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The Community Dynamics of Major Bioleaching Microorganisms During Chalcopyrite Leaching Under the Effect of Organics

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Abstract To determine the effect of organics (yeast extract) on microbial community during chalcopyrite bioleaching at different temperature, real-time polymerase chain reaction (PCR) was employed to analyze community dynamics of major bacteria applied in bioleaching. The results showed that yeast extract exerted great impact on microbial community, and therefore influencing bioleaching rate. To be specific, yeast extract was adverse to this bioleaching process at 30°C due to decreased proportion of important chemolithotrophs such as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. However, yeast extract could promote bioleaching rate at 40°C on account of the increased number and enhanced work of *Ferroplasma thermophilum*, a kind of facultative bacteria. Similarly, bioleaching rate was enhanced under the effect of yeast extract at 50°C owing to the work of *Acidianus brierleyi*. At 60°C, bioleaching rate was close to 100% and temperature was the dominant factor determining bioleaching rate. Interestingly, the existence of yeast extract greatly enhanced the relative competitiveness of *Ferroplasma thermophilum* in this complex bioleaching microbial community.

Introduction

The recent years witnessed serious depletion of copper resources, especially the high grade ones. As a result, it is inevitable for people to process great number of intractable low grade ones. A lot of researches concerning the copper processing technology have been conducted [23–25]. In comparison with the conventional methods, bioleaching shows a number of advantages including simple process flows, low investment and operation cost, and environmentally friendly character [29].

The bioleaching microorganisms can be classified into three categories according to their adaptation to temperature: mesophiles, moderate thermophiles, and extreme thermophiles [1, 12]. Because various kinds of bacteria show distinct metabolic activity at different temperature, the environmental temperature exercises great impact on the function and structure of microbial community. Apart from the factor of temperature, the effect of organics should never be overlooked. Yeast extract often serves as the energy source for obligative or facultative heterotrophic microorganism [4]. In some experimental researches, to evaluate the effect of organics on bioleaching process, yeast extract is usually adopted as the typical organics [22]. According to some researches, a certain amount of organics is adverse to some autotrophic bioleaching bacteria [3, 5]. It is also reported that relatively high concentration (0.2% w/v) of yeast extract would inhibit the growth of some facultative autotrophic bacteria [20]. On the contrary,

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heterotrophic and facultative microorganisms can use organics to support their growth [34]. Obviously, the role played by organics in bioleaching is very complicated. It is the fact that bioleaching microbial community is extremely complex in actual situation, which consists of the autotrophic, heterotrophic, and facultative microorganisms. In this artificially assembled microbial community, what the role played by the organics in the complex bioleaching microbial community? How it influence the structure and function of the complex bioleaching microbial community? So far, others only focused on one strain to study the effect of organics on bioleaching [15]. And no comprehensive and systematic researches have been conducted to clarify the effect of organics on the structure and function of complex bioleaching microbial community within so large temperature range.

High levels of bacterial diversity make quantifying and characterizing microbial communities a daunting task. In recent years, real-time PCR has emerged as a strongly and widely used method for biological investigation for its outstanding advantages [27]. This method provides very accurate and reproducible quantization of gene copies and can detect and quantify extremely small amounts of specific nucleic acid sequences. Besides, because the real-time PCR does not require post-PCR sample handling, it can prevent potential PCR product carry-over contamination, resulting in much faster and higher throughput assays. Moreover, real-time PCR method has a very large dynamic range of starting target molecule determination [9]. As a result, the real-time PCR method has been widely applied to assess microbial community in some particular environment [6, 31]. Also, the application of real-time PCR has been demonstrated valid and accurate to monitor population dynamics of mixed bioleaching bacteria [32].

In this study, yeast extract was adopted to study the effect of organics on the population dynamics of bioleaching microbial community including *Acidithiobacillus ferrooxidans*, *Leptospirillum ferriphilum*, *Acidithiobacillus thiooxidans*, *Acidithiobacillus caldus*, *Acidiphilium* spp., *Ferroplasma thermophilum*, *Acidianus brierleyi*, and *Sulfobacillus thermosulfidooxidans* using the real-time PCR method. The structure and function of complex bioleaching microbial community were determined under the effect of temperature and organics.

