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Biodegradation of chlorpyrifos by scaling up method in controlled environmental conditions and plant growth enhancement

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Abstract

Biodegradation study of chlorpyrifos was done by the bacterial strains of species *Pseudomonas aeruginosa*. Scaling up the concentration of chlorpyrifos from 15mg/l up to 100mg/l was done, and GC-MS recorded an amount of chlorpyrifos degradation at a constant interval of 15days. The results show that in this scaling up processes 15-50mg/l concentration of chlorpyrifos was readily degraded by *Pseudomonas spp.* But as we started increasing the level from 75-100mg/l, the biodegradation rate starts decreasing up to 25% at 100 mg/l concentration. Intermediate such as 3, 5, 6 trichloro-2-pyridine,2,4-bis (1, 1 dimethyl ethyl) phenol and 1, 2 Benzenedicarboxylic acid persisted during bioremediation, but after day 18-20 these subsequently get convert to CO₂, biomass and nutrients. This strain also shows some significant plant growth promoting activities such as IAA production, p-solubilization, ammonia production both in presence and absence of chlorpyrifos. Confirmed when inoculated with chlorpyrifos degrading strain *Pseudomonas aeruginosa*, results showed enhanced plant growth in terms of plant height parameters. Thus it is concluded that *Pseudomonas aeruginosa* easily degrades chlorpyrifos at 50-75mg/l chlorpyrifos concentration, but its potential gets inhibited at a higher level of chlorpyrifos. This study also clearly indicated the role of *Pseudomonas aeruginosa* (AP2016) in practical bioremediation of chlorpyrifos and simultaneously increasing the production of pesticide-contaminated agricultural fields.

Keywords: Biodegradation, p-solubilization, ammonia production, pesticide contamination

Introduction

Chlorpyrifos (O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate) is one of the most extensively applied organophosphorus insecticides all around the world, which controls a broad-spectrum of insects of economically important crops. Chlorpyrifos is moderately toxic organophosphate insecticide, used to restrict the use of highly toxic organophosphate compounds (Tomlin and Council, 1994; McConnell *et al.*, 1994) [17, 25]. This insecticide is having high residual concentrations (0.01-0.62 mg/kg) and long half-life (60-120 days) which contaminates aquatic and terrestrial ecosystems and human habitats (Ngan *et al.*, 2005; Nawaz *et al.*, 2011) [13, 12]. Chlorpyrifos inhibits the acetylcholinesterase activity irreversibly and causes the death of insects (Karalliedde and Senanayake, 1989) [9], this affects the broad range of non-targeted insects also as acetylcholinesterase is present in all vertebrates (Sogorb *et al.*, 2004) [16]. The biodegradation of chlorpyrifos yields TCP, which is Co-metabolized with CP as a sole carbon source by many bacteria (Singh *et al.*, 2011) [15]. TCP is a crucial metabolite having antimicrobial properties which inhibits the bioremediation of CP (Feng *et al.*, 1997; Cáceres *et al.*, 2007) [5, 3]. Bioremediation is a quick and environmentally cleaner process to clean up the polluted environment. Till date, several works on biodegradation of chlorpyrifos has been conducted, and several bacterial strains have been isolated from different genera. Such some microorganism include *Bacillus pumilus* C2A1 (Anwar *et al.*, 2009) [23], *Enterobacter* strain B-14 (Singh *et al.*, 2004), *Stenotrophomonas* sp. YC-1 (Yang *et al.*, 2006), *Bacillus pumilus* C2A1 (Anwar *et al.*, 2009) [23], *Synechocystis* sp. Strain PUPCCC 64 (Singh *et al.*, 2011) [15], *Pseudomonas* Iso 1-4, *Agrobacterium* Iso 5-6, and *Bacillus* Iso 7 (Maya *et al.*, 2011), *Cupriavidus* sp. DT-1 (Peng Lu *et al.*, 2013), *Sphingobacterium* sp. JAS3 (Jayanthi Abraham and Sivagnanam Silambarasan, 2013).

In the present study, bioremediation of chlorpyrifos was done with the help of isolated species of *Pseudomonas aeruginosa*. Inoculation of *Pseudomonas* sp. in okra (*Abelmoschus esculentus* L.), tomato (*Lycopersicon esculentum* L.), and African spinach (*Amaranthus* sp.) showed the enhanced plant growth stimulating properties (Adesemoye and Ugoji, 2006).

