Screening and Characterization of Emulsifying Hydrocarbon-Degrading Bacteria from Coastal Waters of the Caspian Sea[1]

Probir i karakterizacija emulgirajućih bakterija koje razgrađuju ugljikovodike iz obalnih voda Kaspijskog mora

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DOI 10.17818/NM/2021/2.2
UDK 628.355:54
Original scientific paper / *Izvorni znanstveni rad*
Paper accepted / *Rukopis primljen:* 25. 8. 2020.

Summary

As a result of 400 cultures screening isolated from the contaminated coastal zones of the Caspian Sea, 4 new strains were selected that had a stable growth and utilized oil with NaCl concentration close to that of seawater. *Stenotrophomonas chelatiphaga* wkal49, *Stenotrophomonas chelatiphaga* wkal51, *Sphingobacterium kitahiroshimense* wkar54, and *Achromobacter sp*. wkar55 were identified based on an analysis of the direct nucleotide sequence of the 16S rRNA gene fragment. The degree of oil degradation by these strains was above 50%. The hydrophobicity of the cell surface, emulsifying activity, the degree of influence on the viscosity of crude oil, and also the ability to produce surfactants were the four key factors that made up the studied parameters for the selected strains. The studied strains formed an emulsion layer on the surface of the culture medium. The most active producers of extracellular bioemulsifiers were the *Stenotrophomonas chelatiphaga* wkal49 and *Stenotrophomonas chelatiphaga* wkal51 strains. Both demonstrated the highest hydrophobicity, emulsification index, and the highest value for decreasing oil viscosity.

KEY WORDS

hydrocarbon-degrading bacteria
emulsifying activity
hydrophobicity
bioremediation
biosurfactants
biodegradation

Sažetak

Kao rezultat probira 400 kultura izoliranih iz kontaminiranih obalnih područja Kaspijskog mora, odabrana su 4 nova soja koja su mogla stabilno rasti i koristiti se uljem s koncentracijom NaCl koja je približna koncentraciji u morskoj vodi. Stenotrophomonas chelatiphaga wkal49, Stenotrophomonas chelatiphaga wkal51, Sphingobacterium kitahiroshimense wkar54 i Achromobacter sp. wkar55 identificirani su na temelju analize izravnog nukleotidnog slijeda fragmenta gena 16S rRNA. Stupanj razgradnje ulja tim sojevima bio je iznad 50%. Hidrofobičnost stanične površine, aktivnost emulgiranja, stupanj utjecaja na viskoznost sirove nafte, kao i sposobnost proizvodnje površinski aktivnih tvari bila su četiri ključna čimbenika, odnosno proučavani parametri za odabrane sojeve. Ispitivani sojevi tvorili su emulzijski sloj na površini medija za kulturu. Najaktivniji proizvođači izvanstaničnih bioemulgatora bili su sojevi Stenotrophomonas chelatiphaga wkal49 i Stenotrophomonas chelatiphaga wkal51. Oba su pokazala najveću hidrofobnost, indeks emulgiranja i najveću vrijednost za smanjenje viskoznosti ulja.

KLJUČNE RIJEČI

bakterije koje razgrađuju ugljikovodike
aktivnost emulgiranja
hidrofobnost
bioremedijacija
biosurfaktanti
biorazgradnja

1. INTRODUCTION / *Uvod*

The Caspian Sea is one of the oldest petroleum-producing basins in the world. In Azerbaijan, oil production began more than 150 years ago on the Absheron Peninsula, and industrial development on the shelf began as early as 1924. The North Caspian basin shelf located in Kazakhstan and Russia offers the over-salt deposits of Gran, Martyshy, Zhanatalap, Buzachi, Karazhanbas, and Kalamkas, which have been exploited for more than 30 years, together with the subsalt oil and gas Tengiz field (Kaiser & Pulsipher, 2007) since 1993.

Under a constant threat of pollution, the Caspian Sea suffers from pollution sources that include river flow, onshore industrial and municipal wastewater, and offshore and onshore oil extraction. Also, wells located in the flooded areas of the Kazakhstan part of the Caspian make up additional pollution sources. These pollutions reach the sea more easily due to sea-level growth, which causes coastal zone flooding where many oil wells are still in production. Consequently, water quality has deteriorated at Kalamkas field areas and also at the

[1] This article was prepared with financial support from the Ministry of Education and Science of the Republic of Kazakhstan (project grant number 0115RK00372) and the Ministry of Education and Science of the Republic of Kazakhstan and World Bank (grants program for senior scientists' group, subproject: APP-SSG-16-0555).

flooded wells of Kulaly Island (Tasmagambetova et al., 2019). Oil production, transportation, and spills are dramatically obtrusive to the surrounding environment, not only on the Kazakhstan part of the Caspian region but on the entire water area of the middle and northern basin of the Caspian Sea, which is a serious problem (Tasmagambetova et al., 2019).

The increasing number of oil spills in the sea requires effective solutions for environmental restoration. Petroleum, which has been the most dominant energy source for a long time, is a complex mixture of hydrocarbons, including more than 70% of alkanes along with aromatic substances, naphthenes, and resins. The long-chain alkanes present in crude oil remain stable because of a non-volatile nature, becoming a serious threat to terrestrial and marine ecosystems (Das & Chandran, 2011; Bao et al., 2014).

For removing oil residues on the affected shorelines, bioremediation techniques have become a major mechanism. Despite various existing bioremediation techniques (biostimulation, augmentation, phytoremediation, and rhizoremediation) for enhancing the natural biodegradation achieved by indigenous microorganisms, the usage of new microbial strains with high degrading ability has attracted the most interest (Varjani, 2017; Ossai et al., 2020).

