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EXPERIMENTAL ARTICLES

Effect on the Medium Salinity on Oil Degradation by Nocardioform Bacteria

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Abstract—Oil degradation by cultures of *Rhodococcus erythropolis* and *Dietzia maris* was found to depend on the NaCl concentration in the medium. Optimal utilization of turbine oil by *R. erythropolis* and *D. maris* was observed at 0.5 and 2 to 5% NaCl concentration, respectively. Mineral oil and a mixture of paraffins (C_{14}–C_{18}) were utilized within a broader range of the medium salinity. As shown by fluorescent microscopy, *D. maris* colonies formed on the oil drop surface, whereas *R. erythropolis* cells penetrated the drops. The strains studied may populate various ecological niches in oil-containing ecosystems. They are promising for the development of microbial preparations for cleaning the environment from oil pollution.

Key words: nocardioform bacteria, oil utilization, respiration, GLC, halotolerance, biodiversity, environmental oil pollution.

Hydrocarbon-oxidizing microorganisms have been used to develop preparations applicable for cleaning soils and water bodies from the effects of oil pollution [1]. Microorganisms utilizing oil paraffins [2] and other oil fractions [3, 4] have been isolated. Hydrocarbon-oxidizing bacteria naturally inhabit oil fields with various degrees of formation water mineralization [5], as well as oil-polluted grounds and soils differing in their salinity and halotolerance of the resident microflora [6, 7].

We have previously described the nocardioform bacteria capable of degrading oil, including oil fractions that are multicomponent and difficult to oxidize [8]. Respiration of hydrocarbon-oxidizing bacteria cultivated on synthetic medium containing glucose or acetate as the carbon and energy source was dependent on the medium salinity [6]; the rhodococcal growth kinetics and respiration in the presence of high salt concentrations were shown to be interrelated [9, 10].

MATERIALS AND METHODS

This study used strains *Rhodococcus erythropolis* INMI 100 and *Dietzia maris* INMI 101 cultivated on a medium of the following composition (g/l): glucose, 5; peptone, 10; yeast extract, 5; NaCl, 5 (pH 7.2).

To study degradation of oil and oil products, the bacteria were grown on Raymond's medium [11] containing from 0.5 to 10% NaCl. Technical turbine oil (TO), purified mineral oil (MO), a mixture of paraffins with a chain length of C_{14}–C_{18} (PAR), and crude Devonion oil from the Romashkinskoe oil field (Tatarstan) served as the carbon source. Bacteria were cultivated at 26–28°C in flasks on a shaker from 7 to 21 days depending on the substrate used.

Methods for Analysis of Oil-Degrading Activity

1. Gas–liquid chromatography. Oil degradation by microorganisms grown for 7 days on a medium containing TO, MO, or PAR (0.3 vol %) as a single carbon source was assessed from the microbial respiratory activity (CO_2 release as a result of bacterial metabolism). The bacteria, cultivated in hermetically sealed 30-ml flasks containing 10 ml Raymond's medium, were sampled at regular intervals. The inoculum was 2 vol %. Gases were analyzed on a model 3700 chromatograph equipped with a heat-conductivity detector. Argon was used as the carrier gas. The temperature was 50°C. To determine CO_2 emission, a metal 3.5-m column packed with Chromosorb 101 (80 mesh) was used.

To evaluate bacterial oxidation of the aliphatic oil fraction, the *R. erythropolis* and *D. maris* strains were cultivated with oil (2 vol %) for three weeks. Then, the amount and composition of oil carbohydrates was analyzed chromatographically using the *iso*-alkanes pristane (*i*19) and phytane (*i*20) as internal standards. This analysis employed a capillary 25-m column with an internal diameter of 0.32 mm, apieson as the immobile phase, hydrogen as the carrier gas, and linear temperature programming within a range of 100–320°C at a heating rate of 2°C/min.

