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ISOLATION AND CHARACTERIZATION OF NAPHTHALENE DEGRADING BACTERIA FROM SOILS IN WARANGAL

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ABSTRACT: Bacteria capable of utilizing naphthalene, as their sole source of carbon and energy for growth were isolated from three different sites in warangal,Andhra Pradesh. By standard bacteriological methods, these bacteria were characterized taxonomically as belonging to the genus Pseudomonas, Burkholderia or Actinomycetes. Two of the isolates, which showed the highest growth during screening as demonstrated by an increase in their optical densities at 600 nm and named as NITWDBT1 and NITWDBT3.In isolate NITWDBT1, the lowest optimum growth of 0.189 at 600 nm was observed when the level of anthracene was lowest (50 ppm); while the highest optimum growth of 0.578 was recorded when the level of anthracene was highest (300 ppm). Similarly, the lowest optimum growth in isolate NITWDBT3 was 0.173 at anthracene level of 50ppm, while the highest was 0.380 when the level of anthracene was 250 ppm (OD600). These two isolates were identified as *Pseudomonas aeruginosa* and *Burkholderia cepacia* respectively, were also able to grow in anthracene and carbazole, but not very much so in 2,4-dichlorophenol and D-camphor. The isolates showed a concentration-dependent growth in all the compounds they were grown. There were visible changes in the colour of the growth medium of the isolates during their incubation, suggesting the production of different metabolites. There were also changes in their medium pH during growth. These studies demonstrate the possession by the bacterial species of novel degradative systems.

Key words: *Pseudomonas aeruginosa, Burkholderia cepacia, polycyclic aromatic hydrocarbons, persistence, recalcitrance, biodegradation, bioavailablity*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) have found applications in a variety of industries including dyes, drugs, semiconductors, fluorescent reagents, chemiluminescent reagents, and as polychromatic and antistatic additives for plastics. Polycyclic aromatic hydrocarbons are composed of carbon and hydrogen; the carbon atoms being arranged in a series of adjoining six-membered benzene rings. Most of the PAHs are introduced into soil from atmospheric depostion after local and long-range transport, which is supported by the presence of PAHs in soil of regions remote from any industrial activity. Other potential sources of PAHs in soil include disposal from public sewage tretment, irrigation with coke oven effluent, leachate from bituminous coal storage sites, and use of soil compost and fertilizers Thus, all PAHs have in common a singular feature that is based on two or more fused benzene rings (Chaudry, 1994).

PAHs are ubiquitous contaminants of aquatic and terrestrial ecosystems whose presence is attributable to a number of petrogenic and pyrogenic sources, which had increased since the end of the Second World War (Laflamme and Hite, 1978; NAS, 1983; Jonsen *et al*., 2005).). Environments contaminated with PAHs are considered hazardous as studies using animals have shown the specific carcinogenic, mutagenic and teratogenic effects of some PAHs (Miller and Miller, 1974; Moore *et al*., 1989; Autrup, 1990). Their biochemical persistence in the environment arises from dense clouds of π-electrons on both sides of the ring structures, making them resistant to nucleophilic attack (Jonsen *et al*., 2005). Even though higher molecular weight PAHs such as those containing four or more benzene rings are considered to be responsible for the majority of the potential hazards of these compounds to the environment and human health (EPA, 1984), lower molecular weight types such as naphthalene (the simplest containing two benzene rings), anthracene and phenanthrene (both of which contain three benzene rings) are known to have health effects that though are comparatively mild could be potentially hazardous (Klaasen, 2001). Furthermore, some like phenanthrene is considered as a model substrate in environmental PAHs degradation studies because its structure is found in the nucleus of carcinogenic PAHs such as benzo[a]anthracene and 3-methylcholanthrene (Cerniglia and Yang, 1984). As a result of these hazardous effects of PAHs, there is much interest in their environmental effects. Although some physical processes such as volatilization, leaching, chemical and photo oxidation are often effective in reducing the environmental level of PAHs (Bossert and Bartha, 1984; Heitkamp *et al*., 1988), biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of some inherent advantages such as its cost effectiveness and more complete cleanup (Pothuluri and Cerniglia, 1994). Moreover, the physical processes are often limited to aquatic environments only. The microorganisms should possess all the necessary enzymes needed to degrade PAHs. It is known that selection or adaptation of PAHdegrading microorganisms as with other chemicals occur as a result of their previous exposure to this substances in the environment (Lewis et al., 1984; Spain et al., 1980). However, these adaptations occur slowly, and usually depend on the recalcitrance or biodegradability of the particular substance involved (Spain et al., 1980). This is especially so considering that PAHs usually have low aqueous solubility and thus, are poorly available (low bioavailability) for microbial utilization. (Jonsen et al., 2005). A lot of isolated microorganisms have been successfully utilized in major hazardous waste clean-up processes, as for example, in industrial process streams and effluents (Levinson et al., 1994). Unfortunately, most of these studies were carried out in Western countries, and to a limited extent in South America and Asia (Kiyohara et al., 1982; Ghoshal et al., 1996; Prantera et al., 2002). In this work, we report the isolation and characterization of some PAH (naphthalene)-degrading bacteria from soils in Warangal environment, and their course of growth in naphthalene and other aromatic compounds.

