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PROPERTIES OF STRAIN *PSEUDOMONAS PANIPATENSIS*

СВОЙСТВА ШТАММА *PSEUDOMONAS PANIPATENSIS*

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Abstract. A strain of aerobic bacteria of the genus *Pseudomonas* is isolated from oil–contaminated water. Its properties and phylogenetic characteristics have been studied. Also, the influence of strain on the activation of the biological degradation of oil and oil products was tested.

It is found that the method of introduction of the resulting strain at contaminated sites helps shorten the petroleum hydrocarbon degradation in the soil and aquatic ecosystems.

Depending on the ambient temperature a 5 days recycling oil in an aqueous medium under the influence of the strain reaches 40–96%.

Аннотация. Из водного объекта, загрязненного нефтепродуктами выделен штамм эробных бактерий рода *Pseudomonas*. Изучены его свойства и филогенетическая характеристика. Исследовано влияние штамма на активацию биологической деструкции нефти и нефтепродуктов. Установлено, что метод интродукции полученного штамма в загрязненные объекты способствует сокращению сроков деградации нефтяных углеводородов, как в почвенных, так и водных экосистемах. За 5 суток утилизация нефтепродуктов в водной среде под влиянием штамма достигает 40–96%, в зависимости от температуры окружающей среды.

Keywords: microorganisms, hydrocarbons, oil, soil, strain, *Pseudomonas panipatensis*.

Ключевые слова: микроорганизмы, углеводороды, нефть, почва, штамм, *Pseudomonas panipatensis*.

Currently, the pollution of the environment with petroleum hydrocarbons (HC) is a relevant environmental issue. The problem of rehabilitation of disturbed areas after accidental oil spills is especially important for the Far North, where the main oil and gas fields operated by the Russian Federation.

Being highly organized substance, oil alone degrades very slowly, oxidation processes are inhibited by other structures, transformation of certain compounds occurs by way of acquiring sustainable hardly oxidized forms [1, p. 557].

Without the use of additional measures for the rehabilitation of disturbed lands, self–healing process of oil–contaminated soils in regions with favorable climatic conditions takes 10–25 years, while the destruction of oil and its derivatives in the North may last up to 50 years or more [2, p. 7–31; 3, p. 140–159; 4, p. 1–10].

Among the known solutions that can effectively recover the soil of the northern regions from oil, most environmentally justified considered biological processes, based on the intensification of the microbial degradation of oil hydrocarbons [5, p. 198–201].

Thus, the search for highly effective, non–toxic and non–pathogenic strains of hydrocarbon–oxidizing microorganisms (LCS), looking for the restoration of disturbed lands is an urgent task.

The aim of the present study was to obtain a new non–pathogenic strain of the LCS, promising to clean up soil contamination from oil and oil products.

Objectives of research:

1) to extract not pathogenic strain of bacteria capable for degradation of petroleum hydrocarbons from environmental objects;

2) to analyze the nucleotide sequences of the 16Sp RNA gene fragments and determine the phylogenetic position of the selected strain;

3) to study cultural, morphological, physiological, biochemical and chemo–taxonomic characteristics of the strain;

4) to test the resulting strain for the environmental remediation from oil and oil products.

The materials and methods of research

Strains *Pseudomonas panipatensis* C71 (IPNG–ELA–3) deposited in the Russian National Collection of Industrial Microorganisms (VKPM) GosNIIgenetika FSUE (Moscow) are studied.

A method of liquid enrichment cultures in the mineral medium by Muntz was used for culturing the strain [6, p. 1024–1030].

Talakan oil, containing 0.82% wax and 12.4% of resinous substances, was used as a sole carbon source [7, p. 165–170].

Evaluation indicator of viability of the strain was carried out by the cup Koch method [8, p. 40].

Identification of the isolated strain was carried out on the basis of study of their morphological, cultural, physiological and biochemical properties [9, p. 830; 10, p. 800] involving analysis of nucleotide sequences of the 16Sp RNA gene.

Isolation of DNA for PCR was conducted by the method of Ribosomal Database Project II [11, pp. 14–15].

PCR was performed on a GeneAmp PCR System 2700 instrument [12, pp. 146–157; 13, pp. 97–99].

Kinship trees were built up with the help of the technical capabilities of the website (Ribosomal Database Project II: http://rdp.cme.msu.edu).

The resistance of the strain to antibiotics was determined by agar diffusion method [14, p. 448].

The presence of oil in the aquatic environment was determined by spectrometry using concentratometer of oil products (FR.1.31.2007.03234 MIM 01.02.117 procedure of measurement of mass concentration of petroleum products in drinking, natural and wastewater IR–spectrometric method using concentratometer “IRH–025”).

