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RESEARCH ARTICLE

Synergistic PAH biodegradation by a mixed bacterial consortium: based on a multi-substrate enrichment approach

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Abstract

Polycyclic aromatic hydrocarbon (PAH) contamination in the environment involves multiple PAHs and various intermediates produced during the microbial metabolic process. A multi-substrate enrichment approach was proposed to develop a mixed bacterial community (MBC) from the activated sludge of a coking wastewater plant. The degradation performance of MBC was evaluated under different initial concentrations of PAHs (25–200 mg/L), temperature (20–35 °C), pH (5.0–9.0), salinity (0–10 g/L NaCl), and coexisting substrates (catechol, salicylic acid, and phthalic acid). The results showed that the degradation rates of phenanthrene and pyrene in all treatments were up to (99 ± 0.71)% and (99 ± 0.90)% after incubation of 5 days, respectively, indicating excellent biodegradation ability of PAHs by MBC. Furthermore, 16S rRNA gene amplicon sequencing analysis revealed that *Pseudomonas* was dominant, while *Burkholderia* had the largest proportion in acidic (pH = 5.0) and saline (10 g/L NaCl) environments. However, the proportion of dominant bacteria in MBC was markedly affected by intermediate metabolites. It was shown that MBC had a higher degradation rate of PAHs in the coexisting matrix due to the timely clearance of intermediates reducing the metabolic burden. Overall, our study provided valuable information to help design an effective strategy for the bioremediation of PAHs in complex environments.

Keywords Mixed bacterial community · Multi-substrate enrichment approach · Biodegradation · Intermediate metabolites · Metabolic burden · Polycyclic aromatic hydrocarbons

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds comprising two or more aromatic rings with different structural configurations (Nzila 2018). As a typical persistent organic pollutant, PAHs are widely present in environmental media, such as soil, sediments, and water bodies, because of natural and human activities, including wildfires, volcanic explosions, oil refining, and petrochemical industries (Zhang et al. 2022). Given their high toxicity, resistance to biodegradation, and high bioaccumulation, the USEPA has listed 16 PAHs, namely naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k] fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd] pyrene, dibenz[a,h] anthracene, and benzo[g,h,i] perylene as priority PAHs (Gou et al. 2020).

Generally, PAH contamination involves multiple PAHs and various intermediates, such as catechol (CA), salicylic acid (SA), and phthalic acid (PA), produced during the microbial metabolic process (Patel et al. 2019). Despite the high degradability of PAHs, the consortium may lack the ability to efficiently degrade the metabolites produced during biodegradation of PAHs; the accumulation of metabolites can inhibit the biodegradation process (Meng et al. 2014). The opening up of PAHs is the hardest link in the biodegradation process, and it is also a key factor that limits the mineralization effect of pollutants. The intermediate metabolites that are produced as a result can induce some

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enzyme activities (e.g., catechol 1,2-dioxygenase, catechol 2,3-dioxygenase) of microbiota, which are used as the primary energy and carbon source for the initial microbiota. This reduces the adaptation time of microbiota to the provided carbon source and accelerates their growth (Gou et al. 2020).

A mixed bacterial consortium was constructed for bioprocessing using "top-down" or "bottom-up" methods (Gao et al. 2020). The "bottom-up" approach focused on identifying microbial interaction patterns and utilizing this information to understand microbial communities that combined two or more isolated and characterized strains as inoculums to degrade pollutants. The microbial consortia generally lack stability and metabolic diversity (Iwabuchi et al. 2002). The function drive approach was described as a "top-down" approach, in which the design of the community was based on the overall function, stability, and performance needs that were prioritized (Gao et al. 2020). The synthetic community can lead to unforeseen and undesired interactions, adversely affecting community performance and functionality. Considering that many different interactions (e.g., co-metabolism, inhibition, and cross induction) have been identified among PAHs and their metabolites, it is significant to learn from "top-down" and "bottom-up" approaches to develop a new mixed bacterial consortium with high PAH-degrading ability that can remove intermediates in time.

In this study, a mixed bacterial community (MBC) was developed from the activated sludge of a coking wastewater treatment plant in Huayu Gas (Xuzhou, China). This was achieved through a multi-substrate enrichment approach, in which CA, SA, and PA were selected as intermediate metabolites of PAH degradation pathways; moreover, phenanthrene and pyrene were the target contaminants. The degradation of phenanthrene and pyrene by MBC and its microbial community structure under varying environmental conditions (pH, temperature, and salinity) was investigated in intermediate metabolites of PAH degradation pathways (CA, SA, and PA) after biodegradation.

Materials and methods

Culture media and chemicals

Phenanthrene (97%), pyrene (97%), SA (99%), PA (99%), and CA (99%) were purchased from Macklin Biochemical (Shanghai, China). HPLC-grade acetone and methanol were procured from Aladdin Industrial Corporation (Shanghai, China). Mineral salt medium (MSM) consisting of 2.0 $g \cdot L^{-1}$ NH_4Cl, 2.5 $g \cdot L^{-1}$ KH_2PO_4, 0.5 $g \cdot L^{-1}$ K_2HPO_4, 1.0 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 120 $mg \cdot L^{-1}$ $FeCl_3$, 50 $mg \cdot L^{-1}$ H_3BO_3, 10 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 10 $mg \cdot L^{-1}$ KI, 45 $mg \cdot L^{-1}$ $MnSO_4 \cdot H_2O$, 20 $mg \cdot L^{-1}$ $NaMoO_4 \cdot 2H_2O$, 75 $mg \cdot L^{-1}$ $ZnCl_2 \cdot 4H_2O$, 50 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, 20 $mg \cdot L^{-1}$ $AlK(SO_4)_2 \cdot 12H_2O$, 13.25 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, and 10 $mg \cdot L^{-1}$ NaCl purchased from Nanjing Chemical Reagent (Nanjing, China). Stock solutions of a mixture of phenanthrene and pyrene (each 50 mg/L) were prepared in acetone.

