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Isolation and characterization of acidophilic bacterium from Dongxiangshan Mine in Xinjiang Province, China

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Abstract: One bioleaching bacterium, named as strain DXS, was isolated from acid mine drainages (AMDs) of Dongxiangshan Mine of Hami, Xinjiang Province, China. The strain DXS is gram-negative and rod-shaped with a size of (0.40±0.05) μm×(1.3±0.5) μm. The optimal temperature and pH for growth are 30 ℃ and pH 2.0, respectively. It can grow autotrophically by using ferrous iron, elemental sulfur and $Na_2S_2O_3$ as sole energy sources. In the phylogenetic tree, strain DXS has similarity with *Acidithiobacillus ferrooxidans* type strain ATCC 23270 with 99.57% sequence similarity. The cloning and sequencing of Iro protein gene (*iro*) and tetrathionate hydrolase gene (*tth*) reveal that strain DXS is completely identical in *iro* gene sequence to *A. ferrooxidans* LY (DQ166841), and almost identical in *tth* gene sequene to *A. ferrooxidans* (AB259312) (only two nucleotides change). The bioleaching experiments of marmatite and pyrite reveal that the leached zinc and iron concentrations reach 3.01 g/L and 2.75 g/L, respectively. The strain has a well potential application in industry bioleaching.

Key words: *Acidithiobacillus ferrooxidans*; bioleaching; *iro* and *tth* gene; 16S rRNA

1 Introduction

Bacterial leaching of metal sulfides has developed rapidly in the last decades, and it is often considered more environmentally friendly and economical than other available technologies [1−4]. Recovering metals from sulphide minerals by acidophiles has developed into a successful and expanding area of biotechnology. Up to now, many bioleaching microbes have been found, such as the genus *Acidithiobacillus*, *Leptospirillum*, *Ferroplasma*, *Sulfobacillus* and *Sulfolobus*. The classical leaching bacteria now belong to the genus *Acidithiobacillus*, mainly including *A. ferrooxidans*, *A. thiooxidans*, *A. albertensis* and *A. caldus* [5−7].

Acidithiobacillus ferrooxidans (*A. ferrooxidans*, formerly named *Thiobacillus ferrooxidans*) is an acidophilic chemolithotrophic gram-negative bacterium, which widely exists in mesophilic bioleaching systems. *A. ferrooxidans* has attracted great interest because of its use in industrial mineral processing and unusual physiology. It can oxidize ferrous iron, elemental sulfur, reduced sulfur compounds and sulfide minerals. This ability makes it suitable for biomining to recover metals such as copper, gold and uranium [8].

The remarkable feature of the species *A. ferrooxidans* is that it derives energy from the oxidation of ferrous iron or reduced sulfur compounds. This special metabolism is important in highly acidic environments and metal bioleaching.

Based on the great importance of *A. ferrooxidans* in hydrometallurgy, the isolation of more efficient strains for the practical applications is urgent affair especially for complicated metal sulfide minerals. A number of *A. ferrooxidans* were isolated from acidic water samples such as acidic mine drainages and acidic hot springs all over the world, and these, as a result, improved leaching rate of metal sulfides [9−11].

In present work, a study was undertaken to identify a newly isolated *A. ferrooxidans* strain DXS from the acid mine drainages of Dongxiangshan Mine of Hami, Xinjiang Province, China. A series of morphological and biochemical characterizations as well as the analysis of 16S rRNA sequences were done.

2 Experimental

2.1 Strain isolation

A water sample was collected from acid mine drainages of copper mines with a temperature of 20 ℃

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and pH 3.0, located at Hami, Xinjiang Province China. Isolation and purification were done as follows: the sample was inoculated onto the ferrous-agarose solid medium plate, and incubated at 30 ℃ for 8−10 d until the colonies appeared. A single colony was then picked out for next inoculation. The transfer was repeated until the pure culture was obtained. Their identities were confirmed based on their phenotypic characteristics and 16S rRNA sequences.

