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Research Article

Optimization of Crude Oil Biodegradation by *Brevibacterium* sp. Isolated from the Native Sponges of the Persian Gulf

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Background: The native sponges of Persian Gulf are unique species facing difficult climate conditions and environmental contamination. It is necessary to investigate these native sponges because global warming most probably destroyed many of these creatures. Therefore, the study of the microorganisms associated with sponges will introduce new bacterial strains with various industrial and environmental applications and, in this way, a part of the Persian Gulf biodiversity will be preserved for posterity.

Objective: The aim of this study was the isolation and molecular identification of bacteria associated with the ability of biodegrading crude oil from the native sponges of the Persian Gulf. Also, optimization of crude oil biodegradation was done for one of the most efficient bacterial strains.

Materials and Methods: Isolated species were compared in terms of E24 index and growth rate in a culture medium containing at least 2% of oil as the sole carbon source. Molecular identification was done for five bacterial strains. Using the Taguchi experimental design, the effects of 4 factors, namely, carbon source auxiliary, organic and inorganic nitrogen sources, salinity and pH, were evaluated at 3 levels. GC-Mass analysis was performed on the remaining oil in the culture medium..

Results: In the initial screening of two native species of sponges, 22 bacterial strains were isolated which were capable of decomposing oil. Five bacterial strains showed the best results and were recorded in NCBI with access numbers KY283126, KY283128, KY285290, KY285289, and KY285288. *Brevibacterium* sp. (KY283128) showed the highest level of oil degradation (about 97%) and growth rate. The results showed that the optimal oil degradation occurs in the absence of carbon source auxiliary, at 0.5% of salinity, with NH_4Cl as the nitrogen source and at a pH of 6.5.

Conclusions: This bacterial strain can be used for biodegradation in oil-contaminated areas and oil refineries. By isolating the oil degrading gene in this bacterial strain and cloning it in other bacterial strains, the efficiency of eliminating oil contamination can be increased.

Keywords: Associated bacteria, Dictyonella Sp, Gas Chromatography, Taguchi method

1. Background

Oceans, seas and coastal areas have been contaminated in different ways, but contamination through oil pollutants has become a common phenomenon. These pollutants are created caused by human activities, and are regarded as the biggest threat for the survival of marine ecosystems (1-3).When oil spills into the sea, it is its compounds change over time. These changes include evaporation of low molecular weight compounds, dissolving of water-soluble compounds, mixing of oil-droplets with water, optical oxidation, and biodegradation. Oil compounds evaporate at a boiling point below 25 °C. Thus, N-alkanes with a chain shorter than C14 and aromatic hydrocarbons decrease by evaporation or dissolution in water. The dispersion of oil droplets in water columns is caused by waves, and, when the oil contains polar compounds, oil emulsion is created in water and is called chocolate mousse. After evaporation of light portions, the remaining heavy oil accumulates and forms a tar ball

that can be microscopic or several centimeters in size. There are various ways for removing oil contamination from the sea. Biological clean-up or bioremediation is one of the least risky methods. Bioremediation is an interdisciplinary technology, and its success depends on the special expertise in microbiology, ecology, or biochemistry and on the right information about such matters as the native microorganisms, type and concentration of the oil, ecological conditions of the coastal areas, content of nutrients, pH and climate conditions(4-6). Iran, as a major oil producer, has always faced the problem of oil pollution of its aquatic ecosystems, which has increased in recent years. Also, given that about 60% of the world's oil transit is currently taking place through the Persian Gulf, oil spills are very common and will take years to eliminate. Therefore, attention to the biological methods of treating oil pollution is a priority of environmentalist. The Persian Gulf is a semi-enclosed sea whose average salinity and temperature are higher than the global average. It is also noteworthy that, in the last 30 years, the Persian Gulf has experienced three wars during which the largest amount of oil contamination in history was caused when Kuwaiti oil wells caught fire in 1991. Since hydrocarbons are a source of carbon and energy, hydrocarbon degrading microorganisms are always distributed in marine environments after oil spills, and each one can break down certain compounds and naturally clean the oceans. The hydrocarbon decomposing bacteria account for 90% of microbial population in oil spills. Marine microorganisms are very diverse and have developed unique protective mechanisms in the process of their evolution (7-9).

