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Full Length Article

Growth Linked Biodegradation of Chlorpyrifos by *Agrobacterium* and *Enterobacter* spp.

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Abstract

The extensive use of insecticide, chlorpyrifos, is creating contamination of soil and water environments. This study was aimed at isolating bacteria utilizing chlorpyrifos as sole carbon source and evaluating their growth linked biodegradation. A total of 32 bacterial isolates were collected from chlorpyrifos contaminated soil on mineral salt medium spiked with chlorpyrifos as the sole carbon source. Incubation conditions were optimized to achieve maximum degradation of chlorpyrifos by the most efficient bacterial strains. The selected bacteria were able to degrade up to 92% of the spiked chlorpyrifos (100 mg/L) within 18 days under shaking conditions. A neutral pH (7) and incubation temperature of 30°C were found optimum for biodegradation of chlorpyrifos in the broth medium. High performance liquid chromatographic (HPLC-UV) analysis revealed that the biodegradation of chlorpyrifos continued gradually throughout the incubation till the termination of incubation. A statistically significant linear positive correlation (r^2=0.98**) between cell densities of the bacteria and biodegradation of chlorpyrifos under optimized and varying incubation conditions revealed that the biodegradation was a growth-linked process. The 16S rRNA gene analysis indicated that the most efficient bacterial strains, SGB2, SWLC1 and SWLC2 had greater than 97% similarity to the genus *Enterobacter*, while the strain SWLH2 was almost similar to the genus *Agrobacterium*. The findings of this study reveal that *Agrobacterium* and *Enterobacter* spp. exhibited growth linked biodegradation of chlorpyrifos and such efficient bacterial strains could be employed effectively for the degradation of organophosphate type of pesticides including chlorpyrifos to reduce water and soil pollution.

Keywords: Bacteria; Biodegradation; Cell growth; Chlorpyrifos; Factors

Introduction

The use of organophosphorus pesticide is increasing all over the world because of its high effectiveness against target pests and this class of pesticides proved good substitute for organochlorine and carbamate pesticides. Chlorpyrifos [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate] is a moderately toxic, broad-spectrum chlorinated organophosphate insecticide (Singh and Walker, 2006). It is widely used for controlling pests of important crops like cotton, rice, cereals, tobacco, fruits, vegetable crops, pasture, and ornamental plants (Kale *et al*., 1999; Mallick *et al*., 1999). However, its extensive use in agriculture sector is also resulting in contamination of soil, air and ground and surface water, rivers and lakes. Many researchers have reported that large scale use pollutes different components of the environment, including aquatic and terrestrial ecosystems (Sapozhnikova *et al*., 2004; Yang *et al*., 2005; Yu *et al*., 2006). In Pakistan, chlorpyrifos residues have been detected in fruits, vegetables and in cattle meat (Parveen *et al*., 2004; Muhammad *et al*., 2010), implying that there is a potential danger to human health because of its entry in food chain (Cochran *et al*., 1995; Martınez-Vidal *et al*., 1998).

Among remediation techniques, the use of microorganisms is considered one of the very promising approaches. Compared to other approaches, bioremediation is relatively a low cost and easy to use technique, which does not pose threat of secondary pollution to the environment (Racke *et al*., 1990). To date, various researchers have identified bacteria that are able to degrade different types of insecticides (Xu *et al*., 2007, 2008; Fang *et al*., 2008; Vidya-Lakshmi *et al*., 2008; Anwar *et al*., 2009; Zhu *et al*., 2010; Maya *et al*., 2011). Previously, chlorpyrifos has been reported to be resistant to enhanced degradation (Racke *et al*., 1990). Now it has been reported that the biodegradation of chlorpyrifos occurs co-metabolically (Serdar *et al*., 1982; Mallick *et al*., 1999; Guha *et al*., 1997; Horne *et al*., 2002). Several attempts to isolate bacteria using chlorpyrifos as sole carbon source have not been successful (Racke *et al*., 1990; Mallick *et al*., 1999). Recently, some bacterial isolates have been found, which could use chlorpyrifos as sole source of carbon but these isolates could transform chlorpyrifos to 3,5,6-trichloro-2-pyridinol (TCP), which is itself toxic and has antimicrobial properties (Singh *et al*., 2004; Li *et al*., 2007). It has been reported that TCP prevents the proliferation of chlorpyrifos degrading microorganisms (Racke *et al*., 1990).

