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Genetic Basis of Naphthalene and Phenanthrene Degradation by Phyllosphere Bacterial *Strains Alcaligenes* faecalis and *Alcaligenes* sp. 11SO

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Abstract

Two bacterial strains, *Alcaligenes feacalis* and *Alcaligenes* sp. 11SO isolated from the phyllosphere of four ornamental plant species, *Ixora chinensis*, *Ervatamia divaricata, Hibiscus rosa-sinensis* and *Amaranthus cruentus* found in five highly polluted sites in Sri Lanka, showed a higher level of phenanthrene and naphthalene degradation ability. Both these strains harbor plasmids conferring them resistance to ampicillin. Curing of these strains of their plasmid drastically reduced the ability to degrade the hydrocarbons. Upon transformation of these plasmids into *E.coli* JM109 enable it to degrade the two hydrocarbons efficiently. Plasmid encoded phenanthrene and naphthalene degradation suggested the presence of required catabolic genes in these plasmids. PCR amplification with degenerate primers and comparison of their nucleotide sequences with Genbank sequences indicated that plasmids of those bacterial strains harbor the genes *nahR*, *nahU* involved in naphthalene degradation and *phnG* for phenanthrene degradation. RFLP and nucleotide sequence comparison of *nahU* and *nahR* amplicons revealed that both of these genes in two bacterial strains are homologous. But, *phnG* gene copies of two bacterial strains exist as two distinct alleles.

Keywords: Plasmid; *nahR* gene; Phyllosphere bacteria; Phenanthrene; Naphthalene; *Alcaligenes* sp.11SO

Introduction

Polyaromatic hydrocarbon (PAH) pollution is a highly concerned environmental problem in the world. Naphthalene and phenanthrene are the highly abundant PAHs in the ambient air due to the vehicular emission, industrial processes and oil refining processes. Naphthalene is the simplest polycyclic aromatic hydrocarbon member of a widespread, well-studied class of environmental pollutants [1,2]. PAHs in the air, deposit on the ground level by the wet deposition and dry deposition and phyllosphere is one of the major exposing surfaces to these PAHs. The microorganisms colonizing the phyllosphere of these polluted areas are able to degrade polyaromatic hydrocarbons. Out of many phyllosphere microorganisms, bacterial strains have been shown to degrade environmental contaminants and their degradation genes are often located on the catabolic plasmids [3]. For example, *P. putida* NCIB 9816 is a well-characterized bacterium capable of utilizing naphthalene as sole carbon and energy source [4-7]. This trait is conferred in *P. putida* NCIB 9816-4 by an 81 kb plasmid, pDTG1. That encodes key enzymes in early enzymatic steps in naphthalene degradation [5-8]. The structural genes encoding naphthalene-degrading pathway enzymes in a variety of bacteria are highly conserved [6,9-11].

The NAH7 naphthalene catabolic plasmid in *Pseudomonas putida* G7 has been well characterized [6,8,12,13]. Analysis of the NAH7 plasmid [13] showed that *nah* operons are divided into two clusters. The genes of upper operon have genes encoding enzymes that convert naphthalene to salicylate and the lower operon has genes encoding enzymes that convert salicylate to tricarboxylic acid.

Isolated a phenanthrene degrading bacterium [14], *Alcaligenes faecalis* AFK2 which was able to degrade phenanthrene but it was unable to degrade naphthalene. The phenanthrene degradation genes of *Alcaligenes faecalis* AFK2 have since been sequenced and are unique with regard to both the gene organization and sequence similarity of genes when compared to other published sequences. Furthermore, the *phn* genes of *Burkholderia* sp. strain RP007 encode the enzymes for phenanthrene degradation [15].

However in Sri Lanka there are no such recorded studies. So that the present study was carried out with an attempt to investigate the presence of *nahR* and *nahU* genes involved in naphthalene degradation and *phnG* gene required for phenanthrene degradation in *Alcaligenes feacalis* and *Alcaligenes* sp.11SO, phenanthrene and naphthalene degrading two bacterial strains isolated from the phyllosphere of some ornamental plants (*Ixora chinensis*, *Ervatamia dervaticata*, *Hibiscus rosasinensis* and *Amaranthus cruentus*).

