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Biodegradation of Polycyclic Aromatic Hydrocarbon Compound by Bacterial Cultures

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ABSTRACT: In the present study biodegradation of Polycyclic Aromatic Hydrocarbon (PAH) compound Naphthalene by four bacterial cultures *Bacillus subtilis* PD6, *Bacillussp.* PD9, *Enterobactersp.* PD11 and *Bacillussp.* PD14 has been targeted. Biodegradation of Naphthalene by these four selected bacterial cultures was analysed by HPLC (High Performance Liquid Chromatography) technique. HPLC analysis revealed biodegradation of naphthalene by all the four bacterial cultures within a span of six days. Highest biodegradation 78.1% has been shown by *Bacillus subtilis* PD6 while other bacterial cultures *Bacillus sp.* PD9 has shown 77.90%, *Enterobacter sp.* PD11 showed 74.4% and *Bacillus sp.* PD14 exhibited 73.5% biodegradation of naphthalene.

KEYWORDS: Bacterial degradation, Biodegradation, Polycyclic Aromatic Hydrocarbons, HPLC, High Performance Liquid Chromatography, PAHs, Naphthalene biodegradation, Bioremediation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of fused-ring aromatic compounds that are ubiquitous environmental pollutants (Balaram Mohapatra and Prashant S. Phale, 202S1). PAHs constitute a large and diverse class of organic compounds and are generally described as molecules which consist of three or more fused aromatic rings in various structural configurations (Kanaly*et al.*, 2000).Polycyclic aromatic hydrocarbons, such as naphthalene, are widespread in the environment and pose a notable health hazard due to their toxic, mutagenic, and carcinogenic properties (Kanaly*et al.*, 2000; Singleton, 2009;Hamme van*et al*., 2003;Veronika, 2008). PAH molecule stability and hydrophobicity are two primary factors which contribute to the persistenceof HMW PAHs in the environment (Sarma, 2004). PAHs are also known to exert acutely toxic effects and/or possess mutagenic, teratogenic, or carcinogenic properties (Cerniglia, 1989; Phillips 1983). Due to their lipophilic nature, PAHs have a high potential for biomagnification through trophic transfers (Twiss*et et al* 1999). Some PAH compounds have been classified as priority pollutants by U. S. Protection Agency (Keith, 1979). Increase in environmental persistence of PAH compounds has been attributed to the size of the PAH compounds i. e. greater the molecular weight or number of rings in the PAH compound more the environmental persistence. In addition to this, increment in PAH genotoxicity has also been observed with increase in the number of rings up to four or five benzene rings. (Cerniglia,1992; Kanaly, 2000). The relationship between PAH environmental persistence and increasing numbers of benzene rings is consistent with the results of various studies correlating environmental biodegradation rates and PAH molecule size (Banerje *et al.*, 1995, Bossert *et al.*, 1986). For example, reported half-lives in soil and sediment of the three-ring phenanthrene molecule may range from 16 to 126 days while for the five-ring molecule benzo[a]pyrene (B*a*P) may range from 229 to 1,400 days (Shuttleworth *et al.*, 1995).

PAHs, present as natural constituents in fossil fuels, are formed during the incomplete combustion of organic material, and are therefore present in relatively high concentrations in products of fossil fuel refining (Bos e*t al.*, 1984; Deschênes,*et al.*, 1996;). Release of petroleum hydrocarbons into the environment is caused due to refining and transportation from the source to destination. Discharge of industrial effluents and accidental release is responsible for such localized loadings of raw and refined products. However, release of PAHs into the environment may be attributed to many sources, including gasoline and diesel fuel combustion (Lim *et al.*,1999;) and tobacco smoke (Gündel *et al*., 1996). PAHs are detected in air (Lim *et al.*, 1999; Koeber. *et al*., 1999**)**, soil and sediment (Coates *et al.*, 1997), surface water, groundwater, and road runoff (Boxall, *et al.*, 1997) are dispersed from the atmosphere to vegetation (Wagrowski *et al.*, 1997) and contaminate foods (Edwards1983). Anthropogenic and natural sources of

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PAHs in combination with global transport phenomena result in their worldwide distribution. Hence, the need to develop practical bioremediation strategies for heavily impacted sites is evident (Fantroussi *et al.,* 2005). PAH concentrations in the environment vary widely, depending on the proximity of the contaminated site to the production source, the level of industrial development, and the mode(s) of PAH transport. Soil and sediment PAH concentrations at contaminated and uncontaminated sites ranging from 1 µg/kg to over 300 g/kg have been reported (Potter,1999; Wilson, 1993).

