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Evaluating efficiency of co-culture of two isolated *Pseudomonas aeruginosa* strains for removal of floating crude oil from oil-polluted wastewater

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ABSTRACT

Two strains of *Pseudomonas aeruginosa* were isolated from crude oil of Isfahan oil refinery and used in a lab scale fermenter for removal of floating crude oil pollution from water. Maximum stable removal efficiency of 92% was reached despite the stepwise increase in the culture medium crude oil concentration from 0.1 g/l to 0.3 g/l and maximum biodegradation rate of 98.6 mg/l h was obtained. During 91 d mixed liquid suspended solid (MLSS) changed between 4000 and 8000 mg/l and sludge volume index (SVI) changed between 20 and 90 mg/l. The performance was investigated during a 91 d continuous operation of a fermenter which was intermittently fed with oil-polluted water. It seems biodegradation of this mixed culture is high removal efficiency (92%) in low time (7.5 d).

Keywords: Floating oil; Biodegradation rate; *Pseudomonas aeruginosa*; Removing; Wastewater

1. Introduction

Crude oil is a mixture of thousands of organic compounds which is a potential source of environmental pollution. 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. Oily wastewater is produced during crude oil and natural gas production processes, at both onshore and offshore operations [1]. This wastewater is a significant source of pollution for local water resources because of its high crude oil and the suspended solids (SS) content, high temperature, and high salt concentration [2].

Crude oil contains saturated compounds, aromatics, resins and asphaltenes. Aromatic compounds, having higher water solubility, are of main concern [3]. Other parts float in water in form of very small and large droplets [4].

Chemical and mechanical methods can be applied for removing large oil droplets or floating crude oil, but these methods are not effective for soluble crude oil and very tiny crude oil droplets. Investigations on biological treatment of crude oil techniques have revealed biodegradation to be a promising cost effective technological alternative [5]. Many surfactants have shown to enhance biodegradation, however, the extent of mineralization may not be affected [6].

This study was aimed to evaluate floating oil biodegradation performance of a mixed culture of two bacterial strains isolated from crude oil of Isfahan oil refinery. The performance was investigated during a 90 d continuous operation of a fermenter which was intermittently fed with oil-polluted water. The crude oil content of the medium was increased in three steps from 0.1% to 0.3%.

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2. Materials and methods

2.1. Isolating medium

A published method was used for isolation of desired oil-degrading strains [7]. The isolating medium (IM) was containing 1 g/l $NaNO_3$, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2$, 0.001 g/l $FeSO_4$, 0.5 g/l K_2HPO_4, 0.5 g/l KH_2PO_4. The medium pH was adjusted to 7.2 by adding suitable amounts of 1 M NaOH solution.

2.2. Isolation of strains

One ml of crude oil was added to 300 ml Erlenmeyer flasks containing 100 ml of IM. The flasks were anaerobically incubated for 72 h at 30 °C on a rotary shaker at 160 rpm. One ml of flask contents was added to a new flask containing 100 ml of fresh IM and incubated for another 72 h in the same conditions, 0.1 ml of the medium was cultured on selective IM agar plates which were then incubated at 30 °C for 48 h. Colonies with different characteristics (according to the color and size) were then repeatedly cultured on IM agar plates until pure cultures were obtained. All pure cultures were maintained on IM agar slants at 4 °C and transferred every four months to a new agar slant. The inoculums for the bioreactor were prepared by growing the selected strains on standard nutrient broth at 30 °C for 20 h.

2.3. Bioreactor

The bioreactor was a standard 1.5 l working volume Labfors fermenter (Infors HT, Bottmingen, Switzerland). The bioreactor was equipped with an air sparger and two disk-turbine impellers.

2.4. Culture medium for bioreactor

An isolating medium as described by Angelidaki et al. [8] containing glucose and varying amounts of crude oil as carbon sources was used for the fermentations. The medium pH was adjusted to 7.2 using 1 M NaOH solution.

2.5. Crude oil

The specifications of the crude oil provided by Isfahan oil refinery (Isfahan, Iran) are shown in Table 1.

