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# Optimization the cultivation of neutrophilic iron bacteria in laboratory conditions

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## ABSTRACT

The neutrophilic iron-oxidizing bacteria are different in morphology, physiology and taxonomic status but are united by their ability to oxidize $Fe^{2+}$ at neutral pH and to form insoluble ferric oxides/(oxy)hydroxides. The hydroxides formed on their sheaths are of great interest for application in different nanotechnologies, biomedical and bioengineering applications. The bacteria are typical oligocarbophils which makes them a successful indicator for organic pollution of the water. The main problem is connected with the formation of sheaths. The goal of this study is connected with the optimization of the cultivation conditions to obtain sheaths with ferroxides of neutrophilic iron bacteria. The optimization included investigations of the types of cultivation, used of the different nutrient media and the duration of the cultivation.The analysis of the results shows that the isolates grow poorly at 10°C and 37°C. Optimal growth of the cultures was observed at 20°C under dynamic conditions. The most appropriate for the formation of sheaths was SIGP medium. The formation of the sheaths started after 7 days cultivation period.The analysis of the ferroxides formed shows that the composition of the nutrient medium strongly influences the type of the formed ferroxides.

**Key words:** neutrophilic iron bacteria, sheathed bacteria, *Leptothrix*, nanotechnologies

## Introduction

The neutrophilic iron-oxidizing bacteria are widely spread in different natural habitats. They are different in morphology, physiology and taxonomic status but are united by their ability to oxidize $Fe^{2+}$ at neutral pH and to form insoluble ferric oxides/(oxy)hydroxides.

The interest to these bacteria increased in the recent years because many reasons. The hydroxides formed on the their sheaths are of great interest for application in different nanotechnologies and different biomedical and bioengineering applications as magnetic resonance imaging contrast enhancement, tissue repair, immunoassay, detoxification of biological fluids, drug delivery and in cell separation, etc. (Gupta, 2005). On the other side after the intensive growth of the bacteria and the formation of insoluble ferric hydroxides which are environmental pollutants lead to significant economic losses.

The bacteria are typical oligocarbophils which makes them a successful indicator for organic pollution of the water.

The sheathed bacteria grow as chains of cells in filaments, 0.4-7 μm in width. Gram negative. Filaments grow in a tube

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of extracellular material referred to as a sheath. Sheaths may appear yellow to dark brown because of the deposition of iron and manganese oxides. Sheathed bacteria are found in aquatic habitats. The motile species in pure culture treated in this group have flagella (Holt, 1994).

The sheathed bacteria studied in pure culture are aerobic chemoheterotrophs that use organic acids and sugars as carbon sources. Some deposit iron and manganese oxides in or on their sheaths and may carry out the oxidation of Fe (II) and Mn (II); however, they are not known to obtain energy from this process. The sheathed bacteria are found in aquatic habitats including lakes, streams, and springs. Some are also found in wastewater treatment systems (Holt, 1994).

The cultivation of the iron bacteria under laboratory conditions is quite difficult (Emerson & Chiorse, 1992). The main problem is connected with the formation of the sheaths. The reason for this is probably the inability to fully mimic the natural conditions or still for reasons unknown.

This study is connected with the optimization of the cultivation conditions to obtain the sheaths with ferroxides of neutrophilic iron bacteria.

The optimization included investigations of the types of cultivation, used of the different nutrient media and the duration of the cultivation.

## Materials and Methods

### *Harvesting and inoculation of samples*

As source of harvesting iron bacteria biomass, it was chosen a natural stream located in region in Vitosha Mountain (1783 m altitude). After filtration and homogenization of the biomass, it was used for inoculation of seven selected nutrient media at different types of cultivation, mimicking the natural environment conditions (pH, aeration, temperature, and illuminance). Two types of 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 days to 4 months. The cultivation was carried out at 3 different temperatures - 10ºC, 20ºC и 37ºC. Periodically samples were taken and performed microscopic analysis of the cultures in the process of cultivation. The list of selected nutrient media contains:

