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PAHs-degrading bacteria isolated from oil-contaminated soil of Western Kazakhstan

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ABSTRACT

This work has been aimed at isolating and studying active destructor strains of polycyclic aromatic hydrocarbons. The strains capable of growing on naphthalene and fluorene as the only source of carbon and energy were isolated from oil-contaminated soil of Western Kazakhstan using the Enrichment Culture Technique. Studying the 16S rRNA gene sequence has shown that all active strains belong to the *Pseudomonas* and *Bacillus* genera. Using the gas chromatographic method, it has been shown that naphthalene was completely degraded in two to five days, and fluorene – in 15 days. The *Bacillus haynesii* 21WDT strain has been capable of degrading both naphthalene and fluorene.

Key words: *Biodegradation, Hydrocarbon-oxidizing bacteria, Polycyclic aromatic hydrocarbons, Naphthalene, Fluorene.*

Introduction

Some of the most common environmental pollutants are polycyclic aromatic hydrocarbons (PAHs). This is a ubiquitous group of several hundred chemically-related compounds with various structures and toxicity to all living organisms that are resistant to the environment. PAHs are released into the environment in many ways and are usually in the form of a mixture containing two or more compounds. PAHs are poorly soluble, easily absorbed by soil particles, and hardly degraded (Bansal and Kim, 2015).

PAHs mainly originate from anthropogenic sources associated with urban development, such as vehicle exhaust emissions, fossil fuel combustion, chemical production, operation of oil refineries, spills of oil and refined petroleum products, waste incineration, sewage deposition, etc. (Callén, 2013). Since PAHs are poorly soluble, easily absorbed by soil particles, and hardly degraded, they tend to accumulate in the soil (Ping, 2007).

PAHs can have carcinogenic and mutagenic effects and are potent immunologic depressants (Tobiszewski and Namieœnik, 2012). They are associated with skin and lung cancer, diabetes, and cardiovascular diseases (Burstyn, 2005).

The U.S. Environmental Protection Agency (USEPA) has identified 16 PAHs, including naphthalene and fluorene, as the top pollutants.

Various physical, chemical, and biological methods are currently used for purifying soil contaminated with hardly soluble pollutants, such as PAHs. Bioremediation is an economically and environmentally friendly alternative.

Microorganisms play the main role in xenobiotics degradation. They can use PAHs as the source of carbon and energy. Examples of the bacteria that degrade xenobiotics are *Pseudomonas, Gordonia, Bacillus, Moraxella, Micrococcus, Escherichia,*

Sphingobium, *Pandoraea*, *Rhodococcus*, *Klebsiella*, *Pelatomaculum*, *Desulphovibrio*, *Methanospirillum*, *Methanosaeta desulfotomaculum*, *Syntrophobacter*, and *Syntrophus* (Varsha, 2011).

In this study, the growth of the bacteria isolated from oil-contaminated soil of Western Kazakhstan on naphthalene and fluorene and their biodegradation were studied.

Materials and Methods

The soil samples were taken from the oil-contaminated site at the Zhanatalap oilfield in Western Kazakhstan from the 0 – 30 cm layer.

Naphthalene and fluorene were obtained from Sigma-Aldrich (purity 98 – 99%).

The culture media were as follows: nutrient agar (Titan Biotech Ltd, India), agar-agar (Himedia, India), and a mineral medium of the following composition, g/l: NH_4NO_3 – 1.0, K_2HPO_4 – 1.0, KH_2PO_4 – 1.0, $MgSO_4$ – 0.2, $CaCl_2 \cdot 6H_2O$ – 0.02, $FeCl_3$ – traces, NaCl – 1.0, and pH = 7.0 – 7.2.

