

Isolation, Molecular, and Metabolic Profiling of Benzene-Remediating Bacteria Inhabiting the Tannery Industry Soil

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Abstract

Benzene is a pervasive contaminant and human carcinogen. Its remediation from environmental resources using conventional procedures has always been challenging due to high cost and incomplete benzene degradation. The present study was designed to explore highly efficient bacteria with benzene degrading potential from tannery industry soil, which might be used as an alternative to these conventional benzene removal remedies. Bacterial isolation was performed using benzene (80 µl/1,000 ml) supplemented with minimal salt media (MSM). Characterization of isolates was carried out by performing growth curve analysis, Gram staining, biochemical characterization via Remel RapID™ NF PLUS System (Thermo Scientific™, Thermo Fisher Scientific, Inc., USA), antibiotic sensitivity profiling, 16S rRNA gene sequencing, benzene removal efficiency estimation assay, FTIR, and GC-MS profiling. Five bacteria isolated in the present study were identified as *Paracoccus aestuarii* PUB1, *Bacillus tropicus* PUB2, *Bacillus albus* PUB3, *Bacillus subtilis* PUB4, and *Bacillus cereus* PUB6. All of these fast-growing bacteria were Gram-positive except *P. aestuarii* PUB1. Maximum benzene removal efficiency (30 mg/l per 25 h) was found in *B. tropicus* PUB2. Comparing the FTIR spectra of bacterial culture supernatant versus control revealed the peaks shifting corresponding to benzene ring bonds breaking. GC-MS analysis identified the metabolic intermediates from benzoate methylation and benzaldehyde pathways. These bacteria can be employed for benzene degradation via enzyme-based nanoparticle synthesis or cloning of relevant genes in eco-friendly expression systems.

Key words: benzene, bioremediation, GC-MS, 16S rRNA gene, whole genome sequencing

Introduction

The United States Environmental Protection Agency (EPA) has declared benzene a pervasive contaminant being identified at a large number of sites on the National Priorities List (NPL) (İşinkaralar and Erdem 2022). It has intrinsic resistance to physical and chemical degradation and tends to persist in the environment for a longer time (Varjani et al. 2017). It is a human carcinogen with a half-life of 210 days (Lawrence et al. 2016). Chemical processes like gasoline pyrolysis, catalytic reformers, and toluene hydro-dealkylation

contribute to the bulk production of benzene (Syman et al. 2023). Other sources include fossil fuel combustion, accidental spills, toluene hydro-dealkylation, wood combustion, vehicle emissions, and leaks from underground storage tanks (Sun et al. 2023; Wang et al. 2024a). Benzene has no threshold value for carcinogenesis induction (Rana et al. 2021). A wide range of health hazards are associated with benzene, like lymphocytic neoplasms, lymphoid leukemia, non-Hodgkins lymphomas, males associated hematologic malignancies, and lung and bladder cancer (Shala et al. 2023; Wang et al. 2023b; Wan et al. 2024). Exposure to even small

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concentrations may lead to a fast heartbeat, sleepiness, unconsciousness, tremors, migraines, headache, and dizziness (Höberg and Järnberg 2023).

Environmental benzene decontamination is the greatest challenge in the present era. Various physico-chemical techniques, i.e., burning, thermal conversion, landfilling, chemical oxidation and incineration and skimming, dissolution, booming, and dispersion, have been employed so far to remove benzene from soil, air, and water, respectively (Zhu et al. 2009; Lam et al. 2015). Being costly, incapable of complete benzene removal, and cause of land disturbance, these conventional strategies proved insufficient. They are being gradually replaced with the least intrusive and cost-effective alternative, i.e., bioremediation, which can be exploited in different ways like via *in situ* microbial addition or bio-augmentation (Muter 2023) or accelerating the existing microbes degradative potential through addition of substances that enhance metabolism or biostimulation (Srivastava et al. 2023). Optimization of bioremediation needs *de-novo* synthesis of benzene-degrading microbial cultures and comprehensive insight into the indigenous microbial communities (Tayyeb et al. 2024).

Various benzene metabolizing bacteria have been explored in literature including *Pseudomonas* and *Hydrogenophaga* strains from contaminated aquifer (Fahy et al. 2008), *Variovorax* sp. from coal-tar contaminated groundwater (Posman et al. 2017), *Acidobacterium* and *Polaromonas* sp. from benzene exposed soil (Xie et al. 2011), *Cupriavidus metallidurans* CH34 (Alviz-Gazitua et al. 2022), *Candidatus Nealonobacteria* (Chen et al. 2023), *Geobacter metallireducens* (Zhang T. et al. 2013), *Bacillus cereus* (Dou et al. 2010), *Ferroglobus placidus* (Holmes et al. 2011), *Pelotomaculum* (Dong et al. 2017), *Pseudomonas mendocina* KR1, *Ralstonia pickettii* PKO1 (Tao et al. 2004), *Rhodococcus opacus* (Na et al. 2005) and *Marinobacterium aestuarii* ST58-10^T (Baek et al. 2018). The majority of these bacteria are slow-growing, exhibit moderate efficiency of benzene degradation, and possess virulence (Mukherjee et al. 2019; Mohammadpour et al. 2020). These limitations are a big hurdle in their application in bioremediation.

Literature has also reported multiple benzene degradation pathways in bacteria, including benzene degradation via benzoate route in *Pelotomaculum* (Abu Laban et al. 2009), via methylation in *Desulfatiglandales* (Zehnle et al. 2023), meta cleavage pathway in *Ideonella benzenivorans* and *Pseudomonas putida* ML2 (Fong et al. 2000; Bedics et al. 2022) and carboxylation pathway of benzene ring activation in *Peptococcaceae* family (Luo et al. 2014). Some bacteria oxidize it into catechol, some into homogentistae, and others into 2-naphthol or 3-hydroxyfluorene (Ladino-Orjuela et al. 2016). A recent study reported the degradation of benzene via formation of catechol which might be

further degraded via two routes. One involves formation of 2-hydroxymuconate semialdehyde, 2-oxopent-4-enoate, 4-hydroxy-2-oxopentanoate and acetaldehyde. At the same time, the other route follows the formation of *cis,cis*-muconate, muconolactone, 3-oxoadipate enol lactone, 3-oxoadipate, 3-oxoadipyl-CoA and ultimately acetyl-CoA. This mechanism was seen in uncultivated bacteria of contaminated environments. Same study reported an anaerobic mode of benzene degradation involving the formation of benzoate, benzoyl-CoA, cyclohexa-1,5-diene-1-carbonyl-CoA, 6-hydroxycyclohex-1-ene-1-carbonyl-CoA, 6-oxocyclohex-1-ene-1-carbonyl-CoA, 3-hydroxypimeloyl-CoA, 3-ketopimeloyl-CoA, glutaryl-CoA, glutaminyl-CoA, protocoal-CoA, (S)-3-hydroxy butanol-CoA, acetoacetyl-CoA, and acetyl-CoA (Zhang et al. 2024).

The tanning process in tannery industries has been categorized as the most polluting activity as it involves using a wide range of persistent organic and inorganic pollutants, including benzene, for skin conversion into leather (Hira et al. 2022). These are also considered the major source of benzene pollution in the environment (Rastogi et al. 2008). Although several sources have been explored in literature, but no one has explored the tannery industries of Southern areas of Pakistan yet.

Despite this diversity in modes of bacterial degradation of benzene, these bacteria cannot be fully exploited because of their slow growth, moderate efficiency of benzene degradation, and virulent nature (Mukherjee et al. 2019; Mohammadpour et al. 2020). They take weeks or even months in complete benzene removal (Mohammadpour et al. 2020; Irshaid et al. 2024). In most cases, the virulence status of bacteria is also unknown, which restricts their exploitation in eco-friendly mode. Current study was designed considering this knowledge gap of benzene degradation associated bacteria and the tannery industry as sources of such microbes. This investigation focused on isolating and characterizing fast-growing and highly efficient benzene-degrading bacteria from soil in the tannery industry. Getting insights into the genomics and metabolomics of these bacteria might equip us with multiple strategies for the environmental decontamination of benzene.