- Vinogradski's medium (VM) (Winogradsky, 1891) - Ferric ammonium citrate - 10 g; $NH_4NO_3$ - 0.5 g; $K_2HPO_4$ - 0.5 g; $MgSO_4.7H_2O$ - 0.5 g; $CaCl_2$ - 0.2 g; $dH_2O$ - 1000 ml; pH 7.0
- Lieske's medium (LM) (Lieske, 1919) – Saturated solution of $Mg(HCO_3)$, 1:10 - 100 ml; $(NH_4)_2SO_4$ - 0.01 g; $K_2HPO_4$ – traces; $MgSO_4.7H2O$ – traces; $dH_2O$ - 1000 ml; pH 6.8 – 7.4
- Fedorov's medium (FM) (Leathen et al., 1956) - $(NH_4)_2SO_4$ - 1.5 g; $MgSO_4.7H_2O$ - 0.05 g; $K_2HPO_4$ - 0.05 g; KCl- 0.05 g; $Ca(NO_3)_2$ - 0.01 g; $dH_2O$ - 1000 ml; pH 7.0
- Adler's medium (AM) (Ellis, 2003) – modification - Sodium lactate - 40.0 mg; Yeast extract - 1.0 g; Ascorbic acid - 0.1 g; $MgSO_4.7H_2O$ - 0.2 g; $K_2HPO_4$ - 0.01 g; $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ - 0.01 g; $dH_2O$ - 1000 ml; pH 7.0
- Isolation medium for bacteria of the group *Sphaerotilus - Leptothrix* (IM) (M622) (Eaton et al., 2005) – Glucose - 0,150 g; $(NH_4)_2SO_4$- 0,5 g; $Ca(NO_3)_2$ - 0,01 g; $K_2HPO_4$ – 0,05 g; $MgSO_4.7H_2O$ - 0,05 g; KCl - 0,5 g; Vitamin B12 - 0,00001g; Thiamin - 0.0004g; $dH_2O$ - 1000 ml; pH 7.0
- SIGP (silicon iron glucose peptone) (Sawayama et al., 2011): 1 g glucose, 1 g Bacto peptone (BD, France), 0.2 g $Na_2SiO_3\_9H_2O$, 0.044 g $CaCl_2\_2H_2O$, 0.041 g $MgSO_4\_7H_2O$, 0.076 g $Na_2HPO_4\_12H_2O$, 0.02 g $KH_2PO_4.2H_2O$, 2.838 g HEPES (N-2-hydroxyethelpiperazine-N'-2-ethanesulfonic acid) and 0.05 mM $FeSO_4$ в 1000 ml $dH_2O$ (pH 7.0).

To determine the most appropriate source of iron used were iron cuttings, iron carbonate, ferrous sulphate and ferric chloride.

### *Morphological and Physiological analysis*

After isolation of pure cultures they were subjected to morphological and physiological characteristics according classical taxonomic scheme. The key morphological characteristics that were analyzed were a) cell shape – (with scanned electron microscopy - JEOL JSN-5510, JEOL, Japan) 2) Gram – stain 3) motility test 4) presence of capsule. The list of growth characteristics includes: 1) ability to grow on different selective media, 2) ability to oxidize $Fe^{2+}$, 3) Preferable source of $Fe^{2+}$, and 4) Ability to oxidize $Mn^{2+}$.

### *PCR detection assay*

Bacterial cells were harvested by centrifugation (4 500rpm/10 min), cell pellet was washed with PBS and subjected to DNA isolation with Prep Mini Spin Kit (GE Healthcare). As a specific target for PCR detection of

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*Leptothrix spp*. was chosen the published sequence of *mofA* gene (GenBank № Z25774.3). Specific primer was constructed with Primer-Blast Software. F1_thrix e 5'-TGT-TCG-AGC-CGG-TGT-TCG-GC-3', and R1_thrix 5'-GAA-TCG-ATC-GCG-ACC-GGC-GT-3'. The PCR mixture contains 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 и 5 µl (10-100 ng) total DNA (Ready-To-Go PCR kit (GE Healthcare). Total volume of single reaction is 25 µl. The PCR programme consists of initial denaturating step 95ºC/5min, followed by 35 cycles (95ºC/1min; 54ºC/1min; 72ºC/1min) and final extension step at 72ºC for 5 min. All reactions were carried on Eppendorf Thermocycler (Eppendorf).

