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Investigation of energy gene expressions and community structures of free and attached acidophilic bacteria in chalcopyrite bioleaching

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Abstract In order to better understand the bioleaching mechanism, expression of genes involved in energy conservation and community structure of free and attached acidophilic bacteria in chalcopyrite bioleaching were investigated. Using quantitative real-time PCR, we studied the expression of genes involved in energy conservation in free and attached *Acidithiobacillus ferrooxidans* during bioleaching of chalcopyrite. Sulfur oxidation genes of attached *A. ferrooxidans* were up-regulated while ferrous iron oxidation genes were down-regulated compared with free *A. ferrooxidans* in the solution. The up-regulation may be induced by elemental sulfur on the mineral surface. This conclusion was supported by the results of HPLC analysis. Sulfur-oxidizing *Acidithiobacillus thiooxidans* and ferrous-oxidizing *Leptospirillum ferrooxidans* were the members of the mixed culture in chalcopyrite bioleaching. Study of the community structure of free and attached bacteria showed that *A. thiooxidans* dominated the attached bacteria while *L. ferrooxidans* dominated the free bacteria. With respect to available energy sources during bioleaching of chalcopyrite, sulfur-oxidizers tend to be on the mineral surfaces whereas ferrous iron-oxidizers tend to be suspended in the aqueous phase. Taken together, these results indicate that the main role of attached acidophilic bacteria was to oxidize elemental sulfur and dissolution of chalcopyrite involved chiefly an indirect bioleaching mechanism.

Keywords Chalcopyrite · Bioleaching · Attachment · Energy genes · Community structure

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Introduction

Chalcopyrite is the most abundant copper sulfide mineral. However, obtaining copper from low-grade chalcopyrite with pyrometallurgical methods is cost-ineffective. Bioleaching of low-grade chalcopyrite ores and tailings is a copper extraction process with low capital and operating cost, and also has environmental benefits [5, 23]. Therefore, bioleaching is an emerging technology to recover metal from low-grade chalcopyrite [20]. During the process of bioleaching, bacterial attachment plays an important role in enhancing the bioleaching rate [36]. Many studies have examined the factors that influence attachment, including particle size [30, 31], growth substrate, types of mineral surface [25], as well as extracellular polymeric substances (EPS) [7, 8, 26, 38]. However, little is known about the specific roles of bacteria attached to the mineral surface play in elemental sulfur and ferrous iron oxidization during chalcopyrite bioleaching. In the indirect contact bioleaching mechanism, the role of attached bacteria is to oxidize ferrous ions to ferric ions. Two other bioleaching mechanisms exist. They are the indirect mechanism and the direct contact mechanism. In the indirect mechanism, unattached bacteria oxide ferrous ions in the liquid medium. In the direct contact mechanism, attached bacteria oxidize the mineral directly by biological means without any involvement of ferric or ferrous ions in solution [6, 18, 19,

32]. In chalcopyrite bioleaching, Shrihari et al. thought the leaching initially took place by a predominantly direct contact mechanism and in the later stages by the indirect mechanism [30]. However, whether a direct contact mechanism exists is under discussion [6]. In 2003, Rodríguez et al. [22] concluded in their paper that the bioleaching of chalcopyrite concentrate is a collaborative effort involving indirect contact mechanism and an indirect mechanism. However, the degree to which each mechanism contributes to chalcopyrite bioleaching and the main role of attached bacteria are unknown. Discovering the main energy sources used by free and attached bacteria is of great help in understanding the main role that free and attached bacteria play in bioleaching.

Based on the energy sources used by acidophilic bacteria, leaching microorganisms are divided into ferrous- and sulfur-oxidizing microorganisms, ferrous-oxidizing microorganisms and sulphur-oxidizing microorganisms, therefore pure culture of *Acidithiobacillus ferrooxidans* and mixed culture of sulfur-oxidizing *Acidithiobacillus thiooxidans* and ferrous-oxidizing *Leptospirillum ferrooxidans* were used in this study.