Naphthalene is released into the environment in complex mixtures of coal tar and coal tar products such as creosote. Many diverse groups of bacteria that degrade naphthaleneare widely distributed in nature (Cerniglia, 1980;Eaton, *et al.,* 1992, Van Hamme *et al*., 2003).

Various types of PAH compounds are released into the environment from different sources such as petroleum products. Crude oil (Chikere *et al*., 2009), automobile vehicular washing are those sources which contain large number of different PAH compounds present. Toxic effects of these PAH compounds are well known and bring about drastic changes in the gene structure by intercalating between the nucleotide base pairs, leading to cancer.

Over a period of time microorganisms have developed a mechanism to utilize PAH compounds as carbon and energy source. Many different bacteria are known to be capable of degrading, and in many cases, completely mineralizing, various individual xenobiotic compound such as (PAHs) making them good candidate species for site remediation applications. The ability to degrade low molecular-weight PAH compounds, such as naphthalene and phenanthrene, is widespread, and numerous researchers have identified bacteria capable of utilizing these compounds for growth. Growth on PAH containing four fused aromatic rings (e.g. chrysene, fluoranthene, pyrene, benz[a]anthracene) is somewhat more rare, although organisms are known that can utilize each of these as growth substrates (Churchill *et al.,* 1999). Currently, only a few bacterial isolates have been reported to degrade five-ring PAHs (e.g. benzo[a]pyrene); furthermore, this generally occurs through co-metabolism, during growth on simpler substrates (Bogan *et al.,* 2003).

Toxicity of Naphthalene

Naphthalene, also known as naphthalin, is a crystalline, aromatic, white, solid hydrocarbon with formula $C_{10}H_8$ and structure of two fused benzene rings (Figure 1). It is best known as the traditional, primary ingredient of moth balls. It is volatile, forming an inflammable vapour, and readily sublimes at room temperature, producing its characteristic odour. It's insoluble in water, somewhat soluble in methanol/ethanol, soluble in organic solvents and very soluble in ether, chloroform, or carbon disulfide (Franco, 2009).

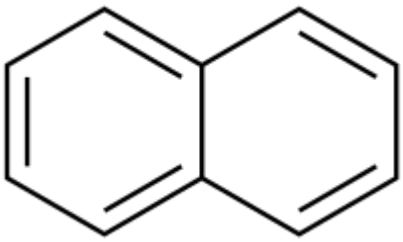

Figure 1: Structure of Naphthalene

Naphthalene is a toxic substance and its toxic effects vary from individual to individual (in adults, ingestion of 6 grams has led to significant toxicity or no symptoms at all; in children, absorption occurs rapidly, a reported dose of 2 grams has been fatal) and they act at on both local and a systemic level. The systemic effects of naphthalene occur chiefly in the erythrocytes causing haemolysis, with subsequent blocking of renal tubules by precipitated haemoglobin. Haemolysis is more likely to occur in individuals with a hereditary deficiency of glucose-6-phosphate dehydrogenase, sickle cell anaemia and sickle cell trait. Besides that, Naphthalene has noxious effects on other targets such as the gastroenteric system, the liver (hepatic necrosis may occur), the urinary system, the brain and the eye (cataract). Local effects are not that serious. It's all about the skin (Contact Dermatitis) and the cornea (corneal lesion). Exposure to large amounts of naphthalene may damage or destroy red blood cells. Humans, particularly children, have developed this condition, known as hemolyticanemia, after ingesting mothballs or deodorant blocks containing naphthalene. Symptoms include fatigue, lack of appetite, restlessness, and pale skin. Exposure to large amounts of naphthalene

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maycause confusion, nausea, vomiting, diarrhea, blood in the urine, and jaundice (yellow coloration of the skin) (Balaram Mohapatra and Prashant S. Phale, 2021).

