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Isolation and characterization of bacterial isolate's from PAH contaminated soil

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Abstract

Screening of polyromantic hydrocarbon degrading microorganisms from oil contaminated soil was studied and the samples were obtained from nearly ten different sites like mechanical workshop, petrol pumps, coal depots, coal tar, washing centres, bus depot. The microorganisms were isolated using streak plate method after selective enrichment techniques. All sample were cultured in Minimal Salt Media with no external carbon sources. The enriched sample were cultured onto nutrient agar plates to isolate the hydrocarbon utilizers from the collected sample. Nearly 20 different isolates were selected and were further identified based on their morphological, biochemical characteristics and on molecular basis. The isolated strains according to biochemical tests belong to the genera Pseudomonas, Bacillus, Yersinia and Shigella. The ability of organism to survive is associated with the ability of the organisms to use the hydrocarbons in polluted soil which is of importance in bioremediation of oil spills, hydrocarbon pollution of soil.

Key Words: PAH, bacterial isolates, characterisation.

1. Introduction

Polycyclic aromatic hydrocarbons are organic compound containing only carbon and hydrogen, that are composed of multiple aromatic rings. The simplest such chemicals are naphthalene, having two aromatic rings and the three rings' compounds and phenanthrene.

Polycyclic aromatic hydrocarbons (PAH) are a group of more than 100 chemicals that are also called polynuclear aromatic hydrocarbons PAHs have also been found in facilities where petroleum, petroleum products, or coal are used or where wood, cellulose, corn, or oil is burned. People living near waste sites containing PAHs may be exposed through contact with contaminated air, water, and soil.

PAH are among persistent pollutant; they cannot be removed or destroyed from matrices contaminate Eating is the main route of exposure to PAH, exceeding the share other routes such as inhalation and skin contact. PAHs are mutagenic and carcinogenic substances, hazardous not only because of their tendency to accumulate in food chain of the hundreds of PAH, sixteen were identified as priority pollutants by the environmental protection agency of the United States of America. Their hydrophobic character and persistence, PAHs can accumulate in the soil, so that soil contamination with PAHs can be consider to be an indicator of environmental pollution because of human activity; hence research on soil contamination with PAHs have become an integral part of the strategies to evaluate the human exposure risk. PAHs presence in the environment has become increasingly important due to the use of wastewater for irrigation, the use of sludge as fertilizer and to pollution caused by automobiles and industrial activities. (2) Polycyclic aromatic hydrocarbons (PAHs) are organic compounds that are mostly colourless, white, or pale-yellow solids. They are a ubiquitous group of several hundred chemically related compounds, environmentally persistent with various structures and varied toxicity. They have toxic effects on organisms through various actions. Generally, PAHs enter the environment through various routes and are usually found as a mixture containing two or more of these compounds, e.g. soot.

Some PAHs are manufactured in the industry. The mechanism of toxicity is interference with the function of cellular membranes as well as with enzyme systems which are associated with the membrane. It has been proved that PAHs can cause carcinogenic and mutagenic effects and are potent immune-suppressants. Effects have been documented on immune system development, humeral immunity and on host resistance [1], [2]. PAHs can be formed both during biological processes and as products of incomplete combustion from either natural combustion sources (forest and brush fires) or man-made combustion sources (automobile emissions and cigarette smoke). Thus, PAHs are commonly detected in air, soil, and water. Therefore, PAHs are considered ubiquitous in the environment [3]. [4] . The ubiquitous nature of PAHs in the environment has been well summarized by Menzie et al. [5]. The major source of PAHs is the incomplete combustion of organic material such as coal, oil, and wood. PAHs are not synthesized chemically for industrial purposes. Nevertheless, there are a few commercial uses for many PAHs. They are mostly used as intermediaries in pharmaceuticals, agricultural products, photographic products, thermosetting plastics, lubricating materials, and other chemical industries [13]

2. Methodology

Sample collection

The soil samples were collected from different polyaromatic hydrocarbon polluted soil areas like bus depot, servicing centre, petrol pump, bike servicing, road asphalt, oil mills, chemical plant, coal depot, from Aurangabad region. The moist soil was collected and stored in polythene bags properly tied and tagged with the name of sample. pH was examined and noted.