2.2 Growth conditions

If not stated otherwise, strain DXS was cultivated in 250 mL flasks with 100 mL modified medium 9K. 9K medium was prepared as follows: solution A (700 mL): 3 g/L $(NH_4)_2SO_4$, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.1 g/L KCl, 0.01 g/L $Ca(NO_3)_2$; solution B (300 mL): 44.7 g/L $FeSO_4·7H_2O$ or 5.0 g/L S^0. Solutions A and B were mixed after being autoclaved separately. The initial pH of the medium was adjusted to 2.0 and 2.5. Ferrous-agarose solid plates were prepared by adding 1% agar powder to the liquid 9K medium above. When a parameter that influenced the growth of the *A. ferrooxidans* was changed, other parameters were kept at optimum. Strains were cultivated in 250 mL flasks containing 100 mL of 9K medium on a shaker at 180 r/min and 30 ℃. The inoculum size comprised 2% (volume fraction) of total culture and the bacteria were cultivated to the stationary growth phase.

2.3 Microscopic studies

Cell motile behavior was observed using Olympus CX 31 optical microscope, and Gram staining was performed using a Gram stain reagent kit (Haitai Biotech, China). Fine morphological features were revealed by scanning electron microscope (SEM, JEOL JSM−6360 LV) as described previously [12].

2.4 Biochemical and physiological experiments

The optimum pH and temperature of the strain DXS were determined in pH- and temperature-controlled cultures, respectively. The isolated DXS grew 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 iron. Growth was estimated after incubation for 72 h. The possible substrates used were as follows: 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%), pyrite (0.5%) and marmatite (0.5%), respectively. All supplements were asepticized.

2.5 Sulfur or ferrous iron oxidizing activity

The oxidizing activity of ferrous iron and elemental sulfur was comparatively studied with the *A. ferrooxidans* ATCC 23270. When ferrous iron was used as the sole energy source, the content of Fe^{3+} in the medium was determined as described by YANG et al [13]. When elemental sulfur was used as the sole energy source, the concentration of sulfate of the medium was measured as described by ZHANG et al [14].

2.6 Genomic DNA extraction and phylogenetic analysis of 16S rRNA

Genomic DNA was extracted using an EZ−10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc., Canada) according to the introduction of the kit. The primers for amplification of 16S rRNA were designed based on the previous report [15]. The PCR amplification was carried out according to the method described by PENG et al [16]. When the PCR program was finished, the PCR product was separated by gel electrophoresis on a 1.0% agar gel in tris/acetate-buffer and analyzed by staining with ethidium bromide (EB) under UV light. The band of the expected size (approximately 1 400 bp) was cut off and purified with a commercial kit (Gel Extraction Kit, Promega, USA). The purified PCR product was ligated into pGM-T vector(Tiangen Biotech, China). The positive clone containing insert was screened out by colony PCR and sequenced by Sunbiotech Company (Beijing, China).

The nucleotide sequence was searched for homology by BlastN at the NCBI server (http:// ww.ncbi.nlm.nih.gov/BLAST/Blast) and submitted to Genbank (Table 1). All available subsets of 16S rRNA gene sequences were selected, analyzed and aligned with CLUSTALX 1.8 [17], and the final phylogenetic tree was generated by MEGA 4.0.

