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# Screening and batch treatment of wastewater containing floating oil using oil-degrading bacteria

A. Talaie[a], M. Beheshti[b,*], M.R. Talaie[b]

[a]*Chemical Engineering Department, Jami Institute of Technology, Delijan, Iran*
[b]*Chemical Engineering Department, University of Isfahan, Isfahan, Iran*
*Email: masbeh@yahoo.com*

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ABSTRACT

Oil pollution is one of the important environmental problems in the world. In order to degrade floating crude oil in the wastewater, two soil samples were taken from the oil-contaminated soils in Ahwaz (Iran) and an oil refinery complex in Tehran (Iran). Fourteen strains of bacteria isolated from these samples. Among these bacteria a strain marked as A-14 could treat 91% of total petroleum hydrocarbons and 83% of aromatic compounds in the floating crude oil after 10 d. Also the optimum time to reach the maximum removal percent (90%) was found to be 7 d. A-14 was identified to be *Pseudomonas aeruginosa*. All isolated bacteria in this study were able to produce biosurfactant. A-12 resulted in the best emulsification index that was about 36%. However, this strain could remove only 46% and 37% of the total petroleum hydrocarbons and aromatic compound, respectively. Most of the isolated microorganisms in this study had higher efficiency for the degradation of total petroleum hydrocarbons (TPH) than aromatic compounds. In most of the previous studies, only dissolved or tiny droplet of crude oil were degraded but the pure culture of *P. aeruginosa* that we isolated them from the oil contaminated soils could degrade floating crude oil with high removal efficiency (90%).

*Keywords:* Biodegradation; Floating oil; Oil pollution; *Pseudomonas aeruginosa* bacteria; Biosurfactant

## 1. Introduction

Oil pollution can be generated as a result of spillage, leakage, discharge, exploration, production, refining, transport and storage of crude oil and fuels in the environment. Wastewater containing oil is one of the main wastes generated by the oil and gas industries. This wastewater is produced concurrently during crude oil and natural gas production, at both onshore and offshore operations [1]. Oilfield produced water is the largest part of the wastewater containing oil. This kind of wastewater is separated from produced oil in oilfields. In the countries where oilfields are in the mid- or final-stage of development, the produced water is the major source of the oily wastewater [2].

Reducing oil either in regularly discharged water effluent, or in the surface water contaminated by accidental fluid releases, is a significant challenge. Due to the characteristics of oily waste water, however, it is difficult to treat it using commercial methods. Crude oil floating in the water can easily be removed by filtration or an alternate physical system. Dissolved hydrocarbons inspite of their small volume, are highly toxic and not easily broken down in the environment. If the wastewater is to released as surface water, it must be treated to remove not only floating oil, but also virtually all of the dissolved oil component that contribute to the high Chemical Oxygen Demand (COD) of the water.

*Corresponding author.

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However, for tiny oil droplets and dissolved oil, physical removal methods are not effective and may be expensive.

