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Formation of artificial sheaths of *Leptothrix* sp. under laboratory conditions

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ABSTRACT

The object of this research are the neutrophilic sheath forming iron bacteria from genus *Leptothrix* isolated from natural stream located in Vitosha Mountain. These bacteria facilitate the iron mineralization and formation the insoluble ferric oxides/(oxy)hydroxides after Fe²⁺-oxidation at neutral pH. The aim of this investigation is establishing the conditions for the formation of tubular structures (sheaths) typical for these bacteria. For this goal cultivation on an elective growth medium and the characterization of the structures formed by optical microscopy and SEM, SEM/EDX, TEM and XRD were performed. The formation of the sheaths was observed at 20°C using a flask-shaking technique. It started after 7 days of the cultivation. Light micrograph images and SEM unveiled that the average size of the sheaths is around 7 – 10 µm, and the average diameter is up to 1 µm. SEM/EDX revealed the elemental composition of the tubular structures. The XRD data showed single-phase composition of the iron oxides on the sheaths obtained. TEM micrographs unveiled the shape of the biogenic nanoparticles. The bacterial mediated formation of iron containing tubular structures and their magnetic properties are of great interest for application in the nanotechnologies and different biomedical and bioengineering applications.

Keywords: *Leptothrix* sp.; iron-oxidizing bacteria; sheath forming bacteria; iron oxide

Introduction

The bacteria from genus *Leptothrix* belong to the neutrophilic β-proteobacteria (Spring, 2006; Takeda et al., 2005; Van Veen et al., 1978). These filamentous Fe/Mn-oxidizing bacterial species have the capability to form microtubular sheaths, and the precipitation of copious amounts of oxidized Fe or Mn distinguish the genus from other phylogenetically related genera (Spring, 2006; Rogers and Anderson, 1976). The *Leptothrix* species are ubiquitous in aqueous environments, especially at sites characterized with neutral pH (around 7), an oxygen gradient and a source

of reduced ferrous ions (Spring, 2006; Takeda et al., 2005). The iron-oxidizing bacteria facilitate iron mineralization by having surface ligands that promote Fe(II) oxidation, although it is not believed that they gain energy from the process (Emerson, 2000). The most common visible inhabitant of many freshwater, low-oxygenated iron seeps is *Leptothrix ochracea*. This chemoheterotroph frequently forms thick filamentous layers comprising a mass of tubular sheaths encrusted in iron. The bacteria use sheath secretion to avoid becoming permanently fixed in the mineral matrix (van Veen et al., 1978).

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The ferric hydroxide is most widespread iron biomineral. It forms in any environment where Fe(II)-bearing waters come into contact with O₂. This includes mine wastes, springs and iron seeps, lakes, streams, swamps, freshwater and marine sediment, soils and subsurface fractured rock, hydrothermal vents, and water distribution systems ect. (Konhauser, 1998, Mulder, E. G. 1989).

Although the bacteria from genus *Leptothrix* is quite difficult to be isolated (Ghiorse, 1984), four species (Emerson et al., 2010) and seven strains have been distinguished on the basis of phenotypic characteristics and/or 16S rRNA gene sequence (Spring, 2006; Sawayama et al., 2011). Axenic cultures of *Leptothrix* tend to lose their sheath-forming capacity after repeated transfers (Van Veen et al., 1978; Ghiorse, 1984; Adams et al., 1986; Emerson and Ghiorse, 1992).

The mechanism of this type of sheath formation is closely associated with the capacity of these organisms to accumulate and oxidize Fe and Mn in aquatic environments (Veen, W.L. et al., 1978; Ghiorse, W.C., 1984).

Many early reports have shown that the *Leptothrix* sheath matrix is a complex hybrid of bacterial exopolymers and aqueous-phase inorganic elements (Takeda et al., 2005; Emerson and Ghiorse, 1993; Emerson and Ghiorse, 1993 (b); Ghiorse, 1984; Sakai et al., 2010). Ghiorse (1984) noted that the sheath of *Leptothrix* comprises a metal-impregnated organic matrix that may be synthesized and excreted from the surface of the bacterial cell envelope.

