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Isolation and Depiction of Petroleum Hydrocarbon Degrading Bacterial Species from polluted shoreline of Upper Lake, Bhopal

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ABSTRACT

Uncontrollable discharge of Petroleum-hydrocarbon compounds (PHC'S) are prone carcinogenic, mutagenic, hemorrhagic and persuasive immune-toxins to the environment. They are acknowledged as a serious threat to the well-being of the living system especially humans and animals. Bioremediation of the soil and marine environment contaminated by these lethal hydrocarbons has emerged to be a competent, commercial, adaptable and eco-friendly regimen. The point of supply for the water and sediment sample was the shallow region near the shore of Upper Lake, Bhopal in summers of the year 2018. The colonies of the bacteria isolated were identified with the help of the Enrichment Media like Bushnell Hass, Mineral Salt, Starch Agar and Biochemical tests like iMVIC, Catalse, Oxidase and Urease. Some mechanical processes were used for the isolation of DNA from the sample. The peculiarity and clarity of the isolated DNA was stable and reliable and have wide applicability in exploring functional genes responsible for degrading lethal hydrocarbons. This manifest that the above observed bacterial species were advantageous in degrading diesel provided as the primary carbon source thus giving a purposeful and leading solution for Bioremediation of hydrocarbon defilement environment.

KEYWORDS: Hydrocarbon, Bacteria, Bioremediation, Contamination, Upper Lake, Sediments, Enrichment Media.

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INTRODUCTION:

Water supports life on earth and it is a web, which interconnects the life strongly of the entire living organism present on this earth. Due to unplanned urbanization and industrialization we are facing serious problem today because most of the water resources have reached to a point of crisis and every form of life, from microbes to man are suffering because of this. Fresh water resources are premeditated to be the prosperity of the nation, but unplanned exploitation of these resources have led to depreciation and cause them to lose their importance.[1] Availability of clean and potable water has become a major issue in many developing countries. Water should be safe and uncontaminated for human consumption, pleasant to taste and should be usable for domestic purpose.[2] UPPER LAKE also known as Bada talab is situated on the western side of Bhopal city (M.P).[3] It was built in 11th century by Paramara Raja Bhoj , king of Malwa by constructing a dam (now known as Bhadbhada dam) to cure his skin disease.[4] It is a profound and secured ecosystem for about 900 years and serves 39.7% of the natives with 30 million gallons of potable water.[3,5] It covers 31.5 km sq. of the total area.[3]

LONGITUDE: 77®18' TO 77®24'E

LATITUDE: 23®13' TO 23®16'N.[6]

PHCs are naturally occurring unpurified products made up of hydrocarbon deposits and other organic matters found in crude oil .Diesel and petroleum are the major examples of PHCs and are the main source of primary energy and fuel resource.[7] Diesel is lethal to ecosystem due to its higher content of light hydrocarbons.[8] Diesel hydrocarbons disrupt biochemical processes of many organisms leading to carcinogenesis, mutagenesis and hemorrhages in disclosed population.[9] BIOREMEDATION has been verified to be the major and ultimate natural method for complete mineralsation of PHC pollutants to CO_2 and water.[10,11,12,13] Various bacterial and fungal species are capable of degrading PHCs effectively like bacterial genera named *Pseudomonas* , *Micrococcus* , *Arthrobacter* and some fungal genera namely *Candida* , *Penecillium* , *Aspergillus*.[10,14,15]

