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Bacterial Identification From Hydrocarbon Polluted Sources And Amplification Of Their Hydrocarbon Degrading Genes

Hayder Fadhil Wadi
M.Sc Microbiology
University College of science,
Osmania University, India

Falah Hassan Kadhum
M.Sc Clinical Pathology
Head of Community Health Department in
Technical Teaching Institutite-Iraq

Akram Jawad Humadi
M.Sc Biotechnology
University College of science,
Osmania University, India

P. Acharya Nagarjun
Assitant Professor (Sr.)
Department of Microbiology
University College of science,

ABSTRACT

The present work deals with identification of bacteria present in different polluted sources followed by the amplification of their genes responsible for hydrocarbon degradation.Hydrocarbon plays a major role in environmental pollution leading to global warming, ozone depletion etc. Two different sources of hydrocarbon polluted samples viz Soil contaminated with Benzene and Water with Kerosine were undertaken for the identification of bacteria present in them. Following Bergey's manual of determinative bacteriology different biochemical tests were performed for bacterial identification along with gram staining and four different species of bacteria were identified as *Micrococcus luteus, Streptococcus pyogenes, Bacillus subtilis and Corynebacterium kutsceri*.The extent of degradation after 30 days was also estimated by PetroSensePHA-100WL.Using the literature search, respective genes responsible for hydrocarbon degradation were identified and amplified in these bacteria.

Keywords: bacteria, Bergey's manual, environmental pollution, Hydrocarbon, PetroSensePHA-100WL.

INTRODUCTION

Our environment is composed of atmosphere, earth, water and space. In absence of pollution, it remains clean and enjoyable. The interaction of the atmosphere, lithosphere, hydrosphere and biosphere is continuing for years together. On account of the various activities of man, the composition and complex nature of environment gets changed. These activities include industrialization, construction and transportation. Such activities, although desirable for human development and welfare, lead to generation and release of objectionable materials into the

environment thus turning it foul, and rendering our life miserable.Environmental pollution is caused by many factors like solid waste (example metals, plastics, etc.), human waste, industrial effluents, domestic waste, etc. Among all these, presence of hydrocarbon molecules make its complex to get decompose.

Hydrocarbons are economically important because major fossil fuels such as coal, petroleum and natural gas, and its derivatives such as plastics, paraffin, waxes, solvents and oils are hydrocarbons. Hydrocarbons along with Nitroxide and sunlight – contribute to the formation of tropospheric ozone and greenhouse gases. The majority of hydrocarbons found naturally occur in crude oil, where decomposed organic matter provides an abundance of carbon and hydrogen which, when bonded can catenate to form seemingly limitless chains.

Contaminated soils are threat to the human and the ecosystem. Biodegradation of such contaminated soils is required by convention method that is affordable and eco friendly [1, 2]. Many bacteria are capable of utilizing and degrading the oil constituents in a better way. Petroleum hydrocarbons are not easily degradable. Microorganisms have the capacity to degrade majority of hydrocarbon components, the saturated and unsaturated alkanes, monoaromatic and low molecular weight polycyclic aromatic hydrocarbons (PAHs). The organism must be in contact with their substrate to utilize and degrade the hydrocarbon. Individual microorganisms can metabolize only a limited quantity of hydrocarbon substrates. So the mixed cultures of microorganisms are required to increase the rate of petroleum biodegradation. When the organism grows in the contaminated soil, they utilize these constituent hydrocarbons as substrates. Such hydrocarbon utilizing microorganism shows emulsifying activity [3, 4]. MTCC bacterial culture Pseudomonas sp, Bacillus sp, Micrococcus sp, were used as oil degrading organisms. These microbial consortia use the hydrocarbon as substrates and degraded it. These organisms have high capability of degrading the hydrocarbons. The majority of hydrocarbons are found in Diesel, Petrol, and Kerosene. The degradation rate is affected by several physico, chemical and biological parameters such as pH, temperature, nutrient, quantity of hydrocarbon. The hydrocarbon presented in contaminated soil samples were analysed using GC-MS. The biodegradation of the hydrocarbons are challenging as they are complex in structure. The use of detergents as a hydrophobic compound for the hydrocarbon makes it possible for the microorganism to use as a substrate. These make them useful in land farming.

Hydrocarbon contamination

The extensive use of petroleum products leads to the contamination of almost all

compartments of the environment, and biodegradation of the hydrocarbons by natural populations of microorganisms has been reported to be the main process acting in the depuration of hydrocarbon-pollution environments[23], the mechanism of which has been extensively studied and reviewed[1]. It often results in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components of the ecosystem if not restored[1]. Oil spills have become a global problem particularly in industrialized and developing countries. Poor miscibility of crude oil accounts for accumulation of free oil on the surface of groundwater and this may migrate laterally over a wide distance to pollute other zones very far away from the point of pollution. Industrial and municipal discharges as well as urban run-offs, atmospheric deposition and natural seeps also account for petroleum hydrocarbon pollution of the environment (Baker, 1983). Attention has been focussed on the marine environment, because oceans and estuaries have generally been the sites of the largest and most dramatic spills. Apparently inevitable spillages, which occur during routine operations of crude oil production, refining, distribution and as a consequence of acute accidents, have generated continuous research interest in this field. The parameters typically measured in laboratory tests of bioremediation efficacy include enumeration of microbial populations, determination or fate of hydrocarbon degradation of disappearance rates[14]. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities and these microorganisms are omnipresent and widely distributed in the nature of the compounds within the petroleum mixture and on environmental determinants[22]. Hydrocarbon enters into the environment through waste disposal, accidental spills, as pesticides and via losses during transport, storage and use. Hydrocarbon(Petroleum and Diesel) degrading bacteria is reportedly ubiquitous in the environment and were widely distributed in marine, freshwater, soil habitats and their use in bioremediation of hydrocarbon-contaminated soils, which exploits their ability to degrade and/or detoxify organic contaminants, has been established as an efficient, economical, versatile and environmentally sound treatment due to extensive increase in the environmental pollution, numerous biodegradative bacteria have been isolated in the past, and their physiology, biochemistry, and genetics have been intensively studies.[1]

Biodegradation, which is the destruction of organic compounds by microorganisms, is carried out largely by diverse bacterial populations, mostly by *Pseudomonas* species.[6] Bioremediation is a biotechnological approach of rehabilitating areas degraded by

pollutants or otherwise damaged through mismanagement of ecosystem. It is the ability of microorganisms to degrade or detoxify organic contaminated areas by transforming undesirable and harmful substances into non-toxic compound (Bioremediation overview, 2003).

