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Research Article

Modeling and Optimization of Chlorpyrifos by Fungi Isolated from Agricultural Soil, Elucidating their Degradation Pathways by LC-MS-Based Metabolomics

Bharath M.[1], Ashashree N. R.[1], Yogesha P.[1], Santhosh M. Sosale[2] and Raju N. S[*].

Department of Studies in Environmental Science, University of Mysore,

Manasagangotri, Mysore -570 006, Karnataka, India

*Corresponding Author E-mail: nsr@envsci.uni-mysore.ac.in

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ABSTRACT

Chlorpyrifos is a moderately hazardous insecticide to humans (Class II) by the World Health Organization (WHO) due to its acute toxicity still we are using cost-competitive and widely available suitable for various agro-climatic conditions, especially in arid regions of India. Our study explores the sustainable biodegradation pathways of biodegradation of chlorpyrifos-contaminated soils so that a novel chlorpyrifos-degrading fungus was isolated and identified as Aspergillus and Fusarium with the unique capability to degrade Chlorpyrifos. Identification of C1 and C2 two fungal strains, followed by isolation and characterization of CP and TCP degrading fungi through efficient biodegradation method. By LC-MS analysis the m/z values of sample TCP detected in culture medium and soil Aspergillus and Fusarium were able to completely mineralize Chlorpyrifos and its metabolite TCP from C1 and C2 culture grown in Mineral medium after 7 days of incubation soil without formation of any known intermediates. This study supports the idea that soils contaminated with Chlorpyrifos could be remedied effectively using fungi that break down Chlorpyrifos. Hence, this fungal consortium can be a valuable bio-augmenting agent to clean up Chlorpyrifos contaminated sites and helps in the sustainable management of natural resources.

Keywords: *Chlorpyrifos, Biodegradation, Fungal Strains, Contaminated soil.*

INTRODUCTION

More than 60 to 70 per cent of India's population depends on agriculture, making it a predominantly agricultural nation. Through industries and urban encroachments, a significant piece of our arable land that is already under cultivation is being rapidly depleted. Water, fertilizers, seeds, and pesticides are the four essential components that modern agriculture depends on.

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The use of pesticides is essential in modern agriculture. Insects, weeds, and illnesses account for about 35–34 per cent of crop production losses, and crop storage losses account for 35 per cent of crop losses (Salami et al., 2010).

India has the world's lowest pesticide consumption per capita at 0.6 kg/ha. China and the United States each consume 13 kg/ha and 7 kg/ha of pesticides per person, respectively. Low farmer purchasing power and limited land holdings are the main causes of India's low per capita pesticide consumption (Oerke, 2012). Although marginal farmers own the bulk of agricultural farmland, they also contribute the majority of the output. There is room to expand the per capita usage of pesticides in India as large-scale agriculture is on the rise (Horrigan et al., 2002).

According to the Food and Agriculture Organization (FAO), pesticides are any substances or combination of compounds designed to prevent, eliminate, or manage pests. The word "code" is a derivative of the Latin verb "to kill." (White, 2015).