***X-ray Diffraction (XRD)***

X-ray diffraction was used for characterization of the obtained crystalline iron containing materials. Filtered and dried at room temperature biomass from the cultivation vessels was used.

## Results and Discussion

The constructed cultivation system allows obtaining enriched cultures from the samples after 14 days. The isolated pure cultures were subjected to classical typing scheme for identification of iron bacteria. From total count of 24 pure cultures, 15 of them were used for typing. The analysis, based on cell morphology revealed presence of bacteria, belonging to the genera *Leptothrix, Sphserotilus, Pedomicrobium, Crenothrix, Siderococcus, Gallionella and Ochrobium*. The typical cell morphology was confirmed by light and electron microscopy.

On the basis of applied classical taxonomic scheme, it was found that 12 of the isolates belong to genera *Leptothrix* and 3 to genera *Sphaerotililus*.

PCR amplification with *mofA* primers was positive for 12 of the isolates (Figure 1), which confirms the results of classical typing scheme.

Sheaths have been formed by the bacteria of the genus *Leptothrix* only on SIGP medium under dynamic cultivation conditions (Figure 2a, b).

XRD data showed that on IM revealed the presence of α-FeOOH (goethite) and γ-FeOOH (lepidocrocite). The samples taken from the cultivation on AM and LM contained α-FeOOH (goethite), γ-FeOOH (lepidocrocite) and $Fe_3O_4$ (magnetite). Their sizes are below 30 nm.

The analysis of the results shows that the isolates grow poorly at 10°C and 37°C. Optimal growth of the cultures was observed at 20°C under dynamic conditions and ferrous sulphate as source of iron. Most suitable isolation medium for obtaining pure cultures of neutrophilic bacteria it is IM. The most appropriate for the formation of sheaths was SIGP medium. The formation of the sheaths started after 7 days cultivation period.

The analysis of the ferroxides formed shows that the composition of the nutrient medium strongly influences the type of the ferroxides.

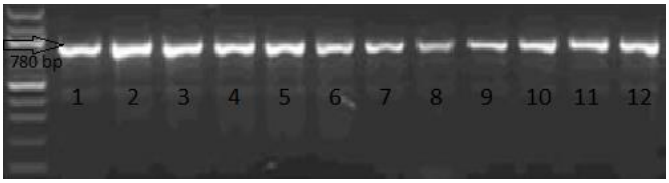


**Figure 1.** *Amplification profile of mofA – PCR*

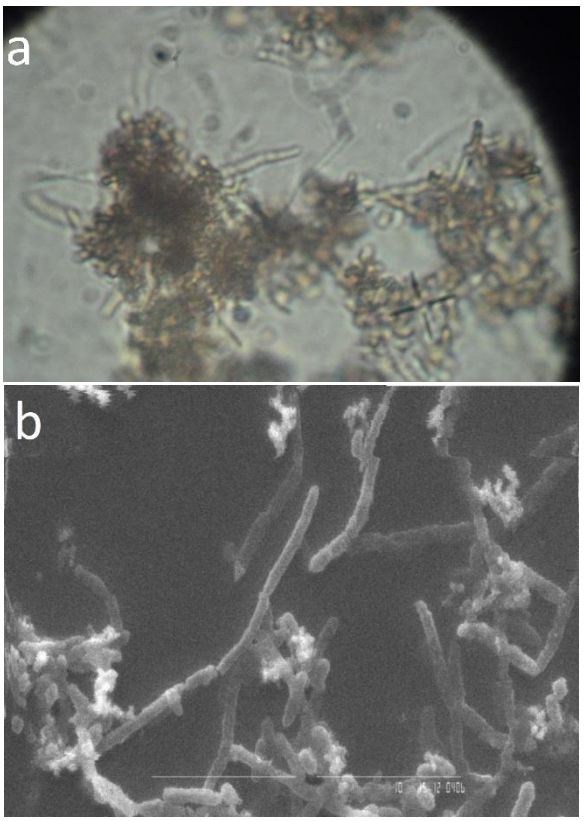


**Figure 2.** *Microscopic images of sheaths of the genus Leptothrix in SIGP medium: (a) light microscopy image, (b) SEM (5000x)*

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