RESEARCH ARTICLE

Quantification of Naphthalene Dioxygenase (*NahAC*) and Catechol Dioxygenase (*C23O*) Catabolic Genes Produced by Phenanthrene-Degrading *Pseudomonas fluorescens* AH-40

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Abstract: **Background:** Petroleum polycyclic aromatic hydrocarbons (PAHs) are known to be toxic and carcinogenic for humans and their contamination of soils and water is of great environmental concern. Identification of the key microorganisms that play a role in pollutant degradation processes is relevant to the development of optimal *in situ* bioremediation strategies.

Objective: Detection of the ability of *Pseudomonas fluorescens* AH-40 to consume phenanthrene as a sole carbon source and determining the variation in the concentration of both *nahAC* and *C23O* catabolic genes during 15 days of the incubation period.

Methods: In the current study, a bacterial strain AH-40 was isolated from crude oil polluted soil by enrichment technique in mineral basal salts (MBS) medium supplemented with phenanthrene (PAH) as a sole carbon and energy source. The isolated strain was genetically identified based on 16S rDNA sequence analysis. The degradation of PAHs by this strain was confirmed by HPLC analysis. The detection and quantification of naphthalene dioxygenase (*nahAc*) and catechol 2,3-dioxygenase (*C23O*) genes, which play a critical role during the mineralization of PAHs in the liquid bacterial culture were achieved by quantitative PCR.

Results: Strain AH-40 was identified as *pseudomonas fluorescens*. It degraded 97% of 150 mg phenanthrene L^{-1} within 15 days, which is faster than previously reported pure cultures. The copy numbers of chromosomal encoding catabolic genes *nahAc* and *C23O* increased during the process of phenanthrene degradation.

Conclusion: *nahAc* and *C23O* genes are the main marker genes for phenanthrene degradation by strain AH-40. *P. fluorescence* AH-40 could be recommended for bioremediation of phenanthrene contaminated site.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) spread widely by the incomplete combustion of fossil fuels, high-temperature pyrolysis of coal, oil and wood, pharmaceutical processes, *etc*. They are raising increasing concerns because they have toxic effects on ecosystems and human health. Because of the high thermodynamic stability of the benzene moiety, PAHs are relatively persistent in the environment [1]. Some are even mutagenic or carcinogenic due to their long-term bioaccumulation and biomagnification [2]. Because of their recalcitrance and health hazards associated with them, the US Environmental Protection Agency (US EPA) has classified 16 PAHs as "priority pollutants" (US EPA 1998) [3]. These properties have prompted researchers to find efficient ways of removal of aromatic contaminants from the environment. There are many efforts and strategies for safely removing such compounds from the environment such as using chemical oxidation and precipitation. However, no effective method for the removal of existing PAHs in the environment has yet been developed [4, 5]. Most of the recent researches have attempted the biological degradation of PAHs by bacteria, fungi, yeasts and higher plants [6-8]. Bacterial degradation of PAHs is more preferable as it is fast, can be aerobical, anaerobical and under variable environmental conditions [9]. Many literature studies discussed the variable capabilities of *Pseudomonas, Achromobacter, Sphingomonas* genus, *Alcaligenes, Mycobacterium, Rhodococcus*, and *Bacillus* in degrading different PAHs compounds [7, 10-14].

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Biodegradation of PAHs is catalyzed by multicomponent enzymes produced by microbes which are encoded by many genes. Naphthalene dioxygenase (*NahAc*) gene is considered a key gene mainly responsible for attacking the aromatic ring structure of PAHs under aerobic conditions [15, 16]. It catalyzes the addition of two oxygen atoms on the aromatic ring of PAH molecule which consequently results in aromatic ring cleavage. *NahAc* gene can not only degrade naphthalene rings, but also mediate the degradation of phenanthrene, anthracene, dibenzothiophene, fluorine, and methylated naphthalenes [17, 18]. It is a highly conserved gene among different Gram-negative bacterial strains, and it has been thought that natural horizontal gene transfer has occurred between different bacterial species that carry this gene [19]. This is supported by the observation that PCR with degenerate primers designed from the *nahAc* gene in *Pseudomonas putida* PaW736 (NCIB 9816) could be detected not only in *Pseudomonas* species but also detected in *Mycobacterium*, *Sphingomonas*, *Gordona*, *Xanthomonas*, and *Rhodococcus*. These strains could degrade low molecular weight-PAHs including naphthalene phenanthrene, anthracene, and higher molecular weight-PAHs such as pyrene and fluoranthene [10, 20].

