Desalination and Water Treatment
www.deswater.com

28 (2011) 46–54
April

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doi: 10/5004/dwt.2011.2199

Biodegradation of heavy crude oil in wastewater by an efficient strain, ERCPPI-1

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Received 1 February 2009; Accepted 1 July 2010

ABSTRACT

The potential biodegradation of heavy crude oil from wastewater was assessed based on the development of a fermentative process with a new strain of *Enterobacter cloacea* (ERCPPI-1) which was isolated from heavy crude oil in south of Iran, when cultured in a basal mineral medium using heavy crude oil as the sole carbon source. The effects of heavy crude oil concentration, temperature, pressure, pH and salinity on the growth rate of ERCPPI-1 in the presence of heavy crude oil as the sole carbon source were investigated. The results showed that ERCPPI-1 has a good potential for biodegradation of heavy crude oil in the concentration ranges of 0.25–10%, temperatures up to 70 °C, pressures up to 6000 psia, pH ranges of 4–10, and salinity up to 15%. However, as the concentration of heavy crude oil is increased from 0.25% to 10%, the percentage of degradation by the strain is decreased from 76.3% to 19.1%. Temperature of 40 °C and pH of 7.0 were found to be the optimum conditions for maximum biodegradation rate. The experiments also showed that the strain ERCPPI-1 was able to produce a type of biosurfactant, using heavy crude oil as the sole carbon source, with high oil spreading and emulsification properties. The experiments performed in piston–cylinder systems demonstrated that the efficiencies of oil recovery and biodegradation of hydrocarbons are at a reasonable rate. These results suggest that strain ERCPPI-1 has the ability to degrade heavy crude oil under ex situ and in situ conditions.

Keywords: Enterobacter cloacea; Heavy crude oil; Biodegradation; Biosurfactant; Ex situ; In situ

1. Introduction

Petroleum hydrocarbon is the principle source of energy in the world and its wide scale production, transport, use and disposal has made it a major contaminant in both prevalence and quantity in the environment [1]. Crude oil components are of environmental concern due to their toxic, mutagenic, and carcinogenic properties [2–4]. Consequently, crude oil is deleterious to a wide spectrum of plants, animals and microbial communities, through oxygen stress and direct toxic effects [5]. On the other hand, the concentrations of the toxic components in heavy and extra-heavy crude oils are higher and the problem is more severe. The traditional treatment of oil-contaminated environments, such as containment and collection using floating booms, adsorption by natural or synthetic materials, etc., cannot degrade the crude oil thoroughly [6]. Previous studies have established that microbial degradation is an important process in determining the fate of spilled oil, implying that microbial biodegradation is one of the principal processes for removal of non-volatile crude oil components from oil-contaminated environments [7]. On the other hand, biodegradation-based alternatives are among suitable

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Presented at the 1st International Conference on Water and Wastewater Treatment, April 21–22, 2010, Isfahan, Iran

options, because of their low cost and ability to convert contaminants to harmless end products. Biological methods can also have an advantage over the physicochemical treatment methods in removing spills as they offer in situ biodegradation of oil fractions by the microorganisms.

Less research has been done with biodegradation of light and heavy crude oil under extreme environmental conditions [1,8–13]. Since the fate of petroleum hydrocarbons in the environment is largely determined by the local environmental conditions, which influence rates of microbial growth, this research was carried out to explore the potential of the isolated bacterial strain to degrade Soroosh heavy crude oil at extreme temperatures, pressures, pH, salinity and heavy crude oil concentrations under ex situ and in situ conditions.

2. Materials and methods

2.1. Chemicals

Benzene, glycerol, glucose, sucrose, fructose, toluene, ethyl benzene, xylene (mixture of isomers), chloroform, and ethanol, were provided from Merck Company, all in analytical grade. Olive oil is purchased from Aceites Benja s.a. Company, spain. Gachsaran (API = 32.1) and Soroosh crude oils (API = 18.5) that were used throughout this study had been selected from the oil fields in south of Iran. All other chemicals were also of analytical grade and purchased from Merck Company.

2.2. Bacteria

The bacterial strain that used in this study, ERCPPI-1, was isolated from samples of Soroosh heavy crude oil and Soroosh heavy crude oil contaminated soil in south of Iran. The isolation, screening and selection program of the biosurfactant producing bacteria from heavy crude oil performed by the Shiraz Institute of Biotechnology in Iran. Heavy crude oil samples were collected in pre-sterilized glass bottles and transported to the laboratory for analyses. Enrichment and isolation of oil degrading bacterial cultures were done using mineral salts medium with heavy crude oil as substrate and a serial dilution-agar plating technique on nutrient agar medium. The isolated bacteria were characterized by using standard biochemical tests and 16S rDNA analysis (API 20E kit, Biomérieux, Mercy, France).

