

**BIODEGRADATION OF PETROLEUM HYDROCARBONS
USING INDIGENOUS HYDROCARBONS-DEGRADING
BACTERIA FROM HEAVILY CONTAMINATED SOILS**

BY

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DEDICATION

To my beloved parents, this journey has been one of countless challenges and triumphs, and through it, your unwavering support and encouragement have been my guiding lights. Your belief in me has been a source of strength that propelled me forward even when the path seemed insurmountable. I owe an immeasurable debt of gratitude to my father, whose wisdom and resilience have been the cornerstone of my spirit, and my mother, whose love and sacrifice have shaped the essence of my being.

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LIST OF ABBREVIATIONS

TPH: Total petroleum hydrocarbons

TOC: Total organic compounds

PAH: Polycyclic aromatic hydrocarbons

MBS: Mineral basal salts

GC: Gas chromatography

PPM: Parts per million

HRT: Hydraulic retention time

COD: Chemical oxygen demand

GPS: Global positioning system

RPM: Revolution per minute

DO: Dissolved oxygen

OM: Organic matter

ABSTRACT

Full Name: Ali Mohammed Hussain AlMutawa

Thesis Title: BIODEGRADATION OF PETROLEUM HYDROCARBONS USING
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This study aimed to evaluate the extent of the environmental degradation of petroleum hydrocarbons by isolating and identifying bacterial species capable of efficiently reducing total organic carbon (TOC) in contaminated soil. The assessment involved a series of procedures, including sample collection, extraction, and subsequent analysis, within a controlled laboratory setting. Emphasis has been placed on utilizing indigenous bacterial populations to efficiently degrade petroleum hydrocarbons.

Several indigenous bacterial strains were isolated and tested using crude oil as the sole carbon source. The efficiency of the biodegradation process was quantitatively measured after a period of five weeks. The results indicated a highly effective degradation rate, achieving an average efficiency of 38% for the reduction of TOC. This finding underscores the potential of using indigenous bacterial species in environmental remediation strategies, particularly for the efficient breakdown of organic pollutants in contaminated areas.

Keywords: indigenous bacterial degradation; bacterial enzymes; soil contamination; soil bioremediation; biodegradation; hydrocarbon components; bacteria; petroleum waste.

ملخص الرسالة

الاسم الكامل: علي محمد حسين المطوع

عنوان الرسالة: التحلل البيولوجي للهيدروكربونات النفطية باستخدام البكتيريا الأصلية المحللة للهيدروكربونات في التربة شديدة التلوث

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الدراسة هذه تهدف إلى تقييم مدى المعالجة الحيوية من خلال عزل وتحديد أنواع البكتيريا القادرة على تحلل الهيدروكربون الكربون العضوي الكلي في تلوث التربة بكفاءة. يتضمن هذا التقييم مجموعة من الإجراءات المنهجية، بما في ذلك جمع العينات، استخلاصها، وتحليلها لاحقاً في بيئة مختبرية مُسيطر عليها. يتم التركيز على استخدام البكتيريا الأصلية في التربة لغرض التحلل البيولوجي.

تم عزل عدة سلالات بكتيرية محلية، وتم اختبارها على النفط الخام كمصدر كربون وحيد. تُقاس فعالية عملية التحلل البيولوجي كميّاً بعد فترة خمسة أسابيع. تشير النتائج إلى معدل تحلل فعال للغاية، حيث تم تحقيق كفاءة متوسطة بنسبة 38% في تقليل المركبات العضوية. تُبرز هذه النتيجة البارزة إمكانية استخدام أنواع البكتيريا المحلية في استراتيجيات المعالجة البيئية، وبشكل خاص لتفكيك الشوائب العضوية بفعالية في المناطق الملوثة.

كلمات دالة: البكتيريا الأصلية للتحلل، إنزيم البكتيريا، تلوث التربة، الترميم البيولوجي للتربة، التحلل البيولوجي، مكونات الهيدروكربون، البكتيريا، نفايات البترول.

CHAPTER 1 - INTRODUCTION

In recent decades, Saudi Arabia has experienced rapid growth in its industrial sectors. The industrial value significantly increased rapidly from 28.3 billion Saudi riyals in 2010 within six years to 213.4 billion Saudi Riyals in 2016 (Alarenan, 2020). This escalation of industrial sectors has increased the level of waste and pollution in the Kingdom. Currently, the amount of waste from the industrial sectors in Saudi Arabia is approximately 64–72 million tons and the estimated increase by 2031 is 125 million tons annually (Radwan, 2021).

The largest industrial cities in Saudi Arabia are located in the Eastern Region, owing to the presence of oil, natural gas, and petrochemical industries. According to [Ministry of Investment \(MISA\)](#) in the third quarter of 2021, there were 1,877 active factories located in the Eastern Region of Saudi Arabia. These industries have a significant impact and great potential to pollute the surrounding environment, especially the soil, which is a vital and essential source of nutrients for plants and resource for the protection of groundwater quality. Therefore, it is crucial to control industrial waste soil contamination in terms of total petroleum hydrocarbons (TPHs) and total organic carbon (TOC) through waste treatment and remediation.

TPHs are among the most prevalent environmental pollutants. This group includes an extensive family of hydrocarbon substances initially derived from petroleum and utilized to create petroleum-based products. Owing to long-term leaks, unintentional spills, or operational errors, the extensive and widespread application of crude oil and other petroleum products, such as heating and cooling, industry, and transportation, results in their discharge into the environment. (Kuppusamy, et al., 2019). Approximately 12,000 spills have occurred in the Niger Delta alone since the 1970s, and have permanently ruined the local mangrove ecosystems and poisoned wells with benzene levels thousands of times greater than the safe limit set by the World Health Organization (Peterson, 2003). Numerous physical, chemical, thermal, and biological techniques and methods have been used for in situ soil remediation of petroleum hydrocarbon contamination. Soil washing is an example of physical treatment. This is achieved by injecting clean water into the contaminated soil and pumping wastewater, which requires human effort and special equipment. Chemical oxidization-reduction is a chemical method for TPH removal which requires

large amount of different chemical oxidants such as MnO_4^- , $\cdot\text{OH}$ -, $\cdot\text{SO}_4$, O_3 , Fe_2^+ , $\text{S}_2\text{O}_8^{2-}$, H_2O_2 , and O_2 . The thermal treatment method involves the use of heat, such as hot air sparging, to volatilize pollutant particles in contaminated soils (Ambaye, 2022). However, owing to the high operational costs and multiple stages of treatment, biological remediation is a cost-effective and environmentally friendly technology that is the most feasible solution for the complete degradation of pollutants (Das and Kumar, 2020).

The environmental consequences of petroleum hydrocarbon contamination pose significant challenges to ecosystems and human health. Accidental oil spills, leakages from industrial facilities, and improper disposal of hydrocarbon-rich waste have led to the widespread contamination of soils and aquatic environments. In response to this environmental crisis, researchers have sought innovative and sustainable solutions for remediating petroleum hydrocarbon-contaminated sites. One promising research avenue involves the utilization of indigenous hydrocarbon-degrading bacteria, which have evolved in heavily contaminated soils, as natural resources for mitigating hydrocarbon pollution. These microorganisms possess a remarkable capacity to utilize petroleum hydrocarbons as sources of carbon and energy, making them invaluable candidates for bioremediation strategies (Das and Chandran, 2011).

In this study, we explored the biodegradation of petroleum hydrocarbons using indigenous hydrocarbon-degrading bacteria isolated from heavily contaminated soils. These bacteria have adapted to thrive in harsh environments with high hydrocarbon concentrations and have developed various metabolic pathways to efficiently degrade complex hydrocarbon mixtures. Monooxygenases and dioxygenases play pivotal roles among the key enzymes involved in the degradation process. These enzymes are responsible for initiating the breakdown of hydrocarbons by introducing oxygen atoms into hydrocarbon molecules, making them more susceptible to subsequent microbial degradation (Leahy and Colwell, 1999).

