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The Prospects of Using Bacteria of the Genus *Rhodococcus* and Microbial Surfactants for the Degradation of Oil Pollutants

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Abstract—The possibility of accelerating oil degradation by an enrichment culture of oil-oxidizing microorganisms in the presence of bacteria of the genus *Rhodococcus* and microbial surfactants was studied. It was shown that the degree of consumption of crude oil (2vol %) after 192 h of enrichment culture growth reached 84%. Inoculation of the active hydrocarbon-oxidizing strain *Rhodococcus erythropolis* EK-1 and exogenous surfactants produced by *Pseudomonas* sp. PS-27 increased this degree to 90 and 93–94%, respectively. On the grounds of these results, efficient methods of purification of the environment from oil pollutants can be developed.

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Bacteria of the genus *Rhodococcus* are the most active degraders of oil products in natural biotopes of polluted sites and within biotechnological preparations [1–4]. For example, the Devoroil association when applied to bioremediation of soils from oil products and other organic pollutants, includes five microbial strains, three of which belong to the genus *Rhodococcus*. The cell wall of rhodococci is associated with lipid components glycolipids and peptidolipids. The cell wall is lipophilic, meaning it has a high affinity for hydrophobic substrates. The initial step of interaction between oil pollutants and the microorganism involves a direct contact between them. This direct interaction is dependent on the structure of the cell wall, that is, its surface hydropathicity [5–7]. During direct contact, hydrocarbons penetrate into the cell as submicroscopic drops. Surfactant activity and hydrophobicity favor the interaction between the microorganism and the insoluble substrate, overcoming the diffusion limitation during the substrate transport to the cell. The optimum transport of nutrients can be ensured by the adhesion of cells to hydrophobic substrates in the early growth stages.

The activation of natural microflora in soils and water polluted with oil products is one of the remediation methods. This method is the most advisable for lasting pollutions. Natural self-remediation mechanisms favor the development of specific oil-oxidizing microflora at sites long exposed to pollutants. The economic aspect of such methods is a notable advantage, because the use of natural microflora reduces the cost of remediation significantly, demanding neither large-scale production of biological preparations nor treatment-associated expenses. However, at high pollutant concentrations in soils and waters, natural bioremediation proceeds too slowly.

Surfactants, particularly those produced by microorganisms, are a potent factor affecting the activity of a microbial population, whether laboratory or natural [8, 9]. Emulsification (solubilization) of hydrocarbons with surfactants favor the influx of hydrophobic organic pollutants (HOPs) from soils and water to microbial cells and, thereby, their degradation [10]. Thus, surfactants act as mediators between the cell and HOPs. Therefore, it is reasonable to apply surfactants, or cultures intensely producing them, to technologies and preparations for the purification of soil and water from oil products [11, 12].

The goal of this work was to research the possibility of intensifying oil degradation by the enrichment cultures of oil-oxidizing microorganisms supplemented with active strains of *Rhodococcus* bacteria and microbial surfactants.

MATERIALS AND METHODS

Microorganisms. Experiments were performed with enrichment cultures of oil-oxidizing microorganisms isolated from oil-polluted samples of soils and bottom sediments of slime tanks of the oil field Dolinanaftogaz, Ivano-Frankovsk Region, Ukraine, and strains formerly isolated by us: *Rhodococcus* sp.

no. 1, *Rhodococcus* sp. no. 2, *R. erythropolis* EK-1, and *Pseudomonas* sp. PS-27 [13, 14].

Development and growth of enrichment cultures of oil-oxidizing microorganisms. Enrichment cultures utilizing oil as a carbon source were obtained by the inoculation of 0.5 g of a soil or sediment sample to 10 ml of mineral medium (g/l): $Na_2HPO_4 \cdot 12H_2O$ (2.0); KH_2PO_4 (1.0); NH_4NO_3 (3.0); NaCl (1.0); Na_2CO_3 (0.2); $MgSO_4 \cdot 7H_2O$ (0.2); $MnSO_4 \cdot 5H_2O$ (0.02); $CaCl_2 \cdot H_2O$ (0.01); $FeSO_4 \cdot 7H_2O$ (0.01). Oil (density, 0.9 g/cm^3) from the Dolina field, Ivano-Frankovsk Region, was used as the sole carbon source at concentrations of 1 and 2 vol %. Cultivation was performed at 30°C, pH 7.0 for 168 h. For serial inoculations, 10% of the liquid culture volume was inoculated into the medium for the next culture. To obtain enrichment cultures, three to five serial inoculations were performed.

