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Bioelimination of phenanthrene using degrading bacteria isolated from petroleum soil: safe approach

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ABSTRACT

Polyaromatic hydrocarbon contamination is considered as serious pollution resulted from industrial activities. Physical or chemical methods used for remediation of hydrocarbons might cause the transfer of the remediated compounds from the polluted sites into other clean locations. Our study is concerned by the bioremediation of phenanthrene, using microbial degraders that will eventually, result in the release of non-toxic structures, as end products. Successfully, three bacterial isolates; *Enterobacter cloacae*, *Bacillus* sp. and *Bacillus thuringiensis* with accession numbers; MK559694, MK561601 and MK559693, respectively, showed higher phenanthrene biodegradation ability. The examined conditions indicated that the isolates were active in a wide range of temperatures, pH and hydrocarbon concentrations. The gas chromatography-mass spectrum analysis of the formed by-products showed the existence of intermediate structures with varied carbon values, ranged from $C_4H_8O_2$ to $C_{12}H_{22}O_{11}$. However, these by-products did not show cytotoxic effect against Vero cell lines, compared with the tested undegraded phenanthrene. The obtained results revealed that these bacterial isolates can be used as potent phenanthrene degraders with safe and non-toxic end products, which allow the treated water to be used for non-potable water applications.

Keywords: Phenanthrene; Bioremediation; Cytotoxicity; Vero cell lines; Chromatographic investigation; End products

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), are widespread compounds that might negatively threaten the environment [1,2]. Significant levels of PAHs have been detected in waters, soils, air and sediments [1,3]. In water, PAHs may be diluted when large quantities of water exist and may be transported to other locations of the environment through the flow of water bodies. Wherever PAHs in the soil are generally adsorbed to soil particles which make their degradation almost limited [4]. Mono-aromatic hydrocarbons are almost known for their toxicity to some microorganisms, as they could dissolve the cell membranes, but in low concentrations, they are easily aerobically degraded [5]. However, phenanthrene is a three-ring aromatic hydrocarbon that is commonly found in water bodies as well as contaminated soils and is considered as the model for biodegradation studies of hydrocarbons [6–8].

Several PAH compounds have been reported to cause toxicity and cancer in a plethora of animal species, including

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humans. Most of the published literature on the effects of PAHs on human health have mainly focused on their potential to cause cancers for skin, lung, breast, scrotum, bladder, and colon, etc. [9,10]. Recently, PAHs have proved to cause reproductive, developmental, and neuro toxicities [11,12].

Bioremediation is a strategy for removing PAHs, because some microorganisms can metabolize PAHs to inert substances, CO_2, and water. Microbial remediation of PAHs is usually occurred through successive oxidations, catalyzed by mono and dioxygenase enzymes that require iron as a cofactor (catalyst). Moreover, bio-surfactant production by PAHs-degrading bacteria, may also, enhance the bioavailability of PAHs in the environment [13].

The PAH-degrading microorganisms could be bacteria, fungi, or algae that can bio-transform the organic compounds into less complex metabolites, and/or mineralize them into a gas (CH_4) under anaerobic conditions, or inorganic minerals (H_2O and CO_2) under aerobic conditions. The extent and rate of biodegradation depend on many factors including; microbial population, accessibility of nutrients, oxygen, pH, temperature, acclimation degree, the compound chemical structure, cellular permeability, and chemical partitioning in the growth medium [14]. However, microbial growth and activity during hydrocarbon biodegradation are almost controlled by physical factors such as; pH, temperature and salinity [15–17]. Thus, the successful bioremediation system depends on the optimization of these factors.

Little attention has been paid for metabolites and pathways of PAHs degradation, by halophilic bacteria [18,19]. Moreover, in some cases, the overall toxicity of produced end-products is potentially more than the parent compounds [20,21]. It is much important to trace the risk assessments that might originate from the overall concentration of a specific hydrocarbon, or the toxicity arose by its intermediate and final end products [22].

The present work is mainly concerned with the isolation of phenanthrene-degrading bacteria. Screening of the isolated species for their efficiency in degradation and investigating the environmental factors that affect the phenanthrene-biodegradation process was representing a priority to our research. The work was also extended to investigate the cytotoxicity of phenanthrene, its intermediates and the dead-end products that could be originated through the biodegradation process. Normal kidney epithelial cells (Vero cell lines) were used, where the toxicity of phenanthrene decreased after the degradation process due to the formation of a safe by-product. The degradation of phenanthrene was followed via measuring the cell density during the incubation period, and the by-products were determined, using the gas chromatography-mass spectrum (GC-MS) at the end of the experiment.

2. Material and methods

2.1. Chemicals

All used chemicals are of an analytical grade. Phenanthrene was purchased from Aldrich® Company (USA, purity above 98%) and its ethyl acetate stock solution at 10.000 ppm was prepared and stored in a brown bottle, while other concentrations were prepared by dilution. Sodium hydroxide and hydrochloric acid were used to adjust the pH. The phenanthrene-degrading strains were routinely grown at 30°C in mineral salts medium (MSM) consisting of (mg/L): K_2HPO_4, 250; $MgSO_4 \cdot 7H_2O$, 300; $CaCl_2 \cdot 2H_2O$, 150; $(NH_4)_2CO_3$, 120; $FeSO_4$, 3.5; $ZnSO_4 \cdot 7H_2O$, 1.3; $MnSO_4 \cdot 4H_2O$, 0.018; $Na_2MoO_4 \cdot 2H_2O$, 0.013 and L-Ascorbic acid 0.005 [23]. Luria-Bertani (LB) broth medium consists of the following ingredients (g/L): Tryptone 10.0; yeast extract 5.0; NaCl 5.0; distilled water 1,000.0 ml was also used [24].

