Bull Environ Contam Toxicol (2016) 97:684–688
DOI 10.1007/s00128-016-1924-2

Characterization of Crude Oil Degrading Bacteria Isolated from Contaminated Soils Surrounding Gas Stations

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Received: 22 January 2016 / Accepted: 15 September 2016 / Published online: 21 September 2016
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Abstract A total of twenty bacterial cultures were isolated from hydrocarbon contaminated soil. Of the 20 isolates, RAM03, RAM06, RAM13, and RAM17 were specifically chosen based on their relatively higher growth on salt medium amended with 4% crude oil, emulsion index, surface tension, and degradation percentage. These bacterial cultures had 16S rRNA gene sequences that were most similar to *Ochrobactrum cytisi* (RAM03), *Ochrobactrum anthropi* (RAM06 and RAM17), and *Sinorhizobium meliloti* (RAM13) with 96%, 100% and 99%, and 99% similarity. The tested strains revealed a promising potential for bioremediation of petroleum oil contamination as they could degrade >93% and 54% of total petroleum hydrocarbons (TPHs) in a liquid medium and soil amended with 4% crude oil, respectively, after 30 day incubation. These bacteria could effectively remove both aliphatic and aromatic petroleum hydrocarbons. In conclusion, these strains could be considered as good prospects for their application in bioremediation of hydrocarbon contaminated environment.

Keywords Bacteria · Bioremediation · Contaminated soil · Petroleum hydrocarbons · 16S rRNA

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Crude oil is a major energy source all over the world. It is a complex mixture of wide variety of different compounds including normal alkanes (n-alkanes), cyclic alkanes (c-alkanes), polyaromatic hydrocarbons (PAHs), and non-hydrocarbon compounds (Liang et al. 2012). Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Holliger et al. 1997). Contamination with petroleum hydrocarbons pose a significant threat to terrestrial and marine ecosystems, tourism and recreation activities (Zhang et al. 2012). Gas stations are potential sources of environmental contamination with hydrocarbon due to either underground storage tanks leakage or spills from fuel dispensing activities. The most abundant volatile organic compounds (VOCs) in gasoline, commonly known as BTEX, comprises benzene, toluene, ethylbenzene, and xylenes in diesel, are carcinogenic and very toxic substances which can involve a serious risk for ecosystem and human health (Marć et al. 2015).

The removal of petroleum hydrocarbons (PHs) contamination can be carried out by physical and chemical treatments, which allows the recovery of the adsorbent and adsorbed, though it is a technique that requires a lot of expenses (Daifullah and Girgis 2003). Various conventional methods like land filling, incineration, air spurging, etc. have been applied to remove these hydrocarbons since long time for remediation of oily waste (Mandal et al. 2011; Vidali 2011). However, these technologies are expensive and can lead to incomplete decomposition of contaminants. Bioremediation presents a promising alternative to traditional cleaning techniques, especially for treating large scale polluted areas. Nevertheless, biological treatment is an efficient, a non-destructive, and cost-effective technology for both ex-situ and in-situ remediation of environments contaminated by hydrocarbons (Liu et al. 2011).

Bioremediation is a natural process carried out by soil and aquatic microorganisms mostly bacteria and fungi whereby organic wastes are biologically degraded under controlled conditions to a harmless state, or to levels below concentration limits (Li et al. 2009). Soil microbes have the catabolic capacity to attack, transform and/or mineralize most of organic compounds. Oxygenases and Peroxidases are the main enzymes, which are used, in intracellular attack of inorganic pollutants and activation as well as incorporation of oxygen in the enzyme key reaction (Fritsche and Hofrichter 2000). The hydrophobic nature of petroleum oil hydrocarbons makes them poorly bioavailable. Thus, biosurfactant production is a desirable characteristic in oil degrading microbes as the biosurfactant increasing the bioavailability of these hydrocarbons and thus facilitate their assimilation by microbial cells (Pacwa-Płociniczak et al. 2016). In order to overcome this obstacle and increase the bioavailability of PHs in soil, the application of hydrocarbon-degrading and biosurfactant-producing bacteria for bioremediation technology is proposed. This advantage is employed for bioremediation of contaminated environments, especially in case of crude oil and oil products contamination, since soil bacteria can benefit from hydrocarbons by using them as carbon source and electron donor (Li et al. 2009). Therefore, the purpose of this study was to explore and compare the efficiency of bacterial cultures in bioremediation of crude oil contaminated soils.