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Xu *et al*., (2008) reported for the first time the isolation of *Paracoccus* sp. that could use chlorpyrifos as a carbon source and completely mineralized it. However, relationship between the growth of chlorpyrifos utilizing bacteria and biodegradation has been studied rarely. The bacteria capable of utilizing chlorpyrifos as sole carbon source and exhibiting growth linked biodegradation can proved to be more efficient in the biodegradation of contaminated sites than those that biodegrade it co-metabolically.

The present study was primarily focused on the isolation of efficient bacterial strains that were able to use chlorpyrifos as sole source of carbon, exhibiting their growth in response to biodegradation. Through repetitive enrichment culture and successive subcultures, efficient bacterial strains were examined for their potential to degrade chlorpyrifos in liquid medium under optimized environmental/incubation conditions.

Materials and methods

Reagents and Chemicals

Technical grade chlorpyrifos (97% pure) was obtained from Ali Akbar Enterprises, Lahore, Pakistan. Analytical grade chlorpyrifos (99.5%) and chlorpyrifos (40% emulsifiable concentrate) were obtained from Dow Agro Sciences, Karachi, Pakistan. Acetonitrile, methanol and water were of HPLC grade. Composition of the mineral salt medium (MSM) in g/L used for isolation was: KH_2PO_4, 4.8; K_2HPO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $Ca(NO_3)_2 \cdot 4H_2O$, 0.04 and $FeSO_4$, 0.001.

Collection of Soil and Water Samples

Fourteen soil samples having history of repeated chlorpyrifos applications and three water samples were collected from different sites of Punjab Province of Pakistan (Table 1). The soil samples were collected from top soil layer (0-15 cm) and air dried at room temperature and mixed thoroughly after passing through a 2 mm sieve. Soil and water samples were stored in polythene bags and polyethylene bottles, respectively in refrigerator at 4°C. The physical and chemical properties of the collected soil samples used for isolation of chlorpyrifos degrading bacteria are presented in Table 1.

Enrichment Culture for Isolation of Chlorpyrifos Degrading Bacterial Strains

Enrichment culture technique was used for isolation of bacterial strains capable of utilizing chlorpyrifos as the sole C source. Bacterial inocula for enrichment culture were prepared by shaking 20 g soil overnight in 100 mL nutrient culture medium at 30°C and 150 rev/min. A settling time of 1 h was provided for solid particles. The supernatant was separated through paper filtration (Whatman No. 42). One mL aliquot was used for the inoculation of 9 mL MSM (50 mL Erlenmeyer flask) enriched with chlorpyrifos at the rate 100 mg/L. In the case of water samples, 1.0 mL water from each sample was added to the MSM in the flasks. These flasks were incubated at 30°C for 10 days under shaking (150 rev/min). This process was repeated twice by taking 0.1 mL of chlorpyrifos culture in the fresh flasks containing 9 mL MSM media enriched with 100 mg/L chlorpyrifos. The bacterial cultures were obtained by spreading the suspension on the agar plates containing MSM plus chlorpyrifos. The plates were incubated at 30°C for 48 h. About 32 discrete colonies of bacteria showing prolific growth were selected for subsequent studies.

The isolated bacteria were cultured in fresh medium containing 100 mg/L chlorpyrifos and then incubated at 30°C for one week under shaking conditions. The cell suspensions were centrifuged at 8000×*g* for 2 min. The cell pellets were washed four times with fresh medium. The cells were re-suspended in sterilized water to achieve an optical density of 0.80±0.02 at 600 nm.

Growth of Bacteria and Degradation of Chlorpyrifos in Liquid Culture Media

The bacterial isolates were examined for their potential to degrade chlorpyrifos in liquid medium. For this purpose, the MSM (pH 7) in 50 mL Erlenmeyer flasks was autoclaved at 121°C for 20 min. To each flask, 20 mL of autoclaved medium was spiked with 100 mg/L chlorpyrifos. The inocula of uniform cell density (OD 0.8) at the rate of 800 µL per flask were added. These inoculated flasks were incubated for 18 days at 30°C on an orbital shaker at 150 rev/min. Uninoculated flasks were also prepared to check for the abiotic degradation under similar conditions. The experiment was conducted with three replications. The biodegradation of chlorpyrifos and bacterial growth were examined at different intervals. Bacterial optical densities were determined by spectrophotometer at 600 nm.

