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Isolation and characterization of acidophilic bacterium from Gaofeng Mine in China

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Abstract: An acidophilic, chemolithotrophic and ferrous oxidizing bacterium strain GF was isolated from the acid mine drainage (AMD) of Gaofeng Mine, Guangxi Province, China using 9K enrichment medium, and then purified on solid ferrous-agarose medium. The physiological experiments show that it can use ferrous or sulfur as sole energy and a low level (0.1%, w/v) of peptone can accelerate the growth of the isolated strain. The optimum pH and temperature for growth are 2.0 and 30 ℃, respectively. The isolated strain shares 99.64% identities of 16S rRNA gene with the type strain *Acidthiobacillus ferrooxidans* ATCC 23270 and 100% identities of *iro* gene (CDS) with *A. ferrooxidans* strain Fe-1. These results show that the strain can be considered as *Acidthiobacillus ferrooxidans*. Because of the high activity of oxidizing ferrous and sulfide mineral, strain GF was used in bioleaching of marmatite. The Zn concentration is 0.273 g/L under the steriled control and 7.30 g/L with adapted GF strain incubated after 29 d in leaching marmatite. The isolated strain GF can be used to leach marmatite in industry application.

Key words: acid mine drainage; *Acidthiobacillus ferrooxidans*; iron-oxidizing bacteria; bioleaching; marmatite

1 Introduction

Highly acidic environment formed by the oxidation of pyrite and other sulfidic minerals is known to be populated by a range of acidophilic and acid-tolerant prokaryotic and eukaryotic life forms. The most studied is *Acidithiobacillus ferroxidans*. *A. ferrooxidans* is an acidiophilic chemolithotrophic gramnegative bacteria, which can derive energy from the oxidation of ferrous, elemental sulfur and its reduced compounds, and sulfides. This peculiar metabolism is important in highly acidic environments[1], metal bioleaching[2−5], and desulfurization of coals [6−7]. In spite of the interest in *A. ferrooxidans* as a potentially useful microorganism for bioleaching, its low growth rate and environment tolerance limited its biotechnological applications. Thus, to isolate more active strains for the practical applications described above is the urgent affairs especially for complicated metal sulfide mines.

In this work, the isolation, characterization and bioleaching of one *A. ferrooxidans* GF was investigated, which was found to be a sulfur-oxidizer and ferrous-oxidizer from Gaofeng Mine, Guangxi Province, China.

2 Experimental

2.1 Enrichment and isolation

Strain GF was isolated from the sample collected aseptically in the acid mine drainage(AMD) of Gaofeng Mine located in Guangxi Province, China. Filter-sterilized 9K medium[8] of pH 2.0 containing inorganic salts with ferrous iron was used for enrichment and maintenance. The bacterium was also grown in iron-free 9K medium of pH 3.0 with 1% sulphur (w/v) as energy source. Erlenmeyer flasks of 250 mL capacity containing 100 mL sterile 9K medium were incubated at 30 ℃ under rotary shaking condition(170 r/min). Then the ferrous-agarose solid medium plate was used for isolation.

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2.2 Analytical determination

Ferrous iron concentration was determined by complex metric titration using potassium dichromate[9]. The pH value was determined by PHS-25 pH meter (CADIHO, Shanghai, China). The population of the bacteria was counted by haemacytometer under binocular microscope for enumerate generation. Gram straining was done according to routine method. The scanning electron micrograph(SEM) observation was taken in College of Life Science, Hunan Normal University, China.

2.3 Physiological experiments

The optimum pH and temperature of the strain GF were determined in pH- and temperature-controlled cultures. The isolated GF strain was grown in 9K medium with the culture maintained at pH 2.0 (to determine the optimum temperature) or 30 ℃ (to determine the optimum pH). The following organic compounds were tested as possible substrates at different concentrations with or without ferrous. Growth was estimated after incubation for 48 h. Isolated GF grew in basal salt medium (9K without ferrous) supplemented with peptone (0.1%), glucose (0.1%), sodium thiosulfate (1.0%), sulphur powder (5%), $FeSO_4·7H_2O$ (14.7%), $FeSO_4·7H_2O$ (14.7%)+peptone (1.0%), $FeSO_4·7H_2O$ (14.7%)+glucose (0.1%), respectively. All supplements were asepticized.

