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Analysis of the phylloremediation capability of *Mangifera indica* in hydrocarbon polluted area: An outlook study

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Abstract
Environmental pollution is reaching such great heights that it is a matter of global concern now. A phenomenon called phylloremediation was observed in plants, especially the ones that grow in polluted areas. Usually, plants growing in polluted areas themselves develop mechanisms to reduce the harmful pollutants in the surrounding to less harmful substances. This is done by the leaves and the microbes associated with the leaves either on their surface or inside the leaves. This project aims to analyze the phylloremediation capability in collected leaf samples from *Mangifera indica* growing in hydrocarbon polluted areas and to understand the various microbes associated with the leaves which are responsible for the degradation of air pollutants, the mechanisms involved and the genes or gene clusters which are responsible for this phenomenon. The procedure includes isolation of hydrocarbon-degrading bacteria from leaf samples, followed by its DNA and plasmid isolations, sequencing, and comparison with known gene sequences in databases using BLAST analysis. The whole process could be further enhanced by genetically engineering the selected microbe. Various genes that are already found to produce certain enzymes which can degrade one or more pollutants can be introduced into our selected organism's genome to improve the phylloremediation capability.

Keywords: Phylloremediation, *Mangifera indica*, hydrocarbon degraders, gene clusters, blast

1. Introduction
The contamination of the air by harmful or poisonous substances such that it greatly affects the health of all the living organisms on earth and at the same time harms the abiotic components of the ecosystem was referred to as air pollution. Environmental pollution is reaching such great heights that it is a matter of global concern now. The major reasons for such alarmingly increasing pollution rates are industrialization, increasing population, economic development, etc. The harmful effects of air pollution are not limited to health alone. It also affects the agriculture and economy which in turn have a great impact on human life.

Some of the major air pollutants are sulphur dioxide, nitrogen dioxides, carbon monoxide, ground-level ozone, particulate matter, and volatile organic compounds (Wei *et al*., 2017) [47]. There has been a lot of effort being put into by various sectors of the society in trying to minimize the air pollution, but still, it seems to be just increasing day by day. Hence, we direct our attention to the remediation of air pollutants by exploiting the capabilities of plants, their different parts, and microorganisms associated with the plants.

Air pollutants can be mitigated through biological means, i.e., bioremediation. Bioremediation is defined as the process whereby toxic substances are biologically degraded under controlled conditions to non-toxic limits established by regulatory authorities (Mueller *et al*., 1996) [28]. For effective bioremediation, enzymatic action of the microorganisms must take place. The major limitation of bioremediation is that it can take place only if the environmental conditions are suitable for microbial growth. Thus, there is a need for the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Sharma, 2012) [44].

We are all familiar with phytoremediation. Phytoremediation is the use of plants to clean up soil, water, and in some cases, air, polluted with hazardous contaminants. The term is a combination of the Greek word-Phyto (plant) and the Latin word remedium (restoring balance).

It can be mainly employed for the removal of contaminants such as polycyclic aromatic hydrocarbons (PAHs), munition waste such as TNT, salt (NaCl), etc. Although it is a proven technology, its global usage as of now is very much limited. This is mainly because although theoretically, it might seem efficient, in reality, there hasn't been able to redress any of the environmental issues to a significant extent (Gerhardt *et al*, 2017).

Phytoremediation is mainly employed in the clearing of soil and stagnant water bodies that are contaminated with substances like heavy metals (Cadmium, Lead, Arsenic, etc) (Feng *et al*., 2013) [15]. Despite its encouraging results in the past years, many challenges have been encountered in phytoremediation in the field.

Phytoremediation is increasingly adopted in the treatment of soil and water pollution. However significant advances in the efficiency of the method are to be made for making phytoremediation a viable, economical option for pollution control.

All the above processes of phytoremediation are meant to reduce or eliminate mostly pollutants or contaminants in water and soil. In other words, phytoremediation mainly concentrates on the reduction of water pollution and soil pollution. In later researches, a different form of phytoremediation, called phylloremediation was observed in plants, especially the ones that grow in polluted areas. Usually, plants growing in polluted areas themselves develop mechanisms to reduce the harmful pollutants in the surrounding to less harmful substances. This is done by the leaves and the microbes associated with the leaves either on their surface or inside the leaves. Both leaves and the microbes can individually control air pollution but their combined effect enhances the process. Thus, phylloremediation is meant to reduce air pollutants whereas phytoremediation could deal with water and soil contaminants.

Phylloremediation is defined as “a natural process of bioremediation of air pollutants through leaves and leaf-associated microbes, not the microbes or leaves alone.” (Wei *et al*., 2017) [47].

During the last three decades, air pollution is increasing due to economic growth and urbanization. Various policies and practices have been brought for the remediation of this pollution, but the air is still polluted. In this review, we are finding a way to reduce pollution using the potential of plant leaves and leaf-associated microbes. Plant leaves are capable of absorbing air pollutants or adsorbing them onto their surfaces and microbes on leaves are capable of converting the pollutants into non-toxic molecules (Wei *et al*., 2017) [47].

2. Review of literature

A variety of microbes are found in the various parts of the plants. They include bacteria, fungi, yeast, algae, protozoa, nematodes. These microbes can either be present inside the leaf tissues or on the surface of leaves. Phyllosphere is the aerial habitat colonized by microbes. Though all types of microbes are found on the leaves, the majority of them are bacteria. Leaves provide a vast surface area for microbes. The surface area provided by leaves alone for microbes is around 6.4x10^8 km sq. (Lindow and Brandl, 2003) [8].

Epiphytic bacterial populations differ sharply in size among and within plants of the same species, as well as nearby. These variations in population sizes are due to large fluctuations in the physical and nutritional conditions of the phyllosphere (Lindow and Brandl, 2003) [8].

2.1 Alteration of plant surface properties

Bacteria can produce compounds with surfactant properties. Hence, they increase the wettability of leaves (Neu *et al*., 1990; Hutchison *et al*., 1993; Bunster *et al*., 1989) [29, 23, 9]. The cuticle is hydrophobic. Thus, the increased wettability of the leaf surface helps improve the solubility and diffusion of substrates. These can prove to be beneficial to the epiphytes. The bacterial movement on the surface of the leaves is also facilitated. The watery layer on the leaf surface produced by these surfactants helps the bacteria to move to places where nutrients are abundant.

Another factor commonly observed in some species which are mostly found as epiphytes are the production of the toxin, syringomycin by Pseudomonas syringae pv. syringae (Quigley *et al*., 1994) [33]. This toxin induces the formation of ion channels. The plant metabolites are released and it leads to cell lysis (Hutchison *et al*., 1995) [24]. Nonpathogenic strains of P. syringae produce syringomycin at levels that won't cause cell death but are enough to trigger the release of plant metabolites (Hutchison *et al*., 1995) [24].

Many of the bacterial colonisers of the phyllosphere can produce indole-3-acetic acid, which is a plant growth regulator (Brandl *et al*., 2001) [8]. This is of great importance as the bacteria colonising the leaves produce growth regulators essential for the plants and which are similar to the auxins produced by plants themselves (Lindow *et al*., 2003) [8].

2.2 Development of phylloremediation technologies

So, as mentioned above, it is established that plants and microbes can individually contribute to the minimization of air pollutants, but their combined effort can accelerate this process. A series of steps have been introduced to develop phylloremediation technology which is as follows:

1. Plant selection
2. Microbe selection
3. Evaluation and compatibility between plant leaves and microbes
4. Implementation (Wei *et al*., 2017) [47]

In their work, Wei and others selected plants from four categories: trees, shrubs, ground cover plants for use in outdoor environments, and foliage plants for outdoor environments.

