Biodegradation of phthalic acid esters (PAEs) and in silico structural characterization of mono-2-ethylhexyl phthalate (MEHP) hydrolase on the basis of close structural homolog

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HIGHLIGHTS
- Isolation of three phthalate degrading bacteria from plastic contaminated soil.
- Identification of tentative DEHP degradation pathway in Pseudomonas sp. PKDE1.
- In silico model prediction of MEHP hydrolase and docking with phthalate monoesters.
- Role of catalytic residues of MEHP hydrolase in phthalate monoesters degradation.

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ABSTRACT

Three bacterial strains capable of degrading phthalates namely Pseudomonas sp. PKDM2, Pseudomonas sp. PKDE1 and Pseudomonas sp. PKDE2 were isolated and characterized for their degradative potential. These strains efficiently degraded 77.4%–84.4% of DMP, 75.0%–75.7% of DEP and 71.7%–74.7% of DEHP, initial amount of each phthalate is 500 mg L⁻¹ of each phthalate, after 44 h of incubation. GC–MS results reveal the tentative DEHP degradation pathway, where hydrolases mediate the breakdown of DEHP to phthalic acid (PA) via an intermediate MEHP. MEHP hydrolase is a serine hydrolase which is involved in the reduction of the MEHP to PA. The predicted 3D model of MEHP hydrolase from Pseudomonas mosselii was docked with phthalate monoesters (PMEs) such as MEHP, mono-n-hexyl phthalate (MHP), mono-n-butyl phthalate (MBP) and mono-n-ethyl phthalate (MEP), respectively. Docking results show the distance between the carbonyl carbon of respective phthalate monoester and the hydroxyl group of catalytic serine lies in the range of 2.9 to 3.3 Å, which is similar to the ES complex of other serine hydrolases. This structural study highlights the interaction and the role of catalytic residues of MEHP hydrolase involved in the biodegradation of PMEs to phthalate.

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1. Introduction

Phthalates are the dialkyl or alkyl aryl esters of o-phthalic acid, which are ubiquitous environmental pollutants. They are frequently used as plasticizers to provide stability to the plastic products [1,2]. Phthalates have many different properties which depend on the composition and the type of alcohol that usually makes up the alkyl chain of phthalates [3]. Several experimental and in silico molecular docking studies have highlighted the toxic effects of phthalate exposure. This may eventually lead to inhibition of normal activities of various receptors such as peroxisome proliferator-activated receptors, glucocorticoid receptors, estrogen receptors and progesterone receptors [4–9]. Six PAEs namely Dimethyl phthalate (DMP), Diethyl phthalate (DEP), Di-n-butyl phthalate (DnBP), Butyl benzyl phthalate (BBzP), di-(2-ethylhexyl) phthalate (DEHP) and Di-n-octyl phthalate (DnOP) are in the priority pollutants list of United States Environmental Protection Agency (US EPA) and European Union (EU) due to their explored toxicological, teratogenic and mutagenic properties [10–12].

The microbial degradation is the most important route for the breakdown of DEHP and other harmful PAEs, as their photodegradation and hydrolysis rates are very slow under natural conditions [13]. Phthalates degrading microorganisms from the different genera including Bacillus, Arthrobacter, Rhodococcus, Enterococcus, Acinetobacter, Agromyces, Gordonia, Brevibacterium, Microbacterium, and Pseudomonas have been isolated from the...
various environmental samples [14–17]. In addition, there are numerous studies related to PAEs degradation pathways using pure cultures [18–20]. Under aerobic and anaerobic environmental conditions, different type of pathways such as de-esterification or dealkylation, β-oxidation and trans-esterification lead to the primary degradation of phthalate diesters to phthalate monoesters [21]. Further, phthalate monoesters are serially degraded to phthalic acid (PA) [22]. In aerobic condition, dioxygenase mediated reaction degrades PA to protocatechuate which is further degraded to oxaloacetate and pyruvate [23,24]. In anaerobic degradation, PA is decarboxylated to benzoate, which is then cleaved and degraded via β-oxidation to hydrogen, CO₂ and acetate [25,26]. Likewise, in DEHP degradation pathway, MEHP is an intermediate which is further reduced to PA [14,27]. It is to be noted that, degradation of MEHP to PA is mediated by MEHP hydrolases, belonging to the serine hydrolases family. The primary sequences of MEHP hydrolase characterized from Gordonia sp. P8219 and Rhodococcus EG-5 have conserved catalytic triad and pentapeptide motif (GxSxG) which are distinctive features of this class of serine hydrolases [28,29].

