

Bioremediation of land oil spills: diversity of microorganisms degrading oil hydrocarbons

A.M. Boronin^{1,2}, A.E. Filonov^{1,2}, I.A. Kosheleva^{1,2},
A.N. Shkidchenko^{1,2}, A.B. Gafarov¹, S.L. Sokolov¹, I.F. Puntus¹,
V.G. Grishchenkov¹, V.V. Dmitriev¹ & M.U. Arinbasarov¹.

¹ *Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences, Russia.*

² *Pushchino State University, Russia.*

Abstract

Environmental pollution by petroleum is an increasing global problem. In Russia this environmental problem is of such a large scale that it renders the excavation and off-site treatment of oil-contaminated soils and industrial wastes hardly possible. Therefore, *in situ* methods are attractive alternative. Bioremediation is a cost-effective and contaminant-specific approach. At the Institute of Biochemistry and Physiology of Microorganisms (Pushchino, Russia) there is a large collection of microorganisms degrading oil products. Their survival, stability, competitiveness, biodegradative activity, and efficiency under varying conditions have been investigated. Polycyclic aromatic hydrocarbons (PAH) belong to a recalcitrant group of environmental pollutants. They are components of oil and often occur in industrial wastes. The biochemical pathways of PAH catabolism and their genetic control in different strains were studied. It was shown that different combinations of host bacteria and plasmids changed growth characteristics and competitiveness of *Pseudomonas* strains. The transfer of degradative plasmids may enhance the biodegradative potential of indigenous microorganisms. Model systems were elaborated to determine the efficiency of PAH degradation [1]. All these approaches can be used for the development of biotechnologies for clean up of oil-contaminated soils.

Isolation and characterization of oil-degrading microorganisms

The necessity of protection and cleaning of tremendous territories of Russia contaminated by oil and oil products is obvious. Microorganisms of different taxonomic groups play the main role in the utilization of individual oil component. Isolation of oil-degrading microorganisms from environment and their systematic selection form the basis for the development of biopreparations degrading oil components under different conditions.

Microorganisms growing on different oil fractions, including persistent heavy oil, were isolated from soil contaminated by oil and oil products. Their ability to grow on various oil hydrocarbons was studied. From the oil- contaminated sites of the Moscow, Tula, Tver, Kirov and Tyumen regions, Tatarstan, Bays Baku (the Caspian Sea) and Biscay, 250 strains were isolated, characterised, and systematized by their cultural and trophic characteristics. The most active and widespread of them belong to the genera *Pseudomonas*, *Rhodococcus*, *Arthrobacter* and *Acinetobacter* [2, 3]. The active oil degraders were studied in accordance with the following characteristics:

- Ability to utilize oil products (crude oil, black oil, asphaltenes, diesel fuel)
- Ability to utilize polycyclic hydrocarbons (naphthalene, phenanthrene, anthracene, acenaphthene, pyrene, dibenzothiophene)
- Ability to utilize monocyclic hydrocarbons (salicylate, gentisate, phenol)
- Ability to utilize *n*-alkanes (hexadecane, octane)
- Growth at different temperatures (4 - 40°C)
- Growth at different pH values (4.5 - 7.0)
- Halotolerance (growth on rich media containing 2.5%; 5%; 10% NaCl)
- Ability to form biosurfactants

With the use of these characteristics a cluster analysis was performed. The resulting dendrogram (Fig. 1.) demonstrates a similarity in the degrading profiles of the strains studied. Such dendrogram can help to form oil-degrading associations as the basis of biopreparations to clean up contaminated sites.

Temperature is a crucial element of the biological destruction of oil products. In cold climate regions, e.g. North of Russia, psychrophilic and psychrotrophic microorganisms obviously play the main role in this process. A screening of the collection of degrading strains allowed for selecting bacterial cultures that utilize oil and its components at 6°C.

