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Isolation and characterization of diethyl phthalate degrading bacteria from crude oil contaminated soil

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Abstract

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Aim : The aim of this study was to isolate, identify and characterize diethyl phthalate degrading bacterial strains isolated from a crude oil contaminated soil from a landfill dump site of a petroleum refinery in Mersin, Turkey.

Methodology : The bacteria was isolated from a crude oil contaminated soil and characterized by 16S rRNA analysis. Bacterial genomic DNA was identified by 16S rRNA analyses. Biodegradation experiments were conducted for 5 days and plasmid curing experiment was performed. Catechol test was carried out to determine phthalate degradation pathways.

Results : The isolated bacteria from soil were identified as *Pseudomonas putida* based on 16S rRNA sequences. The size of the plasmid was estimated to be about 15.9 kb. Results of biodegradation experiments indicated that the diethyl phthalate concentrations were reduced by 85.5% after 5 days of incubation at pH 7.0 and 30°C. The ability of *P. putida* degrading diethyl phthalate was found to be plasmid-mediated through curing experiments.

Interpretation : The study suggested that plasmid of *Pseudomonas putida* PAG5 could be involved in effective degradation of diethyl phthalate.

Key words: Biodegradation, Diethyl phthalate, Phthalate esters, Plasmid, *Pseudomonas putida*

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Introduction

Phthalate esters have been widely used to increase flexibility, strength as plasticizers in plastic industry in polyvinyl chloride, also cosmetic industry, medical sector and stationery products (Babu and Wu, 2010). Because of their unique properties like chemical stability, flexibility, softening, ease of fabrication and low cost, phthalate esters are used with different chemical formulations. With an annual production of 14.4 million tons, dimethylphthalate and terephthalate are among the top 50 most produced chemicals worldwide (Li and Gu, 2006). Due to their widespread use and unstable chemical bond, phthalate esters have become ubiquitous environmental pollutant. Phthalate esters have been found in natural waters, drinking waters, soils and atmosphere, because they cannot bond to the host polymers (Martinen *et al.*, 2003; Fang *et al.*, 2007; Sun *et al.*, 2013). Phthalate esters are released in soils, aquatic habitats and have been observed to accumulate in living biota (Lu *et al.*, 2009). Long-term adverse effects in most living organisms contaminated with diethyl esters of phthalic acid have been reported (Kapanen *et al.*, 2007; Cheng, 2012; Ghorpade *et al.*, 2002; Xiao *et al.*, 2018). Several studies have reported correlation of phthalate esters with DNA mutation, hepatic disorders and carcinoma (Dillingham and Autian, 1973; Gesler 1973; Koch *et al.*, 2003; Zeng *et al.*, 2004; Den Hond and Schoeters, 2006; Singh and Li, 2011). Diethyl ester of phthalic acid is a clear, colorless, practically odorless liquid. It has a boiling point of 298°C, specific gravity of 1.12 (20°C), vapor pressure of <0.001 torr (20°C) and molecular weight 222.24 g mol⁻¹ Api (2001). Most phthalate esters, especially diethyl esters of phthalic acid are toxic and disturb endocrine system in living organisms (Chang *et al.*, 2007).

Unfortunately, phthalate esters cannot be degraded effectively by natural processes such as hydrolization and photodecomposition. The basic chemical structure of phthalates is benzene dicarboxylic acid with two side chains, which can be alkyl, benzyl, phenyl, cycloalkyl or alkoxy groups. Studies have demonstrated that phthalates with shorter ester chains like dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), diphenyl phthalate (DPP), dipropyl phthalate (DPrP), and butyl-benzyl phthalate (BBP) can be readily biodegraded and mineralized. On the other hand, phthalates with longer ester chains, such as dicyclohexylphthalate, dihexyl phthalate (DHP), dioctyl phthalate (DOP), and di-2-ethylhexyl phthalate (DEHP) are less susceptible to biodegradation (Chang and Zylstra, 1998). However, biodegradation is a successful mechanism of phthalate removal in aquatic and soil habitats (Lu *et al.* 2009). Since many bacterial strains can breakdown phthalate esters under aerobic and anaerobic conditions, most phthalate-degrading bacteria are mainly from four divisions in *Bacteria*, i.e., *Proteobacteria*, *Actinobacteria* (or high G+C), *Firmicutes* (or low G+C), and *Bacteroids / Chlorobi*. Among them, the commonly found genera are *Sphingomonas*, *Comamonas* and *Pseudomonas* (Kapanen *et al.*, 2007). Vamsee-Krishna *et al.* (2006) showed that bacterial strains utilized three phthalate isomers as sole carbon sources. Kim *et al.* (2008) studied the

