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DEGRADATION OF PHENANTHRENE AND PYRENE USING GENETICALLY ENGINEERED DIOXYGENASE PRODUCING *Pseudomonas putida* IN SOIL

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Bioremediation use to promote degradation and/or removal of contaminants into nonhazardous or less-hazardous substances from the environment using microbial metabolic ability. *Pseudomonas spp.* is one of saprotrophic soil bacterium and can be used for biodegradation of polycyclic aromatic hydrocarbons (PAHs) but this activity in most species is weak. Phenanthrene and pyrene could associate with a risk of human cancer development in exposed individuals. The aim of the present study was application of genetically engineered *P. putida* that produce dioxygenase for degradation of phenanthrene and pyrene in spiked soil using high-performance liquid chromatography (HPLC) method. The *nahH* gene that encoded catechol 2,3-dioxygenase (C23O) was cloned into pUC18 and pUC18-nahH recombinant vector was generated and transformed into wild *P. putida*, successfully. The genetically modified and wild types of *P. putida* were inoculated in soil and pilot plan was prepared. Finally, degradation of phenanthrene and pyrene by this bacterium in spiked soil were evaluated using HPLC measurement technique. The results were showed elimination of these PAH compounds in spiked soil by engineered *P. putida* comparing to dishes containing

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natural soil with normal microbial flora and inoculated autoclaved soil by wild type of *P. putida* were statistically significant ($p<0.05$). Although adding N and P chemical nutrients on degradation ability of phenanthrene and pyrene by engineered *P. putida* in soil were not statistically significant ($p>0.05$) but it was few impact on this process (more than 2%). Additional and verification tests including catalase, oxidase and PCR on isolated bacteria from spiked soil were indicated that engineered *P. putida* was alive and functional as well as it can affect on phenanthrene and pyrene degradation via *nahH* gene producing. These findings indicated that genetically engineered *P. putida* generated in this work via producing C23O enzyme can useful and practical for biodegradation of phenanthrene and pyrene as well as petroleum compounds in polluted environments.

Keywords: catechol 2,3-dioxygenase, HPLC, phenanthrene, pyrene, *Pseudomonas putida*

Abbreviations: *Pseudomonas putida*: *P. putida*; C23O: Catechol 2,3-dioxygenase; HPLC: High-performance liquid chromatography; GEMs: Genetically engineered microorganisms

INTRODUCTION

In recent decades, genetically engineered microorganisms (GEMs) have been used for many approaches such as bioremediation. Bioremediation is a degradation of environmental pollutants by microorganisms (including yeast, fungi, or bacteria) or their enzymes to remove or neutralize pollutants from a contaminated site to provide an economic and safe alternative (HARMS *et al.*, 2011; KUMAR *et al.*, 2011). This process can be done in aerobic and anaerobic conditions. Bioremediation is safer and cheaper than conventional physicochemical treatments (VIDALI, 2001; THAPA *et al.*, 2012). *Bacillus*, *Mycobacterium*, *Alcaligenes*, *Flavobacterium*, and *Pseudomonas* genera are common bacteria in oil degradation activity (KARIGAR and RAO, 2011; DAS and CHANDRAN, 2011). *Pseudomonas putida* (*P. putida*) is a member of *Pseudomonadaceae* family, Gram-negative, flagellated and rod-shaped gamma-proteobacterium that is found throughout various environments such as soil and freshwater environments (WU *et al.*, 2011; THOMAS *et al.*, 2013).

For increasing and developing the bioremediation activity of *Pseudomonas* spp. molecular engineering techniques can be used (ZUO *et al.*, 2015). One of the important enzymes in the *Pseudomonas* spp. use for degradation of polycyclic aromatic hydrocarbons (PAHs) is catechol 2,3-dioxygenase (C23O). Dioxygenases are non-heme iron enzymes that biodegrade recalcitrant compounds, such as catechol and derivatives, released into the environment by modern industry (MESQUITA *et al.*, 2013). This enzyme catalyzes the conversion of catechol to 2-hydroxymuconic semialdehyde by breaking the C–C bond at the 2,3-(meta) position, which is part of the α-ketoadipate pathway. The functional enzyme is composed of large (α) and small (β) subunits (ZHAO *et al.*, 2011). A *nahH* gene exist in some *Pseudomonas* strains and consist of 924 nucleotides that encoded catechol 2,3-dioxygenase with 36 kDa molecular weight containing 307 amino acid residues (JIANG *et al.*, 2004; CHANG *et al.*, 2015). This gene is one of important genes of naphthalene catabolic plasmid NAH7 in *P. putida* (NEILSON *et al.*, 1999). This plasmid has 83-kb length with two clusters and containing nah1 and nah2 operons and it's related to growth of *Pseudomonas* on polluted environment by naphthalene as a sole carbon source (HEDLUND *et al.*, 2001; COITINHO *et al.*, 2011).

PAHs are environmental contaminants and ubiquitously found as soil, air and water contaminants (ARBABI *et al.*, 2009; CHOUYCHAI *et al.*, 2009). These compounds could be release during incomplete combustion of natural processes like burning coal and wood or forest fires as well as organic materials during industrial and other human activities such as combustion of natural gas, aluminium smelters, vehicle traffic, cooking, tobacco smoking, urban sewage, garbage, power plants chemical, asphalt works, petrochemical and oil industries (MAHVI and MARDANI, 2005; NASSERI *et al.*, 2010; MAHVI *et al.*, 2015). Fluoranthene, chrysene, fluorene, phenanthrene, and pyrene are important PAH compounds (MAHVI and MARDANI, 2005; MORDUKHOVICH *et al.*, 2010). PAHs are associated with health hazardous and can react with pollutants such as ozone, nitrogen dioxide, hydroxyl radicals, and sulfur dioxide in the atmosphere (SROGI *et al.*, 2007). PAHs are carcinogenic, mutagenic, and teratogenic substances (MAHVI and MARDANI, 2005; NASSERI *et al.*, 2010). Biological degradation of these compounds in pollutant environments using genetically modified bacteria is significant. The aim of this study was to evaluate the capability of genetically engineered dioxygenase producing *P. putida* for degradation of phenanthrene and pyrene in polluted soil using high-performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Bacterial strains and growth conditions

