Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by *Bacillus pumilus* strain C2A1

Samina Anwar, Fauzia Liaquat, Qaiser M. Khan, Zafar M. Khalid, Samina Iqbal *

National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan

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ABSTRACT

A bacterial strain C2A1 isolated from soil was found highly effective in degrading chlorpyrifos and its first hydrolysis metabolite 3,5,6-trichloro-2-pyridinol (TCP). On the basis of morphology, physiological characteristics, biochemical tests and 16S rRNA sequence analysis, strain C2A1 was identified as *Bacillus pumilus*. Role of strain C2A1 in the degradation of chlorpyrifos was examined under different culture conditions like pH, inoculum density, presence of added carbon/nutrient sources and pesticide concentration. Chlorpyrifos was utilized by strain C2A1 as the sole source of carbon and energy as well as it was co-metabolized in the presence of glucose, yeast extract and nutrient broth. Maximum pesticide degradation was observed at high pH (8.5) and high inoculum density when chlorpyrifos was used as the sole source and energy. In the presence of other nutrients, chlorpyrifos degradation was enhanced probably due to high growth on easily metabolizable compounds which in turn increased degradation. The strain C2A1 showed 90% degradation of TCP ($300 \, \mathrm{mg \, L^{-1}}$) within 8 days of incubation.

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1. Introduction

Pesticides were introduced to agriculture to fulfill the increased food needs of the growing global population. By now use of pesticides has become a necessary evil although this practice is playing chaos with human and other life forms. Residues of applied pesticides stay in the environment (air, soil, ground and surface water) for variable periods of time [1–3]. Due to the long persistence of organochlorines (lindane, heptachlor, dichlorodiphenyltrichloroethane (DDT), etc.) and their tendency to bioaccumulate and their potential toxicity towards non-target organisms, this group of pesticide has been replaced by relatively less persistent and yet effective organophosphorus (OP) compounds. Though organophosphates degrade faster than the organochlorines, this class of pesticide has acute neurotoxicity which is due to their ability to suppress acetylcholine esterase (AChE). AChE serves as a regulating enzyme of nervous transmission by reducing the concentration of acetylcholine at the synaptic junction. When AChE is inactivated, e.g., by an organophosphate pesticide, the concentration of acetylcholine in the junction remains high, and continuous stimulation of the muscle or nerve fiber occurs, resulting eventually in exhaustion and tetany [4].

Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)phosphorothioate) is one of the most widely used OP insecticides effective against a broad spectrum of insect pests of economically important crops. It is also used for the control of mosquitoes (larvae and adults), flies, termites and various soil and household pests. The half-life of chlorpyrifos in soil is usually between 60 and 120 days, but can range from 2 weeks to over 1 year, depending on the soil type, climate, and other conditions [5]. Initially, degradation of pesticide was observed in alkaline soils and phenomenon was related to its hydrolysis at high pH. However when the several high pH soils were sterile, complete inhibition of chlorpyrifos hydrolysis was observed which indicated the involvement of soil microorganisms [6]. Later, same results were confirmed by Singh et al. [7].

It has been problematic to isolate a pure bacterial strain capable of degrading chlorpyrifos because this organophosphate is resistant towards enhanced biodegradation. Several fruitless attempts were made to isolate a chlorpyrifos degrading microbial system by repeated treatments or enrichment of soil samples [8,9]. Generally, soil microorganisms that repeatedly or continuously encounter synthetic toxic chemicals develop capabilities to degrade such chemicals and such microorganisms with newly evolved traits have been implicated in the rapid inactivation of pesticides in problem soils [10]. A possible reason of for resistance of chlorpyrifos to enhanced biodegradation was attributed to toxic effects of 3,5,6-trichloro-2-pyridinol (TCP), the hydrolytic product of chlorpyrifos [11].

Singh et al. [12] isolated six chlorpyrifos degrading bacteria from an Australian soil showing enhanced degradation of chlorpyrifos.

* Corresponding author at: Environmental Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan. Tel.: +92 41 2651475x294/292; fax: +92 41 2651472.
E-mail address: siqbal@nibge.org (S. Iqbal).

Out of six, one strain *Enterobacter* sp. was able to hydrolyze this pesticide. Li et al. [13] isolated a strain belonging to the genus *Sphingomonas* capable of hydrolyzing chlorpyrifos ($100 \, mg \, L^{-1}$) into 3,5,6-trichloro-2-pyridinol (TCP).

