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BIODEGRADATION OF CHLORPYRIFOS AND THEIR INTERMEDIATE METABOLITES IDENTIFIED BY LIQUID CHROMATOGRAPHY MASS SPECTROSCOPY (LC-MS)

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ABSTRACT

Chlorpyrifos , O,O-diethyl O-(3,5,6-trichloro-2-pyridinol) phosphorothioate, is a broad spectrum moderately toxic organophosphorus insecticide. The widespread use of these pesticides is harmful to the environment and also poisonous to mammals, thus it is essential to remove the same from the environment. In the present study from the chlorpyrifos contaminated soil, six morphologically different bacterial strains were isolated. Among those isolates two bacterial strains which were more efficient were developed as consortium. The two bacterial isolates namely *Pseudomonas* sp, and *Brevibacillus* sp present in the consortia were identified on the basis of Bergey's manual of determinative bacteriology. The biodegradation studies of chlorpyrifos (500mg/L) were conducted in neutral pH and temperature 37 °C. During the degradation some metabolites *viz*., chlorpyrifos oxon and diethylthiophosphoric acid were identified by mass spectral analysis. The results of merit this consortium is used to remediate chlorpyrifos contamination sites.

KEYWORDS: Chlorpyrifos, *Pseudomonas* sp, *Brevibacillus* sp, LC-MS

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INTRODUCTION

Chlorpyrifos (O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is a broad spectrum, moderately toxic organophosphorous insecticide [1]. It is a broad-spectrum organophosphate insecticide and acaricide, and is widely used for pest control on grain, cotton, fruit, and vegetable crops, as well as, lawns and ornamental plants [2]. It has high soil-absorption coefficient, but low water solubility ($2mgL^{-1}$) [3]. The environmental fate of chlorpyrifos has been studied extensively and its degradation may involve a combination of photolysis, chemical hydrolysis and microbial degradation [1]. Chlorpyrifos was resistant to biodegradation and remained effective for up to 5–17 years [4]. It was suggested that the accumulation of 3,5,6-trichloro-2-pyridinol (TCP) which is the hydrolytic product of chlorpyrifos has anti-microbial properties and this prevents the proliferation of chlorpyrifos degrading microorganisms [3]. Bioremediation is considered as an efficient and cheap biotechnological approach to clean up the polluted environment [1,5]. A chlorpyrifos-degrading bacterium *Bacillus pumilus* strain has been recently isolated from soil for bioremediation purpose [6]. Chlorpyrifos was reported to be co-metabolically degraded in liquid media by the pure cultures of *Flavobacterium* sp.[7] and *Escherichia coli* clone [8] . However, these organisms do not utilize chlorpyrifos as an energy source . A pure fungal strain, *Acremonium* sp., utilized chlorpyrifos (83.9%) as a source of carbon and nitrogen [9]. Similarly two soil fungal isolates *Aspergillus niger* and *Trichoderma viride* were evaluated for the degradation of chlorpyrifos [10]. A blue green microalga was reported to degrade 80 ppm of chlorpyrifos using alkaline phosphatase as an enzyme source [11]. However fewer studies were there on the degradation of Chlorpyrfos using bacterial consortium. In the present study, a consortium consists of *Pseudomonas* sp, and *Brevibacillus* sp was isolated from chlorpyrifos contaminated agricultural soil. The work was carried out to study the possible application of isolated consortia in the degradation of chlorpyrifos and analyze their intermediate metabolites by LC-MS Method.

MATERIALS AND METHODS

(i) Chemicals

Technical grade chlorpyrifos 97% was purchased from sigma Aldrich Pvt Ltd Bangalore, India. All other chemicals are analytical grade purchased from HiMedia Pvt Ltd,Mumbai.

(ii) Sample collection

Soil sample was collected from Groundnut field of cuddalore district, Tamilnadu, India where chlopyrifos is sprayed extensively. Soil sample was stored at 20°C for further use.

