Biodegradation of black oil by microflora of the Bay of Biscay and biopreparations

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Abstract

Recent research has shown that the bioremediation of oil-polluted soil by activation of microflora is not always advantageous when compared with the introduction of oil degrading microorganisms. Although microflora isolated from oil-polluted sites is adapted for growth under the above conditions, this does not imply a high rate of oil degradation. Indeed, it has been shown that the hydrocarbon-oxidative activity of isolated strains to black oil was 13.1–17.3% (incubation for 10 days at 24 °C), while the activity of a mixture of three aboriginal strains was 17.8%. At the same time, the hydrocarbon-oxidative activity of associations of the strains isolated from other regions was 24.0–30.0% (in 10 days).

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1. Introduction

In the spring of 2000, a black oil-carrying tanker was shipwrecked near the French shore in the Bay of Biscay. As a result, about 10,000 tonnes of black oil ran out into the ocean polluting the near-shore water area, cliffs and sand beaches of the Bay of Biscay.

Microorganisms play the main role in biodegradation of oil hydrocarbons, along with physicochemical processes. More than 70 genera of microorganisms capable of utilizing hydrocarbons as a sole carbon source are known at present [1–3].

There are two principal approaches to bioremediation of oil pollutions

- stimulation of the activity of aboriginal hydrocarbon-oxidative microflora by improvement of aeration, watering, introduction of biogenic elements and biologically active compounds [4,5];
- introduction of active strains of hydrocarbon-oxidative microorganisms and their associations into polluted objects as a biopreparation [6–8].

Introduction of oil degrading strains is most advisable under unfavourable conditions, e.g. in the northern regions with a short vegetation period or when oil gets into water, where the enrichment culture based on the natural microflora forms very slowly even under optimal conditions [3]. The introduction of biopreparation decreases the pollutant concentration and may be used at a pollution level of 20%.

At the same time, a number of scientists reject the introduction of biopreparations into a polluted environment, supposing it to disturb the natural ecological situation [9].

Soils with a low initial concentration of pollutant (up to 101/m2 oil), in the opinion of some researchers, can be remediated only by stimulation of the activity of aboriginal hydrocarbon-oxidative microflora by loosening, watering, and introduction of the sources of biogenic elements and bioactivators [7–11].

In the opinion of some researchers, the aboriginal hydrocarbon-oxidative microflora of polluted sites has an indisputable advantage over the introduced microorganisms, because it is most adapted for growth under the prevailing conditions [9].

One of the methods of bioremediation is the use of biopreparations containing oil-degrading microorganisms isolated from oil-polluted soil samples and the growth of their
biomass followed by introduction into a polluted object from where they had been isolated.

The goal of the present work was to study the hydrocarbon-oxidative activity of the aboriginal microflora of oil pollutions in the Bay of Biscay and to choose an optimal method of bioremediation of this pollution.

2. Materials and methods

The strains of aboriginal oil-degrading microorganisms have been isolated by a standard method of enrichment cultures from samples taken in the accident area a month after the tanker wreck.

The following strains from the collection of the sector “Biopreparations” of the Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (IBPM RAS): Pseudomonas putida Sh-1 and Rhodococcus sp. Sh-5 (isolated from oil-polluted soil samples of Tyumen), which constituted Biopreparation 1, and the strains from the collection of the Department of Ecoligical Biotechnology, State Research Center of Applied Microbiology (SRCAM): Pseudomonas sp. strain B, Flavobacterium sp. 415, and Bacillus sp. (isolated from water samples of the White Sea (the region of Arkhangelsk) polluted with oil products), which constituted Biopreparation 2, have also been used in experiments. All strains are able to actively assimilate diesel fuel, oil and black oil as the sole carbon and energy source.

Oil-degrading activity was determined by culturing the strains isolated in the course of research, as well as Biopreparations 1 and 2, in flasks with a fluid synthetic nutrient medium containing black oil as the sole carbon and energy source. The medium composition has been described in the previous paper [12]. Black oil in the amount of 1 wt.% was introduced into flasks after sterilization of the nutrient medium. Then the flasks were inoculated either with microorganisms sorbed on a carrier (later sorbed by biopreparation) or with a suspension of microorganisms. Cultivation proceeded at 24 °C on a shaker at 180 rpm during 10 days.

