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The use of microscopy techniques to analyze microbial biofilm of the bio-oxidized chalcopyrite surface

Jiang Lei [a,*], Zhou Huaiyang [b], Peng Xiaotong [b], Ding Zhonghao [a]

[a] Wuhan University of Science and Engineering, Wuhan 430073, China
[b] Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China

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ABSTRACT

This paper deals with the biofilm formed on the surfaces of chalcopyrite during the bio-oxidation process, with microscopy techniques (scanning electron microscope, fluorescence stereo microscope and transmission electron microscope) and in situ chemical analyzes (energy dispersive spectrometer). SEM images showed that this type of structured community of *Acidithiobacillus ferrooxidans* was made up of some bacteria and floccules. Moreover, TEM images indicated that these bacteria were wrapped by EPS. However, almost no EPS can be found in the suspending bacteria in the solution. In addition, large amounts of jarosite and element sulfur were determined in the bio-oxidation process, and the biofilm was covered with the deposition.

1. Introduction

One of the focuses in process engineering studies is bacteria–mineral interaction. The bacteria, including sulfur oxidization bacteria (e.g., *Acidithiobacillus thiooxidans*, *Acidithiobacillus caldus*) and ferrous iron oxidization bacteria (e.g., *Leptospirillum ferrooxidans*, *Ferroplasma acidarmanus*) (Harneit et al., 2006; Edwards et al., 2001), have been extensively examined for their interactions with sulfide minerals. Among them, *Acidithiobacillus ferrooxidans* is highlighted owing to its ability to oxidize Fe^{2+} ions, elemental sulfur, hydrogen (Kai et al., 2007) and hydrogen sulfide (Oprime et al., 2001; Quatrini et al., 2006) in acidic solution.

In the presence of *A. ferrooxidans,* some approaches have been used to improve the leach kinetics. Previous results indicated the positive correlation between oxidation rate of sulfide mineral and dissolved oxygen, concentration of ferric iron, and the number of bacteria on surface of sulfide minerals (Gleisner et al., 2006; Holmes and Crundwell, 2000; Shrihari et al., 1995; Monroy et al., 1995) and further established the rate equation of bio-oxidation on sulfide minerals. In addition to the study on solution chemicals, analyzes of the mineral surface before and after reaction may provide important information on the chemical change of reaction interfaces (Jones et al., 2003). Studies of *A. ferrooxidans* on sulfide surface and surface morphological changes of mineral have attracted attention and biofilm formation is also a well-known phenomena. Biofilms formed on the surface of artificial or natural substances in the photolytic layer of lakes or rivers are complex communities, composed mainly of photoautotrophic (algae) and heterotrophic microorganisms (bacteria, fungi, protozoa). These organisms were embedded in their extracellular polymeric substances (EPS) (Kröpfl et al., 2006). The EPS-matrix was a dynamic system, which filled and formed the space between the cells and it was responsible for the architecture and morphology of the biofilm (Lewandowski et al., 1994). The EPS of *A. ferrooxidans* and other leaching microorganisms mediated the attachment of cells to sulfide minerals. They also played a pivotal role in indirect leaching of base and precious metals via the contact mechanism (Harneit and Sand, 2007). As mentioned before, the bacterial oxidation of chalcopyrite and the chemical characters of biofilms have been studied extensively under different conditions; however, the study of biofilm formation process involving deposition for bioleaching purposes is still incipient. This work's aim is to improve our knowledge on the biofilm formation process during chalcopyrite bacterial oxidative dissolution utilizing microscopy techniques (scanning electron microscope, fluorescence stereo microscope and transmission electron microscope) and in situ chemical analyzes techniques (energy dispersive spectrometer).

2. Experimental

2.1. Preparation of mineral samples

The chalcopyrite samples used for this study were ground in an agate mortar to the size of −80 meshes. The mineral grains were dipped in distilled ethanol for 2 h and then rinsed twice with

* Corresponding author.
E-mail address: andy0920@163.com (J. Lei).