The presence of chlorpyrifos residues in the cultures was examined on HPLC (Schimadzu LC 10A/Waters Alliance), using ODS Hypersil column (250 × 4.6 mm) and UV-VIS detector (300 nm). Acetonitrile, water and glacial acetic acid (82:17.5:0.5, *v/v*) were used as mobile phase. Analytical grade chlorpyrifos (99.5%) was used as a standard for the computation of residual concentration of chlorpyrifos in the liquid medium. Chlorpyrifos from the medium was extracted through an addition of acetonitrile to enrichment media, followed by a vigorous shaking. For this purpose, 2 mL of each culture were taken in separate flasks and the same volume of acetonitrile was added to these flasks. The flasks were shaken for 30 min, using a reciprocating shaker at 150 rev/min. After centrifugation, the samples were filtered through 0.2 µm nylon membrane filters using Swinney stainless syringes (Millipore™).

To study the extraction efficiency of the methods established, a recovery experiment was conducted in the MSM (20 mL) spiked with known concentrations of chlorpyrifos (1, 10 and 100 mg/L). Extraction and analysis

were performed in triplicate according to the procedures described above.

Effect of pH, Temperature and Shaking on Biodegradation of Chlorpyrifos by the Efficient Bacterial Strains

The effect of varying pH (4−9) and temperature (20−45°C) was examined on the biodegradation of chlorpyrifos by the selected bacterial strains (SGB2, SWLC1, SWLC2 and SWLH2). The biodegradation of chlorpyrifos in liquid culture was also studied under static vs. shaking incubation (150 rev/min) to examine the effect of aeration on the biodegradation efficiency of the selected strains. These studies were performed by using the same conditions as described above except the factor under study. The experiments were performed keeping three replications for each factor/level.

Characterization and Identification of Chlorpyrifos Degrading Bacteria

The most efficient bacterial strains such as SGB2, SWLC2, SWLC1 and SWLH2 were identified by using morphological and biochemical tests such as Gram stain, motility, indole, methyl red, voges-proskauer, citrate, oxidase, catalase, urease, nitrate reductase and phosphatase (Holt *et al*., 1994). Utilization of glucose, citrate and mannitol by the selected strains was also examined (Table 2). The efficient four strains (SGB2, SWLC1, SWLC2 and SWLH2) were Gram negative bacteria and they exhibited catalase and phosphatase activity positive but urease activity negative. Other biochemical traits varied with the type of strain.

To identity bacteria, 16S rRNA gene analysis was performed. The extracted DNA was amplified by using universal primers, 27f (5'-AGA GTT TGA TCH TGG CTC AG-3') and 1492r (5'-TAC GGH TAC CTT GTT ACG ACT T-3') (Gurtler and Stanisich, 1996). PCR was performed in a Thermocycler DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD), using the conditions: 1 cycle of 4 min at 94°C; 39 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and final extension step at 72°C for 5 min. The purified 16S rRNA amplicons were sequenced using automated DNA sequencing system (Applied Biosystems, 3730XL USA). The gene sequences were deposited in the GenBank database having the Bank number 1489974 and the given accession numbers are JN 966740, JN 966741, JN 966742, and JN 966743 for SWLH2, SGB2, SWLC2 and SWLC1, respectively. All the 16S rRNA sequences were compared to the known nucleotide sequences through BLAST search *(http://www.ncbi.nlm.nih.gov/BLAST).* To construct phylogenetic trees, multiple alignments were carried out using ClustalX (Thompson *et al*., 1997) and the data obtained were processed using the Neighbor joining method (Perriere and Gouy, 1996).

Table 1: Physical and chemical properties of soil samples collected from different sites

Sample code	Sample description	Texture	% O.M	pH
FSD	Vegetable growing field	Loam	0.36	7.71
SJA	Wheat-sugar cane growing field	Loam	0.72	8.25
SJB	Wheat-Rice growing field	Loam	0.72	8.10
SGA	Citrus orchard	Loam	0.83	7.77
SGB	Wheat growing in citrus orchard	Sandy loam	0.88	7.95
DGK	Fallow field	Sandy loam	0.83	8.56
LHA	Rice growing field	Sandy Loam	0.72	7.97
LHB	Cotton growing field	Sandy loam	0.26	8.64
RP	Cotton harvested field	Sandy loam	0.88	8.86
SWLC	Sugar cane growing field	Loam	0.62	8.00
SWLH	Capsicum growing field	Loam	0.57	7.85
MNA	Cotton growing field	Clay loam	0.67	8.10
MNB	Cotton growing field	Loam	0.83	8.10
MNC	Cotton growing field	Loam	0.78	8.30
SJC	Water pond near wheat growing field	-	-	-
SGC	Pond water near wheat growing area in citrus orchard	-	-	-
MNW	Sewerage water sample from pesticide formulating plant	-	-	-