Isolation of pure cultures. Enrichment cultures were inoculated by the Koch method on agar medias: beef-extract agar (BEA), glucose-potato agar (GPA), and the mineral medium with 0.01 vol % oil or liquid paraffins. Cultural, physiological, and biochemical features of strains were determined by conventional tests [15]. Microorganisms were identified according to [16].

Bacterium cultivation. Bacteria of the genus *Rhodococcus* were grown in liquid mineral medium (g/l) $K_2HPO_4 \cdot 3H_2O$, 2; KH_2PO_4, 1.2; NH_4NO_3, 4.0; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 8×10^{-4}; $FeSO_4 \cdot 7H_2O$, 5×10^{-6}, NaCl (1.0); yeast extract (1.0); peptone (1.0); pH 6.8–7.0. Bacterial strain *Pseudomonas* sp. PS-27 was grown in liquid mineral medium of the following composition (g/l): $K_2HPO_4 \cdot 3H_2O$ (2); KH_2PO_4 (1.2); $NaNO_3$ (3.0); $MgSO_4 \cdot 7H_2O$ (0.5); sodium citrate (5.0); pH 6.8–7.2. The following carbon and energy sources were added: hexadecane, 2 vol %, liquid paraffins (*n*-alkanes, C_{10}–C_{14}) manufactured by the Galichina oil refinery, (Drogobych, Lviv Region), 2 vol %; and oil of the Dolina field, Ivano-Frankovsk Region (density, 0.9 g/cm^3), 1 and 2 vol %.

Bacteria were grown in 750 ml Erlenmeyer flasks with 200 ml of medium, shaking at 200 rpm, and 30°C for 168 h.

For inoculation, we used 1- to 2-day cultures grown on GPA or cultures at the log phase (24–48 h) grown in the mineral medium with liquid paraffins (0.5 vol %).

Biomass was assayed according to the value of 540 nm of the cell suspension, and the amount was recalculated to dry cell weight (according to a standard plot) or gravimetrically.

Emulsifying activity was determined in [8]. The following hydrophobic phases were used: kerosene, liquid paraffins, light oil (density, 0.80 g/cm^3), and rapeseed and soybean oils. The value of the emulsifying index (E_{24}) was determined after 24 h as a percentage of the emulsion layer thickness (relative to the total height of the liquid column in a test tube). This index was determined in the culture liquid obtained by growing *Pseudomonas* sp. PS-27 in a medium with hexadecane (2 vol %) for 168 h.

Oil degradation by enrichment cultures. Enrichment cultures were grown in the presence of *R. erythropolis* EK-1 or surfactants produced by *Pseudomonas* sp. PS-27 in the mineral medium described above, containing 2 vol % oil (density, 0.9 g/cm^3), for 192 h. The volume of the inoculum was 10% of the volume of the medium to be inoculated.

The effect of *R. erythropolis* EK-1 on the degree of crude oil consumption by microbial enrichment cultures was studied. The strain was sampled at the log phase (24 h) of growth in the mineral medium with 0.5 vol % liquid paraffins. The volume of the sample of *R. erythropolis* EK-1, inoculated into the medium for the enrichment cultures, was 5% of the medium volume.

In experiments on the effect of exogenous surfactants on the degree of oil degradation by enrichment cultures, culture liquid obtained by growing *Pseudomonas* sp. PS-27 in the medium with hexadecane (2 vol %) for 168 h was used. The volume of the culture liquid of *Pseudomonas* sp. PS-27 was 10% of the volume of the medium for the enrichment cultures.

Residual oil was assayed gravimetrically and by IR spectroscopy with an AN-1 oil-product analyzer (Russia) after extraction from culture liquids with hexane.

RESULTS AND DISCUSSION

Growth of bacteria on hydrophobic substrates. We studied the ability of strains *Rhodococcus* sp. no. 1, *Rhodococcus* sp. no. 2, *R. erythropolis* EK-1, and *Pseudomonas* sp. PS-27 to consume hydrophobic substrates: liquid paraffins, hexadecane, and oil. The data on biomass accumulation by the oil-oxidizing bacteria under study during growth on hydrocarbon substrates are shown in Fig. 1. In our experiments, all strains consumed liquid paraffins, hexadecane, and oil. Biomass concentrations of the strains under study reached 1.6–4.6 g/l after 168-h cultivation. It is worth noting that the growth curves of strains *Rhodococcus* sp. no. 1, *Rhodococcus* sp. no. 2, and *Pseudomonas* sp. PS-27 were virtually identical (Fig. 1). By the end of cultivation, the biomass concentrations of these strains were 1.6–2.9 g/l.