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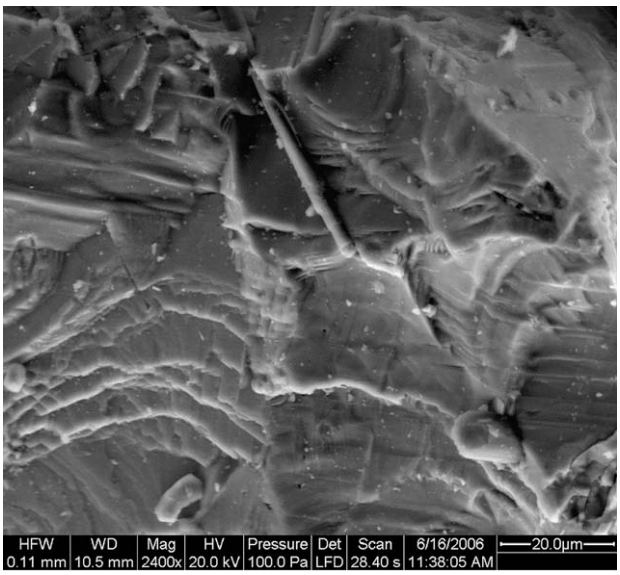

Fig. 1. SEM image of chalcopyrite surface before being oxidized.

deionized water, and dried in a vacuum drying incubator at 40 °C. The SEM images revealed smooth surfaces of minerals, on which no oxidized traces were observed (Fig. 1). Samples were ground further to the size of −200 meshes for chemical analysis, and the results were shown in Table 1. X-ray diffraction analysis revealed minor amounts of pyrite and pyrrhotite as accessory minerals in this sample.

Table 1
Chemical analysis of Chalcopyrite sample

	Concentration (wt%)
SiO_2	7.90
TiO_2	0.18
Al_2O_3	0.89
Fe	27.23
Pb	0.54
Zn	0.92
Cu	23.50
MgO	5.67
CaO	1.03
Na_2O	0.16
S	31.48
Total	99.50

2.2. Bacterial strain and media

A. ferrooxidans was cultured in 9 K medium (Sliverman and Lundgren, 1959) that contained per liter: 3 g $(NH_4)_2SO_4$, 1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.001 g $Ca(NO_3)_2$, and 44.2 g $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 2.0 with H_2SO_4. *A. ferrooxidans* was subcultured thrice before inoculation for experiments. The cultures were filtered through Whatman 17 filter paper to remove the suspended solid material. The cells were then harvested from filtrate by centrifugation (2376*g*) to eliminate residual ferric ion and washed twice with H_2SO_4 solution of pH 2.00. The suspending cells were counted using a haemacytometer. The suspending bacteria concentration for the experiment was 4×10^8 cells/mL.

2.3. Bioleaching experiments

The experiments were conducted in a 250 mL Erlenmeyer flask. The pulp density was 3.8%. Samples were put into 9 K culture medium without Fe^{2+} (Sliverman and Lundgren, 1959), added with *A. ferrooxidans* (inoculated amount of 10%). The flasks were then placed into a 30 °C constant-temperature incubator for 39 days.

2.4. Analytical methods

2.4.1. Reaction product analysis

After the samples were bio-oxidized for 39 days, solid minerals were collected, dried in a vacuum drying incubator at 60 °C, and qualitatively analyzed using a Rigaku D/max-1200 X-ray Diffractometer.

2.4.2. Chemical analysis of mineral particles

Mineral particles were observed using a Dutch FEI Quant 400 environmental scanning electron microscope (ESEM). In a low vacuum mode, the mineral particle samples, without any surface processing (spraying), were directly observed, and chemical components in micro-area of the mineral surface were analyzed using an EDAX Energy Dispersive Spectrometer equipped to the same ESEM.

2.4.3. Microscopic observation through fluorescence staining

After being biologically oxidized, the surface of sulfide mineral was stained with DAPI staining solution. DAPI reagent used in the experiment was manufactured by MDbio Corporation. Stock solution was filtered by a 0.22-μm filter film to remove bacteria, and then prepared as 1 mg/mL with deionized water. Staining solution for subsequent use was prepared by diluting the stock solution to 10 μg/mL. Mineral particles were washed twice, then added to DAPI staining solution; the solution was mixed well with a Vortex mixer, and stored for 15 min at 4 °C, protected from light; finally,

