Isolation and Identification of Bacteria Associated with Petroleum-Based Hydrocarbons from Soil

Dissertation submitted in partial fulfilment of the requirements for the degree of

Master of Science

in

Biotechnology

by

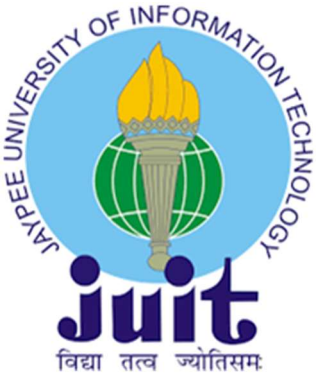
Navdeep Sharda (235111012)

Under the supervision

of

Dr. Jitendraa Vashistt

to



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May, 2025

CANDIDATE'S DECLARATION

I hereby declare that the work presented in this report entitled "**Isolation and Identification of Bacteria Associated with Petroleum-Based Hydrocarbons from Soil"** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biotechnology** submitted in the **Department of Biotechnology & Bioinformatics**, Jaypee University of Information Technology, Waknaghat is an authentic record of my work carried out over a period from August 2024 to June 2025 under the supervision of **Dr. Jitendraa Vashistt** Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.

I also confirm that I have carried out the above-mentioned project work under the proficiency stream.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the work reported in the M.Sc. Microbiology thesis entitled "**Isolation and Identification of Bacteria Associated with Petroleum-Based Hydrocarbons from Soil"** which is being submitted by **Mr. Navdeep Sharda (235111012)** in fulfilment for the award of Masters of Science in Biotechnology by the Jaypee University of Information Technology, is the record of candidate's work carried out by him under my supervision. This work is original and has not been submitted partially or fully anywhere else for any other degree or diploma.

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List of Abbreviations

TPH	Total Petroleum Hydrocarbons
PAH	Polycyclic Aromatic Hydrocarbon
DNA	Deoxyribonucleic acid
(°C)	Degree Celsius
BTEX	Benzene, Toluene, Ethylbenzene (E) and p-Xylene
Approx.	approximately
ppm	Parts Per Million
RNA	Ribonucleic acid
s.g	specific gravity
MAH	Monocyclic aromatic hydrocarbons
bbl	*Barrel*
ITOPE	*The International Tanker Owners Pollution Federation*
IHR	Indian Himalayan region
kJ	Kilojoule
g, gms	gram, grams
Et al.	*and others*
sp.	Species
TCA	Tricarboxylic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
acetyl-CoA	Acetyl-coenzyme A
β-oxidation	Beta-oxidation
Lat	Latitude
Long	Longitude
ml, mL	*millilitre*
cm	*centimetre*
Kg	*kilogram*
pH	*Potential of Hydrogen*
m^3	*cubic meter*
L, ltr.	litre
w/v	Weight per volume

v/v or vol/vol	Volume per volume
psi	Pounds per square inch
r.p.m, rpm	Revolutions per minute
BHB	Bushnell Haas broth
BHA	Bushnell-Haas agar
CFM	Carbon-free media
NB	Nutrient broth
NBA	Nutrient agar
DDB (1,2,3,4)	Diesel degrading bacteria (1,2,3,4)
LAF	Laminar Air Flow
KOH	Potassium hydroxide
100X	100 times
PHB	Polyhydroxybutyrate
TD	Technical data
MR test	Methyl Red test
nm	Nanometre
UV-Vis	Ultraviolet-Visible
µL	microlitre
approx..	Approximately
OD_{600}	Optical density at 600 nm
OD	Optical density
CFU	Colony-forming unit
™	Trademark
AST	Antimicrobial susceptibility testing
PCR	Polymerase Chain Reaction
-ve	Negative
+ve	Positive
C+M	Culture + Media
D+M	Diesel + Media
C+D+M	Culture + Diesel + Media
µg	microgram
mm	millimetre

S/R	Susceptible/Resistant
gDNA	Genomic DNA
MID	Molecular identification
bp	Base pair
m.w	Molecular weight
F and R primers	Forward and Reverse primers
Seq	Sequence
RT	Room temperature
i.e.	Id est, (that is)
µm	micron (micrometre)

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ABSTRACT

Fossil fuels are composed of hydrocarbons with varying composition and structures, which depend on the fuel type and source. This study investigates indigenous bacterial strains that can degrade petroleum-based hydrocarbons, specifically targeting diesel contamination in soil from the Himalayan region near JUIT, Solan. Hydrocarbon contamination is primarily caused by the anthropogenic activities such as oil spills, leakage from vehicles and machinery and industrial effluents etc., which poses significant ecological hazards. Soil sample from a petrol pump was obtained and subjected to a stepwise enrichment technique with minimal salt media supplemented with diesel as the sole carbon source. Four morphologically diverse bacterial isolates were isolated, with strain DDB2 showing significant growth and diesel-degrading capacity. Diesel utilization and diesel tolerance tests further proved its potential for bioremediation. The biochemical characterization suggested that the bacterial isolate might belong to genus *Pseudomonas*. Furthermore, molecular identification by *16S rRNA gene* sequencing was done to identify the isolated bacteria. The work emphasizes on the bioremediation capabilities of indigenous bacterial strains in petroleum contaminated soil, especially within fragile mountainous regions.

Keywords: *Himalayan region, Hydrocarbons, Diesel, Bioremediation, Degradation, Environment*

CHAPTER 1

INTRODUCTION

1.1 Introduction

The most abundant class of organic compounds in the bio-geosphere are hydrocarbons [1]. These compounds are among the most stable substances found in nature, as they can preserve their structure for very long periods. Scientists use hydrocarbon biomarkers to trace back and estimate the age of ancient life forms, including bacteria, archaea, and eukaryotes [2].

Hydrocarbons primarily consist of hydrogen and carbon, which are naturally found in crude oil. Economic activities are fundamentally dependent on fossil fuels, comprising hydrocarbons and various other substances. These energy-rich chemical compounds are formed from dead plants and animals over time by natural processes in the Earth's crust. A great amount of energy for transport, civil and industrial purposes is provided by hydrocarbons. Due to the population explosion, the global demand for energy, thus fossil fuels, is increasing rapidly, which has resulted in the establishment of more petrochemical industries, resulting in more frequent accidental oil spills, increased industry emissions and vehicle combustion, all of which are contributing to the air, water and soil pollution [3].

Petroleum is generally composed of a mixture of hydrocarbons in gaseous, liquid, and solid states, accompanied by trace quantities of various non-hydrocarbon substances that include nitrogen, oxygen, sulfur, and trace metallic elements. Further classification of hydrocarbons present in petroleum can be done into paraffins, cycloparrafins, and aromatics [4].

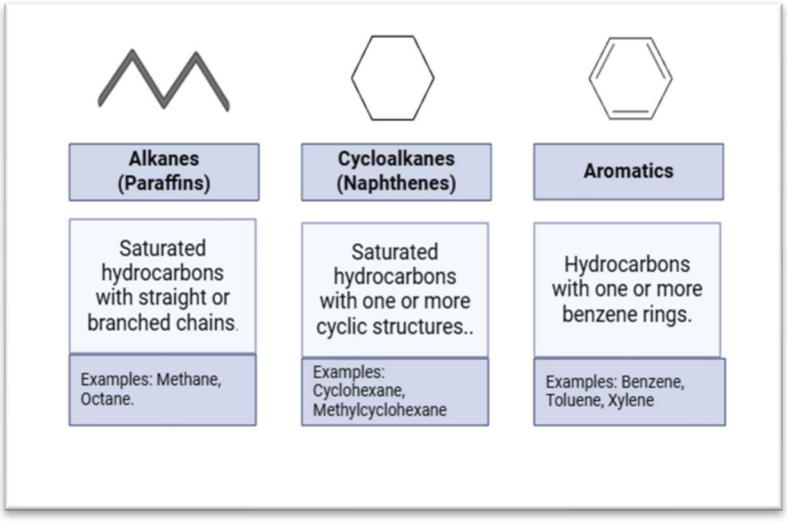

Figure 1.1: Classification of petroleum hydrocarbons based on chemical composition.

Söhngen and Kaserer in 1906, for the first time, did a study on the utilization of hydrocarbons by bacteria. Söhngen isolated a bacterium called Methanobacterium, which was later used by

Orla-Jensen in 1909 as the type culture for the genus *Methanomonas* and later renamed as *Methanomonas methanica.* A similar type of bacteria, which was methane-oxidising, was studied by Münz in 1920, and he classified this isolated microorganism as a facultative autotroph. *Bacillus hexcarbovorum* was isolated by Störmer in 1908, which was able to utilise xylol, toluene, illuminating gas, along methane. Söhngen in 1913, reported some bacteria of the genera *Mycobacterium* and *Pseudomonas* which could oxidise gasoline, kerosene, paraffin oil and paraffin wax into Carbon dioxide gas, water, along with some organic acids in trace quantities [5]. At present, a lot of hydrocarbon-degrading strains of bacteria are isolated from different places, belonging to nearly 43 bacterial genera [6]. Species within the genus Pseudomonas are important contributors to the degradation of these xenobiotics, owing to their diverse adaptive strategies, including the flexibility of carbon metabolism. This genus has been documented in nearly all locations that are contaminated with hydrocarbons [7].

This study primarily focuses on the isolation, screening, and biochemical and molecular identification of hydrocarbon-degrading microbes from a region near our university, i.e., JUIT, Solan, which is located in the outer Himalayan region. The Himalayas have a fragile ecosystem, and diesel spills from various transport activities, tourism, etc, give rise to a threat to alpine soil and water. Biodegradation of such pollutants using indigenous bacteria gives us eco-friendly options to tackle such pollution without corrupting the local soil microbiota with foreign species. Also, clean soil and water promote eco-tourism and the overall health of the masses.

1.2 Aims and Objectives

a) Aim:

- To isolate, identify and characterize hydrocarbon-utilising bacteria from petroleum-associated soil.

b) Objectives:.

- To isolate hydrocarbon-utilising bacteria from petroleum-associated soil.
- To identify the isolated bacteria using biochemical characterization and molecular characterization.

CHAPTER 2

REVIEW OF LITERATURE

2. Literature Review

2.1 Importance of hydrocarbon degradation

Oil spills frequently take place during the extraction, transport, and utilization of petroleum products. These incidents result in the discharge of several toxic organic substances known to have harmful impacts on both human health and the environment. Total petroleum hydrocarbons (TPHs), in particular, are persistent in nature, exhibiting minimal structural alteration over time and limited bioavailability, which makes their natural degradation a challenging process [8]. Total petroleum hydrocarbons (TPHs) exhibit a strong affinity for adsorption onto fine-grained soil particles, which contributes to their persistence in the environment. In the past few decades, emphasis has been placed on the development of cost-effective and practical methods aimed at minimising contaminant exposure to levels below established regulatory limits. Soil washing was one such method for remediation of contaminants from the soil by using water only, or surfactants, acid and alkali agents along with water [9].

Petroleum hydrocarbons are widely recognised for their toxic, carcinogenic, and mutagenic effects, posing significant risks to human health. When crude oil contaminates soil, it alters key physicochemical characteristics such as pH, moisture content, soil structure, and nutrient availability, ultimately reducing the soil's fertility and agricultural productivity. Moreover, these contaminants can leach into and degrade groundwater quality. Their harmful impact on plant germination and growth stems from their interference with the uptake of essential water and nutrients from the soil, leading to impaired development [10]. Polycyclic aromatic hydrocarbons (PAHs) consist of two or more interconnected benzene rings and are pervasive environmental contaminants. They are recognised for their cancer-causing, mutation-inducing, and toxic effects on living organisms, along with a range of other harmful biological impacts. Microbial activity serves as the main pathway through which these compounds are broken down in the environment [11].

So, the remediation of these pollutants is very urgent to ensure a safe environment for all life forms and the overall health of our ecosystem. Biodegradation or other bioremediation approaches which utilise hydrocarbon-degrading microbes are practical and cost-effective to be adopted for such purposes.

2.2 Type of petroleum hydrocarbons

The word petroleum is derived from two Latin words - *petra* and *oleum*, meaning rock oil [12]. Petroleum is a complex mixture of many hydrocarbon-based compounds found naturally under the sedimentary rocks in various states, like gases, liquids, solids and semi-solids and includes additional elements such as nitrogen, oxygen, and sulfur, along with small amounts of various metals. These are categorised according to their distinct physical and chemical characteristics. One such parameter is gravity, which is used to classify them as heavy or light oils, and another is odour, which is used to classify them as either containing or not containing sulfur [13].

Table 1: Proportions of elements in petroleum adopted from James G. Speight [14].

Element(s)	Typical range
Carbon	Major constituent, typically ranging between 83 to 87%
Hydrogen	Ranges from 10 to 14%
Nitrogen	Found in trace amounts, approx. 0.1 to 2.0%
Oxygen	Occurs in very small quantities, around 0.05 to 1.5%
Sulfur	Varies significantly, usually between 0.05 to 6%
Metals (Ni, V - Trace)	Detected in concentrations typically < 1000 ppm

Petroleum hydrocarbons are composed of short-chain hydrocarbons such as paraffins, alicyclics, and aromatics, along with various non-hydrocarbon substances including phenols, thiols, naphthenic acids, asphaltenes, metalloporphyrins, and nitrogen- and sulfur-containing [15].

The different boiling points of the components in crude oil are essential for separating individual hydrocarbons into distinct fractions during the refining process [16].

Via distillation, petroleum is separated into the following four major fractions [17]:

1. Straight-run gasoline having a boiling point of less than 200° Celsius (°C);
2. Middle distillate having a boiling point ranging approximately 185-135° Celsius (°C); Middle distillate is further fractioned into kerosene, heating oils, and other types of fuels for diesel, jet, gas turbine and rocket engines;

3. Wide-cut gas oil of higher boiling points ranging from 345 to 540° Celsius (°C); temperature. This fraction is subsequently refined to produce waxes, lubricating oils, and feedstock used in catalytic cracking for gasoline manufacturing.
4. Residual oil, probably asphaltic.

Petroleum comes with a diverse range of chemical and physical properties, depending upon the origin or source, ranging from liquids without any colour (majorly gasoline) to heavy, black, tar-like material high in asphalt. The specific gravity (s.g.) of petroleum ranges between 0.82-0.95 [17].

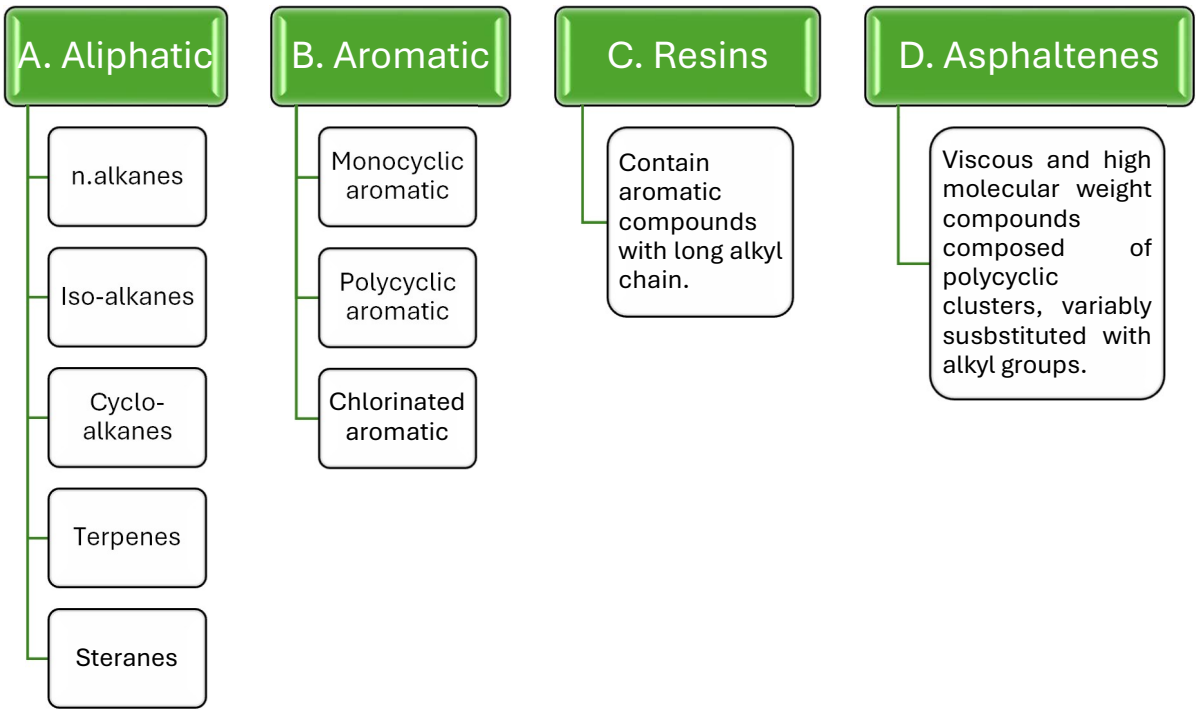

Figure 2.1: Categories of petroleum hydrocarbon compounds on a chemical basis.

Petroleum hydrocarbons are classified into four main groups: alkanes, aromatics, resins, and asphaltenes (Fig. 2.1). Among these, alkanes constitute the largest portion and are considered to be less toxic and more readily biodegradable compared to the others. Solid PAHs have high boiling points and melting points, are very less soluble, and have low vapour pressure, ranging from low and high molecular weights. As the number of rings in polycyclic aromatic hydrocarbons (PAHs) increases, their solubility in water decreases, while their solubility in organic solvents increases because of their hydrophobic nature. Hydrocarbon compounds,

asphaltenes and resins, are high in many other elements such as oxygen, nitrogen, and sulphur. [18]

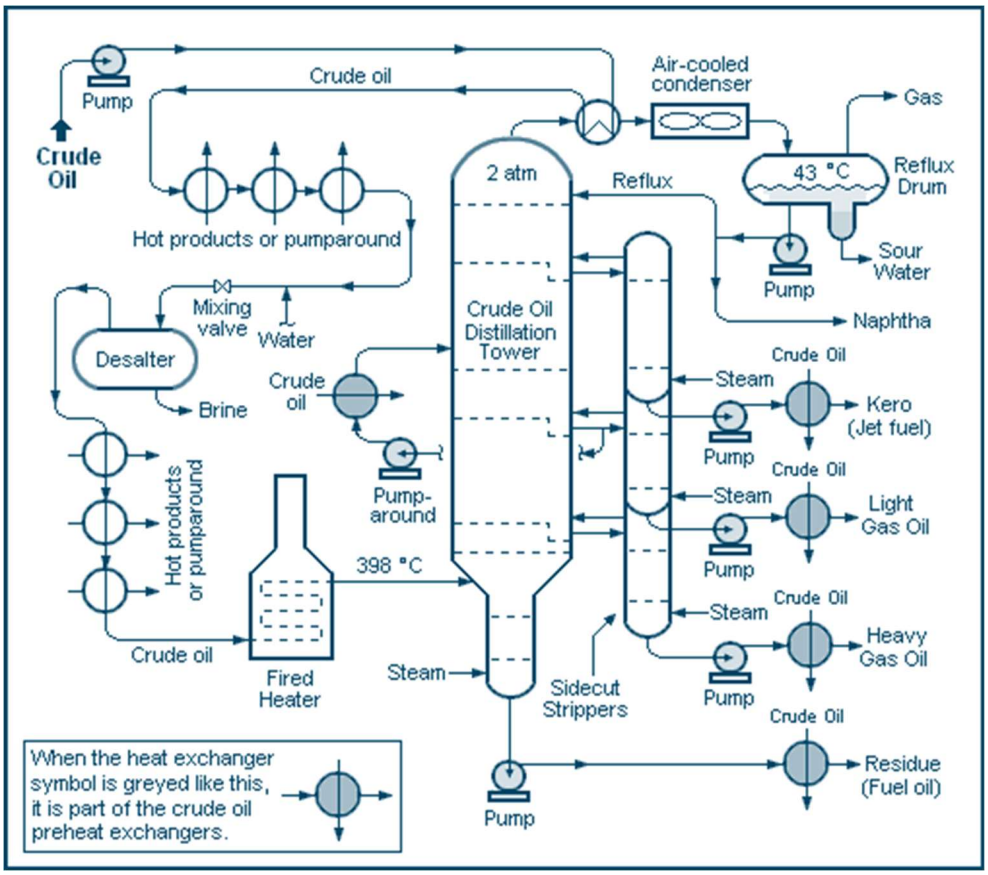

Figure 2.2: Illustration of a traditional crude oil distillation system (Author: Mbeychok, Licencing CC, [19]).

A) **Aliphatic hydrocarbons:** They represent one of the most abundant components of crude oil. Aliphatic hydrocarbons consist of open-chain structures that may be either straight (n-alkanes) or branched (isoalkanes), and also include cyclic structures such as cycloalkanes (also known as naphthenes). Also, the biologically derived hydrocarbons, like terpenes and steranes, belong to this group. Aliphatic hydrocarbons can be either saturated (with only single bonds) or unsaturated (containing one or more double bonds) [20].

n-Alkanes, which represent a significant subgroup of hydrocarbons, are commonly categorised into four groups according to their molecular weight:

a. Alkanes in gaseous form,

b. Alkanes with lower molecular weights (carbon range C_8 to C_{16})

c. Alkanes of intermediate molecular weight (ranging from C_{17} to C_{28}), and

d. High molecular weight alkanes (>C_{28}). [20]

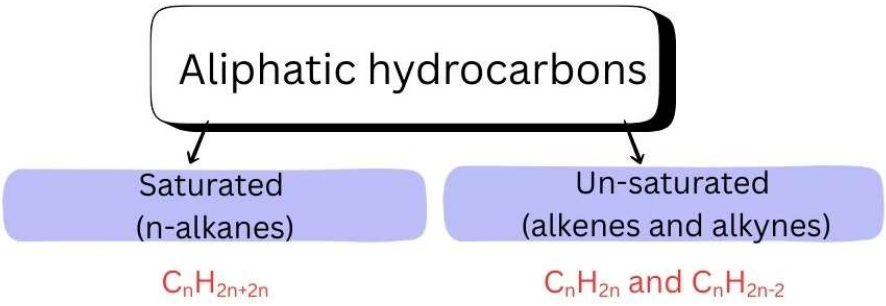

Figure 2.3: Aliphatic hydrocarbons' types and their general formulas.

B) Aromatic hydrocarbons: Almost every petroleum mixture in the world contains aromatic hydrocarbons, a significant type of hydrocarbon. Aromatics are cyclic, unsaturated hydrocarbons with double bonds that alternate. Benzene is the simplest aromatic hydrocarbon. Because they are fragrant compounds, they are also referred to as "aromatic compounds." Because of aromaticity, a unique kind of stability brought about by the delocalisation of π-electrons across its ring structure, benzene is a reasonably stable chemical compound even though it contains three carbon-carbon double bonds. But the fact that benzene causes cancer is widely known. As a result, regulations in a number of countries limit the amount of benzene present in petroleum products such as fuel oil and gasoline [21] [22].

Benzene, toluene, ethylbenzene, and xylene—collectively known as BTEX—are among the monocyclic aromatic hydrocarbons (MAHs), which comprise the majority of aromatic petroleum hydrocarbons (Figure 2.4). These compounds are extensively studied and typically make up about 2 to 20% of crude oil [3].

Organic substances with two or more fused aromatic (benzene) rings are known as PAHs [23]. While multi-ringed PAHs (five rings or more) are usually associated with particles, low-molecular-weight PAHs (two and three rings) are mostly found in the atmosphere's vapour phase. Depending on the temperature of the environment, intermediate-molecular-weight PAHs (four rings) divide into the vapour and particulate phases [24]. Particle-bound PAHs are thought to be extremely dangerous to human health. Benzo[a]pyrene contributes significantly

to the total carcinogenic potential (reportedly between 51 to 64 per cent in one study), and it is frequently employed as a marker for total exposure to carcinogenic PAHs [25]. PAHs can be classified into high molecular weight and low molecular weight PAHs depending upon benzene rings present (molecular weight) (figure 2.5).

