

Studies on Utilization and Degradation of Anthracene by Bacteria Isolated from Industrial Waste Water Areas in Egypt

Mohamed T. Shaaban¹, Hamdy A. Abdelazim², Gehad H. Zanon^{1*}

¹Botany and Microbiology Department, Faculty of science, Menoufia University, Egypt.

²Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt.

*Corresponding author:

Gehad Hassan Zanon

Botany and Microbiology department

Faculty of science

Menoufia University,

Egypt.

Email: ghhz.science45@gmail.com

HISTORY

Received: 17th Feb 2018
Received in revised form: 27th of March 2018
Accepted: 20th of May 2018

KEYWORDS

anthracene
polycyclic aromatic hydrocarbon
Lysinibacillus fusiformis
Bacillus subtilis
Serratia liquefaciens

ABSTRACT

Industrial areas in Egypt has become one of the major routes of some wastes production as it discharges a large amount of petroleum hydrocarbons which possess severe hazardous effects on the surrounding environment. Polycyclic aromatic hydrocarbon (PAHs) is one of the most toxic organic pollutants, among this organic hydrocarbon is anthracene, which is highly carcinogenic. The environmental threat of anthracene to humans due to exposure through industrial effluent discharge and other combustion activities into the environment beside expensive nature of the physical and chemical remediation techniques initiates this research. In this study anthracene was degraded by bacteria isolated from industrial waste water areas and degradation was carried out in liquid minimal salt media (MSM). From the fourteen bacterial strains isolated only three isolates were selected that show high potency for degrading anthracene. Isolates GH1, GH17 and GH21 were identified biochemically and genetically by 16S rRNA sequence as *Lysinibacillus fusiformis*, *Bacillus subtilis* and *Serratia liquefaciens*, respectively. Some environmental factors which effect anthracene degradation were also studied in this research to optimize its degradation process. The intermediates formed during anthracene degradation were identified by GC-MS spectroscopic analysis, which revealed anthracene degradation pathway.

INTRODUCTION

Global industrialization with its rapid development in last decade led to the contamination of soil, water and air [1]. In 2010, it is estimated that the waste pollutants contributed to about 3% to the loss of annual life [2]. Certain recalcitrant hazardous chemicals that are carcinogenic and mutagenic result from rapid industrialization by discharging some industrial wastes or by some accidental spill of these chemicals led to environmental contamination to soils and ground water [3]. These pollutants have a variety of hazardous organic chemicals including poly aromatic hydrocarbons (PAHs) and their derivatives [4]. These aromatic hydrocarbons can be decomposed by microbes enzymatically and use it in their cellular metabolism [5].

Contaminations by aromatic hydrocarbons have been the subject of human health and continuous environment threat concern [6]. Aromatic hydrocarbons found naturally in coal and crude oil, From these hydrocarbons, Polycyclic aromatic hydrocarbons (PAHs) are the most subsurface contaminating chemicals [7]. Anthracene is a type of PAHs which persist,

bioaccumulate in the environment and has toxicological effect. The increased level of anthracene as contaminant in soil, sediments and aquatic systems had been a major concern in the world as a result of its highly toxicity effect, anthracene consists of three fused benzene rings which persist in nature, the European Chemicals Agency (ECHA) has been recently included it among the Substances of Very High Concern list (SVHC) due to its toxicity nature on the environment, anthracene produces from combustion processes and industrial effluent discharge, and human exposes to it through contaminated food with combustion products of petroleum hydrocarbon and founds in tobacco smoke which causes damage to the skin, itching and edema, headaches, nausea, loss of appetite, inflammation or swelling of the stomach and intestine [8].

Microorganisms approved their high adaptability to a wide range of recalcitrant poly aromatics led to the formation of some microbial consortium able to degrade many hydrocarbon wastes. Among these microbial community, bacteria have different enzymes that decompose these aromatic hydrocarbon to less or

non-toxic compounds, a feature which makes them outstanding among all other microbes [9,10]. The diversity of bacterial enzymatic metabolism and their role to face environmental disaster provide a biological reservoir for these recalcitrant aromatic hydrocarbon through biochemical activities of it [11,12].

