Biodegradation of petroleum hydrocarbons by mixed bacterial cultures isolated from local contaminated sources

Thesis

submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated

To

All Living Beings Affected by Pollution

May our efforts to understand and mitigate petroleum hydrocarbon pollution lead to a healthier and more harmonious world for every living creature.

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CERTIFICATE

This is to certify that the work contained in the thesis entitled **"Biodegradation of petroleum hydrocarbons by mixed bacterial cultures isolated from local contaminated sources"** submitted by **Rahul Kumar** (Roll No. 166107116) for award of the degree of Doctor of Philosophy, has been carried out under my supervision and this work has not been submitted elsewhere for award of any degree.

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ABSTRACT

Petroleum hydrocarbons, vital for industry, transportation, and daily life, often lead to environmental issues due to spills during production, refining, and transportation of crude oil and its derivatives, including diesel, engine oil, and gasoline. Such pollution harms soil, water ecosystems, human health, and wildlife. Diesel, a significant hydrocarbon, poses threats to both aquatic and terrestrial environments. Offshore oil drilling, transportation, accidents, and wastewater discharge contribute to aquatic pollution, creating persistent, light-blocking films in seawater. Insoluble fractions sink, impeding oxygen and light access, causing harm to aquatic life. Similarly, diesel spills during land transport, fuelling, and industrial discharge contaminate soil, altering its properties and hindering plant and microorganism growth. Diesel's recalcitrance allows it to persist in soil and groundwater for extended periods. To address hydrocarbon pollution, three approaches are available: physical, chemical, and biological methods. Physical treatment, although effective, is costly and requires specialized equipment. Chemical methods, while efficient, necessitate further handling of residual chemicals and sludge. In contrast, biodegradation offers a practical, economical, and environmentally friendly solution. Bacteria, fungi, and algae have demonstrated the ability to degrade diesel, with bacteria being particularly effective. Various bacterial species, such as *Pseudomonas, Bacillus, Acinetobacter, Actinomycetes, Klebsiella, Rhodococcus, Staphylococcus, Micrococcus, Achromobacter*, and *Flavobacterium*, have been isolated from diesel-contaminated sites. Using mixed cultures of these isolated strains enhances diesel degradation, leveraging their diverse metabolic pathways.

The objective of the thesis was to develop an effective bacterial mixed culture using newly isolated bacteria from locally contaminated sources for the degradation of petroleum hydrocarbons and remediation of diesel pollution in soil and water. Emphasis was given on isolating strains from local sources to develop more adaptable and effective strains for local use. For the degradation of petroleum-based hydrocarbons, four bacterial strains were isolated, with two obtained from hydrocarbon-contaminated soil at a local petrol pump and repairing workshop, and the other two from petroleum hydrocarbon-contaminated soil and sludge from a refinery. The selected strains were studied for their ability to degrade various hydrocarbons, and conditions for maximum degradation were determined. Detailed analysis was conducted on the degradation of diesel by the selected strains. Furthermore, the effectiveness of the two strains showing the highest degradation was evaluated in soil, marine water, and freshwater ecosystems. The performance of the mixed culture of these strains was also studied in all three

ecosystems, with the expectation of increased degradation efficiency. A simulated field study was conducted using a mixed culture of all four bacteria in diesel-contaminated water and soil. The treated soil and water were subsequently used for a phytotoxicity test, using *Brassica nigra* (black mustard) plants, to determine the usability of the treated soil and water.

Firstly, two bacteria were isolated from a local petrol pump and repairing workshop. The sourced soil was contaminated by diesel, petrol, and engine oil. Two highest performing bacterial were selected for further study. These two bacteria were identified and named as *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19. These bacteria were found to have a significant ability to degrade diesel, kerosene, engine oil and light paraffin oil. They showed lower degradability for petrol and heavy paraffin. Lower degradation might be due to the toxicity of the compounds present in petrol and the high viscosity of heavy paraffin. The effects of process parameters on degradation of petroleum hydrocarbons were investigated. The degradation was observed to be higher for *Acinetobacter baumannii* IITG19. These isolated bacteria gave the best growth at 35°C, pH of 7, and inoculum concentration was 1 % v/v. At a substrate concentration of 4-5%, the *Acinetobacter baumannii* IITG19 and *Klebsiella michiganensis* RK, showed the highest. growth. For diesel after 15 days of incubation period, *Klebsiella michiganensis* RK *and Acinetobacter baumannii* IITG19 showed overall degradation percentages of 60 and 76 %, respectively. For the significant (≥0.5) compounds in diesel, the degradation reached up to 77 and 90% for *Klebsiella michiganensis* RK *and Acinetobacter baumannii* IITG, respectively. It was also observed that isolated bacteria were able to degrade paraffins more than the naphthenic and aromatics.

Another set of two diesel-degrading strains isolated from local refinery waste. The strains showing highest degradation and growth for diesel were identified and names as *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20. They were isolated from the soil and sludge of a refinery effluent treatment plant, respectively. The degradation efficiency was observed to be higher for diesel, kerosene, and engine oil compared to that of gasoline. The most suitable conditions for growth and diesel degradation for the selected strains and their mixed culture were found to be 4% v/v initial diesel concentration, 37°C temperature, pH 7, and 1% v/v initial inoculum concentration. *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 showed diesel degradation of 70% and 76%, respectively, during 15-day incubation period under the most suitable conditions. When a mixed culture of these two strains was used, degradation increased further to 85%. *Providencia vermicola* IITG20 exhibited

higher degradation for n-alkanes and aromatics, while *Pseudomonas aeruginosa* IITG21 showed higher degradation for branched alkanes and naphthenes. Degradation was further increased for all types of components of diesel in the presence of mixed culture. *Pseudomonas aeruginosa* IITG21 showed lower degradation of n-alkanes (69%) compared to *Providencia vermicola* IITG20 *(77%)*, but the mixed culture increased degradation to 84%. For branched alkanes, degradation percentages were 77, 74, and 87% for *Pseudomonas aeruginosa* IITG21, *Providencia vermicola* IITG20, and mixed culture, respectively. Naphthenes degradation percentages were 84, 75, and 84% for *Pseudomonas aeruginosa* IITG21, *Providencia vermicola* IITG20, and mixed culture, respectively. For aromatics, *Pseudomonas aeruginosa* IITG21 exhibited lower degradation (61%) compared to *Providencia vermicola* IITG20 (87%), but the mixed culture increased degradation to 93%. For the degradation of individual components of diesel, bacterial mixed culture was found to be most effective as degradation was in the range of 84 – 93%. The rate of diesel degradation was highest in presence of bacterial mixed culture as compared to pure cultures. Rate constant k was found to be highest for bacterial mixed culture (0.154 day^{-1}) compared to the pure culture of both bacteria. Bacterial mixed culture also showed lowest half-life as compared to pure culture of both bacteria. So, bacterial mixed culture was more effective for diesel degradation compared to pure culture.

The study extended to assess the effectiveness of *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 in soil and water ecosystems. These bacteria were selected due to their high degradation efficiency for diesel. The study of diesel degradation was conducted in soil, marine water, and freshwater ecosystems. The study included the assessment of bacterial growth, diesel degradation, and kinetics in each ecosystem. Additionally, their mixed culture was used for the same purpose. The experimental conditions consisted of an initial diesel concentration of 4% v/v, a temperature of 35°C, a pH of 7, and an inoculum concentration of 1% v/v, with an incubation period of 15 days. Both the individual bacteria and the mixed culture showed effectiveness in all three ecosystems, with the freshwater ecosystem showing the highest degradation. *Acinetobacter baumannii* IITG19 showed higher degradation than *Providencia vermicola* IITG20 in all ecosystems. *Acinetobacter baumannii* IITG19 showed degradation percentages of 59%, 62%, and 76%, while *Providencia vermicola* IITG20 showed 31%, 57%, and 69% in soil, marine water, and freshwater, respectively. Degradation of alkanes was higher compared to that of naphthenes and aromatics for both strains. However, *Providencia vermicola* IITG20 showed higher selectivity towards the degradation of naphthenes and aromatics. The higher degradation observed in aquatic ecosystems compared

to terrestrial ecosystems may be attributed to the lower bioavailability of hydrocarbons and the slower growth of bacterial strains in the latter. The presence of intermediate compounds, such as primary alcohols, secondary alcohols, ketones, and esters, confirmed the alcoholic degradation pathways of alkanes and the cyclo-alcoholic pathway of naphthenes. The mixed culture, consisting of both bacteria in equal proportions, showed higher efficiency in diesel degradation compared to the individual strains. Overall, the degradation reached 66, 66, and 81% in the soil, marine water, and freshwater ecosystems, respectively, with over 90% degradation achieved for alkanes. The values of the rate constant (k) for both bacteria and their mixed culture were highest in the freshwater ecosystem and lowest in the soil ecosystem. It was observed that the rate constant (k) was highest for the mixed culture, followed by *Acinetobacter baumannii* IITG19, and lowest for *Providencia vermicola* IITG20 across all ecosystems. The higher range of k values (0.057-0.095 day^{-1}) for *Acinetobacter baumannii* IITG19 compared to *Providencia vermicola* IITG20 (0.025-0.082 day^{-1}) across all ecosystems indicates faster kinetics for the former. An increased range of k values was observed for the mixed culture (0.066-0.11 day^{-1}). This higher value of k suggests higher degradation and a shorter half-life of diesel. The half-life for *Providencia vermicola* IITG20 was 27.4 days in soil, 14 days in marine water, and 8.5 days in freshwater. *Acinetobacter baumannii* IITG19 showed a half-life of 12.1 days in soil, 10.5 days in marine water, and 7.4 days in freshwater. The half-life was further reduced when the mixed culture was used for diesel degradation, resulting in a half-life of 10.6 days in the soil ecosystem, 8.9 days in marine water, and 6.3 days in freshwater ecosystems.

Furthermore, a mixed culture containing all the four newly isolated *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21, and *Klebsiella michiganensis* RK, was used for the remediation of diesel contaminated soil and water. In-Lab experiments highlighted the superior efficacy of this mixed culture, showing enhanced diesel degradation compared to the pure cultures. During in-Lab experiments, the mixed culture showed 90% degradation of 4% v/v diesel over 15 days, with 35°C temperature, pH 7, and 1% v/v inoculum concentration.

Subsequently simulated field studies were carried out using the same mixed culture four newly isolated bacterial strains to study their effectiveness in atmospheric environment. The overall diesel degradation by the mixed culture was observed to be 96% in soil and 99% in water. For different type of hydrocarbons, the mixed culture showed over 97% degradation of n-alkanes

and branched alkanes, and more than 96% degradation of naphthenes and aromatics in soil. In water, over 99% degradation was observed for all type of hydrocarbons.

Analysis of degradation kinetic confirmed first-order degradation with a rate constant (k) of 0.144 day^{-1} in-Lab conditions. Field studies yielded values of 0.196 day^{-1} for soil and 0.288 day^{-1} for water. Mixed culture showed half-lives of 4.7, 3.5, and 2.4 days in in-Lab conditions, soil field and water field, respectively. This study underlined the potential of the mixed bacterial culture for diesel contamination mitigation by integrating simulated field studies and phytotoxicity tests.

Phytotoxicity study on *Brassica nigra* seed growth in various conditions showed the efficacy of a mixed bacterial culture in remediating diesel-contaminated soil and water. Uncontaminated soil and water supported maximum growth, yielding 80% seed germination, a final shoot height of 13.4 cm, and a root length of 5.4 cm after 15 days. whereas, contaminated soil showed no seed germination due to diesel toxicity. Mixed culture-treated soil and water exhibited positive growth with 68% and 70% germination rates, respectively, indicating the successful removal of diesel toxicity. The growth in mixed culture-treated conditions, although slightly lower than uncontaminated ones, showcased the potential for reusing treated soil and water for agricultural purposes. Abiotic control samples without mixed culture treatment showed no seed germination, emphasizing the crucial role of bacteria in effective remediation.

The present work bridges laboratory experiments with real-world applications, paving the way for effective environmental solutions. The study successfully isolated highly effective four bacterial strains: *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21, and *Klebsiella michiganensis* RK. A mixed culture of these strains achieved almost 100% degradation of diesel in the simulated field study. The mixed culture of these strains can be effectively used for the remediation of diesel contamination in soil and water ecosystems. The findings confirmed the ecological compatibility of treated soil and water as they can be further utilized successfully for growing agricultural plants. It is noteworthy that *Klebsiella michiganensis* RK is reported for the first time for hydrocarbon degradation applications. In the future, a pilot-scale study can be performed on petroleum hydrocarbon-contaminated soil and water. The pilot study can include industrial bioreactors or actual contaminated sites.

RESEARCH OUTPUTS

Publications

1. Kumar, R., & De, M. (2023). Enhanced degradation of petroleum hydrocarbons by *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 isolated from local soil sources. *International Journal of Environmental Science and Technology*, 1-12.
2. Kumar, R., & De, M. (2024). Enhanced Bioremediation of Commercial Diesel Contamination by Mixed Culture of Newly Isolated Providencia vermicola IITG20 and Pseudomonas aeruginosa IITG21 from Local Refinery Waste. *International Journal of Environmental Research*, *18*(4), 1-21.
3. Kumar, R., & De, M. (2024). Simultaneous bioremediation of diesel-contaminated soil and water ecosystems using mixed culture of Acinetobacter baumannii IITG19 and Providencia vermicola IITG20. *Environmental Technology*, 1-18.
4. Kumar, R., & De, M., Efficient Bacterial mixed culture for remediation of diesel contaminated soil and water: field study and phytotoxicity test (under review)

Conferences

1. Rahul Kumar and Mahuya De, International Conference on Engineering Sciences and Technologies for Environmental Care (ESTEC 2020), "Isolation and Screening of Petroleum Hydrocarbon Degrading Bacteria." (20-22 February 2020).
2. Rahul Kumar and Mahuya De, International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health (BREEECH 2021), "Exposure of Hydrocarbons on *Klebsiella Michiganensis* and its effect on the petroleum hydrocarbon utilization "(01-04 December 2021).
3. Rahul Kumar and Mahuya De, BioSangam 2022- an International conference on Emerging Trends in Biotechnology, "Removal of Toluene from contaminated water by using isolated bacteria *Klebsiella* sp. And *Acinetobacter* sp." (10-12 March 2022).
4. Rahul Kumar and Mahuya De, 62nd Annual International Conference of Association of Microbiologists of India (AMI), "Surfactant Assisted biodegradation of petroleum hydrocarbon by bacterial consortia" (21-23 September 2022).
5. Rahul Kumar and Mahuya de, International Conference on petroleum, hydrogen & decarbonization (ICPHD 2023), "Degradation Kinetics Study of Diesel by Mixed Culture of *Acinetobacter baumannii* IITG19and *Klebsiella michiganensis* RK (3-5

November 2023).

Sequence Submitted to NCBI database

1. Kumar,R. and De,M., *Klebsiella michiganensis* strain RK, 16S ribosomal RNA gene, partial sequence, SUB6614979 Klebsiella_michiganesis_RK **MN749814**, Dec 30, 2020 ;(https://www.ncbi.nlm.nih.gov/nuccore/1779613826).
2. Kumar,R. and De,M., *Acinetobacter baumannii* strain IITG19, 16S ribosomal RNA gene, partial sequence, SUB6614873 Acinetobacter_baumannii_iitg19, **OK310719,** Dec 31, 2022;(https://www.ncbi.nlm.nih.gov/nuccore/OK310719).
3. Kumar,R. and De,M., *Providencia vermicola* strain IITG20, 16S ribosomal RNA gene, partial sequence, SUB11908050 Providencia_vermicola_IITG20, **OP174960,** Mar 24, 2023;(https://www.ncbi.nlm.nih.gov/nuccore/ OP174960).
4. Kumar,R. and De,M., *Pseudomonas_aeruginosa* strain IITG21, 16S ribosomal RNA gene, partial sequence, SUB11907977 Pseudomonas_aeruginosa_IITG21, **OP174927,** Mar 24, 2023;(https://www.ncbi.nlm.nih.gov/nuccore/ OP174927

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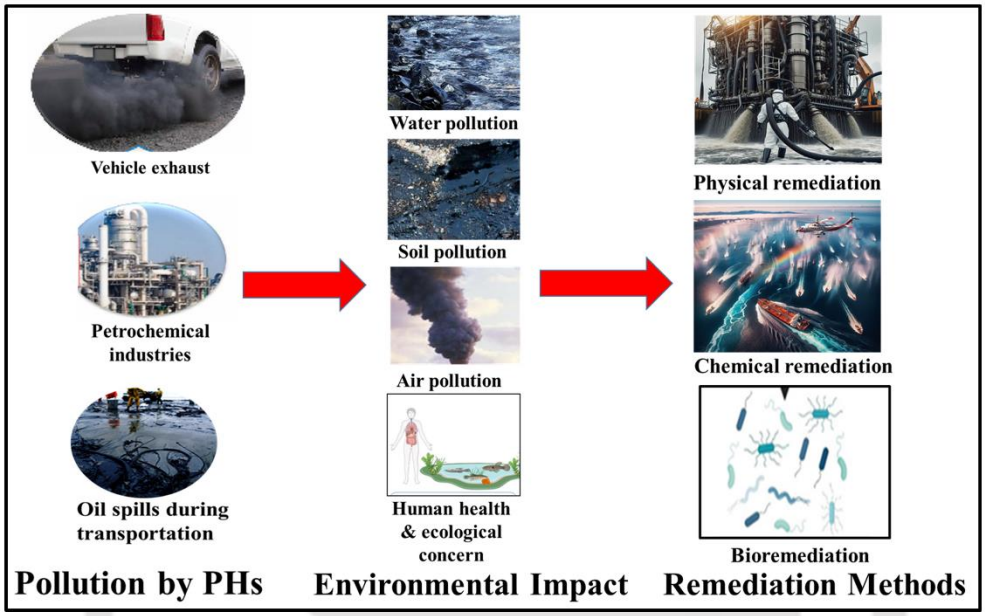
List of Abbreviations

BH	Bushnell Hass
GCMS	Gas chromatography Mass spectroscopy
LB	Luria Bertani
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
PHs	Petroleum Hydrocarbons

Chapter 1

Introduction



Chapter 1 discusses various petroleum hydrocarbons (PHs), their sources and types, which have harmful impacts on the environment. Remediation methods have also been discussed. Bioremediation, especially microbial biodegradation, offers a promising solution to address petroleum hydrocarbon contamination by harnessing the capabilities of microorganisms to restore the environment.

Keywords: Petroleum hydrocarbons, Pollution, Bioremediation, Microbial biodegradation, Bacteria

Chapter 1: Introduction

1.1 Pollution by petroleum hydrocarbons

Petroleum hydrocarbons (PHs) are regarded as the main source of energy and raw materials for various industries (Ambaye et al. 2022). They are also the main sources of industrial and transportation fuels. However, use of petroleum hydrocarbons in various applications are associated with many environmental hazards. Petroleum hydrocarbons are major source of environmental contaminants, commonly resulting from substantial inland and offshore activities such as production, transportation, storage, and refining. In addition, unintentional accidents have caused huge contamination in limited zones (Al-Hawash et al. 2018). Municipal run-offs, petroleum products, such as petrol and diesel spills, and industrial processes, all contribute to PHs pollution as well (Zhang et al. 2019). Frequent inadvertent and/or illegal oil waste discharge at sea also result in large PHs contamination. All these contaminations negatively influence the ecosystem and cause direct or indirect health risks to diverse life forms (Sajna et al. 2015, Fatehi et al. 2021). Petroleum hydrocarbons are thus hazardous substances that are regarded as priority pollutants (Costa et al. 2012).

Among these hydrocarbons, diesel has extensive use as fuels in both transportation and industry. Activities, such as offshore oil drilling, transportation, wastewater discharge containing diesel, accidents spills, and indirect contamination from ship disassembly, contribute immensely to the pollution of the aquatic ecosystem. (Hazaimeh et al. 2021). Diesel spills during land transportation, fuel station filling, industrial wastewater discharge containing diesel etc., also collectively cause diesel pollution in the soil ecosystem (Koshlaf et al. 2020). The urgency of addressing these issues is pressing, as it not only impacts the environment but also has implications for human health and well-being.

1.2 Sources of petroleum hydrocarbon pollution

Petroleum hydrocarbon pollution has emerged as a significant environmental concern, originating from various sources. The release of these compounds into ecosystems can results in extensive environmental degradation, impacting quality of soil, water bodies, and air (Manisalidis et al. 2020). Regular incidents of leaks and accidental spills are common in various stages of petroleum-related processes, including exploration, production, refining, transportation, and storage. Kuppusamy et al. (2020) estimated that approximately 600,000 metric tons of natural crude oil seepage occurs annually. Whether these occurrences are accidental or a result of human activities, the discharge of hydrocarbons into the environment

persists as a leading factor in water and soil pollution. Fig. 1.1. shows different sources of petroleum hydrocarbon pollutions that are caused both by of human activities and natural processes.

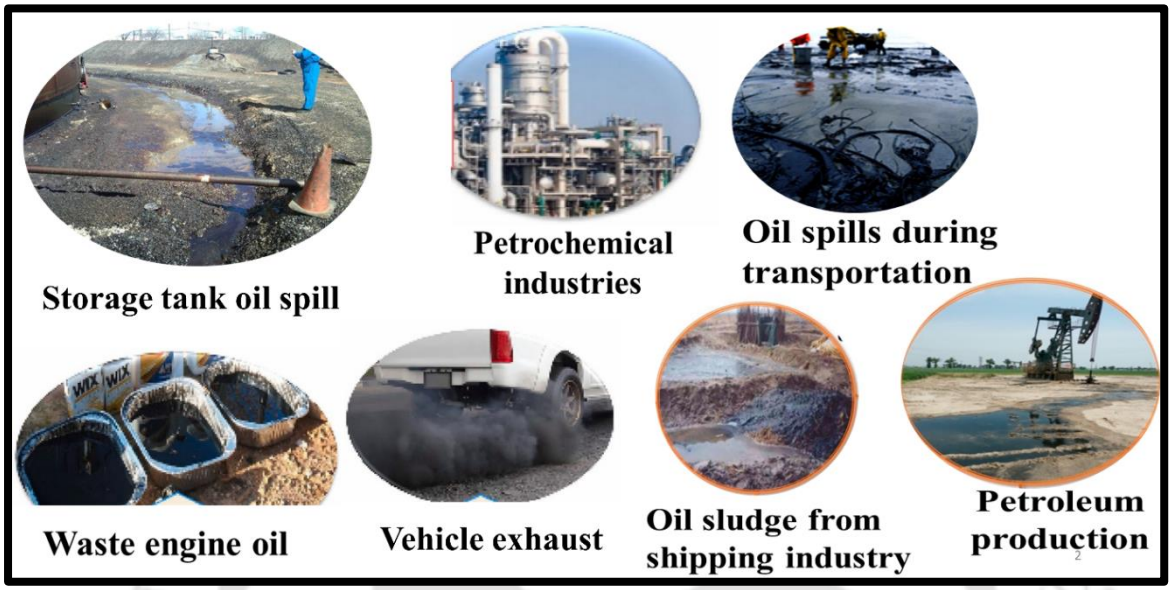


Fig. 1.1. Different sources of petroleum hydrocarbon pollution.

1.2.1 Industrial activities

Industrial activities significantly contribute to petroleum hydrocarbon pollution. Processes like oil refining, petrochemical manufacturing, and the operation of heavy machinery are prone to accidental spills, leaks, and emissions. Inadequate disposal of waste from these industries can release hydrocarbons into the environment, impacting both terrestrial and aquatic ecosystems (Speight 2020).

1.2.2 Transportation

The transportation sector, including automobiles, trucks, ships, and airplanes, is a primary source of hydrocarbon pollution. Combustion of fossil fuels in engines releases hydrocarbons as well as other pollutants into the atmosphere. Additionally, spills during the transportation of petroleum products, such as oil tankers and pipelines, can lead to the direct contamination of water bodies (Bhattacharjee et al. 2022).

1.2.3 Urban and residential activities

Urbanisation and residential activities contribute to hydrocarbon pollution through vehicular emissions, heating, and cooking. These activities release volatile organic compounds (VOCs) and other hydrocarbons into the atmosphere, affecting air quality in urban areas. Runoff from roads and paved surfaces can transport hydrocarbons into water bodies, exacerbating contamination (Zhou et al. 2022).

1.2.4 Natural sources

While human activities are major contributors to hydrocarbon pollution, natural sources also play a role. Natural seepage of oil and gas from underground reservoirs, as well as the decomposition of organic matter, can release hydrocarbons into the environment. While these natural sources have existed for millennia, human activities have accelerated the release of hydrocarbons, leading to imbalance in ecosystems (Patel et al. 2020).

1.2.5 Accidental spills and disasters

Accidental spills during the extraction, transportation, and storage of petroleum products can result in large-scale pollution. Major oil spills from offshore drilling platforms, tanker accidents, and pipeline ruptures can have disastrous effects on marine and coastal environments, causing long-lasting ecological and economic damage (Ogolo et al. 2022).

1.3 Environmental impact of petroleum hydrocarbon pollution

Petroleum hydrocarbon pollution may impact all ecosystems. The soil, water, and air quality can be affected intensely, posing ecological and health challenges.

1.3.1 Soil pollution

Petroleum hydrocarbon pollution severely degrades quality of soil. Contaminated soil experiences reduced fertility, permeability, water retention, and binding capacity (Banerjee et al. 2016). Hydrocarbon deposition alters soil properties. Water retention and gas exchange properties reduce, disrupting microbial activity essential for nutrient cycles. Contamination poses risks to local ecosystems, causing harm to plant and animals, and polluting groundwater. Certain hydrocarbons are carcinogenic and neurotoxic (Das and Chandran 2011), exacerbating long-term soil degradation and impacting agricultural productivity and biodiversity.

1.3.2 Water pollution

Petroleum hydrocarbons in aquatic ecosystems imperil water quality. Accidental spills, runoff, and industrial discharges contaminate rivers, lakes, and oceans, endangering aquatic life. Oil spills, from transportation or accidents, threaten coastlines. Floating hydrocarbons form surface films, affecting oxygen transfer and natural matter. Heavier fractions settle in sediment, impacting bottom-feeding species (Fowzia et al. 2018). Insoluble oil film on ocean surface prevents penetration of light and oxygen, leading to species extinction and reduced microbial populations (Beyer et al. 2016).

1.3.3 Air pollution

Volatile hydrocarbons, released into the atmosphere, contribute to air pollution. Vehicle emissions, industrial activities, and hydrocarbon evaporation from spills are primary sources of air contaminations. The interaction between hydrocarbons and nitrogen oxides under sunlight conditions has the potential to generate ground-level ozone. This ozone, a harmful component of smog that negatively impacts respiratory health and ecosystems (Saxena et al. 2019).

1.3.4 Ecological disruption

The intricate interplay of organisms within ecosystems is disturbed by hydrocarbon pollution. Microbial communities that play pivotal roles in nutrient cycles and ecosystem balance are adversely affected. In aquatic systems, hydrocarbon contamination can lead to poisoning of fishes, reduced biodiversity, and altered species composition. Terrestrial ecosystems experience shifts in plant communities and decreased soil health, influencing the overall stability of ecosystems (Truskewycz et al. 2019).

1.3.5 Human health concerns

The far-reaching consequences of petroleum hydrocarbon pollution extend to human health. Inhalation of volatile organic compounds (VOCs) and airborne particulates, coupled with exposure through contaminated water and food sources, poses risks to human well-being (Marris et al. 2020). Health concerns range from respiratory issues and skin irritation to more severe conditions such as cancer and developmental abnormalities. These pollutants have the potential to harm diverse human body systems, such as circulatory, nervous, endocrine and immune systems. Such damage can lead to various metabolic or hormonal disorders and diseases (Kuppusamy et al. 2020).

1.4 Petroleum hydrocarbon pollutants

Petroleum hydrocarbons sourced from crude oil, are diverse compounds, shaping modern civilisation. Petroleum hydrocarbons consist of aliphatic, naphthenic and aromatic compounds, each with distinct degradation challenges. Subsections explore these petroleum hydrocarbon categories.

1.4.1 Crude oil

Crude oil is a complex mixture of hydrocarbons, including various paraffins, naphthenes, aromatics and asphaltics fractions, in addition to compounds containing nitrogen, oxygen and sulfur. Crude oil is classified into three main categories based on its weight: light, medium, and heavy components (Scholz et al. 1999). Lighter fractions such as alkanes with low carbon number readily degrade, while heavier fractions such as bitumen, due to higher molecular weights and lower solubility, resist microbial degradation. Different hydrocarbons that commonly causes pollutions are discussed below.

1.4.2 Diesel

Diesel, a vital fuel for transportation and industrial processes, comprises a mixture of aliphatic and aromatic compounds, along with additives. The complexity of diesel hydrocarbons poses challenges for degradation due to the presence of aromatic compounds and long-chain alkanes (Fowzia et al. 2018).

1.4.3 Engine oil

Engine oil, essential for lubrication in machinery and vehicles, consists of base oils and additives. The base oils can be mineral-based or synthetic, influencing their biodegradability. Additives, while enhancing engine performance, can introduce complexity to the oil composition. The presence of synthetic components and additives can pose challenges to rapid biodegradation (Ismail 2022).

1.4.4 Kerosene

Kerosene, used in aviation and domestic applications, consists of aliphatic and aromatic hydrocarbons, with varying degradation potential. The presence of simpler aliphatic compounds aids microbial degradation, but persistent aromatics pose challenges (Lednev et al. 2021).

1.4.5 Petrol (Gasoline)

Petrol, a widely used automotive fuel, primarily consists of volatile aliphatic hydrocarbons, enabling faster evaporation and combustion due to its simpler composition compared to diesel. This characteristic, along with a lower boiling point, renders petrol less persistent in the environment making it easier to degrade than diesel. The predominance of simpler aliphatic hydrocarbons in petrol contributes to its enhanced biodegradability (Yang et al. 2020).

1.5 Remediation methods for petroleum hydrocarbons

To address petroleum hydrocarbon contamination, diverse remediation methods, including physical, chemical, and biological approaches, have been developed. Bioremediation stands out as an eco-friendly solution, effectively degrading pollutants.

1.5.1 Physical remediation methods

Physical remediation methods involve removing or containing contaminated materials, using techniques like excavation, dredging, and soil vapour extraction (SVE). Excavation displaces polluted soil, typically followed by treatment or disposal. Dredging removes contaminated sediments from water bodies. SVE uses vacuum systems to extract volatile hydrocarbons. These methods, though effective, can be disruptive and resource-intensive. It may not guarantee complete pollutant removal, leaving residual contamination concerns (Ansari et al. 2018).

1.5.2 Chemical remediation methods

Chemical remediation methods involve altering the chemical structure of hydrocarbon pollutants to reduce their impact. Chemical oxidation uses reactive compounds like hydrogen peroxide or ozone to break down hydrocarbons into non-toxic by-products. In situ chemical oxidation introduces reactive chemicals directly into the contaminated area. Though effective, these methods may produce secondary pollutants, requiring careful management to prevent unintended ecological consequences and ensure overall pollution mitigation effectiveness (Parach et al. 2017)

1.5.3 Bioremediation methods

Bioremediation harnesses living organisms to address environmental contamination, offering eco-friendly and sustainable pollution management (Ra et al. 2019). It relies on natural processes, breaking down pollutants into harmless by-products while minimising site

disturbance and promoting ecosystem restoration. Bioremediation demands fewer resources than traditional methods, yielding cost savings. Its diverse techniques include phytoremediation using plants, microbial remediation with microorganisms, land farming in controlled conditions, in situ bioremediation at the contamination site, and ex situ bioremediation in controlled environments, all capable of long-term pollutant degradation and contaminant specificity (Jimoh et al. 2022).

1.6 Microbial bioremediation

Microbial bioremediation is a potent and sustainable solution to mitigate petroleum hydrocarbon contamination. It depends on the metabolic ability of microorganisms, using their capacity to convert pollutants and restore the environment (Aboulkacem et al. 2023). Microbes thrive on hydrocarbon degradation, utilising these contaminants for growth, reproduction, and energy, with their activity influenced by environmental factors. Various microorganisms show enzymatic capabilities for the degradation of hydrocarbons. Few can degrade alkanes, while others are more capable for degradation of aromatic compounds, with shorter alkanes and less toxic aromatics being more easily biodegradable (Hamme et al. 2003). The effectiveness of bioremediation depends on the synergistic presence of different microorganisms, capable of degrading different hydrocarbons (Santisi et al. 2015).

Microorganisms perform bioremediation by metabolising hydrocarbons into less harmful compounds (Pandey et al. 2009). Their adaptability, rapid growth, and versatile degradation capabilities make them indispensable. Microbial bioremediation is further enhanced by mechanisms like bio-stimulation and bio-augmentation, which promote degradation rates, rendering it a durable, flexible, and effective strategy for pollution remediation (Abatenh et al. 2017). Microbial bioremediation can be further classified as follows:

1.6.1 Biotransformation

Biotransformation, a pivotal process within microbial bioremediation, involves the modification of pollutant molecules into forms that are less hazardous or harmless, rendering them less toxic or easier to degrade (Sakshi and Haritash 2020). This transformation reduces their ecological and human health risks.

1.6.2 Mineralisation

Mineralisation is a type of microbial bioremediation, in which complete degradation of organic pollutant into inorganic elements such as carbon dioxide (CO_2) or water (H_2O) occurs. This ultimate transformation contributes to the closure of the pollutant cycle, converting complex hydrocarbons into simple end products. Mineralisation represents a key objective in achieving the comprehensive removal of pollutants from the environment (Gaur et al. 2018).

1.6.3 Biodegradation

Biodegradation, a fundamental mechanism of microbial bioremediation, involves the degradation of organic contaminants into smaller organic or inorganic molecules. Microorganisms metabolise complex hydrocarbons, breaking them down into simpler compounds through enzymatic actions, also capable of completely degrading hydrocarbons to CO_2 and water (Hussain et al. 2018). This process not only reduces the concentration of pollutants but also facilitates their eventual integration into natural biogeochemical cycles. Various microorganisms such as bacteria, fungi, and algae, demonstrate the capability to utilise petroleum hydrocarbons as their exclusive source of energy in metabolic processes.

Fungi, a diverse group of microorganisms, including genera like *Aspergillus, Cephalosporium, Penicillium, Pichia, Amorphoteca, Neosartorya, Graphium, Talaromycesw, Candida*, and *Yarrowia,* contribute significantly to microbial biodegradation. They thrive in contaminated environments and secrete enzymes that break down hydrocarbons, making them potent agents in bioremediation (Imam et al. 2022). However, their relatively slower growth and sensitivity to environmental variables may limit their applicability in certain scenarios (Adeleye et al. 2018; Mahmoud and Bagy 2018; Sharma et al. 2019).

Algae, mainly found in aquatic environments are promising for water remediation. They can effectively absorb and metabolise hydrocarbon pollutants (Touliabah et al. 2022). Their rapid growth and photosynthesis detoxify water contaminated by petroleum (Ravishankar and Ambati 2019). However, challenges include hydrocarbon specificity and algal blooms (Alazaiza et al. 2022). Algae like *Chlorella vulgaris*, *Scenedesmus* sp., *Selenastrum capricornutum, S. costatum*, and *Nitzschia* are reported for hydrocarbon degradation (García et al. 2021; Othman et al. 2023).

Bacteria has been reported to have high efficiency for biodegradation of petroleum hydrocarbons. Compared to fungi and algae, bacteria often exhibit faster degradation rates and

efficient conversion of hydrocarbons into less harmful by-products (Gaur et al. 2018). Bacteria, diverse and adaptable, are pivotal in microbial biodegradation, serving as prime degraders of petroleum contaminants present in the soil and water ecosystems (Brooijmans et al. 2009; Quintella et al. 2019). Many bacteria such *as Arthrobacter*, *Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, Rhodococcus Actinomycetes, Klebsiella,* and *Acetobacter*, have ability to degrade petroleum hydrocarbons (Jones et al. 2008; Imron et al. 2020).

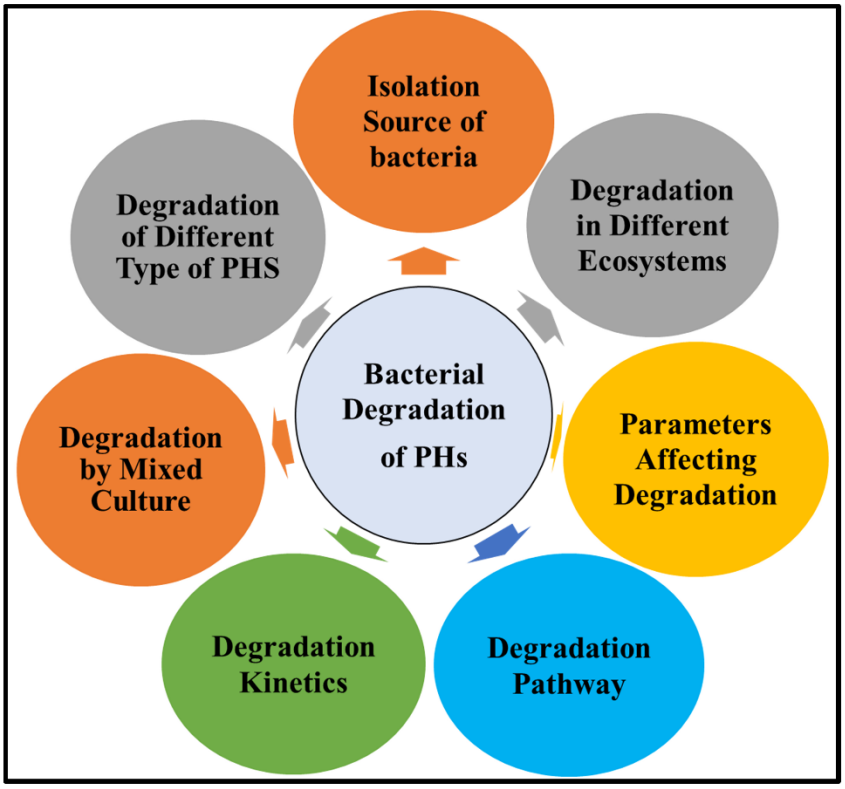
Bacterial communities, including species like *Achromobacter, Acinetobacter, Alkanindiges, Cycloclasticus, Marinobacter,* and *Thallassolituus*, play a pivotal role in degrading petroleum pollutants within ecosystems (Shapiro et al. 2021). These specialised microorganisms thrive in oil-rich environments, with their abundance and diversity closely tied to pollutant levels and environmental factors (Ratnasari et al. 2021; Travkin and Solyanikova, 2021). These bacteria utilise oxidation processes, often facilitated by diverse enzymes, to degrade different hydrocarbon components, including aromatic and aliphatic compounds, as well as polyaromatic hydrocarbons (Abbasian et al. 2015).

Summary

Petroleum hydrocarbons, derived from crude oil, serve as vital energy and material sources for multiple industries but give rise to environmental hazards through production, transportation, refining, and accidental spills. Pollution sources such as industrial activities, transportation, urban processes, natural seepage, and spills, lead to substantial ecological and health consequences. The environmental impact encompasses soil degradation, water and air pollution, ecological disturbances, and health concerns. Diverse petroleum hydrocarbon pollutants pose unique degradation challenges and can be addressed through physical, chemical, and bioremediation methods. Bioremediation, particularly microbial bioremediation, utilising fungi, algae, and bacteria, plays a significant role in mitigating contamination. Bacteria efficiently degrade petroleum hydrocarbons into non-toxic compounds.

Chapter 2

Literature Review



Chapter 2 presents a brief overview of studies reported on bacterial degradation of hydrocarbons in the presence of various bacterial strains. The chapter discusses the importance of sources for bacterial isolations and diesel degradation in various ecosystems. It explores the influence of parameters such as community composition, temperature, and pH on hydrocarbon degradation. The superior performance of mixed bacterial cultures has been highlighted. The degradation kinetics and pathways have been discussed. The detailed discussion has helped to identify the crucial research gaps and formulate the objectives of the work. This chapter serves as a foundation for developing effective bioremediation strategies.

Keywords: Bacterial degradation; process parameters; different ecosystems; Mixed culture; Degradation kinetics

Chapter 2: Literature Review

2.1 Bacterial degradation

Bacterial degradation of petroleum hydrocarbons is a crucial process in bioremediation, particularly in the context of oil spills and contaminated sites. Certain bacteria are able to degrade hydrocarbons by utilizing them as cellular nutrients. The energy stored in hydrocarbon bonds is harnessed by the cells to produce adenosine triphosphate (ATP), which in turn powers various cellular processes. Hydrocarbons also serve as a carbon source for biosynthesis, contributing to the construction of cell components (Bharali et al., 2023). Bacteria can degrade hydrocarbons without generating hazardous materials, making this process environmentally friendly (Ławniczak et al. 2020). Various bacteria, including *Acinetobacter, Burkholderia, Pseudomonas, Staphylococcus, Streptobacillus, Rhodococcus, Streptococcus* showed significant degradation for various petroleum hydrocarbons such as diesel oil, crude oil, alkanes, aromatic compound and poly aromatic compounds (Margesin et al. 2003; Jin et al. 2012; Chaerun et al. 2004; Nie et al. 2014; Xue at al. 2015; Vajrani and Upasani 2017).

Various studies have examined the biodegradation of diesel by different bacterial species and reported degradation is in the range of 27-90%. Differences in the degradation is due to variation in the different parameters such as temperature. pH, initial hydrocarbon concertation and type of ecosystem (soil, marine water or freshwater) (Bacosa et al. 2022). Some of the reported studies on bacterial degradation have been outlined in Table 2.1. Borah and Yadav (2014) found that *Bacillus cereus DRDU1* achieved 71% degradation of diesel in 4 weeks at initial diesel concentration of 2%. Nkem et al. (2016) reported 58 % degradation of diesel oil *by Acinetobacter baumannii* in 2% v/v initial concentration and 10 days of incubation period. You et al. (2018) reported 58 % degradation of diesel by *Pseudomonas aeruginosa* in 14 days at initial concentration of 1.5 % v/v. Sun et al. (2018) observed that *Pseudomonas* sp. *CQ2* degraded 55 % diesel in 14 days when 2% v/v initial concentration of diesel was used. Bekele et al. (2020) observed similar extent of degradation, 84 and 83.4% for *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively, at initial diesel concentration of 3%. The incubation time was maintained at 15 days for this study.

Acinetobacter sp. K-6 showed 59% degradation of diesel in 14 days when an initial concentration of 0.24% v/v diesel was used (Chaudhary et al. 2020). Jerin et al. (2021) reported 52 and 61% degradation of diesel by *Cedecea davisae* and *Acinetobacter baumannii*, respectively in 21 days. For their study, an initial concentration of 10% v/v diesel was used.

Gao et al. (2021) reported 72% degradation of diesel by *Bacillus megaterium MJ4* in 5 days when 1 % initial diesel concentration was used. *Pseudomonas aeruginosa* AHV-KH10 demonstrated 70% degradation of 1% v/v diesel in 35 days at 31 °C (Pourfadakari et al. 2021). About 14% degradation of diesel was observed by the *Pseudomonas* sp in 7 days when 4% v/v initial concentration of diesel was used (Hossain et al. 2022).

Acinetobacter baumannii has shown degradation for crude oil, in the range of 42 to 58% in studies by Mishra et al. (2004), Chang et al. (2011), and Zhang et al. (2021). Additionally, Nnabuife et al. (2022) reported that *Pseudomonas aeruginosa* W15 and *Providencia vermicola* W8, isolated from crude oil-contaminated soil, demonstrated 48% and 46% crude oil degradation, respectively, for a 3% (v/v) crude oil concentration over 28 days at 30°C.

Table 2.1: Reported studies on bacterial degradation of diesel.

S.N.	Bacteria	Conc. of diesel (v/v) and degradation time	Degradation %	Ref.
1	*Bacillus cereus DRDU1*	2% and 28 days	71	Borah and Yadav 2014
2	*Acinetobacter baumannii*	2% and 10 days	58	Nkem et al. 2016
3	*Acinetobacter* sp. K-6	0.24% and 14 days	59	Chaudhary et al. 2020
4	*Acinetobacter baumannii*	10% and 21 days	61	Jerin et al. 2021
	Cedecea davisae		52	
5	*Bacillus megaterium MJ4*	1% and 5 days	72	Gao et al. 2021
6	*Pseudomonas sp*	4% and 7 days	14	Hossain et al. 2022
7	*Pseudomonas aeruginosa*	3% and 15 days	84	Bekele et al. 2022
	Bacillus subtilis		83	

As observed by the above studies, efficiency of bacterial degradation of petroleum hydrocarbons is influenced by several key parameters. Higher initial concentrations of hydrocarbons result in slower degradation rates, as showed by studies by Jerin et al. (2021) and Nkem et al. (2016). Additionally, the duration of the incubation period and the temperature at which the degradation occurs play significant roles, with longer incubation periods and

temperature above 30 °C, generally leading to increased degradation, as observed in studies by Pourfadakari et al. (2021) and Hossain et al. (2022).

2.2 Degradation of different types of petroleum hydrocarbons

Various bacteria are actively involved in the degradation of petroleum hydrocarbon compounds, including n-alkanes, branched alkanes, naphthenes and aromatics (Chunyan et al. 2023). Alkanes are saturated hydrocarbons, which are easily degraded by several bacteria (Liu et al. 2020). Branched alkanes are more resistant to biodegradation than n-alkanes due to their higher degree of branching and lower water solubility (Englert et al. 2019). Naphthenes are difficult to degrade due to the presence of cyclic structure (Reineke et al. 2023). Aromatic compounds are more recalcitrant than aliphatic compounds due to their aromaticity and low water solubility (Ren et al. 2019). Reported studies on degradation of different types of petroleum hydrocarbons by bacteria are summarised in Table 2.2.

Streptomyces sp. have demonstrated proficiency in degrading n-alkanes (C_6-C_{30}), aromatic compounds, and polycyclic aromatic hydrocarbons (PAHs), with degradation of up to 95% within seven days (Baoune et al. 2018). *Pseudomonas aeruginosa* NCIM 5514 is known for its ability in crude oil degradation, specifically in the range of C_8-C_{36}, achieving a 61% degradation in 60 days (Varjani and Upasani 2016). *Bacillus subtilis* Strain DM2 showed its ability in petroleum degradation, degrading compounds with carbon numbers such as C_{12}, C_{14}, and C_{15}, yielding degradation of 54% within 4.7 days (Li et al. 2019).

Elumalai et al. (2017) found that *Geobacillus thermoparaffinivorans*, *Geobacillus stearothermophillus*, and *Bacillus licheniformis*, demonstrated efficient degradation of long-chain n-alkanes (C_{32} and C_{40}). The strains were more effective in degrading C_{40} (up to 87%) in 20 days incubation period. *Pseudomonas aeruginosa* san ai, as reported by Medić et al. (2019), demonstrated remarkable degradation efficiency for n-alkanes (n-hexadecane, n-nonadecane), These compounds were degraded with efficiencies of 80%, 98%, respectively, within seven days at initial concentrations of 20 mg L^{-1}. Furthermore, Kiamarsi et al. (2019) found that *Pseudomonas resinovorans, Plantibacter auratus, Bacillus subtilis, Staphylococcus pasteuri*, and *Bacillus atrophaeus* exhibited substantial degradation capabilities. They degraded 86.0%, 61.3%, 81.1%, 55.0%, and 76.2% of aliphatic compounds in a medium containing crude oil (1% v/v) over 21 days, respectively.

Lv et al (2022) reported that *Bacillus cereus* LY-1 has shown higher degradation efficiency for branched alkanes with high carbon number (C_{15}–C_{24}). *Methanothermobacter* sp. degraded C_9 branched alkanes (2-methyl, 3-methyl, and 4-methyloctane) efficiently (Chen et al. 2019). Siddique et al. (2020) reported that 3-methylhexane, 4-methylheptane, 2-methyloctane and 2-methylheptane were completely degraded by *Desulfotomaculum*, *Smithella, Methanosaeta and Methanoregula* in 1700 days. 2-methylpentane was degraded by bacterial community composed of *Anaerolineaceae, Syntrophaceae, Peptococcaceae, Desulfobacteraceae,* and *Desulfobulbaceae* (Shahimin, & Siddique 2023). *Colwellia* and *Roseovarius* has showm degradation for methylcyclohexane.(Li et al. 2023).

Pseudomonas sp. W10 utilized about 20% and 90%, of pyrene, fluoranthene, respectively, after 30 days of incubation at 37 °C and 180 rpm (Chebbi et al. 2017). Various other bacteria, such as *Stenotrophomonas* sp. DBK3 (degrading p-cresol) and *Klebsiella oxytoca* (degrading pyrene), have demonstrated their efficiency in degrading specific hydrocarbons, highlighting roles of bacteria in mitigating hydrocarbon pollution (Bera et al., 2019; Alfaify et al., 2022). Goswami et al. (2018) reported that maximum degradation of 92, 82 and 81% was achieved by *Rhodococcus opacus* for naphthalene, phenanthrene and fluoranthene, respectively at initial concentration of 200 mg L^{-1}.

Aeribacillus pallidus, *Bacillus axarquiensis*, *Bacillus siamensis* and Bacillus subtilis exhibited significant degradation capabilities for a mixture of polycyclic aromatic hydrocarbons (anthracene, fluorine, phenanthrene, and pyrene). *Aeribacillus* sp. achieved relatively higher degradation at 50°C, with anthracene degradation 92%, fluorine degradation 83%, phenanthrene degradation 16% and pyrene degradation 51% (Mehetre et al. 2019). *Enterobacter* sp. MX1 had shown degradation of pristane, naphthalene, and phenanthrene (Zhang et al. 2022).

Table 2.2: Reported studies on degradation of different type of petroleum hydrocarbons by bacteria.