2.2. Sample collection

Crude oil contaminated soil samples were collected from Cairo Petroleum Refining and Norbetco® Petroleum Companies in Cairo Governorate, Egypt. The contaminated soils were supposed to have some microbial strains with potent ability to degrade PAHs under optimized and controlled conditions.

2.3. Adaptation and isolation of hydrocarbon degrading strains

For adaptation, 25 g from each soil sample was added to 100 ml of MSM and were incubated overnight in a shaking incubator at 30°C with 150 rpm to activate the indigenous microbes for the new cultivation conditions [25]. Then, 10 ml of adapted bacteria were added to 100 ml of MSM amended with 100 ppm phenanthrene and incubated at 30°C with 150 rpm for 6 d to activate the adaptation of existed microbes for the sole amended hydrocarbon.

2.4. Isolation and purification of phenanthrene-degrading bacteria

The isolation process depended on the ability of microbes to grow on mineral salt medium amended with phenanthrene as a sole carbon and energy source. In this case, 100 μl of the adapted soil bacteria were spread over mineral salt agar plates containing 100 ppm of phenanthrene. The plates were incubated at 30°C for one week until the obvious existence of bacterial colonies, which were presumptively considered as phenanthrene degraders. The grown colonies were purified on nutrient agar plates and subsequently preserved in 4°C till use.

2.5. Biodegradation study

To investigate the ability of isolated bacteria to use phenanthrene, as sole carbon and energy source; each bacterial isolate was cultivated in nutrient broth for 18 h, before transferring to the hydrocarbon amended broth. After incubation, 5 ml of each overnight culture was inoculated into 50 ml MSM supplemented with 50 ppm phenanthrene in 250 ml conical flasks. The flasks were incubated at 30°C and 150 rpm, shaking for 6 d with interval sampling every 24 h. In addition, the control flasks included 50 ml MSM supplemented with 50 ppm phenanthrene without bacterial inoculations were prepared. The growth of bacteria was assayed as the ratio of the absorbance reading of their optical densities at 600 nm after incubation to the absorbance reading at zero time (i.e. immediately after inoculation) (I/Io). It worth mentioning that, all assays were carried out in triplicates.

2.6. One variable at time optimization

The effect of different parameters on the biodegradation of phenanthrene by the tested organisms was investigated [26].

2.6.1. Effect of phenanthrene concentration

Different phenanthrene concentrations; 50, 100, 200, 300, 400, and 500 ppm were separately amended to 50 ml MSM followed by the addition of 5 ml overnight culture of each isolate in a nutrient broth. All flasks were incubated at 30°C with 100 rpm shaking for 72 h. After incubation, the hydrocarbon residues from each tested concentration were also measured and the optimum concentration of the hydrocarbon was determined.

2.6.2. Effect of temperature

Temperature is an important factor in utilizing hydrocarbons, as sole carbon sources, by microbes. In batch experiments; different temperatures; 15°C, 25°C, 30°C, 35°C, and 45°C were applied to investigate the biochemical behavior of the tested bacteria towards the tested hydrocarbon. Each 50 ml MSM supplemented with 100 ppm of phenanthrene was inoculated with 5 ml of overnight nutrient broth culture from each isolate. At each temperature, the flasks were shaken at 100 rpm, then followed by incubation for 72 h. At the end of the experiment, the optical density at 600 nm was measured, and the optimum temperature was determined.

2.6.3. Effect of static/shaking conditions

Shaking of microbial cultures, is almost, accompanied by increasing oxygen level and distribution, and hence affects the total process negatively or positively according to the required aerobic or anaerobic conditions. In this experiment, the prepared MSM flasks containing 100 ppm phenanthrene, were inoculated with 5 ml overnight nutrient broth (NB) culture of each bacterial isolate, and then submitted to different shaking conditions; static (0), 100 and 200 rpm. After 72 h, flasks were removed, and the optical densities of the tested isolates were determined.

2.6.4. Effect of pH

Various pH values ranged from 3.5 to 9.5 were used to investigate optimum pH for microbial isolates to degrade 100 ppm of phenanthrene, in 50 ml MSM broth. The pH value was adjusted using either 1 N HCl or 1 N NaOH, before medium sterilization. Overnight NB culture of each isolate was added to each flask with a certain pH. All flasks were incubated at 100 rpm, and 30°C for 3 d. After the specified incubation period, the optical densities of all isolates at each pH value were measured at 600 nm, and the optimum pH value was investigated.

