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BIODEGRADATION OF ORGANOPHOSPHOROUS PESTICIDE, CHLORPYRIFOS BY SOIL BACTERIUM - *BACILLUS MEGATERIUM* RC 88

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Key words *: Organophosphorous Pesticide, Chlorpyrifos, Bacillus megaterium, LC-MS*

Abstract – Chlorpyrifos (CP), an Organ phosphorous pesticide is used around the world to control pest insects in agricultural sector. CP is registered for use in nearly 100 countries and applies to approximately 8.5 million crop acres each year. The crops with the most intense CP use are cabbage, cotton, cucumber, and other vegetable crops. A bacterial strain RC 88 capable of degrading CP was isolated from soil collected from agricultural field of Nanjangud Taluk of Mysore district, Karnataka, India by enrichment method. It was observed that, the strain RC 88 was able to utilize the CP as a sole carbon and energy source grown in Mineral salt medium containing 100mgL^{-1} of CP at 37^{0}C at 150 rpm in a rotary shaking incubator for about 7-14 days. The culture was extracted for CP at a frequency of the 7th and 14th day of incubation and was examined by LCMS. The strain RC 88 showed the ability of CP degradation by Hydrolyzing it into 3,5,6-trichloro-2-pyridinol (TCP). On the basis of morphology, physiological characterization and phylogenetic analysis of 16S rRNA sequence, the isolate was identified as *Bacillus megaterium.* The result obtained suggests that, the *Bacillus megaterium* sp *strain* RC 88 will be potentially useful in abatement of Chlorpyrifos contaminated soil.

INTRODUCTION

Chlorpyrifos [O, O-diethyl-O (3, 5, 6 -trichloro-2-pyridyl phosphate)] an Organophosphorous pesticide (OP) used to control foliage and soil borne insect pest on a variety of food and feed crops. CP has been used as pesticide since 1965 and it is used in agricultural sectors like corn, soybeans, fruits and nuts, broccoli, cauliflower, etc., and non-agricultural sectors like golf courses green houses and turf. It is also registered for the direct use on animal domestic dwellings and commercial establishment (Racke *et al*., 1996). It is included in the second round of chemicals selected for review under the National Registration Authority Existing chemicals Review Program, CP was accorded high priority for review because of its toxicity to birds and aquatic organisms (Environment protection group, Australia 1999). Availability of different pesticides in the field provides exposure of several different kinds of microorganisms to pesticides. Most of the organisms die under toxic effect of pesticides, but few of them evolve in different ways and use pesticide compounds in metabolism. Several reports are available indicating degradation of different pesticides when they are available in nature in excess (Horvath *et al*., 1972). Biotransformation of CP from aerobic consortia in aqueous medium to 3, 5, 6 trichloro-2- pyridinol was reported by Lakshmi *et al.,* 2008. *Pseudomonas aeruginosa* is the most widespread Gram negative bacterium found in soil, isolates of this genus have been found to have potential to degrade CP (Fulekar and Geetha, 2008)

Improved degradation of chlorpyrifos by *Enterobacter* strain B-14 was reported by (Singh *et al*., 2011). A CP degrading *Flavobacterium* sp is reported by (Jilani and Khan, 2004). In this present experimental study, isolation and identification of CP degrading bacteria from agricultural field of rhizosphere soil, which had history of the practice of CP application for many years was carried out.

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MATERIALS AND METHOD

The soil samples were collected from the surface area reaching about 10-15 cm depth and near the rhizosphere region of plants of agricultural fields at Nanjangud Taluk of Mysore District 12.09^0 N 76.77E, Karnataka, India. The soil has the history of extensive use of Organophophorous pesticides for the control of crop pests. The soil sample was air dried at room temperature to the remove excess moisture and it was passed through a 2mm sieve to remove the unwanted debris and stored in a polyethylene bags at 4°C for further experimental study.

