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Isolation and Identification of Pyrene Degrading Bacteria and its Pathway from Suez Oil Processing Company, Suez, Egypt.

Mervat A. M. Abo-State[1*], *Farida M. S. E. El-Dars*[2] *and Bahaaeldin A. Abdin*[2]

[1] Department of Radiation Microbiology, National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt.
[2]Department of Chemistry, Faculty of Science, Helwan University, Ain Helwan, Helwan, Cairo 11795, Egypt.

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Abstract: An indigenous bacterial isolate MAM-P39 was isolated from petroleum polluted soil by selective enrichment with pyrene as the sole source of carbon and energy. During growth on pyrene, the bacterial cell density was monitored by measuring the $O.D_{600}$. Extracellular protein and bacterial count were determined. The degradation percentage of pyrene was quantified by high-performance liquid chromatography (HPLC).The isolate MAM-P39, which has been identified by 16S rDNA, was the best pyrene degrader. This isolate was identified as *Pseudomonas panipatensis* MAM-P39 with accession number MF150314. It could degrade 90.5% and 66.03% of 500 and 2000 µM pyrene. Degradation products of pyrene were identified by gas chromatography-mass spectrometry (GC-MS) analysis.

Keywords: Isolation, Degradation,Pathway, 16S rDNA, GC-MS.

1 Introduction

Petroleum hydrocarbon pollutants are recalcitrant compounds and categorized as priority pollutants [1].

Crude oil is classified into four broad fractions (a) Saturates (aliphatics), (b) Aromatics (ringed hydrocarbons), (c) Resins and (d) Asphaltenes. Aromatics are ringed hydrocarbon molecules they can be mainly divided as (a) monocyclic aromatic hydrocarbons (MAHs) and (b) polycyclic aromatic hydrocarbons (PAHs) [2].

Polycyclic aromatic hydrocarbons (PAHs) are aromatic hydrocarbons with two or more fused benzene rings from natural and anthropogenic sources, such as forest and rangeland fires or the imperfect combustion of fossil fuels and petroleum [3] Waste incineration or as by-products of industrial processes such as petroleum refining, coal gasification, production of aluminum, iron and steel [4].

Polycyclic aromatic hydrocarbons (PAHs) are a category of over 90 various chemicals released from numerous combustion sources. The ubiquity and toxicity of PAHs have posed high health risks on human beings populations [5]PAHs have the possibility of inducing malignant tumors that mostly affect the epidermis and other epithelial tissues as they have a great affinity for nucleophilic center of macromolecules like RNA, protein and DNA [6] PAHs-induced genotoxicity, mutagenicity, and carcinogenicity have been proven in several living organisms or cellular lines both in the research laboratory and natural environment [7].

The manifestation of polycyclic aromatic hydrocarbons (PAH) in refinery effluents is of great concern globally due to its persistence, recalcitrance, and carcinogenicity [8]Petroleum refinery effluents are wastes from industries, mainly involved in refining crude oil and production fuels, lubricants and petrochemical intermediates [9].

Polycyclic aromatic hydrocarbons (PAHs) constitute one group of priority environmental contaminants, which can be of great matter over their toxicity, carcinogenicity, teratogenicity as well as recalcitrance in the environment [10] United States Environment Protection Agency (USEPA) has enlisted 16 of them as priority contaminants [11].
Green technology for cleanup of pollutants by biological means is being used for bioremediation of petroleum polluted site(s) [12] Bioremediation can be explained as the application of living organisms to degrade/detoxify contaminants [13,14] This technology is an effective, economic, versatile and environmentally sound strategy [2]. Biodegradation offers environmentally friendly and cost-effective option way for cleaning-up different environments polluted with hydrocarbons with the good thing about the large range in-situ application like the marine environment

*Correspondingauthore-mail:abostatem@yahoo.com, ch.bahy@gmail.com

[15].
Bioremediation has turned into a major method used in the restoration of petroleum hydrocarbon polluted environments, which makes use of natural microbial biodegradation activity. Removal of petroleum hydrocarbon contaminants from the environment using oleophilic microorganisms (individual isolates/consortium of microorganisms) is eco-friendly and economical [16].
Microbial bioremediation is a vastly used technique for treating petroleum hydrocarbon pollution in both terrestrial and aquatic ecosystems [17]Bioremediation, the utilization of bacteria to eliminate pollutants, has lately been received an attention as the utmost appropriate method bioremediation of crude oil because it is cost-effective and produce safe by-products [18] Microbial remediation has many advantages over physicochemical methods since it can decompose or mineralized harmful pollutants into less hazardous or non-toxic substances with better safety and less environment disruption [19].
Numerous research studies pertaining to biodegradation of hydrocarbon pollutants have been done in the previous ten years [12, 13, 14] Comprehensive studies have been done on the biodegradation of isolated bacteria from the environment resulting in the isolation of some bacteria which have the ability to use PAHs compounds as the sole carbon and energy source [20].
Sundry microbial populations, such as bacteria, fungi, and algae, can act in crude oil degradation. Most researchers consider bacteria to be the most important group of petroleum-degrading organisms since it has swift and numerous metabolic rates of organic degradation [21,22] The prior literature reported that *Pseudomonas aeruginosa* is the most effective in crude oil biodegradation [23].
Pyrene is a tetracyclic high-molecular-weight PAH compound, it has been extensively used as a model compound to review degradation of high-molecular weight PAHs [24]Pyrene is often detected in environmental samples and crude oil resulting from imperfect combustion and used as an indication for PAH-contaminated waste monitoring. It has low biodegradability, high persistence in the environment and has been outlined as a priority pollutant by the United States Environmental Protection Agency (US EPA) [25].
Indigenous hydrocarbon degrading microorganisms play a substantial role in bioremediation process [14] Microorganisms such as bacteria, fungi, algae are reported because of their potential to degrade hydrocarbon pollutants [26]In the past decades, a huge variety of bacterial strains that can degrade PAHs have been effectively isolated from highly PAH-contaminated environments [27]
Bacterial sp. of genera *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Ochrobactrum*, *Pseudomonas*, *Stenotrophomaonas* and *Vibrio* are reported as hydrocarbon degraders [2, 12, 14, 28].

2 Experimental

2.1 Sampling Site

Soil contaminated with sludge was collected from deposits of petroleum field, which are either chronic or recent from the Suez Oil Processing Company (SOPC), Suez, Egypt as indicated in Fig. (1)

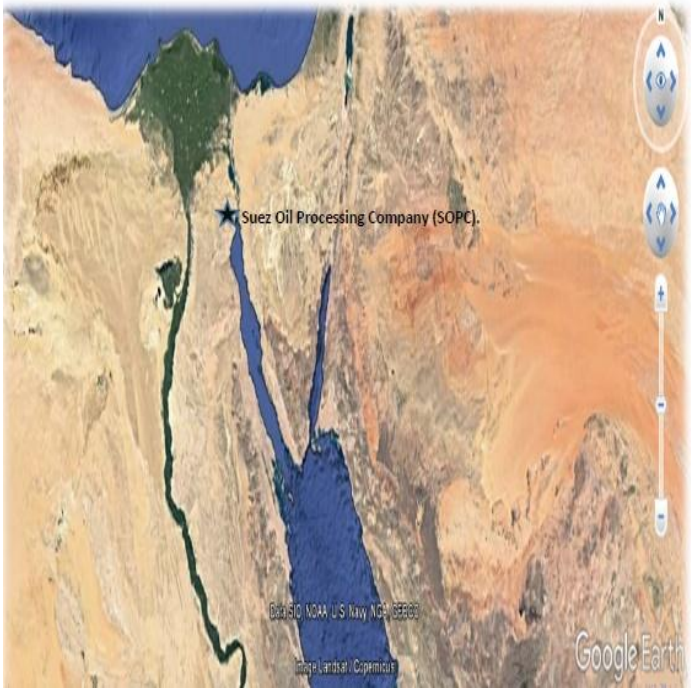

Figure 1. Sampling Site.

2.2 Sampling

Soil contaminated with petroleum crude oil sludge was collected in sterile plastic bags, shipped on ice and stored at 4^{o} C to be used within 4 hours.

2.3 Chemicals

Pyrene and Folin reagent product of Sigma (Aldrich, USA). Chloroform was HPLC grade, obtained from BDH, England. Bovine serum albumin (BSA) obtained from Sigma, USA.

2.4 Culture Medium

2.4.1. Basal Salt Medium (BSM)

The composition of basal salt medium (BSM)was (g/L): $(NH_4)_2SO_4$ 1.1, K_2HPO4 2.2, KH_2PO_4 0.9, $MgSO_4.7H_2O$ 0.1, $MnSO_4.6H_2O$ 0.025, $FeSO_4.7H_2O$ 0.005, L-ascorbic

acid 0.005, deionized water 1000 ml. For use, the following supplements were added to 1 liter of the cooled basal medium: 1 ml of trace elements and 0.1 ml of vitamin solution. Trace element (mg/L): H_3BO_3 0.3, $CoSO_4$ 0.4, $ZnSO_4.7H_2O$ 0.1, $MnCl_2.4H_2O$ 0.03, $NaMoO_4.2H_2O$ 0.03, $NiSO_4.6H_2O$ 0.02, $CuSO_4.5H_2O$ 0.01, HCl 50 ml, deionized water 950 ml. Vitamin solution (mg/L): Biotine 2.0, Folic acid 2.0,Pyridoxal hydrochloride 10.0, Riboflavine 5.0, Thiamine 5.0, Nicotonic acid 5.0, Ca-Panthothenate 5.0, Cyanocobalamine 5.0, P-aminobenzoic acid 5.0, Deionized water 1000 ml [29].

