2.12	2.01
5	3.60	2.00	2.30	2.45	2.45	2.15	2.08
6	3.10	2.00	2.17	2.35	2.40	2.07	1.98

Prepared shaken flasks were incubated 24 ±1 ºC incubator at 150 rpm. Their pH values were measured periodically in the experiments, but any adjustment was not made. The samples were taken intermittently in order to determine the total cell number and ferric ion concentration.

During 16 days of incubation, color changes in the medium were also observed and recorded. At the end of 16 days, the bacteria population was determined by the "Cell Counts by Hemocytometer" method (Mather and Roberts, 1998). Fe(II) analyses were made by the Shimadzu UV160A

spectrophotometer with the standard methods (Fransan 1985; Karamanev et al., 2002). Fe(III) concentrations were determined by the EDTA titration method (Gülensoy, 1984; Qiu et al., 2006). The color change of bacteria was analyzed visually; its activations and developments were examined microscopically.

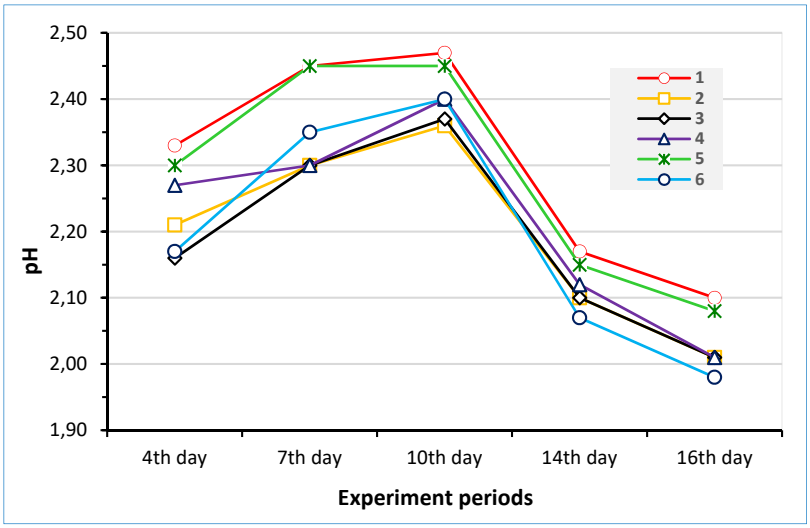

Fig. 1. pH changes in mediums

Table 3. Fe oxidation efficiency obtained in modification experiments

9K medium number	Fe efficiency (%)				
	4th day	7th day	10th day	14th day	16th day
1	22.7	23.2	28.2	31.7	60.5
2	22.2	23.2	27.2	31.3	61.0
3	25.2	25.7	25.9	26.2	52.4
4	11.2	11.2	21.6	23.4	45.8
5	10.1	10.4	23.5	34.2	50.1
6	14.4	14.8	32.3	55.2	86.7

3. Results and Discussions

Before the modification process, as a result of several cultivations, the samples were taken from AMD, and it was observed that 2,04x106 bacteria intensity was reached with the method of "Cell Counts by Hemocytometer" (1/400 mm^2-1/25 mm^2).

After the inoculation process, at the end of the analytic and microscopic investigation of the samples treated in incubation, it was determined that they reached this number in approximately 3-5 days. These bacteria, firstly, were inoculated in Silverman and Lundgren's 9K medium. Since K_2HPO_4 added to this medium increased the pH of the solution and formed a precipitate, the modification process was carried out. In the modification processes, by using different quantities of KH_2PO_4 instead of K_2HPO_4, the disappearance of precipitation and the pH increase were prevented.

Because, the changes in di-potassium hydrogen phosphate concentrations affected bacterial activation (Tuovinen and Kelly, 1973; Lazaroff, 1963). Besides, by using 33g/L $FeSO_4.7H_2O$ instead of 14,7 g/L $FeSO_4.7H_2O$, both bacterial activation and the increase of oxidation speed were provided. pH values in the course of the modification process were given in Table 2.

