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Effect of electromagnetic fields on the denitrification activity of *Paracoccus denitrificans*

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Abstract

Enzymatic activity (denitrification) of *Paracoccus denitrificans* was estimated electrochemically by reduction of duroquinone (DQ). Graphite electrodes covered with whole bacterial cells behind a dialysis membrane were used for measurement. *P. denitrificans* reduce nitrate and/or nitrite under anaerobic conditions to nitrogen gas. DQ acts as an electron mediator. After donation of the electrons to the respiratory system of the bacteria, produced DQ is reduced to durohydroquinone on the electrode surface electrocatalytically. *P. denitrificans* were exposed to low-frequency magnetic field (10 mT, 50 Hz) for 24 min. In comparison with the control samples, the reduction peak of *I–E* curves that represent denitrification activity of the cells decreased significantly after magnetic field exposure. The decrease of the peak current was about 20%. The CFU-colony forming units-method was used to estimate the number of surviving bacteria. After 24 min exposure of 10 mT magnetic field *P. denitrificans* culture on electrode indicates 21% bacterial death.

Keywords: Low-frequency electromagnetic field; Denitrification; *Paracoccus denitrificans*; Graphite electrodes; CFU number

1. Introduction

In the latest few decades, many studies concerning electromagnetic fields effects on biological objects were carried out [1,2]. A lot of them were focused on DNA and gene expression [3–5], transport of ions [6,7], enzymatic activity [8–10], viability and proliferation [11,12]. There are epidemiological studies with controversial results too [13,14]. Bacteria can be a good object for the study of extremely low-frequency electromagnetic fields (ELF EMF) effects [15,16].

The purpose of the present study was to investigate the possible effect of ELF EMF on denitrification activity of *Paracoccus denitrificans*. We used electrochemical method to determinate enzymatic activity [17,18]. Electrode modified with whole cells of bacteria functions as a biocatalyst electrode in the presence of DQ. Denitrification is a process where nitrate is reduced via nitrite, nitric oxide, and nitrous oxide to the nitrogen gas. Series of reductases in the respiratory chain of *P. denitrificans* are associated with this process. Nitrate reductase is the most important electron acceptor for DQ. After donation of the electrons to the respiratory chain, DQ is reduced to durohydroquinone on the electrode surface electrocatalytically. Thus, the electrode will respond to nitrate and/or nitrite as a final electron acceptor under anaerobic condition. This method can serve as a powerful tool to estimate the enzymatic activity of *P. denitrificans*.

2. Experimental

2.1. Instrumentation

Magnetic field was created by a cylindrical coil powered by an autotransformer. Maximal current in the coil was 1.9 A, which corresponds to maximal magnitude of magnetic induction $B_m = 10$ mT. Frequency of the current used was 50 Hz. Parameters of the coil are given in Table 1 and the distribution of the magnetic induction inside the coil is shown in

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Table 1
Cylindrical coil parameters

Diameter	235 mm
Inner diameter	205 mm
Length	210 mm
Number of threads	880
Diameter of wires	2 mm
Weight	5.7 kg

Fig. 1. During the exposure, the room temperature (24–26 °C) was maintained with an airflow and controlled by a thermometer. The samples were placed on the nonconductive stand in the centre of the coil, where uniformity of field is maximal.

For electrochemical measurements, three-electrode system was used. Working electrodes were different types of graphite electrodes. The platinum wire served as a counter electrode, as a reference electrode Ag/AgCl/3 M KCl was used. The cyclic voltammetric (CV) measurements were performed with the EcoTribo Polarograph (Polaro-Sensors, Prague, Czech Republic). AFM measurement was done using Accurex II.L microscope (Fig. 2).

