



## ISOLATION AND CHARACTERIZATION OF A BACTERIAL STRAIN FOR BENZENE DEGRADATION

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### ABSTRACT

This study aims to find a potential solution to the problem of environmental pollution by Industrial effluents – aromatic hydrocarbons being the major constituent. In this study, bacteria belonging to the genus *Pseudomonas* were isolated from the terrestrial and aquatic sites (sewage water) in and around petrol stations in Anantapur, India. The obtained isolate of *Pseudomonas* was acclimatized to benzene stress and a relevant concentration of benzene that supported maximum growth was identified. A Specific strain of acclimatized *Pseudomonas* was obtained by growing it on a selective medium – a medium containing benzene as the sole source of carbon and energy. The presence of benzene degrading gene(s) on the plasmid was confirmed by Plasmid curing using acridine orange as a selective inhibitor. This was followed by isolation of the plasmid. After checking the purity of the plasmid DNA preparation by Agarose Gel Electrophoresis, a basic strain of *E.coli* – DH5 $\alpha$ , was transformed with the isolated plasmid using the Calcium Chloride method to induce artificial competence. Plasmid succeeded to be transferred to *Escherichia coli* DH5 $\alpha$  strain. The transformed strains were screened for their ability to degrade benzene using a selective medium, which contained benzene as the sole source of carbon and energy. So it was deduced that the gene responsible for the degradation process was encoded by this plasmid.

**KEYWORDS:** *Pseudomonas putida*, Plasmid curing, Benzene degradation, 16 s RNA gene.

### INTRODUCTION

Rapid industrialization and improper discharge of industrial effluents, wastes, accidental spills or deliberate release of certain hazardous chemicals that are mutagenic, carcinogenic and recalcitrant, pose a serious threat to environment including soils, groundwater as well as open water bodies (Tani *et al.*, 1998). These effluents have a variety of unusual chemicals including a range of aromatic hydrocarbons and their derivatives (Van der Meer *et al.*, 1992) which the microbes enzymatically decompose and utilize in cellular metabolism (Phale *et al.*, 2007). Benzene, toluene, ethylbenzene, and xylenes (BTEX) are common groundwater contaminants largely because of leaking underground gasoline storage tanks. Among them, benzene represents a particular risk to humans due to its carcinogenicity. Vast quantities of epidemiologic, clinical, and laboratory data link benzene to aplastic anemia, acute leukemia, and bone marrow abnormalities (Kasper *et al.*, 2005; Emil 2008). Benzene targets liver, kidney, lung, heart, brain and can cause DNA strand breaks, chromosomal damage, etc. Benzene has been shown to cause cancer in both sexes of multiple species of laboratory animals exposed via various routes (Huff 2007; Rana and Verma 2005). Some women having breathed high levels of benzene for many months had irregular menstrual periods and a decrease in the size of their ovaries. Benzene exposure has been linked directly to the neural birth defects spina bifida and anencephaly (Lupo *et al.*, 2005). Men exposed to high levels of benzene are more likely to have an abnormal amount of chromosomes in their sperm, which impacts fertility and

fetal development (Xing *et al.*, 2010). Outdoor air may contain low levels of benzene from automobile service stations, wood smoke, tobacco smoke, the transfer of gasoline, exhaust from motor vehicles, and industrial emissions. To understand benzene bioremediation options at contaminated sites, many aerobic benzene degrading isolates have been obtained and examined. Commonly identified isolates appear to fall within the phylum, the Proteobacteria. Proteobacteria include, for example, microorganisms within the genera *Pseudomonas* (Alagappan and Cowan 2003; Bertoni *et al.*, 1996). Although many of these microorganisms are present in mixed cultures and at contaminated sites undergoing benzene degradation it remains technically challenging to prove which are the active and dominant *in situ* benzene degraders within such mixed community samples. Thus, the work was designed to identify the organism, *Pseudomonas putida*, capable of degrading Benzene isolated from the soil and identify the species using microbial staining, biochemical analysis, and to check the biodegradative ability of the strain for benzene.

### MATERIALS AND METHODS

#### Sample Collection

Samples of sewage mixed with sediment particles near petrol bunk rich in Benzene were delivered to the laboratory within one hour after their collection.

#### Sample Collection

Samples of sewage mixed with sediment particles near petrol bunk were aspirated from the bottom of sewers using sterile catheters applied to 150 ml sterile syringes. Samples were delivered to the laboratory within one hour after their collection.

### Isolation and Screening of *Pseudomonas* for Benzene Degradation

10 ml of the sewage water sample taken in test tube and used as a stock. This sample was then serially diluted from  $10^{-1}$  to  $10^{-6}$  dilution and plated on a *Pseudomonas* media (Hi-Media). After confirming the isolated microorganism was *Pseudomonas* based on morphological and biochemical analysis then tested the *Pseudomonas* for benzene degradation capacity by spreading on nutrient agar benzene media containing half strength carbon source and secondary screening by growing in Bushnell – Haas broth that lacks carbon source but it is supplemented with benzene as sole carbon source.