This experimental study was conducted from January, 2015 up to July, 2015. The lyophilized stocks of *E. coli* Top10F and *P. putida* strain ATCC 12633 (Persian Type Culture Collection (PTTC) No. 1694) were obtained from Pasteur Institute of Iran and Iranian Research Organization for Science and Technology (IROST), respectively. The bacterial stocks were cultured in Luria-Bertani (LB) broth and incubated for 24 hours at 37˚C and 26˚C for *E. coli* and *P. putida*, respectively. For antibiotic susceptibility testing both bacterial strains were cultured on LB agar medium containing Ampicillin (50 to 150 mg/mL) and Kanamycin (25 mg/mL) antibiotics. Antibiotics resistance testing was showed that *E. coli* Top10F do not have any resistance to these antibiotics. *P. putida* strain ATCC 12633 had no resistance to Kanamycin, but Ampicillin at concentration of 1500 X (150 mg per mL of culture medium) was suitable for bacterial transformation.

Bacterial DNA extraction

For bacterial DNA isolation from *P. putida* boiling method was used. First, the bacterium was cultured in 5 mL of LB broth medium at 26˚C for 24 hours and then was centrifuged for 10 minutes in the 6000 rpm. The culture supernatant was discarded and bacterial sediment was mixed in 120μL water injection and was placed in water bath for 15 minutes at 95˚C. Finally, the suspension was centrifuged for 10 minutes at 13000 rpm for DNA extraction. The supernatant containing bacterial DNA were transferred to new micro-tube and the concentration and quality of extracted bacterial DNA was measured and quantified by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) at a wavelength of 260/280 nm according to the method described by SAMBROOK and RUSSELL, 2001. Extracted DNA was stored in a freezer at -20˚C for further testing.

Gene amplification

The sequence of *nahH* gene of NAH7 plasmid was obtained from NCBI and primers for gene amplification were designed using software Oligo Primer Analysis Software version 7 and Gene Runner version 3.05 and accuracy of binding of primers to target gene were analyzed by basic local alignment search tool (BLAST) in GenBank data and DNASTAR Lasergene version 10 software. Sequence of primers and their amplified fragment length is shown in Table 1.

Table 1. The sequence of primers used for nahH gene amplification

Gene	Primers name	Sequence	Product length (bp)	Accession number
nahH	NahH-F	5´ATGAACAAAGGTGTAATGCGC-3´	924	X06412
	NahH-R	5´-TTAGGTCATAACGGTCATGAATC-3´		

PCR reaction was performed in a total volume of 25 µL containing 2.5 µL of 10X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP mix (all Fermentas, Germany), 1 µg of isolated bacterial DNA or purified plasmid, 0.25 µM of each primer, and 1 unit of *Taq* DNA polymerase (Roche Applied Science, Germany). The negative control contained all reagents without DNA (2 µL of sterile ultrapure deionized water was used instead). PCR amplification was performed in a Thermal Cycler Astec Co, Ltd under the following conditions: initial denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 50 s, annealing at 62°C for 50 s, extension at 72°C for 50 s, and final extension (72°C for 5 min) was done at the end of the amplification. For confirmation the existence of amplified *nahH* gene (924 bp) 6 µL of PCR products and 1 kb DNA ladder (Fermentas, Germany) were applied to the 2% agarose gel electrophoresis to determine the length of the amplified fragments. After electrophoresis at 80 V for 30 min, the ethidium bromide gel staining was done and gel was examined using UVIdoc gel documentation system (Uvitec, UK) for image obtaining.

Plasmid and engineered vector preparation

Vector pUC18 with catalog number: SD0051 was purchased from Thermo Fisher Scientific Inc., Germany. This cloning and expression vector is a small, high copy number, with 2686 bp in length and *Pseudomonas* and specially *E. coli* are important hosts of this vector. Plasmid sequence was obtained from Addgene cite and restriction enzyme patterns were identified using DNASTAR® Lasergene 10 software. The plasmids were transformed in *E. coli* Top10F strain using T/A Cloning Kit (Fermentas, Germany) according to the manufacturer's protocol for preparing necessary amount of plasmid. Since, the PCR on extracted DNA from *P. putida* showed that *nahH* gene (encoding catechol 2,3-dioxygenase) and NAH7 plasmid was not exist in this bacterium. Therefore, *nahH* gene synthesis and gene cloning into pUC18 vector was ordered to GeneCust Europe (Dudelange, Luxembourg) and engineered pUC18-nahH recombinant vector was sent to Iran. The schematic diagram of engineered pUC18 vector containing *nahH* gene inserted between *Eco*RI and *Bam*HI restriction sites is shown in Figure 1.

Cloning verification of pUC18 vector containing *nahH* gene were done by GeneCust Europe using enzymes digestion (*Eco*RI and *Bam*HI restriction enzymes) and sequencing method.

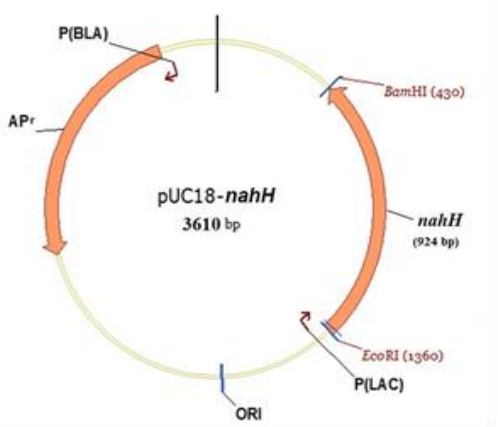

Figure 1. Schematic diagram of recombinant pUC18 plasmids containing *nahH* gene

Transformation of pUC18-nahH recombinant vector

First pUC18-nahH recombinant vector were transformed into *E. coli* Top10F using Ins TA Clone PCR Cloning Kit (Thermo Scientific, Lithuania) according to the manufacturer's instruction to obtain necessary amount of vector. Then, plasmids were extracted from bacterial cells using GeneJET Plasmid Miniprep (Fermentas, Germany) according to the manufacturer's protocol. After confirmation of transformation of recombinant vector by *Hind*III enzyme digestion and PCR technique, the engineered plasmid was transformed again into *P. putida* (150 mg per mL of Ampicillin was used in culture media) using above mentioned kit. After plasmid purification from engineered *P. putida* (containing the recombinant vector) and negative control (non-engineering *P. putida*), for determination the presence of the vector containing *nahH* gene PCR technique was used.