In the present study chemicals like, Chlorpyrifos (99.8%) were purchased from Sigma Aldrich Co USA, Luria Bertani Hi Veg Broth (LB) and Nutrient agar (NA) was purchased from HI-Media, Mumbai, India, TLC plate (M/s S.D Fine Chem Ltd, India). Mineral salt medium (MSM; pH 6.8-7.0) was prepared by using the following chemicals: Dihydrogen Phosphate Na_2HPO_4, 5.8g/L; Potassium dihydrogen Phosphate KH_2PO_4, 3.0g/L; Sodium Chloride NaCl, 0.5g/L; Ammonium Chloride NH_4Cl, 1.0g/L; and Magnesium Sulfate $MgSO_4.7H_2O$, 0.25g/L obtained from Nice chemicals Pvt Ltd, India.

Enrichment and Isolation of CP degrading bacteria from soil samples was carried out by using MSM medium for isolation of bacteria. About 10 g soil sample was added into 250 mL Erlenmeyer flask containing 100 mL MSM medium supplemented with 100 mgL^{-1} CP as the sole carbon source. The flask was incubated at 37°C at 150 RPM for 7 days in a rotary shaking incubator (Hally instruments, India). At the 7th day 10 mL of the culture was sub cultured in fresh 100 mL MSM medium added with 100 mgL^{-1} of CP and incubated for about one week. The incubated culture was serially diluted and spread on Nutrient agar plates and later incubated at 37°C for 48 hours. The bacterial strains obtained were further sub cultured by a streak plate method to get pure colonies. Further the obtained pure colonies were tested for growth and CP degradation by inoculating them in Mineral salt medium supplemented with CP 100 mgL^{-1} as sole carbon source.

In biodegradation studies, the flask containing the media and the pesticide which was incubated at 37°C at 150 RPM for 7 days in a rotary shaking incubator was extracted by Shake flask method by using AR grade n- Hexane and evaporated to dryness and re-dissolved in HPLC grade acetonitrile to 2mL and stored in eppendorf tube and the samples were analyzed for Liquid chromatography Mass Spectroscopy (LC-MS) system (Water's

Fig. 1. Map showing the location of Mysore district

Synapt-G2-S, USA) equipped with an auto sampler column: acquity UPLC-BEH C18 1.7um 1.0 × 50 mm, run time of 8min, flow rate 0.3 mL/min, injection volume 2µL. Mass spectroscopy (MS) was performed using a Synapt G2 HPMS MS (Waters, USA) equipped with Electron spray ionization (ESI) detector. The operating condition was Capillary (kV)-3.00, Sampling Cone-40.00, Extraction Cone-4.00, Source Temperature (^oC)-100, Desolvation Temperature (^PC)-200, and Desolvation Gas flow (L/Hr)-500.0. Tentative identification of strain RC 88 was carried out based on Morphological and Biochemical characterization according to the Bergey's Manual of Determinative Bacteriology (Robert S Breed *et al.,* 1957).

Hi PurA Genomic DNA purification kit from Mol Bio Hi-Media was used to extract and purify DNA. The molecular characterization includes the extraction of bacterial DNA and PCR amplification of 16s rRNA sequences in the DNA. The genomic DNA was isolated using the modified protocol of Doyle and Doyle's method. Bacterial cultures were inoculated into the LB broth and incubated at 35^oC in a shaker for 48 hrs. 2mL of the culture broth was transferred into an Eppendorf tube and centrifuged at 10,000 RPM for 15 min. Supernatant was discarded and the pellets were treated with 500 µL of extraction buffer (100mM Tris, 20mM EDTA, 500mM NaCl and 5% SDS) and incubated for 45min at 65^oC. The bacterial culture was then treated with 25 µL RNAse and incubated at room temperature for 10 min. 25µL Proteinase K added to this and incubated for 10min in Room temperature. This was then centrifuged at 10,000 RPM for 15min. The supernatant was transferred to fresh tube and pellet was discarded. Equal volume of chloroform and isopropanol mixture in the ratio 24:1 was added and centrifuged at 12,000 RPM for 10 min. After centrifugation, the upper aqueous layer was transferred into a fresh tube and 1/20th volume of chilled sodium acetate was added with equal volume of chilled 95% ethanol and Incubated overnight at -20^oC. After incubation, the tube was centrifuged at 12,000 RPM for 10 min. The supernatant was discarded and the pellets were washed with 70% ethanol, air dried and dissolved in 40µL of elution buffer and stored at -20^oC.

