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Original Research

Characterizing Microbial Populations in Petroleum-Contaminated Soils of Swat District, Pakistan

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Abstract

Soil samples from petroleum-contaminated soil were collected from 25 different petroleum filling stations and automobile workshops in the district of Swat, Khyber Pakhtunkhawa, Pakistan. A total of seven bacterial genera were isolated. All of the isolates were Gram-positive bacteria. The genera identified by the culture and cell morphological characteristics were: *Bacillus, Streptococcus, Staphylococcus, Micrococcus, Corynebacterium Arthrobacter*, and *Streptomyces*. Lipolytic and saline activities of the selected isolates were studied. Among the isolates, *Arthrobacter, Staphylococcus, Bacillus, Micrococcus, Corynebacterium*, and *Streptomyces* produced lipase enzymes, while no lipase was produced by *Streptococcus*. Dense growth of *Bacillus* and *Streptococcus* was observed at 1% NaCl. Dense growth of *Streptomyces* was observed at strength of 2% NaCl. At 3% NaCl concentration, dense growth of Staphylococcus, Micrococcus, *Corynebacterium*, and *Arthrobacter* was observed, indicating that they were moderately halotolerent. In our study, *Bacillus, Arthrobacter*, and *Streptomyces* showed optimum growth at pH 8.0, and *Streptococcus*, *Staphylococcus*, and *Micrococcus* showed optimum growth at pH 7.0. Only *Corynebacterium* showed optimum growth at pH 9.0, indicating that it is tolerant of higher pH conditions.

Keywords: Hydrocarbons, Bioremediation, *Arthrobacter, Staphylococcus, Bacillus, Micrococcus, Corynebacterium, Streptomyces*

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Introduction and Background

Petroleum is widely used across the globe as a primary source of energy and fuel [1]. In 2003 its estimated consumption was about 13.1 billion liters per day and is increasing daily [2]. Hydrocarbons are the main constituent of petroleum and are the major cause of environmental pollution [3]. Hydrocarbons are biological in origin [4]. Land disposal of pollutants is the simplest and easiest method, although it may be re-exposed to the environment by landsliding or other activities. Similarly, burning of pollutants may produce carbon dioxide, which is a potential hazard to the environment in the forms of air pollution and global warming. Furthermore, both land disposal and incineration are time-consuming and not safe for living organisms [5].

Bioremediation is the process by which much environmental pollution (including petroleum products) is converted to less toxic or harmless substances by using microorganisms or their metabolic capabilities. The resulting products can be carbon dioxide, water, and other simpler compounds that do not affect the environment [6].

The rate of pollutant degradation is affected by several physical, chemical, and biological factors such as pH, temperature, nutrients, water and oxygen availability, type of organism, and nature of pretreatment [7]. In soil environment the highest degradation rates generally occur in the range of 30-40ºC, while in some freshwater environments it is 20-30ºC and in marine environments it is 15-20ºC with a normal pH of 6-8 under aerobic conditions [8, 9] for isolated *Pseudomonas, Staphylococcus, Micrococcus, Salmonella, Cellobiococcus,* and *Pneumonia* from oil stations and auto mechanic workshops. These genera obtained optimal growth at different temperatures between 20ºC and 90ºC and at different pH levels, ranging from acidic to alkaline. Similarly, bacterial genera *Pseudomonas* and *Rahnella* showed a higher ability to degrade naphthalene at 4ºC [10]. *Micrococcus* spp., *Pseudomonas* spp., and *Bacillus* spp. can degrade hydrocarbons at normal pH (68) and temperature (25-40ºC) under aerobic conditions [8].

Various types of bacteria such as *Acinetobacter, Aeromonascaviae, Bacillus*, *Bravibacterium*, *Citrobacter, Citrobacterkoseri, Corynebacterium, Enterobacter*, *Erwinia, Eschericha*, *Gordonia, Klebsiella, Maltophilia Micrococcus, Micromonospora*, *Mycobacterium, Neisseria, Nocardia, Proteus, Pseudomons, Rhodococcus, Sphingobacterium, multivorum, Stenotrophomonas,* and *Streptococcus* could degrade a high percentage of hydrocarbon pollution [11-14]. In fungi, i.e., *Penicillin* spp., *Aspergillus* spp., *Rhizopus* spp., *Alternaria* spp., and *Cladosporium* spp. could degrade petroleum hydrocarbons [7]. Individual microorganisms can degrade only a low quantity of hydrocarbon pollutants. Mixed culture of microbes can be used to increase the rate of hydrocarbon biodegradation [15]. It was observed that bacterial spp such as *Lysini bacillus, Brevibacillus, Paenibacillus, Alcaligenes, Delftia, Achromobacter,* and *Brevibacteriu motitidis* showed maximum hydrocarbon degradation rates in mixed culture [16].

