Impact Factor 6.1

Journal of Cyber Security

ISSN:2096-1146

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ISOLATION AND CHARACTERIZATION OF NAPHTHALENE DEGRADING BACTERIA

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Abstract

Halogenated organic compounds and crude oil contains polyaromatics hydrocarbon (PAH) responsible for causing widespread pollution. The chief advantage of bioremediation is its reduced cost of remediation and is often a permanent solution. The present study was aimed to focus on the biological methods for the removal of hazardous PAHs employing to isolate naphthalene degrading bacteria from petroleum contaminated soil samples. Isolates were identified and characterized by morphological and biochemical test. *Pseudomonas (*60%*), Bacillus sp (*30%*).* and *Actinomycetes* (10%) were isolated from 10 samples*. P. aeruginosa* was chosen for further studies. *P. aeruginosa* showed fast growth rate with lag phase of 12-18 hours and achieved highest growth within 24 hours. The naphthalene degrading bacteria was able to grow on different concentration of naphthalene showing the degradation ability of the isolated bacteria. The results reveal that with increasing concentration of naphthalene growth of microorganisms also increased. Then, the rate of naphthalene biodegradation was dependent upon concentration. It was observed that maximum growth was observed with a naphthalene concentration of 200 ppm recommend that bacterial growth rates were better in higher concentration of naphthalene.

Introduction

The efficient degradation of polycyclic aromatic hydrocarbons can be preferably achieved using microorganisms. This process of biodegradation using microorganism has ingrained superiority like efficiency and cost effectiveness (Pothuluri and Cerigilia, 1994). The microorganisms have many enzymes responsible for degradation of PAHs. Previous exposure of bacteria to the PAH substances present in soil is important for the selection or adaptation of PAH-degrading microorganisms (Spain et al., 1980, Lewise et al., 1984). These adaptations occur at very slow

pace and directly depend upon the nature of substance involved for biodegradability (Spain et al., 1980). Recently, ecologists have discovered many bacterial and fungal species effective in degrading hydrocarbons from natural environments. Bacterial species isolated from sea coast heavily contaminated with hydrocarbons, have shown promising ability to degrade hydrocarbons. Owing to genotypic constituents, these bacteria have the potential to metabolize different aliphatic as well as aromatic hydrocarbons and their chlorinated derivates, bacterial and fungal species can be used for biodegradation process.

Petroleum hydrocarbons can be efficiently degraded through the oxidative metabolic activities of different strains of fungi, yeast, bacteria and microalgae (Bundy et al., 2004). Fungal species responsible for degradation of naphthalene includes *Phanerochaete chrysosporium, Aspergillus niger* , *Pleurotus ostreatus, Lentinula edodes, Candida* and *Y. lipolytica* (Annibale et al., 2004).

Various bacterial species belonging to different genera are known to have good hydrocarbon degrading potential. They tolerate high concentration of the hydrocarbons and have capacity for their degradation. Bacteria belonging to various genera like *Pseudomonas, Sphingomonas, Aeromonas, Alcaligenes, Xantomonas, Bacillus* (Atlas, 1984). Bacteria can efficiently degrade hydrocarbons because they have the potential to utilize hydrocarbons as the sole source of their growth and energy requirements. They can either utilize aerobic or anaerobic metabolic pathways utilizing oxygen and nitrate or sulfate as the primary electron acceptor respectively. Because aerobic pathway for degradation is a more rapid process and requires less free energy, it is considered more effective.

Pseudomonas belongs to proteobacteria of family Pseudomonasdacae. *Pseudomonas* is rod shaped gram negative bacteria characterized by the production of distinguishing water soluble pyocyanin pigment. *P. aeruginosa* can be often seen proliferating in distilled water, suggesting that it has elementary requirements of nutrition. Research reports that it can utilize about seventy-five organic compounds and can survive in the vey less nutrients (Nwankwo and Shuaibu 2010).