Methods and Materials

Sampling sites

Leaves of four plant species, *Ixora chinensis*, *Ervatamia dervaticata*, *Hibiscus rosasinensis* and *Amaranthus cruentus* from five polluted sites in Sri Lanka, Colombo fort, Orugodawattha, Maradana, Panchikawattha and Sapugaskanda were collected to isolate PAH degrading phyllosphere bacteria. Meemure an isolated less polluted remote village was selected as control site.

Isolation of phyllosphere bacteria

Each leaf sample (4 g) was washed with 100 ml of phosphate buffer and then shaken at 200 rev/min for one hour. Then the diluted samples were directly added to the modified mineral salt agar plates. Plates were then incubated at room temperature (28°C-30°C) for five days. Bacterial colonies were streaked on PAH added Bacto-Bushnell Haas medium to select PAH utilizing bacteria.

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Selection of efficient PAH degrading bacteria

The best PAH degrading bacterial strains were selected based on the results obtained from the colorimetric and HPLC methods indicated below.

Colorimetric assay

Each bacterial strain was inoculated into Bacto Bushnell-Haas broth incorporated with PAH compound (1%v/v) and Methylene blue (2%v/v), the redox indicator and incubated at room temperature (28°C-30°C) with constant shaking at 180 rev/min, for 14 days with a control without bacterial inoculation. From broth culture 5 ml sample was centrifuged at 6000 rev/min for five minutes. The recovered supernatant was assayed spectrophotometrically by measuring absorbance at 609 nm for the residual hydrocarbon. Six replicates were done for each bacterial strain and PAH degradation percentage was determined using the following equation [16].

$$\text{Percentage of PAH degradation} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

HPLC determination of PAH degradation

Each bacterial strain was inoculated into Bacto Bushnell-Haas broth incorporated with PAH (phenanthrene and naphthalene) compound (100 ppm). Then it was incubated at room temperature (28°C-30°C) with constant shaking at 180 rev/min, for 14 days with a control without bacterial inoculation.

Residual PAH in the culture was extracted with hexane and acetone containing mixture. Then extract was analyzed by high performance liquid chromatography (HPLC) equipped with UV detector. Analytical column (250 mm long, 4.6 mm diameter) was packed totally porous spherical C-18 material (packed size, 5µm). Acetonitrile-water mixture (75: 25) was used as mobile phase for PAHs at a flow rate of 1.0 ml min^{-1}. Sample (20 µL) was injected into column through sample loop. UV - detector was set at 254 nm for compound detection. The Chromeleon chromatography software was used for quantification of PAHs throughout the experiment. Finally percentage of degradation was determined.

Isolation of catabolic plasmid and confirmation of plasmid encoded PAH degradation

Catabolic plasmids of *Alcaligenes faecalis* and *Alcaligenes* sp.11SO were isolated according to the method of [17] and each plasmid was transformed into the *E.coli* JM109. PAH degrading ability of transformants were tested using colorimetric and HPLC method to determine the plasmid based PAH degradation. Plasmid based PAH degradation was confirmed by curing the plasmid of bacterial cells using acridine orange [18] and then testing their PAH degradation ability by colorimetric and HPLC methods.

