Biodegradation of petroleum hydrocarbons by bioflocculant-producing microorganisms of the aquatic ecosystems in the Arctic region

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Abstract. Currently it is not feasible to completely eliminate the contamination of water bodies with oil, which makes it relevant to perform activities aimed at addressing this environmental challenge. Biological method of cleaning water could be recognized as an effective means of environmental protection. Increasingly, bioremediation uses indigenous microorganisms of specific contamination sites, since these organisms are adapted to the prevailing climatic and environmental conditions, which confers the advantages. Consequently, studies aimed at isolation and selection of the most active microorganisms oxidizing petroleum hydrocarbons and capable of using them are particularly relevant. The aim of this study is the search and selection of effective microorganisms-degraders of petroleum, maximally adapted to the conditions of the Far North. In this study, hydrocarbon-oxidizing features of indigenous bacteria in the Arctic region were examined, and the most effective strains for devising the bioremediation agents were selected. In all strains of bacteria tested that demonstrated steady and intensive growth in the medium with diesel fuel the following characteristics were studied: the percentage of oil degradation in the conditions of laboratory model experiments, the indicator of hydrophobic indicator, and growth potential as well. Strains of microorganisms with the highest degrading ability were selected: A. eucrenophyla, Ps. lundensis, Ps. fragi. These bacterial strains are promising as biodecomposers of hydrocarbons and can be used in bioremediation of environmental contamination. Bacteria from the isolated strains are persistent and dominant components of the natural aquatic ecological communities, thereby the necessity of taking into account the climatic conditions of the region could be avoided.

1. Introduction
Contamination with petroleum products has become an urgent issue so far [1]. In our country, the problem of pollution with substances containing hydrocarbons are most relevant for the Arctic region, where there are many oil refineries, natural and climatic conditions of the area can provide only a low speed of the processes of self-purification of objects contaminated with oil [2]. Microorganisms capable of biodegradation of petroleum hydrocarbons are well aware of which makes it possible to use them for bioremediation of polluted aquatic ecosystems. Thus, the actual issue is the search and selection of bacteria able to grow and perform intense biochemical activity in the environment with a high content of petroleum hydrocarbons, which is likely to cause biodegradation of these chemicals in future.

Intensive development of artificial “microorganisms” or oil-decomposers as well as the selection and use of various natural microorganisms that use oil as a source of carbon and energy and aimed at
application in certain climatic conditions is being carried out. Advantages of using indigenous bacteria isolated from natural habitats are coupled with their resistance to biotic environmental factors and adaptation to it. Thus, for the geographic area it is promising to develop the home biological product on the basis of the indigenous strains of microorganisms [3]. At the same time, the selection of hydrocarbon-utilizing bacteria that inhabit the Northern areas, should be carried out in terms of their adaptation to low temperatures.

Search in this area will allow to find organisms, actively transforming oil technogenic pollutants, to select strains with a high capacity and selectivity of these contaminants, to assess the possibility and expediency of their application for solving the practical problems in the treatment of industrial and sewage polluted aquatic ecosystems and on their basis to create promising biodegradation agents.

The aim of this study was to examine the ability of bilflocculant-producing bacteria *Pseudomonas lundensis*, *Aeromonas eucrenophyla* and *Pseudomonas fragi* to biodegrade petroleum hydrocarbons and to assess the possibility of using them as decomposers of oil pollution in water objects.

2. Materials and methods

Microbial strains with flocculating activity of more than 60 % were selected from the sludge of sewage treatment plants and freshwater watercourses of the Murmansk region. The isolated strains were identified by the analytical method of mass spectrometry as *Ps. lundensis* strain No. 1, *A. eucrenophyla* strain No. 2, *Ps. fragi* strain No. 3-5 [4].

Pure cultures of microbial strains were added to liquid culture media supplemented with 1% diesel fuel of brand "summer". Experimental model nutrient media varied in initial ratios of biogenic elements (carbon/nitrogen, carbon/phosphorus and nitrogen/phosphorus) and corresponded to the original data of the natural habitat of the bacteria isolated.

The nitrogen sources were (NH₄)₂SO₄, NaNO₃ and NaNO₂, diesel fuel served as a carbon source, while yeast extract was added into the composition of the substrate to meet the requirements of microorganisms in vitamins and other growth factors. Similar cultivation conditions were set for all model media and bacterial strains in the experiments without any exception [5].