Experimental

Materials and Methods

Isolation and morphological characterization of benzene metabolizing bacteria. The serial dilution method was employed using a minimal salt medium (MSM) to isolate bacteria. Composition of MSM was KH_2PO_4 , 1 g; Na_2HPO_4 , 1.25 g; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; MgSO_4 ,

0.5 g; CaCl₂, 0.5 g and FeSO₄, 0.005 g/1,000 ml distilled water and 80 µl benzene. Tannery soil (1 g) was suspended in Ringer's solution (10 ml) and kept under shaking conditions (150 rpm, 37°C) overnight. To prepare serial dilutions, overnight incubated Ringer's solution (1 ml) was added to the tube marked as original. Followed this, solution from this tube (1 ml) was poured into the one marked as 10⁻¹. Dilutions were prepared up to 10⁻⁷. These dilutions (200 µl) from each test tube were spread into Petri plates containing MSM medium supplemented with agar and benzene, followed by incubation at 37°C. After 3 days, bacterial colonies appeared. CFUs were calculated as follows;

$$\text{CFUs per ml} = \frac{\text{Number of Colonies} \times \text{Dilution Factor}}{\text{volume (ml)}}$$

Following this streaking, the purified colonies were obtained. The purified colonies were preserved in the form of glycerol stocks at -20°C. Each discrete colony was examined for color, elevation, texture, and margins. Gram staining, capsule staining, and spore staining were also performed.

Molecular analysis. Isolated bacterial cells were cultured and harvested during the log phase. Organic method was used for DNA isolation from the bacterial pellets (Dairawan and Shetty 2020). Genomic DNA of isolated bacteria was extracted from the pellet. PCR was used for the amplification of template 16S ribosomal RNA (rRNA) gene using previously reported primers i.e., forward 5'-AGAGTTTGATCCTGGTCAGAACGAACGCT-3' and reverse 5'-CGTACGGCTACCTTGTTACGACTTCACCCC-3' with melting temperatures (T_m) of 70.4°C and 74.8°C, respectively (Muccee et al. 2019). Primers were synthesized from Macrogen Inc. (Republic of Korea). Nine variable and ten constant regions of the gene were targeted. Amplicon size was 1,300 base pairs (bp). The PCR reaction mixture (25 µl) contained 12.5 µl master mix, 1.0 µl forward (F) primer, 1.0 µl reverse (R) primer, 0.5 µl Taq polymerase, 2 µl template DNA and 8.0 µl nuclease-free water. PCR amplification conditions used are as follows: 94°C and 10 min for initial denaturation, 35 cycles of denaturation at 94°C for 30 sec, 65°C and 40 sec for annealing, and 72°C and 30 sec for extension, followed by a final extension at 72°C for 10 min. Agarose gel electrophoresis was performed to confirm the target DNA amplification. Amplified products were analyzed via Sanger Sequencing at Macrogen Inc. Korea (Republic of Korea).

Bioinformatics analysis. BLASTN analysis (<http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi>) was used for the interpretation of FASTA sequences, which involved alignment of sequences of present study bacteria with those available at NCBI (National Center for Biotechnology Information) database (Chen et al. 2015;

Schoch et al. 2020). Sequences were submitted to the NCBI Genbank database to get the accession numbers assigned. Afterwards, Clustal Omega Multiple Sequence Alignment Algorithm (<http://www.clustal.org/mbed.tgz>) was consulted to perform multiple sequence alignment of identified bacteria and those retrieved from database for tree construction (Sievers and Higgins 2021). Aligned data was used for phylogenetic tree construction. Tree was constructed via Molecular Evolutionary Genetic Analysis version 11 (MEGA 11) software, considering the bootstrap value of 100 and neighbor joining mode (Tamura et al. 2021).

Growth curve analysis. The bacterial inoculum with 10⁷ cells, i.e., with optical density (OD)=0.1, was used to inoculate the benzene-supplemented MSM for growth analysis. The bacteria were cultured at 150 rpm and 37°C. Culture was drawn at regular intervals i.e., 0, 3, 6, 24, 27, 30, 48, and 51 h to measure the absorbance or OD₆₀₀ via spectrophotometer (BioTek Instruments, Inc., USA). To predict the duration of lag, log, static, and death phases in bacteria, growth curves were plotted by taking time and OD₆₀₀ values along x and y-axis, respectively (Domańska et al. 2019).

RapID NF PLUS system. For the characterization of bacteria at the biochemical level, the Remel RapID™ NF PLUS System (Thermo Scientific™, Thermo Fisher Scientific, Inc., USA) was employed. A panel comprising of cavities with dehydrated substrates was uncovered and inoculated with freshly overnight-grown cultures. Following this, the panel was shaken to uniformly distribute the culture among all the cavities and incubated (37°C for 4 h). After incubation, bacterial response to different substrates was assessed by noting the color change. The observed results were compared with the catalog provided along with the kit.

Bacteria were analyzed for nineteen biochemical tests i.e., urease test (URE), arginine dehydrolase test (ADH), ornithine decarboxylase test (ODC), lysine decarboxylase test (LDC), aliphatic thiol utilization test (TET), lipase detection test (LIP), sugar aldehyde utilization test (KSF), sorbitol fermentation test (SBL), β-glucuronidase test (GUR), *p*-nitrophenyl-β-D-galactosidase (ONPG), *p*-nitrophenyl-β-D-xyloside hydrolase test (βXYL), β-nitrophenyl-β-D-glucoside (βGLU), *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide (NAG), maltose fermentation test (MAL), proline β-naphthylamide (PRO), γ-glutamyl-β-naphthylamide hydrolysis test (GGT), pyrrolidine-β-naphthylamide (PYR), adonitol fermentation test (ADON) and tryptophane fermentation test (IND).

Antibiotic resistance profiling. Stock solutions of antibiotics i.e., augmentin, amoxicillin, cephalixin, and cefadroxil were prepared to prepare discs of different conc. i.e., 5 µl, 10 µl and 15 µl. Petri plates were marked to divide them into four quadrants, which were marked

as control, 5, 10, and 15 μ l. The antibiotic discs with placed in the 5, 10 and 15 μ l volume in each labelled quadrant, respectively. Each Petri plate was inoculated with 100 μ l bacterial culture. The plates were incubated for 24 h, at 37°C. Zones of inhibition formed in each plate were measured.

Confirmation of benzene degradation. Benzene removal assay. The benzene removal potential of bacteria was estimated by measuring the absorbance spectrophotometrically at 180 nm. Media with different known concentrations of benzene i.e., 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, and 30 mg/l were prepared. The value of OD₁₈₀ of these solutions was measured and the standard curve was plotted. Linear regression analysis was applied and linearity was estimated by measuring the correlation coefficient (R^2). Current study bacteria were cultured in MSM supplemented with benzene (40 mg/l) until exponential growth was achieved. The culture was harvested and centrifuged by the end of the log phase. Residual benzene was estimated by measuring OD₁₈₀ of the supernatant. Standard curve was consulted to estimate the benzene degradation potential of bacteria in mg/l.

FTIR analysis. MSM broth (500 ml) with 40 μ l benzene was prepared and inoculated with 100 μ l overnight grown culture to prepare samples for FTIR interpretation. A control, containing only MSM and benzene without inoculation, was used. The flasks were kept under shaking conditions (150 rpm) at 37°C till the exponential growth of bacteria was achieved. The experimental and control flask contents (5 ml) were centrifuged to get the supernatant. Capped the vials and sent them for analysis to the Textile and Polymeric Engineering Department of the University of the Punjab (Pakistan).

Results were obtained in the form of FTIR spectra. Spectral peaks obtained in control and bacterial samples were analyzed for the stretching, bending or any other variations. These variations could help us to identify the change in benzene ring structure induced by bacteria.

