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Original Research Article

Bioremediation of hydrocarbon-contaminated soil: A sustainable solution

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Abstract

Background: Soil microorganisms play a very significant role in bioremediation process. To identify organisms which have capacity to degrade hydrocarbons in soil becomes an important approach in environmental remediation.
Aim: Screening of potential hydrocarbon degrading organisms from different soil and to evaluate its potential in environmental oil and petrol spill clean-up.
Materials and Methods: Total 4 isolates were isolated from 10 oil and petrol contaminated soil samples by using minimal media and petrol as sole source of carbon. Biochemical characterization of these isolates and physiochemical properties of soil was done. The genus of the organism was determined.
Results: Biochemical characterization of isolates was done according to key Bergey's Manual of Systematic Bacteriology (second edition, Volume 3); the potent organism was belonging to the genus *Pseudomonas*. Further gene sequencing studies showed that the isolate was belonging *to Pseudomonas montelii.*
Conclusion: The isolated organism, identified as *Pseudomonas monteilii* exhibit potential oil and petrol degrading activities which make it potential candidate for environmental bioremediation.

Keywords: Bioremediation, *Pseudomonas montelli,* Hydrocarbon-degrading enzymes, Bioremediation strategies, DCPIP.

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1. Introduction

Due to increase in demand for energy, pollution with organic pollutants, polycyclic aromatic hydrocarbons (PAHs), poses a problem to all forms of life.[1] Oil spillage contaminations have very serious effect on the ecosystem.[2] Crude oil and its refined products frequently spilt, and cause soil pollution.[3] These contaminants include PAHs indicating a potential environmental pollutant which alters biological properties of soil in turn will affect change in microbial flora.[5,6]

Generally, soil contaminated with petrol can be treated with physical, chemical, and biological methods.[7] Physical and chemical methods have certain limitations because of which their application becomes less.[8] Thus, bioremediation becomes an effective biological method for removal of petroleum pollutants in environments using selective microbial flora.[9]

Due to various hazardous effect of petroleum hydrocarbon on environment as mentioned above needs to search effective remediation methods for the treatment of contaminated soil. Several strategies have been devised to remediate and restore contaminated environment including conventional methods such as physico-chemical methods and biological methods (requires involvement of biological agents or microorganisms). Physico-chemical methods are employed to remediate the petroleum hydrocarbon contamination from soil such as mechanical, evaporation, dispersion, volatilization, sorption, dilution etc.[10] These methods lead to air pollution. Physical and chemical methods

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are more expensive, less effective and also lead to incomplete decomposition of contaminants. Thus, there is a need to develop a technique which is less toxic to the environment and is more efficient in removal of petroleum hydrocarbon in the soil. Biological methods are widely explored, as they are cost-efficient and suitable for the remediation of petroleum hydrocarbons.[11] As compared to other conventional clean up techniques, bioremediation process represents an important alternative for rehabilitating oil contaminated areas.[12]

There are two main approaches of bioremediation technology namely Bio stimulation and Bio- augmentation, involving addition of nutrients and microbial cultures respectively to degrade the soil contaminants.[13]

The physical parameters are temperature, pH, nutrients, moisture content, organic matter, salinity etc. The choice of appropriate microorganism is a very important factor for the degradation of petroleum hydrocarbon at the contaminated site under favourable environmental conditions. This study aims on utilizing the ability of potential microorganisms to degrade hydrocarbon waste.

2. Materials and Methods

2.1. Collection of soil samples

Petroleum hydrocarbon contaminated soil samples were collected from various automobile garage sites of Camp area, Pune city. A total of 10 soil samples were collected. Soil samples were collected at a depth of 5-15cm. Soils were collected in plastic seal bags. Each soil bags were separately named as S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10. An uncontaminated soil sample was also collected which acted as control. Samples collection time, date, latitude, longitude, area name were labelled and the samples were brought immediately to research lab for further analysis. In the laboratory, the soil samples were air dried and sieved to remove the unwanted particles. The soil samples were stored at 4°C for physico-chemical and microbiological characterization.

2.2. Physico-chemical characterization of collected soil samples

The physical properties like texture, colour, moisture content and total dissolved solids along with various chemical properties like pH, electrical conductivity, organic carbon and organic matter, carbonate and bicarbonate content, available sodium and potassium content, chloride content and available nitrogen were determined for all collected soil samples. These samples were compared with their respective control.[14]

