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ISOLATION AND IDENTIFICATION OF HYDROCARBON DEGRADING BACTERIA FROM ENVIRONMENTAL SOURCE

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ABSTARCT

Petroleum hydrocarbons are classified as high-priority pollutants. Due to their high demand, they are transported through terrestrial and aquatic routes. Their accidental spillage may contaminate soil and water. Due to their complex structure, it is difficult to degrade these pollutants. To evaluate bioremediation techniques for these pollutants samples from contaminated environmental sources were collected aseptically. *Pseudomonas aeruginosa* and *Bacillus subtilis* were isolated from samples by evaluating their growth potential in Bushnell Haas (BH) broth. The biostimulation effect was tested by mixing animal dung from open heaps. The measured OD value in test tubes with animal dung was greater than others. Data was analyzed statistically by applying ANOVA. The P-value obtained from one-way ANOVA (P=0.0116) showed that the results are significant.

Key Words: Biodegradation, Bioaugmentation, Biostimulation, Bioremediation, Hydrocarbons, Organic Matter, Decomposition.

INTRODUCTION

Over the last 50 years, significant human population expansion has led to an increased demand for agricultural goods and food. Large-scale oil leaks have happened in Canada and the United States. (Hassan *et al*, 2019). Research has shown the link between oil spills and environmental damage to land and water. In Iraq, where unrecovered spilled petroleum products migrate to streams and rivers and eventually accumulate in soil, contamination by these products is common (Almansoory *et al.*, 2019). Besides incidental adulteration of the ecosystem, large volumes of oil sludge creates serious problems because many of the management processes used in the decontamination of groundwater and soil have limited application and are expensive (Das and Mukherjee, 2007). Petroleum pollution in soil is mutagenic, impairs seed sprouting and shoot development, and restricts reproduction in annelid worms. Ecosystems in colder climates are assumed to be more vulnerable to leaks, and most of the sub-Antarctic flora and fauna have been demonstrated to be prone to petroleum pollution (Errington *et al*, 2018). These hydrocarbon contaminations are harmful to plant health and have carcinogenic, mutagenic, and strong immune-toxic impacts on human and animal health. Because pollutant accumulation in plant and animal tissue can cause mortality or mutations (Hossain *et al.*, 2022). Physical and chemical treatments can be used to remove petroleum hydrocarbon (PH) contamination, allowing for the recovery of the adsorbed materials, albeit this is a costly procedure. For a long time, many conventional procedures such as landfilling, cremation, and air sparging have been used to eradicate these hydrocarbons for the cleanup of oily waste (Abou-Shanab *et al.*, 2016). Recent developments in bioremediation methods have been made during the last 20 years with the ultimate aim of efficiently and inexpensively restoring damaged areas. There is no one bioremediation method that can be used as a "magic bullet" to clean up damaged habitats, even though researchers have created and simulated a variety of bioremediation strategies (Azubuike *et al.*, 2016). The microbiological activities occurring in the soil are frequently ignored by soil quality evaluation methodologies, without them, life on Earth would be impossible. Rhizosphere microbe populations have a role in soil structure creation and organic matter breakdown, as well as nutrient availability and plant production (Borowik *et al.*, 2021). Bioremediation is the process of utilizing the metabolic capacities of microorganisms for TPH uptake and breakdown into less hazardous substances or their removal from polluted soil (Sari *et al.*, 2019). The microbial mechanisms involved in bioremediation are frequently a part of metal redox cycling or carbon cycling and are typically natural constituents of respiration or adaption. As a result, bioremediation frequently takes place without any direct human involvement (Krzmarzick *et al.*, 2018). Bio-augmentation is a well-known approach for bioremediation of contaminated soils. Several studies have demonstrated the effectiveness of microbial groups in the biodegradation of diesel and fuel oil (Chen *et al.*, 2020). Phytoremediation is a low-cost method that may also be used to clean up hydrocarbon spills. However, it is a protracted process that results in competition for limited nutrients between plants and microbes. Microbial

