

Bioleaching of chalcopyrite by UV-induced mutagenized *Acidiphilium cryptum* and *Acidithiobacillus ferrooxidans*

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Abstract: The original strains *Acidithiobacillus ferrooxidans* GF and *Acidiphilium cryptum* DX1-1 were isolated from the drainage of some caves riched in chalcopyrite in Dexing Mine in Jiangxi Province of China. The optimum temperature and pH for growth were 30 °C and 3.5 for *Ac. cryptum* DX1-1, and 30 °C and 2.0 for *At. ferrooxidans* GF, respectively. For *Ac. cryptum* DX1-1, the optimum UV radiating time was 60 s and the positive mutation rate was 22.5%. The growth curves show that *Ac. cryptum* after mutagenesis reached stationary phase within 60 h, which was 20 h earlier than the original strain. For *At. ferrooxidans* GF, the optimum mutation time was 60 s and the positive mutation rate was 35%. The most active UV-mutated strain *At. ferrooxidans* GF oxidized all the ferrous after 48 h. The bioleaching experiments showed that bioleaching with the mixture of UV-mutated strains of *At. ferrooxidans* GF and *Ac. cryptum* DX1-1(1:1) could extract 3.01 g/L of copper after 30 d, while the extracted copper was 2.63 g/L with the mixture of the original strains before UV-mutation. At the end of the bioleaching experiments, the proportion of the cell density in the cultures of *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF was approximately 1:5.

Key words: *Acidiphilium cryptum*; *Acidithiobacillus ferrooxidans*; UV-induced mutagenesis; bioleaching; chalcopyrite

1 Introduction

Recently, some microorganisms, which can grow in the extreme circumstance, have attracted considerable attention because of their unusual physiology and ecology. Yet, only a modest number of iron- and sulfur-oxidizing bacteria have been isolated from sulfide ores environments, characterised physiologically and phylogenetically, and deposited in data banks[1–3]. Among them, iron/sulfur-oxidizing *At. ferrooxidans* is considered to play the most important role in natural leaching of sulfide minerals[4–5] and is widely used in biohydrometallurgical processes[6–7]. In contrast, iron-reducing bacteria have been shown to accelerate the reductive dissolution of ferric iron-containing minerals, such as jarosites and goethite. These include heterotrophic mesophilic bacteria, such as *Acidiphilium* spp.[8–9]. Interestingly, all strains of *Acidiphilium* spp. appear to be able to catalyze the dissimilatory reduction

of ferric iron to ferrous, though in some strains this is constitutive while in others it is inducible[10].

Leaching reactions that are catalyzed by these bacteria above, however, are relatively inefficient because of their slow growth rate, low cell densities, and inhibition of iron oxidation by ferric iron[11]. Such drawbacks demand the improvement of leaching bacteria.

The UV-induced mutagenesis is a frequently-used and effective method for breeding. The pyrimidine bases have strong UV absorption. When UV ray is absorbed by them, the neighboring double thymines in the chain of DNA will form thymine dimer that mainly causes the mutations. The following is the mechanism. When the reproducing of DNA begins, if it is single thymine dimer, the site of the thymine dimer will possibly run over and the gap will be left, which makes the false bases inserted, causing the mutation of AT→GC. As the DNA of *At. ferrooxidans* contains 46%–47% of AT bases[11], while *Ac. cryptum* contains 37%–40% of AT bases in its DNA,

there will be many neighbouring double thymines which possibly induce mutagenesis.

In this work, the original strains *At. ferrooxidans* GF and *Ac. cryptum* DX1-1 are collected from the drainages of some caves rich in chalcopyrite in Dexing Mine in Jiangxi Province, China[12]. The natural pH is 2.0, the temperature is 21 °C, the bacteria quantity is $6 \times 10^9 \text{ L}^{-1}$, and the cupric concentration is 0.1 g/L. These show that these original strains are characterized by natural oxidative activity of copper-containing sulfides and high natural tolerance of cupric ions.

