Evaluating the Efficacy of Two *Leptothrix* Species for Removal of Iron

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**Abstract:** Two strains of *Leptothrix* sp. were used for leachate iron from Abu Tarture mine. Abu Tarture deposits composed mainly of hematite, pyrite, ankerite and jarosite. Mössbauer spectroscopy results indicated that *Leptothrix* AT06 sp. was more efficient in removing different iron phases than *Leptothrix* AT22 sp. Treatment of phosphate ore by 10mM EDTA prior bacterial treatment lead to completely leachate hematite and pyrite, and almost reduced ankerite and jarosite for both organisms. The total iron reduced from 2.91% before to 1.08% after treatment by *Leptothrix* AT06 with chelating agent EDTA. Amino acid analysis of some protein fractions revealed that protein of *Leptothrix* AT06 sp. characterized by the presence of proline, cystine, methionine, isoleucine, leucine, tyrosine, arginine and lysine while protein of *Leptothrix* AT22 sp. was mainly composed of glutamic acid and glycine in the bacterial cells.

**Key words:** *Leptothrix*, Iron phases, Deferration, phosphate ore

**INTRODUCTION**

Iron is an essential element for all life forms. Naturally abundant iron (Fe) minerals exert a significant influence on soil and sediment geochemistry[8]. As a result, much consideration has been vested in iron redox reactions in aquatic and sedimentary environments. Reduction of iron in natural systems mediated by both abiotic and biotic mechanisms has been well documented[9].

This metal is involved in great enzymatic processes including electron transfer in the respiratory chain, redox reactions with inorganic substrates (oxygenase, hydrogenase, ribonucleotide reductase) and DNA cleavage (endonuclease III)[22]. Despite the abundant of this metal in nature, it occurs as an insoluble forms in ferric hydroxide complexes in the presence of oxygen and water[33] except in acid solution[9]. Therefore, under such iron-limiting conditions, microorganisms have developed different solutions to solve the problem of assimilation of the ferric form of iron from the environment. One class of intracellular iron storage compounds is represented by ferritin in eukaryotes and bacterioferritin in prokaryotes[44].

Ward et al.[13] reported that lactoferrin is a member of the transferrin family of iron-binding proteins. Likewise they found that lactoferrin consists of two repeated iron-binding lobes that bind one iron atom each. The second class is siderophores, which have been identified as intracellular iron storage compounds in various fungal systems[22].

The siderophores are biosynthesized by the organisms under negative iron control. They are released to the environment where the ferrisiderophore complexes are formed. The complexes are taken up by microorganisms; siderophore was coined to designate a family of low molecular mass, ferric specific ligands elaborated by microorganisms to combat iron deficiency[12].

Agarwala et al.[2] suggested a possible role for iron in the synthesis of soluble proteins. They also showed that iron deficiency caused a marked decrease in growth, spore formation and soluble protein, coupled with its increase in supplying iron. Makita et al.[21] found that Fe accumulates mainly in electron opaque granules and in the cell wall, both characterized by the presence of polysaccharides and cysteine-rich proteins.

The main problem of Abu Tartur phosphate is the high iron content (4%) which lies above the admissible limit of commercially used phosphate concentrates. This high content of iron degrades the ore and limits it use in industry. So, the idea of the present work is to use *Leptothrix* sp as a tool in leachate or even partially leaching iron from Egyptian phosphate ores as well as their possibilities for biosynthesis of metallothioneins.

**MATERIALS AND METHODS**

**Sample Localities:** Phosphate sample collected from 154 m depth of mine Abu Tartur phosphate deposit, Egypt.

**Organism Used:** Two strains of *Leptothrix* sp. were sheathed bacteria isolated from enriched soil with iron from Aswan Governorate, Egypt. The medium used
for isolation process was ferric ammonium citrate (Winogradsky's medium). The organisms were tentatively identified according to Krieg and Holt et al.[15]. Both strains- Leptothrix AT06 and Leptothrix AT22- oxidized iron and manganese ions.

**Starvation:** A heavy inoculum of the bacteria was inoculated at 50 ml mineral broth in flask 250 ml capacity. The mineral broth was free from ferrous ions. The culture was incubated at 30°C in a shaker (New Bruinsweek, N.J.,) at 180 rpm for 2 weeks.

**Preparation of the Sample:** The phosphate ore are prepared for different treatments by successive stages of crushing to obtain size fractions of the range 50-100μm.

