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PAH Degradation by Two Native Egyptian Strains *Flavobacterium Sp*. And *Pseudomonas putida*

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Abstract: PAH make serious problems in Egypt and all over the world. PAHs present in soil may exhibit a toxic activity towards different plants, microorganisms and invertebrates. Microorganisms, being in intimate contact with the soil environment, are considered to be the best indicators of soil pollution. In addition, biodegradation is one of the most key processes for PAHs to disappear from the environment. So, in Egypt we try to overcome this problem by searching on microorganisms able to degrade and eliminate this hazard compounds from our environment. Solid and liquid samples were collected from Kafer El Zyat City, Gharbia Governorate, Egypt. Bacterial isolation was carried out using enriched media technique. More than 78 isolates were obtained and capability of degradation also was examined using modified real time method. Finger printing for all the isolates using REP-PCR was carried out. The REP_PCR was grouped the isolates into 10 groups, only two isolates were showed high degradation talent and specificity for the two polyaromatic hydrocarbons (naphthalene or phenol). Moreover, the two isolates were subjected to molecular identification using 16SrRNA gene. Actually, The DNA sequence identified them into *Pseudomonas putida* (DQ399838) and *Flavobacterium sp*. (DQ398100). Strain *P. putida* showed degradation rate for naphthalene reached to 50% after 20 hours from incubation but the same result was obtained by *Flavobacterium sp* after only 17 hours from inoculation time. Whenever, we need to increase the amount of the soil degradation rate by adding these mutant bacteria to the soil and amendment of these bacteria, which will help in bioremediation technology.

Key words: PAH, Soil bioremediation and Bacterial PAH degradation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread in nature (i.e. soil, water and sediments) because of several polluting anthropogenic activities[9]. PAHs present in soil may exhibit a toxic activity towards different plants, microorganisms and invertebrates. Microorganisms, being in intimate contact with the soil environment, are considered to be the best indicators of soil pollution. An alteration of their activity and diversity may result and in turn it wills reflect in a reduced soil quality[11]. The dominant PAH-degrading bacterial species isolated from PAH-contaminated soils belong to a limited number of genera and the most frequently observed isolates are from the sphingomonad and mycobacteria groups[24].

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic pollutants, which cause a public concern due to their potency as carcinogens and mutagens. Originated mainly from anthropogenic activities, PAHs are now ubiquitously distributed in natural waters worldwide[25,26]. In the aquatic environment, PAHs tend to sorbs onto the particle phase due to their high hydrophobicity and solid-water distribution ratios[22]. The distribution of PAHs in the aquatic environment is significantly affected by aquatic particulates, which act as aggregates of numerous complicated organic materials[27].

Bacteria are commonly considered to degrade organic substrates only when they are dissolved in water[22] therefore, many studies proposed that the accumulation of PAHs in sediment or soil would reduce their bioavailability to bacteria and thus retard the

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biodegradation process[28]. However, other studies showed that the existence of sediment would lead to a higher biodegradation rate because sorbed contaminants could be degraded by solid-phase bacteria[30]. For example, Poeton *et al*.[29] reported that the biodegradation rates of phenanthrene and fluoranthene by marine bacteria were much higher in the presence of sediment than in the absence of sediment. In order to interpret those controversial results, further studies are clearly needed to probe the mechanisms of the effects of the solid phase on PAHs biodegradation in natural water systems[31].

Industrial residues contaminating the environment usually represent a mixture of organic and inorganic compounds[6]. Polynuclear aromatic hydrocarbon (PAH) compounds are common groundwater contaminants and highly carcinogenic chemicals. Bioremediation has been studied extensively in the last two decades as a means removing PAHs, especially from contaminated soils. Numerous strains of soil microorganisms that are capable of degrading PAHs have been isolated and characterized. The following strains are known to degrade naphthalene, which is one of the most studied PAHs: *Pseudomonas putida* G7, *Rhodococcus* sp[12].

