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Biological Products for Soil and Water Purification from Oil and Petroleum Products

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Abstract: The purpose of the article is to study whether it is possible to clean soils from oil and petroleum products using bacterial strains. Experimental samples of biological products were cultured in Erlenmeyer flasks in Evans mineral medium in the presence of 15% oil (volume/volume) at a temperature of 24°C for 50 days. For 6 weeks of the experiment, the loss of oil in the area with the test sample of the biopreparation was 70%. The association can be used in saline (up to 8% salt) soils in the pH range 4-9, and at low soil humidity (about 10%).

Keywords: hydrocarbons; petrodestructors; oil; biological products; chromatography; bioremidiation

1. Introduction

When developing microbial preparations and technologies for oil purification of soils and waters in a hot arid climate, it should be considered that the climate of such regions is characterised by large daily temperature differences, high rates of water evaporation and, as a consequence, salinity and low soil humidity[1)]. Purification from oil pollution, and resource conservation issues are especially relevant for Kazakhstan, which is one of the ten largest oil-producing countries in the world[2)]. High soil temperature in spring and summer, followed by evaporation of water and spills of saline drilling fluids leads to chronic salinisation of soils[3)].

Bioremediation is one of the most economical and environmentally friendly, widely used technologies of soil reclamation, in case of contamination with crude oil and petroleum products[4)]. This technology is based on the use of microbial associations consisting of two or more strains, since the introduction of a monoculture of hydrocarbon-oxidizing microorganisms into an oil-contaminated environment cannot completely solve the problem of purification. Many scientific papers have been devoted to the study of this problem[5,6)]. Unique bacterial communities adapted to pollution develop in oil-contaminated soils. In the work of Aitkeldiyeva et al.[7)], using the Illumina MiSeq sequencer, the bacterial structure and diversity of the microbial community in samples of oil-contaminated soils of Kazakhstan deposits were studied.

Currently, a number of preparations for the destruction of oil in temperate and cold climates are presented on the market of Russia and CIS countries. The main of this series is the drug biodestructor of oil pollution microbial-enzymatic microzyme(tm) "PETRO TRIT"[8)]. The dry powder form of the biopreparation contains a consortium of (12) strains of living hydrocarbon-oxidizing microorganisms (UOM) in the form of a concentrate of dry spores with a titer of 4x10 in 12 CFU/gr., mineral salts, an environmentally friendly nutrient carrier.

Scientists have developed a number of bacterial strains[9,10)]. The strain of bacteria *Arthrobacter sp.* 12T, *Arthrobacter sp.* 15T, *Dietzia maris* 84T, *Arthrobacter luteus* 43-A actively recycles oil from various fields of the Caspian region in the mineral environment, seawater and soil[16)]. The degree of destruction of oil, respectively, is: 59.3-85.2%; 58.4-84.8%; 72.4-90.6%; 56.4-80.1%. A common disadvantage of biologics containing a single strain is the lack of environmental plasticity, i.e., in unfavourable conditions, the biologics may lose their activity. And also, the lack of information about whether strain cells are able to survive in a wide pH range and temperature range. In that case is use a biological preparation, which includes *Arthrobacter* microorganisms *sp.* VKM As-2272 D and *Rhodococcussp.* VCM As-2045 D; and also (wt. %): glycerin–8-10, KNO_3–0.360-0.367, KN_2 RO $_4$–0.054-0.055, Na $_2HPO_4$-12H_2 O –0.126-0.128. The method of applying the biopreparation in the form of

a culture liquid causes its short shelf life (1-2 weeks), which is its disadvantage.

The authors[11,12] carried out work on the selection of an active bacterial association capable of consuming oil and petroleum products at a temperature range of 4-50°C. Since the soils of the Republic of Kazakhstan are saline and, as a rule, dry, microorganisms resistant to low humidity (below 10%) and increased salt content (up to 8%) in the medium were used to create an association of bacterial strains of oil and petroleum products destructors. The conducted studies revealed the most promising microbial association consisting of a strain of *Pseudomonas sp.* and two strains of the genus *Rhodococcus* – as the basis of a prototype of a biological product. It has been established that the strains of the association are capable of consuming both alkanes (aliphatic hydrocarbons) and polycyclic aromatic hydrocarbons of oil[13].