Naphthalene has been classified as possibly carcinogenic to humans and animals (Group 2B) by the International Agency for Research on Cancer (IARC). According to the IARC acute exposure to naphthalene may result in cataracts in humans, rats, rabbits, and mice. It may also result inhemolytic anemia in children and infants after oral or inhalation exposure or after maternal exposure during pregnancy. Under California's Proposition 65, naphthalene is listed as "known to the State to cause cancer"(ATSDR, 2005; CCOHS, 2005; IARC 2002; IPCS, 2000; J C Wakefield 2007).

Genes involved in NaphthaleneCatabolism

It is amazing to observe that a single bacterial species can utilize number of different xenobiotic compounds. Some are in soluble state and some are sparingly soluble or completely insoluble. For the utilization of compounds occurring in different physical state in the soil or water as a contaminant, bacteria have developed various mechanisms to break it down and derive energy from it. The basis of these mechanisms lies in the presence of various catabolic genes on chromosome or catabolic plasmids (Sayler Gary S. *et. al.*,1990).

The catabolic gene clusters responsible for the degradation of various xenobiotic compounds are located on the bacterial chromosome and catabolic plasmids carried by the bacterial cell (Widada*et. al.*, 2002).

Large catabolic gene clusters are also found on mobile elements integrated into bacterial chromosomes as genomic islands or conjugative transposons.

Bacterial catabolic plasmids carry various genes that empower their host cells to utilize number of natural and xenobiotic compounds as sole sources of carbon, nitrogen, and energy. Most such plasmids are large (>50 kb) and carry genes for their conjugal transfer to other bacterial strains. Such transfer events across taxa, and subsequent mutations and rearrangements of the catabolic genes, have contributed to the rapid adaptation of bacteria to novel chemical compounds. Recent studies have shown that genes for the degradation of xenobiotic compounds, such as atrazine, haloacetate, and 2,4-dichlorophenoxyacetate, are predominantly carried on the incompatibility group P-1 (IncP-1) plasmids, whereas genes that encode the degradation of natural aromatic hydrocarbons, such as phenol, naphthalene, and toluene/xylenes, are usually found on IncP-2, IncP-7, and IncP-9 plasmids.

Experimentation and Result

Degradation of Naphthalene by selected bacterial cultures

Degradation potential of selected four bacterial cultures towards polycyclic aromatic hydrocarbons was assessed as follows.

- To assess the degradation capability of *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 total 15conical flasks of 150 ml, each containing 100 ml of 0.1X M9 medium were prepared. After autoclaving 25 ppm of naphthalene was added to each flask. Experiment was carried out in triplicate and appropriate controls were included in the experimental setup.
- Each of the four bacterial cultures was inoculated in the 150 ml flask containing 100 ml of 2% nutrient broth. All flasks were incubated at 28^{o}C at 100 rpm till the development of desired growth. Sufficient amount of growth was observed within 24 h after inoculation. ODs of these cultures were measured spectrophotometrically at 600 nm. Sufficient amount of culture was withdrawn aseptically,washed with 0.1X M9 minimal medium in order to remove the traces of nutrient medium. This bacterial culture was inoculated into the experimental flask at a concentration of 0.02 OD/ml of 0.1X M9 minimal medium containing naphthalene as a sole source of carbon.
- All experimental and control flasks were kept for incubation at 28^{o}C for 6 days at 120 rpm.
- Samples were withdrawn at regular intervals of 24 h from each flask for the HPLC analysis (Perkin Elmer, USA).

High Performance Liquid Chromatography (HPLC)

Biodegradation of naphthalene by *Bacillus subtilis* PD6,*Bacillus sp.* PD9, *Enterobacter sp.* PD11 and*Bacillus sp.* PD14 was analysed by HPLC technique.

