Bioremedation of Oil and Oil Products Bacterial Species of the Genus P*seudomonas*

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Abstract

In the article the results of investigation of oxidation processes of crude-oil by bacteria of genus Pseudomonas: Pseudomonas mendocina H-3, Pseudomonas sp. H-7, Pseudomonas stutzeri H-10, Pseudomonas aeruginosa H-14, Pseudomonas alcaligenes H-15 and Pseudomonas sp H-16. It was shown that these microorganisms are capable to oxidize oil's hydrocarbons and oil products. It means that the bacteria may be used for bioremediation of oil-pollutant soils.

Introduction

The petroleum industry and the use of its derived products contribute significantly to volatile hydrocarbon release. Emissions reduction has aroused international interest due to direct and indirect impacts on humans, plants and animals. Biological methods to eliminate hazardous products and to achieve odour control are an attractive alternative to phase transfer techniques such as activated carbon [1-2]. Surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described. At present biosurfactants are unable to compete with the chemical surfactants due to their high production cost. As biosurfactants are readily biodegradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counter parts [3].

In oil-producing regions, the environment is inevitably polluted with crude oil and petroleum products despite all safety measures at extraction, transportation and storage sites [4-6]. As crude oil and its different chemical components have considerable toxic effects on living organisms, it is essential to protect nature from oil and if a certain area is already polluted, it will be necessary to clean it, for example, by specific bioremediation and recultivation technologies [7]. This requires the profound knowledge of the contaminated site including geological, physical and chemical data as well as information on the autochthonous microorganisms (e.g. of an oil-extraction site) which are capable of utilizing hydrocarbons from oil as sole source of carbon and energy. These adapted microbes may help to decompose the pollutants in situ and therefore, it is useful to study them and their degradative enzymes in the laboratory [8].

The specific introduction of selected oil-degrading microorganisms ("oil-destructors") into contaminated sites is one modern approach to develop non-polluting remediation technologies. Such microorganisms can be isolated from fresh, sea and ground waters near oil deposits or from oil-polluted soils (e.g. around petrol stations). Under optimum conditions, the isolated microbial consortia are able to degrade the hydrocarbons by converting them into carbon dioxide, water and biomass as well as harmless transformation products (e.g. fatty acids) [9].

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In this study, we present results of a study on the dynamics of bacterial growth on persistent oil from the Ozen oilfield (Kazakhstan) highly contaminated with high-molecular and aromatic hydrocarbons. Specific changes in the hydrocarbon structure were determined by photo colorimetric and IR-spectroscopic methods.

Material and methods

Microorganisms: Cultures of the gram-negative bacteria *Pseudomonas mendocina H-3, Pseudomonas sp. H-7, P. stutzeri H-10, P. aeruginosa H-14, P. alcaligenes H-15* and *Pseudomonas* sp. *H-16* isolated from oil-polluted soils were used in all experiments [10-11]. Synthetic broth medium E8 used for growth of microorganisms, composition of this medium (Distilled water g/l): KH_2PO_4 - 0,7; $(NH_4)_2HPO_4$ - 1,5; NaCl - 0,5; $MgSO_4$ – 0,8; bactoagar – 20. Every component sterilize in autoclave no less 30 minute under pressure of 0,1 atmosphere. Crude-oil from the Ozen city is hear from east region of Caspian sea was added to a synthetic mineral medium and served as source of carbon and energy. The physical-chemical characteristic of the Ozen crude-oil are: maintenance sulfurs – 0,2 %, paraffin – 12,4 %, resins – 20,3 and asphaltenes – 5,5 %; density – 950 kg/m^3 [4]. Un-inoculated mineral medium supplemented with the same oil but without bacteria was used as the control.