Diverse components of crude oil and petroleum such as polycyclic aromatic hydrocarbons (PAHs) have been found in waterways as a result of pollution from industrial effluents and petrochemical products (Beckles, *et al*., 1998). Petroleum hydrocarbon pollution of the environment may arise from oil well drilling production operations, transportation and storage in the upstream industry, and refining, transportation, and marketing in the downstream industry. Petroleum hydrocarbon pollution could also be from anthropogenic sources (Oberdorster and Cheek, 2000). Some non combusted hydrocarbons escape into the environment during the process of gas flaring. Until recently, the bulk of the associated gas produced during drilling in Nigeria, was flared. Sources of petroleum and its products in the environment will also include accidental spills and from ruptured oil pipelines. Today the international oil and gas-pipelines span several million kilometers and this is growing yearly due to inter-regional trade in petroleum products. Just like any other technical appliance, pipelines are subject to ‘’tear and wear’’, thus can fail with time (Beller, *et al.,* 1996). Spilled petroleum hydrocarbons in the environment are usually drawn into the soil due to gravity until an impervious horizon is met, for example bedrock, watertight clay or an aquifer. Poor miscibility of crude oil accounts for accumulation of free oil on the surface of groundwater and this may migrate laterally over a wide distance to pollute other zones very far away from the point of pollution. Industrial and municipal discharges as well as urban run-offs, atmospheric deposition and natural seeps also account for petroleum hydrocarbon pollution of the environment (Baker, 1983). It is worthy of note that groundwater is one of the many media by which human beings, plants and animals come into contact with petroleum hydrocarbon pollution. In the Niger delta area of Nigeria, extensive farm land has been lost due to contamination with crude oil. Also sources of drinking water and traditional occupation such as fishing and water transportation are greatly affected by crude oil contamination.

Bacterial classification based on staining

On the basis of staining there are two types of bacteria i.e. gram positive and gram negative.Gram positive bacteria normally have cell walls that are thick and composed normally of a single 20 to 30nm thick homogeneous layer of peptidoglycan (murein) lying outside the plasma membrane. Peptidoglycan of gram positive bacteria often contains a

peptide interbridge along with a large amount of teichnoic acids (polymers of glycerol or ribitol joined by phosphate groups), which are covalently connected to either the peptidoglycan itself or to plasma membrane and help give the gram positive cell wall its negative charge. Because of the thicker peptidoglycan layer, the walls of gram positive cells are more resistant to osmotic pressure than those of gram negative bacteria. The periplasmic space of gram – positive bacteria lies between the plasma membrane and the cell wall and is smaller than that of gram negative bacteria.

Gram negative cell wall are much complex than gram positive walls. The thin (2 to 7nm) peptidoglycan layer next to the plasma membrane and bounded on either side by the periplasmic space may constitute not more than 5 to 10% of the wall weight. The periplasmic space of gram negative bacteria is also strikingly different than that of gram positive bacteria. It ranges in size from 1nm to as great as 71nm. It may constitute about 20 to 40% of the total cell volume, and it is usually 30 to 70nm wide. The most unusual constituents of the outer membrane are its lipopolysaccharides which contribute to the negative charge on the bacterial surface.

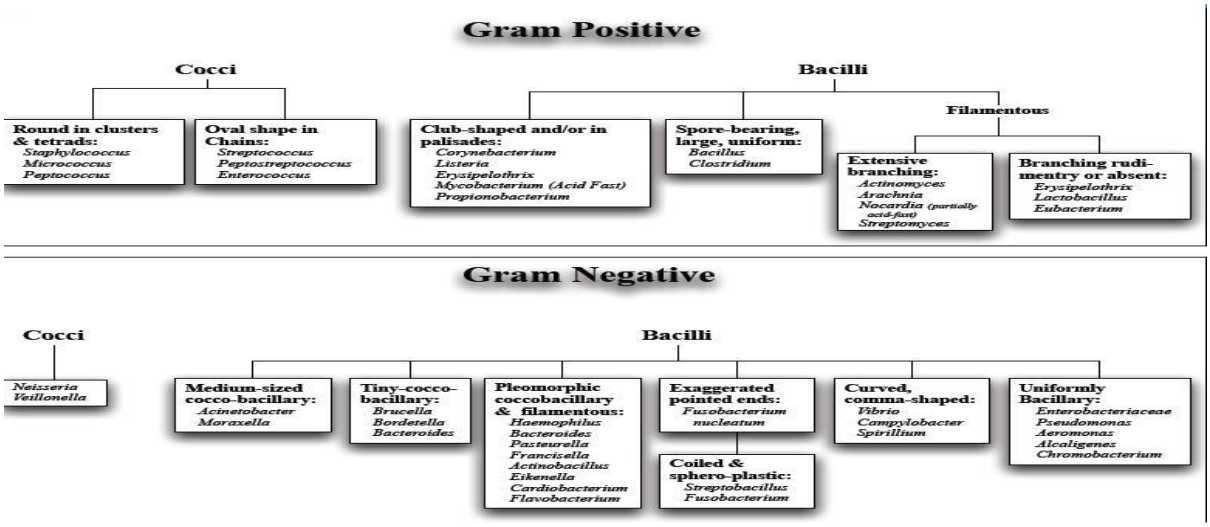


Fig: 1.Bacterial classification based on gram staining

Hydrocarbon utilising microorganisms

Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons. Bacteria and fungi are the key agents of degradation, with bacteria assuming the dominant role in marine ecosystems and fungi becoming more important in freshwater and terrestrial environments. Adapted communities, i.e., those which have been previously

exposed to hydrocarbons, exhibit higher biodegradation rates than communities with no history of hydrocarbon contamination.

The most important(based on frequency of isolation) genera of hydrocarbon utilizers in aquatic environments were *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula, and Sporobolomyces.*

Table: 2. List of known hydrocarbon degrading microorganisms

Crude oil component	Microorganisms
Saturates	*Arthrobacter sp., Acinetobacter sp., Candida sp., Pseudomonas sp., Rhodococcus sp., Streptomyces sp., Bacillus sp., Aspergillus japonicas.*
Monocyclic aromatic hydrocarbons	*Pseudomonas sp., Bacillus sp., b. Stereothermophilus, Vibrio sp., Nocardia sp., Corynebacterium sp., Achromobacter sp.*
Polycyclic aromatic hydrocarbons	*Arthrobacter sp., Bacillus sp., Burkholderiacepacia. Pseudomonas sp., Mycobacterium sp., Xanthomonas sp., Phanerochete chrysosporium, anabena sp., Alcaligenes.*
Resins	*Pseudomonas sp.,* members of *Vibrionaceae., Enterobacteriacee., Moraxella sp.*

Sample description with common bacterial population

S. no.	Polluted sources	Commonly found bacterial population
1.	Polluted river	E. coli, Vibrio sp., Enterococcus sp.,*Mycobacterium avium, Mycobacterium intracellulare, Helicobacter pylori, A. Hydrophila, etc.*
2.	Sewage water	Salmonella, S. Typhimurium, shigella sp.,*Legionella* sp.,Enterococcus sp., E. coli, Vibrio cholerae, Camplyobacter jejuni, Yersinia sp., Dehalococcoides ethenogenes etc.
3.	Phylloplanes of leaves from road side plants	Xanthomonas campestris, Vibrio cholerae, Helicobacter pylori, Mycoplasma-like species, *Pseudomonas fluorescens,Pseudomonas syringae* and *Erwinia* (*Pantoea*) spp., *Pseudomonas aeruginosa, Enterococcus* spp., *Burkholderia cepacia*
4.	Plastic contaminated soil	*Clostridium botulinum, Bacillus Amylolyticus, Bacillus Subtilis, Arthobacter defluvii*

MATERIALS AND METHODS

Serial dilution

The benzene contaminated soil and kerosene contaminated water were subjected for Serial dilution technique under the aseptic and sterile conditions of Laminar Air Flow hood.