The purpose of the article is to study whether it is possible to clean soils from oil and petroleum products using bacterial strains.

2. Materials and Methods

Experimental samples of new biological products created on the basis of the studied microbial associations were used in the work:

– prototype of biological product No. 1, consisting of a strain of the genus *Acinetobacter* No. 7 and two strains *Rhodococcus* (F2/2-H4, F2/1-H4);

– prototype of biological product No. 2, consisting of a strain of *Pseudomonas sp.* K3-H and two strains *Rhodococcus* (F2/2-H4, F2/1-H4).

Biodegradation of individual oil fractions by experimental samples of new biological products in an aqueous medium at 24°C. Experimental samples of biological products were cultured in Erlenmeyer flasks in Evans mineral medium in the presence of 15% oil (volume/volume) at a temperature of 24°C for 50 days. The content of n-alkanes in the residual oil was determined by capillary gas-liquid chromatography. Oil was extracted from the liquid phase by chloroform. The chloroform extract was drained with anhydrous sodium sulfate and evaporated at room temperature to dry. Purification from the polar fraction was carried out on aluminum oxide Al_2O_3. The samples were analyzed on an Agilent 7890 gas chromatograph with a flame ionization detector, DB-1ms column, length 30 m, diameter 0.25 mm, and phase thickness 0.25 μm. For calibration according to individual n-alkanes, a standard mixture of n-alkanes ($C_{11}H_{24}$-$C_{36}H_{74}$) containing a substance and a phytane was used. The content of PAHs (Polycyclic aromatic hydrocarbons) was determined by capillary gas-liquid chromatography.

Oil was extracted from the liquid phase by chloroform. The chloroform extract was drained with anhydrous sodium sulfate and evaporated dry. Purification of the chloroform extract for the determination of PAH was carried out on a Diapak cartridge C. The analysis of the PAH fraction after transfer of acetonitrile heated to 60°C into a chromatographic vial was carried out on an Agilent 1200 liquid chromatograph with a fluorimetric detector, a column of Diasfer 110-C18, length 250 mm, diameter 4 mm, particle size 5 μm. For calibration according to individual PAHs, a standard mixture of PAHs in acetonitrile (13 PAHs) was used. The concentrations were calculated considering the calibration dependence of the peak areas of individual PAHs on their concentration in a standard solution. The calibration curve was constructed and the PAH concentration in the samples was calculated using the ChemStation program.

3. Results and Discussion

To determine the effectiveness of the proposed samples of new biological products based on microbial associations, studies of their properties were conducted. It was found that the prototype of the biological product No. 1, consisting of a strain of the genus Acinetobacter No. 7 and two strains of *Rhodococcus* (F2/2-H4, F2/1-H4) and a prototype of biopreparation No. 2, consisting of a strain of *Pseudomonas sp.* K3-H and two strains of *Rhodococcus* (F2/2-H4, F2/1-H4) showed a pronounced oil-oxidizing ability, accompanied by a decrease in the mass of normal alkanes and an increase in the index of biological degradation. The results are shown in Table 1 and Figure 1.

Table 1. The content of n-alkanes in the studied samples after biodegradation of oil for 50 days at a temperature of 24^0C

Alkanes	A control sample of a biological product	The studied samples of the biopreparation	
		No. 1.	No. 2.
	mcg		
$C_{11}H_{24}$	47532	12851	3430
$C_{12}H_{26}$	89866	26527	7219
$C_{13}H_{28}$	127788	47212	17964