In this review, we discuss the mechanisms and enzymatic processes involved in the biodegradation of petroleum hydrocarbons by indigenous hydrocarbon-degrading bacteria with a particular focus on the pivotal roles of monooxygenases and dioxygenases. By examining the current state of knowledge and recent advances in this field, we aim to provide insights into the potential applications of these natural biodegraders for environmental remediation. Moreover, we highlight

the key resources and research findings that have contributed to our understanding of the biodegradation of petroleum hydrocarbons and the enzymatic machinery involved in this crucial process.

In light of this, recent local and international research related to TPH removal using biodegradation, as well as the variation in materials and methods used in different studies, will be discussed. The purpose of this study was to determine the optimal conditions for biodegradation and the effectiveness of indigenous microorganisms in reducing the total amount of petroleum hydrocarbons in severely contaminated soils by breaking down or digesting the hydrocarbons.

1.1 STATEMENT OF THE PROBLEM

As a result of industrialization, significant change has taken place in the economy, environment, and almost every aspect of society. Soil, water, and air pollution are negative aspects of industrialization. Soil contaminants enter the human body in different ways, such as through direct contact with the skin or through inhalation, which affects quality of life. Therefore, this study aimed to address the problem of soil impurities related to hydrocarbons from petrochemical industries in the 2nd and 3rd Dammam Industrial City and oil field site areas secondary to oil spills or industrial activities that have diesel spills into the soil.

These contaminated areas are left without treatment because of the high cost, lack of effort, and time spent using conventional measures such as physical, chemical, and thermal methods. Biological treatments can effectively mitigate contamination at lower costs. They also have the potential to improve environmental quality over the long term by restoring ecological balance. The effectiveness of biological treatments depends on the appropriate conditions for organisms to thrive, such as temperature, nutrient availability, and pH levels.

1.2 SIGNIFICANCE OF THE STUDY

Biological treatment of contaminated sites is a cost-efficient and effective remediation technique. In the current situation with existing soil-contaminated areas, the findings of this study pertaining to reducing and eliminating TPH by microbial degradation will directly benefit Saudi Vision 2030

towards environmental protection by taking a holistic view of the precious ecosystem and contribute to sustainable development. It provides valuable and measurable information that can be used to improve biological degradation in certain areas. The findings will enhance our knowledge of certain subjects and guide potential implementation.

This study can be used by future researchers as a reference to enhance the use of indigenous bacteria for remediating hydrocarbon contamination.

1.3 OBJECTIVES

The major goal of this study was to evaluate the effectiveness of indigenous hydrocarbon-degrading bacteria in lowering the overall petroleum hydrocarbon levels in highly contaminated soil.

Specific objectives:

- 1- Isolate indigenous bacteria that have degrading capability in the heavily contaminated soil.
- 2- Determine the optimum temperature and pH for the biodegradation process.
- 3- Identify the biodegradation enzymes used by the isolated bacteria.

CHAPTER 2 – LITERATURE REVIEW

Recent research on the use of biodegrading bacteria for contaminant treatment has revealed diverse approaches that focus on different microorganisms and strategies. Various microorganisms, including aerobes and anaerobes, are pivotal for bioremediation at contaminated sites. These microorganisms play crucial roles in eliminating, degrading, detoxifying, and immobilizing hazardous wastes and pollutants.

2.1 TOTAL PETROLEUM HYDROCARBONS (TPHs) IN SOILS

Total petroleum hydrocarbons (TPHs) represent a broad family of chemical compounds originating from crude oil. TPH contamination of soil is a significant environmental concern due to petroleum product spills, leaks, and improper disposal practices. TPH concentrations in soils can vary widely depending on the source and extent of contamination.

Studies have reported soil TPH concentrations ranging from < 100 mg/kg at minimally affected sites to $> 10,000$ mg/kg in heavily contaminated areas. For instance, Smith et al. (2020) reported TPH concentrations at industrial sites averaging approximately 5,000 mg/kg, highlighting the severity of industrial impacts.

TPHs can lead to soil and groundwater contamination, thereby affecting local ecosystems and potentially entering the human food chain. Prolonged exposure to high TPH concentrations has been linked to various health issues, including respiratory problems and skin irritation (Johnson and Stevenson, 2021).

2.2 ADVANCE STUDIES IN BIOREMEDIATION

The field of geomicrobiological engineering has been expanded in recent studies, including the utilization of microorganisms to change and modify the chemical and physical properties of soil for geotechnical applications and bioremediation (Rajasekar, 2021). One promising form of

bioremediation is the treatment of soil contamination using microorganisms and their enzymatic processes to break down contaminants in soil (Barbato, 2021).

2.3 UTILIZATION OF INDIGENOUS BACTERIA FOR BIOREMEDIATION

Many studies have been conducted to break down oil or hydrocarbon substances from soil using indigenous microbes such as *Escherichia coli* (*E. coli*) through their enzymes (xylE, alma, and p450cam), which degrade 60–99% of the target hydrocarbon contamination (French, 2021).

Steliga (2021) studied and monitored the progress of contaminated soil with 50 kg of petroleum substances containing Polycyclic aromatic hydrocarbons (PAH) = 139.3 mg kg⁻¹ dry mass and TPH = 56,371 mg kg⁻¹ dry mass ex-situ under constant temperature and humidity at 20–25 °C and 20–25%, respectively by using indigenous non-pathogenic bacteria (*Actinomycetes*). These bacteria contributed to reducing PAH contamination by 40.3% and TPH content by 86.3% from the initial contamination.

2.4 TEMPRETURE AND pH EFFECT ON THE BIODEGRADATION PROCESS

Gowthaman (2019) found that temperature control played a major role in influencing microbial biotreatment using bacteria isolated from cold regions (*Viridibacillus arvi*, *Lysinibacillus xylanilyticus*, and *Sporosarcina sp*). Ambient temperature is one of the most important factors determining the bacterial efficiency in remediating soil contamination. High temperature hinders bacteria activity at 40 °C and above. The influence of temperature between 10 and 50 °C on *L. xylanilyticus* was investigated for 168 h. The initial pH of the culture medium was essentially neutral (7.1–7.3). The maximum bacterial activity occurs in the temperature range between 20–25 °C and drops gradually when the temperature drops below 20 °C or rises above 25 °C.

Another experiment was conducted to determine the efficiency of *L. xylanilyticus* by exposing the microorganism to a pH range of 5–9. The best effectiveness was found within the weak alkali conditions in the pH range of 7–8.

2.5 USING A VARIETY OF MICROORGANISM FOR BIOREMEDIATION

Al-Dhabaan (2021) used yeast instead of bacteria for oil biodegradation. Only 5 yeasts from the 25 isolated yeasts from an oil field located in Khurais, Saudi Arabia, showed potential for oil degradation. Each yeast showed different degradation efficiencies over 15 days, using the enrichment technique of mineral basal salts (MBS) and medium with 1% (v/v) oil as the only carbon source. Oil breakdown and gas chromatography (GC) were used to analyze the biodegradation processes of the four yeast isolates using spectrophotometry. (Table 1).

Table 1: Percentage of variety of microorganism degradation (Al-Dhabaan, 2021)

Degradation % for five yeast isolates in the presence of 1% (v /v) oil.

Isolates	Identifying	Accession No.	% degradation
A1	<i>Candida tropicalis</i>	MW488263	61
A2	<i>Candida tropicalis</i>	MW488264	56
A3	<i>Rhodotorula mucilaginosa</i>	MW488265	51
A4	<i>Rhodospiridium toruloides</i>	MW488266	33

Ravi (2021) studied the use of fungi to bioremediate soil contaminated with petroleum, which has been increasingly recognized over the last decade. Mycoremediation is a technique that is used to degrade hydrocarbons. Fungi can colonize and adapt to intricate soil matrices under harsh environmental conditions in a variety of heterogeneous habitats. According to previous studies, filamentous fungi have different characteristics that make them preferable for soil bioremediation compared with yeasts and bacteria (Bosco, 2019; Joutey, 2013). The researchers reported that multicellular mycelial growth is the most important type of growth and is stable in the soil, which enhances biotreatment. In addition, the fungal types used for remediation include microfungi, such as *Penicillium* and *Aspergillus* species, and arbuscular mycorrhiza. The most significant degrading fungi were reported to be ligninolytic mushrooms, such as white rot fungi, which have a degradation ability of 99% in diesel-contaminated soils at 20k ppm of PAHs after 8 weeks. According to the investigators, different petroleum compounds exhibit varying levels of fungal degradation.