The carrier used in some experiments for immobilization of the biopreparations was peat with granules of 3–7 mm in size, foamed in a thermal furnace. The mineral composition of a sorbent was as follows: Al2O3: 13–14%, MgO: 13–14%, K2O + Na2O: 2–6%, CaO: 1–3%, H2O: 2–4%, SiO2: 59–69%.

To obtain a sorbed biopreparation, the cultures of oil-degrading microorganisms constituting the biopreparation were inoculated by lawn on LB agar. After 24 h of growth at 24–26°C, cells were washed off with a mineral medium of the above composition and cell suspensions were mixed in equal proportions. This suspension of microorganisms was mixed with peatlite in a ratio 1:1 (by weight) and the sorbent with immobilized biopreparation was dried at 25°C for 24 h. The humidity of the sorbed biopreparation was 1%, and the specific concentration of viable cells was about 10^9–10^10 cells/g sorbent, depending on the biopreparation properties.

For a laboratory soil experiment, 2.5 wt.% black oil as a solution in hexane was introduced into loamy sand and thoroughly homogenized. Hexane was evaporated in an exhaust-hood at room temperature for 3 days with periodical mixing of the soil. Weighed portions of the soil prepared by the above method (40 g) were placed in Petri dishes, where nitrogen, phosphorus and potassium (NPP) salts were added in the form of azophoska as a source of biogenic elements. Tap water in the same volume was added into control variants instead of the salts’ solution.

Biopreparation 2 was used in the soil experiment. The biopreparation immobilized on the carrier was introduced and thoroughly mixed with the soil.

These prepared soil samples were incubated in a thermostat at 23–25°C. Soil humidity during the whole experiment was maintained at 60% total moisture capacity.

Samples for microbiological and chemical analyses were taken immediately after the beginning of the experiment (initial point) and then on Days 15, 30 and 45. The total quantity of microorganisms in the soil was determined by inoculation from dilutions on a surface of enriched agar medium (LB agar) by standard methods [12].

The content and fractional composition of residual hydrocarbons was assayed by the methods described previously [13]. To determine the proportion of black oil in the soil, the entire contents of one dish were used as one repetition (six repetitions per experiment).

3. Results and discussion

The accidental black oil spill resulted in pollution of not only the near-shore water area but also the cliffs and sand beaches of the Bay of Biscay. Such a large-scale ecological disaster could not but affect the microbiological condition of the region of the accident. With regard to the fact that the quantity of aboriginal microorganisms in the sea water, sand soils and on cliffs is usually rather low and that the accident took place in spring (cold season), it may be assumed that the total titre of microorganisms in samples from the site of accident would also be low. However, contrary to these assumptions, the quantity of microorganisms in the samples under study was 10^3–10^5 cells/g sample. Research data are presented in Table 1.

Such a high quantity of microorganisms in the samples under study is probably explained not only by the activation of aboriginal microflora due to the hydrocarbon substrate added but also by the introduction of microorganisms along with black oil. It is interesting that the titre of microorganisms in samples from the cliffs is by an order higher than in other samples. This is probably due to the more favorable thermal and air regimes of the cliffs, which is a significant factor of microflora development, particularly in the cold season.
It is also necessary to mention the high titre of microorganisms in water samples from the near-shore water area. The above data contradict the data of other researchers, since it has been reported that oil-oxidizing microorganisms are actually absent in the open sea. The key role in elimination of oil exposed to the Mediterranean Sea isms are actually absent in the open sea. The key role in elimination of oil exposed to the Mediterranean Sea.

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The data on black oil extraction by petroleum ether, hexane, and diesel fuel are interesting (Table 3). If the amount of admixtures (about 55%) are subtracted from the values given in Table 3, then a certain difference will remain (about 9% for petroleum ether, 35% for hexane, and 40% for diesel fuel). This difference cannot be attributed to the better extractability of black oil by the above solvents. On the contrary, in this case not only dissolution of black oil in solvent but also dissolution of solvent in black oil was observed, because all the above solvents (petroleum ether, hexane and diesel fuel) are hydrocarbons. Thus the solvent–black oil mixtures obtained, in all appearances, are more stable at room temperature than the chloroform–black oil mixture. Therefore the solvents, such as petroleum ether, hexane and diesel fuel are not evaporated completely at room temperature.

3%, sand 2%, and water about 50% of the total weight of black oil. The admixtures were most likely present because black oil for this study was taken not from the tanker (which probably was technically difficult to realize) but from a polluted beach.