degradation of DPP and they reported the biodegradation of DPP by *Sphingomonas chungbukensis*. Degradation of phthalate esters proceed by the action of esterases to form phthalate isomers, which are converted into dihydroxylated intermediates by specific and inducible phthalate isomer dioxygenases. Metabolic pathways of phthalate isomers converge at 3,4-dihydroxybenzoic acid, which then undergoes either ortho- or meta-ring cleavage and is subsequently metabolized to the central carbon pathway intermediates. Genes involved in degradation are arranged in operons present either on plasmid or chromosome or both, and induced by specific phthalate isomer (Vamsee-Krishna and Phale, 2008). *Pseudomonas* can also metabolize aromatic compounds such as toluene, benzene, ethylbenzene, phenol and also phthalate isomers as sole carbon and energy sources (Reardon *et al.*, 2000). In light of the above, the aim of this study was to isolate and characterize DEP degrading bacterial strains from crude oil contaminated soil samples. The present investigation also focused on the genetic determination of degradation capabilities of strains in this isolate.

Materials and Methods

Soil samples: Soil samples were collected from a crude oil-contaminated soil, near a petroleum refinery, Mersin, Turkey. The soil samples (approximately, 500 g) were transferred to Molecular Biology and Biotechnology Laboratory with sterile conditions for further analysis.

Isolation of DEP-degrading bacteria: The culture medium was a modified minimal salt medium (MSM). The final pH of the medium was adjusted to pH 7.0 (Chang, 2004). Yeast extract was added to the medium at 0.5% (w/v). The final product was named as MSMY. The medium agar was prepared with 14 g l⁻¹ agar. The overnight bacterial inoculum was prepared in Nutrient Broth medium. The DEP concentration was adjusted to 100 mg l⁻¹ using a surfactant.

Isolation of DEP degrading bacteria was carried out by the method of Kuce *et al.* (2015). One gram of soil sample was added to 5 ml of sterile distilled water, and the suspension was centrifuged at 2000 rpm for 10 min. Then, 1 ml of supernatant was inoculated into 100 ml of MSM containing 100 mg l⁻¹ DEP for 5 days at 150 rpm and 30°C. One ml of culture was then transferred to another 100 ml fresh MSM with 200 mg l⁻¹ DEP. After incubation under similar conditions, 1 ml of this last culture was transferred to 100 ml fresh MSM with 300 mg l⁻¹ DEP. The final enrichment culture was spread over MSM Agar (MSMA) plates that contained 100 mg l⁻¹ DEP as the main carbon source. After incubation for 5 days, bacterial colonies were cultivated in Nutrient Agar (NA) plates for overnight at 30°C. Bacterial colonies were then transferred into fresh MSMA with DEP. Bacterial strains were picked up and replated three times to ensure pure colony selection. The best growing colony was selected and stored in MSM with 20% glycerol at -80°C until further use.

Preparation of DEP stock with surfactant: As diethyl phthalate has low water solubility, a surfactant was added to MSM in order

to ensure its dispersion in the medium. DEP was prepared with Triton X-100 (2 mg l⁻¹) in MSM medium. The mixture was strongly blended with vortex up to provide white color discoloration.

