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Isolation and characterization of Hydrocarbon-Degrading Bacteria from Oil-Contaminated Soil

PROJECT REPORT

Submitted in fulfilment of the requirements for the award of degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

Under the guidance

of

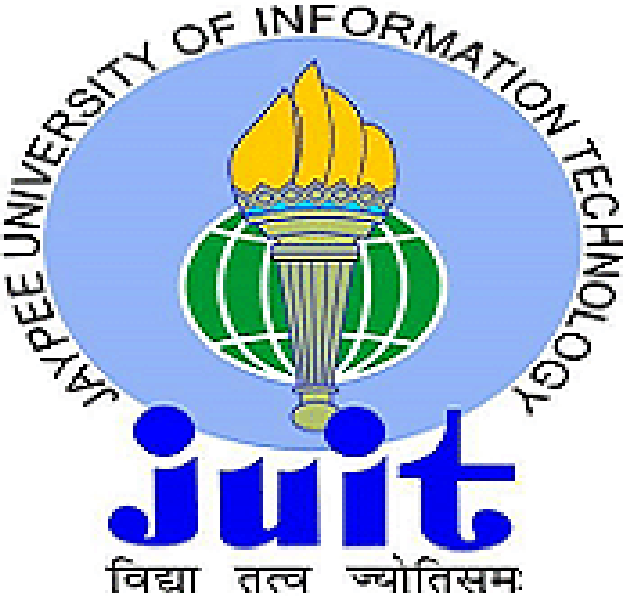
Dr. Saurabh Bansal

Associate Professor

by

Richa Dhiman (211804)

to



DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

Jaypee University of Information Technology, Solan

173234, Himachal Pradesh

DECLARATION

I hereby declare that the work presented in this report entitled '**Isolation and characterization of Hydrocarbon-Degrading Bacteria from Oil-Contaminated Soil**' in the fulfillment of the requirements for the award of the degree of **Bachelor of Technology** in Biotechnology is submitted to the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat. This is an authentic record of my own work carried out over a period from January to May 2025 under the supervision of Dr. Saurabh Bansal (Associate Professor, Department of Biotechnology and Bioinformatics).The matter embodied in the report has not been submitted for the award of any other degree or diploma.

(Student Signature with Date)

Student Name: Richa Dhiman

Roll No.: 211804

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

(Supervisor Signature with Date)

Supervisor Name: Dr. Saurabh Bansal

Designation: Associate Professor

Department: Biotechnology & Bioinformatics

Date

CERTIFICATE

This is to certify that the work which is being presented in the major project report titled "**Isolation and characterization of Hydrocarbon-Degrading Bacteria from Oil-Contaminated Soil**" in fulfilment of the requirements for the award of the degree of B.Tech in Biotechnology and submitted to the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, is an authentic record of work carried out by Richa Dhiman (211804) during the period from January to May 2025 under the supervision of Dr. Saurabh Bansal, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat.

Dr. Saurabh Bansal

Associate Professor

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology, Waknaghat

Date: …….

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Firstly, I express my sincere gratitude and thanks to the Almighty God for His divine blessings, which enabled me to successfully complete this project.

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Richa Dhiman

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ABSTRACT

Oil contamination is a significant environmental concern, primarily resulting from the widespread use and accidental release of petroleum products. These hydrocarbons persist in soil and water, posing long-term risks to ecosystems and human health. Conventional remediation techniques such as mechanical recovery, chemical dispersants, and incineration often fall short due to high costs and secondary pollution. In contrast, bioremediation offers a sustainable, eco-friendly solution by utilizing the natural metabolic capabilities of hydrocarbon-degrading microorganisms.

This project aimed to isolate and characterize bacteria capable of degrading hydrocarbons from oil-contaminated soil. Soil samples were collected and enriched using Minimal Salt (MS) media supplemented with diesel and engine oil as the sole carbon sources. Bacterial growth was monitored through optical density (OD) measurements, and isolates were further examined using Gram staining, biochemical tests (catalase, MR, VP, urease, mannitol, MacConkey), and genomic DNA extraction via the phenol-chloroform method. Two bacterial isolates (A and B) were compared through a 7-day growth curve analysis, and Isolate A demonstrated superior growth. Optimization experiments identified peptone as the most effective nitrogen source, with optimum degradation observed at pH 7 and 37°C.

The results indicate that the isolated strains, likely belonging to the genus *Pseudomonas*, hold strong potential for application in environmental bioremediation. These findings contribute to the development of microbial strategies for the efficient and sustainable cleanup of hydrocarbon-contaminated environments.

CHAPTER 1

INTRODUCTION

Oil Contamination and its Environmental Impact

The problem of oil pollution on a worldwide scale is growing as we are so reliant on petroleum for everything from industry to auto fuel and the products make and use in our homes. Accidental spills, leaking pipelines (often due to old infrastructure), runoff from factories and simply careless throwing way of used oil contribute hydrocarbons (such as diesel, lubricant oil and crude oil) into the surrounding environment. Hydrocarbons are messy and not easy to decompose so can remain in the environment for many years and cause all kinds of problems [1], [2]. Hydrocarbons reach contaminating soil or water and often wreck the natural tiny microbial ecosystem, decrease the growth potential of the soil capability but also make our water supplies dirty. The more time passes, the more the toxic accumulate up the food chain eventually poisoning plants, animals and then humans [1]. Cleaning polluted hydrocarbon sites is extremely difficult. There are some traditional approaches such as skimming the oil off of water, applying chemical dispersants to break it apart, Controlled Burns for hydrocarbons—however these approaches are cost prohibitive, do not remove it all, and do not break all of it into benign products and sometimes just introduce new contaminants to site [3].

The Need for Sustainable Solutions: Bioremediation

As the environmental and economic impacts of typical oil spill response strategies—like chemicals and excavation—become more widely recognized, there is a demand for a more ecological and sustainable approach to the cleanup of oil spills. One of the most exciting green solutions is bioremediation, where microorganisms, specifically bacteria, degrade harmful oil contaminants into relatively harmless substances such as carbon dioxide and water [1], [2].

The beauty of bioremediation is that it can potentially take place on site with minimal site disturbance and no specialized equipment. This can reduce environmental impact and eliminate resource costs. Bioremediation is not a universal solution—its effectiveness is dictated by the type of oil spilled, volume of oil spilled, and environmental conditions, such as soil acidity, temperature, nutrients, and oxygen levels present [3]. Another challenge is that oil can be persistent, with most hydrocarbons being water-repellent making them difficult for microbes to access. Regardless, studies

have shown that given the right conditions, bioremediation can restore oil-affected environments and their ecosystems [4], [5].

Hydrocarbon-Degrading Microorganisms: The Catalysts for Bioremediation

Some terrific microbes, especially bacteria, operate as nature's cleanup crew by using hydrocarbons—the tough stuff in oil—as their food source and energy source. Bacteria such as Pseudomonas, Acinetobacter, and Rhodococcus are the all-stars of bioremediation because they have the ability to break down both simple and complex hydrocarbons into harmless byproducts such as carbon dioxide and water [1], [2]. They do this with special enzymes termed monooxygenases or dioxygenases today we will have to just refer to them as oxygenases because they all do similar things which is chop up those tricky hydrocarbon chains [3]. Another really interesting thing to note is that these bacteria can produce biosurfactants which we can think of as nature's soap. These will help merge oily and water-repelling hydrocarbons with water, which helps the microbes interact with the hydrocarbons and degrade them [4]. This is noteworthy because cleaning up oil spills is difficult because oil does not mix well with water.

Advantages and Challenges of Bioremediation

Bioremediation has some awesome perks that make it a top pick for tackling oil-contaminated areas:

- **Cost-Effectiveness**: It utilizes nature's own microbes, negating the need for expensive machines or chemicals [5].
- **Environmental Friendliness**: It respects the planet avoiding any harmful byproducts—keeping soils and ecosystems healthy [6].
- **Application In Situ**: You can remediate right at the spill site without digging up everything or making an epic mess in the process [7].
- **Survivability:** Your local microbes are already comfortable in their home environment and they can continue to work on the contaminants over time [7].

However, the application of bioremediation is not without significant challenges that require careful consideration and resolution.

Hydrocarbon Toxicity: Excessive amounts of oil can actually kill-off microbes attempting to remediate the oil spill, either slowing-down or stopping them [8].

- **Environmental Conditions:** Conditions like temperature, soil acidity, oxygen, and moisture must be right for microbes to remediate hydrocarbons [9].
- **Bioavailability:** Many hydrocarbons adhere to soils or are water-repellent and inaccessible to microbes. Biosurfactants or additives, like Tween 20, can promote bioavailability, but even then, are complicated [4], [10].
- **Time:** Bioremediation is not a quick solution, as it can take weeks or months to realize any process. This is particularly true when compared to faster chemical methods of remediation [11].

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction to Oil Pollution

Oil pollution is the inadvertent release of crude or refined petroleum products into the environment, generally causing contamination of water and soils. Oil pollution has various causes, usually anthropogenic, for example, oil drilling, oil refining, leaking pipelines, accidents relating to transportation, or industrial spills [1], [2]. The agents of oil pollution are petroleum hydrocarbons, which can be characterized as a large family of organic compounds primarily composed of the elements carbon and hydrogen. The class of hydrocarbons includes simple hydrocarbons (such as alkanes), aromatic hydrocarbons (such as benzene and toluene), and a dangerous subset termed polycyclic aromatic hydrocarbons (PAHs) which includes naphthalene and anthracene [3], [4]. In general, hydrocarbons (especially petroleum hydrocarbons) are troublesome for various reasons:

•They are Toxic: hydrocarbons are a major toxicant to plants, animals, and humans. They can poison cells and disrupt cellular metabolic functions.

• They are Persistent: hydrocarbons may not readily break down and can persist in the environment, sometimes for several years.

• They are non-aqueous: their oily nature makes them sticky and they cling to soil particles, often creating great challenges for clean-up [5].

When the hydrocarbons enter the soil, they can really complicate things:

• Physically, they can clog the soil, causing it to be dense and not allow air and water to it move easily [6].

• Chemically, they disrupt nutrient cycles, causing plants to run out of crucial nutrients such as nitrogen and phosphorus [7].

• Biologically, they kill many of the small microbes in the soil, but leave the oil-tolerant, hardy microbes [8].

Less fertile soil, unhealthy plants, and a major blow to the animal life and biodiversity around the contamination. Most worryingly, toxins may bio-accumulate in the food chain, posing a health risk for both animals and humans [9]. Oil pollution is not simply problematic- it is one of the greatest threats to ecosystems, and human health. This is why greener, sustainable solutions, moves like bioremediation, which uses microbes and provide proper care to another [10], are becoming increasingly popular as an efficient way to recover contaminated sites.

