Enrichment, Isolation and Identification of Polycyclic Aromatic Hydrocarbon Degrading *Rhodococcus ruber*from Sediments

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***Abstract*-** This study focuses on the isolation and biodegradation of PAHs by microorganisms associated with the sediments of automobile workshops of Pondicherry. One of the bacterial isolate selectively enriched in these compounds was identified as *Rhodococcus ruber* based on morphology, 16S rDNA sequencing and phylogenetic characteristics. Biodegradability tests with selected PAHs supplied as sole carbon sourceshowed that Rhodococcus sp. could utilize PAH compoundsin the order of chrysene>dieseloil>benzanthracene>crudeoil>fluorene>fluoranthene>phenanthrene>dibenzothiophene>naphthalene and anthracene. Absorbance change of mineral medium with phenanthreneat 250ppm, 500ppm and 1000ppm concentration showed a linear increase in growth of the isolate in all the concentrations of phenanthrene. At 250ppm of phenanthrene concentration a lowest average growth of 0.05±0.02 (OD_{600}) was observed while the highest optimum growth of 1.02±0.33 (OD_{600}) was recorded at 1000ppm concentration of phenanthrene suggesting that *R.ruber*can endure high concentrations of phenanthrene. Hence,*R.ruber*strain has great application indegradation processes involving petrochemical products.

***Index Terms*-** Bioremediation, PAHs, 16S rDNA and *Rhodococcusruber*.

I. INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) known as toxic contaminants of soil & aquifers and carcinogenic for humans are of great environmental concern. The potential use of microorganisms to clean up contaminated soil, sediments and water can provide efficient, inexpensive and environmentally safe clean-up of waste material [1]. Biodegradation technologies were proved practically on a wider scale during an oil spill of Exxon Valdez tanker [2].

Microorganisms play a pivotal role in the degradation of PAHs in terrestrial and aquatic ecosystems and microbial degradation is the main process in natural decontamination [3]. The microorganisms isolated from hydrocarbon contaminated environments have been found as active as or even more active than those originating from uncontaminated soil [1].Taking into account the fact that each contaminated site can respond in a different way to distinct parameters that affect microbial biodegradation, laboratory-scale bioremediation protocols have been developed in order to determine the effects of different conditions [4].

A better understanding of the diversity of the microbial communities inhabiting PAH-contaminated soil and their response to different biostimulation or bioaugmentation strategies could provide clues about the type of bacteria that are able to adapt and exploit such habitats [5,3]. *Rhodococcus ruber* appears be a promising candidate for microbial remediation studies of gasoline-contaminated sites because of its large degradation range of alkane substrates [6].

II. MATERIALS AND METHODS

Sampling site

Samples were collected fromGorimedu area of Pondicherry (geographically at 11° 95' 82" North and 79° 79' 54" East). This area is situated in the outskirts of Pondicherry where about thirty automobile workshops are located. A composite sample of the soil is collected from the site and transported in ice to the laboratory.

Isolation and culture conditions

Three grams of soil was added to sterile 250-ml Erlenmeyer flasks containing 50 ml of Bushnell Hass Medium (BHM). The bacterial strain was isolated by repeated enrichment cultures adding PAHs as the source of carbon and energy [7]. Each PAH was supplemented at a final concentration of 200 mg/l. The flasks were incubated in the dark on a rotary shaker at 30°C and 200 rpm for 30 days. At the end of this period, the vials were allowed to settle for 1 hr. The supernatant of each vial was collected and re-suspended in phosphate buffer before being added into new 250-ml Erlenmeyer flasks containing 50ml BHM and 200 mg/l of PAH compound. This procedure was repeated five consecutive times totally under the same conditions. Aliquots of every culture were plated on solidified BHM and sprayed with concentrated PAH solutions to produce solid films on the petri dishes. The aromatic degrading candidates were identified by the presence of clearing zones around the colonies that indicates substrate utilization [8, 9]. Subsequently, bacterial growth is monitored by taking the absorbance at 600 nm [10].

