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ORIGINAL RESEARCH

Screening of crude-oil degrading bacteria from gastrointestinal of gobiiformes collected at the Persian gulf: biotechnological importance for remediation of the polluted marine environment

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Abstract Crude oil pollution can decrease the biodiversity in the marine environment. The Persian Gulf in Iran is an ecosystem rich in marine organisms that can effectively degrade crude oil. This study aimed to screen bacterial strains to degrade crude oil from the intestinal microbial flora of Mudskippers fish from the Persian Gulf. The identification of collected fish samples distinguished four diverse genera. *Periophthalmus waltoni* was the most common. Next, biochemical and molecular identification of isolated bacteria was performed. Some biochemical tests, such as catalase, oxidase, and motility, were done. Molecular identification was performed by polymerase chain reaction using general primers. Finally, the ability of bacterial isolates to degrade crude oil was investigated using spectrophotometric, gravimetric, gas chromatography, and FTIR methods. Bacteria identified in this study include *Marinobacter hydrocarbonoclasticus*, *Pseudomonas aestusnigri*, *Thalassospira permensis*, *Microbacterium esteraromaticu*, *Oceanimonas* sp, *Halomonas Salaria*, *Halomonas beimenensis*, *Cobetia marina*, *Tenacibaculum discolor* and *Shewanella chilikensis*. *Halomonas salaria* had the highest growth rate (OD= 1.6) and crude oil degradation (90%) among the studied strains. The results of our studies on five strains of *T. permensis*, *S. chilikensis*, *M. hydrocarbonoclasticus,* and *Oceanimonas* in concentrations of 1, 2.5, 4, 5.5, and 7 g/l crude oil show that with increasing concentration, crude oil reduces the ability of the strains to degradation (decrease degradation from 90 % to 20 %). Other studies have been conducted to recognize fish as an indicator of oil pollution. Still, the results of this research confirmed that crude oil pollution decreases the biodiversity in the intestinal microbial flora of these fish. The isolated bacteria can degrade crude oil and help degrade this pollution in aquatic ecosystems. By applying these bacteria, crude oil pollution in the Persian Gulf can be better managed and decreased.

Keywords Gobiidae . Fish . Bacterial flora . Biodegradation . Bioremediation . Crude oil

Introduction

Gobiiformes are a species of vertebrates identified based on soft tissue characteristics and are present in all aquatic habitats. Eight families have been identified in the order Gobiiformes. Members of the Gobiidae family are the wealthiest species of fish living in the coral reef ecosystem. Crude oil pollution in the marine

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environment is a global problem. This pollution has many effects on the diversity of marine animals and microflora. The Persian Gulf is the maine marine environment in the world, and crude oil pollution occurs many times. Selecting a good strategy for managing crude oil pollution is crucial to decreasing the effect of this contamination on marine animals (Gierl and Reichenbacher 2017).

The microflora of marine fish, especially in the intestine, is essential to the ecosystem. Microbes from seawater enter the intestine of fishes, and an active dynamic occurs between the ecosystem and the intestine's microflora. However, this relationship and community dynamic can be disrupted when stress occurs in marine environments, such as oil pollution. Oil pollution can decrease the biodiversity of microflora. Some sensitive bacteria to the toxic effect of crude oil can die, and prevalent strains that can use crude oil hydrocarbons in the ecosystem. Thus, crude oil pollution in marine environments can change marine fish's microbial community (Ghotbeddin and Roomiani 2020).

Polycyclic aromatic hydrocarbon (PAH) is an organic chemical compound and carcinogen. Some of its components, such as saturated hydrocarbons, are easily degraded; others, such as high molecular weight polycyclic aromatic hydrocarbons and the polar components of crude oil, are persistent and toxic. Hydrocarbons represent a rich source of energy and carbon for microorganisms capable of degrading them and are known as hydrocarbonoclasts. Due to the metabolic capacity of microorganisms and their interactions with the environment, the bioremediation process can be optimized (Singh et al. 2016). One classification in this regard is based on the rate at which microorganisms degrade hydrocarbons, those capable of degrading a wide range of hydrocarbons and those that are only capable of degradation (Cappello et al. 2007).

Bioremediation is generally defined as using the ability of microorganisms to remove or reduce contaminants such as hydrocarbons. Two general approaches have been identified, "In situ bioremediation" and "Ex-situ bioremediation" for bioremediation. Insitue bioremediation, the ecosystem's ability can be used to degrade pollutants. However, in ex-situ bioremediation, the ability of other microorganisms can be used to decrease pollution. In another view, bioremediation is divided into three branches: biosurfactant, bioaugmentation, and biostimulation. Biosurfactants dissolve oil, thereby facilitating the access of microorganisms to petroleum compounds. Bioaugmentation uses the unique abilities of some microorganisms in their metabolic processes to break down contaminants. In biostimulation, attention is paid to the factors that increase the degradation activity of microorganisms (Faizulina et al. 2023).

Approximately 79 bacterial genera capable of utilizing hydrocarbons as an energy source have been identified, along with 9 cyanobacteria, 103 fungi, and 14 algae species that can degrade or convert hydrocarbons. Some examples of research in this field can described as follows:

Cappello et al. (2007) studied bacteria in the gills of bivalves from crude oil-contaminated and uncontaminated sites, noting a higher population density of hydrocarbon-degrading bacteria in contaminated sites. However, the occurrence of heterotrophic bacteria was similar in both locations. D'Costa et al. (2017) investigated the association between microorganisms and marine sponges in natural environments, identifying 349 symbiont bacteria using molecular techniques. Many isolated bacteria belonged to the Vibrionaceae family, including genera Roseobacter, Cobetia, and Pseudovibrio, which exhibit potential hydrocarbon degradation capabilities. Some isolates from this study align with those identified by D'Costa et al. (2017).