2.7 Cloning and sequencing of *iro* and *tth* genes

The *iro* and *tth* genes were amplified by PCR. Two

Table 1 Primers used in this work

Gene	Nucleotide sequences of primers	Genbank accession number
16S rRNA	Forward: 5′-CAGGCCTAACACATGCAAGTC-3′ Reverse: 5′-TACCT GGGCGGWGTGTACAAGGC-3′	DQ427103
iro	Forward: 5′-CTCTGACCGGCGAATCGGG-3′ Reverse: 5′-CCAACCGCATCCGCATATCTTG-3′	FJ640051
tth	Forward: 5′-ATGCCAAGTATTGTACGTAACC-3′ Reverse: 5′-CTAACTGCCATGGCTTATCG-3′	FJ638613

pairs of primers were designed based on the nucleotide sequences of *iro* and *tth* genes of type strain ATCC 23270 (Table 1). The PCR program consisted of one cycle of DNA denaturation for 3 min at 95 ℃, and then 35 cycles were performed as follows: 45 s at 94℃ to denature, 30 s at 55 ℃ to anneal and 60 or 90 s at 72 ℃ to extend. The PCR products were purified and sequenced as described above. The Genbank accession number of each sequence is shown in Table 1.

2.8 Bioleaching of marmatite and pyrite

Marmatite and chalcopyrite used in this experiment were provided by Institute of Minerals Processing Engineering, Central South University, China. The marmatite contains 48.4% of Zn, 20.2% of S^0 and 1.1% of Fe, and the pyrite contains 44.8% of Fe, 47.8% of S^0, and 0.3% of Cu.

Bioleaching tests were carried out in 250 mL flasks containing 100 mL 9K medium. The mineral concentration was 3% (mass fraction). The inoculum of strain DXS culture was 10% (volume fraction), and all the experiments were carried out in triplicate. Abiotic controls were also designed by replacing the bacterial inoculum by an equal volume of related medium. Aliquots of leachate were sampled, and the concentrations of zinc and iron were determined by atomic absorption spectrometry (Hitachi Z−8000) within 28 d of incubation. The lost water in the medium was supplemented with sterilized deionized water after sampling each time.

3 Results and discussion

3.1 Isolation and morphological observation

By streak plate method, a mesophilic acidophilic bacterium named strain DXS was isolated from Dongxiangshan Mine, Xinjiang Province, China. Single colonies appear within 10 d at 30 ℃. Colonies of the isolate are circular, convex and maroon (Fig.1(a)). It is gram-negative, long rod-shaped. The cell of strain DXS is 0.8−1.5 μm in length and about 0.4 μm in diameter. The morphological character is shown in Fig.1(b).

3.2 Biochemical and physiological characteristics

3.2.1 Temperature and pH

The growth status of strain DXS at different temperatures is shown in Fig.2. It is indicated that the strain is mesophilic and able to grow in a temperature range from 20 to 35 ℃, with an optimum at 30 ℃. No growth is detected at 45 ℃ or at the temperature higher than 45 ℃ after 7 d of inoculation. Strain DXS grows in a pH range from 1.0 to 3.0, with optimal growth at pH 2.0 (Fig.3), but no growth is detected at pH lower than 0.5 or higher than 3.5.