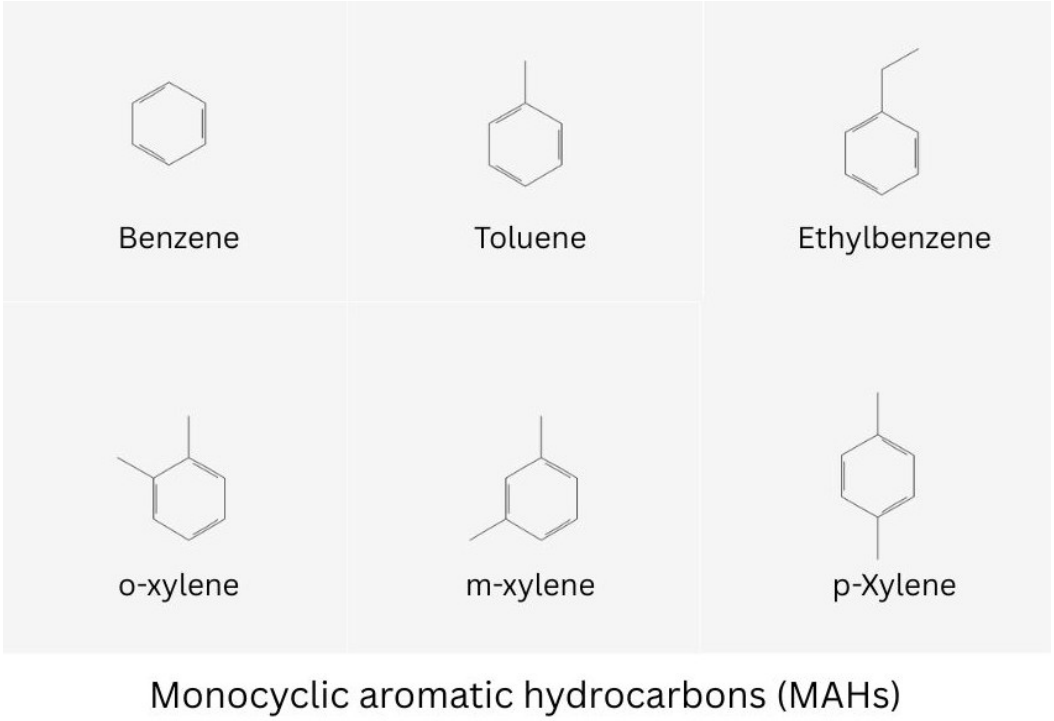

Figure 2.4: Monocyclic aromatic hydrocarbons (structures sourced from [26]).

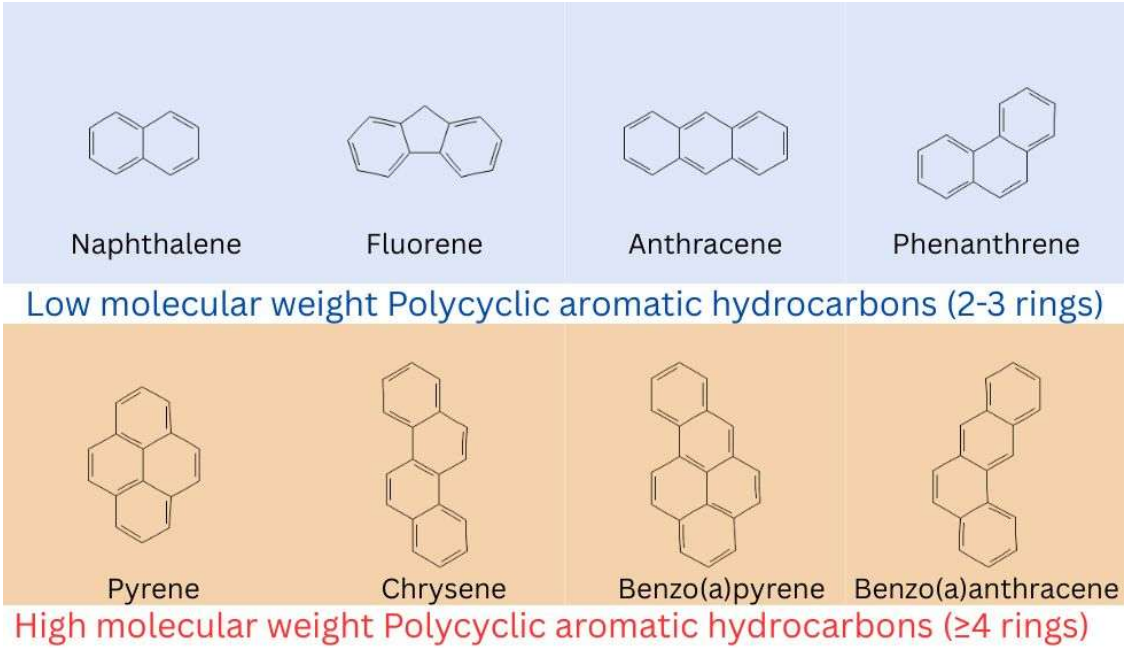

Figure 2.5: Low molecular weight and high molecular weight polycyclic aromatic hydrocarbons (PAHs) (structures sourced from [26]).

C) Resins: Petroleum resins are of low molecular weight, roughly between Mw 500–5,000. Petroleum resins are also known as C_5/C_9 hydrocarbon resins because they are produced from monomers of cracked petroleum fractions, including dicyclopentadiene feedstocks and C5 and C9 [27]. Known as petroleum resins, these thermoplastic hydrocarbon resins are derived from cracked petroleum fractions. They must be differentiated from high polymers such as polystyrene and polypropylene, which are basically pure monomer-based resins made from pure starting ingredients such as propylene and styrene, respectively. Petroleum resin can be used in potentially thousands of applications, such as paints and varnishes, coatings, printing ink, lithographic inks, paper, adhesives, rubber, and compounds that cure concrete. It usually has the tackifying/ sticking effect of adhesives [27] [28].

D) Asphaltenes: The Greeks adopted the term asphaltene, which means "firm," "stable," or "secure," from the word "asphaltu," which means "to split" [29]. The heavy, complex components of crude oil are known as asphaltenes. These particles are brittle, glossy, dark brown to black, amorphous, and solid. Asphaltenes can dissolve in aromatic solvents like benzene and toluene, but do not dissolve in light alkanes like n-pentane or n-heptane. Although their exact chemical composition is unknown, it is generally accepted that they are composed of elements like sulfur, nitrogen, oxygen, and carbon. These substances give some crude oils and residual petroleum products their black hue, particularly when they stay dissolved rather than separating off. Large, polar molecules with extremely high molecular weights are found in asphaltenes [30]. Mostly, they are distributed as colloids rather than dissolved in petroleum [29].

2.3 Environmental behaviour and fate of hydrocarbons

The addition of hydrocarbons into a clean environment instantly alters the state of the environment. These hydrocarbons either kill or inhibit the growth of the numerous microorganism species in the soil, altering the functional states of microbes and the environment [31]. Hydrocarbons are directly toxic to the plant and make the absorption of water and nutrients difficult from the soil because they restrict their flow through the soil matrix, thus reducing the plant productivity [32]. The oil composition changes upon entering the environment. The type and level of weathering vary from site to site. Hydrocarbons, upon entering the environment, are weathered by various biological, chemical and physico-chemical processes [31]. The degradation potential of oil is strongly influenced by weathering. Small

components of petroleum volatilize into the environment under warm conditions if not locked up by other interactions with the environment [31].

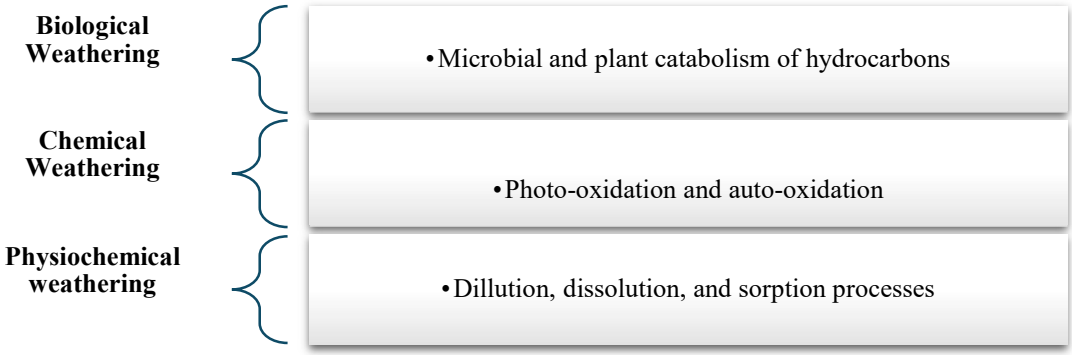

Figure 2.6: Weathering processes of hydrocarbons in the environment.

Lighter aromatic compounds are freed by the volatilisation process (i.e. BTEX and various other structures having simple rings) from complex mixtures of oil. Hydrocarbons having all single bonds between their carbon atoms and one to five rings in their structure are generally considered to be volatile and semi-volatile. An increase in the length of the chain size reduces the volatile nature, while an increase in the temperature increases the volatilisation rate and capacity [33].

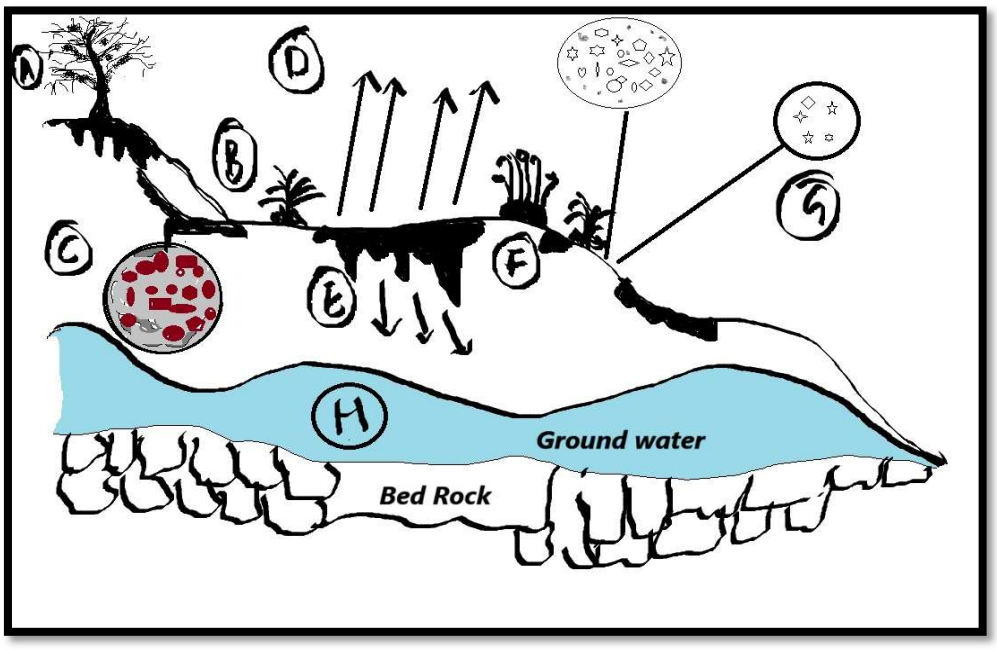

Figure 2.7: Oil's fate after a spill, and changes in the characteristics of the site of the spill. **(A)** represents the death of a plant, **(B)** Anaerobic zones, **(C)** Altered structure of the soil, **(D)**

Volatilization, **(E)** Percolation of hydrocarbons, **(F)** Aerobic zones, **(G)** Initial decrease in the population and types of microorganisms in the soil, **(H)** Groundwater contamination with hydrocarbons.

Dissolution of these hydrocarbons also decreases with the increase in the length of the carbon chain and a number of aromatic rings. The composition of the hydrocarbons greatly impacts the dissolved concentration of hydrocarbons in the water. Chains having fewer than eight carbons are soluble in water at 20° Celsius. However, the presence of polar non-hydrocarbon substances in the environment can enhance the solubility of n-alkane hydrocarbons in the oil, having carbon chains longer than 34 carbon atoms [34].

Many other small aromatic and polycyclic aromatic compounds, like BTEX and naphthalene, are found very commonly in contaminated groundwater [35]. Salinity also affects the solubility of the hydrocarbons. It was reported that 95% of the hydrocarbons can be displaced from the solution at salinities of 3,50,000 ppm. There was a logarithmic decrease in the solubility of the hydrocarbons with an increase in their boiling point [34]. Sorption of hydrophobic hydrocarbons occurs by processes like partitioning of hydrocarbons into the organic matter of soil, diffusion in the small soil pores (in the nano-meter scale) or attachment to some sites present on the organic matter of soil via chemical bonding. Hydrocarbons diffused into the nanopores of the soil are inaccessible to the microorganisms [36]. The presence of more organic matter, more clay content in the soil and high hydrocarbon hydrophobicity increases the sorption of hydrocarbons in the soil [36], [37].

Bioactive compounds released from the microbes and plants can desorb the strongly bound hydrocarbons in the soil. Plant roots can even break the aggregated compounds and desorb the hydrocarbons from the nanopores in the soil [38]. While bioremediating the contaminated site, surfactants are used to decrease the viscosity of the oil, and then the oil is released from the sorbed soil [39]. Increased soil moisture, higher sand content and an increase in temperature also enhance the rate of desorption of the hydrocarbons from the soil matrix [37]. The hydrocarbon contamination also decreases microbial diversity and evenness because some hydrocarbon fractions exhibit a high amount of toxicity to the microbes in the soil and have been linked with the induction of cancer in humans and other health problems in humans [40], [41]. Most of the microbes are not able to survive under the toxicity of the hydrocarbons, but some can be hydrocarbon-tolerant, hydrocarbon-degrading species that will survive [42].

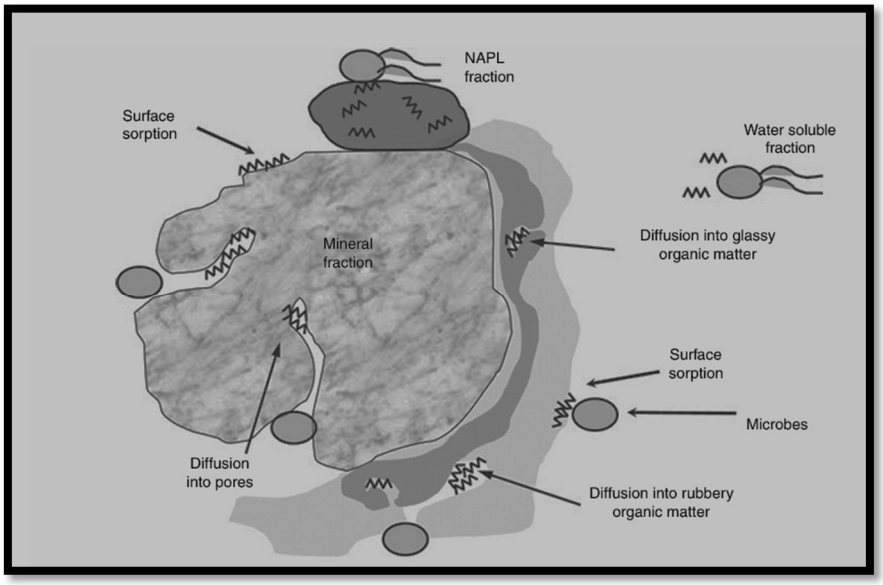

Figure 2.8: Possible interactions between aliphatic hydrocarbons and the soil matrix, adopted from [43].

2.4 Environmental impact of oil spills

Accidental oil spills are known to negatively affect the marine life, habitat and communities and thus leading to huge economic loss [44]. Countries with coastlines are at higher risk of adverse effects due to oil spill disasters. Since 1960, thousands of oil spills have occurred and mainly in hotspots near the Gulf of Mexico, the north-eastern USA and the Mediterranean Sea [45]. Oil spills, depending on the scale or amount of oil leaked, are categorised into three categories - <7 tonnes (small), 7-700 tonnes (medium) and >700 tonnes (large) (<50 bbls, 50-5,000 bbls, >5,000 bbls) by International Tanker Owners Pollution Federation (ITOPF). ITOPE claims twenty major spills in the Atlantic and Indian Oceans since 1979 [46]. For the year 2024, four medium spills and six large spills have occurred. This makes the decade-to-date average of 7.4 spills (>7 tons) per year [47]. Oil has become the lifeline of national economies. In recent trends, overall, global oil trade is increasing while the oil spills are decreasing due to technological advancements, but the mitigation and remediation of oil spills remain a challenge [48].

Oil spills injure almost all forms of life and their habitats. Broadly, the environmental impacts of oil spills are classified into three categories: Immediate (acute) toxicity, mechanical injury and environmental persistence. Volatile components in the oil rapidly mix with the water and

are potent killers of life by poisoning, and this is called acute toxicity. Mechanical injury is the harm they cause to life and habitats by physical impacts (coating, layer formation, etc.). Persistence is how long the components can remain in the environment. Lighter fuels have more toxic components in them, while heavier fuels have less concentration of toxic components.

PAHs are the main contributors to the long-term persistence and chronic ill effects [49]. Thus, aromatic hydrocarbons in oil (PAHs) are the main toxic components in the oil, causing the highest toxicity, while monocyclic aromatics are explosive and thus less persistent and do not accumulate in tissues of marine living organisms, water or sediments [50]. PHAs can bind to the cellular proteins and DNA molecules, thus exhibiting high toxicity to marine organisms [51]. They are less soluble and highly persistent, hence carcinogenic, causing chronic ill effects even in small concentrations to marine life. Not just the toxicity of the PAHs but also their dispersed oil droplets cause sensitivity to some life forms, for example, Atlantic haddock (*Melanogrammus aeglefinus*), which was supposed to have high sensitivity to the dispersed oil droplets, even in small quantities, could lead to abnormalities in cardiac and craniofacial development during developmental stages [52] [53].

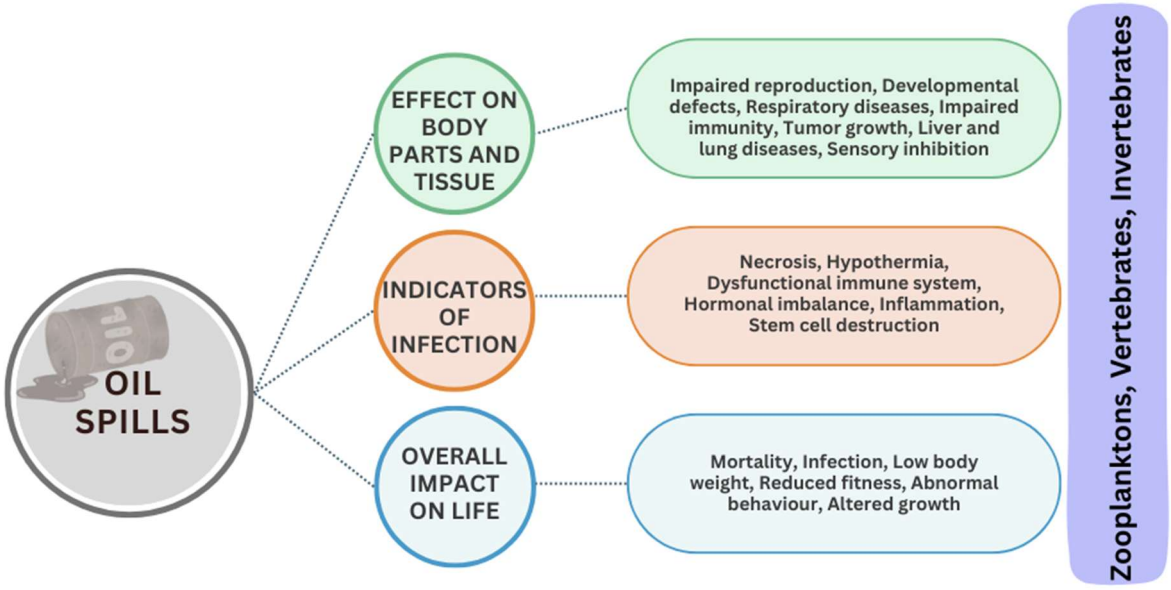

Figure 2.9: Summary- impact of oil spills on marine life.

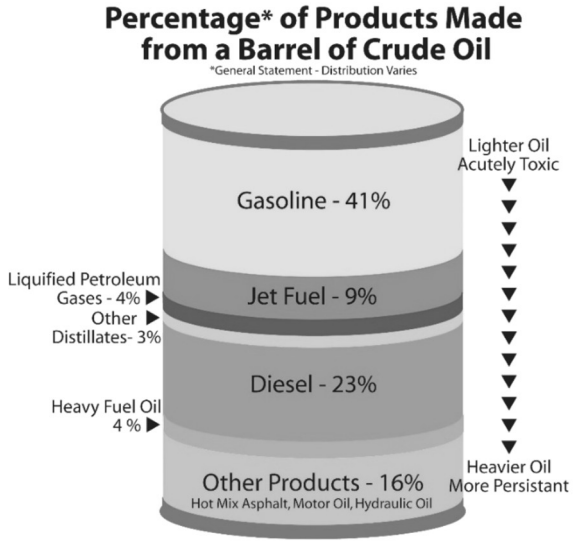

Figure 2.10: Products of crude oil and their comparative toxicity levels.

Table 2: Various oil classes listed by their acute toxicity, mechanical-injury and persistence in the environment, adopted from [49], Symbol (↓) indicating "low" and symbol (↑) indicating "high".

Oil Class	**Acute Toxicity**	**Mechanical Injury**	**Persistence**
Gasoline, Aviation Gasoline, Naphtha	(↑)	(↓)	(↓)
Kerosene, Jet Fuel, Number 1 Fuel oil	Medium	Medium (↓)	(↓)
Diesel Oil, Fuel oil No. 2, Heating oil	Medium	Medium	Medium (↓)
Bunker fuel, Fuel oil No. 6, Intermediate fuel oil	Medium	(↑)	(↑)
Alaska North Slope crude	Medium (↓)	Medium (↑)	(↑)
Biological oil	(↓)	Medium (↑)	Medium (↓)
Motor Oil, Hydraulic oil	(↓)	Medium	Medium
Hot mix asphalt	(↓)	(↑)	(↑)

Shorelines are highly vulnerable to oil spills because oil can penetrate several depths. Sand beaches provide a diverse habitat for many invertebrates. Oil spills lead to decreased diversity in sandy habitats, thus severely affecting the ecological interactions. Recovery of these affected invertebrates varies widely and depends upon the oil type and environmental conditions [54].

