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Oil-Spill Bioremediation, Using a Commercial Biopreparation "MicroBak" and a Consortium of Plasmid-Bearing Strains "V&O" with Associated Plants

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1. Introduction

Oil is the most widely distributed source of energy in the world and the largest-scaled environmental pollutant. Oil, oil products, and oil-containing industrial wastes pollution is ranked second place after radioactive pollution on account of their harmful action to ecosystems (Chernyakhovsky et al., 2004). Oil deposits in Russia are mainly located in the northern regions of the European part and Western Siberia. Therefore oil spills in terrestrial and aquatic ecosystems occur primarily in cold climate regions. Approximately 8 to 9 billion tons per year of oil and oil products are discharged into the environment during extraction, transportation, processing and storing oil. Half of this amount goes into the soil and groundwater, and the other half contaminates surface water and air (Chernyakhovsky et al., 2004). In Western Siberia, in the areas of hydrocarbon accumulations, over 200 thousand hectares of land were polluted with oil reaching depths up to 10 cm in 1995. By 2003, the oil polluted area reached 700 thousand hectares.

The process of natural restoration of polluted environment requires long times. The autoremediation of an oil-spilled soil is a process that requires from 2 to 30 years or more to complete at a level of pollution of 5 g oil/kg soil. The rate of this process is slower in the northern regions. Thus, the aftermath of oil spills is observed over decades (Oborin et al., 1988).

In Russia, this ecological problem is of a special significance because of the scale of the oil spills when soil excavation and restoration *ex-situ* are impossible. Bioremediation, which consists on the microorganisms' ability of utilizing and transforming oil hydrocarbons, plays the main role during *in-situ* remediation. Bioremediation provides an economically

beneficial and high-specific clean-up technology to remove pollutants concentration by targeting individual pollutants or their mixtures. The planning of bioremediation strategies for polluted land and water areas should consider the use of the existing indigenous oil-oxidizing microorganisms with different affinity to oil fractions and their activation by addition of fertilizers (nitrogen, phosphorus, and potassium). The inoculation of effective microorganisms is necessary in the northern regions where the warm season is short and the natural microflora has no time to adapt to the changing environmental conditions, which is the case for Russia located in a zone of cold and moderate climate.

Bacteria such as *Acinetobacter, Alcaligenes, Arthrobacter, Burkholderia, Brevibacterium, Corynebacterium, Micrococcus, Mycobacterium, Nocardia, Pseudomonas, Rhodococcus, Serratia*, and *Bacillus* among others possess significant pollutant degradation potential; it has been shown that these bacteria can use recalcitrant pollutant and xenobiotics as energy source. These microorganisms are often isolated from oil-polluted sites and used to clean-up (bioremediate) oil spills. The remediation of polluted soils requires the study of the microorganisms' diversity in the environment and the determination of the ability of different microbes and their consortia to degrade pollutants in the presence of high salt concentration (Van Hamme et al., 2003; Ventosa et al., 1998). This is important because it has been observed that the introduction of a single oil-oxidizing strain into the oil-spilled environment does not assure a complete clean-up.

The majority of the studies have focused on determining the effect of oil spill on natural populations of microorganisms from different ecological niches (Delille et al., 2002; Prince & Bragg, 1997). However, few studies have addressed the selection of microorganisms capable of degrading oil and oil products at low temperatures.

The research summarized in this chapter describes the screening and collection of microorganisms capable of degrading different hydrocarbons. Several active oil-degrading strains were selected with the following characteristics:

- Psychrotrophic that is the bacteria ability to grow in a wide temperature range (from 4 to 30°C), that is useful for bioremediation of oil-spilled territories in different regions of Russia;
- Halotolerant that refers to bacteria capable of degrading oil and oil products at high salt concentrations (3-7% NaCl). The application of halotolerant bacteria to degrade oil products is of a special importance when conducting bioremediation at seashore, salted water areas, and salted marshes;
- Capacity to produce biosurfactants. The strains must be capable to synthesize biosurfactants when cultivated in mineral media with oil products as carbon and energy sources. The persistence of oil hydrocarbon pollutants in the environment is due to their low water solubility. Therefore, the action of microbial emulsifiers on hydrocarbon pollutants will enhance the strains bioutilization efficiency of these contaminants.
- Capacity to target polycyclic aromatics (PAHs). Microorganisms of the genus *Pseudomonas* bear plasmids that may encode for biodegradation of PAHs. The presence of conjugative plasmids harbouring PAH catabolism genes promotes the increase the PAHs degradation potential because of genes dissemination among indigenous microorganisms.

2. Biopreparation of "MicroBak" and field testing

2.1 Strains selection

A total of 165 strains obtained from the Laboratory of Plasmid Biology of IBPM RAS were examined for their ability to utilize diesel fuel or crude oil as the sole carbon and energy source at temperatures ranging from 4 to 6°C and 24°C. The screening of the most active strains was performed according to the following criteria: their ability to grow on diesel fuel and/or crude oil both at moderate temperatures ranging from 24°C to 32°C and at low temperatures ranging from 2°C to 6°C in the presence of 3 to 10% NaCl and their capability to produce bioemulsifiers when cultivated on hydrophobic substrates. Based on these screening criteria, 9 psychrotrophic strains degrading oil hydrocarbons were selected as follows: *Rhodococcus* sp. S25, *Rhodococcus* sp. S26, *Rhodococcus* sp. S67, *Rhodococcus* sp. X5, *Rhodococcus* sp. X25, *Rhodococcus* sp. Ars38, *Microbacrerium* sp. Ars25, *Pseudomonas* sp. 142NF(pNF142), and *Pseudomonas putida* BS3701(pBS1141, pBS1142) were chosen. The selected microorganisms had different ability to grow on diesel fuel, crude oil, oil fuel, hexadecane, benzoate, benzene, toluene, and naphthalene as a sole carbon and energy source in temperatures ranging from 2°C to 32°C. All the strains were capable of growing in a mineral Evans medium (Evans et al., 1970) in the presence of 3% NaCl, however two of them grew well at NaCl concentrations of 5% and only one bacterium (*Rhodococcus* sp. X5) grew at NaCl concentrations in the range from 7 to 10%. All microorganisms were able to grow in a pH range from 5 to 8.

After determining the main physiological and morphological strait of the strains, the identification of the most active degrader microorganisms was performed. The strains were identified based on their physiological and morphological characteristics. Seven gram-positive strains X5, X25, S25, S26, S67, and Ars38 were identified as representatives of the *Rhodococcus* genus and the strain Ars25 was identified as a *Microbacterium* using nucleotide sequencing of 16S rRNA genes.

The ability of the strains to degrade crude oil was assessed according to the reduction of oil in a liquid medium determined by gravimetric analysis (Baryshnikova et al., 2001). Single strains were able to degrade from 26% to 66% of oil at 24°C, and from 28% to 47% of oil at 4-6°C in 20 days. Eight of the nine strains chosen were more effective in degrading oil at low temperatures (4-6°C) than at a temperature of 24°C (Fig. 1).

Thus, these eight strains, which showed potential for oil bioremediation in cold climates, were used to prepare a consortium of oil degrader strains.

The selection of a mixed consortium of microorganisms was carried out in a liquid mineral medium through batch cultivation with oil as the sole carbon and energy source in the presence of *Eriophorum vaginatum* grass based sorbent or without it. After cultivation, the dominant rhodococci and pseudomonads strains were determined by analysis of the selected population. The representative strains of the genus *Pseudomonas* were distinguished by their cultural and morphological traits. Dominant rhodococci species were determined by performing genotyping of the selected strains because these strains had identical cultural and morphological traits. Random Amplification of Polymorphic DNA (RAPD) PCR-analysis was applied (Fig. 2).

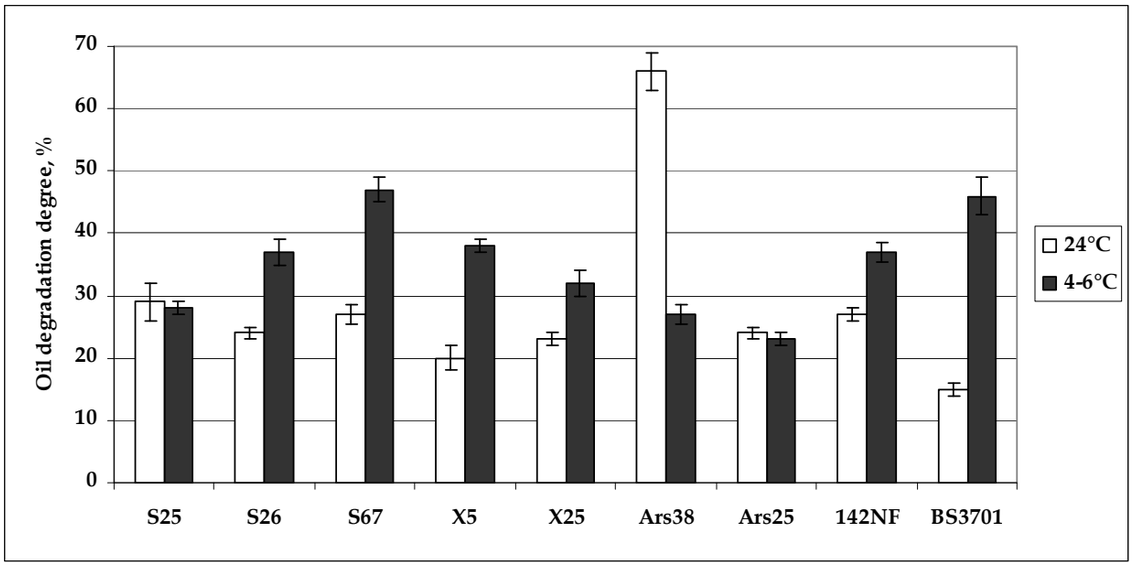

Fig. 1. Oil degradation by single microorganisms strains in Evans liquid medium at different temperatures for a period of 20 days