2. Objectives

When it comes to biological treatments for removing oil contamination, the use of native microorganisms is considered to be a priority. Oil degrading bacteria isolated from the Persian Gulf sponges are mainly comprised of the free bacteria, and no report has been presented about the sponges associated with oil-degrading bacteria from the Persian Gulf so far. The goal of this study was to optimize crude oil biodegradation by bacteria isolated from the native sponges of the Persian Gulf.

3. Materials and Methods

3.1. Sampling and Screening

Healthy living samples of two sponges *(Pachychalina* sp. *and Dichtyonella* sp.*)* were collected by divers from the north coast of the Persian Gulf (Bushehr: Iran) and were transported on ice to the biotechnology laboratory of Persian Gulf Research Institute (Bushehr, Iran). The samples were prepared from the sponges under sterile conditions, based on the method used in the study by Segal *et al*. 2 cm^2 of sponges' mesophyll tissue was isolated using phosphate buffered saline (pH=7 and 0.1 M) and was homogenized in a porcelain mortar. Then, its various dilutions were cultivated in the modified marine agar (HiMedia) (pH=7.6) and nutrient agar (Merck) (pH=7.5) and were incubated at 30 °C for 10 days (Memert incubator model IPP-400). After purifying the colonies, bacteria culture was done in a Merck *minimal salt medium (MSM)* () containing at least 2% of oil (obtained from the Gachsaran oil field with API 28.83) as the sole carbon source and at pH=7.2. The growth rate of the bacteria was determined by measuring the OD at wavelength 620 nm using a spectrophotometer (Perking Elmer Lambda 25)(10-12).

3.2. Emulsion Activity

Bacteria which were able to grow in the MSM were cultured for one week in order to calculate the E24 index. The culture medium was then centrifuged (Eppendorf 5810R), and the supernatant was collected. In the next stage, 6 mL of oil was poured in 50 mL of falcon, and it was stored in the laboratory for 2h in order to reach the ambient temperature. Then, 6 mL of the supernatant was poured on oil, and again the emulsion height was measured. The emulsion was mixed by vortex at a high speed for 2 min and fixed for 24 h in a place. At the end, the properties of the emulsion activity in terms of the bacterial strains were measured by dividing the height of the emulsion layer by the total height in percent according to the following formula (13, 14).

$$E24=\frac{H_{emulsion}}{H_{total}}\times 100$$

3.3. Oil Removal

In order to obtain the degree of oil degradation, the remaining crude oil was extracted using dichloromethane, and absorption was measured at 420 nm by a spectrophotometer, a week after cultivating the bacteria in the MSM. After drying the oil dissolved in dichloromethane, the dry weight was also measured (the crude oil was dried at the laboratory temperature) (15, 16).

3.4. Molecular Identification

The gene of 16SrRNA was extracted using the CTAB method and was amplified by using universal primers with sequences F: AGA GTT TGA TCM TGG CTC AG

and R: TAC GGY TAC CTT GTT ACG ACT T in PCR (Eppendorf l22331). The polymerase chain reaction was done in a volume of 50 µLcontaining 5 µL of buffer (10X), 2 µL of each primer, 2 µL of dNTP, 3 µL of magnesium chloride, 0.6 µL of Taq polymerase enzyme, 4 µL of DMSO, 2 µL of template DNA and 29.4 µL of distilled water. The PCR thermal program consisted of the first stage of 300 S of initial denaturation at 94 °C, which was performed once, the second stage involving annealing, attachment and extension for 30, 60 and 90 S, and at 94, 54, and 72 °C, respectively, which was repeated for 30 times, and the third stage extension of 300 S at a temperature of 72 °C, which was carried out once. Also, 1Kbp was used as the molecular weight marker. It should be noted that the review of the results of DNA sequencing was performed using the software applications Chromas and MAFFT. The similarity of species obtained in this study with other species was checked using the BLAST program at www.ncbi.nlm.nih.gov. MEGA6 was used to calculate genetic distances(17, 18).

