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Investigation of Oil-in-Water Emulsions Treatment by Crude Oil Degrading Bacteria and Coagulation with Cationic Polyacrylamide

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Abstract

Background: Oily wastewater which is released from different industries is one of the most common pollutants. Efficiency of conventional methods such as gravity separation and skimming, dissolved air flotation, demulsifying, coagulation and flocculation are frequently not efficient enough is not acceptable. Therefore, in this study it was intended to use a new strategy that is combined by two methods.

Methods: Nine crude oil degrading bacteria were isolated from oil contaminated sites in the Persian Gulf at Terminal of Bandar Abbas. Choosing cases were cultured in the ONR7a medium supplemented with 1% (v/v) of crude-oil. Two strains that had more growth and higher oil removal were chosen and identified from nine isolated strains for further study. Due to its low price and simple usage of coagulation-flocculation process, the next step of this study was dedicated to this physical treatment method. The coagulants usage has also some limitation because of its toxicity and health hazard, therefore the coagulant content in waste treatment process should be optimized. In this study it is attempted to investigate the efficiency of bioremediation following by coagulation -flocculation process.

Results: The two isolated strains are identified with biochemical and molecular methods as *Alcanivorax* and *Idiomarina*. Then crude oil biodegradation for each strain is determined by spectrophotometry and Gas Chromatography (GC). Cationic polyacrylamide (CPAM) was chosen as flocculants. The crude oil removal was determined by using 30 mg/L, 50 mg/L and 70 mg/L CPAM in jar test for synthetic oily wastewater with three different crude oil content (500 mg/L, 1000 mg/L and 1500 mg/L). The results have shown that the highest removal efficiency is reached by using 70 mg/L of CPAM in the synthetic wastewater with 1000 mg/L crude oil in there.

Conclusion: The study demonstrated that the bio degradation of oily wastewater following by flocculation removes the oil significantly from the synthetic oily wastewater.

Keywords: Oily wastewater; Crude oil degrading bacteria; Cationic polyacrylamide; Flocculation

Introduction

When oil contaminated wastewater appear from kind of sources includes crude oil production, oil refinery, petrochemical industry, metal processing, compressor condensates, lubricant and cooling agents, car washing and restaurants. Oily wastewater has toxic substances like phenols, petroleum hydrocarbons, poly-aromatic hydrocarbons which are inhibitory to plant and animal growth equally mutagenic and carcinogenic to human being. Also, oily wastewater contains high oil content, sulfur, chemical oxygen demand (COD) and color [1-4]. Therefore, attention and developing new technology for cleaning oil pollution has been increased.

There are a lot of technology for the cleaning up oil contaminated areas such as physical removal (booms, skimmers and absorbent materials, chemical methods emulsion breakers (separates water and oil mixtures), gelling agents, burning agents, neutralizing agents, sinking agents, bioremediation chemicals (accelerate oil's natural degradation), viscoelastic additives and herders) and biological methods [5]. Bioremediation approach is environmentally friendly treatment technology for the removal of hydrocarbons by biological agents such as microorganisms. Bioremediation can be improved by either of the two methods, bio-augmentation and bio-stimulation [6,7]. The oil cleaning up strategy could be more efficient, if the biological treatment is combined with a coagulation process. The second part of this study is coagulation with cationic polyacrylamide. The colloidal particles which are found in nature have normally charges on their surfaces, which stabilize them of suspension. The surface properties of colloids could be changed by the addition of some chemical substances and then the dissolved particles can even be precipitated so as to facilitate the separation of solids by gravity, adsorption by various materials or filtration. The stable state would be changed to unstable during the destabilization process and it is well known by two methods [8-10]:

1) Flocculation and

2) Coagulation

The mostly water-soluble linear polymers with high molecular weight are used as a polymeric flocculent and the adsorption activity can be drastically improved by thermal and chemical treatments. Adsorptive capacity (AC) is determined by activation method which is related to some properties such as: surface area pore size and surface functional groups [11-16]. The cationic and anionic form of this kind of polymers is known as polyelectrolytes and each one has its own characteristic features. Cationic polymers which are soluble in water

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are divided to three different classes: ammonium (including amines), sulfonium and phosphonium quaternaries. There are so many kinds of cationic monomers those can be home or copolymerized with acrylamide to reach the polymer which is soluble with a huge range of charge (1-100%) [9].