The representative central intermediate compounds formed during bacterial metabolism of PAHs are catechols. Bacteria can degrade the produced catechol through two pathways, the meta-cleavage pathway and the ortho-cleavage one, in which ring cleavage reactions are the first steps mediated by catechol 2,3-dioxygenase and catechol 1,2-dioxygenase, respectively [21]. Both of these enzymes are encoded by *C23O* and *C12O* genes [21]. The majority of PAH-degrading Gram-negative bacteria can degrade low molecular weight-PAHs, such as naphthalene, anthracene, and phenanthrene, by classic deoxygenation at the Bay-region. During the phenanthrene degradation process, a Bay-region catechol, 3,4-dihydroxyphenanthrene, can be metabolized by the extradyadic and intradialytic dioxygenase activities induced by *C23O* [22].

The main objective of this study was to detect the ability of *Pseudomonas fluorescens* AH-40 isolated from oil-contaminated soil to consume phenanthrene as a sole carbon source and determine the variation in the copy numbers of both *nahAC* and *C23O* catabolic genes along 15 days of the incubation period.

2. MATERIALS AND METHODS

2.1. Isolation of Phenanthrene-degrading Bacteria

To isolate phenanthrene-degrading bacteria, about 5 g oily sludge sample was suspended in 45 ml mineral basal salt medium (MBS) containing (g/l) 0.8 K_2HPO_4, 0.2 KH_2PO_4, 1.0 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.1 $CaCl_2 \cdot 2H_2O$, 0.005 $FeSO_4 \cdot 7H_2O$, and 1ml of trace elements, pH 7.0 ± 0.2 and supplemented with 150 mgL^{-1} of phenanthrene as a sole carbon source [7] for enrichment of PAHs bacterial species. It was incubated in an orbital shaker at 150 rpm at 37ºC for 7 days. This step was repeated five times to ensure that the most adapted and degradable isolates are obtained.

For preparing phenanthrene-containing solid medium (Phen-MBS), 1.5% (w/v) phenanthrene solution dissolved in acetone was sprayed onto the surface of solid MBS agar

plates, which were dried for 20 min at room temperature. Next, 200 µL of the last enrichment step was spread on the surface of a solid MSM agar plate and incubated at 37ºC. One week later, single colonies were successfully isolated on the phenanthrene layer of the MBS agar plate. The single colony was then streaked on fresh (Phen-MBS) agar medium for purification. Finally, the pure colony was preserved on a (Phen-MBS) slant agar [23].

2.2. PAHs Clearing Zone-spray-plate Technique

Phenanthrene degradation by the bacterial isolates was analyzed by the spray-plate technique using phenanthrene as the sole carbon and energy source. Agar was added to the MBS medium for plating in Petri dishes. After solidification, acetone-dissolved phenanthrene was sprayed on the top of the medium to form a white layer on the surface of the solid medium. The bacterial cells were inoculated on that medium and incubated at 37ºC for 5-7 days. After the incubation period, clear zone formation around growing colonies indicate phenanthrene degradation [24].

2.3. Molecular Identification of Phenanthrene-degrading Bacteria by 16S rDNA

The identification was performed based on 16S rDNA sequence analysis. Genomic DNA was extracted from the isolated bacterium strain according to our previously described method [25]. The 16S rDNA PCR amplification was performed using universal primers: 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-CGGCTACCTTGTTACGACTT-3). The sequences obtained were then aligned with known 16S rDNA sequences in GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), the obtained 16S rDNA sequences were aligned and compared with the known 16S rDNA sequences in Genbank database to check the closest available database sequences. To determine the taxonomic position of the isolates, the phylogenetic tree was constructed with MEGA version 4.0 using a neighbor-joining algorithm, plus the Jukes Cantor distance estimation method with bootstrap analyses for 100 replicates was performed [25]. The sequence of the strain has been deposited in the GenBank nucleotide sequence database.

