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# Isolation and Identification of Phenanthrene Degrading Marine Isolates from Indian Ocean

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**ABSTRACT**
Marine water contamination by hydrocarbon and its by-products are currently a worldwide issue. Microorganisms that can ingest hydrocarbons for growth, sustenance, and metabolic processes, classified as Hydrocarbonoclastic bacteria are identified as promising biocontrol agents for the degradation of hydrocarbons. Thus, in the present study, an attempt was made to isolate hydrocarbonoclastic bacterial species that are capable of utilising and degrading Phenanthrene, a polycyclic aromatic hydrocarbon as the sole carbon source. A total of 16 bacterial isolates were obtained as pure cultures from the samples collected at varying depths of 3 to 3500 m from Indian Ocean by the sequential isolation process with Phenanthrene incorporated into the growth media. Growth specificity of the isolates was estimated by subjecting the isolates to different ranges of pH, salinity and temperature. Based on the specificity tests, 3 isolates were selected as potential candidates that can grow well in Phenanthrene. Further, molecular characterization of these isolates by 16s rRNA sequence revealed the identity as *Alcanivorax dieselolei, Rhodococcus pyridinivorans* and *Halomonas titanicae.*

**Key words:** Hydrocarbon, Hydrocarbonoclastic bacteria, Phenanthrene, Bioremediation, Polycyclic Aromatic Hydrocarbons, 16s rRNA

## INTRODUCTION

The scale of marine pollution in the Indian Ocean, particularly in recent years, is dramatically increasing, causing considerable concerns. The major causes of contamination are chemicals, oil spillage and plastics. In particular, 40% of the world's offshore oil production is from the Indian Ocean region. A crude oil spill is a common issue during offshore oil drilling, transport and transfer to onshore. Secondly, the production of petroleum refinery effluent is known to cause pollution due to its toxic effluent discharge. Marine habitats and onshore soil biota are affected by the petroleum hydrocarbons (Sayed et al. 2014), mainly by the Polycyclic Aromatic Hydrocarbons (PAHs) which are recognized as the priority organic pollutants of serious environmental concern (Dhar et al. 2023). Among the PAHs, Phenanthrene (PHE) is a highly reactive compound that belongs to the low-molecular-weight aromatic hydrocarbons group, having three aromatic rings per molecule (Pedetta et al. 2013). It exhibits high toxicity to aquatic organisms and has a substantial impact on ocean health (Yifei et al. 2023). Several studies have demonstrated Phenanthrene toxicity in fish, algae, and superior organisms. It has been listed as 1 of the 16 PAHs of priority pollutants (Marquez-Villa et al. 2023).

Several marine bacteria that specialize in the degradation of hydrocarbons have been isolated from polluted seawater (Das and Chandra 2011). It is reported that around 175 prokaryotic genera in seven phyla of Bacteria and Archaea, and a similar number of fungal genera, can use hydrocarbons as their sole or major carbon source (Hazen et al. 2016). Biodegradation of PAHs, especially phenanthrene, by bacteria and fungi has been reported in several studies (Nzila et al. 2018, Mai et al. 2021, Cerniglia and Sutherland 2010) and is considered promising in eradicating such contamination. Nevertheless, there is always a demand for the search of microbial consortium that can be applied in the process of bioremediation. Thus, the present study was focused on the isolation, identification, and characterization of phenanthrene degrading bacterium from PAH contaminated marine ecosystem, thereby exploring the remediation potential of the isolates.

## MATERIAL AND METHODS

### Sample collection

Oil contaminated water samples were collected aseptically from the Indian Ocean, Equator region (Latitude: 4.22º N / Longitude: 66.69º E), at a depth of 3 to 3500 m, using Oceanographic Vessel of National Institute of Ocean Technology. After collection, the samples were stored at 4°C and transported to the laboratory for further studies.

