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Isolation and Identification of Anthracene Degrading Bacteria Isolated from Polluted Soil in Iraq

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Abstract: Anthracene is a one of polycyclic aromatic hydrocarbon, it's considered slow decomposition pollutants in nature, and therefore they are treated in several ways and removed from the environment. The most important of these methods is biological treatment by microorganisms that are already present in the areas of presence of these pollutants. Soil samples were collected from oil-contaminated areas of Al-Dora and Sheikh Omar refinery area with a depth of (5-10) cm and a series of dilutions from (10^{-1}) to (10^{-8}) were made for them, then 0.1 ml was cultured on solid culture medium (nutrient agar). Twenty bacterial colonies were isolated and then cultured inside wells in Bushnell Haas Media agar that contain Anthracene as the sole source of carbon and energy. They were incubated at a temperature of 37°C for 7 days, then the growth zone was observed on two dishes. The two isolates grown on liquid Bushnell Haas Media were incubated in the shaker incubator at a temperature of 37°C and 150 rpm for 7 days. Then it was examined by HPLC High-performance liquid chromatography to know the percentage of bacteria degradation of anthracene. After this, the most efficient bacterial isolates were diagnosed with the VITEK 2 device. *Serratia ficaria* and *Bacillus spp.* showed the high degradation rates for anthracene. *Serratia ficaria* degraded anthracene by 86.6%, and *Bacillus spp.* degradation was 70.64%.

Keywords: Anthracene, Degrading, Bacteria, Pollution.

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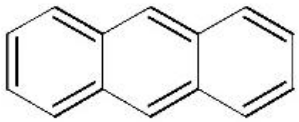
Introduction

Anthracene is a one of polycyclic aromatic hydrocarbons (PAHs), it's pollutants which are ubiquitous (1). Anthracene (ANT) with formula $C_{14}H_{10}$, is a non-mutagenic and non-carcinogenic, low-molecular-weight polycyclic aromatic hydrocarbon present in the environment. Its toxicity can be dramatically increased after solar-light exposure (2). Anthracene is yellowish white particle with mild aromatic odour and are commonly found in petroleum products, cigarette and coal smokes, automobile exhaust, and generated during other combustion and disposal of fossil fuels (3). Despite the substantial existent literature PAHs toxicity, there are very limited data on anthracene however, it is considered a mild irritant of the skin, nose, throat, and eyes (4). Though still considered an unverified carcinogen to human (5). Anthracene also called paranaphthalene or green oil, is a solid polycyclic aromatic hydrocarbon (PAH), consisting of three fused benzene like rings. It is the simplest tricyclic aromatic hydrocarbon from coal tar; melts at 218°C and boils at 354°C (6). Although it is insoluble in water, it is soluble in most organic solvents such as carbon disulfide, alcohols, benzene, chloroform and hydronaphthalenes (7).

About 10 – 500ng have been reported for naphthalene in cigarette (8). Anthracene is on the EPA's priority pollutant list. It has been identified in surface and drinking water, ambient air; exhaust emissions, smoke of cigarettes and cigars, in smoked foods, fossils and edible aquatic organisms. Its presence is found in cigarette smoke and some industries. It may cause tumorigenesis of lungs and other organs (9). Human exposure to anthracene has also been associated with headache, nausea, loss of appetite, inflammation of the gastrointestinal tract, slow reactions, and weakness (10). Oxidation of anthracene yields anthraquinone, the parent substance of a large class of dyes and pigments (11); hence, used in the production of the red dye alizarin and other dyes (12), smoke screens, scintillation counter crystals and in organic semiconductor research. It is also used in wood preservatives, insecticides and coating materials. The United States Environmental Protection Agency (US EPA) has listed these substances as priority contaminants in natural resources However, a wide number of microorganisms slowly degrade these compounds (13,14.15). Six main ways of dissipation, i.e. disappearance, are recognized in the environment: volatilization, photooxidation, chemical oxidation, sorption, leaching and biodegradation. Microbial degradation is considered to be the main process involved in the dissipation of PAH (16). Thus, more and more research Interests are turning to the biodegradation of PAHs. Some microorganisms can utilize PAHs as a source of carbon and energy so that PAHs can be degraded to carbon dioxide and water, or transformed to other nontoxic or low-toxic substances (17). Compared with other physical and chemical methods such as combustion, photolysis, landfill and ultrasonic decomposition, biodegradation is expected to be an economic and environmentally friendly alternative for removal of PAHs (18). Our study aimed to isolate most efficient bacterial strains from two sides that degrade Anthracene and diagnoses them.



