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RESEARCH ARTICLE

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BIOREMEDIATION OF CHLORPYRIFOS BY BACTERIA ISOLATED FROM THE CULTIVATED SOILS

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ABSTRACT

In this study, chlorpyrifos degrading capability of four bacterial monocultures (*RCC-2, GCC-1, GCC-3* and *JCC-3*) and two bacterial mixed-cultures (*GCE345* and *GCC134*) was investigated in terms of treatment duration and culture volume, using soil slurry medium. Among the bacterial mono-cultures, *RCC-2* was found to be most efficient with 21, 37, 54 and 77% of chlorpyrifos degradation in 5, 10, 15 and 30 days of treatment duration, respectively. Out of two bacterial mixed-cultures, *GCC134* was more effective and resulted in 24, 38, 56 and 85% degradation of chlorpyrifos in 5, 10, 15 and 30 days of treatment, respectively. Chlorpyrifos degradation was higher by increasing the culture volume of respective bacterial cultures, from 10% to 25% (v/v), for 10 days of treatment at room temperature. The Pearson correlation between treatment duration and % degradation of chlorpyrifos were 0.953 and 0.988 with bacterial mono- and mixed-cultures, respectively.

KEYWORDS

Bioremediation, Chlorpyrifos, Mono-cultures, Mixed-cultures, Degradation

INTRODUCTION

Organophosphate pesticides constitute a group of widely used, very heterogeneous compounds that share a phosphoric acid derivative chemical structure[1]. The wide use of organophosphorus pesticides has created numerous problems, including the pollution of the environment[2]. Chlorpyrifos is a type of organophosphorus pesticide and its chemical name is *O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate. It is used worldwide as an agricultural insecticide. The reported half-life of chlorpyrifos in soil varies from 10 to 120 days, with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. The higher level of TCP prevents the proliferation of chlorpyrifos-degrading microorganisms in soil. Attempts to isolate chlorpyrifos-degrading bacteria from chlorpyrifos-treated soils have not been very successful. However, chlorpyrifos has been shown to be degraded cometabolically in liquid media by bacteria[3,4,5].

Different bacteria degrade different pesticides, insecticides and herbicides. Chlorpyrifos is one of the most commonly and widely used commercial insecticides[6]. Extensive use of chlorpyrifos contaminates air, ground water, rivers and lakes. If the pesticide is not degraded or detoxified rapidly enough, the risk of its off-site migration may pose a health risk to humans[7]. Catabolism and detoxification metabolism occur when a soil microorganism uses the pesticide as a carbon and energy source. The biodegradation of organophosphorus pesticides by soil microorganisms has been reported by many workers. It has been suggested that cultures of bacteria with the ability to degrade specific compounds can be used for bioremediation process of pesticide polluted sites[8,9].

MATERIALS AND METHODS

Soil used for degradation study:

The soils collected from the cultivated fields (mainly cotton, groundnut and vegetables cultivating fields) in Rajkot district of Gujarat State were used for biodegradation experiments of chlorpyrifos using bacterial mono- and mixed-cultures. The collected soils were first passed through a 2 mm sieve and the sieved fraction was collected and preserved in air tight plastic containers for biodegradation experiments.

Pesticide, chemicals and media:

The technical grade of chlorpyrifos (Lethal, EC 20%), a type of organophosphorus pesticide was selected for the present study. The pesticide was purchased from the local pesticide supplier. This commercial formulation of chlorpyrifos was dissolved in sterile distilled water for amendments to soil samples. The analytical grade chemicals and reagents used in this work were purchased from Hi-media and Qualigens. The culture media used were Nutrient Broth (NB), Luria Bertani (LB) and M9.

Sterilization of media, solutions and apparatus:

All the media and solutions used were sterilized by autoclaving at 121^0C temperature, 15 psi pressure for 20 minutes. The glassware and other apparatus were sterilized in an oven at 180^0C for an hour. After sterilization, the media and solutions were cooled to room temperature and then stored under refrigeration for their subsequent use. The sterilized glassware were stored separately in an oven at 60^oC and cooled to room temperature before their subsequent use.