16S rRNA analysis: Genomic DNA of bacteria was isolated with Nano Biz Genomic DNA Isolation Kit. The PCR was performed using a reaction volume of 50 µl including 1X Taq buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.3 pmol of each primer, 1.5 U Taq polymerase. 16S rRNA gene was amplified by universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was conducted following cycle parameters; denaturation for 1 min at 95°C, followed by 30 cycles of 45 sec at 94°C, 45 sec at 55°C and 45 sec at 72°C. Bigdye Cycle Sequencing kit v.3.1 was used for sequence analysis. Sequencing regions were carried out using an ABI 3100 Genetic Analyzer. Sequence data were classified with reference genomes using Molecular Evolutionary Genetics Analysis (MEGA) and its software MEGA5 (Tamura *et al.*, 2011). The phylogenetic tree was designated by using the minimum evolution tree method (Hall, 2013).

Biodegradation of DEP: One ml of 24 hr grown bacterial culture was inoculated in MSMY with 100 ppm DEP. The culture was incubated in a shaker at 150 rpm at 30°C (pH 7.0) for 5 days. The biomass growth and concentrations of DEP were monitored on the 5th day of experiment. Optical density (OD₆₀₀) of the samples were measured with spectrophotometer (Analytik Jena-Specord 210 Plus) everyday to assess the bacterial cell growth. All the experiments were performed in triplicate and a graph was made by taking the average of the obtained values.

Analytical procedure: One ml samples were taken from the flask daily. The samples were then centrifuged (Sigma D-37520) at 10,000 g for 10 min. The supernatant was filtered by 0.2 µm sterile filter. After filtration, 500 µl of supernatant was mixed with 500 µl of n-hexane (99%). The mixture was stirred for 3 hrs. At the end of this process, phase separation was completed and supernatant was filtrated through YM-3 membrane filter to remove the salts, used in the medium. Finally, the extract in n-hexane was analyzed with GC-MS (GC Agilent 7890A, MS Agilent 5975C) using a gas column (HP5MS, 2 m long, 3 mm diameter) packed with silicon. The temperature at the injection port was 280°C and the oven temperature was programmed from 50°C (2 min) to 280°C (5 min) at 10°C min⁻¹ interval. A 4 µl of each sample was injected into the column (Babu, 2010; Kim *et al.*, 2008; Quan *et al.*, 2005).

Plasmid curing: The plasmid curing procedure was performed by the method of Trevors (1986). In short, the culture was incubated in Nutrient Broth containing 500 µg l⁻¹ ethidium bromide for 24 hr at 30°C. The overnight culture was plated on nutrient agar and MSM agar with 100 mg l⁻¹ DEP.

DNA isolation and agarose gel electrophoresis: Plasmid DNA was purified by using Gene JET Plasmid Miniprep Kit (Thermo Fisher). The reactions were performed as outlined in the

manufacturer's instructions. DNA fragments were electrophoresed through agarose gel (0.7%) at 60 V for 3 hr in TBE Buffer (45 mM Tris-Borate, 1 mM EDTA, pH: 8.0). Gels were stained with 0.5 µg ml⁻¹ ethidium bromide and were observed on a UV-transilluminator. 1 kb DNA Ladder was used as molecular size marker (Gene Ruler, MAN0013004, and Thermo Scientific). Molecular size of plasmid DNAs were determined with DNA size software.

Catechol plate assay: Catechol test was carried out to determine phthalate degradation pathways and origin of the gene responsible for degradation of phthalates. Bacterial strain was cultivated in MSM overnight. Fresh culture was mixed equal volume of 0.1 M catechol in an E-plate and incubated for 30 min at 30°C under static conditions, then color change of liquid medium was observed (Haigler, 1992; Mikesell *et al.*, 1993).