Soil preparation and characteristics

A sandy-clay soil with poorly graded clay mixture from the depth of 50 Cm in sterile dishes was collected from Iran with latitude N 31° 45′08″ and longitude E 50° 33′04″. This soil compositions including gravel, 1%; sand, 73%; silt, 15%; clay, 11%; moisture, 30%; organic carbon, 6.45%; characterized with pH 6.9, bulk density, 0.8%; uniformity coefficient (Uc), 4.1; coefficient of curvature (Cc), 0.45; effective size, 0.085 mm. In this study both autoclaved and natural soil was used.

Soil spiking for pilots preparing

In the present work, soil spiking was performed according to the method of BRINCH *et al.*, (2002). Phenanthrene and pyrene that used in this study were obtained from Merk Co., Germany with a purity of 99.9%. First, all equipments and dishes were autoclaved. 500 gr of autoclaved and natural soil were added to each dishes. For spiking 25% fractions (125 g) of the soil sample were used. Phenanthrene (500 mg/kg) and pyrene (500 mg/kg) separately or combined were added into 100 mL acetone (Merk, Germany) as solvent and were shacked for

each dishes. Each solution was added to 125 g of soil specimen and mixed completely. This mixture for evaporation of solvent was maintained at ambient temperature for 16 hours. Finally, each treated soils were mixed completely with the remaining 75% (375 gr) of the soil sample for pilot preparation.

Culture of P. putida for soil inoculation

Engineered and wild *P. putida* (non-engineered) were cultured in 900 mL of LB broth medium (containing 150 mg per mL of Ampicillin antibiotic) and in 150 mL medium without antibiotic, respectively. The media were incubated at 26°C and 220 rpm for 24 hours in a shaking incubator. Optical density of each cultured bacteria were measured by a spectrophotometer (UNICO UV-2100, Shanghai, China) at 600 nm. A *P. putida* cell density of 8×10^8 to 1.2×10^9 CFU/mL was used to inoculate of soil. Then, in 50 mL Falcon the precipitation of engineered and wild type bacteria was taken separately by centrifuging at 6000 rpm for 10 min. The supernatant was discarded, but 5 mL of medium was remained for feeding and adaptation of bacteria. Finally, sedimentation of bacteria was resuspended in an equal volume of isotonic saline solution (containing 0.9% NaCl) and 150 mL of this solution containing bacteria was soaked to each dish. The pilot plan is shown in Table 2. In dish 5 chemical nutrients including nitrogen (N) and phosphor (P) was used for helping the better growing and degradation of PAHs by engineered *P. putida*. Dishes 6 and 8 containing soil with natural microbial flora (non sterile soil) for comparison and evaluation the effects of bacteria for degradation of PAH compounds in natural conditions.

Table 2. Pilot plan used for investigation the degradation of phenanthrene and pyrene by genetically engineered dioxygenase producing P. putida in spiked soil

Pilot plan Dishes contain 500 g soil, 30% moisture	
Dish	Components
1	Pyrene and phenanthrene 500 mg/kg Autoclaved soil, evaporation percent
2	Phenanthrene 500 mg/kg Ps. GEMs + Autoclaved soil
3	Pyrene 500 mg/kg Ps. GEMs + Autoclaved soil
4	Pyrene and phenanthrene 500 mg/kg Ps. GEMs + Autoclaved soil
5	Pyrene and phenanthrene 500 mg/kg Ps. GEMs + Autoclaved soil + Nutrients N, P
6	Pyrene and phenanthrene 500 mg/kg Ps. GEMs + Soil with natural microbial flora
7	Pyrene and phenanthrene 500 mg/kg Autoclaved soil + wild *P. putida*
8	Pyrene and phenanthrene 500 mg/kg Soil with natural microbial flora

Ps. GEMs: genetically engineered *P. putida* microorganisms

Sampling and extraction method

Before soil inoculation with bacteria and after this process sampling and extraction for measurement of evaporation and degradation of phenanthrene and pyrene from each dish were done. Sampling and extraction for evaluation the degradation of phenanthrene and pyrene by engineered *P. putida* in dishes were performed in each 5 days for three months. Two gr mix soil from each dish were sampled and dried at room temperature for 12 h. Soil was worn out and 2 mL acetonitrile added and phenanthrene and pyrene were extracted using ultrasonic bath sonicator (CD-4820, China) for 30 min. Then, samples were centrifuged at 3000 rpm for 10 min and supernatant using polytetrafluorethylene (PTFE) membrane filter were filtered. In final, 20 µL of each extracted samples were injected to HPLC.

HPLC analysis

In this study, standard solutions of measurement method of HPLC for phenanthrene and pyrene used as a serial dilution concentration were included 100, 50, 25, 12.5, and 6.25, respectively (Table 3). Then, calibration curve for phenanthrene and pyrene was prepared with correlation coefficient equivalent 0.99998 and 0.99937, respectively (Figure 2). Mobile phase of HPLC was included 80% acetonitrile and 20% H_2O with flow rate 1 mL per min, detector UV scan at wavelength equivalent 254 nm.