(iii) Enrichment isolation

Soil enrichment was carried out in Minimal Salt Medium (pH 7.0) containing (g L^{-1}) K_2HPO_4, 1.5; KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.5; $MgSO_4$, 0.2; $CaCl_2$, 0.05; $FeSO_4$, 0.02. 1 g of soil was added to an Erlenmeyer flask (250 mL) containing 100 mL MSM supplemented with chlorpyrifos ($200mgL^{-1}$) as the sole carbon source and incubated at 37°C on a rotary shaker at 150 rpm for 7 days. After 7 days 5 mL culture was recovered from each replicate and transferred to fresh MSM containing chlorpyrifos as the only carbon source and incubated for 7 days. One week following the last transfer, 10 fold dilutions of cultures was prepared and 100µL of each dilution was spreaded on nutrient agar plates. Isolated colonies were purified by repeated streaking. After purification all isolates were tested for growth and chlorpyrifos utilization by inoculating them in MSM containing chlorpyrifos ($500mgL^{-1}$) as sole carbon and energy source.

(iv) Biodegradation of chlorpyrifos

Shake flask studies were carried to work out the chlorpyrifos degrading capacity of the isolated strains. Seed culture of each isolated strains were grown in nutrient broth containing chlorpyrifos (500 mg/L). Following 24 h of incubation, 1% inoculum of the cultures were inoculated in MSM (200 mL) containing 800 mg/L chlorpyrifos and incubated at 37°C and 180 rpm on a rotary shaker. MSM flask without inoculum was kept as control.

(v) Extraction efficiency

Samples were recovered from culture flasks at respective time intervals (0, 12 and 24 hours) and centrifuged at 8000 rpm for 10 min to obtain cell free medium. The supernatant thus obtained was added to the separating funnel and chlorpyrifos residues were extracted from supernatant using equal volume of dichloromethane [6]. After partitioning, organic layer of dichloromethane was evaporated by solvent evaporator to obtain a powdery residue of the organic compound.

(vi) LC-MS Analysis Of Intermediate Metabolites

LC–MS (SHIMADZU) analysis was carried with the following conditions: column: Atlantis C18 (150 x 9 4.6 mm), detector: programmable variable wavelength UV detector, flow rate: 1 mL/min, mobile phase: acetonitrile + water (90:10), injection volume: 20µL chlorpyrifos was detected with a programmable variable-wavelength UV detector at 254 nm. The retention time for chlorpyrifos was found to be 18.57 min. The Mass spectroscopy was performed using a Finnegan model MS (Themo electron corporation, USA). API-ESI (Atmospheric Pressure ion trap detector-Electro spray ionization) method was used for quantification in negative ionization mode, the operating conditions were Spray Voltage (kV): 5.02, Capillary Voltage (V):16.96 Capillary Temperature (°C): 275.00, Dynode (kV): -14.86, Multiplier (V):821.20.

