Biodegradation (2010) 21:637–644
DOI 10.1007/s10532-010-9331-6

ORIGINAL PAPER

# Degradation of Chlorpyrifos by an alkaline phosphatase from the cyanobacterium *Spirulina platensis*

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Received: 6 April 2009 / Accepted: 18 January 2010 / Published online: 3 February 2010


**Abstract** *Spirulina* is a photosynthetic, filamentous, spiral-shaped, multicellular, blue-green microalga. The two most important species are *Spirulina maxima* and *Spirulina platensis. Spirulina* is considered an excellent food, lacking toxicity and having corrective properties against viral attacks, anemia, tumor growth and malnutrition. We have observed that cultures of *Spirulina platensis* grow in media containing up to 80 ppm of the organophosphorous pesticide, Chlorpyrifos. It was found to be due to an alkaline phosphatase (ALP) activity that was detected in cell free extracts of *Spirulina platensis*. This activity was purified from the cell free extracts using ammonium sulphate precipitation and gel filtration and shown to belong to the class of EC 3.1.3.1 ALP. The purified enzyme degrades 100 ppm Chlorpyrifos to 20 ppm in 1 h transforming it into its primary metabolite 3, 5, 6-trichloro-2-pyridinol. This is the first report of degradation of Chlorpyrifos by *Spirulina platensis* whose enzymic mechanism has been clearly identified. These findings have immense potential for harnessing *Spirulina platensis* in bioremediation of polluted ecosystems.

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**Keywords** Alkaline phosphatase · Chlorpyrifos · Cyanobacteria · *Spirulina platensis* · Bioremediation

## Introduction

Indescriminate use of pesticides has resulted in severe contamination and destruction of biodiversity and ecological systems. Large amounts of pesticides reach the soil either as direct application or from aerial spraying, or as plant and animal remains. From the soil these residues can enter into the bodies of invertebrates, get transported into water or air and get broken down to less harmful substances. Chlorpyrifos is a widely used broad-spectrum organophosphate effective in controlling a variety of insects. It is used as an insecticide on grain, cotton, vegetable crops, lawns and ornamental plants as well as a termiticide. It is also registered for direct use on animals, domestic dwellings and commercial establishments (Racke 1993). Chlorpyrifos is widely used in developing countries like India. In the year 2000, it was the fourth highest consumed pesticide after monocrotophos, acephate and endosulfan (Ansaruddin and Vijayalakshmi 2003). In view of the toxicity of these compounds, it is of paramount importance to have efficient means of cleaning the environment of these chemicals.

Biodegradation using microorganisms is one of the safest and cheapest ways of reducing pesticide levels

in the environment. As early as 1975 Munnecke and Hsieh reported the ability of parathion hydrolase, an organophosphorus ester-hydrolyzing enzyme isolated from a mixed microbial culture. Subsequently Jones and Hastings (1981) reported the metabolism of 50 parts per million (ppm) Chlorpyrifos to 3, 5, 6-trichloro-2-pyridinol (TCP) in cultures of several forest fungi (*Trichoderma harzianum, Penicillium vermiculatum, and Mucor* sp.). Since then several microorganisms have been shown to possess the ability to degrade or metabolise pesticides. During this process the microbes either completely metabolise the pesticide in a process referred to as mineralization wherein the energy released is used by the organism or the molecule undergoes co-metabolism with no direct energy benefits.

Though there are a large number of bacteria and fungi that have been shown to be useful for degradation of many of the organophosphorous pesticides in liquid cultures as well as in the soil, there are limited reports on the use of cyanobacteria for biodegradation (Baskaran et al. 1999; Singh and Walker 2006). The data presented in this paper show that the cyanobacterium *Spirulina platensis* is capable of degrading Chlorpyrifos to its primary metabolite TCP in laboratory cultures. A monoesterase acting under alkaline conditions (alkaline phosphatase) has been purified from cell free extracts and shown to be able to hydrolyze Chlorpyrifos in vitro.