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Hence this bacterium has been taken for the present study. Khorshidi *et al.* (2011) had also shown that the application of fertilizers with *Pseudomonas fluorescens* and *Azospirillum lipoferum* had a significant effect on rice yield. *Pseudomonas aeruginosa* was fed up with chlorpyrifos as a sole carbon source in minimal media. The result of varying pH and absorbance was also monitored and measured under the controlled condition to assess the biodegradability of the test organism; further, the strain was classified and characterized through a different confirmatory biochemical test and 16S RNA study.

Materials and Methods

Chemicals

Technical grade chlorpyrifos 20% emulsifiable concentrate procured from Isagro (Asia) Agrochemical Pvt. Ltd., Mumbai. All other chemicals and microbiological media used during this investigation were of A.R. grade and supplied by E. Merck, (India), Himedia (India) and S. D. Fine Chemicals (India).

Culture medium

The MS media was prepared using the standard procedure and ingredients (g/l): 1.0 KH₂PO₄, 1.0 K₂HPO₄, 1.0 NH₄NO₃, 0.2 MgSO₄, 0.02 CaCl₂, 0.01 FeSO₄ and 20.0 Agar Powder. The pH was adjusted to 7.0, using 0.01 N HCl/0.01 N NaOH solutions. The MS media was blended with 1 ml of trace elements solution (Focht, 1994). The Focht trace element solution contained (in mg/l): 169 MgSO₄.H₂O, 288 ZnSO₄.7H₂O, 250 CuSO₄.5H₂O, 26NiSO₄.6H₂O, 28CoSO₄, and 24Na₂.MoO₄.2H₂O

Sampling

Soil samples used in this study was collected from chlorpyrifos treated composite surface soil samples (0-15cm) from Norman E. Borlaug Crop Research Centre, Pantnagar at 29°00'40.5"N79°28'43.3"E. Sampling site has been in use for intensive agriculture practices since long and soil has been exposed to chlorpyrifos for several years. Soil samples were air dried and sieved at 5 mm and preserved at 4 °C, and further used for isolation of chlorpyrifos degrading bacteria.

Microorganisms

A pure culture of *Pseudomonas aeruginosa* (AP2016), a phenol degrading strain was procured for the bioremediation work from the experiment conducted in eco-technology lab GBPUAT, Pantnagar. The culture was maintained on nutrient agar slants. Technical grade chlorpyrifos was used for the bioremediation study.

Identification of chlorpyrifos degrading bacterial strain

Microbiological and biochemical characterization of the chlorpyrifos-degrading bacterial strain (AP2016) was performed, according to Holt *et al.* (1994) [26]. For molecular characterization, genomic DNA of AP2016 was extracted according to bazzicalupo and Fani (1995) [27]. Amplification of 16S rDNA was done using Universal primers, 50-AGAGTTTGATCCTGGCTCAG-30, and 50-TACCTTGTTACGACTT-30. PCR conditions used for the present study were initial denaturation at 95 °C for 1 min, annealing temperature 55-51 °C (touchdown) followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and extension at 72 °C for 1 min, with the last cycle followed by 10 min extension at 72 °C. After gel electrophoresis, the PCR product was eluted using an

extraction kit (Gene). After quantification, the sample was sent to Advanced Biotechnology Centre (CIF), Delhi University South Campus, India, for sequencing. Obtained sequences were compared with the GenBank database using the BLAST program. Multiple sequence alignment for the homologous sequence was done by MEGA 5.0 software, and a phylogenetic tree was constructed using the neighbor-joining method (Tamura *et al.* 2007) [28].

Sterilization of media, solutions, and apparatus

All the solutions and media were sterilized by autoclaving them at 121 °C temperature, 15psi pressure for 20 minutes. The glass wares were sterilized in an oven at 180 °C for an hour. After sterilization, the media was used as per requirement and rest stored under refrigeration when not in use; the sterilized glass wares were also kept in an oven at 60 °C and cooled for further use.

Pesticide spiking

For this Erlenmeyer flask (250ml) and nutrient culture media was autoclaved for 25 minutes at 121 °C. Separate aliquots of 500µl acetone containing chlorpyrifos were aseptically added to the autoclaved and dried Erlenmeyer flasks, allowing the acetone to evaporate. After complete evaporation, 100ml culture media was added aseptically to reach the desired pesticide concentration of 15,25,50,75 and 100mg/l (Brinch *et al.*, 2002) [2].