Sampling and multi-substrate enrichment process

Activated sludge, the microbial source, was collected from a Huayu Gas coking wastewater treatment plant in Xuzhou, Jiangsu Province, China. The wastewater contained a variety of PAHs; thus, it was assumed that PAH-degrading microbiota were present in the activated sludge.

The acclimation process of the mixed PAH-degrading bacterial community was based on a multi-substrate enrichment approach, as illustrated in Fig. 1. The specific operation was as follows: Activated sludge suspension was diluted with saline (v/v, 1:2) and aerated in a plastic bucket for 7 days at 25 °C. Acclimation began with the addition of intermediate metabolites of PAH degradation pathways as primary carbon sources. CA, SA, and PA were selected as common intermediate metabolites of PAH degradation pathways (Patel et al. 2019). The supernatant (5 mL) was added to an Erlenmeyer flask (250 mL) provided with 45 mL sterilized MSM, 200 mg/L CA, and 50 mg/L of phenanthrene and pyrene (each 25 mg/L) and incubated for 7 days (25 °C, 150 rpm). Successive transfers were performed with a gradual increase in the concentration (200 mg/L with each transfer after 7 days of incubation) of CA from 200 to 1000 mg/L. Similar acclimation was repeated with the addition of SA and PA. After acclimation to the addition of intermediate metabolites of PAH degradation pathways, 5 mL of bacterial suspension (15 mL in total) was transferred to 45 mL of sterilized MSM with 100 mg/L phenanthrene and pyrene (each 50 mg/L) as the sole carbon and energy source. With successive transfers, the concentration of phenanthrene and pyrene increased from 100 to 400 mg/L (50 mg/L with each transfer after 7 days of incubation), and a mixed PAH-degrading bacterial community was obtained (designated as "MBC"). The consortium was stored in 25% glycerin at − 80 °C after centrifugation for long-term preservation (Yang et al. 2020).

Effects of initial concentration on PAH degradation

After evaporation of acetone in sterilized 50-mL flasks containing different volumes of stock phenanthrene and pyrene solution (5000 mg/L), the degradation experiments were carried out in flasks containing 9 mL MSM and 1 mL inoculum ($OD_{600} = 1$, measured with a visible light spectrophotometer, 722), provided with different initial concentrations of PAHs (25 °C, 150 rpm): (1) 25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L of phenanthrene and pyrene separately; (2)

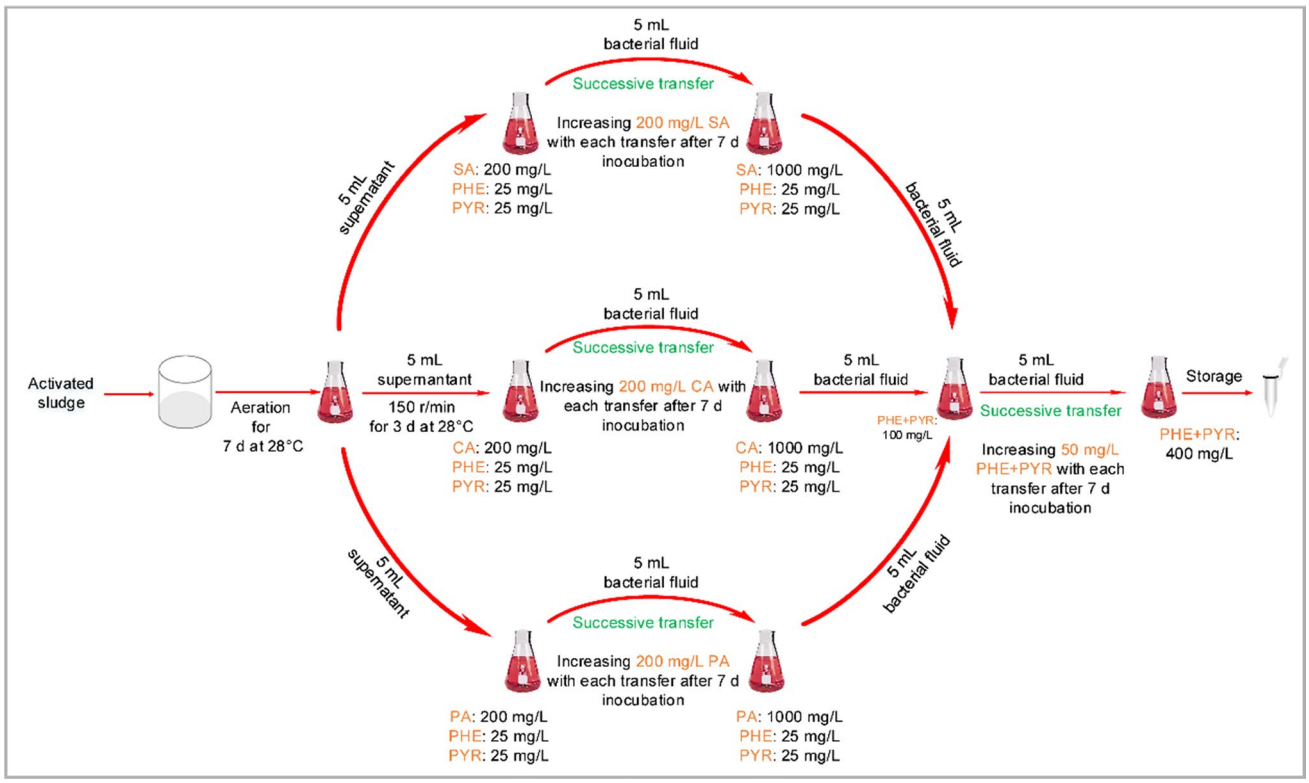

Fig. 1 Schematic illustration of the multi-substrate enrichment process. PHE = phenanthrene; PYR = pyrene; CA = catechol; SA = salicylic acid; PA = phthalic acid

25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L of phenanthrene and pyrene mixture. For inoculum preparation, pregrown MBC was centrifuged at 6000 rpm for 6 min at 4 °C, washed with fresh MSM, and then resuspended in fresh MSM. The initial pH of the degradation system was adjusted to 7.0 with 1 mmol/L NaOH and HCl solution. Three replicate flasks were collected on day 5 for the degradation of phenanthrene and pyrene. Uninoculated flasks containing only MSM supplemented with PAHs on day 5 were used as controls.