The naphthalene and fluorene degrading bacteria were isolated using the Enrichment Culture Technique. For this purpose, 20 g of oil-contaminated soil was placed into flasks with 200 mL of the mineral medium, and the appropriate hydrocarbons were added. Naphthalene was added in the amount of 1 %, and fluorene – in the amount of 0.1 %. The enrichment cultures were incubated on a rotation shaker at 180 rpm. About 14 days later, 20 mL of culture liquid was transferred to 200 mL of fresh medium with naphthalene and fluorene and incubated under the same condition. The hydrocarbon-oxidizing microorganisms were isolated by inoculating the culture liquid into Petri dishes with the nutrient agar.

The isolated cultures were inoculated in an agarized mineral medium in Petri dishes and grown in naphthalene vapors. Fluorene had been previously dissolved in chloroform and applied to the surface of the medium in an even thin layer. The studied strains' ability to consume aromatic hydrocarbons was visually assessed by the growth rate (no growth, weak, moderate, or good growth).

To study the growth of the selected bacterial strains on PAHs five milliliters of the cell suspension was added to flasks with 100 mL of the mineral medium. Naphthalene was added in the amount of 1 g/L, and fluorene – in the amount of 200 mg/L. The ability to grow was assessed by the changes in the culture liquid optical density on a PD-303 spectrophotometer at the wavelength of 540 nm.

For identifying the strains of microorganisms, the genomic DNA was extracted from one – two-day-old cultures of bacteria using a PureLink Genomic DNA Purification Kit, following the manufacturer's recommendations (Invitrogen, Carlsbad, USA). The DNA concentration in the samples was measured on a Qubit® 2.0 fluorimeter with a Qubit™ dsDNA HS Assay Kit (Life Technologies, Oregon, USA). To amplify a segment of the 16S rRNA gene, the following reaction mixture was prepared: 12.5 µL of Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., USA), a pair of universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 806R (5'-GGACTACCAGGGTATCTAAT-3') (Vegas *et al.*, 2006) in the concentration of 1.2 µL per 10 µM, the DNA matrix, and water. The total volume of the reaction mixture was 25 µL. The amplification conditions included the following cycles: one cycle at 95 °C for five minutes, followed by one cycle at 95 °C for 30 seconds, one cycle at 55 °C for 40 seconds, 30 cycles at 72 °C for 50 seconds, and one cycle at 72 °C for 10 minutes.

The fragments of the 16S rRNA bacterial gene were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the the manufacturer's recommendations (BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol, Applied Biosystems, USA), followed by the separation of fragments in an automated 3500 DNA Genetic Analyzer (Applied Biosystems, Hitachi, Tokyo, Japan).

The results of sequencing were processed in the SeqA application (Applied Biosystems). The homologous nucleotide sequences of the 16S rRNA gene were searched for using the BLAST (Basic Local Alignment Search Tool) application in the Gene Bank international database of the USA National Center for Biotechnology Information (htpp://www.ncbi.nlm.nih.gov). The phylogenetic analysis was performed using the MEGA 6 software (Kumar, 2004). The nucleotide sequences were aligned using the ClustalW algorithm. The phylogenetic neighbors were identified using the BLASTN Neighbor-Joining (NJ) method (Altschul, 1997).

To study naphthalene and fluorene degradation, they had been previously dissolved in acetone and added to flasks with 50 mL of sterile mineral medium until the final concentrations were achieved (200 mg/L and 50 mg/L, respectively). The suspen-

sions of the studied cultures were introduced in three-milliliter portions. The flasks were tightly closed and incubated on a shaker at 180 rpm at 28°C for five (for naphthalene) and 15 (for fluorene) days. The control was a mineral medium with naphthalene and fluorene.

To determine the content of naphthalene and fluorene, 50 mL of the sample was extracted with 20 ml of chloroform for 20 min. After the extraction, the organic phase was separated in a separatory funnel, and the total volume of the extract was measured by cylinder. The samples were analyzed by the method of gas chromatography-mass spectrometry (GC-MS) on a gas chromatograph with a 7890D/5977A mass spectrometric detector (Agilent, USA). Chromatography was performed using a DB-35MS (30 m × 0.25 mm, 0.25 ìm film thickness, J &W Scientist Inc., Folsom, CA, USA). To decipher the obtained mass spectra, the Wiley 7th edition and NIST'02 libraries were used (with the total number of the spectra in the libraries exceeding 550 thousand).

