

## XRF analysis of coal bioleaching by chemolithoheterotrophic *Alicyclobacillus* HRM5 and chemolithoautotrophic *Acidithiobacillus ferrooxidans*

S.S. Etemadzadeh<sup>1</sup>, G. Emtiazi<sup>1,2\*</sup> and Z. Etemadifar<sup>1</sup>

1. Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

2. Department of Biotechnology, Faculty of Biological Science and Technology, Shahid Ashrafi Esfahani University, Isfahan, Iran

Received 10 April 2017; received in revised form 22 October 2017; accepted 18 November 2017

\*Corresponding author: emtiazi@sci.ui.ac.ir (G. Emtiazi).

### Abstract

Most studies on sulfur bioleaching from coal depend on an autotrophic microorganism with a low growth and a long leaching time. For this reason, heterotrophic heat and acidic pH-resistant *Alicyclobacillus* was used as the growing and resting cells for the sulfur and iron removal from coal. The results obtained were analyzed by XRF. The data showed that 26.71% of sulfur was removed by *Alicyclobacillus* in a few days; however, 49.07% of sulfur was removed by *Acidithiobacillus* in 30 days. This was interesting since the leachings of zinc, strontium, titanium, and iron by *Alicyclobacillus*, obtained in a few days, were almost the same as the leachings by *Acidithiobacillus* in 30 days. The results obtained also showed that the *Alicyclobacillus* cells growing at 55 °C removed most of the coal impurities without any change in the carbon content of this fuel. To the best of our knowledge, coal leaching by *Alicyclobacillus* is reported for the first time.

**Keywords:** *Acidithiobacillus Ferrooxidans*, *Alicyclobacillus*, Coal Bioleaching, Heterotroph Bioleaching, XRF.

### 1. Introduction

Inexpensive and readily available coal has revolved it as the single largest fossil energy source used worldwide [1]. Coal plays a key role in electricity generation, and it is the input to most iron and steel production and cement units [2]. Due to these factors, coal is expected to be used as the energy source for at least the next two to three decades [3]. The full utilization of coal as a resource is limited by the presence of high levels of ash and sulfur in its major deposits [2]. Coal is a mixture of heterogeneous compounds such as silicon, sulfur, iron, potassium, calcium, strontium, copper, and zinc, and has been demonstrated by the X-ray fluorescence spectrometer analysis of coal and ash from coal, which causes an increase in the amount of ash [4]. Ash is the residue generated by coal combustion, and is one of the largest types of industrial waste [5]. Also the direct combustion of coal containing

sulfur results in several environmental problems such as emission of oxides of sulfur, which leads to respiratory illness and acid rain that causes erosion of building materials and corrosion of steel structures [6, 7].

The sulfur content of coals is known to vary widely from 0.5% to 11% [8]. Sulfur present in coal exists as both the inorganic and organic forms. The inorganic form is mostly pyritic sulfur [FeS<sub>2</sub>], and the organic sulfur is aliphatic and aromatic heterocyclic forms such as sulfides, disulfides, thiols, thiophenes, and dibenzothophenes. Although sulfate and elemental sulfur are also observed, their contents are low [9, 10]. However, physical techniques result in an incomplete removal of the coal pyrite, and, on the other hand, the chemical methods are energy intensive and generate secondary waste products [11, 12]. Compared to the conventional physical

and chemical desulfurization methods, biodesulfurization can selectively oxidize organic and inorganic sulfur and even remove the finely disseminated pyrite in a coal matrix [13].