In this paper, expression of genes involved in energy conservation in attached and unattached *A. ferrooxidans* was monitored with quantitative real-time PCR (qPCR). Additionally, the distribution of *A. thiooxidans* and *L. ferrooxidans* in a mixed culture between the surface of chalcopyrite and the liquid phase during bioleaching chalcopyrite was analyzed by denaturing gradient gel electrophoresis (DGGE).

Materials and methods

Ore

Chalcopyrite collected from Dexing Copper Mine (Jiangxi Province, China) was used in all the experiments. The total chemical analysis displayed that the ore had a total copper content of 32.02 % and a total iron content of 30.90 %. The mineralogical composition was 80 % chalcopyrite, 5 % pyrite, 5 % silicate, and others. The chalcopyrite was crushed into a particle size no larger than 75 μm.

Microorganisms, media, and growth conditions

The following strains of *A. ferrooxidans* (ATCC23270), *A. thiooxidans* (DSM622), and *L. ferrooxidans* (DSM2391) were used in this study. *A. ferrooxidans* strain ATCC23270 was purchased from the American Type Culture Collection (ATCC), USA. *A. thiooxidans* strain DSM622 and *L. ferrooxidans* strain DSM2391 were both obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. All experiments were carried out in triplicate 250-ml flasks containing 100 ml of solution at 30 °C with a shaker operating at 170 rpm, at an initial pH 2.0 and a 3 % w/v pulp density. Iron (II) sulfate grown *A. ferrooxidans*, and *L. ferrooxidans* were cultivated in 9 K medium [33], which contained 3.0 g l^{-1} $(NH_4)_2SO_4$, 0.1 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1} K_2HPO_4, 0.01 g l^{-1} $Ca(NO_3)_2$, and iron (II) sulfate (44.7 g l^{-1}). Sulfur-grown *A. thiooxidans* were cultured in 9 K media supplemented with sulfur powder (3 %, w/v) respectively. Chalcopyrite-grown strains were developed through successive replacement of iron (II) with chalcopyrite in subcultures. Cells grown under different conditions were filtered through Whatman Grade 1 filter paper to remove cells from the suspended solid materials. The liquid containing the cells was then centrifuged at 10,000×*g* for 20 min. The cell pellet was washed twice in dilute sulfuric acid (pH 2) in order to remove any trapped ions and then stored for use. Unless otherwise specified, all strains used in the studies below were grown on chalcopyrite.

Growth curve and chalcopyrite dissolution

Free and attached cells of *A. ferrooxidans* in chalcopyrite cultures were enumerated as follows. At specified times, flasks with cultures growing on chalcopyrite were removed from the shaker and vigorously agitated by hand. Aliquots of 1.5 ml were then immediately removed and transferred to sterile centrifuge tubes. The tubes were centrifuged at 2,000 × *g* for 2 min. Then, a small amount of supernatant was removed and its concentration of copper determined by atomic absorption spectrometry. Unattached (free) cells in the remaining supernatant were enumerated microscopically using a Petroff-Hausser counting chamber. The precipitate in the centrifuge tube was added to 0.1 % Tween-20 solution [13], vortexed three times, and centrifuged at 2,000 × *g* for 2 min. After three times of vortexing, almost all of the attached population was removed. The cells in the resultant supernatant, representing the attached cells, were then enumerated [39].

Real-time PCR detection of energy gene expression

Using TRIZOL® Reagent (Invitrogen), total RNA was extracted from *A. ferrooxidans* attached to chalcopyrite and from unattached *A. ferrooxidans*. The extracted RNA was purified using RNeasy kit (QIAGEN). The purified RNA, devoid of DNA, was first retro-transcribed into cDNA with Reverse Transcriptase (RevertAid™) and random hexanucleotide primers (RevertAid™) following the manufacturer's instructions. According to the annotation of *A. ferrooxidans* ATCC 23270 genes from Comprehensive

