Molecular Profiling of Hydrocarbon Degrading Microbial Communities of Petroleum Contaminated Soil in Noakhali Region, Bangladesh

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Abstract

Petroleum hydrocarbon contamination represents a persistent environmental challenge due to its ecological toxicity and long-term impacts on soil health and microbial communities. Despite increasing vulnerability to petroleum pollution in Bangladesh, particularly in coastal and transport-linked regions, the microbial ecology of contaminated soils in Noakhali remains poorly understood. This study investigated the diversity, functional potential, and antimicrobial resistance profiles of indigenous microbial communities inhabiting petroleum-contaminated soils from selected filling stations in Noakhali, Bangladesh, using an integrated culture-dependent and 16S rRNA gene-based metagenomics approach.

Selective enrichment using Mineral Salt Medium supplemented with diesel resulted in the isolation of 33 potential hydrocarbon-degrading bacterial strains. Morphological, Gram-staining, and biochemical characterization identified *Pseudomonas spp*. as the dominant genus, including *Pseudomonas aeruginosa*, alongside *Klebsiella, Acinetobacter, Enterobacter, Hafnia, Bacillus, Staphylococcus, Micrococcus*, and *Streptococcus* species. Functional screening revealed strong lipase and biosurfactant production by *Pseudomonas aeruginosa*. Antimicrobial susceptibility testing demonstrated site-specific resistance patterns, with the highest resistance observed at Sonapur Filling Station, suggesting localized anthropogenic pressure.

High-throughput sequencing of the 16S rRNA gene identified 172 unique Amplicon Sequence Variants (ASVs). Alpha diversity analyses revealed the highest microbial richness at Alexander Filling Station (AFS). Taxonomic analysis revealed that the microbial communities were predominantly composed of Bacteroidota and Proteobacteria, with notable representation of hydrocarbon-degrading lineages such as *Pseudomonas, Rhodococcus,* and *Acinetobacter,* while *Prevotella* and *Pseudomonas* emerged as the most abundant genera. Predicted functional profiling indicated enrichment of key metabolic pathways and genes involved in alkane and aromatic hydrocarbon degradation, nitrogen and sulfur cycling, and antibiotic resistance, with AFS exhibiting the highest hydrocarbon-degrading potential.

This study provides the first comprehensive molecular insight into petroleum-contaminated soils of Noakhali and underscores the ecological significance of indigenous microbial communities for site-specific, sustainable bioremediation strategies in Bangladesh.

Certification

This is to certify that the student bearing Student ID: **ASH2305MS106M** has carried out the thesis entitled, **Molecular Profiling of Hydrocarbon Degrading Microbial Communities of Petroleum Contaminated Soil in Noakhali Region, Bangladesh**, for the partial fulfillment of the Masters of Science Degree in Microbiology from Noakhali Science and Technology University, Noakhali, Bangladesh, under my academic supervision in the Department of Microbiology, Noakhali Science and Technology University.

The thesis is the outcome of the student's original work, and is deemed worthy of submission for the degree of Master of Science in Microbiology under the Faculty of Life Sciences of Noakhali Science and Technology University.

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Table of Contents

List of Figures

List of Tables

CHAPTER 1: INTRODUCTION

Petroleum hydrocarbons (PHC) are the most widespread environmental pollutants worldwide due to the extensive use, transportation, accidental release of crude oil and refined petroleum products. PHC include alkanes, aromatics, polycyclic aromatic hydrocarbons (PAHs), and petroleum derived xenobiotics, which can persist in terrestrial and aquatic ecosystems and pose serious ecological and public health risks with toxic, mutagenic and carcinogenic properties [1, 2]. In the soil environments, petroleum hydrocarbons significantly affect physicochemical properties, reduce nutrient availability, inhibit seed germination and plant growth, retard soil fertility, and alter the natural structure and functioning of microbial communities [3]. Because hydrocarbons are chemically diverse and resistant to natural degradation, their accumulation leads to long-term ecological damage unless active remediation strategies are implemented.

Several studies on petroleum contamination exerts strong selective pressure on soil microorganisms, suppressing sensitive taxa and enriching bacteria those are capable of tolerating or metabolizing hydrocarbons [4]. Research on chronically polluted geographical regions such as Nigeria, Italy, Iran, Antarctica [5], and Spain consistently demonstrate that petroleum contamination leads to decreased microbial diversity coupled with the proliferation of hydrocarbon-degrading bacteria [6, 7]. Microorganisms play a vital role in the biodegradation of petroleum hydrocarbons. Numerous bacterial taxa including *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Acinetobacter*, *Marinobacter*, *Bacillus*, and *Alcanivorax* possess metabolic pathways that enable the oxidation and breakdown of aliphatic and aromatic hydrocarbons [8]. These bacteria synthesize a range of catabolic enzymes, such as alkane monooxygenases (alkB), aromatic ring-hydroxylating dioxygenases, catechol 1,2- and 2,3-dioxygenases, and cytochrome P450 systems etc., enabling them to utilize petroleum compounds as carbon and energy sources [8]. The structure and functional capacity of hydrocarbon-degrading microbial communities are influenced by contaminant type, concentration, redox conditions, and local environmental parameters such as salinity, pH, and nutrient availability [9].

Bangladesh is vulnerable to petroleum contamination due to increase in demand of energy (oil consumption overall 64.4% in 2019), growing industrialization, and expanded transportation of fossil fuels across coastal and inland regions [10]. Different events of petroleum pollution such as oil tanker accidents in the Sundarbans [11], leakage from inland storage facilities, and chronic contamination associated with ship-breaking industries in Chittagong [12] have raised considerable concern about the ecological consequences of hydrocarbon pollution. Although the

Noakhali region is not traditionally recognized as a petroleum hotspot, its coastal geography, proximity to industrial transport routes, and increasing urban development make it susceptible to petroleum leakage, accidental spills, and diffuse contamination from anthropogenic activities. A study on coastal water pollution have highlighted the potential risk of hydrocarbon accumulation in soils across coastal districts of Bangladesh, due to unregulated industrial waste disposal and increased vehicular activity. Despite the environmental importance of this region, systematic studies on the microbial response to petroleum contamination in Noakhali remain limited. Studies on hydrocarbon-degrading bacteria has focused predominantly on the Sundarbans region [11], coastal marine systems [13], and industrial sites [14]. However, there is a significant research gap regarding microbial community structure in petroleum contaminated soils of Noakhali, a region expanding transport networks, and increasing industrial activity. Molecular characterization of soil microbial communities in this area is essential not only for understanding ecological impacts of hydrocarbon contamination but also for developing region-specific microbial indicators and bioremediation strategies. Culture-based techniques provide only a partial view of microbial diversity, as the majority of environmental microorganisms are unculturable under standard laboratory conditions. To overcome these limitations, molecular methods particularly 16S rRNA gene sequencing have emerged as powerful tools for profiling complex microbial communities and identifying bacterial taxa involved in hydrocarbon degradation. High-throughput sequencing platforms, such as Illumina MiSeq enable revealing both abundant and rare taxa that respond to petroleum contamination [15]. These molecular approaches have been applied in oil-polluted environments including marine sediments [16], mangrove soils, oilfields [17], and refinery-impacted sites [18], but remain underutilized in the context of Bangladeshi ecosystems.

This study therefore applies 16S rRNA gene-based metagenomic sequencing to characterize the hydrocarbon-degrading microbial communities in petroleum-contaminated soils of the Noakhali region, Bangladesh. The research aims to profile the taxonomic composition of soil bacterial communities, assess diversity patterns, and infer the potential functional capacity of indigenous microbes involved in hydrocarbon degradation. By generating the first molecular dataset on petroleum impacted soils in Noakhali, this study provides crucial baseline information that may support pollution monitoring, ecological risk assessment, and the development of bioremediation frameworks tailored to the coastal environments of Bangladesh.

CHAPTER 2: LITERATURE REVIEW

2.1 Petroleum hydrocarbon

Petroleum hydrocarbons are complex mixtures primarily composed of carbon and hydrogen, forming the basis of crude oil and its refined products. There are four main groups of these substances: saturates or aliphatics (30–60% of crude oil), which include long-chain n-alkanes (C10 to C44), branched isoalkanes (like pristane), and cycloalkanes or naphthenes; the aromatics group (20–50%), which include volatile low-molecular-weight compounds (like benzene, toluene, ethylbenzene, xylenes (BTEX), and polycyclic aromatic hydrocarbons (PAHs) like naphthalene and phenanthrene); asphaltenes (5–20%), which are complex polar structures which consist of phenols, fatty acids, ketones, esters, and porphyrins; and resins (5–20%), which are nitrogen- and sulfur-containing compounds that include pyridines, quinolines, carbazoles, sulfoxides, and amides [19]. Different sources give different amounts of each type of crude oil. For example, paraffinic have plenty of saturates, naphthenic have a large amount of cycloalkanes, and aromatic crudes have a substantial amount of ring structures. API gravity is another way to group crude oil. Light oils (31° API, density <0.87 g/cm³) are volatile, have an abundance of n-alkanes, and break down quickly. Medium oils (22-31° API) have equal fractions. Heavy oils (22° API, density >0.91 g/cm³) are thick, have a large amount of asphaltenes, and stay in the environment for long periods of time [20].

PHCs are basic substances for industrial uses. About 40% of the world's demand for oil is for fuels like gasoline, diesel, jet fuel, and heating oil. These fuels are used for heating and transportation. Petrochemical naphtha makes ethylene and propylene, which are used to make plastics, synthetic rubber, and fibers (about 25% of the time). Recent estimates say that global production meets demand for more than 100 million barrels per day. Natural seepage adds about 600,000 metric tons a year, while spills from people add about 0.6 million tons a year [21]. Oil extraction produces a huge 70 billion barrels of water a year around the world. This water often has total petroleum hydrocarbons (TPH) levels of 200 to 7,220 mg/L, which makes contamination worse in hypersaline environments [22]. Diesel fuel has taken part of transportation and industry around the world. It is used to provide power to trucks, ships, generators, and heavy machinery, and is used in large quantities every day, with demand for liquid fuels expected to reach 105 million barrels per day by 2025 [23]. Because diesel is so common and stays in the environment for a long time.

2.2 Characteristics of hydrocarbons in diesel

Diesel fuel consists primarily of a complicated mixture of hydrocarbons derived from the refining of crude oil. The boiling points of these hydrocarbons typically range from 180°C to 360°C. Diesel contains between 11 and 25 carbon chains. Diesel has three main types of hydrocarbons: saturated, unsaturated, and aromatic. [24]. The non-hydrocarbon constituents of diesel include sulfur (S), nitrogen (N), and oxygen (O), which are non-metallic elements, as well as nickel (Ni), iron (Fe), and vanadium (V), which are metallic elements sometimes incorporated as additions. Diesel's hydrocarbon composition generally comprises paraffins, olefins, and specific aromatics such as naphthenes. Moreover, diesel's chemical makeup consists of 24% aromatic hydrocarbons, 74% aliphatic hydrocarbons (C8 to C26), and 2% other compounds [25]. N-alkanes are linear aliphatic hydrocarbons characterized by a range of saturate chains of carbon. When an ionized functional group, such as a carboxyl group, is attached, non-polar organic molecules, or hydrocarbons, become hydrophilic despite their insolubility in water. Generally, a bond's polarity and solubility in water diminish with increasing atomic complexity, therefore reducing the bond's boiling point. [26]. Hydrocarbons are considered detrimental to living organisms, including humans. The level of toxicity varies based on multiple variables, including the properties of the chemicals, density, tension at the surface, additives and volatility. Aromatic hydrocarbons are more detrimental than aliphatic hydrocarbons.

2.3 Sources of pollution and their effects on environment

Transportation sector will remain a major contributor to hydrocarbon pollution due to its continued dependence on fossil fuels for energy. Hydrocarbons enter the ecosystem due to fuel spills, petrol stations, powerplant combustion and motor cleaning procedures. Another issue is the way fossil fuels are produced. Environmental contamination can also result from offshore oil exploration and the transportation of its products. Since diesel is the primary energy source for the majority of enterprises, diesel spills are often a problem in industrial regions [27]. Hydrocarbon contamination escalates when untreated wastewater containing fuel is discharged. Polycyclic aromatic hydrocarbons (PAHs) are the predominant designation for the aromatic compounds included in fuel. They are recognized as toxic, carcinogenic, and mutagenic to organisms due to the presence of two to six aromatic rings. Reports indicate that PAHs may affect nervous system by impairing the immune system, damaging the kidneys and serving as a principal source of mutations and

cancer. [28]. When diesel concentrations exceed 15 g/kg, it adversely affects plant growth due to its toxicity and may lead to diminished soil fertility. [28] . Since diesel is considered a chemical oxygen demand material, its presence in an aquatic environment might limit dissolved oxygen and light penetration. Diesel alters the photosynthesis of autotrophic species, reducing light penetration and so upsetting the stability of underwater ecosystems [29]. Diesel's hydrophobic properties lead it to bioaccumulate in the fat tissues of living things, including people. This bioaccumulation will affect some of the enzymatic reactions and reduce the amount of energy available following specific enzymatic bypasses and alterations. A living organism's natural defenses against diesel's toxicity will be activated when it builds up in its fat tissues due to its poisonous properties. This will cause mutagens to appear and cancer cells to proliferate [24].

2.4 Biodegradation of aliphatic hydrocarbon

The aerobic biodegradation of hydrocarbon occur via the peripheral and central metabolic pathways. In peripheral pathways larger molecules converted into a few essential intermediates. Molecular oxygen (O_2), increases water solubility and chemical reactivity of the petroleum hydrocarbon, is introduced into the hydrocarbon molecule as the initial step in aerobic hydrocarbon degradation. Monooxygenases and dioxygenases are the two types of oxygenase enzymes that catalyze this step [30]. Monooxygenases incorporate single oxygen into the substrate reduce the second oxygen atom to H_2O. Since the essential cofactors, NADH or NADPH, are unstable outside of the cell, they can act on both aliphatic and aromatic hydrocarbons, starting oxidation reactions that take place inside bacterial cells.

Aliphatic hydrocarbons undergo aerobic biodegradation processes primarily via terminal or sub-terminal oxidation processes. Bacteria target terminal methyl group ($-CH_3$) of alkanes in the terminal oxidation pathway. This is changed converted into a primary alcohol by oxygenases, which is then further oxidized to an aldehyde and, ultimately, to a fatty acid. Since oxidative reactions are dependent on the utilization of reduced NADH or NADPH, can only take place within a cell of bacteria, as these coenzymes are prone to rapid degradation in the extracellular environment. Following β-oxidation, the fatty acid is converted to acetyl-CoA, which then enters the TCA cycle to undergo complete oxidation to form CO_2 and H_2O. A secondary alcohol is created by oxidation at a secondary carbon atom rather than the terminal one in the sub-terminal oxidation pathway. Acetyl-CoA is the primary metabolic intermediate that both pathways

converge on, enter into the TCA cycle for biosynthesis and energy production [31]. An example of Hexadecane degradation is attached to explain the degradation pathways (Figure 1).

2.5 Biodegradation of aromatic hydrocarbons in diesel

Figure 2 illustrates aromatic hydrocarbons degradation via more intricate pathways than alkanes, cycloalkanes, or alkynes. Because there are many different enzymes involved, the breakdown process of aromatic hydrocarbons is more complicated than that of aliphatic hydrocarbons, as seen in Fig 2.2. When two oxygen atoms connect with a benzene ring, the dioxygenase enzyme initiates the initial breakdown of aromatic hydrocarbons. In order to start the enzymatic breakdown of hydrocarbon rings, molecular oxygen must be present [32]. The enzyme catalyses the union of oxygen with the substrate molecule, which hydroxylates the benzene and yields cis-benzene dihydrodiol, a less stable chemical. The enzyme cis-diol dehydrogenase will de-hydrogenate this molecule to yield catechol.

The following stage involves breaking the catechol's ring through an interaction between the extra-diol (meta) and intra-diol dioxygenase enzyme (ortho), which adds oxygen molecules to terminate the aromatic ring. It is transformed into cis, cis muconic acid by the intra-diol dioxygenase enzyme such as catechol 1,2-dioxygenase and to 2-hydroxymuconic semialdehyde by the extra-diol dioxygenase enzyme such as catechol 2,3-dioxygenase. Carboxylic acids or aldehydes produced from this pathway will undergo a process analogous to that of aliphatic hydrocarbons, as the reaction yields a linear hydrocarbon. The B oxidation activities in this route produce Acetyl-CoA, which subsequently enters the Krebs cycle and other key metabolic systems of the bacteria. ATP is the energy source for the bacterial metabolism and is produced when the hydrocarbon molecules are completely broken down by the bacterium. Since these processes take place in an aerobic environment, they also generate CO2 and H2O as byproducts [33]. Alkanes > branched alkanes > monoaromatics > cycloalkanes > polyaromatics is the decreasing order of diesel compounds' propensity to biodegrade [34]. N-alkanes are thought to be less poisonous, unstable, and simple for bacteria to exploit as a carbon source [35]. Compared to other types of hydrocarbon, they have been found to be more sensitive and prone to bacterial attack.