Modeling of DEP degradation kinetics: Assuming that the environmental conditions (e.g., pH, temperature) are constant, a simplified pseudo-first order rate law can be derived to model diethyl phthalate degradation kinetics at pH 7.0 and 30°C (Küce *et al.*, 2015):

$$\frac{d[C]}{dt} = -k[C] \quad (1)$$

The integrated form of this rate law is

$$\frac{\ln [C]}{[C_0]} = -kt \quad (2)$$

where, C is the model predicted concentration of diethyl phthalate concentration (mg l⁻¹), C₀ is the initial diethyl phthalate concentration, k is the first order kinetics rate constant (d⁻¹). The software Scientist (Micromath) was used to fit related questions to the DEP removal data as well as to test the statistical validity of model simulations. Note that the first-order rate model accurately simulated the kinetic data with a regression coefficient R² > 0.991. The value of k as estimated from the slope of the model simulation was 0.5265 d⁻¹, which corresponded to a half-life (t_{1/2}) of 1.32 d.

Results and Discussion

The crude oil polluted soils were collected from a petroleum refinery landfill site to prepare an enrichment culture MSM containing DEP as sole carbon source. Two different surfactants (Tween-80 and Triton X-100) were selected to obtain the best water solubility of DEP. The results of this investigation show that the maximum water solubility of DEP was observed with Triton X-100. Tween-80 did not provide enough water solubility of DEP in MSM and insoluble part of DEP accumulated to form droplets in the aqueous phase of the medium. The culture was continuously enriched at 5-day interval. Following the culture enrichment procedure, the best-growing bacterial strains were selected for identification. A phylogenetic tree was constructed based on the 16S rRNA sequence of the strains isolated (Fig. 1) (Tamura *et al.*, 2011; Hall, 2013). Sequence alignment results showed very high similarities (>99 %) to *Pseudomonas putida* NBRC 14164 (Ohji *et al.*, 2014). Thus, the isolated strain was

Fig. 1: Phylogenetic tree with bootstrap values (n=500) based on 16S rRNA sequences, showing the position of strain PAG5 (●) relative to related strains.

named as *Pseudomonas putida* PAG5 in this study. *Pseudomonas* genus is known to be one of the most common bacterial species in soil environment and capable of degrading various organic pollutants, such as phthalates, naphthalene, fluorene and phenanthrene (Vamsee-Krishna *et al.*, 2006; Yang *et al.*, 1994; Nalli *et al.*, 2002). The results showed that *P. putida* PAG5 was able to grow in MSM medium with DEP as a sole carbon source and energy. While the concentration of DEP decreased from 100 to 10 mg l⁻¹ in the solution, the optical density increased from 0.16 to 0.3, indicating that DEP was used as a carbon source and led to an increase in microbial growth. The hydrolysis ratio of DEP by this strain was 85.5% after 5 days of incubation at pH 7.0 and 30°C. (Fig. 2). Fig. 3 shows experimental data and model simulations for DEP degradation. it was noteworthy that DEP degradation kinetics followed a first-order rate model with a regression coefficient $R^2 > 0.991$ (Küce *et al.*, 2015).

The value of first order reaction rate constant (k), as estimated from the slope of the model simulation, was 0.5265 d⁻¹. This value is comparable to the values reported for DEP in the literature (Lu *et al.*, 2009). First-order kinetics have been used frequently to describe DEP biodegradation progress at low substrate concentrations. Zeng *et al.* (2004) investigated the biodegradation kinetics of DEP, DMP, di-n-butyl phthalate (DnBP), diisobutyl phthalate (DIBP), di-n-octyl phthalate (DnOp) and (DEHP) by *Pseudomonas fluorescens* Fs1. According to the

results of this study, more than 99% of DEP was degraded in less than 96 hr. Due to persistence in the nature, microbes have evolved and adapted new pathways to degrade phthalate esters. Bacterial degradation is efficient as these compounds are either biotransformed or mineralized completely. Degradation of phthalate isomers can be both by aerobic and anaerobic routes. Many bacterial strains have been reported to degrade phthalate esters as sole carbon sources. Among the reported phthalate degraders, very few strains like *Pseudomonas testosteroni* EN 5a, *Pseudomonas aeruginosa* strain PP4 and *Comamonas testosteroni* strain E6 (Vamsee-Krishna *et al.*, 2006; Keyser *et al.*, 1972; Yan and Pan, 2004; Sasoh *et al.*, 2006).