Table 1 Primers used for quantitative real-time PCR

Locus (AFE number)	Gene symbol	Sequence		Size of product (bp)
		Forward primer (5′–3′)	Reverse primer (5′–3′)	
3180	*cyc1*	GGCAACAGAAACTCCAAGC	GAGAAGCACCCGCATT	153
3179	*cyc2*	GCAACGGCAACAGAAACT	GAAGCACCCGCATTAGAG	156
3186	*rus*	CAAGGGATTCGGTCATAGT	GTCGGATGCCAGGTAAA	150
2979	*p21*	AAGAGCGACAGCATTCCC	GGCGTCATAACCGAGCAT	105
2761	*sqr*	AATACCAGGCTTTGTTGCG	CCAGATGACCGATGTAGGG	183
2996	*tth*	CGAGTCCTGTCGGAAATAA	ATCGGCAATAAACAAGGTG	266
0254	*16S*	AATCCAAGAAGAAGCACCG	CCACTGATGTTCCTCCAG	238

Microbial Resource of The Institute for Genomic Research (TIGR, http://cmr.tigr.org/tigr-scripts/CMRGenomePage), primers were designed using Primer Premier 5.0. Oligo 6.0 was used to check for primer dimer and hairpin loops (Table 1). Each real-time PCR mixture (final volume 25 µl) contained 12.5 µl of SYBR® Green Real-time PCR Master Mix (Toyobo Co., LTD., Osaka, Japan), 0.5 µl of a 10 mmol l^{-1} sense/anti-sense primer, 5 µl of cDNA template, and 6.5 µl of nuclease-free water. The real-time PCR was carried out with the iCycler iQ Real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA): 1 cycle of 95 °C for 90 s, and then 40 cycles of 95 °C for 40 s, 60 °C for 30 s, and 72 °C for 50 s. At the completion of each run, melting curves for the amplicons were measured by raising the temperature in increments of 0.5 °C from 60 to 95 °C while monitoring fluorescence. The specificity of the PCR amplification was checked by examining the derivatized melting curve for Tm, its symmetry, and the lack of non-specific peaks. All tests were conducted in triplicate. Absolute copy numbers for genes were determined by using standard curves obtained by serial dilutions of genomic DNA. Copy numbers of genes of free and attached *A. ferrooxidans* relative to 16S rRNA [37] were determined from three independent experiments and the mean values and SD are shown. The gene-expression ratio was calculated from the quantified gene expression in cells attached to chalcopyrite divided by that in unattached cells [3, 17, 35]. Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0 J for Windows (SPSS Inc., Chicago, IL, USA) and $p < 0.05$ was defined as statistically significant.

Quantitative analysis of elemental sulfur present on chalcopyrite surfaces

In order to remove any oxide layer that may have formed on the chalcopyrite surface in air, the mineral samples in this experiment were specially treated with a 50 % solution of HCl for 3 h. Then, the mineral was placed in 10 ml of carbon disulfide for 1 h and finally rinsed twice with an additional 5 ml [14]. *A. ferrooxidans* was cultured on this specially treated chalcopyrite for 1, 3, 5, 7, 9, and 11 days. The culture liquid was decanted from respective culture flasks after the specified times, leaving enough of the liquid to avoid removal of any of the chalcopyrite residue. Perchloroethylene (25 ml) was added to each culture flask containing the chalcopyrite residue and remaining spent culture liquid to extract elemental sulfur presented on chalcopyrite surface [15]. The denser perchloroethylene, which displaces the aqueous phase at the bottom of the unstirred flask, was left in contact with the chalcopyrite residue overnight (8–16 h) at room temperature. After completion of the extraction, the liquid contents of the reaction flask were poured into a separatory funnel, and an aliquot of the bottom perchloroethylene layer was drawn off for analysis. HPLC analyses were performed on an Elite P230 high-performance liquid chromatography system with 20 µl injection volume and a UV230 + absorption detector operating at 254 nm. A SinoChrom ODS-BP 5 μ C_{18} reverse-phase column (4.6 × 200 mm) was used with an eluent comprised of 95:5 methanol:water at a flow rate of 1 ml min^{-1}. Data were collected on a PC running the EC2000 Data System.