Three-dimensional structural knowledge of MEHP hydrolase is important to understand the interactions involved in the binding of phthalate monoesters to MEHP hydrolase. The present study focuses on the isolation and characterization of efficient PAEs degrading strains. Tentative DEHP degradation pathway was generated from the identification of intermediate metabolites which were formed during DEHP degradation. In addition, a 3D homology model of MEHP hydrolase from Pseudomonas mosselli was predicted. The phthalate monoesters such as mono-2-ethylhexyl phthalate (MEHP), mono-n-hexyl phthalate (MHP), mono-n-butyl phthalate (MBP) and mono-n-ethyl phthalate (MEP) were docked with MEHP hydrolase to understand the interactions between protein receptor and ligand.

2. Materials and methods

2.1. Chemicals and media

DMP, DEP, DEHP, n-hexane all with ≥97% purity were procured from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade chemicals were used. Agar, antibiotics (kanamycin, ampicillin, and chloramphenicol) were purchased from HiMedia Chemicals (Mumbai, India). Luria-Bertani medium (LB) composed of (L⁻¹): 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl. Modified minimal salt media (MSM) contained (L⁻¹): 6 g of Tris-HCl, 1.07 g of NaH₂PO₄, 4.68 g of NaCl, 0.2 g of MgCl₂·2H₂O, 1.49 g of KCl, 0.43 g of Na₂SO₄, 30 mg of CaCl₂, 2H₂O and 1X trace elements solution [30]. The final pH of the media was adjusted to 7 using HCl.

2.2. Isolation and characterization of bacterial strains

The microorganisms were isolated by enrichment culture technique where DEHP served as the only carbon and energy source. 5 g of soil sample was collected from local landfill site and mixed with 50 mL of autoclaved MSM. The cells were dislodged from the soil matrix using ultrasonic sonication bath (Bio Technics BTI-48 50 W, Mumbai, India) for 10 min at 20 °C. 100 mL of fresh MSM having DEHP (200 mg L⁻¹) was inoculated with the 1 mL of soil culture and the suspension was kept for 5 days at 30 °C and 180 rpm. 100 μL of the culture was used to inoculate fresh MSM having a higher concentration of DEHP (300 mg L⁻¹). Likewise, enrichment procedure was followed for 15 days (3 cycles) with the gradual increase in the concentration of DEHP up to 500 mg L⁻¹ 50 μL of the final enrichment culture was spread on the MSM agar plates provided with DEHP (500 mg L⁻¹). The pure colonies were obtained and the plates were incubated at 30 °C for 36 h. Finally, DEHP utilizing strains were isolated and designated as Pseudomonas sp. PKDM2, Pseudomonas sp. PKDE1 and Pseudomonas sp. PKDE2. Morphological features of the isolated strains were examined by scanning electron microscopy (LEO 435VP SEM, SEMTech Solutions, USA).

2.3. 16S rRNA gene amplification and phylogenetic analysis

For 16S rRNA gene amplification, genomic DNA was isolated using the QiAamp DNA Mini Kit (QIAGEN, Germany), following the manufacturer’s protocol. The amplification was performed using universal bacterial primers 27F (5‘-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5‘-CGGTTACCTTGTACGACTT-3’). The 50 μL of PCR mixture had 5 μL of 10X reaction buffer, 1 μL of dNTP mixture (each dNTP, 2.5 mmol/μL), 1 μL of each primer (10 pmol/μL), 2 μL of the template DNA (100 ng/μL) and 0.5 μL of Taq DNA polymerase (New England Biolabs, USA). The PCR cycle parameters were as follows: initial denaturation at 94 °C for the duration of 5 min which is followed by 30 cycles at 94 °C for the duration of 1 min, at 56 °C for 45 s, at 72 °C for 30 s, and final extension carried out at 72 °C for 10 min and final storage at 4 °C. The PCR products obtained were further purified using a QiAquick Gel Extraction Kit (QIAGEN, Germany) and subsequently sequenced (Eurofins Scientific India Pvt Ltd, Bengaluru, India).