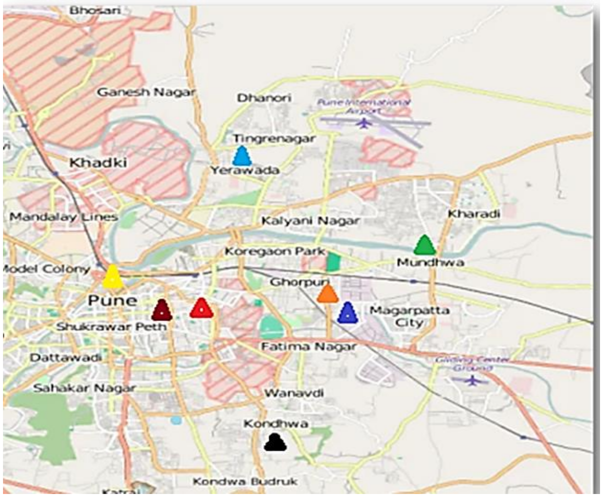

Figure 1: Soil sample collection sites, Pune

Figure 2: Nitrogen estimation of soil samples

Figure 3: Alkalinity estimation of soil samples

2.3 Isolation and identification of microorganisms from collected soil materials and methods

2.3.1. Sample collection

The soil samples were mixed in sterile phosphate-buffered saline and were serially diluted up to 10^{-4}. The dilutions were then plated onto sterile Minimal agar plates supplemented with 1% petrol and oil and were incubated at 37°C for 24 hrs.

2.3.2. Isolation of bacteria

The soil samples mixed in sterile phosphate-buffered saline, were diluted up to 10^{-4}. 0.1ml sample was spread on sterile Minimal agar plates supplemented with 1% petrol and oil and incubated at 37°C for 24 hrs. After the incubation, 4 distinct colonies were obtained and were selected based on their colony characteristics. To get pure cultures the colonies were streaked on minimal agar medium plates. These isolated colonies were then identified up to genus level as per the guidelines provided in Bergey's Manual of systemic Bacteriology second edition Volume 3.

The tests included:

1. Catalase test: In this test the ability of organism to produce enzyme catalase is detected, which breaks down hydrogen peroxide into water and oxygen.
2. IMViC tests: Indole, Methyl Red, Voges-Proskauer, and Citrate utilization test.
3. Nitrate reduction test: To check ability of bacteria to convert nitrate to nitrite.
4. Gelatin liquefaction: To check whether organism produce enzyme gelatinase to liquefy gelatin by certain bacterial species.
5. Sugar fermentation test was carried out. Sugars used in fermentation tests includes 1% Xylose, Maltose, arabinose, sucrose, glucose, lactose, fructose, Trehalose, Mannitol, Sorbitol, and Raffinose.

2.3.3. Bacterial identification by 16S rRNA sequencing

The culture was further subjected to 16S rRNA sequencing to determine the genus and species of the organism.

2.4 Pure hydrocarbon degradation method DCPIP method

To assess the petrol utilization capacity of bacterial isolates, bacterial isolates were inoculated in minimal media broth and incubating them at 37°C for 24 hours. After incubation, centrifuge the culture at 3000 rpm for 10 minutes to obtain cell pellets. The pellets were resuspended in sterile saline, adjusting the optical density (OD) to 0.8 at 600 nm to standardize the bacterial concentration. For the reaction mixture, add 8ml of MS medium, 1ml petrol (1% v/v), and 0.1ml of the prepared bacterial suspension to an Bumper tube Additionally, add 1ml of 0.01% DCPIP (2,6-dichlorophenolindophenol) redox indicator to the mixture to track the bacterial activity. An uninoculated control was prepared with the same components but without bacterial inoculum. The tubes are incubated at 30°C with shaking at 150 rpm for 5-7 days, and the decolorization of the DCPIP is monitored as an indicator of petrol degradation by the bacteria. The extent of decolorization reflects the bacterial capacity to utilize petrol.[15-20]

3. Result

The physicochemical analysis of the hydrocarbon-contaminated soil samples (**Figure 2** and **Figure 3**) revealed several key parameters that influence soil quality and microbial activity. These parameters were consistent with previous studies (**Table 1**), showing a correlation between soil conditions and the presence of hydrocarbon-degrading microorganisms.

Table 1: Physico-chemical analysis

Samples	pH	E.C (µS)	T.D.S (mg/l)	Moisture Content %	% Carbon	% Org Matter	N_2 %	Available N_2 (Kg/Ha)	Chloride Content (mg/l)	CO_3/ HCO3 (mg/l)	Na ppm	K ppm
Control	6.92	0.056	100	9.42	60.12	99.7	0.05	14,800	6.3	12.3	69.1	75.4
S1	8.04	0.041	200	0.17	20.15	34.73	0.0028	6,272	6.94	4	57	38.8
S2	8.32	0.041	400	0.37	19.36	33.37	0.0014	3,136	7.94	6	41.82	54.6
S3	8.10	0.043	1000	0.64	19.36	33.37	0.0042	9,408	11.91	16	34.87	23.5
S4	7.43	0.040	200	1.41	56.62	97.61	0.0070	15,680	12.90	4	30.02	64.93
S5	7.05	0.041	1200	2.14	16.47	28.39	0.0056	12,544	7.94	6	34.07	22.1
S6	7.15	0.042	600	0.41	24	41.37	0.0056	12,544	18.85	12	38.84	27.3
S7	7.55	0.034	1600	0.41	15.1	26.03	0.0014	3,136	19.85	50	64.25	23.8
S8	7.50	0.036	1400	1.01	12.31	21.22	0.0014	3,136	34.74	16	38.59	26.8
S9	7.18	0.035	200	0.25	31.67	54.59	0.0042	9,408	15.88	8	30.31	71.50
S10	7.81	0.041	400	0.53	30.15	51.97	0.0028	6,272	33.74	10	33.53	44.2

