Enhanced biodegradation of mixed PAHs by mutated naphthalene 1,2-dioxygenase encoded by *Pseudomonas putida* strain KD6 isolated from petroleum refinery waste

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are a group of environmental pollutant that are given top priority to maintain water and soil quality to the most amenable standard. Biodegradation of PAHs by bacteria is the convenient option for decontamination on site or off site. The aim of the present study was to isolate and identify naturally occurring bacteria having mixed PAHs biodegradation ability. The newly isolated *Pseudomonas putida* strain KD6 was found to efficiently degrade 97.729% of 1500 mg L⁻¹ mixed PAHs within 12 days in carbon-deficient minimal medium (CSM). The half-life ($t_{1/2}$) and degradation rate constant (k) were estimated to be 3.2 and 0.2165 days, respectively. The first-order kinetic parameters in soil by strain KD6 had shown efficient biodegradation potency with the higher concentration of total PAHs (1500 mg kg⁻¹ soil), $t_{1/2} = 10.44$ days⁻¹. However, the biodegradation by un-inoculated control soil was found slower ($t_{1/2} = 140$ days⁻¹) than the soil inoculated with *P. putida* strain KD6. The enzyme kinetic constants are also in agreement with chemical data obtained from the HPLC analysis. In addition, the sequence analysis and molecular

docking studies showed that the strain KD6 encodes a mutant version of naphthalene 1,2-dioxygenase which have better Benzpyrene binding energy (-9.90 kcal mol⁻¹) than wild type (-8.18 kcal mol⁻¹) enzyme (chain A, 1NDO), respectively, with 0.00 and 0.08 RMSD values. The mutated naphthalene 1,2-dioxygenase *nahAc* has six altered amino acid residues near to the ligand binding site. The strain KD6 could be a good bioresource for in situ or ex situ biodegradation of polycyclic aromatic hydrocarbon.

Keywords Mixed-PAHs biodegradation · Mutant *nahAc* · In silico study · *Pseudomonas putida* KD6

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of semi-volatile environmental pollutants usually having two or more fused aromatic rings (Peng et al. 2016). PAHs are ranked in top ten threatening compounds to human health (Ifegwu et al. 2012). The ubiquitous persistence of PAHs has gained importance due to varied toxicity and structures (Rengarajan et al. 2015). The toxicity of PAHs increases with increasing number of aromatic rings, usually two to four rings PAHs are called light weight PAHs (LPAHs) and more than four aromatic rings containing PAHs are called heavy PAHs (HPAHs); HPAHs are more dangerous than LPAHs (Abdel-Shafy and Mansour 2015). PAHs can be formed in the environment naturally from organic substances but they contribute a little to the toxicity (Guo et al. 2011). The common anthropogenic, petrogenic, pyrogenic and diagenetic activities contribute most of the released PAHs to the environment (Samanta et al. 2002; Lee and Vu 2010). The hydrophobic nature of PAHs makes them most dangerous to human exposure through water contamination and

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accumulation in fatty tissue via food contamination (Diggs et al. 2011). In general, PAHs are teratogenic, carcinogenic and mutagenic and they might induce cancer specifically in skin, lungs, and bladder. In addition, the exposure to HPAHs can suppress the host immune system and also induce the oxidative stress-related tissue damage (Valavanidis et al. 2006). The metabolic activation of PAHs leads to the formation of diol epoxides which interact with DNA and create DNA adducts which is an important step for cellular carcinogenesis (Baird et al. 2005). A significant amount of PAHs had been detected in drinking water reported by WHO (World Health Organization 2010). It is not feasible to determine a level of PAHs exposure to the human body below which a threat is minimal (Yang et al. 2015). Hence, PAHs had been enlisted in priority pollutants list by US-EPA and European Commission (Albuquerque et al. 2016). PAHs usually get released to the environment as a mixture of these chemicals (Keshavarzifard et al. 2014). Therefore, decontamination of mixed PAHs is important to eliminate the threat in the environment (Tobiszewski and Namiesnik 2012). However, biodegradation or biotransformation of mixed PAHs by naturally occurring bacteria is not well documented (Pérez-Pantoja et al. 2012; Zhu et al. 2016; Qi et al. 2015). In the past few years, our laboratory is dedicatedly focussed on isolation and identification of indigenous bacteria having mixed PAHs biodegradation capabilities. In view of isolation and identification of new strains, we have successfully isolated a Novel strain of *Pseudomonas putida* designated as KD6. The strain KD6 is capable of efficiently degrade mixed PAHs in soil and water with simultaneous production of rhamnolipid during metabolism of mixed polycyclic aromatic hydrocarbons. In addition, the sequence analysis revealed that *P. putida* strain KD6 produced a mutated version of naphthalene 1,2-dioxygenase.