## Materials and methods

All Chemicals were purchased from Sisco Research Laboratories, Mumbai, India. *Spirulina platensis* ARM728 was obtained from Indian Agricultural Research Institute, Delhi, India and was maintained and grown in inorganic SOT medium containing Trace metal mixture A5 and B6 (Ronald 1997).

### Culture

*Spirulina* was cultured in sterile SOT medium. Pre-inoculum was added to get an OD of 0.01 at 560 nm. The medium was stirred at a constant speed of 60–80 rpm. The organism was allowed to grow at 28 ± 4°C under cool white light of 4–6 Klux with constant stirring. Growth was monitored by measurement of OD at 560 nm and wet weight.

### Purification of alkaline phosphatase (ALP) activity

The biomass was recovered by ultra filtration carried out using two filters of 80 and 20 $\mu$ meshwork in series under vacuum pressure created by a pump with a capacity of ¼ H.P. and efficiency of 1 l $min^{-1.}$

The cells were harvested on the 14th day as described above, washed three times with 50 mM Tris–Cl buffer, pH, 7.0 and suspended in sonicating buffer (100 mM Tris–Cl, pH 7.0 containing Aprotinin—Trypsin inhibitor from bovine lung 50 µg/ml, Phenylmethyl sulfonyl fluoride 1 mM) at a concentration of one gm pellet in 5 ml of the buffer. Cell free extract was prepared by sonicating the suspension at 14 microns amplitude using 10 s on and 30 s off for 15 cycles in ice and centrifuged at 12,000 rpm for 20 min. The supernatant referred to as crude sonicate was assayed for ALP activity. The sample was subjected to 80% ammonium sulphate saturation at 4°C and centrifuged at 10,000 rpm for 15 min in the cold. The pellet was exhaustively dialysed against 1 mM Tris–Cl, pH 7.0 with 5 changes for 72 h to remove ammonium sulphate. Completion of dialysis was confirmed by absence of ammonium sulphate in the dialysis buffer using Barium chloride.

### Gel filtration

This sample was loaded on a column of Sephadex G-100 (100 × 1.5 cm; bed vol. 100 ml). Flow rate was adjusted to 35 ml per hour using Pharmacia peristaltic pump P-1, Uppsala, Sweden. The column was eluted with 50 mM Tris–Cl buffer pH 7.0. 1 ml fractions were collected using Pharmacia Frac-100 fraction collector and assayed for ALP and protein. The activity was found to elute as a sharp peak. The peak fraction exhibiting maximum specific activity was used for 2D gel electrophoresis and MALDI-TOF as described below.

### 2D Polyacrylamide gel electrophoresis

2D PAGE was carried out as described in Handbook of 2D electrophoresis using immobilized pH gradients (GE Healthcare Biosciences Ltd., India). The peak fraction of ALP from the gel filtration column was lyophilized and protein content estimated by 2D Quant kit (GE Healthcare Biosciences Ltd., India).

IsoElectricFocussing (IEF) was carried out using IPG strips of 13 cm length and a pI gradient from 3 to 10 at 70 V for 18 h followed by SDS PAGE in the second dimension. The sample was treated with the chaotrope 8 M urea, 2% CHAPS, reduced with DTT and the sulphydryl groups blocked by iodoacetylation. Following separation on 2D gels, the sample was stained for protein with silver (Maniatis et al. 1989).

### Matrix-assisted laser desorption/ionization time of flight (MALDI TOF)

The silver stained protein band was excised manually and transferred into an Eppendorf tube. The gel pieces were washed with deionised distilled water and reduced by using the reduction buffer [100 mM $NH_4HCO_3$, 10 mM dithiothreitol (DTT)] to disrupt disulfide bonds. The gel pieces were alkylated using alkylation buffer (100 mM $NH_4HCO_3$ and 55 mM iodoacetamide) and placed in the dark for 30 min at room temperature to prevent disulfide bonds from reforming. Subsequently the gel pieces were dehydrated with 50 µl of 100% acetonitrile, and dried under vacuum using a Speedvac concentrator. The dried gel pieces were submerged in 40 µl digestion solution consisting of (100 ng/µl trypsin) in 50 mM of $NH_4HCO_3$ and incubated overnight at 37°C. The peptides were extracted from the digested mixture by adding 100 µl of extraction buffer (50% acetonitrile containing 5% trifluoroacetic acid). This process was repeated twice and the supernatant collected after centrifuging at 10,000 rpm. The resulting peptide mixture was further concentrated in a Speedvac concentrator. The sample (1 µl) was mixed with matrix solution dissolved in 50% acetonitrile with 0.1% trifluoro acetic acid (1 µl) and vortexed gently. A volume of 2 µl of the mixture were loaded on a stainless steel plate and air-dried. Proper care was taken to prevent any keratin contamination. The sample was subjected to analysis by MALDI-TOF. Proteins were identified using the public domain Mascot search engine by incorporating the standard parameters (http://www.matrixscience.com).