MATERIALS AND METHODS

Waste water samples were collected from different contaminated industrial sites in Egypt such as a paper company drainage, Pesticide Kafr-Zaiat company drainage, Petrochemical industries and oil refinery in Shubra El-Kheima Qalyubia Governorate and 6-October city Cairo Governorate that contains a complex mixture of poly aromatic hydrocarbons (PAHs), and heterocyclic aromatic hydrocarbon including anthracene, and other aromatic hydrocarbon. The samples were collected in glass jars and preserved in a refrigerator at 4 °C until using for isolation purposes.

Chemicals and reagents

Chemicals that were used in this study were purchased from Sigma Aldrich and Fischer that have a purity of 99%. Anthracene was purchased from Fermentas Co.

Culture media

Liquid Minimal salt medium (MSM) used in this research is carbon-free source before anthracene was added after autoclaved at 121 °C for 15 minutes, was composed of phosphate buffer 2x which include ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 14 g, KH_2PO_4 2 g) and topped up with distilled water H_2O to 1L. The pH was adjusted to 7.4, sterilization by autoclaving and trace elements solution which was composed of (HCL (25%) 1.3 ml, ZnCl_2 70 mg, (MgCl_2) $4\text{H}_2\text{O}$ 100 mg, H_3BO_4 62 mg, (CoCl_2) $6\text{H}_2\text{O}$ 190 mg, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 17 mg, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 24 mg, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 36 mg, distilled H_2O up to 1 liter, sterilized by filtration (syringe filter). A 100 x Salt-solution which consists of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 20 g, FeIII-ammonium-citrate (28 %) or 1g, trace elements solution 100 ml was made up by distilled H_2O to 1 liter and sterilized by filtration. Liquid medium was prepared in the flask by putting 10 ml of Phosphate buffer and 100 μl from the stock of major salts 100x and trace elements salts. The solid minimal medium was assembled by sterilization of 15 g agar in 900 ml distilled water, then supplemented with 100 ml buffer 2X and 10 ml from the salt solution

Bacterial isolation

One milliliter from each source was transferred to 50 ml of sterilized broth minimal salt medium (MSM) containing anthracene at the initial concentration of 100 ppm in acetone solvent and sterilized after filtration by 0.25 micrometer filter, stored in a refrigerator. A certain volume of the stock solution was then transferred to the flask bottom prior to the medium addition of a thin film of anthracene that was formed in the flask bottom. The flasks were incubated for one month at 30 °C on a rotary shaker at 150 rpm after several enrichment processes 1. using new minimal media each week [13].

After the incubation period, 0.1 ml of the culture growth from flasks that showed turbidity was plated on solid minimal salt agar plates supplemented with anthracene dissolved in acetone and sprayed at the surface of the agar plates by spray plate technique to form a thin layer of anthracene, the culture inoculum was added after evaporation of acetone by heating the plate near a heat source for minutes [14]. The plates were incubated at 30 °C for one week. Bacterial colonies that appeared in plates that show clear zone around anthracene were

streaked on LB plates for obtaining pure colonies [15,16]. The isolated bacterial strains were preserved in 25% (v/v) glycerol solution at -20 °C and sub-cultured at an interval of 30 days.

Screening of the best anthracene degrading bacterial isolates

The best anthracene-degrading bacterial isolates were screened by the agar plate assay, among all of the colonies, only the colony exhibiting the highest zone of anthracene utilization was selected for further works.

Morphological and molecular identification of the isolates

Some morphological characteristics such as colony morphology, Gram stain, spore stain and motility test were made to the selected bacterial isolates, and molecular identification were also made by isolating DNA of the isolate, the isolated strains were characterized genetically by sequencing the 16S rRNA gene with the universal bacterial primers 8F (5'AGAGTTTGATCMTGGCTCAG) and 1492R (5'GGYTACCTTGTTACGACTT-3'). Similarity, searches of the 16 sRNA sequences were performed compared with sequences available at the NCBI data base using the BLAST algorithm.

Parameters affecting the utilization of anthracene by bacteria

The best selected bacterial isolates that showed higher biodegradation ability were used for optimization condition of biodegradation and utilization rate of anthracene, the parameters that were optimized are temperature, pH, salinity, anthracene concentration, carbohydrate utilization and incubation periods. All these experiments were made in liquid MSM containing the anthracene as a sole carbon and energy source, and the optical density (OD) of the isolates were measured in a spectrophotometer at 650 nm.