2.7. Analytical determination of phenanthrene residues

Phenanthrene residues that were remaining after the biodegradation of different concentrations by different microbial isolates were determined according to the method of [27]. At the end of the experiment, each culture broth including its components was transferred to a separating funnel and mixed with equal volumes of n-hexane. After mixing for 5 min, the resulting organic phase was used for spectrophotometric readings at 275 nm. The highly concentrated samples were diluted by hexane to be within the range of the calibration line (1–10 ppm).

2.8. Molecular identification of the bacterial isolates

2.8.1. Deoxyribonucleic acid extraction

Total deoxyribonucleic acid (DNA) extraction from each bacterial isolate was performed, using Amshag kit® (SRTA-City, Alexandria, Egypt) following the manual instructions. The extracted DNA was checked through migration in 1% agarose gel, using 1X TBE running buffer, followed by photographing using the Gel Documentation system (SynGene®, UK).

2.8.2. Amplification and sequencing of 16S rRNA gene

The amplification of 16S rRNA gene, of the three bacterial isolates, was achieved using universal primers F984 (5′-AACGCGAAGAACCTTAC-3′), and R1378 (5′-CGGT GTGTACAAGGCCCGGGAACG-3′), according to [28], with some modifications. The polymerase chain reaction (PCR) components, composed of 25 μl reaction, containing; 12.5 μl of PCR master mix (Takara®, Japan); 1 μl of each primer; 1 μl of the extracted DNA; and 9 μl of pure injection water. The PCR program was adjusted as follows: activation and denaturation step, at 94°C for 5 min, followed by 35 cycles of 94°C, for 15 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension step, at 72°C for 7 min. The PCR products were detected through migrating the PCR bands in 1% agarose gel, in 1X TBE buffer, using a gel electrophoresis unit, at 150 V for 30 min. The gel was then examined, using a gel documentation system and the formed PCR bands were photographed under the transilluminator. The rest of the amplified PCR products were then submitted for a nucleotide sequencing process (Sigma®, Germany). The obtained sequences were compared with genes deposited in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Moreover, the obtained sequences were deposited in GenBank, with new accession numbers. After releasing the accession numbers, the phylogenetic tree of the isolates, and related strains were constructed, using MEGA 5® software version 5.1.

2.8.3. Gas chromatography-Mass spectrum analysis

Phenanthrene metabolites were analyzed using GC-MS. After 3 and 7 d of incubation, cultures were adjusted to pH 2, with 1 M HCl, and then extracted twice with an equal volume of ethyl acetate. Extracts were then combined dehydrated with anhydrous Na_2SO_4, and rota-evaporated at 35°C. The extraction residues were dissolved in methanol [29].

The GC-MS analysis was carried out using Finnigan SSQ 7000 GC® (United States), equipped with a quartz capillary DB5 (30 m × 0.25 mm, 0.25 μm of film thickness). Helium was used as a carrier gas. The following oven temperature program was used: 25°C for 7 min, then increased to 100°C at 10°C/min, and finally increased to 315°C at 20°C/min, then

maintained for 10 min. Constant temperatures of 280°C, 220°C, and 260°C were kept at the injector, ion source and interface, respectively. Mass ranges from 50–500 were used [30].

2.9. Determination of sample cytotoxicity on cells (MTT protocol)

The cytotoxicity test was performed for the culture broth residues, after the incubation time, specified for the biodegradation test. The purpose of this test was to investigate the cytotoxicity of the residual phenanthrene or its metabolites on normal cell lines. Successive fold dilutions of each hydrocarbon residues were tested against kidney epithelial cells (Vero cell lines). In the beginning, the 96 wells tissue culture plate was inoculated with 1×10^5 cells/ml (100 µl/well) and incubated at 37°C for 24 h to develop a complete monolayer sheet. After the formation of a confluent sheet of cells, the Roswell Park Memorial Institute (RPMI) growth medium was decanted from each well of the microtiter plate. The formed cell monolayer was washed, sequentially for two times with uncultivated RPMI media. Two-fold dilutions of each tested sample were made in RPMI medium with 2% fetal bovine serum (maintenance medium). After that, 0.1 ml of each dilution was tested in three wells leaving it as a control, receiving only maintenance medium. The plate was then, incubated at 37°C for 24 h, and followed by microscopic examination. The cells of each well were checked for any morphological toxicity signs, for example, complete or partial loss of the monolayer, shrinkage, rounding, or cell granulation. On the other hand, tetrazolium dye (MTT) solution was prepared with a concentration of 5 mg/ml in phosphate buffer saline (PBS) buffer (BIO BASIC CANADA INC.®) and 20 µl of it were added to each well. The plate was transferred to a shaking table at 150 rpm, for 5 min to thoroughly mix the MTT substance into the media. The plate was then, incubated at 37°C and 5% CO_2 for 4 h, to allow the MTT substance to be metabolized. The media were dumped off, and the plate was dried on paper towels, to remove residues. The formazan (MTT metabolic product) was resuspended in 200 µl dimethyl sulfoxide (DMSO) and the plate was transferred again to a shaking table at 150 rpm, for 5 min, to mix formazan thoroughly with the solvent. The optical density was measured at 560 nm and subtracted from background OD at 620 nm. The obtained optical density reading was directly correlated with cell quantity [31].

2.10. Scanning electron microscopy

Scanning electron microscopic analysis and surface characterization of M3 isolate in LB broth and in phenanthrene amended MSM broth was carried out using energy-dispersive analysis (JOEL JSM 6360 LA, Japan).

