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ISOLATION AND STUDY OF THERMOTOLERANT OIL-OXIDIZING MICROORGANISMS

Abstract. Climatic conditions limit the effectiveness of the use of the most known remediation methods in regions with hot climates. In the oil-producing regions of Kazakhstan, the climate is characterized by seasonal and daily temperature differences, high rates of water evaporation, salinity and low soil moisture. In this regard, the problem of the development and application of technologies adapted to the above conditions is relevant for Kazakhstan and other countries. Thermotolerant hydrocarbon-oxidizing microorganisms, adapted to extreme climatic conditions, are capable of oxidizing petroleum hydrocarbons at elevated temperatures.

The purpose of the research was isolation and selection of cultures of thermotolerant oil-oxidizing microorganisms, as well as the study of their activity.

From the contaminated soil of the Zhanatalap field (Atyrau region), 72 cultures were isolated by the method of enrichment cultures. Among them 15 cultures showing good and moderate growth at 35^{o}C were selected, 7 cultures – at 40^{o}C and 12 cultures – at 50^{o}C. Their oil oxidizing activity was studied. It was shown that during the cultivation of isolates in a liquid mineral medium with oil, the degree of its destruction at 35^{o}C was 18.7-52.0%, at 40^{o}C – 22.7-31.5%, and at 50^{o}C – 17.7-33.8 %.

Keywords: oil, oil pollution, thermotolerant oil-oxidizing microorganisms, oil destruction.

Numerous disturbances of the ecological balance in nature ultimately lead to environmental disasters. Currently, among the various man-made disturbances of nature, one of the most serious and difficult to repair is oil pollution. Oil and its components (aromatic, naphthenic and paraffinic hydrocarbons) are among the most dangerous pollutants entering the soil in the processes of extraction, transportation, processing and storage. Increasing environmental pollution with oil and petroleum products leads to serious disturbances in natural ecosystems, biological balance and biodiversity. In areas of oil production, the anthropogenic impact on land resources is increasing, which leads to the emergence of man-made changes in the state of the soil. Oil and petroleum products cause almost complete depression of the functional activity of flora and fauna, adversely affecting the links of the biological chain [1, 2]. In the oil-producing regions of Kazakhstan, environmental problems are aggravated by the intensive development of the gas and oil refining industry, which has a negative impact on public health. Allergic diseases such as dermatitis, bronchial asthma and the like are widespread [3].

According to estimates by the United States Environmental Protection Agency (EPA USA), the volume of contaminated soil by oil and oil products exceeds 1 billion cubic meters. Only in the EU there are more than one and a half million polluted areas, for the cleaning of which more than 85 billion euros are needed [4, 5]. In Kazakhstan, in the areas of oil production, the presence of more than 200 thousand hectares of oil-contaminated land has been established [6].

The main risk of pollution by oil and oil products are the regions where the deposits are located and oil is being produced. About 60% of the world's oil reserves are located in countries with hot climates. Temperature conditions are the limiting factor in the remediation of oil-polluted soils and suggest a careful approach to the choice of methods for cleaning contaminated land [7, 8].

One of the main problems of remediation of territories in a hot climate is the fact that high temperatures reduce the viscosity of oil and, thus, accelerate its diffusion deep into the soil. In addition, the evaporation of light fractions of oil at elevated temperatures leads to air pollution by toxic products, while the remaining non-volatile components with high molecular weight form films that are poorly biodegradable. At the same time, elevated average daily temperatures are responsible for the rapid evaporation of water from the soil and from the surface of water reservoirs, which leads to their salinization [9].

A significant contribution to the process of biological destruction of oil is made by hydrocarbon-oxidizing microorganisms, which are a permanent component of soil biocenoses [10]. Promising remediation agents for oil-polluted areas in regions with high temperatures are thermotolerant microorganisms that are resistant and adapted to the lack of water in the soil and the elevated salt content in the area being treated.

Depending on the temperature, bacterial activity and rates of biodegradation may vary seasonally. The effect of temperature on the growth of microorganisms is due to its effect on the rate of chemical reactions in the cell and the state of cellular macromolecules (membrane viscosity, protein conformation, etc.).