3.5. Gas Chromatography by Mass Spectrum

In order to obtain an accurate analysis of the crude oil degradation by the KY283128 bacterial strain after 7 days of culture, the medium containing the bacteria and the one containing no bacteria were analyzed by gas chromatography. The method of sample preparation for GC-MS analysis was as follows. 50 mL of dichloromethane was added to the culture medium. Then the organic phase containing the extracted oil with the separator funnel was poured into the Erlenmeyer flask. Then, 3g of sodium sulfate anhydrous was added to it to absorb the remaining water, and it was stored at the laboratory temperature for a night. In the next stage, the Erlenmeyer flask contents were passed through Whatman paper No. 2 and were placed in a rotary evaporator to evaporate dichloromethane. Finally, the dried samples were analyzed by GC-MS. For performing GC, 10 mL of dichloromethane was added to the dried samples. After the oil dissolved, 1 mL was taken from the samples and was added to 100 µL of the standards. Then, dichloromethane was added, and the volume reached 2. Samples were passed through a 0.45µ filter and were injected into the GC machine (Agilent 6890). The temperature program was as follows: 3 min at 70 °C, 70-250 °C at 10 °C/min, and finally, 40 min at 250 °C (19, 20).

3.6. Optimization Experiment using Taguchi Method

In order to obtain the best conditions, oil analysis was done for bacterial strain KY283128. The Taguchi experimental design was applied using Qualitek4 software with 4 factors and 3 levels. The conditions used in the Taguchi experimental design included salinity at three levels: 0.5, 1.25, and 2%; pH at three levels: 6.5, 7.5 and 8; auxiliary carbon source at three levels: glycerol, glucose, and no auxiliary source; and the source of nitrogen at three levels: peptone, $(NH_4)_2SO_4$, and NH_4Cl. Two culture media were used as control. One medium contained bacteria without crude oil (positive control), and the other medium contained crude oil without bacteria (negative control).

4. Results

4.1. Screening

The growth chart for the isolated bacterial strains cultivated in the minimal salt medium containing 2% of petroleum is presented in **Figure 1**, and the graph related to the E24 index is presented in **Figure 2**.

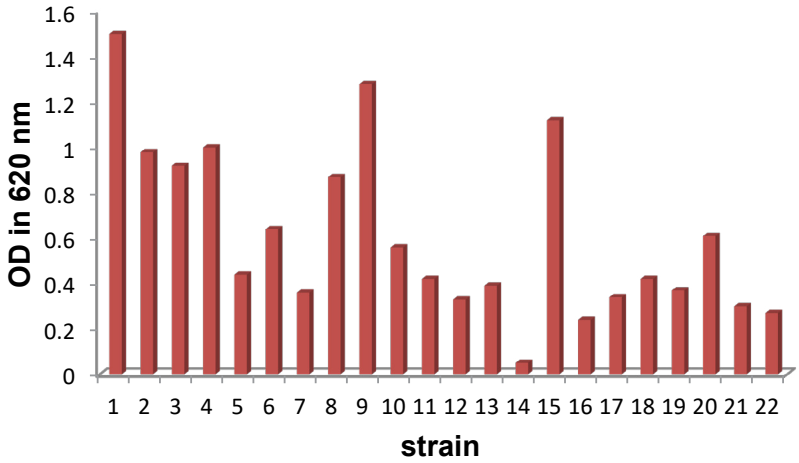

Figure 1. Growth chart of isolated bacterial strains in culture medium with 2% oil

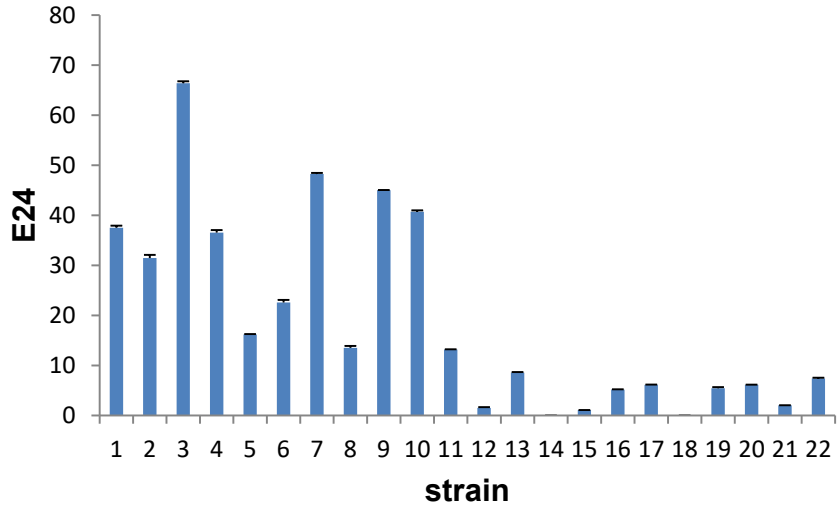

Figure 2. Graph of E24 index of isolated bacterial strains

4.2. Emulsion Activity

In this study, the height of the emulsion layer was measured and expressed in percentage. This index was obtained after performing the triplicate tests with a mean and standard deviation of 66.38±0.4.