Extraction of metabolites. Two present study bacteria (PUB1 and PUB4) with optimum benzene degradation potential were selected for the metabolic profiling. These two isolates were cultured individually in MSM medium supplemented with benzene under shaking conditions at 37°C and 150 rpm until exponential growth was achieved.

Extracellular and intracellular metabolites were extracted using a previously reported method (Muccee et al. 2019). Supernatants of isolated bacterial cultures were used as a source of extracellular metabolites and mixed with methanol in equimolar concentration. Centrifugation was performed for 20 min at 4°C at 8,000 rpm. The top layer (10 ml) of supernatant was carefully collected in a glass vial and incubated for

a week at 50°C. After evaporation of the solvent, the metabolites extract was achieved.

In case of intracellular metabolites, bacterial pellet was used. The pellet was washed with NaCl solution (0.9%) and resuspended in a mixture of deionized water, chloroform, and methanol (800 μ l). Sonication was performed for 1 h at 4°C to optimize the bacterial cell lysis. Centrifugation was performed for 20 min at 10,000 $\times g$ to separate intracellular metabolites, resulting in the separation of three layers. The upper and lower soluble parts were comprised of polar and nonpolar metabolites, respectively. Extracts of extracellular and intracellular metabolites were sent to the Textile Testing International Laboratory, Quaid-e-Azam Industrial Estate, Lahore (Pakistan), for GC-MS analysis.

GC-MS analysis of extracted metabolites. To perform GC-MS analysis Agilent 7890B/5977A (Agilent Technologies, Inc., USA) was used. Parameters considered were inlet temperature (280°C), injection volume (1 μ l), DB-5MS 30 m column (dimensions 0.25 mm $\times$ 0.25 μ m), helium as carrier gas with flow rate (1 ml/min), and oven temperature (50–290°C over a run time of 51.133 min). MS scan mode (35...500) with a solvent delay of 4.8 min was selected to allow the detection of compounds with a specific m/z ratio. Solvent delay ensured the elution of desired analytes before the unwanted peaks.

Confirmation of benzene degrading genes in bacteria via whole genome sequencing. A consortium comprising an equimolar concentration of DNA extracted from the present study benzene metabolizing bacteria and the xylene and phenol degrading bacteria was constructed in a separate research project to confirm the benzene degrading potential of current study bacteria at the genetic level. Consortium construction was performed according to a previously reported study (Muccee and Ejaz 2020). This consortium was analyzed via whole genome sequencing (WGS) at Macrogen Inc. Korea (Republic of Korea). The fastq files obtained were submitted to NCBI Sequence Read Archives (SRA), and an accession number was assigned. The bioinformatics analysis was performed to identify the genes and pathways involved in the breakdown of these compounds. The detailed pipeline involved in in-silico analysis is shown in Fig. S1.

Results

Soil samples from two tannery industries were processed for the isolation of benzene-degrading bacteria. Initially, sixty-four colonies were picked and subjected to growth curve analysis. Five bacteria (Fig. S2) with the fastest growth rate were selected for further characterization.

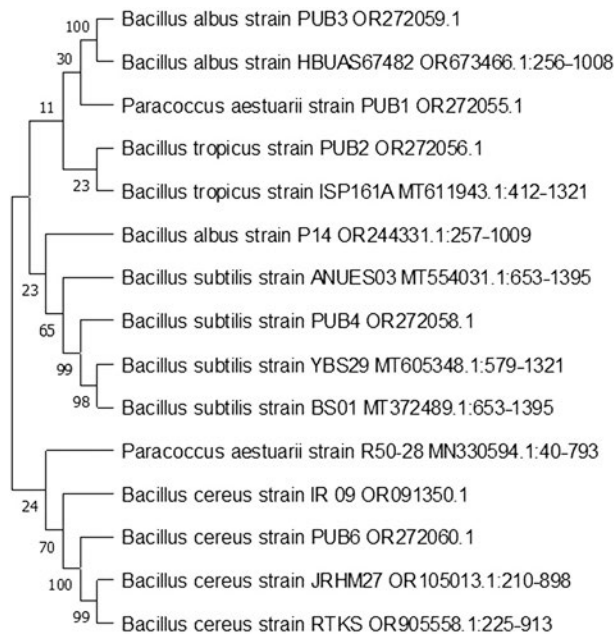


Fig. 1. Phylogenetic tree constructed for present study bacteria using MEGA11 software.

Molecular characterization. Genomic DNA extracted from bacterial isolates and PCR amplicons were resolved on agarose gel. A total of five benzene-metabolizing bacteria were isolated. These showed top BLAST sequence homologies with *Paracoccus aestuarii*, *Bacillus tropicus*, *Bacillus albus*, *Bacillus subtilis* and *B. cereus*, respectively (Table SI). Strains of these bacteria were named PUB1, PUB2, PUB3, PUB4, PUB5, and PUB6, respectively. PU is an abbreviation of the University where research was performed, and B stands for benzene. The numbers 1, 2, 3, 4, and 6 showed the sequence of bacterial isolation. Accession numbers assigned to the present study bacteria are as follows: OR272055 (*P. aestuarii* PUB1), OR272056 (*B. tropicus* PUB2),

OR272059 (*B. albus* PUB3), OR272058 (*B. subtilis* PUB4) and OR272060 (*B. cereus* PUB6).

Phylogenetic analysis. The evolutionary tree constructed for the present study bacteria is shown in (Fig. 1). According to this analysis, *P. aestuarii* PUB1 and *B. albus* PUB3 were closely related to *B. albus* strain HBUAS6748 via bootstrap values of 30 and 100, respectively. *B. tropicus* PUB2 shared the same clade with *B. tropicus* strain ISP161A through a bootstrap value of 23. *B. subtilis* PUB4 showed relatedness with *B. subtilis* strains YBS29 and BS01 via bootstrap value of 99. *B. cereus* PUB6 shared the same clade with *B. cereus* strains JRHM27 and RTKS through strong bootstrap value of 100.

Morphological analysis and bacteria enumeration. Three bacteria *P. aestuarii* PUB1, *B. tropicus* PUB2, and *B. albus* PUB3 were isolated from sample I with CFUs 4.75×10^3 , 9.9×10^3 , and 1.4×10^4 per ml, respectively. Two bacteria *B. subtilis* PUB4 and *B. cereus* PUB5 were isolated from sample II with CFUs 3.2×10^2 and 6×10^2 per ml, respectively.

Isolated bacterial color, shape, margins, elevation, and texture were documented (Table SII and Fig. S3). All bacterial strains were Gram-positive except *P. aestuarii* PUB1. Only *B. tropicus* PUB2 and *B. albus* PUB3 were capsulated while the rest were non-capsulated. The *B. albus* PUB3, *B. subtilis* PUB4 and *B. cereus* PUB6 were spore forming bacteria.

Growth curve analysis. All bacteria were fast-growing, with the log phase starting at 23 h in *B. tropicus* PUB2 and 25 h in *P. aestuarii* PUB1, *B. albus* PUB3, *B. subtilis* PUB4, and *B. cereus* PUB6. *P. aestuarii* PUB1, *B. subtilis* PUB4 and *B. cereus* PUB6 exhibited longer exponential phases i.e., 25–47 h. In PUB1, *B. subtilis* PUB4, and *B. cereus* PUB6, the stationary phase started at 48 h and lasted for 3 h and 1 h, respectively (Fig. 2).