Methods and materials

Isolation site, culture media, chemicals and growth conditions

Soil samples were collected from petroleum refinery wastes near Indian Oil Refinery, Haldia, West Bengal, India. Naphthalene, phenanthrene and pyrene purchased from Sigma Aldrich (Bangalore, India) were used as sole source of carbon and energy in carbon-deficient minimal media (CSM). Glycerol carbon-deficient medium (GCSM) was used as transition media prior to use of CSM. All chemicals available for a preparation of CSM and GCSM were purchased from the HiMedia laboratory (Mumbai, India). CSM and GCSM were prepared according to the previous report (Iyer et al. 2013). The HPLC and GC grade solvents were purchased from the Fisher Scientific (USA).

Enrichment isolation of PAH degrading bacteria

One gram air-dried soil sample was suspended in 50 mL Luria–Bertani broth (HiMedia, India) and incubated at 30 °C for 2 days. An aliquot amount (100 µL) of clear supernatant was inoculated in 5 mL CSM amended with naphthalene (500 mg L⁻¹), phenanthrene (500 mg L⁻¹), and pyrene (500 mg L⁻¹) and rest of the protocol was carried out as described previously (Iyer et al. 2013).

Gene sequences analysis

The polymerase chain reaction (PCR) product using the 27F and 1492R universal primers was used for molecular identification and phylogenetic position of the isolated strain KD6. Naphthalene 1,2-dioxygenase (*nahAc*) was amplified in thermal cycler (Eppendorf, Germany) using forward (5'-CCGCGGAAAACCTTTGTGGGGGA-3') and reverse primer (5'-GCCCAAACGTACGCTGAACCGA-3') followed by the Sanger sequencing. The rhamnolipid production was detected by the PCR amplification of *rhlB* gene using forward (5'-ACGCCATCCTCATCGCCATC-3') and reverse (5'-GCTTGAAGCGCTCGATGCAG-3') primers. Standard reactions contained 1× Phusion High-Fidelity PCR master mix with the HF buffer (Thermo Scientific, USA) respective primer pairs and template DNA. The thermocycling for PCR was for 30 s at 98 °C for the initial denaturation followed by 25 cycles of 30 s at 98 °C, 45 s at 57 °C and 30 s at 72 °C and a final extension for 10 min at 72 °C. The nucleotide sequences retrieved from 16S rDNA and *nahAc* gene were used in the NCBI mega BLAST search to find similarity and phylogenetic position of the gene. The phylogenetic tree was drawn using MEGA7 as described previously (Kumar et al. 2016). The dissimilarity in nucleotide sequences was expressed by Sequence Logo using Web Logo as described previously (Crooks et al. 2004).

Tertiary structure of enzymes

The three-dimensional model of naphthalene 1,2-dioxygenase (*nahAc*) encoded by the *P. putida* KD6 was built using the SWISS MODEL online server (Biasini et al. 2014). The model obtained was then compared with wild type enzyme using Chimera 1.11.2 (Pettersen et al. 2004). The RRDist Map analysis was conducted by previous method (Chen et al. 2015).

Molecular docking studies

The molecular docking studies were conducted using AutoDock (v4.2.1) to estimate difference in binding free energies, ligand efficiency and inhibition constant between

the wild type and mutant enzymes involved in biodegradation of mixed PAHs. The GA-LS algorithm (Genetic algorithm with Lamarckian search) was chosen to find best conformers by setting the docking parameters to the default values. The center grid dimensions were set to $20.273 \times 61.999 \times 87.175$ with grid spacing of $0.375 \text{ \AA}$. The virtual screening was repeated 10 times with unaltered docking parameters having $2.0 \text{ \AA}$ cluster tolerance. The binding site interactions were analyzed by setting VDW scaling factor to 1.27 (Sanner 1999).

