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Research Article

Efficient phenanthrene biodegradation by nitrogen-fixing marine diazotrophs isolated from the Musa Estuary

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Abstract

Polycyclic aromatic hydrocarbons are persistent environmental pollutants that pose significant risks to aquatic ecosystems. Microorganisms capable of degrading hydrocarbons under nitrogen-limited conditions may provide an effective and sustainable approach for the bioremediation of contaminated environments. In this study, four putative diazotrophic bacterial isolates were obtained from water and sediment samples of the Musa Estuary (Persian Gulf). The isolates were identified as *Bacillus cereus* strain BP1, *Bacillus subtilis* strain BP2, *Microbacterium* sp. strain BP3, and *Microbacterium trichothecenolyticum* strain BP4, based on morphological, biochemical, and 16S rRNA gene analyses. HPLC quantified phenanthrene biodegradation. All isolates were able to grow in a nitrogen-free medium supplemented with phenanthrene and exhibited high phenanthrene degradation efficiencies, ranging from 85.56% to 93.44%. Among the tested isolates, *Microbacterium* sp. strain BP3 achieved the highest degradation efficiency, whereas *Bacillus subtilis* strain BP2 exhibited the lowest. These findings highlight the potential of indigenous estuarine bacteria adapted to nitrogen-limited conditions for the biodegradation of PAHs and suggest their possible application in the bioremediation of contaminated marine environments.

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Introduction

Microbial biodegradation is widely recognized as one of the most sustainable and cost-effective approaches for the remediation of polycyclic aromatic hydrocarbons (PAHs) in contaminated environments (Watanabe, 2001; Ali *et al.,* 2022; Vijayanand *et al.,* 2023). The efficiency of PAH biodegradation is governed by multiple environmental and biological factors, including microbial community composition, nutrient availability, nitrogen availability, pH, temperature, and oxygen concentration (Parmanath *et al.,* 2021; Fouad *et al.,* 2022). Among these factors, nitrogen availability plays a pivotal role in hydrocarbon biodegradation. In nitrogen-limited environments, insufficient nitrogen restricts microbial growth and metabolic activity, thereby reducing the efficiency of hydrocarbon degradation (Walworth *et al.,* 2007; Wang *et al.,* 2022; Wang *et al.,* 2023). As a result, microorganisms that can survive in nitrogen-deficient environments may have a distinct ecological advantage in degrading hydrocarbons. In this context, diazotrophic (nitrogen-fixing) bacteria have garnered significant attention due to their dual role. Not only do they help break down organic pollutants, but they also biologically fix nitrogen, which enhances its availability in the environment (Omotayo *et al.,* 2017; Aso and Obuekwe, 2024; Lin *et al.,* 2024). These microorganisms are widespread in marine environments and play a crucial role in the nitrogen cycle through biological nitrogen fixation (BNF) (Halbleib and Ludden, 2000; Bellenger *et al.,* 2020; Lindström and Mousavi, 2020; ; Zeng *et al.,* 2024). Due to the significance of diazotrophic bacteria in environments contaminated by hydrocarbons, identifying native strains from polluted marine ecosystems could offer valuable resources for future bioremediation applications.

The Musa Estuary, located in the northwestern Persian Gulf, is one of the largest estuarine systems in Iran and is heavily influenced by industrial and petrochemical activities. Inputs from petrochemical complexes, oil transfer facilities, shipping operations, urban wastewater, and agricultural runoff have contributed to elevated levels of environmental pollution, including PAHs and other contaminants (Keshavarzifard *et al.,* 2020; Saadati *et al.,* 2020; Agah *et al.,* 2023; Madadi *et al.,* 2023; Jourtani *et al.,* 2024). These conditions make this estuarine ecosystem an important site for investigating indigenous microorganisms with potential applications in bioremediation. Despite growing interest in the microbial degradation of PAHs, information on indigenous putative diazotrophic bacteria capable of degrading phenanthrene in the Persian Gulf remains scarce. Therefore, this study aimed to isolate and identify putative diazotrophic bacterial strains from water and sediment samples collected from the Musa Estuary and to evaluate their ability to grow and degrade phenanthrene under nitrogen-limited conditions. The results provide valuable insight into the potential use of native bacterial resources for the bioremediation of PAH-contaminated marine environments.

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Materials and methods

Sample collection

The sampling stations in the Musa Estuary were selected based on the intensity of anthropogenic activities and pollution load. During the winter season, water and sediment samples were collected from three stations within the estuary. Station 1 (Samaili Estuary) is mainly affected by fishing activities. In contrast, Stations 2 (Jafari Estuary) and 3 (Ahmadi Estuary) are located near petrochemical industrial complexes and are exposed to industrial discharges (Fig. 1). Winter sampling was conducted to minimize seasonal fluctuations and provide relatively stable environmental conditions for microbial isolation. Water samples were collected using sterile glass bottles immersed to a depth of 20 cm below the surface. In contrast, Sediment samples were obtained from a depth of approximately 2 m using a Van Veen grab sampler. Each site was sampled in triplicate to ensure reproducibility. All samples were stored in ice boxes and transported to the laboratory for future analyses (Marzuki *et al.,* 2021; Sandhu *et al.,* 2022). At the time of sampling, water temperature ranged from 17.3 to 21.4 °C, salinity from 41 to 44 ppt, and pH from 7.81 to 8.48.