2. Fluorescent method. A suspension of cells grown for 3 days on Raymond's medium containing from 0.5 to 10% NaCl and TO as the carbon source was stained on a slide with 0.01% acridine orange using a

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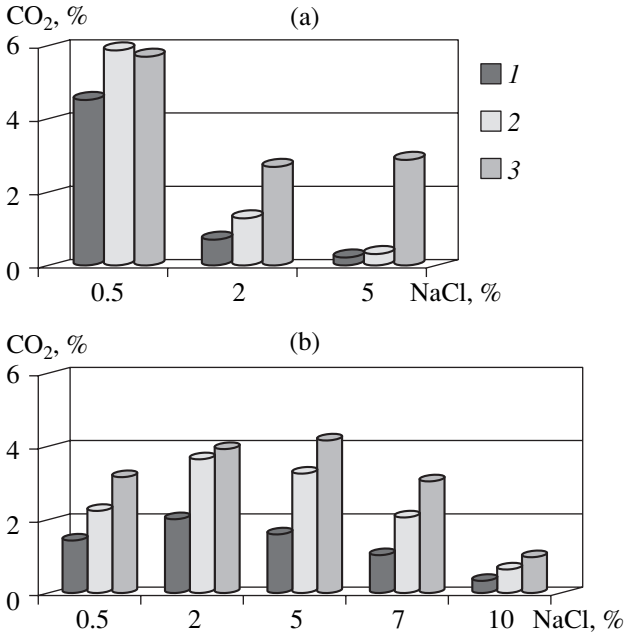

Fig. 1. Oxidation of turbine oil by the (a) *R. erythropolis* and (b) *D. maris* strains at various medium salinities by the (*1*) third, (*2*) fifth, and (*3*) seventh day.

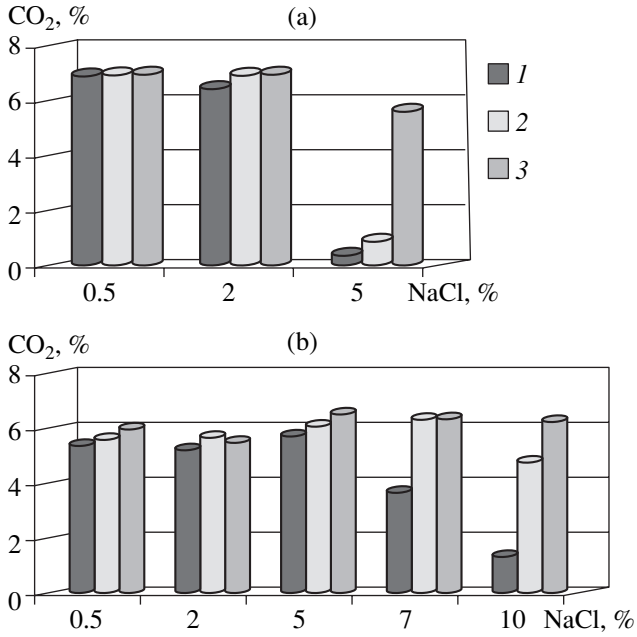

Fig. 2. Oxidation of a mixture of paraffins by the (a) *R. erythropolis* and (b) *D. maris* strains at various medium salinities by the (*1*) third, (*2*) fifth, and (*3*) seventh day.

conventional technique [12]. The stained preparations were examined under a LUMAM-I2 fluorescence microscope. To induce fluorescence of the stained preparations, a FS-1-4 light filter was used in combination with a heat-protecting light filter.

RESULTS AND DISCUSSION

Microbial Oil Degradation Determined from Microbial Respiratory Activity at Various Medium Salinities

Both TO and PAR utilization was found to depend on the medium salinity and on the specific oil-degrading strain. Strain *R. erythropolis* INMI 100 exhibited low halotolerance. On a TO-containing medium, it displayed a significant degradative activity only at a 0.5% medium salinity, whereas at NaCl concentrations from 2 to 5%, the oil-degrading activity of this strain decreased dramatically (Fig. 1a). When PAR served as the substrate, the *R. erythropolis* degradative activity was observed in a broader salinity range, reaching its maximum (6.5–7.0% CO_2) on the third day of cultivation at a NaCl concentration from 0.5 to 2%. At a 5% NaCl concentration, the PAR-degrading activity was low for five days (0.5 to 1% of CO_2 in the gas phase), but it increased significantly (up to 7% CO_2) on the seventh day of cultivation (Fig. 2a).

Strain *D. maris* INMI 101 utilized both substrates within a broader salinity range as compared to *R. erythropolis*. TO utilization was observed at NaCl concentrations ranging from 0.5 to 7%, reaching its maximum at a 2 to 5% salinity. On the third and fifth days of the experiment, from 3.3 to 4.2% CO_2 was determined in the gas phase. At a medium salinity higher than 7%, oil degradation was decelerated and ceased at a 10% NaCl concentration (Fig. 1b). When grown with PAR, *D. maris* displayed significant oil-degrading activity as soon as the third day at a salinity ranging from 0.5 to 5% (5.3 to 5.8% CO_2); at 7 and 10% NaCl concentrations in the medium, oil utilization was decelerated, but it significantly increased on the fifth, and especially on the seventh, day of cultivation (Fig. 2b).