Community structure of free and attached bacteria

To initiate the experiment, *A. thiooxidans* and *L. ferrooxidans* were collected separately. Then the cell pellets were resuspended in 9 K media and the cell number under the optical microscope was counted. The same quantity of *A. thiooxidans* and *L. ferrooxidans* were added into the flasks. After culturing for 0 (2 h), 4, 7, 11, and 13 days, the free and attached cells were collected. Genomic DNA of free and attached samples were extracted for DGGE-PCR amplification. 16S rDNA fragments were amplified with primers 341F (GC) and 518R [16]. PCR amplification reactions were performed with the reagents supplied with T-Gradient Thermoblock (Biometra, Göttingen, Germany). A touchdown PCR was performed as follows: 1 cycle of 95 °C for 5 min, 20 cycles of 94 °C for 1 min, 65 °C for

1 min decreased by 0.5 °C every cycle, and 72 °C for 1 min, and then 15 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and finally 1 cycle at 72 °C for 10 min. DGGE analysis was performed using a model DGGE-1 2001 electrophoresis system (C.B.S Scientific Company, San Diego, CA, USA) with a denaturing gradient of 45–60 % in a 7.5 % polyacrylamide gel, following the manufacturer's instructions. PCR products were mixed with 1/3 volume of 10× sucrose loading buffer. The DNA fragments were separated for 6 h at 200 V and 60 °C. The gel was stained for 20 min with ethidium bromide and documented using a UV transillumination and VisiDoc-It imaging systems (UVP). Then, the DGGE band intensity was analyzed quantitatively by the software Quantityone-1-D (Version 4.6.2) [9, 10]. DGGE band intensity was related to the relative abundance of the corresponding phylotypes. DGGE band intensities were normalized by standards whose populations were already known.

Results

Growth curve and chalcopyrite dissolution

The changes of bacterial population over time are shown in Fig. 1. A lag phase of 3 days was observed before free cells started to grow, and then the stationary phase was reached after linear growth between 4 and 10 days. Also, during the first 3 days, the attached bacterial population increased slowly during the first 3 days. Then the cells grew quickly until the ninth day.

Figure 2 shows the copper concentrations in the flask as a function of time. Copper extraction increased readily in the first 9 days. We can see from the third day to the fifth day the copper concentration in the solution increased quickly.

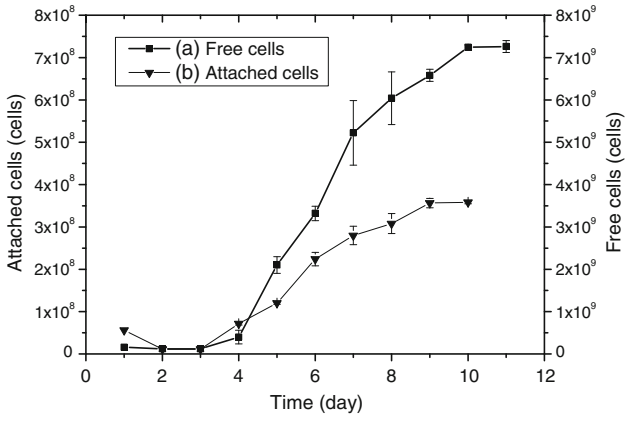

Fig. 1 Cell numbers of attached and free *A. ferrooxidans.* **a** Growth curve of *A. ferrooxidans* free in the solution during bioleaching of chalcopyrite. **b** Cell numbers of *A. ferrooxidans* attached to the chalcopyrite surface

