

Whole Genome Shotgun Sequencing-Based Insights into the Benzene and Xylene Degrading Potentials of Bacteria

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Submitted 7 April 2025, accepted 11 May 2025, published online 16 June 2025

Abstract

Due to their hazardous effects on human health and air quality, benzene and xylene constitute the primary pollutants. Coupling the physicochemical strategies with bacterial bioremediation is an emerging mode of decontamination. Considering the limited understanding of benzene and xylene degradation pathways in the genus *Bacillus*, failure of earlier documented bacteria to degrade these compounds due to poor optimization and complicated real-world contamination scenarios, we initiated the current project. It is an attempt to explore the gene repertoire and pathways associated with the bioremediation of benzene and xylene in new and efficient bacteria. Eleven bacteria were isolated from tannery industry soil in a previous study. Bacterial DNA was extracted by the organic method. To prepare a sample for whole genome sequencing (WGS) analysis, a mixture of genomic DNA was made by adding DNA from each isolate in equimolar concentration (100 ng). The sample was subjected to WGS. Results obtained as FASTq files were submitted to Sequence Read Archives (SRA), NCBI, to get the accession number assigned. Taxonomic profiling revealed that the sample was composed of phyla Proteobacteria (76%), Firmicutes (16%) and unclassified phyla (8%). Functional annotation unraveled the presence of benzoate, *m*-, *p*- and *o*-xylene isomers, benzene, aminobenzoate, 2-, 3- and 4-fluorobenzoate, toluene, chloroalkane and chloroalkene, naphthalene, polycyclic aromatic hydrocarbons (PAHs), dioxin, caprolactum, atrazine, styrene, and chlorobenzene and chlorocyclohexane degradation enzymes and pathways. It is the first ever study documenting the benzene degradation pathway similar to Gram-negative bacteria, in the genus *Bacillus*, inhabiting the tannery soil and coexistence of metabolic pathways for multiple organic pollutants.

Key words: metagenome, *Bacillus*, taxonomic profiling, bioremediation, benzene, xylene

Introduction

Benzene is declared carcinogenic by the International Agency for Research on Cancer (IARC) and the Environmental Protection Agency (EPA) of the USA (Li et al. 2021; Matheson et al. 2023). Xylene is non-carcinogenic with risk class 3 (moderate risk), but when it is found in the form of benzene, toluene, ethylbenzene, and xylene (BTEX), it proves hazardous (Li et al. 2025). These two pollutants adversely affect nervous, respiratory, reproductive, endocrine, urinary and cardiovascular systems (Caron-Beaudoin et al. 2022).

Given the ubiquitous nature, persistence, carcinogenicity, and other associated health hazards, extended range of transport, highly volatile nature, high emission rate, and internal resistance to degradation, ben-

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zene and xylene are the significant concerns today. Multiple approaches, including catalytic oxidation, soil vapor extraction, photocatalysis, and thermal incineration, have been employed (Tabakova 2024). However, as per the previously documented research, these conventional methods are not eco-friendly due to high carbon footprint and are highly expensive (Unegg et al. 2023; Sornaly et al. 2024; Kaur and Sood 2025). In comparison, bioremediation using microbes is found to be more significant due to being economically profitable, eco-friendly, capable of degrading the pollutants in dilute amounts, simple operation, least energy consumption, low carbon footprint, and reduced formation of secondary pollutants (Kuppan et al. 2024). Literature documents various case studies that successfully bio-remediated the water and soil contaminated with oil, polycyclic aromatic hydrocarbons (PHCs) and polychlorinated biphenyls (PCBs) (You et al. 2014; Schad 2016). Hence, bioremediation is capable of diluting the pollutants in environmental resources even without the use of conventional techniques. However, this will require optimizing the bioremediation process, selecting the right microorganism, adaptive management, and sufficient ongoing monitoring. In contrast, physicochemical and mechanical methods cannot completely remove pollutants when employed without bioremediation (Sales da Silva et al. 2020). However, a strategic combination of conventional and biological methods improves the outcome synergistically (Aparicio et al. 2022; Kumar et al. 2022; Nagda et al. 2022; Sornaly et al. 2024; Ha and Tan 2025).

Bacteria, adaptable to various environmental conditions and nutritionally versatile, are the most suitable candidates for bioremediation. The biodegradable potential of bacteria is reflected in their genomes. Whole Genome Sequencing (WGS) could provide a holistic view of the genes of these environmentally competent bacteria concerning bioremediation. In addition, it is also helpful in tracking the changes that occur in microbial communities during the biodegradation process, thus providing insights into its effectiveness and possible ways of optimizing the technique (Chettri et al. 2024).

Ortega-González et al. (2013) documented the genes toluene 1,2-dioxygenase (todC1), and xylene monooxygenase (xylM) in a bacterial sample comprising *Cellulomonas hominis*, *Ralstonia insidiosa*, *Serratia marcescens*, and *Burkholderia kururiensis* from a contaminated area in Southern Mexico. In another investigation, bacteria from *m*-, *o*- and *p*-xylene de-

grading enrichments, from BTEX contaminated soil in Hungary, were found to exhibit catechol 2,3-dioxygenase (C23O) encoding genes. Prominent genera of the bacterial sample were *Pseudomonas*, *Acidovorax*, and *Hydrogenophaga* (Banerjee et al. 2022a). Another study also documented the same C23O gene in related bacteria (Táncsics et al. 2020). The same gene was also identified via Illumina sequencing in microaerobic enrichment dominated by *Rhodoferrax*, *Pseudomonas*, and *Acidovorax* (Bedics et al. 2022). A bacterial sample from petrochemical contaminated water, predominated by Proteobacteria, was found to exhibit toluene 2-monooxygenase (dmpK), glutaryl-CoA dehydrogenase (gcd), and benzoyl-CoA reductase (bamB) genes. These genes were detected using metagenome-assembled genome (MAG) and contig-based genome (Zhang et al. 2024). Other bacteria explored so far include *Pseudomonas aromaticivorans* sp. nov., *Hydrogenophaga*, (Banerjee et al. 2022b). Metagenome analysis based functional annotation of a bacterial sample unraveled the presence of enzymes toluene methyl monooxygenase, benzoate/toluene 1,2-dioxygenase, aryl alcohol dehydrogenase, benzaldehyde dehydrogenase, dihydroxycyclohexadiene carboxylate dehydrogenase, catechol 2,3-dioxygenase, catechol 1,2-dioxygenase, muconolactone D-isomerase, muconate cycloisomerase, 3-oxoadipate enol-lactonase, 2-hydroxymuconate-6-semialdehyde dehydrogenase, 2-hydroxymuconate semialdehyde hydrolase, 4-oxalocrotonate tautomerase, 2-keto-4-pentenoate hydratase, 2-oxo-3-hexenedioate decarboxylase, toluene 4-monooxygenase (T4MO), 4-hydroxy-2-oxovalerate aldolase, 3-methylcatechol 2,3-dioxygenase, acetaldehyde dehydrogenase, 2-hydroxy-6-oxo-octa-2,4-dienoate hydrolase and 2,3-dihydroxyethylbenzene 1,2-dioxygenase in addition to toluene, benzene, ethylbenzene, and xylene mono- and dioxygenases (Eze 2021).

The current study has targeted the genomes of eleven bacteria, which were identified in two separate projects. One project isolated benzene, and another project targeted the xylene-degrading bacteria. Ten of these eleven isolates belonged to the genus *Bacillus* and were Gram-positive.

Although we can find many benzene and xylene-metabolizing bacteria in previously published data, among these, only the pathways of Gram-negative bacteria are well characterized, while Gram-positive, particularly genus *Bacillus*, is the least understood in this regard (Wongbunmak et al. 2020). In addition, *Bacillus* species explored so far have not been opti-

mized for factors affecting bioremediation like temperature, pH, and concentration of contaminants (Mohammadpour et al. 2020). This is uncommon among Gram-positive bacteria reported so far to metabolize benzene and xylene under anaerobic conditions as compared to Gram-negative microbes (Weelink et al. 2010; Banerjee et al. 2022a). Previously reported Gram-positive bacteria associated with degradation of these two pollutants have been documented as more susceptible to environmental constraints like the presence of toxins, including hydrogen sulfide, low temperature, and electron acceptors like sulfate and nitrate (Kaur et al. 2023; 2025).

