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Article

Nonylphenol biodegradation by *Pseudomonas umsongensis* bacterial strain in aquatic medium and soil microcosm

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Abstract. Environmental pollution with nonylphenols (NPs) is a serious environmental problem due to their persistence, toxicity and ability to have a negative impact on the endocrine system of humans and animals. Biodegradation of NPs is the most environmentally safe and effective way to reduce their content in water and soil ecosystems. A novel bacterial strain, *Pseudomonas umsongensis* 16, capable of degrading NP in aquatic media and soil microcosm, was isolated from soddy-podzolic soil contaminated with NP. The strain 16 exhibits the ability to degrade NP in a wide range of temperatures (from +5 to +35°C), pH (5–9), and xenobiotic concentrations up to 500 mg/l. Bioaugmentation of nonylphenol contaminated soil by cells of *P. umsongensis* 16 leads to an increase in the efficiency of NP destruction in the soil microcosm. The degree of pollutant degradation increases by 1.9 times with a 6.4-fold reduction in the time of its half-removal as compared with the soil microcosm without the introduction of the destructor strain cells. It has been established that as a result of bioaugmentation in soil contaminated with NP, the activity of the soil enzyme urease is restored to the level of uncontaminated soil. The results obtained can be used in the development of biotechnology for the purification of environmental objects contaminated with nonylphenols.

Keywords: endocrine disruptors, water and soil ecosystems, biodegradation, bioaugmentation, enzymatic activity

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Introduction

In recent years, much attention has been paid to the study of the negative impact on ecosystems of xenobiotics that disrupt the human endocrine system, including long-chain alkylphenols AP – octyl- and nonylphenols.

Nonylphenols (NPs) are widely used in the production of lubricating oil additives, resins, pesticides, pharmaceuticals, surfactants and others. NPs enter the environment mainly with wastewater and are found in water, soil and air (Bhandari et al., 2021). The content of NPs in water can reach hundreds of μg/l (Sole et al., 2000), in soil – 1 g/kg (Jiang et al., 2018).

NPs have a toxic effect on aquatic and soil organisms: bacteria, fungi, algae, crustaceans, mollusks, earthworms, fish, plants etc. (de Bruin et al., 2019; Domene et al., 2009; Ivey et al., 2018; Jiang et al., 2019; Kuzikova et al., 2020; Shirdel and Kalbassi, 2016; Spadoto et al., 2017; Zaytseva et al., 2015).

Ecosystems contaminated with NPs are characterized by low species composition, decreased sustainability in general (Kuzikova et al., 2019, 2022; Wang et al., 2015). Due to their bioaccumulative capacity, NPs accumulate in plants, tissues of aquatic and soil organisms (Careghini et al., 2015; Diehl et al., 2012; Gautam et al., 2015; Jiang et al., 2019). Moving along the food chain, NPs enter the human body causing a serious threat to health, as nonylphenols cause allergic diseases, disrupt endocrine functions of the body, have cytotoxic effect on liver cells, have neurotoxic, mutagenic and carcinogenic properties (Acir and Guenther, 2018; Forte et al., 2016; Ismanto et al., 2022; Lofti et al., 2021; Noorimotlagh et al., 2020; Paolella et al., 2021; Suen et al., 2012). For these reasons, a number of EU and US countries have imposed restrictions on the production and use of NPs and regulated their content in environmental objects (Laht and Volkov, 2011; Ribeiro et al., 2015).

In the environment, NPs can be transformed to form less toxic compounds through hydrolysis and photolysis. However, the main reason for the reduction of NP content in natural ecosystems is their biological degradation, mainly microbiological (Bandari et al., 2021). Bacterial and fungal cultures capable of degrading NPs and using them as a source of carbon and energy have been isolated from bottom sediments, soils, and activated sludge from sewage treatment plants (Kuzikova et al., 2020; Ma et al., 2018).

The aim of this study was to isolate from soil and identify a highly active bacterial strain-destructor of NP, to study the processes of NP destruction by the isolated strain in liquid media and soil, and to reveal the influence of the isolated strain on the enzymatic activity of nonylphenol-contaminated soil.

Material and methods

Samples of upper horizons of sod-podzolic loamy soil (Priozersky district, Leningrad Oblast) and technical nonylphenol (NP) produced by Sigma-Aldrich (USA) were used as research objects.

Soil NP degrading bacteria were isolated from nonylphenol contaminated soil (300 mg NP/kg dry soil (d.s.)) and incubated for 90 days using an enrichment culture method in liquid minimum mineral medium (MMM) with the following composition (g/l): $(NH_4)_2SO_4$ – 4.0; KH_2PO_4 – 1.5; K_2HPO_4 – 1.5; $MgSO_4 \times 7H_2O$ – 0.2, containing NP (50 mg/l) as a selective agent. Enrichment cultures were incubated on a Certomat BS-1 rotary shaker (Sartorius Stedim Biotech, Germany) at 230 rpm, 28 °C under dark conditions. Every 4 days, the enrichment culture (10% vol.) was passed into fresh medium of the same composition. Bacterial cultures were isolated after the 5th passage by the Koch method on agarized medium of the following composition (g/l): $(NH_4)_2SO_4$ – 4.0; KH_2PO_4 – 1.5; K_2HPO_4 – $MgSO_4 \times 7H_2O$ – 0.2, agar – 2.0, glucose – 5.0, yeast extract – 2.0, containing 50 mg/l NP. The cultures were incubated at 28 °C for 3 days. Morphologically different bacterial colonies were selected for further study of their ability to degrade NP.

