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**ISOLATION, CHARACTERIZATION AND BIOREMEDIATION POTENTIAL OF  
HYDROCARBON DEGRADING BACTERIA FROM PETROLEUM  
CONTAMINATED SOIL**

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**ABSTRACT**

The previous few decades have seen a surge in petroleum products. This pollution led to significant problems. Microorganisms are used in bioremediation process to clean petroleum contamination, it is able to break down petroleum hydrocarbons. The current research sought to identify the microorganisms found in oil-contaminated sites from two different geographic locations (Vadodara and Mandavi (Surat), Gujarat, India), to examine the development of isolated bacteria and their properties. The research used the enrichment culture technique to isolate bacteria. Bushnella-Hass (BH) broth supplemented with crude oil as a sole carbon source. Based on morphological and biochemical characterization, the isolates were identified as *Bacillus spp.* The isolates were morphologically identified as gram's negative, short rod shaped and red in colour. The growth of bacterial population showed by growth curve. Biodegradation assay were performed at lowest (1%) and highest (10%) concentration for 78 hrs by using selected bacterial culture. The ability of isolates to degrade oil was 52% done by

S1 and 60% done by S2 assessed using the gravimetric analysis. In growth condition, maximum growth was optimized at 1% inoculum size after incubation and in optimization test maximum growth was enhance at pH 8.0 and temperature 35 °C after 48 hrs incubation. Antibiotics sensitivity test performed for bacterial growth, isolates were responsive to drugs and have no antagonise impact of soil-friendly microorganisms. The current study was to isolate, characterise, and assess the bacterial population's capacity to degrade oil. This study aims to make some recommendations for the development of bacterial remediation.

**Keywords: Bioremediation, Potential, Petroleum Hydrocarbon, Hydrocarbon degradation, antagonise impact**

## INTRODUCTION

The most frequent environmental contaminants are petroleum hydrocarbons, and oil spills provide a serious threat to both habitats on land and in the ocean. Anytime oil is produced, transported, stored, or processed, oil contamination could happen accidentally or as a result of operations or used on land or at water. Oil spills are a serious environmental threat since they significantly harm the local ecosystems [1]. Petroleum hydrocarbons are toxic chemicals, which enter the environment in large volume through numerous routes. One of the main routes that petroleum oil enters the oceanic environment is by seepage from natural sources. Global economic growth has accelerated during the past ten years, driving increased consumption of crude oil from 86.4 million barrels per day in 2010 to 96.5 million barrels per day in 2021. In 2019, almost 84% of the energy used worldwide is still derived from fossil fuels [2]. The Arabian Gulf region, including both land and offshore wells, produces more than 50%

of the crude oil used worldwide. There were 550 oil spills recorded. 14,000 barrels were spilled in events from 1995 to 1999, and 11,000 barrels were spilled from 2000 to 2003. Various activities in the petroleum sector, both onshore and offshore, have the potential to contaminate aquifers and soil with petroleum products [3]. Crude oil can adsorb the rocks surface and immigrate through porous surfaces which may cause aquifer contamination. The rock's density and the oil's movement in terms of viscosity determine. Characteristics with regard to permeability and porosity. Rainfall may facilitate the spread of contaminants into groundwater and agricultural territory [4]. The contamination of soils results in significant changes to the microbial, physical and molecular characteristics of the soil [5]. The effects of these pollutants on the environment, such as contaminated soil and ground water, have a negative influence on both human and animal health as well as the health of the entire ecosystem [6]. As a

consequence of substantial soil, river and stream, and groundwater pollution brought on by oil extraction, the indigenous and rural inhabitants their experienced cancer outbreaks, birth deformities, miscarriages, and other illnesses [7].

As a bioremediation technique, composting is now regarded as the best ways to remove oil pollution because it has a significant benefits, such as cheap initial and ongoing costs design and functioning simplicity. Decomposition has shown to breaking down fossil fuel compounds in a field or laboratory setting [8]. In-vessel composting is acted upon in closed systems, enabling the choice and maintenance of optimal working conditions for microbial activity and contaminant degradation, as well as temperature, moisture level, and mixing ratios [9].