2 Materials and methods

2.1 Culture media and microorganisms

At. ferrooxidans GF(DQ062115) and *Ac. cryptum* DX1-1 (DQ529311) used in the experiments were isolated and conserved by our laboratory from acid mine drainage(AMD) taken from a reservoir in Dexing Mine, Jiangxi Province, China. Strain GF was grown in medium 9K (containing the following reagents: $(\text{NH}_4)_2\text{SO}_4$ 3 g/L, KCl 0.1 g/L, K_2HPO_4 0.5 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L, $\text{Ca}(\text{NO}_3)_2$ 0.01 g/L) with initial pH value of 2.0 and with 4.5% FeSO_4 as the energy source. Strain DX1-1 was maintained in 9K medium (pH 3.0) with 1.5% glucose as the energy source. Serial dilution was adopted to isolate the microorganism on the agar solid medium (1.5%) (w/v). The appropriately diluted samples were monitored by counting with Hematocyte Counter.

2.2 UV mutagenesis of *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF

The experiment was carried out under the following conditions. The UV wavelength was 2537 Å, the power was 15 W and the distance was 30 cm, and the radiation time was 0, 30, 60, 120, 180 and 240 s, respectively. The cultures were radiated by UV and stirred with bent rod. 1 mL of culture treated by UV was transferred to plate for kill rate assaying. 1 mL of culture treated by UV was transferred into 100 mL of liquid medium in a 250 mL of Erlenmeyer flask added with 0.3% lithium chloride to assist the mutagenesis. The effectiveness of the positive mutation for strain *Ac. cryptum* DX1-1 was determined by the generation time, and for strain GF by the oxidative rate of ferrous ion. The ability of strain *At. ferrooxidans* GF to oxidize Fe^{2+} was tested in 250 mL shake flasks with 100 mL medium at 30 °C, pH 2.0. In the Fe^{2+} oxidation experiment, 30 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was added into the medium (with 6.08 g/L Fe^{2+}).

2.3 Bioleaching

The mineral sample used in the experiments contains about 60.8% chalcopyrite and the component

is listed in Table 1. The mineral was crushed, and subsequently washed with acetone and ethanol[13].

Table 1 Component of chalcopyrite (mass fraction, %)

CuFeS_2	FeS_2	CaCO_3	SiO_2
60.8	20.7	8.4	4.6

Bioleaching tests were carried out in 250 mL flasks containing 100 mL medium. The 9K basal salts medium without iron was used in sulfide minerals bioleaching experiments. The mineral concentration was 5% (w/v). The experiments were carried out in triplicate.

Cells were harvested by centrifugation and washed twice in distilled water adjusted to pH 2.0 with sulfuric acid. The cells were then suspended in basal salts medium without energy sources. Bioleaching tests at 30 °C with initial pH 2.5 were performed using the following six inocula: 1) *Ac. cryptum* DX1-1 after UV-induced mutagenesis, 2) *At. ferrooxidans* GF before UV-induced mutagenesis, 3) *At. ferrooxidans* GF after UV-induced mutagenesis, 4) the mixture of the two strains (1:1) before UV-induced mutagenesis, 5) the mixture of the two strains (1:1) after UV-induced mutagenesis, and 6) control.

The cell density of each organism in culture medium after inoculation was about 1×10^7 cells/mL. The abiotic controls were also designed.

2.4 Analytical methods

Physicochemical analyses of the water samples were performed at the Testing Center of Central South University, China. Flame atomic absorption spectrometry was used for measuring metal ions. pH value was measured with a pH meter (pHs-25). Copper ion concentrations were determined by atomic absorption spectrometry within 30 d of incubation. The oxidative rate of ferrous ions was determined by titration with potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$), the kill rate by plating, and the effectiveness of the positive mutation of *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF by the generation time and the oxidative rate of ferrous ions, respectively.

2.5 Analysis of community structure in leachate at end of bioleaching

2.5.1 DNA extraction and purification[14]

The microorganisms of samples were isolated after filtration of leachate through 0.2 µm nylon filters. DNA was extracted using EZ-10 Spin Column Genomic DNA Isolation Kit (Bio Basic Inc) and purified using Wizard DNA Clean-Up Kit (Promega).

