Biological treatment of PAHs using genetically modified local bacterial isolates

Kazem M. Kazem and Amal A. Hussein

1 Applied Science Department, University of Technology, Baghdad, Iraq
2 Collage of Science Department, Al-karkh University of Science, Baghdad, Iraq

E-mail: Kazemmehti474@gmail.com

Abstract. Bioremediation technology by microorganisms, which already presents in the contaminated soils, is considered one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment. Twelve decomposing bacterial isolates were isolated from three polluted sites (Al-Dora oil refinery, Middle Refineries Company from oil wells, and Karbala oil refinery). This study showed high efficacy polycyclic aromatic hydrocarbons (PAHs) of isolated bacteria appropriate to polycyclic aromatic hydrocarbons decomposition. Primary and secondary screening of bacterial isolate has been performed using experiments based on the colour change in the medium resulting from the degradation of hydrocarbons in the nutrient medium. The screening results were three isolates that were characterized according to the basis of morphological and biochemical features and verified by Vitek 2, Pseudomonas aeruginosa, Escherichia coli and Sphingomonas paucimobilis. The mutagenesis process has been carried out by UV irradiation with a wavelength of 254 nm on selected bacterial strains. Experiments have been conducted on PAHs (naphthalene, phenanthrene and acenaphthene. According to the Biodegradation Efficiency result for 28 days, the best phenanthrene degrading bacteria was (Pseudomonas aeruginosa wild type (73.4%), Escherichia coli wild type (71.5%) and Sphingomonas paucimobilis wild type (68.8%). For naphthalin metabolism, the best degradation efficiency was (Pseudomonas aeruginosa wild type (86.2%), Sphingomonas paucimobilis wild type (69%) and Escherichia coli wild type (63%). At the same time, acenaphthene great degradation efficiency was Sphingomonas paucimobilis wild type (72%), Pseudomonas aeruginosa wild type (71%) and Sphingomonas paucimobilis mutant type (66%). We conclude that random mutation’s effect did not increase the degradation ability for most bacterial isolate.

Keywords: Bioremediation, PAHs, mutant bacteria, Pseudomonas, E.coli, GC-Mass

1. Introduction

One of today's major environmental problems is hydrocarbon pollution from activities related to the petrochemical industry, oil exploration and chemical factories wastes. Hydrocarbon compounds are the most popular waste compounds that have been classified as neurotoxic and carcinogenic organic pollutants. [1]. It is known that polycyclic aromatic hydrocarbons are difficult to degrade because of their low reactivity; The USEnvironmental Protection Agency (US EPA) has listed these substances as priority contaminants in natural resources. However, a wide number of microorganisms slowly degrade these compounds [3]. The biodegradation process of hydrocarbon in contaminated sites by hydrocarbon-degrading bacteria was studied [4]. Biodegradation for mixtures of hydrocarbons usually involves the collaboration of more than one type of microorganisms. This process is certainly relevant in contaminants that contain several different compounds, such as crude oil or petroleum, and complete CO2 and H2O mineralization is required in the final process [5].
With the aid of a diverse community of microorganisms, especially the indigenous bacteria found in the soil, a hydrocarbon-contaminated site's microbial bioremediation is completed. These microorganisms will degrade a wide variety of desired constituents present in oily sludge. A great number of strains of Pseudomonas able to degrade PAHs were isolated from the soil. Other petrochemical degrading bacteria include Yokenella spp, Alcaligenes spp, Rosemonas spp, Stenotrophomonas spp, Acinetobacter spp, Flavobacter spp, Corynebacterium spp, Streptococcus spp, Povidencia spp, Sphingobacterium spp, Capnocytophage spp, Moraxwilla spp and Bacillus spp. Bacteria can degrade Branched hydrocarbons, aliphatic saturated, saturated hydrocarbons and cyclic hydrocarbons through aerobic and anaerobic pathways.