GC-MS analysis for metabolites products of anthracene biodegradation

The metabolites formed during anthracene degradation was qualitatively analyzed by GC-MS spectroscopic analysis by using numbers of test tubes experiments, each test tube contained 4 ml of sterilized liquid MSM medium (pH7), 100 μl of microbial inoculum and 50 μl of anthracene. The extraction of this sample was carried out by mixing the content of one test tube (approximately 5 ml) with 1 ml of hexane [17]. This mixture was shaken thoroughly to emulsify its content and allowed to resettle for five minutes. The top layer was isolated and transferred to a clear vial for further use. The hydrophobic layer containing microorganisms was filtered prior to injection to GC-MS, 1 μl was then injected onto the column using a 1:20 split ratio. A standard mixture of hydrocarbons was used to quantify each analyte by determining its mass spectra as well as its retention times.

RESULTS AND DISCUSSION

Isolation and characterization of anthracene-degrading bacteria from different sample sources

By sampling and inoculating from different industrial waste water samples to liquid minimal media, forty bacterial strains were isolated in regarding to their ability to degrade anthracene through the formation of clear zone in minimal agar plates. From these isolates, only three isolates GH1, GH17, GH21 were selected that have been shown as high utilizing and biodegradation capacity for anthracene according to their high clear zones diameter around anthracene. The morphological characteristics of the isolates were identified and were listed in **Table 1**.

Table.1. The Morphological characteristics of the bacterial isolate GH1, GH17 and GH21.

isolates	colony morphology	Gram stain	spore stain	motility test
GH1	white, round, moist, irregular margin	+ve rods	sporulated	non- motile
GH17	white, rhizoids, dry, irregular margin	+ve rods	sporulated	non- motile
GH21	off white, round, moist, regular margin	-ve rods	no spore	motile

Optimization condition of the most potent anthracene tolerant by three bacterial isolates GH1, GH17, GH21

Many parameters like temperature, pH, salinity, anthracene concentration, incubation periods, and carbohydrate utilization have been studied in this research which influences the biodegradation rate of anthracene by its effective role to affect or inhibit the bacterial growth rate, these factors were optimized and the results obtained after measuring the OD at 650 nm were shown in Fig. 1(A to F) for each parameter.

Effect of temperature

Fig. 1A shows the effect of temperature on the degradation of anthracene. It was observed that the high anthracene degradation was at ambient temperature of about at 30 °C.

Effect of PH:

Different pH values were tested on anthracene biodegradation process, Fig. 1B reveals that the optimum biodegradation of anthracene was found at pH 9.0, which means that anthracene degradation was best at alkaline medium than at neutral.

Effect of salinity

Variety of NaCl salt concentration were added to different minimal liquid media supplemented with anthracene. It was observed that the maximum anthracene degradation rate was at 1% (w/v) of NaCl concentration (Fig. 1C).

Effect of different concentration of anthracene

Different concentrations of anthracene (ppm) were tested to determine the best one among them. The results in Fig. 1D showed that strains GH1, GH17, GH21 could degrade low concentrations of anthracene (50 ppm), the growth of these strains was increased gradually by increasing anthracene concentration until 500 ppm concentration, after that a dramatic decrease was obtained after this concentration. It can be concluded from this figure that the optimum concentration of anthracene for these strains was 500 ppm.

Effect of different incubation periods:

Anthracene degradation was measured at different incubation periods along 4 weeks; the OD was measured at 650 nm after every 4 days. In Fig. 1E it was shown that the degradation was increased along time until it reached 20 days at this point the degradation of anthracene was ceased or decreased slightly.

Effect of different sugars on anthracene degradation:

Different carbohydrate utilization by isolated bacterial strains besides anthracene degradation was tested for its ability to degrade anthracene as in Fig. 1F, it was observed that all isolated bacterial strains have the ability to degrade anthracene efficiently when any sugar was added to the minimal medium.

