# Potential of the rhizobacterium *Ensifer meliloti* for the phytoremediation of mixed PAH–heavy metal contamination

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**Abstract.** The potential of the rhizobial strain *Ensifer meliloti* P221 as a bacterium capable of degrading phenanthrene in the presence of a heavy metal (nickel) and exhibiting plant-growth promoting activity toward *Sorghum bicolor* under mixed contamination condition was studied. It was shown that nickel at a concentration of 0.1 mM inhibited the microbial growth and phenanthrene degradation by 43 and 34%, respectively. Analysis of the enzymes involved in the phenanthrene degradation by the rhizobium revealed the activity of the phenanthrenequinone reductase (PQR) and protocatechuate 2,3-dioxigenase (2,3-PCD). It was found that nickel at concentrations up to 0.2 mM did not affect significantly the PQR activity, but inhibited it by 30% when the metal concentration increased to 5 mM. Nickel at concentrations up to 5.0 mM did not affect significantly on the activity of 2,3-PCD. *E. meliloti* promoted elongation of sorghum seedling roots by 69% in medium co-contaminated with phenanthrene and nickel. The results obtained assume that the rhizobial strain studied can exhibit its PAH-degrading and plant-growth promoting activities in the environment co-contaminated with nickel and phenanthrene. This microorganism may be promising for bioremediation and phytoremediation of mixed PAH–heavy metal contamination.

## 1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) and heavy metals are hazardous environmental pollutants and are often found simultaneously in polluted sites that are the result of natural (volcanic eruptions, forest fires, etc.) and/or man-made (mining and processing of coal or oil, metallurgy and electronic waste) processes. Interacting with each other and with soil components, PAHs and metals form complexes and have a more harmful effect on the environment than the same pollutants present separately [1-3], and therefore reliable ways to address them are urgently needed.

Recently, the use of the bioremediation potential of plants and their associated microorganisms to cleaning up the environment from organic and inorganic pollutants has

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evolved into a modern and increasingly widely used technology named a phytoremediation. Being environmentally friendly and cost-effective, this biotechnology provides several positive effects associated with improving soil quality thruought decontamination from the pollutants, enrichment with available carbon and/or other macro- and microelements, biomass production (e.g., for biofuel production), as well as maintaining biodiversity of soil microorganisms influenced by plants [3-4]. Moreover, the microbe-assisted phytoremediation is considered a more preferable approach than the sole use of plants [6], because the microorganisms associated with its root zone can affect both bio-mass production and soil cleanup, promoting plant growth through improved plant nutrition, phytohormone production and/or through a change in the bioavailability of pollutant [7, 8].

The physiological and biochemical characteristics of rhizobia (root nodule bacteria), as well as the recent intensification of studies aimed at studying their bioremediation potential, suggest that these bacteria can become successful candidates for bioremediation/phytoremediation of complex soil pollution with PAHs and heavy metals [9]. The production of extracellular polymeric compounds contributes to the protection of rhizobia cells from the toxic effects of metals [10-12], ensuring their resistance to these pollutants [13] and their destructive activity towards PAHs [14-16] and other organic compounds suggests the possibility of using these bacteria for the remediation of complex contaminants. Rhizobia may refer to the so-called "bifunctional bacteria" [17] capable of exhibiting resistance to heavy metals and, at the same time, degrading PAHs, and the evolutionarily established close interactions of rhizobia with plants make them promising for use as inoculants for phytoremediation of contaminated soils.

The example of the joint presence of PAHs and heavy metals is oil pollution. PAHs content in crude oil can vary from 20 to 50%, and the content of nickel as one of the most essential crude oil associated metals can reach 100 mg/l [18]. *Sorghum bicolor* (L.) Moench. plants have previously been shown to be successful for phytoremediation of oil-contaminated soils [19], as well as the resistance of this plant to heavy metals [20] and its potential for mixed pollution [21]. An effective partner of sorghum for the remediation of PAH pollution can be the previously isolated phenanthrene degrading *Ensifer meliloti* strain P221 [16, 22]. The objective of present study was to evaluate the effect of nickel on the growth and degradation of PAH by the rhizobacterium *Ensifer meliloti*, and the effect of this microorganisms on the of sorghum seedlings development under conditions of complex pollution.

