ISOLATION AND IDENTIFICATION OF DIESEL-DEGRADING BACTERIA FROM OIL CONTAMINATED SOIL IN MANSEHRA, PAKISTAN

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ABSTRACT: - The present work was conducted to isolate and identify bacteria from oil-contaminated soil to evaluate their role in biodegradation of commercial diesel under laboratory conditions. Diesel fuels are used by different vehicles, diesel generators and especially heavy transport vehicles. Its manufacturing, transportation, utilization and disposal have the threat to pollute the surrounding environment. Biodegradation is one of the biological processes to remediate the pollutants. This is the cheaper and easy method as compared to other methods like direct burning, land foaming and bioventing because diesel smoke has a carcinogenic effect.

In the current exploration ordinary bacterial species that degrade diesel were isolated from selected areas of Mansehra. Six sites were selected and a total of 60 samples were collected. These sites were coded according to their names. For the isolation of diesel degrading bacteria soil samples were analyzed. The isolation of bacteria was done on nutrient agar, nutrient broth and mineral salt medium (MSM), and IR spectroscopy was used to observe the degradation of diesel. Twenty samples were found having bacterial growth. Further analysis showed that four samples BHG, SHNK, TAK and KMOR had diesel degrading capability as these showed positive results. The isolated bacteria were identified by morphological and biochemical features, while the degradation of diesel was determined qualitatively by interpreting the intensity of the peaks of IR spectroscopy. The intensity of the peaks in the spectrum of control was compared with the intensity of the peaks in the spectrum of the test samples which indicated degradation of diesel. Three bacterial species, namely, *Bacillus cereus, Bacillus subtilis* and *Pseudomonas aeruginosa* were *identified* using Berge's manual method. The bacterial species were tested for their capability to degrade commercial diesel presented at different concentrations i.e. 1000 ppm and 5000 ppm.

The highest degradation capability of commercial diesel was exhibted by *Bacillus cereus* followed by *Bacillus subtilis* and *Pseudomonas aeruginosa*.

The results indicate that *Bacillus cereus, Bacillus subtilis* and *Pseudomonas aeruginosa* have the potential of *in situ* bioremediation of diesel-contaminated soils.

Key words: *Diesel, Biodegradation, Spectroscopy, Bacillus cereus, Bioremediation, Bacteria pseudomonas auerginosa.*

INTRODUCTION

Mansehra District is located at the eastern border of the Khyber Pakhtunkhwa Province, three hours away from Islamabad and four hours from Peshawar.

In the current exploration diesel-degrading bacteria were isolated and identified. Diesel fuel, in general, is any liquid fuel used in diesel engines. The most common is a specific fractional distillate of petroleum fuel oil, but alternatives that are not derived from petroleum, such as biodiesel, biomass to liquid (BTL) or gas to liquid (GTL) diesel, are increasingly being developed and adopted. To distinguish these types, petroleum-derived diesel is called petro diesel. Petroleum-derived diesel is composed of about 75% saturated hydrocarbons (primarily paraffins including *n*, *iso*, and cycloparaffins), and 25% aromatic hydrocarbons (including naphthalene and alkyl benzenes). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging approximately from $C_{10}H_{20}$ to $C_{15}H_{28}$.

The commercial diesel oil is the mixture of normal, branched, cyclic and aromatic substances having the characteristics of low water solubility, high adsorption and strong stability of aromatic rings (Dean *et al* ., 2002). With the increase in world population the need of petroleum and diesel fuel is increasing. Use of huge quantities and consumption of these products directly affects the surrounding environment (Raven *et al.,* 1993).The common major global environmental pollution is due to the combustion of diesel fuel, crude oil and petroleum compound. These hydrocarbons are mostly used as primary source of energy. There is a need of large quantities of fuel to run auto mobile and power industry and also used for home heating. During storage, transportation and transformation leakage accidents the environment gets polluted (Watanabe, 2001; Surridge, 2007).

Huge hydrocarbon contaminants are caused by oil spills from marine accidents during shipping and transport of crude oil (Ghosh *et al*., 2006). Diesel fuel contaminants having very high carcinogenicity and mutagencity serve as strong toxicants causing various human and animal health problems (Boonehan *et al* ., 2000 ; Samante *et al* ., 2002). There are various methods for cleaning ground environment from petroleum and diesel hydrocarbon. Among these methods bioremediation is more effective and approachable method to remove hazardous substances, than other commercially available techniques (Alexander, 1994;Ojo, 2006).The process of bioremediation is nowadays used in many countries of the world to clean up our environment from chemical contaminants (Skladany and Metting, 1992). Bioremediation is an emerging field for investigators to present the biological treatment of environmental pollutant, by means of microbes. There are numerous organisms which have the ability to utilize diesel hydrocarbon as rich source of carbon and convert these organic contaminants to harmless compounds (Atlas and Philp, 2005; Koren *et al*., 2003).

