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BIOGENIC IRON OXIDES FROM LABORATORY CULTIVATED *LEPTOTHRIX* SP. FOR APPLICATION IN THE BIONANOTECHNOLOGY

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Abstract: The bacteria from the genus *Leptothrix* have the ability to oxidize Fe^{2+} at neutral pH. As a result, insoluble iron oxides/(oxy)hydroxides are formed on the bacterial surface called sheath. Such structures and oxides are of great interest for application in nanotechnology.

The goal of this study is to obtain iron oxides/(oxy)hydroxides and sheaths after cultivation of the bacteria under laboratory conditions. The pure cultures of the bacteria isolated from natural habitats and identified by the methods of classical and molecular taxonomy as strains of the genus *Leptothrix* are used. Few elective media as well as different conditions of cultivation are used. Four media are found to be the most appropriate for bacterial growth and obtaining of the iron oxides/(oxy)hydroxides. The sheaths are formed only on SIGP medium. They are about 7 μm in length, and up to 1 μm in width. The average size of the biogenic nanoparticles is up to 30 nm.

The characterization of the oxides/(oxy)hydroxides and the sheaths formed is performed by different physical methods.

The composition of the growth media as well as the cultivation procedure have strongly influenced the type of ferric oxides formed.

INTRODUCTION

The sheathed bacteria from genus *Leptothrix* belong to the physiological group of the neutrophilic iron bacteria. They can be found in different aquatic habitats as lakes, streams, springs, swamps, iron seeps, and springs rich in iron and manganese, as well as in wastewater treatment systems (Bergey and Holt, 1994).

The insoluble ferric oxides/(oxy)hydroxides formed after oxidation of the ferrous iron are incorporated on the bacterial surface forming specific sheath structures. These structures as well as ferric oxides formed are of great interest for application in different nanotechnologies, biomedical and bioengineering applications as pigments, adsorbents, ferrofluids, magnetic resonance imaging contrast enhancement, detoxification of biological fluids and others (Gupta and Gupta, 2005, Sawayama et al., 2011).

Goethite (α-FeOOH) obtained by laboratory cultured *Leptothrix* bacteria, can be used as precursor for synthesis of electrochemically active nanosized α-Fe_2O_3. The nanoparticles of bio-αFe_2O_3 can be easily applied for realization of composite electrodes with activated carbon (Veleva et al., 2014). The hybrid battery–super capacitor system (bio-Fe_2O_3+AC)//$LiBF_4$//AC shows high reproducibility of charge/discharge processes, stability and high value of capacity (80 Fg^{-1}), excellent cycle ability and high efficiency (above 95%) at prolong cycling as well as a number of advantages in the performance in comparison to the symmetric AC//$LiBF_4$//AC super capacitor (Veleva et al., 2014).

The bacterial mediated formation of iron containing tubular structures, their magnetic properties and behavior are currently poorly understood. It is also not clear why many of the strains do not form sheaths during the cultivation under laboratory conditions (Emerson and Chiorse, 1992).

In this paper the some results about iron oxides/(oxy)hydroxides formed after cultivation of the bacteria under laboratory conditions are given.

MATERIALS AND METHODS

Sampling region

The bacterial samples are taken from streams in Vitosha Mountain, located at altitude of 1783 m. Subject to the sampling (figure 1) are deposits with typical texture and reddish color (Bergey and Holt, 1994).

Figure 1. The sampling region with the typical texture and reddish color streams in Vitosha Mountain.

Isolation, identification and cultivation of iron bacteria

After microscopic analysis of the samples taken from Vitosha Mountain for the presence of iron bacteria on the basis of their specific morphology elective media were used for obtaining of enriched cultures and isolation of the pure ones. Roux and Fernbach flasks as well as special bioreactor with aeration and flask- shaking technique were used for cultivation of the samples. The following elective media were used:

Lieske's medium (LM) (Lieske R., 1919) – Saturated solution of $Mg(HCO_3)$, 1:10 - 100 ml; $(NH_4)_2SO_4$ - 0.01 g; K_2HPO_4 – traces; $MgSO_4.7H_2O$ – traces; dH_2O - 1000 ml; pH 6.8 – 7.4.