The activity of consumption of “Biscay” black oil by an association of two strains (Sh-1 and Sh-5, Biopreparation 1) was studied in one of the experiments. In this experiment, the biopreparation was used in two forms: fluid (cell suspension) and sorbed (immobilized on pearlite). The results of these experiments are given in Table 5.

The analysis of the data from Table 5 shows that the degree of black oil degradation by Biopreparation 1 was almost 10% higher than in the association based on aboriginal strains.

As might be expected, the quantity of microorganisms in relation to “Biscay” black oil showed the degree of destruction (the titre of microorganisms immobilized on pearlite was 2 × 10^{10} cells/g of dry weight). It is also known that the immobilized form not only significantly reduces the concentration of oil and oil products but also improves hydro-aerial, physical and agrochemical properties of the soil [11]. Besides, the sorbed biopreparation is more convenient for transportation than its fluid forms, therefore in some cases it is more preferable to use the biopreparation immobilized on a carrier.

Comparative analysis of the above data showed that the activity of Biopreparation 2 was a little higher than Biopreparations 1 and 2 was used in further investigation.

The next stage of research was to study the hydrocarbon-oxidative activity of Biopreparation 2 in a model soil system. As the accidental black oil spill in the Bay of Biscay resulted in the sand shore pollution, non-sterile sandy loam was used in a laboratory soil experiment. The results of the soil experiment are given in Fig. 1.

As might be expected, the quantity of microorganisms in the variant with the biopreparation was appreciably higher than in other variants. The initial titre of microorganisms in the control was low, which was most likely determined by depletion of aboriginal microflora in the sandy loam used in the experiment.

### Table 4

<table>
<thead>
<tr>
<th>Strain</th>
<th>Titre of microorganisms (cell/ml of medium)</th>
<th>Biodegradation (%)</th>
</tr>
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<tbody>
<tr>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>3.4 × 10^{8}</td>
<td>1.7 × 10^{11}</td>
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<tr>
<td>F2</td>
<td>3.8 × 10^{8}</td>
<td>5.4 × 10^{10}</td>
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<tr>
<td>F3</td>
<td>1.4 × 10^{9}</td>
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<td>Mixture</td>
<td>8.4 × 10^{9}</td>
<td>1.5 × 10^{11}</td>
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</table>

### Table 5

<table>
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<tr>
<th>Variant</th>
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<th>Biodegradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Fluid</td>
<td>4.0 × 10^{8}</td>
<td>2.7 × 10^{11}</td>
</tr>
<tr>
<td>Adsorbed</td>
<td>3.3 × 10^{8}</td>
<td>5.3 × 10^{10}</td>
</tr>
</tbody>
</table>
Fig. 1. Microorganisms kinetics and quantity of black oil in a laboratory soil experiment: (1) biopreparation + NPK; (2) NPK (the left axis of ordinates on the figure shows the quantity of microorganisms in 1 g of soil (graphs), the right axis of ordinates—biodegradation of black oil in percentage (histograms), the axis of abscissas—the time after the first inoculation of microorganisms into soil in days; dotted line shows the quantity of microorganisms in the control).

The titre of microflora was shown to increase in all variants during the experiments, indicating the assimilation of black oil hydrocarbons by microorganisms. By the end of the experiment, on day 45 of incubation, there was a certain slowdown in the growth of the quantity of bacteria in all variants. The highest rate of black oil destruction was noted in the variant with the biopreparation, where the level of black oil biodegradation in 45 days of incubation was 33.1% (Fig. 1). The method of stimulation of aboriginal microflora was inefficient in this case and the hydrocarbon-oxidative activity of microorganisms in the variant without the biopreparation did not exceed 3%. Besides, the quantity of microorganisms in this variant actually did not differ from the quantity of the control.

4. Conclusions

The method of activation of aboriginal hydrocarbon-oxidizing microflora is far from always being advantageous as compared with the introduction of oil degrading microorganisms. Although the aboriginal microflora isolated from oil-polluted sites is the most adapted for growth in the above conditions, this does not imply that it has the highest rate of oil destruction.

Therefore, the choice of the optimal method of bioremediation of oil-polluted objects needs, first, a complex analysis of remediation conditions and, second, comparison of the efficiency of different methods of bioremediation, such as:

- stimulation of aboriginal microflora;
- introduction of a biopreparation of oil degrading microorganisms;
- isolation of active aboriginal strains-destructors followed by production of their biomass and introduction into the polluted object where they had been isolated from.

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