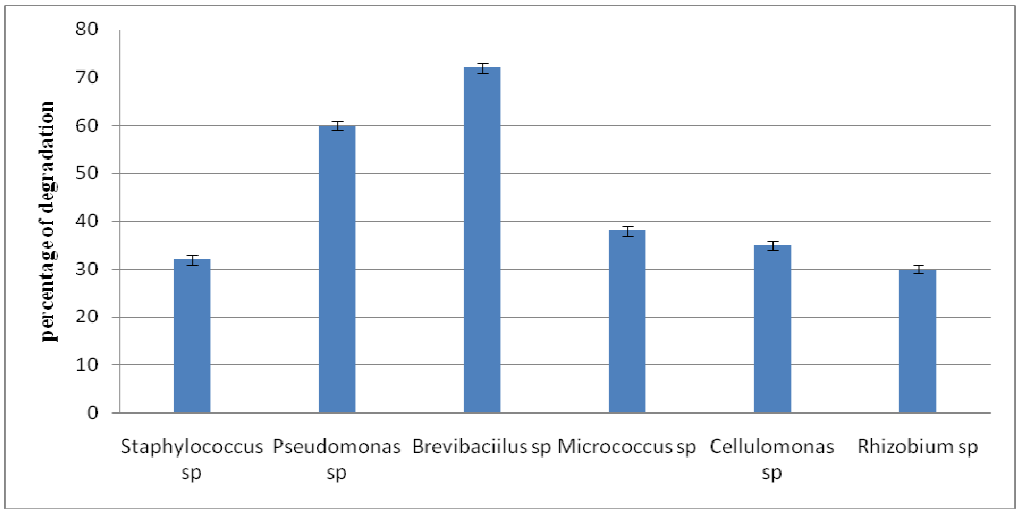
RESULTS

1. Isolation and characterization of chlorpyrifos degrading Bacteria

Based on the enrichment study six bacterial strains were obtained and identified as according to Bergey's Manual of Determinative Bacteriology [12]. The result showed that the bacterial strains were *Staphylococcus* sp, *Pseudomonas* sp, *Brevibaciilus* sp, *Micrococcus* sp, *Cellulomonas* sp, and *Rhizobium* sp. The percentage of chlorpyrifos degradation was denoted in Graph 1. Two bacterial strains namely *Pseudomonas* sp, and *Brevibaciilus* sp, which showed highest degradation ability (>50%) were selected and developed in to a consortium for further study.

Graph 1

Chlorpyrifos degradation efficiency of bacterial consortium obtained by enrichment culture Values are three replicates ± Standard Error Mean (SEM)



2. LC-MS Analysis of intermediate metabolites

The dichloromethane extracted samples were analyzed by liquid chromatography. As shown in (Fig 1a). The chlorpyrifos sample at 0 hrs showed a major peak with the retention time of 18.57 min with m/z 350.59 (Fig 2a). After 24 hrs of incubation the sample was subjected to

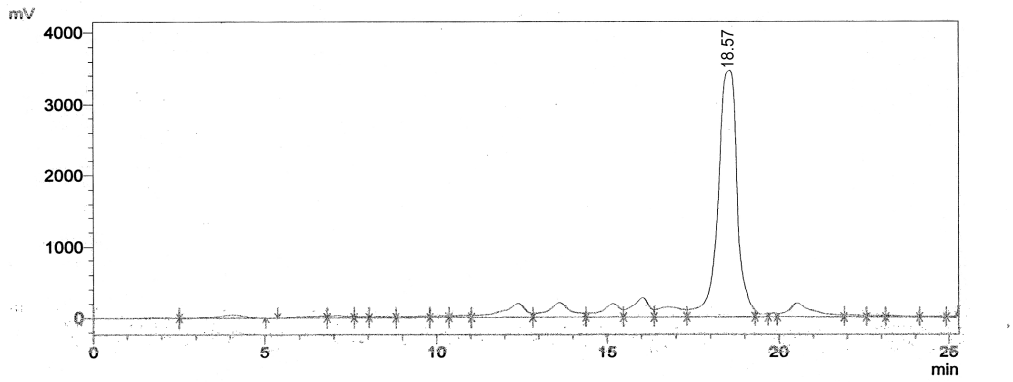
similar studies, one new peak was observed while other peaks disappeared at a retention time of 15.78 min (Fig 1b) with the m/z value of 343.45 (Fig 2b). This mass value is similar to the mass value of chlorpyrifos-oxon [O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphate] [13]. When the spectrum was taken for the same sample after 48 hrs, it was quite interesting to note that the peak at m/z 343.45 was disappeared instead new peaks at a retention time of 15.73 (Fig 1c) with m/z value 155.22 (Fig 2c). Were observed. comparing this spectrum with the spectrum at 0th hour shows that the chlorpyrifos is still present in the medium, since still a peak at m/z ratio of 350.04 is visible in the spectrum. At the end of 48hrs, the formation of Diethylthiophosphoricacid (DETP) at retention time 10.43 min (Fig 1c) with m/z 155.22 (Fig 2c) was observed, the metabolites found during chlorpyrifos degradation were listed in (Table 1). According to this spectrum with the spectrum at 0th hour showed that the chlorpyrifos was still present in the medium but the percentage of degradation was found to be 70%. It is well established that the most common pathway of hydrolytic degradation of chlorpyrifos involves formation of 3,5,6-trichloro-2-pyridinol which is accelerated under alkaline conditions.

