[Article ID] 1003- 6326(2001)05- 0795- 05

UV-induced mutagenesis of oxidation activity of ferrous ion of *Thiobacillus ferrooxidans* ①

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[Abstract] An excellent strain named T. f_6 was isolated and screened, the dose and other condition for the UV-induced mutagenesis were studied and the richened positive mutant m^+ T. f_6 was applied in the column leaching of copper-containing sulfides. The results show that T. f_6 is characterized by rapid oxidation of ferrous ion and cupric sulfide, high tolerance of toxic ion and short generation time. The best mutagenic effectiveness can be obtained under the dose of low kill rate of UV and low temperature treatment, under which the best richened m^+ T. f_6 can be shortened 1.4 h. It was shown by the column leaching of copper that the leaching rate can be enhanced by at least 11% compared with the original one by the mutants.

[Key words] *Thiobacillus ferrooxidans*; mutagenesis; bacterial leaching
[CLC number] TD925.5 [Document code] A

1 INTRODUCTION

T. ferrooxidans is the dominant species in most bacterial leaching places[1,2]. As known, both direct and indirect mechanism are responsible for bacterial leaching of iron-containing sulfide ores[3~5]. Whether direct or indirect mechanism, the intermediate product, ferrous sulfate is assumed to be produced and the bacterial oxidation is followed except these sulfides without iron, for example, cupric sulfide. It was reported by ZHANG and GUAN[6,7] that ferrous sulfate was detected during the whole period of bacterial leaching. The ratio of Fe^{3+} to Fe^{2+} is low (about 0.3 ~ 0.4) during early period (before 11 d), then high and constant (about 37~ 38) during middle or late period (after 11 d). It was observed in our experiments on the bacterial leaching of chalcopyrite[8] that the oxidation of original ferrous ion in pulp solution is completed within 3~ 4 d and no ferrous ion can be detected during the rapid period of bacterial leaching, but ferric iron can be found to rise sharply. We supposed that the oxidative activity of the bacterial strains used by us is higher. All these have shown that the bacterial oxidation of ferrous ion is related to the effective leaching of chalcopyrite. Whether the positive mutation of the oxidative activity of ferrous ion can accelerate the bacterial leaching effectively and how about the potential of induced mutagenesis of *T. ferrooxidans* are the most important questions to be answered in this study, because it is concerned to the importance of industrial breeding of *T. ferrooxidans* by mutation of oxidative activity of ferrous ion.

The growth of *T. ferrooxidans* can be supported easily by solid or liquid medium with ferrous ion, but hardly by medium with sulfide or its intermediates. *T. ferrooxidans* can be cultivated by medium with sodium thiosulfate and form colonies in early study. But our study showed that the medium with sodium thiosulfate doesn't suit for the growth of *T. ferrooxidans* very well even when pH is over 4.5. When pH is below 4.5, at which sodium thiosulfate is decomposed into elemental sulfur and sodium sulfite, many colonies will be formed when *T. thooxidans* is inoculated, but *T. ferrooxidans* will not. Therefore, the oxidative activity of ferrous ion of *T. ferrooxidans* was chosen as the object of screening and breeding partly also because of the limit of solid culture technique.

2 MECHANISM OF UV-INDUCED MUTAGENESIS[9]

The UV-induced mutagenesis is a frequent and effective method for breeding. The pyrimidine bases have strong absorption of UV lay. When UV lay 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 $\rightarrow$ GC. As the DNA of *T. ferrooxidans*

① [Foundation item] Project (59925412) supported by the National Outstanding Youth Foundation of China
[Received date] 2000- 12- 21; [Accepted date] 2001- 05- 10

contains 46% ~ 47%[10] of AT bases, there will be many neighbouring double thymines which possibly will be induced mutagenesis.