Naphthalene is an aromatic hydrocarbon having two benzene rings and has a characteristic odor pleasant odor (Klassan, 2001). It forms sublimable colorless crystals. It burns in air with the formation of much soot. Environmental Protection Agency (EPA) of US has declared

naphthalene as a primary irritant and toxic pollutant (EPA 1986, 1994), exposure to which can cause severe damage to normal functioning of human body. Because of high solubility, simple structure and a regular constituent of PAH contaminated environment, naphthalene is employed for assessing PAH metabolism by bacteria. Various physiochemical methods to degrade the petroleum contaminated site are economically not viable (Heitkamp et al., 1988), therefore, research now-a-days is diverted towards the use of microorganisms for degradation of these pollutants. The present study was aimed at isolating the *Pseudomonas* sp. that has the potential to degrade naphthalene with the following objectives of identification and characterization of isolates and to evaluate the result of the variation in growth of bacterial isolates as effected by the concentration of naphthalene.

Material and Methods

Sample collection for isolating the naphthalene degrading bacteria

About 5g of soil sample from petroleum contaminated site was taken using a sterile loop and stored in sterile polythene bags. The sample were collected from sites with frequent oil spills like petrol loading station (2), petrol pumps (5) and service station (2). All samples were stored at 4°C until used.

Naphthalene degrading bacteria were isolated using the method described by Nnamchi et al. 2006. Enrichment media was prepared by dissolving all the following components : ammonium sulphate (2.5 g), magnesium sulphate (0.5 g), cobalt chloride (0.005), calcium chloride (0.001 g), potassium dihydrogen phosphate (0.0005 g), manganese sulphate (0.0001 g) in 1000ml distilled water in Erlenmeyer flask and autoclaved at 15 psi (121˚C) for 15 min. The medium was cooled to room temperature and naphthalene (0.65 g/l) was added. Thirty ml of Enrichment medium was then dispensed in 150 ml Erlenmeyer flasks. 1.0 g soil was inoculated into enrichment medium and incubated at 30°C at 120 rpm. After 7 days of incubation, 1.0 ml of sample was further transferred to fresh Enrichment medium and again incubated. The enrichment was done thrice.

The sample showing microbial growth in Enrichment media were inoculated on Nutrient agar (NA) medium (HiMedia, Mumbai) and incubated for 48 hours at 30°C. Isolated colonies were purified by streak inoculating on NA plates. Pure cultures were observed for colony type and preserved in nutrient agar slants at 4°C.

Characterization of isolates

The potential isolated bacteria were identified based upon their biochemical and morphological characteristics as described by Bergey's manual of determinative bacteriology (1957) and Cappuccino and Sherman (1999).

Assessment for growth pattern of isolates with time

The growth pattern of all the isolates in the medium containing naphthalene was determined. The isolates were inoculated in Enrichment medium containing naphthalene (0.65 g/l). The bacteria was inoculated at 30° C at 120 rpm. After 2 hrs intervals, 5 ml of each culture was shifted to a test tube under sterile conditions and growth was measured at taking OD at 600nm using spectrophotometer (Systronics, India).

Effect of naphthalene concentration on the growth of the bacteria

The impact of different naphthalene concentration on the growth of bacterial cultures was studied. Enrichment medium with different conc. of naphthalene at 50, 100, 150, 200, 250 and 300 ppm was prepared. The bacterial isolates were inoculated in Enrichment medium with varying naphthalene concentration. After incubation of 3 days at 120 rpm at 30°C, 5 ml sample from each formualtion was analysed for the microbial growth by taking OD at 600 nm using spectrophotometer (Systronics, India).

Result and Discussion

In the present study, the bacteria which can degrade naphthalene by utilizing it as the sole carbon source were isolated followed by their characterization. The soil samples were processed for isolation of potential bacterial isolates and the isolates obtained were identified. Biochemical characterization and morphological analysis revealed the isolates belonged to *Pseudomonas, Actinomycetes* and *Bacillus* sp. After biochemical characterization, it was found that 6 isolates (60%) belonged to *P.aeruginosa,* 1 *(*10%*)* belonged to *Actinomycetes* and 3 (30%) belonged to *Bacillus sp.* (Fig 1). All the isolated bacteria showed the potential to grow on naphthalene containing media. *P. aeruginosa* were chosen as a potential isolate because of its rapid growth rate when cultured in naphthalene containing media within three days.