2.3. MSM medium

Nutrient-rich agar medium containing 1% yeast extract, 1.5% nutrient broth, 1% ammonium sulfate and 2% agar and Luria-Bertani (LB) medium, consisting of 1% Bacto tryptose, 0.5% Bacto yeast extract, and l% NaCl were used to culture isolates. Minimal salt medium (MSM) was used throughout the study and contained of the following basal components (per liter): KH_2PO_4, 2.7 g, K_2HPO_4, 13.9 g, $NaNO_3$, 1.0 g, NaCl, 1.0 g, yeast extract, 0.5 g. The basal minimal medium was supplemented with 10 ml of a stock solution of 0.5% (w/v) $MgSO_4 \cdot 7H_20$ along with 10 ml of 1% (w/v) $(NH_4)_2SO_4$ which were sterilized by filtration through 0.2 μm membrane filters (Millipore Corp., Bedford, MA, USA). A trace element solution (10 ml) consisted of following composition (per 1000 ml): EDTA 0.5 g, $MnSO_4 \cdot H_2O$ 3.0 g, NaCl 1.0 g, $ZnSO_4 \cdot 7H_2O$ 0.10 g. $CuSO_4 \cdot 5H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$, 0.10 g, $AlK(SO_4)_2$ 0.01 g, $CaCl_2 \cdot 2H_2O$ 0.10 g, $Na_2MoO_4 \cdot 2H_2O$ 0.01 g, $NiCI_2 \cdot 6H_2O$ 0.003 g, H_3BO_3 0.01 g which was sterilized by filtration and added to the medium. Initial pH of the medium was adjusted to 7.0.

2.4. OD measurement

The total content of the shaking flask was mixed with 100 ml of toluene and shake vigorously for 2 min on a vortex. The mixtures were centrifuged at 10,000 rpm and 4 °C for 15 min using a high-speed refrigerated centrifuge (MPW, 351R, Germany). The toluene phase was collected for determining hydrocarbon content, and the process was repeated for two more times. At the end, the aqueous phase containing cell pellets was used for measurement of OD_{600}.

2.5. Screening of the microorganisms for biodegradation of Soroosh crude oil

A loopful of the bacterial cultures (12 h old) were inoculated in MSM medium with 1.0% w/v Soroosh crude oil as carbon source. They were kept in a shaker at 150 rpm at 40 °C for a period of 14 d. Time-course samples of culture medium were drawn at appropriate time intervals and cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer (Optima, SP3000 Plus, Japan). Isolate with the highest percentage of degradation of crude oil was selected.

2.6. Potential of the isolated strain for biosurfactant production

The very low water solubility of heavy crude oil has the potential to limit the capacity of bacteria, which generally exist in aqueous phases, to access and degrade the substrate. Therefore, for increasing the solubility and emulsification of the immiscible phases and bioavailability of the insoluble substrate for microorganisms, a chemical surfactant (Tween 80, 0.1% w/v) had been used. As shown in Section 3, Tween 80 has an inhibitory effect on the growth of the ERCPPI-1 in contact with

Soroosh crude oil as the carbon source. The potential of the ERCPPI-1 for production of biosurfactant in contact with benzene, glycerol, glucose, olive oil, sucrose, fructose, toluene, ethyl benzene, xylene, Gachsaran crude oil (a relatively light crude oil) and Soroosh crude oil as the sole carbon (all in 1.0% w/v) had been investigated. Time-course studied were carried out by determination of bacterial cell growth (OD_{600}), dry cell weight (DCW, g/l), biosurfactant production (BP, g/l), surface tension (ST) and interfacial tension (IFT) measurements (mN/m), oil displacing area (ODA, cm^2) and emulsification index (E_{24}, %). Bacterial cell growth was monitored by measuring the optical density of the broth at 600 nm. Dry cell weight was determined by centrifugation of a 20 ml culture broth and cell pellets were washed with distilled water twice and dried by heating at 50 °C until constant weight attained. Oil displacing area by cell-free samples was measured according to Morikawa et al. [14]. Emulsification index (E_{24}) of ERCPPI-1 strain was evaluated according to Cooper and Goldenberg [15]. The interfacial tension was measured at 40 °C using a digital tensiometer (Krüss, SITE 100, Germany) using the spinning drop method. Surface tension measurements were carried out against Gachsaran crude oil by pendant drop method (Krüss, DSA 100, Germany). The isolation and purification of produced biosurfactant was done according to Bagheri et al. [16].

