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Isolation and Characterization of Chlorpyrifos Degrading Bacteria from Chlorpyrifos Contaminated Soil

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Chlorpyrifos is aorganophosphorus insecticide widely used for crop protection. Chlorpyrifos is toxic to environment, exploration of various chlorpyrifos-degrading bacteria to clean-up the pesticides is of immense importance. Six bacterial isolates were successfully isolated from chlorpyrifos contaminated soil and were named CP-1, CP-2, CP-3, CP-4, CP-5 and CP-6. The isolates were capable of utilizing chlorpyrifos (Cp) as the sole source of carbon and were able to grow at 100 mg/L concentrations of chlorpyrifos. Effect of environmental conditions like temperature, pH and NaCl concentration was determined for all bacterial isolates.Most of bacterial isolates showed susceptibility for different antibiotics in antibiotic sensitivity test. Growth kinetics experiments showedthat the bacterial isolates were able to grow in medium containing the individual pesticide as the carbon source. Out of six bacterial isolates CP-3was identified based on16SrRNA sequence analysis.

Key words: Chlorpyrifos, Organophosphorus, Growth kinetics, 16SrRNA.

Indian agriculture sector has been growing rapidly over the years. In order to obstruct the insect from attacking their crops, farmers use pesticide. Pesticides are applied to lower damages on crops and forestry products from pests, pathogens, weeds, mites, nematodes, rodents and regulating plant growth. They are used to lower the cost of agricultural products, and greatly reduce the losses caused by pests and diseases, creating great economic benefits. However, pesticide residues can adversely affect ecosystems and human health and also cause serious environmental pollution.

The production of pesticides started in India in 1952 with the establishment of a plant for the production of BHC near Calcutta, and India is now the second largest manufacturer of pesticides in Asia after China and ranks twelfth globally (Mathur, 1999). There has been a steady growth in the production of technical grade pesticides in India, from 5,000 metric tons in 1958 to 102,240 metric tons in 1998.

Chlorpyrifos, the active ingredient of which is O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is one of the most widely used pesticides and its half-life in soil varies from 10 to 120 days (Getzin, 1985). The molecular formula of chlorpyrifos is $C_9H_{11}Cl_{13}NO_3PS$. Chlorpyrifos was developed by the U.S. chemical company Dow Agro Sciences in 1965. Chlorpyrifos is formulated as a number of different commercial products. The most commonly available formulations include emulsifiable concentrates (EC), granulars (GR) and wettable powder (WP). Granular formulations are commonly employed for controlling soil insects.

Pesticides and their degradation products, generally get accumulated in the top soil and influence not only the population of various

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groups of soil microbes but also their biochemical activities like nitrification, ammonification, decomposition of organic matter and nitrogen fixation (Agnihotri*et al.*, 1981). Microorganisms play an important role in degrading synthetic chemicals in soil (Alexander, 1981). They have the capacity to utilize virtually all naturally and synthetically occurring compounds as their sole carbon and energy source.

In soil, chlorpyrifos may remain biologically active for periods ranging from days to months. Dosage rates, soil type, soil moisture and organic matter content, temperature and insecticide formulation are among the factors which influence biological persistence (Read, 1976; Tashiro and Kuhr, 1978). Chemical persistence is equally variable, with initial half lives ranging from less than a week to a month or more (Agnihotri*et al.*, 1981). It is moderately persistent in nature as its residues were detected in soil even after 3 months and hence causes potential environmental hazards (Chapman *et al.*, 1984). Considerable residues of chlorpyrifos were found in tomatoes (Aysal*et al.*, 1999), cotton seed and oil of oilseed crops like groundnut, safflower and mustard (Bhatnagar and Gupta, 1992).

The environmental fate of chlorpyrifos has been studied extensively. Degradation in soil involves both chemical hydrolysis and microbial activity. In most cases, the aerobic bacteria tend to transform chlorpyrifos by hydrolysis to produce diethylthiophosphoric acid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP). It was suggested that the accumulation of TCP, which has antimicrobial properties, prevents the proliferation of chlorpyrifos degrading microorganisms in soil (Racke*et al.*, 1990). The degradation of chlorpyrifos was very slow in acidic soil but the rate of degradation increased considerably with an increase in soil pH.