Environmental factors such as physical and chemical characteristics of the substrate, nutrients status, pH, temperature and biotic factors such inoculum density prejudice the accomplishment of any bioremediation process [14,15]. It was reported that a *Pseudomonas* sp. was less successful in degrading atrazine in soil with lower pH and higher organic matter [16]. While high pH of soil played an important role in biodegradation of chlorpyrifos [7].

Similarly inoculum size has been identified as a possible reason for the failure or success of bioremediation process of pesticide contaminated sites when inoculated with species able to degrade the contaminant [15]. Inoculum level of 10^6–10^8 cells g^{-1} soil was recommendable to use for the decontamination of pesticide contaminated sites [14]. However in a similar study, Struthers et al. [17] found that inoculum levels of an *Agrobacterium* strain as low as 10^5 cells g^{-1} were adequate to rapidly degrade atrazine. Pesticide concentration was another reason for bioremediation failure [18]. Singh et al. [19] also studied the influence of different environmental conditions on bioremediation of chlorpyrifos and fenamiphos in soil and water to study the bioremedial potential of isolated strains for these two pesticides.

The major hydrolysis product of chlorpyrifos, 3,5,6-trichloro-2-pyridinol (TCP) has greater water solubility than chlorpyrifos and causes the widespread contamination in soils and in the aquatic environment. TCP is not only persistent towards degradation by microorganisms but also limits the biodegradation of chlorpyrifos owing to its antimicrobial activities [9,20,21]. In contrast to the importance of TCP degradation issue, studies regarding its fate and degradation in the environment are scarce.

In the present study, a bacterium *Bacillus pumilus* C2A1 capable of degrading not only chlorpyrifos but also TCP was isolated. Biodegradation of chlorpyrifos and TCP in liquid culture under different environmental factors like different concentrations of pesticide, pH of media and inoculum size of the isolated strain (in liquid culture) were investigated to optimize the conditions for biodegradation of chlorpyrifos by this strain. The study aims at elucidating a possible application of isolated bacterial strain for remediation of chlorpyrifos contaminated environment.

2. Materials and methods

2.1. Chemicals

Analytical grade Chlorpyrifos (98.4%) obtained from Dr. Ehrenstorfer GmbH (Germany) was used as a standard. Technical grade Chlorpyrifos (95%) used in this study was obtained from Pak China Chemicals, Lahore. Trichloropyridinol (TCP, 99%) was purchased from Chem. Service (Web Chester). HPLC grade acetonitrile was procured from Merck. All other chemicals used were purchased from Sigma–Aldrich, Merck or BDH.

2.2. Enrichment, isolation and selection of bacterial strains

Soil samples collected from cotton fields at National Institute for Biotechnology and Genetic Engineering NIBGE (where the pesticide in question is sprayed extensively) were used for enrichment of pesticide degrading strains at $30 \, mg \, L^{-1}$ and $60 \, mg \, L^{-1}$ pesticide (chlorpyrifos) concentrations individually. Microbial isolations were carried out in minimal salt medium (MSM, pH 6.8–7.0) containing (g L^{-1}) Na_2HPO_4, 5.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1; and $MgSO_4$, 0.25. Approximately 20 g soil was added to 50 mL MSM

containing chlorpyrifos and cultured in 250 mL Erlenmeyer flasks on a rotary shaker (at 100 rpm) incubated at room temperature. After two weeks, 5 mL culture was recovered from each replicate and transferred to fresh MSM containing chlorpyrifos ($30 \, mg \, L^{-1}$ and $60 \, mg \, L^{-1}$ separately). Afterwards, three successive transfers were carried out in fresh MSM containing chlorpyrifos as the only carbon source by sub culturing 5 mL inoculum every time and incubating for 2–4 weeks each. Moisture contents were maintained throughout the experiment by regular addition of sterile distilled water.

Two weeks following the last transfer, 10-fold dilutions of cultures were prepared and 100 μL of each dilution was spread on nutrient agar (NA) plates containing $60 \, mg \, L^{-1}$ chlorpyrifos. Isolated colonies were streaked onto NA plates containing chlorpyrifos and purified by repeated streaking. Once all isolates obtained were purified, they were tested for growth and hence chlorpyrifos utilization and by streaking them on MSM agar plates containing $50 \, mg \, L^{-1}$ chlorpyrifos as sole carbon and energy source. At the same time their growth was also monitored in MSM containing chlorpyrifos in shake flasks incubated at 37 °C and 100 rpm. Residual concentration of chlorpyrifos at this stage was determined by HPLC. One strain, designated C2A1, which possessed the highest degrading capacity, was selected for further chlorpyrifos degrading studies.