Table 2: Results of IMViC TEST

Isolates	Indole	Methyl Red	Voges-Proskauer	Citrate Utilization
B-ISO-1	-	-	-	-
B-ISO-2	+	-	-	+
B-ISO-3	-	+	-	-
B-ISO-4	-	-	-	+

Results biochemical test

Table 3: Summarizes the results of the biochemical tests

Isolate	Nitrate reduction test	Gelatin liquefaction	Urease test	Starch hydrolysis	Oxidase test	Catalase test
B-ISO-1	-	-	-	-	-	-
B-ISO-2	-	-	-	-	+	+
B-ISO-3	+	-	-	+	-	-
B-ISO-4	-	+	-	-	-	-

Results of sugar fermentation test

Table 4: Summarizes the ability of the isolates to utilize and ferment sugars

Sugar	B-ISO-1		B-ISO-2		B-ISO-3		B-ISO-4	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
Xylose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+
Raffinose	--	--	--	--	--	--	--	--

Key: + Acid/Gas produced --: Acid/ Gas not produced

The **Table 2-Table 4** summarizes the results of the biochemical tests conducted on various isolates, including Indole, Methyl Red, Voges-Proskauer, and Citrate utilization. B-ISO-1 does not show any reaction for any of the tests, B-ISO-2 has a positive reaction on the indole test, while both B-ISO-3 and B-ISO-4 have no positive reactions towards methyl red and indole. There is only negative reaction for citrate utilization for B-ISO-4.

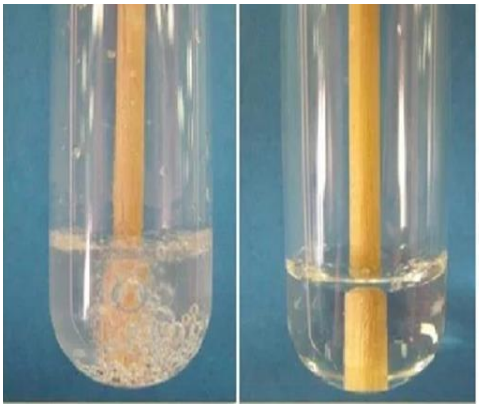

Figure 4: Catalase test

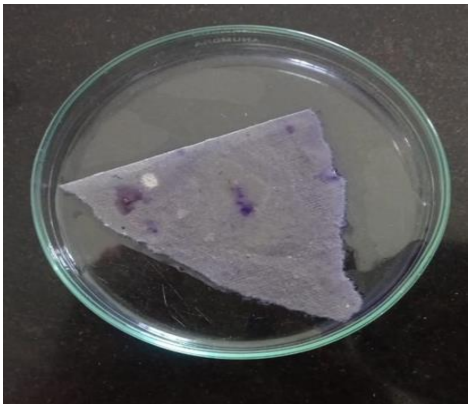

Figure 5: Oxidase test

4. Discussion

The present study focused on isolation and characterization of hydrocarbon degrading organisms present in the soil. The physiochemical parameters (**Figure 2** and **Figure 3**) of hydrocarbon contaminated soil samples were carried out. These parameters determine the quality of soil which in turn influences the microbial communities to degrade petroleum compounds and their derivatives. Our findings (**Table 1**) showed relevance to the previous work done in context with various physiochemical soil parameters.[20]

Amongst the four isolates obtained, the isolate B-ISO-2 showed positive results for catalase (**Figure 4**) and citrate utilisation indicating its ability to decompose hydrogen peroxide and utilize citrate as sole carbon source. These results showed similarity with the guidelines of Bergey's Manual of Systematic Bacteriology (second edition, Volume 3), identifying the isolate's B-ISO-2 as probable *Pseudomonas* spp, 16S rRNA gene sequencing revealed that the isolate B-ISO-2 as *Pseudomonas monteilii*. This species is a part of the *Pseudomonas putida* group and is recognized for its role in biodegradation and bioremediation, particularly in environments contaminated with hydrocarbons,[21] making it suitable for growth on minimal media supplemented with petroleum hydrocarbons. Further DCPIP results confirmed qualitative hydrocarbon degradative ability of *Pseudomonas monteilii.*

5. Conclusion

The isolate obtained during the study B-ISO-2 was identified as *Pseudomonas monteilii* through biochemical profiling and 16S rRNA gene sequencing, confirming its classification within the *Pseudomonas putida* group. The biochemical tests revealed positive oxidase, catalase and citrate utilization, with negative results for MR-VP, nitrate reduction, and gelatin liquefaction, aligning with the characteristics of this species. *Pseudomonas monteilii* is recognized for its biodegradation potential, particularly in hydrocarbon-contaminated environments. Its ability to grow on minimal media supplemented with petroleum hydrocarbons also unique capacity to degrade DCPIP highlights its suitability for bioremediation applications, demonstrating its ecological and environmental significance in addressing hydrocarbon pollution challenges.

6. Source of Funding

None.

7. Conflict of Interest

None.

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