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EXPERIMENTAL ARTICLES

The Properties of Hydrocarbon-Oxidizing Bacteria Isolated from the Oilfields of Tatarstan, Western Siberia, and Vietnam

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Abstract—Eleven strains of hydrocarbon-oxidizing bacteria, isolated from oilfields and representing the genera *Rhodococcus, Gordonia, Dietzia*, and *Pseudomonas*, were characterized as mesophiles and neutrophiles. Rhodococci were halotolerant microorganisms growing in a media containing up to 15% NaCl. All the strains oxidized *n*-alkanes of crude oil. An influence of the cultivation temperatures (28 or 45°C) and organic supplements on the degradation of C_{12}–C_{30} *n*-alkanes in oxidized oil by two bacterial strains of the genus *Pseudomonas* was shown. The introduction of acetate, propionate, butyrate, ethanol, and sucrose led mainly to decreased oxidation of petroleum paraffins. At certain cultivation temperatures, the addition of volatile fatty acid salts increased the content of certain *n*-alkanes in oxidized oil as compared to crude oil.

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Key words: hydrocarbon-oxidizing bacteria, cultivation temperature, pH, salt dependence, petroleum biodegradation.

The recent development of environmental biotechnologies and the biotechnologies of increasing recoverable oil [1] account for the growing interest in aerobic hydrocarbon-oxidizing microorganisms. Oilfields are natural reservoirs formed over a long geological time and had been isolated from external influence until the onset of their oilfield development. Long-term use of flooding technologies in oil mining is associated with the continual introduction of surface microorganisms, oxygen, and certain biogenic elements into oil pools. Hydrocarbon-oxidizing bacteria (HOBs) are one of the main components of this community. A detailed study of the species composition and physiological properties of this group of microorganisms in the areas of oil production, have been initiated by our laboratory [2, 3]. Studies conducted in oilfields with moderate temperatures (of up to 40°C) showed that stratal waters with a mineralization of 3–272 g/l abound in HOBs related to the genera *Rhodococcus, Arthrobacter, Micrococcus, Mycobacterium, Psychrobacter, Paracoccus, Spirillum, Acinetobacter, Flavobacterium*, and *Bacillus* [3, 4]. Rhodococci [3, 4] and the halophilic archaebacteria *Halobacterium* and *Haloferax* [5, 6] were mainly found in highly mineralized waters (over 150 g/l). In oilfields with a high temperature (more than 45°C), the thermophilic representatives of the genus *Geobacillus* dominated [7].

This work is a continuation of the study of the aerobic HOBs properties inhabiting flooded oilfields. Contact of surface ecosystems with oil-bearing strata, in which HOBs are carried with pumped water, makes how HOB function in this environment a topical problem. The study of HOB adaptation to habitat conditions in oilfields offers an approach to solving this problem. The clarification of specific characteristics of HOB metabolism in flooded oilfields (the stratal waters of which normally contain acetate) was of special interest. Earlier model experiments showed that bacterial oxidation of hexadecane in the porous matrix was associated with the formation of a series of fatty acids, from acetate to palmitate [8].

The main goal of our investigation was to study the taxonomic identity and biological properties of the aerobic microorganisms isolated, allowing them to adapt to the habitat conditions (the oil-bearing pool) and determine the changes in the composition of *n*-alkanes (observed on oxidation of oil by pseudomonads under the conditions of oxygen deficiency). In order to reach this goal, we determined (1) the temperature parameters of growth and (2) characteristics of the medium (salinity and pH values), which enable HOBs to function in an oil-bearing stratum, as well as (3) the specific features of oxidation of oil *n*-alkanes by pseudomonads at different temperatures of culturing in a media with

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Table 1. Temperature and salinity parameters and the pH values for the growth of hydrogen-oxidizing bacteria

