(Review Article)

E-ISSN: 0975-8232; P-ISSN: 2320-5148



PHARMACEUTICAL SCIENCES



Received on 31 March 2020; received in revised form, 29 December 2020; accepted, 10 January 2021; published 01 February 2021

AN UPDATE ON BIOLEACHING TECHNOLOGY: IRON BACTERIA AS A SOURCE OF OXIDIZING IRON TRACES FROM WATER SAMPLES

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Keywords:

Iron oxidizing bacteria, Mass multiplication, Bioleaching, Iron oxidation, Iron enriched water samples

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ABSTRACT: The removal of metals by the use of microbes is known as bioleaching in terms of oxidation or degradation of metal from complex raw materials or depots. Such microbial oxidation or bio-removal occurs in terms of sulphur degradation and oxidation (for example, sulphur oxidation, iron oxidation etc.). This process is thus known as bioleaching, which is related to the all metal oxidation processes like sulphur oxidation or iron oxidation. These microbes are mild, moderately thermophilic, iron-mineral, sulphur oxidizing bacteria, and extremely thermophilic. The iron bacterium occurs in nature in different genera viz. Thiobacillus ferroxidans, Leptospirillum ferroxidans, Ferrovum myxofaciens and Acidiphilium or Acidithiobacillus. These microbes are the source of enzymes and metabolites which are of industrial significance. The commercial exploitation of such features of iron bacterial consortia may be utilized in water treatment for the removal of iron. The use of such microbes *via* fermentation technology may be fruitful in the removal of iron from water. Few studies have been done in this aspect. The hypothesis of this concept, although it should be experimented with for significant results.

INTRODUCTION: The 'iron bacteria' are considered to be the first prokaryotes considered to be bacteria that catalyzed the oxidation of iron II (Fe²⁺, ferrous iron) to iron III (Fe³⁺, ferric iron), often causing the latter to precipitate and accumulate as extensive, ochre-like deposits although the definition of what constitutes an 'iron bacterium' has been extended to include prokaryotes that, like Geobacter spp., catalyze the dissimilatory reduction of ferric to ferrous iron. Iron-oxidizing bacteria are considered to be important in the global iron cycle and industrial applications (chiefly biomining) ¹.



DOI: 10.13040/IJPSR.0975-8232.12(2).744-53

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(2).744-53

The oxidation of iron occurs at neutral pH in micro-aerobic and anaerobic environments. Iron-oxidizing bacteria occur in a number of phyla within the domain Bacteria, including the Nitrospirae and the Firmicutes, the majority are included within the largest bacterial phylum, the Proteobacteria. Different iron-oxidizing bacteria have different physiologies in terms of their response to oxygen (obligate aerobes, facultative and obligate anaerobes) and pH optima for growth (neutron-philes, moderate and extreme acidophiles).

2. Bioleaching (Microbial Leaching): Bioleaching has always been focused on achieving effective recovery of valuable metals by improving the efficiency of bioleaching microorganisms ² which is related to sulfur oxidation activities of sulfur-oxidizing microbes and the speciation of intermediate compounds formed during bioleaching processes ³⁻⁶. The initial characterization of isolates

was based on growth studies with iron and sulphur substrates and on the comparison of the whole cell. Three groups of bacteria were isolated and studied: moderately thermophilic iron-mineral sulphideoxidizing bacteria, moderately thermophilic sulphur oxidizers and extremely thermophilic Sulfolobus like organisms ⁷⁻¹⁰. Both moderately and extremely thermophilic acidophiles were isolated from hot spring and coal pile samples. A most common form of leaching is dump leaching, which involves the use of low-grade ore containing a variety of ore fragment sizes. The solution having metallic ions is sprinkled on and allowed to percolate through the dump and recovered in basins. The target metal is then removed and the solution recycled back to the dump. Dump leaching occurs over a period of years, which is much different in comparison to the second method, heap leaching, which has a leach cycle measured in months 11-17. Heap leaching occurs with crushed or uncrushed ore but of a higher grade than in dump leaching. The bacterial leaching of metal sulphides can occur by the effect of microbes on metal sulphide during oxidation.