Each plant responds to pollutants differently. Their tolerance levels vary and this is evaluated using the air pollution tolerance index (Singh *et al*., 1991) [45]. Plants from each category were evaluated to check their tolerance against pollutants like PMs, O_3, SO_2, NOx, and VOCs and their capability to assimilate these (Wei *et al*., 2017) [47]. Evaluation is done by exposing the seedlings to various pollutants at different concentrations and the analysis is made on factors like stomatal conductance, photosynthetic rate, morphological analysis, the maximum quantum efficiency of photosystem II, etc. (Wei *et al*., 2017) [47].

After analysis, suitable plants with greater abilities to assimilate or absorb or adsorb pollutants and those with greater tolerance levels are selected. Thereafter, a suitable microbe is to be selected which is compatible with the selected plant. Some bacteria reside on the surface of the leaves and some others, called the endophytes, reside inside the plant tissues without harming the plants. Since endophytes are not washed away easily by any forms of precipitation, these are more desirable (Wei *et al*., 2017) [47].

The whole process could be further enhanced by genetically engineering the selected microbe. Various genes that are

already found to produce certain enzymes which can degrade one or more pollutants can be introduced into our selected organism's genome to improve the phylloremediation capability. An example of this is the work done by Barac and others. When appropriate degradation pathways were introduced into the endophytic bacteria, they showed improved degradation of toluene. They introduced the pTOM toluene-degradation plasmid of *Burkholderia cepacia* G4 into B. cepacia L.S.2.4, a natural endophyte of yellow lupine. The engineered endophytic bacteria strongly degraded toluene (Barac *et al*., 2004) [6].

Once the microbes are selected, they could be domesticated by exposing them to varying pH, carbon source, temperature, and oxygen. Their phylogenetic relationships with other microbes should be determined. Other factors to be determined and evaluated in the laboratory include their utilization of organic compounds, the decomposition rate of pollutants, adaptability, competition, and growth rate (Wei *et al*., 2017) [47].

The major step following the selection and evaluation of both the plants and microbes in the evaluation of the compatibility between them. The microbes are inoculated into the selected plants to see if they can grow well on the leaf surfaces and at the same time to check if the inoculation affects plant growth (Wei *et al*., 2017) [47]. Once compatible combinations are obtained, they are further tested by exposure to pollutants. The desired microbes can be propagated using bioreactors and plants by tissue culture or cuttings. For real-world testing, these plants could be transplanted to outside polluted environments or greenhouses (Wei *et al*., 2017) [47].

2.3 Hydrocarbon pollution

Any compound which consists of carbon and hydrogen atoms is termed a hydrocarbon. They are organic compounds and some of the forms are gasoline, petroleum, coal, kerosene, charcoal, natural gas, etc. The majority of the air pollution is caused by the automobiles that run on these hydrocarbon-based fuels. It is the incomplete combustion of these hydrocarbon fuels that causes pollution. Hydrocarbons react with oxides of nitrogen to form gases which when are hit by the sun's rays leads to the formation of ozone, which is a heavy form of oxygen and settles down in the atmosphere. Hence, the polluted air feels heavy. The incomplete combustion of hydrocarbons thus affects the quality of air in several ways and causes severe health issues.

2.4 Hydrocarbon degrading bacteria

Hydrocarbons are present on the surface of plant parts, either as by-products or end products of metabolism. Leaves have a waxy coating that contains hydrocarbons (Ilori *et al*., 2006).

Microorganisms with the capability to oxidize various hydrocarbons are widely distributed. The presence of hydrocarbon on leaves attracts more hydrocarbon degraders to colonize it. Thus, there is an increase in the populations of hydrocarbon degraders. These microbes end up on these surfaces by the action of rain, wind, vector, or brush contact (Atlas, 1978) [5]. Thus, such phylloplane act as training sessions for such hydrocarbon-degrading bacteria (Ilori *et al*., 2006).

The large number of hydrocarbon degraders on the leaves studied indicate that these organisms have been exposed to hydrocarbons well in advance, which would be mainly from the leaves. Organisms, however, reach the leaf surfaces through dust, air current, and rain splashes. The presence of nutrients such as exudates or waxes from the cuticle is the driving factor for the establishment of these microbes on the new phylloplane (Rao, 1977) [34].

The seven different genera of hydrocarbon degraders isolated from the different plants that Ilori and others studied were: Acinetobacter, Flavobacterium, Bacillus, Pseudomonas, Alcaligenes, Corynebacterium, and Micrococcus (Ilori *et al*., 2006).

Out of these, the ones that were isolated from *Mangifera indica* and thus found to be compatible with the phylloplane of *Mangifera indica* were: Pseudomonas sp., Alcaligenes sp., and Micrococcus sp. (Wei *et al*., 2017) [47].

2.5 *Pseudomonas citronellois*

Pseudomonas citronellolis can degrade hydrocarbons such as isoprenoid compounds including citronellol (Seubert *et al*., 1960) [43]. And hence it got its present name. It was also found to be able to degrade complex oily sludge contaminations (Bhattacharya *et al*., 2003) [7]. Thus, the species might have the potential to serve as bioremediation organisms to clean up oil-contaminated sites.

The strain P3B5 was isolated from plant leaf material. Plant leaves are covered by a cuticle. This cuticle consists of an esterified aliphatic polymer called cutin. It is overlaid by intra- and epi-cuticular waxes consisting of very-long-chain aliphatic compounds, including alkanes, alcohols, and fatty acids (Remus-Emsermann *et al*., 2016) [35]. *P. citronellolis* can degrade aliphatic compounds too. Thus, there is a possibility that the bacterium may be able to exploit aliphatic compounds as a nutrient source during leaf surface colonization (Remus-Emsermann *et al*., 2016) [35]. Furthermore, *P. citronellolis* were also found to have the ability to degrade terpenoid compounds (Forster-Fromme and Jendrossek, 2006) [16].

Two Alk-B-like alkane monooxygenase genes are present in the genome of *Pseudomonas citronellol*is, which might have the potential to nutritionally access long and very-long-chain alkanes available on plant leaves (Remus-Emsermann *et al*., 2016) [35].

Alkane 1-Monooxygenase 1

Gene sequence which encodes the protein Alkane 1-monooxygenase 1 is given below

ATGAAAGCGCTGCATTATCTGAAATATTTTCTGTTTC
ATGCGGTGGGCCTGCTGAGCGCG
GCGGCGCTGCTGGCGGGCGGCAGCTGGATTGCGTGC
GGCCTGGCGGCGGTGCTGACCATT
TATGTGCTGGGCGATCTGGTGTGCGGCGATGATACC
AGCACCCCGGAATTTCATCATCCG
GGCATTCTGACCTTTCAGCTGTGGCTGGCGCTGCCGC
TGCTGTGCTTTATTGTGTTTGCG
GCGCTGTGGGGCGTGAGCGATGGCGATTTTCTGGGC
TTTGGCGCGGCGCTGGGCCAGGCG
ACCGGCTATGATCTGATTGCGGCGCGCGATGCGACC
AGCCTGGGCCAGCATGTGTGCGCG
TGGATTCTGACCGGCCTGGTGATTGGCACCATTGGC
ACCATTACCGCGCATGAACTGACC
CATCGCACCTGGGATCCGGTGAGCATGCTGATTGGC
CGCTGGCTGCTGGCGTTTAGCTTT
GATACCATTTTTAGCATTGAACATGTGTATGGCCATC
ATCGCTATGTGAGCACCACCGAA
GATCCGGCGACCGCGCCGCGCGGCCGCAACGTGTAT
GCGCATGTGCTGATTAGCACCGTG
CGCGGCAACCTGAGCGCGTGGCATATTGAAAAACAG
CGCCTGGCGCGCAAAGGCTATTGG
CTGCTGGGCTGGCGCAACGCGGTGCTGCGCGGCCAT