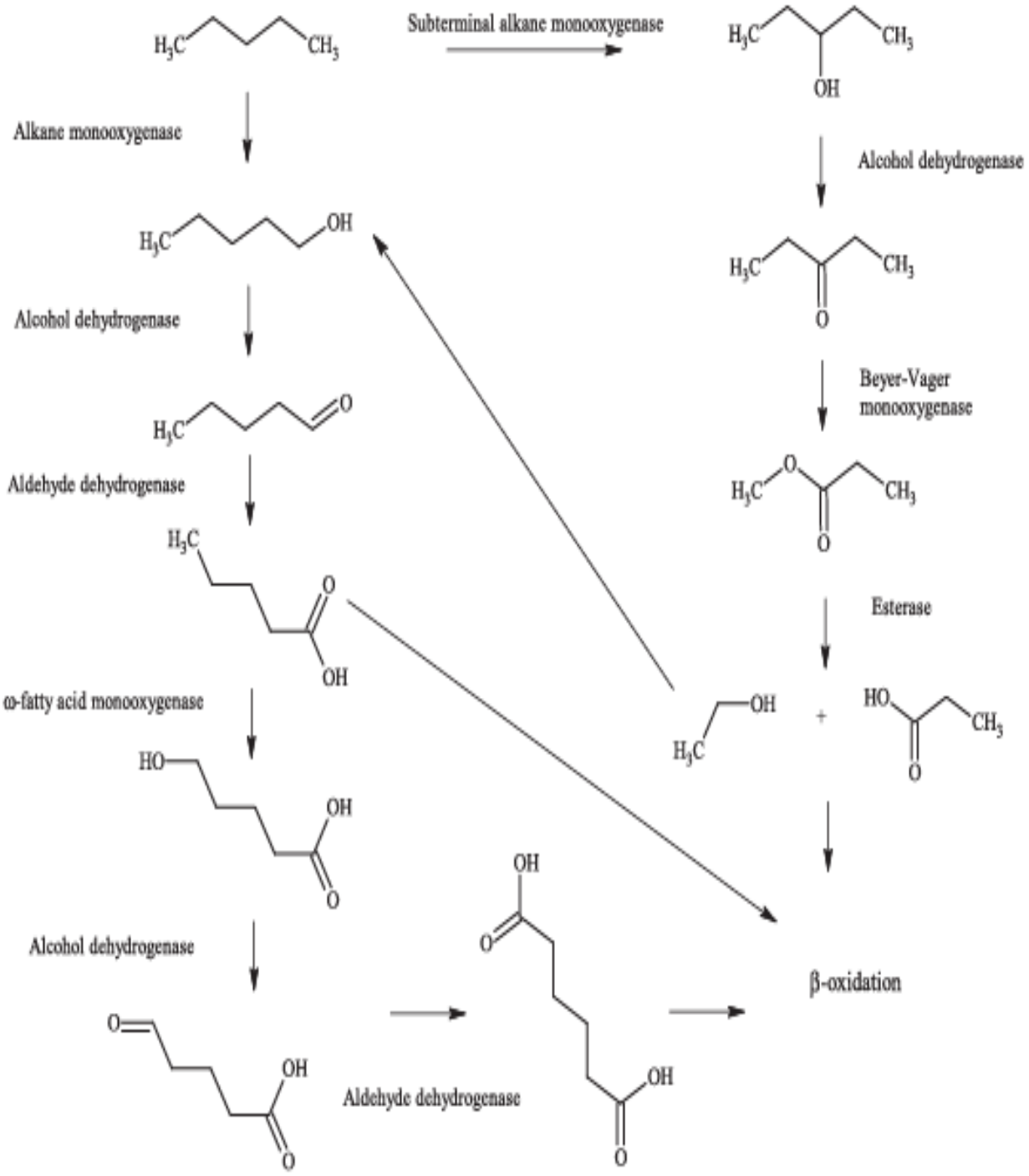

Figure 1: Aliphatic hydrocarbon degradation pathway [36]

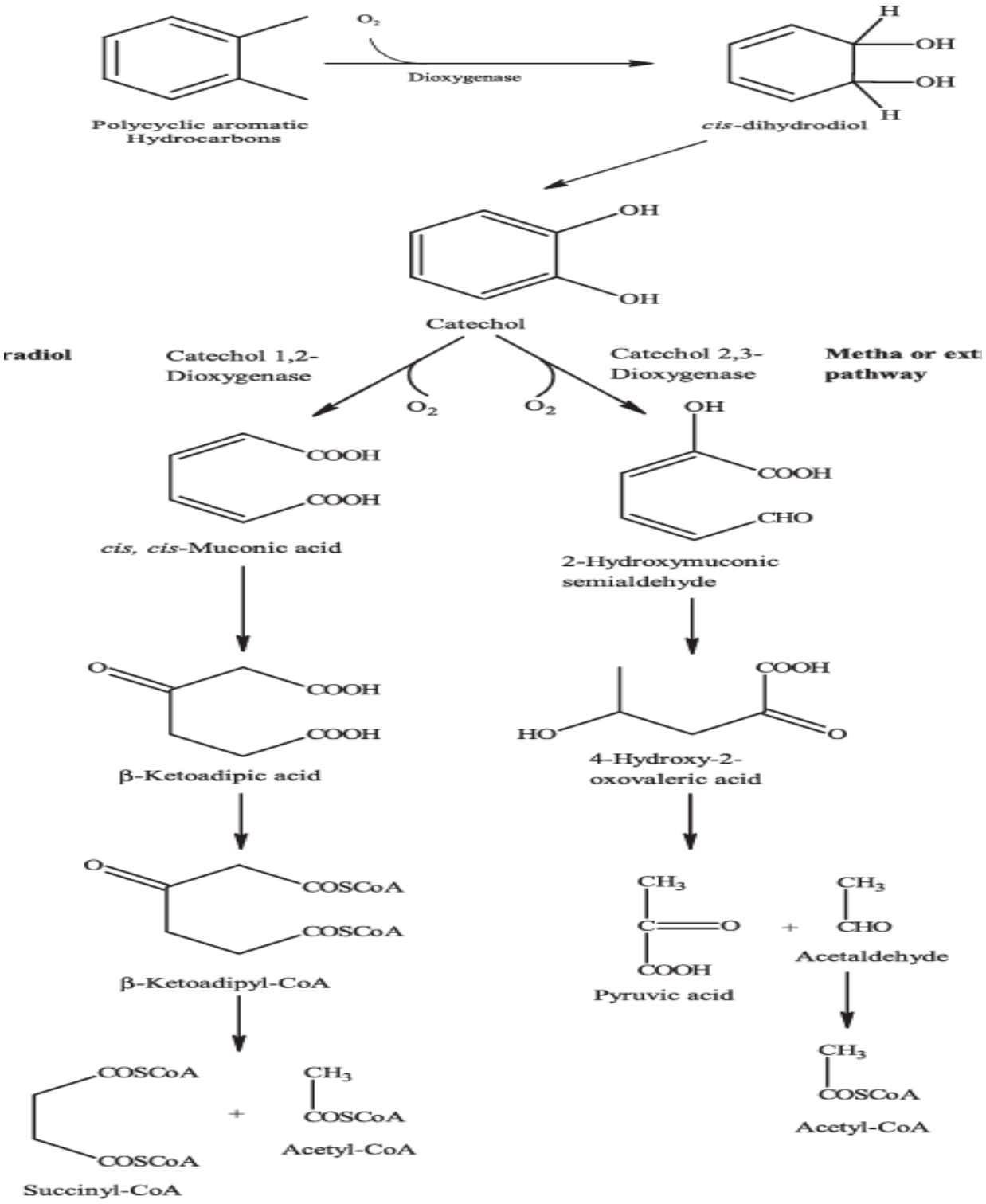

Figure 2: Biodegradation of aromatic hydrocarbons in diesel [36]

2.6 Anaerobic biodegradation

Anaerobic biodegradation of petroleum hydrocarbons is another natural process that takes place in oxygen deficient environments, like deep sediments, aquifers, oil reservoirs, and wastewater treatment systems. Anaerobic degradation is different from aerobic degradation as it uses microorganisms that use nitrate (NO_3^-), sulfate (SO_4^{2-}), ferric iron (Fe^{3+}), or carbon dioxide (CO_2) as terminal electron acceptors [37] shown in (fig 2.4). Aerobic degradation, on the other hand, uses oxygen as a terminal electron acceptor. These pathways permit aliphatic and aromatic hydrocarbons to be transformed down into simpler molecules like carbon dioxide (CO_2) and methane (CH_4) through the use of specialized enzymatic systems and syntrophic microbial interactions [38, 39].

The activation of hydrocarbons is the first step in anaerobic biodegradation (figure 3). This is often the most energy-intensive step because C–H bonds are chemically stable. Microorganisms break down hydrocarbons without oxygen by using fumarate. Enzymes such as benzylsuccinate synthase (BSS) for aromatic hydrocarbons and alkylsuccinate synthase (ASS) for alkanes accelerate this process [40]. This reaction results in benzylsuccinate or alkylsuccinate intermediates that are the first steps in the degradation mechanism. Carboxylation (the addition of a carboxyl group to produce benzoate intermediates), hydroxylation (making phenol derivatives), and methylation are additional ways to activate. Methylation occurs in some methanogenic consortia [39].

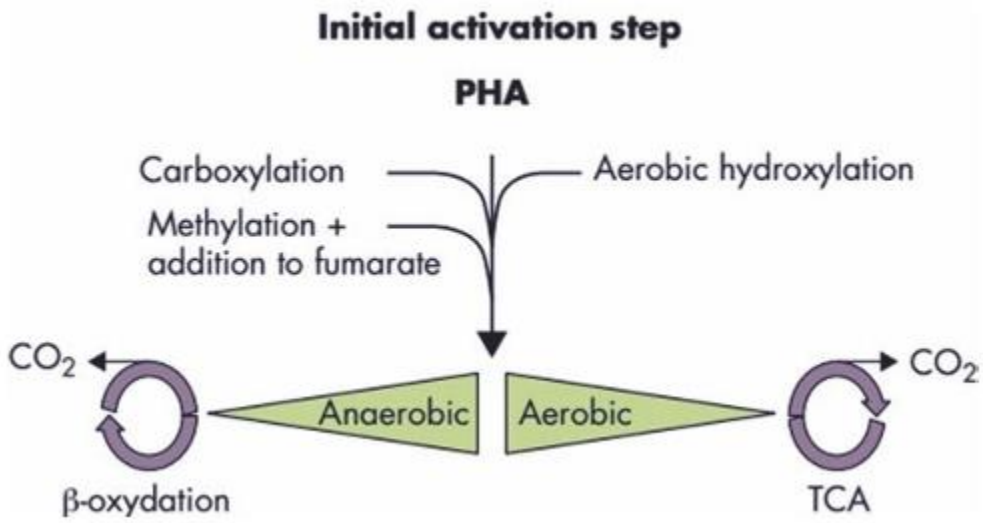

Figure 3: Activation processes of anaerobic PAH's degradation [41]

If hydrocarbons are started to degrade, they turn into central intermediates like benzoyl-CoA (for aromatic compounds) and alkylsuccinates (for aliphatic hydrocarbons). These intermediates undergo a number of enzymatic reactions and changes before entering the β-oxidation pathway, where they are turned into acetyl-CoA. Following that, acetyl-CoA is oxidized to CO_2 and H_2, which makes reducing substances which promote respiration through pathways that reduce nitrate, sulfate, or iron [42]. For instance, *Thauera* and *Azoarcus* species oxidize toluene through benzoyl-CoA during nitrate reduction, while *Geobacter metallireducens* uses Fe(III) as an electron acceptor to break down benzene and toluene [43]. *Desulfatibacillum aliphaticivorans* and *Desulfobacterium anilini* oxidize hydrocarbons and turn sulfate into hydrogen sulfide (H_2S) in sulfate-reducing systems [44]. These reactions are not as energetically favorable as aerobic oxidation, however they allow degradation to proceed in anoxic conditions [45].

In methanogenic conditions without any external electron acceptors, degradation takes place through syntrophic reaction between fermentative bacteria and methanogenic archaea. Bacteria that break down hydrocarbons, like Smithella and Pelotomaculum, turn hydrocarbons into intermediates like acetate, formate, and hydrogen. Methanogens like Methanosaeta and Methanobacterium utilize these compounds and convert them into methane (CH_4) and carbon dioxide (CO_2). This syntrophic reaction occurs only when the concentrations of hydrogen and acetate are very low. The balance can be maintained by methanogens utilizing these intermediates as they form [39].

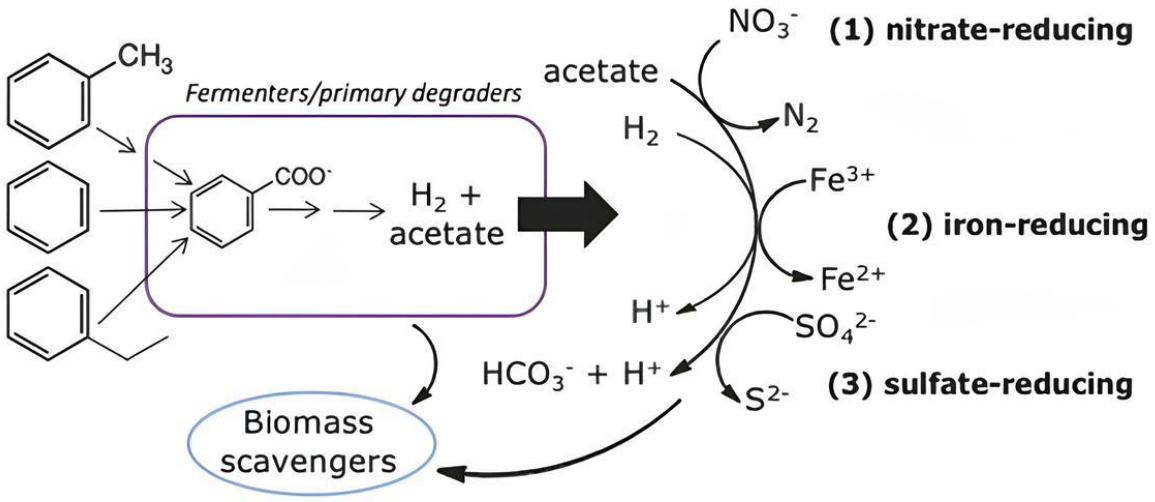

Figure 4: Proposed model of syntrophic anaerobic biodegradation [46]

2.7 Petroleum hydrocarbon degrading bacteria

Native bacteria eventually break down or metabolize the majority of petroleum hydrocarbons found in the environment due to their energy and carbon requirements for development and reproduction, as well as their need to alleviate physiological stress brought on by the presence of petroleum hydrocarbons in the microbial bulk environment [35]. Numerous normal and extreme bacterial species have been identified and used as petroleum hydrocarbon biodegraders. Various petroleum hydrocarbons (such as polyaromatics and aliphatics) have been demonstrated to degrade by oxidising reactions; however, due to the distinct oxygenases present in various bacterial species, these degradation pathways vary substantially. Certain alkanes, for example, can be metabolised by certain bacteria, whilst aromatic or resin fractions of hydrocarbons are broken down by others. This phenomena has to do with the chemical makeup of the hydrocarbon components found in petroleum. Recent studies have identified bacteria from more than 79 genera that are capable of degrading petroleum hydrocarbons ; several of these bacteria such as *Achromobacter, Acinetobacter, Alkanindiges, Alteromonas, Arthrobacter, Burkholderia, Dietzia, Enterobacter, Kocuria, Marinobacter, Mycobacterium, Pandoraea, Pseudomonas, Staphylococcus, Streptobacillus, Streptococcus,* and *Rhodococcus* have been found to play vital roles in petroleum hydrocarbon degradation [47]. Interestingly, "conditionally rare taxa" in soil, such as *Alkanindiges* sp., have been reported to exhibit rare-to-dominant bacterial shifts that are strongly affected by environmental constrains such as diesel pollution. Similarly, some obligate hydrocarbonoclastic bacteria (OHCB), including *Alcanivorax, Marinobacter, Thallassolituus, Cycloclasticus, Oleispira* and a few others (the OHCB), showed a low abundance or undetectable status before pollution, but were found to be dominant after petroleum oil contamination. These phenomena suggest that these microorganisms are crucial to the degradation of petroleum hydrocarbons, and that they significantly influence the transformation and fate of petroleum hydrocarbons in the environment. Although some bacteria have been reported to have a broad spectrum of petroleum hydrocarbon degradation ability, *Dietzia sp*. DQ12-45-1b utilizes n-alkanes (C6–C40) and other compounds as the sole carbon sources and *Achromobacter xylosoxidans* DN002 works well on a variety of monoaromatic and polyaromatic hydrocarbons almost no bacteria can degrade the entire petroleum hydrocarbon fraction. This variation can be ascribed to distinct catalytic enzymes present in different indigenous bacteria, resulting in diverse roles in oil-contaminated environments. This indicates that the cleanup of petroleum hydrocarbon pollution necessitates the

collaborative effort of several functional microorganisms to get optimal environmental purification outcomes [48].

2.8 Restriction of physical contact between bacteria and petroleum hydrocarbon

The biodegradation rate in the environment is often restricted since most petroleum hydrocarbons are hydrophobic and have poor water solubilities. This is due to the fact that bacterial membrane bound oxygenases, which necessitate direct and efficient contact between bacterial cells and petroleum hydrocarbon substrates, frequently participate in the initial stage of the breakdown process of petroleum oil. The following are the main variables limiting the effectiveness of petroleum hydrocarbon biodegradation: (1) Petroleum hydrocarbons have a limited capacity to be bioavailable to bacteria. (2) The necessity that bacterial cells make contact with hydrocarbon substrates prior to the functional oxygenases introducing molecular oxygen into molecules. Bacteria, on the other hand, have developed defences against petroleum pollutants, such as enhancing cell adhesion by changing the components of their surface and secreting bioemulsifiers to improve their access to hydrocarbon substrates. These kinds of bacteria are frequently chosen for use as environmental restoration agents because they can remove petroleum hydrocarbon contaminants from the environment more quickly [49].

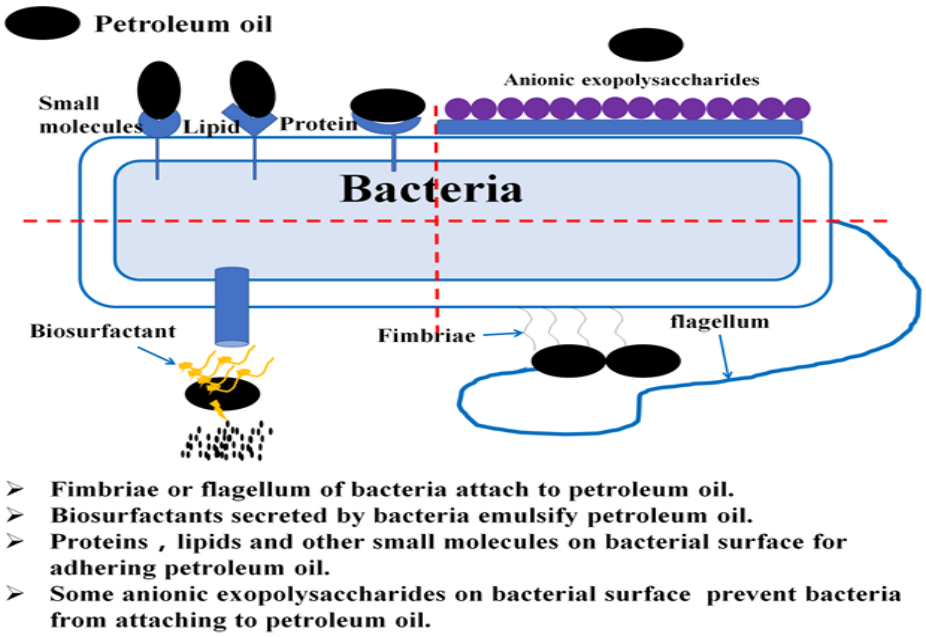

Figure 5: Physical contact between bacteria and hydrocarbons [24]

Effective biodegradation of hydrophobic hydrocarbon substrates depends on the characteristics of bacterial surfaces (Figure 5)**,** and their adhesion mechanisms are crucial. It has been discovered that hydrophobic pollutants' adherence to bacterial cells is primarily linked to hydrophobic fimbriae, fibrils, outer-membrane proteins and lipids, as well as specific small molecules found on cell surfaces like gramicidin S and prodigiosin [50]. Fimbriae on bacterial surfaces were discovered to be beneficial for bacterial adherence, assimilation of hydrophobic substrates, and metabolic activity, and they were crucial for the growth of Acinetobacter sp. RAG-1 when C16 alkane was used as the carbon source. Attaching bacterial cells to specific substrates is not required, however it can improve the biodegradation of hydrophobic hydrocarbons. This is due to the fact that bacteria that have a high surface hydrophobicity can sometimes readily cluster and form biofilms, which might lead to possible hazards like illnesses. It's true that not only hydrophobic bacteria can biodegrade hydrophobic pollutants, but a number of solvent-resistant hydrophilic bacteria can also metabolize these pollutants. This could be due to the solvent-resistant bacteria's initial colonization and dominance of the bacterial surface due to changes in lipopolysaccharides or porines. Consequently, it appears that using hydrophilic microbes rather than hydrophobic ones to remove hydrocarbon contaminants is more beneficial. One viable strategy to increase the bioavailability of petroleum hydrocarbons is the use of surfactants, which may speed up the rates of dissolution or desorption that result in the solubilisation or emulsification

of petroleum hydrocarbon contaminants. As rhamnolipids were present, Bacillus sp. DQ02's adhesion to hydrocarbon rose by 44%, and the breakdown of n-hexadecane increased by 11.6% as compared to treatment without rhamnolipids. In view of this, the selection of appropriate surfactants is of great importance for pollution remediation and the prevention of secondary pollution. Bioemulsifier-producing bacteria, which have attracted much attention, generally have the following two physiological aspects: (1) the ability to enhance the complexation and solubilization of non-polar substrates, thereby promoting the bioavailability of substrates, and (2) the ability to improve affinity between cell surfaces and oil-water interfaces through metabolism, promoting deformation of the oil-water interface film [51].