### Microbial and Biochemical characterization of the isolate *Pseudomonas*

The isolated bacteria was analyzed using different staining techniques such as Gram's staining, motility test, capsule staining and different biochemical techniques such as catalase test, Urease test, Citrate utilization test, Methyl red and Voges-Proskauer test, Hydrogen sulphide production test, Oxidase test, Nitrate reduction Test, Gelatin Liquefaction test.

### Molecular identification

Molecular characterization by polymerase chain reaction (PCR) amplification and partial sequence analysis of the 16S rRNA gene. The amplification was carried out by using PCR master mix kit (Promega) according to the manufacturer's instructions. Universal

*Pseudomonas* genus-specific primers (forward: 5' - GAGTTTGATCCTGGCTCAG-3' and reverse: 5'-AGAAA GGAGGTGATCCAGCC-3') were designed based on the homologous regions specific to *Pseudomonas* genus were used to amplify ~1500 bp. These primers are used to amplify the 16S rRNA gene of most eubacteria (Weisburg *et al.*, 1991). PCR products were purified by Wizard® SV Gel and PCR cleaning up system kit (Promega) following the protocol provided by the supplier and then resolved by electrophoresis on 1% agarose gel. The purified products were sequenced. The sequence of the 16S rRNA of the strain was compared with another 16S rRNA sequences that were published on the NCBI database [<http://www.ncbi.nlm.nih.gov>]. That was done using the BLAST program to determine the nearest phylogenetic neighbors and to compare it with previously published sequences.

### Plasmid curing

Before isolation of plasmid having susceptible degradation gene, plasmid curing suggested by Hardy (1993) was performed to ensure that the gene degrading benzene is plasmid encoded or chromosomal encoded. This is used to determine whether the benzene degradation is plasmid encoded. For this, the strain was cultured in 4 ml nutrient broth overnight. Then 0.2 ml of culture was added in 4 ml nutrient broth and placed in shaking incubator at 29° C for 2 - 4 h. The log phase 200 mL culture was then added in each tube with 2 ml broth containing different concentrations of ethidium bromide (20 mg/ml to 10 mg/ml). Positive control containing only cells (without the curing agent) while negative

control containing only ethidium bromide were also run and all tubes were incubated (in dark) at 29° C overnight. Tubes with highest concentrations of ethidium bromide (in which growth was manifested) and bacterial growths were selected. These were then serially diluted with sterilized distilled water. Following the spreading technique, equal volumes of inoculums from different dilutions were spreaded on nutrient agar plates and also on PNR media containing Benzene of 1400 ppm (in which rich growth and clear zones of hydrolysis of culture were manifested). Plates were then incubated at 37° C for 24 h and then colonies were counted. Colonies were then replica plated on nutrient agar and transferred to selective media containing benzene.

### Plasmid DNA isolation

Plasmid DNA was isolated from *Pseudomonas putida* strain by using Wizard® Plus SV Minipreps DNA purification system kit (Promega) following the manufacturer's recommended protocol. Isolated plasmid DNA (usually 10 µl) was analyzed by gel electrophoresis (0.7% [wt/vol] agarose).

### Transformation experiment

Isolated plasmids carrying the degrading gene (s) from *Pseudomonas putida* strain, was strongly suggested to be responsible for Benzene degradation. Therefore, the plasmid DNA was transferred to *E. coli* DH5a strain by transformation. Two microliters of the plasmids were transferred into *E. coli* DH5a competent cells. After transformation the cells were plated on a Luria Bertani (LB) agar plates containing Benzene (100 ppm) and allowed to grow for 16 hrs at 37°C. Benzene was used to select *E. coli* with plasmid (s) which had the ability to degrade it.

## RESULTS AND DISCUSSION

### Isolation and Screening of *Pseudomonas* for Benzene Degradation

The organism *Pseudomonas* species was isolated by plating the serially diluted samples on *Pseudomonas* media at  $10^{-6}$  dilution and enriching the same with Benzene as the sole carbon source.

### Microbial and Biochemical characterization of the isolate *Pseudomonas*

The results of the isolated bacterium with reference to microbial and biochemical characterization was given in Table 1. Microbial and Biochemical characterization of strain revealed that it is gram+ positive, aerobic, motile, oxidase negative and catalase positive. Partial sequence analysis of the amplified 16S rRNA gene confirmed the classification of the isolate as a member of the genus *Pseudomonas* where it showed the highest degree of similarity (99%) to that of *Pseudomonas putida*. Based upon this finding, the isolate was designated as *Pseudomonas putida*. It has been reported that *Pseudomonas putida* is able to degrade many aromatic compounds and to detoxify high molecular weight polycyclic aromatic hydrocarbons. Therefore, it possesses a potential for soil decontamination (bioremediation).