3. Results and discussion

3.1. Isolation of phenanthrene-degrading bacteria

Contaminated soil samples collected from Cairo Petroleum Refining and Norbetco® Petroleum Companies in Cairo Governorate, Egypt were used for the isolation of hydrocarbon-degrading bacteria. However, a pre-adaptation process was initially performed using phenanthrene as the sole carbon source. The cultivated plates showed the ability of six bacterial colonies to mineralize the used phenanthrene, forming pale clear zones around each colony. A loopful from each bacterial colony was aseptically transferred to sterile nutrient agar (NA) plate for purification and subsequent molecular identification.

3.2. Screening of bacterial isolates

The actual ability of the isolates to degrade the specified hydrocarbon was confirmed through the ability of the isolates to degrade 100 ppm of the phenanthrene in liquid culture. The optical density measurement of each bacterial isolate was the adopted evidence for the honest biodegradation process. Results in Table 1 show that almost all isolates succeeded to degrade phenanthrene in liquid media, with different efficiencies. The most potent isolate was M6 that showed an OD of 1.5 followed by M4 and M3 isolates with OD 1.0 and 0.7, respectively. On the other hand, the other isolates (M2, M8 and M9) were recorded as weak phenanthrene degrading bacteria and showed an OD that almost represented about two-thirds of the lowest potent phenanthrene degrading bacteria.

3.3. Optimization of biodegradation parameters

3.3.1. Effect of phenanthrene concentration

The ability of M3, M4 and M6 isolates to mineralize different concentrations of phenanthrene was investigated. As shown in Fig. 1a, all isolates were able to effectively degrade phenanthrene at lower concentrations. However, this ability was gradually decreased with higher concentrations of the tested hydrocarbon. The three isolates were almost able to degrade phenanthrene with a total uptake percentage, ranged from 60% to 80%, at 50 and 100 ppm concentrations. The obtained data revealed that all the isolates shared the same degradation behavior at lower and moderate concentrations. The isolate M6 was unable to degrade phenanthrene, at 500 ppm concentration, and recorded a zero-degradation percentage. While, M3 and M4 isolates were moderately interactive with phenanthrene at 500 ppm concentration, and showed an uptake percentage of 30 and 20%, respectively. Ling and his colleagues, showed an increase in the degradation of PAHs, at high concentrations >200 ppm [32].

3.3.2. Effect of temperature

The temperature's effect, on the growth of the three tested bacterial isolates at the presence of phenanthrene as sole carbon and energy source, was investigated. As shown in Fig. 1b, the optimum temperature for M4 and M6 isolates was 30°C, consistent with the results obtained by [14]. While the temperature of 25°C was most preferred for the growth of isolate M3. It is worth mentioning that, the three bacterial isolates, showed a detectable growth, at the five tested temperature ranges, indicating the wide range application of the tested isolates as phenanthrene bio-degraders, at different environmental conditions. Isolate M6, showed a sharp growth rate at 30°C, while a moderate growth rate, ranged from 25°C to 37°C was showed by isolate M4. On the other hand, M3 isolate could maintain its growth rate to 45°C.

Table 1
Screening of bacterial isolates for their ability to degrade phenanthrene, at an initial concentration of 50 mg/L

Isolate "No."	Zero	24 h		72 h		144 h	
	O.D (Io)	O.D (I)	I/Io	O.D (I)	I/Io	O.D (I)	I/Io
M2	0.235	0.32	1.361702	0.426	1.812766	0.54	2.297872
M3	0.297	0.452	1.521886	0.851	2.86532	0.7	2.356902
M4	0.379	0.591	1.559367	1.1	2.902375	1	2.638522
M6	0.312	1.02	3.269231	1.3	4.166667	1.5	4.807692
M8	0.326	0.402	1.233129	0.522	1.601227	0.535	1.641104
M9	0.181	0.225	1.243094	0.416	2.298343	0.4	2.209945

O.D means "Optical Density".

