Article

Bioleaching of chalcopyrite by pure and mixed cultures at low temperature

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Abstract: Low-temperature biohydrometallurgy is implicated in metal recovery in alpine mining area, but our knowledge on its mechanism has been limited. To this end, an *Acidithiobacillus ferrivorans* strain and a mixed culture were used for chalcopyrite bioleaching at 6 °C. The passivation of the mineral surface was analyzed using X-ray photoelectron spectroscopy (XPS), the microbial community structure of the mixed culture-mediated system was tested using high-throughput sequencing technology, and the extracellular polymeric substances (EPS) and ferric iron on the microbe-mineral interface were measured. A higher copper extraction rate was achieved using the mixed culture but it did not relieve the passivation of the mineral surface. *Acidithiobacillus* spp. and *Sulfobacillus* spp. were the two major lineages in the mixed culture-mediated system. In the fast-leaching stage, more EPS and ferric iron were extracted in the mixed culture-mediated system. In conclusion, *Sulfobacillus* spp. can relieve the inhibition of organic components to *Acidithiobacillus* spp., maintaining the robustness of the leaching system and more Fe(III) complexed by elevated EPS can enhance chalcopyrite bioleaching by the mixed culture.

Keywords: Chalcopyrite bioleaching; low temperature; microbial community; passivation; extracellular polymeric substances; ferric iron

1. Introduction

Bioleaching as a green biotechnology offers an alternative to traditional pyrometallurgical methods for extraction of valuable metals from sulfide minerals [1,2]. Studies on bioleaching have been extensively carried out using mesophilic, moderate thermophilic and extremely thermophilic acidophiles [3-6]. On the contrary, bioleaching at low temperature was only discussed in several documents. It was reported that the microbially mediated dissolution of sulfide minerals can happen at temperature as low as 0 °C [7]. It was also found that chalcopyrite leaching rate at 4 °C was higher using the cold-adapted *Acidithiobacillus ferrivorans* than the mesophilic *A. ferrooxidans* [8].

One of the topics of interest is to obtain a mixed culture in bioleaching of sulfide minerals. It has been shown that using a mixed culture achieves a higher metal extraction rate than using a pure culture [9,10]. On mesophilic and/or moderate thermophilic conditions, *A. ferrooxidans*, *A. thiooxidans*, *Leptospirillum* spp., *Sulfobacillus* spp., *A. caldus* and *Ferroplasma* spp. were frequently detected in mixed cultures [11,12]. Nonetheless, it was observed that the copper recovery was comparable using the cold-adapted *A. ferrivorans* strain SS3 and the T7 mixed culture [13]. Bacterial community analysis revealed the mixed culture was dominated by *A. ferrivorans* [8,13]. Similar result was obtained by Halinen et al. [14].

Bioleaching is a complex process, which involves a series of (bio)chemical reactions and most reactions happen at the microbes-mineral interface [15]. Microorganisms in bioleaching cultures can secrete extracellular polymeric substances (EPS) and form biofilm at the microbe-mineral interface. Biofilm plays an essential role in the interfacial reactions, which has been the interest of research in recent years [15-18]. It was inferred that EPS has two main functions: mediate bacterial attachment to the mineral surface, and concentrate ferric ions, the major oxidant in chalcopyrite bioleaching [19,20]. To date, EPS formation at low–temperature bioleaching cultures has been described only in few studies. It was observed that cell adhesion at 4 °C was correlated with polysaccharides production [21].

In the present work, an *A. ferrivorans* strain YL15 and a bacterial consortium were respectively used for chalcopyrite bioleaching at low temperature. The microbial community structure in the leaching system by the mixed culture was analyzed. The passivation of mineral surface and the accumulation of EPS and ferric iron on the cell-mineral interface in the two systems were investigated. The data were used to explain the enhancement of chalcopyrite bioleaching by the mixed culture at low temperature. The study would provide new knowledge into the mechanisms of chalcopyrite bioleaching at low temperature.