Molaei (2021) used an anoxic packed bed biofilm reactor (AnPBR) injected with in situ-contaminated petroleum wastewater for the biodegradation of biosurfactant-producing bacteria.

The initial TPH concentration was 99% and chemical oxygen demand (COD) was 96%. Reduction of hydraulic retention time (HRT) from 6 to 24 h has resulted in increased hydrocarbon utilization of 0.45 to 1.66g TPH/g biomass. The utilization of hydrocarbons decreased the performance from 99% to 37.6% because of the lack of nitrate availability after ten days. A gas chromatography/flame ionization detector (GC/FID) effluent chromatogram showed that aromatic hydrocarbons, aliphatic compounds, and cyclic aliphatic compounds were efficiently degraded. (Figure 2).

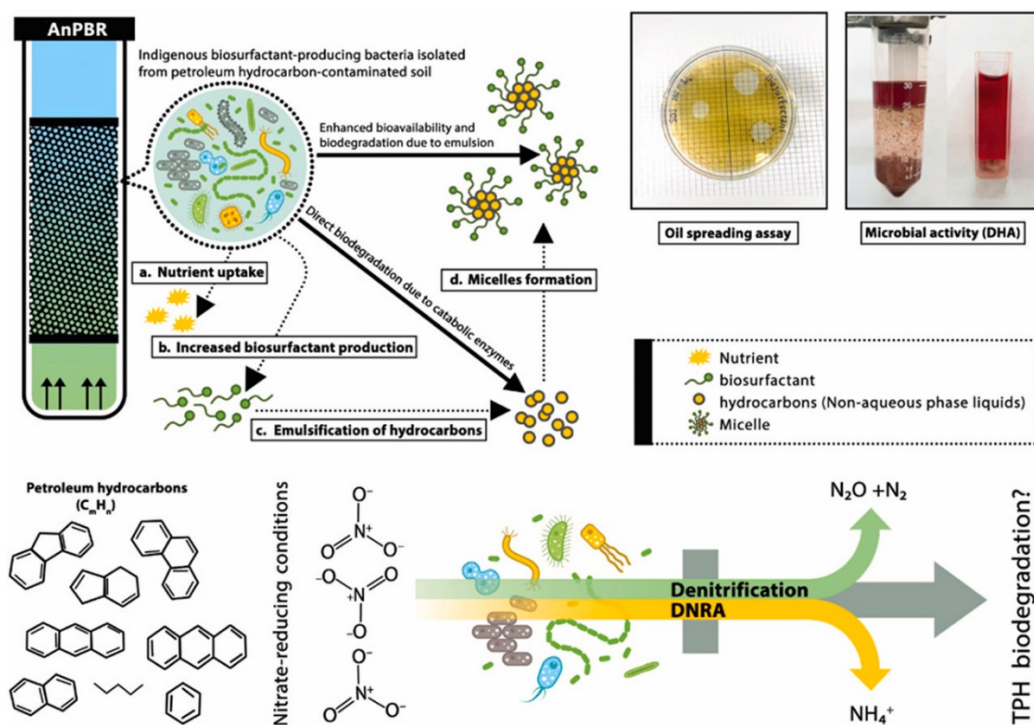


Figure 1. TPH biodegradation process (Molaei, 2021)

Jones (2020) focused on identifying and isolating eight bacteria (*Pseudomonas aeruginosa*, *Corynebacterium kutscheri*, *Bacillus subtilis*, *Micrococcus agilis*, *Serratia odorifera*, *Staphylococcus aureus*, *Flavobacterium aquatile*, and *Micrococcus luteus*) based on the colony characteristics of soil contaminated with waste engine oil. Each microorganism showed different hydrocarbon utilization after 15 days of incubation. The collected samples contained a variety of living microorganisms due to ecological factors that affect the ability of soil microbes to survive,

which is crucial for their ability to decompose and recycle nutrients. Two of the eight bacteria (*C. kutscheri* 52% and *S. aureus* 8%) showed the highest hydrocarbon degradation activities.

Petroleum is a mixture of hydrocarbon components, and no single microbe can degrade all types of hydrocarbon components. One study was conducted on the degradation of hydrocarbons by drill cutting, in which fungi and bacteria were cultured together. The results demonstrated that fungi and bacteria worked synergistically to destroy complex compounds. Fungi provide bacteria with stable surfaces for adherence to this type of biofilm. However, the bacteria do more than just reside on the mycelial surface; they also develop a unique relationship with the fungus. By producing extracellular polymeric substance (EPS), bacteria that form biofilms can quickly colonize air-liquid interfaces by producing extracellular polymeric substances. (Priyamba, 2019).

2.6 IDENTIFICATION AND ISOLATION OF BACTERIA FOR HIGHLY EFFECTIVE BIODEGRADATION

This study compared hydrocarbonoclastic bacterial species with individuals of species based on of the highest oil utilization, and showed that indigenous hydrocarbon-degrading bacteria were more efficient in the biodegradation of contaminated soil than a group of diverse microorganisms (microbial consortium). The hydrocarbon component was removed effectively by individual indigenous bacteria at rates of 73.6% and 69.3%, whereas oil removal by the microbial consortium was between 20% and 30%. Even in extremely contaminated saturated oils, the environment is greatly aided by naturally occurring bacteria that break down the oil. This is due to the fact that oil bioremediation is not significantly improved by a hydrocarbonoclastic microbial consortium bacterial species with high potential of oil degradation (Ali, 2022).

2.7 MONOOXYGENASE AND DIOXYGENASE ENZYMES IN BACTERIA

Dioxygenases catalyze the incorporation of oxygen atoms from O₂ into their substrates, often leading to the cleavage of aromatic rings in a process that is crucial for the degradation of persistent environmental pollutants. There are two types of dioxygenases based on their reaction mechanisms: intradiol and extradiol dioxygenases, which cleave the aromatic ring at different positions relative to the substituent groups (Vaillancourt, 2006). Their role in degrading aromatic

compounds makes them invaluable for processes, such as soil bioremediation and the global carbon cycle (Sharma, 2015).

In contrast, monooxygenases incorporate only one of the two oxygen atoms from O_2 into the substrate, while the other is reduced to water and requires a co-substrate, typically NADH or NADPH, for the transfer of the electrons necessary to activate O_2 (Montellano, 2010). They can be categorized into flavin-dependent monooxygenases, which use flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as cofactors, and P450 monooxygenases, which utilize a heme iron center for oxygen activation. These enzymes are pivotal in bacterial metabolism for the biosynthesis of essential metabolites and detoxification of xenobiotic compounds, contributing to pharmaceutical applications and environmental cleanup efforts as shown in Figure 2 (Morcomb, 2003).

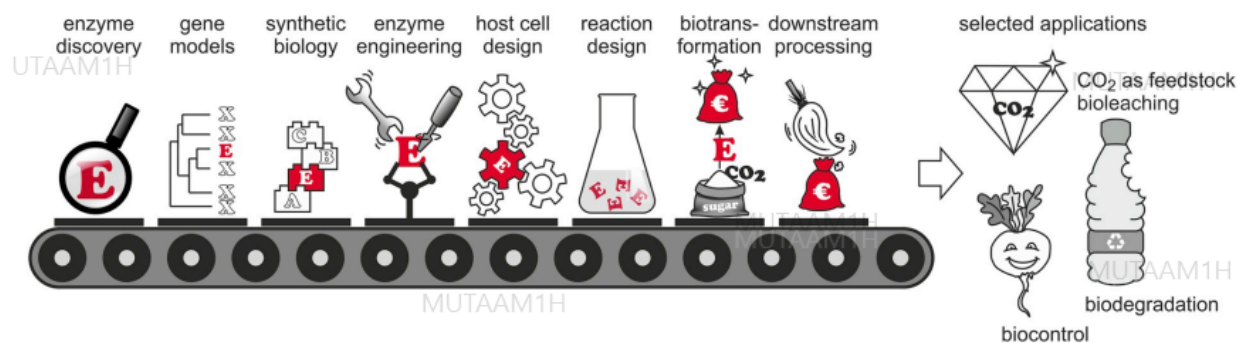


Figure 2. Applications of bacterial metabolic enzymes (Morcomb, 2003).