### Degradation of Chlorpyrifos using *Spirulina platensis*

A stock solution of Chlorpyrifos was made in acetone. Appropriate amounts were added to 250 mL flasks to get final concentrations of pesticide ranging from 10 to 120 ppm and spiked. This was followed by addition of the media and inoculum. Chlorpyrifos was extracted by Liquid Liquid Extraction method (Acidified Acetone LLE) [Acetone: Phosphoric acid: water 98:1:1], the suspension was shaken and stirred for 4 h. Following centrifugation (20 min at 10,000 rpm) the supernatant solution was filtered through glass fiber filter membrane of 0.45 µm and then collected by evaporation. The extraction recoveries were 87%. Though the optimum pH of the enzyme is 10.5, degradation of Chlorpyrifos was carried out at pH 7.0. At that pH, abiotic degradation (Control) gave insignificant degradation of Chlorpyrifos (<5%), 95% of Chlorpyrifos remaining in controls was assumed to be 100% and the tests were representated as percentages. The experiments were repeated at least thrice in triplicates.

### High performance liquid chromatography

Chlorpyrifos and TCP were analysed by the method of Mauldin et al. (2006) using a Jasco HPLC system, UK, equipped with a UV–Vis detector (UV at 230 nm) using HiQ Sil $C_{18}W$ column (4.6 mm $\phi$ × 250 mm) manufactured by Kya Tech, Japan. Samples were chromatographed with mobile phase comprising of 75% acetonitrile and 25% 1 mM phosphate buffer (pH, 4.5) at room temperature (28°C). The flow rate was fixed at 1 ml $min^{-1}$ for at least 25 min extended up to 30 min.

### Assay of alkaline phosphatase

Enzyme activity was assayed using the artificial colourless substrate *p*-nitrophenyl phosphate, which is hydrolysed to coloured *p*-nitrophenol. Deprotonation of the phenolic group gives rise to the yellow coloured *p*-nitrophenylene anion. The concentration of this anion in solution is determined by measuring the absorbance at 405 nm. The reaction mixture consisted of 100 µl of 100 mM carbonate-bicarbonate buffer pH, 10.5, 100 µl of 5 mm pNPP prepared in 10 mM Carbonate-bicarbonate buffer containing 1 mM $MgCl_2$, enzyme and distilled water to get a final volume of 500 µl. At the end of 60 min at 37°C, the reaction was terminated by the addition of 3 ml of termination buffer (0.1 N NaOH with 5 mM EDTA).

One International Unit [IU] of ALP was defined as amount of the enzyme required to liberate one micromole of *p*-nitrophenol in 1 min at 37°C. The enzyme was also assayed by using sodium $\beta$-glycerophosphate and measuring the inorganic phosphate released by the method of Fiske and Subbarow (1925).

### Protein estimation

Protein was estimated by the Folin phenol method of Lowry et al. (1951) using BSA as standard. Specific activity was expressed as IU/mg protein.

### Determination of optimum pH

The activity of ALP was assayed with buffers varying in pH from 3.0 to 10.5. Citric acid-trisodium citrate buffers was used in the range of 3.0–7.0. Carbonate–Bicarbonate buffer was used in the pH range of 7.5–10.5.

### Determination of thermo-stability

The enzyme was incubated in water bath from 37 to 100°C. Aliquots were drawn every 20 min from the tube and the temperature of the residual enzyme was raised by 5°C. The tubes drawn were immediately immersed in ice and assayed for ALP activity.