Enrichment of Culture

1 g of soil sample was added and contents the suspension was used as inoculum to inoculate 100 ml MSM in 250 ml Erlenmeyer flask for enrichment of microbial populations present in the soil samples. The flasks were incubated at 27°C on an orbital shaker at 1800 rpm for 7 days.

The cell suspension was used as inoculum to further enrich the microbial populations. At each transfer, 1 ml of sample was withdrawn and serially diluted and 0.1 ml of the appropriately diluted culture broth was spread plated onto nutrient agar plates

Incubation was carried out for 24 h at 37°C on nutrient agar plate.

Isolation and identification of microorganisms:

All colonies that had grown on was sub cultured on Nutrient broth (NB) and were incubated at 37°C for 24 h. After 24hr, dilutions culture broth was spread onto on NA incubated at 37°C for 24 hrs. to obtain a pure culture. Screening of the isolates was carried out based on colony morphology, size, shape, color, Gram staining and biochemical tests. The isolates were identified according to the descriptions in the Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984), Vol. 2 (1986). API 24 E (Commercial Kit) was used for identification of Gram-negative bacteria.

Oxidase Test:

Dry Filter Paper Method: Since the oxidase reagent is unstable and must be freshly prepared for use, this method is convenient. Strip of Whatman's No. 1 filter paper are soaked in a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride. After draining for about 30 seconds, the strips are freeze dried and stored in a dark bottle tightly sealed with a screw cap. For use, a strip is removed, laid in a petri dish, and moistened with distilled water. The colony to be tested is picked up with a platinum loop and smeared over the moist area.

Glucose Fermentation

Aseptically inoculate glucose broth test tube with the test microorganism using an inoculating needle or loop. Incubate tubes at 35-37°C for 18-24 hours. Observe change in colour.

Fluorescent diffusible yellow pigment.

Pseudomonas F Agar is used to detect pigment production by Pseudomonas species. Pseudomonas produce a variety of pigments, and fluorescein is commonly produced by Pseudomonas aeruginosa. If growth is observed on Pseudomonas F Agar, fluorescein production is determined by visual examination of the plates under ultraviolet lighting.

Pseudo P Agar

Pseudomonas Agar (For Pyocyanin) is recommended for the detection of pyocyanin production by Pseudomonas species.

Principle and Interpretation Pseudomonas Agar is based on the formulation described by King et al (1) and as recommended in U.S. Pharmacopoeia (2) for detecting pyocyanin, a water-soluble pigment by Pseudomonas species.

Cultural response was observed after an incubation at 35-37°C for 18-48 hours.

Nitrate reduction test:

A heavy inoculum of test organism is incubated in a broth containing nitrate. Incubate at an appropriate temperature for 24 to 48 hours Add one dropper full of sulfanilic acid and one dropper full of a α-naphthylamine to each broth.

At this point, a color change to RED indicates a POSITIVE nitrate reduction test. No color change indicates the absence of nitrite.

Add a small amount of zinc (a toothpick full) to each broth. Zinc catalyses the reduction of nitrate to nitrite.

Lactose fermentation test:

Most used is phenol red lactose broth. The medium is a nutrient broth to which 0.5-1.0 An inoculum from a pure culture is transferred aseptically to a sterile tube of phenol red lactose broth.

The inoculated tube is incubated at 35-37 C for 24 hours and the results are determined.

Indole test:

Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.

Incubate at 37°C for 24-28 hours in ambient air.

Add 0.5 ml of Kovac's reagent to the broth culture.

Urease Test:

The Christensen's Urease broth medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.

Leave the cap on loosely and incubate the test tube at 35 °C in ambient air for 18 to 24 hours; unless specified for longer incubation.