MATERIALS AND METHODS

Collection of soil samples about 5 g soil samples were asceptically collected with a sterile scoop from soils in Rampur and Lingampally Village, both in Warangal locality and also from around the National Insyiyute of Technology Warangal area. All samples were placed into sterile polythene bags and stored at 4 °C immediately they were brought to the laboratory,Department of Biotechnology,NIT warangal.

Isolation and screening of bacteria from the soil samples

Bacteria were isolated from the soil samples using an enrichment medium containing naphthalene. The medium consisted of (g /L) NH_4SO_3, 2.5; Na_2HPO_4, 1.0; $MgSO_4$, 0.5; $Fe_2(SO_4)3$, 0.01; $CoCl_2$, 0.005; $CaCl_2$, 0.001; KH_2PO_4, 0.0005; $MnSO_4$, 0.0001; $(NH_4)_6Mo_7O_2.4H_2O$, 0.0001 and naphthalene, 0.65. The naphthalene was added after autoclaving the medium. The medium was first dispensed in 30 mL volumes into 150 mL Erlenmeyer flasks and autoclaved at 121°C and 15 psi for 15 minutes. Next 1.0 g of each soil sample was inoculated into each flask of the medium and incubated at 120 rpm at 30 °C in a orbital shaker for one week. Then 1.0 mL sample was taken from each culture and transferred into fresh enrichment medium, followed by incubation as described above for one week.

The enrichment procedure was repeated for the third time, before their bacterial contents were isolated using a solid medium containing the enrichment medium and 15.0 g/L of pure agar. Inoculated plates were purified by repeatedly subculturing. Pure cultures obtained by this procedure were stored in slants of enrichment medium with 15.0 g/ L pure agar, and also in nutrient agar, and stored at 4 ºC. A loopful of each isolate was inoculated into large test tubes containing 25 mL of screening medium. The screening medium was the same as the enrichment medium, except that 15 mg of naphthalene dissolved in dimethyl sulfoxide was added to each tube after autoclaving, as sole source of carbon. Thereafter, the test tubes were statistically incubated by keeping on the laboratory bench at room temperature (23 – 25 ºC) for three days. The ability of each isolate to utilize naphthalene was indicated by an increase in turbidity of the medium measured at 600 nm using an UV spectrophotometer.

Identification, characterization, standardization and effect of the concentration of Naphthalene on the growth of the isolates of isolates