By creating covalent bonds between neighbouring pyrimidine bases, mutation by irradiation or UV exposure harms DNA. In the double helix configuration of DNA, this pyrimidine dimer does not match well and prevents replication and translation. Dimer development normally results in a mutation in deletion. Many types of radiation (based on intensity and wavelength) may have a range of effects, but there are often insertions/deletions. Purine dimers frequently occur. This study aims to isolate, screen, and identify local bacterial isolates isolated from the contaminated site with hydrocarbons. Using UV light irradiation in mutagenesis of bacterial isolates to obtain mutant efficient bacterial strains degrade PAHs by measuring biodegradation efficiency (BE %).

2. Methods

2.1. Sample Collection

Oil contaminated soil samples were collected from Al–Doura oil refinery in Baghdad, Al-Wasat Refineries Company/Alkout and Karbala’a oil refinery in the duration (5/11/2019 – 17/12/2019). Samples were collected and put in the sterile zip-lock polythene bags and transferred to the laboratory for further study.

2.2. Isolation of Bacteria

Hydrocarbon degrading bacteria were isolated from soil samples using Bushnell Hass broth medium (BH) (0.2 g of MgSO4, 0.02 g of CaCl2, 1.0 g of KH2PO4, 1.0 g of K2HPO4, 1.0 g of NH4NO3, 0.05 g FeCl3, in 1000 mL of distilled water) containing sterilized crude oil 1% (v/v) as a sole source of carbon.

One gram of soil sample was introduced in 500 mL Erlenmeyer flask containing 100 mL of BH medium supplemented with 1 ml (1% v/v) crude oil filtered by Millipore unit using 0.45µm filter paper and incubated at 37°C, 150 rpm for one week. Then 0.1 mL from 10^1 and 10^8 serial dilutions spread on BH agar with sterilized crude oil (1% v/v) as the sole source of energy. The plates were incubated at 37°C for 7 days. Pure colonies were obtained, and each of the selected colonies was sub-cultured and purified by grown on a nutrient agar plate.

2.3. Screening of oil-degrading Bacteria

The purified is isolates were grown on BH broth in the presence of naphthalene (1% wt/v) as the sole source of carbon and incubated at 37°C, 150 rpm for 7 days. After 7 days, the OD of the bacterial growth culture was measured using a spectrophotometer at 600nm for each isolate.

Then 0.5 ml (from grown isolates only) has been spread on the BH agar plate and added 0.5 ml of crude oil as a drop on the plate to illustrate the growth of degrading bacterial isolates only as a conformation step.

The bacteria's ability to degrade hydrocarbons was verified using modified Simmon citrate medium based on Bromothymol blue indicator (also known as bromothymol sulfone phthalein and BTB). Simmons citrate medium include [Sodium Chloride (NaCl) 5.0g /L, Sodium Citrate (dehydrate) 2.0g/L, Ammonium Dihydrogen Phosphate 1.0g/L, Dipotassium Phosphate 1.0g/L, Magnesium Sulfate (heptahydrate) 0.2g/L, Bromothymol Blue 0.08 g/L, Agar 15.0 g/L] [18, 21]. The only difference in this
experiment was the substitution of Sodium Citrate with naphthalene as the sole source of carbon to ensure the bacteria's ability to degrade the PAHs compound. Bacterial isolate, which grown on crude oil, were inoculated (100 μL) in test tubes containing 4 ml of modified Simon citrate broth medium supplemented with 0.04 g (1 % w/v) of naphthalene and bromothymol indicator and incubated at 37 °C and green colour disappearance. Yellow colour forming was observed daily [9].

2.4. Identification of bacterial isolates
Pure colonies which give positive result (yellow colour) were identified and characterized based on their morphological characteristics and biochemical properties according to the identification scheme of Bergey's Manual of Determinative Bacteriology [19]. Isolates were diagnosed for confirmation by the VITEK 2 system for microbial identification.

2.5. Preservation of bacterial strain
The identified bacterial isolates preserved as wild type for other experimental procedure in preservation broth medium by adding 40 ml of glycerol to every 100 ml of brain heart infusion broth for long period preservation, 10 ml dispensed in each well-capped screwed test tube, sterilized by autoclaving, cooled to 37º C and used for preserving bacterial isolates [20].