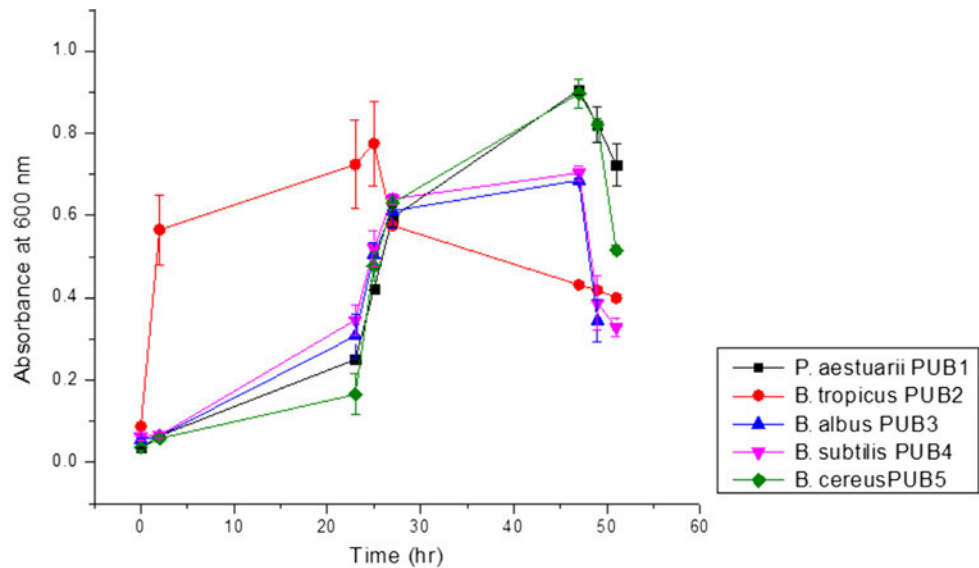


Fig. 2. Growth curve analysis of present study benzene degrading bacteria.

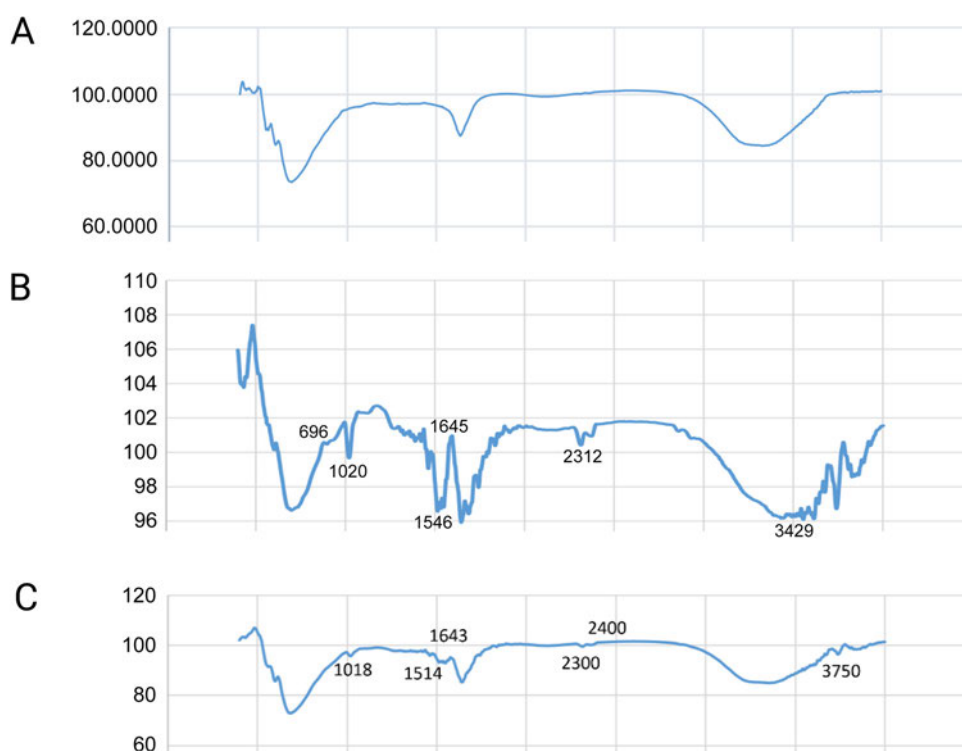


Fig. 3. Comparison of FTIR spectra of control and present study benzene degrading bacteria to analyze the variation of the peaks contributed by bacterial benzene degradation.

A) Control, B) *Paracoccus aestuarii* PUB1, C) *Bacillus tropicus* PUB2, D) *Bacillus albus* PUB3, E) *Bacillus subtilis* PUB4, F) *Bacillus cereus* PUB6.

Biochemical characterization. *P. aesturii* PUB1, *B. albus* PUB3, and *B. cereus* PUB6 showed the same results for biochemical analysis and were positive for ADH, ODC, LDC, TET, LIP, KSF, SBL, PRO, GGT, PYR and ADON. *B. tropicus* PUB2 and *B. subtilis* PUB4 showed different biochemical characteristics. Positive response for TET, LIP, KSF, SBL, PYR, and ADON was recorded in *B. subtilis* PUB4. While *B. tropicus* PUB2 was positive for ODC, LDC, LIP, KSF, SBL, GUR, PRO, GGT, PYR and ADON (Table SIII and Fig. S3).

Antibiotic sensitivity profiling. Present study isolates showed sensitivity to all four antibiotics except *B. subtilis* PUB4, which was resistant against amoxicillin at a concentration of 5 μ l and 10 μ l. Zones of inhibitions in all four strains were recorded (Table SIV) and compared. In the case of cephalexin and cephadroxil, the highest zone was observed in *P. aesturii* PUB1 and *B. subtilis* PUB4. i.e., 4 and 3.4 mm, respectively. *B. tropicus* PUB2 exhibited the highest zone of inhibition for augmentin (2.9 mm) and amoxicillin (2.4 mm).

Benzene removal efficiency estimation. Benzene removal efficiencies of present study bacteria were assessed using the standard curve (Fig. S4) plotted between known concentrations of benzene stock solution versus OD₁₈₀ as maximum UV absorption of benzene occurs at 180 nm. The efficiencies were estimated as *P. aesturii* PUB1 (31 mg/l per 47 h), *B. tropicus* PUB2 (30 mg/l per 25 h), *B. albus* PUB3 (34 mg/l per 47 h),

B. subtilis PUB4 (32 mg/l per 47 h) and *B. cereus* PUB6 (31 mg/l per 47 h).

FTIR analysis. Compared with the control, *P. aesturii* PUB1 and *B. cereus* PUB6 FTIR spectra showed significant changes in the peaks throughout the spectra (Table I). While *B. tropicus* PUB2 and *B. albus* PUB3 showed shifting of peaks in the regions of 400–750 cm^{-1} , 1000–1900 cm^{-1} , 2200–2500 cm^{-1} and 3000–3800 cm^{-1} (Fig. 3). Sharp predominant peaks in FTIR spectra were observed that reflected the metabolites identified through GC-MS spectra i.e., for **toluene** peaks at 1452 cm^{-1} (asymmetric stretching frequency of C=C), 2851 cm^{-1} and 2920 cm^{-1} (stretching vibrations of =C–H and –C–H), were observed. In case of **benzyl alcohol** peaks at 3427 cm^{-1} (O–H stretching), 3010 cm^{-1} (unsaturated C–H) and 2900 cm^{-1} (saturated C–H stretching), **benzaldehyde** peaks at 1637–1733 cm^{-1} (C=O stretching vibrations), **benzoate** peaks at 3427 cm^{-1} (–OH stretching) and 1271 cm^{-1} (C–O–C bonds stretching), catechol peaks at 3427 cm^{-1} (OH stretching), 1658 cm^{-1} (C=C stretch), 1250 and 1281 cm^{-1} (C–O stretch vibrations), 746 and 850 cm^{-1} (out of plane bending of =C–H bond of aromatic ring), **cis-1,6-dihydroxy 2,4-cyclohexadiene-1-carboxylic acid** peak at 2,800–3,300 cm^{-1} (C–H bond stretching), **phenol** peaks at 3427 cm^{-1} (OH bond stretch) and 1650 cm^{-1} (C=C stretch), **cis, cis-muconate** peaks at 1540–1650 cm^{-1} (asymmetric stretching vibrations