Bacillus species explored so far exhibited a narrow substrate range and poor degradation efficiencies (Handayani et al. 2019). Lastly, degradation of these pollutants has never been explored in the native area of Multan, Pakistan. These limitations have been a big hurdle in optimizing the degradation potentials of bacteria isolated and characterized.

So, considering these constraints of earlier reported bacteria, we initiated a current research project hypothesizing that we might identify unusual and novel pathways and associated genes in benzene and xylene metabolizing bacteria isolated from the tannery industry of Multan.

Experimental

Materials and Methods

Isolation and characterization of bacteria. Ingredients of minimal salt medium (MSM) employed for isolation of benzene metabolizing bacteria included KH_2PO_4 (1 g/l), Na_2HPO_4 (1.25 g/l), $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/l), MgSO_4 (0.5 g/l), CaCl_2 (0.5 g/l), FeSO_4 (0.005 g/l), and benzene 80 μl /100ml (Dairawan and Shetty 2020). MSM used for isolation of xylene degrading bacteria composed of KH_2PO_4 (1 g/l), K_2HPO_4 (1 g/l), NH_4NO_3 (1 g/l), MgSO_4 (0.2 g/l), CaCl_2 (0.02 g/l), FeCl_3 0.5M (2 drops/l) and xylene 1 ml/100ml (Doley and Barthakur 2022). Following this, 16S rRNA gene sequencing analysis was performed to determine the genus and species of bacteria (Schumann and Pukall 2013). For amplification of the gene, forward (5'AGAGTTTGATCCTGGTCAGAAC3' and $T_m = 70.4^\circ\text{C}$) and reverse (5'CGTACGGCTACCTTGTTACGACTTCACCCC3' and $T_m = 74.8^\circ\text{C}$) primers were used. Growth phases were predicted based on measuring the optical density

at 600nm (OD_{600}) spectrophotometrically at different time intervals (0, 3, 6, 24, 27, 30, 46, 49, 51, and 54 hours) of isolates cultures incubated at 37°C and 150 rpm (Brewster 2003). To biochemically characterize the benzene and xylene metabolizing isolates, the Remel™ RapID™ NF PLUS System and the Remel™ RapID™ STR (Thermo Scientific™, Thermo Fisher Scientific, Inc., USA) system were used, respectively (Evangelista et al. 2001; Mayz et al. 2013). The degradation of organic compounds was confirmed via removal assays and gas chromatography-mass spectrometry (GC-MS) analysis (Mohamed et al. 2017; Raju and Kumar 2020).

A benzene removal assay was performed by preparing benzene stock solutions of known concentrations ranging between 5–30 mg/l. The OD_{180} of these solutions was estimated to plot the standard curve. To find out linearity, linear regression analysis was performed. Bacteria were cultured in MSM containing benzene (40 mg/l) as the only carbon supplement until log phase. Benzene concentration in supernatant was determined by measuring OD_{180} followed by its comparison with the standard curve (Hussain et al. 2025).

A stock solution of known concentrations of xylene, ranging between 100–900 mg/l, was prepared to perform the xylene removal assay. The OD_{265} of solutions was spectrophotometrically measured, and a standard curve was plotted. Correlation coefficient (R^2) was calculated. Xylene-degrading bacteria were cultured in MSM, which contained xylene as the only supplemental carbon source. Bacteria were harvested at log phase. Residual xylene in the supernatant of each isolate was determined by measuring OD_{265} and comparing it with the standard curve (data is submitted somewhere else for publication).

Next generation sequencing. DNA sample preparation. Cultures of individual bacteria, harvested at the end of the log phase, were pelleted out via centrifugation ($6,000 \times g$, 4°C , and 20 minutes). Pellet was processed for DNA extraction by an organic method based on phenol : chloroform : isoamyl alcohol (PCI). Initially, the pellet was thoroughly suspended in lysozyme (100 μl) and TE lysis buffer (500 μl) and incubated at 37°C for 2–3 hours. Afterwards, the pellet was treated with sodium dodecyl sulfate (SDS) (30 μl) and proteinase K (5 μl) and again incubated for 2 hours at 37°C . This incubation was followed by centrifugation at $14,000 \times g$ and 4°C for 20 minutes. This resulted in the formation of three layers. The topmost aqueous layer contained DNA collected carefully into a separate Eppendorf. The remaining organic and interface layers

were discarded. Sodium acetate (70 μ l) and chilled PCI (400 μ l) were added to the aqueous layer. The tube was incubated at -20°C for 24 hours. Following this incubation, centrifugation ($14,000 \times g$ and 4°C) for 10 minutes was carried out to pellet out the DNA. Pellet was washed 2–3 times with 70% ethanol via centrifugation. After washing, the pellet was dried by placing the Eppendorf in an inverted position on tissue paper. Dried DNA was suspended in low TE buffer (pH = 8.0).

Quality estimation by agarose gel electrophoresis.

A qualified DNA sample for library construction was selected by running the sample on agarose gel electrophoresis. The 1% agarose gel was prepared by dissolving 0.5 g agarose powder in 50 ml of $1\times$ TAE buffer with pH = 8.0. Heated the mixture for 1 minute and poured it into a gel casting tray containing a comb to create wells. Ethidium bromide was added to the gel mixture as a DNA intercalator. Following solidification, the gel was suspended in $1\times$ TAE buffer contained in the electrophoresis tank. The 5 μ l DNA to be tested was mixed with tracking dye bromophenol blue (1 μ l) and loaded into wells. The gel was run for 45 minutes at 90 volts. It was followed by visualization of the gel using the Gel Documentation System, and bands were visible under UV light. A good-quality DNA sample was processed for library construction.

Library construction. Nextera[®] XT DNA Library Preparation Kit (Illumina, Inc., USA) was used to prepare the libraries. The library protocol used was Nextera[®] XT DNA Library Prep Kit Reference Guide (15031942 v03). Genomic DNA (1 ng) was fragmented and tagged with an adapter. After tagmentation, the Nextera[®] indexed primer was used to attach indexes with PCR. Finally, qPCR was used to quantify the purified product as per the qPCR quantification protocol guide (KAPA Library Quantification Kits for Illumina Sequencing platforms) and was qualified using the TapeStation D5000 ScreenTape (Agilent Technologies, Inc., USA). Following this, sequencing was performed using HiSeq[™] (Illumina, Inc., USA).

Raw data. Illumina sequencer generated raw images using sequencing control software for system control and base calling through integrated primary analysis software called Real Time Analysis (RTA). The base call (BCL) binary files were converted into FASTq files using bcl2fastq, an Illumina-provided package. Adaptors were not trimmed away from reads. FASTq files were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) to get the accession number assigned.

Raw data comprised the date of total number of bases sequenced, total number of reads, for paired end

sequencing it is the sum of read 1 and read 2, ratio of GC content (GC %), ratio of AT content (AT %), ratio of bases that have phred quality score of over Q20 (%) and ratio of bases that have phred quality score of over Q30 (%).

Metagenome assembly. WGS reads were assembled into contigs/scaffolds using a new versatile metagenomic assembler, metaSPAdes (Nurk et al. 2017). It is part of SPAdes (St. Petersburg genome assembler), an assembly toolkit containing various assembly pipelines. The metagenomic contigs generated by the metaSPAdes assembler were distributed in 'bins' using the MetaBAT2 program (Kang et al. 2019). MetaBAT2 is a robust statistical framework for reconstructing genomes from metagenomic data. It is an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies.

QUAST quality analysis. Quality Assessment for Genome Assemblies was done by the Quast program (Gurevich et al. 2013). Parameters considered included contigs with several contigs greater than > 10,000 bp, more than or equal to 5,000, 10,000, 25,000, and 50,000 bp. Number of largest contigs. Total length of contigs. Total length of contigs with more than or equal to 1,000, 5,000, 10,000, and 25,000 bp. Low-quality reads were removed using Trimmomatic v0.39 (Bolger et al. 2014). Sequences obtained were assessed and subjected to taxonomic and functional profiling.