**Treatment of the Sample by Sheathed Bacteria:** The bacterial strains inoculated at concentration 10⁶ cell/ml in 5% (w/v) crushed phosphate ore. The phosphate ore was suspended in 50 ml mineral broth. The culture was incubated in incubator shaker at 180 rpm and 30°C for 2 weeks. After a proper incubation period the culture was filtered through Whatman N° 1 filter paper to get rid of water for about 3 hours and then centrifuged at 3000 g for 5 minutes. The supernatant was drained off and residue of phosphate ore was subjected for Mössbauer spectroscopy (Type MR-260/MR= 360) for detection of iron fraction. Total iron content was determined by atomic absorption spectroscopy.

**Treatment of the Sample by Chelating Agent Prior Sheathed Bacterial Treatment:** EDTA was used as chelating agent of phosphate. 5 g of phosphate ore was suspended in 10mM EDTA solution (100 ml). The solution was shaked over night at 200 rpm. The ore was sieved (60 mesh) to get rid of EDTA residues, then phosphate ore was added to mineral broth at concentration 5% (w/v), and flasks inoculated with sheathed bacteria. The rest of experiment was preceded as mentioned above.

**Amino Acid Determination:** Amino acid composition was determined using an automatic amino acid analyzer model LC 3000 eppendorf Biotronik, Germany in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo.

**Protein Determination:** Total cellular protein of the bacterial strain was determined using Folin-phenol reagent according to Lowery et al.[20]; bovine serum albumin was used as a standard protein.

**Gel Filtration:** A Pharmacia column (1.5x30cm) packed with Sephadex G 50 equilibrated with phosphate buffer of pH 7.1 was used for the fractionation of the bacterial protein. The void volume and the uniformity of packing were determined using blue Dextran 2000 and the bromophenol blue. The concentrate of bacterial protein was applied to the column and allowed to pass into the gel by running the column. Phosphate buffer was then added without disturbing the gel surface, 14 fractions, each of 2 ml was collected from the effluent of Sephadex G 50 column.

**RESULTS AND DISCUSSION**

**Results:**

**Identification of Leptothrix sp.:** Both isolates formed smooth colonies. Microscopically, they were rod shaped, single or in pairs with sheath formation (Fig. 1)-assignment as *Leptothrix* AT06- or very long sheath (Fig. 2)-assignment as *Leptothrix* AT22. Sheath formation asserted via a dilute crystal staining solution[29]. Gram-negative and chemoorganotroph. Glucose and peptone were the optimum carbon and energy sources respectively. The only difference between the two iron bacteria that *Leptothrix* AT06 was tolerated incubation temperature up to 40°C and utilized glycerol while *Leptothrix* AT22 not recorded growth at 37°C or utilized glycerol. Poly-β-hydroxybutyrate granules were detected inside the cells. Both isolates have the ability to oxidize and deposit Mn²⁺[20].

Two properties distinguish genus *Leptothrix* from genus *Sphaerotilus*[21]. *Leptothrix* species oxidize Mn²⁺ and are usually found in oligotrophic, iron -and manganese- rich sediments, while *Sphaerotilus* species did not oxidize Mn²⁺ and typically thrives in rich organic nutrients. However, these organisms according to Boone and Castenholz[6], are a member of the Betaproteobacteria class II of the phylum Proteobacteria.

