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The Bioremediation Potential of Native Microorganisms of the Southern Chernozem

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Abstract—In the course of this study, the main groups of soil microorganisms in the southern chernozem were identified. The resistance of isolates to the action of oil in the concentration range of 15–25%, the possibility of using it as a carbon source, the ability of soil microbiota to biodegrade oil in contaminated soil, and the resistance of bacteria to low temperatures, high NaCl concentrations, with acid and alkali resistance were established. Fifteen genera (31 species) of heterotrophic bacteria were isolated from uncontaminated soil samples of the southern chernozem subtype. Our assessment of the abundance dynamics of microorganisms isolated from laboratory contaminated soils showed that as a result of oil exposure there was a significant decrease in the numbers of microorganisms: by the 180th day of our experiment, ten bacteria species belonging to three genera had been isolated, namely *Bacillus*, *Micrococcus*, and *Serratia*. Among the bacteria isolated, resistance to the action of the pollutant at a concentration of 25% was established for *B. coagulans*, *B. mojavensis*, *B. megaterium*, and *M. luteus*, as well as for the museum strain of *B. pumillus* KM. By cultivating the studied bacterial strains on the carbon-free medium M9 with 15 and 20% oil added, their ability to use petroleum hydrocarbons as the sole carbon source was established; however, when the concentration increased to 25%, only *M. luteus*, *B. mojavensis*, and *B. pumillus* KM retained this ability. The presence of hydrocarbon-oxidizing bacteria in soil samples contributed to the 42% decrease in the oil mass concentration in 180 days. The most significant decrease in the concentration of petroleum products occurred in the period from the 10th to the 30th day and amounted to 25%, which is probably due to the increase in the numbers of heterotrophic bacteria. The ability to grow at a temperature of 4°C was established for representatives of the genus *Bacillus*, including the museum strain of *B. pumillus* KM; four strains of bacilli remained viable in an acidic environment (pH 5), seven strains of bacilli and *M. luteus* and *S. plymuthica* remained viable in an alkaline environment (pH 9). The bacterial strains studied were grown on a GRM-agar with an NaCl concentration of 7%; the ability to grow at an NaCl concentration of 15% was preserved only by the museum strain of *B. pumillus* KM. The obtained results open the prospects for the use of hydrocarbon-oxidizing bacteria with a high adaptive potential as potential oil destructors capable of biodegradation at low temperatures, in conditions of high salinity, and in a wide range of pH of the medium.

Keywords: oil, native bacterial strains, bacterial adaptation, biodestruction
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INTRODUCTION

Oil and oil products belong to the group of priority pollutants, the large-scale release of which into the environment contributes to the growth of environmental instability throughout the world (Barabanshchikov and Serdyukova, 2016; Elinskii et al., 2020). As regards the chemical composition, oil is a mixture of complex hydrocarbons containing a wide range of impurities, which are characterized by a cumulative ability, having a toxic effect on living organisms, and are capable of causing mutations and stimulating carcinogenesis (Orudzhev and Jafarova, 2017). Oil pollution most strongly affects the soil into which hydrocarbons enter during production, transportation, and various technological processing schemes. Penetrating into the soil, oil is distributed along its profile, leading to a change in the agrochemical properties and disruption of the respiratory and enzymatic functions (Galitskaya et al., 2021; Melekhina et al., 2021).

The soil microbiota shows variable sensitivity to the action of pollutants, which is not the same for different physiological groups of microorganisms, and also depends on the composition of oil products and the duration of exposure (Alimbetova et al., 2016; Polyak et al., 2018). On the one hand, oil components can have a toxic effect on microorganisms and, on the other hand, they can act as a source of carbon nutrition. The level of toxic effects depends on the predominant fractions of the oil product and decreases in the series aromatic hydrocarbons > cycloparaffins > olefins > paraffins (Novoselova et al., 2014). The presence of oil hydrocarbons in the soil leads to an increase in the amount of nitrogen fixers, ammonifiers, and denitrifiers and a decrease in the numbers of actinomycetes and cellulose-decomposing bacteria (Kirienko and Imranova, 2015; Mikhailova et al., 2016; Alotaibi et al., 2018). A special group is made up of hydrocarbon-oxidizing bacteria (HOBs), for which oil products have a stimulating effect, leading to an increase in their abundance and long-term persistence in contaminated areas (Gogoleva and Nemtseva, 2012; Mikolasch et al., 2019). HOBs are isolated from soils with chronic oil pollution and are predominantly represented by saprophytes belonging to the genera *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Micrococcus*, *Mycobacterium*, *Pseudomonas*, etc. They play a decisive role in the processes of biodegradation of petroleum products in the soil (Shapiro et al., 2018; Korshunova et al., 2019; Safarov et al., 2019; Al-Hawash et al., 2018; Puustinen et al., 2020).