Strain name	Hydrocarbon type	Carbon number	Initial concentration and incubation period	% degradation	Reference
Pseudomonas aeruginosa NCIM 5514	crude oil	C_8-C_{36}	3% v/v, 60 days	61	Varjani and Upasani 2016
Streptomyces spp	Algerian light petroleum oil	C_6-C_{30}	1%v/v, 7 days	95	Baoune et al. 2018
Bacillus subtilis DM2	Alkanes	C_{12}, C_{14}, and C_{15}	2% v/v, 5 days	54	Li et al. 2019
Bacillus licheniformis MN6	n-alkanes	C_{32} and C_{40}	0.1% v/v, 20days	80 and 87	Elumalai et al. 2017
Pseudomonas aeruginosa sanai	n-alkanes	C_{16} and C_{19}	20 mg $L^{-1,}$ 7days	80 and 98	Medić et al. 2019
Desulfotomaculum, Smithella, Methanosaeta and *Methanoregula*	Branched alkanes	C_7-C_9	0.4% v/v and 1700 days	100	Siddique et al. (2020)
Methanothermobacter sp	Branched alkanes	C_9	0.1% v/v, 367 days	100	Chen et al. 2019
Colwellia and Roseovarius	Naphthenes (methylcyclohexane)	C_7	0.52 mM and 18 days	100	Li et al. 2023
Pseudomonas sp. W10	Aromatics (pyrene, fluoranthene)	C_{16}	180 ppm, 30 days	20 and 90	Chebbi et al. 2017
Rhodococcus opacus	Aromatics (naphthalene, phenanthrene and fluoranthene)	C_{10}, C_{14}, C_{16}	600 mg L^{-1}, 21 day	92, 82 and 81	Goswami et al. 2018

2.3 Isolation source of bacterial cultures

The source of isolation significantly influences the petroleum hydrocarbon degradation capabilities of bacteria. Bacteria isolated from specific contaminated sources tend to adapt to the prevailing conditions and develop enzymatic pathways designed to the types of hydrocarbons in those sources (Ramírez-García et al. 2019). These strains exhibit a higher affinity for degrading the hydrocarbons they have encountered, potentially leading to enhanced degradation efficiency. Additionally, bacteria from the same source may develop synergistic interactions that can boost their collective degradation capabilities (Yu et al. 2019). Transferring strains to different environments can pose challenges due to their lack of adaptation. Therefore, the source of bacterial strains is vital while assessing their potential in petroleum hydrocarbon degradation.

The bacteria capable of degrading hydrocarbons can be isolated from a hydrocarbon-rich environment. The bacteria isolated from diesel-contaminated locations have been reported to possess a unique metabolism that makes them more efficient at breaking down diesel (Horel and Schiewer 2014). *Pseudomonas* sp., *Bacillus* sp., and *Acinetobacter* sp. are examples of bacteria obtained from contaminated environments that are more efficient at degrading diesel (Chen et al. 2017; Muangchinda et al. 2018). *Actinomycetes* sp., *Klebsiella* sp., *Acetobacter* sp., *Rhodococcus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Micrococcus* sp., *Bacillus* sp., *Achromobacter* sp., *Flavobacterium* sp. are some of the reported isolates from diesel contaminated habitats for diesel degradation (Imron et al. 2020). Crude oil contaminated sites were also reported for the isolation source of petroleum hydrocarbon degrading bacteria. Ehiosun and Usman (2018) isolated *Streptomyces albus* from crude oil-contaminated soil in the Niger Delta region, which has ability to degrade varying concentrations of crude oil (1, 3, 5, and 7.5%).

Some local contaminated sites such as petrol pump, mechanic workshops have also been reported for the source of hydrocarbon degrading bacteria. Chinenye (2018) isolated 15 bacteria from contaminated soil of mechanic workshops for biodegradation in crude oil-contaminated soil, with *Staphylococcus epidermis*, *Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp., and *Micrococcus* sp. identified as main hydrocarbon degraders. Athar et al. (2014) isolated 19 hydrocarbon degrading bacteria from soil of a motor repairing workshop. These bacteria were able to degrade hydrocarbon compounds such as xylene, benzene, naphylamine, and

diphenylamine. Mandri and Lin (2007) isolated *Flavobacterium* sp., *Acinetobacterium calcoaceticum*, and *Pseudomonas aeruginosa* from soil contaminated with engine oil at a mechanic workshop, showing their capability to utilize used engine oil. Akoachere et al. (2008) isolated bacteria from auto-mechanic workshops and petrol filling stations, which have shown degradability of engine oil.

2.4 Bacterial degradation of diesel in different ecosystems

The characteristics of the ecosystem where contamination occurs also matter. Different ecosystems, such as soil, freshwater, or marine environments, have unique physical and chemical properties that can influence the availability of hydrocarbons, nutrient levels, and the types of bacteria present (Sattar et al. 2022). Bacterial communities and their effectiveness in hydrocarbon degradation can vary significantly between ecosystems. Diesel-degrading bacteria have been reported in diverse ecosystems, including soil, marine water, and freshwater environments.

2.4.1 Freshwater

Various bacteria, such as *Acinetobacter* sp. *K-6, Pseudomonas* sp., and *Bacillus subtilis* were found to be effective degraders of diesel in freshwater ecosystem (Chaudhary et al. 2020; Sun et al. 2018; Fosso-Kankeu et al. 2017). *Vibrio alginolyticus* has shown 27% degradation of 10% v/v diesel in 14 days (Imron et al., 2019). The *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were reported to degrade 1.5 % v/v diesel by 45 and 58%, respectively, in 10 days of incubation (You et al. 2018). Jerin et al. (2021) reported that 61 and 52 % degradation were observed for 10 % v/v diesel in 21-day incubation period in freshwater ecosystem by using *Acinetobacter baumannii* and *Cedecea davisae*, respectively.

2.4.2 Soil ecosystem

Some of the most reported bacteria for degradation of diesel in the soil include *Bacillus* sp. and *Acinetobacter lwoffi*. *Bacillus* sp. has shown 72% degradation for 2% v/v diesel in 15 day incubation period (Elenga-Wilson et al. 2021). *Pseudomonas* sp. *DTF1* has been reported to show 55 % degradation of 1% v/v diesel in soil in 15days incubation period (Yang et al. 2023). Kim et al. (2018) reported that 57% degradation was observed for 1 % v/v diesel by *Thiobacillus* in 120 days. An *Arthrobacter* sp. strain AQ5-05 was also found to be effective in

the soil ecosystem and showed 57 % degradation for 0.5% v/w diesel in 7 days (Abdulrasheed et al. 2020).

2.4.3 Marine water

Many bacteria have been reported for degradation of petroleum in the marine system with high degradation efficiency. Zhou et al. (2021) reported that *Vibrio* sp. *LQ2* gave 54 % degradation of 1% v/v diesel in the marine water during 7 days incubation period. 50% degradation was observed for 0.4% v/v diesel by *Halomonas* sp. in 12 days, when the salt concentration was 4% w/v (Yang et al. 2020). *Bacillus megaterium* showed 27 % degradation for the 1% v/v diesel in the marine water system after 5 days of incubation (Gao et al. 2021). *Delfia* sp. *NL1* have shown 67 % degradation in 2 M saline water for 5% v/v diesel in 7-day incubation period (Lenchi et al. 2020). Dahal et al. (2017) reported 46% degradation of oil (diesel, kerosene and gasoline mixture) in solution mixture contains 7% v/v NaCl by *Acinetobacter halotolerans* sp.nov. at 28°C temperature, pH 7 in 14 days incubation time, when initial oil concentration was 0.15%.

2.5 Parameters affecting bacterial degradation

Microorganisms play a crucial role in environmental remediation by degrading various organic contaminants due to their metabolic capabilities and adaptability for challenging conditions (Joutey et al., 2013). However, their effectiveness depends on factors common to both soil and water environments, including the presence of microbial consortia capable of degrading the pollutant, the bioavailability of the contaminants to bacterial attack, and conditions such as temperature, pH, oxygen levels, moisture, and the presence of electron acceptors, nutrients, and potential substrate composition (Joutey et al., 2013; Ukiwe et al., 2013). Bioavailability is a critical factor affecting bioremediation in contaminated soil and water. It is influenced by physico-chemical properties of the contaminant. These properties include medium's characteristics such as texture, composition, cation exchange capacity, moisture content, and pH. These factors, along with the residence time of contaminant, are especially important for hydrophobic pollutants like polycyclic aromatic hydrocarbons (PAHs) (Mohan et al. 2006).

To achieve effective bioremediation in both soil and water, it is essential to establish and sustain conditions that facilitate enhanced hydrocarbon degradation. This promote the presence of appropriate hydrocarbon degrading bacteria by maintaining adequate levels of nutrients and oxygen, and ensuring the pH falls within the typical range of 6 to 9 (Das and Chandran, 2011).

Moreover, the success of bioremediation in both soil and water is influenced by various factors. These includes the physicochemical properties of the contaminant, as well as its available surface area. Key parameters such as bacterial community, hydrocarbon concentration, temperature, pH, type of ecosystem and initial inoculum volume play pivotal roles in degradation of petroleum hydrocarbon (Fig. 2.1).

2.5.1 Bacterial community

The composition and diversity of the bacterial community in a given environment are crucial factors in petroleum hydrocarbon degradation. Different bacterial species possess varying metabolic capabilities, enabling them to degrade various types and concentrations of hydrocarbons. A diverse bacterial community increases the likelihood of having the necessary microbial species to efficiently break down hydrocarbon pollutants. The selection of bacterial species significantly influences the diesel degradation (Ghoreishi et al. 2017). Species such as *Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., and *Vibrio* sp. are found to be effective in hydrocarbon degradation due to their superior characteristics (Imron et al. 2020). Additionally, *Bacillus subtilis*, *Brochothrix thermosphacta*, *Vibrio alginolyticus*, and *Pseudomonas aeruginosa* have also showed effective biodegradation (Kurniawan et al. 2018; Titah et al. 2018). Different species uses distinct degradation mechanisms, making the choice between single or mixed-species applications important. Mixed bacterial communities can form symbiotic relationships that lead to more efficient degradation (Zhang et al. 2021).

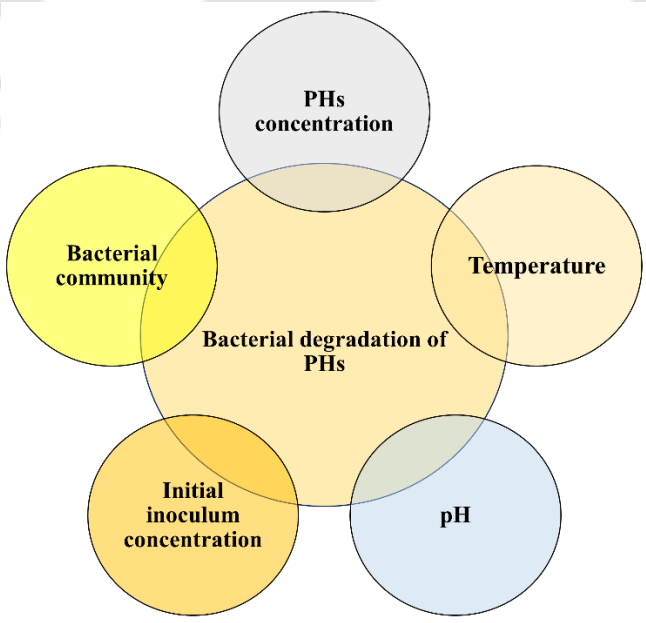


Fig. 2.1. Factors affecting the biodegradation of petroleum hydrocarbons (PHs).

2.5.2 Hydrocarbon concentration

The concentration of hydrocarbons in the environment significantly affects bacterial degradation. Low concentrations support bacterial growth and degradation, while high concentrations can inhibit microbial activity (Ahmed and Fakhruddin 2018). Optimal degradation occurs within a specific range of hydrocarbon concentrations, where microorganisms can effectively utilize the pollutants as carbon and energy sources. Higher concentrations may require increased microbial activity for efficient pollutant breakdown, but excessive levels can overwhelm the microbial population, resulting in slower degradation rates or inhibition of the process (Englert et al. 2019).

Singh and Tiwary (2017) reported that *Pseudomonas stutzeri* P2 gave maximum degradation of phenanthrene (PAH) at 0.5% v/v concentration of phenanthrene. *Pseudomonas mendocina* showed maximum degradation of acenapthene and naphthalene at 35°C (Barman et al. 2017). Sphingomonas *olei* KA1 and *Acinetobacter radioresistens* KA2 have given maximum degradation of crude oil by 71% when initial concentration of crude oil was 2%. Higher or lower concentration than 2% resulted decrease in degradation (Koolivand et al. 2019). Roslee et al. (2019) reported that *Rhodococcus* sp. strain AQ5-07 has shown maximum degradation of diesel at 1% v/v initial concentration. *Enterobacter cloacae*, *Bacillus* sp. and *Bacillus thuringiensis* showed maximum degradation of phenanthrene when initial concentration of phenanthrene was in the range of 50- 100 ppm. At high concentration of 500 ppm, degradation was absent (Abdel-Razek et al.2020). Gao et al. (2021) reported maximum degradation of diesel oil at the initial concentration of 2.5 % v/v by *Bacillus* sp. MJ4.

2.5.3 Temperature

Temperature significantly influences the biodegradation of petroleum hydrocarbon impacting both their physical and chemical properties (Al-Hawash et al. 2018). Lower temperatures are generally associated with reduced degradation rates, and this can be attributed to decreased enzymatic activity levels in bacteria (Bisht et al. 2015). On the other hand, hydrocarbon metabolism rates peak within a temperature range of 30 to 40°C (Kebede et al. 2021). Warmer temperatures generally enhance microbial growth and enzymatic activity, expediting hydrocarbon breakdown (Bai et al. 2023). Conversely, excessively high temperatures can denature enzymes and hinder microbial function. Thus, maintaining an optimal temperature range is crucial for efficient degradation. Different bacteria have distinct optimum temperature

for growth and metabolism, with many hydrocarbon-degrading bacteria thriving at moderate temperatures. Extreme can impede bacterial degradation processes (Rajkumari et al. 2018). Temperature variations can influence hydrocarbon viscosity and pollutant diffusion rates, impacting bacterial degradation. Lower temperatures generally slow down the degradation process as hydrocarbon viscosity decreases, making them less soluble and less accessible to bacteria (Kebede et al. 2022)

For different ecosystems, specific optimal temperature ranges have been observed for maximum degradation (Imron et al. 2020). Diesel biodegradation rates were reported to be highest at approximately 30 to 40 °C in soil, 20 to 30 °C in freshwater, and 15 to 20 °C in marine water (Imron et al. 2020). While biodegradation can occur across a wide temperature range, the rate generally decreases as temperature decreases (Alegbeleye et al. 2017). Notably, psychrophilic environments have showed significant hydrocarbon biodegradation (Varjani and Upasani 2017).

Vaidya et al. (2017) observed that mixed bacterial culture of *Pseudomonas* sp., *Burkholderia* sp., and *Rhodococcus* sp. exhibited maximum pyrene degradation at 37°C. *Enterobacter cloacae* KU923381 demonstrated its highest diesel degradation at 35°C, as reported by Ramasamy et al. (2017). Wang et al. (2019) reported that *Bacillus subtilis* BL-27 showed the maximum crude oil degradation at 45°C. Similarly, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus velezensis* achieved their highest crude oil degradation at 37°C, as reported by Prakash et al. (2021). Premnath et al. (2021) reported that *Klebsiella pneumoniae* exhibited its maximum degradation for Anthracene, Acenaphthene, Fluorene, Naphthalene, and Mixed polycyclic aromatics (PAHs) at 37°C. For the degradation of phenanthrene, Kebede et al. (2022) found the optimum temperature to be 30°C for *Enterobacter cloacae* and *Bacillus* sp., while *Bacillus thuringiensis* showed its highest degradation at 25°C. These findings suggest that most of the bacteria shows maximum degradation in the temperature range of 25-45°C.

2.5.4 pH

The pH levels significantly affect bacterial degradation processes, influencing microbial activity, enzymatic efficiency, and overall bioremediation success (Kurniawan et al. 2018). Different bacterial species exhibit varying pH tolerances, each with a preferred pH range for optimal growth and enzymatic activity. Maintaining a suitable pH range is critical for promoting microbial growth and enhancing hydrocarbon degradation. Most hydrocarbon-

degrading bacteria grow well in a neutral to slightly alkaline pH range, typically around pH 6 to 9 (Zhang et al. 2021). Extreme pH levels can inhibit bacterial growth and metabolic activity, negatively impacting degradation rates. (Al-Hawash et al., 2018).

Different optimum pH has been reported in previous studies. Ibrahim (2016) revealed that diesel degrades more slowly when the pH is acidic (6). Liu et al. (2019) reported that the highest degradation was observed when the pH was neutral or slightly alkaline. Imron et al. (2020) reported that the ideal pH range for diesel breakdown processes falls between 6.5 and 8, as the carboxylation reaction occurred in neutral to slightly alkaline environment.

Khodaei et al. (2017) reported that *Pseudomonas* sp. BTEX-30 has shown maximum degradation of BTEX (benzene, toluene, ethylbenzene and xylenes) compounds at pH 7.6. Optimum pH for *Pseudomonas stutzeri* NA3 and *Acinetobacter baumannii* MN3 was reported 8 and 7, respectively (Parthipan et al. 2017). *Klebsiella pneumoniae* ATCC13883 had given highest degradation of crude oil at pH 7 (Bilen and Seyis 2019). Poorsoleiman et al. (2020) reported that mixed culture of *Actinobacteria,Deinococcus-Thermus, Bacteroidetes* and *Firmicutes*. had given highest performance at pH 7 for the remediation of crude oil contamination. Marine petroleum degrading bacteria Halomonassp.nmyj-1 has shown maximum degradation of diesel at pH 7.39 (Yang et al. 2020). Premnath et al. (2021) reported that maximum degradation of Acenaphthene (48.40%), Fluorene (43.56%), Anthracene (43.31%), Naphthalene (33.54%), and mixed PAHs (27.64%) was observed at pH 8 for *Klebsiella pneumoniae*. These studies suggest that most of the bacteria shows highest performance in the pH range of 6-9.

2.5.5 Inoculum concentration

The concentration of bacterial inoculum, which involves introducing microorganisms to the contaminated site, is crucial for effective degradation (Chaudhary et al. 2019). Maintaining an adequate concentration of active bacterial cultures is essential to ensure efficient pollutant degradation (Yang et al. 2020). A low inoculum concentration may result in slow degradation, while a higher initial inoculum concentration generally speeds up degradation because it provides more microbial biomass to metabolize the hydrocarbons. However, excessive inoculation may not always be beneficial, as it can lead to competition for resources and may not necessarily enhance degradation rates (Otiniano et al. 2022).

Varjani and Upasani (2017) reported that the optimum inoculum concentration for *Pseudomonas aeruginosa* NCIM5514 was 1% v/v for the degradation of crude oil. The optimal bacterial inoculum size for the biodegradation of polycyclic aromatic hydrocarbons using *Mycobacteria confluentis* was found to be 7.5 mL, as reported by Muna Ibrahim et al. (2018). *Bacillus licheniformis* has shown maximum degradation of crude oil at 10% v/v inoculum concentration (Khanpour-Alikelayeh et al. 2021). Tirkey et al. (2021) reported that *Pseudomonas* sp. strain SA3 showed the maximum naphthalene degradation at a 1% v/v inoculum concentration. *Pseudomonas aeruginosa* AHV-KH10 has showed the maximum degradation of diesel at a 1.5% v/w inoculum concentration (Pourfadakari et al. 2021). Nkem et al. (2022) reported that a mixed culture of *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii*, and *Pseudomonas balearica* achieved the maximum degradation of diesel at a 2.5% v/v inoculum concentration. These studies suggest that the optimum inoculum concentrations for the bacterial degradation of petroleum hydrocarbons are in the range of 1 to 10% v/v.

2.6 Degradation by mixed bacterial culture

A mixed culture consists of multiple bacterial strains. When isolated strains used in a mixed culture can synergize their pathways, they can enhance degradation performance (Xu et al. 2018). These cultures comprise diverse microbial communities with varying metabolic capabilities, which collectively contribute to the breakdown of complex hydrocarbon pollutants. Utilising mixed bacterial cultures holds distinct advantages over relying on single strains for the degradation of complex hydrocarbons. The diverse enzymatic machinery of different bacterial species allows for the breakdown of a wider range of hydrocarbon components. Mixed cultures possess the flexibility to adapt to varying environmental conditions, ensuring effective degradation across fluctuating factors.

Various bacterial strains possess the capacity to break down alkanes, though only a limited number show the capability to degrade complex substances like polycyclic aromatic hydrocarbons. While certain bacterial species may target or metabolize select hydrocarbons individually, the presence of a mixed microbial community often yields a synergistic impact (Varjani and Upasani, 2017). Petroleum crude, composed of mixture of hydrocarbons, can undergo degradation by various indigenous bacterial species, each with the ability to utilize or break down specific compound groups (Khalid et al. 2022).

Morales-Guzmán et al. (2017) reported that individual strains like *Citrobacter freundii* CCC4DS3 and *S. marcescens* C11S1 achieved 17% and 10% degradation, respectively, of 1% v/v diesel in 8 days. However, a mixed culture of the same strains achieved a significantly higher degradation rate of 97% within the same period. Safitri et al. (2018) found that a consortium of *B. subtilis InaCC B289* and *P. aeruginosa InaCCB290* degraded 25% v/v diesel by 58%, whereas single cultures achieved only 37-38% degradation in a 25 days incubation period. In the case of 1% v/v crude oil, Parthipan et al. (2018) observed that *B. subtilis B5, B. pumilus B1, S. parvus B7*, and *B. megaterium B6* strains achieved individual degradation rates of 55, 66, 82, and 52%, respectively, in 20 days. However, when used as a mixed culture, the degradation rate increased to 90% within the same period. Furthermore, in a study conducted by Muthukumar et al. (2023), a mixed bacterial culture of *Pseudomonas aeruginosa* PP3 and *Pseudomonas aeruginosa* PP4 achieved a 65% degradation of 8.5% v/w crude oil during a 40-day incubation period.

Bidja et al. (2020) observed that *Serratia marcescens* strain *PL* and *Raoultella ornithinolytica PS* individually achieved 75% and 65% degradation of 0.4% v/v crude oil in a 20-day incubation period. However, when their mixed culture was used, degradation increased significantly to 97% within the same incubation period. Chaudhary et al. (2021) reported 89% degradation of 6% v/v diesel oil in 2 months using a mixed culture of *Rhodococcus* sp., *Burkholderia* sp., *Pseudomonas*, and *Paenarthrobacter* sp., which was higher than the degradation achieved by pure cultures of each strain. Similarly, Rizzo et al. (2018) observed that when *Alcanivorax* strain A53, *Pseudomonas* strain A6, and *Joostella* strain A8 were used individually, degradation of 53, 38, and 27% were achieved, respectively, for 2% v/v diesel in 20 days. However, their mixed culture achieved a much higher degradation rate of 90% in the same conditions. The presence of different strains in the consortium with varied degradation capabilities for different components contributes to the overall enhancement of degradation efficiency.

2.7 Degradation kinetics

The degradation kinetics in terms of rate constant and half-life give knowledge about the efficiency of any degradation process. Higher rate constant or lower half-life suggests faster degradation process (Bilen and Seyis 2020). The bacterial degradation of organic compounds has been reported to follow first order kinetics for wide range of initial diesel concentration

from 0.2-10% v/v, in different ecosystems (Zhou et al. 2017; Ambaye et al. 2022; Khalid et al. 2022).

For soil ecosystem with 0.2% v/v concentration of diesel, Chen et al. (2019) reported first order degradation for a mixed bacterial culture (*Comamonas testosteroni*, *Gordonia alkanivorans, Pseudomonas aeruginosa*, *Alcaligenes sp.*, *Gordonia terrae*, *Gordonia desulfuricans*, and *Rhodococcus erythoropolis)* with k value of 0.01 day^{-1}. In same soil ecosystem for higher concentration of 10% v/v diesel, the rate constant (k) was reported as 0.0388 day^{-1} using *Bacillus salmalaya139S*. The corresponding degradation half-life was obtained as 18 days (Dadrasnia et al. 2020). A bacteria isolated from seawater showed first order degradation with k value of 0.0284day^{-1}. The initial diesel concentration for in the marine water ecosystem was 1% v/v (Xue et al. 2017). However, a significantly higher k value (0.4535 day^{-1}) was reported for a mixed bacterial culture isolated from seawater for 0.2% v/v diesel by Xia et al. (2009) in marine ecosystem.

For a freshwater ecosystem, *Bacillus pumilus* and *Paenibacillus lautus* displayed k values of 0.079 and 0.009 day^{-1}, respectively, for 2.4% v/v diesel, with corresponding half-life of 9 and 76 days (Mauricio-Gutiérrez et al. 2020). When freshwater was contaminated with 2% v/v refinery sludge and treated with *Stenotrophomonas* sp. *IRB19*, a k value of 0.036 day^{-1} and a half-life of 19 days were observed (Behera et al. 2020). Chaudhary et al. (2021) investigated the biodegradation kinetics of 4.5% v/v diesel using a mixed culture comprised of *Rugosibacter, Nevskia, Rhodanobacter, Parvibaculum, Pseudomonas, Paraburkholderia*, and *Herbaspirillum*. The resulting rate constant and half-life for diesel degradation were determined to be 0.0118 day^{-1} and 59 days, respectively. Furthermore, Osinowo et al. (2020) reported that when a mixed culture of *Micrococcus luteus* trpE16 and *Bacillus subtilis* DNK was used to treat soil contaminated with 10% v/v diesel for 84 days, the rate constant and half-life for diesel degradation were found to be 0.1732 and 4 days, respectively. Moreover, Fu et al. (2021) reported a rate constant of 0.1947 day^{-1} for the degradation of 0.75% v/v diesel within a 5 days incubation period using *Pseudomonas sp.* YT-11. These studies suggest that the degradation kinetics of petroleum hydrocarbons by bacterial cultures vary significantly depending on factors such as initial concentration, bacterial species, and ecosystem type. The rate constants (k values) reported in the studies are in range of 0.009 to 0.4535 day^{-1}.

2.8 Degradation pathway

Fig. 2.2 shows the steps involved in bacterial degradation of diesel oil. When diesel compounds come in contact with bacteria, then absorption of the components occurs on the cell membrane surface of bacteria. After absorption, diesel uptake in the cell occurs by endocytosis, which involves active and passive transports (Hua and Wang. 2014). Diesel degradation occurs by intracellular pathways and end products are used in the TCA (Tricarboxylic acid) cycle. TCA is the important metabolic process that generate biomass for the microbial growth (Khalid et al. 2021).

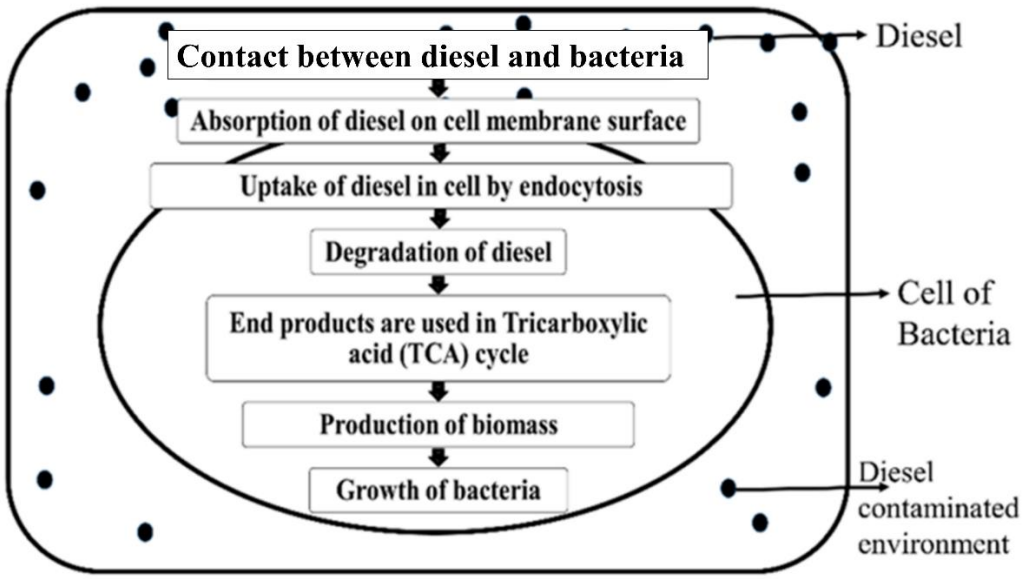


Fig. 2.2 Steps involved in bacterial degradation of diesel oil.

Fig. 2.3 shows the reactions scheme for degradation of different hydrocarbons present in the diesel sample, that is alkanes and naphthenes. The n-alkanes and branched alkanes are reported to degrade by dioxygenase, which acts as the electron acceptor that attacks the diesel compounds (Yap et. al. 2021). The n-alkanes and branched alkanes are degraded by oxidation of the terminal or sub terminal methyl group producing primary and secondary alcohols (Park and Park 2018). Primary alcohols are further dehydrogenated to aldehydes and then to fatty acid or carboxylic acids. The fatty or carboxylic acids further undergo β-oxidation and acetyl coenzyme is produced. This is utilized in tricarboxylic acid (TCA) cycle (Khalid et al. 2021). The secondary alcohols dehydrogenate into the ketone, which is mono-oxygenated to acetyl ester. Acetyl ester is converted to acetate and primary alcohols (Moreno and Rojo 2017).

Acetate is used in the TCA cycle and primary alcohols undergo degradation as discussed earlier.

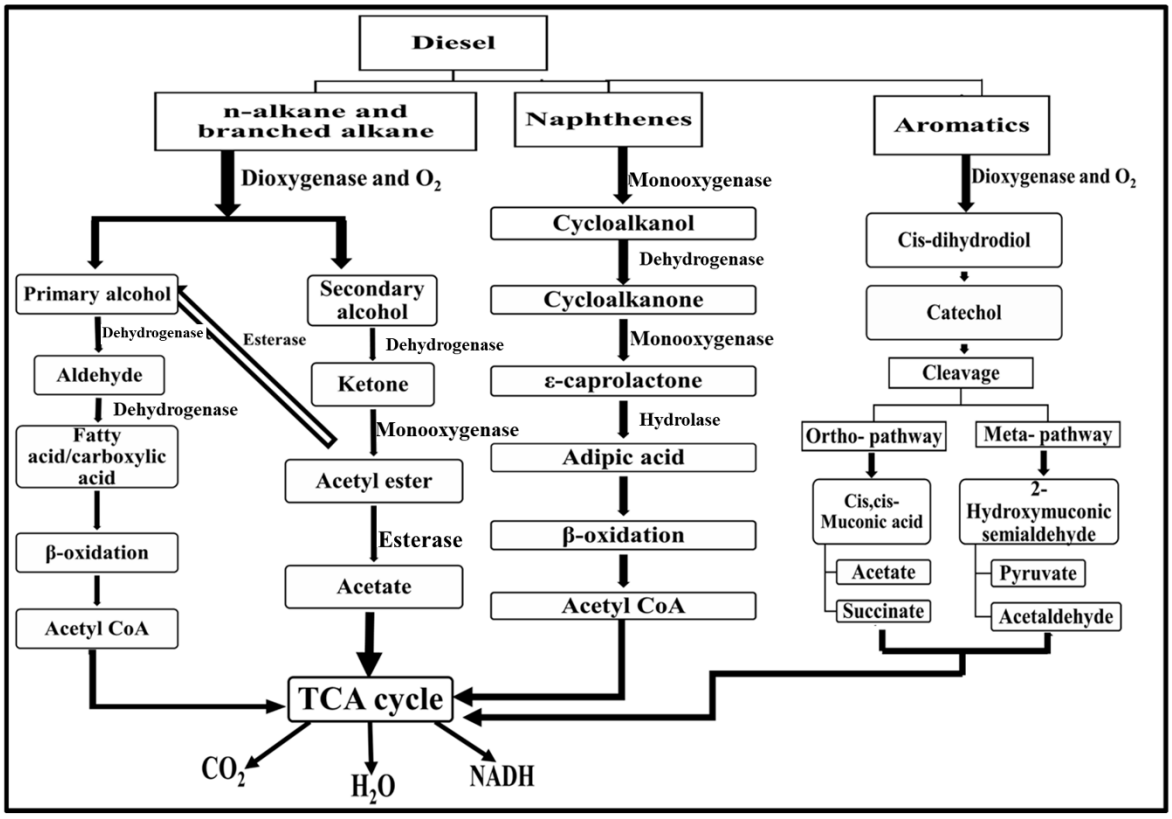


Fig. 2.3 Reported bacterial degradation pathway for diesel oil (Varjani 2017).

The naphthenic compounds are first oxidized to cycloalkanol by using mono-oxygenase in the presence of oxygen (Yap et al. 2021). Formation of cycloalkanol occurs when an enzyme mono-oxygenase attacks an alkyl group in naphthenes. Further, dehydrogenation of cycloalkanol results in naphthenic ketone, which are then converted with the help of the oxygenase to ε-caprolactone. Thereafter, ε-caprolactone is converted to adipic acid by the addition of water molecule (Khalid et al. 2021). Then adipic acid undergoes β-oxidation, which produce Acetyl-CoA used by bacteria in TCA cycle (Luong et al. 2018).

The degradation pathway for aromatic follows either ortho or meta pathway after formation of cis- dihydrodiol, which is further converted to catechol (Brakstad et al. 2017). By ortho pathway, cic, cis- Muconic acid is produced which is further converted into acetate and succinate. Meta pathway produces 2-hydroxymuconic semialdehyde, which further converted into pyruvate and acetaldehyde. The acetate, succinate, pyruvate and acetaldehyde are further used by bacteria in tricarboxylic acid (TCA) cycle, which produces energy for bacterial cell

(Vajrani 2017). Carbon-dioxide and water are released as end products. These pathways suggest that the diverse enzymatic reactions involved in the degradation of alkanes, naphthenes, and aromatics, ultimately leading to the generation of intermediates utilized in the tricarboxylic acid (TCA) cycle.

2.9 Research Gap

Based on previous studies, several research gaps have been identified in the area of bacterial hydrocarbon degradation that require further investigation. One of the prominent areas of concern is the efficiency of bacterial strains, which varies significantly across different ecosystems, thereby raising questions about their adaptability and effectiveness. Comprehensive research is needed to understand the performance of these strains in diverse ecosystems and develop strategies to enhance their versatility. Additionally, bacterial strains often face limitations when dealing with higher concentrations of hydrocarbons, thereby restricting their application in highly contaminated scenarios. Investigating the factors hindering degradation at increased levels and finding viable solutions to this challenge is imperative.

Moreover, many bacterial strains exhibit selectivity in the types of hydrocarbons they can effectively degrade, emphasizing the need for research aimed at broadening their substrate specificity or enhancing their capacity to handle a wider range of hydrocarbon compounds. Time is another critical factor, as bacterial degradation for bioremediation purposes can be time-consuming, posing challenges for urgent clean-up operations. Research efforts are directed towards finding ways to accelerate the degradation process without compromising its efficiency.

In addition to these concerns, other research gaps include the need for comprehensive studies to isolate and characterize novel bacterial strains tailored for specific ecosystems, as well as exploring the adaptability of mixed bacterial cultures across various ecological settings, particularly in practical field scenarios. Furthermore, in-depth investigations into the ecological consequences of degradation products, specifically their phytotoxicity and impact on plant life, are required. Rigorous field studies to validate laboratory findings using mixed bacterial cultures in practical hydrocarbon-contaminated site remediation are also needed for the advancement of the field and the development of more effective bioremediation strategies. Addressing these research gaps is vital for advancing our understanding of bacterial

hydrocarbon degradation and enhancing its practical applications in environmental remediation efforts.

2.10 Objectives of thesis

The overall objective of the present study was to develop an effective bacterial mixed culture using newly isolated bacteria from locally contaminated sources for the degradation of petroleum hydrocarbons and remediation of diesel pollution in soil and water. Specifically, the study aimed to achieve the following objectives:

1. Isolation of petroleum hydrocarbon degrading bacteria from local sources and their identification and characterisation.
2. Growth study of isolated and identified bacteria in presence different hydrocarbons.
3. Determination of most suitable growth conditions such as temperature, pH, hydrocarbon concentration and inoculum concentration.
4. Investigation on bacterial degradation of commercial hydrocarbon diesel.
5. Effectiveness of bacterial degradation in different ecosystems (soil, fresh water and saline water) using diesel contamination.
6. Bioremediation of diesel contaminated water and soil by bacterial mixed culture.
7. Kinetics study for diesel degradation.

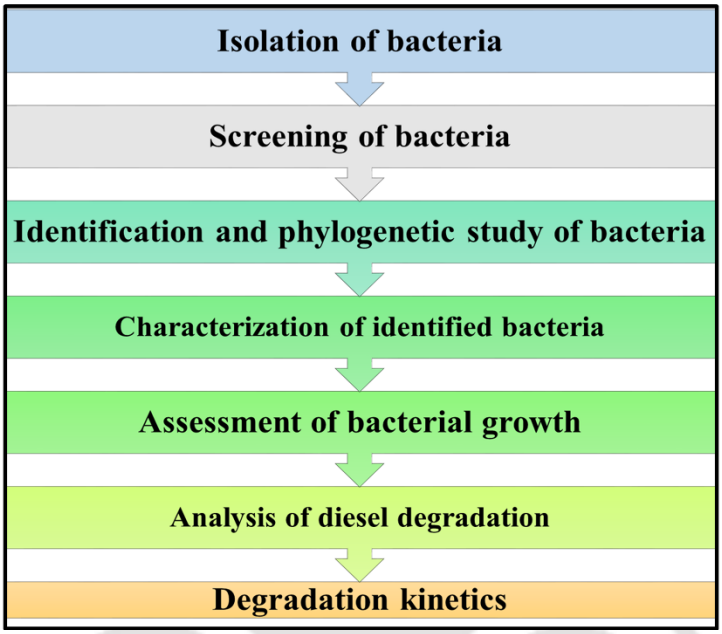
2.11 Organization of the thesis

The thesis has been organized into 8 chapters as described below:

Chapter 1: Introduction
Chapter 2: Literature Review
Chapter 3: Experimental Details
Chapter 4: Degradation of petroleum hydrocarbons by *K. michiganensis and A. baumannii*
Chapter 5: Degradation of petroleum hydrocarbons by *P. vermicola and P. aeruginosa*
Chapter 6: Degradation of diesel in Soil & Water ecosystems by *A. baumannii* and *P. vermicola*
Chapter 7: Simulated field study using mixed culture and Phytotoxicity test
Chapter 8: Conclusions and recommendations

Chapter 3

Experimental Details



Chapter 3 outlines the experimental framework for bacterial hydrocarbon degradation. It describes the detailed methods of bacteria isolation, screening, identification, and characterization. The assessment details of bacterial growth and protocols to determine the effect of process parameters on diesel degradation have been discussed. It also discusses the experimental details for determining the degradation efficiency of different hydrocarbons including diesel by bacteria, and corresponding degradation kinetics. The detailed methods to study the effect of light sources on bacteria and their diesel degradation have been included.

Keywords: Isolation; Screening; Identification; Growth assessment; Degradation; Kinetic study.

Chapter 3: Experimental Details

3.1 Materials

Bushnell-Hass broth (1 L containing 0.2 g $MgSO_4$, 0.2 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_3NO_2, and 0.05 g $FeCl_3$) obtained from Sisco Research Laboratories Pvt. Ltd. (SRL), India, was used for the selective growth of hydrocarbon-degrading bacteria. Luria-Bertani broth (composed of Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, and Sodium chloride 10 g L^{-1}) procured from SRL, India, was used to prepare the master culture for inoculation. Agar from SRL, India, was used to prepare solid media in the perti plates. Luria-Bertani Agar media (from SRL, India) containing 1.5% agar was used to store the bacterial strains. For studying biochemical characteristics, biochemical kit I and II were obtained from SRL, India. Diesel oil, obtained from local petrol pump, served as a carbon substrate unless otherwise specified. Petrol, kerosene and engine oil were procured from the local market. Heavy paraffin and light paraffin oils were obtained from SRL, India. n-Hexane (from SRL, India) was used as a solvent for extraction of oil from water and soil. Water and soil were collected locally.

3.2 Isolation of bacteria

The step involved in the isolation of bacteria is illustrated in Fig. 3.1. For the isolation of bacteria, 1 g of sample was taken from soil or sludge source and mixed with 100 mL of Luria Brentani (LB) media in the 250 mL conical flask. The mixture was kept at 37°C for 5 days, and agitation was maintained at 120 rpm. As LB media is a general type media, all bacteria present in source sample grew efficiently. After 5 days, when turbidity was visible, 5 mL culture from incubated LB media was used as the inoculum. This inoculum was poured into 100 mL Bushnell Hass (BH) broth, supplemented by 1% v/v diesel as the carbon source. As BH is a specific medium, only the bacteria that utilized diesel as the carbon source grew. BH broth was kept at the same conditions as that of LB broth. Only the incubation period was increased to 10 days. After the incubation period, a 5 mL aliquot of this BH broth was taken out and used as the inoculum to repeat the BH broth culture phase, keeping the conditions the same but increasing the diesel content to 2% v/v. Then 1 mL aliquot from this BH broth was taken and mixed with 9 mL of autoclaved distilled water. This was serially diluted up to 10^{-8} dilutions. Even number of dilution were used as the inoculum and they were spread-plated on BH agar plate supplemented with 1% v/v diesel. These BH plates were kept at 37°C for 3 days until clear colonies appeared. About 6 colonies each from soil and sludge sources were selected based on morphology and were plated individually on LB agar plates. The conditions were

maintained same as that of BH agar plates. After 1 day, visible growth of individual colonies was observed and plates were stored at 4°C for further use.

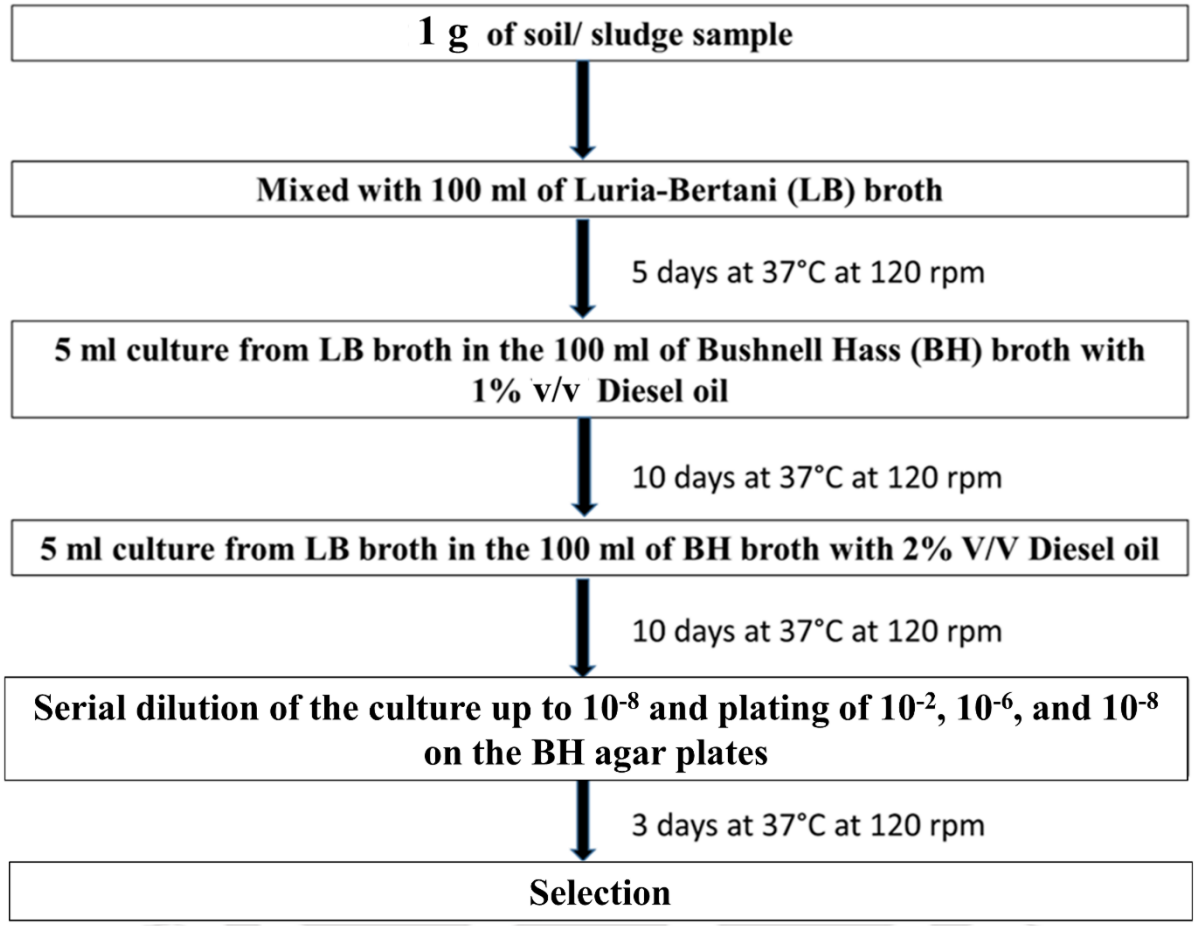


Fig. 3.1: Steps used for isolation of the bacteria.

3.3 Screening of bacteria

To evaluate the effectiveness of the isolated strains under different conditions, screening was done in four sequential stages, using four different techniques: 1. LB broth containing petroleum hydrocarbon oil, 2. LB Agar well diffusion method, 3. Spread plate technique, 4. Growth on BH agar media. The methods are schematically shown in Fig. 3.2 and discussed below.

The first screening stage was performed using LB broth containing petroleum hydrocarbon to determine hydrocarbon tolerance of bacteria. This method used 10 mL of liquid state (broth) of Luria Bertani medium (general purpose growth media) supplemented with 1% v/v of diesel oil. The bacterial isolates were inoculated separately and incubated at 37°C for 24h. The strains

which showed growth were selected for the next stage of screening. Bacterial growth was detected following the increase of turbidity in the media.

The LB agar well diffusion technique (Al-Mujaini et al. 2018) was performed in the second screening stage to determine bacteria's ability to utilise petroleum hydrocarbons as the carbon source. In this LB agar plate was used. 100 µl of diesel oil was spread on the surface of LB agar media. Then wells of equal diameter were created on the LB agar media. Wells were filled with a fresh bacterial culture of about 100 µl. Sample plates were then incubated at 37°C for 24h. As growth was observed around the wells, it was concluded that the selected bacteria were able to utilise the hydrocarbon as the carbon source for their growth.

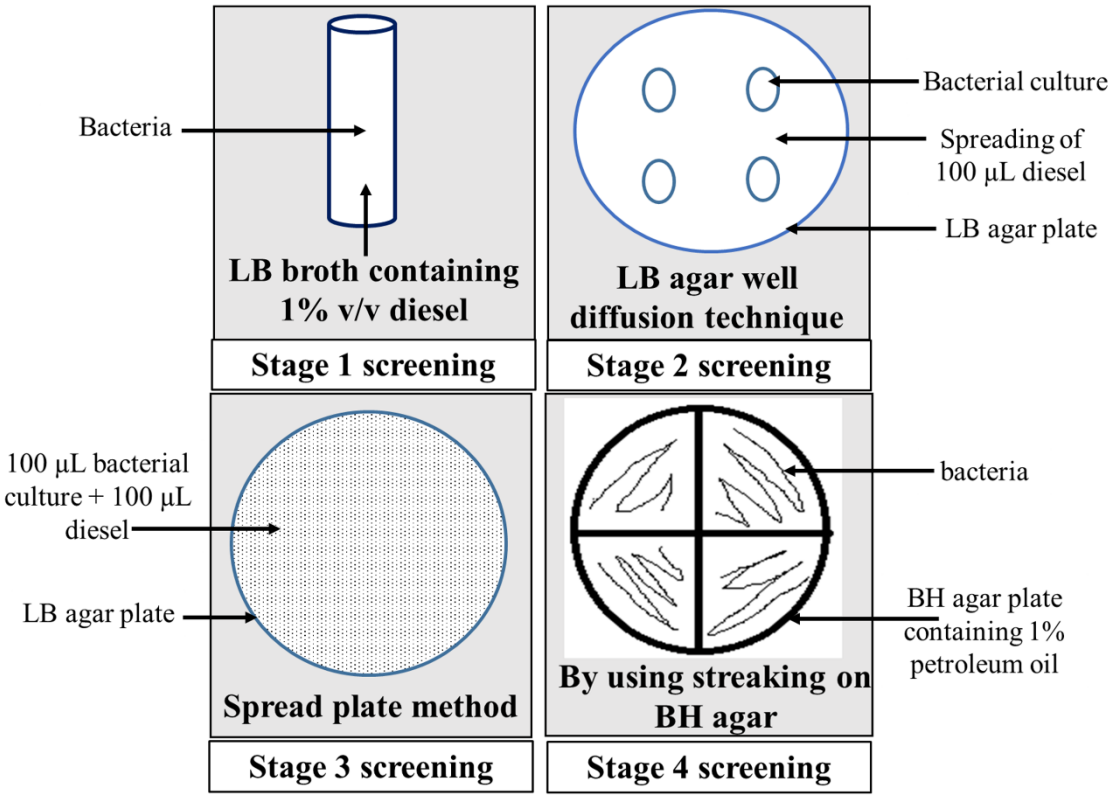


Fig. 3.2 Screening of hydrocarbon degrading bacteria using different methods.

The spread plate method was performed for the third stage of screening. This experiment was done to find the tolerance of bacteria towards a high concentration of hydrocarbon. In this experiment, bacteria were brought in direct contact with high concentration of the diesel. Bacterial inoculum of 100 µl was mixed directly with 100 µl of diesel oil. The mixture was

spread on the Luria Bertani (LB) agar plates, and growth was observed. The bacteria that showed growth were selected for the next stage of screening

The fourth and final screening stage was performed to detect bacteria that can utilise hydrocarbon as the sole source of carbon (Tian et al. 2018). Bushnell Hass (BH) media is specially designed for the study of bacterial growth using external hydrocarbon sources. BH media have all the essential components for the growth except any carbon source. The carbon source needs to be added separately as it is essential for the growth and metabolism of bacteria. As the source of carbon, hydrocarbons were added separately to the medium for the experiment. Both diesel and petrol were used as the carbon sources. Three types of BH agar plates were prepared by adding 1.5 % agar (solidifying agent) and three carbon sources (1% of diesel, 1% of petrol, and an equimolar mixture of petrol and diesel) in the BH medium. Streaking of bacterial isolates was performed on the BH plates, and incubation was done at 37°C for 72h. The bacteria that showed growth on all three types of BH agar plates were selected and stored for further study.

