J Ind Microbiol Biotechnol (2008) 35:1241–1245
DOI 10.1007/s10295-008-0420-z

ORIGINAL PAPER

The reduction of wax precipitation in waxy crude oils by *Pseudomonas* species

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Received: 23 January 2008 / Accepted: 29 July 2008 / Published online: 20 August 2008

Abstract Crude oil with different concentrations was subjected to *Pseudomonas* species at 37 °C and various incubation periods. The results showed that *Pseudomonas* species grew faster at 1% (v/v) concentration of crude oil and exhibited high biodegradation ability within 1 week. On measuring the emulsification activity and emulsion stability during different stages of growth, in various immiscible hydrocarbons, it appeared that the species was able to produce a stable emulsion with a maximum at the end of stationary phase of growth. The gas chromatography analysis of the saturated hydrocarbons of crude oil showed that, an increase in concentration of iso-alkanes in the range of C_{15}–C_{20}, and a bioconversion of heavy iso-alkanes in the range of C_{21}–C_{22+}. Chemical analysis of crude oil by liquid chromatographic technique before and after growth showed that, the saturated alkanes were more degradable than aromatic and asphaltenic compounds. Treatment by *Pseudomonas* species may possibly be an effective method for the biodegradation of heavy paraffinic hydrocarbon leading to an enhancement in crude oil properties.

Keywords *Pseudomonas* · Crude oil · Biodegradation · Gas chromatography · Emulsification activity

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Introduction

In the recent years, much attention has been directed toward biosurfactant due to its broad-range application and also because of its advantage over the chemical surfactants. The amount of biosurfactant producer is mainly affected by the strain type, composition of culture medium and growth conditions [1–3]. Several studies have demonstrated the successful use of biosurfactants for the degradation of organic pollutants in soil, dispersion of oil spills and enhancing crude oil recovery process and the transportation of crude oil [4–10]. Lazer et al. [11] reported the isolation of biosurfactant-producing microorganisms, and had proven that microorganisms could be used as an effective alternative to conventional methods to prevent and remove paraffin damage. The microorganisms used for such purposes are simply hydrocarbon utilizing, non-pathogenic microorganism, the by-products of which are alcohols, gases, acids, biosurfactant and polymers, which cause marked change in the physical–chemical properties of the crude oil as well as on well-bore area [10–14]. Hao et al. stated that the microbial growth on crude oil resulted in losses of aromatic hydrocarbons, resins, and asphaltenes, and the bioconversion of crude oil leads to an enrichment in lighter hydrocarbons and an overall redistribution of these hydrocarbons [13].

The aim of this study is to isolate a bacterial consortium from natural environment that has the ability of producing biosurfactants, which could minimize the paraffin deposition along the production flow line and also enhance the crude oil properties.

In this work, *Pseudomonas* species, which was isolated from marine sediment [15] was screened for its ability to grow on crude oil and *n*-hexadecane. Besides monitoring growth, the bioconversion and biodegradation of crude oil

by means of gas–liquid chromatography were investigated. The bacterial ability to emulsify different hydrocarbons and the emulsion stability at room temperature were also examined. The data presented here demonstrated that *Pseudomonas* species was greatly able to emulsify different hydrocarbon sources and degrade iso-alkanes more than *n*-alkanes, whereas, throughout the literature, *Pseudomonas aeruginosa* was introduced as one of the effective microorganisms in the biodegradation of heavy paraffin hydrocarbon compounds [12].

In addition, two crucial indicator parameters of oil potential depositing paraffin and flow resistance were investigated; wax appearance temperature (WAT), temperature at which the first crystals appear in the bulk of the fluid and the viscosity which represents the resistance of a fluid to flow.

Materials and methods

Source of microorganisms

The microorganism used in this study was obtained by the enrichment culture technique from marine sediment which was contaminated with hydrocarbon. Marine sediment samples were obtained from different locations at the Alharka terminal in the eastern part of Libya.

