Indian J. Agric. Res., **47** (5) : 381 - 391, 2013

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com

ISOLATION AND CHARACTERIZATION OF CHLORPYRIPHOS DEGRADING BACTERIA

Niti Chawla, Sunita Suneja and Kamlesh Kukreja*

Department of Microbiology,
CCS Haryana Agricultural University, Hisar – 125 004, India

Received: 07-11-2012 Accepted: 06-06-2013

ABSTRACT

Twenty six bacterial isolates were obtained from chlorpyriphos contaminated soil samples by enrichment culture technique. Out of these, four isolates showed growth up to 30,000 ppm and four up to 40,000 ppm chlorpyriphos amended in Mineral salt medium (MSM) containing glucose (0.2%). Out of these eight isolates, four produced yellow coloured colonies on MSM agar plates containing chlorpyriphos (50 ppm) and bromo thymol blue (BTB) indicator and also showed the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) in MSM that confirmed their chlorpyriphos degrading capability. These four isolates were tested for different carbon and nitrogen source utilization pattern on MSM agar plates (containing 100 ppm chlorpyriphos). With all the isolates, good growth was observed in presence of five carbon and five nitrogen sources. Therefore, these carbon and nitrogen sources were used for chlorpyriphos utilization in liquid medium. Bacterial count and protein content was found to be more in the medium amended with 100 ppm chlorpyriphos containing glucose as carbon source and ammonium chloride as nitrogen source as compared to medium containing other carbon and nitrogen sources with all the four isolates at 7 days of growth. Maximum utilization of chlorpyriphos was observed with the isolate SB1 (80.1 %) followed by HIC2 (76.2 %), SGB2 (65.2%) and HIIGA2 (58.1%) in liquid medium. Two isolates (SB1 and HIC2) which showed more utilization of chlorpyriphos in liquid medium as compared to others, were characterized using morphological, biochemical and physiological tests. Isolate SB1 was identified as *Pseudomonas* and HIC2 as *Xanthomonas*.

Key words: Biodegradation, Chlorpyriphos, Enrichment culture, *Pseudomonas*, *Xanthomonas*.

INTRODUCTION

Importance of organophosphorus pesticides is increasing in pests control because of their rapid decomposition and less likely accumulation in environment. They are still of great concern however, because of their high solubility in water and excessive usage. The presence of these pesticides in soil and water can directly affect the health of aquatic and terrestrial organisms and may present a threat to humans through contamination. These are acutely toxic and act by inhibiting acetylcholinesterase, an important enzyme in the nervous system (Kanekar *et al.*, 2004). On exposure to organophosphorus pesticides, the enzyme is unable to function causing accumulation of acetylcholine which interferes with the transmission of nerve impulses at the nerve endings.

Chlorpyriphos is the one out of about 100 organophosphorus pesticides in the market today. It is a broad spectrum commercial pesticide used for insect control since 1960. The target insects of chlorpyriphos are white flies, plant louses, termites, cockroaches, ants and mosquitoes (Fang *et al.*, 2006). It is moderately persistent in soils and its half-life is usually between 60 and 120 days but can range from 2 weeks to 1 year depending on the soil type, climate and other conditions. Considerable residues of chlorpyriphos have been reported in apples, tomatoes, cotton seed and oil of oilseed crops like ground nut, safflower and mustard (Aysal *et al.*, 1999; Gupta *et al.*, 2001; Blossom *et al.*, 2004; Aleagha *et al.*, 2011). Microbial degradation has been considered to be the most important degradation method because it is the main factor impacting degradation of chlorpyriphos residues

*Corresponding author's: Email: kkukreja@hau.ernet.in

(Racke *et al.*, 1996). It has been reported to be resistant to enhanced degradation due to the accumulation of its antimicrobial degradation products in the soil. Its microbial degradation results in formation of two products namely chlorpyriphos oxon and 3,5,6,trichloro-2-pyridinol (TCP) (Robertson *et al.*, 1998). It has large effect on public health and environment resulting from its long persistence period in soil and water (Mohan *et al.*, 2004). Therefore, its contamination and degradation have been paid close attention (Racke *et al.*, 1988; Yang *et al.*, 2005). It has also been shown to be degraded co-metabolically in liquid media by bacteria (Mallick *et al.*, 1999; Horne *et al.*, 2002). Several bacteria capable of co-metabolizing chlorpyriphos as sole source of carbon and energy (Qian *et al.*, 2007; Anwar *et al.*, 2009) are known. The present study describes isolation and characterization of chlorpyriphos degrading bacteria from chlorpyriphos contaminated soils.

MATERIALS AND METHODS

Enrichment and isolation of chlorpyriphos degrading bacteria: Soil samples contaminated with chlorpyriphos were collected from the cotton and sugarcane fields of Haryana Agricultural University Farm Hisar and cotton field of shahpur village, District Hisar. These soil samples were partially air dried overnight and then sieved through 2mm mesh sieve and kept in refrigerator until further use.

Chlorpyriphos (20EC, Dursban), Dow Agrosciences India Pvt. Ltd. was purchased from the local pesticide supplier. Above soil samples were treated with 0.8 ml of chlorpyriphos three times at intervals of 10 days to achieve the final concentration of 0.025 ml g^{-1} of dry soil to maximize the degrading capability of the soil. These soils were then used for several rounds of enrichment in mineral salt medium (MSM) containing chlorpyriphos (0.025 ml g^{-1} soil) in the absence or presence of glucose (0.2%). From the final enriched samples, twenty six bacterial isolates were obtained by serial dilution technique on three types of media; Mineral salt medium (MSM) containing 0.2% glucose, Nutrient agar medium (NA) and Mineral salt medium supplemented with nitrogen (MSMN) containing 0.2% glucose and chlorpyriphos and purified by streaking on fresh medium plates. After purification, isolates were transferred to Nutrient agar slants and after growth; slants were stored in refrigerator until further use.