Petroleum and diesel hydrocarbon degradation is not a new concept. It is 50 years old phenomenon in which different bacteria having the ability to degrade petroleum and diesel hydrocarbons have been isolated from contaminated soil, storage tanks and from oil spills environment (Huy *et al*., 1999).There are many microorganisms which have the ability to degrade these pollutants from the environment and many scientists are working to get solution for removing such kind of pollutants

Degradation of hydrocarbon in contaminated soil is a safe, competent, domestic and economical method to degrade harmful contaminants (Mehrashi *et al.*, 2003). Natural population of microbes promotes breakdown of petroleum and diesel hydrocarbon by converting hazardous compounds

into less toxic substances and remediation of contaminants from the environment is completely approachable (Atlas *et al*., 1981).

There are different types of bacteria that are capable of degrading diesel and petroleum hydrocarbons. Only one bacterial species is unable to degrade hydrocarbon molecules. Among naturally occurring bacteria *Pseudomonas spp* are able to utilize carbon as energy source because they contain plasmids, and the relevant genes for degradation (Jawetz *et al.,* 1991). Different bacterial species are isolated successfully from Soil samples from oil contaminated sites. Bacteria of the genus *Staphylococcus* were found as the major degrading microorganisms (DeRito *et al.,* 2005). It can be postulated that gram positive organisms are in the family of such microorganisms that take a part in breakdown of complex hydrocarbon. Some Gram-positive bacteria, mostly *Rhodoccoci* have the ability to break alkanes (Kaplan and Kitts, 2004).

The main objective of this paper is to clearly describe the bacteria isolated from the oily contaminated soil and also to identify those bacterial species which may have potential to be exploited as agents for degradation of commercial diesel.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from oil contaminated soils around the storage tanks inside the filling stations, bus/trucks stand, car wash station and auto workshops from different localities in District Mansehra, such as General bus stand, Shinkiari, Takkra, etc.

The soil was contaminated with diesel and other petroleum fuel spilled and at least 30 soil samples approximately 20 g were collected randomly from the top soil layer up to 10-30 cm in depth using a hand auger followed by bulking in the selected areas. After removal of surface

litter, stone and big soil debris, the samples were passed through a 2 mm sieve and stored at 4 °C in sterile plastic bags. Samples were immediately transported to the laboratory for further analysis.

Enrichment medium

All microbial enrichment was performed in the mineral salt medium (MSM).This medium was used for the growth of such bacteria having the capability to degrade petroleum hydrocarbon. The enrichment medium is also called Basal Salt medium (BSM) and their composition are mentioned below.

(g/L):$NaNO_3$ (7); K_2HPO_4 (1); KH_2PO_4 (0.5); KCl (0.1); $MgSO_4.7H_2O$ (0.5); $CaCl_2$ (0.01); $FeSO_4.7H_2O$ (0.01); H_3BO_4, (0.25); $CuSO_4.5H_2O$ (0.5); $MnSO_4$. H2O (0.5) and $ZnSO_4$. $7H_2O$ (0.7), containing 25 to 100 mg of yeast extract or tryptone **(**Mercade *et al.*, 1996).

All the ingredients of the medium were dissolved in distilled water and shake properly.

The enrichment medium was prepared in 1000 ml flask. The medium was then autoclaved at 121^oC for 15 minutes for sterilization and the pH of the medium was adjusted to 7.3 with a drop of HCl or NaOH.

Cultivation of diesel fuel-degraders

The mineral salt medium (MSM) was used for cultivation of diesel degrading bacteria as describe by Mercade *et al.*, (1996).

Bacterial enrichment was carried out in 250 mL Erlenmeyer flasks containing 100 mL of MSM.

First transfer

From the prepared Enrichment medium 100 ml media was transferred to 250 ml Erlenmeyer flask with commercial diesel as sole source of carbon and energy. One gram of the diesel

contaminated sieve soil was added to 100 ml of the enrichment medium and incubated at 35 ºC by using orbital incubator (120 rpm) for the period of 15 days.

Second transfer

After the completion of initial cultivation one ml of enriched culture was transferred into newly prepared enrichment medium and incubated at the same temperature in the orbital shaker (120 rpm) for the 15 days. The third transfer was same as second as describe by Singh and Lin, (2008).

Isolation of Suspected Diesel Degraders

After the completion of enrichment process 1 ml culture was serially diluted to six levels (10-fold) were made in sterile water from the third enrichment culture. For isolation of suspected diesel degraders 100 µl of diluents was streak on nutrient agar plates from the last dilution (6^{th} level) and the plates were covered with 100 µl of diesel oil before streaking and incubate at 30^{o}C for 48 hours. The nutrient agar (NA) and broth was used for isolation, numeration and maintenance of pure cultures of diesel degraders.

Bio-degradation Analysis

Bio-degradation of commercial diesel fuel was performed in different low 1000 ppm and high 5000 ppm concentration as the sole source of carbon and energy.