Table 1
The degraded metabolites of chlorphyrifos by bacterial consortium.

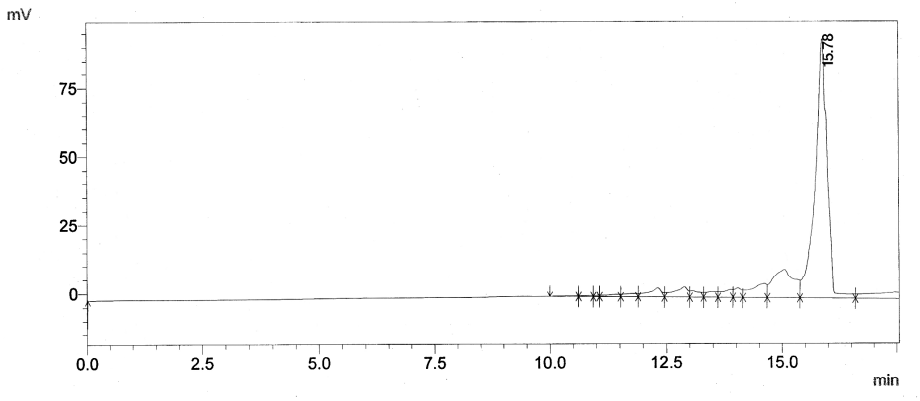
Serial No	Time in (hours)	Retension Time (RT) minutes	Atomic mass (*m/z*)	Metabolites
1	24	15.78	340.32	Chlorpyrifos-oxon
2	48	10.43	157.13	Diethylthiophosphoric acid

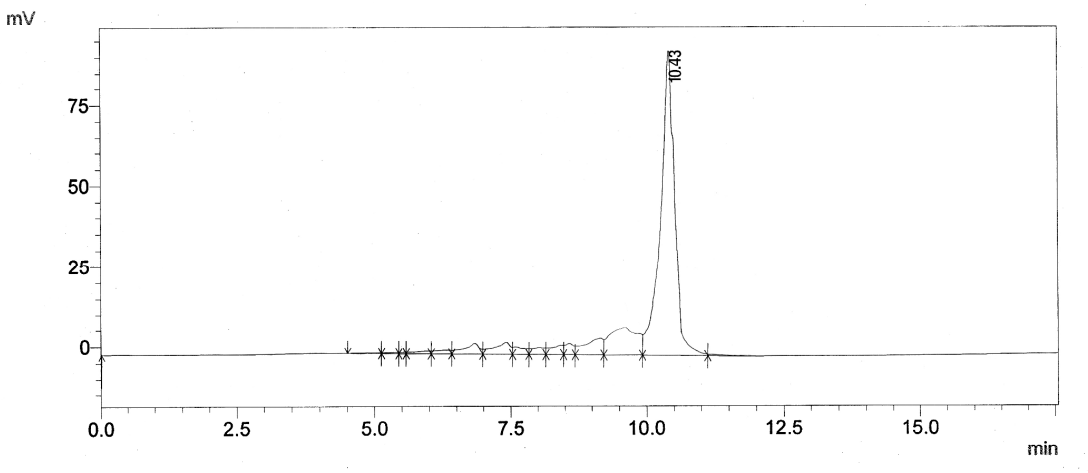
Figure 2
Gaschromatogram of Chlorfyrifos a) 0 hrs b) 24 hrs c) 48 hrs

a)



b)

