Biodegradation of Aromatics Such as Benzene, Toluene and Phenol by *Pseudomonas* Strain

Mahesh Arvind, Sneha Bhatt, and Nichith K. R.

**ABSTRACT**

The environment is home to thousands of life forms and the day to day human activities are posing a serious threat to these organisms and leading them to the path of extinction. A wide range of pollutants are seen contaminating resources such as air, water and land. One major issue of water pollution is due to industrial effluents which includes a combination of phenolics and aromatic hydrocarbons. These organic compounds are said to be toxic to life even in minor quantities. An effective approach to detoxify these contaminated water sources and make it sustainable is by bioremediation. Microbes bioremediate the environment as they biodegrade the pollutants to obtain energy. Among aromatics, phenols and their derivatives from the principal group of environmental contaminants. Even at low levels they are toxic and they pose a threat to the biosphere because of their recalcitrant nature. *Pseudomonas* was the bacteria chosen for the study due to its versatile nature. The bacteria were isolated and characterized using biochemical tests. It was then subjected to degrade organic compounds such as benzene, toluene and phenol. The bacteria showed optimal growth in the presence of these compounds and could metabolize them effectively. A concentration of up to 6% benzene, 2% toluene and 0.5% phenol was found to be degraded. The aromatics were found to be assimilated by the end of 24 hours. The bacteria were successful in biodegradation of these aromatic hydrocarbons and proves to be a novel strain capable of biodegradation of these aromatics at such high concentrations and thus, proves to be a promising solution to decontaminate water resources that are affected due to them.

**Keywords:** Biodegradation, Aromatic Hydrocarbons, *Pseudomonas*, Bioremediation, Recalcitrant

I. INTRODUCTION

Aromatics such as benzene, toluene and phenol are produced as by-products of various industrial processes which prove to be serious environmental contaminants. The derivatives of phenol are said to be labelled as priority pollutants [1] due to their wide distribution and detection in industrial effluents [2]. Phenols have been found to have high toxicity to most living organisms and also cause considerable damage to the environment [2]. These compounds can cause severe health hazards upon entering into the living system. Till date, various physical, chemical and biological techniques have been employed to remove these toxic contaminants from water resources.

Bioremediation is a process by which microbes degrade target pollutants at the site of contamination. This can be done by altering the environmental conditions and providing them with the required nutrients. Bioremediation is a new treatment technology based on fundamental processes of microorganism to utilize synthetic organic molecules as a sole source of energy. Bioremediation usually involves redox reactions where either an electron acceptor is added to stimulate oxidation of a reduced pollutant or an electron donor is added to reduce an oxidized pollutant. Bioremediation exploits the catabolic diversity of microorganism to transform contaminants into eco-friendly products. Many species of bacteria and fungi have evolved the metabolic capacities to degrade aromatic compounds [3]. However, the event of biotechnology for the removal of aromatics from the commercial effluents remains to be adequately addressed even today. Various approaches are being developed to treat the aromatic effluents. The constraints are the availability of suitable microorganism that can overcome their culturing limitations from their natural habits to the effluent conditions.

*Pseudomonas* is a genus of aerobic, non-sporulating, motile Gram-negative bacilli. This genus is found to have considerable heterogeneity [4]. This genus of bacteria is well known for its metabolic versatility allowing it to inhabit a range of environments and utilize an unusually wide range of organic compounds [5].

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Mahesh Arvind*, Sneha Bhatt, and Nichith K. R.
Department of Biochemistry,
Vijaya College,
Bangalore, Karnataka, India
(e-mail: drmhsharv@gmail.com)

*Corresponding Author
Previous studies have shown *Pseudomonas aeruginosa* to be capable of degrading diesel, crude oil, n-alkanes and polycyclic aromatic hydrocarbons (PAHs) in petroleum [6]. *Pseudomonas* is manifested with the capacity to degrade a number of aliphatic, aromatic, polyaromatic hydrocarbons and various derivatives, among a vast variety of miscellaneous organic compounds [7]. *Pseudomonas fluorescens* PU1 has been shown to degrade 800 ppm and 1000ppm of phenol in 72 hours [8]. *Pseudomonas aeruginosa* is a predominant microbial species for phenol degradation [9]. *Pseudomonas aeruginosa* KBM13 exhibited maximum degradation of phenol at a concentration of 500 mg/L [10].