Effects of environmental factors on PAH degradation

To determine the effects of environmental factors on the degradation of 100 mg/L of phenanthrene and pyrene (50 mg/L each) by MBC, experiments were performed at different temperatures (20 °C, 25 °C, 28 °C, 30 °C, and 35 °C), pH values (5.0, 6.0, 7.0, 8.0, and 9.0), and salinities (0 g/L, 1 g/L, 3 g/L, 5 g/L, and 10 g/L NaCl). The inoculation was similar to that described in the "Effects of initial concentration on PAH degradation" section. The pH of MSM was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 using 1 mmol/L NaOH or HCl solution; the initial pH of the degradation system was adjusted to 7.0. Three replicate flasks were collected on days 1, 3, and 5 for the degradation of phenanthrene and pyrene. On day 5, another flask was collected for DNA extraction at each temperature, pH, and salinity condition. Uninoculated flasks containing only MSM supplemented with PAHs on days 1, 3, and 5were used as the controls.

Effects of intermediate metabolites on PAH degradation

To investigate the effects of intermediate metabolites on the degradation of 100 mg/L of phenanthrene and pyrene (50 mg/L each) by MBC, experiments were performed with the addition of 0.05% (w/v) of CA, PA, and SA, respectively. The inoculation and incubation conditions were similar to those described in "Effects of initial concentration on PAH degradation" section. Phenanthrene and pyrene degradation were monitored on days 1, 3, and 5 of incubation. The flasks without intermediates were kept under similar conditions to serve as controls. Samples from each treatment group under the same conditions were used to determine the OD_{600} of the bacterial consortia. All experimental groups were set up in triplicates. On day 5, another flask was collected from each group for DNA extraction.

DNA extraction and Illumina MiSeq sequencing

According to the manufacturer's protocol, total genomic DNA was extracted from the original activated sludge and the consortium using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). All DNA samples were quality checked, and the concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Bacterial 16S rRNA gene fragments (V3-V4) were amplified from the extracted DNA using the primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Zeng & An 2021). The PCR conditions were as follows: 3 min of denaturation at 95 °C, 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C for 27 cycles, and a final extension at 72 °C for 10 min (Laothamteep et al. 2021). PCRs were performed with 4 μL of 5 × *TransStart* FastPfu buffer, 2 μL of 2.5 mM deoxynucleoside triphosphates (dNTPs), 0.8 μL of each primer (5 μM), 0.4 μL of *TransStart* FastPfu DNA Polymerase, and 10 ng of extracted DNA. Additionally, ddH_2O was used to make up to 20 μL. Agarose gel electrophoresis was performed to verify the amplicon size. Amplicons were subjected to paired-end sequencing on the Illumina MiSeq sequencing platform using the PE300 chemical at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Amplicon sequence processing and analysis

After demultiplexing, the resulting sequences were merged with FLASH (v1.2.11) (Magoc & Salzberg 2011) and quality-filtered with fastp (0.19.6) (Chen et al. 2018). The high-quality sequences were de-noised using the DADA2 (Callahan et al. 2016) plugin in the Qiime2 (Bolyen et al. 2019) (version 2020.2) pipeline with recommended parameters, which resulted in single-nucleotide resolution based on error profiles within samples. DADA2 de-noised sequences are called amplicon sequence variants (ASVs). To minimize the effects of sequencing depth on alpha and beta diversity measures, the number of sequences from each sample was reduced to 4000, which still yielded an average Good's coverage of 97.90%. Taxonomic assignment of ASVs was performed using the Naive Bayes consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S rRNA database (v138). Analyses of the 16S rRNA microbiome sequencing data were performed using the free online Majorbio Cloud Platform (www.majorbio.com).

Determination of PAHs

The entire content of the flasks (10 mL) was added to 20 mL of methanol using ultrasonic dissolution promotion to extract residual phenanthrene and pyrene (Gu et al. 2015). After filtration through a 0.22-μm polytetrafluoroethylene membrane, the residual phenanthrene and pyrene in the mixed solution were determined using a Flexar Quaternary LC Pump Platform (Flexar LC, PerkinElmer, Singapore) fitted with a C18 column (Brownlee C18, 5 μm, 150 × 4.6, PerkinElmer) and a UV/Vis detector (PerkinElmer). Phenanthrene and pyrene were eluted using a mobile phase (methanol:water, 80:20) at a 1 mL/min flow rate and monitored at 254 nm. The column temperature was 30 °C, and the injection volume was 50 μL. Chromatograms were recorded and integrated with system software (Chromera 2.1, PerkinElmer). The average recovery rates of phenanthrene and pyrene were (100 ± 0.23)% and (84 ± 0.05)%.

Statistical analysis

All experiments were carried out in triplicate. The values of the degradation ratio were calculated as the mean ± standard deviation (SD), which were represented in the error bar to show variation within the same experiments. Statistical analyses were performed using the Origin 2021 software program (OriginLab Corporation, USA).

Results and discussion

Dynamics of the bacterial community during the multi-substrate enrichment process

The bacterial communities in the activated sludge and consortium during the multi-substrate enrichment process were characterized using high-throughput sequencing of the 16S rRNA gene amplicons, as shown in Fig. S1. The species richness and diversity indices were calculated (Table S1). The Shannon and Simpson indices revealed that the bacterial community of each sample varied, and the bacterial diversity decreased as multi-substrate enrichment proceeded. Proteobacteria (91.6–98.8%) was the most abundant in all the samples, followed by Actinobacteria (0.9–5.6%). Previous studies have also implicated a higher relative abundance of Proteobacteria and Actinobacteria and a positive correlation with PAH degradation in PAH-contaminated sites (Lee et al. 2018; Liu et al. 2022).

