Bio-reduction of Fe(III) ores using three pure strains of *Aeromonas hydrophila*, *Serratia fonticola* and *Clostridium celerecrescens* and a natural consortium

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1. Introduction

Nowadays, biotechnological solutions result, in many cases, the most adequate answers to environmental, biological and other problems related with materials or health. Recent examples of this great variety of functions and applications of microbial are the employment of *Pseudomonas* for the bioremediation of petroleum-contaminated soils (Karamalidis et al., 2010); the use of *Clostridium butyricum* for improving the processing of cellulose (Yung-Chung et al. 2010) or the mobilization of ferrous iron by anaerobic reducing bacteria (Miot et al., 2009).

The active role of microorganisms in promoting the leaching of metals from naturally occurring ores is well known. Some bacterial species and consortia have been identified as the major players in the aerobic oxidation of ores has been studied in depth and its mechanisms widely discussed. Two fundamental contributions have led to the implementation of bioleaching processes: the use of Archaea and bacterial thermophiles and the development of heap bioleaching technology (Brierley, 2007).

In spite of this important advance in aerobic biomining processes, little is known about anaerobic bioleaching, and bio-beneficiation of oxides.

Currently, acid-generating aerobic bioleaching systems for the recovery of copper and gold are being discussed in the light of economic and environmental constraints. Anaerobic bioleaching technology circumvents these limitations since no oxygen is required and generation of acid is avoided (Haoran et al., 2005). In fact, iron reducing microorganisms play an important role for alkalinity-generating processes in mining lakes with low pH (Porsch et al., 2009). Biotechnologically speaking, the use of dissimilatory Fe(III)-reducers for iron extraction from recalcitrant ores represents a low-cost and environmentally acceptable technology (Hitzman, 2001).

Two different approaches have been considered for the implementation of anaerobic bioleaching of iron ores: (1) using aerobic microorganisms such as *Acidithiobacillus ferroxidans* that are force to grow under anaerobic conditions, (Li et al., 2006) and (2) using anaerobic microorganisms with the ability to dissolve ores through the dissimilatory pathway (Aralah et al., 2008). Two representative equations showing the use lactate as electron donor and iron (soluble or as solid hematite) as electron acceptor are the following:

\[
\begin{align*}
4 \text{Fe}^{2+}(aq) + \text{CH}_3\text{COO}^- + 6\text{H}^+ & \rightarrow 4 \text{Fe}^{3+}(aq) + \text{CH}_3\text{CO}_2^- + 2\text{H}_2\text{O} \\
2 \text{Fe}^{3+}(aq) + \text{CH}_3\text{CHOHCOO}^- + \text{H}_2\text{O} & \rightarrow 2 \text{Fe}^{2+}(aq) + \text{CH}_3\text{CO}_2^- + \text{HCO}_3^- + 4\text{H}^+
\end{align*}
\]

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In the dissimilatory Fe(III) reduction process, microorganisms transfer electrons to an external source of ferric iron that is reduced to ferrous iron but without assimilation of iron. At neutral pH and under oxidizing conditions, Fe(II) is the stable oxidation state of iron, more soluble and therefore biologically more available than ferric iron (Arnold et al., 1988). Since Fe(III) is insoluble, Fe(III) oxides are the primary repository of oxidized iron in near-surface environments.

Dissimilatory Fe(III)-reducing microorganisms can be divided into two major groups: those that support growth by conserving energy from electron transfer to Fe(III) and those that do not. The *Clostridium* and *Serratia* genera are fermentative bacteria belonging to the latter group whereas *Aeromonas hydrophila* belongs to the former group. The biotechnological possibilities of *A. hydrophila* has been described yet. An example is the selection of the bacteria for the recuperation of polyhydroxalkanoates (PHA) with different applications as bio-plastics or bio-implant materials (Jian et al., 2018).

