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Biodegradation potential of petroleum hydrocarbons by bacteria and mixed bacterial consortium isolated from contaminated sites

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Abstract: The aim of this study was to isolate, characterize, and evaluate the potential of petroleum hydrocarbon (PHC)-degrading bacterial strains from oil-contaminated soil in the Meerut region. Among 59 oil-degrading bacterial cultures isolated from the oil-contaminated soil samples, 1 *Bacillus* species, 2 species of *Pseudomonas*, and 1 species of *Micrococcus*, identified on the basis of biochemical and 16s rDNA sequencing, were found to have the ability to utilize PHCs such as benzene, diesel, toluene, anthracene, and naphthalene. These strains were selected for further study to measure the quantitative determination of PHC metabolization. Along with these selected strains, a mixed bacterial consortium was formulated and used for PHC degradation. Among the individual strains, *Pseudomonas* sp. APHP9 performed better than the other bacterial isolates. Maximum biodegradation of benzene and toluene was done by the bacterial consortium. The mean growth rate constant (K) of soil isolates also increased with a successive increase in PHC concentration. Moreover, *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9, *Pseudomonas* sp. APBP1, *Micrococcus* sp. APIO4, and the consortium resulted in a 54.8%, 60.2%, 40.9%, 32.5%, and 66.2% decrease in benzene concentration and a 61.2%, 68.4%, 53.7%, 39.3%, and 75.4% decrease in diesel concentration, respectively, after 6 days of incubation as estimated by HPLC analysis.

Key words: Bacteria, consortium, biodegradation, hydrocarbons

1. Introduction

Petroleum and petroleum product spills both offshore and onshore can lead to severe environmental pollution. For instance, large-scale accidental spills in oceanic regions pose a great threat to the marine ecosystem. Similarly, in the case of any large-scale onshore oil spill, the petroleum components may reach the water table before becoming immobilized in the soil. Those components that reach the water table may rapidly spread horizontally on the ground water surface and ultimately partition into groundwater and soil pore space along the transport path, causing the contamination of large volumes of soil and groundwater. An efficient way of remediating the oil-contaminated sites could be employment of special microorganisms, such as bacteria, microalgae, and fungi, either isolated from a different environment and introduced into contaminated sites or isolated and enhanced from the organisms already present in the same environment [1].

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The contamination of the environment with petroleum and petroleum-based hydrocarbons has had significant effects on environmental and human health. Much attention has been paid to novel approaches for bioremediating these oil-based contaminants. The techniques of bioremediation are at present receiving encouraging promotion and exposure as potentially effective approaches for hydrocarbon bioremediation. Furthermore, the biological approaches and techniques could have an advantage over the commonly applied physicochemical management system in eradication of oil spills, as they are more cost-effective for the biodegradation of petroleum hydrocarbons (PHCs). Bioremediation can be defined as the alteration of chemical compounds by living beings, in particular microbes, into cell biomass, energy, and natural waste commodities [2].

Among the major hydrocarbon products, benzene is of major concern as it is a stable, water-miscible, highly mobile, poisonous, and cancer-causing aromatic compound. Successful degradation of benzene by microorganisms in an aerobic environment has been reported; however, under anaerobic conditions its rate of biodegradation is observed to be very slow and poor [3,4]. The common bacterial genera exploited for benzene bioremediation are *Pseudomonas*, *Bacillus* [5], *Acinetobacter* [6], *Gammaproteobacteria* [7], and *Marinobacter* [8]. The other bacterial species identified for diesel biodegradation were *Pseudomonas aeruginosa* [9] and *Staphylococcus aureus* [10].

The employment of microorganisms in the biodegradation of hydrocarbons over chemical or conventional treatment is preferred for many reasons. First of all, the potential and selected microbes can alter raw and crude oils in beneficial ways and the resulting end products are comparatively safer to the environment and all living beings. Microorganisms have been employed for bioremediation of hydrocarbon-rich waste material products, along with their various recalcitrant noxious compounds, which are finally converted into environmentally friendly products. These microbes utilize waste material as carbon substrate, increase their population, and ultimately biodegrade hydrocarbon products to nontoxic products, such as H_2O and CO_2 [11].