2.4. Phenanthrene Biodegradation Assay

A pre-grown bacterial colony on phen-MBS agar plate was scratched, re-suspended in phosphate buffer saline (PBS) of pH 6.8 and then washed twice. An aliquot of OD 600 =0.03 of bacterial cells was dispersed in fifty millilitres of sterile MBS supplemented with acetone-dissolved phenanthrene at a final concentration 150 mgl^{-1} followed by evaporation of acetone with gentle shaking. After 3 days intervals, aliquots of 5 ml were withdrawn for the determination of bacterial growth and phenanthrene degradation. Aliquots of MBS medium containing phenanthrene served as a negative control [10]. The growth of bacteria was monitored by OD 600 and colony-forming Unit (CFU/mL) as well. A tenfold of serially diluted samples were plated on Luria-Bertani broth (LB) agar plate. The plate was then incubated for 24 h at 37°C, and the bacterial colonies were enumerated

as CFU/mL. Each experiment was carried out in triplicate, and the average of the results of each experiment was used for statistical analysis [26].

For detection of the remaining concentration of phenanthrene, another aliquot of phenanthrene growing bacterial sample (5ml) was withdrawn. It was extracted twice with an equal volume of ethyl acetate and passed through anhydrous sodium sulphate to remove any traces of water. The ethyl acetate extract was then introduced for HPLC analysis. HPLC model Waters 600E equipped with autosampler waters 717 plus and dual wavelength UV detector model Waters 2487 (set at 254 nm) were used. The conditions of operation were as follows: Mobile phase: acetonitrile (A): water (W) isocratic program, HPLC grade Water %: 40% Acetonitrile % : 60%. Column: SUPELCOSIL ™ LC-PAH, 15 cm x 4.6 mm, 5μm Injection volume: 2.0 μl [23].

Different concentrations of phenanthrene (5-2000 mgml^{-1}) were dissolved in ethylacetate for plotting the calibration curve (concentration *versus* peak area). The peak area of known concentration of phenanthrene was determined by using HPLC with the same conditions mentioned above.

2.5. Quantification of Phenanthrene-degrading Genes Using qPCR

The copy number of naphthalene dioxygenase *nahAc* and catechol 2,3 dioxygenase *C23O* genes were determined as the method described by Park and Crowley [3]. Genomic DNA was extracted from an aliquot of 5 ml of phenanthrene growing bacterial cells each 3 days of incubation. The amount and purity of DNA were quantified by NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), based on the 260 nm absorption and the 260/280 ratio, respectively. The fragments of *nahAc* and *C23O* genes were amplified using the primer sets mentioned in Table **1** as published by Cébron *et al*. (2008), The PCR reaction mixture and annealing temperture as well as the expected band size are given in Table **1**.

nahAc and *C23O* genes were quantified using SYBR Green I qPCR. Firstly, the PCR products of *nahAc* and *C23O* were separately cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Then, the plasmids carrying the *nahAc* and *C23O* genes were extracted and purified using PureLink Quick Plasmid Miniprep Kit (Invitrogen Corporation, Carlsbad, CA). Then, the amount of *nahAc* and *C23O* genes in each aliquot was measured by conducting qPCR using the following program: an initial hold of 10 min at 95°C, followed by 35 cycles consisting of 45 s each of denaturation at 95°C, annealing at 57°C and extension at 72°C.

In order to quantify the genes copy number, 10-fold serial dilutions were prepared from the DNA of isolated strain AH-40 to construct five-point standard curves for both *nahAc* and *C23O* genes. These curves were designed by plotting the cycle of threshold (Ct) values *versus* log of initial *nahAc* or *C23O* copy numbers. After that, the Ct value of an aliquot sample (every 3 days of incubation) was adopted to calculate the number of *nahAc* and *C23O* gene copies.