remediation (biostimulation and bioaugmentation), in contrast to these methods, may provide faster effects and is far more efficient and cost-effective (Aziz *et al.*, 2020). Successful bioremediation necessitates the selection of an appropriate algal, bacterial, or fungal strain, as well as the identification of microbial ecology, type of pollution, and other environmental conditions. Microbes are also involved in the synthesis of biosurfactants, which have a wide range of applications in the environment, particularly in bioremediation (Naeem and Qazi, 2020). Because of its advantages over other remediation approaches microbial remediation is gaining popularity because of their high efficiency cost-effectiveness, and environmentally favorable treatments. The use of bacteria along with fungi in monocultures or co-culture consortia is a common bioremediation strategy and these bacterium-fungi interactions have been well documented. Surprisingly most bio-surfactant-producing bacteria ensure that biodegradation is more successful by breaking target hydrocarbons therefore boosting their availability to the bacteria (Atakpa *et al*., 2022).

MATERIALS AND METHODS

Sample Collection

Soil samples contaminated with diesel and engine oil hydrocarbons were collected from petrol pumps, automobile workshops, and contaminated parts of vehicles in sterilized polythene bags in the district of Toba Tek Singh. Sample from decomposing organic matter was also collected from rural areas of district Toba Tek Singh. Collected samples were transported to the BSL-II lab Institute of microbiology, University of Agriculture Faisalabad and used for the isolation of hydrocarbon-degrading bacteria.

Media preparation

Bushnell Haas broth, mineral salt agar, Cetrimide agar and nutrient agar were prepared according to normal composition. The appropriate components were put in distilled water and heated until they were thoroughly dissolved. The broth and media's pH was kept at 7.5 throughout this process. Following that, the broth and medium was autoclave sterilized at 121 °C for 20 minutes before being poured into sterilized flask and Petri plates. Plates were arranged on a flat surface, let to harden, and then kept in storage at a temperature of 4°C.

Isolation of Hydrocarbon Biodegrading Bacteria

Samples from each source were inoculated in Bushnell Haas broth supplemented with 0.7 ml diesel and engine oil and incubated at 37 °C for 7 days. OD values of each inoculated sample were measured by spectrophotometer at 600 nm. After seven days inoculum was grown on nutrient agar and mixed colonies were observed.

Identification of Bacterial isolates

Microscopically, bacteria were identified by looking at colony morphology, surface pigmentation, shape, margin, and surface on nutrient agar plates. Gram staining was used to identify staining behavior, shape, cell arrangement, and granulation, and spore staining was used to look at bacteria that produce spores.

Morphological characteristics

Morphological features of bacteria were determined under microscope after Gram staining (Cruickshank, 1979).

Biochemical identification of isolates

For identification of isolates biochemical tests were performed by following Berge's manual.

Purification of Bacterial isolates

Based on morphological and biochemical identification bacterial colonies isolated on nutrient agar were cultured on Cetrimide agar and mineral salt agar. *Bacillus subtilis* and *Pseudomonas aeruginosa* were purified from the hydrocarbon contaminated samples which were able to utilize diesel and engine oil as carbon and energy source for their growth.

Data analysis

All data examined using one way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS), (Shahaby *et al.*, 2015). Variances among mean values for treatments at $P<0.05$ were evaluated using Post hoc test (Tukey's test). The data is presented as mean ± standard error. Time course of growth for bacterial isolates cultured in In Bushnell Haas broth supplemented with 0.7 ml petroleum hydrocarbon for 7 days

RESULTS

Isolates from soil samples

Soil samples were inoculated in Bushnell Haas broth. Colonies of bacteria were cultured on Nutrient agar and were identified by culturing on mineral salt media and cetrimide agar, Grams staining and biochemical tests. Genus *Bacillus* and *Pseudomonas* were isolated and identified (Table 1-5).

Table 1. List of bacteria isolated from different soil samples.

