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Research Paper

ISOLATION AND CHARACTERIZATION OF HYDROCARBON DEGRADING BACTERIA

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Abstract

Hydrocarbons and their derivatives have been found to pose a serious environmental threat. These compounds are difficult to degrade and may persist in the environment. The present work aims to isolate hydrocarbon degrading organisms from the contaminated sites. These isolates were able to degrade hydrocarbons and can later be used in biotechnology for environments depollution. Isolation and characterization of Hydrocarbon degrading organisms from different Gas stations in India resulted in collection of 15 different species. Soil samples collected were subjected to selective enrichment technique by growing the organisms on α-naphthol medium. Comparative studies were carried out by growing the isolates on both the medium containing phosphate and devoid of phosphate. It was found that PO_4 enhances the rate of degradation of lubricant oil. All the isolates were subjected to morphological and biochemical tests and identified on the basis of Bergey's Manual. Further confirmation is to be carried out by 16s rRNA sequencing.

Key words: *hydrocarbons, depollution, 16s rRNA sequencing*.

INTRODUCTION

Hydrocarbons are currently the main source of energy for civilizations.[1] They are divided on the basis of their structure and the bonding. Aliphatic hydrocarbons are of three types: alkane, alkenes, and alkynes. Saturates are defined as hydrocarbons containing single bonds. They are categorized according to their chemical structures into alkanes and cycloalkanes.[2] Saturated hydrocarbans represents highest percentage of crude oil constituents.[3]

Petroleum, one of the major source of energy, consists of different mixtures of hydrocarbons and other organic compounds. Petroleum hydrocarbons are released into the environment via oil spills, leaking or unplugged oil wells, oil refinery sites, incomplete combustion of fossil fuels etc. Oil is an unusual pollutant, which is slowly degraded weathering. Thus, has most pronounced effects on the flora and fauna in the affected sites.[4]

Biodegradation of hydrocarbons by potential microorganisms allows the conversion of hazardous substances into non-toxic products and represents one of the important techniques by which petroleum and diesel products are degraded from the environment

inexpensively and noninvasively.[5] The process of bioremediation - use of microorganisms to detoxify or remove the pollutants in the environment is an evolving technique for the degradation of many environmental pollutants including the petroleum products.[6] Many soil organisms are able to degrade hydrocarbons by using various metabolic pathways.[7] The hydrocarbon degrading heterotrophs undergoes degradation either by aerobic pathway (i.e. use of O2 as a primary electron acceptor) or anaerobic pathway (i.e. use of alternative electron acceptor such as nitrate or sulphate).[8] Biosurfactants released by bacteria, has the ability to reduce the surface tension and allows the emulsification of hydrocarbons which improves the uptake by facilitating adhesion and allows the formation of biofilms as well as enhances the cell motility on solid surfaces.[9]

There are various factors which are responsible for the effective biodegradation of hydrocarbons. Hydrocarbon's chemical structure affects its rate of degradation. Usually, larger the structure of hydrocarbon, are slowly oxidized.[10] The important determinants that influences the rate of bioremediation are potential microbes, physical chemical properties of soil or water, nutrients, temperature, pH, moisture content, dissolve oxygen, inhibitory substrates etc.[11] Biostimulation and Bioaugmentation are the two important approaches of oil bioremediation.(a)Biostimulation, in which the growth of oil degraders is enhanced by addition of nutrients or growth stimulators such as PO_4 or N_2.(b) Bioaugmentation, in which oil degradation of soil is enhanced by addition of oil degrading organisms to the existing population of microbes.[12]

Most PAH are reported to be carcinogenic and toxic to other living beings and remain persistent in the environment.[13] The use of recombinant DNA techniques will play a crucial role in elimination of PAH. These advanced techniques and tools should be employed more frequently to achieve higher rate of degradation of hydrocarbons in the environment.[14] In this paper, the isolation and characterization of 15 different isolates was performed by morphological and biochemical tests. Also, their ability to degrade the hydrocarbons in the presence and absence of PO_4 was performed. These organisms isolated from the petroleum sites can be used as the seed organisms for efficient degradation of hydrocarbons at the contaminated sites. The pure culture need to be confirmed by 16S rDNA gene nucleotide sequence analysis.