The copy number of each *nahAc* and *C23O* genes per ml of plasmid solution was calculated based on the following equation:

$$\text{Copy number (molecules/ μl)} = \frac{DNA(g/μl)}{\text{Plasmid length (bp)} \times 660^a} \times A^b \qquad (1)$$

Where: 660^a = the average molecular weight of one base pair

A^b = Avogadro's number = 6.02×10^{23}

3. RESULTS AND DISCUSSION

3.1. Isolation, Molecular Identification and Phylogenetic Analysis of Bacteria

Ten bacterial isolates were isolated from oily sludge sample (Table **2**). These isolates were variable in shape arrangement and growth rate on Phen-MBS agar plates. Six isolates were gram -negative and four isolates were gram-positive. Based on fast growth on Phen-MBS agar plates, AH-40 was selected among the bacterial isolates for detailed studies as shown in Table **2**. This isolate showed a clear zone around its growing colony on the MBS agar with phenanthrene as a sole source of carbon. Based on the morphological shape and some biochemical activities, it was observed that AH-40 is short rod shaped, gram-negative, non-spore forming bacteria. It could produce catalase and oxidase enzymes. In addition, it is carbohydrate oxidative and/or fermentative bacteria.

A strain AH-40 was genetically identified based on the comparison of the 16S rDNA sequences and phylogenetic analysis. The alignment results showed that the 16S rDNA sequences of the strain AH-40 were highly homologous with 100% similarities to *Pseudomonas fluorescens* KU898262.1. To confirm the position of the bacterial strain AH-40 in phylogeny, the number of sequences representing some other *Pseudomonas* species were selected from the Genbank database for the construction of the phylogenetic tree. The tree indicated that AH-40 and *Pseudomonas fluorescens* KU898262.1 shared a one clad cluster (Fig. **1**). Therefore, the strain AH-40 was identified as *Pseudomonas fluorescens*.

Table 1.   Characteristics of PCR primer sets used in this study.

Primer	Sequence (5` to 3`)	Expected Size (bp)	Annealing Temperature (°C)	References
***nahAc*F**	5-`TGGCGATGAAGAACTTTTCC-3`	900	57	[3]
***nahAc*R**	5`-AACGTACGCTGAACCGAGTC-3`			
***C23O*F**	5`-AAGAGGCATGGGGGCGCACCGGTTCGA-3`	487	53	[43]
***C23O*R**	5`-TCACCAGCAAACACCTCGTTGCGGTTGCC-3`			

Table 2.   General morphological characters and some biochemical activities of phenanthrene degrading isolated bacterial strains form oily sludge sample.

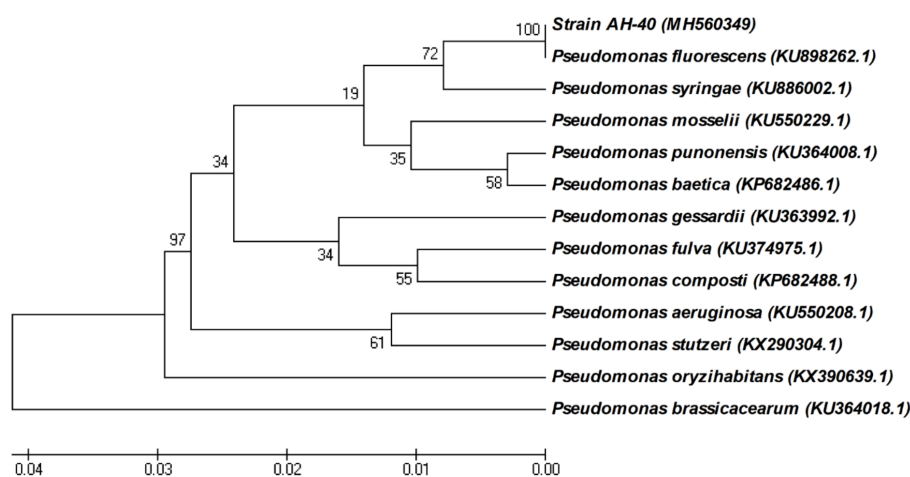| Isolates	Cell Shape	Clear Zone	Gram Staining	Capsule	Spore	Acid Fast	Catalase	Oxidase	Carbohydrates O/F
AH-05	short rods	-	-	-	-	-	+	+	O
AH-10	rods	-	-	+	-	-	+	-	O/F
AH-15	bacilli	-	-	-	+	-	+	-	-
AH-20	short rods	-	-	+	-	-	+	-	O
AH-25	filamentous	-	+	-	+	+	+	-	-
AH-30	rods	-	+	+	-	-	+	-	O
AH-35	bacilli	-	+	-	-	-	+	-	O/F
AH-40	**short rods**	+	-	-	-	-	+	+	**O/F**
AH-45	cocci	-	+	-	-	-	+	-	O
AH-50	rods	-	-	-	-	-	+	-	O/F

O; oxidative , F; fermentative.