Table 2: Morphological and biochemical characteristics of selected strains of bacteria capable of degrading chlorpyrifos in liquid medium efficiently

Type of tests	Bacterial strains capable of degrading chlorpyrifos			
	SGB2	SWLC1	SWLC2	SWLH2
Gram's reaction	-	-	-	-
Motility	+	-	+	+
Indole test	+	+	-	-
Methyl red	+	+	-	-
Voges-Proskauer test	+	+	+	+
Catalase	+	+	+	+
Citrate utilization	-	-	+	+
Oxidase	+	-	+	+
Urease	-	-	-	-
Nitrite reduction	+	+	-	+
Casein hydrolysis	-	-	-	-
Starch hydrolysis	-	-	+	+
Growth on MacConkey agar	+LF	+NLF	+NLF	+NLF
Phosphatase	+	+	+	+
Utilization of glucose and manitol as C source	+	+	+	+
Utilization of arginine, alanine and lysine as N source	+	+	+	+
H_2S Production	-	-	+	+

Data Analysis

Percent biodegradation was calculated on the basis of the fraction of spiked amount of chlorpyrifos disappeared in the treated samples during the incubation time. The means and standard errors of three replicates were computed using data analysis tools of software MS-Excel® 2007. The statistical analysis was done by using Minitab 15 software (Minitab® Institute, USA).

Results

From fourteen soil and three water samples, only 32 isolates

Table 3: Biodegradation of chlorpyrifos in mineral salt medium by the efficient bacterial strains

Bacterial strains	Biodegradation of chlorpyrifos (%)[a]			
	3 d	7 d	14 d	18 d
Uninoculated control (abiotic)	-	10.9[a] ± 2.8[b]N	13.2 ± 1.6N	14.8 ± 0.8N
Enterobacter sp. SGB2	27.7 ± 4.7L	54.4 ± 3.0IJ	67.4 ± 3.4F	86.0 ± 1.5BC
Enterobacter sp. SWLC1	21.4 ± 6.6M	51.7 ± 3.8J	65.5 ± 2.7FG	81.2 ± 2.8DE
Enterobacter sp. SWLC2	35.8 ± 1.8K	61.5 ± 1.5GH	83.7 ± 2.8CD	93.7 ± 2.6A
Agrobacterium sp. SWLH2	33.8 ± 3.4K	58.9 ± 2.1HI	77.0 ± 3.0E	89.1 ± 1.3AB

Table 4: Change in optical density of the culture as a result of chlorpyrifos degradation by the efficient strains of bacteria over a period of time

Bacterial strains	Optical density (Absorbance at 600 nm)[a]			
	3 d	7 d	14 d	18 d
Enterobacter sp. SGB2	0.26 ± 0.03[b]	0.51 ± 0.07	0.58 ± 0.05	0.71 ± 0.08
Enterobacter sp. SWLC1	0.19 ± 0.03	0.39 ± 0.05	0.57 ± 0.05	0.70 ± 0.05
Enterobacter sp. SWLC2	0.29 ± 0.02	0.56 ± 0.04	0.65 ± 0.04	0.78 ± 0.03
Agrobacterium sp. SWLH2	0.28 ± 0.04	0.48 ± 0.03	0.61 ± 0.05	0.74 ± 0.04

[a]The data are average of three replicates
[b](±) represents standard error

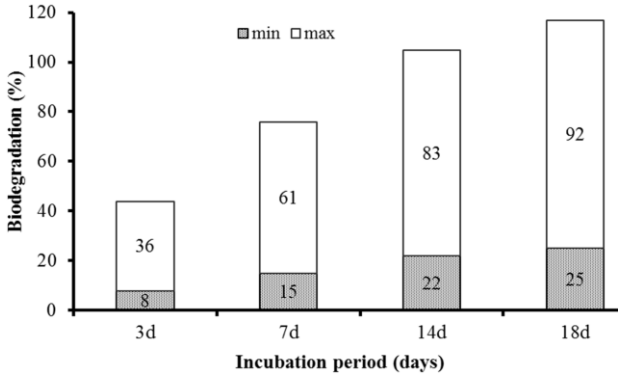

Fig. 1: The range of biodegradation (%) of chlorpyrifos by bacteria isolated from different sites over a period of time. The data are average of three replicates

were selected to test the biodegradation potential (data not shown). The isolates from Sargodha and Sahiwal soils proved to be the most promising strains responsible for chlorpyrifos biodegradation. The isolation from water samples was not so successful owing to low solubility of chlorpyrifos in water samples. The potential of 32 isolates to degrade chlorpyrifos in broth culture varied substantially (Fig. 1). Up to 36% biodegradation of the spiked amount (100 mg/L) of chlorpyrifos was observed after 3 days of incubation, which reached 61 and 83% after 7 and 14 days, respectively. After 18 days, maximum biodegradation of chlorpyrifos was 92%.