Scaling up technique

In the scaling up processes fresh one milliliter of culture from the chemostat was transferred to 250ml Erlenmeyer flask containing 10mg/l concentration of chlorpyrifos. Then the inoculated flasks (in triplicates) were incubated in an orbital shaker at 150 rpm at 37 °C temperature for 15 days. After 15 days, 1ml of the inoculum with 15mg/L pesticide was taken and put into culture media with a pesticide concentration of 25mg/l. The flasks were again kept on the orbital shaker incubator at 150 rpm, 37 °C for 15 days, after which 1 ml was further transferred to culture media with 50mg/l pesticide, and subsequently to 75 and 100mg/l, each stage passing through shaker at 150 rpm, 37 °C for 15 days. The entire scale-up period lasted 90 days after which the bacteria were found to be well adapted to chlorpyrifos at 25, 50 and 75 mg/l, which the bacterium could access and utilize as the sole source of carbon. After 15 days, the samples with the different concentrations of pesticide were removed and analyzed with GC-MS for subsequent biodegradation of chlorpyrifos and their intermediates. In this whole study period, the microbial biomass was also calculated by the UV-visible spectrometer at 550nm.

Determination of auxiliary characteristics

Indole acetic acid (IAA) production

Media

LB broth (50 mL), supplemented with 25, 50, 75, 100, 150 and 200 µg/L tryptophan, was inoculated with 50 µL of cell suspension (OD₆₀₀= 0.5) taken in triplicate and incubated at 32 °C, 150 rpm for 84 h. At 24, 48, 72 and 84 h intervals, the culture was centrifuged, and 3 mL of the supernatant was mixed with 6 mL of Salkowski's reagent. And the intensity of the color was taken as OD₅₃₅, and the amount of IAA produced was quantified using a standard curve. The effect of chlorpyrifos on the IAA production capability of the microbes was also evaluated by supplementing the LB-broth with 15, 25, 50, 75 to 200 mg/L of chlorpyrifos in the presence of 150 µg/L tryptophan (Loper and Scroth, 1986) [10].

Phosphate solubilization

Phosphate solubilization potential of bacterial strain determined by plating the bacteria on Pikovskaya agar medium. After one week of incubation at 35 °C, the clear zone around the bacterial colony indicated phosphate solubilization. Phosphate solubilization was analyzed by computing the Solubilization Index (SI), which is the ratio of total diameter (colony + halo) to colony diameter. The effect of chlorpyrifos on the phosphate solubilization ability of the bacteria quantified by supplementing Pikovskaya agar plates with 100 and 200 mg/L of chlorpyrifos (Gaur, 1990)^[7].

Ammonia production

Ammonia production measured by McCartney bottles containing 10 mL peptone water which were inoculated with freshly grown bacterial culture in triplicate and incubated at 35 °C for 96 h. The NH₃ production was detected by adding Nessler's reagent, and observing the formation of yellow color in the bottle, the intensity of the color produced noted. The addition of 100 evaluated the effect of CP on ammonia production by bacterial strains under study-200 mg/L chlorpyrifos to peptone water (Cappuccino and Sherman, 1992)^[4].

Results and Discussions

In this present study, the microbial degradation of chlorpyrifos studied by the potential degrader *Pseudomonas aeruginosa* (AP2016) which was isolated and screened from the soil. The selected isolate was gram-negative rods; the biochemical characteristics showed that indole formation, methyl red test Voges-Proskauer tests were negative, citrate test was positive, H₂S not produced, catalase, gelatin hydrolysis, and oxidase tests were positive (Table 1). A pure culture of *Pseudomonas aeruginosa* strain was feed with different concentration of chlorpyrifos i.e.; 15, 25, 50, 75 and 100mg/L (scale up) and the results were quantified by GC-MS under controlled environmental conditions as shown in figure 1 (a, b and c). Based on GC-MS analysis, it confirmed that benzene, 1, 3-bis (1, 1-dimethylethyl) was the metabolite during the chlorpyrifos and its metabolite TCP degradation by AP2016 strain of *Pseudomonas aeruginosa* as shown in Fig. 1a, initially, the sample showed m/z value of 350.76, which indicated that chlorpyrifos is still present in the medium. At the end of the 10th day, the formation of TCP with m/z of 199.06 [(M+1H)⁺, where M = 198] was obtained (Fig. 1b). After incubation for 20 days, a final metabolite of chlorpyrifos was identified with mass ion at m/z of 191 [(M+1H)⁺, where M = 190] which corresponds to benzene, 1, 3-bis (1, 1-dimethylethyl) by Mass Spectrometry (Fig. 1c).