Screening of bacterial isolates for chlorpyriphos utilization: All the isolates were screened for chlorpyriphos utilization on MSM agar plates in the presence (0.2%) or absence of glucose. Actively growing cultures were spotted on chlorpyriphos amended MSM agar plates with (0.2%) and without glucose. Plates were incubated at 29± 1^0C in a BOD incubator for 3-4 days. Eight isolates able to grow in the presence of highest concentration (30,000 and 40,000 ppm) of chlorpyriphos were referred as CPY^+. Actively growing CPY^+ bacterial isolates were spotted on chlorpyriphos amended MSM agar plates containing BTB indicator (prepared by adding 0.5%BTB in 0.2N NaOH) and incubated for 3 days at 29± 1^0C. The colour change produced by the bacterial isolates on the medium was observed. Out of eight, four bacterial isolates which showed chlorpyriphos utilization ability on MSM agar plates were checked for chlorpyriphos utilization in MSM containing chlorpyriphos (100 ppm) as described by Yu and Yu (2000). Bacterial isolates were inoculated in MSM containing chlorpyriphos (100 ppm) and incubated at 30^0C in a shaking incubator for 5-6 days. Then 1ml of freshly prepared 2,3,5-triphenyl tetrazolium chloride (TTC, 0.02%) was added to each flask and flasks were incubated at 29± 1^0C in a BOD incubator. After 4 hr of incubation, flasks were observed visually for the appearance of red colour in the broth (due to the formation of formazone). Appearance of red colouration is the indicative of positive test.

Determination of carbon and nitrogen source utilization pattern: Selected isolates which were able to utilize chlorpyriphos in liquid medium in the presence of 0.2% glucose were further tested for chlorpyriphos utilization in presence of different carbon and nitrogen sources on MSM agar plates. For confirmation of chlorpyriphos utilization in the presence of these carbon and nitrogen sources in liquid medium, MSM supplemented with either of these carbon and nitrogen source and chlorpyriphos was prepared. For inoculum preparation, actively growing cultures were transferred to Nutrient broth and kept on a rotary shaker at 29± 1^0C for 18 hr. Finally flasks were inoculated with 3% fresh inoculum of 1.0 O.D and incubated at 29± 1^0C for 7 days. Viable count in culture broth was determined by dilution plating on Nutrient agar plates and protein

content was determined by Lowry's method (Lowry *et al*., 1951).

Chlorpyriphos extraction from liquid medium: Chlorpyriphos was extracted from the liquid medium by the method of Jain *et al*., 1974. For this, one ml of the sample broth was transferred to eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was separated out and extracted with 2 ml of chloroform. The aqueous phase was removed and the solvent phase containing chlorpyriphos was transferred to a test tube and evaporated to dryness followed by addition of 0.2 ml each of 2 percent 4- (p-nitrobenzyl) pyridine solution and 2 percent cyclohexylamine solution prepared in acetone. The lower part of the tube was immersed in a oil bath maintained at 175-180°C for 3 minutes and cooled for a few seconds in a cold water bath. The absorbance was read at 520 nm after diluting to 3 ml with ethyl acetate within 10 minutes. The chlorpyriphos concentration was estimated by referring to the standard curve. Standard curve was prepared by using gradient concentrations of chlorpyriphos (0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ppm) and developing colour after adding the reagents and measuring the absorbance at 520 nm.

Analysis of chloride released from chlorpyriphos Chloride release by bacterial isolates in MSM containing chlorpyriphos (100 ppm) was analysed at 7 days of growth following the Argentometric method (Greenberg *et al*., 1992). This is indirect method of measuring chlorpyriphos degradation.

Identification of bacterial isolates: Bacterial isolates were grown at 29± 1^{0}C for 24 hr on Nutrient agar medium slants/plates. The bacterial cultures were examined for various morphological, biochemical and physiological characteristics as per procedure described in Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994). The inter-relationship of the microorganisms in each section of Bergey's Manual is based on characteristics such as morphology, staining reactions, nutrition, cultural characteristics and biochemical test results for specific metabolic end products.

RESULTS AND DISCUSSION

Isolation of bacteria from enriched soil samples Twenty six bacterial isolates were retrieved from chlorpyriphos contaminated soil samples by enrichment culture technique (Table 1). Our results were in accordance with Zhu *et al*. (2010) who isolated a strain ZHU-1 capable of utilizing chlorpyriphos as the sole carbon and energy source from chlorpyriphos contaminated soil by enrichment culture technique and Awad *et al*. (2011) who isolated five bacterial isolates capable of degrading chlorpyriphos from pesticides contaminated soil in Egypt using enrichment culture technique. Recently, Kavikarunaya and Reetha (2012) isolated bacterial isolates capable of degrading chlorpyriphos from the soil sample collected from the paddy field of Annamalai Nagar which was having a history of repeated pesticide applications.

TABLE 1: Bacterial isolates retrieved from chlorpyriphos (CPY) contaminated soil samples on different media.