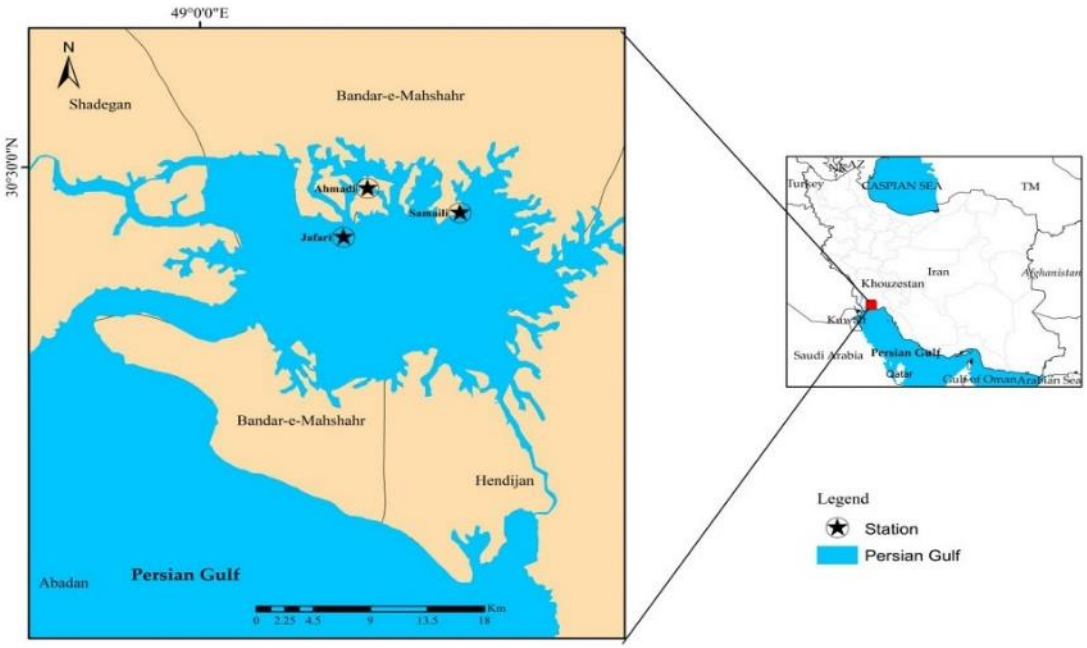

Figure 1: Location of the three sampling stations in Musa Estuary (Persian Gulf, Iran).

Culture medium

Burk's nitrogen-free (N-free) medium was used for the enrichment and isolation of putative nitrogen-fixing bacteria. The medium was prepared according to the method described by Stella and Suhaimi (2010).

Isolation of nitrogen-fixing bacteria

Water and sediment samples were enriched in Burk's N-free liquid medium and subsequently plated onto Burk's N-free agar following procedures Stella and Suhaimi (2010). Colonies growing on the nitrogen-free medium were purified by repeated streaking. To screen nitrogen-

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fixing bacteria, the isolate's growth on Burk's N-free agar was compared with growth on nutrient agar. Isolates that demonstrated sustained growth on the nitrogen-free medium were selected for further analysis. Since acetylene reduction assays (ARA) and molecular detection of nitrogen-fixation genes were not available, growth on nitrogen-free medium was used as a preliminary screening criterion rather than a definitive confirmation of nitrogen-fixing ability. Purified isolates were preserved in 40% (v/v) glycerol at −80°C until further characterization (Sultana *et al.,* 2021). Well-grown and morphologically distinct colonies were further subcultured on Burk's N-free agar for morphological characterization, biochemical tests, and DNA extraction (Augustine and Joseph, 2018; Ayal *et al*., 2024; Purwanti *et al*., 2024).

Phenotypic characterization of bacterial isolates

The morphological characteristics of the bacterial colonies, including color, shape, margin, and elevation, were examined on nutrient agar plates. Gram staining was performed, and representative isolates were chosen for further characterization (Al-Dhabaan, 2019).

Biochemical characterization

Biochemical profiling of the isolates was carried out following the procedures described in Bergey's Manual of Systematic Bacteriology. The tests included the methyl red (MR) and Voges–Proskauer (VP) tests, indole production, motility, Simmons' citrate utilization, the potassium hydroxide (KOH) test, and hydrogen sulfide (H_2S) production (Siddiqui *et al*., 2024).