2.9 Factors affecting diesel biodegradation

The degradation processes of diesel are influenced by multiple factors, as outlined in Fig. 3. The parameters (bacterial species, oxygen, bioavailability of diesel, temperature, pH, nutrients, salinity) are significantly connected with the optimal conditions for bacterial survival in contaminated environments.

2.9.1 Bacterial species

The type of bacteria is the main indicator of the efficacy of diesel biodegradation. *Bacillus* sp., *Acinetobacter* sp., *Pseudomonas* sp., and *Vibrio* sp. exhibit exceptional capabilities in the degradation of hydrocarbon contaminants. *Vibrio alginolyticus*, *Bacillus subtilis*, *Brochothrix thermosphacta* and *Pseudomonas aeruginosa* have demonstrated superior biodegradation capabilities compared to laboratory cultures. *Bacillus sp*., *Vibrio sp*., *Flavobacterium sp*., *Pseudomonas sp*., *Acinetobacter sp*. and *Rhodococcus sp*. are recognized for their capacity to synthesize biosurfactants. [24].

2.9.2 Diesel bioavailability

Diesel bioavailability is crucial for biodegradation, as bacteria can only decompose water-soluble contaminants. Bioavailability refers to the ability of a substance to be assimilated by living organisms. Bacteria must directly encounter diesel to utilize it in their metabolic processes. Regarding biodegradation, bioavailability can be described as the water solubility of fuel, as microorganisms can utilize mainly water-soluble chemicals. The ability to dissolve of fuel

escalates with temperature, and elevated temperatures, primarily due to solar exposure, can diminish the viscosity, hence reducing the surface tension between water and diesel [52].

2.9.3 pH

Diesel degraded more slowly when the starting pH was acidic (pH 6), as most heterotrophic bacteria prefer to grow in neutral to alkaline pH. Furthermore, the pH has to be neutral or slightly alkaline to get the highest rate of biodegradation [53].

2.9.4 Temperature

Variations in temperature can improve the diffusion rates of pollutants by decreasing the viscosity of hydrocarbons. Bacteria have an easier time breaking down a spread contaminant. Degradation generally proceeds more slowly in colder temperatures. As the temperature drops, hydrocarbons become less viscous [54].

2.9.5 Oxygen availability

Both aerobic and anaerobic conditions can be used for diesel biodegradation. Nevertheless, diesel does not biodegrade as quickly in anaerobic environments as it does in aerobic ones [55].

2.9.6 Nutrient availability

Since nutrients are the primary component of cell development in diesel biodegradation, their availability is crucial. Bacteria use carbon and a few other elements as their energy source during metabolism in order to create new cells. Essential nutrients must be readily available in order for the bacterial metabolism to function normally. Diesel can produce hydrogen, carbon, and oxygen through the hydrocarbon structure's breakdown. While only little amounts of sulfur, magnesium, potassium, calcium, and iron are needed and can be obtained naturally, additional nitrogen and phosphorous are sometimes needed since they are needed in tiny amounts. The optimal ratio of essential nutrients for diesel biodegradation, often known as the C:N:P ratio, ranges from 100:15:3 to 120:10:1. The optimal N/P ratio for the hydrocarbon breakdown process ranged from 2.4 to 10. When N and P were introduced at a ratio of 10, the diesel-contaminated medium may degrade by 75.8%; however, when the ratio was 20, it may only degrade by 52.4%. A N/P proportion of 10 can speed up hydrocarbon breakdown by as much as 95% at a starting concentration of 100 mg/L [54].

2.9.7 Salinity level

For many non-halophile bacteria, the breakdown routes and enzymes involved in the aerobic metabolism of hydrocarbon molecules are typically documented. The mechanism and enzymes involved in the biodegradation of hydrocarbons in high salinity environments, however, are poorly understood. The osmotic pressure in the medium increases with increasing salinity of the water, which destabilizes intracellular osmotic pressure and prevents bacteria from synthesising macromolecules, which in turn hinders the enzymes involved in the biodegradation process. Furthermore, a high salinity level in water will deprive microorganisms of oxygen, which will slow the rate of bacterial development [56].

2.10 Environmental and human health implications

When petroleum-based hydrocarbons are discharged into the environment due to inadvertent releases from industries or as byproducts from commercial or privately owned processes, and/or into the earth's surface by leaks or spills, they flow across the topsoil toward groundwater. Some species can decompose certain pollutants into tiny portions [57] while others may volatilize into the atmosphere. Furthermore, certain components will remain in the soil for prolonged times and may be decomposed by other soil-living microbes, ultimately resulting in detrimental health impacts [58]. According to the Environmental Protection Agency (USEPA) [59], releases of TPH into the environment pose significant threats to public health and safety by contaminating drinking water, increasing risks of fire and explosion, degrading air and water quality, undermining agricultural productivity, damaging recreational areas, destroying habitats and food sources, and depleting non-renewable resources. Once the soil is contaminated by petroleum hydrocarbons (PHCs), remediation may require several years. Yang et al. reported that the Qingdao oil pipeline incident, which led to soil contamination, was caused by human error, reflecting inadequate planning and maintenance of the pipeline, as well as negligence following the discovery of a breach [60]. Soil contaminated with petroleum hydrocarbons presents a concern for several reasons. First, upon release into the soil, the volatility of PHCs can present fire and explosive risks, particularly when vapors accumulate in confined spaces. Contaminants may disrupt the transmission of nutrients and water, resulting in land degradation. Petroleum residuals may adhere to soil particles and persist within the soil for extended periods. Although contaminants may be degraded by carbon sources, they remain toxic to the preponderance of soil biota. PHCs may compromise the

aesthetic quality by generating objectionable odors, tastes, or appearances in environmental media [61]. Lastly, PHCs may present a potential risk to the environment.

Some marine environments where oil spills have occurred, natural attenuation processes, such as biodegradation, have led to partial ecosystem recovery [62]. However, the speed and effectiveness of this recovery are often dependent on several factors, including the type of hydrocarbons, the presence of favorable conditions for microbial activity, and the severity of contamination. In contrast, some regions, such as those affected by the Exxon Valdez spill in 1989, have shown prolonged recovery times, with some ecosystems still exhibiting reduced biodiversity decades late [63]. The 2010 Deepwater Horizon spill saw microbial blooms degrade 50-60% oil within months, restoring pelagic fish stocks by 2014 but persistent oxy-PAHs in sediments caused 20-year benthic deficits and dolphin strandings [64]. Conversely, rapid coastal recovery via bioremediation, with PAH levels dropping 80% in 5 years, enabling ecosystem rebound without long-term harm [62]. These underscore balancing degradation speed with metabolite monitoring for sustainable remediation.

The United States Environmental Protection Agency [59] states that in order to determine the danger of petroleum contamination, it is necessary to bring together information about exposure and hazards into a synthesis. The potential for exposure by skin contact or ingestion is associated with the propensity of a certain substance to be absorbed into soil particles as well as into plants by means of absorption via the roots, which might potentially enter the food chain [65]. In 1991, a study conducted in Australia by Christie et al. examined cancer incidence within the Australian oil industry from 1981 to 1989 [66]. The study observed approximately twice the expected number of cancer cases among employees across various oil companies operating in the country, including refineries, production facilities, warehouses, and distribution centers. The most prevalent cancer types identified were lung cancer and pleural cancer, both of which have been associated with PAH exposure, along with non-Hodgkin lymphoma, multiple myeloma, and leukemia, the latter primarily linked to benzene exposure. Benzene is also an aromatic compound derived from oil refining; however, it is characterized by its distinctive properties. Volatile substances, along with PAHs, have been extensively associated with the development of cancer. Similar findings were reported by Järvholm et al. in Sweden, where a cohort study was conducted involving 4,319 Swedish laborers (4,128 men and 191 women) with a minimum of one year of employment in the energy industry [67]. Cancer incidence among these workers was compared to that in the general

population, and the results indicated that refinery operators exhibited a statistically significant increase in the occurrence of leukemia, with six cases reported. Versus 1.7 expected, with a 90% confidence interval [CI] for the relative risk, in comparison to the general population.

Furthermore, the likelihood of being exposed to a chemical by inhaling it is connected to how likely it is to evaporate either directly from the soil or from bodies of water that have been contaminated after the substance has been released [65]. In addition, the solubility and the specific gravity of a compound have an effect on the extent to which a chemical may influence surface or groundwater supplies. This is because these two factors affect the degree to which a chemical can impact these water sources, and as a result, they also affect the degree to which exposure via these channels poses a danger [57]. The intake of a combination of chemicals that are vaporized from soil that is polluted has a negative impact on the health of humans [68]. When a group of people are exposed to pollutants, the kind or method of attack of the pollutants may have a detrimental impact on their health [69].

Death may occur in the event that exposures are at a high level [57]. Fatigue, headache, nausea, and sleepiness are all possible side effects of breathing in toluene at concentrations that are higher than 100 parts per million (100 ppm) over a period of time that exceeds several hours. The Centers for Disease Control and Prevention (CDC) has indicated that cessation of exposure will result in symptom resolution; however, persistent exposure may lead to irreversible harm to the central nervous system. Moreover, excessive amounts of benzene are correlated with a higher risk of leukemia. [70]. Chlorinated solvents may induce liver dysfunction, renal impairment and neurological degeneration and other consequences [57]. Other health problems, including nausea, eye irritation, skin rash and exhaustion are of great concern. Headaches are also a potential health impact.

2.11 Metagenomics

Metagenomics enables comprehensive analysis of microbial communities by sequencing genetic material directly from environmental or clinical samples, without cultivation and revealing uncultivable diversity. This approach has transformed microbial ecology, identifying novel pathogens, functional pathways, and microbiome-host interactions across ecosystems like soils and human microbiomes [71]. Next-generation sequencing have scaled metagenomics for high-

throughput applications, integrating shotgun and targeted methods to profile taxonomy, resistance genes, and metabolic potential simultaneously [72]. Recent high-throughput advancements have made metagenomics essential for linking microbial composition to environmental perturbations, such as pollution impacts on community structure.

16S metagenomics targets the 16S rRNA gene, a conserved bacterial marker with hypervariable regions that allow taxonomic profiling at genus-to-species resolution through PCR amplification and sequencing. Unlike shotgun metagenomics, 16S focuses on ribosomal sequences for rapid community surveys, normalizing for abundant taxa while identifying rare operational taxonomic units (OTUs) via clustering algorithms [73]. This method reconstructs phylogenetic trees from amplicon data, facilitating comparative analyses across diverse habitats [74]. Shotgun metagenomics offers comprehensive functional profiling, including viral and eukaryotic components, with higher resolution for metabolic reconstructions compared to marker-gene surveys. 16S metagenomics provides cost-effective, high-throughput taxonomy at depths exceeding 10^5 reads per sample, with standardized pipelines minimizing technical variability [73]. 16S metagenomics has successfully profiled thousands of microbiomes, correlating community shifts with oil spills and validating bioremediation candidates through OTU tracking over time [75]. Landmark studies achieved >95% species recovery in mock communities when using full-length sequencing, outperforming short-amplicon V4 regions. Its application in global soil databases has linked alpha-diversity to carbon sequestration, demonstrating predictive power for ecosystem services [76].

CHAPTER 3: MATERIALS AND METHODS

3.1 Sampling site

The petroleum contaminated soil samples were taken from three distinct filling stations and one garage in Noakhali, Bangladesh: Sonapur Filling Station (22.824951081800855, 91.09817074122229), Haque Filling Station (22.83021019139196, 91.10077186887327), Mesars Firoz Alam and Sons ($22^{0}39'58"N$, $90^{0}54'39''E$) and Mayer Dowa (22.8414° N, 91.0984° E).

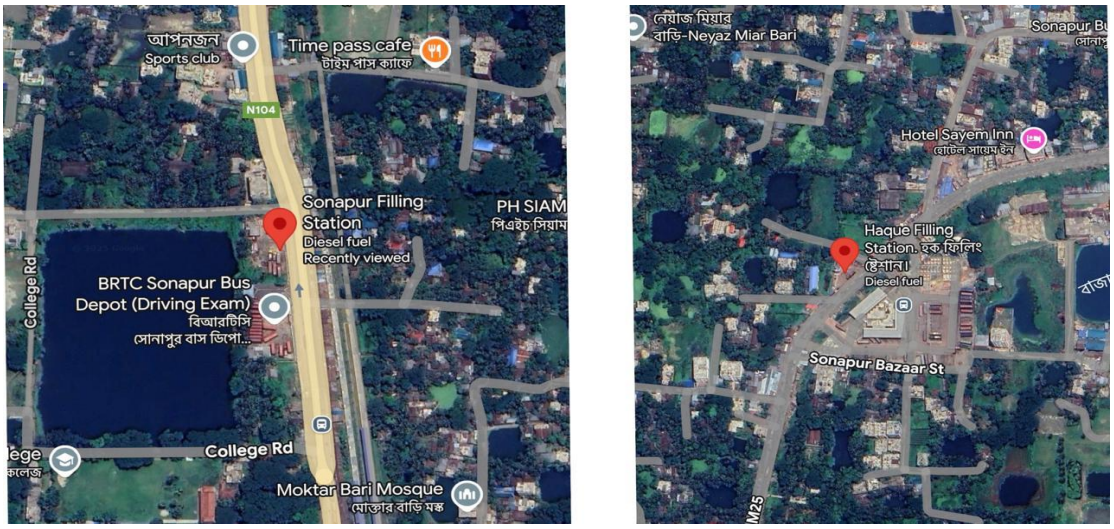

Figure 6: Sampling Location

3.2 Sample collection

A sterile spatula was used to collect roughly 100g of petroleum-polluted soil samples from several locations in Noakhali. After removing over 3 cm of the soil's surface, the samples were collected from each collection site. The samples were kept in a tight sealed, sterile falcon tube. The tube was then promptly taken to the laboratory and stored in an ice box.

Figure 7: Sample Collection

3.3 Isolation of petroleum degrading bacteria

To isolate hydrocarbon degrading bacteria, soil samples were enriched by transferring 2 gm into 250 ml Erlenmeyer flask containing 50 mL of mineral salt medium (MSM) and 2% (v/v) diesel as sole source of carbon [77] and incubated in a rotary shaker at 30^0 C and 110 rpm for 72 hours [78]. Filter paper with a pore size of 0.45 μm was used for the sterilization of diesel. As petroleum oils do not dissolve in water, tween80 was used in the media to improve solubilization. 0.005% tween80 (v/v) was used in the medium [79]. After incubation the culture was serially diluted upto 10^{-1} -10^{-7} fold. Sole carbon source was applied as 200 ul of petroleum (diesel) as sole source of carbon was spread onto the prepared mineral salt agar media [80]. Again, 100 ul of diluted cultures (10^{-5}-10^{-7}) were spread on mineral salt agar (MSA) media. The composition of MSA were listed in appendix B . The culture plates were incubated at 30^o C for 72 hours. The bacterial population grown in the MSA plate was considered to be capable of diesel degradation. Morphologically different colonies were sub-cultured on Mineral Salt Agar media. Finally, the pure colonies of each isolate were sub-cultured in LB media for further experiments and preserved as glycerol stock at -80 °C for future use.

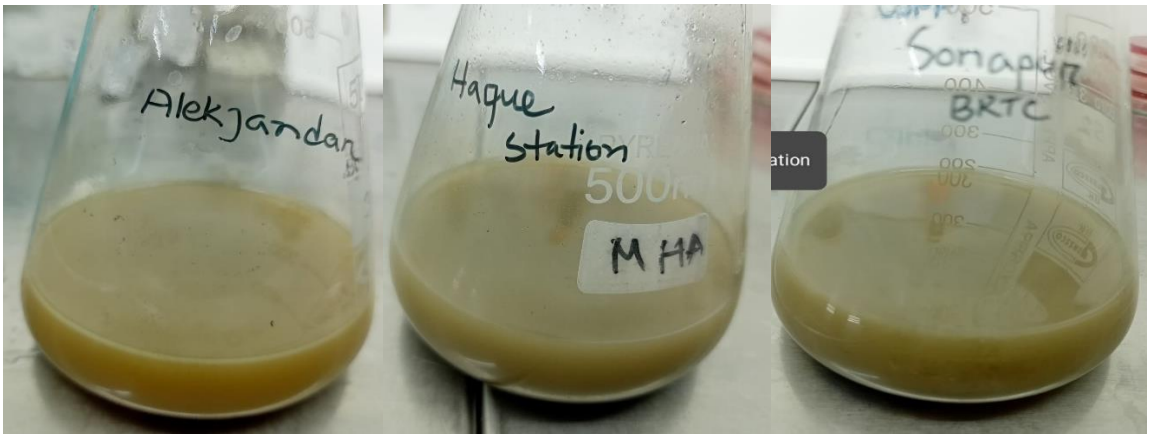

Figure 8: Enrichment of soil sample in mineral salt media

3.4 Identification and characterization

3.4.1 Morphological characterization

Gram staining was performed on presumptive isolates as follows: a smear of the presumptive isolate was made on a grease-free glass slide and waited for drying. The smear was then covered

with crystal violet for about 1 minute and rinsed with tap water. Following that, iodine was applied on the smear and wait for 1 minute. Then glass slide was rinsed with tap water. The smear was decolorized by adding solution of alcohol and acetone for 10- 30 seconds, then washed with water. The final dye safranin was used for 10-30 seconds, and rinsed with water and allowed to dry. The Gram-stained slides were then examined using an oil immersion objective under a microscope (100 X). If the bacteria exhibit a purple coloration, they are classified as Gram positive. If the bacteria exhibit a pink or red coloration, they are classified as Gram-negative.

3.4.2 Biochemical characterization

3.4.2.1 Triple sugar iron (TSI)

Test Triple Sugar Iron (TSI) agar was used to differentiate bacteria based on their ability to ferment sugars (lactose, sucrose, and glucose) and produce hydrogen sulfide (H_2S) (figure: 9) . To perform the test, a TSI agar was inoculated first by stabbing the butt of the medium with a sterile needle and then streaking the slant surface of the medium. After incubation o the test tube at 35–37°C for 18–24 hours, results were interpreted according to color changes and gas or H_2S production compared to control.

