**ABSTRACT**

Increasing soil pollution all over the world has instigated global concerns as enormous quantities of toxic chemicals and heavy metals like cadmium, lead, mercury, petrochemicals, insecticides, polycyclic aromatic hydrocarbons (PAHs) and chlorophenols are finding their way into the environment, affecting the land and soil, causing soil pollution and thus posing a threat and menace to health and well-being of people and ecosystem. The ubiquitous dissemination, low bioavailability, high perseverance of contaminants like poly-hydrocarbon and metals in soil have the potentially destructive effects to human health, envisages to study the biodegradation of PAHs (polycyclic aromatic hydrocarbons) and PACs (polycyclic aromatic compounds). The diversity of micro-organisms that diminish the PAHs/PACs can be utilized in the advancement of bioremediation techniques. The role of metal-tolerant, (PAH)-degrading bacteria helps in the biodegradation of organic compounds at miscellaneous polluted sites. The isolation of (PAHs)-degrading bacteria from contaminated soil samples collected from garages and petrol pumps of Delhi and NCR region was carried out in the present study. Also, the bacterial samples were tested for the tolerance towards 4 heavy metals- arsenic (As), lead (Pb), cadmium (Cd), and mercury (Hg). Morphological studies and biochemical tests were conducted to find the genera of the bacterial samples. The study indicates that hydrocarbons were degraded by the isolates P1, P2, P4, P5, P5*, G1, G3. These isolates were also found to be tolerant at a high concentration of metals (Arsenic, Cadmium, Mercury, and Lead) as minimum inhibitory concentration (MIC) was also calculated. Antibiotic susceptibility of the isolates was tested against various antibiotics. Thus the study suggests that the isolates identified as Pseudomonas aeruginosa, Acinetobacter baumanii, and Klebsiella pneumoniae are not only PAH-degrading but metal-tolerant and antibiotic-resistant too and are of immense potential for bioremediation of contaminated soils.
uble in water and soil, gets adsorbed onto the particulate materials, resulting in their degradation slowly. According to Oyenekewa (2011), degradation in sediments is a gradual process. Therefore the soil is considered as a major environmental sink for hydrocarbons. Phillips (1999) and Luch (2005) reported in their study about the toxicity and carcinogenicity of aromatic hydrocarbons in human beings and potentially deleterious effects on human health. Although few physical processes such as leaching, volatilization, photo, and chemical oxidation are known for decreasing the levels of PAHs from the environment (Heitkamp et al., 1988), but are usually confined to aquatic environments.

Pollution by hydrocarbons also results in the accumulation of metals in soil and, eventually, the uptake and distribution of the same in plants (Vwioko et al., 2006). Studies reported by Olugboji and Ogunwole (2008); Ipeaiyeda and Dawodu (2008) at auto garages or workshop sites suggest that spent engine oil finally seeps into water bodies after it enters into the environment. These sites largely contaminated with heavy metals as well as hydrocarbons, often referred as hazardous site are of chief concern in the current scenario (Tremaroli et al., 2009; Ramakrishnan et al., 2011) and remediation of the same through conventional techniques-physical, mechanical and chemical is not so effective.

Bioremediation provides an enhanced technique for treating and removing the contaminants. Microorganisms act as an important crusader in bioremediation of soil contaminated intermittently with heavy metals and hydrocarbons. Some microbes ubiquitously distributed in nature possess the ability of utilizing hydrocarbons as the only carbon source to provide energy for different metabolic activities and therefore, can be used as a remedial measure against contaminants (WHO, 1987; Lee and Shim, 2007) proving effectual for the enhancement of clean-up process and designing of potential strategies for bioremediation (Lloyd et al., 2005). According to (Adeline et al., 2009), the microorganisms degrading the hydrocarbons depend on the length and structure of the carbon chain constituting the organic compound as well as the environment factors.

Metals, when present in soil contaminated with hydrocarbon, hinder the degrading activity of bacteria by interacting/interfering with enzymes, damaging cell membranes or DNA as reported by Thavamani et al. (2012a,b). Certain microbes, through some mechanisms, survive even at high metal concentrations Bruins et al. (2000); Thavamani et al. (2015); Tiku and Asikong (2016) suggested the role of metal-tolerant, poly-aromatic hydrocarbon-degrading bacteria acting as an inexpensive, important and effective tool in the remediation of mixed contaminated sites.