### Sequential Isolation of hydrocarbonoclastic bacteria

Sequential isolation of hydrocarbon degrading bacteria was carried out by processing the samples in an enrichment media, Bushnell and Haas Mineral Salt (BHMS) supplemented with Phenanthrene (250 mg $L^{-1}$). To the prepared broth medium, the collected sample was inoculated at a volumetric ratio of 1:0.25 (Kumar et al. 2019). The pH was subsequently adjusted to 7±0.2 and incubated at 28ºC for 15 days on a rotary shaker at 120 rpm till the growth was observed (turbidity). After incubation, Phenanthrene degrading Hydrocarbonoclastic bacteria were isolated by plating 0.1 mL from the incubated growth medium to BHMS agar medium supplemented with Phenanthrene (Yin et al. 2020). The plates were incubated at 28ºC for 7 days to allow prominent growth. The colony morphology was observed and further purification was performed by streaking onto fresh agar plates. The Phenanthrene degrading isolates were purified to monoculture and stocked for further studies.

### Growth specificity of phenanthrene degraders

For in vitro selection of bacteria for bioremediation applications, the tolerance of each bacterial strain at varied pH, salt, and temperature fluctuations was examined (Kumar et al. 2014). Bacterial cultures were grown in Zobell Marine Broth (Himedia Ltd., India) with varying parameters such as pH levels ranging from 5 to 10, temperature conditions between 10-45ºC and salt concentration at 1 to 10% w/v. The growth of isolates was assessed by plating onto Zobell Marine Agar (ZMA) medium (Himedia Ltd., India).

### Growth efficacy of the isolates in varying concentrations of Phenanthrene

To evaluate the Phenanthrene tolerance and utilization capability of the isolates, four different concentrations (25, 50, 75, 100 mg) of Phenanthrene was supplemented to the growth medium (Minimal salt agar). The plates were incubated and observed for growth pattern.

### Molecular characterisation of the bacterial isolates

DNA was extracted from fresh cultures of the selected bacterial isolates by using IGB (ImmuGenix Biosciences) Bacterial DNA Extraction kit (Chennai, India). A PCR targeting 16S rRNA gene was performed in Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). The amplicons were resolved along with DNA markers in 1% agarose with ethidium bromide (10 mg/mL) by gel electrophoresis for ~15min at 135 V using Mupid-exU system (Takara, Japan) and gel was analysed by BioGlow UV Transilluminators (Crystal Technology, USA). The resultant product size was 1500bp. The 16S rRNA PCR product was sequenced using ABI PRISM® BigDye™ Terminator and ABI 3730XL sequencer (Applied Biosystem, USA) using forward primer. After sequencing, the sequence chromatogram files were examined for quality and the low-quality ends of 16S rRNA sequences were trimmed by using Bio-Edit version 7.0.9 (Isis Pharmaceuticals) Species identification of the bacteria was achieved using the Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene sequences of the bacterial isolates were deposited in the NCBI GenBank database and accession numbers were obtained.

## RESULTS

### Isolation of Phenanthrene degrading bacteria

From the initial isolation procedure, bacterial growth was observed in all the collected samples. Prominent growth was seen in the samples collected from 3 to 1500 m and comparatively a nominal growth was noticed in 2500 to 3500 m (Table 1). The growth of Phenanthrene degrading bacteria was observed in Phenanthrene incorporated growth media. Overall, from the colonies developed, 16 morphologically

Table 1. Growth of isolates collcted at varying depths

| Sample depth | Bacterial growth |
|---|---|
| 3 m | +++ |
| 120 m | +++ |
| 400 m | +++ |
| 900 m | +++ |
| 1500 m | +++ |
| 2500 m | ++ |
| 3000 m | ++ |
| 3500 m | ++ |

+++ - Prominent growth, ++ - Nominal growth

distinct and most commonly observed colonies (named as ISO1 to ISO16) were chosen for further studies. The colony morphology presented a wide variety of bacterial consortium in the marine environment.