2.3. Taxonomic identification of the bacterial strain

For sequencing of the 16S rRNA gene, total genomic DNA was extracted from the bacterial strains as described by Wilson et al. [22]. The 16S rRNA gene was amplified using the universal primers, FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′; *Escherichia coli* bases 8–27) and RP1 (5′-ACGGHTACCTTGTTTACGACTT-3′, *E. coli* bases 1507–1492) [23].

PCR products consisting of the amplified 16S rRNA gene fragment were purified with QIAQuick spin column (QIAGEN) and cloned into pTZ57R TA cloning vector. The cloned vector was transformed into *E. coli* Top10 and plasmids were obtained by using a Fermentas Miniprep DNA Purification Kit for nucleotide sequencing.

2.4. Inoculum preparation

Seed culture of strain C2A1 was grown in nutrient broth medium, harvested by centrifugation at $4600 \times g$ for 5 min, washed with autoclaved N-saline (0.9% NaCl in distilled water) and resuspended in N-saline to set an $OD_{590 \, nm}$ of 0.7. Colony forming units (CFU mL^{-1}) of this suspension were quantified by the dilution plate count technique. Two percent of this suspension was used as inoculum for chlorpyrifos biodegradation studies until otherwise mentioned.

2.5. Extraction of samples (pesticide residues) for HPLC analysis

Samples (5–10 mL) were recovered from culture flasks and centrifuged at $7200 \times g$ for 10 min to obtain cell free medium. Chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) residues were extracted from supernatant using equal volume of dichloromethane (DCM) twice. Organic layers of DCM were aspirated, pooled and evaporated at room temperature under nitrogen. The residues were dissolved in HPLC grade acetonitrile (1 mL) and then filtered through flouropore™ filter membrane (0.45 μm FH) to remove any particles. Before HPLC analysis, these samples were diluted if required. Pesticide and TCP concentrations of the filtrates were measured by HPLC as described in Section 2.6 and those of the culture medium was calculated.

2.6. HPLC conditions and analysis

Extracted samples were analyzed on a Varian HPLC equipped with a ternary gradient pump, programmable variable-wavelength UV detector, column oven, and electric sample valve and ODS2 C_{18} reversed-phase column. The pesticide residues analyses were conducted using a gradient mobile phase of acetonitrile:water:acetic acid as described earlier [24]. Sample injection volume was 20 μL, the mobile phase was programmed at flow rate of 1 mL min^{-1} and chlorpyrifos was detected at 290 nm and 320 nm wavelengths. The retention time for chlorpyrifos and 3,5,6-trichloropyridinol was 15 min and 7.8 min respectively.

2.7. Biodegradation of chlorpyrifos

Shake flask studies were carried to work out the chlorpyrifos degrading capacity of the best-isolated strain so far, i.e. C2A1. MSM (30 mL) containing 50 mg L^{-1} chlorpyrifos (as sole source of carbon and energy) were inoculated (2% inoculum) with C2A1 and incubated at 37 °C and 100 rpm on a rotary shaker for 5 days. At 24 h intervals, samples from the culture medium were recovered aseptically and residual pesticide concentration determined using HPLC. Uninoculated flasks with MSM containing 50 mg L^{-1} chlorpyrifos served as controls. The experiment was conducted in duplicate. Biodegradation of chlorpyrifos by strain C2A1 at high concentrations, i.e. 100 mg L^{-1}, 200 mg L^{-1}, 300 mg L^{-1}, 500 mg L^{-1} and 1000 mg L^{-1} was studied in 500 mL flasks containing 150 mL MSM (in duplicate). Duplicate flasks without inoculum were kept as controls. Samples (10 mL) were recovered at 24 h intervals from flasks containing 100 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1} chlorpyrifos for 10 days of incubation. In case of 500 mg L^{-1} and 1000 mg L^{-1}, sampling was done after each 4 days of incubation. Extraction of pesticide residues from representative samples and HPLC analysis for quantitative determination of the pesticide was done as described in Sections 2.5 and 2.6.