3 SCREENING OF OUT-SET STRAIN

The original strains were collected from the drainage of some caves rich in chalcopyrite in Dabao Mountain in Gangdong Province. The natural pH was 1.9, and the bacterial quantity was 6×10^9/L, the cupric concentration was 1.988 g/L. These showed that these original strains were characterized by high natural oxidative activity of copper-containing sulfides and high natural tolerance of cupric ion. The 9K medium was modified as follows: ferrous sulfate 22.2 g/L, other basal salts as same as 9K, pH 2.0 in liquid medium, pH 3.0 in solid medium, purified agar 15 g/L. It was shown that there will have the most colonies under that condition[11]. An excellent strain was isolated after repeated plating and screening of the largest colonies which is supposed to be high oxidative activity of ferrous ion and short generation time. Its characteristics are shown as Fig. 1 and Fig. 2, and the strains' generation time in rotary culture is shown in Table 1.

Table 1 Generation time of strains in rotary culture

Strains	Generation time/h	Strains	Generation time/h
0	7.8	8	6.2
1	5.8	9	5.8
2	6.3	10	6.0
3	5.7	11	7.4
4	7.1	12	6.3
5	5.8	13	6.5
6	5.6	14	6.0
7	7.0	15	5.7

0 is the original strain.

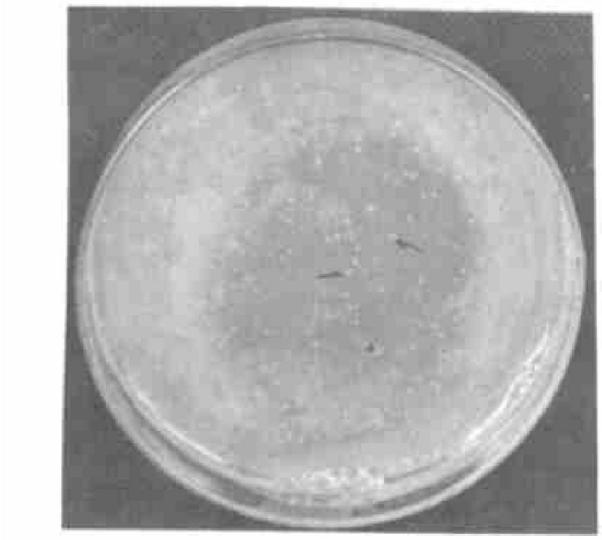

Fig. 1 Characteristics of colonies of T. f_6

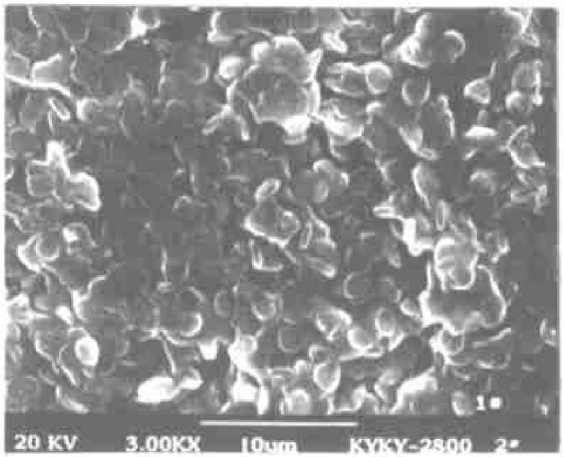

Fig. 2 Characteristics of T. f_6 in colonies

The relationship between the bacterial growth and the oxidation of ferrous ion at 2×10^8 ~ 3×10^8 bacterium/L of the inoculum that was transferred continuously to culture is exhibited in Fig. 3. From Fig. 3 it can be demonstrated that: 1) the bacteria amount rises from 10^8 bacterium/L to 10^9 bacterium/L when the oxidative rate of ferrous ion is about 15%, then to 10^{10} when the rate is about 45%. The delay period can almost be omitted and the logarithm period is typical. 2) It is the typical stable period when the rate is between 65% ~ 75%, during which the amount keeps at 10^{10} bacterium/L. 3) the contabescence period begins when the rate is over 80%, during which the amount is below 10^{10} bacterium/L gradually. Therefore, the generation time can be estimated by logarithm period and the stable period can be acted as the inoculating time, at which the amount of the inoculum can keep relative stable and the activity of enzyme of ferrous oxidase in a unit of live cells can be acted as an index for screening after being mutated. The generation time of T. f_6 was measured to be 5.6 h in liquid medium with ferrous ion.