Table 3. Serial dilution concentrations used for standard solutions of measurement method in HPLC

Signal 1: VWD1 A, Wavelength=254 nm

RetTime [min]	Sig	Lvl	Amount [ng/ul]	Area	Amt/Area	Ref	Grp	Name
3.372	1	1	6.25000	2175.24000	2.87325e-3			Phenanthrene
		2	12.50000	4591.74000	2.72228e-3			
		3	25.00000	9245.23000	2.70410e-3			
		4	50.00000	1.82122e4	2.74541e-3			
		5	100.00000	3.64239e4	2.74545e-3			

Peak Sum Table

Signal 1: VWD1 A, Wavelength=254 nm

RetTime [min]	Sig	Lvl	Amount [ng/ul]	Area	Amt/Area	Ref	Grp	Name
4.415	1	2	6.25000	643.75281	9.70870e-3			Pyrene
		3	12.50000	1294.51270	9.65614e-3			
		4	25.00000	2450.40234	1.02024e-2			
		5	50.00000	4774.40430	1.04725e-2			
		6	100.00000	8957.70020	1.11636e-2			

Peak Sum Table

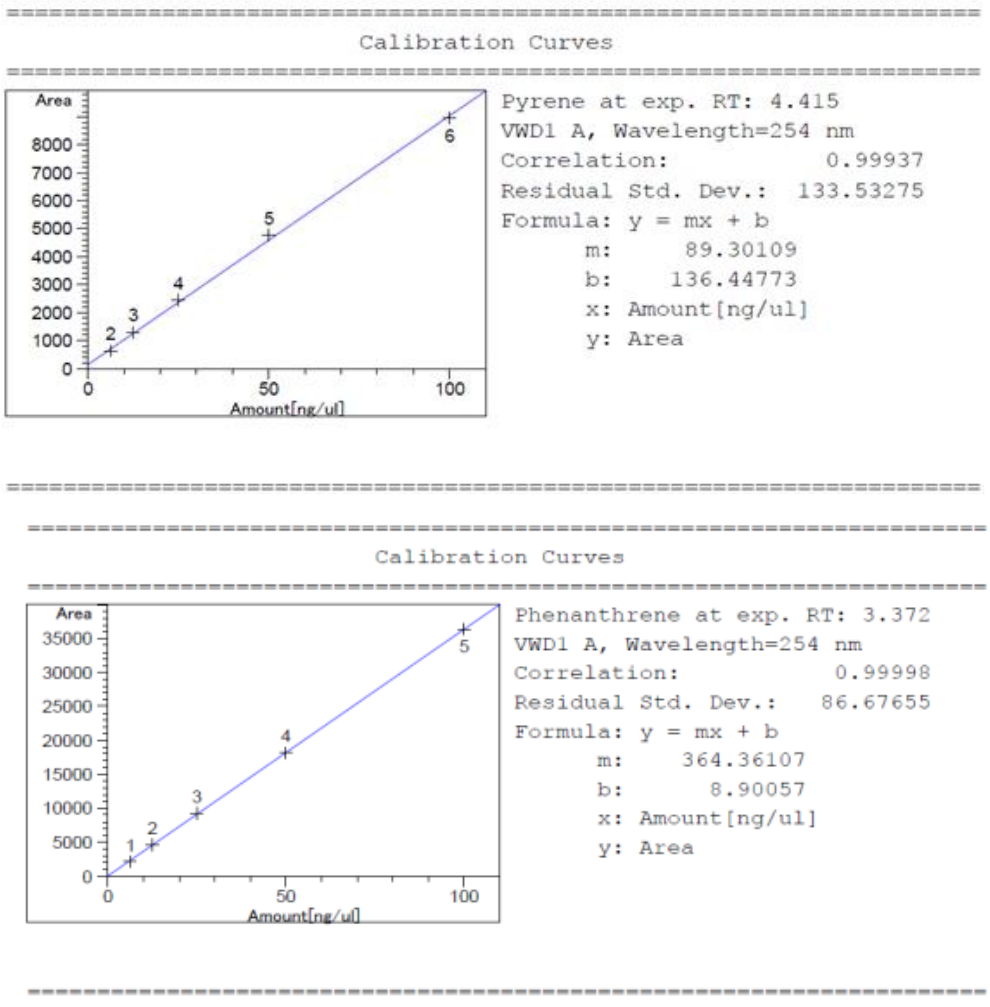

Figure 2. HPLC calibration curves of phenanthrene and pyrene

Confirmatory tests

Every 30 days from beginning 1 gr of contaminated soil by phenanthrene and pyrene containing the engineered *P. putida* was mixed in sterile water injection and passed it through from MESH filter to achieve soil extraction. Then, 40 µL of soil extraction in sterile conditions were cultured on LB agar plates containing ampicillin (150 mg per mL) for 24 hours at a temperature of 26°C to obtain colonies. CFU (Colony Forming Unit) were determined every 10 days from 1 gr contaminated soil by serial dilution in sterile saline and were plated by drop plating and culturing on LB agar media. Plate count agar using Quebec colony counter (Bibby Sterilin Ltd, Stratfordshire, UK) was done and colony counts expressed as CFU g −1. The

colonies were used for catalase and oxidase tests as well as DNA extraction and *nahH* gene amplification by PCR. In catalase test some of colonies of the cultured bacteria were mixed in fresh drop of hydrogen peroxide (H_2O_2) 3% and the release of oxygen indicates that the test is positive. In addition, some colonies of *P. putida* for oxidase test were transferred on filter paper soaked with tetra-methyl-p-phenylenediamine dihydrochloride solution and production of indophenol and purple color indicates the test is positive.

Statistical analysis

All studies were performed in at least three times and data were collected in Statistics programs for the Social Sciences software, version 20 (SPSS, Inc., Chicago, IL, USA). The differences between the degradation of phenanthrene and pyrene by engineered *P. putida* in the period of experiment were analyzed using one-way ANOVA (Analysis of variance) followed by Tukey HSD post-hoc test for comparing the significant difference among means of various treatments. The *p*-value less than 0.05 (5%) were considered statistically significant between groups.

RESULTS

Cloning confirmation

Cloning verification of *nahH* gene in pUC18 vector was confirmed by *BamH*I and *EcoR*I restriction enzymes digestion (Figure 3) and indicating the presence of the gene in vector. Furthermore, in order to verify the gene synthesis and cloning of the *nahH* gene, engineered plasmid (pUC18-nahH) was sequenced by GeneCust Europe (Dudelange, Luxembourg) using Sanger sequencing method (Figure 4).