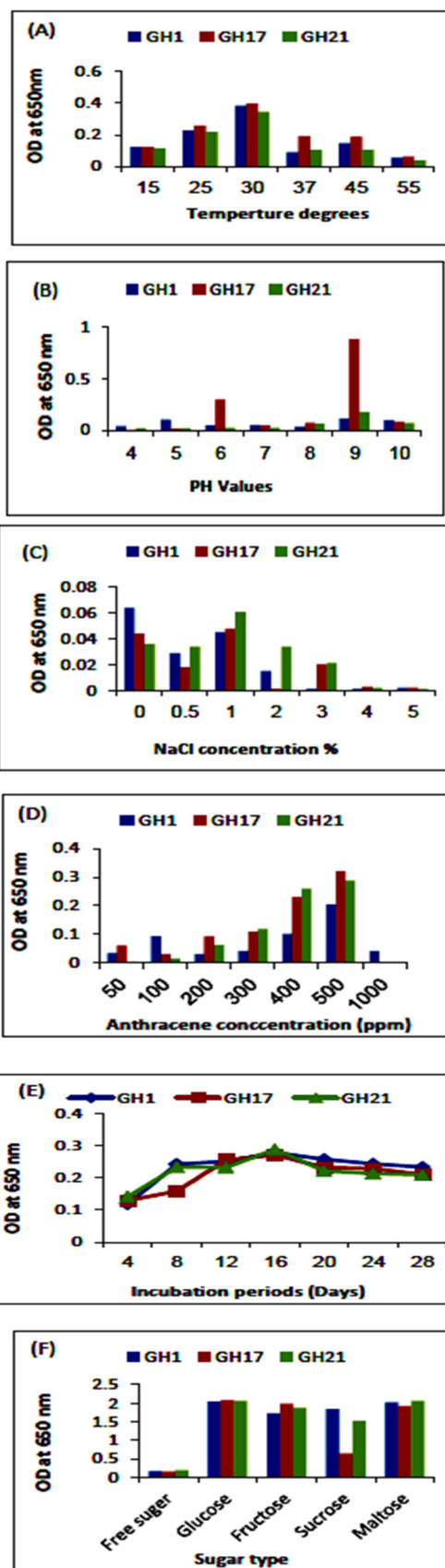


Fig. 1. Influence of environmental parameters on anthracene degradation, (A) temperature, (B) pH, (C) salinity, (D) anthracene concentration, (E) incubation periods and (F) different sugar type.

Molecular identification of bacterial isolates

The bacterial isolates were identified genetically by sequences of 16S rRNA gene from isolated DNA obtained from BLAST and were confirmed using NCBI sequence similarity search tool. The results confirmed that GH1 strain shows 98% similarity to *Lysinibacillus fusiform*, GH17 shows 99% similarity with *Bacillus subtilis* and finally, GH21 shows 99% similarity with *Serratia liquefaciens*.

GC-MS instrumental analysis for degradation studies

GC-MS spectroscopic analysis was carried out to determine the metabolites formed during the biodegradation of anthracene. Some metabolites were detected in the anthracene degradation pathway (Fig. 2).

