The Using of *Pseudomonas* Cells for Bioremediation of Oil Contaminating Soils

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Abstract

The article describes results on the oxidation of crude-oil by bacteria of genus *Pseudomonas*: *Ps. mendocina* H-3*, Ps. sp.* H-7*, Ps. stutzeri* H-10*, Ps. aeruginosa* H-14*, Ps. alcaligenes* H-15 and *Ps. sp.* H-16. These microorganisms isolated from oil-contaminated soils in Kazakhstan were found to be capable of growing on crude oil components and oxidizing the hydrocarbons to different extent. Therefore, they may be useful for the bioremediation of oil-polluted soils and waters.

Introduction

The petroleum industry and the use of its products contribute significantly to the release of volatile and hazardous hydrocarbons. Emission reduction of crude-oil hydrocarbons has aroused international attention and interest due to direct and indirect impacts on humans, plants and animals. Biological methods to eliminate hazardous products and to improve the quality of soil, water and air (odor) are an attractive alternative to classical phase transfer techniques such as binding on activated carbon [1-2]. Surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic and food industries to mobilize compounds insoluble in water. Most of these compounds are chemically synthesized but there are also surface-active molecules of biological origin, which become more and more attractive as environment-friendly substitutes. At present, most biosurfactants are still too expensive and cannot compete with the chemical surfactants due to their high production cost. As biosurfactants are readily biodegradable and can be produced from renewable and cheap raw materials, they could replace their chemically synthesized counter parts in future [3].

In oil-producing regions, the environment is inevitably polluted with crude oil and petroleum products despite of 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. Moreover, 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

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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].

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.

Materials and Methods

The microorganisms used included cultures of *Ps. mendocina* H-3*, Ps. sp.* H-7*, Ps. stutzeri* H-10*, Ps. aeruginosa* H-14*, Ps. alcaligenes* H-15 and *Ps. sp.* H-16 isolated from oil-polluted soils which were deposited in the culture collection of the Al-Farabi Kazakh National University [Almaty, Kazakhstan]. The synthetic liquid medium E8 used for all growth studies consisted of following components (in distilled water): 0.7 g/l KH_2PO_4, 1.5 g/l $(NH_4)_2HPO_4$, 0.5 g/l NaCl, 0.8 g/l $MgSO_4$ and 20 g/l bactoagar (State scientific Center of Apply Microbiology, Moscow, Russia). It was sterilized in an autoclave for 30 minutes. Crude-oil from the Ozen city (near the Eastern region of Caspian sea) was added to the synthetic mineral medium and served as source of carbon and energy. The physical-chemical characteristics of the Ozen crude-oil are: sulfur content – 0.2%, paraffin-naphtene and aromatic oils – 61.6%, hard paraffin – 12.4%, resins – 20.3% and asphaltenes – 5.5%; density – 950 kg/m^3 [4]. Uninoculated mineral medium supplemented with the same oil but without bacteria served as the control.

Dynamics of growth of bacterial cultures was studied in 500 ml flasks containing 100 ml a medium (E-8) under aerobic conditions. Oil was added as the sole source of carbon and energy at concentrations of 40 and 100 g·l^{-1} after sterilization. Cultivations were carried out in triplicate on a rotary shaker at 220 rpm at room temperature 20°C. Increase in biomass was controlled by the change in optical density (*OD*) at 540 nm using the spectrophotometer AP-101 (Apel Co., Ltd., Japan). Bacterial biomass can be calculated from these data using calibration curves. *OD* measurements were done after 3 hrs of incubation and afterwards, every 24 hrs over a period of 15 days.

Changes in the composition of storage-pit oil were performed by infra-red (IR) spectroscopy 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 (Germany) and FTIR Satellite (Mattson, USA) in the range from 400 to 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.

The group structure of an organic part of the oil contaminated soil defined by changed VNIINP method, based on Marcusson adsorption-chromatographic method consisting of asphaltene sedimentation by petroleum ether. Various sorption abilities of oils and tars by silica are used for their division.

Results and Discussion

All bacterial strains tested grew well in the presence of crude oil added to the mineral medium as the sole source of carbon and energy (see Fig. 1a and 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 *Ps. 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 maximum biomass caused an increase in ΔOD_{540} of 1.15 that corresponds to a 2.5- to 3-fold increase in the initial biomass added. Comparison of the biomass production of *Ps. sp.* H-7 and *Ps. stutzeri* H-10 shows that the former species has a higher degradative potential. Thus, the maximal increase in ΔOD_{540} of *Ps. stutzeri* H-10 cultures amounted to 0.46-0.52 at both oil concentrations whereas in cultures of *Ps. sp.* H-7, increases of more than 1.0 were observed (see Table 1).