In addition, the species richness of the top 15 dominant genera in all samples during the multi-substrate enrichment process was reflected using a heat map (Fig. 2). The dominant genus in the original sample of AS was *Pseudomonas*. *Ralstonia*, *Delftia*, and *Achromobacter* were dominant in CA, SA, and PA samples, respectively. It has been established that *Ralstonia* (Oie et al., 2007) and *Achromobacter* (Chi et al., 2021) have the ability to degrade CA and PA, respectively; however, *Delftia* has not been associated with the biodegradation of SA. Other genera that contributed to PAH biodegradation were *Stenotrophomonas* (Zang et al.

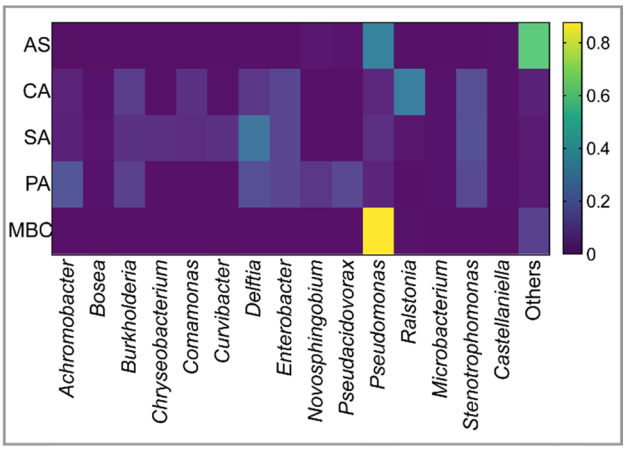

Fig. 2 Heat map analysis of the 15 dominant genera of bacterial microbiota during the multi-substrate enrichment process. AS: activated sludge; CA: the consortium domesticated by catechol; SA: the consortium domesticated by salicylic acid; PA: the consortium domesticated by phthalic acid; MBC: the consortium after the multi-substrate enrichment process

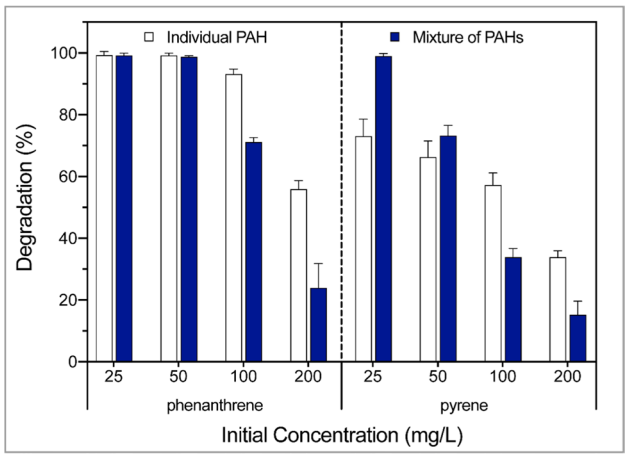

Fig. 3 Biodegradation of PAHs (phenanthrene and pyrene) when they were provided as individuals and as a mixture of PAHs under initial concentrations (25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L) by consortium MBC after incubation of 5 days (28 °C, 150 rpm). The degradation was calculated in percentage, and error bars represented the standard deviation of triplicate independent measurements

2021), *Pseudacidovorax* (Dealtry et al. 2018), *Novosphingobium* (Fida et al. 2017), *Enterobacter* (Lors et al., 2012), *Comamonas* (Qin et al. 2019), *Chryseobacterium* (Xiao et al. 2019), and *Burkholderia* (Morya et al. 2020). After acclimation with phenanthrene and pyrene, the dominant genus in the MBC sample was *Pseudomonas.*

Biodegradation of individual PAHs vs. a mixture of PAHs under different initial concentrations

Figure 3 illustrates phenanthrene and pyrene degradation by MBC, individually and as a mixture, at different initial concentrations (25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L). When the initial concentrations of phenanthrene were 25 and 50 mg/L, the degradation ratio of phenanthrene was almost 100.0%, individually and when mixed with pyrene. When the initial concentrations of pyrene were 25 and 50 mg/L, the degradation rates of pyrene were 99.0% and 73.3%, respectively, when provided in the mixture, and were 73.1% and 66.3% when supplemented as individual PAHs. When the initial concentrations of both phenanthrene and pyrene were 100 mg/L and 200 mg/L, the degradation rate of the individual PAHs was higher than a mixture of PAHs. Li et al. (2021) found that in a phenanthrene (500 mg/L)-pyrene (10 mg/L) mixture system, the biodegradation efficiency of pyrene increased from 17.8% (in a single substrate system) to 96.2% after 7 days because phenanthrene served as a co-metabolic substrate to significantly improve pyrene biodegradation. Competitive inhibition and increased toxicity levels were revealed at higher initial concentrations of mixed PAHs (Yuan et al. 2018). In addition, phenanthrene was quickly degraded, followed by pyrene, at the above four initial concentrations. This was consistent with previous studies, in which the degradation ratios generally increased with decreasing molecular weight (Geng et al. 2022).

Effect of environmental factors on PAH degradation

To evaluate the potential degradation capacity of MBC for mixed PAHs, a range of degradation tests were conducted at various temperatures, pH values, and salinities. Figure 4A reveals that MBC exhibited good biodegradation efficiency between 20 and 35 °C on day 5. However, there was no impact on pyrene degradation between 25 and 35 °C. In comparison, the degradation rates of phenanthrene and pyrene at 20 °C were lower for the first 3 days, possibly due to lower PAH solubility and bacterial metabolic activity (He et al. 2022). Typically, increasing the medium temperature increases the solubility of PAHs, thereby increasing their bioavailability and mass transfer into cells (Kumar et al. 2021). Moreover, microbial activities increased with increasing temperature in the appropriate range due to the enhancement of enzymatic activity and microbial metabolism, which facilitated the degradation efficiency of PAHs (Liu et al. 2017). Although the enrichment of MBC was carried out at 25 °C, the results of this study showed that it could adapt well to temperature changes.