The strain *R. erythropolis* EK-1 showed higher biomass concentrations when grown on hydrocarbons: 3.5–4.6 g/l (Fig. 1). As biomass concentration correlates with the rate of hydrocarbon consumption, this strain can be recommended for increasing the degree of hydrocarbon degradation by microbial enrichment cultures. Another reason why such studies with strain *R. erythropolis* EK-1 are viewed as being promising is that it produces surfactants possessing emulsifying properties [13]. Chemically, surfactants produced by *R. erythropolis* EK-1 are lipid complexes with polysaccharide–proteinaceous substances. The lipids have

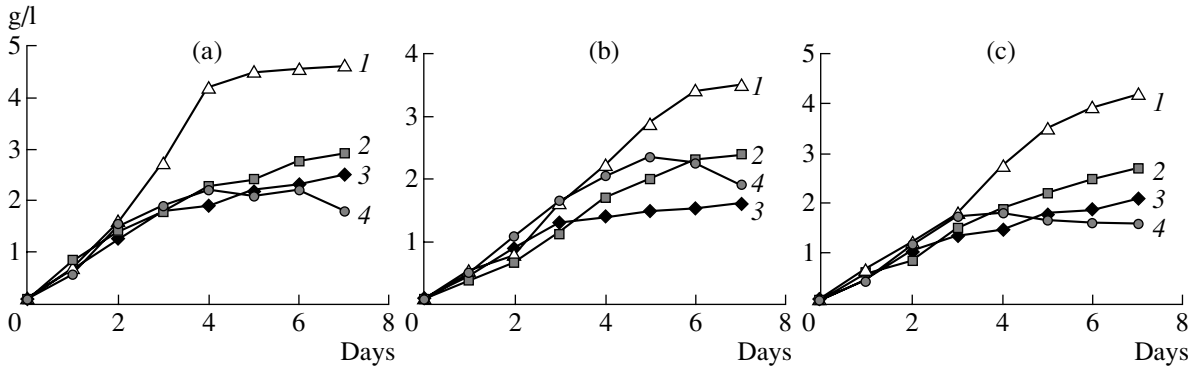

Fig. 1. Growth (g/l) of oil-oxidizing bacteria in the medium with (a) liquid paraffins, (b) hexadecane, and (c) oil. *1, Rhodococcus erythropolis* EK-1; *2, Rhodococcus* sp. no. 1; *3, Rhodococcus* sp. no. 2; *4, Pseudomonas* sp. PS-27.

been found to contain glycolipids (trehalose mono- and dicorynomycolates) and common lipids (cetyl alcohol, palmitic acid, methyl n-pentadecanoate, triglyceride, mycolic acids, etc.) [13].

Emulsifying properties of *Pseudomonas* sp. PS-27 surfactants. In addition to *R. erythropolis* EK-1, we chose the bacterial strain *Pseudomonas* sp. PS-27 for the following reasons: (1) *Pseudomonas* sp. PS-27 can grow on hydrophobic substrates (Fig. 1); (2) this strain produces extracellular surfactant glycolipids (rhamnolipids) and alginate polysaccharides [14]; (3) it is a sufficiently active producer of surfactants. The amounts of rhamnolipids and polysaccharides produced by *Pseudomonas* sp. PS-27 are 7.5–8.0 and 4.5–5.0 g/l, respectively.

Emulsification of organic substances (in particular, oil and petroleum products) with surfactants is crucial for a fast biotechnological purification of water and soil. Studies of emulsifying properties of the culture liquid obtained by growing *Pseudomonas* sp. PS-27 with hexadecane showed that the liquid efficiently emulsified various hydrophobic substrates with emulsification indices varying from 65 to 90% (Fig. 2).

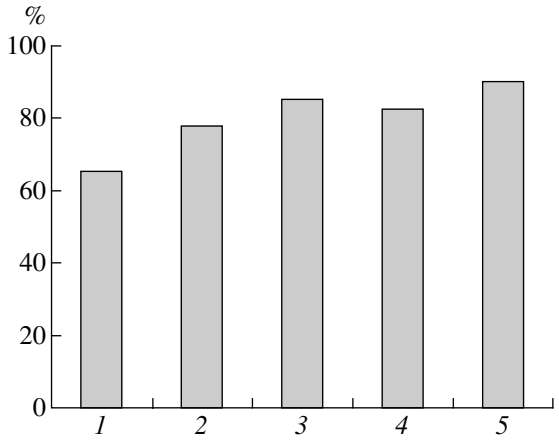

Fig. 2. Emulsifying properties of surfactants produced by *Pseudomonas* sp. PS-27. Emulsified substrate: *1*, kerosene; *2*, liquid paraffins; *3*, oil; *4*, rapeseed oil; *5*, soybean oil.