2.7. Determining the degradation percentage

The total hydrocarbons in the treatments were determined as described by Rahman et al. [17]. The calibration curve for determination of Soroosh heavy crude oil concentration in toluene is shown in Fig. 1.

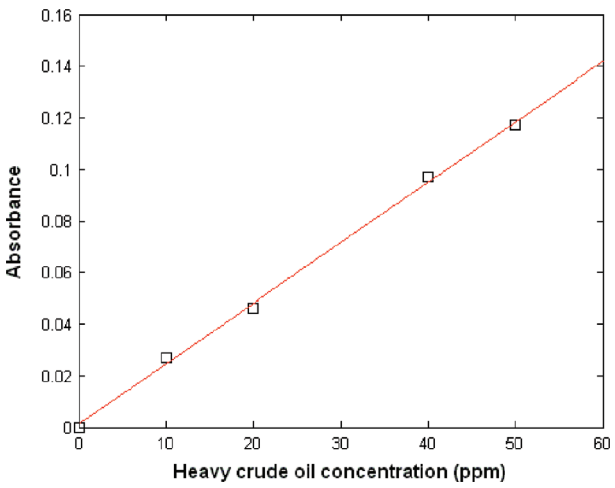

Fig. 1. Calibration curve for determining petroleum hydrocarbon content.

2.8. Biodegradation of heavy crude oil

The bacteria from overnight culture at the log phase of growth were transferred to 500 ml flasks, each containing 100 ml of sterile defined MSM medium with 0.5% w/v of Soroosh crude oil. The flasks were incubated in a shaker at 150 rpm and 40 °C for 21 d (three weeks). The bacterial growth, percent of degradation, sulfur and heavy metals removal from Soroosh crude oil were determined. Hewlett–Packard gas chromatograph equipped with atomic emission detector was used for the determination of metal complexes. The total sulfur content was determined by X-ray fluorescence spectroscopy.

2.9. Effect of pH

The effect of pH on growth and degradation of 0.5% (w/v) Soroosh crude oil was studied. MSM medium with Soroosh crude oil was prepared at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using 6N HCl/1N NaOH. The flasks were inoculated with bacterial culture and incubated at 40 °C and 150 rpm for 21 d. The bacterial growth, percent of degradation, sulfur and heavy metals removal from Soroosh crude oil were determined.

2.10. Effect of temperature

The influence of temperature (30, 40, 50, 60 and 70 °C) on growth and degradation of crude oil by the bacterial isolate was studied using MSM medium with 0.5% (w/v) of Soroosh crude oil at pH 7.0 and 150 rpm for 21 d. The bacterial growth, percent of degradation, sulfur and heavy metals removal from Soroosh crude oil were determined.

2.11. Effect of concentration of Soroosh crude oil

Biodegradation of Soroosh crude oil with ERCPPI-1 was performed with various concentrations of crude oil (0.25, 0.5, 1, 2.5, 5, 7.5 and 10% w/v). For all the concentrations, the experiment was conducted at 40 °C, pH 7.0 and 150 rpm. The inoculated flasks were incubated and the bacterial growth, percent of degradation, sulfur and heavy metals removal from Soroosh crude oil were determined.

2.12. Effect of pressure

To investigate the potential of the strain ERCPPI-1 under in situ conditions and various pressures, the tests performed in piston–cylinder systems that are shown in Fig. 2. The influence of pressure (1500, 3000, 4500 and 6000 psia) on growth and degradation of heavy crude oil by the ERCPPI-1 was studied using MSM medium with 0.5% (w/v) of Soroosh crude oil at 40 °C, pH 7.0 and 150

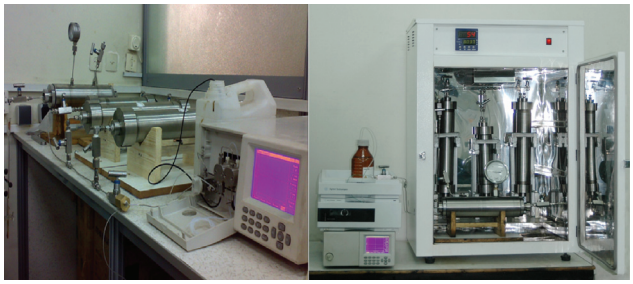

Fig. 2. Piston–cylinder system.

rpm. The bacterial growth, percent of degradation, sulfur and heavy metals removal from Soroosh crude oil were determined.