2.5.2 16S rRNA gene amplification and cloning

Two primers were used to amplify 16S rRNA gene

fragment: forward primer 27F (5'-AGAGTTTGTATCC-TGGCTCAG-3') and reverse primer 1492R (5'-GGT-TACCTTGTTACGACTT-3')[15]. The PCR products were visualized by 1.0% low-melting-point agarose gel electrophoresis, and purified using Wizard DNA Clean-Up Kit (Promega). The purified 16S rRNA gene fragments were ligated into the vector PCR2.1 TOPO, then transformed into *E. coli* TOP10F' competent cells according to the manufacturers' instructions (Invitrogen). Recombinants were identified based on blue-white screening, and grown overnight in Luria-Bertani (LB) agar plates containing appropriate amounts of ampicillin, X-gal and IPTG at 37 °C. Next, 60 white colonies were randomly selected, and the recombinant plasmids containing 16S rRNA gene fragments were reamplified by PCR using the vector primers M13F (5'-GTAAAA-CGACGGCCAGTG-3') and M13R (5'-GGAAACAGC-TATGACCATG-3').

2.5.3 Amplified ribosomal DNA restriction analysis (ARDRA)

The amplified rRNA PCR products were digested with *Hind*I and *Msp*I (Fermentas) overnight at 37 °C.

The resulting ARDRA products were separated by gel electrophoresis in 3.0% agarose. The ARDRA patterns were visualized by UV excitation, and were compared with the ARDRA patterns from YANG et al[12].

3 Results and discussion

3.1 Geochemical analyses of AMD

There was 4.401 g/L sulfur in the AMD which was one of the substances that supplied energy for *Ac. cryptum* DX1-1 in the former experiments[13]. Fe^{3+} was one of those substances too, which was one reason for *Ac. cryptum* DX1-1 enhancing bioleaching. The pH value was 2.0 and temperature was 21 °C when AMD samples were collected.

3.2 Microorganism strains

The study on effects of temperature and pH on the growth of strains *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF are shown in Figs.1 and 2, respectively. It shows that the optimum condition was 30 °C, pH 3.5 for strain DX1-1, while that was 30 °C, pH 2.0 for strain

