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Biodegradation of Hydrocarbons via Bacteria Isolated from Polluted Soil: A Case Study, Dammam; Saudi Arabia

Mohamed M. Ibrahim[1,2]*, Ameena Al-Turki[3], Duna Al-Suwaidi[3] and Gehan A. El-Gaaly[1]

[1]King Saud University, Science College, Botany and Microbiology Department, Riyadh 11451-P.O. Box 2455, Sauid Arabia.
[2]Botany and Microbiology Department, Faculty of Science, Alexandria University, 21511, Alexandria, Egypt.
[3]Al-Bassam Schools, Ministry of Education, Dammam, P.O. Box 945, Saudi Arabia.

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Five pure aerobic microbial isolates were found from polluted soil samples of two areas of Eastern region, Dammam; Saudi Arabia. All isolates were gram positive and three of them were identified and found belong to coryneform and Nocardioform bacteria. Five isolates were examined for their capability to grow on mineral salt agar plates accompanied with one of the following typical n-alkanes or aromatic hydrocarbons: hexane, heptane, paraffin, benzene, toluene, naphthalene and kerosene. Cell hydrophobicity, the capability to yield glycolipids and extracellular emulsifying action were also detected and evaluated on the basis of growth of soil isolates on hydrocarbons. This study revealed degraders with broader abilities to grow on both types of hydrocarbons, good production of glycolipids and emulsifying activity. On this basis, isolate S2 with good emulsifying activity and producing glycolipids was proposed for bioremediation applications of hydrocarbon polluted soil environments.

Key words: Bioremediation, Dammam, Hydrocarbons, Pollution.

Environmental contamination by petrol derivatives has been a subject of study over the past four decades. The leakage of these derivative oils, such as lubricant oils, is capable of harming the environment in many ways (Atlas, 1995; Okoh *et al*., 2006). A major concern for petroleum hydrocarbon bioremediation is the presence of heavy compounds such as polycyclic aromatic hydrocarbons (PAHs), asphaltenes and many branched compounds with 20 or more carbon atoms (Winkelmann *et al*., 2009). These heavy hydrocarbon constituents are not easily metabolized by microorganisms and are considered potential health risks due to their possible carcinogenic and mutagenic actions (Baheri and Meysami, 2002). Furthermore, 1% of all oil consumption is used to produce lubricants (Bartz, 1998; Perelo, 2010) and all procedures in lubricant oil production and transport can generate environmental impacts (Amund, 1996; Parish *et al*., 2004). Moreover, lubricant oil can persist for more than six years in some ecosystems, resulting in chronic problems to the biota (Burns *et al*., 1996; Plohl *et al*., 2002).

Lubricant discharge in nature causes continuous concern due to its non-quantified impact and its potential chronic damage (Wright, 1993; Husaini *et al*., 2008). In this context, residual lubricant oil contained in commercial bottles

* To whom all correspondence should be addressed.

causes significant contamination, because one ton of this automotive oil corresponds to the waste produced from the domestic sewers of 40,000 inhabitants per day. Besides this, only 1.0 L of lubricant is able to deplete the oxygen of 1,000,000 L of water, therefore a city discharge of 200,000 inhabitants is the same as 47 L of residual oil, which affects 47 million L of water (Lopes *et al*., 2008; 2010).

The high rate of growth in the petrochemical industries locally and globally cause significant environmental problems as a result of harmful residues resulting from the fields of manufacturing , in addition to the hydrocarbon residues resulting from the use of these products. Saudi Arabia producing 50 % of the petrochemical products in the Gulf region and considered as one of the top petrochemical producing countries and by 2015 will produce nearly five total world output of petrochemical products. The Arabian Organization for Industrial and Mining Development reported that Saudi Arabia has about 70 percent of the petrochemical industry in Arab world. Moreover, the annual report of the " Union Gulf of the Petrochemicals and Chemicals" documented that the production capacity of the petrochemicals in Saudi Arabia has reached 86.4 million tons in 2012 , in a striking increase from previous year's levels of 80.5 million tons.