## 2 Materials and methods

### 2.1 Microorganism and cultivation conditions

The subject of our study was rhizobacterium *Ensifer meliloti* (former *Sinorhizobium meliloti*) strain P221 (IBPPM383) from the Collection of Rhizosphere Microorganisms, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (http://collection.ibppm.ru).

To estimate the growth and phenanthrene degradative activity of *E. meliloti* P221 bacteria were cultivated in 300 mL Erlenmeyer flasks with 50 mL MSM medium [23] containing sodium succinate (1 g/L) and phenanthrene at the concentration of 0.2 g/L as carbon and energy sources. Bacterial growth was determined turbidimetrically at 440 nm, (Evolution 60, Thermo Scientific, USA). Degradative activity of the microorganism toward phenanthrene was expressed in percent of reduction of the initial phenanthrene concentration.

To define the resistance of *E. meliloti* P221 to nickel ions the maximum tolerant and minimum inhibitory concentrations of the metal (MTC and MIC) in the cultivation medium

were determined. The microorganism was cultivated in test tubes with 5 ml of LB medium supplemented with nickel (in the form of $NiSO_4 \times 7H_2O$) at different concentrations (0, 0.2, 0.5, 1, 2, 3, 4, or 5 mmol/L) at 29°C for 5 days. Bacterial growth was determined turbidimetrically at 440 nm, (Evolution 60, Thermo Scientific, USA), determining the MIC and MTC of nickel.

To assess the impact of nickel on growth and phenanthrene degradation *E. meliloti* P221 was cultivated in 300 mL Erlenmeyer flasks with 50 mL MSM medium [23] containing phenanthrene at the concentration of 0.2 g/L and nickel at concentrations of 0, 0.2, 0.5, 1, 2, 3, 4, or 5 mmol/L. Biomass grown in Petri dishes on LB was washed off with mineral medium was used as inoculum at the initial dose corresponding to $OD_{440} = 0.2$. Cultivation was carried out at the aeration (orbital shaker, 130 rpm) and 29°C for 14 days. To assess the bacterial growth, the optical density of microbial suspension was measured turbidimetrically at 440 nm (Evolution 60, Thermo Scientific, USA).

## 2.2 HPLC analyses of phenanthrene and its metabolites

After cultivation, phenanthrene and its metabolites were extracted from the cultivation liquid with solvents. Phenanthrene was extracted with chloroform (5 mL per 50 mL, three times for 5 min each), extracts were combined, the solvent was evaporated, and the residues were re-dissolved in acetonitrile before analysis. Metabolites were extracted with ethyl acetate (10 ml per 50 ml of culture medium, three times for 5 min each), first from the native culture medium and then after acidifying the culture medium to pH 2 by adding 1 M HCl. Neutral and acid extracts were dried over sodium sulfate and concentrated by solvent evaporation at room temperature.

The residual concentration of phenanthrene and its metabolites were determined by high-performance liquid chromatography (HPLC) on an Agilent Technologies 1220 Infinity II LC chromatograph (Agilent Technology, Waldbronn, Germany) equipped with a 4.6 × 150-mm ZORBAX Eclipse PAH 5-Micron (phenanthrene determination) and 4.6 × 150-mm ZORBAX Eclipse Plus C18 5-Micron (metabolites determination) columns and a 254-nm UV detector. The solvent system was $H_2O$ : acetonitrile, the linear gradient was 40–100% acetonitrile, and the time was 17 min to determine the residual phenanthrene. The solvent system was $H_2O$ (acidified to pH 2.5) : acetonitrile, the linear gradient was 40–100% acetonitrile, and the time was 17 min to determine the metabolites. The PAHs and their metabolites were analyzed by comparing the retention times with those of standard compounds.

