Research in Biotechnology, 3(2): 08-13, 2012 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

Isolation, Identification, Resistance profile and Growth kinetics of Chlorpyrifos resistant Bacteria from Agricultural soil of Bangalore

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Although there are benefits to the use of pesticides, there are also drawbacks, such as potential toxicity to humans and other animals and change in the ecobalance due to residual effects. The soil microflora (under persistence pesticide stress) are able to detoxify/degrade these toxic compounds into nontoxic products. Chlorpyrifos (a trichloropyridinyl phosphothioate) is one of the most widely used pesticides that exert broad based toxic effects. The present study involves the isolation, identification and characterization of the chlorpyrifos resistant bacterial isolates from cabbage cultivated soil of a private agricultural farm in Bangalore, India. Out of 15 isolates 3 chlorpyrifos hyper resistant bacteria were finally selected for follow up studies. Three isolates viz., *Bacillus stearothermophilus*, *Bacillus circulans* and *Bacillus macerans* were found resistant to 50mg/L, 55mg/L and 60 mg/L of chlorpyrifos.

Key words: Chlorpyrifos, *Bacillus stearothermophillus, Bacillus circulans, Bacillus macerans.*

Chlorpyrifos [o,o-diethyl o-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is an organophosphorous insecticide applied to soil to control pests in agricultural fields (Racke *et al.,* 1994). Pesticides are necessarily poisonous but they play an important role in the availability of plenty of cheap and consistent supplies of food to the world population (Akhtar & Ahmed, 2002). The pesticides reach soil by one way or the other. Many are applied directly on the surface or injected into the upper layers of the soil (Akhtar & Solangi, 1990). However, from the aspect of environmental pollution, extensive use of pesticides and other agrochemicals not only limits plant growth but may also induce mutagenic and carcinogenic effects on non target microorganisms. Although pesticides may not be universally toxic to all species of microorganisms, they have the potential of disturbing microbial events/activities in the environment, polluted by these chemicals (Pimental, 1971). There are many pesticides and insecticides to which pests and insects are resistant. As a result they are not degraded in the environment by routine processes. These undegradable compounds however are degradable by bacterial activity (Roberts *et al.,* 1993). Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemicals (Michelic & Luthy, 1988). Rates of pesticide degradation in a soil are a function of multiple factors including population densities and activity of pesticides degrading microorganisms, pesticide bioavailability and soil parameters

Received : February 08, 2012; Accepted : March 15, 2012

such as pH, soil water content and temperature (Parkin & Daniel, 1994). The soil microorganisms are responsible for processes like conversion of organic matter, formation of humus, decomposition of phosphorous and other elements from complex parent compounds, fixation of nitrogen and transformation of nitrogen interlocked inorganic matter to soluble compounds for plant assimilation (Akhtar *et al.,* 1990).

Different bacteria degrade different pesticides, insecticides and herbicides. Chlorpyrifos is one of the most commonly and widely used commercial insecticides (Kuperberg *et al.,* 2000). Its microbial degradation results in higher concentration of 3,5,6-trichloro-2-pyridinol (TCP), a major metabolite of chlorpyrifos (Robertson *et al*., 1998). In alkaline soils, chlorpyrifos is hydrolysed readily to TCP which is further degraded by microbial activity. Chlorpyrifos hydrolysis was greatly accelerated under low moisture conditions, both in acidic and alkaline soils (Racke *et al.,* 1996).

Materials and Methods

Microorgnaisms: Fifteen bacterial strains were isolated from cabbage cultivated private agricultural farm, Bangalore, Karnataka, India.

Chlorpyrifos: Commercial-grade insecticide chlorpyrifos (20% E.C.) was procured from Rangaswamy and co, Bangalore and the same was used throughout the experiment.

Media: Nutrient agar, Nutrient broth, 1% Tryptone broth, Glucose phosphate broth, Simmons citrate agar, Urea broth, Nitrate broth, Starch agar media, Sugar fermentation broth.

Reagents: Gram‘s staining reagents, Endospore staining reagents, Distilled water. Kovac‘s reagent, Methyl red reagent, Barritt‘s reagent, alpha-Naphthalamine, Sulphanilamide, Hydrogen peroxide, Oxidase reagent, Gram‘s iodine

Miscellaneous: Inoculation loop, Bunsen burner, Cover slip, Vaseline, Cavity slide, Filter paper, Staining tray, Microscope

Isolation and characterization of chlorpyrifos degrading bacteria

Soil bacteria capable of degrading chlorpyrifos were isolated from R.K. farms, hosur road, Bangalore, Karnataka, India. Soil samples from cabbage grown agricultural farm land were collected, serially diluted and inoculated into Nutrient agar media in the petriplates and incubated for 24h at 37^0c (Aneja, 2003). Bacterial isolates were subjected to morphological, cultural and biochemical studies.

Selection of chlorpyrifos resistant bacteria: Isolated colonies were picked from nutrient agar plates and screened for their resistance to chlorpyrifos by replica plating (Lederberg *et al.,* 1952) on nutrient agar plates containing of different concentration of chlorpyrifos (50-80 mg/L).