Materials and Methods

The bacterial isolates used in this study were isolated from soil collected nearby different gas stations by enrichment cultivation. All cultivations were carried out at 30°C in (MBSM) modified basal salts medium (Hu et al. 2007; Al-Wasify and Hamed 2014). Five gram of soil was inoculated into 100 mL MBSM containing 2% (vol/vol) crude oil as the sole source of carbon, and incubated with shaking at 150 rpm for 7 days. After five cycles of enrichment, 1 mL of the culture was serially diluted and 100 µL was spread on 1.5% agar MBSM crude oil-coated plates, and incubated for 7 days. The selected bacterial isolates based on the phenotypic variations were cultivated overnight in LB broth. The washed bacterial cells were used to inoculate 300 mL flasks containing 25 mL of MBSM supplemented with 1 mL crude oil. All flasks were incubated at 30°C with shaking at 150 rpm/min for 13 days. The estimation of crude oil degradation was determined gravimetrically (Sakalle and Rajkumar 2009) and the best crude oil degraders were selected for further investigations. Experiments were performed in triplicate, and data are expressed as mean standard deviation (±SD).

Genomic DNA of selected bacterial isolates were extracted from 10 mL bacterial cultures grown overnight according to Ausubel et al. (1999). Oligonucleotide primers were used to amplify the 16S rRNA gene fragments (Abou-Shanab et al. 2010). A Perkin-Elmer 377 DAN sequencer in combination with Dye Deoxy Terminator Cycle Sequencing Kit was used for sequencing the amplified PCR products. A BLAST search of GenBank database was used to identify named bacterial species. The nucleotide sequences determined in this study have been deposited in the National Center for Biotechnology Information (NCBI) database.

The selected isolates were tested for their ability of emulsifying oil by measuring the emulsion index and the surface tension. Each bacterial isolate was grown on MBSM containing 2% glucose for 48 h and then filtrated. The emulsifying activity was determined using a modification of the method described by Bosch et al. (1988). The surface tension was measured with a ring-transiometer (TD1 LAUDA).

Batch experiment was performed in 100 mL flasks to investigate the ability of selected bacterial strains for crude oil degradation in both aqueous media (25 mL) and soil (20 g) amended with 4% crude oil. Flasks were inoculated with 2 mL (~7.4×10^8 colony forming unit/mL) of each bacterial isolate and the inoculum was thoroughly mixed into the soil and aqueous media. Un-inoculated flasks were used as control and all flasks were incubated at 30°C, for 30 day. The residual crude oil was extracted by solvent mixture (acetone/methylene chloride) (1: 1, vol/vol). The organic layer was taken out and evaporated at room temperature. The extracted residue was re-suspended in 1 mL n-hexane from which 1 µL was injected in a gas chromatography (GC) for analysis (Ho-Sang and Oh-Seung 2000).

Results and Discussion

Total culturable crude oil utilizing bacteria as a colony forming unit g^{-1} soil (85×10^{11}) were determined in soil samples collected nearby gas stations. Out of these cultures 20 bacterial isolates were selected based on colony morphology and size to determine their ability for 4% crude oil degradation (Fig. 1). The biodegradation rates of crude oil (4%) ranged from 10% to 93%. The highest degradation (93%) was achieved by isolate RAM13 followed by RAM03 (89%), RAM06 (73%) and RAM17 (69%). While, the lowest degradation rates (10%) was obtained by isolate RAM04. This might account for the varying ability of these isolates to produce biosurfactants/bioemulsifiers and hence effect on crude oil degradation. In many studies the concentration of oil was accepted to be 1%–2% and generally microorganisms can tolerate the crude oil concentration below 5% in their culture medium (Minoui et al. 2008). Out of the 20 isolates, four bacterial cultures (RAM03, RAM06, RAM013,

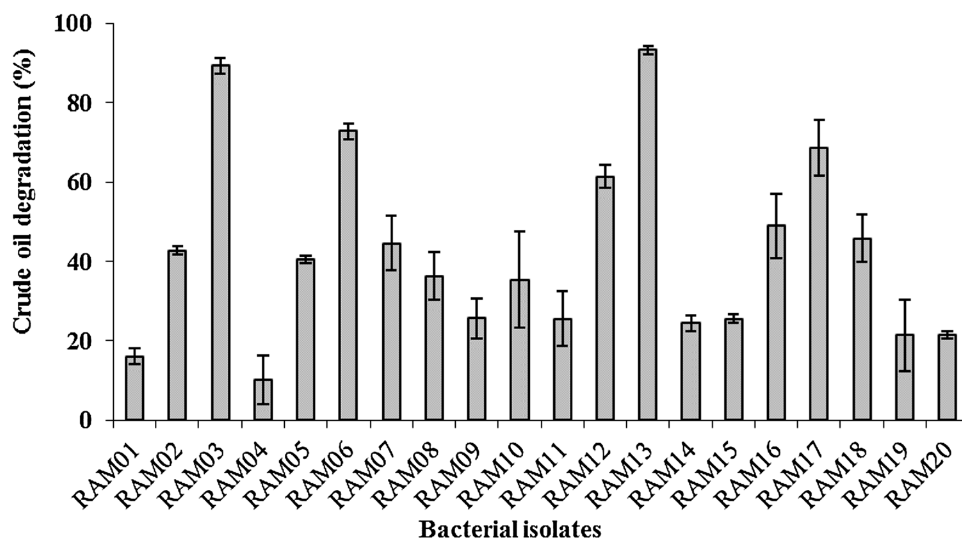

Fig. 1 Crude oil degradation by different bacterial isolates growing in MBSM amended with crude oil (4 %) after 13 day incubation at 30°C and 150 rpm. Error bars represent standard error of the mean (n = 3)