Free energy calculation using GBSA method

The sequential rigid-flexible molecular docking was performed by the MOE (molecular operating environment) docking algorithm (Chemical Computing Group Inc 2011). The London-free energy of binding (ΔG_{London}) and $\Delta G_{\text{GBVI/WSA}}$ (generalized-born volume integral/weighted surface area) were employed as scoring functions to identify more favorable docked poses and correctly estimate the binding affinity of the analyzed enzyme–substrate complexes.

Biodegradation of PAHs in liquid medium

Biodegradation study using naphthalene, phenanthrene and pyrene as sole source of carbon and energy was conducted by the protocol described previously (Akbar et al. 2014). The conical flasks were incubated at 30°C with 150 rpm and un-inoculated conical flask was used as a control. Culture medium was collected at regular intervals of 24 h for degradation and growth kinetic study.

Enzyme kinetic assay

Enzyme kinetic assay using cell-free extract (CFE) of the strain *P. putida* KD6 was conducted using previously described methods (Lin et al. 2011) with following modifications. The strain KD6 was grown in 250 mL of CSM amended with mixed PAHs (500 mg L^{-1} each) as sole source of carbon and energy at 30°C with 150 rpm for 72 h and centrifuged at $4000g$ for 20 min. The cell pellet was then washed and resuspended in 50 mmol L^{-1} potassium phosphate buffer (pH 7.5) and 0.5 mM NADH at 4°C and sonicated for 30 s. Cell pellet was then centrifuged at $11000g$ (Centrifuge 5804R, Eppendorf, Germany) at 4°C for 20 min. The clear supernatant obtained was used for different enzyme assays and protein concentration was measured using standard biochemical methods (Lowry et al. 1951). The CFE was incubated with H_2O_2 (0.4 mM) prior to the determination of enzyme activity for complete deactivation of the enzyme (Lee 1999). The enzymatic activity was estimated spectrophotometrically at

30°C and pH 7.5. One unit enzyme activity for naphthalene, phenanthrene and pyrene was defined as the amount of enzyme catalyzing the substrate at 1 mol min^{-1} . Naphthalene, phenanthrene and pyrene were detected at 260 nm (UV-Vis-1800, Shimadzu, Japan). The kinetic constants of the CFE for naphthalene, phenanthrene and pyrene ($0.5\text{--}10 \text{ }\mu\text{M mL}^{-1}$ each) such as maximum reaction rate (V_{max}), Michaelis–Menten constant (K_m), turnover rate (K_{cat}) and the specificity constant (K_{cat}/K_m) were determined by the Lineweaver–Burk plot.

Determination of bio-surfactant producing ability

The quantity of biosurfactant produced by *P. putida* strain KD6 was estimated using oil displacement test and emulsification assay described previously and expressed by emulsification index (E24) (Govarthanan et al. 2016).

Biodegradation of PAHs in soil

Biodegradation study in different microbiologically active soils was conducted by the procedure described previously (Akbar et al. 2014). Soil samples were removed at 7-day interval for degradation and growth kinetic study.

Measurement of PAHs biodegradation

High pressure liquid chromatography analysis

Biodegradation of naphthalene, phenanthrene and pyrene by *P. putida* strain KD6 was analyzed on a 1260 infinity series (Agilent, USA) HPLC system, equipped with a Quaternary Pump (G1311C), programmable variable-wavelength UV detector (G1314B), Auto sampler (G1364C), and Zorbax SB-C18 reversed-phase column ($4.6 \times 12.5 \text{ mm}$, $5 \text{ }\mu\text{m}$). Detector output was processed by Chem Station Open Lab (v2.1) data system. PAHs concentration analysis was conducted using isocratic elution conditions with the mobile phase 80:20 (v/v) methanol: water at a flow rate of 1 mL min^{-1} . The detection was performed at 254 nm. Sample injection volume was $20 \text{ }\mu\text{L}$. The retention time of naphthalene, phenanthrene and pyrene was 4.03, 4.98 and 5.57 min, respectively (Fig. 4). The calibration curves of PAHs were made from the serial dilutions of the authentic standard samples dissolved in 100% methanol. The linear range and the equation of linear regression were obtained sequentially at $0\text{--}500 \text{ mg L}^{-1}$ at an interval of 20 min. Mean areas generated from the standard solution were plotted against concentration to establish the calibration equation. The concentration of naphthalene, phenanthrene, pyrene and the metabolic intermediates were determined on the basis of the peak areas in the chromatograms.