Figure 2 shows time course for degradation of naphthalene by four bacterial isolates while Figures 4a to 4f show chromatograms for initial and final levels of naphthalene after growth of bacterial isolates for 6 days. The chromatograms clearly show decrease in peak heights representing naphthalene in case of all isolates with a corresponding formation of bio-transformed

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products as indicated by appearance of new peak in chromatogram. However negligible change in peak height was observed in case of control flasks.

Results of HPLC analysis revealed that naphthalene was degraded by all the four bacterial cultures, but efficient biodegradation was found to be shown by *Bacillus subtilis* PD6 (78.1%) and *Bacillus sp.* PD9 (77.90%). *Enterobacter sp.* PD11 showed 74.4% and *Bacillus sp.* PD14 exhibited 73.5% degradation of naphthalene (Figure3).

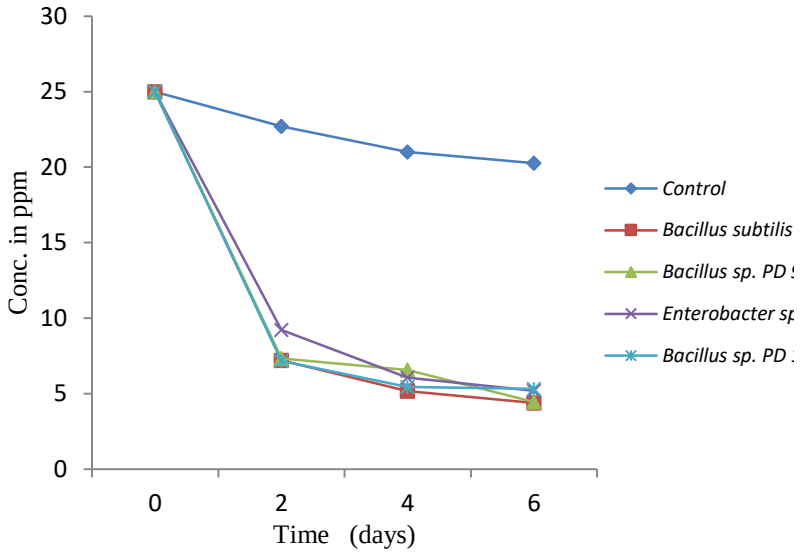

Figure 2: Naphthalene degradation by selected four bacterial cultures

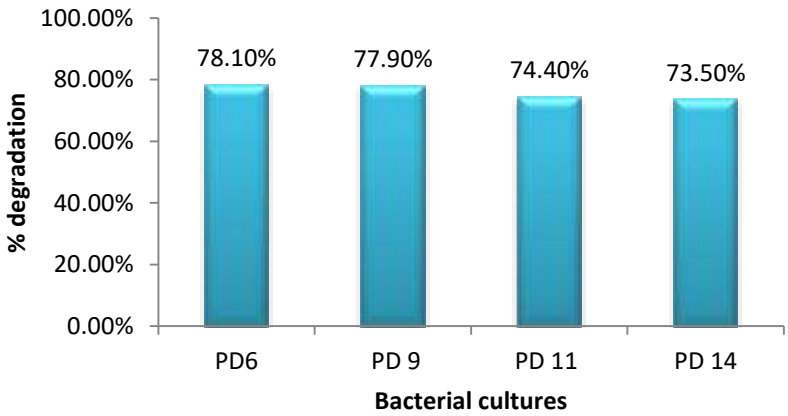

Figure 3: Percent degradation of naphthalene on 6th day.