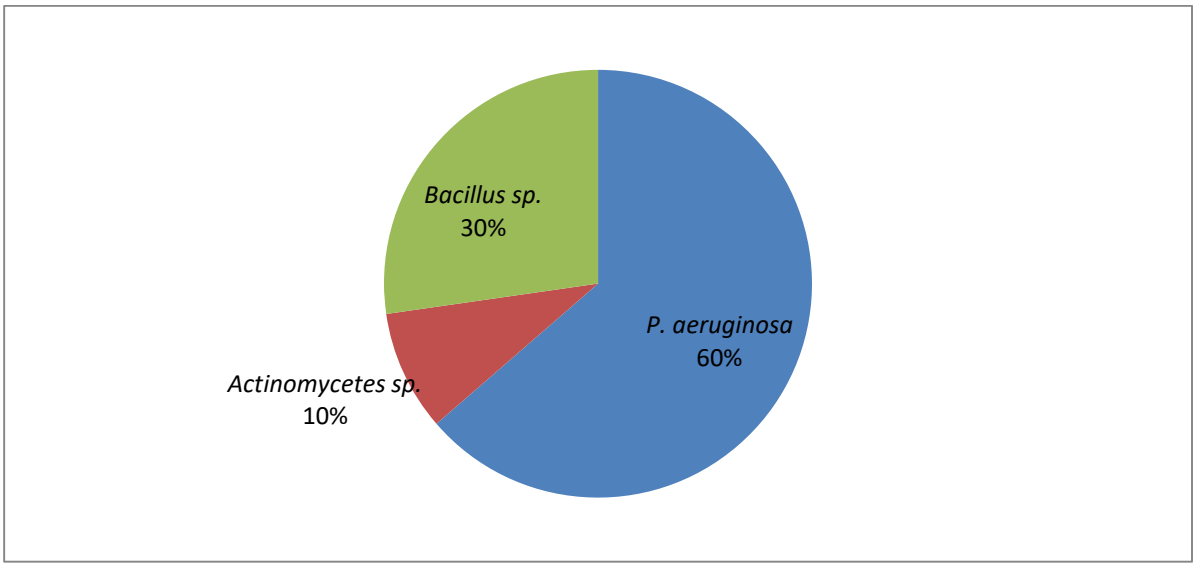

Fig. 1. Prevalence of naphthalene degrading bacteria isolates in soil samples

The time taken by the different bacterial isolates for the utilization of the naphthalene is depicted in Table 1 (Fig. 2). It was observed that following inoculation all the isolates grow well on naphthalene. Among all the isolates, *P. aeruginosa* exhibited highest growth showing maximum growth within 18 hours.

Table . 1. Time course of growth of *P. aeruginosa*

Time Sample no.	0 hrs	2 hrs	6 hrs	12 hrs	18 hrs	24 hrs	36 hrs	72 hrs
ND1	0.00	0.011	0.042	0.045	0.11	0.587	0.500	0.465
ND 2	0.00	0.012	0.073	0.073	0.15	0.314	0.285	0.217
ND 3	0.00	0.025	0.059	0.059	0.12	0.353	0.324	0.284

ND 4	0.00	0.015	0.054	0.054	0.13	0.524	0.473	0.398
ND 5	0.00	0.012	0.092	0.097	0.19	0.529	0.425	0.329
ND 7	0.00	0.017	0.065	0.065	0.12	0.464	0.369	0.268
ND 10	0.00	0.124	0.073	0.057	0.13	0.374	0.365	0.252

All the isolates exhibited short lag phase of 12-18 hours and showed high growth rates within the first 24 hrs. ND1 show highest OD at 600nm wavelength after 24 hrs incubation. After that decrease in OD was observed with time.