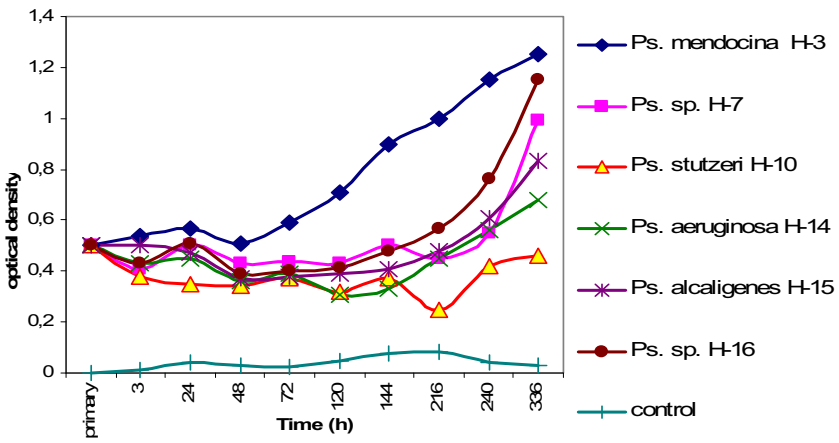
Dynamics of growth of bacterial cultures was studied in with volume 500 ml under aerobic conditions in a liquid mineral medium (E-8). Oil was added as the sole source of carbon and energy at concentrations of 40 and 100 g l^{-1} oil addition in medium after sterilization. Cultivations were carried out in three times on a rotary shaker at 220 rpm at room temperature 20º C. Increase in biomass was controlled by the change in optical density (OD) at 540 nm using photocolorimeter KVK-2 MP. It is possible to calculate the bacterial biomass from these data, i.e. does a calibration curve exist comparing OD and actual biomass. OD measurements were done after 3 h of incubation and afterwards, every 24 h over a period of 15 days.

Chemical analysis of diesel fuels was performed using an Agilent 6890N gas chromatograph equipped with an Agilent 5973N mass detector (Agilent, Germany). Separation of diesel hydrocarbons was carried out on a DB-XLB GC-column (30 m x 0.25 mm, 0.50 µm particle size) using the following temperature program and conditions: 40ºC – 10 min., with 2ºC min^{-1} to 250 ºC – 20 min; ion range – 10-300 Da, injection volume – 0.2 µl with flow separation 1:50; carrier gas – helium at a flow rate of 1 ml min^{-1}.

Changes in the composition of storage-pit oil by Infra-red (IR) spectroscopy were estimated by comparing the spectra determined in the beginning of the experiment and after 7 as well as 14 days of bacterial growth. IR spectra were recorded using a two-beam automatic spectrometer UR-20 in the range 400-4000 cm $^{-1}$. Analyses were carried out with disks of potassium bromide (KBr) in which the oil was incorporated under pressure. The thickness of the absorbing layer was 0.01 mm.

Results and discussion

All bacterial strains tested grew well in the presence of crude oil added to a mineral medium as the sole source of carbon and energy (see Fig. 1 (a and b)).



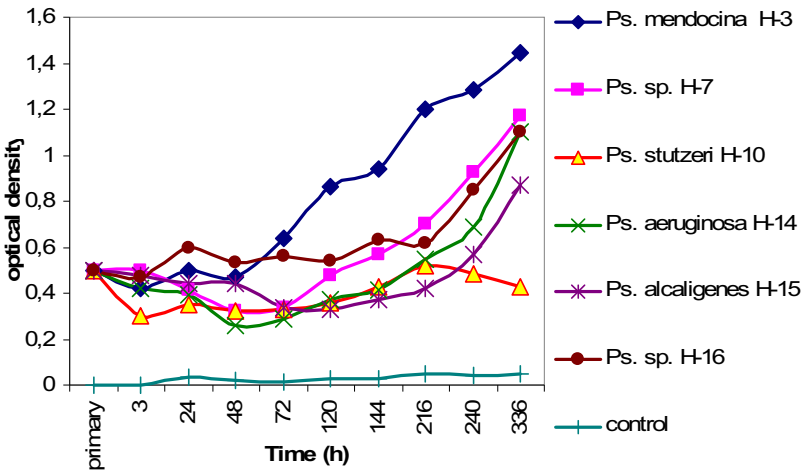


Fig. 1. Growth of different bacterial strains of the genus *Pseudomonas* in a mineral medium with crude-oil hydrocarbons (Ozen oilfield) as sole source of carbon and energy. Oil concentration 40 gl^{-1} (a) and 100 gl^{-1} (b).