2.4 DNA extraction and amplification

The sample was prepared from 150 mL iron medium culture, harvested and washed several times with H_2SO_4(pH=2.0) to remove precipitates, then the chromosomal DNA was extracted by using the Genomic DNA Isolating Kit(SK1201) of Sangon Company (Shanghai, China).

The fragments of *iro* were amplified by PCR using TGRADIENT (Biometra, Germany). Primers (YT1 and YT2) were designed via the reference sequence (accession number: E03451)[10] and synthesized by Sangon Company (Shanghai, China). The conditions used for PCR were: 10×dNTPs(2 mmol/L each) 5 μL, 10×PCR buffer 5 μL, target DNA (0.1 u g/μL) 2 μL, enzyme mixture(Taq DNA polymerase: Pfu DNA polymerase=1:1) 1 μL, primers 10 pmol and the reaction mixture made to a final volume of 50 μL with deionised water. Thermal cycling was as follows: an initial step of 5 min at 94 ℃, followed by 35 cycles of 45 s at 94 ℃, 30 s at 59 ℃, 40 s at 72 ℃, and then 7 min at 72 ℃.

Primers:YT1: 5'-CTCTGACCGGCGAATCGGG-3'; YT2: 5'-CCAACCGCATCCGCATATCTTG-3'.

The 16S rRNA gene was amplified from the genomic DNA using universal bacterial primers F27 and R1492[11]. The conditions used for PCR were: 10× dNTPs(2 mmol/L each) 5 μL, 10×PCR buffer 5 μL, target DNA(0.1 μg/μL) 2 μL, enzyme mixture (Taq DNA polymerase: Pfu DNA polymerase=1:1) 1 μL, primers 10 pmol and the reaction mixture made to a final volume of 50 μL with deionised water. Thermal cycling was as follows: an initial step of 5 min at 94 ℃, followed by 30 cycles of 45 s at 94 ℃, 45 s at 65 ℃, 90 s at 72 ℃, and then 7 min at 72 ℃.

2.5 Cloning and sequencing

The PCR products were purified by E.Z.N.A^{TM} Gel extraction Kit(OMEGA,USA). The purified products were cloned into PCR2.1 vector using TA Cloning Kit (Invitrogen, San Diego, CA). Clones were sequenced by Sunbiotech Company (Beijing, China), as M13 forward and reverse primers were used in sequencing reactions. *Iro* gene sequences were analyzed and compared with the reference sequence (E03451) by Clustal X. 16S rDNA sequences were also analyzed and aligned with other sequences deposited in Genbank by Clustal X[12]. The sequence data were compared with 16S rRNA gene sequences deposited in Genbank by using the BLAST search program.

2.6 Bioleaching of marmatite

The oxidation of marmatite by isolated strain GF was assessed by growing cultures in 250 mL shaking flasks, each of which contained 5% marmatite(w/v). Incubate 10 mL isolated strain GF of 10^7 (cell/mL) in 90 mL liquid cultures shaken at 170 r/min, at 30 ℃. The granularity of marmatite was 104−147 μm. The composition of the marmatite is shown in Table 1.

Table 1 Component of marmatite (mass fraction, %)

Zn	Fe	S	Others
47.4	17.17	34.43	1

3 Results and discussion

Strain GF was isolated from the water sample of pH value 1.75. The water sample was rich of elements of Fe, As and Zn, and the idiographic result is shown in Table 2. The color of the pool for sampling was maroon (shown in Fig.1).

