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Effects of dissolved low molecular weight organic acids on oxidation of ferrous iron by *Acidithiobacillus ferrooxidans*

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ABSTRACT

A few researchers have reported on work concerning bioleaching of heavy-metal-contaminated soil using *Acidithiobacillus ferrooxidans*, since this acidophile is sensitive to dissolved low molecular weight (LMW) organic acids. Iron oxidation by *A. ferrooxidans* R2 as well as growth on ferrous iron was inhibited by a variety of dissolved LMW organic acids. Growth experiments with ferrous iron as an oxidant showed that the inhibition capability sequence was formic acid > acetic acid > propionic acid > oxalic acid > malic acid > citric acid. The concentrations that R2 might tolerate were formic acid 0.1 mmol L^{-1} (2 mmol kg^{-1} soil), acetic and propionic acids 0.4 mmol L^{-1} (8 mmol kg^{-1} soil), oxalic acid 2.0 mmol L^{-1} (40 mmol kg^{-1} soil), malic acid 20 mmol L^{-1} (400 mmol kg^{-1} soil), citric acid 40 mmol L^{-1} (800 mmol kg^{-1} soil), respectively. Although R2 was sensitive to organic acids, the concentrations of LMW organic acids in the contaminated soils were rather lower than the tolerable levels. Hence, it is feasible that R2 might be used for bioleaching of soils contaminated with metals or metals coupled with organic compounds because of the higher concentrations of LMW organic acids to which R2 is tolerant.

1. Introduction

The contamination of soils with heavy metals continues to be a challenge when remediating many industrial sites [1]. Soil washing with acidic aqueous solution and organic solvents has been studied. Although the process is efficient for heavy metal recovery, the overall cost and difficulty of recovery of the solvent are the main limitations of this technique [2]. It is necessary to develop inexpensive and environmentally friendly extraction processes to remove heavy metals from contaminated soils or sediments. Currently, bioleaching appears to be more attractive due to its lower chemical consumption and low impact to the environment. Several studies have indicated the feasibility of remediating contaminated soils using *Thiobacilli* spp. [3,4].

The main microbial species associated with the leaching process are *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. During the bioleaching process, metal solubilization can be achieved by acidification through ferrous iron oxidation by *A. ferrooxdians* or sulfur oxidation by *A. thiooxidans*. However, the sulfur based bioleaching process is inhibited by residual sulfur, which might lead to secondary pollution such as reacidification [5]. In the iron-based bioleaching process, *A. ferrooxidans* can oxidize ferrous ions, producing ferric ions, sulfate ions and sulfuric acid (Eq. (1)) [6]:

$$4FeS_2 + 15O_2 + 2H_2O \rightarrow 4Fe^{3+} + 8SO_4{}^{2-} + 4H^+ \quad (1)$$

A. ferrooxidans is a gram-negative acidophilic chemolithoautotroph, which uses CO_2 as a carbon source and obtains its energy for growth from the oxidation of ferrous iron, sulfur, and reduced sulfur compounds [7]. The bacterium is of great importance for the leaching of metals mostly from ore deposits, mine tailings [8], sewage sludge [9–14], sediment [15–18] and fly ash in the incineration of municipal waste [19,20]. Only a few efforts have been made to apply bioleaching techniques to remediate contaminated soils [3,21–23]. This is mainly due to the sensitivity of the leaching bacterium *A. ferrooxidans* to a wide variety of organic substances, especially the dissolved low molecular weight (LMW) organic acids often present in soils [24,25]. Since Zagury et al. [26] demonstrate some strains of *Thiobacillus* are tolerant to organic substances, we attempted to investigate the tolerance to LMW organic acids of *A. ferrooxidans* isolated from soil contaminated with heavy metals, and to assess whether the isolate might be useful for remediating metal-contaminated soils.

The specific objectives of this study are as follows: (1) to isolate *A. ferrooxidans* from soil contaminated with high levels of

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heavy metals; (2) to identify the strain on the basis of phylogenetic analysis of PCR-amplified 16S rDNA sequences; (3) to investigate the influence of certain low molecular weight organic acids on oxidation of iron by *A. ferrooxidans* R2; and (4) to determine concentrations of organic aids in soil samples to assess the practicality of bioleaching using *A. ferrooxidans* R2.

