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RESEARCH

Degradation and metagenomic analysis of 4-chlorophenol utilizing multiple metal tolerant bacterial consortium

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Abstract
Chlorophenols are persistent environmental pollutants used in synthesizing dyes, drugs, pesticides, and other industrial products. The chlorophenols released from these processes seriously threaten the environment and human health. The present study describes 4-chlorophenol (4-CP) degradation activity and metagenome structure of a bacterial consortium enriched in a 4-CP-containing medium. The consortium utilized 4-CP as a single carbon source at a wide pH range, temperature, and in the presence of heavy metals. The immobilized consortium retained its degradation capacity for an extended period. The 4-aminoantipyrine colorimetric analysis revealed complete mineralization of 4-CP up to 200 mg/L concentration and followed the zero-order kinetics. The addition of glycerol and yeast extract enhanced the degradation efficiency. The consortium showed both ortho- and meta-cleavage activity of catechol dioxygenase. Whole genome sequence (WGS) analysis revealed the microbial compositions and functional genes related to xenobiotic degradation pathways. The identified genes were mapped on the KEGG database to construct the 4-CP degradation pathway. The results exhibited the high potential of the consortium for bioremediation of 4-CP contaminated sites. To our knowledge, this is the first report on WGS analysis of a 4-CP degrading bacterial consortium.

Keywords Bacterial consortium · Biodegradation · 4-Chlorophenol · *Paracoccus* · *Proteobacteria* · Whole genome sequencing

Introduction

Environmental pollution due to industrial development is a serious hazard to human health. Chlorophenols are chlorinated aromatic compounds that are widely employed in industry, agriculture, and medicine. They are found in wastewater, surface water, soil, and sediments, in bodies of aquatic organisms, animal milk, adipose tissue, and urine (Olaniran and Igbinosa 2011; Zada et al. 2021). In strongly contaminated groundwater the chlorophenol concentrations have been reported to range from 100 to 200 mg/L (Ettala et al. 1992). As per recommendations from the World Health Organisation (WHO) and United States Environmental Protection Agency (USEPA), the concentration of chlorophenol in drinking water and industrial effluent should not exceed 0.5 µg/L and 100 µg/L, respectively (Zada et al. 2021). More than 100,000 tonnes of chlorophenols are used worldwide each year (Nowak and Mrozik 2018). The extensive use of these compounds and their release into the environment, primarily through inappropriate disposal, pose a concern to humans, aquatic life, and wildlife due to their acute toxicity, carcinogenicity, and refractory nature (Nowak and Mrozik 2018; Zada et al. 2021). In particular, exposure to 4-chlorophenol (4-CP) causes a range of adverse health effects including diarrhoea, tremors, internal organs (kidney, lungs) dysfunction, mutations, abnormalities, muscular weakness, and coma in living organisms (Kwean et al. 2018; Swain et al. 2021). The 4-CP can be generated from paper industry during chlorine bleaching of pulp and wastewater treatment, as well as from the breakdown of more highly chlorinated phenols and phenoxy herbicides (Bjerketorp et al. 2018). The USEPA has classified 4-CP as a primary pollutant (Dan et al. 2021). Consequently, 4-CP pollutants ought to be managed carefully before being released into a public space

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Various methods for the removal of 4-CP comprise adsorption, photocatalysis, electrochemical oxidation, etc. (Adhikari et al. 2017; Allaboun and Abu 2016; Duan et al. 2013; Saravanakumar et al. 2023). Although highly efficient, these techniques have limited use due to high cost and in some cases production of harmful intermediates. Bioremediation, on the other hand, is a cost-effective and environmentally friendly method for the reclamation of 4-CP contaminated environments (Patel and Kumar 2016; Arora and Bae 2014). It involves the use of microorganisms to degrade or transform pollutants into less toxic forms. Further, the bioremediation which can be conducted *in situ* reduces the need for costly excavation and transportation of contaminated material. Several bacterial species such as *Bacillus* sp., *Pseudomonas* sp., *Rhodoccocus* sp., *Arthrobacter* sp., and *Alcaligens* sp., have been reported to degrade 4-CP (Farrell and Quilty 2002; Konovalova et al. 2009; Nordin et al. 2005; Sandhibigraha et al. 2020; Swain et al. 2021). Swain et al. (2021) reported that *Bacillus flexus* was able to remove 78.12% of 4-CP at a concentration of 50 mg/L. According to Patel and Kumar (2016), a bacterial consortium consisting of *Pseudomonas aeruginosa* GF, *Kocuria rhizophila* 11Y, *Bacillus endophyticus* CP1R, and *Bacillus cereus* 3YS degraded 3-CP and 4-CP. However, alone none of these strains demonstrated chlorophenol degradation activity thus underscoring the importance of bacterial consortia for bioremediation of polluted environments. Environmental factors play a vital role in the biodegradation of pollutants in contaminated habitats which also require optimization to enhance the biodegradation efficiency (Fletcher et al. 2011; Swain et al. 2021; Wang and Sun 2021; Zhao et al. 2018).

The information on the degradation of chlorophenols using naturally developed bacterial consortia is limited. Therefore, the aim of present study was to characterize the 4-CP degradation by a laboratory developed bacterial consortium. The degradation activity was assessed at different 4-CP concentrations and in response to various environmental factors e.g., temperature, pH, heavy metals, and extra carbon sources. Further, whole genome sequencing (WGS) approach was used for the taxonomic and functional diversity analysis of the consortium.

Materials and methods

Chemicals and selection of bacterial consortium

All the chemicals and reagents were analytical grade. Catechol, 4-aminoantipyrine reagents, and 4-CP were obtained from Sigma-Aldrich (India). HPLC grade ethyl acetate, glycerol, and sucrose were purchased from SRL, India. Bushnell Haas medium (BHM), heavy metal salts, glucose, fructose, and yeast extract were purchased from Himedia, India.

BHM (Bushnell and Haas 1941) supplemented with 40 mg/L 4-CP was inoculated with 1.0 g soil sample collected from a pesticide contaminated rice field in Umshing area of Shillong, India (Latitude-25.61 N and Longitude-91.90 E). The inoculated flasks were incubated for two weeks in an incubator shaker at 110 rpm at 25±1 °C. The developed bacterial consortia were enriched by ten successive transfers every fifth day into fresh medium and by gradually increasing the 4-CP concentration from 40 to 100 mg/L. A total of five bacterial consortia with the capacity to utilize 4-CP as a carbon source were developed by enrichment method. A consortium showing the best degradation efficiency was selected and maintained in 100 mg/L 4-CP supplemented BHM for further study.

Microbial culture condition

All the experiments were carried out in a 250 mL Erlenmeyer flask containing 100 mL BHM supplemented with 4-CP. Acclimated microbial consortium were inoculated at initial $OD_{600} = 0.04$. The inoculated flasks were incubated at 25±1 °C in an incubator shaker (110 rpm). Uninoculated control flasks were incubated in parallel.