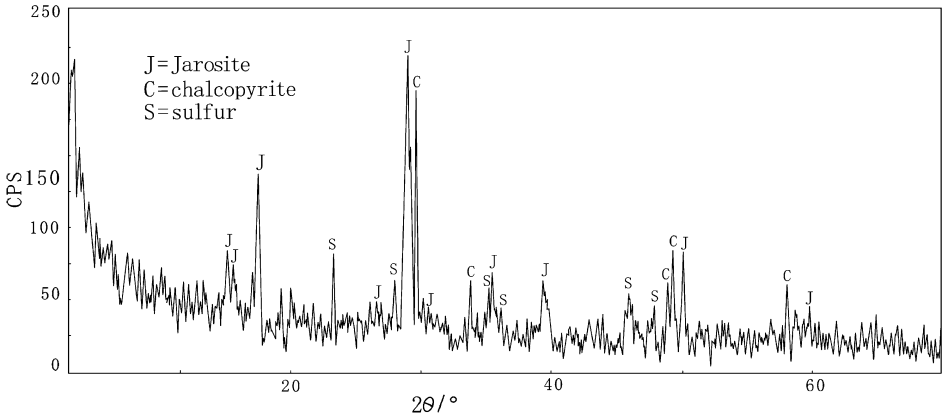

Fig. 2. X-ray diffractometer analysis of solid mineral of chalcopyrite with 39-days-bio-oxidation.

the mineral particles were placed on slide, and dried; bacteria attached to the surfaces of mineral particles were observed through ultraviolet fluorescence using a Leica MZ FLIII fluorescence stereo microscope.

2.4.4. Transmission electron microscope observation of bacteria

The suspending bacteria collected by centrifugation (2376*g*) from the solution with 39-days-bio-oxidation of chalcopyrite were concreted by 0.4% agar solution, and cut into 0.15 cm × 0.45 cm slices. The minerals with 39-days-bio-oxidation of chalcopyrite were processed in the same way. These slices were then fixed with 2% glutaraldehyde for 2 h, and washed by PBS thrice for 10 min each time. Subsequently, samples were immersed in 1% osmic acid at 4 °C for 2 h, and then were stepwise dehydrated in ethanol with concentration of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% for 10 min, respectively. After that, samples were transferred through epichlorohydrin and embedded in epoxy resin. Finally, samples were cut into ultrathin section and dyed with uranyl acetate and lead citrate. The inner structure of bacteria and the relationship of mineral were observed under a JEM-2010HR Transmission Electron Microscope with an accelerating voltage of 100 kV, and chemical components in micro-area were analyzed using an OSFORD INCA energy dispersive spectrometer equipped to the same TEM.

3. Results and discussion

3.1. Reaction solid product identification

In general, the process for *A. ferrooxidans* to oxidize Fe^{2+} to Fe^{3+} was accompanied by deposition of jarosite (Daoud and Karamanev, 2006). The deposition generally existed all through the bio-oxidation process of sulfides. With 3-days-bio-oxidation of chalcopyrite, yellowish deposition appeared in the reaction solution, but the deposition changed to yellow brown after day 6. X-ray diffraction analysis reveals that the deposition phases include jarosite and elemental sulfur (Fig. 2).

3.2. Surface characteristics of chalcopyrite after bio-oxidation

As shown in Fig. 3A, the chalcopyrite surface has been covered by a smooth film but some part has fallen off with-15-days-bio-oxidation. On the spot where the film fell off, honeycomb holes can be found (Fig. 3B). The contour of elliptical holes is quite similar to the shape of *A. ferrooxidans*. The original erosion pits generated under the effect of the attachment bacteria (Rodriguez-Leiva and Tributsch, 1988). So the form of honeycomb may be directly related to the attachment of bacteria. However, almost no bacteria are found on the smooth film through SEM images. Meanwhile, the ultraviolet fluorescent image also shows only several bacteria on the surface of chalcopyrite with 15-days-bio-oxidation (Fig. 4, the blue-fluorescence lightening ones are bacteria. Once the bacteria stained by DAPI, the DNA of the bacteria under ultraviolet fluorescence would lighten blue). An EDS analysis on the film covering the chalcopyrite surface and the part uncovered (Fig. 5) reveals great differences in their elemental compositions. The uncovered chalcopyrite remains as it is, while the elemental sulfur and oxygen of the film have obviously increased, and Fe has reduced and Cu almost completely disappears.