2.2. Conventional Methods of Oil Remediation

Oil spills wreak havoc on the environment by releasing crude oil or petroleum products onto soils and into waters. Many spills are caused by human error, such as drilling accidents, ruptured pipelines, or shipping failures [1],[2]. The spillage of thousands to millions of gallons result in a toxic soup of hydrocarbons. There are the simple alkanes, aromatic hydrocarbons (i.e. benzene), and polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, each which are toxic to ecosystems [3],[4]. Traditional cleanup approaches, that have been in use for years, are costly, often damaging to the environment, and land typically remains impaired from contamination. Bioremediation, defined as utilizing naturally-occurring microbes/biota to degrade oil, continues to present as a more cost-effective, landscape-friendly alternative. Ultimately, this paper will highlight the traditional cleanup approaches, the deficiencies of traditional remediation, and the recent developments in bioremediation as alternative options to oil-contaminated land, supported by citations up to [37].

Traditional Cleanup Methods: The Old-School Fight Against Oil Spills

For at least the last few decades, we have utilized physical and chemical and stabilization techniques to clean up contaminated oil-soaked soils. While physical remediation techniques do exist in a small landfill, these rarely work and come with a lot of costs to the environment if they do work.

A. Physical Methods

These methods are about physically removing or separating the oil from the soil:

These methods involve the physical removal, or separation, of oil from contaminated soil. This category includes:

•**Digging It Up (Excavation):** Workers remove contaminated soil with bulldozers and treat it off-site or disperse it to landfills. For small spills, this is a rapid, if not cheap, remedy. However, it tears up the terrain, disrupts wildlife, and generates considerable cost (fuel for trucks, labor,

landfill tipping fees, etc. [1]). For large spills in remote locations, however, it takes an incredible number of trucks to remove the oil – to paraphrase the phrase, it's like trying to empty a lake with a spoon.

•**Soil Washing:** Soil washing is a very clever way to flush oil from soil using water or chemical solutions. In theory, it could be very useful, but in order for it to work a considerable amount of toxic wastewater is created which is in itself requires cleanup. In addition, washing the oil from the soil is a hit or miss operation. Again, in clay-heavy soils and organic-rich soils, washing is particularly challenging and oil will often stick like glue to the soil [2].

•**Cooking the soil (Thermal treatment):** This method uses some significant heat - sometimes up to 500 degrees or more - to either burn off the oil or vaporize it. This can be very effective at destroying most contaminant, however, it is extremely costly and energy-inefficient, the useful microbes and nutrients in the soil are destroyed, and the soil is sterilized like a wasteland [3].

B. Chemical Methods

These rely on chemical reactions to tackle the oil:

• **Chemical Oxidation:** Strong oxidizers such as hydrogen peroxide or ozone are injected into the soil to mineralize the oil into less hazardous materials. While the concept is valuable, these chemicals can alter the pH of the soil, eliminate beneficial soil microbes, and they cannot mineralize every type of oil, because, for example, thick or heavy oils respond poorly. [4]

• **Dispersants & solvents**: Dispersants and solvents operate in a similar way to dish soap by dispersing or breaking up the oil into small particles that can be rinsed away or biodegraded. The problem is that dispersants are generally toxic themselves and release pollutants back into the environment, possibly harming plants, animals impacted, and water systems. [5]

C. Stabilization and Solidification Technology

This technique utilizes materials such as lime, cement, and/or clay to incorporate oil into a solid mass that will prevent the oil from spreading. It is like putting the oil in a straitjacket. However, the oil is not actually removed, and the resulting treated soil will often be hard, lifeless, and completely unproductive for supporting crops or ecosystems. [6]

Why These Technologies are Losing

The traditional remediation approaches are blighted with flaws that make them less than ideal:

- They're Eye-Wateringly Expensive: For excavation and thermal treatments, it takes heavy machinery, professional labor, and a lot of money. Excavating or thermal treating, tons of soil, or running an industrial oven isn't cheap [7].
- They're a Gigantic Energy Hog: Heating soil to evaporate away the oil uses fossil fuels and releases greenhouse gases, which makes climate change worse [8].
- They Create More Mess: Chemical methods, particularly dispersants, sometimes leave behind toxic residues, exchanging one problem for the next [9].
- They're Not Effective for Large Spills: They work well with small and contained spills, but not for large, although not exclusive to, remote sites like rural areas or developing countries with limited means [10].
- They Destroy the Soil: Many of these methods destroy the soil structure, nutrient profile, microbiological communities, leaving soil that cannot even support plants or wildlife [11].
- They Can Be Band-Aids: Stabilization can trap the oil, but doesn't destroy it, so the issue can re-emerge [6].

All of these headaches have led scientists and communities to continue to seek alternative eco-friendly and sustainable options. Enter bioremediation and nature's little janitors that simply live their lives while cleaning up oil spills with less environmental impact.

2.3. Concept of Bioremediation

Bioremediation is akin to allowing nature’s superheroes—microbes—to come in and clean up our oily messes! It takes advantage of living organisms (mostly bacteria but some fungi and plants), in order to break down, detoxify or transform harmful hydrocarbons into safe byproducts such as carbon dioxide, water, and microbial biomasses. These microbes have been eating hydrocarbons for millions of years by using them as an energy source and food source for growth [12], and we are just now learning how to use them to help us fix anthropogenic disasters

Why Sidle is Such an Exciting Environmentally Friendly Option

- It’s Kind to the Planet: With no toxic chemicals or energy-gobbling machinery, that’s a big win for the environment [13].

- It's Easier on the Pocketbook: Compared to traditional methods, it's frequently less costly—especially for cleaning up large areas where digging up or burning contaminated material would be expensive [14].
- It Works On-site: Cleanup can occur right at the spill site, which saves land disturbance and reduces transportation costs for hauling contaminated soil across the state [15].
- It Keeps Going: Once the correct conditions are established, microbes continue their activity—like a self-propelled cleanup team [16].
- It Heals the Land: Unlike deeds that leave soils and land devoid of life, bioremediation may recover life and fertility for microbial assists to repair ecosystems [17].
- It's Flexible: It can flow from a small gas station spill, to large-scale ocean spills [12].

Types of Bioremediation Strategies

To make bioremediation even better, scientists use two main tricks:

A. Bio augmentation

This is comparable to having a crew of ultra elite oil-eating microbes. The ultra specially selected or manufactured bacteria capable of hydrocarbon degradation are introduced into the soil.

• **Why bother?** A life saver when the deficient microorganisms in the soil, are too little to remediate, or they are too mature to remediated oil at a pace that yields the desired decontamination [18].

• **Where do they come from**? These rockstars are primarily sourced from other oil-impacted contaminated sites contaminated sites that they have learned to thrive upon hydrocarbons, and they've lived there for a long time [19].

• **Pretty Sweet Deal!** The new microbes may not adapt well to the local soil micro environment, they may get out competed by resident microbes, or they may actually be reacting negatively for other reason, like the cold, and also possibly insufficient nutrients to facilitate growth [20].

B. Bio stimulation

This is about enhancing the existing microorganisms in the soil to help them be as effective as possible:

- **What they do:** Scientists provide essential nutrients like nitrogen and phosphorus, maintain moisture in the soil, inject oxygen for aerobic microbes, and sometimes change the pH to create a good place for the microbes to do their thing [21].
- **Why It's Good (Solution):** It is sustainable in the sense that it works with the locals—microbes that are already tuned to the site conditions [22].
- **How Well It Works**: Bio stimulation can remediate and hasten clean up when the contaminants are limited by nutrient availability, and usually does a better job than bio augmentation because there are no new players involved [23].

Taking Out Oil Pollutants

Some of the bacteria have pretty good range in their ability to degrade oil, from simple alkanes up to tough PAHs. The aces include Pseudomonas, Bacillus, Rhodococcus, Mycobacterium, and Acinetobacter, and they each have a part of the oil complex that they can degrade best [24].

2.4. Microbial Degradation of Hydrocarbons

Bioremediation is an elegant process whereby microbes convert hydrocarbons to simpler, less toxic products, by utilizing an enzyme system to degrade hydrocarbons, in the presence of O2 (aerobic) or in absence of O2 (anaerobic), with each of the pathways having different potentials [25].

Aerobic Degradation

In the presence of molecular oxygen, microbes launch the degradation process by using oxygen-dependent enzymes.

- Alkanes: These linear or branched hydrocarbons are attacked by alkane monooxygenases which convert these alkanes to their alcohols, then to their aldehydes, and finally to acids which undergo β-oxidation to produce energy, CO2, and water [26].
- Aromatic Hydrocarbons: More complex compounds, like benzene, toluene, or PAHs, are attacked by dioxygenases which cleave the ring structure and produce intermediates such as catechol that are decomposed to produced non-toxic compounds that enter the energy cycle of the cell [27].

- What is Left-over: Mostly, CO2 and water, with some microbial biomass at high levels of hydrocarbon pollution, and all done in an environmentally-sound way [28].

Anaerobic Degradation

- In places with little molecular oxygen available for microbes to use, such as deep soils or wetland soils, microbes move onward, using whatever is there to produce energy by using electron acceptors such as nitrate, sulfate, or iron (using steel scrap or slag):
- It's Slower: Since it is less efficient without molecular oxygen, it just takes longer to degrade hydrocarbons [29].
- Who is Doing It: Bacteria like Desulfovibrio (sulfate-reducers) and Geobacter (iron-reducers) are dominant bacteria [30].
- How the Degradation is Established: Anaerobic hydrocarbon degradation may involve specialized reactions such as fumigate addition or hydroxylation dependent on the hydrocarbon [31].
- This slower process makes a good chance for biodegradation in anaerobic places that tend to stay anaerobic, deep sediments and aquifers being good potentials [32].

What Are the Factors That Influence Cleanup?

Ultimately, microbes are really good at breaking down hydrocarbons, provided that:

- Hydrocarbon Class: Simple alkanes are more readily degraded than complex PAHs.
- Oxygen Availability: Aerobic processes have a rapid degradation rate, but anaerobic processes are slow and necessary in environments that limit oxygen availability.
- Nutrients: Nitrogen and phosphorus serve as nutrients for microbes.
- Temperature and pH: Most microbes thrive in a temperature range of 20–40°C with a near neutral pH (6.5–8.5).
- Pollutants: Heavy metals or other contaminants can also disrupt the degradation process [33].
- Establishing these conditions is like crafting the perfect play for microbes to perform in.

2.5. Types of Hydrocarbon-Degrading Bacteria

Bacteria are the superheroes of bioremediation, converting toxic oil to harmless byproducts because hydrocarbons are like candy to them. There are various types of bacteria that have preferences for different hydrocarbons [34].

2.5.1. Major Genera Involved in Hydrocarbon Degradation

Pseudomonas spp.