Place of soil sample collection	Mineral salt medium(MSM)*	Nutrient agar(NA) medium	Mineral salt medium supplemented with nitrogen(MSMN)*
Shahpur	2	3	1
Shahpur**	1	2	2
HAU –I	2	-	2
HAU –I**	2	1	-
HAU –II	1	1	2
HAU –II**	2	-	2
Total	10	7	9

Total No. of isolates: 26; *: 0.2 % glucose amended in plating medium; **: 0.2 % glucose amended in enrichment medium; Shahpur: Soil collected from rhizosphere of cotton field; HAU-I: Soil collected from rhizosphere of cotton field; HAU-II: Soil collected from rhizosphere of sugarcane field

Screening of bacterial isolates for utilization of chlorpyriphos on MSM agar plates: In the further step, it was interesting to know the ability of the bacterial isolates retrieved from chlorpyriphos enriched soils to tolerate and grow on higher concentration of chlorpyriphos. Such ability has been recently studied by Bhagobaty and Malik (2008). They observed that cultures could tolerate upto 3200 ppm of chlorpyriphos amended in Minimal salt medium plates. In our study, out of 26 bacterial isolates, four isolates (SB3, SC, HIC2, HIIC1) showed growth upto 30,000 ppm and four isolates (SGB2, SB1, HIIGA1, HIIGA2) upto 40,000 ppm of chlorpyriphos amended in MSM agar plates containing 0.2% glucose (Table 2). Out of these eight isolates, four isolates (SB1, HIC2, SGB2 and HIIGA2) produced yellow coloured colonies (Fig 1)

TABLE 2: Screening of bacterial isolates for chlorpyriphos utilization on MSM agar plates with and without glucose.

Bacterial isolates	-G + CPY (1-50ppm)	G (0.2%)	G (0.2%) + different concentrations of chlorpyriphos (ppm)						
			100-1000	3000	5000	10,000	20,000	30,000	40,000
SA1	-	+	+ +	+ +	+ +	+ +	-	-	-
SA2	-	+	+ +	+ +	+	+	-	-	-
SB1	-	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +
SB2	-	+	+ +	+ +	+ +	+	-	-	-
SB3	-	+	+ +	+ +	+ +	+ +	+ +	+ +	-
SC	-	+	+ +	+ +	+ +	+ +	+ +	+ +	-
SGA	-	+	+ +	+ +	+ +	+	-	-	-
SGB1	-	+	+ +	+ +	-	-	-	-	-
SGB2	-	+	+ +	+ +	+	+	+	+	-
SGC1	-	+	+ +	+ +	+	+	-	-	-
SGC2	-	+	+ +	+ +	+	-	-	-	-
HIA1	-	+	+ +	+ +	-	-	-	-	-
HIA2	-	+	+ +	+ +	-	-	-	-	-
HIC1	-	+	+ +	+ +	+	-	-	-	-
HIC2	-	+	+ +	+ +	+ +	+ +	+ +	+	-
HIGA1	-	+	+ +	+	+	+	+	-	-
HIGA2	-	+	+ +	+	-	-	-	-	-
HIGB	-	+	+ +	+ +	+	-	-	-	-
HIIA	-	+	+ +	+ +	-	-	-	-	-
HIIB	-	+	+	+	+	+	+	-	-
HIIC1	-	+	+ +	+ +	+	+	+	+	+
HIIC2	-	+	+ +	+	+	-	-	-	-
HIIGA1	-	+	+ +	+ +	+ +	+	+	+	+
HIIGA2	-	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +
HIIGC1	-	+	+ +	+ +	+	-	-	-	-
HIIGC2	-	+	+ +	+	-	-	-	-	-

- No growth; + medium growth; + + heavy growth

Isolates details:

SA: Isolated from Shahpur cotton field soil and plated on MSM (+ o.2%glucose)
SB: Isolated from Shahpur cotton field soil and plated on NA
SC: Isolated from Shahpur cotton field soil and plated on MSMN (+ o.2%glucose)
SGA: Isolated from shahpur cotton field soil amended with 0.2% glucose and plated on MSM (+ o.2%glucose)
SGB: Isolated from shahpur cotton field soil amended with 0.2% glucose and plated on NA
SGC: Isolated from shahpur cotton field soil amended with 0.2% glucose and plated on MSMN (+ o.2%glucose)
HIA: Isolated from HAU cotton field soil and plated on MSM (+ o.2%glucose)
HIC: Isolated from HAU cotton field soil and plated on MSMN (+ o.2%glucose)
HIGA: Isolated from HAU cotton field soil amended with 0.2%glucose and plated on MSM (+ o.2%glucose)
HIGB: Isolated from HAU cotton field soil amended with 0.2%glucose and plated on NA
HIIA: Isolated from HAU sugarcane field soil and plated on MSM (+ o.2%glucose)
HIIB: Isolated from HAU sugarcane field soil and plated on NA
HIIC: Isolated from HAU sugarcane field soil and plated on MSMN (+ o.2%glucose)
HIIGA: Isolated from HAU sugarcane field soil amended with 0.2% glucose and plated on MSM (+ o.2%glucose)
HIIGC: Isolated from HAU sugarcane field soil amended with 0.2% glucose and plated on MSMN (+ o.2%glucose)

on MSM agar plates containing chlorpyriphos (50 ppm) and BTB (5ml/l). Cells utilize chlorpyriphos and chloride produced by them decreases the pH which is indicated by the change in colour of the indicator dye from green to yellow. Such indicator media have been used for the rapid screening of bacteria capable of degrading diethyl formamide (Veeranagowda *et al.*, 2002), chlorpyriphos (Anuja, 2005) and pentachlorophenol (Nandish, 2005).

