World Journal of Pharmaceutical Science and Research
www.wjpsronline.com
Review Article
ISSN: 2583-6579
SJIF Impact Factor: 3.454
Year - 2024
Volume: 3; Issue: 3
Page: 156-170

STUDY ON ISOLATION AND CHARACTERIZATION OF BACTERIA FROM SOIL SAMPLES OF FUEL STATION

V. Asha Ranjani*, G. Tulja Rani, B. Jithendar, K. Serafina and K. Jeevana Sri

Malla Reddy Pharmacy College, Maisammguda, Dhulapally, Medchal District, Hyderabad.

Article Received: 14 May 2024 | | ***Article Revised: 06 June 2024*** | | ***Article Accepted: 28 June 2024***

Corresponding Author: V. Asha Ranjani
Malla Reddy Pharmacy College, Maisammguda, Dhulapally, Medchal District, Hyderabad.
DOI: https://doi.org/10.5281/zenodo.12630517

ABSTRACT

Petroleum products pose a significant threat to marine life and surroundings, necessitating natural decontamination methods. A study investigated the ability of sediment microorganisms to degrade crude oil, a key hydrocarbon, using a 12-month period of sediment samples. Out of 113 degrading bacteria, three were identified as efficient, with the highest degradation rate of 55% recorded by Pseudomonas aeruginosa I5 isolate.[1] This systematic review identifies and analyzes hydrocarbon-degrading bacteria in Colombia, focusing on Pseudomonas and Bacillus. The largest number of publications in 2018 was in Colombia, with Pseudomonas and Bacillus being the most frequently identified bacterial genera. Studies show that hydrocarbon degradation is more efficient when bacterial consortia are used. This information will be useful for future studies in this field. The study characterized 26 hydrocarbon-degrading endophytic bacteria from Lotus corniculatus L. and Oenothera biennis L. collected in petroleum hydrocarbon-polluted sites. The isolates were classified into Proteobacteria and Actinobacteria, with most belonging to the genera Rhizobium, Pseudomonas, Stenotrophomonas, and Rhodococcus. Over 90% of the isolates could grow on diesel oil, and 20% could use n-hexadecane as a sole carbon and energy source. The study found that these endophytic bacteria have the potential to improve phytoremediation of petroleum hydrocarbon-polluted soils.[2][3] The increasing population and modernization have led to increased environmental pollution from petroleum hydrocarbons. Bacteria degrading these compounds are often used for bioremediation. However, microbial remediation technology faces challenges due to environmental factors. This paper reviews recent literature on bacteria as biodegraders, discusses implementation barriers, and offers suggestions for future developments. The study investigated the impact of light crude oil on bacterial communities during an oil spill in the North Sea and mesocosms. Results showed no oil-induced changes in the bacterial community, but a decrease in the dominant SAR11 phylotype and an increase in Pseudoalteromonas spp. in the oiled mesocosms. 216 strains were isolated from hydrocarbon enrichment cultures, revealing the susceptibility of SAR11 to oil pollution. Crude oil-derived hydrocarbons are the largest environmental pollutants globally, causing a growing interest in their removal. Bioremediation is the most economically justified method for clean-up. This review aims to explain the formation of crude oil, its abundance, and the bacterial ability to use them as energy. It also discusses the impact of nutrient limitations on biomass growth, the formation of aerobic and anaerobic conditions, and the role of surfactants in biodegradation. The review serves as a starting point for further debate on bioremediation strategies.[4][5][6]

KEYWORDS: Crude oil, hydrocarbon, Pseudomonas aeruginosa, Bacillus. These bacteria have phytoremediation, modernization, environmental, pollution, petroleum hydrocarbons.

INTRODUCTION

India's coastline spans 5500 km, with diverse ecosystems like mangroves and coral reefs. However, oil spills, ship accidents, and offshore oil production damage these habitats, causing irreversible damage. To preserve these habitats, research is needed for bioremediation of oil spills, identifying efficient degrading microbes, and accelerating biodegradation rates.

Petroleum hydrocarbons, derived from organic matter, are used for industrial energy production worldwide. The petroleum industry in Colombia has grown significantly, with reserves of 1.5 billion barrels, accounting for 26% of the country's exports. However, petroleum sources contribute to pollution, land use changes, and water utilization issues. Colombia has experienced numerous terrorist attacks resulting in hydrocarbon spills.

Biodegrading bacteria, such as Escherichia coli, Alcaligenes sp., and Thiobacter subterraneus, can reduce pollutants and contribute to ecosystem remediation. Monooxygenases, oxygen-dependent enzymes, allow bacteria to use hydrocarbons as substrates, allowing them to survive in hydrocarbon-polluted environments.

The first study on bacterial degradation in Colombia was published in 1996, and since then, several studies have been published on hydrocarbon-degrading bacteria (HDB) in Colombia. This review aims to identify all studies on HDB conducted in Colombia and provide an analysis of their hydrocarbon degradation capability, aiming to better understand bioremediation challenges and plan future studies.

