

# Surface alteration of realgar ( $\text{As}_4\text{S}_4$ ) by *Acidithiobacillus ferrooxidans*

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**Summary.** The chemical and physical characteristics of realgar (an arsenic sulfide mineral that occurs in several crystalline forms) in the presence of *Acidithiobacillus ferrooxidans* BY-3 were investigated in this work. Grains of the mineral were incubated for 10, 20, and 30 days with *A. ferrooxidans* cultured in 9K medium at 30 °C and at 150 rpm agitation. Abiotic control experiments were conducted in identical solutions. The effect of bioleaching on the surface properties of realgar was characterized by scanning electron microscopy (SEM), energy-dispersive spectroscopy (EDS), inductively coupled plasma atomic emission spectroscope (ICP-AES), X-ray diffraction (XRD), and Raman spectroscopy. SEM and EDS analyses confirmed the ability of *A. ferrooxidans* to modify surfaces of realgar and to efficiently enhance its dissolution. ICP-AES showed the dissolution and precipitation of realgar during bioleaching. Based on the XRD pattern and the Raman spectra, the decrease in arsenic in the liquid phase was due to co-precipitation of the mineral with Fe(III) or Fe(III) compounds (e.g., jarosite or goethite). Thus, not only did Fe(III) alter the surface of realgar, but it also promoted its dissolution during bioleaching. [*Int Microbiol* 2012; 15(1):9-15]

**Keywords:** *Acidithiobacillus ferrooxidans* · realgar (arsenic sulfide) · bioleaching · Raman spectroscopy · X-ray diffraction

## Introduction

Realgar is a red, semiconductor, arsenic sulfide mineral that exists in several crystalline forms. It is often found in association with orpiment, another arsenic sulfide. Natural realgar occurs in a low-temperature phase termed  $\alpha\text{-As}_4\text{S}_4$ . A high-temperature polymorph,  $\beta\text{-As}_4\text{S}_4$ , can be obtained by heating realgar above 252 °C. Both forms are based on the same

cage-like molecule even though they differ in their molecular packing, which involves two distinct monoclinic lattices [4]. Throughout history, realgar has had many applications in the manufacture of fireworks and leather, in pesticides, and as a medicinal agent [16]. Recently, realgar was reported to be clinically effective in the treatment of various forms of cancer both in vitro and in vivo [3,20,27].

The preparation of realgar is achieved using various traditional and modern methods, such as acid extraction, calcination, membrane dialysis, mechanical milling, cryo-grinding, and the chemical synthesis of quantum dots [1,21]. However, realgar is poorly soluble in aqueous and most organic solvents due to its high intrinsic lattice energy. Consequently, these technologies are often expensive and

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environmentally deleterious; moreover, their success may be hindered by ineffective dissolution and storage difficulties [7]. Additional problems are the high toxicity and poor bioavailability of realgar, which have seriously limited its use in the clinical setting.

Bioleaching, a long-standing technology in hydrometallurgy, has recently been applied to prepare realgar, with the aim of increasing its bioavailability [7]. In the case of realgar production, the first report of biological precipitation was that of Huber et al. in 2000 [6,12]. Those authors described bioleaching of the mineral by the hyperthermophile *Picrobacterium arsenaticum*. Under organotrophic conditions and in the presence of thiosulfate and arsenate, *P. arsenaticum* forms realgar [5]. By taking advantage of an arsenic-resistant strain of indigenous *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) BY-3, we applied this bioleaching method to develop a bio-arsenic aqueous solution from coarse realgar and confirmed its in vitro and in vivo anticancer activities [26].

*Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*) is a mesophilic and acidophilic chemolithotrophic bacterium and the most well-studied acidophilic organism [8]. Due to its bioleaching capabilities, it is an important member of microbial consortia involved in the iron cycle. In fact, *A. ferrooxidans*, *Acidiphilium* spp., and *Leptospirillum* spp. were reported to account for 80% of the prokaryotic diversity in the Río Tinto ecosystem (Huelva, Spain), where ferric iron and sulfates are abundant [9,10,19]. Even though arsenic can be toxic to *A. ferrooxidans*, the bacterium can be adapted to tolerate much higher arsenic concentrations than it does in nature [Mandi C (2003) The effects of arsenic on *Thiobacillus ferrooxidans*. Columbia University, Master Thesis], with some strains resistant to arsenic at concentrations in the milligram per liter range [23]. Compared to the traditional above-mentioned methods, the bioleaching of realgar produces extraordinary increases in its solubility and bioavailability while decreasing its toxic effects [3].