Several oxyhydroxides of varying crystallographical and chemical composition have been dissolved via anaerobic bioleaching. Bose et al. (2009) described the bio-reduction of hematite by *Shewanella oneidensis* strain MR-1. Other oxyhydroxides (ferrihydrite, lepidocrocite, goethite and hematite) have also been tested using dissimilatory iron reducing strains of *Shewanella putrefaciens* (Bonneville et al., 2004).

One key factor for the industrial implementation of anaerobic bioleaching of oxides is the recovery of the metal of interest in a soluble form (Kim et al., 2002). Thus, one of the most interesting aspects of applying dissimilatory Fe(III) reduction would be the production of soluble products after mineral dissolution (Arnold et al., 1988).

As in the case of aerobic processes, the anaerobic bioleaching of iron ores can promote the formation of different precipitates during microbial growth. In fact, most of the Fe(II) produced from microbial Fe(III) reduction is found in solid phases. Different insoluble phosphates and carbonates, such as vivianite (Fe₃(PO₄)₂·8H₂O) and siderite (FeCO₃), can be formed in bacterial cultures grown on soluble Fe(III). When oxyhydroxide is used as a source of Fe(III), the final product formed may be maghemite (Fe₂O₃) (Lovley, 2006). In concrete, the precipitation of magnetite requires a combination of passive and active mechanisms. A hypothesis is that the precipitation occurs as a consequence of active production of Fe⁷⁺(aq) when bacteria utilize ferrirhodite as a terminal electron acceptor, and the pH rise probably due to the bacterial metabolism of amino acids (Pérez-Gonzalez et al., 2010).

This research is focused on the anaerobic bioleaching of iron ores with emphasis on two aspects: screening of bacterial species, and their adaptation to improve solubilization of iron compounds. The kinetics of dissimilatory Fe(III) reduction was investigated using three different pure strains (identified as *A. hydrophila*, *Serratia fonticola* and *Clostridium celerecrescens*) and a natural consortium. The aim of isolation was to obtain not strictly anaerobic species but facultative anaerobes with greater flexibility and versatility to facilitate upscaling from culture conditions to industrial bioreactors. A complementary study on the nature of the soluble Fe(II) complex formed in *A. hydrophila* cultures is also reported.

The most novel aspect described here concerns the behaviour of *A. hydrophila*. Both *A. hydrophila* and the natural consortium cultures showed faster growth kinetics and higher Fe(II)-bioreduction (100% in less than 48 h) than the other two isolates (*Serratia* and *Clostridium*). An additional advantage of *A. hydrophila* cultures was that Fe(II) remained in solution at circum-neutral pH values. Neither *Serratia* nor *Clostridium* exhibited this behaviour. Instead, two precipitates (Fe₂O₃ and FeCO₃) were identified in cultures of *Serratia* and *Clostridium* respectively. In the microbial consortium “B-orilla”, ferrous iron also precipitated as vivianite (Fe₃(PO₄)₂) and siderite (FeCO₃). In addition, the ability of *A. hydrophila*—not yet reported—to grow at low pH values is another promising result related to the implementation of the ore dissolution process.

### 2. Methods

#### 2.1. Sampling site

The samples for the microbial study were collected from the edge of an open-pit lake surrounding an extinct mine site named “Brunita” (formerly a source of Pb–Zn ores) near La Unión (Murcia, Spain). The artificial lake, formed after the shutdown of the mineral activity, has a capacity of approximately 622,000 m³ of water at pH 3.0–3.5 and with a high concentration of MgSO₄ (Robles-Arenas, 2007).

The enrichment cultures were started in situ as follows: samples (6 g ca.) were taken with a box-corer and immediately transferred to sterile plastic tubes containing deionized and deoxygenated water (20 ml). The mixture was then vigorously shaken and inoculated (10% v/v) using sterile syringes in vials containing a specific basal medium for dissimilatory ferric reducers (as described below). Vial headspaces were previously bubbled with a sterilized gas mixture of N₂/CO₂ (80/20, v/v).