$C_{14}H_{30}$	136074	65651	32840
$C_{15}H_{32}$	161664	98819	59382
$C_{16}H_{34}$	141217	91476	51692
$C_{17}H_{36}$	148669	106831	68787
pristan	57062	56210	50632
$C_{18}H_{38}$	132421	107295	76820
fitan	45819	45092	40538
$C_{19}H_{40}$	144174	123256	96241
$C_{20}H_{42}$	135708	117235	94333
$C_{21}H_{44}$	137091	120092	98092
$C_{22}H_{46}$	115617	102582	84211
$C_{23}H_{48}$	115727	103007	83955
$C_{24}H_{50}$	81487	72842	57892
$C_{25}H_{52}$	73462	65116	50532
$C_{26}H_{54}$	51689	45658	35433
$C_{27}H_{56}$	43587	38001	29097
$C_{28}H_{58}$	24256	20966	15419
$C_{29}H_{60}$	22746	19062	13972
$C_{30}H_{62}$	12498	10299	7288
$C_{31}H_{64}$	8338	6758	522
$C_{32}H_{66}$	4273	2803	2371
$C_{33}H_{68}$	197	182	118
$C_{34}H_{70}$	1508	1098	809
$C_{35}H_{72}$	307	323	550
$C_{36}H_{74}$	712	463	387
the amount of alkanes	2061489	1507707	1080525
index (dimensionless)	0.4	0.5	0.6
Degradation of alkanes	%		
		27	48
Fractions of petroleum products	mcg		
medium boiling	3370672	2921897	2376099
high boiling	2355042	2189018	1742117
sum of fractions	5725713	5110915	4118216

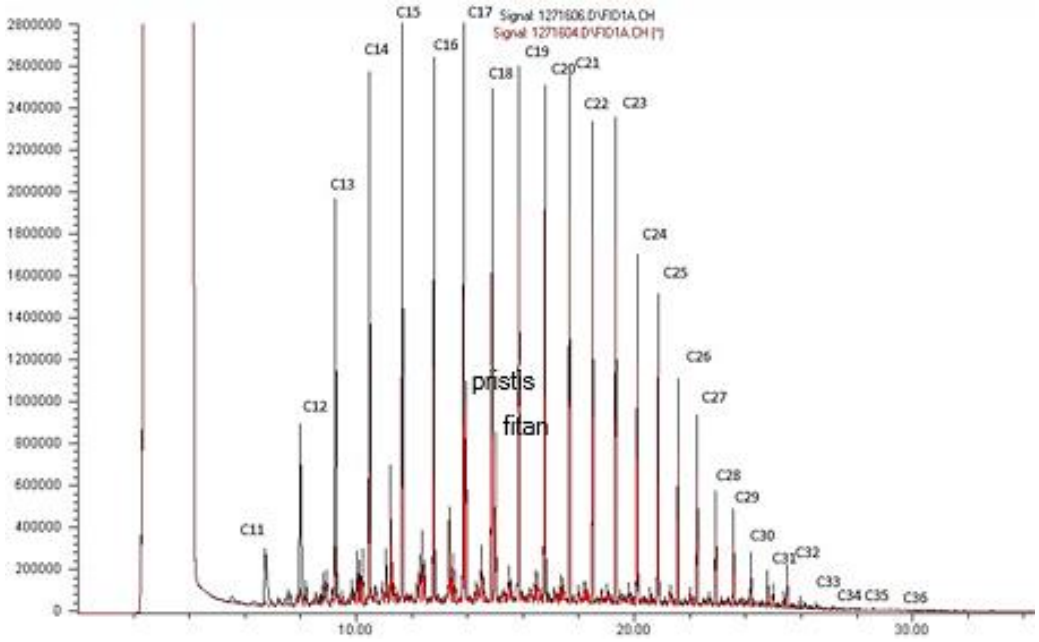

Fig. 1. Chromatogram of residual n-alkanes after biodegradation of oil by a prototype of biological product No. 2 (red) compared to the control (black color)

It was shown that the most effective biological preparation No. 2 (F2/1-H4, F2/2-H4, K3-H) degraded 48% of the studied n-alkanes compared to the control (medium with oil without microorganisms). For biopreparation No. 1, this indicator was 27%. The paper also investigated the differences in the consumption of individual n-alkanes by the studied associations. Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous stable pollutants contained in wastewater and gas emissions from coke, gas and petrochemical industries[14]. Recently, pollution of soils and water systems in industrially developed areas of the world has been a serious problem, since many PAHs belong to the class of carcinogens and mutagens. We carried out a quantitative assessment of the residual content of various PAHs (with the number of rings from 3 to 6) in samples obtained after oil degradation by the experimental samples of biological products under study: No. 1 and No. 2. Biopreparations were cultured in Erlenmeyer flasks in Evans mineral medium in the presence of 15% oil at a temperature of 24°C for 50 days. The results are shown in Table 2 in Figure 2.

