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Isolation and Screening for Efficiency of Organic Phosphorus Pesticide (Chlorpyriphos) Degrading Bacteria from Different Crops

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ABSTRACT

In the present study total eight bacterial isolates were isolated from vegetable crops like tomato, brinjal by the enriched MSM broth with a supplement of chlorpyrifos source. These isolates were characterized morphologically, cultural and biochemically. Isolates of the microbial strains for identifying those having high chlorpyrifos degradation capabilities in liquid culture were undertaken as well. Among the eight chlorpyrifos bacterial isolates, CDB-1 showed the high population count at different incubation periods (1st day to 5th day) compared to all CDB inoculates. The CDB-1 isolate utilized the pesticide (chlorpyrifos) effectively and showed maximum growth bacterial count 5.8 cfu $\times 10^6$ mL^{-1}. Efficient CDB-1 isolate and chlorpyrifos degrading capacity were determined with measurement of chlorpyrifos residual concentrations at different days of intervals using gas chromatographic method. The degradation of chlorpyrifos at different concentrations (20, 30 and 40 mgL^{-1}) was examined in the MSL medium. By this degradation percentage study of chlorpyrifos revealed that the chlorpyrifos degrading bacterial isolate (CDB-1) degrade the chlorpyrifos effectively. These isolates used the OP compounds as a carbon source.

INTRODUCTION

Different agrochemicals, such as pesticides, were introduced in agri-ecosystem to fulfil the increased food needs of more growing population. But now the use of pesticides has become a necessary evil. Residues of applied pesticides stay in the environment (air, soil, ground and surface water) for variable periods of time. Due to the long persistence of organchlorine pesticide molecules, tendency to bioaccumulate and their potential toxicity towards non-target organisms (biomagnifications), this group of pesticides was replaced by relatively less persistent and yet effective organophosphorus (OP) compounds.

Organophosphorus compound pesticides are degradable. Environmental pollution caused by pesticides and their degradation products is a major ecological problem (Guliy et al. 2003). It has been documented that, organophosphorus pesticides (OP) constitute the largest group of pesticides used globally and account for about 38% of the total pesticides used worldwide (Singh & Walker 2006). Environment preservation is one of the aims of the sustainable development. Environmental pollution has increased in many regions due to industrialization. In India, alarming levels of pesticides have been reported in air, water, soil as well as in food and biological materials. The most important pollutants among the toxicants in India are organochlorine and organophosphorus pesticides. Modern agriculture is a capital and technology intensive affair that is highly reliant on extensive chemical inputs in order to enhance the production. Consequently, a huge variety of chemical pesticides are popularly used across the globe for pest control purposes.

The wide application of organophosphorus (OP) insecticides such as chlorpyrifos, phorate, malathion, dichlorvos, are employed for plant protection against insect pests. This organophosphorus pesticide is one of the major chemicals responsible for the contamination and deterioration of soil and groundwater, particularly in the close vicinities of agricultural fields (Jayashree & Vasudevan 2006). Owing to their high toxicity and persistence in the environment, most of them are banned all over the world.

Microorganisms are important in maintaining soil fertility and are also important agents which detoxify pesticides in soil. Pesticide biodegradation is a ubiquitous environmental process. Pesticide biodegradation has been documented in a wide range of habitats, including soils, sediments, surface and groundwater and sewage sludges etc. The ubiquity of pesticide degradation suggests that bioremediation strategies can play an important role in the treatment of pesticide wastes. Insecticides and their degradation products generally get accumulated in the top soil and influence not only the population of various groups of soil microbes, but also their biochemical activities like ni-

trification, ammonification, decomposition of organic matter and nitrogen fixation. Microorganisms play an important role in degrading synthetic chemicals in soil. They have the capacity to utilize virtually all natural and synthetically occurring compounds as their sole carbon and energy source.

Several researchers reported potential bacterial strains like *Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp., *Klebsiella* sp., *Serratia marcescens, Enterobacter* sp., *Stenotrophomonas* sp., *Sphingomonas* sp., *Flavobacterium* sp. etc., fungal strains such as *Phanerochaete chrysosporium*, *Aspergillus terreus, Verticillium* sp., *Pseudomonas, Trichoderma harzianum,* etc. and cyanobacteria like *Anabaena* sp., *Aulosira fertilissima, Phormidium valderianum* for organo phosphorous pesticides (chlorpyrifos and phorate) degradation (Dhanya 2014).

