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Biological Degradation of Naphthalene: A New Era

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Abstract

Naphthalene is a simplest Polycyclic Aromatic Hydrocarbon (PAH). PAHs are major contaminants of environment, associated with common anthropogenic activities such as oil refineries and incomplete combustion of fossil fuels. PAHs are toxic, mutagenic and carcinogenic. Isolation of naphthalene degrading bacteria is recommended using a complex ONR 7a medium. Present work includes a modified medium with Naphthalene as a sole source of carbon. Four isolates were screened from marine sample collected from Mumbai as well as petroleum soil sample from Trimbak road Satpur, Nashik. Further characterization using morphological and biochemical tests showed resemblance with Gram positive bacteria as well as Gram negative bacteria, belonging to genus such as *Micrococcus spp*, *Bacillus spp, Staphylococcus spp*, *Pseudomonas spp.* These strains were further grown in modified broth for 45 days as well as on ONR 7a agar medium. In turbidometric assay *Bacillus spp* showed significant growth at 1 mg/ml of naphthalene concentration. Catechol which is a intermediate product which generated through biodegradation of naphthalene was detected by Winkelmann modified Arnow's method. All four isolates efficiently degraded naphthalene which was confirmed by Arnow's test. These naphthalene degraders could be further checked and explored for their efficiency in bioremediation of polluted marine environment and in oil contaminated fields.

Keywords: Naphthalene; ONR 7a; *Bacillus;* Bioremediation; Catechol

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed contaminants in diverse environments because of their common association with many anthropogenic activities such as petroleum refining and incomplete combustion of fossil fuels[1]. PAH bioremediation is considered an effective and environmentally benign cleanup technology as it involves the partial or complete bioconversion of these pollutants to microbial biomass, carbon dioxide and water[1]. A successful bioremediation strategy will require an in-depth understanding the factors that influence the biodegradation process and the ecology of pollutant-degrading bacteria[1].Naphthalene, the simplest PAH, has long been used as a model compound in PAH bioremediation studies. Common naphthalene-degrading bacteria include *Pseudomonas* spp, *Vibrio* spp, *Mycobacterium* spp,*Marinobacter spp*, and *Sphingomonas spp*. *Micrococcus spp* [2]. Although many naphthalene-degrading bacteria have been isolated, these bacteria may thrive in one environment but may not be able to compete with other micro-organisms in another environment, as environmental conditions impose a selection pressure on specific types of bacteria. PAH are soluble in non-polar organic solvents. Thus, the existence of these pollutants in aquatic environments is very toxic and dangerous for humans and other creatures, it is also harmful for useful microorganisms of plants in contaminated soils. Since, the oil compounds are very resistant to evaporation, due to having aromatics derivatives in their structures, they can remain in the environment for a long time; however, the microbial population in contaminated sites results in an increase in the degradation rate of these stable compounds. Recently, many physical-chemical and biological methods have been used to clean up the contaminated sites. But these methods are not economical, or they lead to the formation of other toxic compounds in the environment. Therefore, a bioremediation method is considered as an economical and safe approach for the environment. Bioremediation is the use of microorganisms to remove environmental contamination. This method is used for harmful toxic substances and also for oil contamination cleanup. Bacteria convert the pollutant organic compounds into less harmful compounds by aerobic and anaerobic respiratory reactions, fermentation, co metabolism, and dehalogenation; using them as the only source of carbon and energy. Furthermore, indigenous bacteria have been shown to out compete artificially introduced strains in several bioremediation investigations [3]. Therefore, implementation of a successful bioremediation strategy should necessitate a detailed evaluation of the roles of the indigenous bacteria [4]. This study describes the isolation and characterization of three different strains of naphthalene-degrading bacteria obtained from oil-contaminated sea water due to oil spillage and one isolate from petroleum soil.

