CHARACTERIZATION OF CRUDE OIL DEGRADING BACTERIA ISOLATED FROM CONTAMINATED SOILS SURROUNDING GAS STATIONS

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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 broth basal salt medium amended with 4% crude oil (as a sole source of carbon), emulsion index, surface tension, and degradation percentage. These bacterial cultures had 16S rRNA gene sequences that were most similar to *Ochrobactrum cytisi* (RAM03), *Ochrobactrum anthropic* (RAM06 and RAM17), and *Sinorhizobium meliloti* (RAM13) with 96, 97 and 97, and 97% similarity. We assessed the capability of these bacterial strains to degrade crude oil in culture medium and soil. The tested bacterial strains revealed a promising potential for bioremediation of petroleum oil contamination as they could degrade more than 84% and 54% of total petroleum hydrocarbons (TPHs) in modified basal salt medium and soil, respectively, amended with 4% crude oil after 30 day incubation. These bacteria could effectively remove both aliphatic and aromatic petroleum hydrocarbons, and they are able to produce bio-surfactant. These data indicate that these isolates may have the potential for use in bioremediation of petroleum hydrocarbon contaminated soil.

Keywords: Bacteria, bioremediation, crude oil, petroleum hydrocarbons, contaminated soil, 16SrRNA

1. Introduction

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). Contamination with petroleum hydrocarbons pose a significant threat to terrestrial and marine ecosystems, tourism and recreation activities (Zhang *et al*., 2012). The removal of petroleum hydrocarbons 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. It is observed that none of the conventional methods is environment friendly solution (Sood *et al*., 2009). Nevertheless, biological treatment is an efficient, environment-friendly, and cost-effective technology for both *ex-situ* and *in-situ* remediation of environments contaminated by hydrocarbons (Liu *et al*., 2011).

Biodegradation 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 and /or mineralize most of organic compounds. 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 a source of carbon (Li *et al*., 2009). Although, extensive

research for decades on the biological effects and degradation of hydrocarbons in soil, transformation of laboratory-developed bioremediation technique *in-situ* gives often unsatisfactory results. Therefore, the purpose of this study was to explore and compare the efficiency of bacterial cultures in bioremediation of crude oil contaminated soils. In addition, the identification of these isolates was confirmed by the partial sequence of the 16SrRNA genes.

2. Materials and methods

2.1. Enrichment culture and biodegradation assay

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^{o}C in (MBSM) modified basal salts medium (Hu *et al*., 2007). 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 25ml of MBSM supplemented with 1ml crude oil. All flasks were incubated at 30^{o}C with checking at 150 rpm/min for 13 day. The estimation of crude oil degradation was determined gravimetrically (Sakalle and Rajkumar, 2009) and the best crude oil degraders were selected for further investigations.

2.2. Bacterial identification

Genomic DNA of selected bacterial isolates were extracted from 10 ml bacterial cultures grown overnight according to Ausubel *et al*., (1987). Oligonucleotide primers were used to amplify the 16SrRNA 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.

2.3. Surface active properties

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 48h 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).

2.4. Gas chromatographic analysis of residual crude oil

The ability of selected strains to degrade aerobically crude oil was determined in 100 ml flasks containing 25ml of MBSM and in Falcon tube (50ml) contains 20g soil, both MBSM and soil amended with 4% crude oil and inoculated with 2ml of bacterial suspension with optical density 0.9 at 600nm. Un-inoculated flask and tube were used as control and experiment was incubated at 30^{o}C, 150 rpm/min 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 GC for analysis (Ho-Sang and Oh-Seung, 2000).

3. Results and discussion

3.1. Enrichment and isolation of crude oil utilizing bacteria

An enrichment culture has been widely accepted as the method of choice for isolating bacteria expressing specific phenotypes and has been used successfully to isolate bacteria capable of degrading hydrocarbons (Al-Wasify and Hamed, 2014). Total culturable crude oil utilizing bacteria as a colony forming unit/ g soil (85 x 10^{11} cfu/g) were determined in soil samples collected nearby gas stations. Out of these cultures 20 bacterial isolates were selected based on colony morphology and size from MBSM containing 2% crude oil as a sole carbon source.

3.2. Selection of oil degrading bacteria

To evaluate the degradation potential of these isolates, crude oil degradation in culture media was determined gravimetrically after 13 day of incubation (see Figure 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 survive in a given concentration of crude oil. In many studies the concentration of oil was accepted to be 1 to 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, RAM13 and RAM17) were chosen based on their degradation rates.