The elimination of oil pollution can be carried out in various ways; however, the use of biotechnological methods is the most effective and economically advantageous (GOST R 57447-2017; Frank et al., 2020; Plekhanova and Kholkin, 2021). To perform remediation measures, preference has recently been given to native HOB strains, for the activation of which two approaches are used: in situ biostimulation and in vitro biostimulation followed by reintroduction (Thapa et al., 2012). In the first case, optimal conditions for HOBs are created directly at the site of contamination by introducing the necessary macro- and microelements, aeration, etc.; in the second case, they are biostimulated in the laboratory with subsequent introduction to the contaminated areas. For most HOBs, the optimal conditions for growth and reproduction are a temperature of 28°C, pH values close to neutral (6.7–7.4), and NaCl content in the medium of up to 2%. However, under environmental conditions, these indicators differ significantly from optimal ones. Therefore, in the case of manmade accidents that occur during oil production, the efficiency of natural bioremediation can be quite low.

The goal of this work is to study the adaptive abilities of oil degrading microorganisms to the action of physical and chemical environmental factors.

MATERIALS AND METHODS

Research was carried out in the period from 2016 to 2021. The object of this study was the soil of the southern chernozem subtype, which is widespread on the right bank of Saratov oblast, where it accounts for more than 10% of the area (Boldyrev, 1997). Soil samples were taken in sterile containers according to the recommendations and delivered to the laboratory during the day (GOST 17.4.3.01-2017, GOST 17.4.4.02-2017). The oil of the Sokolovogorskii oil field (Volga–Ural oil basin, Saratov, Russia) was used as the pollutant. The oil of this field is light, low-viscous, low-sulfureous (class I), low-resinous, the yield of light fractions is high, paraffin (type P2) (Trebin et al., 1980).

Prepared weighed soil samples of 500 g were placed in containers. Oil in an amount of 10% of the soil mass was introduced into the experimental sample, and the mass fraction of oil products in the soil extract was measured. The soil without the addition of the pollutant was used as the control. Soil samples were placed in a KS-200 climatostat (SKTB, Russia) at a temperature of 20°C. Humidity was maintained at a constant level, watering samples with deionized water.

To identify heterotrophic and hydrocarbon-oxidizing microorganisms and study their resistance to the action of the pollutant, the corresponding nutritional media were used: the GRM-Agar (Federal Budgetary Institution of Science, State Scientific Center for Applied Microbiology and Biotechnology, Russia) and the GRM-Agar and the M9 medium with the addition of 15, 20, and 25% of oil. The quantitative indicators of the soil microorganisms were determined using the methods of sequential cultivation and surface inoculation of dense nutrient media (*Praktikum po mikrobiologii*, 2005). Incubation was carried out in a thermostat at a temperature of 28°C for 1–3 days, and then the quantitative accounting of the grown colonies was carried out. Inoculation was carried out on the 1st, 10th, 30th, 60th, and 180th days from the moment of introducing the pollutant into the soil.

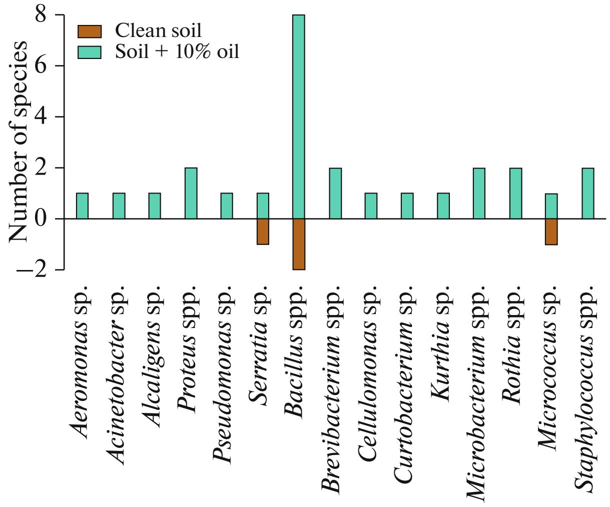

Fig. 1. Number of bacterial species isolated from soil samples of the southern chernozem.