Biological system can remove dissolved oil, tiny oil droplets and floating oil together. Biological treatment is an effective and economical way that can be used in oil de-emulsification and wastewater treatment [3]. Crude oil degrading microorganisms, namely bacteria, yeast and fungi, which can grow using crude oil as carbon source, have been reported as oil degraders [4]. Biodegradation of crude oil by these indigenous microorganisms is one of the primary choices for removal of petroleum and other hydrocarbons from the environment. Several researchers have studied the use of microorganism to decompose petroleum products and have shown this to be a promising technological alternative [5–8]. For instance, Li et al. [2] have studied the biodegradation of wastewater produced from oilfield with an oil-degrading bacterium. They could remove 80% of crude oil based on COD. Tellez et al. [1] evaluated the performance of an activated sludge system for removing total petroleum hydrocarbons (TPH) from oilfield produced water. This activated sludge process could remove approximately 98% of TPH after filtration and sedimentation with solid residence time of 20 d. In another study [9] the effect of iron, nitrogen and phosphorus on the biodegradation of petroleum in the seawater was studied and it was shown that nitrogen and phosphorus were necessary for biodegradation of crude oil. However, the presence of iron did not influence biodegradation rate which can be explained by the high levels of iron in seawater. Oh et al. [10] evaluated the effects of nutrients on the various parts of crude oil biodegradation in the upper intertidal zone with two different isolated bacteria. For one of the bacteria the rate of removal of the aliphatic and aromatic hydrocarbons reached its maximum value during 0–21 d and for the other it took at least 91 d to reach the maximum rate. The presence of rhamnolipid was shown to be vital for the onset of biodegradation of crude oil by *P. aeruginosa*. When glycerol was used as carbon source, the higher amount of rhamnolipid was produced during biodegradation process [11]. The oily sludge biodegradation was optimal at a pH of 7.5–7.8, temperatures of 20 °C or above, and C/N and C/P ratios of 60:1 and 800:1, respectively. The effect of four dispersants on biodegradation and growth of bacteria in the presence of crude oil as the only source of carbon was investigated. Good growth of microorganisms was observed when only one of the dispersants was used. Vieira et al. [8] studied the effects of fuel concentration (diesel and gasoline), nitrogen concentration and culture type on the biodegradation of synthetic effluent. An experimental design with two levels and three variables was used. The nitrogen concentration of 550 mg/l and the fuel concentration of 4% (v/v) were found to be the optimum levels. The biodegradability study showed the TPH removal of 90 ± 2% during 49 d.

The main objective of the present study was to investigate the capabilities of 14 bacteria, isolated from different environmental sources, to remove floating crude oil from wastewaters. Also the ability of these bacteria to emulsify their environment was examined by using emulsification index (E24) test.

## 2. Materials and methods

### *2.1. Sampling procedure*

Two soil samples were taken from the oil-contaminated soils around a gas station in Ahwaz (Iran) and an oil refinery complex in Tehran (Iran). These soils have been contacted with oil for several decades. Also two oil-contaminated water samples were obtained from the effluent of processing wastewater and sanitary wastewater treatment systems of Tehran refinery complex. Soil samples were taken from surface layer (0–5 cm) and collected in sterile two-liter glass bottles. Water samples were taken from 80 cm depth in two-liter sterile plastic containers. The samples were maintained at 4 °C.

### *2.2. Microorganisms screening and cultivations*

5 g of each soil sample was mixed with 450 ml sterile physiological serum (9 g/l NaCl) in sterile Erlenmeyer flasks. The mixture was shaken using a rotary shaker at 160 rpm for 30 min.

The medium for isolating (MI) oil degrading bacteria contained g/l: $NaNO_3$ 1, $MgSO_4$ $7H_2O$ 0.1, $CaCl_2$ 0.01, $FeSO_4$ 0.001, $K_2HPO_4$ 0.5, $KH_2PO_4$ 0.5 and pH of this medium was adjusted to 7.2 by adding suitable amounts of NaOH 0.5 M. Isolation of the oil degrading bacteria was performed as follows: water samples and supernatant from soil samples were inoculated into 300 ml Erlenmeyer flasks containing 100 ml of MI and 1 ml crude oil. The flasks were shaken at 30 °C on a rotary shaker at 160 rpm. After 72 h, 1 ml of each flask contents was inoculated into a new 300 ml Erlenmeyer flasks containing 100 ml of MI and 1 ml crude oil. The mixtures was shaken at 160 rpm and 30 °C. After 72 h enrichment, 0.1 ml of the supernatant was inoculated onto selective MI agar plates. The plates were incubated at 30 °C for 48 h. Colonies with different characteristics (color and size) were then repeatedly transferred to the MI agar plate until pure cultures of each kind of colonies were obtained. All pure cultures were maintained on MI agar slants at 4 °C and transferred every four months.