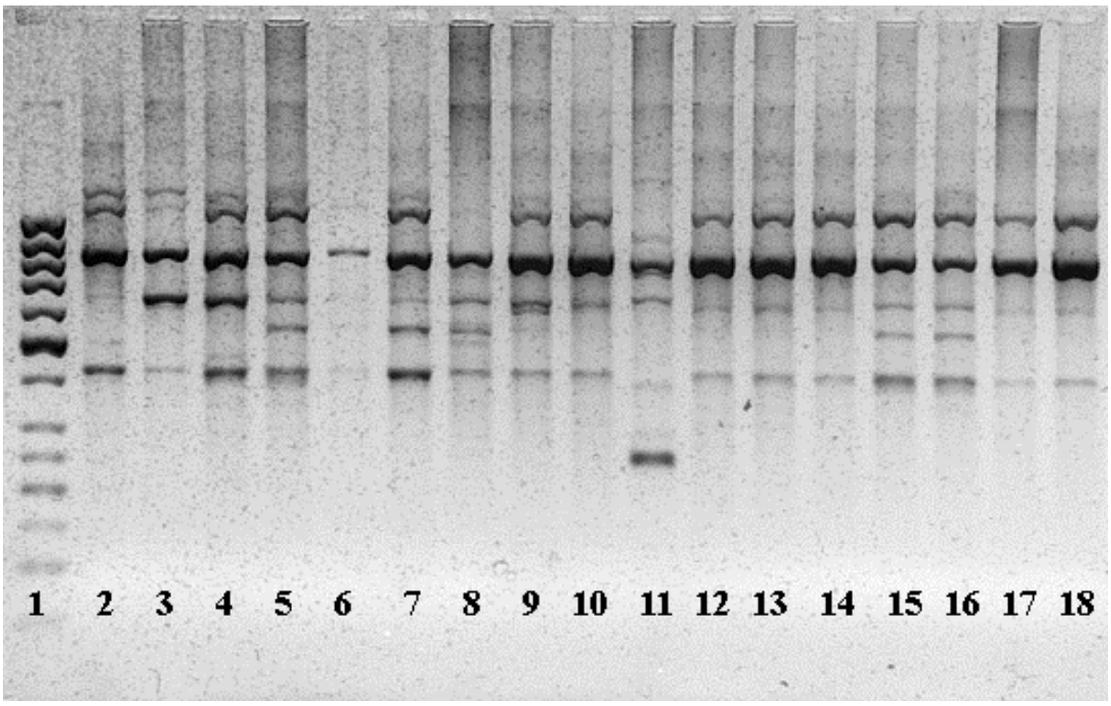

Fig. 2. RAPD PCR-analysis of rhodococci strains with OA20 primer (GTTGCGATCC):
1 – 50 bp Ladder (Fermentas);
2 – *Rhodococcus* sp. S25; 3 – *Rhodococcus* sp. S26; 4 – *Rhodococcus* sp. S67; 5 – *Rhodococcus* sp. X5; 6 – *Rhodococcus* sp. X25; 7 – *Microbacterium* sp. Ars25; 8 – *Rhodococcus equi* Ars38; 9-18 – clones presenting the largest population after cultivation in liquid mineral medium

Thus, a consortium of microorganisms including *Rhodococcus* sp. X5, *Rhodococcus* sp. S67, *Pseudomonas* sp. 142NF(pNF142), and *Pseudomonas putida* BS3701(pBS1141,pBS1142) was selected. This consortium was used as the basis for the biopreparation to target the bioremediation of soils polluted by crude oil and oil products. Characteristics of the microbial consortium are: psychrotrophy, halotolerant (3-5% NaCl), capability to synthesize bioemulsifiers, carrier of plasmids in pseudomonades capable of targeting the biodegradation of polycyclic aromatic hydrocarbons.

2.2 Role of catabolic plasmids in oil biodegradation

Many examples of plasmid genes encoding for hydrocarbons degradation, including short chain alkanes, substituted and non-substituted aromatic hydrocarbons, and other xenobiotics are known (Harayama et al., 1990; Wallace et al., 1992). The concentration of aromatic compounds in oil hydrocarbons depends on the crude oil and it could range from 10% to more than 50%. Aromatic compounds are the most toxic and recalcitrant components in the crude oil. The role of catabolic plasmids in oil biodegradation was determined by gravimetric analysis of the oil degradation efficiency of *Pseudomonas* strains bearing PAH biodegradation plasmids and their plasmid-free variants. It was revealed that the presence of naphthalene degradation plasmid pBS216 in the *Pseudomonas chlororaphis* PCL1391 strain promoted a significant increase (10-fold) of oil degradation in 7 days if compared with the plasmid-free strain. However, the presence of plasmids pOV17 or pNF142::Tc in the strain did not have the same effect (Fig. 3). Plasmids pBS216 and pOV17 are known to have similar structures but different activities of catechol-2, 3-dioxygenase (Volkova et al., 2005). Genes encoding for the enzyme are often localized in one of the plasmids from a bacterial host bearing different plasmids that influences the oil degradation capability. The results obtained show that the catabolic potential of microorganisms in oil degradation is given by a combination of "host bacterium - plasmid". The presence of pBS1141 plasmid in the strain *Pseudomonas putida* BS3701 induced the removal of oil hydrocarbons (up to 3-fold) in comparison with an eliminant BS3701E, which does not present the PAH biodegradation plasmid pBS1141.

Figure 4 indicates that the cell biomass growth in oil shows a higher increase for the plasmid-bearing bacteria than the cell biomass growth of plasmid-free microorganisms. These plasmid genes have the ability of using different aromatic hydrocarbons (naphthalene, phenanthrene etc.) in comparison with plasmid-free strains. For example, naphthalene dioxygenase encoded by genes of naphthalene biodegradation plasmid catalyzes about 76 reactions (deoxygenation, monooxygenation, dehydratation, O- and N-dealkylation and sulfooxidation).

Crude oil and oil products are complex multicomponent pollutants containing hundreds of chemical compounds. During oil degradation both quantitative (content decrease) and qualitative (transformation of fractions composition) changes take place, for instance, selective degradation or transformation of single oil components occurs. Fractionation of the residual oil on silica gel separate oil components in three fractions: the hexane fraction containing paraffin, naphthene, and aromatic hydrocarbons, the benzene fraction containing polycyclic aromatic hydrocarbons; and the alcohol-benzene fraction contains naphthenic acids and tars (Babaev et al., 2009). The plasmid-bearing strains evaluated in this study showed that the naphthalene biodegradation plasmids increased the degree of degradation of hexane, benzene, and benzene-alcohol fractions.

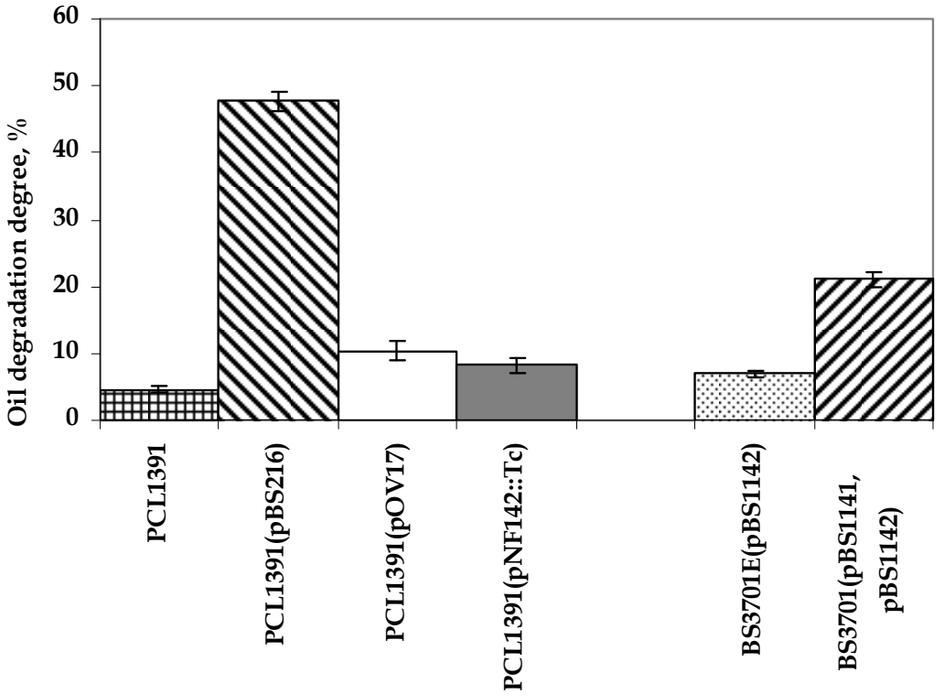

Fig. 3. The degree of oil destruction by native plasmid-bearing strains and their plasmid-free variants in Evans medium in 7 days at 24ºC

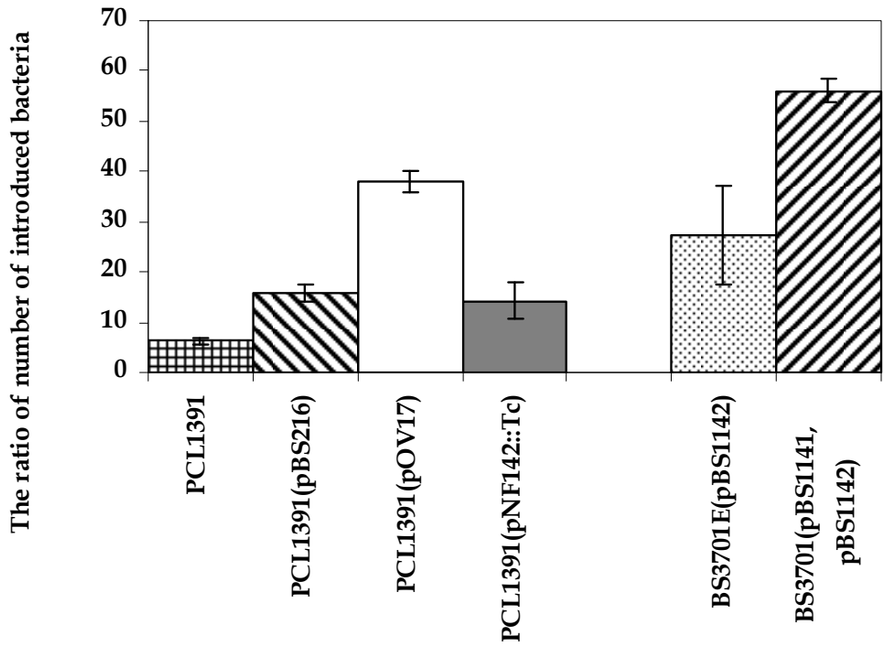

Fig. 4. The ratio of the final to the initial number of microorganisms for a testing period of 7 days

The highest degradation of paraffin-naphthene, mono- and polyaromatic hydrocarbons, asphalthenes and tars was detected in the sample containing the strain *P. chlororaphis* PCL1391(pBS216), in which the degradation degree for the three fractions was 38%, 31% and 26% respectively, causing a total oil degradation of 48%. Thus, the presence of PAH degradation plasmids in host strains promotes the increase of oil degradation.

2.3 Evaluation of biosurfactants

The capacity of bacteria to produce surface-active compounds (biosurfactants) during hydrocarbon degradation is one of the most important mechanisms allowing microorganisms to use oil components poorly soluble or insoluble in water (Desai & Banat, 1997). Biosurfactants contribute to the solubilization of hydrocarbons by forming emulsions that facilitates the contact of microbial cells with the hydrophobic substrate and its influx into the cell. Currently biosurfactants are a subject of research. Advantages of biosurfactants over synthetic surfactants are their effectiveness, low toxicity, and biodegradability. The appropriate knowledge of biosurfactant properties, structures, and their biosynthesis conditions will promote the formulation of biopreparations (Banat et al., 2010). Despite of the numerous reports on new strains-producers of surface-active compounds a unified methodological approach for the screening and identification of these organisms from native samples has not been developed yet. The general accepted criteria for the evaluation of the surface activity of microbial cultures are still unknown.

Different strains were screened to determine the most efficient producers of biological surface-active agents. The microorganisms chosen for this evaluation were the following: gram-negative *Pseudomonas fluorescens* 142NF (pNF142), *Pseudomonas putida* BS3701 (pBS1141,pBS1142) (which bear plasmids of biodegradation of mono- and polycyclic aromatic hydrocarbons); gram-positive *Rhodococcus* sp. S67, *Rhodococcus* sp. X5, and *Rhodococcus* sp. S26. These strains are highly effective oil destructors.

When carrying out the selection of microorganisms capable of producing surfactants, the surface tension of the culture broth is usually estimated (Satpute et al., 2010). Simple and rapid methods based on measuring the emulsification of hydrophobic compounds such as hexadecane are applied that allow measuring the index of emulsification and the degree of emulsification activity. The determination of glycolipid biosurfactants is performed using photocolorimetry reactions such as the Molisch reaction.