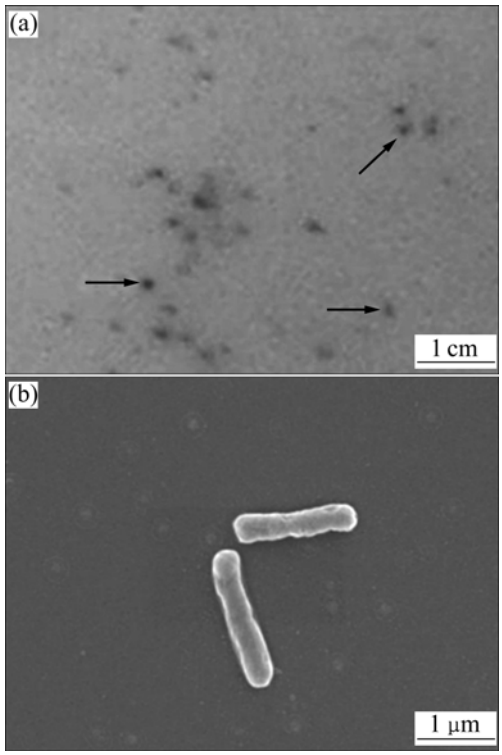

Fig.1 Colonies (a) and scanning electron micrograph (b) of strain DXS

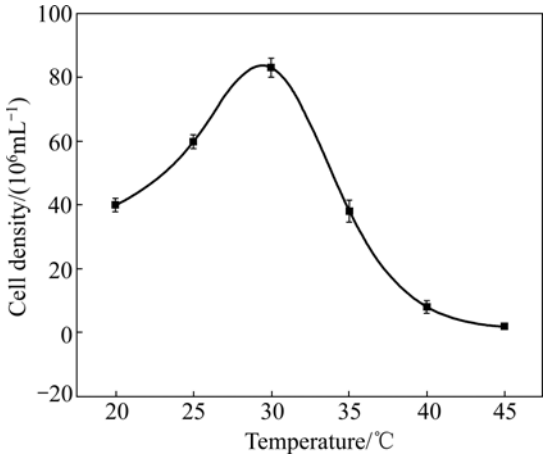

Fig.2 Effect of temperature on growth of strain DXS

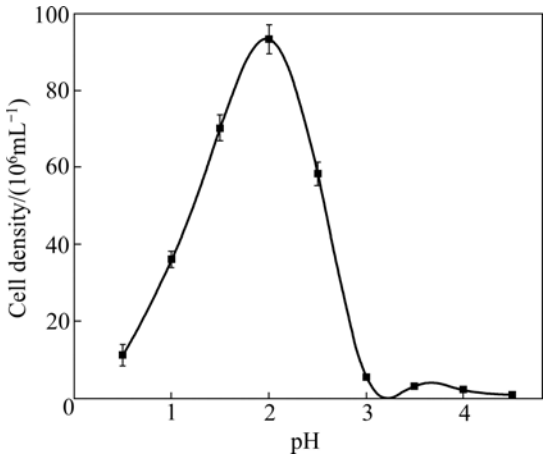

Fig.3 Effect of pH on growth of strain DXS

According to the optimum growth parameters as mentioned above, experiment was carried out in the optimal growth condition of strain DXS. The experiment performed at 30 ℃, pH 2.0 and 180 r/min, 5% of the seed culture of stable growth stage was inoculated to medium with addition of 14.7% $FeSO_4·7H_2O$ or 0.5% S^0. The growth curves of isolate DXS are shown in Fig.4. The data show that the strain can be activated in a short time, and the number of cells reaches the maximum (about 5.2×10^7 mL^{-1}) after 40 h when grown on $FeSO_4·7H_2O$. When grown on S^0, the strain requires 160 h to reach the maximum, however, the bacterial density reaches 10.1×10^7 mL^{-1}, which is much higher than that grown on $FeSO_4·7H_2O$.

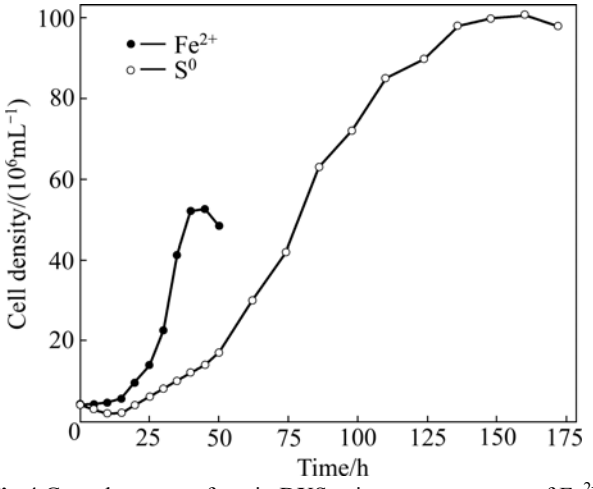

Fig.4 Growth curves of strain DXS using energy source of Fe^{2+} or S^0

3.2.2 Utilization of substrates

Strain DXS can grow autotrophically by using ferrous iron or elemental sulfur as sole energy source. The strain is sensitive to organic acids and other small molecular organic compounds, which can inhibit the growth of these microbes. In addition, strain DXS can grow by oxidizing pyrite and marmatite.