There is an increased interest in promoting environmental methods in the process of cleaning oil-polluted sites. These methods are less expensive and do not introduce additional chemicals to the environment. Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are biodegradable(Huang *et al*., 2004).

Due to hydrocarbons being widespread pollutants, their hydrophobicity causes a low bioavailability and therefore a particular persistence against bioremediation measures (Anderson *et al*., 1995; Azadpour *et al*., 1997). However, numerous terrestrial and aquatic species of microorganisms possess the ability to degrade these hydrocarbons. In order to increase the bioavailability of these otherwise hardly accessible substrates, microorganisms use strategies like increasing the hydrophobicity of their surfaces or the production of biosurfactants (Niescher *et al*., 2006).

The aim of the present study was to isolate microorganisms from the eastern region, Dammam Saudi Arabia with greatest acting hydrocarbon degraders with good emulsifying activity and producing glycolipids. Based on this results our isolate could be proposed for treatment of soil environments polluted with different hydrocarbons.

MATERIALS AND METHODS

Isolation of microorganisms

A survey of petroleum-degrading bacteria was carried out in soil polluted with hydrocarbons at the study area to evaluate distribution of naturally occurring petroleum-degrading aerobic bacteria. Different soil samples (Eastern region, Dammam, Saudi Arabia) were used for microbe isolation. One gram of soil from each site was homogenized and rinsed with 20 ml of physiological solution. Serial dilutions were made and 0.05 ml of them were spread onto plates with appropriate media as beef extract-pepton agar; Kuster agar; Czapec agar; mineral agar No 1; maize extract agar No 6 (Gauze *et al*., 1983).

Media and growth conditions

All cultivations were performed in mineral salts medium (MSM) which contains (g^{-1} D^{-1}): $(NH4)_2SO_4$ (2.0); KH_2PO_4 (6.0); $Na_3C_6H_5O_7 \cdot 2\,H_2O$ (1.0); $MgSO_4 \cdot 7\,H_2O$ (0.1), supplemented with 2 mm $CaCl_2$ (pH 7.0). Isolated pure cultures were tested for their ability to grow on solid MSM with 1.5% agar and with % of one of the following model hydrocarbons: alkanes D-hexane, heptane and paraffin; aromatics D- benzene, toluene and naphthalene; and kerosene, a mixture of aromatic hydrocarbons and alkanes. Hydrocarbons were sterilized by filtration through 0.2-µm membrane filters (Millipore Corp., USA). Agar plates were incubated for 20 to 30 days at 20°C or 28°C. Isolate was cultivated in 300 ml Erlenmeyer flasks containing 40 ml liquid MSM supplemented with 2% (v/v) of one of the following hydrocarbons: heptane, paraffin, benzene and kerosene as a sole source of carbon and energy. As inoculum was used culture after 24 h of cultivation in liquid meat-

peptone broth (MPB). Flasks were incubated shaking (130 rpm) for 16 days at 25°C. Growth was monitored by measuring the optical density at 570 nm (OD570).

Identification of microorganisms

The classification and determination of hydrocarbon-degrading strains isolated from polluted soil was performed in the central Laboratory of dental college, King Saud University, Riyadh; KSA. An automated test system VITEK API 20E (Bio Merieux, Inc. Hazelwood, Mo., USA) was used for determination and identification of isolates. It detects bacterial growth and metabolic reactions in the micro wells of plastic test cards by measuring fluorescence. Isolates were subcultured onto agar and incubated for 24 h at 37°C before testing on the VITEK system. The microorganisms were identified according to general principles of microbial classification, using selective media and macro- and microscopic examination of morphological characters.

Cell surface hydrophobicity test

Cell hydrophobicity was measured by microbial adherence to heptane according to a method of Rosenberg*et al*. (1980) with slight modification. The cells were washed twice and resuspended in PUM buffer, pH 7.1 (22.2 g $K_2HPO_4 \cdot 3\,H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7\,H_2O$ and distilled water to 1000 ml), to an initial absorbance of the cell suspension at 550 nm of 0.5-0.6. The cell suspension (1.2 ml) with heptane (0.2 ml) was vortexed in a test tube at high speed for 2 min and equilibrated for 1 h. The optical density of the bottom aqueous phase was then measured at 550 nm. Hydrophobicity was expressed as the percentage of adherence to the hydrocarbon calculated as follows: 100 ×(1-OD of the aqueous phase/OD of the initial cell suspension).