PCR amplification of *nahR*, *nahU*, *phnG* and *phnAc* regions in catabolic plasmid

Plasmid isolation, gel electrophoresis, transformation and amplification of DNA by PCR were performed by standard procedures [17]. The conserved regions of G7-*nahR* (*P. putida* G7), *nahR* (*P. stutzeri* AN10) and NCIB-*nahR* (*P. putida* NCIB 9816-4) (GenBank accession no.s M22723, AF039534 and AF491307, respectively) have been used to design the degenerate primers for PCR amplifying *nahR* genes. The conserved regions of pND6-*nahU* (*P. putida* ND6) (GenBank accession no AAP44249) genes has been used to design the degenerate primers for amplifying *nahU* gene and the conserved region of GZ38-*phnG* (*P. putida* G7) (GenBank accession no AF112137) has been used to design the degenerate primers of PCR for amplifying *phnG* genes. The conserved region of *Burkholderia* sp. strain RP007 (*phnAc* (AF061751) and *Alcaligenes faecalis* AFK2 (*phnAc* (AB024945) have been used to design phnAc primers. Sequence of these primers are, nahR-F, 5«-CGCGAATTCATGGAACTGCRTGAYCTGGA-3«,nahR-R,5«-CGCGAATTCTCAATCMGWAAACAGSTCRAAC-3«. nahU-F, 5<<- GGAGACATCATATGCAAAATTCTACTTCTGCTCTGAA-3',nahU-R, 5'-CCAATCTCGAGGGCCGCTTGCGCGC-3', phnAc-F, 5<<- TTCGAGCTGGAATGTGAGC-3', phnAc-R 5<<- AATAACCG-GCGATTCCAAAC-3', phnG-F, 5<<- GGAAGGATCCGAATTCAT-TAAAGAGGAGAAATTAACTATGCACGCAGATACCGCGACCC-GCCAGCACTGGATGTCCG-3', phnG-R, 5<<- TGGTTGGGATCCC-GAGCCATGGTTATTAATGGTGATGGTGATGGTGTGCGTT-GTCTCCGCGAACCATCGTAAAG AGTCGACCC A-3'.

Plasmid DNA templates of *Alcaligenes faecalis* and *Alcaligenes* sp.11SO were amplified using above primers. Then PCR products were visualized and their size estimation was done by gel electrophoresis.

Sequencing of PCR products

Automated sequencing was carried out using applied Biosystems automated sequencer (ABI3730XL) at Macrogen, Seoul, Korea. Purified PCR products representing *nahR*, *nahU*, and *phnG* gene segments were sequenced directly using appropriate primers.

Restriction digestion of PCR products

PCR amplicons of *nahR, nahU* and *phnG* genes were digested using *HindIII* restriction enzyme to determine their RFLP patterns.

Computer analysis

Nucleotide sequences of *nahR*, *nahU* and *phnG* gene segments were aligned with gene sequences of Genbank using BLAST [19].

Results and Discussion

PAH degrading phyllosphere bacterial population of the leaf samples collected from five highly polluted sites were much higher compared to that of less polluted control site. Colorimetric test and HPLC analysis results revealed that out of isolated 38 bacterial strains twenty could degrade one or both of tested two PAH compounds, naphthalene and phenanthrene. Eight of these PAH degraders belong to five genera *Alcaligenes, Pseudomonas*, *Serratia*, *Bacillus* and *Acenatobacter* which showed relatively higher degradation ability of these chemicals (Figure 1). Furthermore, *A.feacalis* (KT356811) had the highest naphthalene (92.9%) and phenanthrene (89.6%) degradation

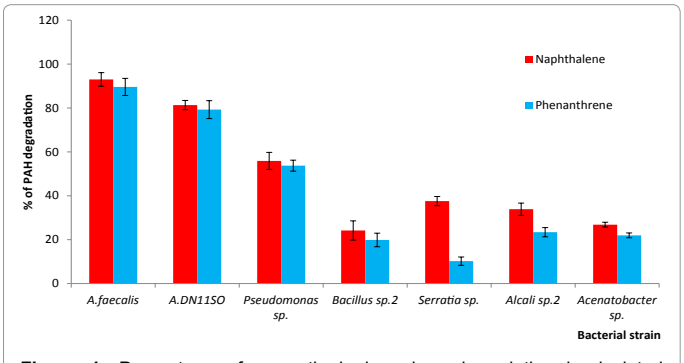

Figure 1: Percentage of aromatic hydrocarbon degradation by isolated bacterial strains, determined by HPLC method, N=10.

ability (Figure 1). *Alcaligenes* sp.11SO (KT356809) also had higher naphthalene (81.32%) and phenanthrene (79.24%) degradation ability compare to other bacterial strains. According to the literature [20] most of the naphthalene degraders were *Pseudomonas* sp. and *Alcaligenes* sp. were the predominant phenanthrene degraders. But the present investigation showed significantly high efficiencies of the two isolated *Alcaligenes* sp. in degrading both naphthalene and phenanthrene.