In relation to temperature, microorganisms are divided into the following groups: psychrophilic (minimum – about 0^{o}C, maximum – below 20^{o}C); psychroactive / psychrotrophic (minimum – about 0^{o}C, optimum and maximum – above 20^{o}C); mesophilic (minimum – above 0^{o}C, maximum – up to 45^{o}C); thermophilic (maximum above 45^{o}C).

Thermophilic microorganisms, in turn, depending on the temperature range, are divided into 5 groups:

1. Thermotolerant (minimum - + 10^{o}C; optimum - + 35-40^{o}C, as in mesophiles; maximum - + 55-60^{o}C);

2. Facultative thermophiles (minimum - below + 20^{o}C; maximum - + 55-65^{o}C);

3. Obligate thermophiles (minimum - + 40^{o}C; optimum - adjacent to the upper limit (+ 65-70^{o}C); maximum - above + 70^{o}C);

4. Extreme thermophiles (minimum - above + 40^{o}C; optimum - + 70-75^{o}C; maximum - above + 90^{o}C);

5. Hyperthermophiles (minimum - about + 70^{o}C; optimum - above + 80^{o}C; maximum - above + 100^{o}C) [11].

The enzymes used by thermotolerant bacteria to destroy hydrocarbons operate at higher temperatures than the enzymes of common mesophilic destructors. Therefore, thermotolerant microorganisms can and should be used in the remediation of oil-contaminated soils and waters under conditions of high ambient temperatures. However, the diversity of thermotolerant bacteria, their metabolic pathways of degradation and transformation of hydrocarbons are poorly studied.

In this connection, the isolation and study of thermotolerant microorganisms-destructors of petroleum hydrocarbons is current.

The purpose of this work was isolation and selection of cultures of thermotolerant oil-oxidizing microorganisms, as well as the study of their activity.

Materials and methods. The isolation of new thermotolerant oil-oxidizing microorganisms was carried out by the method of enrichment cultures. In Erlenmeyer flasks with 200 ml of Voroshilova-Dianova (VD) mineral medium, 20 g of oil-contaminated soil and 10 ml of Zhanatalap field oil were added. The flasks were placed in thermostatic shakers at temperatures of 35^{o}C, 40^{o}C, 50^{o}C. After 14 days, the first passage was conducted on a fresh medium with oil, another 14 days later - the second passage.

Isolation of oil-oxidizing microorganisms was carried out by seeding the culture liquid in Petri dishes with nutrient agar. Grown up individual colonies were seeded with streak plating to check purity. Pure cultures were re-seeded on slant nutrient agar.

The ability of the isolated cultures to grow on oil was studied in a liquid mineral VD medium. 5 ml of the cell suspension of each strain studied and 1 ml of Zhanatalap oil (Atyrau region) were added in flasks with 100 ml of medium. The flasks were incubated in thermostatic shakers (120 rpm) at appropriate temperatures for 7 days. The destruction of oil was judged visually by the change in oil slick and biomass accumulation.

Quantitative oil consumption was determined by gravimetric [12], and the component composition – by gas chromatographic methods [13]. In flasks with 100 ml of mineral medium, 2% of oil was added.

Then 5 ml of suspension of the studied cultures was added. Cultivation was carried out in thermostatic shakers at appropriate temperatures for 14 days. The residual oil was extracted with chloroform.

All experiments were performed in 3 replicates.

Statistical processing of the research results was carried out according to generally accepted criteria of variation statistical analysis with the calculation of average values (M), arithmetic average error (m) using the computer package Microsoft Excel, 2010. The statistical significance of differences in average values was estimated using the standard method [14]. The differences with $p < 0.05$ were considered statistically significant.

Results and discussion. Oil-oxidizing microorganisms growing at elevated temperatures were isolated from oil-contaminated soils of Western Kazakhstan by the method of enrichment cultures. In total, 34 cultures were isolated from the initial enrichment cultures and two subsequent passages at a temperature of 35°C, 24 isolates – at 40°C, and 14 isolates – at 50°C. Bacterial cultures differed in shape, size, and surface of colonies. Non-pigmented colonies were met mostly.

Selected cultures were tested for the ability to grow in a liquid mineral medium in the presence of 1% oil (table). Their growth was evaluated visually by changes in the oil slick and biomass accumulation.