The DNA obtained samples were diluted with DNAse free water to the required concentration. The DNA was used as a template to amplify variable region of bacterial 16s rDNA gene by using the universal primers 16sF (5^o-CCAGACTCCTACGGGAGGCAGC - 3^o) and 16sR (5 - GCTGACGAGAGCCATGCAGCACC-3^o). The PCR amplification of 16s rDNA were as follows: volume (concentration)-Template 2 µL (100 ng µL^{-1}), dNTP's 1 µL (10 mM), Taq Buffer 2.5 µL (10 x), Nuclease free water 14.5 µL, Taq Polymerase 1 µL (5 units/µL), Forward primer 2 µL (10^{o}µL^{-1}) and Reverse primer 2 µL (10 pl^{-1}), final total reaction volume is 25 µL. The amplification program was operated as below: Initial denaturation at 95^{0}C for 5min, 1 Cycles; Denaturation at 95^{0}C for 30sec, 35 Cycles; Annealing 60^{0}C for 30sec, 35 Cycles; Extension 72^{0}C for 45 sec, 35 Cycles; Final extension 72^{0}C for 10min, 1 Cycles; Hold 18^0 C for 1 min, 1 cycle. The PCR amplification products were electrophoresed on 1.2 % Agarose gel for purity check. The PCR product was sequenced by Chromous Biotech, Bangalore.

RESULTS AND DISCUSSION

In the present enrichment study the total of eight representative bacterial strains were obtained from soil samples enriched with CP and were coded as RC 53, RC 88, RC 10, RC 8, RC 12, RC 13, RC 16 and RC 18 and it was observed that all the bacterial stain showed growth in MSM medium containing 25 mg L^{-1} and 35 mg L^{-1} of CP. At 75 mg L^{-1} of CP the bacterial strain coded RC 53, RC 88, RC 10 and RC 8 only showed growth in the mineral medium. Further the concentration of CP was augmented to 100mg L^{-1} to confirm the tolerance, growth limit and it was observed that the bacterial strain RC 88 only showed the growth. All the other bacterial strain was unsuccessful to grow in 100mg L^{-1} of CP. Finally the bacterial strain RC 88 was used for the degradation of CP, the results are shown in the below Table 1.

Bacterial strain RC 88 was able to endure in higher concentration of CP (100mg L^{-1}) in mineral salt medium and sub cultured, the morphological and physiological characteristic of the isolate was observed and the colonies were slow growing rhizoidal, flat, filiform, rough, translucent, pale white. The biochemical tests like catalase and oxidase test showed +ve whereas the Indole, MR/VP, citrate utilization, urease production test showed –ve result. This implifies the bacterial strain to be *Bacilli* species. Molecular Identification was carried out to identify the bacterial species. The PCR amplification products were electrophoresed on 1.2 % Agarose gel for purity check. The PCR product was sequenced by Chromous Biotech, Bangalore, as

Table 1. Showing bacterial strains growth on Mineral salt medium at different concentrations of chlorpyrifos, where the strain RC 88 showing positive results at 100 mg L^{-1}

Sl No.	Strain Code	Bacterial growth on MSM containing Chlorpyrifos as the sole carbon and energy source			
		25 mg L^{-1}	35 mg L^{-1}	75 mg L^{-1}	100 mg L^{-1}
1	RC 53				
2	RC 88	++	++	++	-+
3	RC 10	+	+	-	-
4	RC 8	+	-	-	-
5	RC12	+	+	-	-
6	RC 13	+	-	-	-
7	RC 16	+	-	-	-
8	RC 18	+	+	-	-

Note: - '+' Indicates growth of bacteria
'-' Indicates No growth of bacteria

per the nucleotide blast analysis, The strain RC-88 was showing 99% similarity to species *Bacillus megaterium*Y-1 of Acession no. KF483225.1. *Bacillus megaterium* is a gram positive endospore forming, rod shaped aerobic bacteria. It is normally found in soil and considered as a saprophyte. It belongs to the family the *Bacillaceae*.

