Isolation and Characterization of Oil Degrading Bacteria from Soil Contaminated with Used Petroleum Products

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Objective: Certain petroleum products (such as crude oil, engine oil, diesel etc.) from appliances including generators, engines and automobiles can cause environmental pollution. It was hypothesized that oil rich environment could contain microbial communities that are specifically adapted to use hydrocarbons as sole source of energy for the metabolic activities. Therefore, this study was planned to isolate certain bacteria that have potential to use hydrocarbon for their growth and other metabolic activities. In this study, we had evaluated the microbiological and physicochemical characteristics of soil that is contaminated with used petroleum products in Faisalabad Metropolitan area. Sampling soil sites include filling stations, automobile mechanic workshops, surroundings of power generators etc. Culture-able bacteria were isolated through serial dilution method using nutrient agar medium. For the isolation of hydrocarbon utilizing bacteria, Bushnell Hass (BH) medium supplemented with different concentrations of hydrocarbons was used as sole carbon source. The plates were kept at 37 °C until visible colonies were formed. On the basis of colony characteristics and biochemical tests, bacterial isolates were identified. Out of 32, four bacterial isolates exhibited the growth in BH medium supplemented with different concentration of diesel and used engine oil as sole source of carbon.

Keywords: Bushnell Hass; Metropolitan area; contaminated.

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Introduction:

Petroleum oil is a major supply of energy for vehicles and industries. It's also used for domestic purpose. Because the use of crude oil expands with industrialization, crude oil becomes a larger potential supply of contaminants within the soil and water environments (Bento et al., 2003). Contamination of soils, groundwater, sediment, surface water and air with crude may be a major threat for globe. Certain diseases specifically cancer, bone marrow damage,

kidney and liver diseases are related with exposure to high concentration of petroleum oil (Olukunle et al., 2013).

The effect of oil pollution is fatal and devastating not solely to human, but also to animal and agricultural lands. Some animals, birds, mammals, and fish are killed if petroleum hydrocarbons are eaten. Several may die from consumption of oil contaminated food products. Birds and insects may die if oil coats the feathers and wings, as that may hinder free locomotion and therefore the ability to remain warm. In addition, oil produce owns a nasty odour from water and leaves a sticky film on the surface of water creating it unsafe and ugly to consume (Farrington and McDowell, 2004; Jain et al., 2011). It is so necessary to seek out solutions to resolve these environmental issues.

The increasing industrial enlargement has led to growing riot of the ecosystem and also the existence of different types of pollution. The crude oil is an important article of trade. The employment of petroleum oil as fuel has led to intensive economic development globally. Starting within the 1950s, oil and gas became the most important sources of primary energy for increasing world population. The great need for this energy supply has led to the gradual collapse of natural oil reserves. The increasing industrial development has led to emergent disturbance of the natural ecosystem and has caused different types of contamination. The world depends mostly on petroleum fuel that has led to serious economic expansion worldwide. The clear benefits of petroleum consumption, however, can carry major environmental impacts that may be local or global in scale. Those impacts include but not limited to air pollution, global climate change, and oil spills. Environmental pollution with petroleum and petrochemical products has been recognized as a significant and serious problem around the globe. It causes long-term damage to aquatic and soil ecosystems, human health and natural resources. Many of these contaminants have demonstrated to be toxic and carcinogenic and are also easy to incorporate into the food chain (Farrington and McDowell, 2004). However high portion of the hydrocarbons found in petroleum products are biodegradable.

The indigenous microbial flora in oil contaminated soil is capable of mineralizing these pollutants in the environment to safe and acceptable levels. It has been reported that oil-degrading bacteria are abundant in soils contaminated with used petroleum products. These can be exploited for the bioremediation of contaminated soils. *Pseudomonas sp*, *Micrococcus sp*, *Bacillus sp*, *Salmonella sp*, *E.colisp*, *Klebsiella sp*, *Streptococcus sp* are amongst the mostly reported species that have been isolated from the petroleum-derived product spill contaminated soil.