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, performed 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 the microbes.

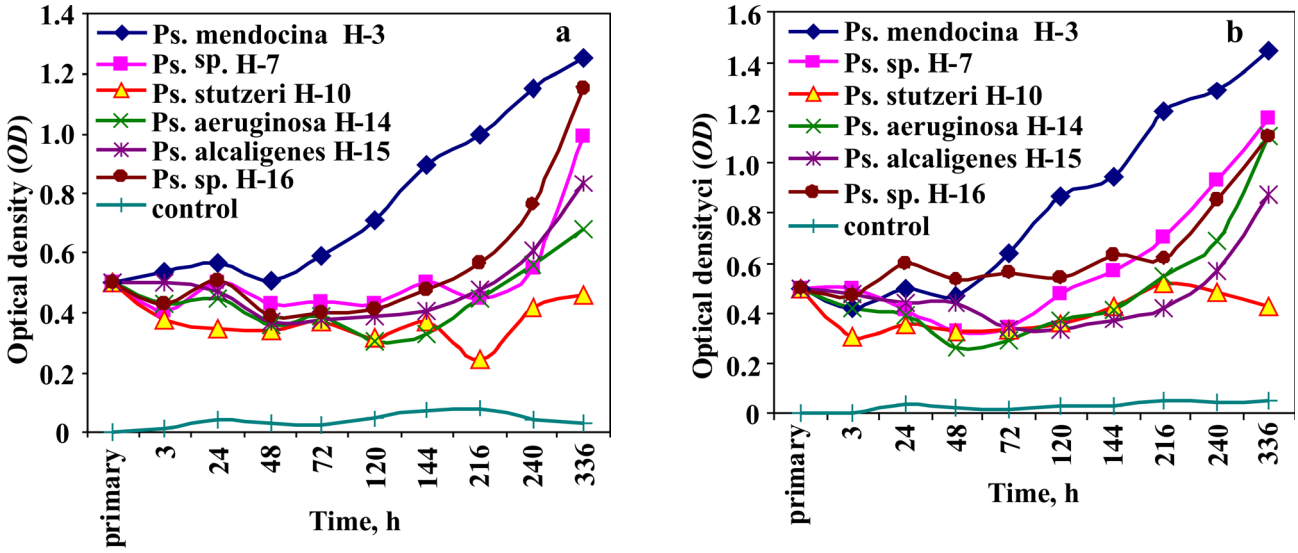


Fig. 1. Growth of different bacterial strains of the genus *Pseudomonas* in a mineral medium with crude-oil hydrocarbons (Ozen oilfield) as sole source of carbon and energy. Oil concentration 40 g·l^{-1} (a) and 100 g·l^{-1} (b).

It turned out that, in microbiologically treated oil samples, the linear and branched paraffin structures and isomers 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 consider-

Table 1

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

Tests	C_xH_y conc., g·l^{-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 g·l^{-1}	0.45	0.70	0.50	0.25	0.48	0.87	0.30	0.36
	100 g·l^{-1}	0.54	–	–	0.37	0.48	–	–	0.34
Ps. sp. H-7	40 g·l^{-1}	0.43	–	–	0.30	0.38	0.71	0.27	0.38
	100 g·l^{-1}	0.47	–	–	0.41	0.48	1.15	0.25	0.22
Ps. stutzeri H-10	40 g·l^{-1}	0.41	–	–	0.30	0.49	0.89	0.31	0.34
	100 g·l^{-1}	0.40	–	–	0.28	0.50	0.50	0.23	0.46
Ps.aeruginosa H-14	40 g·l^{-1}	0.41	–	–	0.30	0.50	0.63	0.28	0.44
	100 g·l^{-1}	0.38	0.52	0.20	0.38	0.50	0.52	0.18	0.35
Ps.alcaligenes H-15	40 g·l^{-1}	0.39	–	–	0.27	0.44	0.85	0.28	0.32
	100 g·l^{-1}	0.52	0.65	0.19	0.29	0.53	0.57	0.20	0.35
Ps. sp. H-16	40 g·l^{-1}	0.44	–	–	0.34	0.50	0.47	0.20	0.42
	100 g·l^{-1}	0.50	0.50	0.22	0.44	0.54	0.57	0.27	0.47
Control	40 g·l^{-1}	0.47	0.50	0.15	0.30	0.38	0.36	0.12	0.33
	100 g·l^{-1}	0.52	0.40	0.18	0.44	0.42	0.55	0.22	0.40

ably 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 [10-11].