Bacterial cultures used:

Four bacterial mono-cultures viz. *RCC-2* (*Pseudomonas sp.*), *GCC-1* (*Staphylococcus sp.*), *GCC-3* (*Flavobacterium sp.*) and *JCC-3* (*Streptococcus sp.*) were used in this study. All these four bacterial cultures were obtained from the previous work on isolation, characterization and identification of chlorpyrifos degrading bacteria from the cultivated soil (details not shown). Each of the two mixed-cultures, viz. *GCE345* and *GCC134* were prepared by mixing three bacterial monocultures in equal proportion.

Degradation studies:

The bacterial mono- and mixed-cultures to be assayed for their degradation of chlorpyrifos were first sub-cultured in Luria broth nutrient medium and then transferred into soil slurry medium. Erlenmeyer flasks (250 mL) in triplicate containing 100 mL soil slurry medium were stoppered with cotton wool plugs and autoclaved before use. For determining the effect of treatment duration, 10 mL of the full grown bacterial cultures in Luria broth was transferred into flasks. To study the effect of culture volume on chlorpyrifos degradation, 10, 15, 20 and 25 mL of cultures were transferred into the respective labeled flasks. The degradation assays were performed in sealed flasks spiked with 20 mg/L of chlorpyrifos. Uninoculated flasks were used as controls. The flasks were incubated at room temperature and samples for pesticide residue analysis were taken aseptically.

Extraction of chlorpyrifos from samples:

After the bioremediation by respective bacterial cultures, it is important to recover chlorpyrifos from soil slurry to determine the extent of chlorpyrifos degradation. The extraction of chlorpyrifos was carried out using chloroform as a solvent. The autoclaved soil slurry medium containing 10g soil per 100 mL of double distilled water in a sterile flask was mixed vigorously to secure homogeneous medium. The medium was supplemented with 20 mg/L of chlorpyrifos for degradation assay. After incubation period, 10 mL of soil slurry was taken in a centrifuge tube and centrifuged at 4000 rpm for 20 minutes. After this, 5 mL of supernatant was transferred into another tube and an equal volume of chloroform was added, shaken gently and allowed to settle for 30 minutes. The upper water layer was discarded and 2 mL of bottom layer containing the undegraded pesticide (chlorpyrifos) was taken in an eppendorf tube. The sample was stored at 4^{o}C till analyzed by GC-MS.

GC-MS analysis:

A GC-MS-QP2010 system equipped electron capture detector (ECD) was used to analyze the residual chlorpyrifos in the sample after bacterial degradation. One micro-litre of each chloroform extract was manually injected. BPX5 column (30m, 0.25mm) was fitted and a temperature program (180^{o}C for 1.5 min, 260^{o}C for 20 min, at the rate of 10^{o}C/min) used. Nitrogen was used as carrier gas at a column head pressure of 95.6 kpa giving a linear carrier flow of 36.4 cm/s and column flow was 0.90 mL/min. The injection mode was split, injector temperature was 260^{o}C and detector temperature was 280^{o}C. The chromatographic analysis was done for 22 minutes. The chromatograms were recorded using a computer and compared with standard library of NIST-07 mass spectral database. External standards were used for the quantification residual concentration of chlorpyrifos in the sample.

Effect of treatment duration and culture volume:

Chlorpyrifos degradation capability of bacterial mono- and mixed-cultures was determined in terms of treatment duration and culture volume. To study the effect of treatment duration on chlorpyrifos degradation, the experimental system containing 10 mL of respective active bacterial culture per 100 mL of soil slurry supplemented with 20 mg/L of chlorpyrifos was incubated at room temperature for 5, 10, 15 and 30 days, respectively. For determining the bioremediation capability of bacterial cultures in

terms of culture volume, the experimental system was prepared using 100 mL of soil slurry containing 20 mg/L of chlorpyrifos and inoculated with 10, 15, 20 and 25 mL of respective bacterial culture separately, and incubated at room temperature for 10 days. In both cases, 10 mL broth was taken out in the sterile tube after incubation period and used for the estimating residual chlorpyrifos by GC-MS.

Statistical analysis:

The data obtained from the study of samples were analyzed using statistical features available with MS-Excel worksheet. All the data were average of three replicates. The analyzed data were used to prepare result table and graphs. The useful inferences were derived from the interpretation of such result table and graphs.