These two bacterial strains harbor an approximately 23 kb plasmid. Upon transformation of these plasmids into *E.coli* JM109 strain, it's PAH degradation ability was similar to that of original organism. Further, after curing of plasmids, the two *Alcaligenes* sp. lost their PAH degradation ability. These results revealed that PAH degradation ability of *Alcaligenes faecalis* and *Alcaligenes* sp.11SO was a plasmid encoded character. Therefore, these plasmids should harbor naphthalene and phenanthrene catabolic genes *nah* and *phn* respectively.

nahR gene fragment analysis

Expected 921 bp *nahR* gene fragment [21] was observed in gel electrophoresis of PCR amplicons of *nahR* genes of *Alcaligenes faecalis* and *Alcaligenes* sp.11SO (Figure 2). Therefore, both of these *Alcaligenes* strains may harbor *nahR* gene on their catabolic plasmids.

According to the literature, *nahR* gene was predominant in the plasmids of *Pseudomonas* strains. For example *Pseudomonas putida* G7 haboured *nahR* gene on their NAH7 plasmid [22,23] *P. putida* NCIB 9816- 4, harboured *nahR* gene in their plasmid pDTG1 [6,7]. The two phyllosphere bacterial strains, *Alcaligenes faecalis* and *Alcaligenes* sp.11SO were the best AH degraders having plasmid born *nahR* genes responsible for naphthalene degradation.

PCR amplicons of *nahR* gene were sequenced and their nucleotide sequences were compared with the nucleotide sequences of *nahR* genes deposited in Genbank. Plasmid- haboured *nahR* gene of *Alcaligenes* sp.11SO had 79% sequence similarity to the *nahR* gene located on the pDTG1 plasmid of *P. putida*. Nucleotide sequence of *nahR* amplicon of *Alcaligenes faecalis* is almost close (88% sequence similarity) to the *nahR* gene of plasmid pND6 in *Pseudomonas* sp. *nahR* genes of two *Alcaligens* sp. were similar to the *nahR* gene located on the plasmid of

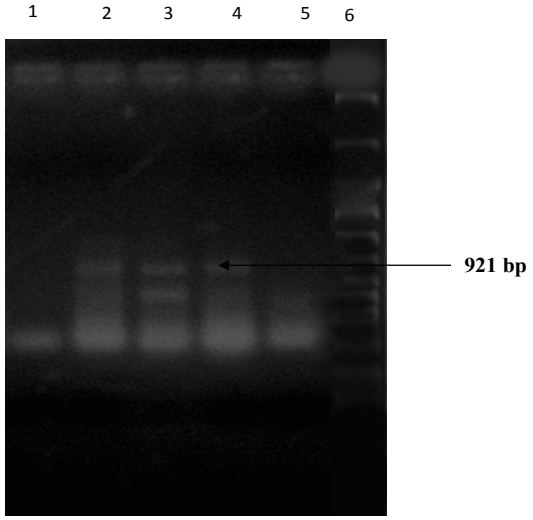

Figure 2: Gel electrophoresis of PCR products obtained with primers for *nahR* gene and plasmid DNA templates Lane 1-Control, Lane 2 - *Alcaligenes faecalis*, Lane 3 - *Alcaligenes* sp.11SO and Lane 6- Lambda *HindIII* ladder.