The present study reports that biodegradation of aromatic compounds such as benzene, toluene and phenol by *Pseudomonas* strain. This is a new agent for bio degradation of pollutants and is capable of mineralizing benzoid compounds as the sole source of carbon and energy. The study shows its applicability in bioremediation of effluents containing aromatic compounds.

### II. OBJECTIVES

- To study the growth of isolated bacteria *Pseudomonas*, in the presence of phenols.
- To study the degradation of phenol by the isolated strain.
- To identify the metabolites in the degradation of the phenols.

### III. MATERIAL AND METHODS

#### A. Chemicals:
All chemicals used were of analytical grade and purchased from commercial suppliers.

#### B. Isolation of microorganisms from source:
Soil samples were collected from hydrocarbon contaminated site around Peenya industrial area and was used to isolate microorganism adopting selective enrichment techniques. The bacterial strain was grown on mineral salt medium supplemented with varied concentrations of phenol as the sole source of carbon and energy.

#### C. Culturing of bacteria:
The organism was maintained and propagated on nutrient-agar and substrate-mineral salt media. For purification of the bacterial strain, the microorganism was grown on nutrient agar medium. For metabolic studies the bacterial strains were grown on mineral-salt medium (MSM) containing (g/L): K2HPO4, 1.6; KH2PO4, 0.2; (NH4)2SO4, 1.0; MgSO4·7H2O, 0.2; NaCl, 0.1; CaCl2·2H2O, 0.02; FeSO4·H2O, 0.01; Na2MoO4·2H2O, 0.5; MnSO4·H2O, 0.5; Na2WO4·2H2O, 0.5. The growth substrate was supplemented to the sterilized mineral salt medium aseptically. The flasks were then inoculated with 5% of inoculum aseptically and were incubated at 25°C (± 2°C) on a rotary shaker for 24 hours. Uninoculated flasks were incubated in parallel as controls [11].

### D. Identification and characterization of bacteria:
These are a series of different tests performed in order to identify and differentiate bacteria.

1) **Gram staining**
This is the most common technique used to differentiate bacteria into two large groups based on the composition of the cell wall i.e., Gram positive and Gram negative. This technique was developed by Hans Christian Gram and is the preliminary step used in the identification of bacteria [12].

2) **Motility test**
The motility of the bacteria is largely credited to the presence of hair-like appendages called flagella. The motility of the bacteria can be observed under a light microscope using the hanging drop technique [13].

3) **IMViC test**
It is a group of important tests performed to differentiate coli forms. The coli form group of bacteria includes both aerobic and facultative aerobic bacteria which are Gram negative and non-sporulating. The classical species include *Enterobacter* and *Escherichia*. IMViC stands for the first letter of each test in the series which includes Indole test, Methyl red test, Voges-Proskauer test and citrate utilization test. This test is mainly performed in order to distinguish *E. coli* from *Enterobacter aerogenes* [14,15].

4) **Nitrate reduction test**
This test helps differentiate bacteria on their ability to reduce nitrate to nitrite and other nitrogenous gases thus, grouping them into nitrate positive and nitrate negative organisms. Nitrate reduction may be coupled to anaerobic respiration in some species [16,17].

5) **H2S production test**
Some bacteria possess the ability to reduce sulfur containing compounds during metabolism which is used as a test to identify bacteria [18,19].

6) **Catalase test**
It is a test performed to check the ability of the bacteria to produce the catalase enzyme. This enzyme helps in the breakdown of hydrogen peroxide to water and oxygen, which is produced as a result of aerobic respiration [20].

7) **Oxidase test**
This test is used to detect the presence of cytochrome C in mitochondria which produces the enzyme cytochrome C oxidase [21].

8) **Urease test**
This test is performed to check the ability of the bacteria to produce the enzyme urease. Urease is a hydrolytic enzyme involved in the cleavage of the amide linkage in urea to liberate ammonia and water [22,23].