2.8. Effect of alternate carbon source/nutrients on biodegradation of chlorpyrifos

Glucose, nutrient broth and yeast extract were used to examine the effect of nutrients and/or alternate carbon source on the chlorpyrifos degrading capability of the strain C2A1. Minimal salt medium (30 mL) supplemented with glucose, nutrient broth and yeast extract (separately) and 50 mg L^{-1} chlorpyrifos inoculated with C2A1 (in duplicate). All the three nutrients were added aseptically in MSM to the final concentration of 1 g L^{-1} separately. The flasks were incubated at 37 °C and 100 rpm on a rotary shaker and pesticide concentration was determined. Bacterial growth in terms of colony forming unit (CFU) was also monitored.

2.9. Effect of inoculum density on biodegradation of chlorpyrifos

Liquid cultures of strain C2A1 in nutrient broth containing chlorpyrifos (50 mg L^{-1}) were prepared. After 24 h of incubation, culture medium was centrifuged at 4600 × *g* for 5 min, washed with autoclaved N-saline and resuspended in N-saline to set an OD$_{590 nm}$ of 0.8. CFU of the suspension was determined and inocula containing 1.5×10^9 CFU mL^{-1}, 1.5×10^7 CFU mL^{-1}, and 1.5×10^5 CFU mL^{-1} were prepared by adding appropriate amount of N-saline. These inocula (2%) were added to MSM containing 50 mg L^{-1} chlorpyrifos. Residual pesticide concentration was determined at 24 h intervals.

2.10. Effect of pH on biodegradation of chlorpyrifos

Chlorpyrifos degrading ability of C2A1 was also determined at three different pH conditions of minimal salt media, i.e. acidic (5.5),

neutral (7.0) and basic (8.5). Solutions of Na$_2$HPO$_4$ (5.8 mg L^{-1}) and KH$_2$PO$_4$ (3.0 mg L^{-1}) were mixed in such proportion to achieve buffer solutions in MSM (containing NaCl, 0.5 g L^{-1}; NH$_4$Cl, 1 g L^{-1}; and MgSO$_4$, 0.25 g L^{-1}) at pH 5.5, 7.0 and 8.5. MSM media at different pH containing chlorpyrifos (50 mg L^{-1}) were inoculated to study the effect of pH on chlorpyrifos degradation.

2.11. Degradation of 3,5,6-trichloro-2-pyridinol (TCP)

Different concentrations of TCP (100 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1}) in MSM were inoculated with and incubated at 37 °C and 100 rpm on a rotary shaker. TCP residues were extracted using dichloromethane after 2, 4, 6, and 8 days of incubation and residual TCP was determined by HPLC.

3. Results and discussion

3.1. Isolation and characterization of chlorpyrifos degrading bacterium

Soil samples collected from cotton fields of NIBGE (already exposed to pesticides belonging to different chemical classes, i.e. organophosphates, carbamates, nitro-guanidine and pyrethroids) were enriched in the presence of chlorpyrifos to isolate the chlorpyrifos degrading bacteria. From this enrichment culture, 22 morphologically different strains were isolated on nutrient agar plates containing chlorpyrifos. The chlorpyrifos utilization ability of these strains was checked on MSM/agar plates containing chlorpyrifos (40 mg L^{-1}) as sole source of carbon and energy. Of 22 strains tested, 11 strains were capable to grow on MSM/agar plates supplemented with chlorpyrifos. These strains were inoculated in MSM containing 50 mg L^{-1} chlorpyrifos and incubated at 37 °C. Pesticide utilization ability was confirmed by quantitative HPLC analysis of the residual pesticide. Two strains C2A1 and C2A2 were able to grow in this medium utilizing 74% and 69% chlorpyrifos within 3 days of incubation. In the present studies, strain C2A1 (showing highest degradation) was employed for detailed chlorpyrifos degradation studies under various culture conditions. Aislabie and Jones [25] proposed that bacteria present in the field are able to utilize the pesticide as a substrate for growth and their numbers are enhanced on subsequent application. This is related to an increase in the biological capacity of the soil to degrade these products due to the proliferation of microorganisms using the pesticide as a source of carbon and energy. This phenomenon is called enhanced biodegradation of pesticides [10,26].

3.2. Identification of strain C2A1

Colonies of strain C2A1 on nutrient agar plates appeared circular with convex elevation and entire (even) margin. This strain was motile, gram positive and small rod shaped. Biochemical tests for strain C2A1 are described in Table 1. Sequence analysis of 16S rRNA gene using BLAST showed that strain C2A1 was *B. pumilus*. To our knowledge, this is the first report for biodegradation of chlorpyrifos by *B. pumilus*, a gram positive bacterium. Previously, *B. pumilus* has been reported to degrade carbofuran [27] and bisphenol A [28].

