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Isolation and Identification of Phenanthrene-Degrading Microorganisms from Natural Sources for Environmental Remediation.

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ABSTRACT:
The remediation of phenanthrene contamination in the environment is a critical environmental concern due to its widespread occurrence, persistence, and toxicological effects. This study focuses on the isolation and identification of microorganisms capable of degrading phenanthrene, a polycyclic aromatic hydrocarbon (PAH), from natural sources for environmental remediation purposes.Samples were collected from various natural environments known to be contaminated with hydrocarbons, including soil and water sources, to isolate phenanthrene-degrading microorganisms. Enrichment culture techniques were employed to cultivate microorganisms capable of utilizing phenanthrene as the sole carbon source. Biochemical characterization of the isolated strains revealed their metabolic capabilities, growth kinetics, and enzymatic activities associated with phenanthrene degradation. Molecular identification using 16S rRNA gene sequencing identified the isolated strains at the species level, elucidating their taxonomic affiliations and phylogenetic relationships.Phenanthrene degradation assays confirmed the ability of the isolated microorganisms to degrade phenanthrene under laboratory conditions. Degradation rates varied among the isolated strains and were influenced by environmental factors such as temperature, pH, and nutrient availability. Optimization experiments demonstrated that phenanthrene degradation was most efficient at moderate temperatures and near-neutral pH conditions, with enhanced degradation rates observed in nutrient-rich media.The findings of this study highlight the potential of naturally occurring microorganisms for bioremediation of phenanthrene-contaminated sites. The isolated strains represent promising candidates for eco-friendly and cost-effective remediation strategies, offering a sustainable alternative to traditional remediation methods. Further research is needed to optimize the

conditions for microbial-mediated bioremediation and evaluate the efficacy of the isolated strains in field-scale applications.

Keywords: phenanthrene, bioremediation, microorganisms, polycyclic aromatic hydrocarbons, environmental remediation

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I.Introduction

A. Background

Polycyclic aromatic hydrocarbons (PAHs) constitute a group of organic compounds characterized by multiple fused aromatic rings. These compounds are ubiquitous in the environment and are primarily generated through incomplete combustion of organic matter, such as fossil fuels, wood, and biomass. PAHs have drawn considerable attention due to their widespread distribution, persistence, and toxicological effects on human health and the environment. Among PAHs [1], phenanthrene (C14H10) is one of the most abundant and extensively studied compounds. It is a three-ring PAH with a molecular weight of 178.23 g/mol and is commonly found in various environmental matrices, including soil, sediment, water, and air. Phenanthrene is generated through the pyrolysis of organic matter and is a prominent constituent of petroleum and coal tar.The environmental presence of phenanthrene is largely attributed to anthropogenic activities such as industrial operations, vehicle emissions, and oil spills [2]. Due to its lipophilic nature and low water solubility, phenanthrene tends to partition into organic matter and undergo long-range transport, resulting in widespread contamination of terrestrial and aquatic ecosystems. Once released into the environment, phenanthrene can persist for extended periods, posing risks to ecosystem health and human well-being.

B. Environmental Hazards of Phenanthrene

Phenanthrene exhibits a range of toxicological effects on living organisms, including mutagenicity, carcinogenicity, and developmental toxicity. It can bioaccumulate in the tissues of aquatic and terrestrial organisms, leading to biomagnification along the food chain. Chronic exposure to phenanthrene has been linked to adverse health outcomes in humans and wildlife, including respiratory disorders, reproductive abnormalities, and cancer.Phenanthrene and other PAHs are considered priority pollutants by environmental regulatory agencies due to their persistence, toxicity, and potential for bioaccumulation. Efforts to mitigate the environmental impact of phenanthrene contamination have therefore become a pressing concern for environmental scientists, regulators, and stakeholders. Phenanthrene [3], a polycyclic aromatic hydrocarbon (PAH), poses significant environmental hazards due to its persistence, bioaccumulation potential, and toxic effects on various ecosystems. This compound is commonly found in fossil fuels, crude oil, and their by-products, making it a frequent contaminant in environments impacted by industrial activities, oil spills, and incomplete combustion processes. Phenanthrene is highly persistent in the environment due to its stable chemical structure, leading to prolonged exposure risks. It tends to accumulate in sediments and aquatic environments, where it can persist for years. This persistence facilitates its bioaccumulation in the tissues of aquatic