When MO served as the substrate, the respiratory activity of the two strains was similar to that observed during the cultivation with PAR within the same salinity range. When cultivated on a medium containing a mixture of paraffins (PAR) or purified mineral oil (MO), both cultures showed a higher oil-degrading activity than during cultivation with turbine oil (TO). This supports our previous data on the substrate specificity of nocardioform hydrocarbon-oxidizing bacteria with respect to oil of different composition [8].

Oil Degradation by the R. erythropolis and D. maris Strains

After incubation for three weeks with crude oil, both strains completely oxidized *n*-alkanes and *iso*-alkanes with the chain length C_{11}–C_{30} and C_{14}–C_{18}, respectively. The qualitative composition of intact oil and the oil incubated with the *D. maris* strain are shown in Figs. 3a and 3b, respectively. A similar chromatogram was obtained after oil degradation by the *R. erythropolis* strain.

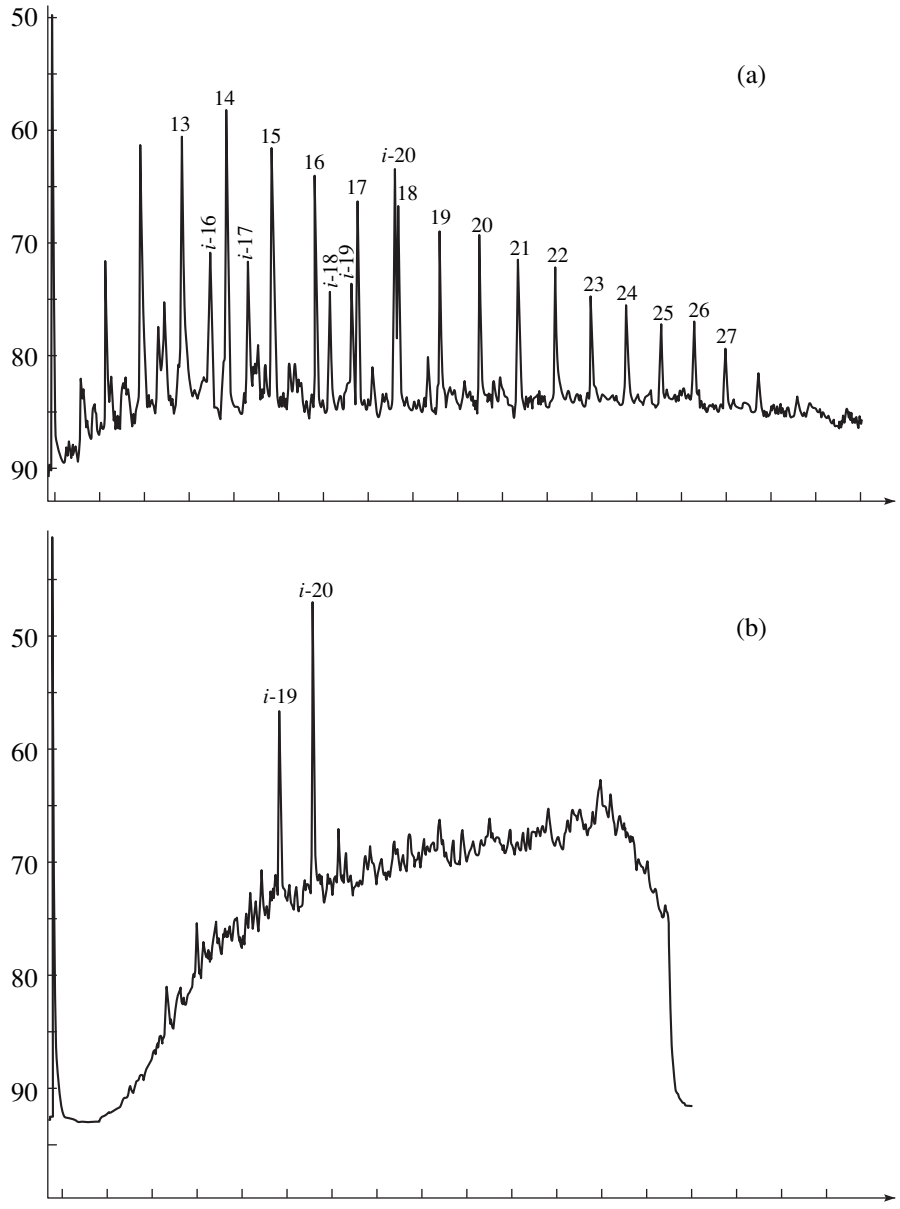

Fig. 3. Oxidation of oil hydrocarbons by nocardioform bacteria. Chromatograms of (a) the control sample of crude oil and (b) oil incubated with *D. maris.*