GGCATGAGCCTGCTGCTGGTGGCG
GCGGCGTATGCGATTGGCCAGTGGCCGGCGGCGCTG
TTTTTTATTGCGTGCGCGCTGTGG
GGCAAAGCGCTGCTGGAAATTGTGAACTATATGGAA
CATTATGGCATGGTGCGCAACCCG
GCGACCCCGGTGCAGCCGCGCCATAGCTGGAACACC
AACAAACGCATTAGCAGCTGGACC
ATGTTTAACCTGACCCGCCATAGCCATCATCATGCG
CAGGGCGAAGTGCCGTATCAGGAT
CTGAAACCGTTTCCGGATGCGCCGATGATGGTGGGC
GGCTATCTGACCACCATTATTGTG
GCGATGCTGCCGCCGCTGTGGCATCGCCTGATGACC
CCGAAAGTGCTGGCGTGGGATCGC
GAATATGCGAGCGCGGAAGAACGCCAGCTGGCGGC
GACCGCGAACGCGCGCAGCGGCATT
CCGGCGCTGATGAAATATAGCAACTATCCGAGCCCG
AGC
(Retrieved from https://www.bioinformatics.org/sms2/rev_trans.html)

The fasta format of the protein Alkane 1- monooxygenase 1 is given below

MKALHYLKYFLFHAVGLLSAAALLAGGSWIACGLAA
VLTIYVLGDLVCGDDTSTPEFHHPGILTFQLWLA
LPLLCFIVFAALWGVSDGDFLGFGAALGQATGYDLIAA
RDATSLGQHVCAWILTGLVIGTIGTITAHELT
HRTWDPVSMLIGRWLLAFSFDTIFSIEHVYGHHRYVST
TEDPATAPRGRNVYAHVLISTVRGNLSAWHIE
KQRLARKGYWLLGWRNAVLRGHGMSLLLVAAAYAI
GQWPAALFFIACALWGKALLEIVNYMEHYGMVRNP
ATPVQPRHSWNTNKRISSWTMFNLTRHSHHHAQGEVP
YQDLKPFPDAPMMVGGYLTTIIVAMLPPLWHRL
MTPKVLAWDREYASAEERQLAATANARSGIPALMKY
SNYPSPS
(NCBI, accession no. AMO76609)

Alkane 1-Monooxygenase 2

Gene sequence which encodes the protein Alkane 1-monooxygenase 2 is given below:

ATGTTTGCGTTTCTGAGCCCGGCGTGGATGCTGCGCC
TGAAAAAAGCGGGCTATTGGATT
TGGCTGGTGCCGGTGTTTGGCATTCCGCTGAGCTATT
GGTGGAGCTATGGCAGCGGCCAT
CCGAACGCGTGGGCGTGGTTTGTGATTGCGGTGGTG
TTTCTGGTGATTCCGGTGCTGGAT
GCGATTGTGGGCCGCGATCCGGCGAACCCGGATGAA
GCGAGCGAAGTGCCGGCGCTGGAA
CGCGAAGGCTATTATCGCTTTCTGAGCCTGATGACC
GTGCCGCTGCTGCTGGGCATGCTG
GTGTATGGCGGCTGGGTGATTACCCATTATGAAGCG
TGGAACTGGGTGGGCAAACTGGGC
TGGACCCTGAGCGTGGGCACCGTGATGGGCGCGATT
GGCATTACCGTGAGCCATGAACTG
ATTCATAAAGATCCGCAGCTGGAACAGCGCGCGGGC
GGCCTGCTGCTGGCGGCGGTGTGC
TATGCGGGCTTTAAAGTGGAACATGTGCGCGGCCAT
CATGTGCATGTGAGCACCCCGGAA
GATGCGAGCAGCAGCCGCTATGATCAGAGCCTGTAT
GCGTTTCTGCCGCATGCGTATAAA
CATAACTTTCTGAACGCGTGGCGCCTGGAAGCGCAG
CGCCTGAAACGCAAAGGCCTGCCG
GCGCTGCATTGGCGCAACGAACTGATTTGGTGGTAT
GCGGTGAGCGCGCTGTTTCTGCTG
GGCTTTAGCCTGGCGTTTGGCTGGCTGGGCGCGGTG
TATTTTCTGGGCCAGGCGGCGATG
GCGTTTACCCTGCTGGAAATTGTGAACTATGTGGAA
CATTATGGCCTGCATCGCCGCCGC
CTGGATGATGGCCGCTATGAACGCACCACCCATGAA
CATAGCTGGAACAGCAACTTTCTG
CTGACCAACCTGTTTCTGTTTCATCTGCAGCGCCATA
GCGATCATCATGCGTATGCGAAA
CGCCGCTATCAGGTGCTGCGCCATTATGATGATAGC
CCGCAGCTGCCGAACGGCTATGCG
GGCATGATTGTGCTGGCGCTGTTTCCGCCGCTGTGGC
GCGCGGTGATGAACCCGCGCGTG
CGCGCGTATTATGCGGGCGAAGAATATCAGCTGAGC
GATACCCAGCGCGCG

(Retrieved from https://www.bioinformatics.org/sms2/rev_trans.html)

The fasta format of the protein Alkane 1-monooxygenase 2 is given below

MFAFLSPAWMLRLKKAGYWIWLVPVFGIPLSYWWSY
GSGHPNAWAWFVIAVVFLVIPVLDAIVGRDPANP
DEASEVPALEREGYYRFLSLMTVPLLLGMLVYGGWVI
THYEAWNWVGKLGWTLSVGTVMGAIGITVSHEL
IHKDPQLEQRAGGLLLAAVCYAGFKVEHVRGHHVHVS
TPEDASSSRYDQSLYAFLPHAYKHNFLNAWRLE
AQRLKRKGLPALHWRNELIWWYAVSALFLLGFSLAFG
WLGAVYFLGQAAMAFTLLEIVNYVEHYGLHRRR
LDDGRYERTTHEHSWNSNFLLTNLFLFHLQRHSDHHA
YAKRRYQVLRHYDDSPQLPNGYAGMIVLALFPP
LWRAVMNPRVRAYYAGEEYQLSDTQRA

(NCBI, accession no. AMO75838)

P. citronellolis P3B5 contains two putative AlmA-like monooxygenases responsible for the degradation of very-long-chain alkanes (>C30) (Remus-Emsermann *et al*., 2016) [35].