Bacterial growth in polycyclic aromatic hydrocarbon (PAH)-contaminated soils is dominated by the low bioavailability and often long-term persistence of these compounds[21]. Nonetheless, significant bacterial communities that are able to degrade PAHs develop in such environments and a range of phenotypic properties has been proposed to play a critical role in partially overcoming the low bioavailability of PAHs to allow growth. These properties include enhanced cell-surface hydrophobicity, biofilm formation, surfactant production, motility and chemotaxis and the ability to use multiple and mixed substrates[22]. Microorganisms inoculated into PAH-contaminated soil environments must find and mobilize PAHs before degradation can take place and motility and chemotaxis are therefore thought to be desired properties. Indeed, motility also contributes to soil survival in general—motile *Pseudomonas fluorescens* strains, for example, survive better in agricultural soils than nonmotile strains, probably due to motile populations finding favorable microsites in the soil matrix and avoiding predation[23].

Several bacterial species, such as *Mycobacterium* sp., *Pseudomonas* sp., *Stenotrophomonas maltophilia* and *Rhodococcus* sp. have been reported to be pyrene degraders[1]. *P. putida* G7 was reported to be attracted to the pollutant naphthalene[9,15].

Pseudomonas bacteria capable of degrading naphthalene, anthracene and phenanthrene can be readily isolated from PAHs contaminated soils[13].

All *Pseudomonas* strains tested capable of naphthalene degradation catabolize this substrate to salicylate via similar biochemical pathways[14].

In this study we focused on isolation of bacterial isolates from industrial polluted area. The petroleum wastes, pesticides and factories wastes make serious problems in Egypt for environment, plants, animals and the human. So, we try to find Egyptian native strains have the efficiency to eliminate or at least decrease the growth of the environmental pollution. Whenever, isolation, adaptation, identification and examination such bacteria to degrade the polyaroamtic hydrocarbon was carried out, that it the aim target of this study. For that reasons we select Kafer Elzayat City as a source of these bacterial isolates because this city one of the biggest industrial city in Egypt.

MATERIALS AND METHODS

Enrichment and isolation of phenol and naphthalene-degrading bacteria: Polyaromatic hydrocarbon degrading bacteria were isolated from liquid and solid wastes of (petroleum tanks, Paper Company, Insecticides and Pesticides Company, Housing wastes and Agriculture wastes) Kafer El-Zayat area. Kafer El-Zayat, Gariah, Egypt. Bacterial enrichment cultures were set up in 500 ml cotton-plugged Erlenmeyer flasks containing 50 ml of minimal basal salt medium (MSM) using phenol naphthalene, as a sole source of carbon. Contaminated solid and liquid wastes were used as sources of the inoculums for the enrichment cultures. Stock solution of phenol and naphthalene (20 mg ml^{-1}) were prepared in acetone and added to the mineral salt medium at an initial concentration of 10 ppm. The phenol and naphthalene concentration in the mineral slat medium were slowly increased to about 500 ppm over a period of 57 days and were maintained at this level for the duration of the enrichment at 30°C with shaking 200 rpm. After one weeks of enrichment, sub-samples from the flasks were streaked on plat containing solid medium. Plates were prepared with MSM that had been solidified with 2% agar and sprayed with a 20 mg ml^{-1} phenol and naphthalene solution dissolved in acetone. The plates were incubated at 30°C temperature. The colonies were grown on the plates were picked and streaked on new minimal agar plates.

DNA fingerprinting for the isolated bacteria: To examine the relatedness between these isolates, REP-PCR was carried out using two primers; the first one is BOXA1R primer according to[16] (5`-CTA CGGCAAGGCGACGCTGACG-`3). The second primer used in this study is the REP1R-I according to[20] (5`-CGGICTACIGCIGCIIII -`3). PCR mixtures were prepared with 1µl of genomic DNA, 5 µl

of Taq buffer 10x, 2.5 mM of MgCl2, 200 µmol of each deoxynucleoside triphosphate, 20 pmol each primer, 5 µg of bovine serum albumin, 1% of formamide and 2.5 U Taq polymerase (Promega, Germany) and sterile filtered milliQ water to a final volume of 50 µl. The PCR program was as follows: primary denaturation at 95ºC for 5min, 40 cycles was applies as follow: 94ºC for 30 Sec, 47 ºC for 30 Sec and 72ºC for 1min in case of REP1R-1 but 7min in case of BOXA1R. Final extension step at 72ºC for 7 min and PCR reaction kept on 4ºC until removing the tubes. PCR products were separated on agarose gel electrophoresis using 1.5% w/v agarose in 0.5 x Tris-borate EDTA buffers. Size of each band was estimated by using DNA molecular weight marker. Finally, gel was photographed by using gel documentation system.