Interaction of Oil-Degrading Microorganisms and Oil Drops Examined by Fluorescence Microscopy

Microscopy of the preparations stained with acridine orange showed that the interaction of *D. maris* cells with TO drops and their propagation depended on the NaCl concentration in the medium (Figs. 4a–4f).

At a 0.5% NaCl concentration, the bacterial cells adhered to the oil drops, although they did not propagate on the third day of growth (Fig. 4a). On the fifth day, a tendency toward colony formation on the drop surface was observed (Fig. 4b). At 2 and 5% NaCl concentrations, cell adhesion and propagation on the substrate surface was maximum. As early as the third day, high numbers of adhered cells and microcolonies were seen (Fig. 4c), and they increased on the fifth day (Fig. 4d). Microcolonies often enlarged significantly on the drop surface; the substrate migrated from the drops into microcolonies, and the drops lost their spheric shape. Increasing the medium salinity to 7 and especially to 10% caused significant inhibition of microorganism propagation, although cell adhesion to the drop surface was virtually not affected (Figs. 4e and 4f).

The cell size and shape of the bacteria studied depended on the NaCl concentration in the medium. At the optimal salinity (2–5%), the *D. maris* cells were rods 0.7 to 1.0 μm long (Figs. 4c and 4d), which corresponds to the minimal cell size under standard condi-