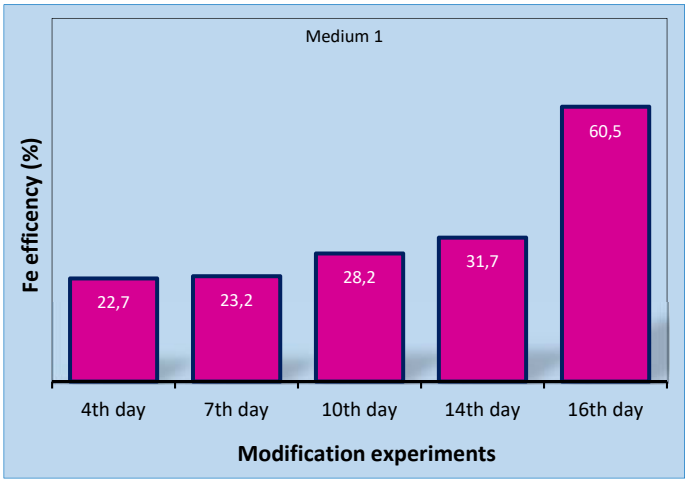

Fig. 2. Fe efficiency (%) in Medium 1

As can be understood from Table 2, pH values were high in the medium of K_2HPO_4 which was 0,5 g/L in contrast to the medium with KH_2PO_4. When Fig. 1 is examined; the minimum pH values were observed in the mediums using 0.1 g/l KH_2PO_4 and 33 g/l $FeSO_4.7H_2O$ during the 0-16-day experiment, while the maximum pH values were observed in the mediums using 0.5 g/l K_2HPO_4 and 33 g/l $FeSO_4.7H_2O$.

More (approximately 40-50% by volume) sulfuric acid was used (compared to other mediums) to lower these pH values to the experiment starting value. As it was seen, isolated bacterium shows more rapid specific growth in lower pH values. So, the high pH value of the medium at the beginning affected the growth and adaptation time.