The selected bacterial isolates were incubated in liquid MMM medium containing NP (100 mg/l) on a rotary shaker (230 rpm, 28 °C) in the dark for 7 days, then samples were taken to determine the NP content and to select the most active strains-destructors.

Phenotypic traits of the NP-destructor bacterial strain were identified using generally accepted techniques (Krige and Padgett, 2011).

Sanger sequencing of the 16S rRNA gene sequence fragment (rrs) was used to identify *Pseudomonas umsongensis* strain 16. The primers fD1 (5'-AGAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CTTAAGGAGGAGGTGATCCAGCC-3') were used for amplification of the 16S rRNA gene fragment (about 1500 bp) (Weisburg et al., 1991). The nucleotide sequence of the PCR product was determined on an ABI 3500xl genetic analyzer (Applied Biosystems, USA). Identification of strain 16 was carried out in the Departmental Collection of useful microorganisms for agricultural purposes (VKSM, St. Petersburg).

Strain 16 was cultured in depth on an MMM medium containing NP on a Certomat BS-1 rotary shaker at 230 rpm, 28 °C under dark conditions. Nonylphenol was added to the nutrient medium as ethanol solutions, creating NP concentrations of 50.0–500.0 mg/l. The duration of cultivation was 7 days.

The effect of temperature on the degradation of NP (50 mg/l) was detected under static conditions in the dark in the temperature range of 5–35 °C. The effect of pH on NP degradation (50 mg/l) was studied under dark conditions on a Certomat BS-1 rotary shaker (230 rpm, 28 °C) at initial pH levels of 5.0–9.0. Abiotic controls without bacterial cells were used to evaluate NP loss under abiotic conditions.

In experiments to study the degradation of NP by strain 16 in soil, the pollutant was applied to soil in the form of acetone solutions according to the method described previously (Kuzikova et al., 2019), creating a concentration of 100 mg NP/kg d.s. Nonylphenol-contaminated soils not containing cells of strain 16 were used as control groups: Control 1 – soil samples sterilized by autoclaving at an overpressure of 1 atm for 1 h 20 min, Control 2 – unsterilized soil samples. Incubation of soil samples (control and containing strain 16) was carried out under dark conditions at 24 ± 2 °C for 30 days and 60% soil moisture with periodic stirring.

Suspensions of cells grown at 28 °C for 48 h on agarized MMM medium were used as inoculum in experiments on NP degradation both in liquid medium and in soil. Bacterial suspensions were added to liquid MMM medium and soil containing NP, creating initial cell concentrations of 2 ± 1×10^8 cells/ml and 4 ± 1×10^8 cells/g d.s., respectively.

The NP content in soil samples, culture fluids (cells + medium), and abiotic controls was determined by high-performance liquid chromatography on a Hewlett-Packard H 1090 chromatograph (USA) according to methods presented previously (Kuzikova et al., 2019, 2020).

The kinetics of NP degradation in the fast phase under different conditions of bacterial cultivation was analyzed according to the first-order model. NP loss was estimated by the duration of the period of 50% degradation of pollutant T_{50} and by the value of the first-order reaction rate coefficient k (day^{-1}) calculated using linear regression analysis with the equation

$$C = C_0 \cdot e^{-kt},$$

where k is the first-order kinetic constant of the reaction, C is the NP concentration at time t, mg/l, C_0 is the initial NP concentration. The parameter k was obtained by linear regression between the rate of pollutant loss (C/C_0) and time (t) (Wang et al., 2019).

The urease activity of soil samples was determined colorimetrically on a Genesys 10uv scanning spectrophotometer (Thermo Spectronic, USA) according to the Nessler method (Jayaraman, 2011). Urease activity was expressed in milligrams of $N\text{-}NH_4$ per 1 g d.s. In determining urease activity, soil samples not contaminated with NP and not containing cells of strain 16 were used as controls.

Statistical processing of the data was performed using the Statistica software suite (version 6). Statistical significance was established at $p \leq 0.05$. The obtained data are presented as the mean value ± standart deviation of three independent repetitions of each variant.

Results and discussion

Seventeen bacterial strains with the ability to grow on agarized medium containing NP (50 mg/l) were isolated from enrichment cultures obtained from soil samples incubated with NP for 90 days.

Among the most active NP destructors strain 16 was noted: under dark conditions the degree of pollutant loss (initial concentration of 100 mg NP/l) after 7 days of its cultivation in deep conditions amounted to 77%, while in the abiotic control (without bacterial cells) the NP content in the medium did not decrease.