MATERIALS AND METHODS

Sample collection and processing: Soil samples were collected from 4 different gas stations 1) Raj Gas Station. 2) Bhawani Gas Station. 3) Akashwani Gas Station 4) Railway Station Gas station, located in Aurangabad district of Maharashtra, India. About ½ kg of top layered soil was collected from each Gas station in different sterile plastic bags. Each soil sample was mixed properly and was sieved through 2mm pore size siever. The sieved soil was used for the isolation purposes.

Media acquisition: All the media used for the experimental purposes were procured from Hi media PVT.LTD, Mumbai, India.

Enrichment, isolation, purification and identification of microorganisms:

Enrichment: The processed Soil samples were enriched by adding alpha naphthol to it. These samples mixed with alpha naphthol were incubated for 4 days at 30^0C.[15]

Isolation: After 4 days of incubation the 1g of each enriched soil sample was inoculated on the sterile alpha naphthol medium plates. These enriched soil samples was incubated at 37^0C for 3 days.[16]

Purification: The bacterial colonies on the surface of alpha naphthol medium were isolated and purified on sterile plates of nutrient agar containing alpha naphthol.

Colonies of different morphology were selected and sub cultured on sterile nutrient agar plates to obtain pure cultures.

Identification: Primary screening by Gram's staining and microscopic examination was performed to eliminate apparently similar organisms. Phenotypic and biochemical characterization of pure cultures were performed according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology and the purified isolates were identified.

Hydrocarbon degrading abilities of the isolates: The isolates were grown on minimal medium supplemented with 0.1%(w/v) alpha naphthol to examine the degradation of hydrocarbons. The plates were incubated at 37^0C and the absorbance at 600nm was measured. The isolates from Beed bypass gas station (5a.2, 10.1, 10.2, S1.1, S2.1, S2.2) and the isolates from Bhavani gas station(3.1, 3.2, 7.1, 7.2, 11.1, 11.2) was showing better degradation than the isolates from Akashwani gas station (1.1, 2.1, 4.1).

Petroleum Oil degrading abilities of the isolates by growing them on a diverse media: Two different Media with 0.2% lubricant oil, one with phosphate and the other devoid of phosphate were prepared.[17] The isolates were inoculated in both the inoculums and the inoculated mediums were incubated at 37^0C on shaker for 10 days. Absorbance was measured at 600nm and the results were compared.[18]

RESULTS AND DISSCUSSIONS

In the above experiment, soil samples were gathered from the different gas stations (oil contaminated sites). Original samples from each location were characterized with a high diversity of microorganisms. The metabolic diversity of microorganisms in the natural environment is an important factor in biodegradation of hydrocarbons. The extensive degradation of oil is accomplished by mixed bacterial population. The hydrocarbon degrading organisms isolated from each individual soil sample were enriched on the minimal media containing alpha naphthol medium. After few days of cultivation the bacterias were isolated from the surface of alpha naphthol medium and purified on nutrient agar medium containing alpha naphthol colonies of different morphologies were selected and recultured. Primary screening was performed by undergoing Gram's staining and microscopic examination.

Apparently similar organisms were eliminated. After the second enrichment technique, high diversity of microbial population was obtained which were able to degrade oil. It resulted in collection of six bacterias from Bhavani Gas station, Three colonies from Akashwani Gas station, and six from Beed bypass Gas station. Total 15 different pure bacterial isolates were collected.

Colony characteristics: Colony characterization of the purified samples was carried out and the results obtained are shown in the table below (table 1).