Anthracene

Materials and methods

Sample collection

Twenty soil samples were collected from Al-Doura refinery in Baghdad. Samples were collected with a depth of 5-10cm by sterilized zip-lock polythene containers and moved to laboratory and kept at 4°C.

Isolation of bacteria

After serial dilution 0.1 ml of 10^{-5} dilution of soil suspension was poured and spreaded over the nutrient agar plates by using sterile L rod. After incubation for 24 hrs. at 37°C, mucous colonies were formed over the plates and 20 colonies taken to study on.

Screening of bacterial isolates

Primary screening (By Solid Media)

Bushnell Haas Medium was prepared and glucose was added by 1% for adaptation of bacteria to consume anthracene and agar-agar was added to the medium to solidify then sterilized by autoclave at 121°C for 15 minutes. Then, it is left to cool and anthracene was sterilized by 0.45 Millipore filter and added to the medium as sole source of carbon the media was poured into plates and left to solidify. Make wells inside the dish and fill the wells with bacterial broth to see the size of bacterial growth zone around each well which indicates the ability of bacteria to degrade anthracene.

Secondary screening

Preparation of secondary screening samples

Bushnell Haas media broth was prepared in 100ml and sterilized by autoclave at 121°C for 15 minutes, after cool the Anthracene was added by 0.45 millipore filter for sterilization then separated into 50ml in two flasks each one inoculated with one bacterial strain based on primary screening results, these two flasks were incubated for 7 days at 37°C and 150rpm.

Secondary screening by HPLC

The concentrations of anthracene, naphthalene and phenanthrene were calculated before and after treatment with bacterial isolate using (HPLC) according to (170), under the following conditions as shown in Table (1).

Table (1): Conditions of HPLC analysis

Pump model	S 2100 Quaternary Gradient Pump
The mobile phase	acetonitrile–water (70:30 v\v)
Column	C18-ODS
Column dimension	25 cm × 4.6 mm× 5 µm
Detector UV	220 nm
Flow rate	0.8 ml/min
Sample volume	100 µl
Temperature	30°C

The equation below was used to calculate the concentration of the compound in the plant:

$$C.\ sam = \frac{Cst * Asam}{Ast} * \frac{D\ .\ F}{Wt}$$

C. sam= concentration of sample, **Cst**= concentration of standard, **Asam**= area of sample, **Ast**= area of standard, **D.F**= dilution factor, **Wt**= weight of sample.

The percentage of PHAs was calculated using the equation below:

$$\mathbf{C}_{\mathbf{(Sample)}} = \frac{\mathrm{C}_{(\mathrm{Standard})} * \mathrm{A}_{(\mathrm{Sample})}}{\mathrm{A}_{(\mathrm{Standard})}}$$

$$\textbf{Sample concentration percentage} = \frac{\mathrm{C}_{(\mathrm{Sample})}}{\mathrm{C}_{(\mathrm{Control})}} * 100\%$$

$$\textbf{Degradation percentage} = 100 - \text{Sample concentration percentage}$$

Conventional diagnosis of bacteria

The growing colonies of bacteria isolates were initially diagnosed depending on:

Microscopic examination

The reaction pattern of bacterial cells with gram-stain had been studied. This include the examination of shape, Gram stain reaction, and arrangement of cells (19).

VITEK 2 compact identification System

The pure isolates to be identified were culture on Brain heart agar (BHA); the plates were incubated in a incubator at 37°C for 18-24 hrs. Test Card Setup procedure included (20):

1. Inoculums were prepared from a pure culture.
2. Aseptically transferred 3.0 ml of sterile saline aqueous (0.45% to 0.50% NaCl, pH 4.5 to 7.0) into a clear plastic test tube (12 mm × 75 mm) polystyrene test tube. The turbidity is adjusted and measured using turbidity meter called the DensiChek.
3. A sterile swab or applicator stick is used to transfer a sufficient number of morphologically similar colonies to the saline tube prepared in step 2. A homogenous organism is prepared by the suspension with a density equivalent to the appropriate McFarland standard using the VITEK 2 Densi CHEK plus.
4. In a second tube containing 3.0 ml of saline, transferred 280 µl of the suspension prepared in step 3 for AST-GP cards. Then place this tube in the cassette with a susceptibility card. The tube with the initial bacterial suspension could also be used for the inoculation of an identification card. Microorganisms were then identified by VITEK later, the tube with the initial bacterial suspension could also be used for the inoculation of an identification with the automated phenotypic method.