4.3. Growth Rate of the Bacteria and Percentage of the Crude Oil Degradation

Bacterial strain No. 3 with access number KY283128 showed the best results for oil degradation and E24 index. After 48 h, bacterial strain No. 3 with access number KY283128 showed the highest growth rate, but its growth rate gradually decreased and, on day 5, entered a rest state. The oil degradation was evaluated by reading the concentration of residual oil at a wavelength of 420 nm and measurement of the dry weight. The KY283128 bacterial strain had a low weight and absorption rate. The amount of the sample absorption was subtracted from, and divided by, the control absorption. Then, the amount of oil degradation was determined and expressed in percentage. For measuring the dry weight of the sample, the amount of the dried oil was measured and subtracted from the weight of the dried control oil, and net amount of the extracted oil was determined. The results are shown in the **Table 1.**

4.4. Molecular Identification

Of the 22 bacterial strains obtained from the native sponges, 5 strains with higher growth rates and better oil degradation capability were selected to perform molecular identification using 16srRNA gene amplification. Then, the product of 1400 bp produced by the PCR was sequenced after purification. After blasting and obtaining a genetic distance between the most similar bacterial strains, the bacterial strains closest to the studied bacteria were identified. The sequences of the identified bacterial strains were recorded in NCBI. Two bacterial strains were obtained from sponge *Dictyonella* sp. with access numbers KY283126 (bacterial strain No. 1) and KY283128 (bacterial strain No. 3), and 3 bacterial strains were obtained from sponge *Pachychalina* sp. with access numbers KY285288 (bacterial strain No. 7), KY285289 (bacterial strain No. 9) and KY285290 (bacterial strain No. 10), which are available in NCBI.

4.5. GC-MS

Seven days after the cultivation of the bacteria in the MSM containing at least 2% of crude oil, the residual oil was extracted by dichloromethane and was sent to the laboratory for GC-MS analysis. The light crude oil evaluated in this study contained 23 compositions

Table 1. Bacterial growth rate and crude oil degradation percentage

	Control	KY283128 bacterial strain
Growth rate at 620 nm after 48 hours	0	0.97±0.05
Residual oil absorption at a wavelength of 420 nm	1.89±0.02	0.61±0.02
Oil degradation percentage based on absorption at 420 nm	0	67.90±0.8
Dry weight (g)	2.54	0.53
Oil degradation percentage based on dry weight	0	79.1

