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ADVANCES IN BIOTECHNOLOGY

ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM CASPIAN SEA RESPONSIBLE FOR OIL DEGRADATION

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ABSTRACT

About 400 cultures of microorganisms were isolated from water and soils samples of oilfields Kalamkas and Karazhanbas (the Caspian Sea). The 57 cultures of them showed stable growth on mineral medium Voroshilova - Dianova (VD) with 1% crude oil as the sole carbon source. Several characteristics allowing to assess the bioremediation potential of cultures were studied: the concentration of residual petroleum products based on fluorimetric analysis, the ability to emulsify oil and the cell-surface hydrophobicity based on adhesion of cells to hydrocarbons. Obtained data allowed to select 5 bacteria cultures capable to degrade oil. The nucleotide sequencing of the 16S rRNA gene showed the highest similarities of these cultures with *Ochrobactrum sp. Achromobacter sp., Roseomonas mucosa, Stenotrophomonas sp.* and *Sphingobacterium sp.* These microorganisms have the potential in hydrocarbons degradation and could be very useful for further creation on their basis consortia for bioremediation of environmental pollution.

Keywords: bacteria, the Caspian Sea, oil degradation.

INTRODUCTION

The Caspian Sea is the unique natural complex with rich flora and fauna as well as rich in oil and gas reserves. The development of hydrocarbon raw materials, intensification of industrial production, urbanization of regions lead to heightened pollution of the hydrosphere and soils by oil products that poses a threat to the environmental safety of these territories. In recent decades, there was a deterioration of the Caspian Sea ecological state. The mass death of fish and seals, the presence of the parasitic and pathogenic bacteria in marine organisms, the appearance of physiological and morphological abnormalities in sturgeon and other fishes are evidenced about this [1], [2].

So, the creation of an effective and affordable ways to eliminate the dangerous oil pollutions is an actual problem. Microbial oxidation of hydrocarbons is one of the leading factors in the oil elimination. The microorganisms' activity leads to transformation of oil pollution to simple compounds and, thus, the inclusion of hydrocarbon components to the total carbon cycle. The main advantages of bioremediation in comparison to mechanical, chemical and physical methods are: more

complete purification degree, environmental safety, minimum energy expenditure on mass unit of removed compounds, high efficiency and others [3].

Hydrocarbon-oxidizing microorganisms which degrade oil derivatives *in vivo* are widely distributed in nature and belong to different systematic groups. These include the genera: *Pseudomonas, Rhodococcus, Achromobacter, Brevundimonas, Sphingomonas, Acinetobacter, Arthrobacter, Corynebacterium, Bacillus, Ochrobactrum, Stenotrophomonas, Burkholderia, Xanthomonas; Aspergillus, Penicillium, Beauveria, Acremonium, Cladosporium, Fusarium, Trichoderma, Amorphoteca, Talaromyces, Graphium*; *Candida, Criptococcus, Rhodotorula* and others [3].

Therefore, the study of indigenous oil oxidizing microorganisms and creation on their basis of products for territories purification from oil derivatives is an actual problem [4].

The aim of work was to isolate and identify of indigenous microorganisms from Kalamkas and the Karazhanbas oilfields capable to oil degradation that might be useful for further commercial use.

MATERIALS AND METHODS

Collection of samples

Water and soils samples of oilfields Kalamkas and Karazhanbas were collected from surface horizon in accordance with [5]. 0,2 l or kg of 3-5 surface point samples were collected in the coastal part of the Caspian sea (0-20 cm under the surface). These point samples were mixed and the average sample by weight of 0,5 l or kg were prepared. For average soils samples preparation the method of the envelope was used. Water samples were collected at the same area over the same distance. The distance between the areas - 200-300 m. Samples were labeling with information about collection conditions. Prior to analysis samples were store in ice.

Isolation of microorganisms [6]

To isolate the hydrocarbon-oxidizing microorganisms the samples were suspended into the mineral medium Voroshilova – Dianova (VD). This medium contained (g/l): K_2HPO_4 - 1; KH_2PO_4 - 1; NH_4NO_3 - 1; $MgSO_4$ - 0,2; $CaCl_2*6H_2O$ - 0,01; $FeCl_3*6H_2O$ - 3 drops, NaCl – 10. As the sole carbon source 1% sterile oil from Kalamkas and Karazhanbas was added.