Microorganism	Source of isolation	Temperature, °C*	pH*	NaCl, %*
Romashkinskoe and Bondyuzhskoe oilfields (mineralization of stratal waters 3–272 g/l; pH 5.2–8.5; T = 30–35°C)				
D. maris H-2	oil	5–37 (28)	6.0–9.0 (7.0)	0–15 (5.0)
D. maris 7824 A	stratal water from the producing well	5–37 (28)	5.5–9.5 (7.0)	0–7 (0.5)
D. maris 4-3	stratal water from the producing well	5–37 (28)	6.0–9.0 (7.0)	0–10 (0.5)
R. ruber B	stratal water from the producing well	5–45 (28)	5.0–9.0 (7.0)	0–15 (5.0)
Gordonia sp. B	stratal water from the producing well	5–30 (25)	5.0–9.0 (7.0)	0–10 (0.5)
Mykhpaiskoe oilfield (mineralization of stratal waters 24–32 g/l; pH 6.8–8.4; T = 20–80°C)				
Rhodococcus sp. C A	stratal water from the producing well	5–30 (25)	5.0–9.0 (7.0)	0–10 (5.0)
Rhodococcus sp. C B	stratal water from the producing well	5–37 (25)	4.5–9.0 (7.0)	0–12 (5.0)
White Tiger oilfield (mineralization of stratal waters 35 g/l; pH 7.0–8.3; T = 37–50°C)				
P. stutzeri A	stratal water from the injection well	10–45 (37)	5.0–9.0 (7.0)	0–5 (0.5)
P. aeruginosa 202	stratal water from the injection well	10–45 (37)	5.0–9.0 (6.0)	0–5 (0.5)
P. pseudoalkaligenes 5	stratal water from the injection well	10–50 (37)	6.0–9.0 (7.0)	0–5 (0.5)

* The optimal value is in brackets.

restricted access to oxygen, containing organic compounds.

MATERIALS AND METHODS

Oil-oxidizing bacteria were isolated from the oil and stratal waters of Bondyuzhskoe and Romashkinskoe oilfields (Tatarstan), Mykhpaiskoe oilfield (western Siberia), and White Tiger oilfield (Vietnam). The stratal waters of the oilfields in Tatarstan were characterized by a low oil stratum temperature (30–35°C) and a wide range of salinity (from 3 g/l in the injection well bottom zone to 290 g/l in the pool brine). The stratal waters of Mykhpaiskoe oilfield were characterized by low mineralization (25–30 g/l) and a wide temperature range (from 20°C in the injection well bottom zone to 80°C in the stratal waters of the producing wells). The microorganisms of White Tiger oilfield were isolated from the hole clearance of the injection well, where the temperature was 37–50°C, and the pumped water salinity (35 g/l) did not change appreciably throughout the long-term development period (Table 1). The above oilfields were previously characterized in greater detail [2, 9, 10].

The isolation of pure cultures was carried out according to [2]. In identifying the microorganisms, the data and methods described in Bergey's Manual of Determinative Bacteriology [11], as well as in the works of Nesterenko *et al.* [12], Rainey *et al.* [13], and Suzuki *et al.* [14], were used. To determine salinity, temperature, and pH optima of growth, the bacteria were cultivated (stationary conditions) at 28°C in test tubes with Raymond's mineral medium [2] (containing 5 g/l glucose as the organic substrate) for 14 days. Biomass increment was determined by the value of the optical density at 540 nm. The same parameter was used to detect the growth of microorganisms in media with sodium salts of volatile fatty acids and ethanol (5 g/l). In a number of experiments, the GPY medium was used, which had the following composition (g/l): glucose, 5; peptone, 10; yeast extract; 5, NaCl, 5.

In experiments studying the effect of pH (4.0–10.0) on the growth of bacteria, the following dilute solutions were used as buffers: citric acid–Na_2HPO_4 (pH 5.0); MES–NaOH (pH 6.0); HEPES (pH 7.0); Tris (pH 8.0–9.0); and sodium carbonate–sodium bicarbonate (pH 10.0).