The disappearance of chlorpyrifos from the inoculated medium by the four most efficient bacterial strains (SGB2, SWLC1, SWLC2 and SWLH2) at different incubation times is shown in Table 3. These strains showed a high potential to degrade chlorpyrifos in liquid medium, which ranged from 81−94% within 18 days. Among all the tested strains, SWLC2 isolated from the soil of sugarcane field was the most efficient degrader. The strains SGB2, SWLC1 and SWLH2 were able to degrade 86, 81 and 89% chlorpyrifos in 18 days, respectively. The calculated recovery efficiencies ranged from 97−99% (data not shown), which indicated that HPLC had a high accuracy for chlorpyrifos determination and extraction procedure was efficient in extracting the chlorpyrifos residues from the mineral salt medium. The cell density of the cultures increased with the increase in biodegradation of chlorpyrifos (Table 4). The highest cell density (OD 0.78) was recorded in the case of bacterial strain SWLC2, followed in descending order by SWLH2 (OD 0.74), SGB2 (OD 0.71) and SWLC1 (OD 0.70). There was a significant linear positive correlation (r^2=0.98) between percent biodegradation of chlorpyrifos by the selected bacterial strains and cell densities in the broth medium.

Data regarding the effect of pH on biodegradation of chlorpyrifos in MSM by the efficient bacterial strains are presented in Fig. 2. Maximum biodegradation (up to 92%) was observed between pH 7 and 8. A sharp decrease in biodegradation was observed as pH decreased to below pH 7. However, there was a slight decrease in the biodegradation of chlorpyrifos as the pH increased to 9. Strain SWLC2 showed better performance in degrading chlorpyrifos at all studied pH levels than other three strains. Abiotic degradation was greatest in the alkaline pH range (7−9). The bacterial biomass of four strains at different pH was in line with the biodegradation rate of chlorpyrifos and the highest cell densities were observed over a range of pH 7 to 9 (Fig. 3). The optimal temperature for biodegradation of chlorpyrifos by the efficient four strains was 30°C (Fig. 4). A slight decrease in the biodegradation was observed when the temperature decreased from 30 to 25°C or increased from 30 to 35°C. However, the biodegradation sharply decreased when the temperature further changed on either side, i.e., lesser than 25°C or greater than 35°C. Abiotic degradation was higher at high temperature levels. The strain SWLC2 was the most efficient degrading strain at different incubation temperatures as compared to other three strains. The change in bacterial cells growth followed almost the same pattern as observed with biodegradation by these strains at different temperatures (Fig. 5). The optimal temperature range for bacterial growth as indicated by optical densities was 30 to 35°C. Under shaking (aeration) conditions, the biodegradation of chlorpyrifos by four strains was about 87−92% after 18 days compared to 50−56% biodegradation under static condition (Fig. 6). Strain SWLC2 showed the highest

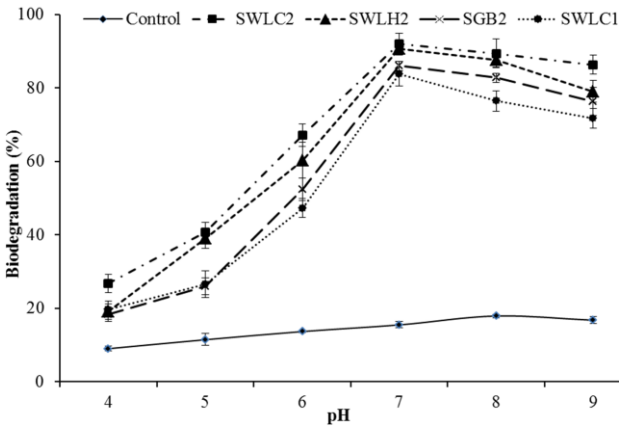

Fig. 2: Biodegradation of chlorpyrifos by the most efficient bacterial strains at different pH after 18 days of incubation. The data are average of three replicates