Composition of normal saline solution

S. no.	constituents	Amount for 100 ml (in gram)
1	NaCl	0.85

1. 20 (10 each) sterile, clean and grease free test tubes were taken and were marked as 10^{-1} to 10^{-10}, separately for both samples so that two sets of 10 test tubes were ready.
2. 10ml of freshly prepared normal saline solution (0.85% NaCl) was poured into each tube.
3. 1ml of thoroughly mixed water sample was poured into first tube of the 1^{st} set marked as 10^{-1} and 1gm of soil sample was added to the first tube of the 2^{nd} set marked as 10^{-1}.
4. Solution presentin first tube was mixed well using micropipette and 1ml from it was transferred to second tube marked as 10^{-2}.
5. Solution presentin second tube (containing 10^{-2} dilution) was mixed well using micropipette and 1ml from it was transferred to next tube marked as 10^{-3}.
6. Subsequent transfer of solution was done in ascending order of the tube's number (or in descending order of the dilution) till the solution was transferred to the last tube marked as 10^{-10}.Steps 4 to 6 were performed for both the samples in their respective set of ten tubes.

Culturing/Plating of samples

Spread plate technique for Mother Plate Preparation:

Composition of growth media

S. no.	Constituents	Amount for 100 ml
01	Peptone	0.5gm
02	NaCl	0.3gm
03	Benzene/kerosine	0.5ml
04	Agar-agar	2.5gm
05	Distilled water	For dilution upto100 ml

1. 100ml of nutrient agar media was prepared with Benzene/kerosene as the sole source of Carbon replacing the yeast extract and was autoclaved for 15 minutes at 121^{o}C and 15lbs in order to sterilize.

2. Sterilized media were poured onto 4 sterile petri plates (25ml approximately in each) in LAF hood and allowed to solidify.
3. From the last two tubes of both the sets 100µl of inoculums was pipette out and poured onto separate petri plates having solidified nutrient agar media.
4. With the help of a spreader, the inoculum was spread over media uniformly in clockwise and counter clockwise directions.
5. Petri plates containing the inoculums was kept in upside down position in bacteriological incubator at 37°C for 24 hour incubation.
6. Petri plates were observed after 24 hour for single isolated colonies.

Mannitol fermentation test:

Composition of mannitol fermentation broth

S. no.	Constituents	Amount for 100 ml (in grams)
01	Casein	0.5
02	Peptone	0.5
03	Mannitol	0.5
04	NaCl	1.5
05	Phenol red	Pinch
06	Distilled water	100 ml

1. A clean and sterile test tubes were taken and 5ml of Mannitol broth was poured in it and autoclaved at 120°C at 15lbs for 15 minutes.
2. Autoclaved broth containing tube was aseptically allowed to cool to room temperature in a LAF hood.
3. With the help of sterile inoculating loop, a small amount of inoculum was taken from the pure culture plate which showed catalase test positive (A) and was used to inoculate the test tube.
4. Inoculated broth was incubated for overnight at 37°C in bacteriological incubator.
5. After 24 hour of incubation the tubes were observed for any change in pH (indicated by change in color).
6. Change in color from red to orange/yellow indicates positive test for mannitol fermentation otherwise no change in color indicates negative result.

Yellow pigmentation in the colonies:

1. This culture that was subjected to Mannitol was further observed for the coloration/pigmentation in its colony in pure culture plate.

Glucose fermentation test:

Composition of glucose fermentation broth

S. no.	constituents	Amount for 100 ml (in grams)
01	Peptone	0.5
02	Mannitol	0.5
03	Nacl	1.5
04	Phenol red	Pinch
05	Distilled water	100 ml

1. Glucose fermentation broth was prepared and 5ml of it was transferred to a test tube and autoclaved at 118°c &15 lbs for 15 minutes.
2. Autoclaved tube was allowed to cool to room temperature in aseptic conditions of LAF hood.
3. Culture showing mannitol fermentation test negative and yellow pigmentation in its colony was taken for inoculation of glucose broth.
4. Inoculum from respective culture was taken with the help of sterile inoculating loop and was inoculated into respective tubes marked as the culture name.
5. The inoculated tube was incubated in bacteriological incubator for overnight at 37°c.
6. After 24 hour of incubation, the tube was observed for any change in pH(indicated by change in color).
7. Change in color from red to orange/yellow indicates positive result for glucose fermentation and no change in color indicates negative result.

Assay for gram positive rod bacteria.

I Spore forming and aerobic

Starch hydrolysis test:

Composition of starch agar media

S. no.	constituents	Amount for 100 ml (in grams)
01	Peptone	0.5
02	Beef extract	0.15
03	Nacl	0.5
04	Starch	0.3
05	Agar-agar	2.5
06	Distilled water	100ml

1. A clean grease free and sterile test tube was taken for gram positive, aerobic and spore forming rod shaped bacteria (C).

2. 25 ml of starch agar media was prepared and the autoclaved media was allowed to solidify in a petri plate.
3. The plate was streaked with respective culture in zigzag fashion.
4. Streaked plate was kept in bacteriological incubator for overnight incubation at 37°c.
5. After overnight incubation, 2-3 drops of iodine solution was added to and spread around the colony.
6. Plate was observed for any color change i.e. violet color appearance or no change.

. Voges Proskauer test:

Composition of glucose peptone phosphate water

S. no.	constituents	Amount for 100 ml (in grams)
01	Peptone	0.7
02	Dextrose	0.5
03	K_3PO_4	0.5
04	Distilled water	100ml

1. 10 ml of autoclaved glucose phosphate peptone broth was transferred to a clean, grease free and sterile test tube.
2. Each tube was inoculated with respective culture (C) and incubated for 48 hours at 37°C in bacteriological incubator.
3. 5 drops of 0.3% (w/v) methyl red indicator solution were added to tube and mixed well.
4. Tube was observed for immediate color change as bright red color indicated positive test while yellow indicated negative.

Citrate test:

Composition of simmon's citrate agar media

S. no.	Constituents	Amount for 100 ml (in grams)
01	Ammonium dihydrogen phosphate	0.1
02	Potassium dihydrogen phosphate	0.1
03	Nacl	0.5
04	Sodium citrate	0.2
05	$MgSO_4$	0.02
06	Agar-agar	2.5
07	Bromomethyl blue	0.08
08	Distilled water	100ml

1. Simmons's citrate agar media were prepared and autoclaved and 5ml of it was kept in Autoclaved tube in slanting position to prepare slant.
2. Prepared (solidified) slant was streaked with culture having positive VP test (C) and incubated for 48 hours at 37°c in bacteriological incubator.
3. Incubated slant was observed for any change in color
4. Change in color from light green to blue indicates positive test.