MATERIALS AND METHODS

Sample collection:

A total of six (06) samples of contaminated soil from petroleum products of different mechanic workshops, filling stations and motorcycle workshops located in different areas of Faisalabad city were collected in sterilized sample containers with a spatula. These samples were transported to the Department of Microbiology laboratory (Government College University, Faisalabad GCUF) for physiochemical analysis within 6 hours after collection and then the samples were stored at refrigeration temperature i.e.,4°C for further analysis.

Isolation and biochemical characterization:

Soil samples were plated on the general media (nutrient agar) as well as on differential/selective media to isolate the bacterial species from the contaminated soils. Serial dilutions and pour plate methods were used to enumerate microbial population density. The isolated colonies were subjected to microscopic examination and various biochemical identification procedures according to the standard microbiological procedures. All biochemical tests were performed by using commercially available QTS Rapid Bacterial Identification Kit according to the kit manual.

Hydrocarbon degradation potential:

Steps:

- Sample collection
- Sample processing
- Enumeration of total heterotrophic bacteria and fungi
- Enumeration of total hydrocarbon- utilizing bacteria and fungi
- Characterization and identification of hydrocarbon- utilizing bacteria
- Determination of physiochemical properties of contaminated soil

RESULTS

Isolation of bacteria from contaminated soil

Bacteria were isolated on nutrient agar medium from collected soil samples using serial dilutions plate techniques in which 0.1 mL of supernatant was inoculated on enriched nutrient agar using the streak plate technique. A wide range of morphologically different colonies were obtained in each of the dilutions of soil samples i.e.10^{-2}, 10^{-3} and 10^{-4} after incubation of 24 hours at 37°C.The data obtained were reported in table no.1

Table No.1. Colony morphology of isolated bacteria in different dilutions of soil samples contaminated with used petroleum products.

1.	Zone 1 (Sargodha road)	10^{-2}	Mixed growth, Yellow & white small colonies
		10^{-3}	Medium sized & large yellow colonies
		10^{-4}	Small & large white colonies
2.	Zone 2 (Jaranwala road)	10^{-2}	2 typed white small colonies
		10^{-3}	White small 1 typed colonies
		10^{-4}	Large numerous colonies
3.	Zone 3 (Jhang road)	10^{-2}	Pale colonies small & large
		10^{-3}	Large & medium sized colonies
		10^{-4}	No Growth
4.	Zone 4 (Jinnah colony)	10^{-2}	Mixed growth
		10^{-3}	Large colonies
		10^{-4}	No Growth

Pure Cultures obtained from streak plate method

From the cultures obtained from pour plate method, morphologically distinct colonies were carefully chosen and streaked on nutrient agar plates. These plates were placed in incubator at 37°C for 24-48 hours.

Study of Colonial Morphology

After 24 hours, total 32 purified isolates were obtained on the basis of different morphological characters. The colony morphology observed was as under:

Form: (circular, filamentous and irregular)

Elevation: (flat and convex)

Margin: (entire, undulate and filamentous)

Optical feature: (transparent, opaque and translucent) which are shown in Fig .1 & 2.

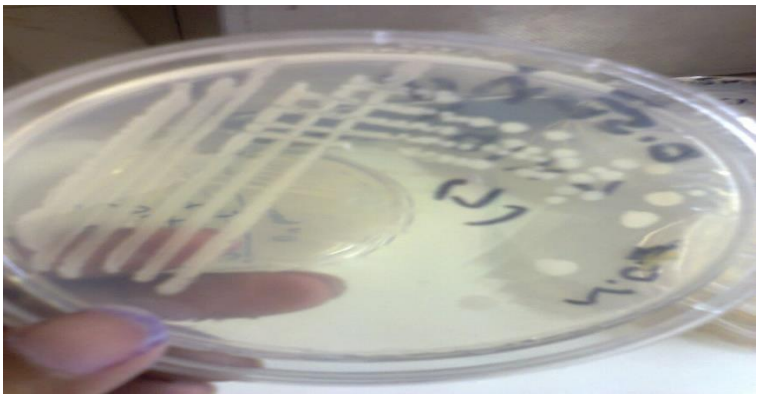

Figure.1 Circular, convex, entire margined and translucent colonies of different isolates obtained from soil samples