Bioleaching involves a chemical microbial-driven dynamic process of oxidation and dissolution, which converts insoluble metal sulfides into soluble forms [14]. Bio-processing of coal is done for the following aims: (i) coal cleaning—removal of sulfur and other trace elements by microbial processes, (ii) coal conversion—microbial liquification, microbial gasification, and methane production [2]. Mesophilic moderately thermophilic and extremely thermophilic microorganisms exhibit the ability to enhance pyrite oxidation [15, 12]. The most widely used microorganism is the meso-acidophilic bacteria, namely *Acidithiobacillus ferrooxidans* [16]. The mesophilic and moderately thermophilic autotrophic leaching bacteria are distributed among proteobacteria (*Acidithiobacillus*, *Acidiphilium*, *Acidiferrobacter*, *Ferroplasma*); *Nitrospirae* (*Leptospirillum*); firmicutes (*Alicyclobacillus*, *Sulfobacillus*); and actinobacteria (*Ferrimicrobium*, *Acidimicrobium*, *Ferrithrix*) [17-19]. Also leaching within archaea (*Sulfolobales*, extremely thermophilic) and thermoplasmales (*Ferroplasma acidiphilum* and *Ferroplasma acidarmanus*) have been reported [18, 19]. Heterotrophic organisms like *Pseudomonas aeruginosa*, *P. putida*, *Beggiatoa*, and some fungi (like *Aspergillus* sp.) are also able to remove sulfur from coal when they use glucose as the only carbon source, and coal as the only source of sulfur [20, 21].

The object of this work was to investigate the ability of *Alicyclobacillus*, a thermo-acidophilic gram-positive bacterium [22] in coal bioleaching, which has not been studied and compared with *Acidithiobacillus ferrooxidans* for coal biodesulfurization and impurity removal.

## 2. Materials and methods

### 2.1.1. Coal sample

The Iranian coal includes a high sulfur content (reported by various authors). For example, the Mazino coal in central Iran at the Tabas coalfield has a high sulfur content including sulfate sulfur, pyritic, and organic sulfur. The sulfur content of the coal has been obtained to be 6.7%. The major minerals in the Iranian coal has been determined as 70-80% of silica (20); this impurity promotes the transportation price. Also the iron oxides and iron hydroxides are in the Tabas coalfield.

The coal sample used in this work was collected in a clean container from the Zobahan factory in Isfahan, Iran; it is obtained from the Tabas era. This sample was kept in a clean, cool, and dry room for further experiments. Its elemental composition was determined by XRF (X-ray fluorescence is a non-destructive analytical technique used to determine the elemental composition of materials) and reported in Table 1.

### 2.1.2. Microorganism

In this work, *Alicyclobacillus* HRM5 (KM983424.1) [22] was used for bioleaching of coal, and was compared with the common biodesulfurization bacteria *Acidithiobacillus ferrooxidans* (PTCC 1746).

## 2.2. Media and culture

### 2.2.1. Malt Extract Agar (MEA)

MER, obtained from Merck (Germany), being an ingredient of malt extract and nutrient (42 g/L), was used and autoclaved at 121 °C for 15 minutes.

### 2.2.2. Modified Basal Salt Medium (BSM)

100 mL of a sulfur-free medium including 2.44 g  $\text{KH}_2\text{PO}_4$ , 14.05 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 2.00 g  $\text{NH}_4\text{Cl}$ , 0.001 g  $\text{CaCl}_2$ , 10 g glucose (or 20 g yeast extract), and 1 g of coal powder were added to 250-mL flasks, which were autoclaved at 110 °C for 20 minutes (or 121 °C for 15 minutes) [20].

### 2.2.3. 9k Medium

3.00 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.10 g KCl, 0.50 g  $\text{K}_2\text{HPO}_4$ , 0.50 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.01 g  $\text{Ca}(\text{NO}_3)_2$  were added to 1 L of distilled water, the solution pH was adjusted by  $\text{H}_2\text{SO}_4$  10 N (pH = 3.0), and the solution was then autoclaved at 121 °C for 15 minutes. At last, 44.22 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was added to this medium in sterile conditions [23, 12].

### 2.2.4. Modified autotrophing medium for bioleaching (CI medium)

The CI medium included 1.10 g  $\text{NH}_4\text{Cl}$ , 0.10 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 3.00 g  $\text{KH}_2\text{PO}_4$ , and 0.14 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . The solution pH was adjusted by HCl (pH = 3.0) [24]. 15 g of coal powder was added, and the mixture was autoclaved at 121 °C for 15 minutes.

## 2.3. Analytical methods

### 2.3.1. Assay of sulfur in supernatant solution

Sulfate concentrations can be determined by barium chloride ( $\text{BaCl}_2$ ). In this turbidimetric procedure, sulfate ions in an acidic medium are precipitated by barium chloride to form barium sulfate. The resulting suspension was measured using a photometer at 420 nm or a turbidity-meter [25].