Plant-endophytic bacteria associations have gained attention in recent decades due to their beneficial interactions and ecological balance. Endophytes, which reside in plant tissues without any noticeable harmful effects, have been shown to increase plant growth and development, facilitate the availability of nutrients, produce phytohormones, and protect plants against pathogens. They also increase plant tolerance to contaminants by their degradation and detoxification, and reduce plant stress by producing 1-aminocyclopropane-1-carboxylic-acid-deamylase(ACC). Phytoremediation is a green technology that uses plants and their microorganisms to clean up polluted soils and groundwaters. Non-regulated disposal of oil sludge, oil extraction, refining, and leakage during storage and transport have led to significant soil contamination and accumulation of aliphatic and aromatic hydrocarbons in the environment. To successfully phytoremediate, plants that tolerate high levels of contaminants, in combination with beneficial plant-associated endophytic bacteria capable of degrading pollutants, are essential.

Plant species like Lotus corniculatus and Oenothera biennis, which grow abundantly in soils heavily polluted with petroleum hydrocarbons, are useful tools for phytoremediation purposes. Their high tolerance to hydrocarbon contamination may indicate that the tissues of these plants are colonized by hydrocarbon-degrading endophytes, offering potential for detailed studies and applications.[4][5]

Petroleum oil is a crucial resource for countries, but its use leads to environmental deterioration. Large spills and discharges of petroleum hydrocarbons occur due to various accidents, such as blowouts, leaks, and equipment overhauls. Contamination is a constant threat, especially in extreme environments like polar regions, deep sea areas, deserts, and wetlands. Petroleum hydrocarbon-degrading bacteria have evolved to treat oil pollution, and they have been used to degrade waste products from various industries. This low-cost and eco-friendly technology has become a

promising method for addressing petroleum hydrocarbon pollution. The development of microbial remediation technology has attracted attention for future development of bacterial remediation of petroleum hydrocarbons.

Crude oil pollution has caused significant damage to marine habitats and socio-economic implications. The Deepwater Horizon oil spill in the Gulf of Mexico was the largest offshore spill in US history. Crude-oil components are toxic and stressful to marine organisms, including microorganisms. Many microbes have developed pathways for hydrocarbon metabolism, some thriving only in the presence of crude-oil components. Obligatory hydrocarbonoclastic bacteria, such as Alcanivorax, Cycloclasticus, Thalassolituus, and Oleibacter, typically bloom and become dominant members of prevailing microbial communities after oil contamination. However, oil floating on the surface of marine waters is broken down into small droplets, dispersed, and integrated into the water column. The study hypothesizes that below the oil-water interface, specialized hydrocarbonoclastic bacteria are replaced by generalist marine bacteria better adapted to grow on low levels or more soluble components of crude oil. Comparative studies of bacterial community changes in the water column during a small experimental spill of light crude oil in the North Sea and oil-enriched 1-m3 seawater mesocosms have been conducted.[2]

Petroleum hydrocarbons are a major environmental pollutant, with public concerns about contamination from crude oil-related products. Hydrocarbons are toxic substances that can be used as substrates by living organisms, making them a potential source of pollution. Microorganisms have the ability to decompose hydrocarbons, making them useful in bioremediation processes. However, there is still a need for further research and misconceptions about the relationship between microorganisms and hydrocarbons. Understanding the history of petroleum and the interactions between microorganisms and hydrocarbons is crucial for developing effective decontamination methods. This mini-review aims to provide a background on the involvement of microorganisms in crude oil formation and biodegradation, identify factors limiting bacteria growth in hydrocarbon-rich environments, and evaluate strategies for improving hydrocarbon decontamination processes.

Diesel, a complex compound of alkanes and aromatic hydrocarbons, is a major global issue causing soil and seawater pollution. Common causes include oil spills, accidents, offshore oil platforms, and refineries. The World Health Organization sets 50 μg/L as the maximum level of hydrocarbon in the marine environment. Diesel accumulates in marine biota, which are toxic, carcinogenic, and mutagenic compounds to living organisms. Treatment of diesel-contaminated environments can be physical, chemical, or biological. Physical treatment requires extensive equipment and can be expensive. Chemical treatment is effective but requires further treatment for chemical residues or sludge. Biological treatment is considered promising and environmentally friendly. Bacteria can degrade diesel by using it as a carbon source, with over 30 species capable of completely degrading hydrocarbon compounds. Understanding the process of diesel degradation by bacteria isolates can lead to enhanced application in real contaminated environments. This paper discusses the limitations, challenges, and prospects of using bacteria isolates to treat diesel and oil contamination, focusing on soil and marine environments.[3]

Lignin, an aromatic heteropolymer, is a significant challenge in biomass utilization. This paper investigates lignin-degradation bacteria from rotten wood in Qinling Mountain. Petroleum, or crude oil, is a naturally occurring liquid composed of hydrocarbons, and its products are widely used in various industries. Petroleum pollution occurs during production, storage, processing, refining, and transportation, leading to environmental issues such as leakage, accidents, and oil spills. Human activities, such as urban run-off and industrial effluents, contribute to petroleum pollution. The

chemical components of petroleum products are toxic and carcinogenic to living organisms, affecting human health. Remediating soil from petroleum contaminants is crucial to prevent harmful effects on living beings. Mechanical and chemical methods are used for soil remediation, but these methods can lead to incomplete degradation and complex structures. Biodegradation by indigenous microorganisms is a reliable method for removing petroleum hydrocarbons, and bioremediation is cost-effective. Many bacterial species have been used in the degradation of petroleum hydrocarbons, and this study focuses on lignin-degradation bacteria isolated from contaminated soil.