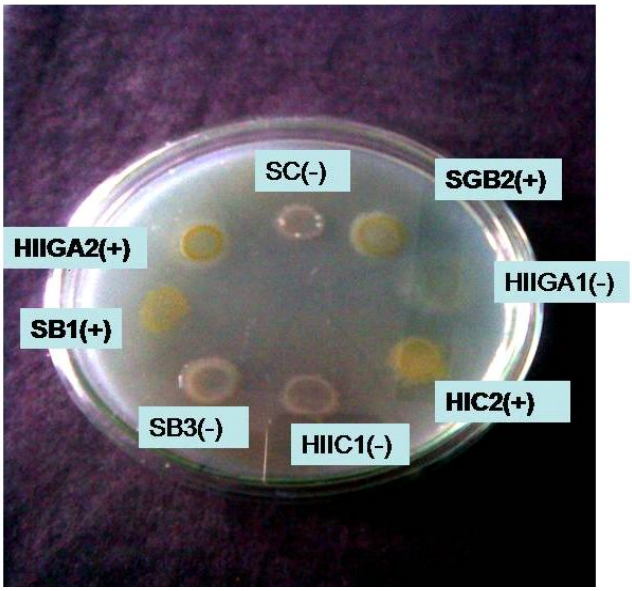

FIG 1:Growth of different bacterial isolates on MSM agar plates containing chlorpyriphos (50 ppm) and BTB.
+ Yellow coloured colony; - White coloured colony

Screening of bacterial isolates for utilization of chlorpyriphos in liquid medium: Bacterial isolates which showed chlorpyriphos degrading ability on MSM agar plates were checked for chlorpyriphos utilization in MSM containing chlorpyriphos (100 ppm) and 2,3,5-triphenyl tetrazolium chloride(TTC, 0.02%) as described by Yu and Yu (2000) and later on used by Bhagobaty and Malik (2008). All the four selected isolates showed the reduction of TTC in the presence of chlorpyriphos which was observed visually with the appearance of red colour (Fig 2) in the broth. Actually, TTC can be used as colorimetric indicator of biochemical oxidation of organic substrates. Bacterial oxidation of a substrate generates reduced NAD; if the e- is donated to an e-transported system such as TTC which acts as artificial e- acceptor and reduces to highly coloured product formazone which can be observed visually (Yu and Yu., 2000). These tests confirmed the biodegradation ability of these four bacterial isolates.

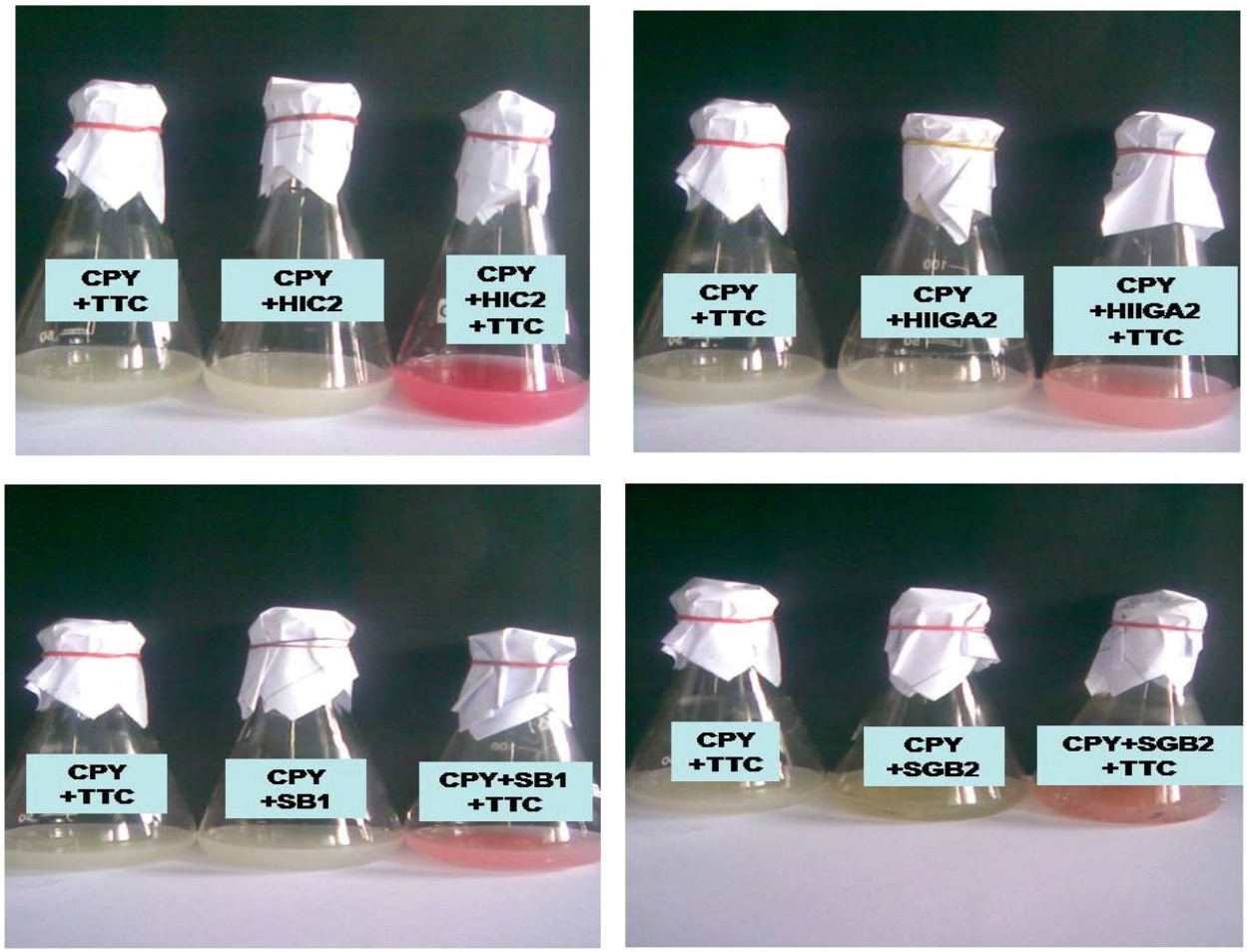

FIG 2: Reduction of 2,3,5-triphenyl tetrazolium chloride(TTC) by the bacterial isolates