- **Where They Reside**: This group is widespread in soil and water and has a non-specific relationship with oil-soaked locations.
- **What They Do**: They are versatile, degrading a range of alkanes, aromatics, and heterocyclic compounds, including the legendary *Pseudomonas putida's* ability to degrade toluene, xylene, and aliphatic hydrocarbons [35].

Alcanivorax spp.

- **Where They Reside:** Generally, in the oceans, especially after an oil release.
- **What They Do**: These species are specialists for alkane degradation, with *Alcanivorax borkumensis* being the most recognized oil-degraders in the marine environment that degrade the fractions of crude oil [36].

Rhodococcus spp.

- **Where They Reside**: Found in both contaminated soils and oil reservoirs.
- **What They Do**: Being favorable with both aliphatic hydrocarbons and aromatics (like PAHs), in contrast to some of the other members of the oil degrading group. *Rhodococcus erythropolis* is recognized for its versatility, particularly in degrading naphthalene [37].

Acinetobacter spp.

- **Where They Reside:** These species are commonly present in soil and water environments where they are known to degrade a diverse array of pollutants.
- **What They Do**: These species can degrade both aliphatic and aromatic hydrocarbons which makes them nice fit for mixed oil releases [34].

Mycobacterium spp.

- **Where They Grow:** Present in soil and water and are notorious for slow-growth, however, they are very effective.
- **What They Do:** They have been shown to degrade some of the most persistent and biodiverse resistant high-molecule weight compounds which are PAHs that can persist soil and water for eons [34].

2.5.2. Enzymatic and Genetic Superpowers

Hydrocarbon-degrading bacteria display distinct genetic features that allow them to exist in, and flourish, in contaminated environments. Many of these hydrocarbon-degrading bacteria possess genes encoding enzymes responsible for hydrocarbon degradation. Examples of these enzymatic processes are:

- Monooxygenases: Initiate the process by adding oxygen to hydrocarbons.
- Dioxygenases: Cleave aromatic rings like a boss!
- Alkane Hydroxylases: Convert alkanes to alcohols as an initiation into the degradation event [26].

A lot of these talents are encoded in plasmids — portable gene packets that can be shared or passed among bacteria adding to their ability to rapidly increase their oil degrading prowess like a flash mob [35].

2.5.3. Why They Matter to Nature

These bacteria are important for maintaining ecosystems by detoxifying oil and allowing ecosystems to be restored from recent avarice. The marine bacterium *Alcanivorax borkumensis* is an aquatic hero after hydrocarbon spills because, with favorable nutrient conditions and no competition, it can perform hydrocarbon degrading magic! On land, *Pseudomonas spp*, and *Rhodococcus spp* can amend more robust hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), and start the process of restoring soil fertility and fostering soil biodiversity [36].

Example: *Alcanivorax borkumensis*

When an oil spill occurs, this hydrocarbon degrading marine bacterium can be a clean-up hero! It can be present (with fresh oil) in large concentrations (~10^6 -10^7 /mL) alongside most oil spills and can thrive in conditions with excess nitrogen and phosphorus in order to outcompete other microbes to use the alkanes in crude oil. It is basically a natural superhero for marine remediation [36].

2.6. Environmental Factors Influencing Biodegradation

Microbes can be picky about their habitat. Here's what can help or hurt them achieve their oil-degrading objectives:

pH Level

Most oil-degrading bacteria prefer a pH of 6.5–8.5. Above or below this range and they basically shut down. Soil acidity can further hinder access to hydrocarbons (33).

- **Acidic Conditions**: At low pH, many microorganisms can become stressed and inactive. The acidic environment may also impede the solubility of certain hydrocarbons—making them functionally unavailable to bacteria.

Temperature

Microbes are most active at temperatures that range from 20° C to 40° C. Colder temperatures slow microbes down and hotter temperatures denature their enzymes and reduces community diversity (11).

- **Optimum Temperature:** Most hydrocarbon degrading bacteria are organism is at its optimum metabolic activity which include hydrocarbon oxidation is between 20° C and 40° C (12).
- **Low Temperatures**: When the temperature is reduced, the rate of microbial degradation declines due to low metabolic activity; some bacteria have adapted to cold ecosystems, such as those in the polar region; they still degrade hydrocarbons at lower rates than bacteria found in warmer, temperate climates (11).
- **High Temperatures**: While the higher temperature can facilitate solubility of hydrocarbons (thereby allowing hydrocarbon degrading bacteria to thrive); it also negatively affects bacterial growth, denatures enzymes, reduces microbial diversity, and slows the process of degradation (12).

Oxygen Availability

Oxygen is like rocket fuel for aerobic microbes like *Pseudomonas*, powering fast cleanup. In low-oxygen zones, anaerobic microbes step in, but they're slower [15].

- **Aerobic Conditions**: In aerobic conditions, alkanes and aromatic hydrocarbons can be degraded by different bacteria or groups of bacteria, such as *Pseudomonas* or *Alcanivorax*. bacteria utilize oxygen as an electron acceptor to degrade hydrocarbons into smaller and less toxic compounds [14,15].
- **Anaerobic Conditions**: In oxygen-deficient environments, degradation to hydrocarbons may take much longer and is more difficult. Some bacteria have adapted to anaerobic conditions and instead use an alternative electron acceptor such as nitrate, sulfate, or iron for hydrocarbon degradation [15].

Nutrient Levels

Nitrogen and phosphorus are the microbes' energy drinks. Without enough, they can't grow or work. Biostimulation often adds these to speed things up [8].

- **Nitrogen and Phosphorus:** Nitrogen and phosphorus are important to microbial growth and available nitrogen and phosphorus can affect the rate of hydrocarbon degradation. Bio-stimulation is often used for the addition of nitrogen and phosphorus to the environment to promote the growth of hydrocarbon degrading bacteria [8,9].

Surfactants (e.g., Tween 20)

Surfactants (or surfactants) break oil into small droplets, similar to soap, which allow microbes to attack it. Tween 20 is a commonly used surfactant as it is non-toxic and increases clean-up [5].

- **Mechanism of Action**: Surfactants are added to the environment to emulsify the hydrocarbons, which break the hydrocarbons down into smaller droplets which increases the surface area available for attack by the microorganisms. This helps make the hydrocarbons more bioavailable to the microorganisms and greatly enhances biodegradation rates [5].

- **Usage in Bioremediation**: The most common application of surfactants is for bioremediation of oil spills and contaminated soils, especially if the hydrocarbons are in a non-aqueous phase or difficult to remediate (due to their hydrophobic nature) [5,6].

Hydrocarbon Concentration

Too much oil can either inhibit or starve microbes of oxygen. Moderate levels help and biostimulation returns a rhythmic equilibrium back into the process [16].

Other Factors

- **Salinity:** Ocean spills require salt-tolerant bacteria.
- **Co-Pollutants:** Heavy metals or other toxins environmental are added stressors to microbes, slowing their assimilation [17].

Mediation of these parameters resembles creating the ideal workspace for microbes reasonability to excel in their task.

2.7. Enrichment and Isolation of Oil-Degrading Bacteria

The isolation of bacteria capable of degrading oil from contaminated environments is the initial step of researching and utilizing microbial communities for bioremediation. These bacteria can utilize hydrocarbons (e.g., diesel, engine oil, and crude oil) as their carbon and energy source. Using enrichment and isolation techniques promotes the exclusive growth of specialized microbial populations.

Enrichment Culture Technique

Enrichment is a method used to stimulate the growth of selected microorganisms, by setting up favorable conditions for that specific microorganism's survival and growth. For example, with hydrocarbon degraders, we usually start with a Minimal Salt Media (MSM) as the base media. This will be free of complex carbon sources, meaning it will only allow microbes capable of metabolizing the hydrocarbon that is added to the MSM to develop and grow [34].

- **MSM Make Up:** MSM will provide basic inorganic salts such as Na_2HPO_4, KH_2PO_4, NH_4Cl, and $MgSO_4$. Note that it does not provide any available carbon sources for organisms [34].

- **Hydrocarbon as Sole Carbon Source:** In order to enrich hydrocarbon degrading microbes, the MSM is supplemented with diesel, used engine oil, or crude oil as the only carbon and energy source. This will select for only those organisms capable of using hydrocarbons to survive and grow [35].

- **Incubation:** After, the mixture will be incubated under appropriate conditions (i.e., 30-37°C shaking, ~150-180 rpm) for several days to allow for active degradation and enrichment of the microbes that can degrade oil. This gives an oil degrading bacteria a competitive advantage in the culture.

Serial dilution and plating

Serial dilution is used to decrease microbial load and allow colonies to be isolated after enrichment. This process is carried out by either spread plating or pour plating on agar media amended with hydrocarbons [35].

- Serial dilution: The broth culture enriched is diluted systematically (e.g., 10^{-1} to 10^{-6}) with sterile saline or distilled water.
- Amended hydrocarbon agar: Appropriate aliquots of the desired dilution are spread onto MSM plates, with specific hydrocarbons if desired (e.g., 1% diesel or engine oil) either mixed into the media or spread on top of the agar medium.
- Selective growth: Only the bacteria capable of metabolizing hydrocarbons will grow. Incubation conditions is on average between 24-72 hours, with longer times for microorganisms with slow growth rates.

Potent degrader screening

Once all colonies have grown, screening emphasizes identifying a specific strain that most effectively degrades hydrocarbons [36].

- Colony morphology: When choosing which specific colony types, size, shape, texture, color, and spread will more often than not be deciding factors. Hydrocarbon degraders regularly appear as opaque, shiny, or oily colonies.
- Zone of clearance: In some uncommon modified agar methods, bacteria that can degrade hydrocarbons display clear zones or haloes of any kind around positively identified colonies. The zone of clearance demonstrates that a bacteria is active at degrading hydrocarbons.

Characterization of Isolates

After isolation, it is important to perform a variety of characterizations to determine the identity and functionality of the bacterial strains.

- Gram Staining: One of the first tests performed to separate bacteria types as either Gram-positive or Gram-negative; this basic differential stain is indicative of cell wall structure.
- Colony Morphology: Observed on agar plates during exposure to standard growth conditions, the colonies are examined for the characteristics of margin, elevation, opacity, and pigmentation.
- Biochemical Assays: These tests may include:
  - Catalase and Oxidase
  - Starch and lipid hydrolysis
  - Nitrate reduction
  - Use of carbon sources

The enrichment and isolation of oil-degrading bacteria from hydrocarbon-biased minimal salt media is an extremely important bioremediation research process. This allows for the selection of microorganisms capable of metabolizing petroleum hydrocarbons, and the study of the selected organisms, which by definition, will be able to degrade hydrocarbons (Rosenberg, 1992).