Annotation of metagenomic contigs for taxonomic and functional profiling. For taxonomic profiling, annotation of metagenomic contigs was done by the Kraken program. Kraken is an ultrafast and highly accurate program for assigning taxonomic labels to metagenomic DNA sequences (Wood and Salzberg 2014).

After concatenating qualified reads, the binning approaches MaxBin2 and MetaBAT2 algorithms were employed to reconstruct the good-quality draft genome. By default, values of minimum completion (70%) and contamination (10%) were considered. For functional profiling, metagenomic contigs of each bin were analyzed by Prokka v.1.14.6 (Seemann 2014; Neely et al. 2020). KofamScan v.1.3.0 (Aramaki et al. 2020) was used to annotate Kyoto Encyclopedia of Genes and Genomes (KEGG).

Sequence deposition. The FASTq files are submitted in Sequence Read Archives, SRA NCBI database, under the accession number PRJNA976120.

Results

Isolation and characterization of isolates. Among the total bacteria characterized in the current study, six were benzene-metabolizing and five were xylene-degrading. These were identified as belonging to *Paracoccus aestuarii* (01), *Bacillus tropicus* (01), *Bacillus albus* (01), *Bacillus subtilis* (01), *Bacillus thuringiensis* (02), *Bacillus cereus* (04), and *Bacillus mycoides* (01). NCBI accession numbers assigned to these isolates are, OR272055, OR272056, OR272059, OR272058, OR272061, OR272060, OR272064, OR272063, OR272072, OR272071, and OR272073. Biochemical profiling exposed presence of enzymes arginine dihydrolase (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), γ -glutamyl- β -naphthylamide hydrolysis test (GGT), adonitol fermentation test (ADON), aliphatic diol test (TRD), triglyceride test (EST), urea test (URE), glucose test (GLU), proline- β -naphthylamide test (PRO), pyrrolidonyl- β -naphthylamide (PYR) and sodium nitrate test (NO_3). Benzene and xylene removal efficiencies. Benzene degradation efficiency measured between 30 m g/l per 23 hours and 32 m g/l per 47 hours. Xylene metabolizing potential was estimated, ranging between 30 m g/l per 48 hours and 65 m g/l per 48 hours (Table SI).

GC-MS based profiling identified the metabolic intermediates of multiple previously documented benzene degradation pathways. i.e., benzene methylation pathway, benzene degradation via benzaldehyde and via carboxylation, meta and ortho-cleavage pathways and benzene degradation via phenol. The intermediates were toluene, benzyl alcohol, benzaldehyde, benzoate, catechol, *cis*-1,6-dihydroxy-2,4-cyclohexadiene-1-carboxylic acid, phenol, *cis,cis*-muconate, 3-oxoadipate enol lactone, 3-oxoadipate, palmitate, chlorobenzene, *p*- and *o*-xylene, cyclopentasiloxane, decamethyl and dodecamethyl, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethylcyclotetrasiloxane, 6-fluoroindole, TMS derivative, methyltris(trimethylsiloxy) silane, 1,1,1,3,3,5,5,5-heptamethyltrisiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethylhexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethylcyclotetrasiloxane, 1,4-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, mercaptoacetic acid, 2TMS derivative, hexasiloxane, tetradecamethyl, 1,1,1,5,7,7,7-heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane, oxazepam, 2TMS derivative, *N*-ben-

zyl-*N*-ethyl-*p*-isopropylbenzamide, cyclononasiloxane, octadecamethyl, bis(2-ethylhexyl) phthalate, cyclononasiloxane, octadecamethyl, cyclononasiloxane, octadecamethyl, octadecanoic acid and *n*-hexadecanoic acid etc.

In xylene degrading bacteria, metabolic intermediates of earlier documented pathways, i.e., xylene degradation via benzoate formation and anaerobic oxidation pathway, were identified. Like, methylbenzyl alcohol, methyl benzaldehyde, methyl benzoate, 1,2-dihydroxy-methyl-cyclohexa-3,5-diene carboxylate, *p*-methylbenzyl alcohol, *p*-toluic acid, *p*-tolualdehyde, 4-methylcatechol, 4-methylcyclohexa-3,5-dien-1,2-diol-carboxylic acid, 3-methyl-*cis*-muconate, 4-methyl muconolactone, 3-methylmuconolactone, 4-methyl-3-oxoadipate, oleic acid, 1,3,5-cycloheptatriene, *cis*-vaccenic acid, *n*-hexadecanoic acid, toluene, bis(2-ethylhexyl) phthalate, *cis*-octadecenoic acid, octadecenoic acid, glycerol 1-palmitate, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, octadecenoic acid, 2,3-dihydroxypropyl ester, octadecenoic acid, 2,3-dihydroxypropyl ester, octadecenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, glycerol 1-palmitate, 7-chloro-1,3,4,10-tetrahydro-1-[[3-[[2-hydroethyl]amino]propylimino]-3-[4-(trifluoromethyl)phenyl], ethanone, 2-(2-benzothiazolylthio)-1-(3,5-dimethylpyrazolyl), *N*-(3-methoxyphenyl)-4-piperidinamine-*N'*-trimethylsilyl, 1,2-cyclohexanedicarboxylic acid, methoxyacetic acid, heptadecyl ester, tributyl-octadecyl-stannane, 7-chloro-3-[2,4-dichlorophenyl]-3,4-dihydro-10-hydroxy-1,9(2*H*,10*H*)-acridinedione, 1-(*N,N*-dimethyl) hydrazone, 1,2-cyclohexanedicarboxylic acid, heptyl nonyl ester, 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, beta-alanine, *N*-(1-naphthyl)- decyl ester, 2-bromo-4,5-dimethoxycinnamic acid, 2-(pyridin-2-ylformamido)acetic acid, 2TMS, 9-iodo-8-methyl-3-oxo-2-oxabicyclo(4.4.0) dec-4-ene-6,8-carbolactone, 1*H*-imidazole-2-methanol, 1-decyl, 2-bromo-4,5-dimethoxycinnamic acid, 2-methyl-7-phenylindole, 2-Bromo-4,5-dimethoxycinnamic acid, and 4,4'-Methylenedi-2,6-xlenol, bis(tert-butyl dimethylsilyl) ether.

Next generation sequencing. Quality control analysis of a sample. The quality control analysis of the sample was performed by the 260/280 ratio and agarose gel electrophoresis. The 260/280 ratio was estimated as 1.338. DNA sample resolved on agarose gel is shown in Fig. S1.

Table I
Stoichiometry of reactions involved in benzene, xylene and toluene degradation identified in the current study bacteria based on WGS.

No. of reaction	Stoichiometry of reactions	
	Reactants	Products
Benzene degradation pathway		
I	$C_6H_6 + H_2 + CO_2$ benzene	$C_7H_8O + [O^-]$ benzyl alcohol
II	C_7H_8O	$C_7H_6O + H_2$ benzaldehyde
III	$C_7H_6O + [O^-]$	$C_7H_6O_2$ benzoate
IV	$C_7H_6O_2 + [O^-] + H_2O$	$C_7H_8O_4$ <i>cis</i> -1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate
V	$C_7H_8O_4$	$C_6H_6O_2$ catechol
VI	$C_6H_6O_2 + 2 [H^+]$	$C_6H_5O_4^- + 3 [H^+]$ <i>cis,cis</i> -muconate
VII	$C_6H_5O_4^-$	$C_6H_6O_4$ muconolactone
VIII	$C_6H_6O_4$	$C_6H_5O_4 + [H^+]$ 3-oxoadipate enol lactone
IX	$C_6H_5O_4$	$C_6H_5O_5^{2-}$ 3-oxoadipate
X	$C_6H_5O_5^{2-} + [H^+] + C_{21}H_{36}N_7O_{16}P_3S$	$C_{27}H_{42}N_7O_{20}P_3S + [O]$ 3-oxoadipyl-CoA
XI	$C_{27}H_{42}N_7O_{20}P_3S + 5H_2O$	$C_{23}H_{38}N_7O_{17}P_3S + 4CO_2 + 7H_2O$ acetyl-CoA
<i>p</i> -Xylene degradation pathway		
I	$C_8H_{10} + [O^-]$ <i>p</i> -xylene	$C_8H_{10}O$ 4-methylbenzyl alcohol
II	$C_8H_{10}O + CO_2$	$C_9H_{10}O_2 + [O^-]$ <i>p</i> -methylbenzoate
III	$C_9H_{10}O_2 + 2O_2$	$C_8H_9O_4 + CO_2 + [H^+]$ <i>cis</i> -1-2-dihydroxy-4-methylcyclohexa-3,5-diene-1-carboxylate
IV	$C_8H_9O_4$	$C_7H_8O_2 + CO_2 + [H^+]$ 4-methylcatechol
V	$C_7H_8O_2 + O_2$	$C_7H_8O_4$ 2-hydroxyl-5-methyl- <i>cis,cis</i> -muconic semialdehyde
VI	$C_7H_8O_4 + [O]$	$C_7H_8O_5$ 2-hydroxyl-5-methyl- <i>cis,cis</i> -muconate
VII	$C_7H_8O_5$	$C_7H_8O_5$ 2-oxo-5-methyl- <i>cis</i> -muconate
VIII	$C_7H_8O_5$	$C_6H_8O_3 + CO_2$ 2-hydroxyl- <i>cis</i> -hexa-2,4-dienoate
IX	$C_6H_8O_3 + H_2O$	$C_6H_{10}O_4$ 4-hydroxyl-2-oxohexanoate
X	$C_6H_{10}O_4 + 2O_2 + H_2O$	$3CO_2 + C_3H_3O_3^- + H_2O$ pyruvate
<i>m</i> -Xylene degradation pathway		
I	$C_8H_{10} + [O]$ <i>m</i> -xylene	$C_8H_{10}O$ 3-methylbenzyl alcohol
II	$C_8H_{10}O$	$C_8H_8O + H_2$ 3-methylbenzaldehyde