Determination of growth and 4-CP degradation

The bacterial consortium was inoculated in 4-CP amended BHM and the growth of the consortium was determined as an increase in cell density at λ_{600} using UV-VIS spectrophotometer (Chettri and Singh 2019). Measurement of residual 4-CP in the culture supernatant was determined by 4-aminoantipyrine colorimetric method (Farell and Quilty 1999). For gas chromatography mass spectrometry (GC-MS) analysis of residual 4-CP, a 5 mL culture supernatant was extracted twice in an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to 1.0 mL final volume and passed through prewashed Isolute Solid Phase Extraction (SPE) column. The filtered extract was analyzed using a Thermo Scientific, Trace GC Ultra gas chromatograph coupled with a mass spectrometer (ITQ 1100 Ion Trap MS, Thermo Scientific) by injecting 1 µL of ethyl acetate extract (Chettri et al. 2016). The degradation percentage of 4-CP was calculated using Eq. (1):

$$\text{Degradation } \% = \left(C_0 - C_t\right)/C_0 \times 100 \quad (1)$$

The degradation of 4-CP was fitted into zero-order kinetics as described in Eq. (2)

$$C_t = C_0 - kt \quad (2)$$

where C_0 is the initial concentration of 4-CP, C_t is the concentration of 4-CP at time t, t is the degradation period in hour and k is the biodegradation rate constant (Gaya et al.

2009). The half-life ($t_{1/2}$) of biodegradation was calculated using the Eq. (3)

$$t_{1/2} = C_0/2k \quad (3)$$

Effect of different process variables on biodegradation

The effect of various factors on the 4-CP degradation was evaluated in a bacterial consortium inoculated in 100 mL BHM containing 100 mg/L of 4-CP and incubated for 36 h under shaking condition (110 rpm); at six different temperatures (15, 20, 25, 30, 35 and 40 °C), five different pH (5, 6, 7, 8, and 9), in presence of 100 µM (Al^{3+}, Cr^{3+}, Fe^{2+}, Mn^{2+}, Pb^{2+}, Sr^{2+}, Zn^{2+}) and 10 µM (Cu^{2+}, Co^{2+}, Ni^{2+}) metal ions, and presence of 0.5 g/L (sucrose, glucose, fructose, yeast extract), and 0.05% glycerol as carbon sources. The concentration of glycerol is mentioned in percentage because it was available in liquid form.

Enzyme assay

The bacterial consortium was inoculated in 100 mg/L 4-CP supplemented BHM. After 24 h of incubation, the cells were collected by centrifuging at 13,000 rpm for 10 min at 4 °C. The pellet was washed twice and re-suspended in 33 mM Tris-HCl buffer (pH 7.6). The suspension was sonicated in an ice bath and centrifuged at 20,000 rpm at 4 °C for 20 min. The cell free extract was used for estimating catechol 1, 2-dioxygenase and catechol 2, 3-dioxygenase activities according to Farell and Quilty (1999). Total protein was assessed using bovine serum albumin (BSA) as the standard (Lowry et al. 1951).

Statistical analysis

Each experiment was repeated three times with three replicates each. The values from replicate experiments were used for calculating the mean ± standard deviation. One-way ANOVA was done in Microsoft Excel.

Whole genome sequencing (WGS)

Genomic DNA from 4-CP degrading bacterial consortium was extracted using c-TAB and Phenol: Chloroform extraction technique, followed by RNase A treatment. The extracted DNA's quality and quantity were assessed using NanoDrop, followed by 0.8% agarose gel electrophoresis. The paired-end sequencing library was created from QC-passed DNA samples using the illumina TruSeq Nano DNA Library Prep Kit. The library was sequenced on the Illumina NextSeq500 platform using 2 × 150 bp chemistry. Covaris M220 fragmented about 200 ng of DNA to produce a mean fragment distribution of 350 bp. Double stranded DNA fragments with 3' or 5' overhangs were generated by Covaris shearing and then subjected to end-repair. The End Repair Mix converts the 3' or 5' overhangs into blunt ends followed by adapter ligation to the fragments. AMPure XP beads were used to size select the ligated products. The ligated products were PCR amplified using the index primer according to the manufacturer's protocol. The PCR amplified library was analyzed on 4200 Tape Station system (Agilent Technologies) using high sensitivity D1000 Screen Tape.

To obtain high quality clean reads, the adapter sequences, ambiguous reads (reads with unknown nucleotides "N" >5%), and low-quality sequences (reads with>10% quality threshold (QV) < 20 phreds score) were removed using Trimmomatic v0.38. A minimum length of 100 nucleotides after trimming was used. The high quality paired-end reads (>20 phreds score) were used for de-novo assembly. These reads were assembled into scaffolds using CLC Workbench (ver.9), and the average size of scaffolds was ~677 bp. Prodigal 2.6.3 was used to predict genes from assembled scaffolds, and the total number of genes was estimated at 89,837, with a minimum gene length of 60 bp and a maximum gene length of 8,340 bp. The WGS sequenced raw data was deposited to the National Centre for Biotechnology Information (NCBI) under BioProject: PRJNA728811 and Sequence Read Archive (SRA): SRS18487309. The data summary can also be accessed at NCBI via https://www.ncbi.nlm.nih.gov/bioproject/PRJNA728811

Results

Effect of 4-CP concentration on growth and biodegradation activity

The bacterial consortium was inoculated in 4-CP (50–300 mg/L) amended BHM showed an increase in cell density with increasing 4-CP concentration up to 200 mg/L (Fig 1a). The maximum growth at 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, and 300 mg/L was observed respectively, at 18 h, 24 h, 36 h, 48 h, and 60 h after incubation. However, growth was least at 300 mg/L concentration.

The amount of residual 4-CP was determined in the supernatant from the aforementioned cultures. The time needed for complete degradation increased with increasing 4-CP concentration (Fig 1b). The consortium required 18 h, 36 h, 60 h, and 120 h for complete degradation at 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L, respectively. At 300 mg/L concentration, the consortium could degrade only 28.8% of 4-CP after 144 h of incubation, resulting in incomplete elimination of the compound. A straight line with slope $=-k$ and intercept $= [4\text{-CP}]_0$ was obtained from