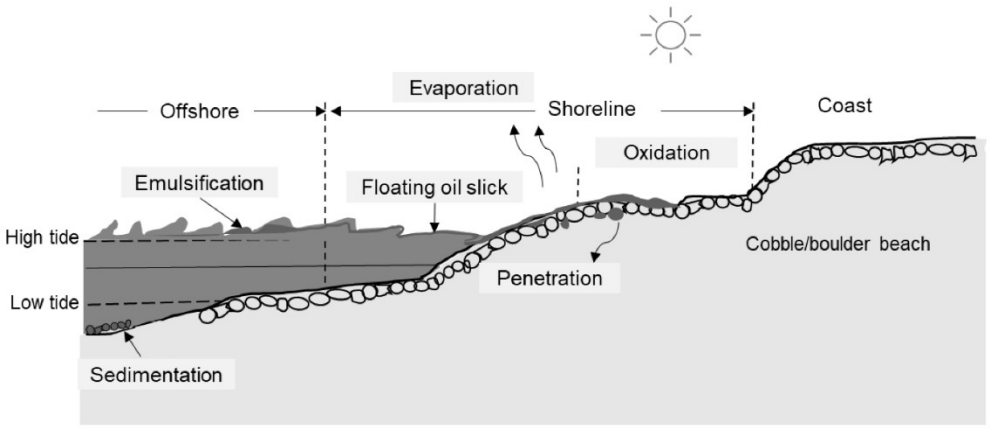

Figure 2.11: Fate of oil on shorelines, adopted from [55]

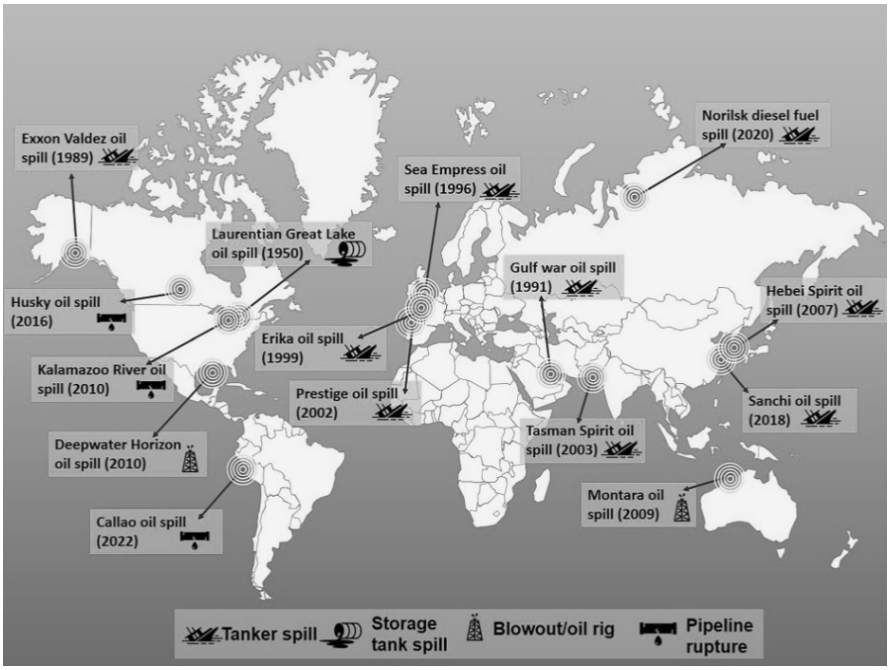

Figure 2.12: Major oil spills recorded in history, adopted from [56]

2.5 Hydrocarbon contamination in mountain ecosystems

PAHs have mutagenic and carcinogenic properties; due to this, they are gaining more and more attention over time for their adverse effects. PAHs are semi-volatile organic compounds which can deposit and accumulate in the soil and water, and their steady release causes a great threat to human well-being and the environment. Colder environmental conditions in high-altitude mountainous areas halt the evaporation of the volatile components. The lipophilic and hydrophobic nature of the PAHs enables them to easily accumulate in the living systems and thus contribute to the biomagnification [57]. Low molecular weight PAHs remain in the gaseous phase and can go to untouched high-altitude areas in the mountains. Combustion

products are transported by air to high-altitude mountainous regions [58][59]. Due to atmospheric deposition and sources from surrounding areas, the mountain surface soil is contaminated or left with residues of PAHs. The subsequent movement or dispersion of PAHs in the global environment is largely influenced by the atmosphere [60]. In a 2015 study by Devi et al., sixteen types of PAHs (summarised for quantification) were found in surface soil samples of Indian Himalayan regions in quantities which were greater than the PAHs amount in mountain soil in other parts of the world, indicating that PAHs accumulated in the mountain soil of IHR. They also concluded that compared to the sum of heavy molecular weight-PAHs, the low molecular weight-PAHs' sum was comparatively lower [61].

Given that the Indian Himalayan region is at a relatively high altitude and is greatly impacted by seasonal variations or fluctuations [62][63] [64] [65][66] [67] and during the Indian monsoon, there is a significant chance that organic pollutants could be carried and accumulated in the Indian Himalayan region over vast distances via the mechanism of long-range atmospheric transport [68] [69] [67] [70]. In a 2016 study in Nepal, the mean PAHs concentration in soil was significantly higher on the southern side of the Central Himalayas than on the northern side. The Himalayan ecosystem is fragile, and more investigations are needed to determine the effects of organic pollutants on it.

2.6 Microbial degradation of hydrocarbons

During evolution, microbes have had a lot of time to adapt to different environmental conditions involving the utilization of hydrocarbons as an energy source. The reason why hydrocarbons are avidly used by microbes is that they have high calorific or energy values. Biologically, fats or lipids are energy-rich molecules for living systems, but hydrocarbons, including aromatic hydrocarbons, have higher energy values. For example, fats (lipids) provide 37 kJ/g energy, about 17 kJ/g energy is provided by proteins and carbohydrates and on the other hand, combustion of crude oil (hydrocarbons) gives 42–47 kJ/g of energy output [71].

A highly specialised kind of bacteria known as methanotrophic bacteria, which are capable of breaking down the simplest hydrocarbon (C_1), i.e, methane, and some non-C_1 compounds using them as a source of carbon and energy. However, hydrocarbons with more carbon atoms prevent these bacteria from growing [72]. PAHs tend to bind with the soil's organic matter and get associated with non-aqueous phases, which limits their bioavailability for microbial breakdown. However, various microbes—including bacteria, fungi, and algae—can metabolise

these bounded PAHs through a diverse range of degradation pathways involving catabolic genes [73]. Heitkamp et al. in 1988 for the first time reported the degradation of pyrene and other polycyclic aromatic hydrocarbons with four rings by *Mycobacterium* sp. Mineralisation of the pyrenes and then their utilization is initiated by the treatment of chloramphenicol due to pyrene-degrading enzymes induced in *Mycobacterium* sp. upon treatment [74]. Following this discovery, several pyrene degraders from the genera *Rhodococcus, Bacillus, Burkholderia, Cycloclasticus, Sphingomonas, Pseudomonas, Mycobacterium and Stenotrophomonas* have also been identified [75], [76]. Numerous scientific studies have demonstrated that a wide range of bacterial populations that come in immediate contact with hydrocarbons quickly change into hydrocarbonoclastic bacteria (a group of prokaryotes which can utilise or degrade hydrocarbons), which are able to break down and use hydrocarbon compounds as sources of carbon and energy [19]. The ability of emulsification of lipophilic molecules (oils) and metabolisation of hydrocarbons, activation of specialised DNA repair mechanisms, production of molecules involved in quorum sensing and biofilm mechanisms, and regulation of efflux pumps and pores to regulate the concentration of hydrocarbons inside a cell are just a few of the adaptive mechanisms that hydrocarbonoclastic bacteria have evolved with time to withstand the presence of hydrocarbons in their interior and exterior environments [77].

Hydrocarbon biodegradation begins with microbial adhesion to the substrate, directly to oil molecules or adsorbed particles, followed by the production of biosurfactants, or bio-emulsifiers, gases, solvents, biopolymers, and organic acids for increasing their bioavailability. Microbes can use certain enzymes to break down hydrocarbons in the environment. A microbial consortium is a group of diverse microorganisms which work together to perform degradation of hydrocarbons more effectively than a single type of bacterium alone. These consortia are typically better at eliminating a variety of hydrocarbon contaminants [78].

The majority of bacteria have plasmids that encode for biodegradation enzymes. When it comes to the presence of biodegradative enzymes, *Acinetobacter* sp. are unique; their chromosomes contain plasmids (episomes) [79] [80] . The Q15 plasmid may have a role in the breakdown of hydrocarbons, according to some authors [81]. Different types of plasmid molecules are found in hydrocarbonoclastic bacteria, which play an important role in breaking down petroleum-based pollutants. Plasmids like Q15, OCT, TOL, NAH7, pND140, and pND160 carry specialised genes such as alkA, alkB, alkM, theA, assA1, assA2, LadA and nahA-M. These genes produce enzymes that play a crucial role in the catabolism of the oils or hydrocarbons

[80] [82] [83]. A wide range of bacteria can break down the simpler compounds of crude oil, but only a small number of species have the potential to break down complex compounds like resins, asphaltenes and PAHs. Bacterial species which utilise a few hydrocarbons as an energy source usually show synergetic effects in consortium and thus utilise more number of hydrocarbons as a preferred source of food [84], [85], [86], [87]. The presence of hydrocarbons in certain bacteria can alter their chemotaxis [7]. Chemotaxis is a behavioural response in which bacteria use chemoreceptors to sense changes in a particular chemical's concentration and change their position in response [88]. Hydrocarbon-degrading bacteria have the ability to move towards hydrocarbon-contaminated areas and regulate their position [88], [89]. In a recent column experiment by Rolando et al., it was discovered that the motile and chemotactic bacterium *Pseudomonas putida* G7 can sorb and cometabolise pyrene, mobilising and rendering it bioavailable for biodegradation [90].

Particular enzyme systems can mediate the breakdown of petroleum hydrocarbons. Usually, the first attack is accomplished via several processes, including (a) microbial cell adhesion to the substrates and (b) the generation of bioemulsifiers, biosurfactants, biopolymers, solvents, acids, and gases [78].

2.7 Metabolic processes for the breakdown of hydrocarbons

Hydrocarbons are broken down by bacteria using both aerobic and anaerobic bacterial catabolic metabolism. Since oxygen is the last electron acceptor, degradation rates are influenced by its content; biodegradation occurs more efficiently and rapidly in aerobic conditions. Under anaerobic conditions, where the final acceptor of electrons may be sulfate, nitrate, or an iron molecule, biodegradation is a little slower or negligible than the breakdown of hydrocarbons under aerobic conditions [91] [92] [93]**.**

Under aerobic conditions, hydrocarbons are directed into degradative pathways that ultimately converge on the tricarboxylic acid cycle, often referred to as the Krebs cycle or TCA cycle, where they completely oxidize the substrate to produce carbondioxide gas and (nicotinamide adenine dinucleotide) NADH.. When bacteria break down PAHs aerobically, oxygen is required for ring hydroxylation, ring cleavage, and final uptake of the electrons. However, anaerobic PAH consumption is based on reductive mechanisms [94] [95]. Because of their fused aromatic ring structures, PAHs are less bioavailable, hydrophilic, and thermodynamically more stable, and this makes them more difficult to degrade. Pyrene mineralisation begins through initial dioxygenase-catalysed attacks at either the C-1/C-2 or C-4/C-5 positions of the

aromatic ring. This results in the formation of dihydrodiols, which subsequently undergo re-aromatisation followed by ring cleavage by dioxygenases. These reactions lead to the generation of phenanthrene dicarboxylate, which undergoes sequential decarboxylation to yield phenanthrene carboxylate. Through a deoxygenation process, phenanthrene carboxylate is further transformed into cis-3,4-dihydroxyphenanthrene-4-carboxylate. This intermediate then re-aromatises to form dihydroxyphenanthrene, which is metabolised to produce hydroxynaphthoate. Additionally, pyrene degradation may also proceed via the phthalate pathway, highlighting the metabolic versatility of the hydrocarbons-degrading microbes [96] [97].

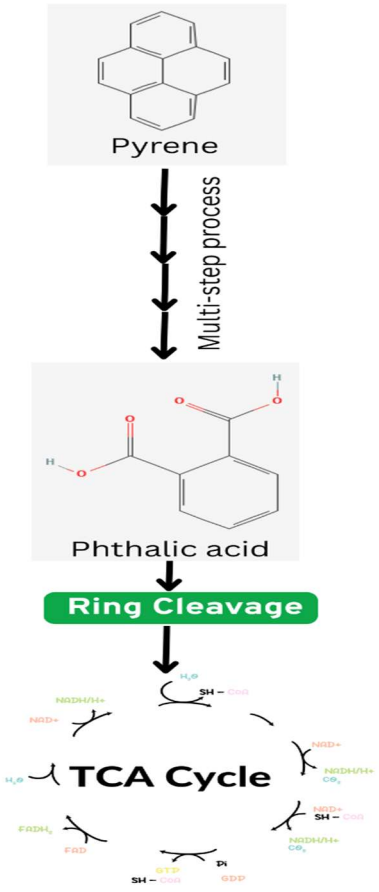

Figure 2.13: Proposed pathway of Pyrene degradation in a consortium of bacteria [97]

Many biochemical changes are involved in aerobic hydrocarbon biodegradation, which is often divided into peripheral and central metabolic routes. The conversion of a wide variety of hydrocarbon molecules into a comparatively small number of important metabolic intermediates is carried out via the peripheral routes. The enzymatic addition of molecular oxygen (O_2) to the hydrocarbon substrate is the first stage of aerobic degradation. This

oxygenation boosts the compound's chemical reactivity and water solubility, which allows further breakdown processes. A class of enzymes called oxygenases catalyses the addition of oxygen as a hydroxyl group (-OH) [98].

The breakdown of hydrocarbons is facilitated by two primary categories of oxygenase enzymes. As the name suggests, monooxygenases add only one oxygen atom (from O_2, molecular oxygen) to the substrate, and the other oxygen atom is reduced to water. Dioxygenases, on the other hand, directly integrate both molecular oxygen atoms into the substrate molecule [98].

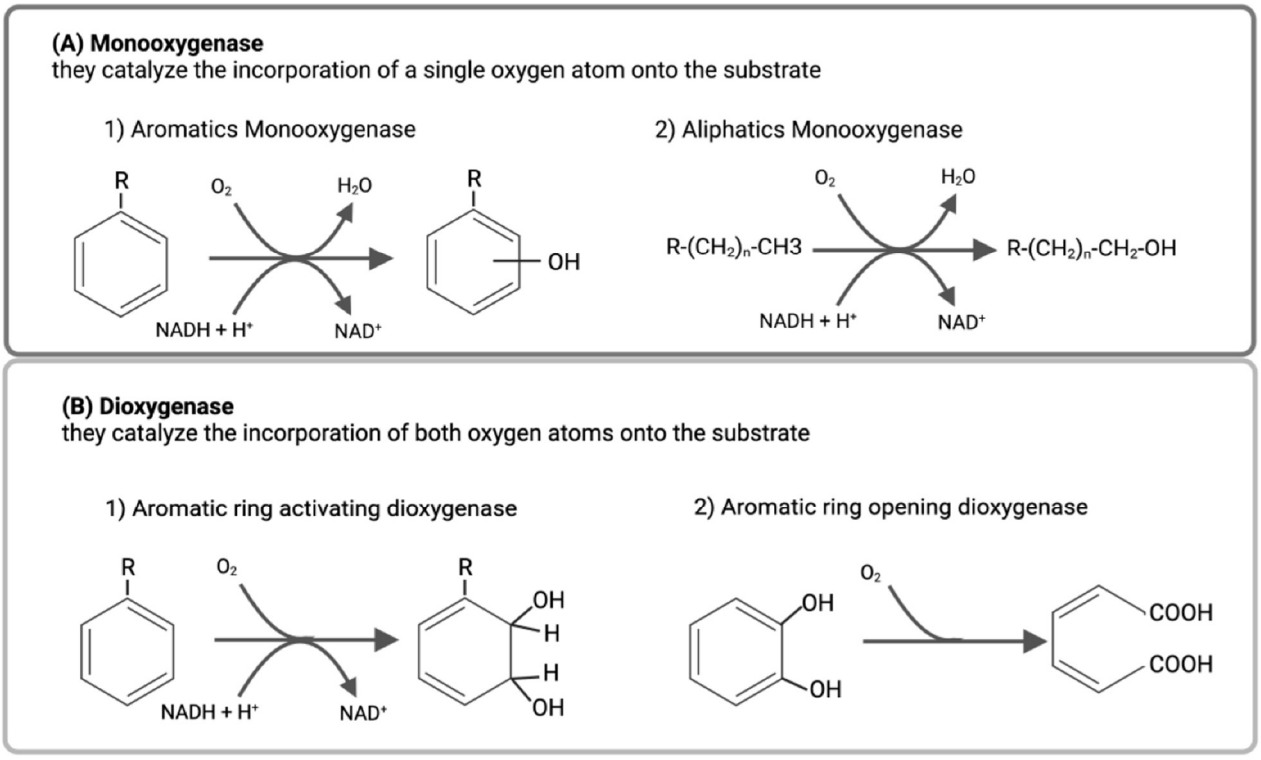

Figure 2.14: Mechanism of action of oxygenase enzymes- monooxygenases and dioxygenases. Dioxygenases work by ring activation (forming dihydroxy compounds) or causing rupture of the ring, which was previously activated, adopted from [98]

Because reduced cofactors like NADH or NADPH are necessary for oxygenation reactions, these processes can only take place inside bacterial cells because these coenzymes are unstable outside of cells. As a result, hydrocarbons must be brought inside the bacterial cell before they can be oxidised, and oxygenase enzymes operate inside the bacterial cells. Both aliphatic and

aromatic hydrocarbons can be acted upon by the versatile enzymes known as monooxygenases. Dioxygenases, on the other hand, are categorised according to their function; some catalyse ring cleavage, while others are engaged in the activation of the aromatic ring. These two types of dioxygenases add two oxygen atoms to the substrate. Notably, pyridine nucleotides in reduced form are not necessary for the working of ring-cleaving dioxygenases [99].

Aerobic biodegradation of aliphatic hydrocarbons:

a) **Sub-terminal oxidation:** To break down alkanes, certain bacteria first add oxygen to a methyl group that is located in the sub-terminal position, which is slightly away from the end of the chain. A secondary alcohol is produced in this step, which is then transformed into a ketone and eventually an ester. After that, the ester undergoes hydrolysis, which produces an alcohol and a fatty acid. Ultimately, the corresponding fatty acid is formed due to further oxidation of the alcohol and fatty acid (Figure 2.15) [100], [101].

b) **Terminal oxidation:** Most bacteria first add oxygen to the methyl group at the end of the chain (terminal position) to break down aliphatic hydrocarbons. The methyl group produces an alcohol as a result of this process. After that, the alcohol undergoes a gradual oxidation process that first produces an aldehyde and subsequently a carboxylic acid. The bacteria convert hydrocarbons into forms that they can further degrade and use through this step-by-step oxidation process (Figure 2.15) [100], [101]**.**

The similarity between the sub-terminal and terminal oxidation pathways lies in their final product—a fatty acid. By the process of β-oxidation, this fatty acid is converted to acetyl-CoA, which then enters the Krebs cycle for energy production, regardless of whether the oxidation is terminal or sub-terminal [100], [102].

Aerobic biodegradation of aromatic hydrocarbons:

Aromatic hydrocarbons can be used as carbon and energy sources by a variety of aerobic organisms. The biodegradation of naphthalene is an example. The first step in this process is the conversion of naphthalene into catechol (ortho-diphenol), an intermediate with a single aromatic ring that contains two hydroxyl groups. By adding two oxygen atoms to the carbon atoms containing the hydroxyl groups, the enzyme catechol 1,2-dioxygenase then catalyses the

breakdown of the aromatic ring at the ortho position. Cis,cis-muconic acid is produced when the bond between carbon atoms 1 and 2 is broken in this step. Ultimately, additional processing of cis,cis-muconic acid results in a lactone, which is subsequently transformed into succinate and acetyl-CoA, two crucial molecules that enter the Krebs cycle to produce energy (Figure 2.16) [103],[100].

The meta-cleavage of the aromatic ring is catalysed by the enzyme catechol 2,3-dioxygenase. This enzyme opens the ring between a hydroxylated carbon atom and its neighbouring non-hydroxylated carbon atom. This reaction produces hydroxymuconic semialdehyde, which is then further transformed into pyruvate and acetaldehyde through a series of metabolic steps [100].

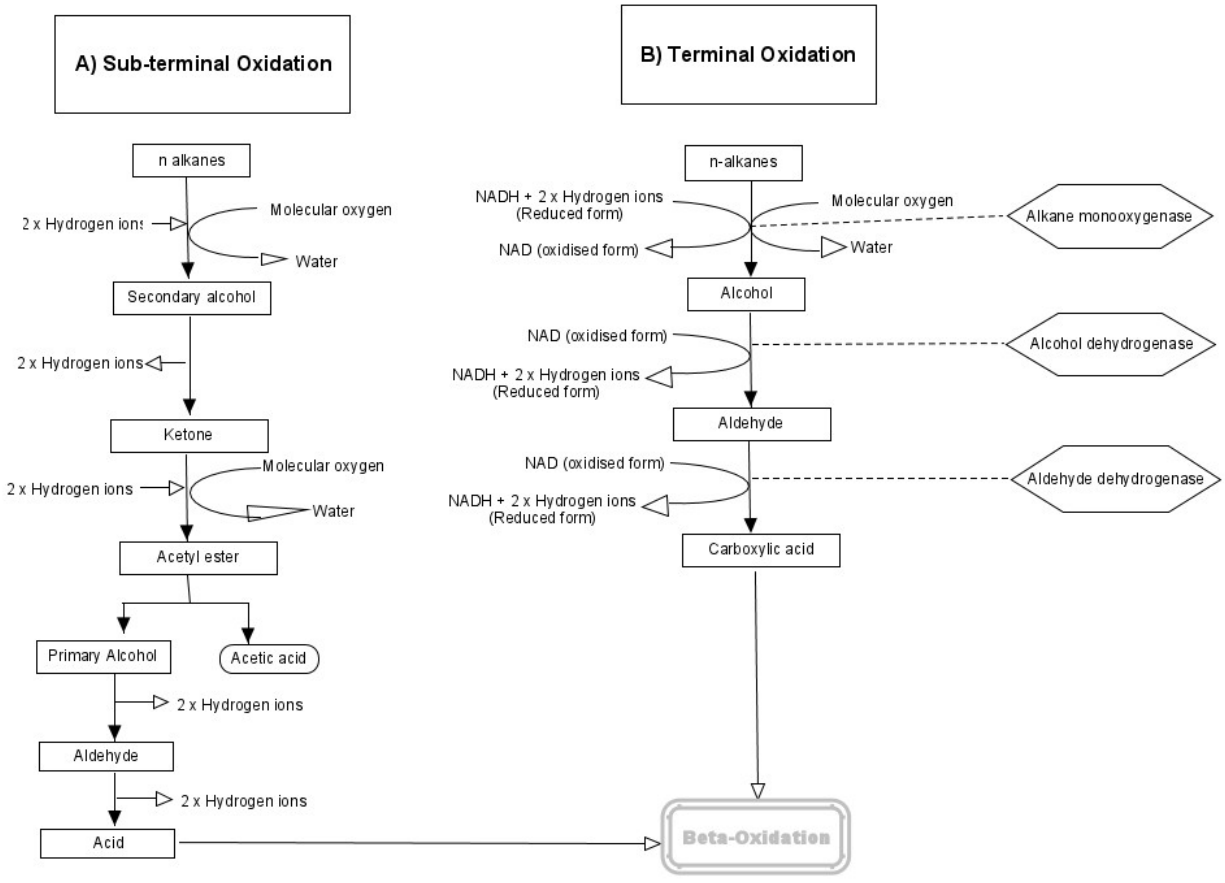

Figure 2.15: **A)** Sub-terminal oxidation of aliphatic hydrocarbons; **B)** Terminal oxidation (oxidation of terminal methyl group) of aliphatic hydrocarbons (Pathway drawn using **PathVisio 3.3.0** [104] software).

