

## Liquid-nitrogen cryopreservation of three kinds of autotrophic bioleaching bacteria

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**Abstract:** Three kinds of autotrophic bioleaching bacteria strains, including mesophilic and acidophilic ferrous ion-oxidizing bacteria *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*), mesophilic and acidophilic sulfur-oxidizing bacteria *Acidithiobacillus thiooxidans* (*A. thiooxidans*), and moderately thermophilic sulfur-oxidizing bacteria *Acidianus brierleyi*, were cryopreserved in liquid nitrogen and their ferrous ion- or sulfur-oxidizing activities were investigated and compared with the original ones. The results revealed that ferrous ion/sulfur oxidation activities of the strains were almost equal before and after cryopreservation. Glycerin was used as cryoprotective agent. In conclusion, liquid-nitrogen cryopreservation is a simple and effective method for autotrophic bioleaching microorganisms.

**Key words:** *A. ferrooxidans*; *A. thiooxidans*; *Acidianus brierleyi*; cryopreservation; liquid nitrogen; growth activities

### 1 Introduction

Bioleaching is an approach of hydrometallurgy to extract metals from ores using microorganisms. This technique can offer a number of advantages in recovering metals from the low grade ore, boundary ore and tailings for its short process, low cost and little pollution. Some autotrophic bacteria play an important role in heap leaching and in-situ leaching of refractory ore[1–2]. There are diverse microbes involved in the bioleaching systems, including autotrophic or facultatively autotrophic bacteria such as *A. ferrooxidans*[3], *A. thiooxidans*, *Leptospirillum ferrooxidans* and *Ferrobacillus ferrooxidans*. And some other acidophilic thermophilic strains such as *Sulfolobus acidocaldarius*, *Sulfobacillus thermosulfidooxidans* and *Acidianus brierleyi*[4] are also widely utilized in bioleaching[5]. Researchers have made great efforts to screen leaching microorganisms from environment. Therefore, how to

preserve the bioleaching microbes becomes an important issue. The previous studies indicated that frequent subculture resulted in variation, decline of the characteristics and even death of the strains[6]. However, currently few investigations have been carried out in the terms of the preservation of bioleaching microorganisms. As far as the conservation of *A. ferrooxidans* is concerned, ZHOU et al[7] explored the effects of dryness preservation on the Fe<sup>2+</sup> oxidation of *A. ferrooxidans*, and ZHANG et al[8–10] investigated several methods for short-term preservation of *A. ferrooxidans*. ZHOU et al[11] preserved *A. caldus* by freeze-dry preservation. Furthermore, CLELAND et al[12] reported the method of using glycine betaine as a cryoprotectant for prokaryotes including *A. ferrooxidans* and *A. thiooxidans*.

Preserving bacteria in liquid nitrogen is a routine method in strain conservation. The cells can be preserved for a long time in the liquid phase of liquid nitrogen pot at –196 °C or in the gas phase at –150 °C. The technique

of cryoconservation in liquid nitrogen is advantageous because of its wide range, long-term storage and little mutation on all the cells. Numerous investigations have been reported on the conservation of heterotrophic bacteria by liquid nitrogen method. However, few studies have been published for autotrophic microorganisms including bioleaching bacteria with the method. In this study, we aim at exploring the feasibility of conserving bioleaching bacteria by liquid nitrogen method.

## 2 Experimental

### 2.1 Microorganisms and culture medium

Mesophilic and acidophilic ferrous ion-oxidizing bacteria *A. ferrooxidans* DC[13] was grown in 9K medium[14] with initial pH of 2.0. Mesophilic and acidophilic sulfur-oxidizing bacteria *A. thiooxidans* DMC was grown in Starky liquid medium with initial pH of 2.0[15]. Moderately thermophilic sulfur-oxidizing bacteria *Acidianus brierleyi* type strain JCM8954<sup>T</sup> was purchased from JCM. The strain was grown in JCM medium 176 with initial pH of 2.0. Both *A. ferrooxidans* and *A. thiooxidans* were incubated at 30 °C and the *Acidianus brierleyi* was incubated at 65 °C. Three strains were on a rotary shaker of 180 r/min. All the cultured media were autoclaved at 121 °C and 103 kPa for 30 min, respectively.