Adler's medium (AM) (Ellis D., 2003) – modification - $C_3H_5NaO_3$ - 40.0 mg; Yeast extract - 1.0 g; $C_6H_8O_6$ - 0.1 g; $MgSO_4.7H_2O$ - 0.2 g; K_2HPO_4 - 0.01 g; $(NH_4)_2Fe(SO_4)_2.6H_2O$ - 0.01 g; dH_2O - 1000 ml; pH 7.0.

Isolation medium for bacteria of the Sphaerotilus - Leptothrix group (IM) (M622) (Eaton, 2005) – $C_6H_{12}O_6$ - 0.150 g; $(NH_4)_2SO_4$ - 0.500 g; $Ca(NO_3)_2$ - 0.010 g; K_2HPO_4 - 0.050 g; $MgSO_4$ - 0.050g; KCl - 0.050 g; $CaCO_3$ - 0.100 g; Vitamin B_{12} - 0.00001 g; Thiamine - 0.0004 g; dH_2O – 1000 ml; pH 7.0.

Silicon iron glucose peptone (SIGP) (Sawayama et al., 2011) – $C_6H_{12}O_6$ - 1 g, Bacto peptone (BD, France) - 1 g, $Na_2SiO_3.9H_2O$ - 0.2 g, $CaCl_2.2H_2O$ - 0.044 g, $MgSO_4.7H_2O$ 0.041 g, $Na_2HPO_4.12H_2O$ - 0.076 g, $KH_2PO_4.2H_2O$ - 0.02 g, HEPES (N-2-hydroxyethelpiperazine-N'-2-ethanesulfonic acid) - 2.838 g, and 0.05 mM $FeSO_4$ in 1000 ml of distilled water; pH 7.0.

As an additional source of iron, iron cuttings are supplemented to all media used. The cultivation is carried out at 20°C and a pH of 7.0. The changes in the pH and the content of Fe^{2+} during the cultivation as well as a microscopic observation are monitored periodically.

The isolation of the pure cultures on IM is carried out by the standard single-cell technique from the samples of the enriched cultures obtained.

The identification of the isolates is performed according to taxonomic schemes of Bergey's Manual of determinative bacteriology, 8th Ed., including morphological, cultural and biochemical characteristics. The confirmation of the

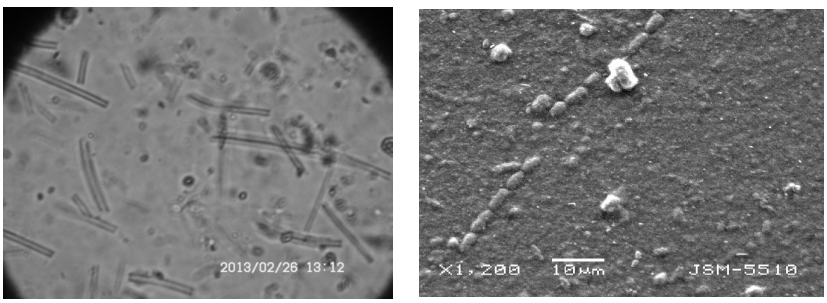
taxonomic status of the isolates is done by the PCR detection assay. The bacterial cells are harvested by centrifugation (4 500rpm/10 min), the cell pellet is washed with Phosphate-buffered saline (PBS) and subjected to DNA isolation with Prep Mini Spin Kit (GE Healthcare). The published sequence of *mofA* gene (GenBank № Z25774.3) is chosen as a specific target for PCR detection of *Leptothrix* sp. The specific primers are constructed with Primer-Blast Software: F1_ thrix is 5'- TGT TCG AGC CGG T TCG GC - 3', and R1_ thrix 5'- GAA TCG ATC GCG AC C GGC GT - 3' (LKB GmbH). The PCR mixture contained 1 µM of each primer (Sense and Antisense), 0,2 mM dNTPs, Taq buffer 1x (Invitrogen), 1,5 mM $MgCl_2$, 2,5 U Taq polymerase and 5 µl (10-100 ng) total DNA. The total volume of single reaction is 25 µl. The PCR program consisted of an initial denaturation step 95ºC/5min, followed by 35 cycles (95ºC/1min; 54ºC/1min; 72ºC/1min) and a final extension step at 72ºC for 5 min. All reactions are carried out on an Eppendorf Thermocycler (Eppendorf).