After completion of the screening stages, number of isolates were obtained, which showed growth and tolerance in the presence of petroleum hydrocarbons. Another growth study was performed to further select bacterial strains with the highest growth. The bacteria showing higher growth may be associated with its higher ability for hydrocarbon utilisation. The growth study was done by comparing the growth of all isolates under similar conditions of temperature, pH, media, substrate concentration and incubation time. The BH broth media, supplemented with 1% of diesel as the carbon source, was used in this experiment. For the growth study, single colony of bacteria was inoculated in different flasks containing BH media of 100 ml. Cultures were incubated at 37°C for 96h. Growth was observed by measuring the absorbance at 600nm, using UV-Vis spectrophotometer. The higher growing strains were selected, and again grown on LB plates and stored at 4°C for further study.

3.4 Identification and phylogenetic study of bacteria

For identification of bacterial isolates, 16S rRNA gene amplification was carried out. The utilization of 16S rRNA sequence analysis is a widely accepted and robust method for bacterial identification and phylogenetic placement (Church et al. 2020). For 16S rRNA gene amplification, DNA of the two isolates was used in Polymerase Chain Reaction (PCR) using the universal primers 16F27 (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1492 (5'-TACGGYTACCTTGTTACGACTT-3') with the following conditions: initial denaturation at

97°C for 4 min, followed by 35 cycles (94°C for 1 min, 52°C for 1 min, and 72°C for 1 min) and a final elongation step at 72°C for 5 min (Jindal & Aggarwal 2023). The amplified 16S rRNA gene fragments were subjected to Sanger sequencing at the National Centre for Microbial Resource, Pune. The obtained 16S rRNA sequences were compared to those in the GenBank database (maintained by National Center for Biotechnology Information, NCBI, USA), using Basic local alignment search tool (BLAST), to identify the closest relative (Zhu et al. 2023).

For further validation of taxonomic classification, phylogenetic analysis of the sequences belonging to the closest hits for each strain was carried out in Molecular Evolutionary Genetics Analysis tool (MEGA) X (Kumar et al. 2018). Multiple sequence alignments were generated using the ClustalW algorithm program in MEGA X with default parameters (Vargas-Ordóñez et al. 2023) and used to generate phylogenetic tree. Phylogenetic tree was constructed using the neighbour-joining method and maximum likelihood method. The neighbour-joining method utilized the maximum composite likelihood model, while the maximum likelihood method employed the Tamura-Nei model (Hu et al. 2022). Bootstrap analysis (with 500 replicates) was performed to test phylogeny. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (500 replicates), has been indicated alongside the branches (Okafor et al. 2021). This phylogenetic positioning reaffirms their taxonomic affiliation and provides insights into their evolutionary history within the diverse bacterial genus (Numberger et al. 2019). Based on the percentage of high sequence similarity and clear phylogenetic clustering in the same branch, each strain was assigned to a species. Furthermore, the 16S rRNA sequences of the bacterial strains were submitted to NCBI GenBank.

3.5 Characterization of identified bacteria

The bacteria were characterized by morphological and biochemical analysis. The morphological study was done by Field Emission Scanning Electron Microscope (FESEM), GEMINI Carl Zeiss. For the FESEM analysis, sample was prepared by taking overnight grown culture. This culture was centrifuged to remove the media component, and the bacteria cell was collected as a pellet. Thereafter, bacterial cell was washed with autoclaved distilled water and drop casted on the glass coverslip. It was then mounted on the sample holder, and gold coating was done. The bacteria were observed at the magnification of 20000, and the image was recorded.

Biochemical tests were performed as per the method describe by Bergey (2001). Biochemical tests included starch hydrolysis, gelatine liquefaction, triple sugar iron (TSI) agar, H_2S production, urease and malonate utilization tests. These tests showed the metabolic capability of isolated bacterial strains for certain type of biochemicals. These tests were performed to differentiate bacterial isolates from each other and confirm their distinct metabolic preferences, thereby determining that they are different strains.

The malonate utilization test was used to determine the ability of bacteria to utilize malonate as a sole carbon source for growth. For this test, a bacterial culture was inoculated into malonate broth and incubated at 37°C for 24 hours. Growth and colour changes were observed to assess malonate utilization; a change in the broth colour from green to blue indicated the utilization of sodium malonate, thus yielding a positive result. Conversely, if the colour changed from green to yellow, it indicated negative results for malonate utilization; the formation of yellow colour occurred due to an increase in acidity in the medium.

The starch hydrolysis test was used to determine the ability of bacteria to hydrolyse starch using the enzyme amylase. Starch hydrolysis tests involved streaking the bacterial culture onto starch agar plates, followed by incubation at 37°C for 2 days and flooding with Gram's iodine solution to detect starch hydrolysis. Colourless zone around the colonies indicates starch hydrolysis. A blue or purple zone indicate starch is not hydrolysed.

The gelatin liquefaction test is conducted to detect bacteria producing the gelatinase enzyme, which degrades or liquefies gelatin. To perform this test, the bacterial culture was inoculated onto nutrient gelatin medium and incubated at 37°C for 2 days. Liquefaction was assessed by placing the nutrient gelatin culture at 4°C for 30 minutes, followed by incubation at 37°C to observe the results. Liquefaction of the media indicates positive results.

Triple sugar iron (TSI) agar test was done to differentiate bacteria based on the ability to utilize sugars and produce hydrogen sulfide (H_2S) gas. This test involved inoculating the TSI agar slant with the bacterial culture and observing for fermentation of sugars, gas production, and hydrogen sulfide (H_2S) production after incubation. If media colour changes from reddish pink to yellow, it indicates positive result. For H_2S production tests, the bacterial culture was inoculated onto TSI media, incubated, and observed for the presence of a black precipitate indicating hydrogen sulfide production.

The urease test was used to determine the ability of bacteria to produce the urease enzyme, which hydrolyses urea to produce ammonia and carbon dioxide. Urease Tests included inoculating the bacterial culture on urea agar slants, followed by incubation at 37°C for 24h. Colour change from yellow to pink or magenta indicates positive result for urease activity.

The Gram's nature of bacterial strains was also identified by using Gram's staining. To conduct Gram's staining, a bacterial culture was spread onto a glass slide and air-dried. Following this, heat fixation was applied to kill and affix the bacteria onto the slide. Subsequently, the slide was immersed in a crystal violet solution, staining all cells purple as the primary stain. After rinsing, the slide underwent treatment with iodine solution (Gram's iodine), forming a complex with crystal violet and enhancing its retention within bacterial cells. Ethanol was then used as a decolorizing agent, removing the crystal violet-iodine complex from gram-negative cells while leaving it intact in gram-positive cells. The slide was counterstained with safranin, staining gram-negative cells pink or red, contrasting with the purple colour retained by gram-positive cells. Finally, the stained slide was observed under a microscope, where gram-positive bacteria appeared purple, and gram-negative bacteria appeared pink or red.

3.6 Assessment of bacterial growth

Assessment of bacterial growth was determined both in water and soil samples. For water samples, 2 mL aliquots were taken and centrifuged at 8000 rpm for 10 min. The bacterial cells were obtained in the form of pellets, and the supernatant were discarded. The bacterial pellets were dissolved in 2 mL of sterile distilled water. Then, dissolved bacteria were placed in quartz cuvettes and placed in sample holder of UV-Vis spectrometer. A different cuvette contain only sterile distilled water was used as the reference. Bacterial growth was measured by determining absorbance at 600 nm.

For soil sample, 2 g of soil was mixed with 20 mL of distilled water and then sonicated for 1 h. After sonication soil settled at the bottom, and bacteria were retained in the supernatant water. This bacteria containing water was centrifuged at 8000 rpm for 10 min. Bacteria were separated as the pellets and supernatant was discarded. The bacterial pellets were mixed with 2 mL of sterile distilled water. Thereafter, growth was measured using absorbance at 600 nm, as described for water sample.

3.7 Growth curve analysis

The growth curves of isolated bacteria were generated using Bushnell Hass broth medium supplemented with 1% diesel as the carbon source. After every 12h, samples were taken out, and their absorbance was measured using a UV-Vis spectrophotometer (Shimadzu). The absorbance was taken at the wavelength of 600 nm. This was done up to 240 h. Then growth curve was plotted between absorbance v/s time in days.

3.8 Degradation assessment of different hydrocarbons

The degradation ability of selected bacteria for different hydrocarbons was investigated using a Bushnell Hass broth medium that initially did not have any carbon source. The petroleum hydrocarbons were provided as the carbon source. Bacterial growth occurred due to utilisation of the externally added hydrocarbons, resulting in their degradation. To check the degradation ability of the isolated bacteria for different hydrocarbon sources, 1% v/v of diesel, petrol, kerosene, engine oil, light paraffin oil, or heavy paraffin oil were added separately to the media as the sole carbon source. The bacterial growth was detected and quantified by measuring optical density at 600nm using UV Vis spectrophotometer. The growth of strains was related to their ability to utilise the petroleum hydrocarbons.

3.9 Determination of diesel degradation

To assess diesel degradation, degraded products were extracted using hexane as the solvent. For hydrocarbon extraction from water samples, 10 mL of hexane was added to the culture, sonicated for 1h, and poured into a separating funnel, allowing it to settle for 6 h. Three layers were formed, with the top layer containing hexane and residual oil components. After separation and centrifugation, to remove water impurity, this process was repeated twice. For soil samples, 10 g was placed in a 30 mL centrifuge tube with 10 mL of n-hexane and 10 mL of water. Sonication for 60 minutes facilitated diesel extraction, followed by centrifugation at 8000 rpm for 20 minutes. The collected n-hexane layer was further analysed by Gas Chromatography-Mass spectrometry (GC-MS) or gravimetric method, to detect hydrocarbons, which were further used to determine extent of degradation.

To assess the extent of diesel degradation in contaminated soil, hydrocarbons were extracted from both the untreated and treated soil samples. A 10 g soil sample was transferred into a 30 mL centrifuge tube along with 10 mL of n-hexane and 10 mL of water. After 60 minutes of sonication, the mixture underwent centrifugation at 8000 rpm for 20 minutes. The resulting n-

hexane layer was carefully transferred into a clean centrifuge tube. This extraction process was repeated twice to ensure thorough extraction, and the collected samples were analysed for degradation, following the same procedures as water samples.

The GC-MS analysis was performed using a PerkinElmer Clarus 680 gas chromatograph equipped with a Clarus 600C mass spectrometer. The MS 5 silica capillary column (60 m x 0.25 mm) from Perkin Elmer was used. The GC injector was kept at 280°C, and the solvent delay time was set to 8 min. At a flow rate of 1 mL/min, helium was used as the carrier gas. The mass spectrometer had a scan range of 50 to 600Da. The present components were identified using the GC-MS library of the National Institute of Standards and Technology (NIST) database.

The degradation of diesel over time was also assessed through the gravimetric method (Wang et al., 2023). Residual hydrocarbons were extracted as described above. The content of the extracted final mixture and the feed mixture was quantified using the gravimetric method, measuring the diesel's weight before and after degradation. The change in weight, indicative of degradation extent, was then compared with the initial weight of the feed diesel. The components in the extracted oil were analyzed using GC-MS. The degradation for diesel was calculated by using equation (1):

$$\text{Overall degradation } \% = \frac{C_0 - C}{C_0} \times 100 \qquad (1)$$

Where , C_0 = Total hydrocarbon content in feed mixture

C = Total hydrocarbon content in final mixture

The degradation of the individual compounds present in diesel was determined by equation (2):

$$\text{Individual compound degradation } \% = \frac{(C_i) - (C_f)}{(C_i)} \times 100 \qquad (2)$$

Where , C_i = Intial concentration of compound in feed mixture

C_f = Final concentration of compound in final mixture

The concentration of metabolites present in the final mixture after degradation was calculated by the following equation (3):

$$\text{Concentration of pathways intermediate product} = \frac{A_p}{A_f} \times 100 \qquad (3)$$

Where A_p is the peak area for intermediate product, and A_f is the total peak area of all the hydrocarbons present.

3.10 Effect of process parameters

The effect of process parameters, such as initial diesel concentration, pH, temperature and inoculum concentration on diesel degradation was studied. The diesel concentration was varied in the range of 1 - 6% v/v, and pH was varied in the range of 5-9. The temperature was changed in the range of 25-40 °C, and three inoculum concentrations (0.5, 1, and 2 % v/v) were used. The experiments were done by the shake flask method. In this method, the culture was inoculated with diesel and kept at specific temperature for 10 days. At regular intervals of 2 days samples were collected and bacterial growth was measured. Thereafter, analysis of solution was done using GCMS and gravimetric method to determine degradation of diesel.

3.11 Photolysis test

The photolysis test was performed to determine the diesel degradation only by light source without using any bacteria. For this experiment 4% v/v diesel was kept under the UV LED of 254 nm wavelength and Vis LED of 400-700 nm wavelength for the 7 days. The percentage degradation of diesel was determined by using GCMS analysis.

3.12 Effect of light source

The effect of different light sources on degradation of diesel was studied by carrying out the experiments in presence of Ultraviolet Light-emitting diode (UV LED) of 254 nm wavelength and Visible Light-emitting diode (Vis LED) of 400-700 nm wavelength using 4% diesel concentration at 35 °C temperature, pH 7. The inoculum volume was 1% v/v. A similar experiment was performed in dark that is in absence of any light source. The degradation was carried out for 7 days in shake flask, and thereafter, analysis was done using GCMS. The growth of bacteria was measured by a UV-Vis spectrometer.

3.13 Degradation kinetics

For determination of degradation kinetics, soil and water samples were collected at regular time intervals. For each sample, extent of degradation was determined as discussed in earlier section. This degradation data was used to determine degradation rate constant and half-life of diesel degradation. First- order kinetic model was used to determine degradation kinetics of diesel. Rate of diesel degradation was expressed by following equation (4):

$$-r_d = -\frac{dC}{dt} = kC \qquad (4)$$

Here r_d is the rate of diesel degradation, t is the time in days, and C is the diesel concentration at any time. With respect to diesel concentration, the degradation was assumed to be first-order, and k is the first-order kinetic constant (day^{-1}). Integrating Equation 4 with the following conditions: at initial time t = 0, C = Co, and concentration was C at any time t.

$$-\int_{C_0}^{C} \frac{dC_0}{C} = k\int_{0}^{t} dt \qquad (5)$$

or

$$\ln \frac{C_0}{C} = kt \qquad (6)$$

The value of the kinetic constant was determined from the slope of the plots ln C_o/C against time in days. The equation (7) was used to calculate the half-life:

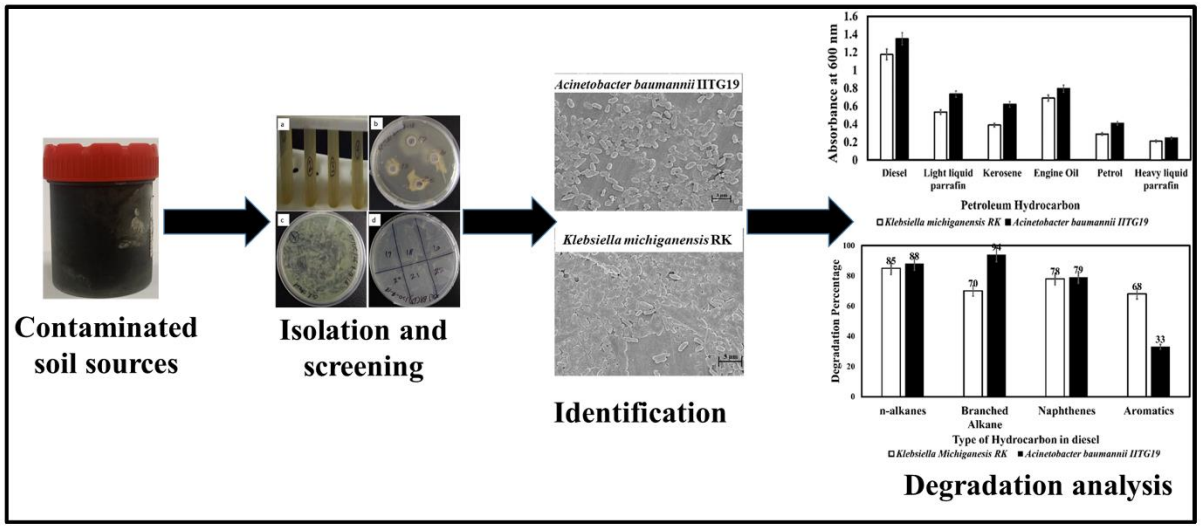
$$t_{1/2} = \frac{(0.5)^{1-n} - 1}{k(n-1)} C_0^{1-n} \qquad (7)$$

Where $t_{1/2}$ is the half-life for degradation of diesel, n is the order of degradation kinetics, C_0 is the initial diesel concentration, and k is the degradation rate constant. For first order degradation kinetics, Eq. 7 was simplified with n = 1 as:

$$t_{1/2} = \frac{\ln 2}{k} \qquad (8)$$

Chapter 4

Degradation of petroleum hydrocarbons by *K. michiganensis and A. baumannii*



In Chapter 4, two newly hydrocarbon-degrading bacteria, Klebsiella michiganensis RK *and Acinetobacter baumannii IITG19, were successfully isolated from local contaminated soil sources. These bacteria demonstrated effective degradation of diesel, kerosene, engine oil, and light paraffin oil, with Acinetobacter baumannii IITG19 exhibiting higher degradation than Klebsiella michiganensis RK. Most suitable growth conditions were observed at 35°C, pH 7, and an inoculum concentration of 1% v/v. Notably, these bacteria displayed superior efficiency in degrading paraffinic compounds (~90%) compared to naphthenic or aromatic compounds. The study marks the first report of Klebsiella michiganensis RK for hydrocarbon degradation.*

Keywords: Biodegradation; Hydrocarbons, Diesel; *Klebsiella michiganensis* RK*; Acinetobacter baumannii* IITG19

Chapter 4: Degradation of petroleum hydrocarbons by *K. michiganensis and A. baumannii*

4.1 Introduction

Petroleum hydrocarbons, crucial for industry and daily activities, may lead to pollution through spillage. Biological treatment, particularly bacterial degradation, is an effective remediation method as discussed in earlier chapters. *Acinetobacter*, *Burkholderia*, *Pseudomonas*, *Staphylococcus*, *Streptobacillus*, *Rhodococcus*, *Streptococcus* are among bacterial strains that have been reported to degradation for various petroleum hydrocarbons such as diesel, crude oil, alkanes, aromatic compound and poly aromatic compounds (Margesin et al. 2003; Chaerun et al. 2004; Jin et al. 2012; Nie et al. 2014; Xue at al. 2015; Sarkar et al. 2017; Varjani 2017; Vajrani and Upasani 2017). Details have been discussed in chapter 2.

Various studies have reported the degradation of diesel by different bacteria. Borah and Yadav (2014) found that *Bacillus cereus* DRDU1 degraded 71% of diesel in 4 weeks at an initial concentration of 2%. Jerin et al. (2022) observed 52% and 61% degradation of diesel by *Cedecea davisae* and *Acinetobacter baumannii*, respectively, over 21 days with a 10% v/v initial diesel concentration. Bekele et al. (2022) reported similar degradation levels, with *Pseudomonas aeruginosa* and *Bacillus subtilis* degrading 84% and 83.4%, respectively, at an initial diesel concentration of 3% over 15 days. Nkem et al. (2016) reported 58% degradation of diesel oil by *Acinetobacter baumannii* in a 2% v/v initial concentration over a 10-day incubation period. *Acinetobacter baumannii* has also shown the degradation ability for crude oil, with reported degradation rates ranging from 42% to 58% (Mishra et al. 2004; Chang et al. 2011; Zhang et al. 2021).

In the present study, strains were isolated from the local contaminated soil sources and screened for their degradation ability of petroleum-based hydrocarbons. The emphasis on the isolation of strains from local sources was given to develop more adaptable and effective strains for local use. The isolated strains were screened using different methods. The selected strains were studied for degradation of various hydrocarbons and the conditions were determined for maximum degradation. The degradation analysis of diesel by the selected strains was done in details. The effect of UV and visible light on bacterial degradation was also compared.

4.2 Experimental details

The contaminated soil samples, used in this study, were collected from two points, local petrol pump and motor repairing workshop. Both the points were exposed to long-term pollution by

petroleum hydrocarbons due to frequent leakage and disposal of petrol, diesel, and engine oil. The samples collected from the local petrol pump and motor repairing workshop were denoted as PP and RW, respectively. Samples were collected from 5 cm below the surface and stored in sterile sample containers. Thereafter, they were aseptically transferred to the laboratory and stored at 4°C.

Bacteria were isolated using culture enrichment followed by the serial dilution method. The isolated strains were screened for their ability for degradation of petroleum hydrocarbons. Screening was performed in 4 stages, Growth in LB broth containing 1% v/v diesel, LB agar well diffusion technique, spread plate method and by streaking on BH agar plates containing diesel. Then based on the highest growth in 1% v/v diesel, bacterial strains were selected. The selected isolates were identified using 16S rRNA sequencing and phylogenetic study. Characterization was performed using biochemical tests, Gram's staining, and morphological studies. Details of isolation and characterization are discussed in Chapter 3, section 3.1-3.5.

Degradation assessment of different hydrocarbons was also done using the 1% v/v of diesel, petrol, kerosene, engine oil, light paraffin oil, or heavy paraffin oil as the carbon source. Growth curves were generated by using 1% v/v diesel and measuring absorbance at 600 nm in 12-hour intervals up to 240 hours. Photolysis test and effect of light source on degradation of diesel by bacteria was also studied as per protocol described in chapter 3, section 3.11 and 3.12.

The effect of process parameters on diesel degradation was explored by varying initial diesel concentration, pH, temperature, and inoculum concentration over a 10-day period using the shake flask method following the methodology outlined in Chapter 3, section 3.10. The diesel degradation analysis was conducted at the most suitable process parameters for 15 days. The control sample was prepared using BH media with same conditions without adding any bacteria. The detailed methodology of the experiments used is outlined in Chapter 3 section 3.9.

4.3 Results and Discussion

4.3.1 Isolation and screening of bacteria

19 bacterial strains were isolated from the soil sample collected near the petrol pump (PP samples), and 10 strains were obtained from the soil sample collected from the repair workshop (RW samples). The isolated strains were streaked on Luria-Bertani agar plates and incubated

for 24 h at 37°C before storage at 4°C. Screening of the isolates (29 strains) was then performed. Fig. 4.1 shows screening of bacterial isolates.

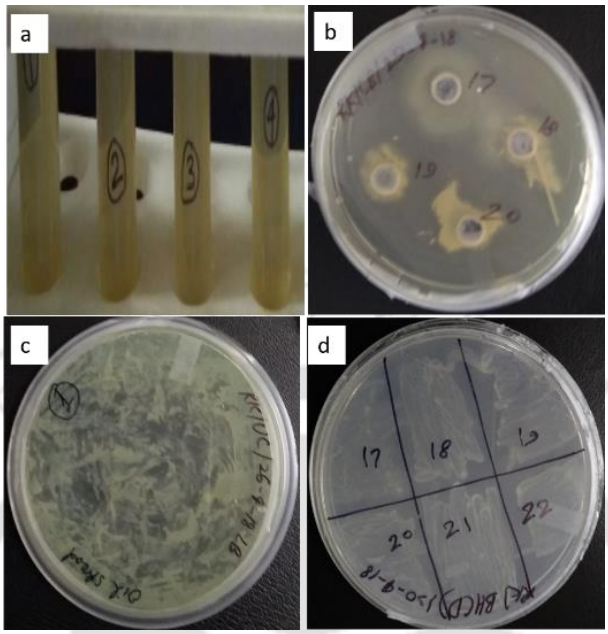


Fig. 4.1 Screening of strains based on their ability to grow in presence of petroleum hydrocarbons under different conditions a) Growth in LB broth containing 1 % of diesel oil, b) Agar well diffusion in presence of diesel on the surface (100 µl), c) spread plate method in presence of equal concentration of inoculum and diesel oil (100 µl each), d) streaking on BH agar plate in presence of 1 % diesel.

In the first screening stage, all 29 isolates showed positive results by showing growth using carbon sources from nutrient-rich LB media and added hydrocarbon. It was also observed that the addition of petroleum hydrocarbon did not adversely affect the bacterial growth. Hence it was concluded that all these bacteria had tolerance for petroleum hydrocarbons. The second stage of screening was done using the agar well diffusion method. It was observed that growth occurred around the wells. This confirmed that all the selected 29 strains could utilise hydrocarbon as the carbon source. In the third stage of screening, 20 out of 29 isolates showed growth in the presence of diesel. In this experiment, nine bacteria isolates did not show any growth suggesting non-tolerance of these strains to direct contact with a high concentration of petroleum hydrocarbons. The remaining 20 isolates, having tolerance for direct contact of high concentration of petroleum hydrocarbons, were subjected to the next screening stage. In the fourth and final screening stage, 13 out of 20 isolates showed growth in presence of all three conditions (1% diesel, 1% petrol and a mixture of 0.5% petrol & 0.5% diesel). The growth

confirmed their ability to utilise diesel as well as petrol as the carbon source. The remaining 7 isolates showed growth either in petrol or diesel or none and were discarded.

Fig. 4.2 shows the comparison of growth of the 13 isolates, surviving the four stages of screenings. Among these 13 isolates, the P10 (isolated from the PP soil sample) showed the highest growth in the Bushnell Hass broth media supplemented with 1% v/v diesel, followed by W4 isolated from the RW soil sample. P10 and W4 were selected for further study.

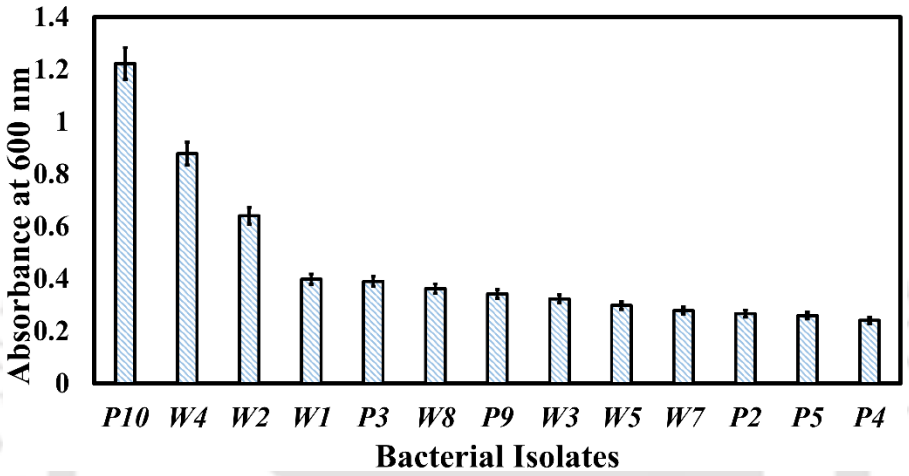


Fig. 4.2 Growth of 13 isolates, that survived the four stages of screenings. Growth was measured by taking absorbance at 600 nm. (Sample names starting with *P* and *W* corresponded to strains isolated from petrol pump (PP) and repairing workshop (RW) soil samples, respectively.)

4.3.2 Identification and Phylogenetic analysis of bacteria

The 16S rRNA amplification was performed for bacterial isolates P10 and W4, yielding sequences of 1338 bp and 1166 bp in length, respectively (Fig. 4.3). Basic Local Alignment Search Tool (BLAST) analysis revealed that bacterial isolate P10 was identified as *Acinetobacter baumannii*, showing 99.93% similarity to *Acinetobacter baumannii* strain CIP 70.34, with nearly full-length sequence and minimal differences from the closest neighbour confirming its identity, and an E-value of zero validating the result. Similarly, bacterial isolate W4 was identified as *Klebsiella michiganensis*, showing 99.14% similarity to *Klebsiella michiganensis* strain W14, with confirmation of its identity through nearly full-length sequence and minimal differences from the closest neighbour, and an E-value of zero confirming the validity of the result. The 16s rRNA sequence of *Klebsiell*a and *Acinetobacter* was deposited in the NCBI gene bank with the names *Klebsiella michiganensis* RK and *Acinetobacter*

baumannii IITG19. GenBank accession numbers MN749814 and OK310719 were obtained for *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19, respectively.

a

TCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAATCGCTGGCAACAAAGGATAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTT
CCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCAT
CGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT
ACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACTCCTCAAGGGAACAACCTCCAAGTCGA
CATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAG
TCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGA
ATTCTACCCCCCTCTACAAGACTCCAGCCTGCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTT
CACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCG
TATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGATGAGGTTATT
AACCTTATCGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCT
GCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGT
TCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACT
AGCTAATCCCATCTGGGCACATCTGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTAT
GCGGTATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCC
GCCACTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCGTTCGACTTGCATGTGTTAGGCCTGCCGCCAGCG
TTCAATCTGAGCCAGGATCAAACTC

b

CTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGG
CTTTTTGAGATTAGCATCACATCGCTGTGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCT
GGCCGTAAGGGCCATGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCACTGGCAGTATCCTTAAA
GTTCCCATCCGAAATGCTGGCAAGTAAGGAAAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCAC
GACACGAGCTGACGACAGCCATGCAGCACCTGTATCTAGATTCCCGAAGGCACCAATCCATCTCTGGAAA
GTTTCTAGTATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGT
GCGGGCCCCCGTCAATTCATTTGAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGTCTACTTATCGCGTTA
GCTGCGCCACTAAAGCCTCAAAGGCCCCAACGGCTAGTAGACATCGTTTACGGCATGGACTACCAGGGTA
TCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCAGCGTCAGTATTAGGCCAGATGGCTGCCTTCGCCATC
GGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCT
CACCAGTATCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATCCGACTTAATAAGCCGCCTACGCA
CGCTTTACGCCCAGTAAATCCGATTAACGCTCGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAG
CCGGTGCTTATTCTGCGAGTAACGTCCACTATCTCTAGGTATTAACTAAAGTAGCCTCCTCCTCGCTTAAAG
TGCTTTACAACCATAAGGCCTTCTTCACACACGCGGCATGGCTGGATCAGGGTTCCCCCCATTGTCCAATA
TTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCGGATCATCCTCTCAGAC
CCGCTACAGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACTTAGGCTCATCTATTAG
CGCAAGGTCCGAAGATCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCATCCCTTTCGAGATGTTGT
CCCCCACTAATAGGCAGATTCCTAAGCATTACTCACCCGTCCGCCGCTAGGTCCAGTAGCAAGCTACCTTC
CCCCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCAATCTGAGCCAG

Fig. 4.3 16s rRNA sequence of a) *Klebsiella michiganensis* RK b) *Acinetobacter baumannii* IITG19.

Phylogenetic tree for both bacterial isolates is shown in Fig. 4.4. The phylogenetic study, using the Neighbor-Joining method for bacterial strains successfully confirmed the molecular relationships among the isolates. According to the analysis, strain P10 was confirmed as *Acinetobacter baumannii*, indicating its close relationship to other reported strains of *Acinetobacter* genus. Similarly, strain W4 was confirmed as *Klebsiella michiganensis*, showing affinity to other *Klebsiella* species.

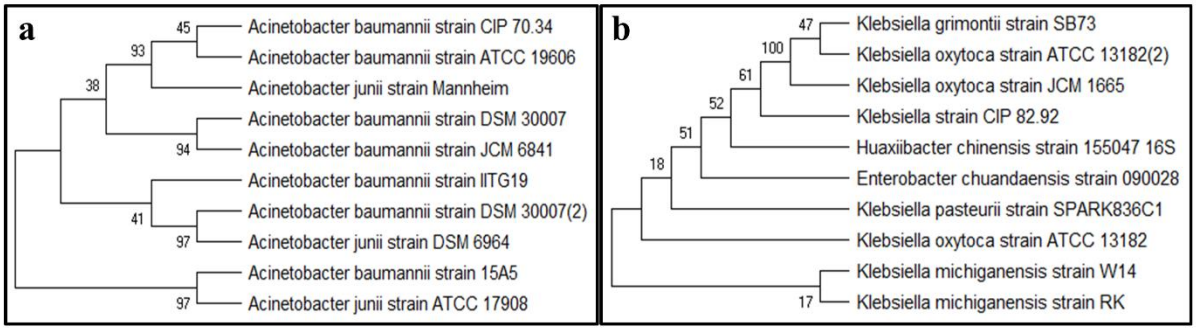


Fig. 4.4 Phylogenetic tree for a) *Acinetobacter baumannii* IITG19, b) *Klebsiella michiganensis* RK.

4.3.3 Biochemical analysis of bacteria

The result of the biochemical test is summarised in Table 4.1. Both strains *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 showed positive result for the malonate utilisation test by changing colour from green to blue using sodium malonate as the carbon source. The pH indicator was bromothymol blue. The colour change occurred due to the formation of sodium hydroxide, which produced alkalinity in the medium. The gelatin liquefaction test was also performed, and both isolates showed a positive result. Both isolates produced gelatinase enzyme, which hydrolysed gelatin and converted the medium from solid to liquid. The starch hydrolysis test was performed to determine their ability to hydrolyse starch by producing the enzyme α amylase. *Klebsiella michiganensis* RK was negative by the dark blue colouration of the media, and *Acinetobacter baumannii* IITG19 was positive for this test by forming clear zones. TSI test was performed to determine the ability of isolates to ferment sugar to produce H_2S and CO_2. The *Klebsiella michiganensis* RK showed a negative result with no change in the colour of TSI media. There was production of H_2S by *Klebsiella michiganensis* RK, which was observed as black precipitation at the bottom of the tube. The *Acinetobacter baumannii* IITG19 showed positive result for sugar fermentation by the colour change of media from red to yellow, and there was no production of H_2S. The Phenyl Alanine Deaminase Test was performed to determine the ability of isolates to produce deaminase enzyme. The *Klebsiella michiganensis* RK showed positive results by producing dark green colour due to oxidative deamination of phenylalanine to phenylpyruvic acid. The *Acinetobacter baumannii* IITG19 showed negative result with no change in media colour. The Urease Test was performed to determine the ability of isolates to produce urease enzyme. The urease enzyme causes hydrolysis of urea into ammonia and CO_2. The *Klebsiella michiganensis* RK showed positive result by changing media colour from yellow to pink. For *Acinetobacter*

baumannii IITG19, no colour change was observed, so it is a negative result for the urease test. Both isolates were found to be Gram's negative in Gram's staining. Thus, the biochemical study differentiated both the isolates, and it was confirmed that both isolates were from different bacteria groups.

Table 4.1 Biochemical tests of bacteria

Biochemical Test	*Klebsiella michiganensis* RK	*Acinetobacter baumannii* IITG19
Malonate utilisation	Positive	Positive
Gelatine Liquefaction	Positive	Positive
Starch Hydrolysis	Negative	Positive
Triple Sugar Iron Agar	Negative	Positive
H_2S production	Positive	Negative
Phenyl Alanine deaminase	Positive	Negative
Urease	Positive	Negative
Gram's Staining	Negative	Negative

4.3.4 FESEM analysis of bacteria

The Fig. 4.5 show the FESEM images of the bacteria before exposure to diesel. The oval rod shape was observed for *Acinetobacter baumannii* IITG19 (Fig. 4.5a) and the elongated rod shape for *Klebsiella michiganensis* RK (Fig. 4.5b). The Fig. 4.6 c and d show the images of the same strains after exposure in diesel. The change in external texture clearly confirmed the uptake of diesel by bacteria.

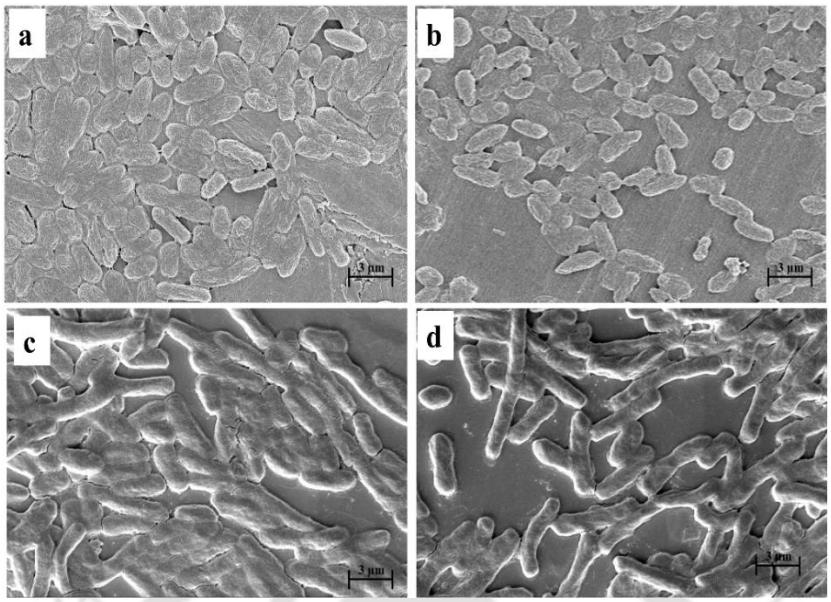


Fig. 4.5 FESEM images before exposure to diesel of a) *Acinetobacter baumannii* IITG19 b) *Klebsiella michiganensis* RK; FESEM images after exposure to diesel for 7 days c) *Acinetobacter baumannii* IITG19 d) *Klebsiella michiganensis* RK.

4.3.5 Degradation assessment for various hydrocarbons

The degrading ability of the two isolates for different hydrocarbons was determined by measuring their growth in culture after 15 days of incubation. The growth was determined as before by measuring absorbance at 600 nm and shown in Fig. 4.6. Both isolates grew in the presence of different hydrocarbons such as petrol, diesel, kerosene, engine oil, light liquid paraffin and heavy liquid paraffin; however, the extent of growth was different. The highest growth corresponded to the highest degradation (Chen et al. 2019b). The overall order of growth and hence degradation was found to be in the order of diesel> light liquid paraffin > kerosene > engine oil > petrol > heavy liquid paraffin. It was also observed that degradation by *Acinetobacter baumannii* IITG19 was higher than that by *Klebsiella michiganensis* RK for all hydrocarbons.

Different constituents of the various petroleum hydrocarbons may explain their different extent of degradation. The diesel contains mainly branched and cyclic alkanes (60 to 90 % v/v), with some aromatic compounds (5 to 40% v/v) and small amount of alkenes (0 to 10% v/v) (Mitter et al. 2021). Further, the alkanes in diesel are mainly mid-range alkanes that are less hydrophobic and less toxic than the aromatic or short and long-chain alkanes. This makes diesel more readily biodegradable (Lui et al. 2021) and hence the highest degradation was observed

for diesel. Both isolates also showed adequate growth in the presence of light liquid paraffin, which agreed with the above observation that both isolates were able to degrade mid-range alkane hydrocarbons. Growth was also observed in the kerosene. The main constituents of kerosene are saturated straight-chain and branched-chain paraffin and cycloparaffins. Hence, it can be concluded that both the isolates were able to utilise naphthenic compounds in addition to alkanes.

In engine oil, the lower growth rate might have been due to the presence of saturated long-chain hydrocarbons (paraffins) of higher carbon number and a higher proportion of naphthenic and aromatic compounds. In addition, the usually higher viscosity of engine oils than diesel or kerosene make them less available to strains. The lower utilisation of higher range paraffin may be attributed to their aquaphobic nature, thus making them less accessible to bacteria than the mid-range compounds (Xu et al. 2018). The second-lowest growth was observed in petrol, despite the carbon number being in the lower range (Wang et al. 2016). It might be explained by presence of a higher amount of aromatics (20-50%) in petrol, which is less biodegradable as compared to alkanes. The short-range alkanes present in petrol might be more bioavailable, but are associated with cell toxicity. This toxicity inhibits bacterial growth by interfering with bacterial metabolism and leads to cell death. Consequently, less growth was observed in petrol resulting in low degradation (Gargouri et al. 2015). The lowest growth was observed for heavy liquid paraffin and might have been caused by the significant presence of compounds of higher carbon number. The higher carbon number and high viscosity might have made heavy liquid paraffin less bioavailable to isolated bacterial strains. The study thus suggested that the isolated bacterial strains were more suitable for degradation of medium range alkanes.

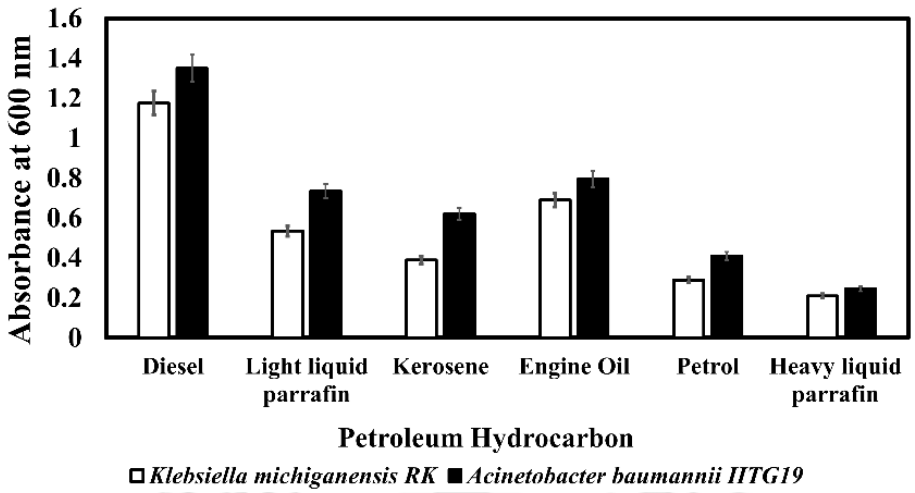


Fig. 4.6 Growth of *Klebsiella Michiganesis* RK and *Acinetobacter baumannii* IITG19 in various hydrocarbons. (at hydrocarbon concentration 4% v/v, pH 7, temperature 35°C and inoculum concentration 1% v/v).

4.3.6 Growth curve analysis of bacteria

The growth of isolated bacteria *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 is shown in Fig. 4.7. It was observed that the lag phase was 12h for both isolates, which suggested that the isolates took 12h to get adapted to hydrocarbon media and get ready to multiply. The growth was slow during this period. Log phase was 12 to 48 h for *Klebsiella michiganensis* RK and 12 to 72 h for *Acinetobacter baumannii* IITG19. The period of the log phase represented the period of the highest growth rate in which the isolates multiplied very fast. The stationary phase was up to 9 days for both. The stationary phase corresponded to the period where the growth rate was equal to the death rate of isolates. After 9 days, the decline phase started when the growth rate was lower than the death rate of isolates. This low growth rate might have been caused by lower availability of growth media components or by the inhibitory effect of any products produced by hydrocarbon degradation. So, from the growth curve study, it can be concluded that these bacteria can be used up to 10 days for bacterial degradation study at the conditions used.

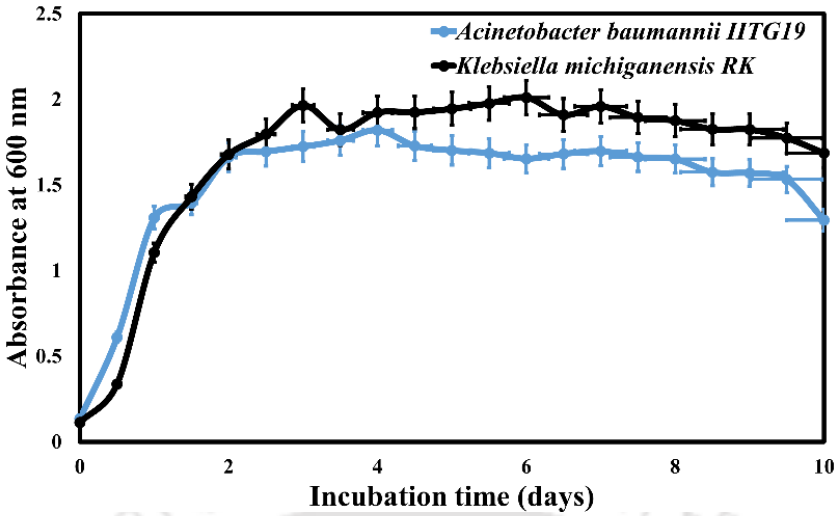


Fig. 4.7 Growth curve analysis of *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 in presence of 1% v/v diesel concentration, pH 7, temperature 37°C and 120 rpm.

4.3.7 Effect of process parameters on diesel degradation

Figs. 4.8 and 4.9 show the effects of different process parameters on growth and hence degradation ability of the two bacteria strains, *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19, for diesel. The effect of diesel oil concentration on growth of strains as function of time is shown in Fig. 4.8 a) and b). It was observed that for the first 3 days the growth of both the strains was similar in all the samples in spite of having different initial concentrations of diesel. Thereafter, there was variance in strain growth depending on diesel concentration. After day 7, it was detected that the strain present in the sample, containing 4% v/v diesel, had undergone the highest growth. The growth of strains added in the sample having 5 % v/v diesel was also comparable. However, the sample having higher concentration of 6% v/v diesel, showed lower growth of strains. The difference in growth rate of strain after 7 days was more prominent for *Klebsiella michiganensis* RK compared to that of *Acinetobacter baumannii* IITG19. At lower diesel concentrations, the growth and hence degradation was low due to less availability of substrate. At higher concentration of 6% v/v diesel, the lower growth or degradation might had resulted from toxic effect. As the concentration of 4% v/v diesel favoured the growth of strains most, it was used in subsequent study.

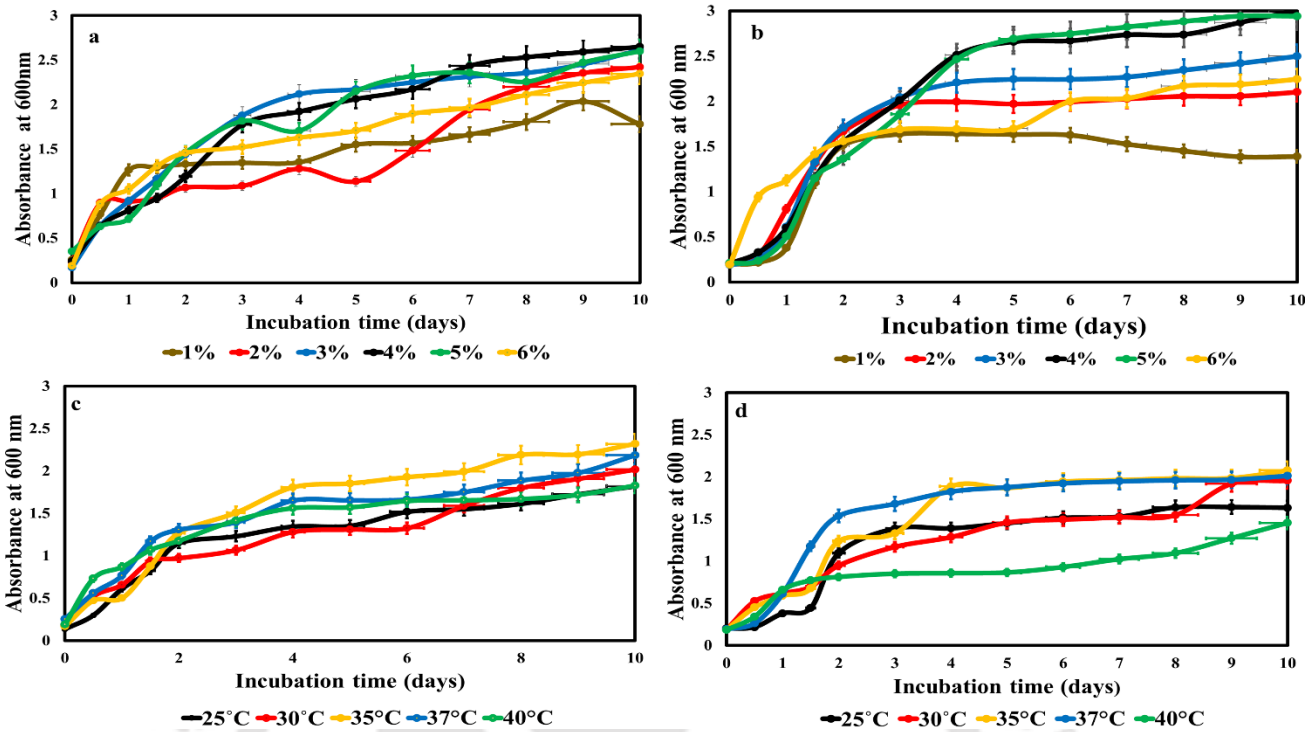


Fig. 4.8 Effect of diesel concentration on growth of a) *Acinetobacter baumannii* IITG19, b) *Klebsiella michiganensis* RK (at pH 7, temperature 37°C, inoculum concentration 1 % v/v); Effect of temperature on growth of c) *Acinetobacter baumannii* IITG19 and d) *Klebsiella michiganensis* RK (at pH 7, diesel concentration 4% v/v, inoculum concentration 1 % v/v).

The effect of temperature on the bacteria's growth with respect to incubation time has been shown in Fig. 4.8c and 4.8d. No difference in growth as function of temperature was observed for first 2 days for both strains. Thereafter, the strain *Acinetobacter baumannii* IITG19 showed highest growth at 35° consistently for the remaining period of study (Fig. 4.6c). The growth decreased at higher temperatures of 37 and 40 °C. Palanisamy et al. (2014) also reported similar optimum temperature of 35ºC for *Acinetobacter baumannii*. For *Klebsiella michiganensis* RK, similar growth was observed at both the temperatures of 35 and 37°C (Fig. 4.3d). When the temperature was further increased to 40°C, decrease in growth was observed. Saha et al. (2013) also reported 35°C as the optimum temperature for *Klebsiella michiganensis*. The results indicated that both the isolates in this study were highly efficient in degrading diesel hydrocarbons at varying temperatures, and their cells were most active at 35°C. Hence, 35°C was used as the most suitable temperature in subsequent studies.