Several techniques, including physical and chemical are presently available to clean up these dangerous hydrocarbons from the environment [10]. However, due to the low expansive, effectiveness, and quick development of microorganisms on growth media, biological techniques are frequently preferred over physical and chemical methods for the biodegradation of contaminants [11]. The chemical components that make up crude oil number in the thousands. Every kind of oil has a different composition, so there are various methods to deal with them using microbes

and flora. Bioremediation can take place spontaneously or additionally with both bacteria and chemicals [12]. Various bacterial strains were used in the bioaugmentation method to recover the oil wastes [13]. Different microbe species attack various petroleum compounds differently, so different petroleum compounds biodegrade concurrently but at various rang. Microorganisms produce enzymes that target the hydrocarbons molecule when carbon sources are present. It takes a different enzymes and metabolic processes to break down the hydrocarbons found in gasoline. But the absence of a suitable Enzymes either defend against assault or serve as a barrier to full hydrocarbon degradation [14].

The problem of pollution brought on by crude oil components is very severe. Hydrocarbon-degrading bacteria are essential economically, but they also pose serious risks to the environment. On the premise of previously research pertaining to recent advancements in the field of bacterial remediation of hydrocarbons, the goal of this study is to offer some recommendations for the creation of bacterial hydrocarbon remediation.

## **MATERIALS AND METHODS**

### **Sample collection of contaminated soil**

The soil samples were collected from garages situated in 2 different sites, Vadodara and Mandavi (Surat), Gujarat,

India. The soil samples were stored in sterile collection bottles for further assays.

### **Culture enrichment and isolation of bacteria**

The selective enrichment method was used to isolate the microorganisms. The petroleum oil used as the primary source in experiments. For the isolation of bacteria, BH broth was used with crude oil which is supplemented with 1 gram of contaminated soil sample. The broth medium incubated at rotatory shaker on weekly basis. The samples from main enrichment (1/100) were transferred to fresh BH broth and streaked on NA media which was incubated at 37°C for 24-48 hrs [15].

### **Identification of bacteria**

The identification of isolates was done by using morphological and biochemical characterization. The colony characteristics of isolates was studied on NA medium. Gram's reactions, biochemical test and carbohydrate fermentations tests were performed to unfold the morphological and metabolic properties of isolates respectively [16].

### **Growth Curve of bacterial isolates**

To study the growth pattern of isolates, the isolates were inoculated in nutrient broth and incubate for 48 hrs at room temperature. After the incubation bacterial growth was observed in every 6 hours using spectrophotometer keeping optical density at 600 nm.

### **Crude oil biodegradation assay**

The degradation assay was performed in lowest (1%) and highest (10%) concentration of crude oil. Flask was subjected for biodegradation assay including one control and all flasks were incubated on a shaker for 5 days. Samples were removed periodically at 24, 48, and 72 hrs of incubation and the final concentration of bacterial growth observed at 600 nm optical density using UV-Vis spectrophotometer.

### **Biodegradation efficacy by gravimetric analysis**

The oil biodegradation ability of isolates was analysed by gravimetric analysis. 100 ml of strain cultures were measured gravimetrically in a conical beaker. In order to stop bacterial activity, (CH<sub>3</sub>)CO and C<sub>6</sub>H<sub>14</sub> (1:1) were mixed with 1N HCL and added in the flasks. After 20 minutes, the solution was separated into three layers: an organic layer on top, an aqueous layer in the centre, and a biome layer at the bottom. The top layer was treated with sodium sulphate [17]. After the organic layers had been broken down, the oil layer had been placed in a hot air oven set to 50°C to enable the solvent to evaporate, the total weight of the beakers was calculated. After the complete evaporation process, the oil residue was weighed and used as the gravimetric value for additional calculations [18].

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% degradation = Amount of oil degraded/  
Amount of oil added in the media × 100

### **Effect of pH and temperature on bacterial growth**

The isolates were cultured on BH broth with oil and the pH of media was adjusted in the range from 5.0, 8.0, and 10.0. Later, the inoculated cultures were incubated at various temperatures such as 25°C, 35°C, and 45°C, to determine the ideal temperature for the greatest growth of the isolated bacteria. The optical density (OD) of all the samples was measured after 24hrs by UV-Vis spectrophotometer at 600 wavelength [19].