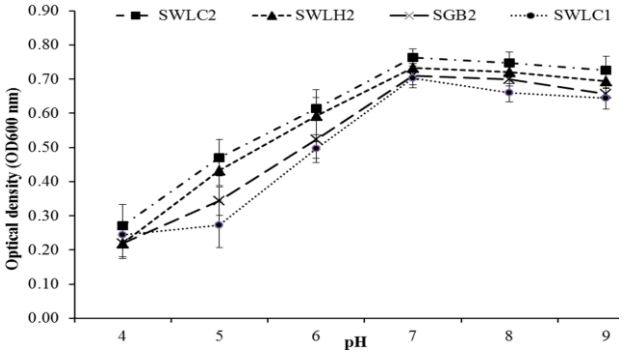

Fig. 3: The change in bacterial cell densities in the culture as a result of biodegradation of chlorpyrifos at different pH after 18 days of incubation. The data are average of three replicates

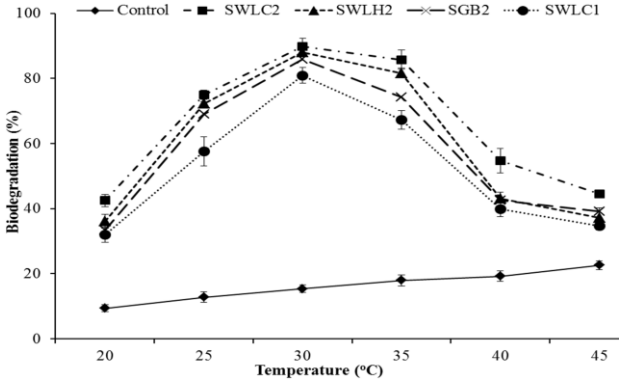

Fig. 4: Biodegradation of chlorpyrifos by the most efficient bacterial strains at different temperatures after 18 days of incubation. The data are average of three replicates

degradation of chlorpyrifos under shaking incubation. Abiotic degradation was also greater under shaking than that observed under static conditions. Optical densities of the

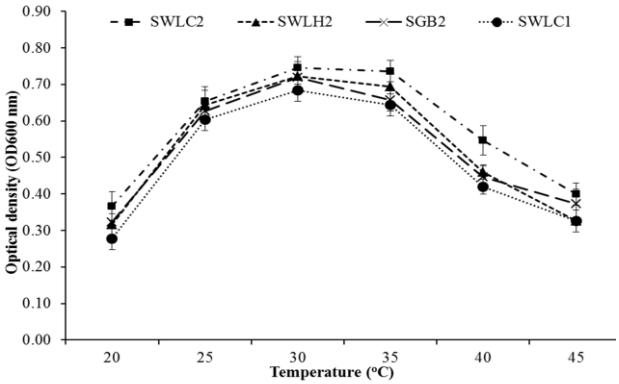

Fig. 5: The change in bacterial cell densities in the culture as a result of biodegradation of chlorpyrifos at different temperatures after 18 days of incubation. The data are average of three replicates

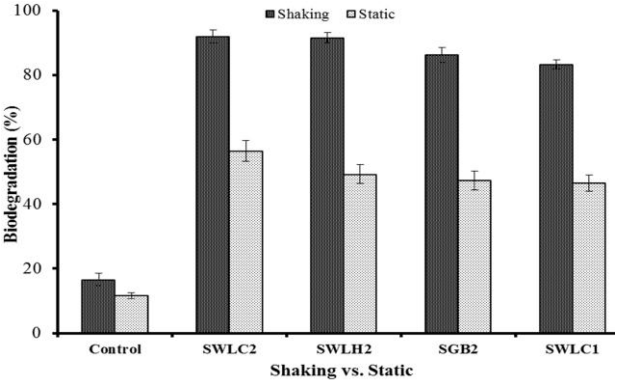

Fig. 6: Effect of shaking (aeration) and static conditions on biodegradation of chlorpyrifos by the most efficient bacterial strains over a period of 18 days. The data are average of three replicates

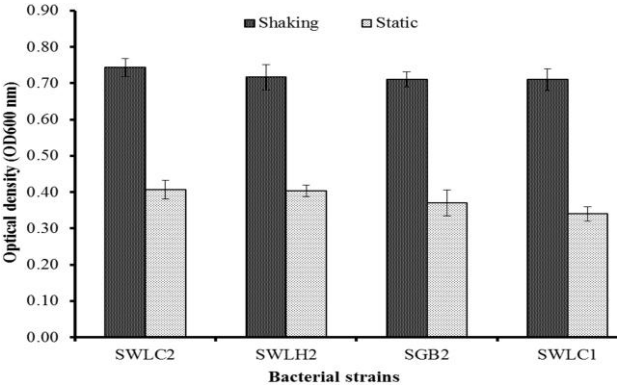

Fig. 7: Optical densities (OD_{600}) of bacterial strains under static vs. shaking conditions

strains were more under shaking condition than under static conditions (Fig. 7).