2.4.2 Luria Broth Medium

The Luria broth medium (LB) composed of the following (g/L): Tryptone 10.0, Yeast extract 5.0, NaCl 5.0, distilled water 1000 ml. The pH was adjusted to 7.1 + 0.2 before sterilization [30].

2.5 Adaptation Technique

According to [31,32,33] with modification, soil samples (75 grams) were added to (225 ml) of BSM and incubated overnight in shaking incubator at 30°C with 150 rpm for adaptation of the microbial communities (Indigenous mixed bacteria). From the preadapted microbial communities, 10.0 ml was used to inoculate 150.0 ml of BSM. The BSM was amended by (500µM) pyrene and incubated at 30°C with shaking incubator (150 rpm) for 3 days.

2.6 Enrichment Technique

The preadapted indigenous bacterial communities were used to inoculate (10% v/v) fresh BSM amended with 500µM of Pyr. and incubated for 7 days at 30°C in a shaking incubator (150 rpm) (First Transfer).The grown bacterial communities from the first transfer were used to inoculate (10% v/v) fresh BSM amended with 1000µM of Pyr. and incubated for 7 days at 30°C in a shaking incubator (150 rpm) (Second Transfer).From the second transfer, the indigenous bacterial communities in BSM were used to inoculate (10% v/v) fresh BSM amended with 1500µM of Pyr. and incubated for 7 days (Third Transfer) at 30°C in a shaking incubator (150 rpm). For each treatment three replicates were used. The bacterial isolates able to use pyrene as a sole carbon and energy source from the third transfer were used for isolation.

2.7 Isolation of Bacterial Isolates

Bacterial communities able to tolerate 1500µM of Pyr. were serially diluted with sterile saline and plated on the surface of L.B agar plates, spread and incubated at 30°C for 48 hours. The separated single colonies were picked up and streaked on the surface of L.B agar slants. These slants were kept at 4°C for further investigation.

2.8 Screening for the Most Potent Bacterial Isolates

The bacterial isolates those were able to tolerate 1500µM of Pyr. were streaked on BSM agar plates amended with the concentrations (500, 1000, 1500 and 2000) µM for pyrene. Three replicates were used for each isolate, the plates were incubated at 30°C. The growth of bacterial isolates was monitored every day for 15 days.

2.9 Growth of the Most Potents Bacterial Isolates on BSM Supplemented with Difference Concentrations of Pyrene

The six most promising pyrene degrading bacteria were grown in LB broth media for 48 hours in shaking incubator (150 rpm) at 30°C. The well-grown cultures were centrifuged at 8000 rpm for 10 minutes. The pellets were washed twice with sterile BSM. The washed pellets were suspended in BSM supplemented with pyrene and incubated in shaking incubator (150 rpm) at 30°C for 3 days for adaptation. Fifteen ml of each of the preadapted six selected isolated bacterial isolates were used to inoculate 150 ml of BSM. The BSM was amended by four different concentrations of pyrene (500, 1000, 1500 and 2000) µM. Three replicates were used for each isolate inoculated in each BSM containing each compound for each concentration.

Growth was determined by measuring optical density (O.D) at 600 nm periodically at zero time (initial), 1, 2, 3, 4, 5, 6, 7, 14 and 21 days [32,33] using spectrophotometer LW-V-200 RS UV/VIS, Germany).Also protein was determined at 720 nm periodically at zero time (initial), 1, 2, 3, 4, 5, 6, 7, 14 and 21 days using spectrophotometer (LW-V- 200 RS UV/VIS, Germany). According to [34] to determine the amount of soluble protein in any culture of the polycyclic aromatic hydrocarbon-degrading bacteria, the following solutions must be prepared. Sol. (A) Cupper sulphate 1.0%, Sol. (B) Sodium potassium tartrate 2.0% and Sol. (C) Sodium carbonate 2.0% + sodium hydroxide 0.4%. Five ml of the reaction solution was added to 1 ml of the diluted sample of the culture filtrate. Distilled water was used as a blank. Then the mixture was allowed to stand at room temperature for 10 minutes. After that 0.5 ml of Folin reagent was added. The reaction tubes were incubated at room temperature for 20 minutes. The absorbance was determined at 720 nm. To determine the concentration of the protein in samples, a standard curve of Bovine serum albumin (BSA) was determined. Quantitative analyses by HPLC were determined at the end of incubation period (21 days). Bacterial count was determined at zero time (initial) and 21 days.

2.10 Analysis

2.10.1 High Performance Liquid Chromagraphy(HPLC)

The quantitative determination of various chloroaromatic compounds was performed using High-Performance liquid Chromatography (HPLC) in Micro Analytical Center, Cairo University-Egypt. The residual concentrations of the target compounds in all the degradation studies were quantified using reverse-phase high-performance liquid chromatography (HPLC) (young line YL9100 system, South Korea, 2014) with UV detector. HPLC analyses were carried out on a reverse phase C-18 column using mobile phase with an acetonitrile to water ratio of 80:20. A constant flow rate of 1.3 ml/min was maintained. The elution profile was monitored at 230 nm, injection volume was 20 µl. pyrene was extracted from the liquid medium before analysis [37].

2.10.2 Gas Chromatography/ Mass Spectrometry (GC-MS)

The qualitative and quantitative determination of various compounds was performed using Gas Chromatography / Mass Spectrometry (GC/MS) in The Regional Center for Food and Feed (R.C.F.F.), Giza, Egypt. The analysis was carried out using a GC (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000 Triple, Quad) equipped with Agilent HP5ms (5%-phenyl methyl polysiloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 µm film thickness) Santa Clara, California, USA. The carrier gas was helium with the linear velocity of 1 ml/min. The injector and detector temperatures were 200º C and 250º C, respectively. The volume injected 1µl of the sample. The MS operating parameters were as follows: ionization potential 70 eV, interface temperature 250º C, and acquisition mass range 50–600 [38]. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching withNIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature.

2.11 Identification of the Most Potent Pyrene Degrading Bacterial Isolate

2.11.1 Phenotypic Characterization of PAH Degrading Bacterial Isolate (Morphological)

The isolate MAM-P39 was characterized as Gram-negative, transparent, large, irregular colonies with olive green pigmentations, short rods/ cocci, flat when grown on L.B agar plates.Colony morphology of the most potent polycyclic aromatic hydrocarbon degrading isolate (MAM-P39) was assessed by monitoring their growth on L.B agar plates. Cellular morphology was examined by light microscope (Leica, Leitz, Labor Luxs, Germany).

2.11.2 DNA Extraction

The genomic DNA of the bacterial isolate was extracted by Wizard® Genomic DNA Purification Kit. According to the manufacturer's recommended procedure. PCR was performed in a thermal cycler (Biometra® cycler personal). The obtained purified DNA was re-suspended in 100 µl of TE buffer [39].

2.11.3 PCR Amplification of Bacterial 16S rDNA

PCR amplification of the 16S rDNA was performed using two universal oligonucleotide bacterial primers, 16S rDNA forward primer: 5-GAG TAA TGT CTG GGA AAC TGC CT-3, 16S rDNA reverse primer: 5-CCA GTT TCG AAT GCA GTT CCC AG-3. PCR reactions mixtures contained 1 µL of a10 µM working solution of each primer, 1 µL of genomic DNA, 12.5 µL of a Dream Taq Green DNA Polymerase (2x) (© 2012 Thermo Fisher Scientific Inc.) and 9.5 µL of Water, nuclease-free following the manufacturer's guidelines. PCR conditions used for the amplification of 16S rDNAwere: 95°C for 5 min., followed by 35 cycles of 95°C for 1.5 min, 59°C for 1 min and 72°C for1.5 min, with final 10 min extension at 72°C. Then DNA molecules were separated in 0.8% agarose-TBE according to [40] (90 mMTris-borate, pH 8.0, 2 mM (EDTA).

2.11.4 Sequencing

The purified PCR product was sequenced in one direction using the previously forward designed universal primer in an automated sequencer ABI Prism 3730XL (Applied Biosystems, Foster City, CA, USA) at Macrogen Inc., Korea. Sequences were analyzed by using Geneious Pro 8.1.1. The 16S rDNA gene sequences were compared to those of the Gene Bank and EMBL databases by advanced BLAST (Megablast) searches from the National Center for Biotechnology Information (NCBI).

2.11.5 Phylogenetic Analysis Tree Construction

The phylogenic relationship of the isolates was determined by comparing the sequencing data with the related 16S rDNA gene sequences in the Gen Bank database of the National Center for Biotechnology Information, via BLAST search. The phylogenetic tree was constructed by the Geneious Pro 8.1.9 program [41].

3 Results and Discussion

3.1 Growth and Degradation of Pyrene by the Most Potent Isolate.

3.1.1 Growth of Isolate (MAM-P1) on Different Concentrations of Pyrene.

The trend of growth of isolate MAM-P1 on different concentrations of Pyrene was indicated in Figure (2).The results revealed that the growth increased at the first day followed by a decrease at the second day followed by gradual increase reached the maximum value at the 14th day on (500 µM) of pyrene. Where the other three concentrations the growth increase from the beginning till the 14th day then decrease. The growth was concentration and incubation period dependent.The extracellular protein secretion profile revealed that there was a gradual increase in protein production from the first day till 14th days for (500, 1000, 1500 and 2000µM) concentrations of pyrene respectively then decrease as indicated by Figure (3). Also, secretion of protein by isolate MAM-P1 was concentration dependent.