2. Materials and methods

2.1 Mineral

Chalcopyrite sample was obtained from Guangzhou in Guangdong province of China. XRD analysis showed that the sample contains 97.83% chalcopyrite and 2.17%. The sample consists of 33.1% copper, 28.7% iron and 35.4% sulfur. Sample was ground and sieved to obtain fractional sizes ≤ 75 μm and sterilized by UV irradiation for 24 h in an aseptic room.

2.2 Cultures

Acidithiobacillus ferrivorans strain YL15 was isolated from acidic water (acid mine drainage pond) of Yulong copper mine in Tibet of China [22]. The strain was routinely cultivated in iron-free 9K medium [23] with chalcopyrite at a pulp density of 3% as a sole energy source at 6 °C. Besides the acidic mine drainage pond sample, another 4 water samples were collected from different sites: an acidic tailing pool, two tailing dumps and an effusion pool. Each sample (including the acidic mine drainage pond sample) was firstly filtered through qualitative filter paper to remove solid particles and then filtered through a 0.22 μm polyethersulfone membrane. After that, microbial cells were washed down and collected by centrifugation at 10,000 x *g* for 10 min. The harvested cells were combined together and then cultivated using chalcopyrite as a sole energy source at 6 °C. The obtained mixed culture was used as a seed in our lab for chalcopyrite bioleaching at low temperature.

2.3 Bioleaching experiments

Bioleaching of chalcopyrite was conducted in 500 mL shake flasks. Chalcopyrite at a pulp density of 3% and iron-free 9K medium (250 mL) were added into each flask. The pure culture of strain YL15 or the mixed culture was centrifuged at 10,000 x *g* for 10 min at 4 °C to harvest cells. The collected cells were washed using aseptic acidified water (pH2.0) for twice and then resuspended with 10 mL 9K medium. Cells were added to the flasks and adjusted to obtain an initial cell density of approximately 2×10^7 cells/mL. The flasks were operated at 160 rpm and the temperature was 6 °C. Bioleaching experiments were carried out in two groups: M-15 and MHJ. Chalcopyrite-grown cells of strain YL15 and the mix culture were inoculated to groups M-15 and MHJ, respectively. An abiotic control was also conducted. All the bioleaching experiments were carried out in triplicate.

Samples were withdrawn at regular intervals to determine the oxidation reduction potential (ORP), pH values and metal ion concentrations. Concentrations of iron and copper were measured using the method as described previously [23]. The pH value was measured with pHS-3E acid meter (LEICI, Shanghai) and ORP (Ag/AgCl) value was assayed against a platinum electrode.

2.4 X-ray photoelectron spectroscopy (XPS) analysis

Ore residues were withdrawn at different stages of bioleaching. Sample preparation and XPS analysis has been described in a previous document [23]. In brief, ore samples before or after bioleaching were filtered, washed and dried in vacuum. XPS spectra of the samples were recorded at constant pass energy of 20 eV and 0.1 eV/step using Al Ka X-ray source. Binding energies were referred to the C 1s level at 284.8 eV. Peaks of S 2p were fitted using Savitsky-Golay model and Smart method (in Avantage 5.52) for line smoothing and for obtaining the background of spectra, respectively. The ratio of each sulfur species was calculated based on the area of each peak [23].

2.5 Sequencing of prokaryotic 16S rRNA gene sequences

Bioleaching samples of group MHJ were withdrawn at different time points. Sessile cells were detached from mineral surface by vigorous vortex for 10 min in the presence of 1 gram of glass beads. The obtained samples contained both free and detached cells and were centrifuged at 2,500 x *g* for 5 min to remove the ore residues. The obtained supernatants were centrifuged at 10,000 x *g*, 4 °C for 10

min to collect total cells. Genomic DNA was extracted as previously described [24]. The integrity of DNA was checked on an agarose gel by ethidium bromide staining. Concentration of DNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, USA).