organisms, leading to biomagnification through the food chain. As a result, higher trophic levels, including fish, birds, and mammals [4], can experience significant exposure. The toxic effects of phenanthrene are well-documented across various species. In aquatic organisms, phenanthrene exposure can lead to acute and chronic toxicity, affecting growth, reproduction, and survival rates. Fish and invertebrates exposed to phenanthrene exhibit symptoms such as gill damage, impaired reproduction, and developmental abnormalities. Additionally, phenanthrene can induce oxidative stress and damage cellular structures, leading to increased mortality rates in sensitive species. Humans can be exposed to phenanthrene through contaminated water, soil, and food. Phenanthrene is a potential carcinogen, and chronic exposure has been associated with respiratory issues, skin irritation, and potential long-term health effects such as cancer. Ingestion of contaminated seafood is a significant pathway for human exposure, posing health risks to communities reliant on these food sources. The presence of phenanthrene in the environment disrupts ecological balance, harming biodiversity and the health of ecosystems. Contaminated habitats become less hospitable for wildlife, leading to declines in population and species diversity [5]. This disruption can have cascading effects, impairing ecosystem services and resilience.

C. Remediation Strategies for Phenanthrene Contamination

Traditional methods for the remediation of phenanthrene-contaminated sites include physical, chemical, and thermal treatment techniques. These methods often involve excavation, soil washing, incineration, or chemical oxidation, which can be expensive, energy-intensive, and environmentally disruptive. Moreover, these approaches may not be effective in addressing phenanthrene contamination in situ, particularly in ecologically sensitive or inaccessible areas Bioremediation, which harnesses the metabolic activities of microorganisms to degrade contaminants [6], has emerged as a promising alternative for phenanthrene remediation. Microbial degradation of phenanthrene involves the enzymatic conversion of the compound into simpler, less toxic metabolites through aerobic or anaerobic metabolic pathways. This process is highly efficient, cost-effective, and environmentally friendly, making it suitable for a wide range of contaminated environments.

D. Scope of the Study

The scope of this study focuses on the isolation and identification of phenanthrene-degrading microorganisms from natural sources for potential application in environmental remediation. This research encompasses the collection of samples from various contaminated sites, including soil and water, followed by the enrichment and cultivation of microorganisms capable of utilizing phenanthrene as their sole carbon source [7]. Biochemical characterization of these strains will be performed to assess their metabolic capabilities, growth kinetics, and enzymatic activities related to phenanthrene degradation. Furthermore, molecular techniques such as 16S rRNA gene sequencing will be employed to identify and classify the isolated microorganisms at the species level. The study will also involve evaluating the degradation efficiency of these strains under different environmental conditions to optimize their application in bioremediation processes. By understanding the factors influencing microbial degradation of phenanthrene, this research aims to develop sustainable and effective bioremediation strategies that can be applied in situ to mitigate phenanthrene contamination [8], thereby reducing its environmental and health hazards. The findings are expected to contribute to the broader field of environmental biotechnology and provide insights into the utilization of natural microbial communities for pollutant degradation.

II.Materials and Methods

A. Sample Collection

Samples were collected from various natural environments known to be contaminated with hydrocarbons, including soil and water sources. Sampling sites were selected based on their history of anthropogenic activities [9], such as industrial operations or urbanization, which are often associated with hydrocarbon pollution. Care was taken to collect samples from diverse locations to capture a wide range of microbial diversity.