In Pakistan, especially in the Swat region of Khyber Pakhtunkhwa, limited research has been available on the bioremediation of environmental pollution caused by petroleum products.

Materials and Methods

Sample Collection

For the current study, 25 samples from petroleum-contaminated soil were collected from 25 different locations of petroleum filling stations and automobile workshops around the Swat Valley from Bahrain to Barikot. The samples were collected in sterilized plastic bags and each bag was labeled to show the date and site of sample collection. The samples were then brought to the university laboratory for further processing.

Media Preparation and Sterilization

Nutrient Agar Medium

Nutrient agar media was prepared by adding 28 g of nutrient agar powder to 1000 ml of distilled water. The constituents of nutrient agar media were peptone 5 g/L, meat extract 1 g/L, yeast extract 2 g/L, NaCl 5 g/L, and agar 15 g/L with a pH 7.0, supplemented with 1% (1 mL/100 mL) cycloheximide. The solution was stirred using a magnetic stirrer to ensure the complete dissolution of the compounds. The media was sterilized in an autoclave for 15 minutes at 15 psi and was poured into 90 mm sterilized Petri dishes.

Nutrient Broth Medium

Nutrient broth medium was prepared by adding 13 g of nutrient broth powder to 1000 ml of distilled water. The chemical constituent of nutrient broth was peptone 5 g/L, meat extract 1 g/L, yeast extract 2 g/L, and NaCl 5 g/L with a pH 7.0. The solution was stirred using a magnetic stirrer to ensure that the compound was completely dissolved. The media was sterilized in an autoclave for 15 minutes at 15 psi and was poured into 10 ml sterilized test tubes.

MacConky Agar Medium

MacConky agar medium was prepared by adding 50 grams of MacConky agar to 1000 ml of distilled water. Its chemical composition was peptone 17 g/L, protease peptone 3 g/L, lactose 10 g/L, bile salt 1.5 g/L, sodium chloride 5 g/L, neutral red 00.3 g/L, crystal violet 0.001 g/L, and agar 13.5 g/L with neutral pH 7.0. The solution was stirred using a magnetic stirrer to ensure that the compound was completely dissolved. The media was sterilized in an autoclave for 15 minutes at 15 psi and poured into 90 mm sterilized Petri dishes.

Effect of Temperature on the Growth of Isolates

The growth rate of the isolates was checked at different temperature ranges from 20ºC to 40ºC for temperature optimization.

Isolation of Bacteria

Soil samples were serially diluted (10^{-1} to 10^{-9}) and 10^{-6} tube samples were inoculated on the plates with the help of a wire loop and kept for 24-48 hours at 37ºC for the growth of bacterial colonies. Bacterial colonies were subsequently streaked three to four time on fresh plates of nutrient agar media for pure colonies isolation, then the fresh culture were kept at 37ºC for 24-48 hours and stored in the refrigerator at 4ºC for future use.

Identification of Bacteria

The following tests and procedures were used to verify the identity of the screened and selected bacteria for hydrocarbon degradation.

Morphological Characteristics

Selected isolates were characterized by colony morphology on nutrient agar, Gram staining, and morphological characteristics like colony size, shape, structure, opacity, elevation, pigmentation, and margin as describe by Holt et. al (1994) for identification of the isolates [17].

Gram Staining Procedure

The Gram staining technique was used for differentiation between Gram positive and Gram negative bacteria. A drop of sterilized water was placed on a neat and clean glass slide, and a single isolated colony of 24-72 hours-old culture was mixed in it. The smear was made by spreading the culture. This smear was air dried and fixed by rapidly passing the slide three times over the flame and flooded with crystal violet for one minute and gently washed with distilled water, then Gram iodine was poured on the slide for another minute and gently washed with distilled water. The slide was drained by acetone for 10-20 sec and gently washed with distilled water. Finally safranin was poured on the slide for one minute and it was gently washed with distilled water, after which the slides were kept under bibulous paper, air-dried, and examined under the oil immersion objective lens (100x) of a light microscope.