Results and discussion

Primary screening by solid media

The incubation of bacterial isolates is done in wells in solid Bushnell Haas Media with Anthracene as sole source of carbon at 37°C for 7 days according to the research (21) to measure the size of growth zone around each well. So, the size of growth zone is an appropriate method to measure the efficiency of bacterial degradation of Anthracene as shown in the Table (2).

The results showed that only two of the 20 plates showed the bacterial growth zone.

Table (2): First Screening by solid media

Bacterial isolate code	PAH type	Growth zone size	Plate picture
Z1	Anthracene	16mm	
Z3	Anthracene	16mm	

Z1 isolate shows 16 mm growth zone and Z3 also shows 16 mm growth zone which means these bacterial steranes are capable of degrading anthracene.

Secondary screening measurement of PAHs degradation using HPLC anthracene

Anthracene HPLC result for control sample showed high purity by 100% for the compound. The test is used to confirm the type of sample, purity and chemical configuration of polycyclic compound. The retention time of anthracene (3.09) with only one peak number as evidence for purity and standard solution concentration was (10 ppm) and corrected areas (1255.58). The previous number is used as control number to explain the biodegradation efficiency of samples contain anthracene.

The HPLC result indicated that the isolate (Z1) degrade (86.6%) of Anthracene.

The HPLC result indicated that the isolate (Z3) degrade (70.64%) of Anthracene.

Secondary screening was done by HPLC device, the bacterial isolates were cultured in Bushnell Haas Media

containing PAHs as sole source of carbon to force bacteria to degrade them.

In addition creating bacteria-free solutions to be tested by HPLC as control solutions and PAHs test after adding the degrading bacteria to know the percentage of degradation, bacteria were added to the solution and incubated in the shaking incubator at a temperature of 37°C and 150 rpm for 7 days according to the research (21).

Table (3): Percentage of PAHs degradation by bacteria

PAH type	Bacteria Code	Degradation percentage
Anthracene	Z1	86.6%
	Z3	70.64%

Isolate Z1 showed degradation of anthracene was 86.6% and Isolate Z3 showed anthracene degradation by 70.64%.

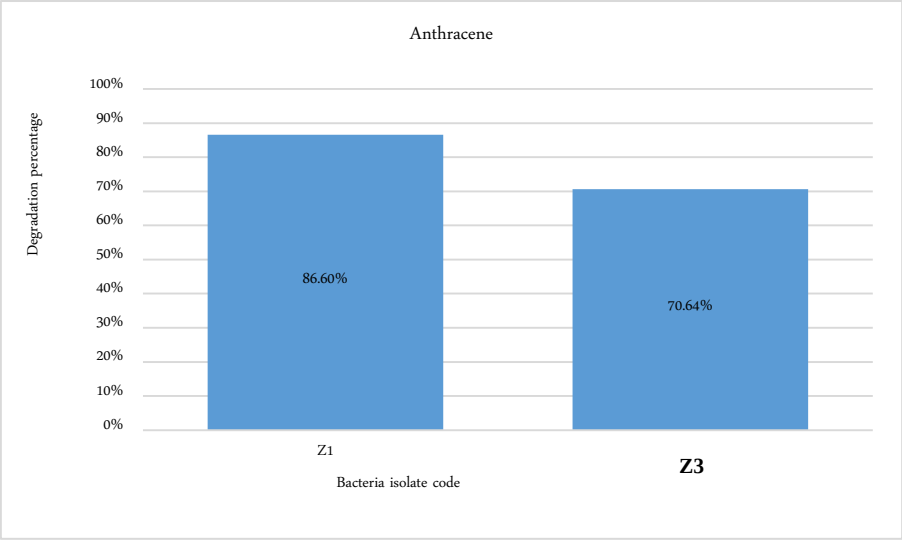


Figure (1): Rates of anthracene degradation by isolates over 7 days of shaking incubation at 150 rpm and 37°C.