#### 2.2. Enrichment cultures

All enrichment cultures were grown in modified ferrous sulfate-free Postgate C medium (BRS) supplemented with ferric citrate (60 mM Fe³⁺). The modified Postgate C medium contained the following salts (g/l): KH₂PO₄, 0.5; NH₄Cl, 1; Na₂SO₄, 4.5; CaCl₂·6H₂O, 0.06; MgSO₄·7H₂O, 0.06; sodium lactate, 6; yeast extract, 1; FeSO₄·0.004; and sodium citrate·7H₂O, 0.3. pH was adjusted to 7.5 ± 0.2.

Dissimilatory Fe(III)-reducing enrichment cultures were prepared in situ by adding 6.0 g (ca.) (wet weight) of soil sample to 60 ml of sterile basal medium containing ferric citrate (3 g/l in Fe³⁺) as electron acceptor and sodium lactate (6 g/l in lactate) as electron donor (Cummings et al., 2000). The cultures were transferred in the dark, in boxes, at ambient temperature (18–20 °C). Once in the laboratory, the cultures were incubated unshaken at 30 ± 2 °C in the dark. Subsequent transfers were carried out, and in each transfer Fe-reducing activity was checked by analysis of Fe(II) with the ferrozine method (as described below).

Standard anaerobic techniques were used throughout this study. Each culture medium was dispensed into glass flasks where the air headspace was replaced with N₂/CO₂ (80/20, v/v). The flasks were capped with butyl rubber stoppers and sealed with an aluminium crimp. Sterilization was accomplished by autoclaving (121 °C, 30 min).

#### 2.3. Characterization of the microbial consortium *B-orilla*

A culture-independent approach based on PCR amplification and denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA gene fragments from both *Bacteria* and *Archaea* was used to analyse the microbial community of the *B-orilla* consortium. The DGGE fingerprint study was performed at the Biotechnology Centre of the Universidad Católica del Norte (Antofagasta, Chile). Details of the experimental procedure can be found in Demergasso (Demergasso et al., 2005).

#### 2.4. Isolation and identification of Fe(III)-reducing bacteria

Bacteria were isolated after ten successive transfers (10% inoculum) of active Fe(III)-reducing enrichment cultures. Isolates were obtained by spreading 100 µl of enrichments onto agar plates
(20 g/l of agar) prepared in BRS medium supplemented with ferric citrate (60 mM in Fe3+). Plates were incubated anaerobically at 30 ± 0.2 °C in an anaerobic jar (GENbox bioMérieux). Carbon dioxide content measured in the jar after 24 h was higher than 15%. Individual colonies were transferred to 10 ml serum bottles containing BRS medium supplemented with Fe3+. Fe(II) production was measured spectrophotometrically using the ferrozine method (described below).

Isolates were re-streaked and purified before being sent to the Spanish Collection of Type Cultures (CECT, University of Valencia, Valencia, Spain) for identification. Partial 16S rRNA sequences (about 1000 nucleotides) were obtained from the CECT microbial identification service. The methodology employed is described in Arahal (Arahal et al., 2008).

2.5. Kinetic tests

The kinetics of ferric reduction was tested in vials containing 54 ml of Postgate C medium supplemented with ferric citrate (60 mM in Fe3+) and 6 ml of an inoculum obtained after more than ten successive transfers. Bacterial cultures were kept in anaerobic and static conditions, in the dark at 30 ± 0.2 °C. In addition, chemical controls prepared with prepared cell-free culture medium to evaluate the extent of aqueous chemical reactions that might also occur (Bose et al., 2009). All reported data are averages of duplicate kinetic tests. In order to avoid any discrepancy caused by physiologically changes of the bacterial culture, all experiments were inoculated with the same inoculum as described by Li (Li et al., 2006).

2.6. Monitoring cultures: sampling and analysis

Dissimilatory Fe(III)-reducing cultures were monitored by taking samples at different times and analyzing ferrous and total iron concentration and the total number of cells. Ferrous and Fe tot concentration were measured in the same acid extraction solution. The total number of living cells was determined in a Thoma counting chamber.