RESULTS AND DISCUSSION

Degradation of chlorpyrifos by bacterial mono-cultures:

The degradation of chlorpyrifos was 21, 37, 54 and 77% after incubation period of 5, 10, 15 and 30 days, respectively, at room temperature, in soil slurry medium containing 20 mg/L of chlorpyrifos and inoculated with 10% (v/v) active bacterial mono-culture *RCC-2*. Under the similar experimental conditions, the chlorpyrifos degradation was 23, 34, 48 and 62% after incubation period of 5, 10, 15 and 30 days, respectively, by culture *GCC-1*. When soil slurry medium was inoculated with culture *GCC-3*, the degradation of chlorpyrifos was 19, 33, 43 and 51% after incubation period of 5, 10, 15 and 30 days, respectively. The bacterial mono-culture *JCC-3* resulted in 16, 33, 60 and 65% degradation of chlorpyrifos under similar experimental conditions, after incubation period of 5, 10, 15 and 30 days, respectively (**Figure-1**). The value of Pearson correlation coefficient between treatment duration and % degradation of chlorpyrifos by bacterial mono-cultures (r = 0.953) was extremely significant (**Table-1**). The positive value of correlation coefficient indicated that the degradation of chlorpyrifos in soil slurry medium by bacterial mono-cultures increases with the increase in treatment duration, till 30 days of treatment. Therefore, the proper optimization of treatment duration could play an important role in the bioremediation process of chlorpyrifos contaminated soil and water.

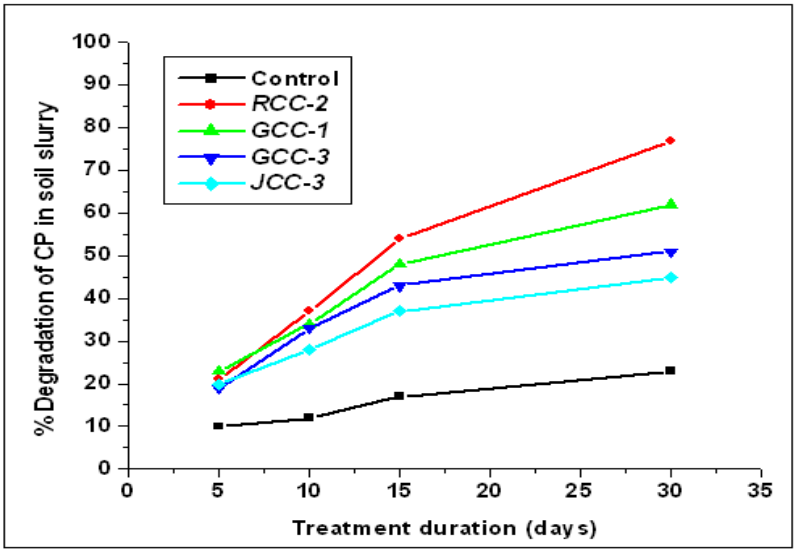

Figure-1

Chlorpyrifos degradation by bacterial mono-cultures in response to treatment duration

When soil slurry medium containing 20 mg/L of chlorpyrifos was separately inoculated with 10, 15, 20 and 25 mL of respective bacterial mono-cultures per 100 mL of medium and incubated at room temperature for 10 days, then the degradation of chlorpyrifos was found to be 34, 50, 62 and 74%, respectively, by isolate *RCC-2*. The degradation was 27, 41, 50 and 62%, respectively, by isolate *GCC-1*. In case of isolate *GCC-3*, the degradation was 22, 25, 29 and 37%, respectively. The chlorpyrifos degradation was 25, 29, 38 and 47%, when inoculated separately with 10, 15, 20 and 25 mL of *JCC-3* mono-culture and incubated at room temperature for 10 days (**Figure-2**). The value of Pearson correlation between culture volume and % degradation of chlorpyrifos by bacterial mono-cultures (r = 0.989) was extremely significant (**Table-1**). The positive value of correlation coefficient indicated that the degradation of chlorpyrifos in soil slurry medium by bacterial mono-cultures increases by increasing the culture volume of bacterial mono-cultures. Therefore, it important to optimize the culture volume of bacterial mono-cultures, prior to bioremediation of chlorpyrifos contaminated soil and water.