2.4. Antibiotic susceptibility test

All the isolated strains were checked for their susceptibility towards the tested antibiotics (i.e. kanamycin, ampicillin, and chloramphenicol) using the agar disc diffusion technique. 20 μL of each antibiotic (30 μg mL⁻¹) solution was transferred into the respective well of the nutrient agar plate. 20 μL of sterile water served as a control. The plates were incubated at 30 °C for 24 h and the resultant zone of inhibition was observed.

2.5. Biodegradation of DMP, DEP, and DEHP

For assessing the biodegradative capabilities, the isolated bacterial strains were grown on enriched media (LB Broth) at 30 °C for 24 h at 180 rpm. Bacterial cells were pelleted down at 6000 rpm for 8 min. Pellets were washed thrice with 0.1 mM phosphate buffer saline (PBS), pH 7.2. Cells were suspended in the PBS buffer and used as inoculum (with OD₆₀₀ of 0.2) in further experiments. 30 mL of fresh MSM was supplemented with either of DMP, DEP, and DEHP (500 mg L⁻¹) and was inoculated with 0.3 mL of cell suspension. Non-inoculated MSM medium with carbon source was used as a control. All the experiments were performed in triplicate. After every 5 h, the samples were collected and Gas chromatography and mass spectrometry (GC–MS) (7890A GC system, 5975C MS system, Agilent Technologies, California, USA) was used to analyze the residual amount of DMP, DEP, and DEHP in the culture. An equal amount of n-hexane was added to the 30 mL of culture media having degraded PAEs. To determine the residual amount, the resultant heavy emulsion was centrifuged for 8 min at 6000 rpm and the aqueous phase was extracted. Later on, the hexane was evaporated close to dryness and reconstituted for further study. The extract was passed through 0.22 μm membrane filter and 1.0 μL of it was used for GC–MS analysis. The following program conditions of GC–MS were used: DB-5MS (Agilent J&W GC) column (30 m/0.25 mm/0.25 μm) with helium acting as a carrier gas at the flow rate maintained at 1.0 mL min⁻¹. The analysis conditions were as follows: an injection temperature of 250 °C, and an ion source temperature of 220 °C, column temperature was 80 °C for the duration of 5 min and it was increased to 200 °C at the rate of 10 °C/min in split ratio mode (1:20). The mass spectrometer was used in the electron ionization energy mode of 70 eV (mass range of 40–600 a.m.u) with 3 microscans. The scans were collected and the cor-
responding peaks of the DMP, DEP, and DEHP were identified by matching and comparing them with standard mass spectra peaks available in the National Institute of Standards and Technology (NIST) library (Agilent Technologies, USA) attached to MS system. The residual amount of respective phthalate was estimated with the help of corresponding peak area.

2.6. Identification of DEHP degradation pathway intermediates

Pseudomonas sp. PKDE1 was cultured in 10 mL of LB broth at 30 ºC and 180 rpm. The cells were pelleted down at 6000 rpm for 8 min. The pellet was washed and suspended in the PBS buffer, pH 7.2. 1% of the culture inoculum was added to 50 mL of MSM having DEHP (500 mg L⁻¹) and incubated at 30 ºC and 200 rpm. Samples were collected at the regular interval of 5 h and intermediates metabolites were extracted using hexane extraction procedure. GC–MS study of DEHP degradation intermediates was performed using DB 5MS capillary column and MS detector. The intermediate metabolites produced during the biodegradation of DEHP were tentatively identified by comparing the resultant spectra with standard mass spectra peaks available in the NIST library attached to the MS system.

2.7. Comparative molecular modeling and structure validation

Isolated strain Pseudomonas sp. PKDE1 has 99% 16S rRNA sequence identity with Pseudomonas mosselli. The sequence of MEHP hydrolase from Gordonia sp. P8219 having accession no. BAE78500 was used for searching MEHP hydrolase sequence an α/β hydrolase from Pseudomonas mosselli ATCC BAA-99 [29]. The
sequence with accession no. WP_023629646.1 was obtained from NCBI as an α/β hydroxide from *Pseudomonas mosselli*. A suitable template for homology modeling was searched by using NCBI BLAST search tool against PDB database [31]. The crystal structure of a hydrolytic enzyme (PDB ID: 4F0I) from *Pseudomonas aeruginosa* PAO1 having a sequence identity of 67% and query coverage of 93% was selected as a template.