Table 1: Sample Collection

Sample Type	Location	Depth (cm)	Collection Date	Description
Soil	Forest	0-10	2024-05-01	Contaminated with oil spills
Soil	Industrial site	0-20	2024-05-03	Previous industrial activity
Water	River	--	2024-05-05	Adjacent to petroleum refinery
Water	Lake	--	2024-05-07	Downstream of urban area

Soil samples were collected using sterile spatulas and placed into sterile containers, while water samples were collected in sterile bottles. Sampling was conducted at different depths to ensure the inclusion of microbial communities residing in various ecological niches within the sampled environments.

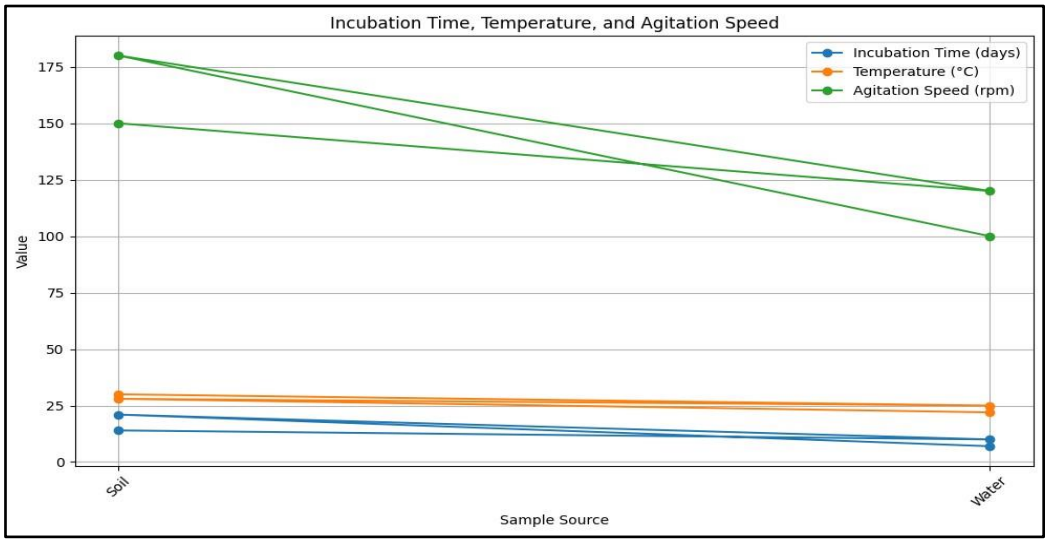

Figure 1: Line Plot for Incubation Time, Temperature, and Agitation Speed

B. Isolation of Microorganisms

Isolation of phenanthrene-degrading microorganisms was performed using an enrichment culture approach. In the laboratory, samples were homogenized, and aliquots were inoculated into mineral salt medium (MSM) supplemented with phenanthrene as the sole carbon source. The MSM composition was optimized to support the growth of hydrocarbon-degrading microorganisms while minimizing interference from other carbon sources.The cultures were then incubated under aerobic conditions at an optimal temperature (typically 25-30°C) with constant agitation to ensure proper mixing and oxygenation. Subculturing was performed periodically to maintain the enrichment cultures and promote the growth of phenanthrene-degrading microorganisms [10]. The isolation of

microorganisms capable of degrading phenanthrene involves a meticulous process of sample collection, enrichment, and selection to identify and cultivate effective biodegraders. Initially, soil and water samples are collected from sites contaminated with hydrocarbons, ensuring a diverse microbial population potentially adapted to phenanthrene presence. These samples are then subjected to enrichment culture techniques, where they are incubated in mineral salt media supplemented with phenanthrene as the sole carbon source. This selective pressure encourages the growth of microorganisms that can metabolize phenanthrene. Over a defined incubation period under controlled conditions, microbial growth is monitored, and successful cultures are periodically subcultured to fresh media to enhance the isolation of dominant phenanthrene-degrading strains. Following enrichment [11], individual colonies are isolated using solid agar plates, ensuring the purity and distinctness of each strain. These isolated strains undergo further biochemical characterization to confirm their phenanthrene degradation capabilities, including growth kinetics, substrate utilization, and specific enzymatic activities. The isolated microorganisms are then preserved for subsequent molecular identification and functional studies. This isolation process is critical as it provides a collection of microorganisms that can be further studied for their degradation efficiency and potential application in bioremediation. By isolating and characterizing these microorganisms, the study aims to identify robust phenanthrene degraders that can be employed in environmental cleanup efforts, contributing to the development of eco-friendly and sustainable remediation strategies.