The number of thermotolerant microorganism cultures growing in mineral medium with oil

Temperature	Total cultures	Growth intensity	
		moderate	good
35 °C	34	10	4
40 °C	24	7	–
50 °C	14	4	8

At 35°C, 4 cultures showed good growth in oil. At the same time, there was no oil film on the surface of the medium, the oil itself was either converted into a homogeneous emulsion, or was in the form of small suspended particles. There was a significant increase in biomass. 10 cultures showed moderate growth. The remaining isolates showed no significant activity.

At 40°C, none of the studied cultures showed good growth. 7 cultures grew moderately in these conditions. A suspension of small particles of oil with biomass was observed in the culture liquid; oil on the surface of the medium was partially preserved as a thin film.

At 50°C from the 14 tested cultures, 8 showed a good growth, 4 cultures showed moderate growth and only 2 cultures grew poorly.

Thus, 15 cultures that showed good and moderate growth at 35°C, 7 cultures at 40°C and 12 cultures at 50°C were selected for further research.

Studying the ability of selected cultures of thermotolerant oil-oxidizing microorganisms to utilize oil at 35°C showed that when they grew in a liquid mineral medium with oil, the degree of its destruction was 18.7-52.0% (figure 1). In most of the cultures tested, oil utilization did not exceed 28%. Three cultures consumed over 35% of oil. The most active were the cultures of P1-35-14 and P2-35-9. When incubated, the destruction of oil was more than 50%.

At 40°C, in most of the studied cultures, the degree of oil destruction was 22.7-25.3% (figure 2). Only in two isolates IP-40-4 and P1-40-8 it exceeded 30%.

At 50°C, the destruction of oil was about the same. The three most active isolates IP-50-3/1, P2-50-5 and P2-50-2 utilized 29.4-33.8% of oil (figure 3). Abiotic losses of oil were 10.1% and 10.8% at 40°C and 50°C, respectively.

Gas chromatographic analysis of oil after cultivation of selected strains of microorganisms showed that, during the experiment, at all temperatures studied, reactions occur, as a result of which the content of the main components of oil changes. According to the data obtained in the experimental samples, a decrease in the number of n-alkanes occurred. Also it was noted a decrease in basic aromatic hydrocarbons. Figure 4 shows the chromatograms of the oil of the control and the test sample after 14 days of cultivation with culture P2-35-9.

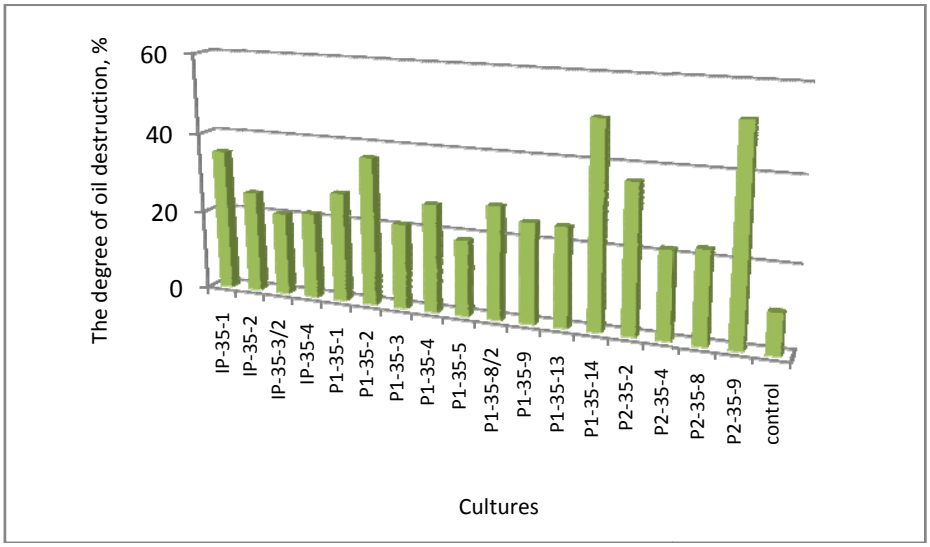


Figure 1 – The destruction of oil by new cultures of thermotolerant oil-oxidizing microorganisms at 35°C