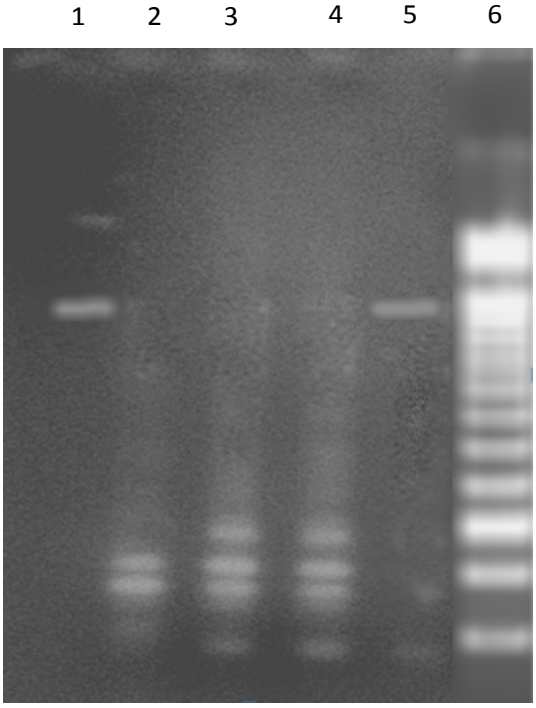

Figure 3: Gel electrophoresis of *HindIII* digested PCR amplicons of *nahU* gene fragment, Lanes: 1, non-digested 712-bp nahU PCR product from *A.faecalis*; Lane-3-digested PCR product from *Alcaligenes faecalis*, Lane-4-digested PCR product from *Alcaligenes* sp.11SO; Lanes-5, non-digested 712-bp nahU PCR product from *Alcaligenes* sp.11SO Lane 6 - 100-bp molecular weight marker.

Pseudomonas sp. Close sequence relationship of *Alcaligenes nahR* gene with the *nahR* genes of *Pseudomonas* sp. suggest that this gene may have transferred from *Pseudomonas* sp. to *Alcaligenes* sp. by conjugation at some stage of evolutionary process.

RFLP pattern obtained with *HindIII* digestion of *nahR* amplicon of *Alcaligenes faecalis* plasmid was similar to that of *Alcaligenes* sp.11SO plasmid. It revealed, *nahR* gene was homologous to each other and same gene type exists in these PAH degrading phyllosphere bacterial population.

nahU gene fragment analysis

Expected 712 bp *nahU* [20] gene fragment was observed in gel electrophoresis of PCR amplicons of both *Alcaligenes faecalis* and *Alcaligenes* sp.11SO. Therefore, both of these *Alcaligenes* strains harbor *nahU* gene on their catabolic plasmids. Presence of similar RFLP patterns in *HindIII* digest of these amplicons (Figure 3) indicating that both *Alcaligenes* sp. harbor similar *nahU* genes. *nahU* gene is an isofunctional gene located on the outside of the lower pathway operon [20].

phnG gene fragment analysis

Expected 652 bp [15] amplicons of *phnG* observed in PCR amplicons of two *Alcaligenes* sp. (Figure 4). Therefore, these two *Alcaligenes* spp. harbored *phnG* gene on their catabolic plasmids. Failures to amplify plasmid templates with *phnAc*-specific primers suggest the absence of *phnAc* gene on these catabolic plasmids.

All *phnG* amplicons of two bacterial strains were digested with *HindIII* enzyme. RFLP pattern of *HindIII* digest with phnG amplicon in *Alcaligenes faecalis* was different from that of *Alcaligenes* sp.11SO