Sediments are naturally produced materials that are broken by the method of weathering and erosion. They are transported by the action of wind, water or force of gravity. Sand and slits are drifted in suspension in river water. Sediments also often settle-out of slow moving or stagnant water in lakes and rivers. Water saturated sediments that are present beneath the river channel often shelter the majority of biomass in the water resource, primarily in the form of microbial bio-films.[16] Sediment microbial communities are capable of metabolic activities in river and stream ecosystems. These communities are highly complex and sensitive to changes by human practices and are responsible for the lake biogeochemical cycling and supplies 76-96 % of total respiration. Organic

pollutants degradation in the sediments are mainly performed by genera like: *Sulfuricurvum*, *Thiobacillus* and *Burkholderia*.[17,18,19,20,21] For the study we used Enrichment Media (Bushnell Haas Agar Media, Mineral salt Media, NAM, Starch Agar Media) and performed Microscopic Observations (Shape, Color Morphology, Size of the developed colonies) and Biochemical Tests (Voges-Proskauer, IMVIC Test, TSI, Litmus Milk Reaction).

The purpose for this research is that the upper lake of Bhopal is shrinking and is being polluted due to various human activities causing ecological damage and it is a major source of potable water for the local people of the area.

MATERIAL AND METHODS:

Collection of sample:

The sample was collected during the summer season in the month of June from the shore of Boat Club, Upper lake Bhopal (Figure.1). It is situated to the western side of the city. The water resource had the following characteristics: pH: 6-7, DO: 7.9%, Temperature: 34.8^{0}C. The sample collected had the composition of water and sediments. The water sample was yellowish brown in color and had an offensive odor near the site of collection. The sample was bought in the sampling bottle to the laboratory for further analysis.

Figure1. Collection of polluted sample at the study site

Chemicals and Media:

The chemicals used in the study includes Indole Stain (Kovoc's Reagent), Catalase(H_2O_2), Methyl Red Stain, Vogas Proskaur(VP-I and VP-II reagents) Oxidase, Urease. Media includes Bushnell Hass Media, Nutrient Agar Media, Mineral Salt Media, Mobility Agar Media, Starch Agar Media, Tryptone broth, MRVP broth.

Isolation of bacterial culture:

(A) Primary Isolation of Bacterial Culture was done by using Nutrient Agar Media:

250ml of Nutrient agar media was prepared by the composition of Beef Extract, Peptone, Agar, NaCl and Distilled Water. The plates were prepared in the Laminar Air Flow by pouring approximately 9ml of the media in each plate and then were placed until they solidify under sterile conditions. Dilution Series were made from the sample taken up to 5 dilutions. After that the sample taken were streaked out on the plates by sterile glass rod. The plates were then incubated at 37^0C for 24hrs for the further growth of the micro-organisms. Colonies different in shape, color, and sizes were selected from different agar plates and sub-cultured for further analysis.

(B) Pure Culture (Sub-Culturing) was done to obtain pure bacterial colonies:

After the growth of different colonies on the NAM Plates the colonies were isolated from the parent culture with the help of inoculation loop and then were streaked by the technique of quadrant streaking on the NAM plates further. The plates were then incubated at 37^0C for 24hrs for the further growth of the micro-organisms after which the plates were examined for colony growth and enumerated with magnifying hand lens. In total 6 colonies from the sediment sample and 4 colonies from the water sample were recorded as shown in Figure 2 and 3.