When the hydrocarbon-oxidizing activity of bacteria was determined, the inspissat oil of Bondyuzhskoe oilfield (2 vol %) was added to Raymond's medium. This oil is classified with high-sulfur type products (2.0–2.2 vol % sulfur) paraffinaceous (waxy) (4.0–4.1 vol %), with a specific gravity of 0.871–0.876 g/cm^3. To demonstrate the possibility of oxidation of the oil *n*-alkanes, gram-positive bacteria were cultivated 120 rpm and 28°C in 250-ml flasks with cotton wool stoppers; the volume of the medium was 70 ml. Pseudomonads were cultivated under stationary conditions in 100-ml flasks with butyl rubber stoppers without additional oxygen supply. The volume of the medium was 20 ml. Media containing (1) oil without the microorganisms and (2) pseudomonads without organic substrates were considered as controls. Sucrose, ethanol, acetate, propionate, and butyrate (20 g/l) were added to the oil-containing mineral medium as additional organic compounds. The cultures were incubated under stationary conditions at 28 and 45°C for 21 days. Hexane extracts of the culture liquid with bacterial cells and residual oil were used for analyzing the hydrocarbon phase.

n-Alkane degradation was determined by HPLC on a 3700 chromatograph with a flame ionization detector and a 25-mm capillary column. Apiesone served as the

Table 2. Results of the chemotaxonomic and molecular–genetic studies of *Dietzia maris* strains isolated

Organism	G + C in DNA, mol %	Glycolate test	Fatty acid composition	Menaquinones	Results of 16S rRNA sequencing	Total cell proteins (SDS-PAGE)
D. maris DSM43672 [13]	73.0	Acetyl	15 : 0, 16 : 0, 17 : 0, 16 : 1, 17 : 1, 18 : 1 (type IE)*	8(H_2)	–	–
D. maris H-2	66.6	Glycolyl	15 : 0, 16 : 0, 17 : 0, 16 : 1, 17 : 1, 18 : 1, 10Me19 (type IE)	8(H_2)	*D. maris* DSM 43672 cluster	*D. maris* DSM 43672 cluster
D. maris 7824 A	65.8	Glycolyl	15 : 0, 16 : 0, 17 : 0, 16 : 1, 17 : 1, 18 : 1, 10Me19 (type IE)	8(H_2)	*D. maris* DSM 43672 cluster	*D. maris* DSM 43672 cluster
D. maris 4-3	66.2	Glycolyl	15 : 0, 16 : 0, 17 : 0, 16 : 1, 17 : 1, 18 : 1, 10Me19 (type IE)	8(H_2)	*D. maris* DSM 43672 cluster	*D. maris* DSM 43672 cluster

* Type IE according to Suzuki and Komagata's classification [16].

stationary phase. Hydrogen was the carrier gas. The column temperature at the beginning of the analysis was 100°C; the final temperature was 320°C; the heating rate was 5°C/min. When quantitating the chromatograms of the aliphatic fraction of crude oil, we used the total length of the phytane and pristane peaks (*iso*-C_{19} + *iso*-C_{20}) as an internal standard. The procedure was considered correct if the standard *iso*-C_{19}/*iso*-C_{20} ratio was retained throughout the experiment. Thus, the calculation allowed estimating relative values of changes in the content of individual *n*-alkanes (chain length, C_{12}–C_{30}) in the culture (expressed as percentages of the oil content).

RESULTS AND DISCUSSION

Study of the taxonomy of the HOBs isolated. Eleven strains of hydrocarbon-oxidizing organotrophic bacteria were selected.