DNA Extraction and 16S rRNA Gene Analysis

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, as described by Moore *et al.* (1999). PCR amplification of the 16S rRNA gene was performed using universal primers 16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (5′-GGTTACCTTGTTACGACTT-3′) (Wang *et al.,* 2022). Each PCR reaction (25 µL) contained 12.5 µl of Amplicon 2X Master Mix (Amplicon, Denmark), 1 µL of each forward and reverse primer (10 µM), 8.5 µl of double-distilled water, and 2 µL of template DNA. Amplification was performed using a MyCycler (Bio-Rad) thermal cycler to optimize target amplicon yield. Thermal cycling was performed for 30 cycles, including an initial denaturation at 96 °C for 3 min, followed by 30 cycles of denaturation at 96°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min (Kassa *et al.,* 2024). To determine the size of PCR products, the amplicons were subjected to electrophoresis on a 1% (w/v) agarose gel at 90 V for 45 min. A 100 bp DNA ladder was used as a molecular size marker to assess fragment sizes, targeting the 16S rRNA gene with an expected size of ~1500bp. The agarose gel, stained with Safe stain, was visualized under UV light using a Gel Doc system, and DNA bands were observed (Hammoudeh *et al.,* 2024). Purified amplicons were sequenced by Sanger sequencing (Faza Biotech Co., Tehran, Iran) (Huang *et al.,* 2024).

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Sequence quality was checked using BioEdit software, and taxonomic identification was performed using BLAST searches against the NCBI database (Yuan *et al.,* 2014; Schnytzer *et al.,* 2018; Protic *et al.,* 2024). Phylogenetic relationships were inferred using the Neighbor-Joining (NJ) method with 1000 bootstrap replicates in Molecular Evolutionary Genetics Analysis (MEGA) software version 10.0. In this analysis, the *Pseudomonas aeruginosa* strain KSG was used as an outgroup (Brait *et al.,* 2022). The obtained sequences were deposited in GenBank under their respective accession numbers (Zheng *et al.,* 2018).

Microbial growth in the presence of PAHs

The ability of the isolates to grow in the presence of phenanthrene was evaluated in Burk's nitrogen-free medium supplemented with phenanthrene (50 ppm) as the sole carbon source (Abdel-Razek *et al.,* 2020). Bacterial growth was monitored by measuring optical density at 630 nm (OD630) using a UV–Visible spectrophotometer (SPECORD 250, Analytik Jena AG, Germany) at 24-h intervals over a 10-day incubation period. All experiments were conducted in triplicate (Toledo *et al.,* 2006; Sun *et al.,* 2014).

Phenanthrene degradation and HPLC analysis

Phenanthrene degradation was evaluated in Burk's nitrogen-free broth with phenanthrene as the sole carbon source. Following incubation, residual phenanthrene was extracted using an n-hexane/acetone solvent mixture. The filtered extracts were directly injected into a high-performance liquid chromatography (HPLC) system equipped with a Knauer K-2600 UV detector at 254 nm. A Nucleosil® LC-PAH column (250 mm × 4.6 mm i.d., particle size 5 µm, pore size 100 A°) was used for the Phenanthrene separation. An abiotic control consisting of Burk's nitrogen-free medium supplemented with phenanthrene (50 ppm) but without bacterial inoculation was included and incubated under the same conditions as the experimental treatments. This control was used to account for any non-biological loss of phenanthrene during the incubation period. Phenanthrene removal efficiency was determined by comparing the residual phenanthrene concentration in inoculated cultures with that of uninoculated controls. All experiments were performed in triplicate (Zafra *et al.,* 2014; Qi *et al.,* 2017).

Chromatographic conditions

All sample injections were held constant at 20 µL using a fixed-volume injection loop. The flow rate of the mobile phase (A) acetonitrile and (B) water was set at 1.5 ml min^{-1}. The Phen was separated using the following gradient program: 35% A was maintained for 2 min; a linear gradient from 35% A at 2 min to 100% A at 14 min; and 100% A was maintained for 10 min. Thereafter, the mobile phase was returned to the initial condition (35% A) and held for 5 min to prepare the column for the next injection. The column temperature was maintained at 25°C, and chromatographic data were acquired at 254 nm.

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Statistical analysis

All experiments were performed in triplicate, and the results are presented as mean±standard deviation (SD). Statistical analyses were conducted using SPSS software version 25. Data normality was evaluated using the Kolmogorov–Smirnov test. Since the data showed a normal distribution, differences among bacterial isolates were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $p<0.05$ (Fu *et al.,* 2018).

Results

Isolation and Selection of Nitrogen-Fixing Bacteria

A total of 24 bacterial isolates were initially recovered from water and sediment samples collected from the Musa Estuary using Burk's nitrogen-free medium. Growth on this medium served as a preliminary criterion for identifying nitrogen-fixing bacteria. To evaluate their adaptation to nitrogen-limited conditions, the isolates were cultured on both Burk's nitrogen-free agar and nutrient agar. Based on their colony morphology and growth characteristics on Burk's nitrogen-free medium, four selected isolates (BP1–BP4) were selected for detailed phenotypic, molecular, and phenanthrene degradation analyses. The selected isolates exhibited slower growth and distinct colony morphologies on Burk's nitrogen-free medium compared to nutrient agar, highlighting their capacity to persist under nitrogen-limited conditions.