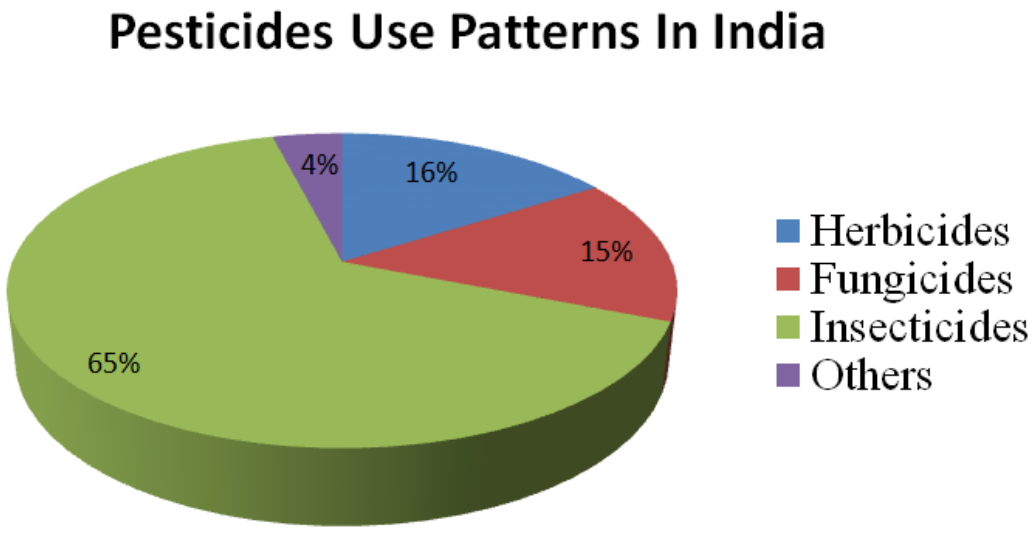

Figure-1: outlook of pesticide consumption in India, Source: Krishi Jagran

Figure-1: outlook of pesticide consumption in India, Source: Krishi Jagran

Chlorpyrifos (CP)

One of the most often used organophosphate insecticides is Chlorpyrifos, also known as O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate. With a production of 3.64 to 4.99 million kg in the agricultural sector during 2007, Chlorpyrifos holds the top position among conventional pesticide active ingredients globally. With 9540 tonnes of formulation, Chlorpyrifos was the second-most utilized agricultural pesticide in India in 2013–2014 [Ministry of Chemicals and Fertilizers, Government of India (2014)] (Solomon, 2014). Chlorpyrifos was initially registered in the United States in 1965, and its first crop use authorizations were granted in 1974. Chlorpyrifos-containing goods have been available on the market for more than 40 years. Chlorpyrifos is approved for use in more than 98 nations around the world to protect more than 50 different crops from harm from a variety of insect and pest damage (Singh & Walker, 2006).

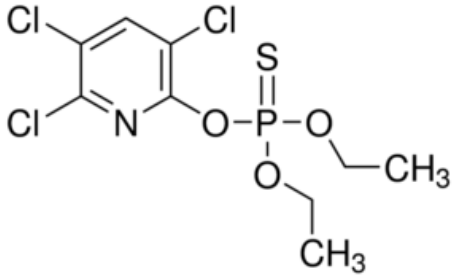

Figure-2: **Structure of Chlorpyrifos**

Uses of Chlorpyrifos

A common organophosphate pesticide with a broad-spectrum insecticidal effect is Chlorpyrifos. It is applied to the soil to kill termites and is used to manage gall midges, leaf folders, and leaf hoppers in rice. Additionally, it works well against a variety of soil insects, including grubs, wireworms, cutworms, and rootworms. It can also manage dull insects in corn, fruits, and other crops through contact exposure. One of the most commonly used pesticides is Chlorpyrifos due to the variety of arthropod pests it may control (Reddy, 2012).

Environmental Fate

Despite being largely immobile in the environment, Chlorpyrifos is only modestly persistent. At very low concentrations (BCF = 2.50 to 3.54), Chlorpyrifos may bioconcentrate in ecological systems.

Biodegradation of Chlorpyrifos

Because it is inexpensive and causes less collateral damage to native creatures, biological degradation is a widely used method for the elimination of organic contaminants. Numerous variables affect it, including temperature, solar radiation, soil type, pH, oxygen or other electron acceptors, nutrients, chemical makeup and bioavailability of the target component, and the quantity and variety of degradation communities (Olaniran et al., 2013). In liquid medium and soil, organophosphate insecticides have been documented to be broken down by several bacterial and fungal species. Thanks to enrichment culture techniques, it has been possible to isolate microbial species that can utilize Chlorpyrifos as their only carbon source (Pointing & biotechnology, 2001). The rate and scope of bioremediation are significantly influenced by the concentration, solubility, and accessibility of pesticides to bacteria. Due to its poor solubility in water (2 mg/L) and strong adsorption affinity to organic materials and soil, Chlorpyrifos has a low bioavailability for microbial breakdown (Gadd & Biotechnology, 2009).