Enrichment culture experiment was carried out in the batch experiment using bioreactor with 1.8 liter of sterilized Enrichment Salts Medium (ESM) [15]. A measure of 10 ml of Marine sediment sample plus 0.5% (v/v) of crude oil were added to the sterilized ESM. The system was operated at the following parameters: temperature 37 °C, pH 7.3, air flow rate 2 l/min and agitation at 250 rpm. The incubation period was left up to 7 days. For isolating pure culture from ESM, a streak plate method on Nutrient Agar plates was used. The plates were incubated at 37 °C for 24 h.

Growth conditions and growth mounting

Pure cultures were aseptically transformed into Mineral Salts Medium (MSM) [16] containing different concentrations of crude oil 1, 5 and 10% (v/v). Cultures were grown in 250-ml Erlenmeyer flasks with 50 ml of medium and incubated with shaking at 150 rpm and 37 °C for 7 days.

The growth rate of the isolated species was determined by measuring the culture turbidity. The turbidity of the culture was determined by measuring the Optical Density (OD) at a wavelength of 540 nm in 2-ml cuvettes using a spectrophotometer (Jenway Series 6105 UV–Vis) [15].

Characterization of bacterial stain

Preliminary identification procedures were carried out according to the Berge's manual of systematic bacteriology, Krieg and Holt [17] and Bradshaw [18] on the basis of morphological features and standard physiological tests.

Emulsification activity and stability measurement

The emulsification activity (E_{24}) and stability of culture broth were determined using kerosene, xylene, toluene and crude oil [19, 20]. In this test 2 ml of hydrocarbon was added to an equal volume of the culture broth in a screw-cap test tube and vortexed at high speed for 2 min. The emulsion stability at ambient temperature and atmospheric pressure was determined after 24 h by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying it by 100 [19].

The emulsification activity of supernatant fluid was considered by measuring the absorbance at 610 nm using a spectrophotometer (Jenway Series 6105 UV–Vis).

Chemical property analysis

Liquid chromatographic analysis

Confirmative experiments were conducted on the residual crude oil by using combined solvent extraction-column chromatographic techniques. Solvent extraction steps were involvedin the removal of inorganic compound and separation of asphaltene compounds. The chromatographic steps were used to fractionate the residual of crude oil to saturated and aromatic fractions in the crude oil; details of these experiments are described in references [21, 22].

Gas chromatographic analysis

The analysis of *n*-alkanes and iso-alkane before and after microbial growth was done using a capillary gas chromatograph (Chrompack Model 439) equipped with a Flame Ionization Detector (FID), a split/splitless injector and cp-sil 5 CB column (50 m, 0.32 mm, 0.12 μm film thickness). A column temperature of 40 °C was held for 2 min and then ramped at a rate of 5 °C/min to a final temperature of 300 °C and held for 30 min.

Physical property analysis

Wax appearance temperature (WAT)

Differential Scanning Calorimetry (DSC) was used to measure WAT of the crude oil samples with cooling rate of 10 °C per min.

Viscosity

Viscosity measurement was performed on rotating viscometer (CV100), which allows the determination of viscosity against the shear rate. Experiment conditions were 50 °C sample temperature, 5 min of shearing time and 12 s^{-1} maximum shearing rate.

Results and discussion

In this study, the potential production of biosurfactant at various incubation periods was assessed based on the emulsification activity percent (E_{24}). Figure 1 showed that, *Pseudomonas* species was able to grow on two different concentrations of *n*-hexadecane. The emulsification activity of culture broth for culture grown on concentration of n-C_{16} 0.3% (v/v) was higher than that of n-C_{16} 1% (v/v). For culture grown on concentration of n-C_{16} 0.3% (v/v), the highest value of emulsification activity was obtained on toluene with E_{24} equal to 100% after 24 h. The specific growth rate and the emulsification index values are similar to the results obtained by *Pseudomonas aeruginosa* which emulsifies kerosene with 60.6% and has specific growth rate 0.25 h^{-1} [23]. From Fig. 2, it is found that the culture supernatant generated stable emulsions of high optical density values, which indicated the production of extra-cellular surface-active compounds; this result was in agreement with that of Lang and Wagner and Santa Anna et al. [3, 24].

Figure 3 shows the growth curve of *Pseudomonas* species, which has a lag phase of approximately 8 h and peak growth at 20 h with a maximum specific growth rate of 0.243 h^{-1}.