Identification of bacterial isolates

MacConkey Agar Test

The MacConkey agar test shows a growth of Z1 isolate (*Serratia ficaria*) colonies which means it is a gram negative bacteria. No growth of Z3 (*Bacillus subtilis*) colonies means it is a gram positive bacteria.

Microscopic examination

The microscopic examination revealed the identity of the two efficient bacterial isolates that analyze PAHs, which are gram negative (*Serratia ficaria* (Z1)) and gram positive bacteria (*Bacillus subtilis* (Z3)) as shown in Figures (2) and (3) and Table (4).

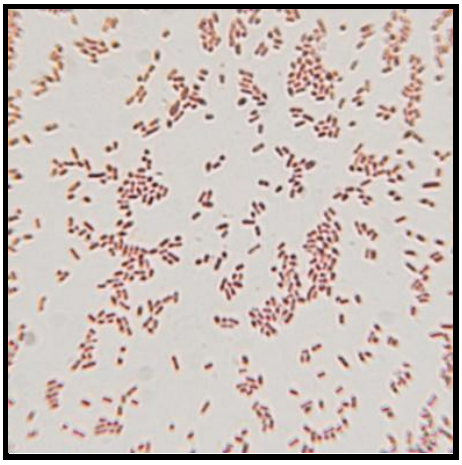


Figure (2): *Serratia ficaria* (Z1).

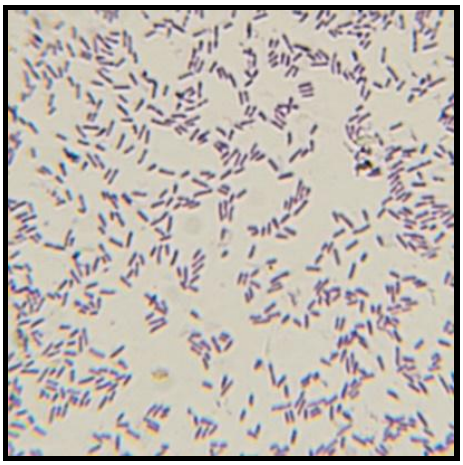


Figure (3): ***Bacillus subtilis*** **(Z3).**

Table (4): Gram stain test results

Name of bacteria	Gram stain
Serratia ficaria **(Z1)**	-
Bacillus subtilis **(Z3)**	+

VITEK 2 identification test

Bacterial Isolates have been identified for the validation by VITEK 2 which has been used to confirm the conventional diagnosis. According to the results from VITEK2 technique the two isolates were identified as *Serratia ficaria* (probability 94%) and *Bacillus subtilis*. (probability 85%).

This study found that *S. ficaria* was able to degrade anthracene by 86.60% and we did not find any research that use this strain of bacteria to degrade anthracene and *Bacillus subtilis* degrade anthracene by 70.64% where in (23) *Rhodococcus* sp. Degraded anthracene by 53% but in (22) anthracene was degraded by only 16%.

Conclusions

Through the study we concluded that *S. ficaria* degrade anthracene by 86.60% and *Bacillus subtilis* degradation was 70.64% and it took short period of time (7 days).