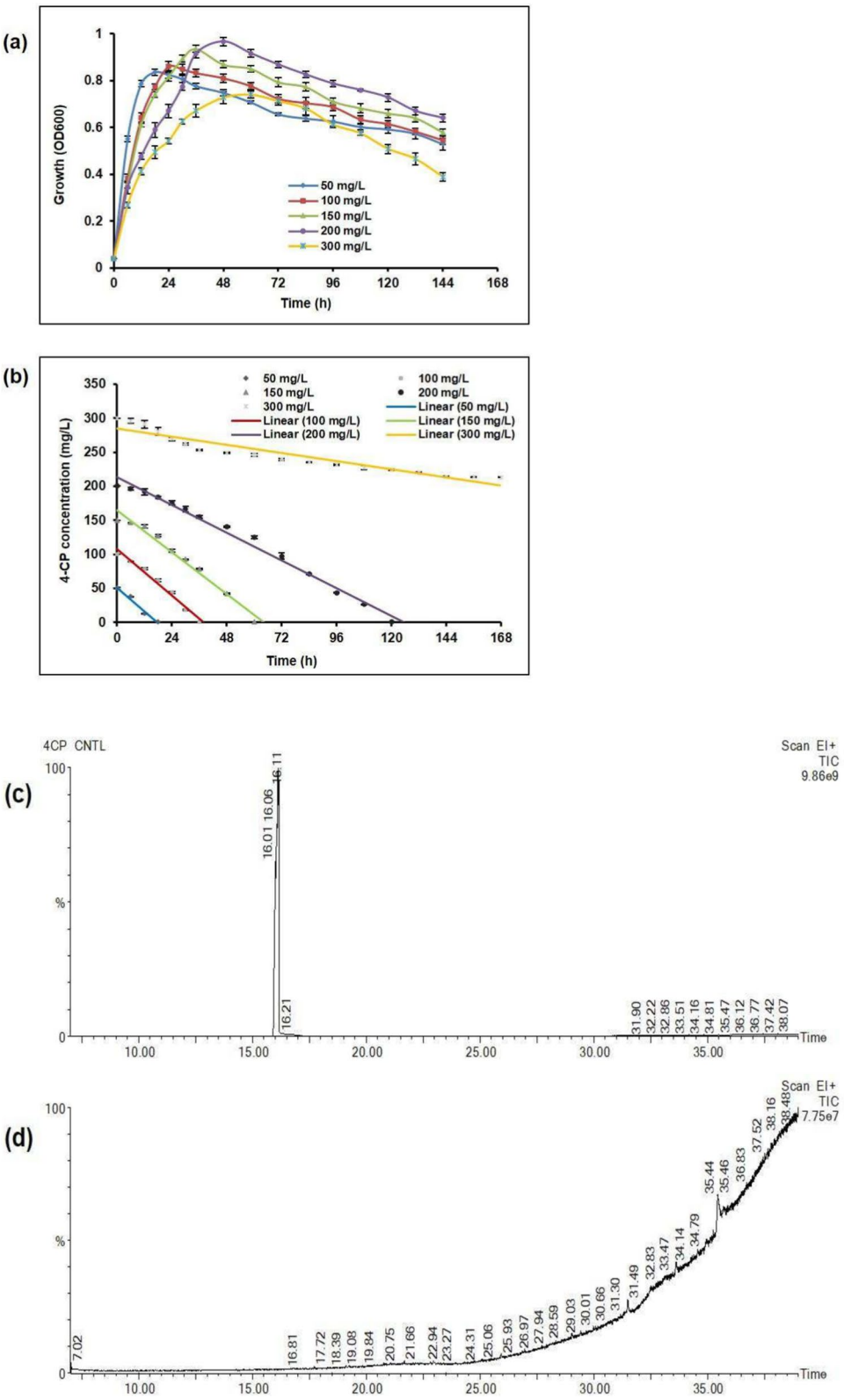

Fig. 1 **a** Growth kinetics of bacterial consortium in 4-CP (50 mg/L–300 mg/L) amended BHM. **b** Zero order kinetic model fitted to the biodegradation of 4-CP. **c** The GC–MS Chromatograph of culture extracts at zero time and **d** 36 h after 4-CP treatment (100 mg/L)

a plot of [4-CP] against time, which conforms to the zero-order kinetics for 4-CP degradation. The values from Fig 1b were used to calculate the biodegradation rate constant (k) and half-life ($t_{1/2}$) of degradation. The k and $t_{1/2}$ values were found to be 2.89±0.0221 mg/L/h and 8.65 h at 50 mg/L; 2.83±0.006 mg/L/h and 17.64 h at 100 mg/L; 2.56±0.007

mg/L/h and 29.30 h at 150 mg/L; 1.70±0.0032 mg/L/h and 58.90 h at 200 mg/L; and 0.5632±0.019 mg/L/h and 266.34 h at 300 mg/L concentrations. The decline in degradation rate from 200 mg/L onward concentration reflects the upper limit of the bacterial consortium to degrade 4-CP. The degradation of 4-CP (100 mg/L) was assessed also by GC-MS based analysis. The absence of metabolite in GC-MS profile revealed complete degradation of 4-CP in 36 h. (Fig 1c and 1d). The consortium retained the 4-CP degradation activity 12 months after preservation in different matrices, e.g., Calcium alginate beads, sawdust, rice husk, charcoal, coconut husk, and firewood ash. The initial degradation activity of the preserved cells was highest for alginate beads. The preserved cells regained degradation ability comparable to freshly grown inoculum after two consecutive transfers.

Effect of temperature, pH, heavy metals, and carbon sources on biodegradation activity

A complete degradation of 4-CP was observed after 36 h incubation at temperatures ranging from 15 and 35 °C with a degradation rate of 2.7±0.02 mg/L/h. At 40 °C, the degradation rate decreased drastically to 0.87 mg/L/h, exhibiting just 19.9% and 31% degradation after 24 h and 36 h respectively (Fig 2a). As shown in Fig 2b, the ideal pH range for 4-CP degradation was between 7 and 9 as complete degradation was achieved within 36 h of incubation. At pH 6, about 82.5% of the total 4-CP was degraded in 36 h. However, when the initial pH was at 5, a drastic inhibition in 4-CP degradation was observed. The degradation rates were 0.03 mg/L/h, 2.11 mg/L/h, 2.66 mg/L/h, 2.75 mg/L/h, and 2.84 mg/L/h at pH 5, 6, 7, 8 and 9 respectively.

Similarly, a complete degradation occurred in Al^{3+}, Cr^{3+}, Fe^{2+}, Mn^{2+}, $Pb^{2+,}$ and Sr^{2+} supplemented conditions with a degradation rate of ~2.7±0.03 mg/L/h. Biodegradation of 4-CP was reduced to 64.96%, 57.47%, and 37.96% respectively in Ni^{2+}, $Zn^{2+,}$ and Co^{2+} treated conditions (Fig 2c). The degradation rate was 1.74 mg/L/h (Ni^{2+}), 1.53 mg/L/h (Zn^{2+}), and 1.04 mg/L/h (Co^{2+}). In contrast, Cu^{2+} treatment caused almost complete inhibition of 4-CP degradation. The addition of glycerol and yeast extract to the culture medium significantly increased the removal rate of 4-CP (Fig 2d). The degradation rates in sucrose, glycerol, glucose, fructose, and yeast extract amended medium were 2.71 mg/L/h, 2.89

