RESEARCH ARTICLE

Degradation of Pesticide Chlorpyrifos by Soil Bacteria *Achoromobacter xylosoxidans* (Accession No. KX817809.1) with Bioremediation Potential

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Abstract

Chlorpyrifos is a very widely used insecticide and considered one of the most frequently used chlorinated organophosphorus pesticides. In all the currently used method for removing such contaminants from the environment, the biodegradation has been shown to be more effective than any other method. Many pesticide degrading bacteria were isolated and identified through the colony, biochemical tests, and further identified by the 16S RNA sequencing method. The most potent strain grow in mineral salt medium (MSM) supplemented with chlorpyrifos as the sole source of carbon (150 to 1,000 µg/mL) and was monitored at an optical density of 600 nm. The growth parameters at different physical and chemical conditions were further optimized. The result showed that *Achoromobacter xylosoxidans* (accession no. KX817809.1) had maximum growth after 8 days. The present study shows that the isolated bacteria can be used for bioremediation of pesticide chlorpyrifos in a contaminated environment.

Keywords: Bacteria, Bioremediation, Chlorpyrifos, Degradation, Pesticide.

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Introduction

A pesticide contaminates the environment mainly through their application in agricultural and horticultural systems for the management of pests, wash-off from manufacturing waste, and accidental spillage into the soil. Similarly, organochlorine (OC) compounds, such as, chlorpyrifos is one of the most widely used insecticides in agricultural soil (Maya *et al*., 2011). Extensive use of pesticides can result in several health and environmental problems, like poisoning in farmers, neurological and skin disorders, cardiopulmonary disorders, and lowering the sperm counts in applicators. Certainly, pesticides have improved crop production, but their potential to persist in the environment may cause adverse effects among different forms of life and ecosystems. Approximately 90% of agricultural pesticide application does not reach its target organisms, contaminating earth's ecosystems, and affecting public health by entering the food chain. Microorganism plays a crucial role in biogeochemical cycles for the sustainable development of the biosphere. Bioremediation, by the help of bacteria to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments (Belal *et al*., 2008). Studies of microbial degradation can be used for the development of bioremediation processes to detoxify pesticides to lower concentrations than the standards established by regulatory authorities (Vidali, 2001). This study aimed at isolating chlorpyrifos degrading bacteria from a contaminated agricultural soil sample, analyzing and screening its growth response in mineral salt medium supplemented with chlorpyrifos in different oncentrations (ranging from low to high).

Materials and Methods

Chemicals

Chemicals used for the preparation of the media were of the highest purity grade and were obtained from HiMedia, Loba Chemie, Merck, and Qualigens.

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Soil Collection

Agricultural soils were taken from different areas of Patna, Bihar, which have past few years of history of chlorpyrifos use in pest control, were selected for this study. Random sampling was done, and the samples were collected from different agricultural fields, i.e., three vegetable fields and three rice crops fields from 12 to 15 cm depth of the field and stored aseptically for further analysis.

Isolation of Chlorpyrifos Degrading Bacteria

For the isolation of microbes from different soil samples, serial dilution was carried out by dissolving 0.1-gram of each soil samples in 9.9 mL of normal saline solution. For isolation of bacteria, 1 mL of different soil dilution were spread over the pre-sterilized Petri plates containing nutrient agar media (pH 7.2–7.4, peptone 5 grams, beef extract 3 grams, sodium chloride 5 grams, agar 15 grams, and D.W. 1,000 mL) from dilution 10^{-6}, 10^{-7}, and 10^{-8}, and culture plates were incubated at 37°C for

24 to 48 hours (Aneja, 2003). Chlorpyrifos utilizing microbes were isolated from soil samples by the enrichment culture technique nutrient culture medium, using chlorpyrifos as the sole source of carbon (150 to 1,000 µg mL^{-1}). This was done by sub-culturing those pure cultures of bacteria pesticides growing on NA media plates and incubated at 37°C for bacterial plates. Chlorpyrifos utilizing microbes were isolated from soil samples by the enrichment culture technique nutrient culture medium, using chlorpyrifos as the sole source of carbon as described by Zhu *et al*. (2010).