2.2. Electrode preparation and measurement

Three types of working electrodes were used: pencil graphite electrode (PGE), surface of 0.79 mm^2; glassy carbon electrode (GCE), surface of 3.14 mm^2 (Metrohm); and pyrolytic graphite electrode with basal orientation (PGEb), surface of 15 mm^2 (GE-Advance Ceramics). All the electrodes were coated with Torr-seal (Varian). Before use, the electrodes were mechanically polished with silicon carbide papers (the minimal diameter of polishing particles was 5 μm) (Struers). Then the electrodes were polished with 1 μm diamond polishing particles in paste (Leco) on Lecloth B polishing cloth (Leco). Before the final use, the electrodes were sonicated for 5 min.

The electrodes were modified with known amount of live bacteria in the following way: harvested bacteria were diluted in basal PBS. A 5-μl solution containing 100 μg of dry cells weight of *P. denitrificans* per 10 mm^2 was placed on the electrode surface and enables to evaporate. As the last step, the electrode was clothed by a dialysis membrane, which was physically attached to the electrode with nylon net.

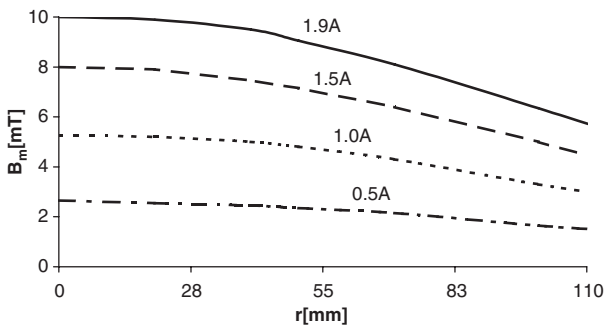

Fig. 1. Dependence of the magnetic field induction B_m in the coil on the distance from the coil axis (which is situated at $r=0$ mm) for different current values.

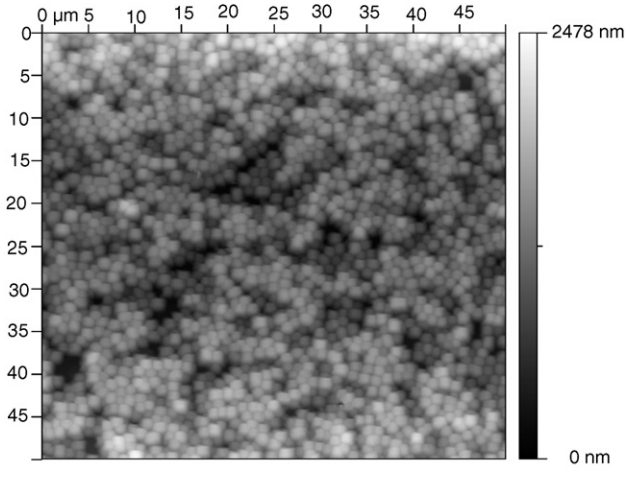

Fig. 2. AFM contact mode image of bcGCE without dialysis membrane.

Measurements were done at the room temperature (24–26 °C) in 3 ml of the basal PBS solution as the electrolyte. For each measurement a newly prepared bacteria covered electrode (bcE) was used. The basal PBS was continuously stirred at 3600 rpm and the surface was continually flushed with argon gas to maintain anaerobiosis.

2.3. Bacteria and chemicals

P. denitrificans CCM 982 (NCIB 8944) was obtained from the Czech Collection of Microorganisms and cultivated in an anaerobic medium, containing 50 mM sodium succinate as the electron donor and 10 mM KNO_3 as the electron acceptor [19]. The pH was adjusted to the value of 7.3 by addition of NaOH. For electrochemical measurement, the cells were harvested by a centrifugation (5000 rpm, 5 min) after 22 h incubation at 30 °C. Then the cells were washed twice in the basal phosphate buffer solution (PBS, pH 7.3). For CFU-colony forming units-measurement was used bacteriological agar No. 1 (Oxoid). We employ two methods of ELF EMF field effect on bacterial viability estimation. First method follows the same procedure as for the bacteria modified electrodes preparation. Bacteria were diluted after magnetic field exposure on electrodes and placed on agar plates in Petri dishes of 90-mm diameter. The other method used bacteria which were diluted from 22 h aged bacterial culture and then dispensed on agar plates. The bacterial culture was ready to endure magnetic field exposure. After 2 days of incubation at 30 °C, CFU were counted. Duroquinone was purchased from Sigma-Aldrich. All other reagents were of the highest grade commercially available.