## 2.3 Enzyme assay

Bacteria were grown on R2A agar medium containing 0.05 g/L of phenanthrene for 3 days. Biomass was scraped and resuspended with 50 mM sodium phosphate buffer (pH 7.0). The cells were centrifuged (12,000 g, 10 min, Eppendorf Centrifuge 5810), then washed twice with 50 mM Tris-HCl buffer (pH 7.5) and centrifugation. The cell biomass (2.2 g) was resuspended in 50 mM Tris-HCl buffer (pH 7.5) and disrupted by ultrasonic treatment (five times for 20 sec. with 1 min intervals, at 22 kHz) by using Ultrasonic Desintegrator UD-20 (Techpan, Poland). The cell debris was removed by centrifugation at 4°C for 15 min at 14,000 g. The obtained supernatant as a cell-free extract was used as crude enzyme preparation. Protein content in cell-free extracts was determined according to [24].

The activity of bacterial enzymes related to PAH degradation was assayed spectrophotometrically by using an Evolution 60 spectrometer (Thermo Scientific, USA). The activity of NADH-phenanthrenequinone reductase (PQR, EC 1.6.5.5) was measured by monitoring the oxidation of NADH at 340 nm according to [25]. Protocatechuate 2,3-

dioxygenase (2,3-PCD, EC 1.13.11.x) was assayed by observing accumulation of 2-hydroxymuconic semialdehyde at 375 nm [26, 27]. Protocatechuate 3,4-dioxygenase (3,4-PCD, EC 1.13.11.3) activity was determined by monitoring the decrease in absorbance at 290 nm as described by [28]. Protocatechuate 4,5-dioxygenase (4,5-PCD, EC 1.13.11.8) was monitored by an increase in absorbance at 410 nm [29]. The activities of all enzymes are expressed in units (U) defined as μmol substrate oxidized per minute and per mg protein.

The effect of nickel on the activity of enzymes was assessed by introducing nickel ions (at a final concentration of 0.1–5 mM) into the reaction mixtures and measuring the residual enzymatic activity.

### 2.4 Plant inoculation with *E. meliloti* P221

Seeds of *Sorghum bicolor* (L.) Moench were previously calibrated, washed, and sterilized with a mixture of ethanol and hydrogen peroxide (1:1), after that they were washed six times with sterilized tape water. The test tubes with 10 mL of 0.6% agar Ruakura medium [30] containing phenanthrene of 0.05 mg/L, or 0.1 mM of nickel (in the form of $NiSO_4 \times 7H_2O$), or both, or without additives were inoculated with suspension of *E. meliloti* (to final concentration $10^7$ cell per mL). Noninoculated tubes were used as control. Sterilized seeds were put on the surface of the medium in the tubes and incubated at 24°C, 8000 lux, and 14/10 h light/dark periods for 14 days. At the end of the experiment, the plants were removed from the vessels, the length of the root system was measured.

### 2.5 Statistics

All experiments were performed in three biological and analytical replicates. All data obtained were subjected to statistical processing, calculating the mean values, for comparison of which the standard deviation, confidence interval at $P \leq 0.05$ were used. Calculations were carried out in Microsoft Excel 2007.

## 3 Results and discussion

### 3.1 Phenanthrene degradation by *E. meliloti* P221

The degradation of phenanthrene by the rhizobacterium *E. meliloti* strain P221 was studied under conditions of co-metabolism of PAHs with succinic acid (1%) as an available source of carbon and energy and as a component of plant root exudates. The succinate ensured the growth of the microorganism in the medium, but did not inhibit the degradation of PAHs (Fig.1).

The log phase of culture growth reached maximum on the second day, and the maximum degradation of phenanthrene (35%) was observed on seventh day of cultivation. Phenanthrene degradation did not increase with the duration of cultivation. Presumably, byproducts of PAH degradation could inhibit this process.

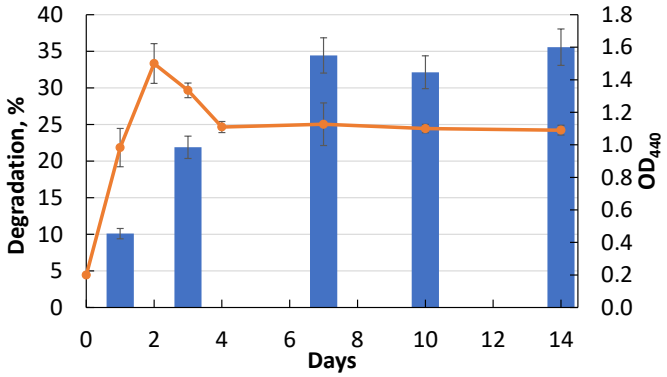


**Fig. 1.** Growth (—) and biodegradation of phenanthrene (▬) by *E. meliloti* P221 in MSM medium.

Among the phenanthrene metabolites formed, the 1-hydroxy-2-naphthoic acid (HNA) was dominant. Figure 2 shows the accumulation of this metabolite in the medium. The maximum content of HNA was observed in 7 days of cultivation, and it did not increase.