Chlorpyrifos has been reported to be degraded co-metabolically in liquid media by bacteria (Mallick*et al.,* 1999). Microorganisms like *Sphingomonas* sp., *Stenotrophomonas* sp., *Bacillus* sp., *Brevundimonas* sp. and *Pseudomonas* sp. Some fungi also degrade chlorpyrifos which include *Trichodermaviridae* and *Aspergillusniger* (Mukherjee and Gopal, 1996) utilize chlorpyrifos as a sole source of carbon. Chlorpyrifos-degrading bacteria may be applied either directly or indirectly in the bioremediation of chlorpyrifos contaminated soils.

MATERIAL AND METHODS

Sample collection

Soil samples were collected from the sludge produced by chlorpyrifos producing industrial plants of Ahmedabad, Ankleshwar, Vadodara, Kalol and different part of Gujarat. Soil samples were also collected from field where chlorpyrifos has been applied for more than 3 years for the control of various insect pests.The collected samples were air dried, ground, passed through 2 mm sieve and stored in the sealed plastic bags at room temperature. These stored samples were used for further experimentation.

Pesticide used

Chlorpyrifos technical (96% purity) was obtained from Crop Life Science Limited, Ahmedabad. Chlorpyrifos (99% purity) were purchased from Sigma-Aldrich, USA.

Enrichment of soil samples

Soil samples were air dried to 20% (w/w) moisture content and passed through a sieve with 2 mm mesh. Soil samples were enriched in 250 ml Erlenmeyer flasks containing 50 ml MSM supplemented with appropriate organophosphorus pesticide and 10 g soil. For enrichment of chlorpyrifos degrading bacteria MSM was supplemented with chlorpyrifos (25 mg/l) as a sole source of carbon, energy. The flasks were incubated on a rotary shaker at 150 rpm for seven days at 30^0C. After a week, 1 ml from the above medium was inoculated to the same fresh medium. This was repeated 5-6 times for selective enrichment of pesticide degrading bacteria.

Isolation and screening of organophosphorus degrading bacteria

For isolation of organophosphorus degrading bacteria the enriched soil samples were serially diluted and spread plated on to MSM containing chlorpyrifos (25 mg/l).After isolation, the representative microorganisms growing on the plates were purified following the four-way streaking method. The chlorpyriphos degrading

bacterial isolates were tested for their ability to grow on higher concentration by inoculating to the MSM containing chlorpyriphos (50, 75 and 100 mg/l) with and without agar. Finally, the strains with the tolerance to the highest chlorpyriphos concentration were selected and different biochemical tests were performed for further identification.

Effect of environmental conditions on bacterial growth

There are many parameters which affect growth of bacterial isolates viz. temperature, pH, aeration, salt concentration, nutrient availability, radiation, presence of heavy metals, carbon source, etc. Out of these, some important parameters viz. pH, temperature, salt concentration and carbon source affecting bacterial growth were considered.

Effect oftemperature on bacterial growth

Effect of temperature on bacterial growth was tested on both nutrient agar medium and nutrient broth. All bacterial isolates were inoculated and incubated at different range of temperature 15°C, 25, 35 45 for 48 hr. After incubation presence or absence of growth on nutrient agar medium was recorded. The absorbance of bacterial isolates from nutrient broth was measured at 600 nm and data were used to plot a graph to determine the effect of temperature on the respective bacterial isolates.

Effect ofpH on bacterial growth

Effect of pH on bacterial growth was tested on both nutrient agar medium and nutrient broth. Nutrient agar medium and nutrient broth were adjusted with different ranges of pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. All bacterial isolates were inoculated and incubated at 37 for 48 hr. After incubation presence or absence of growth on nutrient agar medium was recorded. The absorbance of bacterial isolates from nutrient broth was measured at 600 nm and data were used to plot a graph to determine the effect of pH on the respective bacterial isolates.

Effect of NaCl on bacterial growth

Effect of NaCl on bacterial growth was tested on both nutrient agar medium and nutrient broth. Nutrient agar medium and nutrient broth were supplemented with different ranges of NaCl concentration 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0%. All bacterial isolates were inoculated and incubated at 37 for 48 hr. After incubation presence or absence of growth on nutrient agar medium was recorded. The absorbance of bacterial isolates from nutrient broth was measured at 600 nm and data were used to plot a graph to determine the effect of NaCl on the respective bacterial isolates.