The GC-MS data also showed that chlorpyrifos was degraded up to 68% in MSM containing 75mg/l chlorpyrifos at 12 days, after that up to 21days, there is no significant degradation observed. While in MSM containing 100mg/L chlorpyrifos it was degraded up to only 25% up to 15 days after it shows no significant degradation. In the rest of the concentrations that were evaluated, chlorpyrifos completely degraded. Thus microorganism showed the best result at 15-75 mg/L concentration.

The potential biodegrader *Pseudomonas aeruginosa* (AP2016) was used in the overall study in flasks bioreactor under controlled conditions. The results quantified by GC-MS showed TCP (3, 5, 6-trichloro-2-pyridinol) as the hydrolyzed product in MSM with chlorpyrifos as a sole source of carbon and phosphorus. However, similar metabolization and mineralization of Chlorpyrifos to 3, 5, 6- trichloro-2-

pyridinol (TCP) and 3, 5, 6-trichloro-2-methoxy pyridine (TMP) was reported (Racke *et al.*, 1993). Similarly, in a previous study, TCP was detected as a principal metabolite in chlorpyrifos degradation (Baskaran *et al.*, 2003)^[1]. The GC-MS data showed that chlorpyrifos was completely degraded within 14th day showing .011 mg/L concentration. While at 25 mg/l and 50mg/L concentration, it takes 18 and 21 days respectively for complete degradation. At 75mg/l concentration the degradation is rapid up to 12days, and after that, the concentration varied from 51-50.8 mg/l, this showed an accumulation of intermediates in the flasks reactor. The TCP started accumulating in the medium at 20-50 mg/L concentration, but during the experiment, its start decreasing. At 100mg/L TCP started to accumulate again, and chlorpyrifos degradation slows around 10th to 11th day of study (fig1). The presence of intermediates, e.g., 2, 4-bis (1, 1-dimethyl) phenol and 1, 2 Benzenedicarboxylic acids, were also detected at 25 and 50 mg/l chlorpyrifos in MSM.

Effectiveness of *Azotobacter chroococcum*, *Bacillus mucilaginous*, and *Pseudomonas fluorescense* P469 has been tested in field trials as an effective Biofertilizers (Zhigletsova *et al.* 2010; Kutyova *et al.* 2002). The plant growth was promoting the activity of the *Pseudomonas* spp. Both in the absence and presence of chlorpyrifos was also examined (Table 2). The bacterial spp. Shows a substantial amount of increase in IAA production within 24hrs by stimulating the effect of *Pseudomonas* spp, which reduces the plant uptake of toxic ions and increasing the auxin content of wheat (Hasnain and Sabri 1996). The increase in IAA shows concentration dependent pattern, depending upon the concentration of tryptophan and chlorpyrifos, respectively. At 25, 50, 75,100 and 200 µg/mL, the observed IAA values were 15.39, 18.11, 23.23, 24.27, 24.55 and 25.15 µg/mL respectively which shows significant increases in IAA production by bacterial strain. When the effect of presence and absence of chlorpyrifos was assessed the result shows an initial increase in IAA value from 18.30, 19.02, and 19.68, 19.46µg/mL at 15, 25, 50 and 75 mg/l of chlorpyrifos concentration.

Further, the value of IAA was reduced from 19.46 to 17.88 µg/mL as the chlorpyrifos concentration increased from 75 to 100 mg/l, respectively. Bacterial strain also shows p-solubilization activity, which also reduced as we increased the chlorpyrifos concentration. The further bacterial strain was found positive for ammonia production and showed the same trend as above.

PGP and bioavailability property by *P. chlororaphis* TSAU13 and *P. extremorientalis* TSAU20 was observed in common bean (*Phaseolus vulgaris*) in nutrient-deficient soil of Uzbekistan (Egamberdieva 2011). The growth studies of *V. unguiculata* (cow pea) in controlled conditions were undertaken with *Pseudomonas* spp. (with or without). In the controlled condition, the plant shows normalize growth rate, but with the addition of chlorpyrifos to the soil, there is a reduction in certain plant parameters such as shoot (12.92cm), root (15.26cm) and leaf length (2.95cm) respectively. Plant inoculated with the bacterial strain (AP2016) shows significant growth enhancement. Studies showed the role of combined inoculation of nitrogen-fixing bacteria (*Rhizobium leguminosarum*) with PSB *Pseudomonas* sp. strain 54RB in increasing the dry matter and yield of wheat (Afzal *et al.* 2005). An increase of 16.07 to 17.95cm, 19.26 to 19.85cm and 5.21 to 5.33cm in the shoot, root and leave size is observed at 100, 150 and 200 mg/kg concentration of chlorpyrifos. Naveed *et al.* (2008) reported the improved growth (up to 39%), increased plant height (16%), increase in