FAD-Containing Monooxygenase EthA

Gene sequence which encodes this protein is given below

ATGAGCGTGGAACATCTGGATGTGCTGATTGTGGGC
GCGGGCCTGAGCGGCATTGGCGCG
GCGTATCATCTGATGAAACATTGCCCGGGCAAAACC
TTTGCGCTGCTGGAAGGCCGCGAA
GCGATGGGCGGCACCTGGGATCTGTTTCGCTATCCG
GGCATTCGCAGCGATAGCGATATG
TATACCCTGGGCTATAACTTTAAACCGTGGACCGAT
GCGAAAGCGATTGCGGATGGCCCG
AGCATTCGCCATTATATTGAAGAAACCGCGCAGGAA
AACGGCATTGATCGCAAAATTCGC
TATCGCCATCGCGTGCTGAAAGCGGATTGGAACAGC
GATAGCGCGCGCTGGCTGCTGACC
GTGCAGCGCGGCGATGAACCGGAACCGGTGCGCAT
GAGCGCGCAGTTTCTGCTGATGTGC
ACCGGCTATTATCGCTATGAAGCGGGCTATACCCCG
GAATTTAAAGGCCGCGATAGCTTT
CGCGGCCAGATTATTCATCCGCAGCTGTGGCCGGAA
GGCTTTGATTATGCGGATAAACGC
GTGGTGGTGATTGGCAGCGGCGCGACCGCGGTGACC
CTGGTGCCGAGCCTGACCGATAAA
GCGCGCCATGTGACCATGCTGCAGCGCAGCCCGAGC
TATGTGATTAGCCTGCCGCAGAAA
GATCCGATTAGCAACTTTCTGCGCCGCTTTCTGCCGG
AAACCTGGGTGTATCGCCAGGCG
CGCGCGCGCAACGTGACCATGCAGATGGTGTTTTTT
CTGTTTGCGAAAACCTTTCCGAGC
CTGGCGCGCAAAGCGCTGCTGGGCCTGGCGCGCCAT
CAGCTGGGCAAAGATTTTGATATG
CGCCATTTTAGCCCGCGCTATAAACCGTGGGATGAA
CGCGTGTGCTGCGTGCCGGATGGC
GATCTGTTTAAAGCGCTGCGCAAAGGCAAAGCGAGC

GTGGTGACCGAACATATTGATGGC
TTTTGCGAAAAAGGCATTCGCCTGAAAAGCGGCCAG
GTGCTGGAAGCGGATGTGATTGTG
ACCGCGACCGGCCTGGATCTGGTGATGTTTGGCGGC
GCGGAACTGGCGGTGGATGGCAAA
CCGTTTCAGGTGAACCAGAGCATGGGCTATCGCGGC
ATTATGCTGCGCGATCTGCCGAAC
CTGGCGGCGGTGGTGGGCTATACCAACGCGAGCTGG
ACCCTGAAAGCGGATCTGAGCAGC
GAATATTTTTGCCGCCTGATTAACCATATGGATGCGA
TTGGCATGCGCCAGTGCACCCCG
CGCGCGGGCGCGGGCCAGGTGAAAGAAGAACCGTT
TCTGAACCTGAACAGCGGCTATATT
CAGCGCGCGGCGGATAAAATGCCGAAACAGGGCGA
TCGCACCCCGTGGAAACTGTATCAG
AACTATGCGCTGGATCTGGCGCTGCTGCGCCATGGC
AAAGTGGAAGATGGCTATCTGACC
TTTACCAGCCCGCGCGCGCGCCAGGGCGATGAAGCG
AGCCTGCAGGCGGTG
(https://www.bioinformatics.org/sms2/rev_trans.html)
The fasta format of the protein is given below:

MSVEHLDVLIVGAGLSGIGAAYHLMKHCPGKTFALLE
GREAMGGTWDLFRYPGIRSDSDMYTLGYNFKPW
TDAKAIADGPSIRHYIEETAQENGIDRKIRYRHRVLKAD
WNSDSARWLLTVQRGDEPEPVRMSAQFLLMC
TGYYRYEAGYTPEFKGRDSFRGQIIHPQLWPEGFDYAD
KRVVVIGSGATAVTLVPSLTDKARHVTMLQRS
PSYVISLPQKDPISNFLRRFLPETWVYRQARARNVTMQ
MVFFLFAKTFPSLARKALLGLARHQLGKDFDM
RHFSPRYKPWDERVCCVPDGDLFKALRKGKASVVTEH
IDGFCEKGIRLKSGQVLEADVIVTATGLDLVMF
GGAELAVDGKPFQVNQSMGYRGIMLRDLPNLAAVVG
YTNASWTLKADLSSEYFCRLINHMDAIGMRQCTP
RAGAGQVKEEPFLNLNSGYIQRAADKMPKQGDRTPW
KLYQNYALDLALLRHGKVEDGYLTFTSPRARQGD
EASLQAV
(NCBI, accession no. AMO73862)

P. citronellolis is able to degrade terpenes such as citronellol. A corresponding gene cluster (PcP3B5_19950–20010) was also detected in *P. citronellolis* P3B5, which may be responsible for enabling this species to colonize the phyllosphere (Remus-Emsermann *et al*., 2016) [35].
P. citronellolis P3B5 genome contains genes whose products are involved in the biosynthesis of indole-3-acetic acid (PcP3B5_05210–05220, PcP3B5_17810, PcP3B5_07120–07140). It is a plant hormone of the auxin class and induces plant cell elongation and division, which leads to an increase in locally available nutrients. Thus *P. citronellolis* P3B5 has an impact on the plant host (Remus-Emsermann *et al*., 2016) [35].

2.6 Pseudomonas putida
Pseudomonas putida has been found to colonize the phyllosphere of several plants and its various strains are involved in the active degradation of various hydrocarbons. One of them is the hydrocarbon- naphthalene. Various strains of Pseudomonas putida contain certain plasmids that contain genes that lead to the degradation of naphthalene.

2.7 Naphthalene
Naphthalene is the simplest polycyclic aromatic hydrocarbon with the formula C10H8. It is a white crystalline solid with a characteristic odour, detectable at concentrations as low as 0.08 ppm by mass (Amoore *et al*., 1983) [4]. Its structure consists of a fused pair of benzene rings. Exposure to large amounts of naphthalene may damage or destroy red blood cells (Santucci and Shah, 2000) [36], cause confusion, nausea, vomiting, diarrhea, blood in the urine, and jaundice. The International Agency for Research on Cancer (IARC) classifies naphthalene as possibly carcinogenic to humans and animals. The IARC also points out that acute exposure causes cataracts in humans, rats, rabbits, and mice; and that hemolytic anaemia can occur in children and infants after oral or inhalation exposure or after maternal exposure during pregnancy.

2.8 Pseudomonas putida G7
Pseudomonas putida G7 contains the NAH7 naphthalene catabolic plasmid. The nah operons are divided into two clusters (Undugoda *et al*., 2016) [46]. The genes of the upper operon have genes encoding enzymes that convert naphthalene to salicylate. The lower operon has genes encoding enzymes that convert salicylate to acetyl coenzyme A and pyruvate (Zhou *et al*., 2002) [49].
A Tn5 insertion mutation which occurs immediately upstream from the first gene of the lower operon, nahG, found in the NAH7 plasmid gives the Nah− Sal− phenotype (Schell, 1985; Park *et al*., 2002) [39], which defined the regulatory region containing the gene encoding the NahR protein. The NahR protein is a LysR-type transcriptional regulator (LTTR). This protein is necessary for activation of both the upper (genes nahA-F) and lower (genes nahG-M) operons on the NAH7 plasmid (Huang & Schell, 1991; Schell, 1985, 1986, 1993; Schell *et al*., 1990; Schell & Poser, 1989; Schell & Wender, 1986; Park *et al*., 2002) [22, 39, 40, 41, 42, 37, 38]. Studies have shown that NahR is constitutively expressed at low levels (Cebolla *et al*., 1997) [11]. NahR, bound to its inducer, salicylate, leads to the activation of both nah operons by interacting with DNA enhancer sequences upstream of the promoter region in upper and lower nah operons (Schell & Poser, 1989) [37]. The 5' flanking region of known nahR genes is the divergently transcribed nahG-nahR promoter region and NahR binding in this -60 bp region upstream of the nahG gene transcription start site induces nahG gene transcription, but represses its own expression by negative autoregulation (Schell & Poser, 1989; Schell & Wender, 1986; Yen & Serdar, 1988) [37, 38, 48]. The exact mechanism of autoregulation is yet to be discovered (Yen & Serdar, 1988) [48].