As it was shown by the last reports, anionic and nonionic polyacrylamide gave rise to a poorer oil–water separation and the final oil concentration in the treated water was higher in comparison with the water which was treated by cationic polyacrylamide. Therefore, the cationic polyacrylamide was found more effective in the separation process of oil from water [17]. The mechanism of flocculation that causes this high efficiency is the strong interaction of the cationic functional group with suspended negatively charged particles such as oil droplets. The flocculation is affected by the extent of ionization and interaction with water and these features are derived from the density and distribution of positive charges along the macromolecular backbone [9].

Therefore, it was decided to use polyacrylamide and methacryloxyethyl trimethyl ammonium chloride as cationic agent. In the other word, it was tried to show the function of this cationic polyacrylamide on the treatment process of contaminated water with different concentration of crude oil. The cationic poly acrylamide was synthesized in our laboratory by using low-pressure ultraviolet initiation method through copolymerization with methacryloxyethyl trimethyl ammonium chloride (DMC).

The specific aims of this research were:

1) To study crude oil degrading native bacterial strain isolated from oil-polluted sites in the Persian Gulf.

2) To study the coagulation effect of synthesized cationic polyacrylamide.

Materials and Methods

Sampling

In this study, water and sediment samples were collected by hand (depth range = 5–15 m) in the Persian Gulf at Terminal of Bandar Abbas. Collected samples were transported to the laboratory.

Isolation and selection of crude-oil degrading bacteria

To The ONR7a medium supplemented with 1% (v/v) of crude-oil (Iranian light crude oil) as sole carbon source and energy used for isolation of crude oil degrading bacteria. ONR7a contained (per liter of distilled water) 40 g of NaCl, 11.18 g of $MgCl_2.6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2\_2H_2O$, 1.3 g of TAPS0 {3-[N tris (hydroxymethyl) methylamino]-2 hydroxypropane sulfonic acid}, 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HPO_4\_7H_2O$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27 mg of H_3BO_3, 24 mg of $SrCl_2\_6H_2O$, 2.6 mg of NaF and 2 mg of $FeCl_2.4H_2O$. For solid media, Bacterial agar (15 g/l) was added to the solution [18,19].

Condensed seawater (5 mL) and portion of sediments (10 g) were added to Erlenmeyer flasks containing 100 mL of medium and the flasks were incubated for 7 days at 30°C on rotary shaker (180 rpm, INFORS AG). Then 5 ml were removed to fresh medium. After a series of four further subcultures, inoculums from the flask were streaked out, and phenotypically different colonies on ONR7a agar were purified. Phenotypically different colonies obtained from the plates were transferred to fresh medium with and without crude oil to eliminate autotrophs and agar utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on crude oil were stored in stock media with glycerol at -20°C for further characterization [20].

Identification of isolates

Biochemical characterization: The following characteristics were determined according to the "Bergey's Manual of Determinative Bacteriology: The Gram stain, motility, starch hydrolysis, indole, H_2S production, catalase and oxidase, oxidation/fermentation, reduction of nitrate, Growth and acidification of carbohydrates tests were performed. Furthermore, all strains were tested for their ability to grow in different temperatures (upto 55°C) and salt concentrations (1% to 30%) using Nutrient agar medium [21].