Materials and Methods

Mineral Composition, Bacterial Strains, and Culture Conditions

The low grade mineral contained 0.3% copper and chalcopyrite accounted for 62.2% of the minerals containing copper. The mineral samples were crushed and then sieved through the 75 μm size pore for later use. All strains were provided by China Center for Type Culture Collection (CCTCC). Each strain was cultivated in appropriate medium under different conditions (Table 1). After being harvested by centrifugation at 10,000×*g* for 20 min and being made into bacterial suspension with sterilized water (pH 2.0), these eight bacteria strains served as the inocula for chalcopyrite bioleaching. Then, each strain was equally (1.0×10^7 per/ml) inoculated into 250 ml flask containing sterilized chalcopyrite medium (pH 1.5). We carried out

Table 1 Culture condition for the eight bacteria strains

Bacteria strain	Medium	pH	Temperature (°C)
Acidithiobacillus ferrooxidans F1	3.0 g/l $(NH_4)_4SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l KCl, 44.71 g/l $FeSO_4{\cdot}7H_2O$	1.8–2.0	30
Leptospirillum ferriphilum YSK	3.0 g/l $(NH_4)_4SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l KCl, 44.71 g/l $FeSO_4{\cdot}7H_2O$	1.6–1.7	40
Acidithiobacillus thiooxidans A01	3.0 g/l $(NH_4)_2SO4$, 3.0 g/l KH_2PO4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.25 g/l $CaCl_2{\cdot}2H_2O$, 0.5% sulfur (w/v)	2.0–2.5	30
Acidithiobacillus caldus S1	3.0 g/l $(NH_4)_2SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.05 g/l K_2HPO_4, 0.1 g/l KCl, 5.2 g/l $Na_2SO_4{\cdot}10H_2O$, 1% sulfur (w/v)	2.0–2.5	45
Acidiphilium sp. DX1-1	3.0 g/l $(NH_4)_4SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l KCl, 1% glucose (w/v)	3.5	30
Ferroplasma thermophilum L1	3.0 g/l $(NH_4)_4SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l KCl, 20 g/l $FeSO_4{\cdot}7H_2O$, 0.01%(m/v)yeast extract	1.0	45
Acidianus brierleyi 8954	3.0 g/l $(NH_4)_2SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l KH_2PO_4, 0.1 g/l KCl, 1% sulfur (m/v), 0.02% (m/v)yeast extract	2.5	65
Sulfobacillus thermosulfidooxidans	3.0 g/l $(NH_4)_4SO_4$, 0.01 g/l $Ca(NO_3)_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l K_2HPO_4, 0.1 g/l KCl, 20 g/l $FeSO_4{\cdot}7H_2O$, 0.02%(m/v)yeast extract	1.5–1.6	45

the enumeration through direct counting method using blood counting chamber. The sterilized chalcopyrite medium consisted of the processed minerals (previously mentioned) and sterilized 9K basic medium without $FeSO_4$. The special 9K culture medium included $(NH_4)_4SO_4$ (3.0 g/l), $Ca(NO_3)_2$ (0.01 g/l), $MgSO_4{\cdot}7H_2O$ (0.5 g/l), K_2HPO_4 (0.5 g/l), and KCl (0.1 g/l). After this step, bacterial mixture was cultivated at 30, 40, 50, and 60°C under the aerobic condition at 170 r/min (in the rotary platform). There were two experiment groups at each temperature, one group was added with 0.01% yeast extract and the other group was not. The abiotic controls were also designed at each temperature. The leaching experiments lasted for 28 days. All tests were conducted in triplicate.

Physicochemical Analysis

Samples were collected at regular intervals to be measured for dissolved copper, total dissolved iron, and pH. Copper and total iron concentration in solution were measured by inductively coupled plasma (ICP) total analysis method at 4th, 8th, 12th, 16th, 20th, 24th, and 28th day. The pH of the bioleaching systems was measured with a pH meter, and the pH of the system was kept stable around 1.5.

Extraction of Genomic DNA

Genomic DNA was extracted from bacteria using a TIANamp Bacteria DNA kit (Tiangen, Beijing, China). The acquired genomic DNA stained by ethidium bromide was checked through 1.0% agarose gel electrophoresis. Purified genomic DNA concentration was measured spectrophotometrically using a DanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and adjusted to proper concentration for the PCR use.

Design and Specificity of PCR Primers

Primers used in the study are summarized in Table 2. PCR primers were designed by using Primer Primier 5.0 and then synthesized by Shanghai Sangon Company (China). According to the size of the specific amplified products stained by ethidium bromide through 1.5% agarose gel electrophoresis, the specificity of primers can be determined. DNA sequencing was conducted by Shanghai Sangon Company (China) and BLAST analysis in GenBank. Specificity of these primers was also evaluated by real-time PCR (see below).