the number of grains per spike (11.7%), and grain yield (39%) compared to non-inoculated maize control. Since 200 mg/kg chlorpyrifos was detrimental for the growth of the plant, and it showed a reduction of 16.59, 18.15 and 4.42 cm in shoot, root and plant length respectively (Table 3).

Soil induced degradation led to cumulative degradation by several soil bacteria as an effective method. In a study, C5 consortia are capable of giving 90% degradation of chlorpyrifos (125 ppm) in eight days of incubation under optimized conditions of pH 7 and temperature 30 °C (Elizabeth Mary John *et al.* 2016) [29]

Further bioremediation studies the change of pH at different concentration of chlorpyrifos was observed (Fig2) that shows the pH 7-7.5 optimum for chlorpyrifos degradation. *Pseudomonas putida* showed the 76% degradation of chlorpyrifos at pH 7, temperature 35 °C, pesticide concentration of 2% (Vijayalakshmi P. and Usha M. S, 2012). Initial there is a gradual decrease in the pH up to 7-8th day, but it further increases during the later stages of the study. At 15mg/L chlorpyrifos in MSM, the pH varied from 7.0-5.5 with a low of 5.02 at the 11th day. At 25mg/L chlorpyrifos in MSM, the pH decreased from 7.0 to 5.7 by the last day of the experiment with the lowest value of 5.55 on the 12th day. A decrease from 7.0-5.66 was also observed in 50mg/L flask with a low of 5.44 at 5th day. In the un-inoculated flask, the pH remained constant. In a study Brajesh K. Singh *et al.* 2003 [30], concluded that only soils with a pH of >6.7 were able to maintain this degrading ability 90 days after inoculation, so in this present study also the pH shows high degradation ability at pH>6.5.

At the start of the experiment, the strain *Pseudomonas aeruginosa* (AP 2016) showed exponential growth in the flasks bioreactor in 15-50mg/L concentration of chlorpyrifos. But at 75-100mg/L, the growth rate declines. At 15mg/L concentration, the maximum growth was attained at 5th day and constant phase at 7th day. At 25- 50 mg/L chlorpyrifos, the maximum microbial growth was attained at 6th and 7th day, respectively, and there after the growth decline (Huang, 2000) [8]. And control showing zero growth (Fig3).

As the *Pseudomonas aeruginosa* (AP 2016) was well adapted to 75mg/L of chlorpyrifos concentration in MSM, but from day 12th at 75mg/L of chlorpyrifos, it started becoming detrimental for its growth. Furthermore, intermediates such as 2, 4-bis (1, 1- dimethyl ethyl) phenol and 1, two benzene dicarboxylic acid persisted for the longer duration at higher concentration of chlorpyrifos in MSM.

Conclusion

Plant growth promoting characteristics of *Pseudomonas aeruginosa* revealed the presence of substantial phosphate solubilization, IAA, and ammonia production. Bacteria