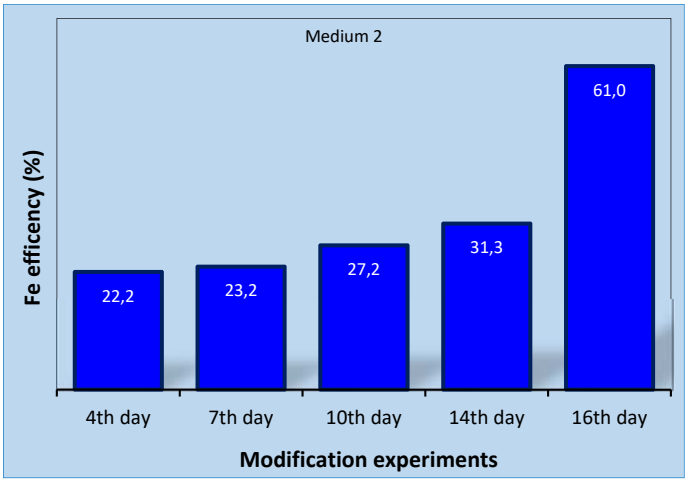

Fig. 3. Fe efficiency (%) in Medium 2

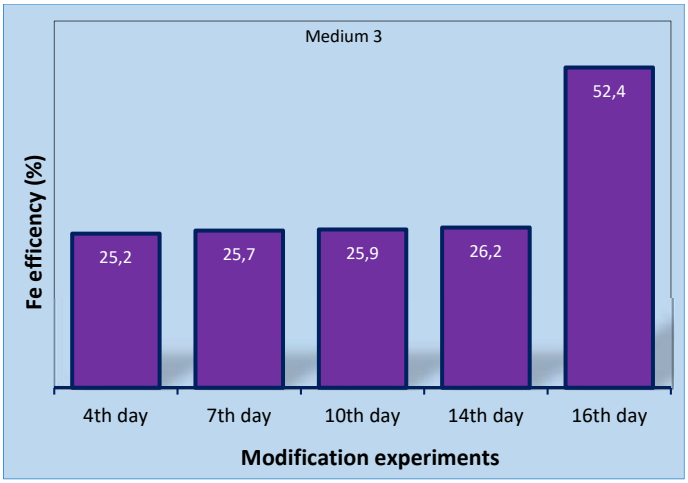

Fig. 4. Fe efficiency (%) in Medium 3

In addition, as the pH of the solution decreases, bacteria affect the iron dissolution rate more. Setting the correct pH value is necessary for the growth of leaching bacteria and the dissolution of metals. pH values between 2.0–2.5 are optimum for bacterial oxidation of Fe(II) and sulfur. pH values below 2.0 cause inhibition of T. ferrooxidans but can be adapted even to lower pH values by increasing acid addition (Bosecker, 1997). According to Table 3, the exchanges of Fe efficiency by different mediums are given in Figs. 2-7.

In the 0-7 days range, the lowest Fe efficiency was obtained in Medium 5, while the highest efficiency was obtained in Medium 3. Between 7-16 days, the lowest Fe efficiency was obtained in Medium 4, and the highest Fe efficiency was obtained in Medium 6.

In other words, the Fe efficiency is minimal in the medium using 33 g/l $FeSO_4.7H_2O$ for 4-16 days (Figs. 2-7). When the ratios of K_2HPO_4 and KH_2PO_4 are compared; While the lowest Fe efficiencies were determined in the mediums using 0.5 g/l K_2HPO_4 and 0.5 g/l KH_2PO_4, the maximum Fe efficiencies were obtained in the mediums with 0.5 g/l K_2HPO_4 between 0-7 days. Between 10-16 days, maximum Fe efficiencies were obtained in the medium using 0.1 g/l KH_2PO_4. In summary, the ratios of 33 g/l $FeSO_4.7H_2O$ and KH_2PO_4 give the best results at maximum efficiencies.

At the end of the experiments, the change in Fe efficiencies is not much until the 7th day. The main reason for this is the adaptation of the bacteria to the medium and medium conditions. It was understood from the values obtained at the end of the first 7 days that the medium with the slowest pH change is Medium 2, Medium 3 and Medium 4 and this was entirely due to KH_2PO_4. The low Fe efficiency is due to the prolongation of the adaptation period of the bacteria. After the 7th day, the adaptation period was completed and the Fe efficiency began to increase.