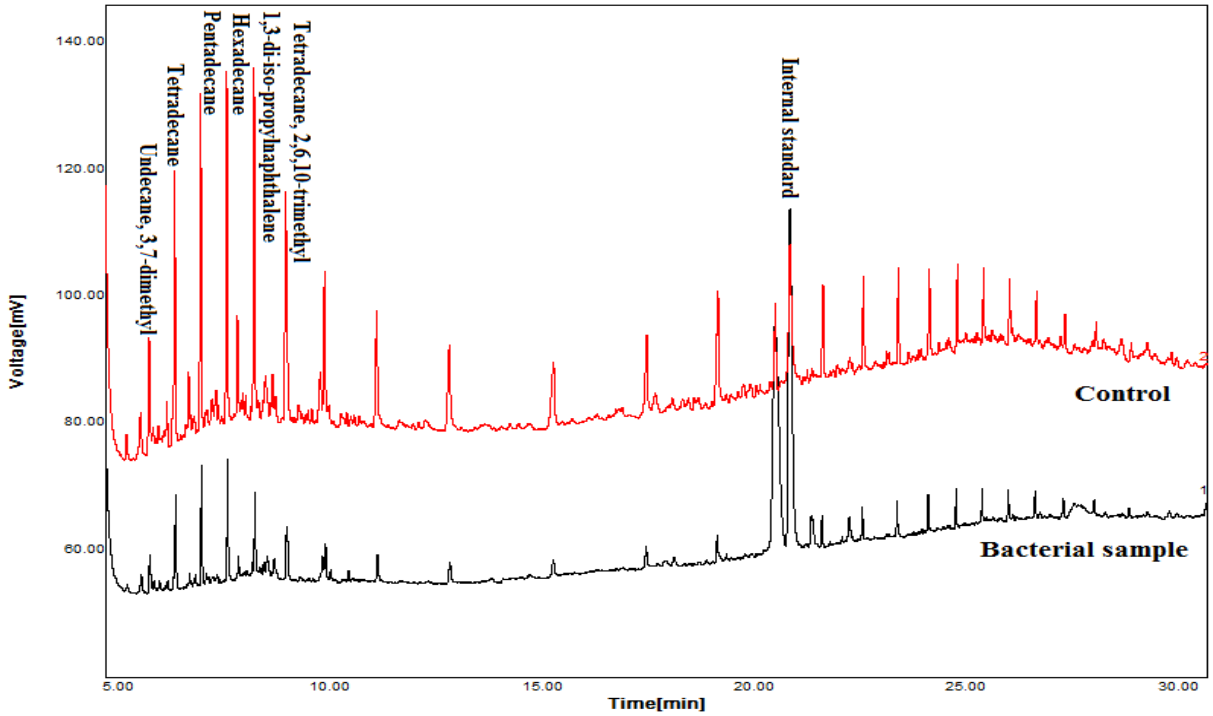

Figure 3. GC-MS diagram for the control sample and the sample containing bacteria based on internal standards

Table 2. ANOVA and the impact of each factor used in Taguchi Method

Factor	Impact Factor %	Pure Sum of Squares (SS′)	Variance (V)	Sum of Squares (SS)	Degree of Freedom (DoF)
Auxiliary carbon	13.746	106.88	53.44	106.88	2
Ph	5.944	46.22	23.11	46.22	2
Salinity	35.695	277.55	138.77	277.55	2
Nirtogen	44.612	346.88	173.44	346.88	2
Total	100	777.556	388.76	777.556	8

which were mostly alkanes. The area under the curve represented a decrease in the peaks of samples containing bacteria, but no reduction in the peaks of the control sample. The chromatography diagram is shown in **Figure 3.**

4.6. Optimization Experiment using Taguchi Method

Crude oil degradation was performed for bacterial strain No. 3 using the Taguchi experimental design with 4 factors and 3 levels with respect to the optimization. After 2 weeks of incubation, the amount of oil degradation based on oil absorption at wavelength 420 nm and dried weight was investigated for 9 culture mediums. The Taguchi experiments were repeated 4 times, and 3 tests were identical. At the end of the incubation period, the percentage of oil degradation was calculated. The data collected were into Qualitek-4 software, and each factor was investigated by statistical analysis. The results obtained by the analysis of variance for oil absorption and dried weight are shown in **Table 2**.

The results showed that the most influential factors in the oil degradation were the nitrogen sources were (44.61%) and salinity (35.62%), while pH had the lowest effect (5.94%). The results showed that carbon and nitrogen had the most interaction (42.18%), the salinity and nitrogen sources had a 3.12% interaction, while nitrogen and pH had no interaction. The optimized interaction of carbon and nitrogen was achieved when the carbon level 2 (glucose) and nitrogen source level 3 (NH_4Cl) were used while the optimized interaction of the nitrogen source and pH occurred when pH Level 2 (7.5) and nitrogen source level 1 (peptone) were used. The results obtained regarding the interaction of the factorial design are shown in **Table 3**.

By investigating the effects of all factors in the analysis of the dry weight and the absorption of crude oil obtained at wavelength 420 nm, an optimum condition

Table 3. Estimated interactions between factors

#	Interactions between Factors	Columns	Col	SI%	Opt
1	Carbone× nitrogen	1×4	5	42.18	(3,2)
2	Salinity ×pH	2×3	1	39.06	(1,3)
3	pH ×carbone	1×2	3	28.12	(3,1)
4	Salinity× carbone	1×3	2	28.12	(3,3)
5	Nitrogen ×salinity	3×4	7	3.12	(3,2)
6	Nitrogen× pH	2×4	6	0	(1,2)

SI: Interaction Severity Index of factors
Columns: The effect of 4 factors used
Col: Levels that can be considered, e.g., the interaction between carbon and nitrogen can be considered at 5 levels
Optimum (Opt): Condition in which the examined factors had the best interaction

was suggested by Qualitek-4 software in which the highest amount of oil degradation would occur. The optimum condition was achieved when the carbon source was not present in the environment, salinity was set to 0.5%, the pH value was 6.5 and NH_4Cl was used as the nitrogen source.