The PCR reactions were carried out under the following condition: 30 s denaturation at 95°C, 30 s annealing at 59°C, and 30 s extension at 72°C, along with an initial 3 min denaturation at 95°C, and a final 10 min extension reaction at 72°C. Then, the PCR products were analyzed through agarose gel electrophoresis and were purified using QIAquick-spin PCR purification kit (Qiagen, Hilden, Germany). The PCR product was cloned to T-Vector for sequence analysis. The real-time PCR was carried out with iCycler iQ Real-time PCR detection system (Bio-Rad, Hercules, Calif.). Each reaction mixture (final volume, 25 μl) contained 12.5 μl of SYBR Green Real-time PCR Master Mix (Toyobo) which consisted of Taq DNA polymerase, dNTP, $MgCl_2$, SYBR Green I dye, 5 pmol/μl the

Table 2 Designed primer used in the study

Target species	Target gene	GenBank no.	Primer name	Length (bp)	Sequences (5′–3′)	Position	T_m (°C)	Product length (bp)
A. ferrooxidans	*gyr*B gene	FJ154525	F1-F	18	CGGTGTCTCGGTAGTGAA	81–98	55.48	160
			F1-R	18	GGGTTTGAAGCGGATAGT	223–240	55.12	
L. ferriphilum	*gyr*B gene	FJ875112	L-F	18	GAAAACACTTGAGGACGG	443–460	53.91	168
			L-R	18	CGGATAAAACGGTTGATT	593–610	53.48	
At. thiooxidans	*gyr*B gene	FJ154526	A01-F	18	GACCCGTACCCTCAATCA	603–620	56.72	175
			A01-R	18	CGGTTTCACTTCACTGGA	760–779	55.44	
At. caldus	arsB gene	DQ810790	AC-F	18	TGCGGCTCGACTTCTCAC	1943–1960	61.32	150
			Ac-R	18	GGGCATAGCGATCAAACG	2092–2075	60.18	
Acidiphilium spp.	16S rRNA gene	EF556220	16S-F	18	TGGCGGCACGCTTAACAC	30–47	64.34	165
			16S-R	18	CTCCTCAGGCGACTTTCG	194–177	59.65	
F. thermophilum	16S rRNA gene	FJ154518	Ft-F	23	CCCACTTTGATGTTGCTTTTCCG	737–759	73.06	248
			Ft-R	19	TGCATGGCCGTCGTCAGCT	966–984	72.5	
A. brierleyi	16S rRNA gene	FJ154515	8954F	18	GGACTGCCGTCGTTAAGA	1114–1131	57.31	293
			8954R	19	GAAGGCAAGAAACCTCACT	1388–1406	54.43	
Sulfobacillus thermosulfidooxidans	arsB gene	EF589669	St-F	18	CCGTGTTATTAGCAGGAG	4034–4051	51.65	261
			St-R	20	CAATTAGAACAGTCGGCATA	4294–4275	53.97	

forward primer, 5 pmol/μl the reverse primer, and 5 μl DNA template. Then nuclease-free water was used to make up the whole system to 25 μl. Under the monitoring of an iCycler iO Real-time PCR detection system, the real-time PCR reactions were carried out under the following condition: initial 3 min denaturation at 95°C, and then 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s. After the completion of each run, melting curves for the amplicons were measured by raising the temperature 0.5°C from 59 to 99°C while monitoring the change of fluorescence rate. All tests were conducted in triplicate.

Determination of Microbial Community Structure

At 4th, 12th, 20th, and 28th day, leaching solution combined with the elution from chalcopyrite surface were added into centrifugal flask, then the bacteria were harvested through centrifugation at 10,000×*g* for 20 min at 4°C in a 5804R centrifuge (Eppendorf, Wesbury, NY). Genomic DNA was extracted using a TIANamp Bacteria DNA kit (Tiangen, Beijing, China). To determine the microbial community structure, real-time PCR method was carried out using specific primers based on *gyr*B gene, 16S rRNA gene, and arsB gene. The real-time PCR mixture and reaction condition was the same just as previously mentioned. The microbial community structure was determined according to the proportion of the targeted gene copies of each strain in the same sample.

Results and Discussion

Evaluation of the Specificity of Primers and Real-time PCR Assay

The quality of the amplified products stained by ethidium bromide was checked through 1.5% agarose gel electrophoresis. Amplified DNA fragments were correct because each amplified products was a single segment matching the expected size (Fig. 1). DNA sequencing was carried out by BLAST analysis in GenBank, and they matched the sequence of the gene that has been deposited in GenBank. So, we do not re-put the existed sequence into GenBank. GenBank numbers of these sequences have been shown in the Table 2. These results suggested that the conserved 16S rRNA gene, less conserved *gyr*B gene, and functional gene such as arsB gene are suitable and accurate to analyze this microbial community [11, 30]. In order to examine the bacteria growth kinetics and microbial community dynamics, copy number of the specific gene can be regarded as a proxy for cell counts using quantitative PCR [8]. No matter the genes are single-copy or multi-copy in their genomes, copy number of the specific gene can serve as the relatively quantitative indicator that can reflect the alternation tendency of this microbial community.