2.8. Recent Advances and Biotechnological Applications in Bioremediation

Although bioremediation has progressed over the past few decades, the explosion of technology in microbiology, molecular biology, and environmental engineering have pushed the boundaries of traditional bioremediation. The intention of this progress and new technology is to refine bioremediation to improve efficiency, flexibility and scale.

Genetic Engineering of Microorganisms

As mentioned before, the tools of genetic engineering now enable researchers to target specific bacterium genes responsible for the hydrocarbon metabolism and enhance their degradation capabilities. By knocking in or knocking out genes related to metabolism of organic hydrocarbons at the level of alkane monooxygenases or dioxygenases, for example, may improve a hydrocarbon degrading bacterium's ability to degrade aliphatic and aromatic hydrocarbons [26].

Application of Omics Technologies

Omics technologies, like genomics, transcriptomics, proteomics, and metabolomics, provide a system level perspective of microbial communities and how they function [6]:

- Genomics reveals the hydrocarbon degrading genes carried in microbial strains.
- Transcripts and proteomics give indication of gene and protein expression when exposed to hydrocarbons.
- Metabolomics tracks intermediate and final products of hydrocarbon degradation pathways.

Metagenomics for Uncultured Microbes

Classic microbiology restricts our perspective to those bacteria that are culturable, which only accounts for a limited subset of the entire microbial community [5]. Metagenomics overcomes the limitation of culturing by investigating DNA directly from sampled environmental materials. This allows us to see how much diversity and functional potential exists in uncultured microbes that are predicted to have important roles in hydrocarbon degradation. The data acquired from metagenomic analyses can be applied to help choose unique microbial consortia (when undertaken at scale) or discover new biodegradation pathways that could be developed biotechnologically (Wang et al., 2016).

Biosurfactant-Producing Strains

Some specific strains of bacteria additionally produce biosurfactants, which are naturally occurring surface-active agents that bind with hydrophobic hydrocarbons and emulsify them to enhance the bioavailability of hydrocarbons for microbes to utilize them. Engineering or enriching for these strains with the potential to produce biosurfactants (e.g., Pseudomonas aeruginosa, Bacillus subtilis) is another increasingly used strategy to enhance the efficiency of remediation, particularly in soil with a high concentration of hydrocarbons [6].

Immobilization Techniques and Bioreactors

To increase microbial stability, survival, and re-usability, researchers have created immobilization techniques that encapsulate bacteria in carriers such as alginate beads, biochar, or synthetic polymers. This encapsulation protects the bacteria from environmental stressors and enables them to degrade pollutants in less erratic manner [14]

.2.9 Research Gaps and Future Directions

Although bioremediation has progressed considerably through fundamental research in laboratory settings and molecular strategies, there still remains significant research gaps which hinder the transition to full scale applications of bioremediation strategies for oil-contaminated soils, while also considerably reducing their success over a protracted period. Addressing these research gaps is paramount to proceed in successfully transitioning from experimental conditions to real world conditions that are environmentally conscious and aligned with policy requirements.

Need for Field-based

Validation Bioremediation uses microorganisms (bacteria and fungi) for practical solutions and has great potential for remediation of oil-contaminated environments due, in part, to its sustainable approach. However, its ability to be appropriately implemented is dependent upon substantial field-testing, extended monitoring, optimization of microbial consortia, the nature of horizontal gene transfer (HGT) within the consortia, and climate resilient microbial agents. Transitioning from controlled laboratory conditions to complex conditions in practice will require addressing substantial research gaps to prove efficacy, safety, and scalability in the real world. The following sections broadly outline these issues in formal (but readable) terms and refer to the evidence explored within the project literature as much as possible.

Necessity of Field-Based Validation

When using bioremediation in a lab, researchers usually create conditions for the right temperature, pH, amount of nutrients and species of microbes [1]. On the other hand, the naturally contaminated sites are described by climate, soils types and co-contaminants, all of which could negatively impact the growth and functioning of the microbes.

In order to fill that gap, there should be comprehensive field trials to see if the lab-created microbial strains are functioning in a natural and outside lab conditions. It is important for such studies to evaluate some elements.

- The ability for introduced or activated microbes to survive and function in natural soils with other microbes.
- Temperature-Resistant Mrollary: How well hydrocarbon degradation works under changes in temperature and rain.

- The biosphere of local ecosystems can be impacted permanently or temporally by extractive practices which may be seen as changes to biodiversity and the soil's ability to function [3].

Field-based validation to demonstrate that bioremediation can work in the real world and improve the process under varied environmental conditions [4].

Importance of Long-Term Monitoring

The Need for Long-Term Monitoring

The goal of bioremediation is the eventual complete restoration of the environment in question. Even after pollution levels have been reduced, continuing to monitor the environment is necessary to help understand the pace of recovery of the ecosystem [5]. Long-term monitoring should focus on two areas:

- Residual Byproducts: Any toxic materials that remain behind or develop as byproducts of an incomplete process.
- Re-Plenishing Soil: The restoring of all necessary elements for soil to be functional for healthy agriculture and environment [6].

The focus of much of the bioremediation work continues to be on the rapid depletion of hydrocarbons, with little emphasis placed on what happens with time. Adequate long-term monitoring emphasizes that ecological improvements actually happen, along with identifying future problems that can arise [7].

Examination of Microbial Consortia

Conventional bioremediation practices rely almost exclusively on single microbial strains for hydrocarbon degradation. In reality, in natural systems, destruction of pollutants is done collectively in microbial communities, with each species contributing its specialized metabolic mechanism for digestion of complex hydrocarbons [8]. These interactions are far powerful than the use of a single strain for contaminant destruction [9].

Future research should prioritize:

- **Identification of Compatible Strains:** Identifying microbial species that have complementary metabolic pathways that will increase degradation efficiency.

- **Interspecies Dynamics:** Examining how microbial communities communicate and cooperate with each other to facilitate optimal community performance.
- **Synthetic Consortia Development:** Development of microbial consortia that mimic natural communities to improve resilience and effectiveness in degrading complex contaminant mixtures [10]. Utilization of microbial consortia can offer more positive bioremediation results, particularly with heterogeneous oil spills, by relying on the combined strengths of various microbial populations [11].

The Role of Horizontal Gene Transfer (HGT)

Bacteria can use horizontal gene transfer to obtain and pass on their ability to metabolize hydrocarbons to other individuals of the same population. The transfer of plasmids, bacteriophages, or transposons allows microorganisms to adapt quickly and begin degrading multiple forms of hydrocarbons. This contributes to increasing community-level cleaning capabilities [12].

Improvements in bioremediation can be achieved by concentrating research on:

- This type of work involves locating and understanding the gene(s) involved in hydrocarbon breakdown.
- Environmental Conditions: Knowing how things like pollution or lack of nutrients enhance HGT.
- I think we have to accept that in our environment there could be some gene dispersal that could contribute to nasty strains [13].

Now if we can understand HGT, we could potentially improve microbial communities for bioremediation while managing the transfer of genes with a sensitivity to the environment [14].

Climate Adaptability of Bioremediation Agents

Climate change presents bioremediation challenges which include higher temperatures, altered rainfall patterns, droughts, flooding and increased salinity of soil, all problematic for microbes [15]. If we are to improve bioremediation's reliability regarding climate change, we need to stabilize the microbial agents on which we rely [16].

The primary research goals include:

- Providing a proactive or engineered strain that can continue degrading wastes even when under duress.
- Testing bacteria and other microbes under multiple climate probes with respect to temperature and water supply.
- Adapting bioremediation techniques to suit different environments like arid locations, and wet coastal locations, [17]. By incorporating climate adaptability, bioremediation approaches may not only be effective but enhanced adaptability supports widespread adoption in the face of degrading global environmental conditions [18].

Integration with Ecological Risk Assessment

Integration with Ecological Risk Assessment

Even though bioremediation approaches are eco-friendly, they must still undergo ecological risk assessments (ERAs) to understand any unintended consequences such as:

- The introducing strains exhibiting invasive behavior.
- A disruption of native microbial diversity.
- The retention of potentially toxic degradation by-products. When ERAs are made part of bioremediation planning, particularly for microorganisms, it allows for an environmentally safe ecology to be implemented while ensuring that the remediation process is consistent with environmental and regulatory protection measures.

Policy and Regulatory Frameworks

The idea of using microscopic microbes (viruses, bacteria, fungi, etc.) to naturally break down toxic pollutants as a sustainable and eco-friendly way to remediate oil polluted environments has immense potential. However, laboratory success alone will not transfer the technology into practice; it takes a significant amount of essential science and a well-defined and supportive regulatory and policy framework for its successful integration. In many parts of the world, legislation concerning microbial remediation is ambiguous and vague, especially with respect to genetically modified bacteria or a microbial consortium in a natural setting [1]. This lack of regulatory clarity is, and will remain if left unresolved, a significant barrier to widespread adoption.

Clear standards and coherence within safety protocols are essential to transition bioremediation from a process applicable to small areas to a safe, reliable, and broadly applicable use in cleanup

operations. "At the same time, the guidelines would ensure safe uses of microbes (for instance, destroying toxic chemicals in the environment, protecting crops against diseases) and protect human health," the authors argue, and while also building public and regulator support for a strategy that some consider controversial or potentially unsafe. Standardized performance metrics for microbial effectiveness will also be needed to create a benchmark of typical performance against which bioremediation can be validated to stakeholder satisfaction. Future scaling up of bioremediation the landscape will be contingent on partnerships among governments, academic institutions, and industry leaders; they together have the expertise and innovation to help capture due diligence and create consistent approaches [3]. This process will entail collaboration with large groups of microbiologists, molecular biologists, environmental engineers and policy makers. However, the collaboration bears relevance as it is the only way to develop bioremediation as a supported, regulated option for cleaning up oil-impacted soils globally, ultimately leading to a less contaminated and healthier planet.

CHAPTER 3
MATERIALS AND METHODLOGY

3.1 Sample Collection

- Soil sample was collected from a petrol pump site located in Bilaspur, Himachal Pradesh (approx. coordinates: 31.3410° N, 76.7575° E) [1].
- 1 g of soil was mixed thoroughly in 100 mL of sterile distilled water.
- The mixture was vortexed and allowed to settle.
- 1 mL of the supernatant was used for inoculating enrichment media [2].

3.2 Preparation of Minimal Salt (MS) Media

- MS media was prepared by dissolving essential salts and trace elements in distilled water [3].
- pH was adjusted to 7.0.
- 0.005% Tween 20 was added to facilitate hydrocarbon emulsification [5].
- Diesel and used engine oil were added at concentrations of 0%, 0.1%, and 0.2%.
- Media was autoclaved at 121°C for 15 minutes.

