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ORIGINAL ARTICLE

Bioremediation of Organophosphorus Pesticide Using Pseudomonas Biofilm: A Sustainable Approach to Environmental Detoxification

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ABSTRACT
The increasing use of organophosphorus pesticides like dimethoate has raised environmental concerns due to their persistence and toxicity. This study focuses on the bioremediation of dimethoate using bacterial biofilms, specifically those formed by Pseudomonas spp., isolated from pesticide-contaminated soil. The isolated bacterium demonstrated growth in the presence of dimethoate, indicating its potential for pesticide degradation. Characterization through Gram staining, IMViC tests, and enzymatic assays confirmed the identity of the organism as Pseudomonas spp. To evaluate its biodegradation efficiency, CO_2 evolution studies were conducted, showing a progressive increase in microbial activity over four days, indicating the utilization of dimethoate as a carbon source. The biofilm-forming ability of Pseudomonas was assessed using tube assays and microtiter plate assays, revealing maximum biofilm formation at 72 hours. A biofilter incorporating Pseudomonas biofilms was designed in a glass column and used for dimethoate degradation. FTIR spectroscopy analysis demonstrated significant structural changes in the pesticide after treatment, confirming effective degradation. These findings suggest that Pseudomonas biofilms can serve as an eco-friendly and efficient bioremediation strategy for organophosphorus pesticide degradation, offering a sustainable alternative for mitigating pesticide contamination in agricultural environments.

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INTRODUCTION

The widespread use of organophosphorus pesticides in agriculture has led to severe environmental and health concerns due to their persistence and toxicity. Dimethoate, a commonly used organophosphorus pesticide, poses significant risks to ecosystems and human health due to its accumulation in soil and water bodies [1]. Conventional methods of pesticide removal, such as chemical treatments and incineration, often lead to secondary pollution and are economically unfeasible for large-scale applications [2]. Therefore, biological approaches like bioremediation have gained attention as sustainable and cost-effective alternatives. Bioremediation employs microorganisms to degrade pollutants into non-toxic components, making it an environmentally friendly method for pesticide degradation [3]. Among various microbial candidates, *Pseudomonas spp*. is well-known for their ability to degrade organic pollutants, including pesticides, due to their metabolic versatility and biofilm-forming ability [4]. Biofilms, structured microbial communities encased in an extracellular polymeric matrix, enhance bacterial resilience and metabolic efficiency, making them highly effective for bioremediation applications [5]. This study focuses on isolating and characterizing a pesticide-degrading Pseudomonas strain from contaminated soil and utilizing its biofilm-forming capabilities to construct a biofilter for dimethoate degradation. The efficiency of degradation was evaluated using CO_2 evolution studies and Fourier Transform Infrared (FTIR) spectroscopy. By demonstrating the potential of bacterial biofilms in pesticide removal, this research contributes to the development of sustainable bioremediation strategies for environmental pollution control. The findings of this study offer a sustainable and eco-friendly approach to mitigating pesticide contamination in agricultural environments. By harnessing the metabolic potential of Pseudomonas biofilms, this study presents a promising biotechnological solution

for the degradation of organophosphorus pesticides like dimethoate. The biofilter system developed in this research can be optimized for large-scale applications, reducing pesticide residues in soil and water, thereby minimizing ecological and human health risks. Furthermore, this study provides a foundation for future research on enhancing microbial biofilm systems for more efficient bioremediation processes.

MATERIAL AND METHODS

Isolation of Pesticide-Degrading Bacteria

Soil samples were collected from pesticide-contaminated agricultural fields and serially diluted before being plated onto nutrient agar. Bacterial colonies capable of utilizing dimethoate as the sole carbon source were selected for further analysis [6]. The selected isolates were maintained on King's B medium to promote characteristic pigment production, confirming the presence of *Pseudomonas* spp [7].

Characterization of the Isolated Bacteria

Gram Staining

Gram staining was performed to determine the cell wall characteristics of the isolated bacteria following the standard methods [8]. The isolate was identified as a Gram-negative bacillus.

Biochemical Tests (IMViC)

The *Indole, Methyl Red, Voges-Proskauer, and Citrate Utilization (IMViC) tests* were conducted following standard procedures to identify the bacterial isolate [9].

