



Application of Genetically Engineered Dioxygenase Producing *Pseudomonas putida* on Decomposition of Oil from Spiked Soil

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Received 2016 April 04; Revised 2017 January 18; Accepted 2017 February 08.

Abstract

Background: It is well known that bioremediation or using microorganism enzymes plays an important role in decomposition and changing of oil pollutants and PAH compounds into nonhazardous or less-hazardous substances. The genetically manipulated bacteria like *Pseudomonas* sp. can enhance the natural biodegradation activity of polycyclic aromatic hydrocarbons (PAHs) in polluted cities. *Pseudomonas putida* (*P. putida*) is a saprotrophic soil bacterium that is found throughout various environments such as soil and freshwater environments. Oil and petroleum industries are important exposure of PAHs in environment that strongly associated with the development of human cancers.

Objectives: In the present work, *P. putida* was genetically manipulated for the biodegradation of oil in spiked soil and its activity was measured using the high-performance liquid chromatography (HPLC) method.

Methods: The catechol 2,3-dioxygenase (C23O) encoded gene (*nahH*) was cloned into pUC18 for generating pUC18-nahH. This recombinant vector was transferred into *P. putida*, successfully, and both wild type and genetically modified *P. putida* were inoculated in spiked soil with oil for pilot plan preparation. Then, biodegradation activity of this bacterium on the elimination of phenanthrene and pyrene as an oil indicator in spiked soil were evaluated by the HPLC measurement technique.

Results: The results showed the biodegradation of these PAH compounds in oil-spiked soil by genetically manipulated *P. putida* in both groups (containing autoclaved soil and dish containing natural soil) comparing to the inoculated group by wild type of *P. putida* were statistically significant ($P < 0.05$). Finally, confirmatory tests (catalase, oxidase, and PCR) were accomplished on isolated bacteria from spiked soil and were indicated that engineered *P. putida* was alive and functional by producing the C23O enzyme for biodegradation activity of oil.

Conclusions: These findings demonstrated a degradable strain of *P. putida* that was generated in this study by genetic engineering can be useful to assess biodegradation of PAHs compounds in oil and petrochemical pollutions.

Keywords: Oil, Catechol 2,3-dioxygenase, HPLC, *Pseudomonas putida*

1. Background

Degradation of oil or petroleum by microorganisms via secretion of enzymes in the contaminated site has been produced. Bioremediation is a use of natural or genetically engineered microorganisms (GEMs) or their enzymes to clean up or neutralize oil pollutants from contaminated soil and groundwater. Bioremediation can be done in aerobic and anaerobic conditions and this technology is more economical and safer than conventional physicochemical treatments (1-3).

Oil composed mostly of diverse aliphatic and aromatic

hydrocarbons and microorganisms such as hundreds of species of bacteria, archaea, and fungi can degrade it as sources of carbon and energy for growth, however, their biodegradation function on each compound may be strong and weak (4). Common bacterial species including *Bacillus*, *Acinetobacter*, *Arthrobacter*, *Mycobacterium*, *Alcaligenes*, *Flavobacterium*, and *Pseudomonas* have oil degradation activity (5, 6). *Pseudomonas putida* (*P. putida*) is a member of Pseudomonadaceae family and this bacterium can be used for the bioremediation process in the environment. This bacterium is a Gram-negative, flagel-

lated, and rod-shaped that is typically found in soil and water (7, 8).

Engineering of *Pseudomonas* sp. by molecular techniques can be used for increasing and developing bioremediation activity of these bacteria (9). *Pseudomonas* sp., by producing catechol 2, 3-dioxygenase (C23O) can utilize and decomposition polycyclic aromatic hydrocarbons (PAHs). C23O is a nonheme iron dioxygenase and a key enzyme in the biodegradation of PAH compounds that is released into the environment by a large number of an industrial process by catalyzing the conversion of catechol to 2-hydroxy-muconic semialdehyde (9-11).

A catechol 2,3-dioxygenase encoding gene (*nahH*) is one of the important genes of naphthalene catabolic plasmid NAH7 (83-kb length) in some *Pseudomonas* strains like *P. putida* and consist of 924 nucleotides that encoded catechol 2,3-dioxygenase with 36 kDa molecular weight containing 307 amino acid residues (12, 13). This plasmid helps the growth of *Pseudomonas* on oil- and -naphthalene contaminated environments as a sole carbon source (14, 15). The molecular degradation pathway of PAHs by dioxygenase that is encoded by the *nahH* gene is shown in Figure 1.

PAHs enter the environment through various routes. PAHs are ubiquitous environmental contaminants and enter the environment through various ways that are found in soil, air, water, and food sources (16, 17). PAH compounds are known as human carcinogens that spread during incomplete combustion of organic matter by industrial and other human activities (e.g., combustion of urban sewage, garbage, natural gas, vehicle traffic, aluminium smelters, tobacco smoking, cooking, power plants chemical, asphalt works, petrochemical, and oil industries) as well as natural processes like burning coal and wood or forest fires (18-21). Natural crude oil and kerosene contain significant amounts of PAHs (22). PAHs containing up to 6 fused aromatic rings and including fluoranthene, chrysene, fluorene, phenanthrene, and pyrene (17, 18).