At the determinate time, the microbial culture was centrifuged at 3000 rpm for 10 min, then 80  $\mu$ L of barium chloride (100 g/L) was added 2 mL of the supernatant solution, whose pH was adjusted. Then the absorbance of the solution was measured at 420 nm.

### 2.3.2. Assay of released iron in supernatant solution

Presence of  $\text{Fe}^{3+}$  (ferric) ions is determinate by adding thiocyanate ions ( $\text{SCN}^-$ ). These react with the  $\text{Fe}^{3+}$  ions to form a blood-red colored complex that can be monitored by a spectrophotometer at 490 nm [26].

At the determinate time, the microbial culture was centrifuged at 3000 rpm for 10 min, then 1 mL of the supernatant solution was placed in a clean test tube, to which was added 1 mL of the thiocyanate solution. After 2 minutes, the absorbance of the solution was measured by a photometer at 490 nm.

### 2.4. Inoculation of bacteria to coal medium for bioleaching

*At.ferrooxidans* was cultured on a solid 9k medium and incubated at 30 °C. After 12 days, the colonies of bacteria were harvested and washed three times with sterile deionized water. The biomass of bacterium ( $\sim$  2 g DCW) was inoculated to a 250-mL Erlenmeyer flask containing 100 mL of the modified bioleaching medium (Cl medium) (without any iron and sulfur sources and containing 15 g coal), and then incubated at 30 °C and 180 rpm for 30 days. After the incubation time, the biotreated coal used for studying and measuring the exact amount of elements was analyzed by XRF.

Coal bioleaching by *Alicyclobacillus* HRM5 was done in both the resting cell and growing cell state, and also with different carbon sources (glucose and yeast extract) and different temperatures.

*Alicyclobacillus* was cultured on malt extract agar and incubated at 40-42 °C. After 48 hours, colonies of bacteria were harvested in sterile deionized water and washed three times. In the resting cell state, 5 mL of the suspension ( $\text{OD}_{600} \sim$  8.0-8.2) was inoculated to a 250 mL Erlenmeyer flask containing 100 mL of the modified basal salt medium (without carbon and iron sources containing 1 g coal; pH = 4, adjusted with HCl) and then incubated at 30, 44, 55 °C and 180 rpm for 14 days. In the bioleaching time, variation in pH, oxidation–reduction potential (Eh), and release of iron and sulfur were measured in the supernatant solution at the determinate time.

In the growing cell state, 2 mL of the suspension ( $\text{OD}_{600} \sim$  0.5) was inoculated to a 250 mL Erlenmeyer flask containing 100 mL modified BSM with 1 g coal and different carbon sources (glucose and pH = 3 or yeast extract and pH = 4, adjusted by HCl); also there was no iron in the bioleaching medium. Afterwards all of them were incubated at 30, 44, 55 °C and 180 rpm for 14 days. In the bioleaching time, variation in pH, oxidation–reduction potential (Eh), and release of iron and sulfur were measured in the supernatant solution at the determinate time. Eventually, after comparing all the results obtained for *Alicyclobacillus*, the best results were selected for the elements analyzed by XRF.

### 2.5. Bacterial removal from coal

The coal samples were separated by a centrifuge (3000 rpm for 10 minutes), and then put in NaOH 1 N for 2 hours. After washing by distilled water, the coal samples were dried at 80 °C.

## 3. Results and discussion

*At.ferrooxidans* is an acidophilic, mesophilic, and chemoautotrophic bacterium that obtains its energy from oxidation of the reduced sulfur compounds such as metal sulfides (MS) and ferrous ion. It can oxidize ferrous irons to ferric irons and gain metabolic energy in the process [12, 27]. This bacterium is commonly used for inorganic sulfur removal from coal, although it does not have the ability to eliminate organic sulfur.

*Alicyclobacillus* is a spore forming leaching bacterium belonging to Firmicutes [19], which was first isolated in 1967 from hot springs, and some have been characterized as mixo- and heterotrophic acidophiles able to oxidize iron and sulfur compounds [14]. This genus was initially characterized as strict anaerobes but it also includes aerobes, facultative anaerobes, and aerobes, found in moderate-to-high temperature habitats such as geothermal hot springs [14, 28].