Table 2 Elements analysis of sampled point

Element	Content/(mg·L^{-1})	Element	Content/(mg·L^{-1})
Cu	104.45	Fe	121 250
Pb	0.95	Zn	18 500
Mn	882.5	As	21 700
Co	34.09	Ni	50
Cd	64	Mg	764.5

Fig.1 Physiognomy of sampling site

Enrichment and purification were used in the isolation of the iron-oxidizing bacteria *A. ferrooxidans*-like strain named GF, which was identified by its cultural, morphological and physiological characteristics. Gram straining showed that strain GF was gram negative. And strain GF grew in both ferrous and sulfur liquid culture. In solid culture with KSCN and ferrous, colonies (pointed by the arrowhead) of strain GF reached a diameter of 0.90−1.20 mm (shown in Fig.2). Similar results were reported by LAVALLE et al[13] for the size of colonies, but the colonies of strain GF became brown in only 48 h but not 2 weeks.

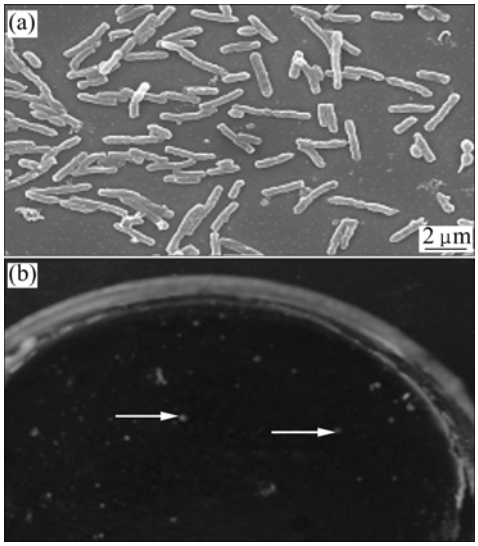

Fig.2 SEM images of strain GF (a) and colonies on plate(b)

The microscopic examination of strain GF isolated from solid medium showed that they were single or in pairs long rod shape cells. Using SEM the cellular dimensions were determined to be (0.4±0.1) μm × (3.5±0.2) μm (shown in Fig.2), which was much larger than that reported before[14].

At the optimal pH of 2.0 for growth, strain GF grew in a temperature range of 25−40 ℃. The results show that the optimum was at 30 ℃ (shown in Fig.3(a)). At the optimal temperature, the strain GF grew in the tested pH range of 1.0−4.0, and the optimum pH of 2.0 is obtained (shown in Fig.3(b)).

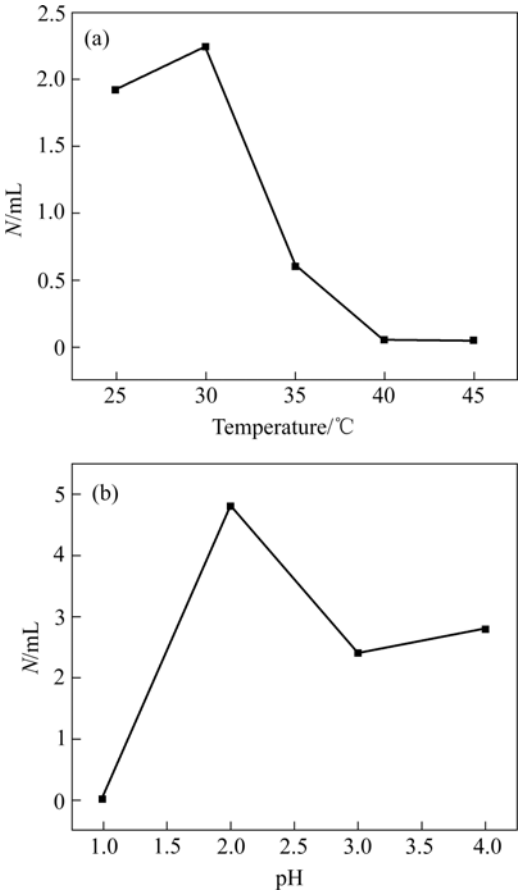

Fig.3 Effects of temperature (a) and pH (b) on growth of strain GF

The specific growth rate μ and generation time G were calculated from the exponential phases by non-lineal regression using the growth yield ($Y_{X/S}$) and the values of ferrous-oxidation rate. The value of growth rate μ and generation time G for this strain were 0.0 139 yh and 5.00 h, respectively. The result is accordant with other reports about *A.ferrooxidans*[3−15].