Detailed procedures for amplification and sequencing of prokaryotic V4 hypervariable region of 16S rRNA gene have been described in a previous document [25]. Briefly, sequences of V4 region of prokaryotic 16S rRNA gene were amplified using the universal primers 515F and 806R [26] linked with barcodes, adapter, a pad and a linker of two bases. The purified PCR products were employed for library construction. The MiSeq 500 kit was used for sequencing (2 x 250 bp paired-ends) on the MiSeq sequencing platform (Illumina, USA).

Raw sequences were split to samples based on their barcodes. The raw sequencing data was deposited at Sequence Read Archive under the accession number SRP133342. After sequence trimming, reads assembly and chimeras sequences checking, operational taxonomic unit (OTU) clustering was performed using UPARSE at a 97% similarity level. Thereafter, taxonomic affiliation of each sequence was analyzed using RDP Classifier against the SILVA 16S rRNA database at a 70% threshold [27].

2.6 Extraction of EPS and ferric iron on the mineral surface

The extraction of EPS from bioleaching system was performed as described in our previous study [28]. To confirm that cells were not damaged during extraction, concentration of glucose 6-phosphate dehydrogenase (G6PDH) in the extracts was measured [28]. The low content of G6PDH indicated the extracts were not contaminated by a significant amount of intracellular components. Contents of polysaccharides, proteins and eDNAs of EPS were determined as previously reported [15]. The EPS-bound iron on the mineral surface was extracted by ultrasonic for 30 °C as described in a previous report [29] and the extraction was performed on ice for 30 min. All experiments were performed in triplicate. Statistical analysis was performed using the Student's *t*-test in R (version 3.6.0, https://www.r-project.org).

3 Results

3.1 Bioleaching of chalcopyrite by the pure and mixed cultures

An *Acidithiobacillus ferrivorans* strain YL15 (group M-15) and a mixed culture (group MHJ) were used for bioleaching of chalcopyrite at 6 °C. As is shown in Figure 1a, pH increased to about 2.5 in the first 4 days in group MHJ, this is due to that protons attacked chalcopyrite and the generation of protons derived from microbial sulfur oxidation is less than the consumption of protons in this stage. After day 12, as microbial sulfur oxidation accelerated, pH started to decrease and achieved ~2.0 on day 24. ORP value in the first 12 days was low, but after that it rocketed up to ~600 mV (Figure 1b). Trends in group M-15 was similar to group MHJ, and the main difference was that pH decreased between days 16-28.

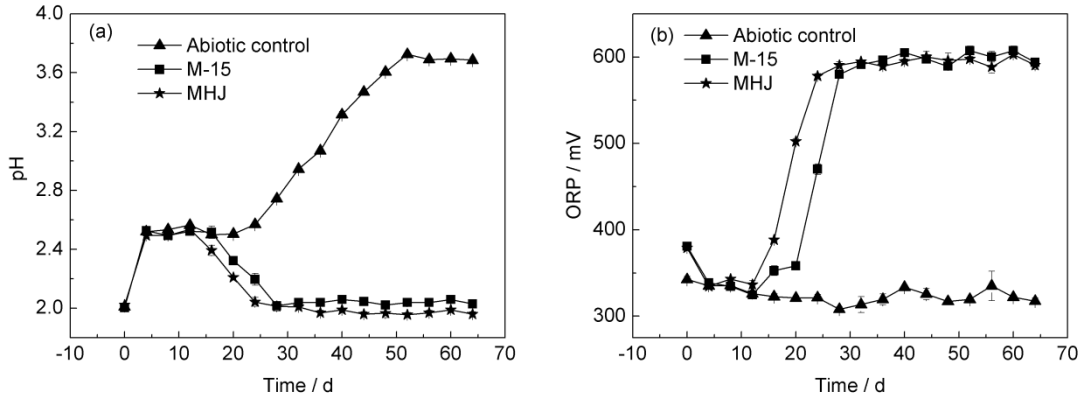

Figure 1. Variations of pH (a) and ORP (b) during chalcopyrite bioleaching.