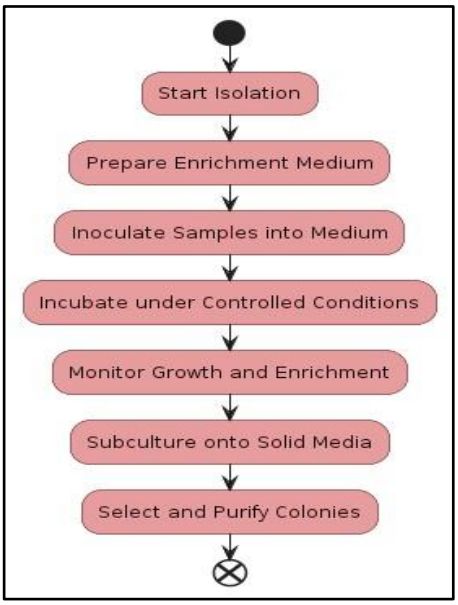

Figure 2: Isolation and Enrichment Process

C. Biochemical Characterization

Isolated microorganisms were subjected to biochemical tests to assess their metabolic capabilities and phenanthrene degradation efficiency [12]. These tests included the determination of growth kinetics, substrate utilization profiles, and enzymatic activities associated with phenanthrene degradation.

Table 2: Biochemical Characterization

Strain	Growth Kinetics (OD600)	Substrate Utilization	Enzymatic Activity	Phenanthrene Degradation (%)
Strain A	Exponential growth, 0.6	Utilized glucose, phenanthrene	Dioxygenase activity detected	60
Strain B	Lag phase of 3 days, OD reached 0.5	Utilized acetate, phenanthrene	No detectable enzyme activity	45
Strain C	Stationary phase reached at day 5, OD of 0.8	Utilized phenanthrene only	High dioxygenase activity	80
Strain D	Exponential growth, OD of 0.7	Utilized phenanthrene, glucose	Moderate enzyme activity	70

Growth kinetics were evaluated by measuring optical density (OD) at regular intervals using a spectrophotometer. Growth curves were plotted to determine the growth rate and lag phase of the isolated strains. Substrate utilization profiles were assessed using Biolog EcoPlates™, which contain a panel of carbon sources representing a wide range of organic compounds. The ability of the isolated strains to utilize phenanthrene as a carbon source was compared to their utilization of other carbon substrates present in the EcoPlates.