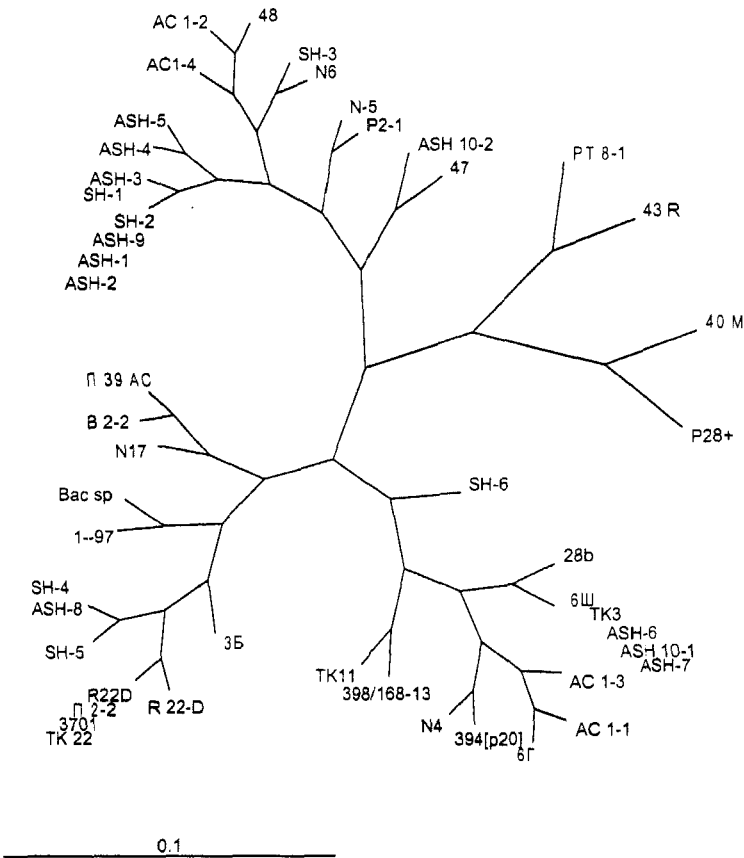


Figure 1. Dendrogram of oil-degrading strains constructed on the basis of comparison of their cultural and trophic characteristics: ability to grow on oil products, individual oil components, at different temperature values, pH and salt concentration in growth medium.

The study of 88 strains able to grow on rich media at 12°C and 6°C demonstrated that in some of them the enzyme systems for utilization of hydrocarbons could function at low temperature. The combination of abilities to utilize a broad range of substrates (Table 1.) and grow within a wide temperature range is of special interest for the comprehension of functioning of microbial genes and enzymes at low temperatures and for development of biotechnologies for cleaning the oil-contaminated environments in cold regions [4].

Forty oil-degrading strains able to utilize crude oil at 6°C were selected among isolated microorganisms; from these forty, 13 the most active ones were selected that degraded more than 25% oil in liquid mineral medium during 10 days. As seen from Figure 2, strains DS2, DS9, DS22 degraded crude oil at 6°C better than at 24°C.

Table 1. Bacterial strains capable of growing on different substrates at low temperature.

Number of strains	Growth temperature	Growth substrates						
		L-agar	Octane	Hexadecane	Diesel fuel	Phenol	Naphthalene	Oil
88	12°C	88	6	25	28	6	18	15
42	6°C	42	2	10	12	1	7	5

In the experiments carried out at 6°C for 90 days, the introduction of some selected degrading strains to soil contaminated by crude oil decreased the pollutant concentration by 22.3%; the indigenous microflora activated by fertilizers (nitrates and phosphates) degraded 14.5% of crude oil and only 12.1% without the nutrients. At the same time, the density of introduced oil-degrading microorganisms in contaminated soil was a 10 to 100-fold decrease in 45 days [5]. Attempts to compose artificial associations of studied strains did not result in enhancing of bioremediation process. Artificial associations of 2 or 3 oil-degrading strains increased the level of oil destruction only in 8-9% in comparison with using of single strains. The results obtained cannot provide criteria for artificial microbial associations composing. Such criteria could be developed during further investigations.

Selection of active degrading strains in model flow systems

Oil and oil products are complex multi-component substrates, and their degradation can be more efficient with the use of microbial consortia rather than individual strains. A promising approach is the selection of such associations of active strains in flow systems. The population dynamics of diesel-degrading microorganisms in four consecutively connected non-sterile bioreactors was studied. It turned out that under such conditions, not all of them were able to survive in the composed consortium (Fig. 2).