**Deferration of Iron by Leptothrix sp.:** The iron ore structure mainly composed of magnite, pyrite and hematite. The ability of sheathed bacteria to deferrate phosphate ore from Abu Tartur was confirmed by Mössbauer spectroscopic analysis. Typical absorption peaks of pyrite (FeS₂), hematite (Fe₂O₃), ankerite Ca(FeCO₃)₂, jarosite KFe₃(AlSO₄)(OH)₃ and total Fe by Mössbauer are presented in Fig. (3). Mössbauer spectroscopic analysis of phosphate ore after sheathed bacteria treatment (Figs. 4 and 5) showed that area of hematite (a, b, c and d) were completely disappeared, that might due to the ability of bacteria to deferrate iron phase of hematite. Also total area of ankerite peaks (e and f) was smaller than control one, that indicated ankerite partially reduced. Alternatively,
Table 1: Mossbauer parameters of treated samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Component</th>
<th>Mossbauer parameter</th>
<th>assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ</td>
<td>ΔH</td>
<td>Δ( rel.)</td>
</tr>
<tr>
<td>Ore without any treatment</td>
<td>a</td>
<td>2.28 1.28 - 0.08</td>
<td>Fe²⁺ in trans position in montmorillonite</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.48 1.22 - 0.03</td>
<td>ankerite</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>1.02 0.35 - 0.17</td>
<td>Fe³⁺ in cis position in montmorillonite</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>0.61 0.32 - 0.65</td>
<td>pyrite</td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>-0.19 0.34 511 0.06</td>
<td>hematite</td>
</tr>
<tr>
<td>Ore+Leptothrix AT06</td>
<td>a</td>
<td>2.79 1.11 - 0.44</td>
<td>Fe²⁺ in trans position in montmorillonite</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>2.07 1.01 - 0.35</td>
<td>ankerite</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>0.61 0.25 - 0.21</td>
<td>pyrite</td>
</tr>
<tr>
<td>Ore+Leptothrix AT22</td>
<td>a</td>
<td>2.32 1.28 - 0.05</td>
<td>Fe²⁺ in trans position in montmorillonite</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>1.5 1.24 - 0.03</td>
<td>ankerite</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>1.02 0.35 - 0.46</td>
<td>Fe³⁺ in cis position in montmorillonite</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>0.59 0.31 - 0.46</td>
<td>pyrite</td>
</tr>
<tr>
<td>Ore+Leptothrix AT06+10mM EDTA</td>
<td>b</td>
<td>0.6 0.31 - 1</td>
<td>ankerite</td>
</tr>
<tr>
<td>Ore+Leptothrix AT22+10mM EDTA</td>
<td>a</td>
<td>2.3 1.28 - 0.07</td>
<td>Fe²⁺ in trans position in montmorillonite</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.6 0.31 - 0.93</td>
<td>ankerite</td>
</tr>
</tbody>
</table>

Fig. 1: A photograph of Leptothrix AT06 sp. (x=1000).

Jarosite (g and h) and pyrite (i and j) peaks not showed change in their transmission (%) or area, whereas the bacteria not utilized these two types of ferrous compounds. Data from atomic absorption of total Fe was not reduced markedly.

Upon deferration of ore after treatment of bacterial culture with chelating agent EDTA at concentration 10mM, bacterial strains showed high efficiency of deferration (Figs. 6 and 7). Peaks of hematite and pyrite completely disappeared whilst peak spectra of ankerite diminished greatly. A jarosite spectrum was still after treatment with 10mM EDTA but in a small range; specifically for Leptothrix AT06 (Fig. 6). Mossbauer parameters and assignments of the spectral components are shown in table (1). Atomic absorption spectroscopy shows that all the used
Fig. 2: A photograph of Leptothrix AT22 sp. (x=1000).

Fig. 3: Mössbauer spectroscopic analysis of phosphate ore from Abu Tartur mine: a, b, c, d= Hematite peaks; e, f= Ankerite peaks; g, h= Jarosite peaks; i, j= Pyrite peaks, k, l= Total Fe peaks.

Fig. 4: Mössbauer spectroscopic analysis of phosphate ore after treatment with Leptothrix AT06 sp.
Fig. 5: Mössbauer spectroscopic analysis of phosphate ore after treatment with *Leptothrix* AT22 sp.

Fig. 6: Mössbauer spectroscopic analysis of phosphate ore after chelation with 10mM EDTA and treatment with *Leptothrix* AT06 sp.

Table 2: Total cellular protein of two *Leptothrix* sp before and after treatment phosphate ore with 10mM EDTA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein content (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leptothrix</em> AT06</td>
<td>230</td>
</tr>
<tr>
<td>10mM EDTA and <em>Leptothrix</em> AT06</td>
<td>764</td>
</tr>
<tr>
<td><em>Leptothrix</em> AT22</td>
<td>271</td>
</tr>
<tr>
<td>10mM EDTA and <em>Leptothrix</em> AT22</td>
<td>890</td>
</tr>
</tbody>
</table>

Bacteria decreased the iron content with different percentages. A reasonable leaching was obtained by *Leptothrix* AT06 with chelating agent EDTA in which the total iron decreased from 2.91% (before) to 1.82% (after). Meanwhile, the cellular protein of *Leptothrix* AT06 and *Leptothrix* AT22 were about 2.5- and three-folds than that before treatment with EDTA respectively (Table 2).

Table 3: Fractionation of *Leptothrix* AT06 sp. protein on Sephadex G50 using a pharmacia column (1.5 x 30 cm) cultivated on phosphate ore at 30°C for two weeks.