The present study primarily focused on the isolation of efficient bacterial strains that were able to use chlorpyrifos and phorate as a sole source of carbon, exhibiting their growth in response to biodegradation. Through repetitive enrichment culture and successive subcultures, efficient bacterial strains were examined for their potential to degrade chlorpyrifos in liquid medium under optimized environmental/incubation conditions.

MATERIALS AND METHODS

Isolation of chlorpyriphos degrading bacteria by enriched soil sample using serial dilution technique: Soil samples were collected from different agricultural fields where vegetable crops like brinjal, tomato, etc., were extensively grown and chlorpyriphos pesticide used extensively. Soil samples were pooled and collected into sterilized polythene bags to avoid external contamination. The soil sample containing polyethylene bags were brought into the laboratory and stored in 4°C to maintain the biological activity.

Air-dried and sieved (<2 mm) soil samples (10 g) from farm lands were collected and suspended in 250-mL Erlenmeyer flasks containing 50 mL of mineral salts medium (MSM medium gL^{-1} :KH_2PO_4: 4.8, K_2HPO_4 : 1.2, NH_4NO_3 : 1.0, $MgSO_4 \cdot 7H_2O$: 0.2, $Ca(NO_3)_2 \cdot 4H_2O$: 0.04, $Fe(SO_4)_3$: 0.001, pH: 7.0.) (Rasul et al. 1988) supplemented with chlorpyrifos (50 mg L^{-1}). The flasks were incubated on a rotary shaker at 250 rpm for 7 days at 300°C. The enriched soil samples were subjected to serial dilution technique and samples were inoculated on enriched nutrient agar plates for obtaining pure cultures.

Maintaining of chlorpyriphos degrading bacterial isolates in nutrient agar medium containing 5 ppm chlorpyriphos: Pure cultures of bacteria used in the present investigation were sub cultured on nutrient agar media plates by cross streaking methods and stored at 4°C. Every time purity was checked by microscopic observation by gram staining procedure.

Morphological, cultural, biochemical characterization and functional properties of pesticide chlorpyriphos degrading bacteria: The isolated bacteria were studied for their morphological characteristics like gram reaction, pigmentation; cultural and biochemical characteristics like indole production, methyl red, Voges Praskaure's test, citrate utilization test, oxidase, catalase and sugar fermentation tests.

RESULTS AND DISCUSSION

Isolation and screening of isolates for chlorpyriphos degradation: Serial dilutions of enriched soil samples ranging from 10^{-1} to 10^{-8} were prepared and spreaded on nutrient agar medium plates. The nutrient agar plates were incubated at 37°C with pH 7 incubator. Bacterial population counts taken at different intervals like 2 days, 4 days and 7 days were 5.25 cfu $\times 10^6$ g^{-1} soil, 7.8 cfu $\times 10^6$ g^{-1} soil, 3.5cfu $\times 10^6$ g^{-1} soil respectively. Similarly in tomato crop bacterial count at 2 days, 4 days, and 7days, were 2.4 cfu $\times 10^6$ g^{-1} soil, 4.2 cfu $\times 10^6$ g^{-1} soil and 1.4 cfu $\times 10^6$ g^{-1} soil respectively. Eight different bacterial colonies were observed on nutrient agar plates at 10^{-4} dilution. These eight different bacterial isolates were named as CDB-1, CDB-2, CDB-3, CDB-4, CDB-5, CDB-6, CDB-7 and CDB-8 isolates. The eight bacterial colonies were purified by streak plate method on enriched nutrient agar medium plates.

Morphological, cultural, biochemical characterization and functional properties of pesticide chlorpyriphos degrading bacteria: The cell morphology, colony morphology, gram reaction, sporulation and shape were studied for eight chlorpyrifos degrading bacterial isolates. Among eight CDB (chlorpyrifos degrading bacteria) isolates, four isolates were gram negative (CDB-3, CDB-6, CDB-7, CDB-8,) and remaining four CDB isolates (CDB-1, CDB-2, CDB-4, CDB-5) were gram positive. All the isolates were rod shaped, no motility, gram positive isolates were having sporulation.

