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RESEARCH ARTICLE

Chlorpyrifos degradation by the cyanobacterium *Synechocystis* sp. strain PUPCCC 64

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Abstract
Background, aim, and scope Indiscriminate use of insecticides leads to environmental problems and poses a great threat to beneficial microorganisms. The aim of the present work was to study chlorpyrifos degradation by a rice field cyanobacterium *Synechocystis* sp. strain PUPCCC 64 so that the organism is able to reduce insecticide pollution in situ.
Material and methods The unicellular cyanobacterium isolated and purified from a rice field was identified by partial 16S rRNA gene sequence as *Synechocystis* sp. strain PUPCCC 64. Tolerance limit of the organism was determined by studying its growth in graded concentrations (2.5–20 mg/L) of chlorpyrifos. Chlorpyrifos removal was studied by its depletion from the insecticide supplemented growth medium, and its biodegradation products were identified in the cell extract, biomass wash, and growth medium.
Results and discussion The organism tolerated chlorpyrifos up to 15 mg/L. Major fraction of chlorpyrifos was removed by the organism during the first day followed by slow uptake. Biomass, pH, and temperature influenced the insecticide removal and the organism exhibited maximum chlorpyrifos removal at 100 mg protein/L biomass, pH 7.0, and 30°C. The cyanobacterium metabolized chlorpyrifos producing a number of degradation products as evidenced by GC-MS chromatogram. One of the degradation products was identified as 3,5,6-trichloro-2-pyridinol.
Conclusion and recommendations Present study reports the biodegradation of chlorpyrifos by *Synechocystis* sp. Biodegradation of the insecticide by the cyanobacterium is significant as it can be biologically removed from the environment. The cyanobacterium may be used for bioremediation of chlorpyrifos-contaminated soils.

Keywords Biodegradation · Bioremediation · Chlorpyrifos · Cyanobacterium · *Synechocystis* · 3,5,6-trichloro-2-pyridinol

1 Background, aim, and scope

Rice is an important cereal crop of the Asian countries. More than two billion people worldwide consume rice as a staple food. In India, rice is cultivated in about 44.3 million hectares producing 141 million metric tons of grains annually (Yadav et al. 2010). More than 70 species of insects, pests, and fungi attack rice crop causing a great loss to yield. The farmers are thus forced to use a large number of pesticides to protect the seedlings and the crop. However, indiscriminate use of pesticides poses a great danger to beneficial microflora of rice fields including cyanobacteria (Kumar et al. 2008; Shen et al. 2009; Galhano et al. 2010). Cyanobacteria are an important component of rice field ecosystems as they contribute to the soil fertility as natural biofertilizers (Kumar and Kumar 1998; Singh and Datta 2006).

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Considerable amount of work has been reported related to pesticide-induced inhibitory effects on growth, photosynthetic pigments, photosynthesis, and nitrogen fixation in cyanobacteria (Mohapatra et al. 2003; Jha and Mishra 2005; Prasad et al. 2005; Singh and Datta 2006; Chen et al. 2007). A few reports are also available for pesticides degradation by cyanobacteria (Lee et al. 2003; Barton et al. 2004; El-Bestawy et al. 2007; Cáceres et al. 2008).

Chlorpyrifos is applied on a large scale in rice fields of Punjab state of India as a broad spectrum organophosphate insecticide for the control of foliar insects. The physico-chemical properties of chlorpyrifos are given in Table 1. Chlorpyrifos remains biologically active in soil for periods ranging from 20 to 90 days and is moderately persistent, with half-life varying from 10 to 60 days (Getzin 1981a; Lakshmi et al. 2008). This range in half-life is due to the fact that degradation of chlorpyrifos in soil is affected by its initial concentration, soil moisture, temperature, and pH (Racke et al. 1994; Awasthi and Prakash 1997). Major routes of chlorpyrifos degradation are volatilization, microbial degradation, and chemical hydrolysis on dry soil surfaces (Getzin 1981a, b; Racke et al. 1988).