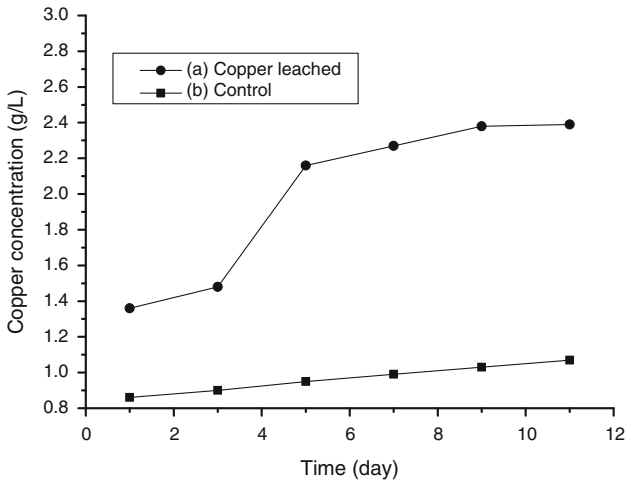

Fig. 2 Concentration of copper leached in the solution. **a** Copper leached by *A. ferrooxidans* and **b** copper leached in a sterile system

Differential expression of energy genes

Results of quantitative real-time PCR detection are shown in Table 2. The fold changes were that attached bacteria up-regulated their sulfur oxidation related genes *sqr* by 15.9-fold and *p21* by 5.9-fold while also down-regulated their ferrous oxidation genes *rus* by 0.48-fold and *cyc1* by 0.17-fold in comparison to free bacteria (ANOVA, $p < 0.01$). The fold change of *tth* and *cyc2* of attached *A. ferrooxidans* to free *A. ferrooxidans* were 0.93-fold and 0.77-fold, which were not significant (ANOVA, $p > 0.05$).

Quantitative analysis of elemental sulfur present on chalcopyrite surfaces

Figure 3 shows that elemental sulfurs were produced in the bioleaching process on the mineral surface. HPLC analysis shows that the pretreatment of the ore with CS_2 removed any elemental sulfur that may have been present on the ore at the very beginning of the experiment. During the first day, significant elemental sulfur was produced on the mineral surfaces. Then the amount of elemental sulfur declined until the seventh day. After this time, there was a slight increase in the amount of elemental sulfur.

Community structure of free and attached bacteria

Community structures of bacteria free in the solution and attached to the mineral surface are shown in Figs. 4 and 5. In attached bacteria community, the initial (2 h) percentage of *A. thiooxidans* is 24.07 % and the initial percentage of *L. ferrooxidans* is 75.92 %. The percentage of

Table 2 Quantification of genes transcripts of *A. ferrooxidans* during bioleaching of chalcopyrite by quantitative real-time PCR

Gene	Attached cells		Free cells		Induction ratio (attached/free)
	Relative copy no.	SD	Relative copy no.	SD	
p21	1.07E + 06	±8.78E + 04	1.81E + 05	±2.94E + 04	5.9
sqr	1.53E + 05	±9.29E + 03	9.61E + 03	±1.46E + 03	15.9
tth	1.34E + 06	±1.45E + 05	1.44E + 06	±2.37E + 05	0.93
cyc2	1.44E + 06	±1.53E + 05	1.87E + 06	±1.76E + 05	0.77
rus	2.31E + 05	±4.95E + 03	4.84E + 05	±1.98E + 04	0.48
cyc1	3.96E + 03	±1.39E + 03	2.40E + 04	±5.83E + 03	0.17

The induction ratios were calculated from the quantified gene-expression in cells attached to chalcopyrite divided by that in unattached cells. Values shown are the mean of three independent experiments. Copy numbers were normalized against house-keeping gene 16S rRNA gene to correct for the sample-to-sample variation

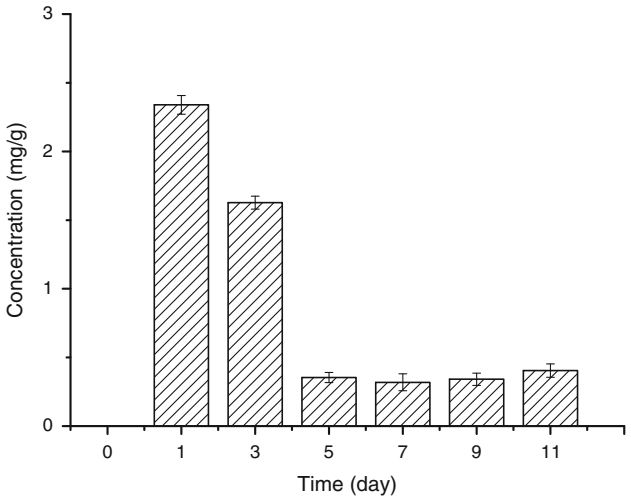

Fig. 3 Elemental sulfur recovered from the surface of the chalcopyrite particles at different times during bioleaching of chalcopyrite. HPLC analysis shows that the pretreatment of the ore with CS2 removed any elemental sulfur that may have been present on the ore at very beginning of the experiment