The liquid medium used for cultivation was nutrient broth. The bacteria were cultivated in 500 ml Erlenmeyer flasks containing 100 ml liquid medium at 30 °C. The flasks were shaken at 160 rpm for 24 h. Cells were separated by 15 min centrifugation at 3000 rpm, and washed with sterile physiological serum to eliminate medium components. The pellet was then suspended in the same volume of sterile distilled water.

### 2.3. Oil-degradation capacity of the bacteria

In order to evaluate the oil-degradation capacity of the bacteria, 500 ml Erlenmeyer flasks containing 100 ml MI with 10% cell suspension solution and 1 ml crude oil were incubated on a rotary shaker at 160 rpm for 10 d. Then residual TPH of the culture mixture was determined by spectrometric method.

### 2.4. Biomass determination

Biomass content was determined by measuring optical density of the culture using the spectrophotometer at 600 nm. The actual biomass in dry weight (g/l) was obtained from the constructed calibration curve.

In order to evaluate bacteria growth rate the following procedure was used:

The bacteria were inoculated into 100 ml of MI containing 1% crude oil. The cultures were then shaken at 160 rpm and 30 °C for 7 d. At the end of this period, the mixture has been kept still for 1 h. At the end, the optical density of the aqueous phase was measured using spectrophotometer at 600 nm.

### 2.5. Emulsification index (E24)

For estimation of the emulsification index, 6 ml of kerosene was added to 4 ml of the culture broth in a graduated tube and was shaken at high speed for 2 min. The emulsion stability was determined after 24 h. The E24 index was calculated from the ratio of emulsion layer thickness to the total thickness.

### 2.6. Spectrometric analyses

In order to measure the amount of oil remaining in the mixture, the pH of the culture was adjusted to 2 or less by adding HCl 0.2 m.The residual oil in the culture was extracted by 25 ml carbon tetrachloride. The mixture was left for 15 min for phase separation and equilibration. Afterwards, 1 ml of the lower organic phase was transferred to a new 100 ml volumetric flask and was made up to 100 ml with carbon tetrachloride. In order to remove any suspended particles, which may interfere with the absorbance measurement, the sample was centrifuged for 15 min at 3000 rpm. TPH concentrations were quantified by using spectrometric at 400 nm at which the maximum absorbance for carbon tetrachloride/crude oil mixture occurs [12]. The calibration curve of the carbon tetrachloride/crude oil mixture at 20°C was obtained as:

$$y = 0.1219x \tag{1a}$$

$$R^2 = 0.994 \tag{1b}$$

Where $x$ is oil concentration (v/v) % and $y$ is the absorbance of carbon tetrachloride/crude oil mixture at 400 nm.

The percentage crude oil removed from the culture was calculated using the following equation:

$$(O_i - O_r)/O_i \times 100\% \tag{2}$$

Where $O_i$ is the initial oil concentration and $O_r$ is the residual oil concentration after biodegradation. All experiments were performed in duplicates and the error was less than 6%. Aromatic concentrations were quantified by using spectrometric ultraviolet (UV) at 254 nm and also between 190 nm to 500 nm using the Jasco V-570 spectrophotometer [13].

### 2.7. Gas chromatography analyses (GC)

Samples at the end of the cultivation were analyzed with GC to verify the spectrometric method results and to identify some of the hydrocarbons of the crude oil. GC analyses were conducted with a Varian GC (CP-3800) equipped with a Varian CP SIL8 capillary column (25 m × 0.32 mm i.d. × 0.1 µm film thickness) and a flame ionization detector (FID). Nitrogen was used as carrier gas. FID and injector temperatures were 325 °C and 285 °C, respectively.

The total run time was 30 min. The initial oven temperature was 30 °C. After 3 min. the oven temperature was increased to 300 °C at the rate of 15 °C/min. The oven temperature was then held at this temperature for 5 min. The oven temperature was again increased to 325 °C at the rate of 15 °C/min and was again kept at this temperature for the remainder of the run time.