Indole Test: Kovac's reagent was added to the culture broth, and the absence of a red ring indicated a negative result.

Methyl Red Test: Methyl red indicator was added, and no colour change indicated a negative result.

Voges-Proskauer Test: Alpha-naphthol and potassium hydroxide were added, and no colour change confirmed a negative reaction.

Citrate Utilization Test: Growth and a colour change from green to blue on Simmon's citrate agar indicated a positive result.

Enzymatic Tests

Catalase Test: A drop of 3% hydrogen peroxide was added to a bacterial smear, and immediate bubbling indicated a positive reaction [10].

Oxidase Test: The bacterial culture was applied to an oxidase disc, and a deep purple colour within 10 seconds confirmed a positive oxidase reaction [11].

Estimation of Microbial Activity by CO_2 Evolution

Microbial activity was assessed by measuring CO_2 evolution over four days. The setup included sealed glass chambers containing bacterial cultures with dimethoate as the sole carbon source. Released CO_2 was trapped using a potassium hydroxide (KOH) solution and quantified by titration following the method [12].

Biofilm Formation Assays

Tube Assay for Biofilm Detection

Pseudomonas cultures were inoculated in nutrient broth and incubated at 37°C. After incubation, the broth was decanted, and the tubes were washed with phosphate-buffered saline (PBS), dried, and stained with 0.1% crystal violet to visualize biofilm formation [13].

Microtiter Plate Assay for Biofilm Quantification

Biofilm formation was quantified using a 96-well microtiter plate assay, where cultures were grown in nutrient broth, stained with crystal violet, and solubilized with ethanol to measure absorbance at 570 nm using a microplate reader [14].

Evaluation of Dimethoate Degradation by FTIR Spectroscopy

A biofilter was constructed using a glass column containing Pseudomonas biofilms. Dimethoate-contaminated water was passed through the biofilter and incubated for 5-6 days. The treated and untreated samples were analyzed using Fourier Transform Infrared (FTIR) spectroscopy to detect changes in functional groups [15].

RESULTS

Isolation and Identification of Pesticide-Degrading Bacteria

The soil sample collected from a pesticide-contaminated agricultural site was screened for bacteria capable of utilizing dimethoate as the sole carbon source. Colonies that grew on nutrient agar supplemented with dimethoate were isolated and subjected to further characterization. Among the isolates, one strain demonstrated strong growth and adaptation to the pesticide environment. When streaked on King's B medium, the bacterial isolate exhibited characteristic fluorescent pigmentation under UV transillumination, suggesting that it belonged to the *Pseudomonas* genus (Figure 2).

Figure 1: A. Colonies of *Pseudomonas sp*. On Nutrient Agar B. Colonies of *Pseudomonas sp*. On Kings B Medium

Characterization of the Isolated Bacteria

Gram Staining

Gram staining of the isolated bacterium revealed that it was a Gram-negative bacillus. The microscopic observation confirmed the characteristic rod-shaped morphology, further supporting its identification as *Pseudomonas* spp. (Figure 3).

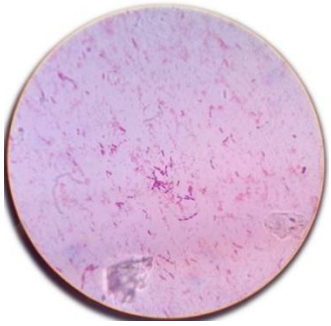


Figure 3: Gram staining of the isolate showing Gram-negative bacilli

Biochemical Characterization (IMViC Tests)

To confirm the identity of the isolate, a series of IMViC tests were performed (Table 1).

- **Indole Test**: The absence of a red ring after adding Kovac's reagent indicated a negative indole test result.
- **Methyl Red Test**: No colour change upon the addition of methyl red suggested that the organism did not perform mixed-acid fermentation.
- **Voges-Proskauer Test**: The lack of a colour change after adding alpha-naphthol and potassium hydroxide confirmed a negative result.
- **Citrate Utilization Test**: The isolate grew on Simmon's citrate agar with a colour change from green to blue, indicating the ability to utilize citrate as a sole carbon source.