6.5% NaCl growth:

Composition of 6.5% NaCl solution

S. no.	constituents	Amount for 100 ml
01	Peptone	0.5gm
02	NaCl	6.5gm
03	Yeast extract	0.3gm
04	Distilled water	For dilution upto100 ml

1. 5 ml of 6.5% (w/v) NaCl solution was poured in clean grease free & sterile tube and inoculated with culture having positive citrate test.
2. Tube was incubated in the bacteriological incubator for 24 hours at 37°C.
3. Inoculated solutions were observed for turbidity.Turbid solutions indicate positive NaCl growth.
4. Culture with positive NaCl growth at 37°c were subjected further to incubation in bacteriological incubator 24 hours at 55°c in fresh 6.5 % (w/v) NaCl solutions.

II Spore forming and aerobic

Pure culture D that was found to be non spore forming was subjected to Catalase and Starch Hydrolysis Tests.

Isolation of DNA (genomic):

The genomic DNA was isolated from all the 4 bacteria as follows:

Composition of lysis buffer

S. no.	Constituents	Amount for 100 ml (in grams)
01	50mM Tris HCl	0.788
02	20mM EDTA	0.584
03	1.5% SDS	1.5
04	Proteinase K (20mg/ml)	200µl
05	50mM NaCl	0.292

1. 1.5 ml of each overnight incubated bacterial culture broth was taken in eppendorf tube.
2. Eppendorf tube was centrifuged at 6000 rpm for 10 minutes.

3. The supernatant from the centrifuged tubes was discarded.
4. 500 µl of freshly prepared lysis buffer was added to the pellet and was vortexed gently.
5. Votexed solution was incubated in water bath at 65°c for 20 minutes.
6. Incubated solution was gently vortexed for 10 minutes.
7. Solution was cooled to room temperature.
8. 50 µl of 3M sodium acetate was added to the cooled solution and was kept in ice-cold condition for 10 minutes.
9. Solution containing lysis buffer and sodium acetate was centrifuged at 10000 rpm for 10 minutes.
10. From the centrifuged tube, supernatant was transferred to fresh and sterile tube and equal volume of ice cold isopropanol was added.
11. Above solution/mixture was incubated at 4°c for 24 hours.
12. Incubated mixture was centrifuged at 13000 rpm for 10 minutes.
13. Pellet was taken and was dissolved in 200 µl of TE buffer.

Gene amplification through conventional PCR (Thermocycler):

1 cycle: 94°C for 5 min (Initial denaturing)

35 cycles: 94°C for 1 min (denaturing)

55°C for 1 min (annealing)

72°C for 2 min (extension)

1 Cycle: 72°C for 10 min (final extension)

RESULTS

PRINCIPLE: The double stranded DNA is denatured to separate into two single strands and allowed to hybridize with a primer and then forms the primer template molecule used for the synthesis of DNA by Taq DNA polymerase enzyme. The PCR mainly involved 3 reactions based on the temperature gradient. They are

1. Denaturation
2. Annealing
3. Synthesis

Materials required

Genomic DNA/Template DNA=2µl (sample)