Materials and Method

Isolation procedure of halophilic naphthalene degraders

Sea water contaminated with marine fuel oil (Mumbai) as well as nearby petrol pump[5] (Trimbak Road) were aseptically collected and stored at –4°C for one month before use. ONR 7a [6] & modified Halophile moderate media were used for isolating naphthalene-degrading bacteria[7]. The direct isolation method and the enrichment isolation method were performed at 25°C to 37°C. Isolates were screened to select for bacteria that can grow rapidly on ONR 7a agar plate with naphthalene as sole carbon source. Four of the isolates exhibited relatively faster growth rates than the rest and were picked and chosen for further study [6]. To select the best and strongest degrading strains, the polycyclic aromatic hydrocarbons (PAHs) and the isolated bacteria with the desired substrate for concentration range were cultured in mineral culture base(ONR7a). Bacteria that started growing fast and had a high turbidity in the vicinity of the

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aromatic compounds were selected as appropriate microbial strains. Naphthalene is difficult to be used in its crystal structure, as it cannot be dissolved directly in broth or agar so a special treatment has to be given. To prepare stock solution of naphthalene that can be added, an excess of naphthalene was transferred to bottle containing Methylene dichloride, and Methylene dichloride was removed by evaporation [1]. After that it was introduced in agar and broth in sufficient quantity.

Media

An artificial sea water mineral salts medium (ONR7a) based on the ionic composition of sea water was used in this study. This medium contained all of the major cations and anions that are present at concentrations greater than 1 mg/liter in sea water. Nitrogen was provided in the form of NH_4Cl, and phosphorous was provided in the form of Na_2HPO_4, ONR7a contained (per liter of distilled or deionized water) 22.79 g of NaCl,11.18g of $MgCl_2$,$6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2 2H_2O$, 1.3g of TAPS0{**3**-[N-tris(hydroxymethyl) methylamino]-2-hydroxypropanesulfonicacid}**,** 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HPO_4.7H_2O$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27 mg of H_3BO_3, 24 mg of $SrCI_2.6H_2O$, 2.6 mg of NaF, and 2.0 mg of $FeCl_2.4H_2O$,naphthalene 10mg/ml. To prevent precipitation of ONR7a during autoclaving, three separate solutions were prepared and then mixed together after autoclaving when the solutions had cooled to at least 50°C; one solution contained NaCI, Na_2SO_4, KCl, NaBr, $NaHCO_3$, H_3BO_3, NaF, NH_4Cl, Na_2HPO_4, and TAPSO (pH adjusted to 7.6 with NaOH), the second solution contained $MgCl_2$, $CaCl_2$, and $SrCI_2$, (divalent cation salts), and the third solution contained $FeCl_2$. For solid media, agarose was added to the first solution.

Other media were also tried for naphthalene degradation such as Minimal medium and Bushnell Hass medium.

Modified Halophile Moderate medium (mHM)

ONR 7a is very complex and rare medium, so to tackle with this problem a new medium was devised and named it as modified Halophile Moderate medium (mHM). The carbon source from the original halophile moderate medium i.e. glucose and yeast was substituted with naphthalene as sole carbon source. This medium was used for further inoculation and incubation.

Sea water was inoculated on mHM agar plates as well as mHM broth for 30 days at 37°c.The colonies obtained were further used by subculturing in mHM broth for 30 days again at alternate shaking conditions at room temperature and at 37°c.

Morphological and biochemical characterization

Gram staining was performed for all the colonies from the plates incubated as well as of the broth. Growth was confirmed by colony formation on agar plates containing the naphthalene as sole carbon source and compared with control plates without the naphthalene as substrate. Various other biochemical tests[8-10] were performed such as catalse, oxidase, glucose utilization, amylase ,and turbidometric [2] assays for naphthalene utilization at different concentrations ranging from 1mg/ml , 2mg/ml , 3mg/ml , 4mg/ml and 5mg/ml. All the tubes were kept on shaker and results were recorded at 615nm wavelength by comparing with the control (table 1).

Determination of the time course for growth

Biodegradation ability of Microorganisms were studied using 10mg/ml concentrations of naphthalene in to the 200 ml mineral base medium(ONR7a) of Erlenmeyer flasks whose heads were covered with cotton Each of the flasks were inoculated with isolated strains and incubated at 30° C on shaker. [10] Within 24 hours, 5.0 ml sample was collected from each flask and assayed for OD at 600 nm in a UV spectrophotometer. (Structure 1).