Suzuki et al. (Suzuki, T. et al., 2011; Suzuki, T. et al., 2011 (b)) have demonstrated using electron energy-loss spectroscopy that sheath-fibers of *L. ochracea* have a carbon core of bacterial exopolymers and that aqueous-phase iron interacts with oxygen at the surface of the carbon core, resulting in the deposition of iron oxides at the surface. These reports support the hypothesis presented by Chan et al. (2004) that organic polymers play important roles in ecosystems by accumulating biologically important elements and that microbial polymers could scavenge iron oxide particles and induce the crystallization of unexpected phases.

Many researchers have reported that the major inorganic component of the sheaths is in the form of iron oxides that bind other inorganics such as Si, P, and often Ca and S (Emerson and Ghiorse, 1993; Emerson and Ghiorse, 1993 (b); Ghiorse, 1984; Sakai et al., 2010; Suzuki et al., 2011).

Hashimoto et al. (2012) have proposed a structural model for the iron oxides produced by *Leptothrix ochracea* in which the XRD-amorphous structure of the basic texture of the sheath is constructed of an FeO₆ network intermingled with SiO₄, suggesting that Si could be a key unit of the XRD-amorphous structure. Although these studies are provided further insights into the structural and spatial associations among the constitutional elements in the sheath of Fe-oxidizing bacteria including *Leptothrix*, more information is needed to understand the initiation and construction of such unique hybrid structures.

The quality and quantity of the natural sheaths, however, are inevitably influenced by environmental changes such as temperature, quantity of supplied groundwater or spring water, concentrations of inorganics in water, as was noted by Vollrath et al. (2013).

This biologically derived organic/inorganic hybrid is chemically and physically active and thus is considered as a future-oriented promising functional material (Furutani, 2011). Further, the artificial synthesis of biogenic iron oxides is energy and cost effective. Laboratory obtained biogenic iron oxides/(oxy)hydroxides are promising byproducts for catalysts (Shopska et al., 2013), as precursor for synthesis of the hybrid battery–super capacitor system „bio-Fe₂O₃+AC//LiBF₄//AC“ (Veleva et al., 2014), for removal of dyes (Safarik et al, 2015), Fe²⁺-ions (Rentz et al., 2009) and heavy metals (Pokhrel and Viraraghavan, 2009; Rentz et al., 2009; Brij Kishor, 2004).

In the present study the data for sheath formation of *Leptothrix* sp. during the cultivation on elective medium are given.

Materials and Methods

A pure culture from genus *Leptothrix* isolated from a natural stream in Vitosha Mountain, located at altitude of 1783 m was used (Angelova et al., 2014; Angelova et al., 2015). For the formation of tubular structures (sheaths) the cultivation was performed on a SIGP medium (Sawayama et al., 2011). The isolate was inoculated into SIGP medium with iron cuttings and glass plates on the bottom serving for attachment of the bacteria and cultivated under dynamic conditions (70 rpm) at 20°C. During the cultivation the dynamic of pH and Fe²⁺ ions as well as microscopic analysis were carried out.

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The characterization of the structures formed by light microscopy and scanning electron microscopy (SEM) (JEOL JSN-5510, JEOL, Japan) was performed. The elemental composition of the microtubules produced was analyzed with an EDX-equipped SEM (Philips ESEM XL30 FEG). XRD measurement showed the composition and the size of the iron oxides on the tubular structures obtained. TEM micrographs unveiled the shape of the biogenic particles.

Results and Discussion

The cultivation of *Leptothrix* sp. on SIGP medium resulted in increase in the fluffy, ochreous deposits in the

flasks, mainly on the iron cuttings and glass plates. Light microscopy revealed the dynamic of the formation of the sheaths during the cultivation.