### **Effect of the inoculum size on bacterial growth**

The different size of the inoculum affects the bacterial growth. Therefore, in inoculum size test, nutrient broth media was prepared in three different concentrations of culture viz. 1%, 2%, 3% with one control (without addition of an inoculum). Later, all the flask were incubated on shaker for 24 hrs. After the incubation, the growth was observed at 600nm optical density using by UV-Vis spectrophotometer.

### **Antibiotics sensitivity test for bacterial growth**

The antibiotics activity was performed against test organisms. In this study antibiotic susceptibility was checked using antibiotic discs. The antibiotics that were widely accessible: Ampicillin/Cloxacillin

(10 µg/disc), Streptomycin (25 µg/disc), Chloramphenicol (50 µg/disc), Kanamycin (5 µg/disc). The bacterial activities were estimate by measuring zone of inhibition on Muller Hinton agar plates.

## **RESULTS AND DISCUSSION**

### **Sample collection of contaminated soil**

The contaminated soil samples were collected from two different. One was from Vadodara and another was from Mandavi (Surat), Gujarat, India (**Figure 1**). The samples were collected from garage and transferred to the lab to examine their physiological properties. The physiological properties of soil sample 1 and sample 2 are illustrated in **Table 1**.

### **Isolation of oil degrading bacteria**

There were 2 potent bacterial isolates were obtained from 2 different samples using BH medium. After 5 days incubation of samples in BH medium, the oil degrading bacteria were isolated on nutrient agar medium. A total of 6 bacterial colonies were observed, however, the 2 most potent were selected for further assays (**Figure 2**).

### **Identification of oil degrading bacteria**

The bacteria were identified based on morphological and biochemical characteristics. Both the isolates S1 and S2 showed creamy white colour colony on Nutrient agar. Gram's staining revealed that both the isolates are Gram positive, and short rod shaped. Biochemical test, carbohydrate fermentation characterization

of the isolates was performed with the procedure outlined in Bergey's Manual of Systematic Bacteriology. The results are illustrated in **Table 2**. The Gram's staining and biochemical analysis proposed that isolates might belong to *Bacillus spp.*

#### **Growth of bacterial isolates:**

The bacterial growth pattern of isolates was studied by bacterial growth curve. The lag phase represents the inoculum of cells into fresh medium, therefore, the population remain unchanged. In exponential phase the cells begin to increase, and determining the maximum growth rate and doubling time of bacterial population. As shown in **Figure 3**, both isolate 1 and isolate 2 had approximately similar growth pattern in terms of time and OD. At last, no net growth of bacterial population was observed in stationary phase.

#### **Crude oil biodegrading assay**

Estimation of biodegrading assay for the two isolates at two different concentration is represented in **Figure 4**. A reaction with a lowest (1%) concentration of oil showed a highest rang of oil degradation, whereas a reaction with a highest (10%) concentration of oil showed a lowest range of oil degradation at the conclusion of the test reaction with bacterial isolates.

#### **Biodegrading efficacy by gravimetric analysis**

The degrading efficacy of the isolates was determined by gravimetric analysis. The

biodegradation of oil was observed to be as 52% by S1 and it was found to be 60% by S2.

#### **Effect of pH and temperature on bacterial growth**

In this test the growth condition of the isolates was optimized. Isolated bacteria were cultured in various pH and temperature conditions, as well as incubated for varying amounts of time viz., 24h, 48h, 72h, in order to explore the ideal growth conditions. The isolates strain showed highest growth at pH 8.0 after 48 hrs of incubation and to detect optimum temperature for their highest growth, showed after 48 hrs incubation at 35 °C (**Figure 5**).

#### **Effect of inoculum size on bacterial growth**

To identify the effect of inoculum size of the isolates, the was isolates were inoculated in different inoculum size viz. 1%, 2%, and 3%. It was observed that both the isolates showed maximum growth at 1% inoculum size and lowest growth was observed at 3% inoculum size (**Figure 7**).

