International Journal of Researches In Biosciences, Agriculture & Technology | Dec. 2014, Special Issue | ISSN No. (Online) 2347-517X

Evaluation of Degradation of Napthalene by *Pseudomomonas Species* Isolated From Soil

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Abstract:

Due to worldwide environmental problem associated with contamination of PAH and their products there is always scope to isolate the bacterial consortia able to degrade PAH. Naphthalene, a model compound representing PAH selected for this purpose. The present work describes the isolation, characterization and naphthalene degradation potential of microbial consortia isolated from contaminated soil of oil depot, Nagpur, India. We also detected the presence of some metabolites thereby indicating the possible fate of degradation of naphthalene by isolated bacterium.

Keywords:
Napthalene, Pseudomanas, Mineralisation, Catechol.

Introduction:

Polycyclic aromatic Hydrocarbons (PAH) are widely distributed in the environment and are global environmental pollutants contaminating agricultural lands, soil and groundwater (Zeinali et al., 2007). Incomplete combustion of fossil fuels, organic matter and waste particularly from industries associated with petroleum gas, wood preservatives, cocking of coals etc constitutes major anthropogenic entry route for PAHs into the environment along with vehicular emission, petroleum spills. Naturally occurring phenomenon such as volcanic eruptions, forest fire, etc are also adds to this (Al Turki, 2009; Bamforth and Singleton, 2005; Mahony et al., 2006). Low solubility, low Volatility and hydrophobic nature leads to persistence of PAH in receiving environment. Number of rings, structual angularity make PAH recalcitrant to degradation. This class of pollutant is of increasing public concern due to its toxic carcinogenic, mutagenic and teratogenic properties (Warith et al., 1999; Mumtaz et al., 1995; Mastraland Callen, 2000; Thiele-Bruhn and Brummer, 2005).

PAH is considered one of the most acutely toxic compounds in water soluble fraction of petroleum. Accumulation of PAH in soil can lead to contamination of the food chain, which then cause exposure to plants and humans directly or indirectly. US EPA has listed 16 PAH as priority pollutants. Although PAH undergoes abiotic reduction by photolysis, chemical oxidation and volatilization, microbial degradation is the major process affecting their persistence fate in the environment (Atlas, 1981**,** Yuan et al, 2002). Usually decontamination occurs by physical or chemical methods such as precipitation, extraction, oxidation or combustion. However, microbiological treatment is promising technique for decontaminating water, soil, effluent. Bioremediation is, cost effective, efficient alternative to physicochemical treatments used for remediation of hydrocarbon contaminated sites (Ulrici, 2000).

Naphthalene is among the 16 PAHs classified as priority pollutants by Environmental Protection Agency (EPA) of United States, is a commonly recognized pollutant in nature. This aromatic hydrocarbon and its methylated derivatives are considered some of the most noxious compounds in the water-soluble fraction of petroleum.(Keith and Telliard, 1979). It is a frequent micro pollutant in potable water. Diverse organic compounds present in pesticides, fungicides, detergents, dyes and

mothballs contain certain PAHs like naphthalene and phenanthrene (Samanta, 2002). Studies with laboratory animals reported the toxicity and cataractogenic activity of naphthalene. Binding of this compound covalently to the molecules in the liver, kidney and lung tissues enhances its toxic effects. (Goodmann et al., 2001)

Material and methods:

Chemicals: Napthaalene (98 %,) was obtained from across organics (New Jersey, USA). Methanol was purchased from qualigen. Acetone, Bushnell Hass Agar and Bushnell Has broth, Luria Bertani media (LB), Nutrient agar (NA) obtained from Hi-Media. Ethyl acetate, and all other chemicals and reagents, used for the study, were of analytical grade. 2,3 dihydroxybenzoic acid (99 %,), Cis-cismucccinic acid (98%,), pthalic acid (99%), catechol (99%) , 2,3 dihydroxynapthalene (98%), 3-hydroxy-2-napthoic acid (98%), Salicyldehyde (99%), 1,2 dihydroxyanthraquinone (Alizarin) (97%), Acetaldehyde (99.5%), 2-carboxybenzaldehyde (99%), 2-aminophenol (99%), and other metabolites were also purchased from Acrosorganics.NAD, NADH, FAD, FADH were purchased from Sigma.

Sample collection and Site:- The soil sample used for isolating naphthalene degrading microorganism was collected from Indian oil Corporation bulk petroleum depot (IOCL), Khapri, Nagpur, India. The soil contaminated with petroleum oil for years is used for the isolation of PAHs grader bacterial culture. After removal of stones surface soil was taken from 10 different locations, mixed and stored at 4^0C and bring to the laboratory in ice box.