2.6. Biodegradation of PAH by bacterial strains.
The three efficient bacterial strains (S.D1, S.D2 and S.K1) used to degrade PAHs material as the sole source of energy and carbon in BH broth media. 20 ml of BH medium prepared with 0.2 g (1% wt/v) of each (naphthalene, acenaphthene and phenanthrene). Then 20 μm from (S.D1, S.D2 and S.K1) inoculated separately in liquid BH medium and then incubated at 37°C, 150 rpm for 28 days. The optical density (OD) of bacterial culture measured at 600 nm every 5 days to estimate bacterial growth [7].

2.7. Mutation of bacterial isolates
The three efficient bacterial strains (S.D1, S.D2 and S.K1) were activated in LB broth at 37°C for 24 hr. Then 500 μm of each bacterial strain spread on three LB agar plates according to the time of mutation (10 min, 30 min and 60 min) by UV light radiation at 254nm wavelength from 40 cm distance. The effect of mutation by UV light was studied on morphological (size, shape and arrangement) and biochemical scale (hemolysis and lactose ferments test).

The clear individual colony of each irradiated plate of bacteria was selected to inoculate in BH media supplemented with 0.2 g of PAH to estimate the biodegradation of PAH.

2.8. Biodegradation of PAHs by mutant bacterial strains
Twenty μl of mutated bacteria inoculated in 20 ml BH media [with 0.2 g of (naphthalin, phenathrin and acenphthene) as the sole source of carbon] for 28 days (at 37oC, 150 rpm). The optical density measured every 5 days to estimate and observe the bacterial growth state.

2.9. Biodegradation efficiency measurement
The culture media, after 28 days, were dried by the oven (dry heat) in a glass petri-dish. The precipitate was collected and weighed to estimate the Biodegradation Efficiency for both wild and mutated biological process.

\[
BE\% = \frac{Cwt - Swt}{Cwt} \times 100.
\]

Where,

- Cwt: is the control weight.
- Swt: is the sample weight after 28-day treatment.

The powder prepared for FTIR and GC Mass analysis.

2.10. GC Mass condition analysis for residual Hydrocarbons
In the examination of PAHs (Naphthalene, Acenaphthene and phenentrene), the GC Mass (Agilent, model 7820 A) device was used under the following conditions:

**Column:**

DB-5ms Ultra Inert, 122-5532UI, 30 m x 0.25 mm, 0.25 μm, Carrier: Helium constant flow 30 cm/s Oven: 40 °C (1 min) to 100 °C (15 °C/min), 10 °C to 210 °C (1 min), 5 °C/min to 310 °C (8 min). Injection: Split/splitless, 260 °C, 53.7 mL/min total flow, purge flow 50 mL/min on at 0.5 min, gas saver flow 80 mL/min on at 3.0 min. Detector: MSD source at 300 °C, Quadrupole at 180 °C, Transfer line at 290 °C, Scan range 50-550 amu.

### 3. Results and Discussion

Bacterial strains were collected and isolated from soil contaminated with oil. Incubation of unknown-bacterial isolates in broth Bushnell Hass broth medium (BH) with crude oil as the sole source of carbon provide an appropriate method to reduce the number of isolates depending on the efficiency of HC degradation. This step will reduce the number of bacterial isolates to five unknown isolates.

Another confirmation step uses a modified Simmon citrate medium with another carbon source instead of citrates PAHs in this case (naphthalene) to select the efficient HC degrading bacteria. The addition of bromothymol blue indicator in BH media confirms positive results by changing media from green to yellow due to acidic compound metabolites from oxidation of naphthalene, as shown in Fig (1) below. Bacterial isolates have been identified for validation by VITEK 2, which has been used to confirm the conventional diagnosis. According to the results from the VITEK2 technique, the three isolates (S.D1, S.D2 and S.K1) were identified as *Pseudomonas aeruginosa* (probability 99%), *E.coli* (probability 89%) and *Sphingomonas paucimobilis* (probability 93%), respectively.

