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Diversity, activity, antibiotic and heavy metal resistance of bacteria from petroleum hydrocarbon contaminated soils located in different sites

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Abstract

The main aim behind this project was to obtain a collection of hydrocarbonoclastic bacteria that might be suitable for bioremediation processes as soil inoculums to remove hydrocarbons in presence of heavy metals. To achieve this soils from different sites have been selected like Hindustan Petroleum of RAIPUR, BILASPUR & BACHELI strongly contaminated solely with hydrocarbons and for comparison non-contaminated soil of RAIPUR. To reveal affect of pollutants on endogenous micro biota and the bacterial shifts resulting as a consequence of different types of pollution. Different molecular biological and microbiological techniques were used. Individual samples were tested:

1. For their ability of degrading different types of hydrocarbons (aliphatic, aromatic & polycyclic aromatic hydrocarbons)
2. For their capability of resisting heavy metals (Cu2+, Zn2+, Pb2+)
3. If they proliferate in presence of antibiotics.

Results that were observed was increased activity of hydro carbon clastic bacteria due to the significant Co2 production and elevated hydrocarbon degrading bacterial counts. And the bacterial communities that are diversified in number of species were influenced by the pollutants present in it was tested by the T-RFLP. Strains of highest antibiotic resistance and heavy metal tolerance were isolated from the sample. Moreover, some test indicated significant correlation between heavy metal tolerance/antibiotic resistance and antibiotic resistance/hydrocarbon degradation ability of the isolates.

Keywords: Polyaromatic Hydrocarbon, Benzene, Toluene, Anthracene.

INTRODUCTION

Soil contamination may take place due to production, refinery, transportation or storage of crude oil or their derivatives by incidents like accidental leakages or such like that with the BTEX compounds (benzene, toluene, ethyl-benzene,& xylene), poly aromatic hydrocarbons (i.e. PAHs). The main sources of contamination are observed to be the oil wells, petroleum plants etc. since trace heavy metals are common constituents of crude oil, petroleum derivatives(Pb^{2+},leaded gasoline, lubricating oils or greases,Zn^{2+},Cd^{2+}. The area with an increased long term hydrocarbon polluted, heavy metal contaminations of them are chosen .These compounds in the environment leads to serious health risks, due to carcinogenic and mutagenic effects. Because antibiotic resistant & heavy metal resistant genes are to be found in some mobile genetic element, metal pollution often promotes antibiotic resistance emergence in exposed organisms that also has a growing concern in natural and clinical settings. Thus, remediation of these areas was the great interest.

Multiple treatment methods have been applied. Among them, physical and chemical approaches eliminated a broad spectrum of contaminants, with a drawback of high energy consumption and need of additional chemicals. With physico-chemical treatment, for example incineration, pollutants may transfer from one environmental compartment to another. As a result, BIO-Remediation was found to be cost-effective, applicable in large fields. In this method, metabolic activity of microorganisms leads to complete breakdown of organic compounds into non-toxic compounds potentially ending in their mineralization. It's a time consuming process and its success depends on many factors such as pH, temperature, availability of O_2, nutrients. For evaluation of the petroleum contaminated hydrocarbon environment, if a microbial community of the contaminated environment has a metabolic potential to remove the contamination.

MATERIALS AND METHOD

Site description and soil sampling

Soils samples were collected from four different places. They were Hindustan petroleum of Raipur, Bacheli, Bilaspur & for comparing non contaminated soil from Raipur as a Control. Among all these places, Raipur is in 22˚33'N to 21˚14' N & 82˚6' to 81˚38' E in centre of Chhattisgarh. Bilaspur is located in the eastern part of Chhattisgarh, situated within the latitude of 21˚47 to 23˚8' & 81˚14' to 83˚15' longitude and the last Bacheli, within latitude of 18˚42' 13.36" & longitude of 81˚14' 17.65". Hydrocarbon contaminated soil samples were collected in vicinity of above ground or underground. Samples as a negative control is been taken from non-contaminated areas. The top 1 cm of soil was collected using sterile spatula into sterile flasks with cotton plugs for the microbiological analysis.