Emulsifying activity

The emulsifying activity of the supernatant fluids after growth of soil isolates on glucose was determined using the test of Berg*et al*.(1990) with slight modification. Samples (0.2 ml, after suitable dilution) were mixed with 0.5 ml of TM buffer (20 mm Tris [Tris (hydroxyl methyl)-amino methane]/ HCl buffer, pH 7.0 and 10 mm$MgSO_4$) and then 0.1 ml of kerosene was added. The tubes were then vortexed at room temperature and at high speed for 1 min. The turbidity of the water phase was measured at 550 nm after standing for 1 min. One unit of emulsifying activity was defined as the amount of the emulsifier producing an absorbance of 1.0 at 550 nm in the assay (EU ml^{-1}).

RESULTS

Isolation of soil microorganisms

Five different aerobic microbial colonies were isolated from polluted soil samples. On solid media some strains showed rod morphology. Dependent on the growth phase in liquid media (exponential or stationary) the cells passed from rods to cocci. Morphological and some biochemical properties of the strains give us a means to determine them as coryneform a genus of Gram-positive, rod-shaped bacteria (Table 1). Other strains have features of actinomycetes and belong to following genera: *Nocardia* and related Nocardioform group.

Growth of isolates on solid media with some model hydrocarbons

Isolated pure cultures of soil microorganisms were screened for their ability to grow on MSM with 1.5% agar and with 2% of each one of the model n-alkanes or aromatic hydrocarbons (1% for naphthalene) used as a sole carbon source (Table 2). As can be realized, only two isolates, S3 and 5 were unable to grow on the hydrocarbons used. All other isolates showed growth (more or less) on both aromatic and aliphatic hydrocarbons tested, especially strain 2. Three of the isolates formed dark blue halos on agar plates indicating a production of anionic glycolipids (Table 2).

Hydrophobicity and emulsifying activity of soil isolates

Glucose-grown soil isolates were tested for their cell surface hydrophobicity to heptane and for extracellular emulsifying activity. Of the 5 strains studied, strain S3 had the lowest (9.3%),

Table 1. Characterization of isolates from Petroleum polluted soils from the eastern region , saudi arabia

Isolates	Isolation Temp	Gram Staining	Strain Identification
S_1	29	Gram positive	*Nocardioform*
S_2	28	Gram positive	Coryneform
S_3	28	Gram positive	Nonidentified
S_4	27	Gram positive	*Nocardioform*
S_5	28	Gram positive	Nonidentified

Table 2. Growth and Glycolipid production of microbial isolates from Petroleun polluted soils from the eastern region, Saudi arabia on solid media with 2% of indicated hydrocarbons as a sole carbon source

Isolates	Heptane	Hexane	Paraffin	Benzene	Touene	Naphthalene	Kerosene	Glycolipid Production
S_1	+	-	++	-	-	++	+	+
*S_2	+++	++	+	+++	+	+	+++	+
S_3	-	-	-	-	+	-	-	-
S_4	+	+	-	+	++	++	-	+
S_5	-	-	+	-	-	-	+	-

- : No formation of colonies observed
+: Formation of weak colonies <1 mm diameter
++: Formation of colonies between 1 to 3 mm
+++: Formation of colonies between 3 and 5 mm and more

Table 3. Hydrophobicity and emulsifying activity of microbial isolates from Petroleum polluted soils from the eastern region, Saudi Arabia after growth on 2% glucose

Isolates	Hydrophobicity (%)	*Emulsifying activity [EU ml^{-1}]
S_1	47.6±2.1	0.43±0.15
S_2	75.6±5.5	2.18±0.22
S_3	9.3±1.6	0.26±0.17
S_4	28.6±3.6	0.30±0.11
S_5	10.0±3.9	0.31±0.19

*Emulsifying activity to kerosene. EU: Emulsifying units as absorbance at 550 nm per ml. Values are mean of three separate experiment ±S.D

and strain S2 had the highest (75.6%) hydrophobicity values (Table 3). All isolates showed extracellular emulsifying activity after growth on glucose (Table 3). It was highest for isolates 2 (2.18) although strains S3 and S5 did not show growth on most of the hydrocarbons used.