The effect of mutation by UV-C light was studied, as shown in Fig (2) decrease in colony size for (*E.coli* and *Sphingomonas*) while the colony size increased gradually with irradiation duration *Pseudomonas*. Lacto-fermenting bacteria (*E.coli*) show moderate resistance toward UV light for the initial amount. The mutated type of (*E.coli*) acquired high resistance after irradiation due to their strong and stable gene expression, fig [3]. The optical density measurement of wild and mutant bacteria at 600nm wavelength by spectrophotometer every 5 days for 5 weeks. For each sample indicate bacterial growth with PAH as the sole source of carbon in BH media, wild isolate shows high adaption ability with the increased curve (approximately one week). In contrast, mutated isolates took two weeks to adapt and start the growth stage.

Through the gravimetric method that depends on the sample weight difference before and after biological treatment, approximate biodegradation ratios were obtained, depending on the law:

\[
BE\% = \frac{Cwt - Swt}{Cwt} \times 100
\]

- BE%: is the control weight.
- Swt: is the sample weight after 28-day treatment.

According to the numbers illustrated in Table (0), initial results can be attained, indicating each bacteria's ability to decompose a specific substance. Concerning naphthalene, the wild *Pseudomonas aeruginosa* are better decomposer for naphthalene among the rest of the species by up to 88%. In the mutagenic type, they have broken down by up to 75%. It was followed by the wild *Sphingomonas paucimobilis*, at 75%, and then by 58% for the mutated type. Then *E.coli* bacteria were considered the least bacteria capable of degrading naphthalene, by 70% for the wild type and 59% for the irradiated type. Before treating naphthalene adopted in the equation, the sample's weight is relatively less than its weight in the medium because naphthalene is a volatile substance, especially during the long study period (28 days).

The tricyclic phenanthrene was degraded with the highest amount through *Pseudomonas aeruginosa* bacteria by 73% for the wild type and 57% for the irradiated type. That follows it in the second set is *E.coli* wild type as it degrades the phenanthrene by 71% and the mutated type, which dissolved 65% better than the mutated type. Simultaneously, the *Sphingomonas paucimobilis* bacteria are less than hydrocarbon decomposition by 68% for the original isolate and 64% for the mutated type.
The triple benzene ring acinaphthene is decomposed by the wild *Sphingomonas paucimobilis* by 72% and mutagenic type by 66%, which is the highest among the original mutagenic isolates of the rest of the species. In comparison, the *Pseudomonas aeruginosa* bacteria have dissociated the compound relatively less by 71% for the wild type and 33% for the mutated type, which is the lowest percentage among the rest of the mutagenic isolates. *E.coli* bacteria degraded the material with a ratio close to both the original and mutagenic isolates, reaching 64% and 60% for the original and mutagenic isolates, respectively.

Through the above table data, we can see the effectiveness of the decomposition of the material for each bacterium. As each material was measured in its pure state and the area of its peak was calculated. The pure naphthalene substance has a peak area of 7179468783 and is considered control. After processing and using it as a source of carbon and energy by the wild type of *E. coli* bacteria for 28 days, the sample was measured in the GC Mass device. The total area of the peaks decreased to 163557919, meaning that the bacteria degraded the material at a rate of 97.7%. As for the mutated type *E. coli*, the peaks' total area was 336196087, which is relatively less than the original type. Nevertheless, it degraded naphthalene by up to 95.3%. The result of its treatment is considered good because it was exposed to radiation and was mutated.

Phenanthrine compound with the three rings, the peak area in its pure state was 324904536, and it is considered to control. Then it was slowly decomposed by the wild type *Sphingomonas paucimobilis* bacteria, as the total areas of the peaks after examining the treated sample were 183193411, meaning that the bacteria consumed the compound by 43.6%. The mutagenic sample gave a peak area of 267739318, meaning that it consumed the compound less. By only 17.5%, which is the lowest among the samples, this may be because the mutagenic bacteria have relatively lost their ability to produce the analytic enzymes.