Reports are available on chlorpyrifos residues in food chain (Aysal et al. 2004). Maximum chlorpyrifos residue level of 0.5 mg/kg for rice, a supervised trials median residue of 0.12 mg/kg, and a highest residue level of 0.28 mg/kg were estimated from data of supervised trials on rice conforming to Good Agricultural Practise conducted in Columbia, the Philippines, Thailand, Vietnam, and India (WHO and FAO 2004). Chandra et al. (2010) have detected residual chlorpyrifos in the range of 0.024–0.07 mg/kg of cauliflower and 0.018–0.021 mg/kg of brinzal. The LD_{50} for chlorpyrifos in rat has been reported to be 82–270 mg/kg body weight (Berg 1986; US Environment Protection Society 1984). These reports indicate that indiscriminate use of chlorpyrifos may cause serious human health problems. There is growing concern about the toxicological and environmental risks associated with chlorpyrifos residues. The persistent nature of the insecticide is a health hazard, and thus, there is a need to detoxify this moiety (Mukherjee et al. 2004).

Cyanobacterial biofertilizers are added to rice fields to increase the fertility of soil and to minimize dependence on chemical fertilizers. The aim of the present study was to investigate whether *Synechocystis* sp. growing in rice fields is able to degrade chlorpyrifos so that this strain can be recommended for inoculation in rice fields along with cyanobacterial biofertilizers to minimize deleterious effects of chlorpyrifos.

2 Material and methods

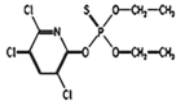
2.1 Chemicals

All the chemicals used in the media preparation and assay of insecticide were obtained from Merck, India. Commercial grade chlorpyrifos (Chlorvip 20%, *w/v*) used during the present study was manufactured by Godrej India Ltd. and purchased from the local market. Standards of chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) were obtained from Sigma-Aldrich Co., USA.

2.2 Isolation, identification, and culture conditions

Synechocystis PUPCCC 64 was isolated from a rice field of the village Derabassi (30° 58′ 72″ N; 76° 8′ 28″ E) of Patiala district of Punjab state, India. Isolation and purification of the organism were performed by serial dilution and plating method (Stanier et al. 1971). The organism was identified following Komárek and Anagnostidis (1998), and its identification was confirmed on the basis of partial 16S rRNA gene sequence. Genomic DNA extraction was done by HiPurA™ plant genomic DNA Miniprep Purification Spin kit (HIMEDIA®, Mumbai, India). The 16S rRNA gene was amplified using cyanobacteria-specific primers namely CYA359F and CYA781R (Nübel et al. 1997). The total 50 μL PCR reaction mixture was comprised of 200 μM dNTPs, 50 μM of each primer, 1× PCR buffer, 3 U *Taq* polymerase, and 100 ng genomic DNA. The thermocycling procedure involved an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for

Table 1 Physico-chemical characteristics of chlorpyrifos (Venkta Mohan et al. 2004)

Chemical name	Molecular weight	CAS registry number	Formula	Structure	Vapour pressure at 25 °C (MPa)	Activity	Soil sorption coefficient (mg/g)	Solubility at 25 °C in water (mg/L)
O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate	350.62	2921-88-2	$C_9H_{11}Cl_3NO_3PS$		2.49	insecticidal	849	1.39

1 min, 52°C for 1 min, 72°C for 2 min, and final extension at 72°C for 8 min. The gel-purified product was obtained using Real Genomics™ Gel DNA Extraction kit (Real Biotech Corporation, Taipei Country, Taiwan). The sequencing was done using BigDye®Terminator v3.1 cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA). The sequence was analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm and aligned to near neighbors. Phylogenetic tree was constructed using MEGA4 software package (Tamura et al. 2007).

The organism was grown photoautotrophically in batch cultures in modified Chu-10 medium (Safferman and Morris 1964) containing potassium nitrate (10 mM) as nitrogen source in 250 mL Erlenmeyer flasks. The stock and experimental cultures were maintained in a culture room at 28°C±2°C. The surface of culture vessels was illuminated with fluorescent tubes giving photon flux of 44.5 μmol m^{-2} s^{-1} with light/dark cycle of 14/10 h. The culture vessels were hand-shaken four to five times daily to keep the cultures in homogenous state.