For Biodegradation studies, Chlorpyrifos (100 mg L^{-1}) was amended in mineral salt medium at different pH 5.5 ± 0.2 , 5.9 +/-0.3, 6.0+/-0.2, 6.9+/-0.2, 7.0+/-0.2, and 8.5+/-0.1 and inoculated with the strain RC 88 and was incubated for about 7-14 days in rotary shaking incubator at 150 RPM at 37^0C. After 14th day of incubation period, the cultures were extracted by liquid-liquid phase extraction by separating funnel using AR grade n- Hexane. The lower phase containing chlorpyrifos was evaporated at room temperature and redissolved with equivalent volume of HPLC grade acetonitrile and bring to final volume of 1.5 mL and stored at -20^0C and it was analyzed by LC-MS for the identification of chlorpyrifos metabolites, the *m/z* at different pH is tabulated in table 6 out of which the metabolite obtained after degradation was TCP and chlorpyrifos oxon. The chlorpyrifos standard and degraded liquid cultures were analyzed by LC-MS. The LC-MS chrmotogram of chlorpyrifos standard showed (M+) value 351.9 (Fig. 3). At the seventh day

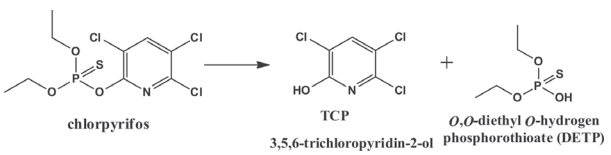

Fig. 2. The chlorpyrifos cleavage after degradation by bacterial strain RC 88 forming TCP

of the incubation a portion of the culture was extracted and analysed by LCMS the the peak showing mz value 336.32 (M+2H)+ Where M 334 is the chlorpyrifos oxon value and at the 14th day incubated culture showed (M^+H^+) value 199.96 where M is 198.44 which is 3,5,6 trichloro-2-pyridinol, (Fig. 4, 5). This clearly indicates that chlorpyrifos degradation has taken place to chlorpyrifos oxon and 3,5,6- Trichloro-2-pyridinol (Table 2) which is less harmful to the environment.

Table 2. Showing the metabolities of chlorpyrifos degradation

Sl No	Days	*m/z*	Chemical name
01	07	336.32	Chlorpyrifos oxon
02	14	197.96	3,5,6- Trichloro-2-pyridinol

Table 3. Physico-chemical properties of standard chlorpyrifos (FAO specification and Evaluation for chlorpyrifos pg 15, Food and agricultural Organisation of United Nations)

Parameter	values and Conditions
Vapour pressure	1.0 x10^{-3} Pa at 25^0C
Melting point	42-44^0C
Solubility in water	0.39 mg L^{-1} at 19.5^{o}C

Similar to our findings, *Bacillus subtilis* strain was isolated from Jerar village bridge water sample, Egypt for degradation of CP (Ehab *et al.*, 2014) and other aerobic consortia capable of degradation of chorpyrifos as a sole carbon source in aqueous medium (Vidya *et al.*, 2008). The metabolism of chlorpyrifos by microorganism in soil has been reported by many scientists. Similarly, *Pseudomonas*