Molecular identification: An analysis of *16S rRNA* was performed to taxonomically characterize the isolated strains. TotalDNA extraction of bacterial strains was performed with the CTAB method. PCR amplification of *16S rRNA* genes was performed using the general bacteria primer F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer R (5′-TACGYTACCTTGTTACGACTT-3′). The amplification reaction was performed in a total volume of 25 µl consisting, 2 mM $MgCl_2$ (1 µl), 10x PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 µl), 2 mM each dNTP (2 µl), 0.15 mM each primer (1 µl), 1U (0.5 µl) taqDNA polymerase (Qiagen, Hilden, Germany) and 2 µl of template DNA (50 p). The distill water was added for remaining of reaction (15 µl). Amplification for 35 cycles was performed in a thermocycler Gene Amp 5700 (PE Applied Biosystems, Foster City, CA, USA). The temperature profile for PCR was kept, 94°C for 5 min, 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, 30 cycles; then 72°C for 10 min and finally storage at 4°C. The *16S rRNA* amplified was sequenced with a Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Similarity rank from the Ribosomal Database Project RDP) and FASTA Nucleotide Database Query were used to determine partial *16S rRNA* sequences to estimate the degree of similarity to other *16S rRNA* gene sequences. Analysis and phylogenetic affiliates of sequences was performed as previously described protocols [22].

Growth and crude oil removal assay

The bacterial isolates were grown at 30°C for 1 week on rotator shaker (180 rpm). The growth of the isolates was routinely assessed indirectly by measuring the turbidity (OD600 nm) using a UV–Visible spectrophotometer (Shimadzu UV-160, Japan). The crude oil removal assay was carried out by dissolving the residual crude oil in the medium in dichloromethane (DCM) and reading the optical density of the oil extract against a blank at a wavelength of 420 nm [23].

Measure oil degradation with GC

Oil degradation was investigated by GC-FID. The residual crude oil extracted in the samples in studies was quantified according to previous described protocols. This procedure was treated with anhydrous sodium sulfate (Na_2SO_4) to remove residual water. Extracts were concentrated by separator funnel. Analyses were done by GC (Varian 3800 model, USA) equipped with a SE-54 capillary column (25 m × 0.32 mm × 0.1 µm) and flame ionization detector (FID). Helium was used as the carrier gas (30 ml/min). The oven was programmed as follows: 100°C (1 min) then increased to 300°C (2 min) at a rate of 30°C (1 min) [24].

Measure of emulsification activity, bacterial adherence to hydrocarbons (BATH)

The emulsification activity (E24) was determined by the addition of hexadecane to the same volume of cell free culture broth. After mixing with a vortex for 2 min and leaving to stand for 24 h, the E24 index is

given as percentage of height of emulsified layer (in millimeters) divided by total height of the liquid column (in millimeters) [25]. Measurement of the bacterial adhesion to hydrocarbon was performed as described by Pruthi and Cameotra [26].

Polyamide preparation

The cationic polyacrylamide (CPAM) solution including acrylamide monomer and methacryloxyethyl trimethyl ammonium chloride (DMC) monomer was prepared. The mass ratio of the monomers determined as following:

mAM: mDMC = 3:2

In this case the CPAM solution was prepared by dissolving 0.2 g of mixture AM and DMC in 200 ml deionized water; therefore a 1.0 mg/ml of CPAM was obtained. The pH of the solution was adjusted on 4 using 0.1 mol/l NaOH or 0.1 mol/l HCl. Then, 0.1 g of IR2 (Figure 1) as photo-initiator was added to the solution under nitrogen stream during continues stirring [27]. Then copolymerization was done UV radiation with low-pressure mercury lamps at room temperature for three hours.

Crude oil was the hydrocarbon composition used as an oil phase. It was chosen to prepare emulsions, because evaporation does not occur during coagulation and flocculation, and its solubility in water is insignificant. The crude oil emulsion was prepared in water with three concentrations of 500 mg/L, 1000 mg/L and 1500 mg/L. The mixture was prepared using a mechanical stirrer at 1500 rpm for 15 min and a stable emulsion was formed. In addition, the best isolate (BHA25) was inoculated to Erlenmeyer flasks containing water with different concentrations of crude oil, and the flasks were incubated for 48 hours at 25°C on rotary shaker (120 rpm).