There is little research on the microbial flora of degrading bacteria in the fishes, especially in the Persian Gulf. This study aims to screen, isolate, and identify crude oil-degrading bacteria from the intestines of Gobform fishes. Also, selecting the best strain and optimizing optimal degradation conditions is another purpose of this study.

Materials and methods

Collect and identify fish

Fish samples were randomly collected in 2019 from five regions of the northern Persian Gulf, Iran. These regions were selected because, according to previous research (Hassanshahian 2012a; 2012b; 2013), high oil pollution occurs in these regions. Thus, according to adaptation theory, the chance of delicate, robust, degrading bacteria in regions with high pollution is higher than in other regions. The caught fish were transferred to the laboratory under temperature control. In the laboratory, after identification and biometrics of the fish, the skin of the abdominal area of the samples was disinfected with ethanol. In the abdominal area, it was cut from the anus to the thoracic girdle, and the intestine was separated. Identification of fish species

was carried out by Murdy (1989), Carpenter and Niem (2001), Nelson et al. (2016) and Mury (1989).

Isolation of bacteria

The tested samples were first enriched separately under sterile conditions in 100 ml of phosphate-buffered saline (PBS) for one hour in a shaker at 160 rpm and 30 °C to isolate the crude oil-degrading bacteria. After the enrichment period, 10 ml of this suspension was added to 100 ml of ONR7a medium containing 1% crude oil as the only carbon and energy source. They were kept for two weeks. The enrichment process in ONR7a was selected because, in the collected samples, some associated bacteria cannot withstand crude oil degradation. Then, these two steps of enrichment can reduce the microbial load and select degrading bacteria (Hassanshahian et al. 2014a).

Finally, from the final passage, 10^{-4} and 10^{-5} dilutions were prepared from the medium, and 100 µl was transferred to the marine agar medium. After two days, unique colonies were selected from each culture medium and moved to a new marine agar culture medium to prepare a pure colony. Bacteria isolated in the ONR7a medium were cultured with oil as the single carbon source to screen for excellent oil-degrading bacteria. The bacterial strains with the highest optical density (OD) were selected for initial identification (Hassanshahian et al. 2014b).

The negative control for this analysis was ONR7a medium with crude oil and without any sample inoculation. The experiment used this control to control possible contamination during the enrichment process.

Emulsifying properties (E_{24}) and bacterial adhesion to hydrocarbon (BATH) activity

Crude oil-degrading bacteria were first cultured in marine broth (MB), and after the isolates reached their logarithmic growth, 4 ml of the culture medium was poured into a test tube containing 6 ml of sterile kerosene and vortexed at high speed for 2 min. It was then allowed to stand at room temperature for 24 hours (Pruthi and Cameotra 1997). The emulsifying activity was calculated by Eq 1.

$$\text{Eq 1: E 24} = \frac{\text{Altitude Area emulsified}}{\text{The total height of liquid}} \times 100$$

To evaluate BATH, there are two OD needs: the first OD is before the bacteria come into contact with hydrocarbons, and the second OD is after contact with hydrocarbons. The first 24-hour culture of the strains was prepared in a marine broth culture medium. All samples were adjusted by OD 0.3 to 0.4 nm using a spectrophotometer. After centrifugation of the samples at 500 rpm, the precipitate was poured into 9 ml of ONR7a culture medium. After the vortex of the prepared solution, its OD was recorded at 600 nm (OD_1). Later, 200 µl of hexadecane was added. After stirring for 2 minutes, they were kept at room temperature for 45 minutes to separate the hydrocarbon phase, and the turbidity of the aqueous phase was measured (OD_2). The results were determined as aqueous phase adsorption after treatment compared to the initial adsorption of the bacterial suspension according to Eq 2 (Hassanshahian and Emtiazi 2008).

$$\text{Eq 2: BATH} = \frac{\text{OD1}-\text{OD2}}{\text{OD1}}$$

Biochemical and molecular identification of bacteria

Biochemical detection was performed according to the protocol described by Bergey for catalase, oxidase, motility, etc. tests. Molecular identification was performed by polymerase chain reaction using primers specific for 16S rDNA and the conditions described in Table 1. Bacterial genomic material was extracted using the phenol-chloroform method, and then the OD of the PCR product at 260 nm was measured using a Shimadzu-UV-160A spectrophotometer. Cinna-Gen Company prepared the primers used, and also, according to the size of the PCR product in this study, a 1 Kb ladder was used (Sambrook and Russell 2006). The PCR product was loaded on 1% gel agarose, and then the 1400 bp band was extracted from the gel agarose and sent for sequencing. Sequencing results in blasted gene banks and their homology percentage were investigated. The phylogenetic dependence of the sequences was investigated using the method previously explained by Yakimov et al. (2007). Draw the phylogenetic tree using MEGA software version 10.2.5 was performed.

Crude oil degradation by bacteria

The ability of bacterial isolates to degrade crude oil was investigated by Spectrophotometric, Gravimetric, Gas chromatography (GC), and Fourier-transform infrared (FTIR) spectroscopy.