There is a need to ascertain the antibiotics susceptibility pattern of the microorganisms when used for bioremediation of hydrocarbon-heavy metal-polluted environment in other to avoid the spread of antibiotics resistance genes in the environment. Therefore, this research work is aimed at analysing the hydrocarbon biodegradation potential, heavy metal tolerance, and antibiotic resistance among bacterial isolates from petroleum polluted soil samples. The current study included isolation of bacteria from petroleum-contaminated areas, their screening for degradation of petrol and tolerance of these potential bacterial strains towards heavy metals like cadmium, lead, arsenic, and mercury and resistance towards antibiotics. Further, the potential bacteria were isolated and characterized depending on morphology, cultural, biochemical, and physiological features.

MATERIALS AND METHODS

Chemicals
Analytical grade chemicals were used for the experiments.

Collection of samples
In the present study, to isolate novel strains of petroleum degrading and metal tolerant microorganisms, soil samples were collected from various petroleum oil-contaminated sites such as petrol pumps, garage, and automobile workshops from different localities of Delhi and NCR. The samples were collected in sterilized polythene bags, from a depth not exceeding 6 inches. Each polythene bags was well labelled with the name of the site of collection before being processed.

Isolation of petrol degrading bacteria
Isolation of bacteria from oil-contaminated soil samples were followed in Erlenmeyer flask containing Bushnell Hass (BH) broth medium (MgSO$_4$, 0.20g; CaCl$_2$, 0.02g; KH$_2$PO$_4$, 1.00g; K$_2$HPO$_4$, 1.00g; FeCl$_2$, 0.05g; NH$_4$NO$_3$, 1.00 g; per liter of distilled water and 1% petrol). These 50 ml Erlenmeyer flask containing 20 ml BH medium were sterilized by autoclaving at 15 lbs pressure for 20 min at 121°C. 1 g of soil with 1% petrol and a set of the flask containing 1% petrol in BH medium without soil sample were also run as substrate control. All flasks were then incubated at 37°C, 150 rpm for 3 days on orbital rotatory shaker [21] (Deziel et al., 1996), and observed for development of turbidity in the
Table 1: Morphological tests of bacterial isolates

| Gram Staining | Endospore Staining | Motility |
|---------------|--------------------|----------|
| Positive (+ve) P3*, G2 | P1, G1, G3 | P1, G1, G2, G3 |
| Negative (-ve) P1,P2,P3,P4,P5,P5*, G1, G3 | P2,P3,P3*,P4,P5,P5* |

Table 2: Biochemical Characterization of bacterial isolates

| MacConkey Agar Test | Starch Hydrolysis | Catalase Test | Citrate Utilization | Mannitol Salt Sugar Test | Urease Test | Methyl Red Test | VP Test |
|---------------------|-------------------|---------------|--------------------|--------------------------|-------------|----------------|---------|
| Positive | P1, P2, P3, P4, P5, P5*, G1, G3 | P3*, P4, P5, P5* | P1, P2, P3, P3*, P4, P5 | P4, P1, G1 | P3 | P1, P3, P3*, P4, G2 |
| Negative | P3*, G2, P1, P2, P3, G1, G2, G3 | G1 | P1, P2, P3, P4, P5, P5*, G1, G2, G3 | G1, G2 | P1, P2, P3, P4, P5, P5*, G1, G2, G3 | P2, P5, P5*, G1, G3 |

Table 3: Antibiotic sensitivity of the isolates

| Ampicillin | Kanamycin | Rifampicin | Vancomycin | Tetracycline | Gentamicin |
|-----------|-----------|------------|------------|--------------|------------|
| Resistant | P1, P2, P3, P3*, P4, P5, P5*, G1, G2, G3 | P1, P2, P3, P4, P5, P5*, G1, G3 | P1, P2, P3, P4, P5, P5*, G1, G2, G3 | P1, P3, P3*, P4, P5, P5*, G3 | P1, P2, P3, P3*, P4, P5, P5*, G1, G2, G3 |
| Sensitive | P3* | P3, P3*, P4, G2 | P2, P3* | P2, G1, G2 | P1, P2, P3, P3*, P4, P5, P5*, G1, G2, G3 |

medium. 1 ml aliquot of broth medium from all flasks was serially diluted up to 10^{-5} and vortexed for a few minutes. Then, 0.1 ml of these dilutions (10^{-3}, 10^{-4}, 10^{-5}) were spread aseptically on freshly prepared BH agar plates and were incubated at 37°C for 48 hours. The microbial colonies that appeared with characteristics of bacterial morphology were isolated and purified on the BH agar medium.