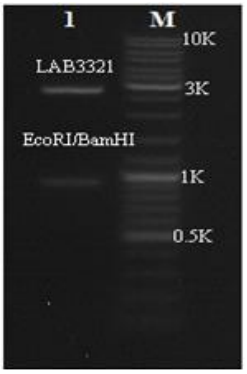

Figure 3. Digestion of pUC18 recombinant vector contain *nahH* gene using restriction enzymes for cloning confirmation (Lane M is 100 bp molecular weight markers (Fermentas, Germany), and lane 1 is digested pUC18 recombinant vector (2686 and 924 bp) by *BamH*I and *EcoR*I restriction enzymes, respectively)

After extraction of pUC18 recombinant plasmids containing *nahH* genes from engineered *E. coli*, accuracy of transformation using PCR method and *Hind*III enzyme digestion (were revealed 3004 and 606 bp fragments) was evaluated and indicated the successfully transformation (Figures 5 and 6).

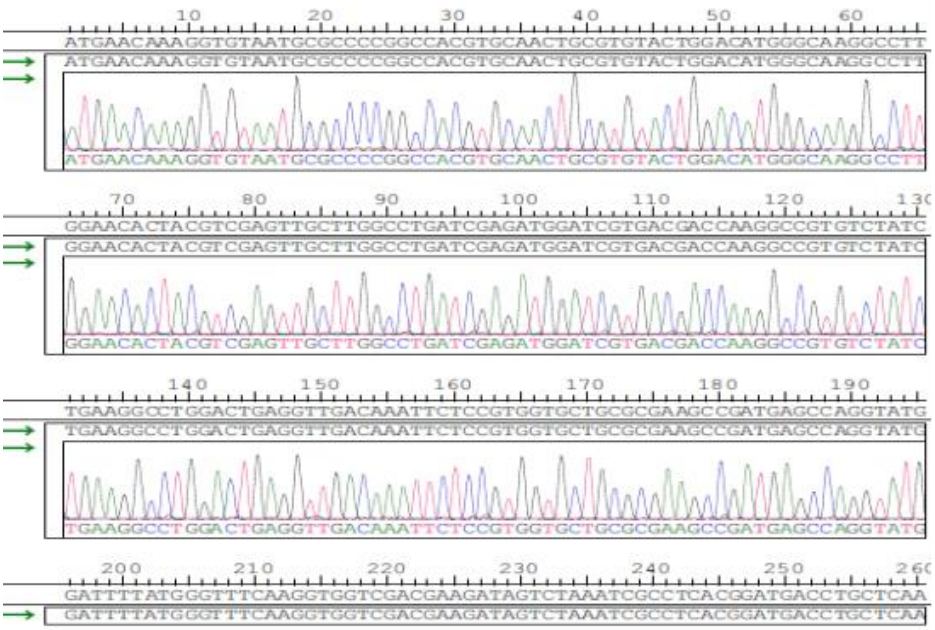

Figure 4. A diagram's of *nahH* gene sequencing for confirmation of gene synthesis and cloning in pUC18 vector

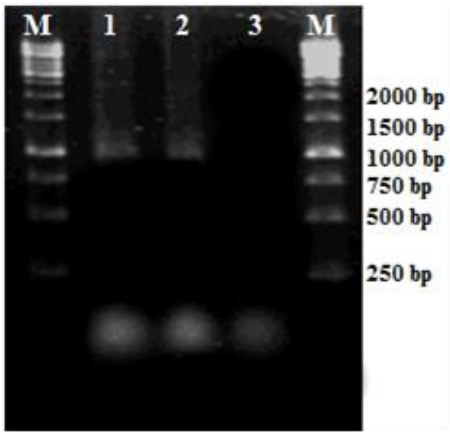

Figure 5. Amplified *nahH* gene of isolated recombinant pUC18 vector from engineered *E. coli* on 1% agarose gel electrophoresis (Lane M is 1 kb molecular ladder (Fermentas, Germany), lanes 1 and 2 are amplified gene (924 bp), and lane 3 is negative control (without DNA), respectively)

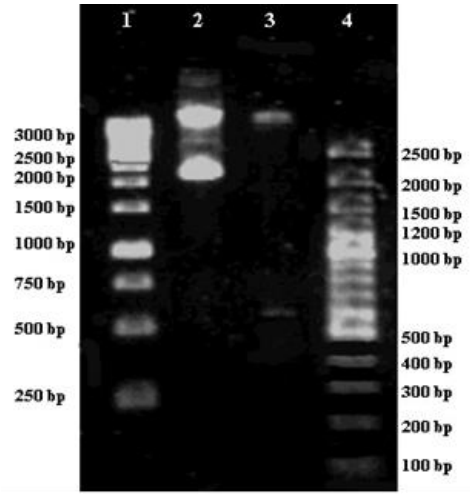

Figure 6. An 2% agarose gel electrophoresis for confirmation of recombinant pUC18 plasmid containing *nahH* gene by *Hind*III restriction enzyme digestion (Lanes 1 and 4 are 1 kb and 100 bp molecular weight markers (Fermentas, Germany), respectively, lane 2 is uncut recombinant vector (3610 bp), lane 3 is digested recombinant vector by *Hind*III restriction enzyme (3004 and 606 bp fragments), respectively)

Recombinant vector (pUC18- nahH) using Ins TA Clone PCR Cloning Kit was transformed again into *P. putida*, successfully. DNA was extracted from engineered *P. putida* and negative control (non-engineering *P. putida*) and PCR technique for amplification of *nahH* gene was positive (924 bp) in engineered bacteria (Figure 7).