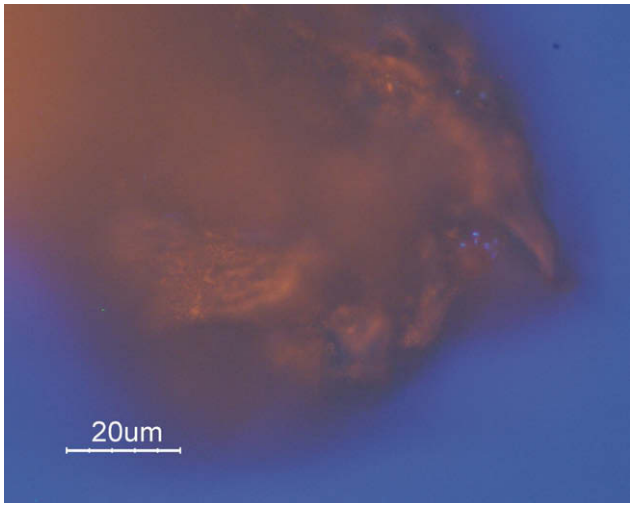

Fig. 4. Fluorescence staining image of chalcopyrite surface with 15-days-bio-oxidation.

As shown in Fig. 6A and B, with 15-days-bio-oxidation of chalcopyrite, some film-like substances exist among the mineral grains. The thickness of such substances is about 2–5 μm, representing the film falling off the grain surface. A large amount of bacteria can be found on the backside of the film (Fig. 6C and D). After 39 days, most of the films covering the chalcopyrite surface have also fallen off and the chalcopyrite surface becomes rough and much holey.

3.3. Evidence of the existence of biofilms on chalcopyrite surfaces

Lilova and Karamanev used the conception "biofilm" in their study of copper sulfide bio-oxidation by *A. ferrooxidans*. Strictly speaking, this "biofilm" was not a real biofilm, but a film on the biofilm reactors (Lilova and Karamanev, 2005). Current definition of a bacterial biofilm is that a structured community of bacterial cells is enclosed in a self-produced polymeric matrix and attaches to an inert or living surface (Costerton et al., 1999). According to

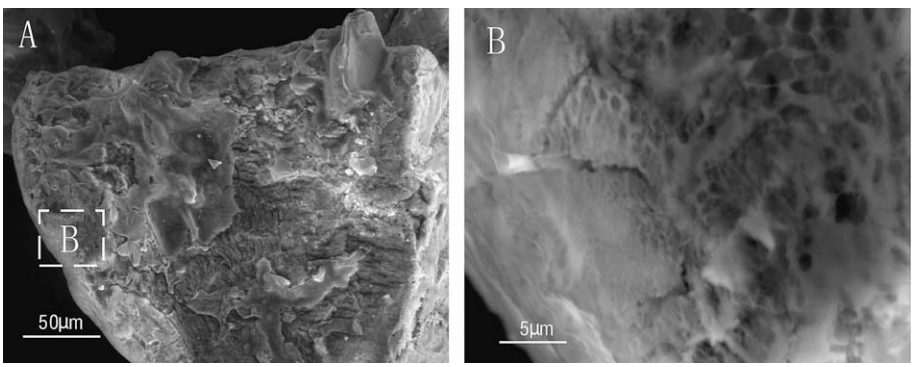

Fig. 3. Surface characteristics of chalcopyrite with 15-days-bio-oxidation.