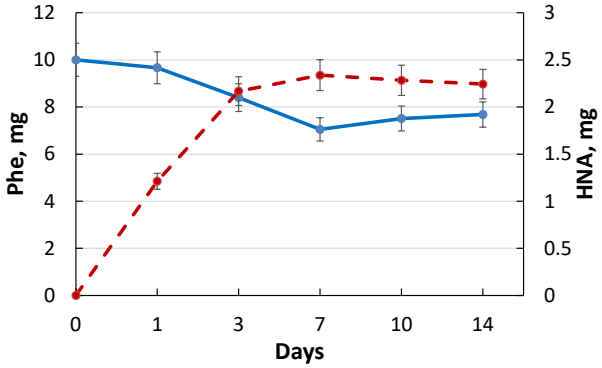


**Fig. 2.** Degradation of phenanthrene (—) and formation of HNA (– –) by *E. meliloti* P221.

Additional experiments have shown that the *E. meliloti* strain P221 does not use HNA for growth, but can degrade it to some extent with the formation of subsequent metabolites [16]. At the same time, the accumulation of this metabolite that is resistant to subsequent degradation in the medium can cause limited degradation of the original PAH.

### 3.2 Nickel resistance of *E. meliloti* P221

A study of the resistance of the *E. meliloti* P221 to nickel showed (Fig. 3) that nickel at a concentration of up to 0.5 mM did not inhibit the growth of the microorganism in the LB medium, while at a concentration of 1.0 mM, bacterial growth was inhibited by 32.5%.

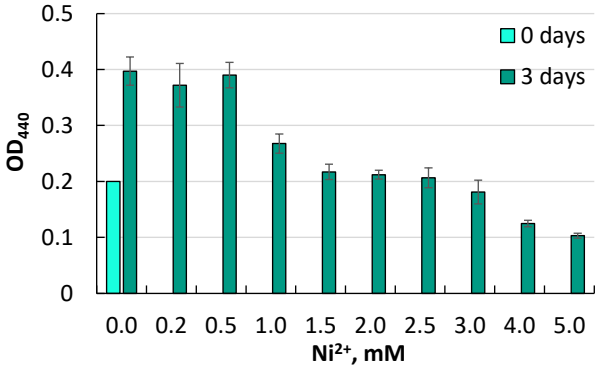


**Fig. 3.** Effect of nickel on growth of *E. meliloti* P221 in LB medium.

Thus, the MIC and MTS values of nickel for the strain studied were determined as 0.5 and 1.0 mMб respectively.

### 3.3 Effect of nickel on phenanthrene degradation by *E. meliloti* P221

Based on the data on the resistance of the *E. meliloti* P221 to nickel, metal concentrations of 0.1, 0.2, 0.5, and 1.0 mM were selected to study the effect of the nickel on the phenanthrene degradation. At the above concentrations nickel inhibited both growth and PAH degradation in different extend (Fig. 4).