One of the features of surfactants most important for their practical use is their ability to emulsify hydrocarbons over a broad pH range. It was shown that surfactants produced by *Pseudomonas* sp. PS-27 could form fine stable emulsions with hydrophobic substrates at a pH from 5.5 to 10. This property makes *Pseudomonas* sp. PS-27 surfactants promising for the treatment of oil-polluted sites under various environmental conditions.

Surfactants are relatively new biotechnological products. In recent years, many studies aimed at searching for new active surfactant producers have been reported. Many of them show the ability of new surfactants to accelerate oil-product degradation under both laboratory and field conditions [9, 17–19]. Data from the literature indicate that this ability is not limited to surfactants of rhodococcal origin [3, 4, 9], but also inherent in surfactants produced by *Candida antarctica* [17], unidentified bacterial strains (supposedly of the genus *Bacillus* [18]), and *Flavobacteruim* sp. MTN11 [19].

Oil degradation by the enrichment culture in the presence of rhodococci and surfactants. We studied the ability of the enrichment culture of oil-oxidizing microorganisms (no. 3), which was by us, to utilize crude oil in the presence of strain *R. erythropolis* EK-1 and surfactants produced by *Pseudomonas* sp. PS-27.

We studied ten oil-polluted samples and isolated six enrichment cultures, consuming 50–80% of the substrate when cultivated on the mineral medium with 2 vol % oil for 192 h. Enrichment culture no. 3 proved to be the most active. It was chosen for subsequent experiments. We found that this culture consisted of six bacterial species and identified them by morphological, cultural, physiological, and biochemical tests as representative of the genera *Acinetobacter*, *Nocardia*, *Arthrobacter*, *Mycobacterium*, and *Rhodococcus*.

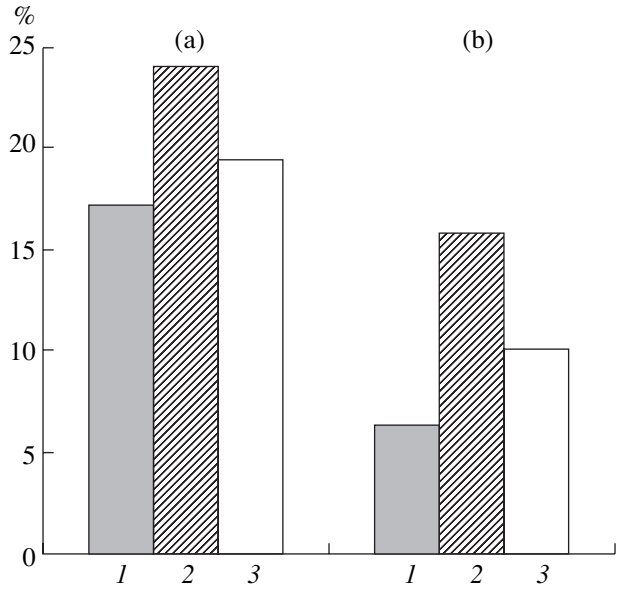

Fig. 3. Effect of *Rhodococcus erythropolis* EK-1 and surfactants produced by *Pseudomonas* sp. PS-27 on the degree of oil degradation (% residual oil) by oil-oxidizing enrichment culture no. 3 after (a) 72 and (b) 192 h of cultivation. Initial oil concentration in the medium: 2 vol %; *1*, enrichment culture + *Pseudomonas* sp. PS-27 surfactants; *2*, enrichment culture; *3*, enrichment culture + *Rhodococcus erythropolis* EK-1.

The data presented in Fig. 3 indicate that surfactants of *Pseudomonas* sp. PS-27 accelerated oil consumption by the enrichment culture under study. After 72 h of enrichment culture growth, the content of residual oil was 24.1% of the starting one, and with the presents of the surfactants, it was as low as 17.2%. The addition of strain *R. erythropolis* EK-1 to enrichment culture no. 3 also accelerated oil consumption. The enrichment culture, when cultivated with the rhodococcus, reduced the content of residual oil to 19.5% after 72 h. After 192 h, the enrichment culture reduced the oil content to 15.8%, whereas in the presence of *R. erythropolis* EK-1 and *Pseudomonas* sp. PS-27 surfactants reduced to 10.0 and 6.4%, respectively.

Our study demonstrates that oil degradation by an enrichment culture can be accelerated by adding active hydrocarbon-oxidizing strain *R. erythropolis* EK-1 and surfactants produced by strain *Pseudomonas* sp. PS-27.

The experimental data provide grounds for developing efficient methods of purification of soil and water from oil pollution.

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