### E. Study of the growth curve:
The bacterial cells isolated by substrate enrichment culture were used for the growth study. Various phenolic compounds served as source of carbon and energy. The cells were freed from adhering substrate by centrifugation (5000 rpm) at 5º for 20 min. The cells were repeatedly washed with 0.05M phosphate buffer (pH 7.0) and centrifuged. The cell pellet obtained was finally re-suspended in the sterile mineral-salt medium. Suitable aliquots (2.0 ml) of this cell suspension were inoculated to the flasks containing different compounds (2,4,6 and 8% each of benzene and toluene and miscellaneous organic compounds) [7].
0.2, 0.5, 1 and 2% each of phenol). The growth of the organism was measured turbidimetrically by monitoring the OD at 660 nm at different incubation periods. Benzene, phenol and toluene utilization was followed by estimating residual substrate colorimetrically at 660 nm [11].

F. Isolation and Identification of Metabolites:
Metabolic intermediates were isolated using solvent of the spent culture medium. The spent media was examined for the accumulation of metabolic intermediates at regular intervals. The metabolic intermediates were isolated from the spent broth using ethyl acetate. It was performed on silica gel plates (0.25mm thickness using 2-propanol; Ammonia; water (20:1:2 V/V) solvent system [24]. Preparative TLC was performed to purify the metabolites. The spots on the chromatogram were visualised under UV lamp or by spraying with a mixture of potassium ferricyanide and 2% FeCl3 solution to detect hydroxylated metabolites. The separated metabolites were scraped off from chromatograms, eluted with methanol and subjected to Ultra-violet (UV) spectral analysis (UVVIS Spectrophotometer, Model SL 159, ELICO, India) [3].

IV. RESULTS
The bacteria were isolated from the source and characterized in order to identify them. They were then subjected to the minimal media to study their growth kinetics in presence of benzene, toluene and phenol. The spent media was later used to study the metabolites produced as a result of biodegradation of benzene, toluene and phenol.

A. Isolation and characterization of bacteria:
The bacteria were isolated and cultured in nutrient agar medium as they are the basic media and enhance the growth of non-fastidious bacteria due to the presence of nutrients in abundance. The identification and characterization of bacteria is an important as well as a systematic procedure that has to be performed. The biochemical tests and gram staining categorize bacteria using various properties like composition of cell wall (Gram staining), production of hydrolytic enzymes (Indole test, Urease test), ability to ferment glucose (Methyl red test and Voges Proskauer test), ability to use citrate as the sole carbon source (Citrate test), and the ability to reduce compounds like nitrate and sulphate (Nitrate reduction and H+H2 production test) respectively. The motility of the bacteria was determined by the hanging drop technique. All the results together have indicated the isolated organism to belong to the strain of *Pseudomonas*. The results are tabulated in Table I.

IV. RESULTS

B. Study of growth curve of bacteria:
Upon characterisation and identifying the bacteria to be *Pseudomonas*, they were cultured in the Minimal salt medium in presence of varying of concentrations of benzene, toluene and phenol. The bacteria were also cultured in the minimal salt media in the absence of benzene, toluene and phenol, which were used as controls. After incubating them at a temperature of 37°C ±2°C for 24 hours, the optical density was measured using a colorimeter at a wavelength of 660 nm as a measure of the bacterial growth and its ability to use organic compounds like benzene, toluene and phenol as a sole carbon source. The results proved to be promising as the bacteria was able to grow at concentrations of 6% in both benzene and toluene. The results are depicted in Table II and Fig. 1.

Whereas, *Pseudomonas* showed highest growth at a concentration of 0.5% phenol which has been tabulated in Table III and Fig. 2.