<table>
<thead>
<tr>
<th>Fraction N°</th>
<th>Iron Conc. (µg/ml)</th>
<th>Protein Conc. (µg/ml)</th>
<th>Fe/Protein Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Each (5 ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>0.435</td>
<td>17</td>
<td>25.53</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>1.957</td>
<td>501</td>
<td>3.913</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>0.802</td>
<td>115</td>
<td>7.63</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>0.164</td>
<td>15</td>
<td>10.93</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>0.106</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fraction 8</td>
<td>0.393</td>
<td>88</td>
<td>4.53</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>0.384</td>
<td>338</td>
<td>1.13</td>
</tr>
<tr>
<td>Fraction 10</td>
<td>0.338</td>
<td>143</td>
<td>2.43</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>0.206</td>
<td>313</td>
<td>0.63</td>
</tr>
<tr>
<td>Fraction 12</td>
<td>0.177</td>
<td>119</td>
<td>1.53</td>
</tr>
<tr>
<td>Fraction 13</td>
<td>0.00</td>
<td>30</td>
<td>0.00</td>
</tr>
<tr>
<td>Fraction 14</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Fig. 7: Mössbauer spectroscopic analysis of phosphate ore after chelation with 10 mM EDTA and treatment with *Leptothrix* AT22 sp.

Fig. 8: Amino acid analysis of fraction N° 2 for *Leptothrix* AT06 sp.
Protein Fractionation of Two Leptothrix Species Cultivated on Phosphate Ore: Data presented in Tables (3 and 4) showed that the protein content of the Leptothrix AT06 and Leptothrix AT06 with chelating agent EDTA is scattered over a wide range of fractions. Leptothrix AT06 shows cellular protein in 10 fractions only while Leptothrix AT06 with EDTA exhibited protein in all fractions. The highest protein content of Leptothrix AT06 was detected in fraction number 2 followed by fractions number 9, 11 and 10 while fractions number 5, 6, 7 and 14 had no detectable amounts of proteins. Iron was found to be associated with ten fractions only. On the other hand, Leptothrix AT06 after treatment of phosphate ore with 10mM EDTA exhibited protein content and iron in all fractions. The highest protein fraction recorded in numbers 7 and 8, while the highest iron content was recorded in fraction number 6.

Fraction number 2 for Leptothrix AT06 sp contains high concentration of protein that chelated a high level of iron ions. Its hydrolyzate revealed the presence of 8 amino acids; (Fig. 8) shows their concentrations, µg/ml. The most characteristic one was lysine. More likely, fraction number 6 for Leptothrix AT06 with EDTA
Leptothrix and characteristic amino acids, (Fig. 9). proline, cystine, isoleucine, leucine and arginine are revealed 13 amino acids in its structure. There were proline, cystine, isoleucine, leucine and arginine as characteristic amino acids, (Fig. 9).

Results of protein fractionation of Leptothrix AT22 and Leptothrix AT22 with chelating agent EDTA are presented in Tables (5 and 6). Leptothrix AT22 shows cellular protein in 9 fractions and iron in 6 fractions only. The highest protein content was detected in fraction number 10 and followed by fraction number 9. Leptothrix AT22 with chelating agent EDTA exhibited cellular protein and iron in 12 fractions, each. The highest protein content and iron concentration were detected in fraction 9.

Fraction 10 for Leptothrix AT22 showed least number of amino acid between all tested fractions. It recorded 4 types of amino acids with nearly equivalent percentage of serine and glutamic acids, and in other side glycine and valine, (Fig. 10). Similarly, fraction number 9 for Leptothrix AT22 with chelating agent EDTA showed 5 amino acids in its structure with the presence of citrulline as characteristic amino acid, (Fig. 11).

**Discussion:** Iron is the most abundant transition element in the Earth's crust. Approximately one-third of the Earth's mass is estimated to be iron. Its concentration is relatively high in most crustily rocks (lowest in limestone, which is more or less pure calcium carbonate)\[12\].

The idea of the present work depends mainly on the fact that some aerobic microorganisms are capable of oxidizing the iron from ferrous to ferric:

\[4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O\]

The oxidation of the iron takes place rapidly inside the cell. Two species of Leptothrix were isolated from nische enriched with magnate, pyrite and hematite; consequently reflect a specific environmental condition. Regulation of uptake of Fe metal would seem reasonable in order correctly to fulfill this need. Temperatures inside Abu Tartur mine between 30 and 35°C that it suitable for growth of Leptothrix species, particularly Leptothrix AT22. On the other hand, these bacteria and other various bacteria are able to acidify the medium, which ensures a better solubility of ferric ions and enables iron uptake\[23\].

