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Study of Products from Neutrophilic Iron Bacteria by Prompt Gamma/Neutron Activation Analysis and X-Ray Diffraction

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SUMMARY

The Fe(II)-oxidizing bacteria (FeOB) and Fe(III)-reducing bacteria (FeRB) were among the first groups of microorganisms to be recognized for carrying out a fundamental geological process - the bacterial iron redox cycling. The deposition of iron ions by the bacteria from the Sphaerotilus-Leptothrix group of neutrophilic FeOB is extracellular in the form of biogenic products contained in tubular structures (sheaths). We report on the determination of the concentration of elements in these products and the relative amounts of biogenic iron oxides/(oxy) hydroxides resulting from the bacterial metabolism. The Fe (II)-oxidizing organism was isolated from freshwater wetland surface sediments in Vitosha Mountain. Biogenic nanostructured materials were obtained after growing the genus Leptothrix in SIGP and Adler's nutrient media. Formation of sheaths was observed only in case of dynamic cultivation in SIGP medium. High enrichment level of iron was found by the PGAA and NAA techniques in the products of cultivated isolates as compared to the reference sample (product of nature). Three iron oxide phases were found after cultivation in Adler's medium: lepidocrocite (γ-FeOOH), non-stoichiometric magnetite (Fe3-xO4) and goethite (α-FeOOH). The cultivation in the SIGP medium yielded a single phase bacterial product – lepidocricite of poor crystallinity.

Introduction

Iron oxides/hydroxides are widespread in geological, technological and environmental locations (Cornell and Schvertmann, 2003). A large part of sedimentary iron deposits can be attributed directly or indirectly to microbial activity and Banded Iron Formations (BIF), for instance, is thought to have formed from bacterial-mediated precipitation of iron from the Earth's ancient oceans (Schieber, 2004).

The Fe(II)-oxidizing bacteria (FeOB) and Fe(III)-reducing bacteria (FeRB) were among the first groups of microorganisms to be recognized for carrying out a fundamental geological process - the bacterial iron redox cycling (Weber *et al*., 2006). The notable feature of some FeOB is are the unique morphological structures they produce, such as powders, sheaths or stalks, that act as organic matrices upon which the deposition of hydrous ferric oxides can occur. They are capable of accumulating metals by binding them as cations to the cell surface in a passive process as well gaining energy for growth from the oxidation of ferrous iron with O_2 as terminal electron acceptor. Emerson *et al*. (2010) provided a historical overview of research on circumneutral bacterial Fe(II) oxidation, as well as the physiology and systematics of known lithotrophic FeOB.

Sheath-forming iron- and manganese-depositing bacteria belonging to the Sphaerotilus-Leptothrix group (SLG) are widespread in natural and artificial water systems. Known requirements for their growth include the presence of organic substrates and molecular oxygen (Bergey and Holt, 1994). They are capable of oxidizing Fe^{2+} and Mn^{2+} and as a result of their metabolism, they form biogenic iron oxides/(oxy)hydroxides accumulated in their “sheaths”. The sheaths may appear yellow to dark brown because of the deposition of iron and manganese oxides. The efforts to mimic the growth conditions in nature, however, often fail to lead to the formation of sheaths in laboratory conditions.

In this report we complement the evidence on the biogenic oxides’ phases and the capabilities for accumulation of iron in the bio-products of bacteria from the genus Leptothrix grown in the elective nutrient media known as Adler’s medium (AM) also for the silicon–iron–glucose–peptone (SIGP) medium, the only nutrient medium wherein we observed that the formation of sheaths has noticeably occurred (Angelova *et al*., 2014).

Materials and Methods

The natural biomass was collected from a stream in Vitosha Mountain where deposits with characteristic texture and brown-red colour were formed (Figure 1, a).