3. Results

3.1. Enzymatic activity

Response for nitrate (i.e. the reduction of the electron mediator DQ and the enzymatic activity of reductases series) of *P. denitrificans* was recorded using three types of bacteria-modified electrodes. Basal PBS was used as electrolyte, 0.5 mM

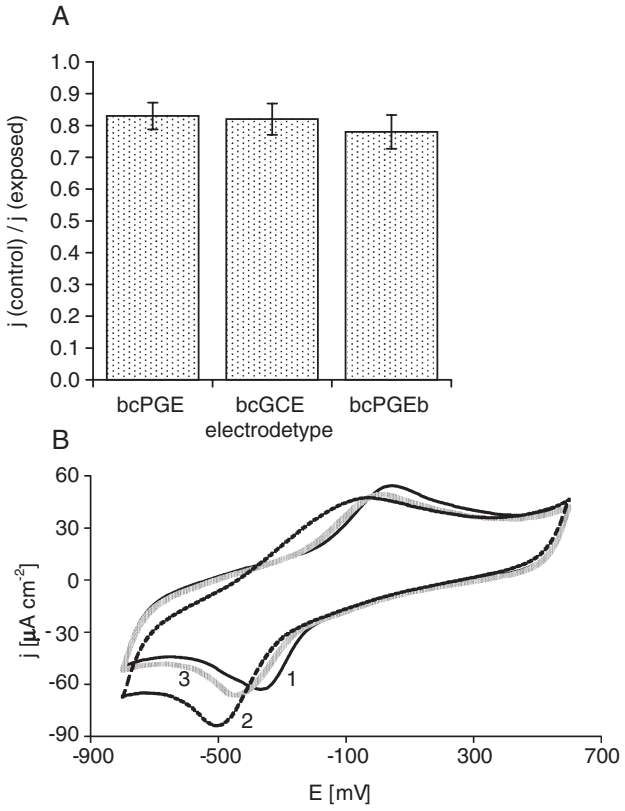

Fig. 3. (A) Decrease of the voltammetric peak current j after magnetic field exposure (B_m=10 mT, t=24 min, f=50 Hz) for different types of electrodes. (B) Cyclic voltammogram of exposed bcPGE, scan rate 500 mV s^{-1}. Curve 1 denotes electrolyte (PBS) with 0.5 mM DQ, curve 2 represents the situation 5 min after KNO_3 addition (sooner is the system not fully stabilized) and curve 3 is the same system after exposure to magnetic field.

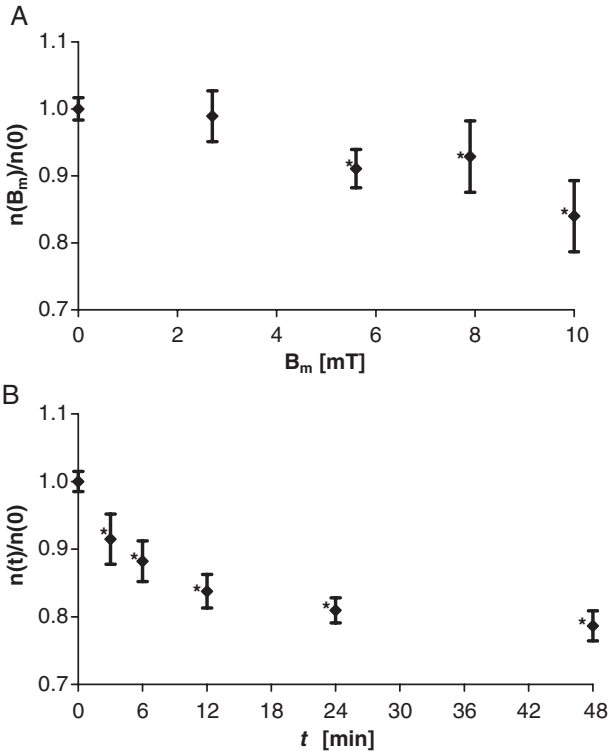

Fig. 4. Dependence of CFU on magnetic field exposure. (A) Dependence of the relative number of CFU on the value of the magnetic field induction for t=12 min. (B) Dependence of the relative number of CFU on the duration of exposure for B_m=10 mT, * denotes relative CFU numbers, which are statistically significant different at the 0.95 level of significance from the control one.