Each medium was made supplied with 1 ml of culture of the strains containing 0,6×10⁹ cells. Cultivation temperature was 7±2 °C. The incubation time lasted 21 days. The initial features of microbial growth (turbidity of the medium, the bottom or wall growth, the formation of flakes) was observed. Changes in the concentrations of bacterial cells of inocculators were determined using McFarland standard. This method provides standardization of the number of bacteria in suspension without the use of spectrophotometric methods.

Mass concentration of hydrocarbons was determined using fluorimetric method on the apparatus "Fluorat-02". The suspension of microorganisms was incubated with hydrocarbons (1% diesel) in a nutrient medium for 21 days [6].

Mass concentration of oil products in the sample was calculated by the formula:

$$X = \frac{(X_1 \times V_g \times K)}{V}$$

where X is mass concentration of petroleum products in the sample, mg/l; $X_1$ – mass concentration of oil products in the hexane extract of the sample, mg/l; $V_g$ – volume of hexane, taken for extraction, ml; K – dilution factor of the extract; $V$ – volume of sample, ml

The residual concentration of diesel fuel in the study sample was recalculated into the percentage, taking the control sample for one hundred percent. After that, the concentration of petroleum products decomposed with microorganisms, was calculated by subtracting the residual concentration of petroleum products out of a hundred.

The hydrophobic indicator of the obtained microorganism strains was defined. In determining the hydrophobicity of the bacteria cell surface Serebryakova's method was used [7]. The hydrophobic indicator is a crucial physico-chemical characteristics which shows the ratio of hydrophilic and
hydrophobic components of the cells in the surface layers of their shells. This indicator is one of the key characteristics of the cell surface that determines the adsorption immobilization of microorganisms. Hydrophobic-hydrophilic nature of the microorganism surface as an integral indicator of its structure should be considered in biological research when studying the adhesion processes of cells and surfaces of different structures, as well as their growth on hydrocarbons associated with the direct consumption of the substrate. When interacting with hydrocarbon substrate, oil-oxidizing bacteria are known to contact with hydrocarbons directly due to the hydrophobic cell surface and the presence of lipid components [8, 9].

Chloroform was used as the hydrocarbon phase for defining the hydrophobic indicator. Bacterial biomass was washed off the agar with phosphate buffer. One milliliter of cell suspension was diluted ten times, then optical density (OD_0) was measured using photoelectrocolorimeter KFK-3. Bacterial suspension of chloroform was shaken and defecated for separation of the phases "hydrocarbon-water".

The hydrophobic indicator (%) was calculated using the formula
\[ IH = 100 - \left( \frac{OD_1 \times 100\%}{OD_0} \right) \] (2)

where OD_0 – initial optical density of bacterial suspension, standard unit;
OD_1 – optical density of the suspension after shaking with chloroform, standard unit.

This method of determining the hydrophobicity is based on the assessment of the degree of cell attachment to the surface of the phase separation of hydrocarbons and water after mixing and subsequent separation of the biphasic system "hydrocarbon-water".

3. Results
The perspectivity of microorganisms - crude oil degraders is determined by their physiological and biochemical properties: primarily by their ability to rapidly utilize petroleum hydrocarbons, high hydrophobicity, ability to adapt to the environment and abundance in aquatic ecosystems.

Bacterial strains of *Pseudomonas lundensis*, *Aeromonas eucrenophyla* and *Pseudomonas fragi* had positive growth dynamics in all nutrient media (NM) supplemented with 1% diesel fuel as the only carbon source (Figure 1). The first signs of microorganism growth in nutrient media were observed on the 15th day of cultivation of all cultures except *A. eucrenophyla* in the reference nutrient medium. The adaptation of the culture to the environment conditions, induction of enzyme systems, activation of the microorganisms occurred in the period of the lag phase. In the process of cultivation enzymes of hydrocarbon oxidation gradually accumulate which causes further utilising of hydrocarbons.
Figure 1. The growth dynamics of the examined bacterial strains of *Ps. lundensis*, *A. eucrenophyla* and *Ps. fragi* on model nutrient media (NM) with diesel fuel

Maximum growth rate was observed in the culture *Ps. fragi* No. 3 on all model nutrient media. This strain was isolated from municipal wastewater. The strain *Ps. lundensis* was characterized by the lowest values. The largest increase in the number of microorganism cells was observed in the nutrient medium of NM No. 2 (Figure 1).

Forming flakes is a significant ability of microorganisms - crude oil decomposers in biodegradation of petroleum hydrocarbons. Flocculation was observed from the 15th day of cultivation. Cultural liquids: *Ps. lundensis* in NM No. 3, *A. eucrenophyla*, *Ps. fragi* No. 4 and No. 5 in all NM, *Ps. fragi* No. 3 in NM No. 2. did not develop flocculation.