2.9 Pseudomonas putida NCIB 9816
P. putida NCIB 9816 is capable of utilizing naphthalene as the sole carbon source. An 81 kb plasmid, pDTG1 containing nahR gene conferred this trait in this strain. The plasmid encodes key enzymes in early enzymatic steps in naphthalene degradation (Undugoda *et al*., 2016) [46].
A few of the gene segments present on the plasmid pDTG1 and the proteins coded by them are mentioned below:

nahR Gene
The Gene sequence of the nahR gene found in plasmid pDTG1 in Pseudomonas putida NCIB 9816-4 is given below
ATGGAACTGCGTGATCTGGATTTAAACCTGCTGGTG
GTGTTCAACGAGCTGCTGGTCTGCAGACGCGTCT
CTATCGTTGCCGAGAACCTGGGCCTGACCCAGCCTG
CTGTCAGCAATGCGCTGAAACGCCTGCGCACAGC
GCTACAGGACCAACTTTTCGTGCGCACCTACCAGGG
TATGGAACCCACACCCTACGCCGCGAACTTGGCC

GAGCCCGTCGCCTTGGCCATGCACGCCCTGCGCGAA
GCCCTGCAGCACCATGAGCGCTTCGATCCGCTGA
CCAGCGAGCGTACTTTCACCCTGGCCATGACCGACA
TCGGCGAGATCTACTTCATGCCGCGGCTGATGGA
TGCGATTACTCTTCAAGCCCCCAATTGCGCGATCAG
CACCGTGCGCGACAGTTCGATGAGCCTGATGCAA
GCCTTGCAGAACGGCACAGTGGACCTAGCCGTGGGC
CTGCTGCCCAACCTGCAGACTGGCTTCTTCCAGC
GCCGGCTGCTCCACAATCACTACGTGTGCCTGTGTC
GCAAGGACCATCCGGCCACCCGCGAACCCCTGAC
TCTTGAGCGCTTCTGTTCCTATGGCCACGTGCGTGTC
ATCGCCGCTGGCACAGGCCACGGCGAGGTGGAC
ACATACTTGACGAAGGCCGGCATCCGGCGCGACATC
CGCCTGGAGGTGCCGCACTTCGTCGCCGTTGGCC
ACATACTCCAGCGCACCGAGCTGCTCGCCACTGTGC
CGATACGTTTCGCCGACTGCTGCGTAGAGCCCTT
CGGTCTGAGCGTCTTGCCGCACCCAGTCGCCTTGCC
GGAAATCGCCATCAACATGTTCTGGCATGCGAAA
TACCACCAGGACCTCGCCAATATCTGGTTGCGGCAA
CTGATGTTCGACCTGTTTTCTGATTGA
(Retrieved from NCBI; Accession ID: AF491307)

The protein encoded by this gene is a naphthalene degradation LysR-family transcriptional activator.

MELRDLDLNLLVVFNELLVCRRVSIVAENLGLTQPAVS
NALKRLRTALQDQLFVRTYQGMEPTPYAANLA
EPVALAMHALREALQHHERFDPLTSERTFTLAMTDIGE
IYFMPRLMDAITLQAPNCAISTVRDSSMSLMQ
ALQNGTVDLAVGLLPNLQTGFFQRRLLHNHYVCLCRK
DHPATREPLTLERFCSYGHVRVIAAGTGHGEVD
TYLTKAGIRRDIRLEVPHFVAVGHILQRTELLATVPIRF
ADCCVEPFGLSVLPHPVALPEIAINMFWHAK
YHQDLANIWLRQLMFDLFSD
(NCBI; Accession ID: AAM09539)

2.10 Naphthalene dioxygenase enzyme

The first step in the aerobic degradation of naphthalene is catalyzed by naphthalene dioxygenase (NDO) enzyme system (EC 1.14.12.12) from Pseudomonas sp. strain NCIB 9816-4. In this reaction, both atoms of oxygen are added to the aromatic nucleus of naphthalene by NDO, forming cis-naphthalene dihydrodiol (Jeffrey *et al*., 1975; Jerina *et al*., 1971) [25, 26]. The NDO system consists of three components, each of which has been purified and characterized (Rebecca *et al*., 2000). An iron-sulfur flavoprotein reductase and an iron-sulfur ferredoxin, both, transfer electrons from NAD(P)H to the catalytic oxygenase component (Ensley and Gibson, 1983; Ensley *et al*.,1982; Haigler and Gibson,1990) [14, 13, 19]. The oxygenase is composed of a large subunit called α and a small subunit called β, in an α3β3 configuration (Kauppi *et al*., 1998) [27]. Naphthalene dioxygenase is a member of a large family of oxygenases whose α subunits contain a Rieske [2Fe-2S] center and mononuclear nonheme iron (Butler and Mason, 1997). Electrons are transferred from the Rieske center of the ferredoxin to the Rieske center of the oxygenase α subunit. The reduced Rieske center in one α subunit transfers an electron to mononuclear iron at the active site in an adjacent α subunit (Kauppi *et al*., 1998; Parales *et al*., 1999) [27, 50]. His-208, His-213, and Asp-362 coordinate the active-site iron, forming a 2-His-1-carboxylate facial triad. Asp-205 in the catalytic domain of the NDO α subunit is hydrogen-bonded to His-208 and His-104 in the adjacent α subunit. His-104 is one of the Rieske center ligands. Asp-205 is required for efficient electron transfer from the Rieske center to the active-site iron (Parales *et al*., 1999) [50].