Media: -Minimal media used for enrichment and testing hydrocarbon degrading ability was Bushnel Hass Broth (BHB). It is composed of (L^{-1}): Magnesium Sulphate 0.20 g, Ammonium Nitrate 1.00 g, Calcium chloride 0.02 g, Ferric Chloride 0.05 g, Monopotassium Phosphate 1.00 g, Dipotassium Phosphate 1.00 g. To this trace element solution was added at (L^{-1}): $CuSO_4$ 0.4mg, KI 1.0 mg, $MnSO_4.H_20$ 4.0 mg, $ZnSO_4.7H_2O$ 4.0 mg. H_3BO_3 5.0 mg, $H_2MoO_4.2H_2O$ 1.6 mg. The pH was adjusted to 7±0.2. Solid minimal media plates were prepared by addition of 20 g of agar agar (L^{-1} BHB).To investigate the ability of *P. stutzeri* to utilize the pathway intermediates, cells were cultivated in BHB in the presence of various substrates singly as sole carbon source under identical conditions.

Enrichment of Naphthalene degrading Bacteria:-

Minimal media (BHB) was sterilized by autoclaving (121^0 C for 20 min). 1 g of soil sample was suspended in 100 ml of BH Broth containing 250 mg Lit $^{-1}$ of naphthalene as an enrichment substrate and incubated with shaking at 120 rpm at 28^0 C. After 7 days, 5 ml aliquot was transferred to 100 ml of fresh BHB containing the same amount of naphthalene and incubated under same condition as mentioned above. After four such enrichments potential bacterial isolates able to degrade naphthalene were screened using spray plate method. Incubation was always carried at 30 ^{0}C unless otherwise stated.

Screening and isolation of naphthalene degrading bacteria:-

Spray-plate technique of kiyohara was used to screen, isolate naphthalene degraders. Cultures from enrichment flask after appropriate dilution was spread on solid BH Agar plates and sprayed with hexane containing 0.2% naphthalene dissolved in it as sole carbon source. After incubation in dark at 30^0 C for 7 days, naphthalene degrading bacteria were visualized by a distinct sprayed-coated clear zone surrounding individual colonies. Among these, representative colonies forming large clear zones were aseptically removed and pure bacterial strains obtained by streaking and restreaking on NA and were kept on LB slant at 4 ^{0}C. Many researchers employed this method as fast and effective way for initial isolation of bacteria that use PAH as the sole carbon and energy source. (Kiyohara et al., 1982; Kastner et al., 1994; Madsen and Kristensen, 1997).

Bacterial identification and phylogenetic analysis of 16S rDNA sequences

1. Morphological and Biochemical Identification

Morphological and phenotypic identification of the napthalene degrading bacteria was initially done by Gram-staining, and other classical tests. Various biochemical tests were done as follows: oxidase, catalase and urease activity, voges–Proskauer and methyl red reaction, sugar fermentation, and indole production, citrate use, gelation liquefaction, starch, lipid and nitrate reduction, etc.

2. 16S rDNA sequence and phylogenetic analysis

Genomic DNA was extracted from liquid culture using the Nucleo-Pore DNASure Tissue mini Kit (Genetix) as described by manufacturer instructions,16S rDNA gene was PCR amplified with bacterial primers (Primers obtained from SciGenom Lab, Cochin, Kerala, India).

16sF 5'-AGAGTTTGATCCTGGCTCAG-3 and 16sR- 5' ACGGCTACCTTGTTACACTT-3'. Amplification cycle was as follows: initial denaturation at 94 ^{0}C for 5min followed by 30 cycles of denaturation at 94 ^{0}C for 2 min, annealing at 55 ^{0}C for 1 min, elongation at 72 ^{0}C for 2 min, and final elongation at 72^{0}C for 14 min (PCR assembly). Amplicon was electrophoresed in a 1% agarose gel, visualized under UV, purified using nucleospin purification column (Macherey-Nagel) and sequenced in ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The bacterial 16S gene sequence analysis was carried out using bioinformatics tool BLAST of NCBI (http://www.ncbi.nlm.nih.gov). Based on maximum identity score first few sequences were selected and aligned using multiple sequence alignment software MultAlin. Dendrogram was constructed using CLC genomic software.

PAH degradation assays:

Naphthalene degradation experiments were conducted in triplicate.The ability of the isolated bacterial strain to degrade napthalene was assessed in liquid culture. Stock solution of napthalene in acetone was added in respective flasks to have final concentration of 250 ppm. Flasks were kept for overnight to completely vent off acetone before addition of BH broth in order to minimize the effect of acetone used to dissolve PAHs in the stock solution.100 ml of autoclaved BH broth then aseptically added to the experimental flasks. 1ml aliquot of fresh cultures of the isolated strain was used for inoculating the experimental flasks containing naphthalene. The inoculums preparation was done by previously growing culture separately on BHB amended with naphthalene.For inoculation cells were collected by centrifugation at 6000×g for 10 min and washed twice with 0.5 M phosphate buffer (pH7) and resuspended in the same buffer to have OD of 1 at 600 nm. 1ml of this preparation was used to inoculate experimental flasks. BH broth with only naphthalene was used as control. Flasks then subjected to incubation on rotary shaker at 120 rpm at 28 ^{0}C. Culture were withdrawn at intervals of two days and analyzed for remaining naphthalene concentration using HPLC from which percent naphthalene degradation was calculated

Extraction of naphthalene and its metabolites

Culture in experimental flask, in duplicate, extracted every 48 hours upto 8 days. Culture extracted twice with equal volume of ethyl acetate after acidification to pH 2 with 1 N HCl. The organic phase extractions combined, dried over anhydrous sodium sulfate and evaporated to dryness. It is re-dissolved in methanol, filtered and 10 µl of this was analyzed by high pressure liquid chromatography. Based on the naphthalene concentration remains percent degradation was calculated. Naphthalene and its metabolites were identified by comparison of the retention time with those of authentic standards.