It is well known that the carbon source may have a significant impact on biosurfactants synthesis (Muthusamy et al., 2008). Biosurfactant synthesis is often observed in various microorganisms during their growth on hydrophobic substrates such as carbohydrates and vegetable fats. On the other hand, intensive biosurfactant synthesis is detected during the growth of microorganisms (representatives strains of *Pseudomonas aeruginosa*) on hydrophilic carbon sources (glucose, glycerol) (Abdel-Mawgoud et al, 2010). In this study, biosurfactants-producer microorganisms were compared by their cultivation on two different substrates: hexadecane as a hydrophobic substrate and glucose as a hydrophilic substrate.

It was found (Table 1) that hexadecane stimulates the intensive synthesis of biosurfactants. In this case, the content of biosurfactants in the culture broth for all the strains evaluated was high and a significant decrease of the surface tension was observed from 77 mN/m

down to 34-31 mN/m (control). The largest values of the emulsification index and degree of emulsification activity were detected. For instance, rhodococci strains appeared to be more effective producers of biosurfactants compared to pseudomonades when cultivated on hexadecane.

Strain	Glycolipids content[4], mg/l	Surface tension[5], mN/m	Index of emulsification, %		Emulsifying activity[4] (λ=540 nm), units of optical density
			CFS[4]	CB[5]	
P. fluorescens 142NF	190±10	34±1	50±4	50±5	0,9±0,2
P. putida BS3701	250±20	34±1	53±6	53±5	0,6±0,1
Rhodococcus sp. S67	310±20	33±1	78±9	78±6	0,7±0,1
Rhodococcus sp. X5	400±30	31±1	75±6	75±7	1,0±0,2
Rhodococcus sp. S26	740±50	32±1	47±3	47±3	1,5±0,2

[4] The value measured for the cell-free supernatant (CFS)
[5] The value measured for the culture broth (CB)

Table 1. Surface-active properties of microorganisms' growth in the medium with hexadecane.

Biosurfactants can be extracellular (exo-type) and cell-bounded (endo-type). The index of emulsification and the emulsifying activity were determined for only the solubilized biosurfactants (exo-type) in the broth after cells precipitation. Nevertheless, there was some effect of the endo-type biosurfactants on the index of emulsification in the non-centrifuged culture broth.

The results show that the indices of emulsification in the culture broth and in the cell-free supernatant for the strains cultivated on hexadecane were the same (Table 1). Thus, the five strains producing extracellular biosurfactants were evaluated when using hexadecane as the sole carbon and energy source.

In the case of glucose as the source of carbon and energy, the content of glycolipids did not exceed 50 mg/l, and the values of surface tension obtained were much higher than for the case of hexadecane. This suggests that the growth of microorganisms on the hydrophilic substrate (glucose) was not accompanied by the intensive formation of exo- biosurfactants as in the case of hexadecane. The emulsification activity and emulsification index determined during the growth of *Pseudomonas* on glucose indicates the synthesis of exo-type biosurfactants (Table 2). At the same time, the emulsification index values appeared to be 0% for cell-free supernatants of rhodococci, and 7-29% - for the culture broth. Moreover, cell suspensions of *Rhodococcus* were able to stabilize hexadecane/water emulsions effectively (Table 3). Emulsification was not observed for the *Pseudomonas* cells at the same conditions. Thus, rhodococci produced endo-type biosurfactants, when glucose was used as a growth substrate.

Biosurfactants were extracted from the culture broth using a mixture of chloroform : methanol (3:1), followed by the evaporation and purification of the organic phase, using

silica gel column chromatography. The identification of the glycolipid biosurfactants was performed by thin layer chromatography (TLC) on plates of silica gel through a specific reaction to sugar: treatment with phenol and sulfuric acid followed by heating. The presence of carbohydrate in the biosurfactant molecule resulted in a blue-violet coloration of the compound.

Strain	Glycolipids content, mg/l[4]	Surface tension, mN/m[5]	Index of emulsification, %		Emulsifying activity[4] (λ=540 nm), units of optical density
			CFS[4]	CB[5]	
P. fluorescens 142NF	10±2	54±1	33±4	33±4	1,1±0,2
P. putida BS3701	19±2	61±1	33±3	33±3	0,5±0,1
Rhodococcus sp. S67	49±9	53±1	0	7±2	0,2±0,1
Rhodococcus sp. X5	42±8	56±1	0	29±4	0,2±0,1
Rhodococcus sp. S26	19±5	49±1	0	29±3	0,2±0,1

[4] Emulsification index measured for the cell-free supernatant (CFS)
[5] Emulsification index measured for the culture broth (CB)

Table 2. Surface-active properties of microorganisms' growth in the medium with glucose.

Strain	Medium	Emulsifying activity, %
P. fluorescens 142NF	Evans medium + glucose	0
	Luria-Bertany medium[6]	0
P. putida BS3701	Evans medium + glucose	0
	Luria-Bertany medium	0
Rhodococcus sp. S67	Evans medium + glucose	35±4
	Luria-Bertany medium	40±5
Rhodococcus sp. X5	Evans medium + glucose	38±4
	Luria-Bertany medium	46±5
Rhodococcus sp. S26	Evans medium + glucose	46±6
	Luria-Bertany medium	48±6

[6] Carhart & Hegeman, 1975

Table 3. Emulsification properties of cell suspensions cultivated in agar media

According to TLC results it could be assumed that two strains of *Pseudomonas* produced the same rhamnolipids. The biosurfactants samples of *Pseudomonas* had only one spot with a flow rate (R_f) of 0.32 (Fig. 5). It is well known that, when performing TLC analysis under the conditions described, the glycolipids of pseudomonads are separated according to the number of rhamnose residues contained in the molecule. Mono-rhamnolipids have a greater mobility, with the known values for the retention reaching 0.7 (Zhang & Miller, 1994; Robert et al., 1989), and for di-rhamnolipids R_f reported values are 0.32 (Matsufuji et al., 1997) and 0.45 (Zhang & Miller, 1994).

If the results obtained in this work are compared to the published data, then it can be reasoned that the strains *P. fluorescens* 142NF and *P. putida* BS3701 produce di-rhamnolipids. Another common type of biosurfactants, which are produced by microorganisms of the genus *Pseudomonas*, are lipopeptides (Desai & Banat, 1997). Consequently, TLC analysis was performed by staining the chromatograms with ninhydrin to test the presence of lipopeptides in the extracts. The absence of a characteristic violet-pink color indicated the absence of lipopeptide biosurfactants in the extracts.

TLC of trehalose lipids showed the presence of four (strain *Rhodococcus* sp. S67) or five (strains *Rhodococcus* sp. X5 and S26) components with the following values of R_f: 0.32, 0.46, 0.51 (absent in the strain S67), 0.57, and 0.63. The identification of the trehalose lipids produced by the representatives of the genus *Rhodococcus* using the retention values was difficult because there is no enough published data available that could be used for comparison purposes. The TLC analysis of the succinoyl trehalose lipids that are produced by the strain *Rhodococcus* sp. MS11 show a single spot with R_f of 0.41 (Rapp & Gabriel-Jurgens, 2003). This study revealed distinctive retaining values for the compounds isolated; the spots with R_f of 0.32 and 0.46 are the only compounds There is no published data on R_f for trehalose lipids in the system chloroform : methanol : water (65:15:2). It should be noted that the separation of rhodococci biosurfactants on four or five components rather than trehalose lipids by TLC had not been previously described.

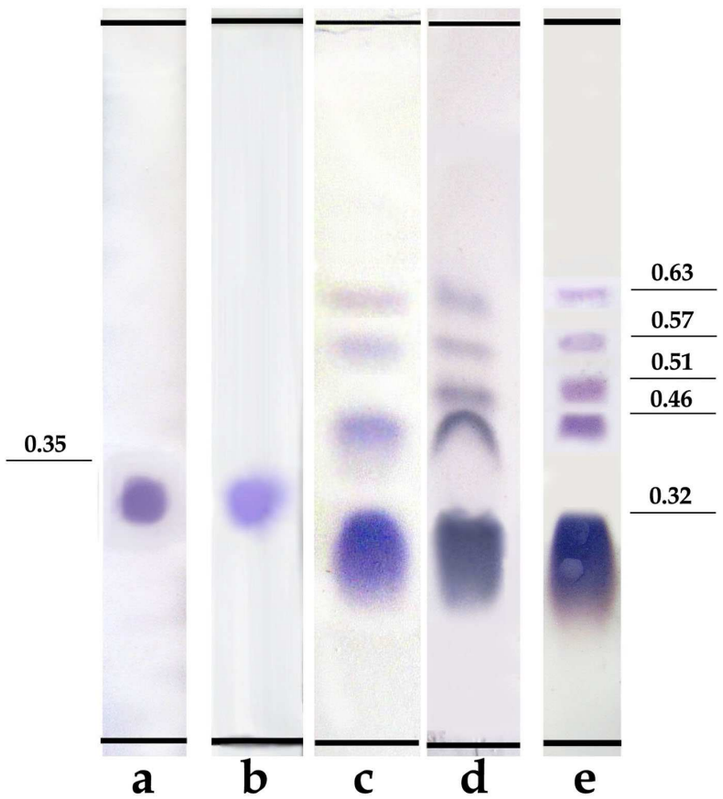

Fig. 5. Chromatograms of purified samples of biosurfactants synthesized by microorganisms (R_f.is shown): a) *Pseudomonas fluorescens* 142NF(pNF142); b) *Pseudomonas putida* BS3701(pBS1141,pBS1142); c) *Rhodococcus* sp. S67; d) *Rhodococcus* sp. X5; e) *Rhodococcus* sp. 26

The biosurfactants samples obtained were characterized using mass-spectrometry with electron spraying in a positive ionization mode. For glycolipids of the strains of *Pseudomonas fluorescens* 142NF and *Pseudomonas putida* BS3701 a series of signals was observed in a range of 700-900 Da (Fig. 6).