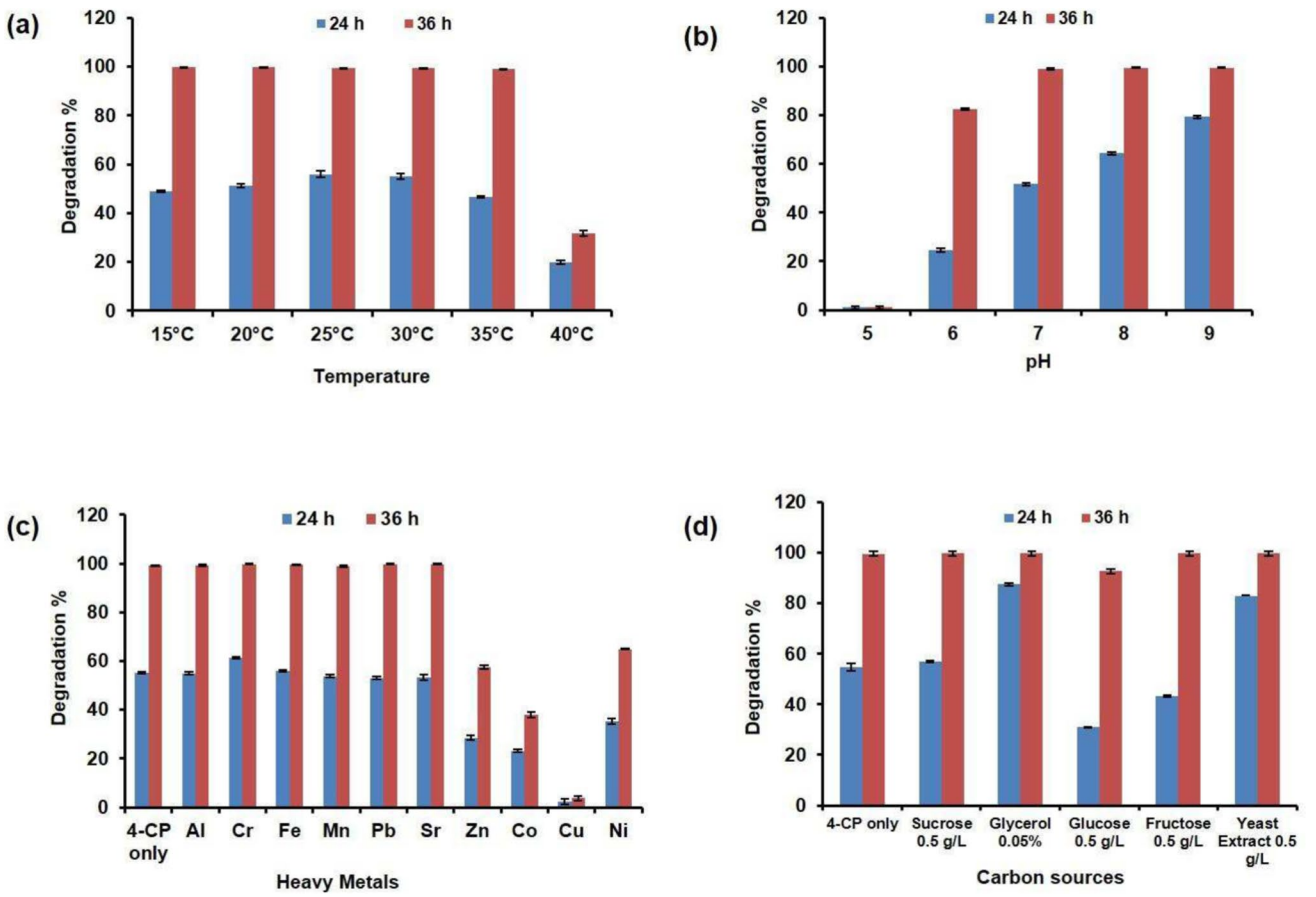

Fig. 2 Effect of various parameters on the removal of 4-CP (100 mg/L) in BHM by bacterial consortium: **a** temperature, **b** pH, **c** heavy metals, and **d** carbon sources

mg/L/h, 2.38 mg/L/h, 2.63 mg/L/h, and 2.86 mg/L/h respectively. After 36 h incubation, complete removal of 4-CP was achieved in each carbon source amended media except for glucose.

Catechol dioxygenase activity

The bacterial consortium grown with 100 mg/L of 4-CP showed both catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities at 0.029±0.003 µmoles cis,cis-muconic acid/min/mg protein and 0.027±0.002 µmoles 2-hydroxymuconic semialdehyde/min/mg protein respectively.

Microbial community structure

The WGS statistics of the consortium generated a total of 89,837 high quality filtered reads and summary of metagenomics sequencing statistics were listed in Table 1. Taxonomic analysis of the predicted genes was carried out using Kaiju (kaiju.binf.ku.dk). The high throughput metagenomics sequencing data revealed the level of microbial diversity of the consortium. The microbial consortium was dominated by Bacteria (87.19 %), followed by unclassified sequences (12.73%), Eukaryotes (0.04%), Archaea (0.03%), and Viruses (0.01%).

The microbial community abundance of the consortium at phylum and genus level is presented in Fig 3. The bacterial domain was dominated mostly by the phyla *Proteobacteria* (73.27%) and *Bacteroidota* (10%), constituting more than 83% of the total bacterial community. At the class level, *Alphaproteobacteria* (42.13%), *Betaproteobacteria* (25.92%), and *Gammaproteobacteria* (4.41%) accounted for 72.46% of the total bacteria (Fig S1a). *Burkholderiales* (25.65%), *Rhizobiales* (20.22%), and *Rhodobacterales* (16.26%) were found to be the most common order (Fig S1b). The dominant bacterial families were *Rhodobacteraceae* (16.12%), *Comamonadaceae* (12.01%), *Alcaligenaceae* (11.43%), and *Rhizobiaceae* (10.66%) (Fig S1c). The bacterial genera at >1% abundance comprised *Paracoccus* (13.24%), *Achromobacter* (10.93%), *Hydrogenophaga* (6.74%), *Rhizobium* (5.27%), *Leadbetterella* (4.41%), *Brevundimonas* (4.16%), *Acidovorax* (3.76%), *Mesorhizobium* (2.79%), *Shinella* (2.58%), *Aquamicrobium* (1.34%), *Sphingobacterium* (1.32%) and *Thermomonas* (1.23%) (Fig 3b). *Achromobacter denitrificans* (8.72%), *Leadbetterella bysophilia* (4.41%) and *Paracoccus denitrificans* (4.22%) were the three most abundant species (Fig S1d).

Functional annotation

Functional annotation of genes was carried out using Cognizer (metagenomics.atc.tcs.com/cognizer), a comprehensive stand-alone framework that is capable of simultaneously providing clusters of orthologs genes (COG) and Kyoto encyclopedia of genes and genomes (KEGG) subsystem annotations to individual sequences constituting metagenomic datasets. Cognizer's innovative 'direct search' feature considerably reduces the total compute needs involved with functional analysis.