Previous investigations revealed that ferrous iron and elemental sulfur exert important effects on metal extraction during the bioleaching of realgar, but the chemical and physical changes that take place at the realgar surface have yet to be thoroughly studied [7]. Several analytical investigations of the inorganic alteration process of realgar surfaces have provided information about their oxidation kinetics and the light-induced degradation of realgar as well as electrochemi-

cal effects and the surface properties of realgar nanoparticles in the absence of bacteria [2,14,15,22]. These studies have improved our understanding of realgar alterations in the presence of air, water, acidic, neutral, and other abiotic surroundings. To date, however, surface analytical techniques, such as Raman spectroscopy, have not been applied in the examination of realgar after its reaction with *A. ferrooxidans*. In a previous study, the reactivity of realgar with iron-oxidizing bacteria was monitored by scanning electron microscopy (SEM) and energy dispersive spectrometric (EDS) [17].

The aim of this study was to observe the chemical and physical changes occurring at powdered realgar surfaces in the presence of the bacterium *A. ferrooxidans*. The long-term goal is to achieve the necessary modifications of the mineral that will facilitate its effective use in biotechnological and clinical applications. Accordingly, change in the realgar surfaces were analyzed using several complementary techniques: SEM, EDS, powder X-ray diffraction (XRD), and Raman spectroscopy.

## Materials and methods

**Realgar.** The investigation was carried out using realgar  $As_4S_4$  (99.01 % purity) from Shimen County, Hunan Province, China, which was purified by traditional methods according to the Chinese Pharmacopoeia [Chinese Pharmacopoeia Committee (2010) Pharmacopoeia of the People's Republic of China, China People's Press, Beijing, pp 316]. The raw realgar was crushed to 200 mesh size (approximately  $75 \pm 10 \mu\text{m}$ ) and then analyzed chemically, using an inductively coupled plasma atomic emission spectroscope (ICP-AES, IRIS Advantage, Thermo Jarrell Ash Corporation, USA), and mineralogically by XRD.

**Microorganism and bioleaching experiments.** The native *A. ferrooxidans* strain BY-3 (CCTCC-M203071) was previously isolated from an abandoned copper mine in Baiyin, Gansu Province, China. The bacterium was cultured in 9K medium [18], consisting of (per liter): 3.0 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 g KCl, 0.5 g  $\text{K}_2\text{HPO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g  $\text{Ca}(\text{NO}_3)_2$ . Bioleaching experiments were performed in 250-ml conical flasks containing 100 ml of 9K medium (with 44.69 g of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  per liter) and 0.5 g of realgar at an initial pH of 1.7 (adjusted with sulfuric acid). The flasks were incubated at 30 °C, with shaking at 150 rpm. A sterile flask without bacteria but subjected to identical experimental conditions was included as a negative control. The experiments lasted for 30 days. All experiments were performed in duplicate at a minimum. The average values are reported.

**Analytical procedures.** In bioleaching experiments, soluble arsenic concentrations were measured by ICP-AES [7]. Surfaces of realgar before and after bioleaching were coated with gold and observed with a JEOL scanning electron microscope (JSM-5600LV, Tokyo, Japan) operated at 20 kV and a JEOL field emission scanning electron microscope (FESEM; JSM-

6701F, Tokyo, Japan) operated at 5–8 kV and using image slave software for image capture. The ratio of arsenic to sulfur was analyzed by EDS (Thermo KeveX) at a JSM-5600LV workstation. Sample preparation for SEM analysis was previously described [17]. XRD analyses of the powder form of the raw sample and residual bioleaching were conducted on a multipurpose X-ray diffraction system (X'Pert-Pro MPD, Philips), using Cu K $\alpha$  radiation ( $\lambda = 0.15406$  nm) and operated at 40 kV and 40 mA. The sample preparation method for XRD was previously described [23]. Raman spectroscopy measurements were carried out with a high-resolution Raman spectrometer (Horiba-Jobin, Yvon, HR-800) using a He–Ne laser ( $\lambda = 532$  nm) as the excitation source.