Biodesulfurization is a clean alternative method used to remove sulfur from coals, where microbes can catalyze the biochemical reaction in an aqueous medium [29], and *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* are the bacteria frequently used for the removal of inorganic sulfur compounds in coal [12]. Recently, bioleaching of other trace elements have been studied by heterotrophic bacteria like *Pseudomonas mendocina* and *Bacillus* species, and fungi such as the *Aspergillus* and *Penicillium* species [30].

Here, we used a serotype of *Alicyclobacillus* (HRM5) for bioleaching coal by oxidizing sulfur and other metals. The results obtained confirmed that *Alicyclobacillus* could bioleach iron, sulfur, and other metals. Also the results obtained for bioleaching by *Alicyclobacillus* were compared with those for a typical bioleaching bacterium, *At.ferrooxidans*.

X-ray fluorescence spectrometry is an instrumental method that was used for the analysis of major, minor, and trace elements in the virgin and biotreated coal samples. The amount of elements in virgin coal are shown in Table 1. As shown in this table, the virgin coal has impurities of silica, sulfur, and iron by 30.40, 19.98, and 16.10%, respectively.

**Table 1. Amount of impurities in virgin coal by XRF analysis.**

Compound	Concentration (%W/W)
Si	30.40
Fe	19.98
S	16.10
Al	13.00
K	7.40
Ca	6.35
Ti	2.22
P	1.45
Cl	0.840
Sr	0.76
Na	0.55
Mg	0.430
Cu	0.360
Zn	0.160
<b>Total</b>	<b>100</b>

### 3.1. Coal bioleaching by *Alicyclobacillus* HRM5

Different studies have demonstrated that *Alicyclobacillus*, a mesophilic or moderate thermophile microorganism, is suitable for coal biodesulfurization [14, 31].

The figures were plotted using Excel in both the resting and growing states for different temperatures and carbon sources. After comparison, it was found that the highest amount of bioleaching by *Alicyclobacillus* HRM5 endospore forming bacteria had been placed in the growing state with glucose, as the carbon source, at 55 °C (Figures 1 and 2). Figure 1a illustrates the growth curves for the *Alicyclobacillus* HRM5 bacteria, which after 72 h entered the logarithmic phase, and the stationary phase occurred after 9 days. The comparison of leachings by the microscopic diagram showed that the virgin coal

particles were more black, and contained larger particles compared to the leaching one (Figure 3). The pH value of the solution in a given bioleaching operation was determined by the balance between the acid-producing and acid-consuming reactions. At the beginning of the bioleaching reaction, the pH value changed slightly to alkaline but after 72 h, consistent with bacterial growth phase, pH was reduced (Figure 1b). During leaching of sulfur, sulfuric acid was produced and the pH of the medium was changed to acidic.

The rate of oxidation–reduction potential (ORP) is related to the amount of ions such as  $Fe^{+3}$  and  $Fe^{+2}$  in the solution, and alteration in the amount of ions due to the change in the rate of Eh. ORP increased from 9.5 to 20.7, while these changes were low and irregular at the beginning of bioleaching (Figure 1c).

Studying Figure 1d (iron assay) and Figure 1e (sulfur assay) showed that leachings of iron and sulfur were almost the same and both of them were variable at the end of incubation.

### 3.2. Result of coal bio-demineralization by XRF analyzer

The coal sample (coal bioleaching with *Alicyclobacillus* HRM5) was analyzed by XRF, and the exact amount of elements was determined (Table 2). This result was compared with the amount of elements in the virgin coal (Table 1). The XRF results demonstrate that leaching of sulfur was 26.71% and iron was 31.93, while assaying the released iron did not locate iron elimination. Also bioleaching of other elements like strontium (64.34%), copper (67.22%), zinc (57.5%), potassium (37.43%), calcium (72.13%), titanium (25.68%), phosphorus (24.83%), and chlorine (71.43%) occurred, causing ash reduction. The data was obtained by comparison of %W/W of the elements in the treated and untreated samples. In the leaching reactor, the organism dissolved some compounds but since Si was not soluble, in the XRF analyses, %W/W increased for this element.