A series of twenty two Erlenmeyer flasks of 300 ml capacity were used for this experiment. Out of these twenty two flasks ten flasks were used for 1000 ppm and ten flasks were used for 5000 ppm and two flasks each was used for reference/controls. Each flask contained 100 ml of 8 g/l of nutrient broth and was sterilized by autoclaving (121 ºC for 15 min). After autoclaving 100 ml of 1000 ppm diesel solution from stock solution was added in to each of the 11 flasks and similarly 100 ml from 5000 ppm diesel solution was added to the next series of 11 flasks.

The suspected diesel degrading bacteria in nutrient broth were shaken and the flasks were inoculated by transferring 2 ml of pre-culture (about 3×10^8 cells/ml) of bacteria and initial pH was adjusted to 7.3. The experimental flasks were incubated under aerobic condition in an orbital incubator (120 rpm) at temperature (30 °C) for 20 days.

Qualitative Determination of degraded diesel

Samples were randomly removed for qualitative determination by Fourier transform infrared spectroscopy (FTIR) Version 1.30 Shimadzu Corporation and bacterial augmentation were monitored by viable counts on nutrient agar plates (Amund and Igiri, 1990).

Identification of Isolated Diesel Degraders

After the completion of degradation analysis the suspected diesel degrader's growth was monitored by viable counts on nutrient agar plates and then the diesel degraders were identified by various methods.

i. Morphological identification
ii. Biochemical identification

Morphological identification

Basic morphological identification was performed including colony morphology, shape and color of colony and Gram-reaction to identify the bacterial diesel degraders (Balows *et al*., 1992). For colony morphology, shape and colour of samples were examined through compound microscope and the results were recorded.

Biochemical identification

Biochemical identification is based on biochemical tests including catalase, coagulase, urease, oxidase activities, Simmons citrate, methyl red voges prosker (MRVP) and indol productions were checked as recommended by Smibert and Krieg (1994).Triple Sugar Iron Agar is used for

the differentiation of microorganisms on the basis of dextrose, lactose and sucrose fermentation and hydrogen sulfide production (Kligler**,** 1918).

RESULTS AND DISCUSSION

Properties of soil

The physico-chemical properties of the soil samples like texture and pH along with their codes are presented in Table 1.

Cultivation of diesel fuel-degraders

The cultivation of diesel fuel degraders were carried out in 250 mL Erlenmeyer flasks containing 100 mL of MSM and few drops of commercial diesel as sole source of carbon and energy as describe by Mercade *et al*., (1996).One gram of the diesel contaminated sieve soil was added to 100 ml of the enrichment medium and incubated at 35 ºC by using orbital incubator (120 rpm) for the period of 15 days. After the completion of initial cultivation 5 ml of enriched culture was transferred into newly prepared enrichment medium and incubated at the same temperature in orbital shaker (120 rpm) for the 15 days. After the completion of 2^{nd} transfer of bacterial cultivation finally transfer 2 ml of enrichment culture in to the third newly prepared medium and incubated at 35 ºC by using orbital incubator (120 rpm) for the period of 15 days, Singh and Lin, (2008).

Isolation of Diesel Degraders

After the completion of cultivation process, a 10-fold serial dilution of the culture up to 6^{th} level was made and 100 µl of diluents was streak on nutrient agar plates from last dilution as describe in materials and methods.

A total of 30 samples were run, 18 samples shows growth. Five samples from each selected area were collected. From Shinkiari and Bhagwar areas 10 samples were collected and run and only 6 samples shows growth. From Athar shesha 5 samples were collected and only 2 samples shows growth and then the total of 5 samples were collected from Takkra and only 4 samples shows growth. From Kalgan moare and Mansehra General Bus stand a total of 10 samples were collected and only 2 and 4 samples show growth respectively. The details of the total samples and the number yielding bacterial growth are given in Table 2.

The samples which showed 40 to 112 colonies were examined per plate on the basis of colony growth appearance were inoculated in nutrient broth in order to sub cultured for obtaining pure cultures that were further use for degradation and identification process. These colonies have distinct features such as rods and become wrinkled, convex or flat surface and ranging from brownish, greenish and greenish, yellowish were selected. All samples demonstrated a successful growth in nutrient broth as viewed by turbidity of the cultures, as shown in figure 1.

Table No 2: Distribution of samples yielding or not yielding bacterial growth by sites.

S.No	Origin of samples	Samples code	Total no of samples	Samples yielding growth	Samples not yielding growth
1	Bhagwar	BHG	10	3	7
2	Shinkiari	SHNK	10	6	9
3	AhtarShesha	ASM	10	2	8
4	Takkara	TAK	10	4	6
5	Kalgan Moare	KMOR	10	2	8
6	Mansehra Gen bus stand	MGBS	10	3	7

Total	**60**	**20**	**40**

Figure 1. Petri plates showing growth of bacterial strains after 5 days incubation.

(a) (b). (c)

Figure 2. Significant growths in nutrient broth of collected soil samples were shown.

Degradation analysis of commercial diesel by isolated bacterial species

Degradation of commercial diesel by isolated bacterial species was confirmed by Fourier transform infra-red (FTIR). Commercial diesel was presented to the test organisms in two concentrations 1000 ppm and 5000 ppm for 15 days as described in materials and methods.