Carbon and nitrogen source utilization pattern on MSM agar plates: Selected bacterial isolates were tested for chlorpyriphos utilization in the presence of different carbon and nitrogen sources on MSM agar plates. Out of 8 carbon sources tested, good growth was observed with 5 carbon sources (glucose, mannitol, sucrose, mannose and arabinose) in all the four isolates (Table 3). While out of 8 nitrogen sources tested, good growth was observed with five nitrogen sources such as urea, ammonium chloride, glycine, tryptophan and sodium nitrate (Table 4). Our results are in accordance with Bhagobaty and Malik (2008) who studied the chlorpyriphos utilization by the four bacterial isolates in the presence of different carbon (glucose, mannitol, sucrose, mannose, fructose, raffinose adonitol, dulcitol, adonitol, maltose and arabinose) and nitrogen (arginine, glycine, histidine, threonine, methionine and tyrosine) sources on MSM agar plates containing 100 ppm chlorpyriphos and supplemented with 1g/l of either of carbon and nitrogen source. All the isolates showed similar properties in utilizing glucose and sucrose. Different isolates showed different utilization pattern of different nitrogen sources. Recently, Kavikarunaya and Reetha (2012) studied the growth of the three chlorpyrifos degrading isolates *viz.*, *Pseudomonas fluorescens, Bacillus subtilis* and *Klebsiella sp.* in minimal salt broth containing 50 ppm of chlorpyrifos and different carbon (dextrose, fructose, lactose, galactose and mannose) and nitrogen (malt extract, peptone, yeast extract, casein and beef extract) sources. The growth of bacterial isolates was maximum in the presence of dextrose followed by fructose, galactose and mannose and malt extract followed by peptone, yeast extract and casein.

TABLE 3: Chlorpyriphos utilization by bacterial isolates on MSM agar plates with different carbon sources.

Carbon source(0.2%)	Bacterial isolates			
	SB1	SGB2	HIC2	HIIGA2
Glucose	+ + +	+ +	+ +	+ + +
Mannitol	+ +	+ + +	+ +	+ + +
Fructose	+	+	+	+
Lactose	±	±	±	±
Sucrose	+ +	+ +	+	+ +
Mannose	+	+ +	+ +	+
Arabinose	+ +	+ +	+	+ +
Maltose	+ +	+ +	+	±

+ small growth ; + + medium growth ; + + + heavy growth; ± negligible growth

TABLE 4: Chlorpyriphos utilization by bacterial isolates on MSM (0.2%glucose) agar plates amended with different nitrogen sources.

Nitrogen source(0.07M)	Bacterial isolates			
	SB1	SGB2	HIC2	HIIGA2
Glycine	+ +	+ +	+ +	+ +
Tryptophan	+	+	+	+
Tyrosine	+ +	+	-	-
Potassium nitrate	+	±	±	-
Sodium nitrate	+ +	+ +	+	+ +
Ammonium sulphate	-	-	-	-
Ammonium chloride	+ + +	+ +	+ +	+ +
Urea	+ +	+	+	+

+ small growth ; + + medium growth ; + + + heavy growth; ± negligible growth

Carbon and nitrogen source utilization pattern in liquid medium: For confirmation of chlorpyriphos utilization in the presence of five selected carbon sources in liquid medium, isolates were grown in MSM supplemented with either of the carbon source (0.2 %) along with 100 ppm chlorpyriphos(Table 5). Bacterial count and protein content was found to be more in medium amended with chlorpyriphos and carbon source as compared to the medium containing only carbon source and highest bacterial count i.e. 1760.0 x 10^7cfu ml^{-1} and protein content 364.4 µg ml^{-1} were observed in medium amended with chlorpyriphos along with 0.2 % glucose with the isolate SB1. Similar results were observed with other bacterial isolates i.e. bacterial count and protein content were found to be more in medium amended with chlorpyriphos and carbon source and highest bacterial counts i.e. 1696.0 x 10^7 , 234.0 x 10^7 and 50.0 x 10^7 cfu ml^{-1} and protein contents i.e. 468.5, 312.2 and 214.4 µg ml^{-1} were observed in medium amended with chlorpyriphos along with 0.2 % glucose with bacterial isolates HIIGA 2, SGB 2 and HIC 2 as compared to medium amended with other carbon sources. As with no other carbon source, better growth than glucose was observed, therefore glucose at the concentration of 0.2 % was used as the carbon source in the medium for further experiments.

For confirmation of chlorpyriphos utilization (100 ppm) in the presence of five selected nitrogen sources in liquid medium, isolates were grown in

TABLE 5: Growth of bacterial isolates in MSM with different carbon sources.

Treatments	Bacterial Isolates							
	SB1		SGB2		HIC2		HIIGA2	
	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})
Glucose	1130.0	276.1	187.0	259.9	29.0	152.8	1392.0	413.9
Glucose + CPY	1760.0	364.4	234.0	312.2	50.0	214.4	1696.0	468.5
Mannose	11.0	32.0	2.0	36.7	10.0	108.3	120.0	148.4
Mannose + CPY	17.0	40.7	1.0	46.1	15.0	128.9	126.0	187.8
Mannitol	168.0	112.7	107.0	155.6	12.0	98.3	62.0	83.9
Mannitol + CPY	188.0	137.0	118.0	172.8	14.0	113.8	71.0	91.6
Arabinose	32.0	46. 8	72.0	115.0	4.0	72.2	166.0	245.6
Arabinose + CPY	48.0	54.4	84.0	168.9	10.0	98.6	174.0	268.2
Sucrose	173.0	141.1	140.0	198.3	26.0	139.4	123.0	172.7
Sucrose + CPY	194.0	146.2	166.0	221.1	37.0	144.6	134.0	201.1
CD at 5%	30.01	8.85	5.75	5.17	2.81	5.23	6.47	3.12
SE(m) ±	7.62	2.98	1.96	1.74	0.95	1.76	2.17	1.05

CPY: Chlorpyriphos (100 ppm); carbon sources (0.2%)