According to Singh et al., there are toxic trace elements in coal like Cd, Cu, Cr, Ni, Pb, and Zn, which can be removed by mixed bacterial consortium [32]. Suarez et al. used XRF for analyzing coal and ash from coal, and demonstrated the major, minor, and trace elements in both of them [4].

Removal of the elements like Si, Pb, Cu, Mg, and Al from coal has been done by the fungi *Aspergillus* species [20, 33, 34].

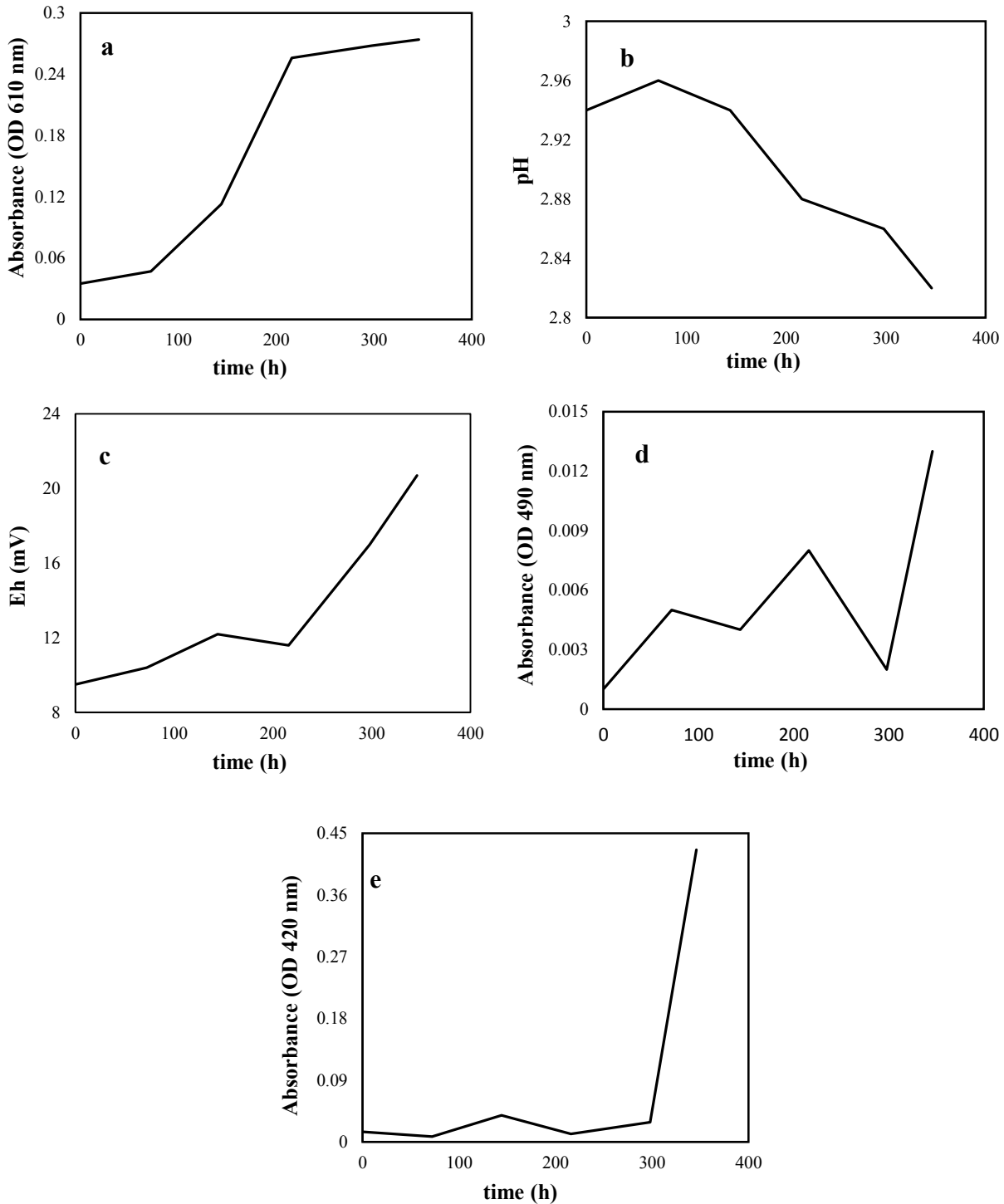


Figure 1. Charts of coal bioleaching by *Alicyclobacillus* HRM5 in growing state with glucose, as the carbon source, at 30 °C in 14 days. a Growth amount of *Alicyclobacillus* HRM5 in bioleaching medium. b Variation in pH by *Alicyclobacillus* HRM5 in bioleaching medium. c Variation in Eh by *Alicyclobacillus* HRM5 in bioleaching medium. d Iron leaching from coal by *Alicyclobacillus* HRM5 in bioleaching medium. e Sulfur leaching from coal by *Alicyclobacillus* HRM5 in bioleaching medium.