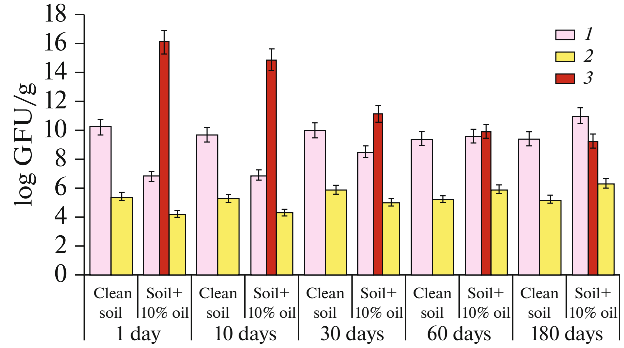

Fig. 2. Abundance dynamics of soil microorganisms depending on the oil concentration in the soil: *1*, heterotrophic bacteria; *2*, hydrocarbon–oxidizing bacteria; *3*, mass fraction of petroleum products.

The identification of isolated strains of microorganisms was carried out on the basis of studying phenotypic properties according to the Bergey determinant of bacteria (*Bergey's Manual*, 2007).

The determination of the mass fraction of oil products in soil samples was carried out according to the standard method using IR spectrometry (PND F 14.1.272-2012). The measurements were carried out immediately after the introduction of the pollutant into the soil and 10, 30, 60, and 180 days after the pollution.

The study of adaptive abilities was carried out in relation to the isolated HOBs as well as the museum strain of *B. pumillus* KM from the collection of the Department of Microbiology and Plant Physiology, Chernyshevskii Saratov State University, for which the previously conducted studies made it possible to establish hydrocarbon-oxidizing activity (Uspanova et al., 2020).

The ability of microorganisms to adapt to oil contamination was studied by inoculation of the GRM-agar containing 15, 20, and 25% of oil. The ability of microorganisms to use oil hydrocarbons as a single carbon source was determined on the carbon-free medium M9, to which 15, 20, and 25% of the pollutant were added.

To identify the resistance of bacteria to low temperatures, cultivation was performed at 4°C. Salt resistance was determined on the GRM-agar with a NaCl concentration of 7, 10, and 15%, and acid resistance and alkali resistance were determined on the GRM-agar (pH 5 and 9).

Statistical processing of the material included methods of descriptive statistics. The correlation of variation of the abundance of bacteria with the mass fraction of oil in the soil was analyzed using the Pearson correlation coefficient. The correlation was considered significant at $p \leq 0.05$. Statistical data processing was performed in MS Excel 2000 programs (Microsoft Corp.) and Statistica 6.0 (StatSoft Inc., OK, United States).

RESULTS AND DISCUSSION

To select native strains that are resistant to oil and capable of its biodegradation, it was of interest to evaluate the species composition of heterotrophic bacteria in the control and experimental soil samples. During the microbiological examination of the clean soil of the southern chernozem subtype, 15 genera of heterotrophic bacteria were isolated, which were attributed to 31 species. The species variety of heterotrophic bacteria in the oil-contaminated soil decreased by 68% by the 180th day of the experiment and amounted to ten species of bacteria belonging to three genera (Fig. 1).

Most of the isolated bacteria (eight species) belonged to the genus *Bacillus*. According to the published data, representatives of this species are characterized by a high level of resistance to various adverse environmental factors due to the capability of spore formation. In addition, representatives of the *Bacillus* genus have a wide set of enzyme systems that allow them to utilize a wide range of organic substrates, involving them in their metabolic processes (Korshunova et al., 2019; Fagbemi and Sanusi, 2017). The studied samples of oil-contaminated soil were used to isolate one species of both the *Micrococcus* and *Serratia* genera. The results are consistent with the published data, according to which the bacteria of these genera are some of the most resistant to oil (Fedorova et al., 2009; Balandina and Eremchenko, 2018; Obayori and Salam, 2010; Rajasekar et al., 2011).

Table 1. Ability of heterotrophic bacteria to reproduce on the GRM-agar with different oil concentrations

The studied bacterial strains	The oil concentration in the medium, %		
	15	20	25
Bacillus circulans	–	–	–
B. coagulans	+	+	+
B. halmapalus	+	+	–
B. megaterium	+	+	+
B. mojavensis	+	+	+
B. niacini	+	+	–
B. pumilus	+	+	–
B. simplex	–	–	–
Micrococcus luteus	+	+	+
Serratia plymuthica	+	+	–
B. pumilus KM	+	+	+

+, there is visible growth; dash, no visible growth.