Two primers i.e. forward primer= 2µl and Reverse primer=2µl

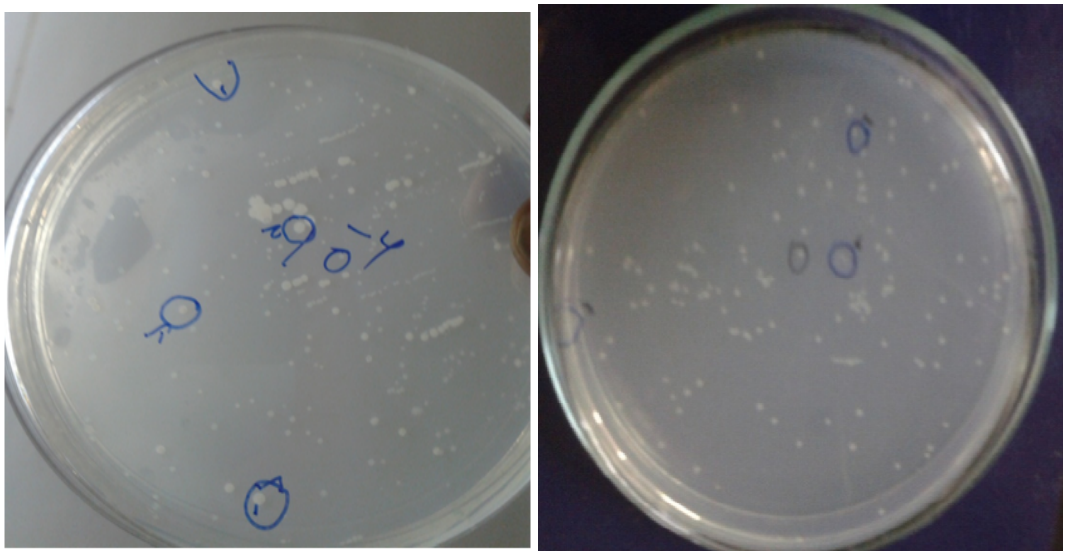
PCR buffer=4µl

DNTPs =4µl

Taq-DNA polymerase enzyme =0.2µl

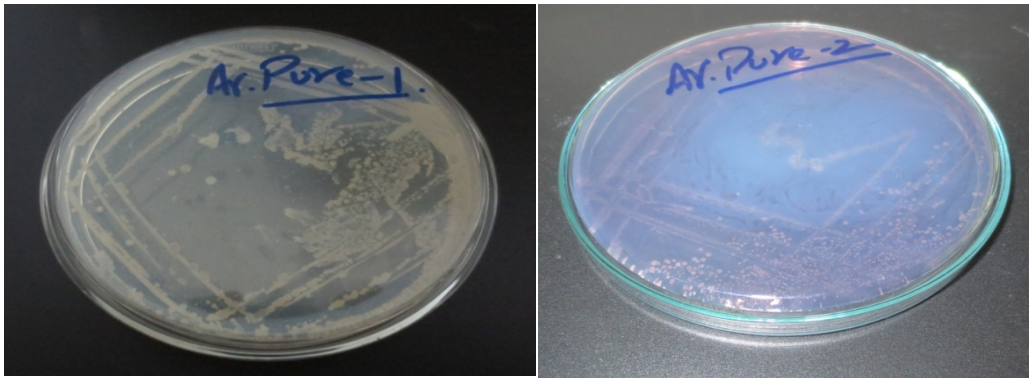
MgCl2 =2µl

Nuclease free water for making volume upto 25 µl



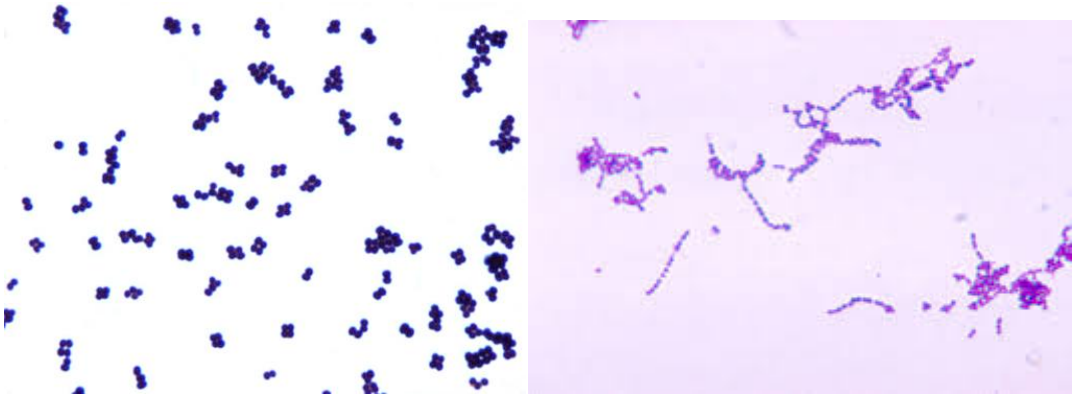
Mother plates from soil sample

Mother plates from water sample

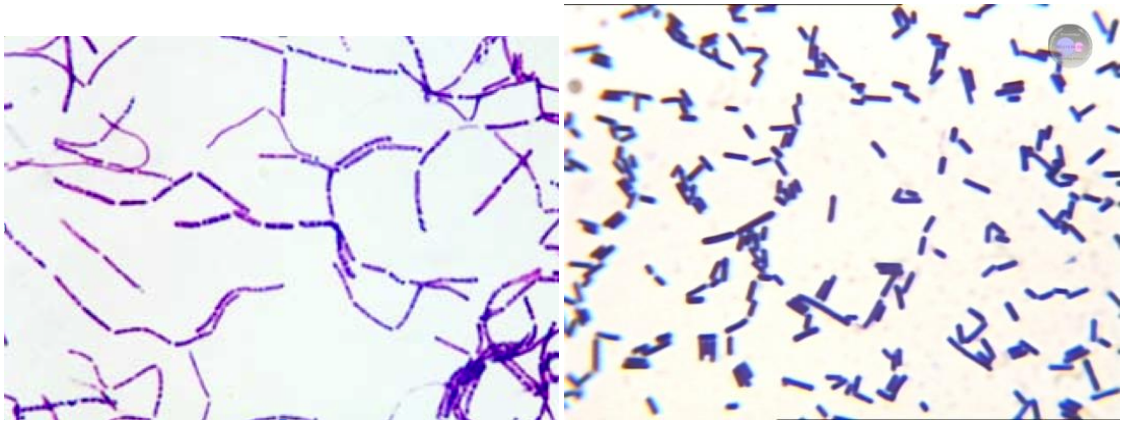


Pure plates A and B from Soil samples

Pure plates C and D from Water samples



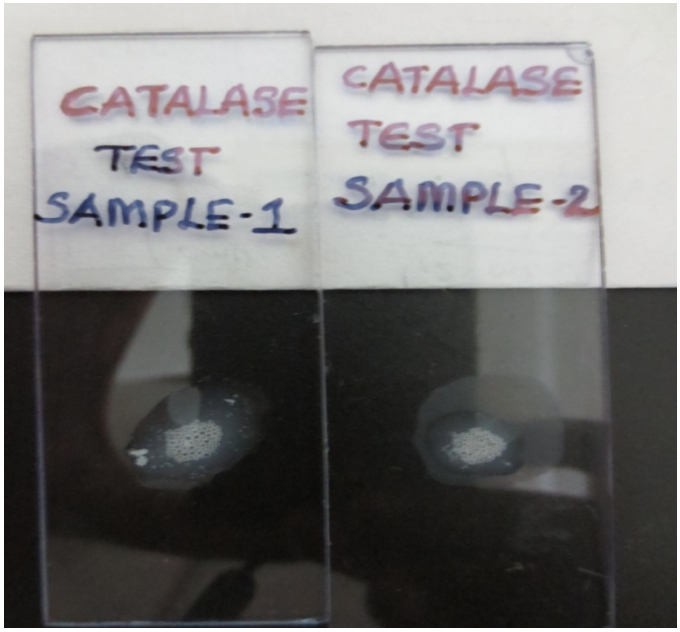
A(Gram positive cocuus in tetrad) B (Gram positive cocuus in chain)



C (Gram positive Spore bearing Rod) D (Gram Positive Non Spore bearing Rod)

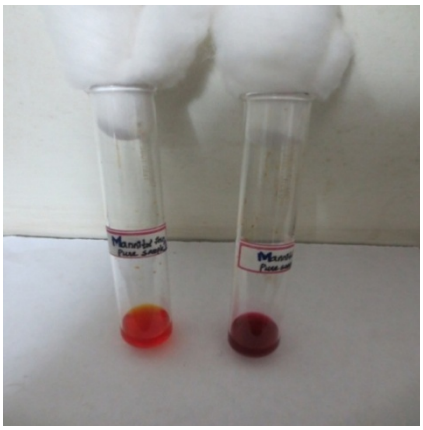
Identification of bacterial cultures:

Catalse test



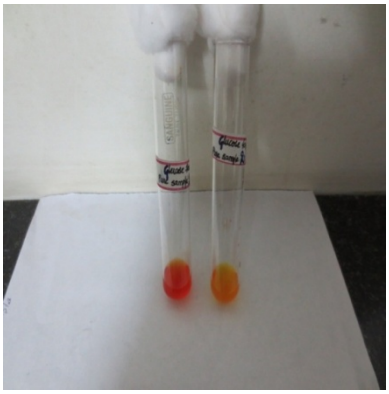
Catalse test was performed on samples A, B and D. A showed positive result with Bubble production and effervescence and B and D Showed negative result with no bubble formation.

Mannitol Test



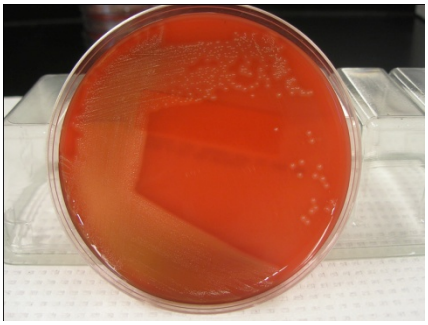
Mannitol test was performed on Sample A and it showed negative result as red colour was retained and not change to yellow (Left tube in the figure is the Sample A)

Glucose fermentation



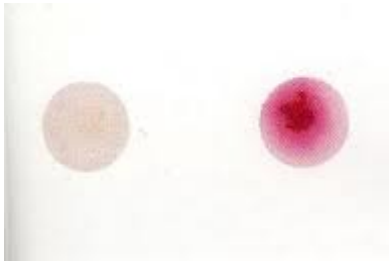
Glucose fermentationtest was performed on Sample A and it showed negative result as red colour was retained and not change to yellow (Left tube in the figure is the Sample A and the Right tube is a demonstration of Positive result in a reference sample)

Haemolysis Test



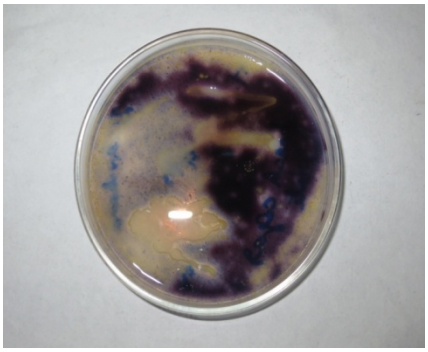
Haemolysis Test was performed on Sample B snd it showed β haemolysis with a non green colony growth.