## Results

*Spirulina platensis* was observed to be growing in media containing up to 80 ppm Chlorpyrifos. The intensity of the pigment colour of the cultures was inversely proportional to pesticide content in the range 1–80 ppm. It was observed that cultures containing the pesticide showed a lag in development of colour compared to control. This was overcome after the first few days presumably due to conversion of the pesticide to the less toxic TCP. It is known that Organophosphorous (OP) compounds do not adversely affect bacteria because they do not possess acetylcholine esterase that can be inhibited by OP compounds (Singh 2009).

When *Spirulina* was cultured in media containing a range of Chlorpyrifos from 10 to 120 ppm, it was observed that at concentrations below 40 ppm, only TCP could be detected by HPLC at the end of 24 h (Figs. 1, 2). However, at concentrations above 60 ppm, both TCP and Chlorpyrifos peaks could be detected. These results indicate that *Spirulina* is not only tolerant to Chlorpyrifos but is also capable of detoxifying it to TCP.

Incubation of 25 and 50 µl aliquots of crude sonicates with 100 ppm Chlorpyrifos followed by analysis on HPLC revealed that about 50% of the Chlorpyrifos was transformed to TCP at the end of 1 h while at the end of 2 h more than 85% of Chlorpyrifos was converted to TCP. These results indicated an intracellular localization for the Chlorpyrifos degrading activity. Hence a search for a Chlorpyrifos degrading activity resulted in the identification of an ALP, which was purified and found to be capable of degrading the pesticide in vitro.

Using pNPP as the substrate, a phosphatase activity was detected in the sonicate of a 14-day culture. The sonicate was concentrated by precipitation with 80% ammonium sulphate and subjected to gel filtration. The ALP activity eluted as a sharp peak on Sephadex G-100 (Fig. 3). This peak fraction resolved into 4 spots on 2D gel electrophoresis (Fig. 4). The protein identity of the spots on MALDI revealed it to be an ALP. This purified enzyme was also capable of releasing inorganic phosphate from the organic ester sodium $\beta$-glycerophosphate.

The purified ALP activity showed an optimum temperature of 40°C. The enzyme was found to be stable to heat up to 60°C with 60% of the enzyme activity being retained (Fig. 5). Total loss of activity was observed above 70°C. The activity was stable to storage at room temperature for 4 months with just 18% loss. The enzyme lost up to 55% activity after repeated freezing and thawing.

When assayed at varying pH values, four peaks for optimum rate of enzymatic reaction were observed. Maximum activity was observed with a sharp peak at pH 10.5. In addition three smaller peaks were observed (Fig. 6). At pH 9.5, a peak exhibiting 60% of the optimum activity was seen while at pH 7.0, & 8.0, peaks having 40% of the optimal activity were observed. It is probable that these four peaks correspond to the 4 spots of ALP activity observed on 2D PAGE.

When 25 (25 µg) and 50 µl (50 µg) preparations of the final purified preparation of ALP was incubated with 100 ppm Chlorpyrifos and subjected to HPLC analysis, it was observed that 56 and 22% of residual