The eight strains isolated from the oil and stratal waters of Tatarstan and western Siberia oilfields were gram-positive bacteria. Based on the physiological and morphological characteristics, they were identified as representatives of the genera *Rhodococcus, Gordonia*, and *Dietzia* (Table 1). Some of the strains isolated were close in physiological and biochemical characteristics to the genus *Rhodococcus* and, in particular, to the strain that was known earlier as the strain type *R. maris* [12]. Later, Rainey *et al.* [13] provided a detailed substantiation of classifying *R. maris* DSM 43672 with the genus *Dietzia.* A number of our strains were assigned to the cluster of *Dietzia maris* (data generated by T.P. Tourova), as evidenced by the results of 16S RNA sequencing (the same conclusion was also made based on the results of SDS-PAGE of total cell proteins of these strains [15]). The data allowing us to assign the strains isolated to the genus *Dietzia*, presented in Table 2. The main distinctions from strain type *D. maris* DSM 43672 consist in the lower G + C content, with the presence of 10 methyl-octadecanoic (10-Me-C_{19}) acid in the composition of cellular fatty acids, and in the glycolyl type of the cell wall of our strains.

The three strains isolated from the stratal water of the bottom zone of injection well 202 (White Tiger oilfield) were identified by the results of 16S rRNA sequencing (data generated by T.P. Tourova) as gram-negative *Pseudomonas* spp.: *P. stutzeri* A, *P. aeruginosa* 202, and *P. pseudoalkaligenes* 5. The latter bore maximum resemblance to *P. pseudoalkaligenes* and *P. mendocina.* However, the absence of the yellow pigment gave us grounds to attribute this microorganism to *P. pseudoalkaligenes* [11].

Temperature and saline growth parameters, pH range, and growth in the presence of oxidized organic substrates. The results of Table 2 showed that all the microorganisms studied were mesophiles and neutrophiles. The gram-positive isolates assigned to the genera *Rhodococcus, Gordonia*, and *Dietzia* grew, with some exceptions, at lower temperatures (5–37°C, with an optimum at 28°C) than pseudomonads. The values of pH for most of the isolates were in the range of 5.0–9.0 (Table 2). *D. maris* strains and rhodococci exhibited greater halotolerance than pseudomonads. Most strains from the genera *Dietzia* and *Rhodococcus* grew in a medium with glucose both in the absence of NaCl and at NaCl concentrations of up to 10–15%. Their optimum growth was observed at NaCl concentrations equal to 0.5 and 5%. It should be noted that maximum salinity of the medium, at which the growth of rhodococci and *Dietzia* strains was observed, varied with the substrate used. For example, strain *D. maris* 7824A grew at a NaCl content of up to 7% (in medium with glucose), 10% (in medium with C_{14}–C_{16} *n*-alkanes), and 15% (in GPY medium and in media with acetate and glutamate). Many microorganisms are known to accumulate glutamate during osmotic stress [17], and the yeast extract contains glycine-betaine [18], which is an osmoprotector.

Table 3. Growth of hydrocarbon-oxidizing bacteria in different oxidized organic substrates

Microorganism	Optical density(OD_{540}) in media with substrates*					
	Formate	Acetate	Propionate	Butyrate	Ethanol	Sucrose
D. maris H-3	0.20	2.48	0.85	0.66	0.70	2.05
D. maris 7824 A	0.23	1.50	0.54	0.64	1.44	1.38
D. maris H-2	0.20	1.32	0.48	0.66	0.95	2.34
R. ruber B	0.10	0.72	0.66	0.48	0.45	2.28
Gordonia sp. D	0.10	2.00	3.70	2.90	1.50	1.02
Rhodococcus sp. C A	0.20	2.00	2.10	3.50	5.00	3.00
Rhodococcus sp. C B	0.20	1.62	0.75	0.90	1.05	3.60
P. stutzeri A	0.52	1.52	1.68	2.24	2.0	1.60
P. pseudoalkaligenes 5	0.14	1.00	1.50	1.25	1.35	1.2
P. aeruginosa 202	0.24	1.25	1.65	1.20	1.50	1.08

* Increment relative to the control (the initial value).

All the microorganisms studied grew at the expense of acetate oxidation; propionate, butyrate, ethanol, and sucrose (Table 3) served as additional substrates in oil oxidation experiments. An increase in the optical density of formate-containing medium was observed only with the culture of *P. stutzeri* A (Table 3). In addition, all the isolates oxidized *n*-alkanes of crude oil (the length of carbon chains varied from C_{12} to C_{28}–C_{30}).