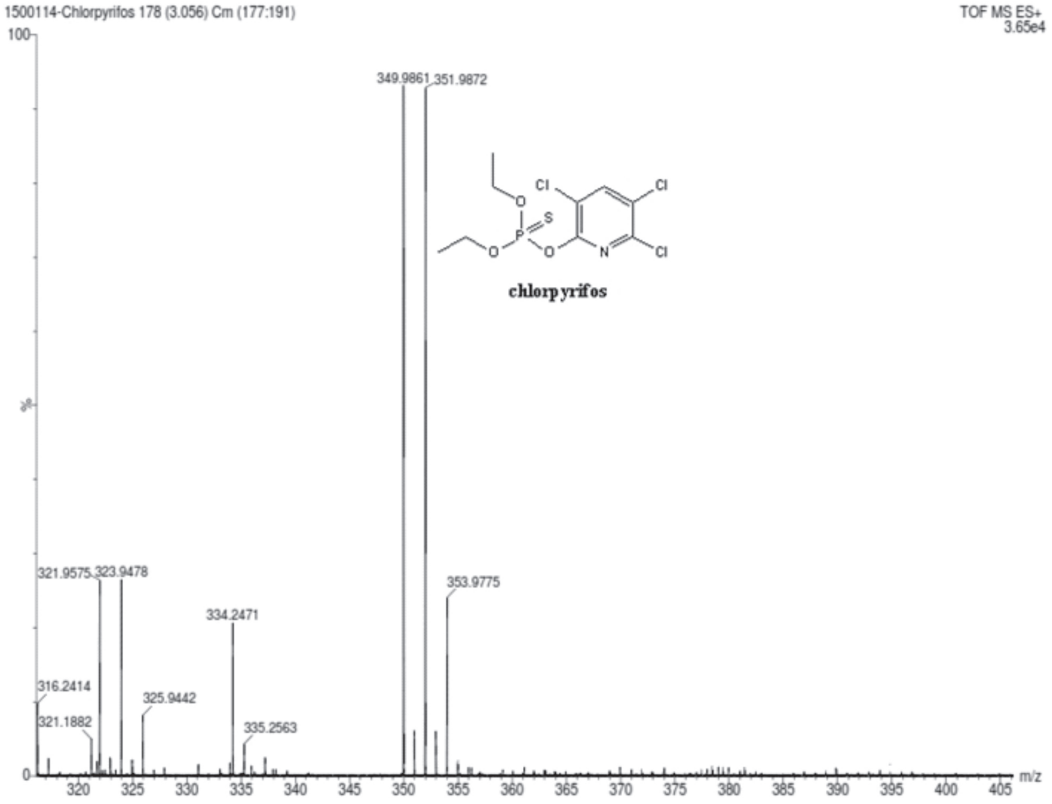

Fig. 3. LC-MS chromatogram of the Standard Chlorpyrifos showing (M+) value 351.9

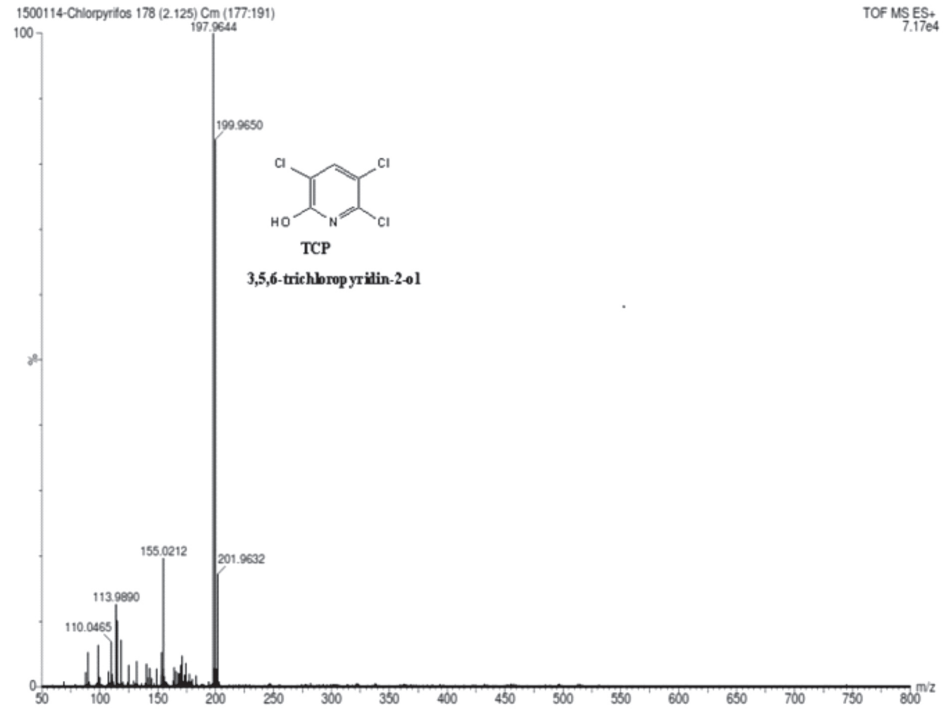

Fig. 4. LC-MS chromatogram of the bacterial metabolite of chlorpyrifos, 3,5,6-trichoropyridin-2-ol (TCP) obtained after degradation study by *Bacillus megaterium* strain