Figure 2. Endospore in *Alicyclobacillus* (*Alicyclobacillus sendaiensis* [000.000.659] | BACTERIA | Pinterest | Sendai and Microbiology).

Table 2. Amount of impurities in bio-demineralization coal by *Alicyclobacillus* HRM5 after 14 days by XRF analysis.

Compound	Concentration (%W/W)
Si	44.193
Al	18.50
Fe	13.60
S	11.80
K	4.63
Ca	1.77
Ti	1.65
Na	1.60
P	1.09
Mg	0.470
Sr	0.271
Cl	0.240
Cu	0.118
Zn	0.068
<b>Total</b>	<b>100</b>

XRF analyzes of coal treated by *At.ferrooxidans* after 30 days incubation showed sulfur and iron removal, respectively, to be 49.07% and 30.73% (Table 2). Leaching of other elements like strontium (70%), copper (68.89%), potassium (6.49%), calcium (53.7%), titanium (37.39%), and chlorine (47.61%) was done. Also zinc and sodium were eliminated completely.

Table 3. Amount of impurities in bio-demineralization coal by *At.ferrooxidans* after 30 days by XRF analysis.

Compound	Concentration (%W/W)
Si	44.00
Al	17.91
Fe	13.84
S	8.20
K	6.92
P	3.54
Ca	2.94
Ti	1.39
Mg	0.48
Cl	0.44
Sr	0.228
Cu	0.112
<b>Total</b>	<b>100</b>