3.3. Surface active properties

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 to the degrading microbes (Sepahi *et al*., 2008). The initial surface tension was 58 mN/m in control (in the absence of bacteria), while the presence of bacterial strains (RAM03, RAM06, RAM13 and RAM17) reduced the surface tension to 29, 38, 39 and 40 mN/m, respectively (see Figure 2). The maximum emulsion index (60%) with lower surface tension (29 mN/m) was achieved by *Ochrobactrum cytisi* (RAM03). A similar observation was made by Ferhat *et al*. (2011) who found that *Ochrobactrum intermedium* and *Brevibacterium lutescens* reduced the surface tension below 31.5 mN/m. 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).

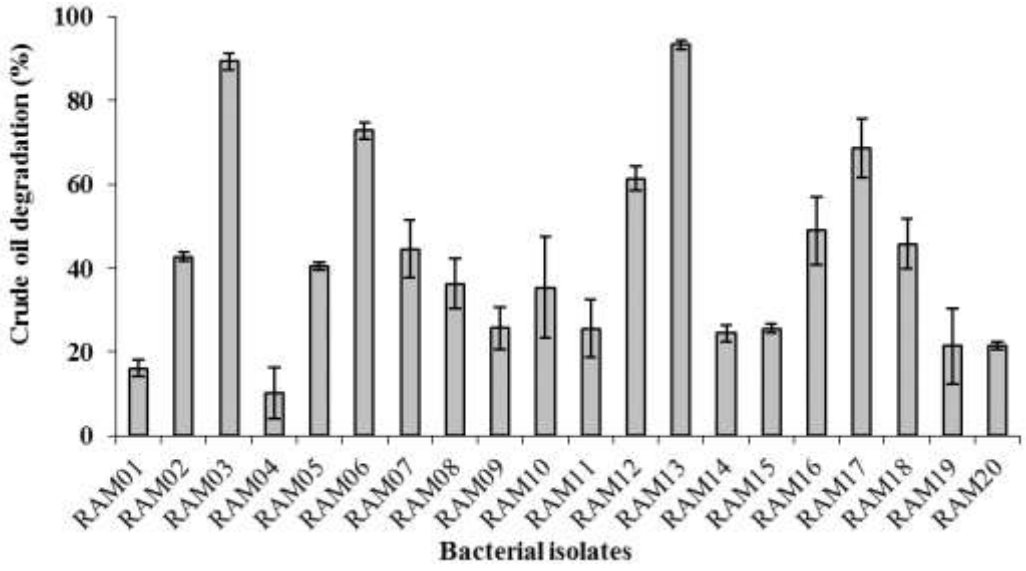

Figure 1: Crude oil degradation by different bacterial isolates growing in MBSM amended with crude oil (4%) after 13 day incubation at 30°C and 150 rpm.

3.4. Biodegradation of total petroleum hydrocarbons (TPHs)

The GC analysis of crude oil residues in culture media revealed that *O. cytisi* (RAM03), *O. anthropi* (RAM06), *O. anthropi* (RAM017), and *S. meliloti* (RAM013) degraded 93, 88, 90, and 85% of TPHs, respectively. In crude oil contaminated soil RAM03, RAM017, RAM013, and RAM06 degraded 54, 49, 44 and 20% of TPHs after 30 day incubation (see Figure 2). Indigenous microbes of soil may not be efficient degraders of complex compounds of crude petroleum-oil therefore, introduction of efficient hydrocarbon degraders would be necessary in order to effectively degrade these complex hydrocarbons. 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.