MATERIALS AND METHODS

Chemicals and media

A local pesticide store in the Mysore area of Karnataka, India, sold commercial-grade insecticide CP (20 per cent E.C). Both 3,5,6-Tri chloro-2-pyridinol and Chlorpyrifos, both of analytical grade 99 percent, were bought from Sigma Aldrich, USA. The only other substances and materials employed in this research were analytical-grade materials from Hi-Media Pvt, Ltd. in Mumbai, India (Bhattacharjee et al., 2010). The following elements were present in the mineral salts medium (MSM) in grams per liter: K2HPO4, 1.75, KH2PO4, 0.75, MgSO4, 0.13, NH4Cl, 0.5, Fe2 (SO4)3, pinch, and CaCl2, pinch. The mixture also contained 1 milliliter of a trace element solution. The amount of each trace element in the solution was as follows: MnCl2.4H2O, 0.198; ZnCl2, 0.136; CuCl2.2H2O, 0.176; CaCl2.6H2O, 0.024; and NiCl2.6H2O, 0.024% (all in mg/L). The MSM employed in this study was created to evaluate pesticide CP as a sole carbon source for bacteria (Yang, 2005).

Soil sample

In a polythene bag, soil samples were taken from the top 0–20 cm of the agricultural soil in the hamlet of Shyadanahalli in the Pandavapura taluk of the Mandya district of Karnataka, India. The gathered soil samples were gently air-dried in the lab at room

temperature, sieved through 2mm mesh, and then preserved for further use (Pansu & Gautheyrou, 2006).

Enrichment Procedure and Isolation of Fungal Strain

Fungal isolates degrading CP were obtained by enrichment culture. 10g of soil samples were inoculated into MSM medium amended with 100µL CP and cultured in 250ml conical flasks. The cultures were incubated on a rotary shaker at 30ºC at 150 rpm for 7 days. After 7 days, the fungal colony was isolated by streaking the enriched sample on the PDA (Potato Dextrose Agar) media. Out of the 4 isolates, strains C1 and C2 were selected for further study due to their ability to degrade CP and had higher tolerance to CP (Gomaa & Technology, 2013).

Identification of Fungal strain

Based on morphological appearance and observation under a microscope, the fungi C1 and C2 were identified as *Aspergillus* and *Fusarium* respectively.

Biodegradation of CP by Fungal strain in mineral medium

Degradation of CP was performed in 250ml conical flasks containing 100ml of mineral medium supplemented with 50µL CP as the sole carbon source and inoculated with a 1ml spore suspension of C1 and C2. Flasks were incubated at 30±2ºC on a rotary shaker at 120 rpm. Uninoculated flasks, spiked with CP acted as control (Akbar & Sultan, 2016).

Biodegradation of CP by Fungal strain in soil

The ability of a particular fungal strain to degrade CP in the same soil that produced the fungi isolates. To eliminate pebbles and other objects, the top 0–20 cm of the soil was taken, air dried at room temperature, thoroughly mixed, and sieved through a 2 mm filter. Before being used for analysis, the soil samples were sterilized by being autoclaved three times for 30 minutes at 120°C (Chaillan, 2004). Then, two treatments were carried out: (1) adding pesticide, isolated fungal spores (C1 and C2), and nutrients (Carbon, Nitrogen, and Phosphorous); and (2) adding pesticide, isolated fungal spores (C1 and C2) but not nutrients. In sterile conditions, 100 grams of soil samples were deposited in 250 ml conical flasks together with 30 ml of a solution containing 108 fungal spores/ml (C1 and C2) and 100 l of CP, nitrogen, phosphorus, and glucose (Singh & Jha, 2016). Using the formula C/N/P 100:10:1, the amounts of carbon, nitrogen, and phosphorus were computed. Glucose, (NH4) 2.SO4 and K2HPO4 served as the sources of carbon, nitrogen, and phosphorus, respectively. Autoclave-sterilized samples were employed as a soil test control (Guildford et al., 2000).