Motility test:

Hanging drop preparation is a special type of wet mount. In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility.

Triple Sugar Iron Agar:

With a sterilized straight inoculation needle touch the top of a well-isolated colony

Inoculate TSI Agar by first stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant.

Leave the cap on loosely and incubate the tube at 35°C in ambient air for 18 to 24 hours

Ornithine decarboxylase

Moeller decarboxylase base-4 tubes with lysine, ornithine and arginine hydrochloride 1% and control. Inoculate the test medium, overlaid with sterile paraffin oil. Incubate and read daily for four days

TEST FOR Gm + ve rods.

Thioglycolate Broth Method:

It is a media that consumes oxygen and permits growth of anaerobes. Tubes containing sterile Thioglycolate broth were inoculated with the Gm +ve cultures and they were sealed by paraffin tape .The tubes were than incubated at room temperature for 24 hours after which growth was observed in form of turbidity.

Starch Hydrolysis Test:

Sterile Nutrient agar plates containing starch were inoculated with test organisms and incubated for 24 hours at room temperature. Results were seen in the form of clear zones surrounding colonies after addition of iodine solution.

Indole test:

Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth. Incubate at 37°C for 24-28 hours in ambient air.

Add 0.5 ml of Kovac's reagent to the broth culture.

Methyl Red Test:

This test is used to detect acid production. Methyl red acts as pH indicator.

Test organism is inoculated into glucose phosphate broth at incubated at room temperature for 48 hours. Few drops of MR reagent is added to detect acid production by change in colour.

Voges Proskauer Test:

This test detects butylene glycol producers.

Test organism is inoculated into glucose phosphate broth at incubated at room temperature for 48 hours.0.6 ml of alpha naphthol and 0.2 ml of 40 % KOH is added to the tubes and allowed to stand for 15 min. Colour change is observed.

Citrate Utilisation test:

Detects the ability of organisms to utilise citrate as sole carbon source.

Test organisms are inoculated on Simmon's Citrate agar and incubated at room temperature for 24 hours. Change is colour of agar is observed.

Glucose Fermentation:

A liquid broth containing glucose and pH indicator like phenol red is sterilised and inoculated with test organisms, incubated at room temperature for 24 hours. Change in colour of broth observed.

Oxidase Test:

Used to determine whether organism possesses cytochrome c oxidase enzyme.

Strip of Whatman's No. 1 filter paper are soaked in a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride.

After draining for about 30 seconds, the strips are freeze dried and stored in a dark bottle tightly sealed with a screw cap.

For use, a strip is removed, laid in a petri dish and moistened with distilled water.

The colony to be tested is picked up with a platinum loop and smeared over the moist area.

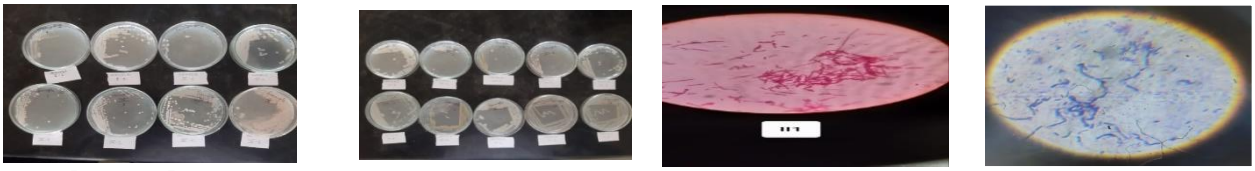
Motility test:

Hanging drop preparation is a special type of wet mount. In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility.

3. Results

Morphology:

Figure 1



Biochemical Tests:

1.Oxidase Test

Oxidase positive organisms are those which belong to Pseudomonas genera and those which are negative fall under Enterobacteriaceae. Pseudomonas are further subjected to Glucose fermentation and Enterobacter's are subjected to Lactose fermentation.