The effect of pH on bacterial growth with respect to incubation time is shown in Fig. 4.9 a and b. After the 3rd day, the highest growth for isolates was observed consistently at pH 7. Higher

or lower pH values than 7 led to a reduction in bacterial growth. Similar optimum pH conditions have been reported (Marvi-Mashhadi et. al. 2022). Fig 4.7a also shows that the effect of variation of pH on strain growth was more distinct for *Acinetobacter baumannii* IITG19. The effect of pH on diesel degradation is usually attributed to its role in dissolving the nutritional substances in the medium, its influence on substrate ionisation, and substrate availability for the microorganism. Since for both the strains, the highest growth was observed at pH 7, it was used in further studies.

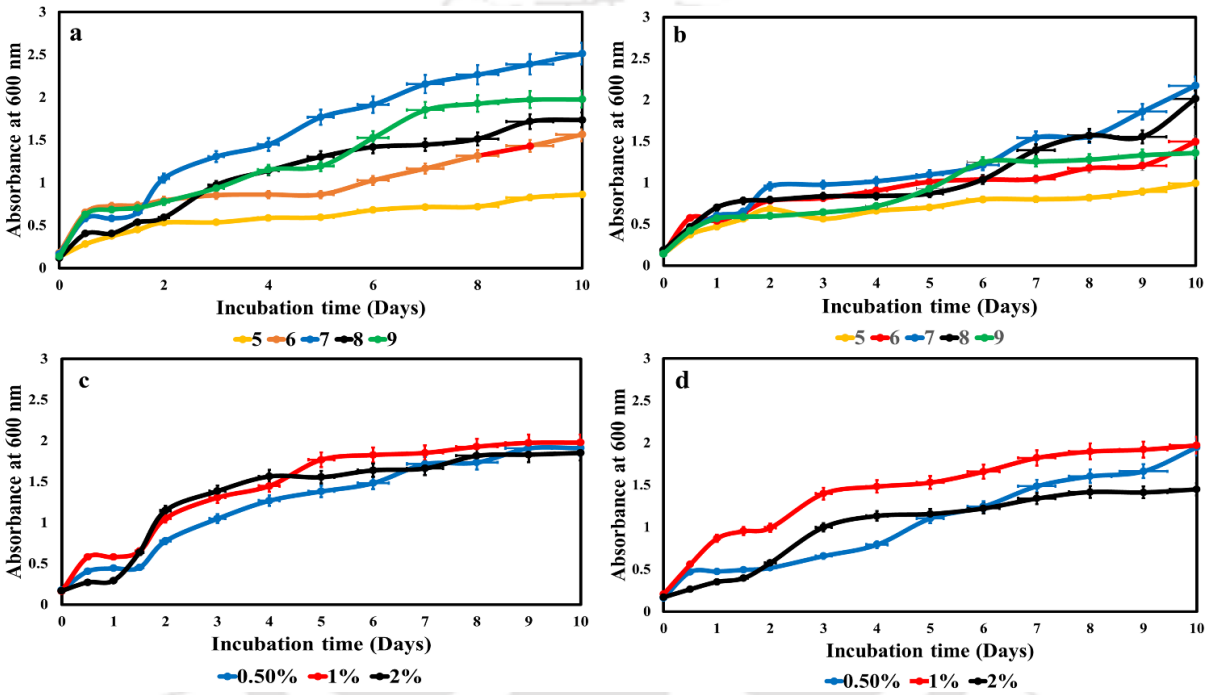


Fig 4.9 Effect of pH on growth of a) *Acinetobacter baumannii* IITG19, b) *Klebsiella michiganensis* RK, (at diesel concentration 4% v/v, inoculum concentration 1 % v/v , temperature 35°C). Effect of inoculum concentration on growth of c) *Acinetobacter baumannii* IITG19 and d) *Klebsiella michiganensis* RK (at diesel concentration 4% v/v, pH 7, temperature 35°C).

The effects of initial inoculum concentration on growth of bacteria and hence diesel degradation with respect to incubation time for *Acinetobacter baumannii* IITG19 and *Klebsiella michiganensis* RK are shown in Fig. 4.9 c and d, respectively. The highest growth was obtained at 1% v/v of inoculum for both isolates. The growth of strains was restricted at a lower initial inoculum concentration of 0.5 % due to fewer bacterial cells. The lower growth observed at the higher inoculum concentration of 2% may be attributed to the increased production of inhibitory compounds at higher inoculum levels. Additionally, higher bacterial

density can lead to competition for limited resources, such as nutrients and oxygen, creating stress among cells and reducing overall metabolic efficiency. This phenomenon, sometimes referred to as self-inhibition, suggests that 1% inoculum provides an optimal balance, allowing sufficient space and resources for maximum degradation efficiency. Hence, an initial inoculum concentration of 1% v/v was turned out to be most suitable for the degradation of petroleum hydrocarbons using the two selected strains. In this study, the most suitable conditions for the growth of the selected strains were thus determined as temperature of 35°C, pH 7 and inoculum concentration of 1% v/v. At 4% diesel concentration maximum degradation was obtained for *Acinetobacter baumannii* IITG19 and 5% for *Klebsiella michiganensis* RK.

4.3.8 Effect of different light sources

Fig. 4.10 shows the results of photolysis test for degradation of diesel in presence of only light source after 7 days of exposure. About 4 and 18% diesel degradation occurred in presence of visible light and UV light, respectively. Higher degradation occurred in presence of UV light compared to the visible light. Fig 4.11 also compares the extent of degradation of different types of hydrocarbons present in diesel in presence of different light sources. Degradation of 17, 13, 10 and 4% were observed for n-alkanes, branched alkanes, naphthenes, and aromatics fraction, respectively under the visible light. When exposed to UV light, the degradation for diesel fractions enhanced up to 52, 45, 33 and 16 % for n-alkanes, branched alkanes, naphthenes and aromatics, respectively. The higher degradation under the UV light might be explained by increase in oxidation under UV light (Borecki et al. 2022). Hence UV photolysis was more effective for the degradation of diesel in used conditions of study.

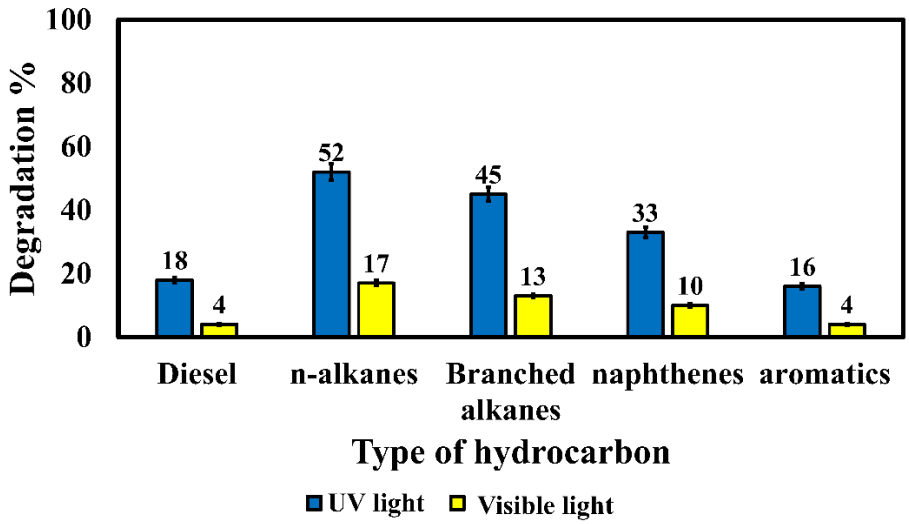


Fig. 4.10 Photolysis test for diesel degradation in 7 days (at diesel concentration 4% v/v, pH 7, temperature 35°C).

Bacterial growth of *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 in dark as well as in the presence of UV and visible light is shown in Fig. 4.11. Both bacteria showed the highest growth under visible light. When the light source was removed (i.e. in dark), bacterial growth was slightly lower, possibly due to the absence of light-enhanced enzyme activity or light-induced breakdown of diesel components that support metabolism. In contrast, exposure to UV light resulted in bacterial death, leading to the lowest growth observed. This reduced growth under UV light is consistent with previous studies that reported decreased bacterial concentrations following UV exposure (Pullerits et al. 2020; Rezaie et al. 2020).

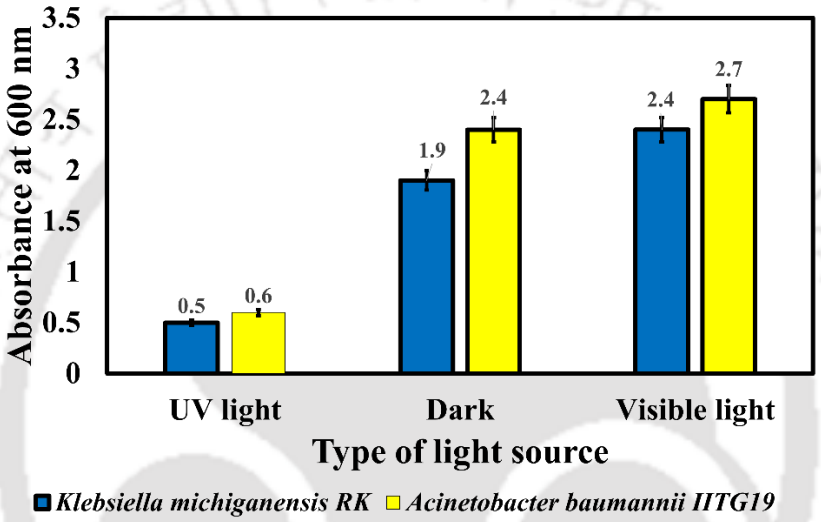


Fig 4.11 Effect of light source on growth of *Klebsiella michiganensis RK* and *Acinetobacter baumannii IITG19* (at pH 7; temperature 37°C; inoculum concentration 1 % v/v) and 4% v/v diesel concentration).

The effect of light source on degradation of diesel by bacteria *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 after 7 days of exposure is shown in Fig. 4.12. Under the UV light, 20 and 22 % degradation of diesel was incurred by *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19, respectively in 7 days of incubation period. On exposure in visible light, the degradation of diesel increased to 33 and 46 % by *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 respectively in the same incubation period. In presence of bacteria, the increase in degradation under the visible light exposure might be attributed to better growth of both bacteria in visible light compared to that under UV light source as shown in Fig. 4.12.

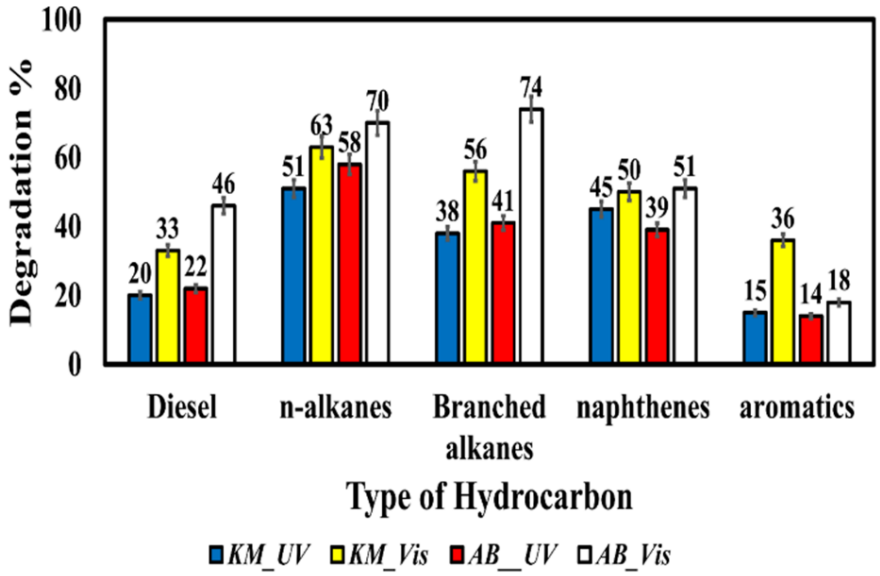


Fig. 4.12 Effect of light source on diesel degradation by bacteria in 7 days (at diesel concentration 4% v/v, pH 7, temperature 35°C and inoculum concentration 1% v/v).

(Note: *KM_UV* represent *Klebsiella michiganensis* RK under UV light, *KM_Vis* represent *Klebsiella michiganensis* RK under visible light, *AB_UV* represent *Acinetobacter baumannii* IITG19 under the UV light and *AB_Vis* represent *Acinetobacter baumannii* IITG19 under the visible light.)

In presence of *Klebsiella michiganensis* RK, degradation of n-alkanes, branched alkanes, naphthenes and aromatics were 51, 38, 45 and 15 %, respectively under the UV light, while that was 63, 56, 50 and 36, respectively under the visible light (Fig. 4.12). For the *Acinetobacter baumannii* IITG19, degradation of 58, 41, 39 and 14% were observed for n-alkanes, branched alkanes, naphthenes and aromatics, respectively under the UV light, which increased to 70, 74, 51 and 18 %, respectively under the visible light. Hence visible light source was better for the bacterial growth and consequently hydrocarbon degradation of diesel.

4.3.9 Degradation of diesel

The diesel used in this study consisted mainly of alkane compounds. The naphthenic and aromatic content of diesel was low. The main components in the feed diesel and their relative concentrations are listed in appendix Table A1. The compounds that were 0.5% or higher in the feed have been included in the Table. Among the considered components, about 36% was n-alkane and 54% was branched alkanes. The naphthenes content was 9% and aromatics was only 1%. The corresponding detected carbon number range was C_{12} to C_{26} for n-alkane, C_{11} to C_{29} for branched alkanes, C_{10} to C_{15} for naphthenes and C_{12} for aromatics.

Table 4.2 Degradation of main hydrocarbons (>0.8%) of feed diesel by the isolated strains after 15 days of incubation.

Hydrocarbon name	Molecular formula	Degradation for *Acinetobacter baumannii* (%)	Degradation for *Klebsiella michiganensis* (%)
Paraffins (n-alkanes)			
1-fluorododecane	$C_{12}H_{25}F$	87.7	85.9
pentadecane	$C_{15}H_{32}$	96.0	82.5
heptadecane	$C_{17}H_{36}$	92.1	95.0
nonadecane	$C_{19}H_{40}$	95.2	85.6
icosane	$C_{20}H_{42}$	96.2	90.9
henicosane	$C_{21}H_{44}$	90.4	90.2
tricosane	$C_{23}H_{48}$	83.7	66.0
pentacosane	$C_{25}H_{52}$	93.4	91.7
hexacosane	$C_{26}H_{54}$	94.6	76.7
hentriacontane	$C_{31}H_{64}$	10.2	56.2
Paraffins (Branched alkanes)			
4,5-dimethylnonane	$C_{11}H_{24}$	94.9	93.3
7,7-diethylheptadecane	$C_{21}H_{44}$	95.9	72.8
5-ethyl-5-methylnonadecane	$C_{22}H_{46}$	91.9	91.9
2,6,10,14-tetramethyloctadecane	$C_{22}H_{46}$	98.1	92.2
2,6,10,14-tetramethylnonadecane	$C_{23}H_{48}$	91.7	86.7
11-methyltricosane	$C_{24}H_{50}$	95.6	69.2
2,6,10,14-tetramethyl-7-(3-methylpentyl)pentadecane	$C_{25}H_{52}$	90.1	72.7
11-methylpentacosane	$C_{26}H_{54}$	91.9	93.3
2-methylhexacosane	$C_{27}H_{56}$	85.5	79.3
2-methylheptacosane	$C_{28}H_{58}$	89.3	74.1
13-methylheptacosane	$C_{28}H_{58}$	95.8	78.8
1-iodooctacosane	$C_{28}H_{57}I$	94.8	81.2
2-methyloctacosane	$C_{29}H_{60}$	84.9	68.8
Naphthenes (Cycloalkanes or cycloparaffins)			
1-pentylcyclohexene	$C_{11}H_{20}$	88.0	71.7
heptylcyclohexane	$C_{13}H_{26}$	92.3	81.3
tridecan-6-ylcyclohexane	$C_{19}H_{38}$	27.7	81.5
1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	$C_{10}H_{18}$	90.3	79.7
Aromatics			
2,6-dimethyl-1,2,3,4-tetrahydronaphthalene	$C_{12}H_{16}$	33	68.00

Table 4.2 summarises bacterial degradation of significant compounds (>0.8%) of feed diesel. The corresponding spectra have been given in appendix Fig. A1. Fig. 4.13 shows the overall

degradation of diesel and different types of hydrocarbons present in diesel by the two isolated bacteria. Both the strains showed significant degradation for all types of hydrocarbons but degradation was higher for alkanes compared to that for naphthenes or aromatic compounds. The *Acinetobacter baumannii* IITG19 showed highest degradation for branched alkanes followed by that of n-alkanes. The degradation of both normal and branched alkanes was higher in the presence of *Acinetobacter baumannii* IITG19 compared to that shown by *Klebsiella michiganensis* RK. On the other hand, the *Klebsiella michiganensis* RK showed higher degradation for aromatic compounds as compared to that shown by *Acinetobacter baumannii* IITG19. The degradation for naphthenes by two strains was similar.

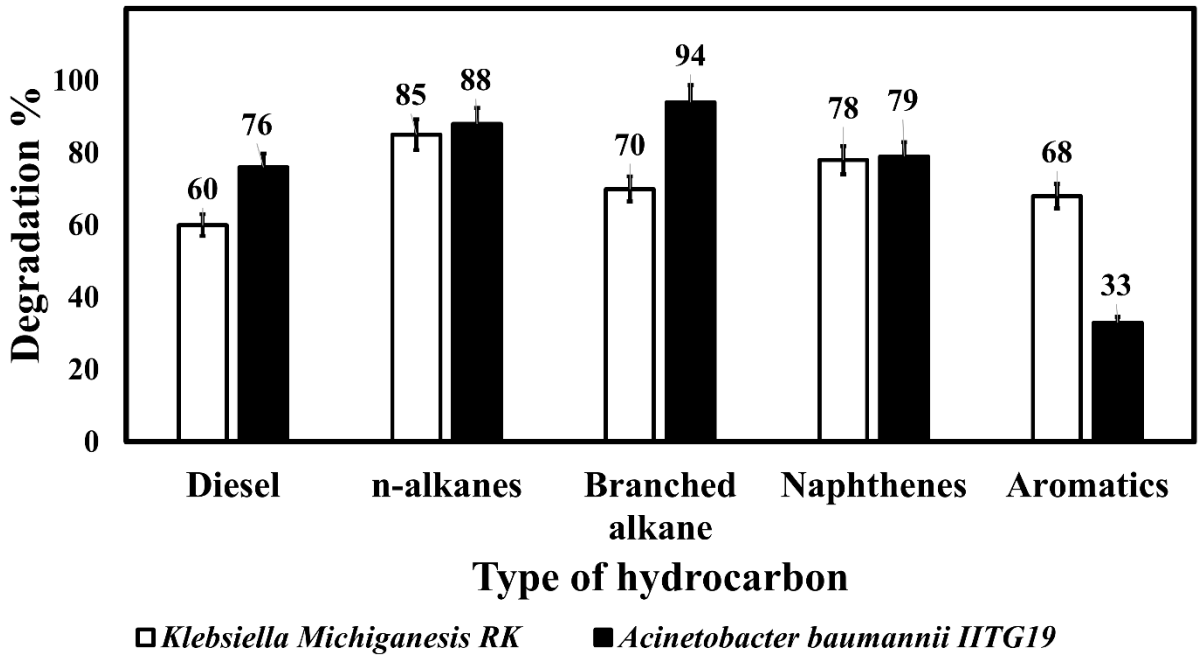


Fig. 4.13 Degradation for different types of hydrocarbons present in diesel in 15 days (at diesel concentration 4% v/v, pH 7, temperature 35°C and inoculum concentration 1% v/v).

Aromatics supported lesser growth of both bacteria, hence degraded less compared to that of paraffin and naphthenes. The overall degradations were 60 and 76 % for *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19, respectively for initial diesel concentration of 4% v/v after 15 days at 35°C, pH 7. When only significant compounds **(**>0.8%) were considered, the degradation percentage was higher at 90% for *Acinetobacter baumannii* IITG19 and 77% for *Klebsiella michiganensis* RK. The extent of degradation by the two isolated strains was higher or at par, compared to the reported biodegradation of diesel (Table 4.3).

Table 4.3 Comparison of present study with reported studies for bacterial degradation of diesel.

S.N.	Bacteria	Conc. of diesel (v/v) and degradation time	Degradation %	Ref.
1	*Bacillus cereus DRDU1*	2% and 28 days	71	Borah and Yadav 2014
2	*Acinetobacter baumannii*	2% and 10 days	58	Nkem et al. 2016
3	*Acinetobacter* sp. K-6	0.24% and 14 days	59	Chaudhary et al. 2020
4	*Acinetobacter baumannii*	10% and 21 days	61	Jerin et al. 2021
	Cedecea davisae		52	
5	*Bacillus megaterium MJ4*	1% and 5 days	72	Gao et al. 2021
6	*Pseudomonas* sp	4% and 7 days	14	Hossain et al. 2022
7	*Pseudomonas aeruginosa*	3% and 15 days	84	Bekele et al. 2022
	Bacillus subtilis		83	
8	***Klebsiella michiganensis RK***	**4% and 15 days**	**60**	**This study**
	Acinetobacter baumannii IITG19		**76**	

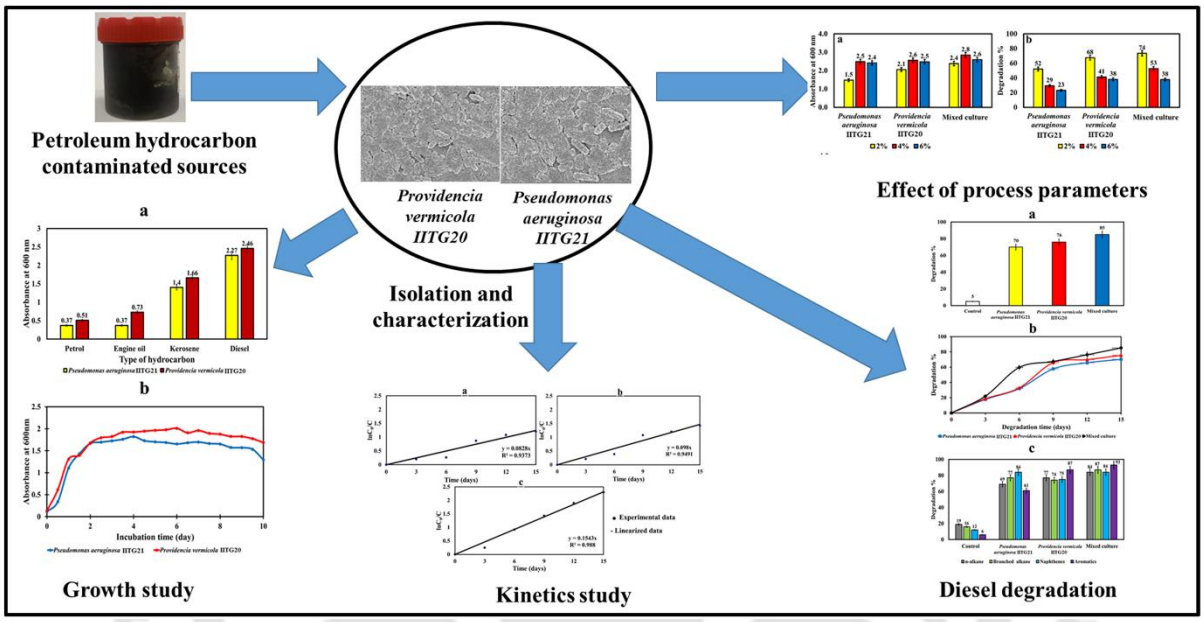
4.4 Summary

In this study, two new hydrocarbon-degrading bacteria, *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19, were successfully isolated from the two local soil sources contaminated with diesel, petrol and engine oil. These bacteria were found to have a significant ability to degrade diesel, kerosene, engine oil and light paraffin oil. They showed lower degradability for petrol and heavy paraffin. Lower degradation might be due to the toxicity of the compounds present in petrol and the high viscosity of heavy paraffin. The degradation was observed to be higher for *Acinetobacter baumannii* IITG19. These isolated bacteria gave the best growth at 35°C, pH of 7 and inoculum concentration was 1 % v/v. At substrate concentration of 4-5%, the *Acinetobacter baumannii* IITG19 and *Klebsiella michiganensis* RK, showed highest growth. UV Photolysis was more effective for degradation of diesel compare to visible light photolysis. The degradation of diesel by UV light was 18% and that by visible

light is 4% after 7 days of exposure. However, bacterial degradation of diesel was more effective in presence of visible light compared to that in presence of UV light in the same time period. This might be attributed to better growth of the bacteria in visible light compared to that under the UV light source. For diesel, after 15 days of incubation, *Klebsiella michiganensis* RK *and Acinetobacter baumannii* IITG19 showed overall degradation of 60 and 76 %, respectively. For the significant (≥0.8) compounds in diesel, the degradation reached up to 77 and 90% for *Klebsiella michiganensis* RK *and Acinetobacter baumannii* IITG, respectively. It was also observed that isolated bacteria were able to degrade paraffins more than the naphthenic and aromatics. To best of our knowledge, the *Klebsiella michiganensis* RK is reported for the first time for hydrocarbon degradation application.

Chapter 5

Degradation of petroleum hydrocarbons by *P. vermicola and P. aeruginosa*



Chapter 5 explores Providencia vermicola IITG20 and Pseudomonas aeruginosa IITG21, isolated from local refinery waste sludge and contaminated soil. They were effective in utilizing various hydrocarbons for growth. Their degradation potential was studied under specific conditions: 4% v/v initial diesel concentration, 37°C temperature, pH 7, and 1% v/v initial inoculum concentration, over 15 days of incubation period. Degradation of 70% and 76% for diesel were recorded for Pseudomonas aeruginosa IITG21 and Providencia vermicola IITG20 pure cultures, respectively. Their mixed culture achieved higher degradation of 85%, with lower half-life of 4.5 days, indicating synergistic effects.

Keywords : *Providencia vermicola* IITG20; *Pseudomonas aeruginosa* IITG21; mixed culture; Degradation kinetics.

Chapter 5: Degradation of petroleum hydrocarbons by *P. vermicola and P. aeruginosa*

5.1 Introduction

Numerous bacteria, particularly those isolated from diesel-contaminated sites, exhibit efficient diesel degradation (Horel and Schiewer 2014; Chen et al. 2017; Muangchinda et al. 2018). *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp., and others have demonstrated significant diesel-degrading capabilities (Imron et al. 2020; You et al. 2018; Sun et al. 2018; Nnabuife et al. 2022; Pourfadakari et al. 2021; Yang et al. 2023). Mixed bacterial cultures have shown enhanced degradation, harnessing synergies for improved hydrocarbon breakdown (Xu et al. 2018; Safitri et al. 2018; Parthipan et al. 2018; Morales-Guzmán et al. 2017; Muthukumar et al. 2023). Details have been discussed in Chapter 2.

This study aimed to isolate bacterial strains having high degradation ability for petroleum hydrocarbons having higher carbon numbers, such as found in diesel. The bacterial strains were isolated from contaminated soil and sludge samples obtained from a local refinery. The local source was used to isolate the bacterial strains, to increase their effectiveness in controlling local hydrocarbon based pollutions. The performance of the mixed culture of the bacterial strains was also studied. The study on the degradation kinetics by the isolated bacterial strains gave more insights regarding their effectively.

5.2 Experimental details

The study utilized contaminated soil and sludge samples obtained from the waste treatment plant of Indian Oil Corporation Limited Refinery, Guwahati, India. The samples were stored at 4°C until utilized for isolation of bacteria. Bacteria were isolated through culture enrichment followed by serial dilution methods as discussed in Chapter 3, section 3.2. The isolated strains were further screened for their petroleum hydrocarbon degradation abilities in four stages: growth in LB broth with 1% v/v diesel, LB agar well diffusion, spread plate, and streaking on BH agar plates containing diesel, as discussed in Chapter 3, section 3.3. Strains showing the highest growth in 1% v/v diesel were selected, identified via 16S rRNA sequencing and phylogenetic analysis, and characterized through biochemical tests, Gram's staining, and morphological studies by the following methodology outlines in Chapter 3, section 3.4 and 3.5. Degradation assessment of various hydrocarbons, including diesel, petrol, kerosene, and engine oil was conducted at 1% v/v concentration as discussed in Chapter 3, section 3.8. Growth curves were studied by measuring absorbance at 600 nm in 12-hour intervals over 240 hours

in diesel-containing media by the following methodology outlines in Chapter 3, section 3.7. The effect of process parameters on diesel degradation, such as initial diesel concentration, pH, temperature, and inoculum concentration, was investigated over a 10-day period using the shake flask method as discussed in Chapter 3, section 3.10. Diesel degradation analysis was further studied under optimal process conditions for using 15 days of incubation period by the following methodology outlines in Chapter 3, section 3.9. The control samples were prepared using BH media under identical conditions without bacterial inoculation.

Further mixed culture was prepared by mixing equal volume of 0.5 mL from each bacteria. Diesel degradation by mixed culture was assessed. Degradation kinetics study was performed by using both bacteria as pure culture as well as mixed culture as discussed in Chapter 3, section 3.13.

5.3 Results and Discussion

5.3.1 Isolation and screening of bacteria

Total 12 bacteria were isolated, 6 from soil and 6 from sludge sources. Fig. 5.1 shows representative images of screening experiments. During screening experiments, all 12 isolates showed good diesel tolerance and utilization ability. Growth of all 12 bacteria was observed in all four stages of screening. In the first stage of screening (LB broth supplemented with diesel), all bacteria grew, as confirmed by the increase in turbidity. In the second stage of screening (LB agar well diffusion), it was observed that all the 12 isolates were able to grow around the wells created in agar plates. The addition of diesel in LB agar did not stop the growth of bacteria. In third stage of screening (spread LB agar plates) as well, growth of all bacteria were observed despite of mixing inoculum with equal amount of diesel. In fourth and final stage of screening, by streaking on BH agar media containing 1% v/v diesel or petrol or equimolar mixture of diesel and petrol, again growth was observed in all the plates by using added hydrocarbon oil as the carbon source. Hence, these experiments confirmed that all the studied 12 bacteria, could tolerate and utilize diesel, petrol and their mixture successfully as the carbon source, in the used concentration range.

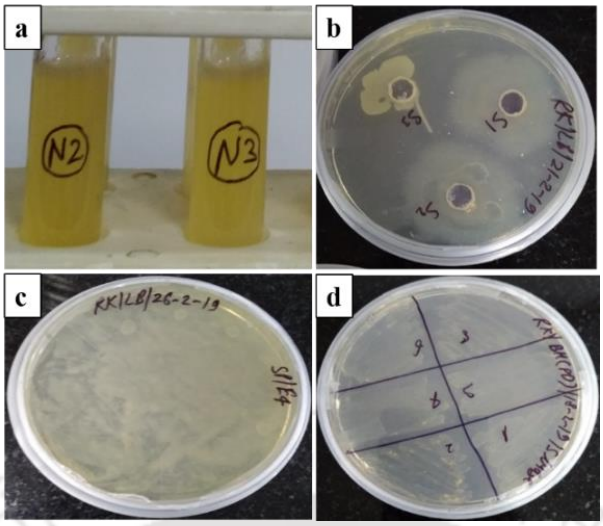


Fig. 5.1 Screening for the hydrocarbon degradation and tolerance a) LB broth with diesel, b) agar well diffusion, c) spread plate, d) growth on BH agar.

Fig. 5.2 shows the comparison of growth of all the 12 bacterial isolates using 1% v/v diesel concentration after 96 h of incubation period. It was observed that the bacterial isolates had different growth pattern. The strains isolated from the soil source (N1 to N6) in general showed higher growth than that of isolates obtained from sludge source (S1 to S6). The results suggested that bacterial strains isolated from contaminated soil possibly had more suitable metabolic pathways for utilization of diesel as a carbon source as compared to those isolated from sludge (Xu et al. 2018). Thus isolates from different sources did not have equal potential for diesel degradation. Even among same sourced isolates, there was difference in growth suggesting that they might have been strains of different lineage. Among, bacterial strains isolated from contaminated soil source, N2 showed the highest growth while, S3 was the highest growing bacterial isolate among those derived from sludge source. Hence, N2 and S3, isolated from two different sources, soil and sludge, respectively, were selected for further study.

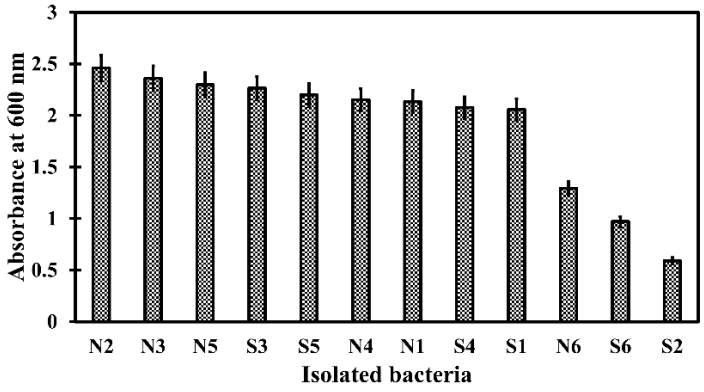


Fig. 5.2: Growth of bacteria using 1% v/v diesel concentration, pH 7, temperature 37°C and 120 rpm at 96 h incubation period. (N1, N2, N3, N4, N5 and N6 are the isolates from the contaminated soil. S1, S2, S3, S4, S5 and S6 are the isolates from the refinery sludge).

5.3.2 Identification and phylogenetic study

The 16S rRNA amplification for both bacteria was done and sequences were obtained. Fig. 5.3a and b shows 16S rRNA sequences of *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively. Sequences for both bacteria were approximately 1200 bp in length. As per the BLAST analysis bacterium N2 was identified as *Pseudomonas aeruginosa*, with its 16S rRNA gene sequence showing a high similarity of 99.85% to *Pseudomonas aeruginosa* strain JU_CHE_01. Whereas, bacterium S3 was identified as *Providencia Vermicola*, with a 99.93% similarity to *Providencia vermicola* strain OF6. Nearly full-length sequence and minimal differences from the closest neighbours confirmed the identity of both bacteria. The E-values (error value) for all BLAST results were zero, confirming their validity.

a

GCAGGCCTAACACATGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAG
TAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCT
GAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
GGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAAAGCCTGATCCAGC
CATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTA
ATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC
GAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTG
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TTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTG
ATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCC
TTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
TAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAAT
GGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGC
AGTCTGCAACTCGACTGCGTGAAGTCGGAATC

b

CTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAACAGGGGAAGCTTGCTTC
TCGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACCACTGGAAA
CGGTGGCTAATACCGCATAATCTCTTAGGAGCAAAGCAGGGGAACTTCGGTCCTTGCGCTATCGGATGAACC
CATATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
ATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCCTAGGGTTGTAAAGTACTTTCAGTCGGG
AGGAAGGCGTTGATGCTAATATCATCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
TGATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAGACTGGTCAGCTAGAGTCTTG
TAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGC
GGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCTGTAAACGATGTCGATTTGAAGGTTGTTCCCTTGAGGAGTGGCTTTCGGAGCTAACGCGTTAAAT
CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATTTAGCAGAGATG
CTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCG
GTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGC
TACAATGGCGTATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTACGTCGTAGTCCGG
ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATA
CGTTCCCGGGCCTTG

Fig. 5.3. 16s rRNA sequence of a) *Pseudomonas aeruginosa* IITG21, b) *Providencia vermicola* IITG20.

The phylogenetic study by Maximum Likelihood method and Neighbor-Joining method for both the bacterial strains are shown in Fig.5.4. These analyses successfully determined the molecular relationships of the isolates, identifying their closest genetic relatives. According to the phylogenetic analysis, classification of bacterium N2 was confirmed as *Pseudomonas aeruginosa*, a member of the *Pseudomonas* genus within the gram-negative *Pseudomonadaceae* family of gammaproteobacteria class. This finding suggested that *Pseudomonas aeruginosa* evolved from the common ancestor of all *Pseudomonas* sp, reaffirming its place within this genus. The identity of bacterium S3 was confirmed as *Providencia vermicola,* a member of the gram negative *Providencia* genus in the *Providenciaceae* family, also belonging to the gammaproteobacteria class. *Providencia vermicola* was found to be related to other *Providencia* species and shared common ancestors

within this taxonomic group. The 16s rRNA sequences of isolated strains were deposited in the NCBI GenBank as *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively, with GenBank accession numbers OP174927 and OP174960, respectively.

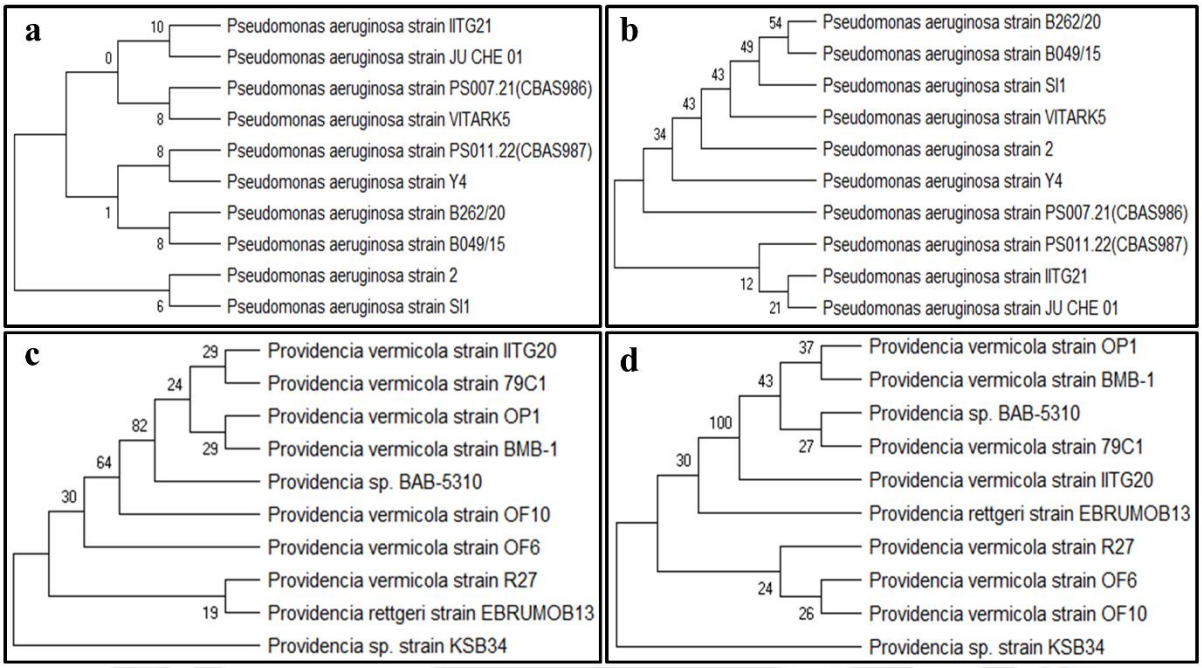


Fig 5.4 Phylogenetic tree for isolated bacteria *Pseudomonas aeruginosa* IITG21 using a) Maximum Likelihood method, b) Neighbour-Joining method; Phylogenetic tree for *Providencia vermicola* IITG20 using c) Maximum Likelihood method, d) Neighbour-Joining method.

5.3 Characterization of identified bacteria

Fig. 5.5a and b show the FESEM images for both the selected strains before exposure of diesel. *Pseudomonas aeruginosa* IITG21 was of elongated rod shape (Fig.5.5a), and *Providencia vermicola* IITG20 showed oval rod shape (Fig.5.5b). Morphology of the strains suggested both to be bacilli because of rod shape. The average length of *Pseudomonas aeruginosa* IITG21 was 3.3 µm, while that of *Providencia vermicola* IITG20 was 2.6 µm. Changes in the external morphology of both bacteria were observed after exposure to diesel, as shown in Fig. 5.5c and d. After exposure to diesel, the average length of *Pseudomonas aeruginosa* IITG21 decreased to 2.7 µm. while that for *Providencia vermicola* IITG20 to 2.1 µm. The images also suggested possible formation of a lining around the cell wall on exposure to diesel. These reductions in size and formation of lining possibly was caused by stress, induced by uptake of diesel (Pátek

et al. 2021). These observations confirmed that bacterial cells were interacting with the carbon sources. The morphological changes observed in cells also suggested adaptive response behaviour of them, undergoing structural modifications on exposure to hydrocarbons.

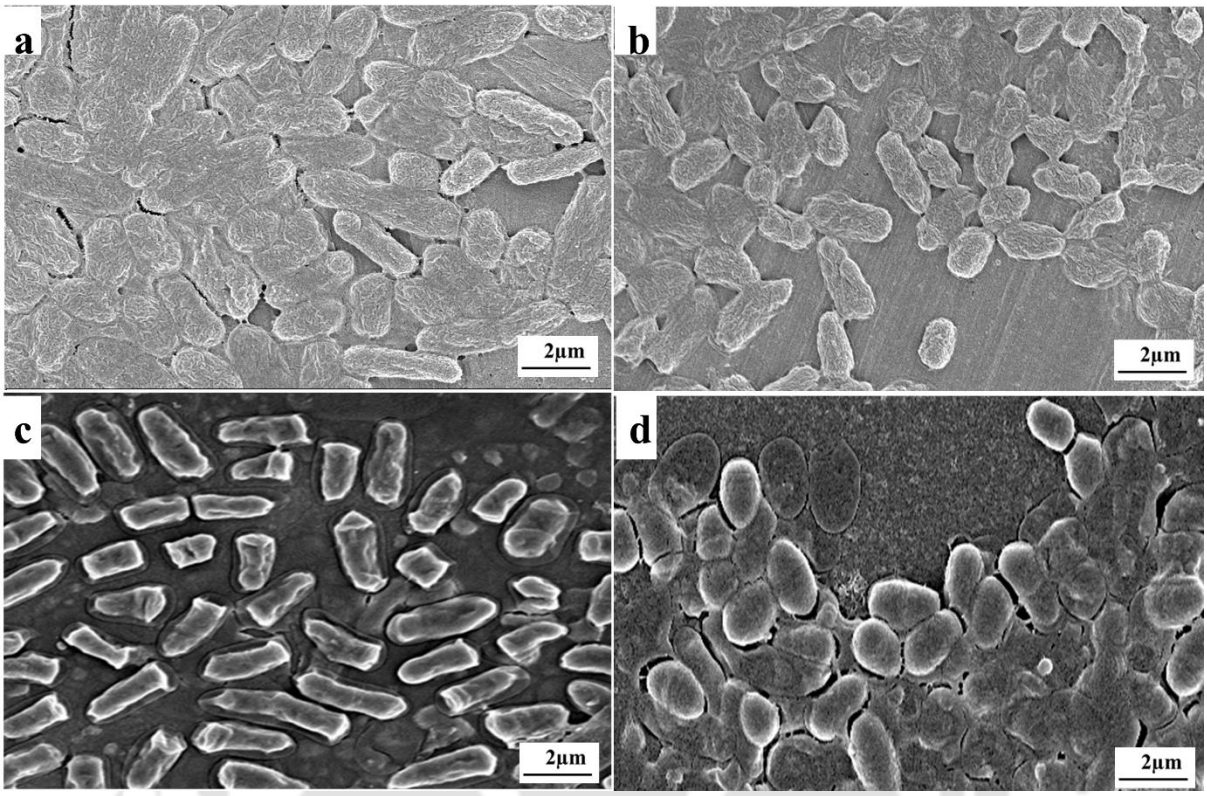


Fig. 5.5. FESEM images of cells before diesel exposure for a) *Pseudomonas aeruginosa* IITG21, b) *Providencia vermicola* IITG20; FESEM images of cells after exposure to diesel for 15 days, c) *Pseudomonas aeruginosa* IITG21, d) *Providencia vermicola* IITG20.

Table 5.1 shows the results of the biochemical tests of identified bacterial strains. These metabolic capabilities are often used to differentiate bacterial species based on their ability to utilize specific substrates. Both strains showed positive results for the starch hydrolysis, gelatine liquefaction and urease tests. These positive results indicated that both *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 possessed the enzymatic machinery required to utilise starch, hydrolyse gelatin, and breakdown urea. However, for the phenyl alanine deaminase test, they were negative. This result suggested that neither *Pseudomonas aeruginosa* IITG21 nor *Providencia vermicola* IITG20 were able to deaminate phenylalanine. Differences in results were observed during malonate utilization analysis and Triple Sugar Iron Agar analysis for the two bacterial strains. Malonate utilization and Triple Sugar Iron Agar test results were negative for *Pseudomonas aeruginosa* IITG21, while the results were positive for

strain *Providencia vermicola* IITG20. Similarly, in the Triple Sugar Iron Agar test, *Pseudomonas aeruginosa* IITG21 showed negative results, as there was no observable change in the colour of the TSI medium. The *Providencia vermicola* IITG20, on the other hand, showed positive outcome signified by the transition of the medium from red to yellow. Furthermore, the hydrogen sulfide (H_2S) production test results also differed for the two bacterial strains. *Pseudomonas aeruginosa* IITG21 showed positive results for H_2S production test, while *Providencia vermicola* IITG20 showed negative results. These observable difference of behaviour of the two bacterial strains for these tests further confirmed them to be of different species. Gram's staining showed both strains to be Gram's negative. The results of biochemical tests confirmed that the *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 possessed distinct metabolic profiles, as evidenced by differences in their responses to various tests.

Table 5.1. Biochemical test of isolated strains

Biochemical Test	***Pseudomonas aeruginosa* IITG21**	***Providencia vermicola* IITG20**
Malonate utilization	Negative	Positive
Starch hydrolysis	Positive	Positive
Gelatine liquefaction	Positive	Positive
Phenylalanine deaminase	Negative	Negative
Triple sugar iron agar	Negative	Positive
H_2S production	Positive	Negative
Urease	Positive	Positive
Gram's staining	Negative	Negative

5.3.4 Growth study in different hydrocarbons

The bacterial strains *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 had shown growth in all four types of petroleum hydrocarbons oil and their growth is presented in Fig. 5.6a. This experiments were performed in the BH broth, which does not have its own carbon source. Hence the growth of the bacteria can be attributed entirely to the utilization of the external hydrocarbon oils added to the system. The growth of *Providencia vermicola* IITG20 was slightly higher compared to that of *Pseudomonas aeruginosa* IITG21 in the presence of all the hydrocarbons. The growth of both bacteria in different hydrocarbons varied in the same order of petrol < engine oil < kerosene < diesel. This suggested that the both bacteria possessed similar preferences and metabolic capabilities for these hydrocarbon

substrates. Growth of bacterial strains in diesel was highest and that in presence of petrol was lowest. Since higher growth is associated with higher degradation, two bacterial strains were expected to be most effective for the degradation of diesel.

The degradation efficiency of bacterial strains varied among diesel, petrol, kerosene, and engine oil due to differences in hydrocarbon composition. Diesel, high in mid alkanes, showed the highest degradability, while kerosene exhibited the second-highest growth attributed to n-alkanes and branched alkanes, despite containing less degradable cycloalkanes. Engine oil, with higher carbon numbers, was less bioavailable, resulting in lower growth. Petrol, containing aromatic compounds, showed the lowest growth due to cell toxicity. Both bacteria showed higher efficiency in degrading alkane-type hydrocarbons present in diesel and kerosene.

Different constituents within petroleum hydrocarbons contribute to variations in their degradation rates as details explained in the Chapter 4. Diesel showed higher biodegradability due to its predominance of mid-range alkanes, which are less hydrophobic and toxic (Lui et al., 2021). Both isolated bacterial strains showed growth in kerosene, indicating their capability to degrade mid-range alkane hydrocarbons, including naphthenic compounds. Engine oil, with its higher proportion of long-chain hydrocarbons and viscosity, hindered bacterial access and growth rates. Petrol, containing a higher proportion of aromatics, exhibited lower degradation rates due to their reduced biodegradability and cell toxicity (Wang et al., 2016; Gargouri et al., 2015). Thus both isolated strains are suitable for degrading medium-range alkanes.

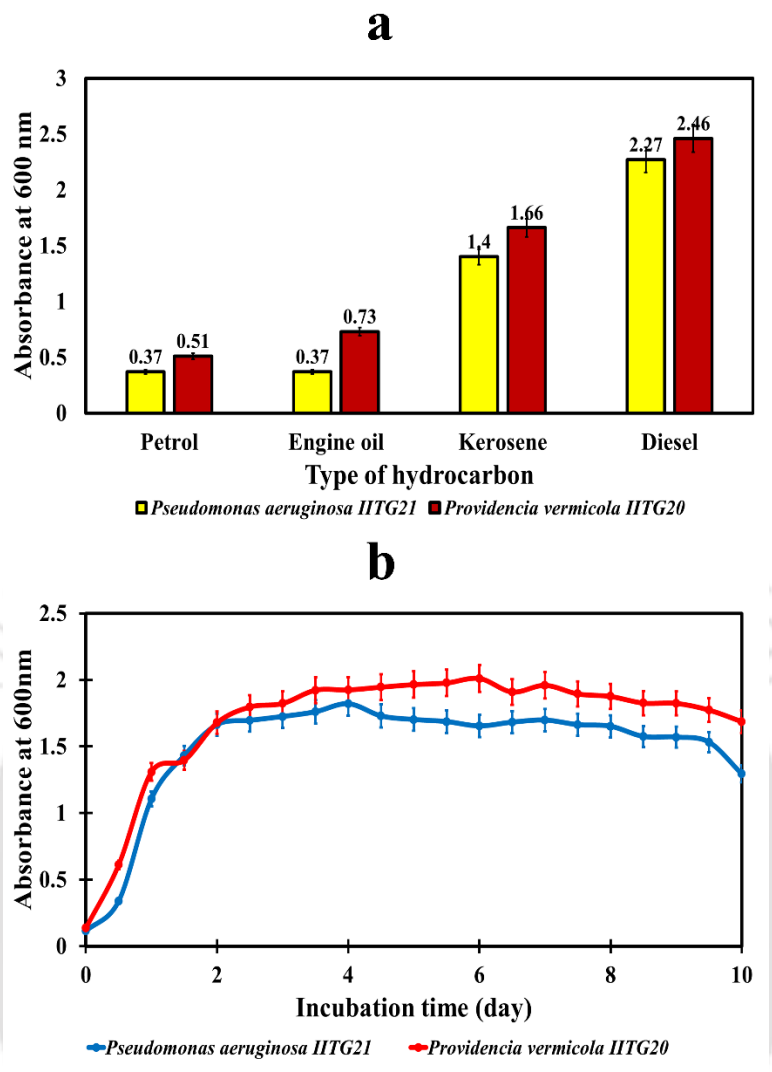


Fig. 5.6. a) Growth of *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 in the presence of various hydrocarbons at 1% v/v concentration after 10 days incubation. (pH 7, temperature 37°C, 120 rpm and 1% v/v inoculum concentration), b) Growth curve study of *Pseudomonas aeruginosa* IITG21, and *Providencia vermicola* IITG20 at initial diesel concentration of 1% v/v, pH 7, temperature 37°C, 1% v/v inoculum concentration and 120 rpm.