The emulsion stability was determined after 1 and 24 h of emulsion preparation using toluene, xylene and kerosene; the stabilization of biosurfactant was indicated by the emulsification percentage of activity of the culture broth.

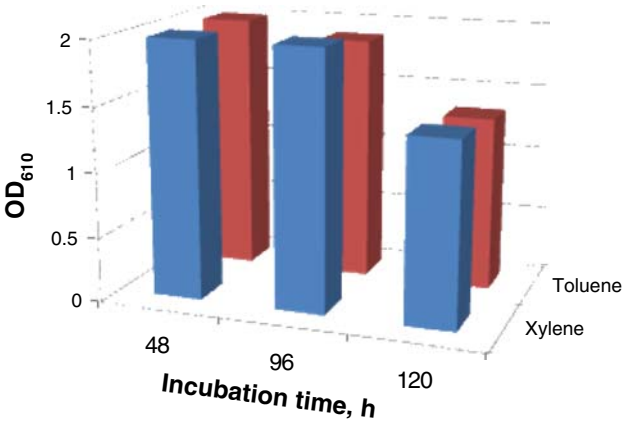


Fig 2 Emulsification activity of supernatant fluid (cell free broth culture)

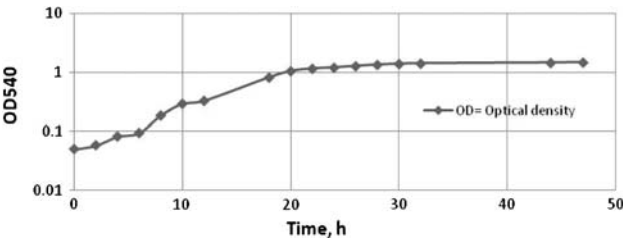
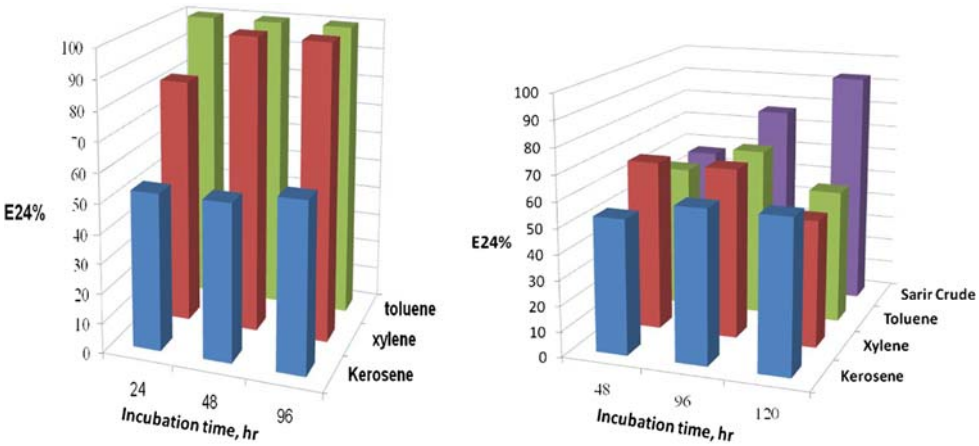


Fig. 3 Growth curve of *Pseudomonas* species

The results shown in Fig. 4 demonstrated that the isolated species was better able to produce a stable emulsion on toluene and xylene than kerosene. Also measurement of the emulsifying activity during different stages of growth showed that, the activity was higher and the stability was best during the end of stationary phase of growth.