Table 2. Residual content of PAHs (mcg) and their degree of destruction (%) in the studied samples according to the results of high-performance liquid chromatography

PAU	A control sample of a biological product	The studied samples of the biopreparation			
		No. 1.	No. 2.	No. 1.	No. 2.
	mcg			Degradation, %	
fluorene	180	120.6	90	33	50
acenaphthene	205.2	136.8	108	33	47
phenanthrene	876.2	709.2	576	19	34
anthracene	176.4	115.2	77.4%	35	56
fluoranten	194.0	177.8	154.8	8	20
pyrene	127.1	60.5	57.6	52	55
benzo(a)anthracene	0	0	0	0	0
chrysene	135	95.8	77.4%	29	43

benzo(b)fluoranthene	496.1	414	312.5	17	37
benzo(k)fluoranten	24.0%	12.6	9.0	48	62
benzo(a)pyrene	(21.2)	8.9	6.8	58	68
dibenz(a.h)anthracene	66.6	57.6	59.4	14	11
benzo(g.h.i)perylene	24.1%	22.0	15.4	9	36
PAH amount	2526.0	1930.9	1544.3		
degradation of PAHs (by amount), %	-	23.6	38.9%		

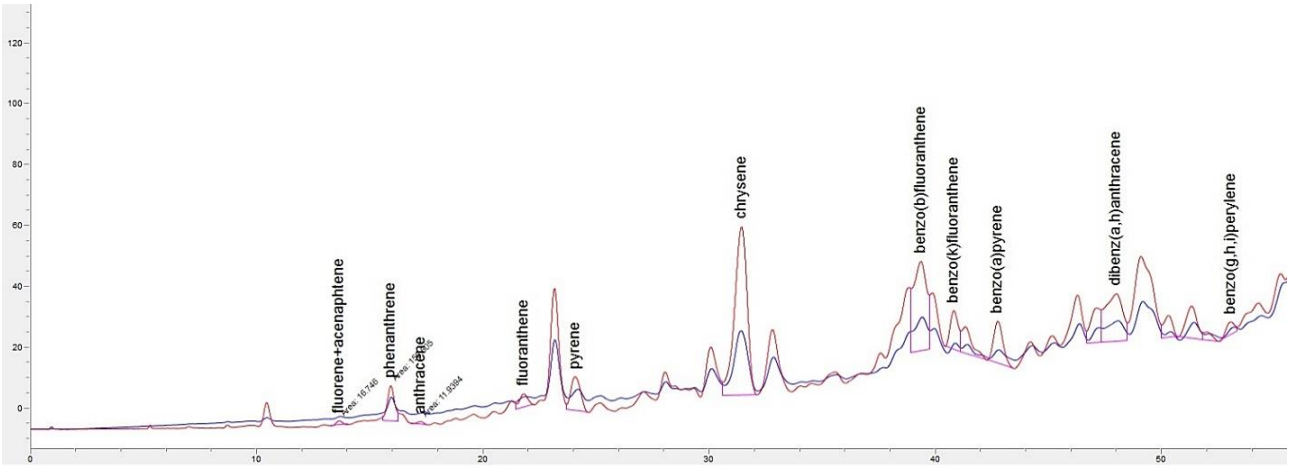

Fig. 2. Chromatogram of residual polycyclic aromatic hydrocarbons after biodegradation of oil by experimental samples of biological products No. 1 (red) and No. 2 (blue)

The PAH content was determined by capillary gas-liquid chromatography. The concentration of individual 13 PAHs was determined in the studied samples[15]. It is shown that PAHs are most effectively consumed by biopreparation No. 2. This biological product effectively decomposes polycyclic hydrocarbons (the number of rings is 3-6): PAHs were degraded by 38.9% relative to the control. For biopreparation No. 1, this indicator was 23.6%. Based on the analysis of the results obtained, a prototype of biopreparation No. 2 was selected for further work, consisting of a strain of *Pseudomonas* K3-H and two strains *Rhodococcus* F2/1-H4, F2/2-H4. The three bacterial strains included in the association are further designated *Rhodococcus erythropolis* KZ1*, Rhodococcus erythropolis* KZ2*, Pseudomonas putida* KZ3[16].