Checking the PCR products is performed on 3% agarose gel (Agarose low EEO, AppliChem, Germany). To establish the size of the amplified fragments marker (Gene RulerTM, 100 bp DNA Ladder Plus) is used.

The analysis of the formed iron oxides was achieved by X-Ray Diffraction (XRD), magnetic measurements by a physical properties measurement system (PPMS, Quantum Design), Light and Scanning Electron Microscopy (SEM). All measurements are conducted on samples taken after 40 days of the cultivation of the isolates.

RESULTS AND DISCUSSION

The analysis of the samples from the sampling area showed the presence of the neutrophilic iron bacteria with typical morphology and sheaths (figure 2).



a) b)

Figure 2. Analysis of the samples for the presence of iron bacteria:
a) Light microscopy ×400; b) SEM ×1200.

After the isolation of the pure cultures, their identification is established by the methods of the classical taxonomy. Six morphological, 8 physiological and 18 biochemical properties of the isolates have been checked. According to the results obtained nine of the isolates belong to the genus *Leptothrix*. This was confirmed by the PCR- assay (figure 3).

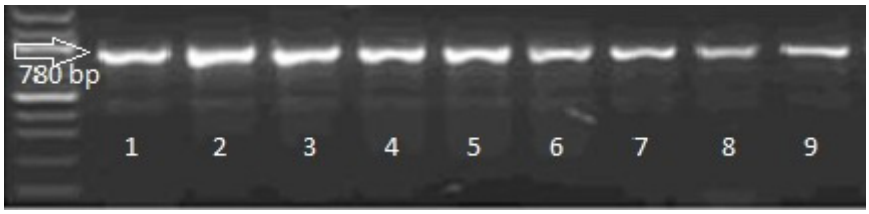
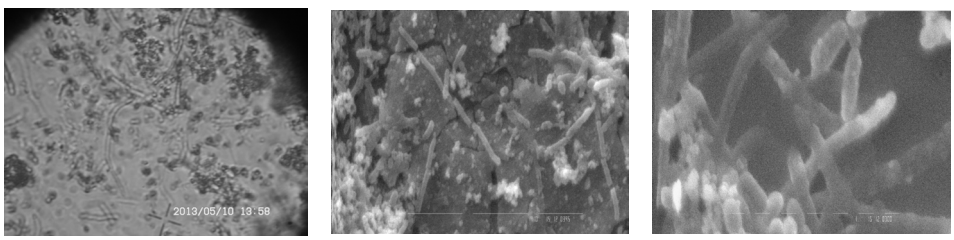


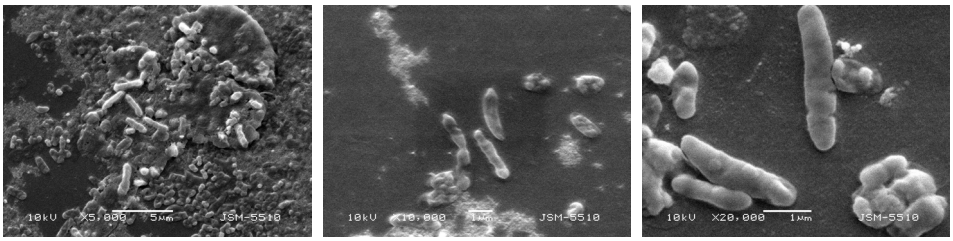
Figure 3. PCR detection assay: an amplification profile of *mofA* gene of *Leptothrix* sp.

The bacteria formed the typical sheaths (figure 4) during the cultivation on SIGP medium, while on AM, LM and IM media such structures are not found (figure 5). According to the results obtained the SIGP medium proved to be the most appropriate one for the formation of sheaths compared with the other media used. The formation of the sheaths started after seven days cultivation. Their average diameter is in the range of about 1 μm, with the length reaching approximately 7 μm. The structures disintegrated completely approximately 90 days after the cultivation. It is obvious that the formation of the sheaths strongly depends on the medium used. The optimal growth of the cultures is observed at 20°C under flask- shaking technique of cultivation.



a) b) c)

Figure 4. Sheaths formed on SIGP medium: a) Light microscopy ×400;
b) SEM ×3500; c) SEM ×10 000.



a) b) c)

Figure 5. SEM of bacteria from the *Leptothrix* sp. cultivated on different media: a) AM ×5000; b) LM ×10 000; c) IM ×20 000.