III	$C_8H_8O + CO_2 + H_2$	$C_9H_{10}O_2 + H_2O$ <i>m</i> -methylbenzoate
IV	$C_9H_{10}O_2 + CO_2$	$C_8H_8O_4 + H_2$ 1,2-dihydroxy-3-methylcyclohexa-3,5-dienecarboxylate
<i>o</i> -Xylene degradation pathway		
I	$C_8H_{10} + [O^-]$ <i>o</i> -xylene	$C_8H_{10}O$ 2-methylbenzyl alcohol
II	$C_8H_{10}O$	$C_8H_8O + H_2$ 2-methylbenzaldehyde
III	$C_8H_8O + CO_2 + H_2$	$C_9H_{10}O_2 + [O]$ <i>o</i> -methylbenzoate
IV	$C_9H_{10}O + CO_2$	$C_8H_{10}O_4$ 1,2-dihydroxy-6-methylcyclohexa-3,5-dienecarboxylate
V	$C_8H_{10}O_4$	$C_7H_8O_2 + CO_2 + H_2$ 3-methylcatechol
VI	$C_7H_8O_2 + O_2$	$C_7H_8O_4$ <i>cis,cis</i> -2-hydroxyl-6-oxohept-2,4-dienoate
VII	$C_7H_8O_4 + [O^-]$	$C_5H_6O_3 + 2CO_2 + H_2$ <i>cis</i> -2-hydroxy penta-2,4-dienoate
VIII	$C_5H_6O_3 + H_2O$	$C_5H_7O_4^- + [H^+]$ 4-hydroxyl-2-oxopentanoate
IX	$C_5H_7O_4^- + [O^-]$	$C_3H_3O_3^- + 2CO_2 + 2H_2$ pyruvate
Toluene degradation pathway		
I	$C_6H_5CH_3 + [O]$ toluene	$C_6H_5CH_2OH$ benzyl alcohol
II	$C_6H_5CH_2OH$ benzyl alcohol	$C_6H_5CHO + H_2$ benzaldehyde
III	$C_6H_5CHO + [O]$ benzaldehyde	$C_7H_5O_2 + [H]$ benzoate

Raw data statistics. In the present study, 14,782,932 reads were produced in the bacterial DNA sample, and total read bases were 2.2 Gbp. The GC, AT, Q20, and Q30 contents were estimated as 54.9, 45.1, 93.9, and 86.2 (Table SI and Fig. 1).

QUAST quality assessment parameters calculated by metaSPAdes program. The QUAST quality parameters and their values calculated via metaSPAdes program are shown in detail in Table II.

Taxonomic profiling. Assigning taxonomic labels to metagenomic contigs showed that 75% of contigs belonged to the genus *Bacillus*, 13–15% to genus *Pseudomonas* and 3–5% to genus to *Ochrobactrum*. Moreover, 49% of sequences matched with *B. cereus*, 20% with *B. thuringiensis*, 13% with *Pseudomonas stutzeri*, and 3% with *Ochrobactrum anthropi* (and 8% unclassified sequences) (Fig. 2).

Functional annotation of aromatic compounds degrading genes. Gene annotation by the Prokka program revealed 4,992 metagenomic contigs with 27,559,880 bases and 27,021 protein-coding regions.

Functional annotation of benzene and xylene degradation genes retrieved from the KEGG database is described in Table III. The genes were found to involve in xylene, benzene, toluene, benzoate, aminobenzoate, and fluorobenzoate.

Among the total genes identified in the current bacterial community, a maximum number of genes were identified to be associated with benzoate and xylene metabolism. i.e., 28 and 14%, respectively. Followed this, genes found were associated with metabolism of xenobiotics by cytochrome P450 (6%), aminobenzoate (6%), chloroalkane and chloroalkene (6%), styrene (6%), dioxin (6%), caprolactum (5%), fluorobenzoate (4%), toluene (4%), steroid (4%), chlorocyclohexane and chlorobenzene (3%), atrazine (3%), naphthalene (3%) and polycyclic aromatic hydrocarbon (2%) (Fig. 3). Stoichiometry of the reactions of pathways associated with the degradation of benzene, *o*-, *m*-, *p*-xylene and toluene is given in detail in Table I.

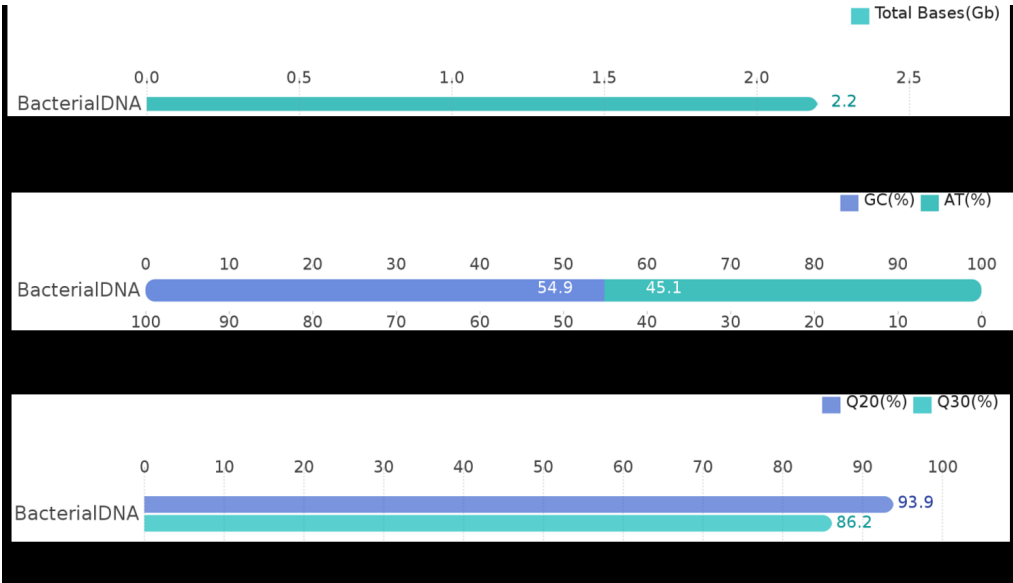


Fig. 1. Total bases, GC and AT contents, and Q20 and Q30 ratios of total reads calculated during WGS analysis.

Discussion

WGS strategies are proving a source of insights into the biodegradation capabilities of bacteria (Oyewusi et al. 2021). It decodes both the uncultivable and cultivable microbes. The current study attempts to decode the underlying benzene and xylene-metabolizing genes and pathways of cultivable bacterial species. Ten out of eleven bacteria analyzed belonged to the genus *Bacillus*.