Determination of naphthalene degradation efficiency of isolates

Naphthalene degrading [11] ability of isolates were monitored by the spectrophotometric assay.ONR 7a media were prepared containing 10mg/ml of naphthalene as sole source of carbon. 1 ml cultures (24hrs) from enriched broth of isolates were used. Ability of each isolates to degrade naphthalene was monitored spectrophotometrically by taking absorbance at 365 nm against uninoculated media as a control. (Structure 2).

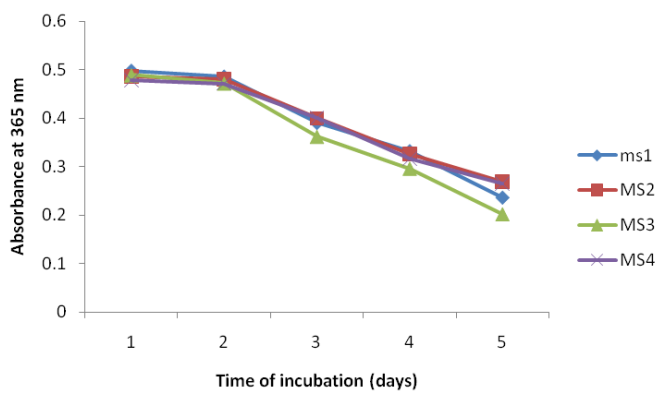
Correlation of naphthalene degradation and growth of isolates

Efficiency of isolated organism to degrade naphthalene and its relation with the growth was studied [10,12] by estimating the residual naphthalene after 24 hours of interval. To estimate the residual naphthalene after time course standard graph were prepared using 1 to 10 mg /ml of naphthalene, while extrapolating the value on standard graph after specific time interval residual naphthalene concentration was estimated and same suspensions were observed for the growth reading at 600nm.while comparing the data generated it was found that MS3shows significant growth as well as efficiency of naphthalene degradation (Structure 3 & Structure 4).

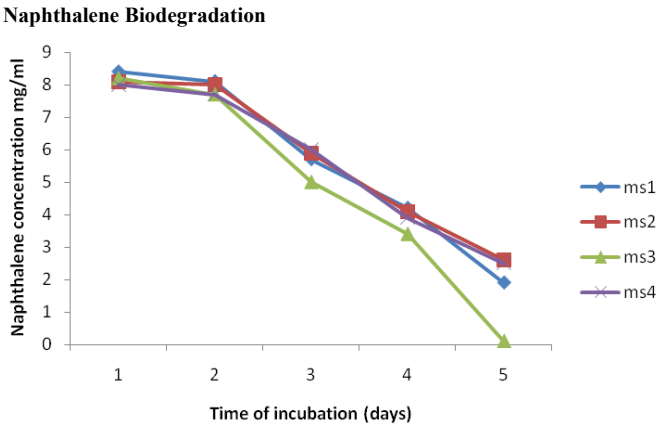
Test	Isolate I	Isolate II	Isolate-III	Isolate-IV
Shape	circular	Blunt end rods	Circular	Circular
Gram character	Gram positive cocci	Gram negative short rods	Gram positive cocci	Gram positive cocci
Amylase	–	–	+	–
Glucose utilization	+	+	+	+
Gelatinase	–	–	+	+
β-Galactosidase	+	+	–	+
Catalase	+	+	+	+

('+'Positive test, '-' negative test)
Table no. 1: Biochemical characterization of isolates

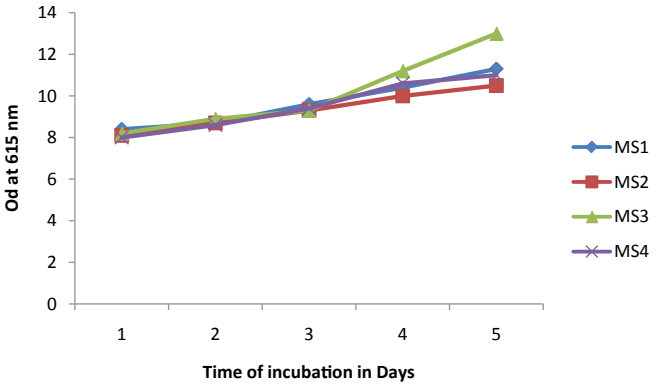
Table 1: Morphological and biochemical characterization.