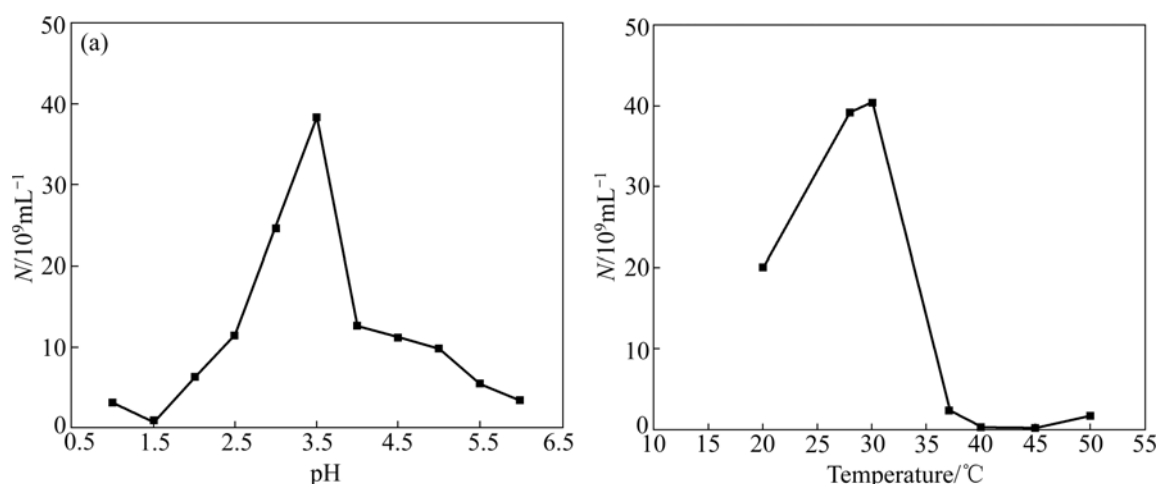


Fig.1 Effects of pH and temperature on growth of strain *Ac. cryptum* DX1-1

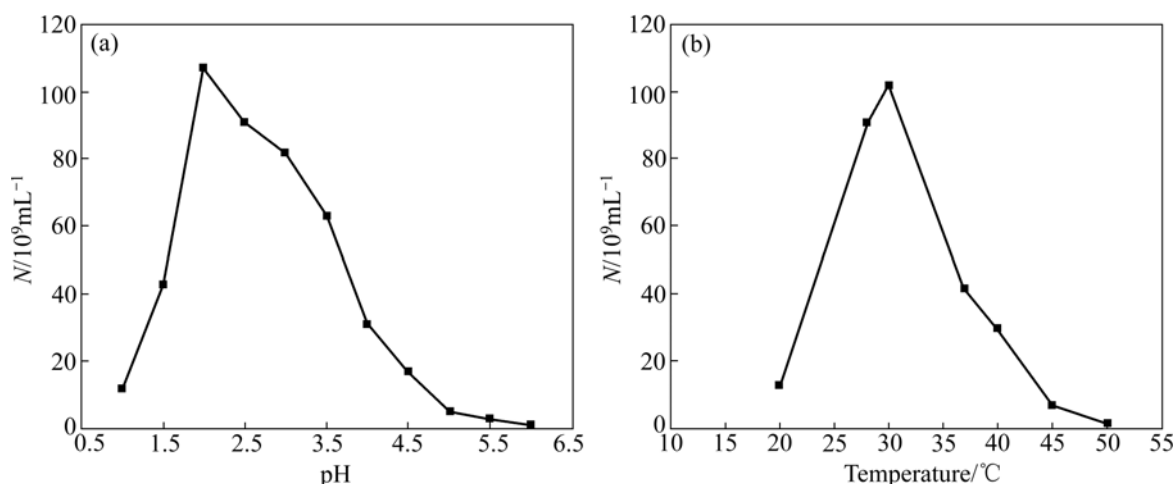


Fig.2 Effects of pH and temperature on growth of strain *At. ferrooxidans* GF

GF). *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF had different optimum pH values. We chose pH 2.5 in the bioleaching experiment because *At. ferrooxidans* GF plays more important role in bioleaching. Phylogenetic tree based on 16S rDNA sequences shows the location of the two strains (Fig.3). GenBank accession numbers are provided in parentheses.

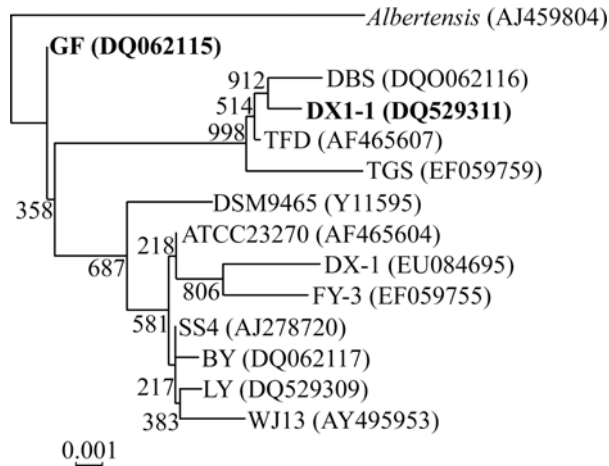


Fig.3 Phylogenetic tree based on 16S rDNA sequences, showing location of strain DX1-1 and strain GF (GenBank accession numbers are provided in parentheses)

3.3 UV-induced mutagenesis of *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF

3.3.1 UV-induced mutagenesis of *Ac. cryptum* DX1-1

The inhibitive rates of colonies (UV kill rate) and the positive mutation rate for *Ac. cryptum* DX1-1 are listed in Table 2. It is shown that the longer the radiation time of UV, the higher the kill rate of the strain, i.e. the less the alive. For *Ac. cryptum* DX1-1 the optimization time was 60 s and the positive mutation rate was 22.5%. In this study, the positive mutated colonies had higher cell density at stationary phase than the original strain.