For example, biomass of *Pseudomonas mendocina H-3* increased three-fold (according to the OD) during the growth on crude-oil at concentrations of 40 and 100 g l^{-1} within a cultivation period of 9-10 days. Cultures of *Pseudomonas* sp. *H-16* were also able to efficiently use oil hydrocarbons as growth substrate and more than double their biomass within 14 days of cultivation. The maximal increase in biomass caused an increase in OD_{540} of up to 1.15 that corresponds to a 2.5- to 3-fold increase in the initial biomass added. Comparison of the biomass production of *Pseudomonas* sp. *H-7* and *Pseudomonas stutzeri H-10* shows that the former species has a higher degradative potential. Thus, the maximal increase in OD_{540} of *Pseudomonas stutzeri H-10* cultures amounted to 0.46-0.52 at both oil concentrations whereas in cultures of *Pseudomonas* sp. *H-7*, increases of more than 1.0 were observed.

The analysis of spectral changes (in the infrared range) of the residual oil after microbial treatment is of general scientific and practical interest since it allows to draw conclusions regarding the oxidative attack of enzymes on the different oil fractions (hydrocarbons). The spectroscopic studies, perfo-rmed in the present paper with oil that was exposed to 6 different strains of the genus *Pseudomonas*, led to interesting findings regarding the chemical changes caused by microbes.

It turned out that, in microbiologically treated oil samples, the linear and branched paraffin structures and isostructural were enriched including long-chain molecules (1465, 1380, 720 cm^{-1}).

There were also indications for the presence of aromatic structures (1600 cm^{-1}), the quantity of which, however, was considerably smaller than that of paraffins. In control samples of storage-pit oil, we found an absorption band at 1700 cm^{-1} that proves a negligible oxidation of the native material [12-13].

To compare the IR characteristics of the oil samples with each other and with the controls, specific spectral indices were calculated. Following ratios/indices corresponding to specific structural properties were calculated: aromatic hydrocarbons *vs. n*-aliphatic paraffins (A_1), aromatic *vs.* aliphatic hydrocarbons (A_2), the degree of branching of paraffins (B) as well as the length of aliphatic hydrocarbons (P).

The spectral indices are summarized in table 1 and indicate that – 7 days after inoculation with bacteria – the degree of branching slightly increased while the hydrocarbon length decreased. Among the microorganisms tested, *Pseudomonas mendocina H-3* was the most active one that caused a noticeable relative increase in aromatic and branched aliphatic hydrocarbons (at 40 g l^{-1} oil). Within 14 days, all samples with bacteria showed a similar behavior and the degree of branching raised while the degree of aromatic rings considerably increased in comparison to control samples. Next to *Pseudomonas mendocina H-3*, *Pseudomonas stutzeri H-10* was the most active bacterial strain at an oil concentration of 40 g l^{-1}.

Table 1
Changes in the composition of oil hydrocarbons after bacterial treatment of Ozen crude-oil (40 gl^{-1}) and (100 gl^{-1}) in a liquid mineral medium