MSM supplemented with either of the nitrogen source (0.07 M). All the four isolates showed more bacterial count and protein content in the presence of ammonium chloride indicating more utilization of chlorpyriphos in the presence of this nitrogen source (Table 6). In the isolate SB1, maximum bacterial count (288.2 x 10^7 cfu ml^{-1}) and protein content (207.6 µg ml^{-1}) was observed with ammonium chloride. The similar trend was observed with the isolates SGB 2, HIIGA 2 and HIC 2 that showed maximum bacterial count (226.6 x 10^7 , 224.3 x 10^7, 186.0 x 10^7 cfu ml^{-1}) and protein content (294.0, 323.0, 394.2 µg ml^{-1}) with ammonium chloride. Therefore, ammonium chloride was selected as nitrogen source in the medium for degradation studies. Utilization of chlorpyriphos in the MSM was enhanced when the medium was supplemented with glucose and ammonium chloride. Our results are in agreement with Ivashina (1986) who studied chlorpyriphos degradation by several microbial cultures maintained in liquid medium containing 10 ppm chlorpyriphos. Dissipation was more rapid in a sucrose-supplemented medium containing *Trichoderma sp*. and glucose supplemented media containing *Bacillus sp*. than in control medium containing no microorganism. Anwar *et al*. (2009) examined the effect of nutrients and/or alternate carbon sources such as glucose, nutrient broth and yeast extract in minimal salt medium on the chlorpyriphos utilizing capability of the *Bacillus pumilus* strain C2A1 and observed increased pesticide utilization in the presence of these nutrients. Enhancement in chlorpyriphos utilization in presence of these nutrients may be probably due to more growth which in turn increased degradation. Recently, Hindumathy and Gayathri (2013) also studied the chlorpyriphos utilization (100 ppm) by the bacterial isolate in the liquid medium with additional glucose (1000 mg/l). The authors concluded from the results that the presence of glucose supports more biomass, which in turn brings about higher degradation and dissipation of the pesticide. Maximum dissipation of 84.5% was observed with bacterial isolate in presence of glucose as compared to 73.3% dissipation in absence of glucose. Farhan *et al*. (2013) also observed increased chlorpyriphos utilization in the presence of glucose *by the Klebsiella* sp.

Residual chlorpyriphos in MSM at 7 days of growth: To study the utilization of chlorpyriphos by the above isolates in MSM, medium was amended with 100 ppm chlorpyriphos and bacterial isolates were grown in this medium. After 7 days of bacterial growth, residual chlorpyriphos level was determined. The residual chlorpyriphos level was 13.2 ppm with the isolate SB1, 15.8 ppm with the isolate HIC2, 23.0 ppm with the isolate SGB2 and 27.8 ppm with the isolate HIIGA2 respectively. Percent utilization of chlorpyriphos was maximum with the isolate SB1 (80.1 %) followed by HIC2 (76.2 %), SGB2 (65.2%)

TABLE 6: Growth of bacterial isolates in MSM with different nitrogen sources

Treatments	Bacterial Isolates							
	SB1		SGB2		HIC2		HIIGA2	
	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})	Bacterial count (cfu x 10^7 ml^{-1})	Protein (µg ml^{-1})
Glycine	225.4	166.3	174.6	215.3	98.0	291.0	128.0	188.2
Glycine + CPY	246.3	179.4	188.0	264.5	115.3	317.4	164.0	235.2
Tryptophan	43.0	49.2	36.0	94.5	46.0	206.0	73.0	96.3
Tryptophan + CPY	60.2	67.0	42.3	120.3	63.6	233.9	94.2	113.3
Sodium nitrate	90.6	106.7	102.0	175.4	78.0	264.8	101.0	128.0
Sodium nitrate + CPY	114.3	124.7	124.4	186.8	90.0	285.2	148.3	213.0
Ammonium chloride	234.0	183.2	178.2	236.4	136.0	345.6	183.2	272.3
Ammonium chloride + CPY	288.2	207. 6	226.6	294.0	186.0	394.0	224.0	323.0
Urea	205.2	143.8	112.0	183.3	88.0	273.2	92.3	107.7
Urea + CPY	226.4	156.6	146.0	186.2	97.0	295.4	113.4	137.8
CD at 5%	5.26	5.25	6.85	5.72	6.39	4.35	3.25	4.56
SE(m) ±	1.79	1.76	2.23	1.72	2.01	1.32	1.09	1.53

CPY: Chlorpyriphos (100 ppm); nitrogen source (0.07M)

and HIIGA2 (58.1%) respectively (Table 7). Our results are similar to Anuja (2005) who reported degradation of chlorpyriphos (50 ppm) by the nine bacterial isolates in Mineral salt medium in presence of glucose (1 g/l) after 7 days of growth. Isolate JA-15 showed 100 % degradation while JA-8 showed 96.91% and the least degradation i.e 66.2 % was shown by the isolate JA -12.

Chloride release by bacterial isolates in MSM with chlorpyriphos (100 ppm) at 7 days of growth : The ability of the bacterial isolates to degrade chlorpyriphos was also assessed indirectly in terms of chloride released by bacterial isolates in MSM containing chlorpyriphos (100 ppm) (Fig 3). The chloride release was maximum with the isolate SB1 (7.1 ppm) followed by HIC 2 (6.9 ppm), SGB2 (6.7 ppm) and HIIGA2 (6.4 ppm) respectively. Biologically mediated dehalogenation has been reported in the degradation of several chlorine-

TABLE 7: Effect of different isolates on residual chlorpyriphos in MSM at 7 days after growth

Bacterial Isolate	Residual chlorpyriphos (ppm)	Utilization of chlorpyriphos (%)
Control (uninoculated)	58.2	12.2
SB1	13.2	80.1
SGB2	23.0	65.2
HIC2	15.8	76.2
HIIGA2	27.8	58.1
CD at 5%	3.76	
SE(m) ±	1.18	