Fig. 5.6 b shows the growth curves of *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20. These growth curves exhibited distinct phase characteristics of bacterial growth. The growth was very low for both strains in the first 12 h, representing the lag phase. In the lag phase, as it is known that the strains get adapted to the surrounding media, which is diesel in this case. For *Pseudomonas aeruginosa* IITG21, an increase in growth was observed between 12 to 48 h (2 days) and for *Providencia vermicola* IITG20 same was observed between 12 to 36 h (1.5 days). These periods were, therefore, the log phase for respective strains. At

this phase, growth of bacterial strains was higher than their decaying, resulting in an increase in overall growth. After log phase, up to next 9 days no effective increase or decrease in growth was observed for both the strains. This stabilization in growth suggested that the bacterial populations reached a state of equilibrium, where the number of dying bacterial cells was balanced by the number of multiplying cells. That is the growth rate and decaying rate of strains became almost equal during this period. This period represented the stationary phase. However, after 9 days, growth was found to decrease for the both the strains indicating initiation of decline phase. In this phase, number of dying bacteria cell was higher than number of multiplying bacteria cell, thereby, resulting in an overall decrease in growth. This decrease in growth might had been caused by exhaustion of the carbon source present in the system or by detrimental effect of any accumulated by-products.

The comparison of growth curves of the two bacterial strains showed that *Providencia vermicola* IITG20 had lower log phase, thereby attaining the stationary phase earlier in 1.5 days compared to 2 days for *Pseudomonas aeruginosa* IITG21. In addition, it showed higher growth compared to that of *Pseudomonas aeruginosa* IITG21, almost in the entire range of incubation period of 10 days. However, for both the bacterial strains, initiation of decline phase started at similar time. These observations suggested *Providencia vermicola* IITG20 had faster and higher growth with compared to that of *Pseudomonas aeruginosa* IITG21 and later analysis further confirmed the same.

5.3.5 Effect of process parameters

The effects of various process parameters on the growth and biodegradation capabilities of *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21 and their mixed culture for diesel are shown in Fig.5.7 and 5.8. Fig. 5.7a and 5.7b show the effect of initial diesel concentration on bacterial growth and diesel degradation, respectively. For both bacterial strains and their mixed culture, the growth increased as the initial diesel concentration increased from 2 to 4 % and thereafter, again decreased slightly at 6%. Growth was highest at 4% v/v and lowest at 2% v/v. The results suggested that a certain threshold concentration of diesel was required to support the robust bacterial growth and in this case it was 4% v/v. The slight decrease in bacterial growth at diesel concentration of 6% v/v, suggested that this concentration of hydrocarbon was high enough to induce slight toxicity for the strains, thereby negatively affecting their growth.

In contrast to growth, the percent degradation continuously decreased, on increasing the initial diesel concentration from 2 to 6% in presence of both the bacterial strains, at same initial inoculum concentration of 1%. Degradation percentages for both bacteria and their mixed culture were highest at the initial diesel concentration of 2% v/v and lowest at 6% v/v diesel. This phenomenon may be attributed to the availability of the carbon substrate. At lower diesel concentrations, the hydrocarbon molecules were more dispersed and readily available for bacterial utilization, leading to more efficient degradation. However, at higher diesel concentrations, the hydrocarbon molecules were expected to become more congested, thereby limiting their accessibility to the bacteria. Consequently, interaction of hydrocarbons with bacteria was reduced, decreasing the percentage degradation (Jabbar et al. 2022). Another reason may be that, with increasing concentration of hydrocarbons at same inoculum concentration, later became the limiting factor, thereby reducing the percentage degradation of hydrocarbons. The concentration of 4% v/v diesel thus favoured the growth of strains most, in addition to giving moderate degradation. Thus this concentration balanced between providing sufficient carbon sources for growth but avoiding presence of excessive of hydrocarbons. This concentration thereby was selected for subsequent studies.

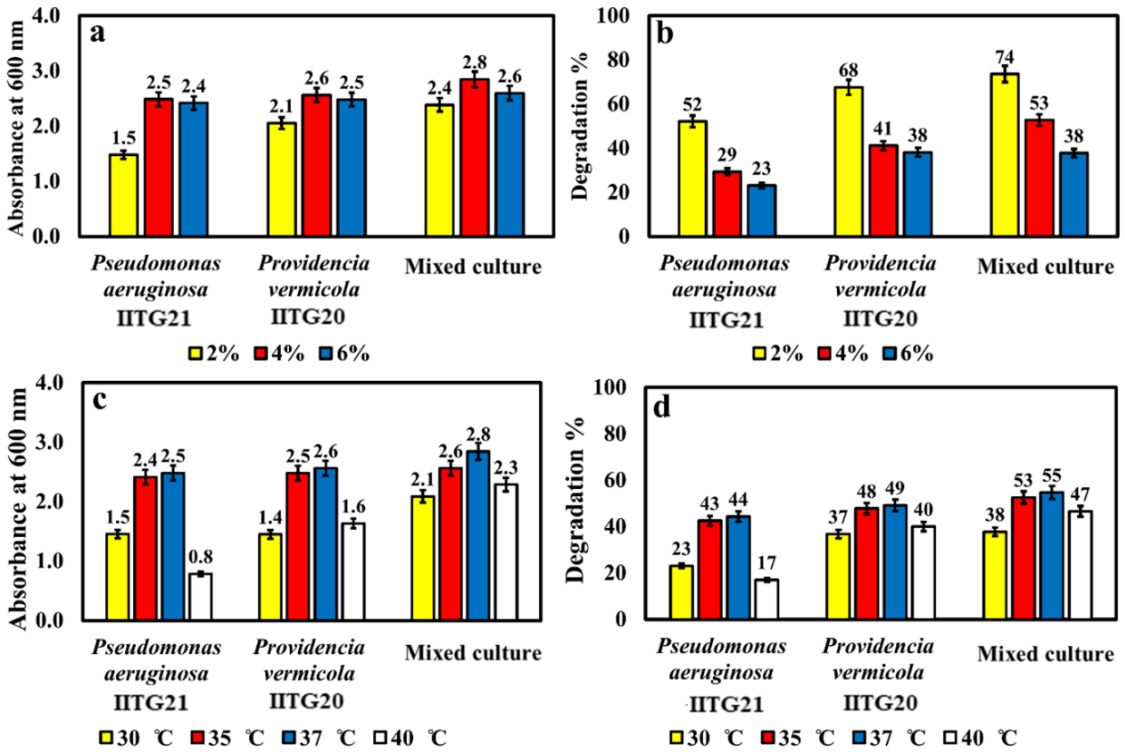


Fig. 5.7 Effect of initial diesel concentration on a) growth, b) degradation of diesel (at 37°C, pH 7 and inoculum concentration 1%); Effect of temperature on c) growth and d) degradation of diesel (at 4% v/v initial diesel concentration, pH 7 and 1% v/v inoculum concentration).

Fig. 5.7c and d show the effect of temperature on the growth and degradation of diesel, respectively, by *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21 and their mixed culture. The bacterial growth and degradation of diesel were observed to be favoured at 35 and 37 ^{o}C compared to that at lower temperature of 30^{o}C or higher temperature of 40^{o}C. The growth and degradation at 37°C was just slightly higher than that at 35°C for both bacterial strains. This suggested that that the temperature range of 35-37^{o}C supported the metabolic activities of the strains most suitably. Low temperatures can slow down or inhibit the metabolic activities of bacteria, including the enzymatic processes that are required for diesel degradation. Bacteria may also produce fewer enzymes at lower temperatures, which are necessary for breaking down the complex hydrocarbons in diesel. Conversely, high temperatures can denature the enzymes and disrupt bacterial cellular structures, reducing their effectiveness in diesel degradation.

At 30°C, the growth of the two bacterial strains was similar but at 40°C the detrimental effect of temperature was more for *Pseudomonas aeruginosa* IITG21. It showed the lowest growth and diesel degradation at 40°C, indicating that the high temperature significantly weakened the metabolic activities and enzymatic processes essential for diesel degradation, for this strain. The *Pseudomonas aeruginosa* IITG21 was thus more temperature sensitive, particularly at higher temperature. For mixed culture, the effect of variation of temperature on growth and degradation was significantly reduced. This suggested that synergy between the two bacterial strains were able to develop a more sustainable metabolic pathway for bacterial growth and thereby degradation of diesel. The mixed culture exhibited higher growth and percent diesel degradation compared to that of the pure cultures at all temperatures used. The findings suggested that the two bacterial strains and their mixed culture were highly effective in degrading diesel in temperatures range of 35-37^{o}C with the highest activity at 37°C. Therefore, 37°C was selected as the most suitable temperature for subsequent experiments.

Fig. 5.8a and b show the effect of pH on bacterial growth and diesel degradation, respectively. The highest growth and degradation for both bacteria and mixed culture was observed at a neutral pH 7, with a reduction in bacterial growth and degradation in acidic (pH 5) or basic (pH 9) conditions. Compared to neutral pH 7, the *Providencia vermicola* IITG20 showed significantly lower growth at acidic pH 5 or basic pH 9 as can be observed from Fig. 5a. *Pseudomonas aeruginosa* IITG21 also showed significant decrease in growth at pH 5. These results agreed with reported observations that extreme pH can disrupt microbial activities due

to adverse effects on enzyme function and cellular processes (Roslee et al. 2020). In literature, similar optimal pH values were observed for *Pseudomonas aeruginosa* (Deivakumari et al. 2020; Ravi et al. 2022) and *Providencia vermicola* (Vijayalakshmidevi & Muthukumar 2015; Mukherjee et al. 2017). However, for mixed culture, though the effect of change of pH on growth was less significant but clear decrease in degradation was observed at acidic pH 5 (37%) and basic pH 9 (48%) compared to that at neutral pH 7 (64%). This emphasizes the complex interactions and potential mutual adjustments that can occur within mixed bacterial cultures. In such cultures, the metabolic activity of one strain may potentially compensate for the other's performance under changing pH conditions. As the highest growth and degradation was observed at pH 7 for both strains and their mixed culture, it was selected for further studies.

Fig. 5.8c and d show the effect of initial inoculum concentration on the growth and diesel degradation, respectively, in presence of individual bacterial strains and their mixed culture. All strains exhibited maximum growth and degradation at an initial inoculum concentration of 1% v/v. This observation suggested that this concentration of bacterial cells was most suitable for efficient utilization of diesel as the carbon source at the studied diesel concentration of 4% (v/v). At a lower inoculum concentration of 0.5% v/v, the growth of the strains was less and might be due to overwhelming presence of hydrocarbons generating toxic effect on strains. The diesel degradation was also low at this concentration due to lower availability of bacterial cells to carry out the degradation process effectively. The process of degrading diesel depends on specific enzymes and metabolic pathways. In situations with a low bacterial population, these resources might become limited, resulting in slower degradation. On the other hand, lower growth and degradation at higher concentration of 2% v/v possibly resulted from formation of inhibitory compounds (Goveas et al. 2022). Therefore, an initial inoculum concentration of 1% v/v was deemed most suitable for degradation of 4% v/v initial concentration of petroleum hydrocarbons using the bacterial strains and their mixed culture. The 1% v/v initial concentration of inoculum allowed for high growth and efficient diesel degradation, without the limitations seen at lower concentrations or the potential inhibitory effects at higher concentrations.

The parametric study also showed that at all conditions *Providencia vermicola* IITG20 exhibited better growth and degradation compared to that by *Pseudomonas aeruginosa* IITG21. The later was observed to be more sensitive to change in process parameters, showing worst performance at extreme conditions. It was also noted that the mixed culture of the two bacterial

strains showed significantly improved growth and degradation performance as compared to both the individual strains, at all the conditions studied. The effect of change of process parameters on growth and degradation performance was further neutralized in use of the mixed culture, that is the effect of change of parameters on growth and degradation was lower in presence of mixed culture compared to that observed for either of the two individual bacterial strains. Based on the above results, most suitable conditions for growth and degradation for the two bacterial strains and their mixed culture, were determined to be 4% v/v initial diesel concentration, 37°C temperature, pH 7, and 1% v/v initial inoculum concentration.

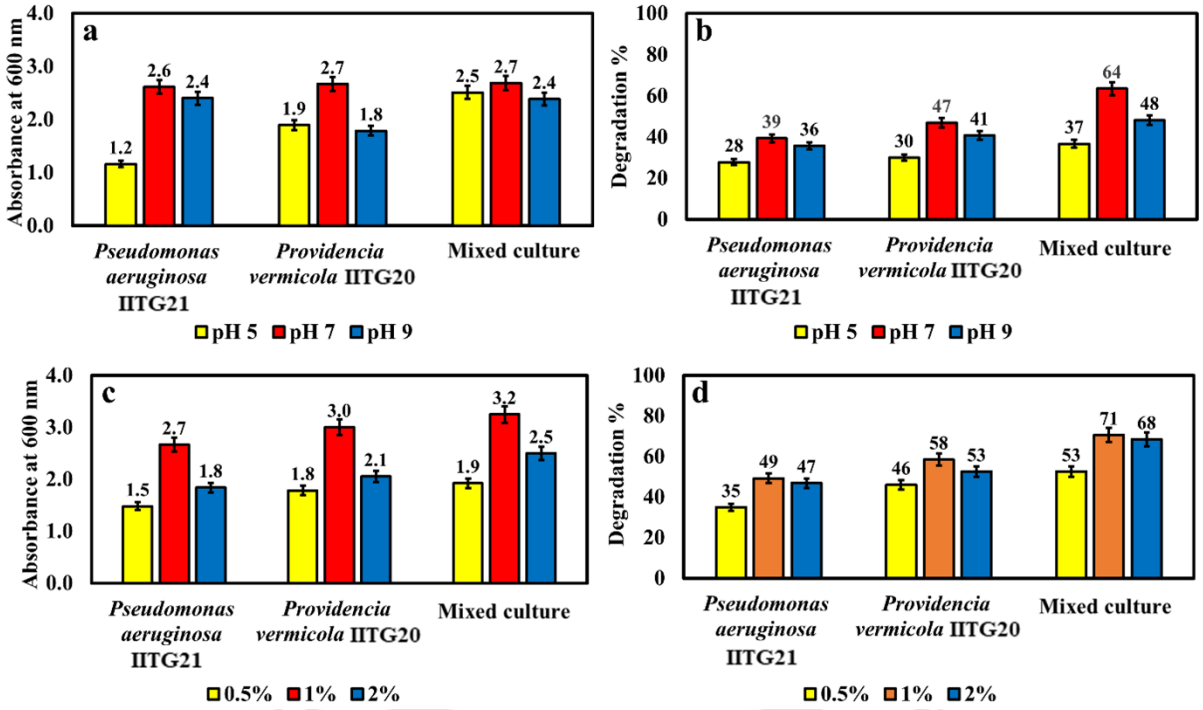


Fig. 5.8 Effect of pH on a) growth, b) degradation of diesel (at 4% v/v initial diesel concentration, 37°C temperature, and inoculum concentration 1%); Effect of inoculum concentration on c) growth and d) degradation of diesel (at 4% v/v initial diesel concentration, pH 7 and 37°C temperature).

5.3.6 Growth assessment of bacteria at most suitable conditions

Fig. 5.9 compares the growth of individual bacterial strains and their mixed culture as function of incubation time, at the most suitable conditions determined above. It was observed that growth for all the cultures was significantly increased in first 3 days. This rapid initial growth phase is characteristic of bacteria when they adapt to their environment and start utilizing

available resources efficiently. Thereafter, the growth of all cultures stabilized and remained relatively steady. This phase of steady growth suggested that the bacteria had successfully adapted to the conditions and were actively metabolizing diesel as their carbon source. During this period, bacterial populations were likely in a balanced state of multiplication and decay. It was also observed that highest growth was obtained in the presence of the mixed culture of *Providencia vermicola* IITG20 and *Pseudomonas aeruginosa* IITG21 compared to their pure form in entire incubation time. The higher growth of strains in mixed culture suggested that both strains could co-metabolize together, facilitating their growth. Though the *Providencia vermicola* IITG20 showed growth less than mixed culture but its growth was higher than that of *Pseudomonas aeruginosa* IITG21 during entire incubation time. Both *Providencia vermicola* IITG20 and *Pseudomonas aeruginosa* IITG21 showed steady growth from 3rd to 12th day, and thereafter growth declined from 12th to 15th day. For mixed culture, growth was steady up to 9 days and then started decreasing from 9th to 15th day. As nutrients become scarce, the rate of cell division decreased, leading to a gradual reduction in overall growth. Simultaneously, waste products generated during metabolism might hinder bacterial proliferation, contributing to the observed decline for all strains. The early decline observed in bacterial growth in presence of mixed culture can be attributed to faster exhaustion of nutrient and accumulation of inhibitory metabolites due to faster and higher growth in presence of them. Both the phenomena limited the bacterial growth at later stage thereby shortening the stationery phase.

On comparison of the growth curves studied at initial diesel concentration of 1% (v/v) (Fig. 5.7b) and that in 4% (v/v) (Fig.5.10) showed increased log and stationery phases for individual bacterial strains at higher concentration of diesel. In case of lower concentration of 1%, the initial growth was observed for 1.5-2 days, while it was 3 days for both the strains at 4% concentration. The extended log phase on using higher concentration of diesel might be associated with delay in adaptation by strains under the conditions. Similarly, steady phase continued up to 12 days for 4% v/v initial diesel concentration, in contrast to 9 days observed on using 1% diesel concentration. The extended stationery phase observed at higher initial concentration of diesel, might had resulted from more availability of carbon sources to sustain the bacterial strains for increased time.

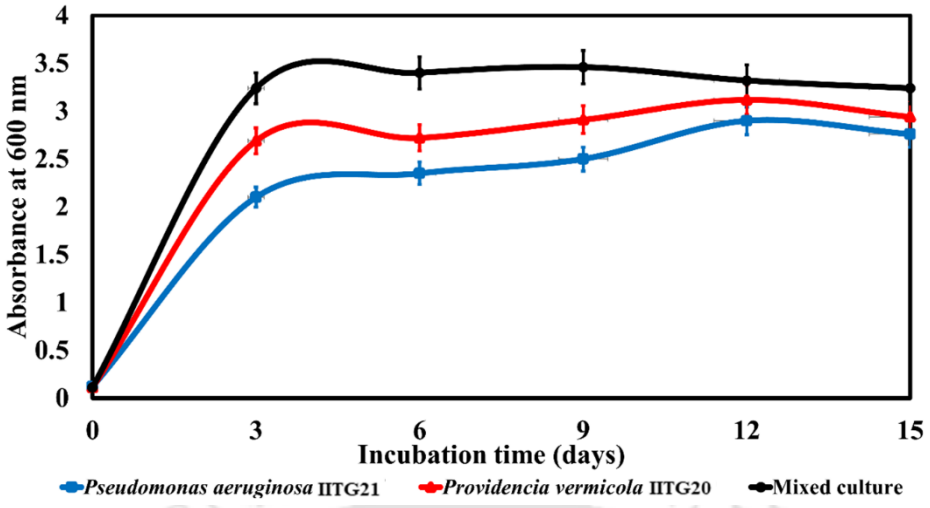


Fig. 5.9. Growth of bacteria at most suitable conditions in presence of individual bacterial strains and their mixed culture at diesel concentration of 4% v/v. (pH 7, temperature 37°C and 120 rpm) in different incubation period.

5.3.7 Degradation of diesel at most suitable conditions

Fig.5.10a compares the degradation of diesel, in presence and absence (control sample) of bacterial strains for initial diesel concentration of 4% v/v. The control setup, which did not have any bacterial source, gave only 5% degradation of diesel. This degradation was attributed to abiotic factors, such as photolytic degradation induced by light (Ossai et al. 2020), degradation induced by temperature (Li et al. 2022) or degradation by chemical reactions occurring in absence of microbial activity (Restrepo-Flórez et al.2014). In presence of the bacterial strains, the overall degradation of diesel increased significantly, confirming the positive role played by the two strains in degrading diesel components. The overall diesel degradation was 70% and 76% for *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively, in 15 days of incubation time. In the presence of mixed culture, containing two strains in the ratio of 1:1, the degradation was enhanced to significantly to 85% in the same incubation period.

The degradation of diesel with respect to time at most suitable conditions by two bacterial strains and their mixed culture is shown in Fig.5.10b. *Providencia vermicola* IITG20 showed higher degradation as compared to that shown by *Pseudomonas aeruginosa* IITG21 but lower than that observed for the mixed culture during entire incubation period. This consistent higher degradation by *Providencia vermicola* IITG20 in comparison to *Pseudomonas aeruginosa* IITG21 may be attributed to its specialized enzymatic and metabolic pathways suitable for

diesel hydrocarbon breakdown. This agreed with the higher growth observed for this strain compared to that observed for *Pseudomonas aeruginosa* IITG21 (Fig. 5.9). The overall degradation of diesel by mixed culture was higher than the both the individual bacterial strains, over the entire range of studied incubation time as shown in figure. The enhanced degradation by mixed culture might be explained by synergistic effect of two strains, enabling them to utilize hydrocarbons and other metabolites more rapidly as mentioned earlier (Zhang et al. 2021). This cooperative degradation is facilitated by a broader range of enzymes in the mixed culture and potential symbiotic interactions between the strains (Cao et al. 2022).

The degradation of different types of hydrocarbons present in diesel by the individual and mixed culture of two bacteria, as well as in the control sample (without bacteria) after 15^{th} day of incubation, is shown in Fig.5.10c. For the control sample, it was observed that n-alkanes were the most degraded component as 19% degradation was observed in 15 days. Branched alkanes were the second most degraded component (16%), and naphthenes were the third highest degraded component (12%). Aromatics were the least degraded diesel component, as only 6% degradation was observed. As explained earlier, the abiotic degradation might be attributed to factors such as temperature, exposure to light, or chemical reactions that may happen without microbial support. The n-alkanes were the most degraded components suggesting that these straight-chain hydrocarbons were more susceptible to abiotic degradation processes. This could be due to their simpler chemical structures, making them more prone to photolytic and chemical breakdown in the presence of environmental factors like light and temperature. Branched alkanes exhibited slightly lower degradation, indicating that branching may confer some degree of resistance to abiotic degradation. Naphthenes, being cyclic hydrocarbons, showed a comparatively lower degradation rate, possibly due to their increased structural stability (Adedeji et al. 2022). Aromatics, with their more complex and stable ring structures, exhibited the least degradation, further supporting their resilience to abiotic degradation (Imam et a. 2022).

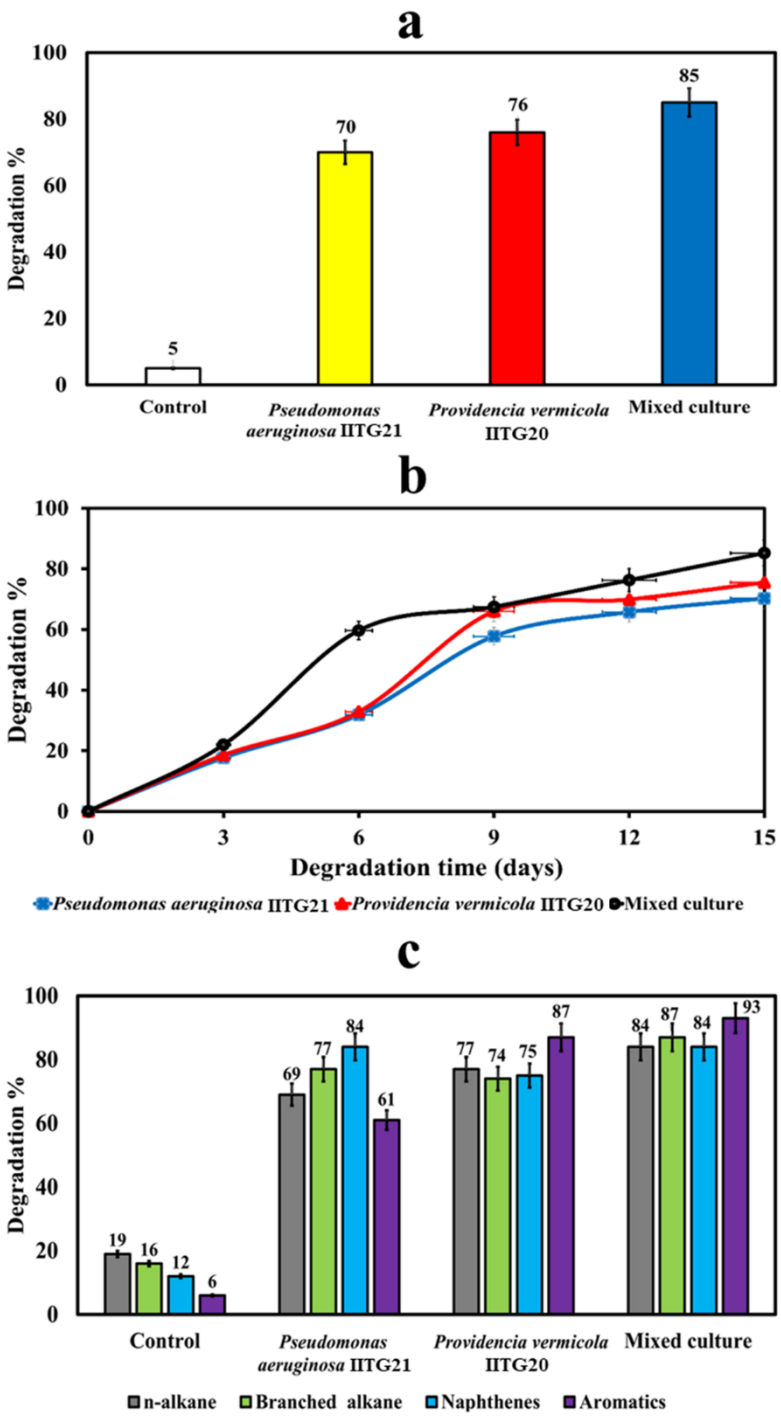


Fig 5.10. a) Overall degradation of diesel after 15 days incubation, b) Degradation of diesel with respect to time, c) Degradation for different types of hydrocarbons present in diesel, by individual bacterial strains and their mixed culture (initial concentration 4% v/v, pH 7, temperature 37°C, 120 rpm, 15 days total incubation).

In presence of strains the degradation was significantly enhanced for all the components of diesel (Fig. 5.10c). The enhancement emphasized the significant positive role played by the

bacterial strains. Pure culture of both the strains showed significant degradation for all types of hydrocarbons. The *Pseudomonas aeruginosa* IITG20 showed the highest degradation for naphthenes (84%), followed by branched alkanes (77%). For n-alkanes, degradation was higher (69%) than aromatics but lower than naphthenes and branched alkanes. Aromatic compounds were the least degraded compounds (61%). It was also noticed that *Providencia vermicola* IITG20 had a higher degradation for aromatic components (87%) compared to other types of components, including n-alkanes (77%), naphthenes (75%), and branched alkanes (74%). Thus, the degradation of branched alkanes and naphthenes were higher in the presence of *Pseudomonas aeruginosa* IITG21 and that for aromatic compounds was higher in presence of *Providencia vermicola* IITG20. The observed differences in the degradation capabilities of two strains suggested their distinct metabolic pathways and enzymatic systems. *Pseudomonas aeruginosa* IITG21 having higher proficiency in degrading naphthenes and branched alkanes, might be predominantly associated with enzymes such as naphthenes monooxygenases or alkane hydroxylases, which are known to facilitate the degradation of cyclic and branched hydrocarbons, respectively (Chunyan et a.2023). On the other hand, *Providencia vermicola* IITG20 excels in degrading aromatic compounds, indicating its possession of enzymes like dioxygenases or monooxygenases that target aromatic rings (Kotoky et al. 2022). Both the strains however, exhibited substantial degradation capabilities for all types of hydrocarbon, emphasizing their versatility in degradation of diesel hydrocarbons.

In the presence of the bacterial mixed culture, degradation of all the components increased compared to the pure culture. In the bacterial mixed culture, degradation for aromatics reached up to 93%, followed by 87% for branched and 86% for naphthenes. For n-alkanes, degradation was also increased up to 84%. These results showed that though the bacteria had different preferences for different types of compounds, but when they grew together in mixed culture, their synergistic effect promoted degradation of all types of hydrocarbons. This increased degradation might be assigned to cooperation between enzymes produced by the bacteria. Bacterial mixed culture has a wide variety of enzymes compared to pure culture. In addition, the symbiotic association between bacteria might also be responsible for higher degradation as one bacteria may utilize hydrocarbons, and others may enhance the metabolism (Cao et al. 2022).

The degradation of the individual components in diesel after 15 days of incubation period has been summarised in Table 5.2. The corresponding spectra is shown in appendix Fig. A2. From

Table 5.2, it can be observed that use of mixed culture enhanced the degradation of most of the compounds as compared to that observed in the presence of pure culture. Particularly the components which were difficult to degrade in presence of single bacterial strain was easily degraded by mixed culture. For example, henicosane (22%) and icosane (60%) were the lowest degraded compounds by *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively. In the presence of mixed culture, degradation values increased to 66 and 96 %, respectively. Among branched alkanes, the lowest degradation was observed for 2,6,10,14-tetramethyl-7-(3methylpentyl) pentadecane (27%) and 7,7-diethylheptadecane (18%) by *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively, which increased to 92% and 90% in the presence of mixed culture. The 1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene (28%) and heptylcyclohexane (32%) were the lowest degraded naphthenes by *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively. In the presence of mixed culture, degradation for both compounds increased respectively, by 87 and 80%. This observed increase in the degradation in presence of mixed culture might have resulted from the synergetic action of both the bacteria as also discussed earlier.

In this analysis it was also observed that molecules with simpler structure was more degraded than that having complex structure. Among naphthenes, tridecan-6-ylcyclohexane underwent highest degradation, about 80%, by *Pseudomonas aeruginosa* IITG21. On the other hand, the naphthene 1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene with more complex structure, showed lower degradation of only 43% with same bacterial strain. Lower degradation due to the increased complexity of substrate molecular structure might have arisen from the need of requiring more enzymatic steps. Further, it was noted that 1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene with multi-cyclic rings were difficult to be degraded by *Pseudomonas aeruginosa* IITG21, showing only 28% degradation, while compounds with attached hydrocarbon chains such as heptylcyclohexane were more easily degraded (61%) by same strains, though the carbon number was higher for the latter. The *Providencia vermicola* IITG20 showed reverse degradation performance, with 81% degradation for former component and 32% degradation for the later. The higher branching increased difficulty in its degradation by *Pseudomonas aeruginosa* IITG21. The 2,6,10,14-tetramethyl-7-(3methylpentyl) pentadecane was degraded only 27%, while 11-methylpentacosane was degraded by only 48% in presence of same strain. However, it was observed that *Providencia vermicola* IITG20 were more effective in degrading the same

molecules with higher branching. The components were degraded by 81 and 94%, respectively with the same strain. It has been already discussed above how aromatic compounds were more degraded by *Providencia vermicola* IITG20. The2,6-dimethyl-1,2,3,4-tetrahydronaphthalene was degraded by 61 and 87 % by *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, respectively. Thus the behaviour of the two strains shows that they can complement each other performance. Increased in degradation performance in presence of mixed culture confirm the same. Many of the constrains were minimized by use of mixed culture, displaying metabolic synergy and specialization, thereby, excelling at degrading hydrocarbons difficult to disintegrate.

Table 5.2. Degradation of major hydrocarbons present in diesel by *Pseudomonas aeruginosa* IITG21, *Providencia vermicola* IITG20 and bacterial mixed culture after 15 days incubation period.

Hydrocarbon name	Molecular formula	Degradation %		
		Pseudomonas aeruginosa IITG21	*Providencia vermicola* IITG20	mixed culture
Paraffins (n-alkanes)				
dodecane	$C_{12}H_{26}$	45	77	81
pentadecane	$C_{15}H_{32}$	40	80	92
heptadecane	$C_{17}H_{36}$	89	80	85
nonadecane	$C_{19}H_{40}$	66	89	88
icosane	$C_{20}H_{42}$	92	60	96
henicosane	$C_{21}H_{44}$	22	78	66
tricosane	$C_{23}H_{48}$	61	75	84
pentacosane	$C_{25}H_{52}$	71	79	87
hexacosane	$C_{26}H_{54}$	71	77	86
Paraffins (Branched Alkane)				
4,5-dimethylnonane	$C_{11}H_{24}$	90	92	94
4,6-Dimethyldodecane	$C_{14}H_{30}$	78	88	94
2,6,11-trimethyldodecane	$C_{15}H_{32}$	74	40	55
5,6-dipropyldecane	$C_{16}H_{34}$	68	81	84
2,6,10,15-tetramethylheptadecane	$C_{21}H_{44}$	77	77	87
7,7-diethylheptadecane	$C_{21}H_{44}$	74	18	90
5-ethyl-5-methylnonadecane	$C_{22}H_{46}$	90	88	88
2,6,10,14-tetramethyloctadecane	$C_{22}H_{46}$	96	90	99
2,6,10,14-tetramethylnonadecane	$C_{23}H_{48}$	89	95	88
(Z)-5-methyldocos-5-ene	$C_{23}H_{46}$	79	50	74
11-methyltricosane	$C_{24}H_{50}$	56	80	82
2,6,10,14-tetramethyl-7-(3methylpentyl) pentadecane	$C_{25}H_{52}$	27	81	92
11-methylpentacosane	$C_{26}H_{54}$	48	94	96
2-methylhexacosane	$C_{27}H_{56}$	90	74	97
2-methylheptacosane	$C_{28}H_{58}$	93	94	97
13-methylheptacosane	$C_{28}H_{58}$	71	76	84
1-iodooctacosane	$C_{28}H_{57}I$	70	76	97
2-methyloctacosane	$C_{29}H_{60}$	71	81	87

Naphthenes (cycloalkanes or cycloparaffins)				
1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	$C_{10}H_{18}$	28	81	87
1-pentylcyclohexene	$C_{11}H_{20}$	61	85	93
methylcyclodecane	$C_{11}H_{22}$	70	65	84
heptylcyclohexane	$C_{13}H_{26}$	61	32	80
1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene	$C_{15}H_{24}O$	43	87	75
tridecan-6-ylcyclohexane	$C_{19}H_{38}$	80	92	85
Aromatics				
2,6-dimethyl-1,2,3,4-tetrahydronaphthalene	C12H16	61	87	93

5.3.8 Degradation kinetics

Plotting ln(Co/C) against time in days resulted in a linear relationship for degradation in the presence of individual strains and their mixed culture, as shown in Fig. 5.11. This confirmed the validity of the first-order kinetics for diesel degradation under the used conditions for the studied bacterial strains. Fig. 5.12 shows plot of diesel concentration against the time that further confirmed the first order kinetics. The value of kinetic constant was determined from the slope of the plots and given in Table 5.3. Similar first-order degradation kinetics have also been reported in the literature (Osinowo al. 2020; Balseiro-Romero et al. 2017). For *Providencia vermicola* IITG20, the value of k was higher at 0.098 day^{-1} compared to 0.083 day^{-1} for *Pseudomonas aeruginosa* IITG21, suggesting degradation kinetics was faster for former. These findings agreed with the earlier observations that *Providencia vermicola* IITG20 had faster and higher growth and degradation compared to that of *Pseudomonas aeruginosa* IITG21. The rate constant for degradation further increased significantly to 0.154 day^{-1} with use of mixed culture of the two bacterial strains. This enhancement in the rate of degradation in presence of mixed culture may be attributed to the synergistic effect of the two bacterial strains co-existing in the mixed culture as discussed earlier.

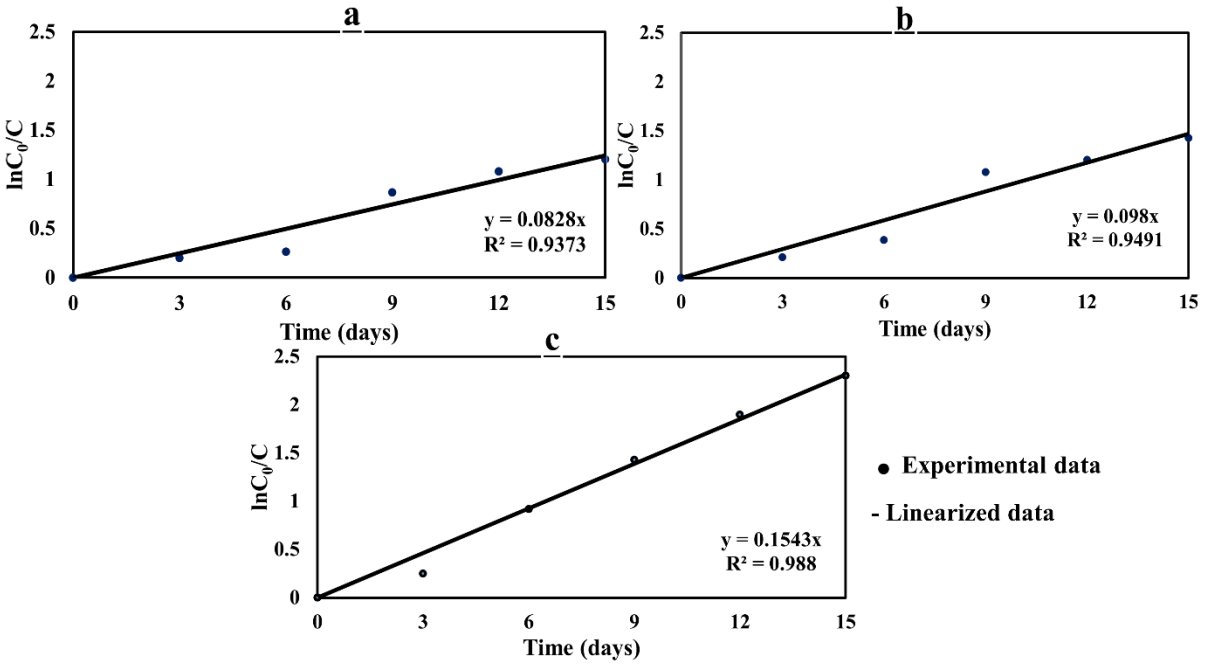


Fig. 5.11. Graph depicting first order kinetics for degradation of diesel in presence of a) *Pseudomonas aeruginosa* IITG21, b) *Providencia vermicola* IITG20, c) mixed culture.

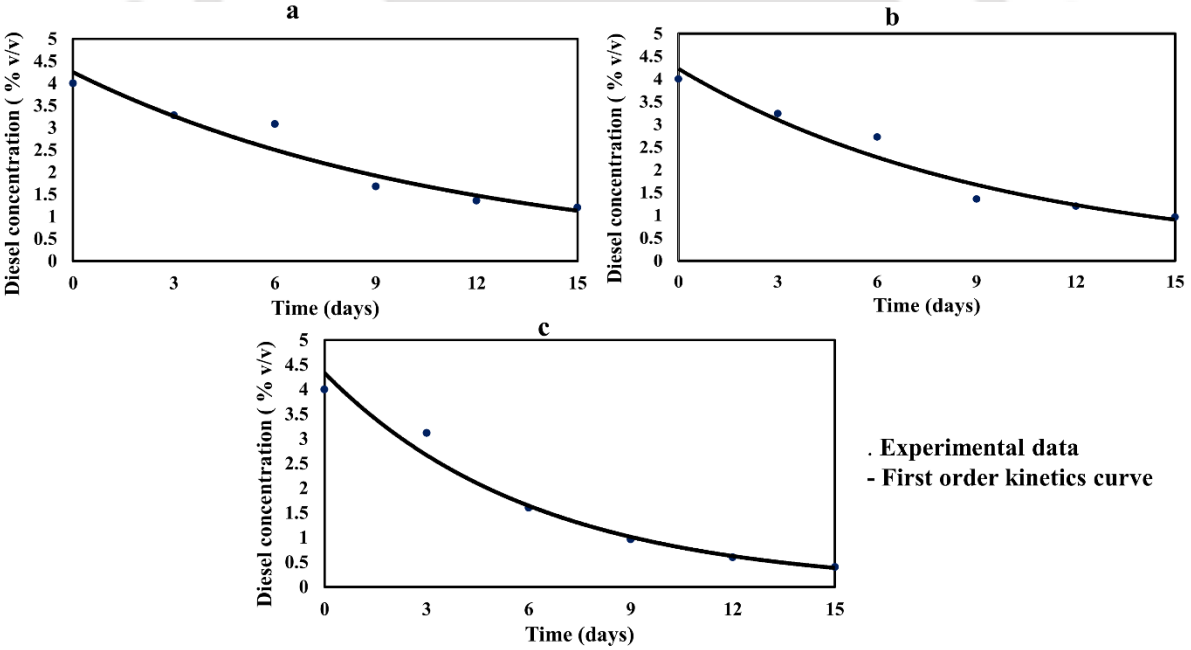


Fig.5.12. Validation of experimental results using the first order kinetics for a) *Pseudomonas aeruginosa* IITG21, b) *Providencia vermicola* IITG20, c) mixed culture

Table 5.3. Kinetics parameters for biodegradation of diesel.

Bacteria	k day^{-1}	R^2	Theoretical Half-life	Experimental Half life
Pseudomonas aeruginosa IITG21	0.083	0.94	8.3 day	8.1
Providencia vermicola IITG20	0.098	0.95	7.1 day	7.5 day
Bacterial mixed culture	0.154	0.99	4.5 day	5 day

The value of k corresponds to half-life for degradation (Dadrasnia et al. 2020). The theoretical half-life was calculated putting n = 1 in the Eq. 1:

$$t_{1/2} = \frac{\ln 2}{k} \qquad (5)$$

This theoretical half-life provided an estimate of the time required for degradation of 50% of the diesel by first-order kinetics. The theoretical half-life, calculated using Equation 5, has been tabulated in Table 5.3. The half-life for *Providencia vermicola* IITG20 was 7 days compared to 8 days for *Pseudomonas aeruginosa* IITG21. This suggested that *Providencia vermicola* IITG20 degraded 50% of the diesel in a shorter time compared to that by *Pseudomonas aeruginosa* IITG21. This aligned with the earlier observation that degradation was faster and higher for *Providencia vermicola* IITG20 than that for *Pseudomonas aeruginosa* IITG21. The half-life further decreased to 4.5 days for bacterial mixed culture, corroborating even faster degradation in the presence of mixed culture. This reduction in the half-life highlights the potential for accelerated diesel degradation in practical applications, particularly on using a mixed culture of the bacteria used in this study.

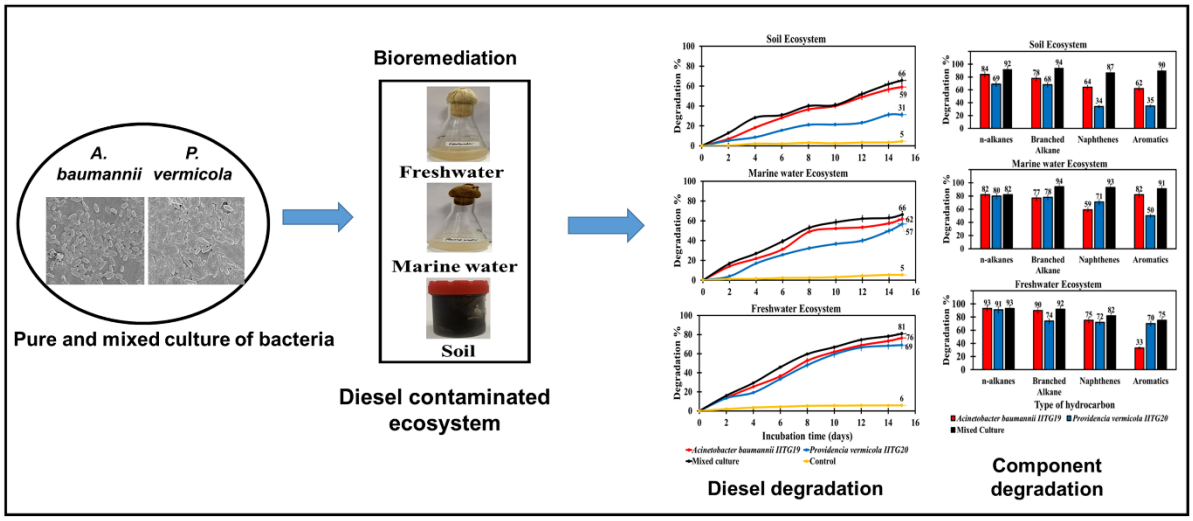
The experimental half-life was calculated from fig.5.10b by determining the days required for 50% degradation or the time in which concentration of diesel became half of the initial concentration. The values are included in Table 5.3. The values of experimental half-life agreed closely with that of theoretical values for the respective degraders (Table 5.3). The close agreement between experimental and theoretical half-life values for diesel degradation also validated the first order kinetics assumed for the degradation reactions.

5.4 Summary

Pseudomonas aeruginosa IITG21 and *Providencia vermicola* IITG20 were successfully isolated from the soil and sludge of a refinery effluent treatment plant, respectively. These bacteria proved to be highly capable of degrading petroleum hydrocarbons. Degradation was higher for diesel, kerosene, and engine oil more compared to that for gasoline. The most suitable conditions for growth and diesel degradation for the selected strains and their mixed culture were found to be 4% v/v initial diesel concentration, 37°C temperature, pH 7, and 1% v/v initial inoculum concentration. *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 showed diesel degradation of 70% and 76%, respectively, during 15 days of incubation period under the most suitable conditions. When the mixed culture of these two strains was used, degradation increased further to 85%. *Providencia vermicola* IITG20 exhibited higher degradation for n-alkanes and aromatics, while *Pseudomonas aeruginosa* IITG21 showed higher degradation for branched alkanes and naphthenes. Degradation was further increased for all types of components of diesel in the presence of mixed culture. *Pseudomonas aeruginosa* IITG21 showed lower degradation of n-alkanes (69%) compared to *Providencia vermicola* IITG20 *(77%)*, but the mixed culture increased degradation to 84%. For branched alkanes, degradation percentages were 77, 74, and 87% for *Pseudomonas aeruginosa* IITG21, *Providencia vermicola* IITG20, and mixed culture, respectively. Regarding naphthenes, degradation percentages were 84, 75, and 84% for *Pseudomonas aeruginosa* IITG21, *Providencia vermicola* IITG20, and mixed culture, respectively. For aromatics, *Pseudomonas aeruginosa* IITG21 exhibited lower degradation (61%) compared to that by *Providencia vermicola* IITG20 (87%), but the mixed culture increased degradation to 93%. For the degradation of individual components of diesel, bacterial mixed culture was found to be most effective as degradation was in the range of 84 – 93%. The rate of diesel degradation was highest in presence of bacterial mixed culture as compared to pure cultures. Rate constant k was found to be highest for bacterial mixed culture (0.154 day^{-1}) compared to the pure culture of both bacteria. Bacterial mixed culture also showed lowest half-life as compared to pure culture of both bacteria. The enhancement in rate of degradation in presence of mixed culture may be attributed to the synergistic effect of the two bacterial strains co-existing in the mixed culture.

Chapter 6

Degradation of diesel in soil and water ecosystems by *A. baumannii* and *P. vermicola*



Chapter 6 discusses diesel degradation and bacterial growth in soil, marine water, and freshwater ecosystems using Acinetobacter baumannii IITG19, Providencia vermicola IITG20, and their mixed culture. Acinetobacter baumannii IITG19 showed higher degradation rates than Providencia vermicola IITG20 in all ecosystems. Higher diesel degradation was observed in freshwater compared to other ecosystems. The mixed culture showed the highest degradation of diesel at 66,66 and 81% in soil, marine water, and freshwater, respectively. The highest rates were observed in freshwater (0.11 day^{-1}), followed by that in marine water (0.078 day^{-1}), and soil (0.066 day^{-1}). Corresponding half-life values were 6.3, 8.9, and 10.6 days for the mixed culture in freshwater, marine, and soil ecosystems, respectively.

Keywords : Diesel degradation; *Providencia vermicola*; *Acinetobacter baumannii*; Mixed culture, Soil ecosystem; Marine water and freshwater

Chapter 6: Degradation of diesel in soil and water ecosystems by *A. baumannii* and *P. vermicola*

6.1. Introduction

The reported studies showed that specific bacteria were effective only in a specific ecosystem. Some bacteria were reported to be effective in soil, while some other bacteria were found to be effective in fresh water. Again different bacteria were effective in marine water. Various bacteria, such as *Acinetobacter* sp. K-6, *Pseudomonas* sp., and *Bacillus subtilis*, are reported to show effective diesel degradation in freshwater (Chaudhary et al. 2020; Sun et al. 2018; Fosso-Kankeu et al. 2017). In soil ecosystems, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* demonstrated degradation (You et al. 2018). In marine water, *Vibrio* sp. LQ2 showed degradation (Zhou et al. 2021). Microbial mixed cultures, such as *Joostella* sp. A8 and *Pseudomona*s strain A6, showed enhanced degradation compared to respective pure cultures (Rizzo et al. 2018). Degradation kinetics, in terms of rate constants and half-life, revealed variations in efficiency across ecosystems and bacterial strains (Bilen and Seyis 2020; Ambaye et al. 2022; Khalid et al. 2022; Chen et al. 2020; Dadrasnia et al. 2020; Mauricio-Gutiérrez et al. 2020; Behera et al. 2020). Detailed discussion on reported literature has been included in Chapter 2.