Fig. (1). Phylogenetic relationship between strain AH-40 and other 16S rDNA sequences of published strains belonging to *Pseudomonas* sp. In the phylogenetic tree, AH-40 and *Pseudomonas fluoresences* were clustered together as one clade. GenBank accession numbers are given in parentheses.

The nucleotide sequence of 16S rDNA sequences of isolated strain AH-40 reported in this study has been deposited in DDBJ, EMBL, and GenBank nucleotide sequence databases under the name *Pseudomonas fluorescens* and accession Number: MH560349.

The 16S rDNA sequences and phylogenetic analysis were considered as powerful tools for the identification of bacterial isolates [7, 27]. Most PAHs bacterial degraders belong to the gram- negative group such as *Pseudomonas* sp., *Sphingmonas* sp. and *Achromobacter dentrificanse* [7, 10, 28]. *Pseudomonas* sp is one of the most popular bacterial species that could be isolated from different contaminated sources [29]. In addition, *Pseudomonas* sp can degrade many types of pollutants like PAHs [11, 23], Aliphatic hydrocarbons [30] and industrial dyes [29]. Moreover, Ortega-Calvo *et al*. [31] isolated phenanthrene degrading *P.fluorescens* from the soil and Abbasnezhad *et al*. [32] studied the en-

hancement of phenanthrene degradation by *P. fluorescens* LP6a when 1-dodecanol was added to the medium.

3.2. Growth Linked Phenanthrene Degradation Pattern

The data in Fig. (**2**) showed the capability of strain AH-40 to degrade 150 mgL-1 of phenanthrene within 15 days of incubation and consume it as a sole carbon and energy source for growth.

It was noticed that the bacterial growth exhibited a short lag stage (3 days) on phenanthrene followed by a long exponential one that extended from days 3 (3.3 x10^7) to 12 days (2.8x10^8 CFU/ml) incubation with logarithmic increase. Then, the growth showed a static trend.

Regarding the phenanthrene degradation rate, the HPLC results, plotted in Fig. (**2**), showed that there was no significant decrease in the concentration of phenanthrene results
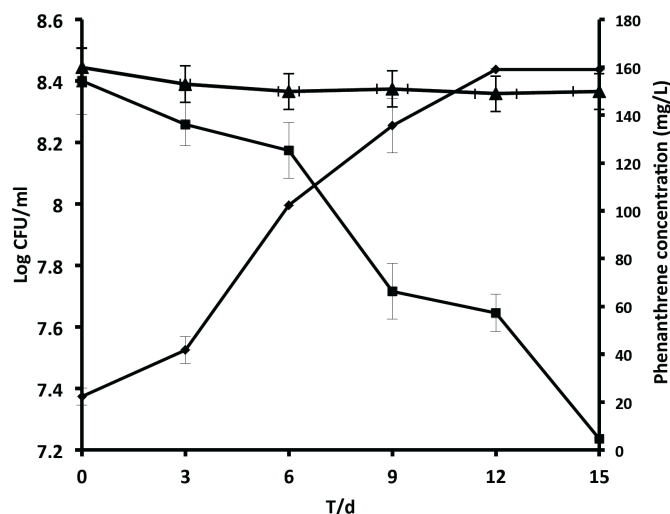
Fig. (2). The growth profile of strain AH-40 (Log CFU/ml) (◆), the remaining concentration of phenanthrene (mg/L) (■) during the incubation period of 15 days at 30°C. Phenanthrene concentration in negative control (▲) Error bars represent the standard deviation (SD±) of three replications.

from the initial time to 6 days of incubation. On the other hand, it significantly ($P < 0.05$) decreased gradually to reach 4.6 ± 0.6 mg/L^{-1} of phenanthrene after 15 days of incubation compared to the remaining phenanthrene concentration results at 6 days (125.3 ± 11 mg/L^{-1}) as well as the negative control samples. Moreover, it was noticed that the concentration of phenanthrene (mg/L^{-1}) in negative control samples was not significantly changed during 15 days of incubation as illustrated in Fig. (**2**).