HPLC Analysis

HPLC analysis was performed on Shimadzu HPLC system (Shimadzu SPD-20A prominence), equipped with uv-visible detector. For analytical purpose separation was

achieved using a capillary column Luna 5u C18 (2) 100A, with dimension of 150 × 4.6 mm (Phenomenex), under binary mode with total flow of 1.00 ml/min. End time 15 min, wavelength 254 nm. Max. Pump pressure limit 320, lamp D2, polarity +, response 1.00 sec. injection volume 10 µl. For the detection of naphthalene and its metabolites, mobile phase consists of mixture of methanol and water (70:30) at a flow rate of 1.0 mL/min to elute the column (mob phase mixed sonicated to remove air bubble and filter through 0.02 filter), using a sample injection volume of 10 µL. Standard solutions of naphthalene at different concentrations were used for reference. Based on the remaining PAHs present in the sample, the percentage degradation of PAHs by the organisms was calculated

Preparation of cell-free extract: Logarithmic growth phase culture with naphthalene as sole carbon source were harvested by centrifugation (4000 g 15 min)**,** washed twice with 50 mM phosphate buffer (cold, pH 7.5) and pellets were re-suspended in 5 ml of the same buffer. Cells suspension disrupted by sonicationin an ice bath. Crude extract obtained after centrifugation at 12,000 x g for 30 min at 4^0C.The supernatant obtained used as cell-free enzymes for further studies. Protein measured by the Lowry's method (Lowry et al., 1951) with BSA as a standard. Enzymatic transformations of various substrates were carried out by recording cell-free-extract-catalyzed changes in uv–visible spectra using SL 210 double beam spectrophotometer (Elico) using 1 cm path-length quartz cuvettes. Data were analyzed by the spectra treat software. Spectrophotometer also used to measure OD for bacterial growth.

Growth on intermediates: Further metabolic characterization of isolate was done by growing cells separately with salicylic acid, gentisic acid and catechol, cis-cismuconate, some of the known intermediates in naphthalene degradation as sole soure of carbon and enegy (Grund et al 1942; Eaton and Chapman, 1992). Enzyme assays were also performed on the crude cell extract in order to characterize the possible metabolic pathway involved in naphthalene metabolism by the isolated *P. stutzeri* strain.

Enzyme assays: Salicylate hydroxylase was estimated according to procedure of White-Stevens and Kamin 1972. Naphthalene dioxygenase was detected by the formation of blue indigo pigmentation on BH plates containing 1 m Mindole (Ensley et al., 1983).1,2 dihydroxynapthalene dioxygenase and salicylaldehyde dehydrogenase activities were measured by the methods reported (Shamsuzzaman and Barnsley 1974) . Catechol-1,2-dioxygenase (C120) was analyzed by the method of (Hegeman 1966). Catechol 2,3-dioxygenase activity was identified as described by (Feist and Hegmann 1969). Boiled crude extracts of cells were used as negative controls. Crude extract protein contents were measured by the lowry's method .

Growthconditions: The influence of pH, temperature, substrate concentration on the degradation of naphthalene was assessed using BH broth, three replicates. Concentration of napthalene used to study effects of various environmental conditions was 250 ppm unless otherwise stated.

Effect of pH: A series of degradation testswere carried out at three different pH. The autoclaved medium was adjusted to pH 6.5, 7.0 and 7.5 using predetermined amounts of filter sterilized (0.22 micron) 1 M HCl or 1 M NaOH and incubated at 30^oC.

Effects of Temperature: The incubation temperatures used were 25^0C, 30^0C and 35^0C maintained using water bath incubator shaker.

Effect of PAH concentrations: PAH biotransformation by the bacteria under different concentrations was determined in parallel by using conical flasks containing PAH range from 250, 500, 750 and 1000 ppm, respectively Control experiment was conducted without bacterial isolates to check loss due abiotic conditions.

Result and discussion:

Enrichment, Isolation and screening of naphthalene degrading bacterial cultures:

During enrichment positive growth was determined by an increase in the turbidity of the experimental flasks containing naphthalene as a sole carbon and energy source compared to control flasks. After 4 weeks enrichment, the PAH-degrading strains isolated in this study were selected preliminary on the basis of their ability to form zone in a spray plate method. Five different indigenous bacterial cultures able to degrade naphthalene were obtained and named as N1 to N5.Among the cultures, isolates N3 formed large spray-coated clear zone in a spray-plate test (degrading upto72 % of naphthalene under experimental conditions.) hence, it was selected for further degradation and metabolic studies.

