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

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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].

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 benzenoid 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/lt: K_2HPO_4 , 1.6; KH_2PO_4 , 0.2; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.02; $FeSO_4 \cdot H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.5; $Na_2WO_4 \cdot 2H_2O$, 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 ($\pm$ 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 mobility 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) H_2S 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 5° 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

0.2, 0.5, 1 and 2% each of phenol). The growth of the organism was measured turbidometrically 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 H₂S 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.

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 ₂ S production	Negative
Catalase	Positive
Oxidase	Positive
Urease	Negative

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 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

Growth curve

Fig. 1. Growth curve of *Pseudomonas* in presence of benzene and toluene

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

Growth curve

Fig. 2. Growth curve of *Pseudomonas* in presence of phenol

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.

Fig. 3. The biodegradation pathway of benzene, phenol and toluene.

The key enzymes involved in the following degradation of aromatics include monooxygenases, 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 phenols. 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 monooxygenases. 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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REFERENCES

- [1] S.Shashirekha, L.Uma and G.Subramanian, " Phenol degradation by the marina Cyanobacterium Phormidium valderianum BDU 30501", Journal of Industrial microbiology and biotechnology, Vol 19, pp 130-133, 1997.
- [2] Butani Naresh, Parekh Honey and Saliya Vaishali, Biodegradation of phenol by a bacteria; strain isolated from a phenol contaminated site in India (2012) International Research Journal of Environmental Sciences.
- [3] Phenol degradation by immobilized cells of *Arthrobacter citreus* Chandrakant Karigar, Aravind Mahesh, Manjunath Nagenahalli1 & Dae Jin Yun; Biochemistry Division, Department of Chemistry, Central College Campus, Bangalore University, Bangalore, 560001, India; Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660701, Korea.
- [4] Özen, A. I., & Ussery, D. W. (2012). Defining the *Pseudomonas* Genus: Where Do We Draw the Line with *Azotobacter*? *Microbial Ecology*, 63(2), 239–248. doi:10.1007/s00248-011-9914-8
- [5] Stanier RY, Palleroni NJ, Doudoroff M. The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol*. 1966; 43:159–271.
- [6] Zhang X, Young L. Y. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* (63): 4759-4764 (1997)
- [7] Palleroni* N.J., Pieper D.H., Moore E.R.B. (2010) Microbiology of Hydrocarbon-Degrading *Pseudomonas*. In: Timmis K.N. (eds) Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Heidelberg
- [8] Mahiuddin M, Fakhruddin AN, Abdullah-Al-Mahin. Degradation of Phenol via Meta Cleavage Pathway by *Pseudomonas fluorescens* PU1. *ISRN Microbiol*. 2012; 2012:741820. Published 2012 Jan 23. doi:10.5402/2012/741820
- [9] A. M. Ahmed, G.F. Nakhla & S. Farooq (1995) Phenol degradation by *pseudomonas aeruginosa*, Journal of Environmental Science and Health. Part A: Environmental Science and Engineering and Toxicology, 30:1, 99-107, DOI: 10.1080/10934529509376188

- [10] Kassim, Kais & Rahal, B.S. & Mohamed, M.M. (2017). Biodegradation of phenol by *Pseudomonas aeruginosa* isolated from soil contaminated with diesel fuel. *Bioscience Research*. 14. 713-720.
- [11] Mahesh Arvind, P. C. Shreedharan, and S. R. Ambika, "Bioremediation for Environmental Management," *International Journal of Environmental Science and Development* vol. 6, no. 7, pp. 555-558, 2015.
- [12] Colco R. Current Protocols in Microbiology. 2005.
- [13] Jain A., Jain R., Jain S. (2020) Motility Testing – Hanging Drop Method and Stab. In: Basic Techniques in Biochemistry, Microbiology and Molecular Biology. Springer Protocols Handbooks. Humana, New York, NY
- [14] MacFaddin J F. Biochemical Tests for Identification of Medical Bacteria, 2nd ed. Williams and Wilkins, Baltimore.1980.
- [15] MacFaddin J F. Biochemical Tests for Identification of Medical Bacteria, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA. 2000.
- [16] Knapp JS, Clark VL. Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. *Infect Immun* 1984; 46:176-181.
- [17] Skerman VBD. 1967. p.218 – 220. A guide to the identification of the genera of bacteria. The Williams & Wilkins Co., Baltimore, MD.
- [18] Tille, P. M., & Forbes, B. A. (2014). *Bailey & Scott's diagnostic microbiology* (Thirteenth edition.). St. Louis, Missouri: Elsevier.
- [19] Cappuccino J.G. and Sherman N. 2008. *Microbiology: A Laboratory Manual*, 8th ed. Pearson Benjamin Cummings, San Francisco, CA, USA.
- [20] Facklam R and Elliott J A. *Clin.Microbiol. Rev*, 1995; 8(4):479.
- [21] Vashist, Hemraj & Sharma, Diksha & Gupta, Avneet. (2013). A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Science*. 1. 1-7.
- [22] Bailey, W. R., and E. G. Scott. *Diagnostic microbiology*, 4th ed. Mosby, St. Louis, MO. 197
- [23] Christensen, W. B. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol.* 1946, 52:461–466.
- [24] Mapana J Sci, 12, 4 (2013), 9-18 ISSN 0975-3303|doi:10.12723/mjs.27.2 9 Biodegradation of Phenolic Pollutants Mahesh Arvind *
- [25] Alexander (1999) *Biodegradation and Bioremediation*, Academic Press, San Diego, CA.
- [26] Eltis, L & Bolin, J. (1996). Evolutionary relationships among extradiol dioxygenases. *Journal of Bacteriology*. 178. 5930-5937. 10.1128/jb.178.20.5930-5937.1996.
- [27] Zechendorf B. Sustainable development: how can biotechnology contribute? *Trends Biotechnol.* 1999;17(6):219–225. doi:10.1016/s0167-7799(98)00297-9.