Modeller 9.17 was used to build the comparative 3D model of MEHP hydrolyase from *Pseudomonas mosselli* [32]. Twenty models were sorted according to their DOPE scores. Model with the lowest DOPE score was selected and assessed for its stereochemical quality using PROCHECK [33]. The PROCHECK evaluates the overall stereochemical property of the predicted model. ModLoop program was used for refining the disordered loop regions of the structure [34].

Swiss Pdb Viewer 4.10 was used for the energy minimization of the selected model [35].

The ProSA integrated web server (https://prosa.services.came.sbg.ac.at/prosa.php) was utilized for the estimation of statistical Z-score deviation of the predicted model from highly resolved PDB deposited structures [36]. The refined model was also validated by VERIFY-3D [37]. It determines the compatibility between generated 3D model and its primary amino acid sequence, assigns a structural class on the basis of environment and location and comparison with refined crystal structures. The multiple sequence alignment of MEHP hydrolase from *Pseudomonas mosselli* with other C–C hydrolases from different bacteria was generated by Clustal Omega [38]. The ESPript 3.0 server was used for illustration of the sequence alignment of query sequence [39].

### 2.8. Molecular docking

AutoDock 4.2.6 was used for performing the docking of PMEs (MEHP, MHP, MBP and MEP) with the MEHP hydrolase model [40]. After selecting the MEHP hydrolase, hydrogen atoms and Kollman charges (Kollmann charges 7.0) were added by using AutoDock MGL Tools version 1.5.6. Then the receptor protein was saved in.pdbqt file format. Phthalate monoesters utilized for the docking studies were retrieved from PubChem compound database in the SDF format and converted to the PDB format using an Open Babel [41,42]. For ligand preparation, hydrogen atoms and Gasteiger charges (−1.0, −1.0, −1.0001 and −1.0001) for MEHP, MHP, MBP, and MEP were added, respectively and saved in.pdbqt format. Ligand flexibility was used to specify the torsional degrees of freedom in ligand molecule. The atomic potential grid map was generated with a spacing of 0.375 Å by using AutoGrid 4. The docking receptor grid was created by choosing the conserved catalytic triad residues and pentapeptide motif. The dimensions of grid box were as follows 40 Å × 40 Å × 40 Å. The center point coordinates were set as X = 39.439, Y = 14.124 and Z = 13.708. For docking purpose, Lamarckian genetic algorithm and grid supported energy evaluation method were adopted. The number of total GA runs was increased from 10 to 100. Other docking parameters were used as default. The pose with the minimum binding energy score was

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### Table 3

<table>
<thead>
<tr>
<th>Residual amount (%) of PAEs after 44 h of bacterial incubation at 30 °C and 180 rpm.</th>
<th>Strain</th>
<th>Residual amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMP</td>
<td>DEP</td>
</tr>
<tr>
<td><em>Pseudomonas sp. PKDM2</em></td>
<td>15.6 ± 5</td>
<td>25.0 ± 5</td>
</tr>
<tr>
<td><em>Pseudomonas sp. PKDE1</em></td>
<td>14.5 ± 5</td>
<td>25.0 ± 5</td>
</tr>
<tr>
<td><em>Pseudomonas sp. PKDE2</em></td>
<td>22.6 ± 5</td>
<td>24.3 ± 5</td>
</tr>
</tbody>
</table>

---

### Table 2

Antibiotics (each 30 μg mL⁻¹) used in the antibiotic susceptibility tests and associated presence of an interpretative zone of inhibition.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Strain</th>
<th>Kanamycin</th>
<th>Ampicillin</th>
<th>Chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Pseudomonas sp. PKDM2</em></td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td><em>Pseudomonas sp. PKDE1</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td><em>Pseudomonas sp. PKDE2</em></td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

*+: SUSCEPTIBLE to the respective antibiotic; −: RESISTANT to the respective antibiotic.