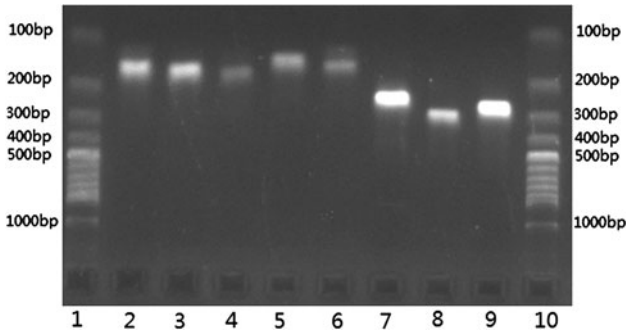

Fig. 1 PCR amplification products of selected genes deriving from eight strains on 2% agarose gel: *lane 2 A. ferrooxidans* F1, *lane 3 L. ferriphilum* YSK, *lane 4 At. thiooxidans* A01, *lane 5 At. caldus* S1, *lane 6 Acidiphilium* spp. DX1-1, *lane 7 F. thermophilum* L1, *lane 8 A. brierleyi*, *lane 9 Sulfobacillus thermosulfidooxidans*, and *lanes 1*, *10* marker

The Effect of Yeast Extract on Microbial Community Structure at Different Temperature

The Microbial Community Shift Under the Effect of 0.01% Yeast Extract at 30°C

In the group without being added yeast extract, *L. ferriphilum* YSK accounting for 51% of the whole community and *At. thiooxidans* A01 accounting for 28% of the whole community were the dominant strains at 4th day. Then, the population proportion of *L. ferriphilum* YSK and *At. thiooxidans*, respectively, decreased to 34 and 18% at 12th day. The microbial community structure became increasingly simple in the following period. *L. ferriphilum* YSK was the only dominant strain in later period together with little number of other strains such as *At. thiooxidans* A01, *At. caldus* S1, and *F. thermophilum* L1 (Fig. 2a). The predominance of *L. ferriphilum* at 30°C was because it optimally utilizes energy from oxidizing Fe^{2+} at warm temperatures (25–42°C) [18]. Under the effect of 0.01% yeast extract, microbial community structure was different (Fig. 2a). *F. thermophilum* L1 accounting for 23% of the whole community, *At. thiooxidans* A01 accounting for 29% of the whole community, and *Acidiphilium* spp. DX1-1 accounting for 35% of the whole were the dominant strains at 4th day. Total seven strains except for *A. brierleyi* were found at 4th day. From 4th day, the number of *F. thermophilum* L1 began to increase. As a result, this strain became the dominant strain in the following period. This was because yeast extract could promote the growth of *F. thermophilum* L1 that belongs to a kind of facultative bacteria [33]. It was found that the microbial community under the effect of 0.01% yeast extract was more complex

