Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Bacterial Species Isolated from Oily Polluted Soils

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Abstract

Twenty five bacterial isolates were isolated from three types of oily polluted soils which were Al-Najaf Oil Refinery/Najaf and two fuel stations in Hilla which were Ishtar and Al-Hilla Al-Jadida. These isolates were purified and identified according to phenotypic and microscopic properties in addition to biochemical tests. The results showed that these isolate belonging to five different bacterial species, three of them are gram negative bacteria which were: Pseudomonas aeruginosa, Pseudomonas putida and Acinetobacter baumannii, while two of them were gram positive bacteria which were Staphylococcus epidermidis and Bacillus sp. Preliminary experiment was done to show the ability of these isolates to grow on hydrocarbons mixture (1%) as a sole source of carbon and energy, by denotation of viable bacterial count (CFU/ml) for seven days of incubation period. The results explained that the gram-negative bacterial isolates have such ability, while the gram-positive bacterial isolates didn't have this ability. Three parameters were used to evaluate the biodegradation efficiency of the isolates hydrocarbons consumption percentage, reduction of surface tension and production of biosurfactant. The most efficient isolate was P. putida which consumed 78, 76.6 and 55.5% of phenanthrene, anthracene and pyrene, respectively, at 37°C, pH=7 with agitation. Also it reduced the surface tension of the medium to 44.1 dyns/cm when it consumed phenanthrene and 53.2 dyns/cm for anthracene and 55.2 dyns/cm for pyrene compared with 72.2 dyns/cm of control sample. In the same conditions it produced biosurfactant at concentration of 40, 37.1 and 31.1 mg/L for phenanthrene, anthracene and pyrene, respectively, while P. aeruginosa consumed 70, 66.5 and 50.1% at the same condition from phenanthrene, anthracene and pyrene, respectively, also it reduced the surface tension of the medium to 46.9, 54.5 and 57.2 dyns/cm and it produced biosurfactant at concentration of 34.5, 32.5 and 28.2 mg/L when it consumed phenanthrene, anthracene and pyrene, respectively. On the other hand A. baumannii consumed 56% from phenanthrene, 54.1% from anthracene and 43.2% from pyrene and it reduced the surface tension of the medium to 49.3, 55.3 and 61.3 dyns/cm for phenanthrene, anthracene and pyrene, respectively, while it produced biosurfactant at concentration of 30.1, 29.2 and 22.3 mg/L when it consumed phenanthrene, anthracene and pyrene, respectively. Consortium of three efficient isolates (P. putida, P. aeruginosa and A. baumannii) was made. The results explained that the consortium efficiency increased more than the individual isolates when consumed 100, 95 and 88% of phenanthrene, anthracene and pyrene, respectively, at the seventh day of incubation.

Keywords: Biodegradation/PAHs/Consortium/Pseudomonas/Acinetobacter.
Introduction

Carbon and hydrogen involve in forming all of the petroleum organic compounds, when these two elements interact, it would be form a large group of organic compounds which were called Hydrocarbons. It form most of petroleum compounds, these compounds involve a huge group of linear (Paraffins) and cyclic (Aromatics) compounds which consist of one or more of fused aromatic rings (Jaffe and Manning, 2000). Polycyclic aromatic hydrocarbons (PAHs) are important pollutants found in air, soil and sediments and are of environmental concern because of their toxic, mutagenic and/or carcinogenic effects, so exposure to PAHs may represent a significant health risk to human populations, and therefore their fate in nature is of great environmental concern. Microorganisms with the ability to degrade many PAHs have been described, and their mechanisms of action have been studied (Mittal and Singh, 2009). PAHs consist of two or more of fused aromatics rings which involve: naphthalene, anthracene, benzo[a]anthracene, benzo[a]pyrene, phenanthrene, chrysene, fluoranthene, benzo[a]fluoranthene, fluorene and pyrene (Alvarez and IIIman, 2006). PAHs are formed during incomplete combustion or pyrolysis of organic material and in connection with the worldwide use of oil, gas, coal and wood in energy production. Data from animal studies indicate that several PAHs may induce a number of adverse effects, such as immunotoxicity, genotoxicity, carcinogenicity, reproductive toxicity (affecting both male and female offspring), and may possibly also influence development of atherosclerosis. However, the critical endpoint for the health risk evaluation is the well-documented carcinogenicity of several PAHs Many PAHs are capable of producing tumors in experimental animals. Benzo[a]pyrene (BaP) has been used for many years as a model compound in a variety of different carcinogenicity bioassays, epidemiological studies in coke-oven workers and coal-gas workers provide sufficient evidence of the role of inhaled PAHs in the induction of lung cancer. In vitro experiments have demonstrated the cytotoxicity of BaP and various other PAHs to cells from the lungs of experimental animals and humans. Severe, long-lasting hyperplasia and other adverse effects (WHO, 2000).
Anthracene is a solid PAH; its molecular formula $C_{14}H_{10}$ consisting of three fused benzene rings in linear form, derived from coal-tar or other residues of thermal pyrolysis. Anthracene is colorless or pale white and it is not carcinogenic with water solubility 0.7mg/L. Bacteria like *Pseudomonas*, *Sphingomonas*, *Nocardia*, *Rhodococcus* and *Mycobacterium* could use it as a sole source of carbon and energy (Dean-Ross et al., 2001; Moody et al., 2001). In Iraq, there is a few studies about this issue. Al Taher et al 2008 tried to compose such bacterial consortium have ability of hydrocarbon degradation in order to use it the treatment of wastewater in Al – Dora Oil Refinery.