The results of FTIR analysis showed that the introduction of bacterial species enhanced the degradation of commercial diesel as compared to the samples that were not supplemented with any bacterial species. Commercial diesel degradation was analyzed by using FTIR. First of all a control sample was run having commercial diesel concentration of 1000 ppm and 5000 ppm in the absence of bacterial species. It showed IR spectroscopic peak at 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ corresponds to the stretching vibration of hydrogen and carbon bonds which was

recorded on a graph paper (Figure 2). The intensity of the above peaks in the spectrum of control was compared with the intensity of the peaks in the spectrum of the test samples. This response was used as standard/reference for comparison with results of test samples offered diesel at a concentration of 1000 ppm.

To validate the degradation of diesel in 10 samples with 1000 ppm concentration were tested in FTIR separately. The 1000 ppm concentration control/standard intensity of peaks at 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ was 10.1, 7.11, 4.22 upon calculations the overall intensity was 21.42 which was high as compared with the intensity of a spectrum of the samples.

It has been found that only 4 samples showed degradation of commercial diesel as the IR spectroscopic peaks at 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ have low intensity as compared with standard/control. The sample taken from BHG showed degradation of diesel which has low intensity which was 16.53 that indicates the degradation of diesel as it was different from 21.42 of standard peak of diesel as shown in Figure 3 and table 3.

The samples taken from TAK and SHNK also showed degradation of diesel. The intensities of these samples were recorded as 13.1, 18.89 and 17.05 respectively. The results of FTIR spectrum peaks obtained on graph paper were shown in Figures 4, 5, and 6. The values of IR spectroscopic peaks and the low intensity recorded in these samples were the clear signal of diesel degradation compared with standard/control as shown in tables 4,5 and 6.

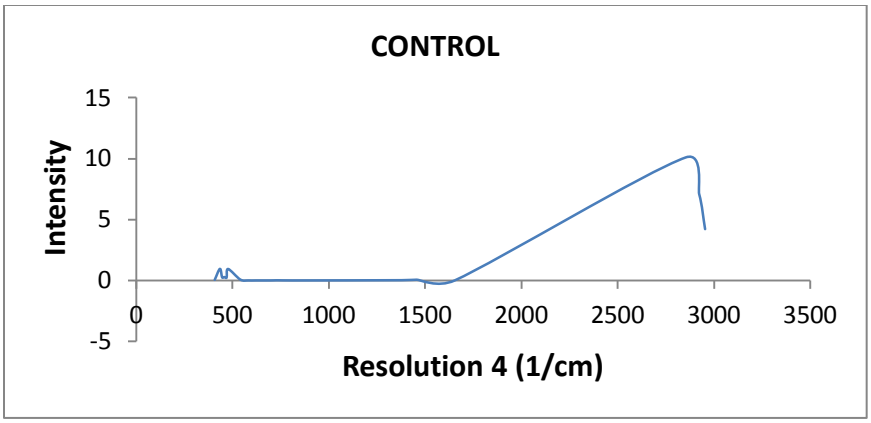


Figure 2. Standard IR spectroscopic peak of Diesel without any bacterial species at 1000 ppm

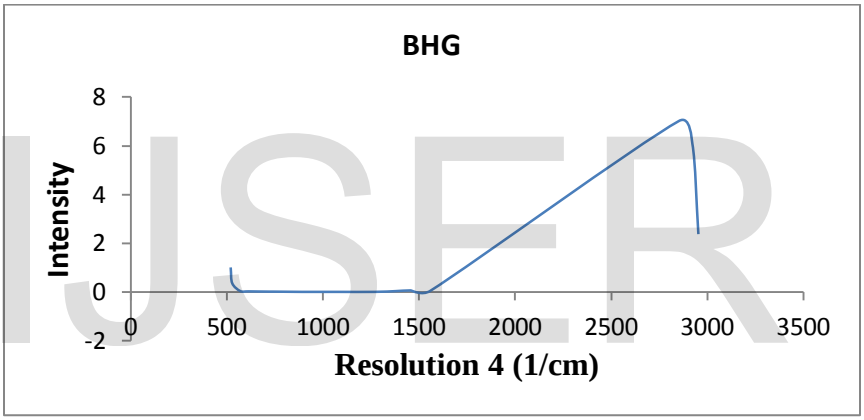


Figure.3. IR spectroscopic peak of Sample BHG with *Bacillus cereus* at 1000 ppm

Intensity of control (1000 ppm)			Intensity of sample BHG (1000 ppm)	
S. no	Peaks	Intensity	Peaks	Intensity
1	2852.72	10.01	2852.72	7.02
2	2922.16	7.10	2922.16	6.08
3	2953.02	4.22	2953.02	2.43
Total		21.42	Total	16.53