Enzymatic Tests

Catalase Test: The bacterial culture showed effervescence upon the addition of hydrogen peroxide, indicating a positive catalase test.

Oxidase Test: The culture turned deep purple within 10 seconds of contact with the oxidase disc, confirming a positive oxidase reaction (Figure 5).

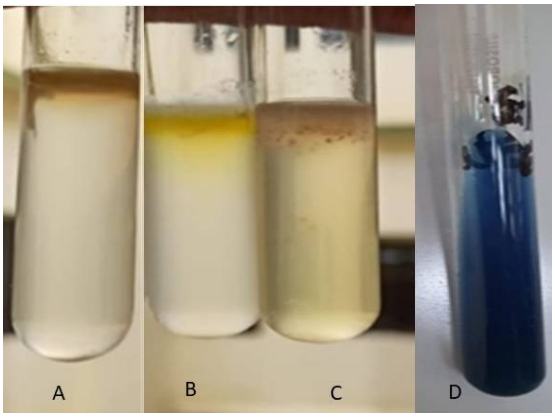


Figure 4: IMViC test results

A. Indole: Negative**; B.** Methyl Red: Negative**; C.** Vogues Proskauer: Negative**; D.** Citrate: Positive

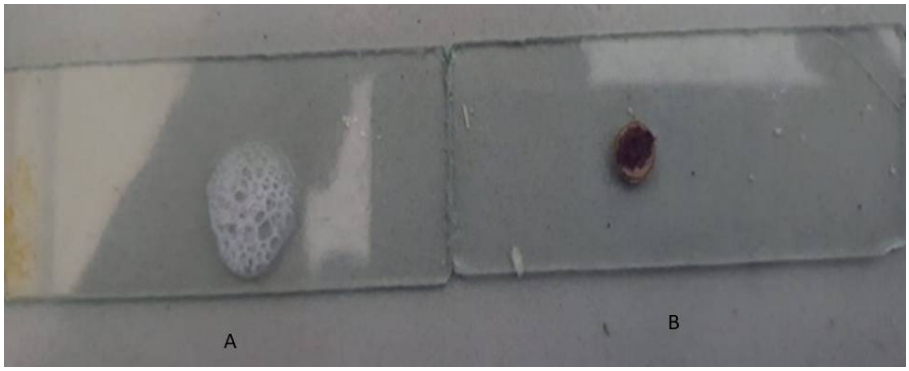


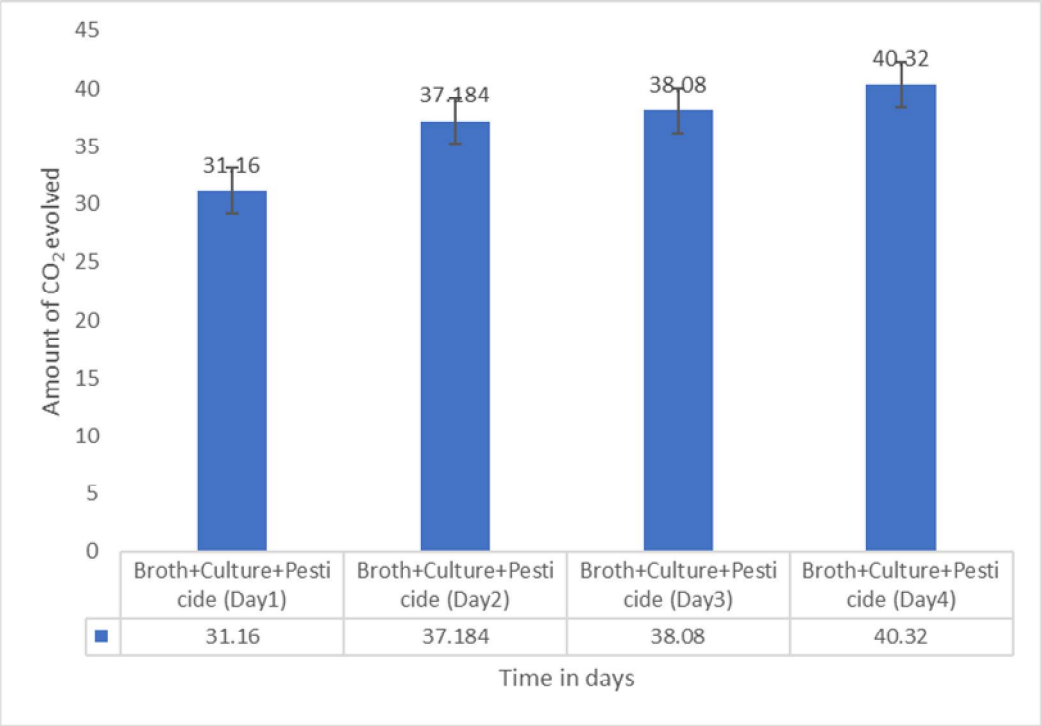
Figure 5: A. Catalase and B. Oxidase test results