Several PAHs in oil and petroleum products are carcinogenic, mutagenic, and teratogenic substances and associated with health hazardous and skin carcinogens (18, 23).

PAH biodegrade bacterial species that are genetically modified are useful with high efficiency for degradation of these compounds in contaminated environments by oil and petroleum products (24, 25).

2. Objectives

In this study, *P. putida* was genetically modified to produce dioxygenase and biodegradation capability of oil in

spiked soil by this bacterium, which was evaluated by using the high-performance liquid chromatography (HPLC) method.

3. Methods

3.1. Preparation of Bacterial Strains and Culture Conditions

The present experimental study was performed from January to July 2015. The lyophilized stocks of *P. putida* (ATCC 12633, Persian Type Culture Collection (PTTC) No. 1694) and *E. coli* strain Top10F were obtained from Iranian Research organization for science and technology (IROST) and Pasteur Institute of Iran, respectively. Both bacterial stocks were suspended and cultured in the Luria-Bertani (LB) broth and incubated for 24 hours at 37°C for *E. coli* and 26°C for *P. putida*. Antibiotic susceptibility testing was performed on both bacterial strains by culturing on LB agar medium containing Ampicillin (50 to 150 mg/mL) and Kanamycin (25 mg/mL) antibiotics and showed that *E. coli* Top10F and *P. putida* strain ATCC 12633 were not resistant to these antibiotics, except Ampicillin, at concentrations 1500 X (150 mg per mL of culture medium), was suitable for *P. putida* transformation.

3.2. DNA Isolation

Bacterial DNA was extracted from *P. putida* colonies using the boiling method. The optical density of extracted bacterial DNA at a wavelength of 260/280 nm was measured by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) according to the method described by Sambrook and Russell, 2001 (26). The DNA samples were stored in the freezer at -20°C for further testing.

3.3. Recombinant Plasmid Preparation

In this study, pUC18 cloning and expression vector (2686 bp) with catalog number: SD0051 was purchased from Thermo Fisher Scientific Inc., Germany. *Pseudomonas* and especially *E. coli* are important hosts of this small plasmid for high copy number multiplying. The sequence of pUC18 plasmid was obtained from the Addgene site (www.addgene.org) and using DNASTAR® Lasergene software version 10 restriction enzyme patterns were identified. T/A Cloning Kit (Fermentas, Germany) was used for transferring of a plasmid into *E. coli* Top10F strain according to the manufacturer's protocol.

In the present work, *nahH* gene synthesis and its cloning into the pUC18 vector were ordered to GeneCust Europe (Dudelange, Luxembourg) and engineered pUC18-*nahH* recombinant vector was generated. The accuracy of cloning of pUC18-*nahH* recombinant vector was found

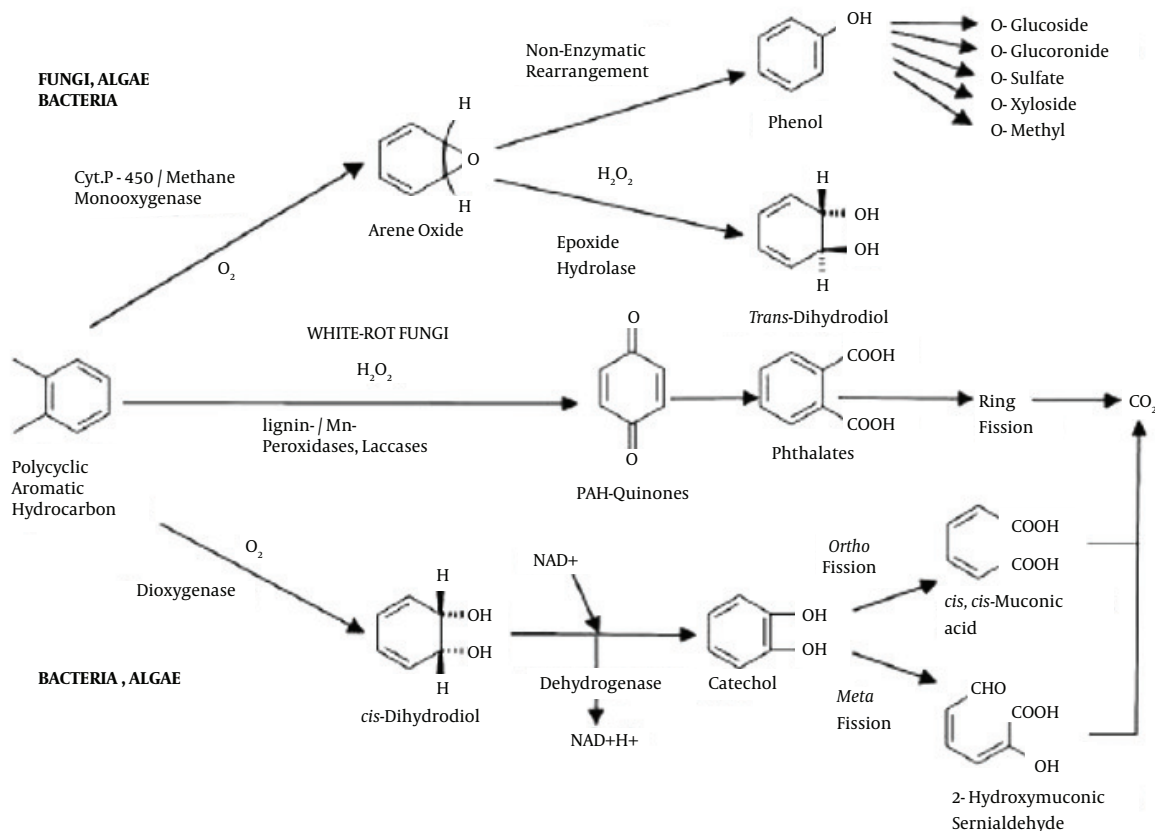


Figure 1. Molecular Degradation Pathway of PAHs by Dioxygenase (Encoded by *nahH* Gene)

using enzymes digestion (*EcoRI* and *BamHI* restriction enzymes) and sequencing technique.