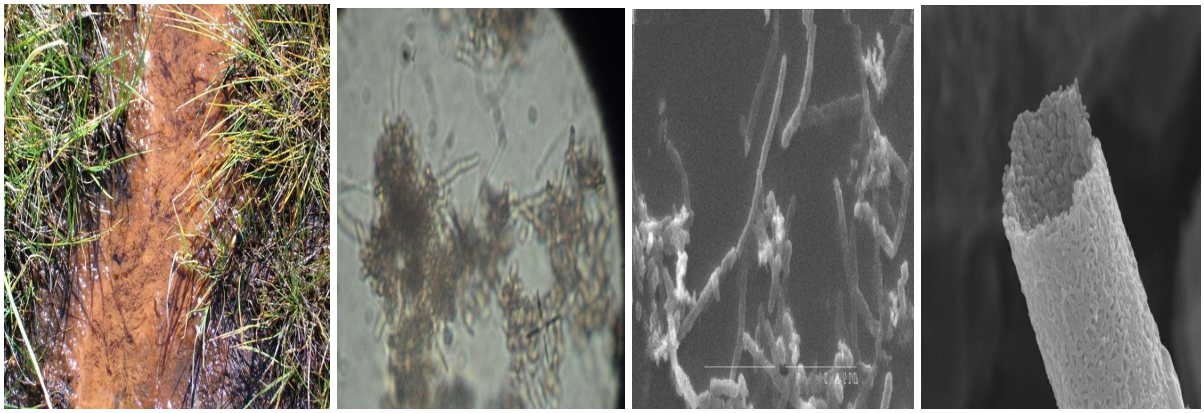

Figure 1 *(a) Typical bacterial deposits in the water flow in Vitosha Mountain (Aleko locality, 1783 m altitude); SEM images (JEOL, x5000) of Leptothrix sp. bacteria cultivated in SIGP (b) and AM (c).*

Two types of bacterial cultivation – static and dynamic were carried out. The dynamic cultivation was achieved both in Erlenmeyer flasks by shake at 70 rpm and in specially constructed fermenter with additional aeration. The period of cultivation was from 7 to 120 days. The cultivation was carried out at 3 different temperatures - 10oC, 20oC и 37oC. The static cultivation lasted 120 days in Roux

flasks. Periodically, liquid samples were taken and microscopic analyses of the cultures in the process of cultivation were performed. Samples of the biomass cultivated in different feeding media were collected and are noted further in the text according to the name of the used medium as follows:

- Reference sample: biomass picked from a natural source in Vitosha Mountain;
- Adler's medium (AM): Sodium lactate - 40.0 mg; Yeast extract - 1.0 g; Ascorbic acid – 0.1 g; MgSo4·7H2O; K2HPO4 – 0.01 g; (NH4)2Fe(SO4)2·6H2O – 0.01 g; in 1000 ml distilled H2O (pH 7.0); iron cuttings;
- SIGP: monobacterial Leptothrix with nutrient silicon–iron–glucose–peptone (SIGP) medium (Sawayama *et al*., 2011): 1 g glucose, 1 g Bacto peptone, 0.2 g Na2SiO3·9H2O, 0.044 g CaCl2·2H2O, 0.041 g MgSO4·7H2O, 0.076 g Na2HPO4·12H2O, 0.02 g KH2PO4·2H2O, 2.838 g HEPES (N-2hydroxyethelpiperazine-N'-2-ethanesulfonic acid) and 0.05 mM FeSO4 in 1000 ml distilled H2O (pH 7.0); iron cuttings.

The analysis of the population of the iron bacteria in the natural biomass was carried out on the basis of the specific morphology of the bacteria according to the literature by microscopy of fresh and fixed preparations. After isolation of pure cultures they were inspected with respect to morphological and physiological characteristics and identified following the classical and molecular taxonomic scheme (Angelova *et al*., 2015). The key morphological characteristics analysed were: a) cell shape; b) Gram*stain; c) motility; d) presence of capsule. The list of growth characteristics includes: 1) ability to grow on different selected media; 2) ability to oxidize Fe^{2+}; 3) Preferable source of Fe^{2+}.