At the initial stage of the sheath formation, the tubular structures were thinner and shorter. Approximately from 7 to 24 day of the cultivation process was observed abundance of tubular structures (Figure 1a – 1d). After this period of time their number were progressively decreasing (Figure 1e - 1h). The structures disintegrated completely to the end of the cultivation, when additionally fresh medium and iron source were not added.

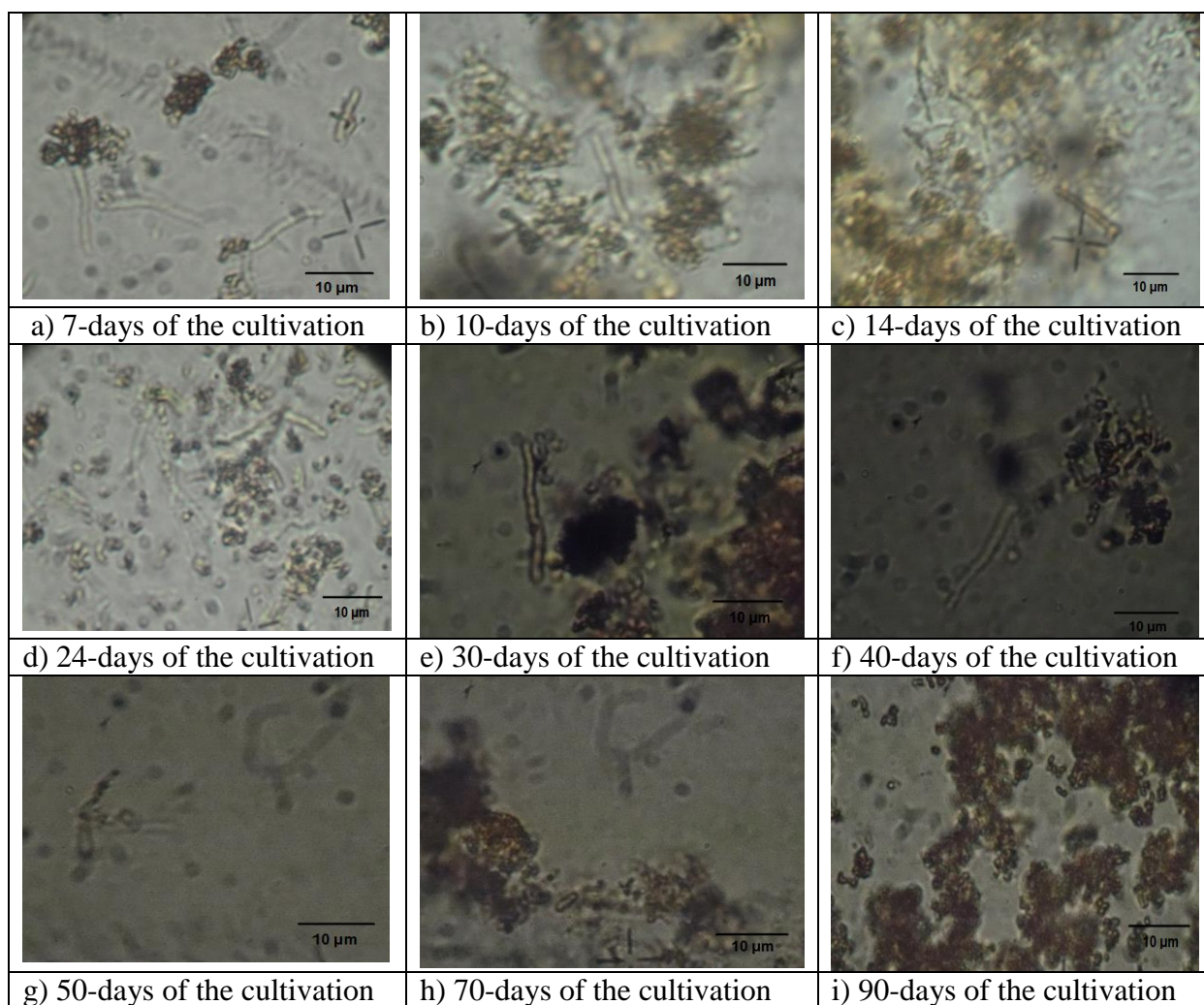


Figure 1. *Forming tubular structures and their changes during the cultivation.*

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Light micrograph images and SEM unveiled that the average size of the sheaths formed were around 7 – 10 μm and the average diameter were up to 1 μm (Figures 1 and 2). Their diameters and lengths varied probably depending on their maturity (Sawayama *et al.