The cells of strain 16 are Gram-negative non-spore-forming motile bacilli. On the surface of meat-peptone agar, the strain forms rounded milk-colored colonies with a diameter of 2.5–3.5 mm, having a flat, slightly convex profile, even edge, smooth, shiny surface, fine-grained structure, and film-like

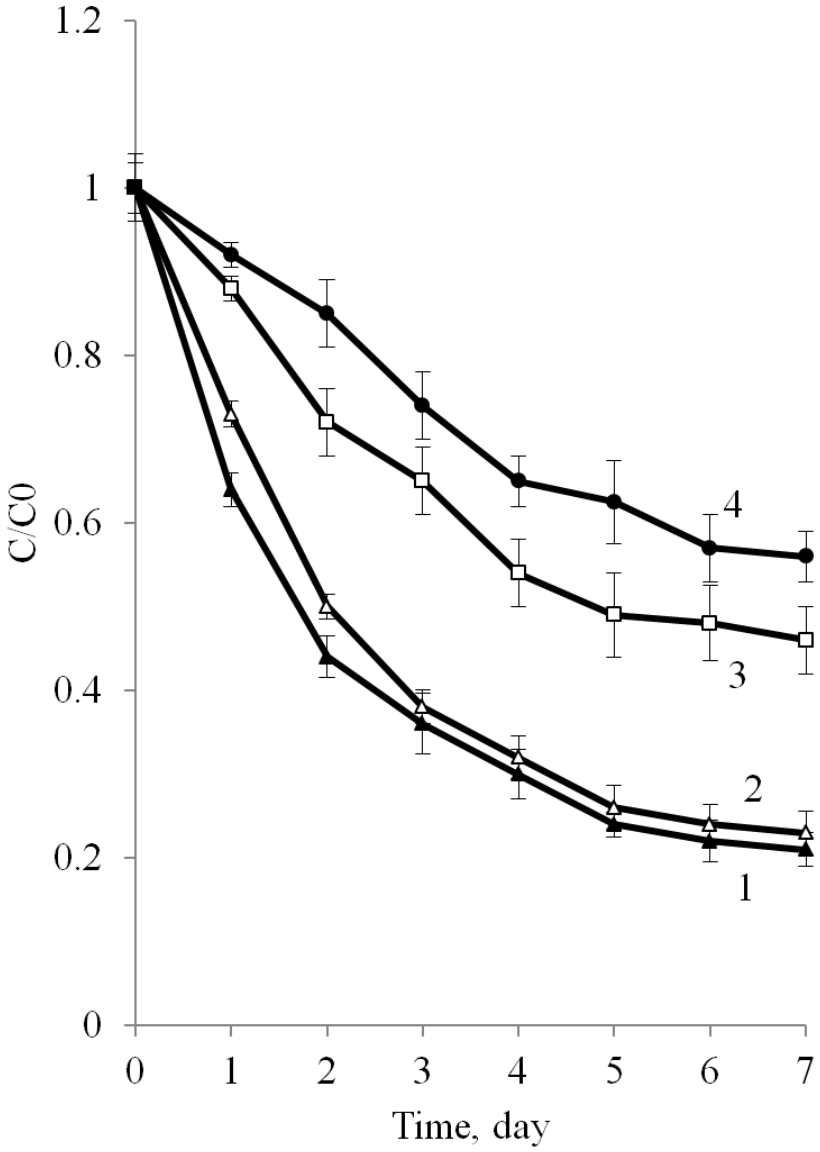

Fig. 1. Dependence of NP content in the medium on time of *P. umsongensis* 16 cultivation. Initial concentrations of NP: 1 – 50 mg/l; 2 – 100 mg/l; 3 – 300 mg/l; 4 – 500 mg/l.

Table 1. Parameters of kinetics of nonylphenol degradation by *P. umsongensis* strain 16 depending on its initial concentration in the medium.

NP concentration, mg/l	Equation of biodegradation kinetics	k, h^{-1}	T_{50}, h	R^2
50	$\ln (C/C_0) = -0.014 \cdot t - 0.056$	0.014 ± 0.001	45.5 ± 3.9	0.975
100	$\ln (C/C_0) = -0.014 \cdot t - 0.001$	0.014 ± 0.003	49.4 ± 4.2	0.996
300	$\ln (C/C_0) = -0.006 \cdot t + 0.009$	0.006 ± 0.001	117.0 ± 11	0.994
500	$\ln (C/C_0) = -0.004 \cdot t + 0.004$	0.004 ± 0.001	174 ± 19	0.955

consistency. Strain 16 is catalase- and oxidase-positive and aerobic. The Voges-Proskauer reaction is negative and it does not form indole. The strain is incapable of urea utilization, assimilates atmospheric nitrogen. It is capable of denitrification, consumes nitrogen of mineral salts, catabolizes galactose, mannose, mannitol, glucose (with acid formation), lactose, rhamnose, arabinose, fructose, dulcite, sorbitol, xylose, inositol, starch. The strain 16 has proteolytic activity against casein, it does not liquefy the gelatin. The strain exhibits amylolytic and lipolytic activity, capable of growth in a wide range of temperatures (+5–+36 °C), with growth retarded at +36 °C. It grows at pH 5–9, with weak growth at pH 5 and no growth at pH 3. It grows well in the presence of 2.5–5% NaCl (very weak growth at 6.5–10% NaCl).