Cultivation of strain A-13 with some hydrocarbons in batch system

Strain S2 with the best growth on agar plates with some model hydrocarbons was cultivated in liquid MSM with 2% of each one of the hydrocarbons used as a sole carbon source. The strain showed best growth on kerosene and extracellular emulsifying activity after growth on paraffin and kerosene. It was observed an increase in cell hydrophobicity after cultivation of the strain on each one of the hydrocarbons used (Table 4).

Table 4. Hydrophobicity and emulsifying activity of strain A-13 after growth in liquid mineral salts media with 2% of each one of the hydrocarbons used as a sole carbon source

Hydrocarbon	Growth (OD_{570})	Hydrophobicity (%)	Emulsifying activity [EU ml^{-1}]
Glucose (Control)	0.46±0.13	53.6±1.3	1.13±0.26
Heptane	0.87±0.14	66.4.±2.3	0.23±0.11
Paraffin	0.12±0.03	34.3±1.6	0.75±0.12
Kerosene	1.68±0.10	87.6±2.6	0.82±0.33
Benzene	0.63±0.15	44.5±1.8	0.18±0.09

* Strain was cultivated at 25°C with agitation
** Emulsifying activity to kerosene. EU : Emulsifying units as absorbance at 550 nm
Means values from three separate experiments are given ± S.D

DISCUSSION

Biosurfactants are produced by a wide variety of microorganisms and have different natural roles in the growth of microorganisms (Ron and Rosenberg, 2001). Most of biosurfactants are different types of glycolipids (Desai and Banat, 1997; Chaillan *et al*., 2006). Secreted surface-active compounds improve cell growth and bioavailability of hydrophobic compounds thus accelerating their degradation (Ron and Rosenberg, 2002).

Hydrophobicity is an important factor in the initial adhesion of microorganisms to the interface between the non-aqueous phase liquid (NAPL) and the aqueous phase. A number of species of bacteria are able to degrade liquid hydrocarbons after adhering to the surfaces of droplets (Stelmack *et al*., 1999; Niescher *et al*., 2006). This direct contact between a bacterial cell and a target hydrocarbon can significantly increase the rate of diffusion into the cell, thereby enhancing growth and increasing the apparent rate of dissolution of the hydrocarbon. Such correlation between high initial cell hydrophobicity and good growth on hydrocarbons exists for S2 isolate, while isolates 1 and 4 growing on the hydrocarbons tested were with middle or low hydrophobicity. In the present study, all soil isolates with different initial hydrophobicity showed extracellular emulsifying activity. Isolates with highest activity, S1 and S2 were very different in their initial hydrophobicity. Similar observations have been reported by other authors that both hydrophilic and hydrophobic bacteria were able to produce surfactants when grown on glucose or hexadecane (Bouchez-Naitali *et al*., 1999).

The significant increment in cell hydrophobicity after cultivation of the strain on each one of the hydrocarbons used can be induced to change in the presence of combination of both excreted surfactants and slightly soluble substrate (Zhang and Miller, 1994).During growth on soluble carbon source glucose high emulsifying activity of isolate A-13 was observed indicating production of biosurfactants.

This suggests a broader role for biosurfactants than just hydrocarbon uptake. A likely possibility is the more general participation in adhesion and de-adhesion interactions between microorganisms and interfaces (Chaillan *et al*., 2006).

CONCLUSION

The results presented here will be particularly useful in choosing strains for environmental applications involving the implantation of microorganisms in the soil matrix (bioaugmentation). As contaminated sites usually contain heterogeneous hydrocarbons, it is promising to use for bioaugmented clean-up strains with broad abilities to grow on different hydrocarbons. For this purpose isolate S2 with good emulsifying activity and producing glycolipids was proposed for hydrocarbon waste treatment of polluted soil environments.

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