*, 2011). Spring [30] reported that golden-brown and highly refractive sheaths of *Leptothrix* sp. are brittle and easily broken into shorter fragments. Therefore, measurements of the length of the microtubules may not be reliable for these structures.

The XRD data showed single-phase composition of the iron oxides on the sheaths obtained (lepidocrocite), as well as the size of the iron particles - 8 nm (Figure 3). TEM micrograph unveiled the needle-like shape of the biogenic nanoparticles (Figure 4).

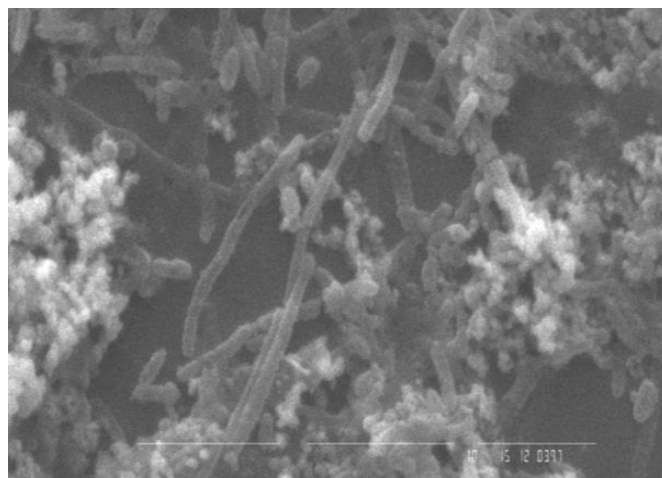


Figure 2. SEM of well formed tubular structures after 7-days of the cultivation; magnification $\times 3500$, scale bar = 10 μm .

