Isolation and identification of oil degrading bacteria from oil sludge in Abadan oil refinery

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Abstract

Oil sludge, a rich source of hazardous pollutants that cause soil and water pollution, can be treated by biodegradation. The aim of this study was isolation of native bacteria from oil sludge produced in Abadan refinery, Iran. Native bacteria were isolated and screened based on their potential in reduction of total petroleum hydrocarbons, hemolysis, oil spreading technique and gas chromatography analysis. They were identified by 16S rRNA sequencing. Optimum N and P sources were also investigated. *Pseudomonas stutzeri* AOR_1, through biosurfactant production, and *Klebsiella pneumoniae* AOR_2, via degradation of long chain hydrocarbons, had the most potential in oil recovery. $NaNO_3$ and Na_2HPO_4 were the optimum N and P sources for *P. stutzeri,* and NH_4Cl and $(NH_4)_2HPO_4$ for *K. pneumoniae*. It can be concluded that the introduced bacterial isolates have great potential in oil recovery from oil sludge, which makes them candidates for biodegradation and bioremediation purposes.

Key words: biodegradation, bioremediation, oil recovery, oil sludge.
Abbreviations: PAH, polyaromatic hydrocarbons.; TPH, total petroleum hydrocarbons.

Introduction

Oil, as the most demanded energy resource, has a vital role in industrial countries. Use of oil as an energy resource necessitates standard guidlines to be regarded for use in those countries. Progressive economic growth has increased demands for oil, which must be met by new exploration and production technologies (Bao et al. 2009). Traditional oil recovery (chemically enhanced oil recovery) can approximately recover 40 to 45% of the oil during primary and secondary recovery (Sen 2008).

Crude oil is composed of several compounds, such as aliphatic, aromatic and polyaromatic hydrocarbons (PAH), and also sulfur, oxygen and nitrogen containing compounds. PAH compounds are toxic and can be carcinogenic. Oil waste management in oil refineries needs to be focused on diminishing waste and selecting suitable strategies in order to prevent soil damage. High concentrations of these pollutants, due to their toxic and carcinogenic nature, can affect cell metabolism (Tanti et al. 2009).

Oil sludge is one of the plentiful pollutants that is routinely produced in oil drilling and refinery processes; for example oil refineries in India produce nearly 28 000 tons of sludge annually (Joseph, Joseph 2009). Several immunotoxic and carcinogenic compounds are present in oil sludge (Mishra et al. 2001). Most of this sludge is released into environment without any treatment, which can cause serious environmental complications. The sludge hydrocarbons can slowly diffuse deep in to soil and cause pollution of bedrock and underground water resources. On the other hand, light hydrocarbons evaporate and form a layer of oil-contaminated dust leading to air pollution (De-qing et al. 2007). The pollution produced by oil sludge creates altered ecological conditions by influencing physico-chemical properties of soil. This negatively affects seed germination, plant growth and development. Furthermore, soil aeration will be inhibited at oil sludge accumulation sites and cause damage to the soil rhizosphere. Finally, these changes destroy plant flora in the polluted regions, sometimes cause complete destruction of habitats (Tanti et al. 2009).

One of the main problems in oil refineries is safe elimination of oil sludge produced during oil refining. While burning of oil sludge is an easy and simple method for its removal, it causes greater air pollution emissions (De-qing et al. 2007). Among the vast technologies that can be applied for decontamination of polluted sites, in situ bioremediation by native microorganisms is widely used. This treatment can reduce the threat of underground water contamination and increases biodegradation (Mishra et al. 2001). This is a clean technology that can be more compatible with environment (Ayotamuno et al. 2007).

Different bacterial species have been reported that have biodegradation potential. For example *Bacillus* spp. (Joseph, Joseph 2009), *Bacillus pumilus* strains (Calvo et al. 2004), *Pseudomonas* sp. and *Bacillus* sp. (Ayotamuno et al. 2007)

Pseudomonas aeruginosa and *Rhodococcus* sp. (Cameotra, Singh 2008), *Pseudomonas* sp. and *Klebsiella* sp. (Makut, Ishaya 2010) and Pseudomonas spp. (Barathi, Vasudevan 2001; Mishra et al. 2001) have been found to have potential in oil sludge biodegradation. These introduced bacterial species mainly act through degradation of long chain hydrocarbons or production of biosurfactant. The aim of the present study was isolation of native bacteria from oil sludge produced in Abadan refinery, in Khouzestan, south west of Iran, for bioremediation of oil sludge and oil sludge polluted sites.

