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Microbial community shifts during the process of marmatite bioleaching

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ABSTRACT

Predefined mixed cultures of *Acidithiobacillus ferrooxidans*, *Acidithiobacillus caldus* and *Acidithiobacillus thiooxidans* strains were used to stimulate marmatite bioleaching under different temperatures. Changes in this simple bacterial community over time during the leaching process were evaluated using denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments. *A. ferrooxidans* and *A. caldus* were detected in all samples taken at 5-d intervals during a 41–46 days bioleaching process. The presence of *A. thiooxidans* in the initial inoculum stimulated bioleaching, however the relative population of *A. thiooxidans* gradually declined, and this species was not detected after 30 day (at 30 °C) or 15 days (at 35 °C). Scanning electron microscopy (SEM), X-ray diffraction (XRD) and energy dispersive X-ray analysis (EDX) analyses revealed the production of S^0 and jarosite during bioleaching. Bacteria were observed to be attached to mineral surfaces. Results suggested that *A. ferrooxidans* and *A. caldus* together could perform marmatite bioleaching, and that *A. thiooxidans* could promote the bioleaching of marmatite by *A. ferrooxidans* and *A. caldus*. This study improves the understanding of marmatite bioleaching at different temperatures.

1. Introduction

Acidophilic microorganisms play an important role in bioleaching, the process whereby metals are released through the oxidation of metal sulfides by Fe^{3+}. The process is catalyzed by the action of acidophilic Fe and S oxidizing organisms (Brierley and Brierley, 2013; Vera et al., 2013). Understanding the microbiology of this process is a key to advancing commercial bioheap operations (Brierley, 2001; Rawlings, 2002). Dominant acidophiles in this process are generally thought to be *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, *Acidithiobacillus caldus* (Hallberg and Lindström, 1994), *Sulfobacillus thermosulfidooxidans* and other related species. Therefore, these iron and sulfur oxidizing microorganisms are widely used in the bioleaching of sulfide ores and flotation concentrates (Rawlings and Johnson, 2007).

Marmatite is an important source of Zn ore in China, but is difficult to process effectively by traditional technologies due to its high Fe content (Shi et al., 2005). Some recent research has focused on bioleaching of marmatite (He et al., 2009; Lan et al., 2009). However, the microbial community shifts that occur during marmatite bioleaching are poorly understood and little effort has been made to characterize the microbiological components of this process.

In addition, bioleaching rates are affected by temperature, which is a major selective pressure for the organisms that inhabit a bioleaching operation (Franzmann et al., 2005).

Therefore, in this study bioleaching of marmatite was investigated using mixed cultures of widely used acidophiles, and the effect of temperature on microbial diversity during the bioleaching process was evaluated.

2. Materials & methods

2.1. Ore sample

The marmatite sample used in this experiment was provided by the Key Laboratory of Biohydrometallurgy of the Ministry of Education, Central South University, China. The sample was ground and sieved to a particle size of 0.074–0.147 mm. Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) identified the main elemental composition of the sample as (wt.%): Zn 44.5, S 29.6, Fe 13.0, As 5.56, Pb 1.65, Sn 0.71 and Si 0.61. The sample surface was cleaned in an ultrasonic bath before use (300 W, 20 min at a mode of 10 s work + 10 s pause).

2.2. Microorganisms and culture conditions

The three microorganisms used in this study were *A. ferrooxidans* DX, *A. thiooxidans* DMC and *A. caldus* YN6, which were isolated from acid mine drainage in Dexing, Jiangxi Province, China, a coal heap drainage in Chenzhou, Hunan Province, China (Fu et al., 2008) and an acidic hot spring mine sample in Tengchong, Yunnan Province, China, respectively. The strains were identified by morphological and physiological characteristics and by comparison of their 16S rRNA gene sequences

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with 16S rRNA gene sequences in the GenBank database using the basic local alignment search tool (BLAST).

The 16S rRNA gene sequences of *A. ferrooxidans* DX, *A. thiooxidans* DMC, and *A. caldus* YN6 were submitted to the GenBank with the accession numbers DQ529310, EF091849 and DQ431196, respectively. Optimal growth pH and temperature for *A. ferrooxidans* DX were 2.0 and 30 °C, for *A. thiooxidans* DMC were 2.0–2.5 and 30 °C (Fu et al., 2008), and for *A. caldus* YN6 were 2.5 and 45 °C, respectively. Bacteria were first cultured in basal medium with the addition of $Fe_2SO_4 \cdot 7H_2O$ (44.7 g/L) as an energy source for *A. ferrooxidans* and S^0 (10 g/L) for *A. thiooxidans*/*A. caldus*. The composition of the basal medium was as follows (per L): 3 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.01 g $Ca(NO_3)_2$. All reagents were of analytical grade. The initial pH value was adjusted to 2.0 using dilute sulfuric acid. The basal medium was sterilized by high-pressure steam sterilization (121 °C, 0.1 MPa, 20 min). 44.7 g of $Fe_2SO_4 \cdot 7H_2O$ was dissolved in 200 mL sterile basal medium and sterilized by filtration (using a 0.22 μm filter) before being added into 800 mL sterile basal medium.

2.3. Leaching experiments

Bacterial cells of individual pure cultures were harvested during the log phase of growth and re-suspended together in the sterile basal medium at approximately equal numbers of cells. The final density of the mixed inoculum was 3.96×10^6 cells/mL.