Gas chromatography–mass spectrometry analysis

The intermediate products of naphthalene, phenanthrene and pyrene biodegradation by *P. putida* KD6 were analyzed using GC–MS (GC Trace GC Ultra, MS-Polarisq, Thermo Scientific India Pvt. Ltd) equipped with a capillary column (TR-WaxMS, 30 m × 0.25 mm [ID] × 0.25 µm film thickness). The inlet and the interface temperature were kept at 280 °C with split ratio for 10 for 2 min. The oven temperature program was 80 °C for 1 min, then raised up to 270 °C at 7 °C min^{−1} for 5 min. The carrier gas (helium) flow rate was 1 mL min^{−1}. The mass spectrometer was operated at electron ionization energy 70 eV with scan range 50–500 *m/z* (mass to ratio). Total run time for sample analyses was 33 min. The entire analyses were performed in electron ionization mode at full scan. The metabolite identification was based on mass spectra comparison with the NIST library (v2.0, 2008).

Statistical analysis

The first-order kinetic model was used to describe the rate of total reduction of PAHs in CSM and soil using $C_t = -C_0 \times e^{-kt}$ (Akbar et al. 2014). The algorithm as expressed in was used to determine the half-life ($t_{1/2}$) values of PAHs in soil, where, $t_{1/2}$ = half-life of PAHs. Results were assessed by the Origin® 2016 (California, USA) and Graphpad Prism® 6.01 (San Diego, USA) software packages for windows.

Ethical consideration

The study was approved by ethical committee meeting at Vidyasagar University.

Results and discussion

Enrichment isolation of mixed PAHs degrading bacteria

After seven constitutive subcultures in CSM, one strain was obtained. The strain was gram negative, rod shaped with distinguished colony morphology on CSM agar plate amended with naphthalene (500 mg L^{−1}), phenanthrene (500 mg L^{−1}) and pyrene (500 mg L^{−1}). The previous studies on isolation of PAHs degrading microbial consortium have demonstrated isolation from oily sludge (Gallego et al. 2007), refinery sludge (Arvanitis et al. 2008) and soil with a prior report of PAHs contamination (Jacques et al. 2008).

Gene sequence analysis

The nucleotide sequences retrieved from *nahAc* (Figure S4) and 16S rRNA by conventional Sanger di-deoxy sequencing were used for further analysis. The phylogenetic tree of 16S rRNA gene (Fig. 1b) was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.60276577 is shown (Fig. 1b). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. The evolutionary distances of *nahAc* gene were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. The optimal tree with the sum of branch length = 3.75728405 is shown (Fig. 1a). The percentage of replicates trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. All ambiguous positions were removed for each sequence pair. The evolutionary analyses were conducted in MEGA7. The sequence logo (Fig. 1c) was created by multiple sequence alignment by careful searching of two adjacent amino acid residues of the mutated positions E200 (AEN), G210 (VGW), M250 (GMG), G256 (DGY), D264 (ADL) and K334 (EKD). Glutamic acid at 200, Glycine at 210, Methionine at 250, Glycine at 256, Glutamic acid at 264 and Lysine at 334 were found in chain A of 1NDO, 2HMN, 2HMJ, 4HJL and 2BMO (except D263). In addition Glycine at 210, 255 were also found in chain A of 5BRC, 2GBW, 2CKF and Glutamic acid at 263 and Lysine at 334 were also present in chain A of 5BRC.

Structural difference between wild type and mutant type enzyme

The tertiary structure of mutant naphthalene 1,2-dioxygenase was found to have dissimilarity with the wild enzyme (1NDO) (Fig. 2a). The RRDist Map of 1NDO and mutant model shows difference = −0.726 (#0:224.A/#0:277.A, #1:224.A/#1:277.A). However, mutant model has significant similarity with 2HMN (Fig. 2b) and has difference = 0.266 (#0:224.A/#0:277.A, #1:224.A/#1:277.A).