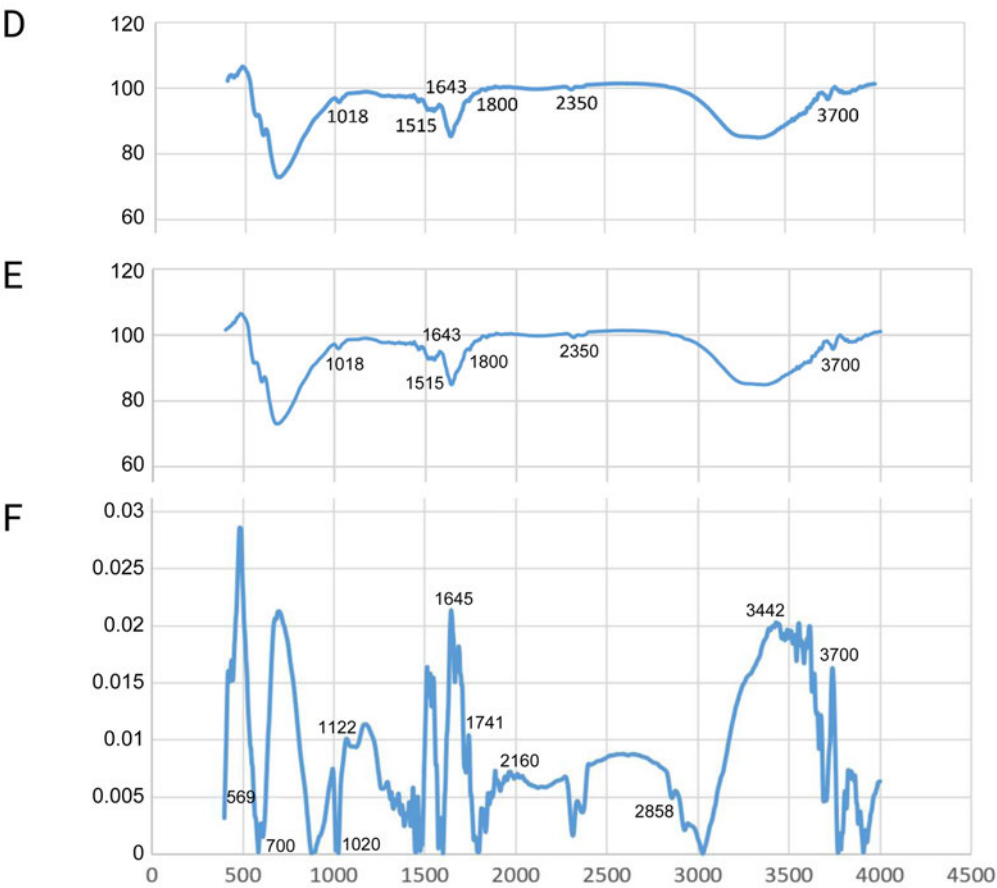


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of CO_2^-) and $1360\text{--}1450\text{ cm}^{-1}$ (symmetric stretch of CO_2^-), **3-oxoadipate enol lactone** and **3-oxoadipate** peaks at $1637\text{--}1733\text{ cm}^{-1}$ ($\text{C}=\text{O}$ stretch vibrations) and 3427 cm^{-1} (OH stretching) and **palmitate** 685 and 723 cm^{-1} ($-\text{OH}$ swinging vibrations), 939 cm^{-1} ($-\text{OH}$ out-plane stretch vibrations) and 1299 cm^{-1} ($-\text{OH}$ in-plane stretching), 2848 cm^{-1} ($-\text{CH}_2$ symmetrical stretching), 2913 cm^{-1} ($-\text{CH}_3$ symmetrical stretch vibrations), 1695 cm^{-1} $\text{C}=\text{O}$ stretch vibrations, and 1464 cm^{-1} ($-\text{CH}_2$ and $-\text{CH}_3$ deformation vibrations).
GC-MS-based metabolites identification. Among the present study bacteria, isolates with efficient benzene degradation (*P. aestuarii* PUB1 and *B. subtilis* PUB4) were selected for GC-MS-based profiling. Fifty-five compounds were identified based on the comparison of GC-MS spectra obtained with the NIST library (Fig. S5) and the comparison of m/z ratios of spectral peaks with masses of benzene degradation intermediates and products reported in the literature (Fig. S6). They included the metabolic intermediates of the benzene methylation pathway, benzene degradation via benzaldehyde, carboxylation, and phenol pathway and β -keto adipate pathway. Pathways involving the formation of these intermediates are shown in Fig. 4 and 5. In addition to

these compounds, some other metabolic intermediates were also identified and given in Table SV.
WGS and identification of benzene degradation associated genes. WGS analysis of the present study bacterial consortium predicted their taxonomic composition. Assigning taxonomic labels to metagenomic contigs showed that 75% of contigs belonged to the genus *Bacillus*. This is consistent with the ribotyping analysis results of the present study, as out of five isolates, four belonged to *Bacillus*.
Functional profiling revealed the presence of multiple enzymes associated with benzene degradation. i.e., benzoate/toluate 1, 2-dioxygenase subunit alpha [EC:1.14.12.10], dihydroxycyclohexadiene carboxylate dehydrogenase [EC:1.3.1.25], catechol 1,2-dioxygenase [EC:1.13.11.1], muconate cycloisomerase [EC:5.5.1.1], muconolactone D-isomerase [EC:5.3.3.4], 3-oxoadipate enol-lactonase [EC:3.1.1.24], 3-oxoadipate CoA-transferase, alpha subunit [EC:2.8.3.6], acetyl-CoA acyl-transferase [EC:2.3.1.16], 3-oxoadipyl-CoA thiolase [EC:2.3.1.174], catechol 2,3-dioxygenase [EC:1.13.11.2], 2-hydroxymuconate-semialdehyde hydrolase [EC:3.7.1.9], 2-keto-4-pentenoate hydratase [EC:4.2.1.80], 4-hydroxy 2-oxovalerate aldolase [EC:4.1.3.39], acetaldehyde

Table I

Metabolites identified in the present study bacteria, through comparison of m/z ratios of GC-MS spectra with molecular weights of earlier reported benzene degradation metabolites.

Metabolite identified	Metabolites unidentified	PUB4	PUB1	Fragmentation ion (<i>m/z</i>)	Molecular weight (M/w)	FTIR spectral peaks (cm ⁻¹)
Benzene methylation pathway						
Toluene	<i>cis</i> -benzoate dihydrodiol, <i>cis</i> -benzene dihydrodiol	I	I	39, 51, 65, 77, 91, 92	92.14	1452 (aromatic deformation), 2851 and 2920 (=C–H and –C–H)
Benzyl alcohol		I		65, 77, 79, 91, 107, 108	108	3427 (OH), 3010 (unsaturated CH), 2900 (saturated CH)
Benzaldehyde		I	I	29, 39, 51, 52, 77, 78, 105, 106	106	1637–1733 (C=O vibs)
Benzoate		I	I	176, 121 , 93, 77, 65	121	3427 (OH), 1271 (C–O–C stretching)
Catechol		I	I	81, 66, 53, 110	110	3427 (OH), 1658 (C=C), 1250 and 1281 (C–O), 746 and 850 out of plane bending of =C–H bond of aromatic ring
Benzene degradation via benzaldehyde						
Benzyl alcohol	benzoate, benzoyl-CoA, acetyl CoA	I	I	65, 77, 79, 91, 107, 108	108	3427 (OH), 3010 (unsaturated CH), 2900 (saturated CH)
Benzaldehyde		I	I	29, 39, 51, 52, 77, 78, 105, 106	106	1637–1733 (C=O vibs)
Benzene degradation via carboxylation						
Benzoate	<i>cis</i> -1,6-dihydroxy-2,4-cyclohexadiene- 1-carboxylic acid	I	I	176, 121 , 93, 77, 65	121	3427 (OH), 1271 (C–O–C stretching)
<i>cis</i> -1,6-dihydroxy-2,4-cyclohexadiene- 1-carboxylic acid		I	I	44, 73, 149	156	2800-3300 (C–H stretch)
Catechol		I	I	81, 66, 53	110	Same as above
Benzene degradation via phenol						
Phenol		I	I	17, 38, 39, 40, 63, 65, 77, 93, 94	94	3427 (OH), 1650 (C=C stretch)
Catechol		I	I	81, 66, 53, 110	110	Same as above
β-ketoadipate pathway						
Catechol	3-oxoadipyl CoA, succinyl CoA, succinate, fumarate	I	I	81, 66, 53, 110	110	Same as above
<i>cis,cis</i> -muconate		I	---	77, 106, 122, 126, 138, 171, 195, 228	126	1540–1650 (asymmetric stretch of CO ₂ ⁻), 1360–1450 (symmetric stretch of CO ₂ ⁻)
3-oxoadipate enol lactone		I	---			1637–1733 (C=O vibs), 3427 (OH),
3-oxoadipate		I	---	40, 44, 73, 84, 87, 101, 114, 115, 160	160	1637–1733 (C=O vibs)
Palmitate		I	I	41, 43, 57, 73, 89, 99, 117, 127, 141, 169, 183, 201, 215, 229, 239, 257, 271, 285, 299, 313	255	2848 and 2913 (–CH ₃ and –CH ₂ vibs), 1695 (C=O), 1464 (–CH ₂ and –CH ₃), 939 (–OH out plane vibs) and 1299 (–OH in-plane vibs), 723 and 685 (–OH swinging vibs)