5. Discussion

The samples prepared from sponges were used to be identified in terms of morphological characteristics and belonged to the genus *dictyonella*, family *dictyonellidae*, order *halichondrida*, class *demospongiae,* and phylum *porifera*. (21).

The performance of the biosurfactant produced by the bacteria was evaluated for measuring the emulsion activity index (13). This index increased with increases in the bacterial growth rate and the production of biosurfactant, which represented the degrading capability of the bacteria. Ahmed *et al.* investigated this index for the bacteria *Enterobacter cloacae*E1, a petroleum-degrading bacterium, and showed that the emulsion activity index for this bacterium was 62% (22). Sepahi *et al.* studied this index for 15 species of oil degrading *bacillus*. Two strains, *Bacillus*S35 and *Bacillus*S6, showed the highest level of emulsification activity index, namely, 85% and 71%, respectively (23). Onuoha *et al.* studied the emulsion activity index for three strains of bacteria belonging to the genera *Bacillus* sp.*, Pseudomonas* sp., and *Corynebacterium* sp. The results showed that the emulsification activity was equal to 77.40% for *Corynebacterium* sp. in 1% of oil, 96.30% for *Bacillus* in 0.25% of oil, 40% for *Pseudomonas in* 0.25% of oil (24). In the present study, the emulsification activity index was 66.38±0/4%.

In this study, the rate of crude oil degradation was obtained by spectrophotometry, measurement of dry weight, and GC-MS analysis. Latha *et al*. extracted the residual oil using N-hexane, dehydrated it using anhydrous sodium sulfate, and dried it using a low-pressure evaporator. The results showed that the rates of oil degradation were higher in samples with lower dry weights (25).

Santisi *et al.* and Hassanshahian *et al.* measured the rate of crude oil degradation using spectrophotometry. In these studies, the residual oil was extracted by toluene and dichloromethane in a culture medium, and its absorption was measured at 420 nm. The samples which showed high degradation had a lower absorption (1, 26). In this study, the residual oil was extracted by dichloromethane, and absorption was measured at wavelength 420 nm. After using spectrophotometry, the oil extracted at the laboratory temperature was naturally dried, and its weight was measured. The results showed that this bacterial strain had a dry weight of 0.53 g while a dry weight of 2.54 g was reported for the control sample.

The results showed that this bacterial strain had the highest growth rate and the crude oil degradation value. The residual oil in the culture medium involving this strain was analyzed by GC-MS. In this study, 23 compounds of crude oil were identified which were mostly alkanes, and the rates of oil degradation in samples containing bacteria were obtained by calculating the area under the curve for control samples and the samples containing bacteria and by considering an internal standard. Of 23 oil compounds available in the bacteria culture medium, Dodecane,2,6,11-trimethyl, Undecane,4,6-dimethyl, Silane, cyclohexyl dimethoxy methyl, Decane,2,3,5,8-tetramethyl, Dodecane,2,6,10-trimethyl, 2,4-Di-tert-butylphenol, Tetradecane, 5-Methyl and Pentadecane, 7-Methyl were completely degraded, and their amounts in the remaining crude oil were reduced to zero. The amounts of other compounds also significantly reduced. By subtracting the area under the curve of the bacterial culture sample from that of the control sample, the purity of the degradation was reported to be 97.28%. The area under the curve for the control sample and bacteria culture sample were equal to 22.12 and 0.6, respectively.