A wide range of microorganisms are known to have the ability to use hydrocarbons as a source of energy and carbon. These microorganisms, when grown on a hydrocarbon substrate, synthesize a wide range of chemical substances (glycolipids, lipopeptides, phospholipids, and others) with biosurfactants (Wu et al., 2019) active on the surface, the use of which increases the solubility of water-insoluble contaminants. The bioavailability of hydrocarbons is a key bioremediation question, so more attention should be paid to this problem. Increased bioavailability leads to improvement in the accelerated decomposition of pollutants (Ortega-Calvo, 2017), while the search for new strains of microorganisms – key producers of biosurfactants – is important for conducting effective bioremediation of groundwater and the marine environment.

This study aims to isolate and perform screening of hydrocarbon-degrading bacteria using water samples collected from Caspian coastal zones, which have been polluted by hydrocarbons, in order to study their ability to produce surfactants.

2. MATERIALS AND METHODS / *Materijali i metode*

2.1. Sampling / *Uzorkovanje*

The water samples were collected from Caspian coastal zones polluted by hydrocarbons (Karazhanbas and Kalamkas oil fields of Buzachi Island, Kazakhstan, from spring 2015 to autumn 2016, Fig. 1). The Karazhanbas oil field is located 230 km from Aktau city in a northern direction; the Kalamkas oil field is located approximately 60 km away.

Water samples were taken in the coastal part of the Caspian Sea, aseptically in sterile 500 ml Duran Schott glass bottles from different sampling points dipping the bottles at a depth of 30 cm from the surface. Sampling was carried out in accordance with ISO 5667-3:

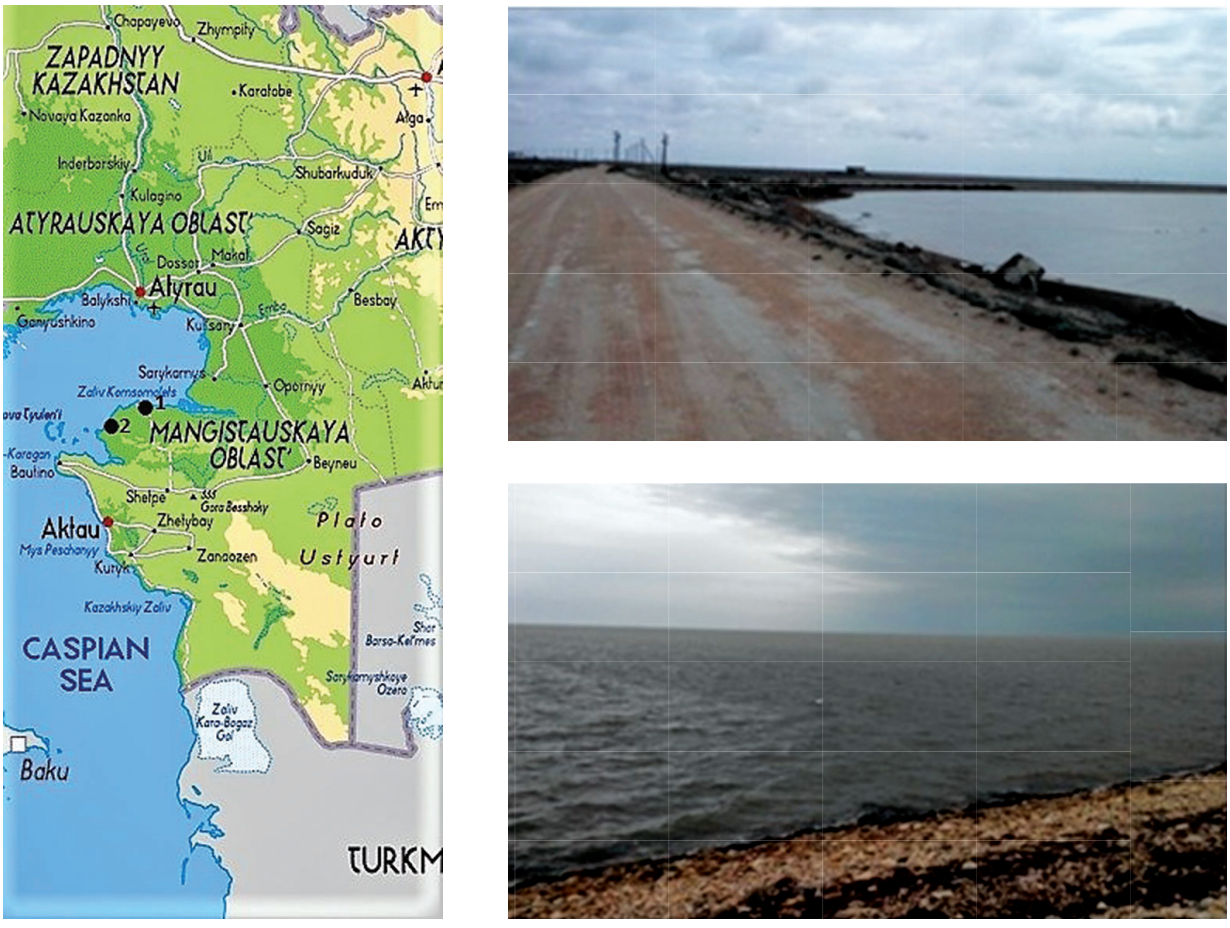

Figure 1. Sampling map at Karazhanbas and Kalamkas oil fields Kalamkas ; 2- Karazhanbas) and sample collection points

Slika 1. Karta uzorkovanja na naftnim poljima Karazhanbas i Kalamkas (1 – Kalamkas; 2 – Karazhanbas) i točke za prikupljanje uzoraka

2012: general requirements for the collection, handling, transport and storage of all water samples, including samples for biological analysis.