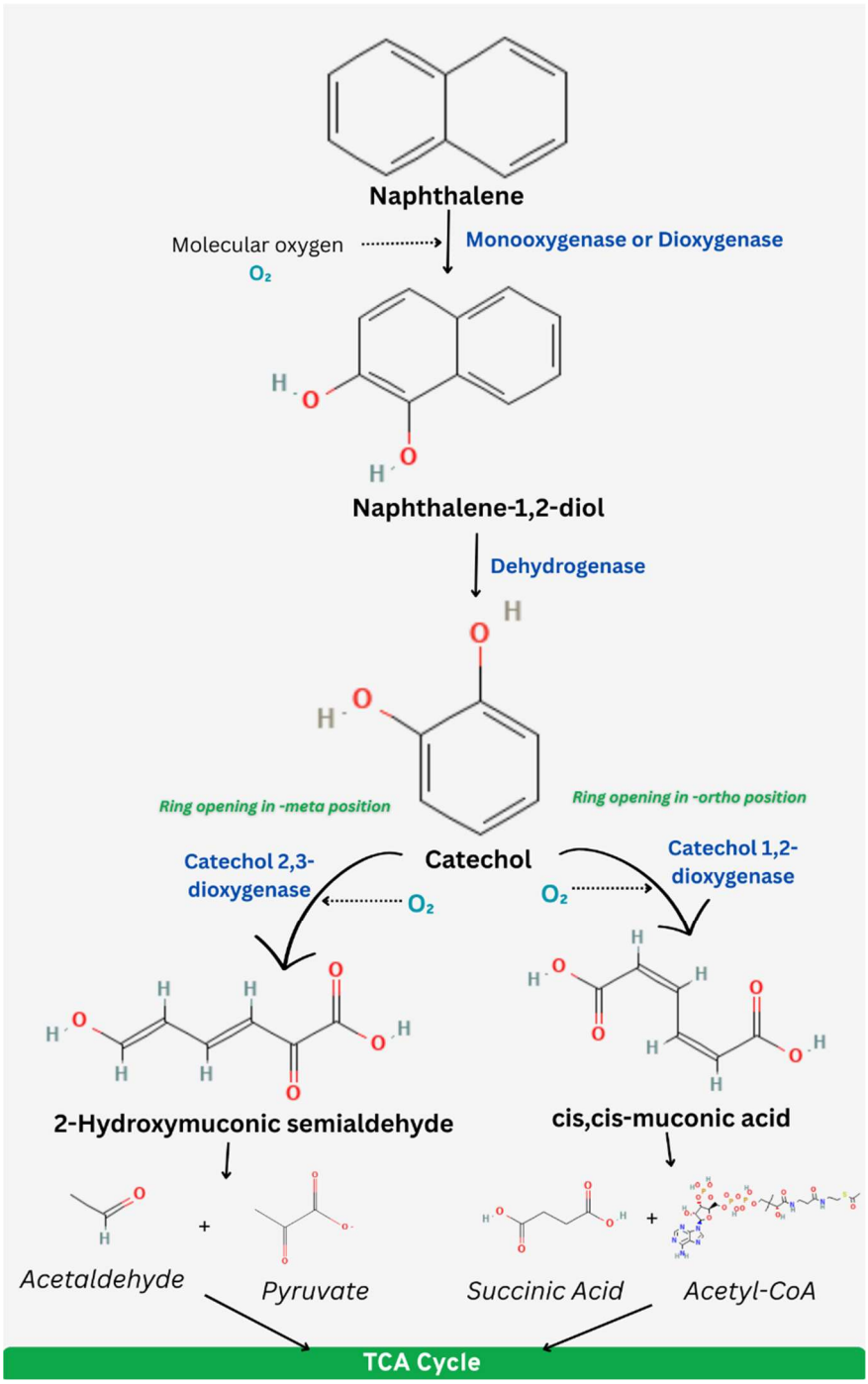

Figure 2.16: Degradation pathway of aromatic hydrocarbons (e.g, naphthalene) by aerobic bacteria. (structures downloaded from https://pubchem.ncbi.nlm.nih.gov/).

2.8 Genes and enzymes involved in hydrocarbon degradation pathways

There are various genes which encode different types of enzymes which actively help in the hydrocarbon degradation by the bacterial system. Genes encoding enzymes responsible for the degradation and metabolism of xenobiotic compounds are typically found on plasmids [105] a

rapid response to hydrocarbon contamination. In Table 3, key enzymes in the biodegradation of hydrocarbons are listed along with their functions.

Table 3: List of important enzymes that break down aromatic and aliphatic hydrocarbons.

Hydrocarbon type	Enzyme	Associated gene(s)	Main function	Reference(s)
Aliphatic	Alkane hydroxylase (sometimes also referred to as alkane 1-monooxygenase)	*alkB, alkG, alkT*	Oxidation of alkanes into primary alcohols.	[106]
	Cytochrome P450 Monooxygenase	*CYP153A*	Terminal oxidation of medium or long-chain alkanes	[107]
	Alcohol Dehydrogenase	*adh*	Converts primary or secondary alcohols to the corresponding aldehydes	[108]
	Aldehyde Dehydrogenase	*aldH*	Converts aldehydes to fatty acids	[109]
Aromatic	Catechol Dioxygenase (Discovered by Osamu Hayaishi [110])	*catA, catB*	Ortho- and meta-oxidative cleavage of catechol	[110], [111]
	Biphenyl Dioxygenase	*bphA, bphB, bphC*	Degradation of biphenyl and polychlorinated biphenyls (PCBs)	[112], [113]
	Naphthalene Dioxygenase	*nahA, nahB*	Degradation of naphthalene (Oxidises naphthalene to form cis-naphthalene dihydrodiol)	[114], [115]
	Toluene Dioxygenase	*todC1, todC2, todD*	Oxidation of toluene and xylene	[116], [117]

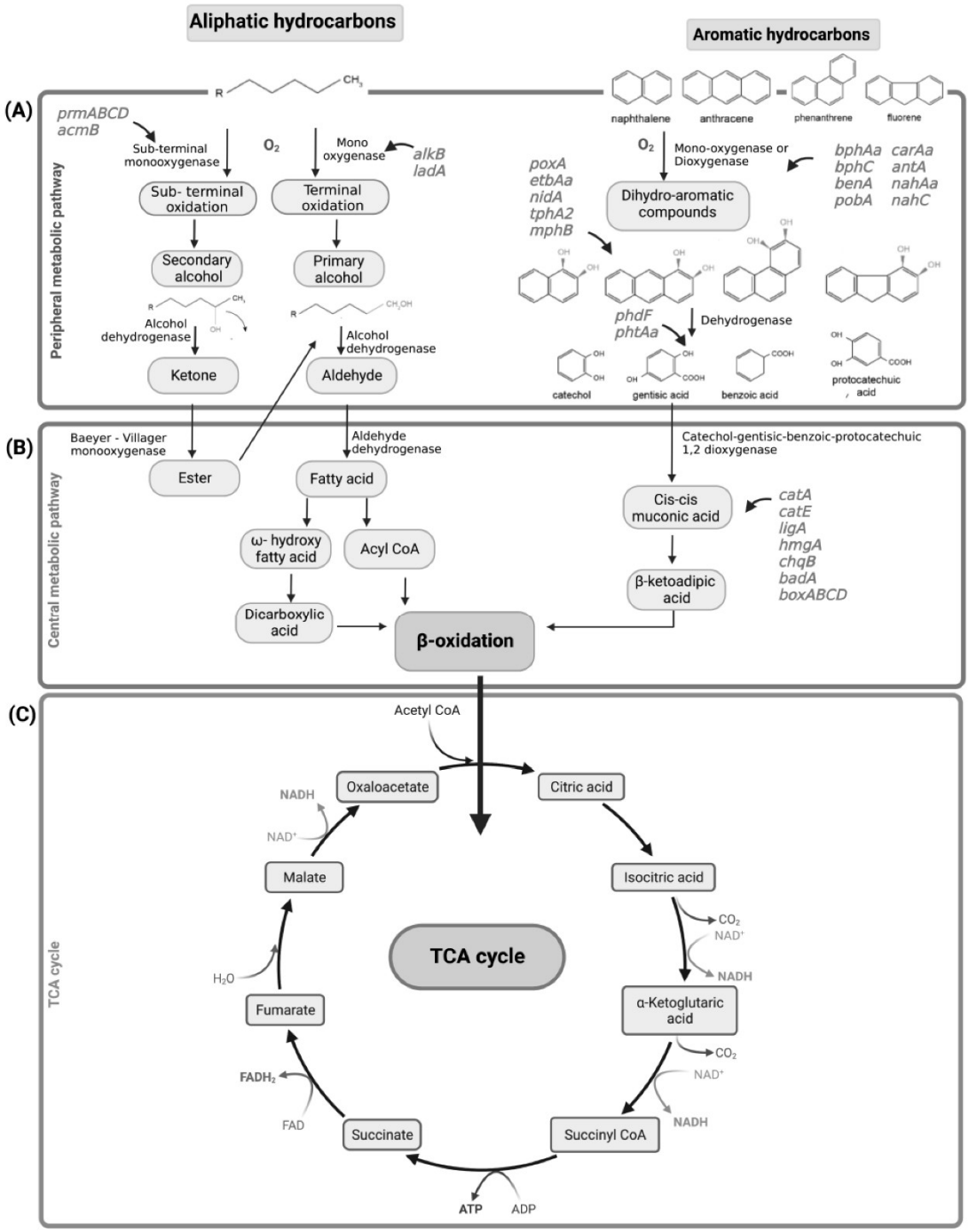

Figure 2.17: Summary of main reactions which lead to the degradation of aliphatic hydrocarbons and aromatic hydrocarbons, adopted from [100]

In conclusion, hydrocarbons are prevalent contaminants that endanger human health and ecosystems. If concentrations aren't too high, bacteria may break them down. Although elimination can be enhanced by techniques including biostimulation, bioaugmentation, and biosurfactants, practical implementation is dependent upon regional environmental conditions.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

We performed the isolation, characterization of a bacterium from soil sample which was collected from a site near to JUIT, Waknaghat, Solan.

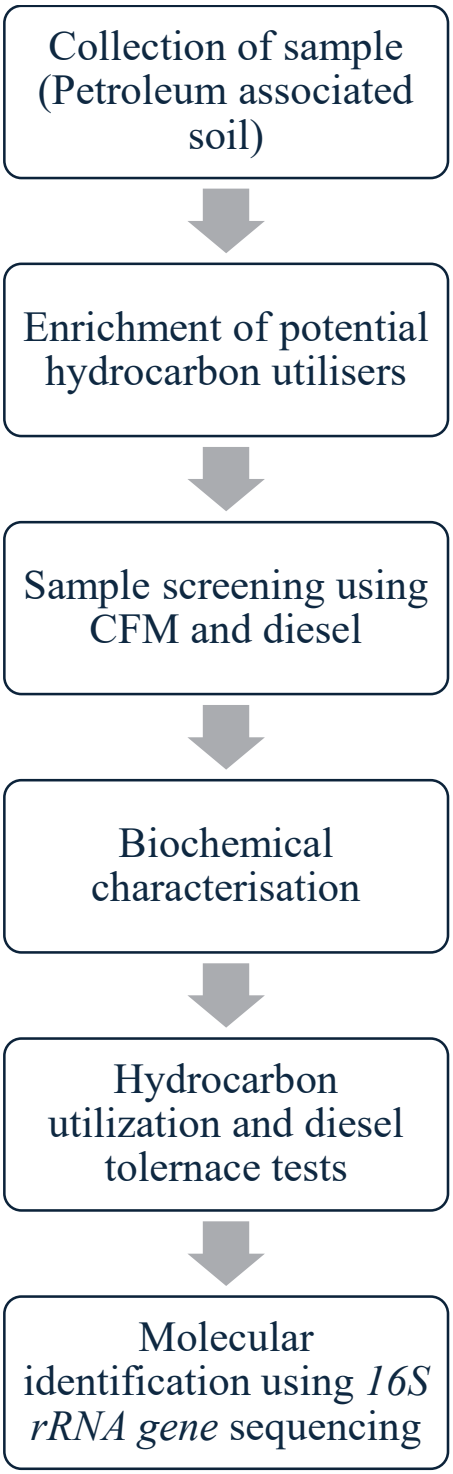

Figure 3.1: Schematic representation of experimental workflow.

3.1 Sampling from a hydrocarbon contaminated site

An oil-contaminated site was identified, and a soil sample was collected from the Wakna Filling Station, IndianOil, Waknaghat, Solan, Himachal Pradesh, India (Lat 31.0106475° Long 77.09087°). The sample was collected in a sterilised Tarson centrifuge tube (50 ml) on a sunny day (20°C) from about 5 cm below the surface soil. The sample was brought to the lab. pH of the soil sample was determined using a 1:1 soil to water (w/v) ratio method [118] (EUTECH Cyberscan PH Tutor Meter – ECPHTUTOR-I). Diesel was also procured from Wakna filling Station, IndianOil, having a density of 821.4 Kg/m^3.

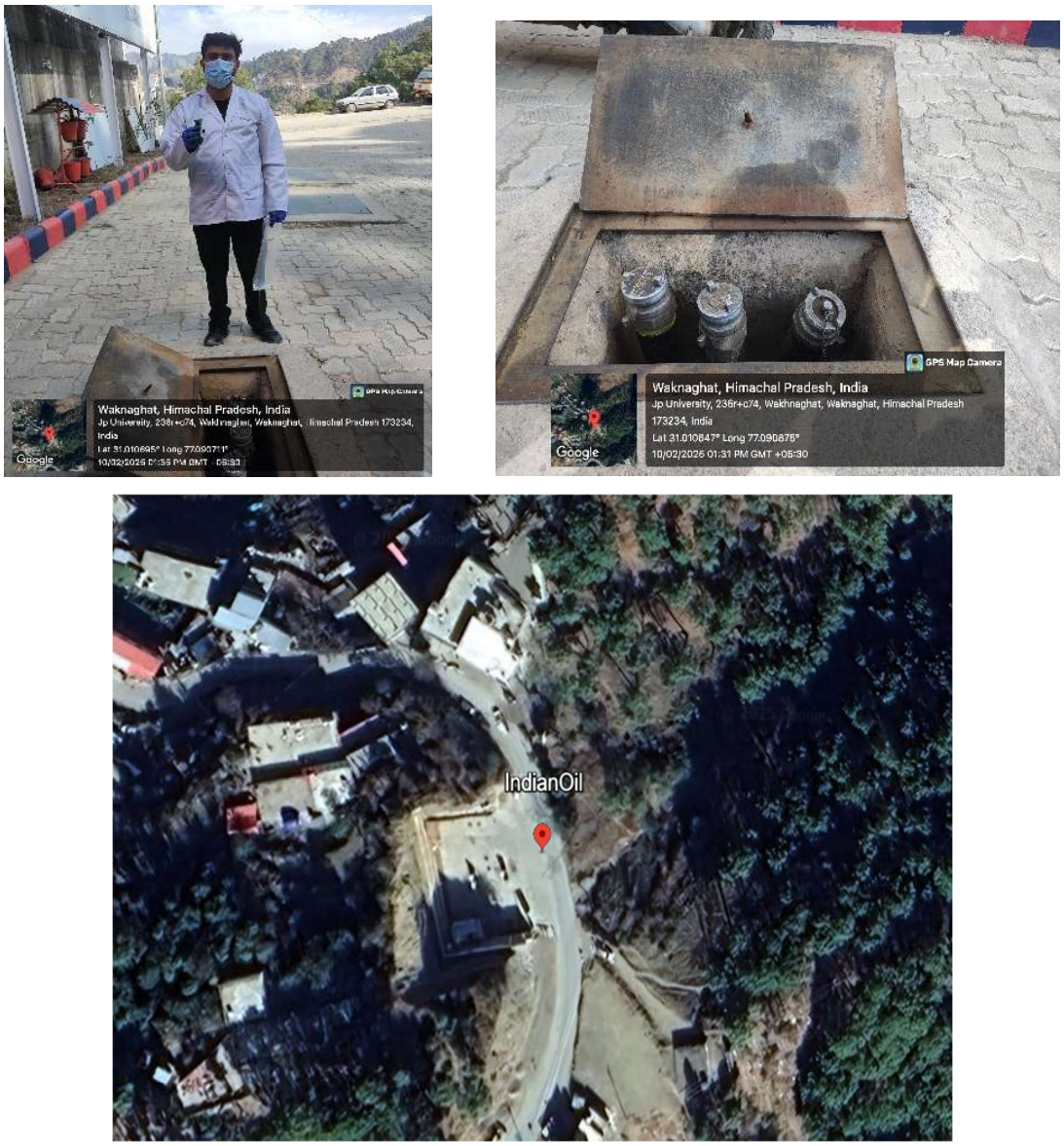

Figure 3.2: Sampling site at Waknaghat, Solan, India.

3.2 Media preparation

Bushnell-Haas broth (BHB), from HiMedia (India): Ammonium nitrate 1.0 gms/ltr., Dipotassium phosphate 1.0 gms/ltr., Monopotassium phosphate 1.0 gms/ltr., Ferric chloride 0.050 gms/ltr., Magnesium sulphate 0.200 gms/ltr., and Calcium chloride 0.020 gms/ltr. was used. The pH of the medium was 7.2. Bushnell Haas agar from HiMedia was used.

Nutrient broth from HiMedia (India), having the following composition- Peptic digest of animal tissue 5.0, Sodium chloride 5.0, Beef extract 1.50, and Yeast extract 1.50 gms/ltr. was used. pH of media was 7.4.

MacConkey agar with bromocresol blue indicator was used for screening the gram-negative and lactose-fermenting bacteria. The broth and agar from HiMedia was prepared, which contained peptic digest of animal tissue 10 gms/ltr., Bile salts 4gms/ltr., Sodium chloride 5gms/ltr., Lactose 30 gms/ltr., Bromo cresol purple 0.12gms/ltr.

Muller-Hinton broth (HiMedia) was used for the antimicrobial susceptibility testing of the bacterial isolate. Ingredients of this broth are HM infusion B from equivalent to beef source 300gms/ltr., Acicase™ 17.5gms/ltr., Starch 1.5gms/ltr. Solidified media was prepared from this broth by adding agar at a concentration of 2% (w/v).

Luria Bertani Broth, Miller (HiMedia) was used for the culturing of bacteria for DNA isolation purposes and other molecular work. Ingredients included Tryptone 10gms/litre, Yeast extract 5gms/litre, Sodium chloride 10gms/litre.

All media were sterilised by autoclaving at 121°C at a pressure of 15 psi for 15-20 minutes.

3..3 Isolation of the hydrocarbons-utilizing bacteria

Methodology for the isolation of hydrocarbon utilizing bacteria was adopted from [119]. Briefly, 2-gram petroleum-associated soil sample was added to 20 ml of 0.9% w/v normal saline solution in a sterilised test tube. After vortexing it for 10 seconds, the suspension was allowed to settle for five minutes to facilitate the sedimentation of most of the soil particles at the bottom of the test tube. 2mL of supernatant as an inoculum was added into BHB in an Erlenmeyer flask, which was supplemented with 1% diesel (v/v). The sample was agitated on a shaker (120 r.p.m) (Labnet 211DS Shaking Incubator) at 30°C temperature for seven days. This was done to enrich only those species of bacteria which can utilise diesel (hydrocarbons)

as a sole carbon source. A control was set up using the nutrient broth (HiMedia, India) inoculated with 1 gms soil per 100 mL to enumerate the original population of the bacteria in the soil sample in a 500 ml Erlenmeyer flask. The flask was kept at 100 r.p.m agitation (Labnet 211DS Shaking Incubator) at 30°C for 5 days. Enumeration of the original bacterial population was performed later.

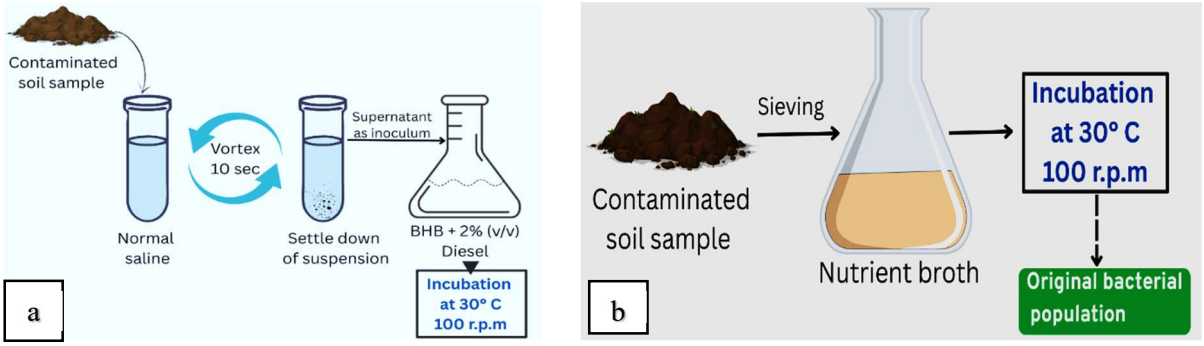

Figure 3.3: A) Methodology followed for hydrocarbon-utilizing bacteria cultivation, B) control, for cultivation of the original population in the soil sample.

3.4 pH measurement of enrichment culture

The pH of the medium was measured after preparing the medium and after the incubation period was completed. The changes in the pH were monitored to analyse the metabolic activity of the microbes.

3.5 Sequential inoculation-based enrichment of diesel-degrading microbes

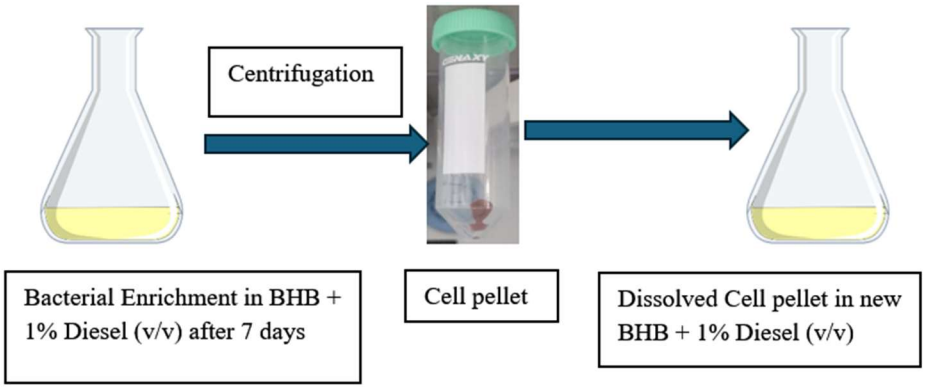

Figure 3.4: Sequential inoculation-based enrichment of diesel-degrading microbes.

The enrichment culture after seven days of incubation was centrifuged (Eppendorf Centrifuge 5804 R) at 5,000 rpm for 5 minutes at room temperature to pellet down the bacterial cells. The supernatant was then discarded, and the cell pellet was dissolved into the freshly prepared Bushnell Haas broth with 1% Diesel (v/v) in an Erlenmeyer flask. The flask was put under the same incubation conditions as above for 7 days. This step was again repeated for two times, and pH was measured at the end of incubation after each step, thus giving three enrichments in total [119].

3.6 Enumeration of the bacteria

To quantify the viable bacteria in the samples, the serial dilution method was used. In this method, 9 mL Millipore (Elix® 3) water containing glass test tubes were autoclaved for sterilisation. Aseptically transferred 1 mL of the bacterial culture into the first test tube (10^{-1} dilution) while pipetting gently for proper mixing under horizontal Laminar Air flow Unit (RH-58 LAF Horizontal, Rescholar Equipment, India). Then, transferred 1 mL from the 10^{-1} dilution tube to the 10^{-2} tube. Subsequent dilutions were made similarly using sterile micropipette tips up to 10^{-10} dilution. 100 µL of solution from each dilution tube was spread onto the nutrient agar plates using a sterile glass spreader, and the plates were incubated at 37°C, overnight (BOD Incubator (Super Deluxe), Macflow Engineering, India). The number of colonies were counted after incubation to estimate the bacteria in CFU/mL in the control, second and third enrichment samples.