Identification of the culture

1. Morphological and biochemical characteristics

Morphological and biochemical identification of bacteria was done according to Bergy`s Manual of Systematic bacteriology (Holt et al 1994). Culture N3 is rod shaped about 2.5 µm × 0.5 µm with wrinkle colony on LB solid plate. Cells are Gram-negative, motile, denitrifier .Utilize maltose, glucose and unable to liquefy gelatin indicate that culture N3 belong to *P. stutzeri*, possibly. Napthalene degraders N2 and N4 were identified as *Corynbacterium* species. Cultures N1, N5 belongs to *P. fluroscence* and *P. aeruginosa* respectively. Biochemical characteristics and morphology of the isolates described in **(Table 1)**

2. 16S rRNA gene sequencing:

The DNA sequencing and BLAST analysis of 16S rRNA gene of the isolate N3, shows significant similarity of 98 % with *Pseudomonas stutzeri* (NCBI sequence **accession number KJ622305**). The phylogeny cluster of isolated N3 *P. stutzeri* along with related Pseudomonas species is depicted in **Fig.** 1. Based on nucleotide homology, phylogenetic analysis, biochemical and morphological characteristic isolate was identified as *P. stutzeri* and used to refer by the same name in this study. Studies by earlier workers also showed the predominance of these bacterial species in biodegradation of polycyclic aromatic hydrocarbons. Genus Pseudomonas is one of the most studied and reported as degrader not only for PAHs but for other organic recalcitrant pollutants too, Though the number of bacterial species had been identified and isolated from diverse environmental samples, (Zhang et al., 2004). Many species of Pseudomonas used to degrade phenanthrene, anthracene, naphthalene as well as long chain compounds (Barathi and Vasudevan, 2001; Puntus et al., 2005, Zhang et al., 2004).

3. Degradation of Naphthalene

The degradation experiment was carried out with initial napthalene concentration of 250 ppm at 28 ^{0}C, pH 7.0. In test flask, none of the other carbon source except napthalene was added to the medium. (Figure 3) depicts the napthalene degradation results. Pseudomonas stutzeri showed 72% of naphthalene degradation in eight days of incubation under experimental conditions. The abiotic loss of naphthalene was only about 0.5 % as evidenced from control flask. Napthalene removal by bacteria increase in accordance with incubation period studied up to eight days, with 10%, 49%, and 65% degradation on second, fourth and sixth day of incubation. Since native bacteria of contaminated areas are constantly in contact with aromatic compounds, these bacteria should somehow be able to degrade the materials surrounding them. Degradability of naphthalene by native bacteria has reported in many studies (Bestetti et al., 2005).*Pseudomonas* sp. HOB1isolated from polluted sediment, showed ability to degrade 2000 ppm of naphthalene within 24 h and also exhibited potential to tolerate high concentration up to 60,000ppm of naphthalene. (Pathak et al., 2009). Strains belonging to Pseudomonas stutzeri are already isolated and studied for PAH degrading

potential and variations on degradative activity of one PAH in the presence of others have also studied (Zhao et al., 2009; Stringfellow and Aitken 1995).

4. Effect of environmental conditions on degradation of PAHs

4.1 pH:A series of degradation tests were carried out at three different pH. 63.2%, 73,5 % and 66.3 % degradation achieved at pH 6.5, 7.0 and 7.5 respectively (**Fig.4a**). Maximum degradation rate for *P. stutzeri* species determined in this study is at neutral pH 7. About 10% increased in degradation from 63.2 to 73.5 % was noted for pH variation from 6.5 to 7.0. Further increase in pH to 7.5 lowers degradation indicating optimum degradation at pH7. *Pseudomonas* species has has very diverse range of pH for mineralization of PAHs some increased naphthalene degradationion in slightly alkaline condition at pH range of 7.5–8.5 (pathak et al., 2009) whereas other such as strain JM2 of *Pseudomonas* showed degradation even under extreme range of pH 4.5 also. (Jing et al., 2012).

4.2 Temperature: 25 ^{0}C, 30 ^{0}C and 35 ^{0}C were temperature range selected for study. Isolated stutzeri species showed maximum degradation of 76.2 % on eighth day at 35 ^{0}C. (**Fig.4b**). This is similar to other studies in which maximum naphthalene degradation was reported in the temperature range of 35–37 ^{0}C. (pathak et al., 2009). Napthalene degradation rate of 63.5 % and 72 % were reported at temperature of 25 ^{0}C and 30 ^{0}C respectively.

4.3. Substrate concentration: Biodegradation experiment for napthalene showed *P. stutzeri*is able to remove napthalene at all the provided concentrations with increasing efficiency as with incubation period of eight days. (**Fig.4 c**). Highest degradation rate of 74.32 % was achieved at 500 ppm followed by 64.66 % degradation at 750 ppm. It is lowest with 59.8 % at 1000 ppm whereas, at 250 ppm 71% degradation rate reported on eighth day. Increased availability of substrate to bacteria may results into enhanced degradation rate observed for the isolate upto 500 ppm, further increase in napthalene concentration lower the rate.