The Identification of the isolated bacteria: According to Edwards *et al*.,[17] and Wilson *et al*.,[18], the 16S r-RNA gene was amplified using specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACT T-3'). The PCR products were routinely analyzed by electrophoresis in 1.0% agarose gels in 1x Tris-Borate-EDTA buffer[10]. The target band was purified using gel extraction kit (Qiagene, Germany) and then subjected to partial sequencing using ABI 377 automated sequencer. Approximately 1000 bp was sequenced and phylogenetic analysis of the polyaromatic hydrocarbon degrading bacteria based on 16S r-RNA genes were carried out by pair-wise comparison using ClustalW and MEGA 3.1

Examination the degradation of the polyaromatic hydrocarbon on line: The degradation of the hydrocarbon was examined using the Real time method according to Winge *et al*.[19] 2% of PAH(s) as stock solution were added to the MSM culture media (Mineral Salt Medium) as a carbon source separate or in combination. A 270ul from MSM containing the desired of hydrocarbon was loaded into 96 well ELISA plate (Coster, Germany) and then bacterial inoculums was added to each well (30 ul from fresh culture). The plate was incubated at the optimum temperature overnight in the Fluoroskan (Labsystem, Finland). The degradation values were monitored directly each one-hour and the data was recorded automatically as Excel sheet.

Upgrading the degradation ability of the selected bacteria: The sixteen isolates were selected to upgrading their degradation abilities for the polyaromatic hydrocarbon. These bacterial isolates were inoculated in ELISA 96 well has MSM medium containing different concentration from the desired hydrocarbon starting with 5ppm and ended with 500ppm. The polyaromatic hydrocarbon degradation rates were examined using Real time method.

RESULTS AND DISSECTIONS

Bacterial isolation and sampling: Egyptian's industrial sector is comprised of more than 20 major industrial sub-sectors, these are: textile spinning and weaving, textile processing, leather, pulp and paper, cement, sugar, fertilizer, petro-chemicals, industrial chemicals, pesticides and insecticides, dies and pigments, pharmaceuticals, food processing, edible oils and fat, dairy, tobacco, steel, automobile, polyester-fiber and yarn and wool & wool processing.

Kafer El-Zayat area, Kafer El-Zayat is an industrial city and has many industrial factories especially petro-chemicals and insecticides. So, it has many contaminated areas easily to isolate different bacterial isolates inhabited or living in this habitat. The bacterial culture capable of degrading naphthalene and phenol was isolated from solid and liquid wastes using enrichment technique[7]. The survival bacteria were then selected and morphological studies were carried out as a first step to distinguish between them. Seventy-eight isolates showed dissimilarities in colony morphology and texture. All of them was inoculated in flasks contains MSM media supplemented 4 different polyaromatic hydrocarbons (phenol, naphthalene, antheratheneand and phenantherene) and after 4 days of inoculation some bacteria survive and fight but others did not show any growth. The isolates, which remain survival, was selected and subjected to further studies.

Fingerprinting for the isolated bacteria: All the isolated bacteria were subjected to REP-PCR using two primers BOX1AR and PRIM1. The data presented in Figure (1 and 2 A&B) revealed that the two primers grouped the selected 15 isolates into 10 groups have two ancestors. The biodiversity, of the isolated bacteria showed the adaptation capability of these organisms to survive and compete in these contaminated areas. Gibson and Sayler[3] 1992 obtained the same results. Tuomi *et al*.[33] also proved that the transformation of environmental contaminants is a complex process that is influenced by the nature and amount of the contaminant present, the structure and dynamics of the indigenous microbial community and the interplay of geochemical and biological factors at contaminated sites. Whenever, Ghazali *et al*.[32] mentioned that PAH biodegradation by pure cultures may not represent the actual behavior of environmental microbes since the cooperation of indigenous strains is damaged.