Two isolates, which gave the highest OD readings, were identified to their species level, while the others were identified to their genus level only, using conventional microbiological and biochemical procedures. The tests were carried out according to the procedures described by Cowan and Steele (1974) and Cheesebrough (1998) and Bergey's manual of systematic bacteriology (1993). All the isolates were code-named; the two referred to above were named NITWDBT1 and NITWDBT3 and subsequently used for further studies. Before usage in subsequent works, cells were washed and standardized to the McFarland nephelometer standard of 0.5 (Baron and Finegold, 1990). In all cases, 1% v/v of standardized inoculum was used according to the volume of medium used. The two isolates were inoculated into 250 mL Erlenmeyer flasks containing 75 mL of sterile enrichment medium in triplicates. The flasks were then incubated in the orbital shaker as previously described for three days. At six-hourly intervals, 5.0 mL sample was collected from each flask and assayed for OD at 600 nm in the UV spectrophotometer. 3000 mL of the enrichment medium was prepared in a 4 L flask and dispensed in 75 mL volumes into thirtytwo 250 mL Erlenmeyer flasks before autoclaving. The flasks were then divided into six sets of six flasks each. Thereafter, the following levels of naphthalene were added to each of the six sets of flasks: 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm. Three sets of flasks were inoculated with isolate NITWDBT1, and the others with isolate NITWDBT3. Inoculated flasks were then incubated as previously described for three days. Five milliliter sample was acceptically collected from each flask and assayed for the level of microbial growth by measuring the OD as described previously and pH using a pH meter.The ability of the isolates to grow on varying amounts of anthracene (a PAH), Carbazole (a heterocyclic aromatic compound), 2,4-dichlorophenol (a chlorophenol), and DL-Camphor was studied. The preparation and sterilization of media as well as the addition of the hydrocarbons were carried out as in the case of naphthalene above. Thereafter, each set of triplicate flasks were inoculated with the two isolates as applicable, and incubated as described before for three days. Samples were also collected at six hourly intervals and measured for optical density.

Statistical analysis

Statistical analysis was performed using the least significant difference test method (Obi, 1990).

RESULTS

The studies have been carried out haphazardly and have usually covered rather not extensive areas. Twenty bacteria isolates were obtained after screening shown in Table 1. Two isolates, NITWDBT1 and NITWDBT3, which were found to degrade naphthalene, better than the rest of the isolates were identified as *Pseudomonas aeruginosa* and *Burkholderia cepacia*. The characteristics of the two bacteria are shown in Table 2. Results also indicate that most of the isolates with low OD were Actinomycetes as most of them produced aerial mycelia, which bore chains of spores, while the remaining isolates were of the genus Pseudomonas and Burkholderia. This is shown in Tables 3 and 4.

Table 1:Screening of bacteria isolates

S.No.	Isolate	OD600
1	Control	0.00
2	KN1	0.01
3	KN2	0.01
4	KN3	0.02
5	KN4	0.03
6	KN5	0.05
7	KN6	0.06
8	KN7	0.07
9	KN8	0.16
10	KN9	0.01
11	KN10	0.02
12	KN11	0.05
13	KN12	0.14
14	YP1	0.39
15	YP2	0.45
16	YP3	0.02
17	YP4	0.03
18	NITWDBT1	0.47
19	NITWDBT2	0.34
20	NITWDBT3	0.48

Time course of growth of isolates NITWDBT1 and NITWDBT3 on Naphthalene and Effect of naphthalene concentrations on the growth of the isolates

The time course of the utilization of naphthalene by the two isolates as their sole source of carbon for growth is shown in Fig. 1. From the Fig.1, it is seen that both organisms grew relatively well in naphthalene. Of the two, isolate NITWDBT1, *Pseudomonas aeruginosa*, grew better, peaking at OD value of 0.51 at 600 nm wavelength after 18 hours, whereas isolate NITWDBT3 *Burkholderia cepacia* peaked at an OD value of 0.34 after 30 h. During the course of their growth in naphthalene, the isolates also produced coloured metabolites, which were greenish yellow for isolate NITWDBT1, and faint yellow for isolate NITWDBT3.There was a correspondingly higher growth of both isolates as the levels of naphthalene were increased from 50ppm to 300ppm (Fig. 2 a and 2b). In isolate NITWDBT1, the lowest optimum growth of 0.16 (OD600) was observed when the level of naphthalene was lowest (50 ppm), while the highest optimum growth of 0.60 was recorded when the level of naphthalene was highest (300 ppm). Similarly, the lowest optimum growth in isolate NITWDBT3 was 0.10 at naphthalene level of 50 ppm, while the highest was 0.55 when the naphthalene level was 250 ppm (OD600). Table 4 illustrates the average optical density measurements of the two isolates grown at different levels of naphthalene. Analysis of variance (ANOVA) of the effect of the different levels of naphthalene on the growth of the isolates showed significant ($p < 0.01$) differences in the growth of the isolates at the different naphthalene levels. Using the calculated F-LSD value of 0.11, it was observed that the differences existed between naphthalene levels of 300ppm against 50ppm, 100ppm and 150ppm; 250ppm against 50 ppm, 100 ppm and 150 ppm; and between 200 ppm against 50 ppm and 100 ppm.