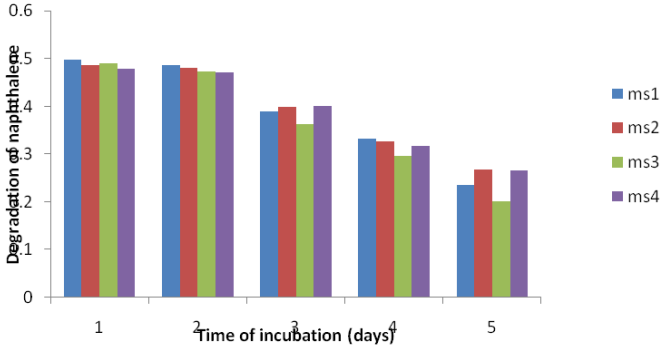
Structure 1: Time course of isolates to degrade naphthalene (absorbance at365nm against time of incubation).



Structure 2: Biodegradation of naphthalene (concentration of naphthalenemg/ml against time of incubation).



Structure 3: Biodegradtion of naphthalene isolate 3 (MS 3) showed significant growth at 1 mg/ml of naphthalene concentration. OD at 615 nm.



Structure 4: Efficiency of isolate to degrade naphthalene in specific period of time((residual naphthalene concentration mg/ml against time of incubation).

Aromatic compound degradation

To study the ability of the isolates to degrade aromatic compounds [13] (phenol, disel, toluene, and xylene) was added to mineral salt medium. Growth of isolates in this media was monitored turbidometrically at wavelength λ365nm.MSM media was supplemented with 1% of test compounds (Structure 5).

Confirmatory test to detect naphthalene degradation

Arnow's method [4] modified by winkelman [4] was used for the detection of catechol generated through degradation of naphthalene [14] (Figure 1).

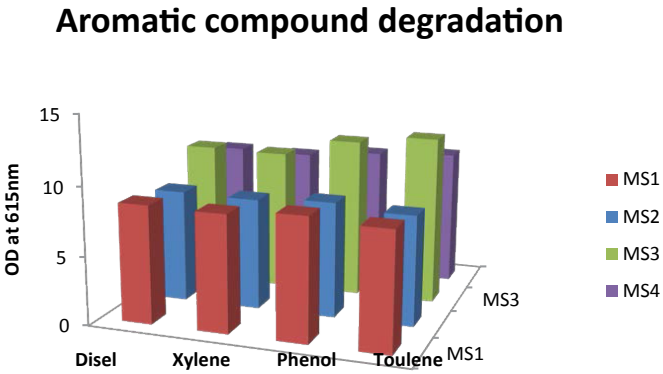
Bioremediation of petrol soil and plant growth promotion

Soil sample from the contaminated region of petroleum industry were collected (Indian oil Manmad). 5Kg of soil was filled in the containers containing soybean seeds and were inoculated along with the 50 ml enriched broth containing naphthalene degrading culture. Along with the normal soil and soya bean seeds as positive and petroleum soil **[15]** and soya bean seed as a negative control. Growth of soya bean crop was monitored in terms of height of plant, nodule formulation, chlorophyll content after 45 days of inoculation

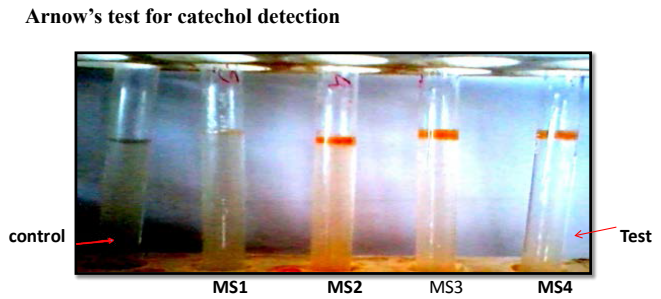
Results

Enrichment and isolation

After the enrichment and culturing the colonies obtained showed different variety of organism like Gram positive bacteria, Gram negative and few filamentous bacteria which may belong to genus *Aquafaciens, Proteobacteria, Spirochetes*. A total of four naphthalene-degrading bacterial strains were isolated from oil-contaminated sea water sample. These are designated as MS1, MS2, MS3 and MS4. These isolates are chosen for further study, since it exhibited relatively fast growth rates on ONR 7a agar plates fed with naphthalene as sole carbon source.