For sampling, 0.5 ml of the sterile gas mixture (N2:CO2, 80/20 v/v) was injected into the anaerobic vial with a deoxygenated, sterile syringe and 0.5 ml of sample was withdrawn. The mixture of gases was sterilized through a sterile filter of 0.22 μm. Samples collected were immediately transferred to the extraction solution to prevent any iron oxidation.

Bacterial culture vials with precipitates were sampled in a different way (García-Balboa et al., 2009): two replicates were taken from the clarified supernatant and another two from culture shaking vials. With this sampling method it is possible to determine the ferrozine–Fe(II) complex at 562 nm after 5 min of colour development (Bose et al., 2009). Standards of ferrous iron for the ferrozine assay were prepared from ferrous ethylene diammonium sulfate tetrahydrate dissolved in 0.5 N HCl (Lee et al., 2007). Any possible reduction by ferrozine of Fe(III) from biogenic nanoparticles that might have passed through the filter was prevented using HEPES at pH 7 (Pullin et al., 2004). Duplicate samples were also taken from cell-viable control blanks prepared to examine the extent of abiotic reduction of Fe3+ in solution.

2.7. X-ray diffraction analysis

Precipitates formed in some culture vials were recovered and preserved for identification. Oxidation was prevented by keeping samples in anaerobic conditions before analysis by bubbling a gas mixture of CO2:N2 (80/20 v/v). In certain cases X-ray diffraction analyses were performed on wet solids.

Mineralogical characterization was carried out by powder X-ray diffraction (XRD) on a Philips X’pert-MPD system with a Cu anode operating at a wavelength of 1.5406 Å (CuKα1) as the radiation source. Specimen were prepared by grinding the samples to a fine powder with a mortar and pestle and then placed on off-axis quartz plates (18 mm diameter × 0.5 mm DP cavity). The scanning range was from 10° to 60° 2θ with an angular interval of 0.05° and 4 s counting time. The crystalline phases were identified using standard cards from the International Centre for Diffraction Data (ICDD, Newtown Square, Pennsylvania) Powder Diffraction File database.

2.8. Growth on Fe(III) ores

Three Fe(III) ores (ferrihydrite, hematite and ammonium jarosite) with different crystallinity properties were used to test the growth of the three isolates and the microbial consortium. Ferrihydrite, an amorphous Fe(III) oxyhydroxide, was chemically synthesized by neutralization of a 0.4 M FeCl3 solution with NaOH followed by washing with deionized water until the chloride concentration as determined by titration with AgNO3 was below 1 mM (Lovley, 2006). The solid obtained was kept constantly humid in anaerobic vessels previously bubbled with the gas mixture of N2:CO2 (80/20 v/v). Ferrihydrite was not dried because its crystalline properties change with the drying process and it is transformed into a more crystalline iron compound, goethite. The amorphous nature of the Fe(III) oxyhydroxide was confirmed by X-ray diffraction analysis. Two additional solids were also tested: analytical-grade hematite (Fe2O3) from Panrec and crystalline ammonium jarosite biosynthesized using a pure culture of Sulfolobus metallicus grown in Norris medium (Norris and Barr, 1985) (MgSO4·7H2O, 0.5 g/l; (NH4)2SO4, 0.4 g/l and K2HPO4, 0.2 g/l) supplemented with 50 mM Fe2+ at pH 1.8, 250 rpm and 70 ± 3 °C. The ammonium jarosites collected were washed with acidified water and dried at room temperature. The composition of the solid that formed was confirmed by XRD analysis.

Tests were performed in vials containing 30 ml of basal solution (Postgate C medium) prepared in anaerobic conditions. The three isolates and the microbial consortium were adapted to the solids by progressively reducing the proportion of iron in the liquid/solid ratio (75:25, 50:50, 15:85 and 0:100).