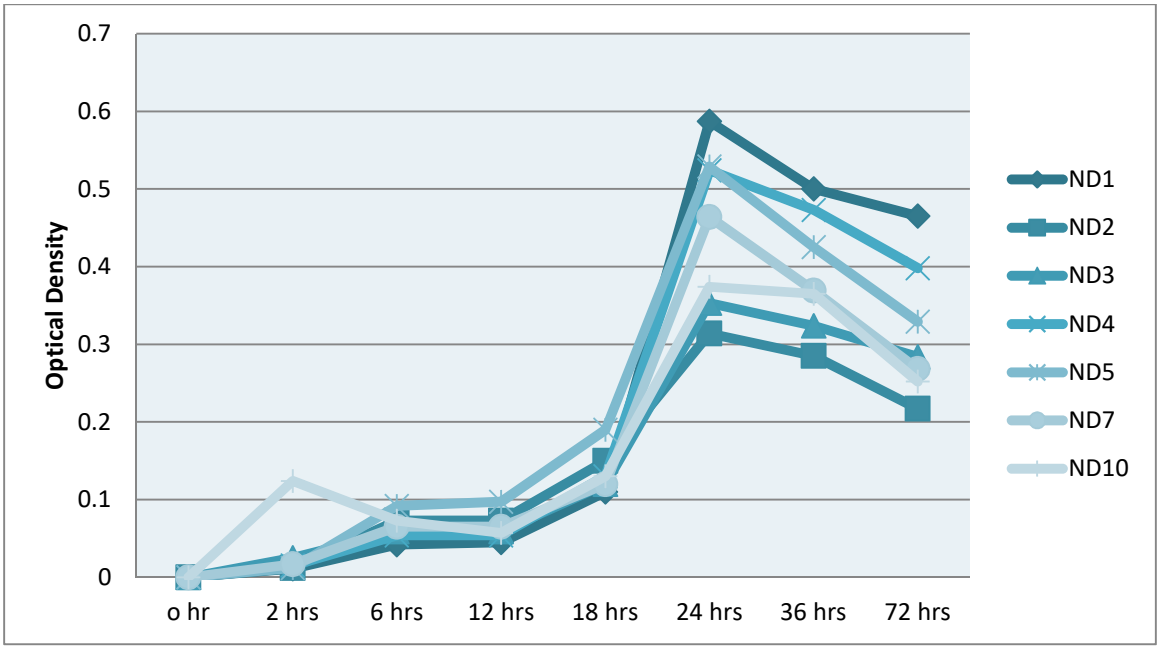

Fig.2. Growth Profile of *P aeruginosa* with time

These results hold great importance because usually naphthalene degradation takes several months (Lee, 1998). This observation may correspond to unique genotype bacterial species.

Effect of naphthalene concentration on bacterial

With the increased naphthalene concentration, higher growth rate of bacteria was observed. Increase in bacterial growth rate in different concentrations of naphthalene ranging from 25 ppm to 200 ppm is shown in Table 2 (Fig.3). The lowest growth was observed when the concentration of naphthalene was lowest i.e 25 ppm, whereas highest growth rate was observed at naphthalene concentration of 200 ppm.

Table.2. Effects of different conc. on growth of isolates

Isolate	Absorbance at 600 nm with different concentration of naphthalene				
	25ppm	50ppm	100ppm	150ppm	200ppm
ND1	0.019	0.045	0.066	0.092	0.104
ND 2	0.012	0.115	0.122	0.076	0.116
ND 3	0.016	0.112	0.022	0.112	0.095
ND 4	0.020	0.017	0.048	0.090	0.048
ND 5	0.019	0.010	0.040	0.105	0.022
ND 7	0.007	0.040	0.109	0.119	0.122
ND10	0.008	0.015	0.043	0.068	0.102

Our results are in agreement with the observations of Bauer and Capone (1985) whose results revealed that with increasing concentration of naphthalene, bacterial growth rate also increases. Literature reports that maximum growth is seen at naphthalene concentration of 200 ppm, confirming that that higher concentration of naphthalene leads to higher growth rate in bacteria. From our observation ND7 isolate shows highest growth rate at 200 ppm. Low growth rate at low concentrations may be due to the fact that bacteria is unable to fulfill its energy sources as too lower the concentration would become a limiting factor for the bacterial growth (Wong et al., 2001).