RC-88, the peak showing ($M+H^+$) value 199.96 of TCP

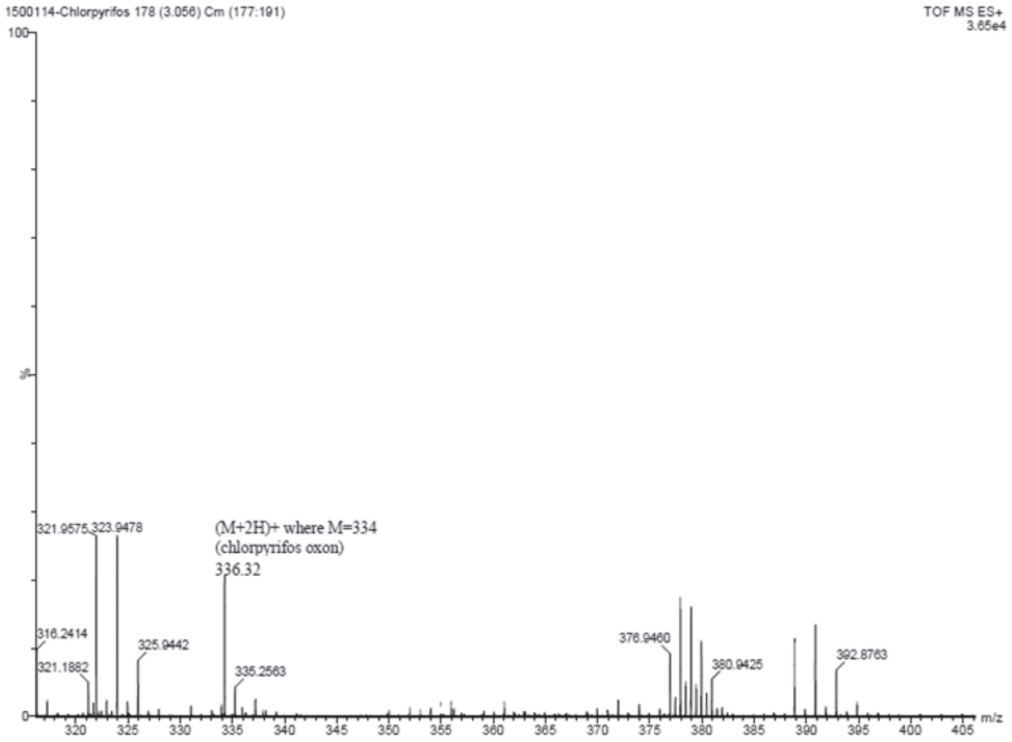

Fig. 5. LC-MS chromatogram for chlorpyrifos extract after degradation study of the culture filtrate at a intervals of time 07th days of incubation, the peak showing *m/z* value 336.32 $(M^{+}2H)^{+}$ Where M 334 is the chlorpyrifos oxon