Morphology, Habitat, Prevalence, and 3. Mechanism of Growth of Iron Oxidizing bacteria: Several bacteria genera and species are found in a variety of soils and aquatic habitats associated with iron. Researchers subsequently investigated the physiology and the ultra-structure of some of these unique microorganisms. Most of the iron bacteria could easily be identified directly by observation under the microscope due to their distinct characteristic sheath secretion. Most of the bacteria are of genera viz. Sphaerotilus and Leptothrix group, while Gallionella are recognized by their elongated helical or twisted stalks, composed of numerous intertwine microfibrils. Iron-oxidizing bacteria colonizes the zone of groundwater where deoxygenated water from an anaerobic environment flows into an aerobic environment. Groundwater having dissolved organic material gets deoxygenated by the microbes which feed on organic material where organic material concentration may exceed the dissolved oxygen.

The density of iron-reducing bacteria reduces insoluble ferric oxide in the soil to soluble ferrous hydroxide with the release of oxygen, which will oxidize the rest of the remaining organic material.

$$4H_2O + 2Fe_2O_3 \rightarrow 4Fe (OH)_2 + O_2$$

(Water) + (Iron [III] oxide) → (Iron [II] hydroxide) + (oxygen)

Deoxygenated water when flows through the source of oxygen, iron-oxidizing bacteria use that oxygen to convert the soluble ferrous iron back into an insoluble reddish precipitate of ferric iron:

$$4\text{Fe (OH)}_2 + \text{O}_2 \rightarrow 4\text{H}_2\text{O} + 2\text{Fe}_2\text{O}_3$$

(Iron [II] hydroxide) + (oxygen) \rightarrow (water) + (Iron [III] oxide)

Genera, Ferrovum is common in several acid mine waters and can be isolated using cultureindependent methods ¹⁸⁻²⁰. Different iron bacterial cultures known are Thiobacillus ferroxidans, Leptospirillum ferroxidans, Ferrovum myxofaciens and Acidiphilium or Acidithiobacillus 21-22. The isolate, Ferrovum myxofaciens is an acidophilic, psychrotolerant obligate autotrophic bacterium, which uses ferrous iron as electron donor and oxygen as electron acceptor ²³. Ferrous ions are the core carbon source of the genera of most of the iron oxidizing bacteria, which generates oxidative stress within the bacterial cell ²⁴. The enhancement of oxidative stress for acidophilic iron-oxidizing bacteria under atmospheric conditions revealed that reduced oxygen concentrations decrease the stress level. Elevated carbon dioxide concentrations also enhance microbial growth; thus, carbon dioxide is also the carbon source of Ferrovum genera. Since carbon dioxide is poorly soluble in acidic aqueous solution. Thus, the cultures, Ferrovum, Acidiphilium are cultivated under different gas phases, lacking oxygen and increased levels of carbon dioxide.

The common bacterium, Iron-oxidizing bacterium, *Thiobacillus ferrooxidans* had a significant role in microbiological leaching of metal sulfide 25 . Under acidic conditions, the bacterium rapidly oxidizes ferrous ions to produce a large number of ferric ions in its environment. Hydrogen sulfide-ferric ion oxidoreductase (SFORase) 26 and a sulfite: ferric ion oxidoreductase 27 in sulfur oxidation of *T. ferrooxidans*. These utilize Fe³⁺ as an electron acceptor in the oxidation of elemental sulfur and sulfite ions, respectively. These enzymes are involved in aerobic sulfur oxidation by this strain

has accumulated ²⁸⁻³⁰. Iron oxidation and bacterial leaching by iron-oxidizing bacteria have a significant role of SFORase. The most important examples of iron-oxidizing bacteria utilizing **SFORase** Leptospirillum ferrooxidans, are Leptothrix, and Gallionella. SFORase activity was also determined in cell extracts by measuring ferrite ion in a reaction mixture ³¹. Laboratory studies showed that microbes could catalyze up to 80% of the Fe oxidation ³². Ferrous iron stimulated growth of the microorganisms, including *Leptothrix* ochracea and Gallionella sp., in the microcosms however, dominant microbes were unicellular, no. The studies suggested that classic iron bacteria such as Leptothrix and Gallionella were important in laying down the matrix of the mat, there was an even larger population of unicellular prokaryotes that might be playing a key role in iron oxidation.