Petroleum is a complex substance composed of aliphatic and aromatic hydrocarbons, with lower concentrations of asphaltenes, resins, and metals. Its composition varies according to geographical localization and physical, chemical, and biological conditions in the environment. Petroleum spills into the environment result from high volumes of petroleum used for energy and chemicals production, accidents during operating processes, transportation, refining, storage, and consumption. The persistence of petroleum hydrocarbons in the environment can compromise water resources, soil quality, and food availability.

Environment-friendly approaches have been proposed to remediate petroleum-contaminated environments, such as bioremediation and phytoremediation. Bioremediation involves the metabolization of pollutants through their complete removal, immobilization, or transformation into less toxic products. In phytoremediation, plants and rhizospheric microorganisms interact to degrade, remove, or stabilize contaminants in soil and water. Rhizodegradation occurs when microorganisms associate with roots, leading to the biodegradation.

Petroleum hydrocarbons are the most common environmental pollutants and pose a great threat to terrestrial and marine ecosystems. Bioremediation is a strategy that utilizes biological activities for quick elimination of environmental pollutants. Growth stimulation of indigenous microorganisms, biostimulation, and inoculation of foreign oil-degrading bacteria is a promising means of accelerating detoxifying and degrading activities at polluted sites.

The study aims to study some bacterial strains isolated from oil polluted sites in the Persian Gulf to evaluate their capability to produce biosurfactants and biodegradation of crude oil.

Crude oil, a mixture of hydrocarbons and organic compounds, poses significant environmental problems when spills occur. Bioremediation is an efficient, economic, and versatile alternative to physicochemical treatment of oil-contaminants. Extensive research has been conducted on oil bioremediation and crude oil degradation using pure culture or mixed bacterial consortia isolated from oil-contaminated soils. However, degradation efficiency is limited by substrates, geological, climatological, and ecological factors. A combined bacterial consortium showed better results due to their synergetic effects.

Characterization of bacterial populations living in oil-contaminated soils and evaluation of their degradation capacities could potentially guide for improving remediation of such environments. Culture-dependent and culture-independent approaches can provide information about viable microorganisms in such environments. A consortium of seven bacteria was studied for its potential bioremediation potential.

Biodegradation by natural populations of microorganisms is the most basic and reliable mechanism for eliminating thousands of xenobiotic pollutants, including crude oil, from the environment. The effects of environmental conditions on microbial degradation of hydrocarbons and hydrocarbon contamination on microbial communities are areas of great

interest. Bioremediation is a strategy to utilize biological activities to the greatest extent possible for the rapid elimination of environmental pollutants.

Oil contamination is a global issue with long-lasting effects and difficult remediation. Various methods of oil degradation have been developed, including land farming, surface heating, and microbial oil degradation. Microbial oil degradation is promising for sustainability and environmental friendliness. Bacteria from different habitats, such as soil and ocean, are screened for their oil degrading properties. This study isolated Urobacterium intermedium strain 2745-2 from the Changing oilfield, a rare strain associated with both human pathogens and the environment. Three draft genome sequences within O. intermedium have been published, but no environmental strain has been sequenced before this study. Comparative genomic analysis is needed between human and environmental isolates of O. intermedium to better understand its adaptation mechanisms. This study aims to investigate the oil-degrading genes of strain 2745-2 and identify distinctions and similarities among the genomes and genes indicating adaptation to specific environments.

MATERIALS AND METHODOLOGY

The study involved collecting surface sediment samples from an oil-contaminated site in Ennore creek, Colombia. The THB population was enumerated using the pour plate method, while the HDB population was enumerated using spread plate technique. The bacteria were isolated and stored at 4°C for further identification. Preliminary screening of crude oil degraders was conducted on hydrocarbon degraders stored on glycerol stock. Isolates that grew on BHA plates but failed to grow on BHA plates were confirmed as non-degraders. The zone of clearance around the degraders was observed. Isolates with greater zone of clearance were subjected to estimation of oil degrading efficiency. The growth was analyzed in terms of biomass and degradation rates by gravimetric method. The estimated crude oil degradation was achieved through gravimetric analysis. Molecular characterization was performed using BigDye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase. The sequences obtained were compared with sequences from the Basic Local Alignment Search Tool (BLAST) search of National Centre for Biotechnology Information (NCBI) data bases. Strains showing more than 97% 16s RNA gene sequence similarity were of the same species. Phylogenetic analysis was performed using MEGA5.1 software, Neighbour Joining and Maximum Likelihood method. The 16s RNA sequence of the efficient degrader was submitted to the Gene bank, NCBI, USA to obtain the accession number.

The average number of heterotrophic bacteria during the study period was in the order of 22.32× 105 CFUg-1, with the highest count recorded in the post monsoon, August 2009, and the lowest in the beginning of summer, March, and April 2010. Similar studies reported THB counts ranging from 9.0×103 to 2.6×106 CFUg-1 in hydrocarbon contaminated surface sediment samples of Gokarna, River state.