The strain' oxidative activity of reduced sulfur oxidase is shown as Fig. 4. It is demonstrated that 10 g/L cupric sulfide (the effective constituent 96%)

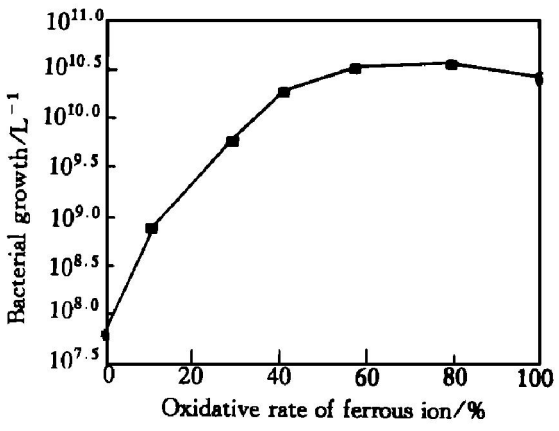

Fig. 3 Relationship between bacterial growth and oxidative rate of ferrous ion of T. f_6

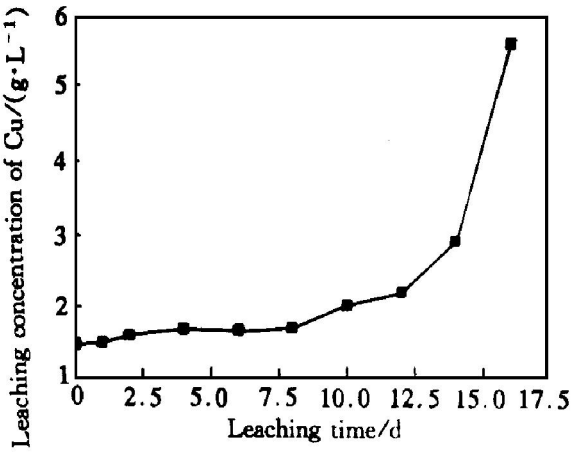

Fig. 4 Oxidation of reduced sulfur in cupric sulfide by T. f_6

had almost been oxidized after leaching 16 d when 1 mL of 10^{10}/L bacteria was inoculated into 100 mL leaching solution without any iron. It was also demonstrated by our other study that the strain is characterized by 0.1 g/L Ag^+ resistance[7] and stronger As^{3+} resistance and rapid oxidative activity of chalcopyrite or arsenopyrite. For example, 10% pulp concentration and 24% grade of chalcopyrite concentrate can be oxidized 37% after 25 d.

4 CONDITIONS OF UV-INDUCED MUTAGENESIS

The experiment was carried out under the conditions as follows: the UV wavelength was 2537 Å, the power was 20 W and the distance was 30 cm, the radiation time was 0, 30, 60, 120, 180, 240 s for number 0#, 1#, 2#, 3#, 4#, 5#, respectively. The bacterial quantity was counted with Petroff-Hausser and Helber counting chamber when it grew to later logarithm period (or 60% oxidation rate of ferrous ion). The diameter of dishes was 9 cm, in which 6 mL culture was transferred. The cultures were radiated by UV as stirred with bent rod. 1 mL culture treated by UV was transferred into 1 mL liquid medium with ferrous ion in 250 mL Erlenmeyer flask added with 0.3% lithium chloride to assist the mutagenesis. The Erlenmeyer flasks were wrapped with black paper and the lids with eight layers of sterile gauze. Two groups were divided in this experiment: the first group was treated at 4 ℃ for 12 h, the second group was inoculated directly into rotary bed at 30 ℃ and 180 r/min. All sterile operations were carried out in ultraclean platform. The oxidative rate of ferrous ion was determined by titration with potassium dichromata, the generation time by the variation of multiplying quantity, the kill rate by plating, and the effectiveness of the positive mutation by the generation time and the oxidative rate of ferrous ion.