2.13. Effect of salinity

The effect of salinity (0, 2.5, 5.0, 7.5, 10, 12.5 and 15% w/v) on growth and degradation of crude oil by the bacterial isolate was studied using MSM medium with 0.5% (w/v) of Soroosh crude oil at 40 °C, pH 7.0 and 150 rpm. The bacterial growth, percent of degradation, sulfur and heavy metals removal from Soroosh crude oil were determined.

3. Results and discussion

The enrichment and isolation procedure resulted in two pure cultures able to grow in MSM medium with heavy crude oil as carbon source. These isolated bacteria belonged to the genera *Enterobacter cloacea and Enterobacter sakazakii* among which the strain *Enterobacter cloacea* (ERCPPI-1) had the highest potential for degradation of heavy crude oil as the sole carbon source. Therefore, this strain was selected for investigating the effects of extreme environmental conditions on the biodegradation of heavy crude oil.

The potential of the strain ERCPPI-1 for biosurfactant production in contact with benzene, ethyl benzene, fructose, glucose, glycerol, olive oil, sucrose, toluene, xylene, Gachsaran crude oil and Soroosh crude oil as the sole carbon source has been shown in Fig. 3. The best biosurfactant production of 1.63 g/l, interfacial tension of 0.8 mN/m against Gachsaran crude oil and surface tension of 31.7 mN/m was obtained when the cells were grown in the presence of olive oil as carbon source. In contact with Soroosh heavy crude oil these results were 0.33 g/l, 7.6 mN/m and 42.4 mN/m, respectively. The obtained results showed that strain ERCPPI-1 is able to produce biosurfactant in contact with a lot of chemicals as carbon source and a wide range of environmental conditions.

The infrared and mass spectroscopy, ^{1}H NMR, ^{13}C NMR and thermal gravimetric analysis (TGA) of the

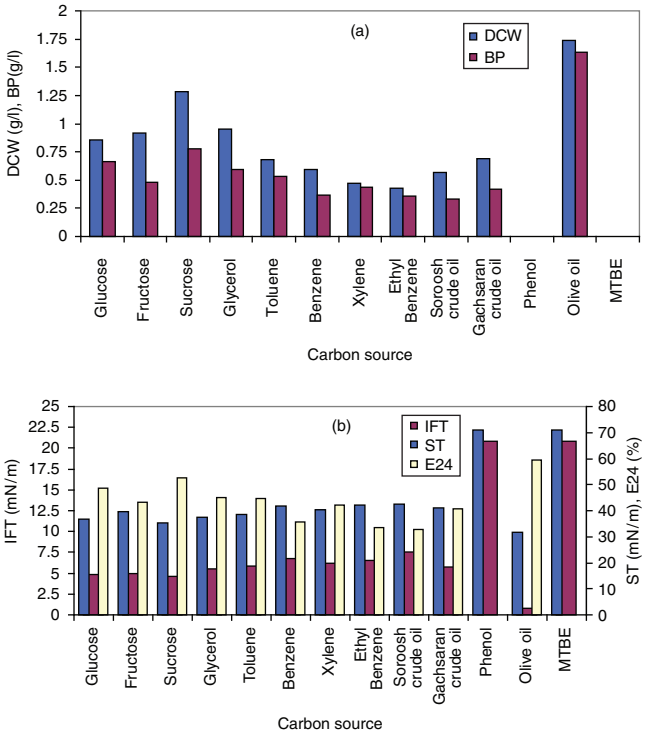

Fig. 3. Effect of various carbon sources on (a) growth and biosurfactant production of the strain ERCPPI-1, (b) surface activity of biosurfactant produced by strain ERCPPI-1 (T = 40 °C, salinity=0.1%, P = 14.7 psia, pH = 7.0, carbon source concentration = 1.0%, rotation =150 rpm).

purified biosurfactant showed that it is a exopolysaccharide type surfactant [16,18].

The experiments were carried out in the presence of the produced biosurfactant by the strain ERCPPI-1, with a chemical surfactant (Tween 80), at the concentration of 0.1% (w/v), and without the surfactant. The results presented in Figs. 4–8 show that the chemical surfactant has an inhibitory effect on the growth rate of the strain and biosurfactant accelerates the degradation rate. Both enhancement and inhibition of biodegradation of hydrocarbons have been observed by use of chemical surfactants [19].