Table 3. Intensity pattern of Sample BHG with *Bacillus cereus* at 1000 ppm

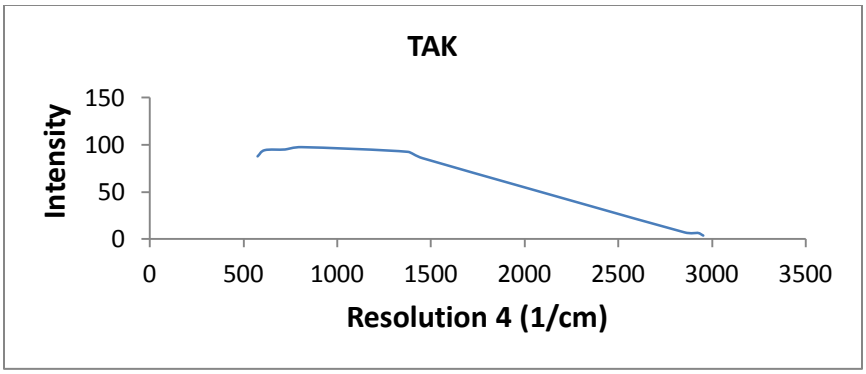


Figure 4. IR spectroscopic peak of Sample TAK with *Bacillus subtilis* at 1000 ppm

Intensity of control (1000 ppm)			Intensity of sample TAK (1000 ppm)	
S. no	Peaks	Intensity	Peaks	Intensity
1	2852.72	10.01	2852.72	7.04
2	2922.16	7.10	2922.16	6.53
3	2953.02	4.22	2953.02	3.63
Total		21.42	Total	17.2

Table 4. Intensity pattern of Sample TAK with *Bacillus subtilis* at 1000 ppm

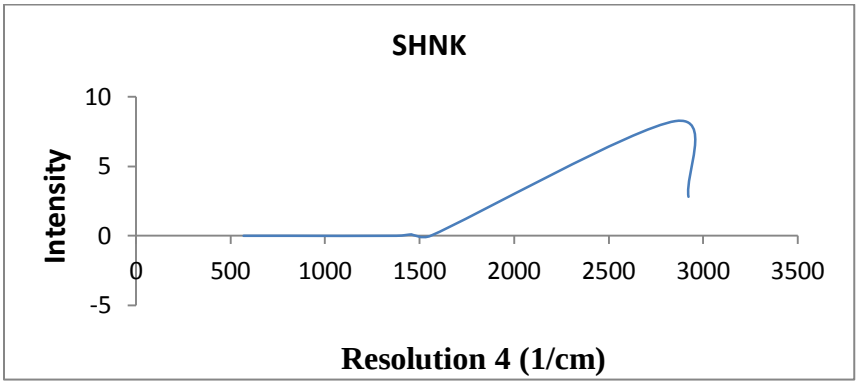


Figure 5. IR spectroscopic peak of Sample SHNK with *Pseudomonas aeruginosa* at 1000 ppm

Intensity of control (1000 ppm)			Intensity of sample SHNK (1000 ppm)	
S. no	Peaks	Intensity	Peaks	Intensity
1	2852.72	10.01	2852.72	8.26
2	2922.16	7.10	2922.16	7.82
3	2953.02	4.22	2953.02	2.81
Total		21.42	Total	18.89

Table 5. Intensity pattern of Sample SHNK with *Pseudomonas aeruginosa* at 1000 ppm

In the second assessment the test samples were presented diesel in concentration of 5000 ppm. A control sample without any bacterial specie was evaluated in FTIR spectroscopy. Its peak was same i.e. 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ but different in the intensity 229.21 was recorded on a graph paper as shown in Figure 6.

A comparative study of diesel degradation was carried out in 10 test samples incubated with diesel at 5000 ppm concentration. In these 10 samples only 3 samples, taken from BHG, SHNK and TAK showed degradation. The diesel degradation of these samples was tested in FTIR spectroscopy. The intensities of the peaks of these samples were recorded on a graph paper and it was found that the sample taken from BHG was low intensity i.e.182.94 while samples taken from SHNK and TAK have intensities of 162.51 and 187.68 respectively. The peaks of these 3 samples evidently indicated the diesel degradation because the intensity of the control was high as compared with these samples as shown in Figures 7, 8 and 9 compared to the standard/control as shown in table. 6, 7 and 8.

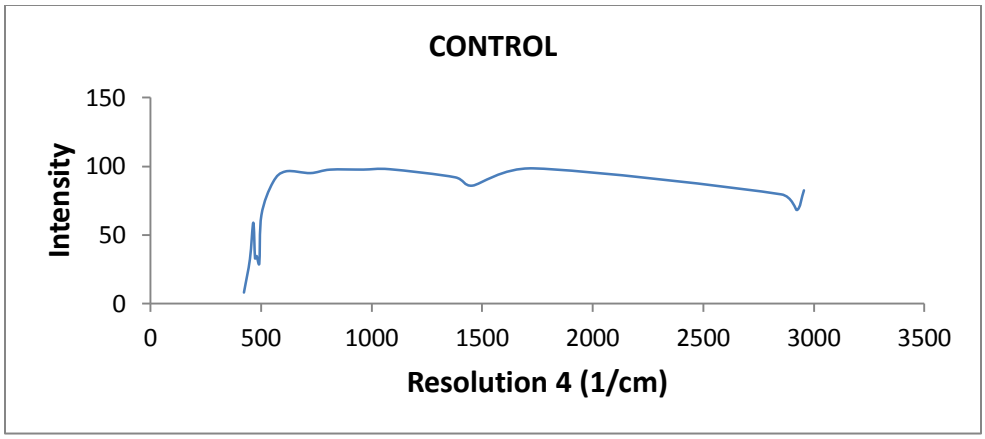


Figure 6. Standard IR spectroscopic peak of Diesel without any bacterial species at 5000 ppm.