Using the sequencing method of a fragment of the 16S rRNA gene sequence, it was shown that the 16S ribosomal RNA gene of strain 16 exhibits high degrees of identity (> 98%) with similar genes of *Pseudomonas* bacteria – *Pseudomonas putida* strain P13 (100%), *Pseudomonas umsongensis* strain 6A2 (100%), *Pseudomonas putida* strain ATCC 12633T (98.06%), *Pseudomonas umsongensis* strain Ps 3-10T (99.66%) and 100% similarity with the analogous gene of the type strain *Pseudomonas umsongensis* strain Ps 3-10T. Based on the combination of phenotypic features and sequencing results, strain 16 was identified as *Pseudomonas umsongensis* strain 16.

Pseudomonas bacteria are members of the phylum Proteobacteria (class Gammaproteobacteria). It has been previously found that Gammaproteobacteria, along with Alphaproteobacteria, become dominant bacterial groups in microbial communities of soils and sediments during their long-term incubation with NPs (Kuzikova et al., 2019, 2022; Wang et al., 2015). It is known that various strains of genus *Pseudomonas* have a high potential to biodegrade xenobiotics of diverse chemical structure (Burlachenko et al., 2021; Huang et al., 2020; Korshunova et al., 2020; Merkova et al., 2018; Ravi et al., 2018; Shah, 2018; Zaytseva et al, 2020), including nonylphenols (Bai et al., 2017; Ma et al., 2018; Qhanya et al., 2017; Watanabe et al., 2012).

P. umsongensis strain 16 was found to degrade NP in the concentration range of 50–500 mg/l (Fig. 1). The analysis of NP loss curves under the conditions of the conducted experiments allowed us to highlight its biphasic character, similar to the previously described process of alkylphenol degradation by other microorganisms (Kuzikova et al., 2020; Medvedeva et al., 2017). Thus, after 96 h of cultivation (in the fast phase of pollutant loss), the degree of NP biodegradation amounted to 35–70% depending on its initial concentration. Then the biodegradation process slowed down, and during the next 72 h no more than 9% of NP was degraded, regardless of its initial content (Fig. 1). The limitation of NP biodegradation processes at the end of the fast phase may be associated with the formation of metabolites toxic for bacteria (Bai et al., 2017).

When the NP content in the medium was increased from 50 to 100 mg/l, no statistically significant differences in the rate of pollutant loss were found. However, an increase in the NP concentration in the medium from 100 mg/l to 300 mg/l and further to 500 mg/l resulted in a significant slowdown of its biodegradation, which was reflected in a statistically significant ($p < 0.05$) increase (2.4 and 3.5 times, respectively) in its 50% removal time and a decrease in the degradation rate coefficient k (Fig. 1, Table 1).

The bacterium we isolated showed higher NP destructive properties compared to some other NP-destructive strains, such as *P. putida* SLY10 bacterium destroying NP at a much lower concentration: 20 mg/l by 50.2% in 72 h (Ma et al, 2018), bacterium *Pseudomonas* sp. destructing NP at concentrations of 50 and 100 mg/l by 60% and 30% in 5 days, respectively (Watanabe et al., 2012). At the same time, the bacterium *P. umsongensis* 16 was less active compared to strains *P. nitroreducens* LBQSKN1, *P. putida* LBQSKN2, *Pseudomonas* sp. LBQSKN5, *Pseudomonas* sp. LBQSKN6, which carried out 41–46% degradation of NP at a concentration of 2.5 mM (550 mg/l) within 12 h (Qhanya et al., 2017).

Among abiotic factors affecting xenobiotic biodegradation processes in aquatic and soil ecosystems, temperature and acidity (pH) are among the most important (Bacosa et al., 2022, Kanwal et al., 2022). Temperature has significant effects on chemical and biochemical reactions, microbial growth and survival, taxonomic composition, enzyme activity, metabolic activity, physiology and diversity of bacterial communities of different ecosystems responsible for degradation of pollutants (Kebede et al., 2021; Pischedda et al., 2019). The pH of the medium affects the physiological properties of microorganisms, the membrane transport, the balance of catalytic reactions in cells, the enzymatic activity of microorganisms and is essential for the biological degradation processes of organic pollutants (Bacosa et al., 2022).

P. umsongensis strain 16 demonstrated the ability to degrade NPs over a wide temperature range: from 5 °C to 35 °C (Fig. 2, Table 2).

It should be noted that in the temperature range of 15–28 °C, no significant differences in the rate of NP biodegradation by strain 16 were found ($p > 0.05$). The degree of nonylphenol degradation in this