3.4. Bacterial Transformation with Recombinant Vector

For obtaining enough pUC18-*nahH* recombinant vector using Ins TA Clone PCR Cloning Kit (Thermo Scientific, Lithuania) this vector was transferred into *E. coli* Top10F according to the manufacturer's protocol. The plasmids were extracted from bacterial cells using the Plasmid Mini Kit (BIONEER, South Korea) and after confirmation of the transferring of pUC18-*nahH* by HindIII enzyme digestion and PCR technique, the genetically engineered plasmid was transferred again into *P. putida* using the above-mentioned kit (LB media containing 150 mg per mL of Ampicillin was used for colonies selection). Then, PCR was done on purified plasmid from engineered *P. putida* (containing the pUC18-*nahH* recombinant vector) and negative control (non-engineered *P. putida*) to diagnose the existence of the vector containing the *nahH* gene.

3.5. *nahH* Gene Amplification

The *nahH* gene sequence was obtained from GenBank of National Center for Biotechnology Information (NCBI). Oligo Primer Analysis Software version 7 and Gene Runner version 3.05 were used for designing primers for gene amplification. The oligonucleotide primers sequence was compared and blasted by basic local alignment search tool (BLAST) in GenBank data and DNASTAR Lasergene software (version 10).

Polymerase chain reaction (PCR) in a total volume of 25 μL consist of 0.2 pM of each primer (NahH-F: 5'-ATGAACAAAGGTGTAATGCGC-3' and NahH-R: 5'-TTAGGTCATAACGGTCATGAATC-3' with Accession number: X06412), 2.5 mM MgCl_2 , 200 μM dNTPs mix, 2.5 μL of 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1 unit of Taq DNA polymerase (all Fermentas, Germany), and 100 ng of isolated bacterial DNA or purified plasmid. For the no-template control (negative), 2 μL of sterile ultrapure deionised water instead of template DNA were used. The

standard PCR reaction was carried out in a Gradient Palm Cycler (Corbett Research, Australia). The thermo-cycling conditions involved an initial denaturation at 94°C for 5 minutes followed by 35 cycles consisting of 50 seconds denaturation at 94°C, 50 seconds annealing at 62°C, 50 seconds of extension at 72°C, and a final extension at 72°C for 5 minutes. The PCR amplification products (6 μ L/lane) and 1 kb DNA marker (Fermentas, Germany) were loaded on the 2% agarose gel electrophoresis. 1X Electrophoresis buffer (TBE): 89 mM Tris-borate, 2 mM EDTA (for 1.0 L of 5X TBE: 54.0 g of Tris base, 27.5 g of boric acid, and 20 mL of 0.5 M EDTA, pH 8.0). The gel was run at a constant voltage of 80 V for 30 minutes for product separation.

The gel was stained with 0.1% ethidium bromide (0.4 g/mL) and images were captured using the UVIDoc gel documentation system images (Uvitec, UK).

3.6. Soil Preparation and Characteristics for Pilot

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

3.7. Method for Spiking Soil Samples

In the present work, soil spiking was performed based on the Brinch et al. 2002 study (27). Oil (Kerosene) was obtained from a refinery. The soil spiking procedure was carried out under sterile conditions. First, 500 g of autoclaved and natural soil were added to each dish. 125 g of soil (25% of each fraction) were spiked to oil (500 mg/kg) that were added into 100 mL acetone (Merk, Germany) as a solvent. This combined fraction was mixed completely by shacking for each dish. This mixture for evaporating the solvent was maintained at ambient temperature for 16 hours. Finally, each treatment (spiked soils) was mixed completely with the remaining 75% (375 g) of the soil sample for pilot preparation.

3.8. Soil Inoculation

Wild *P. putida* (non-engineered) and genetically manipulated and were cultured in 300 mL (150 mL for each dish) of LB broth medium (containing 150 mg per mL of Ampicillin antibiotic) and in 150 mL medium without antibiotic, respectively. The cultures were incubated for 24 hours at 26°C and 220 rpm in a shaking incubator. When the optical density of each cultured bacteria at 600 nm (OD600)

that was measured by a 2100 visible spectrophotometer (Unico, Shanghai, China) were reached to 8×10^8 to 1.2×10^9 CFU/mL, they were used for soil inoculation. The precipitation of wild type and engineered bacteria was taken separately by centrifuging at 6000 rpm for 10 minutes. The supernatant was discarded, but 5 mL of LB medium remained for adaptation and feeding of bacteria.