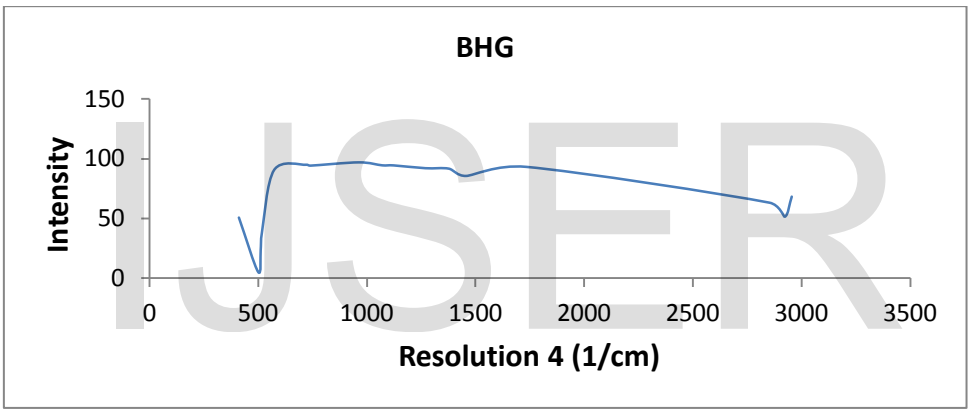
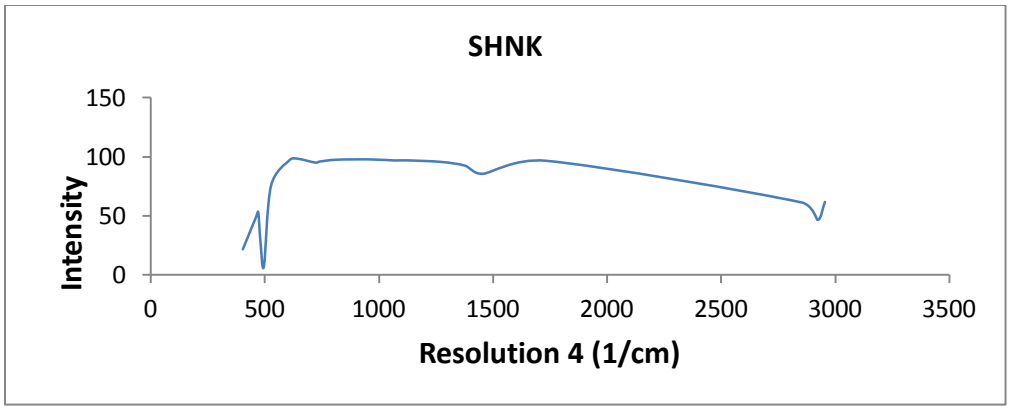


Figure 7. IR spectroscopic peak of Sample BHG with at *Bacillus cereus* 5000 ppm

Intensity of control (5000 ppm)			Intensity of sample BHG (5000 ppm)	
S. no	Peaks	Intensity	Peaks	Intensity
1	2852.72	78.98	2852.72	63.22
2	2922.16	67.69	2922.16	51.43
3	2953.02	82.54	2953.02	68.29
Total		229.21	Total	182.94

Table 7. Intensity pattern of Sample BHG with *Bacillus cereus* at 5000 ppm



285

286 Figure 8. IR spectroscopic peak of Sample SHNK with *Pseudomonas aeruginosa* at 5000

287 ppm

Intensity of control (5000 ppm)			Intensity of sample SHNK (5000 ppm)	
S. no	Peaks	Intensity	Peaks	Intensity
1	2852.72	78.98	2852.72	61.22
2	2922.16	67.69	2922.16	46.43
3	2953.02	82.54	2953.02	61.86
Total		229.21	Total	169.51