**TABLE: 1** Microbial and biochemical characterization of *Pseudomonas*

S.No	Test	Result
1	Gram staining	Positive
2	Motility test	Positive
3	Catalase test	Positive
4	H <sub>2</sub> S production	Negative
5	Gelatin test	Positive
6	Oxidase test	Negative
7	Urease test	Negative
8	Methyl red test	Negative
9	Voges proskauer	Negative
10	Citrate utilization	Positive
11	Nitrate reductase	Negative

**Plasmid curing**

In order to determine the involvement of plasmid-encoded genes in benzene degradation, plasmid curing was performed in which ethidium bromide, a mutagenic compound that rendered the plasmid genes inactive was used. It was observed that 200 out of 250 colonies mutated were unable to utilize benzene and thus failed to grow. So it was concluded that the genes involved in benzene degradation were plasmid encoded. It was noted that cured isolates are unable to degrade benzene.

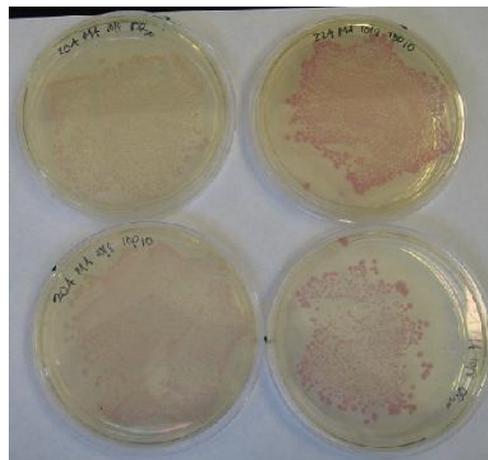
**Plasmid DNA profiling of *Pseudomonas putida* strain**

The plasmid extraction method, previously described, was used to extract the plasmid encoding the benzene degrading gene (s) from strain *Pseudomonas putida*. The size of the plasmids were estimated on the basis of electrophoretic mobility of the isolated fragments as compared to the sizes of Promega supercoiled marker, its molecular weight which was found in the range of ~5 kb Figure 1.

**FIGURE 1:** Agarose gel electrophoretogram of plasmid DNA purified from strain *Pseudomonas putida*. M, Marker DL15000; 1, the plasmid DNA of strain *Pseudomonas putida*.**Transformation of another bacterial strain with the gene bearing-plasmid**

The plasmid was used to transform *E. coli* DH5 $\alpha$  strain. Plasmid (5 kb) was successfully transferred to this strain to

allow it to grow on benzene. Positive transformants were selected on LB media plates that contain 100 ppm benzene. This strain (transformed strain) had the ability to hydrolyze benzene (100 ppm) in LB media and maintain its activity after repeated subculture. The DH5 $\alpha$  bacteria carrying the Plasmid DNA degraded the benzene and utilized as a sole carbon source, the transformed indicates that benzene degradation is a Plasmid dependent process Figure 2.

**FIGURE 2.** Transformed *E. coli* DH5 $\alpha$  carrying the Plasmid DNA degraded the benzene and utilized as a sole carbon source.

Marecik *et al.* (2008) reported that the positive effect of microorganisms in the process of xenobiotic degradation can be explained in terms of a variety of different species and a broad spectrum of produced enzymes. Microorganisms can therefore use different sources of carbon and nitrogen to adapt to different environments. Also, new gene combinations could allow the degradation of related compounds, degradation via different pathways, or recombination between related genes to generate even greater metabolic diversity (Karns, 1990). When this plasmid is genetically transferred to a new host it confers the capacity to metabolize the compound of interest.

Wide varieties of aromatic compounds which are being released into the environment through different human activities are metabolized by soil bacteria. This is of great importance in environmental cleanup technologies (Watanabe *et al.*, 1996). Efficient bioremediation process is very important. This is because the bacteria involved perform a complete degradation pathway to eliminate toxic metabolites from soil (Kazunga *et al.*, 2001). In the present study, a bacterial strain *Pseudomonas putida* isolated from soil of petrol contaminated soils, was tested for its ability to degrade benzene, a toxic chemical which is present in the effluents of many petroleum products and industries. The strain grew well in selected media containing Benzene with glucose and without glucose showing that it readily decomposed it and utilize it as a sole source of carbon and energy. Similar findings on *Pseudomonas sp.* (PN1001) that actively utilized and degraded pentylaniline and aniline as carbon source with no glucose/starch degradation has being reported by Wang *et al.* (2006).

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