The presence of oil in the soil was determined by cold extraction in chloroform. Structural and group composition of the extracts and their fractions was studied by IK Fourier spectroscopy. Group component composition of the extracts was determined by the method of liquid–adsorption column chromatography (RD 52.18.647–2003. HOWTO determine the mass fraction of oil products in soil. Measurement technique gravimetric method).

The results of the study

A strain of bacteria *Pseudomonas panipatensis* C71 (IPNG–ELA–3) have been isolated from water contaminated with petroleum hydrocarbons. The resulting strain is characterized by the following features.

Morphological and cultural characteristics

This is a Gram–negative rod bacterium, the size of 2,0–3,0×0,6–0,8 μm. In smear, it is placed in clusters. It is erobe. It doesn't form spores and capsules.

The resulting strain forms a wet grayish colony in a meat–peptone agar (mass. %: enzymatic peptone — 1,0; sodium chloride — 0.5; agar — 1,0; water meat is rest, pH 7.0 to 7.2). The color of the colonies is changing after 1–2 days. The colonies turn in green, the edge of the colony becomes rough.

In the Saburo medium (wt. %: hydrolyzed fish meal — 1,0; pancreatic hydrolysate of casein — 1.0; yeast extract — 0.2; dehydrogenated sodium phosphate — 0,2; D–glucose — 4,0; agar — 1,0; distilled water — the rest; pH of 6.0±3) it forms large colonies with grey with green pigment, the maximum diameter of which is 0.5 cm.

In meat–peptone broth (wt.%: enzymatic peptone — 1.0, sodium chloride — 0.5, water meat — rest; pH 7.0 to 7.2) it causes diffuse opacity.

In the mineral Muntz medium with oil and oil products (diesel fuel, motor oil, gas condensate, petrol) of the following composition: (wt. %: KNO_3 — 0,4; $MgSO_4 \times 7H_2O$ — 0.08; NaCl — 0.1; —0.14 K_2 HPO_4; KH_2 PO_4, or 0.06; agar — 2.0; oil or petroleum products — 1,0; distilled water — the rest, pH 7,2) the strain grows in the form of lusterless non–transparent grey with green pigment colonies with a diameter of 0.1–0.3 cm.

Physiological and biochemical characteristics

The strain grows at temperatures from +8 to +42 °C. Under aerobic conditions it grows better. In anaerobic conditions, the strain does not die immediately, but develops slower. The optimum growth is under conditions of +20 to +30 °C, pH should be from 6.0 to 8.0. The strain grows in a salt broth with the addition of 0.1–2.0% NaCl.

The strain is catalase positive and oxidase negative. It has metabolism of oxidative type. Biochemical activity of the strain is weak. It is not able to assimilate polyhydric alcohols.

Glucose and maltose is poorly fermented by the strain; other carbohydrates are not fermented at all. The strain does not assimilate lysine and ornithine. It does not hydrolyze gelatin. It does not decompose starch. The strain is Indole negative. Reaction Voges–Proskauer is negative. Phenylalanine Desoxaminase is negative. It can ferment beta galactosidase.

Phylogenetic characteristics

When the variable regions of 16Sp RNA were sequenced, the following derived nucleotide sequence for the tested strain was derived:

ATGCCTAGGAATACAGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAA
GACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATG
AGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTA
ACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAA
GTNACTACCTGCTGTTTGACGT

Further analysis by RDP II 16Sp RNA database showed homology with the same bacterial species.

According to the analysis the phylogenetic tree from homologous strains was constructed (Figure 1).

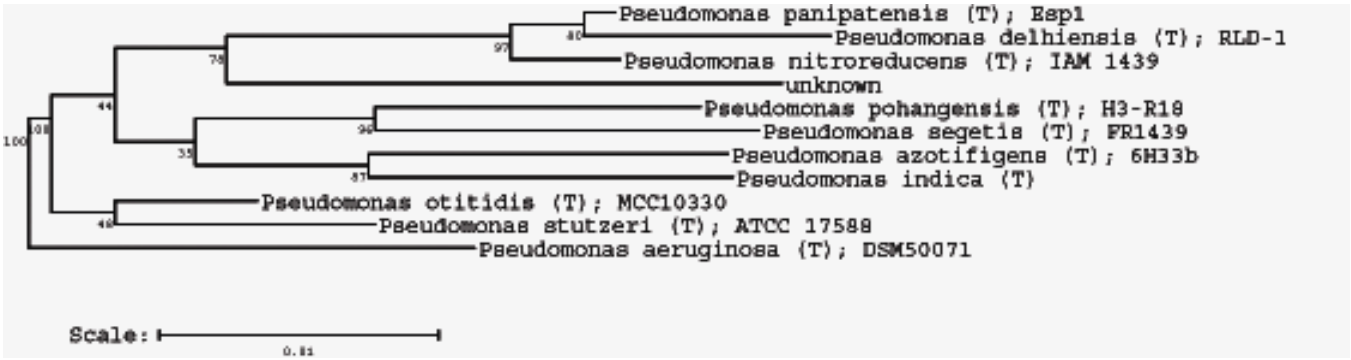

Figure 1. *Pseudomonas panipatensis* phylogenetic tree.