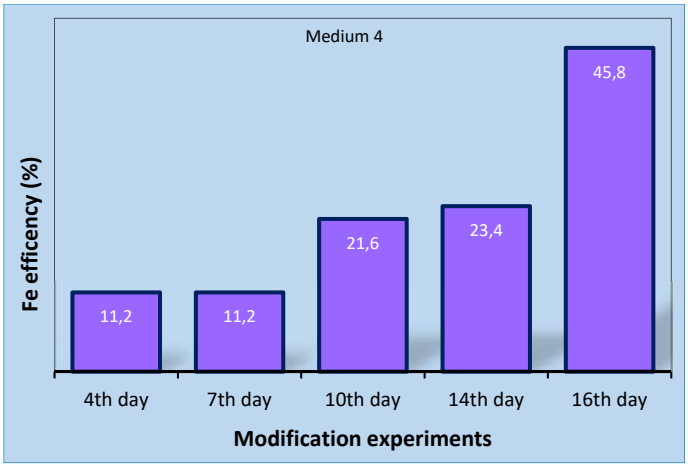

Fig. 5. Fe efficiency (%) in Medium 4

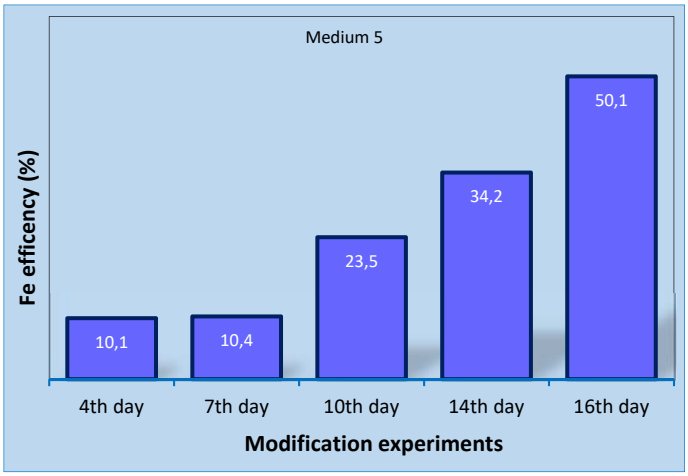

Fig. 6. Fe efficiency (%) in Medium 5

From the 10th day, it was observed that the Fe efficiency was approximately 40% higher than the others, and 86.7% of the

iron amount could be oxidized to Fe(III) in the medium, in which 0.1 g KH_2PO_4 and 33 g $FeSO_4.7H_20$ were used.

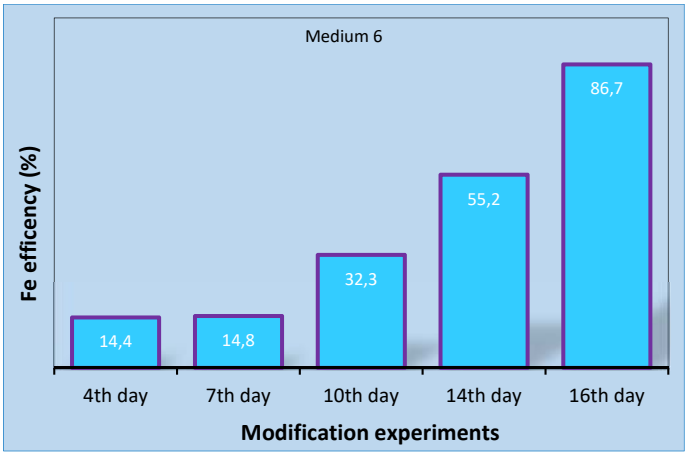

Fig. 7. Fe efficiency (%) in Medium 6

According to the bacterial counts made at the end of the 16th day of the experiments, the number of bacteria growing in the Medium 6 was $2.01x10^6$ bacteria/ml. In contrast, this number was around 1.8-1.9 $x10^5$ bacteria/ml in the other mediums. As it is known, if bacteria grow slowly, high iron precipitation percentages are observed. Bacteria can produce some acids, significantly reducing this iron precipitation.

4. Conclusions

Accordingly, at the end of this study, the following conclusions were reached in general;

- Before pH adjustment in prepared mediums, the highest pH values were measured in the medium where K_2HPO_4 was used. KH_2PO_4, which is used instead of K_2HPO_4, raises the ambient pH less. Thus, it is ensured that the acid consumption to be used for pH adjustment during the bacterial cultivation process is lower.

- The speed of Fe oxidation was obtained the highest from medium-6. At the end of the 16th day, 86.7% of the iron was oxidized in medium 6 using 0.1 g/L KH_2PO_4 and 33 g/L $FeSO_4.7H_20$. In others, this rate is about 40% less. The main reason for this is that the use of large amounts of iron sulfate positively affects the bacterial oxidation rate.

- The best medium for the fastest oxidation and highest growth rate was determined.

- The highest bacterial population was obtained in the Medium 6.

- In light of this knowledge, with the modification process, it was concluded that by using 0.1 g/L KH_2PO_4 instead of 0.5 g/L K_2HPO_4, in 9K medium where Silverman and Lundgren's 9K was used caused a positive impact on oxidation speed and bacteria development and positive results.

- As a result, it has been determined that it has advantages such as low initial pH, (hence rapid growth with low acid consumption) and rapid Fe oxidation of the modified 9K medium according to Silverman and Lundgren's 9K medium.

In light of these results, it was revealed that positive results were obtained on bacterial growth and oxidation rate by using 0.1 g/L KH_2PO_4 instead of 0.5 g/L K_2HPO_4 used in Silverman and Lundgren's 9K medium with the modification process.

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