2. Materials and methods

2.1. Isolation of indigenous A. ferrooxidans

Indigenous *A. ferrooxdians* (strain R2) was isolated from a soil collected from a site near Hong Tou Shan copper mine in the Liao Ning Province, China, using modified Leathen medium $(NH_4)_2SO_4$ 0.45 g L^{-1}, KC1 0.05 g L^{-1}, K_2HPO_4 0.15 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, Ca $(NO_3)_2$ 0.01 g L^{-1}, $FeSO_4{\cdot}7H_2O$ 20 g L^{-1}; adjusted to pH 4.0 using 5 M H_2SO_4 [27]. Soil pH was 2.89 (soil to water, 1:2.5). The soil total concentrations of Cu, Zn and Pb were up to 531.2 mg kg^{-1}, 3097 mg kg^{-1} and 218.4 mg kg^{-1}, respectively.

Isolation of colonies was performed by plating of enrichment cultures onto 1.5% sulfate agar: $(NH_4)_2SO_4$ 3.0 g L^{-1}, KCl 0.1 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, Ca $(NO_3)_2$ 0.01 g L^{-1}, $FeSO_4$ 22.2 g L^{-1}, adjusted to pH 2.5 using 5 M H_2SO_4 [28].

Ten grams of soil sample was shaken with 100 mL sterile saline solution (0.9%) for 2 h at 30 °C in a conical flask. After deposition of the solid phase, 10 mL of the supernatant was taken into 100 mL of sterile medium and incubated on a HDL® rotary shaker (HZQ-C, China) at 160 rpm and 30 °C until pH in the test run decreased to the lowest. Then 10% of the incubated medium solution was mixed with 100 mL of fresh medium, after many times such successive transfers into sterile fresh medium. Then the enrichment solution was spread on solid medium plates. Small rust-colored colonies that appeared after incubation for 20–30 d at 30 °C were selected and inoculated into 100 mL of modified Leathen medium. The cultures were plated again, and single colonies were selected to ensure purity. The morphology of the isolate was checked by microscopy (Olympus, BH-2) and scanning electron microscopy (SEM) (JSM-T300, JEDL, Japan), and uniform morphology was taken as an indication of purity [29].

2.2. Strain identification

Identification of the isolate was performed by phylogenetic analysis of sequenced PCR-amplified 16S rDNA gene [30]. Biomass was harvested by filtration through 0.45-μm Millipore membranes and cells were washed four times with 0.01 N H_2SO_4, transferred to 1.5 mL Eppendorf tubes and added 50 uL TE (pH 8.0). Genomic DNA extraction was completed following the procedures of the Bacterial DNA Kit (BioDev-Tech, Beijing, China). The 16S rDNA genes were amplified using primers F27 (5′-AGAGTTTGATCATGGCTCAG-3′) and R1492 (5′-TACGGTTACCTTGTT ACGACTT-3′). PCR reactions (25 μL) contained 10× buffer 2.5 μL; dNTP 2.0 μL, F27 1.0 μL, 1492R 1.0 μL, DNA template 1.0 μL, Taq enzyme 2.0 μL; two-distilled water 173 μL. Amplifications were performed using a 2400 Perkin–Elmer DNA thermal cycler and included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 10 min. The purity of PCR products was verified using the BioDev-Tech kit (Beijing, China). Sequencing of the purified products was performed at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The sequences were aligned using Clustal x 1.8 software, and phylogenetic trees were constructed using the Phylip 3.65 software using the neighbour-joining method [31].