2.3 Chlorpyrifos exposure

Graded concentrations (2.5–20 mg/L) of chlorpyrifos were prepared in culture medium. The growth experiments were conducted in 250 mL Erlenmeyer flasks with 100 mL cultures. Exponentially growing cultures were concentrated by centrifugation at 5,000×*g* and washed thrice with double distilled sterilized water and inoculated in flasks to obtain initial absorbance 0.1 at 720 nm (2.5 μg chl/mL culture), and cell number was ascertained (1.3×10^7 cells/mL). At regular intervals of 2 days, extending up to 16 days, aliquots were withdrawn and increase in cell population was ascertained by counting the number of cells using a Neubauer hemocytometer (Marienfeld, Germany). The average of 20 counts was taken as cell number data. Percent inhibition of growth was calculated by taking log-phase growth data of day 12. Generation time of the organism in graded concentrations of chlorpyrifos was determined from the linear portion of growth curve following Forlani et al. (2008).

2.4 Chlorpyrifos uptake experiments

Chlorpyrifos uptake experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL cultures supplemented with 5 mg/L chlorpyrifos (double the recommended dose of field application). At this concentration, there is 20% inhibition in the growth of the organism. So the selected concentration, at one hand, is more than the recommended field application dose, and on the other hand, it is not causing profound effect on growth of the organism. Exponentially growing cultures were inoculated to get initial absorbance 0.5 at 720 nm (12.5 μg chl/mL culture). At regular intervals, 3 mL cultures were withdrawn and centrifuged at 5,000×*g*. Chlorpyrifos was extracted three times from the supernatant with dichloromethane, followed by three times with hexane. The collected solvents were allowed to dry and the final volume of extracted chlorpyrifos was made in hexane. The recovery efficiency of chlorpyrifos was 95%. Chlorpyrifos was quantified by injecting 1 μL splitless injection of extracted sample in a gas chromatograph (GC-17 A, Shimadzu, Japan). The GC operating parameters were: capillary column BP (100% dimethyl polysiloxane), length 30 m, internal diameter 0.25 mm; oven temperature 250°C; injection temperature 270°C; detector temperature 290°C; detector, ECD; carrier gas and flow, Helium, 1 mL/min.

2.5 Identification of degradation products of chlorpyrifos

Exponentially growing washed cultures of the organism were inoculated in 500 mL Erlenmeyer flasks containing 250 mL medium supplemented with chlorpyrifos (5 mg/L) to attain biomass load of 50 mg protein/L culture. After 60-h incubation, biomass was harvested by centrifugation (5,000×*g*) and supernatant kept. Cell pellet was washed with distilled water to remove any insecticide residues adhering to the cell surface. The biomass and cell wash were saved for detection of chlorpyrifos products. Cells were disintegrated with the help of a sonicator (Soniprep 150, Sanyo, UK) by giving 30 pulses (5 μm amplitude) each of 1 min, with interval of 30 s. Chlorpyrifos residue was extracted from the supernatant, biomass wash, and cell-free extract as described in section 2.4. Chlorpyrifos and its degradation products were analyzed by capillary gas chromatography–mass spectrometry in selected ion monitoring mode (GC-MS/SIM) (Wong et al. 2010). In this technique, mass spectrometer was set to scan over range of 1 unit. A plot of the ions current resulting from this very small range of mass was detected and plotted. The GC-MS (GC-Trace Ultra, MS-DSQ II) was of Thermo Scientific, USA make fitted with AS3000 injector and Thermo TR.1 capillary column.

Cells were hydrolyzed in 0.1 N NaOH, and protein content was determined following Lowry et al. (1951).

2.6 Statistics

Data in figures and tables are expressed as mean±standard error for three independent experiments with triplicate samples within each experiment. The data were analyzed by applying ANOVA and Tukey's post hoc test at 95% significant level using GraphPad Prism 5 version 5.04.

3 Results and discussion

3.1 16S rRNA gene sequence analysis

A partial nucleotide sequence of 422 bp obtained by amplification and sequencing of 16S rRNA gene fragment showed 100% similarity with *Synechocystis* sp. PCC 6714 (Fig. 1). The organism was identified as *Synechocystis* sp. strain PUPCCC 64. Since it is not a phylogenetic study, we think partial gene sequence is sufficient for identification purpose. The nucleotide sequence has been deposited in the NCBI Genbank data base with accession number GQ907237.