Table2:Identification of two isolates as *Pseudomonas aeruginosa* and *Burkholderia cepacia*

Test Characteristic	Appearance and Reaction NITWDBT1	NITWDBT3
Bacteriological Tests: Shape	Rod	Rod
Gram reaction	Negative	Negative
spore	Negative	Negative
Growth Tests: D-Glucose	Positive	Positive
D-Fructose	Positive	Positive
D-Galactose	Positive	Positive
D-mannitol	Positive	Positive
Sucrose	Positive	Positive
Maltose	Negative	Negative
Arabinose	Negative	Positive
Trehalose	Negative	Positive
Inulin	Negative	Negative
Sorbital	Negative	Positive
Meso-Inosital	Negative	Positive
Cellobiose	Positive	Positive
Acetamide	Positive	Positive
Cetrimide	Positive	Positive
Starch	Negative	Negative
Growth at 41^{0}C	Positive	Positive
Pigmentation	Positive	Positive
Biochemical Tests: Nitrate reduction	Negative	Positive
Glucose fermentation	Negative	Negative
Arginine dehydrolase	Positive	Negative
Galatinase	Positive	Negative
Oxidase	Positive	Positive

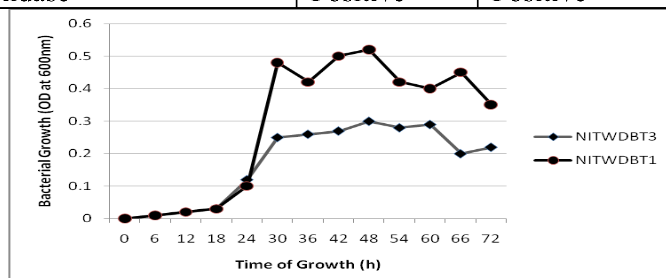

Fig. 1: Time course of growth of the isolates on naphthalene

Table3: Identity of all twenty isolates

S.No.	Isolate	Identity
1	Control	Actinomycete
2	KN1	Actinomycete
3	KN2	Actinomycete
4	KN3	Actinomycete
5	KN4	Actinomycete
6	KN5	Actinomycete
7	KN6	Actinomycete
8	KN7	Actinomycete
9	KN8	Pseudomonas
10	KN9	Pseudomonas
11	KN10	Pseudomonas
12	KN11	Actinomycete
13	KN12	Pseudomonas
14	YP1	Pseudomonas
15	YP2	Actinomycete
16	YP3	Burkholderia
17	YP4	Actinomycete
18	NITWDBT1	Pseudomonas
19	NITWDBT2	Actinomycete
20	NITWDBT3	Burkholderia

Table4: Average OD of isolates grown at different concentrations of naphthalene

	Levels of PAH(Naphthalene) ↓					
Isolate↓	50 ppm	100 ppm	150 ppm	200 ppm	250 ppm	300 ppm
NITWDBT1	0.11	0.18	0.25	0.33	0.31	0.39
NITWDBT3	0.08	0.08	0.12	0.24	0.34	0.22

Effect of the concentrations of some aromatic compounds on the growth of the isolates and effect of different concentrations of Naphthalene on medium pH

The two isolates grew on anthracene, carbazole and marginally on DL-camphor and 2,4-dichlorophenol, using them as their sole source of carbon for growth as illustrated in Fig.s 4 to 7. Of these, the isolates grew well on anthracene and carbazole, and poorly on Dlcamphor and 2, 4-dichlorophenol. The growth of the two isolates on anthracene was also concentrationdependent as observed in the case of the utilization of naphthalene. In isolate NITWDBT1, the lowest optimum growth of 0.186 at 600 nm was observed when the level of anthracene was lowest (50 ppm); while the highest optimum growth of 0.610 was recorded when the level of anthracene was highest (300 ppm). Similarly, the lowest optimum growth in isolate NITWDBT3 was 0.165 at anthracene level of 50ppm, while the highest was 0.420 when the level of anthracene was 250 ppm (OD600).