Cell deposition was re-suspended 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. All dishes of phenanthrene and pyrene as oil indicators (500 mg/kg) were evaluated. The pilot plan including dish 1: oil (Phenanthrene + Pyrene) and *Pseudomonas* + autoclaved soil), dish 2 was involved soil with natural microbial flora (non-sterile) and genetically engineered *P. putida* (Ps. GEMs+ soil with natural microflora) to evaluate the effect of oil biodegradation of bacteria in natural conditions and dish 3 containing oil (Phenanthrene + Pyrene) Ps. GEMs + autoclaved soil.

3.9. Sampling and Extraction Method

The evaporation of phenanthrene and pyrene as an index of oil in spiked soil at the end of 3 months was 16.43% and 13.25%, respectively. Sampling and extraction were each performed in 5 days and were followed for 3 months to investigate the biodegradation of oil by engineered and wild *P. putida* in dishes. For sampling and extraction, 2 g of spiked soil from each dish were selected and dried at room temperature for 12 hours. Then, the soil was grinded and 2 mL acetonitrile was added. After that, oil was extracted using ultrasonic bath sonicator (CD-4820, China) for 30 minutes. Then, samples were centrifuged at 3000 rpm for 10 minutes and supernatant were followed-through from polytetrafluorethylene (PTFE) membrane filter. Finally, 20 μ L of each extracted samples were injected into HPLC.

3.10. HPLC Analysis

In this study, phenanthrene and pyrene as an indicator of oil were used as a serial dilution concentration (including 100, 50, 25, 12.5, and 6.25, respectively) to create standard solutions of the measurement method of HPLC (Figure 2). Then, the calibration curve for phenanthrene and pyrene was prepared with correlation coefficient equivalent 0.99998 and 0.99937, respectively (Figure 3). The mobile phase of HPLC has included 80% acetonitrile and 20% H₂O with flow rate 1 mL per min, as well as the detector measured ultraviolet absorbance at 254 nm.

3.11. Confirmatory Tests

Every 30 days, 1 g of spiked soil by oil that was contaminated by wild type and engineered *P. putida* was mixed in

Signal 1: VWD1 A, Wavelength = 254 nm

Ret Time [min]	Sig	LV1	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	2.74541 e-3			
		5	100.00000	3.64239e4	2.74545e-3			

Peak Sum Table

Signal 1 1: VWD1 A, Wavelength = 254 nm

Ret Time [min]	Sig	LV1	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. The Concentrations of Phenanthrene and Pyrene as an Indicator of Oil Used for Standard Solutions for Measurement Method in HPLC

sterile water injection and follow-through from MESH filter to obtain soil extraction. Extracted soil (40 μ L) were transferred on LB agar plates containing ampicillin (150 mg per mL) in sterile conditions and was cultured at 26°C for 24 hours to obtain colonies. Every 10 days, 1 g of contaminated soil were mixed in sterile saline for preparing serial dilution and were plated by drop plating for 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} . Catalase and oxidase tests, as well as DNA extraction and *nahH* gene amplification by PCR, were done on each colony. Catalase test was done by mixing some colonies into a fresh drop of hydrogen peroxide (H₂O₂) 3% and releasing oxygen gas indicate that this test is positive. In addition, some colonies of *P. putida* were selected for oxidase test by transferring bac-

teria on filter paper that was soaked with tetra methyl-p-phenylenediamine dihydrochloride solution. Production of indophenol and purple color indicates that the oxidase test is positive.

3.12. Statistical Analysis

All experiments were performed at least 3 times. The collected data were analyzed in the Statistics programs for the Social Sciences software, version 20 (SPSS, Inc., Chicago, IL, USA). The differences between the biodegradation of oil by engineered and wild *P. putida* in each plate at the period of the experiment were evaluated using one-way ANOVA (Analysis of variance) followed by Tukey HSD post-hoc test for comparing the significant difference among means of various treatments. P values at or below 0.05 were considered statistically significant between groups.