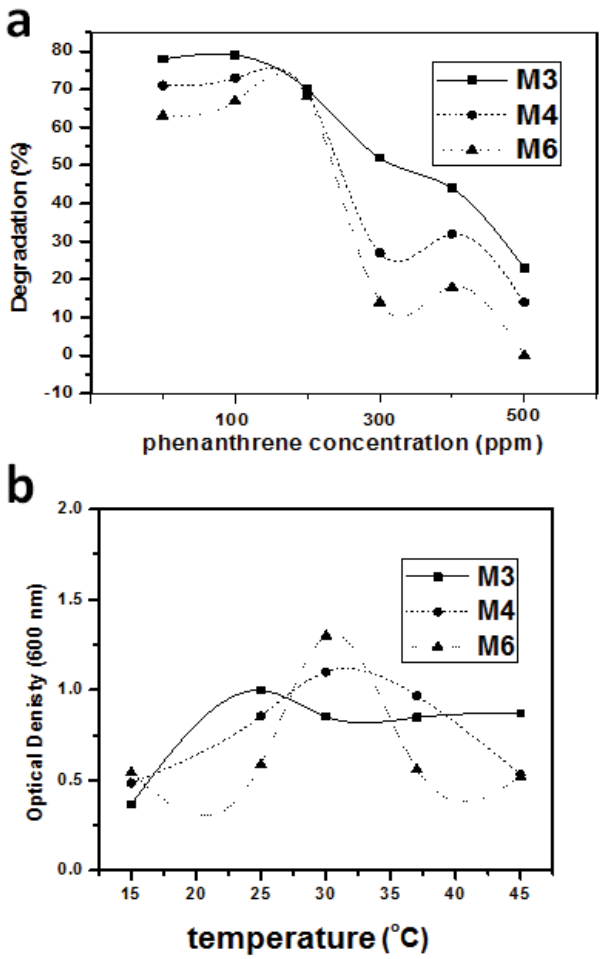

Fig. 1. (a) Degradation percentage of phenanthrene at different concentrations, using selected bacterial isolates and (b) effect of temperature on the growth of bacterial isolates in the presence of phenanthrene as a sole carbon source.

3.3.3. Effect of shaking

Shaking of bacterial strains is an important factor that significantly affects the rate of bacterial growth and subsequently the hydrocarbon biodegradation. It almost affects the percentage of the dissolved oxygen, and determine the aerobic, or anaerobic degradation pathway. As shown in Fig. 2a, three different shaking conditions, have been applied; however, the moderate one was recorded as the most preferred condition among the three studied isolates. The static condition was recorded as an unfavorable condition for the three isolates and its recorded OD was lower than the reported readings, for shaking conditions. On the other hand, an extremely high shaking rate was also, unfavorable for all tested isolates; however, it was preferred then, the static conditions. These observations may be attributed to the block of some pathways responsible for the biodegradation of phenanthrene at the presence of elevated concentrations of dissolved oxygen. Moreover, all the tested isolates preferred the moderate shaking conditions at 100 rpm and recorded the highest growth rates using phenanthrene as a sole carbon source [33].

3.3.4. Effect of pH

The three phenanthrene degrading isolates were tested for their biodegradation capacity, at wide ranges of pHs. As depicted in Fig. 2b, the three tested isolates were able to grow at acidic and alkaline pH values, ranged from 3.5 to 9.5, while the optimum value has been demonstrated at pH 6.5. Isolate M6 was recorded as the best degrader, followed by isolate M4, whenever optimum conditions were available. The ability of the tested isolates to grow and degrade phenanthrene, through wide pH ranges, is considered a good sign for the stable biodegradation capacity of these isolates, even under changed pH conditions, or in extreme environments. However, Simarro et al. [34] showed that the favored pH range for PAHs degradation is between 5.5 and 7.8.

3.4. Molecular identification

The promising bacterial isolates, for the ability to biodegrade phenanthrene, were submitted for DNA extraction, PCR amplification and sequencing of their 16S rRNA genes. As shown in Fig. 3, the amplification of the genes was succeeded and confirmed through the presence of the required 500 bp fragment. The obtained genes were subjected for a sequencing process, to compare their nucleotide sequences, with the sequences submitted in the GenBank, to confirm their identity. The compared sequences revealed that the bacterial isolates M3, M4 and M6, are highly close to the *Enterobacter cloacae*, *Bacillus* sp. and *Bacillus thuringiensis*

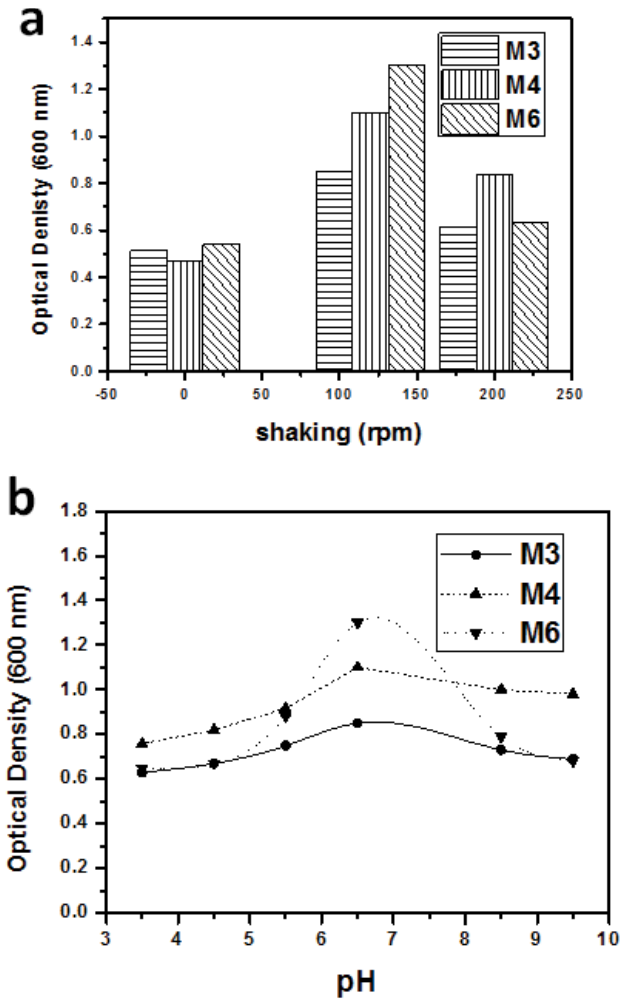

Fig. 2. (a) Effect of static/shaking conditions, on the growth of bacterial isolates, at the presence of phenanthrene as sole carbon source and (b) effect of pH, on the growth of bacterial isolates, at the presence of phenanthrene as a sole carbon source.