The study involved collecting L. corniculatus and O. biennis from a polluted site in Czechowice-Dziedzice, Silesia, Southern Poland. The soil had a high petroleum hydrocarbon content, and the plants were collected with soil adhering to the roots. The endophytic bacteria were isolated using two methods: maceration and liquid enrichment cultures. The macerate was plated on solid M9 mineral medium with crude oil, while the enrichment culture was spread on a solid M9 mineral medium with crude oil. Morphologically distinct bacterial colonies were selected, purified, and stored at -20°C. DNA was extracted from each isolate, and the 16S rRNA gene was PCR amplification using primers 27F and 1392R. Amplified ribosomal DNA restriction analysis (ARDRA) was performed on the PCR products, and representative patterns were selected for sequencing. The 16S ribosomal DNA sequences were compared with

nucleotide sequences in the National Centre for Biotechnology Information (NCBI) database using BLAST. A phylogenetic tree was constructed using the neighbour-joining method and bootstrap analysis was performed. The sequences of 16 selected isolates were submitted to the NCBI GenBank database under accession numbers from KU726257 to KU726272.[9][10]

TYPES OF BACTERIA

Bacteria are small, single-celled organisms found in various ecosystems, including soil, water, and the human body. They have a nucleoid and a cell wall, with their DNA found in a region called the nucleoid. Bacteria have diverse nutritional strategies, including autotrophic and heterotrophic, and can be aerobic, anaerobic, or facultative anaerobes. They reproduce asexually through binary fission and play essential roles in nutrient cycling. Some bacteria form symbiotic relationships with other organisms, such as human gut bacteria that aid digestion and protect against pathogens. Pathogenic bacteria can cause diseases in humans, animals, and plants, producing toxins and disrupting bodily functions. Understanding bacteria is crucial for scientific research, medicine, agriculture, and environmental management.

Bacteria are incredibly diverse, and they can be classified in various ways. Here are some common types based on different criteria:

1. Shape

- Cocci: Spherical-shaped bacteria. Examples include Staphylococcus and Streptococcus.
- Bacilli: Rod-shaped bacteria. Examples include Escherichia coli (E. coli) and Bacillus anthracis.
- Spirilla: Spiral-shaped bacteria. Examples include Helicobacter pylori and Treponema pallidum.
- Spirochetes: Spiral-shaped bacteria with flexible bodies. Examples include Borrelia burgdorferi, which causes Lyme disease.

2. Cell Wall Composition

- **Gram-positive:** Bacteria with a thick layer of peptidoglycan in their cell walls, which retain the crystal violet stain in the Gram staining process. Examples include Staphylococcus aureus and Streptococcus pneumoniae.
- **Gram-negative:** Bacteria with a thin layer of peptidoglycan in their cell walls, which do not retain the crystal violet stain but are counterstained with safranin in the Gram staining process. Examples include Escherichia coli and Pseudomonas aeruginosa.

3. Metabolism

- Aerobic: Bacteria that require oxygen for their metabolism. Examples include Mycobacterium tuberculosis and Bacillus subtilis.
- Anaerobic: Bacteria that can survive and grow in the absence of oxygen. Examples include Clostridium botulinum and Bacteroides fragilis.

4. Pathogenicity

- Pathogenic: Bacteria capable of causing disease in humans, animals, or plants. Examples include Salmonella enterica and Vibrio cholerae.
- Non-pathogenic: Bacteria that do not cause disease. Examples include Lactobacillus acidophilus and Escherichia coli (some strains).

5. Nutritional Requirements

- Autotrophic: Bacteria that can synthesize their own food from inorganic sources. Examples include Cyanobacteria.
- Heterotrophic: Bacteria that require organic compounds as a carbon source. Examples include most bacteria, including those found in the human gut.

6. Environment

- Thermophiles: Bacteria that thrive in high-temperature environments. Examples include Thermus aquaticus.
- Halophiles: Bacteria that thrive in high-salt environments. Examples include Halobacterium salinarum.

7. Specialized Bacteria

- Methanogens: Bacteria that produce methane as a metabolic byproduct. Examples include Methanobrevibacter smithii.
- Nitrogen-fixing bacteria: Bacteria capable of converting atmospheric nitrogen into ammonia. Examples include Rhizobium and Azotobacter.

These are just a few ways to categorize bacteria, and many bacteria may fit into multiple categories simultaneously.[13]

Isolation

Sampling Strategy and Collection of Hydrocarbon-Contaminated Samples

Sampling strategy and collection of hydrocarbon-contaminated samples are critical steps in assessing the extent of contamination, understanding the composition of hydrocarbons, and designing effective remediation strategies. Here is a guide on how to develop a sampling strategy and collect samples:

Isolation Techniques for Hydrocarbon-Degrading Bacteria

Isolating hydrocarbon-degrading bacteria from environmental samples involves selective enrichment and cultivation techniques to isolate bacterial strains capable of utilizing hydrocarbons as carbon sources. Several methods, including serial dilution, spread plate method, and enrichment cultures, are commonly employed in microbial isolation. We employed serial dilution techniques to obtain pure cultures. Where we took the contaminated soil sample – 1gm and added it to the distilled water – 100ml in a conical flask; from this solution, we measured 1ml and added it to another 10ml of distilled water in a test tube, we continued this process for 7 test tubes to isolate pure strains of bacteria.[14]