288 Table 8. Intensity pattern of Sample SHNK with *Pseudomonas aeruginosa* at 5000 ppm

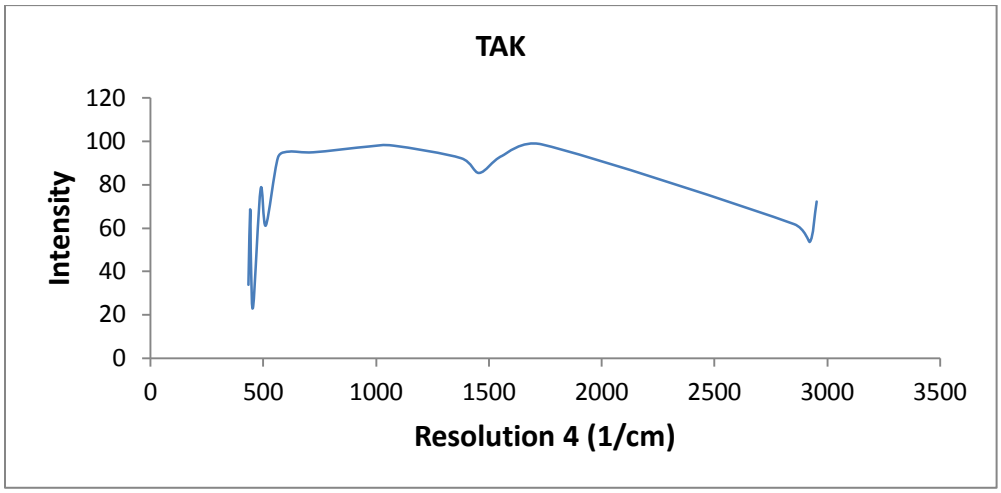


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Figure 9. IR spectroscopic peak of Sample TAK with *Bacillus subtilis* at 5000 ppm

Intensity of control (5000 ppm)			Intensity of sample TAK (5000 ppm)	
S. no	Peaks	Intensity	Peaks	Intensity
1	2852.72	78.98	2852.72	61.83
2	2922.16	67.69	2922.16	53.61
3	2953.02	82.54	2953.02	72.24
Total		229.21	Total	187.68

Table 9. Intensity pattern of Sample TAK with *Bacillus subtilis* at 5000 ppm

Identification of Isolated Diesel Degraders

Four bacterial isolates showed positive results during diesel degradation analysis of the selected samples. These bacterial species were identified based on both morphological and biochemical identification techniques.

Different morphological characteristics were considered during identification. On the basis of these morphological features three bacterial species namely *Pseudomonas aeruginosa, Bacillus subtilis and Bacillus* cereus were tentatively identified. The morphological features of these strains are given in Table 10.

Table 10: Morphological features of bacterial strains

S. no	Sample code	**Growth samples characteristics**			Bacterial species identified tentatively
		Cell shape	colony colour	Gram test	
1	BHG	Smooth, circular	white-cream	Gram +ive	*Bacillus cereus*

2	SHNK	Rod-shaped	blue/green	Gram –ive	*Pseudomonas aeruginosa*
3	TAK	Rod-shaped	Brownish	Gram +ive	*Bacillus subtilis*

The identification of the isolated bacterial species was further confirmed by using various biochemical tests. The results of these biochemical tests are given in Table 11.

Table 11**:** Identification and characterization of diesel-degrading bacteria using various biochemical tests

S. No.	Biochemical tests	*Bacillus cereus* (BHG)	*Pseudomonas aeruginosa* (SHNK)	*Bacillus subtilis* (TAK)
1	Catalase	+	+	+
2	Flurecent Pigment	–	+	–
3	Oxidase	+	+	–
4	Indol	–	–	–
5	Methyl red	–	–	–
6	Motility	+	+	+
7	Simmons citrate	+	+	+
8	Urease	+	+	+
9	voges-proskauer	+	–	+

Table 12: Identification and characterization of diesel-degrading bacteria using TSI

Organism	**Triple sugar iron**			
	Slop	**Butt**	**H2S**	**GAS**
Bacillus subtilis	**R**	**Y**	**-**	**-3**
pseudomonas aureginosa	**R**	**Y**	+	**-**
Bacillus cereus	**R**	**Y**	**_**	**-3**

Key: H_2S=hydrogen sulphide gas (blackening), R= red pink (alkaline reaction), Y=Yellow (acid reaction)

The results stated that Identification on the basis of biochemical test and TSI were confirmed that species characters were similar to *Bacillus subtilis,Bacillus cereus* and *pseudomonas auerginosa* as describe by Smibert and Krieg (1994).

Disscusion

The present exploration was carried out to assess the status of biodegrading bacteria in soil samples collected from various localities in district Mansehra. The results of the current investigation evidently indicated that limited samples gave positive results. The ability of isolated bacterial species to degrade commercial diesel was also evaluated using IR spectroscopy. The method of biodegradation of important chemicals by microorganisms has gained much significance in the field of Biotechnology. Now several researches are being conducted in various parts of the world to isolate valuable biodegrading bacteria and fungi from soil or other sources and then use these species in environmental Microbiology.

Soil contaminated with petroleum hydrocarbon is the most probable resource to isolate diesel degrading microbes. The degradation potential of isolated bacteria was examined. Disclosure to rising point of diesel concentration was used to find out the resistance of isolated strain. The use of microbes to degrade such health perilous compounds is the need of time. Several researches are being conducted to build up techniques to isolate such kind of degrading bacteria from soil or water and then use these strains on commercial basis to hygienic our environment from such carcinogenic compounds.