3.3. Degradation of chlorpyrifos by B. pumilus C2A1

Time course studies for degradation of chlorpyrifos by *B. pumilus* C2A1 indicated that only 6% of the added pesticide was degraded in 24 h after which rapid degradation started. After completion of the experiment 82% degradation was achieved leaving 9 mg L^{-1} chlorpyrifos in culture medium. Also degradation rate decreased

Table 1
Biochemical characteristics of *Bacillus pumilus* C2A1.

Biochemical test	Results
(1) Catalase	Positive
(2) Gelatin liquification	Positive
(3) Voges–Proskauer	Positive
(4) Arabinose utilization	Positive
(5) Mannitol utilization	Positive
(6) O-nitrophenyl-β-galactopyranoside (ONPG)	Negative
(7) Arginine dihydrolase (ADH)	Negative
(8) Lysine decarboxylase (LDC)	Negative
(9) Ornithine decarboxylase (ODC)	Negative
(10) Hydrogen sulfide (H$_2$S)	Negative
(11) Tryptophan deaminase (TDA)	Negative
(12) Indole	Negative
(13) Citrate utilization	Negative

Fig. 2. Degradation of chlorpyrifos by *Bacillus pumilus* strain C2A1 in minimal salt medium (MSM) containing 50 mg L^{-1} chlorpyrifos as the sole source of carbon and energy (▲) and in the presence of other nutrients, i.e. yeast extract (♦), nutrient broth (●), glucose (■) and control (▼).

significantly when a lower concentration was reached which might be due to elevated expression of enzymes at higher concentrations. No degradation was observed in uninoculated control. This showed that strain C2A1 was proficient for biodegradation of chlorpyrifos. When exposed to higher concentrations of chlorpyrifos, *B. pumilus* C2A1 showed efficient degradation. At 100 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1} concentrations, pesticide degradation started after 2, 2 and 3 days of incubation respectively and 73%, 83% and 87% of the added chlorpyrifos was degraded within 10 days (Fig. 1). At relatively higher concentrations, i.e. 500 mg L^{-1} and 1000 mg L^{-1}, chlorpyrifos degradation started after 7 and 10 days of incubation degrading 88% and 89% of pesticide respectively after two weeks (data not shown). There are reports regarding microbial degradation of pesticide like ethoprophos and others at high concentrations [29–31]. However maximal concentration of chlorpyrifos at which microbial degradation of this pesticide has been reported is 250 mg L^{-1} [12]. To our knowledge *B. pumilus* C2A1 is the first reported strain that can tolerate/degrade chlorpyrifos up to 1000 mg L^{-1}. During degradation studies at high chlorpyrifos concentrations, longer lag phase was observed with increasing concentration of pesticide. Karpouzas and Walker [29] proposed that longer lag phase at higher concentration might be because greater number of bacteria are needed to initiate rapid degradation of pesticide. Possibly biodegradation starts slowly and requires an acclimation period before rapid degradation occurs. It has been suggested [32] that at constant biomass or low substrate levels, the degradation rate is proportional to the concentration of residual substrate, which falls off continually.
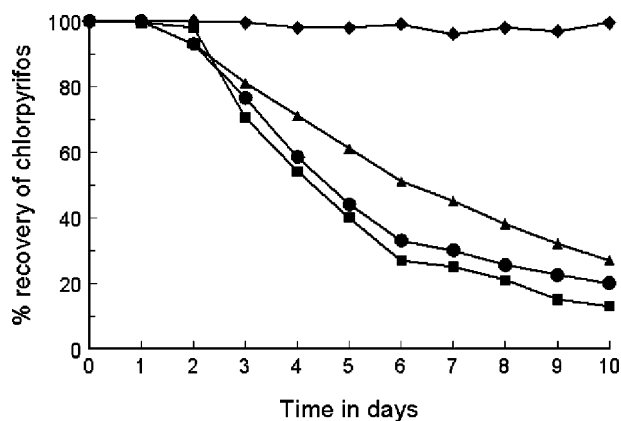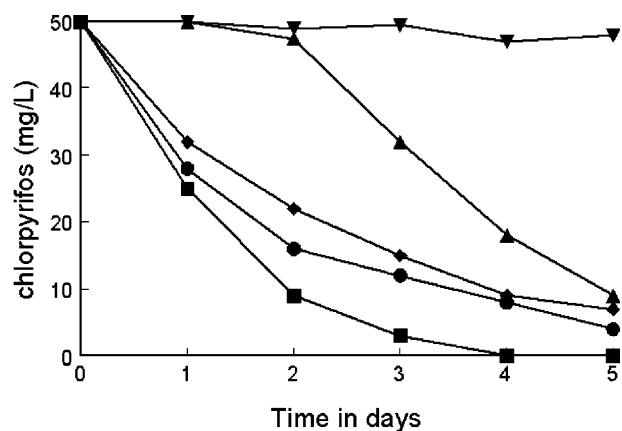
3.4. Effect of alternate carbon source and nutrients on degradation of chlorpyrifos