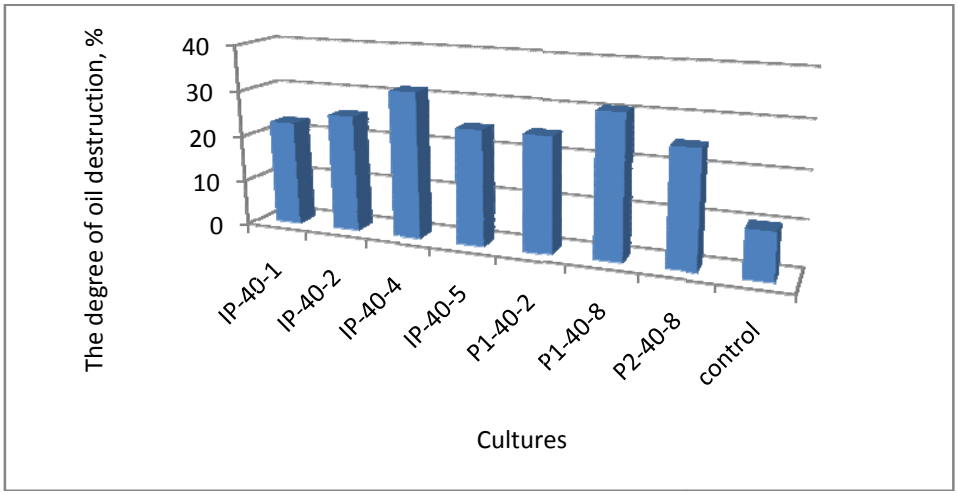


Figure 2 – The destruction of oil by new cultures of thermotolerant oil-oxidizing microorganisms at 40°C