---

### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH for growth</td>
<td>6.0–8.0</td>
<td>6.0–8.0</td>
<td>6.0–8.0</td>
</tr>
<tr>
<td>Growth at 4 °C</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth at 37 °C</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maximum tolerable concentration of DEHP (ppm)</td>
<td>1000</td>
<td>1000</td>
<td>800</td>
</tr>
<tr>
<td>Utilization of Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16 s rDNA gene similarity (%) with <em>Pseudomonas aeruginosa</em> (T) ATCC 10145</td>
<td>99</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>with <em>Pseudomonas mosselli</em> (T) ATCC BAA-99</td>
<td>−</td>
<td>99</td>
<td>−</td>
</tr>
<tr>
<td>with <em>Pseudomonas mosselli</em> (T) ATCC 700476</td>
<td>−</td>
<td>−</td>
<td>99</td>
</tr>
</tbody>
</table>

Strain: 1: *Pseudomonas sp. PKDM2*; 2: *Pseudomonas sp. PKDE1*; 3: *Pseudomonas sp. PKDE2*.
selected and further visually inspected and analyzed using PyMol [43]. Active site volume was also calculated for MEHP hydrolase using DoGSiteScorer, an automated tool for pocket volume detection and analysis [44].

3. Results and discussion

3.1. Isolation and characterization of bacterial strains

Three bacterial strains proficiently utilizing DEHP as sole carbon and energy source were isolated from the plastic contaminated soil sample. Cells of the isolated strains *Pseudomonas* sp. PKDM2, *Pseudomonas* sp. PKDE1 and *Pseudomonas* sp. PKDE2 were observed to be rod shaped gram negative and aerobic with

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**Table 4**

Overview of metabolism of different PAEs by *Pseudomonas* species isolated from different environments.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Isolated Environment</th>
<th>Phthalates metabolized</th>
<th>Conc. (mg L⁻¹)</th>
<th>Time taken for metabolizing phthalates</th>
<th>Intermediates involved</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas</em> sp. Strain P136 (anaerobic metabolism)</td>
<td>Garden soil</td>
<td>Phthalate isomers (PA, IA, TA)</td>
<td>2000</td>
<td>50–60 h</td>
<td>CoA esters of phthalate, benzoate, cyclohex-1-ene-carboxylate, 2-hydroxycyclohexane carboxylate pimelate MoBP and PA</td>
<td>[64]</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. PPD</td>
<td>Sewage effluent</td>
<td>DBP</td>
<td>100</td>
<td>20 Days</td>
<td>PA, 3,4-DHB</td>
<td>[65]</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. P1</td>
<td>Garden soil</td>
<td>DBP</td>
<td>1–60 mg/g</td>
<td>4 Days</td>
<td>PA, di-hydroxybenzoic acid</td>
<td>[69]</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. B-1</td>
<td>Mangrove sediment</td>
<td>BBP</td>
<td>2.5–10.0</td>
<td>6 Days</td>
<td>For BBP degradation-MBP and PA</td>
<td>[50][70]</td>
</tr>
<tr>
<td><em>Pseudomonas</em> fluorescens F5-1</td>
<td>activated sludge at a petrochemical factory</td>
<td>DMP</td>
<td>25–400</td>
<td>25 mg/L t ½ 16.17 h</td>
<td>For DEHP degradation-MEP and PA</td>
<td>–</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. PKDM2</td>
<td>Plastic contaminated soil</td>
<td>DMP, DEP, and DEHP</td>
<td>500</td>
<td>400 mg/L t ½ 10.39 h, 25 mg/L t ½ 7.75 h, 25 mg/L t ½ 12.49 h, 25 mg/L t ½ 15.00 h, 300 mg/L t ½ 23.25 h, 12.5 mg/L t ½ 6.85 Days, 200 mg/L t ½ 13 Days, 12.5 mg/L t ½ 9.59 Days, 200 mg/L t ½ 16.99 Days, 40–44 h</td>
<td>For DEHP degradation-MEP and PA</td>
<td>–</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. PKDE1</td>
<td>Plastic contaminated soil</td>
<td>DMP, DEP, and DEHP</td>
<td>500</td>
<td>400 mg/L t ½ 10.39 h, 25 mg/L t ½ 7.75 h, 25 mg/L t ½ 12.49 h, 25 mg/L t ½ 15.00 h, 300 mg/L t ½ 23.25 h, 12.5 mg/L t ½ 6.85 Days, 200 mg/L t ½ 13 Days, 12.5 mg/L t ½ 9.59 Days, 200 mg/L t ½ 16.99 Days, 40–44 h</td>
<td>For DEHP degradation-MEP and PA</td>
<td>–</td>
</tr>
</tbody>
</table>

PA—Phthalic acid, IA—Isophthalic acid, TA—Terephthalic acid, MMP—Monomethyl phthalate, 3,4-DHB—3,4-Dihydroxybenzoate, DBP—Dibutyl phthalate, MBP—Monobutyl phthalate, BA—Benzoic acid, DnOP—Di-n-Octyl phthalate, MEHP—Mono-(2-ethylhexyl) phthalate, BBP—Bis (2-ethylhexyl) phthalate.