These results strongly suggested that the isolated bacterium belonged to the genus *Pseudomonas*.

Estimation of Microbial Activity by CO_2 Evolution

Microbial metabolic activity was assessed by measuring CO_2 evolution as an indicator of pesticide degradation. The results showed a gradual increase in CO_2 production from Day 1 to Day 4 (Graph 1), indicating that the bacteria were actively metabolizing dimethoate. The increasing trend in CO_2 evolution suggests that *Pseudomonas* effectively utilized dimethoate as its sole carbon source.

Figure 6: Experimental setup for CO_2 evolution



Graph 1: CO_2 evolution over time showing increased microbial activity

Biofilm Formation Assays

Tube Assay for Biofilm Detection

The ability of *Pseudomonas* to form biofilms was evaluated using the tube assay. After incubation and staining with crystal violet, biofilm formation was visible along the walls of the test tubes (Figure 7). Observations over a period of four days showed that maximum biofilm formation occurred on the third day.

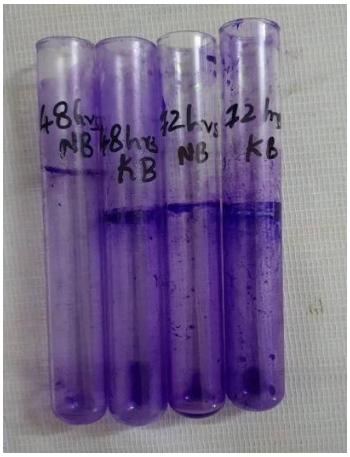
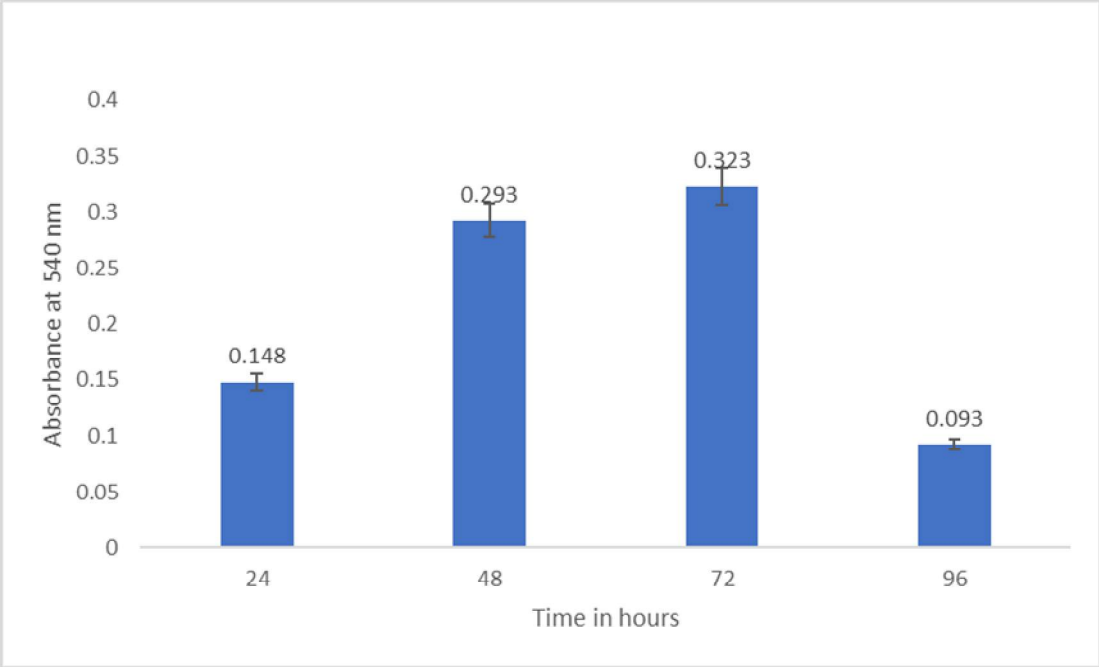