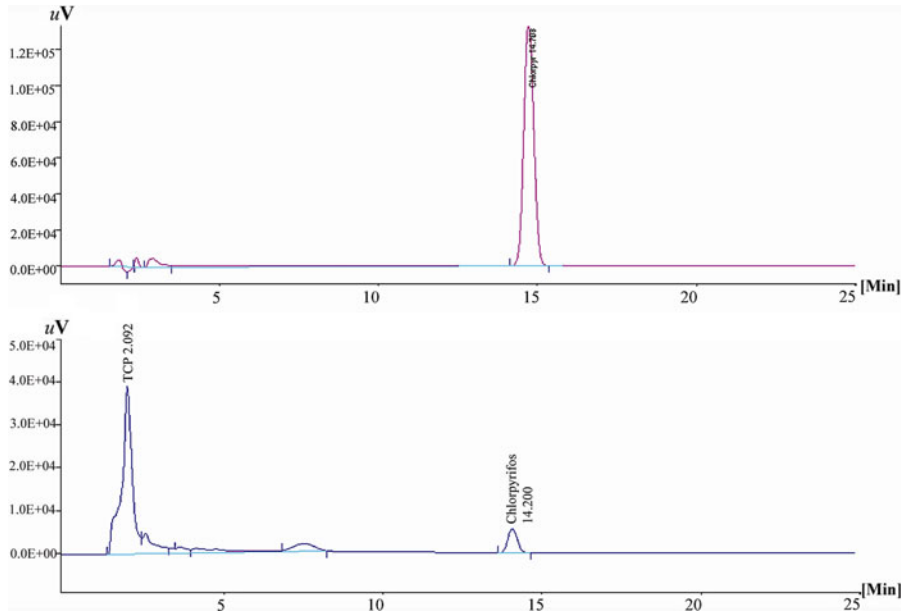


**Fig. 1** Chromatogram showing degradation of Chlorpyrifos: *Spirulina* culture grown in medium containing 80 ppm Chlorpyrifos and analyzed by HPLC as described in "Materials and methods". 95% of Chlorpyrifos is converted to TCP. *Top* Control, *Bottom* test

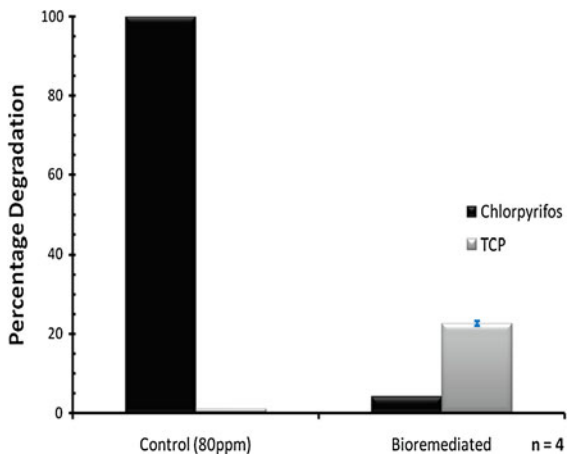


**Fig. 2** Percentage of Chlorpyrifos and TCP in *Spirulina* cultures grown in 80 ppm Chlorpyrifos. 95% of Chlorpyrifos is converted to TCP

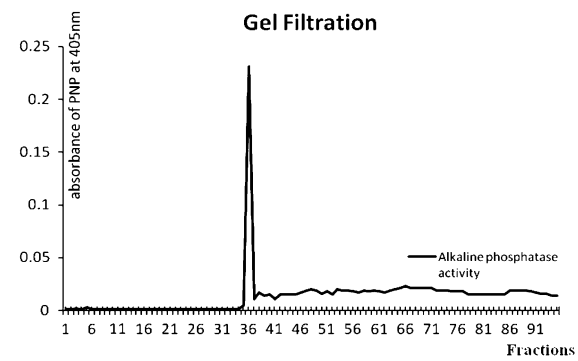


**Fig. 3** Gel filtration of alkaline phosphatase; 80% ammonium sulphate precipitate after dialysis loaded on Sephadex G-100 (100 × 1.5 cm; bed vol. 100 ml) which eluted as a *sharp peak*

Chlorpyrifos was detected at the end of 1 h. This confirms that the enzyme preparation degrades 78% of the 100 ppm Chlorpyrifos in 1 h (Figs. 7, 8).

## Discussion

The results presented in this paper show that *S. platensis* can withstand and grow in media containing up to 80 ppm Chlorpyrifos. The results of the HPLC analysis indicate that Chlorpyrifos is converted to its primary metabolite TCP under the conditions of the experiments described above. Though we have not carried out experiments to study the mineralization of Chlorpyrifos, it is possible that *S. platensis* is capable of complete degradation of the Chlorpyrifos molecule.

