Pak. J. Bot., 37(2): 381-388, 2005.

CHLORPYRIFOS RESISTANT BACTERIA FROM PAKISTANI SOILS: ISOLATION, IDENTIFICATION, RESISTANCE PROFILE AND GROWTH KINETICS

MUNAZZA AJAZ[*], NUSRAT JABEEN[**], SHAHIDA AKHTAR[***] AND SHEIKH AJAZ RASOOL[**]

[*]*Department of Microbiology,*
Federal Urdu University of Arts, Sciences & Technology,
Gulshan-e-Iqbal Campus, University Road, Karachi, Pakistan.
[**]*Department of Microbiology, University of Karachi, Karachi-75270, Pakistan*
[***]*Pesticide Research Institute SARC, Karachi, Pakistan.*

Abstract

Haphazard use of pesticides has been a potential source of ecological imbalance in addition to their genetic activity. The soil microflora (under persistence pesticide stress) are able to detoxify/degrade these toxic compounds into non toxic products. Chlorpyrifos (a trichloropyridinyl phosphothioate) is one of the most widely used pesticides that exert broad based toxic effects. The present study was initiated to isolate, identify and characterize the chlorpyrifos resistant bacteria from cotton cultivated soil of NIAB, Faisalabad, Pakistan (using conventional and API kit methods). Out of 20 isolates 3 chlorpyrifos hyper resistant bacteria were finally selected for follow up studies. The screening was performed by replica device. Three isolates viz., *Ps. putida*, *Aeromonas* sp., and *Klebsiella* sp., were found resistant to 2mg/mL, 4mg/mL and 8mg/mL of chlorpyrifos while *Ps. putida* and *Aeromonas* sp., also resisted the 10mg/mL and 20mg/mL doses. Growth kinetic studies revealed that under highly stressed conditions of chlorpyrifos, the generation time was extended compared to shorter generation time in plain liquid medium. Further studies (including analysis of the chlorpyrifos degraded products) are in progress.

Introduction

Chlorpyrifos [o,o-diethyl o-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is an organophosphorous insecticide applied to soil to control pests in agricultural field (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 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).

The most important factors affecting the activity of the insecticides are: the mechanism of action of the active ingredients; the defense mechanisms of the treated insects; the type of formulation; systemic properties of active ingredients; the mode of application; the type, moisture content and temperature of soils (Malinowski, 2000). It is interesting to isolate and study the biodegradable potential (at molecular level) of the super tolerant bugs that transform the toxins into environment friendly compounds.

Materials and Methods

Bacterial strains: Twenty strains were isolated from cotton cultivated soil of NIAB, Faisalabad, Pakistan.

Media and growth conditions: Nutrient broth (BioM Laboratories, USA) was used for the screening of cultures, chlorpyrifos resistance and for growth kinetic studies. Simmon citrate agar (Difco Laboratories, USA), peptone tryptone broth, Clark's medium and peptone nitrate broth were used for the identification. Chlorpyrifos (Pakistan Agro Chemical Pvt.), Difco agar (2%) was added in order to solidify the medium. All the cultures were maintained in vials by growing them in 3mL of nutrient broth and after 24 h incubation overlaid with 3mL of 40% glycerol. Vials were stored at -70°C.

Isolation and identification of bacteria from soil sample: The soil sample was collected from cotton cultivated field. Dilution (1:10) of 1gm of soil was made in sterile distilled water. Then 10 uL from each dilution was spread on nutrient agar medium and incubated at 37ºC for over night. Following the overnight growth, the isolated colonies were gram-stained and different biochemical tests were performed for further identification.

For the identification of bacterial isolates, the characters such as morpho-colonial bases, utilization of various carbohydrates and synthesis of enzymes like oxidase and catalase were included (Cappuccino & Sherman, 1999; Holt *et al.,* 1994). API 20 E and API 20 NE kits were used for further confirmation.

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 upto 20mg/mL chlorpyrifos.

Studies on growth kinetics of chlorpyrifos resistant isolates: Overnight cultures of chlorpyrifos resistant bacteria were inoculated in a flask containing 100mL medium to obtain 0.1 O.D. at 530nm. The flasks were incubated in a shakobater (Stewart sc, UK) at 37°C (150rpm) and O.D. 530 was recorded periodically until the growth reached the stationary phase (Madigan *et al.*, 1997).