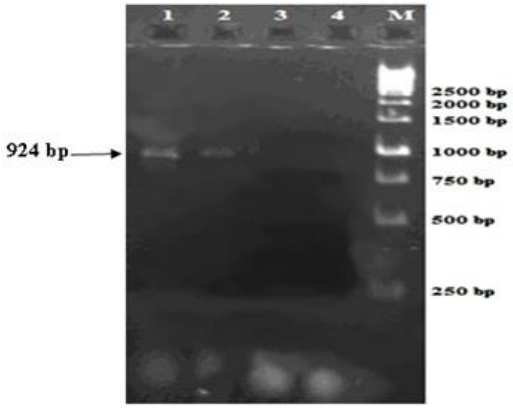

Figure 7. An 1% agarose gel electrophoresis for confirmation of *P. putid* transformation (Lane M is 1 kb molecular ladder (Fermentas, Germany), lanes 1 and 2 are amplified *nahH* gene (924 bp) of engineered *P. putida*, line 3 is wild *P. putid* strain, and line 4 is negative control (no DNA), respectively)

HPLC analyzing data

The degradation of phenanthrene and pyrene by engineered *P. putida* in dishes in each 5 days for three months was analyzed by HPLC technique. Data were evaluated using one-way ANOVA followed by Tukey HSD post-hoc test for comparing the significances (Tables 4 and 5). After evaluation the evaporation of these PAHs compounds in dishes and according to the graphs are shown in Figures 8 and 9, degradation of phenanthrene and pyrene in polluted soil by engineered *P. putida* comparing to dishes inoculated by wild type of *P. putida* as well as dish containing natural microbial flora were statistically significant ($p<0.05$).

Table 4. Comparison the degradation of phenanthrene by natural microbial flora, engineered and wild P. putida, and evaporation of this compound in polluted soil by one-way ANOVA and Tukey post-hoc test

Dishes	Groups	Mean difference ± SE	Sig. *p*-value**
Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	-65.281* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	-64.141* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-65.647* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	-62.575* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	-6.445 ± 8.142	0.985
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	-5.423 ± 8.142	0.994
Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	65.281* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	1.139 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-0.366 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	2.705 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	58.835* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	59.857* ± 8.142	0.000

Dishes	Groups	Mean difference ± SE	Sig. *p*-value**
Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	64.141* ± 8.142	0.000
	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	-1.139 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-1.506 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	1.566 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	57.696* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	58.718* ± 8.142	0.000
Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	65.647* ± 8.142	0.000
	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	0.366 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	1.506 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	3.071 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	59.202* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	60.223* ± 8.142	0.000
Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Soil with Natural Microbial Flora (6)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	62.576* ± 8.142	0.000
	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	-2.705 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Autoclaved Soil (4)	-1.566 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Autoclaved Soil + Nutrients N, P (5)	-3.071 ± 8.142	1.000
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	56.13* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	57.152* ± 8.142	0.000

Dishes	Groups	Mean difference ± SE	Sig. *p*-value**
Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + *Pseudomonas* (7)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	6.445 ± 8.142	0.985
	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	-58.835* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Autoclaved Soil (4)	-57.696* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-59.202* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	-56.13* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	1.022 ± 8.142	1.000
Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	5.423 ± 8.142	0.994
	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	-59.857* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	-58.718* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-60.228* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	-57.152* ± 8.142	0.000
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	-1.022 ± 8.142	1.000

*The mean difference is significant at p<0.05 by one-way ANOVA.

**The significant difference among means of various treatments in each groups using post-hoc Tukey test (p<0.05).

SE: Std. Error

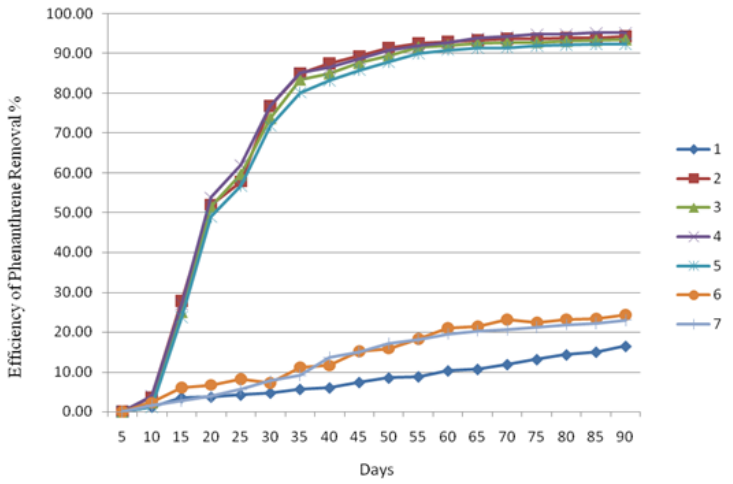

Figure 8. Comparison of phenanthrene removal measured in each dish every 5 days

Table 5. Comparison between pyrene evaporation and degradation of this compound in each dishes analyzed by one-way ANOVA and Tukey post-hoc test

Dishes	Groups	Mean difference ± SE	Sig.*p*-value**
Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	Pyrene 500mg/kg + Ps.GEMs + Autoclaved Soil (3)	-53.8869113* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	-37.542* ± 8.688	0.001
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-48.6* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	-28.733* ± 8.688	0.021
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	-2.735 ± 8.688	1.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	-8.758 ± 8.688	0.951
Pyrene 500mg/kg + Ps.GEMs + Autoclaved Soil (3)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	53.887* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	16.344 ± 8.688	0.497
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	5.286 ± 8.688	0.996
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	25.154 ± 8.688	0.066
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	51.152* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	45.128* ± 8.688	0.000

Dishes	Groups	Mean difference ± SE	Sig.*p*-value**
Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	37.542* ± 8.688	0.001
	Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (2)	-16.344 ± 8.688	0.497
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-11.058 ± 8.688	0.863
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	8.81 ± 8.688	0.95
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	34.808* ± 8.688	0.002
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	28.784* ± 8.688	0.02
Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	48.6* ± 8.688	0.000
	Pyrene 500mg/kg + Ps.GEMs + Autoclaved Soil (3)	-5.286 ± 8.688	0.996
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	11.058 ± 8.688	0.863
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	19.868 ± 8.688	0.259
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	45.866* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	39.842* ± 8.688	0.000
Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Soil with Natural Microbial Flora (6)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	28.733* ± 8.688	0.021
	Pyrene 500mg/kg + Ps.GEMs + Autoclaved Soil (3)	-25.154 ± 8.688	0.066
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Autoclaved Soil (4)	-8.801 ± 8.688	0.95
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Autoclaved Soil + Nutrients N, P (5)	-19.868 ± 8.688	0.259
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	25.998 ± 8.688	0.051
	Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	19.974 ± 8.688	0.253
Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + *Pseudomonas* (7)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	2.735 ± 8.688	1.000
	Pyrene 500mg/kg + Ps.GEMs + Autoclaved Soil (3)	-51.152* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs+ Autoclaved Soil (4)	-34.808* ± 8.688	0.002
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-45.866* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	-25.998 ± 8.688	0.051
	Pyrene&Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	-6.023 ± 8.688	0.993