3.7 Selection of morphologically distinct colonies

After the third enrichment and serial dilution, four morphologically distinct colonies were selected from a 10^{-6} dilution plate. These colonies were chosen based on their shape, edge, colour, and texture. Each of these individual colonies were then isolated and quadrant-streaked in duplicates onto fresh nutrient agar plates to obtain a pure culture. The plates were incubated overnight at 37°C (BOD Incubator (Super Deluxe), Macflow Engineering, India).

3.8 Gram staining of the isolated bacterial strains

Gram staining of the isolates was performed to determine whether the bacteria were gram negative or gram positive and their morphological charcateristics were also observed. The Gram staining protocol by the American Society for Microbiology was adopted to characterize the bacteria [120]. **(Appendix-1)**

3.9 Cryopreservation of isolated bacterial strains

Glycerol stabilises the membranes of the bacterial cells, thus keeping the cells alive. At -80°C, glycerol stocks can be stored for several years. For the long-term preservation, all the bacterial isolates were incubated in sterilised nutrient broth in an Erlenmeyer flask (100 mL) under aseptic conditions (RH-58 LAF Horizontal, Rescholar Equipment, India) and incubated at 37°C overnight with shaking at 100 r.p.m. 2 mL of overnight culture was added to an equal volume of 50% glycerol (v/v) in cryovials and stored at -80°C for further experimentation [121].

3.10 Growth on diesel for screening of hydrocarbon degraders

The modified spread plate method was used to examine the hydrocarbon utilization ability of the bacterial isolates [122]. 100 µL of diesel was spread on the BHA plates using a sterile glass spreader until a uniform surface layer was formed. The bacterial isolates DDB1, DDB2, DDB3, and DDB4 were streaked onto these plates using two distinct methods: continuous streaking (for confluent growth) and quadrant streaking (for obtaining isolated colonies). Abiotic control was set up to check for contamination. These streaked plates and abiotic control were incubated at 30°C for four days (Labnet 211DS Shaking Incubator) without shaking.

3.11 Preliminary screening of the DDB2 isolate on the MacConkey agar

Preliminary screening was done to differentiate between lactose fermenting and non-lactose fermenting gram-negative bacteria by growing it on the MacConkey agar plates. MacConkey agar is a solid differential medium used to isolate gram-negative enteric bacteria and to differentiate lactose and non-lactose-sugar-fermenting bacteria. Only bile salt-resistant bacteria, particularly the members of the Enterobacteriaceae family and the genus

Pseudomonas, can grow, and gram-positive bacteria cannot grow on this medium. Lactose fermenters produce a red to pink colour due to a drop in pH, and neutral red is absorbed by the bacteria, while non-lactose fermenters give a pale to transparent colour. Strong lactose fermenters have a pink halo around colonies due to bile salt precipitation by the acid produced. Weak lactose fermenters may appear red or pink, but don't have a halo around colonies [123].

3.12 Potassium hydroxide string Test

3% KOH was placed on a clean and grease-free microscopic slide. A loop of an isolated, discrete colony was spread on the drop and stirred continuously till a suspension was formed. Formation of a mucoid string indicates that the bacteria is gram-negative, and if the suspension remains fluid it indicates that the bacteria is gram-positive. In gram-negative bacteria, 3% KOH breaks down the cell wall of the bacteria and releases viscid chromosomal materials, which gives the thick string texture [124].

3.13 Biochemical characterisation

a) Catalase test: The catalase test was carried out using the slide drop method. A sterile loop was used to pick a single bacterial colony and spread it on a clean microscope slide. Then, a drop of 3% hydrogen peroxide (H_2O_2) was added using a Pasteur pipette to observe the reaction. The formation of bubbles of O_2 indicates the presence of the catalase enzyme [125].

b) Starch hydrolysis test: To evaluate starch-degrading activity, the bacterial culture was streaked on starch agar plates under aseptic conditions (RH-58 LAF Horizontal, Rescholar Equipment, India). The inoculated plates were incubated at 37 °C (BOD Incubator (Super Deluxe), Macflow Engineering, India), overnight. Following incubation, the agar surface was flooded with Gram's iodine, which interacts with residual starch. A clear halo around the microbial growth signifies the breakdown of starch by amylase, indicating a positive result. In contrast, uniform dark staining without a clear zone denotes a negative reaction, implying the absence of starch hydrolysis [126]. **(A-2)**

c) Detection of PHB inclusions using Sudan Black B Staining

Sudan Black B staining was used to visualize intracellular poly-β-hydroxybutyrate (PHB) inclusions in bacterial cells. A clean glass slide was used to prepare a thin smear of the bacterial

culture, which was then left to air dry. Once dried, the slide was heat-fixed by quickly passing it through a flame. The fixed smear was stained with 0.3% Sudan Black B solution (prepared in 70% ethanol) for a duration of 10 minutes. Following staining, the slide was carefully rinsed with 70% ethanol to eliminate excess dye until the smear background appeared clear. It was then washed with distilled water. Subsequently, safranin as counterstain was applied for 30 seconds to provide contrast. After a final wash with distilled water, the slide was allowed to dry, and microscopic examination was carried out under oil immersion (100x objective lens).PHB granules appear as black to bluish structures [126]. *B. subtilis* was used as a positive control. **(Appendix-3)**

d) Growth at 42° C

To determine the ability of bacterial isolates to grow at elevated temperatures—a distinguishing trait of *Pseudomonas aeruginosa*—cultures were incubated at 42°C (B.O.D. INCUBATOR SHAKER, Macflow Engineering Pvt Ltd.). Under aseptic conditions, a small inoculum of the test isolate was quadrant streaked onto sterile MacConkey agar. The inoculated plates were then incubated at 42°C for 24 to 48 hours [126], [127].

3.14 Diesel utilization test

The diesel utilization test protocol was designed to visually inspect the bacterial growth in the presence and absence of diesel. Abiotic or negative control does not have bacteria in it, while the other control has no diesel but bacteria in it. In this experiment evaluation of diesel as a potential carbon source was done.

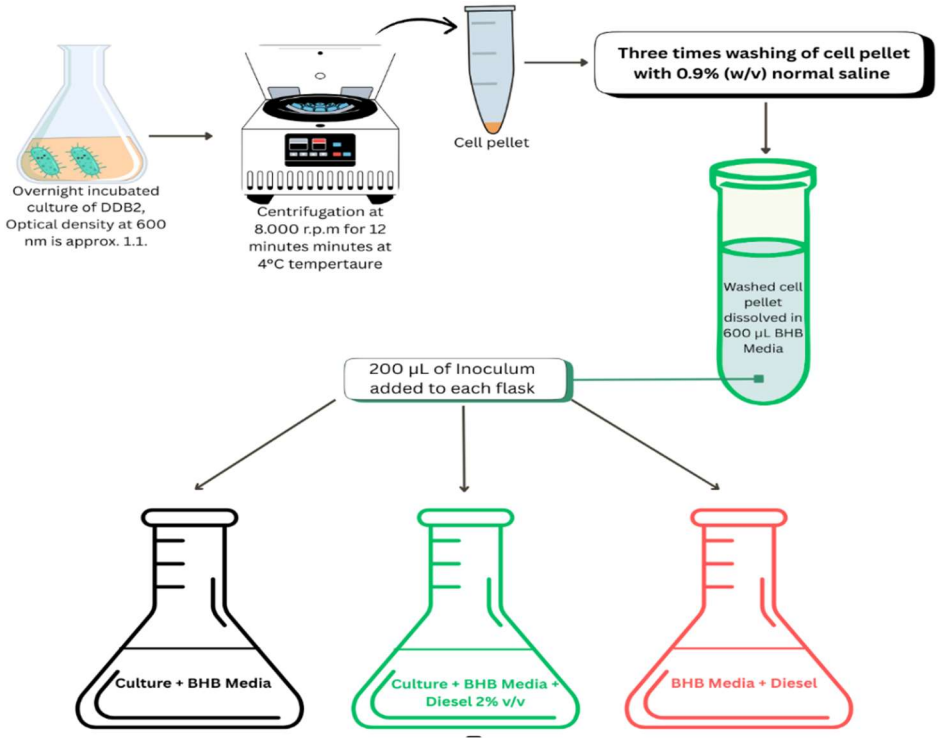

Figure 3.5: Experimental design for hydrocarbons utilization test.

The first step was the inoculum preparation i.e., overnight culture of DDB2 bacteria with an $OD_{600} \approx 1.1$. Centrifugation (Eppendorf Centrifuge 5804 R) of this culture was performed at 8,000 rpm for 12 minutes at 4°C. After that, the cell pellet was washed three times with 0.9% NaCl normal saline. The washed pellet was then resuspended in 600 µL of BHB (Bushnell Haas Broth) media. The second step was the inoculation setup step, in which 200 µL of inoculum was added to each flask:

- **Flask 1 (Biotic Control)**: BHB Media + Culture
- **Flask 2 (Test)**: BHB Media + 2% Diesel (v/v) + Culture
- **Flask 3 (Negative Control or Abiotic Control)**: BHB Media + 2% Diesel (v/v) (no culture)

The third step was to visually inspect the bacterial growth after seven days.

3.15 Diesel tolerance test of DDB2 bacteria

The range of the diesel concentration studied in the tolerance test was between 5% (v/v) to 25% (v/v) of diesel [128]. For the diesel tolerance test, 100 µL of inoculum was added from an overnight-grown fresh culture to different Tarson tubes of size 50 mL, having BH broth and diesel at different concentrations, 5, 10, 15, 20, 25 % (v/v), in triplicates [128] [129] respectively. The cultures were incubated at 30° C in an orbital shaker at 150 rpm (Labnet 211DS Shaking Incubator), ensuring proper oxygenation of the samples. Absorbance of the samples was measured after a seven-day incubation period to check the effect of varying diesel concentration on bacterial growth using a spectrophotometer (SHIMADZU UV-1900i UV-VIS Spectrophotometer). Before the measurement of each sample, the sample was agitated at very low rpm for 20-30 seconds, to ensure that no diesel emulsions form, to get accurate readings in triplicates [130] [131].

3.16 Antimicrobial susceptibility test (AST) of DDB2

To perform the AST of DDB2, the disk diffusion method was employed. For the standardisation of the inoculum, 0.5 McFarland standard having an OD_{600} of 0.098 (0.5 McFarland standard corresponding to approximately 1.5×10^8 CFU/mL [132]) was used. The turbidity of the inoculum, prepared in normal saline, was visually matched to the 0.5 McFarland standard. The standardised inoculum was spread uniformly with sterile cotton swab on Mueller-Hinton agar plate under a laminar air flow hood cabinet (RH-58 LAF Horizontal, Rescholar Equipment, India). Antibiotic disks were placed gently with the help of sterile tweezers. Incubation of the plate was done at 37° C overnight (BOD Incubator (Super Deluxe), Macflow Engineering, India), and the results were noted by measuring the zone of inhibition [133].

0.5 McFarland standard was prepared by mixing 0.05 mL of 1.175% $BaCl_2$ with 9.95 mL of 1% H_2SO_4, and then vortexed to get 0.5 McFarland standard. OD was measured at 600nm, and it was 0.098, which was under the prescribed limits [132].

3.17 Molecular identification of the bacterial isolate DDB2

The bacterial isolate DDB2 was identified using *16S rRNA gene* sequencing; this process was outsourced to Barcode Biosciences, Bangalore, an ISO 9001:2015 certified laboratory. Genomic DNA (gDNA) of high quality was isolated and confirmed by visualisation on a 1% agarose gel. Amplification of the *16S rRNA gene* was achieved using universal primers (*16SrRNA*-F and *16SrRNA*-R), resulting in a distinct amplicon of around 1500 base pairs. The PCR product was then purified and subjected to Sanger sequencing (ABI 3730xl Genetic Analyzer). The obtained forward and reverse sequences were assembled to form a consensus sequence, which was subsequently compared with sequences in the NCBI GenBank 'nr' database using BLASTn [134], [135]. For phylogenetic evaluation, the consensus sequence was analysed in MEGA11 software **(A-6)** [136] using the Maximum Likelihood approach based on the Tamura-Nei model. **(Appendix-4 and Appendix-5)**

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of the hydrocarbons-degrading strains

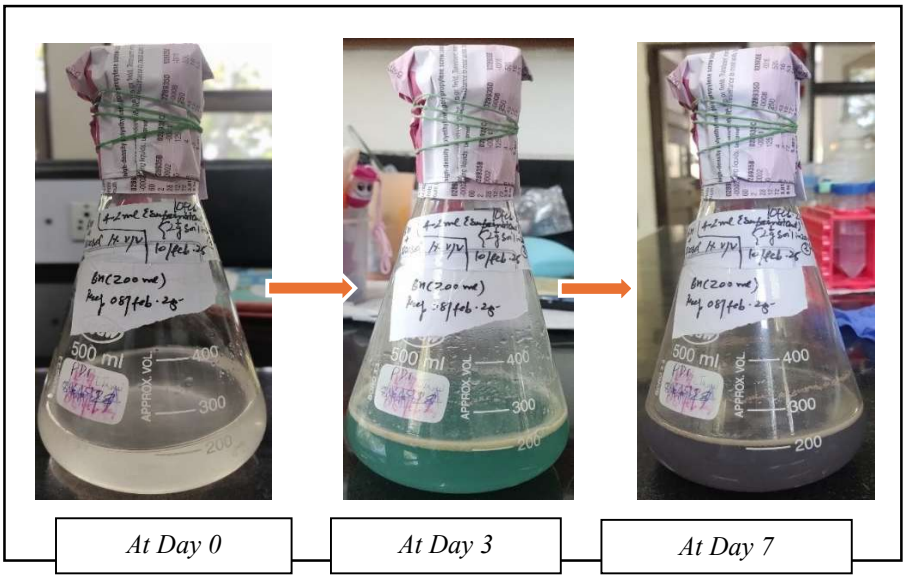

Figure 4.1: Colour change of enrichment medium with time.

pH: pH of the sample soil was 8.16.

On Day 0: The medium's initial visual appearance before microbial activity or expected hydrocarbon degradation was observed to be pale and translucent.

On Day 3: The colour of the media changed to greenish. The greenish appearance of the media indicated the growth of microorganisms and their initial adaptation to hydrocarbons. The proliferation of Pseudomonas species, occasionally creates pigments like pyocyanin [137] under hydrocarbon stress, or the synthesis of certain metabolites, may be connected to this colour shift.

On Day 7: The colour of the media changed to dark brown, possibly due to the production of some pigments or metabolites [137].

4.2 pH measurement of enrichment culture

The pH of the media after seven days of incubation was measured. pH on day 7 (after the first enrichment step) was 5.8. The pH of the media after the second sequential enrichment (after 7 days) was 5.28, and after the third sequential enrichment, the pH of the media was 5.0. The pH change could indicate the formation of some metabolites during diesel degradation, and formation of several organic acids [138].

4.3 Enumeration of the bacteria

Bacteria were enumerated using the serial dilution method for the control sample, after the second and third enrichment steps using selective media. CFU/mL after five days of incubation in the control sample (petroleum- associated soil in NB) was 1.04×10^8, after second enrichment (BH + 1% diesel v/v) was 8.9×10^8, and after third enrichment (BH + 1% diesel v/v) was 2.7×10^8.

4.4 Selection of morphologically distinct colonies

Four morphologically distinct colonies were selected.

Table 4: Observation table for the above-selected colonies.

Colony number and name	Shape	Colour	Margin	Elevation	Opacity and texture	Size	Surface
1 (DDB1)	Circular	White	Smooth (entire)	Raised	Opaque and buttery	Comparatively medium-size	Smooth and shiny
2 (DDB2)	Circular	Cream	Smooth (entire)	Convex	Opaque and buttery	Largest of all	Smooth and slightly matte (but not shiny)
3 (DDB3)	Circular	White/Cream	Entire (smooth and well-defined)	Raised	Opaque and buttery	Comparatively small in size	Smooth and slightly glossy
4 (DDB4)	Circular	White	Entire	Convex	Opaque and buttery	Smallest of all	Smooth and slightly glossy

An important observation was that there was a greenish tint to the white colour of all the colonies.

4.5 Gram staining of the isolated bacterial strains

All four morphologically selected isolates, DDB1, DDB2, DDB3, and DDB4, were gram-negative and appeared pink under the microscope. They did not retain the crystal violet stain and took up the counterstain (safranin), thus appearing pink due to their thin peptidoglycan layer.

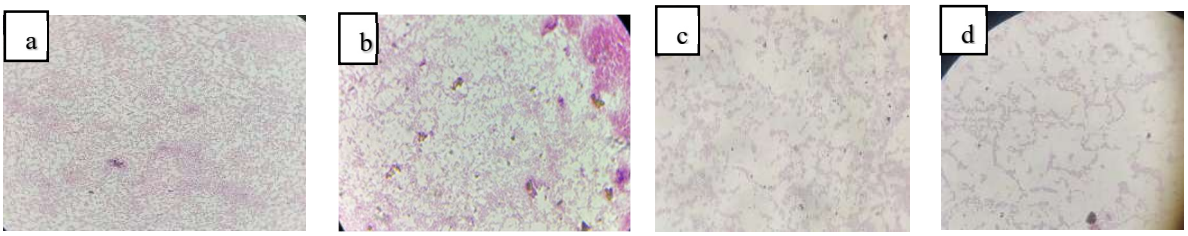

Figure 4.2: Gram-staining results of bacterial isolates under 100x magnification of the microscope. a) DDB1, b) DDB2, c) DDB3, and d) DDB4.

4.6 Cryopreservation of isolated bacterial strains

Figure 4.3: Cryovials containing different isolated bacteria (DDB1 to DDB4).

4.7 Growth on diesel for screening of hydrocarbon degraders

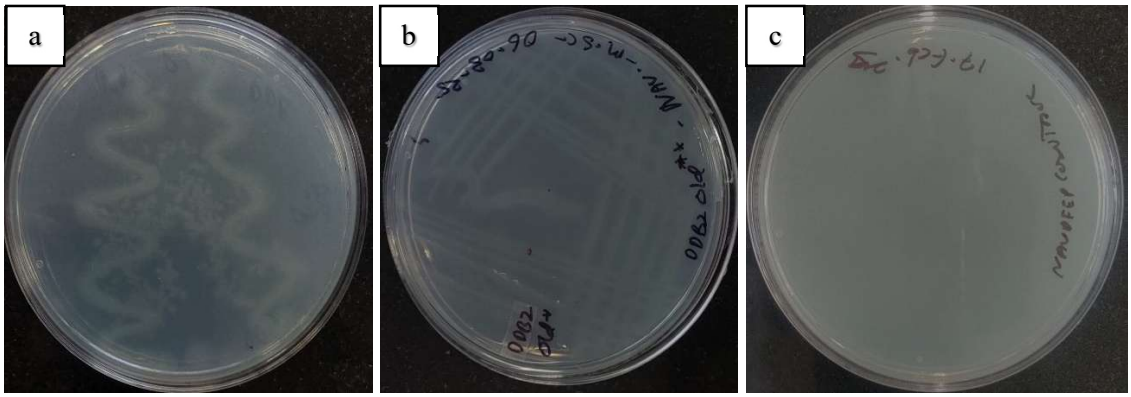

Figure 4.4: a) Continuous streak of DDB2 isolate on diesel spread BH agar plate after four days of incubation; b) Quandrant streak of DDB2 on diesel spread BH agar plate after four days of incubation; c) Negative control *E. coli* on diesel spread BH agar plate.

DDB2 isolate showed positive and fast growth on the diesel-aided BH agar. The DDB2 isolate's growth was observed to extend out toward the nearby surface diesel, growing beyond its original streak path. DDB2 was thus recognised as the most potent hydrocarbon degrader out of other isolates thus it was selected for further analysis.

4.8 Preliminary screening of the DDB2 isolate on the MacConkey agar

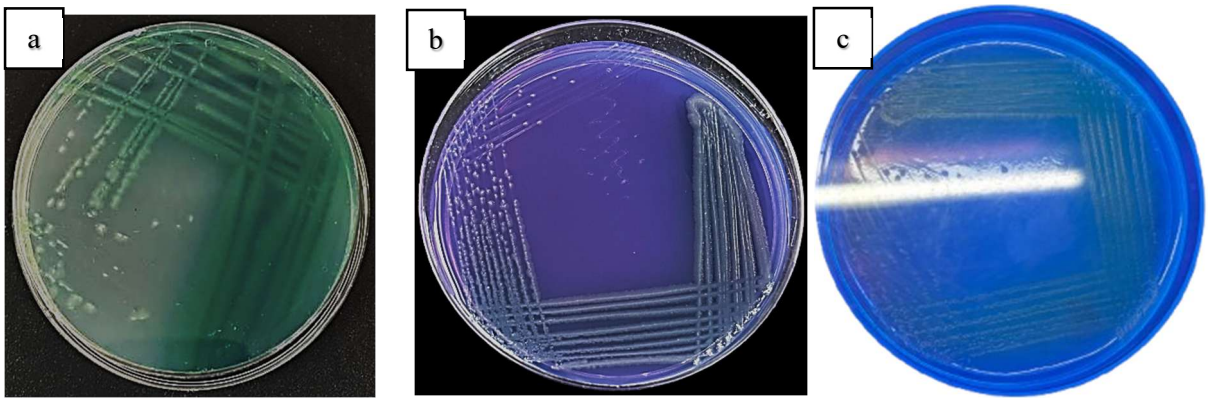

Figure 4.5: a) DDB2 bacterial isolate on nutrient agar showing green tint; b) DDB2 on MacConkey agar with bromocresol blue indicator; c) DDB2 showing fluorescence under UV lamp light

DDB2 produced green coloured pigment on the nutrient agar, which covered the whole plate surface. Some species of Pseudomonas, like *Pseudomonas aeruginosa,* are known to produce commercially viable soluble pigments of various colours. It produces blue coloured pigment pyocyanin, yellow green-coloured pigment pyoverdin, red coloured pyorubin and brown coloured pyomelanin. Pyocyanin pigment has anti-fungal properties [137].

DDB2 produced a green tint, which can be seen around the bacterial growth under ultraviolet radiation. The colonies were pale yellow. *Pseudomonas aeruginosa* is known to produce fluorescent green coloured pigment on MacConkey agar, which can be seen under ultraviolet radiation [139].

4.9 Potassium Hydroxide String Test

All the bacterial isolates were gram-negative bacteria, forming a string during the KOH string test.

4.10 Biochemical characterisation.

a) Catalase test: Bubble formation was observed instantly after hydrogenperoxide was added onto the culture. Thus, all the bacterial isolates (DDB1 to DDB4) were catalase positive.

b) Starch hydrolysis test

Figure 4.6: Negative result of starch hydrolysis test for DDB2, a) bacterial plate before test; b) bacterial plate after test showing no zone of clearance or halo around the bacterial growth.

The starch hydrolysis test for DDB2 yielded a negative result, indicated by the absence of any clear zone or halo surrounding the bacterial growth.

c) Detection of PHB inclusions using Sudan Black B Staining

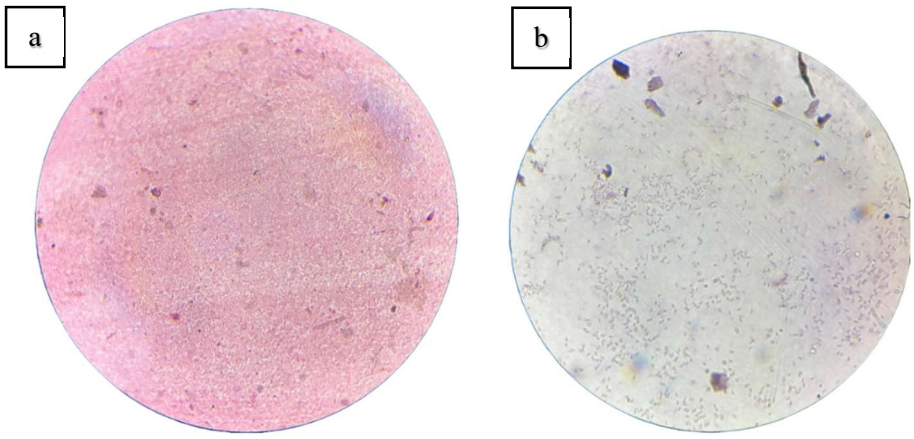

Figure 4.7: a) Sudan Black B staining of DDB2 under 100X magnification of light microscope; b) Sudan Black B staining of the positive control i.e., *Bacillus subtilis* under 100X magnification of light microscope.