As illuminated by the Nernst equation:

$$E = E^0 + \frac{RT}{F} \ln \frac{[Fe^{3+}]}{[Fe^{2+}]} \quad [1]$$

The value of ORP is mainly controlled by the ratio of Fe(III) to Fe(II). Variations of Fe(III) and Fe(II) concentrations are shown in Figure 2a and Figure S1, respectively. The ratio of Fe(III) to Fe(II) was low in the early stage. After that, as microbial oxidation of ferrous iron accelerated, concentration of Fe(III) increased, accompanied with a decrease in the concentration of Fe(II). This was in accordance with the low ORP value in the early stage and its rapid rise thereafter (Figure 1b). The copper concentration in groups M-15 and MHJ was much higher than that in the abiotic control (Figure 2b), suggesting that microorganisms greatly enhanced dissolution of chalcopyrite. Concentration of copper increased significantly after day 12 and reached the highest value on days 44 and 48 in MHJ and M-15, respectively. The highest copper concentration in group MHJ was significantly higher than in group M-15 ($p < 0.05$).

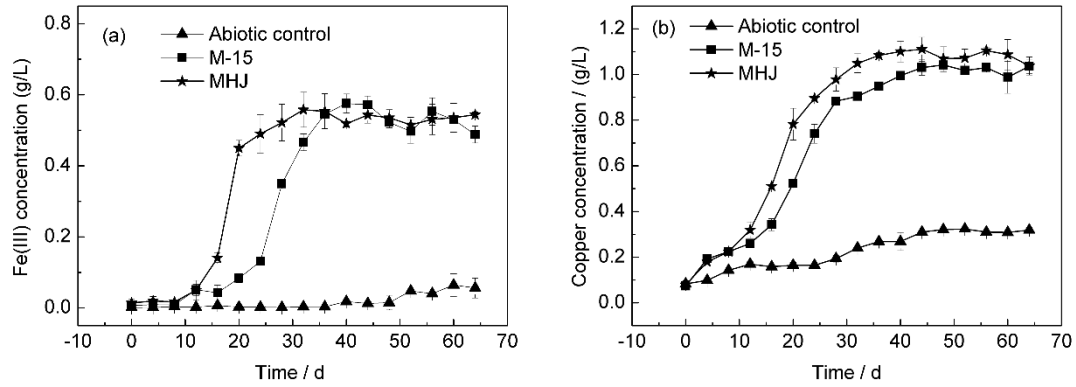

Figure 2. Variations of copper (a) and ferric iron concentrations (b) during chalcopyrite bioleaching.

3.2 XPS analysis

As reported previously, the surface of chalcopyrite would be passivated by sulfur species during bioleaching [30]. XPS is an appropriate tool to detect the intermediate sulfur species on the surface of chalcopyrite [31]. The S $2p_{3/2}$ peaks of chalcopyrite sample before and after bioleaching (day 48 for M-15 and day 40 for MHJ) were analyzed based on binding energy (BE) and full width at half maximum (FWHM). The S 2p spectra of chalcopyrite surface before and after bioleaching were shown

in Figures. 3 and 4. The S 2p spectra for MHJ and M-15 were quite similar (Figure 4). Elemental sulfur (S^0), monosulfide (S^{2-}), disulfide $(S_2^{2})^-$, polysulfide (S_n^{2-}) and sulfate (SO_4^{2-}) were detected on the mineral surface. The content of each component during bioleaching was shown in Figure 5. The content of S^0 was low all the time, ranging from ~6.0% to ~7.5%. Ratios of S_n^{2-} and S^{2-} were higher after bioleaching than in the raw mineral, e.g., percentage of S_n^{2-} was < 10% in the beginning, but increased to > 22.1% and 23.9% after bioleaching in M-15 and MHJ, respectively. On the contrary, the contents of SO_4^{2-} and S_2^{2-} decreased after bioleaching. The ratio of SO_4^{2-} was high (37.6%) before bioleaching, but decreased to 8.5% and 7.2% in groups MHJ and M-15, respectively.