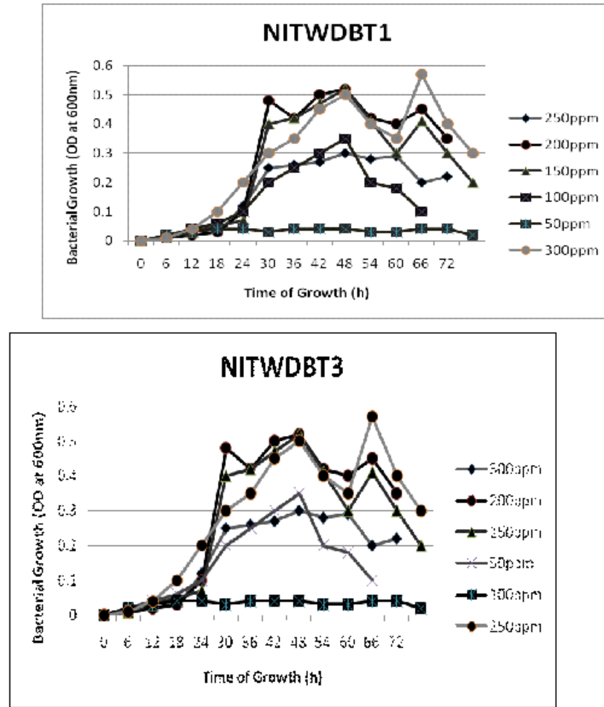

Fig. 2: Effect of different concentrations of naphthalene on the growth of the isolates (2.a. NITWDBT1 and 2.b.NITWDBT3)

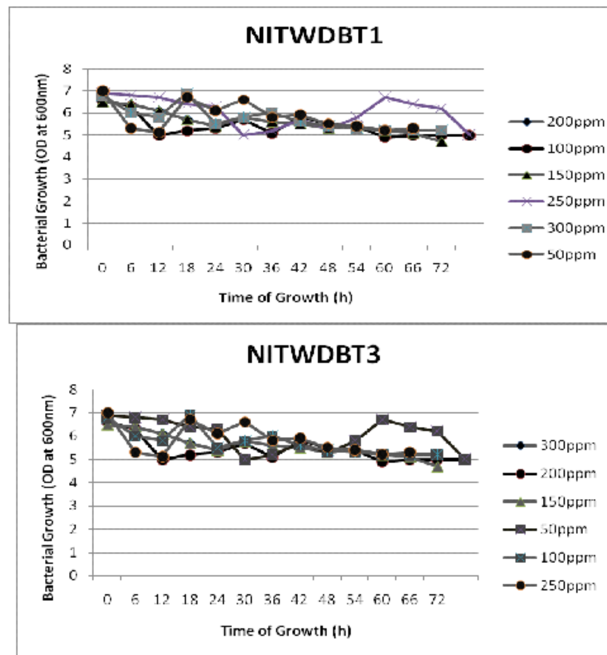

Fig. 3: Effect of the growth of the isolates in different concentrations of naphthalene on medium pH(3.a. NITWDBT1 and 3.b.NITWDBT3)

The growth of the isolates in carbazole only partially followed the concentration-dependent pattern observed in naphthalene and anthracene. Results of the effect of naphthalene on the growth of the isolates on medium pH illustrated in Fig. 3a and 3b showed that there was a fall in pH from a nearly neutral initial medium pH in almost all the concentrations, to acidic and weakly acidic levels (between 5.2 to 6.0 for NITWDBT1, and 4.0 to 6.0 for NITWDBT3) by the end of the experiment.

