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Biodegradation of Polychlorophenols By *Arthobacter Citreus*

Sneha Bhatt[1], Nichith K R[2], Mahesh Arvind[#3]

Associate Professor, Department of Chemistry and Biochemistry, Vijaya College, Basavanagudi, Bangalore-560004, Karnataka, India.

Abstract: *Polychlorophenols are a group of organic compounds widely used in the production of biocides such as pesticides, fungicides, insecticides, etc., The effluents from the biocide industry, thus possess a large amount of these Polychlorophenols contaminating the environment. The Polychlorophenols are highly toxic due to their ability to disrupt the structural and functional integrity of biological membranes, causing lethal repercussions. Bioremediation thus provides a promising and skimping outcome to relieve environmental pollution. Bacteria and fungi possess the ability to degrade organic compounds in their natural environment, either aerobically or anaerobically. Bacteria and fungi can easily metabolize Polychlorophenols to produce intermediates that enter the Krebs cycle. In the present study, the isolated organism was identified as Arthrobacter citreus, and its ability to degrade two important Polychlorophenols, i.e., trichlorophenol pentachlorophenol, was investigated. Trichlorophenol is naturally occurring, whereas; pentachlorophenol is anthropogenic. The bacteria were found to grow at 5mM and 3mM concentrations of trichlorophenol and pentachlorophenol, respectively. The metabolites were also determined by Thin Layer chromatography analysis, which indicated the metabolite, β-ketoadipate, thus ensuring the entry of the metabolized components to the citric acid cycle.*

Keywords: *Arthrobacter citreus, β-ketoadipate, Bioremediation, Pentachlorophenol, Polychlorophenols, Trichlorophenol.*

I. INTRODUCTION

Bioremediation is an emerging process that employs specific strains of microorganisms to degrade organic matter under a constrained environment biologically. The microbes degrade the organic matter, which is potential pollutants, to less toxic and non-hazardous forms. Bioremediation involving microorganisms for the degradation of pollutants is a very efficient technology, also being cost-effective. Microbial degradation can be hindered by various environmental factors [1], but the Arthrobacter species' ability to tolerate various environmental stress helps them in biodegradation [2].

Chlorophenols and its derivatives are persistent environmental pollutants [3] commonly found in pesticide preparations and industrial wastes [4]. They are a broad group of chlorinated organic compounds, including monochlorophenols, polychlorophenols, chloronitrophenols, chloroaminophenols, and chloromethylphenols [3]. Their recalcitrant nature makes them persistent in the environment leading to pollution. Chlorophenols are highly toxic to lifeforms due to their carcinogenic, mutagenic, and cytotoxic properties [3]. Of the many chlorophenols, our study focuses on trichlorophenols and pentachlorophenols. Trichlorophenol and pentachlorophenol are toxic, persistent wood, and cellulose preservatives [5]. They exhibit toxicity due to their ability to penetrate epithelium, causing tissue damage followed by necrosis.

Arthrobacter citreus is a genus of obligate aerobes characterized by their rod coccus growth [6]. They are commonly found in soil, and all species are Gram-positive. They exhibit rod-shaped morphology during their exponential growth phase and cocci in their stationary phase.

Studies have revealed that *A.cystallopoietes* and *A. chlorophenolicus* can reduce hexavalent chromium and 4-chlorophenol levels in contaminated soil, suggesting they may be useful bioremediation [7,8]. Also, *Arthrobacter spp strain R* has been shown to grow on a variety of aromatic compounds, including homocyclic compounds such as hydroxybenzoate, as well as N-heterocycles, including pyridine and picoline [9]. *Arthrobacter species AD26* has been shown to degrade Atrazine [10]. Polychlorinated biphenyls are shown to be degraded by *Arthrobacter SP M5, Arthrobacter spp BPA,* and *Arthrobacter spp BIB* [11,12,13].

The present study reports the biodegradation of Polychlorophenols such as trichlorophenol and pentachlorophenol by *Arthrobacter citreus* strain. This organism can be employed in the biodegradation of pollutants and can use these organic compounds as the sole source of carbon to produce energy. The study aims to show the application of bioremediation of soil contaminated with chlorophenols.

II. OBJECTIVES

- To study the growth of the isolated bacteria, *Arthrobacter citreus*, in the presence of trichlorophenol and pentachlorophenol.
- To study the degradation of trichlorophenol and pentachlorophenol by the isolated strain.
- To identify the metabolites in the degradation of the trichlorophenol and pentachlorophenol.

III. MATERIALS 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 sites contaminated with Polychlorophenols and isolated the microbe by adopting selective enrichment techniques. The bacterial strain was grown on a mineral salt medium supplemented with varying concentrations of polychlorophenol such as trichlorophenol and pentachlorophenol 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 a 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; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.02; $FeSO_4.H_2O$, 0.01; $Na_2MoO_4.2H_2O$, 0.5; $MnSO_4.H_2O$, 0.5; $Na_2WO_4.2H_2O$, 0.5. The growth substrate was supplemented to the sterilized mineral salt medium aseptically. The flasks were then inoculated with 5% inoculum aseptically and were incubated at 25ºC (± 2ºC) on a rotary shaker for 24 hours. Uninoculated flasks were incubated in parallel as controls [14].