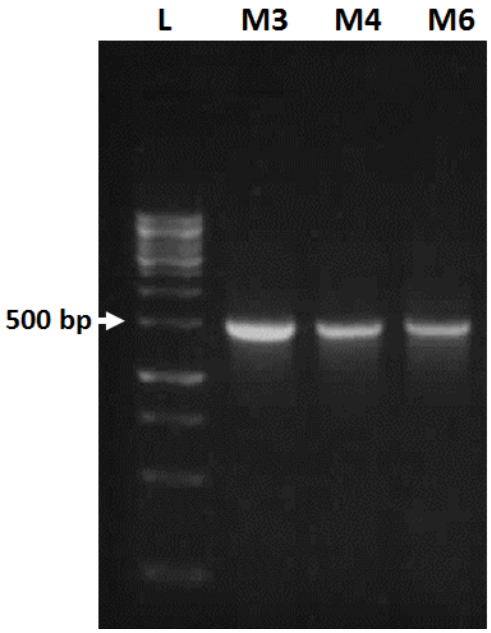

Fig. 3. Gel electrophoresis for PCR product of the three bacterial isolates where; (L) is 100 bp DNA ladder and (M3, M4, and M6) are the 16S rRNA PCR products of the three bacterial isolates.

strains, respectively. The similarity percentages of the three isolates were subsequently, recorded as 97.31%, 99.52% and 99.52%. The three genes were submitted to the GenBank, and serially, got the accession numbers MK559694, MK561601 and MK559693, as a consequence result. The phylogenetic tree of the three isolates and other similar isolates was able to uncover the degree of genetic similarity, among the isolates, and the relative closest ones, as shown in Fig. 4.

The alignment among *Enterobacter cloacae* strain (as an example) and other sequences deposited in GenBank could be shown as follows:

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MH040950.1_886-1275 -CTTGACATCCACAG-AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTG
KY937910.1_531-920----CTTGACATCCACAG-AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTG
KP276148.1_120-509----CTTGACATCCACAG-AACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTG
MK559694.1_1-400------CCCTTGGCCCTTGACATCCTCAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTG
*********************************************
MH040950.1_886-1275 -TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG
KY937910.1_531-920----TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG
KP276148.1_120-509----TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG
MK559694.1_1-400------TGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG
*********************************************
MH040950.1_886-1275 -CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACT
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KP276148.1_120-509----CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACT
MK559694.1_1-400------CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCCTCGGCCGGGAACTCAAAGGAGACT
*********************************************
MH040950.1_886-1275 -GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCA
KY937910.1_531-920----GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCA
KP276148.1_120-509----GCCGGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCA
MK559694.1_1-400------GCCGGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCA
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MH040950.1_886-1275 -GGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG
KY937910.1_531-920 ----GGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG
KP276148.1_120-509---- GGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG
MK559694.1_1-400------ GGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGGAAGCGG
********************************************
MH040950.1_886-1275 -ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
KY937910.1_531-920----ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
KP276148.1_120-509----ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
MK559694.1_1-400------ACCTCATAAAGCGCATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
********************************************
MH040950.1_886-1275 -ATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACG
KY937910.1_531-920----ATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACG
KP276148.1_120-509----ATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACG
MK559694.1_1-400------ATCGCTAGTAATCGTAAATCAAAATGCTACGGTGAATACG
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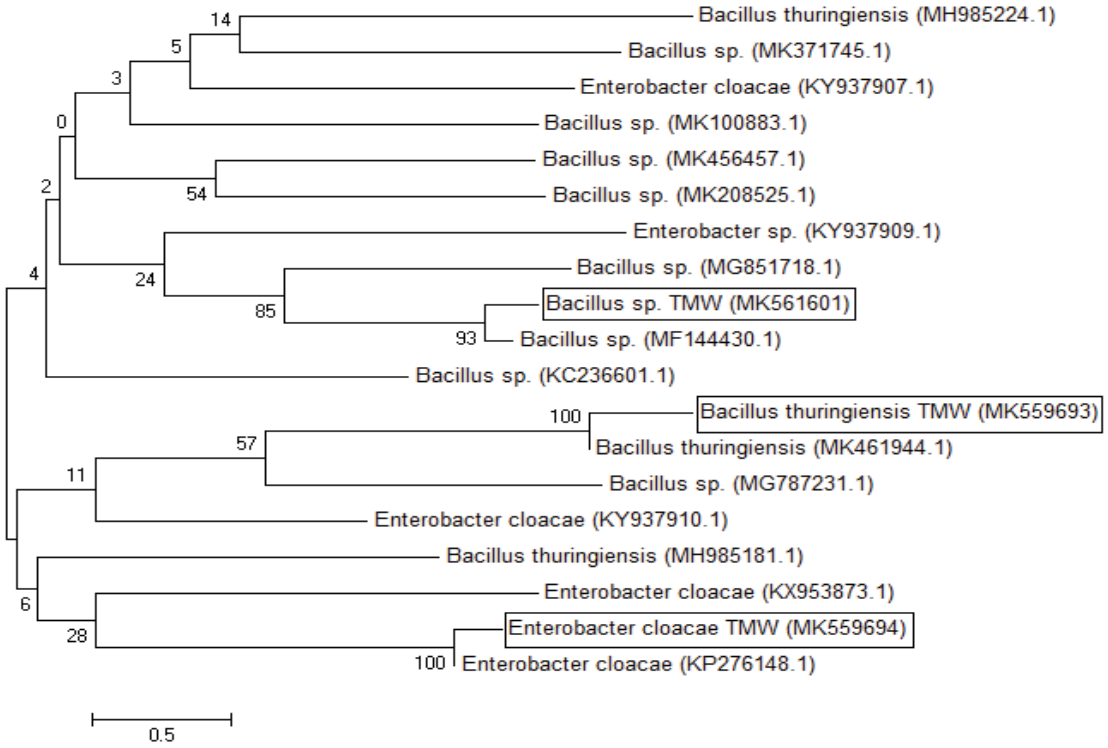