Microbes isolated from contaminated soil vary depending on the type of hydrocarbon contamination type, and ecological factors. Microbial selection depends on the degradation efficiency of microorganisms. The biological remediation process depends on several factors such as temperature, relative humidity, pH, and the abundance of hydrocarbon pollutants.

Many studies have focused on bioremediation because it is cost-effective and safe for eliminating hydrocarbon contamination, and the microorganisms used for remediation can be bacteria, fungi, or yeasts. These microorganisms have different features and select microorganisms depending on many aspects such as the type of pollutant in the hydrocarbon component, ecological factors, and soil properties (physical, chemical, and biological). Thus, indigenous hydrocarbon-degrading

bacteria from contaminated soil are more efficient than any diverse group of microorganisms at degrading heavy contaminants from the soil. However, there is a notable gap in the literature regarding the comparative efficiency of degradation under optimal biodegradation conditions. In addition, comprehensive studies comparing the effectiveness of these indigenous bacteria in hydrocarbon degradation using different strains are lacking. Many studies have also failed to address how environmental factors such as temperature and pH influence the interaction between indigenous bacteria and hydrocarbon degradation. This includes the study of the interactions between these bacteria and other soil organisms.

Screening approaches for monooxygenase and dioxygenase enzymes are critical for understanding their roles in metabolic processes and their applications in biotechnology. Monooxygenases incorporate one atom of the oxygen molecule into the substrate while reducing the other to water, whereas dioxygenases incorporate both atoms into their substrate (Toure et al., 2020). These enzymes are widely used in the synthesis of complex organic compounds, bioremediation, and in the pharmaceutical industry (Smith, 2021).

High-throughput screening (HTS) techniques have been developed to identify and characterize monooxygenases and dioxygenases with the desired specificities and activities. These methods often use colorimetric or fluorometric assays based on the detection of reaction by-products indicative of enzyme activity (Patel, 2019). For monooxygenases, the release of hydrogen peroxide during substrate oxidation can be quantified using chromogenic substrates such as Amplex Red, which reacts with H_2O_2 to produce a measurable signal (Zhang et al., 2018).

The ability of dioxygenases to cleave molecular oxygen can be exploited in screening methods by monitoring the specific transformation of substrates, such as catechol derivatives, which change color upon oxidation (Liu et al., 2019). These substrates are particularly useful for detecting extradiol and intradiol dioxygenase activity, which is crucial for the catabolism of aromatic compounds (Williams et al., 2020).

Phage display technology has also been used to screen monooxygenase enzymes. This involves displaying enzyme libraries on the surface of bacteriophages and screening for those that confer the desired activity on a target substrate. This technology is powerful for identifying enzymes with

high specificity and has been extensively used in the pharmaceutical industry (Greener et al., 2021).

Enzyme-linked immunosorbent assay (ELISA)-based techniques are another common approach, in which antibodies specific to monooxygenase or dioxygenase reaction products are used to detect and quantify enzyme activity (Mehta et al., 2022). This method is particularly sensitive and can be applied to various sample types, including environmental samples, in bioremediation studies.

In situ activity assays using fluorescent or luminescent reporters offer a non-invasive way to measure enzymatic activity within living cells, thus providing insight into the biological functions of these enzymes under physiological conditions (Khan et al., 2017). Such assays have become increasingly important in the field of environmental microbiology, where the activity of these enzymes indicates the presence of pollution-degrading microbial populations (Chen et al., 2021).

Genetic screening is an alternative approach in which mutants or naturally occurring variants of monooxygenase and dioxygenase genes are evaluated for desired traits. This method can be very effective in discovering novel enzyme activities, particularly when combined with mutagenesis and recombinant DNA technologies (Huang et al., 2020).

Overall, these screening methods enhance our ability to discover and engineer monooxygenases and dioxygenases for various applications ranging from synthetic chemistry to environmental sustainability (Ramirez et al., 2023).

CHAPTER 3 - MATERIALS AND METHODS

3.1 SOIL SAMPLING

One-kilogram soil samples were collected from two diesel-contaminated field sites. The sites were located within a 150-kilometer radius of Alhasa City. The first sample was obtained from the Al Jafurah area (GPS coordinates 24.528795, 49.479522). The second sample was obtained from the Al Uqayr area (GPS coordinates 25.664264, 50.006086) (Figure 3).

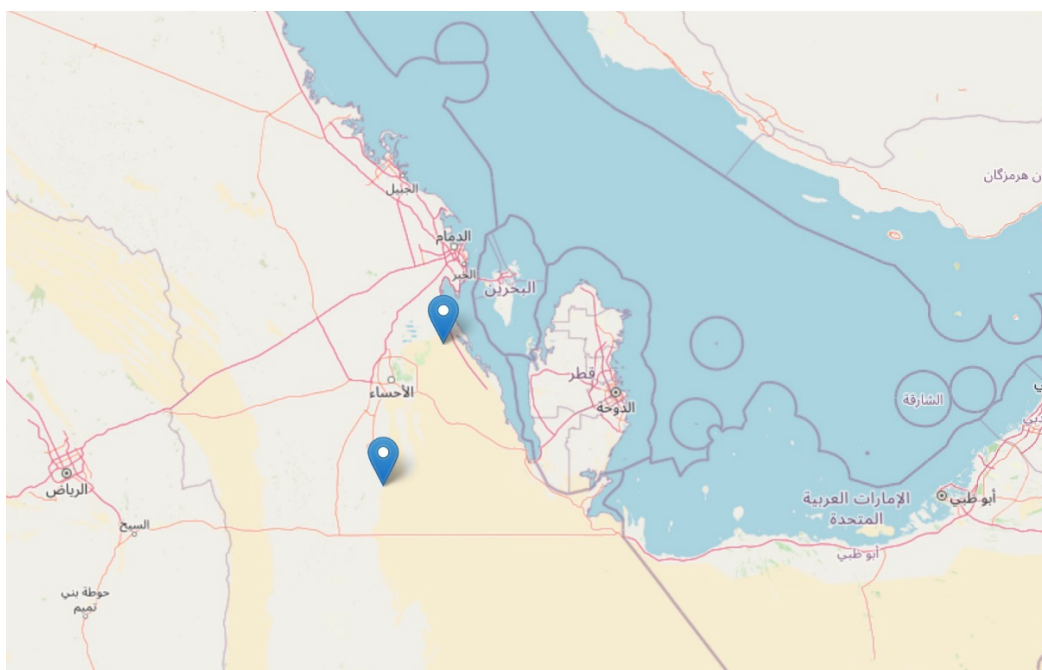


Figure 3. Map of Eastern Region in Saudi Arabia used for sample collection

For laboratory analysis, both samples were securely placed in specialized containers and preserved at low temperature.

3.2 SOIL CHARACTERIZATION

As these samples were collected from the Eastern Region of Saudi Arabia, it is important to note that the soil primarily consisted of sandy soils. This region is predominantly characterized by fine

to coarse sand particles. Additionally, it features sabkha terrains, which are coastal or salt flats that form in arid or semi-arid regions often adjacent to the sea (Fryberger, 2006).

Soil pH was assessed by measuring the pH of the samples using a pH meter. The procedure entailed mixing the hydrocarbon-contaminated soil with distilled water at a 1:2 ratio and placing the mixture in 200 ml flasks (Figure 4).



Figure 4. Contaminated soil samples mixed with distilled water.

These flasks were then placed in a shaker incubator at a temperature of 35°C, and agitated at a speed of 100 rpm for five days. Subsequently, the pH of these samples was measured and found to be between 7.0 and 7.2.

Soil TPHs were measured using gas chromatography with flame ionization detection (GC-FID), which is a powerful analytical method used in analytical chemistry to separate and analyze compounds that can be vaporized without decomposition. The soil-to-water ratio was 1:1, and organic carbon calibration was performed using glucose in the range of 0.5–25mg C. The FID

signal was recorded and analyzed. The peaks in the chromatogram correspond to different compounds, with the area under each peak.