A. thiooxidans increased throughout the bioleaching process while *L. ferrooxidans* decreased. At the end, *A. thiooxidans* dominated the microorganisms that attached to the chalcopyrite. In contrast, in the culture liquid, the percentage of *A. thiooxidans* decreased after the bioleaching process began, while *L. ferrooxidans* increased. At the end, *L. ferrooxidans* dominated the microorganisms in the culture liquid.

Discussion

On the first day, the proportion of attached cells was more than free cells. The reason for this may be that during the bioleaching of chalcopyrite, the microorganism preferred to attach onto the surface of sulfide minerals after being inoculated [1, 8].

From the third day to the fifth day, the dissolution of chalcopyrite increased quickly. At the same time, the population of free and attached bacteria began to increase (Fig. 1). Free or attached bacteria contributed to the dissolution of chalcopyrite. After 9 days of growth, the population of free and attached bacteria became stable and the dissolution of chalcopyrite also became slow. Because chalcopyrite is an acid-soluble sulfide mineral, dissolution of chalcopyrite was also seen in the control experiment.

The genes in this study selected for their involvement of in the process of energy conservation were *cyc1*, *cyc2*, *rus*, *sqr*, *tth*, and *p21*. The first three genes, *cyc1*, *cyc2*, and *rus*, all encode enzymes involved in ferrous iron oxidation [37], and the last three genes encode enzymes that play pivotal roles in sulfur oxidation [11, 21, 29]. The six genes can represent the energy utilization to a great extent.

Because the expression levels of *tth* and *cyc2* were originally high (Table 2), their changes were not significant. The up-regulation of sulfur oxidation genes of attached *A. ferrooxidans* suggested that attached cells preferred to use elemental sulfur as energy source. The mechanism of attached *A. ferrooxidans* to take the role of oxidizing elemental sulfur was by up-regulation of sulfur oxidation genes.

The big change of *sqr* expression level was due to the pathway by which *A. ferrooxidans* degrade chalcopyrite. There are two different sulfide-degradation pathways, the thiosulfate pathway and the polysulfide pathway. Acid nonsoluble metal sulfides such as pyrite, molybdenite, and tungstenite are degraded by the thiosulfate pathway, and acid-soluble sulfide minerals, e.g., hauerite, sphalerite, galena, chalcopyrite, and arsenopyrite, are degraded by the polysulfide pathway [26–28]. In the polysulfide pathway, the main sulfur intermediate is polysulfide and elemental sulfur while thiosulfate is only a by-product [28]. Extracellular elemental sulfur (S_8) is mobilized as persulfide sulfane sulfur by special outer-membrane proteins and oxidized by periplasmic sulfur dioxygenase [24]. Free