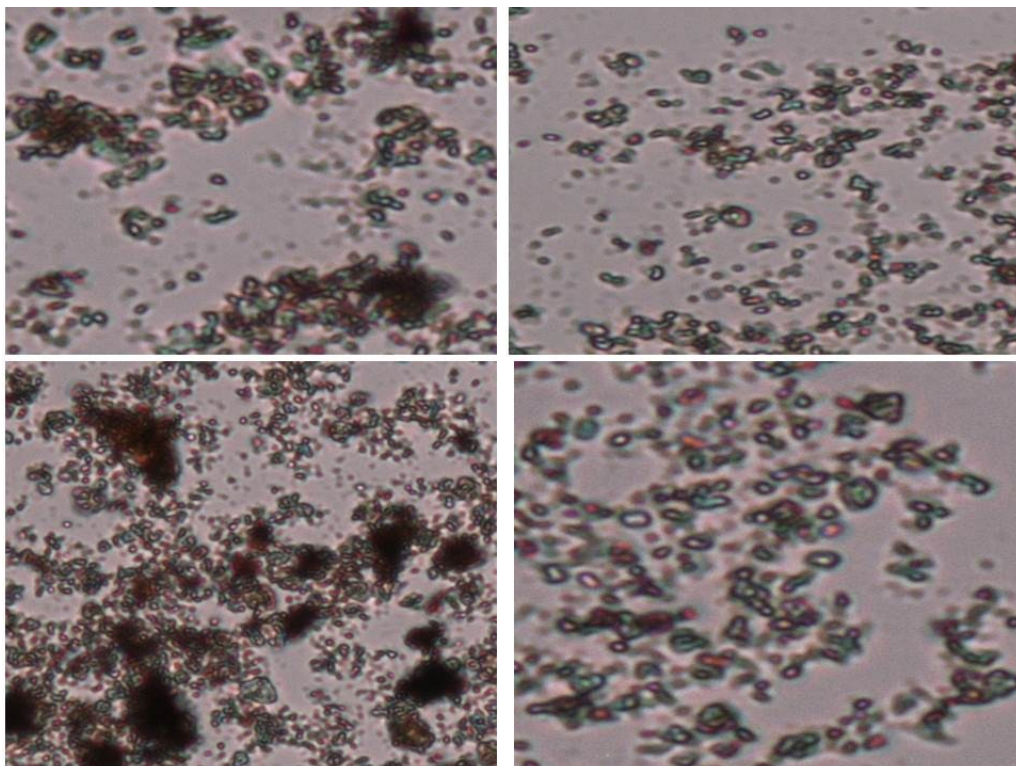


Figure 3. Comparison of microscopic diagrams of control (left) and coal leaching (right).

#### 4. Conclusions

In this work, bioleaching was done by *Alicyclobacillus* HRM5 for the first time. The removal of sulfur was 26.71%, although it was less than biodesulfurization by *At.ferrooxidans* (49.07%) in our experiments. but by leaching in 14 days, removal of iron with *Alicyclobacillus* HRM5 was wonderfully more than *At.ferrooxidans*.

Also *Alicyclobacillus* HRM5 could remove elements such as strontium (64.34%), copper (67.22%), zinc (57.5%), potassium (37.43%), calcium (72.13%), titanium (25.68%), phosphorus (24.83%), and chlorine (71.43%) thus causing ash reduction.

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## آنالیز XRF نمونه‌های زغال سنگ آبشویی شده توسط باکتری کمولیتوتروف آلیسکلوباسیلوس HRM5 و باکتری کمولیتوتروف اسیدی تیوباسیلوس فروکسیدانس

شکوفه سادات اعتمادزاده<sup>۱</sup>، گیتی امتیازی<sup>۱\*</sup> و زهرا اعتمادی فر<sup>۱</sup>

۱- گروه زیست‌شناسی، دانشکده علوم، دانشگاه اصفهان، ایران

۲- گروه بیوتکنولوژی، دانشکده علوم و فناوری زیست‌شناسی، دانشگاه شهید اشرفی اصفهان، ایران

ارسال ۲۰۱۷/۴/۱۰، پذیرش ۲۰۱۷/۱۱/۱۸

\* نویسنده مسئول مکاتبات: emtiaz@sci.ui.ac.ir

### چکیده:

تاکنون بیشترین مطالعات انجام شده پیرامون حذف زیستی گوگرد از زغال سنگ با استفاده از میکروارگانیسم‌های اتوتروف بوده که رشد آهسته داشته و بنابراین نیاز به زمان طولانی برای فرآیند بیولیچینگ بوده است. در این مطالعه برای اولین بار، توانایی باکتری مقاوم به گرما و اسید، آلیسکلوباسیلوس، در دو حالت رشد (growing cells) و استراحت (resting cells) برای حذف آهن و گوگرد از زغال سنگ مورد آزمایش قرار گرفت و نتایج به دست آمده توسط آنالیز XRF تأیید شدند. نتایج نشان‌دهنده حذف ۲۶/۱٪ گوگرد توسط آلیسکلوباسیلوس در مدت چند روز بوده، در حالی که حذف گوگرد به میزان ۴۹/۰۷٪ توسط باکتری اتوتروف اسیدی تیوباسیلوس فروکسیدانس در مدت ۳۰ روز انجام شده است. علاوه بر آن آبشویی روی، تیتانیوم، استرانسیوم و آهن نیز توسط آلیسکلوباسیلوس انجام شده که تقریباً با میزان آبشویی توسط اسیدی تیوباسیلوس در مدت ۳۰ روز مشابه بوده است. همچنین مشخص شد که حالت رشد و دمای ۵۵ درجه سانتی‌گراد بهترین شرایط آبشویی زغال سنگ توسط آلیسکلوباسیلوس است.

**کلمات کلیدی:** اسیدی تیوباسیلوس فروکسیدانس، آلیسکلوباسیلوس، آبشویی زغال سنگ، بیولیچینگ هتروتروفی، XRF.