Figure.2 Dry small circular yellowish opaque bacterial colonies obtained during the isolation of oil degrading bacteria

Identification of isolates on the basis of Gram's staining

All the bacterial isolates obtained from four zones of samples were Gram stained. The isolates observed under 100X lens of compound microscope (IRMECO) were Gram positive bacteria and Gram-negative bacteria on the base of Gram reaction. Cellular arrangements were in chains, bunches, scattered and morphology was as rods and cocci. The gram-stained isolates are shown in the following figures and details for cellular morphology and Gram reaction are given in Table no.3

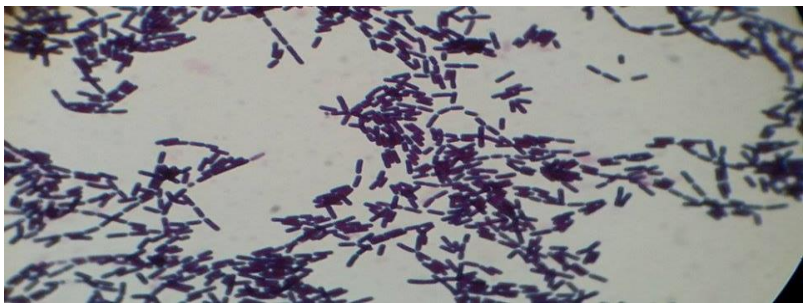

Figure.3 Gram positive rods colonies as observed under microscope (100X)

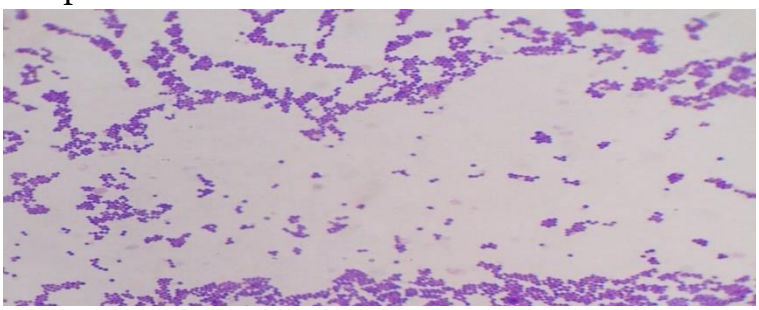

Figure:4 Gram positive cocci colonies of bacterial isolates as seen under microscope (100X)

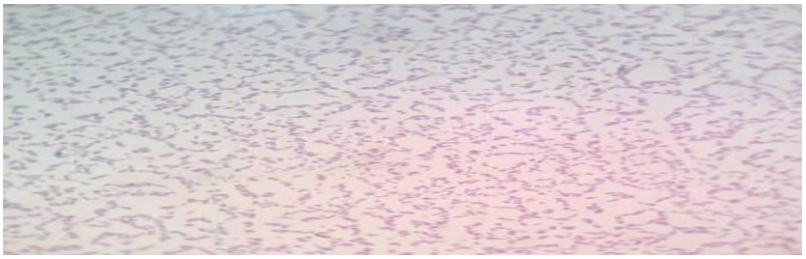

Figure:5 Gram negative cocci bacterial colonies under microscope (100X)

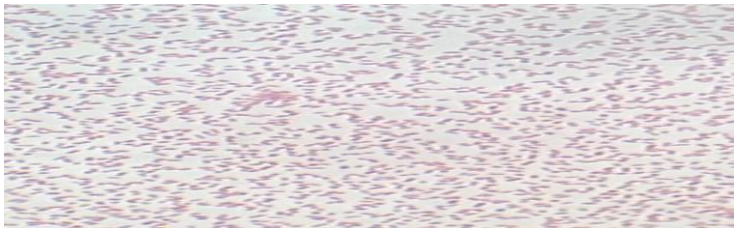

Figure .6.Gram negative rods (clusters) colonies of bacterial isolates obtained from oil contaminated soils

Table no 2. Characteristics of the bacterial isolates based on Gram's reaction.