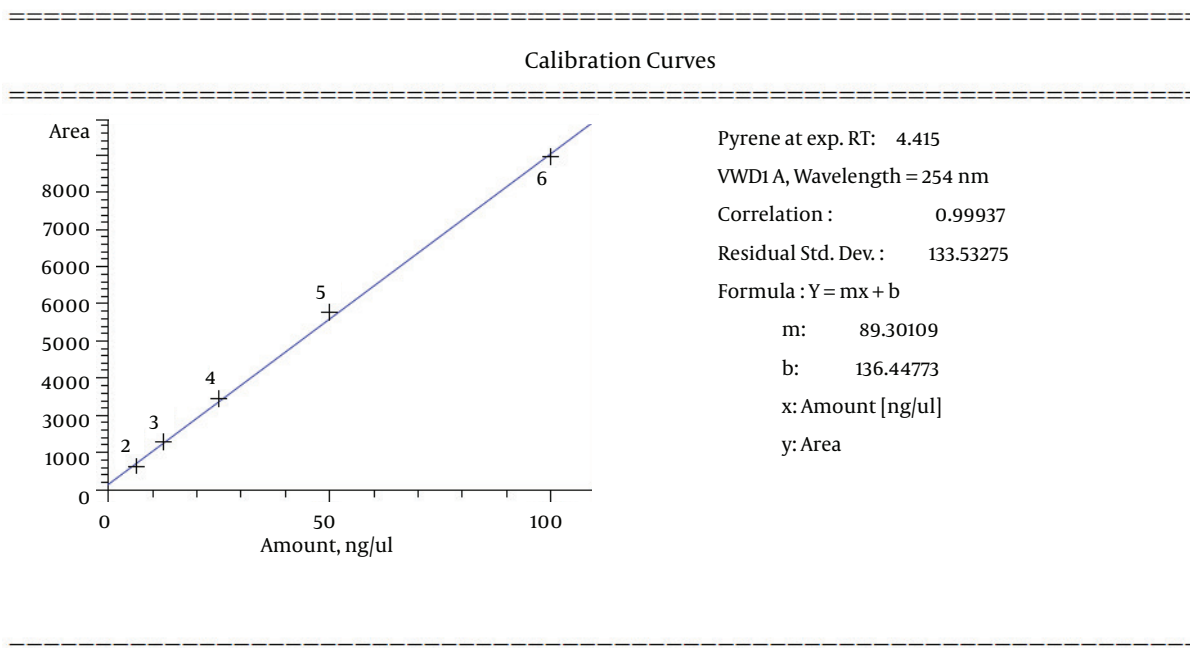
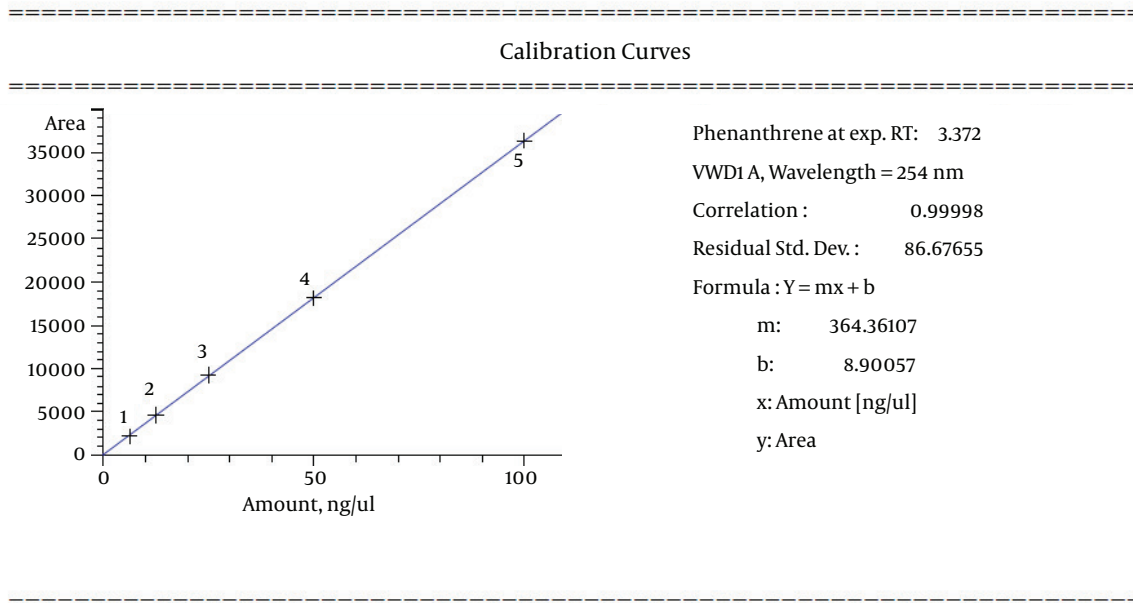


Figure 3. The Calibration Curves of Phenanthrene and Pyrene used for HPLC

4. Results

4.1. Confirmation of Cloning

PCR technique on extracted DNA from *P. putida* strain (ATCC 12633) that was used in this study showed that NAH7

plasmid and *nahH* gene was not a presence in this bacterium. Therefore, synthesis of the *nahH* gene was ordered to GeneCust Europe (Dudelange, Luxembourg). *BamHI* and *EcoRI* restriction enzymes digestion and sequencing using the Sanger sequencing method were done on the engi-

neered pUC18-nahH recombinant vector for the accuracy of *nahH* gene cloning in pUC18 vector and verifying the gene synthesis indicates the presence of this gene in vector (Figure 4A).

For obtaining sufficient amount of pUC18-nahH, the recombinant vector containing *nahH* gene was extracted from engineered *E. coli*. The accuracy of transformation was determined by the PCR method and HindIII enzyme digestion (were revealed 3004 and 606 bp fragments) and was confirmed the successful transformation (Figure 4B and 4C).

The pUC18- nahH recombinant vector using Ins TA Clone PCR Cloning Kit was transferred again into *P. putida*, successfully. Bacterial DNA was extracted from engineered and wild *P. putida* strains. Amplification of *nahH* gene (924 bp) by PCR was positive in engineered *P. putida*.

4.2. HPLC Data Analysis

Biodegradation of oil by wild type and genetically engineered *P. putida* in dishes was analyzed by the HPLC technique each in 5 days for 3 months. Data were evaluated using the one-way ANOVA followed by Tukey HSD ("Honestly Significant Difference") test for comparing the significances (Table 1). Biodegradation of oil in polluted soil by genetically manipulated *P. putida* (Ps.GEMs + soil with natural microbial flora and Ps.GEMs + autoclaved Soil) comparing to inoculated dish by wild-type *P. putida* (*Pseudomonas* + autoclaved soil) were statistically significant ($P < 0.05$). Furthermore, the statistically significance between autoclaved soil and soil contain natural microbial flora (both inoculated by genetically engineered *P. putida*) was not observed ($P > 0.05$).