### 2.8. Identification of microorganism

The best strain was selected based on its capability to decrease TPH and aromatic compounds. This strain was then identified by various methods including Gram staining, oxidase test, cell morphology, colony morphology, cultivation on the nutrient agar and nutrient broth and caloric pigments investigation. Cell morphology, Gram staining and oxidase tests were performed by the methods reported previously [14].

## 3. Result

### 3.1. The capability of the bacteria to remove floating crude oil

Fourteen strains of bacteria isolated from gas station soil in Ahwaz city, processing wastewater, sanitary wastewater and soil containing crude oil of the Tehran oil refinery were able to grow using crude oil as the sole carbon source. The strains marked as A-1 to A-14 were used to treat the wastewater containing crude oil. In Fig. 1 the isolated strains are compared in terms of their ability to remove TPH and aromatic compounds. Differences are evident in the decrease of TPH and aromatics for different microbial inoculums. The highest degradation was obtained for the strain A-14 which decreased about 91% of TPH and 83% Aromatic compounds after 10 d. The ability of the strain A-3 for the removal of aromatic compounds was comparable with that of the strain A-14 but, however, this strain was less efficient for the degradation of TPH compared to the strain A-14 (Fig. 1). The strain A-14 was therefore chosen for further experimental study. After treating the oil containing with the strain A-14, the spectra of the extracts were obtained between 190 nm and 500 nm. Fig. 2 shows the spectra for the extracts of treated and untreated wastewater. The area under the absorption curve in the range of 190 nm–500 nm was reduced significantly after treatment indicating the ability of the bacterium for degradation of oil pollution Alkenes, alkynes, compounds containing carbonyl and thiol groups, organic sulfides and aromatics have the maximum absorbance at 175 nm, 170 nm, 188 nm, 200–220 nm and 254 nm, respectively [15]. Thus, the area under the spectra curve in the range of 190 through 300 nm can be considered as the concentration of crude oil.

In order to evaluate the accuracy of the spectrometric method, three GC analyses were performed for the untreated samples (Fig. 3) and treated samples with the strains A-14 (Fig. 4) and A-3 (Fig. 5). The TPH removal for the strains A-3 and A-14 was 79% and 91%, respectively at the end of the 10 d experimental period.

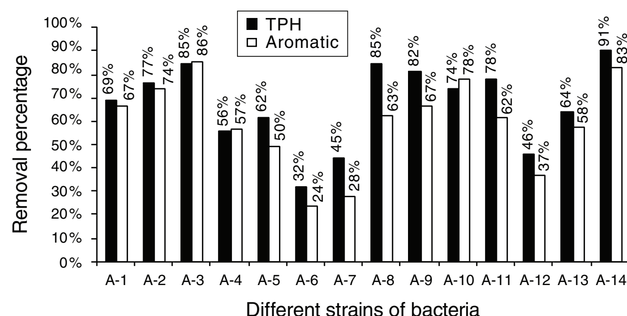


Fig. 1. Removal of TPH and aromatic compounds from wastewater containing crude oil for different isolated strains.

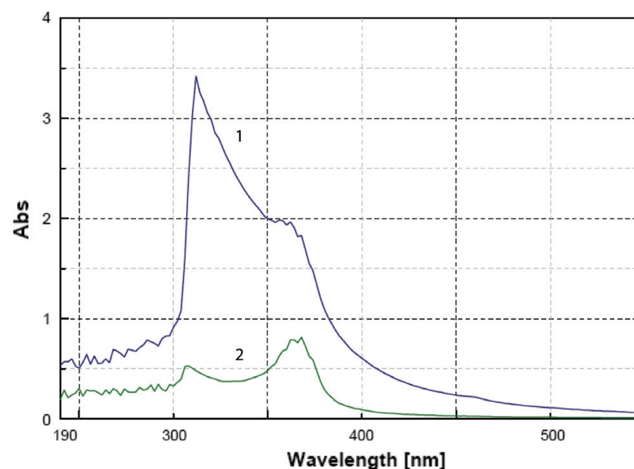


Fig. 2. Changes of spectra of the wastewater before and after treatment with the strain A-14; (1) before treatment and (2) after treatment.