Then, the CPAM was added at three different concentrations (30 mg/L, 50 mg/L and 70 mg/L) to each sample. All experiments were carried out by a six-cell Jar test apparatus (Zagchemie Yaran Co. Ltd.) and in accordance with ASTM 2001 standard under the same conditions of rapid mixing at 120 rpm for 1 minute, then slow down at 30 rpm for 20 minutes, followed by settlement for 15 minutes, and finally, the purified oily water was sampled to determine its turbidity. Determination of turbidity was performed as an indicator of oil contaminants in the emulsions prepared using the HACH 2100 turbidity meter with a maximum sensitivity of 0.01 NTU. The efficiency of the separation of the crude oil emulsion by coagulation, flocculation and then sedimentation was determined by the refining degree (% Re) which was calculated using equation (1):

$$Re\% = [(T0\text{-}T)/T0] \times 100 \quad (1)$$

Whereas T_0 and T are the initial and final turbidity and Re is the percentage of removal of the turbidity of crude oil-containing emulsions respectively.

Results and Discussion

Isolation and identification of bacteria

9 crude oil bacteria were isolated from enrichment cultures that incubated at 30°C for two weeks. Selection of bacteria was based on better ability to grow in presence of crude oil in growth media. Five colonies which showed higher growth rates were selected for further study. These strains were first identified by classical biochemical tests. The results of biochemical identification were showed in Table 1. Molecular identification of isolates was performed for 2 strains by amplification and sequencing the *16S rRNA* gene sequencing and comparing them to the database of known *16S rRNA* sequences. These strains show that in Table 2. All sequences of two bacteria were submitted to the Genetic Sequence Database at the National Center for Biotechnology Information (NCBI). Phylogenetic relationships of the experimental isolates and the closely related species were analyzed using the multi sequence alignment program (MEGA 5) and the results are shown in phylogenetic tree (Figures 2 and 3).

Growth rate and crude-oil removal by the studied strains

All bacterial strains were grown in 1% crude oil for 1 week with shaking. After 1 week, the levels of microbial growth and crude oil biodegradation were analyzed using spectrometry-based methods and GC- FID method respectively. As reported in Table 3, results of the research showed that the amounts of crude oil were decreased in the presence of the studied bacterial strains considerably. It means that the bacterial strains were able to degrade crude oil and consumption of its components.

The GC–FID chromatogram for these strains in compare to blank was shown in Figure 4. As shown in this figure almost peaks in the crude

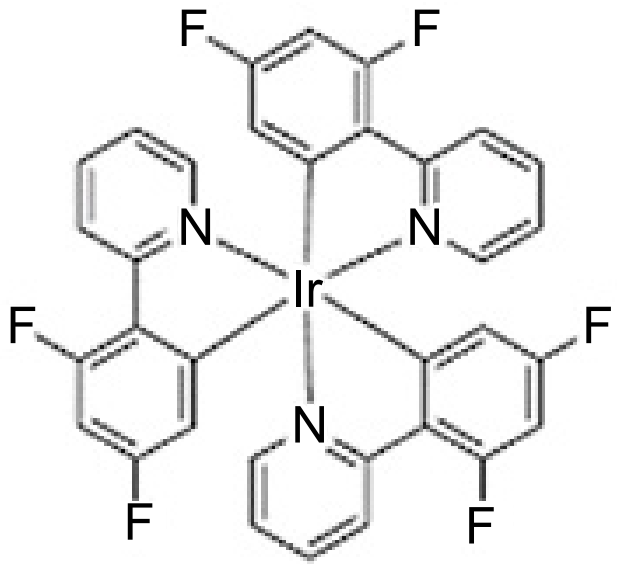

Figure 1: IR2 chemical structure.