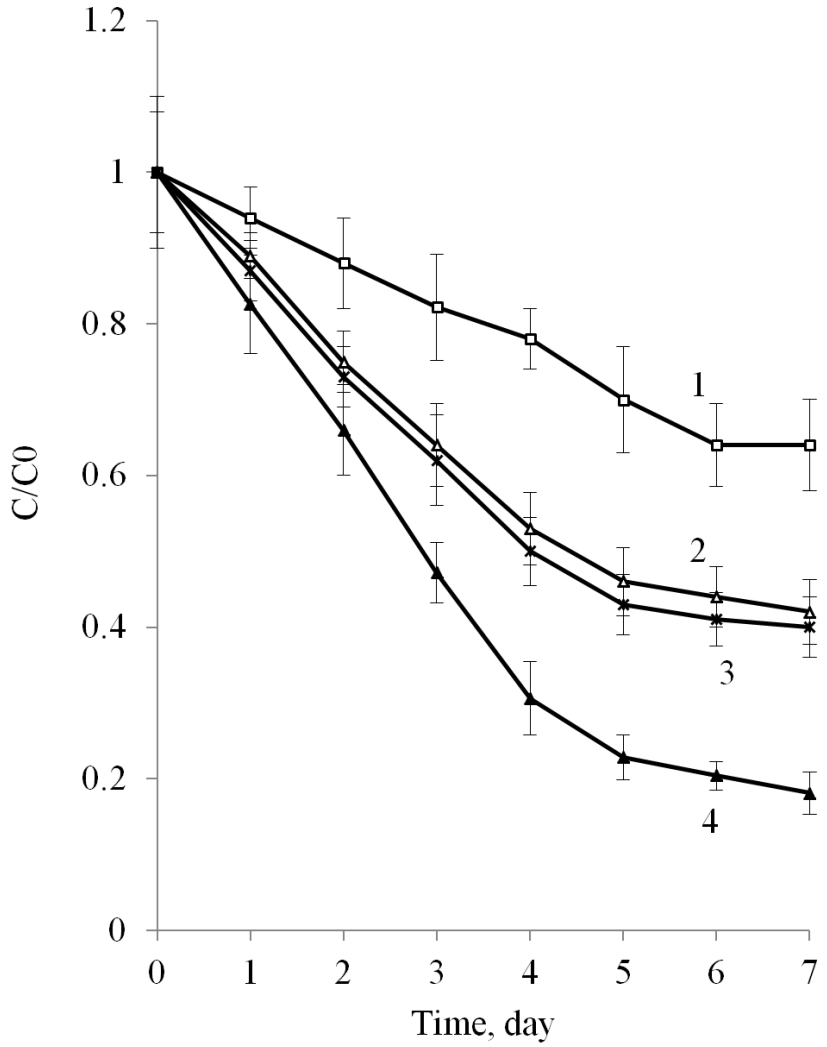

Fig. 2. Dependence of NP content on temperature and time of *P. umsongensis* 16 cultivation 16: 1 – 5 °C, 2 – 15 °C, 3 – 28 °C, 4 – 35 °C.

Table 2. Parameters of kinetics of nonylphenol degradation by *P. umsongensis* strain 16 depending on temperature and initial pH level.

Parameter		Equation of degradation kinetics	k, h^{-1}	T_{50}, h	R^2
Parameter, °C	5	$\ln (C/C_0) = -0.003 \cdot t$	0.003 ± 0.001	>168	0.995
	15	$\ln (C/C_0) = -0.007 \cdot t + 0.024$	0.007 ± 0.002	102.4 ± 9.1	0.997
	28	$\ln (C/C_0) = -0.007 \cdot t + 0.021$	0.007 ± 0.001	102± 7	0.994
	35	$\ln (C/C_0) = -0.012 \cdot t + 0.077$	0.012 ± 0.001	64.2 ± 6	0.974
pH	5.0	$\ln (C/C_0) = -0.015 \cdot t - 0.021$	0.015 ± 0.002	44.8 ± 3.4	0.998
	7.0	$\ln (C/C_0) = -0.014 \cdot t - 0.058$	0.014 ± 0.001	45.4 ± 4.1	0.971
	9.0	$\ln (C/C_0) = -0.023 \cdot t - 0.087$	0.023 ± 0.003	26.3 ± 1.9	0.972