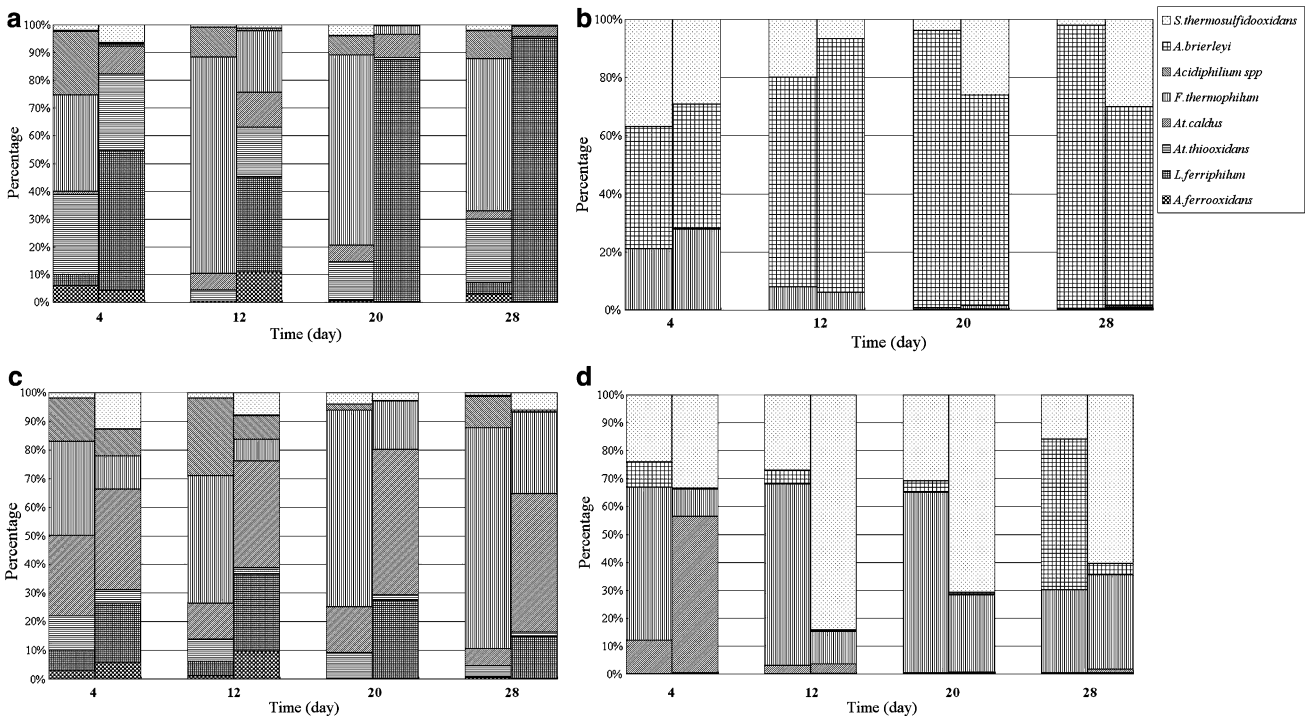

Fig. 2 Microbial community shift under the effect of yeast extract at different temperature. **a** Microbial community shift of the group added with 0.01% yeast extract and the group without being added yeast extract at 30°C; **b** microbial community shift of the group added with 0.01% yeast extract and the group without being added yeast extract at 40°C; **c** microbial community shift of the group added with 0.01% yeast extract and the group without being added yeast extract at 50°C; **d** microbial community shift of the group added with 0.01% yeast extract and the group without being added yeast extract at 60°C. The *left bar* stands for the microbial community composition of the group added with yeast extract at a specific day; the *right bar* stands for the microbial community composition of the group without being added yeast extract at a specific day

than the community without being added yeast extract at 28th day (Fig. 2a). According to the Simpson' diversity index equation, the diversity index value of the community added with yeast extract (0.6320) was much greater than the value of the community without being added yeast extract (0.09674). Obviously, the existence of yeast extract increased the number of facultative heterotrophically bacteria such as *Acidiphilium* spp. DX1-1 and *F. thermophilum* L1, and thereby generating high microbial community diversity index. Interestingly, it seemed that *At. thiooxidans* A01 could perform better under the effect of 0.01% yeast extract compared with other autotrophic bacteria at 30°C.

The Microbial Community Shift Under the Effect of 0.01% Yeast Extract at 40°C

In the group without being added 0.01% yeast extract, seven strains except for *A. brierleyi* were found at 4th and 12th day. The thermophile character of *A. brierleyi* [14] accounts for its absence in the medium at 40°C. *At. caldus* S1 and *L. ferriphilum* YSK were the dominant bacteria at 4th and 12th day and their population proportion, respectively, increased from 34 to 37% and from 21 to 27%. From 12th day, the number of *F. thermophilum* L1 began to increase. Its population proportion increased from 27% at 20th day to 29% at 28th day. In the final period (from 20th to 28th day), *F. thermophilum* L1, *At. caldus* S1, and *L. ferriphilum* YSK were the dominant strains (Fig. 2b). This was because *F. thermophilum* L1 performs optimally at 45°C, *At. caldus* is a moderate thermophile with a growth temperature optimum of 45°C [13], and *L. ferriphilum* YSK has the ability to grow at 45°C [7]. Under the effect of 0.01% yeast extract, microbial community structure shift was distinct (Fig. 2b). At the 4th day, the microbial community, containing *F. thermophilum* L1 (33%), *Acidiphilium* spp. DX1-1 (15%), *At. caldus* S1 (28%), and other little number of strains, was relatively complex. At the 12th day, the dominant bacteria were *F. thermophilum* L1 (45%) and *Acidiphilium* spp. DX1-1 (27%). In the following period, the number of *F. thermophilum* L1 made a further increase, and this strain became the only dominant bacteria. According to Fig. 2b, it is clear that the proportion of *F. thermophilum* L1 was higher in the group added with yeast extract than the other group without being added yeast extract at 40°C. The strong competitive power of *F. thermophilum* stemmed from the fact that

F. thermophilum L1 has a temperature optimum of 45°C and grows well utilizing both ferrous iron and yeast extract [33].

The Microbial Community Shift Under the Effect of 0.01% Yeast Extract at 50°C

Acidithiobacillus caldus S1 (56%) and *S. thermosulfidooxidans* (34%) were the dominant strains at 4th day in the group without being added yeast extract. From the 4th day, the number of *At. caldus* S1 was dramatically decreased, however, the number of *S. thermosulfidooxidans* and *F. thermophilum* L1 was considerably increased. As a result, these two strains became the dominant bacteria (Fig. 2c). This was because *S. thermosulfidooxidans* belongs to moderate thermophile and actively functions in chalcopyrite dissolution at this temperature [28]. Under the effect of 0.01% yeast extract, microbial community structure shift was different at 50°C (Fig. 2c). During the whole experiment period, only four kinds of strains such as *F. thermophilum* L1, *S. thermosulfidooxidans*, *A. brierleyi*, and *At. caldus* S1 were found. *F. thermophilum* L1 (55%) and *S. thermosulfidooxidans* (24%) were the dominant bacteria at 4th day. From the 4th day, the number of *At. caldus* S1 began to fall down, and this strain was almost vanished in the medium finally. From the 20th day, the number of *F. thermophilum* L1 began to fall, however, the number of *A. brierleyi* started to increase significantly. As a result, *A. brierleyi* (54%) became the dominant group together with previous dominant strains, *F. thermophilum* L1 (30%) and *S. thermosulfidooxidans* (16%), at 28th day. This was due to the fact that *F. thermophilum* L1 and *S. thermosulfidooxidans* are moderate thermophiles [10] and *A. brierleyi* belongs to thermophiles, however, other strains might be inhibited by high temperature. Besides, *A. brierleyi* demonstrated strong competitive power at 50°C even its optimal temperature (around 65°C) is far beyond 50°C. This was probably because yeast extract abounding with protein, amino acid, vitamin, and trace element is beneficial for its growth.