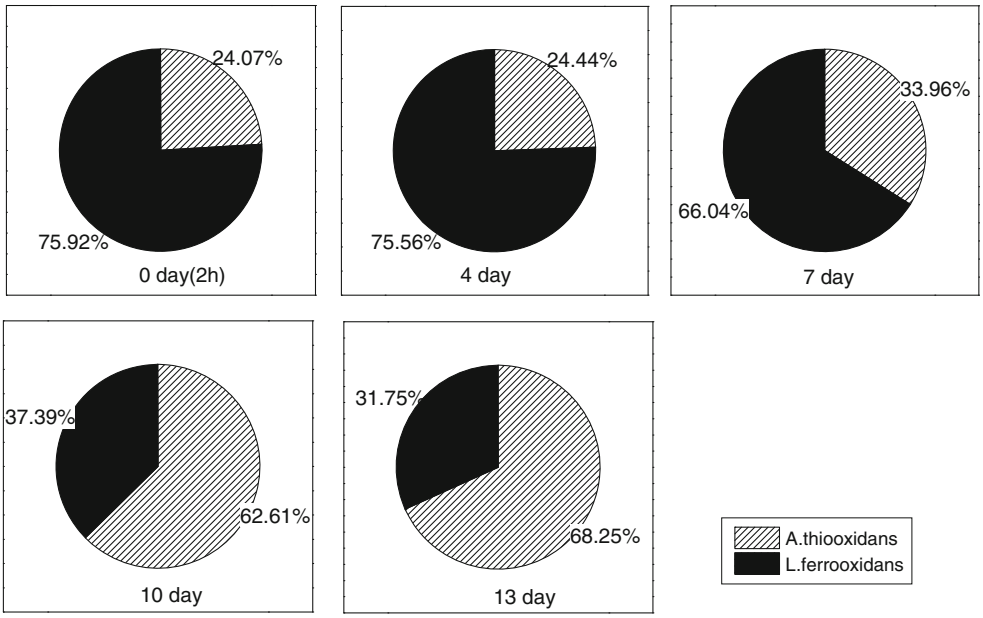

Fig. 4 Community structure with respect to *A. thiooxidans* and *L. ferrooxidans* on the chalcopyrite particle surfaces at different times during bioleaching of chalcopyrite

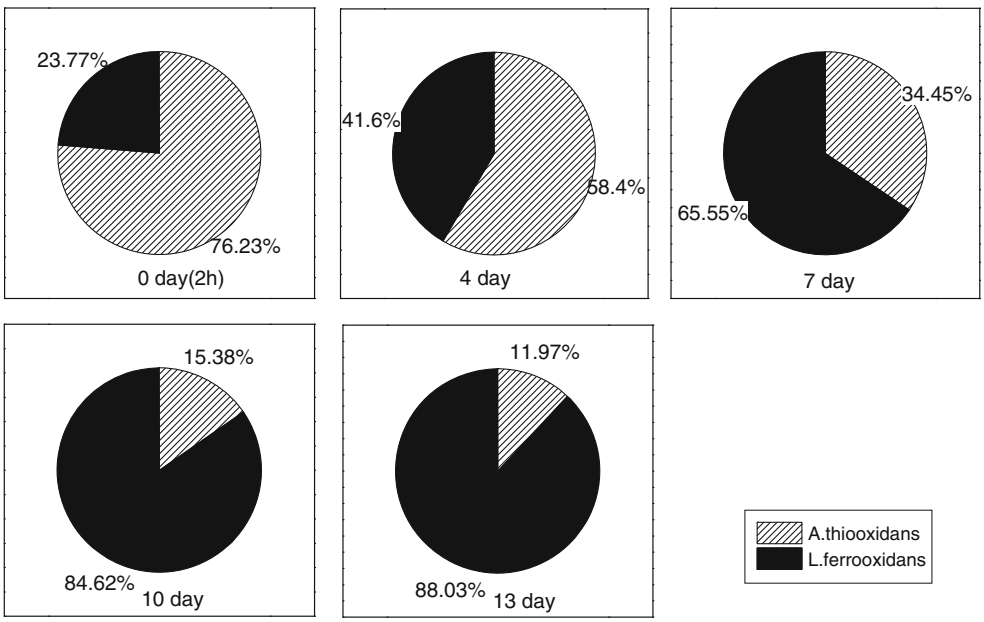

Fig. 5 Community structure with respect to *A. thiooxidans* and *L. ferrooxidans* in the culture liquid at different times during the bioleaching of chalcopyrite

sulfide produced in this process is oxidized by sulfide quinone reductase (SQR). Thus, in our experiments, as chalcopyrite was degraded via the polysulfide pathway, the *sqr* gene was highly expressed. The up-regulation of *sqr* indicted that chalcopyrite was indeed degraded via the polysulfide pathway.