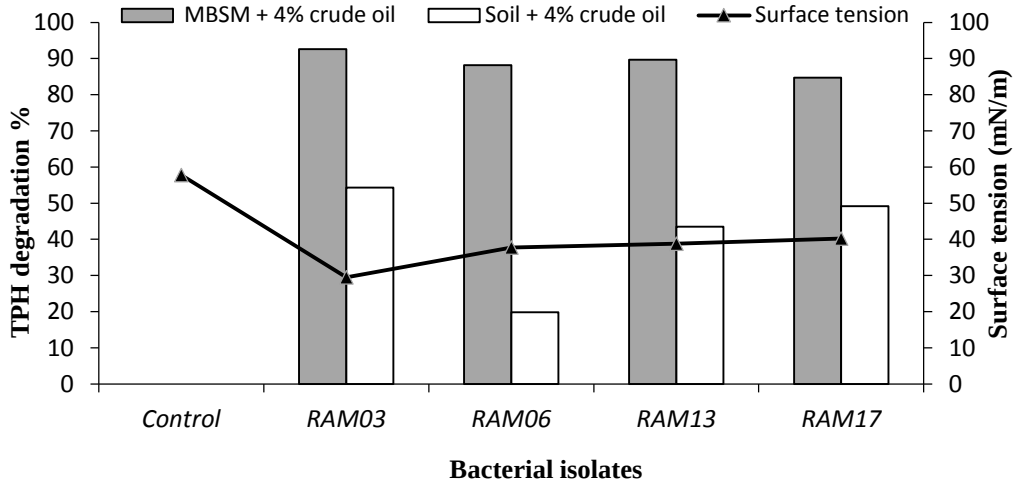

Figure 2: Effect of bacterial isolates on surface tension and TPH degradation in MBSM and soil amended with 4% crude oil after 30 day incubation.

3.5. Aliphatic hydrocarbons degradation

The results of aliphtic hydrocarbons degardation in soil and aqueous media by selected bacterial strains [(*O. cytisi* (RAM03) and *S. meliloti* (RAM13)] were shown in Figure 3. The maximum degrdation rates (from 96 to 100% and from 70 to 100%) of n-alkanes (C14-C30) were achieved by *O. cytisi* (RAM03) growin in aquoues media and soil, respectively. The greater n-alkane degradation was observed in *Rhodococcus*-amended soils compared to control soil, especially for n-alkanes with chain lengths from C18 to C29; which demonstrated 80-100% removal in inoculated soils whereas corresponding control values were 20-40% (Kuyukina *et al.*, 2013).

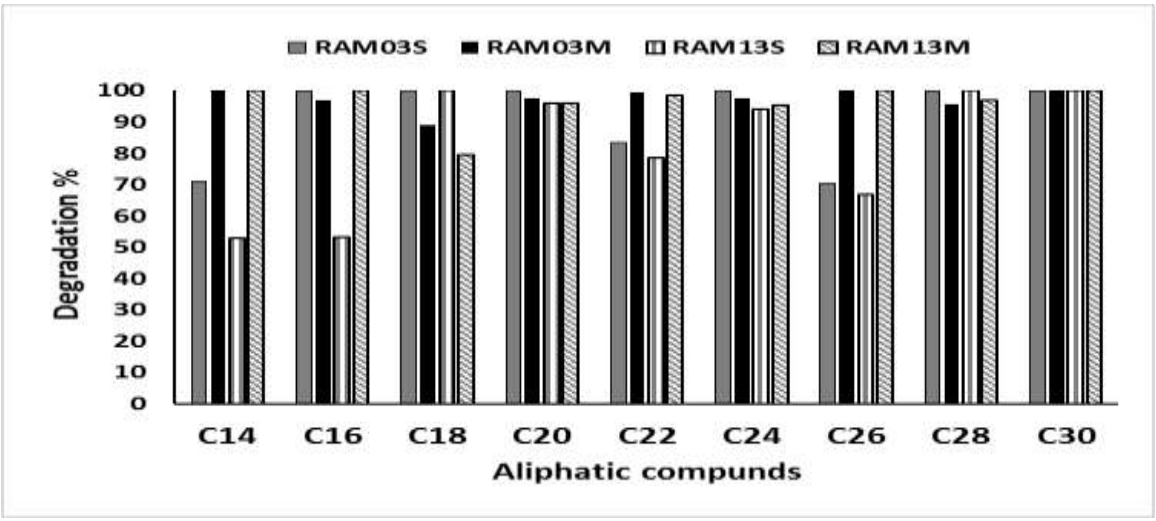

Figure 3: Degradation percentage of aliphatic compounds by bacterial strains in aqueous media (M) and soil (S) supplemented with 4 % crude oil after 30 day incubation.

4. Conclusions

Our results reveal that the oil degredation rates was dependent upon the reduction of surface tenstion and the increase of emulsion index. Both properties lead to increased solubility or mass transfer efficiency of hydrophobic compounds, improving the utilization of these substrates by

Ochrobactrum sp. and *S. meliloti*. These bacterial isolates have a promising potential for bioremediation of petroleum oil contaminated sites.