Taxonomic profiling revealed 49% *B. cereus* species and 20% *B. thuringiensis*. This is a validation of our previously performed 16S rRNA sequencing according to which most of the bacterial community was comprised of *Bacillus* species. This finding is also consistent with earlier literature reporting different species of the genus *Bacillus* as associated with benzene and xylene metabolism. i.e., *B. thuringiensis*, *B. cereus*, *Bacillus amyloliquefaciens*, *Bacillus infantis*, *Bacillus pumilus* (Dou et al. 2010; Undugoda et al. 2018; Wongbunmak et al. 2020; Kesavan et al. 2021; Kaur et al. 2023; 2025; Wu et al. 2023; Jin et al. 2025;). Our findings are not in line with some of the literature because species belonging to *Pseudomonas*, *Sphingobium*, *Azoarcus*, *Cupriavidus*, *Hydrogenophaga*, and *Rhodococcus*, have also been documented as benzene and xylene metabolizing bacteria (Lee et al. 2012; You et al. 2018; Devanadera et al. 2019; Lopez et al. 2022; Sathesh-Prabu et al. 2023). All of these bacteria except *Rhodococcus* are Gram-negative while those documented in current study are Gram-positive. According to literature,

Table II
Statistics of contigs produced by the metaSPAdes program.

No.	Quast quality parameters	Statistics (bp)
1	Contigs > 10,000 bp	4,992
2	Contigs ≥ 5,000 bp	940
3	Contigs ≥ 10,000 bp	431
4	Contigs ≥ 25,000 bp	169
5	Contigs ≥ 50,000 bp	76
6	Largest contig	469,435
7	Total length	30,570,959
8	Total length ≥ 1,000 bp	27,559,880
9	Total length ≥ 5,000 bp	19,294,370
10	Total length ≥ 10,000 bp	15,759,083
11	Total length ≥ 25,000 bp	11,859,173
12	Total length ≥ 50,000 bp	8,511,336
13	N50	10,938

as compared to Gram-positive bacteria, Gram-negative bacteria exhibit greater efficiency of benzene and xylene metabolism (Mazzeo et al. 2010; Chicca et al. 2020; Bacosa et al. 2021). Dominance of Gram-positive isolates in the current project is justified as per the literature finding that Gram-positive bacteria dominate tannery industry soils due to their high adaptability to the heavy metals and organic compounds found in tannery waste (Albokari et al. 2018; Oruko et al. 2019;

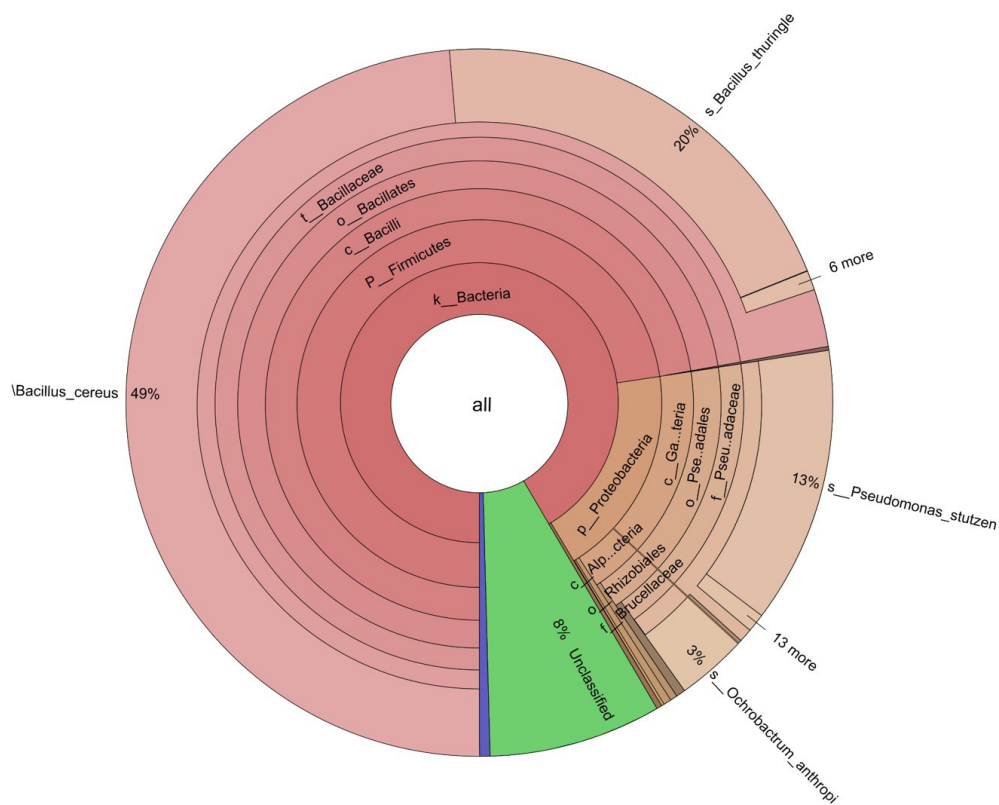


Fig. 2. Krona image of taxonomic assignments to metagenomic contigs by Kraken.

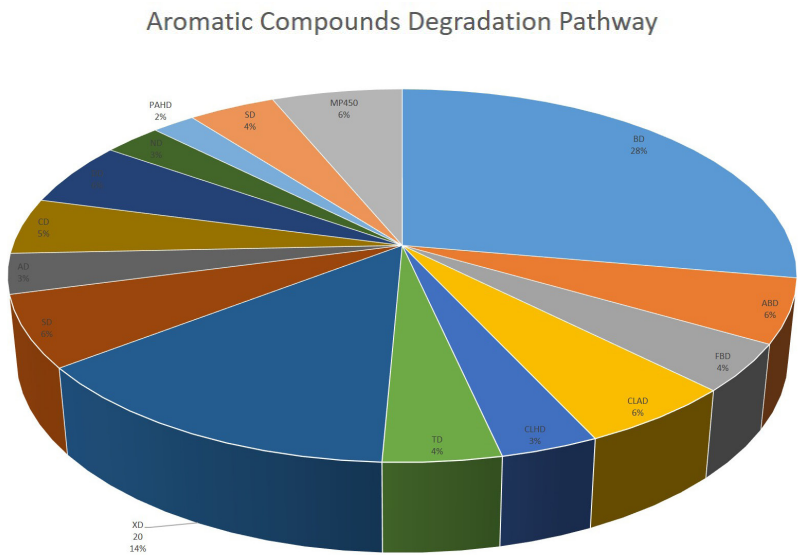


Fig. 3. Pathways associated with aromatic compound metabolism, with their respective abundances, explored in a bacterial consortium using whole genome sequencing.
BD – benzoate degradation, ABD – aminobenzoate degradation, FBD – fluorobenzoate degradation, CLAD – chloroalkane and chloroalkene degradation, CLHD – chlorocyclohexane and chlorobenzene degradation, TD – toluene degradation, XD – xylene degradation, SD – styrene degradation, AD – atrazine degradation, CD – caprolactam degradation, DD – dioxin degradation, ND – naphthalene degradation, PAHD – polycyclic aromatic hydrocarbon degradation, SD – steroid degradation, MP450 – metabolism of xenobiotics by cytochrome P450

Table III

Functional annotation of genes associated with aromatic compound degradation via the KEGG database.