Sr. No	Colony colour	Colony form	shape	Gram stain	Place of collection
1	Brown	Circular	Rods	Negative	Zone 1
2	Yellow	Circular	Cocci	Negative	Zone 1
3	Orange	Circular	Cocci	Negative	Zone 1
4	Cream	Irregular	Rods	Negative	Zone 1
5	Pink	Irregular	Rods	Negative	Zone 2
6	Yellow	Circular	Cocci	Positive	Zone 2
7	Orange	Circular	Cocci	Positive	Zone 2

8	Cream	Irregular	Rods	Negative	Zone 2
9	White	Irregular	Rods	Negative	Zone 2
10	Pink	Irregular	Rods	Negative	Zone 2
11	Yellow	Irregular	Cocci	Negative	Zone 2
12	Orange	Circular	Cocci	Positive	Zone 2
13	Orange	Circular	Cocci	Negative	Zone 2
14	Cream	Circular	Rods	Negative	Zone 3
15	White	Circular	Rods	Negative	Zone 3
16	Brown	Circular	Rods	Negative	Zone 3
17	Yellow	Circular	Cocci	Positive	Zone 3
18	Orange	Irregular	Cocci	Positive	Zone 3
19	Cream	Irregular	Rods	Positive	Zone 3
20	Pink	Circular	Rods	Negative	Zone 3
21	Yellow	Circular	Cocci	Negative	Zone 3
22	Orange	Circular	Cocci	Negative	Zone 4
23	Cream	Circular	Rods	Negative	Zone 4
24	White	Irregular	Rods	Negative	Zone 4
25	Pink	Irregular	Rods	Negative	Zone 4
26	Yellow	Circular	Cocci	Negative	Zone 4
27	Orange	Circular	Cocci	Negative	Zone 4
28	Orange	Circular	Cocci	Positive	Zone 4
29	Cream	Circular	Rods	Negative	Zone 4
30	White	Irregular	Cocci	Positive	Zone 4
31	Yellow	Circular	Cocci	Positive	Zone 4
32	Yellow	Circular	Cocci	Positive	Zone 4

Hydrocarbon degradation potential of bacterial isolates

After the preparation of Modified Bushnell Hass medium enriched withdiesel and used engine oil, 32 selected isolates were taken and streaked on petri dishes separately on modified diesel medium and used engine oil medium with the help of sterile loop. The bacteria were inoculated on these respective media and plates were incubated for 3-6 days (72- 144 hours) at 37°C.The results obtained in this experiment were summarized in Table 3.

Table no.3. Characteristics and hydrocarbon degradation potential of the bacterial isolates on modified BH media.

Sr. No	Gram stain	Morphology	Place of collection	Oil degrading ability on BH media enriched with Diesel	Oil degrading ability on BH media enriched with used engine oil
1	Negative	Circular, Rods	Zone 1	No	No
2	Negative	Circular, Cocci	Zone 1	No	No
3	Negative	Circular, Cocci	Zone 1	No	No
4	Negative	Irregular, Rods	Zone 1	No	No
5	Negative	Irregular, Rods	Zone 2	Yes	Yes
6	Positive	Circular, Cocci	Zone 2	No	No
7	Positive	Circular, Cocci	Zone 2	No	No
8	Negative	Irregular, Rods	Zone 2	Yes	Yes
10	Negative	Irregular, Rods	Zone 2	No	No
11	Negative	Irregular, Cocci	Zone 2	No	No
12	Positive	Circular,	Zone 2	No	No
13	Negative	Circular, Cocci	Zone 2	No	No
14	Negative	Circular, Rods	Zone 3	No	No
15	Negative	Circular, Rods	Zone 3	Yes	Yes
16	Negative	Circular, Rods	Zone 3	No	No
17	Positive	Circular, Cocci	Zone 3	No	No
18	Positive	Irregular, Cocci	Zone 3	No	No
19	Positive	Irregular, Rods	Zone 3	No	No
20	Negative	Circular, Rods	Zone 3	No	No
21	Negative	Circular, Cocci	Zone 3	No	No
22	Negative	Circular, Cocci	Zone 4	No	No
23	Negative	Circular, Rods	Zone 4	No	No
24	Negative	Irregular, Rods	Zone 4	No	No
25	Negative	Irregular, Rods	Zone 4	No	No
26	Negative	Circular, Cocci	Zone 4	No	No
27	Negative	Circular, Cocci	Zone 4	No	No
28	Positive	Circular, Cocci	Zone 4	No	No
29	Negative	Circular, Rods	Zone 4	No	No
30	Positive	Irregular, Cocci	Zone 4	No	No
31	Positive	Circular, Cocci	Zone 4	No	No
32	Positive	Circular, Cocci	Zone 4	No	No