Dishes	Groups	Mean difference ± SE	Sig.*p*-value**
Pyrene & Phenanthrene 500mg/kg + Soil with Natural Microbial Flora (8)	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil, Evaporation percent (1)	8.758 ± 8.688	0.951
	Pyrene 500mg/kg + Ps.GEMs + Autoclaved Soil (3)	-45.128* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil (4)	-28.784* ± 8.688	0.02
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Autoclaved Soil + Nutrients N, P (5)	-39.842* ± 8.688	0.000
	Pyrene & Phenanthrene 500mg/kg + Ps.GEMs + Soil with Natural Microbial Flora (6)	-19.974 ± 8.688	0.253
	Pyrene & Phenanthrene 500mg/kg + Autoclaved Soil + Pseudomonas (7)	6.024 ± 8.688	0.993

*The mean difference is significant at the 0.05 level by one-way ANOVA.
**The significant difference among means of various treatments in each groups using post-hoc Tukey test ($p<0.05$).
SE: Std. Error

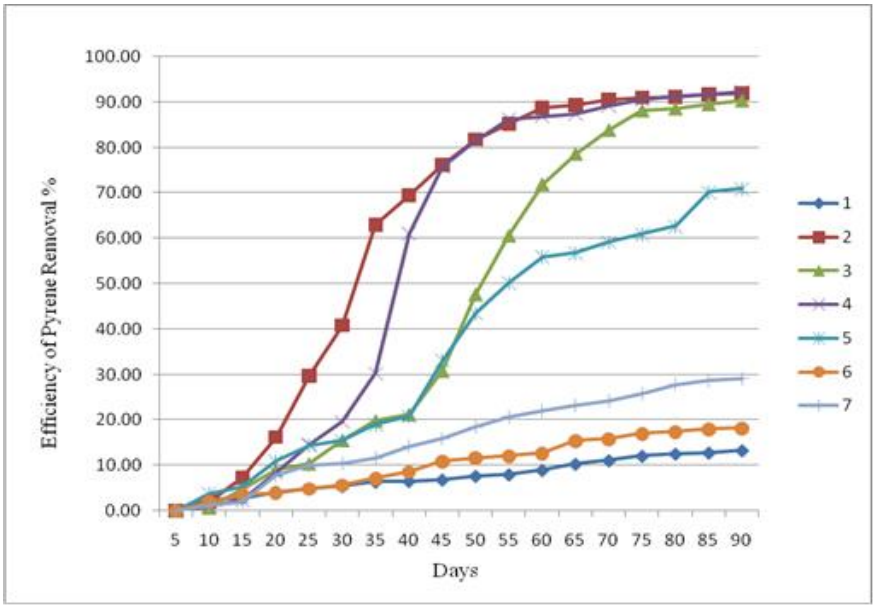

Figure 9. Comparison of pyrene degradation in dishes evaluated in each 5 days

Confirmatory tests on isolated engineered P. putida from spiked soil

Confirmatory tests including catalase, oxidase and PCR were performed on isolated bacteria from spiked soil by phenanthrene and pyrene. Catalase test on isolated bacteria was positive via oxygen producing and confirmed that engineered *P. putida* was active in dishes. Also oxidase test on isolated engineered *P. putida* from contaminated soil was positive by oxidation of tetra-methyl-p-phenylenediamine dihydrochloride and creating dark violet color (Figure 10). C23O producing gene (*nahH*) was amplified (924 bp) in isolated bacteria from spiked soil (Figure 7) and indicated that this gene expressed in engineered *P. putida*. Bacterial population analysis as CFU/g in spiked soil by phenanthrene and pyrene is shown in Figure 11.

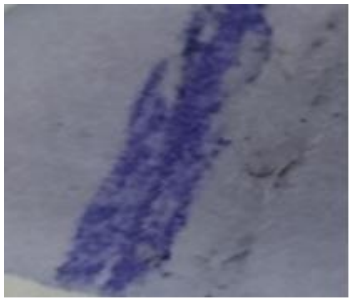

Figure 10. Oxidase test on isolated engineered *P. putida* from contaminated soil was positive (creating dark violet color)

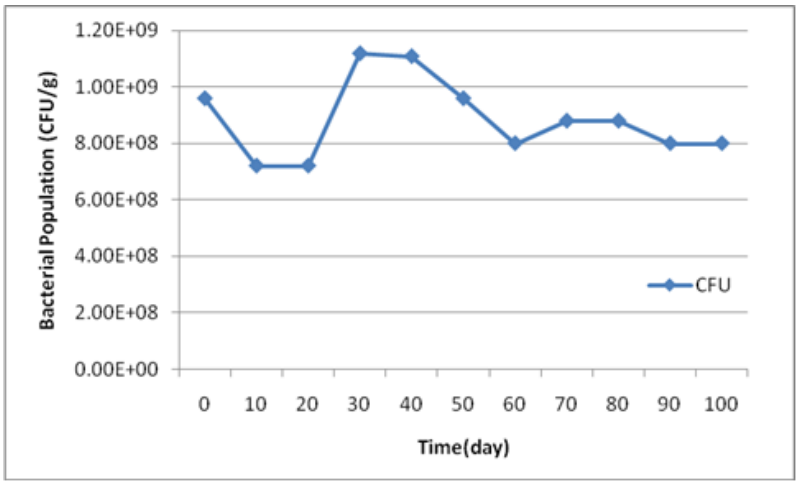

Figure 11. Bacterial population (CFU/g) in spiked soil by phenanthrene and pyrene that evaluated every 10 days (Between 0-20 days after inoculation the *P. putida* population were decreased and it may be related to adaptation of bacteria in new environment, in 20-40 days after inoculation CFU was increased and then the population were decreased again. Between 60 to 100 days CFU was almost unchanged)