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Gram Staining

This method is also known as differential staining. It was invented in 1884 by Danish scientist Hans Christian Gram. Gram Staining involves basic steps: In this bacteria are stained with Crystal violet. It imparts purple color. Some of the bacteria gain Purple color and some remain unstained. Then it is treated with grams iodine solution. Iodine is used as a mordant. Mordant is a substances which increases it efficacy of stains towards biological specimens. A decolorizer is used to remove excess of stain. Here a mixture of alcohol and acetone is used, to remove the extra stain. This is a differential step. Then another, i.e. secondary stain is added Saffranin. Those bacteriae which are remain unstained will get stained by the secondary stain. Bacteria that retain color are classified a gram's positive and that loses color are classified as gram's negative. On the basis of Gram staining and their respective shapes some plates were selected

Table 1.

S.No.	Serial Dilution	Contaminated Site
1	10^{-3}	RNCS (Raipur Non Contaminated Site)
2	10^{-4}	RNCS
3	10^{-5}	BICS (Bilaspur Contaminated Site)
4	10^{-7}	BICS
5	10^{-4}	BCS (Bacheli Contaminated Site)
6	10^{-5}	BCS
7	10^{-3}	RCS (Raipur contaminated Site)
8	10^{-6}	RCS

Enrichment purification and culturing of hydrocarbon degrading bacteria

All the microorganisms isolated from various soil samples of different petroleum sites. For this, identification techniques were applied like Gram staining & biochemical tests like :

- Catalase test
- Urease test
- Hydrolysis of gelatin test
- Cellulase production test
- Hydrogen sulfide test
- Starch hydrolysis test
- Carbohydrate fermentation test
- Imvic tests:
  - Indole test
  - Methyl red & Voges proskaeur test
  - Citrate utilization test
- Casein hydrolysis test.

Hydrocarbon degradation of potential isolates

Identified strains were treated with hydrocarbons like Benzene and Toluene. There were two methods applied for degradation of these hydrocarbons. They are:

Dye Method: Microbes have been cultured in BBH media along with filter sterilized benzene / toluene and a indicator dye resazurine. Resazurine is used as a redox indicator. Test media contained 50ml BBH mineral broth supplemented with 0.2µm filtered sterilized of one of the hydrocarbon and resazurine indicator i.e. 10mg/l. For selecting a strain which posses increase hydrocarbon degradation potential the concentration of applied carbon source was set to 0.5g/l. Test solutions were inoculated with 250µl strain cultured solutions prepared from respective plates. Reading were taken at (OD_{600}= 0.5nm). Initial hydrocarbon degradation changes the blue color of test solution to colorless via pink. The flasks contains the test solution were incubated for a week in rotary shaker at 145 rpm and 28°C. Samples which shows no degradation activity (blue color) were marked "-", minimum microbial activity (bluish pink color) "+", the medium activity pink samples by "++", while some samples showing increased hydrocarbon degradation activity (colorless) were marked "+++".

Turbidity Method: In this, test solution is prepared without addition of indicator. It is incubated for a 4four weeks and reading were taken at (OD_{600}= 0.5nm).

RESULTS

Enrichment purification and culturing of hydrocarbon degrading bacteria

Biochemical tests were done for the identification of microbes present in contaminated soils. From these we come to know that in some of the soil samples *pseudomonas* species were present and in some of the contaminated soils *Enterobacter aerogenes.*

PROPERTIES	RNCS 10^{-4}	RNCS 10^{-3}	BICS 10^{-5}	BICS 10^{-7}	BCS 10^{-5}	BCS 10^{-4}	RCS 10^{-3}	RCS 10^{-6}
Gram stain	+	-	+	-	-	+	+	-
Color	white	white	White	White	white	White	white	white
Shape	cocci	cocci	Rod	Rod	cocci	Cocci	cocci	cocci
Starch hydrolysis	+	+	+	+	+	+	+	+
Urease	+	-	-	-	+	-	+	-
Indole	-	-	+	-	+	-	+	+
Methyl red	-	+	-	-	-	+	-	-
Voges Proskaeur	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Hydrogen Sulphide	-	-	-	-	-	-	-	-
Cellulase	+	+	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+	+	+
Glucose	-	+	+	-	-	-	-	-
Sucrose	+	-	-	-	+	-	-	-
Lactose	-	-	-	+	+	-	-	-
Caseinase	-	-	-	-	-	-	-	-