Studies of oilfields conducted by us over the last ten years [2, 3], together with the results represented in this article, showed that halotolerant microorganisms predominated in both the oil and the stratal waters of different mineralization (3–273 g/l). Bacteria of the genus *Rhodococcus* and the strains of *D. maris* were predominant. Prevalence of rhodococci in oil production regions over a broad geographical range was also noted by other researchers [4]. The results obtained showed that, in oil stratum, gram-positive bacteria migrate with oil from the injection well bottom zones to the oil-producing zones, and they can be detected in the stratal waters recovered. Oil seems to contribute to the cell viability and proliferation. The microorganisms' tolerance of the high salinity of oil stratum brines also contributes to their survival. Thus, part of the microorganisms that gain entrance into the pumped water with aerobic microflora become permanent inhabitants of flooded oilfields. The microorganisms isolated are adapted to the ecological habitat conditions, judging by the temperature, salinity, and pH optima for growth (Table 1). In addition, they are capable of growing due to the oxidation of oil hydrocarbons.

Effects of the cultivation temperature and additions of organic substances on the degradation of *n*-alkanes of oil in cultures of two pseudomonade strains. After three weeks of *P. aeruginosa* 202 cultivation at 28°C (optimum growth temperature), the spectrum of *n*-alkanes changed (Fig. 1a). In the control variant (without the supplements of additional organic compounds), the degradation extent of *n*-alkanes increased with their molecular weight (100% for C_{12} and 0% for C_{28}–C_{30}). A decrease in the oxidation efficiency of paraffin hydrocarbons was more pronounced the longer the carbon chains were; this is inherent in microorganisms from different taxonomic groups [19]. The addition of GPY barely, if at all, increased the efficiency of oil hydrocarbon oxidation, while the introduction of individual organic compounds lowered the oxidation capacity of C_{12}–C_{16} *n*-alkanes (as compared to the oil-containing mineral medium control, Fig. 1a). This may be related to the mechanism whereby the oxidation of hydrocarbons is inhibited by metabolic products [20] or predominant oxidation of the substrates is added.

The cultivation of *P. aeruginosa* 202 at 45°C (maximum or near-maximum growth temperature) increased the efficiency of hydrocarbon oxidation and the residual content of oil *n*-alkanes (chain length, C_{12}–C_{27}), varied from 12 to 39% of the initial value (Fig. 1b). The addition of GPY did not affect the oxidation of alkanes. Individual organic compounds adversely affected paraffin degradation. Moreover, an introduction of salts of lower aliphatic carbonic acids increased the content of *n*-alkanes (chain length, C_{12}–C_{27}) in the culture to levels higher than that characteristic of oil (Fig. 1b).