The results also showed that the increase in the bacterial number was followed by the decrease in the remaining concentration of phenanthrene. The maximum growth was recorded after 12 days however the maximum phenanthrene degradation was detected after 15 days.

The degradation of phenanthrene was initially slow due to its hydrophobic nature which restricts its availability to the bacterial cells. However, after initial degradation, the polarity was probably introduced into phenanthrene by extracellular enzymes such as dioxygenases produced by bacterial strain to enhance its availability as previously explained by Kumar *et al*. [33]. Some bacterial strains, such as *Achromobacter denitrificans,* produced biosurfactant and/or increased their cell surface hydrophobicity in order to overcome the hydrophobicity of PAHs compounds as previously mentioned by Mawad *et al*. [7].

There were several reports available on the bacterial utilization of phenanthrene as a source of carbon and energy by pure bacterial strains [6, 10, 34]. The percentage of phenanthrene degradation (97%) by bacterial strain AH-40 used in this study was higher than the phenanthrene degradation percentage mentioned in otherliterature studies. *Trametes versicolor* degraded 76.7% of phenanthrene when the initial concentration was 100mg/L [35]. *Pseudomonas* strain BZ-3 degraded 75% of phenanthrene at an initial concentration of 50 mg/L^{-1} 7 days [36]. Ca-alginate-immobilized *Sphingomonas pseudosanguinis* strain J1-q (S1) removed 63.2 of phenanthrene within 42 days [37].

Therefore, it can be concluded that there was a close relationship between the degradation of phenanthrene and bacterial growth. The increase in the number of bacterial strain AH-40 enhanced the degradation of phenanthrene.

3.3. Copy Number of *nahAc* and *C23O* Genes

Figs. (**3** and **4**) showed the copy number of *nahAc* and *C23O* genes along 15 days (3 days intervals) of phenanthrene degradation that expressed in copies/ml of spiked sample.

The results in Fig. (**3**) showed that the copy number of *nahAc* gene was not uniform and regular along the 15 days of incubation. It increased at 3 days then decreased after 6 days. From 6 days to 12, the copy number of *nahAc* gene increased by a hundred times. Then, it decreased 10 times at 15 days.

On the other hand, the amount of the *C23O* gene started to increase at 3 days. The copy number of the *C23O* gene increased by over a thousand times at 6 days and remained at this level up to 12 days. After 12 days, the copy number of the *C23O* gene decreased 10 times at 15 days as shown in Fig. (**3**).

It was noticed that the maximum amount of *C23O* gene was 3.5×10^{11} copies/ ml at 9 days of degradation while the maximum amount of *nahAc* was 1.5×1011copies/ml at 12 days. Also, the amount of *nahAc* gene exceeded *C23O* by 6 fold at 3rd day of degradation.

A gene of *nahAc* encoding components of the naphthalene ring-hydroxylating dioxygenase was reported to catalyze the incorporation of both atoms of molecular oxygen into adjacent positions of an aromatic ring [38, 39]. In addition, the high amount of *C23O* gene indicated that meta cleaving was the major pathway for catechol degradation [40].