**Materials and Methods**

**Samples Collection:** Fifteen soil samples were collected from oily polluted soils from which were Al-Najaf Oil Refinery/Najaf and two fuel stations in Hilla which were Ishtar and Al-Hilla Al-Jadida in the amount of five samples for each type of soils (sandy soils). These samples collected at nearby level from soil surface at height 5cm and it brought to the laboratory with dry clean plastics sacks.

**Isolation and Purification of Bacteria:** Microorganisms used in all experiments were isolated by selective enrichment technique Bushnell-Hass Mineral Salts Medium (MSM) was used in the enrichment technique by adding 1gm of polluted soils to 100ml of MSM and incubated for 24hr at 37°C then it would be take a loopfull and culturing it on blood agar medium by streaking methods and incubated for 24hr at 37°C. The bacterial isolates were purified by streaking on blood agar medium three times until purity. Then bacterial isolates were identified by chemical tests and API 20 system (Biomerieux).

**Determination the ability of hydrocarbon degraders:** Preliminary experiment was done to determine the ability of bacteria to use polycyclic aromatic hydrocarbons as a sole source of carbon and energy by calculating the number of (Colony Forming Unit) CFU/ml by mixing 1gm from drying hydrocarbons with 100ml of MSM and inoculated with 0.1ml [concentration 1x$10^8$cell/ml compared with opacity tube (WHO/ England)] from each isolate then the medium was incubated for seven days at 37°C, pH=7 with agitation. A loopfull was taken from the $10^{-6}$ dilution and culturing it in nutrient agar to calculate the number of colonies.

**Efficiency of Bacterial Isolates to Degrade Hydrocarbons:** It was done by inoculating 99ml of MSM containing 2% of (anthracene, phenanthrene, pyrene) individually by 1x$10^8$ cell/ml from each isolates and incubated for seven days (Kumar et al., 2006):

**Calculation of hydrocarbons consumption percentage:** Weight method was used to calculate hydrocarbons consumption according to (Arafa, 2003). The residual of hydrocarbons percentage was calculated according to equation:

\[ R = \left( \frac{A-B}{A} \right) \times 100\% \]

**R=** Hydrocarbons consumption percentage

**A=** Added hydrocarbons

**B=** Residual of hydrocarbons

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Calculation of surface tension: Capillary method was used to calculate the changes of surface tension (dyns/cm units) of culture medium daily according to the following equation (Cooper and Goldenberg, 1987): 
\[ \gamma = \frac{1}{2} rh \rho g \]

\( \gamma \) = surface tension value (dyns/cm)
\( r \) = capillary tube radius (cm)
\( h \) = capillary tube liquid height (cm)
\( \rho \) = liquid density (gm/cm\(^3\))
\( g \) = global acceleration (dyns/cm\(^2\))

Calculation of produced biosurfactant concentration: the biosurfactant calculated according to (Goswami and Singh, 1991) by taking 2ml from the filtrate after medium filtration under sterile condition then add appropriate volume from acetone (1:3) (v:v) and keep it in refrigerator at 4°C for 24-48hr to evaporate the acetone, the weight of precipitate that remain in the bottom represent the biosurfactant concentration:

Biosurfactant conc. = container weight with precipitation – empty container (mg/L)

Consortium Efficiency Test: A consortium from the three efficient bacterial isolates was done by taking 1ml at concentration of 1x10\(^8\) cell/ml from each isolate then added to 250ml flask which contains 97ml from MSM with 2% of phenanthrene, anthracene and pyrene individually. Then incubated for seven days at 37°C, pH=7 with shaking (120 rpm/min) in the shaker incubator (Gallenkamp/England).