The results about the dynamics of the concentration of the ferrous ions and the change of the pH during the cultivation of the bacteria on different media are given in tables 1 and 2.

Table 1. Dynamics of pH during the cultivation.

Sample	pH				
	Days				
	1	**7**	**14**	**30**	**40**
LM Control	7.30	7.30	7.30	7.30	7.30
AM Control	7.20	7.21	7.19	7.23	7.27
IM Control	7.15	7.15	7.14	7.14	7.14
SIGP Control	7.00	7.02	7.08	7.10	7.12
LM	7.30	7.30	7.32	7.33	7.35
AM	7.10	7.09	7.10	6.98	6.95
IM	7.15	7.12	7.08	6.89	6.75
SIGP	7.00	7.18	7.23	7.37	7.45

Table 2. Dynamics of the iron utilization during the cultivation.

Sample	Fe^{2+} (g/l)				
	Days				
	1	**7**	**14**	**30**	**40**
LM Control	2.54	2.54	2.54	2.38	2.38
AM Control	1.78	1.78	1.78	1.58	1.58
IM Control	1.98	1.98	1.98	1.58	1.58
SIGP Control	1.98	1.98	1.98	1.98	1.98
LM	2.57	2.18	1.98	0.99	0.79
AM	1.58	1.18	0.99	0.59	0.37
IM	1.98	1.78	1.58	1.39	1.19
SIGP	1.78	0.99	0.59	0.59	0.59

It is obvious that the rate of chemical oxidation of the ferrous ions is very low in all controls tested. On the other hand the concentrations of the ferrous ions in the samples depend on bacterial growth and the media composition. After 40 days of the cultivation the lowest residual ferrous ions are found in the AM, LM and SIGP media.

In all media above mentioned different iron oxides/(oxy)hydroxides are formed.The data from the XRD analysis are given in table 3.

Table 3. X-ray Diffraction data of the iron oxides/(oxy)hydroxides formed.

Nutrient media	***Type of the oxides/(oxy) hydroxides formed, their percentage ratio and the particle size***
AM	► lepidocrocite (γ-FeOOH) - 59.67 % - 29 nm ► magnetite (Fe_3O_4) - 21.56 % - 24 nm ► goethite (α-FeOOH) - 18.77 % - 12 nm
IM	► goethite (α-FeOOH) - 77.03 % - 10 nm ► lepidocrocite (γ-FeOOH) - 22.97 % - 28 nm
LM	► goethite (α-FeOOH) - 77.02 % - 10 nm ► lepidocrocite (γ-FeOOH) - 14.84 % - 28 nm ► magnetite (Fe_3O_4) - 8.14 % - 27 nm
SIGP	► lepidocrocite (γ-FeOOH) – 100 % - 8 nm

The magnetic measurements done by a physical properties measurement system (PPMS, Quantum Design) of samples from AM shows superparamagnetic behavior typical for the magnetite while the samples from the IM, LM and SIGP show weak magnetic behavior.

CONCLUSIONS

After cultivation of *Leptothrix* sp. on different media, sheaths are formed only on the SIGP medium after 7 days of cultivation. Although each of them (AM, LM, IM and SIGP) is appropriate for obtaining the desired iron oxides/(oxy)hydroxides. The average size of the iron containing particles is up to 30 nm and their composition differs and depends on the medium used. The nanosized particles possess magnetic characteristics.

The results indicate that these nanoparticles with tunable magnetic properties, high surface to volume ratio and a defective amorphous structure are promising candidates for removal of heavy metals from wastewaters.

The analyses revealed a wide range of potential applications of the material obtained as novel industrially functional materials for electronics, catalysts, adsorbents, pigments, ferrofluids, super capacitors and others.

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