2.2. Media / *Mediji*

Luria-Bertani medium containing 10 g/l bacto tryptone (Difco, USA), 5 g/l yeast extract (Difco, USA), 10g/l NaCl served as maintenance and preservation medium (Sambrook et al., 1989). Evans medium (pH 7.0) containing was used as a minimal medium and to determine the ability of microorganisms to grow at different temperatures (24 ° C and 4 ° C) and in the presence of an increased salt concentration (3, 5, 7, and 10%) (Evans et al., 1970). The trace elements solution in 1% HCl contains 0.41 g/l ZnO, 5.4 g/l $FeCl_2 \cdot 6H_2O$, 2.0 g/l MnCl2·4H2O, 0.17 g/l $CuCl_2 \cdot 2H2O$, 0.48 g/l $CoCl_2 \cdot 6H2O$, 0.06 g/l H_3BO_3. Plus 2% (wt/vol) agar (Difco, USA) was added for achieving agar medium.

2.3. Isolation of hydrocarbon-degrading bacteria and studying the ability of strains to grow in the presence of oil / *Izolacija bakterija koje razgrađuju ugljikovodike i proučavanje sposobnosti sojeva da rastu u prisutnosti ulja*

Hydrocarbon-degrading bacteria were isolated via enrichment in Evans medium with crude oil as a sole source of carbon and energy. Crude oil was taken from Karazhanbas and Kalamkas oil fields. Agar (15 g/l) was added prior to autoclaving at 121 °C for 20 minutes to obtain an agar growth medium. The pH was adjusted to 7.0–7.2 with 1.0 M NaOH (Atlas & Bartha, 1992).

The strains were cultured in Erlenmeyer flasks with 100 ml of Evans minimal medium supplemented with oil from 1, 3 to 5% (v/v) of oil. Inoculation of the flasks was carried out with a suspension of microorganisms (a seed dose of $1–5 \times 10^7$ cells / ml). Cultivation was carried out for 7 and 14 days. The microorganisms' growth was assessed by the biomass gain spectrophotometrically (Cintra 6 UV-Visible, GBC Scientific Equipment, Australia). The percentage of oil utilization was measured using the gravimetric analysis.

2.4. Estimation of oil degradability rates by gravimetric analysis / *Procjena stupnja razgradljivosti ulja gravimetrijskom analizom*

Bacterial degradation was measured by the total index of hydrocarbon loss in a liquid medium determined by the gravimetric method. The residual oil was extracted from the culture medium (30 ml) using chloroform in the ratio 2:1; the extract was separated by centrifugation for 30 minutes at 4,000 rpm and dried by stirring over 3 g of anhydrous sodium sulfate. To remove chloroform, tubes with 5 ml of extract were kept at 70–75 °C during 4–5 h, then at 35–40 °C overnight before being weighed.

The percent of oil degradation by weight method was determined using the following formula:

$D = \left((Pc - Ps) \div Pc\right) \times 100\%$ (Latha & Kalaivani, 2012),

where:

D is the degree of oil degradation (%)

Pc is the hydrocarbon concentration in the control

Ps is the hydrocarbon concentration in the samples

2.5. Determination of bacterial cell surface hydrophobicity and emulsifying potential of the biosurfactant / *Određivanje hidrofobnosti površine bakterijskih stanica i potencijala emulgiranja biosurfaktanta*

The bacterial cell surface hydrophobicity was measured using the Rosenberg method (Rosenberg et al., 1980). The optical density of the hydrophilic phase at 670 nm was measured on a spectrophotometer; the ditch length was 1 cm. The hydrophobicity percentage was calculated as the ratio of the difference between the initial and final optical density values to the initial value (Rosenberg et al., 1980).

The emulsification index test was evaluated using the method suggested by Cooper et al. (1987), and Wang et al. (2014). To achieve this, 4 ml of hexadecane was added to 4 ml of cell-free broth in a test tube, which was vortexed at high speed for 2 min and allowed to stand for 24 h. The heights of emulsion, oil, and aqueous zones were measured. The percentage of the emulsification index was calculated using the following equation:

$$\mathrm{E}_{24=}(He \div Hs) \times 100\%$$

where:

E_{24} is the 24-hour emulsification index

He is the height of the emulsion layer

Hs is the total height of the solution

The analysis of the emulsification activity of cell-free supernatant was performed according to the method described by Cirigliano and Carman (1985). Microbial cells were removed by centrifugation (MiniSpin, Eppendorf, Germany) at room temperature, with cell-free aliquots (1.5 ml) transferred to test tubes and diluted with 1.5 ml of 0.05 M phosphate-buffered saline (pH 6.8).

Then, 0.75 ml of hexadecane was added; the mixture was vortexed for 2 minutes at 25°C in shaker tubes and the absorbance of 540 nm wavelength was measured at spectrophotometer. The phosphate buffer was used as a reference solution. One unit of emulsification activity (UEA) was defined as the amount of emulsifier required to produce an emulsion with absorbance equal to one at 540 nm.

The content of glycolipid surface-active compounds was assayed by the phenol–sulfuric acid colorimetric method proposed by Dubois et al. (1956). This method determines total sugars, which are part of glycolipids. Microbial cells were removed from the cultural fluid by centrifugation at 10,000 rot/min for 10 min (Rotanta 460R, Hettich-Zentrifugen, Germany) at 4°C. Cell-free supernatants (2 ml) were transferred to test tubes and diluted with 20 µl of 80% phenol water solution. 5 ml of sulfuric acid was added during intensive mixing. The mixture was heated in a water bath at 25°C for 20 min and the absorbance of 480–490 nm wavelength was measured at spectrophotometer (Cintra 6 UV-Visible, GBC Scientific Equipment, Australia). A comparison solution was prepared in a similar fashion, replacing the sample with distilled water. The content of reference sugar (rhamnose) was found from pre-built calibration dependencies for the corresponding carbohydrate. To calculate the content of the biosurfactant, the obtained sugar concentration was multiplied by a coefficient (4.9) equal to the ratio of the molecular weight of the biosurfactant to the molecular weight of the corresponding carbohydrate.