Selective Media

Gram-negative bacteria were inoculated on MacConky agar media for isolate confirmation and Gram-positive were inoculated on nutrient agar media.

Growth Rate of Isolates at Different Salt Concentrations (Saline Activity)

The isolates were grown at nutrient agar media (as mentioned earlier) supplemented with 1-3% (w/v) NaCl for saline activity.

Lipolytic Activity

Selected isolates were grown at 1% (v/v) Olive oil for the determination of lipolytic activity and lipase production. The bacterial colonies that produced a shallow zone around them were considered lipase positive, while those colonies with no shallow zone around them were considered lipase negative.

Effect of pH on the Growth of Isolates

The growth of isolates was checked at various pH levels (5-10). Diluted HCl was added drop by drop to the growth medium for acidic pH, and basic pH was adjusted by adding NAOH to the medium.

Results

Isolation and Culture Morphological Characteristics of Petroleum Degraders

Among different sampling locations, various bacterial genera like *Bacillus, Streptococcus, Staphylococcus, Micrococcus,* and *Corynebacterium Arthrobacter* were isolated (Table 1). The isolated genera were identified on the basis of morphological characteristics such as colony shape, colony size, colony elevation, marginal outlines, colony opacity, surface, and pigmentation (Table 1).

Cell Morphological and Gram Staining Characteristics of Selected Bacterial Isolates

Cell morphological characteristics like cell size and shape on agar plates were studied and examined. Furthermore, the Gram staining tests were conducted several times for identification of the selected isolates (Tables 2, 3).

Effect of Temperature on Isolate Growth

The effect of Temperature on the growth of selected isolates was checked. Among the *Micrococcus* isolates, *Arthrobacter* and *Corynebacterium* showed low growth at 20ºC, moderate growth at 25ºC, and dense growth at 30-37ºC. Similarly, *Staphylococcus, Bacillus, Streptococcus*, and *Streptomyces* also showed low growth at 20ºC, moderate growth at 25ºC, and dense growth at 30-37ºC. The most suitable temperature selected based on bacterial growth was 37ºC. This temperature (35ºC) was the incubation temperature employed for further investigation (Table 4).

Table 1. Isolation of petroleum-degrading bacteria from different petroleum-contaminated soil sites.

Sample No.	Name of sample location	Name of Isolates
1	Bahrain petrol pump	*Arthrobacter Bacillus*
2	Bahrain Workshop 1	*Bacillus*
3	Barikot Workshop 1	*Bacillus*, *Staphylococcus*
4	Barikot Workshop 3	*Staphylococcus*
5	Barikot Workshop 4	*Micrococcus*
6	Barikot Petrol Pump	*Staphylococcus*
7	Charbagh Petrol Pump	*Bacillus*
8	Fizagut Workshop	*Corynebacterium*, *Arthrobacter*
9	Fizagut Petrol pump	*Bacillus*
10	Khwazakhela Petrol pump	*Streptomyces*, *Bacillus*
11	Khwazakhela Workshop 1	*Bacillus*
12	Khwazakhela Workshop 2	*Bacillus*
13	Manglawar Petrol Pump	*Streptococcus*
14	Matta Petrol Pump	*Staphylococcus*
15	Mingora Petrol Pump	*Bacillus*
16	Mingora Workshop 1	*Arthrobacter*
17	Mingora Workshop 2	*Bacillus*
18	Odigram Petrol Pump	*Bacillus*
19	Qamber Petrol Pump	*Arthrobacter*
20	Rahim abad Petrol Pump	*Streptococcus*
21	Rahim abad Workshop 1	*Bacillus*
22	Rahim abad Workshop 2	*Bacillus*
23	Satal Petrol Pump	*Arthrobacter*
24	Watkay Workshop 1	*Bacillus*
25	Watkay Workshop 2	*Bacillus*, *Arthrobacter*

Lipolytic Activity of Selected Bacterial Isolates

Lipase activity of selected isolates was studied. Among the *Arthrobacter* isolates, *Staphylococcus, Bacillus, Micrococcus,Corynebacterium,* and *Streptomyces* showed lipase activity by producing a shallow zone around their colonies, whereas Streptococcus did not produce a shallow zone around its colony hence it was lipase negative (Table 5).