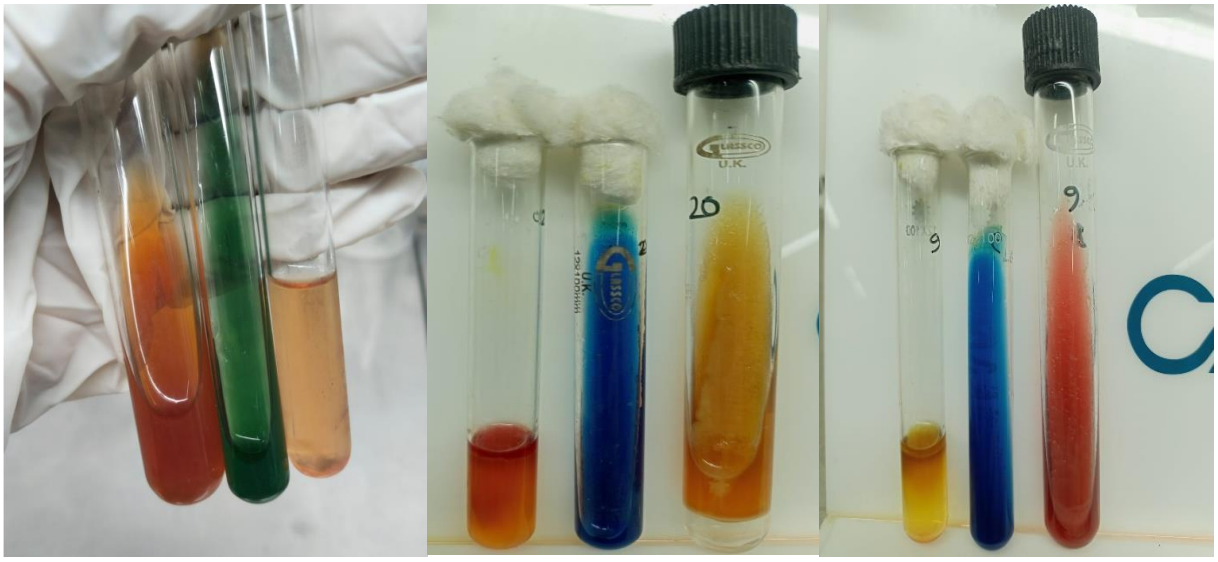

Figure 9: Biochemical Test (Control, test: *Klebsiella* spp, test: *Pseudomonas* spp.)

3.4.2.2 Motility indole urease Test (MIU Test)

In MIU test three tests in a single tube can be conducted that helps to differentiate the bacteria based on their motility, production of urease and indole (figure 9). To perform the test, tested bacterial colony was stabbed into the media by using a sterile needle. Then the test tube was incubated for 18-24 hours at 35–37°C. Following incubation, the test organisms in MIU media exhibit either diffuse growth or turbidity radiating from the stab inoculation line for motile organisms, whereas non-motile organisms display confined growth along the stab line. Furthermore, the urease activity of the bacteria can be assessed by the shift in color of the medium from yellow to pink-red, attributable to the presence of ammonium carbonate. Indole is synthesized from tryptophan found in casein enzymatic hydrolysate by the enzyme tryptophanase. The indole produced interacts with p-dimethylaminobenzaldehyde in Kovac's reagent to yield a quinoidal red-violet molecule.

3.4.2.3 Oxidase test

The oxidase test identifies organisms that generate the enzyme cytochrome oxidase and is useful in categorizing these species during the preliminary stages of identification. Cytochrome oxidase is involved in the process of electron transport chain. The examined colonies of bacteria was put to filter paper already soaked with freshly prepared oxidase reagent. The emergence of a purple-blue color within ten seconds signified a positive oxidase test.

3.4.2.4 Catalase test

The catalase test was performed for the detection of the presence of catalase enzyme in bacterial isolates, which breakdown hydrogen peroxide (H_2O_2) into water and oxygen, producing visible bubbles (figure: 10). A clean, dry glass slide was labeled, and a small amount of bacterial culture aged 18 to 24 hours was smeared onto slide using a sterile wooden stick or platinum loop. The bacterial stain was immediately treated with one or two drops of a 3% hydrogen peroxide solution. A dark background was used for immediate gas bubble monitoring. Whereas the lack of bubbles indicated a negative result, the presence of bubbling was noted as a positive result, suggesting catalase enzyme activity. *Staphylococcus aureus f*unctioned as the test's positive, while *Streptococcus species* acted as the negative control in order to validate the test. This test accurately

distinguishes catalase-positive, such as *Staphylococcus* from catalase-negative, such as *Streptococcus.*

Figure 10: Catalase test

3.4.2.5 Selective media

Selective media, specifically MacConkey Agar, was used to facilitate identification to differentiate between lactose-fermenting and non-lactose fermenting bacteria, as well as to identify possible gram-negative microbes (Figure 11), due to its bile salts and crystal violet, which suppress the development of Gram-positive bacteria.

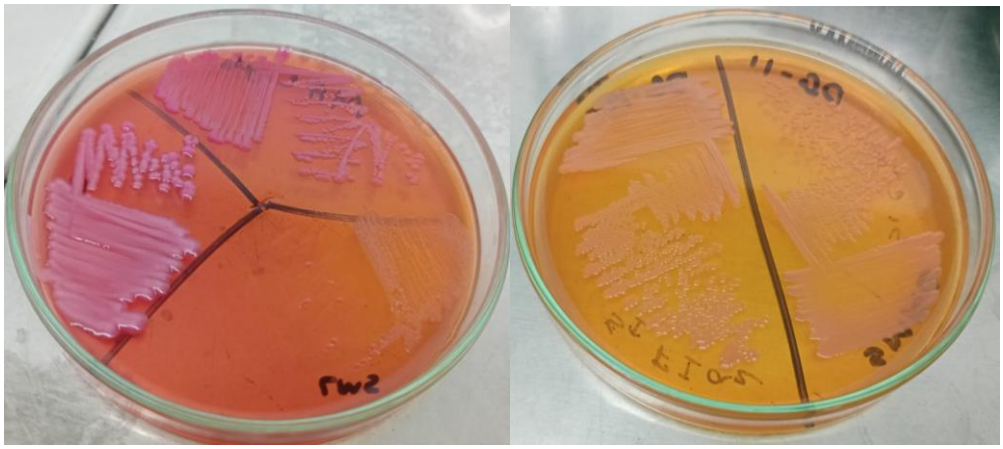

Figure 11: Lactose fermenter and non-lactose fermenter

3.5 Antibiotic sensitivity test

Given that the isolates possess the potential for bioremediation applications, it is essential to assess their susceptibility to identify remedies for any inadvertent human infections. In this investigation, we utilized the following Azithromycin (AT15) 15 mcg/disc, Tetracyclines (TE30) 30 mcg/disc, Chloramphenicol (C30) 30 mcg/disc, Gentamicin (GEN10) 10 mcg/disc, Imipenem (IPM 10) 10

mcg/disc, and Ciprofloxacin (CIP5) 5 mcg/disc correspond to the following classes: Macrolides, Penicillins, Tetracyclines, Chloramphenicol, Cephalosporins, Aminoglycosides, Carbapenems, and Fluoroquinolones. To assess antibiotic susceptibility, the isolates of bacteria were initially enriched in LB media to achieve logarithmic growth phase, with optical density (OD) evaluated between 0.3 and 0.9 using a spectrophotometer at 600 nm. Bacterial cultures were utilized as inoculum and applied to Muller Hinton Agar (MHA) using a sterile cotton swab. The antibiotic discs were aseptically placed on the Mueller-Hinton agar plate and incubated at 30°C for 18-24 hours. Following incubation, the zone of inhibition was quantified (Figure 12).

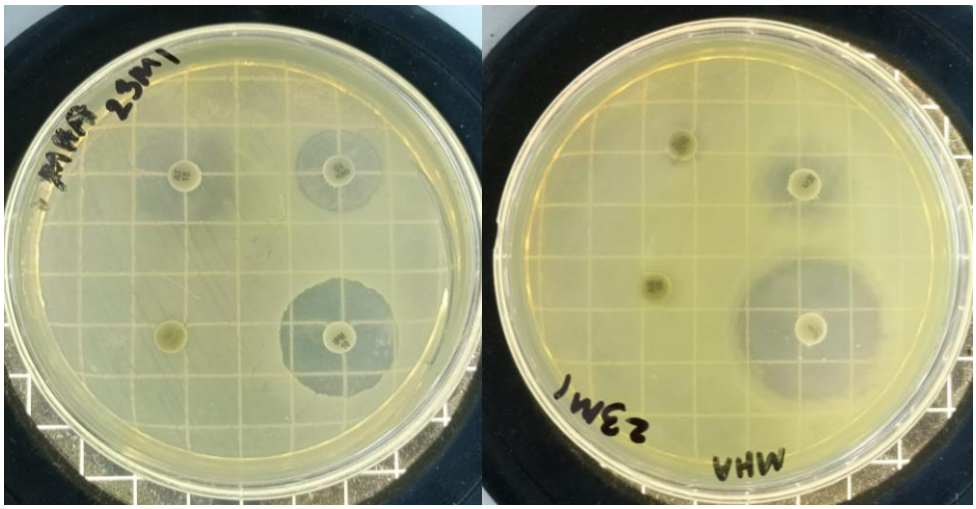

Figure 12: Antimicrobial sensitivity test

3.6 Screening of biosurfactant producing bacteria

To evaluate the biosurfactant-producing bacterial isolates capable of successfully degrading complex hydrocarbon of petroleum, a mineral salt medium was supplemented with 2% of each of the elements that follow: Crude oil (v/v) and glucose (w/v) were utilized (Figure 13). The medium's pH was adjusted to 7.0 and sterilized at 21 pressure for 20 minutes. Bacteria were cultured in Luria Bertani (LB) medium with agitation at 110 rpm at a temperature of 30°C for 24 hours. 1 mL of culture with an optical density at 600 nm (OD600) between 0.6 and 1.0 was transferred to 100 mL of carbon-amended mineral salt media. The infected media was cultured for five days at 30°C and 120 rpm. Following the incubation period, the supernatant without cells was obtained via centrifugation at 6500 g and 4°C for 20 minutes. Filtration was employed to eliminate any residual bacterial cells remaining in the supernatant. The procedure involved utilizing filter paper having a

pore size of 0.22 µm, after which the filtered component had been stored at a temperature of 4°C until its subsequent use [81].

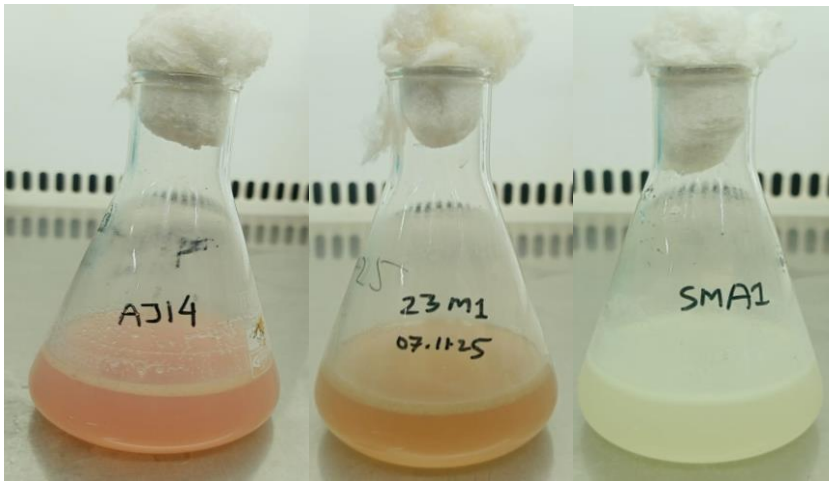

Figure 13: Biosurfactant production test

3.6.1 Blue agar plate method

The preparation of CTAB agar plates was accomplished by dissolving 12 grams of agar, 0.15 grams of cetyltrimethylammonium bromide (CTAB), and 0.005 grams of methylene blue in 1 liter of distilled water. The pH of the solution was then adjusted to 7, and the mixture was sterilized. In the CTAB plates, two holes with a diameter of 6.5 millimeters were made and each of the holes was filled with around 150 microliters of filtered cell-free supernatant. Following 48 hours of incubation at 37°C, the plates were examined for blue halos. The presence of a deep blue halo encircling the culture was considered acceptable. Blue halos surrounding the wells containing cell-free supernatant show the presence of anionic surfactant.

3.7 Lipid hydrolysis test

The lipid hydrolysis test was performed to detect the activity of lipase enzyme in bacterial isolates capable of hydrolyzing Tween 80, a lipid substrate composed of oleic acid esters. Pure bacterial colony were streaked onto agar plates containing 10 g/L peptone, 5 g/L NaCl, 0.1 g/L $CaCl_2 \cdot 6H_2O$, 10 mL (v/v) Tween 80 (added post-autoclaving at 60°C), and 20 g/L agar. Tween 80 hydrolysis by bacterial lipases liberates fatty acids, which precipitate as insoluble calcium salts upon reaction with Ca^{2+} ions, forming visible white precipitates around colonies (positive result) or showing no precipitation (negative result) [82]. This assay confirms the presence of extracellular lipases,

relevant for petroleum-degrading bacteria in bioremediation processes involving lipid-like hydrocarbon breakdown.

3.8 Microbial community analysis via 16S rRNA metagenomics

To analyze the petroleum hydrocarbon degrading bacterial community, collected soil samples were enriched in mineral salt medium. Three samples were conducted for 16S rRNA metagenomics which showed diverse bacterial community in the laboratory condition. To enrich the sample 4g of soil samples were enriched in 250 ml conical flask containing 100 ml mineral salt media supplemented with 2% diesel as carbon source. The culture was incubated at 30o C for 38 hours in rotary shaker at 120 rpm. After incubation, 50 ml of the enriched culture was kept in sterile falcon tube and send to the Genome Centre of International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) for 16S rRNA amplicon sequencing by Illumina MiSeq platform.

3.8.1 DNA extraction and 16S rRNA sequencing

DNA from bacteria was extracted from enrichment of petroleum-contaminated soils obtained from the Noakhali region using the UltraClean® Soil DNA Isolation Kit. The quantity and quality of DNA were evaluated using spectrophotometry and agarose gel electrophoresis to confirm its appropriateness for subsequent PCR amplification. The polymerase chain reaction (PCR) analysis with primers 515F (FWD: GTGYCAGCMGCCGCGGTAA) and 806 (REV: GGACTACNVGGGTWTCTAAT) were intended to amplify the V4 hypervariable region of bacterial 16S rRNA genes, hence enabling microbial community characterization. The PCR procedure had been prepared in an overall volume of 25 µL, consisting of 1 µL of template DNA, 0.5 µL for each primer (10 µM), 10 µL of 2x PCR master mix, and 13 µL of PCR-grade water to achieve the desired volume. The PCR parameters were as follows: 3 minutes at 94°C; 35 cycles of 45 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 72°C, followed by 10 minutes at 72°C and a hold at 4°C. PCR amplification was conducted in triplicate with 25 µL reactions for each sample. Triplicate PCR results from each sample were combined (total volume 75 µL) and confirmed by 2% agarose gel electrophoresis. Amplicons were subsequently measured via the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Equimolar quantities of amplicons were combined to build a sequencing library; 5–10% PhiX control DNA was used to enhance sequence complexity. Sequencing was performed on the Illumina MiSeq platform

utilizing the 2x250 bp paired-end sequencing chemistry in accordance with the technique outlined by Caporaso et al. (2012). The resultant sequence reads underwent quality filtration, chimera elimination, and subsequent bioinformatics analysis for taxonomy and diversity characterization of hydrocarbon-degrading microbial populations.

3.8.2 Data pre-processing

The paired-end sequences were converted to QIIME2 format for baseline data processing, quality control, taxonomic grouping, relative abundance evaluation, and functional assessment using the QIIME2 platform (version 2024.10). The paired-end sequence data preliminary processing has been conducted through the DADA2 plugin in QIIME2 [83]. The raw data was refined to exclude barcodes, ambiguous bases, primers sequences and homopolymers exceeding six bases. Reads less than 251 bp along with an average Phred quality score below 30 were eliminated. DADA2 filtered noisy reads, performed corrections for error on marginal sequences, eliminated chimeras and singletons, combined denoised paired-end reads, and de-replicated the filtered reads. The results obtained from DADA2 are termed as amplicon sequence variants (ASVs).The overall workflow is shown in figure 14.

3.8.3 Taxonomic assessment

The taxonomic classification was done by annotating the amplicon sequence variants (ASVs) that were created from the DADA2 denoising method using a pre-trained Greengenes 13_8 reference database with Naive Bayes classifier [84]. The classifier is constructed using a supervised machine-learning method. In this method, unknown sequences are assigned taxonomy after the Naive Bayes algorithm identifies the reference database's typical nucleotide patterns for each taxonomic classification [84].

The classifier undergoes training using thousands of reference 16S rRNA sequences processed by the QIIME2 developers. From this data, they construct k-mer frequency distributions, which are small subsequences that are useful statistical features for taxonomy prediction. There are several degrees of hierarchical linking between these k-mer patterns and recognized taxonomic labels, from kingdom to species. When trained on ASVs, the classifier takes their k-mer composition into account to determine the likelihood that each ASV is a member of a specific taxon. The taxonomy that corresponds to the taxon with the highest posterior probability is then assigned.

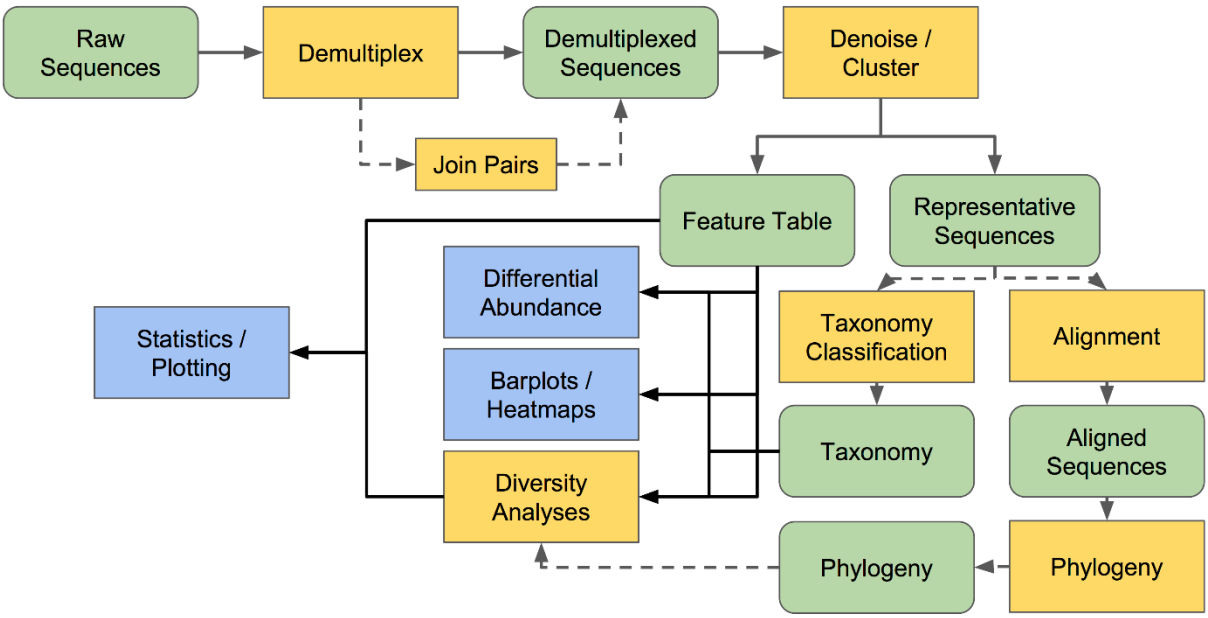

Figure 14 : Data analysis though QIIME 2 pipeline

3.8.4 Diversity analysis

Diversity assessments were conducted in QIIME2 to analyze both within-sample and between-sample microbial community patterns. Alpha diversity measurements included Observed Features (richness), Shannon diversity (richness and evenness), Faith's Phylogenetic Diversity (phylogeny-aware richness), and Pielou's Evenness, providing complementary insights into community composition within samples. Beta diversity was assessed using Jaccard and Bray–Curtis distances (non-phylogenetic) enabling comparison of community similarity across samples based on both taxonomic presence/absence and relative abundance [85]. As most phylogeny-based diversity metrics need an evolutionary historical context, a rooted phylogenetic tree was built based on amplicon sequence variants (ASVs). Phylogeny construction was performed out via the q2-fragment-insertion program [86, 87], which utilizes a reference-oriented SEPP algorithm to insert query sequences into a high-quality core phylogeny. The Greengenes 13_8 database was used as a model tree, ensuring constant proper placement of the short-read 16S rRNA segments. To standardize sampling effort across samples, a rarefaction depth of 13455 sequences per sample was established based on the point at which alpha rarefaction curves exceeded saturation, indicating that increasing sequencing depth did not generate substantial gains in observed richness.