2.3. Culture procedures

Bacterial growth inhibition by iron was determined in 250-mL conical flasks containing 85 mL of modified Leathen medium plus 15 mL inoculum supplemented with an organic acid at a chosen concentration. The modified Leathen medium was autoclaved at 121 °C for 20 min and the 15 mL inoculum was added after cooling. The organic acids were sterilized by filtration through a 0.22-μm Millipore membrane filter, separately. The inoculum was prepared by growing the bacterium in 500 mL conical flasks containing 170 mL of modified Leathen medium and 30 mL of bacterial suspension which was initially activated in fresh medium three consecutive times. The flasks were shaken on a rotary shaker at 150 rpm and 30 °C for 30 h, which was determined in a preliminary experiment to delineate the time required for the highest biological activity. After initial trial experiments, the final selected concentrations for each organic acids were as follows: for formic, acetic, propionic and oxalic acids, 0, 0.1, 0.2, 0.4, 0.8, and 2.0 mmol L^{-1}; for citric and malic acids, 0, 10, 20, 40, and 80 mmol L^{-1}. Triplicate flasks were used at each concentration. The flasks without organic acids were used as controls. Samples were withdrawn from the conical flasks at regular intervals for determination of ferrous iron by using 1,10-phenanthroline method [32] with UNICO™ 7200 Spectrophotometer (Shanghai, China) at 530 nm.

2.4. Calculations

The iron oxidation rate was calculated from the initial and final concentrations of ferrous iron according to Eq. (2),

$$\mathrm{IOR} = \frac{(4000 - C_{Fe^{2+}})}{4000} \quad (2)$$

where IOR represents iron oxidation rate; $C_{Fe^{2+}}$ is the concentration of Fe^{2+} (mg L^{-1}) and 4000 (mg L^{-1}) is the initial concentration of Fe^{2+} [25].

2.5. Determination of LMW organic acids in soils by HPLC

2.5.1. Soil samples

Soils A (a contaminated industrial soil) and B (a contaminated agricultural soil) were obtained from far and close to the Shenyang Smelter (123°49′411″E, 42°07′785″N) and from the Shenyang Zhang Shi Irrigation Area (122°52′21″E, 41°31′11″) (China). These soil samples were air-dried, ground and sieved (<0.25 mm).

2.5.2. Reagents and standards

Malic and citric acids (Guaranteed Reagent, G.R.) were purchased from Merck (Darmstadt, Germany), formic acid (HPLC grade), diammonium hydrogen phosphate and phosphoric acid (G.R.) were obtained from Kermel (Tianjin, China). Acetic, propionic, oxalic and tartaric acids and 1,10-phenanthroline (G.R.) were obtained from Pandeng Ltd. (Shenyang, China). Stock solutions (1 g L^{-1}) were prepared in ultra-pure water (EASYpure RF, Barnstead, USA) and stored in darkness at 5 °C. Deionised water was purified with a Copact Ultrapure water system (USA).

2.5.3. Extraction of dissolved organic matter (DOM)

Procedures for isolation of the DOM are described in detail elsewhere [33]. The DOM was extracted by shaking in the dark for 12 h with deionised water (soil to water, 1:5) at 160 rpm and 20 °C. The suspensions were centrifuged for 30 min at 4000 rpm and filtered through a 0.45-μm cellulose acetate filter. The soil residues were washed with deionised water, centrifuged and filtered. The process was repeated three times and then collected the filtered solutions containing DOM together. The DOM solutions were concentrated

by a freeze-drying (FD-1C-50, China). The dissolved organic carbon (DOC) as representative of soil DOM was determined by a total organic carbon (TOC) analyzer (Multi N/C, 3000). The concentrations of DOM (Soils A and B) were 24.22 mg DOC kg^{-1} and 189.12 mg DOC kg^{-1}, respectively.

2.5.4. HPLC analysis

HPLC analysis of soluble LMW organic acids was done as described in Van Hees et al. [34]. All data were corrected with respect to a blank of deionised water prepared in the same way (centrifugation, filtration, concentration). HPLC (Agilent 1100 Series) conditions were: Zobax C18 column, 250 mm × 4.6 mm; mobile phase 5 g L^{-1} $(NH_4)_2HPO_4$–H_3PO_4 (pH 2.5); flow rate 0.5 ml min^{-1}; temperature 35 °C; injection loop 10 μL; diode array detector (DAD) at 215 nm.