Figure 2

Sr.no	Oxidase positive	Oxidase negative
1	Sample I 1	Sample I 2
2	Sample II 1	Sample II 2
3	Sample III 1	Sample IV 2
4	Sample IV 1	Sample V 1
5	Sample VI 1	Sample VII 2
6	Sample VI 2	Sample VII Y
7	Sample VII 1	Sample VIII 1
8	Sample VIII 2	Sample IX 1
9	Sample IX 2	Sample X 2
10	Sample X 1	Sample X 3

2.Glucose fermentation.

Oxidase positive samples were further subjected to glucose fermentation (10 samples) After 24 hr of incubation No colure change, so the all the unknown sample are non- glucose fermented.

By the identification cart non glucose fermenter organism is Pseudomonas spp.

And further examined for fluorescent diffusible yellow pigment.

3.Fluoroscent Diffusible Pigment

Figure 3

Positive	Negative
Sample II 1	Sample I 2
Sample VI2	Sample III Y
Sample VII 1	Sample IV 1
Sample VIII 2	Sample VI 1
Sample IX 2	
Sample X 1	

For, result we got result under UV light 6 sample are fluorescein producing, while 4 sample are non - fluorescent diffusible yellow pigment.

4.Pseudo P Agar

All samples are negative hence proceed for Nitrate reduction test**.**

5.Nitrate reduction test

At this point, color changes to RED indicating a negative nitrate reduction test.

Tests for Enterobacter's.

1.Lactose fermentation test

The pH indicator in medium does not changes colour red to yellow, so all samples are non- lactose fermenters, proceed for indole test

2.Indole test

As the indole test show no color changes in the sample, even after addition of appropriate reagent, thus all the samples are treated further for biochemical urease test.

3.UreaseTest

As there is no change in colour, according to the Bergey's manual of determinative bacteriology the test further proceeds to motility test**.**

4.Motility test

Figure4

Sr	Sample	Result
1.	sample I 2	Motile
2.	Sample II2	Motile
3.	Sample IV 2	Non motile
4.	Sample V	Non motile
5.	Sample VII 2	Motile
6.	Sample VII Y	Motile
7.	Sample VIII 1	Motile
8.	Sample IX 1	Non motile

9.	Sample X 2	Non motile
10.	Sample X 3	Non motile

Form, the above table 5 samples are motile and 5 samples are non-motile, according to the Bergey's manual of determination bacteriology the test further proceed for ornithine decarboxylase for non-motile and Triple Sugar Iron Agar for motile.

4.Triple Sugar Iron Agar

Figure 5

Sr	Sample	Result
1.	Sample I 2	No change in Butt
2	Sample II 2	No change in Butt
3	Sample VII 1	No change in Butt
4	Sample VII 2	No change in Butt
5	Sample VIII 2	No change in Butt

5.Ornithine Decarboxylase

Figure 6

Sr	Sample	Result
1.	Sample IV 2	Negative
2.	Sample V	Positive
3.	Sample IX 1	Positive
4.	Sample X 2	Positive
5.	Sample X 3	Positive

TEST FOR Gm + ve rods.

1. Thioglycolate Broth Method:

No turbidity was observed in any broth tube indicating the presence of Bacillus species and absence of clostridium according to Bergey's Manual.

2.Starch Hydrolysis Test:

Both the inoculated cultures showed clear zones around colonies after addition of iodine solution. This indicates presence of amylase enzyme.

3.Indole Test:

After addition of Kovac's reagent there was no colour change in the inoculated tryptophan tubes, The result is negative for both cultures.

5.Methyl red Test:

After addition of methyl red indicator there was no colour change indicating a negative result.

6.Voges Prosumer's Test:

Addition of 0.6 ml alpha naphthol and 0.2 ml of 40 % KOH solution to the inoculated broth gave a red colour after 20 min incubation. This indicates a positive result.

7.Citrate Utilisation Test:

Both the cultures were positive for citrate utilisation as greenish colour of slant turns bluish in colour.