and RAM17) were chosen based on their degradation rates for further experiments.

Analysis of the 16S rRNA gene sequences revealed that these isolates (RAM03, RAM06, RAM013, and RAM17) were closely related to *Ochrobactrum cytisi, Ochrobactrum anthropi, Sinorhizobium meliloti*, and *Ochrobactrum anthropi* based on 96 %, 100 %, 99 %, and 99 % similarity in their 16S rDNA gene sequences, respectively. The nucleotide sequence coding for these 16S rRNA genes have been submitted to the GenBank database under the accession numbers KM491174, KT962902, KT958556, and KT962903, respectively. Members of these genera have also been identified as crude oil degraders and biosurfactant producers in a wide range of studies (Sarma et al. 2012; Bezza et al. 2015; Wang et al. 2016).

Therefore these bacterial strains were screened for their ability to produce biosurfactants/bioemulsifiers. The initial surface tension was 58 mN m^{-1} in un-inoculated flasks (control), while the presence of bacterial strains (RAM03, RAM06, RAM13 and RAM17) reduced the surface tension to 29, 38, 39 and 40 mN m^{-1}, respectively (Fig. 2). The maximum emulsion index (60 %) with lower surface tension (29 mN m^{-1}) was achieved by *O. cytisi* (RAM03), while the lower emulsion index (43 %) with higher surface tension (40 mN m^{-1}) was obtained by *O. anthropi* (RAM17). A similar observation was made by Ferhat et al. (2011) who found that *O. intermedium* and *Brevibacterium lutescens* reduced the surface tension below 31.5 mN m^{-1}. The GC analysis of crude oil residues in culture media revealed that *O. cytisi* (RAM03), *O. anthropi* (RAM06), *S. meliloti* (RAM013), and *O. anthropi* (RAM017) degraded 93 %, 88 %, 90 %, and 85 % of TPHs, respectively. While, in crude oil contaminated soil RAM03, RAM017, RAM013, and RAM06 degraded 54 %, 49 %, 44 % and 20 % of TPHs, respectively, after 30 day incubation (Fig. 2). The highest TPHs degradation rates in aqueous media (93 %) and soil (54 %) were achieved by *O. cytisi* (RAM03) most likely as a result of its ability to produce biosurfactants/bioemulsifiers. Johnsen et al. (2005) reported that PH compounds strongly bind to soil particles and thus are poorly accessible to bacterial cells.

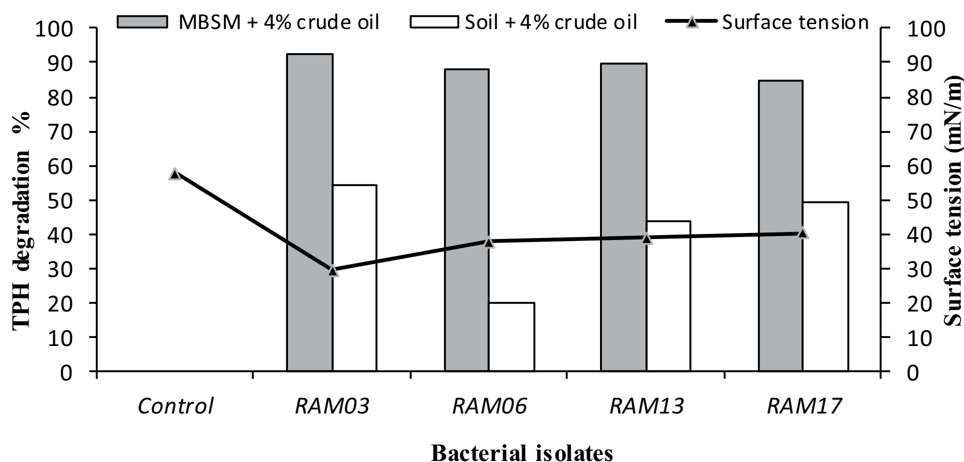

Fig. 2 Effect of bacterial isolates on surface tension and TPH degradation in aqueous media (MBSM) and soil amended with 4 % crude oil after 30 day incubation