DQ as an electron mediator and 1 mM KNO_3 as an electron acceptor. Scan rate of CV was 500 mV s^{-1}. Increase of peak current around of −500 mV for bcPGE and bcPGEb was 20 μA cm^{-2} and 40 μA cm^{-2} for the bcGCE. All three electrodes were tested for time stability (peak current shows no changes during 60 min after addition of KNO_3). Control measurements were done with uncovered electrodes, and there was no change after addition of KNO_3. It was also important that all three electrodes, either uncovered or covered only with dialysis membrane, showed no changes in the peak current after exposure to magnetic field of 10 mT for 24 min.

After exposure of bacteria modified electrodes to magnetic field (10 mT, 50 Hz, exposure time 24 min) a decrease of peak current was observed. A small shift in peak position was also observed. An example of CV curves is shown in Fig. 3, the data are shown in Table 2.

Table 2
Magnetic field effect on three different bacteria-modified electrodes

Electrode	j (control)/j (exposed)
bcPGE	0.831±0.042
bcPGEb	0.784±0.053
bcGCE	0.819±0.049

All 3 experiments were repeated 10 times.

3.2. CFU determination

CFU method was used to determinate bacterial response to magnetic field. We carried out CFU counting after exposition to 10 mT magnetic field for 24 min on electrode surface. There was (21±6)% decrease (statistically significant) in CFU number for bacteria exposed on electrodes compared with unexposed control. We measured the dependence of CFU number after exposure to magnetic field (B_m=10 mT, f=50 Hz) for different times of exposure and the dependence on B_m for 12 min exposure on agar plates (Fig. 4). For statistical analysis of the results, the Student's statistics at the 0.95 level of significance was used. All the experiments were repeated at least 10 times.

4. Discussion

We have found that the reduction peak of DQ decreased for all three types of bacteria-modified electrodes after exposure to magnetic field (B_m=10 mT, f=50 Hz, exposure time=24 min). The decrease was different for each electrode, but in a view of statistical analysis there was no significant difference among these three electrodes. From these results, it follows that the reduction peak current was decreased at about 20%. We measured reaction of bacteria on agar plates (solid bacterial soil) to magnetic field exposure. The results were obtained by counting colony-forming units (CFU). Both the dependence of