Studies on growth kinetics of chlorpyrifos resistant isolates: Overnight cultures of chlorpyrifos resistant bacteria were inoculated in a flask containing 100mL of media. The flasks were incubated in a shaker incubator at 37°C (150rpm) and O.D. at 540 was recorded periodically. The experiment was also performed in the presence of 50 mg/L of chlorpyrifos.

Results and Discussion

Since Chlorpyrifos is one of the most commonly used commercial insecticide (Kuperberg *et al.,* 2000), it is therefore logical that the bacteria from chlorpyrifos contaminated cabbage field could be able to degrade this pesticide. The results indicate and include the gram positive rods to a greater extent (at 60%) followed by gram positive cocci (at 20%) and gram negative rods (at 10%). The results are represented in table 1. A set of biochemical tests have led to the identification of the genus of all the isolated bacteria. The bacterial colonies were identified and the results are represented in the table 2. The three isolates viz., *Bacillus stearothermophilus, Bacillus circulans* and *Bacillus macerans* were found resistant to 50mg/L, 55mg/L and 60mg/L of chlorpyrifos (table 3). Yun Long *et al.,* (1997) also reported the isolation and identification of bacteria from soil that were capable of degrading a number of pesticides.

Table 1: Isolation of bacteria - Colony characteristics

Sam. & D.F	Sl no.	No. of cols.	Size in mm	Margin	Elevation	Optical feature	Texture	Color	Form	Gram's rcn.	Endo spore	Motility
A 10^{-3}	1	20	2 (0.5)	circular	flat	opaque	Smooth	cream	circular	G+ve rods	+ terminal	+
	2	2	7 (0.2)	rhizoid	flat	opaque	Rough	cream	irregular	G+ve rods	+ central	+
A 10^{-4}	3	1	10 (0.1)	irregular	flat	opaque	Smooth	cream	irregular	G+ve rods	+ terminal	+
	4	2	3 (0.3)	lobate	flat	opaque	Smooth	cream	circular	G+ve rods	+ central	+
B 10^{-3}	5	7	2 (0.5)	wavy	flat	opaque	Smooth	cream	circular	G+ve rods	+ terminal	+
	6	30	1 (0.5)	circular	Raised	Transparent	Smooth	white	doted	G+ve cocci	-	-
	7	40	1 (0.5)	circular	Raised	Transparent	Smooth	cream	doted	G+ve cocci	-	-
B 10^{-4}	8	3	5 (0.5)	wavy	Raised	translucent	Smooth	cream	circular	G+ve rods	+ terminal	+
	9	1	8 (0.1)	lobate	Raised	translucent	Smooth	cream	spindle	G-ve rods	-	-
	10	3	1 (0.2)	rhizoid	Raised	translucent	Smooth	cream	doted	G+ve cocci	-	-

+ refers to presence; - refers to absence; A: Control B: Private farm; Figures in the parenthesis refer to standard deviation.

Table 2: Identification of the bacterial genera

Sam. & D.F	Sl no./ Test	I.P.T	M.R.T	V.P.T	C.U.T	C.T	O.T	N.T	G.F.T	A.T	U.T	Genus
A 10^{-3}	1	-	+	-	-	+	+	+	+	+	-	*Bacillus*
	2	-	+	-	-	+	+	+	+	+	-	*Bacillus*
A 10^{-4}	3	-	+	-	-	+	+	+	+	+	-	*Bacillus*
	4	-	+	-	-	+	+	+	+	+	-	*Bacillus*
B 10^{-3}	5	-	+	-	-	+	+	+	+	+	-	*Bacillus*
	6	-	+	-	-	-	-	+	-	+	-	*Staphylococcus*
	7	+	+	-	-	+	-	-	+	+	-	*Staphylococcus*
B 10^{-4}	8	-	+	-	-	-	+	+	+	+	-	*Bacillus*
	9	-	+	-	-	+	-	-	+	-	-	*Shigella*
	10	-	+	-	-	+	-	-	+	-	-	*Staphylococcus*

+ refers to presence; - refers to absence; A: Control B: Private farm;

Table 3: Identification of the bacterial species

Sl no.	8/1/2/5	3	4/4
Growth at 50^{0}c	+	+	-
Growth at 60^{0}c	+	-	-
Growth in 7% NaCl	-	+	-
Growth at pH <6	+	+	-
Identification	*Bacillus stearothermophilus*	*Bacillus circulans*	*Bacillus macerans*

+ refers to a positive test (Resistant); - refers to a negative test (Sensitive)

Table 4: The growth of bacteria at different concentrations of Insecticides

Sample	Concentrations of chlorpyrifos (mg/L)						
	50	55	60	65	70	75	80
Bacillus stearothermophillus	+++	+++	+++	++-	++-	-+-	-+-
Bacillus circulans	+++	+++	+++	+++	++-	-++	--+
Bacillus macerans	+++	+++	+++	+++	+++	++-	+-+

+ refers to presence of growth of the bacteria; - refers to absence of growth of the bacteria