Materials and methods

Oil sludge sampling and analysis

Oil sludge samples were collected from oil reservoir tanks in Abadan oil refinery in south western Iran. Ten oil sludge samples were prepared from different parts of the Abadan oil refinery and then mixed and used as a bulk sample. Total petroleum hydrocarbons (TPH) of these samples were measured by soxhlet-extraction method. For this purpose, TPH content in 10 g of oil sludge was extracted using dichloromethane. Subsequently, this solvent was evaporated at room temperature in a safety fume hood and weight of TPH content was recorded (Mishra et al. 2001; Joseph, Joseph 2009). The carbon content of sludge was measured according to ASTM-D189 standard method (Jones, Pujadó 2006).

Bacterial isolation

A mineral base medium composed of 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.05 g $CaSO_4$ $2H_2O$, 0.5 g $MgSO_4$ H_2O, 0.09 g $FeSO_4$ $7H_2O$ and 1 g $(NH_4)_2SO_4$ (Merck, Germany) was used for isolation of native bacteria from oil sludge samples. This medium was freshly prepared for each test with pH adjusted to 7 by 1 mM solutions of NaOH or HCl. For enrichment of oil recovering bacteria from sludge, a 2-g sludge sample was inoculated to 100 mL mineral base medium in 250 mL Erlenmeyer flasks that had been sterilized at 121 °C for 15 min. This sludge included as the sole source of carbon and energy for bacteria in this medium. Then, 1 g of sludge or sludge contaminated soil was added to prepared flasks and incubated at 130 rpm and 28 °C for 5 days. After incubation, growth of bacteria was assessed by culturing on solid medium. For this purpose, 100 µL of broth was cultured on oil agar plates and incubated at 28 °C for 5 days. The colonies formed on agar plates were purified by subsequent subculturing and finally cultured and stored on nutrient agar medium (Merck, Germany; Joseph, Joseph 2009).

Screening of isolates for oil recovery

All of the colonies on solid medium were screened for their ability in oil recovery from sludge. For this purpose, a 5% (w/w) mixture of oil sludge and washed and sterilized sand was prepared; 250 g of this mixture in 200 mL distilled water was poured in 1L Erlenmeyer flasks. The C:N:P ratio was adjusted to 100:5:1 with respect to C content of sludge. Briefly, the concentrations of these elements were first measured in sludge sample and then urea and $(NH_4)_2HPO_4$, as nitrogen and phosphorus sources respectively, was added to achieve the ratio. An inoculum equal to 0.5 McFarland from fresh culture of isolates was prepared in the flasks. One flask was used as a control. These were incubated at 28 °C and 130 rpm for five days, and then allowed to settle. After this, the supernatant was poured off and the TPH content of deposits was measured as previously described. By comparing the pre- and post-treatment TPH content, the best isolates were selected and complementary studies were conducted (Joseph, Joseph 2009).

Study of oil recovery mechanisms by oil spread method

In this procedure, 1 mL of 24 h culture of the isolate was centrifuged at 13000 rpm for 10 min. Then, 15 µL of cell-free supernatant was added to 50 mL distilled water containing 40 µL sterile crude oil. The appearance of a clear zone on the surface of oil layer was considered as biosurfactant production (Deziel et al. 1996; Desai, Banat 1997; Satpute et al. 2008).

Study of oil recovery mechanisms by sheep erythrocyte haemolysis

This test was also used to assess biosurfactant production by isolates. A streak culture of bacterium was made on blood agar medium containing 5% sheep red blood cells and incubated for 24 h at 30 °C. Any sign of a hemolysis surrounding colonies was considered biosurfactant production (Deziel et al. 1996; Desai, Banat 1997; Satpute et al. 2008).

Study of oil recovery mechanisms by biodegradation of oil sludge saturated content

Biodegradation of saturated compounds was studied by gas chromatography (GC). For this purpose, a culture of isolate was prepared in oil recovery medium (as previously described) and incubated at 28 °C, 130 rpm for 5 days. One µL Supernatant samples (1 µL) from treated oil sludge were used in GC. Furthermore, untreated oil sludge was also extracted and 1 µL of rthe emained liquid was analyzed by GC and resultant diagrams were compared (Fedorak, Westlake 1981).