The genes *cyc2, cyc1*, and *rus* are all located in the rus operon. There are three promoters (PI, PII, and P_{rus}) within the operon [2, 4]. Both *cyc2* and *cy1* are regulated by PI or PII, while *rus* is regulated by P_{rus}. Because *rus* is regulated by P_{rus} alone, it is easy to understand why the expression level of *rus* was different from that of *cyc2* and *cyc1*, but it is challenging to explain that the expression levels of *cyc2* and *cyc1* were different. However, this was consistent with what Yarzábal et al. found in their research [37]. In their study, *cyc2* expressed 4.9-fold higher in Fe(II) than in

sulfur-growth condition, while *cyc1* expressed 23.5-fold higher in Fe(II) than in sulfur-growth condition [37]. Therefore, some transcriptional regulation may be involved in the process.

The presence of elemental sulfur on the surface of chalcopyrite (Fig. 3) is consistent with previous study [12, 34, 40]. Oxidation of chalcopyrite in the presence of acid and ferric ions is often represented as:

$$CuFeS_2 + 4Fe^{3+} \rightarrow 5Fe^{2+} + Cu^{2+} + 2S^0$$

The reaction is initially rapid, but soon slows [12]. As a result, a considerable amount of elemental sulfurs is produced on the sulfide mineral surface. Elemental sulfur as a substrate induces the up-regulation of sulfur oxidation-related genes. After up-regulation, the abilities of attached *A. ferrooxidans* to oxidize elemental sulfur were enhanced. Therefore, as the proportion of attached cells increased (Fig. 1.), the amount of elemental sulfurs on the mineral surface decreased.

The attached bacteria, at the beginning (2 h), the percentage of *L. ferrooxidans* was more than *A. thiooxidans* (Fig. 4), maybe because *L. ferrooxidans* was easier attached to the sulfide mineral surface. AFM measurement has shown that the attach force of *L. ferrooxidans* was bigger than *A. thiooxidans* (data not shown). Later, *A. thiooxidans* can use sulfur as an energy source and begin to grow. The percentage of *A. thiooxidans* increased throughout the bioleaching process while *L. ferrooxidans* decreased. The reason for this may be that the dissolution of chalcopyrite would release both elemental sulfur and ferrous iron for the growth of bioleaching microorganisms. However, the oxidation of elemental sulfur can give more growth energy to the sulphur-oxidizing microorganisms than the oxidation of ferrous iron giving to iron-oxidizing microorganisms [26]. *A. thiooxidans* can thus derive more energy than the iron-oxidizing microorganisms for their growth and propagation. Therefore, *A. thiooxidans* exceed *L. ferrooxidans*. At the end, *A. thiooxidans* dominated the microorganism that attached to chalcopyrite. Because in our experiment *A. thiooxidans* could only use elemental sulfur as an energy source, we can say the main role of the attached bacteria is to oxidize elemental sulfur.

In contrast, in the solution, the percentage of *A. thiooxidans* decreased (Fig. 5) throughout the bioleaching process while *L. ferrooxidans* increased. During bioleaching, both elemental sulfur and ferrous iron were produced. Elemental sulfur was mainly oxidized by attached bacteria, and ferrous irons were released into the solution. Because ferrous irons were used as an energy source, the population of *L. ferrooxidans* increased. At the end, *L. ferrooxidans* dominated the microorganism in the culture liquid.

In this study, after adaptation, both bacteria grew well in the chalcopyrite bioleaching. Therefore, the copper resistance of *A. thiooxidans* and *L. ferrooxidans* may affect their growth to some extent, but was not the limiting factor.

Therefore it can be concluded that acidophilic bacteria that attach to chalcopyrite surfaces prefer to use elemental sulfur as an energy source. The main role of attached bacteria was to oxidize elemental sulfur. The main role of free bacteria was to oxidize ferrous iron. Dissolution of chalcopyrite was mainly through an indirect bioleaching mechanism.

This work contributes to better understand the different roles between free and attached bacteria and knowledge of the bioleaching mechanism. This work also contributes to instruct the selection of acidophilic bacteria in application of mix culture of acidophilic bacteria in bioleaching.

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