Experiments using a mixture of all three cultures (*A. ferrooxidans*, *A. thiooxidans* and *A. caldus*) as the inoculum were conducted at 30 or 35 °C. Experiments with two species (*A. ferrooxidans* and *A. caldus*) as the inoculum were conducted at 35 °C. All experiments were carried out in duplicate 250-mL flasks containing 90 mL of sterile basal medium (pH 2.0), inoculated with 10% (vol/vol) inoculum and supplemented with 3% (wt/vol) surface-cleaned marmatite. The flasks were incubated on a rotary shaker (160–180 rpm) at 30 or 35 °C. The leaching experiments lasted for 41–46 days.

2.4. Analytic methods

Progression of the bioleaching was monitored through the measurement of Fe^{2+}, Fe^{3+}, Zn^{2+}, cell counts, microbial community, and pH every five days. At each sampling time, two sub-samples were prepared for analysis. The ferrous iron concentration was determined by standard potassium dichromate titration (Peng et al., 2006). The concentrations of the soluble Zn and Fe were measured by atomic absorption spectrophotometry.

Cell counts were estimated using an improved Neubauer cell counting chamber and light microscopy (Olympus CX31, Olympus Corporation, Japan). The pH value in the leaching solution was measured with a pH-meter (PHSJ-4A). The microbial community during the bioleaching process was studied using 16S rRNA-based DGGE. To understand the leaching process of marmatite, the surface morphology, elemental content and chemical composition of the mineral were analyzed by scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX) and X-ray diffraction (XRD).

2.5. Statistical methods

Multivariate analysis of variance (MANOVA) (Weinfurt, 1995) was used to assess the statistical significance of the effect of temperature on bioleaching. Temperature was one of the independent variables with two levels (30 °C and 35 °C), and bioleaching time was another independent variable with eight levels (5 days, 10 days, etc.). The pH value, cell count, Zn^{2+} concentration and total Fe concentration were four dependent variables.

2.6. Preparation of DNA

Extraction of nucleic acids was performed according to the procedure described by Zhou et al. (1996). Firstly, the pulp was centrifuged at 10,000 ×*g* for 15 min and the pellet was mixed with sterilized sand and liquid nitrogen, and ground to powder in a mortar. The mixture was then mixed with 13.5 mL of extraction buffer (0.1 M Na_3PO_4 [pH 8.0], 0.1 M Tris–HCl [pH 8.0], 0.1 M EDTA [pH 8.0], 1.5 M NaCl, and 1% hexadecylmethylammonium bromide) and 50 μL of proteinase K (10 mg/mL). This mixture was transferred into a 50-mL centrifuge tube and incubated at 37 °C for 30 min. 1.5 mL of 20% (wt/vol) sodium dodecyl sulfate was added and mixed gently, then incubated at 65 °C for 2 h. The mixture was centrifuged and the supernatant was transferred into a new 50-mL centrifuge tube. The crude DNA was purified using Wizard Plus SV Minipreps DNA Purification System (Promega Corporation, USA) and quantified by ethidium bromide-UV detection on an agarose gel.

2.7. Primers and PCR amplification

16S rRNA gene fragments were amplified with primers 357F (5′-CCTACGGGAGGCAGCAG- 3′) and 517R (5′- GTGCCAGC(A/C)GCCGCGG- 3′), which are specific for bacterial 16S rRNA gene sequences. To provide more stable denaturation behavior of DNA fragments in DGGE, a 40-bp GC-rich sequence (GC-clamp): 5′- CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGG- 3′ was attached to the 5′-end of primer 357F. The 16S rRNA gene fragments (about 230 bp) suitable for subsequent DGGE analysis were obtained by PCR as previously described (Casamayor et al., 2002; Muyzer et al., 2004). PCR amplification reactions were performed on a T-Gradient Thermoblock (Biometra, Germany) using reagents purchased from Tiangen Biotech Co., Ltd. (Beijing, China). A hot start PCR was performed at 95 °C for 5 min and a touchdown PCR was performed for 20 cycles with the annealing temperature initially set at 65 °C and then decreased by 0.5 °C every cycle until it was 55 °C; then 15 additional cycles were carried out at 55 °C. The denaturing step was 1 min at 94 °C, the primer annealing step was 1 min at the touchdown temperatures described above, and primer extension was 1 min at 72 °C. The final extension step was 10 min at 72 °C.

2.8. DGGE analysis

Cells of three microorganisms used in the study (*A. ferrooxidans*, *A. thiooxidans* and *A. caldus*) were first collected separately for DGGE-PCR amplification, and then the three PCR products were mixed as a marker standard. DGGE was optimized by changing run conditions to best distinguish the three species. A gel with a 40%–70% gradient of denaturant, run at 200 V for 6 h resolved the three products well.

DGGE analysis was performed using a model DGGE-1 2001 electrophoresis system (C.B.S Scientific Company Inc., CA.) with a denaturing gradient of 40–70% in a 7.5% polyacrylamide gel, following the manufacturer's instructions. 15 μL of PCR products was mixed with 5 μL of 10× sucrose loading buffer, and DNA fragments were separated for 6 h at 200 V and 60 °C. The gel was stained for 20 min with 0.5 μg/mL ethidium bromide and gel image was captured using UV transillumination on a VisiDoc-It imaging system (UVP, America).

3. Results and discussion

3.1. Variations of pH, cell counts, and metal concentrations

Variations of pH, cell counts and concentration changes of Zn^{2+} as well as total Fe over time are shown in Fig. 1(a, b, c and d).

Fig. 1(a) indicates that temperature had a great effect on pH during the bioleaching process. At 30 °C, the pH value rose to a peak of 3.0 in the first 10 days and then declined afterwards. By the 45th day the pH was below 1.6. At 35 °C, the pH reached a peak value of 2.9 after only