***P. putida* naphthalene dioxygenase gene (nahAa, nahAb, nahAc, and nahAd)**

nahAb gene

ATGGAACTCCTCATACAACCGAACAATCGCATAATT
CCCTTCAGTGCCGGTGCCAACCTTCTGGAAGTGC
TTCGCGAGAACGGTGTAGCTATTTCCTACAGTTGCTT
GTCTGGGCGTTGCGGAACCTGTCGCTGCCGGGT
TATAGATGGCAGTGTCATTGATTCTGGGGCGGAAAA
TGGGCAATCAAACCTCACCGACAAGCAGTATGTG
CTCGCCTGTCAGTCAGTACTCACTGGCAATTGCGCTA
TCGAAGTCCCAGAAGCCGACGAAATTGTCACTC
ACCCGGCGCGAATCATCAAGGGCACAGTGGTCGCAG
TCGAGTCGCCCACTCACGATATCCGTCGCTTACG
CGTACGCCTCTCCAAGCCCTTCGAGTTCTCACCCGGA
CAGTACGCGACACTGCAGTTCAGCCCTGAGCAT
GCGCGTCCGTATTCAATGGCAGGTTTGCCAGATGAC
CAAGAAATGGAGTTCCACATACGCAAGGTGCCGG
GTGGGCGCGTCACGGAGTATGTTTTCGAACACGTCC
GCGAAGGTACAAGCATCAAGTTGAGCGGGCCTCT
TGGTACGGCTTATCTACGTCAGAAGCACACCGGACC
GATGCTGTGTGTAGGTGGCGGGACCGGACTCGCA
CCGGTGCTGTCGATTGTTCGCGGCGCGCTGAAGTCG
GGTATGACGAACCCCATCCTCCTTTATTTCGGGG
TGCGCAGTCAGCAAGACCTCTACGACGCAGAGCGAT
TGCACAAACTCGCCGCTGACCACCCTCAACTGAC
CGTACACACGGTGATTGCAACGGGCCCGATTAATGA
GGGTCAGCGAGCCGGCCTAATTACCGATGTGATC
GAAAAAGACATCCTTTCGCTGGCTGGGTGGAGGGCC
TACCTGTGCGGCGCACCAGCGATGGTTGAAGCGT
TGTGCACCGTCACCAAGCATCTTGGAATATCACCCG
AACATATTTATGCCGATGCCTTCTATCCCGGTGG
GATCTGA
(NCBI; Accession ID: AF491307 REGION: 14536.15522)

It encodes naphthalene 1, 2-dioxygenase reductase component (plasmid)

(*Pseudomonas putida*), whose FASTA sequence is as follows:

MELLIQPNNRIIPFSAGANLLEVLRENGVAISYSCLSGR
CGTCRCRVIDGSVIDSGAENGQSNLTDKQYV
LACQSVLTGNCAIEVPEADEIVTHPARIIKGTVVAVESP
THDIRRLRVRLSKPFEFSPGQYATLQFSPEH
ARPYSMAGLPDDQEMEFHIRKVPGGRVTEYVFEHVRE
GTSIKLSGPLGTAYLRQKHTGPMLCVGGGTGLA
PVLSIVRGALKSGMTNPILLYFGVRSQQDLYDAERLHK
LAADHPQLTVHTVIATGPINEGQRAGLITDVI
EKDILSLAGWRAYLCGAPAMVEALCTVTKHLGISPEHI
YADAFYPGGI
(NCBI; Accession ID: AAA25904)

The purified enzyme catalyzed the reduction of cytochrome c, dichlorophenolindophenol, Nitro Blue Tetrazolium, and ferricyanide. These activities were enhanced in the presence of added FAD. The ability of the enzyme to catalyze the reduction of the ferredoxin involved in naphthalene reduction and other electron acceptors indicates that it functions as a NAD(P)H-oxidoreductase in the naphthalene dioxygenase system. The results suggest that naphthalene dioxygenase requires two proteins with three redox groups to transfer electrons from NADH to the terminal oxygenase (Haigler and Gibson, 1990) [19].

nahAb gene

ATGACAGTAAAGTGGATTGAAGCAGTCGCTCTTTCT
GACATCCTTGAAGGTGACGTCCTCGGCGTGACTG
TCGAGGGCAAGGAGCTGGCGCTGTATGAAGTTGAAG

GCGAAATCTACGCTACCGACAACCTGTGCACGCA
TGGTTCCGCCCGCATGAGTGATGGTTATCTCGAGGG
TAGAGAAATCGAATGCCCCTTGCATCAAGGTCGG
TTTGACGTTTGCACAGGCAAAGCCCTGTGCGCACCC
GTGACACAGAACATCAAAACATATCCAGTCAAGA
TTGAGAACCTGCGCGTAATGATTGATTTGAGCTAA
(NCBI. Accession ID: AF491307 REGION: 15666..15980)
It encodes naphthalene 1, 2-dioxygenase ferredoxin component (plasmid) (*Pseudomonas putida*), whose FASTA sequence is as follows:
MTVKWIEAVALSDILEGDVLGVTVEGKELALYEVEGEI
YATDNLCTHGSARMSDGYLEGREIECPLHQGR
FDVCTGKALCAPVTQNIKTYPVKIENLRVMIDLS
(NCBI; Accession ID: AAA25905)
One of the three components of the naphthalene dioxygenase, the protein contained 2 g-atoms each of iron and acid-labile sulphur. It had an apparent molecular weight of 13,600. The evidence suggests that it is a ferredoxin-type protein that functions as an intermediate electron transfer protein in naphthalene dioxygenase activity (Haigler and Gibson, 1990) [19].

NahAc gene
ATGAATTACAATAATAAAATCTTGGTAAGTGAATCT
GGTCTGAGCCAAAAGCACCTGATTCATGGCGATG
AAGAACTTTTCCAACATGAACTGAAAACCATTTTTG
CGCGGAACTGGCTTTTTCTCACTCATGATAGCCT
GATTCCTGCCCCCGGCGACTATGTTACCGCAAAAAT
GGGGATTGACGAGGTCATCGTCTCCCGGCAGAAC
GACGGTTCGATTCGTGCTTTTCTGAACGTTTGCCGGC
ATCGTGGCAAGACGCTGGTGAGCGTGGAAGCCG
GCAATGCCAAAGGTTTTGTTTGCAGCTATCACGGCT
GGGGCTTCGGCTCCAACGGTGAACTGCAGAGCGT
TCCATTTGAAAAAGATCTGTACGGCGAGTCGCTCAA
TAAAAAATGTCTGGGGTTGAAAGAAGTCGCTCGC
GTGGAGAGCTTCCATGGCTTCATCTACGGTTGCTTCG
ACCAGGAGGCCCCTCCTCTTATGGACTATCTGG
GTGACGCTGCTTGGTACCTGGAACCTATGTTCAAGC
ATTCCGGCGGTTTAGAACTGGTCGGTCCTCCAGG
CAAGGTTGTGATCAAGGCCAACTGGAAGGCACCCGC
GGAAAACTTTGTGGGAGATGCATACCACGTGGGT
TGGACGCACGCGTCTTCGCTTCGCTCGGGGGAGTCT
ATCTTCTCGTCGCTCGCTGGCAATGCGGCGCTAC
CACCTGAAGGCGCAGGCTTGCAAATGACCTCCAAAT
ACGGCAGCGGCATGGGTGTGTTGTGGGACGGATA
TTCAGGTGTGCATAGCGCAGACTTGGTTCCGGAATT
GATGGCATTCGGAGGCGCAAAGCAGGAAAGGCTG
AACAAAGAAATTGGCGATGTTCGCGCTCGGATTTAT
CGCAGCCACCTCAACTGCACCGTTTTCCCGAACA
ACAGCATGCTGACCTGCTCGGGTGTTTTCAAAGTAT
GGAACCCGATCGACGCAAACACCACCGAGGTCTG
GACCTACGCCATTGTCGAAAAAGACATGCCTGAGGA
TCTCAAGCGCCGCTTGGCCGACTCTGTTCAGCGA
ACGTTCGGGCCTGCTGGCTTCTGGGAAAGCGACGAC
AATGACAATATGGAAACAGCTTCGCAAAACGGCA
AGAAATATCAATCAAGAGATAGTGATCTGCTTTCAA
ACCTTGGTTTCGGTGAGGACGTATACGGCGACGC
GGTCTATCCAGGCGTCGTCGGCAAATCGGCGATCGG
CGAGACCAGTTATCGTGGTTTCTACCGGGCTTAC
CAGGCACACGTCAGCAGCTCCAACTGGGCTGAGTTC
GAGCATGCCTCTAGTACTTGGCATACTGAACTTA
CGAAGACTACTGATCGCTAA
(NCBI; Accession ID: AF491307 REGION: 16051..17400)
It encodes naphthalene 1,2-dioxygenase iron sulfur protein component large subunit (plasmid) (*Pseudomonas putida*), whose FASTA sequence is as follows:
MNYNNKILVSESGLSQKHLIHGDEELFQHELKTIFARN
WLFLTHDSLIPAPGDYVTAKMGIDEVIVSRQN
DGSIRAFLNVCRHRGKTLVSVEAGNAKGFVCSYHGW
GFGSNGELQSVPFEKDLYGESLNKKCLGLKEVAR
VESFHGFIYGCFDQEAPPLMDYLGDAAWYLEPMFKHS
GGLELVGPPGKVVIKANWKAPAENFVGDAYHVG
WTHASSLRSGESIFSSLAGNAALPPEGAGLQMTSKYGS
GMGVLWDGYSGVHSADLVPELMAFGGAKQERL
NKEIGDVRARIYRSHLNCTVFPNNSMLTCSGVFKVWN
PIDANTTEVWTYAIVEKDMPEDLKRRLADSVQR
TFGPAGFWESDDNDNMETASQNGKKYQSRDSDLLSNL
GFGEDVYGDAVYPGVVGKSAIGETSYRGFYRAY
QAHVSSSNWAEFEHASSTWHTELTKTTDR
(NCBI; Accession ID: AAO64274)