Filtered and air dried in the laboratory at room temperature biomass from nature (Reference sample) and the cultivation vessels was used in our study. SEM and TEM images using JEOL instruments were taken in studying the products morphology. The biogenic iron oxides/(oxy)hydroxides were identified on powdered materials from laboratory x-ray diffraction (XRD) phase analysis using a Bruker D8 diffractometer in the Bragg–Brentano reflection geometry with Cu Kα radiation.

Neutron activation analysis (NAA) and prompt gamma activation analysis (PGAA), both using the k0-standardization method, were applied for identifying and quantifying the concentrations of major and trace elements in powdered dry residue of initial biomass and bio-products of cultured bacteria. In contrast to coupled plasma mass spectrometry (ICP-MS), atomic absorption spectroscopy (AAS) or other common analytical methods, the nuclear techniques employed are non-destructive and allow analysis of the solid material without sample preparation and standardization problems. PGAA is focused on the detection of the prompt γ- rays (leaving the compound nuclei in 10^{-12}-10^{-9} s) emitted by the target during neutron irradiation whereas NAA exploits the delayed γ-rays from the radioactive daughter nucleus (with short or long half-lives), detected after the irradiation. Consequently, the sensitivity for concrete elements varies between the two methods (Gmeling *et al*., 2014). Essentially, PGAA and NAA are multi-elemental, multi-isotopic techniques providing evidence for the average composition of the irradiated volume. Thus, they can be very accurate for homogeneous samples.

The samples were irradiated in the reactors BRR (Budapest) and FRM-II (Munich). PC-based gamma ray spectroscopy systems coupled to high purity p-type germanium detectors of high efficiency and energy resolution of γ-lines were used for the qualitative and quantitative analysis of about 30 elements and their radionuclides. The scheme for irradiation was chosen according to the half-lives of the element of interest. Short-term irradiations were performed for determination of the short-lived elements such as Al, Ca, Cu, Mg, Mn, S and V. Within a maximum decay period of 120 s the samples were packed into polyethylene capsules and measured for 10 min and again later for 20-30 min. Long-term irradiation was used for the determination of elements producing medium- or long-lived isotopes ($T_{1/2}$≥6 h). Each sample was measured three times. The results obtained by the two nuclear methods showed very good agreement.

Results and Discussion

The genus Leptothrix isolated from the site in Vitosha Mountain and grown in the culture medium SIGP produced the typical microtubules (Figure 1,b) whereas in the AM medium merely bacterial

cells were observed (Figure 1,c). The optimal growth of the cultures was observed at 20 °C under dynamic conditions in SIGP. The formation of sheaths started after a seven-day cultivation period. The structures disintegrated completely approximately after 90 days since cultivation. The sheaths' formation under laboratory conditions depended mainly on the nutrient medium and cultivation types. A change of the dimensions of the tubular structures formed could be observed; the average diameter was in the range of 0.4 - 1 μm with the length reaching approximately 7 μm.

Table 1 presents some selected PGAA/NAA data. The amounts of transition metals in the artificial culture medium increase markedly in comparison with the reference (product of nature). The strong increase in the iron content in the biogenic mat resulting from bacterial growth is manifested clearly; it depends on the culture medium and is superior in the SIGP medium where the enrichment reached a factor close to 5. Additional interest comes from the registered highly selective increase of several essential elements in support to the ability of the PGAA/NAA techniques to reveal and quantify the presence of specific elements in the biosphere. However, the details are out of the scope of the present report and these data will be presented and discussed elsewhere.

Table 1 *PGAA/NAA results for the SIGP and AM samples as compared to the reference.*

Sample/ Elem./Unit	Fe g/kg	Mn g/kg	Cr mg/kg	Co mg/kg	As mg/kg	Ca g/kg	K mg/kg	Na mg/kg	Si w %	P w %	S w %
Reference (Vitosha)	93 ±4	1.28 ±0.09	18.0 ±1.2	7.6 ±0.4	18.3 ±0.9	9.4 ±2.3	3080 ±204	4633 ±271	n.a.	n.a	n.a.
SIGP	453 ±16	4.61 ±0.16	160 ±30	54.8 ±4.3	0.67 ±0.10	1.12 ±0.07	334 ±28	213 ±11	0.13 ±0.10	1.2 ±0.2	0.13 ±0.01
AM	415 ±16	2.53 ±0.09	170 ±14	21.8 ±2.9	-	1.10 ±0.10	500 ±30	420 ±27	0.22 ±0.03	0.88 ±0.07	0.08 ±0.01