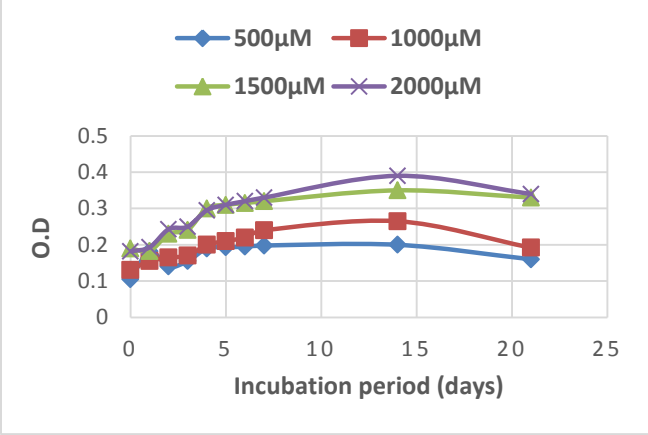

Figure 2. Growth of isolate MAM-P1 on different concentrations of pyrene.

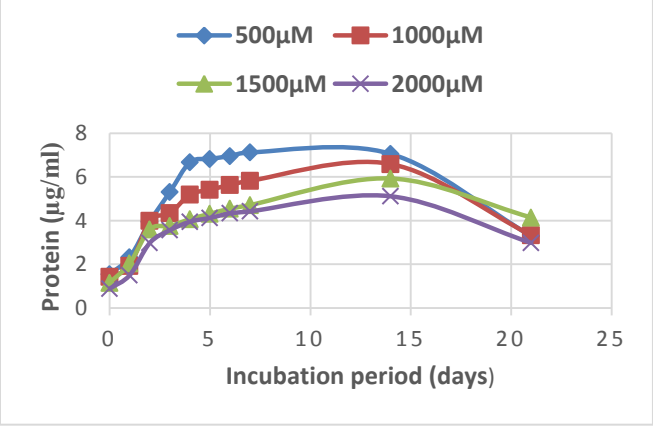

Figure 3. Extracellular protein of isolate MAM-P1 on different concentrations of pyrene.

3.1.2 Growth of Isolate (MAM-P8) on Different Concentrations of Pyrene

In case of isolate MAM-P8 the growth was decreased from the beginning till the first day then increase till 14th day at concentrations (1000µM) pyrene and at (1500 and 2000µM) concentrations decrease till second day then increase till 14th. But at 500 µM concentration growth gradually increase reached maximum at 14th day as in Figure (4). Extracellular protein production by MAM-P8 was shown in Figure (5). The results indicated that there was an increase in protein secretion at the beginning and continue till the 14th day then, began to decrease till the end of the incubation period.

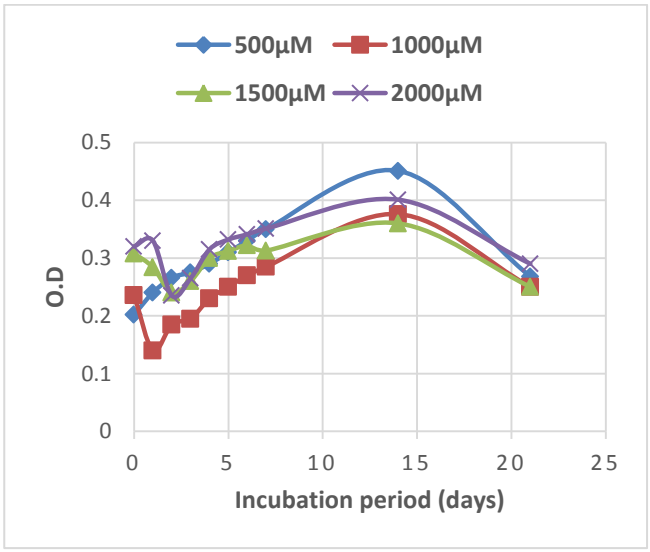

Figure 4. Growth of isolate MAM-P8 on different concentrations of pyrene.

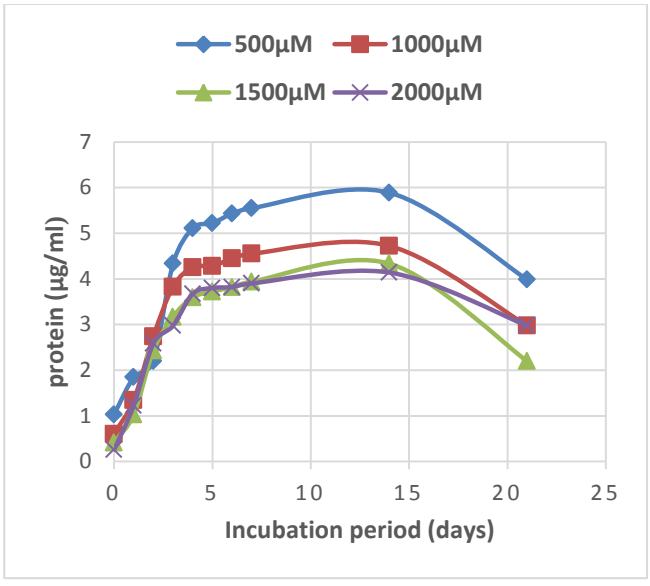

Figure 5. Extracellular protein of isolate MAM-P8 on different concentrations of pyrene.

3.1.3 Growth of Isolate (MAM-P13) on Different Concentrations of Pyrene

In case of isolate MAM-P13, the growth was increased from the beginning till 14th day at the four concentrations ofpyren as shown in Figure (6). However, its extracellular protein for the four concentrations of pyrene showed an increase till the 14th day of incubation and then began to decrease as in Figure (7). Secretion of extracellular protein by isolate MAM-P13 was concentration dependent.

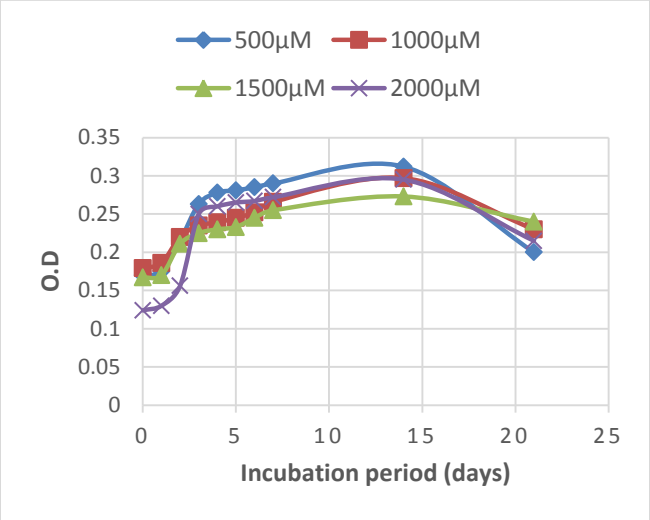

Figure 6. Growth of isolate MAM-P13 on different concentrations of pyrene.

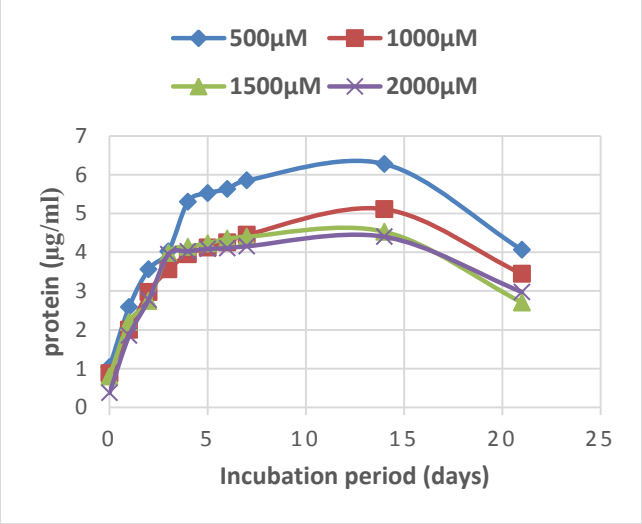

Figure 7. Extracellular protein of isolate MAM-P13 on different concentrations of pyrene.

3.1.4 Growth of Isolate (MAM-P39) on Different Concentrations of Pyrene

In case of isolate MAM-P39, the growth was increased gradually from the beginning for all concentrations of pyrene recorded maximum value at the 14th day of incubation period as in Figure (8). Extracellular protein production by MAM-P39 was shown in Figure (9). The results indicated that there was an increase in protein secretion at the beginningand continue till the 14th day, then began to decrease till the end of the incubation period.

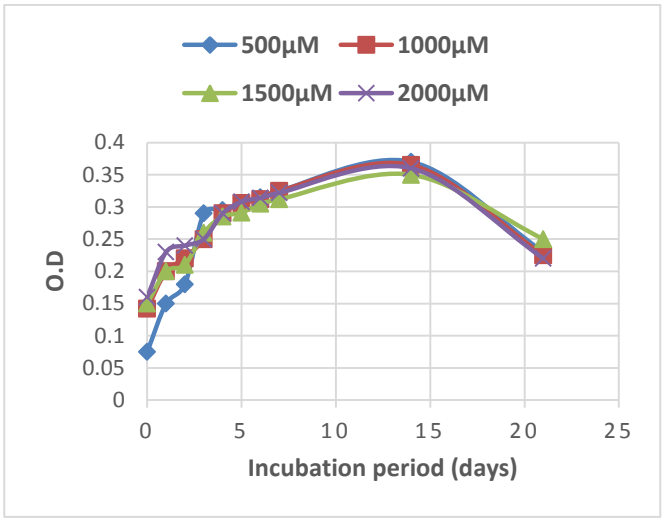

Figure 8. Growth of isolate MAM-P39 on different concentrations of pyrene.