Chemical analysis of phosphate ore show that about 50% of the total iron in the form of pyrite, the rather high relative area of the component of pyrite (~ 70% on the average). However, the amount of dissolved iron in the form of free ferric or its hydrolysis product is extremely low. The actual uptake of iron in the form of pyrite by Leptothrix AT06 and Leptothrix AT22 is confirmed by the measurements of Mössbauer spectroscopy and atomic absorption spectroscopy in which the percentages of total iron before and after each treatment are determined. Accordingly, Theil and Raymond\[30\] stated that occurrence of iron-bearing minerals in phosphate ore makes the Mössbauer spectroscopy a powerful technique for their investigation.
The results indicated that Leptothrix AT06 and Leptothrix AT22 utilized hematite (Fe₂O₃) completely and partially utilized ankerite Ca(Fe)CO₃. In Fig. (3) the relative area indicates that hematite exists in small relative amounts (4-7%). The uptake of these iron phases was probably throughout siderophore. Siderophores are defined by their ferric specific chelating property. The formation of siderophores is induced only under iron stress[24]. The growth of two species of Leptothrix in minimal broth free from ferrous ions was satisfied to produce siderophore prior any treatment with phosphate ore.

Pyrite uptake is achieved firstly by chelation of phosphorus through 10µM EDTA because average phosphorus content (P₂O₅) 25.5% that inhibit growth of majority of bacteria. Uptake hematite, ankerite, pyrite and to great extent jarosite appeared as iron-rich protein which called siderophore or even other biogenic minerals such as ferric oxyhydroxide[19] or goethite[22] or iron hydrogen carbonate[5]. Protein content ascertain hematite and pyrite uptake which increased 2.5-folds for Leptothrix AT06 and three-folds for Leptothrix AT22 than previous treatment. Meanwhile, reduction of total iron content from 2.91 (before) to 1.08% (after) treatment, i.e. about 62.9% reduction, indicated high efficiency of last treatment. Previously, investigations recorded about 20 and 35% reduction of pyrite by Acidithiobacillus ferrooxidans[17,10]. However, similar results were obtained by Tugel et al.[31] when added acid to samples and produced Fe²⁺ recoveries which were four times greater than those without acid.

Iron was found to be associated with different fractions of low and high molecular weight proteins. Induction of high quantities of low and high molecular weight protein to chelate metal ions and reduce its harmful effect on the cell could be
suggested. These results indicated that phytochelation or metallothionein (MT) - like protein formation as a mechanism of iron tolerance has not been synthesized. Detoxification of excess amounts of this metal can be achieved by siderophores or transferrins. They have the capacity to bind 60µM iron\(^{[11]}\). These results are in harmony with those obtained by Möller et al.\(^{[23]}\) and Alavandi and Ananthan\(^{[2]}\) who reported that the presence of iron-binding protein/peptide is one of the mechanisms of metal tolerance.

A high amount of aspartic acid is usually related to the presence of iron in growth media. Certain amino acids especially aspartic acid are conjugated to non-peptide molecules. Sometimes this route is a mean of detoxification\(^{[1]}\). Also, presence of glycine, cystine and methionine (two latter found in Leptothrix AT06) is remarkable. Glycine is one of the amino acids which act as intermediates in incorporating or disposing small molecules and producing the most unexpected biomolecules. It's well known that a variety of biopolymers have potential applications as metal-binding agents, e.g. thiol groups in sulfur-containing amino acids\(^{[16,4]}\).

Ferritins, iron-containing proteins, are also known to bind a variety of divalent metal ions \textit{in vitro} and \textit{in vivo}\(^{[27]}\). The previous authors suggested that ferritin serves as the initial chelator of metal ions, and the synthesis of MT is initiated as a second line of defense. As demonstrated by Schultz and Hutchinson\(^{[28]}\), the MT-like proteins are not the sole representatives for metal tolerance. The results presented in this investigation are in accordance with the previous results and ensured that two species of \textit{Leptothrix} have developed several protective mechanisms to help their survival under the presence of high concentration of iron. Hence, the isolated \textit{Leptothrix} AT06 and \textit{Leptothrix} AT22 can be used in iron deferration from phosphate ore of Abu Tartur mine. This leachate should pretreatment with chelating agent such as 10µM EDTA.

**Fig. 11:** Amino acid analysis of fraction N° 9 for \textit{Leptothrix} AT 22 sp. after treatment of phosphate ore with 10mM EDTA.
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REFERENCES