D. Identification and characterization of bacteria

These are a series of different tests performed to identify and differentiate bacteria.

a) Gram staining is the most common technique used to differentiate bacteria into two large groups based on the cell wall composition, i.e., Gram-positive and Gram-negative. This technique was developed by Hans Christian Gram and is the preliminary step used to identify bacteria [15].

b) Motility test **-** The mobility of the bacteria is largely credited to the presence of hair-like appendages called flagella. The bacteria's motility can be observed under a light microscope using the hanging drop technique [16].

c) IMViC test **-** It is a group of important tests performed to differentiate coliforms. The coliform 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 each test's first letter in the series, which includes the Indole test, Methyl Red test, Voges-Proskauer test, and citrate utilization test. This test is mainly performed to distinguish *E. coli* from *Enterobacter aerogenes* [17,18].

d) 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 [19, 20].

e) H_2S production test **-** Some bacteria can reduce sulfur-containing compounds during metabolism, which is used as a test to identify bacteria [21,22].

f) ***Catalase test*** - It is a test performed to check the bacteria's ability to produce the catalase enzyme. This enzyme helps break down hydrogen peroxide to water and oxygen, which is produced as a product of aerobic respiration [23].

g) Oxidase **test** is used to detect cytochrome C in mitochondria, which produces the enzyme cytochrome C oxidase [24].

h) Urease test – This test is performed to check the bacteria's ability to produce the enzyme urease. Urease is a hydrolytic enzyme involved in the amide linkage's cleavage in urea to liberate ammonia and water [25,26].

E. Study of the growth curve and degradation

The bacterial cells isolated by selective enrichment culture technique were used for the growth study. Trichlorophenol and pentachlorophenol served as a 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 varied trichlorophenol concentrations and pentachlorophenol ranging from 1mM to 5mM. The organism's growth was measured turbidometrically by monitoring the optical density at 660 nm at different incubation periods. Utilization of trichlorophenol and pentachlorophenol was followed by estimating residual substrate colorimetrically at 660 nm [14].

F. Isolation and Identification of Metabolites

Metabolic intermediates were isolated using the 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 [27]. Preparative TLC was performed to purify the metabolites. The chromatogram spots were visualized under a UV lamp or by spraying with a mixture of potassium ferricyanide and 2% $FeCl_3$ solution to detect hydroxylated metabolites. The separated metabolites were scraped off from chromatograms, eluted with methanol, and subjected to Ultra-violet (UV) spectral analysis (UV-VIS Spectrophotometer, Model SL 159, ELICO, India) [28].

IV. RESULTS

The isolated bacteria were cultured and characterized based on morphology and metabolic characteristics. The bacteria with the ability to degrade polychlorophenols were subjected to minimal selective media in the presence of polychlorophenols like trichlorophenol and pentachlorophenol to determine its growth kinetics and also the degradation of the respective polychlorophenols. The remaining spent cultures were used to determine the metabolites of the degradation pathway using thin-layer chromatography.

A. Identification and Characterization of the Organism

The ability of the microbes to adapt to their varying environmental conditions makes them versatile. The microorganisms used in this study were isolated by enrichment culture technique. The microorganisms were isolated from the soil obtained from the contaminated site and were classified based on phenotypic characters and biochemical characteristics. The isolated strain of polychlorophenol metabolizing bacteria was Gram-positive due to a peptidoglycan cell wall's presence and exhibited a coccoid morphology. It was found to be motile and possessed catalase activity but lacked oxidase activity. It was also negative for Indole production, Methyl red, and Voges- Proskauer but showed positive for Citrate utilization. It could not reduce nitrate and sulfur compounds and also does not produce hydrolytic enzymes like urease. These results identify the bacteria to be *Arthrobacter citreus,* according to Bergey's Manual of Determinative Bacteriology [29].

B. Study of Growth Study, Utilization of Polychlorophenols

The growth kinetics of the bacteria, *Arthrobacter citreus,* was studied to understand its ability to use the polychlorophenols as the sole source of carbon. The bacteria were cultured in minimal salt media with varying concentrations of trichlorophenol and pentachlorophenol. The concentration used ranged from 1mM to 15mM in the case of both trichlorophenol and pentachlorophenol. The bacteria cultured in minimal salt media in the absence of trichlorophenol and pentachlorophenol were used as controls. The cultures were then left in a shaker incubator at 37˚C ± 2˚C for 24 hours. The optical density of the cultures was read at a wavelength of 660nm to determine the extent of bacterial growth. The result showed that the bacteria could utilize up to 5mM of trichlorophenol and 3mM of pentachlorophenol in 24 hours. It was also observed that they could tolerate concentrations of 12mM and 15mM of trichlorophenol and pentachlorophenol, respectively, and represented in figure 1.