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 $g \cdot l^{-1}$ oil). Within 14 days, all samples with bacteria showed a similar characteristic 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 $g \cdot l^{-1}$.

Increasing the oil concentration up to 100 $g \cdot l^{-1}$, adversely affected bacterial activity which became evident by minor changes in all spectral indices investigated. In comparison to all other bacteria tested, *Ps. 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.

The results of this study agree with literature data on the use of microorganisms for cleaning oil-polluted soils. The use of consortia of unknown composition may result in poor growth and hence less oil degradation as documented by Arino et al. (1998) [12] and recently, by Adebusoye *et al.* (2006) [13]. According to the data of Kaukova *et al.* (2000) [14], 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) [15].

Moreover, Faizov *et al.* (2003) [16] 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 cyclic or aromatic compounds) and can serve as excellent sources of carbon and energy for specialized microorganisms.

Oil contaminated soil of oil-extracting manufactures from "Chempromservice-Aktobe" JSC mud storage, located in territory Zhanazhol deposit of the Aktyubinsk area had been selected for carrying out of laboratory and field experiments on soil clearing by free cells of hydrocarbon-oxidize microorganisms. *Pseudomonas mendocina H-3* bacteria added as oil destructive cultures.

The content of an organic part in the soil is determined during experiments. The experiments were carried out with use sterile and unsterile soils for a correct estimation of participation native (in structure of soil) and the brought micro flora in oil destruction. Duration of experiences made 180 day.

As shown in the Table 2, the maximum destruction rate of organic substances was in a sample in which microorganisms added in unsterile soil. The concentration of organic substances essentially did not change in control tests with sterile and unsterile soil during all tests.

Table 2

Dynamics of the organic part content in oil contaminated soil at introduction of hydrocarbon oxidizing microorganisms (MO) suspension

Samples	The content of organic part in the soil, mass %				
	initial	15 days	30 days	60 days	180 days
Sterile soil	34.0	34.0	34.0	34.0	34.0
Unsterile soil		33.9	33.5	33.0	33.0
Sterile soil with MO		23.8	20.4	16.7	6.0
Unsterile soil with MO		22.1	18.7	12.6	3.0

The concentration of organic substances has decreased to 20.4 and 18.7% in the samples of sterile and unsterile soil with addition of bacteria for the first month of experiment. The content of organic part has decreased up to 16.7 and 12.6% after 2

months. The quantity of organic substances in polluted soil has made 3 and 6% after 6 monthly experiments, and the degree of their degradation has made 83 and 91%.

The soil samples for the analysis were selected in the beginning and the end of experiences in field experiments. The suspensions of hydrocarbon oxidizing microorganism's cells were used for introduction. Experiments were carried out on the sites in the size 1×1×0.8 m in 5 times multiple frequency. It is necessary to note, that the investigated sites already visually differed from initial color and a smell later 6 months. Light-brown color of soil was characteristic for them, there was no sour organic smell.

The analysis of group structure has allowed to following results (Table 3): in initial soil in lots contains paraffin-naftene oils 32.9% from weight of oil components. On a share of tars 41.3% and on a share of asphaltenes 8.5% from the sum of all components are necessary. Differently, oils a little prevail among an organic part of soil.

Table 3

Influence of Pseudomonas mendocina H-3 cells suspensions on group structure of an organic part of the oil contaminated soil

Group structure components	Organic part of initial soil	Organic part of soil after 6 month
Oils:		
Paraffin-naftene	32.9	27.7
Monocyclic aromatic	1.4	5.4
Bicyclic aromatic	0.3	2.9
Polycyclic aromatic	15.6	1.4
Sum of oils	50.2	37.4
Tars:		
Benzene	33.1	2.4
Ethanol-benzene	8.2	39.0
Sum of tars	41.3	41.4
Asphaltenes	8.5	21.2

The quantity of oils in the organic part has noticeably decreased and concentration of asphaltenes has increased after introduction of microorganisms in 6 months. The concentration of paraffin-naftene and polycyclic aromatic components has decreased in structure of oils, whereas amount of mono- and bicyclic aromatic hydrocarbons has increased. Though tar fractions on a total sum have not changed, concentration of benzene tars has decreased more than in 10 times and has made only 2.4%. The raised concentration of ethanol-benzene tars in an organic part, as is known, can be connected with secondary oxidation of compounds. It is necessary to note, that as a result of biooxidation in structure of an organic part the amount of asphaltenes increases for 2.5 times.