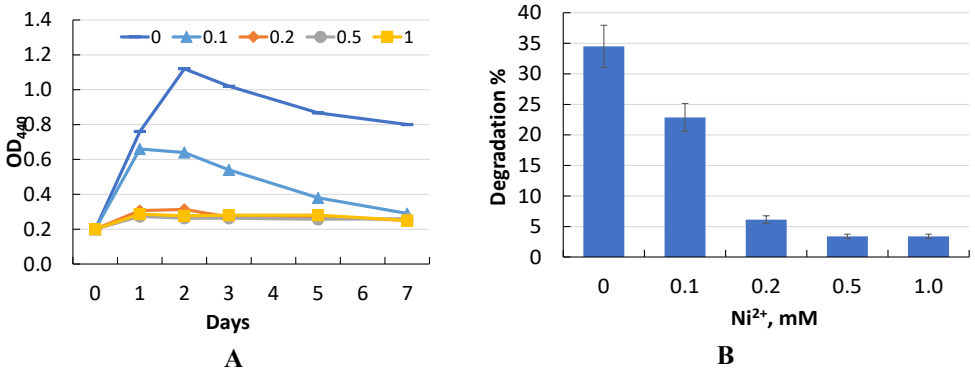


**Fig. 4.** Growth (A) and degradation of phenanthrene (B) by *E. meliloti* P221 at different concentrations of nickel.

At a concentration of 0.1 mM, nickel inhibited the growth of the microorganism by 43%, and the degradation of phenanthrene, by 34%. At a concentration of 0.2 mM and higher, the growth of the microorganism was inhibited by more than 70%, and the phenanthrene degradation by 82% and more. However, in all variants a formation of HNA was observed.

The reduction phenanthrene degradation may be associated with the toxic effect of nickel on enzymes involved both in the growth of the microorganism and directly in PAH degradation [31, 32].

### 3.4 Effect of nickel on the activity of phenanthrene degradation enzymes

A large number of enzymes are involved in the microbial degradation of PAHs at different stages [33]. The enzymes of the "upper" pathways of PAH degradation are characterized by

greater specificity in interaction with the structure of the original compound, and the enzymes of "lower" degradation pathways are involved in the oxidation of less specialized aromatic structures and catalyze the formation of compounds of the TCA cycle. Taking this into account, we searched for the appropriate enzymes in the microorganism studied. The activities of PQR and 2,3-PCD were revealed in cell-free extract of *E. meliloti* P221 (Table 1).

The role of PQRs in PAH degradation is the detoxification of PAH *o*-quinones (formed as a result of autoxidizing of PAH dihydrodiols) that prevent loss of PAH catechols from the PAH degradation [34]. Two bacterial PQRs were purified and characterized from pyrene-degrading *Mycobacterium* sp. strain PYR100 [34]. This enzyme may be considered as enzyme of "upper" pathways of PAH degradation.

In the course of this study the rhizobial PQR was detected for the first time. The activity of this enzyme was predominant in the cell-free extract of *E. meliloti* P221. The results in Table 1 show that the addition of nickel to the reaction mixture had an inhibitory effect on the PQR activity. The lowest activity of the enzyme was found in the presence of 3.5–5.0 mM $Ni^{2+}$, it was reduced by 30% from initial one.

**Table 1.** Effect of nickel on the enzyme activity of *E. meliloti* P221 involved in PAH degradation.

| Nickel concentration, mM | PQR | | 2,3-PCD | |
|---|---|---|---|---|
| | Relative activity, U/mg protein | Residue activity, % | Relative activity, U/mg protein | Residue activity, % |
| 0 | 4.961 ± 0.350 | 100 | 2.20 ± 0.05 | 100.0 |
| 0.1 | 4.912 ± 0.264 | 99.0 | n.a. | n.a. |
| 0.2 | 4.819 ±0.428 | 97.1 | n.a | n.a. |
| 0.3 | 4.598 ±0.189 | 92.7 | n.a | n.a. |
| 0.4 | 4.554 ± 0.052 | 91.8 | n.a | n.a. |
| 0.5 | 4.377 ±0.254 | 88.2 | 2.10 ± 0.03 | 95.7 |
| 1.0 | 4.195 ±0.365 | 84.6 | 2.24 ± 0.32 | 101.8 |
| 1.5 | 4.046 ± 0.054 | 81.6 | 2.00 ± 0.19 | 90.9 |
| 2.0 | 3.923 ±0.305 | 79.1 | 2.03 ± 0.19 | 92.1 |
| 2.5 | 4.362 ±0.189 | 87.9 | 2.28 ± 0.49 | 103.5 |
| 3.0 | 4.117 ± 0.174 | 83.0 | 2.39 ± 0.39 | 108.7 |
| 3.5 | 3.267 ±0.389 | 65.8 | 2.15 ± 0.31 | 97.6 |
| 4.0 | 3.288 ±0.262 | 66.3 | 2.18 ± 0.10 | 99.1 |
| 4.5 | 3.210 ±0.334 | 64.7 | 2.30 ± 0.21 | 104.7 |
| 5.0 | 3.482 ±0.125 | 70.2 | n.a | n.a. |