Safe drinking water is an important and basic fundamental right of a living being. If the drinking water gets contaminated with opportunistic pathogenic microbes, this may lead to health implications for consumers ³³. In rural communities, untreated surface water from rivers, dams, and streams is directly used for drinking and other domestic purposes 34. These unprotected water sources can be contaminated with microbes through rainfall-runoff and agricultural inputs, mixing with sewage effluents and faeces from wild life 35, 36, which makes unacceptable it for human consumption. Infections causing faecal coliforms, aeromonas, and Pseudomonas, are used as indicators of faecal contamination in water ³⁷, and the presence of these pathogens may have severe health implications on consumers, especially those ³⁸. Excessive that are immune-compromised consumption of antibiotics and medicines through agricultural processes and day to day use has been reported ³⁹. The excessive consumption of antibiotics leads to the development of antibioticresistant bacteria, which affect the treatment of infections 40, 41. Antibiotic resistance is thus of major concern in today's times; its presence in all types of water bodies is well reported 42, 43. The spread of pathogenicity of microbes occurs by the poor or lack of ability of resist of the destruction of antibiotics. Today's biological waste disposal in the water bodies leads to antibiotic-resistant bacteria and, thus, the occurrence of multidrug-resistant organisms (MDRs) ⁴⁴.

- **4. Mechanism of Action of Iron Oxidizing Bacteria:** Different strains *viz. Acidithiobacillus* species are potent iron oxidizers ⁴⁵⁻⁴⁸. *Acidithiobacillus* species oxidizes iron, produces electron, which undergoes as per the requirement of the organism. The electron flow reduces oxygen molecules to water, and proton counterbalances the downhill flow of electrons. It is observed that pH changes occur from 2.0 to 6.5/7.0 (the extracellular pH being 2.0 and intracellular being 6.5–7.0) due to the inflow and outflow of protons.
- 5. Removal of Iron from Water by using Iron Oxidizing Bacteria (bioleaching of Iron): The discovery and exploration of Iron bacteria is found as a significant and revolutionary treatment technology for water treatment experts who work on biological solutions-based formulations for water treatment. There are different controversies between the believers who understand that the iron removal cannot be accomplished only by iron bacteria as such while the true concept is that, iron oxidizing bacteria along with filters and carriers framed in specific machinery is able to oxidize and degrade the iron from the depots of iron present in the water and sediments. The sand filters could be either solely biological or iron-oxidizing bacteria play supplementary role a physicochemical iron removal process under certain conditions. The main complication in the growth of iron bacteria is that the organisms grow and thrive well between the pH of about 5 to 9. At these pH values, iron Fe (II) is physicochemically/ non-biologically oxidized to Fe (III), making it a difficult task to decide if the bacteria contributed to the oxidation of Fe (II). The technology of bioleaching can be developed by the combination of the biological phenomenon in the filters, which substantially reduces the iron concentration in the treated water by removal of iron slow and steadily with the continuous operation of machinery or bioreactors. This technology will be able to remove Fe-organic or Fe-silicate complexes and also increase the rate of oxidation of Fe (II) to Fe (III).
- **6.** Metabolism of Iron Removal by Iron Oxidizing Bacteria: The oxidation reaction of ferrous iron to ferric iron by biological means is similar to that of the physicochemical reaction indicated in the following reaction. It is known to be one of the most significant characteristics of iron

oxidizing bacteria, but very little is known about the mechanism involved in initiating and perpetuating this exothermic biochemical iron oxidative processes in drinking water plants.

$$4\text{Fe}^{2+} + \text{O}_2 + 10\text{H}_2\text{O} \rightarrow 4 \text{ Fe (OH)}_3 + 8\text{H}^+ + \text{Energy}$$

Iron oxidizing bacteria derive their essential energy requirements through a strictly chemolithotrophic process. This is enzyme-mediated oxidation of Fe (II) with a concomitant fixation of carbon dioxide into an assimilable nutrient for the iron-oxidizing bacteria. As a result, precipitation of Fe (III) salts occurs either by the enzymatic action autotrophic bacteria (intracellular) or by catalytic action of polymers excreted by the bacteria sheath (extracellular). The original source of carbon dioxide is transformed by anaerobic fermentation in groundwater and by gravity viz. 100 m depth below the ground existing solely in the anoxic zone ⁴⁹⁻⁵¹. Different types of ironoxidizing bacteria may be involved in water treatment systems, but in all cases, the microbial oxidation process can be observed in nature, which operates by rapid oxidation of insoluble ferric hydroxides, which generates precipitates.