3. Results and discussion

3.1. Isolation and identification of bacteria R2

The soil samples collected from the mine site showed a high level of acidity (pH 2.89). Following inoculation into modified

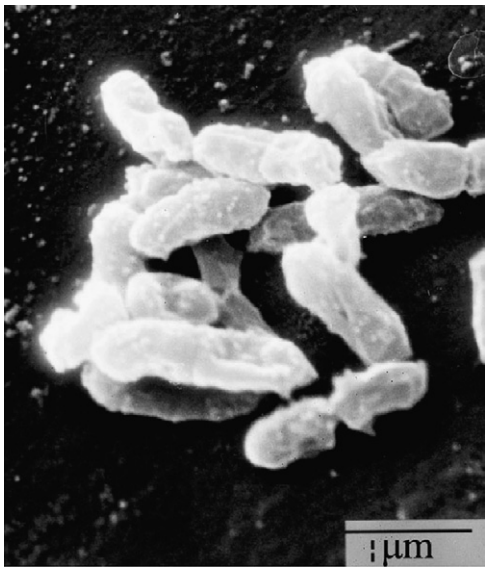

Fig. 1. SEM (scanning electron-microscope) analysis of *A. ferrooxidans* strain R2.

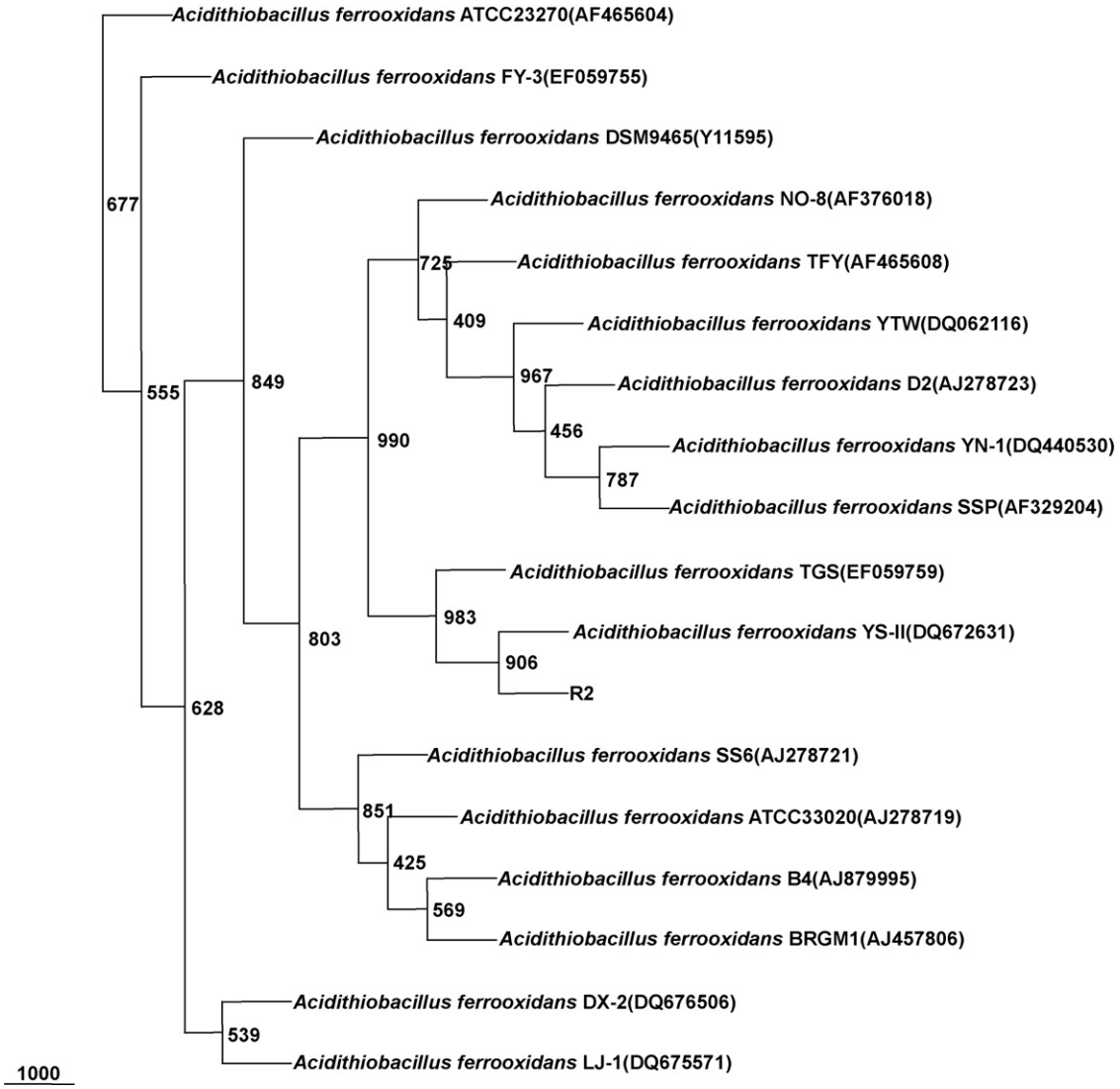