Extraction of Samples (Pesticide Residues) from mineral medium and soil

Aqueous samples in the amount of 25 ml were extracted from culture flasks on the extraction day. A separating funnel was filled with the supernatant, and an equal amount of petroleum ether was added. The petroleum ether was mixed well by shaking the funnel up and down. To dry the extracts over anhydrous sodium sulfate (Na2SO4) and let the solvents evaporate at room temperature, the petroleum ether's organic layer was aspirated and collected. Following that, the leftovers were dissolved in 1 ml of HPLC-grade acetonitrile for further analysis with liquid chromatography-mass spectroscopy (LC-MS) (Ferrer, 2001).

Degradation analysis by LC-MS

An inertial ODS3 column (50 3 mm) and a UV detector connected to the LC-MS were used to look at the remaining CP in the samples following fungal degradation. The cartridges were washed with deionized water that contained 0.1% formic acid and then condition with acetonitrile. (Chaves et al., 2008). With a sample injection volume of 10 L and a flow rate of 0.2 mL/min, acetonitrile and 0.1 percent formic acid in water were used as the gradient mobile phase. The oven temperature was held at 370 C, and the UV detector was adjusted to 230 nm. Under these conditions, CP retained for an average of 18.6 minutes. Mass spectrometry (MS) was carried out using a Finnegan model MS (Thermo electron Corporation, USA). An ion trap detector with an atomic pressure chemical utilization (APCI) source was used for measurement in positive ionization mode. The operating conditions

were as follows: an APCI source, 5.02 kV of spray voltage, 16.96 v of capillary voltage, and 275 oC of capillary temperature. Multiplier (V): 821.2; dynode (kV); ion detection system (Nguyen, 2009).

RESULTS AND DISCUSSION

Chlorpyrifos and its toxic residue TCP are known for bioaccumulation, which can get into the food chain in many ways. Analysis of it in natural soil and water is a subject of public and scientific attention. The present study analyzed Soil samples collected from Shyadanahalli village, Pandavapura taluk, Mandya district, India, for CP and TCP. From Thin Layer Chromatography (TLC) analysis, it was found that, out of 4 samples analyzed, 02 soil samples (S1 and S2) detected CP hydrolyzed metabolite TCP (Varsha et al., 2022). Qualitative analysis by Liquid Chromatography Mass Spectroscopy (LC-MS) revealed that retention time for internal standard CP was found to be 3.18 and for TCP is 2.48 respectively (figures 8 & 10), corresponding to its mass 350 and 198 respectively (figure 7 & 9) (Seiber et al., 2021). Compared to internal standard values, the samples S1 and S2 were detected with TCP in the range of 4.41 and 5.18 µg/kg, respectively (Figures 11 & 12). These findings suggest that, in soil, the applied Chlorpyrifos undergoes Hydrolysis to form TCP (Racke, 1990).

Isolation and characterization of CP & TCP degrading fungi:

From the enrichment culture, 4 fungal strains designated (C1, C2, C3 & C4) were isolated. These strains were further purified and checked for their ability to tolerate TCP (TCP Susceptibility test) at different concentrations (100 to 300mg L^{-1}) by inoculating them in potato dextrose agar media individually. From this experiment, two fungal cultures designated as C1 & C2 were able to tolerate the highest concentration of TCP and hence, selected for biodegradation studies (Midekssa, 2013).

Identification of C1 and C2 fungal strains

C1 and C2 were identified as *Aspergillus* and *Fusarium* sp., respectively. The C1 isolate exhibited a white colony with greenish morphology and C2 isolate exhibited a white colony with brownish morphology on PDA agar with Chlorpyrifos.