### 2.2 Cryoprotective agent

Two cryoprotective agents were used in this study. One of them is glycerin, the other is designated as GP provided by the China Center for Type Culture Collection(CCTCC). The glycerin was diluted to concentration of 60% (volume fraction) with deionized water and 100% GP. Two cryoprotectants were autoclaved at 121 °C and 103 kPa for 30 min, respectively. The different concentrations of the protectants used in the study are listed in Table 1, the suspensions with different protectant combined are listed in Table 2.

### 2.3 Incubation and collection of bacteria

The cells of *A. ferrooxidans*, *A. thiooxidans* and *Acidianus brierleyi* were harvested during late logarithmic

**Table 1** Concentrations of cryoprotectants using for preservation of bacteria

| Cryoprotectant* | Storage concentration   | Final concentration in the supernatant |
|-----------------|-------------------------|----------------------------------------|
| A               | 60% glycerin            | 30% glycerin                           |
| B               | 100% GP                 | 50% GP                                 |
| C               | 30% glycerin+<br>50% GP | 15% glycerin+<br>25% GP                |

\*: A—Glycerin (60%); B—GP(100%); C—Glycerin (30%) and GP (50%).

phase. The cultures of *A. ferrooxidans* and *Acidianus brierleyi* were divided into two test groups, respectively. The jarosite (or sulfur) were filtrated removing for one group and the other was not. For the culture of *A. thiooxidans*, the sulfur was removed by filtration. All the cultures were centrifuged at 10 000 r/min for 15 min and the cells were harvested, respectively. Collected cells were washed twice using H<sub>2</sub>SO<sub>4</sub> (pH=2.0). The cell pellets were resuspended with deionized water and mixed well with equivalent volume of cryoprotective, and then transferred them into the frozen tubes, respectively.

### 2.4 Pre-freezing procedure and conservation

First, the frozen tubes were held at 4 °C for about 30–50 min and then transferred them into –20 °C for another 30–50 min, and then the tubes were kept at –80 °C for 2 h. Finally, the frozen tubes were stored in the liquid phase of liquid nitrogen.

### 2.5 Recovery of frozen strains and Activity measurement

The frozen tubes were taken out from liquid nitrogen after 7 d and immediately thawed in water bath at 37 °C. The cryoprotective agent was removed and washed twice with culture medium without ferrous ions or sulfur. Then recovered cells were inoculated in the related fresh medium. The bacteria were harvested during late logarithmic phase and then inoculated into another corresponding medium. Cellar densities of *A. ferrooxidans*, *A. thiooxidans* and *Acidianus brierleyi* were  $5 \times 10^5$ ,  $3 \times 10^6$  and  $5 \times 10^5$  mL<sup>-1</sup>, respectively. The concentrations of ferrous ion were measured using the

**Table 2** Different combinations of bacteria with cryoprotective additives

| Cryoprotectant | <i>A. ferrooxidans</i> DC |            | <i>A. thiooxidans</i> DMC |            | <i>Acidianus brierleyi</i> JCM8954 |            |
|----------------|---------------------------|------------|---------------------------|------------|------------------------------------|------------|
|                | Filtered                  | Unfiltered | Filtered                  | Unfiltered | Filtered                           | Unfiltered |
| A              | A-Ff                      | A-Uff      | A-Ft                      |            | A-Fb                               | A-UFb      |
| B              | B-Ff                      | B-Uff      | B-Ft                      |            | B-Fb                               | B-UFb      |
| C              | C-Ff                      | C-Uff      | C-Ft                      |            | C-Fb                               | C-UFb      |

potassium dichromate titrimetric method[16]. Change of the sulfur oxidation of *A. thiooxidans* and *Acidianus brierleyi* can be evaluated with pH variation of the medium were measured by precision acidity meter (pHS-3C). All experiments were repeated in triplicate using the original strains as controls and aseptic medium without inoculation as blank control.