DISCUSSION

In the present work, *P. putida* was genetically engineered via transformation of recombinant vector (pUC18-nahH) to produce dioxygenase enzyme, successfully and degradation of phenanthrene and pyrene by this bacterium in spiked soil in each dishes were evaluated using HPLC measurement technique. The results were showed removing of phenanthrene and pyrene in spiked soil by engineered *P. putida* comparing to dishes containing normal soil with natural microbial flora and inoculated autoclaved soil by wild type of *P. putida* were statistically significant ($p<0.05$). In addition, degradation of phenanthrene and pyrene by engineered *P. putida* in dish 5 (autoclaved soil together N and P chemical nutrients) comparing to dishes 2, 3, 4 and 6 (autoclaved soil that spiked by phenanthrene and/or pyrene + Ps.GEMs) were not statistically significant ($p>0.05$), but it was effective more than 2% on degradation of these compounds by engineered *P. putida*. Confirmatory tests including catalase, oxidase and PCR were done on isolated bacteria from spiked soil and were indicated that engineered *P. putida* was alive and functional as well as it can degradate phenanthrene and pyrene via *nahH* gene expressing. Yet, no study has been done on degradation of phenanthrene and pyrene in soil by genetically engineering *P. putida* that producing *nahH* gene. But in similar studies gram-negative bacteria like *Pseudomonas* spp. and other microorganisms as well as fungi has been used for removing of PAH compounds. NIU *et al.,* (2009) was investigated the application of pure culture of *P. putida* ZWL73 in soil contaminated with 4-chloronitoben-zene (4CNB) accelerated the 4CNB degradation in soil microcosms. *Sphingobium chlorophenolicum* used for pentachlorophenol (PCP) removal from soil by DAMS *et al.,* (2007). LIPTHAY *et al.,* (2001) showed that *tfdA* gene carried on plasmid pRO103, to phenol-degrading recipient strains significantly increased the degradation rate of phenoxyacetic acid in sterile and non-sterile soils. Also, in the present study, after transformation of recombinant pUC18-nahH vector into *P. putida* that produce dioxygenase enzyme the degradation of phenanthrene and pyrene in spiked soil was increased statistically significant. In another research by CAO *et al.*, (2012) rhlABRI cassette gene of *P. aeruginosa* BSFD5 was cloned and transformed into the chromosome of *P. putida* KT2440 by a new random transposon vector without introducing antibiotic-resistance marker and genetically generated *P. putida* KT2440-*rhlABRI* could increase the dissolution of pyrene via promoting its degradation by indigenous microorganisms in soil. But in the present study *nahH* gene encoded C23O enzyme was cloned into pUC18 by introducing antibiotic-resistance marker in *P. putida* for removing of phenanthrene and pyrene from soil. In the study of ZHOU *et al.*, (2013) the *C23O* gene was successfully cloned into the plasmid pK4 derived from pRK415 and transformed into *Pseudomonas* sp. CGMCC2953. Their findings showed recombinant *Pseudomonas* sp. CGMCC2953-pK strain, can increased the ability of phenanthrene degradation. In the present study, after cloning of *nahH* gene, pUC18-nahH recombinant vector was successfully created and transformed into *P. putida*. The effects of engineered *P. putida* expressed C23O enzyme on PAHs degradation in spiked soil were showed phenanthrene and pyrene removal was increased statistically significant comparing to other groups. A 1,2,3-Trichloropropane (TCP) degradation activity was evaluated by genetically engineered *P. putida* (SAMIN *et al.*, 2014). In their study *dehalogenase* gene (*dhaA31*) was cloned behind the constitutive dhlA promoter and introduced into the genome of *P. putida* strain MC4 using a transposon delivery system. Similar to this study on increasing of PAHs degradation by genetic engineering *P. putida* their study showed GEMs developed the biocatalyst of a recalcitrant chlorinated hydrocarbon in aerobic bioremediation.

CONCLUSIONS

Biologically degradation of PAHs for decreasing their affects on environment and health problems are important. In conclusion, the recombinant genetically engineered *P. putida* containing pUC18-nahH vector that produced in this work, was biodegradated phenanthrene and pyrene in spiked soil statistically significant. Moreover, using N and P chemical nutrients in soil may not be significant effects on phenanthrene and pyrene removal by engineered *P. putida* but they were useful. The findings of this study revealed that engineered *P. putida* that expressed C23O enzyme is effective microorganism and potentially useful biodegradable agent in polluted soil by PAH compounds.

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DEGRADACIJA FENANTRENA I PIRENA PRIMENOM *Pseudomonas putida* GENETIČKI MODIFIKOVANOG ZA PROIZVODNJU DIOKSIGENAZE U ZEMLJIŠTU

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Izvod

Cilj ovih istraživanja je bio primena modifikovanog soja *P. putida* koji proizvodi dioksigenazu za degradaciju fenantrena i pirena u uzorkovanom zemljištu primenom HPLC metoede. *nahH* gen, koji kodira *catechol 2,3-dioxygenase* (C230) je kloniran u pUC18 i pUC18-nahH. Rekombinovan vektor je generisan i uspešno je izvršena transformacija divljeg soja *P. putida*. Transformisani i divlji soj su inokulirani u zemljište i pripremljena je eksperimentalna kutija (ili Petri kutija) parcela. Rezultati analize korišćenjem HPLC su pokazali eliminaciju PAH komponenti u zemljištu koje je inokulirano genetički modifikovanim sojem *P. putida* u poređenju sa kutijama u kojima se nalazilo prirodno zemljište sa prirodnom florom mikroorganizama kao i inokuliranom autoklaviranim zemljištem sa divljim sojem *P. putida* bili su statistički značajni različiti ($p<0.05$). Izvršen je dodatni test uključujuči katalazu, oksidazu i PCR, na izolovanim bakterjama iz inokuliranog zemljišta koji ukazuje da je modifikovana bakterija živa i funkcionalna. *P. putida* može da se koristi za biodegradaciju fenatrena i pirena kao i petrolejskih komponenata u kontaminiranoj spoljnoj sredini.

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