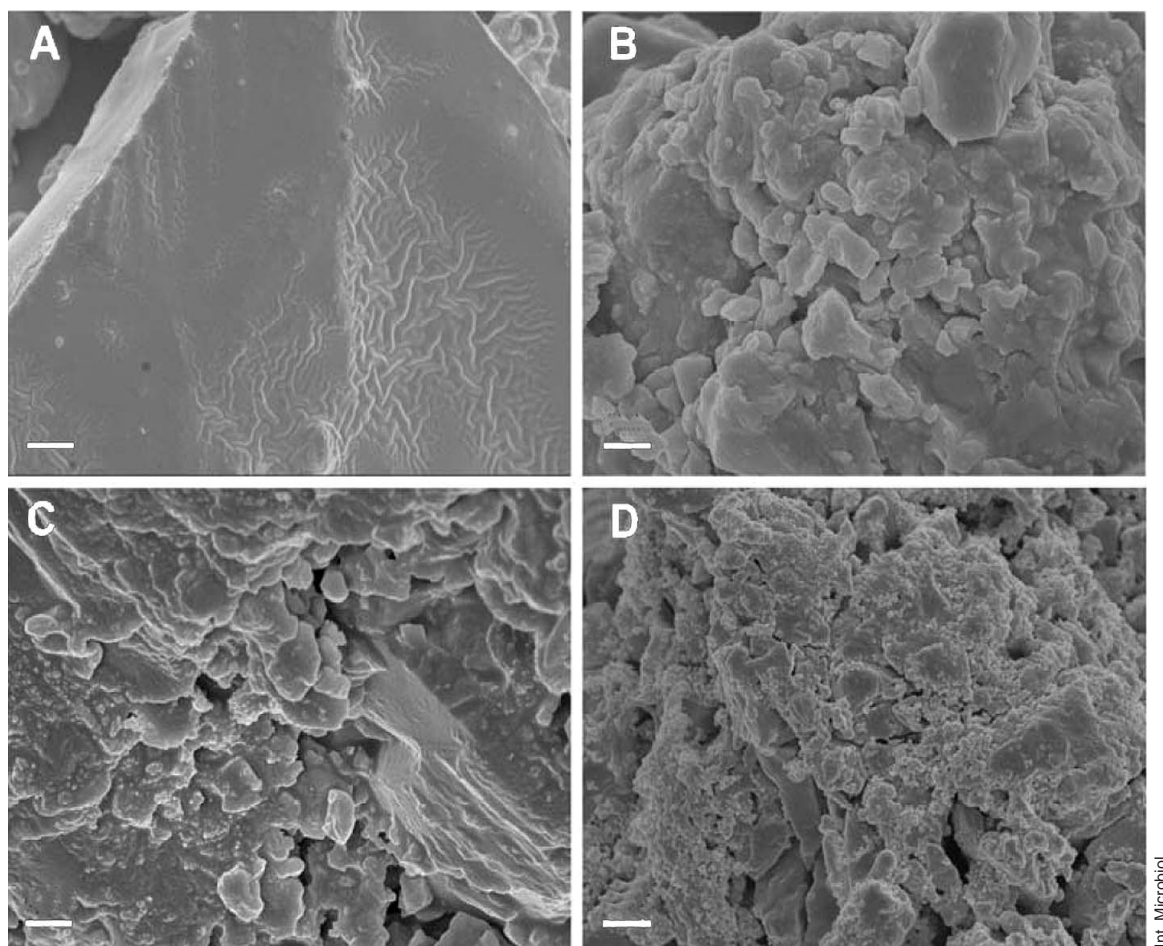
## Results and Discussion

The studied sample mostly consisted of realgar (97 %, w/w), with very small inclusions of arsenolite (3 %, w/w). Chemical analysis of this sample showed that it contained the following elements (expressed as %, w/w): As (68.0), S (31.01),

Ca (0.01057), Fe (0.04015), Mg (0.00293), Hg (0.00323), K (0.00412), Se (0.00598), Al (0.00662), Cd (0.00004), Zn (0.00092), Cu (0.00013), and Ba (0.00028).