Requirements

- K_2HPO_4 – 2.0 g
- $(NH_4)_2SO_4$ – 0.5 g
- KH_2PO_4 – 0.02 g
- $MgSO_4 \cdot 7H_2O$ – 0.05 g
- $FeSO_4 \cdot 4H_2O$ – 0.4 g
- $MnSO_4 \cdot 4H_2O$ – 0.4 g

- $ZnSO_4 \cdot 7H_2O$ – 0.2 g
- $CuSO_4 \cdot 7H_2O$ – 0.04 g
- KI – 0.3 g
- $Na_2MoO_4 \cdot 2H_2O$ – 0.05 g
- $CoCl_2 \cdot 6H_2O$ – 0.04 g
- Agar – 1.5% (for solid medium)
- Diesel and used engine oil
- Distilled water
- Tween 20 – 0.005%

Procedure

- All salts as per composition were weighed and dissolved in 200 mL distilled water.
- pH was adjusted to 7.0 using 1N NaOH or HCl and then 0.005% Tween 20 was added [4,5].
- Media was transferred into flasks and 0%, 0.1%, or 0.2% of diesel/engine oil were added in respective flasks and properly labeled [6].
- The media containing flasks were then autoclaved for 15 minutes at 121°C and 15 psi.
- Following sterilization, the media was cooled down and was used for enrichment culture.

3.3 Enrichment and Isolation of Bacteria

- 1 mL of prepared soil supernatant was inoculated into each MS broth with different hydrocarbon concentrations [7].
- The cultures were incubated at 37°C for 7 days at 180 rpm.

- After incubation, 100 µL from each flask was spread on solid MS agar plates.
- Plates were incubated at 37°C for 3–5 days.
- Morphologically distinct colonies were picked for further characterization.

Requirements

- Enrichment MS media
- Diesel/Engine oil
- Incubator shaker
- Sterile Petri plates
- Micropipette and sterile tips

Procedure

- Inoculate 1 mL of the soil solution into MS broth with hydrocarbons.
- Incubated the flasks at 37°C and 180 rpm for 7 days.
- After incubation, plate 100 µL onto MS agar.
- Incubated and observe for colony formation.
- Isolate and subculture individual colonies.

3.4 Gram's Staining

- Selected isolates were stained using the Gram staining technique to determine cell wall type.

Requirements

- Glass slides

- Inoculating loop
- Bunsen burner
- Crystal violet
- Gram’s iodine
- Decolorizer (ethanol)
- Safranin
- Distilled water
- Microscope

Procedure

- Prepare bacterial smear on a clean slide and heat-fix.
- Stain with crystal violet for 1 minute and rinse.
- Apply iodine for 1 minute and rinse.
- Decolorize with ethanol for 15–30 seconds.
- Counterstain with safranin for 1 minute.
- Observe under a microscope for purple (Gram-positive) or pink (Gram-negative) cells.

3.5 Biochemical Characterization of Isolates

To investigate the metabolic capabilities and enzymatic traits of our hydrocarbon degrading bacterial isolates, we performed a series of biochemical tests using standard microbiological techniques. Each test was conducted in a completely aseptic manner utilizing fresh, pure bacterial cultures and each was performed with precision and reliability reported [15].

Methyl Red (MR) Test

Requirements

- MR-VP broth
- Methyl red indicator
- Test tubes
- Incubator (37°C)
- Micropipette

Procedure

- Inoculate the bacterial isolate into MR-VP broth.
- Incubate at 37°C for 48 hours.
- After incubation, add 5 drops of methyl red indicator to the tube.
- Gently shake to mix.
- Observe the color change: a red color indicates positive MR test (mixed acid fermentation).

Catalase Test

Requirements

- Fresh bacterial culture (colony)
- 3% Hydrogen peroxide (H_2O_2)
- Clean glass slide
- Inoculating loop

Procedure

- Transfer a small portion of the bacterial colony onto a dry glass slide.
- Add 1 drop of 3% H_2O_2 to the smear.
- Observe immediately for effervescence (oxygen bubbles).
- Presence of bubbles confirms catalase-positive bacteria.

MacConkey Agar Test

Requirements

- MacConkey agar plates
- Sterile inoculating loop
- Incubator (37°C)

Procedure

- Streak the bacterial isolate onto a MacConkey agar plate using a sterile loop.
- Incubated the plate at 37°C for 24–48 hours.
- Observe colony morphology and color changes.
- Pink/red colonies indicate lactose fermentation; colorless colonies indicate non-fermenters.

Mannitol Fermentation Test

Requirements

- Mannitol fermentation agar containing phenol red
- Sterile inoculating loop
- Incubator (37°C)

Procedure

- Streak the bacterial isolate onto Mannitol fermentation agar plate using a sterile loop.
- Incubate at 37°C for 24–48 hours.
- Observe the broth for color change.
- Yellow color indicates acid production and positive fermentation.

Urea Hydrolysis Test

Requirements

- Urea agar slants
- Inoculating loop
- Incubator (37°C)

Procedure

- Streak the bacterial isolate on a sterile urea agar slant.
- Incubate at 37°C for up to 5 days.
- Observe daily for color change.
- Pink color indicates positive urease activity due to ammonia production.

Voges–Proskauer (VP) Test

Requirements

- MR-VP broth
- Barritt's reagent A (α-naphthol)
- Barritt's reagent B (40% KOH)

- Test tubes
- Incubator (37°C)

Procedure

- Inoculate the isolate into MR-VP broth.
- Incubate at 37°C for 48 hours.
- Add 1 mL of Barritt's reagent A, followed by 1 mL of reagent B.
- Shake gently and leave undisturbed for 15–30 minutes.
- A pink to red color indicates a positive VP test, showing acetoin production.

3.6 Genomic DNA Isolation

We used the phenol-chloroform method, a widely adopted and reliable method to recover high quality bacterial DNA which we extracted to investigate the molecular characteristics that our hydrocarbon degrading bacterial isolates possessed. It involved growing the bacterial cultures, breaking open the cells to extract their contents, taking pains to separate the DNA from proteins and other impurities and verifying the DNA's quality with agarose gel electrophoresis to see clear, well defined bands [13].

Requirements

- Nutrient broth
- TE buffer (Tris-EDTA)
- Lysozyme
- SDS (Sodium Dodecyl Sulfate)
- Phenol: Chloroform: Isoamyl alcohol (25:24:1)

- Sodium acetate (3M, pH 5.2)
- Absolute ethanol (chilled)
- 70% ethanol
- Nuclease-free water
- Centrifuge tubes
- Microcentrifuge
- Shaking incubator (37°C)
- Agarose powder
- 1X TAE buffer
- Gel electrophoresis apparatus
- UV transilluminator

Procedure

A. Genomic DNA Extraction

- Inoculate the bacterial isolates in nutrient broth and incubate overnight at 37°C with shaking.
- Centrifuge the culture at 8,000 rpm for 10 minutes to collect the bacterial pellet.
- Resuspend the pellet in TE buffer to stabilize and protect DNA.
- Add SDS to lyse cells and release genomic DNA.
- Add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), mix well, and centrifuge at high speed for 10 minutes.
- Carefully transfer the upper aqueous phase containing DNA to a fresh tube.
- Add 2.5 volumes of chilled absolute ethanol and 0.1 volume of sodium acetate to precipitate DNA.

- Incubated at -20°C for 30 minutes, then centrifuge to collect the DNA pellet.
- Wash the pellet with 70% ethanol, air dry, and dissolve in nuclease-free water.
- Store DNA at 4°C for further analysis.

B. DNA Quality Check via Agarose Gel Electrophoresis

- Prepare a 1% agarose gel in 1X TAE buffer.
- Pour into a gel tray with a comb and allow it to set.
- Load DNA samples into the wells and run at 80–100V for 30 minutes.
- Visualize the gel under UV light. Clear, high-molecular-weight DNA bands confirm successful genomic DNA isolation.

3.7 Turbidity Analysis

We performed a turbidity analysis using minimal salt (MS) broth supplemented with hydrocarbons in order to evaluate our ability to use isolated bacterial strains, Isolate A and Isolate B, to degrade hydrocarbons and grow efficiently. Here we use this approach to measure bacterial growth by monitoring the optical density over time which in turn provides us with a clear window into their performance [18].

Requirements

- Minimal Salt (MS) broth
- Diesel (0.1% and 0.2%)
- Engine oil (0.1% and 0.2%)
- Isolate A and Isolate B (actively growing cultures)
- Shaking incubator (37°C, 180 rpm)
- UV-Visible spectrophotometer

- Sterile 1 mL pipettes
- Sterile culture tubes/flasks
- Cuvettes (for spectrophotometer readings)

Procedure

- Prepare 100 mL of MS broth in sterile flasks for each condition.
- Add 0.1% or 0.2% diesel or burned engine oil to each flask as the sole carbon source.
- Inoculate each flask with 1 mL of Isolate A or Isolate B culture, previously grown in hydrocarbon media.
- Incubate all flasks at 37°C in a shaking incubator at 180 rpm for 7 days to ensure aeration and uniform mixing.
- Do not add any additional carbon sources to ensure that any observed bacterial growth is due to hydrocarbon degradation.
- Every 24 hours, withdraw 1 mL aliquots aseptically from each flask.
- Measure turbidity at 600 nm (OD_{600}) using a UV-Visible spectrophotometer.
- Record OD_{600} readings daily for each condition (Isolate A and B with 0.1% and 0.2% diesel/engine oil).
- Plot the OD values against time to generate bacterial growth curves and compare the hydrocarbon degradation efficiency of the isolates.

Fig.1: Bacterial growth of Isolate A and isolate B

3.8 Optimization Analysis

To enhance the growth of hydrocarbon-degrading bacteria, optimization studies were carried out in three successive stages [25]:

- Evaluation of nitrogen sources
- Determination of optimal pH using the best nitrogen source
- Determination of optimal temperature at optimal pH with the selected nitrogen source

3.8.1 Evaluation of Different Nitrogen Sources

Objective:

To identify the most effective nitrogen source for supporting the growth of hydrocarbon-degrading bacteria in diesel-containing environments.

Methodology:

Three nitrogen supplements were selected for evaluation:

- Peptone (organic source)
- Yeast extract (organic source)

- Ammonium sulphate (inorganic source)

Each nitrogen source was individually incorporated into minimal salt (MS) broth containing diesel as the sole carbon source. Equal volumes of MS broth were prepared and sterilized via autoclaving. After cooling, 1 mL of actively growing bacterial culture (previously enriched in similar conditions) was inoculated into each flask [26].

The flasks were incubated at 37°C with shaking at 180 rpm for 7 days. Bacterial growth was monitored daily by measuring optical density at 600 nm (OD600) using a UV-Vis spectrophotometer.

Fig.2: Flasks with nitrogen sources peptone, yeast extract and ammonium sulphate

3.8.2 Optimization of pH Using Peptone as Nitrogen Source

Objective: To determine the optimal pH for bacterial growth in MS broth containing peptone and diesel.