3.2.3 Sulfur and ferrous iron oxidizing activity

The oxidizing activities of ferrous iron and sulfur of strain DXS are shown in Fig.5 and Fig.6, respectively. This indicates that strain DXS shows a higher oxidation activity than the *A. ferrooxidans* ATCC 23270 when using sulfur or ferrous iron, especially ferrous iron.

3.3 Phylogenetic analysis

The nearly complete sequence consisting of 1 395 bases of the amplified 16S rRNA of strain DXS was determined. 16S rRNA sequence analysis indicates that the closest relative of strain DXS is *A. ferrooxidans*, with a 16S rRNA sequence similarity over 99%. Phylogenetic tree based on 16S rRNA sequences of DXS and the species within genus *Acidithiobacillus* was constructed

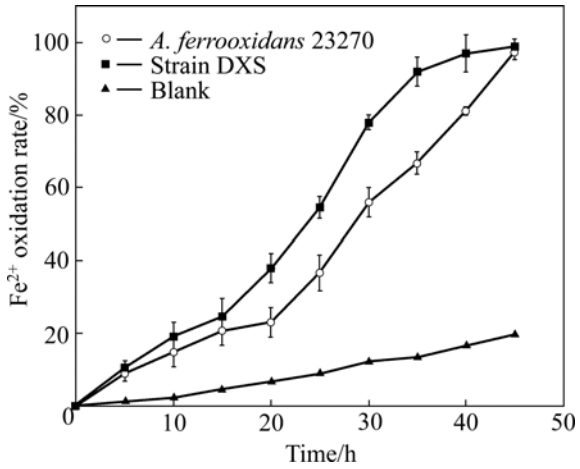

Fig.5 Ferrous iron oxidation of strain DXS

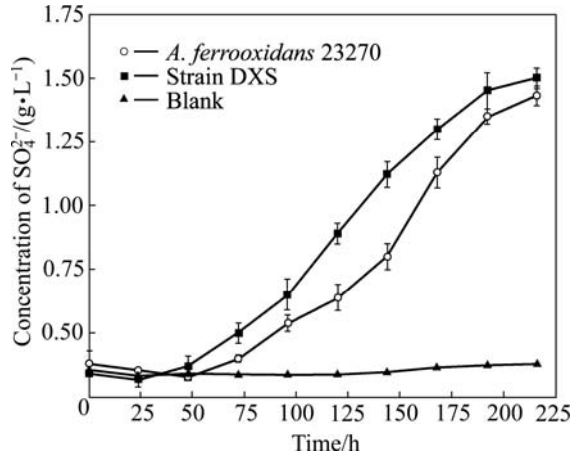

Fig.6 Sulfur oxidation of strain DXS

using softwares of Clustal X1.8 and MEGA 4.0 (Fig.7). The results also clearly show that strain DXS is most closely related to *A. ferrooxidans*.

3.4 Cloning and sequencing of *iro* and *tth* genes

The protein encoded by the *iro* gene (Iro protein) is considered to be a member of the high-potential iron-sulfur protein (HiPIP) family. The Iro protein has been proposed to be the first electron carrier in the ferrous iron respiratory chain between ferrous iron and oxygen [18]. However, recent studies have suggested that the Iro protein is involved in the electron transfer chain by transferring electrons from a cytochrome bc_1 complex to a terminal oxidase [19]. These studies suggested an important role for the Iro protein in the oxidation of Fe^{2+}, although its exact physiological role has not been determined [18−19].