2.6. Bioreactor operation

Bioreactor vessel was filled with 750 ml of medium containing 6 g/l glucose and 0.1 g/l crude oil and sterilized at 125 °C for 15 min. Two hundred and fifty ml of the seed culture was added aseptically to the fermenter and operation was started by onset of aeration. The temperature and air flow rate were maintained at 30 °C and 0.85 l/min, respectively, during the experiment. Each day 200 ml of the bioreactor content was drained and centrifuged at 3000 rpm for 15 min. The precipitated biomass was mixed with 200 ml of fresh sterile medium and added to the bioreactor. A portion of supernatant was used for estimating the extent of crude oil biodegradation. Initially, glucose was used as the carbon source while the crude oil concentration was kept at 0.1 g/l. At day 37 the crude oil concentration in the bioreactor was increased to 0.2 g/l and kept at this value until the day where the crude oil concentration in the bioreactor was again increased to 0.3 g/l.

2.7. Analysis of total petroleum hydrocarbons (TPH)

The pH of 100 ml of the supernatant was adjusted to 2 or less by adding 0.1 M HCl. The supernatant was then mixed with 25 ml of carbon tetrachloride and centrifuged for 15 min. One ml of organic phase was added to 99 ml of carbon tetrachloride and centrifuged at 5000 rpm for another 15 min. Absorbance of clear supernatant was measured at 400 nm which was reported to be λ_{max} for crude oil [9]. TPH of the sample was estimated from its absorbance (x) using the following standard calibration equation which was obtained at 20 °C:

$$y = 0.1219x \qquad (R^2 = 0.994) \tag{1}$$

where x is oil concentration (volume %) and y is the absorbance of carbon tetrachloride/crude oil solution at 400 nm.

The percentage of feed crude oil removed by fermentation was then calculated.

2.8. Determination of suspended solids

Total suspended solids (TSS), mixed liquid suspended solids (MLSS), and settle able suspended solids were measured using standard methods [10].

Table 1
The specifications of crude oil provided by Isfahan oil refinery

Specification	Value	Unit
Sp. gr. (15/15 °C)	0.8575	–
Kinematic viscosity (10 °C)	18.09	C. St.
Kinematic viscosity (40 °C)	6.28	C. St.
Asphaltene	2	wt.%
Wax	11	wt.%
Sulfur	1.3	wt.%
Carbon residue	3.61	wt.%
Vanadium	7.1	ppm

For determination of TSS and MLSS, 100 ml of sample was filtered through a pre-weighed paper filter. The paper filter was the dried and the TSS was calculated by subtracting the final and initial weights.

For determination of settleable suspended solids used in half funnel. Volume of settled solid in half hour was equal amount of settle able suspended solids.

2.9. Measuring of emulsification index (E24)

Emulsification index was estimated for both isolated and mixed microorganisms. Two ml of well-mixed culture medium containing either isolated microorganisms or mixed microorganisms was added to the test tubes Afterwards, 2 ml of gas oil was added to the tubes and the mixture was shaken vigorously at high speed for 3 min. After 24 h the height of the emulsion layer (h1) and total height (h2) were measured. Emulsification index was calculated by dividing h1 by h2 [11].

3. Results and discussion

Two gram negative rod shape bacteria were isolated from crude oil and named as A1 and A2. They both tolerated up to 6% NaCl and were found to be strains of *P. aeruginosa*. E24 index for pure isolates A1 and A2 and a mixture of 50% (w/w) A1 and 50% (w/w) A2 was measured to be 20%, 25%, and 21%, respectively.

Fig. 1 depicts MLSS profile of the bioreactor contents which is an indication of cell density. After a lag phase glucose is consumed and cell density increases. After glucose depletion a second lag phase was observed which was followed by consumption of crude oil and more increase in the cell density and finally steady state is established for 0.1 g/l of crude oil concentration in the medium. Increasing oil concentration from 0.1 g/l to 0.2 g/l caused a sudden drop in cell density most probably due to toxic effects of aromatic compounds at high concentrations. During the following days the isolates were adopted with increased concentration of crude oil and cell density gradually returned to the steady state value. The same trend was observed after second increase in crude oil concentration, although regain of steady state was faster this time. The steady state MLSS was nearly 7500 mg/l in all phases.