The interactions between realgar powder and cultures of *A. ferrooxidans* BY-3 in the presence of ferrous iron were analyzed by SEM and EDS, with the results presented in Fig. 1 and Table 1, respectively. Realgar samples subjected to bioleaching by *A. ferrooxidans* BY-3 for 0 (non-treated), 10, 20, and 30 days, are shown in Fig. 1. During the first 10 days of the experiment, no significant changes in the raw realgar powder were observed (Fig. 1B). However, after 20 days, as shown in Fig. 1C,D, larger cracks or pits appeared on the the mineral surface. Previous investigations suggested that the attachment of *A. ferrooxidans* to the realgar surface is the key step in the bioleaching process [7].

The combined actions of attached bacteria and free bacteria were determined to increase the rate of realgar dissolu-



**Fig. 1.** Scanning electron microscopy (SEM) image of the surface of realgar reacted with *Acidithiobacillus ferrooxidans*. (A) Before leaching; raw realgar powder. (B) After 10 days. (C) After 20 days. (D) After 30 days. Scale bar = 1  $\mu$ m.

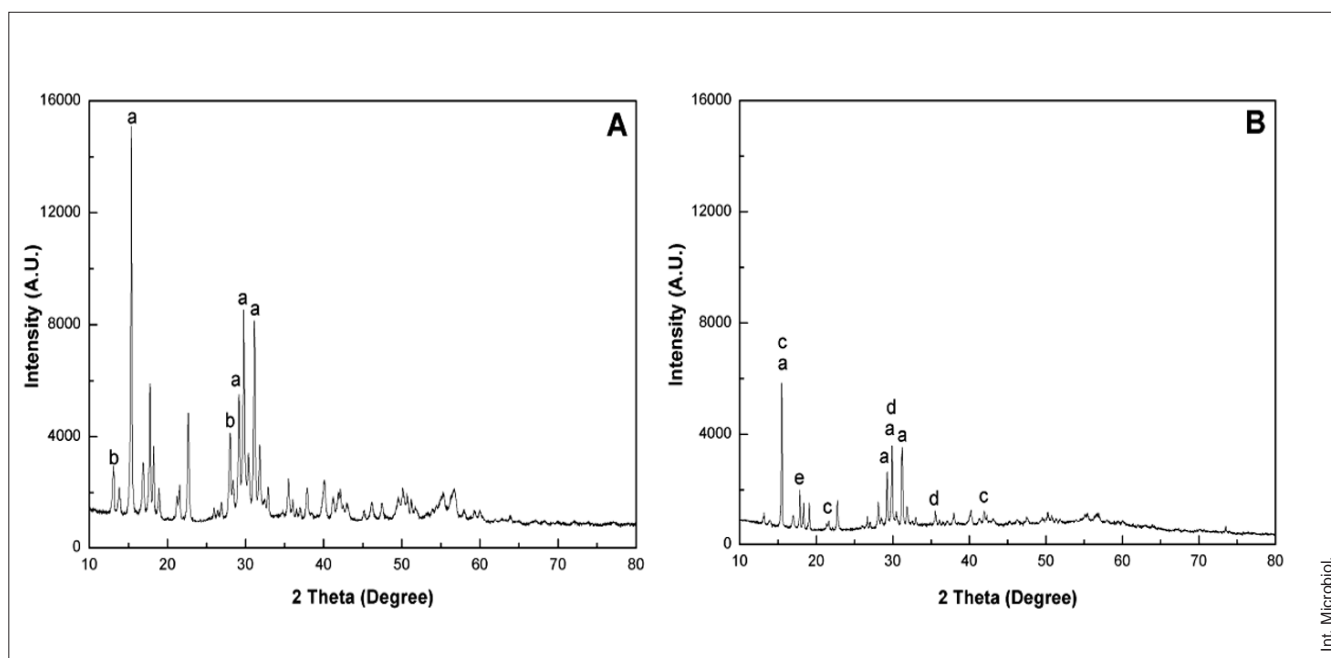
**Table 1.** The EDS analysis of the arsenic and sulfur ratio of realgar

Sample	Time (days)	Element	Weight conc. (%)	Atom conc. (%)	Ratio As/S	Stoichiometric As/S
Before bioleaching	0	As	50.44	30.34	0.44	$As_{0.44}S_{0.56}$
		S	49.56	69.66		
After bioleaching	20	As	48.95	29.10	0.41	$As_{0.41}S_{0.59}$
		S	51.05	70.90		
	30	As	46.85	27.39	0.38	$As_{0.38}S_{0.62}$
		S	53.15	72.61		

tion, confirming that direct bacterial action plays a major role in the modification of realgar surfaces.