Bacterial growth curve

Serial dilution method and colony counts were used to determine growth curves. A bacterial culture was prepared in 100 mL Bushnell-Hass mineral base medium (pH 7) containing oil sludge and incubated at 28 °C and 130 rpm for five days. One mL of this culture was serially tenfold diluted in phosphate buffer and then cultured in duplicate on tryptic soy agar (Merck, Germany). The number of colonies formed on this medium were counted after 24 h

at 28 °C. This process was repeated every 12 h and after six days the growth curve was obtained (Van Hamme et al. 2000). Optimization of N and P sources, for the selected isolates was also done. To do this, the isolates were cultured in Bushnell-Hass mineral base medium with different nitrogen sources including $NaNO_3$ (1 g L^{-1}), NH_4Cl (0.7 g L^{-1}), NH_4NO_3 (1 g L^{-1}) and urea (0.75 g L^{-1}) and the growth curve was determined as above. In a similar way, different P sources including K_2HPO_4 (1 g L^{-1}), Na_2HPO_4 (0.84 g L^{-1}) and $(NH_4)_2HPO_4$ (0.8 g L^{-1}) were also used to find the optimum P source for each isolate (Reda, Ashraf 2010).

Identification based on 16S rRNA

DNA was extracted by a DNA extraction kit (Cinagene, Iran). Then, using FD1 and RP1 universal primers in a 25µl PCR reaction containing Taq DNA polymerase (1.5 u), $MgCl_2$ (2 mM), dNTPs (10 mM), PCR buffer, forward and reverse primers (10 pmol) and template (5 µL), the 1500 bp target of 16S rRNA was amplified as follows: initial denaturation at 94 °C (5 min), then 30 cycles of 94 °C (60 s), 62 °C (40 s) and 72 °C (150 s) and a final extension at 72 °C (20 min). The purified PCR product was sequenced (Macrogen, Korea) and identified based on homology comparison with NCBI databases (Weisburg et al. 1991).

Results

TPH content of oil sludge was determined by soxhlet-extraction method. The total polyhydrocarbon content of 10 g Abadan oil refinery oil sludge was 1.875 g or 185.7 g kg^{-1}. The carbon concentration of sludge according to ASTM-D189 was 13.4 kg per 100 kg. Bacterial isolation from sludge samples during five days of incubation at 28 °C resulted in eight bacterial isolates, further assessed for their oil recovery potential from sludge. After incubation and settling of the recovery medium, oil recovery appeared as a colored patch on the surface of recovery medium. Interestingly, in the control flask no oil separation appeared. The sludge of these recovery mediums was collected and its TPH content was determined (Table 1). Based on the comparison of the pre- and post-treatment, TPH content of oil sludge and the potential of oil recovery of isolates was determined. Isolates AOR_1 and AOR_2 showed 90 and 70% oil recovery, and were selected as the best isolates for further analysis. In oil spreading technique, the supernatant of both isolates caused a clear zone in the oil layer on the water surface, indicating biosurfactant production.

In haemolysis assay for both isolates, no haemolysis appeared on blood agar plates after 24 h of incubation. Fig. 1 shows the GC result of extracted hydrocarbons from oil sludge. Saturated hydrocarbons from C_{10} to C_{40} were present in the oil sludge. The majority of these constituents were C_{18} to C_{35}. The GC result of treated oil sludge with AOR_1 is illustrated in Fig. 2. All normal alkanes of oil sludge were present in treated supernatant with AOR_1. Thus this isolate does not have high potential of hydrocarbon degradation, but through biosurfactant production led to oil hydrocarbon release from oil sludge.

The supernatant of oil sludge treated with AOR_2 was also subjected to GC analysis (Fig. 3). In comparison to untreated oil sludge, C_{35} to C_{40} alkanes were degraded to light weight alkanes,and mainly C_{12} to C_{14} hydrocarbons.