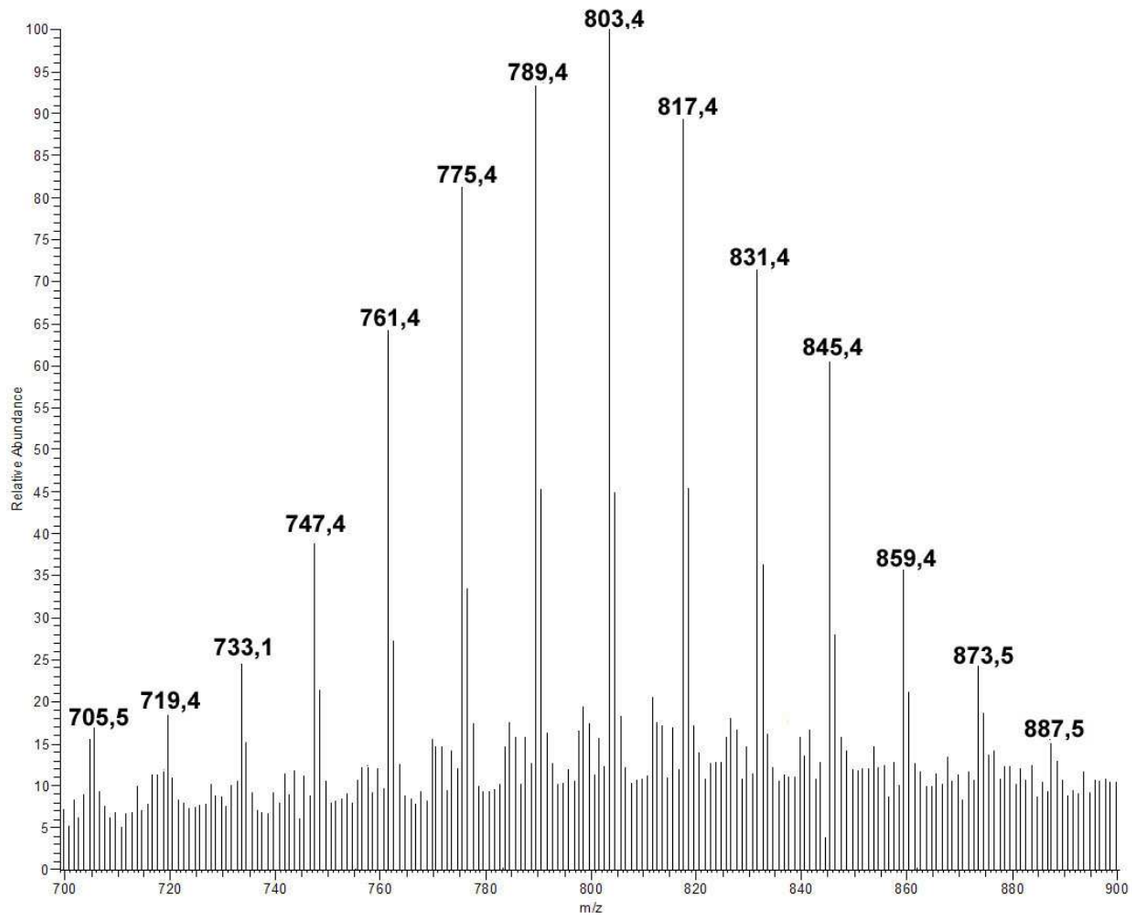

Fig. 6. The mass spectrum of glycolipid biosurfactants produced by the strain *Pseudomonas fluorescens* 142NF

One of the most intense peaks detected corresponded to the pseudo-molecular ion [M + H +] with a mass of 803 Da. It has been reported that ramnolipid B has a molar mass of 802 Da that contains two residua of hydroxydecanoic acid and one residuum of decenoic acid (Fig. 7a) (Abdel-Mawgoud et al., 2010). The difference in the masses for each pair of adjacent peaks was 14 Da that corresponds to the mass of a fragment of -CH_2-. It can be considered that the strains *Pseudomonas fluorescens* 142NF and *Pseudomonas putida* BS3701 produced a mixture of homologous rhamnolipids type B.

The same peaks were detected in the mass-spectrum of the biosurfactants produced by the strains *Rhodococcus* sp. X5 and *Rhodococcus* sp. S26: 866.4, 871.5, 877.2, 894.4, 899.9 (Fig. 8). Previous research on biosurfactants produced by rhodococci indicated that the main signals

in the mass-spectra of electron spray were induced by pseudo-molecular ions [M + Na +] with masses of 871.5 and 899.6 (Rapp & Gabriel-Jurgens, 2003; Tuleva et al., 2008). That corresponded to the homologous succinoyl trehalose lipids: dioctanoyl-decanoyl (848 Da) and octanoyl-didecanoyl (876 Da), differing by 28 Da, i.e. on a double methylene fragment (-CH_2-) (Fig. 7). Thus, the strains *Rhodococcus* sp. X5 and *Rhodococcus* sp. S26 evaluated in this work produced trehalose lipids of the similar structure. The remaining three signals in Fig. 8 could not be identified.

Infrared spectroscopy showed the presence of functional groups typical for the proposed structures of glycolipids in biosurfactants isolated from pseudomonads and rhodococci. A broad absorption band of a hydroxyl group at 3450 cm^{-1} was distinguished on the spectra obtained. Bands of valent oscillations of carbonyl groups of esters and carboxylic acids were observed in the areas of 1745 cm^{-1} and 1630 cm^{-1}, respectively. The peak of 1047 cm^{-1} belonged to the asymmetric valent oscillations of C-O-C bonds. Spectrum peaks of valent oscillations for aliphatic C-H bonds in the area of 2924 and 2852 cm^{-1} were observed, the absorption of deformation vibrations of these bonds was present at 1380 cm^{-1}.

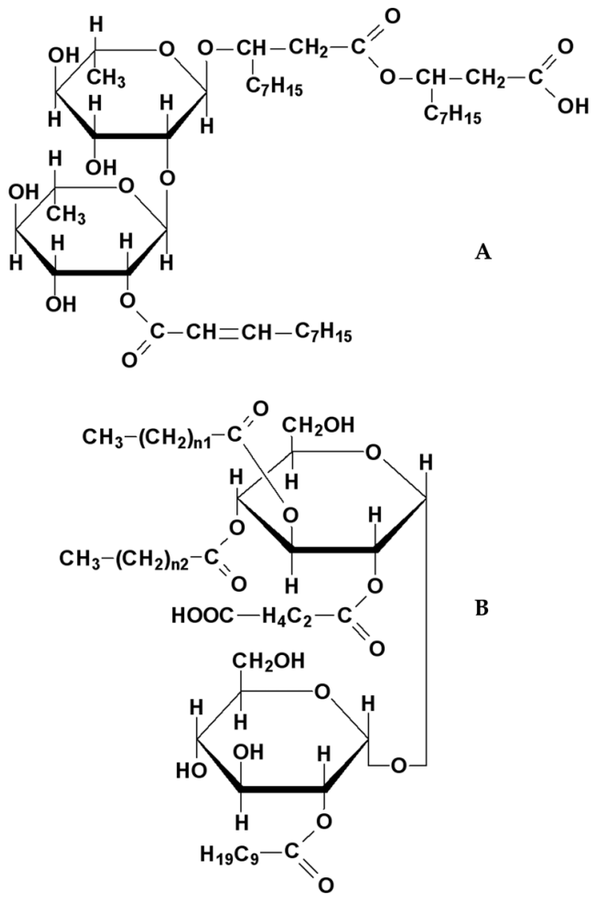

Fig. 7. The proposed structure of glycolipid biosurfactants: A - rhamnolipid B, produced by pseudomonads (Abdel-Mawgoud et al., 2010); B - succinoyl-dioctanoyl-decanoyl trehalose: $n_1 = n_2 = 6$; succinoyl-dioctanoyl-*di*decanoyl trehalose: $n_1 = n_2 = 6, 8$, produced by rhodococci (Rapp & Gabriel-Jurgens, 2003; Tuleva et al. , 2008)

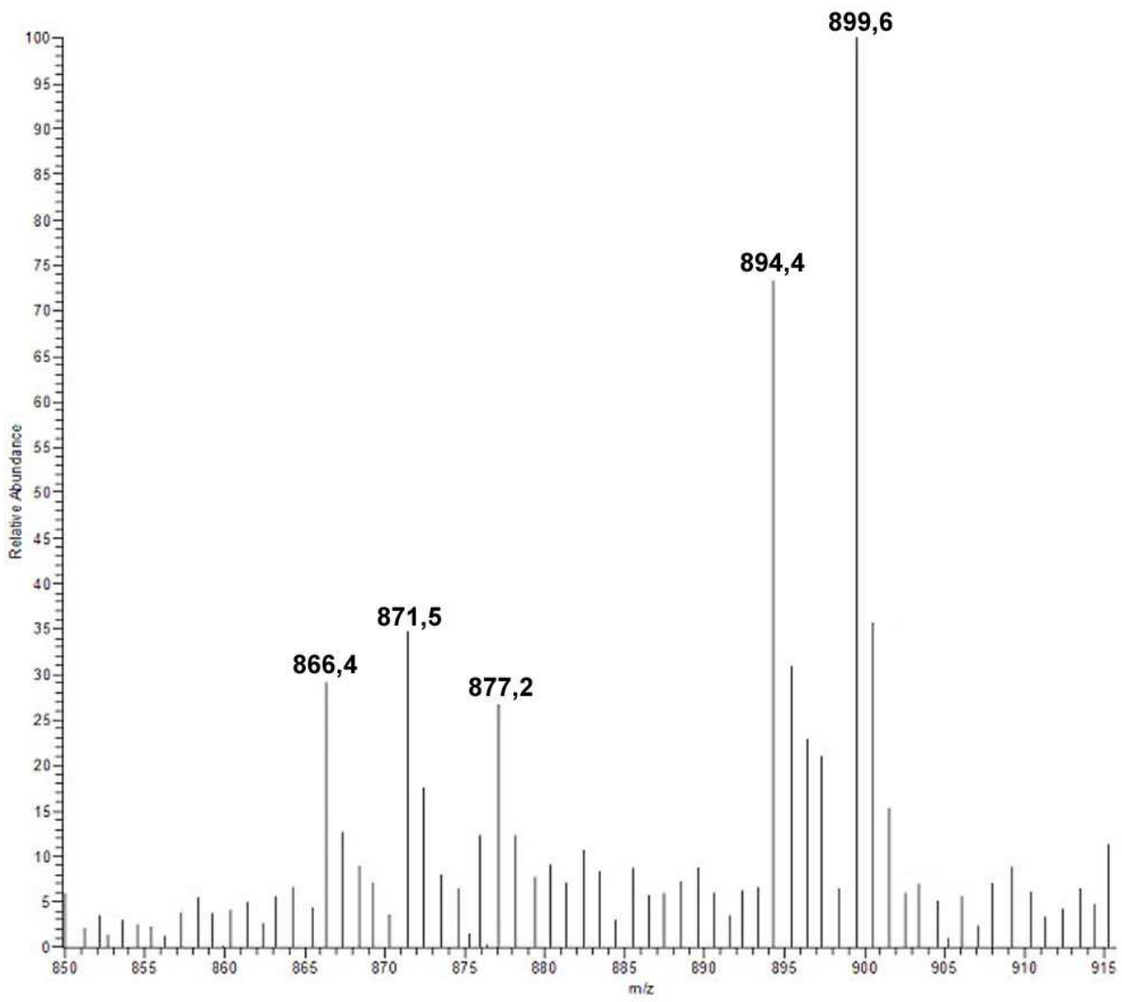

Fig. 8. The mass spectrum of glycolipid biosurfactants produced by the strain Rhodococcus sp. X5

Thus, all the microorganisms evaluated in this study were able to synthesize biosurfactants. The most efficient producers of exo-type biosurfactants were rodococci bacteria when cultivated on hexadecane and when cultivated in glucose they produced cell-bound biosurfactants (endo-type). Pseudomonads produced only extracellular biosurfactants. The formation of homologous di-rhamnolipids by the strain *Pseudomonas putida* BS3701 and the strain *Pseudomonas fluorescens* 142NF was demonstrated. The studied rhodococci spp. strains S67, X5 and S26 produced compounds of glycolipid nature, two of which were related to succinoyl-trehalose lipids.

2.4 Pilot testing of the "MicroBak" biopreparation for *in situ* clean-up of oil-spilled soil

Pilot testing was carried out in an open environment located in the municipal waste refinery of Pushchino from September 2006 to September 2007. The land tested included four plots of 1 m^2 each (Fig. 9). The composition of crude oil used as a model contaminant (25 g oil per 1 kg soil) is presented in Table 4.