Nyyssönen *et al*. [41] detected 4.5×10^6 *nahAc* gene copies /g dry weight soil by Real-Time PCR. Similarly, 7×10^3 copies / g dry weight soil were determined in petroleum
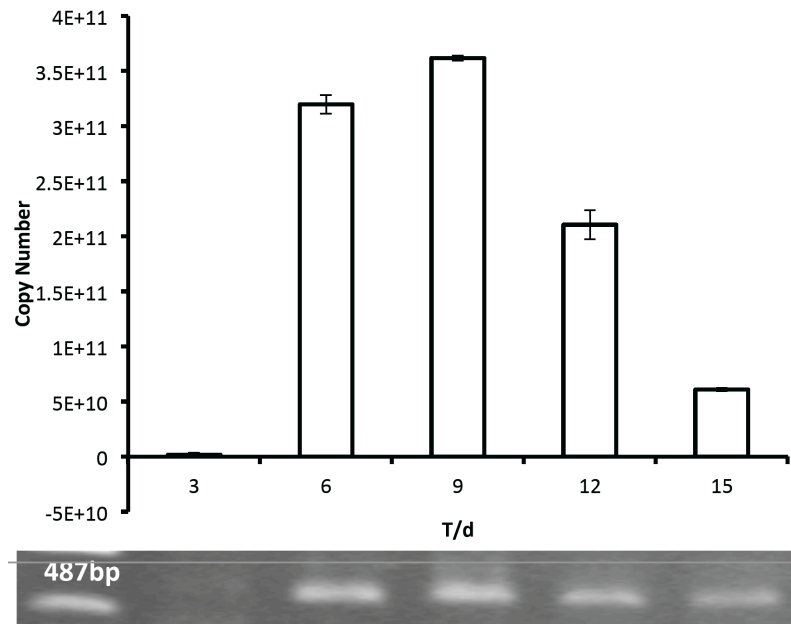
Fig. (3). Agarose gel electrophoresis of PCR products amplified with *Nah-AC* specific primer sets using the genomic DNAs from strain AH-40. The copy number *nahAC* gene for the 5 samples during phenanthrene degradation in 15 days incubation period. Error bars indicate the standard deviation of the two independent PCR runs.
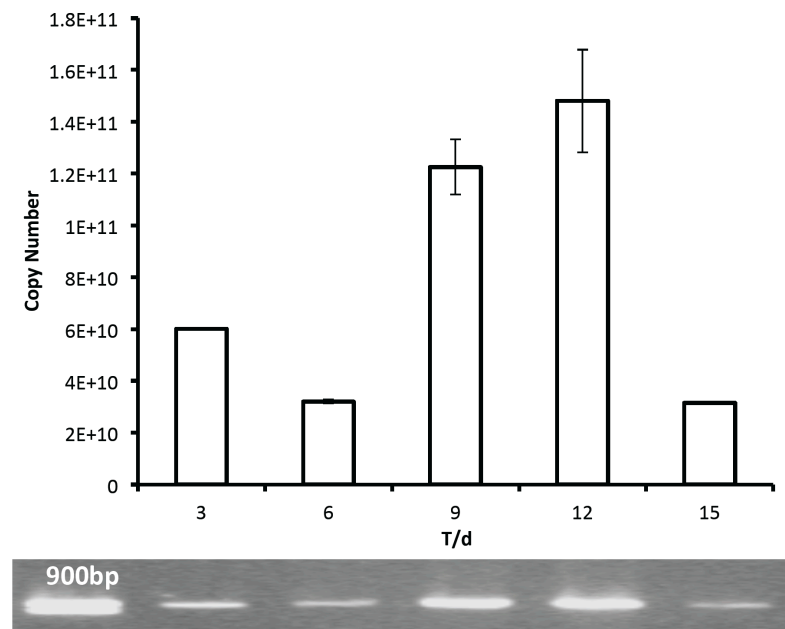

Fig. (4). Agarose gel electrophoresis of PCR products amplified with *Nah-AC* specific primer sets using the genomic DNAs from strain AH-40. The copy number *C23O* gene for the 5 samples during phenanthrene degradation in 15 days incubation period. Error bars indicate standard deviation (SD±) of the two independent PCR runs.

hydrocarbon-contaminated soil [42]. Okuta *et al.* [43] also determined the overexpression of catechol 2,3- dioxygenase in BTEX contaminated environment.

It can be concluded that there is a proportional relation between the level of phenanthrene degradation and the concentration of *nahAc* gene. The *nahAc* and *C23O* genes could be used as an indicator of phenanthrene degradation.

CONCLUSION

The microbial decomposition is a safe and cost-effective process for the removal of PAHs from sediments and surface soils. The present study reveals that *Pseudomonas fluorescence* AH-40 is capable of removing 97% phenanthrene when the initial concentration was 150 mg/L^{-1}. The bacteria could consume phenanthrene as a sole source of carbon to

increase their cell number for 15 days. The *nahAc* and *C23O* genes are considered the main marker genes for the degradation of phenanthrene by strain AH-40. Therefore, *P. fluorescence* AH-40 could be recommended for bioremediation of phenanthrene contaminated site.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available in the GenBank nucleotide sequence database at [https://www.ncbi.nlm.nih.gov/nuccore/MH560349.1?report=GenBank], reference number [MH560349.1].

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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