Morphological and Biochemical Characterization of Isolates

The four selected putative diazotrophic isolates (BP1–BP4) exhibited distinct morphological and biochemical characteristics (Tables 1 and 2). All isolates were identified as Gram-positive, rod-shaped bacteria. There were notable differences in colony morphology among the isolates, particularly regarding pigmentation, texture, and growth patterns on Burk's N-free medium. Specifically, isolates BP3 and BP4 formed transparent, dewdrop-like colonies on Burk's N-free agar, while colonies on nutrient agar appeared opaque (Fig. 2). Biochemical profiles also confirmed the initial classification and differentiated the isolates well. Variations were observed in citrate utilization, motility, and MR–VP reactions, whereas all isolates showed consistent Gram-positive characteristics. Detailed morphological and biochemical features are presented in Tables 1 and 2.

Molecular identification of the bacterial isolates

PCR amplification of the 16S rRNA gene from the four selected isolates confirmed successful DNA extraction, producing bands of approximately 1500 bp on a 1% agarose gel (Figs. 3 and 4). The 16S rRNA gene sequences of the four bacterial isolates were analyzed using BLAST, identifying them as follows: BP1 as *Bacillus cereus* (accession number PX456987), BP2 as *Bacillus subtilis* (accession number PX454376), BP3 as *Microbacterium* sp. (accession number PX457319), and BP4 as *Microbacterium trichothecenolyticum* (accession number PX456114). The phylogenetic tree was constructed using the 16S rRNA gene sequences through the neighbor-joining (NJ) algorithm.

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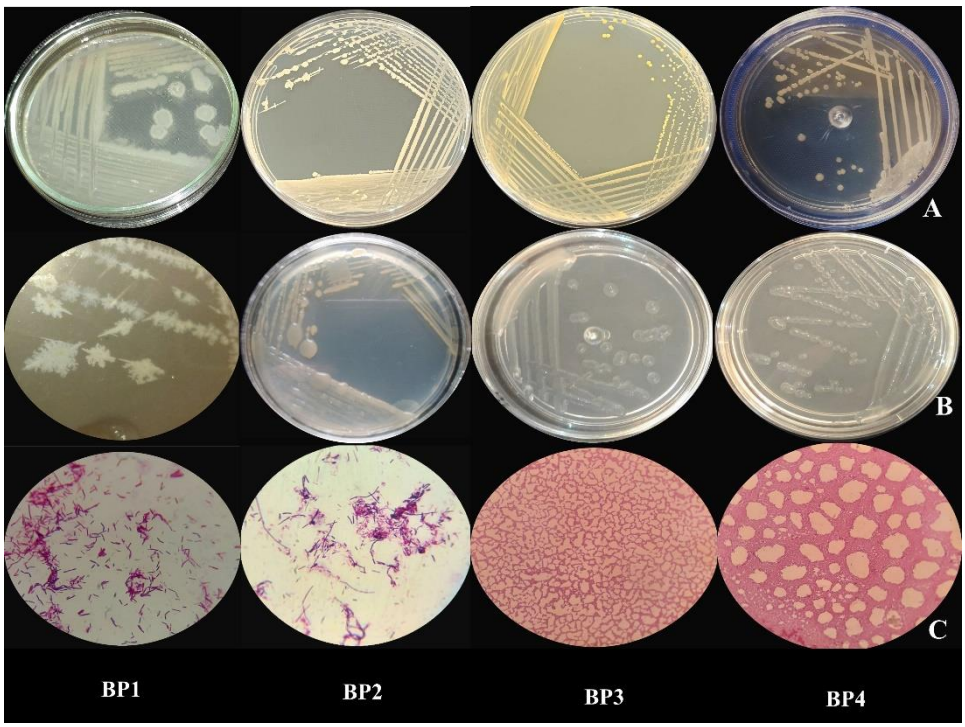

Figure 2: Colony morphology of nitrogen-fixing bacterial isolates on different culture media and their microscopic appearance. (A) Growth of isolates on nutrient agar (nitrogen-rich medium). (B) Growth of the same isolates on Burk's N-free agar (nitrogen-deficient medium). (C) Microscopic observation of representative isolates after Gram staining.

Table 1: Morphological properties of the bacterial isolates on Burk's N-free agar medium.

No	Form	Color	Surface	Margin	Elevation	Texture	Source
BP1	Rhizoid	White	Rough	Filamentous	Flat	Dry	Water
BP2	Circular	Cream	Smooth	undulate	Flat	Mucoid	Sediment
BP3	Round	Clear	Smooth	Entire	Convex	Viscose	Sediment
BP4	Round	Clear	Smooth	Entire	Convex	Viscose	Water

Table 2: Gram reaction and biochemical characteristics of bacterial isolates.