Characterization and Identification of Isolates

The bacterial isolates grown on chlorpyrifos agar were subjected to biochemical tests. The tests carried out, include gram staining, catalase test, citrate utilization, oxidase test, indole production, motility, sugar fermentation, methyl-red test, nitrate reduction, starch hydrolysis, Voges-Proskauer test, and hydrogen sulfide production (Bergey & Holt, 1984).

The best-selected strain with maximum degradation ability was further confirmed on the basis of 16S rRNA sequencing at Yaazh Xenomics (Madurai, Tamilnadu, India). The phylogenic neighbor-joining tree was using a complete 16S rRNA gene sequence analysis.

Effects of various Physicochemical Parameters

The isolated bacterial strain was further subjected to different physical and chemical conditions, like temperature (15, 25, 37, and 50°C), pH (5.5, 7, and 10.5), salt concentration (1, 5, 10, and 15%), nature of carbon source (sucrose, lactose, mannose, dextrose, and glucose), and nitrogen source (peptone, casein, yeast extract, and beef extract) (Yang *et al.,* 2018).

Growth Kinetic of Bacterial Isolates at Different Concentration of Compounds

Growth curve experiments were obtained by subjecting the isolated strain P-7 to different doses of chlorpyrifos (150, 250, 500, and 1,000 µg mL^{-1}) in order to determine the optimum concentration that stimulates the growth of isolates in liquid MSM medium at different concentrations (i.e., 150, 250, 500, and 1,000 µg mL^{-1}) at the interval of 2, 4, 8, 10, and 12 days using a spectrophotometer (Labtronics, LT-29) (Rani *et al*., 2008).

Results

Isolation of Chlorpyrifos-degrading Strain

Isolation of strains in MSM containing Chlorpyrifos (CP) in different concentrations (150 to 1,000 µg mL^{-1}) resulted in 30 isolates from six different agricultural soil samples. After the initial screen, 12 with good potential of degradation of CP were selected for further study after several enrichment transfers and cloning on nutrient agar plates containing CP as the sole carbon source. Among the 12 isolates, strain P-7 was selected for further investigation as it shows good degradation at 1,000 µg mL^{-1} of CP concentration. The colony produced on the nutrient agar plate was white with an oval in shape and the margin was smooth and elevated, as shown in Fig. 1. Microscopic examination after gram staining revealed that the isolated bacterial strain named P-7 was a gram-negative, motile, and rod-shaped, as depicted in Fig. 2.

Biochemical Characterization of the selected Strain

Biochemical tests were performed for all the isolates using Bergey's Manual of Systematic Bacteriology for biochemical characterization. The specified biochemical test was performed on isolates that show a positive result for, catalase, oxidase, and citrate, and identified as *Achoromobacter* sp., as shown in Table 1, which was further confirmed on the basis of 16S rRNA sequencing as *A. xylosoxidans* (accession no. KX817809.1).

Cultural Characteristics and Identification of the Selected Isolate

The isolated strain P-7 was identified up to the genus level as *Achoromobacter* species, which was confirmed as *A. xylosoxidans* (accession no. KX817809.1), on the basis of 16S rRNA sequencing, as at Yaazh Xenomics, Madurai, Tamilnadu (India). The phylogenetic neighbor-joining tree (Fig. 3) was constructed for strain P-7 using an almost complete 16S rRNA gene sequence (784 bp). The 16S rRNA sequence was prepared with the help of the National Center for Biotechnology Information (NCBI) blast similarity search tool. The program MUSCLE 3.7 was used for multiple alignments of sequences. The resulting aligned sequences were cured using