Soil sample	Isolate from soil sample
D1	*Bacillus sp*
D2	*Bacillus sp, Pseudomonas aeruginosa*
D3	*Bacillus sp,*
D4	*Bacillus sp*
E1	*Bacillus sp*
E2	*Bacillus sp*
E3	*Bacillus sp*
E4	*Pseudomonas aeruginosa, Bacillus subtilis*

D1: Soil sample collected from petrol pump, provided with diesel.
D2: Soil sample collected from workshop, provided with diesel.
D3: Soil sample collected from decomposing organic matter, provided with diesel.
D4: Soil sample collected from vehicle parts, provided with diesel.
E1: Soil sample collected from petrol pump, provided with engine oil.
E2: Soil sample collected from workshop, provided with engine oil.
E3: Soil sample collected from decomposing organic matter, provided with engine oil.
E4: Soil sample collected from vehicle provided with engine oil.

Table 2. Cultural, Morphological and Biochemical characteristics of *Bacillus* sp.

Cultural characteristics	
Shape	Round
Color	White/Pale
Surface	Dull, wrinkled
Margin	Irregular
Morphology	
Straight rods	+
Cocci	-
Gram stain	Gram positive
Cell arrangement	Short chain, Single
Spore staining	+
Biochemical tests	
Amylase	+
Glucose fermentation	-/-
Methyl red	-
VP	+
Oxidase	+
Catalase	+

Bacillus sp. are identified by observing their cultural characteristics, their morphology and by performing biochemical tests. Their colonies are round, pale rankled and irregular, their cells are gram positive rods and contain spores (Table 2). Colonies of *Pseudomonas* sp were round, pale, convex and undulate, gram-negative rods, and non-spore-producing bacteria (Table 3).

Table 3. Cultural, Morphological and Biochemical identification of *Pseudomonas* sp.

Cultural Characteristics	
Shape	Round
Color	Pale
Surface	Convex
Margin	Undulate
Morphology	
Straight rods	+
Cocci	-
Gram stain	Gram negative
Cell arrangement	Short chains, single
Spore staining	-
Biochemical tests	
Amylase	+
Glucose fermentation	-/-
Methyl red	-
VP	-
Oxidase	+
Catalase	+

Table 4. Differentiation among isolates of *Bacillus* species.

Bacterial isolates	Gram stain	Shape	Starch hydrolysis	Motility	VP	Citrate
Bacillus subtilis	+	Rods	+	+	+	+
Bacillus cereus	+	Rods	+	+	+	----
Bacillus.megaterium	+	Rods	+	+	-	----

Strains of *Bacillus* sp. were differentiated from each other by performing different biochemical test, positive citrate utilization test showed that the isolated bacterial strain was *Bacillus subtilis.*

Table 5. OD value at 600 nm comparison of seven days incubation.

Sample	Day 1	Day 3	Day 5	Day 7	Average Value
D1	0.384	0.398	0.411	0.438	0.407625
D2	0.378	0.383	0.387	0.396	0.385875
D3	0.381	0.6	0.85	1.209	0.76
D4	0.371	0.38	0.3885	0.406	0.3863125
E1	0.381	0.418	0.455	0.529	0.44575
E2	0.378	0.417	0.4555	0.533	0.4458125
E3	0.381	0.65	0.95	1.306	0.82175
E4	0.371	0.394	0.4165	0.462	0.4108125
			grand mean		0.507992188

D1: Soil sample collected from the petrol pump, provided with diesel.
D2: Soil sample collected from the workshop, provided with diesel.
D3: Soil sample collected from decomposing organic matter, provided with diesel.
D4: Soil sample collected from vehicle parts, provided with diesel.
E1: Soil sample collected from the petrol pump, provided with engine oil.
E2: Soil sample collected from the workshop, provided with engine oil.
E3: Soil sample collected from decomposing organic matter, provided with engine oil.
E4: Soil sample collected from vehicle parts, provided with engine oil.

Table 6. One way ANOVA results on time course of growth of selected bacterial isolates during culturing in BHB supplemented with 0.7 ml petroleum hydrocarbon.