Biochemical test	Oxidation-Fermentation (O/F)	Oxidase	Catalase	Motility	H2S production	Indole production	Nitrate reduction	TSI
T 1-2	-/-	-	+	-	-	-	+	ALK/ A
T 1-1	-/-	-	+	-	-	-	+	ALK/ ALK
T 2-1	+/-	-	+	-	-	-	+	ALK/ ALK
T 2-2-1	+/+	+	+	-	-	-	+	ALK/ ALK
T 2-2-2	-/-	+	+	-	-	-	+	ALK/ ALK
Abbreviation used: + = Growth positive, - = Growth negative, A = Acid, ALK = Alkaline								

Table 1: Biochemical identification of bacterial strains.

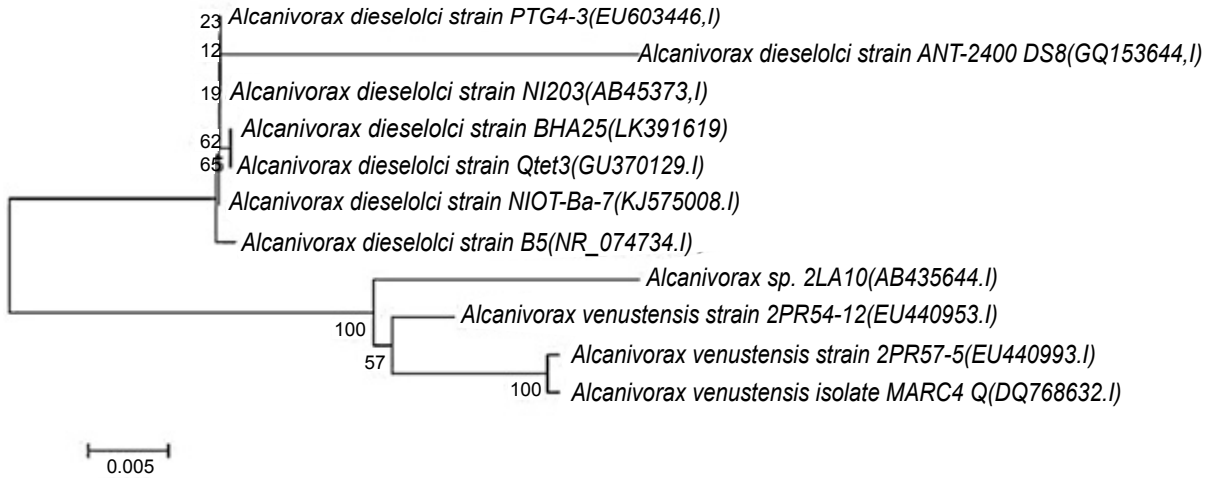

Figure 2: Phylogenetic tree of *16S rDNA* sequences of the T2-1 isolate obtained from Persian Gulf. The tree was constructed using sequences of comparable region of the *16S rDNA* gene sequences available in public databases. Neighbour-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence.