Spectrophotometric

In this method, the bacteria were inoculated into an ONR7a medium containing 1% crude oil and incubated for 15 days. Next, the growth rate of the strains was investigated by recording OD at 600 nm. Then, 50 ml of dichloromethane (DCM) was added to the medium. DCM was mixed well with ONR7a medium and petroleum and separated using an organic and aqueous phase separating funnel. Following the formation of the aqueous phase at the top and the organic phase containing the oil dissolved in DCM, the organic phase was transferred to another vessel. The amount of crude oil removal was ascertained by dissolving 1 ml of the solution taken in 5 ml DCM and then reading the turbidity of the extracted oil in front of the control group at 420 nm and using Eq 3 to measure the crude oil removal percentage (Ansari et al. 2021).

$$\text{Eq 3: oil removal rate} = \frac{\text{Sample absorption rate} - \text{Control absorption rate}}{\text{Control absorption rate}} \times 100$$

Gravimetric

Likewise, after 15 days of incubation of bacterial strains in ONR7a medium with 1% crude oil, the sample was extracted using DCM similarly. The organic phase, which contains crude oil dissolved in DCM, was exposed to air to evaporate DCM. The quantity of oil destroyed was estimated by reducing the residual hydrocarbons from the main weight of the petroleum hydrocarbons as a control (Bayat et al. 2017).

Gas chromatography (GC)

The strains were incubated in an ONR medium containing 1% crude oil for ten days. After incubation, 50 ml of DCM was added to the culture medium and transferred to the separating funnel to separate the organic and aqueous phases. To the organic phase containing the oil dissolved in DCM, 3 g of sodium sulfate was added to absorb the remaining water and incubated overnight at room temperature. The sample was passed through Whatman No. 1 paper and placed at ambient temperature for DCM evaporation. After DCM evaporation, 3 µl of DCM was added to the remaining 1 µl of oil and analyzed by GC (Jakada et al. 2023).

The specifications of the column used are as follows: Varian capillary column cp-sil 5 cb and FID detector (0.25 µm × 320 µm × 30 m) cp8740. Helium was used as the carrier gas. The initial temperature was 100 °C for 1 min, the injection temperature was 280 ºC, the column holding temperature was 70 °C for 3.32 min, then 290 °C for 7 min, the final temperature was 290 °C and the flow rate was 0.7 ml/min. The peaks obtained from GC were compared with the control, and the decomposition percentage was calculated for each strain.

Fourier-transform infrared (FTIR) spectroscopy

This method is an effective and advanced technique for discovering the structure and determination of chemical groups and is mainly used to identify organic compounds. This method for crude oil degradation was selected because during the biodegradation pathway, some intermediate was produced, and this method can predict these intermediates. Infrared analysis was performed in the spectral region of 4000-500 cm^{-1} at a resolution of 50 cm^{-1} and from NaCl or KBr cells at 25 °C (Motamedi et al. 2023).

Table 1 PCR program and primers used for molecular identification of bacterial isolates

Primers	Sequence (5′→3′)	Sequence size (bp)	PCR program* (35 cycle)	PCR components (25 µl)
Uni_1492R	TACGYTACCTTGTTACGACTT	21	Denaturation (1 min, 94 ºC),	10 X PCR Buffer (20 mM Tris, 50 mM KCl): 2.5 µl,
Bac27_F	AGAGTTTGATCCTGGCTCAG	20	Extension (1 min, 72 ºC),	$MgCl_2$ (2 mM): 1µl, Primer (0.12 µM): 0.3 µl, Taq DNA polymerase (1 U): 0.2 µl, dNTP (200 µl each dNTP): 0.5 µl, DNA template: 1 µl, Distilled deionized water: 19.2 µl

* Annealing temperature Uni_1492R (53.6 ºC) and Bac27_F (56.7 ºC).

The effect of crude oil concentration on the rate of degradation

To evaluate the growth rate and crude oil remediation by strains at various oil concentrations, the strains were cultured in ONR7a medium containing 1 to 7% crude oil. After 15 days of incubation at 30°C on the shaker at 160 rpm, the crude oil sample was extracted using DCM similarly. The organic phase, which contains crude oil dissolved in DCM, was exposed to air to evaporate DCM. Before, bacterial growth and crude oil removal were quantified (Lai et al. 2014).

Test halophilicity

Tolerance to salt is an essential index for selecting halophilic bacteria, and this test was done to reach this purpose. Bacteria were tested for halophilic or halotolerant as well as salt concentration. For this purpose, the bacteria were cultured in ONR7a agar medium with 4% salt and without salt and kept in an incubator at 30°C for 24 h (Hassanshahian et al. 2014a).

Results

Fish identification

The collected samples belonged to 4 genera, among which the *Periophthalmus waltoni* was more abundant than the others. Images of fish are displayed in Figure 1, and their other specifications are presented in Table 2.

E_{24} and BATH

The results related to E_{24} and BATH are reported in Table 3. According to these results, the T4 and T7 strains have the highest BATH and E24 values (76 and 34 percent, respectively). The strains with high emulsification activity and better surface hydrophobicity can be selected as robust degrading bacteria be-

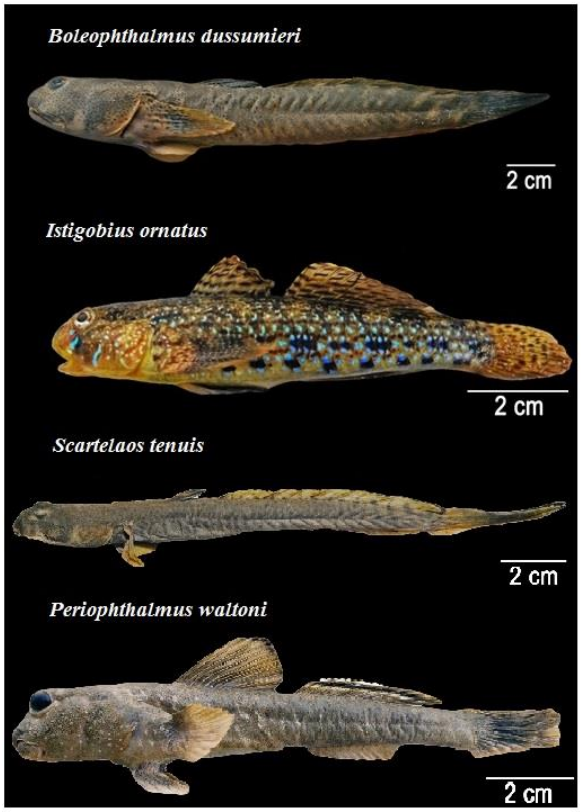

Fig. 1 The macroscopic figure of harvested fish

cause the petroleum hydrocarbons are hydrophobic and immiscible in water. Based on the results of this Table, the best strains (T4 and T7) were selected to continue the studies.