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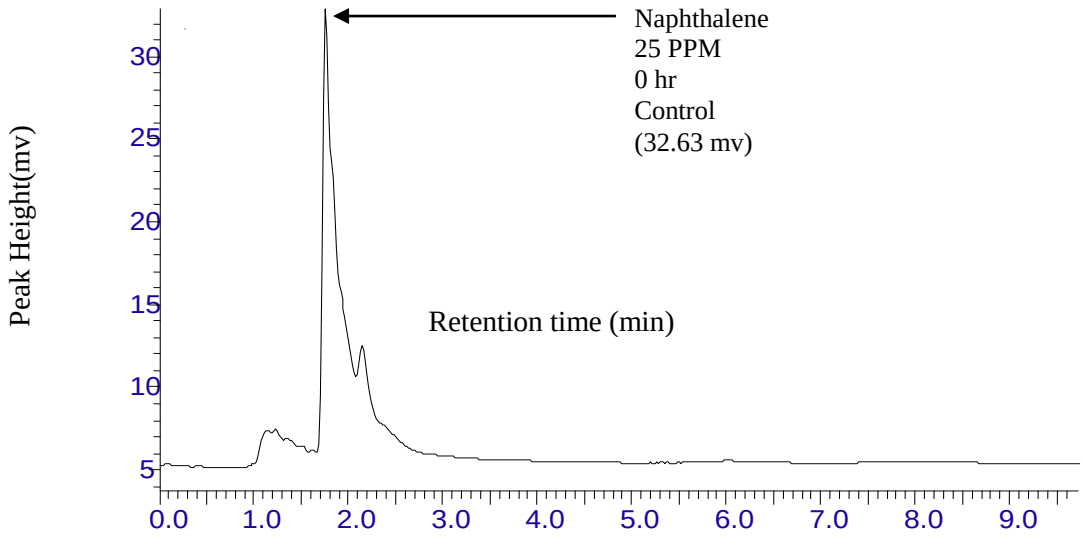

Figure 4a: HPLC chromatogram depicting level of Naphthalene in control flask at 0hr

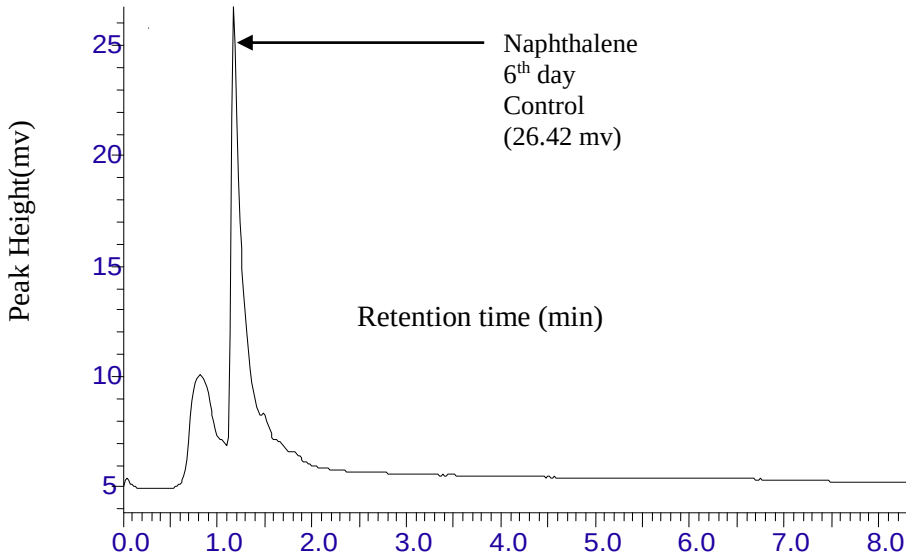

Figure 4b: HPLC chromatograms depicting level of Naphthalene in control flask on 6th day