The inhibitive rates of colonies (UV kill rate) determined by plating were 0, 13.4%, 19.4%, 25.3%, 83.6%, 97.5% respectively. It is showed that the longer the radiation time of UV, the higher the kill rate of the strains, that is, the less the alive. The oxidative rates of ferrous ion of the first group after being cultured in rotary bed for 56 h were 20.2%, 76.3%, 96.6%, 46.7%, 21.67%, 18.5% respectively. It was demonstrated that significant positive mutation had taken place in 1#, 2#, 3#, especially in 2#. The results were also proved by the generation time of 5.6, 5.2, 4.2, 5.0, 5.5, and 5.8 h in turn. The oxidative rates of ferrous ion of the second group were divided into two stages: reducing in turn with the enhance of UV-kill rate during long period, and then the effectiveness of positive mutation was embodied slightly by them. For example, the oxidative rates were 14.0%, 12.1%, 11.3%, 10.4%, 2.3%, 0.7% for 36 h, then 79.0%, 81.7%, 75.4%, 78.3%, 71.5% and 67.3% for 120 h. Therefore, the best one of all positive mutations is 2#, whose average generation time of the richened strains can be shortened by 1.4 h only once UV-induced mutagenesis.

5 APPLICATION OF MUTATED STRAINS IN COLUMN LEACHING

The m^+ T. f_6 strains in the best 2# flask were not separated and screened further, but richened by continuous culture. The m^+ T. f_6 strains were chosen as the leaching strains and T. f_6 as the control. The distinction of the generation time between them was obvious, the former is 1.4 h shorter than the latter. The following was the bacterial leaching conditions: the glass column dimensions were d 5 cm × 25 cm, the mass of samples was 500 g, which were copper-containing sulfides, the average particle size was 6 ~ 10 mm. The quantity of the inoculums was 0.5% (volume fraction). The volume of the leaching solution was 2 L, whose pH was constantly kept to be 2.0, into which only 3 g/L ammonium sulfate and 0.5 g/L dipotassium hydrogen phosphate were added according to the character of the sample. The columns were leached by dilute sulfuric acid (pH 2.0) for 7 d, then by bacteria, recycling one time every day.

The sample of sulfide is mainly composed of chalcocite and chalcopyrite, accounting for 58.2% and 41.8% respectively. The chemical compositions are shown in Table 2, the growth of different strains in the columns in Table 3, and the cupric leaching rates of them in Fig. 5.

It was shown from Fig. 3 that the cupric leaching rates by T. f_6 and m^+ T. f_6 were 51.96% and 61.1% respectively, the latter was 11.0% higher than the former at least. It was shown by Fig. 3 that

Table 2 Chemical compositions of copper-containing sulfides in Yunnan (%)

Cu	S	Fe	As	Al_2O_3	CaO
2.52	0.141	1.79	0.0007	2.97	5.69
MgO	Zn	SiO_2	Au/(g·t^{-1})	Ag/(g·t^{-1})	
1.03	0.0018	60.96	0.178	95	

Table 3 Growth of different strains in column leaching

Strains/turn	T.f_6	m$^+$ T.f_6
0	2.0×10^4	2.0×10^4
11	5.6×10^5	0.8×10^6
20	6.7×10^5	1×10^6
30	6.7×10^5	1×10^6
40	6.67×10^5	5.33×10^6

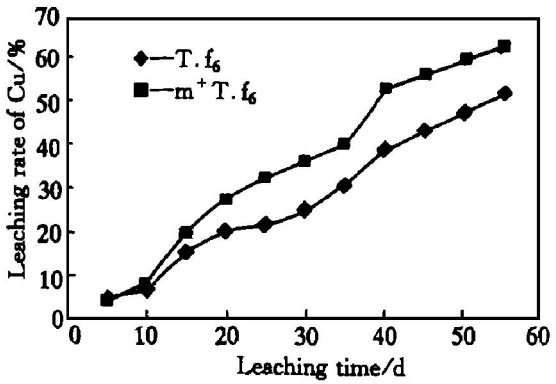

Fig. 5 Leaching of sulfides containing copper by original T.f_6 and m$^+$ T.f_6

the growth of the former was less 0.5~1 order of magnitude than that of the latter. All these demonstrated that enhancing the bacterial oxidative activity of ferrous ion by screening and induced mutagenesis can indeed prompt the bacterial leaching of copper.