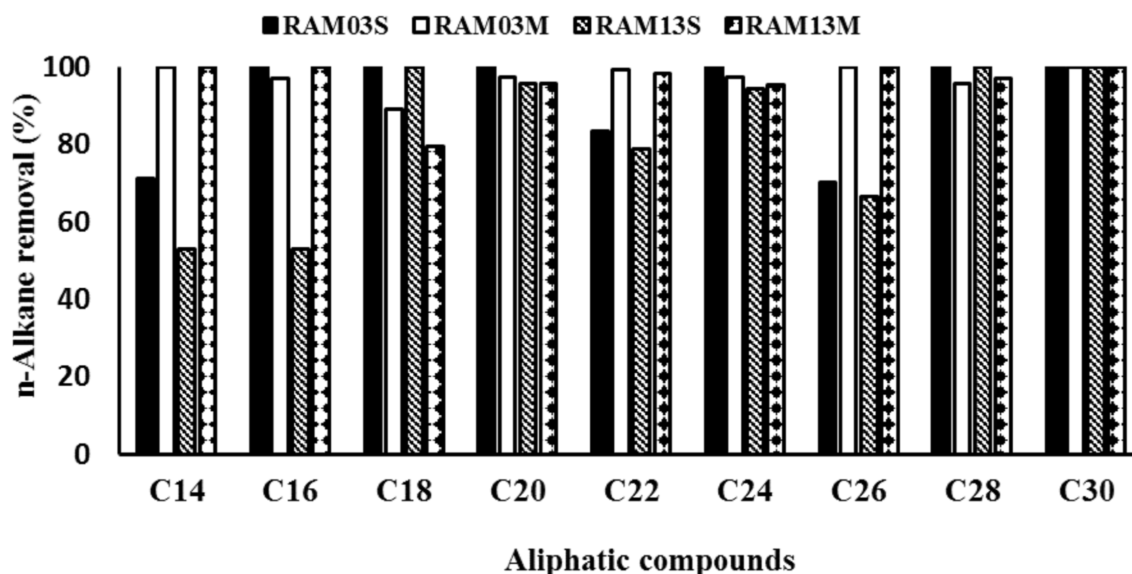

Fig. 3 Degradation percentage of aliphatic compounds by bacterial strains in aqueous media (M) and soil (S) supplemented with 4 % crude oil after 30 day incubation

High emulsification index and low surface tension enhance the bioavailability of hydrophobic compounds to bacterial cells and thus support faster degradation of crude oil (Martino et al. 2012). Nevertheless, emulsification activity is specific for bioemulsifiers and is not always exhibited by biosurfactant-producing strains (Batista et al. 2006). Willumsen and Karlson (1997) observed that emulsion formation did not correlate with a reduction of surface tension. However, the results obtained by other authors showed that the ability of bacteria to form a stable emulsion is not very common (Kumar et al. 2013; Tambekar and Gadakh 2013). Ekpo and Udofia (2008) reported that biodegradation of crude oil in MSM was up to 97 %, 86 % and 72 % by *Pseudomonas aeruginosa, Micrococcus varians* and *Bacillus subtilis*, respectively.

Two bacterial strains [*O. cytisi* (RAM03) and *S. meliloti* (RAM13)] were chosen based on their degradation rates of TPHs. The results of aliphatic hydrocarbons degradation in soil and aqueous media by *O. cytisi* and *S. meliloti* were shown in Fig. 3. We observed exceeding individual n-alkane degradation in aqueoues media and soil inoculated with *O. cytisi* compared to *S. meliloti*. This was most obvious for C18, C20, C22, and C24 for aqueous media; *O. cytisi* was almost always more efficient than *S. meliloti* but with small differences. In soil inoculated with *O. cytisi* a significant difference was detected for C14, C16, C20, C22, C24, and C26, since the performance of *O. cytisi* and *S. meliloti* was almost identical for the rest of PHs. Kuyukina et al. (2013) reported that the higher n-alkane degradation (80 %–100 %) was observed in *Rhodococcus*-amended soils compared to un-inoculated soil (20 %–40 %), especially for n-alkanes with chain lengths from C18 to C29.

The results of this study indicate that from 20 hydrocarbon degrading bacterial isolates, *O. cytisi* and *S. meliloti* showed a high degradation ability of crude oil in aqueous media with n-alkanes (C14–C30) as substrates; *O. Cytisi* demonstrated a slightly better performance in aqueous media with C14 and C16 though. These results indicate that these strains have great potential for bioremediation of PHs contaminated sites. Further studies are justified to determine the functional metabolism and molecular mechanism involved in PHs biodegradation.

Acknowledgments Funding was provided by City of Scientific Research and Technology Applications, Genetic Engineering and Biotechnology Research Institute, Department of Environmental Biotechnology.

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