#### **Antibiotic sensitivity test for bacterial growth**

The oil degrading bacterial isolates were tested against the antibiotics for studying antibiotics susceptibility. The highest zone of the isolate S1 was 18 mm and S2 was 17 mm against kanamycin (5 mcg/disc). The effect of other antibiotic susceptibility test is showed in **Table 3**.



Figure 1: Sample collection of contaminated soil

Table 1: The physiological properties of obtained samples

Physiological properties	Soil sample	
	Vadodara	Mandavi, Surat
Place of sample collected	Vadodara	Mandavi, Surat
Name of sample	S1	S2
Volume	1g	1g
pH	8.0	8.2
Colour	Brownie's black	Black
Consistency	Loose	Semi tightly bound
Moisture content	1.62%	3.09%
Water holding capacity	48.5%	46.5%

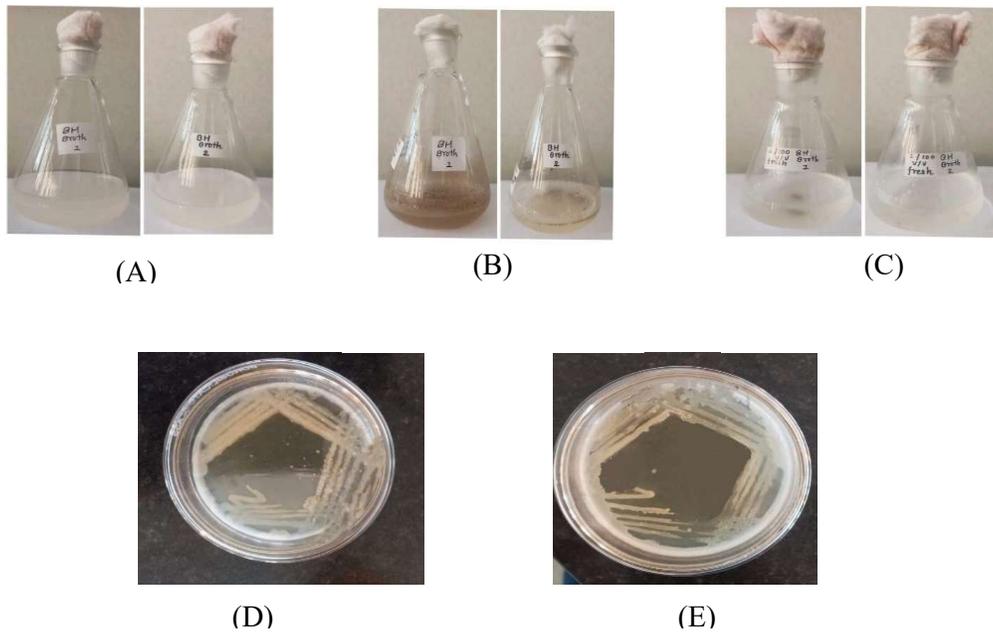
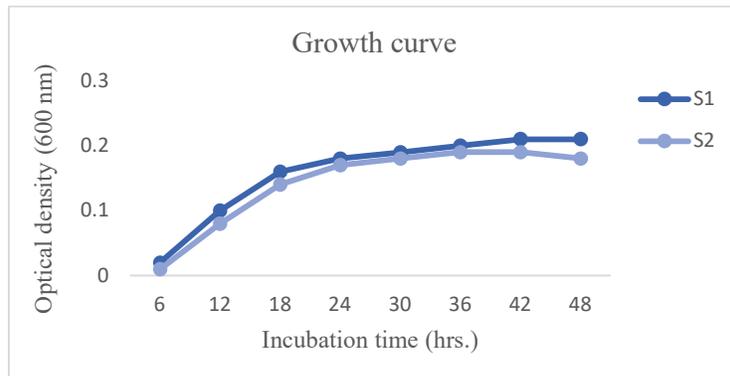