The strain C2A1 inoculated in MSM containing 50 mg L^{-1} started degrading chlorpyrifos after 24 h of incubation. It degraded 82% of pesticide within 5 days of incubation leaving 9 mg L^{-1} of chlorpyrifos. When yeast extract, nutrient broth and glucose were added to the culture medium, bacterium utilized 86%, 92% and 100% of pesticide respectively (Fig. 2). In the presence of all three nutrients nearly 50% chlorpyrifos degraded within 24 h of incubation. Glucose enhanced the degradation rate of isolate as it completely degraded the pesticide within 3 days of incubation. This result contrasts with previous findings of Singh et al. [12] who reported that with addition of carbon sources, an *Enterobacter* strain stopped degrading chlorpyrifos and after 3 days of incubation started utilizing chlorpyrifos again. Our findings revealed that *Bacillus* preferred to utilize chlorpyrifos even in the presence of nutrient rich environment and its degrading ability was positively influenced by the presence of the supplementary nutrient sources. This might be because potentially the chlorpyrifos degrading enzymes in *B. pumilus* are expressed even in the presence of readily available carbon sources. No appreciable degradation of chlorpyrifos was observed in any of the uninoculated samples.

In this experiment, bacterial growth in terms of colony forming unit was also monitored. It was observed that CFU mL^{-1} of cultures containing glucose, nutrient broth and yeast extract (separately) along with chlorpyrifos was higher than cultures containing chlorpyrifos as sole source of carbon and energy (Fig. 3).

3.5. Effect of inoculum density on degradation of chlorpyrifos

Chlorpyrifos was degraded by *B. pumilus* C2A1 during incubation with all the three initial inoculum densities tested. In cultures inoculated with highest initial cell density, i.e. 10^9 CFU mL^{-1}, chlorpyrifos degradation started rapidly as utilization was observed within 24 h of incubation, apparently there was no lag phase and more than 80% degradation was deliberated after 5 days of incubation (Fig. 4). However in cultures with lower inoculum densities, i.e. 10^5 CFU mL^{-1} and 10^7 CFU mL^{-1} of *B. pumilus* C2A1 there were longer lag periods as degradation chlorpyrifos started after 1 and 2 days of incubation and resulted in 50% and 72% degradation of pesticide respectively after 5 days of incubation.

In general, a smaller inoculum density resulted in longer lag phases before rapid degradation of chlorpyrifos started. It has been suggested that the acclimation period during degradation of xeno-
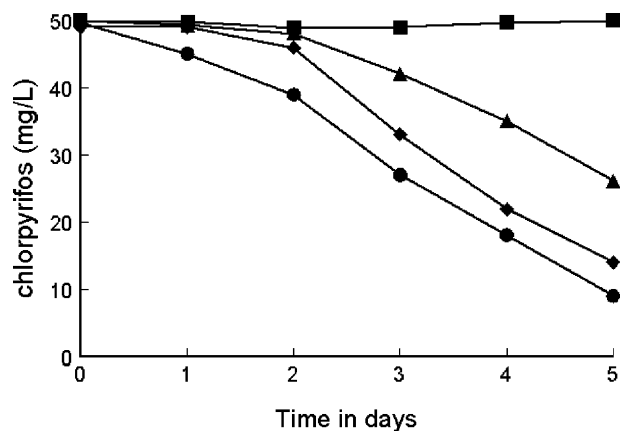

Fig. 1. Degradation of chlorpyrifos by *Bacillus pumilus* strain C2A1 in minimal salt medium (MSM) containing chlorpyrifos as the sole source of carbon and energy at concentrations 100 mg L^{-1} (▲), 200 mg L^{-1} (●) and 300 mg L^{-1} (■), and control without inoculum (♦).