Density, g/cm^3	Water content, %	Salts content, mg/ml	Contamination, %	Sulfur content, %	Composition		
					Hexane fraction, %	Benzene fraction, %	Alcohol-benzene fraction, %
0,868	0,06	45	0,0080	1,42	62,29	13,49	11,21

Table 4. Properties of the crude oil used

Plot 1 Control	**Plot 3** Consortium "MikroBak" fertilizer
Plot 2 Control with crude oil	**Plot 4** Consortium "MikroBak" fertilizer

Fig. 9. The scheme of the pilot field experiment

The degree of oil degradation was assessed in plot 2 (control) and plot 4 (where the consortium "MicroBak" (10^7 colony forming units (CFU)/g soil) and the "Nitroammophoska" fertilizer (3 g per 1 kg soil) were introduced) as indicated in Fig. 10. The pilot testing indicated that the oil degradation degree was higher in plot 4 where microorganisms and the fertilizer were introduced. In plot 4, 34% of the oil was degraded after two months of testing, while only 22% of the oil was degraded in the control plot (plot 2).

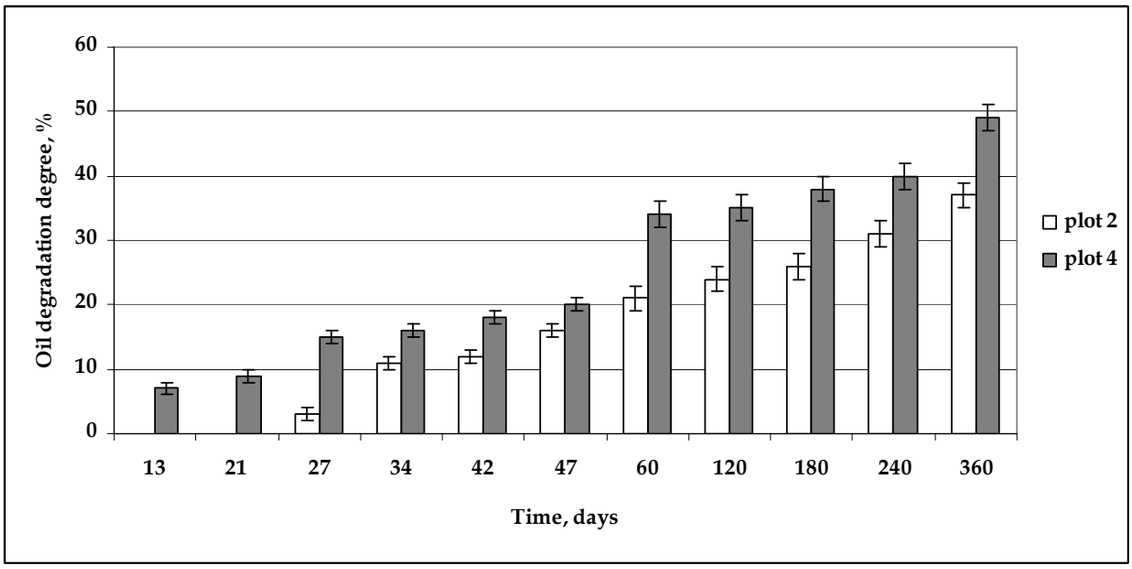

Fig. 10. Oil removal in the pilot field experiment

After 360 days of testing (end of field testing, Fig. 10) the total amount of oil removed was 49% and 38% for plots 4 and 2, respectively.

Soil toxicity evaluation in the previously bio-treated plot (plot 4) compared to the toxicity of the control plot (plot 2) was conducted using oat as bioindicator. The results obtained after a

month of testing demonstrated that the soil toxicity decreased in plot 4 where the consortium "MicroBak" and a fertilizer were introduced (Fig. 11).

Fig. 11. Pilot testing: soil phytotoxicity evaluation. June, 2007. A – Control plot (soil with oil), B – Plot 4 pre-treated with the consortium "MicroBak" and a fertilizer

2.5 Pilot testing of the "MicroBak" biopreparation in an industrial site polluted with hydrocarbons

Pilot testing of the biopreparation was performed in the territory of JSC "Tulskaya Toplivno-Energeticheskaya Compania" (Tula region, Kireevsky rajon, Rozhdestvenka) from September to November, 2007 at temperatures ranging from 0-22°C (Table 5). Soil was polluted by a mixture of oil products (diesel fuel, gasoline, gas condensate, oil fuel) due to the emergency flows during handling and pipelines breaking.

The polluted sites were sanded, and then the sand : soil mix (to 5 cm in depths, 1 : 2) was removed to an open plot where the soil clean-up process from took place. The initial level of oil pollutants in the soil was 14.2 g/kg soil (1.4 wt% oil residuals). The clean-up was performed by introducing "MicroBak" biopreparation (5×10^8 CFU/kg soil) and the mineral fertilizer "Nitroammophoska" (1.5 g/kg soil). After 14 days of testing, additional amounts of biopreparation (5×10^8 CFU/kg soil) and fertilizer (1.5 g/kg soil) were introduced into the polluted soil by ground loosening in depths of 10 to 20 cm.

The bioremediation efficiency of the polluted soil was estimated by determining the residual content of hydrocarbons (Table 5). After one month of testing, the content of hydrocarbons in the polluted soil decreased by 99.6% due to the simultaneous supplementing of the biopreparation and mineral fertilizer into the soil, while in the control site, the percentage of oil components removal was 90.7%.

Sampling date	Air temperature, °C	Soil temperature, °C	pH of soil water extract	Soil humidity, %
September 14, 2007	9	11	7.35	24
September 28, 2007	22	16	7.11	20
October 17, 2007	15	8	7.41	28
November 15, 2007	0	0	7.32	30

Table 5. Characteristics of soil and air during field tests

Sampling date	Residual content of oil products, mg/kg soil		Clean-up degree, %	
	Control plot	Experimental plot	Control plot	Experimental plot
September 14, 2007	13026±4559	11994±4198	-	-
October 17, 2007	1214±425	52.0±23.4	90.7	99.6
November 15, 2007	19.6±8.8	18.0±8.1	99.8	99.8

Table 6. Residual concentration of hydrocarbons in the control plot and in the bio-treated plot

After two months of field testing, the number of heterotrophic and hydrocarbon-oxidizing microorganisms was determined (Fig. 12). Figure 12 indicates that the addition of mineral fertilizers and soil tillage in depths from 10 to 20 cm aided the growth of microorganisms (by two orders of magnitude) compared to the control plot.

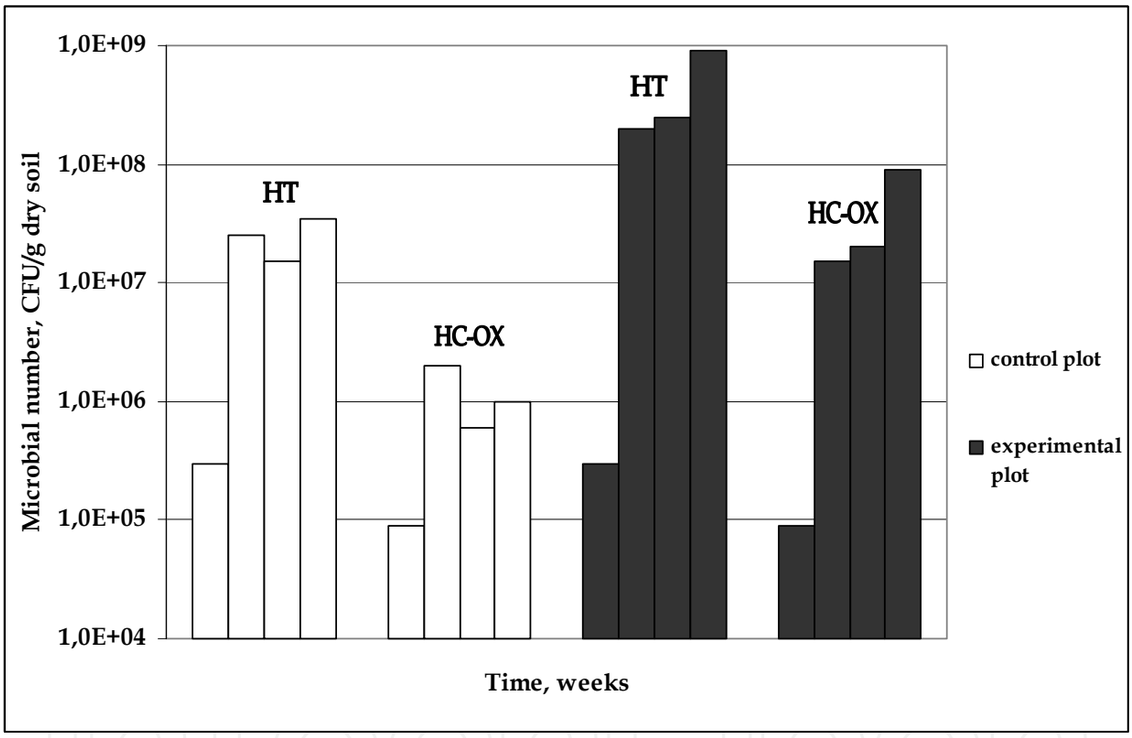

Fig. 12. Kinetics of microbial populations number (HT – heterotrophic microorganisms, HC-OX – hydrocarbon-oxidizing microorganisms)

These pilot test results demonstrate the bioremediation efficiency and the potential *in- situ* bioremediation of the biopreparation at low temperatures (0-15^{o}C).

3. Oil biodegradation by the "V&O" consortium in a wide temperature and pH range at high levels of petro-pollution

Highly-polluted areas are common in sites of oil extraction, oil transportation, and oil storage such as tailings ponds, aged oil fuel spots, and oil storage pits. Thus, the application of specialized microorganisms' preparations capable of degrading high concentrations of oil in the polluted areas in a wide range of temperatures and pH values is of special interest.

3.1 Development of the consortium "V&O"

Seven bacterial strains obtained from the Laboratory of Plasmid Biology of IBPM RAS and ten bacteria obtained from JSC "Biooil" were chosen to formulate the specialized biopreparations.

All the microorganisms were capable of growing on crude oil, diesel fuel, and fuel oil. The strains 142NF and *Pseudomonas putida* F701 demonstrated the ability of using naphthalene or salicylate as the sole carbon and energy source. While the strains *Rhodococcus* sp. S25, *Rhodococcus* sp. S67, *Pseudomonas putida* F701, 5 and *Acinetobacter baumannii* 1B were able to use toluene as the sole carbon and energy source. Microorganisms 1A, 142NF and F701 were not capable of degrading decane, nonane, and hexadecane. The strains S67, *Rhodococcus* sp. X5, *Rhodococcus* sp. X25, 142NF and F701 showed capability of degrading benzoate.

All the strains evaluated (17 strains) showed tolerance to sea salt at a concentration of 5 wt% in a liquid mineral medium polluted with 2% of diesel fuel. Strains 142NF and 2B utilized diesel fuel in the presence of 7 wt% of salt concentration, while the bacterium X5 was capable of degrading diesel fuel at a salt concentration of 10 wt%. Moreover, the strains from IBPM RAS were capable of degrading diesel fuel in a pH range of 5 to 7, with the strains S25, S26, and F701 being resistant to a pH of 8. Most bacteria from the "Biooil" collection, excluding the strains 2C, 3, 4, and 5, were able to grow in the liquid mineral medium containing 2% of oil diesel fuel in a pH range from 5 to 9. The growth of strains 1B, 2A, *Serratia* sp. 6, and the *Acinetobacter baumannii* 7 was observed at acidic conditions (pH 4) and at basic (pH 10) conditions. Almost all the microorganisms studied were able to degrade oil and diesel fuel (2%) in a temperature range of 4°C to 30°C, while strains 4, 2B, and 2C were able to grow in a temperature range of 14 to 30°C. Strains F701, 1B, 6, 7, S26 and S25 demonstrated the capability of pollutant degradation (oil or diesel fuel) at 42°C.