Strain GF cultured for long time in 9K medium plus Fe^{2+} was used as the inoculum in the studies of the adaptation to organic substrates such as glucose and peptone. The result showed that the activity of strain GF descended along with the ascending of concentration of glucose. When the concentration of peptone was at a low level (0.1%, w/v), the growth of strain GF was increased. But the growth dropped quickly when the concentration of peptone exceeded 0.5% (w/v). It is shown that a high concentration of peptone would inhibit the growth of stain GF.

The growth of strain GF on other inorganic substrates such as sodium thiosulfate and sulfur was also studied. Strain GF could not grow on 9K basal salt medium with sodium thiosulfate, indicating that sodium

thiosulfate could not be used as the sole energy source of strain GF. The ferrous oxidation rates of strain GF were evaluated in terms of permanganimetry. Fig.4(a) shows the time course of ferrous oxidation of strain GF in 9K medium, in which ferrous was used as the sole energy source; while Fig.4(b) shows the pH descend in 9K basal salt medium supplemented with sulfur as the sole energy source. It was found that strain GF had a high ability of oxidating ferrous. In the first 8 h, the ferrous oxidation was unconspicuous compared with that of the control. At the following 16 h, it began to represent the difference to the control. From 28 h, the uptrend was very obvious and at 45 h the ferrous oxidation was completed. The curve tally with the growth curve very well, which has lag phase and exponential phase. Furthermore, strain GF was a sulfur-oxidizer and could grow using sulfur as the sole energy source. And the growth could make the pH value of the medium decrease along with the time. It was obvious that the metabolism of the isolated strain GF produced H^+ to make pH descend.

The partial sequence consisting of anterior 838 bases of the amplified 16S rDNA of strain GF (DQ062115.1, Genbank) was determined. It shared 99.64% identities with the type strain of *A. ferrooxidans* ATCC 23270 (AF465604.1, Genbank) for 16S rRNA gene. Phylogenetic analysis was carried out using the sequence of strain GF and the sequences within thiobacillus genus and other close relatives from Genbank. A maximum likelihood tree generated by CLUSTALX is shown in Fig.5. Strain GF was found cluster tightly with the type strain *A. ferrooxidans* ATCC 23270.

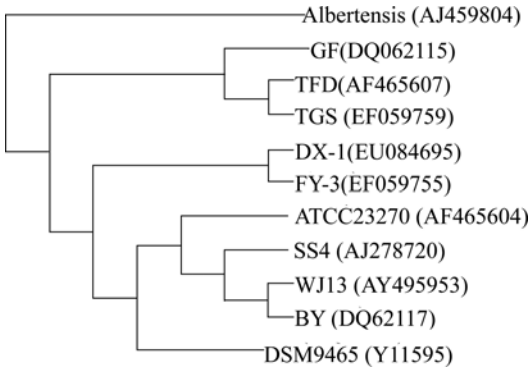

Fig.5 Phylogenetic tree derived from 16S rDNA sequence of strain GF

The *Iro* protein of *A. ferrooxidans* was proposed to be the first electron acceptor in several alternative models of electron transfer chain between Fe^{2+} and oxygen[16]. It played an important role in the energy metabolism of *A. ferrooxidans*[17−19]. So the partial sequence (CDS, DQ062113, Genbank) of *iro* gene was cloned and sequenced. It shared 100% identities with the reference sequence of *A.ferrooxidans* strain Fe-1 (E03451, Genbank)[19].