The three solids employed were ferrihydrite, ammonium jarosite and hematite. The total concentration of Fe(III) was 100 mM.
3. Results and discussion

3.1. The “B-orilla” consortium culture. Isolation and characterization of the isolates

An acidic abandoned mine site was sampled to isolate Fe(III)-reducing bacteria. The screening stage was not centered on strict anaerobic iron reducers but microorganisms able to tolerate low concentrations of oxygen, with wider biotechnological applications.

Molecular analysis (DGGE) of the bacterial prokaryotic consortium “B-orilla” revealed low species richness with a maximum of three different bands both in bacterial and in archaeal fingerprints. The data compiled are shown in Table 1. One explanation could be that the culture employed in the molecular analysis was successively adapted to growth under very restricted conditions and probably contained a "very reduced version" of the originally-present bacterial population. In contrast, the genus Clostridium was the most abundant and one of the isolates corresponded to C. celerecrescens species are flexible in the use of electron acceptors and are dominant with frequency in enrichment cultures (Wang et al., 2009).

After isolation and purification, three Fe(III)-reducing strains were selected and identified as C. celerecrescens, S. fonticola and A. hydrophila. In all cases microbial colonies grown on Fe(III)-citrate medium under anaerobic conditions were typically less than 1 mm in diameter. Fe(III)-reducing colonies of Clostridium and Serratia were similar and easily recognizable by change of colour and the appearance of brownish precipitates of Fe(II) and possibly co-precipitates of Fe(III) over the whitish colonies. The agar in the A. hydrophila plates changed from brown to green, and a light whitish film covered the surface; there were no precipitates on the colonies.

In addition, LB medium plates were prepared to test the ability of the three strains to grow under aerobic and fermentative conditions. Unlike Clostridium, Aeromonas and Serratia grew aerobically in LB medium. Colonies of A. hydrophila were white, domed, entire, and wet whereas Serratia colonies were whitish and creamy.

Growth under anaerobic and fermentative conditions was tested in plates prepared with lactate but without Fe-citrate. The three strains were also able to grow on organic acid (lactate) in anaerobic conditions.

The cells of the three strains are straight rods with rounded ends (bacilli), usually from 0.3 to 1 μm wide and 1 to 3 μm long. All three bacteria are motile due to the presence of polar flagella.

3.2. Kinetics of Fe(III) reduction by the “B-orilla” microbial consortium and the three isolates

The prokaryotic consortium “B-orilla” reduced Fe(III)-citrate through all (1:10) serial transfers as evidenced by the production of Fe(II) with no detectable inhibitory effect. Reduction of Fe(III)-citrate by the bacterial prokaryotic consortium was fast and effective: complete iron bio-reduction was achieved in 36 h. However, Fe(II) did not remain in solution. As growth progressed, the concentration of ferrous iron in solution decreased whereas the Fe(II) concentration measured in the acid extraction solution increased. X-ray analysis of the white precipitate recovered showed a mixture of Fe(II) compounds: vivianite (Fe₃(PO₄)₂·8H₂O) and siderite (FeCO₃).

The three isolated strains (A. hydrophila, S. fonticola and C. celerecrescens) reduced Fe(III)-citrate under anaerobic conditions with lactate as the electron donor (Fig. 1). The generation of Fe(II) coincided with an increase in the number of cells (Fig. 2), which suggests that Fe(III) reduction took place through a dissimilatory pathway (Knight and Blakemore, 1998). The extent of Fe(III) reduction and the final cell yields were directly proportional to the amount of Fe(III) added. In addition, pH maintained circum-neutral values during the microbial growth phase (data not shown), as one would expect in an enzymatic process (Knight and Blakemore, 1998; Weisener et al., 2008). Otherwise, if a fermentative process had been involved in Fe(III) reduction, the recorded pH values would have been around 4.5, consistent with the production of organic acids (Knight and Blakemore, 1998).