Turbidity tests

The tetrazolium salt appeared promising as an indicator of viability in assays as a colorimetric indicator of viability in respiring bacteria [11]. A carbon source utilization study was conducted using chrysene, benzanthracene, dibenzothiophene, fluorene, naphthalene, fluoranthene, crude oil and diesel oil as carbon source [12]. The selected PAHs were added to the

microplates in the following concentrations: 1000µg/ml of acenaphthene and naphthalene; 200µg/ml of fluoranthene, fluorene, dibenzothiophene, crude oil, and diesel: 100µg/ml of phenanthrene and benz (o) anthracene. 50µl of each PAHs compound in acetone was coated on the microplates prior to bacterial inoculum prepared as above [13]. The concentration of p-iodo nitro-tetrazolium formazan (INTF) formed was measured at 405nm in dual mode with 655nm reference filter [14, 15].

Growth on phenanthrene as carbon substrate

The purified and isolated bacterial stock (1ml) was added into the conical flask containing 50ml of BH broth. 1ml of standard phenanthrene stock in acetone was coated into the bottom of the flask in methanol and the solvent is allowed to evaporate under a fume hood prior to addition of BH broth. Three different concentrations of phenanthrene: 250ppm, 500ppm, 1000ppm were prepared in acetone as solvent [16]. The flasks were in an incubator shaker at 30ºC and 180rpm. The optical density of the culture was measured using UV/Vis Spectrophotometer at 600nm.

SEM Analysis

The immobilised bacterial cells were fixed at 24°C for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated with a serial concentration of ethanol. The dehydrated cell samples were coated with gold and examined using a scanning electron microscope (S-3400N; Hitachi Co.)[17].

Genotypic Characterisation

The isolates were identified by 16S rDNA sequencing after extracting DNA. The sequence obtained was compared to find sequence similarity using GenBank program - Basic Local Alignment Search Tool (BLAST). The phylogenetic tree based on 16S rDNA gene sequence was constructed by the neighbour-joining method.

Nucleotide sequence accession number

The sequence obtained in this study was deposited in the GenBank database. The GenBank accession number for the nucleotide sequence reported in this paper is **JF430081** (Isolate 1).

III. RESULTS

The isolated strain is a rod-shaped, gram-positive bacterium forming raised pink coloured colonies on nutrient agar medium. Initially colonies were smooth, opaque convex with slightly irregular edges. A primary mycelium that was on the surface and penetrated into the agar was visible when cultures were examined with a lightstereomicroscope. The Scanning electron micrographs (Figure(a-b)) clearly indicate the hyphal structure of the given *Rhodococcus* sp.

Biodegradation experiments

In the given study*Rhodococcus* sp. showed decolorisation in the microplates due to formazan formation in the order of chrysene>dieseloil>benzanthracene>crudeoil>fluorene>fluoranthrene>phenanthrene>dibenzothiophene>naphthalene and anthracene as the carbon source. The growth of the isolates on PAH was concentration dependent. Here, the lowest optimum growth of 0.01 (OD_{600}) was observed when the level of phenanthrene was higher than 100ppm, while the highest optimum growth of 0.09 was recorded when the level of phenanthrene was 15ppm (Figure 2). Similarly, the lowest optimum growth of 0.05 at anthracene level of 30ppm was observed while the highest absorbance of0.45 was recorded when the anthracene level was 15ppm (OD_{600}). However, no significant growth of isolate was observed under different naphthalene concentrations ranging from 15 - 100ppm.

Degradation of Phenanthrene

The average optical density of *Rhodococcus* spp. grown in Bushnell Haas broth at 250ppm, 500ppm and 1000ppm concentration of phenanthrene was measured at 600nm.There was a corresponding increase in growth of isolate in all the three concentrations of phenanthrene studied from time 0 to three weeks. In all the cases OD values higher than that of the control were observed where phenanthrene is used as additional carbon substrate.The isolate showed significant ($p < 0.01$) differences in the growth of the isolate at different phenanthrene levels[16] when compared with controls (Figure(3a-3c)).At 250ppm of phenanthrene concentration a lowest average growth of 0.05±0.02 (OD_{600}) was observed while the highest optimum growth of 1.02±0.33 was recorded when the level of phenanthrene was highest (1000ppm).