Yang et al. (2005) isolated an *Alcaligenes faecalis DSP3*, capable of degrading both Chlorpyrifos and its primary metabolite TCP. Feng et al. (1997) showed that TCP could be degraded by a *Pseudomonas* species. Singh et al. (2004) studied *Enterobacter*

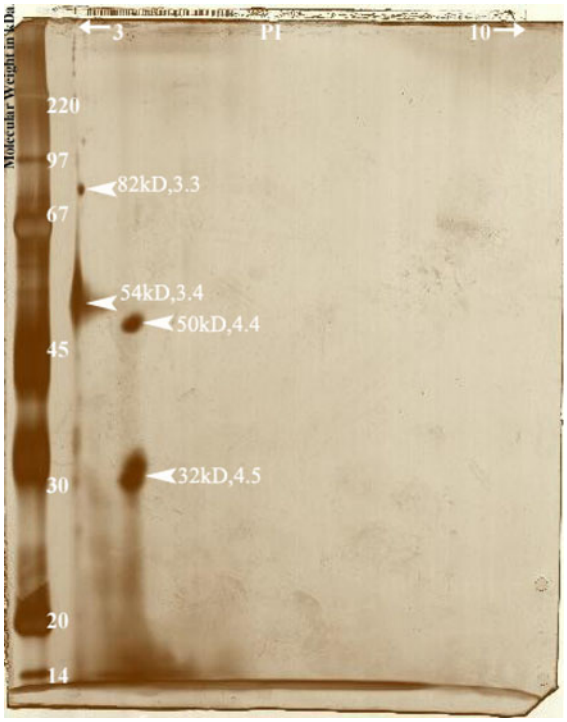


**Fig. 4** 2D gel electrophoresis of alkaline phosphatase; the peak fraction from Sephadex G-100 was subjected to IEF followed by SDS. 4 ALP positive bands developed as 4 protein spots in Silver staining

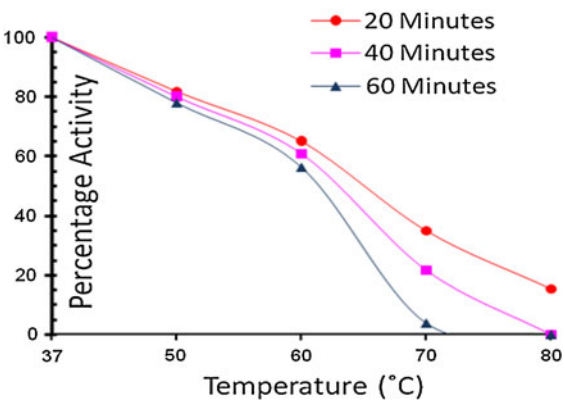


**Fig. 5** Thermal stability for alkaline phosphatase. The enzyme was incubated at increasing temperatures and held for 20, 40 and 60 min, respectively. The curve indicates that 60% activity is retained up to 60°C. Total loss of activity occurs above 70°C

*B-14* and concluded that it bio-transforms Chlorpyrifos up to TCP only. Although the microbial degradation of Chlorpyrifos has been investigated, the existing papers lack information on the genetic and enzymatic aspects involved in its degradation.

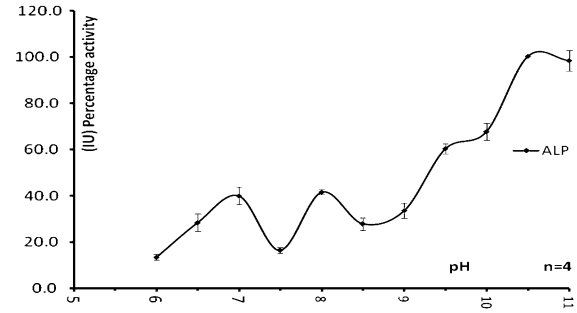


**Fig. 6** pH stability for the enzyme, alkaline phosphatase. Maximum activity was observed with a sharp peak at pH 10.5. At pH 9.5, a peak exhibiting 60% of the optimum activity was seen while at pH 7.0, & 8.0, peaks having 40% of the optimal activity were observed

The experiments reported in this study provide unequivocal evidence for the involvement of an ALP belonging to the class 3.1.3.1. that can accelerate degradation of Chlorpyrifos to TCP. The activity appears to be soluble since it was present in cell free extracts obtained by sonication. This activity on purification reduced pesticide concentration to 22% of the original in 1 h. The ALP activity has been purified and shown to be stable to storage and capable of activity over a wide range of temperature and pH. These properties would make it ideal for the enzyme to be immobilized on a matrix. The temperature stability also indicates that the biomass may be cultivated in outdoor effluent waters in tropical countries like India.