REFERENCES

1. Abou-Shanab R.A.I., van Berkum P., Angle J.S., Delorme T.A., Chaney R.L., Ghozlan H.A., Ghanem K. and Moawad H. (2010), Characterization of Ni-resistant bacteria in the rhizosphere of the hyperaccumulator Alyssum murale by 16S rRNA gene sequence analysis. World J. Microbiol. Biotechnol., 26,101-108.
2. Al-Wasify R.S. and Hamed S.R. (2014), Bacterial Biodegradation of Crude Oil Using Local Isolates. Internat.J.Bacteriol., 2014, 1-8.
3. Ausubel F.M., Brent R., Kingston R.E., Morre D., Seidman J.C. and Struhl K. (1987), Current protocols in molecular bioliogy, Green and Wiley-interscience, New York.
4. Bosch M. P., Robet M., Mercade M. E., Espuny M. J., Parra J. L. and Guinea J. (1988), Surface-active compounds on microbial cultures. Tenside Surf. Deterg., 25, 208-212.
5. Daifullah A. and Girgis B. (2003), Impact of surface characteristics of activated carbon on adsorption of BTEX. Colloids Surf. Physicochem. Eng. Aspects, 214, 181-193.
6. Ekpo M.A. and Udofia V.S. (2008), Rate of biodegradation of crude oil by microorganism isolated from oily sludge environment. Afr. J. Biotechnol., 7, 4495-4499.
7. Ferhat S., Mnifb S., Badis A., Eddouaouda K., Alouaouic R., Boucherit A., Mhirib N., Moulai-Mostefa N. and Sayadi S. (2011), Screening and preliminary characterization of biosurfactants produced by Ochrobactrum sp. 1C and Brevibacterium sp. 7G isolated from hydrocarbon-contaminated soils, Int. Biodeterioration and Biodegradation, 65, 1182-1188.
8. Ho-Sang S.H. and Oh-Seung K. (2000), The Simultaneous Analysis of Benzene, Toluene, Ethylbenzene, o,m,p-Xylenes and Total Petroleum Hydrocarbons in Soil by GC-FID after Ultra-Sonication, Bull. Korean Chem. Soc., 21, 11.
9. Hu Z. F., Dou J. F., Liu X., Zheng X. L. and Deng D. (2007), Anaerobic biodegradation of benzene series compounds by mixed cultures based on optional electronic acceptors. J. Environ. Sci., 19, 1049-1054.
10. Kuyukina M.S., Ivshina I.B., Kamenskikh T.N., Bulicheva M.V. and Stukova G.I. (2013), Survival of cryogel-immobilized Rhodococcus strains in crude oil-contaminated soil and their impact on biodegradation efficiency. Int. Biodeterioration and Biodegradation, 84, 118-125.
11. Li Z., Binazadeh M. and Karimi I.A. (2009), Fast biodegradation of long chain n-alkanes and crude oil at high concentrations with Rhodococcus sp Moj-3449. Enzyme Microb. Tech., 45, 195-202.
12. Liang Y.T., Zhang X., Wang J. and Li G.H. (2012), Spatial variations of hydrocarbon contamination and soil properties in oil exploring fields across China. J Hazard Mater., 241, 371-378.
13. Liu X., Wang Z., Zhang X., Wang J. Xu, G. and Cao Z. (2011), Degradation of diesel-originated pollutants in wetlands by Scirpus triqueter and microorganisms. Ecotox Environ. Safe, 74, 1967-1972.
14. Martino C. D., López N. I. and Iustman L. J. R. (2012), Isolation and characterization of benzene, toluene and xylene degrading Pseudomonas sp. selected as candidates for bioremediation, Int. Biodeterioration and Biodegradation, 67, 15-20.
15. Minoui S., Tehrani M. D., Zare A. and Ahmadi S. (2008), Effect of heavy crude oil on the pattern of respiratory chain of pseudomonas sp., Terrestrial and aquatic environmental toxicology, 2, 34-37.
16. Sakalle K. and Rajkumar S. (2009), Isolation of Crude Oil Degrading Marine Bacteria and Assessment for Biosurfactant Production, Internet J. Microbiol. 7
17. Sepahi A.A., Golpasha I.D., Emami M. and Nakhoda A.M. (2008), Isolation and characrization of crude oil degrading Bacillus spp. Iran. J. Environ. Health. Sci. Eng., 5,149-154.
18. Sood N. and Banwari L. (2009), Isolation of a novel yeast strain Candida digboiensis TERI ASN6 capable of degrading petroleum hydrocarbons in acidic conditions, J. Environm. Management, 90, 1728-1736.
19. Zhang D.C., Mortelmaier C. and Margesin R. (2012), Characterization of the bacterial archaeal diversity in hydrocarbon-contaminated soil, Sci Total Environ., 421, 184-196.