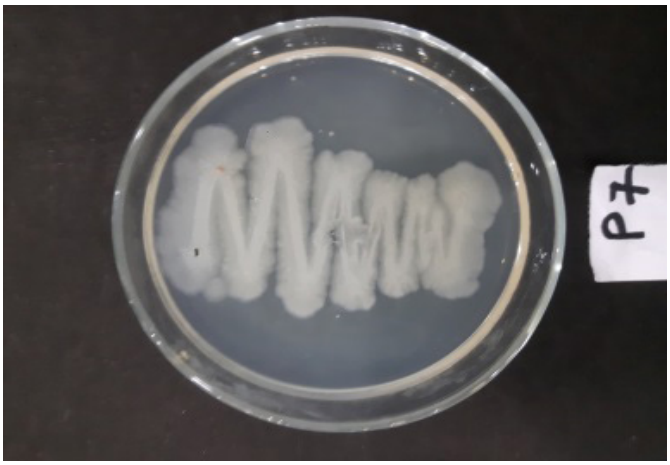

Fig. 1: Colony characteristics of the isolated strain P-7

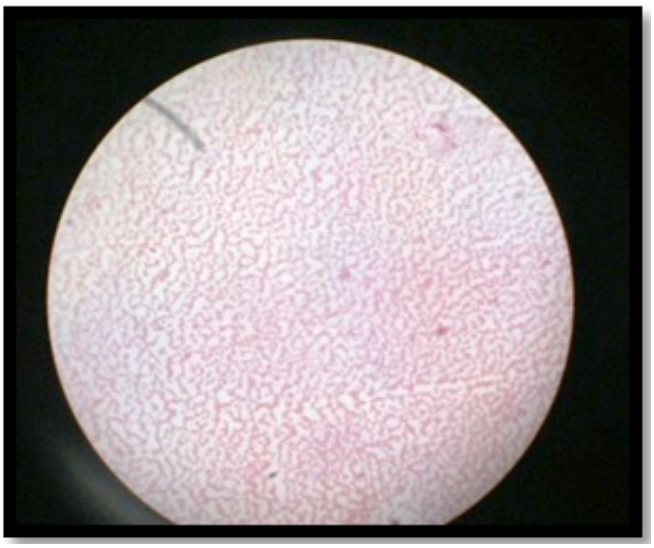

Fig. 2: Microscopic view (40X) of the isolated strain P-7

Table 1: Biochemical test result of isolated strain P-7

S. No.	*Biochemical tests*	*Bacterial strain (P-7)*
1	Amylase	-
2	Casein hydrolysis	-
3	Catalase	+
4	Gelatin hydrolysis	-
5	Nitrate reduction	-
6	Citrate utilization	+
7	Indole production	-
8	Methyl red	-
9	Voges Proskauer	-
10	Lactose	+
11	Dextrose	+
12	Sucrose	+
13	H_2S production	+
14	Citrate utilization	+

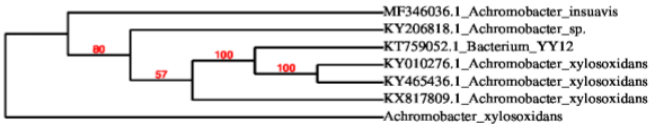

Fig. 3: Phylogenic analysis of strain P-7 based on 16S rRNA analysis; the sequence bar equals 0.02 changes per nucleotide position

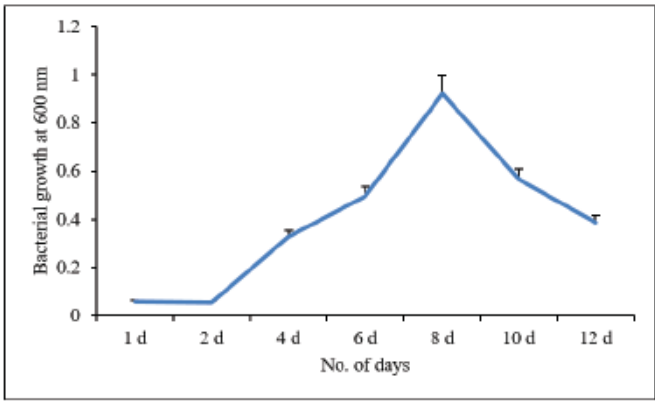

Fig. 4: Growth kinetics of isolates strains P-7 at the chlorpyrifos concentration of 1,000 µg mL^{-1}; all the values are means of 3 replicate (n = 3) ± SD

the program Gblocks 0.91b. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering.

Growth Kinetics of the Selected Strain

The selected strain was grown on MSM containing CP as the sole carbon source at different concentrations (150–1,000 µg mL^{-1}). The bacterial growth was monitored as absorbance at 600 nm. The highest activity was obtained on the 8th day of incubation at 37°C at a concentration of 1,000 µg mL^{-1} (Fig. 4).