3.2 Chlorpyrifos tolerance

Growth of the organism was inhibited by chlorpyrifos in a concentration-dependent manner. The organism could survive in chlorpyrifos up to 15 mg/L and exhibited 20%, 50%, and 77% inhibition of growth in 5, 6.5, and 10 mg/L chlorpyrifos, respectively (Fig. 2). Microscopic examination of cultures from 20 mg/L chlorpyrifos revealed that nearly 99% cells were lysed and pigments were released into the medium. Calculations from growth data also revealed that the organism had 36 h doubling time in culture medium without insecticide. Chlorpyrifos concentration-dependent increase in generation time from 38 h in 2.5 mg/L to 100 h in 12.5 mg/L was observed (Inset Fig. 2). Similar inhibitory effects of insecticides have been reported in *Synechococcus leopoliensis* (Van Donk et al. 1992), *Anabaena sphaerica, Nostoc hatei*, and *Westiellopsis prolifica* (Jha and Mishra 2005), *Phormidium valderianum* (Palanisami et al. 2009) and *Spirulina platensis* (Thengodkar and Sivakami 2010). As per these reports, the level of tolerance to insecticides varied in different cyanobacteria. This may be due to inherent capacity of the organism to detoxify the xenobiotics or may depend on the environment from which a particular organism is isolated.

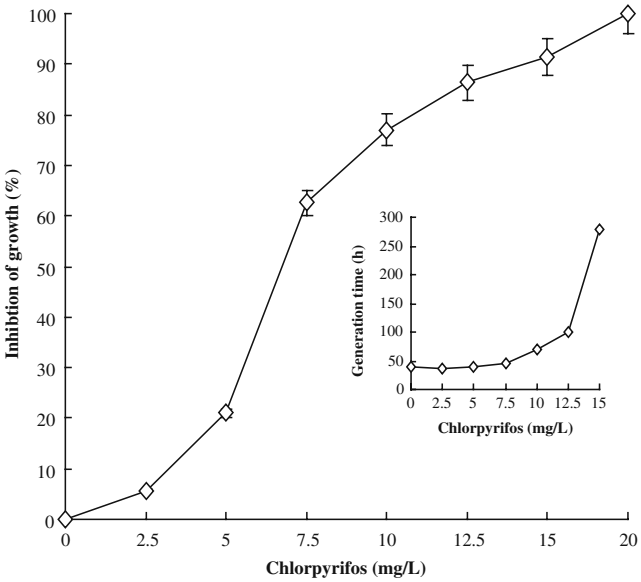


Fig. 2 Growth inhibition of *Synechocystis* sp. strain PUPCCC 64 in presence of chlorpyrifos. (*inset*: effect of chlorpyrifos on generation time). Culture conditions: on day zero, 1.3×10^7 cells were inoculated. On day 12, 3.0×10^8 cells in control cultures were taken as 100%. Generation time was calculated from the growth data between 4 and 12 days. All data in the figure are significantly different at 95% confidence level ($p<0.05$)

3.3 Chlorpyrifos removal

Microorganisms may tolerate insecticide either at uptake level or at intracellular level. Chlorpyrifos uptake by *Synechocystis* was studied to test whether the organism was able to take up and degrade the insecticide intracellularly. The organism removed 3.78 and 4.69 mg/L chlorpyrifos in 4 and 8 days, respectively, from medium containing 5 mg chlorpyrifos/L (Fig. 3). The results revealed rapid initial uptake of chlorpyrifos. Data given in Fig. 3 further revealed that major fraction of the insecticide was taken up by the organism

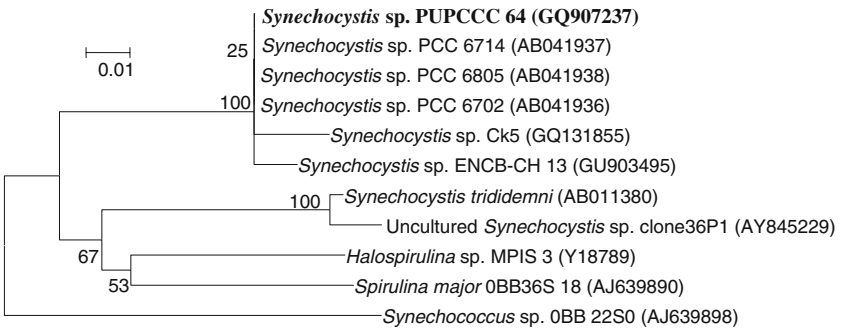


Fig. 1 Phylogenetic tree showing relationship of *Synechocystis* sp. strain PUPCCC 64 with closely related taxa based on partial 16S rRNA gene sequence. The percentage of replicate trees with the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. The evolutionary distances were computed using Kimura two-parameter method and analyses conducted in MEGA4