Of the three isolates tested, A. hydrophila showed the best Fe(III) reduction efficiency. The maximum level of bio-reduction (93%) was reached at day 3 in A. hydrophila culture, but 82% bio-reduction had already been reached by day 2 (Fig. 1). S. fonticola achieved 48% Fe(III) reduction in 15 days and a maximum of 59% in 27 days (Fig. 1). Cultures of C. celerecrescens reduced Fe(III) slowly, and only about 30% bio-reduction was achieved. This maximum value was reached at day 21 and there was no further reduction in the twenty days following (Fig. 2). The behaviour of S. fonticola and C. celerecrescens was similar, then; both were able to reduce Fe(III) via a dissimilatory pathway—as evidenced by the concomitant increase in cells and Fe(II) concentration—but this mechanism had no a real importance and the fermentative pathway was predominant.

A. hydrophila is considered a respiratory organism (Knight and Blakemore, 1998; Lovley, 2006) able to reduce Fe(III) pathway a non-fermentative process. In contrast, S. fonticola and C. celerecrescens are fermentative microorganisms that can use Fe(III) as an electron acceptor (Lin et al., 2007b) and anaerobically reduce Fe(III) primarily through fermentative processes (Scala et al., 2006). These microbes anaerobically reduce iron primarily through fermentative processes, although this reduction represents only a minor pathway for electron flow (Wang et al., 2009). Therefore, these two strains reduce Fe(III) but do not conserve energy from Fe(III) reduction (Lovley, 2006). In fact, the two isolates tested were unable to bior-educate all the ferric iron (Fig. 1). Clostridium in particular has been demonstrated to play a minor role in the bio-reduction of iron in
natural environments (Lin et al., 2007b). However, although dissimilatory Fe(III) reduction is not the principal avenue of survival in Fe(III)-anaerobic environments, both strains were able to reduce Fe(III) via a dissimilatory pathway and both are commonly related
to dissimilatory Fe(III) reduction processes (Adams et al., 2007; Arnold et al., 1988). In addition, the versatile behavior of these facultative Fe(III) reducers makes them interesting (Lin et al., 2007a).

A. hydrophila presented even faster kinetics (100% of bio-reduction in 48 h) than other Fe-reducers with similar metabolisms reported in the literature (Adams et al. 2007; Knight and Blakemore, 1998; Scala et al., 2006).

Then, S. fonticola and C. celerecrescens were not selected to go in depth because these two species did not reduce Fe(III) in a 100% and their kinetic are sensibly slower. In contrast, the prokaryotic consortium and the A. hydrophila isolate were able to reduce all the Fe(III) in less than 48 h and retained this ability through transfers.

Although the microbial characterization of the enrichment cultures was not complete, the results obtained from the identification of the isolates provide useful indirect information. The presence of Clostridium, Serratia and Aeromonas sp. is an indication that fermentative and respiratory processes would take place in real conditions. Although Clostridium and Serratia might not play a key role in iron reduction, their fermentation products can provide excellent substrates for iron reduction by other microorganisms such as Aeromonas. For instance, in sedimentary environments in which Fe(III) reduction is the predominant terminal electron acceptor, the oxidation of organic matter takes place with the strong collaboration of fermentative and respiratory organisms (Lovley, 2006). Furthermore, ferrhydrate reduction by several Geobacter species is expected to be stimulated by the presence of secondary bacteria, including the fermentative Lactococcus lactis (Stroh and Schink, 2004). Microbial synergies between fermentative and Fe-reducing microorganisms have been reported in nature (Scala et al., 2006).

Specific analysis of the concentration of organic acids in the spent cultures would be necessary to fully demonstrate the following hypothesis, but it is very possible that C. celerecrescens (and also S. fonticola) use citrate as a fermentative substrate, so that the Fe(III) is dissociated from the Fe(III)-chelated complex and may precipitate as a non-crystalline form, sometimes together with other Fe(II) solid compounds (Lovley, 2006). As discussed further below, this does not occur in A. hydrophila cultures because, as reported by Knight (Knight and Blakemore, 1998) after HPLC analysis of culture fluids, A. hydrophila does not produce citrate anaerobically.