The study of the abundance dynamics of heterotrophic bacteria and HOBs in the control and experimental samples of the southern chernozem soil was carried out on the 1st, 10th, 30th, 60th, and 180th days. In parallel, the effectiveness of oil degradation by aboriginal strains was assessed by a change in the content of the pollutant in the experimental sample (Fig. 2).

On the 1st day of the laboratory experiment, a general decrease in the abundance indicators of the studied groups of bacteria was revealed. Thus, the numbers of heterotrophic bacteria decreased by 33%, and the numbers of HOBs decreased by 22% compared to the control sample. The decrease in the numbers of native microorganisms is probably due to the inability of the soil microbiota to undergo rapid adaptation in the presence of the pollutant in the soil (Zakaria et al., 2021).

On the 10th and 30th days after the introduction of oil into the soil, the abundance of the studied groups of microorganisms was also lower than the control values.

On the 60th day, there was a gradual restoration of the quantitative indicators of the soil microbiota. The numbers of heterotrophic bacteria did not differ significantly from similar values in the control soil sample. The numbers of HOBs in the oil-contaminated soil was 13% higher than the control. The results are consistent with the data of other authors that oil has a stimulating effect on the growth of microorganisms and the biological activity of the soil, since it acts as an available organic substrate (Gennadiev et al., 2015).

At the final stage of the experiment (180th day), a further increase in the quantitative indicators of soil microflora took place: the numbers of heterotrophic bacteria and HOBs in the oil-contaminated soil was 17 and 22% higher compared with the control samples. High indicators of the numbers of heterotrophic bacteria are most likely associated with their ability to use oil hydrocarbons as a source of carbon nutrition.

Using correlation analysis, based on the calculation of the Pearson correlation coefficient, the average coefficients of positive linear correlation were found between the numbers of heterotrophic and hydrocarbon-oxidizing microorganisms in the control soil sample ($r = -0.64$, $p = 0.048$). In the oil-contaminated soil sample, there was a very high coefficient of positive linear correlation between the studied groups of soil microorganisms ($r = 0.99$, $p = 0.035$).

The effectiveness of oil biodegradation by aboriginal strains of microorganisms of the southern chernozem soil was assessed by a change in the content of the pollutant in the experimental sample. The initial concentration of oil in contaminated soil was 14.3 g/kg. The mass fraction of the oil product was observed to decrease by 7% on the 10th day of the experiment, by 30% on the 30th day, by 38% on the 60th day, and by 42% on the 180th day compared to the initial values. The maximum decrease in the mass fraction of oil products was observed from the 10th to the 30th day (25%), which is probably due to a sharp increase in the numbers of heterotrophic bacteria during this period. The statistical analysis showed a very high coefficient of negative linear correlation between the numbers of soil heterotrophic and hydrocarbon-oxidizing bacteria and the mass fraction of oil in the soil; for both groups of microorganisms, the Pearson coefficient was $r = -0.95$ at $p = 0.041$, which indicates a significant contribution of the hydrocarbon-oxidizing bacteria of the southern chernozem to the process of degradation of the pollutant.

Table 2. Ability of heterotrophic bacteria to utilize oil as the only carbon source on the M9 medium

The bacterial strains studied	The oil concentration in the medium, %		
	15	20	25
Bacillus circulans	–	–	–
B. coagulans	+	–	–
B. halmapalus	–	–	–
B. megaterium	+	–	–
B. mojavensis	+	+	+
B. niacini	–	–	–
B. pumilus	+	–	–
B. simplex	–	–	–
Micrococcus luteus	+	+	+
Serratia plymuthica	–	–	–
B. pumilus KM	+	+	+

+, there is visible growth; dash, no visible growth.

Table 3. Survival rates of heterotrophic bacteria under the influence of negative physicochemical factors

The strain studied	Growth			Growth on the GRM-agar with NaCl		
	at 44°C	at pH 5	at pH 9	7%	10%	15%
Bacillus circulans	+	–	+	+	+	–
B. coagulans	–	+	+	+	–	–
B. halmapalus	–	–	–	+	+	–
B. megaterium	+	–	+	+	+	–
B. mojavensis	+	+	+	+	+	–
B. niacini	–	–	–	+	–	–
B. pumilus	+	–	+	+	+	–
B. simplex	+	–	+	+	+	–
Micrococcus luteus	–	+	+	+	–	–
Serratia plymuthica	–	–	+	+	–	–
B. pumilus KM	+	+	+	+	+	+

+, there is a visible growth; dash, no visible growth.