To determine the average values and the standard deviations of the degree of naphthalene and fluorene degradation, all the experiments were repeated three times. The statistical analysis was performed using Microsoft software (Redmond, Washington, USA), Excel. The differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

Twenty isolates were recovered from the enrichment cultures with naphthalene, and 28 isolates – from those with fluorene. These cultures were tested for the ability to grow in an agarized mineral medium in the presence of naphthalene and fluorene. In naphthalene, good growth was noted for 17 isolates, while moderate growth was noted for 10 isolates, and 15 isolates did not grow on this substrate. Fluorene was well consumed by 31 isolates, moderately consumed by seven isolates, and no growth was noted for three isolates. Almost all the isolates that grew well on naphthalene also showed the same growth rate on fluorene.

The growth of the selected isolates was studied in a liquid mineral medium with naphthalene at the concentration of 1 g/L. Despite the good growth in an agarized medium, not all isolates showed high biomass accumulation with such a content of the substrate in the liquid medium. The highest activity was shown by the P1-nap2-1, P1-nap2-2, and 21WDT strains, the biomass of which increased 2 – 3.5 times (Figure 1). For the P1-nap2-2 strain, the maximum biomass was noted on day two of cultivation. On the next day, the cell growth reached the stationary phase. The biomass of the P1-nap2-1 strain had dramatically increased by day five, and reached its maximum only on day nine. The 21WDT strain reached the stationary growth phase on day seven of cultivation.

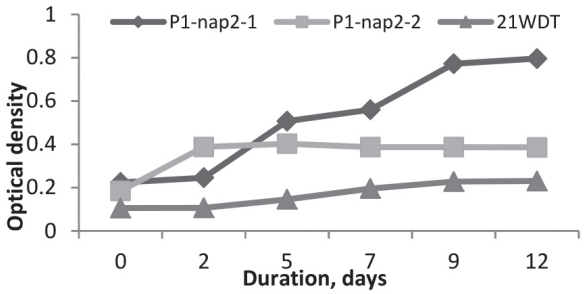

Fig. 1. The growth of the hydrocarbon-oxidizing bacteria on naphthalene

The growth of the selected isolates in a liquid medium with fluorene at the concentration of 200 mg/l was studied. The results of the study showed that five isolates had the highest activity (Figure 2). Their biomass increased 2.0 – 5.0 times. The highest biomass was observed for the 21WDT strain after three days of cultivation, which reached the maximum by day nine. It was noted that with the growth of the 1/2nap and 1/5nap strains, a color change of the culture liquid from colorless to yellow had been observed after one day. Obviously, intermediate fluorene degradation products were formed in the medium.

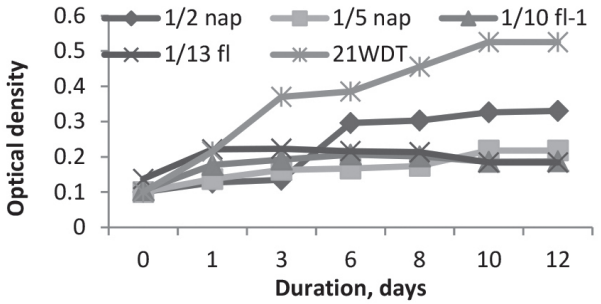

Fig. 2. The growth of the hydrocarbon-oxidizing bacteria on fluorene

The 16S rRNA nucleotide sequence of the studied bacterial cultures was identified, based on which

phylogenetic trees with the homologous strains were built (Figures 3 – 6).