3.3 BACTERIA ISOLATION FROM SOIL CONTAMINATION

A 500 ml Erlenmeyer flask containing 100 g of contaminated soil and 200 ml of autoclaved water was incubated at 30°C. After 24 h, the supernatant was removed and placed into five replicates of 100 mg nutrient broth in 250 ml Erlenmeyer flasks. For three days, the flasks were incubated in a shaker incubator at 30 °C and 150 rpm. Dilutions from each flask were distributed on 0.5% crude oil agar plates and nutrient agar plates after three days of enrichment. Nutrient agar plates were growth tested for 48–72 hours after being incubated for 10 days at 30 °C with crude oil plates.

3.4 BACTERIAL COUNTS

The overnight cultures were collected at an optical density at 600 nm wavelength of 0.50 ± 0.025 . The bacterial cells were harvested by centrifugation at 4000 rpm (rpm) for 10 min at a temperature of 4 °C. Following centrifugation, the cells were washed with sterile 0.9% sodium chloride solution. Serial dilutions ranging from 1:1 to 1:10 were performed. For quantitative and qualitative analysis, 0.1 ml of each dilution were plated onto agar plates supplemented with nutrient broth and crude oil and incubated in duplicate. After incubation for 24 h at a controlled temperature of 37 °C, the resultant bacterial colonies were counted.

After the appearance of distinguishable bacterial strains, subculturing procedures were performed. Bacterial strains were isolated, identified, and characterized based on colony morphology attributes, including color, shape, transparency, form, elevation, and margin (Figure 5).



Figure 5. Distinct strains of bacteria, each characterized by a unique genetic composition and physiological properties

In preparation for subsequent experimental applications, Bushnell Haas broth was formulated using two one-liter flasks, each containing 1 l of distilled water to ensure a pH level of 7, and supplemented with the supplements listed in Table 2.

Table 2: Bushnell Haas broth component

Supplement	Volume	Purity
Magnesium sulphate	0.2 g/l	97%
Calcium chloride anhydrous	0.020 g/l	96%
Dipotassium hydrogen phosphate	1 g/l	99%
Ammonium nitrate	1 g/l	99%
Ferrous chloride	0.05 g/l	97%

The prepared broth was then subjected to autoclaving for 3 h at a temperature of 120 and a pressure of 100 bar. This procedure was designed to facilitate the study of degradation capabilities of the isolated bacterial strains.

3.5 DEGRADATION TEST

To study the degradation process, we prepared 250-ml media flasks using Bushnell Haas broth, to which we added 2 ml of crude oil. This addition was aimed at facilitating the investigation

of degradation processes by the isolated indigenous bacteria, which were also introduced into the medium at a concentration volume of 2 ml. This experiment was designed to assess the impact of varying temperature and pH conditions on bacterial activity. The flasks were maintained at three distinct temperatures: 37 °C, 25 °C, and 10 °C. At each temperature, the media were adjusted to three different pH levels (4, 7, and 10) in a control flask devoid of bacterial inoculation for each set.

Initial TOC and DO measurements were performed before placing the flasks in an incubator. Subsequently, the flasks were incubated for four weeks, during which time the TOC and DO levels were monitored weekly. Notably, some flasks had a foggy appearance in the liquid medium, indicative of bacterial growth.

3.6 SCREENING FOR ENZYMES: MONOOXYGENASES

Bacterial cells were pelleted by centrifugation for 20 min at $2250 \times g$ at 4 °C and resuspended in the reaction mixture (250 µL), which contained NADPH (20 µM), sodium phosphate (20 mM), 1,4-dioxane (1% v/v), sodium cyanide (20 mM), Peroxy Green 1 (250 µM), PTDH (5 µM), FAD (10 µM), Tris-HCl (50 mM, pH 9.0), and glycerol (10 mM). Subsequently, 10 µL of benzyl phenyl sulfide (0.125 M) was dispensed into each well. For activity assays with phenylacetone, the reaction mixture contained the substrate (10 mM), NADPH (50 µM), sodium cyanide (20 mM), Peroxy Green 1 (250 µM), PTDH (5 µM), FAD (10 µM), Tris-HCl (50 mM, pH 7.5), and glycerol (10 mM).

Reactions with phenylacetone were conducted for 1.5 h at 37 °C, 1050 rpm. For benzyl phenyl sulfide, the reaction time was 1.5 or 3 h. Subsequently, the plate was centrifuged for 5 min at $2250 \times g$ at 4 °C. A sample of the supernatant (100 µL) was used to measure fluorescence. Excitation was recorded at 460 nm, emission was recorded at 510 nm, and the sensitivity was set to 45. Mutants with at least 80% fluorescence emitted by the Q152F/L153G/M446G mutant were regarded as uncoupling mutants and were excluded from further analysis. For phosphate quantification, the supernatant (20 µL) was mixed with the molybdate reagent (200 µL), followed by the addition of ascorbic acid (50 µL). After 30 min incubation at 30 °C and 900 rpm, absorbance

was measured at 850, 700, and 600 nm. Phosphate standards containing potassium dihydroxy phosphate (0.2 to 10 mM) were used to prepare a calibration curve for the molybdate assay.

CHAPTER 4 - RESULTS

Several tests were conducted to assess hydrocarbon biodegradation in flask samples, all of which were maintained in a closed condition to prevent the volatilization of TOC. These samples were kept at varying temperatures and pH levels, including a control sample. The initial test was performed at time zero, followed by three subsequent tests at weekly intervals. The results indicated that the rate of degradation differed across flasks and was influenced by the specific combination of temperature and pH. Table 3 presents the findings of the tests conducted for each flask.

Table 3. Biodegradation test results

Temperature °C	10 °C						20 °C						37 °C						Control
pH	4		7		10		4		7		10		4		7		10		TOC mg/l
Time (days)	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	TOC mg/l	DO ppm	
0	2410	7.1	2360	7.31	2330	7.14	2430	6.92	2370	6.84	2470	7.14	2350	6.88	2260	6.81	2190	7.17	2510
7	2410	7.12	2340	7.33	2320	7.15	2300	6.7	2080	6.2	2280	6.6	2190	6.5	2050	6.1	2060	6.4	
14	2380	7.22	2320	7.45	2310	7.18	2040	6.2	1740	5.9	1870	6	1880	6	211.4	5.4	1310	5.7	
21	2350	7.53	2320	7.54	2280	7.24	1870	5.8	118.1	3.5	1740	5.2	211.4	4.8	48.73	3.3	213.2	4.2	
28	2340	7.81	2310	7.56	2280	7.27	1330	4.1	21.79	1.6	91.58	2.4	151.2	3	10.39	1.2	73.22	2.1	

4.1 IMPACT OF TEMPERATURE AND pH ON BIODEGRADATION

The temperature increased from 10°C to 37°C and a marked increase in TOC reduction was observed. Although pH variations at 10 °C did not show a substantial difference in TOC and DO reduction, the impact became more pronounced at higher temperatures. At 20 °C and 37 °C, there is a significant increase in TOC and DO reduction, especially at neutral to alkaline pH levels (pH 7 and 10). This indicated that pH, in conjunction with temperature, played a critical role in the rate of bacterial degradation of organic matter (Figure 6).