Fig. 4. Phylogenetic relationship of the three bacterial isolates, and other related bacteria. The branching pattern was generated by neighbor-joining tree method with 500 bootstrap, and the GenBank accession numbers of the 16S rRNA nucleotide sequences are indicated in brackets.

3.5. Cytotoxicity

The result of the toxicity test showed that the normal cell lines grew without any stress (control sample), as well-formed tissues with a tight junction structure, where there was no disintegration observed (Fig. 5b). On the other hand, the toxicity of the high concentration of phenanthrene (100 ppm) caused a disintegration, which could appear as individual cells (in the highly affected region) or as small holes (in the low affected region). The phenanthrene degradation metabolite showed a very low effect on Vero cells as shown in Fig. 5d. The standard phenanthrene caused damage of 52.9 % for cells (Fig. 5c), while, for degradation, the metabolite of species M3, M4 and M6, caused damage of 4.6%, 0.4% and 1.8%, for the cells, respectively, as shown in Table 2 and Fig. 5a. These results mean that, the metabolite profiles of phenanthrene resulted from isolates (M3, M4 and M6), are safe, and have a very low toxic effect on cells when compared with standards and controls [35].

3.6. Analysis of phenanthrene metabolites using GC-MS

Sample analysis by GC-MS showed the presence of several intermediate (metabolites) decompositions resulting from the degradation of phenanthrene, by the studied bacterial isolates Fig. 6. The molecular formula, retention

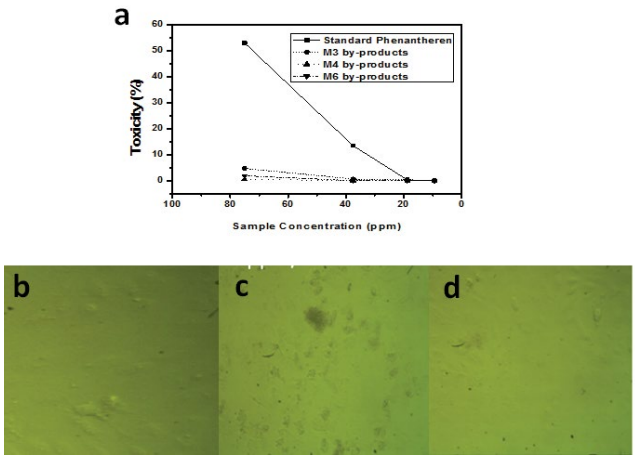

Fig. 5. (a) Toxicity percent of phenanthrene and metabolites produced by studied bacterial species (MTT protocol). Cytotoxicity of phenanthrene and its metabolites, on a normal Vero cell line(s), have been also photographed; (b) Control Vero cell(s), (c) effect of standard phenanthrene, and (d) effect of phenanthrene metabolites.

time and molecular weight of the intermediate metabolites are common in PAHs metabolism, which is given in Table 3. Di-oxygenation in multiple position (1,2–3,4 and 9,10) carbon position [36,37]. Vila and others found that 9,10 di-oxygenation of phenanthrene (diphenic acid) was usually the predominant metabolite [38]. Most of the detected intermediates metabolite were similar to those published in the literature [39,40].

Table 3
GC analysis of phenanthrene by-products

Retention time (RT)	Molecular formula	Molecular weight
4.15	$C_4H_8O_2$	88
8.64	$C_6H_8O_4$	144
10.10	$C_6H_{12}O_6$	180
13.71	$C_7H_{14}O_7$	210
18.03	$C_7H_{14}O_7$	210
18.87	$C_{12}H_{22}O_4$	230
26.96	$C_{12}H_{22}O_{11}$	342
34.79	$C_7H_{14}O_7$	210

For more clarification, interpretation of GC-MS biodegradation of phenanthrene includes some oxidation–reduction reaction, even mono or di oxidation involves cleavage of parent compound, so the less molecular weight which contain (4-6-7-12) carbon atom result from cleavage of phenanthrene or/and an oxidation process which leading to the formation of compound like diethyl ester ($C_4H_8O_2$). While the formation of compounds with molecular weight more than phenanthrene may be due to conjugation between two compounds or addition reaction to a group on another position on the compound.