The current study attempted to identify bacteria that can degrade diesel in different ecosystems. This study intended to determine the effectiveness of *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 for the removal of diesel contamination from soil, marine, and freshwater ecosystems. The effect of the mixed culture of these two strains on the removal of diesel contamination was investigated for the first time. The study also identified the degradation mechanism pathway and conducted a kinetics study to gain further insights into the degradation process. To the best knowledge of authors, no studies on identifying bacteria that can effectively degrade diesel oil in all different ecosystems, namely soil, marine water, and freshwater have been reported yet.

6.2 Experimental details

The diesel degradation experiments were performed in soil, marine, and freshwater ecosystems for both the strains. The effectiveness of mixed culture was also determined in all three ecosystems. All the experiments were done in 250 mL flask at 35°C. For proper mixing, in case of soil sample, manual stirring of the solid feed sample was performed at every 24h. For the water samples, agitation was maintained at 120 rpm. The initial mixture for each

experiment consisted of required amount of feed, 0.33g of Bushnell Hass media and 4 mL of diesel. For soil ecosystem feed consisted of 60g of soil and 35 mL of water.

For marine water ecosystem the feed consisted of 95 mL of prepared artificial marine water (AMW) containing 2.46% NaCl, 0.08% KCl, 0.41% $MgCl_2.6H_2O$, 0.15% $CaCl_2.2H_2O$, 0.61% $MgSO_4.7H_2O$, and 0.02% $NaHCO_3$ (Ferrari et al. 2019). The pH of artificial marine water was maintained at 8. About 95 mL of sterile water was used to emulate freshwater ecosystem. The initial OD of bacterial inoculums were 1 and 1 mL of the same was added to each flask. *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 were used in equal amount to determine the effect of mixed culture on hydrocarbon degradation. About 0.5 mL each of the two inoculums, having 1 OD, was mixed to obtain the mixed culture sample.

The control experiments were also set up to determine the abiotic degradation of diesel in all three ecosystems. The control setups did not have any bacteria, and other conditions are the same as bacterial degradation. All flasks were then sealed and incubated up to 15 days. Growth assessment of bacteria and degradation of diesel were studied at intervals of 2 days' up to 15 days. At the end of 15^{th} day of incubation period, products were analyzed. The degradation kinetics was determined in all three ecosystems in presence of individual strains and their mixed cultures. Assessment of bacterial growth, diesel degradation, analysis of hydrocarbons, and degradation kinetics, were conducted following the procedures outlined in Chapter 3.

6.3. Results and discussion

6.3.1. Assessment of bacterial growth

Fig. 6.1 shows the bacterial growth of *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20 and their mixed culture in soil, marine water and freshwater ecosystems. *Acinetobacter baumannii* IITG19 showed higher growth compared to that of *Providencia vermicola* IITG20, in all ecosystems. Growth of the mixed culture was higher compared to each of the individual strains in all ecosystems. The growth of both the strains and their mixed culture was significantly higher in freshwater than that in other ecosystems. The growth of bacterial strains and their mixed culture was least in soil. The lowest growth of bacteria in soil system might be explained by less availability of oxygen or diesel to the strains as compared to that in water systems. Low porosity of soil might have contributed to this. Amongst water systems, the lower growth of both the bacteria and mixed culture was observed in marine water ecosystem compared to that in freshwater ecosystem. This phenomenon might be attributed to

high salinity of the former. In saline water hydrophobicity of diesel component increases, decreasing bioavailability, and thereby bacterial access to petroleum hydrocarbon. Availability of oxygen is also reduced in saline marine water as it is less soluble in hypersaline environments (Torregrosa-Crespo et al. 2023). Because of less availability of oxygen and diesel, growth of bacteria in marine water was less as compared to that in fresh water.

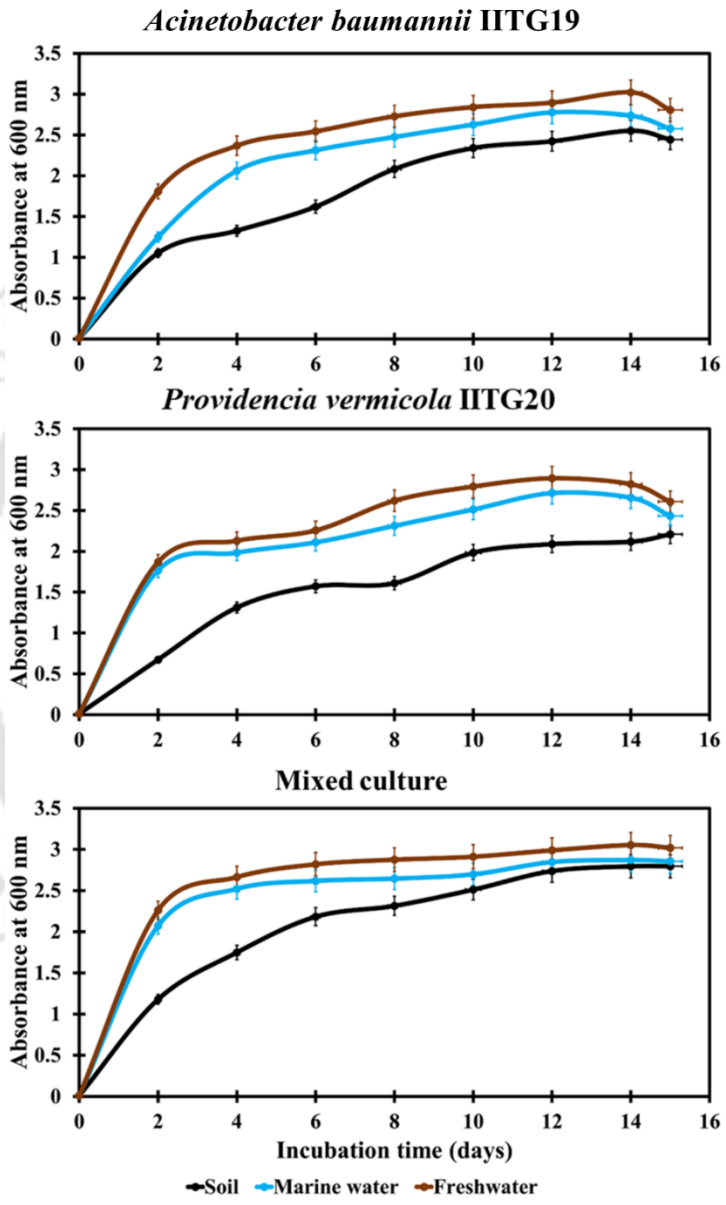


Fig. 6.1. Bacterial growth in soil, marine water and freshwater ecosystems. (at 4% v/v diesel, 35°C temperature and 15 days incubation period).

6.3.2. Analysis of diesel after degradation

Fig.6.2 compares the degradation of diesel with respect to time by bacteria and their mixed culture in soil, marine water and freshwater ecosystems, respectively. The corresponding GC-MS profile has been added as appendix Fig. A3. Both strains and mixed cultures were observed to degrade diesel oil effectively in all the ecosystems. The abiotic degradation of diesel in the control setups is also shown in the figure. It can be observed from the figures that up to 2nd day, the degradation was low and /or similar for all the strains in three ecosystems. This might have resulted as the strains were in the lag phase (adaptation phase). The 2nd day onwards, lower degradation was observed for *Providencia vermicola* IITG20 in all ecosystems compared to that shown by *Acinetobacter baumannii* IITG19. The mixed culture showed higher degradation than the individual strains in remaining incubation time. The degradation was negligible for the control setup in the soil ecosystem, suggesting that only abiotic factors such as light or temperature were not effective for degradation of diesel in any of the ecosystems.

In the soil ecosystem, *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20, and their mixed culture showed an overall degradation of 59, 31, and 66% for diesel, respectively, at the end of 15 days of incubation. In the marine water ecosystem, *Acinetobacter baumannii* IITG19 showed 62% overall degradation of diesel, while *Providencia vermicola* IITG20 showed slightly lower degradation at 59% compared to *Acinetobacter baumannii* IITG19. The use of the mixed culture enhanced the overall degradation of diesel in the marine water ecosystem to 66%.

In the freshwater ecosystem, *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 achieved overall diesel degradation of 76 and 67%, respectively, after 15 days of incubation. The use of mixed culture in freshwater ecosystems enhanced diesel degradation to 81%. The higher effectiveness of mixed culture suggested that *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 can synergise to give better results. Previous researchers (Al-Awadhi et al. 2002; Yang et al. 2019) also reported better degradation ability of mixed culture.

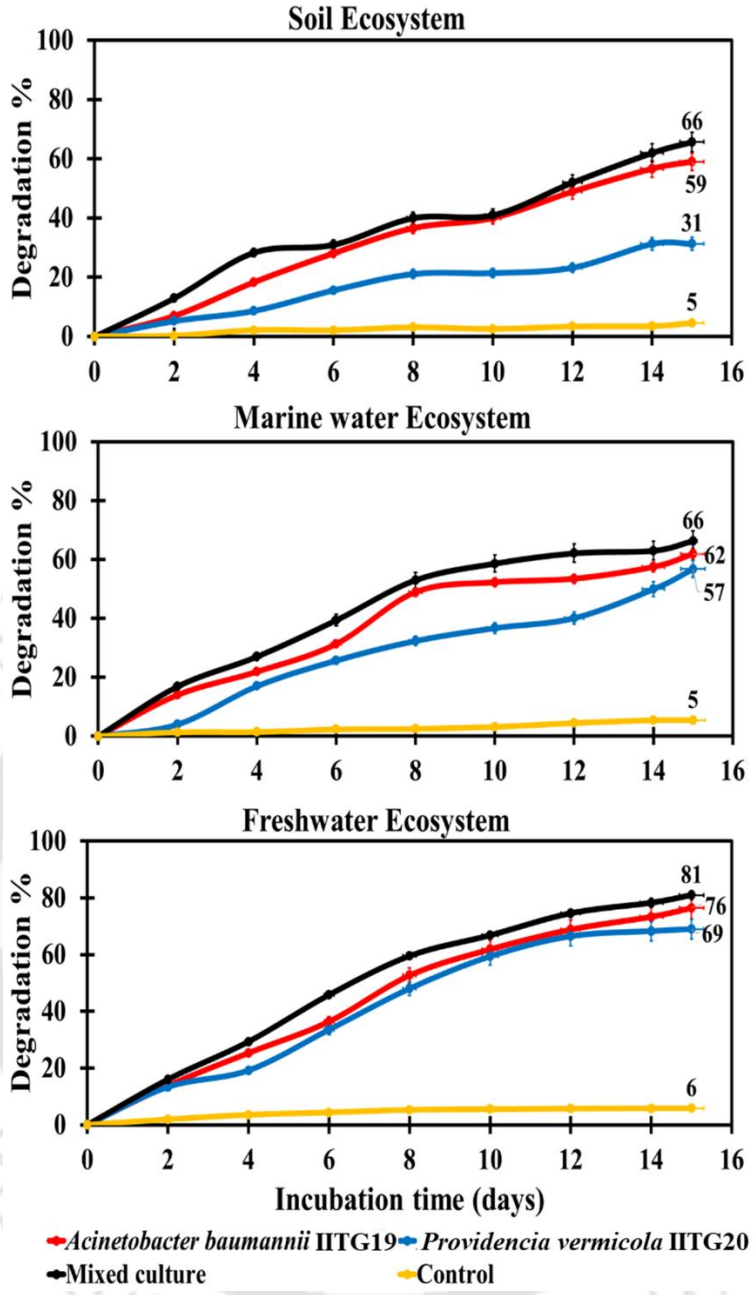


Fig. 6.2. Degradation of diesel with respect to time by individual strains and mixed culture in different ecosystems (at 4% v/v diesel, 35°C temperature).

The extent of degradation for prominent compounds of diesel, in presence of individual strains and their mixed culture after 15 days of incubation time, was compared for all the three ecosystems in Table 6.1.

Table 6.1. Degradation of main hydrocarbons present in diesel by *Acinetobacter baumannii* IITG19, Providencia *vermicola* IITG20 and their mixed culture in soil, marine water and fresh water ecosystems.

Hydrocarbon name	Molecular formula	Degradation by *Acinetobacter baumannii* IITG19 (%)			Degradation by *Providencia vermicola* IITG20 (%)			Degradation by Mixed culture (%)		
		Soil	*marine water*	*fresh water*	*soil*	*marine water*	*fresh water*	*soil*	*marine water*	fresh water
Paraffins (n-alkanes)										
1-fluorododecane	$C_{12}H_{25}F$	91	88	88	68	82	93	81	81	95
dodecane	$C_{12}H_{26}$	91	85	95	83	80	95	98	93	95
pentadecane	$C_{15}H_{32}$	83	88	96	61	91	92	96	80	95
heptadecane	$C_{17}H_{36}$	86	76	92	81	94	94	97	87	98
nonadecane	$C_{19}H_{40}$	91	76	95	67	67	93	97	93	90
icosane	$C_{20}H_{42}$	93	91	96	85	88	91	91	93	97
henicosane	$C_{21}H_{44}$	80	90	90	76	93	93	99	63	88
tricosane	$C_{23}H_{48}$	64	64	84	36	3	66	64	91	81
pentacosane	$C_{25}H_{52}$	77	92	92	60	91	94	94	77	96
hexacosane	$C_{26}H_{54}$	67	44	91	22	22	75	82	96	74
Paraffins (Branched Alkane)										
4,5-dimethylnonane	$C_{11}H_{24}$	89	94	95	66	90	90	97	92	95
4,6-Dimethyldodecane	$C_{14}H_{30}$	44	59	71	23	61	81	93	95	79
2,6,11-trimethyldodecane	$C_{15}H_{32}$	48	59	85	51	53	78	72	89	91
5,6-dipropyldecane	$C_{16}H_{34}$	36	74	76	37	77	81	97	89	87
2,6,10,15-tetramethylheptadecane	$C_{21}H_{44}$	52	84	89	19	80	77	94	96	92
7,7-diethylheptadecane	$C_{21}H_{44}$	72	95	96	68	93	77	64	91	86
5-ethyl-5-methylnonadecane	$C_{22}H_{46}$	79	93	92	76	90	94	99	92	96
2,6,10,14-tetramethyloctadecane	$C_{22}H_{46}$	91	87	98	92	83	97	96	94	92
2,6,10,14-tetramethylnonadecane	$C_{23}H_{48}$	90	88	91	77	86	0	92	95	84
(Z)-5-methyldocos-5-ene	$C_{23}H_{46}$	55	60	62	0	53	64	96	94	83
11-methyltricosane	$C_{24}H_{50}$	93	92	96	80	92	95	80	92	97
2,6,10,14-tetramethyl-7-(3methylpentyl) pentadecane	$C_{25}H_{52}$	73	80	90	12	71	6	96	91	92
11-methylpentacosane	$C_{26}H_{54}$	93	92	92	77	90	84	90	93	85
2-methylhexacosane	$C_{27}H_{56}$	74	81	85	62	79	44	99	97	60
2-methylheptacosane	$C_{28}H_{58}$	74	90	89	67	87	82	96	96	96
13-methylheptacosane	$C_{28}H_{58}$	80	69	96	82	62	80	97	97	97
1-iodooctacosane	$C_{28}H_{57}I$	79	70	95	69	60	83	98	98	97
2-methyloctacosane	$C_{29}H_{60}$	40	56	85	23	47	74	92	96	82

Naphthenes (cycloalkanes or cycloparaffins)										
1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	$C_{10}H_{18}$	76	81	90	18	48	79	89	88	72
1-pentylcyclohexene	$C_{11}H_{20}$	27	50	88	63	77	89	77	92	94
methylcyclodecane	$C_{11}H_{22}$	72	81	52	24	57	78	90	93	89
heptylcyclohexane	$C_{13}H_{26}$	76	78	92	18	86	78	79	95	89
1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene	$C_{15}H_{24}O$	79	34	74	45	75	82	95	90	56
tridecan-6-ylcyclohexane	$C_{19}H_{38}$	64	26	90	50	78	8	93	96	85
Aromatics										
2,6-dimethyl-1,2,3,4-tetrahydronaphthalene	$C_{12}H_{16}$	62	82	33	35	50	75	90	91	70

The Fig. 6.3 compares the extent of degradation experienced by different types of hydrocarbons present in diesel including n-alkane, branched alkane, naphthenes, and aromatics. In the soil ecosystem, n-alkanes showed the highest degradation, with *Acinetobacter baumannii* IITG19 degrading up to 84% and *Providencia vermicola* IITG20 degrading up to 69%. The second highest degradation was that of branched alkanes; 78 and 68% by *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20, respectively, followed by that of naphthenes; 64 and 34%, and aromatics 62 and 35%, respectively. Thus in the soil ecosystem, *Acinetobacter baumannii* IITG19 showed better degradation than that by *Providencia vermicola* IITG20 for all types of diesel components. The *Providencia vermicola* IITG20 demonstrated comparable degradation for branched alkanes and n-alkanes (68-69%), but degradation for naphthenes and aromatics was considerably lower (34-35%) than that shown by *Acinetobacter baumannii* IITG19 (62-64%). When mixed culture was present, there was increase in degradation of each type of components of diesel. The observed degradation was. 92, 94, 87 and 90% for n-alkanes, branched alkanes, naphthenes and aromatics, respectively. The mixed culture thereby, showed higher degradation for all type of compounds as compared to *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 in studied incubation time.

In the marine water ecosystem, *Acinetobacter baumannii* IITG19 showed 82% degradation for n-alkanes and aromatics, followed by 77% for branched alkanes. *Providencia vermicola* IITG20 showed 80% and 78% degradation of n-alkanes and branched alkanes, respectively, comparable to that of *Acinetobacter baumannii* IITG19. However, the degradation of aromatic compounds by *Providencia vermicola* IITG20 was significantly lower at 50% compared to that

82% by *Acinetobacter baumannii* IITG19 (82%). The degradation of naphthenes was, however, higher at 71% for *Providencia vermicola* IITG20 compared to only 59% for *Acinetobacter baumannii* IITG19. The mixed culture of the strains showed only slightly higher or similar degradation rate of 82% for n-alkanes, compared to two individual strains. The mixed culture however, showed 94% degradation of branched alkanes, significantly higher than the individual strains (77-78%). Notably, for naphthenes and aromatics, degradation by mixed culture reached 87% and 90%, respectively, once again higher than the degradation observed for both bacterial strains individually.

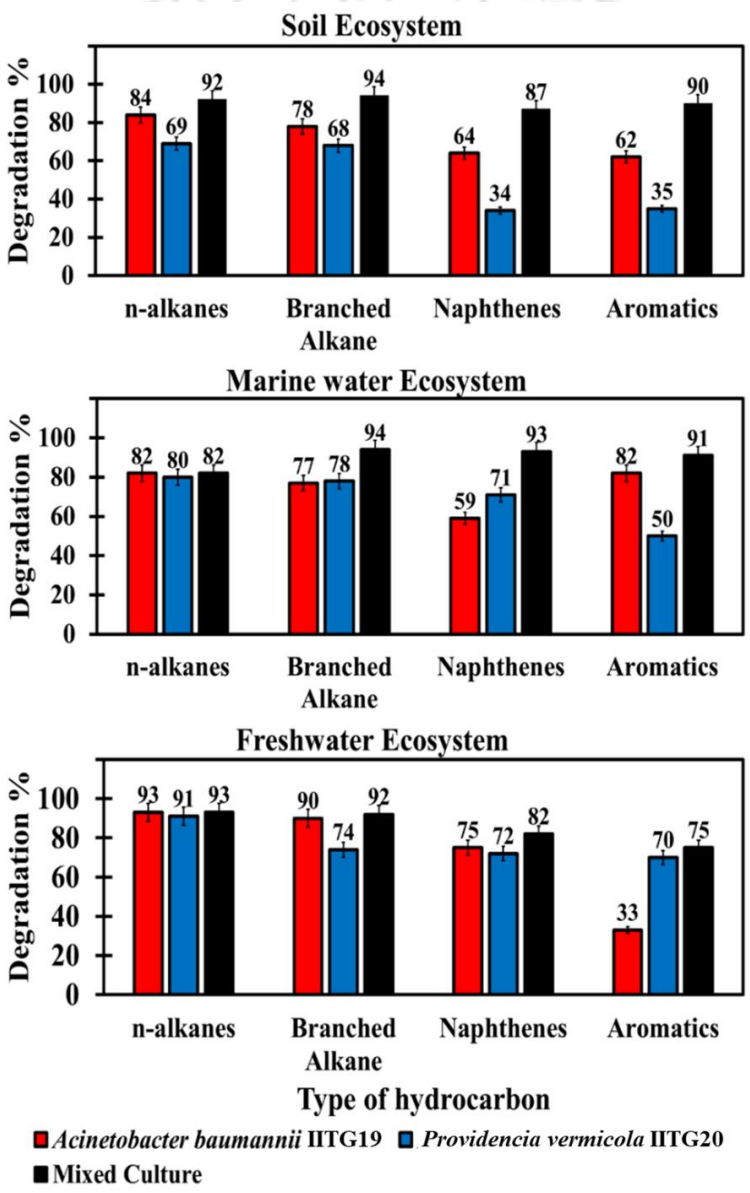


Fig. 6.3. D**egradation of components of diesel by pure and mixed culture in different ecosystems. (at 4% v/v diesel, 35°C temperature and 15-day incubation time).**

In the freshwater, after 15 days of incubation, *Acinetobacter baumannii* IITG19 showed degradation of 93, 92, 75, and 33% for n-alkane, branched alkane, naphthenes and aromatic compounds present in diesel, respectively. The corresponding degradation by *Providencia vermicola IITG20* was 91, 74, 72 and 75%, respectively. Both strains were very effective for the degradation of diesel in fresh water, with *Acinetobacter baumannii* IITG19 performing slightly better. The degradation of n-alkanes and naphthenes was equally effective for both strains, although for branched alkanes, *Acinetobacter baumannii* IITG19 showed higher degradation. *Providencia vermicola* IITG20 showed a much higher degradation of aromatics in freshwater systems. When a mixed culture was used, degradation of diesel components was significantly increased compared to the individual strains. For n-alkanes, branched alkanes, naphthenes, and aromatics degradation reached 93, 92,82 and 75%, respectively. Therefore, the mixed culture proved to be more effective in enhancing the degradation of diesel components in the studied incubation time.

For any ecosystem the overall degradation was higher for *Acinetobacter baumannii* IITG19 than that of *Providencia vermicola* IITG20. For *Acinetobacter baumannii* IITG19 the degradation was in the range of 59 to 76%, whereas *Providencia vermicola* IITG20 showed degradation in the range of 31 to 67% for different types of hydrocarbons in all three ecosystems. The mixed culture showed higher degradation as compared to individual strains in all ecosystems. For different types of hydrocarbons, the degradation ranges for mixed culture was 66 to 81% considering different ecosystems. Better degradation capacity of mixed culture may be attributed to co-culturing of strains in the ecosystems as well as due to synergic effect.

The degradation of diesel has been previously reported for a wide range of initial concentrations, varying from 0.24 to 10%, and varied incubation periods between 7 to 260 days (Table 6.2). However, these studies were mostly limited to a single type of ecosystem. Present study showed effectiveness of isolated bacterial strains in multiple ecosystems, including soil, marine, and freshwater. *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 and their mixed culture showed higher degradation abilities within a relatively short incubation period of 15 days for moderate concentration of diesel at 4% v/v. These results are better than the previously reported studies at similar conditions. The kinetic analysis further confirmed the potential for faster and more efficient diesel degradation for the isolated strains and their mixed culture across different ecosystems.

Table 6.2. Reported studies for bacterial degradation of diesel.

S.N.	Bacteria	Ecosystem	Conc. of diesel (v/v) and degradation time	Degradation %	Ref.
1	*Bacillus* sp	Soil	2% and 15 days	72	Elenga-Wilson et. al 2021
2	*Arthrobacter* sp. strain AQ5-05	Soil	0.5% and 7 days	56.4	Abdulrasheed et al.2020
3	*Thiobacillus* sp.	Soil	1% and 260 days	57	Kim et al. 2018
4	*Pseudomonas* sp. DTF1	Soil	1 % and 14 days	55	Yang et al. 2023
5	*Bacillus megaterium*	Marine water	1% and 5 days	27	Gao et al. 2021
6	*Delftia* sp NL1	Saline water (2M NaCl)	5% and 7 days	66.76	Lench et al. 2020
7	*Vibrio* sp. LQ2	Marine water	0.4 % and 12 day	54	Zhou et al. 2021
8	*Acinetobacter baumannii* *Cedecea davisae*	Freshwater	10% and 21 days	61 52	Jerin et al. 2021
9	*Acinetobacter* sp. K-6	Freshwater	0.24% and 14 days	59.2	Chaudhary et al. 2020
10	*Pseudomonas* sp	Freshwater	4% and 7 days	14.19	Hossain et al. 2022
11	*Halomonas* sp. *and Aneurinibacillus* sp.	Freshwater	1% and 12 days	82.65	Shi et al. 2019
12	*Pseudomonas* sp. CQ2 *Joostella* sp. A8	Freshwater	2 % and14 days	54.7 26.8	Sun et al. 2018
13	*Pseudomonas* strain A6 Mixed culture	Freshwater	2% and 20 days	38.2 90	Rizzo et al. 2017
14	*Bacillus subtilis* *Pseudomonas aeruginosa*	Freshwater	1 % and 56 days	21.61 58.25	Fosso-Kankeu et al. 2017
15	Mixed culture of *Acinetobacter* sp. K-6 *and Rhodococcus* sp	Freshwater	1 % and 40 days	77	Chaudhary et al. 2019
16	***Acinetobacter baumannii* IITG19**	**Soil** **Marine water** **Freshwater**	**4% and 15 days**	**59** **62** **76**	**This Study**
	***Providencia vermicola* IITG20**	**Soil** **Marine water** **Freshwater**		**31** **57** **69**	
	Mixed culture	**Soil** **Marine water** **Freshwater**		**66** **66** **81**	

As shown in Table 6.1, for both the strains, n-alkane was found to be the component of diesel that was most degraded across all the three ecosystems and, aromatic was the least degraded one. Both the strains favoured shorter-chain n-alkanes over longer-chain n-alkanes. Small-sized n-alkanes can pass through bacterial cell membrane-associated lipoproteins more easily, facilitating their degradation. Further, low molecular weight alkanes have a higher cell surface hydrophobicity, which also increased the likelihood of their uptake and degradation (Meng et al. 2018). In terms of toxicant tolerance and cell adherence, bacteria with hydrophobic cell surfaces have distinct advantages over those with hydrophilic cell surfaces. The ability of microorganisms to adhere is critical during the bioremediation of toxic chemicals. Adherence to surfaces is widely accepted as the first step in the process of removing pollutants for microbes. In many cases, hydrophobicity is correlated with adhesion tests (Lim et al. 2023). The degradation rate of pollutants by higher hydrophobic bacteria is reported to be faster than that by low hydrophobic bacteria (Shi et al. 2019).

Longer chain n-alkanes have a lower rate of degradation due to their low solubility (Xiang et al. 2022). Branched alkanes, which have bulkier structures than n-alkanes, are more difficult to degrade as the alkyl branches provide more hindrance to initial diffusion and oxidation (Yusoff et al. 2020). The cyclic structure and higher toxicity of naphthenes or cycloalkanes increased the difficulty level in degradation, making them the third most degraded component (Jaekel et al. 2015). Aromatics with high molecular weights are considered to be recalcitrant in nature. This agreed with the lowest degradation observed for them. It was also observed that both strains degraded certain hydrocarbons more effectively than others. *Acinetobacter baumannii* IITG19 was more effective for the degradation of n-alkane and branched alkanes, while *Providencia vermicola* IITG20, was more effective for naphthenic and aromatic degradation. This behaviour might be correlated to source from where they were isolated. *Acinetobacter baumannii* IITG19 was originally isolated from soil taken from a petrol pump where it was exposed to diesel which contains a larger fraction of n-alkane and branched alkanes. On the other hand, *Providencia vermicola* IITG20 was isolated from oil sludge which contained a larger fraction of naphthenic and aromatic compounds. Since they were exposed to their respective surrounding for long time, they were more compatible with the compounds present there. This might be the reason for higher activity *of Acinetobacter baumannii* IITG19 for the degradation of n-alkane and branched alkanes and that for naphthenic and aromatic degradation for *Providencia vermicola* IITG20. This adaptation phenomenon is the result of three interconnected mechanisms: (i) induction and/or depression of specific enzymes that

degrade hydrocarbons; (ii) genetic changes that result in new metabolic capabilities; and (iii) selective enrichment of microorganisms capable of hydrocarbon transformation (Al-Hawash et al. 2018).

The extent of degradation of the prominent compounds of diesel by mixed culture, when compared with that by single strains (Table 6.2) showed a significant increase. Most of the compounds were degraded by more than 85%, including naphthenes, with maximum degradation was observed up to 97% for alkanes. The results show that the single strains were not equally effective for degradation for all type of components of diesel, but mixed culture resulted in significant degradation for all types of components. For mixed culture, diverse metabolic pathways give a wider range of enzymes than single strains, resulting in a wider range of hydrocarbon degradation (Chuah et al. 2012).

6.3.3. Degradation products

Degradation of diesel can produce several products. These components were not detected in the initial diesel but were present in the mixture after 15 days of incubation. These detected products are shown in Table 6.3. About seven primary alcohols, two secondary alcohols, fourteen esters, three ketones, two acetates, two carboxylic acids, four cycloalkanol and six cycloalkanone were detected in the final hydrocarbon mixture. Details about these product compounds are given in appendix Table A2. The concentration of these compounds were detected in low range (less than 3%) as these compounds usually undergo further reactions (mechanism pathway discussed in Chapter 2, section 2.8). Comparing the three ecosystems, it was observed that their concentration in general was higher in the freshwater ecosystem as compared to the soil and marine water ecosystems. This result agreed with higher degradation rate in the freshwater ecosystem. Ester compounds, in the carbon range of C_{12} to C_{23}, were present in the highest amount in all ecosystems (< 3.4%) in presence of all strains. Esters are present in the degradation pathways of alkanes as explained in chapter 2, section 2.8. Primary alcohols, in the carbon range of $C_5 – C_{16}$, were the second highest (< 3%) detected compounds in the final hydrocarbon mixture. Two secondary alcohols were also identified and were in the 0.44-1.9% range across all ecosystems. Ketone, acetates and carboxylic acids compounds were also detected in the end mixture in presence of both strains and their mixed culture. Cyclic compounds such as cycloalkanol and cycloalkanone were also identified in all ecosystems. Ketone had the third highest concentration (< 2%).

Table 6.3. Compounds detected in final hydrocarbon mixtures after 15 days of incubation period during degradation of diesel

S.N.	Name	Molecular formula
	Primary Alcohol	
1	2,2-Dimethyl-1-propanol	$C_5H_{12}O$
2	2,4,4-Trimethyl-1-pentanol	$C_8H_{18}O$
3	1-Hexanol, 5-methyl-2-(1-methylethyl)-	$C_{10}H_{22}O$
4	2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$
5	1-Dodecanol	$C_{12}H_{26}O$
6	2-Butyl-1-octanol	$C_{12}H_{26}O$
7	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$
	Secondary alcohol	
8	1-Penten-3-ol, 4-methyl-	$C_6H_{12}O$
9	2-Octen-1-ol, 3,7-dimethyl-	$C_{10}H_{20}O$
	Ester	
10	Carbonic acid, nonyl vinyl ester	$C_{12}H_{22}O_3$
11	Methoxyacetic acid, 2-tetradecyl ester	$C_{12}H_{22}O_2$
12	Carbonic acid, dodecyl vinyl ester	$C_{15}H_{28}O_3$
13	Succinic acid, di(dec-4-enyl) ester	$C_{15}H_{26}O_4$
14	Methoxyacetic acid, 2-tridecyl ester	$C_{16}H_{32}O_3$
15	Carbonic acid, prop-1-en-2-yltridecyl ester	$C_{17}H_{32}O_3$
16	Sulfurous acid, 2-propyl tetradecyl ester	$C_{17}H_{36}O_3S$
17	Sulfurous acid, pentadecyl 2-propy l ester	$C_{18}H_{38}O_3S$
18	Carbonic acid, 2-ethylhexyl nonyl ester	$C_{18}H_{36}O_3$
19	Carbonic acid, decyl 2-ethylhexyl ester	$C_{19}H_{38}O_3$
20	Carbonic acid, 2-ethylhexyl undecyl ester	$C_{20}H_{40}O_3$
21	Oxalic acid, cyclobutyl pentadecyl ester	$C_{21}H_{38}O_4$
22	Carbonic acid, decyl undecyl ester	$C_{22}H_{44}O_3$
23	Carbonic acid, eicosyl vinyl ester	$C_{23}H_{44}O_3$
24	5-Hydroxy-4-methyl-6-hepten-3-one	$C_8H_{14}O_2$
25	2-Methyl-2-hepten-4-one	$C_8H_{14}O$
26	4,6-Dimethyloctane-3,5-dione	$C_{10}H_{18}O_2$
27	2-Pentanone, 4-cyclohexylidene-3,3 -diethyl	$C_{11}H_{18}O$
	Acetate	
28	2-Furanmethanol, acetate	$C_7H_8O_3$
29	Hexacosyl acetate	$C_{28}H_{56}O_2$
	Carboxylic acid	
30	1,3-butadiene-1-carboxylic acid	$C_5H_6O_2$
31	Methylenecyclopropanecarboxylic acid	$C_5H_8O_2$
	Cycloalkanol	
32	2-Furanmethanol	C_5H_6O
33	1,4,2,5 cyclohexanetetrol	$C_6H_{12}O_4$
34	Cyclohexanepropanol-	$C_9H_{18}O$
35	1-cyclopentene-1-methanol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{18}O$
	Cycloalkanone	
36	Spiro[3.3]heptane-2,6-dione	$C_7H_8O_2$
37	1-cyclopenten-3-one, 1-(ethoxycarbonyloxy)-	$C_8H_{10}O_4$
38	3-methyl-4,5,6,6a-tetrahydro-1h-pentalen-2-one	$C_9H_{12}O$
39	6-methyl-bicyclo[4.2.0]octan-7-one	$C_9H_{14}O$
40	Cyclopentanone, 2-(1-methylpropyl)-	$C_9H_{16}O$

41	Bicyclo[4.1.0]heptan-3-one, 4,7,7-trimethyl-, [1r-(1.alpha.,4.alpha.,6.alpha	$C_{10}H_{16}O$

In diesel degradation, the final hydrocarbon mixture revealed the presence of primary and secondary alcohols, aldehydes, cycloalkanol, cycloalkanone, and acetate compounds. The detection of primary and secondary alcohols across all ecosystems indicate a common pathway involving the oxidation of methyl groups. The breakdown of primary alcohols into aldehydes, followed by transformations into compounds participating in the tricarboxylic acid (TCA) cycle, was observed. Additionally, the presence of cycloalkanol and cycloalkanone provided evidence of the degradation pathway for naphthenic compounds. The probability of secondary alcohol degradation pathway was noted through the detection of ketones in the final hydrocarbon mixture. Acetate compounds further supported the proposed degradation pathways for both primary and secondary alcohols. However, specific products related to the degradation of aromatic compounds were not identified, possibly due to the low concentration of aromatics in the diesel sample.

6.3.4. Degradation kinetics

ln Co/C (Co: initial concentration and C: concentration at a given time) was plotted against time in days as shown in Fig. 6.4. A linear relationship was observed both in the presence of individual strains and their mixed culture. This linear relationship confirmed the validity of the assumed first-order kinetics for diesel degradation under the used experimental conditions. The kinetic constant values were determined from the slopes of the plots and are provided in Table 6.4. Calculations used for degradation kinetics have been given in appendix Table A3.

As shown in Table 6.4, it was observed that values of rate constant (k) for both bacteria as well as their mixed culture were highest in the freshwater ecosystem and lowest in the soil ecosystem. It was also observed that k was highest for the mixed culture, followed by the *Acinetobacter baumannii* IITG19 and lowest for the *Providencia vermicola* IITG20 across all ecosystems. The higher k value range (0.057-0.095 day^{-1}) for *Acinetobacter baumannii* IITG19 compared to that of *Providencia vermicola* IITG20 (0.025-0.082 day^{-1}) across all ecosystems indicated faster kinetics for former. An increase in the k value range was observed for the mixed culture (0.066- 0.11 day^{-1}) in all three ecosystems. This higher value of k suggested higher degradation and lower half-life of diesel in all ecosystems (Abonyi et al. 2022).

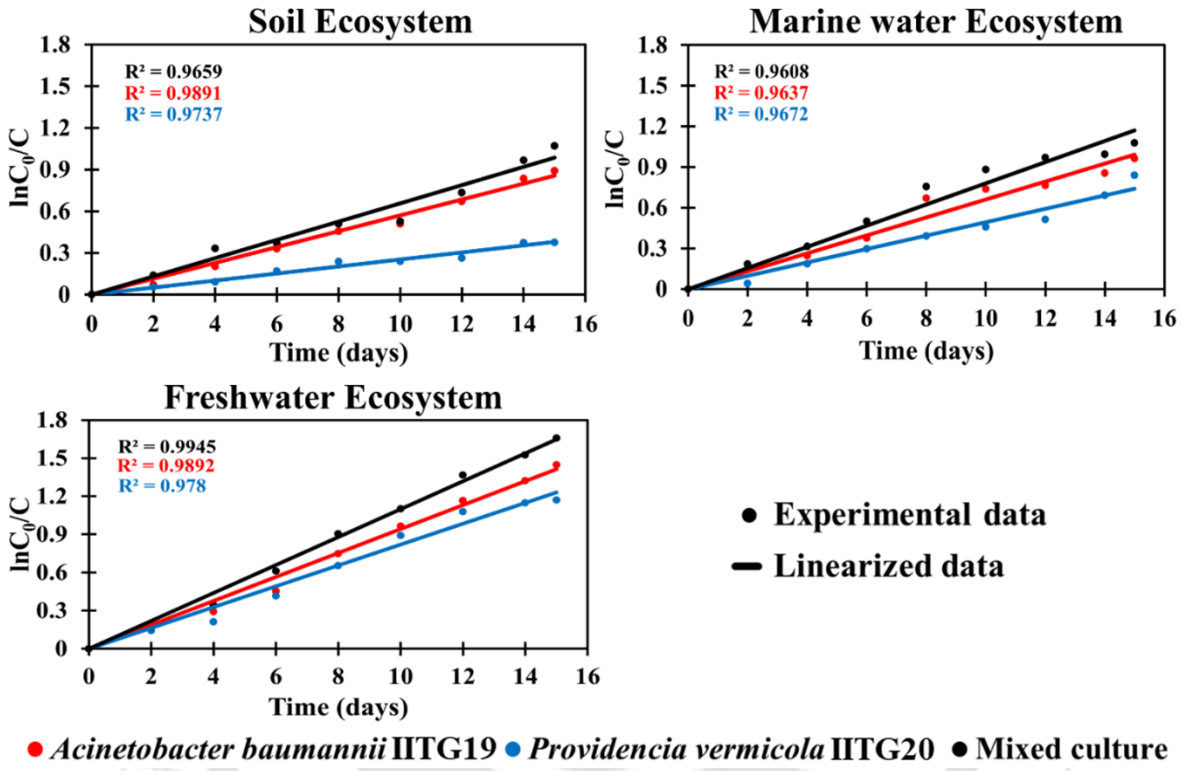


Fig. 6.4. First order kinetics plots for degradation of diesel in different ecosystems.

The experimental results were effectively validated using the first-order kinetics model, as illustrated in Fig. 6.5. These figures provided strong evidence that the first-order kinetic model was suitable for accurately describing the biodegradation process of diesel in used process conditions and in presence of isolated strains.

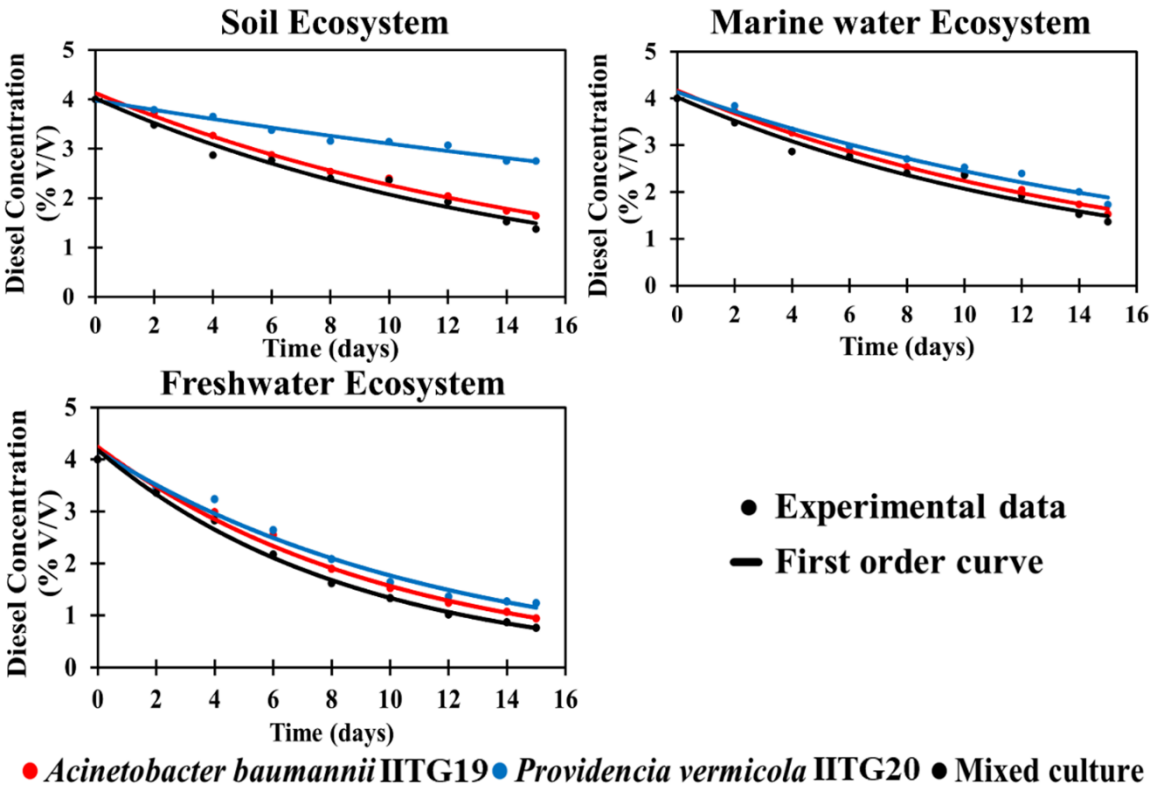


Fig.6.5. Validation of experimental results using the first order kinetics.

The half-life for diesel degradation by both bacteria and their mixed culture in all three ecosystems is shown in Table 3. The half-life for *Providencia vermicola* IITG20 was 27.4, 14 and 8.5 days for soil, marine water and freshwater, respectively. *Acinetobacter baumannii* IITG19 showed lower a half-life of 12.1 days in soil, 10.5 days in marine water, and 7.4 days in freshwater. Half-life was further reduced when the mixed culture was used for diesel degradation. In the soil ecosystem the half-life for mixed culture was 10.6 days, in marine water it was 8.9 and 6.3 days in freshwater ecosystems.

Table 6.4. Kinetics parameters for biodegradation of diesel.

Bacteria	Ecosystem	k (day^{-1})	R^2	Half-life (days)
Acinetobacter baumannii IITG19	Soil	0.057	0.99	12.1
	Marine water	0.066	0.96	10.5
	Freshwater	0.094	0.99	7.4
Providencia vermicola IITG20	Soil	0.025	0.97	27.4
	Marine water	0.049	0.97	14.0
	Freshwater	0.082	0.98	8.5
Mixed Culture	Soil	0.066	0.97	10.6
	Marine water	0.078	0.96	8.9
	Freshwater	0.11	0.99	6.3

Comparison between the rate constant (k) and half-life of the mixed culture, composed of both strains, and values reported in existing literature (Table 6.5) revealed notable findings. Specifically, at a high diesel concentration of 4% v/v, the mixed culture of *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 exhibited a higher k value and a lower half-life compared to reported mixed cultures. Only few studies using lower initial hydrocarbon concentrations showed similar results. This observation was applicable in soil, marine water, and freshwater ecosystems. Hence a mixed culture of *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 showed to be efficient for degradation of higher diesel concentrations, and it has potential for efficient diesel pollution remediation in both water and soil ecosystems.

Table 6.5: Reported studies for first order degradation kinetics for bacterial degradation of petroleum hydrocarbons.

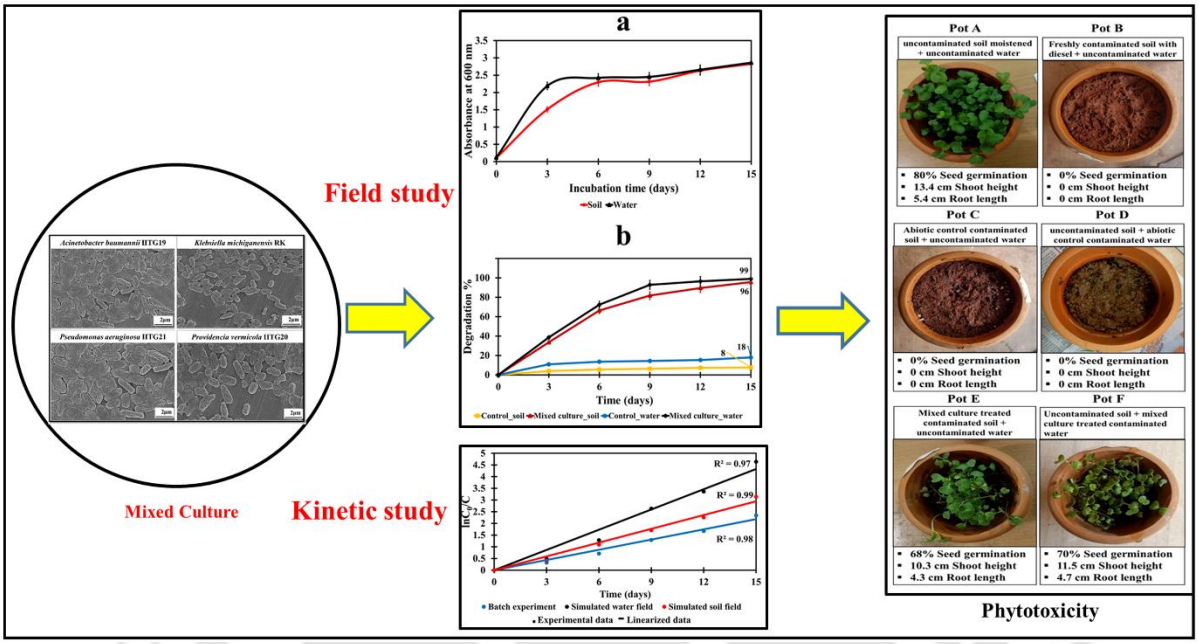
Bacteria	Ecosystem	Hydrocarbons	Rate constant (day^{-1})	R^2	Half-life (day)	Reference
Bacillus *salmalaya*139S	Soil	10 % diesel	0.0388	-	18	Dadrasnia et al. 2020
Mixed culture *(Comamonas testosterone, Gordonia alkanivorans, Pseudomonas aeruginosa, Alcaligenes sp., Gordonia terrae, Gordonia desulfuricans ,* and *Rhodococcus erythoropolis)*	Soil	0.2 % diesel	0.010	-	-	Chen et al. 2019b
Mixed culture *(Acinetobacter junii , Gordonia alkanivorans , Pseudomonas aeruginosa , Exiguobacterium aurantiacum , Serratia marcescens , Gordonia desulfuricans , Rhodococcus erythoropolis , Fusarium oxysporum ,* and *Aspergillus versicolor)*	soil	2 % diesel	0.0072	-	-	Chen et al. 2020
Mixed culture (*Rugosibacter,Rhodanobacter, Nevskia, Parvibaculum, Pseudomonas, Paraburkholderia,* and *Herbaspirillum))*	Soil	4.5 % diesel	0.0118	-	58.6	Chaudhary et al. 2021
Mixed culture *(Streptomyces, Nocardioides, Arthrobacter, Pseudomonas,* and *Bacillus)*	Soil	3.5 % diesel	0.1158	0.96	6	Ambaye et al.2022
Unidentified bacteria isolated from seawater	Marine water	1 % diesel	0.0284	-	-	Xue et al. 2017
Mixed culture of bacteria from seawater	Marine water	0.2 % diesel	0.4535	0.99	-	Xia et al. 2009
Bacillus *pumilus* *Paenibacillus lautus*	Freshwater	2.4 % diesel	0.079 0.009	0.990 0.921	9 76	Mauricio-Gutiérrez et al. 2020
Stenotrophomonas sp. IRB19	Freshwater	2% petroleum refinery sludge	0.036	0.98	19	Behera et al. 2020
Pseudomonas sp. and *Bacillus sp.*	Freshwater	1.5 % crude oil	0.1264	0.98	6	Tian et al. 2018
Pseudomonas sp. HYT	Freshwater	0.14 % diesel	0.272	0.95	-	Zhou et al. 2017
Mixed Culture (*Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20*)*	**Soil** **Marine water** **Freshwater**	**4 % diesel**	**0.066** **0.078** **0.11**	**0.97** **0.96** **0.99**	**10.6** **8.9** **6.3**	**This study**

6.4. Summary

Degradation of diesel was investigated in soil, marine water, and freshwater ecosystems by using *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20 and their mixed culture. Both bacteria and mixed culture were found to be effective in all three ecosystems, with the highest degradation occurring in the freshwater ecosystem. Degradation by *Acinetobacter baumannii* IITG19 was higher than that with *Providencia vermicola* IITG20 in all ecosystems. *Acinetobacter baumannii* IITG19 showed 59, 62, and 76% degradation and *Providencia vermicola* IITG20 showed 31, 57, and 69%, in soil, marine water, and freshwater, respectively. Degradation for alkanes was higher compared to that of naphthenes and aromatics in the presence of both strains. However, *Providencia vermicola* IITG20 showed higher selectivity towards degradation of naphthenes and aromatics. The higher degradation in aquatic ecosystems compared to terrestrial ecosystems may be attributed to less bioavailability of hydrocarbons and lower growth of bacterial strains in the latter. The presence of intermediate compounds such as primary alcohol, secondary alcohol, ketone and esters confirmed the alcoholic degradation pathways of alkane and cyclo-alcoholic pathway of naphthenes. The mixed culture containing both bacteria in equal ratio was more efficient in degradation of diesel compared to that shown by individual strains. The overall degradation reached 66, 66 and 81% in the soil, marine water and freshwater ecosystem, respectively, with more than 90% degradation for alkanes. The degradation rate for mixed culture was higher than that of both the individual strains. The mixed culture had highest degradation rate constant in freshwater at 0.11 day^{-1} followed by that in marine ecosystem at 0.078 day^{-1}. The rate constant was lowest for soil ecosystem at 0.066 day^{-1}. The corresponding half-life were 6.3, 8.9, and 10.6 days for mixed culture in the freshwater, marine and soil ecosystems, respectively.