The 16S rRNA gene analyses indicated that the strains SGB2, SWLC1 and SWLC2 belonged to the family

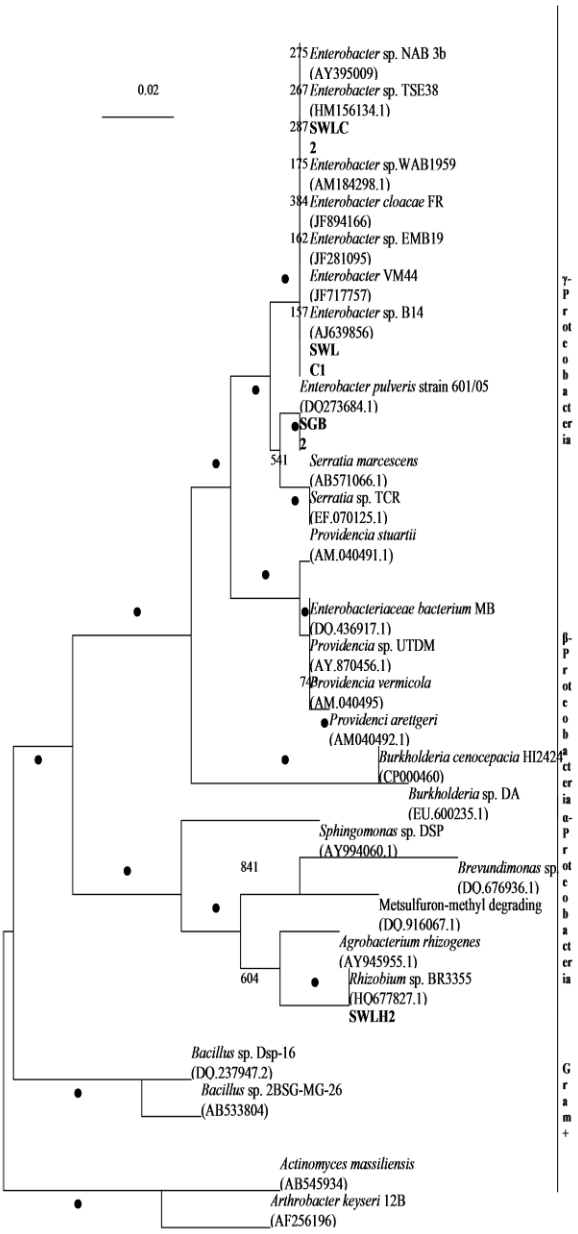

Fig. 8: Phylogenetic tree showing inter-relationship of bacterial strains with closely related species of the bacteria, inferred from 16S rRNA sequences. Tree was generated using the Neighbor-Joining method. Accession number of each type strain is shown in parentheses and the phylogenetic distance is shown on a scale bar

Enterobacteriacea, showing closest resemblance (>97% sequence identity) to the species of genus *Enterobacter* (Fig. 8). Strain SWLH2 belonged to family *Rhizobiaceae* and the highest degree of similarity was found with the genus *Agrobacterium*. The bacterial strains SGB2, SWLC1 and SWLC2 were grouped (i.e., bootstrap > 900%) in a cluster of several *Enterobacter* spp., whereas the strain SWLH2 was grouped in a cluster of α–*Proteobacteria* including *Agrobacterium* sp.

Discussion

This study illustrates biodegradation potential of bacterial strains isolated from chlorpyrifos contaminated soils, using chlorpyrifos as sole source of carbon in the mineral salt medium and their growth in response to biodegradation. Bacteria isolated from contaminated soil used chlorpyrifos as sole carbon source and exhibited the highest efficiency in degrading chlorpyrifos in liquid medium. Some bacterial

strains could degrade about 92% of 100 mg/L of chlorpyrifos in 18 days. The four most efficient strains, identified by 16S rRNA gene sequences, represented two genera including *Enterobacter* (SGB2, SWLC1 and SWLC2) and *Agrobacterium* (SWLH2). These bacteria showed catalase and phosphatase activites positive. It is very likely that the presence of catalase and phosphatase enzymes in these strains might have played a pivotal role in the biodegradation of chlorpyrifos. Thengodkar and Sivakami (2010) have reported that alkaline phosphatase purified from *Spirulina platensis* was able to degrade 100 ppm chlorpyrifos to 20 ppm in one hour.