Thus, from the received results follows, that introduction of hydrocarbon oxidizing bacteria in the oil contaminated soil creates conditions for activization of destruction processes of oil hydrocarbons, that leads to soil improvement. The results received during 6 monthly experiments can form a basis for creation of bioremediation technology of oil contaminated soils.

The infrared (IR) spectroscopic research of an organic part of soil is carried out for determination of change of its structure after biodegradation. IR spectra of an organic part, extracted from initial soil are shown in Fig. 2. The absorption strips in the field of 2953, 2922, 2851, 1456, 1377 cm^{-1} are found out in spectra. Occurrence of these strips in this area specifies presence a lot of the saturated hydrocarbons. Occurrence of the strip at 728 cm^{-1} corresponds to valent and deformation vibrations of the extended normal hydrocarbons. It means that paraffin compounds are present in the crude oil of Zhanazhol deposit. Presence in spectra of absorption strips at 1604 and 1044 cm^{-1} specifies presence of the fragments of the aromatic structures connected with benzene rings in organic part. The strip of average intensity at 1707 cm^{-1} speaks about presence of carbonyl groups, organic acids and their compounds.

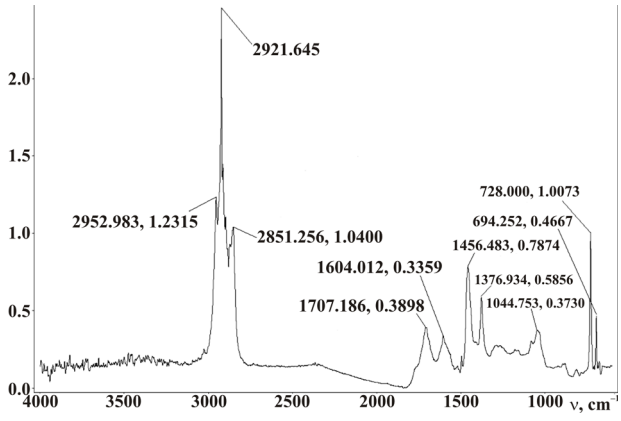


Fig. 2. The IR-spectrum of an organic part of the oil contaminated soil of Zhanazhol deposit.

Occurrence of these strips speaks that the sunlight stimulates processes of growth and oxygenize activity of soil microflora for a long time. Increase of activity oxygen containing components in crude oil is observed.

The estimation of biodestruction processes has shown that there are some changes in samples after bioremediation for 6 months as follows from Fig. 3. After entering microorganisms there are absorption strips of valent and deformation vibrations of group CH_2 and CH_3 of aliphatic structures at 1377, 1462, 2851, 2922, 2954 cm^{-1}. Intensity of these characteristic absorption strips goes down in comparison with initial. The weak strip at 1604 cm^{-1} can be carried to absorption of aromatic hydrocarbons. Also there are wide strips in a range 1073, 1122, 1273 cm^{-1} due to absorption of various oxygen containing compounds (–C–O–, –C–O–C–) alcohols, ethers and complex ethers that are intermediate products of a metabolism at microbic oxidation of normal paraffin. The strip of absorption at 1730 cm^{-1} can be considered also as an attribute of presence of oxygen connections.