The Microbial Community Shift under the Effect of 0.01% Yeast Extract at 60°C

In the group without being added 0.01% yeast extract, *F. thermophilum* L1 (28%), *S. thermosulfidooxidans* (29%), and *A. brierleyi*(43%) were the dominant strains at 4th day. From 4th day, the number of *F. thermophilum* L1 was dramatically decreased, and *A. brierleyi* became the only dominant bacteria together with a little proportion of *S. thermosulfidooxidans* (Fig. 2d). This was because *A. brierleyi* showed high activity at 60°C. Under the effect of 0.01% yeast extract, microbial community comprised only three strains, namely, *F. thermophilum* L1, *S. thermosulfidooxidans*, and *A. brierleyi* (Fig. 2d). In the beginning, these three strains were dominant groups. From 4th day, the number of *A. brierleyi* began to increase, however, other two strains started to decrease. During the mid-term and later period (from 12th to 28th day), *A. brierleyi* grew up to the only dominant strain, which was attributed to the fact that *A. brierleyi* belongs to thermophile that could adapt to extremely high temperature [14]. At 60°C, the existence of yeast extract also decreased competitive power of *S. thermosulfidooxidans*, and temperature was the major factor determining the microbial community structure.

Surprisingly, the *F. thermophilum* L1 was the dominant group at 30, 40, and 50°C and was existed at 60°C in the group added with 0.01% yeast extract. As a result, the existence of yeast extract greatly enhanced the relative competitiveness of *F. thermophilum* L1 in this environment containing available organics and inorganics. This was because *F. thermophilum* L1 has the special ability of utilizing both ferrous iron and yeast extract [33].

Exploration Toward the Effect of Yeast Extract on Fe^{3+} Concentration and Bioleaching Rate Based on Microbial Community Shift at Different Temperatures

The concentration of Fe^{3+} was dramatically increased during the first 4 days (Figs. 3, 4). Fe^{3+} derived from the oxidation of Fe^{2+} [19], during this process bacteria could produce energy for their growth and metabolism. At 30°C, Fe^{3+} concentration of the groups without being added yeast extract was 498, 768, 930, 982, 1014, 1132, and 1290 mg/l, ,respectively at 4th, 8th, 12th, 16th, 20th, 24th, and 28th day. Obviously, it kept increasing all the time, and it was higher than the concentration of the group added with yeast extract (Figs. 3, 4). This could be explained in two parts. First, organics exhibited toxicity to chemolithotrophs that could oxidize Fe^{2+} to Fe^{3+} [16]. To be specific, organics might inhibit oxidation of ferrous iron and sulfur by *A. ferrooxidans* and *At. thiooxidans*, the major functional strains at 30°C [5]. Besides, in the group added with 0.01% yeast extract, the increasing number of *Acidiphilium* spp. (Fig. 2a) reduced Fe^{3+} to Fe^{2+} utilizing energy from organics [12] at 30°C. For the reason that Fe^{3+} can function as oxidizer to accelerate mineral dissolution and exclusive Fe^{3+} is beneficial to mineral oxidation [25, 26], high Fe^{3+} concentration gave rise to the high bioleaching rate (from 17.2% at 4th day to 27.6% at 28th day) of the group without being added yeast extract compared with the rate (from 19.2 to 32.7%) of the group added with yeast extract at 30°C (Fig. 5).