PHCs can be degraded by various microorganisms such as bacteria, fungi, and yeast [12]. Studies have shown that most potential bacteria for PHC degradation have been isolated from areas contaminated with oil [13]. In the biodegradation process of crude oil, the use of microorganisms, and especially the adapted bacterial isolates, modifies the microenvironmental factors to permit microbial growth, which results in a speedy bioremediation process [14]. For survival and proliferation in such environments, microbes rely on nutrients for the production and excretion of exoenzymes for the breakdown of hydrocarbons and their products. The rates of uptake and mineralization of many organic compounds by a microbial population depend on the concentration of the compound [15]. The biodegradation of hydrocarbons is strongly influenced by a large number of local environmental factors that manipulate microorganisms' proliferation and their enzyme-synthesizing abilities. Keeping the above points in view, this study was carried out to explore the possibility and investigate the application of selected potential bacterial strains alone and within a consortium isolated from oil contaminated site to biodegrade benzene and diesel.

2. Materials and methods

2.1. Bacterial isolation

Soil samples from various oil- and diesel-spilled petroleum stations were collected in presterilized glass bottles in Meerut (Table 1) and shipped to the laboratory for further analyses. The enrichment and the isolation of oil-degrading microorganisms were performed using mineral salts medium (MSM) with different PHC products, such as diesel, xylene, anthracene, naphthalene, toluene, and benzene (Table 2). The isolated dominant bacterial cultures were characterized by their morphological and biochemical characteristics [16].

Table 1. Collection of soil samples from oil depots with respect to the site's designation.

Region	Sites	Designation
Partapur Meerut	Hindustan Petroleum Depot	APHP
	Bharat Petroleum Depot	APBP
	Indian Oil Corporation	APIO

Table 2. Growth tests of bacterial isolates from soil on different solid and liquid hydrocarbons as sole carbon substrate.

S. no.	Strain	Diesel[a]	Nap[b]	Ben[c]	Tou[d]	Xyl[e]	Ant[f]
1	APHP1	+++	++	++	W+	++	++
2	APHP2	W+	++	+++	++	W+	++
3	APHP3	+++	+++	+++	W+	++	+
4	APHP4	+++	+++	++	++	++	++
5	APHP5	W+	+	++	++	++	W+
6	APHP6*	+++	+++	+++	++	+++	+++
7	APHP7	-	-	+++	++	W+	W+
8	APHP8	-	+++	++	++	W+	W+
9	APHP9*	+++	+++	++	+++	+++	+++
10	APHP10	W+	+++	++	++	-	W+
11	APBP1*	+++	+++	+++	++	+++	+++
12	APBP2	W+	+++	+++	++	++	W+
13	APBP3	W+	W+	-	W+	++	W+
14	APBP4	W+	++	-	++	W+	W+
15	APBP5	W+	W+	-	-	W+	W+
16	APIO1	W+	W+	+++	+	+	W+
17	APIO2	W+	W+	++	++	++	W+
18	APIO3	W+	W+	++	-	W+	W+
19	APIO4*	+++	++	+++	++	+++	+++
20	APIO5	W+	W+	-	++	++	W+
21	APIO6	W+	W+	W+	-	W+	W+
22	APIO7	W+	W+	+++	-	W+	W+

a: Diesel; b: naphthalene; c: benzene; d: toluene; e: xylene; f: anthracene; W+: weak growth; ++: moderate growth; +++:excellent growth; -: no growth.