The degradation of oil components was evaluated at 7±2 °C under the conditions of cultivation in liquid media. Separate flasks placed with samples were used as control during certain time periods to assess evaporation of light fractions of oil.

Table 1 represents the data of fluorimetric measurement of residual oil content on model culture media after the microorganism growth of the investigated strains. Residual content of oil hydrocarbons was determined in the liquid phase after the certain time of cultivation.

**Table 1.** Experimental and calculated data on the efficiency of cleaning the model nutrient media from diesel fuel

| Model nutrient media | Microorganism strain | Initial concentration of diesel oil, mg/l | Final concentration of diesel oil, mg/l | Efficiency of diesel oil removal, % |
|----------------------|----------------------|-----------------------------------------|----------------------------------------|----------------------------------|
| NM No1               | *Ps. lundensis*      | 25±6                                    | 6,0±1,5                                | 76,0                             |
|                      | *Ps. fragi* No 1     |                                         | 6,4±1,6                                | 74,4                             |
|                      | *Ps. fragi* No 2     |                                         | 6,2±1,6                                | 75,2                             |
|                      | *Ps. fragi* No 3     |                                         | 5,6±1,4                                | 74,5                             |
|                      | *Ps. lundensis*      |                                         | 9,8±2,4                                | 55,4                             |
|                      | *A. eucrenophyla*    |                                         |                                        |                                  |
| NM No2               | *Ps. fragi* No 1     | 22±5                                    | 3,8±1,0                                | 82,7                             |
|                      | *Ps. fragi* No 2     |                                         | 5,3±1,3                                | 75,9                             |
|                      | *Ps. fragi* No 3     |                                         | 7,0±1,7                                | 68,2                             |
Figure 2 presents data on changes in the concentration of decomposed oil products in model nutrient media. Determination of the residual content of diesel fuel components in media after 21 days of cultivation showed that the percentage of degradation varied from 40 to 98%. The highest degradation rate of diesel fuel was observed in A.eucrenophyla strain in NM No. 3 - 97.8%, and the lowest - 40% in Ps. fragi No. 1 in NM No. 4.

![Figure 2. Degree of diesel fuel destruction on model nutrient media (NM) at 7 °C.](image)

![Figure 3. Hydrophobic indicator for the examined microorganism strains.](image)

Experiments on degradation of petroleum hydrocarbons showed that microbial communities have similar degradation activity by the 20th day of the experiment, despite the difference in cell numbers. It should be noted that the results were comparable with previously published data. The degree of biodegradation of oil at low positive temperatures after 20 days of cultivation for different microbial communities ranged from 21 to 70% [10].

The ability of microorganisms to decompose hydrocarbons is largely determined by the structural features of the cell membrane. With the hydrophobicity value of more than 20%, bacteria are selected as producers of biologcal surface active agents. The data presented in figure 3 show that bacteria of the genus Pseudomonas are characterized by high values of hydrophobic indicator.

4. Results
Thus, indigenous microorganisms A. eucrenophyla, Ps.lundensis, Ps. fragi, able to utilize hydrocarbon oil components for their metabolism were selected from different water bodies. The selected bacteria...
are the permanent components of the aquatic biocenoses of the far North, so it is not needed to take into account the climatic conditions of the region.

The change in the number of bacterial strains cells in model media containing diesel fuel as a carbon source was assessed. The bacterial strain *Ps. fragi* No. 3 was revealed to have the maximum growth rate in the environments with petroleum hydrocarbon. For the selected cultures, hydrophobic indicators were identified (the bacteria of the genus *Pseudomonas* has the maximum value). High hydrophobic activity indicates the affinity of the cell wall of the bacteria and the substrate, or petroleum products. Therefore, it facilitates the process of cell interaction with the molecules of petroleum products and enhances the process of hydrocarbon biodegradation due to the activity of the selected microorganisms.

The study found out that the most active hydrocarbon decomposers among the examined cultures were: strains of *A. eucrenophyla*, *Ps. lundensis*, *Ps. fragi* 3, utilized up to 90% of the hydrocarbons of diesel fuel to by the 21st day of cultivation. Thus, a high hydrocarbon-oxidizing capacity allows to use these bacterial strains as a basis for devising a bioremediation agent for oil spills in the Arctic region. This data suggests that cultures of these isolates could be further used for wastewater treatment, and on their basis to devise biological products for cleaning the water bodies from petroleum hydrocarbons contamination.

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