Tests	C_xH_y conc. gl^{-1}	7 days				14 days			
		B= D_{1380}/D_{1465}	A_1= D_{1600}/D_{720}	A_2= D_{1600}/D_{1465}	P= D_{720}/D_{1465}	B= D_{1380}/D_{1465}	A_1= D_{1600}/D_{720}	A_2= D_{1600}/D_{1465}	P= D_{720}/D_{1465}
Ps. mendocina H-3	40 gl^{-1}	0.45	0.70	0.50	0.25	0.48	0.87	0.30	0.36
	100 gl^{-1}	0.54	-	-	0.37	0.48	-	-	0.34
Ps. sp. H-7	40 gl^{-1}	0.43	-	-	0.30	0.38	0.71	0.27	0.38
	100 gl^{-1}	0.47	-	-	0.41	0.48	1.15	0.25	0.22
Ps. stutzeri H-10	40 gl^{-1}	0.41	-	-	0.30	0.49	0.89	0.31	0.34
	100 gl^{-1}	0.40	-	-	0.28	0.50	0.50	0.23	0.46
Ps.aeruginosa H-14	40 gl^{-1}	0.41	-	-	0.30	0.50	0.63	0.28	0.44
	100 gl^{-1}	0.38	0.52	0.20	0.38	0.50	0.52	0.18	0.35
Ps.alcaligenes H-15	40 gl^{-1}	0.39	-	-	0.27	0.44	0.85	0.28	0.32
	100 gl^{-1}	0.52	0.65	0.19	0.29	0.53	0.57	0.20	0.35
Ps. sp. H-16	40 gl^{-1}	0.44	-	-	0.34	0.50	0.47	0.20	0.42
	100 gl^{-1}	0.50	0.50	0.22	0.44	0.54	0.57	0.27	0.47
Control	40 gl^{-1}	0.47	0.50	0.15	0.30	0.38	0.36	0.12	0.33
	100 gl^{-1}	0.52	0.40	0.18	0.44	0.42	0.55	0.22	0.40

Increasing the oil concentration up to 100 g l^{-1}, adversely affected bacterial activity which became evident by minor changes in all spectral indices investigated. In comparison to all other bacteria tested, *Pseudomonas alcaligenes H-15* was the most active strain under these conditions and caused the greatest increase in aromaticity, accompanied by a decreasing amount of *n*-aliphatic hydrocarbons.

Figures 1-3 show the results of the spectrophotometric measurements performed over a time period of 7 days. Both *Pseudomonas* strains were found to grow when diesel fuel was added as the sole source of organic carbon and energy. Optical density (OD_{540}) of the bacterial cultures and hence microbial biomass increased many times over in comparison with the optical density of control flasks with diesel fuels but without bacteria (compare Fig. 1-3). Furthermore, this finding indicates that the growth conditions (temperature, shaking and oxygen supply, mineral content of the medium) were generally suitable for the microbes so that a growth-supporting microenvironment could be established.

The diesel fuels were decomposed and chemically modified during bacterial treatment due to their oxidation and subsequent utilization as growth substrate. As the result, the optical density increased indicating a substantial microbial growth. *P. alcaligenes H-15* showed the best results for all diesel fuels tested and reached the maximal biomass on day 4 of cultivation. *P. mendocina H-3* was less efficient but also degraded the diesel fuels to some extent with a biomass maximum already on the second cultivation day.

The biomass of *P. alcaligenes H-15* increased about 1.5-fold (according to OD_{540} on day 4) in comparison to the initial optical density (OD_{540} = 0.326) in the medium supplemented with diesel fuel of the Joint Stock company (*PetroKazakhstan Oil Products*) both at a con-centration of 5 and 10%. *P. mendocina* H-3 showed the best growth on day 2 of cultivation and the maximal biomass corresponded to an OD_{540} of 0.406 (at a diesel fuel concentration of 5%) and 0.413 (10% diesel fuel). Compared to the initial index of biomass (OD_{540}: 0.354), i.e. a moderate increase of 0.05 units that was also observed for other diesel fuel samples.