In the present case, the microbial consortium might have been expected to reduce Fe(III) more efficiently than the isolates through a cooperative mechanism involving fermentative and respiratory microorganisms present in the "B-orilla" consortium. However, A. hydrophila was able to reduce Fe(III) faster than the consortium and additionally exhibited an ability to retain ferrous ion in solution.

As is well known, an essential requirement for the implementation of anaerobic biodegradation of iron oxides is that ferrous iron remains in solution. However, most of the Fe(II) produced from microbial Fe(III) reduction is found in solid phases such as magnetite, siderite (FeCO3) or vivianite (Fe3(PO4)2·8H2O) (Lovley, 2006). The relative abundance of mineral end products and the relative distribution of these products are strongly dependent on the bacterial species or strain iron reduction (Salas et al., 2010). The formation of these end products is an additional disadvantage because of the need to remove these compounds, and in the case of magnetite, a certain amount of Fe(III) still remains unavailable to the microbial population. Chemical analysis of the precipitates that formed in our S. fonticola and C. celerecrescens cultures revealed the presence of both Fe(II) and Fe(III).

X-ray diffraction analysis of these two precipitates recovered from Serratia and Clostridium cultures allowed to identify the presence of Fe2O3 and FeCO3, respectively. No remaining Fe(III) associated with the solids was identified, possibly because of the amorphous general characteristics of the two solids.
was excellent, comparable to the consortium but with the additional advantage that Fe(II) remained in solution (Fig. 4). As mentioned previously, this is an interesting result in that it shows that iron ores can be reduced without the need of extreme pH and extra energy inputs.

Two additional tests were performed to identify the complexing agent responsible for the solubility of ferrous iron in *A. hydrophila* cultures: (1) in Postage C culture medium but with Fe(II)-pyrophosphate instead of Fe(III)-citrate; and (2) in Postage C culture medium with Fe(III)-citrate but without lactate. Fe(III) reduction was positive in both cases but while in the first case a white solid was formed, no precipitate was observed in the second case. Moreover, ferrous iron concentration was 2.13 g/l with citrate and 0.5 g/l with pyrophosphate after 7 days. In the latter case, a Fe(II)-phosphate, vivianite (Fe(PO4)2·8H2O), was detected in the residue.

Presumably, therefore, the citrate ion is the ligand responsible for the complexation and solubilization of the ferrous iron formed in the reduction step. This is supported by the stability constants of citrate as an iron chelating agent (Virgili, 1996). Table 2 shows that Fe(II)-citrate is less stable than Fe(III)-citrate complex. In such cases, ferrous ion is preferentially “sequestered” by citrate, but as Fe(III) is reduced, Fe(II) is complexed with “free” citrate.

Additionally, Knight (Knight and Blakemore, 1998) demonstrated that *A. hydrophila* was unable to dissimilate citrate anaerobically. If that is so, in our *A. hydrophila* cultures the citrate complex agent should be available after iron reduction.

### 3.4. Bio-reduction of Fe(III)-solids

The “B-orilla” consortium was adapted to grow on ferrihydrite, jarosite and hematite. In all cases the bio-reduction process was incomplete, with a maximum Fe(III) reduction of 50% after one month and the formation of a black precipitate identified as jarosite and hematite.

### Table 2

Stability constant of some iron chelates (log K).