Antibiotics sensitivity test

Sensitivity of bacterial isolates to antibiotics was tested on N-agar media using the octadiscs of antibiotics (Himedia manufactured). All chlorpyrifos degrading isolates were inoculated in nutrient broth and incubated at 37^0C for 24 hr. After incubation 100µl of bacterial culture was spreaded on nutrient agar plate and octadiscs of antibiotics were kept on inoculated plates. The plates were incubated at 37^0C for 48 hr. The zone of inhibition was observed for each disc. The following antibiotics were used for sensitivity test: Tetracycline (TE), Chloramphenicol (C), Ampicillin (Amp), Gentamicin (Gen), Cefazolin (Cz), Cefuroxime (Cxm), Amikacin (Ak), Co-Trimoxazole (Cot).

Growth Kinetics

All bacterial isolates were inoculated in MSM supplemented with two different concentrations of chlorpyrifos(50 and 100 mg/l). The MSM inoculated with bacterial isolates were incubated at 37°C in shaking condition (120rpm).The MSM containing chlorpyrifos(50 and 100 mg/l) and *Escherichia coli*, which is not able to degrade chlorpyrifos, was used as the negative control. The growth kinetics was followed by monitoring the optical density of the medium for 9 days using a UV/VIS spectrophotometer at 600 nm wavelength.

Identification of bacteria

Isolated chlorpyrifos-degrading bacteria were characterized based on 16S rRNA gene analysis. The genomic DNA was extracted using phenol-chloroform method.The 16S rRNA gene was amplified. Sequencing was carried out with an automated sequencer (ABI 3130 XL). 16S rRNA sequences were compared to other 16S rRNA sequences available in the National Center for Biotechnology Information (NCBI) public database by basic local alignment search tool (BLAST) searching. Selected sequences from the database with the greatest sequence similarity to

isolated bacterial sequence were aligned and compared.

RESULTS AND DISCUSSION

Isolation, screening and biochemical characterization of isolates

During primary screening six strains were isolated that were capable of utilizing chlorpyrifos (25 mg/l) as the sole source of carbon.The isolates, designed CP-1, CP-2, CP-3, CP-4, CP-5 and CP-6 were grown in different concentrations of chlorpyrifos (50, 75 and 100 mg/l). Biochemical studies were carried out (Table 1)according to Bergey's Manual of Systematic Bacteriology (Vol I and II).

Effect of environmental condition on bacterial growth

All six isolates showed ability to grow at wide range of temperature, pH and NaCl concentration.The effect of temperature on bacterial growth was seen in the temperature range of 15^{0}C to 55^{0}C with the maximum growth seen at 35±2^{0}C. CP-4 and CP-5 were unable to grow at 45^{0}C whereas all isolates were unable to grow at 55^{0}C. The effect of pH on bacterial growth was observed in the pH range of 4 to 10 with the maximum growth seen at pH 7. All six isolates were unable to grow at pH 4 whereas CP-1 was unable to grow at pH 10. The optimum NaCl concentration for all six isolates was found to be 1 %. All isolates other than CP-4 were grown up to 6 % NaCl concentration. CP-4 was able to grow upto 3 % NaCl concentration.

Antibiotic sensitivity

For antibiotic resistance/ susceptibility profiling, the disc diffusion method was used. The zone of inhibition was measured in millimeter and the resistance and sensitivity of isolated bacteria towards antibiotics used was determined. It was found that all six isolates were found susceptible to Tetracycline, Co-Trimoxazole, Amikacin, Gentamycin, Ampicillin and Chloramphenicol.All isolates were susceptible to Cefuroxime except CP-3 which was resistance. Bacterial isolates CP-4 and CP-5 were susceptible where as other all isolates were resistant to Cefazolin.