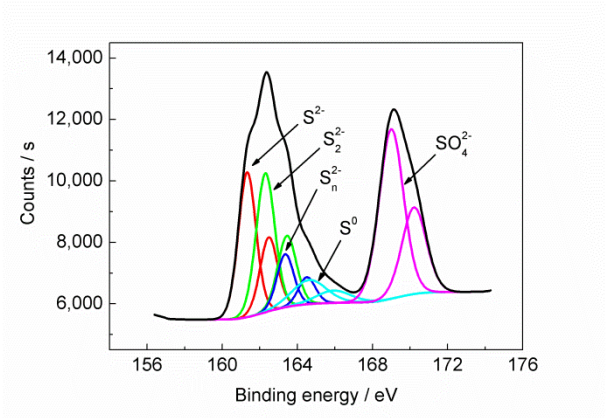

Figure 3. The S 2p spectra of chalcopyrite surface before bioleaching.

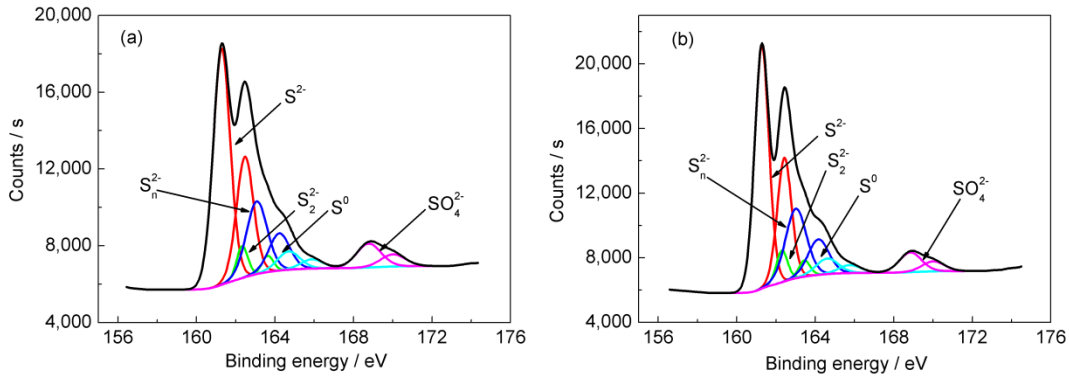

Figure 4. The S 2p spectra of chalcopyrite surface after bioleaching in M-15 (a) and MHJ (b).

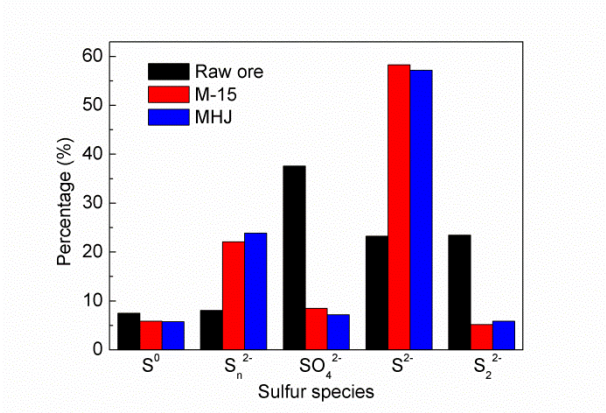

Figure 5. Contents of sulfur species on the mineral surface detected by XPS before and after bioleaching.

3.3 Microbial community dynamics of group MHJ

The microbial community of group MHJ was monitored using high-throughput sequencing of 16S rRNA gene. Samples were withdrawn on days 20, 30 and 50 (designated as MHJ1, MHJ2 and MHJ3). A total of 673,782 pair-end reads were obtained. Rarefaction curves of all samples based on sequencing reads and number of OTUs reached plateaus, indicating that the sequencing depth was appropriate for estimating the microbial diversity.