Screening of petrol degrading ability of isolated bacterial strains

The isolated colonies were further checked for their growth on Bushnell Hass media containing 5% of petrol. The colonies were aseptically streaked on plates containing BH media and petrol as the only source of carbon, followed by incubation for 2 days (48 hours) at 37°C. The resultant colonies that showed the best growth on the media were selected and identified by using Gram’s staining method and by doing biochemical characterization.

Identification of selected bacterial isolates

Morphological and Biochemical Characterization

Selected microbial colonies were characterized by morphological and biochemical characters. Gram’s staining and morphological characterization like motility test and spore staining were done according to Cappuccino and Sherman (1992). Additional biochemical tests were performed according to Aneja (2010) for taxonomic characteriza-
tion, which included starch hydrolysis, catalase test, Methyl Red (MR), Voges- Proskauer (VP) test, citrate utilization, carbohydrate utilization, and urease test.

**Petrol Biodegradation assay of isolated bacterial strains.**

A modified method of Hanson et al. (1993) was utilized for the biodegradation assay. Biodegradation of petrol with selected ten isolates were performed with various concentrations of petrol (1-6%). For all the concentrations, the experiment was conducted at 37°C. The method utilized 2, 6-dichlorophenol indophenol (DCPIP) dye. The ELISA plate was marked, and each well contained BH broth inoculated with culture and incorporated a certain percentage of petrol and dye. Control wells were also prepared, which lacked the microorganism. The degradation of petrol was studied over a duration of 7 days and also monitored daily for color change from deep blue to colorless (from oxidized to reduced state). The readings were taken on a daily basis using ELISA reader. The colour variation happens because of molecular structural change, as the bond between carbon and nitrogen atom breaks from double to a single bond. This change in colour can directly be related with utilization/degradation of hydrocarbons (Bidoia et al., 2010)

**Determination of minimum inhibitory concentration (MIC) of heavy metals**

The isolated colonies were checked for tolerance towards heavy metal. MICs were determined by the plate dilution method against heavy metals Hg, Cd, Pb, and As by constantly augmenting the concentration of the heavy metals on BH media plates, which contain 1% petrol till no colonies grew on the plates. The initial concentration that was used was 2mM and thereby, constantly increasing the concentration every time (2000mM for arsenic and cadmium) and (160mM for mercury and lead) on BH media plates. The minimum concentration restricting microbial growth is contemplated as the MIC.

**Antibiotic sensitivity of the bacterial isolates**

Isolated petroleum degrading and heavy metal resistant isolates were tested for antibiotic resistance and sensitivity, by Kirby- Bauer’s disc diffusion method (Bauer et al., 1966). Once incubated for 18 hours, the micro-organisms were categorized against antibiotics as sensitive or resistant, depending on the diameter of the zone of inhibition as provided in the standard antibiotic disc chart.

**Species identification**

Species identification was done using Biomereux VITEK 2 system (Ligozzi et al., 2002). These isolates were analyzed morphologically and biochemically (Table 1 and Table 2).
The petrol degradation potential of the isolates was studied and quantified. It was found that P5 had maximum degrading ability among all the isolates. It showed a maximum degradation of petrol in 6 days duration. The degradation potential of petrol of all isolates is as follows:

P5 > P2 > P1 > G3 > P5* > G1 > P3* > P4 > G2 > P3 (Graph 1)

The heavy metal tolerance of isolates was also studied and determined for heavy metals lead, arsenic, cadmium, and mercury. P1, P2, P4, P5, P5*, G1, G3 showed high MIC in the range of 1200-1700 mM for arsenic. All the strains had a minimum inhibitory concentration from 1200 -1900 mM for cadmium. MIC for mercury and lead was quite low. Only P5* showed MIC of 150 mM for the lead. So isolates P2, P5*, G1 and G3 showed greater MIC values thus, greater tolerance to heavy metals as compared to other isolates (Graph 2 and Graph 3)