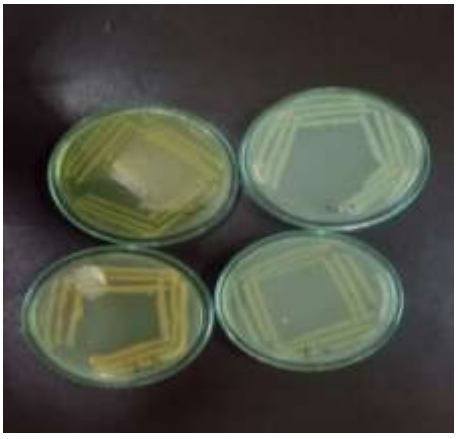


Figure2. Isolation from water sample

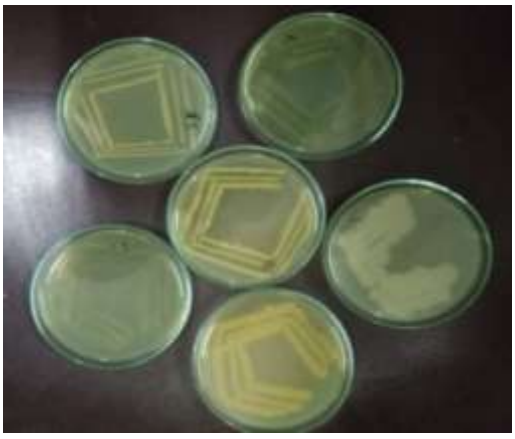


Figure3. Isolation from sediment sample

(C) Selective Media were used for the growth of Hydrocarbon Degrading micro-organism:

Table1. "Media Used For the Experiment"

S. No.	Media	Composition
1.	Bushnell Hass Media	$MgSO_4$: 0.2g, KH_2PO_4: 1g, K_2HPO_4: 1g, $CaCl_2$: 0.02g, NH_4NO_2: 1g, $FeCl_2$: 0.05g, Agar: 20g
2.	Starch Agar Media	Starch: 20g, Beef Extract: 3g, Peptone: 5g, Agar: 15g
3.	Mineral Salt Media	NaCl: 10, KCl: 0.29, $MgSO_4.7H_2O$: 0.42, KH_2PO_4: 0.83, $NaNO_3$:0.42, Na_2HPO_4: 1.25

250ml of the selective media were taken and poured in the plates and let to solidify. The inoculum was taken in the inoculation loop streaked on the enrichment plates. The plates were incubated at 37^0C for 48hrs **(Fig.4)**

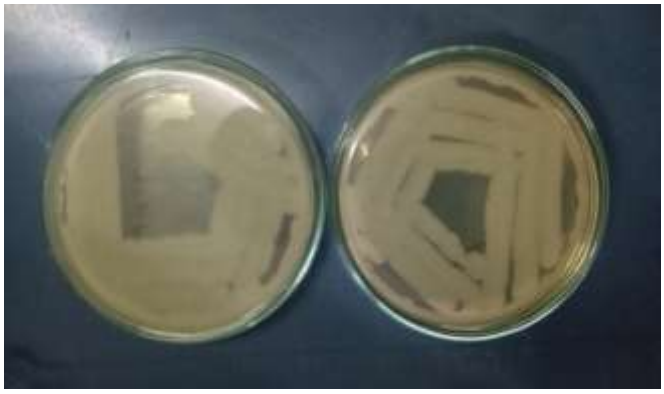


Figure4. Colony obtained from Enrichment media

(D) Isolation of hydrocarbon degrading bacteria:

The bacteria were isolated by inoculating the samples on enrichment medium that contains the autoclaved Bushnell-Haas media (Atlas,1994) supplemented with single hydrocarbon compound as sole carbon source (10% diesel). The medium was incubated at 37^0 C for 5-7 days. After 1 week, 1 ml of enriched media was transferred into freshly prepared enrichment media and incubated at the same conditions as described above. **(Fig.5 and Fig.6)**