Biochemical tests & Gram stain	Isolates			
	BP1	BP2	BP3	BP4
Gram Stain	+*ve*	+*ve*	+*ve*	+*ve*
Lysine Decarboxylase	-	-	-	-
Ornithine decarboxylase	-	-	+	-
TSI	-	+	-	-
MR	-	+	-	-
VP	+	+	-	-
Indole	-	-	+	-
Motility	+	+	-	-
H_2S production	-	+	-	-
Citrate	+	+	+	-
KOH	-	-	+	-
Catalase	+	+	-	-

The resulting phylogenetic tree clearly revealed two major clades corresponding to the genera *Bacillus* and *Microbacterium*. The *Bacillus* cluster comprised *B. cereus* strain BP1, *B. thuringiensis*, *B. paranthracis*, and several *B. subtilis* strains (BP2, R-N-2, and VP4), all of which showed strong bootstrap support values,

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ranging from 93 to 99, indicating a high degree of genetic relatedness within this genus. The *Microbacterium* clade included *M. sediminis*, *M. hydrocarbonoxydans*, *M.* sp. strain BP3, *M. schleiferi*, *M. testaceum*, and *M. trichothecenolyticum* strain BP4, with bootstrap values between 82 and 100, further confirming their close phylogenetic affinity. Overall, the phylogenetic analysis revealed a clear taxonomic distinction between the *Bacillus* and *Microbacterium* isolates. At the same time, the high bootstrap values provided robust statistical confidence in the inferred branching patterns and evolutionary relationships among the studied strains.

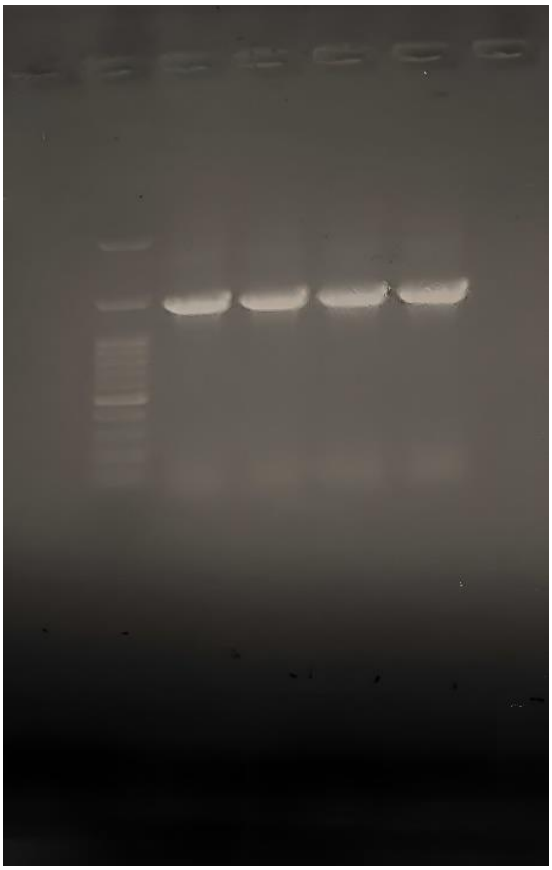

Figure 3: Agarose gel electrophoresis (1%) on PCR-amplified 16S rRNA gene fragments (1500 bp) from four bacterial isolates, using a 100 bp DNA ladder.

Effect of phenanthrene concentrations on the growth of the isolates

The growth of the four selected isolates (BP1: *Bacillus cereus*, BP2: *Bacillus subtilis*, BP3: *Microbacterium* sp., and BP4: Microbacterium trichothecenolyticum) was monitored in Burk's nitrogen-free medium supplemented with 50 ppm phenanthrene over a 10-day incubation period (Fig. 5). All isolates displayed a short lag phase during the first two days, followed by a rapid increase in growth. They reached their maximum optical density between days 6 and 7, after which growth gradually declined. Among the isolates tested, BP3 and BP4 generally showed higher optical density (OD630) values compared to BP1 and BP2 throughout the incubation period. BP4 achieved the highest OD630 and maintained relatively stable growth after reaching its peak, while BP3 experienced a more significant decline after its peak. Additionally, among the Bacillus isolates, BP2 exhibited higher OD630 values than BP1 during the exponential growth phase. The observed growth patterns were determined relative to the uninoculated control treatments and were consistent across the three independent replicates. Statistical analysis (one-way ANOVA followed by Tukey's test, $p<0.05$) indicated significant differences among the isolates during the incubation period.

HPLC determination of PAH degradation

The phenanthrene removal efficiency of the four selected isolates at an initial concentration of 50 ppm was determined using HPLC (Fig. 6).