Classification of genes according to COG Categories

A total of 60,442 genes were assigned to COG categories. The functional annotation distribution of COG is presented in Fig 4. The COG categories at level 1 showed relative abundance of genes involved in Metabolism (46.32%), Poorly characterized (19.78), Cellular processes and signaling (18.55%), and Information storage and processing (15.36%). At level 2, General function prediction only [R] (12.41%) was the dominant function among the 24 categories, followed by Amino acid transport and metabolism [E] (12.26%), Inorganic ion transport and metabolism [P] (7.04%), Transcription [K] (6.90%), Energy transport and conversion [C] (6.80%) and Carbohydrate transport and

Table 1 Summary of metagenomic sequencing statistics of 4-chlorophenol degrading bacterial consortium

High Quality Read statistics	No. of paired-end reads	9,782,002
	Total no. of bases	2,827,837,253
	Data in Gb	~2.83
Metagenome Assembly Statistics	Number of Scaffolds	61,793
	Total size of the assembly (bp)	41,823,913
	Average size of scaffolds (bp)	~677
	Scaffold N50 (bp)	721
	Maximum size of scaffold (bp)	291,401
Gene Prediction Statistics	Number of genes	89,837
	Average gene length (bp)	~419
	Maximum gene length (bp)	8,340
	Minimum gene length (bp)	60

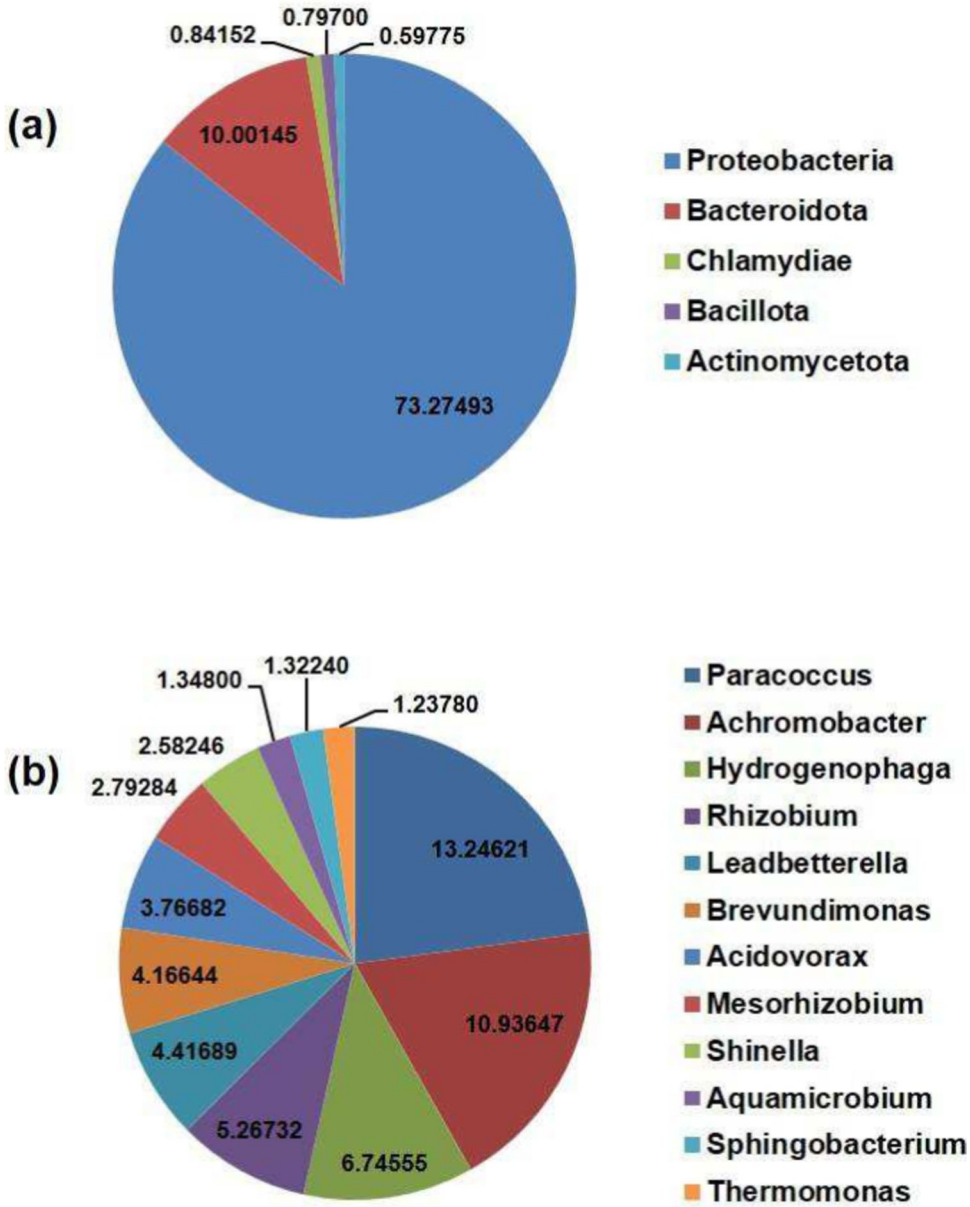

Fig. 3 Pie chart showing the percentage relative abundance at different taxonomic ranks with **a** 0.5 thresholds for phylum level, **b** 1.0 thresholds for genus level

metabolism [G] (6.10%) (Thresholds level 6%). The lowest number of genes were assigned to RNA processing and modification [A] (0.01%), Extracellular structures [W] (0.01%), and Chromatin structures and dynamics [B] (0.04%).

Classification of KEGG functional categories and 4-CP degradation pathway

A total of 87,981 genes were assigned to KEGG orthology (KO). The distribution of KEGG categories at level 1 revealed unclassified genes (40%), metabolism (34%), environmental information processing (16%), genetic information processing (5%), and cellular processes (4%). The five most abundant categories at level 2 of KEGG metabolic pathways were carbohydrate metabolism (15.5%), amino acid metabolism (14.53%), membrane transport (13.54 %), energy metabolism (7.68%), and xenobiotics biodegradation and metabolism (7.66%) (Fig 5). The mapping of obtained KO ids in xenobiotic biodegradation and metabolism pathway revealed 4780 genes corresponding to 308 different enzymes. The number of possible reactions of these enzymes in xenobiotic degradation is presented in Table 2. Majority of these genes were mapped in benzoate degradation (ko-00362) and chlorocyclohexane and chlorobenzene degradation (ko-00361), drug metabolism-other enzymes (ko-00983), xylene degradation (ko-00622), and metabolism of xenobiotics by cytochrome-P450 (ko-00980). Based on the gene mappings in KEGG database, the 4-CP degradation