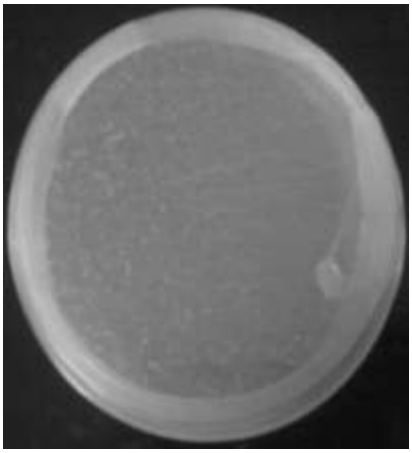


Fig.5: Growth on BH medium with with

10% diesel on sediment sample

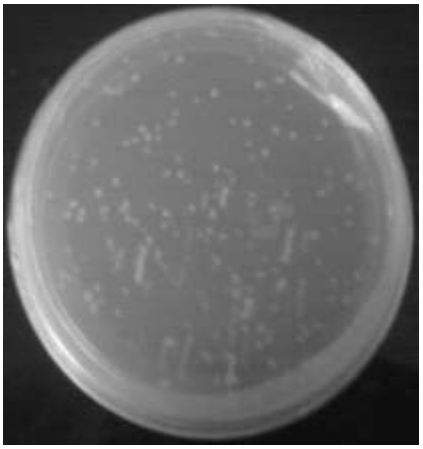


Fig.6: Growth on BH Medium

10% diesel on water sample

(E) Identification of the Bacterial colonies :

The bacteria isolated from the enrichment culture were identified by biochemical test (gram reaction, motility, indole, catalase, urease, oxidase, methyl red, Vogas-poskaur). The observed features were compared morphologically and biochemically using Bergy's Mannual of Determinate Bacteriology by Holt et al (1994) and the scheme of Cheesbrough (2004) **(Table.1 and Table.2)**

Table2. "biochemical tests for water sample"

Organisms	Gram Staining Test	Motility Test	Oxidase Test	Catalase Test	Urease Test	Indole Test	Methyl Red Test	Voges Proskaur Test
Pseudomonas Sp.	Negative (Rod)	+	+	+	-	-	-	+
Bacillus Sp.	Positive (Rod)	+	+	+	-	-	-	+
Enterobacter Sp.	Negative (Rod)	+	-	+	-	-	-	+
Micrococcus Sp.	Positive (Rod)	-	-	+	-	-	+	-
Flavobacterium Sp.	Negative (Rod)	-	-	+	-	+	+	-
Citrobacter Sp.	Negative (Rod)	+	-	+	NA	-	+	-
Actinebacter Sp.	Negative (Rod)	-	-	+	-	-	-	-

Table3. "Biochemical Tests For Sediment Sample"

Organisms	Gram Staining Test	Motility Test	Oxidase Test	Catalase Test	Urease Test	Indole Test	Methyl Red Test	Voges Proskaur Test
Pseudomonas Sp.	Negative (Rod)	-	+	+	+	+	-	-
Bacillus Sp.	Positive (Rod)	+	+	+	-	-	-	+
Enterobacter Sp.	Negative (Rod)	+	-	+	-	-	-	+
Micrococcus Sp.	Positive (Rod)	-	-	+	+	-	-	-
Flavobacterium Sp.	Negative (Rod)	-	-	+	NA	+	+	-
Citrobacter Sp.	Negative (Rod)	+	-	+	NA	-	+	-
Actinebacter Sp.	Negative (Rod)	-	-	+	-	-	-	-

Isolation of DNA:

The DNA of the sample was isolated by applying Bathe's method (Stephan Bathe, et. al, 2001). The supernatant fractions of the DNA sample were extracted with 1 volume of phenol/chloroform/iso-amyl alcohol (25:24:1), after which 1 volume of chloroform/iso-amyl alcohol (24:1)was added and the sample further was precipitated with 0.6 volume of iso-propanol. The isolated fragments were further washed with 70% ethanol and dissolved in 650 µl TE (6.5 ml). The raw and pure DNA extracts were stored at -20^{o}C for further use.

Amplification of DNA:

The isolated 16s rDNA was amplified using forward and reverse primer in a thermo cycler. The electrophoresis techniques used for the analysis of 16s rDNA PCR product helped in separation DNA fragment according to molecular weight in the presence of Agrose in 1x TAE buffer.[22]

Table4. "PCR Cycle for amplification of 16s rdna"

Amplification Stage	Temprature (^{0}C)	Time
Initial Denaturation	94	5 min.
Denaturation	94 (30 cycle)	30 sec.
Annealing	50 (30 cycle)	40 sec.
Extension	72 (30 cycle)	90 sec.
Final Extension	72 (30 cycle)	7 min.