Fig. 3. Growth (colony forming units; CFU) of *Bacillus pumilus* C2A1 in minimal salt medium (MSM) containing 50 mg L^{-1} chlorpyrifos as the sole source of carbon and energy (▲) and in the presence of other nutrients, i.e. yeast extract (♦), nutrient broth (●) and glucose (■).

biotics reflect the time required for multiplication of a small active population to a certain level which is sufficient to rapidly degrade the xenobiotic in question [33]. Similar correlation (as found in the present study) between the length of acclimation period and inoculum density was observed when liquid cultures containing 2,4-D were inoculated with different inoculum densities of *Pseudomonas cepacia* strain BRI6001, a 2,4-D degrading bacterium [34].

3.6. Effect of media pH on biodegradation of chlorpyrifos

One important factor, which influences the degrading ability of microorganisms capable of degrading xenobiotic compounds, is pH. The chlorpyrifos degrading ability of strain C2A1 was studied at three different pH conditions, i.e. acidic (5.5), neutral (6.8) and basic (8.5) in MSM supplemented with 50 mg L^{-1} chlorpyrifos along with control. *B. pumilus* C2A1 showed degradation at all the pH (acidic to alkaline) tested. However, at acidic pH nearly 50% degradation was observed with longer lag phase. At relatively higher pH no lag phase was observed and chlorpyrifos was more efficiently degraded at basic as well as neutral pH whereby more than 80% of the added pesticide was degraded (Fig. 5).

Singh et al. [7] reported rapid degradation of chlorpyrifos by an *Enterobactor* sp. at high pH while it was very slow at acidic pH. However, Karpouzas and Walker [30] reported that two strains of
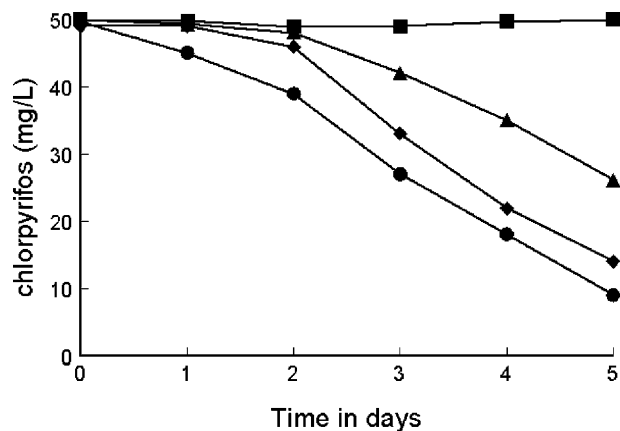

Fig. 4. Degradation of chlorpyrifos by *Bacillus pumilus* strain C2A1 in minimal salt medium (MSM) containing 50 mg L^{-1} chlorpyrifos as the sole source of carbon and energy at various inoculum densities, i.e. 1.5×10^5 CFU mL^{-1} (▲), 1.5×10^7 CFU mL^{-1} (♦) and 1.5×10^9 CFU mL^{-1} (●), and uninoculated control (■).
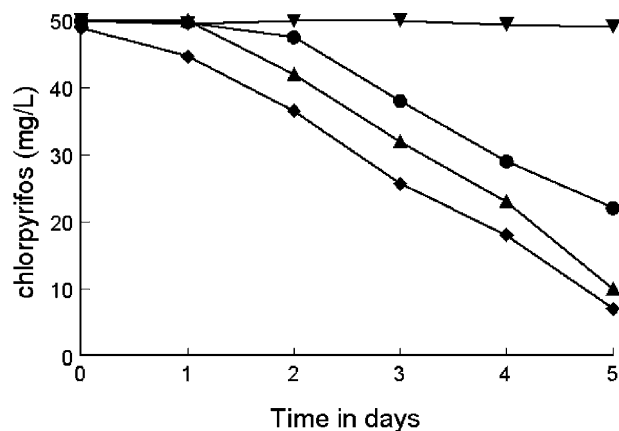

Fig. 5. Degradation of chlorpyrifos by *Bacillus pumilus* C2A1 in minimal salt medium (MSM) containing chlorpyrifos (50 mg L^{-1}) as the sole source of carbon and energy at various medium pH, i.e. 5.5 (●), 7.0 (▲) and 8.5 (♦), and uninoculated control (▼).