isolated from soil rhizosphere are promising plant growth promoter and also good pesticide degrader. Earlier studies conducted on different soil bacteria on thiamethoxam degradation were also found to possess IAA production and N₂-fixation abilities (Zhou *et al.*, 2014) [18]. Significant decrease in PGP activities of chlorpyrifos resistant bacteria was observed in the presence of chlorpyrifos. Chlorpyrifos has been reported to have an inhibitory effect on soil microbes (Fang *et al.*, 2009) [19]. Pesticide hampers the p-solubilization and ammonia production activity of microbes by killing the useful p-solubilizing and cellulolytic bacteria (Ramani, 2001). The growth of *V. unguiculata* is inhibited when soil is supplemented with increasing dose of chlorpyrifos, i.e., 150-200 mg/kg. Introduction of chlorpyrifos to soil also reduced certain plant parameters such as shoot, root, and left size (Table 3). The negative effect of pesticides on plant growth can be attributed to the inhibition of electron flow in the photosynthetic chain. The negative effect of chlorpyrifos to plant growth can be attributed to the inhibition of electron flow in the photosynthetic chain. In photosynthesis, the ATP flow is blocked by uncoupling action of organophosphates and DDT (Mitra and Raghu, 1998) [21]. Plant (*V. unguiculata*) growth was enhanced when soils were inoculated with chlorpyrifos metabolizing bacterial strain. Enhanced rate of plant growth can be attributed to IAA production, phosphate solubilization, and decrease in toxicity of chlorpyrifos owing to its increased degradation. In a greenhouse study, two P-solubilizing bacteria *Serratia marcescens* EB-67 and *Pseudomonas* spp. CDB-35) increased the biomass of maize by 99% and 96%, respectively (Hameeda *et al.* 2006). As observed in the biodegradation studies (Fig1, 2 and 3) chlorpyrifos of 15-75 mg/l concentration was completely degraded, this higher degradation potential is responsible for greater pesticide degrading activity of the inoculated microbes. Here plant-microbe interaction plays an important role: plant secretes his root exudates, which attract maximum microbe's population, which simultaneously enhances the chlorpyrifos degradation.

The study also shows the protection of plant seedlings from toxic effects of pesticide when the soil has been inoculated by pesticide degrading bacteria (Gangadhara and Kunhi, 2000) [22]. In conclusion, these bacteria strain were found to possess not only pesticide degrading capacities that lower the toxic effects of this pesticide on plants but also various other traits that helped in plant growth promotion. The traits include the production of phytohormones such as IAA and p-solubilization that enhance cell growth, solubilization of phosphate for root uptake. Thus the bioremediation of chlorpyrifos by a scale-up technique using potential microorganism has been proved to be very useful in soil and water contaminants.

Table 1: Biochemical Characteristics of the soil isolate

Name of the Test	Result
Indole test	-ve
Methyl Red	-ve
Voges-Proskauer test	-ve
Citrate	+ve
H ₂ S Production	-ve
Catalase	+ve
Oxidase	+ve
Gelatin hydrolysis	+ve

Table 2: Plant growth promoting activities of chlorpyrifos degrading strains in the presence and absence of chlorpyrifos

Bacterial strain	Trp ($\mu\text{g mL}^{-1}$)	IAA ($\mu\text{g mL}^{-1}$)	CP conc. (mg L^{-1})	IAA ($\mu\text{g mL}^{-1}$)	Phosphate solubilization		NH ₃ production
					Zone size	SI	
<i>Pseudomonas</i>	25	15.39 \pm 0.20	Control	17.33 \pm 0.10	16.63 \pm 0.11	4.48	+++
<i>Aeruginosa</i>	50	18.11 \pm 0.31	15	18.30 \pm 0.07	15.26 \pm 0.16	4.34	+++
(AP2016)	75	23.23 \pm 0.85	25	19.02 \pm 0.27	14.30 \pm 0.16	3.79	++
	100	24.27 \pm 0.28	50	19.68 \pm 0.15	13.21 \pm 0.55	3.15	++
	150	24.55 \pm 0.39	75	19.46 \pm 0.23	10.77 \pm 0.33	3.04	++
	200	25.15 \pm 0.34	100	17.88 \pm 0.52	9.91 \pm 0.24	2.51	+

Trp = tryptophan; IAA = indole acetic acid; SI = solubilization index.

The values indicate the mean \pm SD of three replicates.