The tricyclic compound acenaphthene, which contains an ethyl group, was dissolved by *E. coli* bacteria. The pure peak area of the compound reached 411488724 and was adopted as a control during the equation. The sample with the wild type *E. coli* had a peak area of 46588045, i.e. a decrease of 88.6%, representing the compound's degradation rate. As for the sample with mutagenic bacteria, the mutated sample area decreased to 79740172, meaning that the mutated type decomposed the compound by 80.6%, close to the original type.

![Figure 1: bacterial isolates growth in modified BH broth with naphthalene.](image)
Table 1: Biochemical test results of bacterial isolates

| Biochemical test | Ps. aeruginosa | E.coli | Sphingomonas |
|------------------|---------------|--------|--------------|
| Catalase         | Positive      | Negative | Positive     |
| Indole           | Negative      | Positive | Negative     |
| Simmon citrate   | Positive      | Positive | Negative     |
| Tsi              | K/K           | A/A    | K/K          |

Figure 2: Effect of radiation on colony size of bacteria.

| Bacteria | Control | 10 min irrad. | 30 min irrad. | 60 min irrad. |
|----------|---------|---------------|---------------|---------------|
| Mutant E.Coli | Positive | Positive | Positive | Decreased ↓↓ |

Figure 3: Effect of radiation on lacto fermenting ability of bacteria.

Table 2: Biodegradation Efficiency of bacterial strain after 28 day

| Control wt. | Sample weight after 28 day | Biodegradation efficiency |
|-------------|-----------------------------|---------------------------|
| 1- Anthracene 0.2008 gm | 1- E. coli W 0.0571 gm | 71.5 %                     |
|              | 2- E. coli M 0.0695 gm | 65.4 %                     |
|              | 3- Pseud. W 0.0534 gm | 73.4 %                     |
|              | 4- Pseud. M 0.0848 gm | 57.7 %                     |
|              | 5- Sphingo W 0.0625 gm | 68.8 %                     |
|              | 6- Sphingo M 0.0701 gm | 65 %                       |
| 2- Naphthalin 0.0968 gm | 1- E. coli W 0.0349 gm | 63 %                       |
|              | 2- E. coli M 0.0489 gm | 49.4 %                     |
|              | 3- Pseud. W 0.0134 gm | 86.2 %                     |
|              | 4- Pseud. M 0.0468 gm | 51.6 %                     |
|              | 5- Sphingo W 0.0299 gm | 69 %                       |
|              | 6- Sphingo M 0.0496 gm | 48.7 %                     |
| 3-Acenaphthen 0.1758 gm | 1- E. coli W 0.0629 gm | 64 %                       |
|              | 2- E. coli M 0.0698 gm | 60 %                       |
|              | 3- Pseud. W 0.0507 gm | 71 %                       |
|              | 4- Pseud. M 0.1168 gm | 33.5 %                     |
|              | 5- Sphingo W 0.0488 gm | 72 %                       |
|              | 6- Sphingo M 0.0597 gm | 66 %                       |
**Figure 4:** pure phenanthrene GC Mass results.

**Figure 5:** pure naphthalene GC Mass results.
Figure 6: phenanthrene degradation GC Mass result by wild sphingomonas.

Figure 7: phenanthrene degradation GC Mass result by mutant sphingomonas.
Table 3: total area results of PAHs after 28 days.

| Compounds   | Bacteria          | Control total area. | Sample total area. | Biodegradation efficiency (BE %) |
|-------------|-------------------|---------------------|--------------------|----------------------------------|
| Naphthalene | E.coli wild       | 7179468783          | 163557919          | 97.7 %                           |
|             | E.coli mutant     | 7179468783          | 336196087          | 95.3 %                           |
| Phenanthrene| Sphingomonas wild | 324904536           | 183193411          | 43.6 %                           |
|             | Sphingomonas mutant| 324904536          | 267739318          | 17.5 %                           |
| Acenaphthene| E.coli wild       | 411488724           | 46588045           | 88.6 %                           |
|             | E.coli mutant     | 411488724           | 79740172           | 80.6 %                           |