Identification of bacteria

The results for detection isolates using biochemical techniques are shown in Table 4. According to the results presented in this Table, most bacteria are Gram-negative and motile. Also, the Oxidation/ Fermentation (O/F) test reavaled that these bacteria have oxidase enzymes and use these enzymes for the degradation of hydrocarbons. The oxidase test confirms that all isolates can degrade hydrocarbons in the aerobic pathway.

Figure 2 shows the bands formed in gel electrophoresis of 16S rRNA genes. Also, molecular identification was performed by considering 16S rRNA gene sequences, and the results related to the similarity percentage and access number of each isolate are listed in Table 5. According to these results, the bacteria in this research are similar to those in other studies. The implication of these results is the accordance between microbial flora in different zones of the world.

Draw a phylogeny tree using MEGA software version 10. 2. 5 was performed by the Neighbor-Joining method with Bootstrap 1000 and the bacterial genome of *Escherichia coli* (NR 024570.1) as the out-group; the results are shown in Figure 3. The numbers on each node represent the Bootstrap number. The access number of each strain is listed before its name in the phylogenetic tree. This phylogenetic tree shows the similarities of bacteria isolated in this research to another bacteria reported by another researcher in GeneBank. The significance of the current tree is the high similarities of isolated bacteria, which confirmed the accuracy of the results. *E. coli* was chosen as an out-group because the out-group in the phloenetic tree must be a sequence different from that of isolated bacteria.

Table 2 Specifications of examples and synonymous names in this review

Synonyms		Sample collection season	
		Cold	warm
Intestines of *Scartlaos tenuis* Bostanu Port	T1	×	
Intestine of *Periophthalmus waltoni* Bandar e Hasineh	T2	×	
Intestine of *Periophthalmus waltoni* Bostanu port	T3	×	
Sediments of Bostanu Port	T4, T6	×	
Sediments of Bandar e Hasineh	T5, T7	×	
Sediments of Bandar Khamir	T8	×	
Intestine of *Periophthalmus waltoni* Bandar Khamir	T9	×	
Intestine of *Istigobius ornatus* Bandar Lengeh	T10	×	
Intestine of *Periophthalmus waltoni* Ommolkorm Island	T11	×	
Intestine of *Periophthalmus waltoni* Bandar e Hasineh	T12		×
Intestine of *Periophthalmus waltoni* Bandar Khamir	T13		×
Intestine of *Periophthalmus waltoni* Bostanu port	T14		×
Intestine of *Istigobius ornatus* Bandar Lengeh	T15		×
Intestine of *Baleophthalmus dussmieri* Bandar e Hasineh	T16		×
Intestines of *Baleophthalmus dussmieri* Bostanu Port	T17		×
Sediments of Bandar e Hasineh	T18		×
Sediments of Bandar Khamir	T19		×
Sediments of Bostanu Port	T20		×
Intestine of *Istigobius ornatus* Chabahar Port	T21		×

Table 3 Results of BATH test and emulsion activity of isolates

Strains	OD 600 nm	Growth qualitatively	Emulsion type	Dedicated E_{24} (%)	E_{24} (%)	BATH (%)
T_1	1.2	+	No emulsion	32	30.76	21
T_2	1.1	+	No emulsion	33.3	8.3	14
T_3	0.8	++	Full emulsion	3	1.66	12
T_4	1.3	+++	Micro superior	46.15	0	25
T_5	0.7	+++	Micro superior	35	1.66	32
T_6	0.5	+++	Globule and macro superior	21	3.63	36
T_7	0.9	+	Full emulsion	34	76.92	41
T_8	1.5	+++	Micro superior	32	1.53	23
T_9	1.1	++	Micro emulsion	13	6.6	14
T_{10}	0.9	++	Globule	46.15	84.53	37
T_{11}	0.8	+	Incomplete emulsion	21	55	23
T_{12}	1.6	+++	Superior	18	12	22
T_{13}	1.4	+++	Superior	18	10.76	24
T_{14}	1.1	++	Macro emulsion	8	13	11
T_{15}	0.7	++	Incomplete emulsion	50	1.66	35
T_{16}	1.7	+++	Superior	21	23	24
T_{17}	0.9	++	Macro emulsion	12	15	23

-: No growth, +: Low growth, ++: Medium growth, +++: High growth.

The results obtained from the oil degradation of the isolates using the three methods of Gravimetric, GC, and Spectrophotometric are categorized in Table 6. The highest ability to degrade oil was determined according to the three methods studied in T4 and T11 isolates with growth rates of 1.67 and 1.43. These two strains have a higher growth rate than the other isolates. Both isolates were of the genus *Halomonas*.
As presented in Table 6, the best strain is T4, which can degrade 94 % of crude oil, and the lowest crude oil degradation is related to T3 strain.

GC-MS examined the two isolates, T1 and T2, to determine compounds degraded by bacteria in crude oil. The results are shown in Figure 4. According to this picture, aliphatic compounds of crude oil and medium-chain alkanes had the highest degradation compared to long-chain alkanes and aromatic compounds.