Bacterial growth in Evans medium with different oil content was evaluated at temperatures of 24°C and 4°C. The highest growth values at 24°C in 10 days of testing in a medium containing 40% of oil were detected only for the strains S25, S26, 1B, 6, and F701. At 4°C under similar conditions, the best bacterial growth was only detected for the S26 strain. Bacteria S25, S26, 1B, 6, F701, X25, and 7 mineralized oil at a concentration of 30% at room temperature. Strains 7, 6, and S26 were capable of degrading similar oil content at 4°C. Under the conditions of microorganisms cultivation in the liquid mineral medium at concentrations of sea salt of 40% and 3% at 24°C, the best cultivation results were obtained for strains F701,1B, S26, 2C, and 6. At the same cultivation conditions but a temperature of 4°C, microorganisms 1B and S26 were capable of growing. Strains F701, 1B, S26, 2C, 7 and 6 were effective in mineralizing diesel fuel (30% concentration) in the presence of 3% of sea salt at 24°C and at lower temperatures (4°C).

Thus, microorganisms S26, S25, X25, 2C, 1B, 6, F701, and 7 mineralized crude oil and diesel fuel at high concentrations (up to 30%) in the presence of sea salt concentration of 3% in a temperature range of 4 to 42°C and pH values of 4 to 10. These observations indicate that these strains would be effective in degrading oil under natural salinity conditions, for instance in bioremediation applications involving oil-spilled in stratum containing salted water.

The clean-up of acid soils from oil spills is often required (Gemmell & Knowlas, 2000). For these types of applications, the strains evaluated in this work show potential for the successful bioremediation of oil-polluted soils at conditions of high acidity.

The strains *Rhodococcus erythropolis* S26, *Rhodococcus sp.* S25, *Serratia sp.* 6, and *Acinetobacter baumannii* 7 demonstrated the highest efficiency in removing hexane fractions at 24°C. Bacteria *Acinetobacter baumannii* 1B and *Pseudomonas putida* F701 utilized asphalthene-tar oil components effectively. At a temperature of 24°C, bacteria of the genus *Pseudomonas* F701 and 142NF degraded hydrocarbons of benzene fractions. At low temperature (4°C), the ability to degrade benzene-alcohol fraction was revealed in strains *Rhodococcus erythropolis* S26, *Rhodococcus sp.* S25, *Serratia sp.* 6 and *Acinetobacter baumannii* 1B.

An important criterion for choosing microorganisms to formulate a consortium was the presence of catabolic plasmids. From the 17 strains screened, plasmids were discovered in 8 of the strains as follows: *Rhodococcus* sp. X25, *Rhodococcus* sp. S25, *Pseudomonas putida* F701, *Acinetobacter baumannii* 7, *Serratia* sp. 6, *Acinetobacter baumannii* 1B, *Rhodococcus erythropolis* S26, and the strain 4 (Fig. 13).

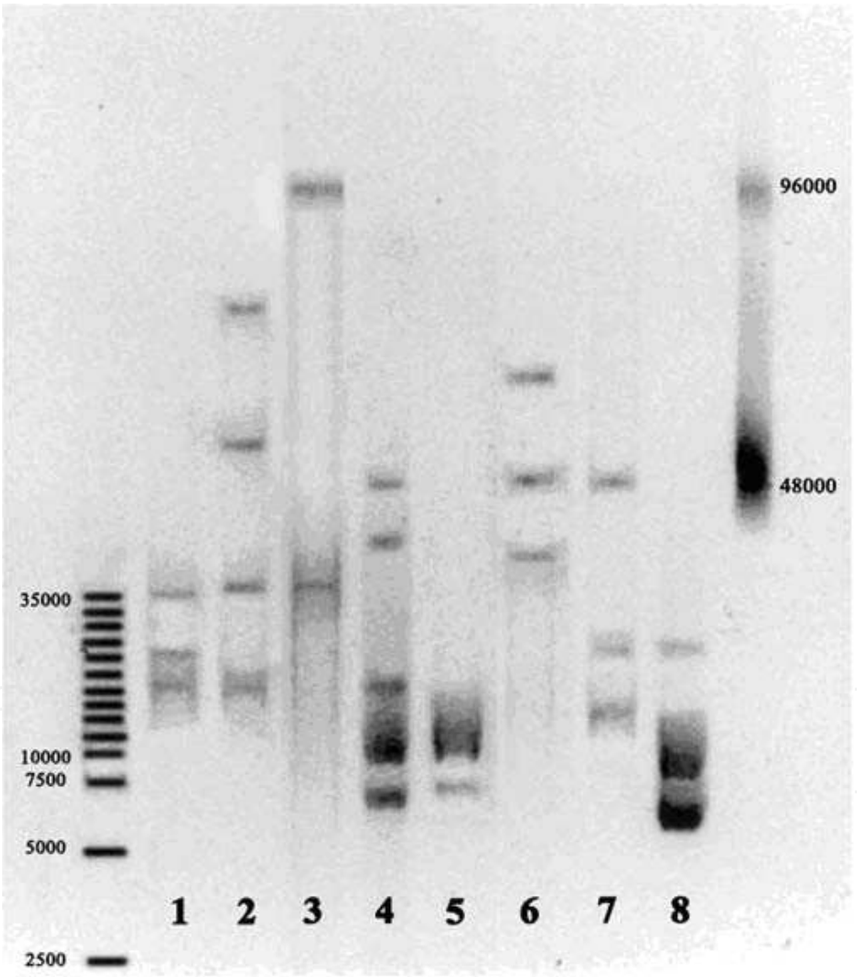

Fig. 13. Electrophoregram of plasmid DNA of degrader strains: *1 kb DNA-marker,* 1 – *Rhodococcus* sp. X25 (35, 21, 17 kb), 2 – *Rhodococcus* sp. S25 (77, 55, 36, 20 kb), 3 – *Pseudomonas putida* F701 (96, 36 kb), 4 – *Acinetobacter baumannii* 7 (50, 42, 20, 12, 6 kb), 5 – *Serratia* sp. 6 (14, 7 kb), 6 – *Acinetobacter baumannii* 1B (67, 48, 34 kb), 7 – *Rhodococcus erythropolis* S26 (48, 30, 15 kb), 8 – strain 4 (30, 8, 6 kb), *λ Ladder PFG*

The naphthalene-biodegradation plasmid pNF142 in the strain *Pseudomonas sp.* 142NF(pNF142) has been previously described (Gomes et al., 2005).

Determination of the localization of naphthalene-biodegradation genes (in the strain F701) or hexadecane-degradation genes (in the strains 1B, 4, 6, 7, S26, X25, S25) was performed through experiments on the spontaneous elimination of plasmids by strains cultivation under non-selective conditions (in Luria-Bertany medium) and through ethidium bromide treatment (10 μg/ml medium). Prolonged cultivation in the rich medium of *Pseudomonas putida* F701 and the strain 4 promoted the appearance of plasmid-free cell in a population. *P. putida* F701 and the strain 4 eliminants were not capable of growing on naphthalene and hexadecane, respectively. The plasmids discovered were stably inherited in strains 1B, 6, 7, S26, X25, and S25.

Ethidium bromide treatment induced the appearance of 1B, 6, 7, S26 and S25 plasmid-free eliminants loosing the ability to grow on hexadecane. Thus, a plasmid control for the hexadecane degradation was proposed.

Mating experiments were performed to discover possible conjugative plasmids. The naphthalene-degrader F701 and hexadecane-degraders 1B, 4, 6, 7, S26, and S25 were used as donors, and *Pseudomonas putida* KT2442 – as the recipient. Microorganisms 7 and F701 were shown to bear conjugative catabolic plasmids that are non-competitive in nature. These conjugative catabolic plasmids could enhance the degradative potential of soil microbial populations by disseminating genes among indigenous bacteria. The question about plasmid conjugativity of strains 1B, 4, S25, 6, and S26 is still open, however they could participate in the distribution of catabolic genes by transformation. The host cell dies and is lysed and the DNA with catabolic genes fluxes into the environment and may stay intact (Prozorov, 1999). Thus, applying microorganisms bearing conjugative plasmids of biodegradation seems to be promising when developing biopreparations for bioremediation technologies.

To create a consortium two steps were carried out: 1) analysis and further combination of physiological, metabolic and degradative traits of microbial properties and the presence of catabolic plasmids in strains as well; 2) selection of a mixed consortium during batch cultivation of the most active microorganisms in a liquid mineral medium with oil as a sole carbon and energy source (Table 7). Based on the traits mentioned above the following bacteria were chosen: *Rhodococcus erythropolis* S26, *Rhodococcus* sp. S25, *Acinetobacter baumannii* 1B, *Serratia* sp. 6, *Pseudomonas putida* F701, and *Acinetobacter baumannii* 7. The microorganisms were inoculated into a liquid Evans medium (pH 4) supplemented with 15% (v/v) of crude oil (Table 4) at temperatures of 4°C or 24°C.

The strains *Rhodococcus erytropolis* S26, *Acinetobacter baumannii* 1B, *Acinetobacter baumannii* 7, and *Pseudomonas putida* F701 are ahead in growth rate, which makes them dominant in the mixed population. This mixture of microorganisms was designated as "V&O" consortium. It should be noted that bacteria *Acinetobacter baumannii* 1B, *Rhodococcus erytropolis* S26, *Acinetobacter baumannii* 7 bear hexadecane degradation plasmids and the strain *Pseudomonas putida* F701 bears naphthalene biodegradation genes on a conjugative plasmid pF701a. Thus, this selected consortium of plasmid-bearing degrader strains is promising for cleaning-up soil and water sites polluted with high concentrations of oil in a wide temperature and pH range.

Criteria	Microorganisms
Medium pH 5 – 8	1A, **1B**, **2A**, 2B, **6**, **7**, S25, S26 and F701
5% NaCl	1A, 1B, 2A, **2B**, 2C, 3, 4, 5, 6, 7, **X5**, X25, S25, S26, S67, **142NF** and F701
Temperature 4 – 42°C	1B, 6, 7, S25, S26 and F701
Oil content 30% at 24°C	**1B**, **6**, 7, X25, **S25**, **S26** and **F701**
Oil content 20% at 2 – 4°C	**6**, **7**, S25, **S26** and F701
Diesel content 30% at 24°C	**1B**, **2C**, **6**, 7, **S26** and **F701**
Diesel content 30% at 4°C	**1B**, 6, 7, **S26** and F701
Biosurfactants active producer	1B, 7, X5, S26, S67 and F701
The presence of catabolic plasmids	1B, 4, 6, 7, S26, S67, X25, S25 and F701

Table 7. Criteria for formulation of a consortium of microorganisms. Strains with the best criteria indicators are bold-typed.

3.2 "V&O" consortium: oil degradation efficiency

The oil degradation efficiency of the selected "V&O" consortium was assessed through crude oil degradation experiments carried out in a liquid Evans medium at temperatures of 4°C, 24°C, and 50°C using IR-spectrometry as the analytical technique. The total testing time was 30 days and crude oil concentration was 150 g/l. The maximum decrease in oil concentration was observed after 15 days of testing (Fig. 14). It is probable that during the first 15 days of testing, the microorganisms rapidly degraded *n*-alkanes (up to C_{12}) and cyclic compounds with one aromatic ring. A drop of degradation rate was observed in the second half of the experiment; in this period microorganisms targeted the destruction of heavy (asphalthene) fractions, which are the most recalcitrant oil components.