Methodology:

MS broth was adjusted to four different pH levels:

- pH 5.0
- pH 6.5
- pH 7.0

- pH 8.5

Adjustments were made using 1N HCl and 1N NaOH, and verified using a calibrated pH meter before autoclaving. After sterilization, each pH-adjusted medium was inoculated with 1 mL of standardized bacterial culture [12].

The flasks were incubated at 37°C with shaking at 180 rpm for 7 days, and OD600 was measured daily. Each pH condition was tested in triplicate.

Fig.3: Flasks with media at pH 5.0, pH 6.5, pH 7.0, and pH 8.5

3.8.3 Optimization of Temperature Using Peptone at pH 7.0

Objective:

To determine the optimal incubation temperature for bacterial growth in MS media supplemented with peptone at pH 7.0.

Methodology:

MS broth at pH 7.0 was sterilized and inoculated with 1 mL of actively growing bacterial culture. The flasks were incubated at the following temperatures:

- 25°C
- 30°C
- 37°C

- 40°C

All cultures were incubated under 180 rpm shaking for 7 days, with OD600 measurements taken daily. Each temperature condition was tested in triplicate.

CHAPTER-4

RESULTS

4.1. Isolation and Enrichment

Soil samples were collected from an oil-contaminated site for isolating hydrocarbon-degrading bacteria. These samples were inoculated into Minimal Salt (MS) broth supplemented with diesel and burned engine oil, followed by incubation at 37°C for 7 days under shaking conditions (180 rpm).

A significant increase in turbidity was observed during incubation. The final optical density (OD_{600}) reached approximately 5.678, suggesting substantial bacterial proliferation and utilization of hydrocarbons as the sole carbon source.

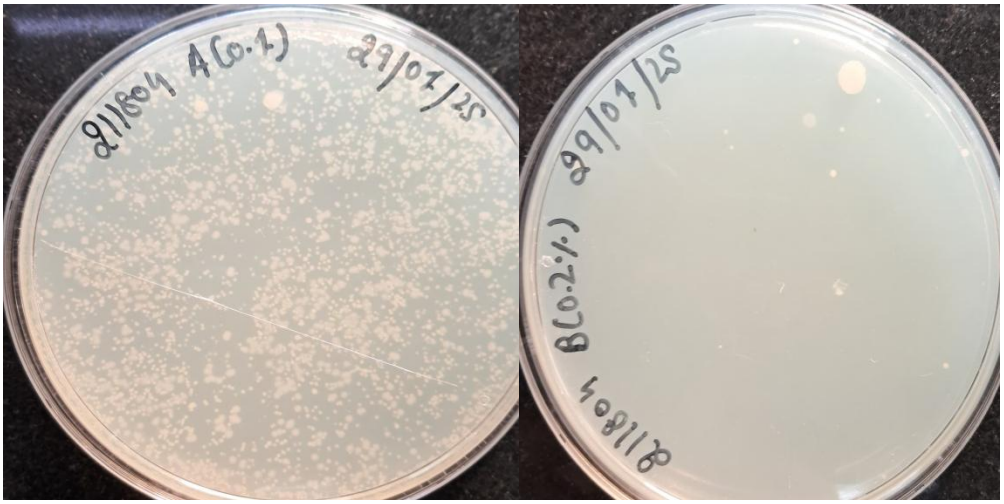

Fig.4: Bacterial colonies of Isolates A and B grown on MS agar plates show dense growth

4.2 Morphological Observation and Gram Staining

Post-enrichment, bacterial cultures were streaked on MS agar plates containing 0.1% and 0.2% diesel and engine oil. Within 3–5 days, visible colonies were observed and picked based on morphological characteristics. Gram staining revealed that all isolates were Gram-negative, exhibiting pink coloration under the microscope. This indicated the presence of organisms with a thin peptidoglycan wall and an outer membrane, typical of oil-degrading bacteria like *Pseudomonas and Acinetobacter*.

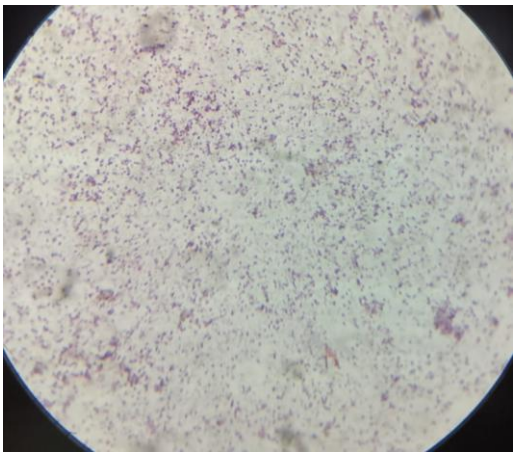

Fig.5: Microscopic view showing Gram-stained bacteria isolated from oil-contaminated soil.

4.3 Biochemical Characterization

To determine the metabolic profile of the isolates, the following biochemical tests were performed:

Table 4.3.1: Biochemical test results showing positive catalase, MacConkey, and mannitol tests for both isolates.

Sr.no	Name of Test	Isolate A	Isolate B
1	Methyl red test	-	-
2	Catalase test	+	+
3	MacConkey Agar test	+	+
4	Mannitol test	+	+
5	Urea Hydrolysis test	-	-
6	Voges-Proskauer test	-	-

These test results, combined with morphological observations, strongly indicate the isolates may belong to *Pseudomonas or Acinetobacter*, which are both capable of hydrocarbon degradation.

4.4 DNA Extraction and Agarose Gel Electrophoresis

Genomic DNA was extracted using the phenol-chloroform method. The extracted DNA was clear, colorless, and had an A_{260}/A_{280} ratio between 1.8 and 2.0, indicating high purity. Agarose gel electrophoresis (1%) confirmed DNA integrity by showing sharp and distinct bands.

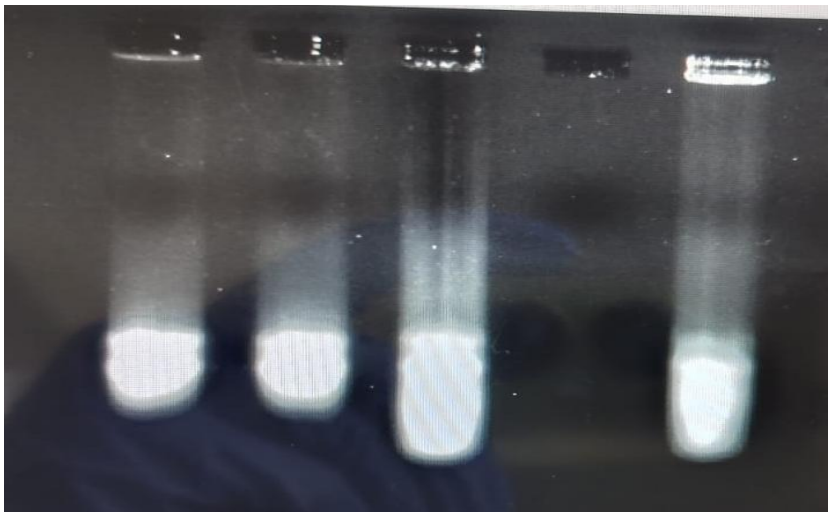

Fig.6: Agarose Gel Electrophoresis of Extracted DNA

4.5 Turbidity Analysis

To assess degradation efficiency, two isolates (A and B) were cultured separately in MS broth with diesel and engine oil, and OD_{600} was recorded for 7 days.

- **Isolate A** showed rapid early growth, peaking at OD ~4.5 on Day 4, followed by a decline likely due to nutrient depletion or toxic metabolite accumulation.
- **Isolate B** demonstrated slower but sustained growth, reaching OD ~4.8 on Day 5, suggesting better adaptation for long-term degradation.

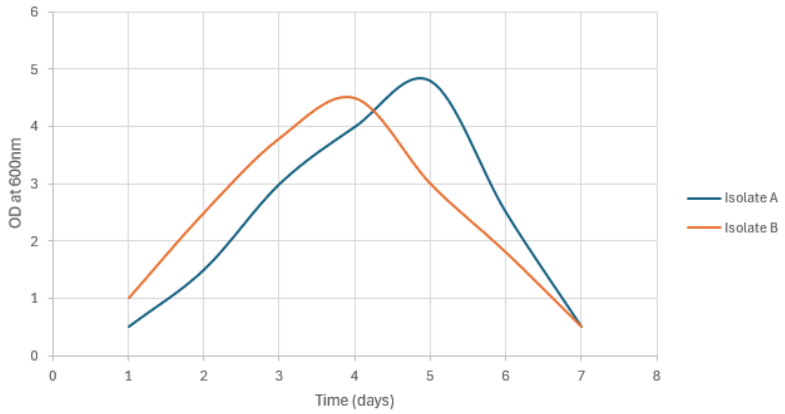

Fig.7: Line graph comparing OD_{600} of Isolate A (peaking at ~4.8, Day 5) and Isolate B (peaking at ~4.5, Day 4) in MS broth with diesel and engine oil over 7 days, showing Isolate B's faster initial growth and Isolate A's higher, sustained growth.

Both isolates show promising bioremediation potential, with A being ideal for quick action and B for sustained degradation.

4.6 Optimization Analysis

4.6.1 Effect of Nitrogen Source

To determine the best nitrogen source for hydrocarbon-degrading bacterial growth, MS broth was supplemented with diesel and one of three nitrogen sources: ammonium sulphate, yeast extract, or peptone. Growth was monitored daily by measuring OD_{600}.

Table 4.6.1: OD_{600} Values for Nitrogen Sources Over 7 Days

Ammonium Sulphate (OD)	Yeast Extract (OD)	Peptone (OD)
1.587	0.576	1.058
3.076	2.076	2.576
5.861	4.567	4.067
5.567	6.587	6.867
4.567	5.068	5.568
3.765	3.654	3.086

Interpretation:

- Peptone supported the highest growth, peaking at OD 6.5 on Day 4, indicating it is the most effective nitrogen source.
- Yeast extract also supported substantial growth (OD 6.0 on Day 4).
- Ammonium sulphate led to moderate growth, peaking at OD 5.5, making it the least effective among the three.