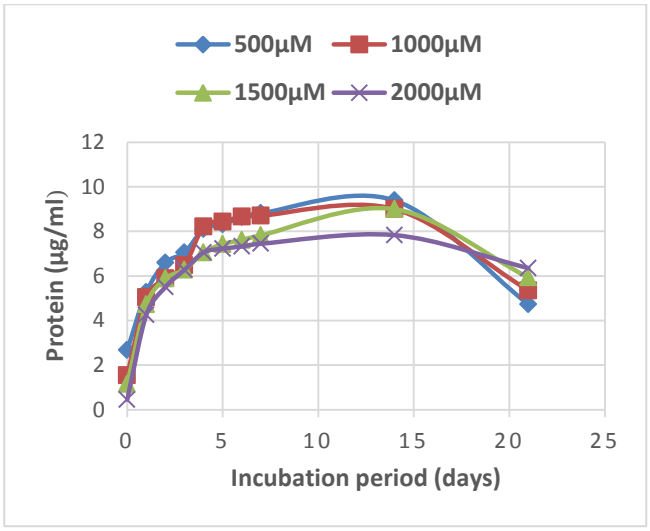

Figure 9. Extracellular protein of isolate MAM-P39 on different concentrations of pyrene

3.1.5 Growth of Isolate (MAM-P43) on Different Concentrations of Pyrene

The growth profile of the isolate MAM-P43 on different concentrations of pyrene was indicated in Figure (10). Results revealed that the growth at (500, 1000 and 1500µM) showed an increase till the 14th day and began to decrease, where at concentration 2000µM the growth was fluctuated increase followed by decrease till the 4th day then increase gradually and reached the maximum at 14th day then decrease. Growth was concentration and time-dependent. The extracellular protein produced by MAM-

P43 indicated that the high protein production was recorded on the 14th day for (500, 1000, 1500 and 2000µM) concentrations then decreased as shown in Figure (11).

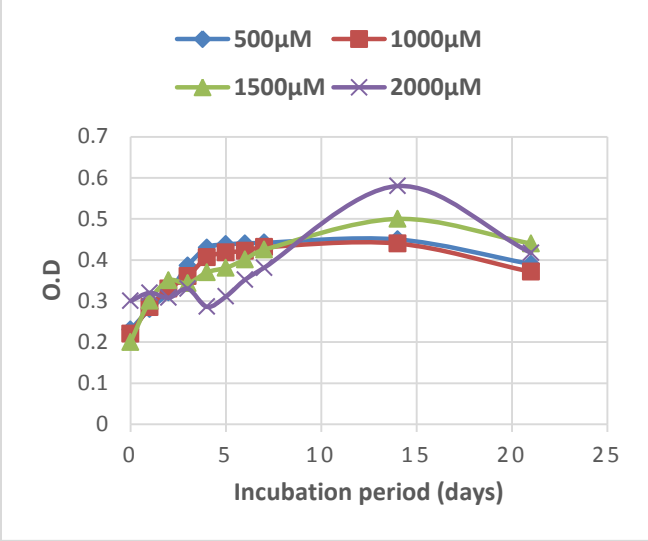

Figure 10. Growth of isolate MAM-P43 on different concentrations of pyrene.

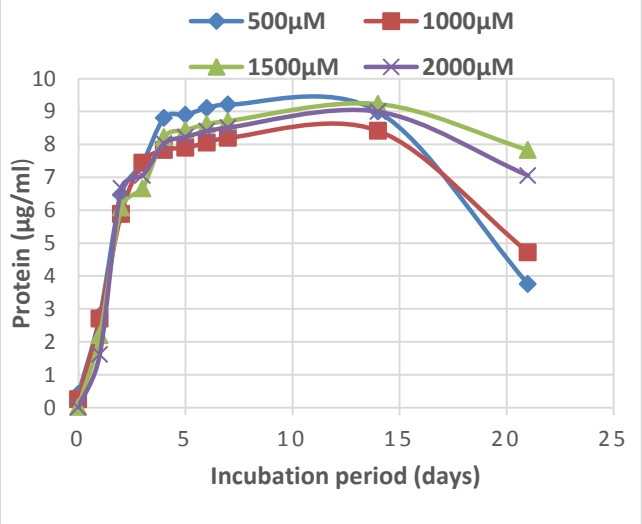

Figure 11. Extracellular protein of isolate MAM-P43 on different concentrations of pyrene.

3.1.6 Growth of Isolate (MAM-P52) on Different Concentration of Pyrene

In case of isolate MAM-P52, the growth was decreased from the beginning for all the concentrations of pyrene and after 1stday the growth gradually increases till the 14th day then decrease as indicated in Figure (12). Extracellular protein productions by MAM-P52 was shown in Figure (13), the results indicated that, there was an increase in protein secretion at the beginning and continue till the 14th day then, began to decrease till the end of the incubation period for all the concentrations.

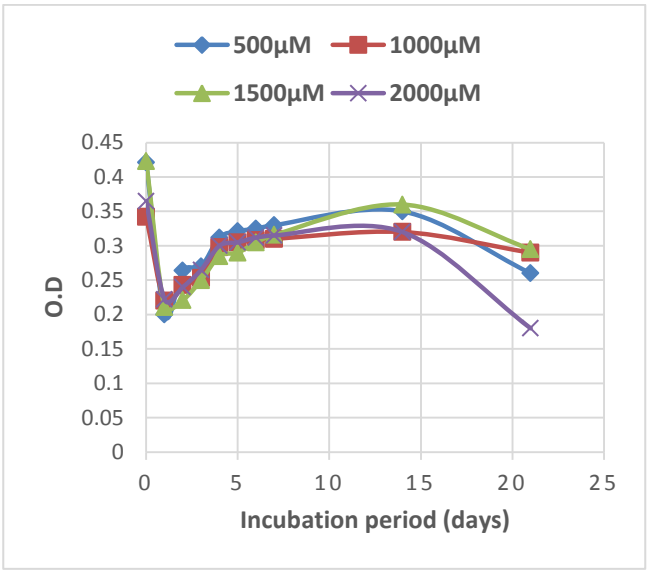

Figure 12. Growth of isolate MAM-P52 on different concentrations of pyrene.

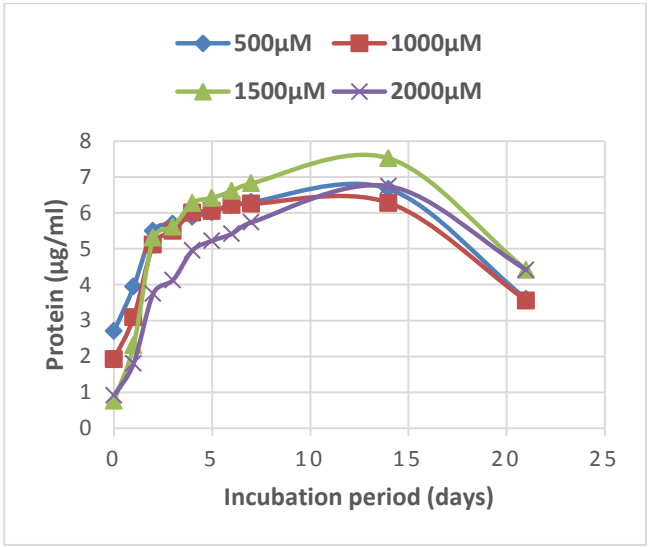

Figure 13. Extracellular protein of isolate MAM-P52 on different concentrations of pyrene.

Pyrene has often been used as a model compound to review biodegradation of HMW PAHs since it is structurally similar to sundry carcinogenic PAHs [42]. Pyrene is often detected in environmental samples and crude oil resulting from imperfect combustion and used as an indication for PAH-contaminated waste monitoring [25]. It is a four-ring (PAH) that has low biodegradability, high persistence in the environment and has been outlined as a priority pollutant by the United States Environmental Protection Agency (US EPA).

Bacteria are reported as the most active agents in petroleum pollutant degradation [28].Enrichment is a method used to isolate microorganisms from their natural environment. It includes inoculating natural sources of bacteria into selective media and then growing under physiological

conditions optimal for the required organisms [43].

Count of different bacterial isolates on different concentrations of pyrene had been shown in Table (1). The initial count was ranging from 7.5 $x10^3$ to 5.5 $x10^5$ CFU/ml and the count after 21 days of incubation period was ranging from 8 $x10^5$ to 25 $x10^6$ CFU/ml Isolates MAM-P1, MAM-P8, MAM-P13, MAM-P39, MAM-P43 and MAM-P52 showed good growth on all the four concentrations of pyrene after 21 days incubation.

Table 1. Count of the selected isolates on different concentrations of pyrene after 21 days incubation period.

Isolate code	Initial count		after 21 days							
			500 µM		1000 µM		1500 µM		2000 µM	
	count	logN	count	logN	count	logN	count	logN	count	logN
MAM-P1	$4.5x10^4$	4.6	$8.6x10^6$	6.9	$3.2x10^6$	6.5	$2.5x10^6$	6.4	$1x10^6$	6
MAM-P8	$2.5x10^5$	5.4	$45x10^6$	7.6	$3x10^7$	7.4	$18x10^6$	7.2	$1x10^6$	6
MAM-P13	$6.5x10^4$	4.8	$35x10^6$	7.5	$18x10^6$	7.2	$50x10^5$	6.7	$12x10^5$	6.1
MAM-P39	$5.5x10^5$	5.7	$38x10^7$	8.5	$35x10^7$	8.5	$33x10^6$	7.5	$25x10^6$	7.3
MAM-P43	$4x10^4$	4.6	$18x10^6$	7.2	$50x10^5$	6.7	$23x10^5$	6.4	$9x10^5$	6
MAM-P52	$7.5x10^3$	3.9	$66x10^5$	6.8	$61x10^5$	6.9	$15x10^5$	6.2	$8x10^5$	6

By comparing the growth and the extracellular protein curves of the six isolates showed that the ability of isolates MAM-P1, MAM-P8, MAM-P13 and MAM-P39 to grow on high concentrations of pyrene compound more than MAM-P43 and MAM-P52 isolates.Thus these isolates were selected to determine the degradation percentage of pyrene in the mineral base medium by HPLC. The degradation rate of pyrene was evaluated by chromatographic analysis after 21 days of incubation as shown in Table (2), the isolates MAM-P1, MAM-P8, MAM-P13 and MAM-P39 could degrade 70.09%, 87.86%, 80.76%, and 90.5% respectively, when grow on 500µM of pyrene and degrade 47.66%, 51.45%, 23.05% and 66.03%respectively when growing on 2000µM of pyrene.