Culture

Identification and Selection of Hydrocarbon-Degrading Bacterial Colonies

Once bacterial colonies have been isolated from environmental samples using serial dilution technique, the next step is identifying and selecting colonies with hydrocarbon-degrading capabilities. This process involves several steps and methodologies to differentiate and screen bacterial isolates based on their ability to utilize hydrocarbons as carbon sources. Below are the critical steps involved in the identification and selection of hydrocarbon-degrading bacterial colonies:

Media Preparation and Optimization for Bacterial Growth

Adequate media preparation and optimization are crucial for supporting hydrocarbon-degrading bacteria growth and metabolic activity in laboratory settings. Properly formulated media provide essential nutrients and environmental conditions required for bacterial growth and hydrocarbon degradation.

Incubation Conditions and Duration

Incubation conditions and duration are critical in supporting hydrocarbon-degrading bacteria's growth and metabolic activity in laboratory cultures. Optimal incubation conditions ensure the efficient utilization of growth media and the successful propagation of bacterial populations. After it was solidified, we kept these petri plates in the incubated at 30℃ for 24 hours.

Sub-culturing Techniques to Maintain Bacterial Cultures

Sub-culturing, passaging, or serial transfer is critical to maintaining bacterial cultures in laboratory settings. Regular sub-culturing prevents culture senescence, maintains culture purity, and ensures the long-term viability of bacterial isolates. Here are the critical sub-culturing techniques commonly employed to maintain bacterial cultures:

Streaking

Using a sterile inoculation loop, we streaked an aliquot from each dilution onto fresh agar slants, selected well-isolated colonies, and streaked them onto new slants to obtain pure cultures, and incubated these slants at 30℃ for 24 hours.

Staining

In microbiology, staining techniques are essential for visualizing and identifying bacterial cells based on morphological and biochemical characteristics. Gram staining and acid-fast staining are two commonly used staining methods for bacterial identification.

We employed the Gram staining method.

Gram Staining

In 1884, Christian Gram developed a differential staining method that divides the bacterial kingdom into two groups - Gram positive and Gram-negative. This method is based on the differences in the composition of the bacterial cell wall. Gram-positive cells have a thick and more cross-linked peptidoglycan layer, while Gram-negative cells have a much thinner layer surrounded by an outer layer of lipopolysaccharide.

Principle: Gram staining is a differential staining technique based on differences in the cell wall composition of bacteria. It divides bacteria into two major groups: Gram-positive and Gram-negative.

Interpretation

Gram-positive bacteria retain the crystal violet-iodine complex and appear purple or blue under the microscope.

Gram-negative bacteria lose the crystal violet-iodine complex during decolorization and take up the safranin counterstain, appearing pink or red.

Applications

Staining results provide valuable information for the preliminary identification and classification of bacteria in microbiology laboratories.[13][14]

Biochemical Tests

Selection of Biochemical Tests for Bacterial Characterization

Biochemical tests are fundamental for characterizing and identifying bacterial isolates based on their metabolic properties and enzymatic activities. Selecting the appropriate biochemical tests is essential for accurate bacterial identification and differentiation. Here is a selection of commonly used biochemical tests along with their principles and applications:

1. Catalase Test

Principle: Catalase is an enzyme that speeds up the breakdown of hydrogen peroxide (H2O2) into water (H2O) and oxygen (O2). When hydrogen peroxide is added to a bacterial colony, catalase activity can be identified by releasing oxygen gas bubbles.

Application: The catalase test differentiates bacteria based on their ability to produce catalase. Catalase-positive bacteria produce bubbles of oxygen gas, whereas catalase-negative bacteria do not.

2. Indole Test

The Indole Production Test determines whether certain bacterial groups can produce the enzyme tryptophanase, which converts the amino acid tryptophan into indole through enzymatic oxidation.

Principle: The indole test detects bacteria's ability to produce indole, a byproduct of tryptophan metabolism. The enzyme tryptophanase catalyses the conversion of tryptophan into indole, pyruvic acid, and ammonia. Indole is detected by adding Kovac's reagent to a bacterial culture, which forms a red colour upon reaction with indole.

Application: The indole test differentiates bacteria based on their ability to produce indole. Indole-positive bacteria produce red after adding Kovac's reagent, while indole-negative bacteria do not change colour.

To detect the presence of indole in the growth medium, Add the Kovac's reagent. Adding Kovac's reagent results in a cherry-red colour if indole is present.

3. Methyl Red Test Voges-Proskauer Test

The Methyl Red - Voges Proskauer (MR-VP) test is used to determine the fermentation of glucose and the production of products by bacteria. There are two groups of bacteria when it comes to glucose fermentation and product production: mixed acid fermenters and 2,3 butanediol. The mixed fermenters produce large amounts of acids, while the second group produces neutral end products called acetoin (acetyl methyl carbinol). Both tests for these products are performed simultaneously and on the same medium (MR-VP broth), which is why they are commonly referred to as the MR-VP test.