2.6. Viscosity measurement / *Mjerenje viskoznosti*

The viscosity of crude oil was measured with a rheometer (MCR 301, Anton Parr, Austria). Microbial strains were cultured in Erlenmeyer flasks with 100 ml of Evans minimal medium supplemented with 3% (v/v) oil. Inoculation of the flasks was carried out with a suspension of microorganisms with $1–5 \times 10^7$ cells / ml. Cultivation was carried out for 14 days. The viscosity of

the oil was measured every three days. From the culture liquid, cells were previously removed by centrifugation for 10 minutes at 10,000 rpm (Rotanta 460R centrifuge, Hettich-Zentrifugen, Germany) at 4 °C. A nutrient medium with oil but without microbial strains was used as the control.

2.7. Identification of isolated microorganisms / *Identifikacija izoliranih mikroorganizama*

Molecular genetic analysis for the identification of isolated strains was performed by the "GosNIIgenetics" National Bioresource Center for All-Russia Collection of Industrial Microorganisms, Federal State Unitary Enterprise, Russia. The identification of bacteria was performed by direct determination of the nucleotide sequence of the 16S rRNA gene fragment, followed by a BLAST search analysis carried out on the EzTaxon-e server (Kim et al., 2012). The chromosomal DNA of strains was isolated by the Kate Wilson method (Clayton et al., 1995). Nucleotide sequences of the selected strains were deposited in GenBank (see the corresponding accession numbers in the text below). *Bacillus cereus* SBUG 2056 and *Rhodococcus erythropolis* SBUG 2052 was originally isolated from collected oil-polluted soil samples, were used as reference strains, and their degradation pathways of oil and naphthalene were previously studied (Mikolasch et al., 2016).

2.8. Statistical processing of results / *Statistička obrada rezultata*

Microsoft Excel 2010 was used to perform statistical results processing. The results are presented as an arithmetic mean and its standard error (M ± m). The significance of differences in the results was evaluated using Student's coefficient (P≤ 0.05).

3. RESULTS / *Rezultati*

3.1. Isolation and screening of hydrocarbon-degrading bacteria and determination of their growth parameters / *Izolacija i probir bakterija koje razgrađuju ugljikovodike i određivanje njihovih parametara rasta*

To isolate hydrocarbon-degrading bacteria and to study their oil degrading activity and ability to produce surfactants, various samples of polluted waters of the coastal zones of the Caspian Sea were used.

Four hundred morphologically distinct microbial colonies were extracted from collected water samples. Microorganism strains, as petroleum hydrocarbons degraders, were grown on a solid medium with 1% petroleum. A streak inoculation of isolates on a solid mineral medium was carried out for this purpose. 225 (57%) of the isolates showed the ability to grow on a medium with oil. 24.6% of the total isolates showed good and intensive growth.

Furthermore, the growth of isolates on media with different concentrations of oil as the sole carbon source was evaluated, as well as their ability to grow on media with mineral salt in concentrations from 1% to 7%. The concentration of 5% of oil corresponds to 45 g/l of hydrocarbons and could be considered as high, which leads to the selection of bacterial strains with the highest growth potential at this concentration (Atlas & Bartha, 1992). The growth rate of microorganisms on a medium containing oil as a carbon source was evaluated on the 5th and 10th day by turbidity, discoloration of the oil, thinning of the oil film, its decay into small particles or grains, and the pigmentation of the medium on a five-point scale. The results of the growth of oil isolates are presented in Table 1.

Table 1 Number of isolates with crude oil growth ability (10th day of growth)

Tablica 1. Broj izolata sa sposobnošću rasta sirove nafte (10. dan rasta)

Assessment scale	Oil concentration, ml/l					
	10		30		50	
	Abs	%	Abs	%	Abs	%
5	62	27.6	33	14.7	15	6.7
4	48	21.3	49	21.8	27	12.1
3	42	18.7	58	25.8	49	21.7
2	39	17.3	39	17.3	58	25.8
1	34	15.1	46	20.4	75	33.3

Abs – absolute number of isolates; 5 – the disintegration of oil into small particles or microparticles; 4 – the disintegration of oil into small grains; 3 – the destruction of oil into flakes; 2 – formation of lumps; 1 – no changes.
Abs – apsolutni broj izolata; 5 – raspadanje ulja na male čestice ili mikročestice; 4 – raspadanje ulja na sitna zrna; 3 – destrukcija ulja u pahuljice; 2 – stvaranje grudica; 1 – bez promjena

Oil consumption by isolates was evaluated qualitatively; the oil color change, the degree and nature of the disintegration of the oil film, and the turbidity of the medium on the 5th and 10th day of growth were all estimated. Out of 225 tested isolates, 110 grew well on mineral media with 10 ml/l of oil and represents 48.9%, while 81 out of 225 isolates (36.1%) grew poorly on medium with this oil content, and 34 isolates (15.1%) lost their ability to grow. Within 110 isolates that grew well in the medium with 10 ml/l of oil (their growth was estimated at 5 and 4 points), in mineral medium with 30 ml/l oil only 33 isolates growth were marked by 5 points and 35 isolates by 4. At a high concentration of oil (50 ml/l), 91 tested isolates out of 225 grew, but microorganisms predominated and their growth was evaluated by 3 points or lower.

The next screening criterion was the biomass of cells, or the number of cells after 7 and 14 days of incubation in a liquid medium with oil (30 ml/l). The number of cells and the rate of growth coincided with the ability of isolates to use oil as the sole source of carbon. Out of 91 selected isolates, 19 showed relatively high growth, while the growth of other isolates was mild or a low number of cells were observed after 1 or 2 weeks of incubation (Fig. 2).

Thus, 19 isolates were selected after various screening methods in Evans medium with crude oil as the sole source of carbon and energy.