Figure 7: Tube assay showing biofilm on test tube walls

Microtiter Plate Assay for Biofilm Quantification

To quantify biofilm formation, a microtiter plate assay was performed. Optical density (OD) measurements at 570 nm confirmed that biofilm formation increased over time, with a peak on the third day, correlating with the tube assay results (Graph 2).



Graph 2: Microtiter assay results, indicating maximum biofilm formation on the third day

Evaluation of Dimethoate Degradation by FTIR Spectroscopy

To confirm the biodegradation of dimethoate, FTIR spectral analysis was performed on treated and untreated samples. The untreated sample exhibited characteristic peaks corresponding to dimethoate's functional groups. However, after treatment with the *Pseudomonas* biofilter, significant peak reductions and shifts were observed, indicating structural degradation of the pesticide (Figure 9).

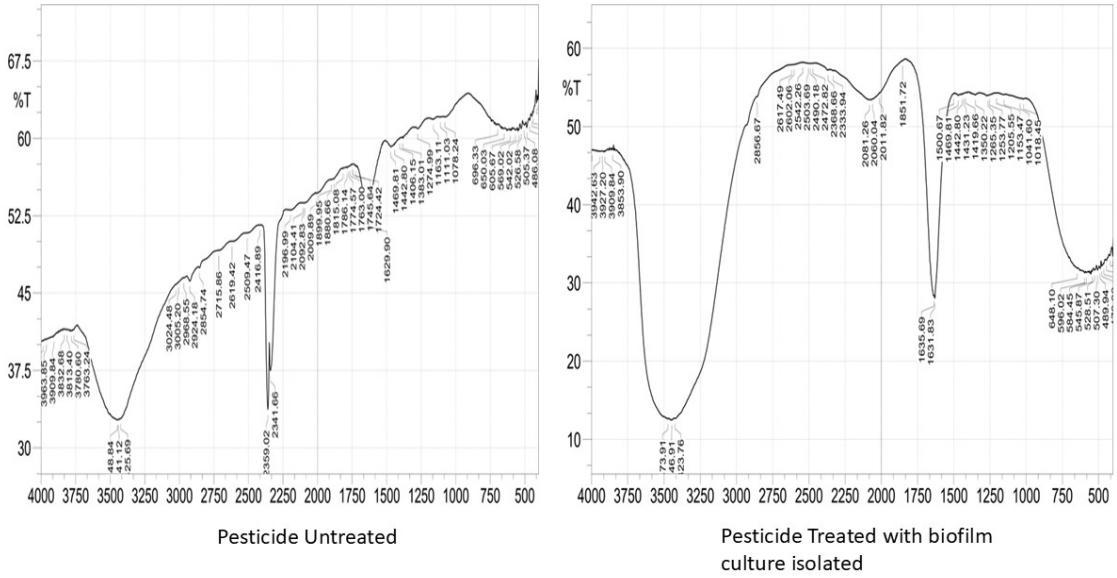


Figure 9: FTIR spectral analysis was performed on treated and untreated sample

Table 2: FTIR spectral analysis results

Sample	Peak Absorption (cm^{-1})	Interpretation
Untreated Dimethoate	Strong peaks	Intact pesticide structure
Treated Dimethoate	Reduced/shifted peaks	Degradation of dimethoate