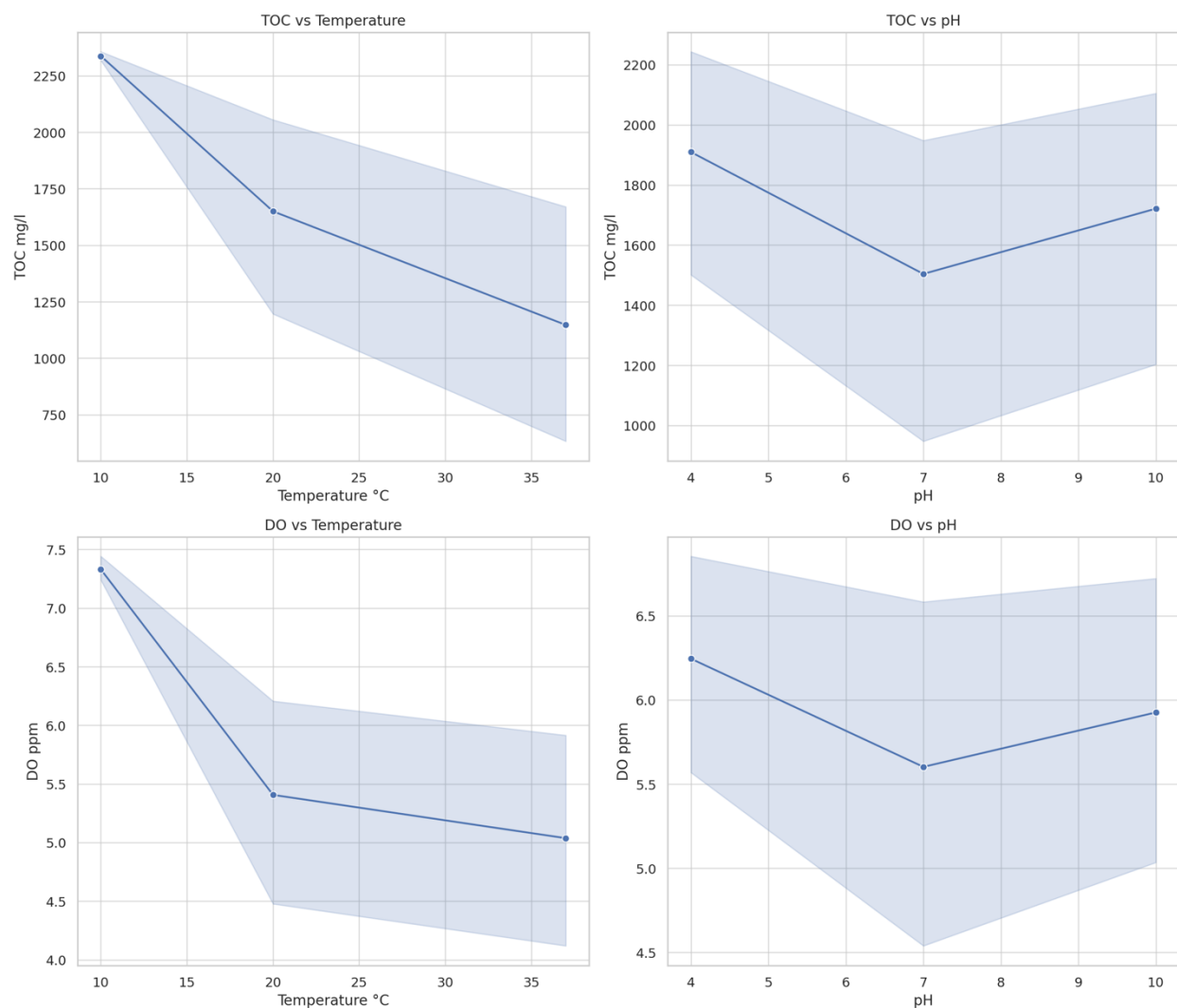


Figure 6. Variations in temperature and pH in biodegradation.

4.1.1 Variation in TOC at 37°C at different pH levels

The impact of temperature and pH on TOC degradation was clearly demonstrated by observing TOC variations over time at 37 °C under different pH conditions. A rapid reduction in TOC levels was evident at pH 7 and 10, as opposed to that at pH 4. This indicates that bacterial activity, which contributes to TOC degradation, was more effective or accelerated at higher pH levels under these conditions (Figure 7).

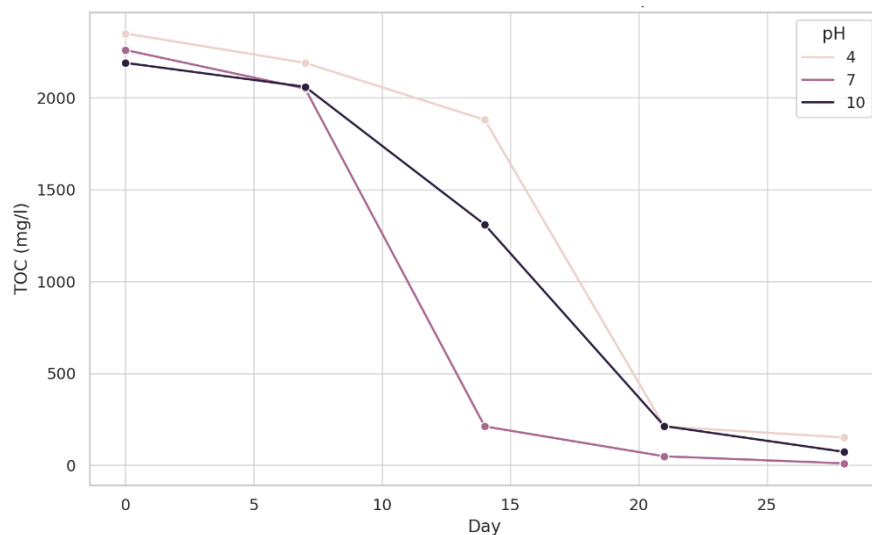


Figure 7. TOC variation over time at 37 °C at different pH levels

This graph underscores the combined effects of temperature and pH on the TOC levels, highlighting the importance of these environmental factors on the dynamics of organic carbon degradation.

4.1.2 Variation in TOC at 20°C at different pH levels

Consistent with observations made at 37 °C, biodegradation at 20 °C also demonstrated distinct trends across different pH levels. This indicated a significant influence of pH on TOC levels at 20 °C. Notably, the rate of TOC degradation at this temperature was lower than that at 37 °C (Figure 8).

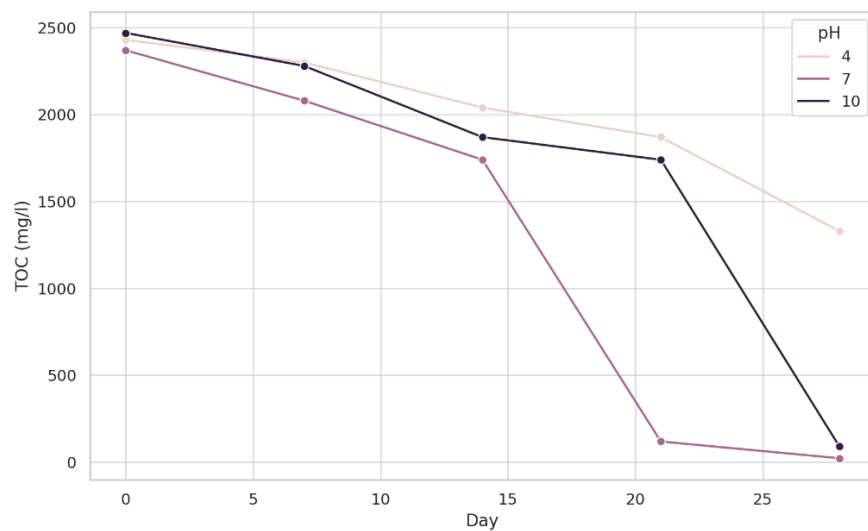


Figure 8. TOC variation over time at 20 °C at different pH levels

4.1.3 Variation in TOC at 30°C at different pH levels

At a temperature of 10 °C, TOC degradation proceeded at a notably slower rate compared to the degradation rates at 37 °C and 20 °C. This variation in degradation efficiency across different temperatures suggests that the bacteria involved in the biodegradation of TOC demonstrated enhanced efficiency at higher temperatures (Figure 9).