### 3 Results

#### 3.1 $\text{Fe}^{2+}$ oxidation activity of *A. ferrooxidans* strain DC

During the recovery culture after cryopreservation, the complete  $\text{Fe}^{2+}$  oxidation time of the three filtered groups (A-Ff, B-Ff, C-Ff) was 6 d, 3 d and 4 d, respectively. Whereas the three unfiltered ones (A-UFF, B-UFF, C-UFF) took 9 d, 5 d, and 7 d to completely oxidize the  $\text{Fe}^{2+}$  under the same condition (Table 3). In the first activation period, the filtered group took little time to oxidize  $\text{Fe}^{2+}$  in the 9K medium compared with the unfiltered one.

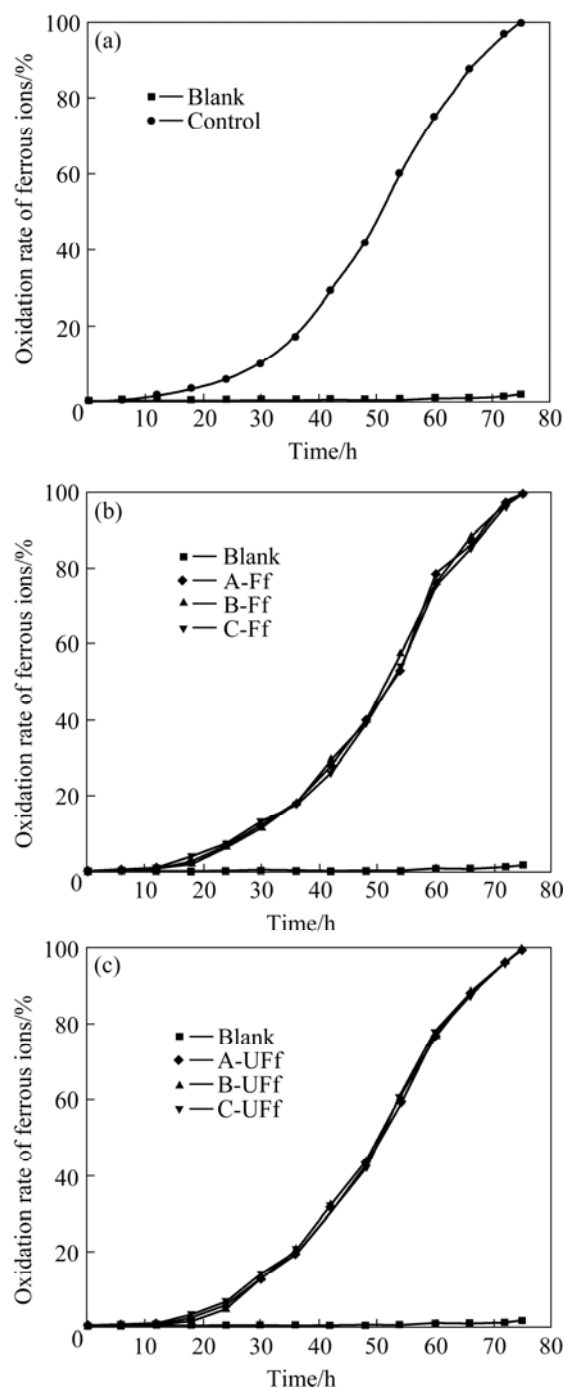
**Table 3** Time of complete  $\text{Fe}^{2+}$  oxidization by *A. ferrooxidans* strain DC

| Group  | A-Ff | B-Ff | C-Ff | A-UFF | B-UFF | C-UFF |
|--------|------|------|------|-------|-------|-------|
| Time/d | 6    | 3    | 4    | 9     | 5     | 7     |

The cryoprotected strain *A. ferrooxidans* strain DC was inoculated in fresh 9K medium and the  $\text{Fe}^{2+}$  oxidation activity is shown in Fig.1. According to the  $\text{Fe}^{2+}$  oxidation curve of the original strain (Fig.1(a)), the bacteria turned into logarithmic phase after 18 h and  $\text{Fe}^{2+}$  was 100% oxidized at 75 h. Interestingly, the filtered groups A-Ff, B-Ff and C-Ff also turned into logarithmic phase after 18 h and  $\text{Fe}^{2+}$  was also completely oxidized at 75 h (Fig.1(b)). However, the unfiltered groups A-UFF, B-UFF and C-UFF entered into logarithmic phase after 24 h and  $\text{Fe}^{2+}$  was also wholly oxidized after 75 h, as shown in Fig.1(c). Moreover, the  $\text{Fe}^{2+}$  oxidation capability was equivalent to the original strain irrespective of the three different cryoprotective agents.