Application: The methyl red test distinguishes between bacteria that perform mixed acid fermentation and produce other fermentation end products. Methyl red-positive bacteria produce a red colour, indicating mixed acid fermentation, while methyl red-negative bacteria produce a yellow colour or remain orange.

The Voges-Proskauer test differentiates between bacteria that produce acetoin as a fermentation product. Voges-Proskauer-positive bacteria produce red after adding alpha-naphthol and KOH, indicating acetoin production, while Voges-Proskauer-negative bacteria do not change colour significantly.

We inoculated the bacteria into the broth and added alpha-naphthol and KOH to it.

Interpretation of Test Results

a. Catalase Test

Positive Result (+): The formation of bubbles (oxygen gas) when hydrogen peroxide is added to the bacterial colony indicates the presence of catalase activity.

Negative Result (-): The absence of bubble formation indicates a lack of catalase activity.

b. Indole Test

Positive Result (+): Formation of a red colour (cherry red or pink) in the supernatant layer after adding Kovac's reagent indicates indole production.

Negative Result (-): The absence of red in the supernatant layer indicates a lack of indole production.

c. Methyl Red Test

Positive Result (+): The development of a stable red colour after the addition of methyl red indicator indicates the production of mixed acids during glucose fermentation.

Negative Result (-): The development of a yellow colour or no colour change indicates the absence of mixed acid fermentation.

d. Voges-Proskauer Test

Positive Result (+): The formation of a red colour after adding alpha-naphthol and potassium hydroxide (KOH) indicates acetoin production.

Negative Result (-): The absence of red or only a slight colour change indicates the absence of acetoin production.[20]

Temperature Viability

Assessment of Bacterial Viability at Different Temperatures

Assessing bacterial viability at different temperatures is crucial for understanding thermal tolerance and growth characteristics. Various methods can determine bacterial viability under different temperature conditions.

Impact of Temperature on Bacterial Growth and Metabolism

Temperature influences bacterial growth and metabolism, shaping microbial ecology, physiology, and biotechnological processes. Understanding the effects of temperature is crucial for various applications, including food safety, clinical microbiology, environmental monitoring, and bioprocessing. Here is an overview of the impact of temperature on bacterial growth and metabolism:

1. Optimal Growth Temperature

Bacteria exhibit optimal growth within a specific temperature range dictated by their metabolic pathways and physiological adaptations.

Psychrophiles thrive at low temperatures (0°C to 20°C), with optimal growth typically below 15°C. Examples include certain species of Pseudomonas and Psychrobacter.

Mesophiles prefer moderate temperatures (20°C to 45°C) commonly encountered in human and environmental habitats. Most pathogenic bacteria, including Escherichia coli and Staphylococcus aureus, are mesophiles, with optimal growth around 37°C. Thermophiles thrive at high temperatures (45°C to 80°C), with optimal growth temperatures typically above 50°C. Examples include species of Thermus and Geobacillus.

2. Impact on Growth Rate

Temperature affects the bacterial growth rate exponentially. Bacterial growth rates accelerate as temperature increases within the permissible range due to higher enzymatic activity and metabolic efficiency.

Conversely, growth rates decline at temperatures outside the optimal range due to enzyme denaturation, membrane destabilization, and metabolic inefficiency.[9] [10]

Antibiotic Test

In future research, antibiotic sensitivity testing will continue to be crucial for guiding effective antibiotic therapy and combating antibiotic resistance, maintaining its pivotal role in clinical practice. The selection of appropriate antibiotics for sensitivity testing will rely on various factors, including infection type, suspected pathogens, and local epidemiological data. In our forthcoming study, to conduct antibiotic susceptibility testing on hydrocarbon-degrading bacteria, utilizing erythromycin, azithromycin, and rifampicin.

The process will commence by pulverizing antibiotic tablets to ensure precise measurement (to 1mg), followed by dilution in 2 ml of distilled water. Subsequently, agar media will be prepared and evenly distributed onto sterile Petri plates using the pour plate method. The supernatant solution containing diluted antibiotics will then be evenly distributed onto the agar media. After solidification, wells will be meticulously made in the agar surface using a borer to accommodate the antibiotics. Each Petri plate will be treated with erythromycin, azithromycin, and rifampicin solutions, ensuring proper placement into pre-made wells. The Petri plates will then be incubated at 30°C for 48 hours to facilitate bacterial growth and enable assessment of antibiotic susceptibility.

This forthcoming study aims to establish rigorous procedures and standardized conditions for evaluating the efficacy of selected antibiotics against hydrocarbon-degrading bacteria. Through systematic testing of multiple antibiotics, our goal is to identify optimal treatment options while contributing to the future understanding of antibiotic resistance mechanisms.

PROCESS OF PETROLEUM HYDROCARBON DEGRADATION

Petroleum hydrocarbon degradation is primarily mediated by microorganisms through various mechanisms, including physical, chemical, and biological processes. Here's a breakdown of these mechanisms:

1. Physical Processes

- **Volatilization:** Some hydrocarbons, particularly lighter ones like gasoline and diesel, can evaporate into the atmosphere.
- **Photooxidation:** Exposure to sunlight can break down hydrocarbons through oxidation reactions initiated by UV radiation.