No.	Associated biochemical pathway	Genes identified
1	Xylene degradation pathway	toluene methyl-monooxygenase [EC:1.14.15.26], aryl-alcohol dehydrogenase [EC:1.1.1.90], benzaldehyde dehydrogenase (NAD) [EC:1.2.1.28], benzoate/toluate 1,2-dioxygenase subunit α [EC:1.14.12.-], dihydroxycyclohexadiene carboxylate dehydrogenase [EC:1.3.1.-, 1.3.1.67, 1.3.1.68], catechol 2,3-dioxygenase [EC:1.13.11.2], 2-hydroxymuconate-semialdehyde hydrolase [EC:3.7.1.9], 2-oxopent-4-enoate/ <i>cis</i> -2-oxohex-4-enoate hydratase [EC:4.2.1.80, 4.2.1.132], 4-hydroxy-2-oxovalerate/4-hydroxy-2-oxohexanoate aldolase [EC:4.1.3.39 4.1.3.43], acetaldehyde/propanal dehydrogenase [EC:1.2.1.10, 1.2.1.87], aminomuconate-semialdehyde/2-hydroxymuconate-6-semialdehyde dehydrogenase [EC:1.2.1.32 1.2.1.85], 4-oxalocrotonate tautomerase [EC:5.3.2.6], 2-oxo-3-hexenedioate decarboxylase [EC:4.1.1.77], 2-keto-4-pentenoate hydratase [EC:4.2.1.80], 4-hydroxy 2-oxovalerate aldolase [EC:4.1.3.39], acetaldehyde dehydrogenase [EC:1.2.1.10]
2	Toluene degradation pathway	toluene methyl-monooxygenase [EC:1.14.15.26], aryl-alcohol dehydrogenase [EC:1.1.1.90], benzaldehyde dehydrogenase (NAD) [EC:1.2.1.28]
3	Benzene	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 acyltransferase [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 dehydrogenase [EC:1.2.1.10], protocatechuate 3,4-dioxygenase, alpha subunit [EC:1.13.11.3], 3-carboxy- <i>cis,cis</i> -muconate cycloisomerase [EC:5.5.1.2], 4-carboxymuconolactone decarboxylase [EC:4.1.1.44], 3-hydroxybenzoate 6-monooxygenase [EC:1.14.13.24]
3	Aminobenzoate degradation pathway	benzaldehyde dehydrogenase (NAD) [EC:1.2.1.28], amidase [EC:3.5.1.4], 4-hydroxybenzoate decarboxylase subunit C [EC:4.1.1.61]
4	Fluorobenzoate degradation pathway	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]

Abate et al. 2021). This adaptability is contributed by thick cell walls containing peptidoglycan.

WGS analysis revealed the enzymes involved in *o*-, *m*- and *p*-xylene catabolism, 3-aminobenzenesulfonate, toluene and 2-, 3- and 4-fluorobenzoate. Based on the identification of enzymes, the xylene degradation pathways suggested in a bacterial sample of the current study are shown in Fig. 4.

The *o*-xylene degradation pathway starts with its oxidation into 2-methylbenzyl alcohol in the presence of toluene methylmonooxygenase, which is oxidized by arylalcohol dehydrogenase into 2-methylbenzaldehyde. After this, 2-methylbenzaldehyde is oxidized in the presence of benzaldehyde dehydrogenase into *o*-methylbenzoate, which is transformed into 1,2-dihydroxy-6-methylcyclohexa-3,5-dienecarboxylate by benzoate 1,2-dioxygenase subunit alpha.

Following this, 3-methylcatechol is produced, which is oxidized into *cis,cis*-2-hydroxyl-6-oxohept-2,4-dienoate in the presence of catechol 2,3-dioxygenase. The *cis*-2-hydroxyl-6-oxohept-2,4-dienoate is then converted into *cis*-2-hydroxypenta-2,4-dienoate, which is acted upon by enzyme 2-oxopent-4-enoate hydratase, and 4-hydroxy-2-oxopentanoate is produced. This compound is then transformed into py-

ruvate and acetaldehyde in 4-hydroxyl-2-oxovalerate aldolase. Pyruvate enters glycolysis, and acetaldehyde is oxidized into acetyl-CoA by acetaldehyde dehydrogenase. Acetyl-CoA then enters the Krebs cycle. Enzymes involved in all these steps were identified in the current study.

The *m*-xylene degradation pathway comprises the initial oxidation of *m*-xylene into 3-methylbenzyl alcohol by the enzyme toluene methyl monooxygenase. It is followed by forming 3-methyl benzaldehyde in the presence of aryl-alcohol dehydrogenase, benzoate 1,2-dioxygenase subunit alpha. Then, benzaldehyde dehydrogenase oxidizes 3-methyl benzaldehyde into *m*-methyl benzoate, which is further oxidized into 1,2-dihydroxy-3-methylcyclohexa-3,5-dienecarboxylate by benzoate 1,2-dioxygenase subunit alpha, which is then transformed into 3-methylcatechol in the presence of dihydroxycyclohexadiene carboxylate dehydrogenase. Further degradation of 3-methylcatechol follows the exact mechanism as documented in *o*-xylene. All enzymes of this pathway were identified in the current study bacterial sample.

During degradation of *p*-xylene, it is first oxidized into 4-methylbenzyl alcohol by toluene methyl monooxygenase.

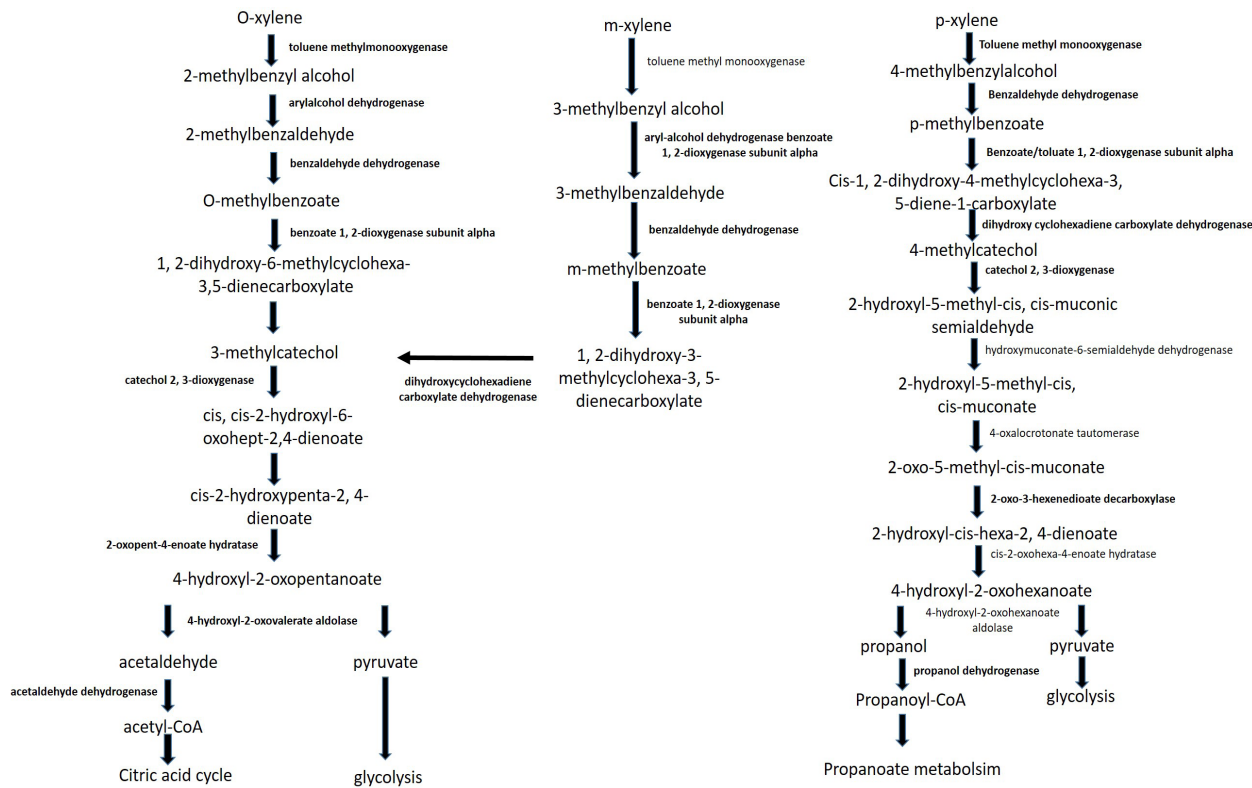


Fig. 4. Reconstruction of ortho, meta and para-xylene degradation pathways based on whole genome shotgun functional annotation.



Fig. 5. Reconstruction of benzene, 3-aminobenzene sulfonate, toluene and 2-, 3- and 4-fluorobenzoate degradation pathways based on whole genome shotgun functional annotation.