Biodegradation of CP and TCP by C1 and C2 strains in liquid culture:

Using fungi C1 and C2, the biodegradation of Chlorpyrifos (100 mg L-1) was evaluated. The elimination of Chlorpyrifos and TCP was observed using Thin Layer Chromatography (TLC) and LC-MS. TLC analyses showed the production of unidentified metabolites. Based on the obtained m/z value, the LC-MS study (figures 13 and 14) demonstrated that the consortium could effectively destroy Chlorpyrifos without the creation of TCP (Nandhini, 2021). According to the latest research, the isolate formed polar metabolites using this molecule as a carbon and energy source for growth. Rare reports of this characteristic in other bacteria that break down Chlorpyrifos exist. According to the majority of cases that have been reported to date, each isolate tended to convert Chlorpyrifos by hydrolyzing the ester linkage to yield TCP, which then accumulated in batch cultures or soils and prevented enhanced degradation from happening because of its antimicrobial properties (Alvarez, 2017).

The work carried out by Silambarasan et al. (2012) showed that *Aspergillus terreus JAS1* was capable of degrading CP and TCP-contaminated soil in the range of 100 and 94.3% respectively. *Alcaligens faecalis* was able to metabolically degrade 100% of CP (250 mg l^{-1}) within 2 days. Similarly, *Bacillus cereus, Bacillus ligniform's* ZHU-1 and *Bacillus pumilus* C2A1 isolated from chlorpyrifos-contaminated soil sites were able to degrade 80%, (150 mg L^{-1}) in 5 days, complete degradation (100 mg L^{-1}) in 14 days and 89% (1000 mg L^{-1}) within 15 days respectively (Briceño, 2012).

In the present study, the two fungal isolates were able to degrade both CP and TCP completely within 14 days. Hence, this fungal consortium can be a valuable augmenting agent to clean up Chlorpyrifos contaminated sites.

Figure-3: Containers of Chlorpyrifos pesticides found in the agricultural field.

Figure-4: Minimal Medium inoculated with fungal strains with Chlorpyrifos as the sole carbon source.

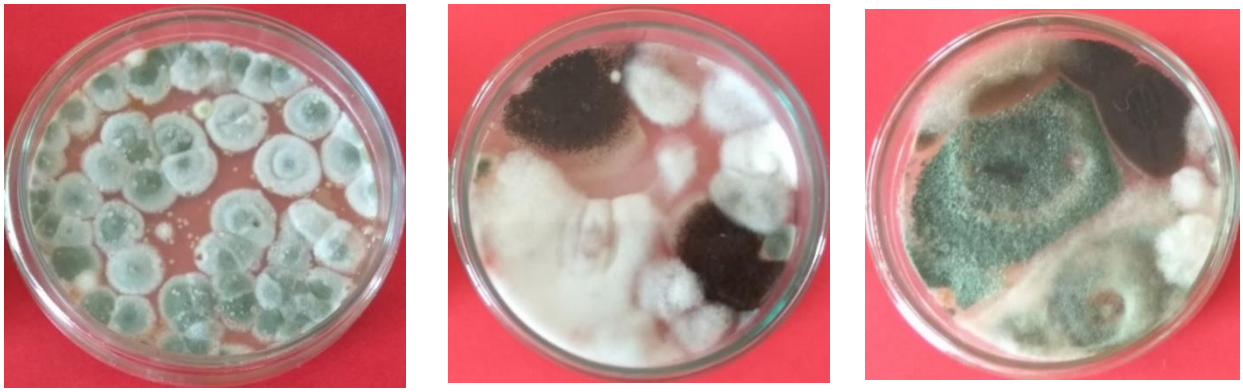

Figure-5: Fungal isolates (C1, C2, C3 & C4) grown on PDA media.