#### 3.2 Sulfur oxidation activity of *A. thiooxidans* strain DMC

*A. thiooxidans* strain DMC was recovered using the same method in Starky liquid medium and achieved the active phase after 8 d, 10 d, and 7 d (Table 4). Obviously, *A. thiooxidans* strain DMC protected by 25% GP+15% glycerin took the least time to achieve the active phase in the first activation period.



**Fig.1** Ferrous ions oxidation rate in 9K medium by *A. ferrooxidans* strain DC: (a) Original strain; (b) Filtered groups A-Ff, B-Ff and C-Ff; (c) Unfiltered groups A-UFF, B-UFF and C-UFF

**Table 4** Time to achieve active phase by *A. thiooxidans* strain DMC

| Group  | A-Ft | B-Ft | C-Ft |
|--------|------|------|------|
| Time/d | 8    | 10   | 7    |

The recovered *A. thiooxidans* strain DMC was then transferred again to another fresh Starky liquid medium. The sulfur oxidation activity and growth curve were

measured (Fig.2). According to growth curve (Fig.2(a)) and the curve of pH variation (Fig.2(b)) of the original strain, the bacteria turned into logarithmic phase for 42 h and went into active phase after 162 h. As shown in Fig.2(c) and Fig.2(d), the times of the activated strain needed to enter the logarithmic phase and active phase are 42 h and 162 h, respectively. The results revealed that the sulfur oxidation rate and growth rate of *A. thiooxidans* strain DMC conserved in liquid nitrogen were relatively equal to those of the original strain after recovered from frozen state.

### 3.3 Sulfur oxidation activity of *Acidianus brierleyi* JCM8954<sup>T</sup>

During the course of recovery cultivation, the filtered groups (A-Fb, B-Fb and C-Fb) took 6 d, 9 d, and 8 d to achieve active phase, respective; while the unfiltered groups (A-UFb, B-Ufb and C-UFb) took only 4 d, 9 d, and 5 d respectively to enter into the active phase (Table 5). It is obviously that in the first activation period, the unfiltered groups took less time to achieve active phase than the filtered groups.

The recovered cells were inoculated again into another fresh medium and the sulfur oxidation activity