This rarefied feature table was utilized as the input for QIIME2's core-metrics-phylogenetic process.

3.8.5 Functional profiling using 16S rRNA datasets

Amplicon sequence variants (ASVs) were reclassified using the GREENGENES reference database (May 2013 release) prior to predict about the metagenome with PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [88]. Using 16S rRNA marker gene sequences, PICRUSt, which is connected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, predicted how many gene families were in petroleum contaminated soil samples. Before prediction and pathway collapse, the number of copies of the 16S rRNA gene was used to normalize the number of ASVs. This was done by ASV count by its predicted copy number. The output was visualized by using R packages.

CHAPTER 4: RESULT

4.1 Isolation of petroleum degrading bacteria

MS (Mineral salt) medium was supplemented with diesel, used for the isolation of the petroleum degrading bacteria. After incubation the cultures were spread onto mineral salt agar media (MSA) plate which was supplemented with diesel. Morphologically different colonies were peaked and sub-cultured onto MSA media supplemented with diesel by streak plate technique. Thirty three were selected as potential candidates for petroleum hydrocarbon (diesel) degradation. As the isolates were grown in media with diesel as sole carbon source for their survival and growth and in the absence of diesel their growth was limited or absence (shown in the figure 15), it may be considered that these isolates are able to uptake diesel as their food and nutrition and thereby are able to breakdown. Moreover, selected isolates also streaked onto Luria-Bertani agar media for pure culture.

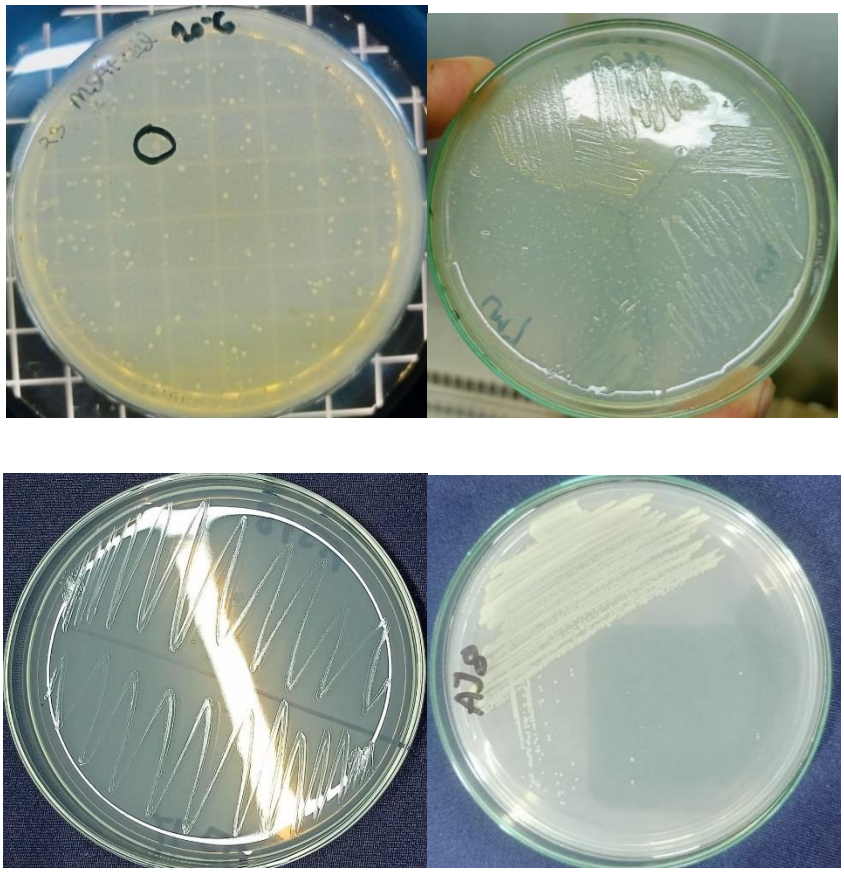

Figure 15: Isolation of petroleum degrading bacteria

4.2 Identification of bacterial isolates

4.2.1 Morphological and gram stain characterization

The primary step of isolation provide a wide range of bacterial isolates with different colony appearances on Luria Bertani (LB) and MacConkey agar culture plates respectively. Gram staining confirmed that there were mostly Gram-negative rods. Many of these rods had unique colony features, as the large, opaque, and flat colonies with uneven edges observed in samples H1 and SLA, and the round, elevated, mucoid colonies shown in samples H5A and H7A. A smaller group of isolates was found to be Gram-positive, such as rods (H2, SLB), tetrads (23M4), and clusters (23M5). These isolates did not grow on MacConkey agar, which differentiated them apart from the Gram-negative enteric bacteria. Colony morphology on Luria-Bertani agar media and MacConkey agar media shown in Appendix A, Table 05**.**

4.2.2 Biochemical characterization

After accomplishment of various tests (triple sugar iron, motility indole urea, citrate, oxidase and catalase) for candidate isolates were recognized morphologically and biochemically. The comprehensive biochemical profiling using Triple Sugar Iron (TSI), MIU, Citrate, Oxidase, and Catalase assays facilitated the probable identification of the isolates to the genus or species level. Biochemical test results of 33 bacterial isolates are presented in Appendix A, Table 06. The findings showed that *Pseudomonas* was the most dominant genus found. Some samples were proven to be *Pseudomonas aeruginosa* (H1, SLA, AJ14), while others were confirmed to be *Pseudomonas spp.* (H7B, AJ6). The study also found a significant amount of other Gram-negative bacteria, such as *Klebsiella spp.* and *Klebsiella oxytoca*, which had acidic TSI responses, as well as *Acinetobacter spp.*, *Hafnia spp.,* and *Enterobacter spp*. The Gram-positive isolates included *Bacillus spp., Staphylococcus spp., Micrococcus spp.,* and *Streptococcus spp*., which were distinguished by their unique biochemical profiles.

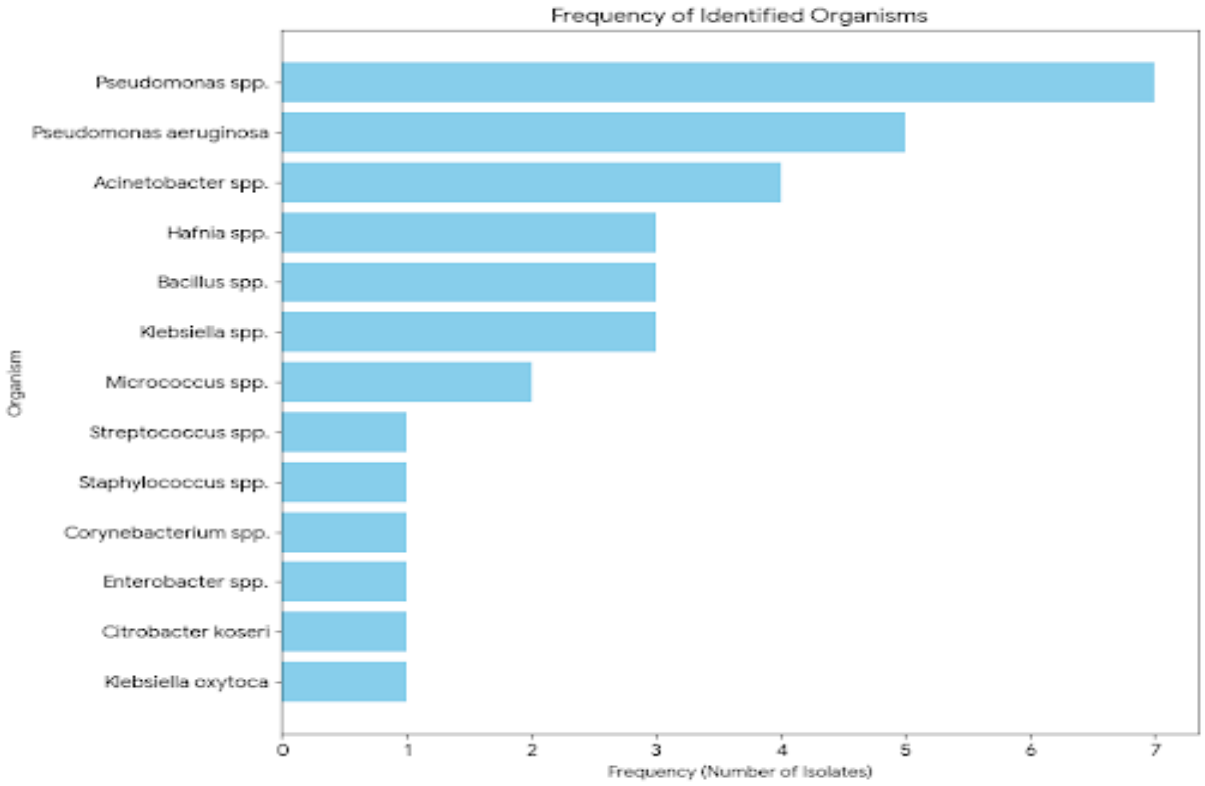

Figure 16: Abundance of bacterial genus according to biochemical test

4.3 Antimicrobial sensitivity test

The antimicrobial sensitivity testing (AST) showed that the four sample sites had different patterns of resistance (Appendix Table 7, Figure 17). Sonapur Filling Station (BRTC) had the highest level of antibiotic resistance, with a resistance rate of 40.0% across all tested isolates and a low sensitivity rate of 30.0%. This means that there are a lot of resistant bacterial strains at this transit hub. Haque Filling Station came in second, with a resistance rate of 38.1%. On the other hand, samples from Alexander were the most sensitive to the antibiotics examined, with a sensitivity rate of 61.9%. This means that resistance mechanisms are less common in this location. Datterhat Garage (DG) likewise had a good profile, with 55.6% sensitivity. However, it had a high intermediate resistance rate of 25.0%, which might mean that it is becoming more resistant.

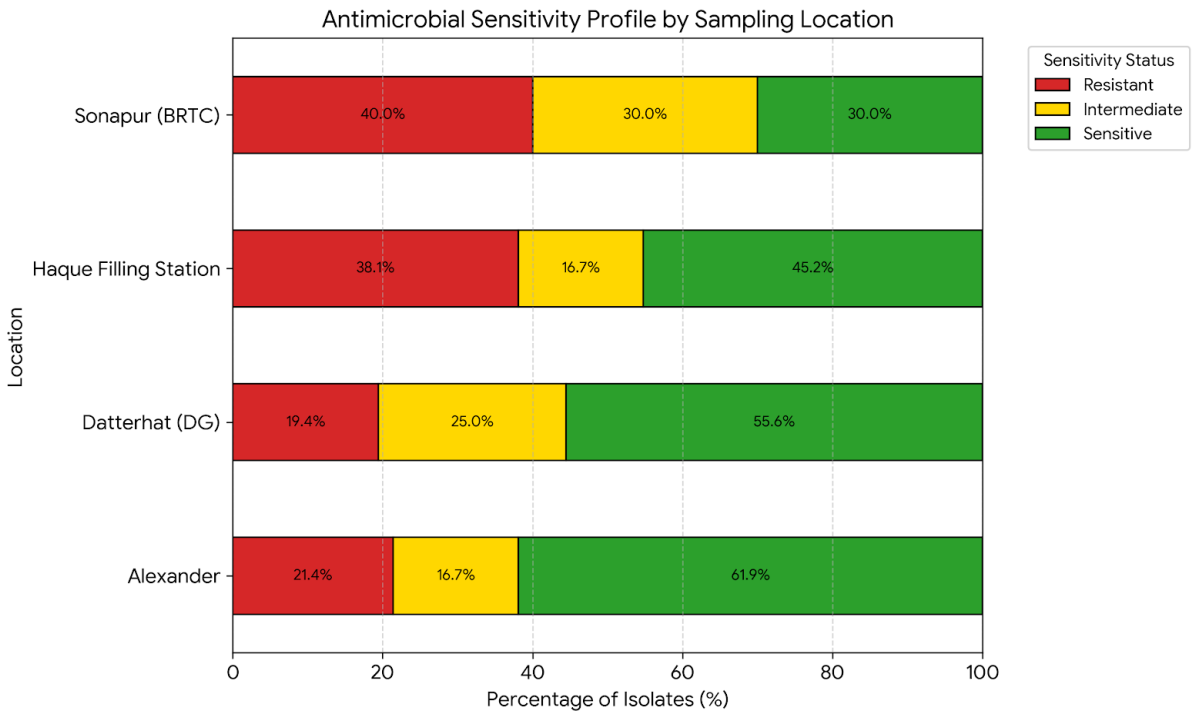

Figure 17: Antimicrobial sensitivity profile by location

The study of the antimicrobial sensitivity test indicates major shifts in resistance patterns across antibiotics and specific bacterial isolates **(**Appendices A**,** Table 07). Tetracyclines (TE30) were the least efficient antibiotic tested, with a very high resistance rate of 83.3% across all bacterial isolates. Azithromycin (AT15) was the second most resistant antibiotic, with 33.3% of isolates exhibiting resistance. Ciprofloxacin (CIP5) and Imipenem (IPM 10) both had a resistance rate of 23.3%. Chloramphenicol (C30), on the other hand, was the most effective antimicrobial drug in this investigation, with just 3.3% of the isolates proving resistant.

4.4 Functional screening

To evaluate the industrial potential of the discovered strains, specific functional tests for lipase and biosurfactant production were performed showed in (Table 04, 05). The lipase production test showed that numerous isolates were positive, including *Pseudomonas aeruginosa* (e.g., AJ14, 23M1) (Figure 18) and other *Pseudomonas species* (e.g., AJ6, LM5), *Acinetobacter spp*. (AJ12), and *Serratia spp*. (SMA1). On the other hand, isolates like *Enterobacter spp. and Klebsiella spp*. did not show any lipase activity. The biosurfactant production test revealed that *Pseudomonas aeruginosa* (samples 23M1 and AJ14) was clearly positive, as shown by a blue halo zone (Fig 19).

Other organisms that were examined, such as *Klebsiella spp*. and *Hafnia spp*., did not exhibit this activity (Fig 19). These results show that the *Pseudomonas* isolates might be used in industry for surfactants and lipases.

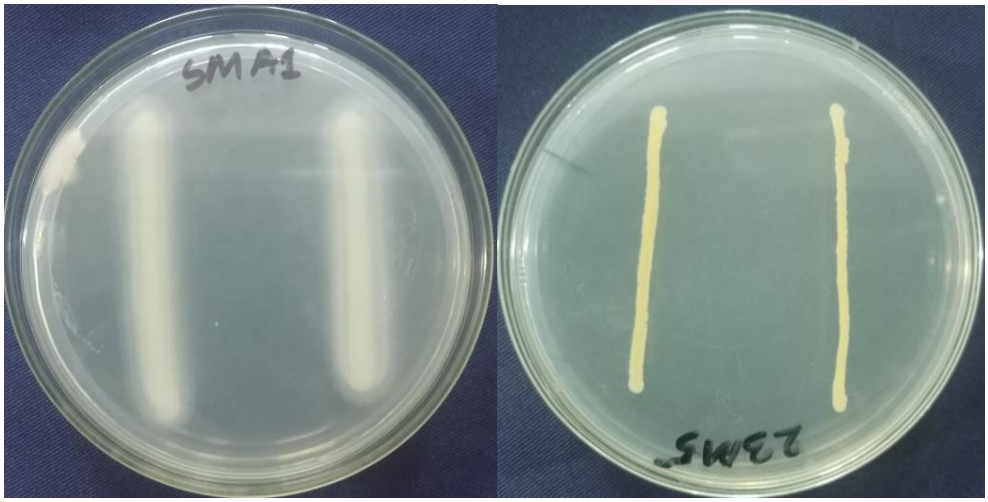

Figure 18: Lipid hydrolysis test (A: positive, B: Negative)

Table 1: Lipase activity profile

ID	Test Result	Organism Name
AJ6	Positive	*Pseudomonas spp*
AJ12	Positive	*Acenetobacter spp*
LM5	Positive	*Pseudomonas*
AJ14	Positive	*Pseudomonas aeruginosa*
23M1	Positive	*Pseudomonas aeruginosa*
AJ9	Positive	*Pseudomonas spp*
SMA1	Positive	*Serratia spp*
LM2	Negative	*Enterobacter spp*
AJ10	Negative	*Klebsiella spp*
23M5	Negative	*Staphylococcus aureus*
SLD2	Negative	*Hafnia spp*

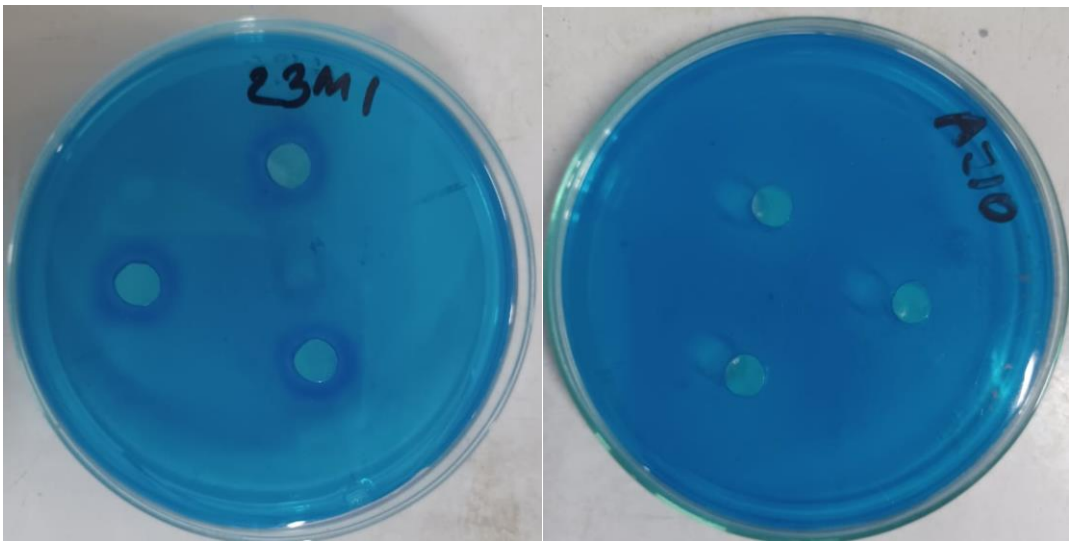

Figure 19: Biosurfactant production test (A: positive, B: Negative)

Table 2: Biosurfactant production test result

ID	Bacterial Name	Test Result (Blue halo zone)
23M1	*Pseudomonas aeruginosa*	Positive
AJ10	*Klebsiella spp*	Negative
SMA1	*Serratia spp*	Negative
AJ14	*Pseudomonas aeruginosa*	Positive
LM4	*Klebsiella spp*	Negative
SLD2	*Hafnia spp*	Negative

4.5 Metagenomics data analysis

4.5.1 Quality assessment, raw data refinement and chimera identification

Sequencing of the 16S rRNA genome from three samples generated 75,148 reads. The paired-end raw data generated in FASTQ files underwent rigorous cutting and filtering. Around eighty-one percent of the data had been retained following multiple quality filtering procedures as seen in Table 03. The high- throughput sequencing of the 16S rRNA gene provided 51,251 raw non-chimeric reads from the three study sites. The Alexander site (AFS_S92) produced the highest sequencing depth with 24750 reads, followed by Sonapur (SFS_S94) with 14,751 reads, and Haque (HFS_S93) with 15770 reads. To mitigate biases associated with unequal sequencing

depths, the dataset was rarefied to the minimum library size of 13,455 reads per sample. The analysis identified a total of 172 unique Amplicon Sequence Variants (ASVs) across all samples. Specific statistics regarding the number of chimeric sequences removed prior to the generation of the ASV table. The number of screened reads each sample following quality processing is listed in the table 04.