3.2. Oil degradability and production of surface-active compounds by selected strains / *Razgradivost ulja i proizvodnja površinski aktivnih spojeva s pomoću odabranih sojeva*

The oil degrading activity of isolates was estimated by growing them in a medium with 30 ml/l of oil. The study of the degree of oil degradation in a liquid mineral environment showed that some bacterial isolates decompose from 1.7 to 17.1% of oil for 10 days. The dry weight of the biomass increased during the cultivation process in the mineral medium with the addition of crude oil as the sole source of carbon and energy (Table 2). The greatest increase in biomass was observed in 9 strains. The degree of oil degradation of 6 isolates was above 47%. For comparison, the degree of oil degradation by reference strains of

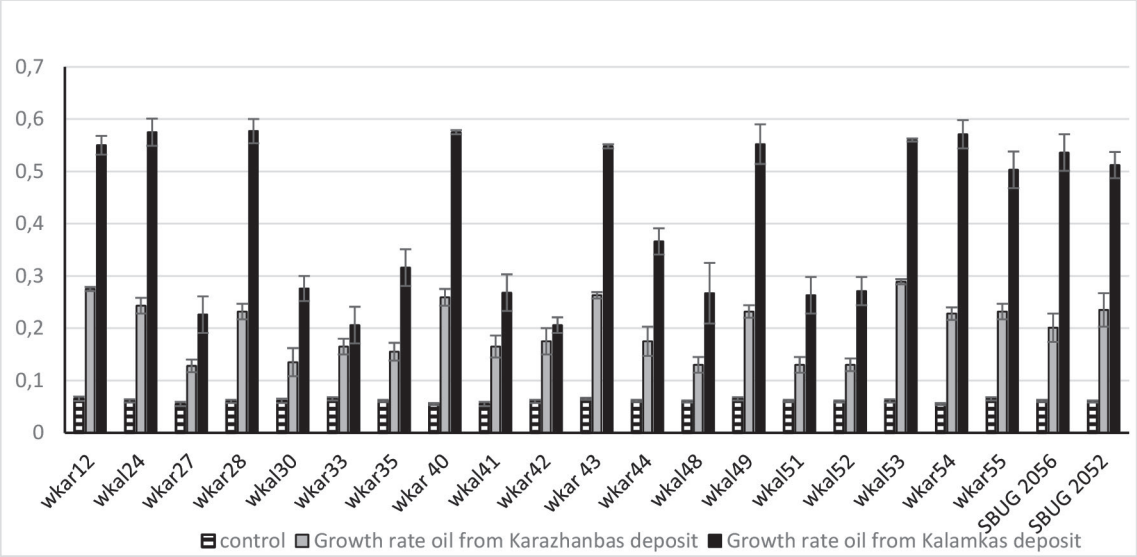

Figure 2. Microbial optical density (h-1) under the 14 days of growth in the presence of oil on a liquid medium (relatively to the control – growth on the medium without oil)

Slika 2. Mikrobna optička gustoća (h-1) tijekom 14 dana rasta u prisutnosti ulja na tekućem mediju (u odnosu na kontrolu – rast na mediju bez ulja)

Table 2 Oil degradability by selected hydrocarbon-oxidizing microorganisms

Tablica 2. Razgradivost ulja odabranim mikroorganizmima koji oksidiraju ugljikovodike

Isolates	Dry biomass, mg/l		Oil degradation, %
	0 days	10 days	
wkar12	0.0257±0.003	0.0757±0.014	3.7±0.5
wkal24	0.0369±0.004	0.1389±0.018	31.1±4.5
wkar27	0.0288±0.003	0.0567±0.014	3.6±0.6
wkar28	0.0550±0.004	0.1052±0.011	1.9±0.4
wkal30	0.0218±0.002	0.0815±0.008	2.9±0.3
wkar33	0.0193±0.001	0.0336±0.003	6.3±0.5
wkar35	0.0227±0.002	0.0656±0.007	17.1±1.8
wkal40	0.0398±0.006	0.1474±0.014	3.8±0.3
wkal41	0.0185±0.002	0.0987±0.001	2.7±0.2
wkar42	0.0268±0.003	0.0943±0.011	8.6±1.0
wkal43	0.0166±0.002	0.0856±0.009	5.0±0.6
wkar44	0.0145±0.001	0.1125±0.011	8.6±1.1
wkal48	0.0381±0.004	0.1201±0.009	**47.5±3.7**
wkal49	0.0319±0.003	0.1567±0.016	**67.7±5.8**
wkal51	0.0140±0.001	0.1100±0.010	**74.1±6.1**
wkal52	0.0298±0.003	0.1639±0.016	**48.3±3.6**
wkal53	0.0341±0.003	0.0653±0.015	1.7±0.3
wkar54	0.0296±0.003	0.1386±0.008	**57.6±5.6**
wkar55	0.0163±0.002	0.0927±0.006	**63.4±3.4**
SBUG 2056	0.0225±0.001	0.1121±0.011	56.6±3.1
SBUG 2052	0.0185±0.002	0.1187±0.001	55.7±3.2

* Cultures, the degree of destruction of petroleum products whereby higher than 47% are shown in bold.

Bacillus cereus SBUG 2056 and *Rhodococcus erythropolis* SBUG 2052 (see details in Materials and Methods section) was above 50%.

The ability to produce biological surface-active compounds was estimated for the 6 selected high oil degradable strains. Four tests were used for screening of hydrocarbon-degrading bacteria with high emulsifying activity: cell surface hydrophobicity, emulsification index, the content of biosurfactants, and emulsifying activity. Four strains have a high hydrophobicity of the cell surface (Table 3), above 50%. Also, these strains demonstrated high emulsifying activity. The maximum content of biological surface-active compounds (310 mg/l) was noted in the wkal51 strain, which was grown on hexadecane. The values of the emulsifying activity measured by the photocolorimetric method in strains grown on hexadecane were 0.2–0.9 units. opt.