One of the important stages in the development of biological products for cleaning the environment from oil pollution is the production of biomass of destructive strains on an industrial scale[17]. In the production of biological products are used *Pseudomonas sp.* K3-H and two strains of *Rhodococcus* F2/1-H4, F2/2-H4, in ANKUM-Fermenter 2M with a volume of 10 liters with a filling factor of 0.6. The media, conditions and cultivation modes were selected for the production of biomass of microorganisms that are part of the biological product[18]. The concentrated suspension of microorganisms was obtained by centrifugation for 30 minutes on a K70 "Janetzki" centrifuge. As a result of the conducted fermentations, a liquid form of a biological product was obtained, consisting of a strain of *Pseudomonas putida* KZ3 and two strains *Rhodococcus erythropolis* KZ1 and KZ2 with the number of microorganisms in the culture fluid 8.3×10^{10} CFU/ml, where 7.0×10^{9} (*Pseudomonas putida*) and 8.2×10^{10} CFU/ml (*Rhodococcus sp.*).

After separation of the culture liquid, a paste with the number of microorganisms in a concentrated suspension of $5.0\times^{10\text{-}11}$ CFU/g was obtained. The dry form of the preparation obtained by freeze-drying with the number of microorganisms 8.0×10^{10} CFU/g was stored at a temperature of 4-8°C for 6 months. Then the number of viable cells was checked. The analysis showed that the number of microorganisms in the sample of the dry preparation was 7.8×10^{10} CFU/g, i.e., when stored at a temperature of 4-8°C for 6 months, there was no loss of viability of microorganisms[19]. Thus, the conditions for storing microbial biomass in both liquid and freeze-dried form have been selected. Pilot-industrial testing of the developed composition of the biological product was carried out at the landfill of oil waste of "K-Kurylys LLP", the Company is engaged in the disposal of all types of oil-containing waste (oil sludge, drilling mud, drilling mud,

smeared soil, etc.). The duration of the pilot test was 2 months; soil moisture was maintained by sprinkling[20].

For field testing, a dry biological product with a microbial population of 8.0×10^{10} CFU/g was used. Two plots of land were plowed at the "K-Kurylys LLP" oil waste landfill with an area of 4 m^2 and a depth of 10 cm for natural soil aeration. Plot: No. 1-control; No. 2-contaminated, for treatment with a biological product (10.34 oil, g/kg of soil). As an organic fertilizer, cow manure was applied to each plot from the calculation of 5 kg/m^2. Suspension with microorganisms with a cell size of 8.0×10^{10} CFU/g was mixed with 4 liters of fresh water (per 4 m^2), 5g of NaCl, 1 g of nitroammophosky and 40 ml of diesel fuel were added, and thoroughly mixed[21]. The drug was activated by aeration (bubbling with a microcompressor, 1 l/min) for 14-18 hours. The diluted drug was used within a day after full activation. On the day of application of the drug, 40 g of nitroammophosky (10g/m^2) was dissolved in 5 liters of fresh water[22,23]. 2.5 liters of the drug and 2.5 liters of nitroammophosky solution were mixed in a container. The total volume in the tank was brought up to 10 liters by adding 5 liters of fresh water. 10 liters of the resulting suspension were applied to a plot of 4 m^2. The control area was watered with 10 liters of fresh water[24),25),26]. Two weeks later, the application of the drug was repeated without adding fertilizers. The ambient temperature during this period was 36-46°C. To determine the oil, averaged samples of 5 g were taken from 3-4 different sites every 2 weeks (Table 3).