Gas chromatographic analysis (GC) was carried out on untreated crude oil, 1 and 5% (v/v) crude oil treated with *Pseudomonas*. GC results indicated that, the microbial degradation of paraffinic hydrocarbon of crude oil at 1% (v/v) was higher than that obtained at 5% (v/v). Figures 5 and 6

Fig. 1 Emulsification activity of culture broth (culture media with microorganisms) grown on different concentrations of n-C_{16}



A - 0.3% (v/v) concentration of n-C_{16} **B** - 1% (v/v) concentration of n-C_{16}

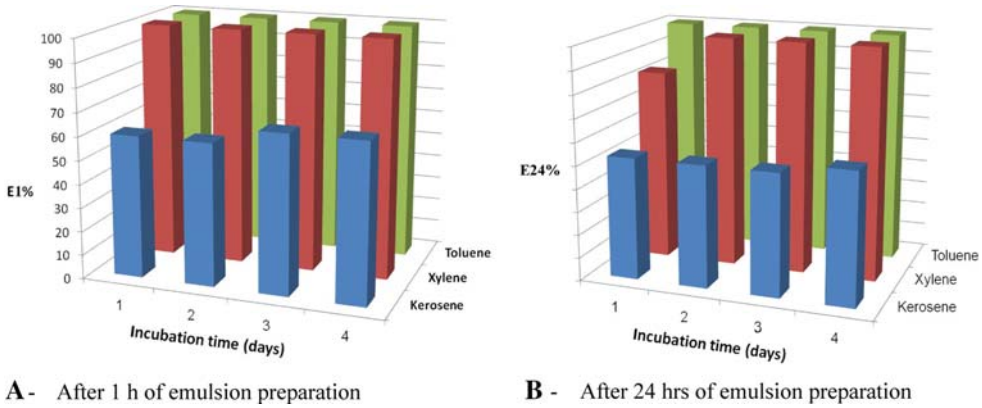


Fig. 4 Emulsions stability measurements on immiscible hydrocarbons during different incubation periods

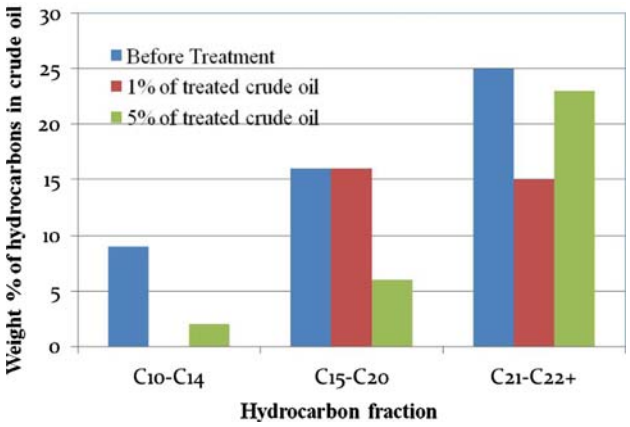


Fig 5 Distributions of *n*-alkane in different concentrations of crude oil before and after treatment with *Pseudomonas*

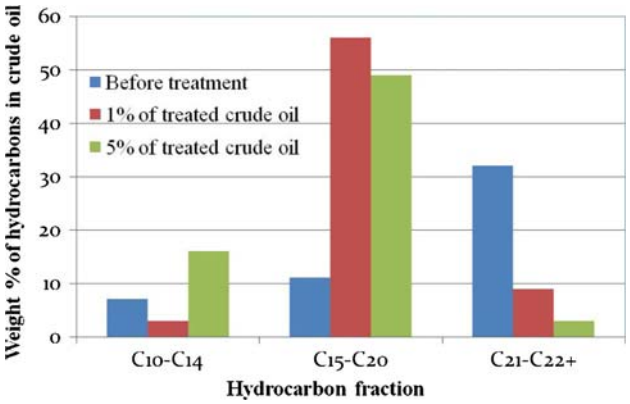


Fig. 6 Distributions of iso-alkane in different concentration of crude oil before and after treatment with *Pseudomonas*

show GC analysis of *n*-alkane and iso-alkane before and after 21 days of microbial growth. At concentrations of 1 and 5% (v/v) crude oil, there was a significant increase in iso-alkane (C_{15}–C_{20}) from 11 to 56 and 49 wt%, respectively. Also, microbial growth resulted in a significant reduction of light hydrocarbon fractions (C_{10}–C_{14}) which may be due to the utilization of the light fraction before the heavy fraction. The same trend had also been observed by Jobson et al. [25].