The growth curve of AOR_1 is presented in Fig. 4. This isolate reached to the end of the log phase after 24 h and then entered the stationary and mortality phase; after 60 h it reached maximum growth again. This was shown in several experiments. In other word, the AOR_1 isolate showed several log phases during a 120 h period. The growth curve of the AOR_2 isolate showed a rapid log phase and reached maximum growth after 24 h, and then entered

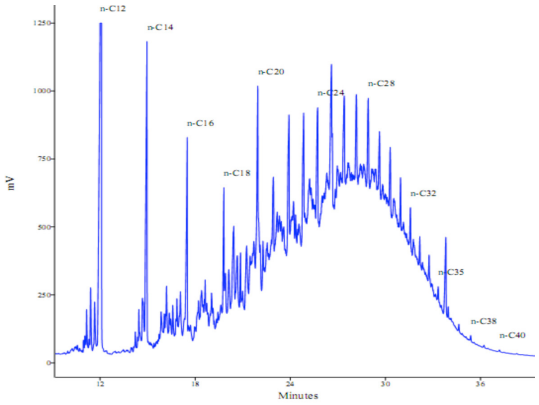

Fig. 1. Gas chromatography analysis of untreated oil sludge.

Table 3. TPH content of oil sludge following treatment with different isolates

Strain	Oil recovery (%)	Oil hydrocarbons of sediment (g)	Sediment (g)	Sludge oil (g)
Control	5	2.2	11.5	12.5
AOR_1	90	0.4	5.2	12.5
AOR_2	70	0.7	5.4	12.5
AOR_3	60	1.0	8.8	12.5
AOR_4	35	1.5	10.0	12.5
AOR_5	20	1.9	9.3	12.5
AOR_6	60	1.0	8.5	12.5
AOR_7	35	1.5	9.0	12.5
AOR_8	40	1.4	8.2	12.5

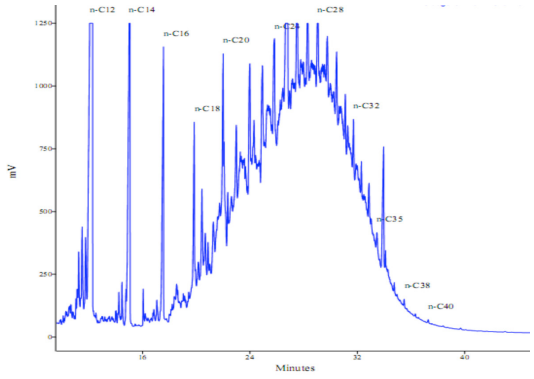

Fig. 2. Gas chromatography analysis of sludge treated with AOR1 strain.

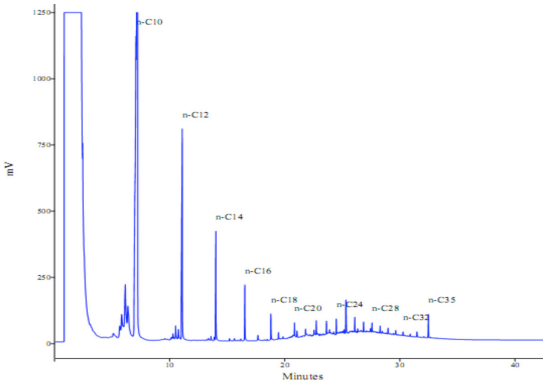

Fig. 3. Gas chromatography analysis of sludge treated with AOR2 strain.

the mortality phase and remained in this phase until 72 h (Fig. 5).

Different N sources were tested for optimum growth of AOR_1. Data in Fig. 6 shows that $NaNO_3$ was the best N source for AOR_1 growth; NH_4NO_3, NH_4Cl and urea had less effect. NH_4Cl showed the best result for AOR_2 growth, followed by NH_4NO_3, $NaNO_3$ and urea (Fig. 6).

Regarding optimization of P source for selected isolates, Na_2HPO_4 was the best P source for AOR_1 (Fig. 7). The two other P sources had minor effect on bacterial growth. Among the used P sources for optimization of AOR_2 growth, $(NH_4)_2HPO_4$ had best effect (Fig. 7).

Molecular identification of AOR_1 and AOR_2 based on 16SrRNA sequencing indicated that these isolates were *Pseudomonas stutzeri* and *Klebsiella pneumoniae* strain, respectively.