The analysis of the phylogenetic relationship built with the use of the type strains of closely related bacteria showed that the studied strains were 98 – 99% homologous to their closest relatives. Based on studying the sequence of the 16S rRNA gene, the 21WDT strain was relegated to the *Bacillus haynesii* species, the 1/10fl-1 strain – to the *Bacillus megaterium* species, the 1/13fl strain – to the *Bacillus thuringiensis* species, the P1-nap2-1 strain – to the *Pseudomonas aeruginosa* species, the P1-nap-2-2 strain – to the *Pseudomonas frederiksbergensis* species, and the 1/2nap and 1/5nap strains – to the *Pseudomonas songnenensis* species.

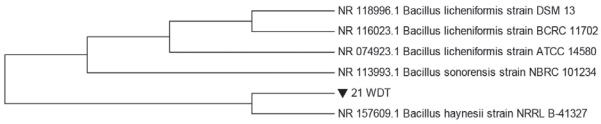

Fig. 3. The phylogenetic tree of the *Bacillus haynesii* 21WDT strain

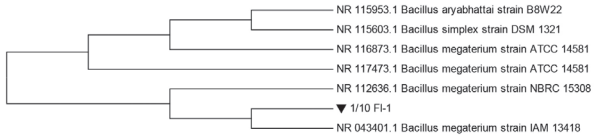

Fig. 4. The phylogenetic tree of the *Bacillus megaterium* 1/10fl-1 strain

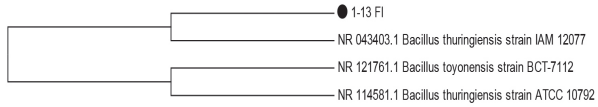

Fig. 5. The phylogenetic tree of the *Bacillus megaterium* 1/13fl strain

It is known that the bacteria of the *Pseudomonas* genus dominate in the environments contaminated with hydrocarbons. They have broad affinity with hydrocarbons and can degrade alkanes, alicyclic compounds, and PAHs (Zhang *et al.*, 2011). The information about naphthalene and fluorene biodegradation by the bacteria of the *Bacillus* genus is sparse (Lateef B. Salam and Oluwafemi S. Obayori, 2014).

Naphthalene degradation (200 mg/L) had been studied for the three strains that showed good biomass growth in the case of cultivation in a liquid mineral medium with this substrate. The results showed that the P1-nap2-1 and P1-nap2-2 strains had completely degraded the studied hydrocarbon as early as on day two (Figure 7). At the same time, complete naphthalene degradation under the influence of the 21WDT strain occurred after five days of cultivation. With that, significant consumption of the substrate by this strain was observed as early as on day two. The natural loss over five days amounted to 14.8 %.

The results showed that two strains that belonged to the *Pseudomonas* genus had a significant ability to degrade naphthalene in 24 hours, and the

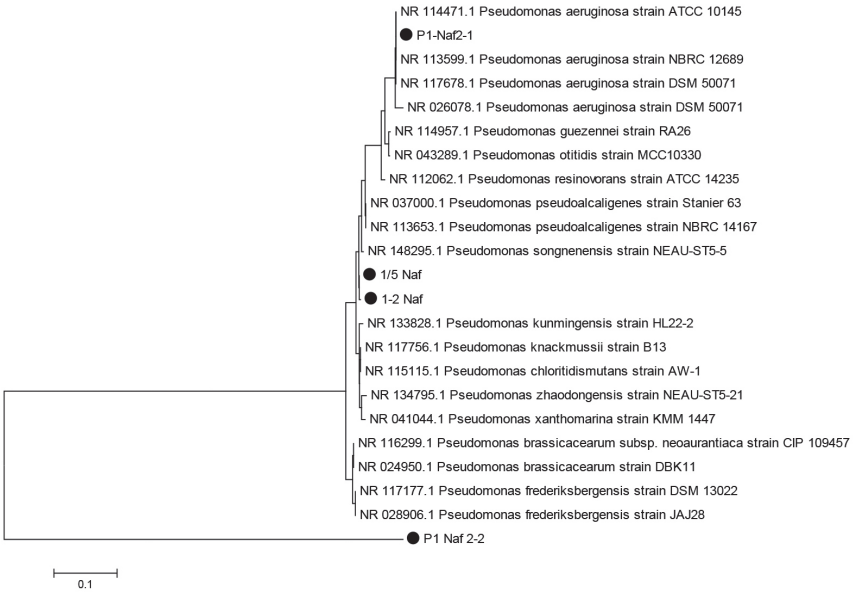

Fig. 6. The phylogenetic tree of the bacterial strains belonging to the *Pseudomonas* genus