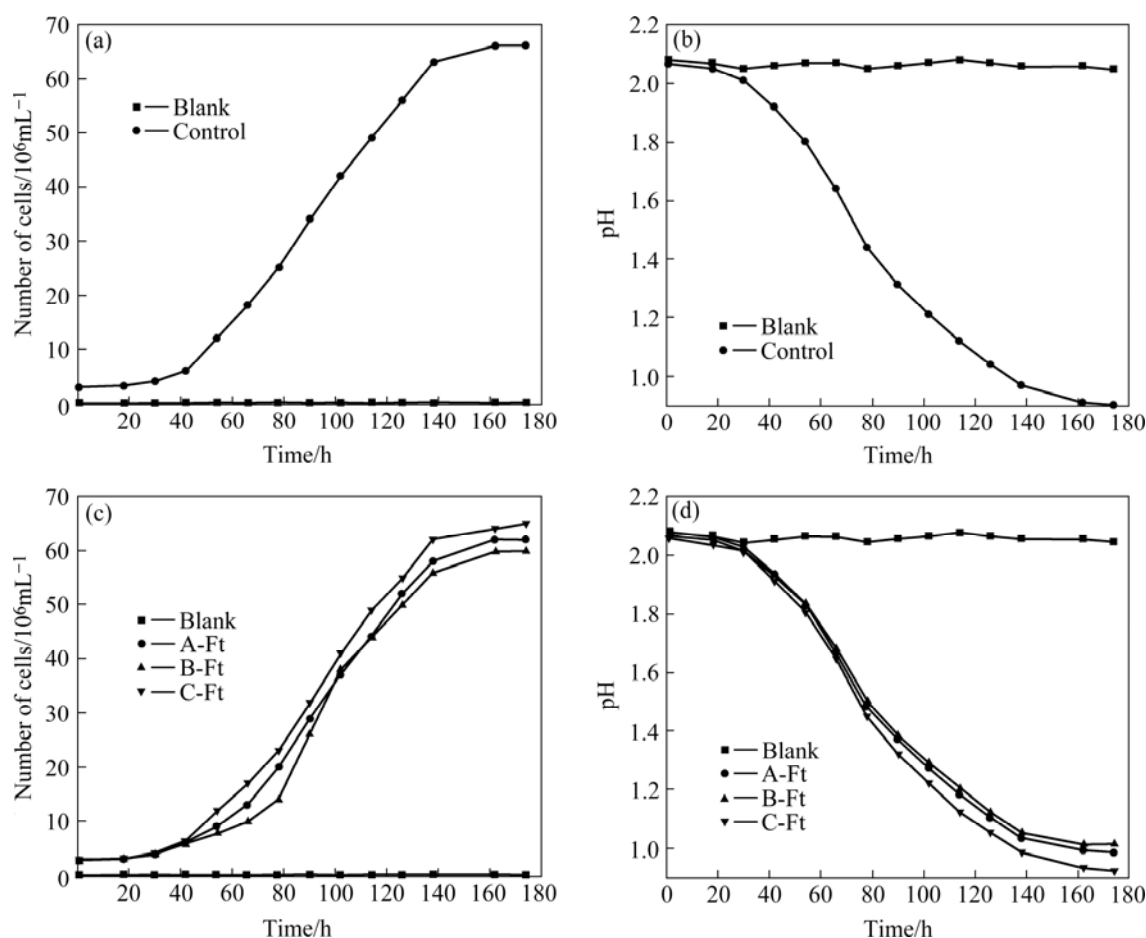
**Table 5** Time to achieve active phase by *Acidianus brierleyi* JCM8954<sup>T</sup>

| Group  | A-Fb | B-Fb | C-Fb | A-UFb | B-UFb | C-UFb |
|--------|------|------|------|-------|-------|-------|
| Time/d | 6    | 9    | 8    | 4     | 9     | 5     |

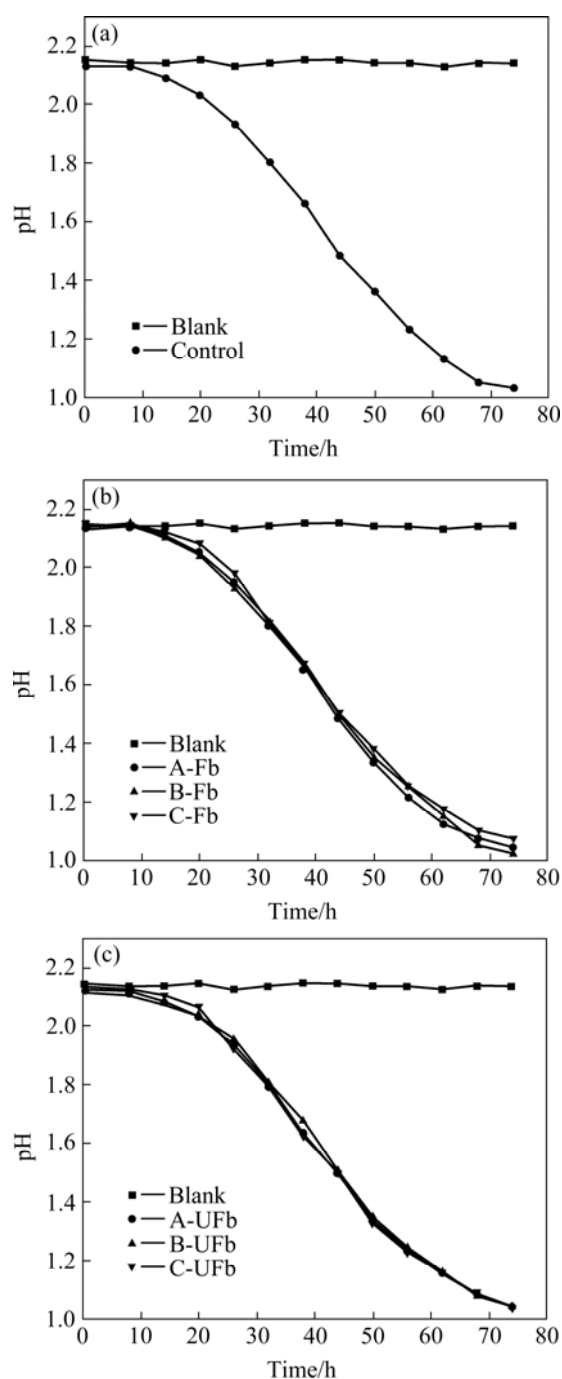
was measured (Fig.3). As indicated by Fig.3(a), the original strain turned into logarithmic phase after 20 h and achieved active phase after 68 h. We can also know from Fig.3(b) and Fig.3(c) that both the filtered groups (4A, 4B, 4C) and the unfiltered groups (5A, 5B, 5C) took almost the same time to turn into the logarithmic phase and active phase with the original strain. The results suggested that the sulfur oxidation capability of *Acidianus brierleyi* JCM8954<sup>T</sup> showed no change. It was irrespective of the kind of cryoprotective additives used.