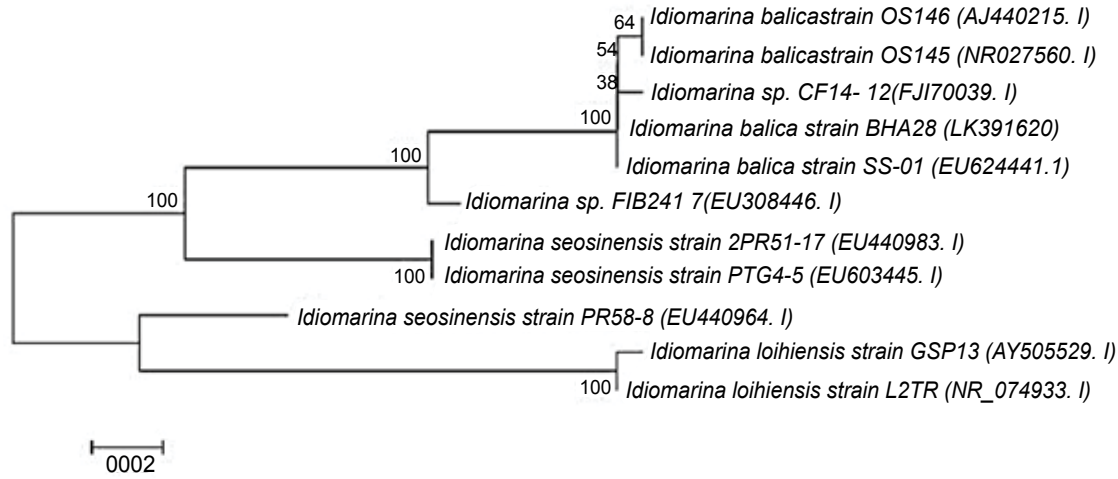

Figure 3: Phylogenetic tree of *16S rDNA* sequences of the T2-2-1 isolate obtained from Persian Gulf. The tree was constructed using sequences of comparable region of the *16S rDNA* gene sequences available in public databases. Neighbor-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence.

oil were decreased dramatically by these strains. The strain BHA25 and strain BHA28 exhibit highest level of crude-oil biodegradation, degrading 93.85% and 50.12% of the oil, respectively (Table 3).

Cell surface hydrophobicity, emulsification activity

The emulsification activity and bacterial adhesion to hydrocarbon (BATH) were investigated for each strain separately. Data obtained from tests were shown in Table 4. The strain BHA25 has the highest values of emulsification activity (E24: 57%) and high values of cell surface hydrophobicity (BATH: 11.5%). Also, bio-surfactants can act as emulsifying agents by decreasing the surface tension and forming micelles. On the other hand, the micro droplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded [28]. Thus, it seems these strains had high emulsification activity and bio surfactant production.

The crude oil removal efficiency by the oil-degrading bacteria and synthesized cationic polyamide

Effect of initial concentration of crude oil: In this study, three different concentrations of oil pollutants in water were 500 mg/L, 1000 mg/L and 1500 mg/L. Comparing the turbidity removal efficiency in each level is seen as an indicator of the system response to changes in the initial concentration of crude oil. The turbidity removal decreases by increasing the inlet concentration. As shown in Figure 5, the highest removal rate is obtained for a concentration of 500 mg/L of crude oil in water. As the concentration of crude oil increases and as a result of increased input load, the turbidity removal decreases, which reduces the response of the system due to reduced ability of CPAM in coagulating heavy loads of contaminants.

Effect of CPAM concentration: As it is shown in Figure 5, increasing concentrations of coagulant from the first level 30 mg/L to the