The effects of Soroosh crude oil concentrations on the growth of the bacterial culture and crude oil degradation were tested. The results as shown in Fig. 4 clearly showed that the isolated bacteria could carry out a maximum of 76.3% of degradation at 0.25% (w/v) concentration of heavy crude oil after 21 d of incubation. At similar operating conditions, the effects of crude oil concentrations were investigated on the percentage of sulfur and heavy metals removal of the crude oil and the obtained results were presented in Fig. 4. As the concentration of crude oil increases, the extent of degradation was inversely decreased [20]. Compounds such as saturates, aromatics and polar compounds present in

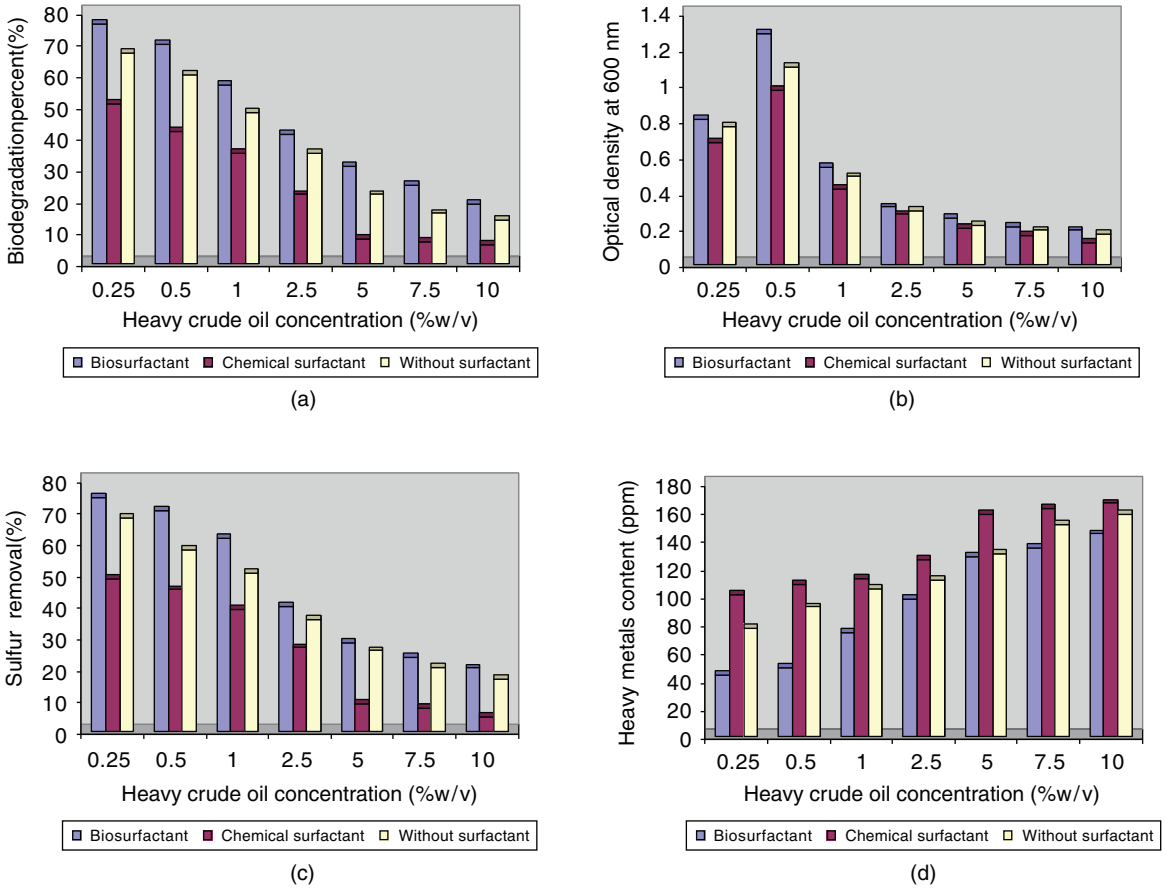

Fig. 4. Effect of heavy crude oil concentration on (a) percentage of biodegradation and (b) growth of the strain ERCPPI-1, (c) percentage of sulfur removal and (d) heavy metals content of Soroosh heavy crude oil (T = 40 °C, salinity = 0.1%, P = 14.7 psia, pH = 7.0, rotation = 150 rpm).