The cultural characters of all isolates were studied on nutrient agar medium plates. All isolates showed different cultural characters on nutrient agar medium. CDB-6, CDB-3 colonies showed yellowish green pigmentation, while CDB-5, CDB-1 were white irregular, CDB-7, CDB-8, CDB-2, CDB-4, were dull white irregular type of colonies on nutrient agar medium plates (Table 1). Biochemical characteristics of chlorpyriphos degrading bacterial isolates are described in the Table 2.

Table 1: Morphological characters of chlorpyrifos degrading bacterial isolates.

Isolate	Gram reaction	Cell shape	Sporulation	Cultural characters
CDB-1	Gram +ve	Rod	Sporulation	Off white, irregular, non-spreading, smooth, flat, opaque, viscid colony with no pigmentation
CDB-2	Gram +ve	Rod	Sporulation	Dull white, irregular, non-spreading, smooth, flat, opaque, viscid colony with no pigmentation
CDB-3	Gram -ve	Rod	Non-Sporulation	Yellowish green, irregular, non-spreading, glistening, con vex, opaque, viscid colony with green pigmentation,
CDB-4	Gram +ve	Rod	Sporulation	Dull white, irregular, non-spreading, smooth, flat, opaque, viscid colony with no pigmentation
CDB-5	Gram +ve	Rod	Sporulation	Off white, irregular, non-spreading, smooth, flat, opaque, viscid colony with no pigmentation,
CDB-6	Gram -ve	Rod	Non-Sporulation	Yellow green, round, non-spreading, glistening, convex, opaque, viscid colony with green pigmentation,.
CDB-7	Gram -ve	Rod	Non-Sporulation	Dull white, round, non-spreading, glistening, convex, opaque, viscid colony with no pigmentation
CDB-8	Gram -ve	Rod	Non-Sporulation	Dull white, round, non-spreading, glistening, convex, opaque, viscid colony with no pigmentation

Table 2: Biochemical characteristics of chlorpyrifos degrading isolates.

S. No	Isolates	Indole-test	MR	VP	Citrat-eutilization	Catalase test	Oxidase test	Starch hydrolysis	Gelatin liquefaction	H_2S test	Carbohydrate utilization						Dinitrification test
											Lactose	Sucrose	Glucose	Fructose	Malate	Mannitol	
1.	CDB-1	+	+	-	+	+	+	-	+	-	-	+	+	+	-	-	+
2.	CDB-2	+	+	-	+	+	+	-	+	-	+	+	+	-	-	+	-
3.	CDB-3	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+
4.	CDB-4	-	+	+	+	+	+	-	+	-	-	+	+	-	-	+	-
5	CDB-5	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
6.	CDB-6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-
7.	CDB-7	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+
8.	CDB-8	-	+	-	+	+	+	-	+	-	-	-	+	-	-	-	+

MR-Methyl Red; VP-Voges Praskaure's

Table 3: Selection of efficient chlorpyrifos degrading bacteria supplementing with MSM broth containing 2% chlorpyrifos.

S.No	Isolates	Bacterial population (CFU×$10^6$$g^{-1}$ soil)				
		1st day	2nd day	3rd day	4th day	5th day
1.	CDB-1	5.8	7.0	7.9	8.2	4.8
2.	CDB-2	2.1	4.0	7.0	1.3	1.3
3.	CDB-3	1.5	1.7	1.7	1.8	1.8
4.	CDB-4	1.8	6.4	7.0	7.6	1.2
5.	CDB-5	0.8	3.2	4.1	6.1	3.8
6.	CDB-6	1.9	3.2	6.0	7.2	3.0
7.	CDB-7	2.0	4.8	6.3	7.0	3.0
8.	CDB-8	1.4	1.5	1.5	1.5	1.6

CDB: Chlorpyrifos degrading bacteria; CFU: Colony forming units; MSM: Minimal salt medium

Chlorpyrifos degrading ability of bacterial isolates: In this present experiment, the growth of eight chlopyriphos pesticide degrading isolates was assessed in mineral salt broth containing 2% of pesticide. Among the eight chlorpyrifos bacterial isolates, CDB-1 showed the high population count at different incubation periods (1st day to 5th day) compared to all CDB inoculates. The CDB-1 isolate utilized the pesticide (chlorpyrifos) effectively and showed maximum growth bacterial count 5.8 cfu ×10^6 mL^{-1} (Table 3).