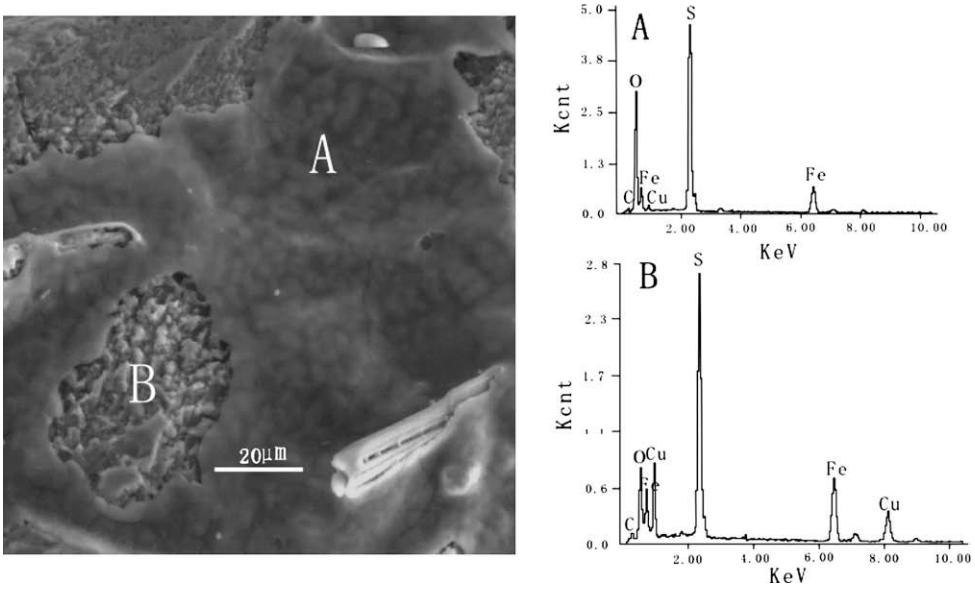

Fig. 5. Energy spectrum analysis of chalcopyrite surface with 15-days-bio-oxidation.

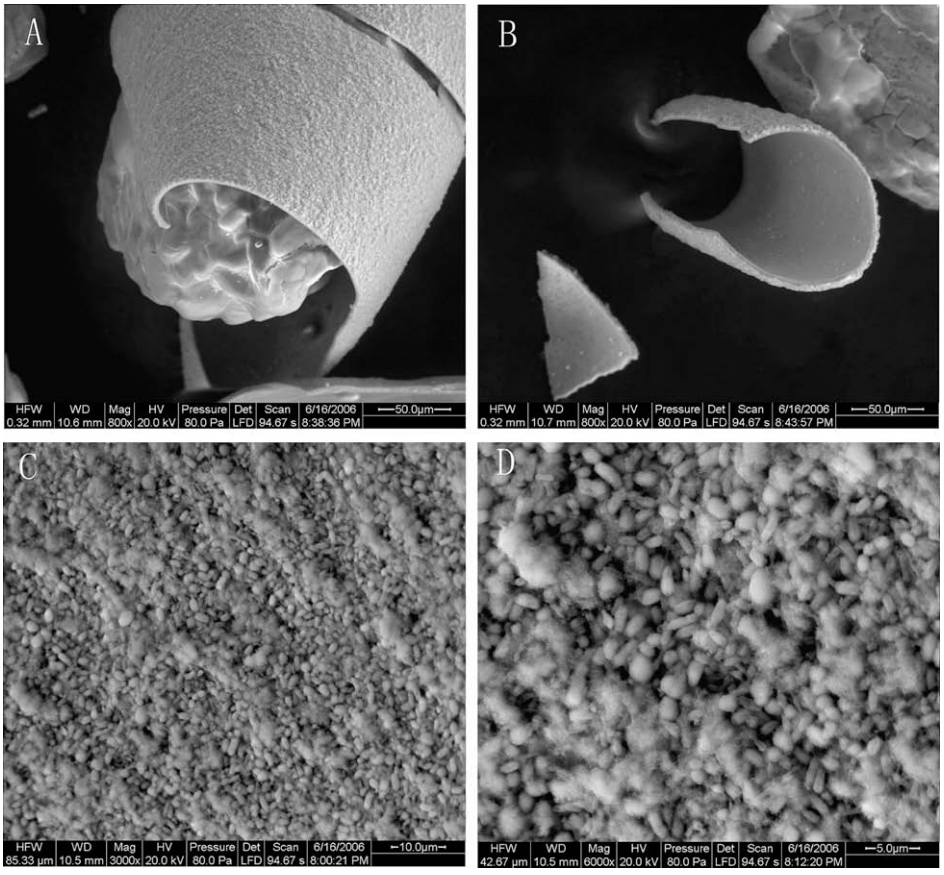

Fig. 6. SEM images of chalcopyrite surface with 15-days-bio-oxidation.

this definition, biofilm should meet at least the following two conditions: (1) bacteria attach to some interface or surface; (2) bacteria are wrapped by the polymeric matrix produced by themselves. Fig. 6C and D show that some bacteria and floccules concentrate on the backside of film. For proving whether it is biofilm formed by *A. ferrooxidans*, further researches are carried out with TEM. The results shown in Fig. 7A and B indicate that the bacterium in the solid mineral formed after bio-oxidation of chalcopyrite is wrapped by EPS. Fig. 8 shows the characteristics of the centrifugally collected suspending bacteria in the solution with 39-days-bio-oxidation. The bacteria take the shape of *Brevibacterium* (the length difference is mainly decided by the angle of slicing). Bacteria contour is in focus and almost no EPS can be found. The synthesis of EPS by cells of *A. ferrooxidans* is strongly influenced by the growth substrate or