Chapter 7

Simulated field study using mixed culture and phytotoxicity test



Chapter 7 explores a mixed bacterial culture of Acinetobacter baumannii IITG19, Klebsiella michiganensis RK, Providencia vermicola IITG20, and Pseudomonas aeruginosa IITG21 for remediation of diesel contaminated soil and water. In-lab experiments demonstrated 90% degradation of 4% v/v diesel in 15 days under specified conditions. Field studies also confirmed significant diesel degradation in soil (96%) and water (99%) by the same mixed culture. Phytotoxicity tests with Brassica nigra seeds confirmed successful remediation. Degradation followed first-order kinetics with varying rates in soil and water ecosystems. The rate constants were 0.144 day^{-1} in lab conditions, 0.196 day^{-1} in soil field study, and 0.288 day^{-1} in water field study.

Keywords : Diesel; Degradation; Mixed culture; Field study; Phytotoxicity

Chapter 7: Simulated field study using mixed culture and phytotoxicity test

7.1 Introduction

Bacterial mixed culture has been shown to be more effective in degrading complex hydrocarbons than single strains. Chaudhary et al. (2021) reported 89% degradation of 6% v/v diesel in 60 days using a mixed culture of *Rhodococcus sp., Burkholderia sp., Pseudomonas*, and *Paenarthrobacter* sp., higher as compared individual strains. Similarly, Rizzo et al. (2018) achieved 90% degradation of 2% v/v diesel in 20 days with a mixed culture, higher than individual strains of *Alcanivorax* strain A53, *Pseudomonas* strain A6, and *Joostella* strain A8, which showed 53, 38, and 27%, respectively. Detailed discussion of the reported literature has been included in Chapter 2.

Microbial survival, environmental stresses, and indigenous microbes can hinder field bioremediation (Bala et al. 2022), making field studies crucial for assessing performance in actual field (Bento et al. 2005; Koshlaf et al. 2020). Ma et al. (2018) conducted a field study using the bacterium *M. gilvum* CP13 for bioremediation on a 700 m² agricultural land contaminated with 2.5 mg kg^{-1} of polycyclic aromatic hydrocarbons. Addition of bacteria to the land showed 62% degradation of polycyclic aromatic hydrocarbons within a period of 183 days. Kim et al. (2018) conducted a field study using a mixed culture of *Pseudoxanthomonas, Sphingomonas, Luteimonas, Cellvibrio, Pedobacter*, and *Variovorax* in the area of 1m × 2m, of experimental plot, contaminated with 5196 mg kg^{-1} total petroleum hydrocarbons. After 4 months, 58% degradation was observed.

Simulated field studies help to bridge the gap between study in laboratory conditions and in actual situations. Simulated field studies are mostly reported for contamination by crude oil. For diesel degradation, simulated field studies are less explored. Vasilyeva et al. (2020) artificially contaminated soil with 5%, 10%, and 15% crude oil in polyvinyl chloride vessels. They were treated under open environmental conditions using a bacterial mixed culture, comprising *Pseudomonas putida B-2187* and *Rhodococcus erythropolis Ac-859*. After one year, degradation of 70, 73, and 73% were observed for the soil initially added with 5%, 10%, and 15% crude oil, respectively. In another study, inter-tidal simulation experiments were performed to simulate 1% v/v heavy oil degradation in marine water in the simulated pool of 5.2 m × 1.7 m× 1.5 m by Dai et al. (2021). Mixed bacterial culture used in this experiments were composed of *Bacillus* sp. DL-13, *Brevibacillus* sp. DL-1, and *Acinetobacter* sp. DL-34. Degradation of heavy oil was reported to be 53% after 100 days.

The degradation kinetics provide insights into the efficiency of degradation processes (Abonyi et al. 2022). Chaudhary et al. (2021) investigated the biodegradation kinetics of 4.5% v/v diesel using a mixed culture comprised of *Rugosibacter, Nevskia, Rhodanobacter, Parvibaculum, Pseudomonas, Paraburkholderia*, and *Herbaspirillum*. The resulting rate constant and half-life for diesel degradation were determined to be 0.0118 day^{-1} and 59 days, respectively. Furthermore, Osinowo et al. (2020) reported that when a mixed culture of *Micrococcus luteus* trpE16 and *Bacillus subtilis* DNK was used to treat soil contaminated with 10% v/v diesel for 84 days, the rate constant and half-life for diesel degradation were found to be 0.173 and 4 days, respectively. Moreover, Fu et al. (2021) reported a rate constant of 0.195 day^{-1} for the degradation of 0.75% v/v diesel within a 5-day incubation period using *Pseudomonas sp.* YT-11.

By treating the diesel-contaminated soil with effective bacteria, the soil's properties and plant growth are expected to be restored. Phytotoxicity test are done to confirm the same. After the bacterial treatment of diesel contaminated soil, a reduction in soil toxicity for plants was observed by various researchers (Dib & Ahmed, 2019; Hawrot-Paw et al., 2020; Mukome et al. 2020, Haider et al. 2021). In soil contaminated with 1% diesel, germination of *Vigna radiata* seeds was nil as reported by Bhattacharya et al. (2020). However, after 10 days' treatment of the contaminated soil with *Stenotrophomonas pavanii* DB1, 75% seed germination occurred, suggesting significant recovery of the contaminated soil. It was also observed that, the root and shoot lengths for control seeds, grown in uncontaminated soil, measured 6 and 7 cm, respectively. In soil treated with *Stenotrophomonas pavanii* DB1, these measurements were slightly reduced to 5.6 cm for the root and 6.1 cm for the shoot. Ambaye et al. (2022) reported a 5% germination rate for *Lepidium sativum L*. seeds in soil contaminated with 0.35% v/v diesel, which increase to 75% after treatment of contaminated soil with bacterial mixed culture. Ali et al. (2021) observed that for maize, 100% seed germination occurred in uncontaminated soil, whereas in 1% diesel contaminated soil, the germination rate was reduced to 67%. After treatment of the 1% diesel contaminated soil with *Bacillus sp. MN54*, seed germination increased to 89%. Furthermore, it was observed that both shoot height and root length showed increments in *Bacillus sp.* MN54 treated soil when compared to the to the contaminated soil.

The research gap in diesel-contaminated soil and water remediation using bacterial mixed cultures lies in the limited integration of simulated field studies and phytotoxicity testing. While prior research shows lab effectiveness, practical applicability is largely unexplored. This

study uniquely addresses diesel degradation in both soil and water, simulating field conditions. Bacterial mixed culture, composed of *Acinetobacter baumannii* IITG19, *Klebsiella michiganensis* RK, *Providencia vermicola* IIT20, and *Pseudomonas aeruginosa* IITG21, was used for the first time. The study includes in-lab experiments, field studies on diesel degradation in soil and water, degradation kinetics, and phytotoxicity testing. This integrated research aimed to provide valuable insights into the practical implementation of bacterial mixed culture for diesel remediation in soil and water ecosystems. To the best of the knowledge of the authors, limited studies on integrated approaches for diesel-contaminated soil and water remediation have been reported.

7.2 Experimental details

Growth and diesel degradation ability were initially compared for the individual bacterial and their mixed cultures using in-lab experiments under controlled conditions. Subsequently, simulated field studies were conducted in presence of mixed culture to investigate its ability to degrade diesel in soil and water ecosystems under uncontrolled environmental conditions, including temperature, humidity and light exposure. Additionally, degradation kinetics were determined to evaluate the rate of degradation in the uncontrolled environmental conditions in soil and water ecosystems. The treated soil and water were then subjected to a phytotoxicity test using black mustard (*Brassica nigra*) as the indicator plant. Prior to the phytotoxicity test, essential soil properties for plant growth were determined.

7.2.1 Diesel degradation in-lab study

The comparative effectiveness of a mixed bacterial culture with respect to individual bacterial strains for diesel degradation were assessed under controlled laboratory conditions. The experiments were performed in the 250 mL conical flask closed with cotton plugs. The working volume was 100 mL. To perform experiments, water was taken and contaminated with 4 %v/v (40 mL / L) diesel. Bushnell Hass (BH) was added as the growth media. Then 1% v/v bacterial inoculum was introduced into the BH broth made up of diesel contaminated water. The strains were then incubated at the optimum temperature of each strain. Temperature of 35°C was maintained for *Acinetobacter baumannii* IITG19 *and Klebsiella michiganensis* RK., while 37°C was maintained for *Providencia vermicola* IIT20 and *Pseudomonas aeruginosa* IITG21. The mixed culture was maintained at 35 °C. All the BH broth samples were subjected to continuous shaking at 120 rpm for 15 days, with pH maintained at 7. A contaminated BH broth without bacterial consortia was used as the abiotic control sample and kept at same condition

as of mixed culture. Aliquot of 4 mL were taken out at every 3 days and growth and degradation with respect to time were determined. Assessment of bacterial growth, diesel degradation, and the study of degradation kinetics, were conducted following the procedure outlined in sections 3.6,3.9 and 3.13, respectively, in Chapter 3.

7.2.2 Simulated field study in soil ecosystem

To assess the effectiveness of mixed culture in remediating diesel-contaminated soil under real-world conditions, simulated field study in soil ecosystem was conducted. The sample soil collected from local agricultural field, was dried at 121°C and crushed to remove large particles, resulting in sterile, moisture-free soil. The 132 g of soil sample was then spread evenly in plastic rectangular trays of dimension of 15 × 23 × 5 cm (Fig. 7.1 a). 60 mL of sterilized water was added to maintain moisture in soil and then 8 ml of diesel was added to contaminate the soil. It was mixed to achieve a homogeneous mixture. At this stage the total weight was 200 g, and contamination level was 4 % v/w diesel. Two trays were prepared; one tray was used as the abiotic control and no bacteria were inoculated in this. In the second tray mixed culture of bacteria was inoculated by 1% v/w. Both sets of trays were placed outdoors, where they were exposed to the natural environment, to facilitate the process of diesel degradation. At intervals of every 3 days, the soil in the tray was gently mixed, and sterile water was sprayed to maintain moisture. The treatment was continued for 15 days. Soil samples were collected every 3 days to determine both bacterial growth and diesel degradation. The evaluation of bacterial growth, diesel degradation analysis, and degradation kinetics followed the methodology mentioned in sections 3.6,3.9 and 3.13, respectively, in Chapter 3.

Fig. 7.1. Experimental setup for diesel degradation studies in soil and water: (a) Trays for soil degradation: the left tray represents the abiotic control (no bacterial

inoculation), while the right tray contains soil inoculated with 1% v/w of the mixed bacterial culture.(b) Buckets for water degradation: the blue bucket represents the abiotic control (no bacterial inoculation), while the green bucket contains water inoculated with 1% v/v of the mixed bacterial culture.

Additionally, both pre-experiment and post-experiment soil samples were analysed to determine the properties such as pH, moisture content and water holding capacity, that are beneficial for agricultural plant growth. The soil properties were studied by using standard methods. For determination of pH of soil, 10 g of air dried soil sample was added in 20 ml of distilled water, mixed well, and then allowed to stand for 1 h at room temperature. The pH of the water post sedimentation of soil was measured using a pH meter (Dadrasnia et al. 2018).

For determining the moisture content of soil, 10 g of soil sample was taken in a pre-weighed beaker, dried overnight at 100°C, and then cooled in a desiccator. The sample was then ground in a mortar, re-dried, and again weighed. The moisture content of the soil was calculated using Equation 1 (Choudhary et al. 2019).

$$\text{Moisture Content } (\%) = \frac{\text{Wt. of moist soil sample - Wt. of dry soil sample}}{\text{Wt. of dry soil sample}} \times 100 \qquad (1)$$

Here, the weight of the moist soil sample is the initial 10 g sample taken that is naturally moist. Weight of dry soil sample is the weight of the same sample after drying at 100^{o}C.

To determine the water holding capacity of soil, the 10 g of overnight dried soil sample was placed in a funnel which was fitted with filter paper. The funnel, with the soil placed in it, was then rested on top of a measuring cylinder, and 50 ml of water was poured through the soil. A fraction of poured water was retained within the soil. The wet soil was rested for 1 hour for removal of any excess water. Water holding capacity (% WHC) was then calculated by equation 2 (Hallam & Hodson 2020):

$$\% \text{ WHC} = \frac{\text{Volume of water retain by soil}}{\text{weight of soil}} \times 100 \qquad (2)$$

The volume of water retained by soil was calculated using equation 3.

$$\text{Volume of water retained by soil} = \text{volume of water poured on soil - volume of residual water collected in measuring cyliner} \qquad (3)$$

7.2.3 Simulated field study in water ecosystems

To evaluate the mixed culture's efficacy in remediating diesel-contaminated water ecosystems under realistic conditions, a simulated field study was performed in a water ecosystem. To prepare artificial diesel-contaminated water, 80 mL of diesel was added to 1920 mL of water, ensuring a uniform mixture and resulting in a contamination level of 4% v/v. 2 L contaminated water was placed in 2.5 L buckets of the diameter 12 cm and 20 cm height. A control experiment was set up in the blue bucket to determine the abiotic loss of diesel (Fig, 7.1b). In the control setup, no bacteria were inoculated. 1% v/v mixed culture inoculum was introduced in the green bucket containing the contaminated water. The 10 mL water samples were collected from each bucket at every 3 days up to 15 days for determination of bacterial growth and diesel degradation in them. pH of water samples was also measured at regular intervals. Bacterial growth was analysed by measuring absorbance at 600 nm using UV-Vis spectrophotometer and the diesel degradation was measured by gravimetric method and GCMS analysis as outlined in Chapter 3, sections 3.6 and 3.9.

7.2.4 Biodegradation kinetics

Biodegradation kinetics study was performed to determine the reaction rate constant of degradation and the half-life of diesel in the presence of the mixed bacterial culture. For the kinetics study, samples were collected at regular intervals of 3 days up to 15 days. Kinetic studies were conducted for both in-lab and simulated field studies as per the methods described in Chapter 3, section 3.13.

7.2.5 Phytotoxicity test

To determine the suitability of mixed culture treated soil and water for agriculture plant growth, experiments were performed using 6 different setups:

i. **Pot A**: using uncontaminated soil and water,
ii. **Pot B**: using contaminated soil
iii. **Pot C**: using abiotic control contaminated soil
iv. **Pot D**: using abiotic control contaminated water
v. **Pot E**: using mixed culture treated soil
vi. **Pot F:** using mixed culture treated water

Brassica nigra seeds were used in this study. 30 numbers of seeds were added to each pots. Percentage of seed germination, shoot height and root length in each pot were determined. The shoot height was monitored at interval of every 3 days for a period of 15 days. After 15 days, root length was measured.

7.3 Results and discussion

7.3.1 Degradation of diesel in-lab study

The growth of the bacterial strains and their mixed culture during in-lab study over 15 days of incubation period, is shown in Fig. 7.2a. The *Acinetobacter baumannii* IITG19 showed the highest growth and *Klebsiella michiganensis* RK the lowest, among the pure cultures. The mixed culture of these four bacterial strains showed the highest growth in the entire range of time studied. The growth for all strains showed an initial rapid increase in the first three days, corresponding to the log phase. Subsequently, from 3rd to 12th day, the growth approached the stationary phase. The initiation of decline phase varied with the bacterial strains. The strains with higher growth showed early declination. Mixed culture, *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 strains showed declination after 13-14 days. This might have been caused by nutrient depletion or the accumulation of metabolic by-products. However, for the slower growing *Klebsiella michiganensis* RK and *Pseudomonas aeruginosa* IITG21 inclination was delayed beyond 15 days of study under studied condition.

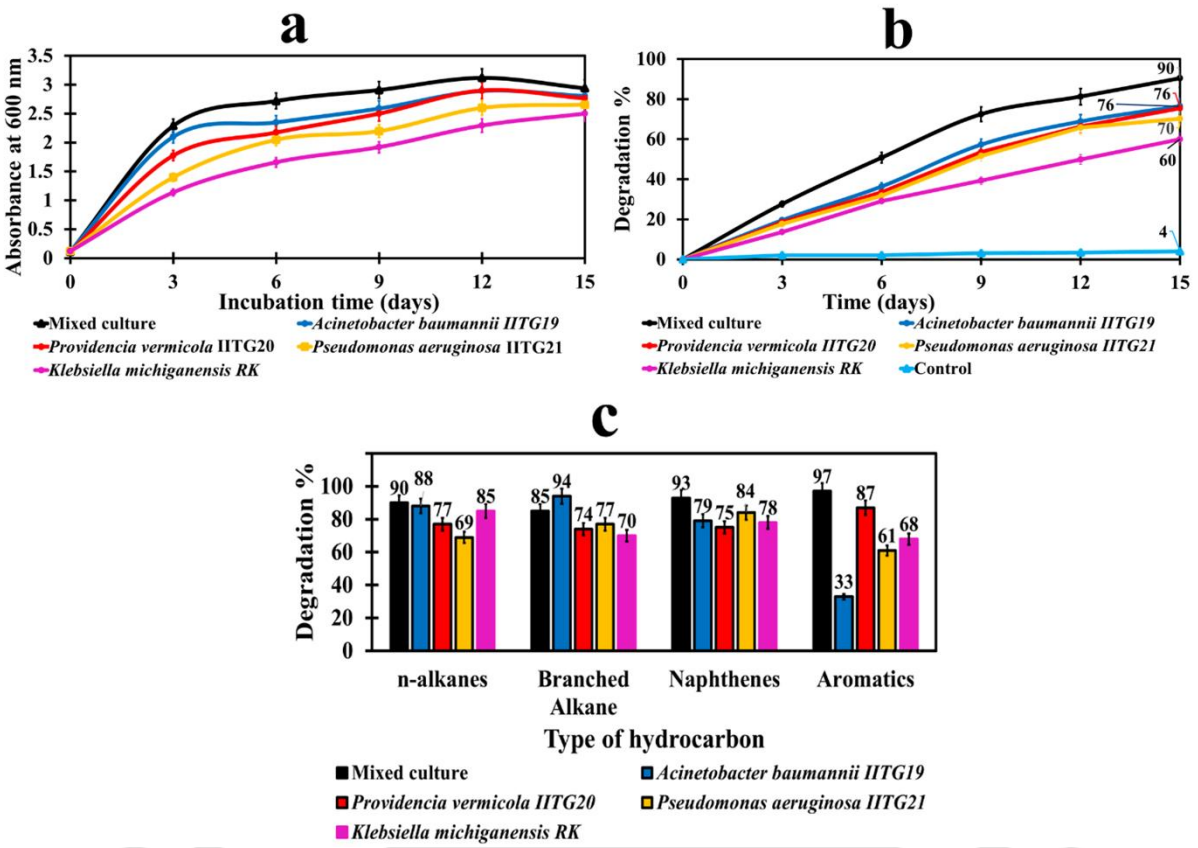


Fig. 7.2. a) Growth of bacterial strains and their mixed culture, b) Diesel degradation by bacterial strains and mixed culture, and, c) Degradation of diesel components at 15th day by bacterial strains and mixed culture (pH 7, 4% v/v diesel, optimum temperature).

Fig. 7.2b shows the degradation of 4% v/v diesel with respect to time for 15 days of incubation period. The corresponding GCMS spectra has been given as appendix Fig. A4. The abiotic degradation in the control setup, which involved degradation only in presence of light and temperature, was negligible (4%). This suggested minimal impact of abiotic factors on diesel degradation in the absence of bacterial activity.

Throughout the entire period of study of 15 days, the mixed culture consistently showed higher degradation compared to all the individual bacterial strains. The degradation of diesel by the bacterial mixed culture reached 90% after 15 days of incubation period, surpassing the degradation observed in presence of individual bacterial strains. The individual *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21, and *Klebsiella michiganensis* RK degraded diesel only up to 76, 75, 70 and 60 %, respectively, in the same period. This enhancement in degradation in presence of bacterial mixed culture can be attributed to the synergistic collaboration among the bacteria present in the mixture.

The degradation of different types of compounds present in diesel by individual and mixed cultures of bacterial strains in 15 days of incubation period, is compared in Fig. 7.2c. Individually the *Acinetobacter baumannii* IITG19 and *Klebsiella michiganensis* RK showed higher degradation for n-alkanes at 88 and 85%, respectively. For same n- alkanes, 77 and 69% degradations were observed in presence of *Providencia vermicola* IITG20 and *Pseudomonas aeruginosa* IITG21, respectively. For branched alkane, Acinetobacter *baumannii* IITG19 showed highest degradation of 94% followed by *Pseudomonas aeruginosa* IITG21 (77%). *Providencia vermicola* IITG20 and *Klebsiella michiganensis RK* showed degradation of 74 and 70% for branched alkanes, respectively. For naphthenes, *Pseudomonas aeruginosa* IITG21 showed better performance than other pure culture with degradation of 84%. *Acinetobacter baumannii* IITG19, *Klebsiella michiganensis* RK and *Providencia vermicola* IITG20, showed 79, 78 and 75% degradation, respectively, for naphthenes. For the aromatics, *Providencia vermicola* IITG20 showed highest degradation of 87% compared to that shown by other pure cultures. *Klebsiella michiganensis* RK and *Pseudomonas aeruginosa* IITG21 showed degradation of 68 and 61% for aromatics, respectively. *Acinetobacter baumannii IITG19* showed lowest degradation of aromatics at 33%.

The varied degradation performance by the bacterial strains, for different type of hydrocarbons, suggested preferred metabolic pathway undertaken by the strains, resulting in preferential consumption of specific hydrocarbons. It was observed that *Acinetobacter baumannii* IITG19 was most effective for degradation of both branched alkanes (94%) and n-alkanes (88%). The *Pseudomonas aeruginosa* IITG21 was found to be the most efficient in degrading naphthenes (84%), while *Providencia vermicola* IITG20 demonstrated the highest efficacy in degrading aromatics (87%) compared to other strains. The *Klebsiella michiganensis* RK had the 2nd highest ability to degrade n-alkane at 85% but showed moderated ability of degradation (70-80%) for all other types of hydrocarbons. The four strains thus showed that preferred degradation for different types of hydrocarbons, making them most suitable for use in mixed culture, to render highest degradation of all types of hydrocarbons. In fact, the mixed culture of these four bacterial strains showed better performance for the all type of the diesel components, except that of branched alkane. The mixed culture showed highest degradation of 90, 93, and 97% for n-alkanes, naphthenes, and aromatics, respectively. For branched alkane. degradation by mixed culture was lower at 85%, compared to 94 % in presence of *Acinetobacter baumannii* IITG19. The lower degradation efficiency for branched alkane might be associated with lower amount of *Acinetobacter baumannii* IITG19 in mixed culture. Each

bacterium's efficiency in degrading specific types of diesel components contributed to an overall synergistic effect, resulting in enhanced degradation.

7.3.2 Simulated field study

7.3.2.1 Analysis of soil

Fig. 7.3a shows the pH of soils at different conditions. The pH of local uncontaminated soil was 6.8. After mixing with 4% v/w diesel in the experimental condition, the pH of the contaminated soil was reduced to 6.2 making it more acidic. When the contaminated soil was exposed to atmosphere in abiotic control condition for 15 days, no significant change in pH of the soil sample was observed as it was still giving pH value of 6.3. However, when the contaminated soil sample was treated with the mixed culture of the four strains for 15 days, pH was observed to again attain the value of 6.9, close to that of uncontaminated soil. The observed recovery of the property of soil due to bacterial treatment suggested that the treatment effectively degraded the contaminating hydrocarbons present in the soil.

The moisture content of soils at different conditions is shown in Fig.7.3b. Moisture content for uncontaminated soil was 25%. After exposure to contamination by 4% v/w diesel, moisture content in soil was reduced to 18%. This reduction can be attributed to alterations in the surface properties of soil particles due to presence of hydrophobic diesel, making them more water-repellent. This contributed to decreasing water retention property of the contaminated soil reducing their moisture content. In abiotic control soil, where the artificially contaminated soil was exposed to atmospheric condition, the moisture content of the control sample increased to 20%. This enhancement suggested partial removal of the contaminating hydrocarbons mainly by evaporation and/or breakdown of diesel present in soil on exposure to sunlight and corresponding heat, thereby slightly improving the property of soil. Treatment of contaminated soil by mixed culture, helped to restore its moisture content to 24%, close to that of uncontaminated soil. This restoration can be attributed to the fast bacterial degradation of diesel contaminants present in soil sample.

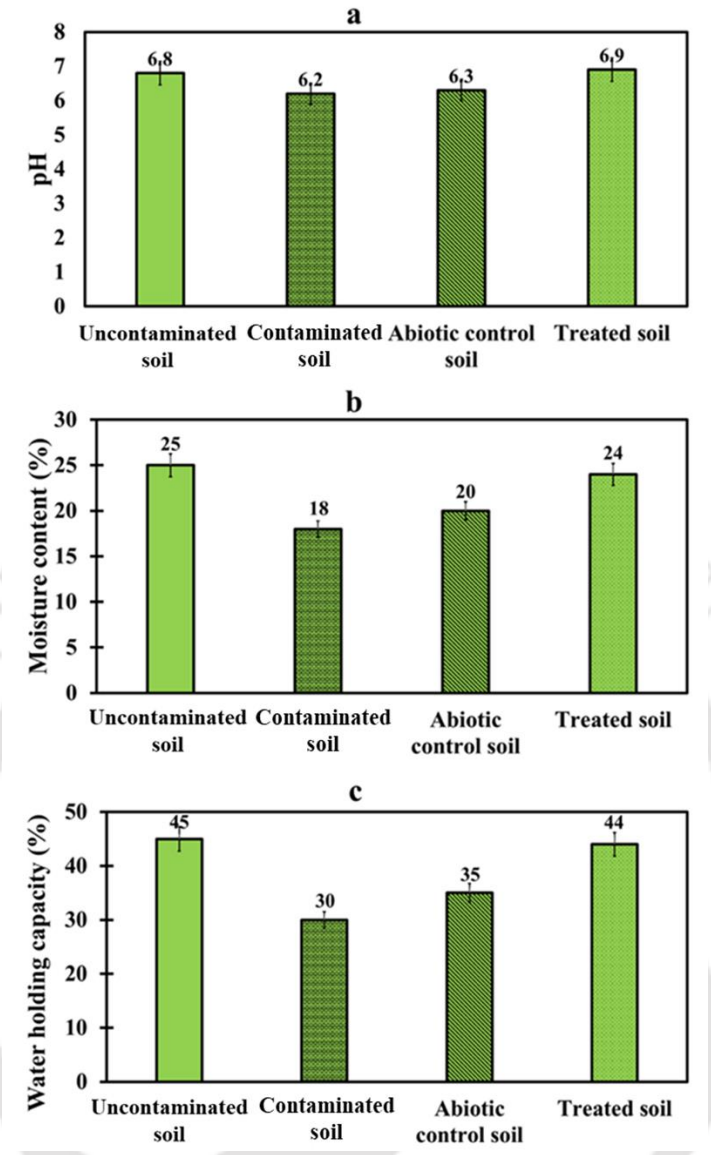


Fig. 7.3. Analysis of soil a) pH, b) moisture content and c) water holding capacity.

Fig. 7.3c compares water holding capacity for the different soil samples. The uncontaminated soil showed water holding capacity of 45%, which on contamination by diesel decreased to 30%. This reduction might be attributed to changes in the surface properties and porosity of the soil caused by presence of diesel contamination. Diesel contamination can induce compaction, reduce porosity, and increase water repellence property. Diesel's hydrocarbons can act as a binding agent, causing soil particles to adhere and compact, reducing number of channels that can hold water. Consequently, capacity of contaminated soil to retain water is decreased. Moreover, the formation of a water-repellent layer of oil on the surface of soil particles prevents water from penetrating the soil, increasing water runoff from the surface (Mohanta et al. 2023). Slight increase in water holding capacity was observed in the abiotic

control soil (35%), although it remained lower than that of uncontaminated soil. The diesel contaminated soil after treatment with mixed culture for 15 days showed water holding capacity of 44%, which closely resembled that of uncontaminated soil at 45%. These observations suggested that the mixed culture treatment of diesel-contaminated soil restored the properties of soil very effectively.

7.3.2.2 Assessment of bacterial growth

Fig. 7.4a shows the growth of bacteria in mixed culture during the degradation of diesel in both simulated soil and water field studies, under uncontrolled environmental conditions. Different patterns of bacterial growth were seen in soil and water samples contaminated with diesel. In the soil, the initial lag phase of 0 to 3 days suggested an adaptation period, followed by a log phase of 3-6 days, indicating active bacterial proliferation. The subsequent exponential growth was observed between 6th and 12th day. Eventually a stationary phase was attained between 12th and 15th day, as resources became limited. In contrast, the water ecosystem showed a faster growth, with quicker adaptation and establishment during the lag and log phases of 0-3 days. The exponential growth phase was also initiated earlier at 3rd day and continued till 12th day, followed by the stationary phase till 15th day corresponding to growth slowdown.

For the initial 12 days, bacterial growth was higher in diesel contaminated water as compared to that observed in diesel contaminated soil. The difference was more significant within the first six days. This difference can be attributed to the enhanced accessibility of hydrocarbons in water, while the porous nature of soil that tends to entrap hydrocarbons, limiting their availability to bacteria. Subsequently, bacterial growth in soil and water converged, exhibiting comparable patterns. By the 12th day, their growth became nearly equivalent and on the 15th day, similar bacterial growth was observed in both water and soil. This can be attributed to the fact that as gradually bacterial populations increased with time, the hydrocarbons became more easily accessible to the strains, having positive effect on their growth. The condition improved particularly in soil ecosystem and the restriction in soil system was minimized. Thereafter, the bacteria followed a similar unhindered growth pattern in soil ecosystem as was observed in water ecosystem. It may be noted from the figure that in mixed culture the bacteria were still growing, though at slower pace, at the end of the 15th day of the study. Thus there was initial dominance of bacterial growth in diesel-contaminated water which may be attributed to enhanced hydrocarbon accessibility, as discussed earlier. Subsequent convergence of growth

patterns of water and soil in later stages, suggested improved accessibility of hydrocarbons over time.

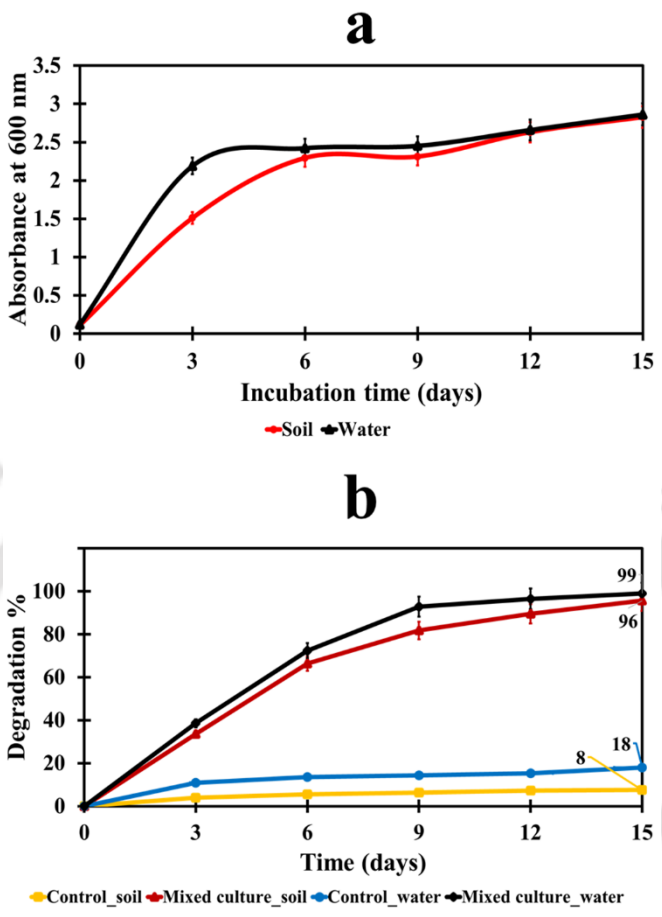


Fig. 7.4 a) Bacterial growth of mixed culture in simulated field study, and b) diesel degradation in simulated field study .

7.3.2.3 Degradation of diesel

Fig. 7.4b shows the time-dependent degradation of 4% diesel in the simulated field study, in presence and absence of mixed culture of the bacterial strains. The corresponding GCMS spectra has been given as appendix Fig. A5. The degradation of diesel was carried out in the simulated field study using both soil and fresh water ecosystems. For comparison, experiment was also done in abiotic control system, without the presence of bacteria. It was observed that diesel degradation in the abiotic control soil reached 8%, while that in the abiotic control water, was 18%, after 15 days of incubation. The degradation of diesel in abiotic control soil and water samples might be attributed to photolytic degradation caused by sunlight or due to evaporative loss to the surrounding atmosphere as mentioned earlier. The degradation of diesel

in presence of mixed culture in soil and water systems was comparable in initial phase of 1-2 days. This might have been the adaptation period of bacteria in soil and water environments. At this stage usually the growth rate and hence degradation rate of diesel are relatively low. However, from the 2nd day onwards and up to the 15th day, a distinctly better degradation performance was observed in presence of mixed culture in water medium compared to that in soil medium. In water, over 90% degradation was achieved by the 9th day, whereas a similar level of degradation in soil was attained only after 12 days of incubation. The degradation of diesel by bacterial mixed culture reached to 99 and 96% in the simulated water and soil field, respectively, at the end of 15th day of incubation. The higher degradation of diesel in water system compared to that in soil system agreed with slower and poorer growth of strains observed in soil system (Fig 7.3a). As discussed earlier, the structural composition of soil reduces hydrocarbons' bioavailability compared to water, resulting in the lower degradation in former. The mixed culture showed almost equal effectiveness in degrading all type of diesel components present in both simulated water and soil fields. The diesel component degraded by mixed culture are shown in appendix Table A4. For n-alkanes, branched alkanes, naphthenes and aromatics, the extent of degradation in soil ecosystem was 97.6, 97.5, 96.2 and 97 %, respectively. Mixed culture performed slightly better in water ecosystem, reaching more that 99% for all type of components. The results of this study was compared with the previous studies as shown in Table 7.1. It can be observed that mixed culture used in this study performed better than many reported ones in terms of higher degradation in lesser exposure period, and also in most cases higher concentration of diesel.

Table 7.1: Comparison of results of simulated field studies for bacterial degradation of diesel of present work with that reported previously.

S.N.	Bacterial mixed culture	Ecosystem	Conc. of diesel (v/v) and degradation time	Experimental setup	Degradation %	Ref.
1	*Pseudoxanthomonas, Sphingomonas, Luteimonas, Cellvibrio, Pedobacter, and Variovorax*	soil	0.5% , 120 days	Area of 1m × 2m	58	Kim et al., 2018
2	*Acinetobacter sp.* K-6 *and Rhodococcus sp.* Y2-2	soil	0.8%w/w, 40 days	Polystyrene container (size: 24 cm × 17 cm × 16 cm)	83	Chaudhary et al., 2019

3	*Bacillus cereus, Bacillus sphaericus, Bacillus fusiformis, Bacillus pumilus, Acinetobacter junii* and *Pseudomonas* sp	soil	Conc. unknown, 84 days	Aluminium pans with a surface area of 441 cm^2 and a volume of 1764 cm^3	84	Bento et. al., 2005
4	Indigenous group of bacteria	soil	10% v/v, 5years	160 m × 85 m	82	Johnsen et al., 2021
5	***Acinetobacter baumannii* IITG19, *Klebsiella michiganensis* RK, *Providencia vermicola* IITG20, *and Pseudomonas aeruginosa* IITG21**	**soil**	**4% and 15 days**	**Rectangular plastic trays of dimension of 15 × 23 × 5 cm**	**96**	**This study**
		water		**Plastic buckets of 2.5 L ,The diameter and height of buckets were 12 and 20 cm**	**99**	

7.3.3 Biodegradation kinetics

Fig. 7.5a shows the plots of ln C_0 / C as function of time for degradation of diesel in-lab study and simulated field studies in presences of mixed bacterial culture. Linear correlation suggested first-order kinetics for diesel degradation. The experimental results of diesel degradation were further validated using first order kinetics as shown in Fig. 5b (remaining diesel concentration v/s time). The exponential curves were fitted to experimental data. Good fitting confirmed the degradation kinetics to be first order. The half-lives were calculated by equation 8 (Chapter 3, section 3.13).

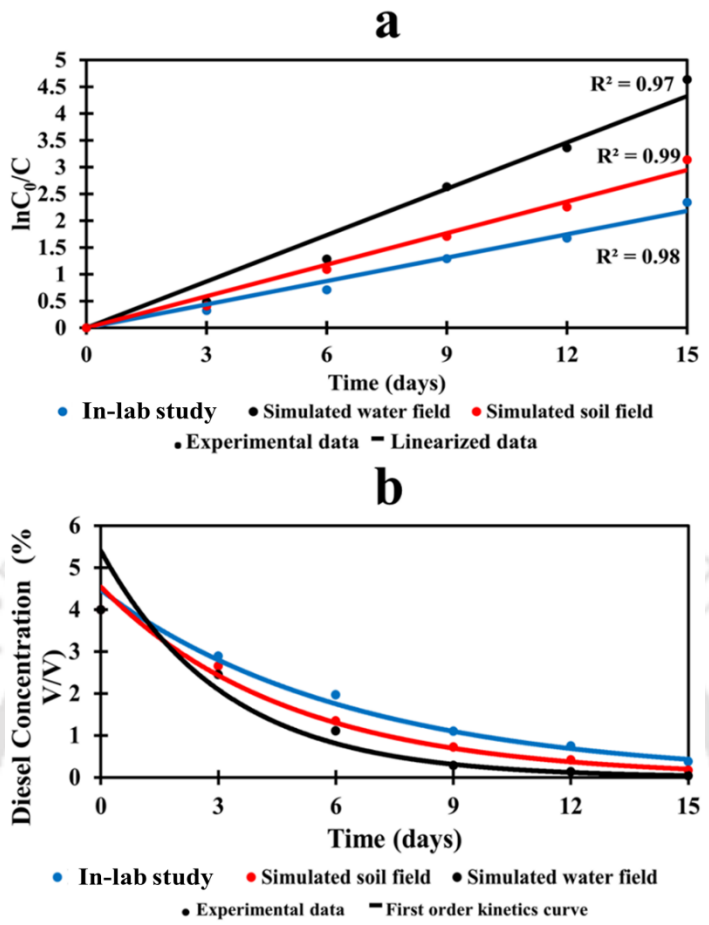


Fig. 7.5. a) Graph showing first order kinetics, b) validation of experimental results using the first order kinetics.

Table 7.2 presents the kinetic parameters for the biodegradation of diesel by the mixed culture in different experiments. In the in-lab experiment, the degradation rate constant (k) was 0.146 day^{-1}, with a high correlation coefficient (R^2) of 0.98, resulting in a half-life of 4.7 days. In the soil field study, the mixed culture showed a higher degradation rate (k = 0.196 day^{-1}) with an improved correlation (R^2 = 0.99) and a shorter half-life of 3.5 days. The water field study showed the highest degradation rate (k = 0.288 day^{-1}), a good correlation (R^2 = 0.97), and the shortest half-life of 2.4 days. These results suggested that the mixed culture was more effective in degrading diesel in water ecosystems, demonstrating faster kinetics and shorter half-lives compared to that in soil environments. The variations in kinetics parameters suggested the adaptability and efficiency of the mixed culture in different environmental systems.

The observed differences in kinetic parameters between soil and water field study can be attributed to several factors influencing bacterial activity and diesel degradation. In water

ecosystems, the availability of nutrients and the accessibility of hydrocarbons are often more favourable for bacterial growth, leading to enhanced degradation rates. The aqueous nature of the medium facilitates better contact between bacteria and diesel, promoting efficient degradation. On the other hand, soil, with its more complex structure and composition, poses challenges for bacterial access to hydrocarbons. The porous nature of soil can entrap hydrocarbons, limiting their availability to bacteria and, consequently, slowing down the degradation process. In-lab experiments exhibited the lowest degradation rate compared to the field studies. This might primarily be attributed to the reduced influence of abiotic factors in the controlled laboratory environment, suggesting the more complex and variable conditions encountered in field experiments.

Table 7.2. Kinetics parameters for biodegradation of diesel.

Bacteria	**Type of experiment**	**k (day^{-1})**	**R^2**	**Half-life (days)**
Mixed Culture (containing *Acinetobacter baumannii* IITG19, *Klebsiella michiganensis* RK, *Providencia vermicola* IITG20, *and Pseudomonas aeruginosa* IITG21)	In-lab Experiment	0.146	0.98	4.7
	Soil field study (Tray)	0.196	0.99	3.5
	Water field study (buckets)	0.288	0.97	2.4

7.3.4 Phytotoxicity Test

Growth of *Brassica nigra* seeds in different conditions with corresponding fraction of seed germination, final shoot height and final root length are shown in Fig. 7.6. *Brassica nigra* seeds showed positive growth in uncontaminated soil moistened by uncontaminated water in **Pot A.** The 80% seed was germinated. The final shoot height and root length after 15 days period were 13.4 and 5.4 cm respectively under this conditions. However, no germination of seed was observed on use of contaminated soil (**Pot B**). This is due to the toxicity of diesel which inhibited the germination of *Brassica nigra* seeds. On using mixed culture treated soil or water, again positive growth of *Brassica nigra* plants was observed as shown in **Pot E and F,** respectively. The corresponding germination were similar at 68% and 70%, respectively. The shoot growth was 10.3 cm and 11.5 cm for mixed culture-treated soil and water, respectively. The root growth was observed to be 4.3 cm and 4.7 cm for mixed culture treated soil and water, respectively. The results suggested that remediation of diesel contamination in soil and water

by studied mixed culture was very effective. Removal of toxicity from soil and water by treatment of studied mixed culture was complete enough to again the support the germination of seed and growth of plants. Both rate of germination and growth of shoots and roots in 15 days of time, though less than uncontaminated condition but encouragingly close to it (Fig. 7.6). This agreed with the results (Fig. 7.3) that showed the properties of contaminated soil such as pH, moisture content, and water holding capacity became close to that for uncontaminated soil after treatment with mixed culture.

To confirm the effect of mixed culture treatment, an abiotic control soil (**Pot C**), representing untreated contaminated soil, and another abiotic control water (**Pot D**), representing untreated contaminated water, were also used for seed germination. No seed germination was observed in these control soil and water samples. This suggested that remediation in the absence of bacteria was not enough to support seed germination or plant growth. This is in agreement with low degradation of diesel observed in abiotic control sample (Fig. 7.4b).

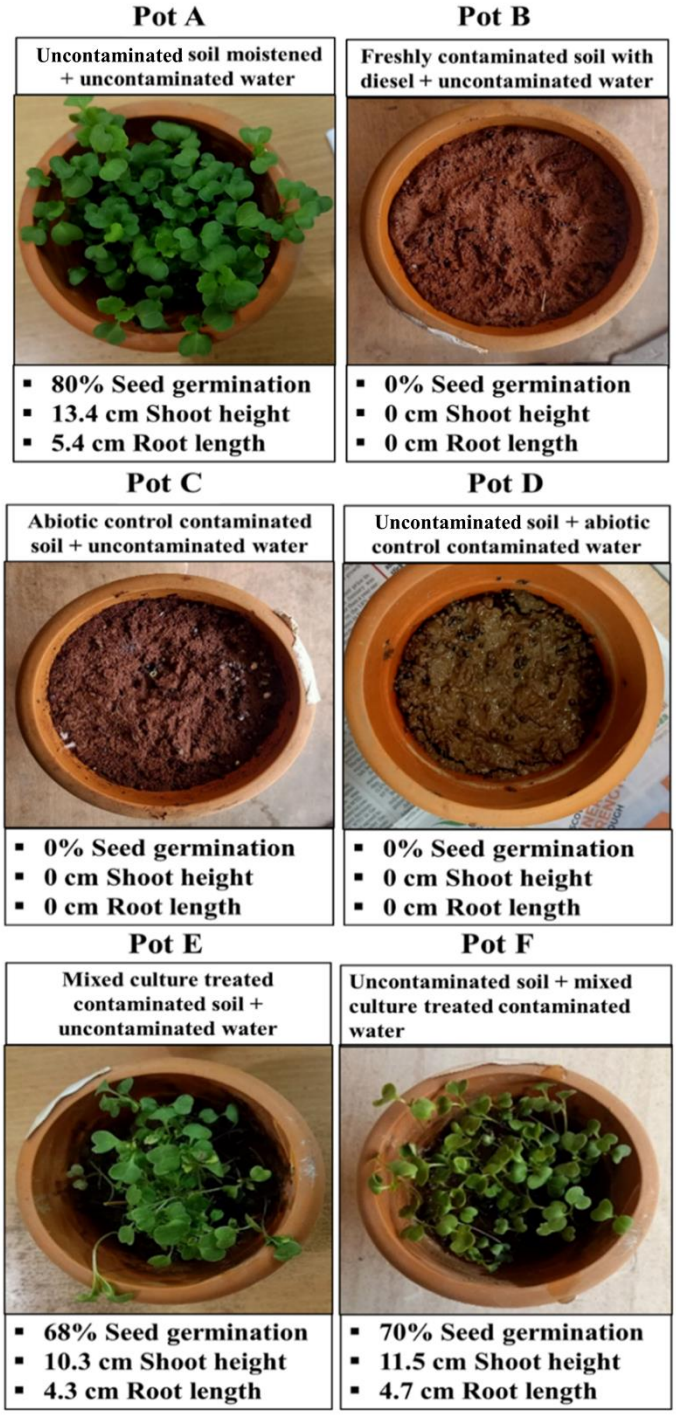


Fig.7.6 Growth of *Brassica nigra* seed in pot A) uncontaminated soil moistened with uncontaminated water, pot B) freshly contaminated soil moistened with uncontaminated water, pot C) abiotic control contaminated soil moistened with uncontaminated water, pot D) uncontaminated soil moistened with abiotic control contaminated water, pot E) mixed culture treated contaminated soil moistened with uncontaminated water, pot F) uncontaminated soil moistened with mixed culture treated contaminated water.

The growth of plants in terms of increase in height of shoot as function of time over uncontaminated soil/water and mixed culture treated soil/water time is compared in Fig. 7.7. It was observed that up for initial 3 days, the growth of plants was comparable in all conditions. However, after the 3rd day, the differences in growth became apparent. The plants grew at faster rate in uncontaminated soil/water compared to that in mixed culture treated soil/water. After 15 days, the growth of plant was highest when uncontaminated soil and water were used, reaching a height of 13.5 ±0.5 cm. In the case of treated water and contaminated soil the final plant height after 15 days was 11.5 ± 0.4 cm. The least growth of 10.3±0.5 cm was observed on use of mixed culture treated soil along with uncontaminated water. The lower growth rate of plant on treated soil than that in presence of treated water agreed with the earlier observation that remediation of diesel contamination in water was more effective than that in soil by studied mixed culture.

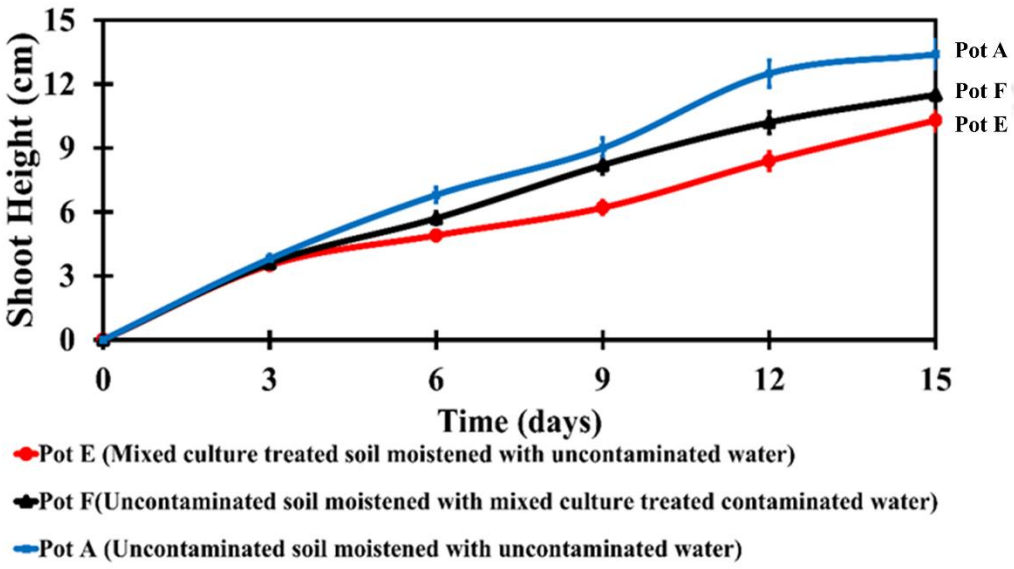


Fig. 7.7. Height of plant shoot as function of time, grown using uncontaminated soil/water, and mixed culture treated soil/water.