The consortium also maintained high degradation activity over a wide range of pH values (5.0–9.0). Most heterotrophic bacteria prefer neutral to alkaline pH for their metabolic activity. According to Govarthanan et al. (2020), PAH biodegradation efficiency can be maximized at pH 7.0 because a neutral to nearly alkaline pH environment is suitable for

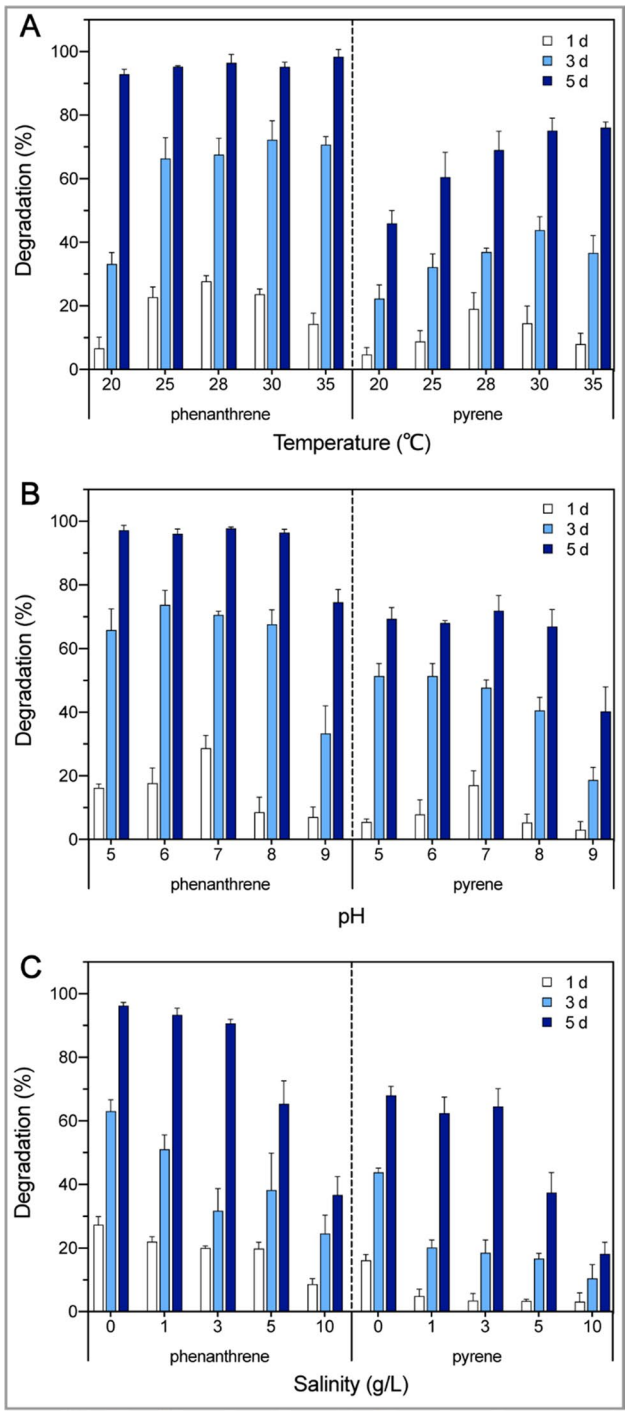

Fig. 4 Biodegradation of phenanthrene and pyrene as a mixture by consortium MBC after incubation of 1, 3, and 5 days under different temperatures (**A**), pH values (**B**), and salinities (**C**). The initial concentration of phenanthrene and pyrene was 50 mg/L

the carboxylation reaction. On day 1, the degradation rates of phenanthrene and pyrene were the highest at pH 7.0. No significant differences in phenanthrene and pyrene degradation were observed at pH values ranging from 5.0 to 8.0 on days 3 and 5. The degradation rate of phenanthrene was the lowest at pH 9.0 and remained > 70% on day 5, suggesting the practical implementation of MBC on a larger scale (Fig. 4B). Our results were consistent with previous reports showing that PAH-degrading bacteria preferred neutral pH for PAH degradation (Vaidya et al. 2017).

The effect of salinity on PAH biodegradation is presented in Fig. 4C. After 5 days of incubation, MBC degraded > 90% of phenanthrene and > 60% of pyrene at NaCl concentrations of 0–3 g/L. When the NaCl concentration was greater than 3 g/L, the degradation rates of phenanthrene and pyrene began to decrease. When the NaCl concentration reached 10 g/L, the degradation rates of phenanthrene and pyrene decreased considerably to < 40% and < 20%, respectively. The prediction of potential functions indicated that high salinity could disrupt the co-metabolism between carbohydrate metabolism and PAH degradation (Shi et al. 2021). These results suggested that MBC had the potential for PAH bioremediation in saline environments.

Overall, the results showed that MBC exhibited excellent PAH degradation performance over a wide range of pH values, temperatures, and salinities, suggesting good bioremediation potential for various contaminated sites.

Effect of environmental factors on the community structure of the consortium MBC during mixed-PAH degradation

At different temperatures, the dominant genus in all treatments after mixed-PAH degradation was *Pseudomonas*; no obvious effect of temperature on bacterial communities in consortium MBC was observed (Fig. 5A). *Pseudomonas* is a well-known degrader of PAHs and has been widely applied in PAH remediation (Rabodonirina et al. 2019). Liu et al. (2021) isolated a newly isolated *Pseudomonas brassicacearum* strain, MPDS, and found that it could effectively degrade PAHs and heterocyclic derivatives, including naphthalene, fluorene, dibenzofuran, and dibenzothiophene.