These results showed the ability of isolates MAM-P1, MAM-P8 and MAM-P39 in the removal of high concentrations of pyrene compound reach to 2000µM is more than MAM-P13 isolate. The most efficient pyrene degrader was isolate MAM-P39.

Table 2. Degradation percentage of pyrene after 21 days by HPLC.

Isolate code	Degradation %	
	500µM	2000µM
MAM-P1	70.09%	47.66%
MAM-P8	87.86%	51.45%
MAM-P13	80.76%	23.05%
MAM-P39	90.5%	66.03%

An isolate of *Cupriavidus* (strain MTS-7) was identified from a long-term PAHs and heavy metals combined contaminated soil with the potential to biodegrade both LMW and HMW PAHs, This strain completely degraded the model 3(150 mg L^{-1}Phe), 4(150 mg L^{-1}Pyr) and 5 (50 mg L^{-1}BaP) ring PAHs in 4, 20 and 30 days, respectively. It might mineralize 90-100% of PAHs (200 mg L^{-1} of Phe and Pyr) within 15 days across pH, which range from 5 to 8 [44].

Lately, many pyrene degraders from the genus *Pseudomonas* was successively isolated from various PAH-polluted sources. Since the variety of the growth environments and the experimental culture conditions, these strains showed a various tolerance, degradation capability and metabolic mechanism for PAHs [45, 46, 47]. Pyrene-degrading bacterial strain *Pseudomonas* sp. JPN2 was isolated from crude oil in Dagang Oilfield, China the degrading percent of the strain JPN2 to pyrene was increased with the expansion of culture time and attain a maximum of 82.88% after 25-day culture [48].

The bacterial strains *Mycobacterium* sp., *Corynebacterium* sp., *Nocardia* sp., *Pseudomonas* sp. *Rhodococcus* sp. and *Micrococcus* sp. were isolated from the soil of the landfills in Shiraz which were potentially competent to degrade pyrene hydrocarbon. The biodegradation values of pyrene after 10 days of incubation, evaluated by high performance liquid chromatography (HPLC) were 89.1%, 79.4%, 75.3%, 68.2%, 62.3% and 56.8% for each strain respectively. Therefore, these bacteria could be utilized to clean the

soilswhich are polluted with pyrene [49].

Pseudomonas putida PL2 isolated from hydrocarbon polluted soil reported as a novel bacterium, which could degrade pyrene and hold great promise for use in PAHs bioremediation in the soil [50].The potential of isolated bacterial strains from petrochemical wastewater in Iran to degrade the polyaromatic hydrocarbon by using batch aqueous system Phenanthrene revealed the highest degradation up to 98%, while 48% and 78% degradation percentage were obtained for anthracene and pyrene, respectively [51].

The degradation percentage of pyrene was 97.7% with an initial concentration of 500 µg g^{-1} in liquid culture within 5 days of incubation without adding any co-metabolism substances or surfactants [52]. *Bacillus cereus* Py5 and *Bacillus megaterium*Py6 were isolated from the consortium and can degrade 65.8% and 33.7% of pyrene with an initial concentration of 50 mg/l within three weeks, respectively [53].The enriched *Escherichia coli* DH5a cells containing the plasmids of YL were demonstrated to degrade 85.7% of the original pyrene concentration at the 21st day [54].

HPLC analysis demonstrated that the degradation rate of pyrene 5 mg/L by the endophytic bacterial strain 12J1 was 83.8% under 28^{o} C for 7 days [55].Biodegradation of pyrene by *Mycobacterium frederiksbergense* was examined in two phases partitioning bioreactor (TPPB). The TPPB achieved complete biodegradation of pyrene, and through the active degradation phase utilization rates of 270, 230, 139, 82 mg/L/d. for initial pyrene loading concentrations of 1000, 600, 400 and 200 mg/L, respectively [56].

3.2 Identification of the Most Potent Pyrene Degrading Bacterial Isolation

From the previous results, we select isolate MAM-P39 isolate that represents the best pyrene degrading bacteria. DNA sequencing of isolate MAM-P39 showed in Figure (14).

The genomic DNA of bacterial strain was extracted by Wizard® Genomic DNA Purification Kit. According to the manufacturer's recommended procedure. PCR was performed in a thermal cycler (Biometra® cycler personal). PCR amplification of the 16S rDNA gene was performed using two universal oligonucleotide bacterial primers, 16S rDNA forward primer: 5-GAG TAA TGT CTG GGA AAC TGC CT-3, 16S rDNA reverse primer: 5-CCA GTT TCG AAT GCA GTT CCC AG-3. PCR reactions mixtures contained 1 µL of a10 µM working solution of each primer, 1 µL of genomic DNA, 12.5 µL of a Dream Taq Green DNA Polymerase (2x) (© 2012 Thermo Fisher Scientific Inc.) and 9.5 µL of Water, nuclease-free following the manufacturer's guidelines. PCR conditions used for the amplification of 16S rDNA gene were: 95°C for 5 min., followed by 35 cycles of 95°C for 1.5 min, 59°C for 1 min and 72°C for 1.5 min, with final 10 min extension at 72°C. Then DNA molecules were separated in 0.8% agarose-TBE according to (90 mMTris-borate, pH 8.0, 2 mM EDTA).

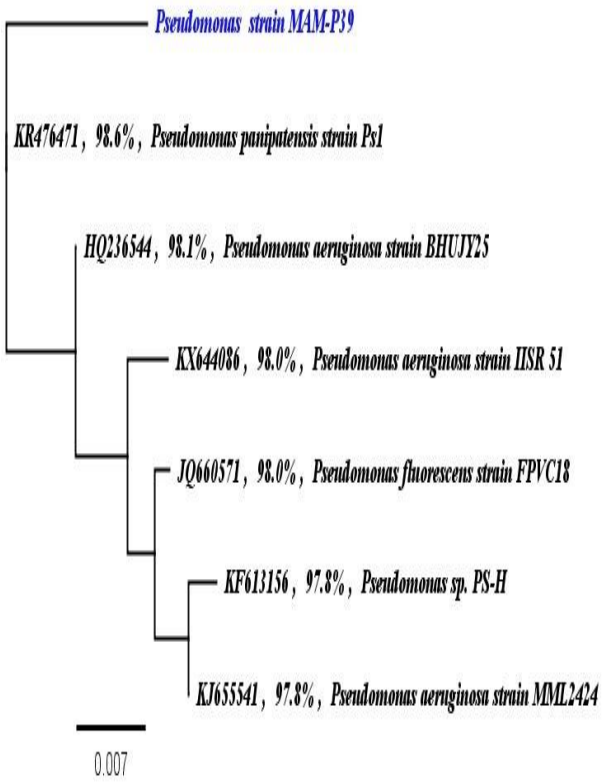

Figure14. Rooted phylogenetic tree showing the relationship of isolated bacterial strain, the 16S rDNA gene sequence aligned inClustalW [57].

The purified PCR product was sequenced in one direction using the previously forward designed universal primer in an automated sequencer ABI prism 3730XL (applied Biosystems, Foster City, CA, USA) at Macrogen Inc., Korea. Sequences were analyzed by using Geneious Pro 8.1.1. The 16S rDNA gene sequences were compared to those of the GeneBank and EMBL databases by advanced BLAST (Megablast) searches from the National Center for Biotechnology Information (NCBI). The phylogenic relationship of the isolates was determined by comparing the sequencing data with the related 16S rDNA gene sequences in the GenBank database of the National Center for Biotechnology Information, via BLAST search. The phylogenetic tree was constructed by the Geneious Pro 8.1.9 program.

DNA of isolate MAM-P39was amplified using universal primers. The purified PCR product of1279 bp was sequenced in one direction using forward universal primer. Based on the alignment of 16S rDNA gene sequences from the GeneBank database, the 16S rDNA gene sequence of the isolate showed the highest identity (98.6%) in the BLAST search to *Pseudomonas panipatensis* strain Ps1 (Figure 14).

Pseudomonas strain MAM-P39 was subjected to analysis of 16S rDNA gene sequences to confirm their identification. The 16S rDNA genes of *Pseudomonas* strain MAM-P39 were highly conserved and showed high similarity to nucleotides sequences that were aligned. Based on the concept of similarity or nucleotides difference between the query nucleotides and those compared, it is recommended when the sequences similarity is more than 90% or the nucleotides different between the query and those compared 1–1.5% (14–22 bp), the query should be categorized as the same species identified by the 16S rDNAgene [58], however most taxonomistsaccept a percent identity score of 97% and 99% to classify a microorganism to genus and species, respectively [59]. A phylogenetic tree of the 16S rDNA gene was performed using geneious Tree Builder option with genetic distance model: Tamura-Nei, tree builder methods: Neighbor-joining [60].