Table 3: Summary of read count after various filtering steps

Sample-ID	No. of raw reads	No. of reads after filtering	Percentage retained
AFS	33129	26903	81.21%
HFS	20639	16550	80.19%
SFS	21380	17370	81.24%

Table 4: Retrieve non-chimeric sequence after denoising and alignment

Sample-ID	Denoised	Merged	Percentage of Input Merged	Non-chimeric	Percentage of Input Non-chimeric
AFS	26740	26300	79.39%	24750	74.71%
HFS	16397	15904	77.06%	14751	71.47%
SFS	17204	16779	78.48%	15770	73.76%

4.5.2 Alpha diversity analysis

Alpha diversity analysis revealed varying microbial richness and evenness across soil samples from three locations (AFSS92, HFSS93, SFS_S94). Observed ASVs were highest in AFS_S92 (102), followed by HFS_S93 (82) and SFS_S94 (89) (figure 20), with Chao1 estimates confirming greater richness potential in AFSS92 (107.62) compared to HFS_S93 (54.81) and SFS_S94 (32.66). Shannon diversity index indicated moderate evenness (AFS_S92: 2.89; HFS_S93: 2.72; SFS_S94: 2.66) (figure 21), while Simpson indices showed comparable dominance patterns (AFS_S92: 0.90; HFS_S93: 0.88; SFS_S94: 0.85).

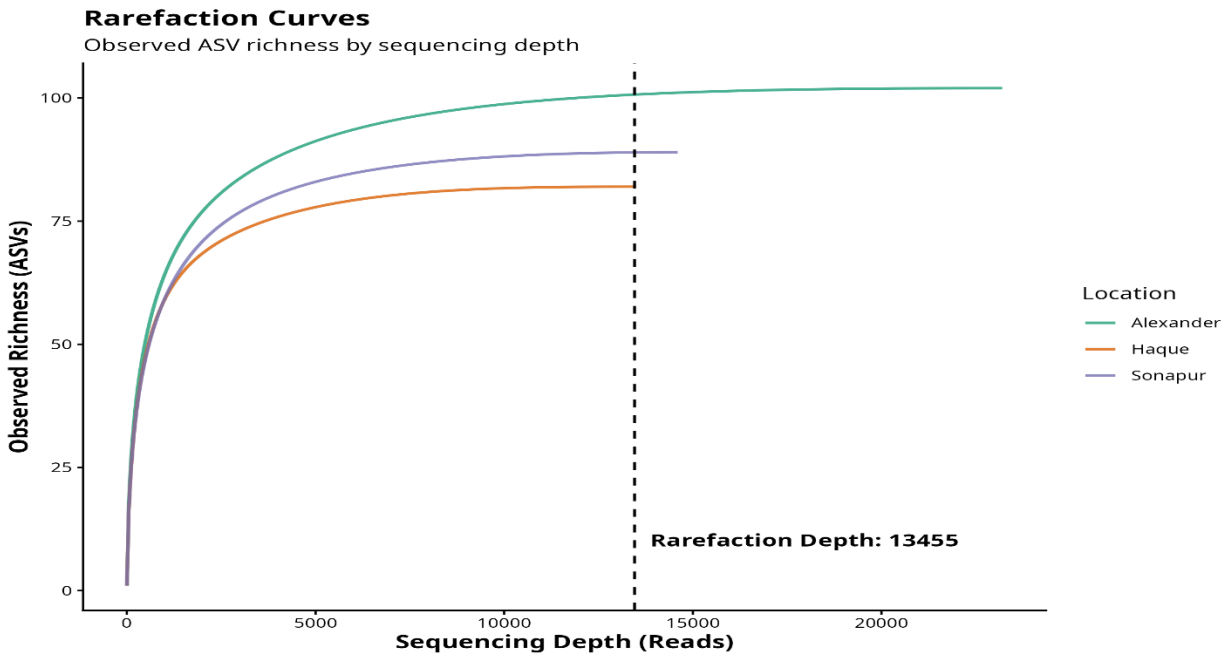

Figure 20: Observed features

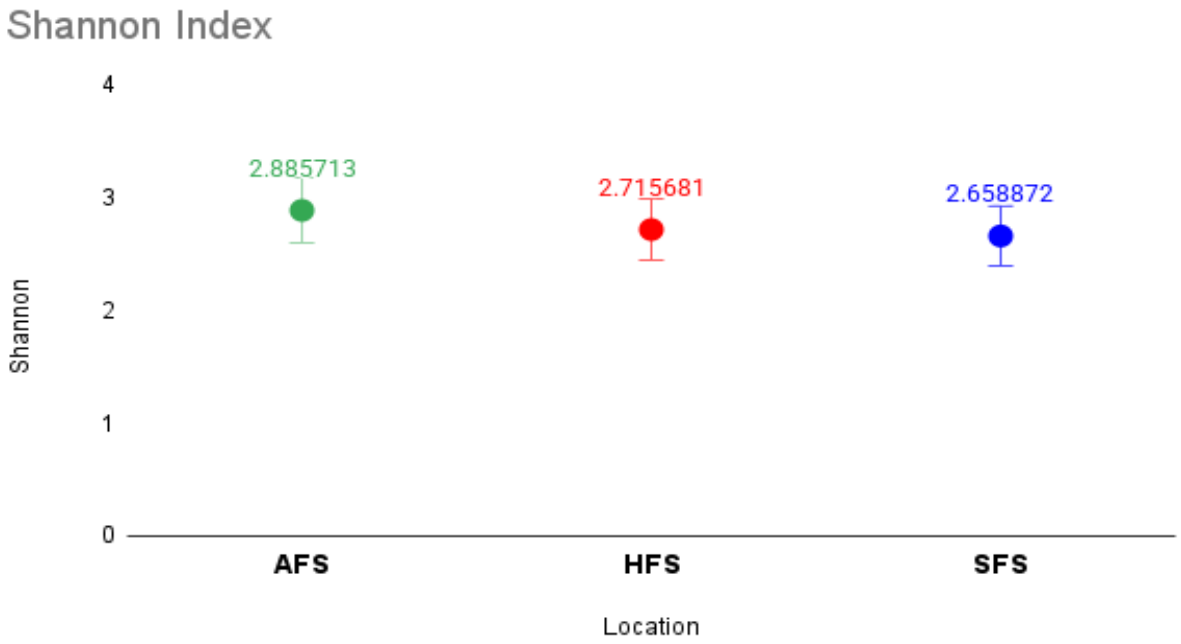

Figure 21: Shannon Index

4.5.3 Diversity profile of the distinct environments

Three petroleum-contaminated soil samples were analyzed to elucidate the structure of bacterial diversity by Illumina MiSeq sequencing of the gene encoding 16S rRNA. Taxonomic profiling at the phylum level indicated that the communities of bacteria were mostly made up of Bacteroidota and Proteobacteria, which together accounted for the majority of the sequences. Bacteroidota was the most abundant phylum in the Haque (60.8%) and Alexander (49.4%) samples, whereas it was less prevalent in Sonapur (43.7%). Conversely, Proteobacteria showed the highest relative abundance in the Sonapur filling station sample (47.8%), compared to 39.1% in Alexander and 27.3% in Haque Filling Station. The phylum Firmicutes was consistently present across all sites, ranging from 6.3% to 9.7% in relative abundance. At the genus level, the communities were driven by the dominance of *Prevotella* and *Pseudomonas*. *C* trend, being the dominant genus in Sonapur with a relative abundance of 38.7%, significantly higher than in Haque (15.2%) and Alexander (31.2%). Other notable genera included *Klebsiella* and *Bacteroides*, which maintained lower but consistent abundances across the study sites.

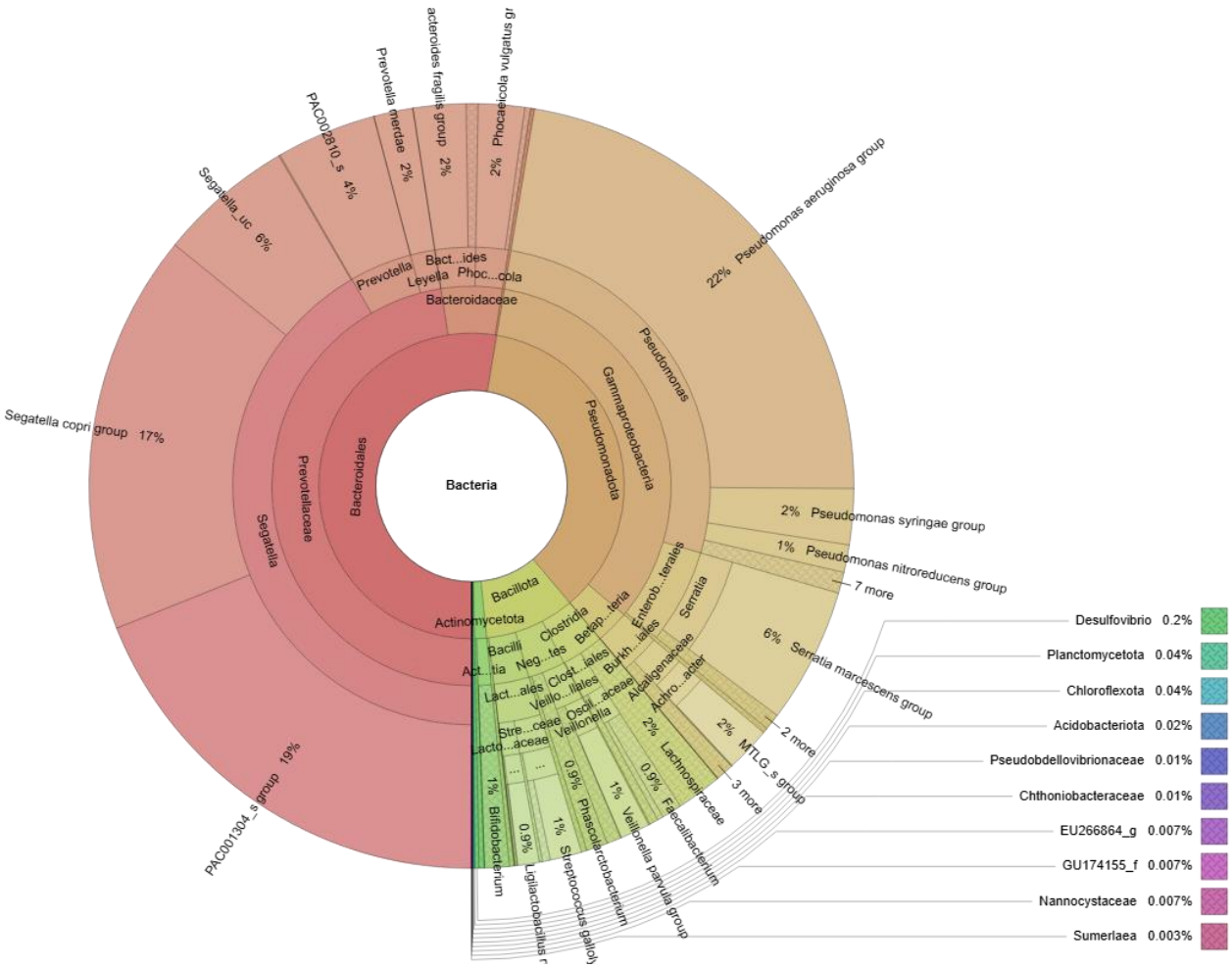

Figure 22: Abundance of Genus in AFS

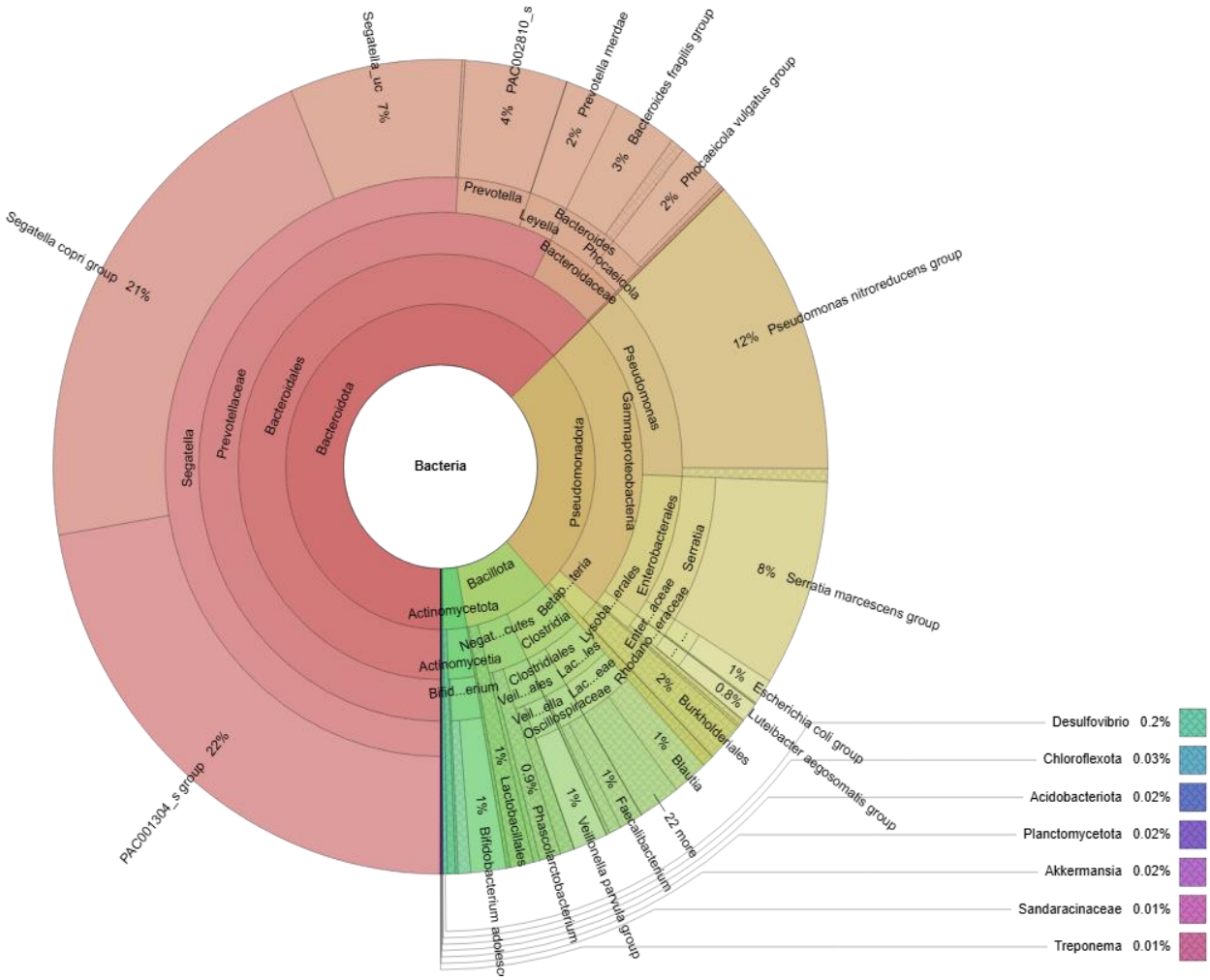

Figure 23: Abundance of Genus in HFS

The abundance of bacterial genus according to their locations are shown in figure 22-24.

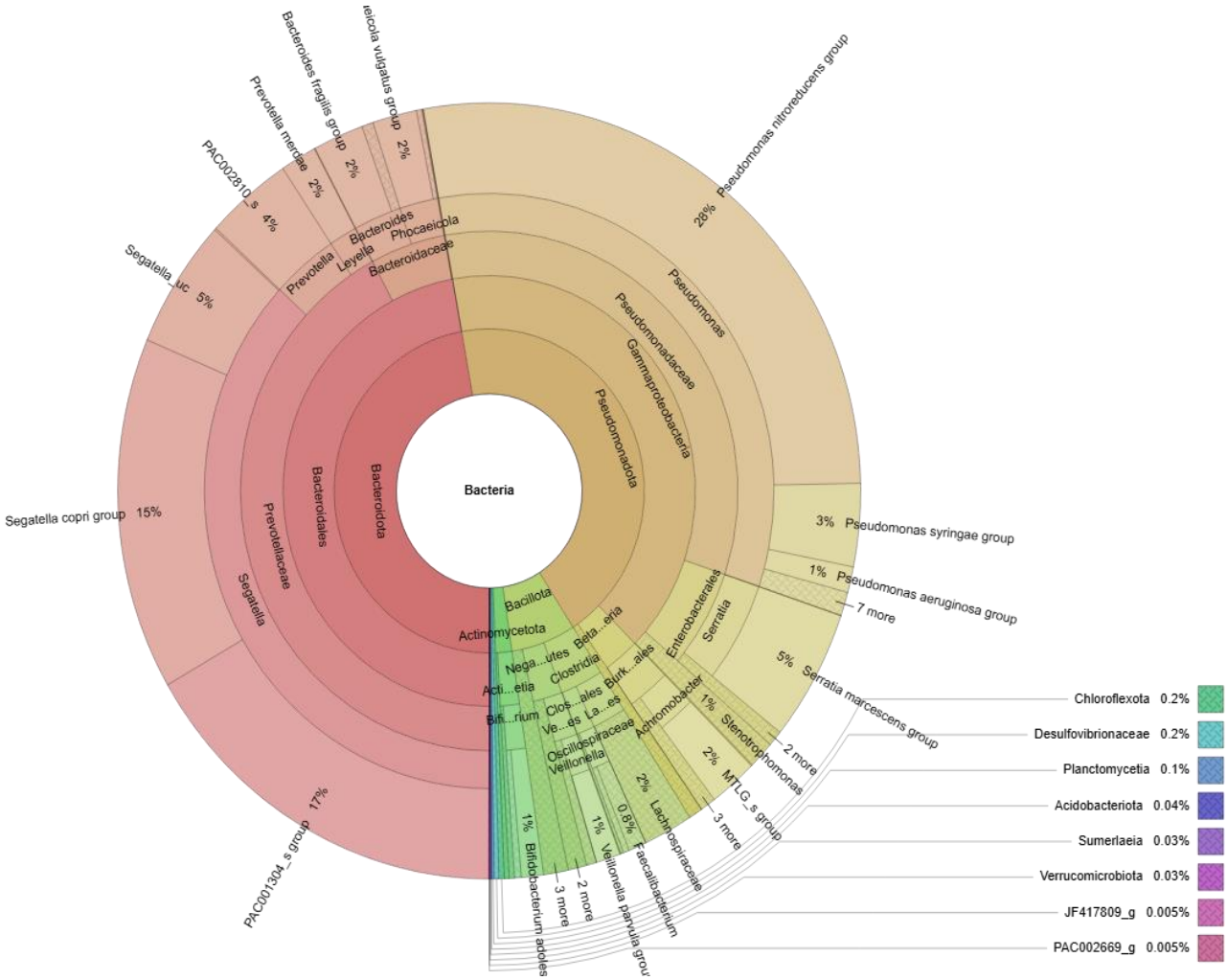

Figure 24: Abundance of Genus in SFS

4.5.4 Predicted functional profiling

4.5.4.1 Functional pathway analysis

Prediction of functional pathway associated with the enzyme commission (EC) numbers and KEGG Orthology (KO) analysis based on the 16S rRNA genes revealed a diverse range of pathways. Total hydrocarbon degradation potential in soil from filling station of Alexander showed highest pathway abundance and Haque filling station showed least comperative to other location showed in figure 25. Figure 26 illustrates the most abundant metabolic petroleum hydrocarbon degrading pathways (Alkane degradation, Catechol ortho-cleavage, CBB cycle, Methane

oxidation, Methanol oxidation, Protocatechuate degradation l, Protocatechuate ortho-cleavage, Toluene degradation). The methanol oxidation pathway exhibited the most significant expression among all soil samples. The toluene hydrolysis pathway showed the lowest expression levels.