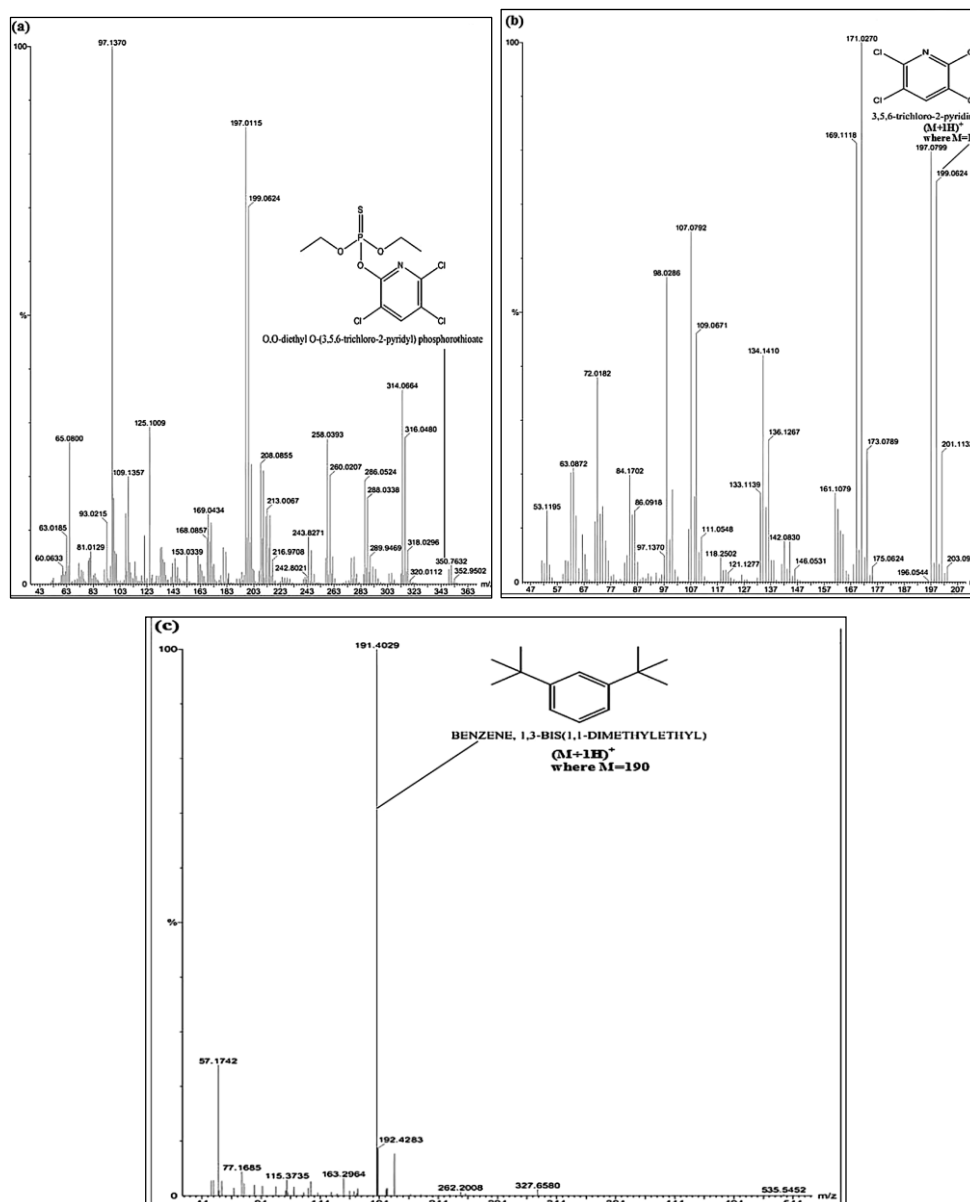
+++ = large amount of ammonia; ++ = moderate amount of ammonia; + = less amount of ammonia.

Table 3: Measurement of growth parameters of *V. unguiculata* at different concentration of Chlorpyrifos (100, 150 and 200 mg /kg).

CP (mg/ kg)	Shoot length (cm)	Root length (cm)	Leaf length (cm)
Control*	15.01 \pm 0.11	16.15 \pm 0.22	3.11 \pm 0.10
CP+ <i>V. unguiculata</i>	12.92 \pm 0.06	15.26 \pm 0.27	2.95 \pm 0.07
CP+ <i>V. unguiculata</i> +100	16.07 \pm 0.44	19.26 \pm 1.27	5.21 \pm 0.17
CP+ <i>V. unguiculata</i> +150	17.95 \pm 0.06	19.85 \pm 0.91	5.33 \pm 0.30
CP+ <i>V. unguiculata</i> +200	16.59 \pm 0.83	18.15 \pm 0.35	4.42 \pm 0.39

*= control (where only growth of *V. unguiculata* was studied)

The values indicate the mean \pm SD of three replicates.