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 cotton field could be able to degrade this pesticide. Out of a total of 20 isolates, 4 were found gram +ve cocci and 3 gram +ve bacilli while 13 were gram –ve bacilli. All the isolates were screened for their ability to resist chlorpyrifos at different concentrations ranging from 2 to 20mg/mL. The resistant isolates were identified on the bases of morpho-cultural and biochemical considerations (Table 1 and 2). Three isolates viz., *Klebsiella* sp., *Pseudomonas putida* and *Aeromonas* sp., offered resistance upto 2mg/mL, 4mg/mL and 8mg/mL of chlorpyrifos while *Pseudomonas putida* and *Aeromonas* sp., also resisted higher concentrations i.e., 10mg/mL and 20mg/mL (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. Morphological and cultural characteristics of isolated strains of bacteria.

S. No.	Isolated organism	Gram-reaction	Cultural characteristics on nutrient agar
1.	*Klebsiella* sp.	Gram-negative rods	Transparent, round, smooth and pinpointed colonies.
2.	*Pseudomonas putida*	Gram- negative rods	Transparent, round and smooth colonies.
3.	*Aeromonas* sp.	Gram-negative rods	Transparent, round, smooth and pinpointed colonies.

Table 3. Effect of chlorpyrifos (added in nutrient agar) on the growth of the resistant isolates.

Isolates	Growth on nutrient agar containing chlorpyrifos (mg/ml)				
	2	4	8	10	20
Klebsiella sp.	+	+	+	-	-
Pseudomonas putida	+	+	+	+	+
Aeromonas sp.	+	+	+	+	+

Key: + = Resistant; - = Sensitive

Table 4. Generation time in minutes of the chlorpyrifos resistant isolates under different conditions.

Isolates	Nutrient broth	Nutrient broth + chlorpyrifos	
		1mg/ml	2mg/ml
Klebsiella sp.	60	184	261
Pseudomonas putida	55	172	247
Aeromonas sp.	68	179	249

Table 2. Biochemical characteristics of the isolates.

Isolated organism	Catalase (production)	Oxidase	Gelatin liquification	Nitrate reduction	Esculin hydrolysis	Urea hydrolysis	Arabinose (fermentation)	Manintol
Klebsiella sp.	+	-	+	+	-	+	+	+
Pseudomonas putida	+	+	-	-	-	-	-	-
Aeromonas sp.	+	+	-	+	+	-	+	+

Key: + = Test is positive; - = Test is negative

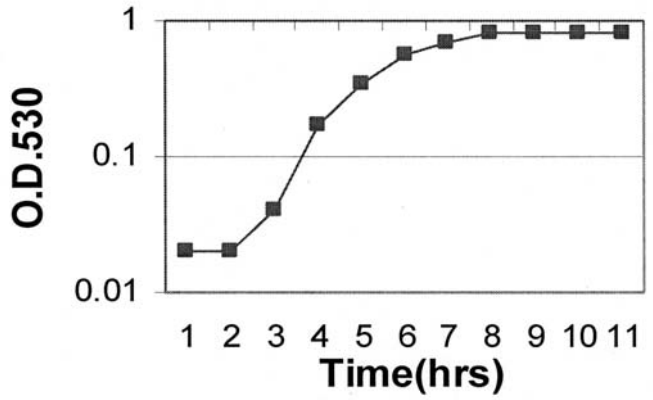

Fig. 1(a). Growth pattern of chlorpyrifos resistant isolate *Klebsiella* sp., in plain nutrient broth.

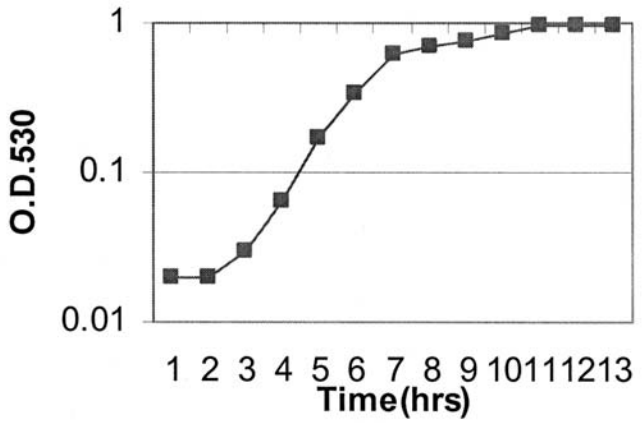

Fig. 1(b). Growth pattern of chlorpyrifos resistant isolate *Pseudomonas putida* in plain nutrient broth.