The GC-MS results presented in Figure 4 confirmed a significant decrease in the peaks of samples compared to blank. The aliphatic compounds such as hexadecane, octadecan, and deodecan are more degraded (90 %) than aromatic compounds such as phenol, naphthalene, and toluene. The light compounds exist first

Table 4 Results of biochemical study of isolates

Strains	Oxidase	Catalase	Citrate	NO_3^- reduction	Indole	H_2S production	Motility	TSI	O/F
T_1	+	-	-	-	-	-	+	A/A	+/+
T_2	+	+	+	-	-	-	+	No ch..	+/-
T_3	+	+	+	+	-	-	+	Alk/Alk	+/-
T_4	-	+	-	-	-	-	-	A/A	+/+
T_5	+	-	+	-	-	-	+	Alk/A	+/+
T_6	-	+	-	+	-	-	-	A/A	-/-
T_9	+	+	-	-	-	-	-	Alk/Alk	-/-
T_{10}	-	+	-	-	-	-	+	Alk/Alk	-/-
T_{11}	-	+	+	-	-	-	+	A/A	+/-
T_{12}	-	+	-	+	-	-	+	A/A	-/-
T_{13}	+	-	-	-	-	-	-	Alk/A	-/-
T_{15}	+	-	-	-	-	-	+	Alk/Alk	-/-
T_{16}	-	+	-	+	-	-	-	A/A	-/-

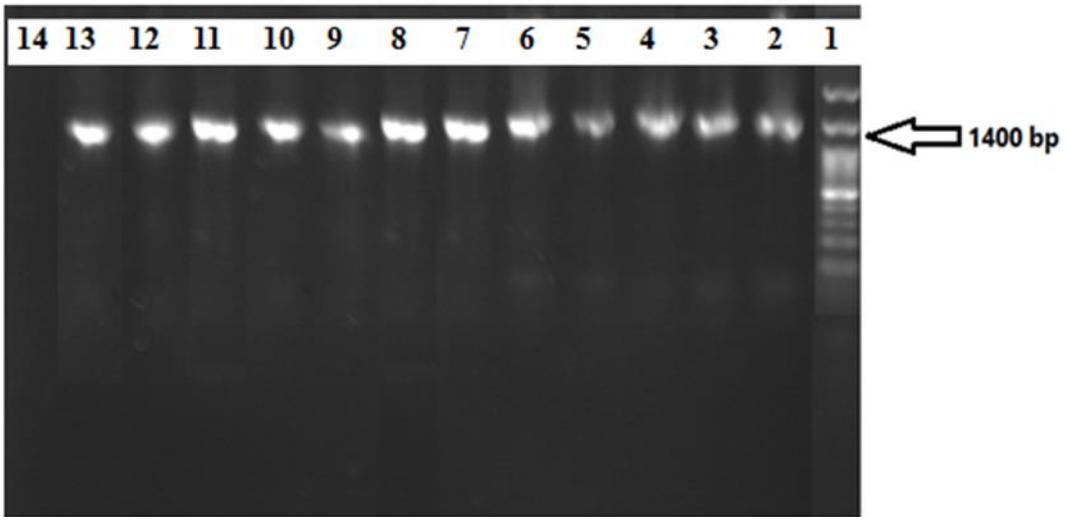

Fig. 2 Gel electrophoresis of PCR products for identification of isolates

Table 5 Molecular identification of isolates

Strains	Similarity (%)	Matching species	Access number
T5	98	*Marinobacter hydrocarbonoclasticus*	MT155932
T3	93	*Pseudomonas aestusnigri*	MT180855
T1	95	*Thalassospira permensis*	MT180856
T6	95	*Microbacterium esteraromaticu*	MT180857
T4	94	*Halomonas Salaria*	MT180858
T11	98	*Halomonas beimenensis*	MT180859
T10	97	*Cobetia marina*	MT180860
T12	98	*Tenacibaculum discolor*	MT180861
T2	96	*Shewanella chilikensis*	MT180862
T9	71	*Oceanimonas*	MT180863