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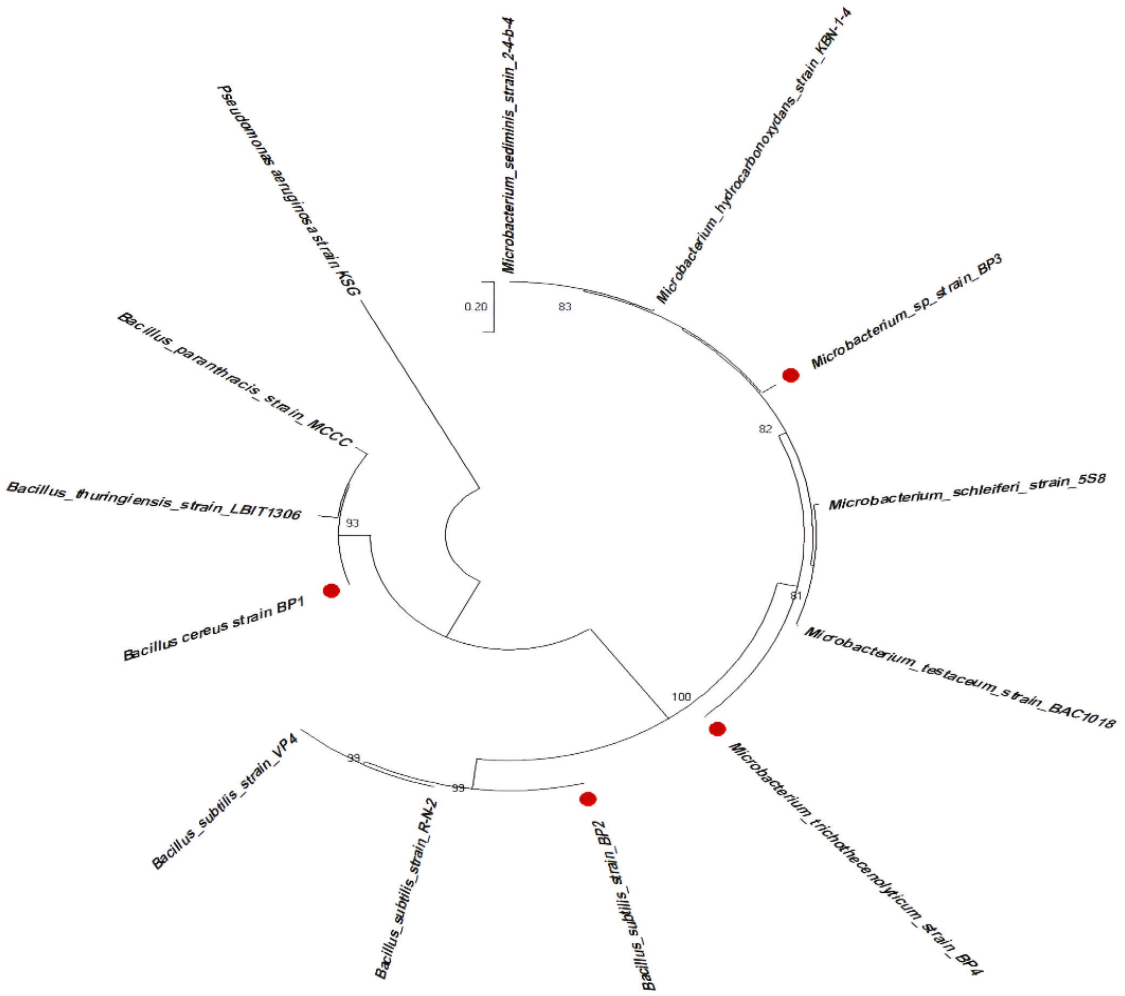

Figure 4: Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences showing the evolutionary relationships between the bacterial isolates obtained in this study and closely related reference strains retrieved from GenBank. The tree was constructed using MEGA v.10.0 with 1000 bootstrap replications. *Pseudomonas aeruginosa* strain KSG was used as the outgroup. Red circles indicate the isolates identified in the present study (BP1–BP4). Bootstrap values (%) greater than 50% are shown at branch nodes.

All isolates demonstrated significant ability to reduce phenanthrene levels. *Microbacterium* sp. (BP3) exhibited the highest removal efficiency at 93.44%, followed closely by *Microbacterium trichothecenolyticum* (BP4) at 91.06%. In contrast, *Bacillus cereus* (BP1) and *Bacillus subtilis* (BP2) achieved removal efficiencies of 87.72% and 85.56%, respectively. Statistical analysis revealed significant differences among some of the isolates ($p<0.05$), as illustrated in Figure 6. These results indicate variability in phenanthrene removal performance among the tested isolates, with BP3 showing the highest degradation efficiency under the conditions of this study.

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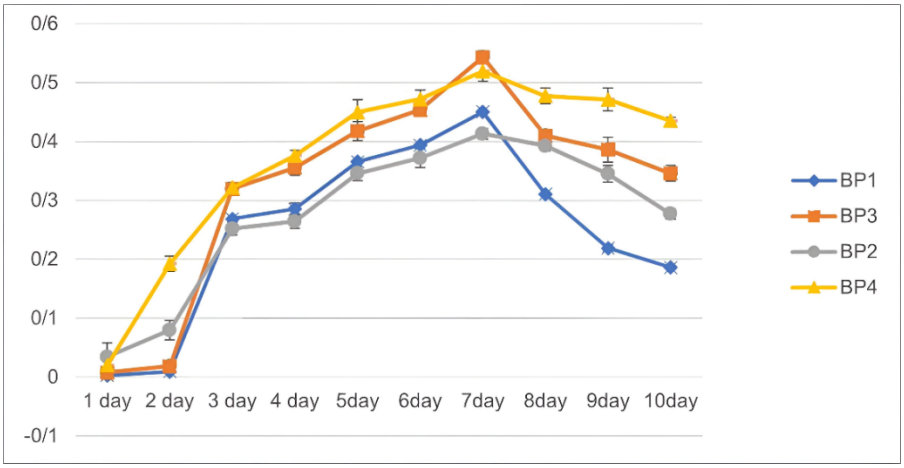

Figure 5: Growth curves of the four selected bacterial isolates cultured in Burk's nitrogen-free medium supplemented with 50 ppm phenanthrene as the sole carbon source over a 10-day incubation period. Values represent the mean ± SD of three independent replicates.