Fig. 2. Phylogenetic tree based on the 16S rDNA sequence of *A. ferrooxidans* strain R2 and the sequences of related species.

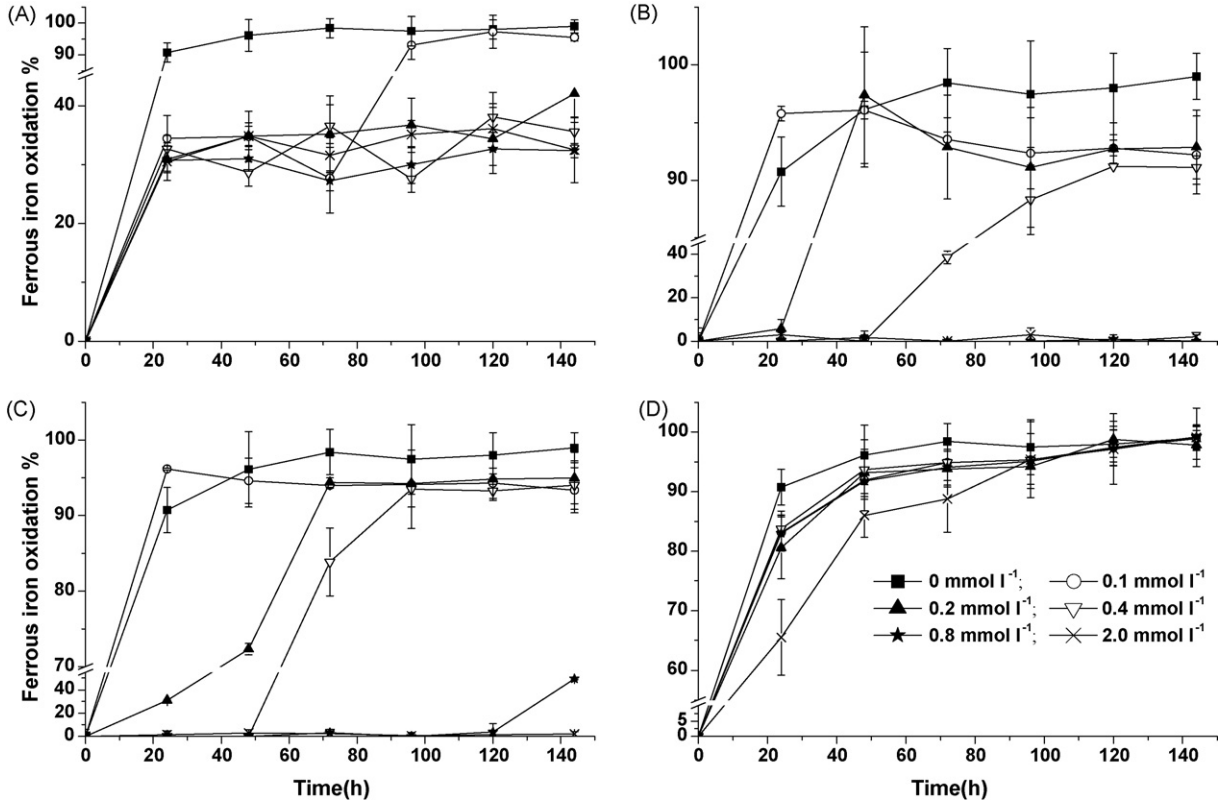

Fig. 3. Effects of formic (A), acetic (B), propionic (C) and oxalic (D) acids on ferrous iron oxidation by *A. ferrooxidans* (mmol L^{-1}): (■) CK; (♦) 0.1; (▲) 0.2; (□) 0.4; (◊) 0.8; (×) 2.

