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Differential fluoride tolerance between sulfur- and ferrous iron-grown *Acidithiobacillus ferrooxidans* and its mechanism analysis

Liyuan Ma[a,b], Xingjie Wang[a,*], Jiemeng Tao[a], Xue Feng[a], Xueduan Liu[a,b], Wenqing Qin[a,b]

[a] School of Minerals Processing and Bioengineering, Central South University, Changsha, 410083, China
[b] Key Laboratory of Biometallurgy of Ministry of Education, Changsha, 410083, China

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ABSTRACT

Toxic ions accumulation in leachate is one of the limitations for bioleaching application. The effects of fluoride ions on microbial growth relevant to bioleaching have been extensively emphasized. However, the reasons that the microorganisms present different fluoride tolerance in bioleaching of different minerals have not yet been proposed. A fundamental exploration on the differential fluoride tolerance of sulfur- and ferrous iron-grown *Acidithiobacillus ferrooxidans* was performed. The results showed that the critical fluoride concentration for sulfur-grown *A. ferrooxidans* was less than 0.1 mM whenever fluoride was introduced. The critical fluoride concentration for ferrous iron-grown *A. ferrooxidans* was 1.0 mM when fluoride was introduced prior to the lag phase, but it was higher than 10.0 mM when fluoride was introduced at the log phase. The ferric ions generated from ferrous oxidation could significantly decrease the concentration of HF and finally reduce the toxicity of fluoride to *A. ferrooxidans*. The changes of concentration of metal ions with strong complex ability to fluoride ions, resulted in an illusion of high fluorine-resistant strain in industrial bioleaching heap. Finally, the feasibility of fluoride removal by ferric ion was put forward. This work has meaningful implications for the industrial bioleaching of high-fluorine containing uranium ore.

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1. Introduction

Bioleaching, the use of microorganisms to recover precious and base metals from low-grade minerals, has developed into a successful and expanding area of biotechnology [1]. Generally, it is applicable to nearly all metals which contains partial sulfide minerals [2]. One of the limitations for the application of bioleaching is the microbial inhibition by toxic ions accumulated in leachate [3,4]. Recently, researches on microbial resistance to Ni^{2+}, As^{3+}, Hg^{+}, and organic pollutants have been investigated in detail [5–8]. Although the microorganisms can adapt to relative higher concentrations of metal ions, anionic species released from the gangue minerals can impact bioleaching even at low concentrations [9]. One of the most popular research areas is the influence of fluoride originating from fluorine-bearing gangue minerals.

Fluorine as fluoride ranks 13th in terrestrial abundance, accounting for 0.09% of the earth's crust [10]. The predominant mineral forms are fluorite (CaF_2), fluorapatite ($Ca_{10}(PO_4)_6F_2$), sellaite (MgF_2), and cryolite (Na_3AlF_6) [11]. The inhibition of *Acidithiobacillus ferrooxidans* by fluoride was firstly reported by Razzell and Trussell in 1963 [12]. A 0.4 mM sodium fluoride caused 30% decrease of iron oxidation by *A. ferrooxidans*. Increasing the sodium fluoride concentration to 1.6 mM resulted in complete inhibition of microbial oxidation of ferrous iron [12]. Recently, it was reported that the fluoride originating from gangue minerals intensively inhibited the bioleaching at concentrations as low as 0.1 mM [13]. Meantime, it has also been demonstrated that the toxic fluoride concentrations at field sites can be significantly higher than the toxic levels reported in the laboratory [14]. The deleterious fluoride ions released from fluorine-bearing minerals strongly affects microbial growth, energy intake, enzyme activity, and metabolism, and in some cases, it killed the cells [15–17]. HF has been verified as the effective form of fluoride to affect the bacterial activity due to its high permeability through phospholipid bilayer [9,18]. When HF transfers into cells, it decomposes into H^+ and F^-. Concomitantly, the cytoplasm is acidified by H^+ meanwhile F^- combines with some biosynthetic endoenzyme, inhibiting microbial growth [19,20] which is similar to that of the organic acids. Microorganisms in extreme environments are normally equipped with

* Corresponding author.
E-mail address: wangxingjie@csu.edu.cn (X. Wang).

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complex metabolic pathways that allow them to adapt to certain stress situations. Regulation of these pathways is activated immediately after the stimulation is introduced [21]. The tolerance mechanism of the bacteria to fluoride involves in regulation of cell membrane and detoxification metabolism [22]. When fluoride stress is introduced, *A. ferrooxidans* maintains the membrane fluidity and permeability by regulating the biosynthesis of diverse membrane components, such as the dermatoplasm, peptidoglycan, polysaccharide and lipoprotein, or by self-tuning the polyunsaturated/saturated fatty acid ratio. Additionally, nitrogen/phosphorus metabolism and expression of the resistance transporter and regulators involved in the detoxification system, which are closely related to protein folding and stability, DNA recombination and repair, are also enhanced to reduce the protein and nucleotide damage. Meantime, different ferrous and sulfur metabolism pathways may be activated depending on the concentration of fluoride present to provide energy for microbial adaptive adjustment [21]. As a whole, the microbial resistance to fluoride directly affects the growth activity and iron- and sulfur-oxidation ability, and subsequently, the leaching performance [13,15].