One Way ANOVA results on time course of growth of selected bacterial isolates.					
	sum of squares	Degree of Freedom (DF)	Mean Square	F	Probability of random F ratio
between groups	0.875713295	7	0.125101899	3.389780014	0.006284234
Within Groups	0.885734641	24	0.03690561		
Total	1.761447936	31			

DISXUSSION

Accidental oil pollution has damaged society and the environment and has become a common occurrence in modern times. In addition to unintentionally harming environments, the enormous quantities of oil sludge produced in refineries by water oil separation arrangements and the accumulation of waste oily materials in crude oil storage tank bases represent a severe hazard. This is because several common decontamination methods for soil and groundwater have constrained uses, are excessively costly, or may only be partially successful (Ferrari *et al.*, 1996; Brown, 2020; Vasudevan and Rajaram, 2001). As a result, despite decades of research, efficient bioremediation of hydrocarbon-contaminated soil is still a challenge. Both on-site and in situ treatment strategies minimize the technical and cost limitations by using microorganisms to break down harmful organic environmental pollutants. It is possible that *Pseudomonas aeruginosa* and *Bacillus subtilis* strains are efficient hydrocarbon degraders given their ability to thrive on a variety of hydrocarbon molecules as sources of carbon and energy. These strains were recovered from a soil sample polluted with petroleum that included components from contaminated cars, workshops, and organic materials in the process of decay.

The goals of this work were to isolate and characterize the bacteria that degrade gasoline's hydrocarbons and to investigate how nutrients affect bacterial growth in the presence of hydrocarbons. The persistence of microbes in petroleum hydrocarbon media after their inoculation is a significant deciding factor in the rate of hydrocarbon biodegradation, whether in the soil or liquid phase. Every bacteria utilized in this study was isolated from a sample of soil that had been polluted with petroleum, therefore they were all able to swiftly adapt to the soil and liquid environments that had been contaminated with oil, as described by other authors (Rahman *et al.*, 2003; (Sugiura *et al.*, 1997). This was shown by the substantial increase in soil *B. subtilis* and *P. aeruginosa* numbers relative to control levels. *Pseudomonas* strains may develop more quickly than *Bacillus* strains because the former strains break down and consume more petroleum hydrocarbons. Additionally, because the native soil bacteria were inefficient degraders of a variety of multifaceted compounds of crude petroleum oil, the introduction of competent hydrocarbon degraders would be required to properly decompose all of the hydrocarbons in a complex petroleum mixture. Since the inclusion of decomposing organic materials as a co-carbon source accelerated the pace. For isolation and identification of bacteria that were able to utilize petroleum hydrocarbons as their source carbon soil samples from contaminated soil with hydrocarbons were collected aseptically in sterilized polythene bags from petrol pumps, automobile workshops, contaminated parts of vehicles and from organic matter decomposing areas. Collected soil samples were diluted in 9 ml of distilled water in test tubes by mixing 1 gram of soil in test tubes. To test if bacteria could break down a hydrocarbon source, Bushnell Haas broth, mineral salt agar, nutrition agar, and cetrimide agar were made. Hydrocarbons are the only sources of carbon in Bushnell Haas broth. To gauge an organism's capacity to break down hydrocarbons, Bushnell-Haas Broth (also known as Bushnell-Haas marine salts broth) is produced in the manner outlined by Bushnell and Haas in their original paper. Since there is no carbon source in its formulation, other hydrocarbons can be added, including light and heavy mineral oils, kerosene, paraffin wax, and gasoline. The Society for Industrial Microbiology Committee on Microbiological Deterioration of Fuels suggested Bushnell-Haas Broth for the microbiological analysis of fuels (McFarlin *et al.*, 2018)

Conclusion

This study analyzed and identified bacteria that were growing in hydrocarbon-contaminated soils, when these bacteria were grown in the laboratory they were able to grow by using hydrocarbon as their source of carbon.

Decomposing organic matter enhanced the growth of bacteria when mixed in growth media provided with a hydrocarbon source.

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