Table 3. Residual oil content and the degree of its loss in model open systems

Date	Control area		An oil-contaminated site treated with a biological product	
	Residual amount of oil, g/kg of soil	Decline, %	Residual amount of oil, g/kg of soil	Decline, %
30.05.2017	2.18	0.00	10.34	0.00
14.06.2017	1.98	9.17	5.60	45.84
29.06.2017	1.98	9.17	4.40	57.45
17.07.2017	1.68	22.94	3.13	69.73

During the 6 weeks of the experiment, the loss of oil in the area with the test sample of the biopreparation was 70%, where the control biopreparation was used, the loss reached only 23%. Thus, the effectiveness of the developed biopreparation KZ1, KZ2 and KZ3 is shown. To determine the total number of microorganisms, averaged samples of 1 g were taken from 3-4 different soil sites every 2 weeks. Samples weighing 1 g were resuspended in 9 ml of phosphate buffer and mixed on a Paramix 2 mixer (Germany) for 1 minute at room temperature and after appropriate standard dilutions were sown on cups with rich medium and minimal Evans medium with the addition of diesel fuel. The cups were incubated at 24°C for 3-7 days. The number of colony-forming units was calculated per 1 g of dry soil (Table 4).

Table 4. Changes in the number of microorganisms in the soil

Date	Control area		An oil-contaminated site treated with a biological product	
	Total number, CFU/g of soil	Number of destructors, CFU/g of soil	Total number, CFU/g of soil	Number of destructors, CFU/g of soil
30.05.2017	7.2×10^6	1.0×10^5	1.2×10^8	9.6×10^7
14.06.2017	4.0×10^6	1.0×10^5	4.7×10^7	8.5×10^6
29.06.2017	3.2×10^7	7.50×10^5	1.4×10^8	5.0×10^6
17.07.2017	3.6×10^6	4.0×10^5	2.0×10^7	1.4×10^6

Over the course of the 6-week experiment, the number of harmful microorganisms in the oil-polluted area was 100 times greater than in the controlled area. This was due

to the introduction of microorganisms from the biological product, which aided in the breakdown of oil by 70% in the polluted area. In contrast, in the controlled area, oil loss only reached 23%. The ambient temperature during this period was 36-46°C. The effectiveness of the developed biopreparation based on strains KZ1, KZ2 and KZ3 is shown.

4. Conclusions

The cultural-morphological, physiological features were determined, the virulence of the strains was studied, the allergenic effect of the sensitizing effect was investigated, the ability to damage the conjunctival membrane of the eye was studied. According to the existing classification of strains, it was concluded that all three studied cultures belong to the 4th hazard class, and are not pathogenic. Bacterial strains intended for the removal of oil and petroleum products from soils and waters in a sharply continental and hot arid climate are deposited in the RSE "Republican Collection of Microorganisms" under individual numbers: *Rhodococcus erythropolis putida* KZ1 numbered B-RKM 0800, *Rhodococcus erythropolis* KZ2 numbered B-RKM 0798, *Pseudomonas putida* KZ 3 numbered B-RKM 0799.

Thus, the research results show that the cleaning of soils from petroleum products and oil using bacterial strains is possible at temperatures from 4°C to 50°C. The association consists of three strains of bacteria *Rhodococcus erythropolis* KZ1, *Rhodococcus erythropolis* KZ2, *Pseudomonas putida* KZ3, which have mycostatic activity (suppresses phytopathogenic fungi). The association can be used in saline (up to 8% salt) soils and waters, in the pH range 4-9, as well as at low soil humidity (about 10%).

The practical value of this article is that it provides information on the characteristics and effectiveness of three bacterial strains, *Rhodococcus erythropolis* KZ1, *Rhodococcus erythropolis* KZ2, and *Pseudomonas putida* KZ3, which can be used for removing oil and petroleum products from soils and waters in a hot and arid climate. Therefore, the findings of this study can be used to develop effective bioremediation strategies for oil and petroleum spills in arid regions, leading to more efficient and cost-effective cleanup efforts.

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