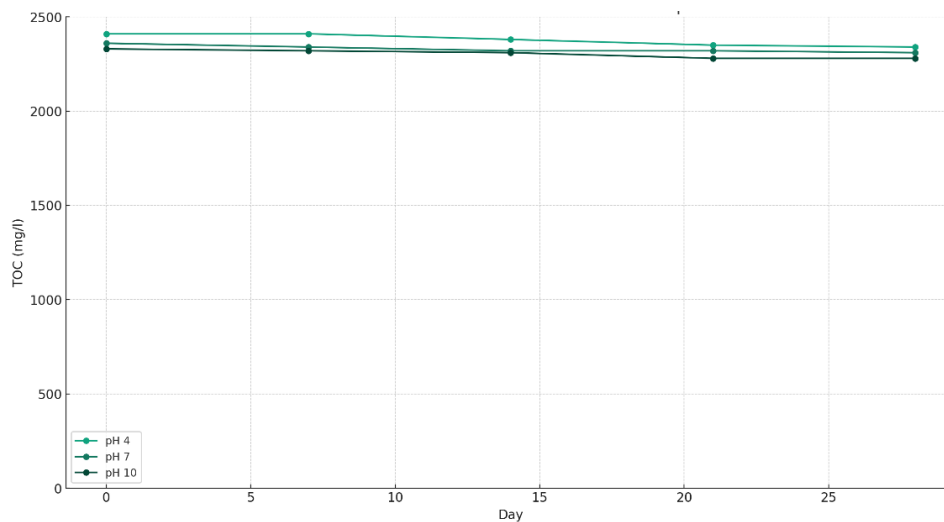


Figure 9. TOC variation over time at 10 °C at different pH levels

4.2 TOC DEGRADATION WITH PH AND TEMPERATURE VARIATION

Average degradation of TOC varied under various pH and temperature conditions. This study aimed to identify optimal conditions for indigenous bacteria to effectively digest and degrade hydrocarbon contaminants. Figure 10 presents a heatmap that offers an extensive visualization of the TOC degradation levels across a range of pH values and temperatures. This heatmap uses a nuanced color gradation scheme, where darker shades signify elevated TOC levels and lighter shades denote lower levels. This visual representation is instrumental in determining the most favorable conditions for the degradation process and contributes significantly to the understanding of bioremediation dynamics under various environmental conditions.

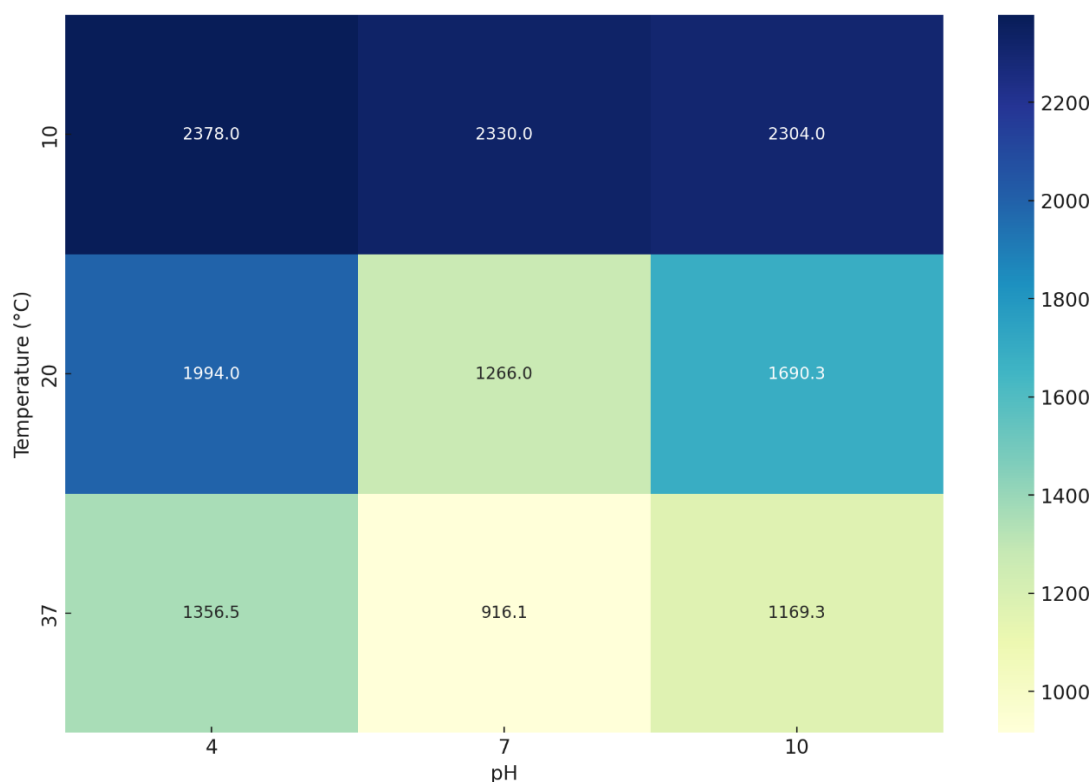


Figure 10. Heatmap visualization of average TOC degradation by color. Darker shades denote high TOC concentration at different level of pH and temperature

Heatmap analysis effectively demonstrated the complex interplay between the environmental parameters and TOC degradation levels. Notably, higher TOC levels, as indicated by lighter colors on the heatmap, were predominantly observed at a temperature of 10 °C and pH 4. This indicates that a significant concentration of TOC remained under these specific conditions. In contrast, the lowest TOC levels, denoted by lighter colors, are found at a temperature of 37 °C

and pH 7, indicating reduced TOC concentrations in these settings due to the bioremediation by bacteria.

CHAPTER 5 – DISCUSSION

The results of this study indicated a significant increase in the efficiency of hydrocarbon removal at higher temperatures. Specifically, at 37°C and a pH 7, the indigenous hydrocarbon soil bacteria demonstrated exceptionally high efficiency in removing TOC, achieving an average reduction of approximately 38% within a 28-day period. Both the temperature and pH significantly influenced the TOC reduction rate. As temperatures increase from 10 °C to 37 °C was associated with a notable enhancement in the rate of TOC degradation. This trend was particularly evident at neutral to alkaline pH (pH 7 and 10), where the reduction in TOC was markedly higher. This phenomenon underscores the interplay between temperature and pH in determining the efficiency of bacterial activity for organic matter (OM) degradation.

Furthermore, the analysis extends to understanding TOC variations at different temperatures, namely 37 °C, 20 °C, and 10 °C. At the highest temperature of 37 °C, TOC degradation was most efficient, particularly at pH levels of 7 and 10. In contrast, at 20 °C, while the degradation trend was similar, the rate was slower. The slowest degradation was observed at 10 °C, indicating a significant temperature dependency of bacterial efficacy. Heatmap analysis illustrating the TOC degradation at various temperatures and pH levels further enhances this understanding. This visual representation, with darker shades indicating lower TOC levels, confirms that optimal bacterial bioremediation occurs at higher temperatures and neutral-to-alkaline pH levels, thereby providing essential insights for environmental management and bioremediation strategies.

In contrast, the removal of TOC was less effective when the temperature was 10 °C and the pH was set to 4 under the same experimental conditions. This disparity in the TOC and DO levels with varying temperatures underscores the profound thermal influence on bacterial metabolic rates and the solubility of oxygen in water. Notably, the study findings suggest that higher temperatures generally stimulate bacterial activity, thereby expediting the decomposition of organic matter, as evidenced by the fluctuations in TOC levels. This observation aligns with previous research that highlighted the critical role of temperature in influencing microbial decomposition rates (Davidson and Janssens, 2006).

The influence of temperature on hydrocarbon removal efficiency has been a focal point in the field of bioremediation. Smith et al. (2018) conducted a study and obtained similar findings to those of this thesis. They demonstrated that the capacity of indigenous bacteria to degrade hydrocarbons increased significantly at higher temperatures. In particular, they found that, as the temperature increased, the rate of hydrocarbon degradation exponentially. This temperature-dependent trend aligns with the observations of the present study, underscoring the critical role of temperature in promoting microbial metabolic activity during bioremediation.

The pH level has also emerged as a crucial determinant of hydrocarbon removal efficiency by microbial communities. Kebede (2021) explored the effect of pH on hydrocarbon-degrading bacteria and reported results consistent with those presented in this thesis. Their study highlighted that neutral to alkaline pH levels in different ranges stimulated higher bacterial growth and enzymatic activity, ultimately leading to more efficient hydrocarbon degradation at a pH of 7.5. This parallel observation underscores the importance of maintaining optimal pH conditions in bioremediation efforts (Kebede, 2021).