Tellez et al. [1] reported removal of approximately 98% of TPH from oilfield produced water with a residence time of 20 d in an activated sludge reactor. In the oilfield produced water some of the petroleum components are dissolved in the water and some other are in the form of tiny oil droplets [1]. In this study we were able to remove floating crude oil in higher concentrations from water. Our data proves that biological treatment could be an economical and effective alternative to the physical methods. Li et al. [12] isolated oil-degrading bacterium capable of removing 80% of chemical oxygen demand (COD) of produced water in 7 d. Vieira et al. [3] reported 90% decrease in TPH of water contaminated with diesel and gasoline in 49 d. In the present study, however, we removed the floating crude oil using the isolated bacteria both at high efficiency (92%) and low residence time (7.5 d) as compared to other studies. Our process demonstrated long-term stability and was able to recover its stable conditions after perturbations made by changes in pollution load.

Removal efficiency followed the same pattern as cell density as is depicted in Fig. 2. At steady state conditions the bioreactor removed 92% of incoming oil despite differences in feed concentration. The isolated strains in this study were not able to grow on crude oil as the sole initial carbon source.

This is possibly due to non-availability of sparingly soluble hydrocarbons to the microorganisms. *P. aeruginosa*

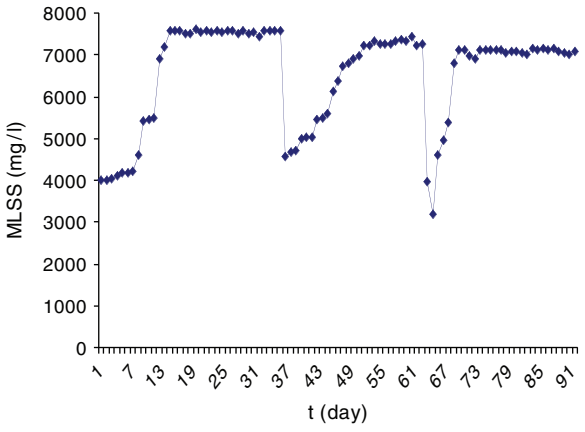

Fig. 1. Profile of MLSS in bioreactor during the experiment.

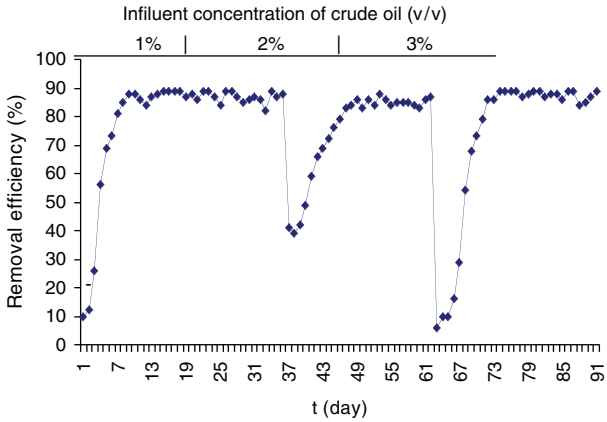

Fig. 2. Profile of crude oil removal efficiencies during the experiment.

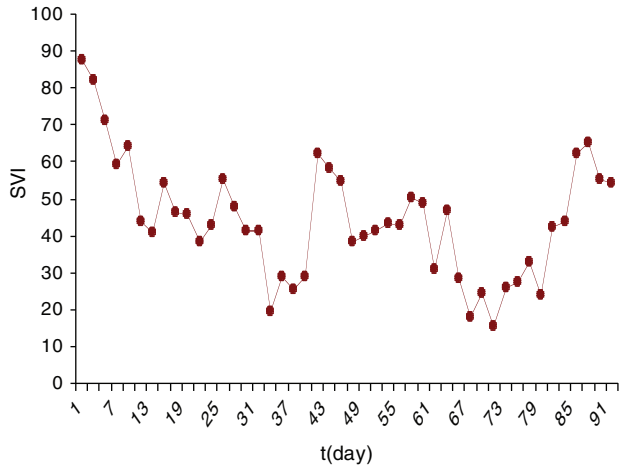

Fig. 3. Temporal variation of SVI in bioreactor during the experiment.