**Growth specificity**

The isolates ISO1, ISO4, ISO5, ISO9, ISO12, ISO13, ISO14 and ISO16 showed maximum growth at a neutral of pH 7, while the isolates ISO2, ISO3, ISO6, ISO7, ISO8 and ISO11 showed maximum growth at pH 8. Two isolates ISO10 and ISO15 showed prominent growth at pH 6 (Fig. 1). Growth estimation at different temperature revealed the influence of temperature on the Phenanthrene degrading bacteria. Very scarce growth was seen at 10°C followed by a gradual increase at 15°C. However, there was a steady and noticeable growth observed between the temperature ranges of 25 to 35°C, after which there was drop in growth at 45°C (Fig. 2). Salinity of the growth medium is considered to have an influential effect on the maximal growth, especially for marine bacteria. The isolates showed significant degrees of tolerance to different salt concentration. It was observed that as the salinity increased from 0 to 5% there was an increase in the growth rate after which it slightly dropped (Fig. 3). The results also revealed the optimal condition to be 3 to 5% for the isolated hydrocarbonoclastic bacteria.

**Growth efficacy in varying concentrations of Phenanthrene**

The isolates were further analysed for their efficiency to grow in different concentration of Phenanthrene. While the isolates ISO1, ISO2, ISO13, ISO14, ISO15 and ISO16 exhibited growth only at 25 mg