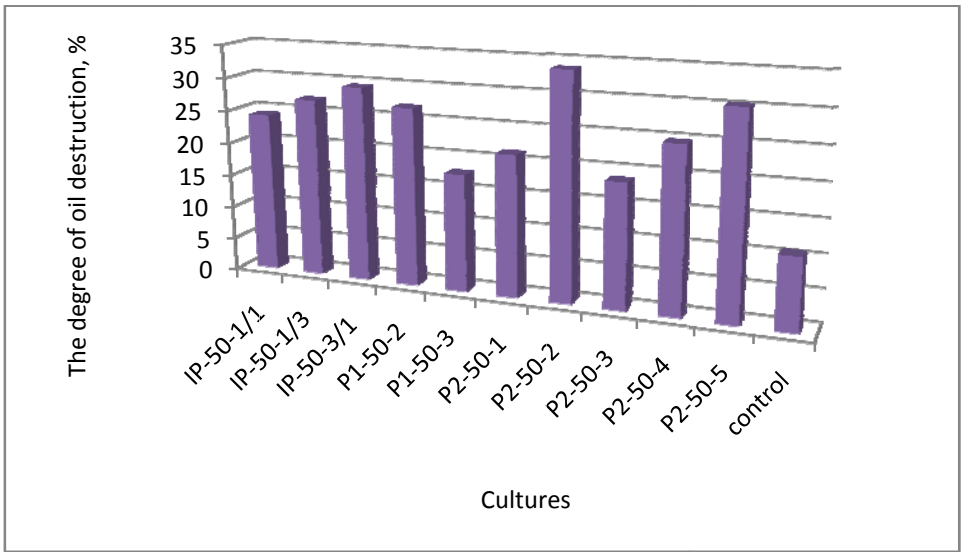
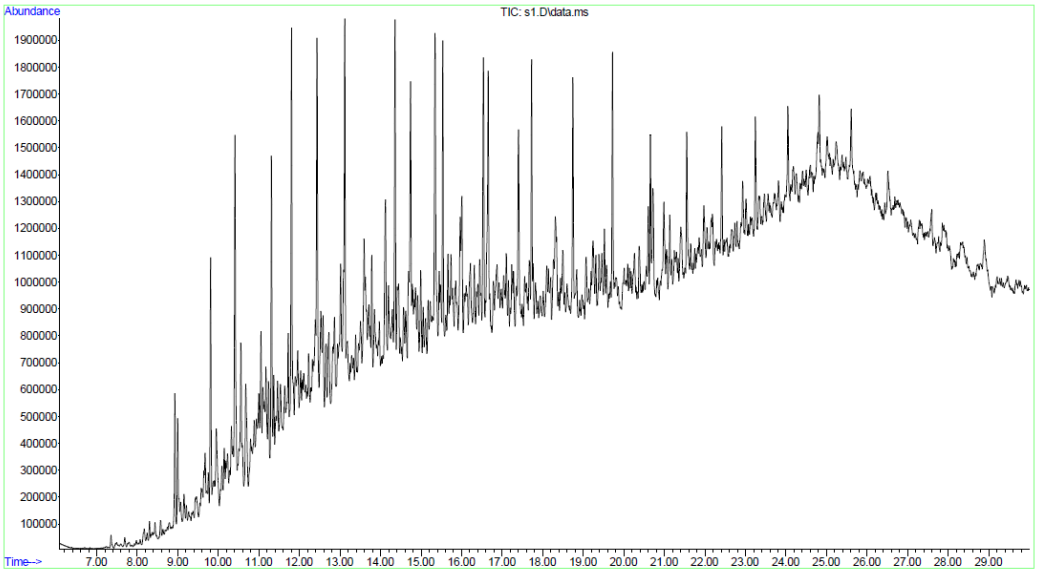
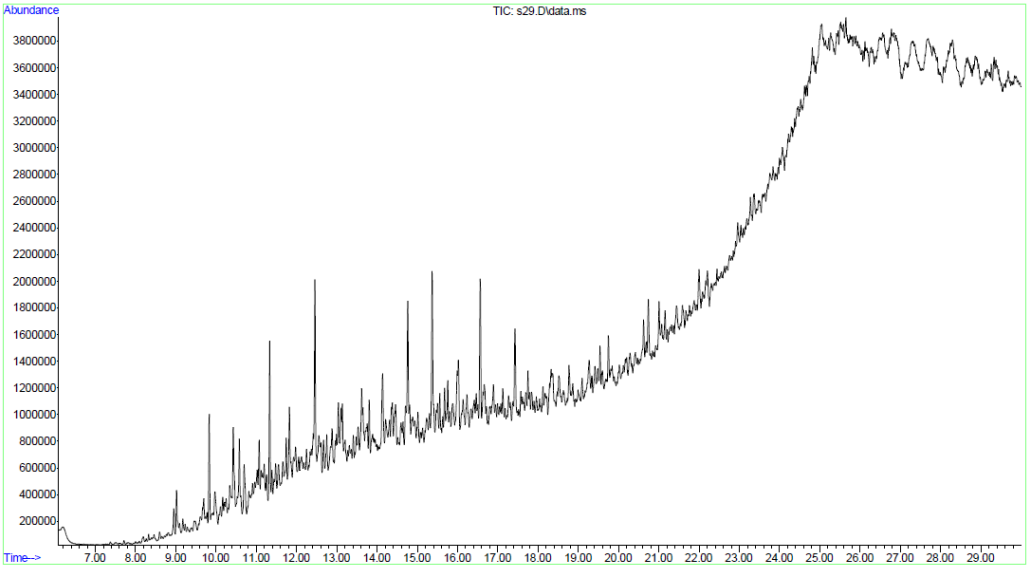


Figure 3 – The destruction of oil by new cultures of thermotolerant oil-oxidizing microorganisms at 50°C



a



b

Рисунок 4 – Chromatogram of the total content of petroleum hydrocarbons
in the control (a) and after cultivation of the strain P2-35-9 (b)

Thus, temperature plays a vital role in the bioremediation of oil pollution. This effect is due to its effect on the metabolic rate of bacteria. Temperature affects both the physical state of the hydrocarbons present in the contaminated area and the microorganisms present in the soil. Research related to the search and study of thermotolerant microorganisms-destructors of oil, is very actual, because they are promising agents for remediation of oil-polluted areas in regions with a predominance of elevated temperatures.