Molecular docking analysis

The best conformations of ligand macromolecular interactions are presented in Table 2. The mutant naphthalene 1,2-dioxygenase encoded by *P. putida* strain KD6 was found to have mutation at 200, 210, 250, 256, 264 and 334 positions of chain A, those are replaced, respectively, by Glutamic acid to Lysine, Glycine to Alanine, Methionine to Isoleucine, Glycine

Fig. 1 Gene sequences analysis of *Pseudomonas putida* strain KD6. **a** The phylogenetic tree of *nahAc* gene encoded by strain KD6 was analyzed using the NCBI Blastx. The PDB data repository was chosen as library. The sequence analysis shows high sequence similarity with chain A of multi-subunit naphthalene 1,2-dioxygenase (1NDO and 2HMN). **b** The phylogenetic tree of the strain *P. putida* KD6 was

analyzed using the NCBI Blastn. *E. coli* K-12 strain was taken as non-homogeneous control. **c** The sequence logo of *nahAc* gene of the strain KD6. The amino acid sequences retrieved from the query sequences were carefully checked and we have found alteration in six positions in chain A (mutant) respectively, at E200, G210, M250, G256, D264 and K334

to Valine, Aspartic acid to Glutamic acid and Lysine to Asparagine (Fig. 1c). This alteration has a positive impact on ligand binding efficiency because E200, G210, M250, G256 and D264 are very close to the ligand binding pocket of naphthalene 1,2-dioxygenase (*nahAc*). In ligand binding pocket N201, F202, D205, H208, V209, L253, V260 etc. are engaged in ligand interactions of 1NDO, mutant and 2HMN (Fig. 3). Glutamic acid and Lysine both are polar amino acids. The amino group of Lysine is highly reactive and most often it is found inside core of the ligand binding pocket (Mendoza and Vachet 2009). Glycine and Alanine are ambivalent amino acids. Valine is a hydrophobic amino acid which replaces Glycine at 256 which is most often is found inside of the protein chain (Lodish et al. 2000) indicating K200, A210 and V256 in the mutant provide better adaptability. In addition, in the mutated position of 264 Glutamic acid is replaced by Aspartic acid and both of these amino acids are negatively charged, which helps to maintain the overall protein charge of the mutant enzyme (Chou and Wang 2015). Lysine at mutant 334 position is replaced by asparagine which has high propensity to form H-bond and is often found on the surface of the protein (Warwicker et al. 2014). The alteration of M250 to I250 does not have impact on ligand–macromolecular

interactions. The mutated version of naphthalene 1,2-dioxygenase with naphthalene, phenanthrene, pyrene as ligand has better benzpyrene binding energy -5.88 , -7.75 and -9.90 kcal mol $^{-1}$ with 0.00 RMSD value indicating the best binding pocket for the ligand (Carugo and Pongor 2008) (Table 2). Moreover, these molecular docking results are also in agreement with the $\Delta G_{GBVI/WSA}$ values calculated by the GBSA approach (Table 3).

Enzyme kinetic assay

Enzyme activity in cell-free extract of *P. putida* KD6 was studied using naphthalene, phenanthrene and pyrene as substrates. The cell free extract from the strain KD6 can degrade naphthalene, phenanthrene and pyrene with different kinetic constants (Table 4) with R^2 ranging from 0.9925 to 0.9971 indicating the experimental data are well correlated with the model. It further indicates that the cell-free enzyme extract of the strain KD6 has certain specificity to the substrates. The K_m , V_{max} and K_{cat} for naphthalene were higher than phenanthrene and pyrene suggesting that the degradation efficiency of cell-free enzyme extract was higher for naphthalene than for that

Fig. 2 RRDist Map analysis of mutated and wild type naphthalene 1,2-dioxygenase. **a** Comparison of tertiary structure and RRDist Map of mutated naphthalene 1,2-dioxygenase (*nahAc*) and 1NDO (light

blue chain). **b** Comparison of tertiary structure and RRDist Map of mutated naphthalene 1,2-dioxygenase (*nahAc*) and 2HMN (light blue chain)

other two PAHs. The specificity constant (K_{cat}/K_m) is the indicator of the specificity of an enzyme to a substrate (Eisenthal et al. 2007). The highest value of K_{cat}/K_m was found for naphthalene ($0.202 \times 10^3 \text{ mL}^{-1} \text{ mol}^{-1} \text{ s}^{-1}$) and lowest for pyrene ($0.567 \times 10^2 \text{ mL}^{-1} \text{ mol}^{-1} \text{ s}^{-1}$) which further validate the chemical data obtained from the HPLC analysis (Table 1).