However, conventional molecular methods (PCR-based technologies, such as gene cloning, terminal-restriction fragment length polymorphism, denaturing gradient gel electrophoresis and in situ hybridization) for assessing microbial community structure and activities are

labor-intensive. Rapid, quantitative and cost-effective tools that can be operated in field scale heterogeneous environments are needed for measuring and evaluating bioremediation strategies and endpoints[8].

The isolates identification: Amplification of the 16S rRNA gene was carried out for only two isolates. These isolates were selected due to their ability to degrade and survive in a high concentration of the polyaromatic hydrocarbons. The PCR product (1.459bp) was subjected to sequence using automated sequencer. The sequence analysis for the two isolates revealed that the two isolates were *Flavobacterium sp.* and *Pseudomonas putida* with the accession numbers DQ398100 and DQ399838, respectively theses results agree with. Whatever, the same way to isolate polyaromatic hydrocarbon degrading bacteria and same isolates or related to our bacterial isolates by[4,5].

The upgrading and evolution of the degrading ability of the two selected strains: All the isolated strains were grown in different concentrations of naphthalene, phenol, antherathene and phenantherene in MSM media as a sole of carbon source and only two isolates showed high degradation abilities. In addition to the high performance of these two isolates in degradation PAHs also they

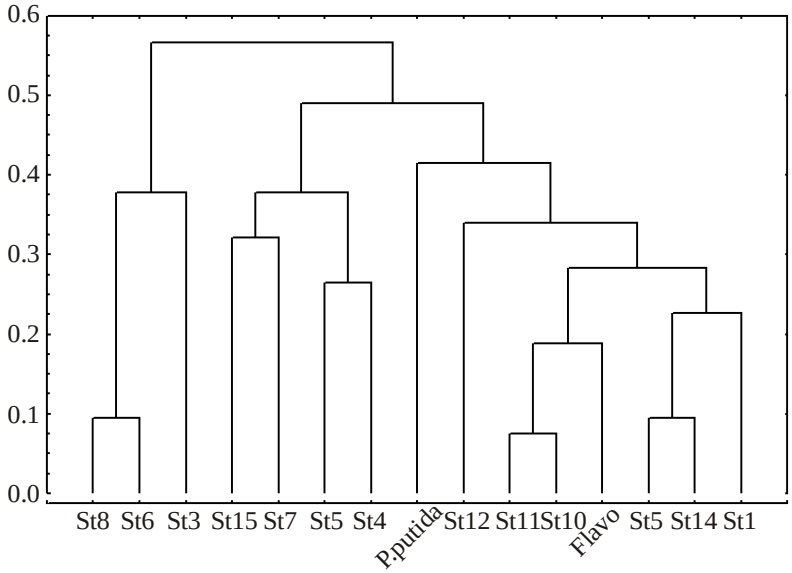

Fig. 1: Dendogram for some of the bacterial isolates based on the REP-PCR.

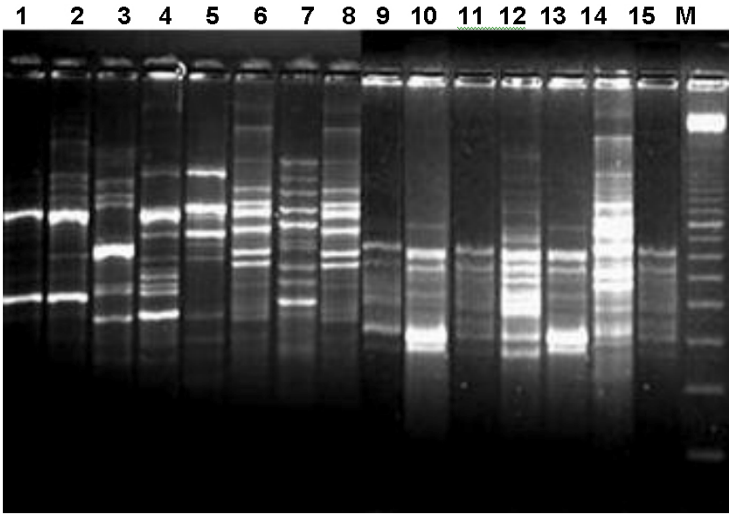

Fig. 2 A: REP-PCR for 15 bacterial isolates as a preventive samples using BOX1AR primer.