Diesel is one of the most ordinary pollutants present in the surroundings. It enters into the soil and water through diverse pathways like leaking the pipelines, car wash stations, auto workshops and oil purifying factories. There are various reports of biodegradation of diesel by the action of different bacterial species that are found in very harsh conditions, Sorkoh *et al*., (1993). In addition to the current research Ijah and Antai, 2003) stated that *bacillus* species was the most prominent isolate of all diesel fuels utilizing bacteria. As Zhuang*et al*., (2002) demonstrated that gram negative bacteria allow them to maintain in the extreme variable diesel contaminated environment. In the present exploration three bacterial species namely *Bacillus subtilis, Bacillus cereus* and *Pseudomonas aeruginosa* were isolated and they showed the potential of degrading commercial diesel under laboratory conditions.

The diesel degrading capability of microbes can be evaluated by using special methods and conventional instruments. Several scientists used Gas chromatograph, GCMS and IR spectroscopy for degradation evaluation. The isolation and augmentation of such microbes on selective media along with commercial diesel has also been considered. In the present work FTIR spectroscopy of Shimadzu Corporation has been used to qualitatively evaluate the determination of commercial diesel fuel presented in two different concentration low and high

1000 ppm and 5000 ppm respectively for 15 days. The FTIR analysis showed that the incorporation of bacterial species that was isolated indicate the degradation of diesel fuel as compared with the samples that were not supplemented with bacteria. The results of IR analyses of TPH shows that the additions of all bacteria strains enhance degradation of diesel fuel compared to the sample that was not supplemented with any bacteria strain Yousefi Kebria *et al.*, (2009).

The results of the FTIR spectrum were plotted on a graph paper. The spectrum taken for a control sample having diesel is given in Figure 4.4. It obviously indicates the qualitative analysis of sample. It showed IR spectroscopic peak at 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ corresponds to the stretching vibration of hydrogen and carbon bonds which was recorded on a graph paper (Figure 4.4). The intensity of the above peaks in the spectrum of control was compared with the intensity of the peaks in the spectrum of the test samples. This response was used as control for comparison with results of test samples offered diesel at a concentration of 1000 ppm. An almost complete removal of diesel components was seen from the reduction in hydrocarbon peaks observed using Solid Phase Micro extraction Gas Chromatography analysis after 5 days of incubation Shukor *et al.*, (2009).The 1000 ppm concentration control intensity of peaks at 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ was 10.1, 7.11, 4.22 upon calculations the overall intensity was 21.42 which was high as compared with the intensity of a spectrum of the samples. It has been found that only 4 samples showed degradation of commercial diesel as the IR spectroscopic peaks at 2852.72 $^{cm-1}$, 2922.16 $^{cm-1}$, 2853.02 $^{cm-1}$ have low intensity as compared with control. In some recent studies done in different countries confirmed the presence of the same bacterial species in different contaminated soil samples as Yousefi Kebria *et al.*,(2009) examined the biodegradation of diesel by *B.cereus* and *B.thuringiensis* strains in 500 and 10000 ppm. It was

found that bacterial species degraded the diesel with increasing the concentration of diesel. In the present study *B.cereus and B.subtilis* was also isolated and showed the capacity of diesel degradation in the concentration of 1000 ppm and 5000 ppm.

Several investigators have reported that inoculation of microbes that acquire the degradative capacity of natural pollutants in cultures may not succeed to function when introduced into the natural environment. With special fertilizers the nutrient sources had a significant collision on diesel degradation of the same isolate. It might be sufficient to harbor microorganisms from a contaminated site, because the microbes have adapted to a contaminated environment and utilizes the contaminant as a sole of carbon and energy (Sohal and Srivastava, 1994; Watanabe, 2002; Ghazali *et al*., 2004; Das and Mukherjee, 2006).The results strongly indicate that the environmental conditions including physical and chemical conditions of the contaminated sites play a crucial role in the degradation even though additional diesel-degrader has been introduced into the contaminated site.

The influence of environmental factors rather than genetic capability of a microorganism have been reported to limit the degradation of pollutants.

The ability of isolated species *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas aeruginosa* as bio-degraders of diesel could be very useful for bioremediation of such petroleum hydrocarbon in areas of Mansehra and other localities in Pakistan.

CONCLUSION

We conclude that diesel-degrading bacteria or other oil degrading bacteria are richly found in the oil contaminated soils in Mansehra and this can be demoralized for large oil-spill clean-up campaigns. This study also provides information about the physico-chemical and biological

requirements for optimum degradation of commercial diesel by these bacteria in the laboratory condition.The microbes isolated from oil contaminated soils in Mansehra were proficient of degrading diesel with a concentration of 1000 ppm and 5000 ppm. All three species were competent to degrade diesel with variations. Among the isolated organisms the *Pseudomonas aeruginosa* were found to degrade diesel up to 1000 ppm and the other isolates namely *Bacillus subtilis, Bacillus cereus* degraded diesel best at 1000 ppm rather than 5000 ppm.

The study purely describes that both gram positive and gram negative bacteria have the capability to degrade diesel fuel. Where ever we use the bacterial consortium in the natural contaminated environment it should be give better results instead of other chemical process.

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