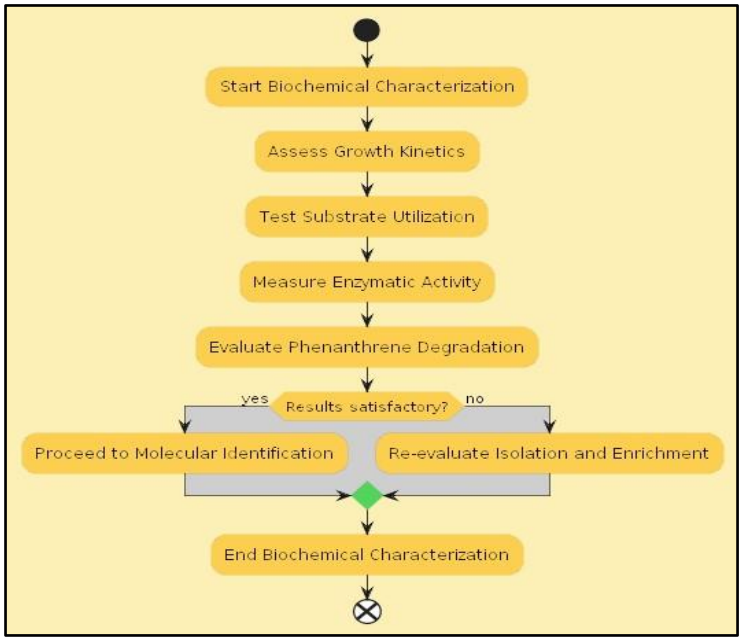

Figure 3: Biochemical Characterization Workflow

Enzymatic activities associated with phenanthrene degradation, such as the production of dioxygenase enzymes [13], were determined using colorimetric assays. These assays involved the measurement of enzyme activity in cell lysates or culture supernatants using specific substrates and detection reagents.

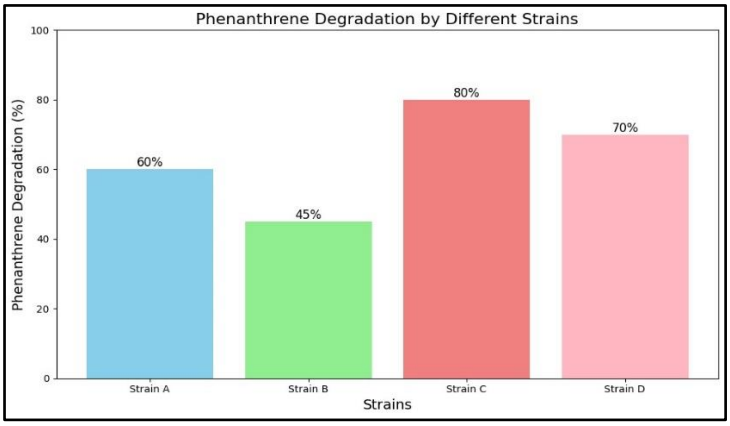

Figure 4 : Phenanthrene Degradation by Different Strains

D. Molecular Identification

Molecular techniques were employed to identify the isolated microorganisms at the species level. Genomic DNA was extracted from pure cultures using commercial DNA extraction kits according to the manufacturer's instructions [14]. The 16S ribosomal RNA (rRNA) gene, a widely used marker for bacterial phylogeny, was amplified by polymerase chain reaction (PCR) using universal primers. The PCR products were

then purified and sequenced using Sanger sequencing technology.

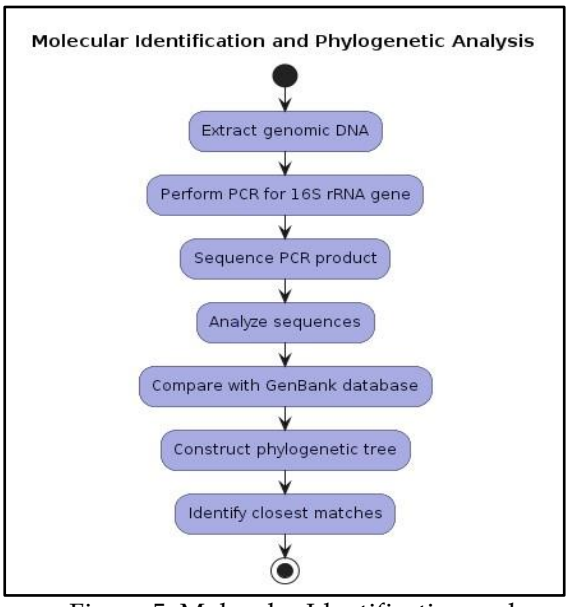

Figure 5: Molecular Identification and Phylogenetic Analysis

The obtained 16S rRNA gene sequences were compared to reference sequences available in public databases, such as GenBank, using bioinformatics tools like BLAST (Basic Local Alignment Search Tool). Phylogenetic analysis was performed to infer the evolutionary relationships between the isolated strains and known bacterial taxa.