Tetrathionate is one of the most important intermediates in dissimilatory sulfur oxidation and can itself be utilized as a sole energy source by some sulfur-oxidizing microorganisms. Tetrathionate hydrolase plays a significant role in tetrathionate oxidation and should catalyze the initial step in the oxidative dissimilation

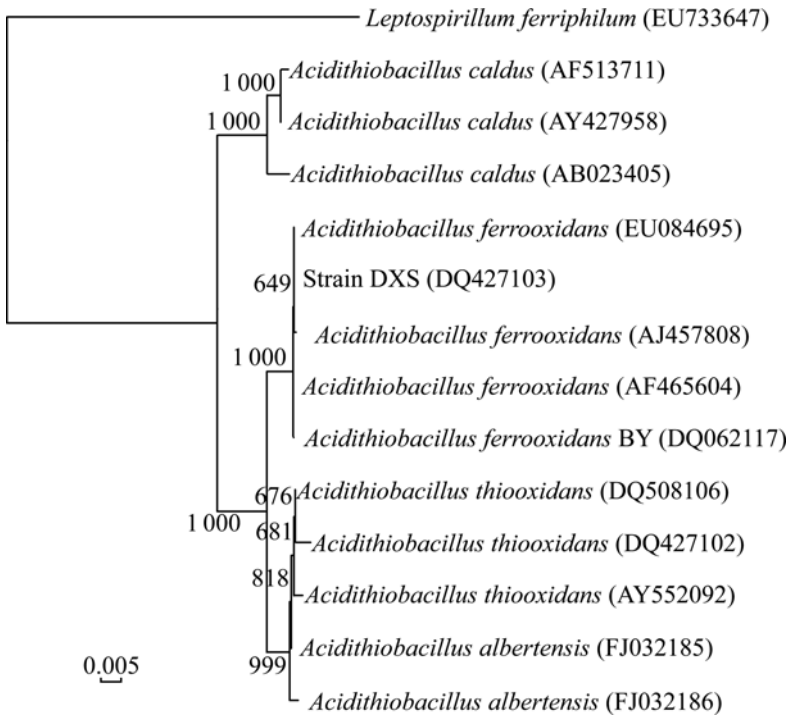

Fig.7 Phylogenetic tree based on 16 rRNA gene sequences of strain DXS and species within *Acidithiobacillus* (GenBank accession numbers are shown in parentheses and numbers depict bootstrap values obtained for a bootstrap sampling of 1 000)

when sulfur-oxidizing bacteria grow on tetrathionate [20]. Investigations of the detailed regulatory mechanism for the expression of *Af-tth* and the synthesis of recombinant Tth enzyme as an active form are now in progress to clarify the mechanism of dissimilatory sulfur oxidation in *A. ferrooxidans*.

In this work, PCR amplification of *iro* and *tth* genes was performed with the gene specific primers. The results reveal that *iro* and *tth* gene fragments of the expected size can be amplified from the genomic DNA of strain DXS, and the nucleotide sequence of the *iro* gene from strain DXS is completely identical to that of strain LY (DQ166841). The nucleotide sequence of the *tth* gene of strain DXS is almost identical (only two nucleotides changed) to that of *A. ferrooxidans* (AB259312). The nucleotide sequences of the *iro* and *tth* genes were submitted to Genbank (Table 1).

3.5 Bioleaching of marmatite and pyrite

Bioleaching of marmatite with *A. ferrooxidans* DXS is shown in Fig.8. In the whole process, zinc extraction rate continuously increases. In the first 18 d, zinc extraction rate increases quickly and after then zinc concentration reaches 2.81 g/L, However, zinc concentration leached by *A. ferrooxidans* ATCC 23270 reaches 1.85 g/L. From the 20th day to the 28th day, zinc extraction becomes slow. The final zinc concentration is 3.01 g/L.