There is only one earlier report on the utilization of organophosphorous pesticides by ten strains of filamentous heterocystous cyanobacteria wherein they have shown the presence of an acid phosphatase connected to polyphosphate degradation rather than an ALP (Subramanian et al. 1994). This acid phosphatase activity was reported to be higher in Pi-minus compared to Pi-plus media. However, the acid phosphatase was not purified or characterized in this study. The reason for the presence of an acidic rather than an ALP in these cyanobacteria is not clear and might reflect an evolutionary difference between heterocystous and nonheterocystous strains of cyanobacteria.

More recently, 6 Chlorpyrifos degrading bacteria were isolated from an Australian soil and their abilities to mineralize Chlorpyrifos were investigated under different culture conditions using uniformly

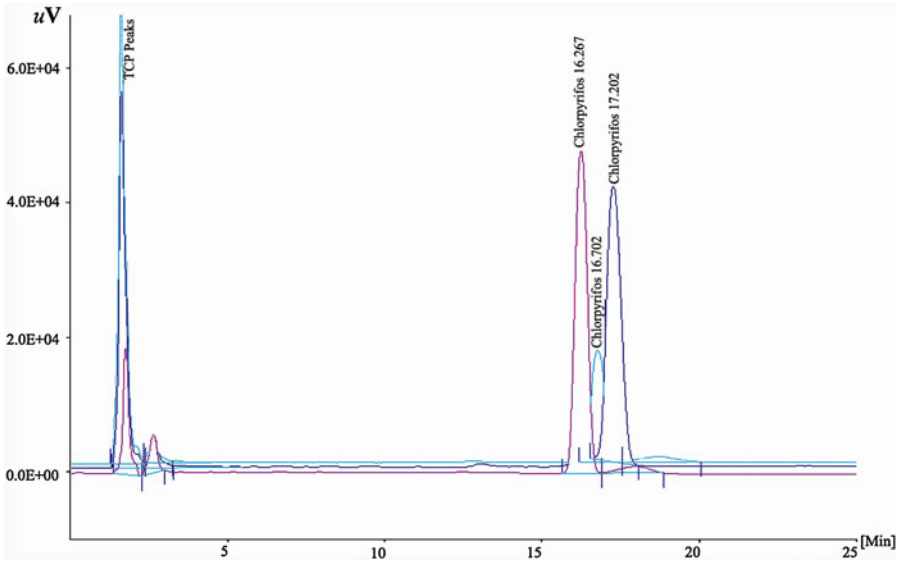


**Fig. 7** Chromatogram showing degradation of Chlorpyrifos by purified alkaline phosphatase; the figure shows increased conversion of Chlorpyrifos to TCP with increase in enzyme concentration. *Red line* denotes 100% Chlorpyrifos with minimal TCP (Control). *Blue line* and *Cyan line* denote residual Chlorpyrifos after incubation with 25 and 50 μg of purified enzyme at 37°C for 1 h. Chlorpyrifos and TCP analyzed by HPLC as described in "Materials and methods" (Color figure online)

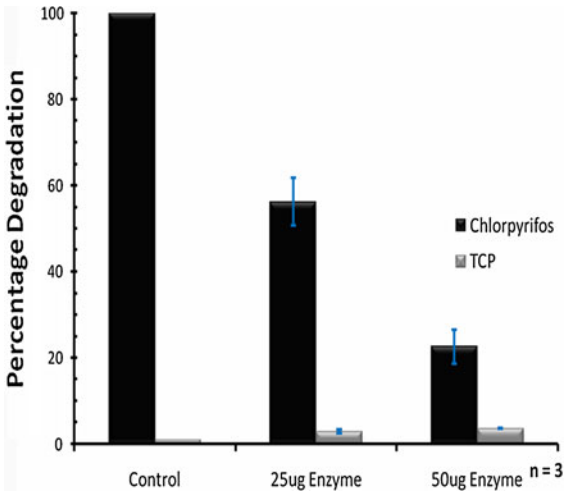


**Fig. 8** Degradation of Chlorpyrifos to TCP by purified alkaline phosphatase at the end of 1-h incubation. Un-degraded Chlorpyrifos was 56 and 22% with 25 μl [25 μg] and 50 μl [50 μg] of the purified enzyme, respectively