Following this, benzaldehyde dehydrogenase performs oxidation and forms *p*-methylbenzoate, which is transformed into *cis*-1,2-dihydroxy-4-methylcyclohexa-3,5-diene-1-carboxylate in the presence of benzoate/toluene 1,2-dioxygenase subunit alpha. Afterwards, dihydroxy cyclohexadiene carboxylate dehydrogenase catalyzes the formation of 4-methylcatechol, which is acted upon by catechol 2,3-dioxygenase and 2-hydroxyl-5-methyl-*cis,cis*-muconic semialdehyde is formed. In a subsequent step, 2-hydroxyl-5-methyl-*cis,cis*-muconic semialdehyde is transformed into 2-hydroxyl-5-methyl-*cis,cis*-muconate in the presence of hydroxymuconate 6-semialdehyde dehydrogenase. Following this, tautomerization by 4-oxalocrotonate tautomerase results in the formation of 2-oxo-5-methyl *cis*-muconate. This is decarboxylated by 2-oxo-3-hexenedioate decarboxylase into 2-hydroxyl-*cis*-hexa-2,4-dienoate. Following this, *cis*-2-oxohexa-4-enoate hydratase catalyzes the formation of 4-hydroxyl-2-oxohexanoate, which is then converted into pyruvate and propanol in the presence of enzyme 4-hydroxyl-2-oxohexanoate aldolase. Pyruvate enters the Krebs cycle, and propanol enters propanoate metabolism through conversion into propanoyl-CoA in the presence of propanol dehydrogenase. All the enzymes of this pathway except hydroxymuconate-6-semialdehyde dehydrogenase, 4-oxalocrotonate tautomerase, *cis*-2-oxohexa-4-enoate hydratase, and 4-hydroxyl-2-oxohexanoate aldolase were identified in the current study bacterial DNA sample. These pathways of xylene degradation are consistent with earlier reported pathways in BTEX-degrading *B. amyloliquefaciens* subsp. *plantarum* W1 (Lee et al. 2019; Wongbunmak et al. 2020).

Pathways identified in the current bacteria involved in benzene, 3-aminobenzene sulfonate, toluene, and 2-, 3-, and 4-fluorobenzoate are shown in Fig. 5. In benzene degradation pathways, benzene is first converted into benzyl alcohol by benzyl alcohol dehydrogenase. Then, benzyl alcohol is converted into benzaldehyde, which is transformed into benzoate by benzaldehyde dehydrogenase. Benzoate is oxidized by benzoate/toluene 1,2-dioxygenase subunit alpha into *cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate, which is transformed into catechol in the presence of dihydroxycyclohexadiene carboxylate dehydrogenase. Catechol is oxidized into *cis,cis*-muconate by catechol 1,2-dioxygenase. The *cis,cis*-muconate is isomerized into muconolactone by muconate cycloisomerase. This is followed by forming 3-oxoadipate-enol lactone in the presence of muconolactone D-isomerase. Afterwards, 3-oxoadipate enol lactonase catalyzes the formation of 3-oxoadipate, which is then transformed into 3-oxoadipyl-CoA in the presence of enzyme 3-oxoadipate CoA-transferase alpha subunit. The 3-oxoadipyl-CoA is then converted into acetyl-CoA and succinyl-CoA by acetyl-CoA acyltransferase and 3-oxoadipyl-CoA thiolase, respectively. Succinyl-CoA then enters the Krebs cycle. All the enzymes of this pathway, except benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, were identified in the current study. This pathway has been documented in literature in *Burkholderia* sp. strain NK8 (Francisco et al. 2001).

During degradation of 3-aminobenzene sulfonate, it is first converted into 3-sulfocatechol in the presence of 2-aminobenzene sulfonate 2,3-dioxygenase.

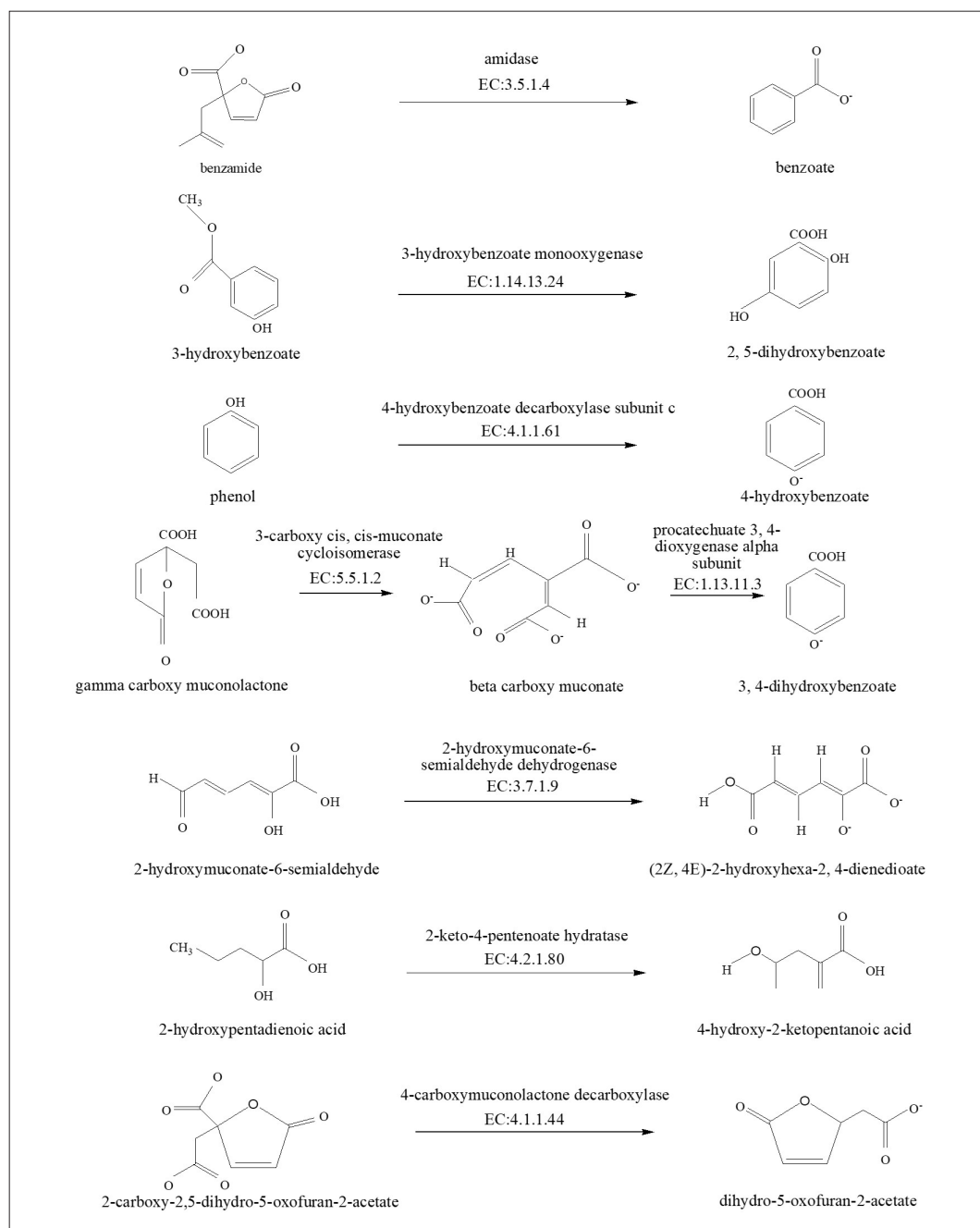


Fig. 6. Reactions of benzene and xylene metabolism catalyzed by bacterial enzymes identified in the current study using whole genome shotgun functional annotation.

Following this, oxidation occurs by catechol 2,3-dioxygenase and 2-hydroxymuconate is formed. Then, 2-hydroxymuconate tautomerizes into gamma-oxalocrotonate by 2-hydroxymuconate tautomerase. Afterwards, decarboxylation occurs in the presence of 2-oxo-3-hexenedioate decarboxylase, and 2-oxopent-4-enoate is formed, which undergoes hydration to form 4-hydroxy-2-oxopentanoate. The hydration reaction is catalyzed by 2-keto-4-pentenoate hydratase. The 4-hydroxy-2-oxopentanoate is further cleaved into acetaldehyde and pyruvate in 4-hydroxyl-2-oxovaler-

ate aldolase. All the enzymes involved in this pathway, except 2-aminobenzene sulfonate 2,3-dioxygenase, 2-hydroxymuconate tautomerase, and 2-oxo-3-hexenedioate decarboxylase, were identified in the current bacteria.