1 g of soil or 1 ml of water samples, 98 ml of the VD medium and 1 ml of oil was added in 250 ml flasks. The flasks were incubated on shaker for 7 days at 220 rpm. Then the microorganisms on different nutrient media (nutrient agar, Saburo medium, Chapek medium) were isolated by the limit dilutions method. Petri dishes were incubated at 28 °C for 1-10 days as fouling. Pure isolates were preserved into test tubes with the nutrient medium slants.

Morphological characteristics

The isolates were characterized by the colonies morphology in solid nutrient medium, by the cells shape using microscopy and by gram staining (for bacteria).

Screening of oil degrading microorganisms

For selection of oil degrading microorganisms each isolate suspended in 100 ml of VD medium with 1% oil. Bacteria and yeast were grown for 7 days, the micromycetes for 10 days on shaker at 220 rpm. The bacteria and yeast ability to grow on oil-contained medium was monitored spectrophotometrically at 590 nm. The micromycetes biomass amount was determined gravimetrically. The suspension was filtered through pre-dried at 80 °C to constant weight filter paper and then again drying to constant weight.

Measurement of mass concentrations of residual oil

Mass concentration of oil derivatives was determined according to [7], [8].

After incubation of the microorganisms suspension in VD medium with 1% oil within 7-10 days, 100 ml of sample was transferred to a separatory funnel. The flask rinsed with 10 ml of hexane and poured in a separatoty funnel. The mixture was extracted by shaking for 1 min. After phase's separation a measurement of upper hexane fraction was performed.

Mass concentration of oil derivatives in the sample was calculated by the formula: X = (Xh * Vh * K) / Vs, where X is mass concentration of oil derivatives in the sample, mg/l; Xh - mass concentration of oil derivatives in the hexane extract of the sample, mg/l; Vh – volume of hexane taken for extraction, ml (10); K – the extract dilution coefficient (if necessary). If the extract was not diluted, then K = 1; Vs – sample volume, ml (100). The residual concentration of oil derivatives in the sample was recalculated into a percentage by taking the control sample as 100%. By subtracting the residual oil derivatives in the sample of 100 the concentration of oil derivatives degraded by the microorganisms was counted.

Measurement of emulsification index

The emulsification index (EI) was determined as described in [9].

2 ml of cell suspension and the same amount of oil were mixed for 2 min and left to stand for one day. The emulsification index was measured in percent as the ratio of height of emulsified layer (mm) to total height of the liquid in the test tube (mm).

Measurement of cell surface hydrophobicity

Cell surface hydrophobicity (CSH) was measured according to the method described by Rosenberg M. in Nikovskya modification [10].

1 ml of suspension with known optical density and 0,5 ml of test hydrocarbon (mineral oil) was mixed and left to stand until complete separation of phases (about 1 h). The optical density of the hydrophilic lower phase was measured at 670 nm. The hydrophobicity percentage was calculated as the ratio of difference between initial and final optical density to initial optical density.

Bacterial identification

Genetic identification of bacterial strains was performed by 16S rRNA gene sequence analysis. DNA extraction was done using Wilson K. method [11], followed by amplification of two 16S rRNA fragments using primers: 5'-AGAGTTTGATCCTGGCTCAG-3' as forward and 5'-GGACTACCAGGGTATCTAAT-3' as reverse [12]. The PCR mix contained 150 ng of DNA, 1 unit of Taq DNA Polymerase, 0,2 mM of each dNTP, 1X PCR buffer (Fermentas), 2,5 mM MgCl2, and 10 pM of each primer. The total volume was 20 µl.

PCR amplification program included a long denaturation at 95 °C for 7 min; 30 cycles at 95 °C - 30 s, 55 °C - 40 s, 72 °C - 1 min; the final elongation was at 72 °C for 7 min. The PCR was performed using a BioRad T100 amplificator. Purification of PCR products from unbound primers was performed by an enzymatic method using Exonuclease I and Shrimp Alkaline Phosphatase (Fermentas) [13]. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by fragments separation on an automatic genetic 3730xl DNA analyzer (Applied Biosystems). The nucleotide sequences of cultures were analyzed using SeqMan (DNA Star) software. Sequences were identified by BLAST algorithm in GenBank. A subsequent nucleotide sequence was aligned with the sequences deposited at the international GenBank database. In cases of dispute, phylogenetic trees were built using the Neighbor-Joining method [14].