The growth of *Pseudomonas* spp. on paraffinaceous diesel fuels (products of Joint Stock company *"PetroKazakhstan Oil Products"* and Russian refinery *"LukOil"*) did not occur permanently and it seems that the bacterial cells oxidized the hydrocarbons rather cyclically in the

so-called catabolic phase (compare the oscillating OD_{540}/biomass curves in Fig. 2-4). In the anabolic phase of metabolism, pure and mixed cultures of microorganisms mainly utilize pre-oxidized products of hydrocarbons with a simpler structure and lower molecular mass (e.g. *n*-alkanols or organic acids). So far as microorganisms cleave a certain number of carbon atoms (one or two) from aliphatic and/or cyclic compounds at each phase of the metabolic cycle, the irreversible destruction of the crude-oil constituents is obvious. Thus at a concentration of 5% fuel, both strains grew well on the diesel hydrocarbons from the Russian refinery (*LukOil*), and at a concentration of 10%, they did so either on diesel fuel from the Pavlodar petrochemical factory (*PPCF*).

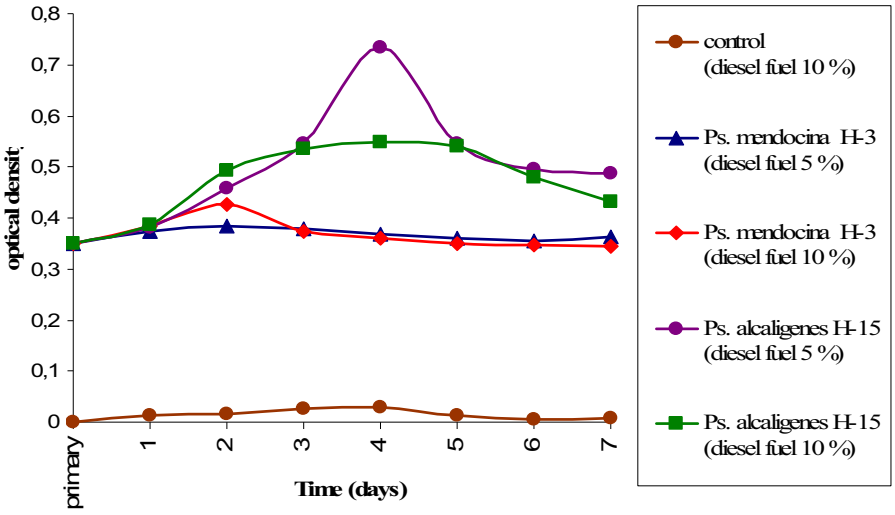


Fig. 2. Dynamics of bacterial growth on diesel fuel of the Joint Stock company *"PetroKazakhstan Oil Products"*

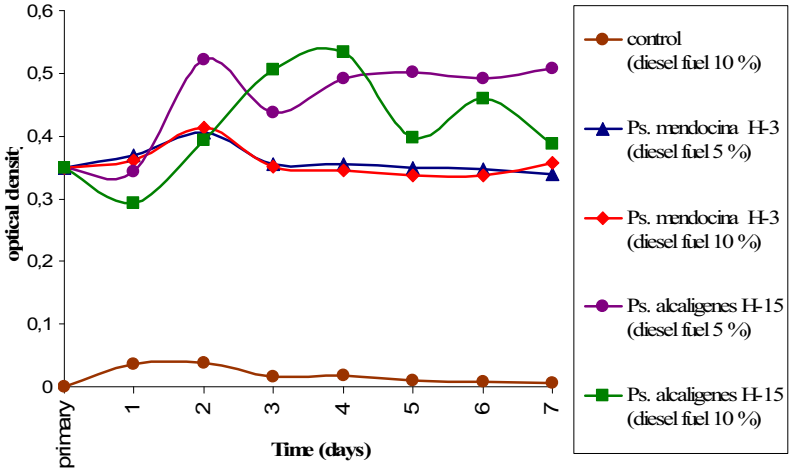


Fig. 3. Dynamics of bacterial growth on diesel fuel of the Pavlodar Petrochemical factory *"PPCF"*

Comprehensive GC-MS analyses of the three original diesel fuels after the 1st and 2nd months of have shown that many distinct peaks of unbranched saturated hydrocarbons (typical *n*-alkanes) appear in the respective chromatograms. Smaller peaks correspond to differently branched paraffins, unsaturated and cyclic alkanes as well as aromatic hydrocarbons. In contrast, the peaks of cyclic and aromatic hydrocarbons are much more pronounced in the gas chromatograms of diesel fuel from the Pavlodar petrochemical factory (*PPCF*) indicating differences in the oil genesis (data not shown).