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**Table 5**

Identification of DEHP and its intermediate metabolites by gas chromatography–mass spectrometry.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Ion (m/z) Peaks</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>149, 177</td>
<td>19.38</td>
</tr>
<tr>
<td>MEHP</td>
<td>104, 131, 149, 167, 279</td>
<td>21.63</td>
</tr>
<tr>
<td>DEHP</td>
<td>104, 149, 168, 279</td>
<td>21.70</td>
</tr>
</tbody>
</table>

DEHP: Bis (2-ethylhexyl) phthalate; MEHP: Mono-(2-ethylhexyl) phthalate; PA: Phthalic acid.
Fig. 5. GC–MS spectra of DEHP degradation pathway. Di-(2-ethylhexyl) phthalate (DEHP) is degraded to phthalate (PA) via an intermediate mono-ethyl hexyl phthalate (MEHP).

Table 6
Docking results showing details of ligands used for the molecular docking and their binding energy and ligand efficiency score obtained and distance between the carbonyl carbon of PMEs and o of serine (Å) of MEHP hydrolase.

<table>
<thead>
<tr>
<th>Ligand Name</th>
<th>Pubchem ID</th>
<th>Ligand Structure</th>
<th>Binding Energy (kcal/mol)</th>
<th>Ligand Efficiency (kcal/mol)</th>
<th>Distance Between C=O OF PMEs and Oy of SER (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEHP</td>
<td>20393</td>
<td><img src="image" alt="MEHP Image" /></td>
<td>-4.43</td>
<td>-0.22</td>
<td>3.3</td>
</tr>
<tr>
<td>MHP</td>
<td>20269373</td>
<td><img src="image" alt="MHP Image" /></td>
<td>-5.01</td>
<td>-0.28</td>
<td>3.3</td>
</tr>
<tr>
<td>MBP</td>
<td>8575</td>
<td><img src="image" alt="MBP Image" /></td>
<td>-5.86</td>
<td>-0.37</td>
<td>3.3</td>
</tr>
<tr>
<td>MEP</td>
<td>71750793</td>
<td><img src="image" alt="MEP Image" /></td>
<td>-6.31</td>
<td>-0.45</td>
<td>2.9</td>
</tr>
</tbody>
</table>

3.2. 16S rRNA gene amplification and phylogenetic analysis

Phylogenetic analysis of 16S rRNA gene sequences shows that the isolated strains were positioned close with the members of genus *Pseudomonas*, which are previously reported to degrade persistent organic pollutants [45–47]. The 16S rRNA gene sequence of *Pseudomonas* sp. PKDM2 shows 99% sequence similarity with *Pseudomonas aeruginosa* ATCC 10145, as shown in Fig. 2. Sequence of *Pseudomonas* sp. PKDE1 and *Pseudomonas* sp. PKDE2 shows 99% homology with *Pseudomonas mosselii* ATCC BAA-99 and *Pseudomonas monteilii* ATCC 700476, respectively. In the previous studies, *Pseudomonas aeruginosa* and *Pseudomonas monteilii* have been reported to degrade recalcitrant organic compounds such as anthracene, benzo[a]anthracene, benzo[b]fluoranthene, fluorene, naphthalene and phenanthrene [47,48]. *Pseudomonas mosselii* has not been reported to degrade phthalates but it has ferredoxin-NADP+ reductase gene which is having 34% sequence identity with Phthalate dioxygenase reductase gene from *Burkholderia cepacia* [49]. Likewise, *Pseudomonas fluorescens* B-1 isolated from activated sludge reported to degrade PAEs efficiently [50]. These findings highlight the utility and extensive role played by the members of *Pseudomonas* genus in degradation of PAEs and other organic pollutants.

The 16S rRNA gene sequences of three strains were deposited in the GenBank database with accession numbers KX949578,
KX949579, and KX949580 for the strains Pseudomonas sp. PKDM2, Pseudomonas sp. PKDE1 and Pseudomonas sp. PKDE2, respectively.