<table>
<thead>
<tr>
<th>Quelant agent</th>
<th>Fe(III)</th>
<th>Zn(II)</th>
<th>Cu(II)</th>
<th>Mn(II)</th>
<th>Ca(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>25.0</td>
<td>14.27</td>
<td>14.87</td>
<td>18.70</td>
<td>13.81</td>
</tr>
<tr>
<td>EDDHA</td>
<td>33.9</td>
<td>14.3</td>
<td>16.8</td>
<td>23.94</td>
<td>–</td>
</tr>
<tr>
<td>HEEDTA</td>
<td>19.6</td>
<td>12.2</td>
<td>14.5</td>
<td>17.4</td>
<td>10.7</td>
</tr>
<tr>
<td>Citrate</td>
<td><strong>11.2</strong></td>
<td><strong>4.8</strong></td>
<td>4.86</td>
<td>5.90</td>
<td>3.70</td>
</tr>
<tr>
<td>Gluconate</td>
<td>37.2</td>
<td>1.0</td>
<td>1.70</td>
<td>36.6</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: stability constants are calculated according to the following equilibria:

metal + quelant chelated – metal; K = [chelated – metal]/[metal] [quelant].
Fe$_2$O$_3$. This precipitate probably slowed down the bio-reduction process.

On the other hand, *A. hydrophila* was able to completely dissolve ferrhydrite in 7 days in cultures with a L/S ratio 75:25. The maximum concentration of Fe$^{2+}$ was 4.51 g/l (total iron was 5.24 g/l). The appearance of the culture was the same as described before when dissolved Fe(III)-citrate was added: no precipitates were observed and Fe(II) and Fe(III) species remained in solution. *A. hydrophila* was likewise able to solubilize ferrhydrite in cultures with a L/S ratio of 50:50, and also ammonium jarosite. Tilt, *A. hydrophila* was unable to solubilize hematite.

Unlike *A. hydrophila*, *S. fonticola* and *C. celercrescens* did not reduce Fe(III) from solid compounds in cultures with L/S ratios of 75:25 and 50:50. In these cases, only the Fe(III)-soluble fraction was reduced.

3.5. Growth adaptation at low pH

The “B-orilla” consortium was grown successfully at pH 4.5, and culture adaptation through successive transfers was achieved at pH values as low as 3.8. The kinetics of iron reduction at this pH became slower (1 week against 3 days at pH 7). This result represents that the adaptation process may be considered concluded.

The successive adaptation of *A. hydrophila* to growth at moderately acidic pH values was also successful. A concentration of 2.5 g/l of Fe(II) was reached in 6 days in cultures grown at pH 5.5 and with 3 g/l of Fe(III)-citrate. Although iron reduction by *A. hydrophila* was faster when Fe(III) was added as a soluble chelate—chelate—than as an iron oxide, however, the fact that the solubilization of ferrhydrite and jarosite took place without precipitation and, in addition, the ability of this strain to grow at low pH values (5.5) is a very promising result for ore dissolution.

*S. fonticola* was adapted to pH 6.0 and a maximum bio-reduction level of 30% was reached in 12 days. *C. celercrescens* adapted at pH 4.8 was able to reduce 30% of ferric iron in 7 days, and a maximum concentration of 1.30 g/l of Fe(II) was measured after 21 days.

Finally, the “B-orilla” consortium presented growth at pH 5 with ferrhydrite as the sole source of Fe(III). Thirty percent of bio-reduction was reached in 4 days and 62% was reached in 25 days. Bio-reduction also took place at pH 4.5. At this pH 30% reduction was also achieved in 4 days. The “B-orilla” consortium also grew with ammonium jarosite at pH 5. 30% bio-reduction was achieved in six days.

In all cases, cultures grown at low pH values tended to increase the pH of the media to reach the pH optimal for each specie, normally around 6.8.

4. Conclusions

The results here described reveal that microbial catalysis of anaerobic iron leaching has commercial potential. To our knowledge, the strain of *Aeromonas* here described is the first published example of a microorganism in which cultures Fe(II) maintains in solution after the dissimilatory iron reduction step. Then, the dissimilatory reduction by *A. hydrophila* may offer an economically and environmentally attractive means of extracting iron from ores. As Fe(II) was yielded in a dissolved and concentrated form in cultures of *Aeromonas*, then, the microbial product should require less redundant during high-temperature conversion to elemental iron in subsequent iron-production steps.

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References