Effect of Different NaCl Concentrations on Growth of Selected Bacterial Isolates

The growth of identified isolates was checked for saline activity on Nutrient agar medium enriched with 1%, 2%, and 3% NaCl (w/v) concentrations. *Bacillus* and *Streptococcus* showed dense growth at 1% NaCl, moderate growth at 2% NaCl, and low growth at 3% NaCl. *Staphylococcus, Micrococcus, Corynebacterium,* and *Arthrobacter* showed low growth at 1% NaCl, moderate growth at 2% NaCl, and dense growth at 3% NaCl, while *Streptomyces* showed low growth at 1% NaCl, dense growth at 2% NaCl, and moderate growth at 3% NaCl (Table 6).

Effect of Different pH Ranges on the Growth of Isolates

Data presented in Table 7 describe the effects of different pH ranges on the growth of selected isolates. *Bacillus*, *Micrococcus*, *Corynebacterium,* and *Streptomyces* spp showed no growth at pH 5.0, while the remaining isolates showed low growth at pH 5.0. All of the isolates showed low growth at pH 6.0 except for *Streptococcus,* which showed normal growth at pH 6.0. *Streptococcus*, *Staphylococcus*, and *Micrococcus* showed optimum growth at pH 7.0; and *Bacillus*, *Corynebacterium,* and *Arthrobacter* showed normal growth at pH 7.0; while only *Streptomyces* showed low growth at pH 7.0. *Bacillus*, *Arthrobacter,* and *Streptomyces* showed optimum growth at pH 8.0, while the remaining isolates showed normal

Table 2. Morphological characteristics of selected bacterial isolates.

Name of isolates	Shape	Size	Elevation	Margin	Opacity	Texture	Pigment
Bacillus	Irregular	Large	Umbonate	Undulate	Opaque	Rough	White
Streptococcus	Round	Large	Flat	Entire	Opaque	Smooth	Bright Yellow
Staphylococcus	Round	Medium	Convex	Entire	Opaque	Smooth	Whitish
Micrococcus	Round	Medium	Convex	Entire	Opaque	Smooth	Yellow
Corynebacterim	Irregular	Medium	Convex	Entire	Transparent	Granular	Yellowish
Arthrobacter	Round	Large	Convex	Entire	Opaque	Smooth	Light Yellow
Streptomyces	Irregular	Medium	Raised	Entire	Opaque	Wrinkle	Light Brown

Table 3. Cell morphological and Gram staining characteristics of selected bacterial isolates.

Name of isolate	Size	Shape	Gram staining
Bacillus	Big	Rod	+
Streptococcus	Small	Cocci	+
Staphylococcus	Small	Cocci	+
Micrococcus	Small	Cocci	+
Corynebacterium	Big	Rod	+
Arthrobacter	Small	Rod coccus	+
Sterptomyces	Big	Filamentous rod	+

+ = Gram positive

Table 4. Effect of temperature on the growth of isolates.

Name of isolate	20ºC	25ºC	30-40ºC
Bacillus	+	++	+++
Streptococcus	+	++	+++
Staphylococcus	+	++	+++
Micrococcus	+	++	+++
Corynebacterium	+	++	+++
Arthrobacter	+	++	+++
Streptomyces	+	++	+++

+ = Low growth; ++ = Moderate growth;
+++ = Dense growth

Table 5. Lipolytic activity of selected bacterial isolates.

Name of isolate	Lipolyticactivity
Bacillus	+
Streptococcus	-
Staphylococcus	+
Micrococcus	+
Corynebacterium	+
Arthrobacter	+
Streptomyces	+

+ = Lipase production and - = No Lipase production.

growth at pH 8.0. Only *Corynebacterium* showed optimum growth at pH 9.0, while *Micrococcus*, *Arthrobacter,* and *Streptomyces* showed normal growth at pH 9.0 and the remaining isolates showed low growth at pH. Similarly, *Streptococcus*, *Staphylococcus*, and *Micrococcus* showed no growth at pH 10.0 and the remaining showed low growth at pH 10.0.

Table 6. Effect of different NaCl concentrations on the growth of selected bacterial isolates.