At the next stage of the work, it was of interest to study the range of resistance of isolated heterotrophic bacteria, as well as the museum strain of *B. pumillus* KM to the action of higher oil concentrations in the medium. It was found that *B. halmapalus*, *B. mojavensis*, *B. coagulans*, *B. niacini*, *B. pumillus*, *B. megaterium*, *M. luteus*, *S. plymuthica*, and *B. pumillus* KM grew on the GRM-agar with 15 and 20% oil concentration; moreover, at a concentration of 15%, the visible growth appeared as early as a day later, and at a concentration of 20% it appeared by the end of the first week of cultivation (Table 1). The greatest sensitivity to the action of the pollutant was shown by two species of bacilli: *B. circular* and *V. simplex*, the growth of which was absent at minimal oil concentrations in the nutrient medium. Under the action of a 25% concentration of the pollutant, viability was retained by *B. coagulans*, *B. mojavensis*, *B. megaterium*, *M. luteus*, and *B. pumillus* KM; however, visible growth of the bacteria was observed only on the 10th day of cultivation.

In order to study the bioremediation potential of the isolated strains, the microorganisms were cultivated in the M9 medium, in which oil hydrocarbons served as the only source of carbon. The ability of the isolated microorganisms to use oil as a single carbon source was studied using the carbon-free M9 medium with the addition of oil. It was established that, at a 15% concentration of oil in the medium, six species of bacteria were capable of using it as a carbon source: *B. mojavensis*, *B. coagulans*, *B. pumillus*, *B. megaterium*, *M. luteus*, and *B. pumillus* KM. With an increase in the oil concentration to 25%, the ability to use it was preserved by three species of bacteria—*M. luteus*, *B. mojavensis*, and *B. pumillus* KM (Table 2).

Thus, at an oil concentration of no more than 10% of the soil mass, the highest oil degradation potential is shown by *B. mojavensis*, *B. coagulans*, *B. pumillus*, *B. megaterium*, *M. luteus*, and *B. pumillus* KM; with an increase in the concentration of the pollutant, its successful degradation is possible using *B. mojavensis*, *M. luteus*, and the museum strain of *B. pumillus* KM.

The assessment of the resistance of the isolated microorganisms to the action of adverse environmental factors showed that only representatives of the genus *Bacillus*, including the museum strain of *B. pumillus* KM (Table 3), were capable of growth at a temperature of 4°C. The ability to grow at pH 5 was shown by three bacilli strains isolated from the soil sample as well as the museum strain of *B. pumillus* KM, and at pH 9 it was shown by nine strains, among which there were seven bacilli strains (including the museum strain) and *M. luteus* and *S. plymuthica*. All isolated heterotrophic bacteria grew on the GRM-agar with a 7% concentration of NaCl; however, an increase in the salt concentration to 10% inhibited the growth of two species of bacilli, as well as *M. luteus* and *S. plymuthica*. The ability to grow at a 15% concentration of NaCl was characteristic only of the museum strain of *B. pumillus* KM.

CONCLUSIONS

At the present time, when developing biological products for the remediation of anthropogenically polluted territories, preference is given to the native strains of microorganisms isolated from soils with chronic pollution, which have acquired selective advantages of resistance to the action of pollutants as a result of natural selection. Among the native bacterial strains isolated from the oil-contaminated southern chernozem soil sample, the highest resistance to the negative effects of physicochemical factors and high pollutant concentrations was shown by *B. mojavensis*, *M. luteus*, and the museum strain of *B. pumillus* KM. The high adaptive potential of *B. pumillus* KM is probably due to the fact that it is a native strain isolated from soils that have been subjected to oil pollution for a long time, which has contributed to the formation of the corresponding resistance mechanisms in it.

The results make it possible to increase the effectiveness of remediation measures for anthropogenically disturbed natural areas by forming associations of native strains of bacteria, taking into account the degree of their resistance to environmental factors and the concentration of oil in oil-contaminated soils.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interes. This article does not contain any studies involving animals or human participants performed by any of the authors.

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