Discussion

Petroleum industries, including refineries, are sources of pollution in the environment. Oil sludge is composed of aliphatic hydrocarbons, aromatic hydrocarbons, polycyclic aromatic hydrocarbons and compounds containing sulfur, oxygen and nitrogen. High concentrations of these compounds are released during refinery processes, and due to their toxic and mutagenic effects can affect cells and their metabolic processes. This type of pollution can change physico-chemical properties of soil and influence seeds germination, growth and development of plants (Tanti et al. 2009). Oil refineries around the world apply different physical, chemical and biological treatments to manage oil sludge produced during refining. Among them, bioremediation using microorganisms is the most applicable method, as it has fewer side effects for the environment and increases biodegradation (Liu et al. 2009). Furthermore, a notable amount of raw petroleum remains in oil sludge. Considering the annual increase of oil prices, use of microorganisms can enhance oil recovery in parallel to reducing environmental contamination (Helmy et al. 2010). Presently there are few studies that have been focused on isolation and identification of bacteria from oil sludge. Bacteria that can tolerate this extreme environment will certainly be able to live in soil polluted with oil and oil derivatives, and they are good candidates for bioremediation.

Eight bacterial isolates were obtained fromthe tested oil sludge. All of these isolates showed potential of oil recovery from oil sludge. *Pseudomonas stutzeri* strain AOR_1 with 90% efficiency and *Klebsiella pneumoniae* strain AOR_2 with 70% efficiency in oil recovery from oil sludge were the best isolates and were selected for further study. Joseph and Joseph (2009), in a similar study, isolated *Bacillus* spp. from oil sludge. Calvo et al. (2004) reported that *Bacillus pumilus* strains isolated from oil sludge had ability of biosurfactant production and oil recovery. Ayotamuno et al. (2007) isolated *Pseudomonas* sp. and *Bacillus* sp. from oil sludge; during six weeks of treatment, TPH reduction by these bacteria reached 63 to 84%. In the study by Cameotra

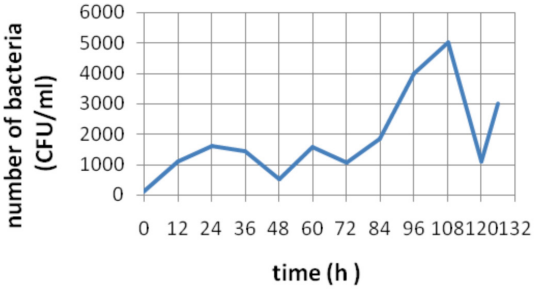

Fig. 4. Growth curve for *Pseudomonas stutzeri* AOR1 strain.

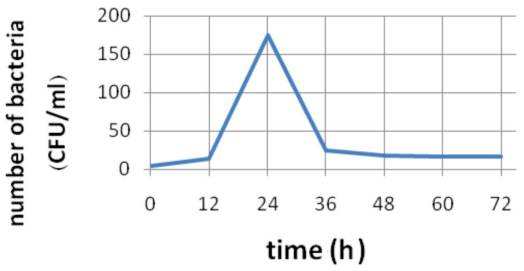

Fig. 5. Growth curve for *Klebsiella pneumoniae* AOR2 strain.

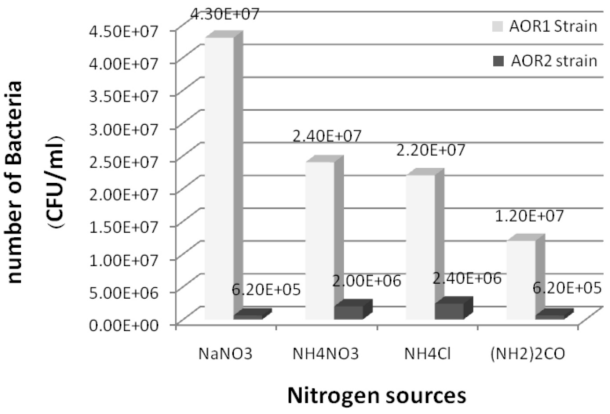

Fig. 6. Effect of different nitrogen sources on bacterial growth.

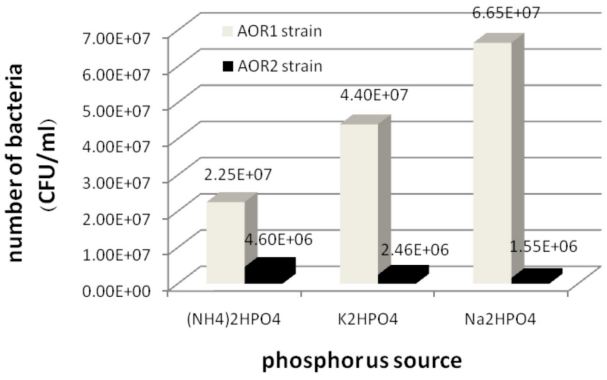

Fig. 7. Effect of different phosphorus sources on bacterial growth.

and Singh (2008) microbial consortium composed of *Pseudomonas aeruginosa* and *Rhodococcus* sp. isolated from soil polluted with oil sludge was used for bioremediation. This consortium was able to degrade about 90% of hydrocarbons during six weeks of incubation, which was at a level similar to the *Pseudomonas stutzeri* strain AOR_1 isolated in the present study.