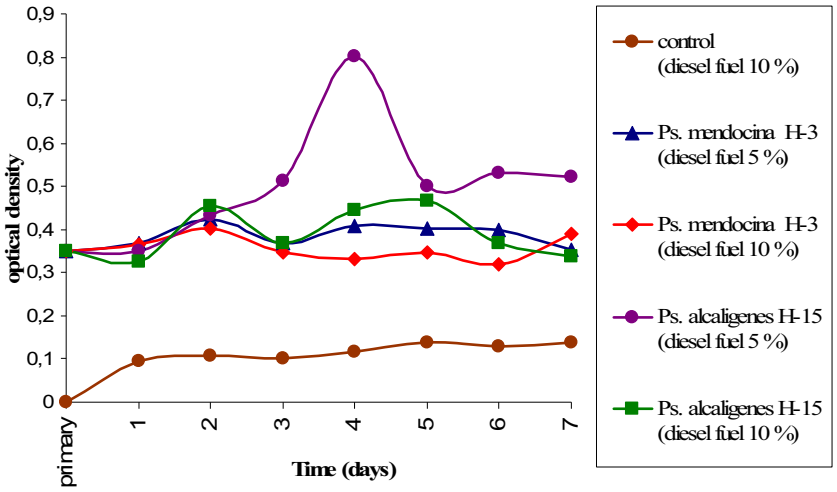


Fig. 4. Dynamics of bacterial growth on diesel fuel of Russian refinery *"LukOil"*

Comprehensive GC-MS analyses of the three original diesel fuels after the 1st and 2nd months of have shown that many distinct peaks of unbranched saturated hydrocarbons (typical *n*-alkanes) appear in the respective chromatograms. Smaller peaks correspond to differently branched paraffins, unsaturated and cyclic alkanes as well as aromatic hydrocarbons. In contrast, the peaks of cyclic and aromatic hydrocarbons are much more pronounced in the gas chromatograms of diesel fuel from the Pavlodar petrochemical factory (*PPCF*) indicating differences in the oil genesis (data not shown).

According to the data obtained, a remarkable feature of the bacterial treatment of diesel fuels is the chemical modification of liquid, saturated light alkanes and the resulting change in the oil composition. For example, the linear, saturated paraffins (*n*-alkanes) up to tetradecane ($C_{14}H_{30}$) in diesel fuels of the Pavlodar petrochemical factory (*PPCF*) and the Joint Stock company (*PetroKazakhstan Oil Products*) as well as the amount of *n*-alkanes up to cetane (*n*-hexadecane, $C_{16}H_{34}$) in diesel fuels of the Russian refinery (*LukOil*) decreased, and the amount of solid, heavy paraffins (e.g. eicosane, $C_{20}H_{42}$ and larger molecules) increased. Furthermore, the total content of saturated hydrocarbons noticeably decreased due to their selective utilization by *Pseudomonas alcaligenes H-15*, while *Pseudomonas mendocina H-3* contributed to decrease of some individual paraffins.