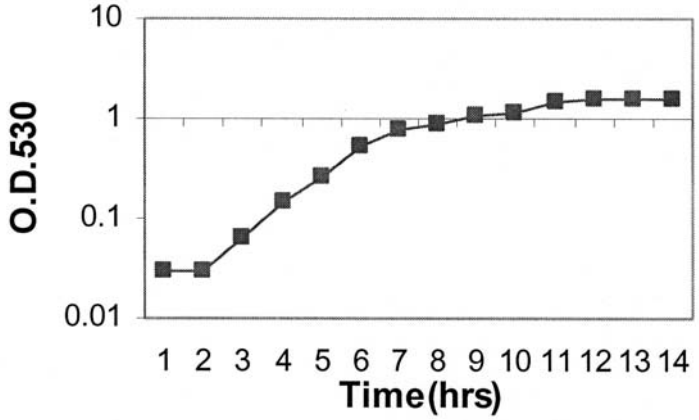

Fig. 1(c). Growth pattern of chlorpyrifos resistant isolate *Aeromonas* sp., in plain nutrient broth.

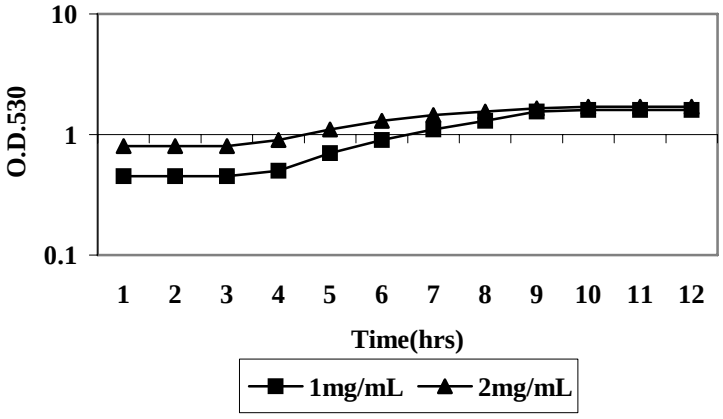

Fig. 2(a). Growth pattern of *Klebsiella* sp., in nutrient broth containing different conc., of chlorpyrifos.

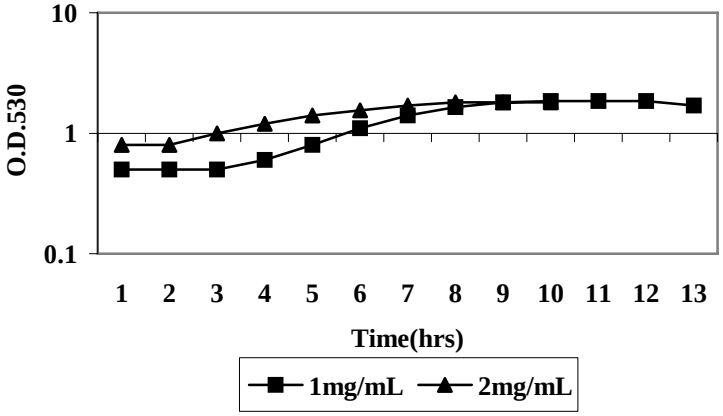

Fig. 2(b). Growth pattern of *Pseudomonas putida* in nutrient broth containing different conc., of chlorpyrifos.

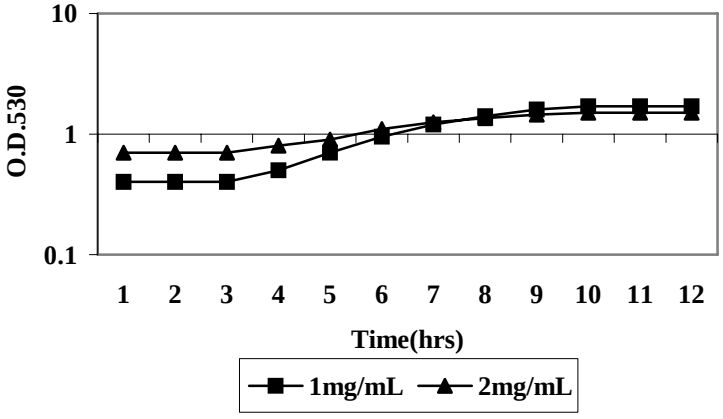

Fig. 2(c). Growth pattern of *Aeromonas* sp., in nutrient broth containing different conc., of chlorpyrifos.