2. Chemical Processes

- **Oxidation:** Hydrocarbons can react with oxygen in the atmosphere or in water to form partially oxidized compounds such as alcohols, aldehydes, ketones, and organic acids.
- **Hydrolysis:** Certain hydrocarbons can undergo hydrolysis in the presence of water, breaking down into simpler compounds.

3. Biological Processes

- **Biodegradation:** Microorganisms, including bacteria, fungi, and some algae, can utilize hydrocarbons as sources of carbon and energy. This process involves enzymatic reactions where specific enzymes break down hydrocarbons into simpler compounds that can be assimilated by the microorganisms.
- **Aerobic Biodegradation:** In the presence of oxygen, aerobic bacteria break down hydrocarbons into carbon dioxide and water through metabolic pathways such as the tricarboxylic acid (TCA) cycle.
- **Anaerobic Biodegradation:** In the absence of oxygen, anaerobic bacteria can degrade hydrocarbons through fermentation or other anaerobic metabolic pathways, producing methane, carbon dioxide, and other byproducts.
- **Cometabolism:** Some microorganisms can degrade hydrocarbons as byproducts while metabolizing other compounds. For example, certain bacteria may degrade hydrocarbons while utilizing simple sugars as their primary carbon and energy source.
- **Syntrophy:** This is a cooperative metabolic process where different microorganisms work together to degrade complex hydrocarbons. For instance, syntrophic bacteria can break down long-chain hydrocarbons into simpler compounds that are then consumed by other microorganisms in the ecosystem.

4. Physicochemical Factors

- Temperature: Higher temperatures generally accelerate the rate of hydrocarbon degradation by enhancing microbial activity.
- pH: Microbial activity and hydrocarbon degradation rates can vary with changes in pH, with neutral to slightly alkaline conditions often being favourable for degradation.
- Nutrient Availability: Adequate supplies of nitrogen, phosphorus, and other nutrients are essential for microbial growth and activity in hydrocarbon-contaminated environments.

Overall, petroleum hydrocarbon degradation is a complex process involving a combination of physical, chemical, and biological mechanisms that are influenced by environmental factors such as temperature, pH, and nutrient availability.[19]

THE ACTIVITY OF BACTERIA IN DEGRADATION OF HYDROCARBONS

The activity of bacteria in the degradation of hydrocarbons is crucial for the natural attenuation of petroleum-contaminated environments and for the success of bioremediation strategies. Bacteria possess the enzymatic machinery necessary to metabolize hydrocarbons, breaking them down into simpler compounds that can be utilized as carbon and energy sources. Here is an overview of bacterial activities involved in hydrocarbon degradation:

1. Production of Extracellular Enzymes

- Bacteria produce extracellular enzymes such as hydroxylases, dioxygenases, and monooxygenases that facilitate the initial breakdown of hydrocarbons.
- These enzymes catalyse reactions that introduce oxygen atoms into hydrocarbon molecules, making them more reactive and susceptible to further degradation.

2. Hydrocarbon Uptake and Metabolism

- Bacteria take up hydrocarbons from their surroundings using specialized transport systems.
- Inside the bacterial cell, hydrocarbons are metabolized through various pathways depending on the structure and complexity of the hydrocarbon molecule.

- Aerobic bacteria metabolize hydrocarbons under oxygen-rich conditions, utilizing pathways such as the tricarboxylic acid (TCA) cycle and beta-oxidation for the complete mineralization of hydrocarbons into carbon dioxide and water.
- Anaerobic bacteria can also degrade hydrocarbons in the absence of oxygen using alternative electron acceptors such as nitrate, sulfate, or carbon dioxide.

3. Formation of Intermediates

- During hydrocarbon degradation, bacteria produce intermediate compounds such as alcohols, aldehydes, ketones, organic acids, and epoxides.
- These intermediates may undergo further metabolism or serve as substrates for other microbial populations within the ecosystem.

4. Energy Production

- Hydrocarbon degradation provides bacteria with carbon and energy for growth and reproduction.
- The energy released during hydrocarbon metabolism is used to drive cellular processes such as biosynthesis, ATP production, and maintenance of cellular homeostasis.[16]

5. Syntrophy and Consortia Formation

- Some hydrocarbons are recalcitrant and require cooperative metabolic interactions among different microbial species for complete degradation.
- Syntrophic relationships involve the exchange of metabolic intermediates between bacteria, where one species metabolizes a hydrocarbon partially and produces intermediates that are utilized by another species for further degradation.
- Consortia formation, where multiple bacterial species work together in proximity, enhances the efficiency of hydrocarbon degradation by facilitating metabolic cooperation and resource sharing.

6. Regulation of Gene Expression

- Bacteria regulate the expression of genes involved in hydrocarbon degradation in response to environmental cues such as substrate availability, oxygen concentration, pH, temperature, and nutrient availability.
- Regulatory mechanisms ensure that hydrocarbon-degrading enzymes are produced and activated when needed, optimizing bacterial metabolism in changing environmental conditions.

Overall, the activity of bacteria in the degradation of hydrocarbons is a dynamic process influenced by microbial community composition, environmental factors, and the physicochemical properties of the hydrocarbon contaminants. Understanding bacterial metabolism and the factors that influence it is essential for designing effective bioremediation strategies and managing petroleum-contaminated environments.