Sudan Black B staining showed the absence of PHB inclusions in DDB2, while the positive control displayed distinctive blue-black granules indicating PHB accumulation.

d) Growth at 42° C

Figure 4.8: a) Plate showing overnight growth of DDB2 at 42° C

Bacterial isolate DDB2 exhibited clear growth following overnight incubation at 42°C, suggesting its thermotolerant nature— a characteristic commonly associated with *Pseudomonas aeruginosa* [127].

Table 5: Distinctive Identification Tests for *Pseudomonas aeruginosa* and their results (Based on Bergey's Manual [127]).

S. no.	Test name	Result
1.	Catalase test	+
2.	Starch hydrolysis test	-
3.	PHB accumulation	-
4.	Growth at 42° C	+
5.	Pigment production	+

Based upon the results (in table 6) and the observed characteristics— including Gram-negative rod morphology, positive test for catalase, growth at 42°C, and the production of a bluish-green pigment accompanied by a fruity, grape-like odour— are indicative of the organism *Pseudomonas sp*. These results aligned with the identification criteria outlined in [127].

4.11 Diesel tolerance test of DDB2 bacteria

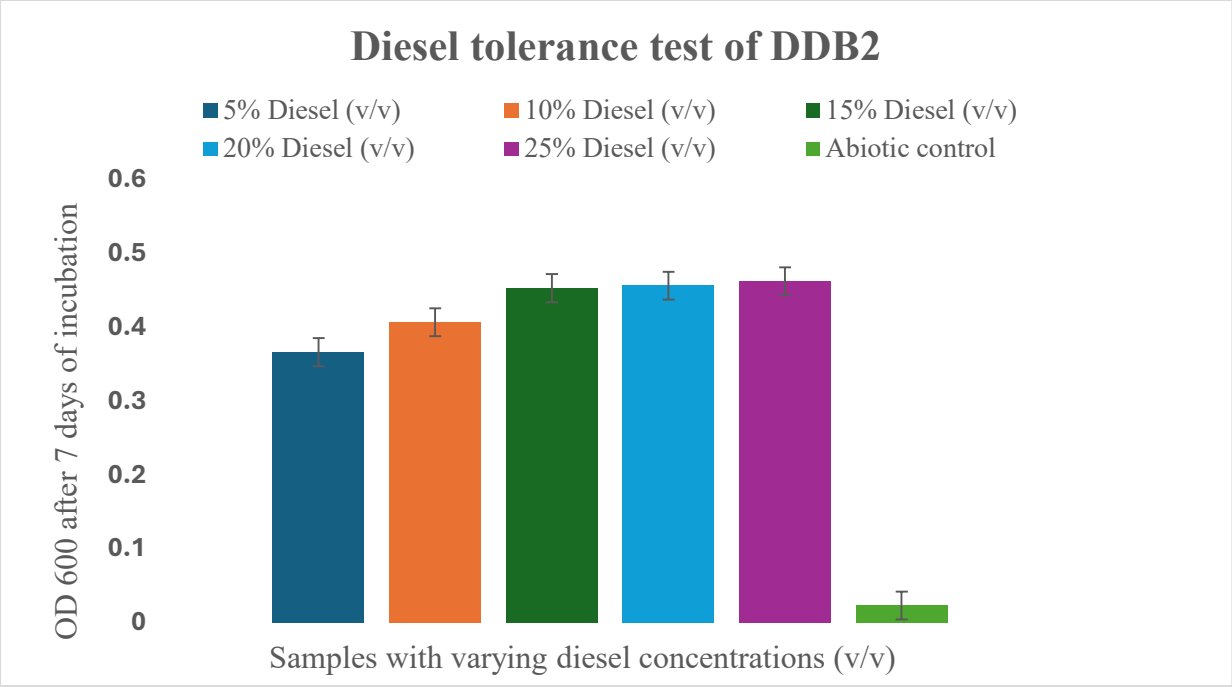

Figure 4.9: Growth response of DDB2 at different diesel concentrations.

The growth of DDB2 increased with diesel concentration from 5% to 25% (v/v), and peaked around 20–25%. This indicated that this bacterial isolate is highly tolerant to the different increasing diesel concentrations. At high diesel concentrations, the bacterial growth was high

due to more carbon source for the utilization as an energy source. A highly tolerant bacterial strain will be very beneficial for further application purposes.

4.12 Diesel utilization test

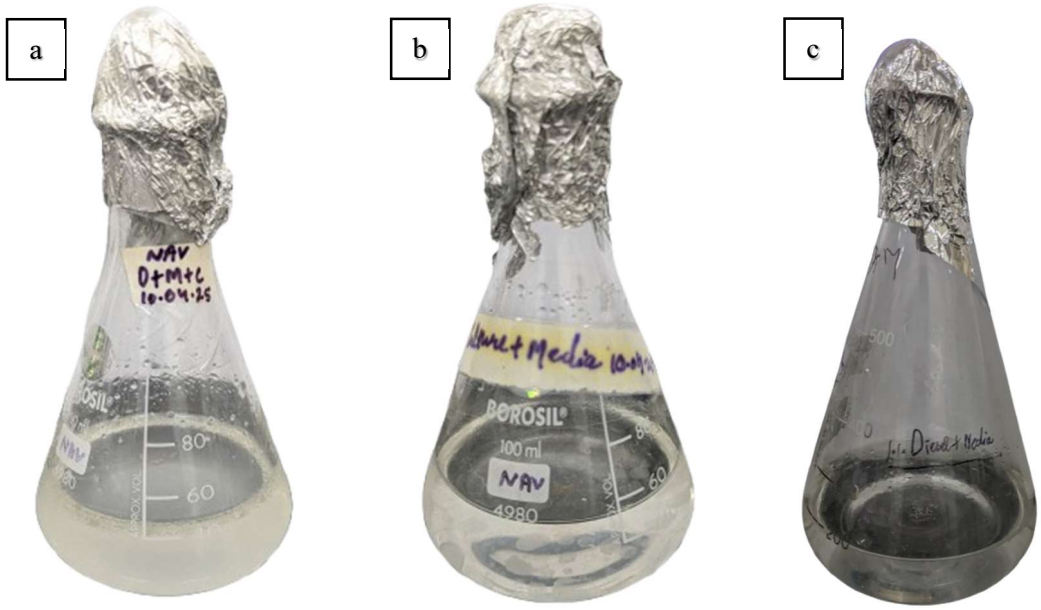

Figure 4.10: a) Turbidity in test flask after 24-hour incubation; b) Clear biotic control after 24 hours of incubation; c) Clear abiotic control after 24 hours of incubation.

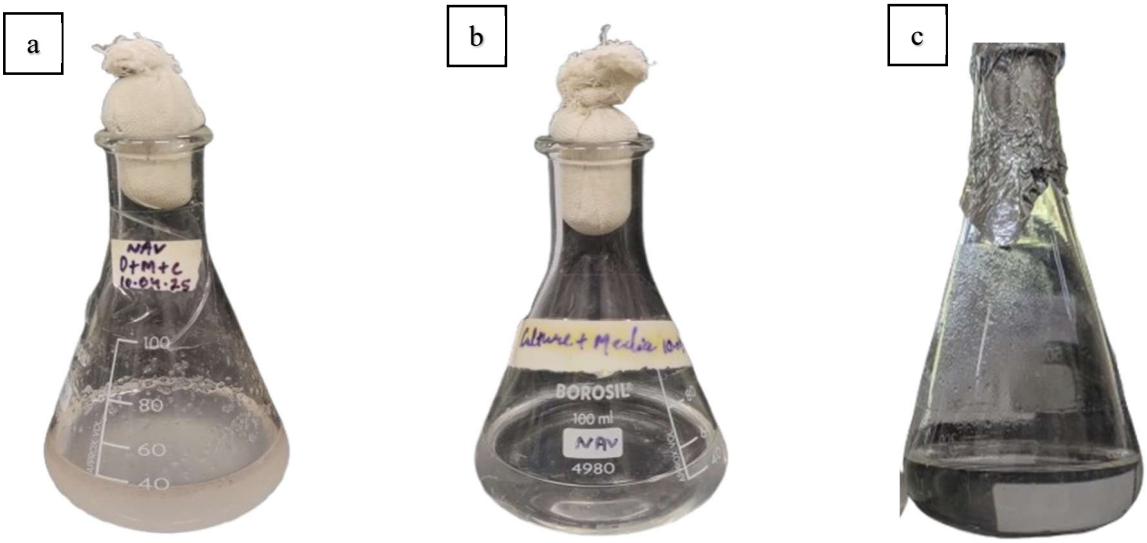

Figure 4.11: a) High turbidity in test flask after five days of incubation; b) Clear biotic control after five days of incubation; c) Clear abiotic control after five days of incubation.

Results: Turbidity is a sign of microbial growth; this is only seen in the test flask containing diesel and the test organism. The lack of turbidity in the biotic control indicated the inability of the organism to grow without diesel. Absence of turbidity in the abiotic control indicated that the procured diesel was not contaminated.

4.13 Antimicrobial susceptibility test (AST) of DDB2

We also conducted the AST to check the strain's ability to grow in the presence of different standard antibiotics. The rationale of this study was to check the pathogenic nature of the isolated bacterium as it belonged to the genus *Pseudomonas* which is known to include opportunistic pathogens [140].

Table 6: AST results for DDB2, diameters of the zones of inhibition of different antibiotics.

Sample ID	Antibiotic	Disc Concentration (µg)	Zone of Inhibition (mm)	Interpretation (S/R)
DDB2	Imipenem	10	35	Susceptible
DDB2	Gentamicin	120	28	Susceptible
DDB2	Doripenem	10	29	Susceptible
DDB2	Ceftazidime	30	13	Resistant
DDB2	Cefepime	30	No zone	Resistant

Interpretations of results according to [141].

Interpretations of results: We found that the strain was susceptible to Imipenem, Gentamicin, and Doripenem. However, resistance was observed for some antibiotics of beta-lactams group-Ceftazidime and Cefepime.

4.14 Molecular identification of the bacterial isolate DDB2

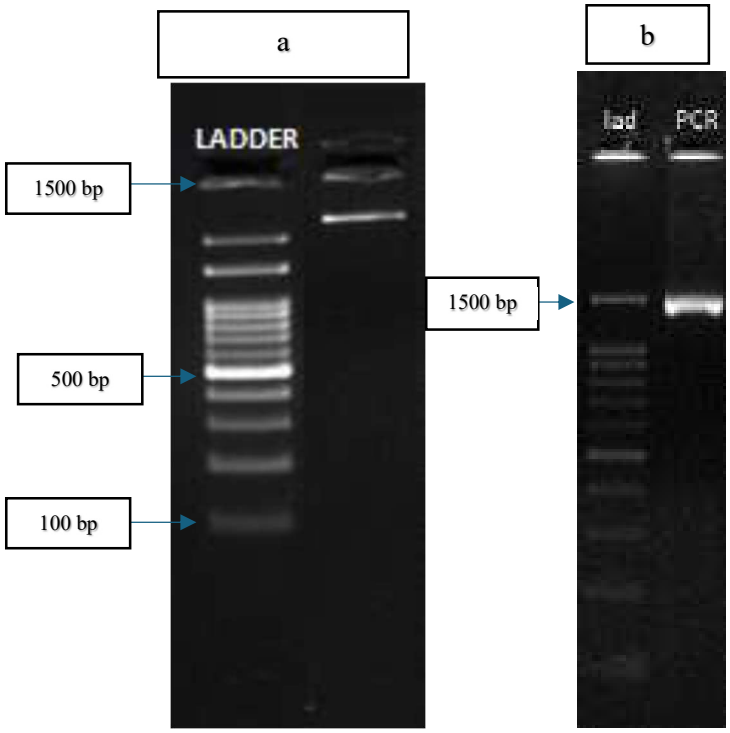

Figure 4.12: a) Isolated gDNA of DDB2; b) PCR product of around 1500 bp **(A-4)**

Sanger sequencing chromatogram data and consensus sequence (A-5)

>Forward Seq data

GGGCCCGTACGAATGTCGACTAGCCGTTGGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCG
CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGG
GAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGC
AACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCA
AGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG
GAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGG
TGAAGTCGTAACAAGGTAACCAGAA

>Reverse complement

ACCCTTAGAGTTTGATTCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAAGG
GAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCC
GGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAG
CCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATC
AGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAA
GCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGT
AAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC
GAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATC
CCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTA
GCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGG
ATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGAGTCCGCCGACC

>Consensus Data

CCTTAGAGTTTGATTCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTC
GAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGA
ATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGA
GGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCT
AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCA
GTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTG
TAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACC
AACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAA
TCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGT
GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAA
GGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATC
TTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCT
TCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCT
AAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGA
GGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGT
GAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGC
AAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACC

a

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas aeruginosa strain PpB3 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2767	2767	100%	0.0	99.93%	1506	PV274339.1
Pseudomonas aeruginosa strain LS8 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2767	2767	100%	0.0	99.93%	1505	PV133734.1
Pseudomonas sp. strain 2B 16S ribosomal RNA gene, partial sequence	Pseudomonas sp.	2763	2763	100%	0.0	99.93%	1503	PP859489.1
Uncultured bacterium clone P7D1-420 16S ribosomal RNA gene, partial sequence	uncultured bacterium	2760	2760	100%	0.0	99.93%	1497	EF509253.1
Streptomyces sp. SCSIO 04777 16S ribosomal RNA gene, partial sequence	Streptomyces sp. SCSIO 04777	2760	2760	100%	0.0	99.87%	1505	KC814697.1
Pseudomonas aeruginosa strain LCS1 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2760	2760	100%	0.0	99.93%	1497	MK430420.1
Pseudomonas aeruginosa strain K3 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2760	2760	100%	0.0	99.93%	1500	EF064786.1
Pseudomonas aeruginosa strain BPT11 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2760	2760	100%	0.0	99.93%	1497	OM541664.1
Pseudomonas aeruginosa strain IHB B 6863 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2760	2760	100%	0.0	99.93%	1500	KF668476.1
Pseudomonas aeruginosa 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2760	2760	100%	0.0	99.93%	1501	DQ115539.1

Figure 4.13: NCBI's nBLAST, showing top ten results [134], [135] .

b

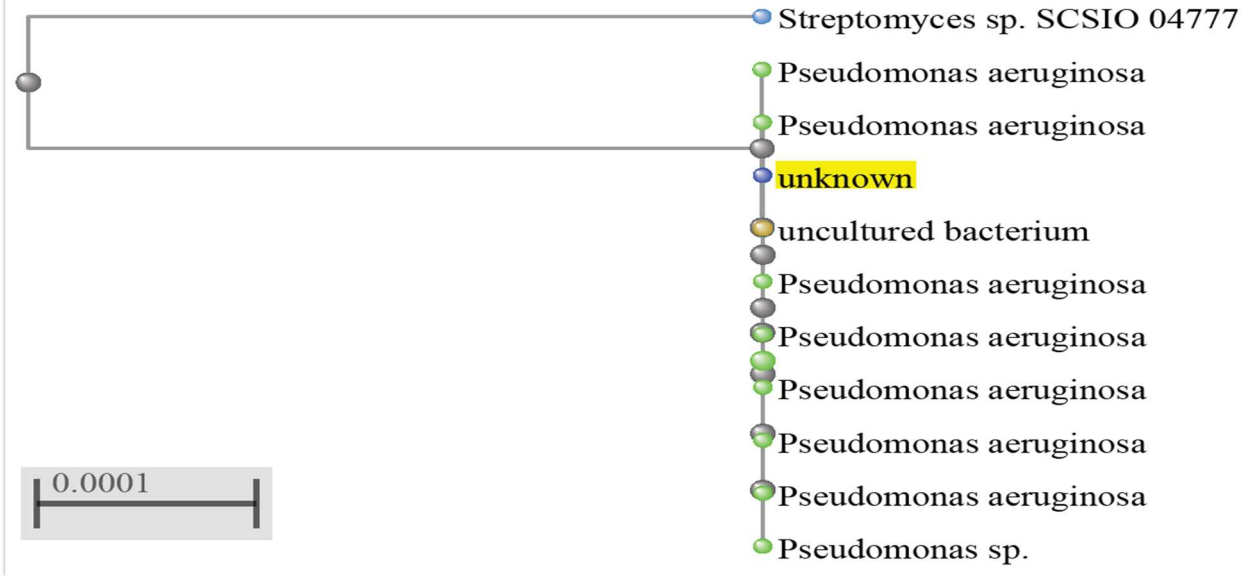

Figure 4.14: Results in BLAST tree view, Query ID: lcl|Query_135185 [134], [135]

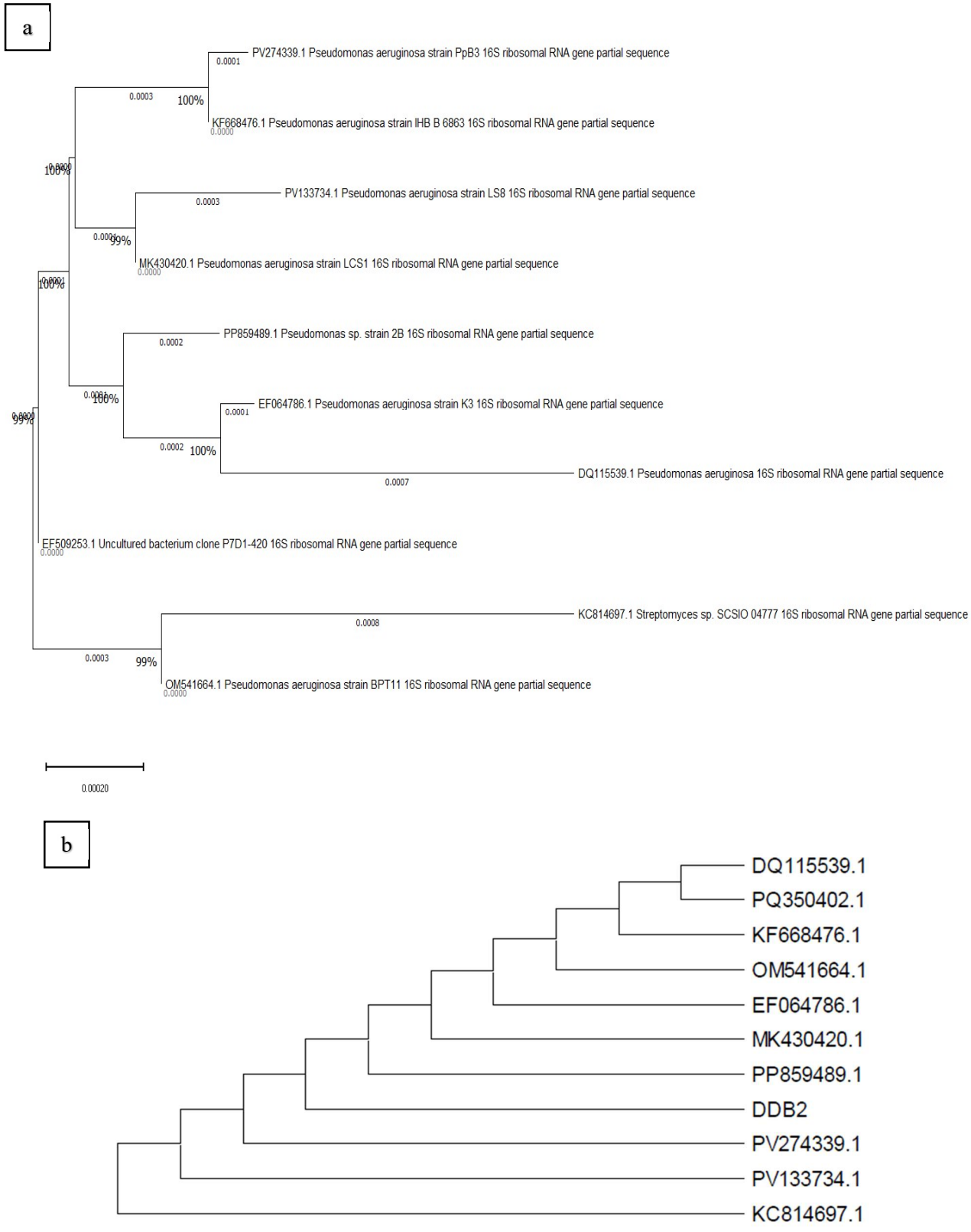

Figure 4.15: a) Phylogenetic tree constructed in MEGA11 software **(Appendix-6)** [136]; b) Phylogeny tree provided in the MID report.

Table 7: Sequences producing significant alignments.

Description	Max Score	Total Score	Query Cover	E Value	% Identity	Accession
Pseudomonas aeruginosa strain PpB3	2767	2767	100%	0.00	99.93%	PV274339.1
Pseudomonas aeruginosa strain LS8	2767	2767	100%	0.00	99.93%	PV133734.1
Pseudomonas sp. strain 2B	2763	2763	100%	0.00	99.93%	PP859489.1
Streptomyces sp. SCSIO 04777	2760	2760	100%	0.00	99.87%	KC814697.1
Pseudomonas aeruginosa strain LCS1	2760	2760	100%	0.00	99.93%	MK430420.1
Pseudomonas aeruginosa strain K3	2760	2760	100%	0.00	99.93%	EF064786.1
Pseudomonas aeruginosa strain BPT11	2760	2760	100%	0.00	99.93%	OM541664.1
Pseudomonas aeruginosa strain IHB B 6863	2760	2760	100%	0.00	99.93%	KF668476.1
Pseudomonas aeruginosa	2760	2760	100%	0.00	99.93%	DQ115539.1
Pseudomonas aeruginosa strain GH01	2760	2760	100%	0.00	99.93%	PQ350402.1

Table 8: Distance matrix, estimates of evolutionary divergence between sequences.

DDB2		0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
PV274339.1	0.000		0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
PV133734.1	0.000	0.000		0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
PP859489.1	0.000	0.000	0.000		0.001	0.000	0.000	0.000	0.000	0.000	0.000
KC814697.1	0.001	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001
MK430420.1	0.000	0.000	0.000	0.000	0.001		0.000	0.000	0.000	0.000	0.000
EF064786.1	0.000	0.000	0.000	0.000	0.001	0.000		0.000	0.000	0.000	0.000
OM541664.1	0.000	0.000	0.000	0.000	0.001	0.000	0.000		0.000	0.000	0.000
KF668476.1	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000		0.000	0.000
DQ115539.1	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000		0.000
PQ350402.1	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	

Result: DDB2 was identified as *Pseudomonas aeruginosa*, exhibiting a high degree of similarity through nucleotide sequence homology and phylogenetic analysis. For strain level identification whole genome sequencing is required as mentioned in the chapter 6.