Growth kinetics and bio-surfactant production during biodegradation of mixed PAHs in liquid

The hydrophobic nature of PAHs makes them low water soluble (Duran and Cravo-Laureau 2016) but rhamnolipid

produced by *P. putida* strain KD6 (Figure S5) reduces the hydrophobicity and increases the bioavailability in water (Pacwa-Plociniczak et al. 2011). The emulsification index (E24) of the strain KD6 was estimated to be 37.126 indicating an effective value (Bonilla et al. 2005). The growth kinetics of *P. putida* strain KD6 was found significantly different ($P < 0.05$) from un-inoculated CSM control medium during biodegradation of mixed PAHs. The growth kinetics and the HPLC analysis show that the strain KD6 was able to successfully metabolize naphthalene, phenanthrene and pyrene as sole and alternative source of carbon and energy (Table 1; Figure S1). However, the strain *P. putida* KD6 can degrade PAHs by a selective way

Fig. 3 Ligand binding pose of wild type and mutant type enzymes. Left to right INDO, mutant and 2HMN. **a** Naphthalene binding pocket. **b** Phenanthrene binding pocket. **c** Benzpyrene binding pocket. 1NDO (**a**) L307, N297, A206, D205, N201, F202, H208, D362, V209, H213, F352, W358. Mutant (**a**) N201, F202, D205, V209, H208, H213, N297, L307, F352, W358, D362. 2HMN (**a**) T308, C309, Y257, M336, E333, V332, L180, I331, G311, M173, L344. 1NDO (**b**) V260, F352, W358, F224, L307, H295, L253, N297, F202, D205,

N201, H208, V209, H213. Mutant (**b**) N201, F202, D205, V209, H208, H213, F224, L253, V260, H295, N297, L307, F352, W358. 2HMN (**b**) V209, H205, H213, H208, V260, N297, L307, F202, V352, E359, N201, D362, W358. 1NDO (**c**) P185, P186, K188, E92, A93, V55, A57, L42, T43, L47, Y329. Mutant (**c**) N201, F202, H205, H208, V209, H213, V260, H295, N297, L307, F352, W358, D362. 2HMN (**c**) D362, H213, H208, D205, A206, N201, W358, E359, V352, F202, L307, N297, H295

which may be based on the number of aromatic rings and complexity of the structures (Figure S1). Biodegradation analysis from HPLC data shows that it first chose naphthalene (bicyclic) followed by phenanthrene and pyrene as sole and alternative source of carbon and energy in CSM medium. In addition, 0.5% sucrose supplementation in CSM medium enhanced biodegradation and cell growth significantly ($P < 0.05$) (Fig. 4a).

Biodegradation of mixed PAHs in different microbiologically active soils

Biodegradation study in different microbiologically active soils revealed significant difference ($P < 0.05$) in mixed PAHs concentrations in sterile soil (SS) and non-sterile non-inoculated soil (nSS). PAHs degradation kinetics follows first-order reaction kinetic with R^2 ranging from 0.883

Table 1 Parameters of first-order biodegradation kinetics of PAHs by *Pseudomonas putida* strain KD6 in CSM and soil

	First-order reaction model ^a	k (day ⁻¹)	$T_{1/2}$ (days)	R^2	Soil treatments ^e	First-order reaction model	k (day ⁻¹)	$T_{1/2}$ (days)	R^2
A ^b	$\ln(C_t/C_0) = -0.167 t$	0.1671	4.1	0.9807	SS ^f + PAHs	$\ln(C_t/C_0) = -0.0032t$	0.0032	213.57	0.9776
B ^c	$\ln(C_t/C_0) = -0.1713 t$	0.1713	4.0	0.9949	nSS ^g + PAHs	$\ln(C_t/C_0) = -0.0049t$	0.0049	140.26	0.9449
C ^d	$\ln(C_t/C_0) = -0.2165 t$	0.2165	3.2	0.9898	SS + PAHs + KD6	$\ln(C_t/C_0) = -0.0663t$	0.0663	10.44	0.9393
A+B+C	$\ln(C_t/C_0) = -0.1224 t$	0.1273	5.4	0.9966	nSS + PAHs + KD6	$\ln(C_t/C_0) = -0.0528t$	0.0528	13.11	0.8834