Ismail *et al.* extracted Arab light crude oil with saturated compounds of N-hexane and investigated it by GC-MS analysis. After comparing the area under the curves, their results showed that the area under the curve reduced for samples containing bacteria (27). Moghaddam *et al.* investigated the rate of Phenanthrene and Florent biodegradation by the bacteria of the different genera using GC-MS. The results showed that in the mixture culture of the different bacteria, 64% of Florent and 58.4% of phenanthrene were degraded in 7 days (28). Linda *et al.* also studied the biodegradation of petroleum products by bacteria using GC-MS. The results showed that the rates of degradation after 21 days were equal to 93.50% and 88.5% for *Pseudomonas aeruginosa* and *Corynebacterium aquaticum*, respectively. (29). Palanisamy *et al*. evaluated the rate of biodegradation of diesel oil by the bacterial strains of *Acinetobacterbaum annii* using GC-MS. Their results showed a reduction in the peak of the curve after 5 days, and this strain was able to degrade the diesel oil by 99%, compared to the control samples (30).

Substances such as phosphorus and nitrogen have a large effect on oil degradation. In this study, three sources of minerals and organic nitrogen were, namely, $(NH_4)_2SO_4$, NH_4Cl and an organic source (peptone). The results of data analysis showed that the best source of nitrogen for oil degradation on level 3 was NH_4Cl, and the percentage of its effect at this level was equal to 63%. Adding an auxiliary carbon source to oil, which is easily consumed by the bacteria, could lead to higher oil degradation. In order to investigate the effect of carbon on oil degradation, 3 levels were used in this experiment, 2 levels with auxiliary carbon sources containing glucose and glycerol in the growth medium and 1 level without an auxiliary carbon source. Unlike most studies, the isolated bacteria in this study provided a higher rate of oil degradation in the absence of auxiliary carbon sources compared to the presence of auxiliary carbon sources such as glucose and glycerol (31).

Enzymatic activities of microbes are influenced by the level of pH. The present study investigated the pH levels of 6.5, 7.5 and 8. The results of analyzing interaction between the factors showed that a pH of 6.5 is the most suitable level for oil degradation (32).

In order to investigate the effect of salt on oil degradation, three concentrations of salt were used: 0.5%, 1.25% and 2%. The results showed that level 2 of salinity (1.25%) had the greatest effect, 63.66%. The results of analyzing interaction between the factors showed that the bacteria were able to degrade the oil mostly at a 0.5% salt concentration (33).

Roozbehani *et al*. evaluated crude oil biodegradation by *Pseudomonas* sp. The optimal condition included 2.5% of salinity, 0.1% of biosurfactant concentration, and a pH of 8.5 (34). Khorasani *et al.* designed and optimized mazut biodegradation by *Enterochacteria cloacae* using the Taguchi method. The results showed that the highest cell biomass was observed at the pH of 8.3, biosurfactant concentration of 4 $g.L^{-1}$, glucose of 4 $g.L^{-1}$, and phosphorus of 9 $g.L^{-1}$ in the span of 10 days (35). Khalifeh *et al*, using the Taguchi experimental design, showed that the pH and the concentration of biosurfactant produced by indigenous bacterial strains in the contaminated areas were the effective factors in biodegradation (36). Castorina *et al.* evaluated biodegradation of oil sludge using the Taguchi method and showed that the optimal condition included a humidity of up to 70%, absence of oxidizing agents, 0.5% of surfactant concentration, and the presence of NH_4Cl as the carbon source (37)

In this study, the nitrogen source, carbon source, and salinity had the greatest effect while pH had the lowest effect. In the culture medium used in the taguchi experimental design, there was no environment in which the proposed conditions were provided, and the culture medium environment was characterized by a salinity of 0.5%, no auxiliary carbon source, a pH of 6.5 and peptone as the nitrogen source. The highest oil degradation would occur in the proposed conditions for the culture medium.

6. Conclusion

In this study, 22 bacteria coexisting with sponges with the capability of degradation of crude oil compounds were isolated from *Dictyonella* sp. and *Pachychalina* sp. Bacterial strain No. 3 showed the highest level of emulsion activity index, which was 66.38%. GC-MS analysis was carried out on this bacterial strain. Most of the compounds in the crude oil were alkanes, and their rate of degradation was equal to 97.28%. The experimental design was performed for bacterial strain No. 3 using the Taguchi method. The results of the experiments showed that the highest rate of degradation occurred in the absence of an auxiliary carbon source and in the presence of a mineral nitrogen source (NH_4Cl), with a salinity of 0.5% and at pH of 6.5. The results of this study showed that the isolated bacterial strain has the ability to degrade light crude oil, and because they are native to the Persian Gulf, they can be used in the process of biodegradation.

Declaration of conflict of interest

The authors declare no conflict of interest.

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