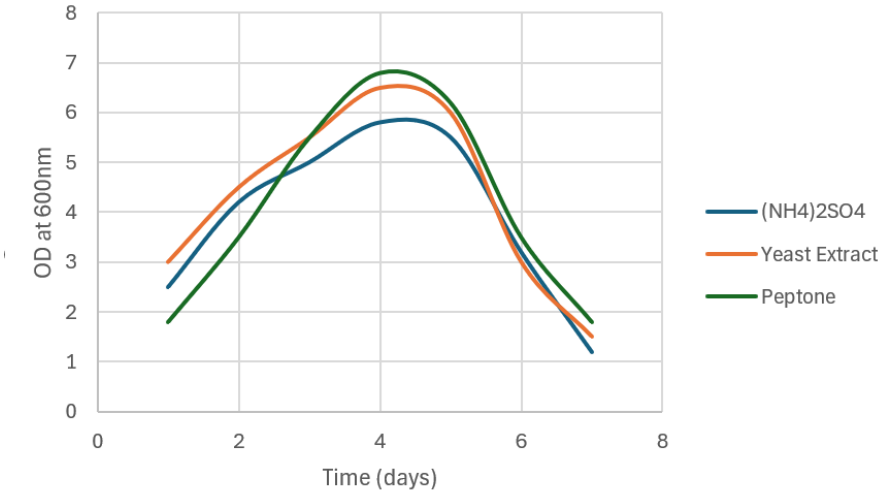

Fig.8: Bacterial growth in MS broth with diesel and nitrogen sources (peptone, yeast extract, ammonium sulphate) over 7 days (OD_{600}); peptone highest (OD ~6.8, Day 4), then yeast extract, ammonium sulphate lowest.

4.6.2 Effect of pH

To determine the optimal pH for the growth of hydrocarbon-degrading bacteria, MS broth containing diesel was adjusted to four different pH levels: 5.0 (acidic), 6.5 (slightly acidic), 7.0 (neutral), and 8.5 (alkaline). Cultures were incubated at 37°C for 7 days, and OD_{600} values were recorded daily.

Table 4.6.2: OD_{600} Values at Different pH Levels over 7 Days

pH 5.0	pH 6.5	pH 7.0	pH 8.5
0.294	0.321	0.671	0.367
0.623	0.534	0.937	1.382
1.483	1.684	2.379	1.827
1.743	2.025	2.969	2.418
2.172	3.567	4.897	3.988
3.897	4.763	5.987	4.987
1.234	2.453	2.765	1.765

Interpretation:

- Neutral pH (7.0) showed the highest OD_{600}, peaking at 5.987 on Day 6, confirming it as the optimal pH for bacterial growth.
- Slightly acidic pH (6.5) supported good growth (peak OD = 4.763), though slightly less than pH 7.
- Alkaline pH (8.5) allowed moderate growth, peaking at OD = 4.987.
- Acidic pH (5.0) resulted in the least growth, with a peak OD of 3.897, and rapid decline after Day 6.

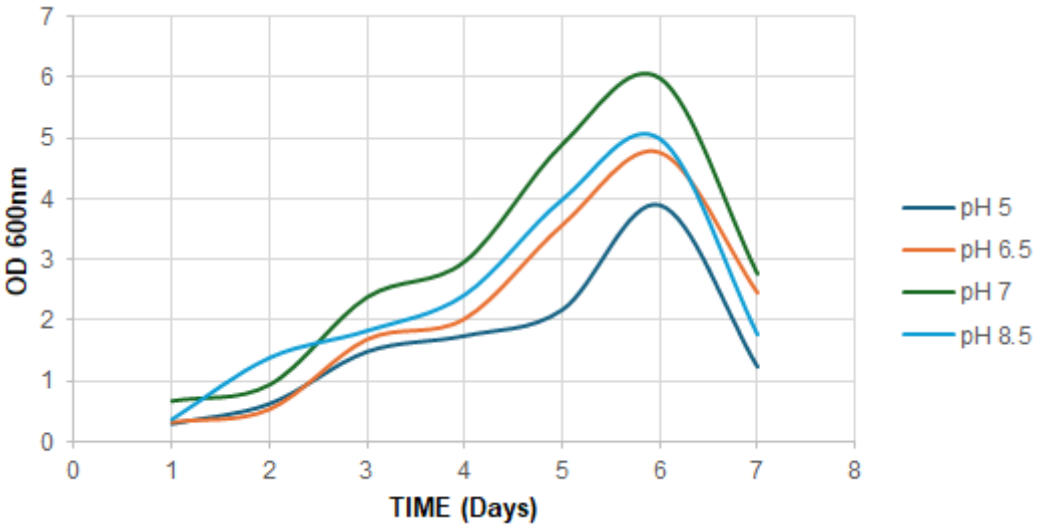

Fig.9: Bacterial growth in MS broth with diesel at pH 5.0, 6.5, 7.0, 8.5 over 7 days (OD_{600}); pH 7.0 optimal (OD ~5.987, Day 6).

The optimal pH for hydrocarbon-degrading bacterial growth was found to be pH 7.0. More acidic (pH 5) and alkaline (pH 8.5) conditions negatively impacted bacterial activity, likely due to enzyme denaturation or membrane instability. Hence, neutral pH conditions are recommended for efficient bioremediation applications.

4.6.3 Effect of Temperature

To investigate the effect of temperature on the growth of hydrocarbon-degrading bacteria, cultures were incubated at four different temperatures: 25°C, 30°C, 37°C, and 45°C in MS broth supplemented with diesel. OD_{600} values were measured daily over a period of 7 days to monitor bacterial growth and determine the optimal temperature for biodegradation activity.

Table 4.6.3: OD_{600} Values at Different Temperatures Over 7 Days

25°C	30°C	37°C	45°C
0.312	0.476	0.651	0.288
0.768	1.024	1.687	0.745
1.254	2.243	3.789	1.389
2.098	3.873	5.234	2.345
2.478	4.121	6.742	3.265
1.876	3.965	5.986	2.198
0.987	2.567	3.789	1.054

Interpretation:

- Optimal growth was observed at 37°C, with a peak OD_{600} of 6.742 on Day 5, confirming it as the most favorable temperature for bacterial activity.
- At 30°C, moderate growth occurred (peak OD = 4.121), indicating suitable but suboptimal conditions.
- At 25°C, bacterial growth was limited, peaking at OD = 2.478, possibly due to reduced enzymatic activity.
- Growth at 45°C was minimal, suggesting heat stress or enzyme denaturation inhibited bacterial metabolism.

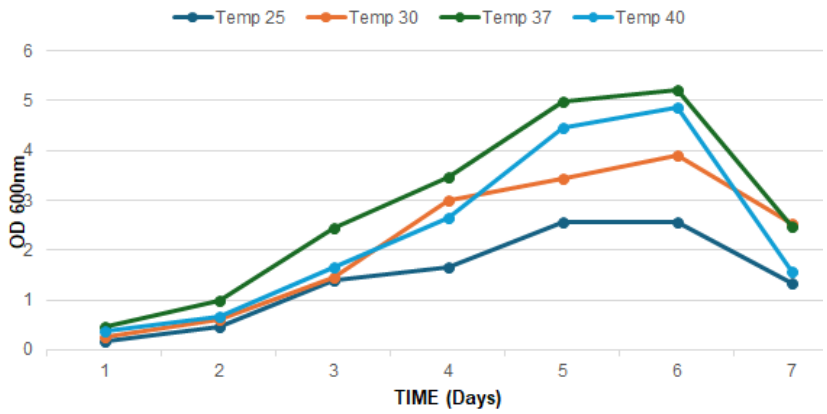

Fig.10: Bacterial growth in MS broth with diesel at 25°C, 30°C, 37°C, 45°Cover 7 days (OD_{600}); highest at 37°C (Tem37, OD ~6.742, Day 5).

The hydrocarbon-degrading bacterial isolates demonstrated maximum growth at 37°C, aligning with typical mesophilic behavior. Higher (45°C) or lower (25°C) temperatures led to suboptimal growth. Thus, 37°C is recommended for effective biodegradation during bioremediation processes.

CHAPTER 5

DISCUSSION

In this investigation I undertook a bold endeavor to elucidate, methodically characterize and finally optimize bacteria from oil contaminated soil for exploitation as agents of environmental restoration via bioremediation. We managed to grow the bacterial strains we isolated to utilize hydrocarbons as the sole carbon and energy source which was promising. Although enrichment cultures were turbid, these isolates formed obvious, robust colonies on minimal salt agar supplemented with diesel and engine oil, providing evidence of their ability to grow on hydrocarbons [1]. However, one isolate performed exceptionally better than all others – optically dense reach $OD_{600} \approx 5.678$ indicating very good growth and high capacity of hydrocarbon degradation [2].

We also used Gram staining to determine that our standout isolate was Gram negative, a trait commonly held by proficient hydrocarbon degrading genera similar to *Pseudomonas and Acinetobacter* [3]. For further elaboration of its identity, we also performed a series of biochemical assays. The isolate showed a positive culture for catalase, mannitol fermentation and MacConkey agar, as well as a negative culture for methyl red, Voges-Proskauer and urease. Based on these findings, we must conclude that these isolates show high degree of affinity with the Pseudomonas genus, known to be highly versatile, metabolically speaking and very suitable for degrading complex pollutants [4] and that we extracted high quality genomic DNA following the phenol-chloroform method, with agarose gel electrophoresis results revealing tight, well defined DNA bands, suggesting this DNA was intact and capable of use in possible future molecular studies [5]. Thus, in a seven day comparative growth analysis, Isolate A fared better than Isolate B but produced significantly higher turbidity, suggesting higher hydrocarbon degradation efficiency [6].
The optimal nitrogen source was found to be peptone, a peak growth at pH 7.0 and 37°C. Growth was also seen at a hydrocarbon concentration of 0.1%, diesel or engine oil, with the isolate growing, but growth declined at 0.2% probably due to toxic effects of higher hydrocarbon levels [7]. The dependencies on other energy sources were confirmed by control experiments without hydrocarbons which showed no growth of this isolate [8].

These results highlight the great promise of these indigenous bacteria in improving remediation of oil contaminated environments at low cost utilizing sustainable, eco-friendly measures and may even pave way for ecological restoration in oil contaminated environments.

CHAPTER 6

CONCLUSION AND FUTURE SCOPE

In order to take advantage of their potential for bioremediation, restoring polluting environments, we set out into a stimulating scientific venture to isolate and characterize bacteria derived from oil polluted soil. We were able to use these efforts to cultivate bacterial strains that used hydrocarbons (the primary components of oil) as sole sources of both nourishment and energy. These were very cloudy cultures (optical density ~ 5.678) for this remarkable group of microbes that can break down oil [1].

The time-honored Gram staining technique was used to determine that our standout bacteria were Gram negative and this trait is often present in highly effective oil degrading genera such as *Pseudomonas* [1]. This identification was further validated by additional biochemical assays in which catalase activity was flagged as positive, with growth on MacConkey agar and in mannitol fermentation; however, methyl red, Voges-Proskauer and urease tests were all negative [3]. We further extracted their DNA by the reliable phenol chloroform method and gel electrophoresis showed clear sharp bands confirming their high quality DNA ready for future genetic exploration [4].