Effect of Temperature and pH

Different temperature and pH, play an important role in affecting cell growth, the efficiency of biodegradation, and the enzymatic activation involved in these metabolic pathways. As shown

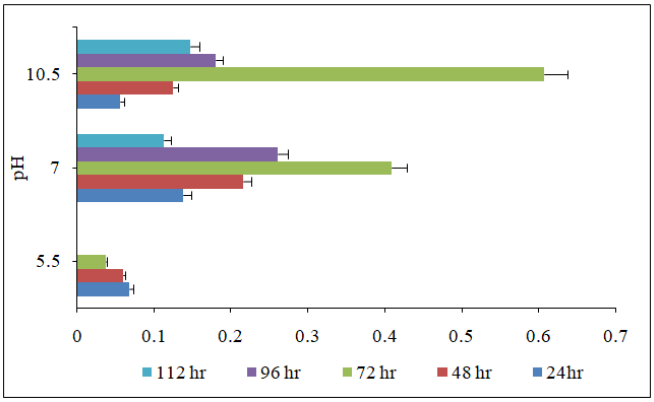

Fig. 5: Showing the effect of different pH on the strain P-7; all the values are means of 3 replicate (n = 3) ± SD

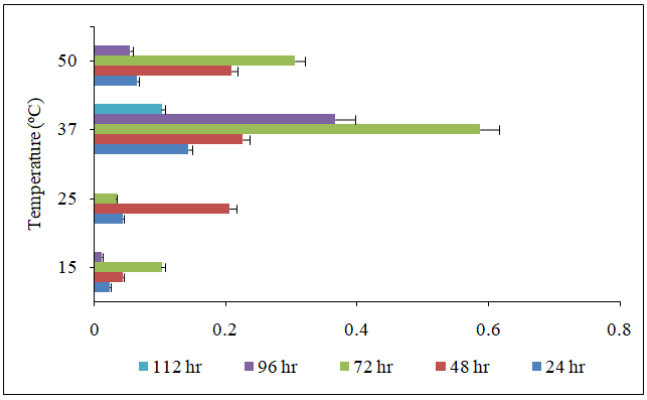

Fig. 6: Effect of different temperatures on the isolated strain P-7; all the values are means of 3 replicate (n = 3) ± SD

in Fig. 5, strain P7 showed different growth rates at different pH values ranging from 5.5 to 10.5, showing maximum activity at pH 10.5. Similarly, the optical density of the strain P-7 was recorded at different temperatures 15, 26, 37, and 50°C, on 2nd, 4th, 6th, 8th, and 10th day of incubation. The highest activity was obtained on the 3rd day of incubation at 37°C (Fig. 6).

Effect of Different Chemical Parameters [Carbon, Nitrogen Source, and Sodium Chloride (NaCl)] Concentration on Growth of Isolates

The selected strain was grown on different carbon (lactose, glucose, mannose, and dextrose), nitrogen (peptone, yeast extract, beef extract, and casein), and NaCl (1, 5, 10, and 15%) condition, and monitored on regular interval till 10th day (Figs. 7 to 9).

Discussion

Bioremediation is an effective tool to restore the polluted ecosystem with the help of biodegradative activities of microorganisms. The strain P-7 isolated and identified in this study is having high osmotic potential with superior salinity tolerance increases, which might affect their metabolic activities (Cortés-Lorenzo *et al.*, 2014). Reports have shown that pH and temperature always impact microbial CP degradation. In this study, we isolated an efficient and halotolerant chlorpyrifos degrading strain, *A. xylosoxidans*, which can grow at a wide range of pH, temperature and utilizes several carbon sources. However,