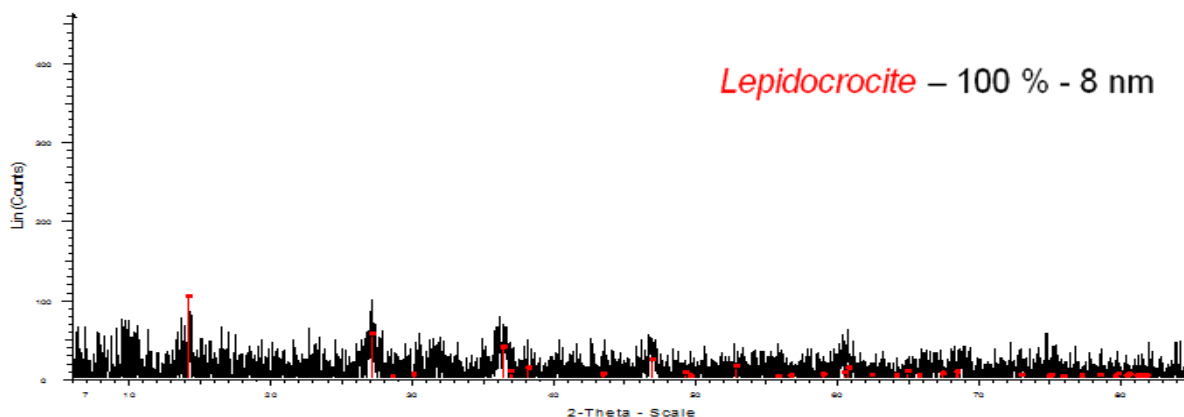


Figure 3. XRD spectrum of the oxide on the tubular structures formed

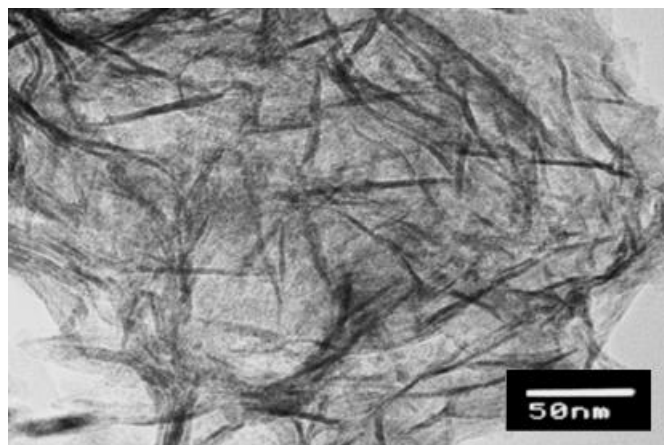


Figure 4. TEM micrograph image of outer surface texture of the lepidocrocite particles with typical needle-like shape

The SEM/EDX analysis revealed that S, O, C, K, Ca, Na, Si, Fe, and P were the inorganic elements of cultured mature microtubules (Figure 5).

During the cultivation unique shaped fibril- and tubule-like structures with characteristic geochemical, electrical, and magnetic properties are formed. These structures are of great interest in terms of engineering novel functional materials. Further detailed geochemical studies of the sheaths are expected to provide an attractive approach to study the broad industrial application of the bacteria-derived iron oxide.

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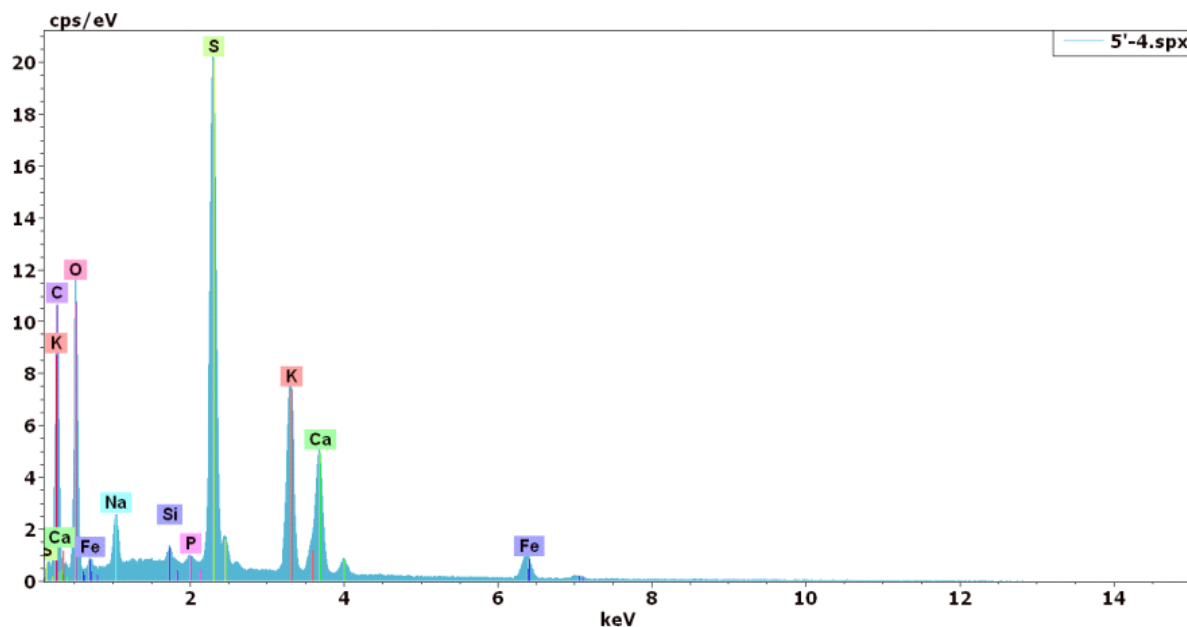


Figure 5. Elemental SEM/EDX analysis of *Leptothrix* sp.-derived sheaths

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