5. HPLC analysis and Naphthalene intermediates:

An HPLC elution profile of the acidic ethyl acetate-extractable metabolites of naphthalene formed by *P. stutzeri* revealed different peaks. For metabolic identification, retention times of these sample peaks were compared with those of authentic compounds known or suspected to be intermediates in napthalene degradation.

Based on this, peak I with retention time of 7.19 min matches with cis-cismuconate**.** Second peak was observed for napthalene that eluted from the column at RT of of10.45 min. Peak 3 with retention time of 12.95 min matches with catechol. Salicylate eluted from column at RT of 16.19 constitutes fourth peak. None of the peaks in chromatogram shows similarity with gentisic acid and or benzoic acid as napthalene metabolites during analysis.

6. Growth on intermediates:

Growth of bacteria on individual metabolites and their utilization confirmed the role of these intermediates in the naphthalene mineralization process. *P. stutzeri* isolated in this study able to grow on salicylate, catechol and cis-cismuconate while gentisic acid failed to serve as sole carbon and energy source. table 2.

7. Enzyme assay results:

Blue indigo pigment formation on BH plates containing 1 mMindole detects presence of napthaenedioxygenase. Activity of nanpthalenedioxygenase reported in cell free extract, an enzyme catalyzing the first reaction of the napthalene degradation patthway and produce 1, 2 diihydroxynapthalne. Increase in OD at 340 nm for NADH was noted during conversion of salicyldehyde to salicylate was observed when checked for salicylate dehydrogenase activity. Salicylate hydroxylase andlactonizing enzyme activity was also noted. Activity of catechol 1,2dioxygenase but not 2,3 dioxygenase was reported in cell free extract.

8. Proposed degradation pathway for napthalene by isolated *Pseudomonas stutzeri:*

Microbial degradation of naphthalene has been studied extensively and the pathway of microbial naphthalene metabolism is well elucidated, aerobically as well as anaerobically (Aranha and Brown, 1981; Rockne and Strand, 2001). The initial step for aerobic degradation in PAHs is the incorporation of two oxygen atoms into the aromatic ring to form cis-dihydrodiols by dioxygenase enzymes (Bamforth and Singleton, 2005; Zhang et al., 2006). Naphthalene is converted to cis-1,2- dihydroxy-1,2-dihydronaphthalene by *Pseudomonas fluorescens* NCIMB 40531 was reported many years ago (Bosetti et al., 1996). This compound is dehydrogenated to 1,2-dihydroxynaphthalene by dehydrogenase. Subsequently, 1, 2-dihydroxynaphthalene is metabolized via 2- hydroxy- 2*H*-chromene- 2-carboxylic acid, *cis-o*hydroxybenzalpyruvate, and 2-hydroxy-benzaldehyde, which is then converted by a series of dioxygenases to salicylic acid (Goyal and Zylstra 1997, Baboshin et al 2008, Denome et al 1993, Kiyohara et al 1994). 1,2-dihydroxynaphthalene is also nonenzymatically oxidized to 1,2-naphthaquinone in some microbes (Auger et al 1995) and to 2-hydroxycinnamic acid through 1,2-dihydroxynaphthalene for the thermophilic bacterium Bacillus thermoleovorans strain TSH1(Zeinali et al. 2008). We didn't get such metabolites in the extract..Cis-2-hydroxybenzalpyruvate, further metabolized via catechol or gentisic acid to carbon dioxide and water (Mrozik et al., 2003; Di Gennaro et al., 2001).

Though we could not detected 1,2 dihydroxynapthalene as a metabolite during HPLC analysis, but ability of isolated *Pseudomonas* species to utilize and grow over it as sole carbon and energy source suggests its role in napthalene mineralization in upper pathway. Further detection of salicylate as metabolic intermediate during analysis with reported activities for enzymes napthalenedioxygenase and 1,2 dihydroxynapthalenedioxygenase indicate that *P. stutzeri* degrade napthalene through a pathway involving 1,2 dihydroxynapthalene and salicylate. Salicylate is common intermediates in the degradation of many PAHs. Its oxidation may follow either a gentisic acid or catechol pathway in order to produce compounds to be integrated in TCA. (Grund et al 1992, Eaton and Chapman1992). Culture failed to grow with gentisate as sole carbon and energy source and its absence as metabolite, ruled out the possibility of salicylate degradation through gentisate pathway. Moreover observed activity for salicyldehyde dehydrogenase and salilcylate hydroxylase in extract with detection of catechol as intermediate suggested further that, salicylate degradation may occurs through catechol. Based on 1, 2 and 2, 3 diooxygenation two different pathways for catechol degradation have been proposed and hence the isolated *Pseudomonas* species was analyzed for the type of ring cleavage dioxygenase. Here in our study isolated pseudomonas sp. showed significantly higher activities of catehcol 1,2dioxygenase (first enzyme of ortho pathway) in cell free extract. This indicates ortho cleavage of catechol. Also detection of cis-cismuconate, a product of 1, 2 dioxygenation of catechol together with lactonising enzyme activity suggests furthercleavage of catecholproceed via cis –cismuconate that undergone lactonisation forming succinate and acetyl CoA. No enzymatic activity was reported when checked for catachol 2,3dioxygenase indicating that isolated *P. stutzeri* strain mineralize catechol via ortho cleavage only.