RESULTS AND DISCUSSION

Sampling of sea water and coastal soils in the area of oil fields Kalamkas and Karazhanbas of Buzachi peninsula was conducted in the spring and autumn of 2015. Based on collected samples more than 400 isolates of microorganisms has been allocated. Ability to stable growth in the liquid and solid mineral VD medium with 1% oil as the sole carbon source showed only 57 monocultures. The colonies morphology and microscopy allowed to determine among them 37 cultures of bacteria, 8 yeast cultures and 12 micromycetes cultures.

Figure 1 shows the results of fluorimetric measurement of oil derivatives concentration. The concentration of degraded oil derivatives by microorganisms ranged from single values up to more than 90%. Cultures skar2, skar4, skar7, skar13, skar20, wkar24, wkar37, wkal45, wkal48, wkal49, wkal51, wkal52 and wkar54 were able to degrade from 42,9 to 91,1% of oil after 7-10 days of cultivation. The rest of the cultures had the low percentage of oil hydrocarbons degradation (less than 40).

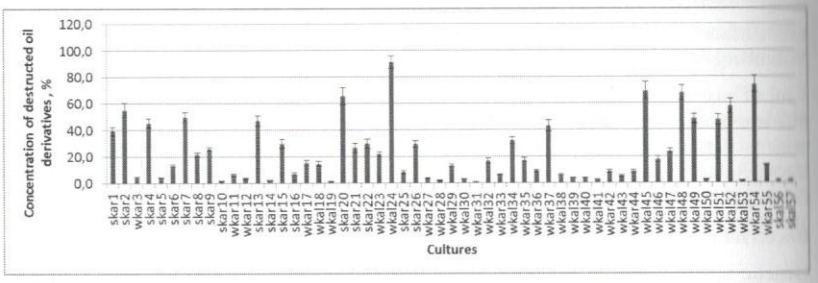

Figure 1 - The concentration of oil derivatives degraded by microorganisms after 7-10 days of cultivation in VD medium with 1% oil

Bioavailability of hydrocarbons for the microbial community is determined by the emulsification index. Many microorganisms synthesize surface active agents -

biosurfactants. They reduce the surface tension between the hydrocarbons and the water surface, whereby for microbial cell is easier to contact and utilize of hydrocarbons [9].

The results of the emulsification index measurement showed good potential of cultures in the formation of an oil emulsion that is showed in Figure 2. 49 of the 57 microorganisms had the value of emulsification index exceeded 40%, with the highest rates in cultures skar4, skar7, skar8, skar9, wkar12, skar21, wkal24, wkal47 and wkal49. The minimum value of the index - below 20% - had five cultures: skar10, skar14, wkal19, wkar28, and wkar31. 3 cultures entered in the middle group (from 20 to 40%): skar15, wkar35, wkar36.

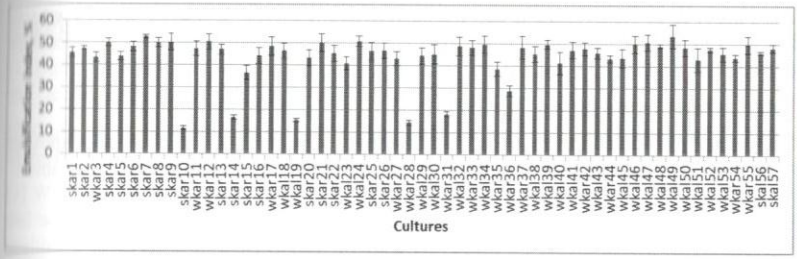

Figure 2 - The emulsification index of microorganisms

The cell surface hydrophobicity of microorganisms as a major indicator of the cells interaction with the substrate was determined by the cell surface ability to adhesion to hydrocarbons [10].