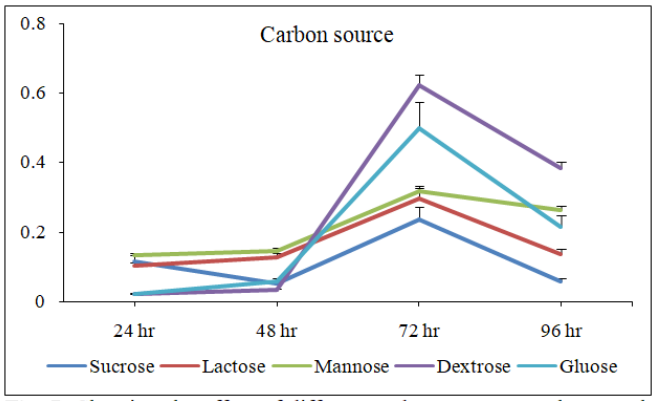

Fig. 7: Showing the effect of different carbon sources on the growth of isolated strain P-7; all the values are means of 3 replicate (n = 3) ± SD

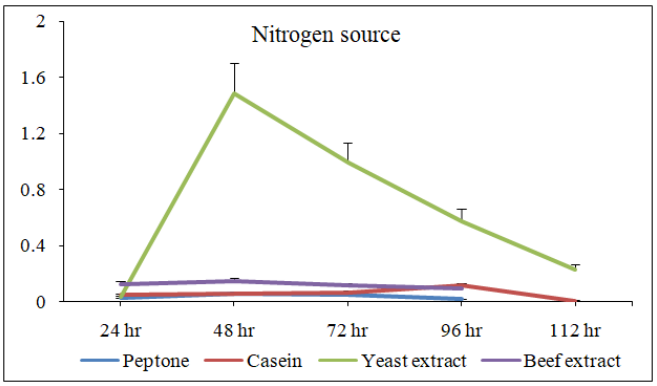

Fig. 8: Showing the effect of different nitrogen sources on the growth of isolated strain P-7; all the values are means of 3 replicate (n = 3) ± SD

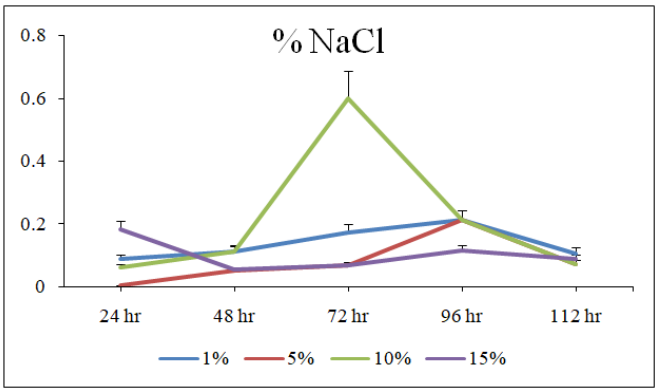

Fig. 9: Showing the effect of different NaCl% on the growth of isolated strain P-7; all the values are means of 3 replicate (n = 3) ± SD

till now reported the available CP-degrading strains are limited. It has been reported by other researchers that the optimization of different physiochemical conditions plays a very important role in accelerating the degradation of chlorpyrifos. It was also reported that bacteria *Sphingomonas* sp. can use chlorpyrifos as its sole source of carbon for growth, by hydrolyzing chlorpyrifos to 3,5,6-trichloro-2-pyridinol (Li *et al.*, 2007). Many authors reported that the most different species, mainly gram-negative bacteria, in the degradation of insecticides, like chlorpyrifos. Vijaylaxmi and Usha (2007) reported many degrader bacteria with the capability to degrade the pesticides, like *Pseudomonas aeruginosa* was the most common gram-negative bacterium found in soil, and this bacterium has been found to have the potential to degrade chlorpyrifos, other reported organisms are *Serratia* sp., *Klebsiella* sp., *Providencia* sp., and *Bacillus* sp. capable of degradation of pesticide chlorpyrifos.

Conclusion

The results presented in this study show that bacterial isolates play a very important role in the degradation and remediation of pesticides. In this study, P-7 isolate was capable of utilizing chlorpyrifos as the sole source of carbon and energy, was identified and further characterization shows that the P-7 is highly halophilic in nature and can tolerate a wide range of pH also. Biodegradation of chlorpyrifos pesticides by bacteria is an effective method of preventing environmental pollution. The result of this study showed that the isolate *A. xylosoxidans* (accession no. KX817809.1) has a wide range of tolerance levels of different environmental conditions and can grow in presence of different chemicals making it an effective solution for removing chlorpyrifos from contaminated soil or other contaminated environments.

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