Table 2 Effect of UV-induced mutagenesis for *Ac. cryptum* DX1-1

Radiation time/s	UV kill rate/%	Number of colony	Number of positive mutated colony	Positive mutation rate/%
20	44	40	4	10.0
60	78	40	9	22.5
100	89	20	3	15.0
140	99	10	1	10.0
200	100	0	0	0

As we chose the big colonies to determine whether it was positive mutation or not, the process was also a rejuvenated process. So, the positive mutation rate we determined was higher than the actual rate.

From the positive mutation, we chose the strain that had the shortest generation time to leach the minerals. The growth curves of *Ac. cryptum* DX1-1 before and after UV-induced mutagenesis are shown in Fig.4. It is shown that the strain after mutagenesis reached stationary phase after 60 h, which was 20 h earlier than the original strain. It had higher cell density (10^{10} /mL) than the original strain (10^9 /mL).

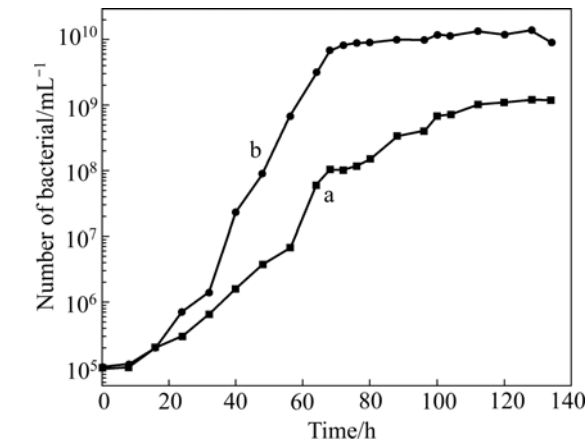


Fig.4 Growth curves of *Ac. cryptum* DX1-1 before (a) and after (b) UV-induced mutagenesis

3.3.2 UV-induced mutagenesis of *At. ferrooxidans* GF

The inhibitive rates of colonies and the positive mutation rates for *At. ferrooxidans* GF are listed in Table 3. It was shown that, like *Ac. cryptum* DX1-1, the longer the radiation time of UV, the higher the kill rate of the strains. The optimization time was 60 s and the positive mutation rate was 35% for *At. ferrooxidans* GF. For *At. ferrooxidans* GF, the positive mutated colonies had higher oxidization rate than the original strain.

Table 3 Effect of UV-induced mutagenesis for *At. ferrooxidans* GF

Radiation time/s	UV kill rate/%	Number of colony	Number of positive mutated colony	Positive mutation rate/%
20	30	20	3	15.0
60	73	20	7	35.0
100	95	15	2	13.3
140	98	10	1	10.0
200	100	0	0	0

From the positive mutation colonies, we chose the strain that had the highest oxidization rate to leach the minerals. The Fe^{2+} oxidization rate curves of the strain *At. ferrooxidans* GF before and after UV irradiation are shown in Fig.5. It is shown that the UV treated strain oxidized all the ferrous ions within 48 h, while the original strain needed 54 h.

The most favorable dose of UV irradiation is related to the character of strains, and to the property of genes as well. It is demonstrated by this study that the low dose of UV irradiation is the most suitable to the gene of *At. ferrooxidans* GF and *Ac. cryptum* DX1-1. The mechanism is probably that middle and high dose of UV irradiation damages the genes more seriously, so that it is hard to rescue the function of these genes, and more negative mutations are induced. At low temperature, not only the activity of the repairing enzymes can be inhibited, but also the time of reproducing, isolation and the forming of homozygous mutants can be satisfied.