The effect of induced bioconversion of crude oil is shown in Table 1. Compared to the untreated crude oil, the relative percentage of saturated hydrocarbon, aromatic hydrocarbon and asphaltene, there was a decrease in the saturated hydrocarbon and an increase in the aromatic hydrocarbon. Aldrett et al. [26] concluded that the speed of the biochemical action on crude oil was faster within the first 7 days, and gradually lowered thereafter. The experimental results of this study as shown in Fig. 7 showed similar observation. After 1 week, there was a noticeable decrease in the heavy fractions and an increase in the light fractions of (C_{15}–C_{20}), from 57 to 22 wt% and from 27 to 65 wt%, respectively, which was explained by the availability of nutrients that were sufficient for microbial growth; there was no high accumulation of poisonous products in the medium during that period of growth, in addition to the biosrfactant production which has significant impact on crude oil degradation. The physical result shows a sharp decrease in viscosity at shear rate below 3 s^{-1}, but after 5 s^{-1} the viscosity remains steady. DSC analysis of the crude oil showed a reduction in the WAT as compared with the WAT before the bacterial growth. WAT was decreased from 50.94 °C before the bacterial growth to 42 °C after the bacterial growth.

Conclusions

The emulsification activity results showed the ability of *Pseudomonas* species to emulsify immiscible hydrocarbon such as kerosene, toluene, xylene and crude oil. Hundred percentage emulsification stability was obtained using toluene and xylene, and 85% stability using kerosene. The amount of biosurfactant produced was highly affected by hydrocarbon concentration in the medium, and its optimum production was at the end of stationary phase of growth. *Pseudomonas* species has a maximum growth rate using 1% (v/v) of crude oil concentration; the maximum reduc-

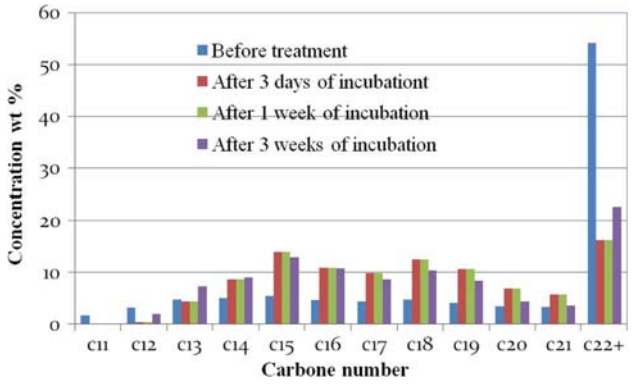


Fig. 7 GC analysis of 5% (v/v) concentration of crude oil treated with *Pseudomonas* at different incubation period

Table 1 Hydrocarbons distribution results from liquid chromatographic analysis

Fraction wt%	Before growth	After growth
Asphaltenes	3.8	3.4
Saturates	64.7	55
Aromatic	35.3	45

tion was observed on heavy iso-alkane fractions after 1 week of incubation period. Liquid chromatography analysis showed a noticeable increase in the aromatic fraction and a remarkable decrease in the saturated fraction. This could be attributed to the microbes which are more capable to degrade paraffinic fraction than aromatic fraction. The marked increase in the light hydrocarbon of iso-alkane (C_{15}-C_{20}) indicates that, *Pseudomonas* species may be an efficient species for reducing paraffin deposition and the reduction in viscosity and WAT indicate the conversion of long-chain alkanes to short ones.

Acknowledgments The work is sponsored by the Libyan Petroleum Institute and the Chemical Engineering Department, AlFatah University, Libya.