Degradation of chlorpyrifos in liquid medium by micro-organism (CDB-1): In this context three varying_concentrations of chlorpyrifos MSM broth were inoculated with efficient CDB-1 isolate and determined the chlorpyrifos degrading capacity with measurement of chlorpyrifos residual

concentrations at different days of intervals using Gas Chromatographic Method. The degradation of chlorpyrifos at different concentrations (20, 30 and 40 mg L^{-1}) was examined in the MSL medium on rotary shaker at 150 rpm, 30°C and optimum pH for isolate (Table 4).

The inoculation of CDB-1 resulted for first 2 days, the chlorpyrifos concentration dropped from 20 to 17.86 mg L^{-1}. After 4th and 6th day, chlorpyrifos declined to 16.16 and 8.03 mg L^{-1}, respectively, and by day 8th and 10th day chlorpyrifos was declined to 5.16 and 3.6 mg L^{-1}. The degradation percentage pattern showed decrease of residues from 2nd day (20 ppm) day to 10th day (3.6 ppm) and residues were dissipated by 10.6, 19.1, 59.8, 74.1, and 81.7 % at 2, 4 ,6 ,8 and 10 days, respectively. The regression equation was Y = -0.0939x+4.491 R^2=0.9654. Half life is 3.24 (Fig. 1).

Second time, the inoculation of CDB-1 resulted in the first 2 days, the chlorpyrifos concentration dropped from 30 to 22.4 mg L^{-1}. After 4th and 6th day, chlorpyrifos was declined to 17.4 and 15.5 mg L^{-1}, respectively, and by 8th and 10th day, chlorpyrifos was declined to 12.9 and 7.5 mg L^{-1}. The degradation percentage pattern showed decrease of residues from 2nd day (30 ppm) to 10th (7.5 ppm) day and residues were dissipated by 25.3, 40.1, 48.1, 56.9, and 74.9 % at 2, 4 ,6 ,8 and 10 days respectively. The regression equation was Y = -0.0546x + 4.4842 R^2= 0.9236. Half life is 5.57 (Fig. 2).

Third time, the inoculation of CDB-1 resulted in the first 2 days, the chlorpyrifos concentration dropped from 40 to 36.7 mg L^{-1}. After 4th and 6th day, chlorpyrifos was declined to 30.4 and 22.3 mg L^{-1}, respectively, and by 8th and 10th day chlorpyrifos was declined to 19.4 and 15.7 mg L^{-1}. The degradation percentage pattern showed decrease of residues from 2nd day to 10th day and residues were dissipated by 8.1, 23.7, 44.1, 52.4, and 60.6 % at 2, 4 ,6 ,8 and 10 days, respectively. The regression equation was Y = -0.0466 x + 4.6563 R^2= 0.9881. Half life is 6.54 (Fig. 3).

By this degradation percentage study of chlorpyrifos revealed that the chlorpyrifos degrading bacterial isolate (CDB-1) degrade the chlorpyrifos effectively. These isolates used the OP compounds as a carbon source. For the duration of the degradation study up to 10th day, the OP compounds in the medium were decreased. Hence, this study reveals that the bacterial isolate (CDB-1) was effectively degrading the chlorpyrifos in the field conditions also. This shows that the application of pesticide degrading bacterial isolates will improve the soil fertility and chemical xenobiotics reduction.

Similar results were found by Fulekar & Geetha (2008), *Pseudomonas aeruginosa* isolate (NCIM 2074) was adapted by subjecting to varying concentrations of chlorpyrifos, i.e.

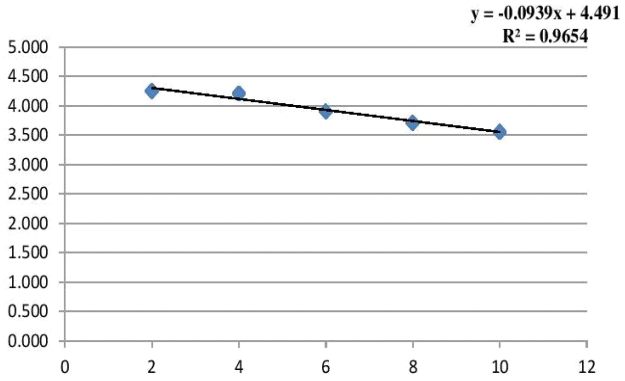

Fig 1: The regression equation (As per text).