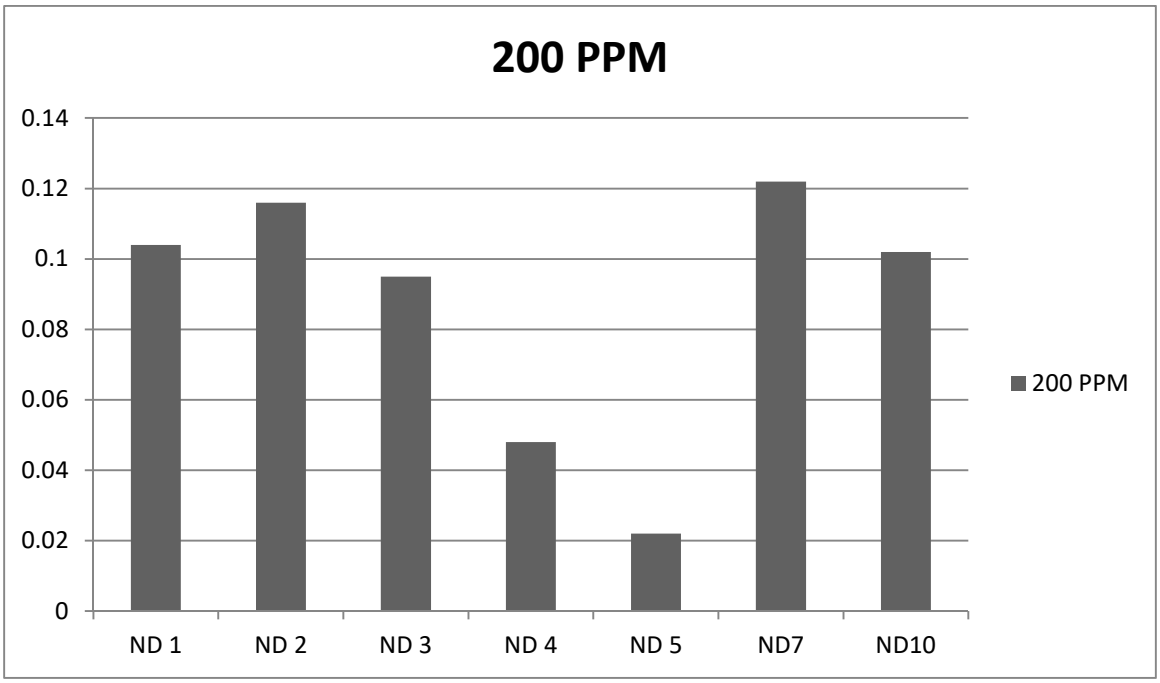

Fig. 3. Growth of *P. aeruginosa* with 200 ppm of naphthalene

In the present study 6 isolates of *P.aeruginosa* were isolated that had the potential to use naphthalene as only source of carbon. All the isolates grew well on the Enrichment media containing naphthalene with a log period of 12-18 hrs. Also when the conc. Of naphthalene was increased the isolates showed increased growth. These microorganisms can be used as bioinoculants in site contaminated with oil spills after confirming their degrading capabilities. As physical and chemical treatment is expensive and time consuming biodegradation using microorganisms is beneficial as it leads to non-toxic compounds**.**

Conclusions

The results revealed that with increasing naphthalene concentration bacterial growth also increases, suggesting that rates of naphthalene biodegradation is dependent on its concentration . It was observed that maximum growth was observed with a naphthalene concentration of 200 ppm proposing that higher concentration of naphthalene usually gave higher bacterial growth. The bioremediation component of this study primary focused on microbial isolation and investigation of degradation ability. In order to enhance the potential of the bacterial isolates as

possible utilizing them for commercial biodegradation purposes, future studies can be planned to optimize and determine various factores that effect their ability and efficiency of hydrocarbon degradation.

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