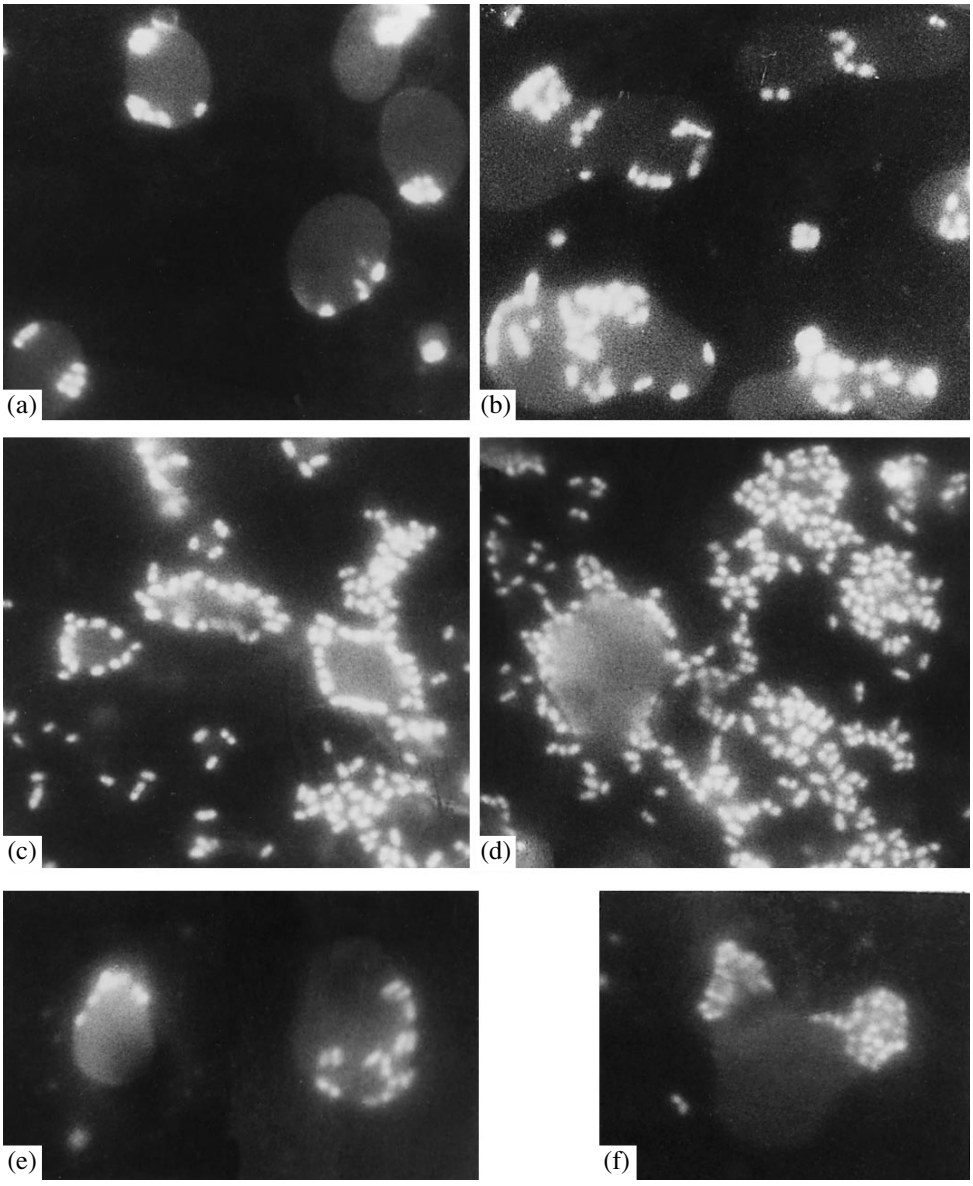

Fig. 4. Interaction of *D. maris* cells with turbine oil drops at various medium salinities: (a) 0.5% NaCl, 3rd day, cell adhesion on the drop surface; (b) 0.5% NaCl, 5th day, cell propagation and microcolony formation; (c) 2% NaCl, 3rd day, active cell adhesion and propagation; (d) 2% NaCl, 5th day, formation of microcolonies; (e and f) 10% NaCl, 3rd and 5th days, unaffected cell adhesion but retarded cell propagation (small projection-like microcolonies on the oil drop surface).

tions [13]. At a 0.5% salt concentration, the cells occurred under hypoosmotic conditions resulting in cell swelling, so that they increased 1.5 to 2-fold in size (to 1.0–1.5 μm) and acquired oval shape (Figs. 4a and 4b). Hyperosmotic conditions (7 to 10% NaCl concentration in the medium) led to plasmolysis of cells, which reduced their size (to 0.6 μm) and retarded cell division (Figs. 4e and 4f).

The oil-degrading strains of *R. erythropolis* and *D. maris* can be used to produce bioformulations for cleaning the environment from oil pollution. These strains utilize not only easily oxidizable *n*-alkanes and *iso*-alkanes, but also oil fractions and products that are not easily available for microorganisms. The oil-degrading microorganisms studied differ in several respects and thus can be used under different conditions. (1) They contain enzymatic complexes differing in substrate specificity [8]; (2) the *R. erythropolis* strain can grow in a weakly acidic medium (pH 5.0), whereas *D. maris* does not grow at pH lower than 6.5; (3) according to Larsen's classification [14], the *R. erythropolis* strain is weakly halotolerant, whereas the *D. maris* strain belongs to moderately halotolerant microorganisms: the oil-degrading activity of the *R. erythropolis* strain is observed within a narrow salinity range (Figs. 1a and 2a), whereas the *D. maris* is

capable of oil utilization at a higher content of NaCl in the medium (Figs. 1b and 2b).

The degradative activity of the *D. maris* strain with respect to turbine oil determined from its respiratory activity by the GLC method correlated with data of the fluorescence method. The morphological changes in rhodococci observed under hypo- or hyperosmotic conditions were undoubtedly associated with changes in their growth and respiration. These changes are reversible [9] and ensure the survival of the microbial population in natural ecotopes under seasonal fluctuations in the habitat salinity.

Fluorescence microscopy showed that the interaction pattern of *D. maris* cells with oil differed from that of *R. erythropolis* cells described earlier [8]. After the adhesion of *R. erythropolis* cells, their propagation was accompanied by penetration into the oil drops and formation of submerged microcolonies. The adhered cells of *D. maris* did not penetrate the drops and formed microcolonies on the drop surface. This suggests that the *R. erythropolis* cell wall can increase its hydrophobicity to a greater extent than the cell wall of the *D. maris* due to the biosynthesis of lipophilic compounds promoting cell penetration into the oil drops. In *D. maris* culture, lipophilic channels are formed within the cell wall [15].