NahAd gene
ATGATGATCAATATTCAAGAAGACAAGCTGGTTTCC
GCCCACGACGCCGAAGAGATTCTTCGTTTCTTCA
ATTGCCACGACTCTGCTTTGCAACAAGAAGCCACTA
CGCTGCTGACCCAGGAAGCGCATTTGTTGGACAT
TCAGGCTTACCGTGCTTGGTTAGAGCACTGCGTGGG
GTCAGAGGTGCAATATCAGGTCATTTCACGCGAA
CTGCGCGCAGCTTCAGAGCGTCGTTATAAGCTCAAT
GAAGCCATGAACGTTTACAACGAAAATTTTCAGC
AACTGAAAGTTCGAGTTGAGCATCAACTGGATCCGC
AAAACTGGGGCAACAGCCCGAAGCTGCGCTTTAC
TCGCTTTATCACCAACGTCCAGGCCGCAATGGACGT
AAATGACAAAGAGCTACTTCACATCCGCTCCAAC
GTCATTCTGCACCGGGCACGACGTGGCAATCAGGTC
GATGTCTTCTACGCCGCCCGGGAAGATAAATGGA
AACGTGGCGAAGGTGGAGTACGAAAATTGGTCCAGC
GATTCGTCGATTACCCAGAGCGCATACTTCAGAC
GCACAATCTGATGGTCTTTCTGTGA
(Source NCBI; Accession ID: AF491307 REGION: 17415.17999)
It encodes naphthalene 1,2-dioxygenase iron sulfur protein component small subunit (plasmid) (*Pseudomonas putida*), whose FASTA sequence is as follows:
MMINIQEDKLVSAHDAEEILRFFNCHDSALQQEATTLL
TQEAHLLDIQAYRAWLEHCVGSEVQYQVISRE
LRAASERRYKLNEAMNVYNENFQQLKVRVEHQLDPQ
NWGNSPKLRFTRFITNVQAAMDVNDKELLHIRSN
VILHRARRGNQVDVFYAAREDKWKRGEGGVRKLVQR
FVDYPERILQTHNLMVFL
(Source: NCBI; Accession ID: AAO64275)

3. Materials and Methods

3.1 Sample collection
Collect the leaves of *Mangifera indica* from the streets where vehicular traffic was high. The leaves were transported to the lab on a sealed cover and stored in the refrigerator until use.

3.2 Isolation of total heterotrophic and hydrocarbon utilizing bacteria from leaf samples
Each leaf sample (4 g) should be washed with 100 ml of phosphate buffer and then shaken at 200 rev/min for one hour. Then the diluted samples should directly be added to the modified mineral salt agar plates. Plates are to be then incubated at room temperature (28°C-30°C) for five days. Bacterial colonies should then be streaked on PAH added Bacto-Bushnell Haas medium to select PAH utilizing bacteria (Undugoda *et al*., 2016) [46].

3.3 Characterization of the isolates
Each isolate should be examined for its size, shape, margin,

consistency, opacity, elevation, pigmentation, Gram reaction, and cell morphology as described by Cowan (1974) [12]. The isolates are to be characterized as described by Holt *et al.* (1994) [21]. Diagnostic properties used should include motility, production of cytochrome oxidase, catalase, indole and urease, gelatin liquefaction, starch hydrolysis, oxidation/fermentation of sugars, methyl red test, Voges Proskaeur test, and growth at 42ºC and 5ºC (Ilori *et al*., 2006).

3.4 Characterization of the isolates

Each isolate should be examined for its size, shape, margin, consistency, opacity, elevation, pigmentation, Gram reaction, and cell morphology as described by Cowan (1974) [12]. The isolates are to be characterized as described by Holt *et al.* (1994) [21]. Diagnostic properties used should include motility, production of cytochrome oxidase, catalase, indole and urease, gelatin liquefaction, starch hydrolysis, oxidation/fermentation of sugars, methyl red test, Voges Proskaeur test, and growth at 42ºC and 5ºC (Ilori *et al*., 2006).

3.5 Selection of efficient PAH degrading bacteria

The best PAH degrading bacterial strains can be selected based on the results obtained from the colorimetric and HPLC methods indicated below:

Colorimetric assay

Each bacterial strain is 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 control without bacterial inoculation. From broth culture 5 ml sample is centrifuged at 6000 rev/min for five minutes. The recovered supernatant is assayed spectrophotometrically by measuring absorbance at 609 nm for the residual hydrocarbon. Six replicates are done for each bacterial strain and PAH degradation percentage is determined using the following equation (Okafor *et al*., 2009) [30].

The absorbance of sample Percentage of PAH degradation = 1-(Absorbance of sample/Absorbance of Control) x 100

HPLC determination of PAH degradation

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

Residual PAH in the culture is extracted with hexane and acetone-containing mixture. Then extract is analyzed by high-performance liquid chromatography (HPLC) equipped with a UV detector. The analytical column (250 mm long, 4.6 mm diameter) is packed porous spherical C-18 material (packed size, 5µm). Acetonitrile-water mixture (75: 25) is used as a mobile phase for PAHs at a flow rate of 1.0 ml min-1. The sample (20 µL) is injected into the column through the sample loop. UV - detector is set at 254 nm for compound detection. The Chromeleon chromatography software is used for the quantification of PAHs throughout the experiment. Finally, the percentage of degradation is determined (Undugoda *et al*., 2016) [46].

3.6 Isolation of catabolic plasmid and confirmation of plasmid-encoded PAH degradation

Catabolic plasmids of the isolated microbes can be isolated according to the method of (Sambrook *et al*., 1989) and each plasmid is to be transformed into the E.coli JM109. PAH degrading ability of transformants are to be tested using colorimetric and HPLC method to determine the plasmid based PAH degradation. Plasmid based PAH degradation is confirmed by curing the plasmid of bacterial cells using acridine orange (Fujiii *et al*., 1997) [17] and then testing their PAH degradation ability by colorimetric and HPLC methods (Undugoda *et al*., 2016) [46].