References

1. Tahzibi A, Fatemeh K, Mahnaz MA (2004) Improved production of rhamnolipids by a *Pseudomonas aeruginosa* mutant. Iran Biomed J 8(1):25–31
2. Kim HS, Yoon BD, Lee CH, Oh HM, Katsuragi T, Tani Y (1997) Production and properties of a lipopeptide biosurfactant from *Bcillus subtilis* C9. J Ferment Bioeng 84:41–46
3. Lang S, Wagner F (1987) Structure and properties of biosurfactants. In: Kosaric N, Cairns WL, Gray NCC (eds) Biosurfactant and biotechnology surfactant science series. Marcel Dekker Inc, New York, pp 21–45
4. Banat IM, Samarah N, Murad M, Hoorne R, Banerjee S (1991) Biosurfactant production and use in the oil tank clean-up. World J Microbiol Biotech 7:80–88
5. Banat IM, Makkar RS, Cameotra SS (2000) Potential commercial applications of microbial surfactant. Appl Microbiol Biotechnol 53:495–508
6. Gutnick Y. Shabtai (1987) In: Kosaric N, Cairns WL, Gray NCC (eds) Biosurfactants and biotechnology, Chap. 8. Marcel Dekker, New York, pp 237–238
7. Maure MA, Dietrich FL, Diaz VA, Arganaraz H (1999) Microbial enhanced oil recovery pilot test in Piedras Coloradas field, Argentina. SPE 53715, pp 1–29
8. Rocky Mountain Oil Field Testing Center (RMOFTC) (1997) December 17, Chemical and microbial paraffin control project. BDM Oklahoma/NIPER, 220N. Virginia
9. Dietrich FL, Brown FG, Zhou ZH, Maure MA (1996) Microbial EOR technology advancement: case studies of successful projects. Soc Petrol Eng SPE 36746:1–17
10. Brochure of CPChem Company (2002). Microbial technology for improvement of oil quality
11. Lazar I, Voicu A, Nicolescu C, Mucenica D, Dobrota S, Petrisor GI, Stefanescu M, Sandulescu L (1999) The use of naturally occurring selectively isolated bacteria for inhibiting paraffin deposition. J Petrol Sci Eng 22:161–169
12. Sadeghazad A, Ghaemi N (2003) Microbial prevention of wax precipitation in crude oil by biodegradation mechanism. Soc Petrol Eng SPE 80529:1–11
13. Hao R, Lu A, Zeng Y (2004) Effect on crude oil by thermophilic bacterium. J Petrol Sci Eng 43:247–258
14. Fedorak PM, Foght JM, Westlake DWS (1983) Comparative studies on microbial degradation of aromatic and saturates in crude oil. In: Zajic JE, Cooper DG, Jack TR, Kosaric N (eds) Microbial enhanced oil recovery. Pennwell Publishing, Tulsa, pp 162–172
15. Asma (2007) Microbial treatment of waxy crude oils for mitigation of wax precipitation. J Petrol Sci Eng 55:111–121
16. Vishniac W, Santer M (1957) The *Thiobacilli*. Bacteriol Rev 21:195–213
17. Krieg NR, Holt JG (1984) Berge's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore
18. Bradshaw LJ (1979) Laboratory microbiology. 3rd edn. W·B. Saunders Company. West Washington Square Philadelphia, 19105, p 19, 42, 88
19. Banat IM (1995) Biosurfactants production and use in microbial enhanced oil recovery and pollution remediation: a review. Bioresour Technol 51:1–12
20. Cooperand Goldenberg (1982) Biosurfactants and enhanced oil recovery. In: Pro. int. conf microbial enhanced oil recovery, Afton, ok, doe conf, 85051. vol 40, pp 112–114
21. Ali LH, Shebi AA, Al·Na, Idh M (2000) Studies on the chemical nature of maltenes separated from sludge deposits. In: 7th inter. conference on stability and handling of liquid fuels (IASH), Graz
22. American Standard Test Method (ASTM D 2549–91) Standard test method for separation of representative aromatics and nonaromatics fractions of high-boiling oils by elution chromatography
23. Benbatta S, Touzi A (1995) Isolation of biosurfactant producing bacteria from oil contaminated soils. New technologies applied to hydrocarbon production. 12–15 Sep., Delft, pp 290–294
24. Santa Anna LM, Sebastian GV, Menezes EP, Alves TLM, Santos AS, Pereira N, Jr., Freire DMG (2002) Production of biosurfactants from *Pseudomonas aeruginosa* PA1 isolated in oil environments. Braz J Chem Eng 19
25. Jobson A, Cook FD, Westlake DWS (1972) Microbial utilization of crude oil. J Appl Microbiol 1082–1089
26. Aldrett S, Bonner JS, Mills MA, Autenrieth RL, Stephens FL (1997) Microbial degradation of crude oil in marine environments tested in a flask. Water Res 31:2840–2848