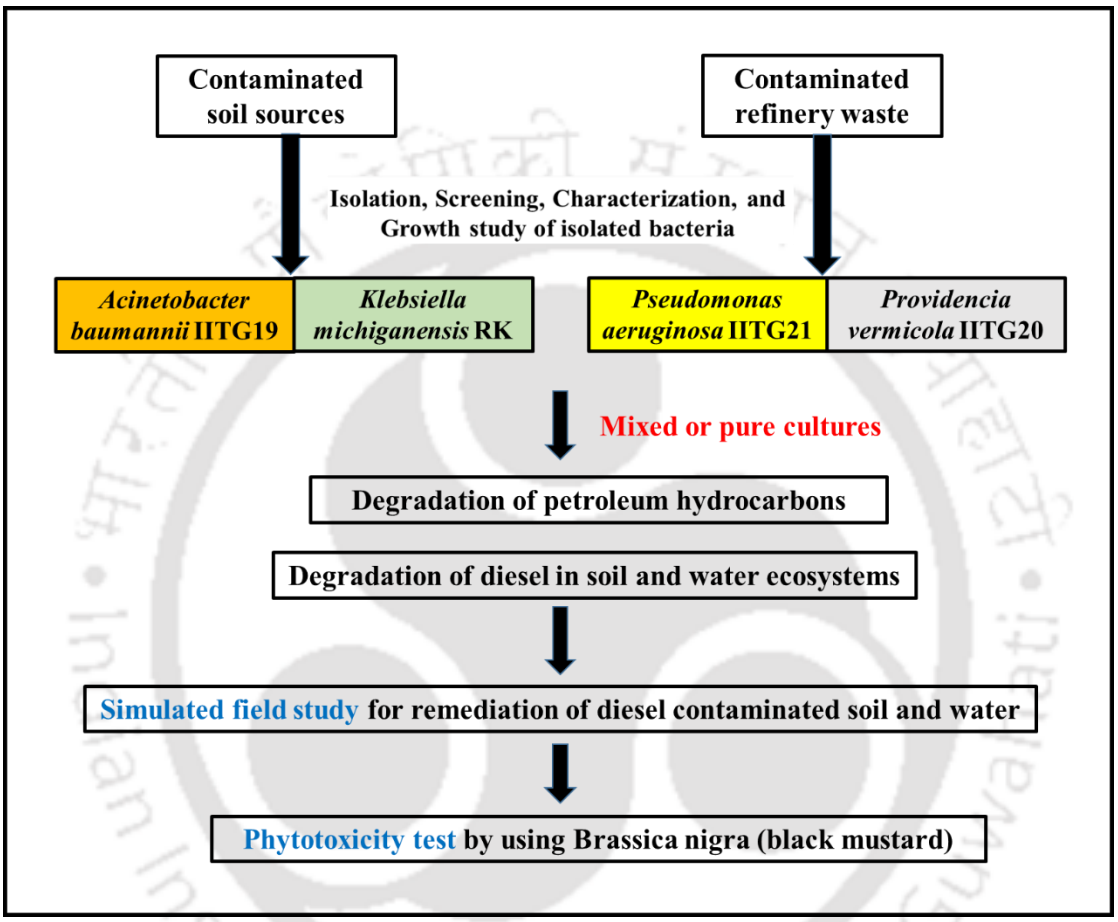
7.4 Summary

In this study, a mixed culture of bacterial strains, including *Acinetobacter baumannii* IITG19, *Pseudomonas aeruginosa* IITG21, *Klebsiella michiganensis* RK, and *Providencia vermicola* IITG20, was used for diesel degradation. In-lab experiments highlighted the superior efficacy of the mixed culture, showing enhanced diesel degradation compared to the pure cultures. During in-lab experiments, the mixed culture showed 90% degradation of 4% v/v diesel over 15 days, with 35°C temperature, pH 7, and 1% v/v inoculum concentration. Subsequent field

studies reveal significant overall diesel degradation by mixed culture in soil (96%) and water (99%). By using mixed culture, over 97% degradation of n-alkanes and branched alkanes, and more than 96% degradation of naphthenes and aromatics were observed in soil. In water, over 99% degradation was observed for all type of components. Phytotoxicity assessments using *Brassica nigra* seeds revealed that contamination caused growth inhibition, while successful growth was recorded in mixed culture-treated soil and water due to remediation. Analysis of the degradation kinetics confirmed first-order degradation with a rate constant (k) of 0.144 day^{-1} in controlled in-lab conditions. Field studies yielded values of 0.196 day^{-1} for soil and 0.288 day^{-1} for water. Mixed culture showed half-lives of 4.7, 3.5, and 2.4 days for in-lab, soil field and water field conditions, respectively. This study showed the potential of the mixed bacterial culture developed in this work for mitigation of diesel contamination by integrating simulated field studies and phytotoxicity tests. This research bridges laboratory experiments with real-world applications, paving the way for effective environmental solutions. The findings confirmed the ecological compatibility of treated soil and water, and endorsed the use of a mixed culture-based approach for bioremediation of diesel-contaminated site, especially at high diesel concentrations. The mixed culture consisting of *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21, and *Klebsiella michiganensis* RK offers an effective solution for diesel contamination mitigation in both soil and water, enabling the reuse of treated soil and water resources.

Chapter 8

Conclusions and Recommendations



This chapter summarizes the major findings of the study and suggests recommendations for future work.

Chapter 8: Conclusions and Recommendations

8.1 Conclusions

This study successfully isolated four new highly effective bacterial strains: *Acinetobacter baumannii IITG19*, *Providencia vermicola IITG20*, *Pseudomonas aeruginosa IITG21*, and *Klebsiella michiganensis RK*. The isolated bacterial strains and their mixed culture showed promising potential for addressing environmental challenges associated with petroleum hydrocarbon contamination in both terrestrial and aquatic ecosystems. A mixed culture of these strains achieved almost 100% degradation of diesel in the simulated field study in 15 days at concentration level of 4% v/v . The mixed culture of these strains can be effectively used for the remediation of diesel contamination both in soil and water ecosystems. The treated water and soil can be further utilized successfully for growing agricultural plants. It is noteworthy that *Klebsiella michiganensis RK* is reported for the first time for hydrocarbon degradation applications. The major findings from study includes:

1. *Klebsiella michiganensis* RK and Acineto*bacter baumannii* IITG19, derived from oil-contaminated soil, showcased remarkable efficiency in the degradation of diverse hydrocarbons, including diesel, kerosene, engine oil, and light paraffin oil. Notably, *Acinetobacter baumannii* IITG19 demonstrated superior degradability, particularly growing under optimal conditions at 35°C, pH 7, and a 1% v/v inoculum concentration. After a 15 day incubation period, both *Klebsiella michiganensis* RK and *Acinetobacter baumannii* IITG19 showed diesel degradation of 60% and 76%, respectively.

2. Strains, *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20, isolated from a refinery effluent treatment plant, showed ability in degrading different petroleum hydrocarbons, such as diesel, kerosene, and engine oil. *Pseudomonas aeruginosa* IITG21 showed a diesel degradation of 70%, while *Providencia vermicola* IITG20 showed 76% degradation during the 15-day incubation period under optimal conditions. The introduction of a mixed culture comprising *Pseudomonas aeruginosa* IITG21 and *Providencia vermicola* IITG20 yielded synergistic effects, enhancing degradation 85%.

3. *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20, both individually and as mixed culture, showcased notable effectiveness in diverse ecosystems, including soil, marine water, and freshwater. Freshwater ecosystems exhibited the highest degradation rates, emphasizing the impact of environmental conditions. The highest degradation in freshwater, as compared to soil and marine water, is attributed to the compact structure of soil, which

reduces the bioavailability of diesel. In marine water, the presence of salinity decreases the solubility of hydrocarbons, resulting in lower degradation compared to freshwater. *Acinetobacter baumannii* IITG19 showed degradation of 59, 62, and 76% in soil, marine water, and freshwater, respectively. *Providencia vermicola* IITG20 exhibited degradation of 31%, 57%, and 69% in the same ecosystems. In comparison, the mixed culture demonstrated superior performance, achieving overall degradation percentages of 66, 66, and 81% in the soil, marine water, and freshwater ecosystems, respectively. Mixed culture also showed higher degradation rate constant of 0.11 day^{-1} in freshwater. The higher effectiveness of mixed culture suggested that *Acinetobacter baumannii* IITG19 and *Providencia vermicola* IITG20 can synergise to give better results.

4. The mixed culture, comprising *Acinetobacter baumannii* IITG19, *Pseudomonas aeruginosa* IITG21, *Klebsiella michiganensis* RK, and *Providencia vermicola* IITG20, demonstrated superior diesel degradation during in-lab experiments, achieving an impressive 90% degradation of 4% v/v diesel over 15 days. All four bacteria showed specialized preference in degrading specific diesel components. *Acinetobacter baumannii* IITG19 showed better performance in degrading branched alkanes (94%) and n-alkanes (88%). *Pseudomonas aeruginosa* IITG21 excelled in naphthenes degradation (84%), while *Providencia vermicola* IITG20 showed the highest efficiency in aromatics degradation (87%) among the strains. *Klebsiella michiganensis* RK was second in n-alkane degradation at 85% but showed moderate efficiency (70-80%) for other hydrocarbon types. The collective preference of these strains for different hydrocarbon types makes them ideal for use in a mixed culture, resulting in the highest overall degradation of diesel by mixed culture as compared to pure culture. The rate constants were 0.144 day^{-1} in lab conditions, 0.196 day^{-1} in soil field study, and 0.288 day^{-1} in water field study.

5. The mixed culture, of *Acinetobacter baumannii* IITG19, *Providencia vermicola* IITG20, *Pseudomonas aeruginosa* IITG21, and *Klebsiella michiganensis* RK, also proved to be a promising solution for diesel contamination in both simulated soil and simulated water field study. Mixed culture showed over 97% degradation of individual compounds found in diesel contaminated soil and 99% degradation of individual compounds in diesel contaminated water in 15 day period with a 4% v/v diesel concentration. The overall degradation of diesel by mixed culture was found to be 96% in simulated soil field study and 99% in simulated water field

study in 15 days. The first-order degradation kinetics analysis revealed a rate constant (k) of 0.196 day^{-1} for soil and 0.288 day^{-1} for water, indicating efficient degradation in field studies.
6. Phytotoxicity study on *Brassica nigra* seed growth in various conditions showed the usability of mixed bacterial culture in remediating diesel-contaminated soil and water. Contaminated soil and water showed no seed germination due to diesel toxicity. while, mixed culture-treated soil and water exhibited positive growth with 68% and 70% germination rates, respectively, indicating the successful removal of diesel toxicity. The growth of *Brassica nigra* seeds in mixed culture-treated water and soil was comparable to uncontaminated soil and water, showing the potential for reusing treated soil and water for agricultural purposes.

8.2 Recommendations

In continuation with the present study, several additional investigations can be conducted in the next phase.

- The bacterial mixed culture can be utilized for **integrated remediation strategies**, including its combination with phytoremediation. Studying plant-microbe interactions, specifically rhizoremediation, where root exudates stimulate microbial activity, could yield synergistic effects that enhance hydrocarbon degradation in soil and foster vegetation growth.
- **Pilot-scale studies** or scale-up studies using the developed bacterial mixed culture can be performed on a larger scale to validate the effectiveness of the mixed culture. For this study, industrial bioreactors and assessments at actual contaminated sites can be conducted for real-world applicability. Field-scale tests across different contaminated sites would validate the culture's performance under varied environmental conditions, such as different soil compositions or contamination levels.
- **Genomic, metabolomic, and enzymatic studies** can be conducted to investigate the genetic basis, metabolic pathways, and enzymatic mechanisms involved in hydrocarbon degradation by isolated bacterial strains. Genomic analysis can identify the genes responsible for producing enzymes and proteins essential for degradation. Metabolomic studies can provide insights into the intermediate compounds formed during hydrocarbon degradation, showing the step-by-step pathways utilized by the bacteria. Enzymatic studies can examine key enzymes to understand their roles and

explore potential enhancements for improving bacterial efficiency in remediating hydrocarbon contaminated environments.

- A comprehensive **techno-economic analysis** can be conducted to evaluate the feasibility and cost-effectiveness of implementing the developed mixed culture. This can include the assessment of operational parameters such as culture preparation, transportation, and application methods. Factors like scalability, resource consumption (e.g., nutrients, water, and energy), and potential environmental impacts can also be considered. Additionally, the analysis can encompass cost estimations for large-scale deployment, including capital and operational expenses, and compare these with the financial benefits of mitigating contamination.
- Long-term **environmental monitoring** can also be performed to track the effects of hydrocarbon degradation on soil and water ecosystems. This can include evaluating residual hydrocarbon, changes in soil and water quality parameters, and the potential accumulation of by-products. Additionally, the impact of inoculated bacteria on native microbial communities, nutrient cycles, and overall ecosystem health can be assessed.

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Appendices

Table A1: Major compounds (Concentration higher than 0.5%) present in diesel.

Hydrocarbon name	Molecular formula	Retention time (min)	Conc. %
Paraffins (n-alkanes)			
1-fluorododecane	$C_{12}H_{25}F$	8.794	2.27
dodecane	$C_{12}H_{26}$	13.26	1.22
pentadecane	$C_{15}H_{32}$	10.935	2.54
heptadecane	$C_{17}H_{36}$	15.246	3.15
nonadecane	$C_{19}H_{40}$	13.12	2.18
icosane	$C_{20}H_{42}$	21.009	2.62
henicosane	$C_{21}H_{44}$	19.203	2.94
tricosane	$C_{23}H_{48}$	35.259	0.86
pentacosane	$C_{25}H_{52}$	24.995	2.57
hexacosane	$C_{26}H_{54}$	32.348	2.12
Paraffins (Branched Alkane)			
4,5-dimethylnonane	$C_{11}H_{24}$	17.277	2.76
4,6-Dimethyldodecane	$C_{14}H_{30}$	16.222	0.71
2,6,11-trimethyldodecane	$C_{15}H_{32}$	21.079	0.80
5,6-dipropyldecane	$C_{16}H_{34}$	14.176	0.94
2,6,10,15-tetramethylheptadecane	$C_{21}H_{44}$	20.338	0.63
7,7-diethylheptadecane	$C_{21}H_{44}$	22.874	2.66
5-ethyl-5-methylnonadecane	$C_{22}H_{46}$	20.288	2.13
2,6,10,14-tetramethyloctadecane	$C_{22}H_{46}$	25.095	5.93
2,6,10,14-tetramethylnonadecane	$C_{23}H_{48}$	23.83	1.64
(Z)-5-methyldocos-5-ene	$C_{23}H_{46}$	22.274	0.54
11-methyltricosane	$C_{24}H_{50}$	26.871	2.32
2,6,10,14-tetramethyl-7-(3methylpentyl) pentadecane	$C_{25}H_{52}$	18.723	1.06
11-methylpentacosane	$C_{26}H_{54}$	31.218	2.32
2-methylhexacosane	$C_{27}H_{56}$	26.986	1.02
2-methylheptacosane	$C_{28}H_{58}$	33.374	1.75
13-methylheptacosane	$C_{28}H_{58}$	29.952	2.40
1-iodooctacosane	$C_{28}H_{57}I$	28.512	2.46
2-methyloctacosane	$C_{29}H_{60}$	34.319	1.23
Naphthenes (cycloalkanes or cycloparaffins)			
1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	$C_{10}H_{18}$	12.57	1.06
1-pentylcyclohexene	$C_{11}H_{20}$	13.786	1.37
methylcyclodecane	$C_{11}H_{22}$	18.468	0.81
heptylcyclohexane	$C_{13}H_{26}$	9.614	1.37
1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene	$C_{15}H_{24}O$	12.29	1.0
tridecan-6-ylcyclohexane	$C_{19}H_{38}$	22.139	0.84
Aromatics			
2,6-dimethyl-1,2,3,4-tetrahydronaphthalene	$C_{12}H_{16}$	17.992	0.89

Table A2: Compounds detected in final hydrocarbon mixtures after 15 days of incubation period during degradation of diesel in different ecosystems (Chapter 6).

name	Molecular formula	Detected Compounds (%)								
		Acinetobacter baumanni IITG19			*Providencia vermicola* IITG20			Mixed culture		
		Soil	*marine water*	*fresh water*	*soil*	*marine water*	*fresh water*	Soil	*marine water*	fresh water
Primary Alcohol										
2,2-Dimethyl-1-propanol	$C_5H_{12}O$	0.610	0.907	0.617	0.748	0.558	0.560	2.287	0.138	0.311
2,4,4-Trimethyl-1-pentanol	$C_8H_{18}O$	0.445	0.932	0.442	0.809	0.474	0.424	0.183	0.955	1.601
1-Hexanol, 5-methyl-2-(1-methylethyl)-	$C_{10}H_{22}O$	0.824	1.019	0.754	0.660	1.126	0.610	0.155	0.251	0.679
2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$	0.747	0.441	0.589	1.376	0.635	0.845	0.086	0.306	0.731
1-Dodecanol	$C_{12}H_{26}O$	0.620	0.506	0.814	0.732	0.511	0.575	0.125	0.297	0.558
2-Butyl-1-octanol	$C_{12}H_{26}O$	0.824	0.943	0.583	0.839	1.896	3.048	0.204	0.214	0.901
1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	1.855	0.386	0.454	1.220	0.442	1.833	0.491	0.180	1.926
Secondary alcohol										
1-Penten-3-ol, 4-methyl-	$C_6H_{12}O$	0.445	0.932	0.442	0.809	0.474	0.424	0.183	0.955	1.601
2-Octen-1-ol, 3,7-dimethyl-	$C_{10}H_{20}O$	1.912	0.553	0.581	1.121	0.616	0.881	0.062	0.154	0.556
Ester										
Carbonic acid, nonyl vinyl ester	$C_{12}H_{22}O_3$	1.880	0.536	0.457	0.985	0.566	1.310	0.077	0.821	0.401
Methoxyacetic acid, 2-tetradecyl ester	$C_{12}H_{22}O_2$	0.611	0.720	0.551	1.542	0.647	0.657	0.098	0.267	0.883
Carbonic acid, dodecyl vinyl ester	$C_{15}H_{28}O_3$	0.688	1.420	2.443	0.790	1.454	3.426	0.317	0.207	0.745
Succinic acid, di(dec-4-enyl) ester	$C_{15}H_{26}O_4$	0.616	0.535	0.430	1.268	1.306	0.681	0.180	0.922	1.545
Methoxyacetic acid, 2-tridecyl ester	$C_{16}H_{32}O_3$	0.653	0.647	0.722	1.134	0.881	0.573	0.122	0.122	0.514
Carbonic acid, prop-1-en-2-yltridecyl ester	$C_{17}H_{32}O_3$	1.115	2.015	1.045	0.871	0.432	0.611	0.087	0.223	0.316
Sulfurous acid, 2-propyl tetradecyl ester	$C_{17}H_{36}O_3S$	0.418	0.510	0.817	0.688	0.527	0.387	0.085	1.511	1.987
Sulfurous acid, pentadecyl 2-propy l ester	$C_{18}H_{38}O_3S$	0.550	0.407	1.614	1.185	0.818	0.527	0.086	0.176	0.466
Carbonic acid, 2-ethylhexyl nonyl ester	$C_{18}H_{36}O_3$	1.115	2.015	1.045	0.871	0.432	0.611	0.223	0.223	0.316
Carbonic acid, decyl 2-ethylhexyl ester	$C_{19}H_{38}O_3$	1.079	0.812	0.421	1.428	0.520	0.627	0.065	0.186	0.717
Carbonic acid, 2-ethylhexyl undecyl ester	$C_{20}H_{40}O_3$	1.284	1.993	0.535	1.094	2.335	1.284	0.206	0.190	0.346
Oxalic acid, cyclobutyl pentadecyl ester	$C_{21}H_{38}O_4$	1.850	0.598	0.561	1.124	0.453	0.570	0.458	0.114	0.471
Carbonic acid, decyl undecyl ester	$C_{22}H_{44}O_3$	0.542	0.708	0.787	0.694	1.173	0.707	0.393	0.140	0.328
Carbonic acid, eicosyl vinyl ester	$C_{23}H_{44}O_3$	0.616	0.535	0.430	1.268	1.306	0.681	0.180	0.922	1.545
Ketone										

5-Hydroxy-4-methyl-6-hepten-3-one	$C_8H_{14}O_2$	0.610	0.907	0.617	0.748	0.558	0.560	0.287	0.138	0.311
2-Methyl-2-hepten-4-one	$C_8H_{14}O$	0.740	1.007	2.143	1.305	0.466	1.385	0.211	0.126	1.640
4,6-Dimethyloctane-3,5-dione	$C_{10}H_{18}O_2$	0.445	0.932	0.442	0.809	0.474	0.424	0.183	0.955	1.601
2-Pentanone, 4-cyclohexylidene-3,3 -diethyl	$C_{11}H_{18}O$	0.699	0.429	0.768	1.571	0.556	2.104	0.072	0.387	0.772
Acetate										
2-Furanmethanol, acetate	$C_7H_8O_3$	0.814	0.800	0.440	1.954	0.456	0.898	0.204	0.214	0.794
Hexacosyl acetate	$C_{28}H_{56}O_2$	0.731	1.663	2.532	0.598	0.445	2.311	0.249	0.130	0.629
CARBOXYLIC ACID										
1,3-butadiene-1-carboxylic acid	$C_5H_6O_2$	0.814	0.800	0.440	1.954	0.456	0.317	0.204	0.214	0.794
Methylenecyclopropanecarboxylic acid	$C_5H_8O_2$	0.551	0.394	1.118	0.601	0.445	0.807	0.458	0.114	0.753
Cycloalkanol										
2-Furanmethanol	C_5H_6O	0.814	0.800	0.440	1.954	0.456	0.898	0.204	0.214	0.794
1,4,2,5 cyclohexanetetrol	$C_6H_{12}O_4$	0.610	0.907	0.617	0.748	0.558	0.560	2.287	0.1378	0.311
Cyclohexanepropanol-	$C_9H_{18}O$	0.963	1.076	0.650	0.712	0.445	0.403	0.072	0.3867	1.519
1-cyclopentene-1-methanol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{18}O$	0.551	0.394	1.118	0.601	0.445	0.400	0.354	0.1968	0.753
Cycloalkanone										
Spiro[3.3]heptane-2,6-dione	$C_7H_8O_2$	0.963	1.076	0.650	0.712	0.445	0.403	0.512	0.862	1.519
1-cyclopenten-3-one, 1-(ethoxycarbonyloxy)-	$C_8H_{10}O_4$	1.222	1.678	0.701	1.011	0.709	0.812	0.137	0.108	0.660
3-methyl-4,5,6,6a-tetrahydro-1h-pentalen-2-one	$C_9H_{12}O$	0.407	0.518	0.569	0.552	0.549	0.638	0.491	0.180	1.386
6-methyl-bicyclo[4.2.0]octan-7-one	$C_9H_{14}O$	0.814	0.800	0.440	1.954	0.456	0.898	0.072	0.387	0.794
Cyclopentanone, 2-(1-methylpropyl)-	$C_9H_{16}O$	0.883	0.718	0.483	1.876	0.642	0.694	0.098	0.267	0.536
Bicyclo[4.1.0]heptan-3-one, 4,7,7-trimethyl-, [1r-(1.alpha.,4.alpha.,6.alpha	$C_{10}H_{16}O$	2.440	1.823	0.690	0.725	0.736	0.443	0.861	0.204	0.426

Table A3. Calculations used for degradation kinetics (Chapter 6)

	Acinetobacter baumannii IITG19				*Providencia vermicola IITG20*				Mixed culture			
Time (day)	**Degradation of diesel (%)**	**Consumed diesel (mL)**	**Remaining diesel (mL)**	**ln(C_0/ C)**	**Degradation of diesel (%)**	**Consumed diesel (mL)**	**Remaining diesel (mL)**	**ln(C_0/ C)**	**Degradation of diesel (%)**	**Consumed diesel (mL)**	**Remaining diesel (mL)**	**ln(C_0/ C)**
Soil Ecosystem												
0	0.00	0.00	4.00	0.00	0.00	0.00	4.00	0.00	0.00	0.00	4.00	0.00
2	7.00	0.28	3.72	0.07	5.25	0.21	3.79	0.05	12.98	0.52	3.48	0.14
4	18.35	0.73	3.27	0.20	8.69	0.35	3.65	0.09	28.33	1.13	2.87	0.33
6	28.11	1.12	2.88	0.33	15.62	0.62	3.38	0.17	31.00	1.24	2.76	0.37
8	36.60	1.46	2.54	0.46	21.13	0.85	3.16	0.24	40.00	1.60	2.40	0.51
10	40.00	1.60	2.40	0.51	21.44	0.86	3.14	0.24	41.00	1.64	2.36	0.53
12	48.89	1.96	2.04	0.67	23.25	0.93	3.07	0.26	52.00	2.08	1.92	0.73
14	56.60	2.26	1.74	0.83	31.25	1.25	2.75	0.37	62.00	2.48	1.52	0.97
15	59.00	2.36	1.64	0.89	31.31	1.25	2.75	0.38	65.69	2.63	1.37	1.07
Marine water Ecosystem												
0	0.00	0.00	4.00	0.00	0.00	0.00	4.00	0.00	0.00	0.00	4.00	0.00
2	14.00	0.56	3.44	0.15	4.00	0.16	3.84	0.04	16.88	0.68	3.33	0.18
4	21.87	0.87	3.13	0.25	17.00	0.68	3.32	0.19	27.00	1.08	2.92	0.31
6	31.31	1.25	2.75	0.38	25.71	1.03	2.97	0.30	39.38	1.58	2.43	0.50
8	48.88	1.96	2.05	0.67	32.37	1.29	2.71	0.39	53.00	2.12	1.88	0.76
10	52.25	2.09	1.91	0.74	36.73	1.47	2.53	0.46	58.62	2.35	1.66	0.88
12	53.44	2.14	1.86	0.76	40.12	1.60	2.40	0.51	62.13	2.49	1.52	0.97
14	57.44	2.30	1.70	0.85	49.95	2.00	2.00	0.69	63.00	2.52	1.48	0.99
15	61.83	2.47	1.53	0.96	56.80	2.27	1.73	0.84	66.00	2.64	1.36	1.08

Freshwater Ecosystem												
0	0.00	0.00	4.00	0.00	0.00	0.00	4.00	0.00	0.00	0.00	4.00	0.00
2	13.82	0.55	3.45	0.15	13.35	0.53	3.47	0.14	16.02	0.64	3.36	0.17
4	25.34	1.01	2.99	0.29	19.18	0.77	3.23	0.21	29.30	1.17	2.83	0.35
6	36.41	1.46	2.54	0.45	34.00	1.36	2.64	0.42	45.80	1.83	2.17	0.61
8	52.68	2.11	1.89	0.75	48.00	1.92	2.08	0.65	59.50	2.38	1.62	0.90
10	61.87	2.47	1.53	0.96	59.00	2.36	1.64	0.89	66.78	2.67	1.33	1.10
12	68.84	2.75	1.25	1.17	66.00	2.64	1.36	1.08	74.53	2.98	1.02	1.37
14	73.35	2.93	1.07	1.32	68.24	2.73	1.27	1.15	78.24	3.13	0.87	1.53
15	76.45	3.06	0.94	1.45	69.00	2.76	1.24	1.17	80.94	3.24	0.76	1.66

Table A4. Prominent compounds degraded during simulated field study of diesel contaminated soil and water by mixed culture (Chapter 7).

Hydrocarbon name	Molecular formula	Degradation %	
		Soil	Water
Paraffins (n-alkanes)			
1-fluorododecane	$C_{12}H_{25}F$	95.90	99.93
dodecane	$C_{12}H_{26}$	93.08	99.60
pentadecane	$C_{15}H_{32}$	97.80	99.90
heptadecane	$C_{17}H_{36}$	98.21	99.91
nonadecane	$C_{19}H_{40}$	94.74	99.84
icosane	$C_{20}H_{42}$	93.20	99.94
henicosane	$C_{21}H_{44}$	96.19	99.91
tricosane	$C_{23}H_{48}$	97.54	99.91
pentacosane	$C_{25}H_{52}$	98.54	98.94
hexacosane	$C_{26}H_{54}$	98.90	99.39
Paraffins (Branched Alkane)			
4,5-dimethylnonane	$C_{11}H_{24}$	96.99	99.90
4,6-Dimethyldodecane	$C_{14}H_{30}$	90.30	99.92
2,6,11-trimethyldodecane	$C_{15}H_{32}$	99.37	99.99
5,6-dipropyldecane	$C_{16}H_{34}$	97.48	99.35
2,6,10,15-tetramethylheptadecane	$C_{21}H_{44}$	89.93	99.87
7,7-diethylheptadecane	$C_{21}H_{44}$	94.40	98.86
5-ethyl-5-methylnonadecane	$C_{22}H_{46}$	98.61	99.24
2,6,10,14-tetramethyloctadecane	$C_{22}H_{46}$	91.15	99.71
2,6,10,14-tetramethylnonadecane	$C_{23}H_{48}$	93.51	99.92
(Z)-5-methyldocos-5-ene	$C_{23}H_{46}$	94.99	99.89
11-methyltricosane	$C_{24}H_{50}$	96.76	99.95
2,6,10,14-tetramethyl-7-(3methylpentyl) pentadecane	$C_{25}H_{52}$	95.04	99.88
11-methylpentacosane	$C_{26}H_{54}$	88.05	99.94
2-methylhexacosane	$C_{27}H_{56}$	98.94	99.25
2-methylheptacosane	$C_{28}H_{58}$	98.85	99.20
13-methylheptacosane	$C_{28}H_{58}$	98.23	99.66
1-iodooctacosane	$C_{28}H_{57}I$	98.20	99.11
2-methyloctacosane	$C_{29}H_{60}$	98.30	99.81
Naphthenes (cycloalkanes or cycloparaffins)			
1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalene	$C_{10}H_{18}$	97.16	99.86
1-pentylcyclohexene	$C_{11}H_{20}$	94.08	99.26
methylcyclodecane	$C_{11}H_{22}$	93.70	99.56
heptylcyclohexane	$C_{13}H_{26}$	96.35	99.92
1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene	$C_{15}H_{24}O$	95.90	99.93
tridecan-6-ylcyclohexane	$C_{19}H_{38}$	98.23	99.87
Aromatics			
2,6-dimethyl-1,2,3,4-tetrahydronaphthalene	$C_{12}H_{16}$	96.96	99.65

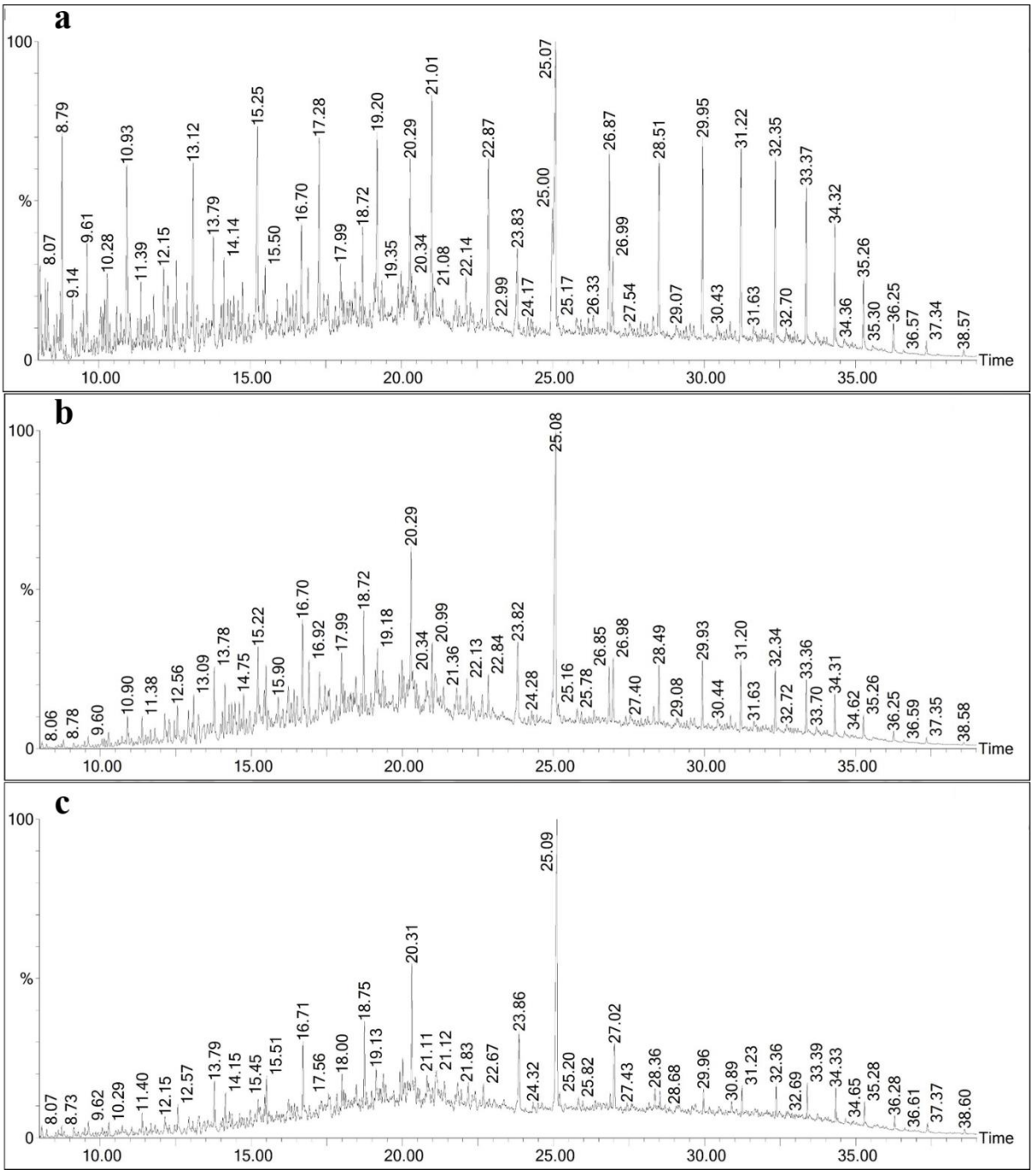

Fig A1: GCMS spectra for, a) feed containing 4% v/v diesel, b) end mixture after 15 days of incubation with *Klebsiella michiganensis* RK, c) end mixture after 15 days of incubation with *Acinetobacter baumannii* IITG19 (Chapter 4).

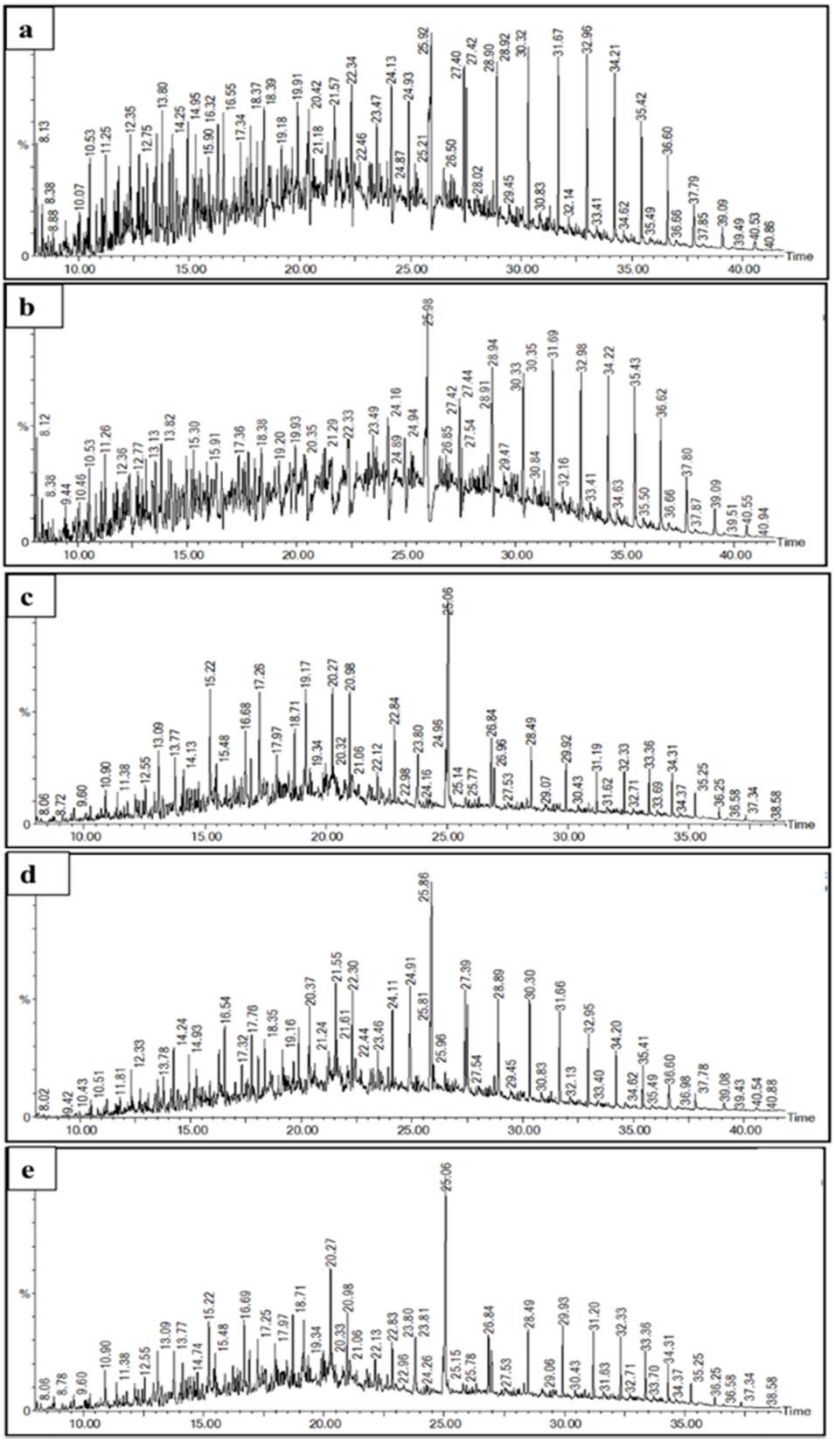

Fig. A2. a) GCMS spectra of Feed diesel; GCMS spectra of final mixture b) in control sample, c) in presence of *Pseudomonas aeruginosa* IITG21, d) in presence of *Providencia vermicola* IITG20, e) in presence of mixed culture (Chapter 5).

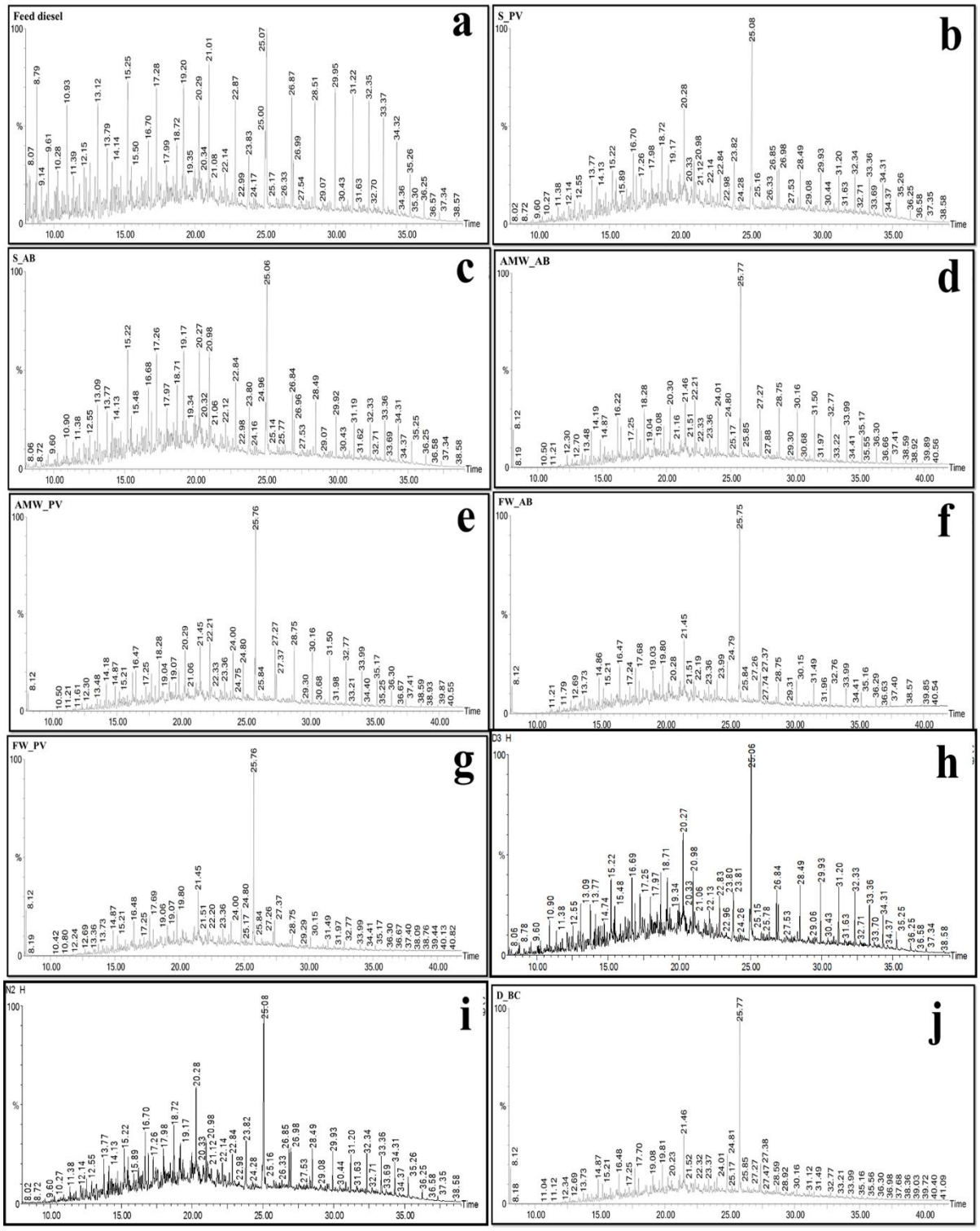

Fig. A3: GCMS profile for a) feed diesel, b) components of *Acinetobacter baumannii* IITG19 treated soil sample, c) components of *Providencia vermicola* IITG20 treated soil sample, d) components of *Acinetobacter baumannii* IITG19 treated marine water, e) components of *Providencia vermicola* IITG20 treated marine water, f) components of *Acinetobacter baumannii* IITG19 treated fresh water sample, g) components of *Providencia vermicola* IITG20 treated fresh water, h) components of mixed culture treated soil sample, i) components of mixed culture treated marine water, j) components of mixed culture treated freshwater (Chapter 6).

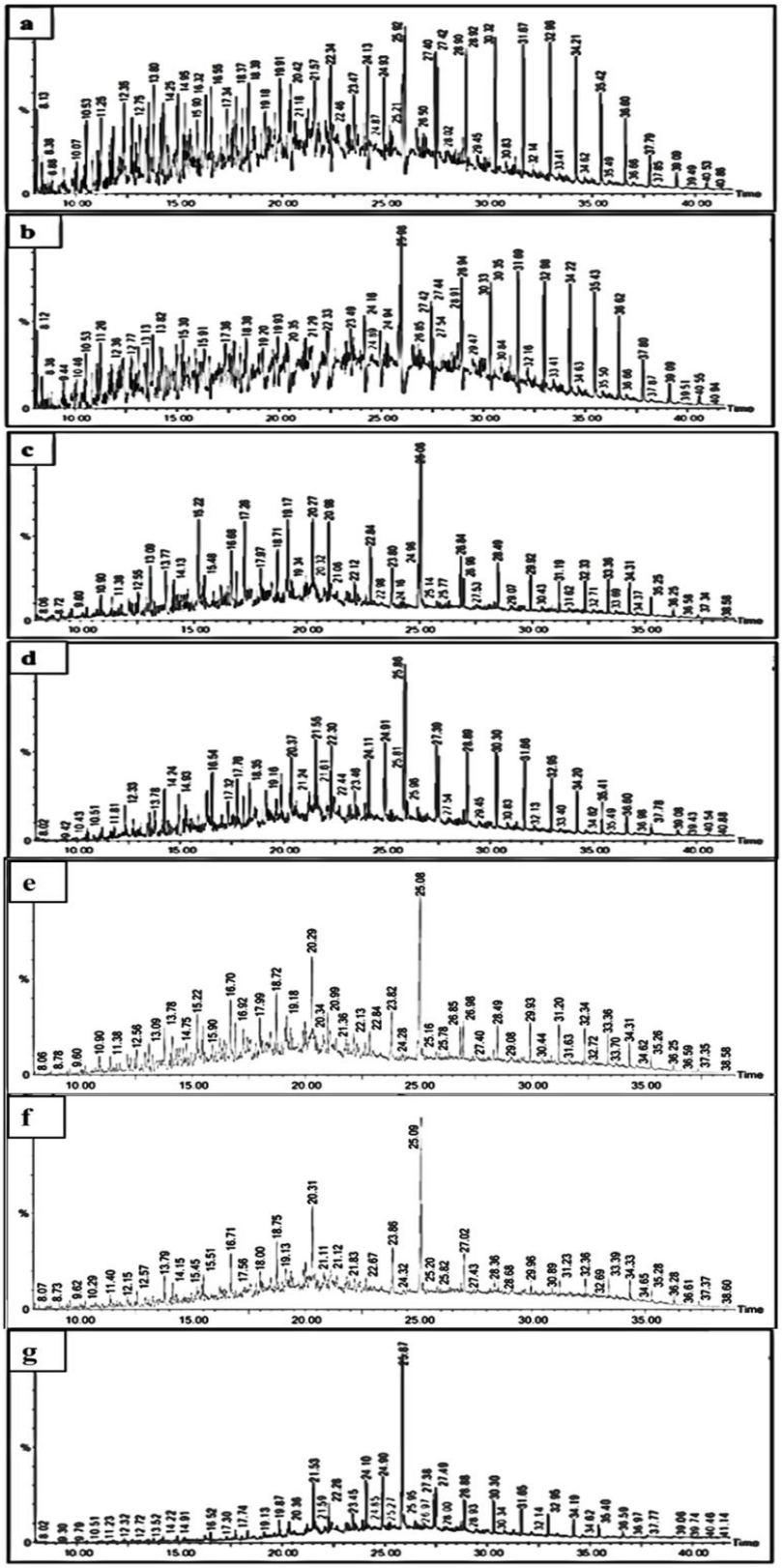

Fig. A4. GCMS spectra of In-Lab experiment a) Feed diesel, b) product of control sample, c) product of *Klebsiella michiganensis* RK, d) product of *Pseudomonas aeruginosa* IITG21, e) product of *Acinetobacter baumannii* IITG19, f) product of *Providencia vermicola* IITG20 and g) product of mixed culture (Chapter 7).

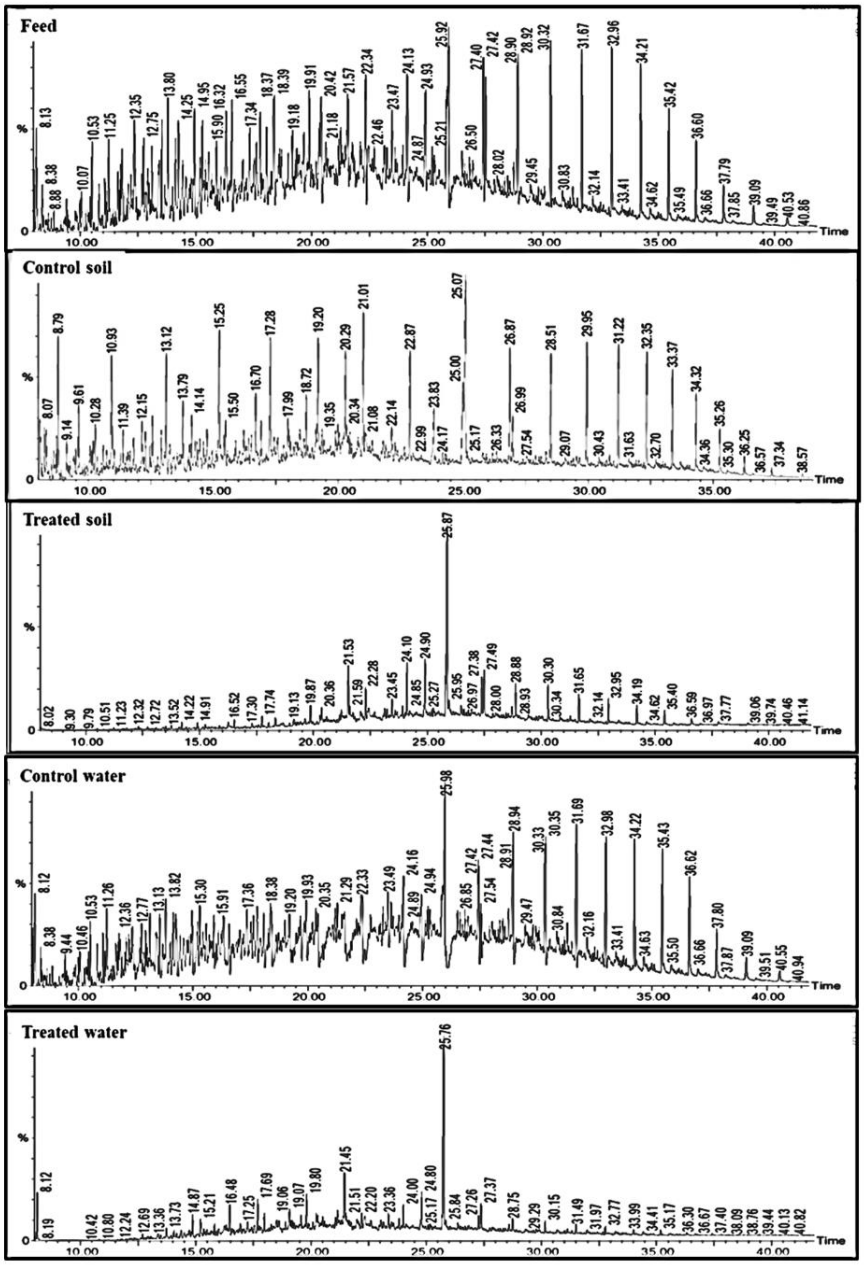

Fig. A5. GCMS spectra of Field Study (Chapter 7).