4. Conclusions
According to the current study and the results given from the experiments, as well as their compatibility with previous studies on the degradation of polycyclic aromatic hydrocarbons by isolating unknown strains of local bacteria in the soil, strains have been screened through some experiments based on the use of crude oil and petrochemical materials as a major source of carbon and energy. Through these laboratory experiments, the number of unknown bacterial isolated from the soil has been reduced. Subsequently, strains with the susceptibility to the decomposition and use of oil and its derivatives from PAHs compounds as the sole source of energy were then used. Some microscopic tests and biochemical diagnostic tests were carried out. The use of VITEK 2 technology to identify the types of isolated strains that the decomposition of oil and PAHs have positively induced compounds through the updated Simmon citrate media. The cultivation of identified bacterial is strains in BH media, with the addition of 0.2 g of naphthalene, phenanthrene and acenaphthene as the sole source of carbon and energy and monitoring the growth rate by measuring the Optical density for 28 days. It was concluded that the bacteria could grow and consume PAHs as an energy source with different growth and degradation levels depending on the type of bacteria. The weight method's rate of degradation of each compound was measured during the 28-day incubation period. The effect of the mutation on the change in bacteria’s susceptibility to growth and decomposition has been observed. It was found that the best species of wild and mutant bacteria with the optimum speed of consumption of the substance were mentioned in Table 2 and 3.

References
[1] Das N, and Chandran, P 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology research international, 2011.
[2] Ghazali F M, Rahman R N Z A, Salleh A B and Basri M 2004. Biodegradation of hydrocarbons in soil by microbial consortium. International Biodeterioration & Biodegradation, 54(1):61-7.
[3] Brewer R, Nagashima J, Kelley M Heskett M and Rigby M 2013. Risk-based evaluation of total petroleum hydrocarbons in vapor intrusion studies. International journal of environmental research and public health, 10(6):2441-67.
[4] AlDisi Z, Jaoua S, Al-Thani1D, AlMeer S and Nabil Zouari1 Department of Biological and Environmental Sciences. College of Arts and Sciences, Qatar University, PoB. 2713, Doha, Qatar.
[5] Kiyohara H, Nagao K and Yana K 1982 Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Applied and Environmental Microbiology; 43: 454-7.
[6] Rasool SA, Ijaz S and Subhan SA Hydrocarbon degradation by marine bacteria: Screening and genetic manipulation. Pakistan Journal of Botany 2003; 35(1): 37-44.

[7] Subathra MK, Immanuel G and Suresh AH 2013 Isolation and identification of hydrocarbon-degrading bacteria from Ennore creek. Bioinformation 2013; 9(3): 150-7.

[8] Chaudhary VK and Borah D 2011. Isolation and molecular characterization of hydrocarbon degrading bacteria from tannery effluent. International Journal of Plant Animal and Environmental Sciences, 1(2):36-49.

[9] Khan JA and Rizvi SHA 2011 Isolation and characterization of micro-organism from oil contaminated sites. Advances in applied science research, 2(3):455-60.

[10] Obuekwe IS and Semple KT 2013. Impact of Al and Fe on the development of phenanthrene catabolism in soil. Journal of Soils and Sediments, 13(9):1589-99.

[11] Kafilzadeh F and Pour FH 2012. Degradation of naphthalene, phenanthrene and pyrene by Pseudomonas sp. and Corynebacterium sp. in the landfills. International Journal of Biosciences, 2(9):77-84.

[12] Ahamed F, Hasibullah M, Ferdouse J and Anwar MN 2010 Microbial degradation of petroleum hydrocarbon. Bangladesh Journal of Microbiology, 27(1):10-3.

[13] Aguilar S, RosadoD., Moreno-Andrés J, Cartuche L, Cruz D, Acevedo-Merino A and Nebot E 2018 Inactivation of a wild isolated Klebsiella pneumoniae by photochemical processes: UV-C, UV-C/H2O2 and UV-C/H2O2/Fe3+. Catalysis Today, 313, 94-9.