Pseudomonas sp. and *Klebsiella* sp. have also been isolated from soils contaminated with oil (Makut, Ishaya 2010). In other studies (Mishra et al. 2001; Barathi, Vasudevan (2001) isolated *Pseudomonas* species were found to br able to degrade petroleum hydrocarbons and was effective in bioremediation. The results of the present study and other similar studies showed that the highest potential of oil recovery from sludge by bacteria was up to 90%. The lack of further recovery may be due to the presence of resins and asphaltenes, which no bacterial isolate will be able to degrade and release.

In order to determine if biosurfactant production is involved in oil recovery and TPH reduction in oil sludge, haemolysis of sheep erythrocytes and oil spreading technique were applied. Although, none of the two selected isolates showed haemolysis, the oil spreading technique was positive for both. However, negative hemolysis does not mean that biosurfactant is not produced, as this method has low specificity and some biosurfactants do not show haemolysis (Walter et al. 2010).

GC analysis of oil sludge suggested that C_{10} to C_{40} normal alkanes and various isoprenoid compounds were present in this sample. GC analysis of supernatant obtained from oil sludge treated with *P. stutzeri* strain AOR_1 did not show presence of C_{10} to C_{40} normal alkanes. This indicated that the degradation potential of this isolate is low and, in regard to 90% TPH reduction in oil sludge, it is a potent biosurfactant producer and through this mechanism can enhance oil recovery. However, some degradation causing reduction in long chain hydrocarbons was observed.

In comparison, GC showed that *K. pneumoniae* strain AOR_2 degraded long chain C_{36} to C_{40} hydrocarbons and other heavy hydrocarbons. GC peaks for light hydrocarbons significantly increased, mostly from C_{10} to C_{14}. This isolate shows a level of biodegradation and hence it has good potential for bioremediation (Pan et al. 2007). Furthermore, pristan and phytane were degraded by this isolate. Thus, degradation of heavy hydrocarbon compounds to light hydrocarbons is the main mechanism of action of this isolate. Behlulgil and Mehmetoğlu (2002) reported that laboratory degradation of petroleum compounds to light hydrocarbons leads to decrease of viscosity and can cause oil recovery. This can produce valuable products from heavy hydrocarbons. *Pseudomonas, Bacillus* and *Arthrobacter* species were the most potent bacterial isolates for this purpose.

An interesting result shown by the growth curve of *P. stutzeri* AOR_1 was that during six days of incubation it showed several log phases and reached maximum growth followed by a stationary and mortality phase. This isolate may selectively consume one carbon source and when this source is depleted it enters the mortality phase. Subsequently, it may utilize other carbon sources and enter the log phase. The second carbon source may be degraded compounds produced during early degradation or perhaps carbon sources that are present in oil sludge but require intracellular enzymes for biodegradation that are not released till bacterial death. Thus, a mortality phase may be required to start a new log phase. In contrast to *P. stutzeri* AOR_1, *K. pneumoniae* AOR_2 had rapid growth and reached maximum growth during 24 h, after which entered the mortality phase. The biomass of *K. pneumoniae* AOR_2 was significantly lower than *P. stutzeri* AOR_1.

Nitrogen and phosphorus are essential elements in protein and nucleic acid structures and also are required for bacterial growth and metabolism. The *P. stutzeri* AOR_1, strain used selectively $NaNO_3$ over other N sources, and thus has more potential in absorption of this resource. In contrast, *K. pneumoniae* AOR_2 had better growth in the presence of NH_4Cl, showing preference for this source of N. Na_2HPO_4 was the best P source for *P. stutzeri* AOR_1, while in case of *K. pneumoniae* AOR_2, $(NH_4)_2HPO_4$ was preferred.

It can be concluded that the isolated bacterial isolates

had a great potential in oil recovery from oil sludge, which makes them candidates for biodegradation and bioremediation. It is possible to enrich contaminated soils with suitable N and P sources found in this survey in order to promote the growth of these isolates in polluted sites. These isolates can be applied as a consortium for maximum biodegradation.

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