spp. has the ability of chlorpyrifos degradation (Xiaohui *et al.*, 2008); (Mukherjee *et al*., 2004) reported that chlorpyrifos was readily hydrolyzed to 3, 5, 6-trichloro-2-pyridinol (TCP) in alkaline soil. Chlorpyrifos hydrolysis were greatly accelerated under low moisture conditions, both in acidic and alkaline soils (Ajaz *et al.,* 2005). *Klebsiella* species isolated by enrichment culture technique also showed positive results by degrading the chlorpyrifos into 3, 5, 6 -trichloro-2- pyridinol (Shenghui *et al.*, 2013). Chlorpyrifos in pure cultures and soil could be degraded by *Aspergillus* sp. Y and *Trichoderma, Alcaligenes faecalis* DSP3, *Fusarium and Bacillus latersprorus* DSP (Fang *et al.,* 2008). *Actinobacteria* were studied for chlorpyrifos degradation, their results showed that the isolates obtained were able to degrade up to 90% of the chlorpyrifos in 50 mgL^{-1} concentration (Bricerio *et al.,* 2012). Comparable TCP metabolites reported the by bacterial strain *Cupriavidu* sp DT-1 (Peng Lu *et al.,* 2013). Finally concludes that the results highlights the bioremediation of chlorpyrifos by using the bacillus species and it is the potential bacteria in cleanup of pesticides contaminated soil , the *Bacillus*

Table 4. Morphological characteristics of Chlorpyrifos degrading bacterial strains

Sl. No	Strain Code	Cell Shape	Gram reaction
1	RC-88	Rod	Gram Positive
2	RC-53	Rod	Gram Negative
3	RC-10	Rod	Gram Positive
4	RC-8	Rod	Gram Positive

Table 5. Biochemical characteristics of Chlorpyrifos degrading bacterial strains

Sl. No	Strain Code	Acid Production	Gas Production	Indole	Methyl Red	Voges Proskauer	Citrate	Urease	Catalase	Probable Genus
1	RC-88	-	-	-	-	-	-	+	-	*Bacillus*
2	RC-53	-	-	-	-	-	-	-	+	*Alcaligenes*
3	RC-10	+	-	-	-	-	+	-	+	*Streptomyces*
4	RC-8	-	-	-	-	-	+	-	+	*Streptomyces*

Table 6. *M/z* value obtained in LC-MS analysis at different ph at which 3,5,6-trichoropyridin-2-ol (TCP) obtained after degradation study by *Bacillus megaterium* strain RC-88, the peak showing ($M+H^+$) value 199.96 is TCP and *m/z* value 336.32 (M+2H)+ Where M 334 is the chlorpyrifos oxon

pH value	*m/z* value
5.5+/-0.2	146
5.9+/-0.3	175
6.0+/-0.2	200
6.9+/-0.2	199
7.0+/-0.2	334
8.5+/-0.1	289

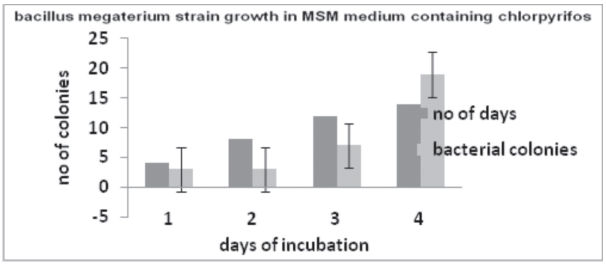

Fig. 6. The graph showing the *bacillus megaterium* strain growth at different intervals of time and its standard deviation.

megaterium RC 88 isolated in the present study completely hydrolyses the organophosphorous pesticide CP into its metabolite 3,5,6-trichloropyridin-2-ol. This bacterial species *Bacillus megaterium* in degradation of CP to its metabolite TCP was first reported as per literature survey. The LC-MS chromatogram confirms the ring cleavage of Chlorpyrifos and results in the formation of chlorpyrifos oxon 3,5,6-trichloropyridin-2-ol. The identified bacterial species represent the further investigation in the biodegradtion study on different pesticides pollutants in the soil. The bacterium is highly useful in abatement of contaminated soil the study is base data for future pilot plant study in cleaning up of contaminated soil.

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