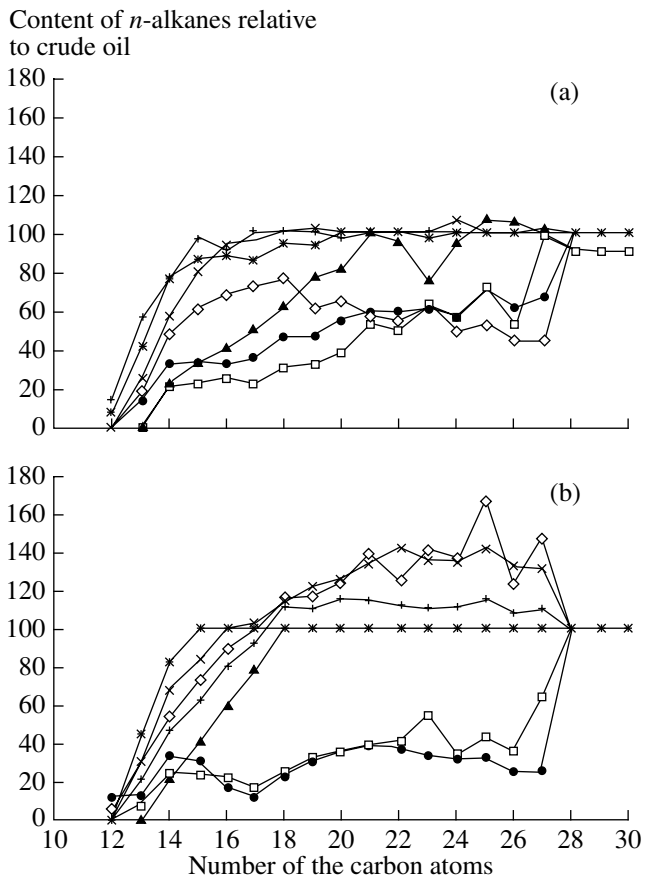

Fig. 1. Change in the relative content of *n*-alkanes in oil during *P. aeruginosa* 202 development: (a) incubation at 28°C and (b) incubation at 45°C (● oil; □ oil + GPY; ▲ oil + sucrose; ◇ oil + acetate; × oil + propionate; + oil + butyrate; ∗ oil + ethanol).

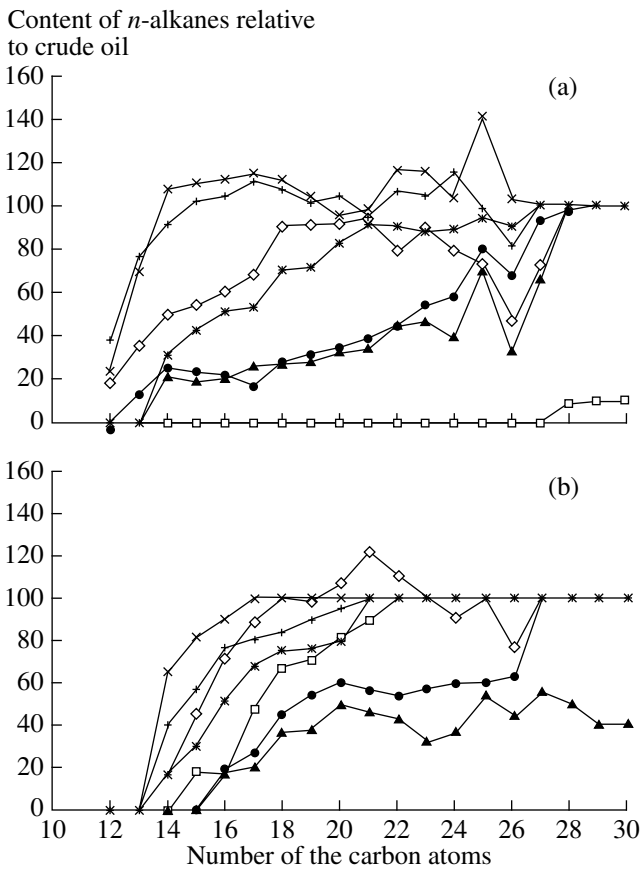

Fig. 2. Change in the relative content of *n*-alkanes in oil during *P. pseudoalkaligenes* 5 development: (a) incubation at 28°C and (b) incubation at 45°C (the designations are the same as for Fig. 1).

When the strain *P. pseudoalkaligenes* 5 was cultivated at 28°C for three weeks, the content of *n*-alkanes changed (Fig. 2a). The character of oil oxidation in the control variant (without the addition of organic substances) was close to the variant with *P. aeruginosa* 202 cultivated at 28°C (Fig. 1a). When GPY was added complete oxidation of almost all of the *n*-alkanes determined was observed, which may be due to the activation of plastic metabolism. The addition of acetate and ethanol decreased the efficiency of paraffin oxidation (compared to the control variant); in variants with an addition of propionate and butyrate, even an increase in the content of C_{14}–C_{26} *n*-alkanes was observed (compared to their petroleum content). The introduction of sucrose did not change the capacity of paraffins for oxidation (as compared to the control variant).