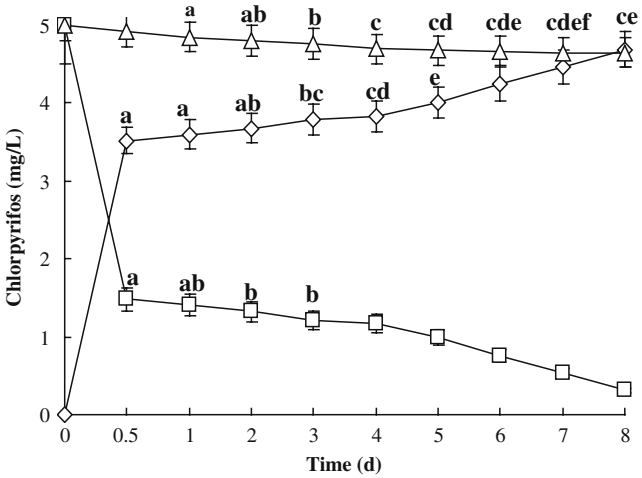


Fig. 3 Chlorpyrifos uptake by *Synechocystis* sp. strain PUPCCC 64. (*triangle*, chlorpyrifos in medium without biomass; *diamond*, chlorpyrifos uptake; *square*, chlorpyrifos in medium). Culture conditions: chlorpyrifos, 5 mg/L; initial biomass, 100 mg protein/L; pH 7.8, and temperature, 28°C±2°C. Data at different time intervals with same *lower case letters* are not significantly different from each other at the 95% confidence level ($p<0.05$)

during the first few hours. The results of short-term uptake experiment revealed linear uptake of chlorpyrifos from first hour (1.04 mg/L) to 12 h (3.52 mg/L) (Fig. 4). From these results, it appears that chlorpyrifos is initially adsorbed on the surface of the biomass followed by slow intracellular uptake. Mehetre et al. (2003) studied the behavior of chlorpyrifos in water flooded model rice ecosystem. Initial rapid decline in chlorpyrifos from the ecosystem was observed during first 10 days and measurable residues were observed up to 30 days. Mukherjee et al. (2004) reported 50–60% chlorpyrifos removal of 50 mg/kg soil on the 20th day

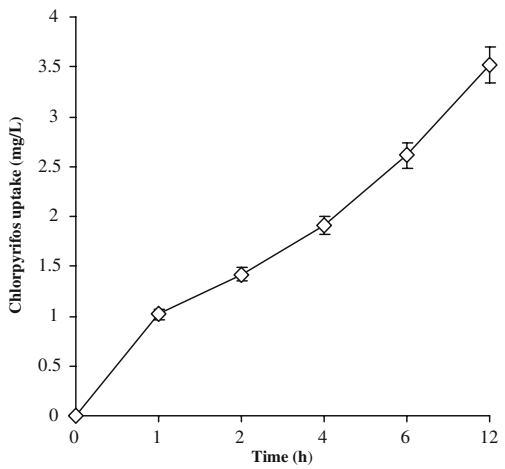


Fig. 4 Chlorpyrifos uptake by *Synechocystis* sp. strain PUPCCC 64. Culture conditions: same as in Fig. 3. All data at different time intervals significantly different from data on zero time and with respect to one another at 95% confidence level ($p<0.05$)

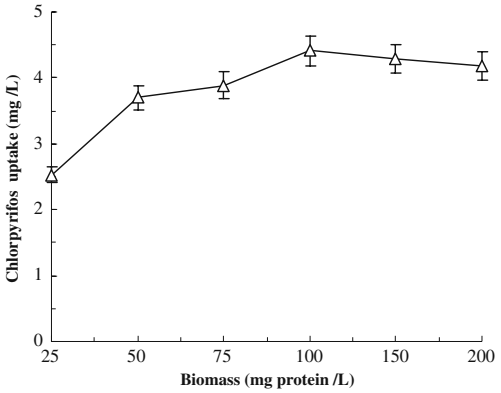


Fig. 5 Effect of biomass on chlorpyrifos uptake by *Synechocystis* sp. strain PUPCCC 64. Culture conditions: chlorpyrifos, 5 mg/L; pH 7.8, temperature, 28°C±2°C, and time, 24-h. All data at different biomass load significantly different from one another at 95% confidence level ($p<0.05$)