Table5. "Primers used for amplification 16s rdna region"

S.NO.	Primers	Sequence
1.	Reverse	(5'- AGAGTTTGATCCTGGCTCAG-3')
2.	Forward	(5'-AAGGAGGTGATCCAGCC GCA-3')

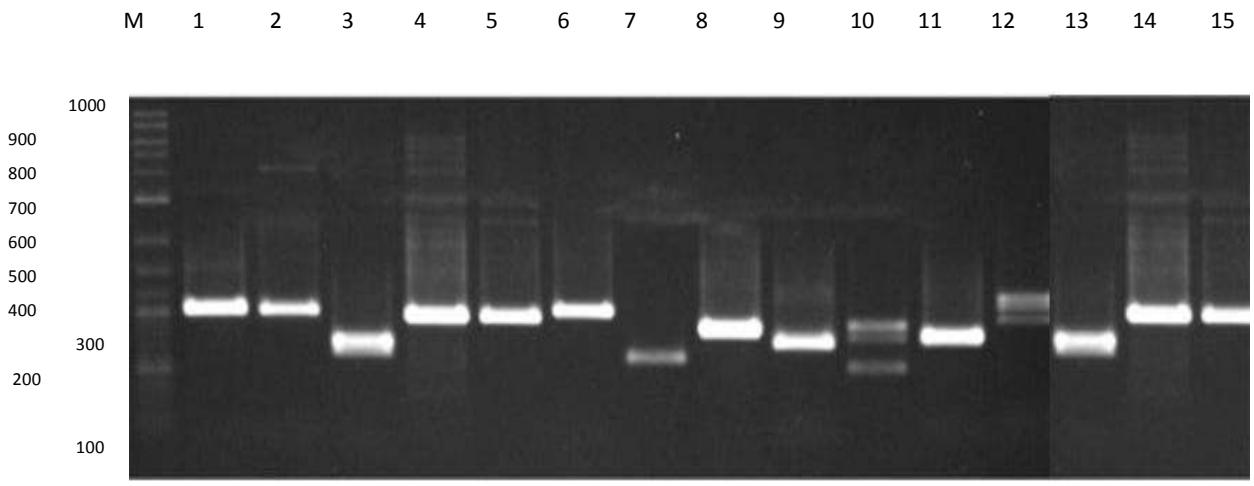


Figure7. 16s rdna PCR Fingerprinting of DNA Sample isolated from shoreline of the boat club of Upper Lake, Bhopal on 2% agarose gel with universal primers.

RESULT AND DISCUSSION

In this study, isolation and identification of the hydrocarbon degrading bacteria in diesel polluted sample collected from the shoreline of Boat Club, Upper Lake, Bhopal has been carried out. Increasing population is leading to production of variety of contamination increases misuse of environment. Petroleum diesel on an average share 21% of oil spills incident.[23] Bioremediation has been recognized as safe method in which using microbes as biological agent is frui1tful.

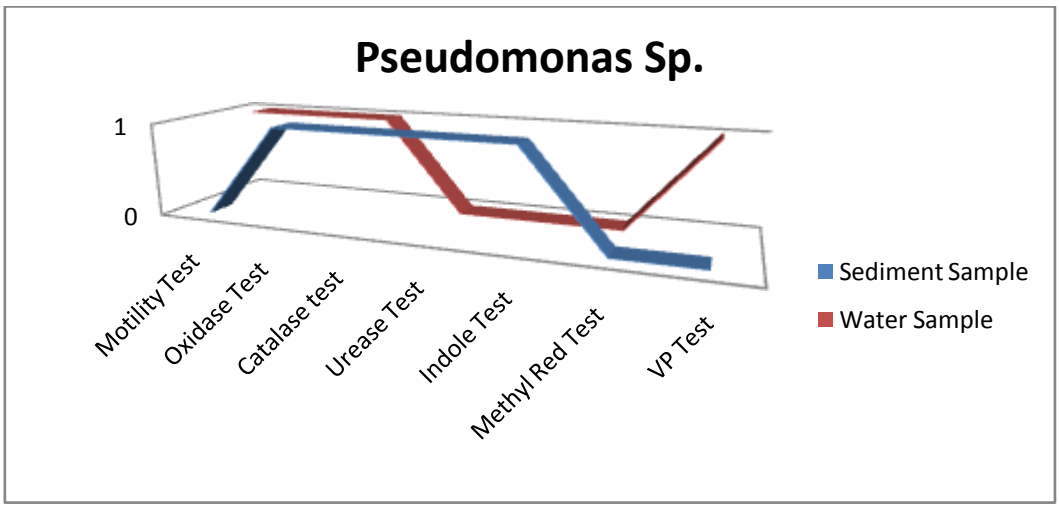
Isolation and identification of bacterial Species:

The hydrocarbon degrading bacteria were isolated and characterized by biochemical methods includes *Pseudomonas sp, Bacillus sp., Actinebacter sp., Enterobacter sp., Micrococcus sp.,*

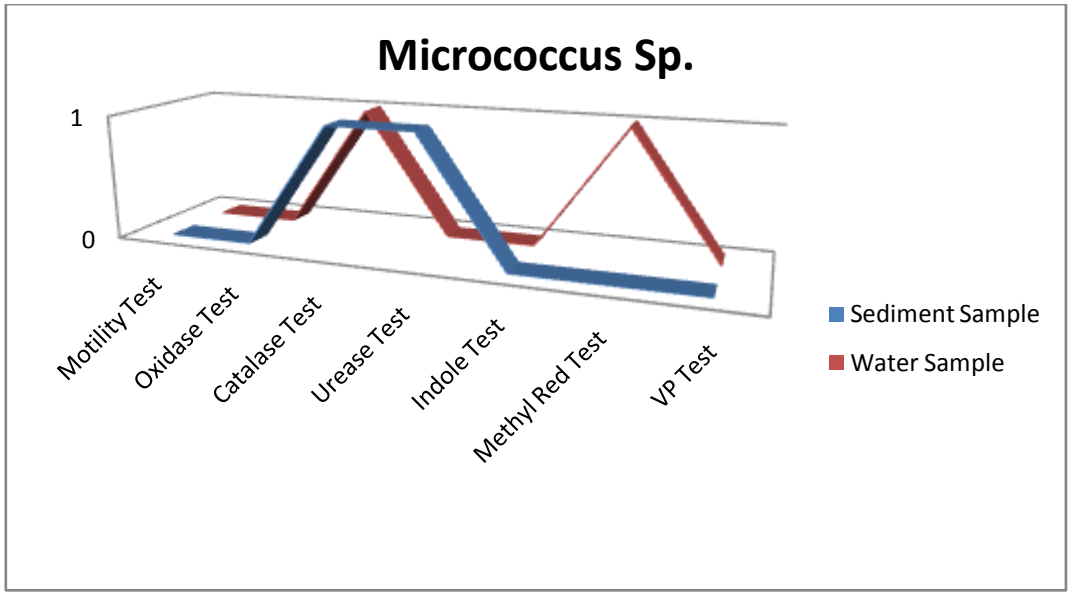
Flavobacterium sp., Citrobacter sp. A sum of seven different bacterial species were isolated which have the ability to degrade different range of petroleum hydrocarbon constituents like Pseudomonas sp. mainly biodegrades Benzene, Toulene, Ethyl benzene, Xylene, Napthalene, Kerosene and Diesel.[24] Bacillus sp. degrades Toulene and Diesel. Micrococcus sp. and Flavobacterium sp. majorly biodegrades Polycyclic Aromatic Hydrocarbons (PAH'S).[25]

Biochemical characterization:

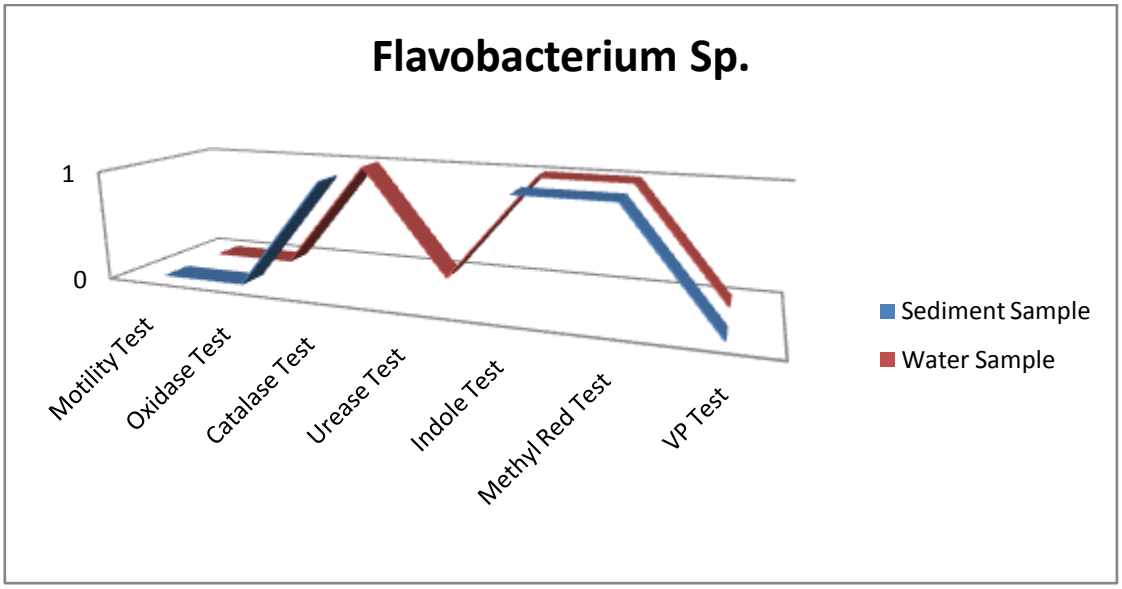
The biochemical characterization of bacterial species obtained from the diesel pollutes water and sediment sample unveils that from the total of seven species two were gram positive and five were gram negative. In the water sample different biochemical test were performed and positive results were shown by four species for motility test, two species for oxidase, all seven for catalase, one specie for indole test, three species for methyl red and VP. None of the observed species gave positive results for urease test. Whereas in the sediment sample positive results were observed by three species for motility, two for oxidase, all seven species for catalase, two for urease, two species for indole, methyl red test and VP test. The graph below shows the four major microbial species which were characterized with the help of biochemical test. **(Graph.1, 2, 3, 4)**