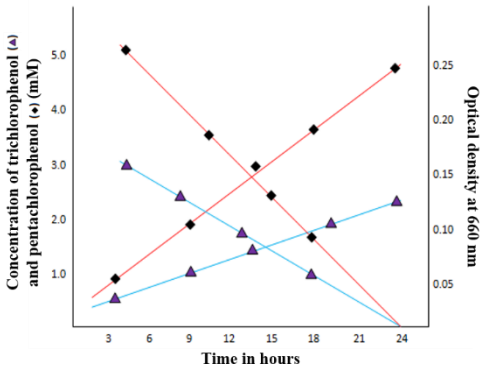

Figure 1: Growth study of Arthrobacter citreus in the presence of Trichlorophenol and Pentachlorophenol.

C. Analysis and determination of metabolites:

Thin layer chromatography was the technique employed to analyze the metabolites of trichlorophenol and pentachlorophenol degradative pathway. The TLC analysis of the spent media indicated the first intermediate of pentachlorophenol degradation to be tetrachloroquinone and dichloroquinol in the case of trichlorophenol. In both cases, the presence of β-ketoadipate indicates the intermediates' entry to the TCA cycle. Thus, trichlorophenol and pentachlorophenol act as a carbon source and are involved in bacteria's energy production.

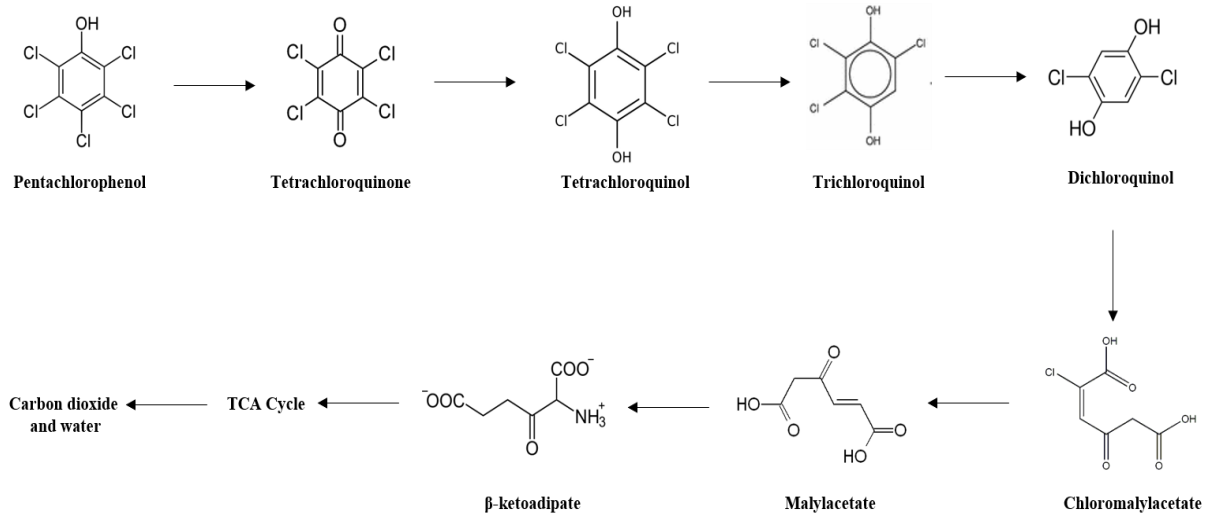

Figure 2: A proposed pathway for the biodegradation of trichlorophenol and pentachlorophenol.

Using these intermediates, the biodegradative pathway of pentachlorophenol was elucidated and is represented in figure 2.

Earlier studies have shown that pentachlorophenol monooxygenase, tetrachloroquinone reductase, tetrachloroquinol dehalogenase, dichloroquinol dioxygenase are the major enzymes involved in the microbial degradation of pentachlorophenol and trichlorophenol [30, 31, 32, 33, 34]. Their presence can be confirmed by assaying these key enzymes' activity; thus, proving the proposed pathway.

V. DISCUSSION

The study aims to demonstrate that the *Arthrobacter citreus* isolated from the polluted site possesses the ability to metabolize Polychlorophenols. The bacteria acquire the ability to degrade these components due to their ecosystems' change as an adaptation. The strain of *Arthrobacter citreus* used in the study is multifaceted as it commands the ability not only to degrade pentachlorophenol and trichlorophenol but also other derivatives of chlorophenols. Bacterial bioremediation is said to be more advantageous than fungal bioremediation due to the faster rate of biodegradation.

VI. CONCLUSION

The use of bioremediation techniques is economical and cost-effective. The microbes isolated from the polluted site are rehabilitated to use the ecosystem's components as the sole carbon source for derivation of energy. The bacteria isolated were identified as belonging to the genus Arthrobacter citreus. It possessed the potential to efficiently degrade Polychlorophenols and tolerate high concentrations of these Polychlorophenols in their habitat. This ability of the bacterium can be exploited to decontaminate sites polluted with Polychlorophenols.

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