2.2. Screening of strains

Dominant bacterial cultures were inoculated in mineral salts broth (MSB) medium, which consisted of 0.7 g NH_4NO_3, 0.1 g K_2HPO_4, 0.1 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.013 g $CaCl_2.2H_2O$, and 0.0013 g $FeSO_4.7H_2O$ per 100 mL of deionized water with 1% various selected PHCs as a carbon source. They were kept in a shaker at 200 rpm at 30 °C for a period of 7 days. The growth was monitored through culture densities, spectrophotometrically measuring the absorption at 620 nm [17]. The isolates were obtained by plating on MSM, using PHCs as the sole source of carbon and energy. The strains that exhibited the heaviest growth density on MSM were subjected to further studies. Potential strains were APHP6 (*Bacillus* sp.), APHP9, APBP1 (*Pseudomonas sp.*), and APIO4 (*Micrococcus* sp.). These were also the dominant organisms isolated from the APHP, APBP, and APIO sites.

For preparation of the mixed consortium system, a loopful of this selected dominant bacterial culture was grown overnight and used to inoculate 100 mL of sterile nutrient broth medium. The inoculated flasks were

kept at 30 °C for 12 h on a shaker at 200 rpm. For preparing the mixed bacterial consortium, equal volumes (with approximately equal densities ranging between 0.81 and 1.0) of culture broth from the selected isolates were used.

2.3. Molecular studies

2.3.1. Isolation of genomic DNA and amplification of 16S rDNA segment by using PCR

Genomic DNA was isolated by using the HiPura Bacterial and Yeast Genomic DNA Purification Spin Kit (HiMedia) using the manufacturer's instructions [18]. 16S rDNA was amplified using universal eubacterial primers fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'). The rp2 (5'-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3') primer set [19] was used for amplification, procured from Genie (India), on an Eppendorf MC thermocycler.

2.3.2. Phylogenetic analysis of isolate

Searches in the EMBL/GenBank/DDBJ/PDB data libraries were performed using the BLAST (BLASTn) [20] search algorithm in order to establish the identity of the isolate. Sequences of the close relatives were retrieved and aligned with the newly determined sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software, version 4 [21]. The phylogenetic tree was constructed by the maximum parsimony method. The Tajima test statistic [22] was estimated using MEGA4. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

2.4. Biodegradation studies

2.4.1. Quantitative degradation of PHC in culture condition

To get fresh bacterial cultures, the mixed bacterial and individual strains were grown for 16 h. To obtain a cell count of 1.0×10^2 CFU/g, the individual and consortium bacterial strains in the log phase of growth were diluted with sterile distilled water. This cell count was poured into a 250-mL conical flask containing 100 mL of sterile defined MSM with a 1:1 ratio of 1% diesel and benzene [17]. All these flasks were incubated for 6 days at 30 °C at 200 rpm. After 48 h, bacterial samples were withdrawn from each set of flasks and poured on plate count agar using the pour plate method for counting bacterial colony-forming units (CFUs). In all treatments, a modified spectrophotometric method [23] was used to measure total hydrocarbons. To do this, 5-mL samples from all the treatments were mixed properly with an equivalent quantity of dichloromethane (1:1) to extract hydrocarbons from the sample. he extracted hydrocarbon samples were then determined using a spectrophotometer at a wavelength of 600 nm. To determine the residual amount of hydrocarbons in all the samples, a standard curve was also prepared using known concentrations of diesel and benzene. The percentage of hydrocarbon degradation was determined by calculating the disparity between the initial and final concentrations of benzene and diesel by applying the following formula:

$$\text{Percent degradation } (\%) = (\underline{\text{initial concentration }} - \underline{\text{ final concentration}}) \times 100.$$

initial concentration

Finally, the extent of biodegradation of PHC by potential isolates was also confirmed by HPLC (Shimadzu) analysis. For this, cultures of potential isolates were separately taken in 250-mL Erlenmeyer flasks

containing 50 mL of MSB amended with 250-mL aliquots of benzene or diesel. The selected bacterial cultures were later incubated at 30 °C in the dark for 6 days at 150 rpm. Using a separating funnel, the contents of each flask were extracted by 99.5% diethyl ether using gentle shaking. From the separating funnel, the analysis of residual hydrocarbons in the upper organic layer was carried out with HPLC analysis using a chromatograph equipped with an octyl silane column. A methanol/water/phosphoric acid mixture (45:55:1) was employed as a mobile phase for hydrocarbon and metabolite determination (flow rate: 1 mL/min at 254 nm).