Leathen medium, turbidity and color changes were observed after 1 week of incubation. The isolate presented as small rust-colored colonies with regular margins after 20–30 d of incubation. Cells of the isolate, named as R2, were gram-negative rods with size of $(0.4\,\mu m \pm 0.2\,\mu m) \times (1.6\,\mu m \pm 0.4\,\mu m)$, singly or in pairs (Fig. 1). Furthermore, a phylogenetic tree was constructed by comparing with the published 16S rDNA sequences of the relevant species. In the phylogenetic tree, strain R2 was most closely related to *A. ferrooxidans* strain TGS and *A. ferrooxidans* strain ATCC33020 with 100% and 99.3% sequence similarity, respectively (Fig. 2). The results strongly suggested that the acidophilic iron-oxidizing bacterium is a strain of *A. ferrooxidans*.

3.2. Inhibition of iron oxidation during A. ferrooxidans growth experiments

3.2.1. Effects of formic, acetic, propionic and oxalic acids on ferrous iron oxidation

The experimental results for the ability of formic, acetic, propionic and oxalic acids to inhibit iron oxidation by *A. ferrooxidans* are given in Fig. 3. Virtually complete iron oxidation could be achieved

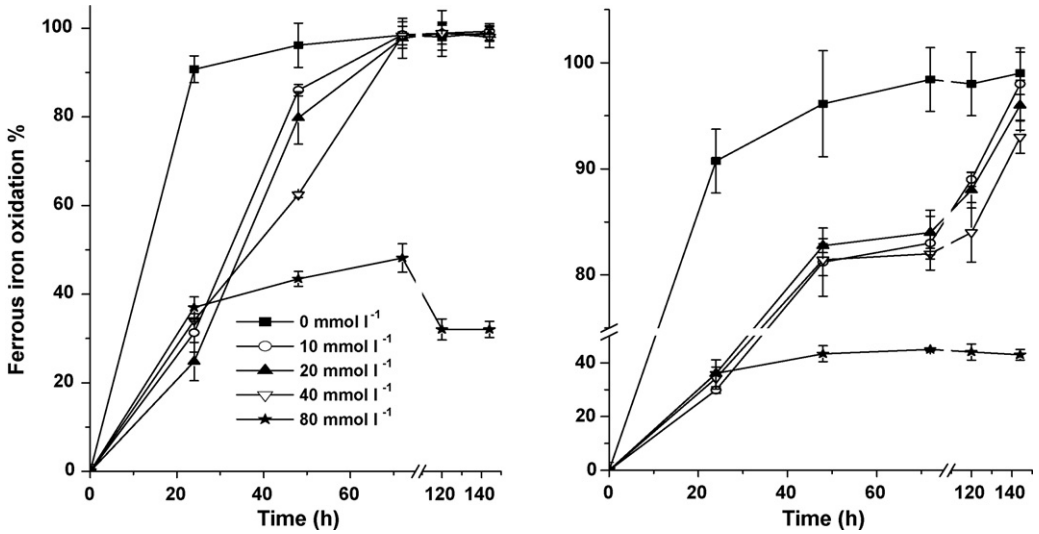

Fig. 4. Effects of citric (A) and malic (B) acids on ferrous iron oxidation by *A. ferrooxidans* (mmol L^{-1}): (■) CK; (♦) 10; (▲) 20; (□) 40; (◊) 80.