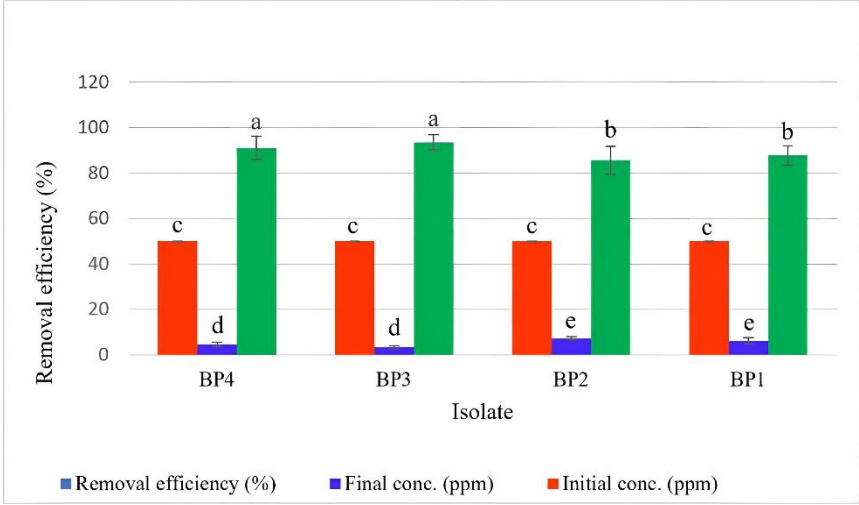

Figure 6: Residual phenanthrene concentration and removal efficiency (%) of the selected bacterial isolates after 7 days of incubation in Burk's nitrogen-free medium supplemented with 50 ppm phenanthrene. Values are presented as mean±SD (n=3). Different letters indicate significant differences among isolates (*p*<0.05).

Discussion

Bioremediation is an important approach for the remediation of environments contaminated with PAHs, using natural microbial biodegradation. Microorganisms naturally biodegrade pollutants, thereby removing them from the environment (Ron and Rosenberg, 2014; Varjani, 2017). Therefore, the identification of indigenous bacterial populations that can survive under nutrient-limited conditions during PAH degradation is of great ecological and environmental interest. In this study, four bacterial isolates with putative diazotrophic potential were selected from a total of 24 isolates obtained from the Musa Estuary. The initial screening involved assessing growth on nitrogen-free medium. Although we did not conduct confirmatory analyses such as the ARA or nifH gene detection, the ability of the selected isolates to grow on Burk's nitrogen-free medium suggests that they have adapted to nitrogen-limited conditions. Additionally, differences in

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growth and colony morphology observed between the nitrogen-free medium and the nutrient agar further support this adaptation. Phenotypic characterization and 16S rRNA gene sequencing identified the isolates as *Bacillus cereus* (BP1), *Bacillus subtilis* (BP2), *Microbacterium* sp. (BP3), and *Microbacterium trichothecenolyticum* (BP4). These isolates belong to the phyla Bacillota and Actinomycetota. Phylogenetic analysis confirmed the taxonomic classifications obtained through BLAST, clarifying the distinctions between the *Bacillus* and *Microbacterium* isolates. The use of nitrogen-free media has been widely recognized as an effective preliminary approach for isolating potential nitrogen-fixing bacteria. For instance, Shieh *et al.* (1988) successfully isolated N_2-fixing bacteria from marine sediments using this type of media, reporting clear and convex colony morphologies that resemble those observed in the current study. Similarly, Yousuf *et al.* (2017) recovered various nitrogen-fixing *Bacillus* species from estuarine environments utilizing nitrogen-free media. In another study, Yousuf *et al.* (2020) described clear, convex, and dewdrop-like colonies among potential diazotrophic isolates from the Arabian Sea. Moreover, the combination of phenotypic characterization with 16S rRNA gene sequencing has become a standard method for identifying bacteria in environmental studies (Uvarova *et al.,* 2020). This approach has also been successfully employed by Kang *et al.* (2015) and Thajudeen *et al.* (2017) to characterize nitrogen-fixing bacteria from marine and estuarine environments. Collectively, these studies emphasize the effectiveness of integrating culture-based screening with molecular identification for characterizing bacterial isolates in environmental contexts.