DISCUSSION AND CONCLUSION

A total of twenty bacterial isolates were obtained and screened for their ability to utilize naphthalene as sole source of carbon and energy. Most of these were identified as Pseudomonas, Burkholderia or Actinomycetes. This conforms to the high degradative ability and ubiquity associated with these bacterial types as it concerns biodegradation of both soil and water environments polluted with petroleum and its many products (Atlas, 1984; Chaudhry, 1994 Jonsen *et al*., 2005). Of this lot, only two species (*P. aeruginosa* and *B. cepacia*) were chosen for further studies as a result of their high growth yield in the relatively short period of three days, as well as other considerations such as their ability to produce coloured metabolites. The two species showed unusually rapid growth rate when cultured in naphthalene – a polycyclic aromatic hydrocarbon. This trait of *Pseudomonas* and *Burkholderia* species both of which formerly belonged to the genus Pseudomonas to grow on highly xenobiotic compounds is made possible by the wealth of catabolic enzymes they possess, and more importantly, by their immense capacity for adaptive change. It is believed that this adaptive capacity is promoted by their inherent patterns of regulation, which allows for the coincidental induction of different catabolic pathways, resulting in novel patterns of biodegradation (Ornston and Yeh, 1982). This probably explains why the two isolates not only grew on and hence metabolised naphthalene, which is considered the simplest and hence, the easiest of all polycyclic aromatic hydrocarbons to degrade, but also to some extent degraded other recalcitrant aromatic compounds that belonged to other classification groups. The ability of *Pseudomonas* and *Burkholderia* species to degrade naphthalene and other PAHs has been reported by several workers (Catterall *et al*., 1971; Cerniglia, 1984; Heitkamp *et al*., 1987; Mueller *et al*., 1990; Kastner *et al*., 1994; Mueller *et al*., 1997; Bosch *et al*., 2000; Jonsen *et al*., 2002; Jonsen *et al*., 2005). In all the cases, the ability of the microorganisms to solely utilize the PAH substrates as sources of both carbon and energy were emphasized. The two bacterial species under consideration showed highly significant ($p < 0.01$) growth on naphthalene. This is reinforced by the intense colouration noticed during their growth. This colouration or pigmentation is believed to result from the production and accumulation of different metabolites during the course of growth of the bacteria (Mueller *et al*., 1990), and suggests significant utilization of the substrates. This probably accounts for the slight fall in the pH of the medium during the growth course, suggesting the possible production of acidic metabolites (Fig. 3a and 3b),this is because of the availability of nutrients (the PAHs), and the ability of the bacteria to break them down to simpler utilizable materials, as well as the intense mixing and aeration in the experimental set-up. In natural oligotropic environments such as soils, this is usually not the case because of the low nutrient concentrations and lack of aeration in such heterogeneous environments (Jonsen *et al*., 2005). The two isolates also showed very fast growth rate and high growth capacity as evidenced in the fact that: (1), they showed very short lag phase of between 12 – 18 h. (Fig.s 1 and 2a & 2b) though isolate NITWDBT3 went beyond that in Fig. 2a and 2b, and (2), they achieved peak growth within the first 48 h. of growth. The importance of this observation becomes evident when it is recognized that on the average, naphthalene usually has a complete biodegradation half-life that runs into several months (Lee, 1998).

This capacity may be attributed to the peculiar genetic make-up of the bacterial species, even though their high exposure to the substrates arising from the many preliminary subculturings and prior exposure to the substrates in the polluted soils from which they were isolated may have contributed. The results also showed that increases in naphthalene

concentration proportionately increased the growth of microorganisms (Fig. 2a and 2b). This agrees with the findings of Bauer and Capone (1985) and others that PAH degradation generally increases with increases in the concentration of PAHs.

According to them, the rates of biodegradation of PAHs are concentrationdependent and conform to Monod's kinetics and first order concentration models (Fu *et al*., 1996; Ghoshal *et al*., 1996). Incidentally, it was observed that isolate NITWDBT3 (*Burkholderia cepacia*) usually reached maximum growth at 250 ppm (which was not the highest naphthalene concentration used during the experiment), suggesting that though higher concentrations of naphthalene usually gave higher bacterial growth, this ceased when a threshold concentration was reached. This agrees with the statement of Alexander (1999) that the acclimation of a microbial community to one substrate frequently results in the simultaneous acclimation to some, but not all structurally related molecules. Also, individual microbial species have the ability to act on several structurally similar substrates, and therefore more easily act on their analogues after the first addition (Bauer and Capone, 1985; Soulas *et al*., 1983; Obrigawitch *et al*., 1983; Mitchell and Cain, 1996). It is therefore not surprising that the two isolates grew on these other organic aromatic compounds, considering that they are all commonly composed of benzene rings as naphthalene.

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