### TABLE I: BIOCHEMICAL TESTS FOR IDENTIFICATION OF BACTERIA

| Biochemical tests        | Results obtained        |
|--------------------------|-------------------------|
| Shape                    | Rod shaped              |
| Gram staining            | Gram negative           |
| Motility                 | Motile                  |
| Indole Production        | Negative                |
| Methyl Red               | Negative                |
| Voges Proskauer          | Negative                |
| Citrate utilization      | Positive                |
| Nitrate reduction        | Positive                |
| H+H2 production          | Negative                |
| Catalase                 | Positive                |
| Oxidase                  | Positive                |
| Urease                   | Negative                |

### TABLE II: OPTICAL DENSITY OF BACTERIA AT 660 NM IN PRESENCE OF BENZENE AND TOLUENE

| Concentration of aromatics (%) | Benzene | Toluene |
|--------------------------------|---------|---------|
| Blank                          | 0       | 0       |
| 2                              | 0.02    | 0.03    |
| 4                              | 0.02    | 0.05    |
| 6                              | 0.04    | 0.06    |
| 8                              | 0.04    | 0.06    |

![Fig. 1. Growth curve of *Pseudomonas* in presence of benzene and toluene](image1.png)

### TABLE III: OPTICAL DENSITY OF BACTERIA AT 660 NM IN PRESENCE OF PHENOL

| Concentration of phenol (%) | Optical density at 660 nm |
|-----------------------------|--------------------------|
| 0                           | 1.02                     |
| 0.2                         | 0.42                     |
| 0.5                         | 0.48                     |
| 1                           | 0.12                     |
| 2                           | 0.09                     |

![Fig. 2. Growth curve of *Pseudomonas* in presence of phenol](image2.png)
C. Analysis of metabolites:

Thin Layer chromatography was performed to analyze the metabolites. TLC analysis of the intermediates suggest that benzene, toluene and phenol are degraded by first being converted to the intermediate catechol as catechol was detected in the cultures of the spent media when run with catechol solution as the standard. Previous studies have indicated that enzymes like phenol hydroxylase, catechol 1,2-dioxygenase and catechol 2,3 dioxygenase are involved in the biodegradation of phenol [3]. The catechol intermediate having been found even in the spent cultures of benzene and toluene indicate the same pathway being followed. Phenol is directly converted to catechol, whereas benzene and toluene undergo hydroxylation to form phenol and o-cresol, respectively. Both phenol and o-cresol thus, obtained is converted to catechol. Catechol undergoes isomerization to form quinol and quinol is metabolized to form a linear structure, beta-ketoadipate which enter the Kreb’s cycle eventually thus, being utilized as the carbon source by the bacteria to release energy. The pathway has been depicted in Fig. 3.

The key enzymes involved in the following degradation of aromatics include monoxygenases, reductases, dehalogenases and dioxygenases. The proposed pathway for degradation can be confirmed by assaying for the key enzymes.

V. DISCUSSION

This study demonstrates that the Pseudomonas strain isolated from contaminated site is able to metabolize benzenoid compounds such as toluene and phenol. Acquisition of degradative abilities by selective enrichment has been seen in laboratory ecosystems for many organic compounds [25]. In consistent with this observation a bacterial strain degrading benzene, toluene and phenol has been isolated from the contaminated site adopting the selective enrichment technique. The strain of Pseudomonas used in this study proves to be a novel strain with the ability to degrade not only aromatics like benzene, toluene and phenol at such high concentrations, but also capable of degrading the derivatives of these aromatics. The advantage of applying the bacterial systems for effluent remediation is that they pose a higher rate of biodegradation than fungi.

In this study, the isolation of metabolites by preparative TLC and the cell free enzyme extract analysis demonstrated that the aromatic compounds are initially hydroxylated to phenol and further to catechol by monoxygenases. Catechol is the substrate for the next enzyme which is a dioxygenase. There are two major mechanisms for ring cleavage of aromatics i.e., the ortho- cleavage and the meta-cleavage [26]. After ring cleavage, the products are transformed and funneled into the TCA cycle, the central metabolic pathway for energy of the cell.

VI. CONCLUSION

The use of biological systems bioremediation is more cost effective than traditional treatment techniques [27]. The microorganism isolated from contaminated site used aromatic compounds as sole source of energy. This was identified and characterized as Pseudomonas strain. The organism had abilities to degrade benzene, toluene and phenols. The study also reveals that Pseudomonas strain can efficiently degrade phenol even at higher concentrations. Since the bacterium is capable of degrading various aromatic compounds there exists a possibility for its use in the development of microbial technology for decontamination of aromatic wastes.

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