Under different pH conditions, the bacterial composition in all treatments after mixed-PAH degradation was dominated by *Pseudomonas*, *Burkholderia*, *Chryseobacterium*, and *Stenotrophomonas* (Fig. 5B). Li et al. (2021) enriched a novel microbial consortium, QY1, in which *Methylobacterium*, *Burkholderia*, and *Stenotrophomonas* were the dominant genera. It was revealed that QY1 degraded 94.5% of 500 mg/L phenanthrene and 17.8% of 10 mg/L pyrene after 7 days. The proportion of *Burkholderia* remarkably increased at pH 5.0, 6.0, and 9.0, which may be due to its adaptability to a wide range of acidic and alkaline conditions (Morya et al. 2020). Garrido-Sanz et al. (2019) isolated a bacterial consortium dominated by *Pseudomonas*, *Aquabacterium*, *Chryseobacterium*,

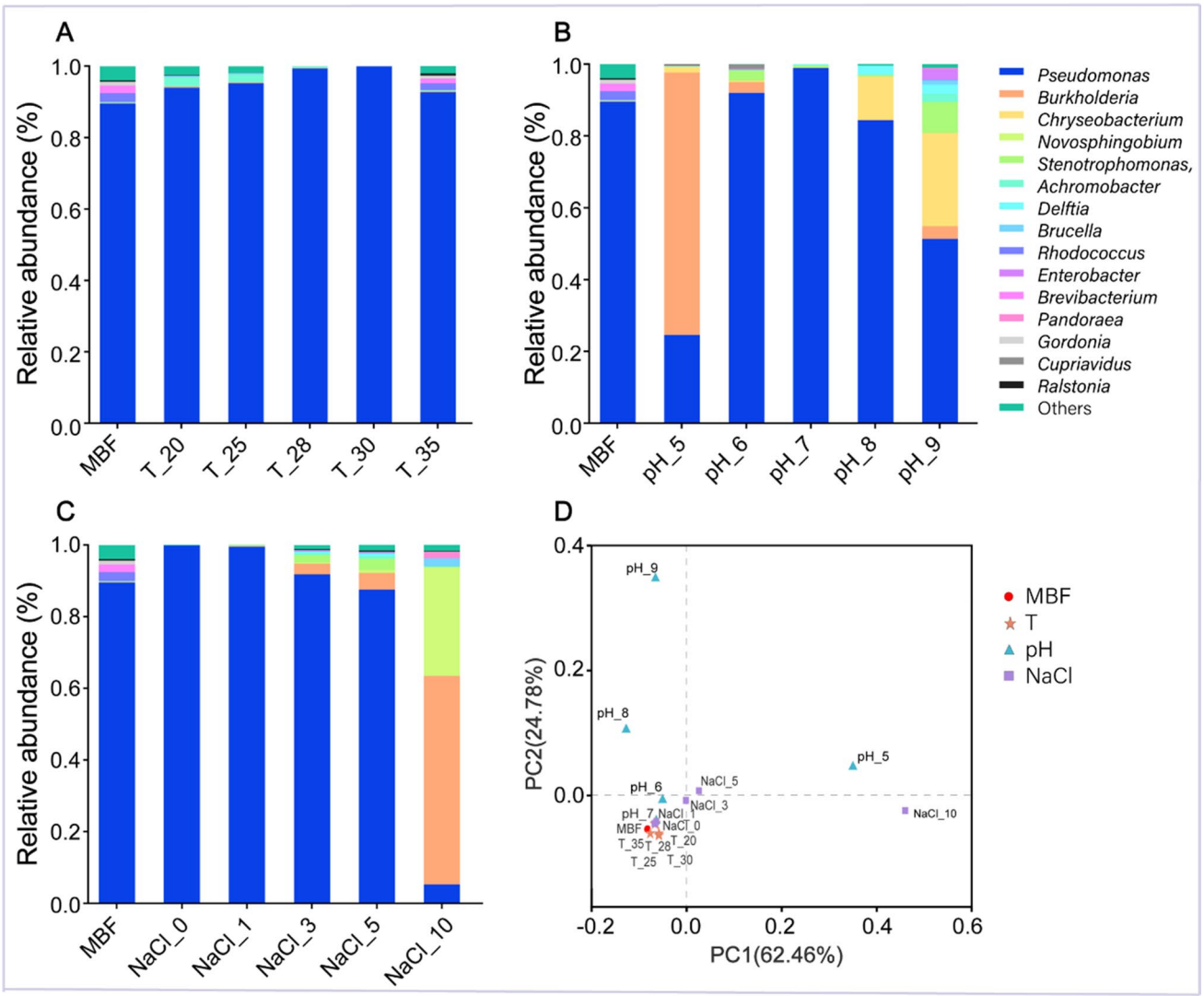

Fig. 5 Bacterial compositions at genus level under different temperatures (**A**), pH values (**B**), salinities (**C**), and principal coordinates analysis (PCoA) based on weighted UniFrac distance (**D**). T_20, T_25, T_28, T_30, T_35: The temperature of the solution was 20, 25, 28, 30, and 35 °C respectively; pH_5, pH_6, pH_7, pH_8, pH_9: The pH of the solution was 5.0, 6.0, 7.0, 8.0, and 9.0 respectively; NaCl_0, NaCl_1, NaCl_3, NaCl_5, NaCl_10: The concentration of NaCl was 0, 1, 3, 5, and 10 g/L; MBC: the consortium after the multi-substrate enrichment process

and *Sphingomonadaceae* that could grow using diesel as well as different alkanes and PAHs as the sole carbon and energy sources.