Growth kinetics

Bacterial growth in MSM supplemented with two different concentrations of chlorpyrifos

Table 1. Biochemical Test Results

Organism	Citrate	Starch	Motility	Oxidase	Catalase	H_2S	Indole	Nitrate	Acid	Gas	Gram	Urease
CP-1	+Ve	-Ve	-Ve	+Ve	+Ve	-Ve	+Ve	+Ve	+Ve	-ve	-Ve	-Ve
CP-2	+Ve	-Ve	-Ve	+Ve	+Ve	-Ve	+Ve	+Ve	+ve	-ve	-Ve	-Ve
CP-3	+Ve	-Ve	-Ve	+Ve	+Ve	-Ve	+Ve	+Ve	+ve	-ve	-Ve	-Ve
CP-4	-Ve	-Ve	-Ve	-Ve	+Ve	+Ve	+Ve	-Ve	-ve	-ve	-Ve	-Ve
CP-5	+Ve	-Ve	-Ve	-Ve	+Ve	+Ve	+Ve	-Ve	+ve	-ve	+Ve	-Ve
CP-6	+Ve	+Ve	-Ve	+Ve	+Ve	-Ve	+Ve	+Ve	+ve	-ve	-Ve	-Ve

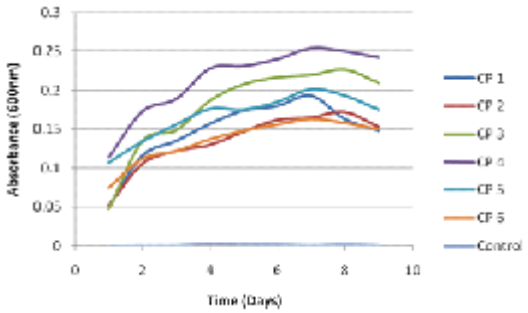

Fig. 1. Growth Kinetics of bacterial isolates in presence of chlorpyrifos (50 mg/l)

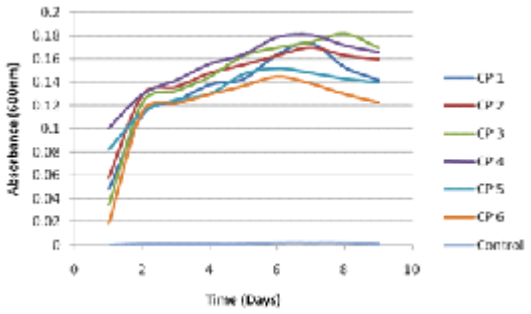

Fig. 2. Growth Kinetics of bacterial isolates in presence of chlorpyrifos (100 mg/l)

(50 and 100 mg/l)is shown in figure 1& 2. As compared to control sample, the growth of all bacterial isolates was significantly stimulated and approximately two to three times faster at the beginning of incubation period (one to four days). Maximum bacterial growth in 50 mg/l was obtained by day seven in CP-1, CP-4, CP-5 and CP-6 where as in CP-2 and CP-3 maximum growth was obtained by day eight. In growth kinetics at 100 mg/l maximum growth was obtained by day six in CP-5 and CP-6 where as CP-1, CP-2 and CP-4 showed maximum growth at day seven. Only CP-3 showed maximum growth by day eight. The growth curve reached a stasis after maximum growth and then decreased. In contrast, the control sample inoculated with *E. coli* showed no change at 600 nm for 9 days incubation.

Identification of bacteria

For the further identification at strain level, CP-3 was identified by 16 S rRNAsequencing. 16 S rRNA gene sequenceof CP-3 was compared with that of referred strains gene sequences in the Genbank. CP-3 shows close homology (98%) with the sequenceof Pseudomonas spp. with accession number KJ778658. The sequence was processedby sequin stand-alone software and deposited it in the GeneBank database with accession number KP340803.

CONCLUSION

All bacterial isolates were able to tolerate high concentration of chlorpyrifos. The isolates also showed growth at wide range of temperature, pH and salt concentration.Most of bacterial isolates were sensitive to antibiotics. In growth kinetics study all isolates showed variation in maximum growth time period. 16S rRNA analysis revealed that CP-3 is related to *Pseudomonas stutzeri*, which is able to participate in efficient degradation of chlorpyrifos. Thus the capacity ofthe isolates to survive and grow in the presence of highconcentrations of the chlorpyrifosmarks them out as goodcandidates for the bioremediation of chlorpyrifospollutedenvironments.

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