Pseudomonas putida (epI and epII) rapidly degraded ethoprophos (an organophosphate pesticide) in minimal medium at pH ranging from 5.5 to 7.6. In the present studies, maximum degradation was achieved at high pH. It is possible that some key enzyme(s) responsible for chlorpyrifos degradation have their optimum enzymatic activity at high pH.

3.7. Biodegradation of trichloropyridinol (TCP)

To study biodegradation of trichloropyridinol (TCP) which is the first metabolite after hydrolysis of chlorpyrifos, *B. pumilus* C2A1 was inoculated in MSM containing 100 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1} TCP. The metabolite utilization by this strain was confirmed by HPLC analysis. It was found that 90% of the added TCP (300 mg L^{-1}) was degraded within 8 days of incubation (Fig. 6). It has been suggested [9] that chlorpyrifos hydrolyzes to TCP and that because the TCP has toxic effects, normally enhanced degradation of chlorpyrifos does not occur. However, in the present studies degradation of chlorpyrifos and TCP were both achieved by the same strain which is a rare finding. Yang et al. [35] reported an *Alcaligenes faecalis* capable to degrade both chlorpyrifos and TCP (100 mg L^{-1}). *B. pumilus* C2A1 showed utilization of both chlorpyrifos and TCP up to a concentration of 300 mg L^{-1} which was three times higher than that reported for *A. faecalis* [35]. Feng et al. [36] reported a *Pseudomonas* sp. that can mineralize TCP in liquid medium but
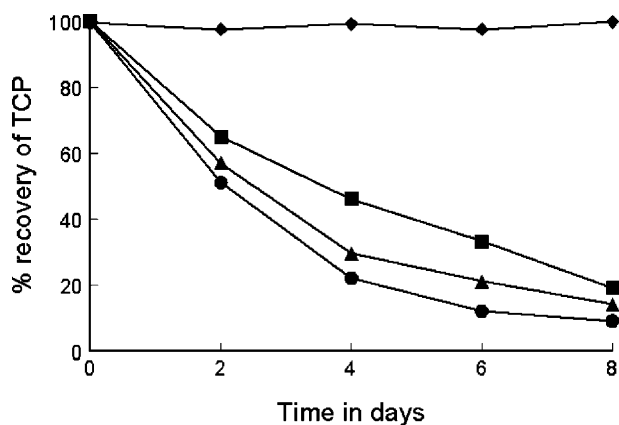

Fig. 6. Degradation of 3,5,6-trichloro-2-pyridinol (TCP) by *Bacillus pumilus* C2A1 in minimal salt medium (MSM) containing TCP as the sole source of carbon and energy at concentrations 100 mg L^{-1} (■), 200 mg L^{-1} (▲) and 300 mg L^{-1} (●), and control without inoculum (♦).

not chlorpyrifos. Owing to its antimicrobial properties, microbial degradation of TCP was rarely observed/reported. Potentially, recalcitrance of TCP might be attributed to the presence of three chlorine atoms on the pyridinol ring which must be removed before the ring breaks [21]. In our study *B. pumilus* C2A1 was found to degrade not only chlorpyrifos but its primary metabolite TCP as well. Moreover, during biodegradation studies of chlorpyrifos (Sections 3.5 and 3.6) under different conditions, no accumulation of TCP was found that showed the efficiency of this strain to degrade both chlorpyrifos and TCP.

4. Conclusion

Present studies revealed that changes in pH of the culture medium and inoculum density affected chlorpyrifos degradation by *B. pumilus* C2A1. Alkaline pH and high inoculum density were essential to achieve maximal degradation in this batch experiment system. *B. pumilus* strain C2A1 was capable to degrade chlorpyrifos over a wide range of pH particularly at pH as low as 5.5. This is an important feature of an organism to be employed for bioremediation of variable environments. *B. pumilus* C2A1 was able to tolerate chlorpyrifos concentration as high as $1000\,mg\,L^{-1}$. Another important feature which is worth mentioning is that this particular strain was capable of degrading TCP. Degradation of this compound by the same strain that degrades chlorpyrifos is very important because TCP is generally antimicrobial, and if it is not degraded it would accumulate in the culture medium and suppress microbial growth and hence degradation of the parent compound through catabolite repression. The information provided here can be used to optimize degradation conditions in the field. However, work continues to study the conditions for *in situ* bioremediation on chlorpyrifos contaminated soils.

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