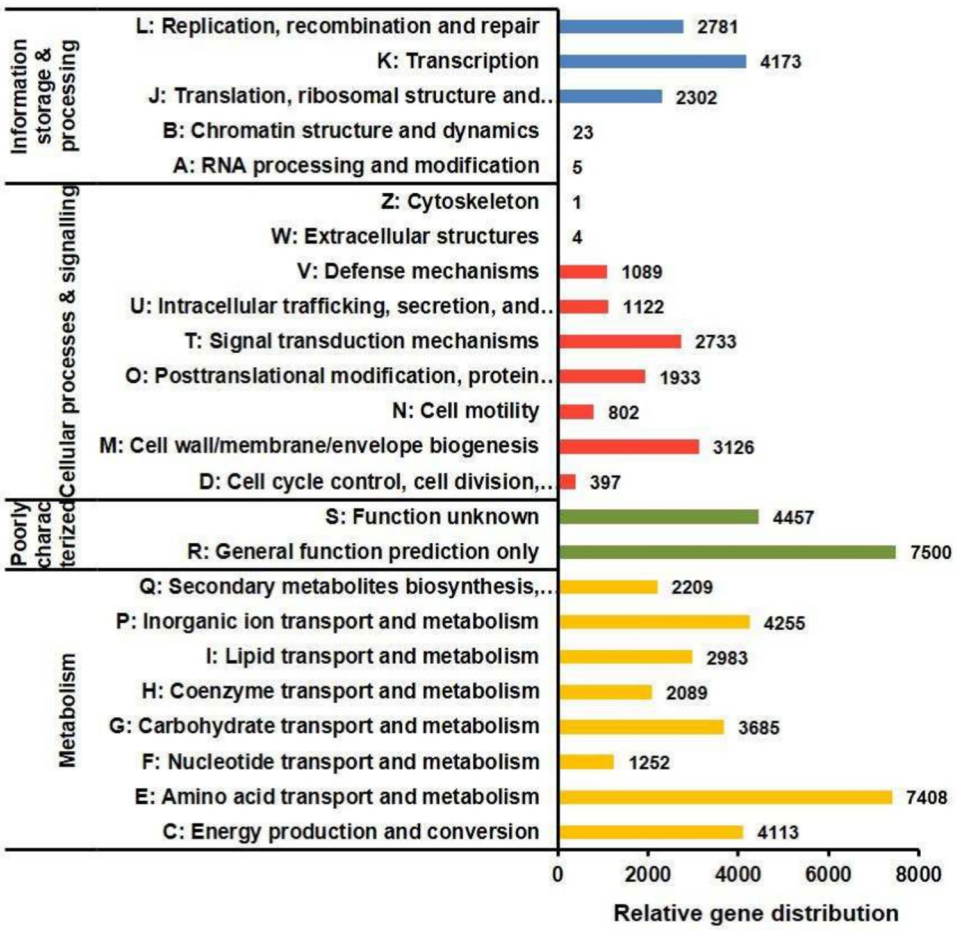

Fig. 4 Relative gene distribution at different levels of COG categories

pathway which is a component of chlorocyclohexane and chlorobenzene degradation (Fig S2), was constructed (Fig 6). The list of enzymes of 4-CP degradation pathway is presented in Table 3. The pathway shows 4-chlorocatechol as the first intermediate in degradation process which is subsequently cleaved through both ortho- and meta-ring cleavage by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase. This result was confirmed by the presence of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities. The 4-CP degradation by microbial consortium followed the chlorocyclohexane and chlorobenzene degradation pathways, and then the benzoate degradation pathways (Fig S3). Finally, the metabolites are metabolized through citric acid cycle.

Discussion

Degradation of 4-CP has been studied using both bacterial isolates and defined bacterial consortium. Swain et al. (2021) reported 78% and 38% degradation by free cells and 90.34% and 49.44% by immobilized cells respectively at 50 mg/L and 200 mg/L of 4-CP by *Bacillus flexus* in 7 days. Patel et al. (2016) using a defined bacterial consortium reported 63% removal of 200 mg/L 4-CP in 168 h. Past studies have focused on using either pure isolates or defined consortium for chlorophenol biodegradation. In our study, the bacterial consortium enriched from pesticide-contaminated soil showed complete biodegradation of 4-CP up to 200 mg/L within 120 h of incubation. Moreover, the retention of 4-CP degradation activity after immobilization for 12 months reflects our consortium's high usefulness for its application under field conditions.

The enriched consortium's ability to degrade 4-CP across a wide temperature range illustrates its resilience to fluctuating temperatures or seasonal variations. Monsalvo et al. (2009) have reported the complete removal of 4-CP (105 mg/L) in acclimated sequencing batch reactors (SBR) industrial sludge at 20 °C–35 °C. At temperatures over 35 °C, the consortium's capacity to breakdown 4-CP decreased significantly, which is most likely caused by inhibition of the cell's multienzyme complex system (Bandyopadhyay et al. 1998).

In soil ecosystems, pH values can be highly variable ranging from 2.5 to 11 (Bartha and Bossert 1984). The consortium's capacity to operate from pH 6-9 enables it to target and remediate 4-CP contaminated sites at wide pH profiles.