by a laboratory-grown green alga *Chlorella vulgaris*. Rapid uptake of herbicides such as atrazine and terbutryn has also been reported for the cyanobacterium *Synechococcus elongatus* and the green alga *C. vulgaris* (González-Barreiro et al. 2006). Orús and Marco (1991) reported the removal of trichlorfon from 300 μg/mL trichlorfon containing culture media by *Gloeothece* PCC 6501, *Plectonema calothricoides*, *Anabaena* PCC 7119, *Nostoc* UAM 2005, and *Chlorogloeopsis* PCC 6912. In the present study, slight decrease in chlorpyrifos was observed when the amount of the insecticide was determined on day 8 in the control flask, which indicated that chlorpyrifos is quite stable under laboratory conditions and its depletion from the medium was biological (Fig. 3).

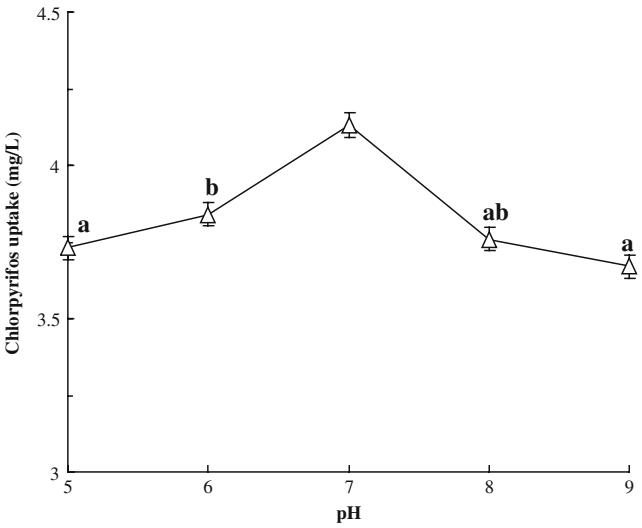


Fig. 6 Effect of pH on chlorpyrifos uptake by *Synechocystis* sp. strain PUPCCC 64. Culture conditions: biomass, 100 mg/L, temperature, 28°C±2°C, and time, 24-h. Data at different pH with same *lower case letters* are not significantly different from each other at 95% confidence level ($p<0.05$)

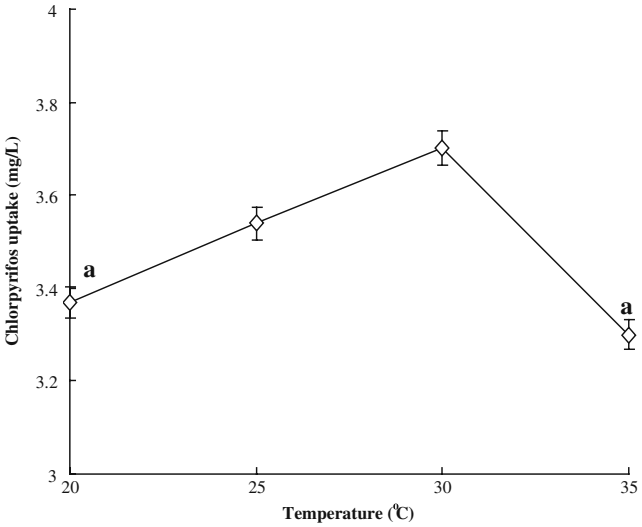


Fig. 7 Effect of temperature on chlorpyrifos uptake by *Synechocystis* sp. strain PUPCCC 64. Culture conditions: chlorpyrifos, 5 mg/L; biomass, 100 mg/L, pH 7.0, and time, 24-h. Data at different temperatures with same *lower case letters* are not significantly different from each other at the 95% confidence level ($p<0.05$)