labeled C14 Chlorpyrifos (Singh and Walker 2006). One *Enterobacter strain B-14* was found to possess mono and diphosphatase activity along with phospho triesterase activity. Further studies revealed that the strain possesses a novel phospho triesterase enzyme system as the gene coding for this enzyme had a different sequence from the well characterized organophosphate degrading gene *opd* (Singh et al. 2004). An *opd* gene has been isolated in different species and has been found to be plasmid based and possessing similar DNA sequences (Singh et al. 2004). While the *opd* gene from *Agrobacterium radiobacter* was found to possess a sequence similar to the *opd* gene from the other bacteria, the *opd* gene from *Enterobacter* was shown to have a unique DNA sequence. It was further suggested that phospho triesterase activity of the *B14* strain degrades the triester bond of the organophosphorus compounds while the phospho diesterases and monoesterases activity are required to make the phosphorus atom available for uptake as a source of inorganic phosphate with the ethanol released being used as carbon source.

It is possible that ALP from *Spirulina* has acquired the ability to hydrolyze Chlorpyrifos promiscuously. Along this line it was proposed by Afriat et al. (2006) that an ability of a progenitor to promiscuously catalyze a low level of the evolving activity would facilitate the divergence of a new function by providing an immediate selective advantage. According to Afriat et al. (2006) newly evolved enzymes generally exhibit sequence homologies to more ancient or even housekeeping enzymes and presumably diverged from them. However, in case of ALP from *Spirulina platensis* this can be confirmed only upon a BLAST search that will yield a result having very high levels of homology with enzymes belonging to the same class or another class. The degree of homology will decide its promiscuous nature and reveal information about evolutionary link. As the

protein sequence for ALP from *Spirulina* is still unknown, a BLAST search using *Spirulina* could not be carried out. Hence the promiscuity of the enzyme can only be speculative. The multiple alignment of ALP from other cyanobacteria shows very limited conservation indicating each of the enzymes to be unique. The sequence of *Arthrospira platensis* is still in progress at Human Genome Center, Beijing, Project ID: 13465 and *Arthrospira platensis* strain Paraca at University of Applied Sciences of Western Switzerland Project ID: 34793.

This is the first report of biodegradation of Chlorpyrifos by an edible alga like *Spirulina*. Since this organism grows very rapidly and under a variety of environmental conditions, using *Spirulina platensis* for biodegradation appears to be an inexpensive and ideal option for reducing Chlorpyrifos levels in the environment. *Spirulina* does not require fertile land but can grow easily on brackish waters as well as fresh water. Besides using less water and producing more protein, it also fixes carbon dioxide and releases oxygen thereby counteracting the greenhouse effect. The *Spirulina* grown under the conditions of biodegradation can also be used as feed and fodder subsequent to degradation. These findings make *Spirulina* a tool for bioremediation by either employing the whole cells in effluent treatment plants or by immobilizing the purified enzyme on a strong matrix to decontaminate polluted environments. Thus, the results presented in this study indicate the use of *Spirulina*, widely grown algae for biodegradation of Chlorpyrifos. In addition a molecular mechanism has been attributed to its degradation.

**Acknowledgments** The authors wish to thank Dr.Vrinda Khole of NIRRH, Mumbai for the 2D PAGE analysis and Dr. Surekha Zingde and Dr. Rukmini Govekar of ACTREC, Mumbai and Dr. S. K. Apte of B. A. R. C., Mumbai for the MALDI-TOF experiments. Technical grade pure Chlorpyrifos was a gift from Dr. M. H. Fulekar, Professor, University department of Life Sciences, Mumbai University, Mumbai.

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