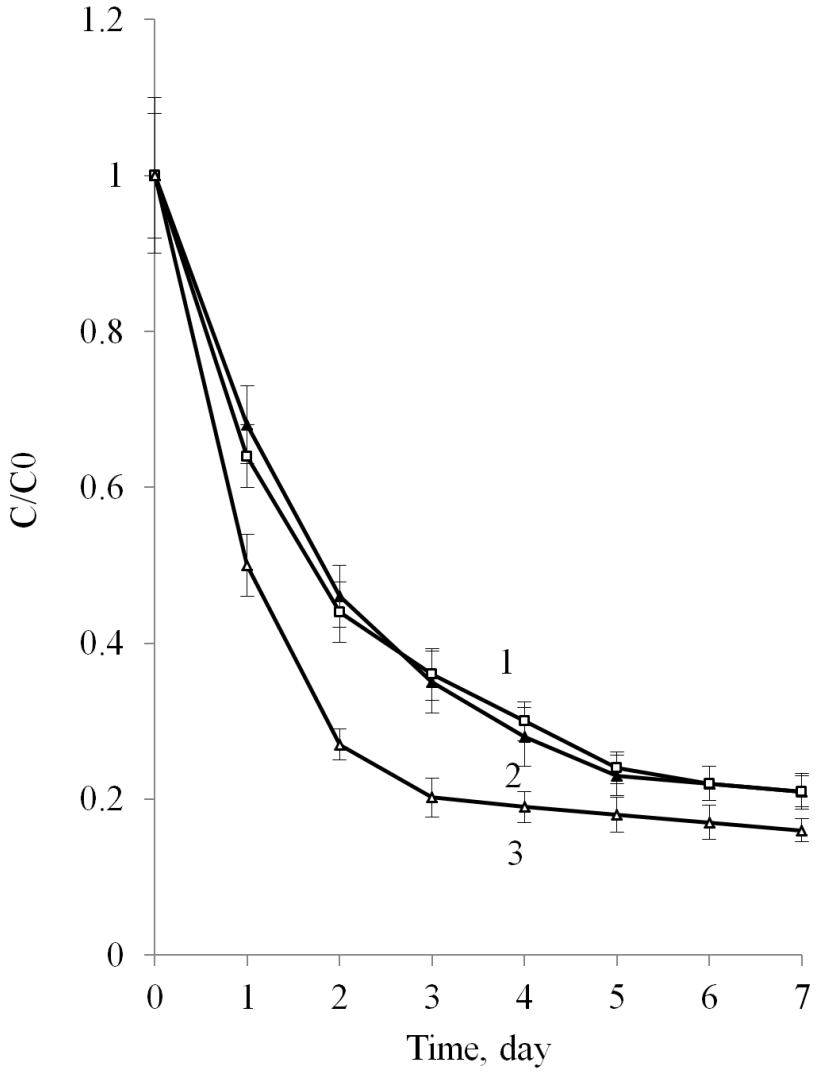

Fig. 3. Dependence of NP content on initial pH of the medium and time of *P. umsongensis* 16 cultivation: 1 – pH 5, 2 – pH 7, 3 – pH 9.

temperature range after 7 days of cultivation of *P. umsongensis* bacteria was 59 ± 1%. At lowering the temperature from +15 °C to +5 °C the process of NP degradation slowed down that was expressed in a 2.3-fold decrease in the value of coefficient k and in an increase in the period T_{50} more than 1.6 times. The degree of xenobiotic degradation after 7 days at +5 °C amounted to 36%. An increase in the cultivation temperature from +28 °C to +35 °C led to acceleration of bacterial destruction of xenobiotic: statistically significant ($p < 0.05$) 1.6-fold increase in the k coefficient, decreased duration of the period of 50% degradation of pollutant, the degree of its destruction after 7 days increased to 82%.

Strain 16 exhibits the ability to degrade NP over a wide pH range: from 5 to 9 (Fig. 3). Our results show that the degradation of nonylphenol by the bacterium *P. umsongensis* 16 occurs more efficiently under alkaline environmental conditions: at pH 9.0, the duration of T_{50} was 26.3 h, and the value of the degradation rate coefficient k was 0.023 h^{-1}. When the pH level decreased from 9.0 to 7.0 and further to 5.0, the duration of NP half-degradation period increased by 1.7 times, and the k coefficient decreased by 1.6 times. It should be noted that there were no statistically significant differences in the NP degradation rate at pH 5 and 7 ($p > 0.05$) (Fig. 3, Table 2).

The influence of acidity level and/or temperature on nonylphenol degradation by *Pseudomonas* sp. bacteria was also shown by other authors. Thus, it was detected that the optimal temperature for nonylphenol degradation by bacteria *P. putida* and *Pseudomonas* sp. was +30 °C (Watanabe et al., 2012; Xie et al., 2015). The highest degree of NP degradation by the bacterium *P. putida* was recorded at pH