Microorganisms exhibit differential responses to pH, a measure of the hydrogen ion concentration in a solution, which significantly influences their metabolic activities and growth. The activity of microorganisms is generally more pronounced in the mid-range of alkalinity due to several biochemical and physiological reasons, while extreme acidity or alkalinity can inhibit microbial activity or even lead to microbial death.

The enzyme activity crucial for microbial metabolism shows optimal performance at specific pH levels, usually within the neutral to slightly alkaline range. Enzymes, which are proteins that catalyze biochemical reactions, have three-dimensional structures that are sensitive to pH changes. At extreme pH levels, either too acidic or too alkaline, the hydrogen or hydroxyl ions can disrupt the hydrogen bonds and ionic interactions within the enzyme structure, leading to denaturation or altered active sites. This conformational change in enzymes at non-optimal pH levels reduces their activity, thus affecting the overall metabolic processes within the microorganism (Madigan et al., 2019).

Moreover, the cell membrane integrity of microorganisms is affected by pH. The cell membrane, composed of phospholipid bilayers and proteins, is crucial for controlling the entry and exit of substances. At extreme pH values, the permeability of the cell membrane can increase, leading to leakage of essential ions and molecules, which can be detrimental to the cell's survival. Additionally, high hydrogen or hydroxyl ion concentrations can disrupt membrane proteins, further impairing cellular function (Brock Biology of Microorganisms, 15th edition).

Furthermore, the solubility of nutrients necessary for microbial growth can be influenced by pH. Certain minerals and nutrients become less available or insoluble at extreme pH levels, limiting their uptake by microorganisms. This nutrient limitation can lead to reduced microbial growth and activity in environments that are too acidic or too alkaline (Atlas and Bartha, 1998).

Monooxygenases are pivotal in the biodegradation of hydrocarbons and display selective affinity towards diverse hydrocarbon substrates in oil. The initial and crucial phases in this process involve the selective binding of the enzyme to hydrocarbons. This binding is predicted based on the strategic positioning of the hydrocarbon molecule within the active site of the enzyme, facilitating effective catalytic activity. The specificity and binding efficiency are significantly influenced by the molecular composition of hydrocarbons, including alkanes, aromatic compounds, and polycyclic aromatic hydrocarbons (PAHs). The geometry of the active site of an enzyme, along with the presence of specific amino acid residues, plays a vital role in determining its substrate affinity (Jones and Voordouw, 2022).

Oxygen is an essential element in the biodegradation process. Monooxygenases catalyze a fundamental reaction involving the incorporation of one oxygen atom into a hydrocarbon substrate, concurrently reducing the other oxygen atom to water. This reaction necessitates the activation of molecular oxygen, a process often supported by cofactors, such as iron or copper, in the active site of the enzyme. Activated oxygen then attacks the hydrocarbon molecule, leading to the formation of an oxygenated intermediate that typically manifests as an alcohol, aldehyde, or ketone. This transformation is critical as it converts hydrophobic compounds into more hydrophilic forms, thereby rendering them more amenable to subsequent microbial degradation (Fernandez-Lafuente, 2021).

Furthermore, the inverse relationship observed between the temperature and DO levels can be attributed to the reduced solubility of oxygen at elevated temperatures, a well-documented physical phenomenon in aquatic systems.

The pH of the samples was another pivotal factor affecting both the TOC and DO levels. Our observations showed that different pH conditions led to variations in TOC, likely due to the pH-dependent nature of the bacterial enzymatic activities involved in organic matter degradation. The degradation of organic compounds by indigenous bacteria is influenced by ambient pH, which can alter enzyme functionality and microbial community composition (Rousk et al., 2010). Similarly, DO fluctuations across varying pH levels can be linked to the pH-dependent solubility of gases and the influence of pH on the biological oxygen demand.

A notable trend in our data was a reduction in TOC levels over time. This trend indicates the active degradation of organic carbon by indigenous bacteria. Bacterial communities play a pivotal role in organic matter decomposition by converting complex organic compounds into simple forms. This process not only reduces TOC but also impacts the overall biogeochemical cycling within the ecosystem (Falkowski et al., 2008). The observed reduction in TOC over the study period aligns with the hypothesis that microbial activity is modulated by environmental factors such as temperature.

CHAPTER 6 – CONCLUSION

Based on a comprehensive analysis of TOC degradation under varying pH and temperature conditions, this thesis presents significant findings regarding the efficiency of bacterial biodegradation in different environmental settings. These results clearly demonstrate that both temperature and pH are critical factors influencing the rate of TOC degradation.

Furthermore, this study underscores the pivotal roles of temperature and pH in determining the rate of bacterial degradation of TOC. These findings have significant implications for environmental biotechnology, particularly for bioremediation. Understanding the optimal conditions for indigenous bacteria to digest and degrade hydrocarbon contaminants is crucial for developing effective strategies to manage and remediate hydrocarbon-polluted environments. The insights gained from this study contribute substantially to broader knowledge of microbial ecology and environmental management.

Considering their substantial toxicity to humans and the environment, hydrocarbon pollutants are a major concern in environmental pollution. Numerous studies have indicated that various enzymes enable microorganisms to decompose hydrocarbons. Recognizing the importance of abiotic factors, including the physicochemical attributes of pollutants and environmental elements, such as bioavailability, nutrients, temperature, pH, aeration, and salinity, is vital for the efficient removal of contaminants. Thus, the optimization of these environmental factors to promote bacterial growth and activity is crucial for successful hydrocarbon biodegradation. Moreover, the development of innovative biosurfactants that enhance the bioavailability of hydrocarbons for bacterial consumption presents a promising avenue for addressing challenges in the microbial uptake of petroleum hydrocarbons. Although this review highlights several key limiting factors, the complex interactions between microorganisms, hydrocarbons, and the environment are not yet fully understood. Continued research is necessary to enhance our predictive knowledge of the fate of hydrocarbon pollutants in the environment and the role of microorganisms in environmental cleanup through biodegradation.

CHAPTER 7 – RECOMMENDATION

This study focused on pH and temperature as key factors in the bacterial biodegradation of TOC, and future research should broaden the scope to include additional environmental factors. These factors include salinity, moisture content, nutrient availability, and presence of other microbial species. Understanding how these variables interact with pH and temperature will enable a more comprehensive determination of optimal conditions for bacterial biodegradation and interactions with other microbial species. Such a comprehensive approach is vital to gain a deeper understanding of the optimal conditions that facilitate bacterial biodegradation.

Moreover, there is a pressing need to delve into the dynamics of the microbial communities involved in biodegradation processes. Diverse bacterial species and their intricate interactions play crucial roles in determining the efficiency of degradation. A thorough understanding of these dynamics is fundamental for developing enhanced bioremediation strategies.

Furthermore, a significant increase in global funding is essential to propel research on bacterial biodegradation. This investment should not only support basic research but also aid in the development of practical applications and the creation of innovative technologies for environmental remediation. Given the complexity of bacterial biodegradation, a multidisciplinary collaboration integrating microbiology, environmental science, chemistry, and engineering is indispensable. Such interdisciplinary efforts are the cornerstone for fostering innovation and acquiring a comprehensive understanding of biodegradation processes.

Additionally, the promotion of partnerships among public institutions, private enterprises, and non-governmental organizations is crucial. These collaborations offer a plethora of perspectives, boost resources, and provide practical environments for testing biodegradation technologies. The synergy of these diverse sectors is instrumental in effectively applying and advancing biodegradation methodologies.

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Objective:

A dedicated in technical services, decarbonization emissions management, energy and environmental support with Saudi Aramco for more than 15 years. I have held pivotal roles as an Environmental and Energy Coordinator. My background encompasses operational management, energy management, and mechanical engineering. Skilled in environmental knowledge, stockholder engagement, project management and net-zero strategy execution.

Workshop and Presentation:

Designed and delivered a comprehensive workshop in Technical Exchange Dhahran Center for executing Aramco's net zero climate strategy across 35+ facilities processing 13 million barrels of oil equivalent per day. Share knowledge with engineering specialists in abatement initiatives and developing comprehensive marginal abatement cost curves (MACCs).