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Isolation and Characterization of Naphthalene Degrading Soil Bacteria

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Abstract

Naphthalene is a type of poly aromatic hydrocarbon that is associated with environmental pollution. Microorganisms are capable of degrading such pollutants because of the metabolic system mainly consisting of the enzymes present in them. Certain bacteria are very efficient to degrade such pollutants and hence this study focuses on isolation of such bacteria and using the same to degrade the environmental pollutant naphthalene. The soil samples were collected from oil contaminated sites and potential bacteria were isolated. These isolates were further characterized upto the genus level using morphological and biochemical tests. In the present study the isolates capable of degrading naphthalene were identified as Acinetobacter sps. (I), Pseudomonas sps.(II), Staphylococcus sps. (III), Bacillus sps. (IV), Alcaligenes sps. (V), Arthrobacter sps. (IV), Micrococcus sps. (VII), Janibacter sps. (VIII), Clostridium sps. (IX) and Pseudomonas sps. (X).

Keywords: Naphthalene Degrading Bacteria, Naphthalene, Bioremediation, Oil Contaminated Sites, Poly Aromatic Hydrocarbon (Pah), *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Arthrobacter*, *Micrococcus*, *Janibacter*, *Clostridium*.

Introduction

Hydrocarbons are broadly classified into two types: aliphatic hydrocarbons and aromatic hydrocarbons. The main structural unit in aromatic compounds is the benzene (C_6H_6) ring. The term Poly Aromatic Hydrocarbons (PAH's) refers to hydrocarbons containing two or more fused benzene rings in linear, angular or clustered arrangements[5]. PAH's are unique environmental contaminants since they enter the environment by incomplete combustion of organic matter. PAH contaminated sites are mostly found in or around cities and are responsible for a number of public health hazards[1]. PAH's are commonly found in areas surrounding petroleum-refining plants, accidental oil spills, pipe leakages and rainwater runoff from roadways. PAH pollution takes place naturally from volcanic eruptions and more recently via man's activities[3]. Naphthalene is one of the hydrocarbon particularly common at oil contaminated sites[2] and is known to enter the environment mostly because of anthropogenic activities such as through industries, burning of organic material, petroleum and wood. Many microbes isolated are capable of degrading naphthalene. Bacteria belonging to different genera have been found useful in the degradation of PAH's including species of *Pseudomonas*[3,4], *Mycobacterium*[3,5], *Micrococcus*[4], *Rhodococcus*[3] etc. Many fungal species are also known to degrade PAH's[3]. Application of such bacteria and fungi to degradation of compounds of PAH's provides an eco-friendly method of eliminating pollutants. Therefore we refer to this process as a Bioremediation, defined as the intentional use of microbes for biodegradation of contaminants to eliminate the environmental pollutants from sites where they have been released. Bioremediation makes use of microorganisms, to detoxify environmental pollutants and transform them to less toxic compounds.

Materials and Methods

Materials: Soil sample from oil contaminated sites, all other requirements for experimental work – such as glassware and basic instruments were obtained from the Biotechnology laboratory. All sterile requirements were kept ready by autoclaving the same at 121˚C for 20 minutes followed by cooling the same prior to commencement of the experiment.

Methods: Soil sample was collected from petrol pump from Vasco – Goa. The composite sample was aseptically collected and brought to the laboratory to be used for further experimentation.

Screening potential naphthalene degrading bacteria: Serial dilution and agar plating technique was used for isolation and screening.

Using solid M9 media with naphthalene: After serial dilution, dilutions 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were plated onto M9 media + naphthalene. The plates were incubated at 37˚C for one week to permit growth of bacteria.

Using liquid M9 media with naphthalene: 1g of the soil sample was taken into a flask containing sterile 100 mL of sterile distilled water to prepare soil suspensions. 0.1mL of the above was then added into sterile 100mL of M9 medium supplemented with 0.1mL of naphthalene (dissolved in ethanol).

The flask was kept on the shaker at 120RPM at 30˚C for one week. After one week of shaker condition, 0.1mL of culture suspension was spread plated on M9 medium supplemented with naphthalene. The plates were incubated at 37˚C for one week to permit growth of bacteria.