3.4 Optimal conditions for chlorpyrifos removal

The effect of biomass, pH, and temperature on chlorpyrifos uptake by *Synechocystis* sp. strain PUPCCC 64 was studied as these factors are reported to influence the pesticide removal or biodegradation by microbial systems (Liu et al. 2003; Wang et al. 2006; Xu et al. 2007). Chlorpyrifos uptake increased with the increase in biomass with maximum uptake (4.41 mg/L) at 100 mg protein/L biomass load (Fig. 5). Increase in uptake with increased biomass may be due to less availability of adsorptive sites at lower biomass load. Thus, 100 mg/L biomass was optimal for maximum removal of chlorpyrifos by the organism from 5 mg chlorpyrifos/L solution. Xu et al. (2007) reported that the bacterium *Serratia* sp. TCR and the fungus *Trichosporon* spp. in co-cultures removed the maximum chlorpyrifos at inoculums size of 0.15 g dry wt./L from a medium containing 50 mg/L insecticide. The results on chlorpyrifos removal over acidic and alkaline range showed maximum removal at pH 7.0 (Fig. 6). Our observations are in agreement with the maximum chlorpyrifos removal by

Table 2 Chlorpyrifos and TCP in different components of *Synechocystis* sp. batch system

Component	Chlorpyrifos (μg)	TCP (μg)
Biomass	113.12±4.5	75.42±3.8
Biomass wash	9.92±2.0	38.16±2.5
Medium	138.33±4.9	613.33±7.9
Total	261.37±4.6	726.91±8.0

The experimental set up contained 250 mL medium with 5 mg chlorpyrifos/L (total 1,250 μg) and biomass having 100 mg protein/L, pH 7.0; temperature 28°C±2°C. After 60 h, biomass was separated from the medium, washed with double distilled water and amount of Chlorpyrifos and TCP was determined

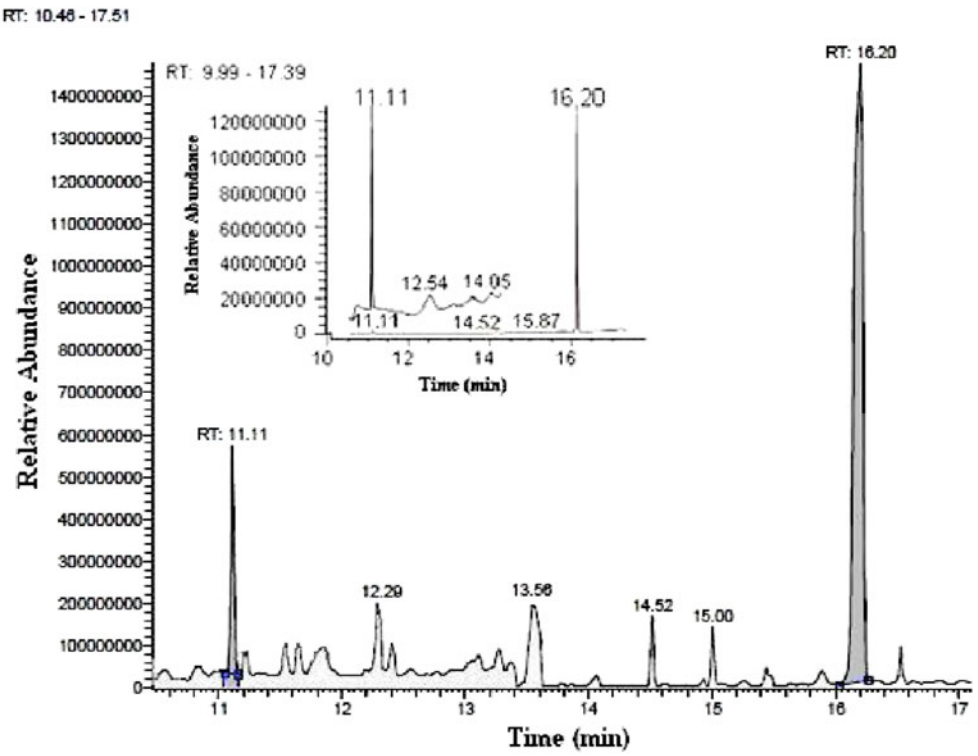


Fig. 8 GC-MS chromatogram of cell extract of *Synechocystis* sp. strain PUPCCC 64. Peaks with RT 11.11 and 16.20 correspond to TCP and chlorpyrifos, *Inset*: GC-MS chromatogram of standard TCP (RT 11.11) and chlorpyrifos (RT 16.20)