In the original diesel fuels, the total amount of *iso*-alkanes was almost equal but after bacterial degradation, their relative amount decreased in diesel fuels of the Pavlodar petrochemical factory (*PPCF*) while they increased in diesel fuels of the Russian refinery (*LukOil*). An increase in the content of *iso*-alkanes was mainly observed for methylalkanes, whereas the fraction of *di*-, *tri*- and *tetra*-methylalkanes decreased. In addition, the bacterial diesel fuel treatment resulted in an increase of the relative amount of long-chain *iso*-paraffinaceous hydrocarbons while that of smaller *iso*-alkanes (up to 2-methyltridecane = *iso*-$C_{14}H_{30}$) diminished. The fate of cycloparaffins also differed in dependence of the individual diesel fuel tested. Thus, their relative amount increased in the diesel fuel of the Pavlodar petrochemical factory (*PPCF*) but the cycloalkanes were degraded in the material from the Joint Stock company (*Petro Kazakhstan Oil Products*). The latter finding is quite important because cycloaliphatic compounds are much more resistant to microbial attack than aliphatic hydrocarbons. Concerning the different cycloalkane fractions, it was observed that the content of smaller cycloalkanes (up to heptilcyclohexane = $C_{13}H_{26}$) decreased (i.e. they were converted by the bacteria) while the fraction of larger cyclic hydrocarbons was enriched.

Diesel fuel of the Pavlodar petrochemical factory chemically differs from the other fuels because of its high content of aromatic hydrocarbons (arenes = benzene, toluene, ethylbenzene, xylenes = BTEX). Its initial arene content amounted to 11 % and that of the two other diesel fuels only to 4%. As the result of biodegradation, the content of aromatic

hydrocarbons increased in all diesel fuels tested. This finding was to expect since aromatic hydrocarbons belong to the most persistent crude oil constituents and their bioconversion requires a number of specific biocatalysts (e.g. aromatic monooxygenases and dioxygenases) which are only produced by a small number of specialized microorganisms [14]. However, it should be noted that the fraction of 1- and 2-methylnaphtalenes anyhow diminished, and that novel aromatic hydrocarbons such as 1,6,7-trimethylnaphtalene and 1,3-dimethylbenzene (*m*-xylene) appeared.

The results of this study agree with literature data on the use of microorganisms for cleaning oil-polluted soils. Consortia of unknown composition might result in poor growth and hence less degradation as documented by S. Arino, R. Marchal and J. -P. Vanbecasteele. (1998) [15] and recently by Sunday A. Adebusoye et al. (2006) [16]. According to the data of Kaukova et al. (2000) [17], the indices of aromaticity and the oxidation state (introduction of oxygen into the hydrocarbons) increased after microbial growth in comparison to native crude-oil whereas the aliphatic character drastically decreased (the relative amount of branched aliphatic hydrocarbons did not change). The methods described here provide an opportunity to design these inocula such that they are ecologically relevant and comprised of organisms known to respond to a particular environmental stimulus or bioremediation treatment [18].

Moreover, Faizov et al. (2003) [19] reported that an enrichment of oxygen in ether bonds and carboxylic groups occurred during the microbial treatment of crude oil. In addition, a considerable decrease in long-chain paraffins ($>C_{16}$) was observed. The effect can be explained by the fact that microorganisms prefer aliphatic hydrocarbons (paraffins) because they are relatively easy to degrade (e.g. compared to cyclaliphatic or aromatic compounds) and can serve as excellent sources of carbon and energy for specialized microorganisms.

Conclusions

To summarize, the data obtained by GC-MS analysis strongly indicate that the content of *n*-alkanes decreases in diesel fuels as the result of bacterial growth while the relative amount of *iso*-alkanes, cycloalkanes and aromatic hydrocarbons tends to increase (even though individual molecule species of the latter fractions may also be degraded by the bacteria). Moreover, the results show that the bacterial strains tested are capable of utilizing crude-oil hydrocarbons from diesel fuels as growth substrate, i.e. they use them as sole source of carbon and energy (productive biodegradation: mineralization of hydrocarbons and conversion into biomass) [20]. Last but not least, our findings support the idea of using bacteria for cleaning the environment by the biological removal of hazardous crude-oil hydrocarbons from contaminated soils. To this end, additional degradation studies and further isolation of bacterial strains from contaminated soil will be necessary. The results presented here demonstrate that bacteria of the genus *Pseudomonas* are capable of degrading and productively utilizing hydrocarbons from a persistent Kazakh crude-oil (Ozen oilfield) which makes them a promising biotechnological target for the development of bioremediation and cleaning technologies.

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