Confirming ability to degrade naphthalene: The colonies obtained in both cases namely using solid media and using liquid media were screened on the basis of their potential to degrade different concentrations of naphthalene[1]. Plates of sterile M9 medium supplemented with different concentrations of naphthalene (1mL, 2mL, 3mL, 4mL and 5mL) were prepared. Different colonies obtained from the previous step were streaked on these plates. These plates were incubated at 37˚C for one week to permit growth of bacteria.

Obtaining pure cultures: Ten bacterial colonies capable of naphthalene degradation were obtained from the previous step and pure culture stock of each isolate was maintained onto plates and slants containing M9 medium supplemented with naphthalene. The plates and slants were incubated at 37˚C for 48 hours for growth. The pure cultures obtained were stored in the refrigerator and maintained as stock cultures.

Characterization of the naphthalene degrading bacteria (Genus level): The pure culture stock in each case was activated by growing a loopful of the bacteria (each isolate) into 100mL of sterile liquid nutrient medium overnight. The activated culture in each case was used for the various tests in the characterization of bacteria.

Characterization of bacteria based on colony characteristics: Each isolate was obtained on sterile solid media and the characteristics were observed and are tabulated in Table-1.

Characterization of bacteria based on organism characteristics: The organism characteristics studied in each case were Gram's character, motility and shape. The results for each were observed and are tabulated in Table-2.

Characterization of bacteria based on staining characteristics: The staining characteristics studied were simple, negative, acid-fast, capsule and endospore staining. The results for each were observed and are tabulated in Table-3.

Characterization of bacteria based on biochemical tests: The various biochemical tests studied for characterization of the bacteria were Indole, Methyl red, Voges–Proskauer, citrate utilization, mannitol, lactose, nitrate reduction test, hydrogen sulphide, gelatin hydrolysis, Hugh-Leifson, haemolytic, amylase, protease, cellulose, lipase, catalase, urease and oxidase tests. The results for each were observed and are tabulated in Table-4.

Characterization of bacteria based on sugar fermentation tests: The various sugars used in the study of fermentation tests for characterization of the bacteria were fructose, xylose, glucose, sucrose, maltose and galactose.

The results for each were observed and are tabulated in Table-5.

Table-1
Colony Characteristics of the obtained ten isolates

Isolates	Colony Characteristics						
	Size	**Shape**	**Colour**	**Margin**	**Elevation**	**Consistency**	**Opacity**
I	Small	Round	Off-white	Entire	Convex	Dry	Opaque
II	Medium	Round	Light Brown	Entire	Convex	Butyrous	Opaque
III	Small	Filiform	White	Filiform	Flat	Dry	Opaque
IV	Small	Round	Orange	Entire	Convex	Butyrous	Opaque
V	Medium	Irregular	Light Brown	Wavy	Convex	Butyrous	Opaque
VI	Small	Filiform	White	Filiform	Flat	Dry	Opaque
VII	Small	Round	Off-white	Entire	Flat	Dry	Opaque
VIII	Small	Round	White	Entire	Flat	Dry	Opaque
IX	Medium	Filiform	Brown	Filiform	Flat	Dry	Tranlucent
X	Medium	Round	Brown	Entire	Convex	Butyrous	Opaque

Results and Discussion

Soil sample was obtained from oil contaminated sites at Vasco, Goa. The soil was used to isolate bacteria that have the potential to degrade naphthalene. Samples containing such bacteria were also obtained in a similar manner by Aislabie *et al*[2]. Screening for potential naphthalene degrading bacteria was done using M9 medium supplemented with naphthalene as done in the studies by Gupta[3]. The ability of the isolates to degrade naphthalene was confirmed by growing the isolates in different concentrations of naphthalene, the method was adopted and improved from studies of Aislabie[2]. Ten isolates with such ability were selected. Pure cultures of the ten isolates were maintained as stock cultures. The isolates were characterized using morphological characterization tests such as Gram character, motility and staining techniques, as well as by using a series of biochemical tests as done in the study by Khan *et al*[6], the results for each are shown in separate tables below, (Table-1 - Colony characteristics), (Table-2 - Gram character, motility and shape of isolates), (Table-3 - Staining characteristics), (Table-4 - Biochemical test results) and (Table-6 - Results of sugar fermentation tests). The 10 isolates were identified as: *Acinetobacter* sps. (I), *Pseudomonas* sps. (II), *Staphylococcus* sps. (III), *Bacillus* sps. (IV), *Alcaligenes* sps. (V), *Arthrobacter* sps. (IV), *Micrococcus* sps. (VII), *Janibacter* sps. (VIII), *Clostridium* sps. (IX) and *Pseudomonas* sps. (X).