Marmatite is an important zinc ore in China, but it is difficult to be processed effectively by traditional technologies due to its high content of iron[20]. The bioleaching of marmatite flotation concentrate by *A. ferrooxidans* has been proved to be very useful in mineral processing[21]. Two leaching runs were used for investigating the leaching efficiency of strain GF: the steriled control and the adapted strain. The experiment results are shown in Fig.6. The extraction of zinc was markedly different between them at the very start. The Zn concentration was 0.273 g/L under the steriled control and 7.302 g/L with the adapted strain GF incubated for

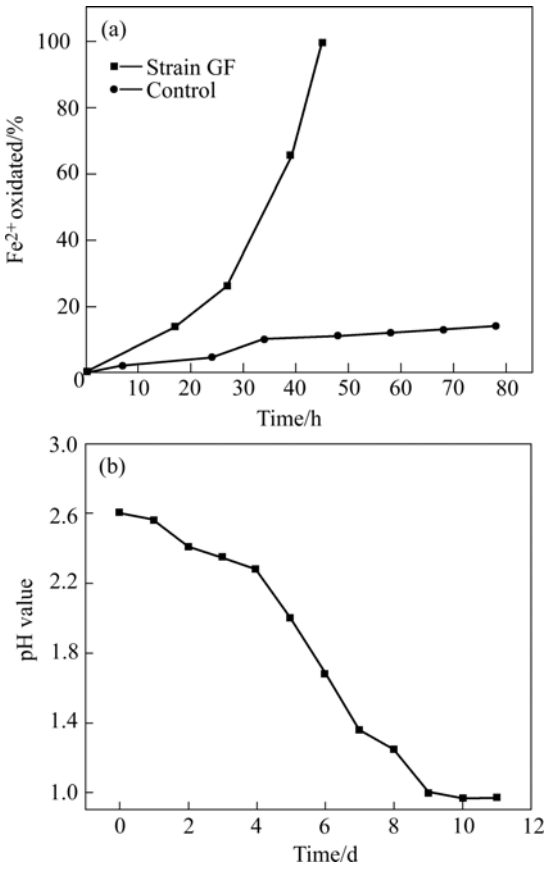

Fig.4 Ferrous iron(a) and sulfur oxidation (b) by strain GF

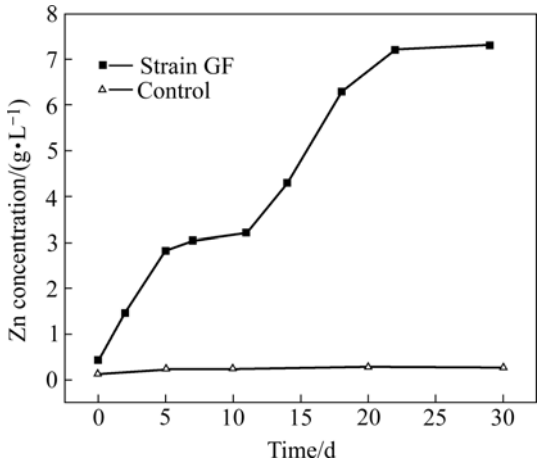

Fig.6 Zinc extraction from marmatite concentrate by strain GF

29 d. Strain GF could increase markedly the leaching rate of marmatite compared with the steriled control.

4 Conclusions

1) One *Acidthiobacillus ferrooxidans* strain GF was isolated from the AMD of Gaofeng Mine, Guangxi Province, China. It was gram negative, and the cellular dimensions was (0.4±0.1) μm × (3.5±0.2) μm. The optimum pH and temperature for growth were 2.0 and 30 ℃, respectively.

2) Strain GF could use ferrous and sulfur as the sole energy source, and a low level (0.1%, w/v) of peptone could accelerate the growth. It had a high ability of oxidating ferrous and sulfate.

3) Strain GF shared 99.64% identities of 16S rRNA gene with the type strain *A. ferrooxidans* ATCC 23270 and 100% identities of *iro* gene (CDS) with *A. ferrooxidans* Fe-1.

4) The adapted strain GF could increase markedly the leaching rate of marmatite compared with the steriled control, and can be used in leaching marmatite in industry application.

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