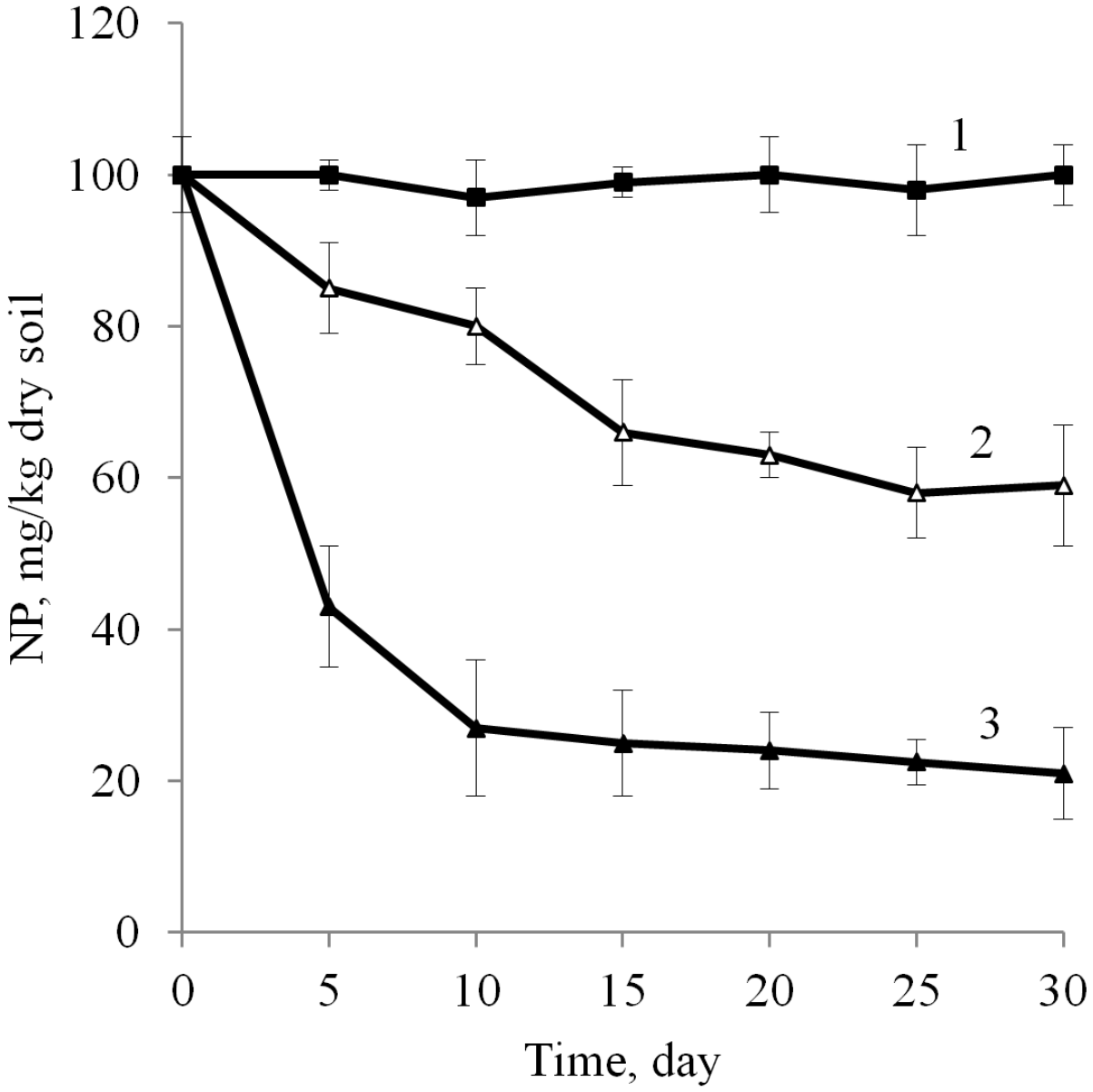

Fig. 4. NP content in soil: 1 – sterile soil; 2 – unsterilized soil; 3 – unsterilized soil containing *P. umsongensis* 16 cells.

Table 3. Parameters of nonylphenol degradation kinetics in soil microcosm.

Soil variant	Equation of degradation kinetics	k, day^{-1}	T_{50}, day	R^2
Control (without *P. umsongensis* 16 cells)	$\ln (C/C_0) = -0.022 \cdot t - 0.018$	0.022 ± 0.005	30.7 ± 2.6	0.936
Treated with *P. umsongensis* 16 cells	$\ln (C/C_0) = -0.13 \cdot t - 0.063$	0.13 ± 0.02	4. 8± 1.1	0.972