7. Biological Conditions for the Precipitation of Iron: Gradual shift from abiotic to biological precipitation is restricted by the use of chemical and the physical properties of the water. The most important criteria for the biological precipitation are enumerated at neutral or slightly acidic pH, a change from negative redox potential to redox potentials up to about 200-320 mV, and oxygen levels changing from zero to 2-3 mg/L, together with a considerable amount of CO2. Thus, redox potential and pH are the main factors that will determine the progression of biotic precipitation of iron ⁵²⁻⁵⁴.

8. Types of Iron Oxidizing Bacteria:

8.1. Leptothrix: The bacterial strain, Leptothrix belongs to the genera; Betaproteobacteria which involves the oxidation of both iron and manganese. There are four recognized species: L. ochracea, L. discophora, L. cholodnii, and L. mobilis. These bacteria are utilized in the production of an extracellular tubular sheath that is occupied by cell filaments. L. ochracea was the first species, most visibly apparent of any of the FeOB in most freshwater environments. It has not been

successfully cultured in the laboratory, nor has it been subjected to a thorough cultivationindependent study to analyze its phylogeny or physiology.

8.2 Leptothrix-Spaerotilus: L. ochracea probably unable to derive energy from the oxidation of Fe (II). L. ochracea share physiology very similar to other *Leptothrix* spp., which are heterotrophs. There is a substantial amount of circumstantial evidence indicating that L. ochracea is a chemolithoautotrophic. First, its abundance in waters indicates that it requires high concentrations of Fe (II) for growth. Second, it produces copious amounts of iron oxides that are deposited on the sheaths. Yet, where it is most actively growing, only approximately 10% of the sheaths contain cells. This is consistent with chemosynthetic growth on a low-yield energy source that results in the production of iron oxyhydroxides, but little biomass. Finally, attempts to culture L. ochracea on typical heterotrophic media that support the growth of other Leptothrix spp. have failed. In addition, attempts to culture it on synthetic media under conditions that support the growth of other lithotrophic FeOB have also been unsuccessful. The cultivated species of *Leptothrix* and the related genus Sphaerotilus includes all oxidize iron and/or manganese (Mn), but they are also obligate chemoorganotrophs, capable of growth on a variety of organic compounds, but lacking evidence for lithotrophic growth on iron or manganese. Although these species do not fit the definition of a lithotrophic FeOB, studies on how they oxidize iron and the nature of the tubular sheaths they produce are informative ⁵⁵⁻⁶⁰.

8.3 Other Freshwater FeOB: The development of gel-stabilized culturing methods that mimic the natural redox boundaries where FeOB grow has led to the isolation of several new species of FeOB. These grow as a band at the oxic-anoxic interface in the gradients. Using this method, carrying out multiple dilution to extinction procedures is possible to obtain pure cultures. Two FeOB isolated using this technique belongs to a novel genus, *Sideroxydans*. Phylogenetically, *Sideroxydans* spp. are close relatives of *G. ferruginea* and *Gallionella* form an order, the *Gallionellales*, within the *Betaproteobacteria* that has in common the ability to grow on iron. *F. radicicola* appears to

be a less common FeOB, because it does not cluster with other known bacteria within the Betaproteobacteria. Morphologically, all three of these species are rod-shaped, unicellular bacteria. Unlike G. ferruginea or L. ochracea, these organisms do not produce recognizable extracellular structures. Oxygenic photosynthetic FeOB. Sideroxydans spp. and F. radicicola are both obligate FeOB that utilize Fe (II) as their only energy source. Rather, when they grow, they produce particulate iron oxy hydroxides of an amorphous morphotype, the cells are closely associated with these particulate oxides, and it is often necessary to utilize a nucleic acid binding fluorescent dye and epifluorescence microscopy to visualize them in the Fe-oxide matrix ⁶¹⁻⁶⁴. The mechanism(s) by which they avoid self-entrapment within the oxide precipitates is not understood. One scenario is that they produce an exopolymer that helps control the precipitation of the iron oxides and prevents them from becoming encrusted.