Figure 2 shows typical XRD patterns. Phase analyses showed presence of iron oxides but manganese oxides were not identified in the samples. The XRD analyses yielded as well the amounts and particle sizes (all below 30 nm) of the resulting oxide products. The SIGP pattern revealed a single-phase of lepidocrocite (γ-FeOOH) with an average diameter of the particles of 8 nm and quite poor crystallinity (small signal-to-noise ratio, rather broad peaks,). Phase and particle size analyses of AM cultivated sample show three distinct phases: lepidocrocite 59.67 % / 29.93 nm, magnetite 21.56 % / 23.860 nm, goethite – 18.77 % / 12.03 nm.

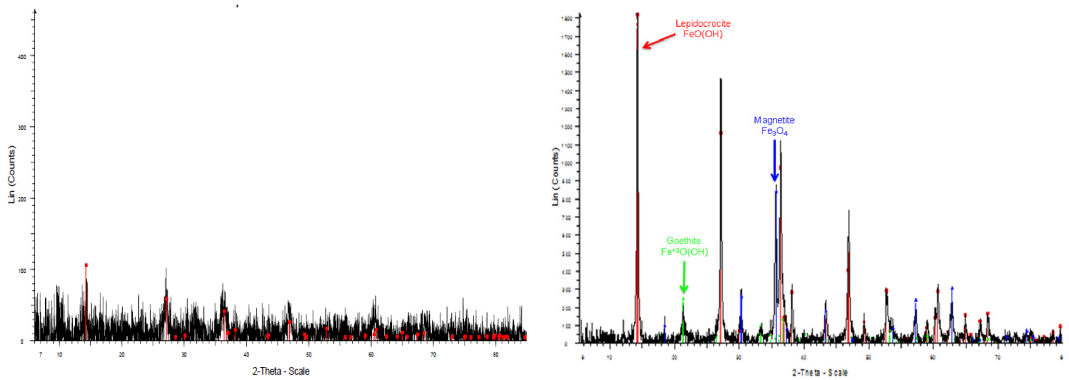

Figure 2 *XRD phase analysis of biogenic powder from cultivated bacteria in an elective medium: (left) SIGP: lepidocrocite (γ-FeOOH); (right) Adler: lepidocrocite (γ-FeOOH), non-stoichiometric magnetite ($Fe_{3-x}O_4$) and goethite (α-FeOOH).*

Here is to note the higher complexity of nanocrystalline minerals because the nanoparticle properties can show marked departures from their bulk analogue materials. The greatest changes are manifested at a few nanometer sizes, where surface effects are likely to dominate chemical bonds, magnetic structure, shape and surface topography as was the case of nanosize goethite (Krezhov, 2009). For instance, nanoparticles of antiferromagnetic materials may have a net magnetic moment due to

uncompensated spins, with implications for the rock magnetic signature. The effects may have important influence on various geochemical and biogeochemical reactions (Liyang *et al*., 2009).

Conclusions

Biogenic nanostructured materials were obtained after dynamic and static cultivation of bacteria of genus Leptothrix in two nutrient media: SIGP and Adler's medium. High enrichment level of iron was found by the PGAA and instrumental NAA techniques in biomass of cultivated isolates as compared to the reference biomass from nature. In contrast to static cultivation in Adler's medium where only bacterial cells were observed, somewhat higher enrichment of iron have exhibited the biogenic products from dynamic cultivation in SIGP medium wherein the formation of sheaths has noticeably occurred. XRD phase analyses indicate distinct presence of nanosize iron oxides/hydroxides but not of manganese oxides. The authors believe that the present experimental evidence contributes to the efforts for understanding of the origin of natural nanomaterials.

Acknowledgements

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