In general, the mechanism of phenanthrene degradation is firstly starting by its oxidation into cis-1,2-dihydroxy-1,2-dihydrophenanthrene or cis-3,4-dihydroxy-3,4-dihydro phenanthrene. This product is further metabolized to 1-hydroxy-2-naphthoic acid followed by oxidative decarboxylation to 1,2-dihydroxynaphthalene with subsequent

Table 2
Effect of different phenanthrene concentrations and it is by-products concentrations on cell growth and the toxicity percent

Dilution	Standard		Sample M3		Sample M4		Sample M6	
	Mean O.D	Toxicity %	Mean O.D	Toxicity %	Mean O.D	Toxicity %	Mean O.D	Toxicity %
75	0.100	52.895	0.203	4.695	0.212	0.469	0.209	1.878
37.5	0.184	13.302	0.212	0.626	0.213	0	0.214	0
18.75	0.213	0.3130	0.213	0.312	0.214	0	0.215	0
9.375	0.212	0	0.211	0	0.214	0	0.215	0

O.D means "Optical Density".

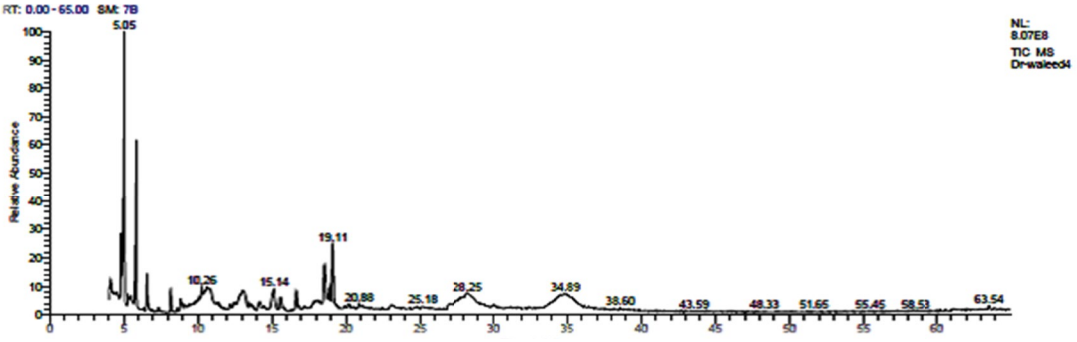

Fig. 6. GC-MS analysis of phenanthrene by-products.

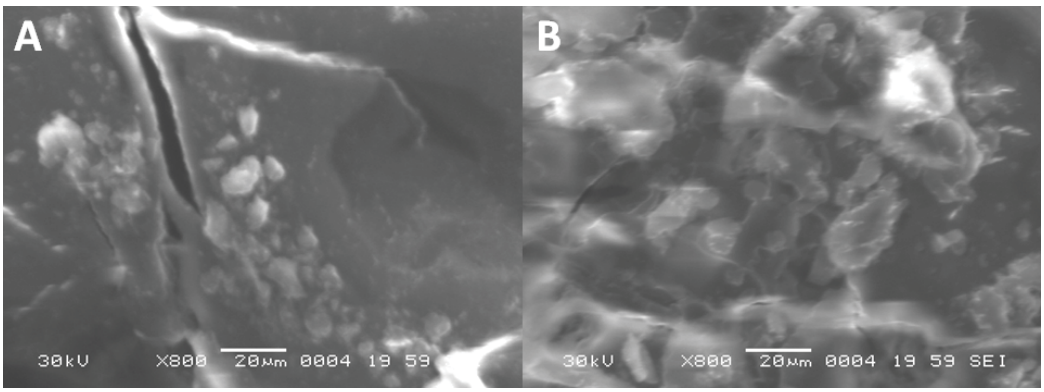

Fig. 7. SEM of isolate M3 after cultivation in (a) LB broth and (b) MSM broth containing phenanthrene.

formation of salicylic acid after meta-cleavage of the ring. The enzymatic machine of the organism can further degrade salicylic acid into catechol or gentisic acid which can be subjected to ring fission forming TCA cycle intermediates [41,42].

3.7. Scanning electron microscopy

The morphological structure of M3 isolate with and without phenanthrene was investigated using scanning electron microscopy (SEM). As shown in Fig. 7, the shape of cells was almost the same rounded and irregular at the presence and absence of phenanthrene. However, intra-structure threads between the microbial cells were appeared in broth cultures depending on phenanthrene as a sole carbon source (Fig. 7b). We could attribute the appearance of these threads to a mutual participation of the microbial cells among each other to assist the biodegradation of complicated structures such as phenanthrene.

4. Conclusion

It could be concluded that the isolated bacteria can degrade phenanthrene at high concentrations and under wide ranges of temperature and pH. The optimum conditions which gave higher degradation rate were; 100 ppm of phenanthrene concentration, temperature 30°C for isolates M6 and M4, and 25°C for M3, most favorable pH 6.5, while the moderate shaking speed (120 rpm) was the best in the degradation process.

The investigation also approved that degradation process leading to the formation of low toxic metabolites.

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