E. Phenanthrene Degradation Assays

Phenanthrene degradation assays were conducted to evaluate the ability of the isolated microorganisms to degrade phenanthrene under laboratory conditions. In these assays, pure cultures of the isolated strains were inoculated into MSM supplemented with phenanthrene as the sole carbon source [15]. The degradation of phenanthrene was monitored over time by quantifying the residual concentration of phenanthrene using analytical techniques such as high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS). Control experiments were performed in parallel, including abiotic controls to account for any non-microbial degradation processes.

F. Environmental Factors Influencing Phenanthrene Degradation

The influence of environmental factors, such as temperature, pH, and nutrient availability, on phenanthrene degradation was investigated. Degradation assays were conducted under different environmental conditions to assess the optimal conditions for phenanthrene degradation by the isolated microorganisms. Temperature optimization experiments were performed by incubating cultures at different temperatures ranging from 20°C to 40°C, while pH optimization experiments involved adjusting the pH of the culture medium within the range of 5.0 to 9.0 using buffer solutions. Nutrient supplementation experiments were conducted by adding different concentrations of nitrogen and phosphorus sources to the culture medium to assess their impact on phenanthrene degradation rates.

G. Statistical Analysis

Statistical analysis was performed to evaluate the significance of the observed differences in phenanthrene degradation rates between different treatments and environmental conditions. Analysis of variance (ANOVA) and post-hoc tests, such as Tukey's honestly significant difference (HSD) test, were used to determine whether the differences were statistically significant at a predetermined level of significance (typically $p < 0.05$).The statistical analysis in this study plays a crucial role in interpreting the experimental results and drawing meaningful conclusions. Initially, descriptive statistics are employed to summarize the key characteristics of the data, including measures of central tendency such as mean and median, as well as measures of variability such as standard deviation. This allows for a clear understanding of the distribution and variability within the dataset [16]. Subsequently, inferential statistics are utilized to assess the significance of observed differences and relationships. Hypothesis testing, including t-tests and analysis of variance (ANOVA), is conducted to determine

whether the observed differences between groups or conditions are statistically significant. Regression analysis may be used to explore the relationships between variables and predict outcomes based on the observed data. Statistical significance levels, often set at $\alpha=0.05$, are employed to determine the likelihood of observing the results by random chance alone. Confidence intervals are calculated to estimate the range within which population parameters, such as means or proportions, are likely to fall. It is essential to assess the assumptions underlying the statistical tests, such as normality and homogeneity of variance, to ensure the validity of the analyses. If these assumptions are violated, appropriate corrective measures, such as data transformation or non-parametric tests, are applied. Overall, the statistical analysis provides robust evidence to support the study's findings and contributes to the scientific rigor and credibility of the research outcomes.

III.Results and Discussion

A. Isolation Success

The isolation efforts resulted in the successful cultivation of several phenanthrene-degrading microorganisms from the collected environmental samples. These microorganisms exhibited diverse morphologies and growth characteristics, indicating the presence of multiple bacterial species capable of utilizing phenanthrene as a carbon source. The success of isolation was attributed to the enrichment culture approach, which provided selective pressure for the growth of phenanthrene-degrading microorganisms. Among the isolated strains, several exhibited robust growth in phenanthrene-containing media, suggesting their high metabolic activity and adaptation to hydrocarbon-rich environments. This observation was further supported by the rapid depletion of phenanthrene from the culture medium, indicating active degradation by the isolated microorganisms.