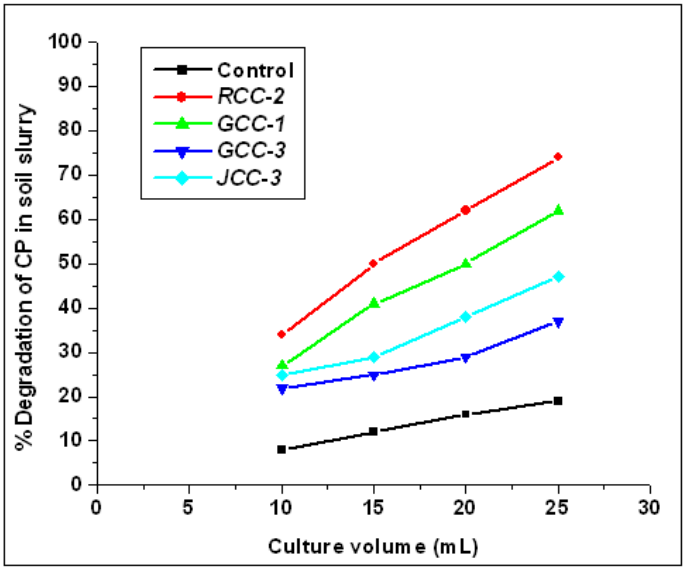

Figure-2

Chlorpyrifos degradation by bacterial mono-cultures in response to culture volume

Table-1

Linear regression analysis between degradation parameters and % degradation of chlorpyrifos by bacterial mono- and mixed-cultures

Degradation parameters		Degradation of chlorpyrifos (%)	
		Pearson correlation	R-square
Treatment duration (days)	Mono-cultures	0.953	0.910
	Mixed-cultures	0.988	0.976
Culture volume (mL)	Mono-cultures	0.989	0.977
	Mixed-cultures	0.986	0.973

Degradation of chlorpyrifos by bacterial mixed-cultures:

The chlorpyrifos degradation was 20, 32, 50 and 74% after treatment duration of 5, 10, 15 and 30 days, respectively, at room temperature, in soil slurry medium containing 20 mg/L of chlorpyrifos and inoculated with 10% (v/v) active bacterial mixed-culture *GCE345*. With mixed-culture *GCC134*, chlorpyrifos degradation was 24, 38, 56 and 85% after treatment duration of 5, 10, 15 and 30 days, respectively, under the similar experimental conditions (**Figure-3**). The value of Pearson correlation between treatment duration and % degradation of chlorpyrifos by bacterial mixed-cultures (r = 0.988) was very significant (**Table-1**). The positive value of correlation coefficient indicated that the degradation of chlorpyrifos in soil slurry medium by bacterial mixed-cultures increases with increase in treatment duration. The results showed that proper optimization of treatment duration using bacterial mixed-cultures could play a significant role during bioremediation of chlorpyrifos contaminated soil and water.

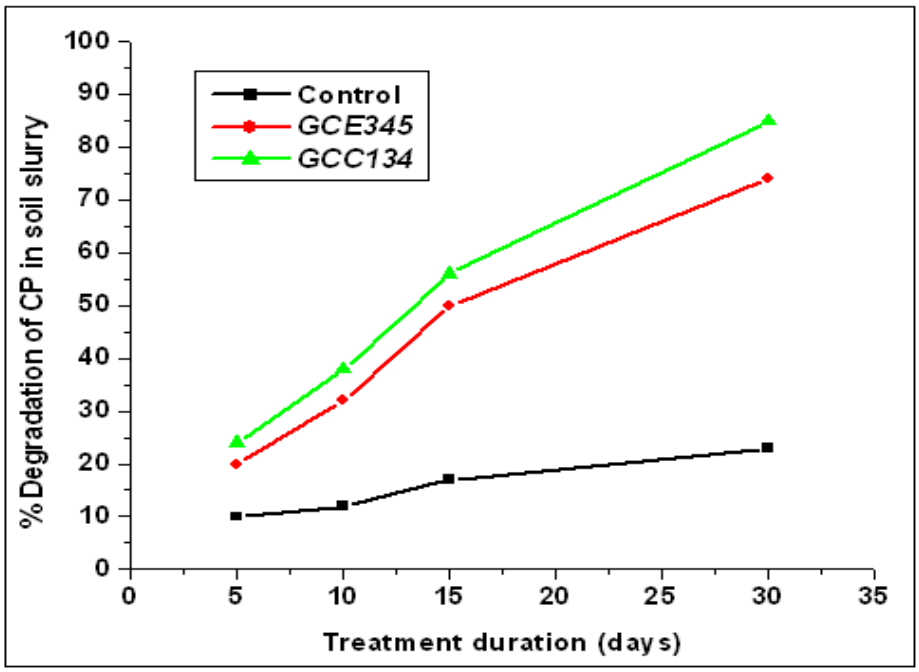

Figure-3

Chlorpyrifos degradation by bacterial mixed-cultures in response to treatment duration