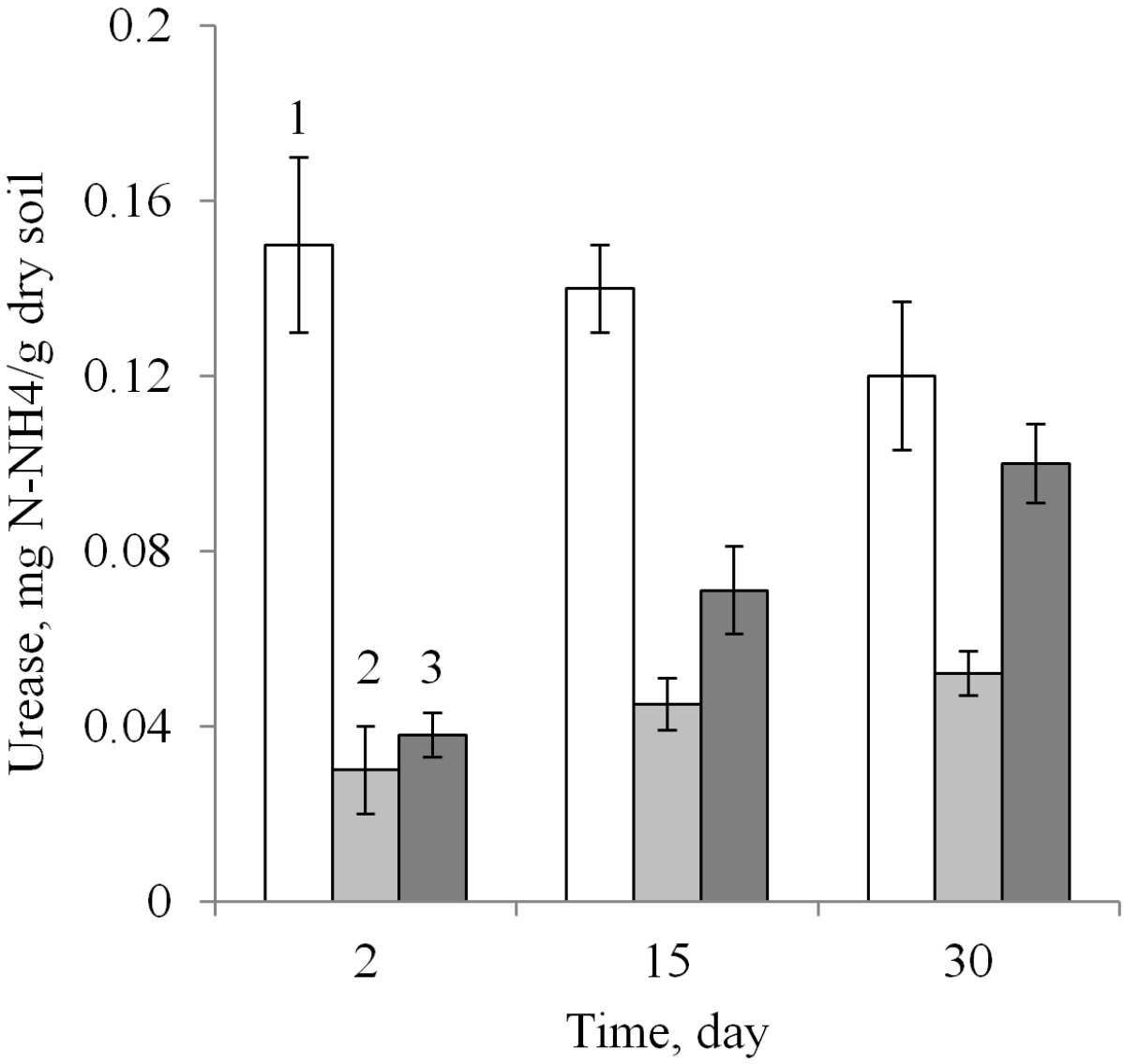

Fig. 5. Effect of nonylphenol (initial concentration of 100 mg/kg d.s.) and *P. umsongensis* 16 on urease activity: 1 – control soil not contaminated with NP and not containing *P. umsongensis* 16 cells, 2 – NP contaminated soil; 3 – NP contaminated soil containing *P. umsongensis* 16 cells.

in the range of 5–7 (Xie et al., 2015). Both an increase and a decrease in temperature and pH relative to the identified optimal levels cause a decrease in the extent of NP degradation, which is associated with an increasing of the activity of enzymes involved in NP degradation processes (Surkatti and El-Naas, 2017).

The ability of our isolated strain to biodegrade NP in wide ranges of temperature (from +5 °C to +35 °C) and pH (5–9) is of interest for its use in the development of biotechnology for the remediation of environmental objects contaminated with this pollutant.

P. umsongensis strain 16 is able to degrade NP not only in the aquatic environment but also in soil microcosms. The 42% decrease in NP content from the initial amount was observed in non-sterilized soil samples contaminated with nonylphenol (Control 2) after 30 days of incubation. It should be noted that in sterile soil samples (Control 1) there is no loss of pollutant, which indicates the biological nature of the NP degradation process by autochthonous microbiota (Fig. 4).

Under bioaugmentation conditions – treatment of nonylphenol-contaminated soil with *P. umsongensis* cells – a significant increase in the degree of NP degradation was observed –from 42% in the control

variant to 79%. The coefficient of degradation rate k increased 5.6 times, the duration of the half-destruction period decreased 6.4 times (Fig. 4, Table 3).

Soil contamination with nonylphenol is known to cause time- and dose-dependent changes in the activity of the pool of enzymes of the nitrogen (urease and protease) and carbohydrate (amylase and cellulase) cycles and redox enzymes (dehydrogenase and catalase) (Zaytseva et al., 2020). Within the framework of this study, the effect of NP and strain 16 on the activity of the soil enzyme urease, the level of which is often considered as an indicator of the health of ecosystem microbial communities was investigated (Sebiomo et al., 2017).

It was previously noted that contamination of sod-podzolic soil with nonylphenol leads to significant inhibition of soil urease activity (Zaytseva et al., 2020). In this work, it was shown that in soil variants both treated and untreated with *P. umsongensis* 16 cells after 2 days of incubation the level of enzyme activity decreased by 75–80% compared to uncontaminated soil (Fig. 5).

Upon further incubation in the soil sample containing cells of strain 16, urease activity was restored, and after 30 days, no statistically significant differences in the levels of this index were found compared to the control sample containing neither NP nor cells of strain 16 ($p > 0.05$). In the sample without *P. umsongensis* 16 cells, the recovery of urease activity over the same period of time was much slower: the level of enzyme activity in this variant was 2.3 times lower than in the control variant (Fig. 5)

Conclusion

Bacterial strain 16 capable of NP biodegradation was isolated from soil contaminated with nonylphenol. Based on phenotypic characteristics and sequencing data of the 16S rRNA gene sequence fragment, strain 16 was identified as *Pseudomonas umsongensis* 16. Its high efficiency in NP biodegradation under aerobic conditions at xenobiotic concentrations up to 500 mg/l in a wide range of temperatures (+5...+35 °C) and pH (5–9) was shown. The isolated strain is able to degrade NP in both the aquatic environment and soil microcosm. Bioaugmentation of nonylphenol-contaminated soil with *P. umsongensis* 16 cells results in increased efficiency of NP degradation and significantly decreased half-life of pollutants compared to soil microcosm without application of *P. umsongensis* 16 cells. It is shown that NP soil contamination leads to a decrease in the activity of the most important soil enzyme of the nitrogen cycle – urease. Upon augmentation with *P. umsongensis* 16 cells, a recovery of urease activity to the level of uncontaminated soil was observed. The results obtained can be used in the development of biotechnologies for remediation of ecosystems polluted with alkylphenols.

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