CHAPTER 5

CONCLUSION

5. Conclusion

The present study primarily focused on identifying and isolating hydrocarbon-degrading bacteria from soil contaminated by diesel near JUIT, which lies in the Himalayan region. The environment and human health are seriously threatened by hydrocarbon pollution, particularly which comes from petroleum-based products. The aim of the study was to isolate hydrocarbon utilising bacteria for their bioremediation potential. Four morphologically different bacterial strains (DDB1–DDB4) were recovered by sequential enrichment in Bushnell Haas broth supplemented with diesel. It was found that all four were catalase-positive and gram-negative. However, the isolate DDB2 showed the strongest growth among these when diesel was used as the only carbon source, suggesting that it has higher hydrocarbonoclastic potential.

Only the DDB2 isolate exhibited significant capabilities in diesel degradation, tolerance to differing diesel concentrations. Furthermore, it exhibited pigment production and fluorescence under UV light. The isolate demonstrated favourable outcomes in the diesel utilization test which indicates its potential to thrive in diverse and challenging environments. Molecular identification through 1*6S rRNA* sequencing validated its taxonomic classification. DDB2 classified as *Pseudomonas aeruginosa*.

This study emphasises the potential of utilising native microbial populations for the biodegradation of petroleum hydrocarbons in ecologically sensitive or fragile areas such as the Himalayas. The use of these bacteria provides an environmentally sustainable and economically viable approach to hydrocarbon pollution remediation while also maintaining the integrity of native microbial diversity by preventing the introduction of non-native species.

CHAPTER 6

FUTURE SCOPES

5. Future Scopes

One may further proceed with the following:

- Conduct whole-genome sequencing of DDB2 to identify the strain and particular genes involved in hydrocarbon degradation.
- Investigate the enzymatic pathways associated with diesel degradation.
- Formulate bacterial consortia to improve bioremediation efficacy.
- Investigate the degradation potential of additional petroleum products, such as polycyclic aromatic hydrocarbons (PAHs) and crude oil.

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APPENDICES

Appendix-1: Gram staining procedure

1. Smear Preparation: Spread a thin layer of bacterial sample onto a clean glass slide and let it air-dry fully.
2. Heat Fixation: Quickly pass the dried slide through a flame two to three times to affix the cells to the glass.
3. Application of Primary Stain: Cover the smear with crystal violet and allow it to sit for 1 minute.
4. First Rinse: Gently rinse the slide with water to remove any unbound stain.
5. Adding the Mordant: Apply Gram's iodine over the smear and leave it undisturbed for 1 minute to help lock in the dye.
6. Second Rinse: Lightly rinse the iodine off with water.
7. Decolourising Step: Apply alcohol or acetone-alcohol for about 10–30 seconds. Stop as soon as the purple dye no longer runs off.
8. Immediate Rinse: Rinse the slide at once with water to prevent over-decolourisation.
9. Counterstaining: Stain the smear with safranin and let it sit for 30 to 60 seconds to colour the Gram-negative cells.
10. Final Wash and Drying: Rinse the slide gently with water again, then blot dry using bibulous or absorbent paper.
11. Microscopic Observation: Examine the slide under a microscope—start at 10x, then switch to 100x oil immersion to observe whether bacteria appear purple (Gram-positive) or pink/red (Gram-negative).

Appendix-2: Starch hydrolysis test procedure

1. Take a starch agar plate that has been sterilized.
2. Inoculate the agar by drawing a single streak of the bacterial sample across the surface, following aseptic techniques.
3. Place the plate in an incubator set at 35–37°C and allow it to incubate for 24 to 48 hours.
4. Once incubation is complete, carefully cover the surface of the agar with iodine solution.
5. Look for a transparent area around the microbial growth to assess starch breakdown.

Appendix-3: Sudan Black B staining procedure

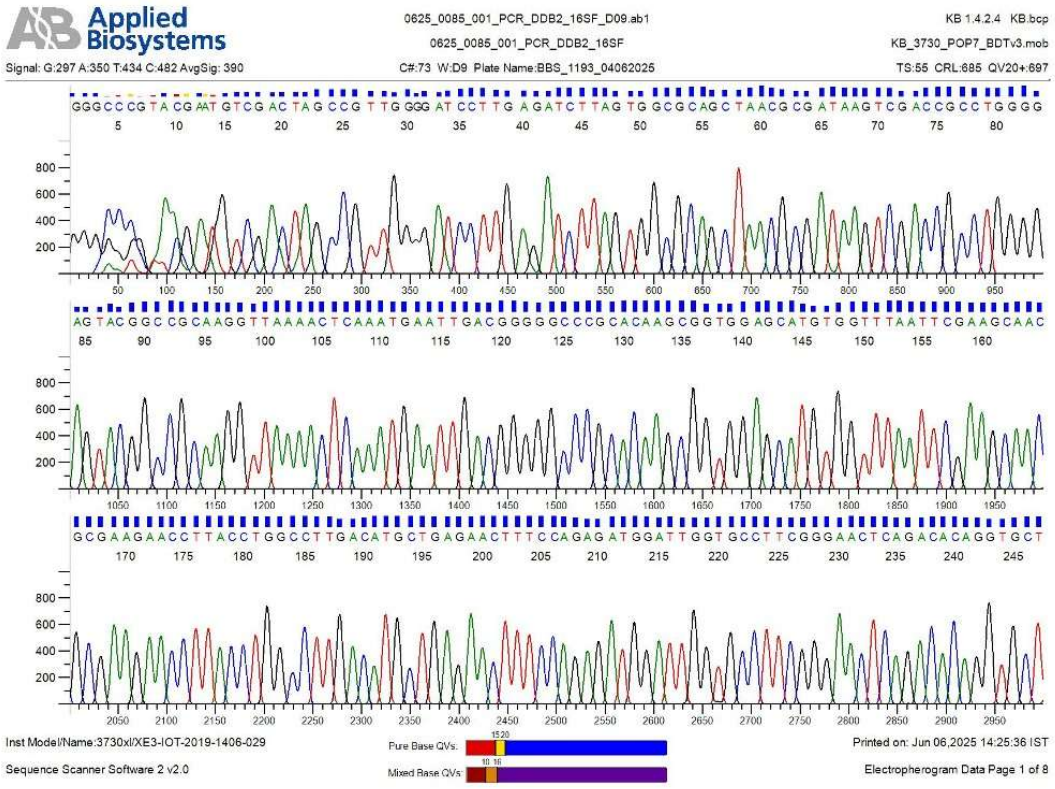
1. Begin by spreading a thin layer of the bacterial culture onto a clean glass slide and allow it to dry.
2. Once dried, fix the smear by swiftly passing it through a flame.
3. Next, cover the smear with 0.3% Sudan Black B solution and let it sit for approximately 10 minutes at RT.
4. After staining, gently wash the slide with 70% ethanol several times (two to three rinses) to remove any unabsorbed dye. Rinse the slide with distilled water to remove remaining ethanol.
5. Apply 0.5% aqueous safranin as a counterstain for 30 seconds to one minute. Rinse the slide again with clean water and blot it dry using bibulous paper.
6. Finally, observe the stained slide under an oil immersion lens (100x). Lipid or PHB inclusions will be visible as dark blue to black spots, while the rest of the cytoplasm will appear pink to light red.

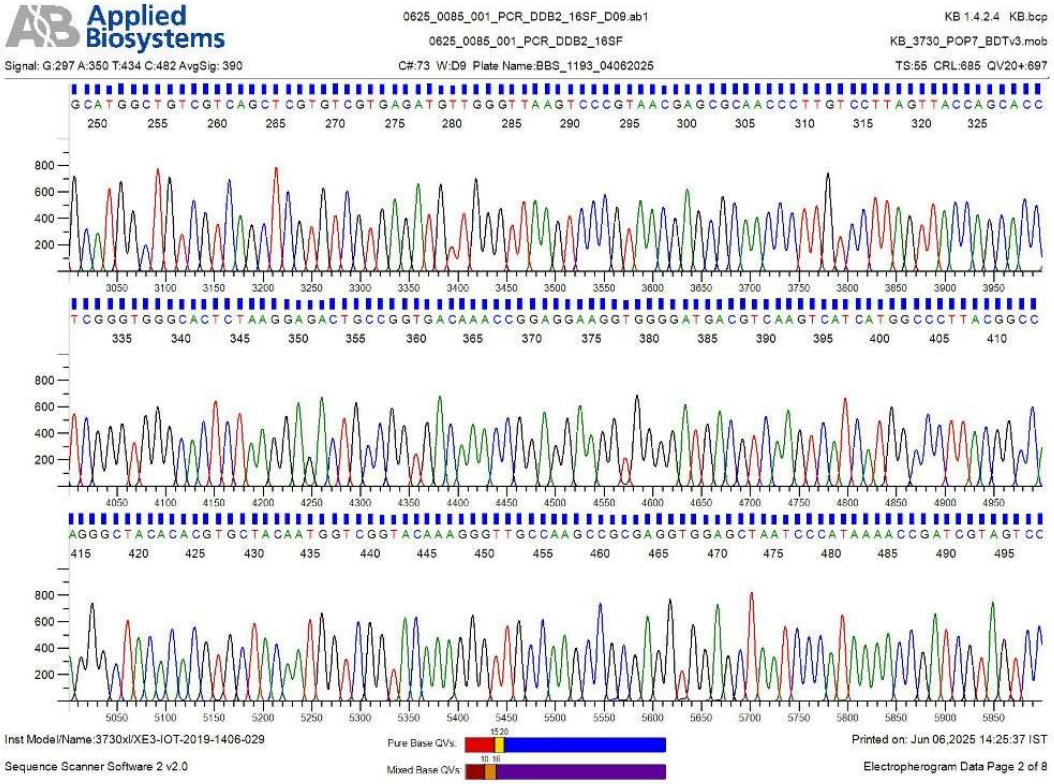
Appendix-4: Procedure of molecular identification using 16S rRNA-based molecular method

1. Genomic DNA was extracted from the bacterial culture "DDB2". Its quality was assessed by electrophoresis on a 1.0% agarose gel, which showed a single band representing high-molecular-weight DNA.
2. The *16S rRNA gene* fragment was amplified using universal primers (*16SrRNA*-F and *16SrRNA*-R). Gel electrophoresis of the PCR product revealed a single, distinct band approximately 1500 bp in length.
3. The amplified product was then purified to eliminate any residual contaminants.
4. Both forward and reverse sequencing of the purified PCR product were carried out using the same primer set (*16SrRNA*-F and *16SrRNA*-R) with the BDT v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer.
5. A consensus sequence of the *16S rRNA gene* was created by aligning the forward and reverse sequencing reads using alignment software.

6. The resulting sequence was submitted to a BLAST search against the NCBI GenBank 'nr' database [134], [135]. The top ten matches with the highest similarity scores were selected for multiple sequence alignment using Clustal W. A phylogenetic tree and distance matrix were then generated using MEGA11 software [136].

Appendix-5: Raw data of Sanger sequencing (Forward and Reverse Sequence) Forward Seq Data-




Applied Biosystems
0625_0085_001_PCR_DDB2_16SF_D09.ab1
KB 1.4.2.4 KB.bcp
0625_0085_001_PCR_DDB2_16SF
KB_3730_POP7_BDTv3.mob
Signal: G:297 A:350 T:434 C:482 AvgSig: 390
C#:73 W:D9 Plate Name:BBS_1193_04062025
TS:55 CRL:685 QV20+:697
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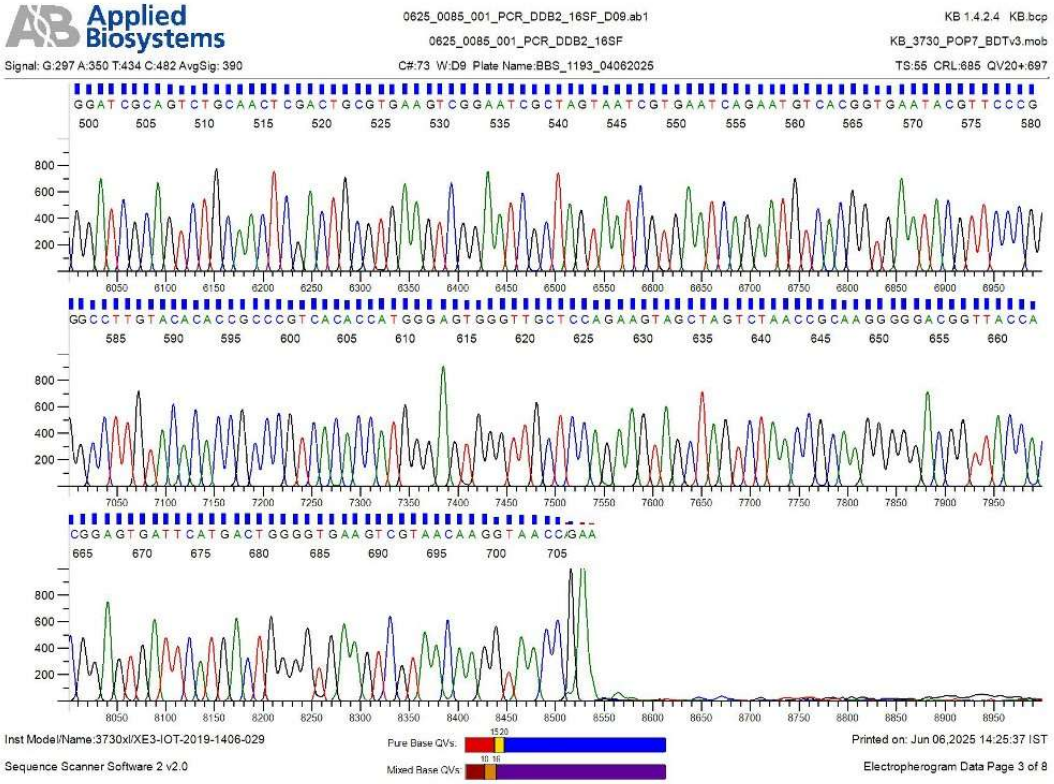
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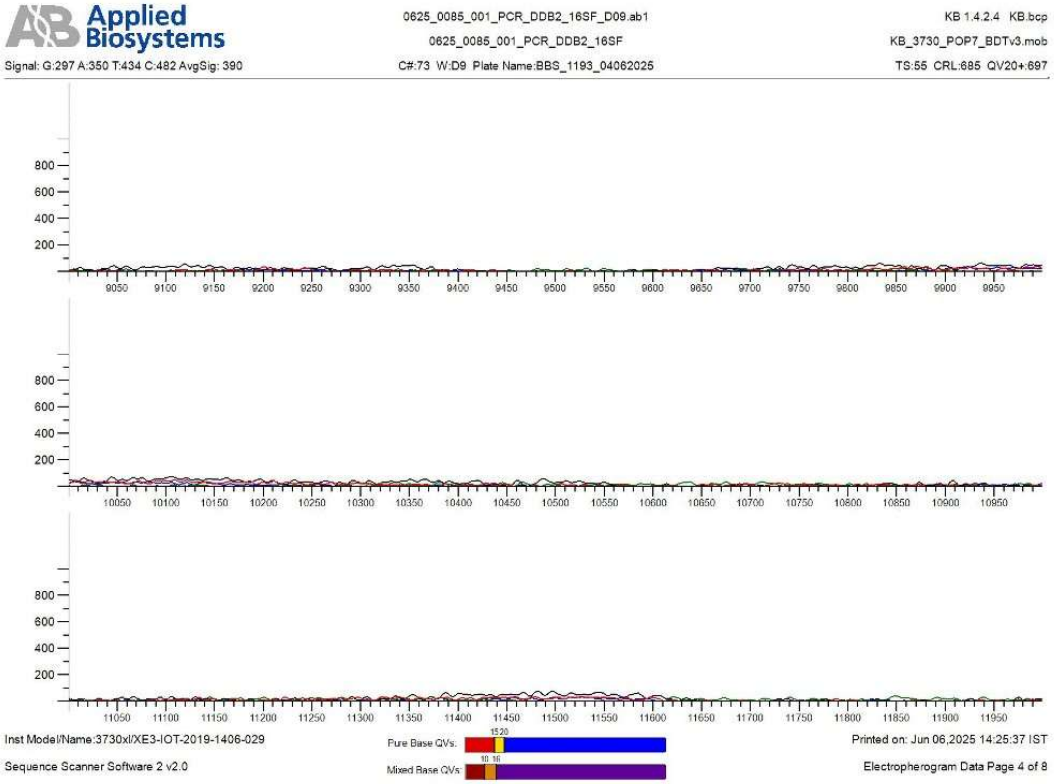
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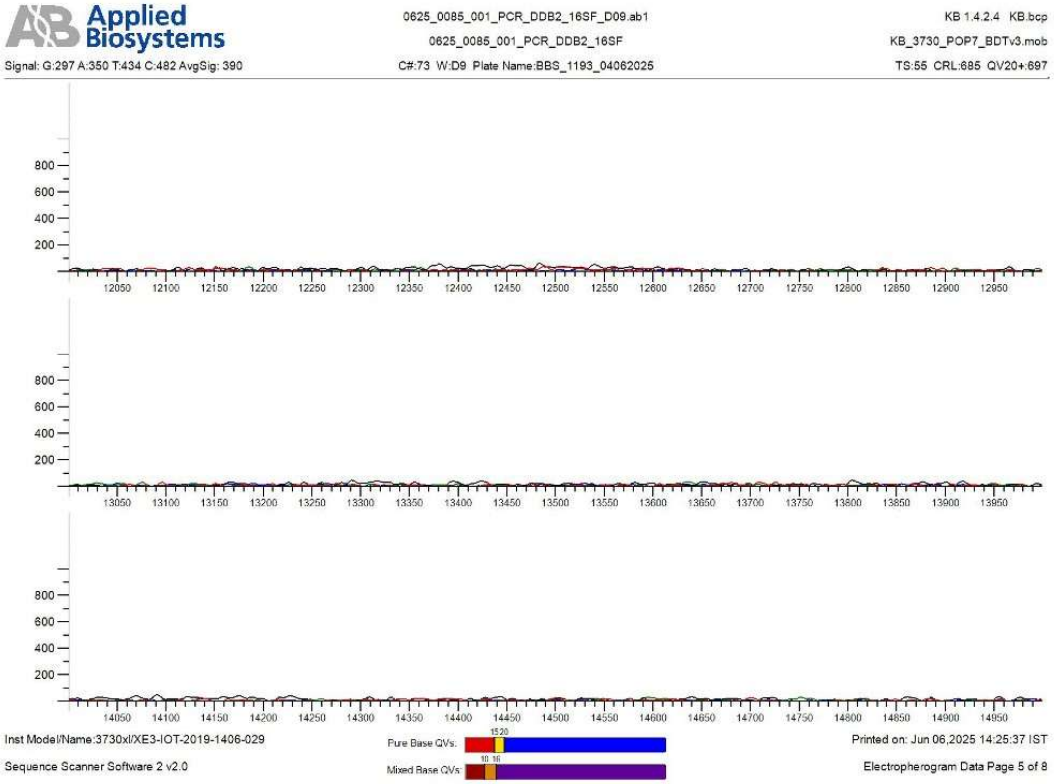
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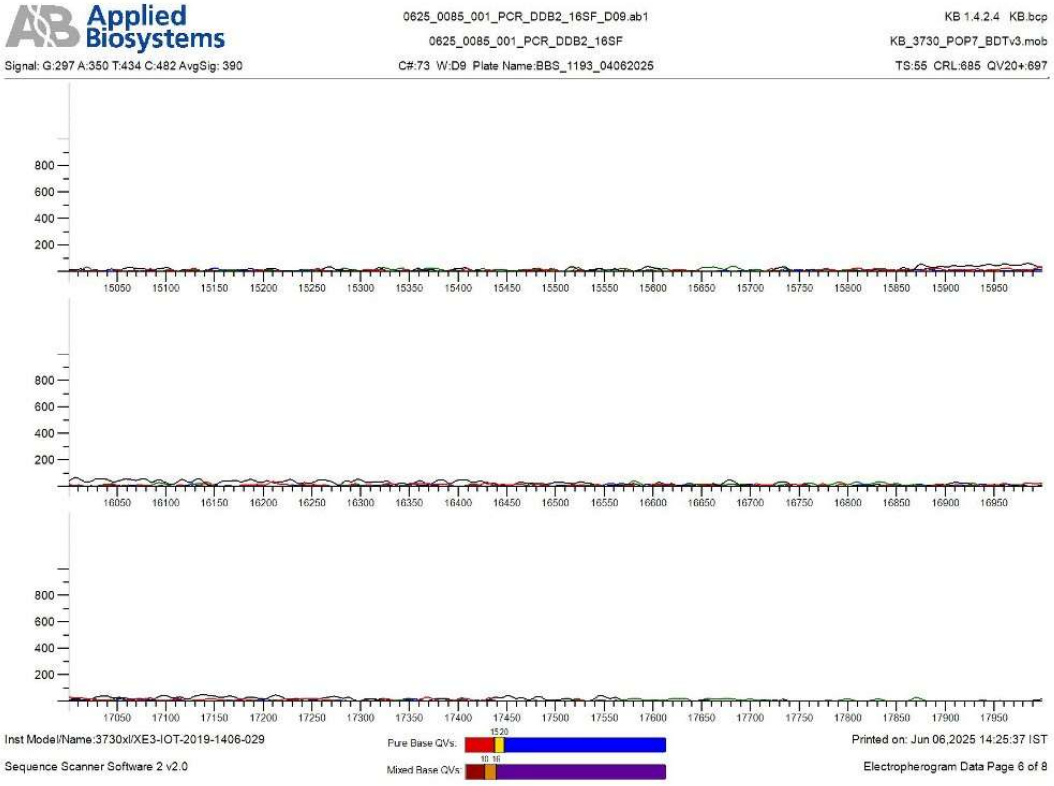
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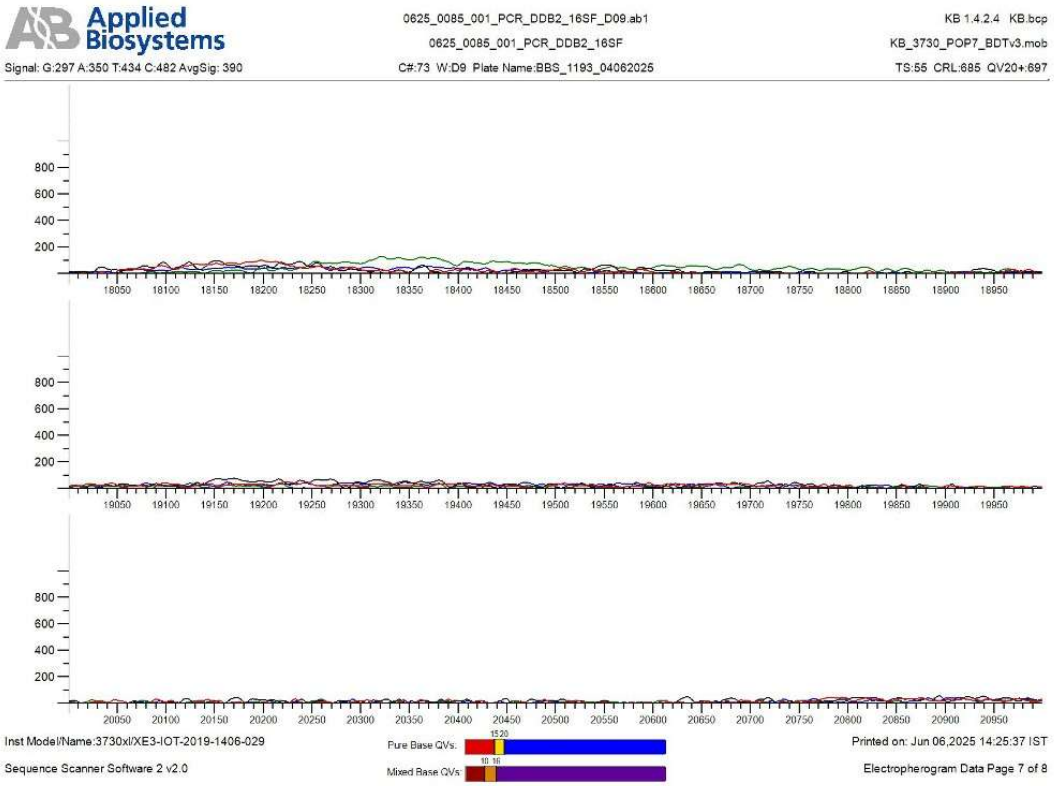
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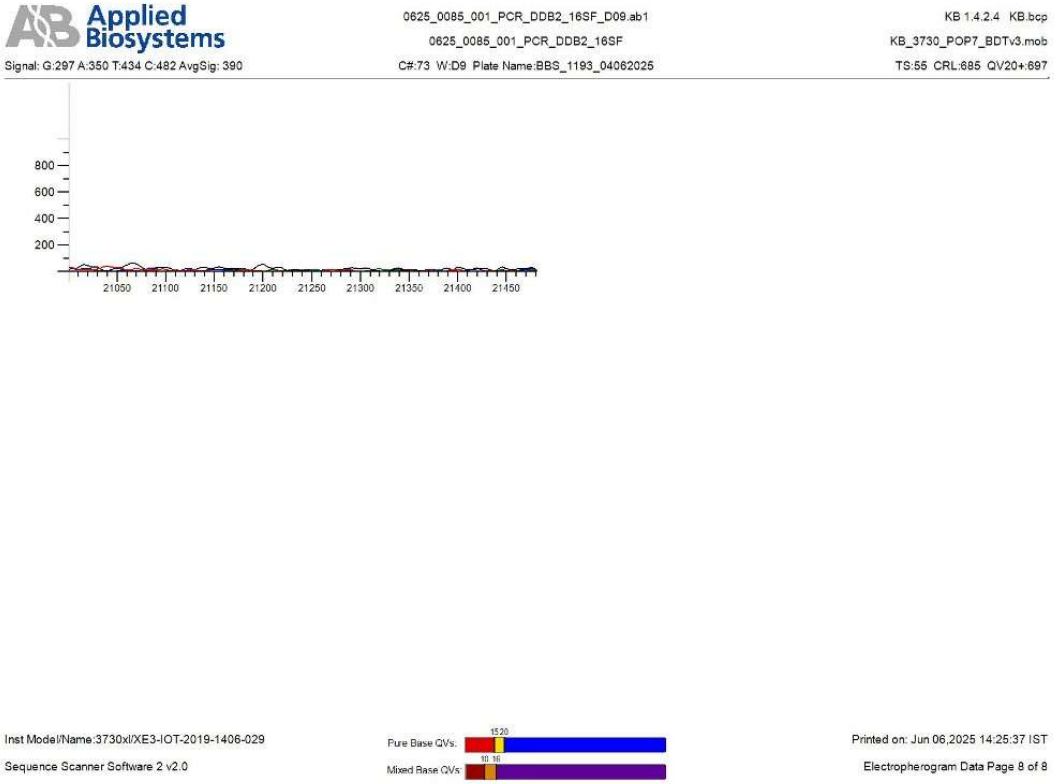