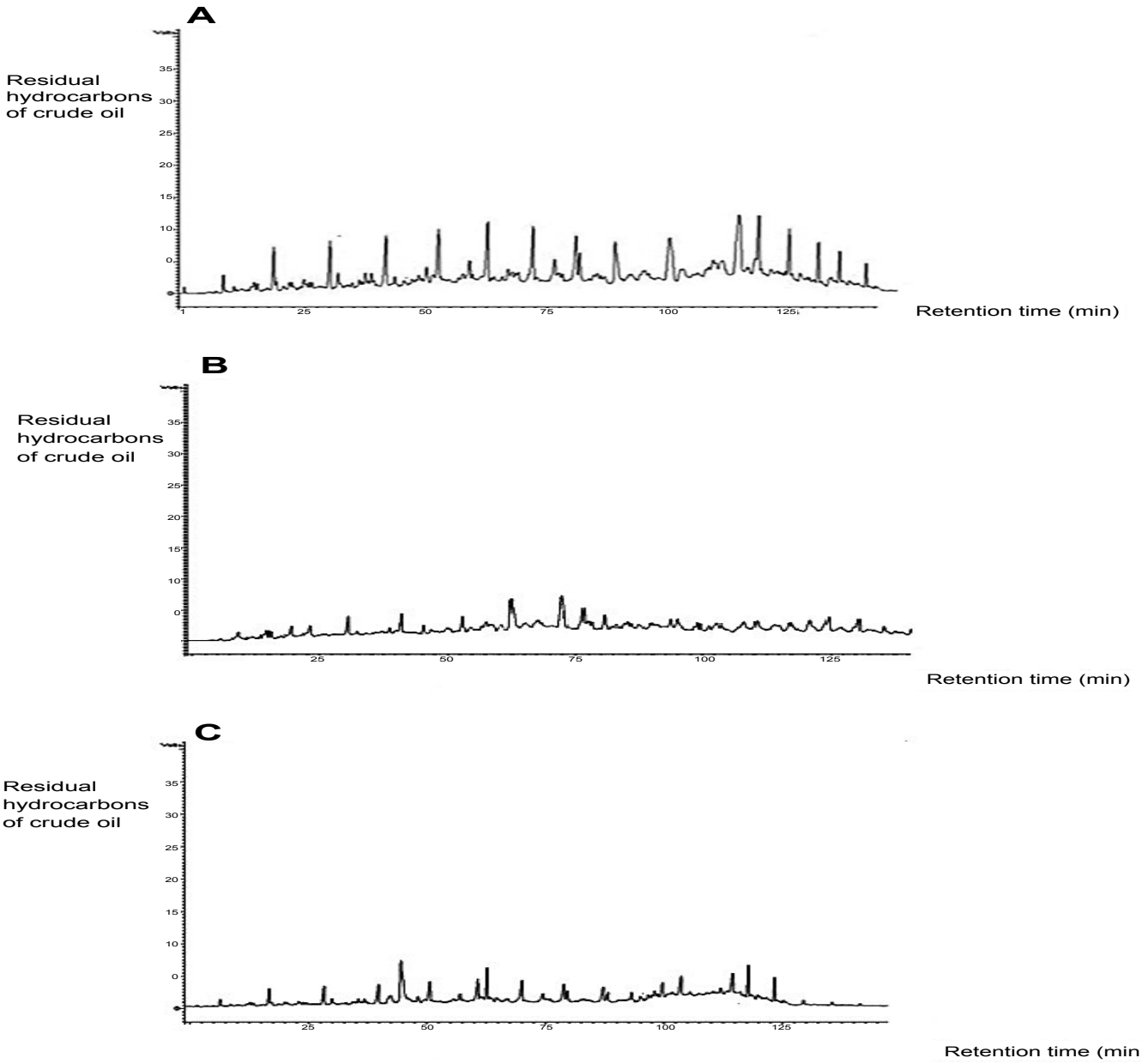

Figure 4: Gas chromatographic profiles of residual hydrocarbons of crude oil in control (A), strains BHA25 (B) and BHA28 (C) flasks.

Intermediate level 50 mg/L, increases the rate of removal of turbidity. With a further increase of the concentration of CPAM from 50 mg/L to 70 mg/L, the turbidity removal efficiency is reduced. Therefore, the optimum concentration for the coagulation of the droplets in the emulsion of crude oil and water is about 50 mg/L.

Discussion

Biodegradation can be described as the conversion of pollutants (hydrocarbons) by micro- organisms (bacteria) into energy, cell mass and biological waste products [29]. The biodegradation of petroleum hydrocarbons is one of the most important processes involved in the weathering and eventual removal of petroleum hydrocarbons from the marine environment. The petroleum hydrocarbons degradation experiment demonstrated that isolated bacteria are useful to assess the potential for natural attenuation of hydrocarbon-contaminated environments [18,19,25,30].

In the presence of oil compounds, various genera of biodegrading and environmental-friendly microorganisms lose their ability to grow and reproduce in polluted soils [31]. In this study, among all the isolated bacteria, *Alcanivorax* and *Idiomarina* had the highest capacity to produce bio surfactant and remove approximately 90% and 53% of crude oil, respectively.