3.7 PCR amplification

Suitable primers are designed for the PCR amplification of the required genes. Plasmid DNA templates are amplified using the above primers. Then PCR products are visualized and their size estimation is done by gel electrophoresis (Undugoda *et al*., 2016) [46].

3.8 Sequencing of PCR products

Automated sequencing should be carried out using applied Biosystems automated sequencer (ABI3730XL). Purified PCR products representing the gene segments are sequenced directly using appropriate primers (Undugoda *et al*., 2016) [46].

3.9 Restriction digestion of PCR products

PCR amplicons of the genes can be digested using the Hind III restriction enzyme to determine their RFLP patterns (Undugoda *et al*., 2016) [46].

3.10 BLAST analysis

Nucleotide sequences of the gene segments are to be aligned with gene sequences of Genbank using BLAST (Altschul *et al*., 1997) [3].

4. Expected results and discussion

The outcomes expected on the completion of the described methodology are.

1. Many species of microorganisms were expected in the Mangifera leaves.
2. There is a chance for hydrocarbon utilizing microorganisms in the isolate.
3. Isolation of DNA from the selected organisms.
4. DNA Sequences.
5. BLAST result.

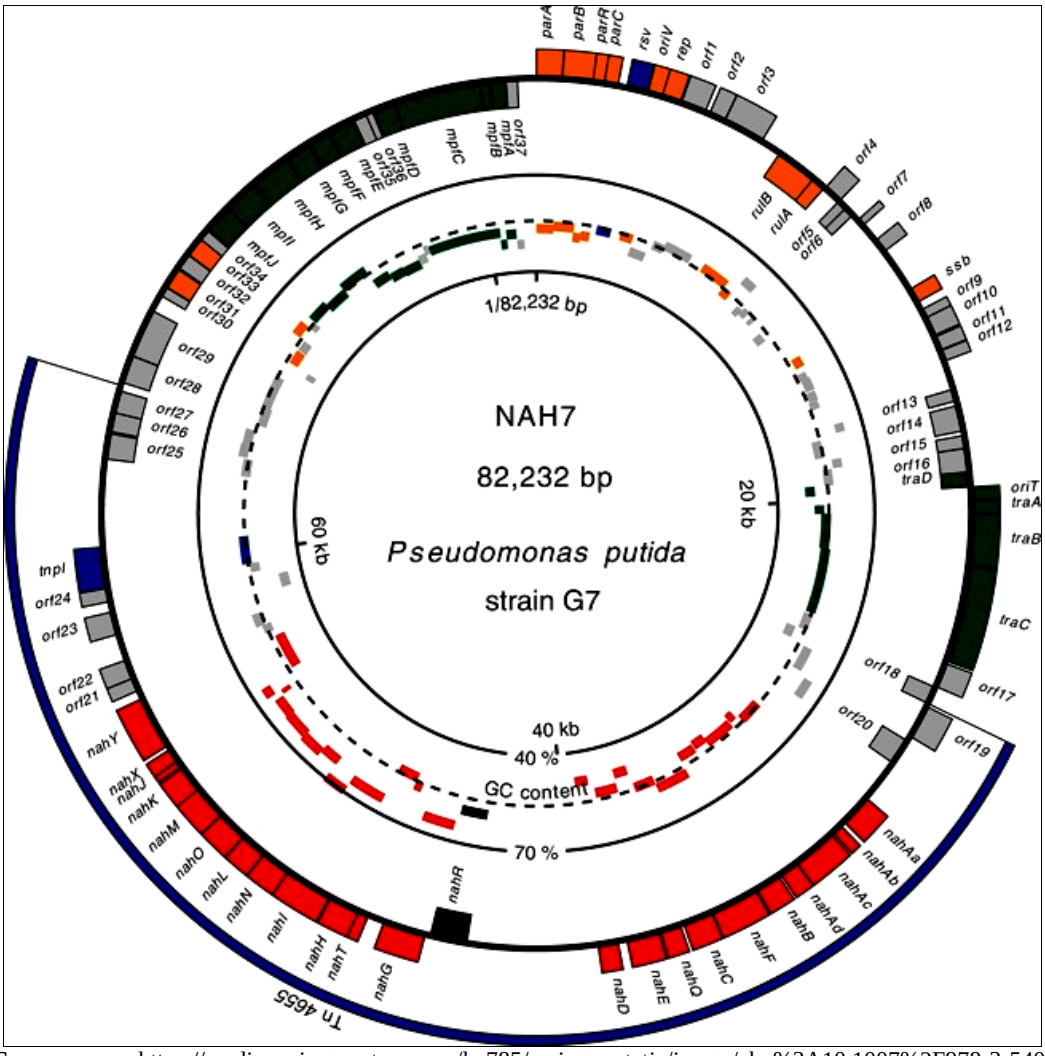
If the genome sequence obtained after the sequencing, matches with any of the bacterial sequences mentioned earlier, it can be able to concluded that the leaves of *Mangifera indica* collected also have one or more of these microbes associated with it and it was developed as a result of heavy hydrocarbon pollution in the area that it was growing in.

And hence it could be said that the plant selected also performs phylloremediation. On the other hand, if the sequences of the microbes isolated from the leaf samples don't match with the gene sequences of the organisms that were earlier found out to perform phylloremediation, it is highly unlikely that the plant selected has phylloremediation capabilities.

5. Conclusions

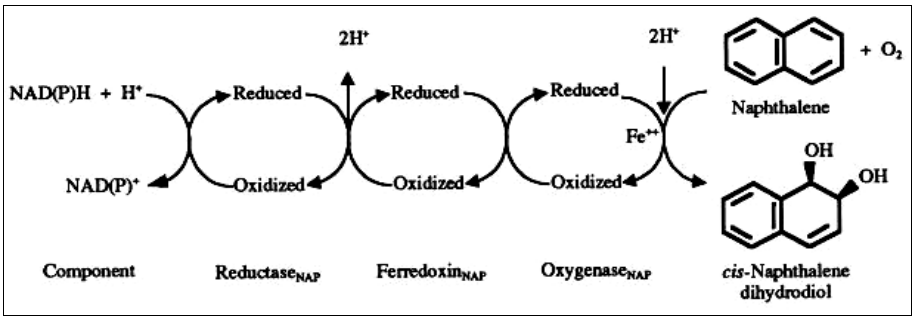
Environmental pollution is reaching such great heights that it is a matter of global concern now. Out of all the measures being taken to reduce this menace, a very cheap and effective approach could be - the use of the concept of phylloremediation of air pollutants, as the aerial surfaces of leaves are estimated to sum up to 4x10^8 sq. km. on the earth (Wei *et al*., 2017) [47]. Objective of this study was to analyse the phylloremediation capability in collected leaf samples from *Mangifera indica* growing in hydrocarbon polluted areas

and to understand the various microbes associated with the leaves which are actually responsible for the degradation of air pollutants, the mechanisms involved and the genes or gene clusters which are responsible for this phenomenon. This information can definitely be used later to induce this natural capability of plants and microbes into the ones that lack it through genetic engineering, followed by tissue culture and micropropagation to produce a large number of genetically modified plants that'll be able to degrade various air pollutants.



Source: https://media.springernature.com/lw785/springer-static/image/chp%3A10.1007%2F978-3-540-85467-8_3/MediaObjects/978-3-540-85467-8_3_Fig1_HTML.jpg

Fig 1: NAH7 plasmid of *Pseudomonas putida* strain G7.



Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC94462/

Fig 2: Reaction catalyzed by the three-component NDO system.

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