To confirm the biodegradation of dimethoate, FTIR spectral analysis was performed on untreated and treated samples. The FTIR spectrum of the untreated sample exhibited characteristic peaks corresponding to dimethoate's functional groups, including strong absorption bands around 2856 cm^{-1}

and 2927 cm^{-1} (C–H stretching), 1745 cm^{-1} and 1774 cm^{-1} (C=O stretching), and peaks near 1087 cm^{-1} and 1078 cm^{-1} related to P=O and P=S stretching vibrations, indicative of its organophosphate structure. In contrast, the spectrum of the treated sample, after biofiltration with *Pseudomonas* sp., showed significant reductions in peak intensities and noticeable shifts in major absorption bands. The disappearance and weakening of peaks associated with key functional groups suggest the structural breakdown of dimethoate. Furthermore, the appearance of minor new peaks indicates the formation of simpler degradation products. These spectral changes confirm that *Pseudomonas* effectively degraded dimethoate by disrupting its critical chemical bonds, thus demonstrating its potential in bioremediation of pesticide-contaminated environments. These results confirm that the *Pseudomonas* biofilm effectively degraded dimethoate over the incubation period, supporting its potential for bioremediation applications.

Figure 9: Biofilter setup using *Pseudomonas* biofilms

Table 2: FTIR spectral analysis results

Sample	Peak Absorption (cm^{-1})	Interpretation
Untreated Dimethoate	Strong peaks	Intact pesticide structure
Treated Dimethoate	Reduced/shifted peaks	Degradation of dimethoate

These results confirm that the *Pseudomonas* biofilm effectively degraded dimethoate over the incubation period, supporting its potential for bioremediation applications.

DISCUSSION

The study successfully isolated and identified a pesticide-degrading *Pseudomonas* strain capable of utilizing dimethoate as its sole carbon source. This finding aligns with previous research highlighting *Pseudomonas* as an efficient degrader of organophosphorus (OP) pesticides due to its diverse metabolic pathways and enzymatic mechanisms [16, 17]. Many *Pseudomonas* species possess genes encoding phosphotriesterases, enzymes that hydrolyze the phosphoester bonds in OP compounds, facilitating their breakdown into non-toxic metabolites [18]. The CO_2 evolution experiment confirmed that *Pseudomonas* efficiently metabolized dimethoate over time. The gradual increase in CO_2 production from Day 1 to Day 4 suggests that the bacteria actively utilized dimethoate as a carbon source, corroborating previous studies that have demonstrated microbial mineralization of OP pesticides through enzymatic hydrolysis and subsequent oxidation [19, 20]. This is particularly significant in the context of bioremediation, as enhanced microbial respiration is a direct indicator of metabolic activity in pollutant degradation. The biofilm assays confirmed that *Pseudomonas* exhibited strong biofilm-forming ability, with maximum biofilm formation occurring on the third day of incubation. Biofilm-based degradation mechanisms offer significant advantages in environmental applications, as microbial biofilms enhance stability, resilience, and prolonged metabolic activity in contaminated sites [21]. Biofilms act as protective matrices for microbial cells, shielding them from environmental stressors while promoting sustained biodegradation of pollutants [22]. The observed robust biofilm formation on Day 3 suggests an optimal adaptation phase where *Pseudomonas* efficiently adhered to surfaces and initiated biofilm maturation, which is crucial for sustained pesticide degradation. This supports findings from previous research on biofilm-mediated bioremediation, where *Pseudomonas* biofilms significantly improved the breakdown efficiency of various organic pollutants [23].

FTIR analysis provided direct evidence of dimethoate degradation by *Pseudomonas*. Structural alterations in the treated sample, compared to the control, indicated successful breakdown of the pesticide. Specific spectral changes, such as the disappearance of characteristic peaks corresponding to organophosphate bonds (P=O and P–O–C) and the emergence of new peaks representing hydrolysis products, are indicative of microbial degradation [24]. These results align with studies demonstrating that OP pesticides undergo microbial hydrolysis and oxidative cleavage, leading to the formation of less toxic metabolites [25]. The observed degradation pattern suggests that the *Pseudomonas* strain harbors specific enzymes capable of cleaving phosphoester bonds, further supporting its potential application in bioremediation [26].
These findings collectively demonstrate that *Pseudomonas* biofilms serve as an efficient and eco-friendly approach to mitigating OP pesticide contamination, offering a promising alternative to conventional chemical or physical remediation methods.

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