Hydrocarbon degrading abilities of isolates: Hydrocarbon degrading abilities of the soil isolates were carried out at 37^oC on the minimal media where alpha naphthol was provided as a carbon source. Absorbance was measured at 600nm. The isolates from Beed bypass Gas station (5a.2, 10.1, 10.2, s1.1, s2.1, s2.2) and from Bhavani Gas station (3.1, 3.2, 7.1, 7.2, 11.1, 11.2) were showing good degradation than the isolates from Akashwani Gas station (1.1, 2.1, 4.1). From the result it was observed that isolates s1.1, 10.2, and 3.1 were efficiently utilizing alpha naphthol as a carbon source in comparison to others.(Fig1)

Lubricant oil degrading abilities of isolates on diverse media (containing Po_4 and devoid of PO_4): Two diverse media were prepared to compare the lubricant oil degrading capabilities of isolates. One media containing lubricant oil (0.2% v/v) and PO_4 and the other containing lubricant oil (0.2% v/v) and distilled water were prepared.

The isolated bacterial cultures were inoculated in both the medium and incubated at 37°C for 10 days. After incubation the lubricant oil degrading abilities of the isolates were compared by measuring the absorbance at 600nm. From the result it was observed that the 11 isolates inoculated on the medium containing PO_4 resulted in higher rate of substrate utilization in comparison to the remaining 4 isolates inoculated on the same medium which resulted in lower rate of substrate utilization. At the same time the utilization of lubricant oil by the isolates was confirmed by increase in the number of viable cells during the incubation period. The finding suggested that supplementation of PO_4 increases the rate of degradation of lubricant oil in most of the organisms. Therefore, the microorganisms isolated from the contaminated sites efficiently utilize lubricant oil as a carbon source in presence of PO_4.(Fig2)

Petroleum oil degrading abilities of isolates on diverse media (containing PO_4 and devoid of PO_4): Bacterial colonies were inoculated in both the medium containing PO_4 and devoid of PO_4 and incubated for 10 days at 37°C. The degrading abilities of the organisms were observed by measuring the absorbance at 600nm. The graphical representation of the growth of organisms in both the media (with and without PO_4) was studied. from the observation it was concluded that PO_4 is essential for the growth of organisms. The organisms were able to degrade the petroleum oil to a greater efficiency in presence of PO_4.(Fig3)

Identification of the isolates: The 15 isolates were subjected to biochemical testing for identification on the basis of Bergy's manual. The isolates showed distinct biochemical reactions which is presented in the table below (table2). The bacteria isolated and which are related to *bacillus* species may be *Bacillus Megaterium, Bacillus Laterosporus. Bacillus circulans,Bacillus Pumilus, Bacillus firmus, Bacillus mycosides and sp*. The isolates need to be confirmed by 16s rRNA sequencing.

Table no. 1 Colony Characteristics

Characters	**Sample 1.1**	**Sample 2.1**	**Sample 3.1**	**Sample 3.2**	**Sample 4.1**	**Sample 5a.2**	**Sample 7.1**	Sample 7.2	Sample 10.1	**Sample 10.2**	Sample 11.1	**Sample 11.2**	Sample S1.1	Sample S2.1	**Sample S2.2**
Size	4mm	3mm	Pin point	3mm	1mm	Pin point	3mm	3mm	1mm	1mm	1mm	Pin point	Pin point	Pin point	Pin point
Shape	Circular	Circular	Punctiform	Circular	Punctiform	Punctiform	Circular	Circular	Punctiform	Punctiform	Punctiform	Punctiform	Punctiform	Punctiform	Punctiform
Surface	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Smooth	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Elevation	Raised	Raised	Flat	Flat	Raised	Raised	Raised	Flat	Flat	Raised	Flat	Flat	Raised	Flat	Raised
Pigmentation	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Viscous	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Gram staining	*Gram +ve coccobacilli*	*Gram +ve streptobacilli*	*Gram +ve Coccobacilli*	*Gram +ve Short rods*	*Gram +ve coccobacilli*	*Gram +ve Short rods*	*Gram +ve* Short rods	*Gram +ve Short rods*	*Gram +ve Short rods*	*Gram +ve* Short rods	*Gram +ve Short rods*	*Gram +ve Short rods*	*Gram +ve Short rods*	*Gram +ve Coccobacilli*	*Gram +ve Short rods*
Motility	Motile	Motile	Motile	Vigorously motile	Vigorously motile	Sluggishly motile	Sluggishly motile	Motile	Motile	Motile	Vigorously motile	Motile	Motile	Motile	Motile
Endospore staining	Non-swollen, Terminal spore	Non-swollen, Central spore	Non swollen, Sub terminal spore	Non-swollen, Terminal spore	Non-swollen, Terminal spore	Non-swollen, Terminal spore	Non-swollen, Terminal spore	Swollen, Central spore	Non-swollen, Terminal spore	Swollen, Terminal spore	Non-Swollen, Terminal spore	Non-swollen, Terminal spore	Non-swollen, Terminal spore	Non-sporulating	Non-swollen, Terminal spore