4.3. Confirmatory Tests

The confirmatory tests including catalase, oxidase, and gene amplification were performed on isolated bacteria from the spiked soil by oil. Catalase test on the isolated wild type and genetically engineered *P. putida* was positive via oxygen producing and confirmed that *P. putida* were active in dishes. Also, oxidase test was positive for isolated engineered *P. putida* from contaminated soil by oxidation of tetra-methyl-p-phenylenediamine dihydrochloride and creating a dark violet color. The amplification of C23O producing gene (*nahH*) in extracted DNA from isolated engineered *P. putida* from the spiked soil by PCR technique (924 bp) was positive and indicated that this gene was expressed in engineered *P. putida*. Furthermore, CFU/g, for analyzing the bacterial population in spiked soil by oil, is shown in Figure 5.

5. Discussion

Application of genetically engineered bacteria (bioremediation) improved biodegradation of pollutants in environments (28). In this study, genetically engineered *P. putida* that produce dioxygenase enzyme was generated via transferring of pUC18-nahH recombinant vector and were used for biodegradation of oil in the spiked soil. Removing of oil from the soil by genetically manipulated and wild *P. putida* was evaluated using the HPLC measurement method. The results indicated that biodegradation of oil from spiked soil using genetically manipulated *P. putida* were statistically significant ($P < 0.05$) comparing to soil contaminated by a wild type of *P. putida*. Also, biodegradation activity of genetically engineered *P. putida* between autoclaved soil and dish that contain natural microbial flora were not statistically significant ($P > 0.05$). Viability and activity of isolated engineered *P. putida* from the spiked soil with oil by culturing, as well as catalase, oxidase, and PCR tests were investigated and explanatory degradation of oil via these bacteria by *nahH* gene expressing in the soil environment.

A number of studies have been performed for biodegradation of PAH compounds by *Pseudomonas* and other gram-negative bacteria as well as fungi such as *Trichoderma/Hypocrea* genus; however, no studies to date have been performed on the degradation of oil (kerosene) in soil by genetically engineering *P. putida*, that produce the *nahH* gene. In one study naturally adapted *P. putida* isolated from oil-contaminated sites in India was isolated and evaluated for biodegradation of crude oil in the open environment (29, 30). In another study, the efficiency of *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains are isolated from a petroleum-contaminated soil sample from North-East India on biodegradation of crude petroleum-oil hydrocarbons in soil and shake flask compared and indicated *P. aeruginosa* strains were more efficient than *B. subtilis* strain and as well as showed *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains could be effective for in situ bioremediation (31). The application of *P. putida* ZWL73 in contaminated soil with 4-chloronitrobenzene (4CNB) was investigated by Niu et al. (32). In another research, it was found that *tfdA* gene located in plasmid pRO103 in phenol-degrading recipient strains leads to significantly biodegradation rate of phenoxo acetic acid in sterile and non-sterile soils (33). In the present study, the pUC18-nahH recombinant vector was generated and transferred into *P. putida* and degradation of oil in polluted soil was increased statistically via evaluation of phenanthrene and pyrene as an indicator. In another study by Cao et al. (2012), *P. putida* KT2440-rhIABRI was genetically generated by cloning and transforma-