Initial level of chlorpyriphos: 100 ppm
Initial detectable chlorpyriphos: 66.3 ppm

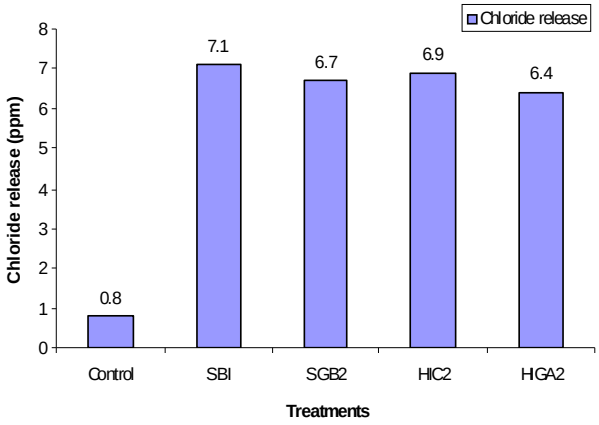

FIG. 3: Chloride release by bacterial isolates in MSM with chloropyriphos (100 ppm) at 7DAG.

containing pesticides (Feng *et al*., 1997; Struthers *et al*., 1998).

Identification of bacterial isolates: Out of four bacterial isolates, two isolates namely SB1 and HIC2 which showed more utilization of chlorpyriphos in liquid medium as compared to others were characterized using morphological, biochemical and physiological tests as described in Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994). Isolate SB1 was identified as *Pseudomonas* and HIC2 as *Xanthomonas* (Table 8). It is well known that *Pseudomonas* is a highly versatile organism with an ability to metabolize even the most complex polymers. Our results were in agreement with the findings of other scientists who isolated *Pseudomonas* spp. which were able to degrade chlorpyriphos (Lakshmirani and Lalithakumari, 1994; Feng *et al*., 1997; Karpouzas and Walker, 2000; Korade and Fulekar, 2009; Farhan *et al*., 2012; Kavikarunaya and Reetha, 2012). Besides that *Xanthomonas* sp. has also been found to degrade organophosphorus pesticides (Tchelet *et al*., 1993).

TABLE 8: Identification of bacterial isolates.

Morphological & biochemical characters	Bacterial isolates	
	SB 1	HIC 2
Colony morphology		
Configuration	Circular	Circular
Margin	Entire	Entire
Elevation	Convex	Convex
Surface	Smooth	Smooth
Opacity	Opaque	Opaque
Pigment	Bluish-green	Yellow
Gram reaction	-ve	-ve
Endospore formation	-ve	-ve
Bacterial morphology	Short rods	Short rods
Bacterial arrangement	Single	Single or Pairs
Growth on MacConkey agar	+	+
Indole production	-	-
Methyl red	-	-
Voges-proskauer reaction	-	+
H_2S production	-	+
Citrate utilization	+	+
Oxidase	+	-
Catalase	+	+
Casein Hydrolysis	+	+
Gelatin Hydrolysis	+	+
Starch hydrolysis	-	+
Cellulose hydrolysis	-	-
Acid production from		
Glucose	+	-
Fructose	-	-
Maltose	-	-
Sucrose	-	-
Galactose	-	-
Physiological characters		
Growth at pH		
pH 5.0	-	-
pH 6.0	+	+
pH 8.0	+	+
pH 9.0	+	+
Growth on NaCl (%)		
2.0	+	+
4.0	+	+
6.0	+	-
8.0	-	-
12.0	-	-
Probable Genus	Pseudomonas	Xanthomonas

REFERENCES

Aleagha, M.M., Pedram, M., Ebadi, A.G., Afsari, H. (2011). Measurement of chlorpyrifos residues (organophosphate insecticide) in apple fruit in Damavand region. *Ameri. J. of Sci. Res.* **13:** 79-81

Anuja, G. (2005). Isolation, Screening and Selection of efficient chlorpyriphos degrading bacteria. M.Sc.(Agri.)*Thesis*, University of Agricultural Sciences, Dharwad.

Anwar, S., Liaquat, F., Khan, Q.M., Khalid, Z.M., Iqba, S. (2009). Biodegradation of chlorpyriphos and its hydrolysis product 3, 5, 6-trichloro-2-pyridinol by Bacillus pumilus strain C2A1. *J. Hazard. Mater.* **168**: 400–405.

Awad, N. S., Sabit, H. H., Salah, E., Abo-Aba, M. and Bayoumi, R.A. (2011). Isolation, characterization and fingerprinting of some chlorpyriphos- degrading bacterial strains isolated from Egyptian pesticides-polluted soils. *Afri. J. Microbiol. Res.*, **5(18):** 2855-2862.

Aysal, P., Gozek, K., Artik, N. and Tuncbilek, A.S. (1999). ^{14}C-Chlorpyriphos residues in tomatoes and tomato products. *Bull. Environ. Contam. Toxicol.*, **62**: 122-125.

Bhagobaty, R. and Malik, A. (2008). Utilization of chlorpyriphos as a sole source of carbon by the bacteria isolated from waste water irrigated agricultural soils in an industrial area of western Uttar Pradesh. *India.Res.J.Microbiol.* **3(5)**: 293-307.

Blossom, Singh, B. and Gaganjyot. (2004). Monitoring of insecticide residues in cotton seed in Punjab, India. *Bull. Environ. Contam. Toxicol.* ,**73**: 707-712.

Fang, H., Yu, Y.L., Wang, X., Shan, M., Wu, X.M., Yu. J.Q. (2006). Dissipation of chlorpyriphos in pakchoi-vegetated soil in a greenhouse. *J. Environ. Sci.*, **18**: 760-764.