Percent of hydrophobicity varied widely to all cultures (Figure 3). From 7,4 in culture skar14 to 92,5% in culture wkal53 on 0 day and from 20,5 in culture wkal52 to 95% in culture wkal53 on 4 day of cultivation.

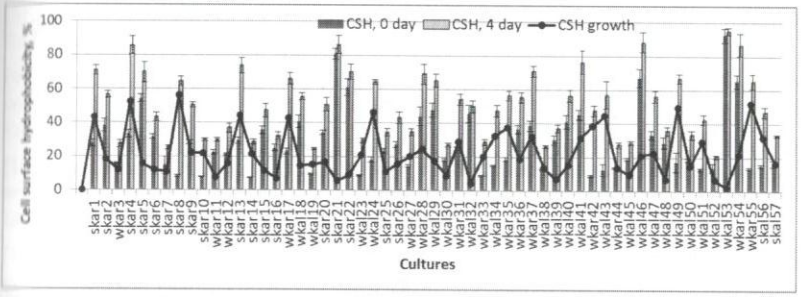

Figure 3 – The CSH and the increase of the hydrophobicity of microbial cells to 4 day of cultivation in VD medium with 1% oil

It was shown that during cultivation with oil the cell surface hydrophobicity in all cultures was increasing at the end of the cultivation (Figure 3). A slight increasing in the hydrophobicity index (up 10%) was observed in cultures wkar11, skar16, skar21, skar22, wkal30, wkal32, wkal39, wkal45, wkal48, wkal52, wkal53. Whereas the maximum increasing in the hydrophobicity index (over 40%) was observed in cultures skar1, skar4, skar8, skar13, wkar17, wkal24, wkal43, wkal49, wkar55. Significant difference of this index in some cultures at the beginning and at end of the cultivation suggests that initially (out of contact with the hydrophobic substrate) a cell surface was hydrophilic which is convenient in biomass manipulations. Increasing of the cell surface hydrophobicity provides the creation of conditions for the effective assimilation and absorption of oil hydrocarbons [15].

The sum of three tests results is that the bacterial cultures skar4, skar13, wkal24, wkal49 and wkar54 had the highest values (Table 1) confirming its potential in oil hydrocarbons degradation.

These bacteria were selected for identification.

Comparison of nucleotide sequences of investigated bacteria with sequences deposited at the GenBank database detected the highest similarities with cultures listed in Table 1.

Table 1 – Characteristics of selected microorganisms

Cultures	Concentration of destructed oil derivatives, %	EI,%	CSH growth, %	Identification result	Accession No.	Homology, %
Skar4	45,2±3,3	50±1,7	52,3	*Ochrobactrum sp.*	KT831449.1	100
Skar13	47,1±3,7	46,9±2,2	44,3	*Achromobacter sp.*	KU644265.1	99
Wkal24	91,1±4,5	50,8±2,3	46,1	*Roseomonas mucosa*	AF538712.1	100
Wkal49	48,3±3,6	53,6±5,4	49,5	*Stenotrophomonas sp.*	LN864633.1	99
Wkar54	74,1±6,1	43,9±1,5	21,4	*Sphingobacterium sp.*	KF928902.1	97

CONCLUSION

About 400 cultures of microorganisms were isolated from water and soils samples of oilfields Kalamkas and Karazhanbas (the Caspian Sea). Only 57 monocultures of them were capable to stable growth in mineral medium with 1% oil as the sole carbon source. The study of the destructive activity and cell surface hydrophobicity of microorganisms showed a variation of the values of these parameters over a wide range for all cultures.

Also the increasing tendency in the hydrophobicity index at the end of cultivation with oil was revealed. At the same time emulsification index was at the high level for most cultures. On the sum of the results of the three tests 5 bacterial cultures with the best values were selected. The index of degraded oil derivatives for these cultures ranged from 45,2±3,3 to 91,1±4,5%; increase the cell surface hydrophobicity was 21,4-52,3%; emulsification index - 43,9±1,5-53,6±5,4%. Identification of these bacteria by 16S rRNA showed that they belonged to *Ochrobactrum sp., Achromobacter sp., Roseomonas mucosa, Stenotrophomonas sp.* and *Sphingobacterium sp.* These bacteria are perspective for the creation of consortia and biological products for water and soils purification contaminated by oil derivatives.

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