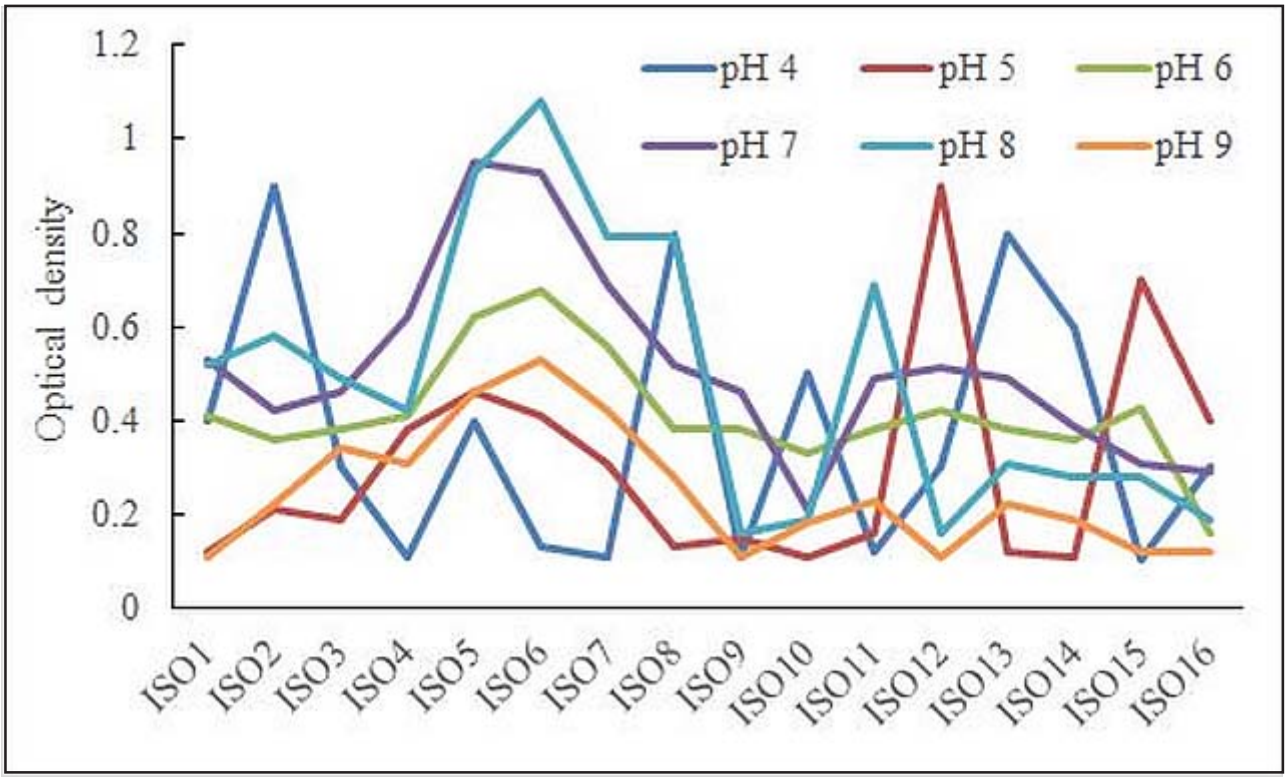


Figure 1. Growth specificity at varying pH levels

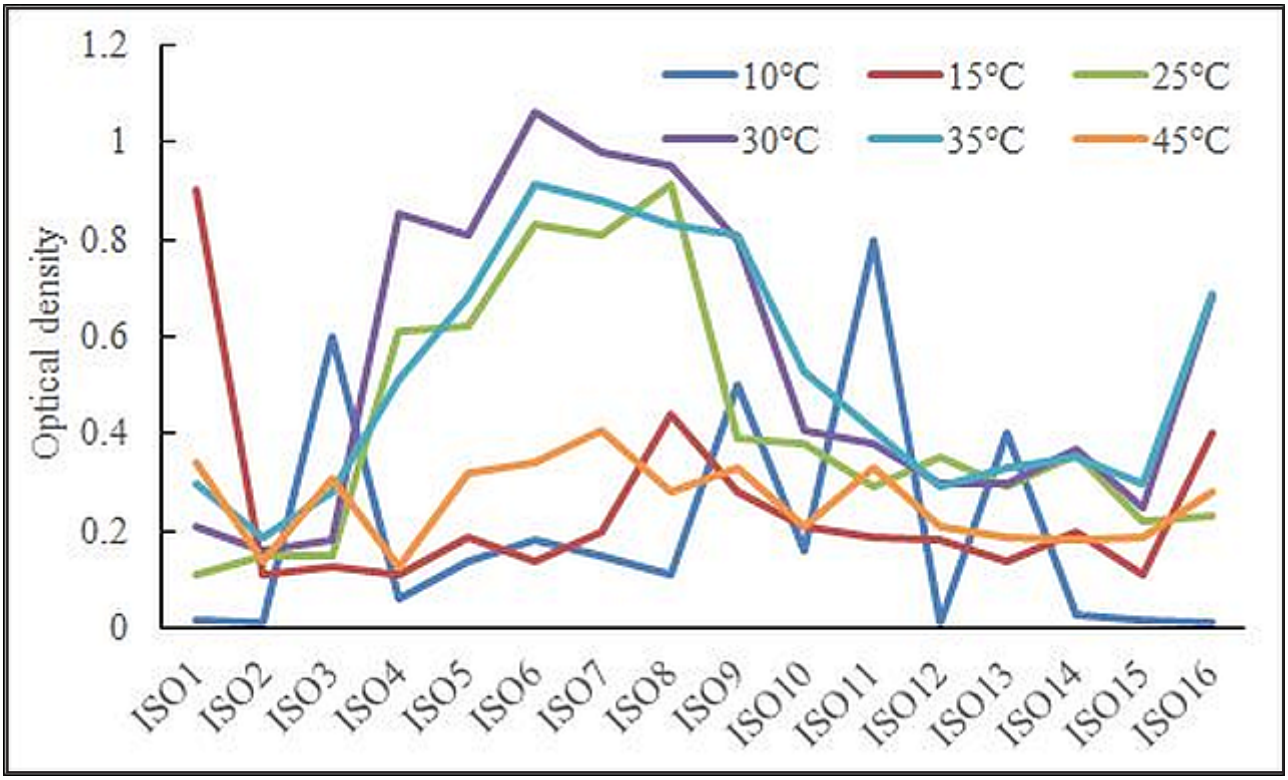


Figure 2. Growth specificity at varying degrees of temperature

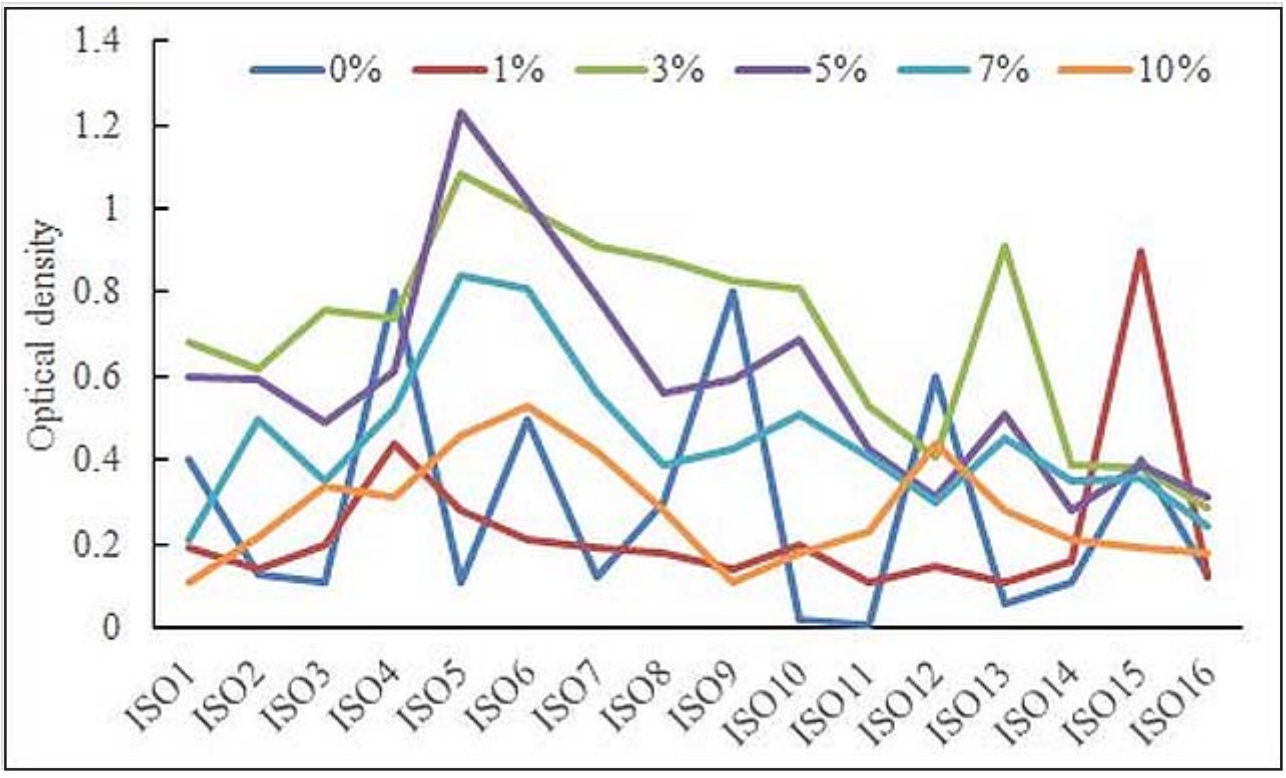


Figure 3. Growth specificity at different salt concentration

Table 2. Growth efficacy of the isolates in varying concentrations of Phenanthrene

| Isolates | Phenanthrene (mg) | | | |
|---|---|---|---|---|
| | 25 | 50 | 75 | 100 |
| ISO1 | +++ | - | - | - |
| ISO2 | +++ | - | - | - |
| ISO3 | ++ | ++ | + | - |
| ISO4 | +++ | +++ | ++ | + |
| ISO5 | ++++ | ++++ | ++++ | ++++ |
| ISO6 | ++++ | ++++ | ++++ | ++++ |
| ISO7 | ++++ | ++++ | ++++ | ++++ |
| ISO8 | +++ | ++ | + | - |
| ISO9 | ++ | ++ | - | - |
| ISO10 | ++ | ++ | + | - |
| ISO11 | +++ | ++ | ++ | + |
| ISO12 | ++ | ++ | + | - |
| ISO13 | +++ | - | - | - |
| ISO14 | +++ | - | - | - |
| ISO15 | +++ | - | - | - |
| ISO16 | +++ | - | - | - |

++++ - Abundant growth, +++ - Prominent growth, ++ - Nominal growth, + - Poor growth, - - No growth

concentration of Phenanthrene, isolates ISO3, ISO8, ISO10 and ISO12 showed considerable growth at 25, 50 and 75 mg and failed to grow in 100 mg concentration. However, the isolates ISO4, ISO5, ISO6, ISO7 and ISO11 showed growth in all the tested concentrations. Overall, three of the sixteen isolates namely ISO5, ISO6 and ISO7 showed abundant growth regardless of the increase in concentration and were identified as the most potential Phenanthrene degrading bacteria (Table 2).