Feng, Y., Racke, K.D. and Bollag, J.M. (1997). Isolation and characterization of a chlorinated pyridinol degrading bacterium. *Appl. Environ. Microbiol.*, **63:** 4096-4098.

Farhan, M., Khan, A. U., Wahid, A., Ali, A. S. and Ahmad F. (2013) Potential of indigenous *Klebsiella* sp. for chlorpyriphos biodegradation. *Pakistan J. Sci.*, ***6*:** 133-137.

Farhan, M., Khan, A. U., Wahid, A., Ahmad, M. and Ahmad, F. (2012). Biodegradation of chlorpyrifos using indigenous *Pseudomonas* sp. isolated from industrial drain. *Pak. J. Nut.*, **11(12):** 1183-89

Greenberg, A.E., Clesceri, L.S. and Eaton, A.D. (1992). Standard Methods for the Examination of Water and Waste Water. 18th ed. APHA, Washington.

Gupta, A., Parihar, N.S. and Bhatnagar, A. (2001). Lindane, chlorpyriphos and quinalphos residues in mustard seed and oil. *Bull. Environ. Contam. Toxicol.*, **67**: 122-125.

Hindumathy, C.K. and Gayathri, V. (2013). Effect of pesticide (chlorpyrifos) on soil microbial flora and pesticide degradation by strains isolated from contaminated soil. *J. Bioremed. Biodeg.*, **4:** 178.

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. 527-533.

Horne, I., Sutherland, T.D., Harcourt, R.L., Russell, R.J. and Oakeshott.,J.G. (2002). Identification of an *opd* (organophosphate degradation) gene in an *Agrobacterium* isolate. *Appl. Environ. Microbiol.*, **68:** 3371-3376.

Ivashina, S.A. (1986). Interaction of Dursban with soil micro-organisms, *Agrokhimia* **8**: 75-76

Jain, H.K., Pandey, S.Y., Agnihotri, N.P. and Dewan, R.S. (1974). Rapid estimation of OP insecticides. *Indian J. Entomol.*, **36**: 145-148.

Kanekar, P.P., Bhadbhade, B., Deshpande, N.M. and Sarnaik, S.S. (2004). Biodegradation of organophosphorus pesticides. *Proc. Indian. Nat. Sci. Academy*, **70**: 57-70.

Karpouzas, D. G. and Walker,A. (2000). Factors influencing the ability of Pseudomonas putida strains epI and II to degrade the organophosphate ethoprophos. *J. Appl. Microbiol.*, **89**: 40-48.

Korade, D.L. and Fulekar, M. H. (2009). Rhizosphere remediation of chlorpyriphos in mycorrhizospheric soil using ryegrass. *J Hazard. Mater.*, **172(2-3)**: 1344-1350.

KaviKarunya, S. and Reetha, D. (2012). Biological degradation of chlorpyrifos and monocrotophos by bacterial isolates. *Inter. J. Pharmaceu. Bio. Arch.* **3(3):** 685-691.

Lakshmirani, N. and Lalithakumari, D. (1994). Degradation of methyl parathion by *Pseudomonas putida*. *Can. J. Microbiol.*, **40**: 1000-1006.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R..J. (1951). Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.*, **193**: 265-275.

Mallick, B. K., Banerji, A., Shakil, N. A. and Sethunathan, N. N. (1999). Bacterial degradation of chlorpyriphos in pure culture and in soil. *Bull. Environ. Contam. Toxicol.*, **62:** 48-55.

Mohan, S.V., Sirisha, K. and Rao, N.C. (2004). Degradation of chlorpyriphos contaminated soil by bioslurry reactor operated in sequencing batch mode: bioprocess monitoring. *J. Hazard. Mater.*, **116**: 39-48.

Nandish, M.S. (2005). Microbial degradation of phenol and pentachlorophenol. *M.Sc.(Agri.) Thesis*, University of Agricultural Sciences, Dharwad.

Qian, B., Zhu, L.S., Xie, H., Wang, J., Liu, W., Xu, Q.F., Song, Y., Xu, R.J. (2007).Isolation and degrading characters of Chlorpyriphos degrading bacteria XZ-3. *Huan Jing Ke Xue.,* **28(12)**: 2827-32.

Racke, K. D., Coats, J. R. and Titus, K. R. (1988). Degradation of chlorpyriphos and its hydrolysis products, 3,5,6-trichloro-2-pyridinol, in soil. *J. Environ. Sci. Health B.,* **23:**527-539.

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

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

Stuthers, J.K., Jayachandran, K. and Moorman, T.B. (1998). Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. *App. Environ. Microbiol.,* **64**: 3368-3375.

Tchelet, R., Levanon, D., Mingelrin, D. *and* Henis, Y.(1993). Parathion degradation by a *Pseudomonas* sp. and a *Xanthomonas* sp. and by their crude enzyme extracts as affected by some cations. *Soil. Biol. Biochem.,* **25***:* 1665–1671.

Veeranagowda, Y., Neelakanteshwar, P.K. and Karegowdar, T.B. (2002). A method for screening of bacteria capable of degrading dimethylformamide. *Curr. Sci.,* **87**: 1652-1654.

Yang, L., Zhao, Y.H., Zhang, BX., Yang, C.H., and Zhang, X. (2005).Isolation and characterization of a chlorpyriphos and 3, 5, 6- trichloro-2-pyridinol degrading bacterium. *FEMS Microbiol Lett.,* **251**: 67-73.

Yu,C.P and Yu, Y.W. (2000). Evaluation of biodegrability by the reduction of tetrazoluim violet in biolog microplates. *Biotechnol. lett.* **22**: 909-919

Zhu, J., Zhao, Y. and Qiu, J. (2010). Isolation and application of a chlorpyriphos-degrading *Bacillus licheniformis* ZHU-1. *Afri. J. Microbiol. Res.,* **4(22)**: 2410-2413.