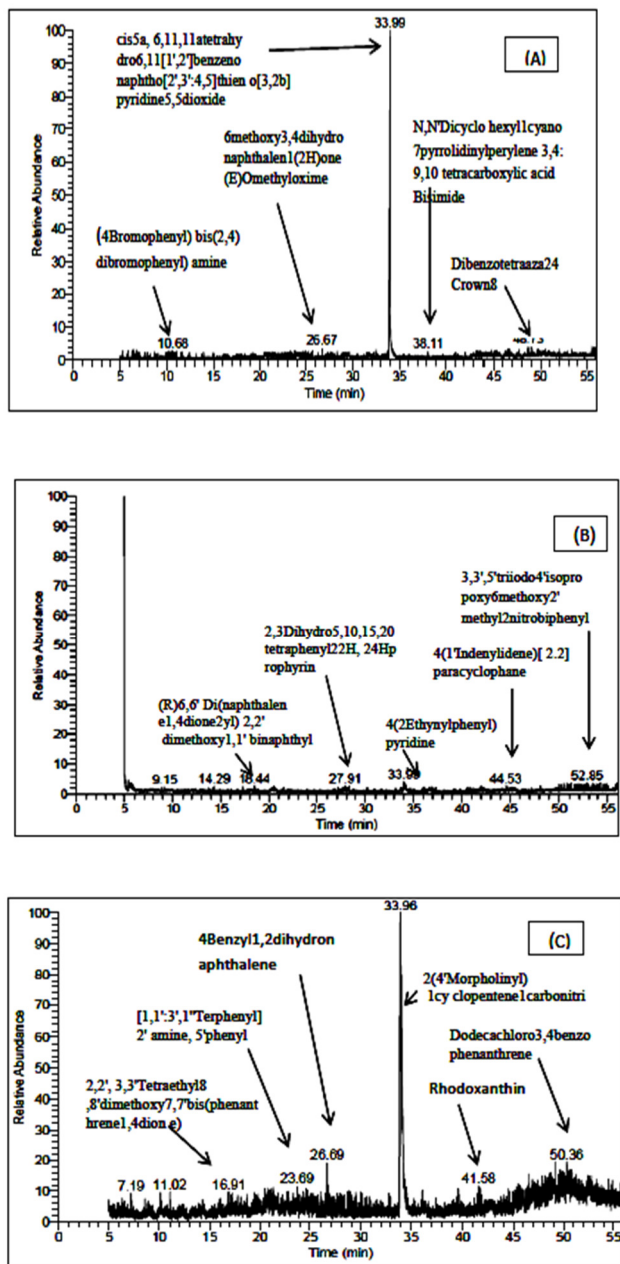


Fig. 2. The GC-mass spectrum of the anthracene in MSM (A) Analysis of anthracene degradation metabolites by Strain GH1 (B) Analysis of anthracene degradation metabolites by Strain GH17 (D) Analysis of anthracene degradation metabolites by Strain GH21.

DISCUSSION

Biodegradation is the major route for most organic pollutants in many environmental waste sites. However, the activity of the degrading microorganism depends on many factors such as contaminant uptake, the concentration of organic pollutant and its toxicity, nutrient availability and activated microbial enzymes [18]. Aromatic hydrocarbons are substances frequently used for human concerns. It often released to the environment accidentally or through some industrial waste discharge, they are widespread contaminants in soil and ground water in industrialized areas [19]. Many microorganisms are known for its efficiency to degrade or mineralize many hazardous organic chemicals; bioremediation is nowadays another alternative way to clean up polluted areas [20].

Anthracene is a tricyclic recalcitrant polycyclic aromatic hydrocarbon (PAHs) that is resistant to degradation [21]. In this study, three isolated bacterial strains GH1, GH17 and GH21 from different industrial sites in Egypt were tested for their ability to utilize and degrade anthracene as it uses it the sole carbon and energy source when supplemented in minimal salt media (MSM). The isolates were identified morphologically, biochemically, genetically. The identification analysis confirmed strain GH1 to was related to *Lysinibacillus fusiform*, strain GH17 was related to *Bacillus subtilis* and strain GH21 was related to *Serratia liquefaciens*.

The rate of anthracene biodegradation is influenced by many factors such as temperature, pH, salinity, pollutant concentration and, chemical and physical characteristics of the contaminated environment [22]. In the previous study the anthracene degradation by three bacterial isolates GH1, GH17, GH21 were degraded efficiently at moderate temperature about at 30 °C as in agreement with Eriksson et al. and Leahy & Colwell, [23,24] polycyclic aromatic hydrocarbons (PAHs) biodegradation rates were slightly higher at moderate temperatures (15–30 °C) due to diffusion, mass transfer and metabolic activity were each increased [25].

pH is another environmental factor that influence the anthracene degradation. The pH may affect the solubility of pollutants, nutrient bioavailability, and the chemical form of the pollutants. From the results, it were showed that the anthracene degradation increased at slightly alkaline PH by three bacterial strains GH1, GH6, GH7 as according to Zhu *et al.* [26] degradation of polycyclic aromatic hydrocarbons (PAHs) increases with increasing pH and slightly alkaline condition consider the optimum for biodegradation of aromatic hydrocarbon. Salinity is an important factor that can render microbial growth and biodegradation of aromatic hydrocarbons [27]. There is an inverse relationship between salinity and hydrocarbon solubility [28].