Although napthalene degradation in some bacterial species proceeds predominately through meta fission of catechol, when supplied as sole carbon and energy source (Garcia–valdez et al., 1989., Williams et al., 1975) but degradation through ortho pathway is not uncommon. Earlier study with *Pseudomonas putida* strain ND6 showed that it able to use both metaandortho cleavage pathway for catechol, during napthalene mineralization process (Li et al., 2012). Other bacterial species such as Pseudomonas sp.s15pl exclusively use only orhto cleavage pathway for

degradation of catechol (kiyoharaand Nagao, 1978). Bacterial species, including *Pseudomonas* and *Alcaligenes* also used ortho cleavage of catechol when grown on either napthalene (Startvolvoiv, 1985) and or its derivative H- acid (1-amino8hydroxy napthalene 3,6 disulphhonic acid) respectively as evidenced in recent study (Usha et al., 2012).

Benzoic acid was also detected in the metabolites of naphthalene by strain TSH1. This product has been found in degradation of many aromatic hydrocarbons (Hamzah and Albaharna 1994; 1 and Suflita, 2000). Generally, benzoic acid was the decarboxylation products of phthalic acid. We not reported phthalate or benzoate as intermediate in napthalene mineralization by *P. stutzeri*.

Table 1: Morphological and biochemical characteristics of isolated PAHs degrading consortia

Napthalene Isolates / **Characteristics**	**N1**	**N2**	**N3**	**N4**	**N5**
Gram staining	Gram Negative,	Gram Positive,	Gram negative	Gram Positive,	Gram Negative
Shape	Bacillus	Rods	Rod	Bacilllus	Bacillus
Motility	+	+	+	+	+
Lipase	_	_	_	_	_
Catalase	+	+	+	+	+
Urea	_	+	_	+	+
Citrate	+	+	_	+	+
MR	_	_		+	+
VP	_	_		+	+
Indole	_	_	_	_	_
Growth on starch			+		
D-maltose	_	+	+	+	_
D-Glucose	+	+	+	+	+
Gelatin Liquification	–	+	_	+	+
Oxidase	+		+		+
Culturres Identified As	*P.fluorescence*	*Corynebacterium*	*P. stutzeri*	*Corynebacterium*	*P. aeruginosa*

Table 2: List of metabolites

Compounds	**Growth of isolates**
Napthalene	Carbon and energy source
Salicylates	Carbon and energy source
GentisateNo growth	
Catechol	carbon and energy source
Cis- cismuconate	carbon and energy source .

Table 3: Specific activity of napthalene metabolizing enzymes in crude extract of *P. stutzeri*isolated from oil contaminated soil.

Enzyme	Specific activity (µmole/min/mg of proteins)
Napthalenedioxygenase	0.072
1,2 dihydroxynapthalenedioxygenase	2.31
Salicyldehyde dehydrogenase	0.574
Salicylate hydroxylase	0.205
Saliylate 5 hydroxylase	-
Catechol 1.2 dioxygenase	0.942
Catechol 2,3 dioxygenase	-
Lactonizing enzyme	0.48

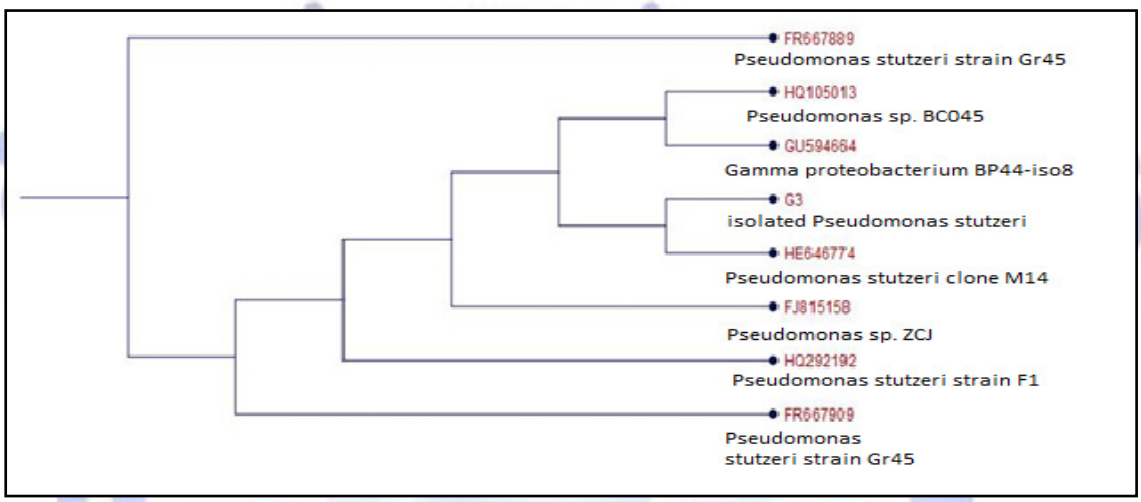
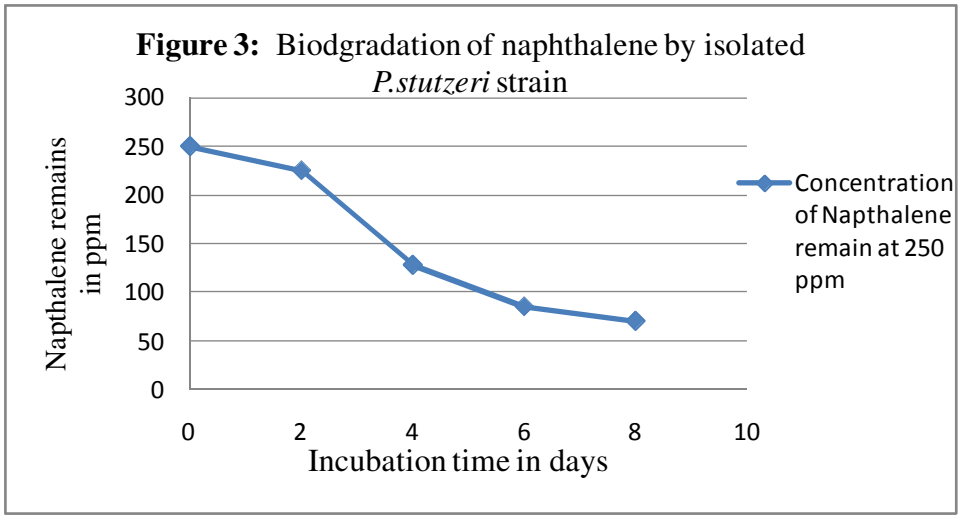