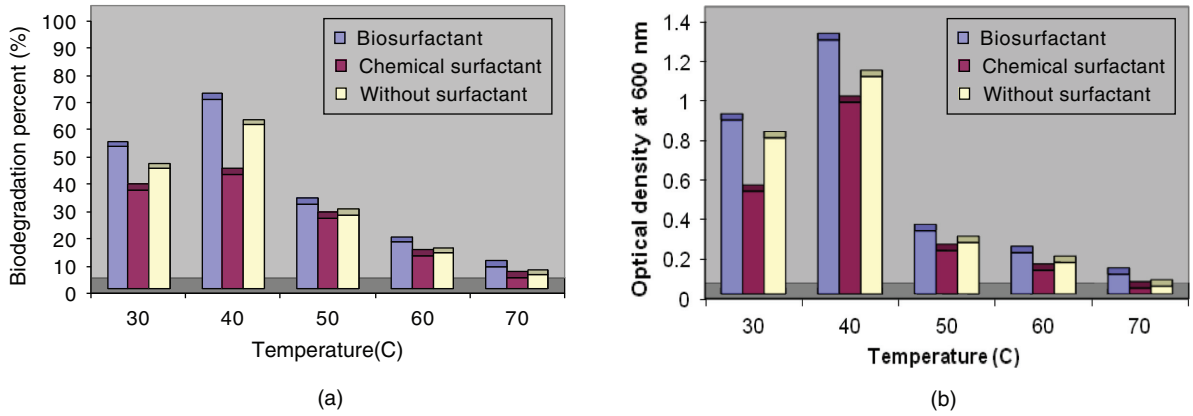

Fig. 5. Effect of temperature on (a) percentage of biodegradation and (b) growth of the strain ERCPPI-1 (pH = 7.0, salinity = 0.1%, P = 14.7 psia, heavy crude oil concentration = 0.5%, rotation = 150 rpm).

different crude oil samples were degraded in different extents [1,21].

Rahman et al. [1] isolated five strains of *Pseudomonas* sp. DS10-129, *Bacillus* sp. DS6-86, *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66 and *Flavobacterium* sp. DS5-73 and utilized them for studying the biodegradation of Bombay High (BH) crude oil. At 1.0% of crude oil concentration, the maximum percentage of degradation

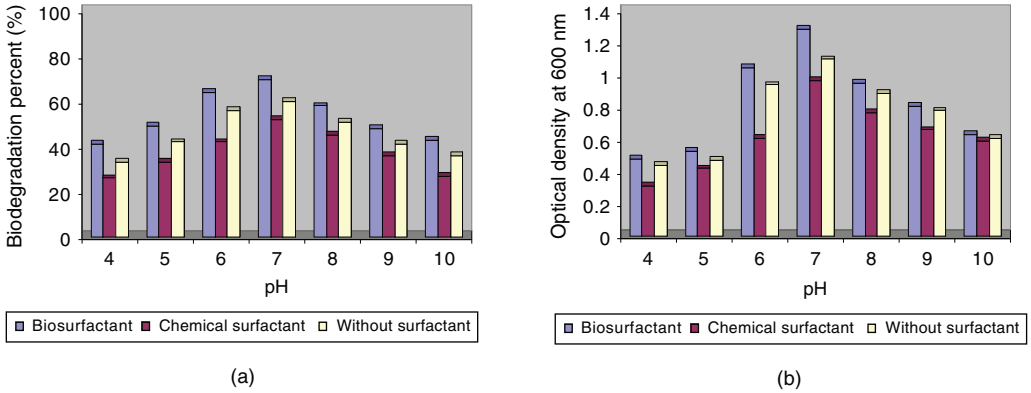

Fig. 6. Effect of pH on (a) percentage of biodegradation and (b) growth of the strain ERCPPI-1 (T = 40 °C, salinity = 0.1%, P = 14.7 psia, heavy crude oil concentration = 0.5%, rotation = 150 rpm).

by each strain was 66%, 59%, 49%, 43% and 41%, respectively. The mixed bacterial consortium degraded a maximum of 78% of BH crude oil. The percentage of degradation by the mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1% to 10%. Sathishkumar et al. [11] reported a maximum of 69% of crude oil degradation by the isolated strain *Pseudomonas* sp. BPS1-8 and 77% by the isolated mixed bacterial consortium. The percentage of degradation by the mixed bacterial consortium decreased from 77% to 45% as the concentration of crude oil was increased from 1% to 12%. Okoh et al. [22] reported about 89% of the heavy crude oil Maya was biodegraded by axenic culture of *Burkholderia cepacia* RQ1 in shake flask. Also, Okoh et al. [23] noted that the amount of heavy crude oil metabolized by some bacterial species increased with increasing concentration of starter oil up to 0.6% (w/v), while degradation rates appeared to be more pronounced between the concentrations of 0.4% and 0.6% (w/v) of oil. Sugiura et al. [24] reported that *Acinetobacter* sp. T4 and 8 mS degraded 20–34% of Arabian light crude oil, 14–27% of Dubai crude oil, 14–25% of Shanghai crude oil and 12–19% of Maya crude oil at 20 °C.