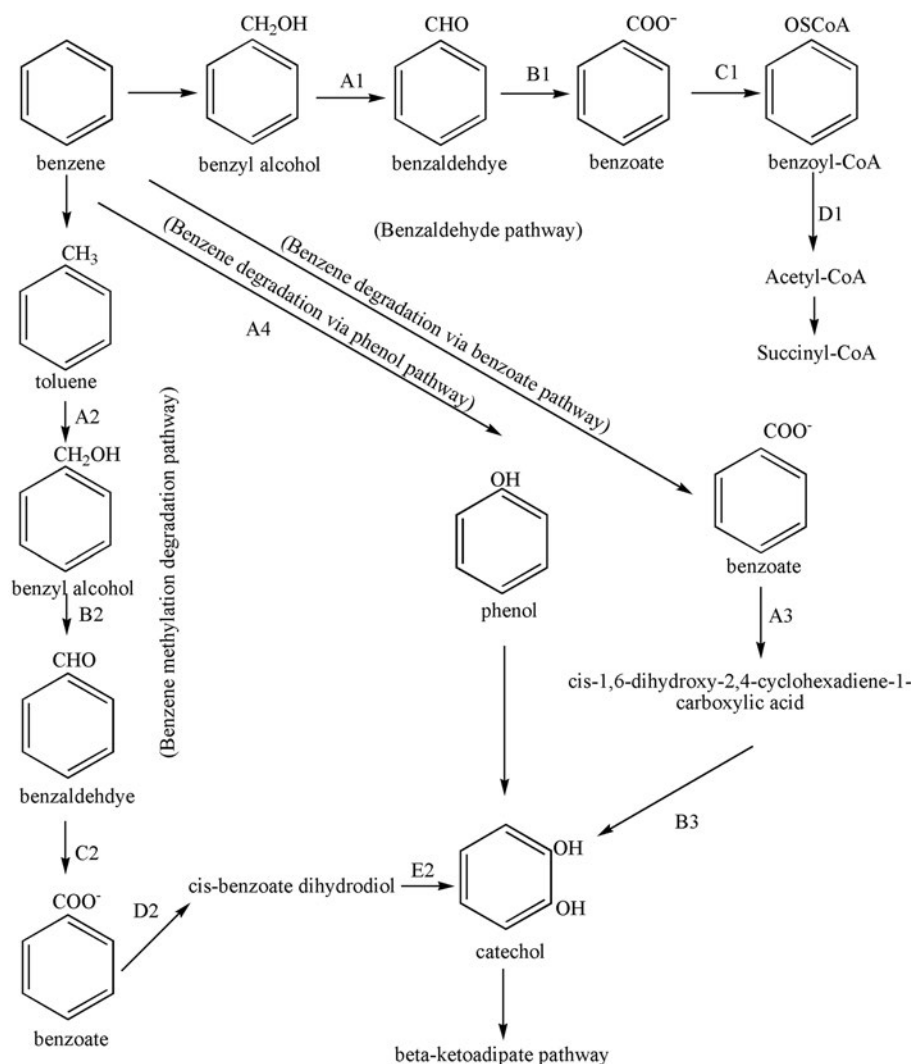


Fig. 4. Four pathways of benzene metabolism suggested in benzene-degrading bacteria in the present study based on GC-MS based identification of metabolites.

A1 – benzyl alcohol dehydrogenase, B1 – benzaldehyde dehydrogenase, C1 – benzoate CoA-ligase, D1 – benzoyl-CoA 2,3-dioxygenase, A2 – methyl monooxygenase, B2 – benzoyl alcohol dehydrogenase, C2 – benzaldehyde dehydrogenase, D2 – benzoate 1,2-dioxygenase, E2 – dihydrocyclohexadiene carboxylate dehydrogenase, A3 – benzoate dioxygenase, B3 – benzoate *cis*-diol dehydrogenase, A4 – benzene phenol monooxygenase

dehydrogenase [EC:1.2.1.10], protocatechuate 3,4-dioxygenase, alpha subunit [EC:1.13.11.3], 3-carboxy-*cis*, *cis*-muconate cycloisomerase [EC:5.5.1.2], 4-carboxy-muconolactone decarboxylase [EC:4.1.1.44], 3-hydroxybenzoate 6-monooxygenase [EC:1.14.13.24]. **Fastq files of WGS sequencing analysis were submitted in SRA NCBI database, under the accession number PRJNA976120.**

Discussion

In the present study, five benzene-degrading bacteria were isolated and analyzed for the prediction of their biochemical, molecular, and bioremediation potentials. Four of these bacteria were Gram-positive, consistent with earlier explored benzene metabolizing Gram-pos-

itive bacteria (Abu Laban et al. 2009; Lazaroaie 2010; Atashgahi et al. 2018). The finding of one Gram-negative bacteria was also reported by some of the benzene degraders in the literature (Lăzăroaie 2010; Varma et al. 2015). The present investigation is the first work that explored the spore and capsule staining characteristics of these types of bacteria.

Data about the biochemical characteristics of benzene-metabolizing bacteria is also scarce in the literature. This research describes for the first time the presence of pyrrolidine- β -naphthylamidase, lipase, aldoses hydrolyzing enzymes, γ -glutamyl- β -naphthylamidases, and adonitol hydrolyzing enzymes in these types of bacteria.

Exponential growth in the present study bacteria started at 23 h (*B. tropicus* PUB2) and 25 h (*P. aestuarii* PUB1, *B. albus* PUB3, *B. subtilis* PUB4 and *B. cereus*