At 40°C, Fe^{3+} concentration of the group added with yeast extract kept increasing during the whole bioleaching

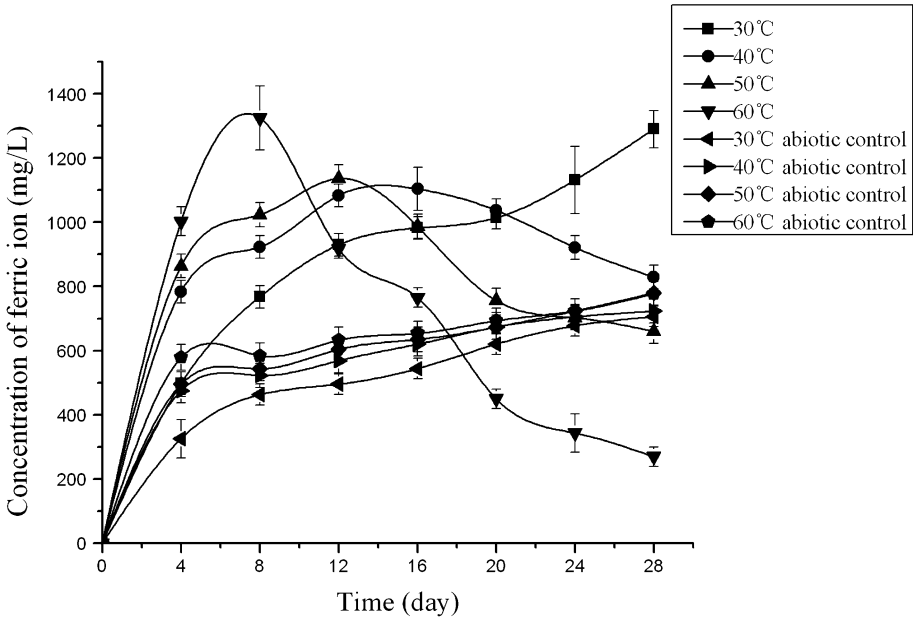

Fig. 3 Variation of Fe^{3+} concentration during bioleaching of chalcopyrite conducted by mixed bacteria without being added 0.01% yeast extract at different temperature

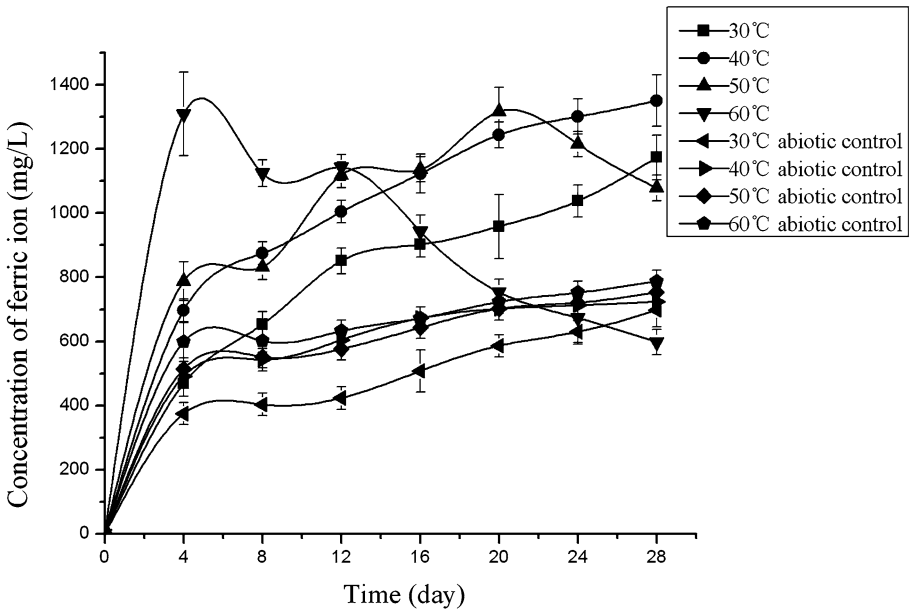

Fig. 4 Variation of Fe^{3+} concentration during bioleaching of chalcopyrite conducted by mixed bacteria under the effect of 0.01% yeast extract at different temperature

process. To be specific, it was 697 mg/l at 4th day, 875 mg/l at 8th day, 1004 mg/l at 12th day, 1124 mg/l at 16th day, 1244 mg/l at 20th day, 1301 mg/l at 24th day, and 1351 mg/l at 28th day. However, Fe^{3+} concentration of the group without being added yeast extract turned to decrease at 16th day, and it was lower than the concentration of the group added with yeast extract all the time (Figs. 3, 4). *F. thermophilum* L1, a strain has a temperature optimum of 45°C and is capable of chemomixotrophic growth on ferrous ion and yeast extract [33], took the dominant place of *At. caldus* S1 that is a sulfur oxidizer [17] under the effect of yeast extract at 40°C (Fig. 2b). For the reason that *F. thermophilum* L1, could oxidize Fe^{2+} into Fe^{3+}, the increasing number of *F. thermophilum* L1 in the group added with yeast extract led to the high Fe^{3+} concentration in solution. Because Fe^{3+} is functional in mineral oxidation and dissolution [25, 26], the relatively high Fe^{3+} concentration promoted the bioleaching rate (from 24.9 to 49.8%) of the group added with yeast extract (Fig. 5).