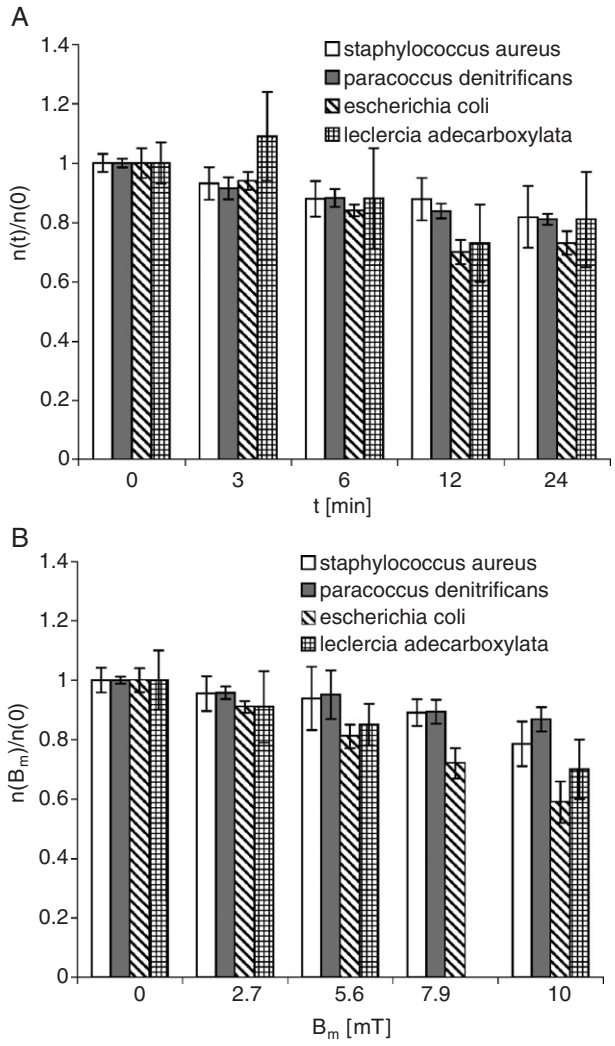

Fig. 5. Comparison of magnetic field effect for four bacterial strains: *E. coli*, *L. adecarboxylata*, *S. aureus* and *P. denitrificans* [17]. (A) Dependence of the relative number of CFU on the duration of exposure for $B_m = 10$ mT. (B) Dependence of the relative number of CFU on the value of the magnetic field induction for $t = 12$ min.

CFU (on the exposure time and magnitude of the magnetic field) showed statistically significant decrease in number of CFU. The bacteria were on solid soil and at the beginning of exposure the number of CFU was the same in both the control and in the exposed sample. CFU counting was performed after 2 days of incubation in the thermostat. After 48 h, all colonies have to be visible. Then we can assume that this decrease of CFU number is caused by bacterial death. This fact is supported by our results achieved with these bacteria and their growth curves—after exposure we observed decreasing number of bacteria in fluid cultivation medium (data not shown).

The counting of CFU number gives us two interesting information: (1) It can be seen from dependence of CFU on the time of exposure, there is some saturation effect. After 3 min of exposure 8.5% of bacteria are dead, after 6 min 12%, after 12 min 16.5%, after 24 min 19% and, finally, after 48 min 21% bacteria are dead. It is clear that the magnetic field does not act in a dose-dependent way, the effect is not linear. This effect was documented for *Staphylococcus aureus* in our previous work [16] as well. (2) The observed decrease of CFU by 19% after 24 min of exposure to magnetic field of 10 mT can be compared with the 21% decrease of bacteria number after magnetic field exposure on electrode and 20% decrease of the reduction peak current for DQ (exposure conditions were the same). There is possible correlation between both effects. The effect detected by electrochemical reduction of DQ is probably caused by the bacterial death. This result corresponds with the results achieved with other bacterial strains. In our previous work, we have accomplished that the magnetic field effect on oxide-reduction activity of the strains used is caused by bacterial death [15].

If data from [16] are used, we are able to compare four bacterial strains and their behavior in magnetic field. These strains are *Escherichia coli* (strain K12 Row, genotype 58–161 *metB1rpsL* $1^{+}F^{def}$P.Fredericq), *Leclercia adecarboxylata* (strain 2177), *S. aureus* (FA 812) and *P. denitrificans*. As it can be seen in Fig. 5, there are some interesting facts. We can assume that the effect of the magnetic field is not only strain dependent, but a morphology effect can be involved as well. Bacteria with rod-like morphology (Gram-negative) are affected by the magnetic fields much more (decrease in CFU number up to 40%) then the spherical bacteria (Gram-positive, decrease in CFU number up to 20%).

The mechanism of acting of EMF on living organisms is not well known yet. There are studies that have observed no magnetic field effect but much more with some sort of effect. Some authors tried to find physical and biological mechanisms of action of electromagnetic fields on living organisms [20,21]. We established that the magnetic field has lethal effect on bacteria *P. denitrificans*, but there was no change in enzymatic activity. We can only assume that EMF does not influence the metabolic activity of bacteria.

Acknowledgements

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