Antibiotic sensitivity of the isolates was studied using antibiotics Ampicillin, Kanamycin, Tetracycline, Vancomycin, Gentamicin, and Rifampicin. P1, P2, P3, P4, P5, P5*, G1, G3 strains showed multiple antibiotic resistance to ampicillin, kanamycin, rifampicin, tetracycline. G2 was sensitive to rifampicin, tetracycline, and gentamycin. This shows that these strains had multiple antibiotic resistance traits. P5 and G3 with high tolerance to arsenic and cadmium were found to be resistant to ampicillin, rifampicin, vancomycin, and tetracycline (Table 3).

Isolates P1, P2, P4, P5, P5*, G1, and G3 were identified by using VITEK2. P1, P5, P5*, G1, G3 were identified as Pseudomonas aeruginosa. P2 and P4 were identified as Acinetobacter baumannii and Klebsiella pneumoniae, respectively.

The cleaning/breakdown of petrol or hydrocarbon in the soil or environment by bacteria, yeast, and fungi is referred to as microbial degradation (Broomjimans et al., 2009). Researchers like (Jan et al., 2003; Atlas, 1992; Yakimor et al., 2007) have shown that mixed population of bacteria with battery of enzymes can degrade hydrocarbon present as contaminant in soil, freshwater, and marine environments through oxygenases which are substrate-specific and act on the hydrocarbon initially. Biodegradation of hydrocarbons can be done either directly by the bacteria by attaching itself to the substrate or through the biosurfactants production (an indirect mechanism) as reported by (Mittal and Singh, 2009). (Das and Mukherjee, 2005) and Patil et al. (2012) reported that Pseudomonas sp produces biosurfactants (surface-active agents) to solubilize aliphatic or aromatic hydrocarbons. Zanaroli et al. (2010) reported in his study about the lack of availability of indigenous microbes useful for the bioremediation process at the polluted site. Wherein Bidoia et al. (2010) suggested in his study a consortium of prospective oil digesting and degrading microorganisms needed for bioremediation.

Bacteria are the most active agents in petroleum degradation. A greater number and percentage of bacteria isolates from the petroleum polluted soils in this study were able to tolerate the heavy metals (Pb, As, Cd, and Hg) tested.

The antibiogram profile of the bacteria isolates from the petroleum polluted samples revealed varying resistance to the antibiotics (Tiku et al., 2016). This can be due to the enzymes produced by the bacterial cell, thus causing changes in the bacterial cell membrane, modification of target site, and development of metabolic pathways by bacteria (Kim et al., 2006). A study by Oyetibo et al. (2010) reported heavy metal resistant and antibiotic-resistant among bacterial isolates to gentamycin, rifampicin, and ofloxacin. The resistance of the organisms to the antibiotics confirms the correlation between resistance metal ions and antibiotics (Oboh et al., 2006).

CONCLUSIONS

Petroleum polluted environments such as automotive workshops soils could serve as a source of microorganisms, especially bacteria that can be useful in the bioremediation of a hydrocarbon-heavy metal-polluted environment. Soil contaminated with hydrocarbon can be a good source of PAHs/PACs degrading bacteria, which can be a potential candidate for bioremediation of such compounds from the environment. The isolates obtained in the present study appears to be well adapted and acclimated to grow in polluted hydrocarbon area. Thus they can be used for decontaminating the hydrocarbon-rich site. The maximum biodegradation efficiency was of P5, and significant degradation was shown by isolates P1, P2, P5*, G1, and G3 after six days of incubation. The isolates P2, P5*, G1, and G3 showed high heavy metal tolerance. Thus, from the study, it can be an inference that the isolates P1, P2, P5, P5*, G1, and G3 are desirable for the preparation of consortium for bioremediation of petrol and heavy metal contamination. Using this consortium will be an effective and promising eco-friendly technology for the degradation of hydrocarbons from crude oil. In addition, the screening of these useful microorganisms for antibiotic resistance is necessary in controlling the spread of antibiotic-resistant genes to other bacteria in the environment, as this could endanger both the pub-
lic health and the environment at large.

**Conflict of interest statement**

Authors declare that they have no conflict of interest.

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