At phylum level, *Proteobacteria* was the dominate lineage and accounted for 81.5%-94.9% of the total biomass, followed by *Firmicutes* (5.04%-18.39%), while the percentages of other phylum, e.g., *Cyanobacteria*, *Thaumarchaeota* and *Bacteroidetes*, were lower than 1% (Figure 6a). Down to the genus level, a total of 27, 35 and 31 OTUs were detected for MHJ1, MHJ2 and MHJ3, respectively, and 20 OTUs were shared by all samples. *Acidithiobacillus* dominated the microbial communities, accounted for 94.8%, 87.0% and 81.1% in MHJ1, MHJ2 and MHJ3, respectively. Sequences of *Sulfobacillus* (affiliated to *Firmicutes*) were detected at a considerable level and increased from 5.04% on day 20 to > 10% on day 50 as bioleaching continued. Contents of other genera, for instance, *Stenotrophomonas* spp., *Pseudomonas* spp. and *Castellanilla* spp., were < 0.1% (Figure 6b).

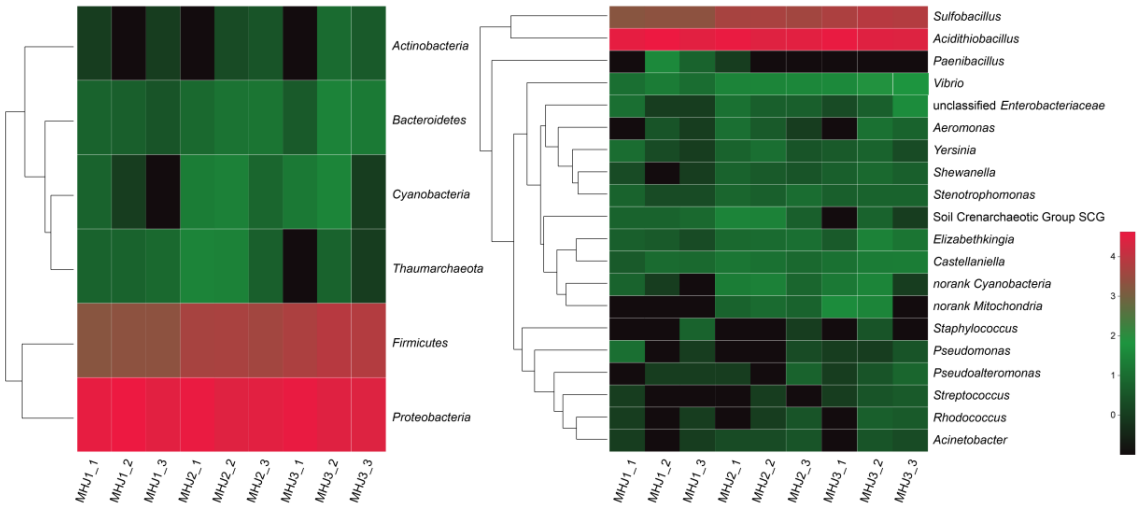

Figure 6. Heatmap of microbial communities on phylum and genus levels of MHJ. MHJ1_1, MHJ1_2 and MHJ1_3, MHJ2_1, MHJ2_2 and MHJ2_3, MHJ3_1, MHJ3_2 and MHJ3_3 were the triplicates of sample MHJ1, MHJ2 and MHJ3, respectively.

3.4 Analysis of EPS components and ferric iron on the mineral surface

The contents of EPS components (proteins, polysaccharides and eDNAs) were analyzed in three stages: adaptive stage, day 8 for M-15 and day 4 for MHJ, early fast-leaching stage, day 18 for M-15 and day 14 for MHJ, middle fast-leaching stage, day 22 for M-15 and day 18 for MHJ, and stationary stage, day 48 for M-15 and day 44 for MHJ. During the adaptive stage, the content of EPS was extremely low, the amounts of proteins and polysaccharides were < 2 mg/10^{10} cells, and eDNAs were below detection limit. During the fast-leaching stage, the content of EPS increased significantly, and all the three components were higher in MHJ than in M-15 ($p < 0.05$). Especially for polysaccharides, the content in MHJ was respectively 22.9% and 27.4% higher than in M-15. In the stationary stage, the amount of

EPS decreased compared with the middle fast-leaching stage, and the content in MHJ was slightly lower than in M-15 (Figure 7a). The content of ferric iron on the mineral surface was higher in MHJ than in M-15 in the fast-leaching stage ($p < 0.05$). For instance, the Fe(III) content in MHJ on day 18 was 25.3% more than in group M-15 on day 22. In the stationary stage, ferric iron on the mineral surface was comparable in the two groups (Figure 7b).