Thus, the members of two different genera of rhodococci differ in the degree of hydrophobicity of their cell surface, and, therefore, they differ in their interaction pattern with the insoluble substrate and in the mode of hydrocarbon utilization.

Thus, the data obtained in this work suggest that the species studied may populate different or partly overlapping ecological niches in oil-containing ecosystems.

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REFERENCES

1. Vel’kov, V.V., Bioremediation: Principles, Problems, Approaches, *Biotekhnologiya*, 1995, no. 3/4, pp. 20–27.
2. Gorlatov, S.N. and Belyaev, S.S., Aerobic Microflora of an Oil Field and Its Capacity for Oil Degradation, *Mikrobiologiya*, 1984, vol. 53, no. 5, pp. 843–849.
3. Sidorov, D.G., Borzenkov, I.A., Milekhina, E.M., and Belyaev, S.S., Microbial Degradation of Mazut in Soil Upon the Application of the Devoroil Biopreparation, *Prikl. Biokhim. Mikrobiol.*, 1998, vol. 34, no. 3, pp. 281–286.
4. Pleshakova, E.V., Biodegradation of Surfactants and Mineral Oils and the Underlying Genetic Mechanisms, *Cand. Sci. (Biol.) Dissertation*, Saratov, 1999, pp. 3–23.
5. Milekhina, E.I., Borzenkov, I.A., Miller, Yu.M., Belyaev, S.S., and Ivanov, M.V., Hydrocarbon-Oxidizing Microflora of Tatarstan Water-Flooded Oil Fields Varying in the Mineralization of Formation Waters, *Mikrobiologiya*, 1991, vol. 60, no. 4, pp. 747–755.
6. Milekhina, E.I., Borzenkov, I.A., Zvyagintseva, I.S., Kostrikina, N.A., and Belyaev, S.S., Ecological and Physiological Characterization of Aerobic Eubacteria from Oil Fields of Tatarstan, *Mikrobiologiya*, 1998, vol. 67, no. 2, pp. 208–214.
7. Plotnikova, E.G., Altyntseva, O.V., Kosheleva, I.A., Puntus, I.F., Filonov, A.E., Gavrish, E.Yu., Demakov, V.A., and Boronin, A.M., Bacterial Degraders of Polycyclic Aromatic Hydrocarbons Isolated from Soils and Bottom Sediments in the Zone of Salt Works, *Mikrobiologiya*, 2001, vol. 70, no. 1, pp. 61–69.
8. Zvyagintseva, I.S., Surovtseva, E.G., Poglazova, M.N., Ivoilov, V.S., and Belyaev, S.S., Degradation of Machine Oil by Nocardioform Bacteria, *Mikrobiologiya*, 2001, vol. 69, no. 3, pp. 321–328.
9. Plakunov, V.K., Arzumanyan, V.G., Voronina, N.A., and Belyaev, S.S., Relationship between Growth and Respiration Rates in Rhodococci Grown at High Salt Concentrations, *Mikrobiologiya*, 1999, vol. 68, no. 1, pp. 40–44.
10. Arzumanyan, V.G., Voronina, N.A., Plakunov, V.K., and Belyaev, S.S., The Degree of Halophily in *Rhodococcus erythropolis* and *Halobacterium salinarium* Depends on the Partial Pressure of Oxygen, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 290–292.
11. Raymond, R.L., Microbial Oxidation of *n*-Paraffinic Hydrocarbons, *Development Ind. Microbiol.*, 1961, vol. 2, no. 1, pp. 23–32.
12. Methods of Taxonomic Analysis, *Metody pochvennoi mikrobiologii i biokhimii* (Methods of Soil Microbiology and Biochemistry), Zvyagintsev, D.G., Ed., Moscow: Mosk. Gos. Univ., 1991, pp. 131–133.
13. Nesterenko, O.A., Kvasnikov, E.L., and Nogina, T.M., *Nokardiopodobnye i korinepodobnye bakterii* (Nocardioform and Coryneform Bacteria), Kiev: Naukova Dumka, 1985, pp. 117–118.
14. Larsen, H., Halophilic and Halotolerant Microorganisms: An Overview and Historical Perspective, *FEMS Microbiol. Rev.*, 1986, vol. 39, no. 1/2, pp. 3–7.
15. Koronelli, T.V., Principles and Methods of Intensification of the Biological Degradation of Hydrocarbons in the Environment (A Review), *Prikl. Biokhim. Mikrobiol.*, 1996, vol. 32, no. 6, pp. 579–585.