A linear positive correlation (r^2=0.98) was observed between cell densities (an indicator of growth) of the bacteria and biodegradation of chlorpyrifos which reveals that the biodegradation was a growth-linked process. In most of the previous studies it has been reported that chlorpyrifos could be degraded by bacteria co-metabolically (Serdar *et al*., 1982; Guha *et al*., 1997; Mallick *et al*., 1999; Horne *et al*., 2002). A good number of the attempts to isolate bacteria using chlorpyrifos as sole carbon source have not been successful (Racke *et al*., 1990; Mallick *et al*., 1999). Although, recently some bacterial strains have been isolated by Singh *et al*. (2004) and Li *et al*. (2007) which could use chlorpyrifos as sole source of carbon but these isolates could transform chlorpyrifos to TCP having antimicrobial properties and may prevent the proliferation of chlorpyrifos degrading microorganisms (Racke *et al*., 1990). In our study, the isolated bacteria utilized chlorpyrifos as a direct source of carbon. It was also noted that initially there was a transient accumulation of TCP, which disappeared quickly (data not shown). This indicates that the tested bacterial strains were also capable of utilizing TCP. This premise of TCP degradation is also supported by the bacterial growth, which increased proportionally with biodegradation, signifying the absence of TCP, which has antimicrobial properties (Racke *et al*., 1990).

As discussed previously, chlorpyrifos has been found to be resistant to biodegradation and most of the attempts to isolate chlorpyrifos degrading microorganisms were not successful but we have been able to isolate bacteria exhibiting growth-linked biodegradation. This might be due to the isolation of bacteria from the soil where chlorpyrifos has been extensively used for many years. This repeated and extensive use of chlorpyrifos might have allowed the opportunistic microorganism to develop capability to use this toxic compound as carbon and energy source. Singh *et al*. (2004) were also able to isolate bacterial strain capable of using chlorpyrifos as sole source of carbon from Australian soil, where this pesticide had been in use for 15 years.

Biodegradation of chlorpyrifos by four strains was optimal at pH 7 with incubation at 30^{o}C under shaking conditions. Such incubation conditions have been reported to favor bacterial growth, which results in an accelerated degradation of the substrate (Yang *et al*., 2006; Abo-Amer 2011). This premise is supported by the results of this study, which indicated a greater cell biomass (bacteria cell densities) in liquid culture under these incubation conditions. The positive correlation between bacterial cell densities and biodegradation at all pH (r^2=0.97**) and temperature levels (r^2=0.95*) and shaking vs. static conditions (r^2=0.98**) further supported the hypothesis that the biodegradation was growth linked. The biodegradation was slow at two extremes of temperatures i.e., 20^{o}C and 45^{o}C. It has been observed that the temperature between 25−30^{o}C is the most suitable for the members of family *Enterobacteriaceae* (Qian *et al*., 2007; Xu *et al*., 2007). The biodegradation under shaking conditions was more pronounced than the static conditions. This might be due to better bioavailability of chlorpyrifos (substrate) to the microbes coupled with physiochemical degradation (Nazli *et al*., 2003; Hussain *et al*., 2007). Alternately, aerobic conditions might be more conducive for growth of tested bacteria or enzyme system involved in the biodegradation. Abiotic degradation was more under alkaline conditions and slower in acidic condition and at lower temperature. This could be due to hydrolysis of chlorpyrifos under neutral to alkaline conditions. The abiotic degradation was also greater under shaking conditions than under static conditions, as the aerobic conditions are relatively more conducive for abiotic (chemical) degradation of chlorpyrifos (Hussain *et al*., 2007).

Furthermore, the biodegradation rate observed in this study was relatively higher than previously reported findings for chlorpyrifos biodegradation, where it was not used as a direct carbon source by the degrading microorganism (Mallick *et al*., 1999; Vidya-Lakshmi *et al*., 2008; Kulshrestha and Kumari 2011). This could be due to the growth linked biodegradation. This hypothesis is also supported by Mallick *et al*. (1999) who reported that *Arthrobacter* sp. used chlorpyrifos as sole carbon source and exhibited rapid biodegradation of chlorpyrifos in liquid medium than *Flavobacterium* sp. that could not grow in response to chlorpyrifos biodegradation.

In conclusion, *Agrobacterium* and *Enterobacter* spp. exhibited growth linked biodegradation of chlorpyrifos. Such efficient bacterial strains can be used successfully for the removal of chlorpyrifos from the contaminated sites.

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