DISCUSSION:

Petroleum is the most valuable liquid ever discovered. There are number of ways by which it can be leaked into the environment. This leakage of petroleum can cause imbalance in the environment component like soil, surface and the underground water reservoirs; therefore, its remediation is necessary to clean the environment (Bhasheer et al., 2014).

There are number of bacteria which can reduce the effects of petroleum in the environment by degrading it. Bacteria do have such metabolic machinery by which they break down the oil and use its carbon as a source of carbon, energy and make environmentally safe and beneficial products. Usually, bacteria exhibit this ability when grown in the environment rich in hydrocarbon like oily soil (Lin et al., 2009). This is because bacteria are able to evolve the system by which they can synthesize the required enzymes to degrade the oil present in the sample. These enzymes are activated only in the environment polluted with hydrocarbons in an uncontaminated ecosystem ecosystem, there is less than 0.1% microbial activity regarding hydrocarbons (Okerentugba and ezeronye, 2003).

In the present study, the samples of oil contaminated soil were collected from different areas of Faisalabad metropolis. For this purpose, whole city was divided into four zones to cover entire metropolis and bacterial isolates were isolated from oil contaminated soils. Apparently similar isolates were obtained by sub culturing the colonies that showed different morphology with respect to color of appearance. Microscopic examination after Gram's staining revealed different clusters of Gram-negative rods (Fig.4.3) and Gram-positive cocci (Fig.4.4). Gram negative bacteria have strong cell envelop that gives those bacteria tolerability to hydrocarbons due to which they are believed to be excellent degraderas also reported in available literature (Ranjan et al., 2014). Another study reported by Shiri et al. (2015) also corroborated our results in which the authors had isolated *Acinetobacter baumannii* and *Pseudomonas sp*. from soils (not contaminated with petroleum products).Even then both these isolates were able to survive oil stress provided in experiments.Similar results were obtained by Nikhil et al. (2013) who isolated both gram positive and gram-negative bacteria from soil contaminated with petroleum products. They had further characterized those bacteria as *Pseudomonas sp*. and *Micrococcus sp*. and suggested that these bacteria could possibly had certain bacterial enzymes involved in hydrocarbon degradation. Due to these enzymes, these bacteria could be able to break down the petroleum products (oil) and could use the carbon as sole source of energy for various physiological and metabolic processes (Ranjan et al., 2014). This further demonstrated that petroleum's degradation could be reliant on the bacteria's natural and acquired potential to degrade petroleum hydrocarbons.

In this study out of 32 bacterial isolates, only three isolates were able to survive in modified Bushnell Hass Agar (BH) medium when supplemented with different concentrations of diesel and engine oil. Modified BH medium was prepared to assess the hydrocarbon degradation ability of these isolates (Table 4.3). In our study; used engine oil and diesel in the culture media negatively affected the microbial population. Our results were in accordance to that obtained by Pattanathu et al. (2002) where authors have reported that less adopted species were eliminated and there was qualitative shift in the population of bacteria. However, this could also attribute to use of mixed populations of microbes since extensive degradation of petroleum products could be generally accomplished by mixed

microbial population rather than single microbial species (Shahaby, 2014). Therefore, it was possible that these isolates might require some metabolites (secreted by other bacteria or microbes) to survive in such extreme environments including hydrocarbon media.
In addition, there is large variation in ability of certain microbes to adopt a specific environment. There are many factors such as enzymes synthesis, pH, temperature, time etc. that help those microorganisms to adopt certain habitats. In our study, it might be hypothesized that these isolates could require more time for proper synthesis of enzymes involved in breakdown of petroleum products (Lemos et al., 2013).

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