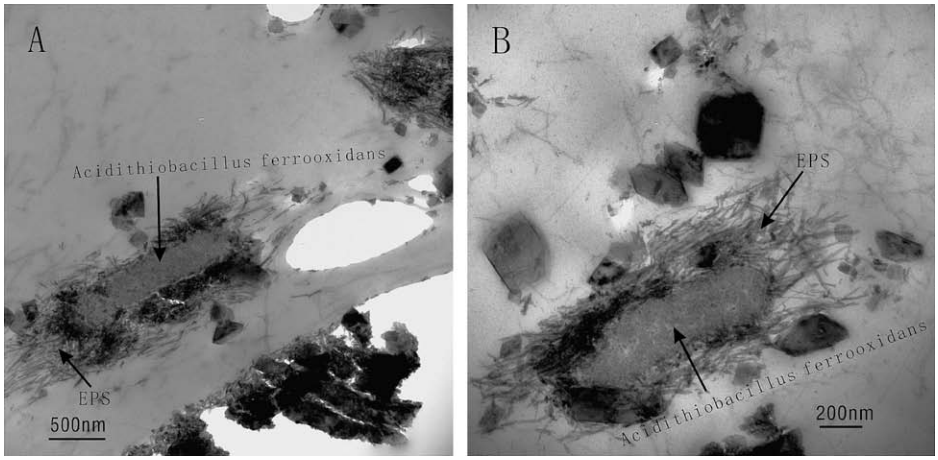

Fig. 7. TEM image of solid minerals of chalcopyrite with 39-days-bio-oxidation.

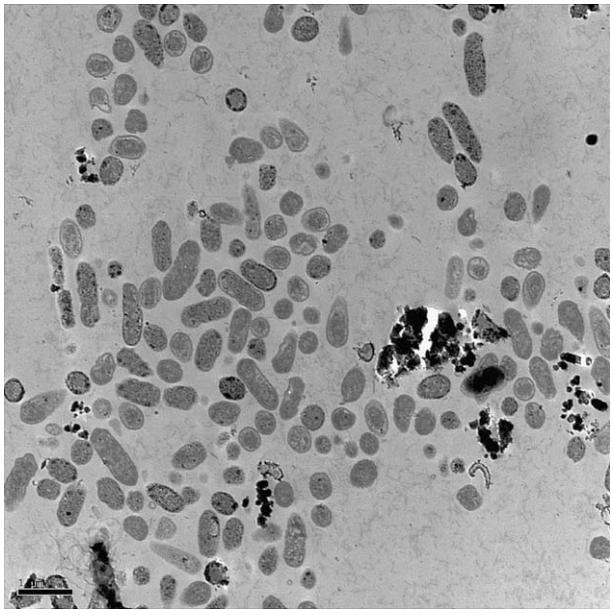

Fig. 8. TEM image of suspension bacteria collected by centrifugation from the solution with 39-days-bio-oxidation of chalcopyrite.

attachment substratum of the cells. Cells growth with soluble Fe^{2+} generally generates less EPS than cells grown with solid chalcopyrite (Harneit and Sand, 2007). Fig. 7 shows that the bacterium is also surrounded by some crystals with side length less than or close to 200 nm, with part of the crystals around the bacteria. Fig. 9 further shows the characteristics of such crystals in large amount. Contrasting the EDS analysis of the vacant area (Fig. 9A) to the crystals (Fig. 9B), we find that the elemental Fe, S and K in the rhombi-structured crystals (Fig. 9B) have obviously increased. And these elements are right those constitute of jarosites. Therefore, with the result of X-ray diffraction analysis (Fig. 2), it can be found that the crystals are the jarosites formed under the effect of *A. ferrooxidans* in the bio-oxidation process of chalcopyrite. Pogliani and Donati suggested a direct relationship existed between jarosite precipitation and the number of attached cells, and the generation of jarosite was mainly affected by pH and temperature (Pogliani and Donati, 2000). Based on the characteristics shown in Figs. 6C and D and 7, it can be confirmed that the community of bacteria and floccules on the backside of film is the biofilm formed by *A. ferrooxidans*.