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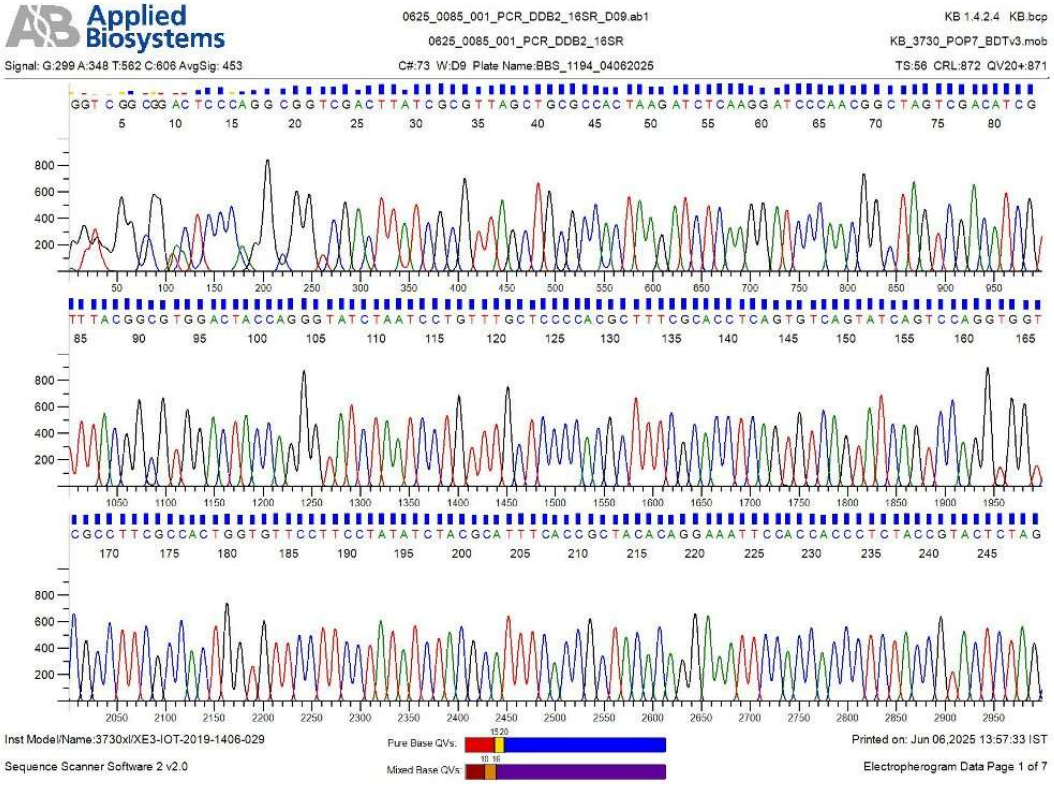

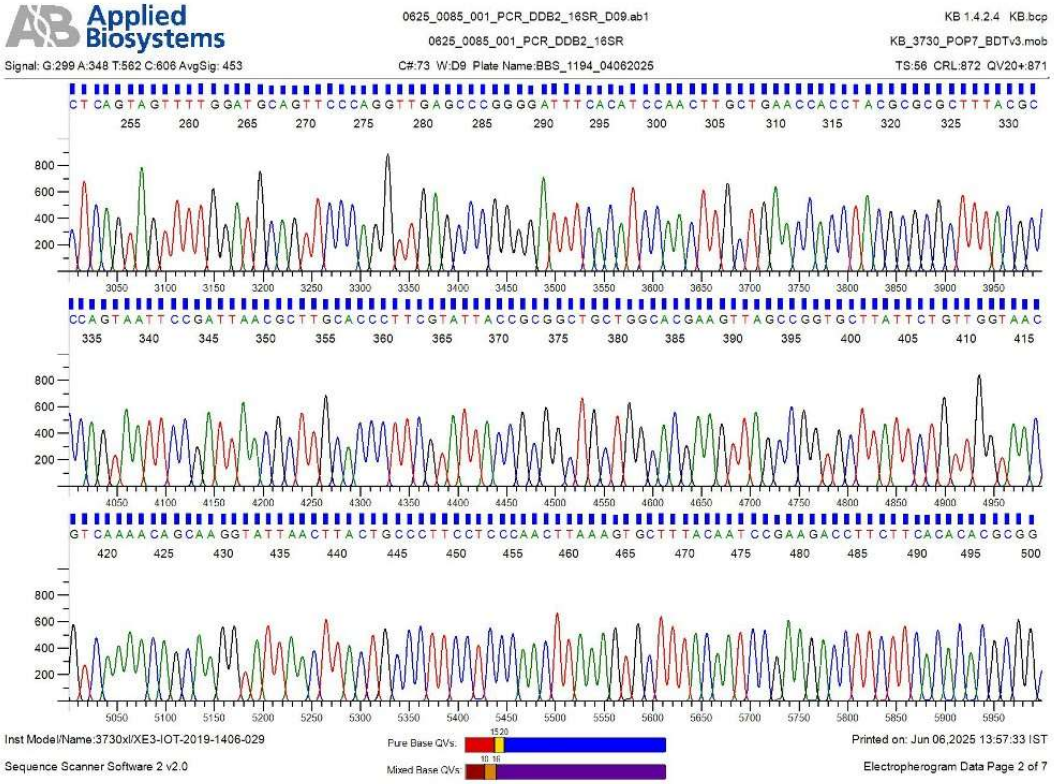
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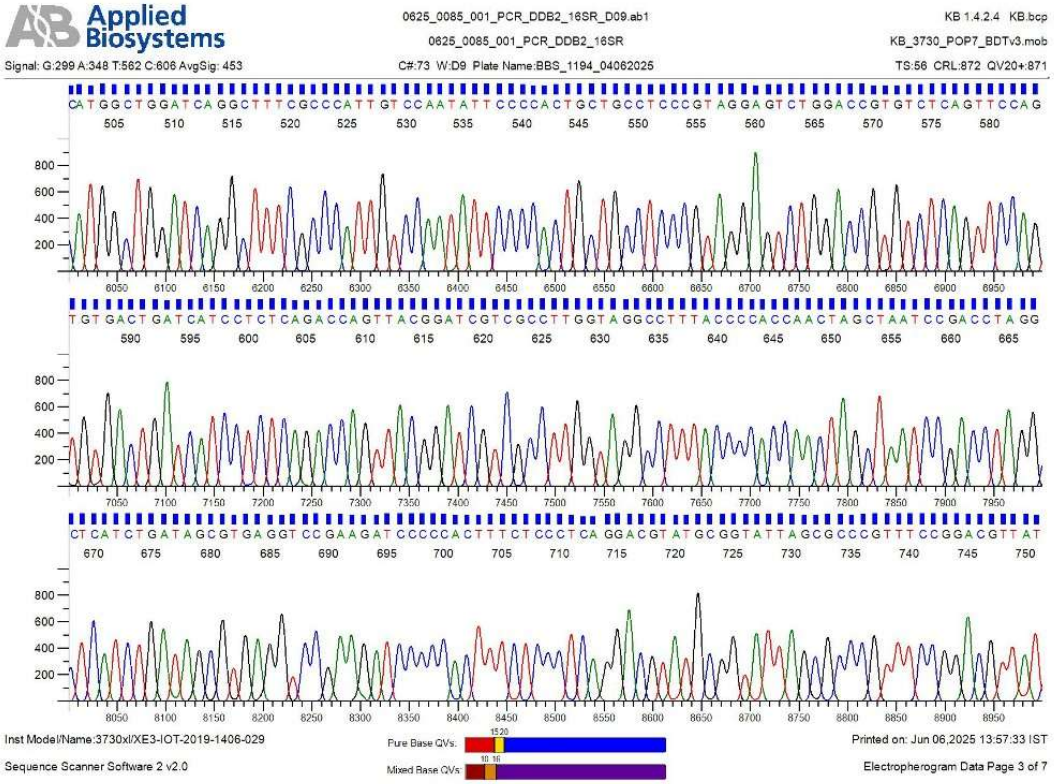
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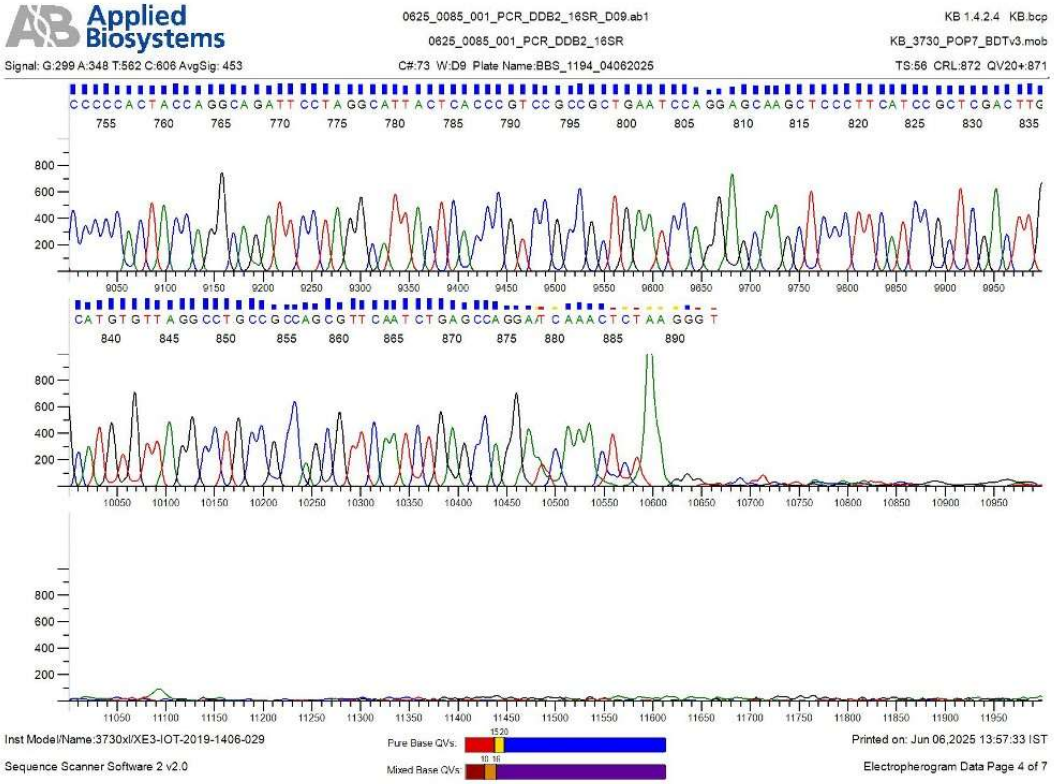
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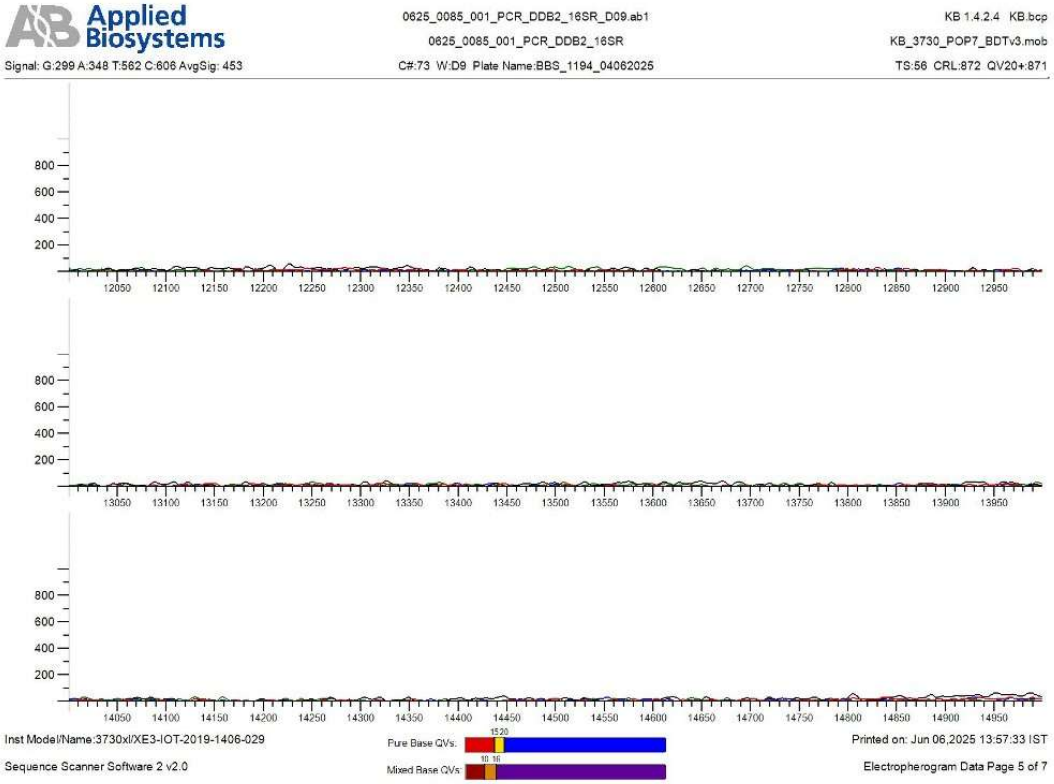
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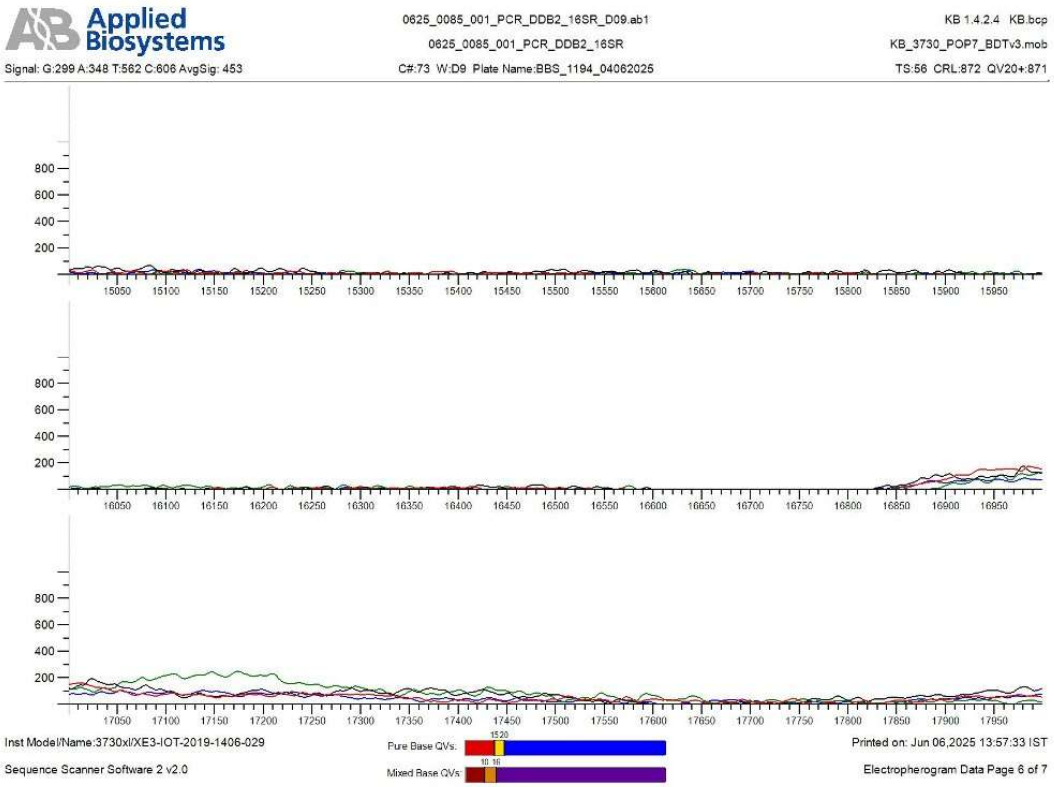
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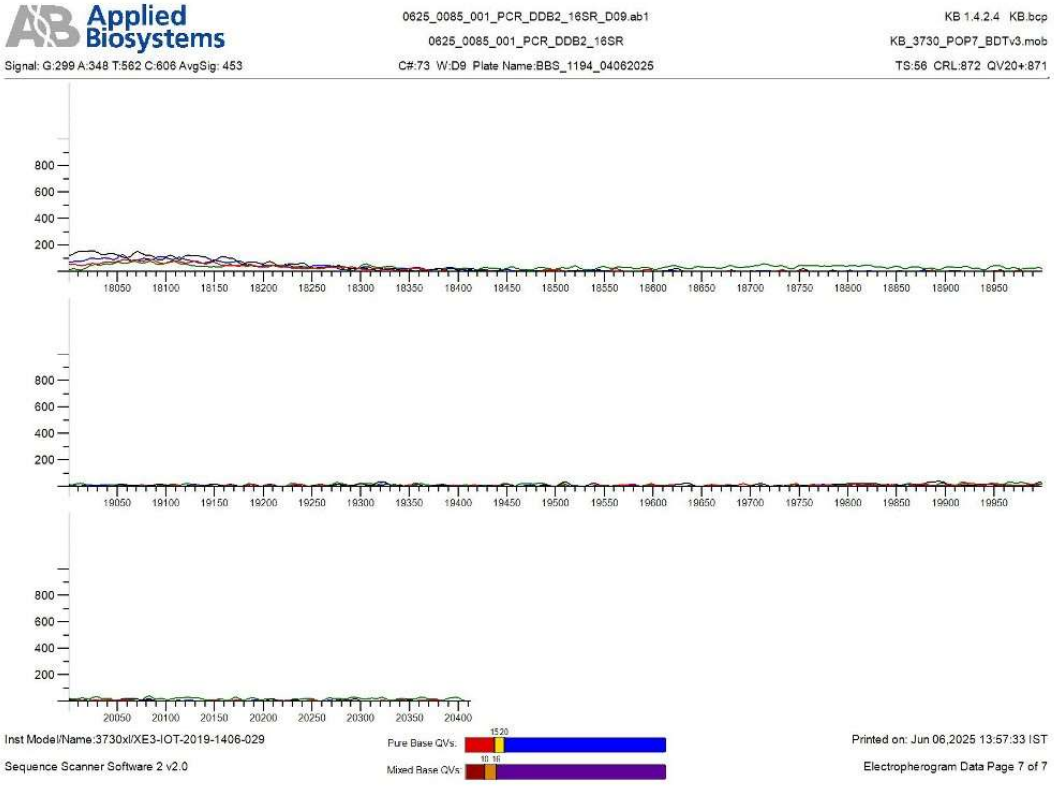
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Appendix-6: Procedure followed for the phylogenetic tree construction in MEGA11 software [136]**:**

1. Installed and opened MEGA11 (64-bit GUI version).
2. Clicked on the “Align” button and selected “Edit/Build Alignment”. In the Alignment Editor popup, chose “Create a new alignment” and clicked OK.
3. Selected “DNA” as the data type for alignment.
4. Imported the downloaded top 10 BLAST sequences in fasta format.
5. Selected all sequences, then in the Alignment tab, clicked on “Align by ClustalW”. A new window popped up. The default ClustalW settings were retained, and OK was clicked.
6. After multiple sequence alignment was completed, the aligned file was saved to the system via the “Data” tab in MEGA format.
7. From the MEGA11 home screen, clicked on the “Phylogeny” tab and selected “Construct/Test Neighbor-Joining Tree”. The previously saved alignment file in MEGA format was opened.
8. Selected only the desired sequences. Then, under the Phylogeny Tree tab, again chose “Construct/Test Neighbor-Joining Tree” using the active file. In the “M11: Analysis Preferences” window, set the Bootstrap value to 1000.
9. Clicked OK, and the phylogenetic tree was successfully constructed.

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Isolation and Identification of Bacteria Associated with Petroleum-Based Hydrocarbons from Soil

Dissertation submitted in partial fulfilment of the requirements for the degree of

Master of Science

in

Biotechnology

by

Navdeep Sharda (235111012)

Under the supervision

of

Dr. Jitendraa Vashistt

to

Department of Biotechnology & Bioinformatics
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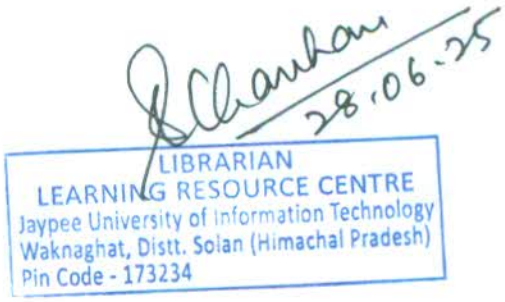

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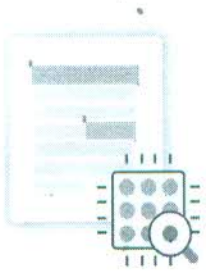

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