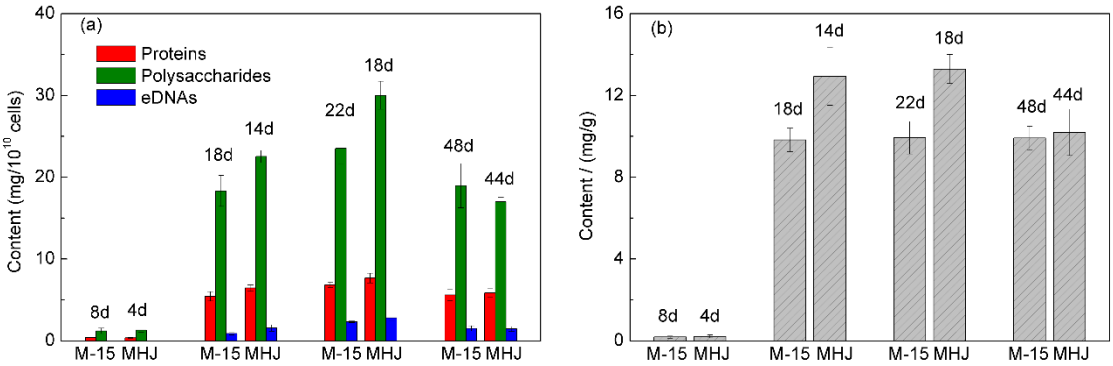

Figure 7. Variations of EPS components and ferric iron on the mineral surface in groups M-15 and MHJ during chalcopyrite bioleaching.

4 Discussion

A pure culture of *A. ferrivorans* strain YL15 and a mixed culture enriched from acid mine drainage in Yulong copper mine were used in chalcopyrite bioleaching (M-15 and MHJ, respectively). The changes of pH, ORP, iron and copper concentrations in both groups were similar. A higher copper extraction rate was achieved in MHJ than in M-15, which is in accordance with previous results that mixed cultures were more efficient in bioleaching of sulfide minerals than pure cultures [9]. To reveal the enhancement mechanisms of mixed culture in bioleaching at low temperature, the microbial community of group MHJ and the passivation substance of the mineral surface were tested, and a comparison of the EPS components and ferric iron at the cell-mineral interface in groups MHJ and M-15 was performed.

Previous work has emphasized the importance of interactions between physiologically distinct acidophilic microorganisms (e.g., autotrophs and heterotrophs) in enhancing sulfide minerals dissolution [32]. To conform this, first of all, the microbial community structure of the mixed culture during bioleaching (MHJ) was monitored. The result revealed an extremely simple bacterial community which consisted of two major lineages: *Acidithiobacillus* and *Sulfobacillus*. In order to find out which species the two lineages belongs to, 16S rRNA gene of sample MHJ3 was amplified using primers 27F and 1492R and the products were transformed to *E. coli* DH5α to build a clone library as described in a previous document [33]. Ten clones were randomly selected, and the inserted fragments were sequenced and aligned using BLAST. It is shown that 9 of the inserted fragments were 100% identical to the 16S rRNA gene sequence of strain YL15 and the rest 1 fragment had a 98% identity to an undescribed *Sulfobacillus sp*. The results unraveled that the *Acidithiobacillus* spp. should be *A. ferrivorans*. The genus *Sulfobacillus* is commonly considered to be moderate thermophilic, although some species can grow at temperatures < 20 °C [34]. However, in a previous report, a mixed culture was enriched at 5 °C with ferrous sulfate as a sole energy and sequences close to *Sb. montserratensis*

were detected at an abundance > 10% [35]. These results indicated that there would be novel *Sulfobacillus* species existing in a low-temperature bioleaching culture.

