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Efficiency of *Pseudomonas mendocina* in biodegradation of chlorpyrifos insecticides

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Abstract: At present, extensive varieties of pesticides are being used but the demand for Organophosphorus pesticide is increasing globally to control insects. Chlorpyrifos is a broad-spectrum, highly toxic, and chlorinated organophosphate insecticide that is synthetic in origin and is normally ester or thiol derivatives of phosphoric. The mode of action involves inhibiting acetyl-cholinesterase leading to the accumulation of acetylcholine causing neurotoxicity. Bacteria capable of degrading the pesticide, chlorpyrifos, were isolated from soil contaminated with pesticides. In this way, three distinct chlorpyrifos degrading strains of *p.mendocina* were isolated, characterized using morphological and biochemical analysis. Strains were exhibited the greatest chlorpyrifos degradation rate reach to 100% and was consequently selected for further investigation. Degradation of chlorpyrifos by strains were rapid at 20 and 37C. bacteria species were able to effectively degrade chlorpyrifos in sterilized medium using high inoculum levels. The maximum degradation rate of chlorpyrifos was calculated as 100% during 6-12 days . Bacteria such as strain PC1, that use chlorpyrifos as a carbon source, could be employed for the biordegradation of sites contaminated with pesticides.

Keywords: Biodegradation; organophosphate; Chlorpyrifos; *pseudomonas putida*; Hplc.

1. Introduction

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichlor- 2-pyridyl phosphorothioate; CPF) is a broad spectrum organophosphate insecticide (OP) that is commercially used to control insects especially termite¹ It was first developed by the Germans in the 1930s and first introduced into the marketplace in 1965, has been widely used globally as an insecticide to control crop pests in agriculture, reduce household pests such as termites, reduce insect damage to turf on lawns, and for mosquito control^{2,3} A variety of studies have shown that exposure to CPF elicits acute toxicity through inhibition of acetylcholinesterase (AChE) throughout the central and peripheral nervous systems, leading to acetylcholine accumulation and long-term stimulation of cholinergic receptors⁴ Chlorpyrifos works basically the same way both in insects and other animals including humans through impairment of nervous system. during development can cause persisting neurobehavioral dysfunction, even with low doses that do not elicit acute toxicity⁵. The fate of chlorpyrifos is affected not only by its own physicochemical properties , but also by characteristics of the soil, management practices and environmental conditions⁶. Its persistence increases with decreased temperature, decreased pH, and decreased light. The half-life of chlorpyrifos showed that it remained stable even after 12 months in the soil⁵ and Chlorpyrifos is moderately soluble in water, the half-life of chlorpyrifos in water ranges from 35 to 78 days² . It has also been reported to have short to moderate persistence in the environment as a result of several non-biological methods including volatilization, photolysis, abiotic hydrolysis, and also by microbial degradation that might occur concurrently⁵ Chlorpyrifos has been of a great concern due to persistence, toxicity and accumulation in soils and ground waters⁷ and It was observed may be causes altered the structure of the gills and liver of fishes⁸. The unsafe misuse of pesticides has caused widespread harm to human health and the environment, because it is one of the most common causes of poisoning around the world⁹, as well as the appearance of its residues in crops and soil, and has proven many reports, including the environmental protection organization, that pesticides cause many types of cancer¹⁰. When pesticides are dispersed in the environment, they become pollutants, with ecological effects that require degradation¹¹. It has become important to treat these pollutants, and there are many different methods of treatment, including chemical and physical treatment, which have faced many criticisms because of the main problems they cause such as the production toxic and radioactive acids