8.4 Role and Application of Iron in Water Treatment for Removal of Nitrogen: Nitrogen is essential for living organisms, while excessive emissions of both organic 65 and inorganic 66 nitrogen species can cause serious environmental problems. As a major contributor to the demand for available oxygen, ammonia is considered a critical pollutant that causes water separation in the aquatic environment ^{67, 68}. Contamination of nitrate in drinking water can increase the risk of non-Hodgkin's lymphoma ⁶⁹, methemoglobinemia ⁷⁰ and other, ovarian ⁷¹ or stomach ⁷² cancer, *etc*. in humans. The nitrate load in the surface water is often considered to be the cause of water quality degradation and eutrophication ⁷³. Large amounts of chemical nitrogen in surface water can affect groundwater due to interaction groundwater and surface water sources ⁷⁴. Nitrogen pollution is mainly caused by the use of nitrogencontaining fertilizers, animal waste, septic system, atmospheric industrial processes from nitrogen oxide emission ⁷⁵, irrigation and storm flow from farms ⁷⁶ etc., which have been a growing global problem, affecting the quality of drinking water, the environment, and the value of aquatic life. For example, two-thirds of rivers and coastal areas in the United States have been moderately reduced or severely damaged by nitrogen pollution 77; More than 85% of lakes and 82% of the 532 major rivers

in China have suffered water depletion and food shortages due to severe N pollution ⁷⁸. As a result of the high levels of pollution caused by excessive nitrogen extraction, strict standards for nitrogen-containing contaminants have been released ⁷⁹. For a long time, due to the various benefits of iron, great interest has been shown in iron-based treatment for wastewater treatment ⁸⁰, sewage disposal ^{81, 82}; air pollution control ⁸³, landfill remediation ⁸⁴, groundwater ^{85,} and wetlands ⁸⁶. In more detail, metal morphologies have been widely used in a variety of ways to degrade inorganic nitrogen ⁸⁷ and natural pollutants, including atrazine ⁸⁸, nitro compounds ⁸⁹, nitrobenzene ^{90,} *etc*. It is very important to emphasize that iron plays an important role in the removal of nitrogen.

In particular, many studies have focused on classification by combining iron with various processes, including abiotic ⁹¹ and biotic processes ⁹². Removal of nitrogen-containing contaminants and the use of iron in water treatment for denitrification have brought widespread concern. Although reviews focusing on the use of iron in the field of the environment and a holistic view of nitrogen removal technology in water treatment have been widely published in recent years, many reviews have focused on metal use or regulation. Few revisions have focused on the direct use of iron in nitrogen removal. Above all, during water preparation, the choice of processes depends on the type of contaminated water and the quality of the water ^{93, 94} incorrect amounts of metal added to the extraction process can lead to unwanted performance, such as the effect of Fe (III) on N₂O product 95.

8.5 Methods Associated with Nitrogen Removal: Many physical or chemical methods have been adopted to convert the iron used in the chemical removal of nitrogen. The main objectives of these approaches are to prevent transmission ⁹⁶, maintain the continuous functioning of the transaction layers ⁹⁷, and provide an accessible environment ⁹⁸ to ultimately improve bulk performance and

When it comes to Fe0 used in nitrate removal, many scholars focus on the use of metal particles of various sizes, including metal implants ⁹⁹, iron craps or powder ¹⁰⁰, micro-size zero-valent iron ¹⁰¹ (mFe0), nano-size nFe0 ¹⁰².

transmission of the electron.

In general, nano-sized metal particles are more efficient than micron-scale powders, which are more likely to be caused by a certain surface area and an increase in surface height ¹⁰³. However, other studies have also shown that there was no specific interaction between Fe0 functionality and specific location ¹⁰⁴.

It is very common to use a variety of iron-based materials in the removal of pollutants containing nitrogen from groundwater ¹⁰⁵, sewage ¹⁰⁶, or industrial wastewater ¹⁰⁷. The characteristics of contaminated water were clear, and the valence of the metal varied from Fe0, Fe (II) and Fe (III) to oxide, to ferrates (IV, V, and VI).