The result were showed that the degradation of anthracene with three bacterial isolates were best at MSM medium free of salt concentration or slightly salt concentration as 1% for anthracene degradation according to the OD at 650 nm of anthracene in MSM liquid medium which agree with Leahy and Colwell [24] that increasing the salinity in the range of 3.3 to 28.4 % led to decrease in the rate of hydrocarbon biodegradation. Another factor that can affect the biodegradation rate is a concentration of hydrocarbon pollutants due to two reasons toxicity and induction. The toxic effects may attributed to disruption of membrane-based energy generation processes [29]. Different concentration of anthracene were studied that the degradation of anthracene by three bacterial isolates were

increased gradually by increasing the concentration of aromatic hydrocarbon from 50 ppm to 500 ppm, until it reached to a certain point that the degradation was decreased at higher concentration 1000 ppm as Chen and Aitken [30] stated that by increasing the concentration dose of the contaminant the toxic effects of these contaminants will increase and this led to that microorganisms will be unable to defend itself from the toxic effect of them. Utilization of carbon source beside the aromatic hydrocarbon is another crucial property for the degradation of them the present study different carbon source such as (glucose, fructose, sucrose, maltose) were added to liquid MSM and the utilization and biodegradation of them were tested.

It is generally accepted that microorganisms do not grow on high-molecular weight (HMW) compounds as sole carbon sources, therefore the microorganisms capable of degrading these pollutants must obtain energy from other source and metabolize these recalcitrant aromatic hydrocarbon. From the above results there is some evidence that the degradation of these high-molecular weight HMW compounds can be enhanced by the addition of another carbon source like sugars. The incubation periods also were studied in this work. The results were indicated that the three bacterial isolates has short generation time capable of demonstrating rapid growth at short incubation periods as the OD increased when the incubation periods was increased then at certain time about after 20 days the OD was decreased slightly and then it was fixed after the 20 days at 24, and 28 days without and change.

The degradation of anthracene and its intermediates were analyzed using gas chromatography (GC-MS) as evident from the result, anthracene was more readily degraded after 28 days of incubation. Results of the GC-Mass spectrum analysis of anthracene biodegradation shown in the Fig (2) indicated that several intermediates were formed during the biodegradation of anthracene which may results from the oxidation process by many bacterial enzymes. Baker and Herson [31] reported that degradation of aromatic hydrocarbons with long chains from C18 C25 proceeds only slowly. In this study isolation of a bacterium capable of degrading this complex aromatic hydrocarbon is highly useful in the bioremediation of hydrocarbon polluted areas.

CONCLUSION

Forty native Egyptian anthracene degrading bacterial strains were isolated from different contaminated industrial waste water sites in Egypt. These bacterial strains were identified as *Lysinibacillus fusiformis* (GH1), *Bacillus subtilis* (GH17), *Serratia liquefaciens* (GH21). Some parameters also studied which affect the biodegradation of the anthracene. Gas chromatography of the anthracene degradation metabolites used for the identification of the intermediates resulted from the bacterial enzymatic attack.

REFERENCES

1. Yang J, Cao J, Xing G, Yuan H. Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous microalgae *Chlorella minutissima* UTEX2341. *Bioresour Technol.* 2015;175:537-44.
2. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the global burden of disease study 2010. *The Lancet.* 2012;380(9859):2224-60.
3. Tani K, Masuhara M, Welikala N, Yamaguchi N, Nasu M. Change in bacterial community during biodegradation of aniline. *J Appl Microbiol.* 1998;84(5):859-64.