and bases, in addition to these methods are uneconomic and ineffective and not suitable for large areas¹². Lately, research activities in this area have demonstrated that microorganisms are potential tool in decaying insecticides into less harmful and non-toxic metabolites through a process known as bioremediation¹³ biodegradation has now emerged as an innovative technology that is critically important for the clean-up of polluted sites. In summary, due to the pervasive nature throughout *Pseudomonas mendocina*, the potential non-pathogenic effect on fish, and efficiency in CPF metabolism, *Pseudomonas* species were considered a vital potential agent for testing in bioremediation. The aim of this study was to determine the extent to which *Pseudomonas mendocina* acts as an effective biomarker, reducing CPF toxicity by breaking down CPF into non-toxic metabolites, and preventing AChE inhibition. *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella* sp., and *Serratiamarscecens* obtained from consortia showed 84, 84, 81, and 80% degradation of chlorpyrifos (50 mg/L) in liquid medium after 20 days and 92, 60, 56, and 37% degradation of chlorpyrifos (50 mg/L) in soil after 30 days. Some recent reports indicate bacterial degradation of chlorpyrifos by *Flavobacterium* sp. ATCC 27551 and *Arthrobacter* sp., isolated from contaminated sources, which degrade chlorpyrifosco-metabolically, and *Enterobacter* strain B-14, *Alcaligenes faecalis*, and *Klebsiella* sp., which degrade and utilize chlorpyrifos as sole carbon source¹⁴. *Bacillus* sp. and *Micrococcus* sp. possess potential to degrade chlorpyrifos².

2. Materials and methods

2.1. sample collection

The soil samples used for the isolation of pesticide degrading bacteria were collected from agricultural fields, residential buildings, and Garden yards from the Iraq-Al Diwanayah where chlorpyrifos pesticide is used extensively. This study included a total of 50 samples were collected at a depth of 5cm to 10cm from different regions. Commercial chlorpyrifos (48%) pesticide was procured from local pesticide shop . Stock solution was prepared and stored in refrigerator for further use. The samples were collected using sterile petri dish and plain tube and 5 grams of soil samples were taken and added Normal line to it, for the purpose of purification and isolation of bacteria.

2.2. The instruments that used in determining removal of chlorpyrifos and detection of bacteria

1. VITEK 2 (Biomérieux, France)
2. High Performance Liquid Chromatography (Hplc) (Knuauer , Germany).

2.3. Preparation of stock pesticide

Commercial grade chlorpyrifos (48%) pesticide was procured from local pesticide shop. Stock solution was prepared and stored in refrigerator for further use. To estimate the amount of residual chlorpyrifos concentrations after removal by bacterial isolates. Standard was prepared for the active ingredient CP by selecting more than one manufacturer of the pesticide containing the active ingredient chlorpyrifos at a concentration of 48%, then 5 mg was taken from each package and analyzed by high performance chromatography according to¹⁵

2.4. Isolation and identification of bacteria

Prepare the soil suspensions by taking 1 gram of soil, and dissolve in 10 ml of normal saline, then mix by the vortex for 10 minutes and centrifuge for 5min at, and then filter with a 0.4 volume filter and use *pseudomonas* agar medium from (Himedia Company, India) to isolate the bacteria that analysis the pesticides. The culture medium consisting of Gelatin peptone 16.00 g / L, Tryptone 10,000 g / L, Potassium sulphate 10.00 g / L, Magnesium Chloride 1.4 g / L, Agar 11.00 g / L, and sterilized by autoclaving at 15lbs (121° C) for 15 min, cool to 45-50 ° C and aseptically add sterile rehydrated contents of one vial of either celrinix supplement (FD029) or CFC supplement (FD036) as desired. The pH was set to 7.1 at 25 ° C. mix well and pour into sterile petri dish plates for selective isolation of *pseudomonas* species. The cultivated bacterial isolates were incubated on the nutrient medium for 24 hours at a temperature of 37 ° C and the bacteria were diagnosed by a modern device called VITEK2 SYSTEM from the company (Biomérieux, France). VITEK2 SYSTEM was examined against three bacteria as shown in (table. 1).

2.5. Treating the medium with the pesticide

Nutrient broth is prepared for the purpose of treatment with chlorpyrifos and consists of peptone 5 g / liter, sodium chloride 5 g / liter, meat extract B 1.5 g / liter, yeast extract 1.5 g / liter. Addison 800 ml of distilled water and dissolve 10.2 grams from the medium, then heat the mixture by microwave and sterilize with an autoclave at 15lbs (121° C) for 15min and then cool to 45° C.