**Fig 1 (a, b, c):** Figures shows the degradation pattern of chlorpyrifos into its degradation product TCP in the culture extracts analyzed by GC-MS

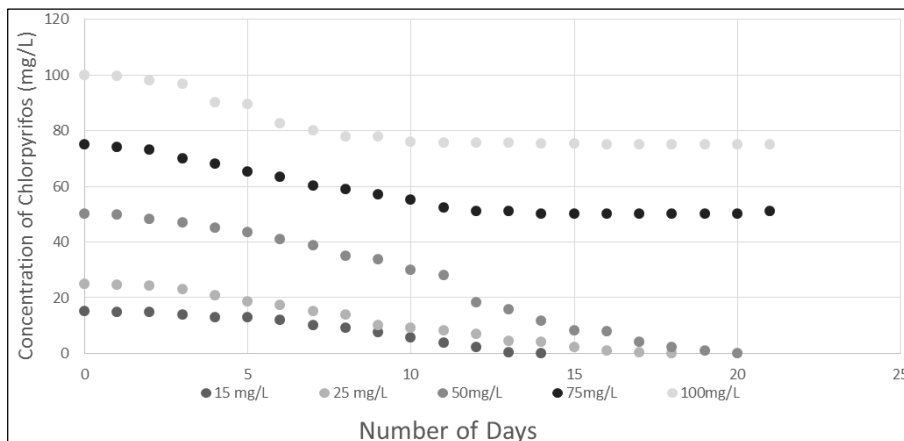


Fig 1: Degradation of chlorpyrifos at different rates at different concentration with respect to no. of days

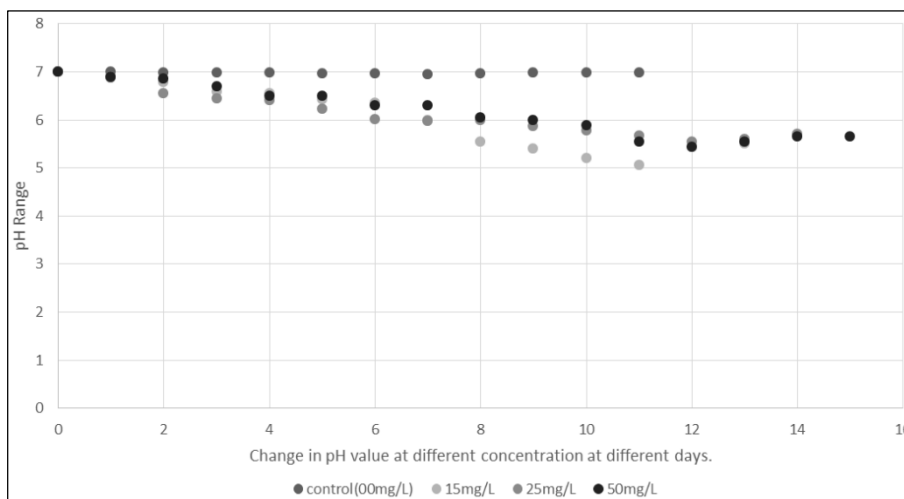


Fig 2: Variation in pH during bioremediation of chlorpyrifos in MSM. Values are expressed as mean \pm SD of experiments in triplicate. (In figure 2, why the data from control group was missing after 11 day?)

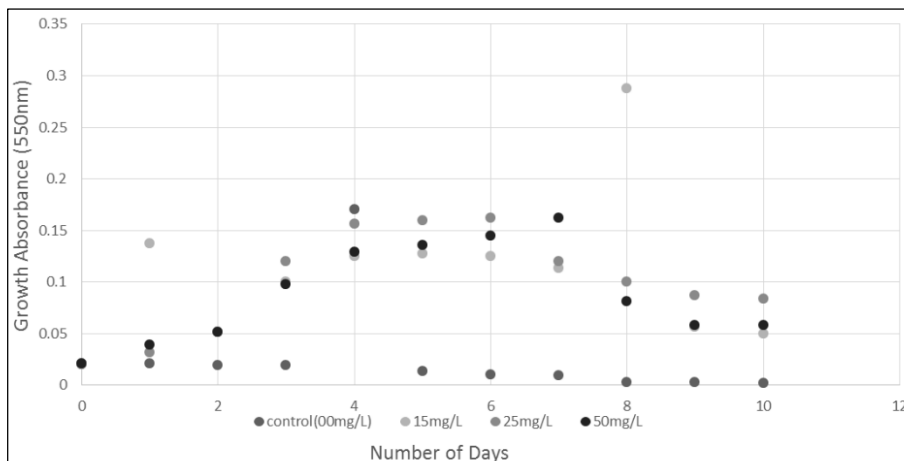


Fig 3: Variation in bacterial growth during bioremediation of chlorpyrifos in MSM. Values are expressed as mean \pm SD of experiments in triplicate. (In figure 3, the treatment of 15 mg/L showed very sudden fluctuations in day 1 and day 8, which were not similar to the general tendency. Are they reliable data? Please explain the reason).

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