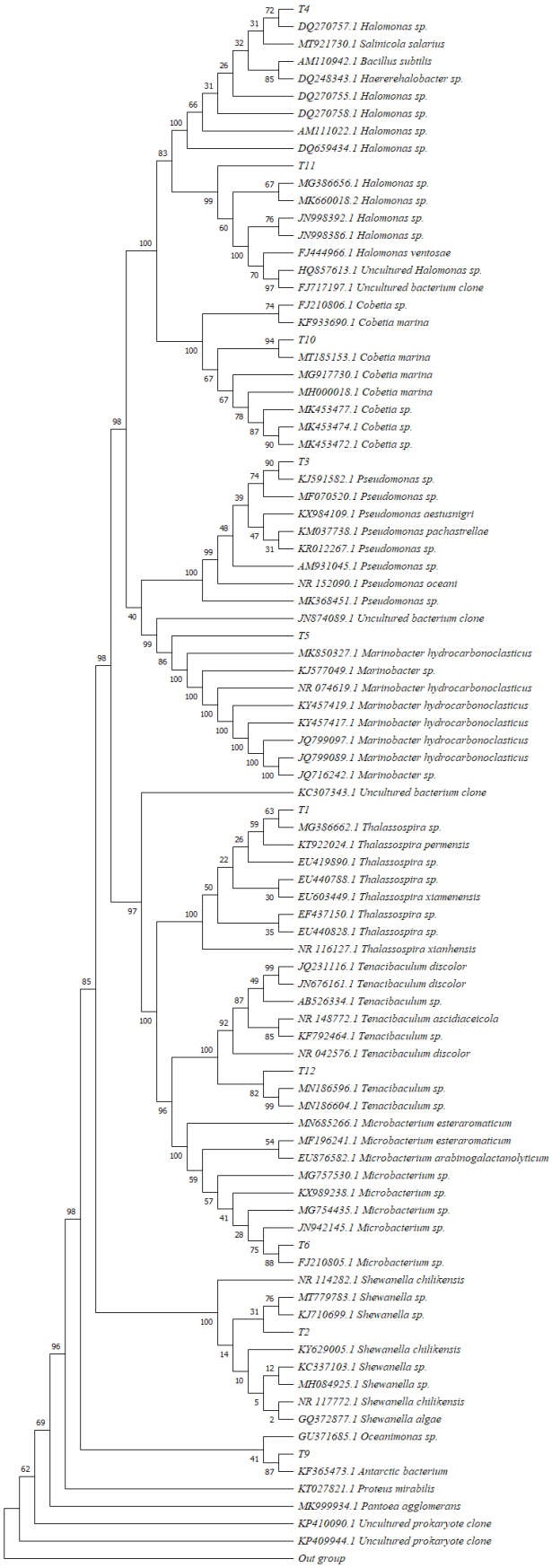

Fig. 3 The phylogenetic tree of isolated strains

from the column of the GC-MS and can be seen in the early retention time. However, heavy compounds exist very slowly from the column of GC-MS and can be seen in the late retention time.

Table 6 The rate of oil removal by isolates

Strains	Growth rate (OD 600 nm)	Oil removal rate		
		Gravimetric	GC	Spectrophotometric
T5	0.77	40.20	56.08	60.25
T3	0.80	25.80	37.13	30.00
T1	1.09	47.12	85.17	66.70
T6	1.14	39.60	74.30	60.70
T9	0.90	34.60	43.50	39.40
T4	1.67	77.30	94.30	87.57
T11	1.43	76.50	88.13	80.41
T10	0.55	32.50	64.13	60.40

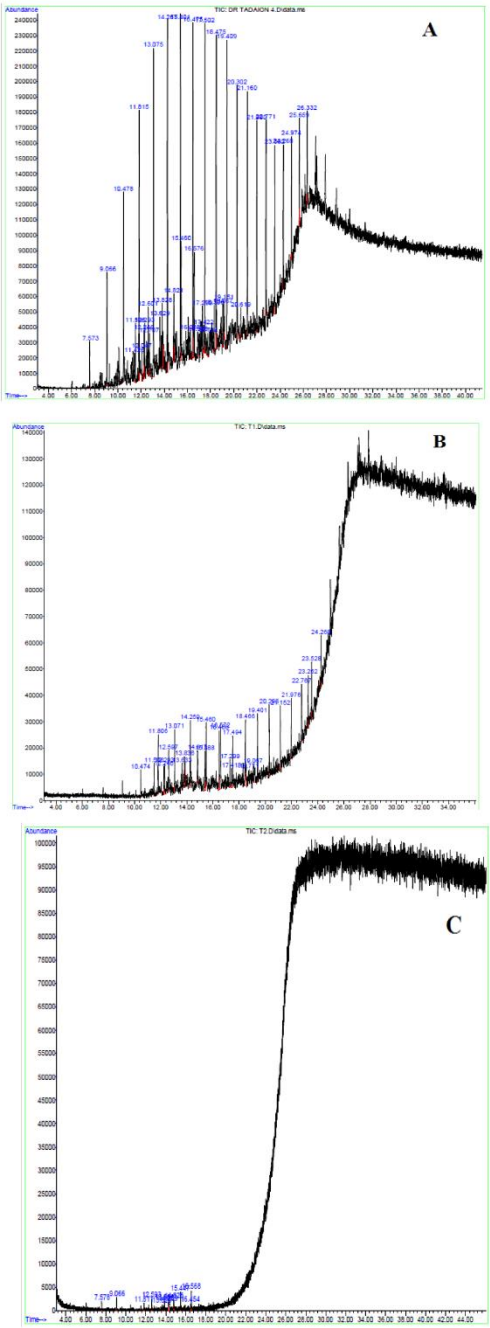

Fig. 4 The GC-MS of degraded oil by selected strains

Figure (5) shows the FTIR analysis. This image shows a comparison between each of the strains in terms of degradation. These peaks also show that the superior degrading strains degrade many crude oil compounds based on the biodegradation path of their choice.

The effect of crude oil concentration on the rate of degradation

The rate of crude oil removal was evaluated by the top 5 strains at different concentrations of crude oil. Each crude oil concentration had a control sample, the bacterial ONR medium. Crude oil removal and adsorption were evaluated at OD_{420}. The results are shown in Figure 6. As can be seen in this Figure, biodegradation has decreased with increasing crude oil concentration. When the concentration increases, the decrease in crude oil degradation can be attributed to the high amount of toxic compounds in the crude oil composition. Since the concentration of these compounds increased, the toxicity of these compounds increased for bacterial cells, and the rate of degradation decreased.

Halophilicity analysis

The results of the halophile or halotolerant strains showed that all tested strains are halotolerant. This means that they can grow without NaCl salt, and as a result, none of the strains were absolute halophiles. However, the results showed that in some strains, growth is significantly reduced by reducing and eliminating salt, and optimal conditions are not created.

The halotolerant properties of the isolated bacteria can help with degradation because the presence of salt can decrease the degradation. The results confirmed that these bacteria also work without salt, which applies to these bacteria.