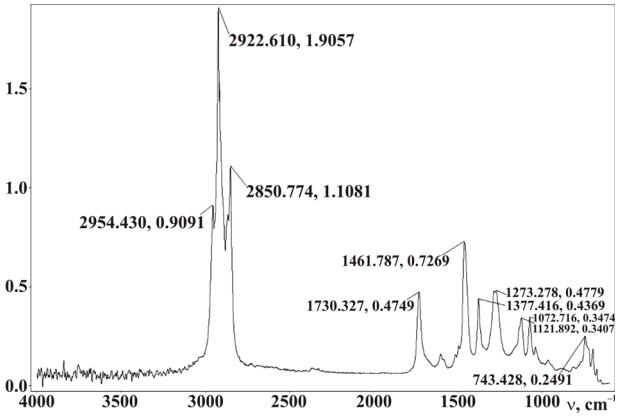
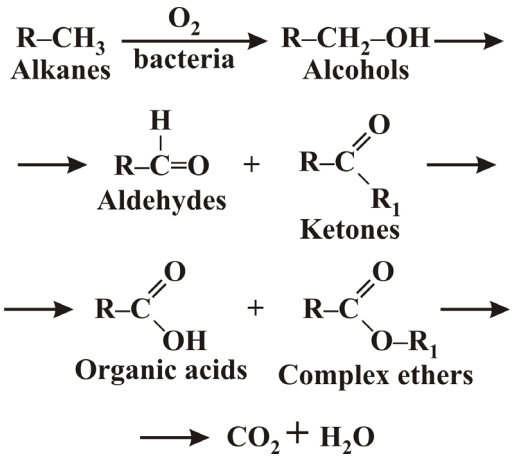


Fig. 3. The IR-spectrum of an organic part of the oil contaminated soil of Zhanazhol deposit after influence of microbic cells

On the basis of the given spectroscopic analyses it is shown, that the quantity of oxygen containing components of oil increases and the quantity of paraffin decreases as a result of bioremediation.

On the basis of results of the IR-spectroscopic analysis of an organic part after biodegradation the Scheme 1 of transformations of hydrocarbons is offered.

Thus, on the basis of the received results of IR-spectroscopy with introduction in crude oil of the specified kinds of microorganisms their effective influence is revealed and the opportunity of their use for clearing of oil contaminated soil is shown.



Scheme 1

Conclusion

The results presented here demonstrate that bacteria of the genus *Pseudomonas* isolated from contaminated soils 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.

References

1. Hamme, J.D.Van., and Ward, O.P., J. Ind. Microbiol. & Biotech., 26:356 (2001).
2. Stehmeier, L.G., Jack, T.R., Voordouw, G., Can. J. Microbiol., 42:1051 (1996).
3. Patel, R.M., Desai, A.J., Let. Appl. Microbiol., 25:91 (1997).
4. Ongarbaev, Ye.K., Mansurov, Z.A., Oil wastes and methods of their utilization. Kazakh University, Almaty, 2003, p. 160.
5. Mansurov, Z.A., Reports of II Intrennational Scientific Nadirov's readings. Almaty-Kyzylorda, 2004, p. 15.
6. Yagofarova, G.G., Mavlutov, M.R., Spivak, A.I., Gatayllina, E.M., Neftyanoe khozyaistvo, 4:67 (1996).
7. Doszhanov, Ye.O., Baybekov, A.S., Shypshibaev, K.K., Zhybanova, A.A., et al., KazNU Vestnik, Ecologic. Ser., 2(13):180 (2003).
8. Ullrich, R., Hofrichter, M., Cell. Mol. Life Sci 64:271 (2007).
9. Ongarbaev, Ye.K., Doszhanov, Ye.O., Mansurov, Z.A., Zhybanova, A.A., Third Symposium (International) on "Physics and Chemistry of Car-

bon Materials / Nanoengineering", The Problem Combustion Institute, Almaty, 2004, p. 113.
10. Terry, J.G., Deborah, T.N., Karen, L.J., Ian, L.P., Biodegradation, 349 (2001).
11. Emtiazi, G., Shakarami, H., Nahvi, I., Mirdamadian, S.H., Afr. J. Biotech., 4(2):172 (2005).
12. Arino, S., Marchal, R., Vanbecasteele, J.P., J. Appl. Microbiol., 84:769 (1998).
13. Sunday, A.A., Matthew, O.I., Olukayode, O.A., Olakunle, D.T., et al., J. Americ. Scien., 2(3):48 (2006).
14. Kaukova, G.P., Egorova, K.V., Gabitova, R.K., Zaripova, S.K., et al., Neftechimiya, 40(2):92 (2000).
15. Supaphol, S., Panichsakpatana, S., Trakulnaleamsai, S., Tungkananuruk, N., et al., J. Microbiol. Meth., 65:432 (2006).
16. Faizov, K.Sh., Jusipbelkov, Y.Zh., Abieva, L., Raimzhanova, M.M., et al., Neft & gas, 2:119 (2003).

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