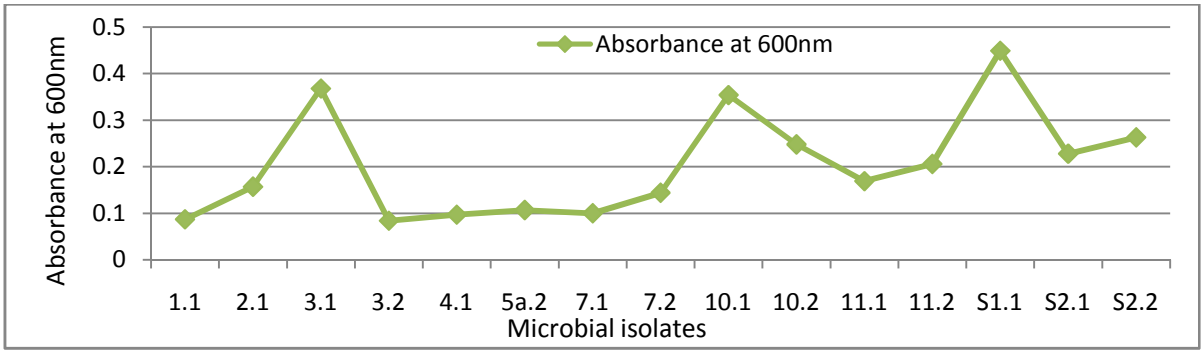

Fig.1 Graphical presentation of hydrocarbon degrading abilities of isolates

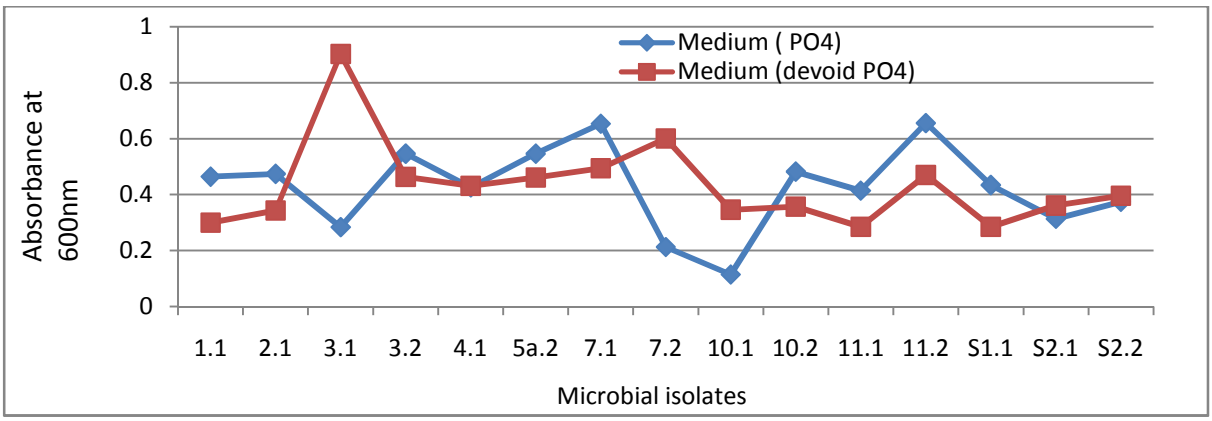

Fig.2 Graphical presentation of lubricant oil degrading abilities of isolates on diverse media