Primary screening of database of GenBank and RDP–II showed that the investigated strain belongs to the following systematic groups of *Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas,* and homology with some species of the genus *Pseudomonas panipatensis is* 97%.

Sequences were aligned with the corresponding sequences of bacterial species nearest available from GenBank data base.

The results of processing of sequences by means of a computer program on the website RDB II (Ribosomal Database Project II), designed to determine the relationship of microorganisms and constructing phylogenetic trees, are presented in graphical form:

S000010427 0.965 0.929 1460 *Pseudomonas aeruginosa* (T); DSM50071; X06684
S000390990 0.965 0.865 1417 *Pseudomonas indica* (T); AF302795
S000428789 0.961 0.900 1368 *Pseudomonas stutzeri* (T); ATCC 17588; AF094748
S000444074 0.953 0.865 1328 *Pseudomonas segetis* (T); FR1439; AY770691
S000514601 0.965 0.886 1444 *Pseudomonas otitidis* (T); MCC10330; AY953147
S000567747 0.953 0.854 1354 *Pseudomonas azotifigens* (T); 6H33b; AB189452
S000626936 0.965 0.872 1427 *Pseudomonas nitroreducens* (T); IAM 1439;
S000639962 0.953 0.840 1364 *Pseudomonas pohangensis* (T); H3–R18; DQ339144
S000639965 0.953 0.872 1418 *Pseudomonas delhiensis* (T); RLD–1; DQ339153
S000824948 0.973 0.947 1344 *Pseudomonas panipatensis* (T); Esp1; EF424401

The criterion for classifying the microorganism to a particular type is considered a homology of at least 97%. According to this criterion, the investigated strain can be attributed to several species of the genus *Pseudomonas*.

Analysis of phylogenetic relationships, built using standard strains of closely related bacteria showed that the closest to the test strain is the kind *Pseudomonas panipatensis*.

The strain is not virulent, non–toxic, non–toxigenic, not phytotoxic (test was conducted on white mice and higher plant seeds).

A study of oil–oxidizing activity of the *Pseudomonas panipatensis* C71 (IPNG–ELA–3) strain found that, in mineral medium the resulting strain for 5 days at a temperature of +8 °C utilizes 40,0–79,6% of oil and oil products; at a temperature of +20 °C – 48,6 – 94,6%; at a temperature of +30 °C — 67,7–96,4%; at a temperature of +37 °C — 81,5–96,2%, depending on xenobiotic type (Table 1).

Table 1.

THE DEGREE OF RECYCLING OF OIL AND OIL PRODUCTS

Option experience		*T °C*			
Xenobiotic, mg/dm³	*The term*	*+8*	*+20*	*+30*	*+37*
Oil	Before experience	1000,0	1000,0	1000,0	1000,0
	after experience	203,96	54,15	35,62	37,50
	% degradation	79,60	94,59	96,43	96,20
Diesel fuel	before experience	1000,0	1000,0	1000,0	1000,0
	after experience	599,54	514,18	322,96	185,4
	% degradation	40,05	48,58	67,70	81,46

The ability of the strain to recycle the petroleum hydrocarbons in the soil has been proved in the experiment set in an open ecosystem. The experiment was laid on a site with permafrost–tundra type soil.

The average temperature of the soil during the whole period of the experiment, on the horizon of 20 cm was +8 °C; on the horizon 10 cm was +13 °C.

A suspension of cells of the strain *Pseudomonas panipatensis* C71 (IPNG–ELA–3), with a titer of at least 1×10^9 cells/cm^3 were introduced into soil contaminated with oil at the rate of 1 liter of the product per 1m^2 of oil–polluted soil.

The soil was mixed thoroughly using shovels and exposed in vivo within 60 days.

Degradation of oil pollution in soil under the influence of the tested strain by the end of the experiment was of 91,7% (Table 2).

Table 2.

THE DYNAMICS OF DEGRADATION OF DIESEL FUEL IN SOIL

Variant of the experiment	*Diesel fuel content before the experiment, mg/kg*	*Diesel fuel content, after the experiment, mg/kg*	*Destruction of diesel fuel, %*
Soil + diesel fuel + cells of strain *Ps. panipatensis*	18351	1521	91,71
Soil + diesel fuel	25823	21247	17,7

Thus, it is shown that a new strain of bacterium *Pseudomonas panipatensis* C71 (IPNG–ELA–3) has high recycling ability, as an oil and oil products in a relatively short time (5 to 60 days). The resulting strain can be used to clean soils, and for purification of water contaminated with oil and oil products (diesel fuel, motor oil, gas condensate) in a wide temperature range from +8 to +37 °C, making it promising for biotechnological production.

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