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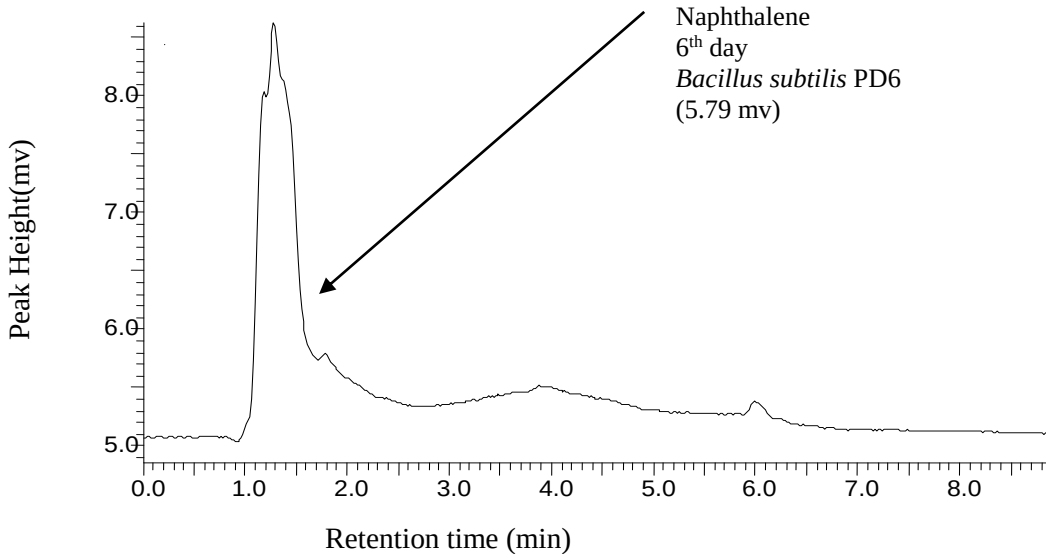

Figure 4c: HPLC chromatogram depicting biodegradation of Naphthalene by *Bacillus subtilis*PD6 on 6th day.

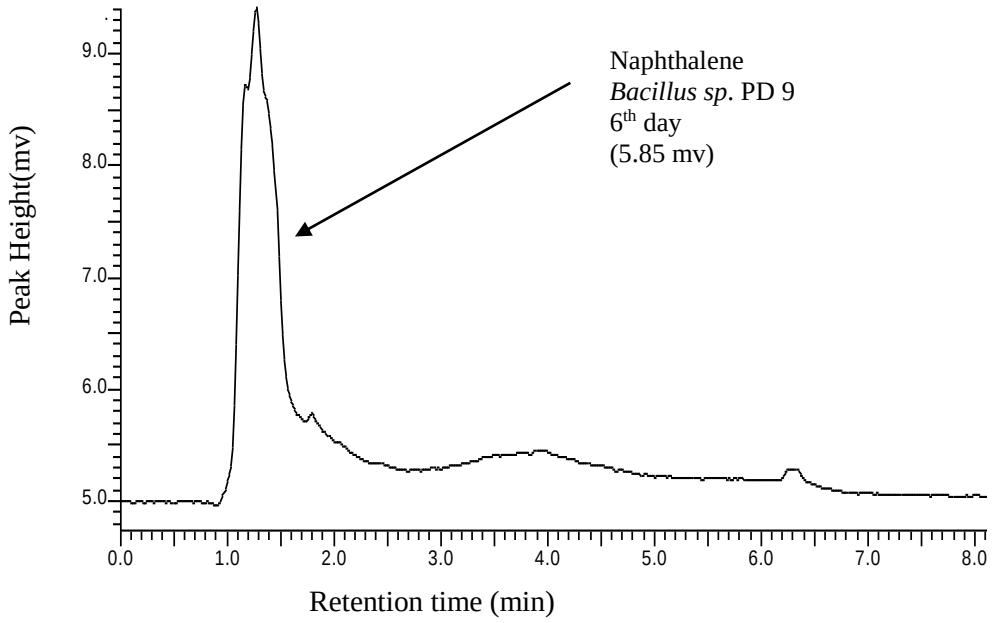

Figure 4d: HPLC chromatogram depicting biodegradation of Naphthalene by *Bacillus sp.* PD 9 on 6th day