Following the phylogenetic analysis, similarity calculations indicated that the closest relatives of isolate MAM-P39 were *Pseudomonas panipatensis* KR476471 (98.6 %), *Pseudomonas aeruginosa* HQ236544 (98.1%), *Pseudomonas aeruginosa* KX644086 (98.0 %), *Pseudomonas fluorescens* JQ660571 (98.0 %), *Pseudomonas* SP. KF613156 (97.8%) and *Pseudomonas aeruginosa* KJ655541 (97.8 %).

The Phylogenetic tree showed that the *Pseudomonas* strain MAM-P39 (601bp), showed high similarity to *Pseudomonas panipatensis* strain Ps1 wich is a Gram negative, motile, rod-shaped, non-sporulating, aerobic bacterial strain that was isolated from oil-contaminated soil [61]Therefore, isolate MAM-P39 was identified as *Pseudomonas panipatensis* MAM-P39 and the nucleotides deposited in the National Center for Biotechnology Information (NCBI) gene bank sequences databases under accession number (MF150314).

3.3 Proposed Pathway of Pyrene Degradation by Pseudomonas Panipatensis MAM-P39

Large number of studies investigated the pathway of the Gram-negative bacteria especially *Pseudomonas* spp.

Pseudomonas panipatensis MAM-P39 was grown in large quantity on 1000µM pyrene for GC-MS analysis after 24 hours incubation. This incubation period had been selected to determine different metabolites formed in degradation of pyrene.

The results of the degradation of pyrene GC/MS as indicatedin Figure (15) and Table (3) revealed that *Pseudomonas panipatensis* MAM-P39 produced 18 intermediate compounds. Proposed pathway of pyrene degradation by *Pseudomonas panipatensis* MAM-P39 indicated in Figure (16).

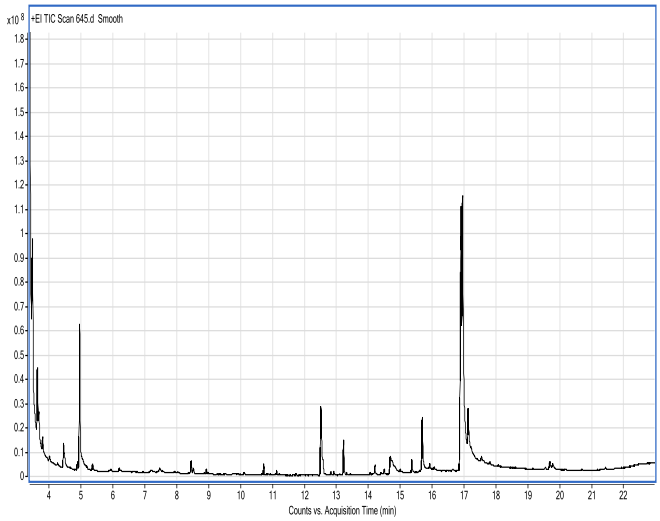

Figure15. Gas chromatography analysis of pyrene degradation by *Pseudomonas panipatensis* MAM-P39.

Table 3. Intermediates determined by GC-MS analysis resulted from biodegradation of pyrene by *pseudomonas panipatensis* MAM-P39 after 24 hours incubation

R.T	Identification	Formula
3.46	3-Methylpenta-1,4-diene-3-ol	$C_6H_{10}O$
3.67	3-Methyl-2-butenoic acid, 3-methylbut-2-enyl ester	$C_{10}H_{16}O_2$
3.778	3-Hexanone	$C_6H_{12}O$
4.01	3-Methyl-2-butenoic acid, 2-pentyl ester	$C_{10}H_{18}O_2$
4.44	Benzene, (3,3-dimethyl-4-pentenyl)-	$C_{13}H_{18}$
4.8	2,6-Dimethyl-8-oxoocta-2,6-dienoic acid, methyl ester	$C_{11}H_{16}O_3$
4.98	Isopropyl (2e)-2-butenoate	$C_7H_{12}O_2$
6.2	2-Xylene	C_8H_{10}
12.49	Ethanone, 1-(3-butyl-2-hydroxy-5-methylphenyl)-	$C_{13}H_{18}O_2$
13.215	Isobutyric anhydride	$C_8H_{14}O_3$
14.6	Diphenylethyne	$C_{14}H_{10}$
15.04	(1-Phenylvinyl)benzene	$C_{14}H_{12}$
15.3	2,6-Di-tert-butyl-para-benzoquinone	$C_{14}H_{20}O_2$
15.67	Octadecanoic acid	$C_{18}H_{36}O_2$
16.9	Farnesol	$C_{15}H_{26}O$
16.94	Pyrene	$C_{16}H_{10}$

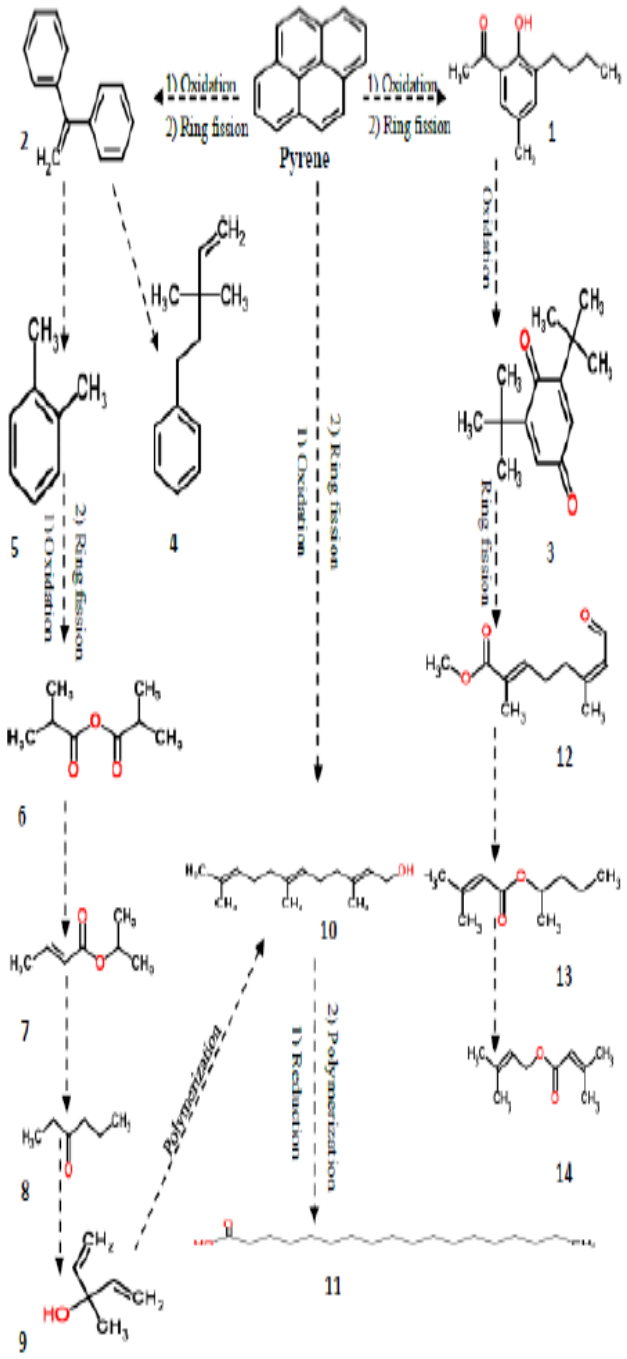

Figure 16. Proposed pathway of pyrene degradation by *Pseudomonas panipatensis* MAM-39 **compound designations**: **1**, Ethanone, 1-(3-butyl-2-hydroxy-5-methylphenyl)**;2**, (1-phenylvinyl) benzene**; 3,** 2,6-di-tert-Butyl-para benzoquinoone**; 4,** Benzene, (3,3-dimethyl-4-pentenyl)**;5**,2-xylene**; 6**, Isobutyric anhydride**;7**, Isopropyl (2e)-2-butenoate**;8**, 3-Hexanone**; 9,** 3-Methylpenta-1,4-diene-3-ol; **10**, Farnesol**; 11,** Octadecanoic acid**;12**,2,6-Dimethyl-8-oxoocta-2,6-dienoic acid, methyl ester**; 13**, 3-Methyl-2-butenoic acid, 2-pentyl ester**; 14**, 3-Methyl-2-butenoic acid, 3-methylbut-2-enyl ester.

Petroleum hydrocarbon pollutants degradation by bacterial species has been well documented and metabolic pathways have been illustrated [26, 28].

Biodegradation pathways encompass the breakdown of organic compounds, being ring fission by intracellular oxidation and hydroxylation the typical initial steps. More specifically, bacteria from the genus *Pseudomonas*, which is present in a large number of diverse natural and contaminated environments, have been the subject of a great scientific concern due to both their high degree of physiological and genetic adaptability and their efficient capacity to aerobically degrade a broad range of aromatic compounds [46].

The microorganisms cleave the benzene ring in different ways by appropriate enzymes [62]. Ortho- or meta-cleavage pathways leading to the formation of central intermediates such as protocatechuates and catechols, which are furthermore, converted to tricarboxylic acid (TCA) cycle intermediates [63].

Usually, aerobic catabolism of PAHs includes a broad variety of peripheral degradation pathways that transform substrates into a small number of common intermediates that can be then processed by a new central pathway to the tricarboxylic acid (TCA) cycle intermediates [42].

Microbial biodegradation of petroleum hydrocarbon pollutants utilizes the enzyme catalytic activities of microorganisms to enhance the rate of pollutant degradation [16].