2.4.2. Growth profile of effective isolates at varying concentration of PHC

Growth profile of isolates in PHC, i.e. diesel- and benzene-amended media, was determined in MSB supplemented with different concentrations (0.5%, 0.8%, and 1.0%) of PHC. The medium was sterilized and inoculated with potential selected individual strains and the consortium strain and incubated at 28 ± 1 °C (160 rev/min) in a shaker incubator. A positive control was maintained in a parallel experiment with a 2% dextrose solution as the sole carbon source. The growth of selected individual and consortium strains was determined by measuring optical density at 600 nm (OD_{600}) after a time interval of 3 h [24]. Mean growth rate (K) was calculated by the following formula:

$$K = 3.322 \log Z_t - Z_0/\Delta T,$$

where K is the mean growth rate constant, Z_t is final growth at time t, Z_0 is initial growth at time 0, and ΔT is difference in time.

3. Results

3.1. Enumeration of total population density and dominant species from each site

It was found that total population density and PHC-degrading populations from each site ranged from 6.4 to 8 CFU/g and 2.3 to 4.7 CFU/g, while dominant species from these sites ranged from 16.6% to 55.7%, respectively. Maximum population density was found from APHP with 8 CFU/g, which was relatively higher than the PHC-degrading population by 57% (data not shown).

3.2. Screening of multiple PHCs such as benzene, toluene, diesel, xylene, and PAH-degrading/utilizing organisms

A total of 59 bacterial strains were isolated from petroleum-contaminated soil. Out of these, 22 isolates were capable of degrading/metabolizing different hydrocarbons, such as benzene, toluene, xylene, and diesel. Furthermore, 4 isolates (APHP6, APHP9, APBP1, and APIO4) were capable of degrading/utilizing all the hydrocarbons used in this study. However, the anticipated number of hydrocarbon-degrading organisms from APBP sites was higher than at APHP and APIO. The abilities of isolates to grow on various hydrocarbons are presented in Table 2, which clearly suggests that isolates APHP6, APHP9, APBP1, and APIO4 had the broader ranges of PHC utilization/metabolization ability.

3.3. Molecular characterization of efficient PAH degrading isolates

The potential isolates used in this study (APHP6, APHP9, APBP1, and APIO4) have the ability to degrade/utilize different PHCs. They were characterized on the basis of different morphological and biochemical parameters as described in *Bergey's Manual of Systematic Bacteriology.* Out of these strains, the 3 strains that

showed appreciable biodegradation capacities were further identified by 16S rDNA gene sequencing as *Bacillus* sp. APH6, *Pseudomonas* sp. APHP9, and *Micrococcus* sp. APIO4 (Figure 1).

3.4. Growth of hydrocarbon-degrading isolates at varying concentrations of PHCs

Isolates APHP6, APHP9, APBP1, and APIO4 were studied for their growth profiles at varying concentrations of benzene- and diesel-amended MSB media. It was found that there was a successive increase in mean growth rate constant (K) values of *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9, and *Pseudomonas* sp. APBP1 isolates with respective increase in the concentration of substrate, while for *Micrococcus* sp. APIO4 there was an increase in K values to up to 0.8 mg/50 mL for both the substrates. In benzene-amended media, the K values obtained were 0.19, 0.48, 0.001, and 0.002 h^{-1} for APHP6, APHP9, APBP1, and APIO4, respectively, which were relatively lower than diesel concentrations. Similar trends were also observed for diesel concentrations where *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9, *Pseudomonas* sp. APBO1, and *Micrococcus* sp. APIO4 had K values of 0.26, 0.37, 0.13, and 0.19 h^{-1}, respectively, in the exponential phase. The average growth rate of all the inoculated bacterial isolates was comparatively higher in the glucose-amended medium (control), where the mean or average growth rate was 0.98, 0.65, 0.29, and 0.66 h^{-1} for *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9, *Pseudomonas* sp. APBP1, and *Micrococcus* sp. APIO4, respectively. The growth profiles of all the isolates at varying concentrations of benzene and diesel with respect to the control are shown in Figures 1–4.