Table-2
Gram character, motility and shape of isolates

Isolates	Gram Staining		Motility Test	
	Gram Character	Cell shape	Hanging-drop	Soft agar stab
I	-	cocci	Non- motile	Non- motile
II	-	bacilli	Motile	Motile
III	-	cocci	Non- motile	Non- motile
IV	+	bacilli	Non- motile	Non- motile
V	-	bacilli	Motile	Motile
VI	-	bacilli	Non- motile	Non- motile
VII	+	cocci	Non- motile	Non- motile
VIII	+	bacilli	Non- motile	Non- motile
IX	+	bacilli	Non- motile	Non- motile
X	-	bacilli	Motile	Motile

+ is a positive result, - is a negative result

Table-3
Staining characteristics results of the isolates

Isolates	Simple staining	Negative staining	Acid- fast staining	Capsule staining	Endospore staining
I	Cocci	cocci	+	-	+
II	bacilli	bacilli	-	-	-
III	Cocci	cocci	+	+	-
IV	bacilli	bacilli	+	-	+
V	bacilli	bacilli	-	-	-
VI	bacilli	bacilli	+	+	-
VII	Cocci	cocci	-	-	+
VIII	bacilli	bacilli	+	+	-
IX	bacilli	bacilli	+	+	+
X	bacilli	bacilli	+	+	-

+ is a positive result, - is a negative result

Table-4
Biochemical test results of the various isolates

Biochemical Tests	Isolates									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Indole test	-	-	-	-	-	-	-	+	-	-
Methyl Red test	-	-	-	-	-	-	+	+	-	-
VP test	-	-	-	-	-	-	+	+	-	-
Citrate utilization	+	+	+	-	+	+	-	+	-	+
Mannitol test	-	-	+	+	-	+	+	-	+	+
Lactose test	-	-	+	-	+	-	+	+	+	-
Nitrate reduction test	-	+	+	-	+	-	-	+	+	+
Hydrogen sulphide test	-	-	-	-	-	-	+	-	+	-
Gelatin hydrolysis	-	-	-	-	-	-	+	+	-	+
Hugh-Leifson test	+	+	-	+	+	+	+	+	+	+
Haemolytic test	+	+	-	-	+	-	+	+	-	+
Amylase test	+	+	+	-	-	+	-	+	-	-
Protease test	-	+	-	+	-	-	+	+	-	+
Cellulase test	-	+	-	+	-	+	+	+	+	+
Lipase test	-	+	+	-	+	-	-	-	-	-
Catalase test	+	+	+	-	+	+	+	+	-	+
Urease test	-	-	+	-	-	-	+	-	-	-
Oxidase test	-	+	-	-	+	-	+	+	-	+

+ is a positive result, - is a negative result

Table-5
Results of sugar fermentation tests

Isolates	Fructose		Xylose		Glucose		Sucrose		Maltose		Galactose	
	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid
I	-	+	+	+	-	+	-	+	-	+	-	+
II	+	+	+	+	+	+	+	+	+	+	+	+
III	+	+	+	+	-	+	-	+	+	+	-	-
IV	-	+	-	+	-	+	-	+	+	+	-	+
V	-	-	+	+	+	+	+	+	+	+	+	+
VI	-	+	-	+	-	+	+	+	-	+	-	+
VII	+	+	+	-	-	+	-	-	-	-	-	-
VIII	-	-	-	-	-	-	-	+	-	-	-	-
IX	-	-	-	-	+	-	-	-	+	-	-	-
X	-	+	-	+	-	+	+	+	-	+	-	+

+ is a positive result, - is a negative result

Conclusion

The main objectives of this study were: To collect soil samples from oil contaminated sites for screening of potential naphthalene degrading bacteria. To isolate pure cultures with naphthalene degrading ability and to maintain them. To identify the isolates on the basis of morphological, biochemical and physiological tests.

All these objectives were successfully completed within a period of six months. Knowledge about such bacteria can allow using the same in bioremediation processes. Further studies can include subjecting the bacteria to phylogenetic studies of the isolates using 16S r(RNA), PCR and sequencing, calculation of biodegradation efficiency of the isolates, comparative study of enzymes produced by the isolates and studying the ability of these isolates in the degradation of other organic contaminants.

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