## 4 Discussion

Bacterial strain is an important microbiological resource. Once a fine strain is obtained, it becomes much imperative to maintain it under feasible condition. *A. ferrooxidans*, *A. thiooxidans* and *Acidianus brierleyi* are all extremophiles and show unique physiological structures. They exhibit strong selectivity as well as great



**Fig.2** Curve of strain growth and pH variation of *A. thiooxidans* strain DMC: (a) Growth of original strain; (b) pH variation of original strain; (c) Growth of *A. thiooxidans* strain DMC cryopreserved; (d) pH variation of *A. thiooxidans* strain DMC cryopreserved



**Fig.3** pH variation of *Acidianus brierleyi* JCM8954<sup>T</sup> in culture medium: (a) Original strain; (b) Filtered groups A-Fb, B-Fb and C-Fb; (c) Unfiltered groups A-UFb, B-UFb and C-UFb

endurance to the living environment. In the process of cryopreservation in liquid nitrogen, cyoprotective agent is a key factor for some bacteria. Otherwise, it will reduce the survival rate of the bacterial cells[17–18].

In the present study, three strains of *A. ferrooxidans*, *A. thiooxidans* and *Acidianus brierleyi* took the least time to turn into active phase when 50% CCPA, 25% CCPA+15% glycerin and 30% glycerin were used as cryoprotectants, respectively. During the first recovery

process, when jarosite (or sulfur) was filtered, the *A. ferrooxidans* strain DC took somewhat shorter time to enter into the active phase than the unfiltered one. Whereas it didn't affect *Acidianus brierleyi* strain JCM8954 no matter the bacteria are filtered or not. In the second activation, three bacterial growth activities remain almost the same compared with the unfiltered one. This means that the autotrophic bioleaching strains may be unfilteredly cryopreserved in liquid nitrogen. Glycerol is a common reagent used in the laboratory. Numerous researches have proved its excellent efficiency in microbial conservation. However, the activities of autotrophic bioleaching bacteria are often inhibited by organic compounds including glycerol, agar, etc. So our results showed that 30% glycerin could be used as a cryoprotective agent to protect bioleaching strains from damage in liquid nitrogen.

## 5 Conclusions

1) Liquid-nitrogen cryopreservation is a simple and effective method for conservation of the unique autotrophic bioleaching bacteria including mesophilic acidophilic and moderately thermophilic bacteria, ferrous iron-oxidizing and sulfur-oxidizing bacteria.

2) 30% glycerin could be used as a cryoprotectant and insoluble sediments in the culture medium were not filtratedly removed.

3) The growth activities and ferrous iron (or sulfur) oxidation capabilities of three strains remained almost unchanged when any kind of cryoprotectant was used.

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