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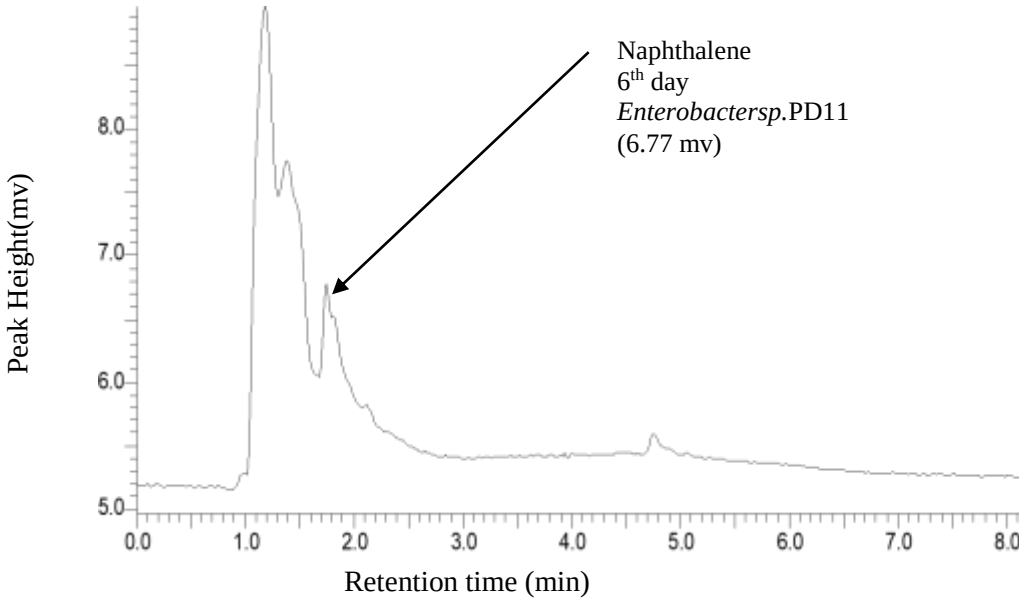

Figure 4e: HPLC chromatogram depicting biodegradation of Naphthalene by *Enterobacter sp.* PD11 on 6th day

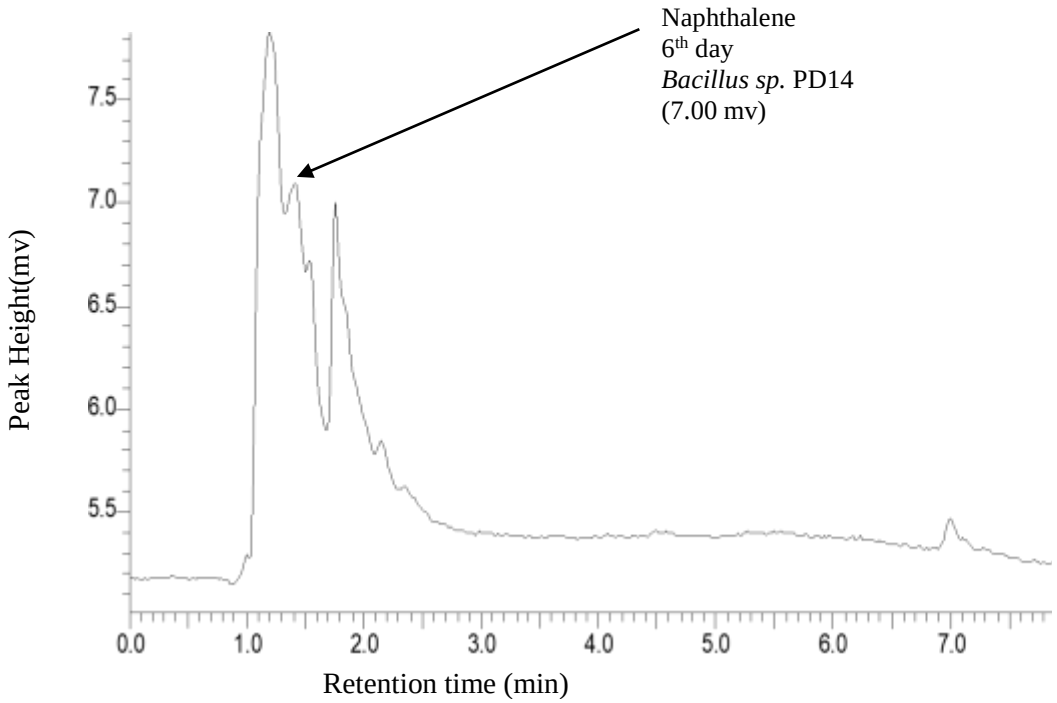

Figure 4f: HPLC chromatogram depicting biodegradation of Naphthalene by *Bacillus sp.* PD14 on 6th day