Figure 1: Dendrogram showing relationship with isolated *P. stutzeri* with closely related sequences collected from the Gene Bank



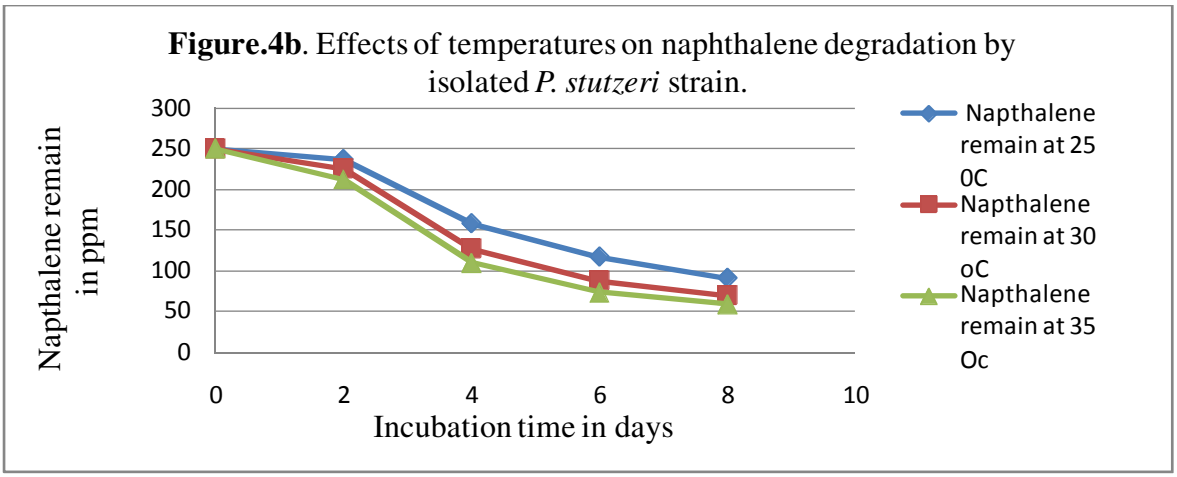


Figure.4b. Effects of temperatures on naphthalene degradation by isolated *P. stutzeri* strain.

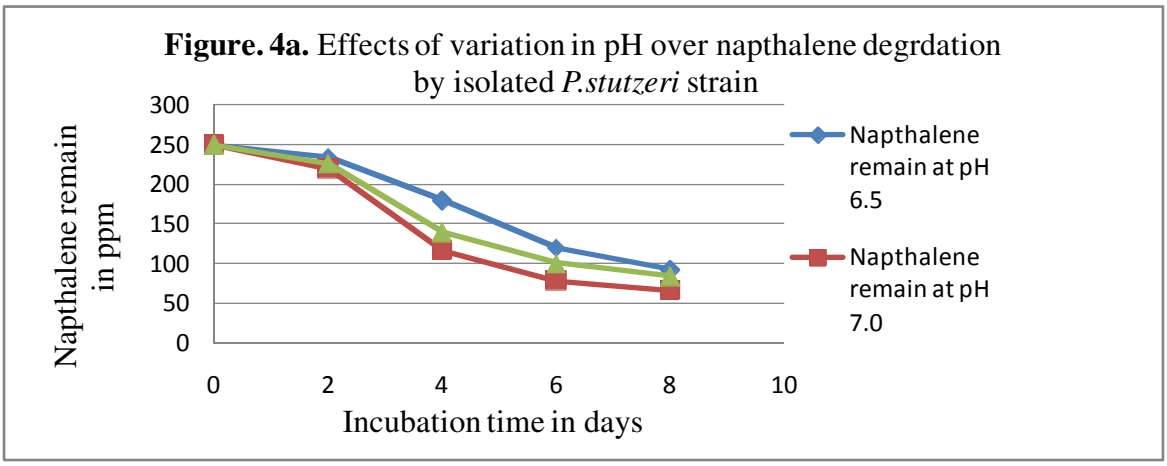


Figure. 4a. Effects of variation in pH over napthalene degrdation by isolated *P.stutzeri* strain