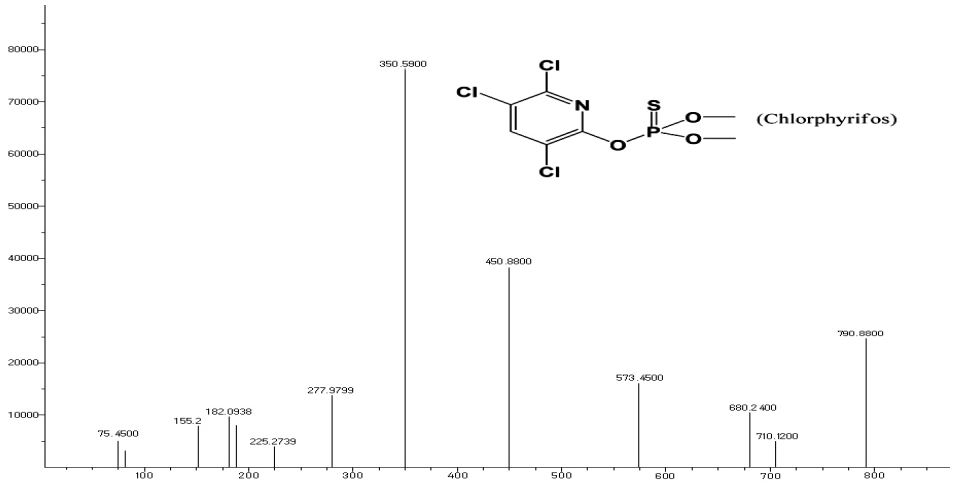




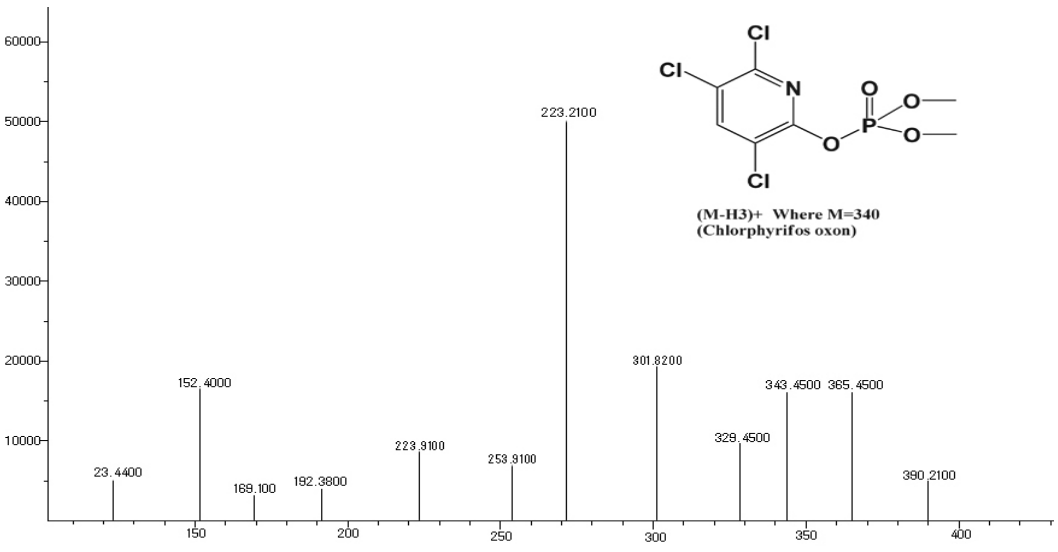
c)