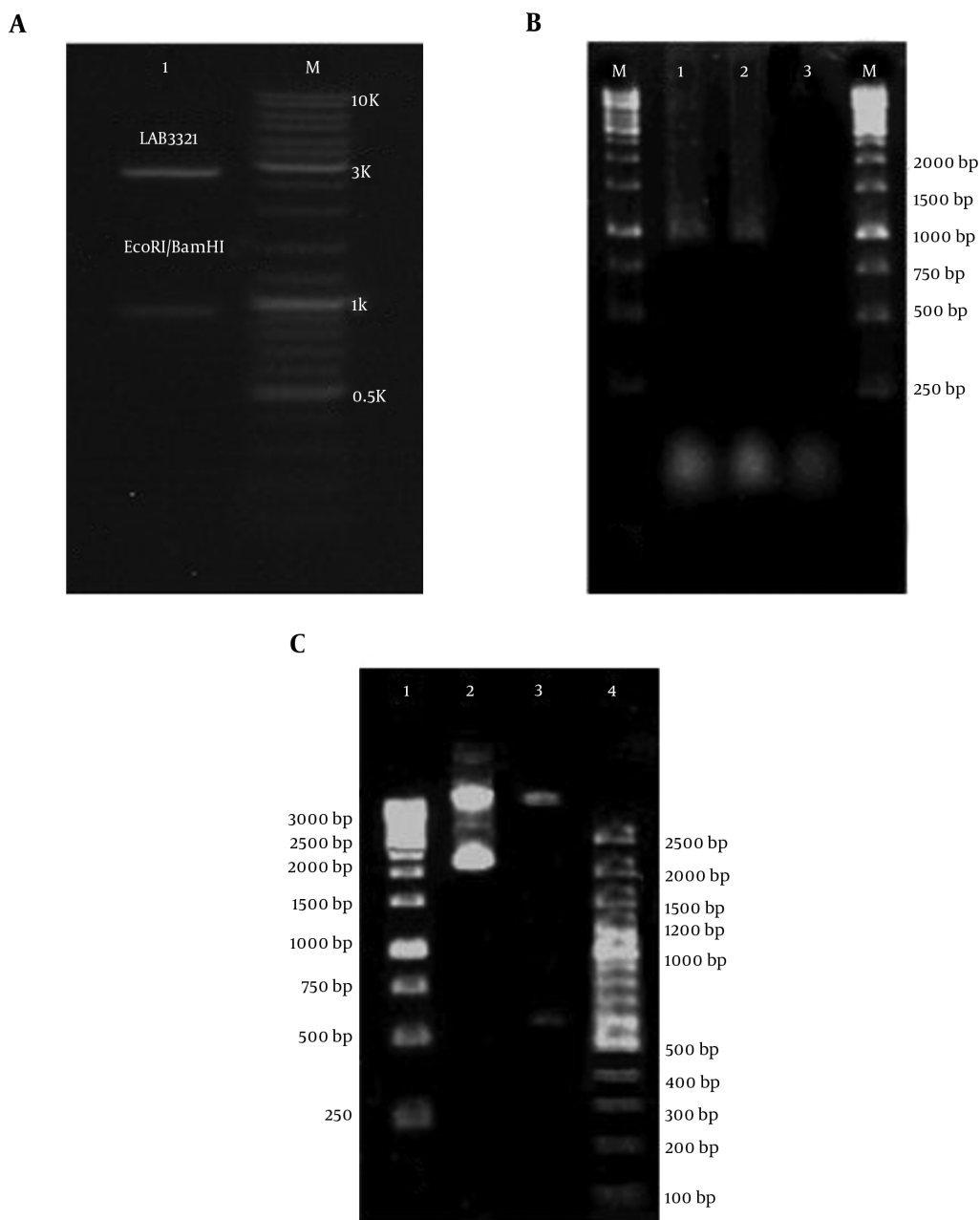


Figure 4. A, Digestion of pUC18-*nahH* Recombinant Vector Using Restriction Enzymes for Cloning Confirmation (Lane M is 100 bp Molecular Weight Markers (Fermentas, Germany), and Lane 1 is Digested pUC18-*nahH* Recombinant Vector (2686 and 924 bp Fragments) by *Bam*HI and *Eco*RI Restriction Enzymes, Respectively); B, Amplified *nahH* Gene on Isolated Recombinant pUC18 Vector from Engineered *E. coli* (Lane M is 1 kb Molecular Ladder (Fermentas, Germany), Lanes 1 and 2 are Amplified *nahH* gene (924 bp), and Lane 3 is Negative Control (without DNA), Respectively); C, Digestion of recombinant pUC18 Plasmid Contain *nahH* Gene by *Hind*III Restriction Enzyme for Confirmation of Transformation (Lane 1 and 4 are 1 kb and 100 bp Molecular Weight Markers (both Fermentas, Germany), Respectively, Lane 2 is Undigested Recombinant Vector (3610 bp), lane 3 is Digested Recombinant Vector (3004 and 606 bp Fragments), Respectively)

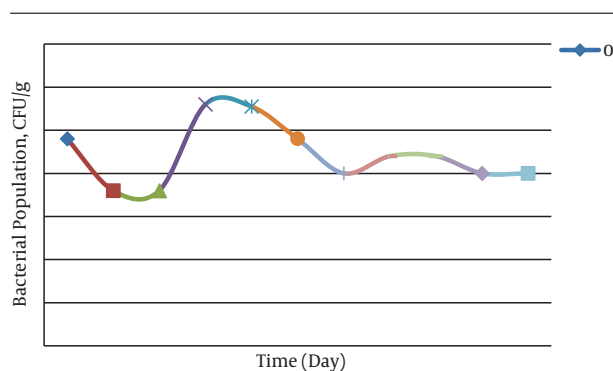
tion of *rhlABRI* cassette gene of *P. aeruginosa* BSFD5 and indicated that this manipulated strain could increase the dissolution of pyrene by promoting its degradation by indigenous microorganisms in soil (34). While in our

study encoded C23O enzyme gene (*nahH*) was cloned and pUC18-*nahH* was generated for transferring into *P. putida* and used for removing of oil from the soil. In the study of Zhou et al. (2013), the C23O gene was cloned into the

Table 1. Comparison the Biodegradation of oil (Phenanthrene and Pyrene as Indicators) by Wild-Type and Genetically Engineered *P. putida* in Dishes Using ANOVA and Tukey Post-Hoc Test