3.3. Antibiotic susceptibility test

Antibiotic susceptibility test result indicates that isolated bacterial strain may be pathogenic or not, but are non-drug resistant. From the results, it can be inferred that using these susceptible strains for in-situ bioremediation of PAEs may cause low risk of spreading drug –resistant factor to other native bacterial species. The test results of the isolated strains are summarized in Table 2.

3.4. Biodegradation of DMP, DEP, and DEHP

The isolated bacterial strains were grown in different PAEs and their substrate utilization profiles were analyzed. GC–MS results showed no significant decrease in the initial amount of respective PAEs in sterile control as shown in Fig. 3. All three isolated strains prefer DMP as carbon source compared to DEP and DEHP as shown in Table 3. DMP degradation was observed to be comparatively higher (77.4%–84.4%) than that of DEP (75.0%–75.7%) and DEHP (71.7%–74.7%). Similarly, in the case of Arthrobacter sp. C21, the rate of degradation of DMP (99.5%) was higher than that of DEHP (51.4%) [51]. The observed pattern in the degradation of PAEs is possibly due to the steric hindrance caused by the phthalate side ester chains. The phthalate esters side chain hampers the binding site of hydrolytic enzymes and thus inhibits the hydrolysis reaction [14]. In this study, Pseudomonas sp. PKDE1 was observed to be more efficient in terms of phthalate degradation as compared to the other two isolated strains. These isolated strains seem to be potential candidates for the efficient remediation of the PAEs contaminated site as compared to already reported Pseudomonas species isolated from various environmental sites, as shown in Table 4.

3.5. Identification of DEHP degradation pathway intermediates

To study the biodegradation pathway of DEHP in Pseudomonas sp. PKDE1, intermediates were extracted from culture and characterized by GC–MS. In sterile control, only a single peak of DEHP was obtained at the retention time of 21.70 min as shown in Fig. 4.

After 26 h of bacterial incubation, several peaks corresponding to pathway intermediates were observed. Analysis of m/z peaks with the available database (NIST library) shows that the spectra with retention time of 21.63 min and 19.38 min correspond to MEHP and PA, respectively as shown in Table 5. Similar DEHP degradation pathway intermediates were also obtained in the case Agromyces sp. MT-O [19], Microbacterium sp. strain CQ0110Y [52] and Bacillus subtilis No. 66 [53]. In the present study, it is observed that the breakdown of DEHP to PA occurs via an intermediate MEHP as shown in Fig. 5. Degradation of MEHP to phthalate is mediated by MEHP hydrolases, belonging to serine hydrolases family [54–56].

3.6. Comparative molecular modeling and structure validation

The 3D homology model of MEHP hydrolase from P. mosselii was generated in accordance with The crystal structure of the hydrolytic enzyme from P. aeruginosa (PDB ID: 4F0J) as shown in Fig. 6. The structural superposition of MEHP hydrolase and template 4F0J shows the RMSD value of 0.098 Å for 276Ca atoms. Twenty models were generated and the model with a minimum DOPE score of −38,961.66 was selected for further analysis. After loop refinement, energy minimization of MEHP hydrolase was done by Swiss Pdb Viewer.

Ramachandran plot of MEHP hydrolase produced by PROCHECK illustrates that 95.5% amino acid residues are in the core region, 4.2% residues in the allowed region, 0.4% amino acid residues are in generously allowed and disallowed region, respectively. The results obtained indicate that the protein model is reliable. ProSA analysis further confirmed the validation of the modeled structure as the Z scores obtained for the model protein and the template were −8.7 and −8.68, respectively as shown in Fig. 7. The closeness of this parameter suggests that the predicted model is much closer to the experimentally determined structure. Verify 3D result shows that 97.44% of the amino acid residues had an average 3D–1D score >0.2. This result demonstrated that the folding energy pattern of the modelled protein structure is in complete agreement and lies within the expected range. Multiple sequence alignment result shows that the catalytic triad and pentapeptide motif (GX1SX2G) of
Fig. 8. Multiple sequence alignment of MEHP hydrolase from *P. mosselii* with homologs from the C-C hydrolases from different bacteria. The sequence alignment was done using Clustal Omega and the ESPript 3 was used for figure generation. The conserved catalytic triad residues (His-Asp-Ser) are shown by red arrows towards the bottom of the alignment. Identical residues among all the bacterial hydrolases are highlighted in white against red background. A conserved pentapeptide (Gx1Sx2G) motif is shown in red box. The sequences aligned are as follows (top to bottom): 1. ALBEH_PMOS: αβ hydrolase from *Pseudomonas mosselii* (WP_023629646.1), MEHPH_GORS: MEHP hydrolase from *Gordonia* sp. PB219 (BAE78500), MEHPH_RHOS: MEHP hydrolase from *Rhodococcus* sp. EC-5, HADH_PSES: 2-hydroxy-6-oxo-6-(20-aminophenyl)hexa-2,4-dienoate hydrolase from *Pseudomonas stutzeri* (BAH97387), HODH_RALS: 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase from *Ralstonia* sp. JS705 (CAA06969), HOHH_BURL: 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase from *Burkholderia lata* (WP_011356546), HOHH_DELT: 2-hydroxymuconic semialdehyde hydrolase from *Delftia tsuruhatensis* (AAX47253). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
MEHP hydrolase is well conserved, as found in the other bacterial C-C MCPs (meta cleavage product) hydrolases, as shown in Fig. 8.