16S rDNA Gene Sequencing

The selected isolate was identified by partial sequencing of 16SrDNAgene (Figure4)byMicrobial Type Culture collection, Chandigarh, India(MTCC). The obtained sequence (1373 bps) was subjected to the BLAST in order to find a homology with other 16S rDNA sequences [18]. Comparison indicated that the isolates are similar to *Rhodococcus ruber* with 99% similarity [19,20].

Phylogenetic tree

Aphylogenetic tree was constructed to know the microbial community structure. The phylogenetic tree was generated by the neighbour-joining method using *MEGA 5*software. Number on bootstrap is >50%. *Dietziameris* ATCC 350153^T was used as an out groupwhere bar in the figure represents 0.05 substitutions per nucleotide positions(Figure 5).GenBank accession number **JF430081** was assigned by National Centre for Biotechnology Information (NCBI) to nucleotide database.

IV. DISCUSSIONS

Indigenous microorganisms, enriched and isolated from automobile workshop sediments were observed to possess ability to degrade broad range of mixtures of substrates ofPolycyclic Aromatic Hydrocarbons (PAHs). A *Rhodococcus* strain capable of degrading broad range of PAHs was isolated and screened in this study. Among the 34 oil-degrading microbial isolates collected from petrochemical contaminated workshop sediments of Puducherry in India five isolates are screened and selected based on their efficiency to mineralize Polycyclic Aromatic Hydrocarbons. The efficiency of biodegradation is assessed using

soil dehydrogenase test to measure microbial activity when supplied with individual PAHs, in addition to different concentrations of phenanthrene. The cells formed a primary mycelium that soon fragmented into rod to coccoid elements. These branched hyphal structures that fragment into irregular rods are typical of *Rhodococci* [15].Strain ZP2 that could not use other PAHs as its sole carbon and energy source except naphthalene, phenanthrene and Tween 80 was studied. The carbon-source utilization experiment conveys some useful information about phenanthrene degradation pathways[21].The chemicals used in our carbon source utilisation tests are main components of crude oil and universally present in contaminated soil. According to our earlier studies *R. ruber* showed a degradation percentage of 86.08 (naphthalene), 81.87 (fluorene), 77.77 (acenaphthene), 78.5 (phenanthrene) and 72.56 (benzanthracene) when analysed using HPLC [15]. The isolate from present studies grew well in liquid medium with phenanthrene as the sole carbon source and its colony was surrounded by a clear zone when grown on the Bushnell has agar plate coated with phenanthrene.

Similar studies undertaken by Amouric*et al.* [22] to investigate the alkane-hydroxylating system of Rhodococcus members revealed that these are highly interesting microorganisms, for their degradation capacities, their hydrophobic cell surfaces, their production of biosurfactants and their resistance to harsh environments.

Scanning electron micrographs which clearly show the fruiting body of the microbe areseen in the Figure 1(b).The results of the studies by Al-Thani*et al.* [23] demonstrated that strains isolated on phenanthrene and anthracene were able to grow better on the three tested PAHs than the strain isolated on naphthalene. Similarly, the PAH degradation ability in combination with rape seed oil by *R. wratislaviensis*was reported byPizzul*et al.* [24].

V. CONCLUSION

PAHs degrading bacterial strain was isolated and identified from contaminated soil collected from automobile workshops of Pondicherry.Biodegradability in the order of chrysene>dieseloil>benzanthracene>crudeoil>fluorene>fluoranthene>phenanthrene>dibenzothiophene>naphthalene and anthracenewas observed. The average optical density of *Rhodococcus ruber* grown in BH broth at 250ppm, 500ppm and 1000ppm concentrations of phenanthrene measured at 600nm showed a correspondingly increased in growth of isolate in all the three concentrations of phenanthrene studied.At 250ppm of phenanthrene concentration a lowest average growth of 0.05±0.02 (OD_{600}) was observed while the highest optimum growth of 1.02±0.33 was recorded when the level of phenanthrene was highest (1000ppm). Among the isolated strains one with better degradation was identified as *Rhodococcus ruber* (JF430081) showing 99% homology. This strain has shown degradation over a broad range of PAHs in turbidity studies.The present study indicates that the isolated strain *Rhodococcus ruber* would prove to be a promising candidate for bioremediation of phenanthrene contaminated sites.