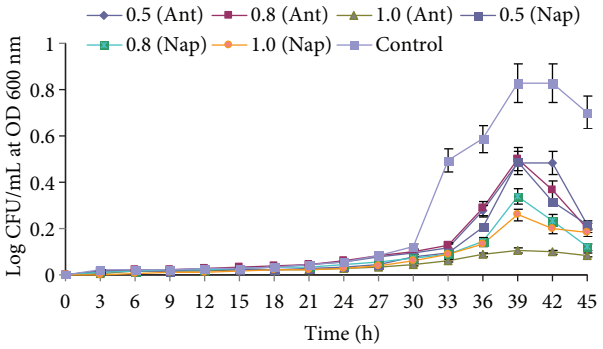

Figure 1. Growth profile of APHP6 in media supplemented with different concentrations of benzene and diesel.

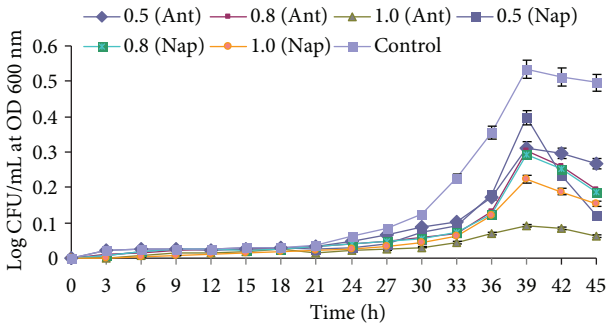

Figure 2. Growth profile of APHP9 in media supplemented with different concentrations of benzene and diesel.

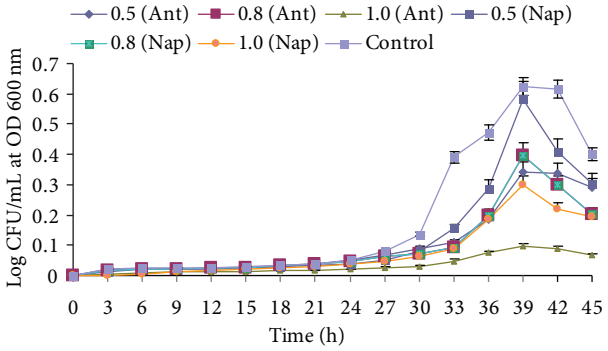

Figure 3. Growth profile of APBP1 in media supplemented with different concentrations of benzene and diesel.

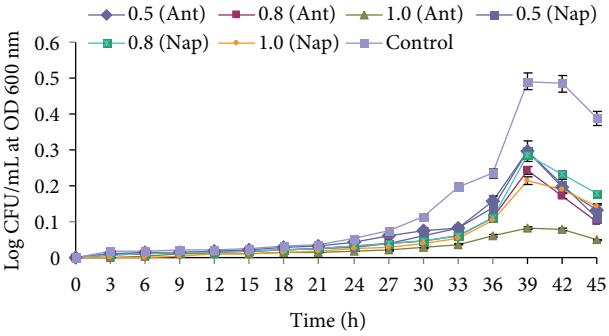

Figure 4. Growth profile of APIO4 in media supplemented with different concentrations of benzene and diesel.