The three chlorpyrifos resistant isolates were monitored for growth kinetics studies in plain nutrient broth and nutrient broth containing two different concentrations (1mg/mL and 2mg/mL) of chlorpyrifos. Fig. 1 a, b and c show the growth kinetics of *Klebsiella* sp., *Pseudomonas putida* and *Aeromonas* sp., in plain nutrient broth, whereas Fig. 2 a, b and c show the kinetics of the resistant isolates under chlorpyrifos stress condition (nutrient broth incorporated with chlorpyrifos). Generation time of all the resistant isolates under different conditions was also calculated (Table 4). In plain nutrient broth the generation time of resistant isolates ranged between 55 to 68 minutes, with an average of 61 min. Nutrient broth is a stress free complete medium containing most of the preformed growth requirements which are directly available to the cells (Madigan *et al.,* 1997). The generation time of all the resistant isolates in nutrient broth containing (1mg/mL and 2mg/mL) chlorpyrifos showed an extended trend because of the chlorpyrifos stress. These observations with reference to generation time under stress of chlorpyrifos in nutrient medium are in agreement with the extended generation time of soil bacteria grown in lab conditions with phenol stress (Ajaz *et al.,* 2004).

References

Ajaz, M., N. Noor, S.A. Rasool and S.A. Khan. 2004. Phenol resistant bacteria from soil: identification-characterization and genetical studies. *Pak. J. Bot.*, 36(2): 415-424.

Akhtar, S. and A. Ahmed. 2002. Pesticides, human health and ecosystem. *J. Baqai. Med. Univ.*, 5(2): 16-19.

Akhtar, S., A.H. Solangi and M.M.H. Baig. 1990. Ill-effect of insecticides on microbial population of cotton field soil. *Pak. J. Agric. Res.*, 11(3): 216-220.

Akhtar, S. and A.H. Solangi. 1990. Effects of pesticides on non-target soil microorganisms- a review. *Progressive Farming*, 10(4): 25-29.

Cappuccino, J.G. and N. Sherman. 1999. *Microbiology Laboratory Manual*, (4th ed.), Addison-Wilsey, California. 1-477 pp.

Holt, G.H., N.R. Krieg, P.N.A. Sneath, J.T. Staley and S.T Williams. 1994. Gram positive cocci. In: *Bergey's Manual of Determinative Bacteriology.* 9th edition. William and Wilkins Baltimore Maryland, U.S.A. pp. 527-533.

Kuperberg, J.M., K.F.A. Soliman, F.K.R. Stino and M.G. Kolta. 2000. Effects of time of day on chlorpyrifos induced alterations in body temperature. *Life Sci.*, 67(16): 2001-2009.

Lederberg, J., L. Cavali and E.M. Lederberg. 1952. Sex compatibility in *E. coli*. *Genetics*, 37: 720-730.

Madigan, M.T., J.M. Martinko and J. Parker. 1997. *Brock Biology Microrganisms*, 8th ed., Prentice Hall International Inc., New Jersey. 1-986 pp.

Malinowski, H. 2000. Impact of more important factors on the activity of insecticides used in forest protection. I. Soil Insecticides. *Sylwan.*, 144(9): 89-100.

Michelic, J.R. and R.G. Luthy. 1988. Degradation of poly aromatic hydrocarbon compounds under various redox conditions in soil water systems. *Appl. Environ Microbiol.*, 54:1182-1187.

Parkin, T.B. and R.S. Daniel. 1994. Modeling environmental effects on enhanced carbofuran degradation. *Pestc. Sci.,* 40: 163-168.

Pimental, D. 1971. Ecological effect of pesticides on non-target species. Executive office of science and technology, Supt. of Documents. U.S.Govt. Printing Office, Stock No. 4106-0029, Washington, D.C.

Racke, K.D., D.D. Fontaine, R.N. Yoder and J.R. Miller. 1994. Chlorpyrifos degradation in soil at termicidal application rates. *Pestc. Sci.,* 42: 43-51.

Racke, K.D., K.P. Steele, R.N. Yoder, W.A. Dick and E. Avidov. 1996. Factors affecting the hydrolytic degradation of chlorpyrifos in soil. *J. Agri. & Food Chem.*, 44(6): 1582-1592.

Roberts, S.J., A. Walker, N.R. Parekh and S.J. Welch. 1993. Studies on a mixed bacterial culture from soil which degrades the herbicide Linuron. *Pestc. Sci.*, 39: 71-78.

Robertson, L.N., K.J. Chandler, B.D.A. Stickley, R.F. Cocco and M.A. Hmetagic. 1998. Enhanced microbial degradation implicated in rapid loss of chlorpyrifos from controlled release formulation suSCon Blue in soil. *Crop Protection*, 17(1): 29-33.

Yun Long, Y., S. FengMing, Z. Zhong, C. HeXing and F. DeFang. 1997. Isolation and identification of a broad-spectrum bacterial strain (*Alcaligenes* sp.) degrading pesticides. *J. Zhe. Agri. U*ni., 23 (2): 111-115.

(Received for publication 3 December 2004)