In terms of temperature, the highest degradation degree was observed in a system at a temperature of 4°C with an oil removal percentage of 44% higher that the oil removal observed in the control test without microorganisms. Oil degradation in the systems studied was assessed against a control test for abiotic oil removal. Since, of course, abiotic removal at 4°C was less than at 24°C, the residual oil content in the control at low temperature was higher that the one at 24°C.

The lowest oil removal (only 1%) was detected in a liquid mineral medium at 50°C (Fig. 14). At a temperature of 50°C, the consortium bacteria grew under critical conditions in the absence of light hydrocarbon fractions that are easily assimilated by the consortium as carbon and energy source. The lack of light hydrocarbon fraction is due to the rapid evaporation of these fractions, which in turn, increases the concentration of heavy compounds, which are more difficult to target during the initial growing stages of microorganisms.

Thus, these results demonstrate the high effectiveness of the "V&O" consortium for oil degradation at low and moderate temperature.

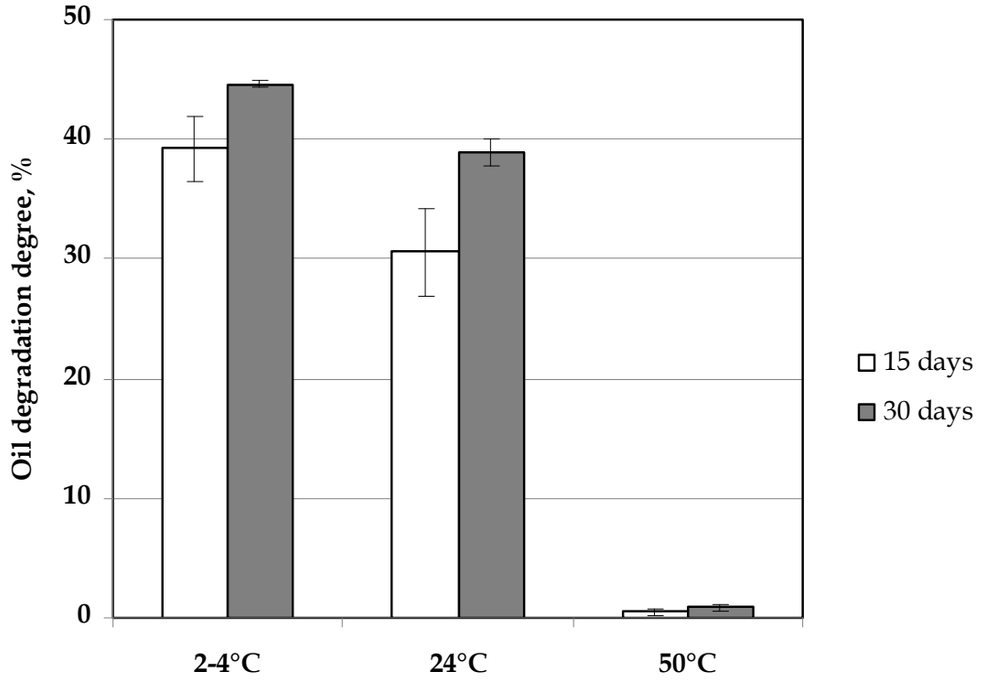

Fig. 14. "V&O" consortium: oil degradation as a function of temperature. Temperatures evaluated were 4°C, 24°C, and 50°C in a liquid mineral medium with 15% (v/v) of oil.

3.3 "V&O" consortium effectiveness versus "Microbak" and "Biooil"

The "Microbak" biopreparation and the "V&O" consortium, which were prepared at the Laboratory of Plasmid Biology (IBPM RAS), and the biopreparation "Biooil", which was provided by JSC "Biooil", were evaluated to determine their effectiveness as oil degraders.

The biopreparation "MicroBak" contains bacteria of the genera *Pseudomonas* and *Rhodococus;* while the biopreparation "Biooil" includes *Bacillus, Sacharomyces, Acinetobacter*, *Enterobacter.*

Assessment of consortia efficiency was performed in a liquid mineral medium containing crude oil (15% v/v) as a sole carbon and energy source (Table N). Infrared spectroscopy was used as the analytical technique to determine the concentration of residual oil in the samples. Figures 15 and 16 summarize the results of oil degradation during cultivation of the three consortia at temperatures of 4°C and 24°C in a period of 30 days. The "V&O" consortium at 4°C rendered the highest oil hydrocarbons removal (44%) after 30 days, while the oil degradation degree in the systems inoculated with the "Biooil" and the "MicroBak" biopreparations reached 36% and 40% after 30 days, respectively (Fig. 15).

At a temperature of 24°C, the V&O consortium induced the highest oil hydrocarbons removal both after 15 days (31%), and after 30 days (39%) (Fig. 16). The "Biooil" and "MicroBak" biopreparations rendered similar oil degradation after 30 days of cultivation with percentages of 36% and 34%, respectively.

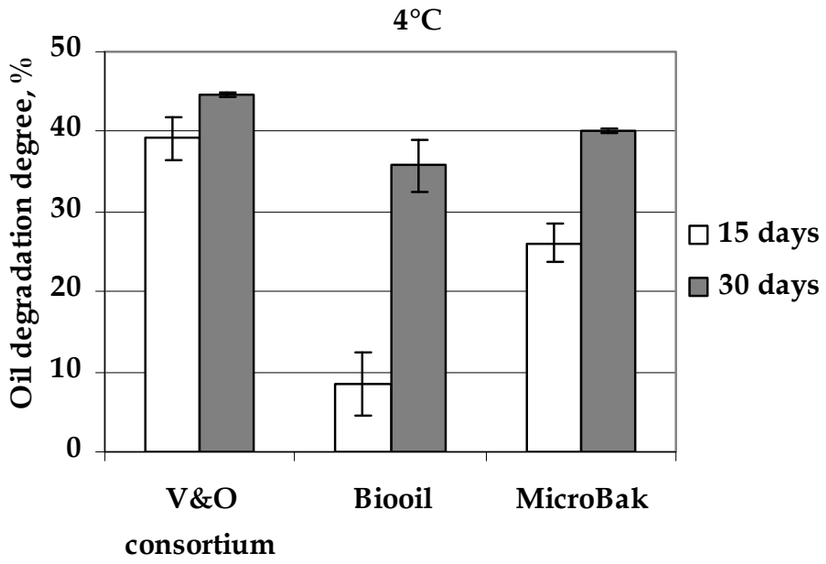

Fig. 15. Effectiveness of "V&O" consortium, biopreparations "Biooil" and "MicroBak" in degrading oil in a liquid mineral medium with 15% (v/v) oil at low temperature (4°C).

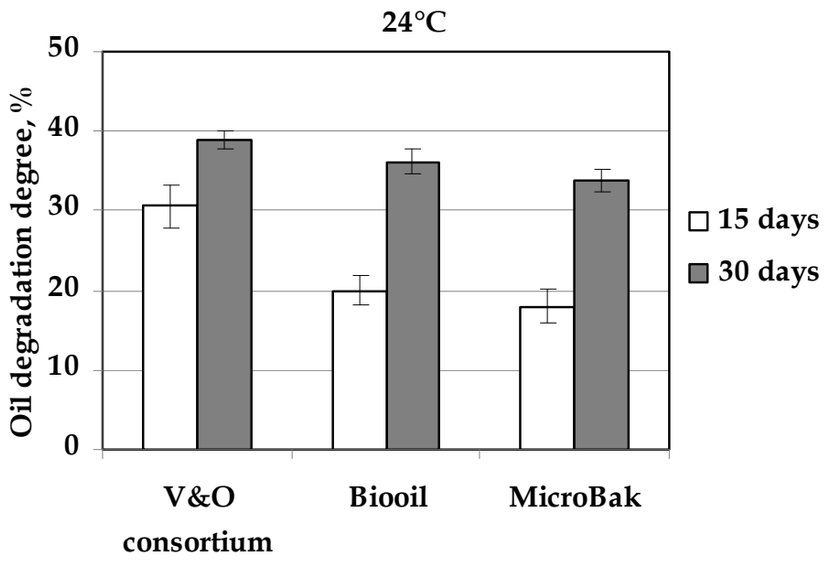

Fig. 16 Effectiveness of "V&O" consortium, biopreparations "Biooil" and "MicroBak" in degrading crude oil in a liquid mineral medium with 15% (v/v) oil at moderate temperature (24°C).

The oil consumption by bacteria in the "MicroBak" and in the "Biooil" biopreparations was slower in comparison with the "V&O" consortium strains, which showed a longer adaptation period to the pollution conditions that was confirmed by bacterial number change (data not shown).

4. Efficiency of plant-microbial consortia

Some plants apply a number of mechanisms to overcome the effect of toxic pollutants. These mechanisms include excretion, conjugation of toxic substances with intracellular compounds and further compartmentalization of conjugates, degradation of pollutants to the cellular metabolites and carbon dioxide. Thus, in the process of soil decontamination from oil and oil products, it seems to be promising to make use of the synergistic approach of microorganisms and plants associations.

Sterile soil experiments were conducted to study oil degradation degree, the interactions between microorganisms within the selected consortium, and to establish the influence of oil hydrocarbons - degrading microorganisms on plants growth.

4.1 Screening of plants to create an effective plant-microbial association

The screening of 20 different plants indicated that sunflower, corn, barley, lawn grass (a mix of grasses with the basic red fescue grass), and some kinds of beans (a string bean, peas) are resistant to petro-pollution (2% (v/w) of crude oil). During the screening process, besides the resistance to toxic oil hydrocarbons shown by the root system of the plants (spur or fibrous), it was also important to consider the branchiness and soil volume coverage of the plants. Thus, it was found that barley and lawn grass roots had a dense biomass and they occupied the highest soil volume. Thus, the associations "V&O - barley" and "V&O – lawn grass" were selected for evaluation in further experiments.

4.2 Removal of oil content by microbial-plant associations

Laboratory tests were carried out to evaluate the oil degradation performance of the "V&O - barley" microbial-plant association. Oil degradation was assessed using a "plant – single strain" association and a "plant – V&O consortium" association in sterile conditions for a testing period of 10 days.

The introduction of microorganisms into soil model systems polluted with oil and seeded with plants (barley) promoted the detoxification of the soil. The degree of detoxification was determined by measuring the shoot length of the plants in the presence of microbial-plant associations and without the microbial-plant associations (Control test: plants + crude oil without microorganisms) (Fig. 17). The detoxification induced by the plant-microbial associations was probably caused by the ability of bacteria to colonize plant roots and rhizosphere with the simultaneous degrading of crude oil, which minimizes the toxic effect of crude oil on plant development.

The number of bacteria in the roots (in water washouts from the roots) was higher (by 1-2 orders of magnitude) than in the rhizosphere (the number of bacteria was determined, using washouts from the near-rhizosphere soil). It has been previously demonstrated that during the simultaneous cultivation of *Rhodococcus* and *Pseudomonas* strains, the number of rhodococci strains decreases in more than one order of magnitude, while the number of pseudomonads strains increases (Baryshnikova et al., 2001). Our results demonstrate the absence of negative interactions between the "V&O - barley" microbial-plant association such as antagonism or allelopathy.