Each figure of the table represents the mean of three replicates. All figures were significantly different at $P < 0.05$

^aInitial concentration = 500 mg L⁻¹

^bNaphthalene

^cPhenanthrene

^dPyrene

^eInitial naphthalene, phenanthrene and pyrene concentration = 500 mg kg⁻¹ soil (each)

^fRefers to sterilized soil

^gRefers to non-sterilized soil with indigenous soil microorganisms

Table 2 Best conformation of wild type and mutant type ligand–macromolecular interactions

Conformation info	Enzymes with PDB ID								
	1NDO (chain A)			Mutant (chain A) ^b			2HMN (chain A)		
	931 ^a	995 ^a	2336 ^a	931	995	2336	931	995	2336
Binding energy ^c	-5.92	-7.6	-8.18	-5.88	-7.75	-9.90	-5.86	-7.83	-10.39
Ligand binding ^c	-0.59	-0.54	-0.41	-0.59	-0.55	-0.50	-0.59	-0.56	-0.52
Inhibition constant ^d	46.14	2.68	1.01	49.38	2.09	55.32	50.5	1.82	24.26
Intermolecular energy ^c	-5.92	-7.6	-8.18	-5.88	-7.75	-9.90	-5.86	-7.83	-10.39
Vdw hb desolv energy ^c	-5.91	-7.6	-8.17	-5.87	-7.75	-9.89	-5.86	-7.83	-10.38
Cluster RMSD	0.00	0.05	0.08	0.00	0.00	0.00	0.00	0.10	0.02
Ref. RMSD	77.29	77.57	55.15	96.32	94.50	94.54	96.04	95.22	95.14

^aPubChem ligand CID 931 = naphthalene, 995 = phenanthrene, 2336 = Benzpyrene

^bMutant enzyme (naphthalene 1,2-dioxygenase) encoded by *Pseudomonas putida* strain KD6

^cEnergy unit = kcal mol⁻¹

^dInhibition constant unit = μ M at 298.15 K temperature

Table 3 Summary of sequential molecular docking for the analyzed wild type and mutant type enzyme–substrate complexes

PubChem ligand ID	ΔG_{London} (kcal mol ⁻¹)			$\Delta G_{\text{GBVI/WSA}}$ (kcal mol ⁻¹)		
	1NDO	Mutant ^a	2HMN	1NDO	Mutant ^a	2HMN
931	-7.16	-7.18	-4.92	-4.53	-4.61	-5.19
995	-8.27	-8.47	-8.64	-5.37	-5.15	-5.34
2336	-3.92	-10.71	-10.26	-5.78	-6.17	-5.82

PubChem ligand CID 931 = naphthalene, 995 = phenanthrene, 2336 = Benzpyrene

^aMutant enzyme (naphthalene 1,2-dioxygenase) encoded by *Pseudomonas putida* strain KD6

to 0.977, indicating the experimental data are well correlated with the model. The rate constant (k) for PAHs in non-sterilized and sterilized soil with strain KD6 was

0.0528 and 0.0663 day⁻¹, respectively, whereas without strain KD6, the k was 0.0049 and 0.0032 day⁻¹, respectively. Furthermore, the rate of mixed PAHs degradation

Fig. 4 Growth and degradation kinetics of mixed PAHs by *Pseudomonas putida* strain KD6 in CSM. **a** Growth kinetics of *P. putida* strain KD6 during biodegradation of mixed PAHs (filled diamond), only naphthalene (blank diamond) as sole source of carbon and energy and mixed PAHs with 0.5% sucrose supplementation (blank round) in CSM. Non-inoculated control (filled round). **b** Biodegradation kinetics of naphthalene (filled round), phenanthrene (filled black triangle) and pyrene (filled square) in CSM. Error bar represents the standard deviation of three replicates. Non-inoculated control (filled gray triangle)