References

1. Li, C.; Li, C.; Yu, H.; Cheng, Y.; Xie, Y.; Yao, W. *et al*. (2021). Chemical food contaminants during food processing: sources and control. Critical Reviews in Food Science and Nutrition, 61(9): 1545–1555.
2. Guiraud, P.; Bonnet, J. L.; Boumendjel, A.; Kadri-Dakir, M.; Dusser, M.; Bohatier, J. *et al*. (2008). Involvement of Tetrahymena pyriformis and selected fungi in the elimination of anthracene, and toxicity assessment of the biotransformation products. Ecotoxicology and Environmental Safety, 69(2): 296–305.
3. Tay, C. C.; Talib, S. A.; Alias, S. and Abidin, A. N. Z. (2017). Occurrence and Bioremediation of Anthracene in the Environment. Journal of Fundamental and Applied Sciences, 9(6S): 214-226.
4. NIOSH, (The National Institute for Occupational Safety and Health) (2019). Registry of Toxic Effect of Chemical Substances (RTECS), Retrieved on 4th December, 2019 from
5. IARC (International Agency for Research on Cancer) (2018). List of Classifications, 1–123. https://monographs.iarc.fr/list-of-classifications-volumes (accessed 5–15 November 2019).
6. Lindsey, J., *et al*. (2014). Anthracene. Photochem CAD. Retrieved 20 February 2014.
7. Haynes, W.M. (2015). CRC Handbook of Chemistry and Physics. 95th Edition. CRC Press LLC, Boca Rato n: FL 2014-2015, p. 3-28, p. 5. 157.
8. Ding, Y. S.; Trommel, J. S.; Yan, X. J.; Ashley, D. and Watson, C. H. (2005). Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes. Environmental Science and Technology, 39(2): 471–478.
9. Johnson, M. O.; Saganuwan, S. A. and Onyeyili, P. A. (2021). Tumorigenic Potential of Dimethyl Sulfoxide, Anthracene in Fish and Anthracene in Dimethyl Sulfoxide in Rattus Norvegicus.
10. Volkova, N.I. (1983). Anthracene and derivatives. In: Encyclopaedia of Occupational Health and Safety, Vol. 1. L. Parmeggiani, Ed. 1983. International Labour Office, Geneva, 162-163.
11. Collin, G.; Höke, H. and Talbiersky, J. (2006). Anthracene in Ullmann's Encyclopedia of Industrial Chemistry, WileyVCH, Weinheim.
12. Adeleye, A. O.; Nkereuwem, M. E.; Omokhudu, G. I.; Amoo, A. O.; Shiaka, G. P. and Yerima, M. B. (2018). Effect of microorganisms in the bioremediation of spent engine oil and petroleum related environmental pollution. Journal of Applied Sciences and Environmental Management, 22(2), 157-167.
13. Wang, S. Y.; Kuo, Y. C.; Hong, A.; Chang, Y. M. and Kao, C. M. (2016). Bioremediation of diesel and lubricant oil-contaminated soils using enhanced landfarming System. Chemosphere, 164: 558-567.
14. Abbasian, F.; Lockington, R.; Megharaj, M. and Naidu, R. (2016). The biodiversity changes in the microbial population of soils contaminated with crude oil. Current Microbiology, 72(6): 663-670.
15. Yuan, S. Y.; Shiung, L. C. and Chang, B. V. (2002). Biodegradation of polycyclic aromatic hydrocarbons by inoculated microorganisms in soil. Bulletin of Environmental Contamination and Toxicology, 69(1): 66–73.
16. Perelo L. W. (2010). Review: In situ and bioremediation of organic pollutants in aquatic sediments. Journal of Hazardous Materials, 177(1-3): 81–89.
17. Toledo, F. L.; Calvo, C.; Rodelas, B. and González-López, J. (2006). Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Systematic and Applied Microbiology, 29(3): 244–252.
18. Titato, G.M. and Lancas, F. M. (2006). Optimization and validation HPLC-UV-DAD and HPLC-APCI-MS methodologies for the determination of selected PAHs in water samples. Journal of Chromatographic Science, 4: 35-40.
19. Mohamed, W.; Sommer, U.; Sethi, S.; Domann, E.; Thormann, U.; Schütz, I., *et al*. (2014). Intracellular proliferation of S. aureus in osteoblasts and effects of rifampicin and gentamicin on S. aureus

intracellular proliferation and survival. European cells and Materials, 28: 258–268.

20. Silva, C. G. D.; Tobouti, N. R.; Zoccoli, C. M. and Silveira, A. C. O. (2017). Evaluation of VITEK 2 Compact and VITEK MS in the identification of coagulase- negative *Staphylococci* isolated from blood cultures. Journal Brasileirode Patologiae Medicina Laboratorial Journal. 53: 293-297.
21. Hossain, M. F.; Akter, M. A.; Sohan, M. S. R.; Sultana, N.; Reza, M. A. and Hoque, K. M. F. (2022). Bioremediation potential of hydrocarbon degrading bacteria: isolation, characterization, and assessment. Saudi Journal of Biological Sciences, 29(1): 211-216.
22. Dean-Ross, D.; Moody, J. D.; Freeman, J. P.; Doerge, D. R. and Cerniglia, C. E. (2001). Metabolism of anthracene by a *Rhodococcus species*. FEMS Microbiology Letters, 204(1): 205-211.
23. Abdul Wahab, T. Y. M.; Padhiar, S. H.; Subhan, S. A.; Baloch, M. N. and Pirzada, Z. A. (2015). Isolation and Characterization of Hydrocarbon Degrading Bacteria from Petrol Contaminated Soil. Journal of Basic and Applied Sciences, 11: 223-231.