After establishing the taxonomic identity of the isolates, their ability to utilize and degrade phenanthrene under nitrogen-limited conditions was investigated. Bacterial growth and phenanthrene removal efficiency were assessed to determine the potential role of these isolates in PAH biodegradation. The growth experiments revealed that all four isolates were able to grow in a nitrogen-free medium containing phenanthrene. The growth responses generally aligned with the HPLC results, as isolates that exhibited more robust growth also showed higher phenanthrene removal efficiencies. The observed degradation efficiencies are consistent with previous studies describing the biodegradation potential of both *Microbacterium* and *Bacillus* species. Members of the phylum Actinomycetota are known for their metabolic versatility and their ability to survive under harsh environmental conditions, making them important contributors to the biodegradation of organic pollutants (Salam *et al.,* 2014; Chaturvedi and Khurana, 2019). Within this phylum, the genus *Microbacterium* has been recognized for its ability to degrade various PAHs under different environmental conditions. For example, Sheng *et al.* (2009) reported that *Microbacterium* sp. removed 98% of phenanthrene within 7 days. Additionally, Li *et al.* (2009) isolated *Microbacterium* strains from mangrove sediments capable of degrading multiple PAHs. Similarly,

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Wongwongsee *et al.* (2013) reported phenanthrene removal efficiencies of 50–76% by *Microbacterium* isolates after two weeks of incubation.

Similarly, *Bacillus* species are among the most frequently reported hydrocarbon-degrading bacteria in both aquatic and terrestrial environments. Their biodegradation potential has been attributed to several characteristics, including ecological adaptability, endospore formation, biosurfactant production, and the secretion of diverse extracellular enzymes (Rooney *et al.,* 2009; Joshi *et al.,* 2016; Parthipan *et al.,* 2017; Bonifer *et al.,* 2019). Several studies have demonstrated the ability of *Bacillus* strains to utilize PAHs as carbon and energy sources. For example, Oyetibo *et al.* (2017) reported phenanthrene degradation by *Bacillus subtilis*. Also, Velayutham *et al.* (2012) observed high PAH degradation rates by *B. subtilis* isolates. Furthermore, Mandree *et al.* (2021) demonstrated complete degradation of naphthalene, phenanthrene, and pyrene by a consortium containing *Bacillus cereus* and *Bacillus subtilis*. Collectively, these studies support the phenanthrene degradation potential observed for the *Bacillus* isolates in the present study. However, because only four isolates were evaluated and no functional analyses of degradation pathways or enzyme activities were performed, the observed differences should be interpreted at the strain level rather than generalized to the entire *Bacillus* or *Microbacterium* genera.

Nitrogen availability is often a limiting factor in the biodegradation of hydrocarbons in contaminated environments (Varjani and Upasani, 2017). In ecosystems contaminated with PAHs, the addition of carbon-rich compounds can alter the carbon-to-nitrogen (C: N) ratio, increasing the microbial demand for nitrogen during biomass production and the degradation of pollutants (Rivera-Espinoza and Dendooven, 2004; Margesin *et al.,* 2007). A disrupted C: N ratio can decrease the efficiency of biodegradation (Welz *et al.,* 2018). In this study, all selected isolates were capable of growing in a nitrogen-free medium and degrading phenanthrene, indicating their adaptation to nitrogen-limited conditions. Such characteristics may be particularly relevant in environments like the Musa Estuary, where petroleum-derived pollutants are prevalent, and nutrient availability can significantly influence microbial degradation processes. However, several limitations must be considered when interpreting the results. First, due to technical and financial constraints, confirmatory analyses of nitrogen fixation, such as the ARA and nifH gene detection, were not conducted. Second, only four isolates were examined, so the observed differences should be interpreted at the strain level rather than generalized across the entire *Bacillus* or *Microbacterium* genera. Third, functional analyses of degradation pathways, catabolic genes, or enzyme activities were not performed, which limits the mechanistic interpretation of the observed phenanthrene degradation. Despite these limitations, the findings provide preliminary evidence that indigenous bacterial isolates from the Musa Estuary may hold potential for future

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bioremediation applications in PAH-contaminated marine environments.

Conclusion

Four bacterial isolates identified as *Bacillus cereus* (BP1), *Bacillus subtilis* (BP2), *Microbacterium* sp. (BP3), and *Microbacterium trichothecenolyticum* (BP4) were collected from the Musa Estuary and assessed for their ability to degrade phenanthrene under nitrogen-limited conditions. All isolates demonstrated high degradation efficiencies, ranging from 85.56% to 93.44%, with BP3 and BP4 achieving the highest levels of performance. Growth in a nitrogen-free medium suggests that these bacteria may have adapted to nitrogen limitation. These isolates may be promising candidates for bioaugmentation in PAH-contaminated marine environments due to their ability to thrive in nitrogen-limited conditions, potentially aiding bioremediation without extensive nitrogen supplementation. However, further studies are needed to confirm nitrogen-fixation activity, assess performance, and evaluate the effectiveness of individual strains and microbial consortia in large-scale remediation.

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Conflicts of interest

The author declares no conflict of interest.

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