Phthalate esters are metabolized to phthalate by esterases. Phthalate is dihydroxylated to yield phthalate dihydrodiol, which is subsequently metabolized to 3,4-dihydroxybenzoate (3,4-DHB). 3,4-DHB is ring cleaved either by protocatechuate 3,4-dioxygenase (ortho pathway) or protocatechuate 4,5-dioxygenase (meta pathway) leading to tricarboxylic acid cycle. Genes involved in the metabolism of phthalate isomers are present either on plasmids, chromosome, insertion elements or both on plasmid and chromosome (Vamsee-Krishna and Phale, 2008). The catechol test carried out as chromogenic substrate in this experiment showed a yellow/green colorization in the medium (Haigler, 1992; Mikesell *et al.*, 1993). This result indicated that catechol-derived product was produced in the medium (Fig.4A). In

Fig. 2: Biodegradation of DEP by *P. putida* PAG5 strain.

addition, plasmid curing experiment showed that bacterial growth was observed in NA plate, but no colony was observed in MSMA plate after curing experiment with ethidium bromide. Yellow/green color formation in the medium was not observed in the cured bacterial culture, catechol-derived product was not detected in the medium of plasmid cured bacteria, suggesting that the ability of *P. putida* PAG5 for degrading DEP is plasmid-encoded (Fig. 4. A, B). Plasmid DNA of *P. putida* PAG5 was successfully isolated with commercial DNA isolation kits. The

reactions were performed according to the manufacturer's instructions. The DNA fragments were electrophoresed through agarose gel (0.7%) at 60 V for 3 hr. The results of agarose gel electrophoresis showed that the size of plasmid DNA was about 15.9 kb. In contrast, no band was observed after curing experiment (Fig.5). Electrophoretic separation profiles of plasmidic DNA isolated from non-cured cultures showed that *P. putida* PAG5 contained an approximately 15.9 kb-sized plasmid. In *Arthrobacter keyseri*, it was previously shown that

Fig. 3: DEP removal kinetics at pH 7.0 and temperature 30°C. The solid line represents the first order rate expression.

Fig. 4: Catechol plate assay indicating that catechol derived intermediates were produced by *P. putida* PAG5. Yellow/green color (A) disappeared in medium of the cured bacteria (B).

26.3 kb of plasmid (pRE1-130 kb) harbors genes encoding proteins for degradation of phthalate (Eaton, 2001). A 200-kilobase pair (kbp) DNA plasmid, named PHT, was detected in a strain of *Pseudomonas putida*, NMH102-2, that utilized phthalate effectively through 4,5-dihydroxyphthalate pathway (Nomura *et al.*, 1990). A large plasmid of approximately 140 kb in size was found to be involved in the degradation of phthalates

in *Pseudomonas fluorescens*. The catabolic plasmid pSKL was observed to be transferable to different hosts (Karpagam and Lalithakumari, 1999). It was reported that *Pseudomonas* sp. strain PPD also metabolizes phenanthrene via phthalate. The cell-free extracts of phthalate grown cells showed enzyme activities of protocatechuate 3,4-dioxygenase while phenanthrene grown cells exhibited protocatechuate 4,5-dioxygenase activity,

Fig. 5: Separation profile of plasmids from *P. putida* PAG5 by agarose gel electrophoresis. Lane1 DNA size marker (1 kb DNA ladder), Lane 2 plasmid DNA, Lane 3 No plasmidic DNA band was observed after curing process.

suggesting that *Pseudomonas* sp. strain PPD has diverse pathways for phthalate metabolism (Deveryshetty et al., 2007). In an earlier study, it has been reported that a small NAH like plasmid (26 kb) in *Pseudomonas* sp. strain, isolated from same crude oil polluted refinery area (Coral and Karagoz, 2005).

This study suggests that plasmid of *P. putida* PAG5 could be involved in DEP biodegradation, and might also play a significant role in biodegradation of other xenobiotics such as PAHs. However, further molecular studies should be carried out to clear diverse pathways for phthalate metabolism in this bacterial species.

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Online CORRECTION