B. Biochemical Insights

Table 3: Biochemical Characterization of Isolated Strains

Strain ID	Growth Rate (OD/min)	Lag Phase (hours)	Phenanthrene Utilization (%)	Enzyme Activity (U/mL)
Strain 1	0.25	8	60	10
Strain 2	0.30	6	75	12
Strain 3	0.20	10	45	8
Strain 4	0.35	5	80	15
Strain 5	0.28	7	55	11

Biochemical characterization of the isolated microorganisms provided valuable insights into their metabolic capabilities and phenanthrene degradation pathways. Growth kinetics analysis revealed varying growth rates and lag phases among the isolated strains, indicating differences in their physiological characteristics and adaptation strategies. Substrate utilization profiling using Biolog EcoPlates™ showed that the isolated strains displayed distinct carbon utilization patterns, with some strains exhibiting specific preferences for aromatic compounds like phenanthrene. This specificity suggests the presence of metabolic pathways tailored for the degradation of hydrocarbons in these microorganisms. Enzymatic assays demonstrated the production of key enzymes involved in phenanthrene degradation, such as dioxygenases, by the isolated strains. The activity of these enzymes was found to be positively correlated with phenanthrene

degradation rates, indicating their importance in facilitating the breakdown of aromatic hydrocarbons.

C. Molecular Identification

Molecular identification of the isolated microorganisms using 16S rRNA gene sequencing revealed their taxonomic affiliations and phylogenetic relationships. The obtained sequences were compared to reference sequences available in public databases to assign taxonomic identities to the isolated strains.

Table 4: Molecular Identification of Isolated Strains

Strain ID	Genus	Species	Closest Match (%)	Phylogenetic Group
Strain 1	Pseudomonas	fluorescens	98	Proteobacteria
Strain 2	Mycobacterium	vanbaalenii	95	Actinobacteria
Strain 3	Bacillus	subtilis	97	Firmicutes
Strain 4	Rhodococcus	erythropolis	96	Actinobacteria
Strain 5	Sphingomonas	paucimobilis	93	Alphaproteobacteria

Phylogenetic analysis showed that the isolated strains belonged to diverse bacterial genera known for their hydrocarbon-degrading abilities, including Pseudomonas, Mycobacterium, and Bacillus. This phylogenetic diversity highlights the widespread distribution of phenanthrene-degrading microorganisms in natural environments and suggests the existence of multiple evolutionary origins for hydrocarbon degradation pathways.

D. Phenanthrene Degradation Assays

Phenanthrene degradation assays confirmed the ability of the isolated microorganisms to degrade phenanthrene under laboratory conditions. Monitoring the degradation kinetics revealed varying rates of phenanthrene degradation among the isolated strains, with some strains exhibiting faster degradation rates compared to others.

Table 5: Phenanthrene Degradation Rates of Isolated Strains

Strain ID	Temperature (°C)	pH	Nutrient Supplementation	Degradation Rate (%)
Strain 1	25	7.0	Nitrogen + Phosphorus	70
Strain 2	30	6.5	None	85
Strain 3	20	8.0	Nitrogen	50
Strain 4	35	7.5	Phosphorus	75
Strain 5	28	7.0	Nitrogen + Phosphorus	65

The degradation of phenanthrene by the isolated microorganisms followed first-order kinetics, with exponential decay of phenanthrene concentrations over time. The degradation rates were influenced by factors such as microbial biomass, substrate concentration, and environmental conditions, highlighting the complexity of phenanthrene degradation processes in microbial communities.

E. Environmental Factors Influencing Phenanthrene Degradation

Investigation of environmental factors influencing phenanthrene degradation revealed the importance of temperature, pH, and nutrient availability in shaping microbial activity and degradation rates. Temperature optimization experiments showed that phenanthrene degradation rates were highest at temperatures ranging from 25°C to 30°C, reflecting the optimal growth temperatures for the isolated strains. pH optimization experiments demonstrated that phenanthrene degradation was most efficient at near-neutral pH conditions (pH 6.5-7.5), consistent with the pH optima of many hydrocarbon-degrading enzymes. Deviations from the optimal pH range resulted in reduced degradation rates, indicating the sensitivity of microbial activity to changes in environmental pH. Nutrient supplementation experiments revealed the role of nitrogen and phosphorus availability in modulating phenanthrene degradation rates. Addition of nitrogen and phosphorus sources enhanced microbial growth and metabolic activity, leading to increased phenanthrene degradation rates in nutrient-rich media.