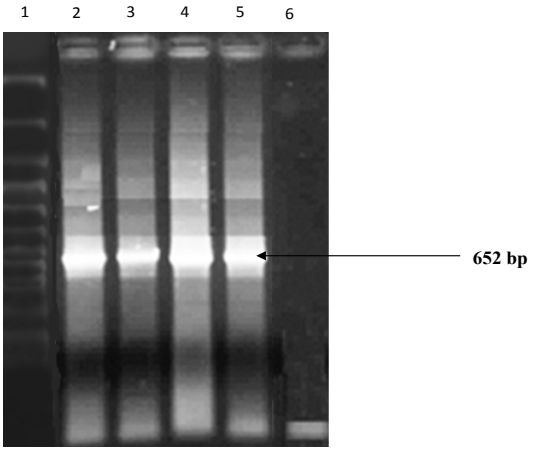

Figure 4: Gel electrophoresis of PCR products obtained with primers for *phnG* gene and plasmid DNA templates Lane 1-Control, Lane 2- *Alcaligenes faecalis*, Lane 3- *Alcaligenes* sp.11SO, Lane 6- Lambda *HindIII* ladder.

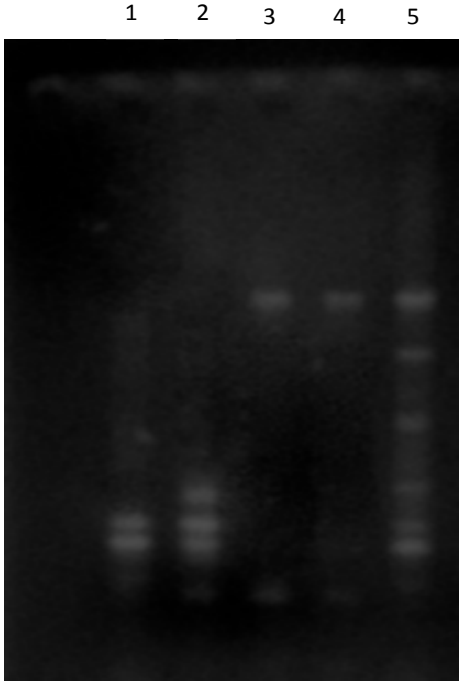

Figure 5: Gel electrophoresis of *HindIII* digested PCR amplicons of *phnG* gene fragment, Lane 1- digested PCR product from *Alcaligenes* sp.11SO, Lane 2- digested PCR product from *Alcaligenes feacalis,* Lanes: 3- non digested 652 bp *phnG* PCR product from *Alcaligenes* sp.11SO, Lanes: 4- non digested 652 bp *phnG* PCR product from *Alcaligenes feacalis* Lane-5 - MW, 100-bp molecular weight marker.

(Figure 5). Thus, *phnG* gene exists as two different alleles in these two strains enabling them to degrade phenanthrene.

The phyllosphere bacterial strains, *Alcaligenes feacalis* and *Alcaligenes* sp.11SO are different PAH degraders. Their phenanthrene and naphthalene degradation ability is a plasmid encoded character. Catabolic plasmids of these two strains harbored naphthalene specific *nahR* and *nahU* genes. The *nahR* and *nahU* genes of these two *Alcaligenes* sp. are homologous to each other. Since naphthalene is a simplest compound, it is easy to degrade to survive under harsh conditions [24,25]. Therefore, it may have limited allele types. But phenanthrene specific isofunctional gene *phnG* exists as two different allele types in the two species of *Alcaligenes*. Phenanthrene is a complex compound. Phenanthrene degradation ability of them was lower than naphthalene degradation. Therefore, they should have several variations in their genes to survive under harsh conditions. Therefore they may have different allele types. Although, these catabolic plasmids lack the gene *phnAc*, their ability to degrade phenanthrene suggests its chromosomal location in these strains. The ability of degradation of two chemicals (PAHs) by one organism is very important when applying them in to the bioremediation. Because, they can survive very well under the harsh conditions created by PAHs.