DISCUSSION

In the present study naphthalene (Singleton, 2009, Van Hamme *et al*., 2003) was chosen as the representative compound for the polycyclic aromatic hydrocarbons (PAH) (Hatzianestis *et al.*, 2002, Veronica, 2008). Since variety of different PAH compounds are present in the petroleum products and automobile waste water released from the automobile repairing garages and

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service stations (Paxéus, 1996), it is extremely important to find out biological agents responsible for the biodegradation of these compounds. During this study biodegradation of naphthalene (Eaton *et al.*, 1992, McKenna, 1976, Van Hamme *et al*., 2003) by *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 was analysed by HPLC. All cultures exhibited biodegradation of naphthalene above 70% (Figure 3). Studies on PAH and Naphthalene degradation is of paramount importance because of its existence in the wastewater released from automobile service stations as was accentuated by the studies conducted by Paxéus, 1996. Research work on biodegradation of PAH compounds is necessary because these PAH compounds may prove to be fatal if taken inside through water or any other source. Some PAH compounds are teratogenic, carcinogenic and mutagenic (Kanaly *et al.*, 2000). In this study four bacterial cultures, *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 were found to be excellent naphthalene degraders eliminating 78.10%, 77.90%, 74.4% and 73.50% naphthalene within 6 days respectively (Figure 3). Naphthalene when added to the 0.1X M9 medium by dissolving in the N, N' Dimethyl formamide solvent, crystals of naphthalene were formed in the medium. These crystals remained present in the control flask but decreased in presence of *Bacillus subtilis* PD6 and *Enterobacter sp.* PD11 as degradation progressed. But in case of *Bacillussp.* PD9 and *Bacillus sp.* PD14 complete solubilisation of the naphthalene crystals was observed next day. This indicated that these cultures might have produced bio-surfactants (Kosaric,2001; Tabatabaee *et al.*, 2005) which assisted in the solubilisation of naphthalene crystals. This observation was made in comparison with control flask in which very little solubilisation of naphthalene crystals was observed. Presence of biosurfactant producing capability in the *Bacillus sp.* was confirmed by Salihu*et al.*, (2009) according to whom *Bacillus sp.* produced the Surfactin as biosurfactant. Removal of naphthalene by *Bacillus* and *Enterobacter sp*. in the present study agrees with the studies executed by Toledo *et al*. (2006) who isolated number of bacterial strains including *Bacillus and Enterobacter sp.* capable of removal of PAH compounds. Results obtained by Molina *et al*., (2009) showed that the *Enterobacter sp*. isolated from the petroleum oil contaminated soil are capable of degrading three PAH compounds namely naphthalene, phenanthrene and fluoranthene. Studies performed by other researchers substantiated the presence of the degradation capability in the *Bacillus* and *Enterobacter sp*. towards other PAH compounds also (Hunter *et al.*, 2005, Laura 2011).

In the present study, *Enterobacter sp.* PD11 belonging to family *Enterobacteriaceae* was isolated and tested for the PAH degrading capability. Enteric bacteria are mainly inhabitants of gut and PAH degrading capability by these bacterial cultures seem to be unusual. However, though few, but reports are available which show that the enteric bacteria are involved in the utilization of aromatic compounds. Such bacteria include genera *Klebsiella*, *Enterobacter*, *Escherichia*, and *Hafnia* (Sarma*et al*., 2005).

CONCLUSION

Four bacterial cultures *Bacillus subtilis* PD6, *Bacillus sp.* PD9, *Enterobacter sp.* PD11 and *Bacillus sp.* PD14 with the ability to biodegrade polycyclic aromatic hydrocarbon compounds were successfully assessed for naphthalene degradation. All the four bacterial cultures were found to be efficiently eliminating significant amount of naphthalene within a period of six days. Two bacterial cultures *Bacillus sp*. PD9 and *Bacillus sp.* PD14 were found to solubilise naphthalene completely within 24 hrs. Thus four cultures with catabolic potential towards polycyclic aromatic hydrocarbons were isolated and tested successfully.

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