As evidenced by EDS analysis, the major constituents of bioleached realgar were As, S, Fe, K, Mg, and Ca. This result was consistent with those of the XRD analysis, which revealed that magnetite and jarosite were the major Fe(III) compounds, while they were not found in the non-treated realgar. According to the EDS results, the ratio of arsenic to sulfur was 0.44:0.56 at the beginning of the experiment; 0.41:0.59 on day 20, and 0.38:0.62 on day 30. The ratios on the latter two days were lower than the stoichiometric ratio,

as described in Table 1. This is in agreement with previous studies showing that the proportion of soluble arsenic gradually increases in the bioleaching process [25]. On day 20, the ratio of arsenic to sulfur decreased to a greater extent in the bioleaching realgar than in the non-treated realgar. Thus, bioleaching not only leads to a more rapid extraction of arsenic, it also causes a relatively severe attack on the realgar surface compared to non-treated realgar. The stoichiometric ratio gradually decreased from 0.41:0.59 on day 20 to 0.38:0.62 on day 30. This decrease in the ratio of arsenic to sulfur was accompanied by an increase in the concentrations



**Fig. 2.** X-ray diffraction (XRD) of: (A) solid realgar; (B) residual realgar after bioleaching by *Acidithiobacillus ferrooxidans* cultures in a medium containing ferrous sulfate. (a) realgar, (b) arsenolite, (c) dimorphite, (d) magnetite, (e) jarosite.

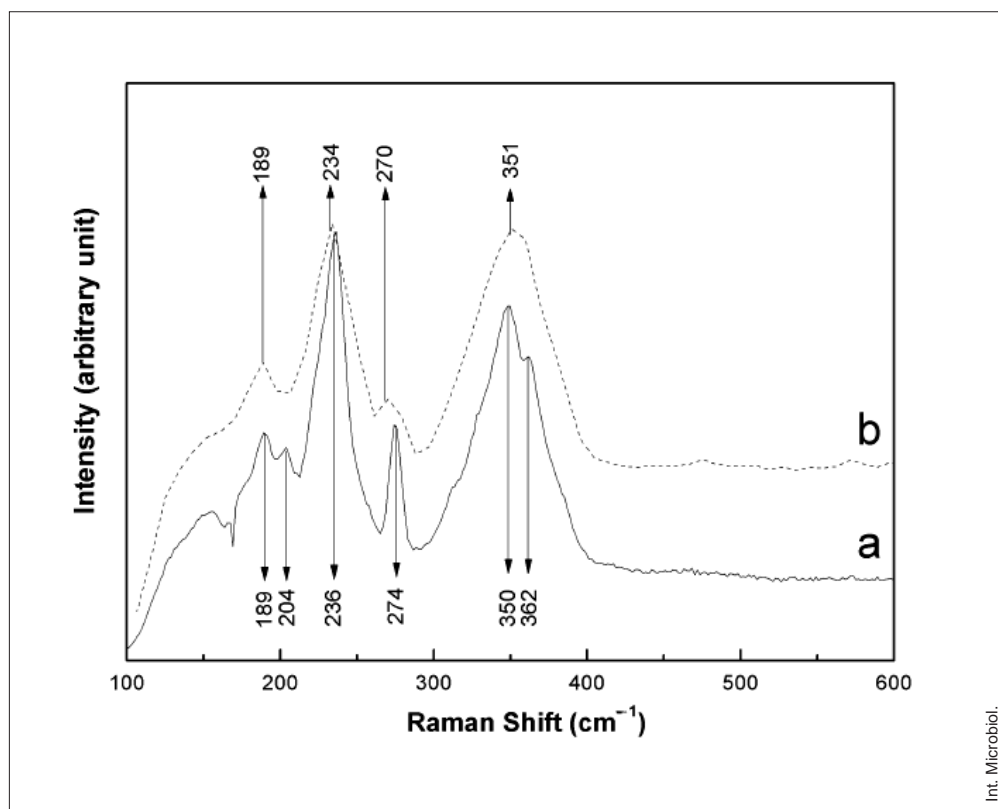


Fig. 3. Raman spectra: (a) realgar powder; (b) residual realgar after bioleaching.

of soluble arsenic in the leach liquor. The results demonstrated that *A. ferrooxidans* can efficiently enhance the dissolution of realgar.