3.4. The formation process of biofilm

Since the steady deposition of jarosites and element sulfur on the mineral surface, it made the biofilm completely covered by deposition. Thus, biofilm was embedded between mineral surface and deposition. Biofilm was a kind of growth pattern of bacteria when they lived on the surface of substance, reflecting an instinct of bacteria. Theoretically, all kinds of bacteria could form biofilm. Whether or not a kind of bacteria could finally form biofilm was

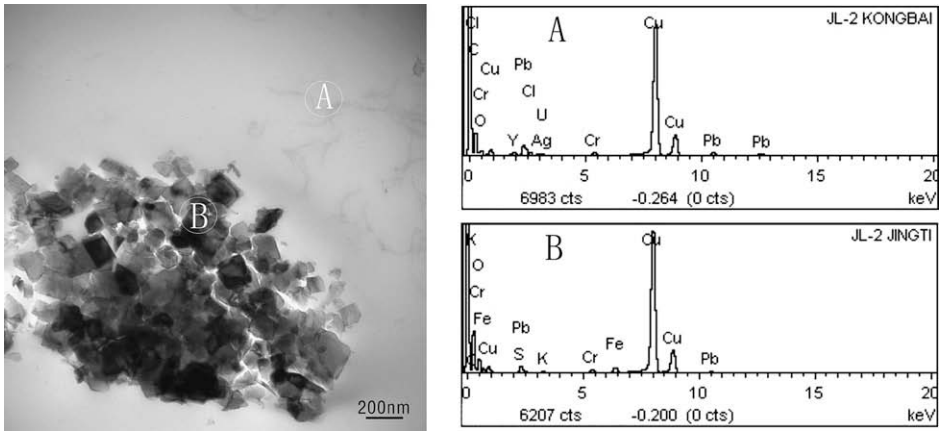

Fig. 9. Energy spectrum analysis of the crystals shown in Fig. 7.

closely linked to various factors of the environment it lived in, e.g., nourishment, temperature, osmotic pressure, pH, Fe ion concentration and redox potential (Li and Zhuang, 2002). There were three stages in the formation of biofilm (Costerton, 1999): (1) the attachment of bacteria to the medium surface; (2) the former bacteria attaching to the surface became firmer under the interaction of the EPS layer produced by the bacteria themselves, and the basic structural unit of biofilm, microcolony with mush-room-like shape, came into being; and (3) the biofilm with highly organizational structure finally occurred. The mature biofilm was heterogenic, and there were many channels around the microcolonies for water transfer, from which nutrient, enzyme and metabolite can be transferred. However, biofilms may have inhibiting effect on metallic surface for corrosion. Their inhibiting effect was generally thought to be caused by oxygen depletion or the formation of passivating layers (Grooteers et al., 2007). Moreover, the biofilm lay between the mineral surface and the deposition that covered the surface of sulfides, even integrating with the upper deposition, so the biofilm may restrain the oxidation of chalcopyrite. Since some films have fallen off from the mineral surface during the bio-oxidation, it is difficult to estimate how the biofilm inhibits or enhances the bioleaching. The generation of biofilm is a dynamic process. Because the bio-oxidation is always in a stirring status, the fall-off of biofilm and upper layer deposition as a whole may be not only caused by physics but also by the death of the attachment bacteria.

4. Conclusions

Microscopy techniques (SEM, TEM and fluorescence stereo microscope) were used to study the character of microbial biofilm during chalcopyrite bio-oxidation. It was apparent that cells would attach to the surface of chalcopyrite during bio-oxidation. However, almost no bacteria were found on the mineral surface through SEM and fluorescence stereo microscope. We deduced the reason was that the attachment bacteria were covered by the large amounts deposition of jarosite and elemental sulfur. Further more, with 15 days oxidation, the chalcopyrite surface was also covered by a smooth film and some part fell off. SEM images further showed that bacteria community existed on the backside of the fallen films. Moreover, TEM images indicated that these bacteria were wrapped by EPS. This study provided the evidence that the community of bacteria and floccules was the biofilm formed by *A. ferrooxidans*, and this biofilm was covered with the deposition generated during the bio-oxidation process.

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