Components of Dishes	Groups	Mean Difference \pm SE	Sig. P Value ^a
Oil (Phenanthrene) 500 mg/kg <i>Pseudomonas</i> + Autoclaved Soil (1)	Oil (Phenanthrene) 500 mg/kg Ps.GEMs + Soil with Natural Microbial Flora (2)	-50.923 ^b \pm 8.373	0.000
	Oil (Phenanthrene) 500 mg/kg Ps.GEMs+ Autoclaved Soil (3)	-60.566 ^b \pm 8.373	0.000
Oil (Phenanthrene) 500 mg/kg Ps.GEMs + Soil with Natural Microbial Flora (2)	Oil (Phenanthrene) 500 mg/kg <i>Pseudomonas</i> + Autoclaved Soil (1)	50.923 ^b \pm 8.373	0.000
	Oil (Phenanthrene) 500 mg/kg Ps.GEMs+ Autoclaved Soil (3)	-9.644 \pm 8.373	0.487
Oil (Phenanthrene) 500 mg/kg Ps.GEMs + Autoclaved Soil (3)	Oil (Phenanthrene) 500 mg/kg <i>Pseudomonas</i> + Autoclaved Soil (1)	60.566 ^b \pm 8.373	0.000
	Oil (Phenanthrene) 500 mg/kg Ps.GEMs + Soil with Natural Microbial Flora (2)	9.644 \pm 8.373	0.487
Oil (Pyrene) 500 mg/kg <i>Pseudomonas</i> + Autoclaved Soil (1)	Oil (Pyrene) 500 mg/kg Ps.GEMs + Soil with Natural Microbial Flora (2)	-43.123 ^b \pm 9.898	0.000
	Oil (Pyrene) 500 mg/kg Ps.GEMs+ Autoclaved Soil (3)	-42.342 ^b \pm 9.898	0.000
Oil (Pyrene) 500 mg/kg Ps.GEMs + Soil with Natural Microbial Flora (2)	Oil (Pyrene) 500 mg/kg <i>Pseudomonas</i> + Autoclaved Soil (1)	43.123 ^b \pm 9.898	0.000
	Oil (Pyrene) 500 mg/kg Ps.GEMs+ Autoclaved Soil (3)	0.78 \pm 9.898	0.997
Oil (Pyrene) 500 mg/kg Ps.GEMs + Autoclaved Soil (3)	Oil (Pyrene) 500 mg/kg <i>Pseudomonas</i> + Autoclaved Soil (1)	42.342 ^b \pm 9.898	0.000
	Oil (Pyrene) 500 mg/kg Ps.GEMs + Soil with Natural Microbial Flora (2)	-0.78 \pm 9.898	0.997

Abbreviation: SE: Std. Error.

^aThe significant difference among means of various treatments in each groups using post-hoc Tukey test ($P < 0.05$).^bThe mean difference is significant at $P < 0.05$ by one-way ANOVA.**Figure 5.** Evaluation of Isolated Bacterial Population (CFU/g) from Polluted Soil by Oil Was Done Every 10 Days (In 0 - 20 Days After Inoculation, *P. putida* Population Were Decreased and It may be Related to Adaptation of Bacteria in New Environment, 20 - 40 Days After Inoculation CFU was Increased and Then (40 - 60 Days) the Population Were Decreased Again, and finally the Bacterial Population Close to Stationary Phase).

plasmid pK4 derived from pRK415 and transferred into *Pseudomonas* sp. CGMCC2953, successfully. The capability of this modified strain was investigated by increasing of phenanthrene decomposition (35). In the present study, the pUC18-*nahH* recombinant vector was successfully created and transferred into *P. putida*. The biodegradation activity of engineered *P. putida* in spiked soil (autoclaved soil and natural soil containing microbial flora) on oil biodegradation showed oil removal compared to wild-type group was increased statistically significant. In the recent study by Samin et al. 2014 genetically engineered *P. putida* via cloning of dehalogenase gene (*dhaA31*) generated for evaluation the 1,2,3-Trichloropropane (TCP) degradation activity (36). Same to the findings of the

present study PAH compound in oil and chlorinated hydrocarbon can effectively biocatalyst by GEMs.

Biodegradation of major oil and PAHs pollutions in soil and water could be more helpful for decreasing of their affects on the environment and its exposures and associated health risks in human. The genetically manipulated *P. putida* containing a recombinant pUC18-*nahH* vector that was produced in this study can effectively biocatalyst and remove oil from spiked soil as well as be used as biodegradable strain in environmental and industrial pollutions. As a suggestion, in future work it would be better if *P. putida* were genetically manipulated for other genes like *nahA* and *nahE* that contribute to PAHs degradation.

Acknowledgments

The present article was obtained from a Ph.D. thesis and financially supported by the center for solid waste research, institute for environmental research, Tehran University of Medical Sciences, Tehran, Iran. The authors would like to sincerely thank the Cellular and molecular research center of Shahrekord University of Medical Sciences (southwest, Iran) for their cooperation.

Footnotes

Authors' Contribution: All authors contributed in all parts of the research. Gashtasb Mardani and Payam Ghasemi-Dehkordi: contribute in bacteriological and molecular tests completely; Gashtasb Mardani: analyzing of HPLC results, data collection and writing the

manuscript; Amir Hossein Mahvi, Morteza Hashemzadeh-Chaleshtori, Simin Naseri and Mohammad Hadi Dehghani: consultation and advice on the process of the research project and analyzing of data.

Financial Disclosure: The authors have no financial interests related to the material in the manuscript.

Conflict of Interest: The authors declare no conflict of interest in this study.

Funding/Support: This project was supported financially by center for water quality research, institute for environmental research, Tehran University of Medical Sciences, Tehran, Iran.

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