Table 1
HPLC calibration data for organic acids

Acid	Concentration ($\mu g\,mL^{-1}$)	Replicates (n)	Correlation coefficient (r)	LOD ($\mu g\,mL^{-1}$)	Recovery ($n=3$) (standard addition 100 $\mu g\,mL^{-1}$)	Retention time (min)
Formic acid	5, 25, 50, 100	3	0.9954	1.0	95 ± 4	6.587
Acetic acid	5, 25, 50, 100	3	0.9991	0.2	96 ± 8	10.104
Citric acid	5, 25, 50, 100	3	1.0000	0.2	99 ± 3	12.755
Oxalic acid	5, 25, 50, 100	3	0.9992	0.00075	102 ± 5	5.358
Malic acid	5, 25, 50, 100	3	0.9990	0.1	97 ± 7	7.495

within 30 h of incubation with no addition LMW organic acid. At concentrations of 0.2 mmol L^{-1}, formic, acetic, propionic, and oxalic acids all caused greater than 20% inhibition of iron oxidation after incubation for 24 h. Of the tested organic acids, formic acid was the most toxic. At a concentration of only 0.1 mmol L^{-1}, formic acid caused greater than 60% inhibition of iron oxidation after incubation for 72 h. However, upon longer incubation, ferrous iron was almost completely oxidized. This phenomenon indicates that the bacterium R2 might be gradually adaptive to formic acid. The presence of 0.2–2 mmol L^{-1} formic acid led to nearly complete inhibition of ferrous iron oxidation in the medium as compared to the control.

Formic acid was also found by Tuttle and Dugan [24] to be the most toxic to *A. ferrooxidans* of the tested simple acids. Similarly, Gu and Wong [25] showed formic acid was the most toxic simple acid with iron oxidation almost completely inhibited at an extremely low concentration of 0.08 mmol L^{-1}. Strain R2 was not sensitive to acetic, propionic or oxalic acids at a concentration of 0.1 mmol L^{-1}; on the other hand, a small quantity of these acids slightly stimulated iron oxidation. At higher concentrations, these acids displayed a delaying inhibitory behavior. Acetic acid was more inhibitory to the bacteria than propionic acid in the range 0.2–0.8 mmol L^{-1}. Almost complete inhibition occurred at the concentrations of 0.8 mmol L^{-1} acetic acid and 2.0 mmol L^{-1} propionic acid after incubation for 144 h.

These results are supported by earlier studies by Gu and Wong [25] who showed that 10.8 mmol L^{-1} acetic acid and 9.88 mmol L^{-1} propionic acid in sewage sludge increased lag period to 6 d and 7 d during the solubilization of Cu and Cr, respectively. Of the four acids, oxalic acid had lower inhibition to oxidation of ferrous iron than formic, acetic and propionic acids. Oxalic acid (0.4 mmol L^{-1} and 2.0 mmol L^{-1}) caused 16% and 35% inhibition after growth of 24 h. Upon further incubation, ferrous iron was almost completely oxidized.

Tuttle and Dugan [24] showed that rather low concentrations (0.001 mmol L^{-1}) of acetic and propionic acids resulted in 35% and 33% inhibition, respectively; oxalic acid (1 mmol L^{-1} and 10 mmol L^{-1}) produced 10% and 100% inhibition. The concentrations that strain R2 might tolerate were formic acid 0.1 mmol L^{-1}, acetic and propionic acids 0.4 mmol L^{-1}, oxalic acid 2.0 mmol L^{-1}, respectively. These observations suggest that strain R2 may be more tolerant to acetic, propionic and oxalic acids and less tolerant to formic acid than the bacterial strains investigated by previous authors.

3.2.2. Effects of citric and malic acids on ferrous iron oxidation

The results as seen in Fig. 4 (A) and (B) indicate that citric and malic acids had the capability to inhibit iron oxidation. The presence of 40 mmol L^{-1} citric acid and 20 mmol L^{-1} malic acid led to lag periods of 2 d and 5 d when iron oxidation was almost completed. After incubation for 24 h, 10 mmol L^{-1} citric and malic acids caused 60% and 55% inhibition of iron oxidation, respectively. Other studies showed that malic acid (1 mmol L^{-1} and 10 mmol L^{-1}) caused 25% and 39% inhibition of iron oxidation [24]. The results found in the present study show that the bacterium R2 could tolerate higher concentrations of citric acid (40 mmol L^{-1}) and malic acid (20 mmol L^{-1}).