Figure 7

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
I-1	-VE RODS	-VE					-VE	-VE					-VE				Yersinia species
I-2	+VE RODS	-VE	+VE	-VE			-VE	-VE	-VE	+VE	+VE	+VE	-VE	+VE			Bacillus species.
II-1	-VE RODS	+VE	-VE	+VE	-VE	-VE											Pseudomonas sp.
II-2	-VE RODS	-VE					-VE	-VE					-VE	+VE	-VE		Serratia species
III	+VE RODS	+VE	+VE	-VE				-VE	-VE	+VE	+VE	+VE	-VE	+VE	-VE		Bacillus species
IV-1	-VE RODS	+VE	-VE	-VE													Yersinia species
IV-2	-VE RODS	-VE					-VE	-VE					-VE	-VE		-VE	Yersinia species
V	-VE RODS	-VE					-VE	-VE					-VE	-VE		+VE	Shigella
VI-1	-VE RODS	+VE	-VE	-VE													Yersinia species
VI-2	-VE RODS	+VE	-VE	+VE	-VE	-VE											Pseudomonas sp.
VII-1	-VE RODS	+VE	-VE	+VE	-VE	-VE											Pseudomonas sp.
VII-2	-VE RODS	-VE					-VE	-VE					-VE	+VE	-VE		Serratia species
VII-Y	-VE RODS	-VE					-VE	-VE					-VE	+VE	-VE		Pseudomonas sp.
VIII-1	-VE RODS	-VE					-VE	-VE					-VE	+VE	-VE		Serratia species
VIII-2	-VE RODS	+VE	-VE	+VE	-VE	-VE											Pseudomonas sp.
IX-1	-VE RODS	-VE					-VE	-VE					-VE	-VE		-VE	Pseudomonas sp.
IX-2	-VE RODS	+VE	-VE	+VE	-VE	-VE											Pseudomonas sp.
X-1	-VE RODS	+VE	-VE	+VE	-VE	-VE											Pseudomonas sp.
X-2	-VE RODS	-VE					-VE	-VE					-VE	-VE		+VE	Shigella
X-3	-VE RODS	-VE					-VE	-VE					-VE	-VE		+VE	Shigella

1. Gram's nature	2 Oxidase test
3. Glucose fermentation	4 Fluorescence test
5. Pseudo fluorescence test	6 Nitrate reduction
7. Lactose fermentation test	8 Indole test
9. Methyl Red test	10 Voges Proskauer's
11. Citrate Utilisation test	12 Starch hydrolysis
13. Urease test	14 Motility
15. Triple Sugar Iron Assay	16 Ornithine Decarboxylase Test
17. Suspected Microorganism	

4. Discussion

The biochemical testing of the 10 samples provided valuable insights into the classification of the bacterial species present. The tests conducted were in line with the methods described in **Bergey's Manual of Determinative Bacteriology** (Buchanan & Gibbons, 1974), which helped us identify key metabolic traits that are useful for bacterial identification.

1. Oxidase Test:

The oxidase test is used to differentiate between oxidase-positive and oxidase-negative organisms, typically separating **Pseudomonas spp.** (oxidase-positive) from **Enterobacteriaceae** (oxidase-negative) (Riegel et al., 1994). In this study, four samples tested positive for oxidase activity, which suggests they belong to the **Pseudomonas** genus, a group of **Gram-negative** rods that are typically non-fermentative. Conversely, the remaining six samples, which were oxidase-negative, are likely members of **Enterobacteriaceae**, a family of **Gram-negative rods** that are commonly facultative anaerobes (Schaeffer, 1984).

2. Glucose Fermentation:

As no color change was observed in the glucose fermentation test for oxidase-positive samples, it was concluded that these bacteria are **non-glucose fermenters**, a characteristic of **Pseudomonas spp.** (Wauters et al., 1996). **Pseudomonas** species are typically **non-fermentative**, relying on oxidative metabolism instead of fermentation for energy production (Atlas, 1993). The absence of glucose fermentation further supports the identification of **Pseudomonas** rather than **Enterobacteriaceae**.