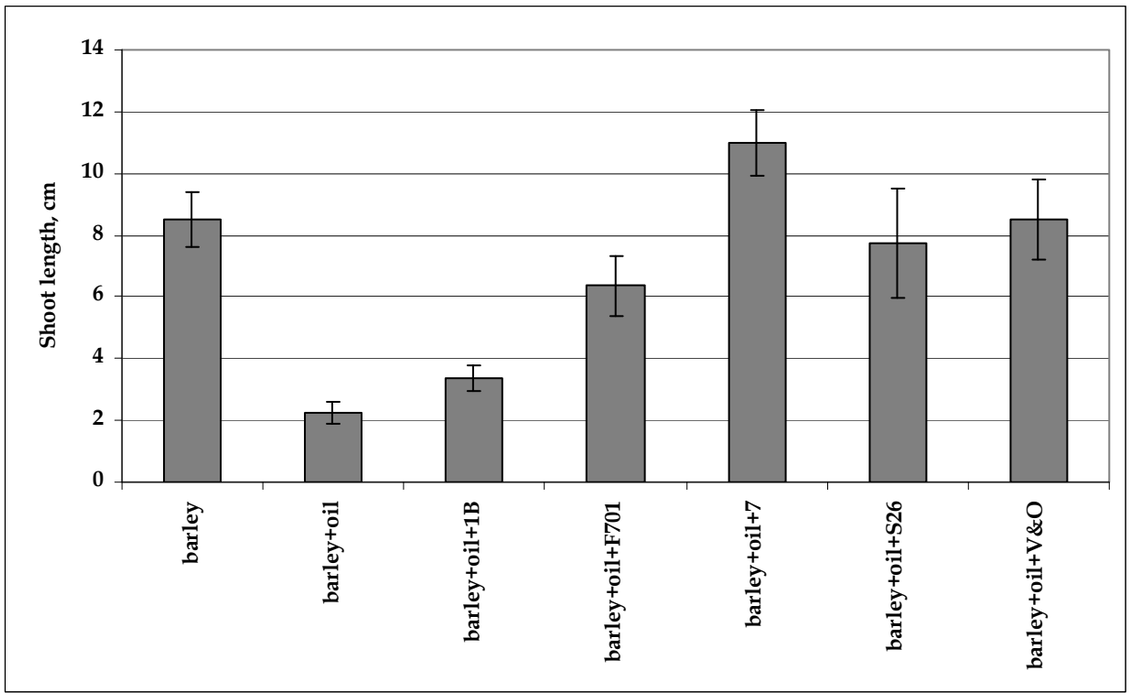

Fig. 17. The length of barley shoots in a sterile model experiment after 10 days of cultivation

In terms of percentage of oil degradation as a result of the application of microbial-plant associations, the experimental results indicate that the highest percentage of oil degradation (19%) was obtained by the application of the "V&O" consortium–barley association (Fig. 18). The other plant-microbial associations evaluated -"*Rhodococcus erythropolis* S26 – barley", "*Acinetobacter baumannii* 1B – barley", "*Acinetobacter baumannii* 7 – barley", and "*Pseudomonas putida* F701 – barley" – rendered a lower percentage of oil removal (< 15%) (Fig. 18).

During the microbial oil biodegradation within microbial-plant associations a cooperation process among bacteria and the plant accelerates the effective utilization of hydrocarbons. The contributions of the plant on the biodegradation of oil are related to enzymatic systems and pollutants detoxification mechanisms. Naumann et al. (1991) reported that the interaction of plant roots with organic compounds (including oil hydrocarbons) induces peroxidase activity that could function in the cell as a protection mechanism and/or influence on the degradation of pollutants present in the environment.

To establish if the microbial-plant association -"V&O – barley"- could be made more effective by the addition of mineral fertilizers non-sterile laboratory experiments were conducted in open systems with oil-spilled soil in the presence of a fertilizer ("Nitroammophoska") and without fertilizer for a period of 14 days. The experimental results demonstrated that supplementing the "V&O – barley "association with fertilizers did not influence on plant growth and did not change the number of the introduced microorganisms.

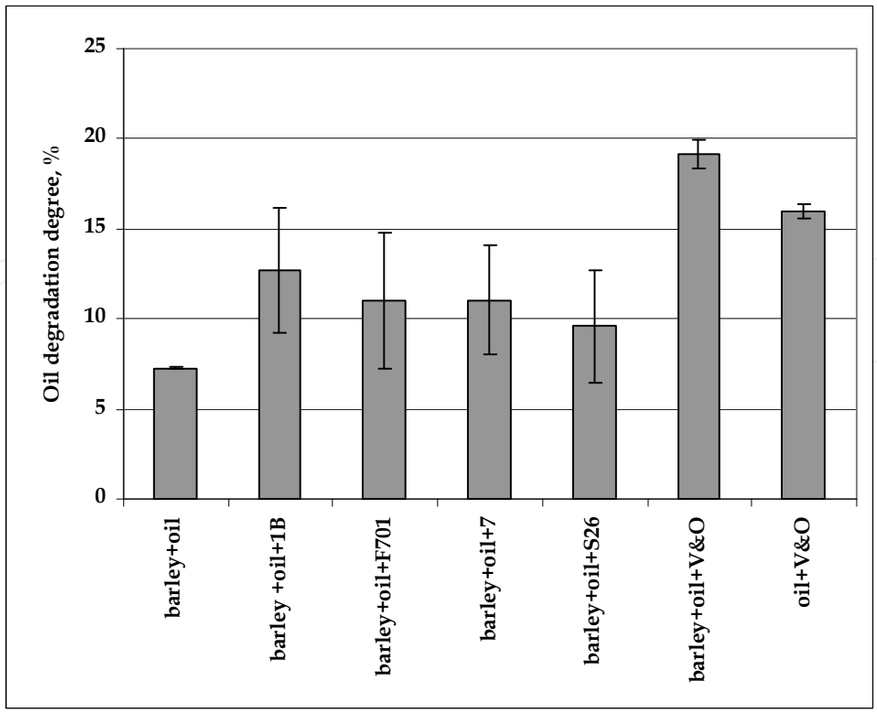

Fig. 18. Oil removal in model soil systems as a function of plant-microbial associations. Testing period: 10 days

5. Field tests of "V&O" consortium and JSC "Biooil" biopreparations

Field experiments on the Yamal-Nenetsky Autonomous Region in Western Siberia on sites of the "Pogranichnoe" oil deposit were conducted during July-August, 2008, with the objective of evaluating the efficiency of the microbial consortium "V&O" and the biopreparations "Biooil-SN" and "Biooil-Jugra" in degrading oil pollutants. The level of soil pollution in the sites ranged from 15 to 110 g/kg soil in an area of 8300 m². In this field testing, favourable conditions for the growth of microorganisms were created by adding mineral and organic fertilizers (calurea), tillaging, and water sprinkling.

After two months of the introduction of the "V&O" consortium the oil clean-up degree on the test site was 80%, while the JSC "Biooil" biopreparations rendered a clean-up ranging from 50 to 70%. The "V&O" consortium show high efficiency in degrading oil hydrocarbons already in a month after treatment (Fig. 19).

Before the experiment, the concentration of indigenous oil-degrading microorganisms was low (5.2×10^1 CFU per g soil) due to the high oil content (>10% of soil mass by weight). However, after the treatment with the "Biooil" preparations and the "V&O" microbial consortium the number of hydrocarbons-utilizing microorganisms increased by 3 orders of magnitude, this increased in microorganism population intensified the biodegradation of oil. These results are confirmed by the oil content removal data presented in Fig. 19.

Chemical toxicity of crude oil for living organisms is not always evident (Pikovsky, 2003). Often recovery of oil-spilled soils is traced by a pollutant change in the soil and sometimes pollutant removal does not show the decrease of its toxicity for living organisms. The soil is

known to be a reservoir that can accumulate high concentration of pollutants. Thus, the soil can be highly polluted but non-toxic and vice versa, low polluted but highly toxic for living organisms, thus it is necessary to control the bioremediation process in terms of both residual oil content and phytotoxicity indicators (Knoke et al., 1999; Phillips et al., 2000). Therefore, phytotoxycity of the soil was visually evaluated by taking photographs of the site after 2 months of bioremediation. The photographs revealed a biomass increase on the plot treated with "V&O" consortium, which is an indication of low phytotoxicity.

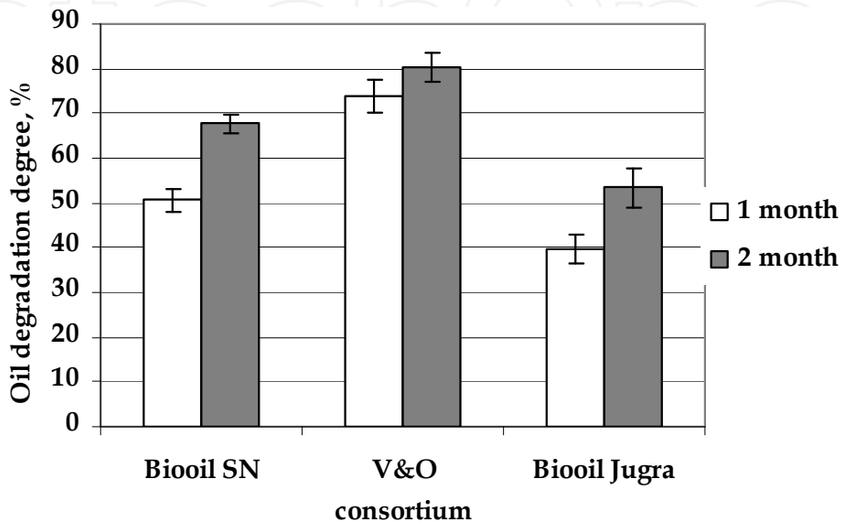

Fig. 19. Oil degradation in a field experiment in Western Siberia with biopreparations and a consortium

6. Conclusions

Results obtained from laboratory and field experiments demonstrated the efficiency of the "V&O" consortium in degrading oil. The percentage of oil removal obtained from the "V&O" consortium is higher (by 10 to 25%) if compared to the efficiency of the JSC "Biooil" biopreparations. Similarly, laboratory and field tests also demonstrated the high oil-oxidizing activity of the consortium "V&O" and the biopreparation "MicroBak". Thus, the consortium "V&O" and the biopreparation "MicroBak" are applicable to clean-up soil and water biotopes from crude oil and oil products spills at temperatures ranging from 4°C to 42°C.

7. Acknowledgment

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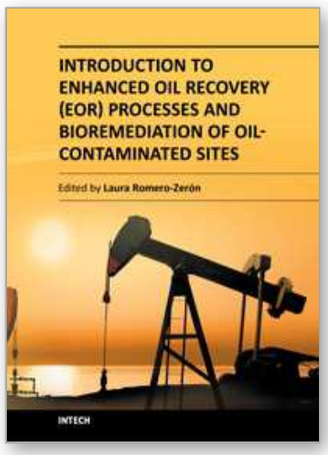

Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites

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This book offers practical concepts of EOR processes and summarizes the fundamentals of bioremediation of oil-contaminated sites. The first section presents a simplified description of EOR processes to boost the recovery of oil or to displace and produce the significant amounts of oil left behind in the reservoir during or after the course of any primary and secondary recovery process; it highlights the emerging EOR technological trends and the areas that need research and development; while the second section focuses on the use of biotechnology to remediate the inevitable environmental footprint of crude oil production; such is the case of accidental oil spills in marine, river, and land environments. The readers will gain useful and practical insights in these fields.

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