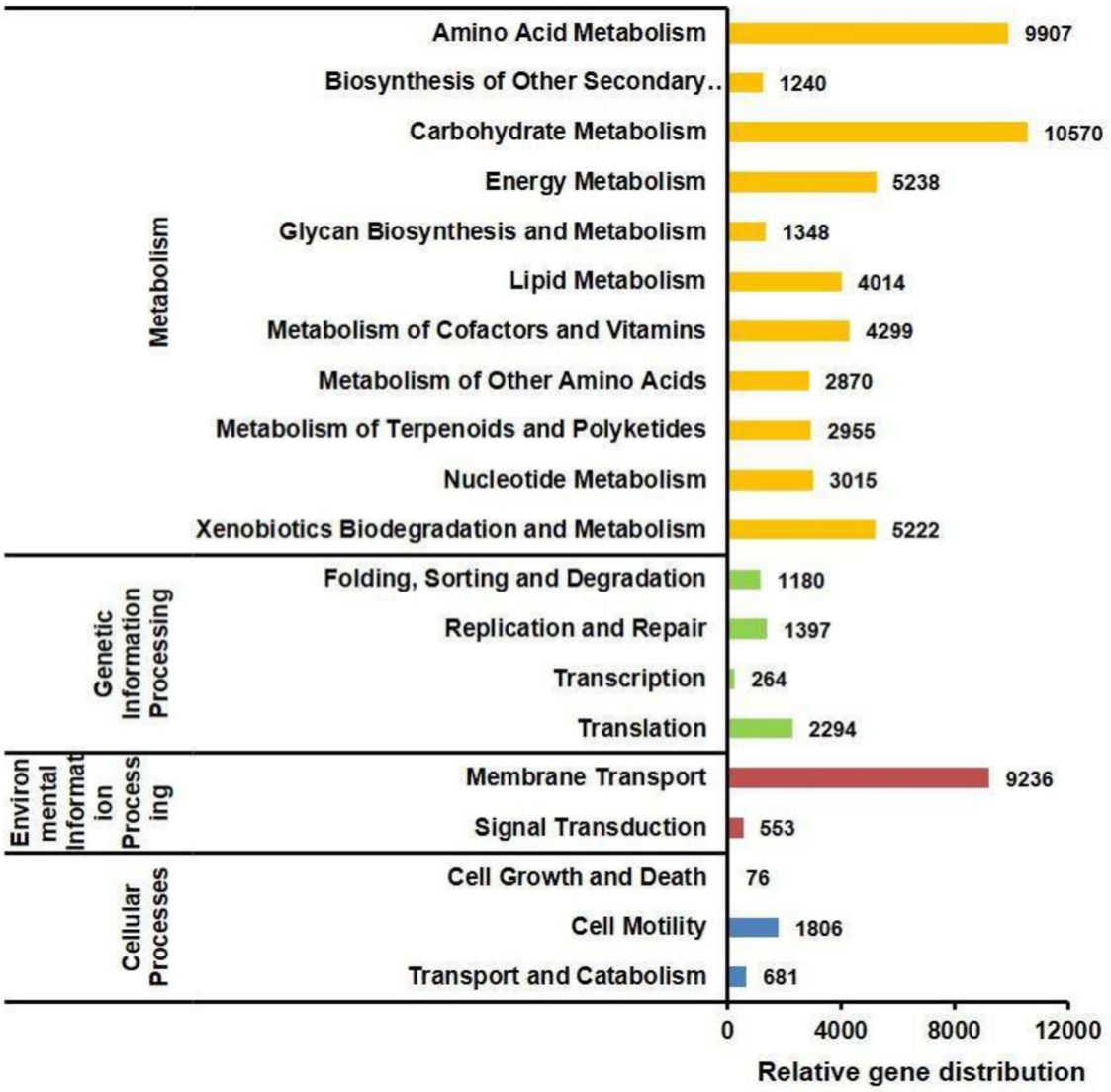

Fig. 5 Functional gene distribution profile of KEGG categories at different levels

Since chlorophenols exist in the unionized hydrophobic form at acidic pH, their toxicity to microorganisms is usually high as they may readily infiltrate the lipid membranes of the microorganisms (Penttinen 1995). This phenomenon explains the low activity of our consortium at pH-5. The result suggests that alkaline pH is more conducive in the removal of 4-CP. Similar observations have been reported for degradation of various chlorophenols by bacterial isolates and defined bacterial consortium (Farag et al. 2021; Gallego et al. 2009).

There are reports on the simultaneous presence of heavy metals in pesticide contaminated soil (Tariq et al. 2016). Heavy metals in contaminated soil affect microbial nutrient cycling activity. Therefore, it is desirable to apply heavy metal tolerant bacteria in bioremediation of contaminated soils. Heavy metals at low concentrations stimulate microbial growth and become toxic at high concentrations (Giller et al. 1998). Our consortium showed 4-CP degradation activity in presence of various metals except Cu^{2+}. Silva et al. (2012) reported stimulation of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities, the key enzymes responsible for 4-CP degradation, in presence of Mn^{2+} and Fe^{3+}. The inhibition of microbial activities in Cu^{2+} treated conditions might be due to an increase in the production of reactive oxygen species, a reduction in the level of enzyme activity, and delay carbohydrate metabolism (Kuo and Genthner 1996; Ma et al. 2022).

Microorganisms display extraordinary diversity with respect to the utilization of carbon sources. Decreased degradation activity in glucose amended medium could be due to microorganism's strong preference for simple carbon sources, resulting in a decrease in pH of the culture medium

Table 2 Mapping of identified enzymes on Xenobiotic degradation and metabolism pathways present in KEGG database

Sl. no.	Xenobiotics degradation and metabolism pathways	Number of reaction mapped
1	Benzoate degradation (ko-00362)	50
2	Aminobenzoate degradation (ko-00627)	22
3	Fluorobenzoate degradation (ko-00364)	14
4	Chloroalkane and chloroalkene degradation (ko-00625)	14
5	Chlorocyclohexane and chlorobenzene degradation (ko-00361)	35
6	Toluene degradation (ko-00623)	19
7	Xylene degradation (ko-00622)	27
8	Nitrotoluene degradation (ko-00633)	2
9	Ethylbenzene degradation (ko-00642)	3
10	Styrene degradation (ko-00643)	12
11	Atrazine degradation (ko-00791)	8
12	Caprolactum degradation (ko-00930)	8
13	Dioxin degradation (ko-00621)	11
14	Naphthalene degradation (ko-00626)	7
15	Polycyclic aromatic hydrocarbon degradation (ko-00624)	5
16	Steroid degradation (ko-00984)	5
17	Metabolism of xenobiotics by cytochrome P450 (ko-00980)	25
18	Drug metabolism cytochrome P450 (ko-00982)	8
19	Drug metabolism-other enzymes (ko-00983)	33

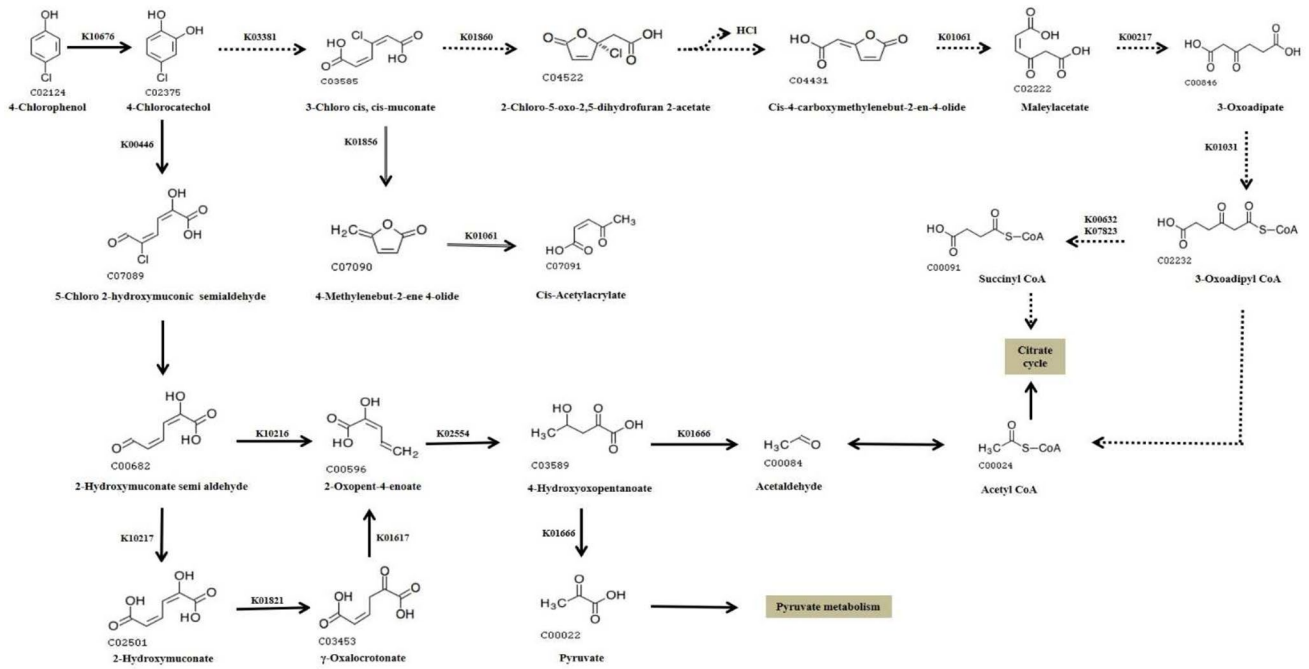

Fig. 6 4-CP degradation pathways constructed by mapping the genes retrieved from whole genome sequencing data in KEGG pathways. Black bold arrows: meta-cleavage pathway, dotted lines: ortho-cleavage pathway

(Fakhruddin and Quilty 2005) and depletion in dissolved oxygen concentration (Wang and Sun 2020a). The high efficiency of carbon sources to stimulate microbial growth and biodegradation of toxic compounds at lower concentrations has been reported (Wang and Sun 2020b; Ziagova et al. 2009).