During a seven day study, one strain, Isolate A, outperformed the second, Isolate B, producing much cloudier cultures and degrading more oil more efficiently [1]. We therefore optimized its environment, meticulously, to establish its environment and learn that it reproduced optimally at pH 7.0 and a temperature of 37°C using peptone as the nitrogen source. However, when oil levels were at 0.1%, the bacteria excelled, but were impaired at higher concentrations (0.2%) when the excess hydrocarbons might be toxic [5].

Future Directions

This work sets a strong basis for using these native bacteria in oil spill clean-up. Forward moving, we are investigating their genetic profile to corroborate their standing to Pseudomonas and to understand the means of oil degradation in them [6]. Additionally, we will further increase our understanding of the enzymes they employ to metabolize hydrocarbons. The combination of these bacteria would have increased impact, so [7] required field trials in real world contaminated sites.

Moreover, there is promising potential by these microbes for restoring oildamaged ecosystems, in a solution of rigorous science and deep environmental stewardship designed on a razor edge of sound science and sensical economics.

BIBLIOGRAPHY

[1] A. Bento, F. C. Camargo, B. Okeke, and F. M. Frankenberger, "Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation," Bioresour. Technol., vol. 96, 9, pp. 1049–1055, Jun. 2005, doi: 10.1016/j.biortech.2004.09.013.

[2] A. Hamzah, A. Tavakoli, and A. Rabu, "Detection of toluene degradation in bacteria isolated from oil contaminated soils," Sains Malays., vol. 40, pp. 1231–1235, 2011. (DOI unavailable)

[3] B. Mishra, R. K. Jyot, R. C. Kuhad, and B. Lal, "Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil," Appl. Environ. Microbiol., vol. 67, 4, pp. 1675–1681, Apr. 2001, doi: 10.1128/AEM.67.4.1675-1681.2001.

[4] C. Holliger et al., "Contaminated environments in the subsurface and bioremediation: organic contaminants," FEMS Microbiol. Rev., vol. 20, 3–4, pp. 517–523, Jul. 1997, doi: 10.1111/j.1574-6976.1997.00334.x.

[5] C. S. Ezeonu, R. Tagbo, E. N. Anike, O. A. Oje, and I. N. Onwurah, "Biotechnological tools for environmental sustainability: prospects and challenges for environments in Nigeria—a standard review," Biotechnol. Res. Int., 2012, doi: 10.1155/2012/450802.

[6] D. Bhattacharya et al., "Detection of catabolic genes in bacterial strains degrading petroleum compounds," Biotechnol. Lett., vol. 25, 22, pp. 1907–1912, Nov. 2003, doi: 10.1023/A:1026281416647.

[7] D. Margesin, C. Zimmerbauer, and F. Schinner, "Monitoring of bioremediation by soil biological activities," Chemosphere, vol. 40, 4, pp. 339–346, Feb. 2000, doi: 10.1016/S0045-6535(99)00218-0.

[8] E. E. Erdogan, F. Sahin, and A. Karaca, "Determination of petroleum-degrading bacteria isolated from crude oil-contaminated soil in Turkey," Afr. J. Biotechnol., vol. 11, pp. 4853–4859, 2012, doi: 10.5897/AJB11.3783.

[9] E. K. Yakimov, K. N. Timmis, and P. N. Golyshin, "Obligate oil-degrading marine bacteria," Curr. Opin. Biotechnol., vol. 18, 3, pp. 257–266, Jun. 2007, doi: 10.1016/j.copbio.2007.04.006.

[10] E. Rosenberg, "The role of microorganisms in petroleum pollution," Microb. Ecol., vol. 23, 2, pp. 97–105, Mar. 1992, doi: 10.1007/BF00172636.

[11] E. W. Liebeg and T. J. Cutright, "The investigation of enhanced bioremediation through the addition of macro and micro nutrients in a PAH contaminated soil," Int. Biodeterior. Biodegrad., vol. 44, 1, pp. 55–64, Jul. 1999, doi: 10.1016/S0964-8305(99)00054-7.

[12] G. Bognolo, "Biosurfactants as emulsifying agents for hydrocarbons," Colloids Surf. A Physicochem. Eng. Asp., vol. 152, 1–2, pp. 41–52, Jun. 1999, doi: 10.1016/S0927-7757(98)00629-2.

[13] H. Bredholt, K. Josefsen, A. Vatland, P. Bruheim, and K. Eimhjellen, “Emulsification of crude oil by an alkane-oxidizing Rhodococcus species isolated from seawater,” Can. J. Microbiol., vol. 44, 4, pp. 330–340, Apr. 1998, doi: 10.1139/w98-020.

[14] H. Harayama, T. Kasai, and A. Hara, “Microbial communities in oil-contaminated seawater,” Curr. Opin. Biotechnol., vol. 15, 3, pp. 205–214, Jun. 2004, doi: 10.1016/j.copbio.2004.04.002.

[15] I. Bossert and R. Bartha, “The fate of petroleum in soil ecosystems,” Environ. Pollut., 1984. (DOI unavailable)

[16] I. M. Banat, R. S. Makkar, and S. S. Cameotra, “Potential commercial applications of microbial surfactants,” Appl. Microbiol. Biotechnol., vol. 53, 5, pp. 495–508, May 2000, doi: 10.1007/s002530000410.

[17] J. D. Desai and I. M. Banat, “Microbial production of surfactants and their commercial potential,” Microbiol. Mol. Biol. Rev., vol. 61, 1, pp. 47–64, Mar. 1997, doi: 10.1128/mmbr.61.1.47-64.1997.

[18] J. E. Clarridge, “Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases,” Clin. Microbiol. Rev., vol. 17, 4, pp. 840–862, Oct. 2004, doi: 10.1128/CMR.17.4.840-862.2004.

[19] J. G. Leahy and R. R. Colwell, “Microbial degradation of hydrocarbons in the environment,” Microbiol. Rev., vol. 54, 3, pp. 305–315, Sep. 1990, doi: 10.1128/mr.54.3.305-315.1990.

[20] J. Holt and S. Williams, Bergey’s Manual of Systematic Bacteriology, vol. 4, Baltimore, MD, USA: Lippincott Williams & Wilkins, 1989. (DOI unavailable)

[21] J. P. Van Hamme, A. Singh, and O. P. Ward, “Recent advances in petroleum microbiology,” Microbiol. Mol. Biol. Rev., vol. 67, 4, pp. 503–549, Dec. 2003, doi: 10.1128/MMBR.67.4.503-549.2003.

[22] M. Deleu, M. Paquot, P. Jacques, P. Thonart, and Y. F. Dufrêne, “Nanometer scale organization of mixed surfactin/phosphatidylcholine monolayers,” Biophys. J., vol. 77, 4, pp. 2304–2310, Oct. 1999, doi: 10.1016/S0006-3495(99)77067-2.

[23] M. Sathishkumar et al., “Biodegradation of crude oil by bacterial strains isolated from oil-contaminated soil and water,” Afr. J. Biotechnol., vol. 7, 24, 2008, doi: 10.5897/AJB2008.000-5143.

[24] M. Watanabe, Y. Kodama, S. Kaku, and A. Harayama, “Molecular characterization of bacterial populations in petroleum-contaminated soil,” Environ. Microbiol., vol. 2, 6, pp. 603–616, Dec. 2000, doi: 10.1046/j.1462-2920.2000.00143x.

[25] M.-E. Ekram, I. Sarker, M. S. Rahi, M. A. Rahman, A. K. Saha, and M. A. Reza, “Efficacy of soil-borne Enterobacter sp. for carbofuran degradation: HPLC quantitation of degradation rate,” J. Basic Microbiol., vol. 60, 5, pp. 390–399, May 2020, doi: 10.1002/jobm.201900626.

[26] N. Das and P. Chandran, “Microbial degradation of petroleum hydrocarbon contaminants: An overview,” Biotechnol. Res. Int., 2011, doi: 10.4061/2011/941810.

[27] P. J. Alvarez and T. M. Vogel, “Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries,” Appl. Environ. Microbiol., vol. 57, 10, pp. 2981–2985, Oct. 1991, doi: 10.1128/AEM.57.10.2981-2985.1991.

[28] R. C. Prince, “Bioremediation of marine oil spills,” Trends Biotechnol., vol. 15, 5, pp. 158–160, May 1997, doi: 10.1016/S0167-7799(97)01033-9.

[29] R. C. Prince, “Oil spill dispersants: Boon or bane?” Environ. Sci. Technol., vol. 49, 11, pp. 6376–6384, Jun. 2015, doi: 10.1021/acs.est.5b00961.

[30] R. K. Hommel, “Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms,” in Physiology of Biodegradative Microorganisms, Springer, 1997, pp. 107–119, doi: 10.1007/978-94-011-3452-1_5.

[31] R. Marchal, S. Penet, F. Solano-Serena, and J. P. Vandecasteele, “Gasoline and diesel oil biodegradation,” Oil Gas Sci. Technol., vol. 58, 4, pp. 441–448, 2003, doi: 10.2516/ogst:2003028.

[32] R. Vidali, “Bioremediation: An overview,” Pure Appl. Chem., vol. 73, 7, pp. 1163–1172, Jul. 2001, doi: 10.1351/pac200173071163.

[33] S. Geetha, S. J. Joshi, and S. Kathrotiya, “Isolation and characterization of hydrocarbon degrading bacterial isolate from oil contaminated sites,” APCBEE Procedia, vol. 5, pp. 237–241, 2013, doi: 10.1016/j.apcbee.2013.05.041.

[34] S. J. Varjani, “Microbial degradation of petroleum hydrocarbons,” Bioresour. Technol., vol. 223, pp. 277–286, Jan. 2017, doi: 10.1016/j.biortech.2016.10.037.

[35] S. R. Peixoto, G. C. Vermelho, and V. M. Rosado, “Bacterial diversity in oil-contaminated soils from mangroves: A case study in Brazil,” Int. Biodeterior. Biodegrad., vol. 89, pp. 73–82, Apr. 2014, doi: 10.1016/j.ibiod.2013.12.012.

[36] S. V. Mohan et al., “Remediation of petroleum-contaminated soils: A review,” Environ. Rev., vol. 14, 4, pp. 261–276, Dec. 2006, doi: 10.1139/a06-006.

[37] T. R. Neu, “Significance of bacterial surface-active compounds in interaction of bacteria with interfaces,” Microbiol. Rev., vol. 60, pp. 151–166, Mar. 1996, doi: 10.1128/mr.60.1.151-166.1996.

[38] W. Wang et al., “Application of metagenomics in bioremediation of petroleum-contaminated environments,” Microb. Biotechnol., vol. 9, 5, pp. 522–534, Sep. 2016, doi: 10.1111/1751-7915.12376.

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