Structure 5: Aromatic compound degradation.



• Arnow's method modified by winkelmann was used for the detection of catechol generated through degradation of naphthalen
• Yellowcolour indicates positive catechol test

Figure 1: Confirmatory test to detect naphthalelne degradation.

Discussion

Bioaugmentation is a common bioremediation strategy that involves introducing indigenous Microorganisms to the contaminated site or bioreactor to detoxify and degrade environmental contaminants. Several successful bioaugmentation cases have been documented[7]. Bioaugmentation result in a significant increase of the hydrocarbon biodegradation in a bench reactor for treatment of oil-contaminated waste water [8] Application of carvone-induced indigenous bacteria in soil was the most effective treatment for mineralizing polychlorinated biphenyls (PCB). Indigenous microbial consortium to bioaugment the aerobic biodegradation of pentachlorophenol (PCP)-contaminated soil in a bioreactor has been used earlier [1]. The results indicated that the bioreactor was very effective in producing a PCP-acclimated biomass after bioaugmentation. These successful cases illustrate that bioaugmentation of indigenous bacteria is a feasible strategy of bioremediation. These isolates could be used further for bioaugmentation studies. Screening for relatively fast-growing naphthalene-degrading bacteria from oil-contaminated marine environment resulted in the recovery of four isolates. Although the isolation methods were unbiased and could select for both Gram-positive and Gram-negative bacteria, three candidate strains were Gram-positive and one is gram negative. This dominance of Gram positive bacteria is demonstrated in the high relative abundances. The dominance of Gram-positive bacteria should not be surprising. Gram positive bacteria have a stronger cell envelope than Gram negative bacteria and this allows them to thrive in the highly variable intertidal sediment environment, where sediment temperatures are high in the day and osmotic pressures and nutrient supply may change periodically over a daily cycle.

Many different species of bacteria with the ability to degrade naphthalene and other PAHs have been isolated, mostly from soil environments. The majority of the PAH degrading bacteria were previously found to belong to the genus *Pseudomonas*, and the PAH-degradative gene clusters in these bacteria were highly homologous to the naphthalene gene (nah gene) cluster from the NAH7 plasmid in *Pseudomonas putida* strain G7[15]. However, recent investigations of contaminated soils have uncovered naphthalene-degrading bacteria that did not hybridize with NAH7-derived gene probes [6], and indicate that there are still many unidentified bacteria with diverse PAH biodegradation pathways that involve hitherto undiscovered genes and gene clusters. The microbial communities in marine environments have generally been reported to be dominated by Gram-negative bacteria reported characterization of phenanthrene-degrading bacteria from San Diego Bay sediments that belonged to the genera *Vibrio, Marinobacter or Cycloclasticus, Pseudoalteromonas, Marinomonas,* and *Halomonas*[5]·However, there is little information on Gram-positive naphthalene-degrading bacteria in marine environments, although PAH-degrading bacteria belonging to the Gram-positive nocardioforms and spore-forming *Paenibacillus* groups have recently been isolated from the rhizosphere of salt marsh plants [7]. The four isolates reported in the current study extend our knowledge of the range of naphthalene-degrading bacteria found in marine environments. This work suggests that Gram-positive bacteria may play a key role in PAH degradation on contaminated tropical beaches.

Further, many different test could be carried out using available kits for API ZYM and API 20E [16] tests that can help in characterization and could be sent for 16 s rRNA sequencing for phylogenetic analysis as the isolates could be novel and can be of tremendous use in bioremediation studies near the shores of Mumbai as the sample was collected from there and also industrial effluents can be treated in waste water treatment.

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