2.6. Biodegradation of chlorpyrifos

After that, add 3 ml of water and use a 0.4 volume filter to sterilize the water with a syringe, add 200µl of chlorpyrifos, and mix well and add the last mixture to the liquid medium in order to inoculate the medium with the isolated and diagnosed bacteria . The residual concentrations were assessed and measured after removal with HPLC and un-inoculated flasks served as controls. Biodegradation experiments were carried out in 250 mL Erlenmeyer flasks containing 60 mL of

medium with 200 µL of CP. The mixture were pour in cup urine 50 ml and inoculated with pseudomonas mendocina and incubated at 37°C. Samples were taken at periodic intervals and the residual pesticide concentration was determined using HPLC. Un-inoculated cup served as controls.

3. Results

The results showed, three distinct chlorpyrifos degrading strains of P.mendocina were highly efficient in the biological removal of organic phosphorous pesticides (chlorpyrifos) at a concentration of 120 µg/l, thus the possibility of using it in the biodegradation of pollutants. PC1 strain removal 97% of CP with at 6 days, the residual chlorpyrifos concentration was 0.37µg/ml to 0.130µg/ml, while PC2 and PC3 degraded CP within 6 days from 0.36µg/ml to 0.137µg/ml and 0.44µg/ml to 0.077µg/ml (table 2). The PC1 could rapidly degrade chlorpyrifos compare with other species during the logarithmic phase of growth. The percentage degradation in first 6 day was higher and rapidly increased thereafter to 100% within 12 day compare with control (Fig. 2,3,4). These results are consistent with many studies and research that have confirmed the ability of microorganisms (bacteria and fungi) to consume a wide range of pesticides and in most cases their ability to consume one or more compounds as a source of carbon and energy[2, 11]. The strain could utilize CP as the sole source of carbon for growth. In HPLC analysis of sample uninoculated (without bacteria) with 200 µl CP, two peaks appeared in HPLC separation (Fig.1), which were further identified as CP by comparing with their authentic standard. HPLC analysis showed that CP could be degraded by PC1 to TCP and diethylthiophosphoric acid.

Table 1. The probability of diagnostic bacterial isolates by Vitek2compact system

NO.	Bacteria	Probability%
1.	P.mendocina C1	98%
2.	P.mendocina C2	96%
3.	P.mendocina C3	99%

Table 2. shows the percentages of residual concentrations after treatment during 6 to 12 days for chlorpyrifos in two experiments.

Bacterial sample	Concentrate Of chlorpyrifos	Residual Concentrate After 6 days	Residual Concentrate after 12 days	control
PC1	120 µg/ml	0.37µg/ml	0.130µg/ml	63.40 µg/ml
PC2		0.36µg/ml	0.137µg/ml	
PC3		0.44µg/ml	0.077µg/ml	

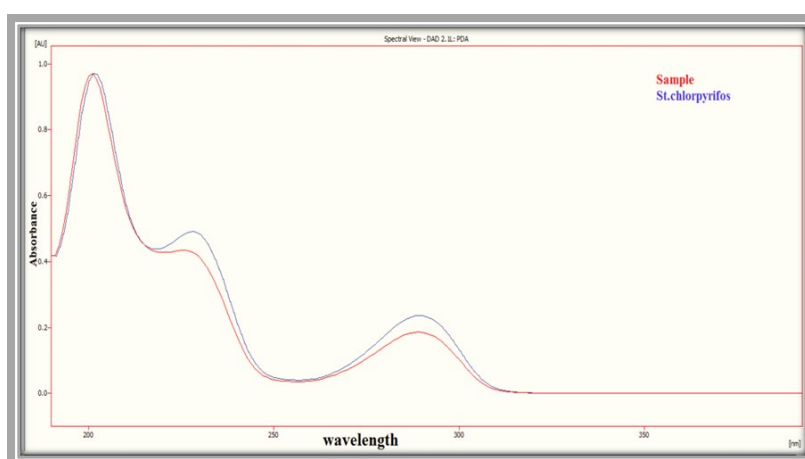


Figure 1. shows the absorbance and spectrum of St.chlorpyrifos and the control (sample uninoculated) treated with the pesticide without bacteria.