References

1. Sutherland JB, Rafii F, Khan AA, Cerniglia, CE (1995) Young LY, Cerniglia CE (eds.) Mechanisms of polycyclic aromatic hydrocarbon degradation. In Microbial Transformation and Degradation of Toxic Organic Chemicals, New York: Wiley: 269-306.
2. Shuttleworth KL, Cerniglia CE (1995) Environmental aspects of PAH biodegradation. Appl Biochem Biotechnol 54: 291-302.
3. Sayler GS, Hooper SW, Layton AC, King JM (1990) Catabolic plasmids of environmental and ecological significances. Microb Ecol 19: 1-20.
4. Cane PA, Williams PA (1986) A restriction map of the catabolic plasmid pWW60-1 and location of some of its catabolic genes. Microbiology132: 2919-2929.
5. Kurkela S, Lehvaeslaiho H, Palva ET, Teeri TH (1988) Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of Pseudomonas putida strain NCIB9816. Gene 73: 355-362.
6. Serdar CM, Gibson DT (1989) Studies of nucleotide sequence homology between naphthalene-utilizing strains of bacteria. Biochem Biophys Res Commun 164: 772-779.
7. Simon M J, Osslund TD, Saunders R, Ensley BD, Suggs S, et al. (1993) Sequences of genes encoding naphthalene dioxygenase in Pseudomonas putida strains G7 and NCIB9816-4. Gene 127: 31-37.
8. Connors MA, Barnsley EA (1982) Naphthalene plasmids in pseudomonads. J Bacteriol 149: 1096-101.
9. Bosch R, Garcia-Valdes E, Moore ER (1999) Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from Pseudomonas stutzeri AN10. Gene 236: 149-157.
10. Bosch R, Garcia-Valdes E, Moore ER (2000) Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from Pseudomonas stutzeri AN10. Gene 245: 65-74.
11. Goyal AK, Zylstra GJ (1996) Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from Comamonas testosteroni GZ39. Appl Environ Microbiol 62: 230-236.
12. Eaton RW (1994) Organization and evolution of naphthalene catabolic pathways: Sequence of the DNA encoding 2-hydroxychromene-2-carboxylate isomerase and trans-o-hydroxybenzylidenepyruvate hydratase aldolase from the NAH7 plasmid. The journal of bacteriology 176: 7757-7762.
13. Schell MA, Wender PE (1986) Identification of the nahR gene product and nucleotide sequences required for its activation of the sal operon. J Bacteriol 166: 9-14.
14. Kiyohara H, Nagao K , Kouno K, Yano K (1982) Phenanthrene-degrading phenotype of Alcaligenes faecalis AFK2. Appl Environ Microbiol 43: 458-461.
15. Laurie AD, Gareth LJ (1999) The phn Genes of Burkholderia sp. Strain RP007 Constitute a Divergent Gene Cluster for Polycyclic Aromatic Hydrocarbon Catabolism. J Bacteriol 181: 531-540.
16. Okafor UG, Tasie F, Okafor FM (2009) Hydrocarbon degradation potential of indigenous fungal isolated from petroleum contaminated soils. The journal of physical and natural science 3: 56-68.
17. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. Cold spring harbor laboratory press. Cold spring harbor, NY.

18. Fujii T, Takeo M, Maeda Y (1997) Plasmid encoded genes specifying aniline oxidation from *Acenetobacter* sp. strain YAA. Microbiology 143: 93-99.

19. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

20. Alquati C, Papacchini M, Riccardi C, Spicaglia S, Bestetti G (2005) Diversity of naphthalene-degrading bacteria from a petroleum contaminated soil. Annals of Microbiology 55: 237-242.

21. Park JW, Knoke KL, Noguera DR, Fox BG, Chambliss GH (2000) Transformation of 2,4,6- trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens*. Appl Environ Microbiol 66: 4742-4750.

22. Dunn NW, Gunsalus IC (1973) Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida. J* Bacteriol 114: 974-979.

23. Saito A, Iwabuchi T, Harayama S (1987) A novel phenanthrene dioxygenase from Nocardioides sp. Strain KP7: expression in Escherichia coli. J Bacteriol 182: 2134-2141.

24. Zhoa NY, Fuenmayor SL, Williams PA (2005) *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. j bacterial 183: 700-708.

25. Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence-analysis programs for the VAX. Nucleic Acids Res 12: 387-395.