The associations studied included active strains of the genera *Spingobacterium*, *Alcaligenes* and *Rhodococcus* isolated from oil-contaminated sites in Moscow, Tula and Kirov regions as well as unidentified indigenous microorganisms. The latter predominated in the first and second bioreactors (80-90% of total microbial population). *Alcaligenes* sp. V2-2 (50 %) and *Rhodococcus* spp. AC1-2, AC1-3, and AC1-4 predominated in the third and fourth ones (50% and 20-25%, respectively), their total concentration was twice

as much than in the first two bioreactors [3]. It is noteworthy that the selected strains utilized aliphatic and polycyclic

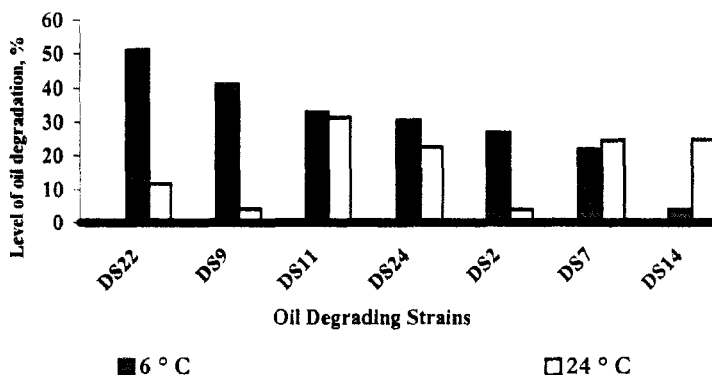


Figure 2. Degradation of crude oil by psychrotrophic microorganisms under 6°C and 24°C.

hydrocarbons and heavy oil fractions (20-25% of black oil). *Rhodococcus* sp. AC1-2 was capable of growing within the temperature range from 4°C to 40°C. In bioreactors, microbial associations degraded 99% of diesel fuel in four weeks. The results obtained point to the possibility of selecting active diesel fuel degrading microorganisms applicable for cleaning oil-contaminated industrial sewages in wastewater treatment plants [6].

Oil containing wastes of petrochemical industry (slimes) is the next serious environmental problem in Russia. The example is one of the largest petrochemical plants of Europe, Nizhnekamskneftekhim located in Tatarstan, with its almost 600 000 m³ of unprocessed oil-slimes.

In bench-level experiments simulating biological cleaning of water from oil slimes, a stable microbial consortium was formed under continuous cultivation conditions in fermentors. From this consortium, 19 microbial strains able to grow on crude and black oil, diesel fuel, *n*-alkanes, phenol, biphenyl, naphthalene or phenanthrene as sole carbon and energy sources were isolated. These strains made up more than 95% of total cultivated microbial population; 70% of them were represented by 4 major strains, which are unidentified yet. However, this microbial association was able to degrade only 30% of hydrocarbons from oil slimes, so the efficiency of bioremediation was insufficient.

In attempts to increase the efficiency of the degradation process, a three-channel laboratory micro-aerotank was constructed, which is analogous to aerotanks for biological cleaning of industrial wastewaters of Nizhnekamskneftekhim. The adapted microbial association degraded up to 70-80% of hydrocarbons of oil-containing slime. The results obtained were used for the further development of the cleaning biotechnology at Nizhnekamsneftekhim.

Plasmid - host bacterium combinations and obtaining of enhanced degrader microorganisms

Polycyclic aromatic hydrocarbons as a component of heavy fractions of oil can serve as model substrates in studies of the biochemical pathways and genetic control of recalcitrant compounds that are persistent pollutants due to their low water solubility. As it has been shown recently, PAH degradation by bacteria is often controlled by large plasmids [7]. Plasmids are known to play an important role in the adaptation of microorganisms to varying conditions of the environment and allow the microorganisms harbouring them to catabolise uncommon and persistent compounds [8]. Many plasmids are conjugative, i.e. they are able to provide a horizontal transfer of genetic information that ensures the propagation of biodegradation features within and between microbial populations [9]. However, biodegradative genes do not express in some host bacteria, despite the fact that plasmids containing these genes can be maintained and replicated in these host background. In turn, plasmids influence the growth, reproduction and survival of bacterial cells. The study of influence of bacterial background on expression of PAH biodegradative genes is of both fundamental and applied importance.