The initial step of upper pathways in aerobic conditions is an oxidation catalyzed by monooxygenases (hydroxylases) or by dioxygenases [64]. The monooxygenases catalyze the cleavage of the oxygen-oxygen bond of O_2, inserting one oxygen atom into the aromatic ring while the other is reduced to H_2O [65].

The upper pathways begin with an oxidation and finish with the formation of central intermediates, which can be catechols or non-catecholic compounds [65]. The former have cis-dihydrodiols groups [45] and the latter are hydroxy-substituted aromatic carboxylic acids [66] resulting from reactions catalyzed by monooxygenases and dioxygenases. The central intermediates that are non-catechols are hydroxy-substituted aromatic carboxylic acids.

The lower pathways refer to the dearomatization of central intermediates and ring cleavage to tricarboxylic acids [65].

Aerobic and anaerobic pathways of microbial degradation petroleum hydrocarbon pollutants include reactions viz. oxidation, reduction, hydroxylation, and dehydrogenation [26, 63].

4 Conclusions

Pseudomonas panipatensis MAM-P39 with accession number MF150314, isolated from soil contaminated with crude petroleum oil sludge was able to degrade 90.5% and 66.03% of 500 and 2000 μM pyrene and can be used as a candidate in degradation of different PAHs.

References

[1] Costa, A.S.; Romao, L.P.; Araujo, B.R.; Lucas, S.C.; Maciel, S.T.; Wisniewski A.J. and Alexandre, M.R.: Environmental strategies to remove volatile aromatic fractions (BTEX) from petroleum industry wastewater using biomass. Bioresour. Technol.,**105**, 31-39, 2012.

[2] Chandra, S.; Sharma, R.; Singh, K. and Sharma, A.:Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. Ann. Microbiol.,**63**, 417-431, 2013.

[3] Haritash, A.K. and Kaushik, C.P.: Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. Journal of Hazardous Materials., **169**, 1–15,2009.

[4] Igwo-Ezikpe, M.N.; Gbenle, O.G.; Ilori, M.O.; Okpuzor, J. and Osuntoki, A. A.: High molecular weight polycyclic aromatic hydrocarbons biodegradation bybacteria isolated from contaminated soils in Nigeria. Res. J. Environ. Sci.,**4**,127-137, 2010.

[5] Liu, B.; Xue, Z.; Zhu, X. and Jia, C.: Long-term trends (1990-2014), health risks, and sources of atmospheric polycyclic aromatic hydrocarbons (PAHs) in the U.S. Environ. Pollut., **220**, 1171-1179, 2017.

[6] Desforges, J.W.; Sonne, C.; Levin, M.; Siebert, U.; Guise, S.D. and Dietz, R.: Immunotoxic effects of environmental pollutants in marine mammals. Environ. Int. **86**, 126-139, 2016.

[7] Jamin, E.L.; Riu, A., Douki, T., Debrauwer, L.; Cravedi, J.P.; Zalko, D. and Audebert, M.: Combined genotoxic effects of a polycyclic aromatic hydrocarbon B(a)P and an heterocyclic amine (PhIP) inrelation to colorectal carcinogenesis. PLoSONE., **3**, 58591- 58599,2013.

[8] Esedafe, W. K.; Fagade, O. E.; Umaru, F. F. and Akinwotu, O.: Bacterial degradation of the polycyclic aromatic hydrocarbon (PAH) -fraction of refinery effluent. Int.J. Environ. Biorem.Biodegrad., **3**, 23-27, 2015.

[9] Nejati, H. and Saien, J.: Enhanced photocatalytic degradation of pollutants in petroleum refinery wastewater under mild conditions.J. Hazard.Mater., **148**, 491-495,2007.

[10]Song, X.; Xu, Y.; Li, G.; Zhang, Y.; Huang, T. and Hu, Z.: Isolation, characterization of *Rhodococcus* sp. P14 capable of degrading high molecular weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. Mar. Pollut. Bull.,**62**, 2122–2128, 2011.

[11]Wei, Y. L.; Bao, L. J.; Wu, C. C.; He, Z. C. and Zeng, E. Y. :Association of soil polycyclic aromatic hydrocarbon levels andanthropogenic impacts in a rapidly urbanizing region: Spatialdistribution, soil-air exchange and ecological risk. Sci. Total Environ., **473**, 676–684,2014.

[12] Varjani, S.J.; Rana, D.P.; Jain, A.K.; Bateja, S. and Upasani, V.N.: Synergistic ex-situ biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat, India. Int. Biodeterior. Biodegrad., **103**, 116-124, 2015.

[13] Sajna, K.V.; Sukumaran, R.K.; Gottumukkala, L.D. and Pandey, A.:Crude oil biodegradation aided by biosurfactants from *Pseudozyma* sp. NII 08165 or its culture broth. Bioresour.Technol.,**191**, 133-139,2015.

[14] Varjani, S.J. and Upasani, V.N.: Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514.Bioresour.Technol.,**222**, 195-201, 2016.

[15] Hassanshahian, M.; Emtiazi, G.; Caruso, G. and Cappello, S.:Bioremediation (bioaugmentation/ biostimulation) trials of oil polluted seawater: a mesocosm simulation study. Mar. Environ. Res.,**95**, 28–38, 2014.

[16] Varjani, S.J.: Microbial degradation of petroleum hydrocarbons.Bioresour.Technol., **223**, 277-286, 2017.

[17] Varjani, S.J. and Srivastava, V.K.: Green technology and sustainable development of environment. Renew. Res. J.,**3**, 244-249, 2015.

[18] Fuentes, S.; Me´ndez, V.; Aguila, P. and Seeger, M.:Bioremediation of petroleum hydrocarbons: catabolic genes, microbial communities and applications. Appl. Microbiol. Biotechnol., **9**, 4781–4794,2014.

[19] Kumar, S.; Upadhayay, S.K.; Kumari, B.; Tiwari, S.; Singh, S. and Singh, P.: In vitro degradation of fluoranthene by bacteria isolated from petroleum sludge. Bioresour.Technol., **102**, 3709–3715, 2011.

[20] Kafilzadeh, F.; Sahragard, P.; Jamali, H. and Tahery, Y.: Isolation and identification of hydrocarbons degrading bacteria in soil around Shiraz Refinery. Afr. J. Microbiol. Res.,**4**, 3084-3089,2011.

[21] Singh, C. and Lin, J.: Bioagumentation efficiency of diesel degradation by *Bacillus pumilus* JLB and *Acinatobacter calcoacetics* LT1 in contanated soils. Afr. J. Biotechnol.,**9**, 6881–6888, 2010.

[22] Milic, J.S.; Beskoski, V.P.; Ilic, M.V.; Ali, S.A.M.; Gojgic-Cvijovic,G.D. and Vrvic, M.M.:Bioremediation of soil heavilycontaminated with crude oil and its products: Composition of themicrobial consortium. J. Serbian Chem. Soc.,**74**, 455-460, 2009.

[23] Saadoun, I.: Isolation and characterisation of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel. J. Basic Microbiol., **42**, 420–428, 2002.

[24] Ling, J.; Zhang, G.; Sun, H.; Fan, Y.; Ju, J. and Zhang, C.:Isolation and characterization of a novel pyrene-degrading *Bacillus vallismortis* strain JY3A. Sci. Total Environ.,**409**, 1994–2000, 2011.

[25] Teh, Z.C.; Hadibarata, T. and Teh.:Enhanced degradation of pyrene and metabolite identification by *Pleurotus eryngii* F032. Water, Air, Soil Pollut., **225**, 1-8, 2014.

[26] Wilkes, H.; Buckel, W.; Golding, B.T. and Rabus R.: Metabolism of hydrocarbons in n-Alkane utilizing anaerobic

bacteria. J. Mol. Microbiol. Biotechnol., **26**, 138-151, 2016.

[27] Bacosa, H.P. and Inoue, C.:Polycyclic aromatic hydrocarbons (PAHs) biodegradation potential and diversity of microbial consortia enriched from tsunami sediments in Miyagi, Japan. J. Hazard. Mater., **283**, 689–697, 2014.

[28] Meckenstock, R.U.; Boll, M.; Mouttaki, H.; Koelschbach J.S.; Tarouco, P.C.; Weyrauch, P.; Dong, X. and Himmelberg, A.M.: Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. J. Mol. Microbiol. Biotechnol., **26**, 92–118, 2016.

[29] Ogawa, N. and Miyashita, K.: Recombination of a 3-chlorobenzoate catabolic plasmid from *Alcaligeneseutrophus*NH9 mediated by direct repeat elements. Appl. Environ.Microbiol., **61**, 3788-3795, **(**1995.

[30]Martin, P.A.W.;Lohr, J.R. and Dean, D.H.: Transformation *of Bacillus thuringiensis* protoplasts by plasmid deoxyribonucleic acid. J. Bacteriol., **145**,980-983,1981.

[31] Abo-State, M.A.M.; Saleh, Y.E.; Gomaa, O. and Khalil, O.A.**:** Isolation and identification of chloroaromatic degrading bacterial strain isolated from indigenous microbial communities of petroleum contaminated soils. World Appl. Sci. J.,**21**, 1341-1355,2013.

[32] Abo-State, M.A.M.; Abdullah, N. and Nader, B.:Biodegradation of high molecular weight polycyclic aromatic hydrocarbons (HMW- PAHs) mixtures by bacteria isolated from soils polluted with petroleum oil. 1st International Conferences on Biological and Environmental Science and Applications. March 23-25, Luxor, Egypt, 2017.

[33] Saleh, Y.E.; Abo-State, M.A.M.; Aziz, N. and Partila, A.M.:Isolation of polycyclic aromatic hydrocarbon degrading bacterial strains isolated from indigenous microbial communities of petroleum contaminated soils. World Appl. Sci. J.,**23**, 554-564, 2013.