PYR Test



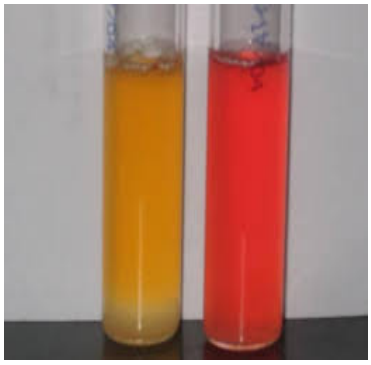
PYR Test was performed on Sample B and positive result was shown as it produced a cherry red colour within a minute.

Starch hydrolysis



Starch hydrolysis was performed on Cand D and both showed positive result with a clear transparent zone around the colony.

Voges Proskauer test



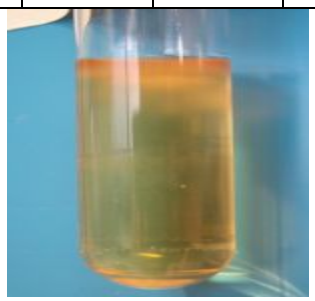
VP was performed on Sample C and it showed positive result as red colour was produceD immediately (Right tube in the figure is the Sample C and the left tube is a demonstration of negative result in a reference sample)

Citrate Test

Citrate Test was performed on Sample C and it showed positive result as the coloue changes from Green to Blue (Left tube in the figure is the Sample C and the Right tube is a demonstration of Negative result in a reference sample).

6.5%NaCl growth Test

Source	Sample code	Catalse	Mannitol	Yellow Pigment	Glucose Fermentation	Haemo-lysis	PYR Test	Bacterium identified
Water contaminated with Kerosine	A	+ve	-ve	+ve	-ve	NA	NA	*Micrococcus luteus*
	B	-ve	NA	NA	NA	β	+ve	*Streptococcus pyogenes*



6.5%NaCl test on both the temperature showed turbity indicating the growth for sample

Observation of biochemical tests for gram positive Coccus bacteria

Observation of biochemical tests for gram positive Rods bacteria

Inference: using biochemical tests of Bergey's Manual of Determinative Bacteriology following 4 bacteria were identified:

Micrococcus luteus, Streptococcus pyogenes, Bacillus subtilis and Corynebacterium kutsceri

Measurement of Hydrocarbons in Nutriant Agar Media (in ppm)

Name of bacteria	After 24 hrs of incubation	After 10 days
Micrococcus luteus	8010	1900
Streptococcus pyogenes	7990	2674
Bacillus subtilis	8005	1698
Corynebacterium kutsceri	7997	6067

Inference: As per the reading it is clear that *Bacillus subtilis*is most efficient in hydrocarbon (Benzene) Degradation while *Corynebacterium kutsceri*showed the least efficiency.*Micrococcus luteus* and *Streptococcus pyogenes*also showed significant degradation of kerosene within 10 days.

Source	Sample code	Spore Forming	Catalase	Starch hydrolysis	V. P.	Cell diameter	Citrate	6.5% NaCl (37°& 55°C)	Bacterium identified
Soil contamina-tedwith Benzene	C	+ve	NA	+ve	+ve	< 1µm	+ve	+ve	*Bacillus subtilis*
	D	-ve	-ve	+ve	NA	< 1µm	NA	NA	*Corynebacterium kutsceri*

Identification of HC degrading gene:

List of HC degrading genes with respective organisms

S. no.	Organism	Gene for HC degradation
1	*Micrococcus luteus*	Prenyltransferase, ubiA
2	*Streptococcus pyogenes*	UDP-glucose 6-dehydrogenase
3	*Bacillus subtilis*	surfactin, sfp
4	*Corynebacterium kutsceri*	catechol 1,2-dioxygenase

Nucleotide (Gene) sequence retrieval:

ubiA prenyltransferase [*Micrococcus luteus NCTC 2665*]

ATGATCCGCACCCTCTTCTGGGTGTCCCGGCCGGTCAGCTGGGTGAACACGGCCTACCCGTTCGCCGCCG
CCGCGATCCTGACCGGGGGGCTGCCCGCGTGGCTGGTGGTCCTGGGCGTCGTGTTCTTCCTGGTGCCCTA
CAACCTGGCCATGTACGGCATCAATGACGTGTTCGACTTCGCCTCGGACCTGCGCAACCCCCGCAAGGGG
GGTGTGGAGGGCTCCGTGCTGGGCGACCCCGCGGTGCGCCGCCGGGTGCTGGCGTGGTCGGTGCTGCTGC
CCGTGCCGTTCGTGGCCGTGCTCGCGGGCTGGTCCGCCGTGCGGGGCGAGTGGGCCGCCGTGCTGGTGCT
CGCGGTGAGCCTGTTCGCGGTGGTGGCGTACTCCTGGGCGGGGCTGCGGTTCAAGGAGCGGCCCTTCCTG
GACGCCGCCACCTCCGCCACCCACTTCGTCTCCCCCGCGGTCTACGGCCTCGCGCTGGCCGGGGCGACCC
CCACGCCCGCCCTGGCGGCGCTGCTGGGGGCGTTCTTCCTGTGGGGCATGGCCTCGCAGATGTTCGGGGC
GGTGCAGGACGTGGTGCCGGACCGGGAGGGGGGCCTGGCCTCGGTGGCCACCGTGCTGGGCGCTCGGCGC
ACCGTCCTGCTCGCCGCCGGCCTGTACGCGGCGGCGGGCCTGCTGCTGCTGGCCACCGACCCGCCGGGCC
CGCTCGCGGCGCTGCTGGCCGTGCCCTACGTGGTGAACACCCTGCGCTTCCGCCGCATCACGGACGCCAC
CTCGGGCGCGGCCCACCGCGGCTGGCAGCTGTTCCTTCCGCTGAACTACGTGACCGGCTTCCTCGTGACC
CTGCTGCTGATCGGGTGGGCGCTGACCCGGGGGGCGGCGGCATGA

SPy_0542 UDP-glucose 6-dehydrogenase [*Streptococcus pyogenes M1 GAS*]