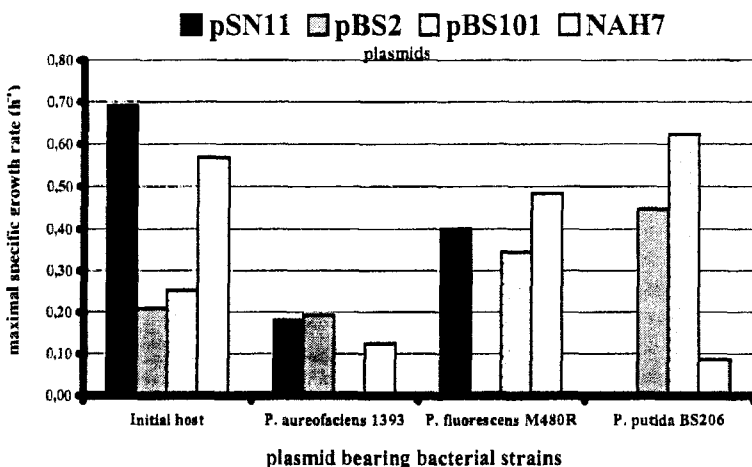


Figure 3. Specific growth rates of plasmid bearing bacterial strains in batch culture on naphthalene. Different plasmids in the same host background.

By conjugal transfer of plasmids NAH7, pBS2, pBS101, pBS216 and pSN11 in the strains *P. putida* BS206, *P. fluorescens* M4-80R and *P. aureofaciens* BS1393, twelve naphthalene-degrading strains were obtained. Their

physiological characteristics were studied. When cultivated on mineral-salt medium supplemented with naphthalene, the transconjugants of *P. putida* BS206 (pBS101), *P. putida* BS206 (pBS2) and *P. fluorescens* M4-80R (pBS101) are characterized by a high specific growth rate ($0.40\text{--}0.62\text{ h}^{-1}$), which is 2-2.5 fold higher than that of the initial host strains of these plasmids (Fig. 3.).

These data demonstrate the possibility of obtaining new "bacterium-plasmid" combinations with the enhanced biodegradative capabilities than the initial isolated strains.

Biopreparations and field testing

The bases of biopreparations are microorganisms or their associations. The selected microorganisms were used to form consortia with purpose of the creation of biopreparations to clean up oil-contaminated soil. To form artificial oil-degrading associations, the strains are selected by their compatibility and high oil-degrading activity. On the basis of cluster analysis (Fig. 1.), 26 associations were composed: 13 of them consisted of 2 microbial strains, 8 of three strains, 3 of four strains, and 2 of five ones. The comparative analysis of individual strains and their associations with respect to black oil and diesel fuel degradation in liquid medium showed that the two-strains (usually *Rhodococcus* - *Pseudomonas*) associations were the most efficient for black oil degradation.

The data obtained indicate that the combinations of microorganisms of different taxonomic groups can be more preferable in developing a biopreparation to clean up black oil contamination. The increase in number of association members to 4-5 strains did not result in the increase of black oil-degrading efficiency. The data obtained indicate that the combinations of microorganisms of biopreparations to clean up environments contaminated by oil products. The population dynamics of individual degrading strains in associations depended on the number and the species of associated strains. Moreover, after microbial degradation the fractional composition of residual black oil change varied significantly depending on the combination of degrading strains. For example, one of the studied three strains - associations was the most efficient degrader of paraffin-naphthene, aromatic and polyaromatic hydrocarbons; while the two 2-strains associations efficiently degraded asphaltic and tarry black oil components.

Using the selected oil-degrading microbial associations and the data of laboratory researches, an experimental preparation NikaOil was developed for the utilization of light and heavy oil fractions. The biopreparations of NikaOil series provide degradation of kerosene and diesel fuel by 85-90%, oil – by 55-60% and black oil – by 25-45% during summer season. The efficiency of NikaOil preparations was demonstrated under field conditions in contaminated sites of Moscow, Tver, Kirov regions and in Western Siberia.

The laboratory researches and field tests of microbial oil-degrading associations demonstrated the possibility of developing highly specialized biopreparations to remove oil contamination in different geographic regions.

Acknowledgements

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