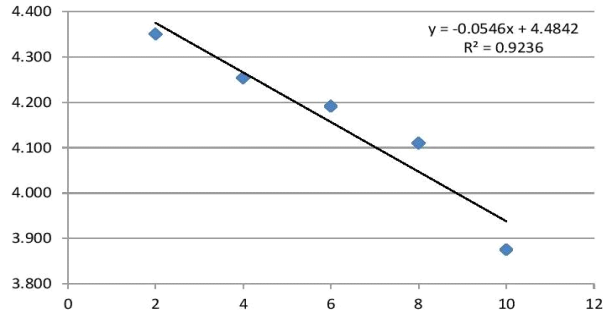

Fig. 2: The regression equation (As per text).

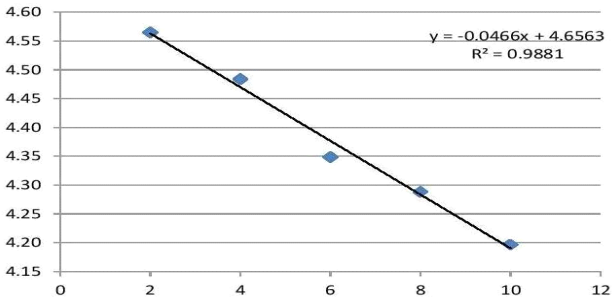

Fig. 3: The regression equation (As per text).

10, 20, 50, 75 and 100 mg L^{-1} in incubator shaker at 37 °C and 150 rpm. An initial 10 mg L^{-1} concentration of chlorpyrifos was supplied in minimal salt medium (MSM) under controlled environmental conditions for 14 days. The biodegradation of chlorpyrifos, as assessed by GC-MS, showed that chlorpyrifos at 10, 25, 50 mg/L degraded completely over a period of 1, 5 and 7 days, respectively. *Pseudomonas aeruginosa* (NCIM 2074) has potential use in bioremediation of chlorpyrifos at concentrations up to 50 mg L^{-1}, but the organism is inhibited by higher concentrations.

Table 4: Degradation percentage of chlorpyrifos at different concentrations in mineral salt liquid medium with an efficient bacterial isolate CDB-1 at 30°C for different time intervals.

Time of interval	CDB-1					
	Concentration (ppm)	Degradation %	Concentration (ppm)	Degradation %	Concentration (ppm)	Degradation %
Initial	20	0.00	30	0.00	40	0.00
2nd day	17.86	10.6	22.4	25.3	36.7	8.1
4th day	16.16	19.1	17.9	40.1	30.4	23.7
6th day	8.03	59.8	15.5	48.1	22.3	44.1
8th day	5.16	74.1	12.9	56.9	19.4	51.4
10th day	3.6	81.7	7.5	74.9	15.7	60.6
Half life	3.24		5.57		6.54	

CDB: Chlorpyrifos degrading bacteria

Maria et al. (2013) reported similar results and the ability of *Streptomyces* strains to remove pentachlorophenol and Chlorpyrifos. Moreover, a pure culture (*Streptomyces* sp. A5) and a quadruple culture showed the highest pentachlorophenol removal percentages (10.6% and 10.1%, resp.), while *Streptomyces* spp. M7 presented the best chlorpyrifos removal (99.2%). Mixed culture of all *Streptomyces* spp. when assayed either as free or immobilized cells showed chlorpyrifos removal percentages of 40.17% and 71.05%, respectively, and for pentachlorophenol it was 5.24% and 14.72%.

CONCLUSIONS

The achieved results were useful to conduct further research through pot culture or field studies on different crops to improve the efficacy of the degradation of the organophosphorus pesticides in the soil. Moreover, this study would be helpful in the practical application of bioremediation of chlorpyrifos-contaminated soils due to its low cost and less collateral destruction of indigenous organisms.

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