4. Meer JR van der, Vos WM de, Harayama S, Zehnder AJ. Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol Rev.* 1992;56(4):677-94.
5. Phale PS, Basu A, Majhi PD, Deveryshetty J, Vamsee-Krishna C, Shrivastava R. Metabolic diversity in bacterial degradation of aromatic compounds. *OMICS J Integr Biol.* 2007;11(3):252-79.
6. Rockne KJ, Chee-Sanford JC, Sanford RA, Hedlund BP, Staley JT, Strand SE. Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl Environ Microbiol.* 2000;66(4):1595-601.
7. Holliger C, Gaspard S, Glod G, Heijman C, Schumacher W, Schwarzenbach RP, et al. Contaminated environments in the subsurface and bioremediation: organic contaminants. *FEMS Microbiol Rev.* 1997;20(3-4):517-23.
8. Oladimeji AT, Ngozi OC, Richard N. Kinetics of degradation of anthracene by the activity of *Corynebacteria* sp and *Pseudomonas putida* in contaminated water. 2010;10.
9. Cappello S, Russo D, Santisi S, Calogero R, Gertler C, Crisafi F, et al. Presence of hydrocarbon-degrading bacteria in the gills of mussel *Mytilus galloprovincialis* in a contaminated environment: a mesoscale simulation study. *Chem Ecol.* 2012;28(3):239-52.
10. Basha KM, Rajendran A, Thangavelu V. Recent advances in the biodegradation of phenol: a review. *J Exp Biol Sci.* 2010;2:219-34.
11. Shingler V. Integrated regulation in response to aromatic compounds: from signal sensing to attractive behaviour. *Environ Microbiol.* 2003;5(12):1226-41.
12. Ellis L. Environmental biotechnology informatics. *Curr Opin Biotechnol.* 2000;11(232).
13. Bola O, Matthew O, Joseph O. Hydrocarbon-degrading potentials of bacteria isolated from a Nigerian Bitumen (Tarsand) deposit. *Nat Sci.* 2006;4(3):51-7.
14. Juhasz AL, Naidu R. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *Int Biodeterior Biodegrad.* 2000;45(1):57-88.
15. Kiyohara H, Nagao K, Yana K. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl Environ Microbiol.* 1982;43(2):454-7.
16. Dean-Ross D, Cerniglia C. Degradation of pyrene by *Mycobacterium flavescens*. *Biochem Eng J.* 1996;49:201-6.
17. Kumari B, Singh SN, Singh DP. Characterization of two biosurfactant producing strains in crude oil degradation. *Process Biochem.* 2012;47(12):2463-71.
18. Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons. In: *Microorganisms to Combat Pollution* [Internet]. Springer, Dordrecht; 1992 [cited 2018 Jul 17]. p. 227-44. Available from: https://link.springer.com/chapter/10.1007/978-94-011-1672-5_16
19. Dua M, Singh A, Sethunathan N, Johri A. Biotechnology and bioremediation: successes and limitations. *Appl Microbiol Biotechnol.* 2002 Jul 1;59(2-3):143-52.
20. Watanabe K. Microorganisms relevant to bioremediation. *Curr Opin Biotechnol.* 2001;12(3):237-41.
21. Johnsen AR, Wick LY, Harms H. Principles of microbial PAH-degradation in soil. *Environ Pollut.* 2005 Jan 1;133(1):71-84.
22. Margesin R, Labbé D, Schinner F, Greer CW, Whyte LG. Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Appl Environ Microbiol.* 2003;69(6):3085-92.
23. Eriksson M, Ka J-O, Mohn WW. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in Arctic tundra soil. *Appl Environ Microbiol.* 2001;67(11):5107-12.
24. Leahy JG, Colwell RR. Microbial degradation of hydrocarbons in the environment. *Microbiol Rev.* 1990;54(3):305-15.
25. Haritash AK, Kaushik CP. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J Hazard Mater.* 2009;169(1):1-15.
26. Zhu X, Venosa AD, Suidan T. Literature review of the use of 1023 commercial bioremediation agents for cleanup of oil-contaminated estuarine environments. 1024 national risk management research laboratory. *Off Res Dev US.* 2004;1025.
27. Hua X, Wang J, Wu Z, Zhang H, Li H, Xing X, et al. A salt tolerant *Enterobacter cloacae* mutant for bioaugmentation of petroleum- and salt-contaminated soil. *Biochem Eng J.* 2010;49(2):201-6.

28. Al-Mailem DM, Sorkhoh NA, Al-Awadhi H, Eliyas M, Radwan SS. Biodegradation of crude oil and pure hydrocarbons by extreme halophilic archaea from hypersaline coasts of the Arabian Gulf. *Extremophiles*. 2010;14(3):321–8.
29. Sikkema J, Poolman B, De Bont J. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev*. 1995;59(2):201–22.
30. Chen S-H, Aitken MD. Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* p15. *Environ Sci Technol*. 1999;33(3):435–9.