[34] Nnamchi, C.I.;Obeta, J.A.N.;EzeoguL.I.:Isolation and characterization of some polycyclic aromatic hydrocarbon degrading bacteria from Nsukka soils in Nigeria. Int. J. Environ. Sci. Tech.,**3(2)**, 181-190, 2006.

[35] Khazi, M.;Aravindan, R. and Viruthagiri, T. (2010): Recent advances in the biodegradation of phenol. Asian J. Exp. Biol. Sci.,**1**, 219–234.

[36] Lowry, H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J.:Protein measurement with the folin phenol reagent.J.Biol. Chem., **193**, 265-275, 1951.

[37] Shafiee, P.;Shojaosadati, S.A. and Charkhabi, A.H.:Biodegradation of polycyclic aromatic hydrocarbons by aerobicmixed bacterial culture isolated from hydrocarbon polluted soils.I.J.C.C.E.,**25**, 73-78,2006.

[38] Oberoi, A.S.; Philip, L. and Bhallamudi, S.M.:Biodegradationof Various Aromatic Compounds by Enriched Bacterial Cultures:Part A–Monocyclic and Polycyclic AromaticHydrocarbons. Appl. Biochemist. Biotechnol., **176**, 1870-1888, 2015.

[39] Sambrook, J. and Russel, D.W.: Molecular cloning: A Laboratory Manual, 3rd Ed. Cold Spring Harbor LaboratoryPress, Cold Spring Harbor, NT, USA, 2001.

[40] Sharp, P. a.; Sugden, B. and Sambrook, J.: Detection of two restriction endonuclease activities in *Haemophilusparainfluenzae* using analytical agarose--ethidium bromide electrophoresis. Biochemistry., **12**, 3055–63, 1973.

[41] Lee, M.Y.; Jeon, H.S.; Lee, S.H. and An, J.: The mitochondrial genome of the long-billed plover, *Charadriusplacidus* (Charadriiformes: Charadriidae). Mitochondrial DNA Part B.,**2**, 122-123, 2017.

[42] Peng, R.H.; Xiong, A.S.; Xue, Y.; Fu, X.Y.; Gao, F.; Zhao, W.; Tian, Y.S. and Yao, Q.H.: Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol.Rev., **32**, 927–955, 2009.

[43] Kaplan, C.W. and Kitts, C.L.: Bacterial succession in a petroleum land treatment unit. Appl. Environ. Microbiol.,**70**, 1777–1786,2004.

[44] Kuppusamy, S.; Thavamani, P.; Megharaj, M.; Lee, Y.B. and Naidu, R.: Polyaromatic hydrocarbon (PAH) degradationpotential of a new acid tolerant, diazotrophic P-solubilizing andheavy metal resistant bacterium *Cupriavidus*sp. MTS-7 isolatedfrom long-term mixed contaminated soil. Chemosphere., **162**, 31-39, 2016.

[45] Ma, J.;Xu, L. and Jia, L.: Characterization of pyrene degradation by *Pseudomonas sp.* strain Jpyr-1 isolated from active sewage sludge.Bioresour.Technol.,**140**, 15-21, 2013.

[46] Moscoso, F.; Deive, F.; Longo, M. and Sanromán, M.:Insights into polyaromatic hydrocarbon biodegradation by *Pseudomonas stutzeri*CECT 930: operation at bioreactor scale and metabolic pathways. Int. J. Environ. Sci. Technol., **12**, 1243-1252, 2015.

[47] Ghosh, I.; Jasmine, J.; Mukherji, S.:Biodegradation of pyrene by a *Pseudomonas aeruginosa* strain RS1 isolated from refinery sludge. Bioresour.Technol.,**166**, 548-558, 2014.

[48] JingnanJin, J.; Yao, J.; Zhang, Q. and Liu, J.: Biodegradation of pyrene by *pseudomonas sp.* JPN2 and its initial degrading mechanism study by combining the catabolic nahAc gene and structure-based analyses. Chemosphere., **164**, 379-386, 2016.

[49] Kafilzadeh, F. and Pour, F.H.: Degradation of naphthalene, phenanthrene and pyrene by *Pseudomonas sp.* and *Corynebacterium sp*. in the landfills. Int. J. Biosci.,**2**, 77-84, 2012.

[50] Ping, L.; Zhang, C.; Zhu, Y.; Wu, M.; Hu, X.; Li, Z. and Zhao, H.:Biodegrading of pyrene by a newly isolated *Pseudomonas putida*PL2.Biotechnol.Bioproc.Eng.,**16**, 1000–1008, 2011.

[51] Shokrollahzadeh S., Golmohammad F, Shokouhi H.: Study of Sphingopyxis Isolates in Degradation of Polycyclic Aromatic Hydrocarbons. Chem. Eng. Trans., **27**, 1-5. 2012.

[52] Zhang, G.Y.; Ling, J.Y.; Sun, H.B.; Luo, J.; Fan, Y.Y. and Cui, Z.J.: Isolation and characterization of a newly isolated polycyclic aromatic hydrocarbons-degrading *Janibacteranophelis*strain JY11. J. Hazard. Mater., **172**, 580–586, 2009.

[53] Li, Y.Q.; Liu, H.F.; Tian, Z.L.; Zhu, L.H.; WU, Y.H. and Tang, H.Q.:Diesel Pollution Biodegradation: Synergetic Effect of Mycobacterium and Filamentous Fungi .Biomed. Environ. Sci., **21**, 181-187, 2008.

[54]Lin, Y. and Cai, L.X.: PAH-degrading microbial consortium and its pyrene degrading plasmids from mangrove sediment

samplesinHuian, China. Mar. Poll. Bull., **57**, 703-706, 2008.

[55] Sheng, X.; Chen, X. and He, L.: Characteristics of an endophyticpyrene degrading bacterium of *Enterobacter sp*. 12J1 from Allium macrostemon Bunge. Int. Biodeterior. Biodegrad.,**62**, 88–95, 2008.

[56] Mahanty, B.; Pakshirajan, K. and Dasu, V.V.: Biodegradation of pyrene by *Mycobacterium frederiksbergense* in a two phasepartitioning bioreactor system. Bioresour.Technol.,**99**, 2694-2698, 2008.

[57] Thompson, J.D.; Higgins, D.G. and Gibson, T.J.: CLUSTALW: improving the sensitivity of progressive multiple sequencealignment through sequence weighting, position-specific gappenalties and weight matrix choice. Nucleic Acids Res.,**22**, 4673-4680,1994.

[58] Suardana, I.W.: Analysis of nucleotide sequences of the 16S rRNA gene of novel *Escherichia coli* strains isolated from fecesof human and Bali cattle. J. Nucleic Acids.2014.

[59] Reller, L.B.; Weinstein, M.P. and Petti, C.A.: Detection and identification of microorganisms by gene amplification andsequencing.Clin. Infect. Dis.,**44**, 1108-1114, 2007.

[60] Drummond, A.J.; Ashton, B.; Buxton, S.; Cheung, M.; Heled, J.; Kearse, M.; Moir, R. S-HS.andThierer, T. W.A. (2014):Geneious v4.8 [Internet]. Available from:http://www.geneious.com.

[61] Gupta, S.K., Kumari, R., Prakash, O. and Lal, R.: *Pseudomonas panipatensis* sp. nov., isolated from an oil contaminated site. Int. J. Syst.Evol. Microbiol., **58**, 1339-1345, 2008.

[62] Li, X.W. and Liu, Z.P.: Microbial biodegradation of petroleum hydrocarbon.Acta.Microbiol.Sinica., **42**, 764-767., 2002.

[63] Abbasian, F.; Lockington, R.; Mallavarapu, M. and Naidu, R.: A Comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria. Appl. Biochem. Biotechnol., **176**, 670–699, 2015.

[64] Huijbers, M.M.E.; Montersino, S.; Westphal, A.H.; Tischler, D.andBerkel, W.J.H.: Flavin dependentmonooxygenases. Arch. Biochem. Biophys., **544**, 2–17, 2014.

[65] Ladino-Orjuela, G.; Gomes, E.; Da Silva, R.; Salt, C. and John R. Parsons.: Metabolic pathways for degradation of aromatic hydrocarbons by bacteria. Rev. Environ. Contam.Toxicol., **237**, 115-121, 2016.

[66] Fetzner, S.:Ring-cleaving dioxygenases with a cupin fold. Appl. Env. Microbiol., **78**, 2505–2514, 2012.

Mervat Aly Mohamed Abo-Stateis currentlyVice Dean of Biotechnology Division,at National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA), Cairo, Egypt.

She holds BSc in Botany and Chemistry from Ain Shams University, Cairo; an MSc and PhD from Cairo University. Her research interests include Biodegradation of Poly cyclic aromatic hydrocarbons (PAHs) and Chloroaromatic compounds.

Farida Mohamed Saad El-Din El-Dars is currently Associate Professor of Environmental Analytical chemistry at the Department of Chemistry, Helwan University. She holds BSc in Chemistry from the American University, Cairo; an MSc from Alberta University, Edmonton, Canada; and a PhD from Ain Shams University, Cairo. During 92-96, Dr. El-Dars was actively involved with the Egyptian Environmental Affairs in the identification of feasible project required for implementation by the national environmental action plan. Her research interests include but are not limited to the application of analytical techniques as well as in green chemistry.

Bahaaeldin Ahmed Abdin is currently working as chemist in Misr Petroleum Company, Cairo, Egypt.

He hold BSc in Chemistry and Microbiology from Sohag University; an MSc from Helwan university, Cairo. His research interests include bioremediation of organic and inorganic wastes and green chemistry applications.