Name of isolate	1% NaCl	2% NaCl	3% NaCl
Bacillus	+++	++	+
Streptococcus	+++	++	+
Staphylococcus	+	++	+++
Micrococcus	+	++	+++
Corynebacterium	+	++	+++
Arthrobacter	+	++	+++
Streptomyces	+	+++	++

+ = low growth; ++ = Moderate growth;
+++ = Dense growth.

Table 7. Effect of different pH ranges on the growth of isolates.

Name of Isolate	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0
Bacillus	-	+	++	+++	+	+
Streptococcus	+	++	+++	++	+	-
Staphylococcus	+	+	+++	++	+	-
Micrococcus	-	+	+++	++	++	-
Corynebacterium	-	+	++	++	+++	+
Arthrobacter	+	+	++	+++	++	+
Streptomyces	-	+	+	+++	++	+

+ = Low growth, ++ = Normal growth,
+++ = Optimum growth, - = No growth

Discussion

Microorganisms like bacteria are important biodegrading agents of petroleum hydrocarbons. Various types of bacteria have been reported that have promising abilities of degradation [18-19]. They break down the complex hydrocarbon chain and utilize their carbon energy sources. There are various external factors like temperature, humidity, oxygen, nutrients, and water availability that can influence their biodegrading ability [20]. The present study confirms various bacterial associations with petroleum hydrocarbons taken from soil samples of different regions of the Swat District in Khyber Pakhtunkhawa.

In the present study, seven different bacterial genera (i.e., *Bacillus, Streptococcus, Staphylococcus, Micrococcus, Corynebacterium, Arthrobacter,* and *Streptomyces*) were isolated from various petroleum-contaminated soils. Our study is in line with Raza et al., (2011), who isolated *Micrococcus*spp.*, Corynebacterium*spp.*,* and *Bacillus*spp from crude oil-contaminated soil [11]. Similar results have been reported by other researchers [9, 21-22], who reported that *Streptococcus* spp., *Arthrobacter* spp, *Staphylococcus* spp., *Micrococcus* spp, and *Bacillus* sp can grow and degrade crude oil.

Various cultural and morphological characteristics of bacteria have been studied by various researchers [23]. In the present study, colony shape, colony size, colony elevation, marginal outlines, and colony opacity, surface, and pigmentation were studied and observed in different genera. Similar reports have been obtained by Ahirwar and Dehariya (2013) and Mhamane et al. (2013) [13, 24]. In the present study the effect of temperature on the growth of bacteria was also studied, which revealed variations in growth and morphological traits. Similar reports have been reported by Sunita et al. (2013) and Rehab et al. (2013) [25, 26].

In our study enriched media with 1% (v/v) olive oil was used for lipase activity of the identified isolates. The results indicated that *Arthrobacter, Staphylococcus, Bacillus, Micrococcus, Corynebacterium,* and *Streptomyces* were lipase-positive, while negative lipase activity was found in *streptococcus*. Our results are in agreement with [27, 28]; Mohan et al., 2008 and Vishnupriya et al., (2010), who reported a potent lipase-producing bacteria, *Streptomyces griseus* (a *bacillus* species), by using different types of oils, including olive, palm, and sunflower. Nisha et al. (2014) also isolated Micrococcus *flavus* and recorded its lipase production at 27ºC to 37ºC [29].

In our study *Bacillus*, *Arthrobacter,* and *Streptomyces* showed optimum growth at pH 8.0, and *Streptococcus*, *Staphylococcus*, and *Micrococcus* showed optimum growth at pH 7.0. Only *Corynebacterium* showed optimum growth at pH 9.0. Our results are in line with the findings of Olajuyigbe and Nisha [30-31], who reported that *Bacillus* spp and *Micrococcus* spp can grow and produce protease and cellulose enzyme at pH 8.0 and 7.0, respectively.

Conclusions

Lots of microorganisms adapt to petroleum-contaminated soil. During this research 25 soil samples were collected from petroleum filling stations and automobile workshops in the Swat District of Khyber Pakhtunkhwa, Pakistan. A total of seven bacterial genera – all Gram-positive – were isolated. These genera were *Streptococcus*, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Arthrobacter*, *Streptomyces,* and *Corynebacterium*. During our research it was found that most of the microorganisms showed optimum growth in saline environments, indicating that they tolerate high pH conditions.

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