Discussion

The presence of microorganisms with appropriate metabolic capabilities is the most critical need for the bioremediation of oil spills, according to Venosa et al. (2002). Marine bacteria can survive in seawater, sediments, and mangrove-related habitats. In these ecosystems, several groups of bacteria use a unique metabolism to produce bioactive compounds and biosurfactants and form biofilms to adapt to different environments (Sing et al. 2016). Microorganisms need nutrients such as nitrogen, phosphate, carbon, and energy to survive. The biodegradation rate depends on the microbes' growth conditions, such as electron

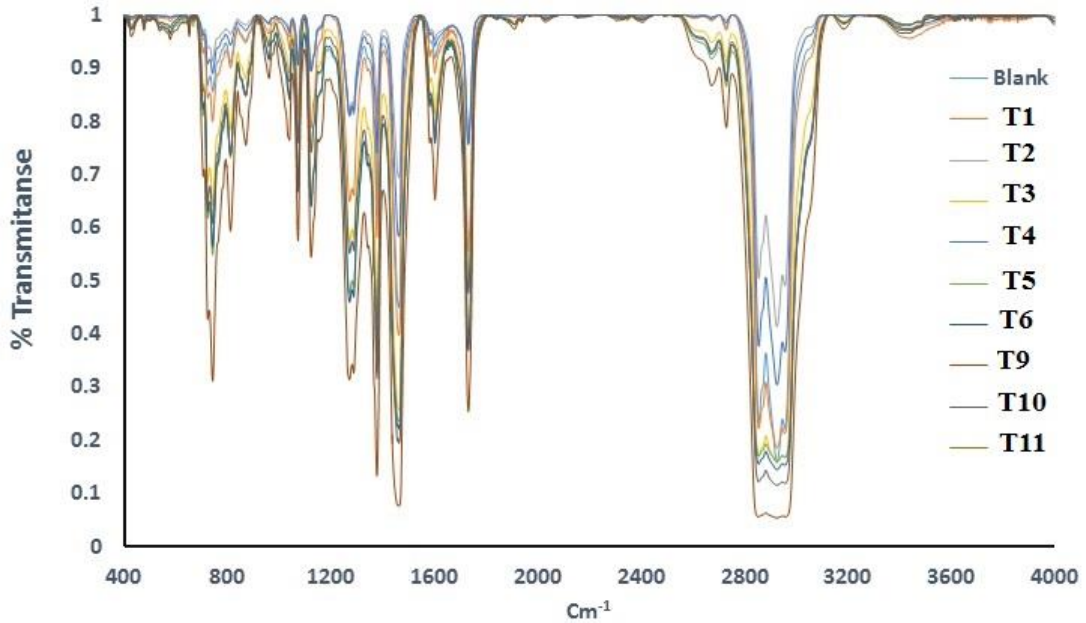

Fig. 5 The FTIR image of degradation of crude oil by isolates

acceptors, oxygen availability, temperature, pH, salinity, and pressure. In this regard, Muñoz and Guieysse suggested that microalgae could be used to produce the oxygen needed by compatible bacteria for the biodegradation of hazardous pollutants such as polycyclic aromatic hydrocarbons, phenols, and organic solvents (Hassanshahian et al. 2012a).

Another study aimed to identify microplastics in sediments and fish of *Periophthalmus waltoni* in mangrove forests in southern Iran. The results showed that most microplastics were polystyrene, polypropylene, and polyethylene terephthalate (Shirani et al. 2014). In another study, Shirani et al. introduced *P. waltoni* as a biomarker in oil-contaminated areas of the Persian Gulf. Also, Shirani et al. investigated the effect of Persian Gulf oil pollution on *P. waltoni* fish for three enzymes: Ethoxyresorufin O-deethylase, glutathione S-transferase, and catalase (Shirani et al. 2014). However, microbial testing has not been performed in these studies.

Nonetheless, Rao et al. (2016) report that *Boleophthalmus* sp. had the lowest accumulation of total petroleum hydrocarbons among the ten fish surveyed off the west coast of India. Also, Soltani et al. report low concentrations of aromatic polycyclic hydrocarbons in three species of fish, *Leuciscus vorax*, *Liza abu,* and *Coptodon zillii*, from Arvand River and the Persian Gulf, which may be due to the presence of decomposing bacteria in these animals. The bacteria identified in this study from the intestines of Persian Gulf mudskippers include *Marinobacter hydrocarbonoclasticus*, *Pseudomonas aestusnigri*, *Thalassospira permensis*, *Microbacterium esteraromaticu*, *Oceanimonas*, *Halomonas Salaria*, *Halomonas beimenensis*, *Cobetia marina*, *Tenacibaculum discolor,* and *Shewanella chilikensis*.

A study by Ma et al. (2018) on the intestinal microbial community of two species of fish, *Eriophthalmus magnuspinnatus* and *Boleophthalmus pectinirostris,* with different sexes (four male and female groups) and other feeding strategies revealed that Proteobacteria are predominant in all four groups. In this phylum, *Aeromonas*, *Acinetobacter*, *Shewanella,* and *Halomonas* are the most identified genera (Ma et al. 2018). They stated that the percentage of each of them depends on the fish diet (Ma et al. 2018). The current study studied the relationship between Gobiiformes intestinal microflora and crude oil degradation. The results confirmed that the microbes in this fish's intestine have the capability for crude oil degradation. The results follow the results reported by other researchers.