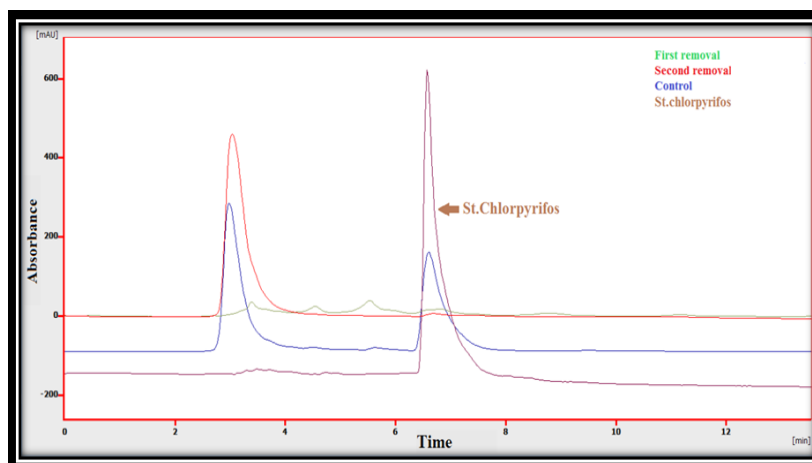


Figure 2. showed growth kinetic for *p.mendocina* C1 on broth media with chlorpyrifos as carbon source and energy. Illustrates peaks Chlorpyrifos (CP) degradation by *Pseudomonas mendocina* C1 strain. 1) Residual CP in inoculated sample after 6 day, 2) Residual CP in inoculated sample after 12 day, 3) CP degradation in un-inoculated control, and 4) standard of chlorpyrifos 48%.

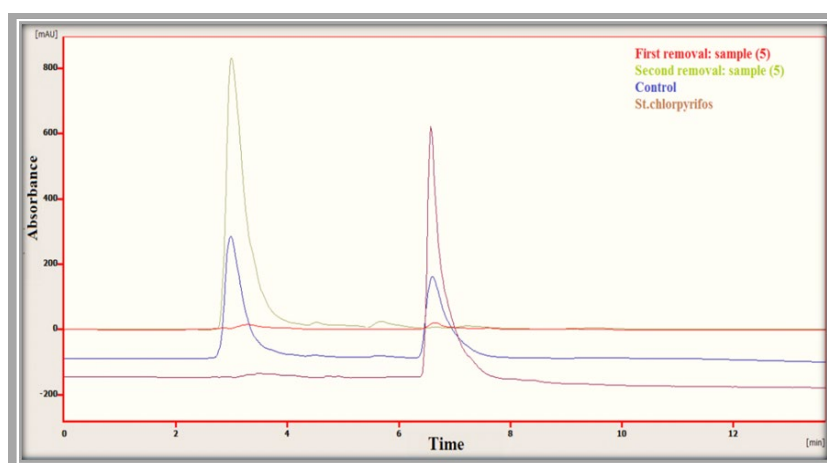


Figure 3. showed growth kinetic for *p.mendocina* C2 on broth media with chlorpyrifos as carbon source and energy. Illustrates peaks Chlorpyrifos (CP) degradation by *Pseudomonas mendocina* C2 strain. 1) Residual CP in inoculated sample after 6 day, 2) Residual CP in inoculated sample after 12 day, 3) CP degradation in un-inoculated control, and 4) standard of chlorpyrifos 48%.