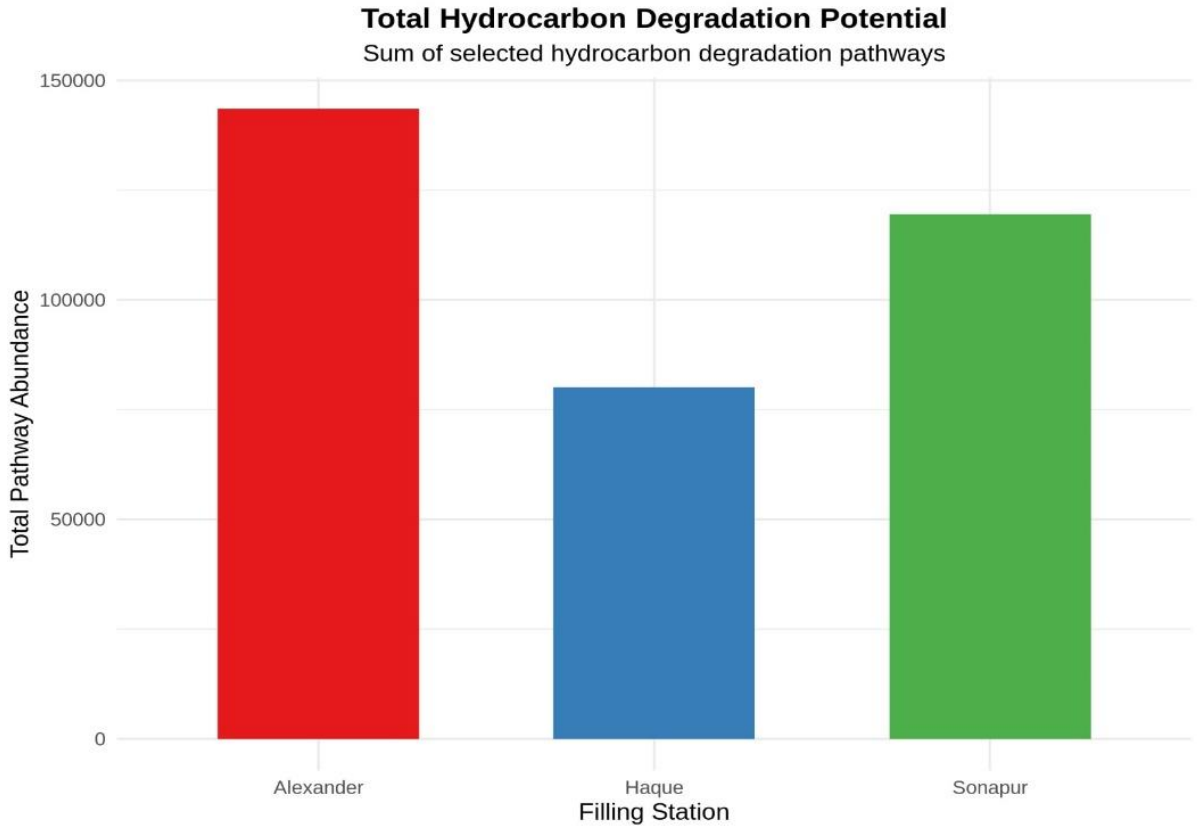

Figure 25: Total hydrocarbon degradation potential

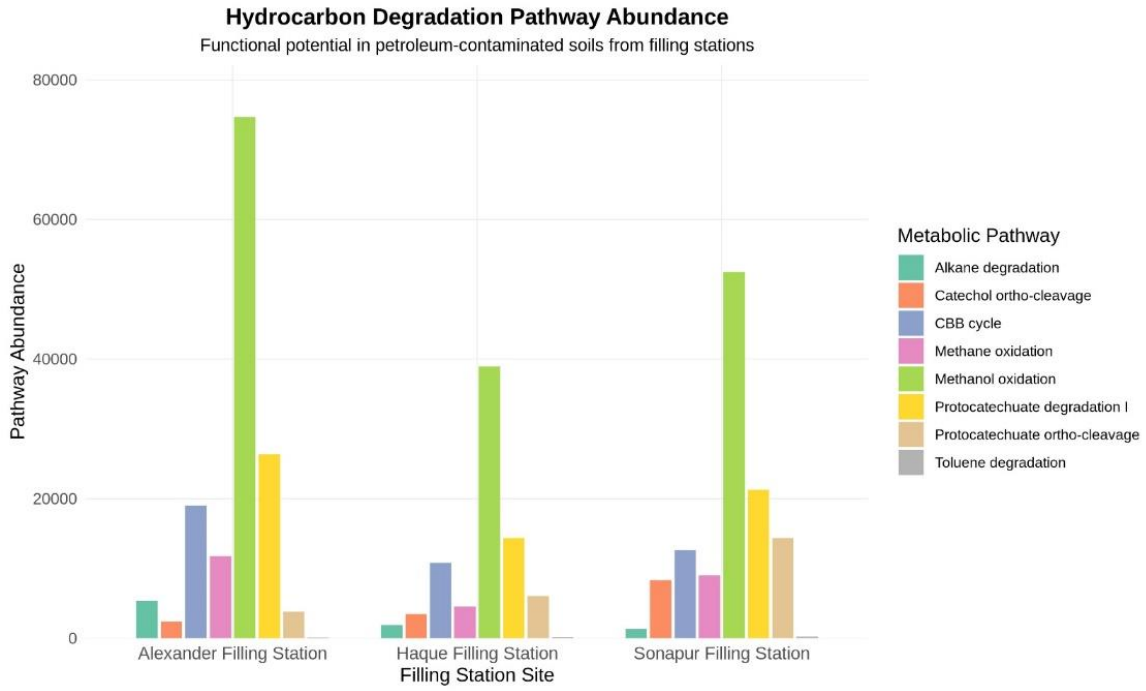

Figure 26: Abundance of petroleum hydrocarbon degradation pathway abundance

4.5.4.2 Prediction of functional genes

AFS soils also have higher potential for several catabolic genes and biogeochemical pathways and HFS and SFS differ in more specialized functions. Genes such as *dsrAB* and *dsrAB*_2, associated with dissimilatory sulfate reduction, showed relatively higher predicted abundance at AFS and SFS but in HFS is comparatively depleted. Hydrocarbon and aromatic-compound degradation genes, including *alkB* (alkane monooxygenase), *tmoA-F* (toluene monooxygenase), *benA/benC, bssA/bssC*, and *nahA-F* variants, tend to be more abundant in AFS and, for some markers, in SFS. Nitrogen-cycling genes such as *nirS/nirK, nirS/nirK_2, nosZ, narGHI*, and *aprAB* showed a mixed pattern with Alexander often enriched for denitrification and nitrate reduction pathways. But HFS and SFS contribute more variably. Finally, genes like *norB*, *bcrABCD*, and various transport or regulator genes show elevated levels at Haque or Sonapur in specific clusters shown in figure 28.

4.5.4.3 Prediction of antibiotic resistance profile

Predicted antibiotic resistance genes varies by location, with Alexander Filling Station (AFS) generally enriched in several resistance determinants compared with Haque Filling Station (HFS) and Sonapur Filling Station (SFS) soils. Imipenem resistance potential is highest at SFS,

intermediate at HFS, and lowest at AFS shown in figure 27. Tetracycline resistance and gentamicin resistance are most pronounced at AFS and decline in HFS and are lowest in SFS. Ciprofloxacin and chloramphenicol resistance signals are detectable but comparatively weak, especially in HFS.

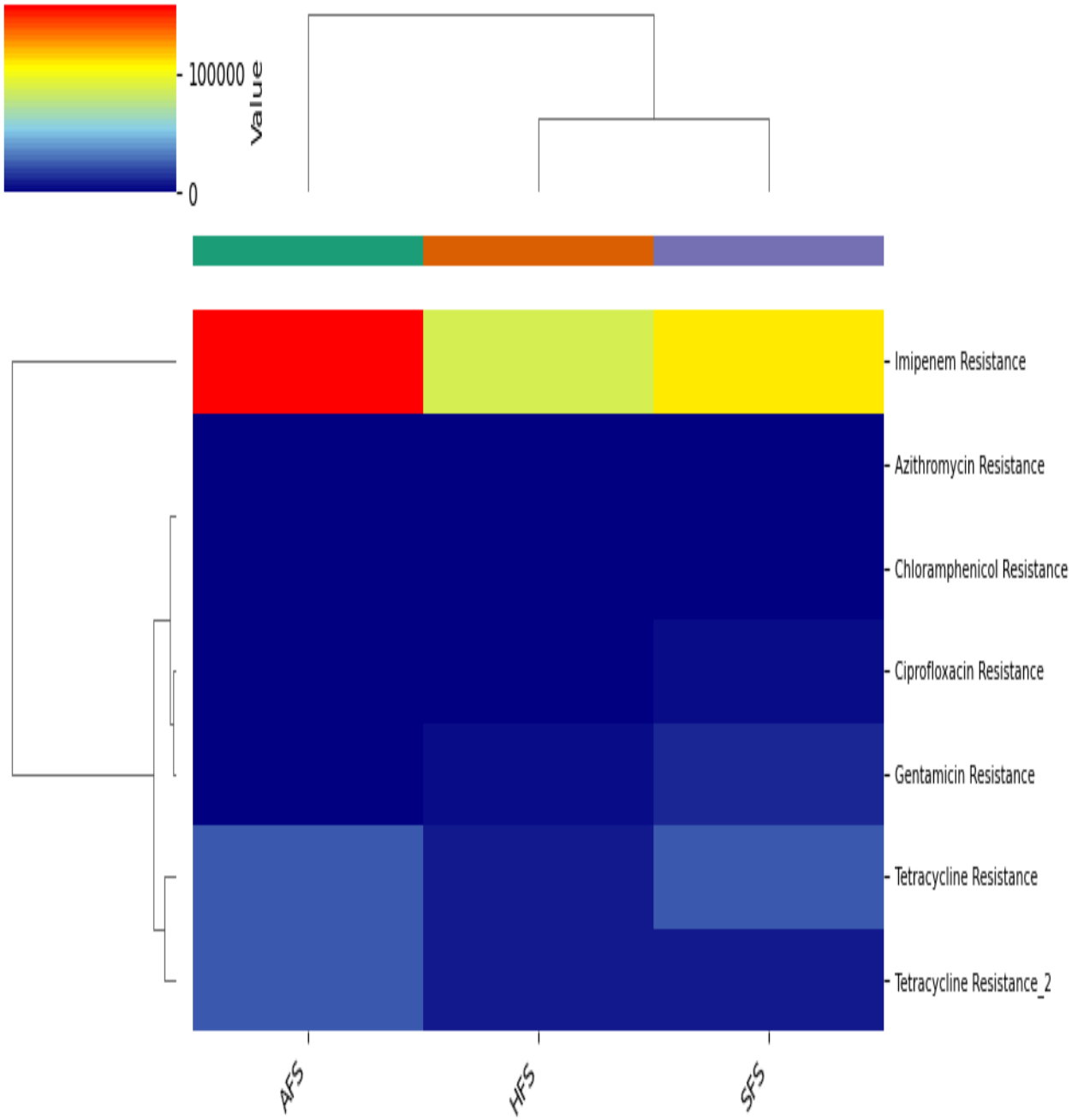

Figure 27: Predicted antimicrobial resistance profile

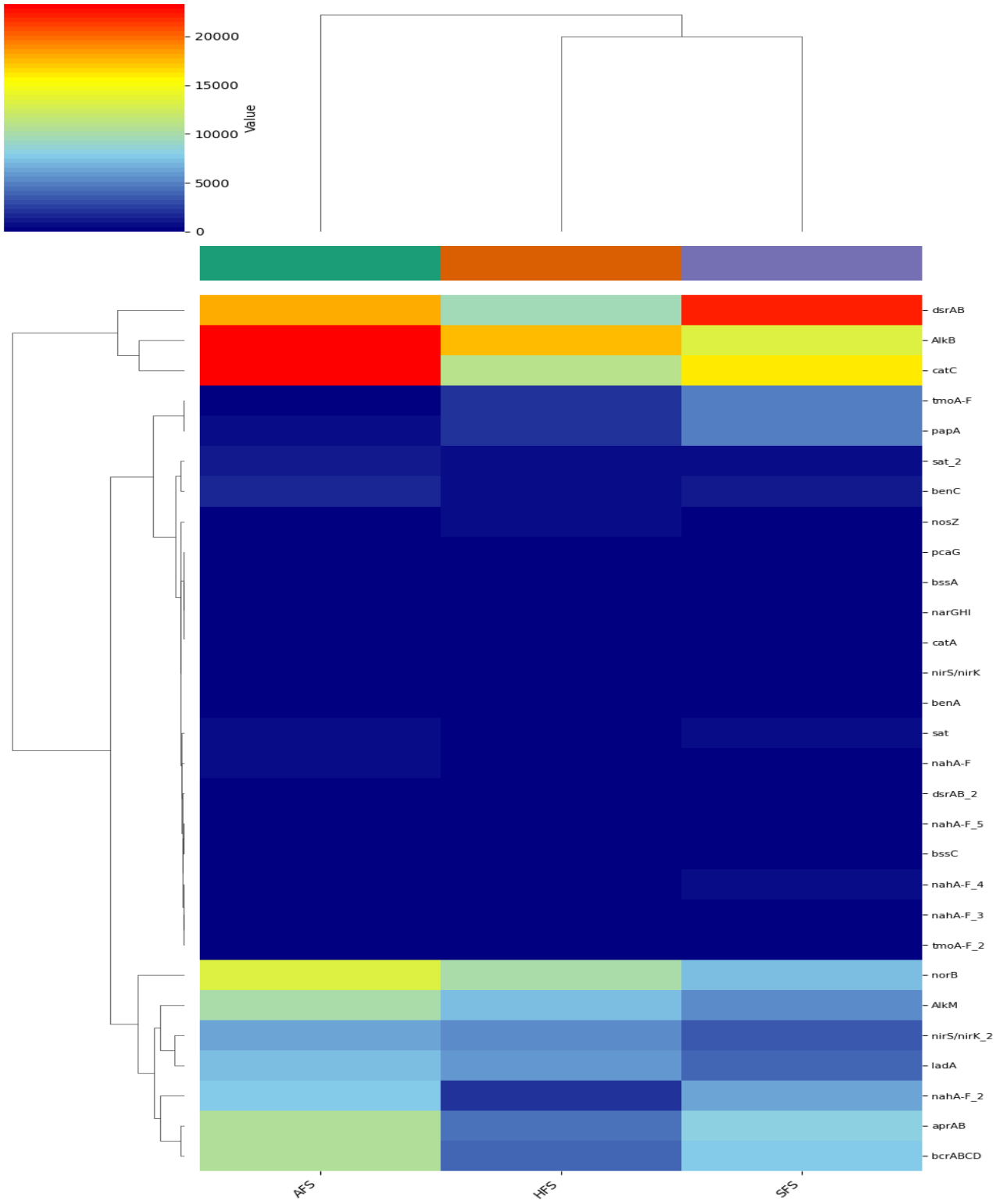

Figure 28: Abundance of gene associated with different degradation pathways

CHAPTER 5: DISCUSSION

This study is a polyphasic approach to determine the microbial ecology of hydrocarbon contaminated soils in the Noakhali region, revealing a robust convergence between phenotypic traits and genotypic potential. The isolation of bacterial colonies on mineral salt media (MSM) utilizing crude oil as the sole carbon source indicated the presence of an active hydrocarbonoclastic community. Biochemical profiling identified *Pseudomonas, Klebsiella,* and *Acinetobacter* as predominant aerobic degraders, determined by their positive catalase reaction. These physiological traits indicate the high abundance of lipid biosynthesis and petroleum hydrocarbon degradation pathways observed by functional metagenomics analysis. It confirms that culture-dependent methods partially captured the functional drivers of remediation, validating these indigenous bacteria as potential candidates for bioremediation (Sutton et al., 2013). Although culture dependent techniques retrieved metabolically active but the high-throughput 16S rRNA metagenomics unveiled more complex community. In our study, Bacteroidota, Pseudomonadota, Bacillota, Verrucomicrobiota, Actinomycetota, Desulfobacterota, Chloroflexota, Acidobacteriota, Planctomycetota were common in all the three locations. But soil of Alexander filling station contain two unique phyla (Bdellovibrionota, Armatimonadota), Haque filing station contain one (Spirochaetota) unique phylum and Sonapur filling station contain unique Mycoplasmatota phylum only compare to others. Bacteroidota and Pseudomonadota are taxonomically dominated bacteria. Distributions of Bacteroidota in Haque filling station (60.8%) and Alexander site (49.4%) and Proteobacteria were most abundant in Sonapur (47.8%). At the genus level, *Prevotella* and *Pseudomonas* structured the communities, with *Prevotella* being most abundant in Haque (50.1%) and Alexander (40.3%) and declined in Sonapur (34.8%). On the other hand *Pseudomonas* showed the opposite pattern in Sonapur (38.7%) and remained lower in Haque (15.2%) and Alexander (31.2%). Other genera such as *Klebsiella* and *Bacteroides* occurred at lower but consistent levels at different relative abundances. *Pseudomonas, Acinetobacter* and *Enterobacter* were also isolated from Chittagong ship-breaking yards [79]. In Daqing Oilfield (China) and chemically contaminated sites in India found the prevalence of *Pseudomonas* and *Acinetobacter* [89]. The taxonomic profile also aligns with the core microbiome of oil spills described in the Niger Delta and the Gulf of Mexico [90].