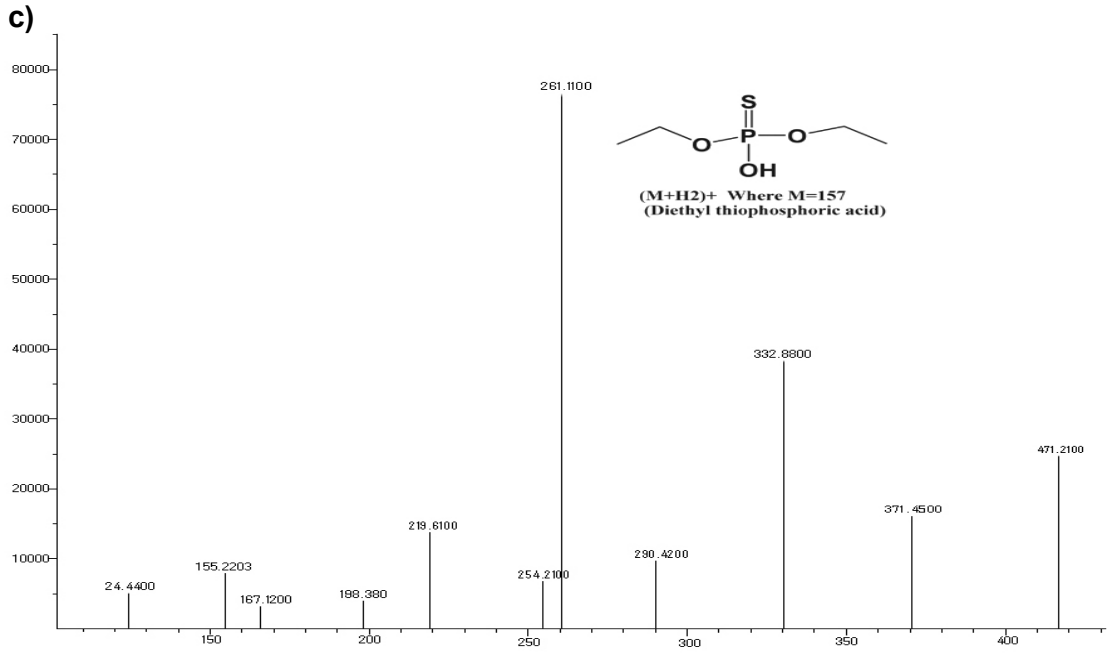
Figure 3
Mass spectram of Chlorfyrifos a) 0 hrs b) 24 hrs c) 48 hrs

a)



b)





DISCUSSION

In the present study the potential bacteria to degrade chlorpyrifos, were isolated and a bacterial consortium was developed from agricultural soil by selective enrichment technique by providing chlorpyrifos as sole source of carbon. The number of isolates was lesser in the soil taken for the study. It might be inhibitory effect of chlorpyrifos on soil microbial population [14]. Degradation of chlorpyrifos by consortium could be due to the combind effect of various isolates. In the present investigation the two bacterial isolates namely *Pseudomonas* sp, and *Brevibacillus* sp, present in the consortia were identified on the basis of Bergys manual of systemic classification. Previously, a chlorpyrifos degrading bacterial strain *Stenotrophomonas* was isolated. The isolate was able to utilize chlorpyrifos as a carbon source [15]. A bacterial consortium was developed from pesticide-contaminated soil of Punjab consisting of *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella* sp and *Serratia marscecens*, that could successfully degrade chlorpyrifos [16]. In the present study it was found that the consortium was able to degrade chlorpyrifos in the medium After 24 hrs of incubation it was found that the consortium formed 3,5,6-trichloro-2-pyridinol in medium and degraded 72% of chlorpyrifos. After 48 hrs of incubation, it was found that the consortium degraded 81% of chlorpyrifos in the medium. In medium the products Diethylthiophosphoricacid (DETP) and 3,5,6-trichloro-2-pyridinol (TCP) was formed as a result of the hydrolysis of the phosphoester linkage in chlorpyrifos. similarly chlorpyrifos-oxon may be formed as a result of the degradation of chlorpyrifos. The degradation mechanism might have involved an inducible enzyme chlorpyrifos hydrolase, an organophosphorus ester-hydrolyzing enzyme, to hydrolyze chlorpyrifos to a non-toxic metabolite[1].

CONCLUSION

The results of the study concluded that the bacterial consortium (*Pseudomonas* sp, *Brevibaciilus* sp,) can be potentially utilized for the bioremediation of chlorfyrifos contaminated soil. Illustrating the pathway of degradation and classifying the genes and enzymes involved in the process represent areas of further investigation.

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