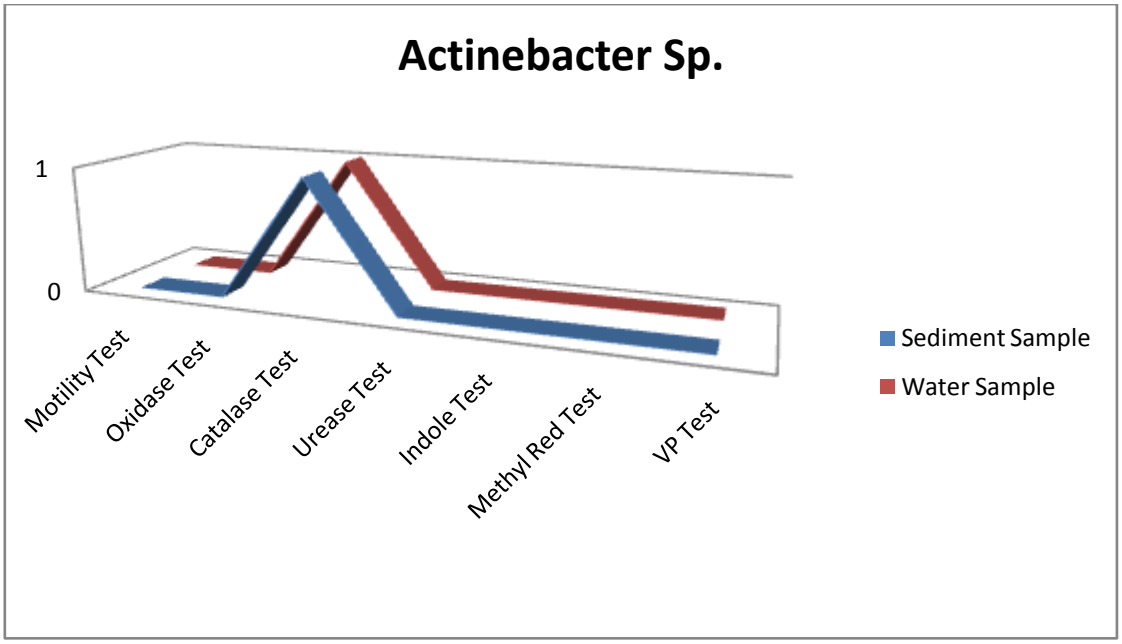
Graph.1: Biochemical Observation of *Pseudomonas* Species in water sample.



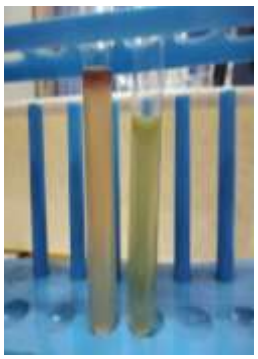
Graph.2: Biochemical Observation of Micrococcus Species in water sample.



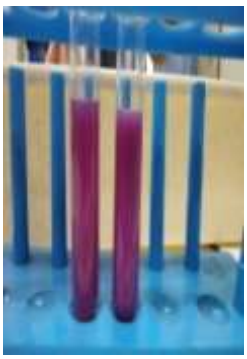
Graph.3: Biochemical Test for Flavobacterium species in sediment sample.



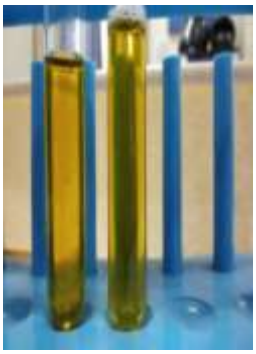
Graph.4: Biochemical Test for *Actinebacter* species in sediment sample.



Indole test



Methyl red test



VP test

Motility test

C atalase test

Isolation and Amplification of DNA:

With the help of PCR Technique, it was well-known that these isolated micro-organisms have genetic capacity to degrade the petroleum hydrocarbons. The effectively growing microbes which were isolated from the diesel polluted water and sediment sample were 16s rDNA sequenced. Since

the amplification of genome is regardless of resemblance and proceeds as random, therefore the number of genomic segment amplified from any sample is not fixed.

CONCLUSION

Upper lake Bhopal is a primary source of drinking water in the city supplying 40 % of the total residents. For the degradation of the hydrocarbons, Bioremediation is the most acceptable and adaptable economical method. The degradation includes micro flora which have the metabolic capabilities to degrade the complex petroleum hydrocarbons in simpler forms. Micro-organisms having the ability to degrade these hydrocarbons are abundant in the contaminated flora. With the help of PCR Technique, it was found that these isolated microbes of Upper Lake Bhopal have genetic capability to degrade the petroleum hydrocarbons and are seen as an alternative to save the biodiversity and the living organism affected by the contamination. This study concludes that there are some specific species of micro-organisms that have a specific characteristic strains and can be used industrially on a large scale for the degradation of the PHC'S thus, neutralizing the alarming threat to the well-being of the living organisms and human beings.

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