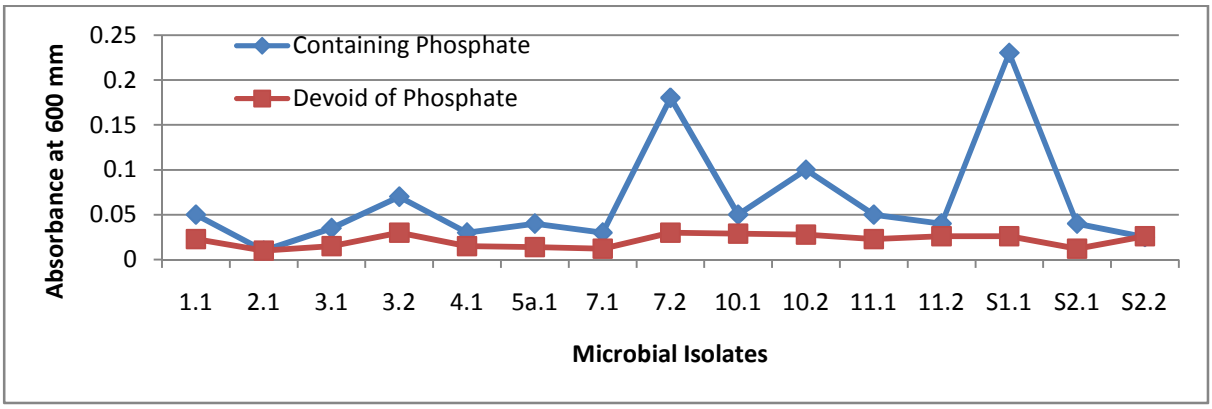

Fig.3 Graphical presentation of petroleum oil degrading abilities of isolates on diverse media

Table 2: Biochemical Tests of the 15 isolates

Sr.no	Isolates/Test	1.1	2.1	3.1	3.2	4.1	5a.2	7.1	7.2	10.1	10.2	11.1	11.2	S1.1	S2.1	S2.2
1.	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.	V.P.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.	Sugar Fermentation															
	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
	Fructose	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+
	Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	Hydrolysis															
	Casein	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	Gelatine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Starch	+	+	+	+	+	-	+	+	-	+	-	-	+	+	+
5.	UtilizationCitrate	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+
6.	Phenyl-alanine De-amination	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7.	Nitrate reduction	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+
8.	Formation of indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	Growth in 7% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.	Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11.	Lysine De- carboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12.	Hydrolysis of Tween80	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+
13.	H2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14,	M.R.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15.	Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CONCLUSIONS

In this study, bacteria that are able to grow on heavy oil as a carbon and energy source were isolated from the petroleum contaminated sites in Aurangabad city of India. The selective enrichment technique was used for the isolation of hydrocarbon degraders. Growth of the isolated bacteria on the medium containing alpha naphthol and lubricant oil demonstrated the hydrocarbon degrading abilities of isolates. The isolates were identified on the basis of morphological, physiological and biochemical characterization. Comparative studies of bacteria on both the medium containing phosphate as well as the medium devoid of phosphate were performed which demonstrated the availability of phosphate enhanced the rate of degradation of hydrocarbons.

Out of the 15 bacteria isolated from petroleum contaminated soil; *Bacillus megaterium, Bacillus pumilus* are promising isolates which utilizes phosphate for their efficient degradation of hydrocarbons. The isolates *Bacillus brevis, Bacillus circulans and Corynebacterium sp*. Are good degraders of hydrocarbon if grown on the medium devoid of phosphates, it can be assumed that these bacteria utilized the minerals present in the oil itself. Additional minerals may be inhibitory to their growth.

It is well known that various indigenous microflora especially those isolated from the contaminated sites possesses the ability to metabolize hydrocarbons based upon their efficiencies. The above isolates obtained from the contaminated soil can be compared with the standard organisms and can be used as seed organisms to the contaminated sites of oil spillage for bio remediation process.

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