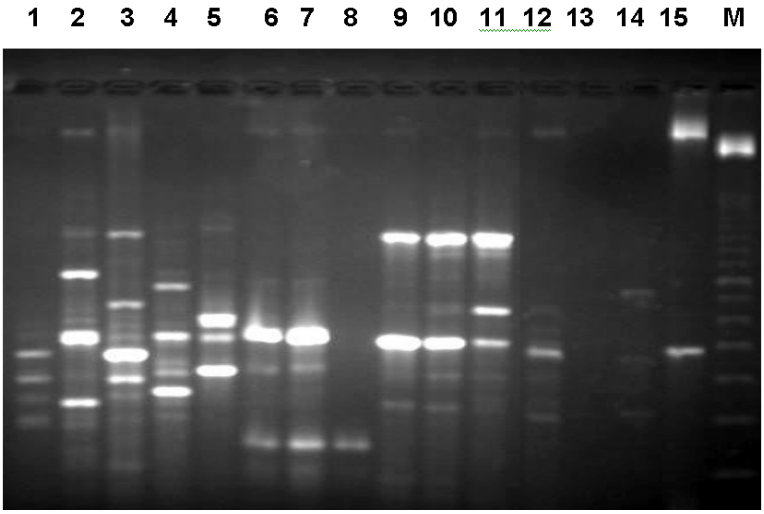

Fig. 2 B: REP-PCR for 15 bacterial isolates as a preventive samples using PRIM1 primer.

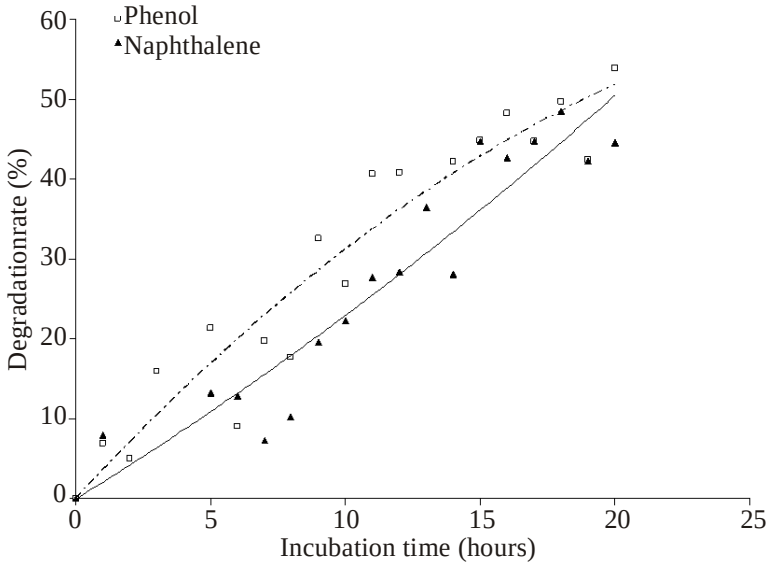

Fig. 3: Degradation rate of the two polyaromatic hydrocarbons (Phenol and Naphthalene) by the two isolates *Flavobacterium* and *P. Putida*, respectively.

showed high specificity for the type of polyaromatic. *Pseudomonas* showed high ability to degrade about 50 % of the total polyaromatic hydrocarbon in the medium after 20 hours from inoculation. Whenever, the *Flavobacterium* strain degrades the same amount of phenol but in 17 hours after inoculation as shown in figure (3). Both of Jilani and Khan,[5] they mentioned that strains are known to degrade naphthalene, which is one of the most studied PAHs: *Pseudomonas putida* G7, *Rhodococcus* sp. Strain B4, *Oscillatoria* sp. strain JCM and *Alcaligenes* sp. strain NP-Alk.

Conclusion: The presence of the *Pseudomonas* bacteria considered as an biosensor for the petroleum soil contamination. On the other hand *Flavobacterium* bacterium also considered as a good indicator for the water contamination with petroleum and insecticides. Now we have to safe our environment from the hazards of pollutions and eliminate most of the problems results in from these contaminations especially in the water and agricultural soil. The continuous increase of the cancers patients in Egypt especially between the farmers. The government should help us to grow these microorganisms and inoculating the contaminated areas

especially in industrials cities to avoid the evolution of this problem in the future.

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