The bioleaching results of pyrite by *A. ferrooxidans* DXS are shown in Fig.9. In the leaching process, total

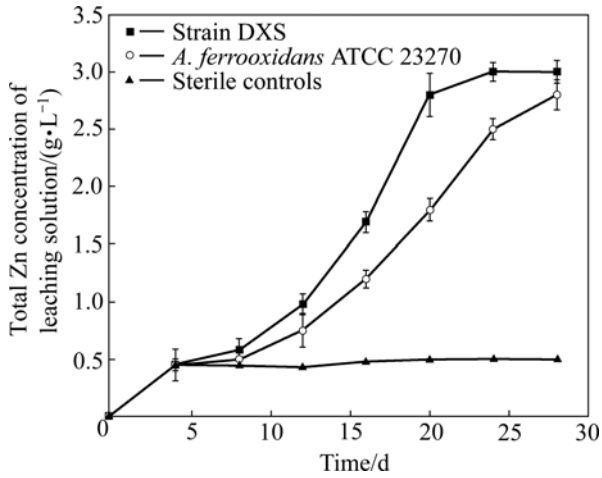

Fig.8 Zinc extraction from marmatite by *A. ferrooxidans* DXS

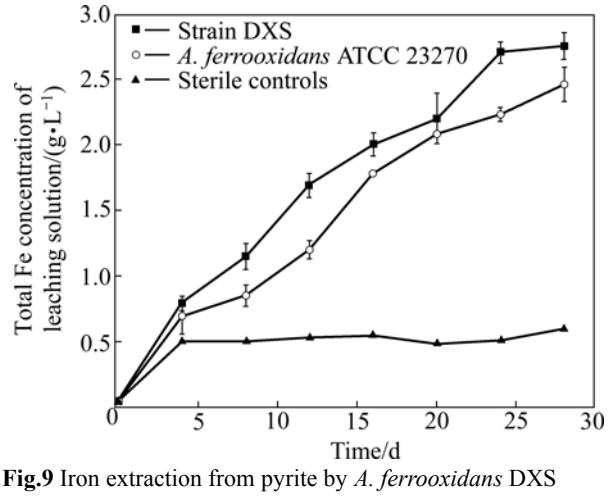

Fig.9 Iron extraction from pyrite by *A. ferrooxidans* DXS

iron extraction continuously increases and finally reaches 2.75 g/L after 28 d. Comparatively, iron concentration leached by *A. ferrooxidans* ATCC 23270 is 2.41 g/L, which is less than that by *A. ferrooxidans* DXS. Almost no soluble iron is detected in sterile controls all the time. The data indicate that *A. ferrooxidans* DXS has great capacity of pyrite leaching. The leaching result is consistent with its high oxidizing activity of sulfur and ferrous iron.

4 Conclusions

(1) *Acidithiobacillus ferrooxidans* DXS, an acidophilic bacterium from acidic mine drainages from Dongxiangshan Mine of Hami, Xinjiang Province, China is isolated. The strain grows at pH value of 1.0−3.0 and temperature of 20−35 ℃, with optimal pH and temperature at 2.0 and 30 ℃, respectively. The cells of the strain are in shape of short rod, with (1.3±0.5) μm in length and (0.40±0.05) μm in diameter. The optimum temperature and pH for strain DXS growth are 30 ℃ and 2.0, respectively. It can use ferrous iron, elemental sulfur and NaS_2O_3 as energy sources. It cannot, however, grow on substrate of glucose, yeast extract, peptone or other alternative organic energy sources.

(2) 16S rRNA sequence analysis indicates that the closest relative of strain *A. ferrooxidans* DXS is the type strain ATCC 23270, with a 16S rRNA sequence similarity over 99.57%. Strain DXS is completely identical in Iro protein gene (*iro*) to *A. ferrooxidans* LY (DQ166841), and almost identical in *tth* gene to *Acidithiobacillus ferrooxidans* (AB259312) (only two nucleotides changed).

(3) *A. ferrooxidans* DXS can oxidize Fe^{2+} and S^0. Bioleaching of marmatite and pyrite with strain DXS can attain good performance.

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