Table 3 The emulsifying activity of selected hydrocarbon-oxidizing microbial strains
Tablica 3. Emulgirajuća aktivnost odabranih sojeva mikroba koji oksidiraju ugljikovodike

Strains	Hydrophobicity, %	Emulsifying index (E_{24}), %	Glycolipids, mg/l	Emulsifying activity	
				visually	optical density
wkal48	35.7±2.4	40	180±10	3	0.3±0.1
wkal49	**66.7±2.6**	**56**	**270±20**	2	**0.7±0.1**
wkal51	**86.8±6.7**	**58**	**310±20**	1	**0.9±0.1**
wkal52	20.5±0.9	39	100± 8	2	0.2±0.1
wkar54	**64.4±1.3**	**52**	**250±20**	**4**	**0.5±0.1**
wkar55	**65.3±4.4**	**52**	**190±10**	**3**	**0.6±0.1**
SBUG 2056	63.7±2.6	52	180±10	4	0.5±0.1
SBUG 2052	62.8±2.3	50	190±18	4	0.6±0.1

*Note: Emulsifying activity was evaluated visually by the height of the emulsion layer to the total height of the liquid in the test tube and was assigned points on a four-point scale depending on the height of the emulsion column (4 – high column; 3 – medium column; 2 – small column; 1 – minor column; 0 – missing column).

density. The maximal emulsifying activity was observed in the wkal51 strain.

The cultures that performed the best on all 4 tests are shown in bold.

Molecular identification of the isolates was performed by amplification and by sequencing the 16S RNA sequences. The molecular identification of selected strains showed that, for the first time, representatives of the *Roseomonas, Stenotrophomonas, Sphingobacterium, Ochrobactrum*, and *Achromobacter* genera were isolated from the coastal waters of the Caspian Sea. Furthermore, the selected microorganisms with high oil degradable activity belong to *Stenotrophomonas chelatiphaga* wkal49, *Stenotrophomonas chelatiphaga* wkal51, *Sphingobacterium kitahiroshimense* wkar54, and *Achromobacter sp.* wkar55.

The degree of crude oil viscosity was studied for 4 strains with high emulsifying activity, which revealed a decrease in oil viscosity (Fig. 3).

Thus, it was demonstrated that strains of *Stenotrophomonas chelatiphaga* wkal49, *Stenotrophomonas chelatiphaga* wkal51, *Sphingobacterium kitahiroshimense* wkar54, and *Achromobacter sp.*, wkar55 all decreased the flow of crude oil.

It was found that the viscosity decrease ranged from 25 to 31% in three days depending on the strain and reached 50% by 7 days of cultivation. This indirectly indicates the production of biologically active substances by these strains. The strain *Stenotrophomonas chelatiphaga* wkal51, which has the highest glycolipid content, provided the highest viscosity reduction.

It was also shown that these strains grew in media with both lower and higher salinity, i.e. all selected strains showed salt tolerance. The optimum salinity of the nutrient medium for their growth was 30 g/l, so very close to sea water (Tables 4 and 5). Emulsifying activity in two strains only (*Stenotrophomonas chelatiphaga* wkal49 and *Sphingobacterium kitahiroshimense* wkar54) slightly changed in the presence of sodium chloride.

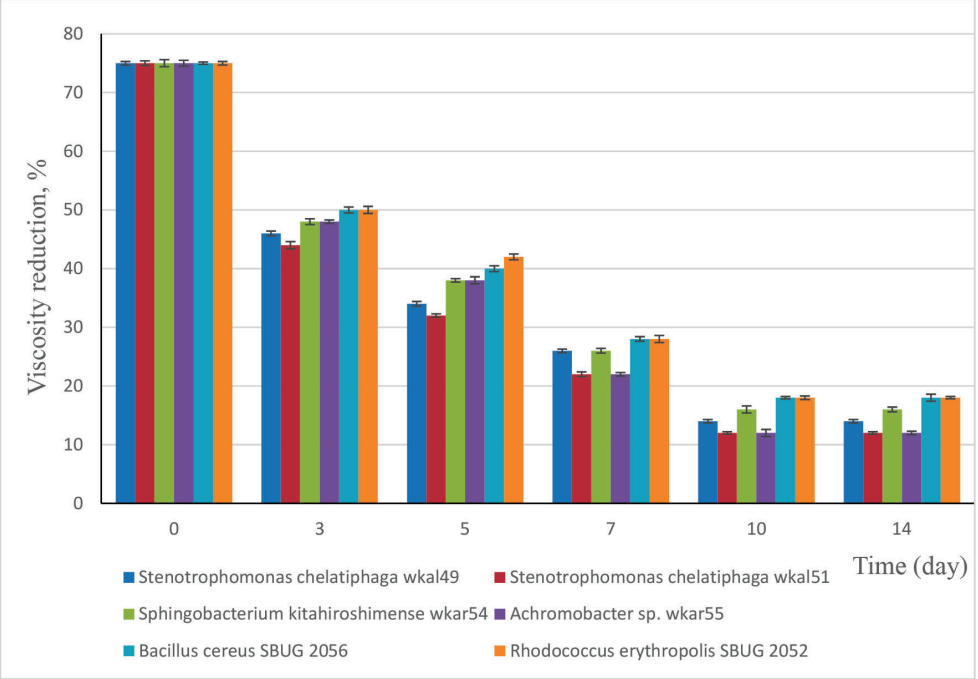

Figure 3 Reduction of crude oil viscosity by strains at 26 °C
Slika 3. Smanjenje viskoznosti sirove nafte sojevima pri 26 °C

Table 4 The ability of selected microorganisms to grow on oil (2%) in the presence of 3, 5, 7% NaCl at 24 ° C and 4 ° C
Tablica 4. Sposobnost razgradivih mikroorganizama da rastu na ulju (2%) u prisutnosti 3, 5, 7% NaCl na 24 °C i 4 °C