6 DISCUSSION

The most favorable dose of UV is related to the character of strains, also to the property of genes. It was demonstrated by this study that the low dose of UV is the most suitable to the gene of ferrous oxidase in *T. ferrooxidans*. The mechanism probably is that middle and high dose of UV damages the genes more seriously so that it is harder to rescue the function of these genes, and more negative mutations produce, even to death. At low temperature, not only the activity of the respairing enzymes can be inhibited, but also the time of reproducing, isolation and the forming of homozygous mutants can be satisfied. At 30 ℃, the normal can reproduce continuously, while at low temperature the reproducing will be inhibited and in-phase growth can be realized, so the direct culture is unfavorable for ample multiplying and express of the positive mutants.

In the bacterium point of view, the bacterial leaching was improved because any variation of the constructions of the enzymes in the operon of ferrous oxidase will lead to the enhancing of oxidation activity, further to the promoting the whole metabolism of the bacterial cell, which causes the generation time shorten, finally causes more bacteria acting on the surface of the sulfide. From the direct and indirect mechanism point of view, the following reaction were taken place during the bacterial oxidation of the copper-containing sulfides:

Direct leaching

$$2Cu_2S+5O_2+2H_2SO_4 \xrightarrow{\text{Bacterium}} 4CuSO_4+2H_2O \quad (1)$$

$$CuFeS_2+4O_2 \xrightarrow{\text{Bacterium}} CuSO_4+FeSO_4 \quad (2)$$

Indirect leaching

$$Cu_2S+2Fe_2(SO_4)_3 \longrightarrow 2CuSO_4+4FeSO_4+S^0 \quad (3)$$

$$CuFeS_2+2Fe_2(SO_4)_3 \longrightarrow CuSO_4+5FeSO_4+2S^0 \quad (4)$$

$$4FeSO_4+2H_2SO_4+O_2 \xrightarrow{\text{Bacterium}} 2Fe_2(SO_4)_3+2H_2O \quad (5)$$

$$2S^0+3O_2+2H_2O \xrightarrow{\text{Bacterium}} 2H_2SO_4 \quad (6)$$

It was shown by the above reactions that the fastened generation time strengthens the direct mechanism quantitatively, but also the indirect mechanism quantitatively and qualitatively because the acceleration of both the oxidation of ferrous ion and the recycling of ferric enforce the reaction of indirect leaching that cause the equation move towards the right according to the kinetics equilibrium theory.

It was demonstrated by this study that *T. ferrooxidans* is characterized by good mutability and the bacterial leaching of copper-containing sulfides was accelerated by its enhancing oxidative activity. This result exhibited that the screening and breeding have practical possibility and potential industrial value. All these questions of effective screening and breeding will be studied further about how to prevent the "passiveness" combining other methods and how to determine the effective related indexes.

7 CONCLUSIONS

1) T.f_6 strain is suitable for the out-set strain of industrial breeding owing to its character of fast oxidative activity of ferrous ion and reduced sulfur, short generation time and high tolerance of toxic ion and good mutability.

2) Low kill rate is the best dose of UV for T.f_6,

that is, 20% ~ 30%, under which the generation time can be shortened by 1.4 h only one time treatment of UV. The positive induced mutagenesis can be promoted by the treatment at low temperature.

3) The bacterial leaching of copper-containing sulfides can be accelerated by the meliorated strains of the oxidative activity of ferrous ion because not only the direct leaching but also the indirect leaching can be strengthened. It was shown that *T. ferrooxidans* is characterized by important potential for industrial application.

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(Edited by YUAN Sai-qian)