From oil-polluted soils of Western Kazakhstan, thermotolerant oil-oxidizing microorganisms have been isolated. Their oil oxidizing activity was studied. It was shown that during the cultivation of isolates in a liquid mineral medium with oil, the degree of its destruction at 35^{o}C was 18.7-52.0%, at 40^{o}C – 22.7-31.5%, and at 50^{o}C – 17.7-33.8 %.

All selected active thermotolerant strains of oil-oxidizing microorganisms can be further used as part of a consortium for cleaning oil-contaminated soils in regions with a hot arid climate.

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ТЕРМОТОЛЕРАНТТЫ МҰНАЙТОТЫҚТЫРҒЫШ МИКРООРГАНИЗМДЕРДІ БӨЛІП АЛУ ЖӘНЕ ЗЕРТТЕУ

Аннотация. Климаттық жағдайлар ыстық ауа райлы аймақтарда көпшілікке танымал ремедиациялық әдістерідің тиімділігін шектейді. Қазақстанның мұнай өндіретін аймақтарында климат маусымдық және тәуліктік температура айырмашылығымен, судың буланuының жоғары деңгейімен, тұзданuымен және топырақтың төмен ылғалдығымен сипатталады. Осыған байланысты жоғарыда аталған жағдайларға бейімделген технологияларды әзірлеу және қолдану мәселесі Қазақстан мен алыс шет елдер үшін өзекті болып табылады. Төтенше климаттық жағдайларға бейімделген термотолерантты көмірсутектотықтырғыш микроорганизмдер жоғары температурада көмірсутектерді тотықтыруға қабілетті.

Зерттеудің мақсаты термотолерантты мұнайтотықтырғыш микроорганизмдердің культураларын бөліп алу және іріктеу, сондай-ақ олардың белсенділігін зерттеу болып табылады.

Жаңаталап (Атырау облысы) кен орнының ластанған топырағынан жинақтау әдісі арқылы 72 изолят бөлініп алынды. Олардың ішінен жақсы және қалыпты өсуін көрсеткен 35°С температурада 15 культура, 40°С температурада 7 культура және 50°С температурада 12 культура іріктеліп алынды. Олардың мұнайды тотықтыру белсенділігі зерттелді. Изоляттарды сұйық минералды қоректік ортада мұнаймен бірге өсіру кезінде олардың ыдырату деңгейі 35°С температурада 18.7-52.0%, 40°С - 22.7-31.5% , ал 50 °С - 17.7-33.8 % құрады.

Түйін сөздер: мұнай, мұнайлы ластаушылар, термотолерантты мұнайтотықтырғыш микроорганизмдер, мұнайды ыдырату.

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ВЫДЕЛЕНИЕ И ИЗУЧЕНИЕ ТЕРМОТОЛЕРАНТНЫХ НЕФТЕОКИСЛЯЮЩИХ МИКРООРГАНИЗМОВ

Аннотация. Климатические условия ограничивают эффективность применения большинства известных ремедиационных методов в регионах с жарким климатом. В нефтедобывающих регионах Казахстана климат характеризуется сезонными и суточными перепадами температур, высокими темпами испарения воды, засоленностью и низкой влажностью грунта. В связи с этим проблема разработки и применения технологий, адаптированных к вышеперечисленным условиям, является актуальной для Казахстана и дальнего зарубежья. Термотолерантные углеводородокисляющие микроорганизмы, адаптированные к экстремальным климатическим условиям, способны окислять углеводороды нефти при повышенных температурах.

Целью исследований было выделение и отбор культур термотолерантных нефтеокисляющих микроорганизмов, а также изучение их активности.

Из нефтезагрязненной почвы месторождения Жанаталап (Атырауская область) методом накопительных культур выделено 72 изолята. Из них отобрано 15 культур, показавших хороший и умеренный рост при 35^{o}С, 7 культур – при 40 ^{o}С и 12 культур – при 50^{o}С. Изучена их нефтеокисляющая активность. Показано, что при культивировании изолятов в жидкой минеральной среде с нефтью степень ее деструкции при 35^{o}С составляла 18,7-52,0%, при 40^{o}С – 22,7-31,5%, а при 50^{o}С – 17,7-33,8%.

Ключевые слова: нефть, нефтяное загрязнение, термотолерантные нефтеокисляющие микроорганизмы, деструкция нефти

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