Strains	Growth on Evans medium in the presence of varying concentrations of NaCl					
	3 %		5 %		7%	
	24°C	4°C	24°C	4°C	24°C	4°C
Roseomonas mucosa wkal24	+	±	+	±	-	-
Ochrobactrum sp. wkal48	+	±	+	±	-	-
Stenotrophomonas chelatiphaga wkal49	+	+	+	±	±	-
Stenotrophomonas chelatiphaga wkal51	+	+	+	±	±	-
Stenotrophomonas sp. wkal52	+	±	+	±	-	-
Sphingobacterium kitahiroshimense wkar54	+	±	+	±	±	-
Achromobacter sp. wkar55	+	+	+	±	±	-

+ growth; ± weak growth; - no growth

Table 5 The emulsifying activity of selected microorganisms in the presence of NaCl
Tablica 5. Emulgirajuća aktivnost razgradivih mikroorganizama u prisutnosti NaCl

Strains	Visual assessment		Optical density	
	Without NaCl	3 % NaCl	Without NaCl	3 % NaCl
*Roseomonas sp.*wkal24	3	2	0.210	0.036
Ochrobactrum sp. wkal48	3	2	0.340	0.031
Stenotrophomonas chelatiphaga wkal49	4	3	0.640	0.340
Stenotrophomonas chelatiphaga wkal51	4	3	0.980	0.340
Stenotrophomonas sp. wkal52	3	2	0.210	0.044
Sphingobacterium kitahiroshimense wkar54	4	2	0.560	0.210
Achromobacter sp. wkar55	3	2	0.740	0.098

4. DISCUSSION / *Rasprava*

Petroleum hydrocarbons are principal pollutants as they are resistant to degradation owing to their low reactivity. At present, various methods are used to restore the environment that has been disturbed as a result of oil production or accidental oil spills, including mechanical, chemical, physicochemical, and biological (bioremediation). Compared to chemical and physical methods (oxidizing agents, adsorbents, chemical surfactants, etc.), which are expensive and require quick screening for further processing, bioremediation using oil-oxidizing bacteria strains is an environmentally-friendly, economical, and promising approach for the prevention and avoid of the negative consequences of oil and oil product spills in the conditions of a hot arid and sharply continental climate, which also increases the salinity of the soil and water (Varjani, 2017; Wu et al., 2019; Xu et al, 2018).

The complete degradation of oil is the result of the activity of various microorganisms. Most of the oil-oxidizing microorganisms isolated from hydrocarbon-contaminated areas of the marine environment belong to the genera *Pseudomonas, Bacillus, Rhodococcus, Arthrobacter, Acinetobacter, Brevibacterium, Gordonia, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia, and Gordonia* (Das & Chandran, 2011; Xu et al., 2018; Li et al., 2018; Hassanshahian & Cappello, 2013).

Various samples of contaminated waters from the Caspian Sea's coastal zones were used in order to isolate oil-oxidizing microorganisms, allowing for an examination of their degrading activity and ability to produce surfactants.

Based on the data analysis of the growth of microorganisms in a medium containing crude oil, 19 out of 440 isolates were selected for further studies, which showed the highest growth on an Evans medium with oil content as the sole source of carbon and energy.

The efficiency of oil hydrocarbon degradation by microorganisms depends on various factors, both physicochemical and biological (Bao et al., 2014). Currently, there are no universal criteria for the selection of oil-degrading microorganisms. Therefore, we selected them based on the following indicators: the hydrophobicity of the cell surface, the ability to produce biosurfactants and emulsifying activity, and the ability of degradable microorganisms to grow on oil in the presence of NaCl when screening strains are isolated from the Caspian Sea's contaminated coastal zones.

Analysis of the oil-degrading ability of 19 isolates actively growing on a medium with oil showed that 6 isolates demonstrated a high degree of oil degradation, ranging from 47.5% to 74.1%. The remaining 13 cultures showed low degrading ability, from 31% to 1.7%.

Four tests were used for the screening of 6 selected isolates with high oil-degrading activity, which were the cell surface hydrophobicity, the content of biosurfactants, the emulsification index, and the emulsifying activity.

The hydrophobicity index is one of the key characteristics of the cell surface, determining the adsorption immobilization of microorganisms. In the process of hydrocarbon oxidation, an important role is played by the direct contact of cells with the substrate. Therefore, the ability or inability of microorganisms to absorb hydrocarbons depends on the composition and structure of the cell wall which, first of all, is determined by the presence of a hydrophobic cell surface. In our experiment, 4 cultures showed a high cell surface hydrophobicity of more than 50%. The maximum hydrophobicity of the cell wall 86.8 ± 6.7% was demonstrated by the wkal51 culture. Increasing the hydrophobicity of the cell surface ensures the creation of conditions for the efficient assimilation and absorption of oil hydrocarbons.

Many authors note that microbial surfactants (biosurfactants) enhance the biodegradation of oil due to the increased bioavailability of hydrocarbons they offer (Hua & Wang, 2014; Khodabhai et al., 2017; Cai et al., 2019).

Biological surface-active compounds (biosurfactants and bioemulsifiers) play an important role in the process of microbial oxidation of water-insoluble compounds, such as alkanes, cycloalkanes, and polycyclic aromatic hydrocarbons (PAHs). Biosurfactants increase the bioavailability of substrates, which makes the studying of the structure, properties, and patterns of formation of these compounds very important. By reducing the surface tension between hydrocarbons and the water surface, biosurfactants make it easier for the microbial cell to contact and dispose of the hydrocarbon. The diversity of biosurfactants makes them an attractive group of compounds suitable for potential use in a wide variety of industrial and biotechnological applications (Hua & Wang, 2014; Duran & Cravo-Laureau, 2016; Santos et al., 2016; Lima et al., 2020).