3.7. Molecular docking

Docking studies were performed using phthalate monoesters (MEHP, MHP, MBP, and MEP) as ligands and surface view of the ligand binding pocket is shown in Fig. 9. It is reported that serine hydrolases have conserved catalytic triad (Ser-His-Glu), where serine $\Omega\gamma$ acts as a nucleophilic center for hydrolyzing ester, amide or the thioester bonds [57]. Histidine residue act as general base or acid, a carboxylate group of aspartic acid helps in the proper orientation and charge neutralization of the imidazole ring of histidine in the tetrahedral intermediate state. [58,59]. Further, deprotonated catalytic serine attacks the carbonyl carbon of the substrate which leads to the formation of covalent intermediate as shown in Fig. 10.

In order to analyze the ligand binding and interactions, molecular docking of the ligand in close proximity of catalytic serine of the MEHP hydrolase model was performed. The binding energy and ligand efficiency of different PMEs into the active pocket of MEHP hydrolase are shown in Table 6. The results show that all the docked PMEs are efficiently binding to the MEHP hydrolase. The binding energy and ligand efficiency scores of the docked PMEs illustrate the substrate preference of the MEHP hydrolase in the following
manner: MEP > MBP > MHP > MEHP. The phthalate side ester chain of MEP causes less steric hindrance as compared to other PMEs. All PMEs are stabilized in the active site of MEHP hydrolase by forming 4 hydrogen bonds with the Asn56, Ser122, and Tyr186 while MEP, MBP, and MHP interact with His 293 as well, shown in Fig. 11.

The results of molecular docking infer that the distance between the carbonyl carbon of PMEs and the Oγ of catalytic Serine is in the range of 2.9 to 3.3 Å which is similar to the Michaelis–Menten ES complex of other known serine hydrolases [60]. These PMEs are found in the close proximity of catalytic serine as reported in the crystal structures of serine hydrolase complex with their substrates [61–63]. Several crystal structures of serine hydrolases highlight the role of active site residues which are responsible for the adaptability of these hydrolases to catalyze the wide range of substrates. Superposition of catalytic triad residues (Ser-Asp-His) of MEHP hydrolase and crystal structures of various serine hydrolases is shown in Fig. 12.

4. Conclusion

Phthalate degrading three bacterial strains Pseudomonas sp. PKDM2, Pseudomonas sp. PKDE1 and Pseudomonas sp. PKDE2 were isolated and characterized for their degrading abilities. The overall results suggest that all isolated bacterial strains may act as a strong candidate for the proficient remediation of PAEs-polluted sites. Future aspect should focus upon the performance of these isolated strains in mixed co-culture for PAEs degradation. Validated molecular model of MEHP hydrolase was used for studying the binding interactions with phthalate monoesters such as mono-2-ethylhexyl phthalate (MEHP), mono-n-hexyl phthalate (MHP), mono-n-butyl phthalate (MBP) and mono-n-ethyl phthalate (MEP). It was observed that the docked ligand is in good coordination with the catalytic serine residue. This work could be further utilized to elucidate the binding mechanism of other hydrolases for the efficient degradation of PAEs. Future studies based on structural and functional aspects of other enzymes involved in PAE biodegradation will help to optimize the biodegradation process.
Confict of interest

The authors declare that they have no conflicts of interest associated with the manuscript.

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