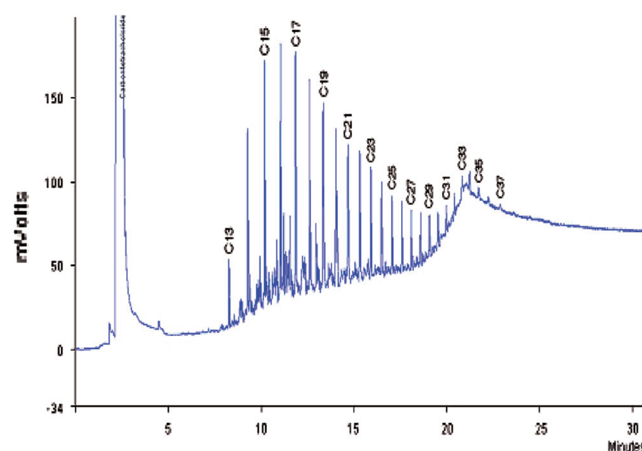


Fig. 3. The GC chromatogram of the extract before treatment.

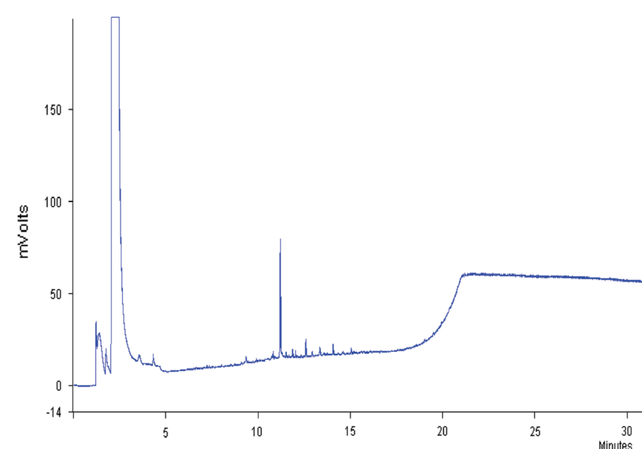


Fig. 4. The GC chromatogram of the extract after treatment with A-14.

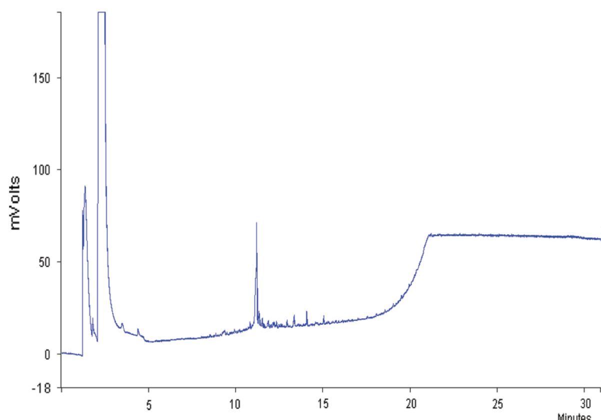


Fig. 5. The GC chromatogram of the extract after treatment with A-3.

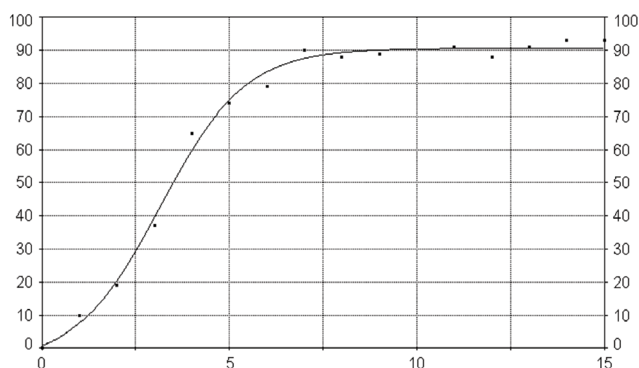


Fig. 6. Profile of oil removal percentage versus time for the samples treated with A-14.