- **8.6 Methods Involved in Chemical Removal of Nitrogen by Iron:** Various dehydration techniques are involved in the chemical removal of nitrogen using iron of different valence. For example, Fe0 and Fe²⁺ are used to reduce NO³⁻ and NO²⁻ and their final products include N₂, N₂O, NH⁴⁺ and NO; NO- can be slowly synthesized into NO³⁻ *via* ferrates (VI, V, and IV)¹⁰⁸ while ammonia can be directly linked to N2 or NO3- *via* ferrate (VI)¹⁰⁸, 109
- **8.7 Methods Involved in Biological Removal of Nitrogen by Iron:** Methods and processes of integrated biological or biochemical processes of process extraction combine with metal. Many types of integrated biological or bio-chemical methods and processes of iron-containing nitrogen removal have been used in many studies, including traditional dehydration procedures and newly developed waste minimization procedures. The methods and procedures most commonly used by researchers are as follows:
- 8.8 System nFe0 and **Hydrogenotrophic Integrated Reinforcement System (nFe0-HIDs):** In an integrated certification process, it has been established that competition exists between nanoparticles of metal and bacteria between the first step of the reduction process. And Fe0 has been proven to have both H₂-related biostimulatory effect produced in the anaerobic corrosion process and an antibacterial effect due to nitrate competition ¹¹⁰. In addition, hydrogenotrophic denitrifying bacteria can also be used to reduce ammonium generation and completely remove nitrates 111.

- 8.9 Anaerobic Ammonium Oxidation (ANAMMOX) Interacts with Iron: In recent years, a unique mechanism of ammonia oxidation produced by ANAMMOX bacteria under a limited oxygen state has been reported, in which high ammonia was synthesized using nitrite as an electron acceptor 112. In addition, ANAMMOX associated with the reduction of ferric iron, called Feammox, was a relatively new cycling process ¹¹³, 114. The mechanisms involved in the unexplained Fe (III) reduction have been demonstrated by adopting isotope sequencing and amplicon-based 16S rRNA sequencing techniques ¹¹⁵. In addition, iron has been used to accelerate the start of the AANMMOX process ¹¹⁶.
- 8.10 Simultaneous **Nitrification** Process, **Denitrification** and **Phosphorus** (SNDPR) **Process:** During the SNDPR process, a temporary decrease in nitrogen removal will occur at the beginning of each cycle of operation. After serval cycles, nutrient removal would no longer be inhibited by Fe³⁺. As iron was continuously added to the reactor, the mud properties and the effect of nitrogen removal would be enhanced especially when the Fe load exceeded 40mg/L ¹¹⁷.
- **8.11 Fe (II) -Mediated Autotrophic Denitrification** (**Fe (II) -MAD):** The Fe (II) -MAD process is another biotechnology that can remove nitrate and iron at the same time as the formation of Fe (III) precipitation. More recently, Fe (II) -MAD has been gaining increasing scientific interest in addition to classical heterotrophic denitrification, especially in the treatment of industrial wastewater which is often carbon-poor^{116, 117}.
- 8.12 The Ferrous **Iron-Based** Chemo **Autotrophic Denitrification (Fe-CAD):** In the Fe-CAD reactor, the sludge has been found to be rich in iron-reducing nitrate-reducing bacteria that reduce bacteria including Rhodanobacter, Mizugakiibacter, Sulfuricella, Comamonas and Gallionella. In addition, in order to improve the function of the Fe-CAD reactor, iron deposits around microbial cells must be removed or inhibited and pH is also a key factor ¹¹⁸.
- **8.13 Effect of Microorganism and Bacterial Community:** There is no denying that iron deposits make a huge difference in the processes or methods of removing excess nitrogen. First, iron

has a profound effect on microorganisms. As mentioned above, iron is an important donor or receiver of the electron. Iron activity of electron modification may be negligible under certain conditions, which, however, promote exoelectrogenic bacteria during the nitrogen removal process ¹¹⁸. In addition, it was reported that the activity and growth of NDAMO bacteria could be significantly enhanced where there is a suitable content of iron and copper ¹¹⁹.

CONCLUSION: The present review regarding iron oxidizing bacteria reveals that, these are the prominent source for oxidizing the iron content in water having predominant iron concentration. Moreover, these can be utilized and further processed *via* mass multiplication and fermentation for reducing the iron content. These bacteria are novel candidates for research, and the exploration of their diversity may lead to new avenues of exploitation in research and other economically useful technologies.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: All authors declare that, there are no conflicts of interest.

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How to cite this article:

Rani U, Gupta S and Kumar V: An update on bioleaching technology: iron bacteria as a source of oxidizing iron traces from water samples. Int J Pharm Sci & Res 2021; 12(2): 744-53. doi: 10.13040/IJPSR.0975-8232.12(2).744-53.

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