Results and Discussion

phenanthrene and anthracene have the same number of rings and the same molecular weight but the results were shown that phenanthrene was the most consumed by bacterial isolates than anthracene this may be due to its solubility which was more than anthracene, while pyrene was little difficult for bacteria to consumed. Twenty five bacterial isolates isolated from three types of oily polluted soils which were Al-Najaf Oil Refinery/Najaf and two fuel stations in Hilla (Ishtar and Al-Hilla Al-Jadida), The preliminary results were shown that the most efficient bacterial isolate was \( P. \) putida by their number of living colony which was 25.1x10\(^8\) cell/ml, while \( P. \) aeruginosa 20.3x10\(^8\) cell/ml and \( A. \) baumannii 17.2x10\(^8\) cell/ml at the seventh day of incubation period while gram-positive bacteria could not grow on hydrocarbons mixture thus Bacillus sp. was not found in the fourth day of incubation period and \( Staphylococcus \) epidermidis was declined in the third day of incubation period (Figure 1).
Graphs showing the growth of P. aeruginosa, P. putida, Bacillus sp., and A. baumannii over time, with the y-axis representing the number of cells/ml x 10^8 and the x-axis representing time in days.
Also the results exhibited that \textit{P. putida} was the most efficient in consuming the three compounds, when consumed 78, 76.6 and 55.5\% from phenanthrene, anthracene and pyrene, respectively, at 37\(^\circ\)C, pH=7 with agitation. At the same time it reduced the surface tension of the medium to 44.1, 53.2 and 55.2 dyns/cm for phenanthrene, anthracene and pyrene, respectively, compared with control sample value which was 72.2 dyns/cm. It also produced biosurfactant at concentration of 40, 37.1 and 31.1 mg/L when consumed the previous mentioned compounds, respectively (Figure 2). The current results came agreed with (Tuleva \textit{et al.}, 2002; Hafez \textit{et al.}, 2006; Kumar \textit{et al.}, 2006; Roy \textit{et al.}, 2007; Bishnoi \textit{et al.}, 2009) whom studied biodegradation of PAHs and biosurfactant production by \textit{P. putida}.
(Figure 2): Biodegradation efficiency of *P. putida* according to PAHs consumed percentage, surface tension reduction and biosurfactant production at 37°C, pH=7 with agitation.

These results explain that bacteria can adapt to changes in the environmental conditions, such as the growth in the presence of hydrocarbon substances by altering the lipid compositions in the cytoplasmic membrane in order to maintaining or adjusting membrane bilayer fluidity (Pinyakong *et al.*, 2000). Alteration of the fatty acids moieties of membrane lipids are thought to be the most effective state membrane, which is essential for optimal membrane function.

The other species *P. aeruginosa* consumed 70% from phenanthrene, 66.5% from anthracene and 50.1% from pyrene, also it reduced the surface tension to 46.9 dyns/cm for phenanthrene, 54.5 dyns/cm for anthracene and 57.2 dyns/cm for pyrene and produced biosurfactant at concentration of 34.5, 32.5 and 28.2 mg/L when consumed phenanthrene, anthracene and pyrene, respectively (Figure 3). These results came in the same direction of (Garcia-Junco *et al.*, 2001; Guo-Liang *et al.*, 2005; Mariano *et al.*, 2008) whom studied isolation and identification of *P. aeruginosa* from polluted soils and waters and test their ability to degrade PAHs.
(Figure 3): Biodegradation efficiency of *P. aeruginosa* according to PAHs consumed percentage, surface tension reduction and biosurfactant production at 37˚c, pH=7 with agitation.

On the other hand *A. baumannii* consumed 56, 54.1 and 43.2% from phenanthrene, anthracene and pyrene, respectively, and reduced the surface tension of the medium to 49.3, 55.3 and 61.3 dyns/cm for phenanthrene, anthracene and pyrene, respectively, also it produced biosurfactant at concentration of 30.1, 29.2 and 22.3 mg/L when consumed phenanthrene, anthracene and pyrene, respectively (Figure 4). The results came agreed with (Malatova, 2005) who studied biodegradation and production of CO\textsubscript{2} and biosurfactant when she isolated *A. baumannii*, *Pseudomonas* sp. and *Serratia marcescens* in New York City.
(Figure 4): Biodegradation efficiency of *A. baumannii* according to PAHs consumed percentage, surface tension reduction and biosurfactant production at 37°C, pH=7 with agitation.

Consortium of the most efficient bacterial isolates (*P. putida*, *P. aeruginosa* and *A. baumannii*) was made. The results showed that this consortium was more efficient than the bacterial isolates individually, so it consumed 100, 95 and 88% from phenanthrene, anthracene and pyrene, respectively, at 37°C, pH=7 with agitation (Figure 4). This observation may be due to Cometabolism phenomenon which means the simultaneous metabolism of two compounds, in which the degradation of the second compound requires the presence of the first compound. Also it is considered a very important operation between microorganisms. These results came agreed with (Igwo-Ezikpe *et al.*, 2010) whom studied biodegradation of PAHs by bacterial consortium. On the other hand, these results came in contrast with Moody *et al.*, 2001 who mentioned that *A. baumannii* had no ability to degrade hydrocarbons, especially polycyclic aromatic hydrocarbons.

(Figure 5): Consumption percentage of PAHs by bacterial consortium grown at 37°C, pH=7 with agitation.
References
Malatova,. (2005). Isolation and characterization of hydrocarbon degrading bacteria from environmental habitats in western New York State. A thesis of Master of Science, Department of chemistry. Rochester,