At 50°C, Fe^{3+} concentration of the group added with some yeast extract reached the peak (1317 mg/l) at 20th day, then it began to fall down (Fig. 4). Fe^{3+} concentration of the group without being added 0.01% yeast extract turned to fall down at 12th day (Fig. 3). Fe^{3+} concentration of the group added with yeast extract was higher than that of the other group in later period probably because organics

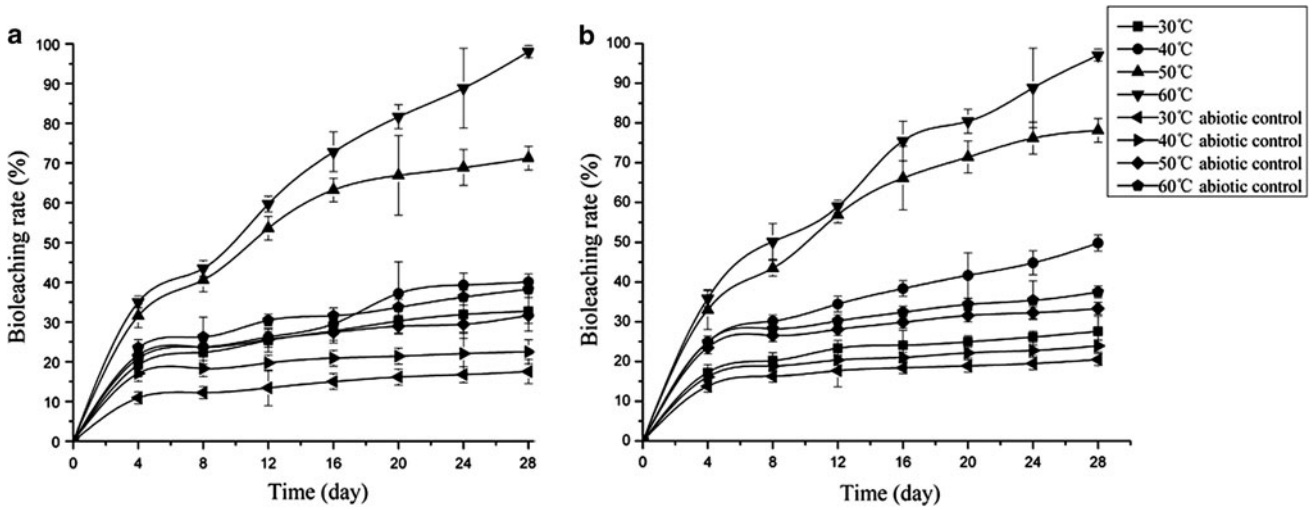

Fig. 5 Comparison of bioleaching rate between the groups added with 0.01% yeast extract and the group without being added 0.01% yeast extract. **a** Change of bioleaching rate of the group without being added 0.01% yeast extract at different temperature; **b** change of bioleaching rate of the group added with 0.01% yeast extract at different temperature

increased jarosite dissolution [2]. The microbial community of the group added with yeast extract mainly consisted of *A. brierleyi*, *F. thermophilum* L1, *S. thermosulfidooxidans*, and the community of the group without being added yeast extract primarily consisted of *F. thermophilum* L1 and *S. thermosulfidooxidans* (Fig. 2c). Obviously, the existence of *A. brierleyi* functioned as the differential factor. It is possible that the combined work of three facultative bacteria strains, namely, *A. brierleyi*, *F. thermophilum* L1, and *S. thermosulfidooxidans* was superior to the work of *F. thermophilum* L1 and *S. thermosulfidooxidans*. This was probably because *A. brierleyi*, a bacteria that is capable of oxidizing sulfur, plays an important role in mineral dissolution. During the oxidization process, chalcopyrite abounded with metal sulfide was broken up under the work of *A. brierleyi*. The solubilization of metal sulfides was to provide sulfuric acid for a proton attack and to keep the iron in the oxidized ferric state for an oxidative attack on the mineral, and therefore improving bioleaching rate [23]. Consequently, the high Fe^{3+} concentration and strong combined work of *A. brierleyi*, *F. thermophilum* L1, and *S. thermosulfidooxidans* might account for the high bioleaching rate (from 33.0 to 78.1%) of the group added with 0.01% yeast extract in comparison with the rate (from 31.5 to 71.2%) of the other group at 50°C (Fig. 5).

At 60°C, Fe^{3+} concentration of the group without being added yeast extract began to decrease from 8th day and Fe^{3+} concentration of the other group started to fall down from 4th day (Figs. 3, 4). The dramatic decrease of concentration might be caused by jarosite formation [21]. Even though microbial community structure was different between the two groups (Fig. 2d), Figure 5 indicated that the bioleaching rate of the two groups were both close to 100% at 28th day, respectively, 97.1 and 98.0%. This was probably because chalcopyrite lattice was broken under the effect of high temperature. Consequently, temperature was the dominant factor determining bioleaching rate at 60°C.

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