The influence of temperature and pH on the extent of biodegradation of Soroosh crude oil has been shown in Figs. 5 and 6, respectively. Temperature of 40 °C showed the maximum percentage of degradation of Soroosh crude oil. Temperature influences crude oil biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community [1,25]. By decreasing the temperature, the viscosity of the oil is increased, the water solubility and the bioavailability of the oil is decreased [1,26]. So, the percentage of degradation is decreased with decreasing temperature. At high temperatures, the situation is inversed and the rate of hydrocarbon metabolism increases with temperature and reaches a maximum. At temperatures higher than the optimum, the membrane toxicity of hydrocarbons is increased [1,27]. The temperature of 30 °C has been reported to be the optimum for microbial growth and PAH degradation [1,28].

Rahman et al. [1] studied the influence of temperature (15, 30 and 45 °C) on degradation of BH crude oil and found that a temperature of 30 °C was favorable for all the five individual isolates and mixed bacterial consortium to obtain the maximum crude oil degradation. Sathishkumar et al. [11] reported a temperature of 35 °C for maximum degradation of crude oil at the investigated temperature range of 25–45 °C. The rate of hydrocarbon metabolism increases to a maximum, typically in the range of 30–40 °C [1].

pH 7.0 was favorable for the strain ERCPPI-1 that showed the maximum percentage of degradation at this pH. A neutral pH of 7.0 has been reported to be optimum for biodegradation [29] and extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons [9].

Rahman et al. [1] investigated the effect of pH (6.5, 7.5, 8.5 and 9.5) on degradation of BH crude oil and reported that pH 7.5 was favorable for all the bacterial isolates and mixed bacterial consortium except for strain *Flavobacterium* sp. DS5-73 that showed the maximum percentage of degradation at pH 8.5. Sathishkumar et al. [11] were found that a pH of 7.0 in the studied pH range of 4.0–7.0 was optimum for maximum degradation. In another study, Okoh [30] reported that a slight alkaline pH of seawater is favorable for petroleum hydrocarbon degradation.

The effect of salinity on the growth of the bacterial culture and crude oil degradation was shown in Fig. 7. As the salinity of the medium is increased, the microbial growth and percentage of biodegradation is decreased

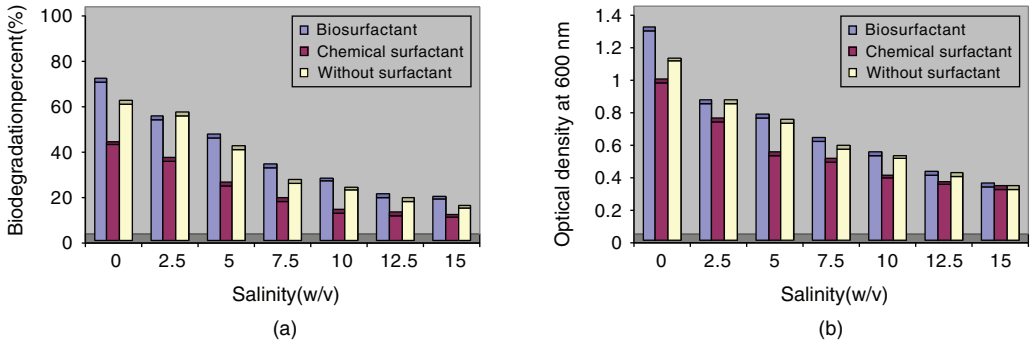

Fig. 7. Effect of salinity on (a) percentage of biodegradation and (b) growth of the strain ERCPPI-1 (T = 40 °C, pH = 7.0, P = 14.7 psia, heavy crude oil concentration = 0.5%, rotation = 150 rpm).