3.5. Determination of residual substrate concentration after biodegradation by HPLC

Bacillus sp. APHP6, *Pseudomonas* sp. APHP9, *Pseudomonas* sp. APBP1, *Micrococcus* sp. APIO4, and a consortium (APH6 + APHP9 + APBP1 + APIO4) of these 4 isolates that have the ability to degrade hydrocarbons were used in this study. Diesel degradation ability was confirmed in solution by HPLC analysis. HPLC analysis of the neutral extracts for *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9, *Pseudomonas* sp. APBP1, *Micrococcus* sp. APIO4, and a consortium from PHC-amended culture media revealed the presence of several metabolites that were eluted at different retention time periods, ranging from 3.0 to 14.2 min. The residual concentrations of diesel were determined by calculating the peak area relative to standard with pure diesel with retention times of 8.933 min and 3.039 min, respectively. *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9, *Pseudomonas* sp. APBP1, *Micrococcus* sp. APIO4, and the consortium resulted in a 54.8%, 60.2%, 40.9%, 32.5%, and 66.2% decrease in benzene concentration (Figure 5) and a 61.2%, 68.4%, 53.7%, 39.3%, and 75.4% decrease in diesel concentration, respectively, after 6 days of incubation as estimated by HPLC analysis (Figure 6).

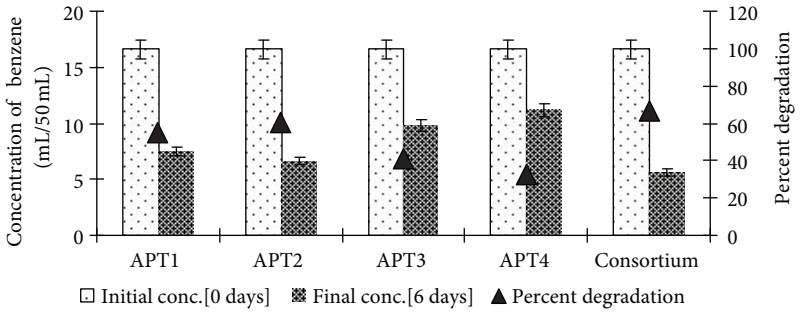

Figure 5. Quantitative degradation analysis of benzene by soil bacterial isolates as estimated by HPLC.

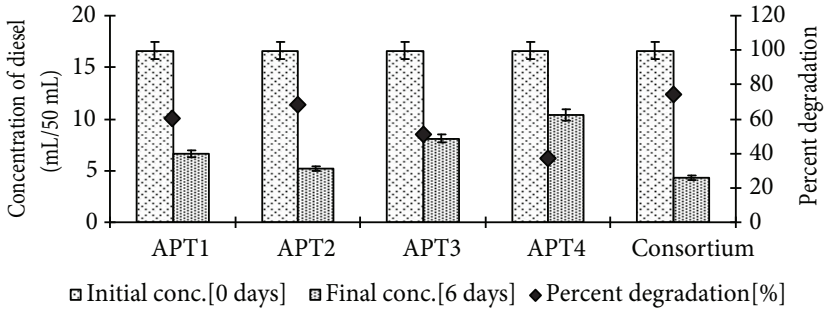

Figure 6. Quantitative degradation analysis of diesel by soil bacterial isolates as estimated by HPLC.

4. Discussion

Screening for relatively fast-growing PHC-degrading bacteria from oil-contaminated soil sediments resulted in the isolation of 4 potential candidate isolates: 1 *Bacillus* sp. (APHP6), 2 *Pseudomonas* spp. (APHP9 and APBP1), and 1 *Micrococcus* sp. (APIO4). Although the isolation methods were unbiased and could select for both gram-positive and gram-negative bacteria, out of all 4 candidate strains, 2 were gram-positive and 2 gram-negative. The 4 strains constituted more than 63% of the total culturable heterotrophic bacteria and more than 69% of the diesel-degrading culturable bacteria. Here, the reason for dominant occurrence of gram-positive

bacteria could be that these bacteria, due to their stronger cell envelope, can proliferate more easily than gram-negative bacteria in the highly variable intertidal sediment environment, where sediment temperatures are high in the day and osmotic pressures and nutrient supply may change periodically over a daily cycle.

A total of 59 bacterial isolates were obtained and screened for their ability to utilize diesel, benzene, and other PHCs as a sole source of carbon and energy. These isolates were identified as 2 *Pseudomonas* spp., 1 *Bacillus* sp., and 1 *Micrococcus* sp. on the basis of 16s rDNA sequencing. This conforms to the high degradative ability and ubiquity associated with these bacterial types as it concerns biodegradation of both soil and water environments polluted with petroleum and its many derivative products [25,26].