The bacterial communities in the MBC after mixed-PAH biodegradation were affected by the addition of NaCl, as shown in Fig. 5C. The proportions of *Burkholderia* and *Novosphingobium* markedly increased with increasing NaCl concentration. It has been reported that 0.5–3% NaCl is optimal for the growth of some *Burkholderia* strains and phenanthrene removal (Liu et al. 2019). Birch et al. (2022) prepared 294 parallel test systems using wastewater treatment plant effluent as inoculum. Passive dosing was used to add a mixture of 19 chemicals at 6 initial concentrations (ng/L to mg/L). Growth of *Novosphingobium* was observed at the highest test concentration (17 mg C/L added).

Additionally, principal coordinate analysis (PCoA), based on weighted UniFrac dissimilarity, was used to compare bacterial diversity among all samples (Fig. 5D). Approximately 87.2% of the bacterial community variance could be explained by the first two principal components. The initial communities (MBC) and the communities in the temperature [the temperature was 20 (T_20), 25 (T_25), 28 (T_28), 30 (T_30), and 35 °C (T_35)], pH (pH = 7.0, pH = 6.0), and salinity treatments [the concentration of NaCl was 5 g/L (NaCl_5) and 10 g/L (NaCl_10)] were clustered in the left bottom quadrant. In addition, the communities in the

temperature treatment showed the least variation with temperature. Among the pH treatments, the communities in the acid group (pH = 5.0) were clearly distinguished from the initial communities (MBC) along the first principal coordinate. In contrast, a separation between the alkaline group (pH = 8.0, pH = 9.0) and MBC was observed along the second principal coordinate. The NaCl_10 treatment exhibited the greatest variation in community composition among all salinity treatments. The above results demonstrated that the MBC community composition responded to changes in environmental conditions. It is worth noting that > 90% of phenanthrene and > 65% of pyrene were removed at pH 5.0, indicating that MBC was able to obtain good biodegradation of PAHs under a wide range of environmental conditions by changing the interactions among members of MBC.

Effect of intermediate metabolites on mixed-PAH degradation by consortium MBC

Figure 6 depicts the effect of the chosen intermediate metabolites on phenanthrene and pyrene degradation by MBC. After 5 days of incubation, the degradation rates of phenanthrene and pyrene in the presence of CA, PA, and SA decreased from 93.8% (2.85 mg/L) to 64.6% (16.28 mg/L), 91.7% (3.82 mg/L), and 89.6% (4.77 mg/L), and from 72.2% (11.54 mg/L) to 53.5% (19.30 mg/L), 68.1% (13.24 mg/L), and 71.8% (11.7 mg/L), respectively. It is worth noting that the degradation of PAHs in the presence of CA was at a minimum on day 3 and maximum on day 5, as compared with that in the presence of SA and PA. The initial degradation rates of phenanthrene and pyrene decreased significantly but

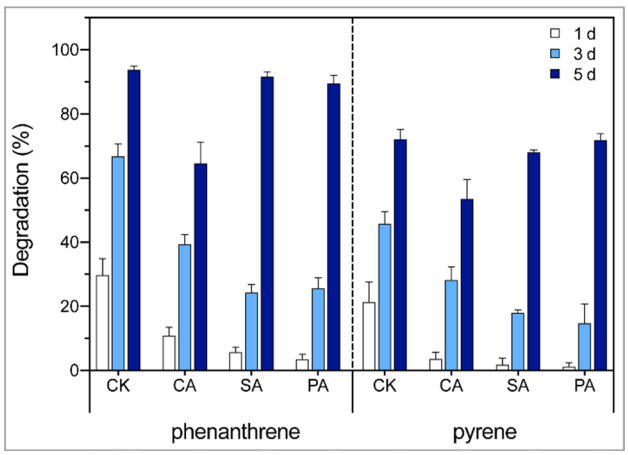

Fig. 6 Biodegradation of a mixture of PAHs (phenanthrene and pyrene) in the presence of intermediate metabolites by consortium MBC after incubation of 1, 3, and 5 days (28 °C, 150 rpm). The degradation was calculated in percentage, and error bars represented the standard deviation of triplicate independent measurements. CK: control check; CA: coexistence of PAHs and catechol; SA: coexistence of PAHs and salicylic acid; PA: coexistence of PAHs and phthalic acid

became less distinct after day 5 in the presence of SA and PA. This may be because the intermediate metabolites of PAH degradation pathways were preferentially utilized as carbon sources due to their simpler structure, resulting in the reduction of phenanthrene and pyrene biodegradation (Patel et al. 2019). In addition, under multi-component contaminated substrate conditions, some key enzymes induced by easily degradable contaminants promoted the simultaneous metabolism of refractory contaminants by microorganisms (Gupta et al. 2015). The regulator NahR, activated by an intermediate of PAH biodegradation, upregulated degradation enzymes, which enhanced the biodegradation of phenanthrene (Cao et al. 2021). As a result, the accumulated intermediate metabolites were promptly scavenged by MBC to degrade PAHs in the mixed matrix.

Effect of pathway intermediates on the community structure of the consortium MBC after mixed-PAH degradation

At the genus level, apparent changes in the composition of microbial communities were observed before and after the addition of intermediate metabolites (Fig. 7). The relative abundance of *Pseudomonas* in the CK sample increased after PAH biodegradation, whereas it decreased in the SA, PA, and CA samples. Additionally, the composition of the microbial communities was markedly different among treatments with intermediate metabolites. The dominant bacterial genera in the SA samples were *Delftia* (37.4%), *Curvibacter* (14.7%), *Chryseobacterium* (13.6%), and *Comamonas* (12.7%). The dominant bacterial genera in the PA samples were *Achromobacter* (27.2%), *Pseudacidovorax* (26.6%), *Novosphingobium* (17.3%), *Delftia* (12.3%), and *Burkholderia* (9.1%). In the CA sample, *Ralstonia* (60.1%), *Comamonas* (14.5%), and *Burkholderia* (7.3%) were predominant. *Pseudomonas* and *Stenotrophomonas* were good biosurfactant producers and tolerant to alkaline pH, with the former being a N-fixing, P-solubilizers and the latter being a mild P-solubilizer (Kuppusamy et al. 2016). Cluster analysis was conducted to better understand the microbial response to PAH biodegradation with the addition of intermediate metabolites. The cluster plot showed that the control sample without intermediate metabolites was clustered with MBC. The bacterial communities of the CA and PA samples were closely linked and clustered into one group, whereas the SA sample was not closely linked to either group.