This study distinguishes itself by identifying specific catabolic pathways, such as alkane degradation, catechol ortho-cleavage, CBB cycle, methane oxidation, methanol oxidation, protocatechuate degradation l, protocatechuate ortho-cleavage, Toluene degradation, and other

aromatic ring cleavage which underscores a functional redundancy. Functional profiling prediction also revealed site-specific metabolic specialization. The Alexander (AFS) site harbored the highest abundance of genes associated with alkane and aromatic degradation (*alkB, nahA-F, benA*), sulfate reduction (*dsrAB*), and nitrogen cycling (*nirS*, *nosZ*), indicating a community with enhanced capacity for both aerobic and anaerobic pollutant degradation. In contrast, the Haque (HFS) and Sonapur (SFS) sites showed lower intensities for these catabolic markers but elevated levels of stress-response genes norB, bcrABCD. This difference suggests that while AFS supports variety of degrading community, SFS and HFS may be driven by specific local environmental conditions or varying contamination ages, leading to niche specialization rather than a uniform functional profile. Moreover, the functional gene diversity observed in SFS and HFS in contrasts with the highly specialized hydrocarbon-degradation seen at AFS. These findings suggest that while some microbial communities adapt to petroleum hydrocarbons as the primary carbon source, others engage in broader environmental processes like sulfur and nitrogen cycling, reflecting their role in overall ecosystem health and resilience.

An important dimension of this study was the assessment of antimicrobial sensitivity. Petroleum hydrocarbon contaminated sites often serve as reservoirs for antibiotic resistance genes due to cross-resistance mechanisms. The predicted resistome showed spatial heterogeneity. AFS displayed the highest abundance of tetracycline and gentamicin resistance genes compared to phenotypic observations. On the opposite SFS exhibited the highest potential for imipenem resistance and resistance to ciprofloxacin, azithromycin and chloramphenicol remains comparatively weak at all sites. This suggests that the busy transit hub at SFS may exert stronger anthropogenic pressure or may be vertical or horizontal gene transfer. However, the phenotypic susceptibility of isolated *Pseudomonas* strains to common antibiotics indicates they remain ecologically safe candidates for field application, minimizing the risk of disseminating multidrug resistance markers [82].

CHAPTER 6: CONCLUSION

In conclusion, by combining both culture-based methods and high throughput 16S rRNA metagenomics sequencing we identified some key bacterial taxa and metabolic pathways crucial for the degradation of petroleum hydrocarbon pollution. This study demonstrates that hydrocarbon-contaminated soils in the Noakhali region harbor a taxonomically diverse and functionally versatile microbiome. This study needs more in-depth investigation to understand the activities and of soil bacterial community and relation between the function and diversity of the microorganisms. Ecosystems with more intra- or inter-specific functional biodiversity are more resilient to changes. This is because new genotypes or species may replace missing capabilities. The predictive metagenomics evaluation found that the community of bacteria in the petroleum hydrocarbon contaminated area mostly depends on petroleum hydrocarbons as sole source of carbon energy. This was due to the abundance of hydrocarbon degradation pathways and the presence of identified oil-degrading bacterial taxa. This study provide valuable information to understand the microbial ecology of petroleum-contaminated soils and provides the framework for future bioremediation strategies in the Noakhali region. It also emphasizes the necessity of combining culture-dependent and culture-independent methods to understand the microbial communities involved in environmental clean-up.

Future Perspective

Genomic characterization of potential candidates, virulence gene identification, their biosurfactant production ability and *in situ* test to understand the biodegradation and application in bioremediation will be performed. Future study should focus on long-term investigations of antimicrobial resistance (AMR) in bioremediation and evaluate the horizontal gene transfer and the possible transmission of resistance genes in microbial communities.

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APPENDICES

Appendix A (Table)

Table 5: Morphological characterization of petroleum degrading bacteria

Sample ID	Colony morphology on culture plate		Gram Stain
	Luria Bertani Agar	MacConkey Agar	
H1	Large, opaque and flat colonies with irregular margins	Colorless, flat, smooth colonies, non-lactose fermenter	Negative, rod
H2	Circular or irregular margins, white in color	No growth	Positive, rod
H5A	Circular, raised, mucoid, and white to opaque	Large, mucoid and pink colonies	Negative, rod
H7A	Circular, raised, mucoid, and white to opaque	Large, mucoid and pink colonies	Negative, rod
H7B	Large opaque and flat colonies with irregular margins	Colorless, flat, smooth colonies, non-lactose fermenter	Negative, rod
H7D	Large opaque and flat colonies with irregular margins	Colorless, flat, smooth colonies, non-lactose fermenter	Negative, rod
H8A	Circular, opaque, non-mucoid	Small, circular, Colorless, non-mucoid	Negative, cocci
SLA	Large, opaque and flat colonies with irregular margins	Colorless, flat, smooth colonies, non-lactose fermenter	Negative, rod
SLB	White circular colony with irregular margins	No growth	Positive, rod
SLC	Circular, white in color and irregular margins	No growth	Positive, rod
SLD1	Small, circular, smooth margin	Circular, colorless, slightly raised	Negative, rod
SLD2	Circular, white mucoid colonies	Lagre, pink, Mucoid colonies	Negative, rod
SMA1	Small, circular with irregular margin	Circular, colorless, slightly raised	Negative, rod
SMA2	Circular, raised, mucoid, and white to opaque	No growth	Positive, bacilli
SMB	Large, opaque and flat col1onies with irregular margins	Circular, colorless, slightly raised, moist surface	Negative, rod

SMC	Flat, opaque and wrinkled colonies with irregular margin	Colorless, flat, smooth colonies	Negative, rod
DG1	Large, flat, irregular, greenish	Small to large, flat, irregular	Negative, rod
DG3	Large, flat, irregular, greenish	Small to large, flat, irregular	Negative, rod
DG7	Small to large, circular	Small, circular, entire white	Negative, rod
DG9	Small, circular, white to yellowish, concave	Small, circular	Negative, rod
DG16	Greenish, large, flat, irregular curve	Irregular, large, greenish circular	Negative, rod
DJ10	Large, circular, white, convex	Medium, large, circular, pinkish	Negative, rod
AJ14	Large, circular, flat, greenish	Whitish, circular, flat, irregular	Negative, rod
AJ15	Small to medium, Circular, raised, entire, white	Circular, raised, entire, transparent	Negative, rod
AJ10	Circular, raised, mucoid, and white to opaque	Large, mucoid and pink colonies	Negative, rod
AJ12	Small, Circular, raised, entire	Small, circular, transparent	Negative, rod
AJ6	Large, raised, Circular, whitish	Pinkish, flat, irregular	Negative, rod
AJ9	Large, raised, Circular, whitish	Pinkish, flat, irregular	Negative, rod
AJ5	Large, raised, Circular, whitish	Pinkish, flat, irregular	Negative, rod
23M4	Small, circular, smooth, convex entire, yellowish	No growth	Positive, tetrads
23M5	Large, round, convex, opaque	No growth	Positive, cluster
23M3	Small, circular, smooth, convex entire, yellowish	No growth	Positive, tetrads
23M2	Small, white, smooth, pinpoint, pairs or chains	No growth	Positive, chain

Table 6: Identification of Bacterial Isolates by Biochemical Characterization

No.	Sample ID	Triple Sugar Iron (TSI)				MIU			Citrate	Oxidase	Catalase	Candidate Organisms
		Slant	Butt	H2S	Gas	Motility	Indole	Urease				
1	H1	K	A/NC	-	+	+	-	-	+	+	+	*Pseudomonas aeruginosa* 100%
2	H2	K	A	-	-	-	-	-	+	+	+	*Bacillus sp* 90%
3	H5A	A	A	-	+	-	-	-	+	-	+	*Klebsiella spp 90%*
4	H6A	A	A	-	+	+	-/+	-	+	-	+	*Citrobacter koseri* 90%
5	H7A	A	A	-	+	-	-	-/s	+	-	+	*Klebsiella oxytoca* 90%
6	H7B	K	NC	-	-	-/+	-	-	+	+	+	*Pseudomonas spp* 90%
7	H7D	K	NC	-	-	-/+	-	-	+	+	+	*Pseudomonas spp* 90%
8	H8A	K	NC	-	-	-	-	- (d)	- (d)	- (d)	-	*Acenetobacter spp* (90%)
9	SLA	K	A	-	-	+	-	-	+	+	+	*Pseudomonas aeruginosa* 100%
10	SLB	K	A	-	-	+	-	-	+	+/-	+	*Bacillus Spp* 90%
11	SLC	K	A	-	-	-/+	-	-/V	+	-	+	*Bacillus spp* 90%
12	SLD1	K	A	-	-/+	+	-	-	+/V	-	+	*Hafnia spp* 90%
13	SLD2	A	A	-	-/+	+	-	-/V	+	-	+	*Enterobacter spp* 90%
14	SMA1	K	A	-	-/+	+	-	-	+/V	-	+	*Hafnia Spp* 90%
15	SMA2	K	A	-/+	-/V	-	-	-	+/V	-	+	*Corynebacterium spp* 90%
16	SMB	K	A	-	-/+	+	-	-	+	-	-	*Hafnia spp* 90%
17	23M1	K	A/NC	-	-	+	-	-/V	+	+	+	*Pseudomonas aeruginosa* 100%

18	AJ14	K	A/NC	-	-	+	-	-/V	+	+	+	*Pseudomonas aeruginosa* 100%
19	AJ15	K	NC	-	-	-/+	-	-	+	+	+	*Pseudomonas sp*
20	AJ10	A	A	-	+	-	-	-	+	-	+	*Klebsiella spp 90%*
21	AJ12	K	NC	-	-	-	-	- (d)	- (d)	- (d)	-	*Acenetobacter spp* (80%)
22	AJ6	K	NC	-	-	-	-	-	+	+	+	*Pseudomonas spp* 100%
23	AJ9	K	NC	-	-	-/+	-	-	+	+	+	*Pseudomonas sp 90%*
24	AJ5	K	NC	-	-	-/+	-	-	+	+	+	*Pseudomonas spp* 90%
25	DG1	K	NC	-	-	-/+	-	-	+	+	+	*Pseudomonas spp* 90%
26	DG7	K	NC	-	-	-	-	- (d)	- (d)	- (d)	-	*Acenetobacter spp* (80%)
27	DG9	A	NC	-	-	-	-	- (d)	- (d)	- (d)	+	*Acenetobacter spp* (90%)
28	DG16	K	A/NC	-	-	+	-	-/V	+	+	+	*Pseudomonas aeruginosa* 100%
29	DG10	A	A	-	+	-	-	-	+	-	+	*Klebsiella spp 100 %*
30	23M5	NC	NC	-	-	-	-	-	+	_	+	*Staphylococcus spp.*
31	23M3	NC	NC	-	-	-	-	-	-	+	+	*Micrococcus*
32	23M4	NC	NC	-	-	-	-	-	-	+	+	*Micrococcus spp*
33	23M2	NC	NC	-	-	-	-	-	-	_	-	*Streptococcus spp*

Table 7: Antimicrobial Sensitivity Profile

Location	**ID**	***Bacteria***	**Chloramphenicol (C30)**	**Gentamycin (GEN10)**	**Imipenem (IPM 10)**	**Ciproflox acin (CIP5)**	**Azithromycin (AT15)**	**Tetracyclines (TE30)**
Haque Filling Station (HFS)	H3B	*Pseudomonas Spp.*	sensitive	sensitive	sensitive	sensitive	sensitive	resistant
	H1	*Acinetobacter*	sensitive	sensitive	sensitive	sensitive	sensitive	sensitive
	H5A	*Klebsiella spp*	sensitive	sensitive	Intermedi-ate	Intermedi-ate	resistant	resistant
	H2A	*Pseudomonas spp.*	Intermedia-te	intermediate	sensitive	Intermedi-ate	resistant	resistant
	H8A	*Acinetobacter spp.*	sensitive	sensitive	sensitive	sensitive	resistant	resistant
	H3A	*Pseudomonas spp.*	Intermedia-te	resistant	sensitive	resistant	resistant	resistant
	H6A	*Klebsiella spp.*	Intermedia-te	resistant	resistant	resistant	resistant	resistant
Alexander (AFS)	AJ5	*Pseudomonas spp.*	sensitive	sensitive	sensitive	sensitive	sensitive	Intermedia-te
	AJ9	*Pseudomonas spp.*	sensitive	sensitive	sensitive	sensitive	sensitive	sensitive
	AJ14	*pseudomonas aeruginosa*	Intermedia-te	sensitive	resistant	sensitive	sensitive	resistant
	AJ11	*Pseudomonas spp.*	sensitive	intermediate	sensitive	Intermedi-ate	sensitive	resistant
	AJ13	*Acinetobacter spp.*	sensitive	sensitive	sensitive	sensitive	sensitive	resistant
	AJ12	*Acinetobacter spp.*	sensitive	resistant	resistant	sensitive	sensitive	resistant
	AJ10	*Klebsiella spp.*	Intermedia-te	sensitive	Interme-diate	resistant	Intermedi-ate	resistant
	23M1	*Pseudomonas aeruginosa*	Intermedia-te	intermediate	sensitive	sensitive	sensitive	resistant
	LM1	*Pseudomonas Spp.*	resistant	sensitive	resistant	Intermedi-ate	sensitive	resistant

Sonapur Filling Station (SFS)	23M5	*Staphylococcus aureus*	sensitive	sensitive	sensitive	intermediate	sensitive	resistant
	LM7	*Staphylococcus spp*	sensitive	sensitive	sensitive	sensitive	resistant	sensitive
	SLD2	*Hafnia spp*	Intermedia-te	sensitive	resistant	Intermedi-ate	resistant	Intermedi-ate
	LM4	*Klebsiella spp*	sensitive	sensitive	Intermedi-ate	Intermedi-ate	resistant	resistant
	LM2	*Enterobacter sp*	sensitive	Intermedi-ate	resistant	resistant	resistant	resistant
	SMA1	*Serratia spp..*	Intermedia-te	resistant	Intermedi-ate	resistant	Intermedia-te	resistant
	SMB	*Serratia spp.*	Intermedia-te	resistant	Intermedi-ate	Intermedia-te	Intermedia-te	resistant
	SLD2	*Hafnia spp.*	Intermedia-te	resistant	resistant	resistant	resistant	resistant
Datterhat Garage (DG)	DG1	*Pseudomonas spp.*	sensitive	Intermedi-ate	sensitive	Intermedia-te	sensitive	resistant
	DG3	*Pseudomonas spp.*	sensitive	Intermedi-ate	sensitive	Intermedia-te	sensitive	resistant
	DG7	*Acinetobacter spp.*	sensitive	sensitive	sensitive	sensitive	sensitive	resistant
	DG9	*Acinetobacter spp.*	sensitive	sensitive	sensitive	sensitive	sensitive	resistant
	DG16	*Pseudomonas aeruginosa*	Intermedia-te	Intermedi-ate	sensitive	sensitive	sensitive	resistant

Appendix B (Media Composition)

A. Mineral Salt Medium (MSA)

Ingredients	Amount (g/L)
K_2HPO_4	1.0 g
$(NH_4)_2SO_4$	0.5 g
KH_2PO	1 g
$MgSO_4$	0.2 g
$FeSO_4.7H_2O$	0.37 g
$MnCl_2.4H_2O$	4 g
$FeCl_3$	0.05 g
KCl	0.7 g
$Na_2MoO_4.2H_2O$	0.5g
$CaCl_2.2H_2O$	4.77 g
Tween 20	0.005%
Agar powder	15g
Diesel	20ml
Distilled water	1000mL

B. Luria Bertani Agar

Ingredient	Amount (g/L)
Tryptone	10 g
Yeast extract	5.0 g
Sodium chloride (NaCl)	10 g
Agar	15.0 g
Distilled water	1000 mL (1 liter)
pH (at 25°C)	Adjust to ~7.4

C. MacConkey Agar

Ingredient	Amount (g/L)
Peptone (Pancreatic digest of gelatin)	17.0 g
Proteose peptone	3.0 g
Lactose	10.0 g
Bile salts (No. 3 or equivalent)	1.5 g
Sodium chloride (NaCl)	5.0 g
Neutral red (pH indicator)	0.03 g
Crystal violet	0.001 g
Agar	13.5 – 15.0 g
Distilled water	1000 mL
pH (at 25°C)	Adjust to ~7.1 ± 0.2

D. CTAB Media

Ingredient	Amount (g/L)
Methylene blue	0.005 g
Cetyltrimethylammonium bromide (CTAB)	0.15 g
Agar	12.0 g
Distilled water	1000 mL
pH (at 25°C)	Adjust to ~7.0 ± 0.2

E. Lipid Hydrolysis Agar

Ingredient	Amount (g/L)
Peptone	10.0 g
Sodium chloride (NaCl)	5.0 g
Tween 80	10 ml (V/V)
Agar	15.0 g
Distilled water	1000 mL
$CaCl_2.6H_2O$	0.1 g

F. Motility Indole Urease (MIU) agar

Ingredients	Amount (g/L)
Casein enzymic hydrolysate	1.0
Dextrose	0.1
Sodium chloride	0.5
Phenol red	10
Agar	0.2

G. Simmon Citrate

Ingredient	Amount (g/L)
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium phosphate (K_2HPO_4)	1.0 g
Sodium chloride (NaCl)	5.0 g
Sodium citrate	2.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g

Appendix C (Reagents)

A. Crystal Violet Reagent

Solution I		Solution II	
Name of Reagents	Concentration	Name of reagents	Concentration
Crystal violet	2.0 g	Ammonium oxalate	0.8 g
Ethanol 95% (v/v)	20 ml	Distilled water	80 ml

B. Gram's Iodine

Reagents	Amount
Iodine	1 g
KI	2 g
Distilled water	97 ml

C. Decoloring Agent: Ethanol 95% (v/v)

I. **Safranin**

Stock Solution: 2.5 g dissolved in 100 ml ethanol 95% (v/v)

II. Working Solution: 10 ml stock solution dissolved in 90 ml distilled water.

D. Kovac's Reagents

Ingredients	Amount
Butyl alcohol	150 ml
p-dimethylaminobenzaldehyde	10 g

E. Oxidase Reagents

Ingredients	Amount
Tetra-methyl—p-phenylenediamine dihydrochloride	1%
Distilled water	99 ml

F. Catalase Reagents

Ingredients	Amount
Hydrogen Peroxide	3%

Appendix D (Apparatus)

Autoclave

Bag sealer

Centrifuge, Eppendorff

Deionizer

Distilled water plant

Disposable micropipette tips

Disposable syringe

Electronic balance

Incubator

Glass wares, Pyrex brand, USA

Microscope

Digital Camera, AxioCam MRc, Carl Zeiss, Germany

Laminar airflow

Magnetic stirrer

Micropipette

Eppendorff

Petridishes

pH meter

Refrigerated superspeed-centrifuge.

Spectrophotometer

Sterilizer

Slide,

Vortex, Fisher

Wafer bath