ATGAAAATTACAGTTGTAGGCATTGGATACGTTGGATTATCGATAGGGCTCCTACTTGCAAAGGAACACG
ACGTCACCTTTTTTGATATTGATAATAAAAAAATTGATTTAATAAATAAAAGGCAATCCCCTCTTAAAGA
AGCAGCTATAAACAAACTTTTATGTAAGGCAAAAAATATTAATGCAACTTCTTCTGAAGAATTAGCATAT
AAGGATGCGACTTTCATAATCTTGTCTTTGCCAACCAACCTAAAATTTAATAAGCTTGATACTTCCATTA
TCGAAATTTCTGTAAGTAATATTTTAAAGATAAACAAAAAGGCTACAATTGTAATAAAGTCGACAGTTCC
AATTGGTTTTACAGAATATTTAAGGAATCGATTTCACTACAACGATATCATTTTTTCACCTGAGTTCCTT
AGGGAAGGATCAACTATTCATGATCAATTGTATCCTTCGAGAACTATAGTTGGAAATGAATCTAGAAATT
CTCAATTATTCTTAGACATACTAACAGATATATCGGTTGAAAAAGACTCGCCATCTTTATTAGTTGGCTC
TTCTGAAGCAGAAGCGATAAAGTTATTTTCGAATGCATACTTGGCACAAAAAATTGCTTTTTTTAATGAG
TTGGATACGTTTGCTGAAATGCAAAATTTGGACTCAAAAAAAATTATTGAGGCTATGGGATATGACCAGA
GAATAGGAAATTCGCACAATAATCCTTCTTTCGGTTTTGGTGGGTACTGTCTTCCTAAGGATATTAAGCA
ATTAGAGTATCATTTTAAAGAAATTCCAGCACCAATTATTACCAGTATAAGTGAATCTAATTTATTAAGA
AAAATTCATATAGCAAAAATGATTTTGAACAGCTCAGCTAAAACAATAGGAATTTATAGAATTAATTCCA
AAAAAGATTCGGATAATTGTAGGGAATCTTCTACAATTGATGTTGCTAAACTTCTAAAAAGCAGTGGTAA
GGATGTTATCATCTTTGAGCCCTTAATTAACCAAAAAAAGTTTTTGGGGTGCCCTTTAAGTAATGATTTT
AATGAATTTATTAAATATTCGGATATTATAGTTGCCAATAGAATAGATGATGCTCTGAGAAAATGTAATT
CAAAAGTTTTTACACGTGATATTTTTCAGTATGATTAA

surfactin, sfp geneBacillus subtilis:

TCAAGCGGAAGCGATAAGCCTTTGCCTTCCTTGTTTGATAAAGCTTTCTTTCATTGACCATAGATGATAA
AAATAGTCTGTCTGCTCGTCCTTGTCTTTTGCTAAAAGGTCGCTGTACTCTGTTTTTGAAAAGAAGCGCT
TGGCGATCTCAAGGCTGATCGGTTTCGTTTTTTCGATATCTATGCCGATCGGCTGTGAATCAAACGCGCA
AATGACCCAGCGTCCGGAGTGAGAAATGTTGAAATGAGCGTCGGGAAGATCAGGGATGCACGGCTTCCCG
TATTCCTGCGTGCTAAAGCGGATATCGGATTTGTCCAACTGATACTGCCTGCTTATGACTGAGCGAACGA
GCACATCTCCCAGCAGGGTGCGGTGAGCATCTTCTTTATGATAAAATCTCCGGCATTTCTCCCGTTTTTC
AGGTGATATGAAAGACATGAACCGTTCATTTTCTTCCTGTGAAAGCGGGCGGTCCATATAAATTCCGTAA
ATCTTCAT

CGTRNA_RS11795 catechol 1,2-dioxygenase [*Corynebacterium glutamicum ATCC 13032*]

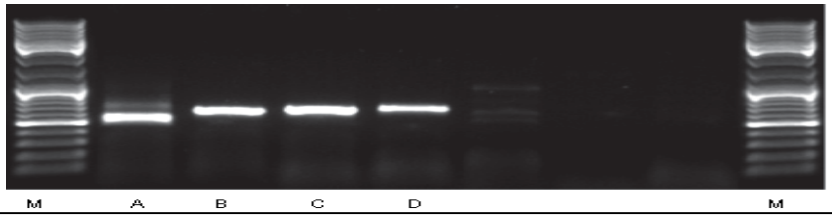
ATGACTTCAGCTGAACAGATCGTTGATCCAACAGCCCACGATTCGGGCAACAAGGCAACTGACAAGTTCA
AGGCAAACCGCGTTTCCTCCGATACCTCCAAGGAACGCGCAAACGCGATCTACGTAGATCTGCTCGCGGC
GATCGCCCAGGTTGCTCACAAGCACGAAGTCACCTACGAAGAGTACGCAGTGCTCAAGCAGTGGATGATC

GACGTTGGAGAATACGGCGAGTGGCCACTGTGGTTGGACGTTTTCGTTGAGCATGAGATCGAAGAGATCA
ACTACAACCGCCACGACTACACCGGAACCAAGGGTTCCATCGAAGGCCCTTATTACGTAGAGAACTCTCC
GAAGCTCCCTTGGGATGCTGAAATGCCAATGCGTGACAAGGACCGCGCATGCACCCCACTGATCTTCGAG
GGGCAGGTTACTGACCTCGACGGCAACGGTCTTGATGGAGCAGAAGTTGAGCTCTGGCACGCAGATGAGG
ACGGATACTACTCCCAGTTCGCGCCTGGAATCCCAGAGTGGAACCTGCGTGGCACCATCGTTACCGATGA
GGAAGGCCGCTACAAGATCAAGACCCTGCAGCCTGCGCCTTACCAGATCCCTCATGATGGCCCAACCGGT
TGGTTCATTGAGTCTTACGGTGGGCACCCATGGCGCCCAGCCCACCTCCACTTGCGCGTTTCCCACCCGG
GCTACCGCACCATCACCACCCAGCTTTACTTCGAGGGTGGCGAGTGGGTCGAAAACGACGTTGCAACCGC
TGTGAAGCCAGAACTGGTCCTGCACCCTGAGACTGGCGAGGATGGTAACCACGTTCACTACCCATTCGTC
CTGGATAAGGAAGACTAG

Primer designing by Primer BLAST:

List of primers with respective genes and the organisms

S. no.	Organism	Gene name	Primer
1	*M. luteus*	prenyltransferase	FP– AAGCCGGTCACGTAGTTCAG RP-TGGCCATGTACGGCATCAAT
2	*S. pyogenes*	UDP-glucose 6-dehydrogenase	FP-GCAAAGGAACACGACGTCAC RP-ACAGTACCCACCAAAACCGA
3	*B. subtilis*	Sfp	FP-CGGAAGCGATAAGCCTTTGC RP – CATCCCTGATCTTCCCGACG
4	*Corynebacterium kutsceri*	catechol 1,2-dioxygenase	FP-TTGCAACGTCGTTTTCGACC RP-CACGACTACACCGGAACCAA



A-prenyltransferase

B- UDP-glucose 6-dehydrogenase

C-sfp

D- catechol 1,2-dioxygenase

Amplicons of the respective genes as run in Agarose

DISCUSSION

The high demand for petroleum and associated products during the last ten decades has made petroleum spills inevitable consequences of oil production and refining. Despite fluctuations in its prices, oil will remain a major source of energy in the next several decades because a reliable alternative has not yet been found. As a result, the problem of pollution during production and transportation of oil would remain a major issue. Microbial degradation appears to be the most

environmentally friendly method of removal of oil pollutant since other methods such as surfactant washing and incineration lead to introduction of more toxic compounds to the environment. Hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater, and soil ecosystems (Atlas and Bartha, 1973). The ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as evidence that those organisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003). In a recent study, Ojo (2006) reported the capability of native bacterial population to mineralize petroleum hydrocarbons in wastewater. Similarly, Okoh (2003) demonstrated degradation rates of different strains of *Pseudomonas aeruginosa* on crude oil with evidence of significant reduction of major peak components of the oil. Virtually all of these studies utilized organisms isolated from petroleum contaminated sites and degradation competencies were only tested on crude oil. In this report however, we chose to isolate hydrocarbon degraders from garden soil and tap water by contaminating them with Benzene and Kerosine respectively.