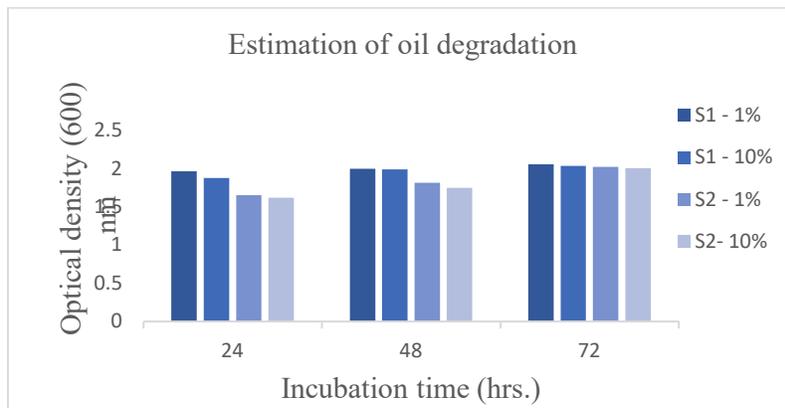
Figure: 2 Isolation of oil degrading bacteria. (A) 100 ml BH broth (B) after 5day incubation at rotatory shaker at room temperature (C) 1/100(v/v) fresh BH broth with 1 ml of old culture in fresh media (D) NA plate with isolate S1 (E) NA plate with isolate S2.

**Table 2: Results of biochemical analysis isolate 1 and isolate 2**

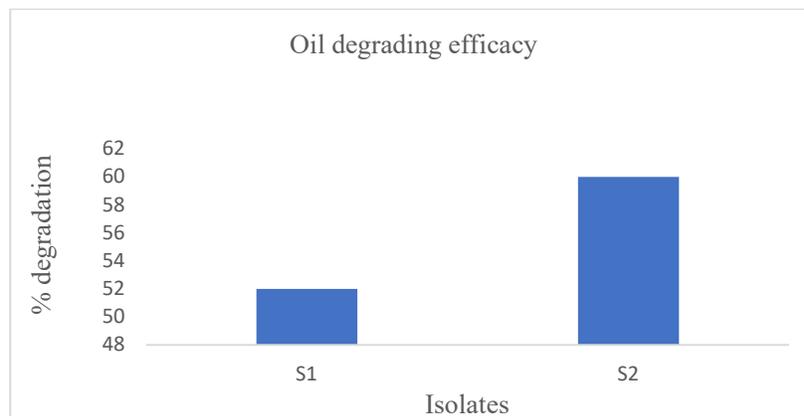
Sr. No	Name of the tests	Results	
		S1	S2
1	Methyl red test	+	+
2	Voges-Proskauer's test	-	-
3	Indole test	-	-
4	Catalase test	+	+
5	Urease hydrolysis test	-	-
6	Citrate test	+	+
7	Triple sugar iron test	+	-
8	Starch hydrolysis test	-	-
9	Carbohydrate fermentation test		
	• Sucrose	-	+
	• Dextrose	+	+
	• Lactose	+	-