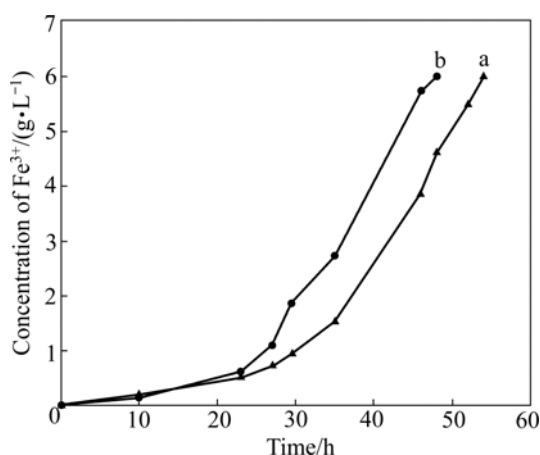
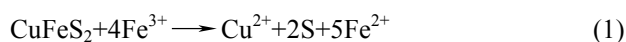


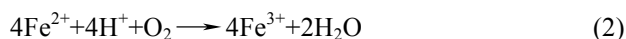
Fig.5 Fe²⁺ oxidation rate curves of strain *At. ferrooxidans* GF before (a) and after (b) UV-induced mutagenesis

3.4 Bioleaching experiment

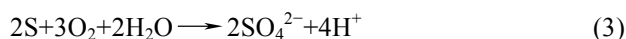
As an acid-soluble metal sulfide, chalcopyrite is probably dissolved by the ferric iron and proton attack [16–19]. Ferric iron oxidizes chalcopyrite to copper, ferrous ions and elemental sulfur in solution. This reaction is illustrated as follows:



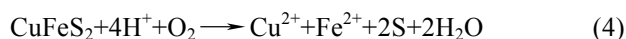
At. ferrooxidans are capable of oxidizing the ferrous ions formed in the leaching reaction by the following reaction:



The sulfur formed in the above reaction can be oxidized to sulfate by *At. ferrooxidans* as the following reaction:



In addition to the bioleaching progress, chalcopyrite may be oxidized as Eq.(4) in sulphuric acid solution, where the sulfur formed by chemical leaching is also oxidized by Eq.(3).



From the above results it can be seen that *At.*

ferrooxidans are propitious to incessancy of the reaction. At the beginning of bioleaching, sulfuric acid is added into reactor to keep pH value around 2.5 for acid consumption. After 6 days, since acid is produced during the oxidation of sulfur and the hydrolysis of ferric iron to ferric species such as Fe(OH)²⁺ and Fe(OH)₂⁺[20], the pH decreases continuously.

As the bioleaching continues, the precipitation of jarosite and sulfur compounds (forming a layer on mineral surface) may hinder chalcopyrite dissolution[21]. *Acidiphilium* is able to reduce a wide range of Fe(III)-(hydr)oxides including akagenite, jarosite, natrojarosite, amorphous ferric hydroxide, and the mixed ferrous/ferric mineral magnetite[10]. The existence of *Acidiphilium* dissolves the hard layer on mineral surface, which leads to further extraction of copper.

The bioleaching result of chalcopyrite with the strains *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF is shown in Fig.6. In the whole process, the copper extraction continuously increases. In the first 15 d, the copper extraction rate increased quickly. From the 16th day to the 30th day, the copper extraction became slow. The first 15 d was the main stage of bioleaching of chalcopyrite, when the copper extraction increased remarkably. The jarosite and sulfur compounds hinder the chalcopyrite dissolution. Therefore, copper extraction in the last 15 d became slow. Bioleaching with strain *Ac. cryptum* DX1-1 after UV-induced mutagenesis could extract copper 0.49 g/L in 30 d. With the strain *At. ferrooxidans* GF before UV-induced mutagenesis, the extraction of copper reached 1.77 g/L in 30 d, while it was 1.98 g/L with the UV-induced mutant strain. Bioleaching with the mixture of strain *At. ferrooxidans* GF and *Ac. cryptum* DX1-1 (1:1) before UV-induced