In case of soil slurry medium containing chlorpyrifos (20 mg/L) was inoculated separately with 10, 15, 20 and 25 mL of respective bacterial mixed-cultures per 100 mL of medium and incubated at room temperature for 10 days, the degradation of chlorpyrifos was found to be 26, 32, 39 and 44%, respectively, by mixed-culture *GCE345*. Under the similar experimental and incubation conditions, the degradation of chlorpyrifos was 33, 42, 56 and 81%, respectively, by culture *GCC134* (**Figure-4**). The value of Pearson correlation between culture volume and % degradation of chlorpyrifos by bacterial mixed-cultures (r = 0.986) was extremely significant (**Table-1**). The positive value of correlation coefficient showed that the degradation of chlorpyrifos in soil slurry medium by bacterial mixed-cultures enhanced by increasing the culture volume of respective bacterial mixed-cultures. Therefore, prior to bioremediation of chlorpyrifos contaminated soil and water, the optimization of culture volume of bacterial mixed-cultures could yield better results.

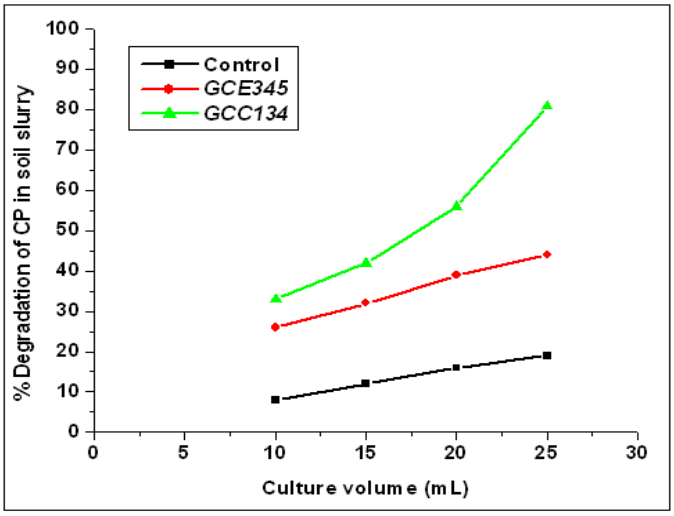

Figure-4
Chlorpyrifos degradation by bacterial mixed-cultures in response to culture volume

GC-MS analysis for toxic intermediates:
After degradation studies, the samples were analyzed by gas chromatographic – mass spectrometry (GC-MS) technique. The GC-MS analysis showed the presence of chlorpyrifos at R.T. of 9.558 minutes. The comparison with standard library of NIST-07 mass spectral database confirmed the matching of mass/charge ratio v/s relative intensity at R.T. 9.558 for the samples to standard spectra of chlorpyrifos. The mass spectra obtained showed that the chlorpyrifos was degraded to some small metabolites which could not be identified using the available library database. The presence of chlorpyrifos was observed at R.T. 9.558 minutes but no any intermediate was identified till R.T. 22 minutes. This indicated that chlorpyrifos is probably completely metabolized by the isolates into smaller intermediates.

CONCLUSION

Compared to bacterial mono-cultures, mixed-cultures could be relatively more effective in bioremediation of chlorpyrifos contaminated soil and water. The positive and extremely significant value of Pearson correlation coefficient between treatment duration or culture volume and % degradation of chlorpyrifos showed that proper optimization of treatment duration and culture volume of bacteria mono- and mixed-cultures, prior to bioremediation process could yield better results. Among the four mono-cultures, the isolate *RCC-2 (Pseudomonas sp.)* was most efficient degrader of chlorpyrifos. Out of two bacterial mixed-cultures, culture *GCC134* was more effective in terms of degradation of chlorpyrifos. The results suggested that the bacterial isolates were not forming any toxic intermediates during the degradation of chlorpyrifos and thus could be effectively utilized for the bioremediation process of chlorpyrifos contaminated soil and water.

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