Sulfobacillus can utilize many organic substances as its carbon source [34]. In group MHJ, the ratio of *Sulfobacillus* increased as bioleaching progressed. This was due to that the accumulated organic substances can promote the growth of *Sulfobacillus*. *Sulfobacillus* can alleviate the inhibition of organic substances to the autotrophic *Acidithiobacillus* and therefore maintaining a more robust microbial community than the pure-culture bioleaching system [36].

It was pointed out in a previous study that using mixed culture can reduce passivation of the mineral surface and therefore enhance bioleaching performance [37]. Therefore, the passivation species on the mineral surface in groups M-15 and MHJ were investigated using XPS. As discussed before, the main component of passivation layer may be jarosite, elemental sulfur or polysulfide [31,38,39]. It was shown that the content of elemental sulfur (S^0) was low during bioleaching, indicating that it was not the main component of passivation layer. The content of SO_4^{2-} (jarosite) was high in the beginning, but decreased significantly in the later stage, suggesting that it should not be the passivation substance. On the contrary, the content of S_n^{2-} (polysulfide) in the raw chalcopyrite was low, but increased greatly after bioleaching. Therefore, polysulfide should be the major component of the passivation layer. However, the S 2p spectra of ore residues in groups MHJ and M-15 were similar and the content of polysulfide was comparable between the two groups (Figure 5). This indicated that the mixed culture did not significantly alleviate the passivation of the mineral surface.

EPS function at the microbe-mineral interface in bioleaching. They are generated by microorganisms and mediate the adsorption of microbes to the mineral surface [40]. Moreover, they can concentrate metallic ions and provide a microenvironment, where the concentrations of metallic ions are higher than in the bioleaching solution [20]. Herein, EPS in different stages of bioleaching in groups M-15 and MHJ were extracted and the contents of the main components were analyzed. It is shown that the contents of polysaccharides, proteins and eDNAs were higher in group MHJ than in M-15 in the fast-leaching stage. Polysaccharides took up for the most part of EPS and change the most (Figure 7a). Some of the compositions in polysaccharides, e.g., the uronic acids, can complex Fe(III) [40]. To this end, the ferric iron on the mineral surface was extracted. The result showed that content of ferric iron was higher in MHJ than M-15 (Figure 7b), which indicated that oxidation of chalcopyrite by ferric iron would be faster in MHJ at the microbe-mineral interface. Therefore, it was hypothesized that more EPS produced in the fast-leaching stage of group MHJ concentrated elevated content of ferric ions and resulted in a higher reactional rate, thereby contributing to a higher copper extraction rate in group MHJ than in group M-15. The study revealed the importance of EPS in bioleaching which was not taken into consideration before when explaining why the mixed culture is more efficient than the pure culture in bioleaching.

5 Conclusions

A pure culture of *A. ferrivorans* strain YL15 and a mixed culture were used in chalcopyrite bioleaching at low temperature. Using the mixed culture resulted in a higher copper leaching rate than using the

pure culture. Microbial community structure analysis unveiled that the mixed culture mainly consisted of *Acidithiobacillus* spp. and *Sulfobacillus* spp. The mixed culture did not significantly changed the sulfur species profile on the mineral surface but may enhance chalcopyrite bioleaching via two pathways: firstly, *Sulfobacillus* spp. can alleviate the toxicity of organic substances to *Acidithiobacillus* spp. and thus sustain a vigorous leaching system; secondly, more EPS secreted by the mixed culture can complex elevated content of ferric iron and accelerate the oxidation rate of chalcopyrite.

Supplementary Material: Figure S1 Variation of ferrous iron concentration during chalcopyrite bioleaching.

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