by indigenous community (nSS+nah) was much slower ($8.912 \text{ mg kg}^{-1} \text{ day}^{-1}$) compared to bacterial strain KD6 ($95.347\text{--}119.731 \text{ mg kg}^{-1} \text{ day}^{-1}$). Half-lives ($t_{1/2}$) for mixed PAHs in non-sterilized and sterilized soils with strain KD6 were calculated from the linear equation obtained from the regression between $\ln(C_t/C_0)$ of the chemical data and time 13.11, 10.44 days, respectively. In contrast, the $t_{1/2}$ for PAHs in non-inoculated soil treatments were 140.26 and 213.57 days, respectively (Table 1). In the non-sterilized and sterilized soils inoculated with strain KD6, about 98 and 91% of total applied PAHs were degraded. In contrast, in non-sterilized and sterilized soil without strain KD6 disappearance of initial PAHs concentration was insignificant, and about 96 and 94% of applied PAHs persisted at the end of the incubation period. In this study, well environmental adaptability was demonstrated by *P. putida* strain KD6 during

Fig. 5 Degradation of PAHs in different microbiologically active soils by *Pseudomonas putida* strain KD6. Non-inoculated control (sterilized soil, square), non-inoculated control with indigenous microbial community (non-sterile, circle), sterilized soil introduced with strain KD6 (diamond down), non-sterilized soil introduced with strain KD6 (diamond up). Error bar represent standard deviation of three replicates

biodegradation of PAHs in different microbiologically active soils (Fig. 5). Biodegradation rate constants (k) in non-sterilized and sterilized soils with *P. putida* KD6 were estimated to be 0.0528 and 0.0663 day^{-1} which represent about 98% and 91% disappearance of the initial PAHs, respectively, at the end of the incubation period ($P < 0.05$).

Identification of D-gluconic acid by gas chromatography mass spectrometry (GCMS) analysis

At the end of incubation period, the metabolic end products of cell-free extract from the strain *P. putida* KD6 were identified by GCMS. The total ion chromatography (TIC) (Figure S2) shows intensities of individual peaks to be low and the retention time of D-gluconic acid was estimated to be 32.22 min (Figure S2). The NIST Library (v2.0, 2008) search shows high similarities of sample mass spectra with NIST MS library spectra (Figure S2). The presence of D-gluconic acid in metabolic intermediate further confirms that *P. putida* strain KD6 was able to utilize PAHs as sole source of carbon and energy in CSM.

Conclusion

The aim of the present study was to isolate and characterize naturally occurring bacteria having significant mixed PAHs biodegradation capability. To pursue this, we have isolated and identified one *P. putida* strain designated as KD6. The strain KD6 shows broad environmental adaptability with efficient biodegradation ability in soil and water. The

Table 4 Apparent enzyme kinetic constants to catalyze naphthalene, phenanthrene and pyrene

Substrate	Kinetic constants ^e				Goodness of fit R^{2d}
	K _m (μmol mL ⁻¹)	V _{max} (μmol min ⁻¹)	K _{cat} (s ⁻¹)	K _{cat} /K _m (mL ⁻¹ mol ⁻¹ s ⁻¹)	
A ^a	0.6471	2.9815	131.308	0.202 × 10 ³	0.9925
B ^b	0.4152	1.1912	30.899	0.744 × 10 ²	0.9971
C ^c	0.2427	0.6652	13.769	0.567 × 10 ²	0.9951

^aNaphthalene^bPhenanthrene^cPyrene^dNon-linear regression between initial substrate concentration 1/[S] and degradation rate constant 1/V yielded regression equation and regression coefficient (R^2)^eThe kinetic constant were determined at 30 °C and pH 7.5 using 0.5–10 μM mL⁻¹ substrate concentration (each) by Lineweaver–Burk plot

chemical data obtained from the HPLC analysis are also in agreement with the enzyme kinetic constants to catalyze naphthalene, phenanthrene and pyrene. The strain KD6 also simultaneously produces rhamnolipid during biodegradation in water. Whereas, the gene sequence (*nahAc*) analysis revealed that the strain KD6 encodes a mutated version of naphthalene 1,2-dioxygenase. The molecular docking studies show the ligand binding energy of mutant enzyme to differ with the wild type enzyme and the mutant has better benzo[a]pyrene binding energy than wild type enzyme (1NDO chain A). The interacting amino acid residues in binding pocket of naphthalene 1,2-dioxygenase have been identified in this study. Identification of interacting amino acid residues in ligand binding pocket of any enzyme is an important step for the rational approach of active site redesigning to have better thermostability and catalytic efficiency.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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