**Figure: 3 Growth of bacterial population**



**Figure: 4 Estimation of oil degradation**



**Figure: 5 Oil degrading efficacy**

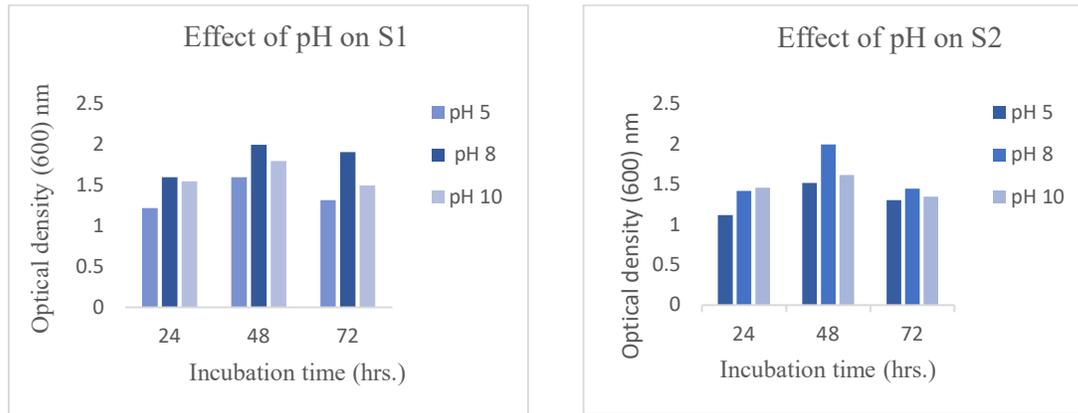


Figure 5: Effect of pH and Temperature on growth of isolates

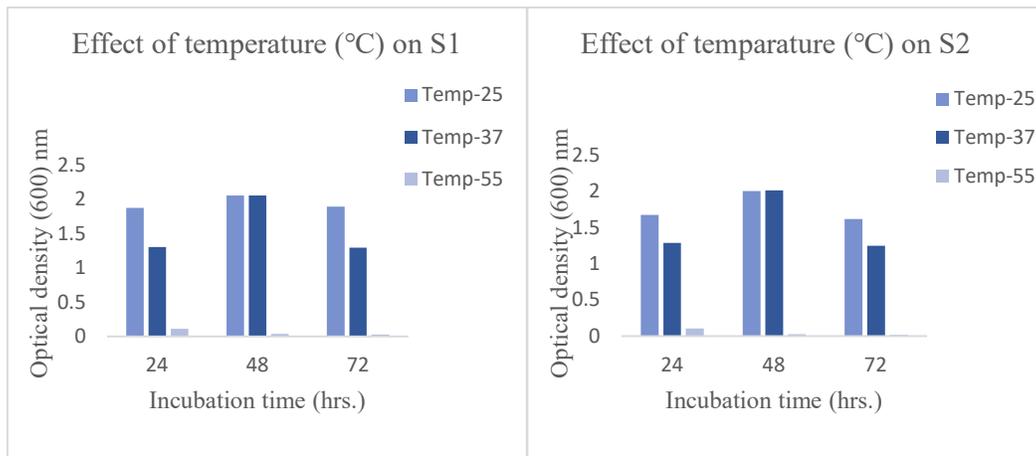


Figure: 6 Effect of Temperature on growth of isolates

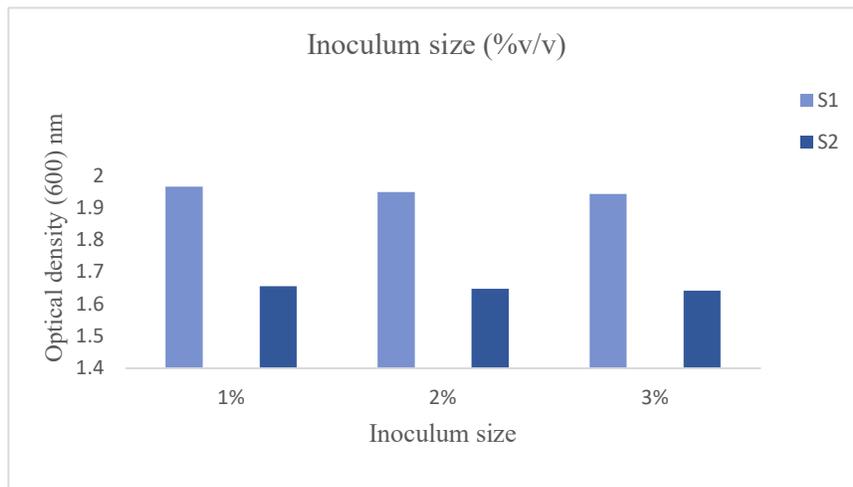


Figure: 7 Effect of inoculum size on bacterial growth

Table: 3 Result of antibiotic sensitivity test

Antibiotics	Zone of inhibition (mm)	
	S-1	S-2
Kanamycin (K 5)	18 mm	17 mm
Streptomycin (S 25)	15 mm	16 mm
Chloramphenicol (C 50)	8 mm	-
Ampicillin/Cloxacillin (AX 10)	-	-

## CONCLUSION

Degradation by microorganisms within the ecosystem is a challenging process for petroleum hydrocarbons as well as other associated pollutants. Numerous microbes assist in the degradation of contaminants like hydrocarbons. In the recent research of the hydrocarbon degrading bacteria were isolated from petroleum contaminated soil. With the help of all the process it can be concluded that the isolated strains of *Bacillus spp.* have bioremediation potential for the oil degradation.

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