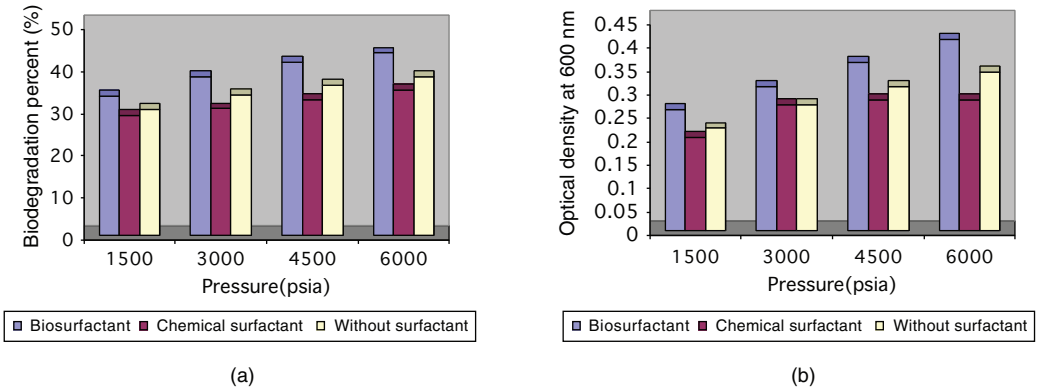

Fig. 8. Effect of pressure on (a) percentage of biodegradation and (b) growth of the strain ERCPPI-1 (T = 40 °C, salinity = 0.1%, pH = 7.0, heavy crude oil concentration = 0.5%, rotation = 150 rpm).

rapidly, because salinity has an adverse effect on the growth of the bacterium [8]. Therefore, as the salinity of the contaminated environment increases, applying lower initial oil concentrations and higher incubation times are necessary to increase the degradation percentage of the crude oil. In most of the previous works, the effect of salinity on biodegradation of crude oil has been limited to marine environments and seawater conditions. Harayama et al. [31] reported that few of the isolated microorganisms for degrading of petroleum components seem to be suitable for petroleum biodegradation in marine environments. Nakamura et al. [12] suggested that *Alcanivorax* sp. becomes dominant in oil-contaminated seawater and plays an important role in the process of biodegradation of oil-contaminated environments in cooperation with other bacteria. Zahed et al. [13] recommended the use of bioremediation for initial oil concentrations of 1 g/l and below in contaminated seawater. Their results also indicated that bioremediation is effective for at least 45 d and hydrocarbon degradation increases greatly as a result of bioavailability and adding dispersant significantly increase bioremediation rate of crude oil in the marine environment.

The influence of pressure on the growth of the bacterial culture and crude oil degradation was shown in Fig. 8. As the pressure of the medium is increased, the percentage of biodegradation is increased, since the substrate bioavailability to the strain had been increased. Upon release into the aqueous phase, heavy crude oil spill will not spread over the oil–water interface as easily as less viscous crude oils and this leads to a very low of contact area between the two phases [32]. By increasing the pressure in the piston–cylinder system, the crude oil spill is spread as a thin layer over the surface of the aqueous phase and the contact area is substantially increased. Since the aqueous solubility of heavy crude oil is very low [33], microorganisms attack to the oil–water interface, mineralize the bulk oil and the created oil droplets are dispersed in the aqueous phase [25,34]. The initial growth of microorganisms during the lag period is associated with increase in cell mass [35] and the production of biosurfactants [36,37] which is produced by the

cell to facilitate the uptake of insoluble hydrocarbons [38]. Production of surfactants by the microorganisms is associated with changes in the droplet size of the oil and subsequent emulsification of the substrate [34,39–41] followed by the uptake of the substrate.

The experiments were repeated for a crude oil with higher API (Gachsaran crude oil) than Soroosh crude oil and the results showed a higher biodegradation percentage and growth at the same conditions. The reason is that as the gravity of crude oil is decreased, the percentage of toxic components and huge molecules in the crude oil is decreased and the bioavailability of the petroleum hydrocarbons and hence microbial activity is increased.

4. Conclusion

Biodegradation has an advantage over the other wastewater treatment methods because it can efficiently destroy the pollutant hydrocarbons present and does not allow the contaminant to accumulate [42]. The present study showed that the isolated strain ERCPPI-1 can efficiently degrade the heavy crude oil components; maximum degradation was achieved at a temperature of 40 °C and pH of 7.0. On the other hand, the isolated strain could produce a type of biosurfactant in the presence of heavy crude oil as the sole carbon source and under extreme environmental conditions. The produced biosurfactant cause a substantial increase in the bioavailability of the hydrocarbon substrate and this leads to a high biodegradation percent. Hence, the use of the isolated bacterium for bioremediation of crude oil-contaminated sites could be suggested under ex situ and in situ conditions.

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