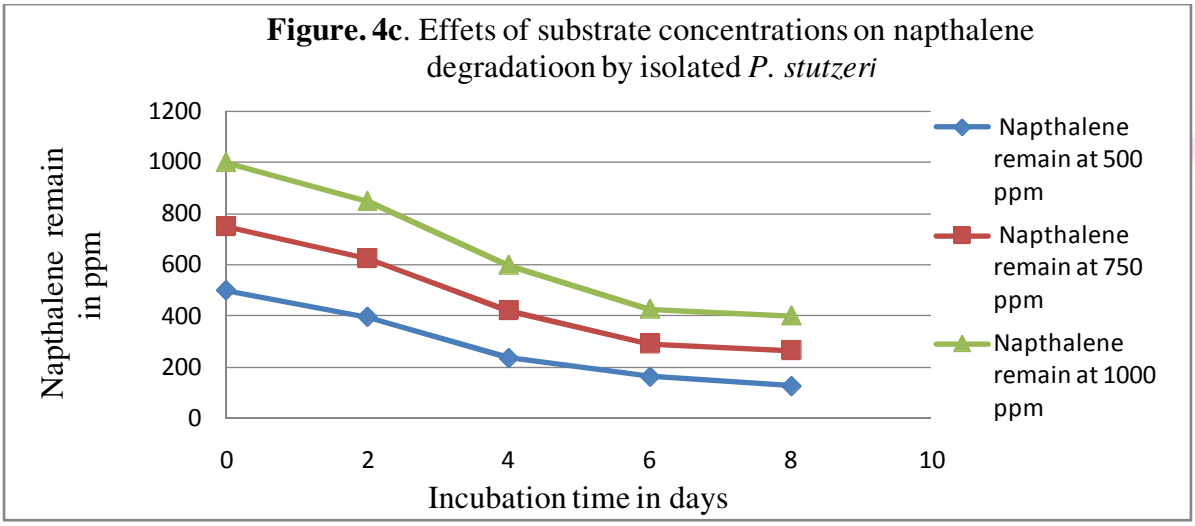


Figure. 4c. Effets of substrate concentrations on napthalene degradatioon by isolated *P. stutzeri*

Conclusion:

The studies aimed at application of suitable bacterial isolate for degradation of naphthalene contained in soil or water for environment remediation and study the biochemical pathways through which uptake, metabolism and degradation would takes

place. Since the study results were to be used for application in the field, the culture was isolated from field samples collected from oil/hydrocarbon contaminated sites. Isolation, identification and enrichment of selected strains were carried out using standard procedures. Bacterial isolates namely *P. stutzeri* were identified as dominant species and were found effective in degrading napthalene studied as representative of polycyclic aromatic hydrocarbons in the laboratory experiments.

Possible metabolic pathways used by isolated culture for degrading naphthalene was also studied Metabolic characterization of strain based on intermediates detected and enzymatic activities reported, suggests napthalene degradation by *Pseudomonas stutzeri* proceed through classical pathway using orhtho cleavage of catechol leading to TCA cycle intermediates.

It is also reported that metabolism leading to mineralization in some microorganisms is not complete and intermediates accumulated as dead end products. However, contrary to this, the isolate under study showed complete metabolism of the subjected substrate.

Environmental conditions influence the potential of cultures to degrade PAH, However under almost all the testing conditions the cultures was able to degrade PAH up to highest tested concentration i.e. 1000 mg/l.The optimal conditions were determined to be at pH 7.0, temperature 35 ^{0}C and napthalene concentration of 500 ppm after eight day of incubation.

The laboratory studies under controlled conditions showed promise by the isolates to degrade naphthalene to reasonable extent 72% at given pH and temperature. However, in the field conditions here ambient temperature vary from 25^0-35^0 C and pH may change depending on the soil conditions, applications of this consortia for remediation of oil contaminated soil will be engineering challenge.

The study result enables the researchers to use these isolate for application to contaminated soil with proper contact and retention time. There are instances of land and water bodies being contaminated by petroleum and crude oil hydrocarbon containing heavy molecular weight compounds mixed with aromatic hydrocarbons.

Though this study could not include technology development for application, the evidence of biochemical degradation of PAH by isolated cultures in the laboratory conditions encourages for its scale up for field sale application. Enrichment followed by mass culture and its application to contaminated site with a proper contact period will be new research area that can be envisaged from this study.

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