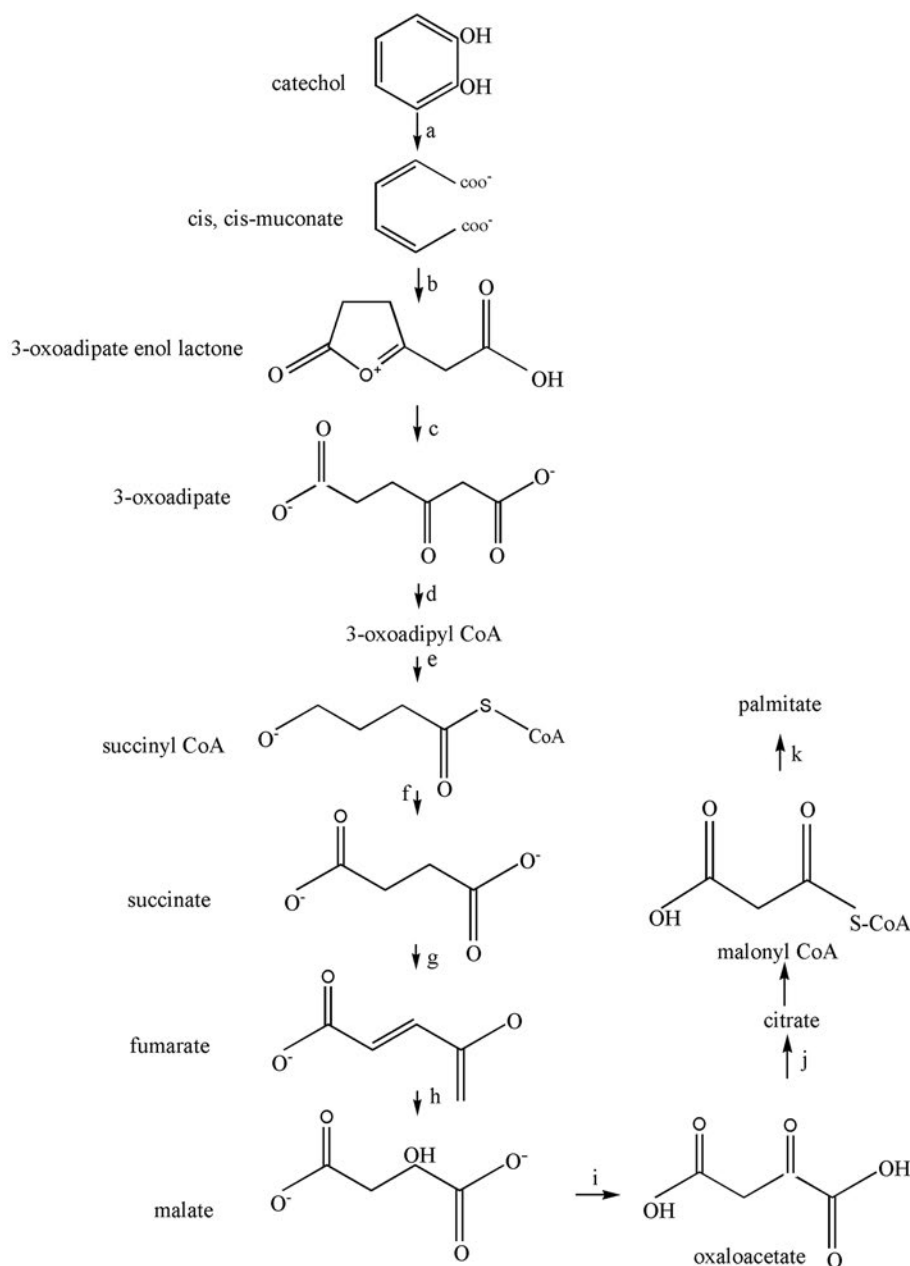


Fig. 5. The β -ketoadipate pathway suggested in the present study benzene degrading bacteria based on GC-MS based identification of metabolites involving this pathway.

a – catechol 1,2-dioxygenase, b – muconate cycloisomerase, c–d – beta-ketoadipate:succinyl CoA transferase, e – succinyl CoA:acetyl CoA C-succinyl transferase, f – succinyl-CoA synthetase, g – succinic dehydrogenase, h – fumarase, i – malate dehydrogenase, j – citrate synthase, k – fatty acid synthase

PUB6), which is showing their fast-growing potential in the presence of benzene. Only a few earlier reported bacteria exhibited this much growth rate (Irshaid and Jacob 2016). However, most of the already explored bacteria are slow growing, with their log phases starting after one week (Meckenstock et al. 2016), taking weeks for benzene degradation like *Marinobacter* sp. (Nicholson and Fathepure 2004). Hence, bacteria explored in this investigation are much better in terms of growth rate than most previously reported bacteria. The highest OD_{600} value reported in the literature for benzene metabolizers is 0.8–0.9 (Zhang et al. 2014). In the present

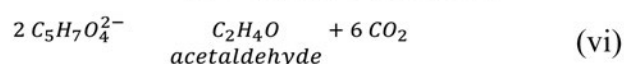
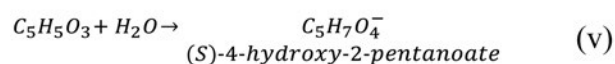
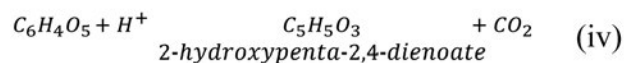
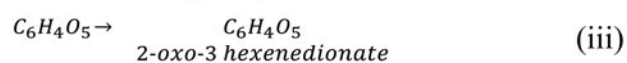
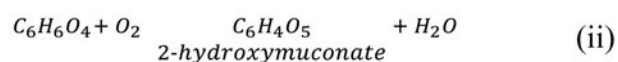
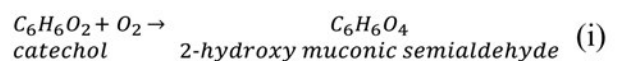
work, *P. aestuarii* PUB1 and *B. cereus* PUB6 also showed consistent values of OD_{600} , while the remaining three isolates showed maximum absorbance from 0.5–0.7.

Antibiotic sensitivity profiling performed in the present work revealed resistance against amoxicillin and augmentin in only *B. subtilis* PUB4. At the same time, the rest of the bacteria were devoid of resistance against all four antibiotics used. These resistant bacteria can be used as a whole cell for safe benzene bioremediation. However, the virulence of these bacteria can be further confirmed through detection of virulence genes. Green remediation exploits only eco-friendly microbes

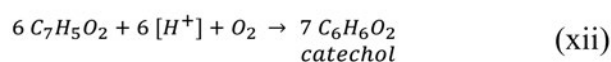
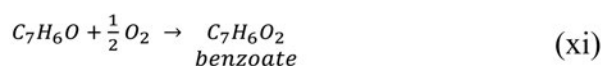
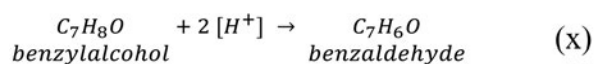
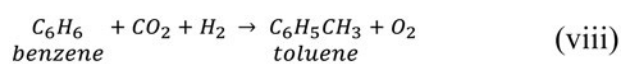
that are non-resistant to antibiotics and don't have virulence genes.

Benzene degradation by the bacteria in the present study was confirmed via FTIR analysis. All the bacteria showed new peaks in spectra at 3000–3500 cm⁻¹ in all isolates, 1100–1800 cm⁻¹ region in PUB6, and 1020 to 1018 cm⁻¹ in PUB1, PUB2, PUB3, and PUB4. The appearance of new peaks in these regions reflected stretching vibrations of C–H and C=C bonds of the benzene ring (<https://webbook.nist.gov/cgi/inchi?ID=C71432&Type=IR-SPE&Index=2>). These FTIR spectral peaks helped us to identify the toluene, benzaldehyde, benzoate, catechol, phenol, benzyl alcohol, *cis*, *cis*-muconate, 3-oxoadipate enol lactone, 3-oxoadipate and palmitate as intermediates of benzene. These spectral peaks have been validated through consulting the previous literature (Aktaş et al. 2003; Kaur and Dhiman 2011; Anantharaj et al. 2015; Smith 2017; 2018a; 2018b; Krishna et al. 2020).

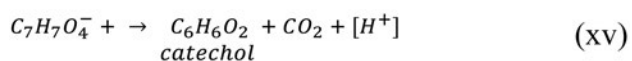
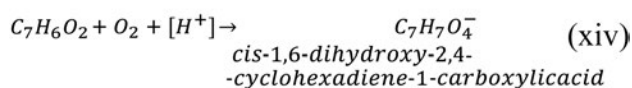
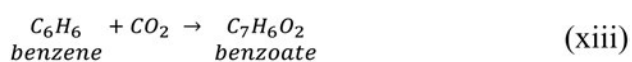
GC-MS-based metabolite identification reflected benzene degradation by PUB1 and PUB4 via the formation of phenol and benzaldehyde and carboxylation and methylation of the benzene ring. Identification of phenol and benzoate in the present study bacteria has also been reported as a metabolite of benzene degradation in *Dechloromonas* strain RCB (Chakraborty and Coates 2005). The route of benzene degradation via phenol formation is by the pathway reported in *G. metallireducens* and *Pseudoxanthomonas spadix* BD-a59 (Zhang et al. 2013). According to this pathway, benzene is converted into phenol in the presence of benzene phenol monooxygenase. Following this, phenol is converted into catechol, which enters the β -ketoadipate pathway. Another mechanism for the conversion of catechol into acetyl-CoA has also been reported, which involves the formation of 2-hydroxy malonic semialdehyde, 2-hydroxymuconate, 2-oxo-3 hexenedionate, 2-hydroxypenta-2,4-dienoate, (S)-4-hydroxy-2-pentanoate and acetaldehyde. Stoichiometry of this pathway is discussed as below.