There are other factors that tend to play an important role in increasing the activation of microorganisms and degradation of crude oil in contaminated areas [32]. In 2017, Ma et al. have shown that the

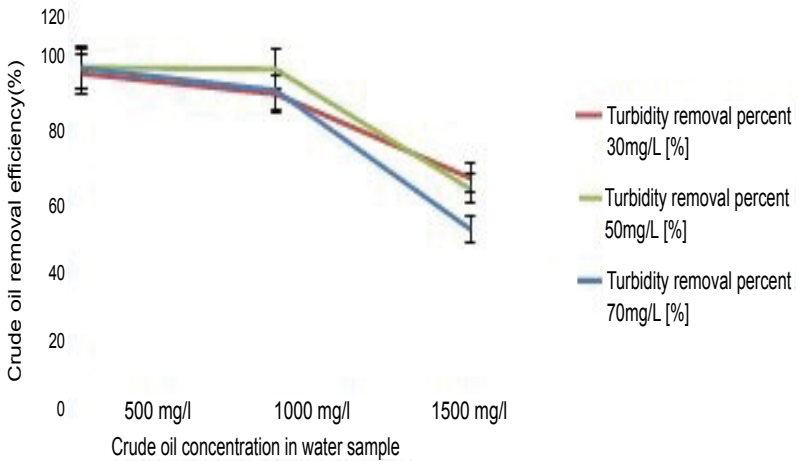

Figure 5: Turbidity removal with 30, 50 and 70 (mg/L) CPAM in three different crude oil concentrations.

Isolates	Closest hit	Accession No
T2-1	*Alcanivorax dieselolei isolate BHA25*	LK391619
T2-2-1	*Idiomarina baltica isolate BHA28*	LK391620

Table 2: Closest relatives of the *16S rRNA* gene sequences of bacteria isolated in this study.

Isolates	Growth rate (OD 600 nm)	spectrometry-based methods	Percentage of oil removal GC
BHA25	0.57	64.46	93.85
BHA28	0.77	63.17	50.12

Table 3: Growth rate and crude oil removal by strains.

Strains	Emulsification activity (E24 %)	Cell Surface hydrophobicity (BATH %)
BHA25	57	11.5
BHA28	56	5.7

Table 4: Measurement of emulsification activity (E24, %), Cell surface hydrophobicity (BATH, %) by strains in this study.

copolymerization of polyacrylamide and methacryloxyethyl trimethyl ammonium chloride (DMC as cationic polymer) has a good effect high turbid water purification [33]. The addition of CPAM coagulant is very beneficial in reducing or overcoming electrostatic barrier, in order to make collisions between individual particles with each other and thus to coagulate them [34].

The second part of this study focused basically on the flocculation efficiency of cationic polyacrylamide by using solution with 30 mg/L, 50 mg/L and 70 mg/L of polyacrylamide and contaminated water with different concentration of crude oil under the condition in which the emulsifiers was broken already by using the isolated bacteria. The hypothesis of coagulation by cationic polyacrylamide is verified by the removal efficiency of crude oil in synthetic wastewater.

Conclusion

The data obtained in the present study investigation advanced our knowledge of petroleum hydrocarbon in culture of *Alcanivorax dieselolei* and *Idiomarina baltica* isolated from sediment samples had good potential for biodegradation of crude oil and can be used for cleanup of oil- contaminated marine environments. In addition to, the observations have shown that the synthesized polymer could be effective to remove up to 90% till the crude oil concretion is less than 1000 mg/L. The 50 mg/L polyacrylamide solution showed the best efficiency for water with 500 mg/L and 1000 mg/L crude oil, but at 1500 mg/L crude oil the best efficiency was obtained by 30 mg/L solution of cationic polyacrylamide. Also, the advantages of using the synthetic polymers as flocculent are: High efficiency, consistency and uniformity and the high stability during the flocculation process. Although they are non-biodegradable, the synthetic polymers are not toxic (even if the associated monomers may be toxic). The analysis illustrated a dramatic fall in the removal efficiency, by increasing the crude oil content in water.

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