CONCLUSION

The biochemical tests discussed are crucial for accurate bacterial identification and differentiation. They provide valuable information about bacterial enzyme production and fermentation processes, aiding in the identification of specific bacterial species. Understanding bacterial viability at different temperatures is essential for various applications such as food safety, clinical microbiology, environmental monitoring, and bioprocessing. Assessing bacterial viability

under different temperature conditions provides insights into thermal tolerance and growth characteristics, which are essential for predicting and controlling bacterial growth in various settings. Bacteria exhibit optimal growth within specific temperature ranges, and their metabolic pathways and physiological adaptations are influenced by temperature, impacting food safety, microbial ecology, and biotechnological processes.

The information presented in this document is valuable for researchers, microbiologists, and professionals working in fields where accurate bacterial identification, differentiation, and understanding of bacterial viability and temperature effects are essential. Applying the principles and applications of the biochemical tests and understanding the impact of temperature on bacterial growth and metabolism can help individuals make informed decisions and take appropriate measures to control and utilize bacterial characteristics effectively. Accurate interpretation of test results and the proper assessment of bacterial viability at different temperatures require attention to detail and adherence to standardized procedures. Ongoing research and advancements in microbiology and biotechnology will continue to enhance our understanding of bacterial characteristics and behaviors, leading to improved techniques and applications in the future.

REFERENCES

1. C Holliger , S Gaspard et al, G Glod Contaminated environments in the subsurface and bioremediation: organic contaminants, 1997 July**.**
2. Yuki Kasai, Akihiro Hara et al. Microbial communities in oil-contaminated seawater, 2004 June.
3. Javier I Medina-Bellver et al, Patricia Marín Evidence for in situ crude oil biodegradation after the Prestige oil spill, 2005 June.
4. Zhengzhi Zhang, Lixue Gai et al. Characterization and biotechnological potential of petroleum-degrading bacteria isolated from oil-contaminated soils, November 2010.
5. Nilanjana Das and Preethy Chandran Microbial Degradation of Petroleum Hydrocarbon Contaminants: An Overview, 2010 Sep 13.
6. Mehdi Hassanshahian, Simone Cappello et al. Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea, January 2012.
7. Mehdi Hassanshahian, Mohammad Saleh Zeynalipour et al. Isolation and characterization of crude oil degrading bacteria from the Persian Gulf (Khorramshahr provenance), 15 May 2014.
8. Mamitha Kumar Subathra, Grasian Immanuel et al. Isolation & identification of hydrocarbon degrading bacteria from ennore creek, 2013 Feb 6.
9. Panagiota-Myrsini Chronopoulou, Gbemisola O Sanni et al. Generalist hydrocarbon-degrading bacterial communities in the oil-polluted water column of the North Sea, 2014 Sep 24.
10. Chai, Lj., Jiang, Xw., Zhang, F. *et al.* Isolation and characterization of a crude oil degrading bacteria from formation water: comparative genomic analysis of environmental Ochrobactrum intermedium isolate versus clinical strains, 14 October 2015.
11. Małgorzata Pawlik, Barbara Cania, Sofie Thijs et al. Hydrocarbon degradation potential and plant growth-promoting activity of culturable endophytic bacteria of *Lotus corniculates* and *Oenothera biennis* from a long-term polluted site, 2017 Jul 6.
12. C-X Yang , T Wang , L-N Gao et al. Isolation, identification and characterization of lignin-degrading bacteria from Qinling, China, 2017 Oct 22.

13. Mariusz Cycoń petroleum, Eric D. van Hellebusch et al. Hydrocarbon-Degrading Bacteria for the Remediation of Oil Pollution Under Aerobic Conditions: A Perspective Analysis, 2018 Dec 3.
14. Hermann J. Heipieper, Derek J. McPhe et al. Microbial Degradation of Hydrocarbons—Basic Principles for Bioremediation: A Review, 2020 Feb 14.
15. Author links open overlay panel Muhammad Fauzul Imron, Setyo Budi Kurniawan et al. Future challenges in diesel biodegradation by bacteria isolates, 1 April 2020.
16. Jessica Aparecida Viesser, Maura Harumi Sugai-Guerios, Petroleum-Tolerant Rhizospheric Bacteria: Isolation, Characterization and Bioremediation Potential, 2020 Feb 6.
17. Diana Carolina Rache-Arce, Maryuris Machacado-Salas et al. Hydrocarbon degrading in Colombia systemic review, 2022 Mar 2.
18. Ashwini Ravi, Mounesh Ravuri et al. Characterization of petroleum degrading bacteria and its optimization conditions on effective utilization of petroleum hydrocarbons, December 2022.
19. Anna Lipphardt , Tobias Karmainski Identification and quantification of biosurfactants produced by the marine bacterium Alcanivorax borkumensis by hyphenated techniques, 2023 Oct 11.
20. Tobias Karmainski , Marie R E Dielentheis-Frenken High-quality physiology of *Alcanivorax borkumensis* SK2 producing glycolipids enables efficient stirred-tank bioreactor cultivation, 2023 Nov 23.