Verticillium sp. DSP at pH 7.0 (Fang et al. 2008). Maximum removal/biodegradation of chlorpyrifos by microorganisms are reported to be at neutral pH or in alkaline range (Singh et al. 2003; Yang et al. 2005, Xu et al. 2007; Fang et al. 2008; Thengodkar and Sivakami 2010). Insecticide removal by the test organism studied at different temperatures showed the maximum uptake of chlorpyrifos at 30°C (Fig. 7). At 40°C, microscopic observations of the cultures revealed cell lysis and loss of pigments into the medium whereas control cultures did not lyse at 40°C. Perhaps, *Synechocystis* could not bear the double stress of pesticide and temperature. The optimum temperature for chlorpyrifos degradation by the mixed culture of the bacterium *Serratia* sp. and the fungus *Trichosporon* sp. was 30°C (Xu et al. 2007).

3.5 Chlorpyrifos degradation

Six major peaks with the retention time 11.11, 12.29, 13.56, 14.52, 15.00, and 16.20 min were observed in the GC-MS chromatogram of cell extracts (Fig. 8). The peak with retention time 16.20 matched with standard chlorpyrifos, whereas peak with retention time 11.11 corresponded to standard TCP (inset Fig. 8). Other unidentified peaks appear to be the products of further degradation of TCP (Fig. 8). Analysis of supernatant and biomass wash for chlorpyrifos degradation products also showed the presence of TCP. In the experimental set up for the chlorpyrifos degradation study, 250 mL cultures contained a total of 1,250 μg chlorpyrifos. After 60 h, chlorpyrifos recovery from cell extract, biomass wash, and medium was 113.12, 9.92, and 138.33 μg, respectively. Along with chlorpyrifos, amount of TCP detected was 75.42, 38.16, and 613.33 μg, respectively (Table 2). The presence of TCP in all extracts indicated that the organism degraded the insecticide intracellularly as well as extracellularly. Rapid depletion of chlorpyrifos from the culture medium may be due to extracellular degradation of chlorpyrifos. Yang et al. (2005) isolated *Alcaligenes faecalis* DSP3 bacterium capable of degrading chlorpyrifos. The primary metabolite was found to be TCP. *Enterobacter* strain B-14 biotransformed chlorpyrifos up to TCP only (Singh et al. 2004). Chungjatupornchai and Fa-Aroonsawat (2008) isolated a gene for organophosphorus hydrolase from *Flavobacterium* sp. and expressed it in *Synechococcus* PCC 7942. They further showed that this enzyme is located both on the surface as well as intracellularly. It is also reported that phosphatases play an important role in the biodegradation of chlorpyrifos (Madhuri and Rangaswamy 2002; Thengodkar and Sivakami 2010). Although few reports on the metabolization of pesticides by cyanobacteria are available (Subramanian et al. 1994; El-Bestawy et al. 2007; Lee et al. 2003), detailed mechanism of chlorpyrifos degradation in cyanobacteria is not known. The mechanism of chlorpyrifos degradation in bacteria and fungi is fairly understood and a number of degradation products such as diethylthiophosphoric acid, TCP, chlorodihydro-2-pyridone, dihydroxypyridine, tetrahydro-2-pyridone, and maleamide semialdehyde have been identified (Singh and Walker 2006). Detection of TCP as one of the chlorpyrifos byproduct during the present study indicates that chlorpyrifos degradation mechanism in cyanobacterium may be similar as in bacteria. Feng et al. (1997) isolated a *Pseudomonas* sp., which mineralized TCP in liquid medium. In subsequent studies based on combined experiments of photolysis and microbial degradation, Feng et al. (1998) suggested the degradation of TCP firstly into chlorodihydro-2-pyridone by reductive dechlorination followed by its degradation into tetrahydro-2-pyridone and then to maleamide semialdehyde, which ultimately got mineralized into water, CO_2, and ammonium. The occurrence of unidentified peaks in the chromatogram of present study may be byproducts of TCP, which need further investigation.

4 Conclusions

This is the first report on degradation of chlorpyrifos by a common rice field unicellular *Synechocystis* sp. strain PUPCCC 64. Since the organism is a rice field cyanobacterium capable of degrading chlorpyrifos, it can be employed for in situ bioremediation of chlorpyrifos in rice field ecosystems.

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