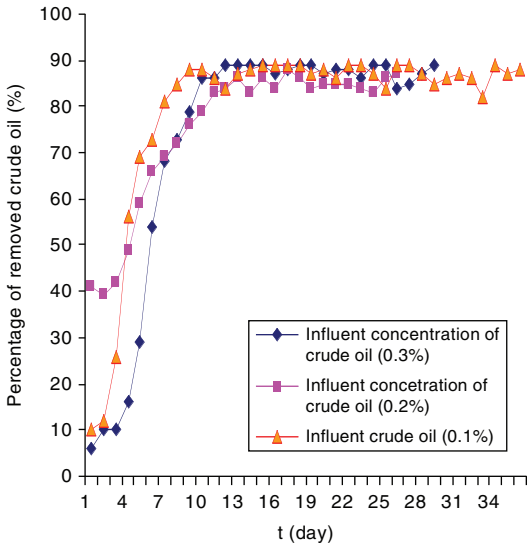

Fig. 4. Relationship between incubation time and removal percentage of crude oil at different concentrations of crude oil.

is able to produce rhamnolipid as a surfactant and addition of glucose to the medium as the initial carbon source probably induces rhamnolipid production and subsequently increases the solubility and availability of hydrocarbons. Liang et al. [13] also observed the necessity of supplementing glycerol or rhamnolipid to the medium in order to induce the growth of the *P. aeruginosa* strains.

During the experiment SVI was also measured. SVI in reactor was low and between 20 and 90 mg/l. A drastic decrease in SVI was observed in the days 37 and 65 where the crude oil concentrations were increased (Fig. 3). The reactor effluent was very turbid probably due to dispersed growth. The low SVI and dispersed growth were most likely because of high concentration of crude oil in the reactor [10]. Seven days after the first increase in the crude oil concentration from 0.1 mg/l to 0.2 mg/l microorganisms again adapted to the new conditions and SVI increased from 20 mg/l to about 60 mg/l. This is in agreement with the data obtained by Tellez et al. [1] who showed that increasing the retention time of microorganisms in the reactor could improve the efficiency of removal crude oil in reactors. A sharp drop in MLSS after the first increase in crude oil concentration followed by a gradual increase and reaching to steady state conditions after 9 d was also observed (Fig. 2).

Incubation times were approximately similar for the crude oil concentrations of 0.1 g/l and 0.2 g/l but the incubation time was increased from 7 d to about 11 d after increasing the crude oil concentration to 0.3 g/l (Fig. 4). Mehrabani [14] studied biodegradation of mono ethylene glycol in the petrochemical wastewater by moving bed bioreactor (MBBR). The incubation time in their study did not change in spite of increasing the organic loading rate from 2.7 kg/m^3 day to 8.5 kg/m^3 day but further increase of loading rate to 12.5 kg/m^3 day was concomitant to substantially longer incubation times.

Biodegradation rates were calculated for each feed (Table 2) and maximum rate was found to be 98.6 mg/l h which was achieved at maximum concentration of oil in feed.

Table 2
Biodegradation rate at different concentrations of crude oil in the medium

Influent concentration of crude oil (mg/l)	Average effluent concentration of crude oil(mg/l)	Incubation time (day)	Removal efficiency (%)	Biodegradation rate (mg/l h)
857.5 (0.1%)	68.973	7	91.96	32.9
1715 (0.2%)	194.35	7	88.66	63.4
2572.5 (0.3%)	205.975	11	92	98.6

4. Conclusions

- Two strains of *P. aeruginosa* that isolated from crude oil of Isfahan refinery could grow using crude oil as the sole carbon source.
- The bioreactor was suitable for the treatment of floating crude oil-containing wastewater, presenting operating stability throughout the experiment. Make of this reactor is very cheap for treatment of oily waste water in comparison to other reactors such as activated sludge or trickling filters.
- The maximum of crude oil removal efficiencies was 92%. Efficiency of the floating crude oil degradation was achieved by applying an HRT equal to 7.5 d for influent crude oil concentrations ranging from 8575 mg/l to 25,725 mg/l.
- Incubation time in initial and second concentration of crude oil was shorter than tertiary organic loading rate due to crude oil.
- A1 and A2 could produce bio-surfactant and they do not need any synthetic surfactants for startup of crude oil biodegradation. They could produce emulsification index about 20% and 25%, respectively. A1 and A2 in mixed culture could produce 21% emulsification index.
- This study was aimed to evaluate floating oil biodegradation performance of a mixed culture of two bacterial strains isolated from crude oil of Isfahan oil refinery. The performance was investigated during a 91 d continuous operation of a fermenter which was intermittently fed with oil-polluted water. It seems biodegradation rate of this mixed culture is fast and favorite.

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