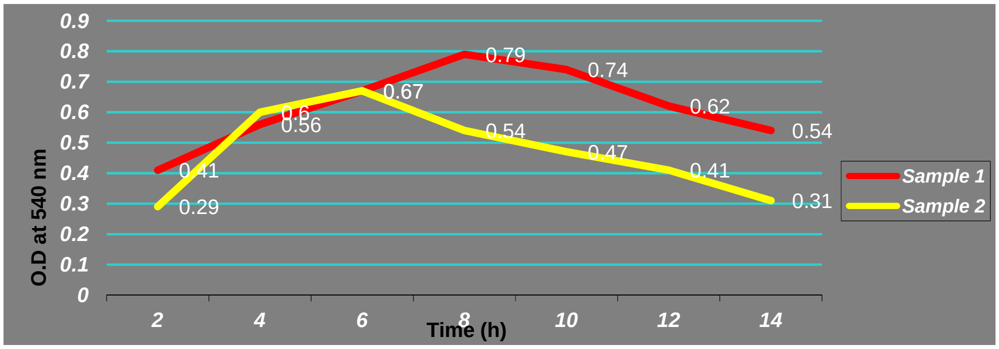

Figure 1: Growth curves of *Bacillus stearothermophilus* in the presence and absence of chlorpyrifos; Samle 1: *Bacillus stearothermophilus;* Sample 2: *Bacillus stearothermophilus* in the presence of chlorpyrifos

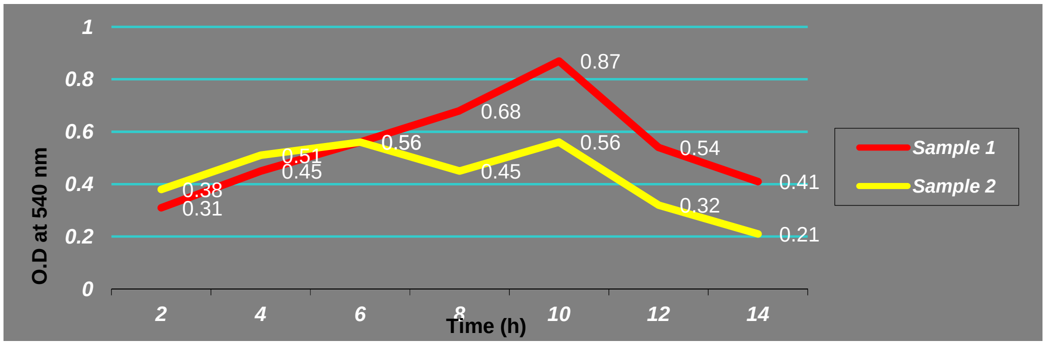

Figure 2: Growth curves of *Bacillus circulans* in the presence and absence of chlorpyrifos, Sample 1: *Bacillus circulans;* Sample 2: *Bacillus circulans* in the presence of chlorpyrifos

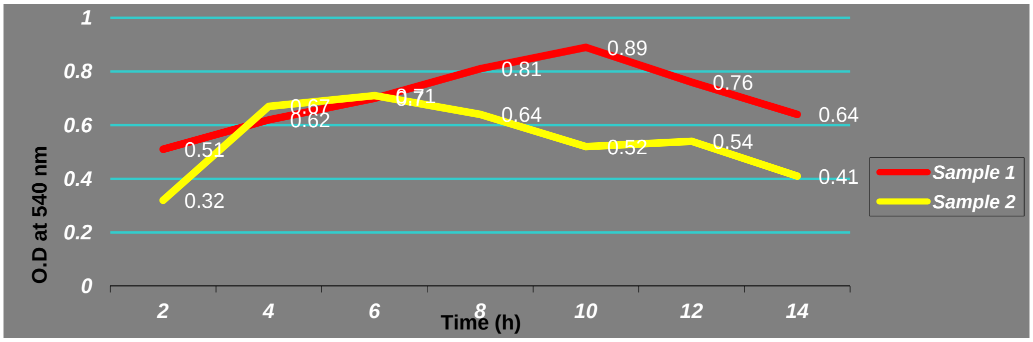

Figure 3: Growth curves of *Bacillus macerans* in the presence and absence of chlorpyrifos Sample 1: *Bacillus macerans;* Sample 2: *Bacillus macerans* in the presence of chlorpyrifos

The three chlorpyrifos resistant isolates were monitored for growth kinetics studies in plain nutrient broth and nutrient broth containing chlorpyrifos. The O.D values for the growth of *Bacillus stearothermophilus, Bacillus circulans* and *Bacillus macerans* in the presence and absence of chlorpyrifos are represented in figures 1, 2 and 3 respectively. The growth curve of all the three isolates shows a slight decline in the presence of chlorpyrifos. However, growth is not completely inhibited and the organisms are able to complete their life cycles. *Bacillus* species found in the mercury contaminated lake along with other bacteria has been reported to show high resistance to mercury (Kafilzadeh and Mirzaei**,** 2008). Since, mercury is one of the most commonly found components in many pesticides, the above report can be considered in favour of our findings. Further, reports of three bacterial isolates viz., *Klebsiella* sp., *Pseudomonas putida* and *Aeromonas* sp., from the Pakistan agricultural soil showing high resistance to chlorpyrifos are available (Ajaz et al., 2005).

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