Reasonable agreement was observed between the results from the GC analysis and spectrophotometric analysis at 400 nm.

The growth of isolated bacteria in oily environment was investigated by inoculating bacteria into mineral environment containing 1% crude oil for 7 d. The value of optical density at 600 nm ($OD_{600}$) was measured at the end of experiment. The growth rate can be considered as medium, high and excellent if the value of $OD_{600}$ is in the range of 0.2–0.4, 0.41–0.6 and 0.61–0.8, respectively [16]. In this study, $OD_{600}$ was 0.661 for the strain A-14. Therefore, this strain can be reported as a high growth rate bacterium. Fig. 6 shows the percentage of degraded oil pollution with time for samples treated with the strain A-14. The optimum time to reach the maximum removal percentage (90%) was found to be 7 d (Fig. 6).

## 3.2. Emulsification index (E24)

The E24 indices of the treated samples were determined for the 14 isolated strains. Fig. 7 shows the aromatic compounds and TPH removal percentage versus E24 indices for the strains.

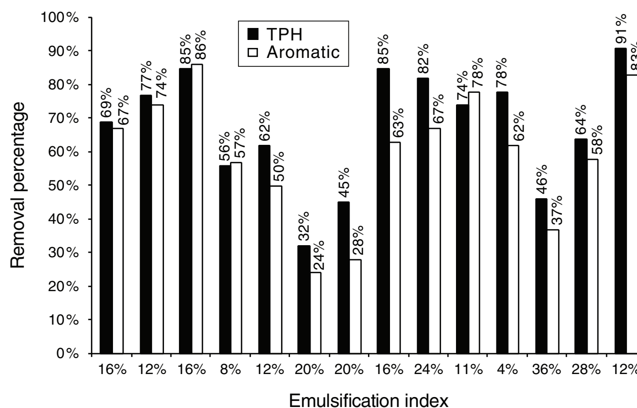


Fig. 7. Removal percentage of TPH and aromatic compound versus emulsification index.

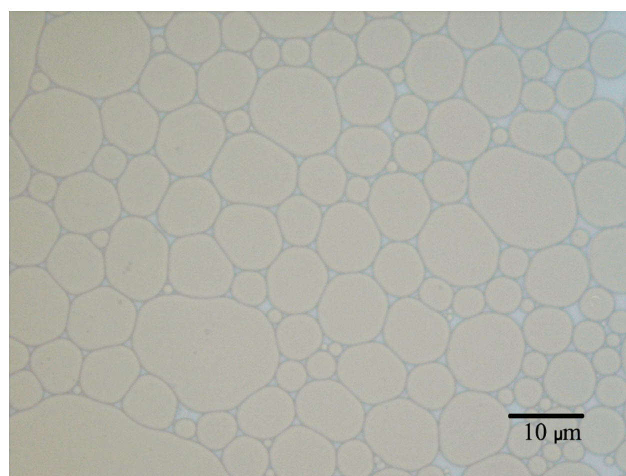


Fig. 8. Tiny oil drops formed due to biosurfactants production.

The highest value of E24 index, which was approximately 36%, belonged to the strain A-12 which eliminated 46% of TPH.

Fig. 8 shows formation of tiny oil drops as a result of biosurfactants production. This picture was taken using light microscope with magnification of 1000. As it can be seen in this picture, most of the oil drops have the size of less than 10 μm.

## 3.3. Identification of isolated bacteria

The bacterium marked A-14 which was isolated from oily soils demonstrated higher efficiency for the removal of TPH and aromatic components compared to the other strains. This bacterium was Gram negative, had a rod shape and was motile. The oxidase and catalase tests for this strain were positive. This strain could not grow in

anaerobic condition, so it is an obligatory aerobic microorganism. The core of A-14 colonies formed by its cultivation in nutrient agar was projected slightly and there was irregular pattern around colonies. After 3 d from incubation on nutrient agar, this bacterium produced green pigments around its colonies. The pigments were soluble in water and acetic acid but could not be dissolved in chloroform. Uniform turbidity was observed due to culture of this bacterium in the nutrient broth. At high temperatures (40 °C) this bacterium produced green pigments in nutrient broth. All of these characteristics can be attributed to the pseudomonas strains and production of green pigment is a common property of *P. aeruginosa*.