In the case of *P. pseudoalkaligenes* 5 cultivation at 45°C, the changes in the spectrum of *n*-alkanes oxidized were of a different character (Fig. 2b). The addition of GPY substantially decreased the efficiency of oxidation of these hydrocarbons. While on the contrary, the introduction of sucrose facilitated the process of biodegradation. The effect of the addition of methanol was similar to what was observed in the experimental variant with the culture grown at 28°C. The introduction of acetate, propionate, and butyrate decreased the efficiency of the degradation of alkanes, but their residual content increased (without exceeding the content in oil; the effect of acetate on C_{20}–C_{21} *n*-alkanes was an exceptional case). The increment in the content of individual *n*-alkanes (relative to their content in crude oil), observed in oil cultures of pseudomonads in the presence of fatty acids (Figs. 1b, 2a, 2b), may be accounted for by their intracellular de novo synthesis. According to the literature data, many bacteria, yeasts, and algae synthesize and accumulate hydrocarbons intracellularly (inside the cells) when grown in different organic substrates, including acetate, and their content in bacterial cells attains 2.8% of dry biomass weight. The amount and composition of intracellular hydrocarbons varied, depending on the medium composition, culture age, species of microorganism, and extraction methods [21, 22].

The pseudomonads studied by us slightly differed in the spectrum of the hydrocarbons synthesized:

P. aeruginosa 202 synthesized C_{18}–C_{27} *n*-alkanes and *P. pseudoalkaligenes* 5, C_{14}–C_{26} *n*-alkanes. This agrees with the literature data on the diversity of intracellular paraffin hydrocarbons of microorganisms and the predominance of C_{16}–C_{32} *n*-alkanes [22]. Theoretically, several mechanisms of intracelluar *n*-alkane synthesis are considered [21]. These include the condensation of two CoA derivatives of fatty acids accompanied by decarboxylation of one of them. Elongation of the carbon chain synthesized by the addition of palmitic (hexadecanoic) acid and its decarboxylation followed by the recovery of the intermediate product is suppose to occur in the process of intracellular *n*-alkane synthesis. We did not observe the synthesis of *n*-alkanes in the cells of pseudomonads grown on fatty acids in the absence of oil. Therefore, oil-derived *n*-alkanes with a shorter carbon chain and, additional salts of fatty acids likely served as growth substrates for the synthesis of C_{14}–C_{27} hydrocarbons in these experiments. According to the data of Kvasnikov *et al.* [23], yeasts grown in media with low-molecular-weight (C_8–C_{10}) n-alkanes accumulated C_{15}–C_{22} *n*-alkanes in the cells, and their amount was greater than that observed when they were grown in a glucose medium. According to the literature data, *n*-alkanes with even and odd numbers of carbon atoms can be synthesized from fatty acids with a different chain length. Interestingly, in the variant with oil and an addition of acetate and butyrate, *P. aeruginosa* 202 (cultured at 45°C) synthesized predominantly *n*-alkanes with an odd number of the carbon atoms (Fig. 1b). In the variant with petroleum and acetate, the culture of *P. pseudoalkaligenes* 5 also accumulated C_{21} *n*-alkane (45°C, Fig. 2b). Propionate stimulated C_{25} alkane formation in the cultures of both pseudomonads grown in the petroleum medium (Fig. 1b, 2a).

Thus, in acting on *n*-alkanes of crude oil, the microorganisms studied oxidized hydrocarbons in a way similar to those documented for other HOBs (short-chain *n*-alkanes were oxidized more efficiently than their long-chain counterparts). In addition, at certain temperatures, high-molecular-weight paraffins were synthesized by pseudomonade cells grown in oil in the presence of fatty acids. The latter result constitutes a new finding, although such studies have not been performed thus far. These observations allow us to consider the vital activity of HOBs to be a factor contributing to the increase in the density and viscosity of residual oil in injection well bottom zones of flooded oilfields.

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