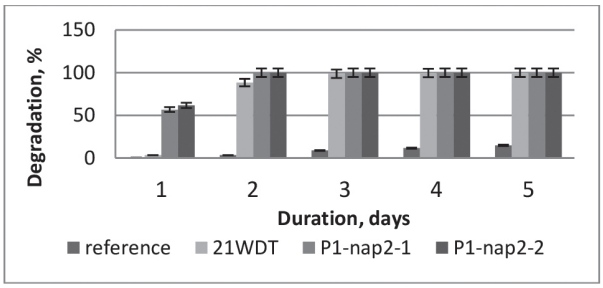

Fig. 7. Naphthalene degradation by active strains

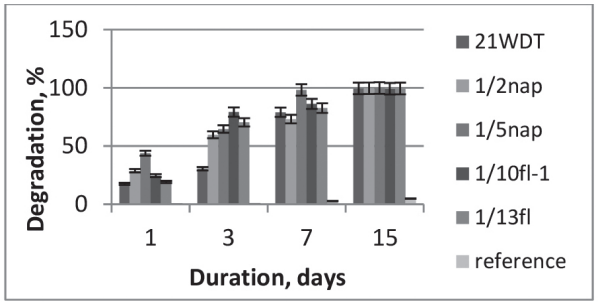

Fig. 8. Fluorene degradation by active strains

Bacillus haynesii 21WDT strain – in 48 hours. The previous studies had confirmed that naphthalene could be quickly degraded in 24 – 48 hours. However, the activity of the isolated strains was higher. They completely degraded naphthalene at the concentration of 200 mg/L in two to five days. The strains isolated by Xuezhu Zhu *et al.* (2016) reduced the concentration of naphthalene (100 mg/L) by 95.3 – 98.0 % in seven days.

According to the USEPA, one of the top pollutants is fluorene. In some studies, it was reported that it could not be significantly biodegraded (Yuan *et al.*, 2000), or could only be degraded cometabolically (Boldrim, 1993). However, the bacteria of the *Arthrobacter* and *Pseudomonas* genera, which used fluorene as the only source of carbon and energy in the aerobic conditions at 25 – 30°C, were isolated (Sokolovská, 2002).

Fluorene degradation (50 mg/L) by five strains of hydrocarbon-oxidizing microorganisms was studied. It was found that after one day, a decrease of the substrate by 17.5 – 43.8 % had been observed (Figure 8). With that, the 1/5nap strain was the most active one. By day three, increased activity was detected in the 1/10fl-1 and 1/13fl strains, which had degraded over 70 % of the substrate. After seven days, under the influence of these and the 1-5nap strain, fluorene degradation amounted to 82.6 – 98.2 %. By day 15, all the strains almost completely degraded the substrate. Throughout the experiment, the natural loss of the studied hydrocarbon was negligible, and by day 15, it was 4.88 %.

The studies of Lateef B. Salam and Oluwafemi S. Obayori (2014) showed that the *Bacillus subtilis* BM1 and *Bacillus amyloliquefaciens* BR1 strains had degraded the same amount of fluorene by 86 and 82% in 21 days.

Conclusion

The strains capable of growing on naphthalene and fluorene as the only source of carbon and energy have been isolated from oil-contaminated soil of Western Kazakhstan. Studying the 16S rRNA gene sequence has shown that all active strains belong to the *Pseudomonas* and *Bacillus* genera. Naphthalene has been completely degraded in two to five days, and fluorene – in 15 days. The *Bacillus haynesii* 21WDT strain has been capable of degrading both naphthalene and fluorene.

Acknowledgments

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