## 4. Discussion

### *4.1. Biodegradation of crude oil by isolated microorganisms*

Biodegradation of crude oil by microorganisms appears to be a natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecular compounds such as biosurfactants. In other words, biodegradation of crude oil contaminants can be described as the conversion of chemical compounds by microorganisms into energy, cell mass and biological products. All isolated microorganisms in this study could grow on crude oil as the sole carbon source. Among the isolated bacteria, the strain A-14 had the highest ability for the removal of crude oil and aromatic compounds. The measurement of the residual crude oil in the samples at different time intervals showed that the optimum time to reach maximum removal percentage (90%) was 7 d (Fig. 6). Tellez et al. [1] achieved a 98% removal of TPH in an activated sludge reactor with residence time of 20 d. They could remove tiny droplets of crude oil and dissolved petroleum compounds in their reactor. Li et al. [2] isolated an oil-degrading bacterium that could remove 80% of oil in produced water after 7 d treatment. Vieira et al. [8] could decrease 90% of TPH (diesel and gasoline) in 49 d using an isolated bacterium In the present study, we isolated a bacterium capable of removing 91% of floating crude oil in 7 d. In comparison with other studies, this bacterium demonstrated higher removal efficiency, lower residence time, and ability to remove high concentrations of crude oil (up to 10 ml/l) in wastewater.

The strain A-14 was identified as *P. aeruginosa*. Many researchers such as Liang et al. [11] have studied biodegradation of crude oil by *P. aeruginosa*. They could remove more than 58% of crude oil by adding a surfactant. The strain A-14, however, removed high concentration of petroleum compounds without need to any surfactant. Li et al. [11] isolated four bacteria for the removal of crude oil in produced water. One of these bacteria was *P. aeruginosa*. They could remove more than 70% of crude oil in 4 d by immobilized microorganisms.

Most of the isolated microorganisms in this study had higher efficiency for the degradation of TPH than aromatic compounds (Fig. 1). This may be because of the existence of nuclear molecules in the aromatic compounds which are more resistant to biodegradation.

### *4.2. Production of biosurfactants*

Bacteria can consume oil as carbon source in various different forms including soluble oil, very small oil droplets, and large oil droplets. Since only 0.02% of crude oil is water soluble, emulsification of crude oil is necessary for improving the bioavailability of insoluble hydrocarbons to microorganisms by increasing the interfacial area between the aqueous- insoluble hydrocarbons. The size of oil droplets suspending in water depends on the concentration of the surfactant agent. Bacteria release different kinds of surfactants for emulsifying oil in water and improving the availability of the hydrophobic hydrocarbons to microorganisms. Biosurfactants are amphiphilic compounds produced by microorganisms which either adhere to cell surfaces or are excreted extracellularly in the growth medium. The role of surfactants on the biodegradation of crude oil contaminated wastewater has been discussed in the literature.

For initiating biodegradation of crude oil, the presence of surfactants in the culture medium is necessary [11]. The E24 indices for the strains A-14 and A-12 were 12% and 36%, respectively. Despite the lower E24 index, the strain A-14 had significantly higher TPH removal (91% compared to only 46% for the A-12 strain). Thus, it is likely that the rate of oil removal is nor proportionally related with the biosurfactant concentration and it seems that a small amount of surfactant is enough for the biodegradation Microscopic study of the samples inoculated with *P. aeruginosa* indicated that produced biosurfactant could make a stable emulsion (Fig. 8).

In summary, in all previous studies, dissolved or tiny droplet of crude oil were degraded but we could degrade floating crude oil by the pure culture of *P. aeruginosa* isolated from environment.

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