Notably, the degradation rates of PAHs (Fig. 6) and the OD_{600} (Fig. S2) of PA were higher than CA on day 5. The total number of bacteria had a significant effect on the degradation rate of PAHs. Therefore, although the bacterial communities of the CA and PA samples were more closely linked, the bacterial number of CA was less than that of PA, resulting in a lower degradation rate of PAHs.

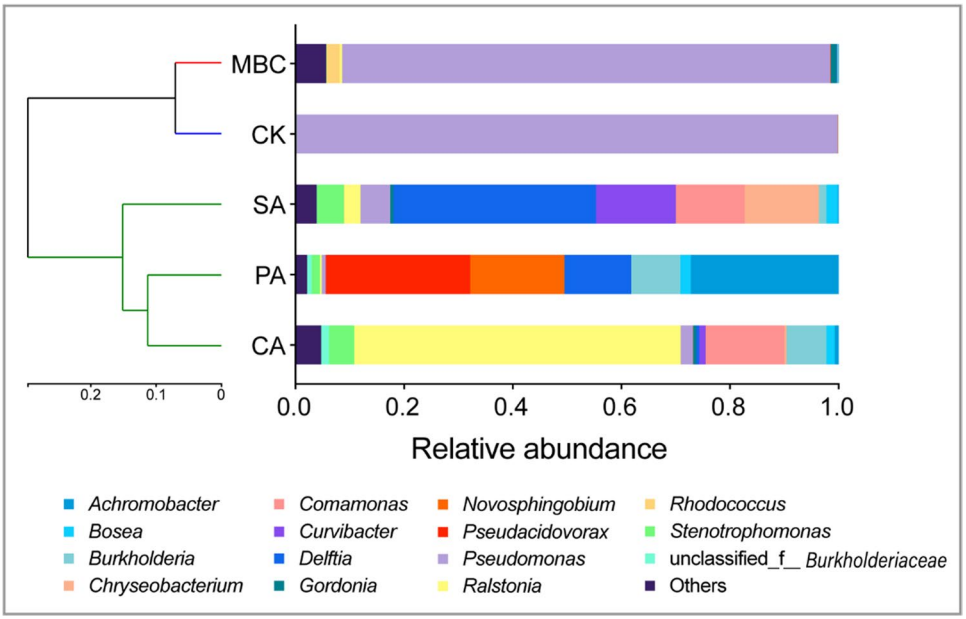

Fig. 7 Bacterial compositions at genus level after biodegradation of phenanthrene and pyrene as a mixture in the presence of intermediate metabolites. MBC: domesticated microbial communities before PAH degradation experiments; CK: control check after PAH degradation experiments; CA: coexistence of PAHs and CA; SA: coexistence of PAHs and salicylic acid; PA: coexistence of PAHs and phthalic acid

Furthermore, despite the different community structures of PA and SA, their inhibitory effects on the degradation rates of phenanthrene and pyrene PAHs were similar (Fig. 6). The microbial community composition after the multi-substrate enrichment process (Fig. 2) and the biodegradation of phenanthrene and pyrene mixture in the presence of intermediate metabolites (Fig. 7) showed that the dominant bacterial genera were similar. The percentage of the dominant genera (*Delftia*, *Achromobacter*, *Ralstonia*) after the addition of intermediate metabolites was less than 10% (Fig. 2). In contrast, the proportion of the three dominant genera increased greatly after the biodegradation of the phenanthrene and pyrene mixture in the presence of intermediate metabolites (Fig. 7). Most identified PAH-degrading bacteria from MGP sites reported in the literature belonged to the genera of *Delftia*, *Achromobacter*, and *Ralstonia* (Chattopadhyay et al. 2022). The draft genome of *Deliftia tsuruhatensis* and *Pseudomonas putida* contains the entire benzoate and near-complete naphthalene and phenanthrene degradation pathways (Ibrar & Yang 2022). Vera et al. (2022) examined microbial community structure in samples from the contaminated sediments and groundwater. The most abundant genera for sediments/microcosms included *Pseudomonas*, *Methylotenera*, *Rhodococcus*, *Stenotrophomonas*, and *Brevundimonas*, and the most abundant for the groundwater/microcosms included *Pseudomonas*, *Cupriavidus*, *Azospira*, *Rhodococcus*, and unclassified *Burkholderiaceae*. It was proved that MBC had the potential to degrade PAHs and their intermediates. The MBC members behaved markedly differently under the stress of different intermediate metabolites. These results revealed that MBC adjusted the proportion of dominant bacteria through the metabolic burden to maintain the PAH degradation performance on multiple substrates.

Conclusion

Multi-substrate enrichment was proposed to develop a bacterial community named MBC from the activated sludge of a coking wastewater plant. MBC degraded mixtures of phenanthrene and pyrene at temperatures of 20–35 °C, pH of 5.0–9.0, salinity of 0–10 g/L NaCl, and in the presence of complex coexisting substrates, such as CA, SA, and PA to reveal excellent tolerance to different environmental stresses. The consortium MBC was dominated by *Pseudomonas* under all environmental conditions after the biodegradation of the mixed PAHs. *Burkholderia* was the major genus in both acidic environments and those with high salt concentrations. Additionally, MBC had a distinct response to the inhibition of intermediate metabolites degraded by PAHs; it adjusted the proportion of dominant bacteria through the metabolic burden. Thus, the findings of this study provide important strategies for practical bioremediation of various PAH-contaminated environments.

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Availability of data and materials The authors declare that data supporting the findings of this study are available within the article.

Declarations

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