Table 3 Summary of enzymes associated with 4-CP degradation screened through KEGG database

Sl. no.	Enzyme	EC number	KO id	Gene name	Gene count
1	2,4-Dichlorophenol 6-monooxygenase	1.14.13.20	K10676	tfdB	27
2	Catechol 1,2-dioxygenase	1.13.11.1	K03381	CatA	18
3	Chloromuconate cycloisomerase	5.5.1.7	K01860		24
4	Carboxymethylenebutenolidase	3.1.1.45	K01061		22
5	Muconate cycloisomerase	5.5.1.1	K01856	CatB	55
6	Catechol 2,3-dioxygenase	1.13.11.2	K00446 K07104	dmpB, xylE CatE	11 13
7	Maleylacetate reductase	1.3.1.32	K00217		9
8	3-Oxoadipate CoA-transferase, alpha subunit	2.8.3.6	K01031	pcaI	13
9	3-Oxoadipate CoA-transferase, beta subunit	2.8.3.6	K01032	pcaJ	10
10	Acetyl-CoA acyltransferase	2.3.1.16	K00632	fadA, fadI	62
11	3-Oxoadipyl-CoA thiolase	2.3.1.174	K07823	pcaF	4
12	2-Hydroxymuconate 6-semialdehyde dehydrogenase	1.2.1.85	K10217	dmpC, xylG, praB	13
13	4-Oxalocrotonate tautomerase	5.3.2.6	K01821	praC, xylH	3
14	4-Oxalocrotonate decarboxylase	4.1.1.77	K01617	dmpH, xylI, nahK	13
15	2-Hydroxymuconate semialdehyde hydrolase	3.7.1.9	K10216	dmpD, xylF	2
16	2-Keto-4-pentenoate hydratase	4.2.1.80	K02554	mhpD	3
17	4-Hydroxy 2-oxovalerate aldolase	4.1.3.39	K01666	mhpE	21
18	Acetaldehyde dehydrogenase	1.2.1.10	K04073	mhpF	5

Chlorocatechols form a key intermediate in aerobic degradation of chlorinated aromatic compounds. The cleavage of the catechol ring occurs via two pathways (1) ortho-cleavage (intradiol) catalyzed by catechol 1,2-dioxygenase and (2) meta-cleavage (extradiol) catalyzed by catechol 2,3-dioxygenase (Farell and Quilty 1999). The bacterium *Rhodococcus opacus* 6a was found to have ortho-cleavage pathway (Konovalova et al. 2009). A microbial consortium comprising of *Pseudomonas* and *Actinomycetes* and a fungus *Trichoderma harzamanium* showed 4-CP degradation via a meta-cleavage pathway (Farrell and Quilty 1999). Presence of enzyme activity of both ortho- and meta-cleavage pathways for 4-CP degradation underscores the high utility of consortium for bioremediation of organic pollutants.

The bacterial consortium developed in presence of xenobiotic exhibits high xenobiotic metabolizing capacity. Metagenomic analysis of such consortium provides direct and in-depth knowledge of relative abundance and metabolic roles of bacterial groups in the metabolism of the xenobiotic. The WGS analysis of our consortium revealed a high abundance of *Proteobacteria* (73.27%) and *Bacteroidota* (10%). Several studies using 16S rRNA metagenomic approach reported a similar composition of 4-CP degrading bacterial community at the phylum level (Zhao et al. 2018). Gómez-Acata et al. (2018) reported the dominance of *Proteobacteria* followed by *Actinomycetota* and *Bacillota* in 4-CP treated SBR inoculated with wastewater sludge from an aerobic wastewater plant. Moreno-Andrade et al. (2020) observed increases in the abundance of *Proteobacteria* and *Bacteroidota* in 4-CP degrading acclimated granules of SBR with a tremendous increase in *Bacteroidota* members from <1% to 40.6%. The dominant genera *Paracoccus*, *Achromobacter*, *Hydrogenophage*, *Rhizobium*, *Leadbetterella*, *Brevundimonas*, *Acidovorax*, *Mesorhizobium*, *Shinella*, *Aquamicrobium*, *Sphingobacterium* and *Thermomonas* have been found coexisting in acclimated granules of SBR degrading 4-CP (Moreno-Andrade et al. 2020). The high abundance of the genus *Paracoccus* in 4-CP fed SBR (Gómez-Acata et al. 2018), and synergistic contribution of *Paracoccus* and *Achromobacter* in degradation of xenobiotics has been reported (Gao et al. 2021). An increase in relative abundance of genus *Leadbetterella* in presence of phenolic compounds was observed by Gómez-Acata et al. (2017). The bacterium *Achromatobacter denitrificans* has been reported in degradation of various xenobiotics (Benjamin et al. 2016; Mawad et al. 2016), thus highlighting the potential of our consortium in bioremediation.

WGS of industrial discharges have provided an in depth understanding of the microbial xenobiotic degradation activity (Pandit et al. 2021; Shah et al. 2013). However, the number of mapped reactions of xenobiotic degradation pathways can vary depending on the sources of microbial samples. In our study, the total number of mapped reactions in degradation of xenobiotics e.g., benzoate, chlorocyclohexane and chlorobenzene, styrene, metabolism of xenobiotics by cytochrome P450, and atrazine respectively were 50, 35, 12, 25, and 8. However, in a study reported by Shah et al. (2013), these values were 24, 5, 1, 16, and 2 respectively. It

is important to note that our consortium was raised in 4-CP amended BHM whereas Shah et al. (2013) used a contaminated environmental soil sample. The presence of a large number of enzymes of xenobiotic degradation (Table 2) mapped in KEGG database reflects the high potential of our consortium in bioremediation of a wide range of xenobiotics including 4-CP.

Conclusion

The enriched bacterial consortium efficiently degraded 4-CP in the presence of heavy metals, carbon sources, at pH 7-9, and temperatures from 15 °C-35 °C. WGS analysis revealed the dominance of phyla *Proteobacteria* and *Bacteroidota*, the bacterial genera *Paracoccus, Achromobacter, Hydrogenophage, Rhizobium, Leadbetterella*, *Brevundimonas, Acidovorax, Mesorhizobium, Shinella, Aquamicrobium*, and the consortium's capacity to devour xenobiotic compounds.

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Author contributions AKS, LK, and NAS designed the study. LK performed the detailed experiments with help from JN. LKand WJL performed bioinformatic analysis. AKS and LK prepared the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors declare that they have no conflicting interests.

Ethical approval Not applicable.

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