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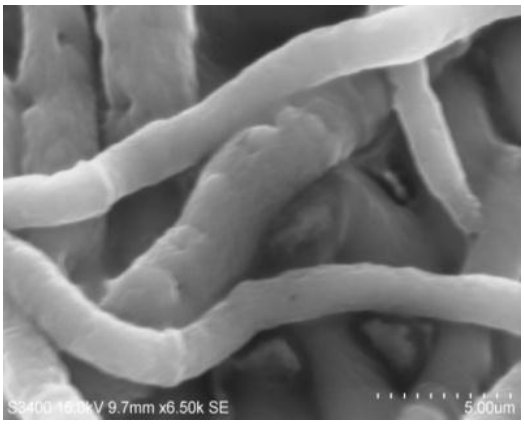
Figures

a)



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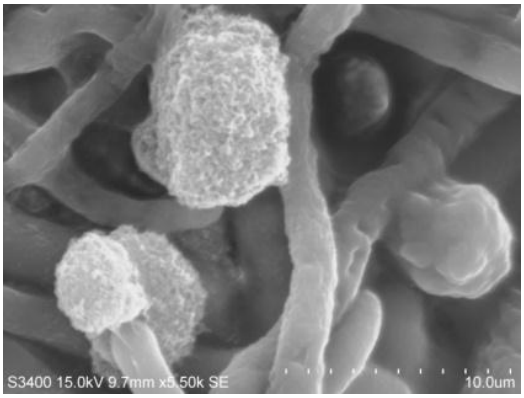


Figure. 1(a-b): Morphology of strain *Rhodococcus* sp. as determined by Scanning Electron Microscope.

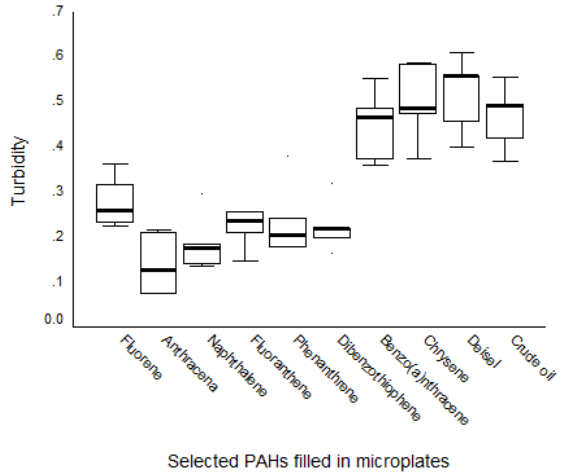
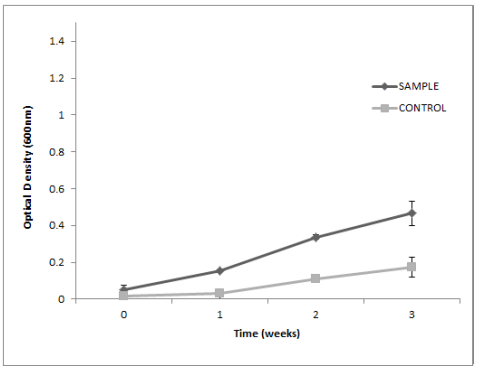


Figure. 2: Biodegradability of selected PAH compounds.

a)



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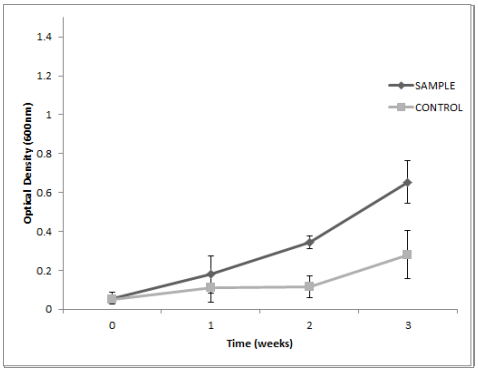