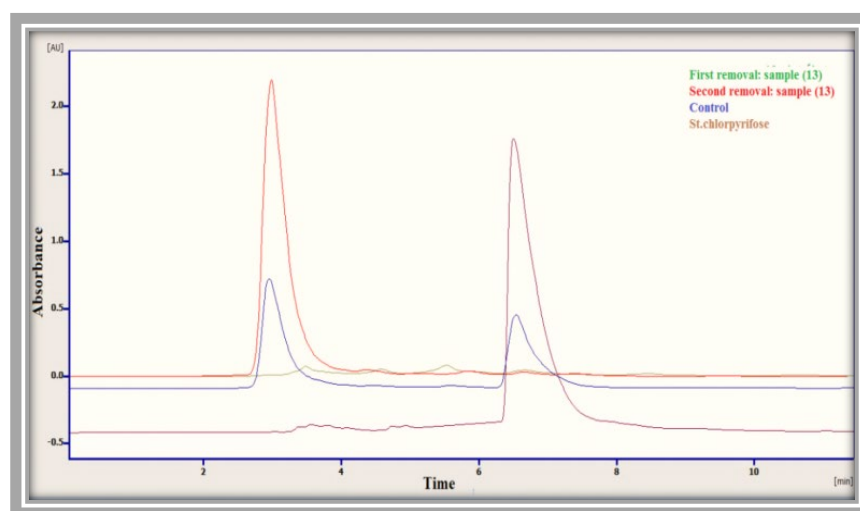


Figure 4. showed growth kinetic for *p.mendocina* C3 on broth media with chlorpyrifos as carbon source and energy. Illustrates peaks Chlorpyrifos (CP) degradation by *Pseudomonas mendocina* C3 strain. 1) Residual CP in inoculated sample after 6 day, 2) Residual CP in inoculated sample after 12 day, 3) CP degradation in un-inoculated control, and 4) standard of chlorpyrifos 48%.

4. Discussion

Only a few organisms capable of degrading chlorpyrifos have been reported till date. The maximum rate of chlorpyrifos removal was observed from 6 to 12 days, which coincided with the period of maximum proliferation. This might indicate that the *Pseudomonas mendocina* utilized CP as a source of energy during its growth phase. It was noted that on reaching lower concentrations of residual pesticide in the medium, the degradation rate decreased significantly as previously observed by. The reported minimum incubation time required for complete removal of chlorpyrifos was 6 h by *Cupriavidus* sp. DT-1 in a liquid medium¹⁶. Most of the common CP degrading bacteria were reported to degrade the pesticide within 48 h in liquid media¹⁷. Chlorpyrifos has been shown to be degraded cometabolically in liquid media by bacteria¹⁴. *P. mendocina* is the most common Gram negative bacterium aerobic, rod-shaped found in soil. Isolates of this bacterium have been found to have potential to degrade chlorpyrifos¹⁸. Since *Pseudomonas mendocina* capable of degradation of various OPs have great potential for bioaugmentation of soil¹⁹ these strain of bacteria with its high degradation potential, fast growth, minimal nutrient requirements and tolerance to high concentrations of pesticide can play a vital role in decontamination of polluted soil. The rapid removal of CP from liquid medium within 6 day by the pB1 strain thus could be one of the fastest reported for effective CP degradation and first of its kind amongst the *Pseudomonads*. To demonstrate the capacity of the recombinant *P. mendocina* strain to degrade CP, the degradation experiments were performed by inoculating the recombinant strain into a nutrient broth medium supplemented with CP. High-performance liquid chromatography (HPLC) analysis indicated that CP were completely degraded within 6 and 12 days respectively. Moreover, the concentration of 3,5,6- trichloro-2-pyridinol (TCP) in the medium increased gradually with the decrease in CP concentration. CP was degraded quickly in the first 6 days, which accounted for 48% of the amount of the initially added pesticides respectively. Maybe a reduction in the degradation rate after 12 days may be due to the accumulation of TCP, which have antimicrobial activity and are toxic to the bacterial growth and metabolism^{20,21}.

5. CONCLUSION

The present study shows that use of the *p. mendocina* culture could effectively degrade chlorpyrifos pesticide. Removal of xenobiotic compounds which are a major threat to environmental pollution are of major concern as they enter the food chain and are the major causes of various diseases. The study shows that the bacterial isolate is able to use chlorpyrifos as sole source of carbon. Therefore these bacteria culture can be used effectively for Bioremediation of contaminated sites. Future studies aim in studying the biotechnology of pesticide degradation and its significance in field conditions.

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