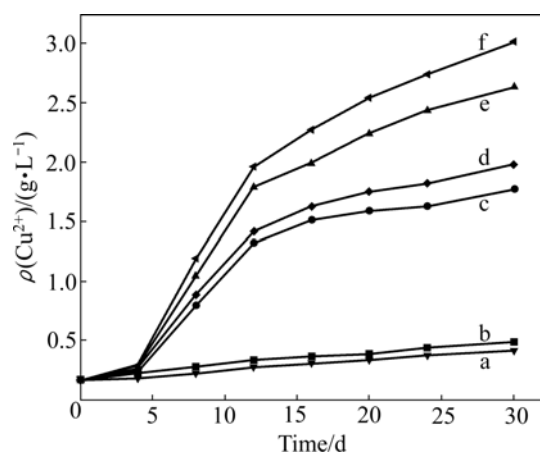


Fig.6 Copper extracted from chalcopyrite by *Ac. cryptum* DX1-1 after UV-induced mutagenesis (b), *At. ferrooxidans* GF before UV-induced mutagenesis (c), *At. ferrooxidans* GF after UV-induced mutagenesis (d), mixture of two strains before UV-induced mutagenesis (e), mixture of two strains after UV-induced mutagenesis (f), and control (a)

mutagenesis could extract copper 2.63 g/L after 30 d, while we got 3.01 g/L copper with the mixture of the two strains after UV-induced mutagenesis. UV-induced mutagenesis is helpful to improving the oxidization activity of *At. ferrooxidans* and makes it propitious to bioleaching. When the two strains were used in bioleaching, the effect was not the simple sum of the effect of bioleaching by the two strains independently, but they can stimulate each other. *Ac. cryptum* accelerated the reductive dissolution of jarosites and goethite, and they also consumed the organic compound which restrained the growth of *At. ferrooxidans*. So, they can assist *At. ferrooxidans* in bioleaching. They do not oxidize ferrous iron[22]. So *Ac. cryptum* had bad performance when they were used as the only bacterium in bioleaching.

3.5 Analysis of community structure in leachate at end of bioleaching

ARDRA patterns are shown in Fig.7. It shows that, at the end of the bioleaching experiments, the proportion of the cell density of *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF became 4:19 in the leaching culture by the two strains before UV-induced mutagenesis and 3:17 in the leaching culture by the two strains after UV-induced mutagenesis. Contrary to that, it is worth to note that at the beginning of the bioleaching experiment, *Ac. cryptum* DX1-1 had the same cell density as *At. ferrooxidans* GF. This means that *Ac. cryptum* DX1-1 is not a prevailing bacterium but an assistant in bioleaching and has a small proportion. So when design bioleaching experiments next, we can prepare a mixture in which *At. ferrooxidans* is in predominated number, and at the same time to which put some *Ac. cryptum* in an approximate proportion of 1:5 to *At. ferrooxidans*.

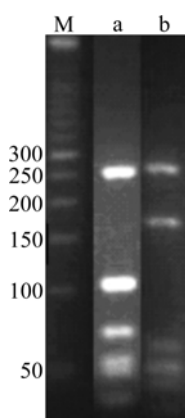


Fig.7 ARDRA patterns of *Ac. cryptum* DX1-1 (a) and *At. ferrooxidans* GF (b) (M was marker)

4 Conclusions

1) Strains *Ac. cryptum* DX1-1 and *At. ferrooxidans*

GF were isolated. They had the optimal growth temperature 30 °C, and the optimal pH values 3.5 and 2.0, respectively.

2) The optimum mutagenesis time was 60 s for both *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF. After the mutagenesis the positive mutation rate for these two strains was 22.5% and 35%, respectively. Strain *Ac. cryptum* DX1-1 after mutagenesis reached the stationary phase after 60 h, which is 20 h earlier than the original strain. It also had higher cell density than the original strain. Strain *At. ferrooxidans* GF after mutagenesis had higher ferrous ion oxidizing capability than the original strain.

3) Bioleaching with the mixture of strains *At. ferrooxidans* GF and *Ac. cryptum* DX1-1 (1:1) after UV-induced mutagenesis could extract copper 3.01 g/L after 30 d, which was higher than that extracted with *At. ferrooxidans* GF only. And also more copper can be leached out by using the mixture after UV-induced mutagenesis than by the two strains before UV-induced mutagenesis.

4) The ARDRA patterns showed that the proportion of the cell density of strains *Ac. cryptum* DX1-1 and *At. ferrooxidans* GF became about 1:5 in the culture at the end of the bioleaching experiments. We can follow this proportion to inoculate these two strains during bioleaching.

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