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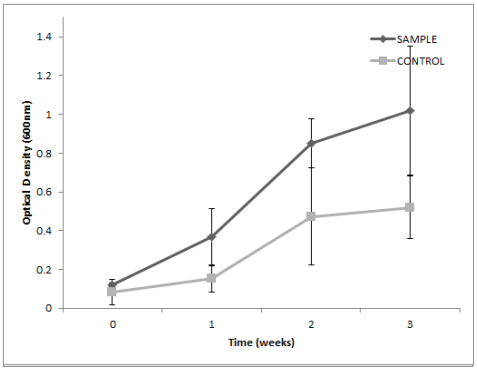

Figure.3(a-c): Impact of 250ppm, 500ppm and 1000ppm concentrations of Phenanthrene respectively on the growth of *Rhodococcus ruber*when compared to control.

CAGTCGAACGATGAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACAC
GTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATA
GGACCTCGGGATGCATGTTCCGGGGTGGAAAGGTTTTCCGGTGCAGGATGGGCCCGCGG
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GAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACG
GCCTTCGGGTTGTAAACCTCTTTCAGTACCGACGAAGCGCAAGTGACGGTAGGTACAGA
AGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGT
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GGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGG
CGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTA
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GATCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGAT
GCAACGCGAAGAACCTTACCTGGGTTTGACATACACCGGAACCGCCCCAGAGATGGGGTT
TCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCACGTAATGGTGG
GGACTCGCAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCAT
CATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCGGTACAGAGGGCTGCGA
TACCGCGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAAC
TCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATAC
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Figure. 4: 16S ribosomal DNA sequence of *Rhodococcus ruber.*

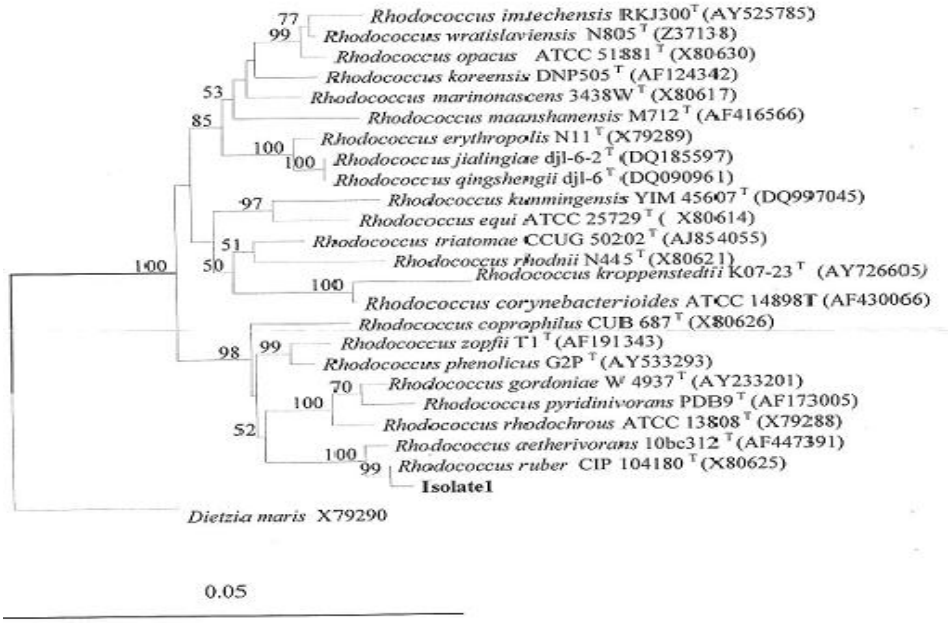

Figure. 5: Phylogenetic relationship of *Rhodococcus ruber* based on sequence analysis and the most closely related bacterial species.