Note. n.a. – did not analyze

The activity of 2,3-PCD as enzyme of "lower" pathways of PAH degradation was presumably revealed in *E. meliloti* P221 (Table 1). This extradiol dioxygenase catalyses *meta*-cleavage (2,3-dioxygenation) pathway of protocatechuate degradation with formation of 2-hydroxymuconic semialdehyde. According to the data obtained, nickel had no significant effect on the activity of 2,3-PCD. The data obtained may indicate the prospects of using the strain studied under conditions of mixed contamination with PAHs and heavy metals.

### 3.5 Effect of *E. meliloti* P221 on sorghum seedlings

The ability of the *E. meliloti* P221 to stimulate plant growth under conditions of mixed PAH–heavy metal contamination was tested with the 14-day-old seedlings of *S. bicolor*. The results are presented in Table 2.

**Table 2.** Effect of inoculation with *E. meliloti* P221 on the root length sorghum seedlings.

| Treatment | Root length, cm | |
|---|---|---|
| | Noninoculated | Inoculated with *E. meliloti* P221 |
| Without pollutant | 15.3 ± 3.2 | 25.5 ± 7.8* |
| Phenanthrene | 9.7 ± 1.7 | 18.0 ±3,8* |
| $Ni^{2+}$ | 10.4 ± 2.4 | 12.3 ± 4.1 |
| Phenanthrene + $Ni^{2+}$ | 8.8 ± 1.2 | 14.9 ± 2.6* |

Note. * – the differences between noninoculated and inoculated plants are significant at $P < 0.05$

Analysis of sorghum seedlings at the end of the experiment showed that both organic (phenanthrene) and inorganic ($Ni^{2+}$) treatments inhibited the development of sorghum roots. At the same time, a significant stimulating effect of the inoculant on root growth was revealed. *E. meliloti* P221 promoted the growth of sorghum roots in medium without any contaminant (by 67%) and in medium with phenanthrene (by 86%), demonstrating greater activity under PAH contamination conditions. In addition, there was a tendency for stimulation by rhizobacterium of seedling roots grown in the medium with nickel, both as the sole contaminant and jointly with phenanthrene. *E. meliloti* promoted elongation of sorghum seedling roots by 69% in medium co-contaminated with phenanthrene and nickel. The results obtained can serve as a basis for field trials of the *S. bicolor – E. meliloti* association to develop approach to phytoremediation of mixed PAH–heavy metal contamination of soil.

## 4 Conclusions

This study demonstrated the potential of the rhizobia *Ensifer meliloti* strain P221 as a "bifunctional" bacterium capable of degrading PAHs (for example, phenanthrene) in the presence of a heavy metal (for example, nickel) and exhibiting plant-growth promoting activity (toward *Sorghum bicolor*) under mixed contamination condition. It was shown that the degradation of phenanthrene at a concentration of 0.2 g/L by the strain continued for 7 days and was limited by the accumulation of HNA metabolite in the medium. Nickel at a concentration of 0.1 mM inhibited the microbial growth and phenanthrene degradation by 43 and 34%, respectively. A higher dose of nickel in the medium caused a more pronounced reduction in the growth and degradative activity of the bacterium. Analysis of the enzymes involved in the phenanthrene degradation by the rhizobium studied revealed the activity of the PQR and 2,3-PCD enzymes. PQR activity in bacteria of the Rhizobiaceae family has been described for the first time. It was found that nickel at concentrations up to 0.2 mM did not affect significantly the PQR activity, and when the nickel concentration was increased to 5 mM, the PQR activity decreased by 30%. The study of the effect of nickel on 2,3-PCD did not reveal a significant inhibitory effect of the metal on the activity of this enzyme. Based on the results obtained, it can be assumed that the rhizobial strain studied can exhibit its PAH-degrading and plant-growth promoting activities in the environment, where the concentration of nickel will be about 0.1 mM, indicating technogenic pollution. Thus, *Ensifer meliloti* strain P221 may be promising for bioremediation and phytoremediation of mixed PAH–heavy metal contamination.

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