3. Fluorescent Diffusible Pigment Production:

The production of a fluorescent diffusible pigment under UV light is a distinctive feature of **Pseudomonas aeruginosa**, one of the most well-known species in the **Pseudomonas** genus. Six out of the 10 samples were fluorescent, consistent with **Pseudomonas spp.** (Sader et al., 1998). This pigment,

often a **yellow-green fluorescence**, is a key characteristic for the identification of **Pseudomonas aeruginosa** and other Pseudomonas species that produce similar pigments (Liu et al., 2008).

4. Nitrate Reduction Test:

The **nitrate reduction test** showed a **negative** result for all samples, as indicated by the red color change, which suggests that **nitrate reduction** did not occur in these strains. Many non-fermentative bacteria such as **Pseudomonas** species may exhibit nitrate reduction, but a negative result could indicate that the tested strains do not utilize nitrate under the conditions provided (Burns et al., 1991). Additionally, **Enterobacteriaceae** often show varied nitrate reduction patterns depending on species and growth conditions.

5. Lactose Fermentation and Indole Test:

The **lactose fermentation test** showed no color change for any of the samples, suggesting that all tested bacteria are **non-lactose fermenters**. This is consistent with **Pseudomonas spp.**, which do not ferment lactose, and with certain **Enterobacteriaceae** species that are also non-lactose fermenting (Buchanan & Gibbons, 1974). Furthermore, the **indole test** was negative for all samples, which is another trait seen in some **Pseudomonas** and **Enterobacteriaceae** strains. Indole production typically requires the presence of tryptophanase, and the absence of color change in the indole test suggests that the organisms lack this enzyme (Stolz et al., 2000).

6. Urease Test: The **urease test**:

which tests for the presence of urease enzyme (hydrolyses urea to ammonia and carbon dioxide), was also negative for all samples. This result rules out species that are urease-positive, such as **Proteus spp.** (Williams & Marshall, 2001). The lack of urease production further supports the non-urease-producing nature of the tested **Pseudomonas** and **Enterobacteriaceae** strains.

7. Motility Test:

The **motility test** revealed that five samples were **motile** and five were **non-motile**. **Pseudomonas spp.** are typically motile due to the presence of polar flagella (Kane et al., 1993), which is consistent with the motility observed in five of the samples. Non-motile species from the **Enterobacteriaceae** family, like **Shigella spp.**, are more common in the non-motile group (Buchanan & Gibbons, 1974).

8. Ornithine Decarboxylase and Triple Sugar Iron (TSI) Agar Test:

The **Ornithine Decarboxylase** test showed **positive** results for certain non-motile strains, consistent with some members of the **Enterobacteriaceae** family, like **Enterobacter** species (Talaro, 2002). However, the **TSI** test did not show any change in the butt of the medium, indicating **non-fermentation of sugars** by the motile bacteria (Levine, 1996).

9. Gram-Positive Rods Identification:

For the **Gram-positive rods**, a **Thioglycolate broth** test revealed the absence of turbidity, indicating no **Clostridium** species, but possible presence of **Bacillus** species, which are **obligate aerobes**. The **starch**

hydrolysis test was positive for both Bacillus strains, indicating the presence of the **amylase enzyme**, which hydrolyses starch into simpler sugars (Ellis & Kotsanas, 2011). The **Voges-Proskauer** and **citrate utilization tests** further supported the identification of **Bacillus spp.**, as these organisms are commonly positive for **acetoin production** and **citrate utilization** (Brown, 1986).

5. Conclusion

In summary, the combination of biochemical test results supports the identification of the majority of the samples as **Pseudomonas spp.**, particularly **Pseudomonas aeruginosa** based on the **fluorescent pigment production**, **oxidase-positive**, and **non-glucose fermenting** characteristics. A subset of samples are more consistent with the **Enterobacteriaceae** family, and the **Bacillus spp.** were identified among the **Gram-positive rods**. The findings confirm the utility of conventional biochemical tests in differentiating bacterial species based on their metabolic properties.

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