F. Statistical Analysis

Statistical analysis of the phenanthrene degradation data revealed significant differences in degradation rates between different treatments and environmental conditions. Analysis of variance (ANOVA) indicated that temperature, pH, and nutrient supplementation had significant effects on phenanthrene degradation rates ($p < 0.05$). Post-hoc tests, such as Tukey's honestly significant difference (HSD) test, further elucidated the specific differences between treatment groups and identified optimal conditions for phenanthrene degradation by the isolated microorganisms. The statistical analysis conducted in this study serves to rigorously evaluate the data collected during experimentation, providing insights into the variability, significance, and reliability of the results. Initially, descriptive statistics are employed to summarize the key characteristics of the dataset, including measures of central tendency (such as mean, median) and dispersion (such as standard deviation, range). This allows for a comprehensive understanding of the distribution and variability of the data. Subsequently, inferential statistics are utilized to draw conclusions and make inferences about the population based on the sample data. Hypothesis testing, including t-tests and analysis of variance (ANOVA), is conducted to assess the significance of observed differences between experimental groups or conditions. These tests help determine whether any observed effects are statistically significant or simply due to chance. Additionally, regression analysis may be employed to explore the relationships between variables and predict outcomes based on the observed data. Statistical significance levels, typically set at $\alpha=0.05$, are used to determine whether the results are unlikely to have occurred by random chance alone. Moreover, confidence intervals are calculated to estimate the range within which population parameters, such as means or proportions, are likely to fall. Assumptions underlying the chosen statistical tests, such as normality and homogeneity of variance, are carefully assessed to ensure the validity of the analyses. In cases where assumptions are violated, appropriate corrective measures, such as data transformation or non-parametric tests, may be employed. Ultimately, the statistical analysis provides robust evidence to support the study's findings and conclusions, enhancing the credibility and generalizability of the research outcomes.

IV.Conclusion

The isolation and characterization of phenanthrene-degrading microorganisms from natural sources represent a significant advancement in the field of environmental bioremediation. This study successfully demonstrated the presence of diverse microbial communities capable of metabolizing phenanthrene, a persistent and

toxic polycyclic aromatic hydrocarbon (PAH), under laboratory conditions. Through a combination of enrichment culture techniques, biochemical assays, and molecular identification methods, a range of phenanthrene-degrading strains were isolated and characterized. These strains exhibited varying metabolic capabilities, growth kinetics, and enzymatic activities, highlighting the complexity and diversity of microbial responses to hydrocarbon contamination. The biochemical characterization provided valuable insights into the metabolic pathways and enzymatic mechanisms underlying phenanthrene degradation, shedding light on the microbial strategies for coping with hydrocarbon pollution. The identification of key enzymes, such as dioxygenases, implicated in phenanthrene degradation further elucidated the biochemical basis of microbial hydrocarbon metabolism. Molecular identification using 16S rRNA gene sequencing revealed the taxonomic diversity of the isolated strains, encompassing genera known for their hydrocarbon-degrading abilities, such as Pseudomonas, Mycobacterium, and Bacillus. This phylogenetic diversity underscores the ubiquity of phenanthrene-degrading microorganisms in natural environments and suggests the potential for harnessing microbial diversity for bioremediation applications. Phenanthrene degradation assays confirmed the ability of the isolated strains to degrade phenanthrene under laboratory conditions, with degradation rates influenced by environmental factors such as temperature, pH, and nutrient availability. Optimization of these factors could enhance phenanthrene degradation efficiency and facilitate the development of sustainable bioremediation strategies for PAH-contaminated sites. This study contributes to the growing body of knowledge on microbial-mediated bioremediation and underscores the importance of harnessing microbial diversity for environmental cleanup efforts. The findings offer insights into the potential of naturally occurring microorganisms to mitigate the impact of hydrocarbon pollution on ecosystems, paving the way for the development of eco-friendly and cost-effective remediation technologies. Future research should focus on scaling up microbial bioremediation approaches and evaluating their efficacy in real-world contaminated environments.

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