Hydrocarbon plays a major role in environmental pollution leading to global warming, ozone depletion etc. The present work deals with identification of bacteria present in different hydrocarbon polluted sources followed by the amplification of their genes responsible for hydrocarbon degradation. Two different sources of hydrocarbon polluted samples viz Soil contaminated with Benzene and Water contaminated with Kerosine were undertaken for the identification of bacteria present in them. Following Bergey's manual of determinative bacteriology different biochemical tests were performed for bacterial identification along with gram staining and four different species of bacteria were identified as *Micrococcus luteus, Streptococcus pyogenes, Bacillus subtilis and Corynebacterium kutsceri*. Using the literature search, respective genes responsible for hydrocarbon degradation were identified and amplified in these bacteria.Bacillus subtilis C9 produces a lipopeptide-type biosurfactant, surfactin, and rapidly degrades alkanes up to a chain length of C19. The addition of surfactin (0.5%, w/v) to the culture of B. subtilis 168 significantly stimulated the biodegradation of hydrocarbons of the chain lengths of 10â€“19; over 98% of the hydrocarbons tested were degraded within 24 h of incubation.*M. luteus* has been the platform for isolation of important enzymes in this most basic of processes, including the cis-prenyltransferase gene, whose gene product carries out the condensation of isopentyl phosphate with allelic diphosphate [Oh *et al.,* 2000].

In a recent work of Satish *et al a*mong the five isolated microorganism from oil

effluent, maximum growth was seen in Streptococcus for an oil concentration of 1% and hence this organism was used for further treatment studies. Among the seven isolated microorganism from oil contaminated soil, maximum growth was seen in Micrococcus sp. for an oil concentration of 1%. Based on the prior research work the hydrocarbon degrading genes of *Micrococcus luteus*and*Bacillus subtilis*were selected and that of *Streptococcus pyogenes* was obtained by KEGG Pathway of aromatic hydrocarbon degradation.However no such gene could be found in*Corynebacterium kutsceri*hence that of a close species *Corynebacterium glutamicum*was taken as reference.In the present study primers of the respective genes were designed by Primer BLAST and a successful amplification was obtained.This also suggests a close homology between catechol 1,2-dioxygenase of *Corynebacterium glutamicum*and its homologous of*Corynebacterium kutsceri.*As per the position of bands in the gel picture the amplicon length ofprenyltransferase was found to be more than those of UDP-glucose 6-dehydrogenase, sfp and catechol 1,2-dioxygenase.

CONCLUSION

The present work deals with identification of bacteria present in different polluted sources followed by the amplification of their genes responsible for hydrocarbon degradation. Following Bergey's manual of determinative bacteriology different biochemical tests were performed for bacterial identification along with gram staining and four different species of bacteria were identified as *Micrococcus luteus, Streptococcus pyogenes, Bacillus subtilis and Corynebacterium kutsceri* from two different sources of hydrocarbon polluted samples viz Soil contaminated with Benzene and Water with Kerosine.The quantitative estimation of hydrocarbon degradation showed that *Bacillus subtilis*is most efficient in hydrocarbon (Benzene) degradation while *Corynebacterium kutsceri*showed the least efficiency.*Micrococcus luteus* and *Streptococcus pyogenes*also showed significant degradation of kerosene within 10 days.

Using the literature search, respective genes responsible for hydrocarbon degradation were identified as prenyltransferase for *Micrococcus luteus,*and sfp for *Bacillus subtilis.*UDP-glucose 6-dehydrogenasewas retrieved from KEGG Pathwayin *Streptococcus pyogenes*. Specific gene for degradation in *Corynebacterium kutsceri* could not be found out. So that of *Corynebacterium glutamicum ATCC 13032* was taken as reference and primers were designed by Primer BLAST. Keeping the PCR Conditions same for all the genes, successful amplification was achieved in these bacteria.

REFERENCES

1. Microbial Hydrocarbon Degradation: Efforts to Understand Biodegradation in Petroleum Reservoirs (http://dx.doi.org/10.5772/55920)
2. Luz AP et al., 2004. A survey of indigenous microbial hydrocarbon degradation genes in soil from Antarctica and Brazil. Can J Microbiol, 2004 May; 50(5): 323 – 33.
3. Int J Environ Res Public Health. Oct 2010; 7(10): 3657–3703.Published online Oct 15, 2010. Water Microbiology. Bacterial Pathogens and Water, João P. S. Cabral
4. Pak. J. Bot., 43(3): 1765-1772, 2011. BACTERIAL AND TOXIC POLLUTANTS IN LAKES OF RIVER INDUS, HUMERAH BANO SHAFIQ1, MUNAZZA AJAZ2 AND SHEIKH AJAZ RASOOL
5. Neff, 1979; CCREM, 1987; NRCC, 1983; USPHS, 1990
6. Story SP, Kline EL, Hughes TA, Riley MB, Hayasaka SS.Degradation of aromatic hydrocarbons by Sphingomonas paucimobilis strain EPA505.Arch Environ Contam Toxicol 47:168-76 (2004)
7. Nojiri H, Habe H, Omori T. Bacterial degradation of aromatic compounds via angular dioxygenation, J Gen Appl Microbiol 47:279-305 (2001)
8. Haritash AK, Kaushik CP. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review, J Hazard Mater 169:1-15 (2009)
9. da Silva M, Esposito E, Moody JD, Canhos VP, Cerniglia CE. Metabolism of aromatic hydrocarbons by the filamentous fungus Cyclothyrium sp. Chemosphere 57:943-52 (2004)
10. Wunder T, Marr J, Kremer S, Sterner O, Anke H. 1-Methoxypyrene and 1,6-dimethoxypyrene: two novel metabolites in fungal metabolism of polycyclic aromatic hydrocarbons. Arch Microbiol 167:310-6 (1997).
11. 1. Aamer Ali Shah, Fariha Hasan, Javed Iqbal AKhter, Abdul Hameed, Safia Ahmed(2008) Degradation of polyurethane by novel bacterial consortium isolated from soil, Annals of Microbiology.
12. 2.Adeline S. Y. Ting, Carol, H. C. Tan and Aw, c. S. (2009). Hydrocarbon-degradation by isolate *Pseudomonas ludenis* UTAR FPE2. Malaysian Journal Of Microbiology, Vol 5(2) pp.101-108.
13. 3. Adriano Pinto Mariano, Ana Paulo de Arruda Geraldes Kataoka, Dejanira de

Frabcheschi de Angelis, Daniel Marcos Bonotto(2007). Laboratory Study on the bioremediation of diesel oil contaminated soil from a petrol station. Brazilian Journal of Microbiology.

14. 4. Anupama Mittal and Padma Singh(2009). Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills. Indian Journal of Experimental Biology.