The detrimental effect of fluoride mostly mentioned in the bioleaching of high-fluorine uranium ores, which account for a large proportion of the world's uranium resources, with more than 60% found in China. The fluorine concentration in the leachate of high fluorine uranium ore can reach 2–4 g/L. In the uranium bioleaching process, iron- and sulfur-oxidizing microorganisms catalyze oxidation through indirect actions. They generate Fe^{3+} by oxidation of pyrite or additional soluble Fe^{2+}. Fe^{3+} readily attacks minerals incorporating U(IV) and converts them to U(VI), which is soluble in dilute sulfuric acid generated by biological oxidation of pyrite or additional sulfur [23]. Since the detrimental influence of fluoride on the bioleaching of uranium was described as exhibiting toxic effects on the bacteria, researches have been dedicated to reduce its adverse effect. The predominant method is improving fluoride-tolerance of the stains. Researchers have performed tests on acclimatizing and culturing the bacteria. After adaption, bacteria can grow in 45 mM F^- [24]. Another alternative would be to develop methods for fluoride removal in the process. The usual method to reduce the concentration of free fluoride ions is the use of metal cations as a complexing agent for fluoride ions, and aluminum is the typical element [25,26].

The influence of fluoride ions on the growth of microorganisms relevant to bioleaching has been extensively addressed. However, the reason that the microorganisms presented differential fluoride tolerance in bioleaching of different minerals has not yet been proposed. Herein, a fundamental exploration on the differential fluoride tolerance of sulfur- and ferrous iron-grown *A. ferrooxidans* was performed to illustrated the function of ferric ions on the detrimental influence of fluoride by monitoring the variation of HF concentration. Meantime, its implication for the bioleaching of high-fluorine containing uranium ore was discussed.

2. Materials and methods

2.1. Strains and culture media

The strain *A. ferrooxidans* ATCC 23270 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The culture medium was prepared using autoclave sterilized 9 K basal medium as described by Silverman and Lundgren [27] at pH 2.00. The 9 K basal medium contained the following components: $(NH_4)_2SO_4$, 3.0 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; K_2HPO_4, 0.5 g/L; KCl, 0.1 g/L, $Ca(NO_3)_2$, 0.01 g/L. The strain was cultivated at 30 °C in successive generations with sulfur (10 g/L) as energy substrate.

2.2. Fluoride tolerance experimental procedure

The fluoride tolerance experiments were carried out in 50-mL shake flasks with 25 mL 9 K basal medium added with ultraviolet sterilized [28] S^0 (10.0 g/L) or ferrous iron (9.0 g/L). They were all inoculated with 2.0×10^7 cells/mL *A. ferrooxidans*. The fluoride stress was introduced both prior to the lag phase or at the log phase. Biotic controls were set up without fluoride. All experiments were cultivated aerobically in triplicate at 30 °C in a rotary platform incubator at 170 rpm. All reagents were analytical grade.

2.2.1. Fluoride tolerance of sulfur grown A. ferrooxidans

In the experiment that fluoride stress was introduced prior to the lag phase, the flasks were treated by adding 0.10 mol/L NaF solution (pH 2.00, adjusted by H_2SO_4), with a final fluoride concentration of 0.10, 0.25, and 0.50 mM (1.0 mM = 1.0×10^{-3} mol/L) before inoculation. In the experiment that fluoride stress was introduced at the log phase, *A. ferrooxidans* was inoculated firstly, and the fluoride was introduced at 66 h according to the earlier experiment results by adding 0.10 mol/L NaF solution (pH 1.65, adjusted by H_2SO_4) to the culture system, with a final fluoride concentration of 0.10, 0.25, 0.50 and 1.00 mM.

2.2.2. Fluoride tolerance of ferrous iron-grown A. ferrooxidans

In the experiment that fluoride stress was introduced prior to the lag phase, the flasks were treated by adding 0.10 mol/L NaF solution (pH 2.00, adjusted by H_2SO_4), with a final fluoride concentration of 0.25, 0.50, 1.00, 1.25 and 1.50 mM before inoculation. In the experiment that fluoride stress was introduced at the log phase, *A. ferrooxidans* was inoculated firstly, and the fluoride was introduced at 22 h according to the earlier experiment results by adding 0.10 mol/L NaF solution (pH 2.00, adjusted by H_2SO_4) to the culture system, with a final fluoride concentration of 2.50, 5.00, 7.50 and 10.00 mM.

2.3. Detection for physiochemical parameters

Cell density was measured by blood cell counting chambers (XB- K-25, QiuJing, Shanghai, China) under a phase contrast microscope (BX-41TF, Olympus, Tokyo, Japan). Redox potential (Ag^0/AgCl reference) and pH were measured by a digital pH/ORP meter (pHSJ-4A, Leici, Shanghai, China). Sulfate ion concentration was quantified by a barium sulfate turbidimetric colorimetric method [29]. Ferrous iron concentration was detected according to the 1,10-phenanthroline method [30]. Values present here were means ± SD (Error bar) of triplicate samples analyzed individually.

2.4. F^- concentration analysis

The Fe-F reactions were carried out by adding 4.0 mL NaF with different concentrations to 4.0 mL $Fe_2(SO_4)_3$ (0.20 mol/L) to achieve different Fe/F ratio. The concentration of F^- ion was analyzed by fluorine ion selective electrode with a Ag^0/AgCl reference (PF-1-01, Leici, Shanghai, China) after the reaction between Fe^{3+} and F^- reaching equilibrium (5 min). The standard line for the electrode potential (Eh) and the concentration of F^- was measured as described by Gao et al. [31]. The pH value of each solution was adjusted to 2.00 or 2.20 before they were used for experiment.

3. Results and discussion

3.1. Fluoride tolerance of sulfur-grown A. ferrooxidans

Different concentrations of fluoride were introduced prior to the lag phase or at the log phase to investigate the influence of fluoride on *A. ferrooxidans* grown on S^0 substrate. Variations of pH

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