During toluene degradation, it is initially oxidized by toluene methyl monooxygenase into benzyl alcohol, which is then oxidized into benzaldehyde in the presence of arylalcohol dehydrogenase. Following this, benzaldehyde is converted into benzoate by benzaldehyde dehydrogenase. All three enzymes involved in

these steps were identified in the bacteria in the current study. This pathway is consistent with earlier reported toluene degradation pathways in BTEX-degrading *B. amyloliquefaciens* subsp. *plantarum* W1 (Lee et al. 2019; Wongbunmak et al. 2020).

Enzyme benzoate 1,2-dioxygenase subunit alpha that catalyzes the transformation of 2-, 3-, and 4-fluorobenzoate into 6-, 3-, 5-, and 4-fluorocyclohexadiene-*cis,cis*-1,2-diol-1-carboxylate, respectively, has been identified in the current study bacteria. Among these compounds, 6- and 3-fluorocyclohexadiene-*cis,cis*-1,2-diol-1-carboxylate are converted into 3-fluorocatechol by enoate reductase and dihydroxycyclohexadiene carboxylate dehydrogenase, respectively. On the other hand, 5- and 4-fluorocyclohexadiene-*cis,cis*-1,2-diol-1-carboxylate are transformed into 4-fluorocatechol in the presence of dihydroxycyclohexadiene carboxylate dehydrogenase. Then, 3- and 4-fluorocatechols are converted into 2- and 3-fluoro-*cis,cis*-muconate by enzyme catechol 1,2-dioxygenase. All the enzymes of these pathways except enoate reductase were identified in the current study. These pathways are consistent with the literature and have been documented in *Sphingomonas* sp. HB-1 and in a microbial sample comprising *Marinobacter*, *Vibrio natriegens*, *Gramella* sp., and *Methyloceanibacter* (Hidde Boersma et al. 2004; Kannan et al. 2023).

In addition to enzymes associated with the above pathways, some additional enzymes were also identified in bacteria, including amidase, 3-hydroxybenzoate monooxygenase, 4-hydroxybenzoate decarboxylase subunit c, 3-carboxy-*cis,cis*-muconate cycloisomerase, procatechuate-3,4-dioxygenase alpha subunit, 2-hydroxymuconate-6-semialdehyde dehydrogenase, 2-keto-4-pentenoate hydratase, and 4-carboxymuconolactone decarboxylase. Reactions catalyzed by these bacteria are shown in Fig. 6.

Enzymes identified in the current study are in accordance with previous literature, as a study focused on WGS of twenty-six different strains of bacteria reported the enzymes associated with benzene, xylene, benzoate, naphthalene, and toluene (Révész et al. 2020; Eze 2021; Bedics et al. 2022; Hossain et al. 2024). Another study reported xylene degrading enzyme catechol 2,3-dioxygenase in a *Pseudomonas* strain (Miri et al. 2022).

The current project documenting the Gram-positive hydrocarbonoclastic *Bacillus* bacteria is a novel addition to the current state of knowledge because the benzene degradation pathway identified in current study have been reported in Gram-negative bacteria in

literature but not in any Gram-positive *Bacilli* except *B. cereus* which was isolated from petroleum contaminated soil (Hussain et al. 2025). Fluorobenzoate degradation pathways identified in the current bacteria have never been documented in any Gram-positive bacteria. However, they are reported in Gram-negative isolates. Toluene and xylene degrading bacteria documented in the current study have been identified earlier either in Gram-negative bacteria or in *B. amyloliquefaciens* subsp. *plantarum* W1 (Wongbunmak et al. 2020). No other *Bacillus* species have been reported to exhibit this xylene degradation pathway. However, the toluene degradation pathway is also reported in another *Bacillus*. i.e., *B. pumilus* MVS3 (Surendra et al. 2017).

In addition, these isolates exhibit the coexistence of metabolic pathways for diverse substrates. i.e., benzene, toluene, *o*-, *m*- and *p*-xylene, 3-aminobenzene-sulfonate and 2-, 3- and 4-fluorobenzoates.

Despite these facts, current data have significance because they document Gram-positive bacteria, which can be more potent bioremediating agents than previously reported Gram-negative bacteria. Due to the unique characteristics of Gram-positive bacterial cell walls, including the absence of an outer membrane, they have a greater tendency to uptake organic pollutants and bind with and accumulate heavy metals from external sources (Liaqat and Sabri 2008). These features render them more potent bioremediation candidates than Gram-negative bacteria (Ganesh and Lin 2009; Nanda et al. 2019). In Gram-negative bacteria, efflux pumps are present, which confer them tolerance against various pollutants like organic solvents, heavy metals, dyes, and antibiotics. Hence, they contribute to the survivability of bacteria in the highly polluted environment. According to the literature, the efflux pumps contribute to the bioremediation potentials of bacteria by allowing them to persist in contaminated environments (Blair et al. 2014).

However, bioremediation is considered an eco-friendly mode of pollutant degradation. A bacterium possessing pumps that confer antibiotics and other multi-drug resistance can be a source of spread of multi-drug resistance (MDR) (Cunningham et al. 2020). According to this fact, we cannot use such bacteria as whole cells for eco-friendly bioremediation. Hence, Gram-positive bacteria can be more potent and sustainable bioremediating agents because they can accumulate large concentrations of pollutants in their cells and have the least chance of developing MDR due to the absence of efflux pumps (Biswas et al. 2021).

Abbreviations

ADH – arginine dihydrolase
 ADON – adonitol fermentation test
 bamB – benzoyl-CoA reductase
 BCL – base call
 BTEX – benzene, toluene, ethylbenzene, and xylene
 C23O – catechol 2,3-dioxygenase
 dmpK – toluene 2-monooxygenase encoding gene
 EPA – Environment Protection Agency
 EST – triglyceride test
 gcd – glutaryl-CoA dehydrogenase
 GC-MS – gas chromatography-mass spectrometry
 GGT – γ -glutamyl- β -naphthylamide hydrolysis test
 GLU – glucose test
 GUR – β -glucuronidase test
 IARC – International Agency for Research on Cancer
 KEGG – Kyoto Encyclopedia of Genes and Genomes
 KSF – sugar aldehyde utilization test
 LDC – lysine decarboxylase test
 LIP – lipase detection test
 MAG – metagenome-assembled genome
 MDR – multi-drug resistance
 MSM – minimal salt medium
 NCBI – National Center of Biotechnology Information
 NO₃ – sodium nitrate test
 OD – optical density
 ODC – ornithine decarboxylase test
 PCBs – polychlorinated biphenyls
 PCI – phenol : chloroform : isoamylalcohol
 PHCs – polycyclic aromatic hydrocarbons
 PRO – proline- β -naphthylamide test
 PYR – pyrrolidonyl- β -naphthylamide
 Q20, Q30 – phred quality scores
 RTA – real time analysis
 SBL – sorbitol fermentation test
 SDS – sodium dodecyl sulfate
 SPAdes – St. Petersburg genome assembler
 SRA – sequence read archive
 T4MO – toluene 4-monooxygenase
 TET – aliphatic thiol utilization test
 todC1 – toluene 1,2-dioxygenase
 TRD – aliphatic diol test
 URE – urea test
 WGS – whole genome sequencing
 xylM – xylene monooxygenase

Availability of data and material

The FASTA sequences of present study isolates are available at NCBI database with accession codes OR272055, OR272056, OR272059, OR272058, OR272061, OR272060, OR272064, OR272063, OR272072, OR272071 and OR272073 and the FastQ files of WGS are available at SRA NCBI under accession ID PRJ-NA976120.

Acknowledgments

The authors extend their appreciation to the Researchers Supporting project (ORF-2025-502), King Saud University, Riyadh, Saudi Arabia, for funding this project.

Author contributions

F.M. perceived idea, supervision and designed methodology, F.M. formal analysis and write up, A.S. formal analysis, A.A. write up, T.A. Data presentation, H.A. formal analysis, S. R. M. formal analysis.

Funding

The authors extend their appreciation to the Researchers Supporting project (ORF-2025-502), King Saud University, Riyadh, Saudi Arabia, for funding this project.

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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