Benzene degradation via the methylation pathway has also been reported in *Alcaligenes xylosoxidans* y234. According to this pathway, benzene is initially transformed into toluene. Toluene is further converted into benzyl alcohol, benzaldehyde, benzoate, *cis*-benzoate dihydrodiol, and ultimately into catechol by the action of enzymes methyl monooxygenase, benzoyl alcohol dehydrogenase, benzaldehyde dehydrogenase, benzoate 1,2-dioxygenase and dihydrocyclohexadiene carboxylate dehydrogenase, respectively. The stoichiometry of pathway is as follows:

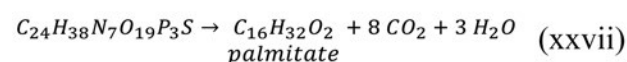
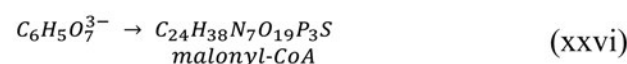
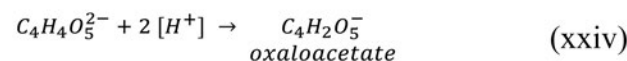
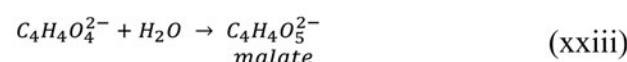
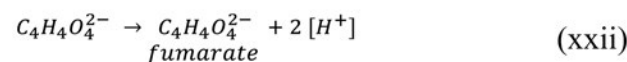
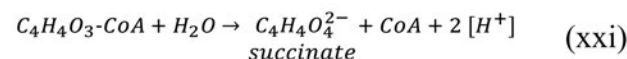
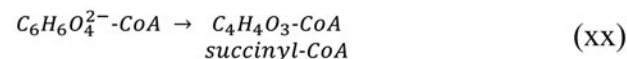
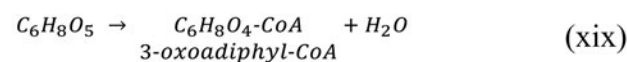
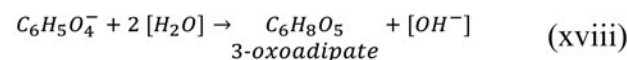
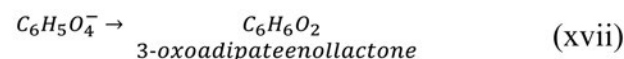
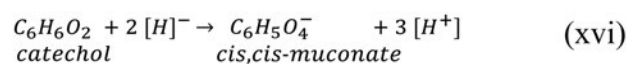


Benzene catabolism through the carboxylation pathway involves the formation of benzoate as an intermediate, which is further cleaved into *cis*-1, dihydroxy acid. Following this, benzoate *cis*-diol dehydrogenase catalyzes the conversion of *cis*-1, dihydroxy acid into catechol. This pathway has also been reported in *Pep-tococcaceae* and *Azoarcus* strains of benzene degraders (Luo et al. 2014).



In addition to these pathways, another mechanism for benzene degradation was identified, which involved the formation of benzaldehyde. This pathway is consistent with the one reported in earlier explored bacteria (Jothamani et al. 2003). According to this pathway, benzene is first converted into benzyl alcohol, which is then transformed into benzaldehyde by benzyl alcohol dehydrogenase. Afterwards, benzaldehyde is transformed into benzoate followed by benzoyl-CoA formation in the presence of enzymes benzaldehyde dehydrogenase and benzoate CoA-ligase, respectively. Benzoyl-CoA

is then converted into acetyl-CoA in the presence of benzoyl-CoA 2,3-dioxygenase. The acetyl-CoA then enters into Krebs cycle. The catechol produced enters the central carbon metabolism via the β -ketoadipate pathway. Some intermediates of this pathway, including *cis,cis*-muconate, 3-oxo adipate enol lactone, and 3-oxo adipate, were identified in the present study bacteria. According to this pathway, catechol is converted into *cis,cis*-muconate followed by the formation of 3-oxoadipate enol lactone, 3-oxoadipate, 3-oxoadipyl-CoA, succinyl-CoA, succinate, fumarate, malate, oxaloacetate, citrate, malonyl-CoA and palmitate in the presence of enzymes catechol 1,2-dioxygenase, muconate



cycloisomerase, beta-ketoadipate:succinyl-CoA transferase, succinyl-CoA:acetyl CoA C-succinyl transferase, succinyl-CoA synthetase, succinic dehydrogenase, fumarase, malate dehydrogenase, citrate synthase and fatty acid synthase, respectively. The stoichiometry of this pathway is given as follows:

This pathway for transforming catechol into fatty acid has been reported in the literature on *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Breisch et al. 2022).

Conclusions

The present study identified and assessed the benzene degrading potential of bacteria under in-vitro conditions. Degradation potential was investigated by culturing bacteria in MSM supplemented with benzene as the only carbon source, benzene removal assay, FTIR and GC-MS-based analysis. Pathways involved have also been confirmed at metabolic and genetic levels. *B. tropicus* PUB2 was found to exhibit the highest removal efficiency. The bioremediation potential of these isolates can be fully exploited commercially by getting insight into the optimum values of physical factors involved, like temperature, pH, pollutant concentration, and oxygen content. Pathogenicity status can be investigated for safe recommendations of isolates for green remediation. Genes of these bacteria can be identified and cloned in eco-friendly expression systems for green bioremediation.

Abbreviations

ADH – arginine dehydrolase test
 ADON – adonitol fermentation test
 BLAST – Basic Local Alignment Search Tool
 CFUs – colony forming units
 EPA – Environmental Protection Agency
 FTIR – Fourier transform infrared spectroscopy
 GC-MS – gas chromatography-mass spectrometry.
 GGT – hydrolysis of γ -glutamyl- β -naphthylamide hydrolysis test
 GUR – β -glucuronidase test
 IND – tryptophane fermentation test
 KSF – sugar aldehyde utilization test
 LDC – lysine decarboxylase test
 LIP – lipase detection test
 MAL – maltose fermentation test
 MEGA – Molecular Evolutionary Genetic Analysis
 MSM – minimal salt medium
 NAG – *p*-nitrophenyl-N-acetyl- β -D-glucosamide
 NCBI – National Center for Biotechnology Information
 NPL – National Priorities List
 OD – optical density
 ODC – ornithine decarboxylase test
 ONPG – *p*-nitrophenyl- β -D-galactosidase
 PRO – proline β -naphthylamide
 PYR – pyrrolidine- β -naphthylamide
 SBL – sorbitol fermentation test
 SRA – Sequence Read Archives
 TET – aliphatic thiol utilization test
 Tm – melting temperatures
 URE – urease test
 WGS – whole genome sequencing
 β GLU – β -nitrophenyl- β -D-glucoside
 β XYL – *p*-nitrophenyl- β -D-xyloside hydrolase test

Availability of data and material

The FASTA sequences of present study isolates are available at NCBI database.

Acknowledgments

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Author contributions

Nadia Hussain, designed methodology, Farhan Mohiuddin: formal analysis and write up, Fatima Muccee: perceived the idea & supervision, Saboor Muarij Bunny: formal analysis, Amal H.I. Al Haddad: formal analysis.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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