Hydrocarbon degradation of potential isolates

Two methods of checking biodegradation have been employed:

Dye method: Microbes have been cultured in BBH media along with filter sterilized benzene / toluene and an indicator dye resazurine. The color of the dye is observed as it changes from purple to colorless via pink. Eight selected colonies in duplicate have been kept on Oct 15th, 2013 in shaking incubator at 140 rpm. One sample from Bacheli (10^{-4}) has shown the pink color as on Oct 21st, 2013 and another from Bacheli 10^{-5} shown pink color on 1st Nov, 2013. Rest of the samples is still kept in shaking incubator for further observation.

Turbidity method: All the eight samples in duplicates also have been kept in BBH media along with filter sterilized benzene / toluene at 35^0C and turbidity was measured using spectrophotometer at regular intervals. Most of the samples have shown an increase in turbidity.

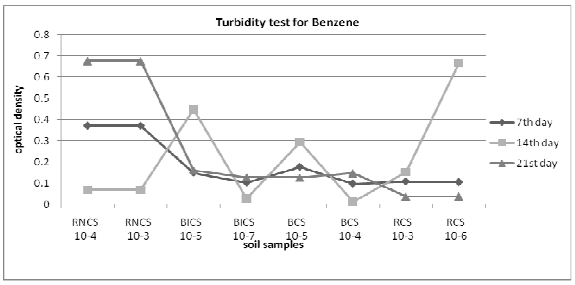
Observation table for Benzene

Soil samples	7th day	14th day	21st day
RNCS 10^{-3}	0.032	0.210	0.474
RNCS 10^{-4}	0.348	0.518	0.552
BICS 10^{-5}	0.530	0.198	0.360
BICS 10^{-7}	0.494	0.405	0.799
BCS 10^{-4}	0.328	0.511	0.414
BCS 10^{-5}	0.330	0.400	0.450
RCS 10^{-3}	0.512	0.530	0.540
RCS 10^{-6}	0.420	0.877	0.563

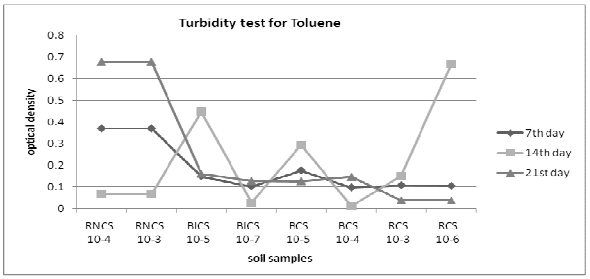
Observation table for Toluene

Soil samples	7th day	14th day	21st day
RNCS 10^{-3}	0.371	0.068	0.678
RNCS 10^{-4}	0.090	0.044	0.167
BICS 10^{-5}	0.150	0.448	0.162
BICS 10^{-7}	0.103	0.028	0.129
BCS 10^{-4}	0.098	0.012	0.148
BCS 10^{-5}	0.177	0.294	0.300
RCS 10^{-3}	0.108	0.152	0.390
RCS 10^{-6}	0.105	0.668	0.581

This shows the increase in turbidity in mid of month and then it declines this just because of the life cycle of bacteria. From the above observation we come to know that bacteriae present in contaminated soil are more potent in degradation of benzene as compared to the toluene.



line Chart for benzene



Line Chart for Toluene

CONCLUSION

Majority of isolated strains were able to successfully degrade the aromatic hydrocarbons. The further degradations of poly aromatic hydrocarbon will be studied as future aspects. The isolated strains were related to pseudomonas species. According to various biochemical test conducted we come to inference that it might be *Enterobacter aerogenes*. In hydrocarbon degradation, it was find that microbes were more potent in degradation of Benzene as compared to the Toluene. One sample from Bacheli (10^{-4}) having benzene have shown the pink color as on Oct 21st, 2013 and another of Bacheli 10^{-4} turn pink on 1st Nov,2013. From this it was concluded that, according to dye method the microbial strains present in that has partially degraded the aromatic hydrocarbon.

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