The changes in arsenic concentration over time in the presence of ferrous iron were also analyzed. In the presence of *A. ferrooxidans* (121.95 mg/l), the maximum level was reached on day 15 and was 7.4 times higher than in the sterile control (16.44 mg arsenic/l). Afterwards, however, the arsenic content began to decrease such that by day 25 it was even lower than in the sterile control. In order to understand these variations in arsenic concentrations, the XRD patterns and Raman spectra of the solid residues were evaluated.

Figure 2 shows the XRD patterns of the realgar particles before and after bioleaching. The many overlapping peaks were accompanied by a tendency toward gradual amorphization as a consequence of microbial action. All identified peaks belonged to different phases. Based on a comparison with the Inorganic Crystal Structure Database (ICSD) data, two phases in the studied raw realgar sample could be ascribed to this form:  $As_4S_4$  (97 %) and  $As_2O_3$  (3 %). In con-

trast, the mineral composition found in the solid residues after bioleaching comprised realgar, dimorphite, magnetite and jarosite, which accounted for 43.0 % (w/w), 23.0 % (w/w), 6.0 % (w/w) and 28.0 % (w/w) of the sample, respectively. These results are consistent with those obtained by SEM and EDS. The most widely used adsorbents are those that are iron-based (e.g., jarosite or magnetite), due to their high arsenic removal efficiency [11]. After 30 days, the presence of jarosite and magnetite was confirmed by the XRD patterns of the solid residues in the bioleaching experiment. However, jarosite was not found in the residue of the culture without *A. ferrooxidans*. The above results suggested that arsenic leached from realgar is adsorbed onto Fe(III) or Fe(III) compounds (including jarosite, goethite, magnetite and hematite), and co-precipitated with Fe(III) extracted from jarosite, as described in [24]. Co-precipitation may have further inhibited the dissolution of realgar. Moreover, previous studies have proposed that the adsorption of As(V) and is competitive, or that ionic As species ( $H_2AsO_4^-$ ) is selectively adsorbed [24]. Consequently, adjustment of the Fe(III)



or Fe(III) compounds is of great importance during the bioleaching of realgar, as it significantly decreases the removal of arsenic from the liquid phase.

The Raman spectrum of raw realgar is presented in Fig. 3a, which shows two As-As stretching vibration peaks at 189  $\text{cm}^{-1}$  and 204  $\text{cm}^{-1}$ , two As-S stretching vibration peaks at 350  $\text{cm}^{-1}$  and 362  $\text{cm}^{-1}$ , and a characteristic As-S-As bending vibration at 274  $\text{cm}^{-1}$ . This spectrum did not change significantly over time, confirming that the material was realgar. The Raman spectrum of realgar modified by bioleaching is shown in Fig. 3b. The relative intensity of the As-As stretching vibration peak at 189  $\text{cm}^{-1}$  is increased; in addition, there are two As-S stretching vibration peaks at 270  $\text{cm}^{-1}$  and 351  $\text{cm}^{-1}$  and a characteristic As-S-As bending vibration at 234  $\text{cm}^{-1}$ . According to Kyono [13], the Raman spectrum of para-realgar is characterized by a pair of strong peaks near 230  $\text{cm}^{-1}$  and a grouping of four distinguishable peaks centered at approximately 340  $\text{cm}^{-1}$ . These phase characteristics are not seen in the spectrum of Fig. 3b. Instead, there was a strong increase in the dissolution of realgar, which retained its original characteristics after bioleaching without alterations in its physicochemical properties.

This study shows that *A. ferrooxidans* is able to modify realgar surfaces and to enhance dissolution of the mineral, as detected by SEM and EDS. Additionally, the ICP-AES results indicated that both dissolution and precipitation occurred during the bioleaching of realgar. As determined from XRD and Raman spectral analyses, the bioleaching of realgar by *A. ferrooxidans* is due to the oxidative properties of ferric iron. Therefore, the addition of Fe(III) and Fe(III) compounds can improve the dissolubility of realgar, in addition to decreasing the arsenic concentration in the liquid phase.

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**Competing interests.** None declared.

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