According to the results obtained from GC-MS in this study, the T1 strain or *Thalassospira permensis* bacterium had a more remarkable ability to reduce crude oil compositions than other identified isolates. The phylogenetic diversity of this bacterium in marine environments has been studied by Lai et al. (2014). Exxon Valdez oil spill bioremediation efforts have shown that light alkanes are removed first, and some

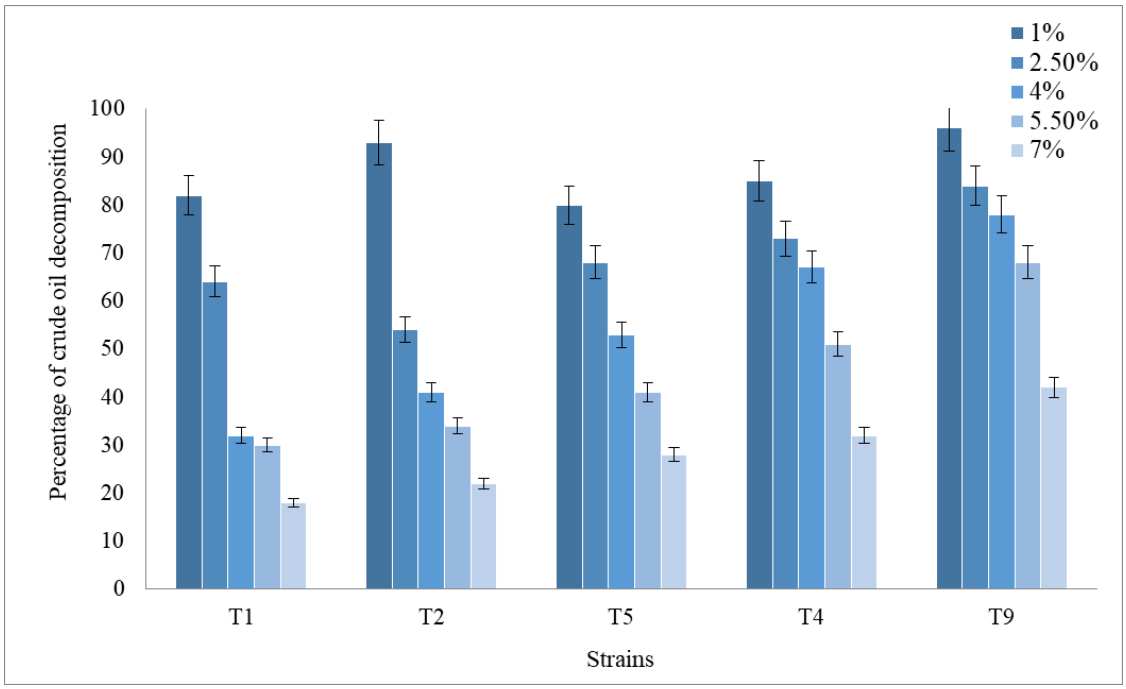

Fig. 6 The effect of crude oil concentration on growth of selected strains

compounds, such as high molecular weight polycyclic aromatic hydrocarbons, may not be degraded at all. The susceptibility of hydrocarbons to microbial degradation can be generally classified as linear alkanes with the highest, followed by branched alkanes, small aromatic compounds, and the lowest, which are related to cyclic alkanes. Therefore, the entry of hydrocarbon-decomposing bacteria into oil-contaminated sites does not guarantee the removal of all crude oil components (Hassanshahian et al. 2014a). The phylogenetic diversity has some effects on the bioremediation capability of isolated bacteria. In this research, a diverse group of bacteria was isolated, and it was confirmed that when the diversity of bacteria in the ecosystem was high, it could contribute to higher biodegradation of pollutants.

Some limitations or challenges were associated with the methodologies employed in the study, such as the sampling zones for these fishes were very difficult to reach and the sampling zones were limited. Also, some limitations exist in crude oil degradation assay. However, this limitation did not have any significant effect on the results.

The duration of degradation of petroleum hydrocarbons in the environment is primarily controlled by factors such as the growth of microorganisms and the enzymatic activity involved in the degradation of petroleum hydrocarbons. Due to the unique conditions in aquatic ecosystems, some environmental factors such as lack of essential nutrients and low temperatures can limit the growth and thus the ability to destroy decomposing bacteria. On the other hand, adding compounds such as nitrogen and phosphorus to increase the growth of microorganisms can facilitate oil decomposition (Nickles et al. 2020). The tests performed in the present investigation showed that the highest growth rate and removal of crude oil among the studied strains belong to the T4 strain or *Halomonas Salaria*. The results of tests in 5 strains of *T. permensis*, *S. chilikensis*, *H. Salaria*, *M. hydrocarbonoclasticus,* and *Oceanimonas* in concentrations of 1, 2.5, 4, 5.5, and 7 g/l crude oil show that with increasing concentration crude oil reduces the ability of the strains to decompose. However, 0.01 g/l of crude oil increases bacteria's abundance and diversity but reduces fungi's abundance (Nirwana and Wedari 2023).

The environmental factors in this research have significant effects on crude oil degradation. For example, an increase in the concentration of crude oil contributes to a decrease in degradation. Some bacteria produce biosurfactants as they grow on petroleum hydrocarbons. This compound reduces the surface tension between the aqueous and organic phases, resulting in hydrocarbon emulsions in water. This process increases biodegradation by increasing the availability of bacteria to petroleum hydrocarbons (Hassanshahian et al. 2012a). Biosurfactants also solve the problem of adsorption and membrane transfer of hydrophobic compounds from the bacterial cell membrane, which is one of the limiting factors of bioremediation. However, the type of surfactant and its concentration affect the quality of biodegradability (Hassanshahian et al. 2012a).

This study investigated the relationship between biosurfactant production and cell surface hydrophobicity and its effect on the decomposition of petroleum hydrocarbons by isolated strains. The results showed that the strains with higher hydrophobic levels produced more biosurfactants and had higher oil removal capability. Six of 11 degrading bacterial strains produced high biosurfactant levels and reduced surface tension to below 40 mN/m. These isolated bacteria can be applied in the field of bioremediation. Some strategies can be applied in this case. Bioaugmentation is a strategy that can be used. In this strategy, the isolated bacteria can increase volume and inoculate in contaminated seawater or sediments.

Finally, some suggestions can be brought for future research. Screening of crude oil degrading bacteria from another fish or discovering the relationship mechanism between these bacteria and fishes are of possible topics to be studied well.

Competing interests The authors declare that they have no conflict of interest.

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