Therefore, one of the main criteria for choosing the most promising oil-oxidizing strains is the possibility of producing biological surface-active compounds. The Kalamkas field oil was used for studying the degrading activity and the ability to produce emulsifying substances. This oil is highly viscous because of its higher content of sulfur, resins, asphaltenes, and other oil components, making it difficult to break down.

Six selected strains of microorganisms were tested for their ability to produce bioemulsifiers by culturing bacteria in a medium with hexadecane. These results proved the presence of a biosurfactant in cell-free culture supernatant. The wkal51 culture showed a maximum of biosurfactant content (310 mg/l).

Also worth mentioning is the fact that all studied strains had emulsifying activity. The cell suspensions of all strains were able to stabilize emulsions, while the results of measuring the emulsification index showed good potential of cultures for the formation of an oil emulsion. In cultures wkal49, wkal51, wkar54, and wkar55, this indicator exceeded 50%.

Molecular identification of the most active strains – oil degraders isolated from the coastal waters of the Caspian Sea – showed that these strains are representatives of the genera *Roseomonas, Stenotrophomonas, Sphingobacterium, Ochrobactrum,* and *Achromobacter*. The four cultures that showed the best indicators for the studied properties (Table 4) (hydrophobicity of the cell wall, emulsifying activity, biosurfactant content, and emulsification index) belong to *Stenotrophomonas chelatiphaga* wkal49, *Stenotrophomonas chelatiphaga* wkal51, *Sphingobacterium kitahiroshersemerse* wkar54, and *Achromobacter sp*. wkar55.

16S rRNA sequences were deposited in GenBank: *Stenotrophomonas chelatiphaga* wkal49, accession no. MH356589; *Stenotrophomonas chelatiphaga* wkal51, accession no. MH356586; *Stenotrophomonas kitahiroshimense* wkar54, accession no. MH356587; and *Achromobacter sp.* wkar55, accession no. MH356582.

In addition, based on the viscosity analysis, it was demonstrated that strains of *Stenotrophomonas chelatiphaga* wkal49, *Stenotrophomonas chelatiphaga* wkal51, *Sphingobacterium kitahiroshimense* wkar54, and *Achromobacter sp*., wkar55 all decreased the flow of crude oil. It is highly probable that the biologically active compounds produced by microorganisms increase the surface area of oil, meaning it becomes more accessible to strains. It is known that various microbial biosurfactants can act as emulsifiers by reducing the surface tension and forming micelles or micro-droplets encapsulated in a hydrophobic cell surface, with the cells absorbing and degrading them (Cai et al., 2019; Sakthipriya et al., 2015; Karlapudi et al., 2018).

One factor that can limit the biodegradation of oil hydrocarbons in contaminated areas is that of high salt content. So, in recent years, the resistance of microorganisms to sodium chloride has attracted the attention of researchers due to the possibility of using them to restore oil-polluted coastal areas. An alternative and eco-friendly method of the remediation technology of environments contaminated with these pollutants is the use of surface-active compounds and the microorganisms associated with them (Duran & Cravo-Laureau, 2016; Pannekens et al., 2019).

It was shown that 6 active strains grew in media with both lower and higher salinity, i.e. all selected strains showed salt tolerance.

So, all the oil-oxidizing microorganisms that we studied – related to the genera *Roseomonas*, *Stenotrophomonas*, *Sphingobacterium*, *Ochrobactrum*, and *Achromobacter* – are able to synthesize biological surface-active compounds. It was noted that all strains with high emulsifying activity and emulsification index are gram-negative bacteria, therefore, it was hypothesized that they produce only extracellular biosurfactants. This is characteristic of representatives of the genus *Pseudomonas* (Gudina et al., 2013). In addition, it was previously shown that microorganisms of the genus *Ochrobactrum* were isolated from environmental objects – soil, plant roots, and clinical material – but they were not previously found in marine or aquatic ecosystems. Nevertheless, there are several reports of strains of *Ochrobactrum anthropi* species that are producers of biosurfactants and can be used for bioremediation from polycyclic aromatic hydrocarbons and other hydrocarbons (Ramasamy et al., 2014). The genus *Stenotrophomonas* includes eight species, which have been isolated from various environmental objects.

The species *Stenotrophomonas maltophilia* (formerly *Pseudomonas maltophilia*) has been well studied, as strains of this species are destructors of various natural and technogenic pollutants (nonylphenol) (Michalska et al., 2020). A new strain of *Stenotrophomonas chelatiphaga* is known, which is an EDTA (ethylenediaminetetraacetic acid) destructor that has been isolated from wastewater (Kaparullina et al., 2009).

5. CONCLUSION / *Zaključak*

As a result of the screening of oil hydrocarbon degrader strains, based on an analysis of their properties, the six most effective oil degrader strains capable of degrading high oil concentrations (up to 30%) in the presence of salt (up to 5% NaCl), as well as bioemulsifiers, were selected. First, we isolated previously undetected oil-degrading microorganisms in the coastal waters of the Caspian Sea – strains of *Roseomonas sp.* wkal24, *Ochrobactrum sp.* wkal48, *Stenotrophomonas chelatiphaga* wkal49, *Stenotrophomonas chelatiphaga* wkal51, *Sphingobacterium kitahiroshimense* wkar54, and *Achromobacter sp*. wkar55.

When growing on a medium with oil, the highest values of emulsifying activity were noted for the *Stenotrophomonas*

chelatiphaga wkal51 strain and for *Achromobacter sp.* wkar55. By analyzing these characteristics, we can conclude that strains of the genus *Stenotrophomonas* are more efficient producers of biosurfactants when grown on a hydrophobic source of carbon and energy. All strains with high emulsifying activity and emulsification index values are gram-negative bacteria, therefore, it was assumed that they produce only extracellular biosurfactants – as is typical for representatives of the genus *Pseudomonas*. The obtained results show that oil viscosity decreased by more than 25% in all cases after treatment with microorganisms, which indirectly indicates the production of biologically active substances by these strains.

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