Inhibition of iron oxidation by organic acids depended on the type of substitution group on the carboxylic acid and to the degree of oxidation of the interior carbons. Longer chain length in straight-chain monocarboxylic acids resulted in decreased inhibition, and greater oxygenation resulted in decreased inhibition for dicarboxylic or multicarboxylic acids.

3.3. LMW organic acids in the soil samples

A satisfactory separation of a wide range of acids could be achieved (Table 1). The performance of the HPLC method was evaluated with regard to limits of detection, calibration and recoveries of standard additions. All calibration graphs were linear and had a correlation coefficient $r > 0.99$. Three replicates were carried out for each analysis with duplicate injections for each standard. Recovery studies were performed by standard addition of 100 $\mu g\,mL^{-1}$ of each acid.

The concentrated solutions from soils A and B were analyzed for water-soluble LMW organic acids using the methods described above (Table 2). The acids were detected by comparing retention times. Acetic, citric, oxalic and malic acids were detected in both soil samples and the concentrations of those acids in soil B were much greater than in soil A. Formic acid and tartaric acid were also observed in soil A. Since the sample B was collected from a polluted agricultural soil, and sample A was obtained from a contaminated industrial soil resulting from smelting activities, no similarity of concentrations of LMW organic acids was expected. The level of oxalic acid in the sample soil B was the highest obtained (3 mmol kg^{-1} soil) of all acids. However, the concentrations of LMW organic acids found in the sample soils were well below those in which *A. ferrooxidans* R2 could tolerate formic acid 0.1 mmol L^{-1} (2 mmol kg^{-1} soil), acetic and propionic acids 0.4 mmol L^{-1} (8 mmol kg^{-1} soil), oxalic acid 2.0 mmol L^{-1}

Table 2
Concentrations of LMW organic acids in the sample soils

Soil	Formic acid	Acetic acid	Citric acid	Oxalic acid	Malic acid	Gluconic acid	Tartaric acid	Total acidity
Acid concentrations in the soils ($\times 10^{-2}$ mmol kg^{-1} soil)								
A	3.68	0.1	0.88	80	4	–	2.8	180
B	–	27	1.96	300	11.8	–	–	815.6

n.d., not detectable, area of peak smaller than intercept of calibration equation or below LOD; tr., traces, peak identified but not integrated.

(40 mmol kg^{-1} soil), malic acid 20 mmol L^{-1} (400 mmol kg^{-1} soil) and citric acid 40 mmol L^{-1} (800 mmol kg^{-1} soil), respectively. Hence, it is likely that the strain R2 could be applied in bioleaching studies of soils contaminated with heavy metals or heavy metals and organic compounds.

4. Conclusions

(1) *Thiobacilli* that tolerate organic compounds were isolated from a sampling site contaminated with heavy metals. The isolate (Strain R2) was identified as *A. ferrooxidans* by phylogenetic analysis of PCR-amplified 16S rDNA sequences.
(2) Iron oxidation was inhibited by all tested organic acids, however formic acid was the most toxic to the activity of strain R2. When concentration of formic acid exceeded 0.2 mmol L^{-1}, iron oxidation was almost entirely inhibited; acetic acid was more inhibitory to bacteria than propionic acid in the range of 0.2–0.8 mmol L^{-1}. After 24 h incubation, 2.0 mmol L^{-1} oxalic acid caused 35% inhibition of iron oxidation; 10 mmol L^{-1} citric and malic acids caused 60% inhibition. On extending the incubation period to 72 h, the bacteria could still oxidize over 80% of the iron present with up to 20 mmol L^{-1} citric acid and up to 40 mmol L^{-1} malic acid. Growth experiments with ferrous iron as an oxidant showed that the inhibition capability sequence was formic acid > acetic acid > propionic acid > oxalic acid > malic acid > citric acid.
(3) HPLC analyses showed that the contaminated soils contained much lower concentrations of the tested acids. Since strain R2 could tolerate higher concentrations of low molecular weight organic acids, the study contributes to recent efforts towards development of cost effective, environmentally safe methods for detoxifying soil contaminated with heavy metals or heavy metals coupled with organic compounds.

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