This trait of *Pseudomonas* and *Bacillus* spp. to grow on highly xenobiotic or recalcitrant compounds is made feasible by virtue of bacterially synthesized catabolic enzymes. Moreover, owing to the presence of catabolic enzymes, bacterial strains are extremely well equipped to make adaptive changes for their survival in adverse environments. This adaptive strategy in bacterial strains is due to their intrinsic and inbuilt patterns of gene regulation system. This innate ability of bacteria also stimulates various catabolic pathways for enzyme production, as and when required, which leads to hydrocarbon biodegradation. This probably explains why the 2 isolates not only grew on but also metabolized diesel and benzene, which are considered the complex, and hence difficult, aromatic hydrocarbons to degrade. The ability of *Pseudomonas*, *Micrococcus*, and *Bacillus* species to degrade diesel, benzene, and other PHCs was reported by several studies [27]. The PHC-degrading microorganisms showed significant differences in their abilities to biodegrade diesel in a solution condition. This probably accounts for the slight fall in the pH (up to 4.5) of the medium during the growth course, suggesting the possible production of acidic metabolites such as organic acids. The PHC degradation also generally increased with increases in the concentration of PHCs, which agrees with the findings by Boshui et al. [28]. Incidentally, it was observed that the consortium of isolated bacterial strains performed better than isolated strains in liquid medium.

Leahy and Colwell [29] reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons, such as crude oil or diesel fuel. Such mixed cultures display metabolic versatility and superiority to pure cultures [30]. Consequently, a microbial conglomerate containing various microbes that manufacture the degradative enzymes for diverse ingredients of the decomposition pathway is considered to be well suited to the degradation of aromatic hydrocarbons. In any biodegradative course, microbes may not be directly implicated in the degradation channel but, for degradation of aromatic hydrocarbons, produce some micronutrients or surface-active agents [31,32]. Sugiura et al. [33] noted that the biodegradation of oil products by microbial consortium was more effective compared to isolated single cultures due to the complex chemical structures of oil products. A variety of microorganisms have the potential to biodegrade diverse forms of petroleum hydrocarbons. Whenever a consortium of potential microbes is employed to degrade a choice of petroleum hydrocarbons such as benzene or diesel, the overall degradation rate is more successful.

Mostly linear and branched chained alkanes with diverse chain lengths are present in diesel oil, as well as a diversity of aromatic compounds. Among such compounds, the linear alkanes are considered to be especially easily biodegradable by microbes, but the low solubility of such organic compounds makes their biodegradation slow and difficult due to sluggish rates of dissolution, absorption, adsorption, or transport across the cell. As a general rule, the transport process across the microbial cell depends upon the bioavailability of hydrophobic compounds, which rely on their absorption and dissolution or their partitioning rates [34]. In this study, it was observed that the amplification of diesel oil biodegradation was enhanced by employing microbes that already existed in an oil-contaminated environment. Applications of specifically acclimatized microorganisms

can bioaugment the process of bioremediation, since indigenous microbes are well adapted to and acquainted with their own ecosystem. Our isolated soil isolates degraded the diesel well under in vitro conditions, as these strains were isolated from the same environment.

5. Conclusion

It is clear from our study that treatment through bioaugmentation technology, i.e. bioremediation by mixed bacterial cultures with different catabolic degradation routes, complements the metabolization and/or mineralization rate of PHCs and does not allow the contaminant to accumulate. Similarly, our investigation also revealed that the mixed bacterial culture of *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9 and sp. APBP1, and *Micrococcus* sp. APIO4 achieved maximum diesel oil and benzene degradation. Moreover, this consortium formulation will be further explored for in situ remediation applications for other recalcitrant pollutants. Hence, it is suggested that the use of above mixed bacterial consortium under optimized conditions be considered as an alternative technology for effective and ecofriendly technology for the degradation of PHCs.

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