**Molecular characterisation of the bacterial isolates**

From the genomic extraction, approximately 25 kb genomic DNA was obtained. The obtained genomic DNA was subjected to the amplification of 16S rRNA. It amplified well and was observed in a 1.2% agarose gel electrophoresis. The expected amplicon size was ~ 1500 bp and the same size was in the obtained amplicon. The amplified product was purified using Exo-sap method and run in an ABI Prism gene sequencer. The sequences were analysed in BLAST program at NCBI website which showed sequences similarities with existing bacterial gene sequences up to 100% sequence similarities. Based on the molecular identification using 16S rRNA region the isolates were identified as *Alcanivorax dieselolei* (ISO5), *Rhodococcus pyridinivorans* (ISO6) and *Halomonas titanicae* (ISO7) belonging to the family Alcanivoracaceae, Nocardiaceae and Halomonadaceae, respectively. The sequences of the isolates were submitted to GenBank and the accession numbers were obtained as OQ520215, OQ520216, and OQ520221 for the three isolates, respectively.

**DISCUSSION**

As a resistant and toxic polycyclic aromatic hydrocarbon, Phenanthrene is largely distributed in aquatic and terrestrial environments with adverse biological consequences. Mineralization of this toxic component via bioremediation has been vastly studied in recent years and might lead to new hope in the economic clean-up of this contaminant (Abbasi et al. 2023). In the present study, Phenanthrene degrading bacteria were isolated from various depths of marine environment. Many study reports have presented the isolation of Phenanthrene degraders from hydrocarbon contaminated marine environment (Tao et al. 2007, Kumar et al. 2023, Manimekalai et al. 2017). Mai et al. 2021, isolated Phenanthrene degrading bacteria from marine contaminated water and presented its degradation metabolism. Six Phenanthrene degrading bacteria were isolated from surface seawater of Pacific Ocean (Iwabuchi 2022). Similarly, the present study demonstrated the isolation of 16 Phenanthrene degraders from the ocean samples. The growth efficacy of the isolates on Phenanthrene supplemented media indicated the ability of the isolates to utilize Phenanthrene as carbon source.

The key factors limiting the overall hydrocarbon biodegradation are generally categorized as biotic factors and abiotic factors. Among various environmental factors, temperature range from 30 to 40°C and pH from 5 to 8 (Kebede et al. 2021) were considered to be optimal. In the present study all the isolates were able to grow at different pH levels, indicating their adaptability to various pH, temperature and salinity. However, the optimal growth temperature was 30-35°C. Moreover, salinity of the growth medium is considered to have an influential effect on the maximal growth, especially

for marine bacteria. Similarly, Kumar et al. (2023) reported the adaptability of bacteria to various conditions like temperature, salinity and pH and revealing the optimum condition for effective degradation.

Effective microbial consortium has attracted the attention of researchers worldwide due to the synergistic interactions among members of the consortium. Phenanthrene was often used as a model substance for microbial metabolism (Wang et al. 2008). Studies in the past have reported the isolation of many Phenanthrene degrading bacteria like *Pseudomonas* (Tian et al. 2002), *Bacillus* (Pourbabaee et al. 2019), *Rhodococcus* (Aitken et al. 1998), *Acinetobacter, Sphingomonas* (Iwabuchi 2022). Thus, in the present study, three bacterial species namely *Alcanivorax dieselolei, Rhodococcus pyridinivorans* and *Halomonas titanicae* exhibited significant growth in higher concentration of Phenanthrene and were identified as potential Phenanthrene degraders.

## CONCLUSION

Polycyclic aromatic hydrocarbons are among the most serious pollutants of the marine environment, causing havoc to the living organism in the ecosystem. The use of bacteria in bioremediation of these toxic contaminants are promising and eco-friendly. Thus, the results of the present investigation offer scientific evidence that isolated bacteria can metabolise the pollutant at various concentrations. Edging forward, the identified microbial consortia can be effectively used in eradicating the pollutants.

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