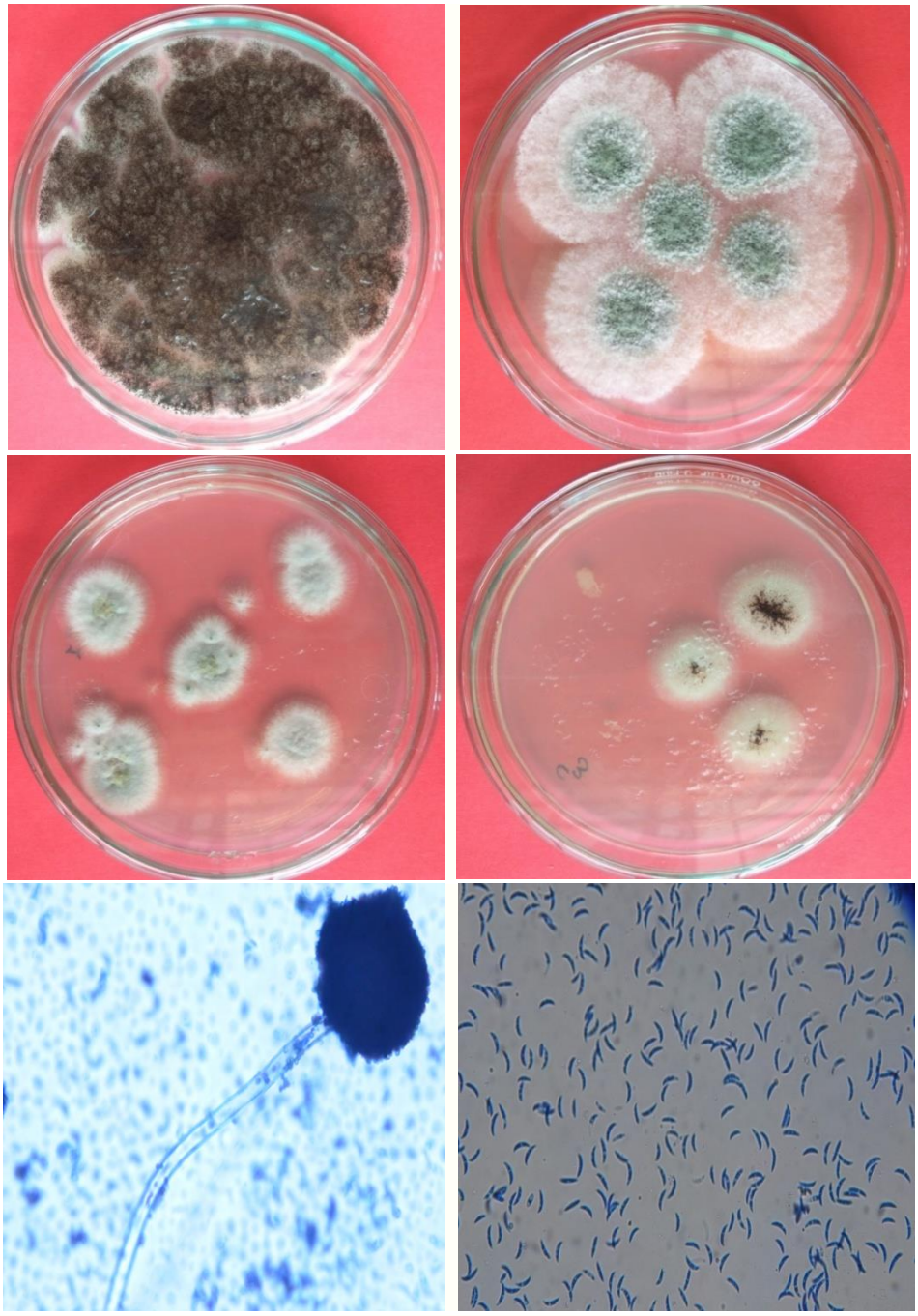

Figure-6: Pure cultures of fungal isolates and microscopic observation of fungal isolates (*Aspergillus* and *Fusarium*).

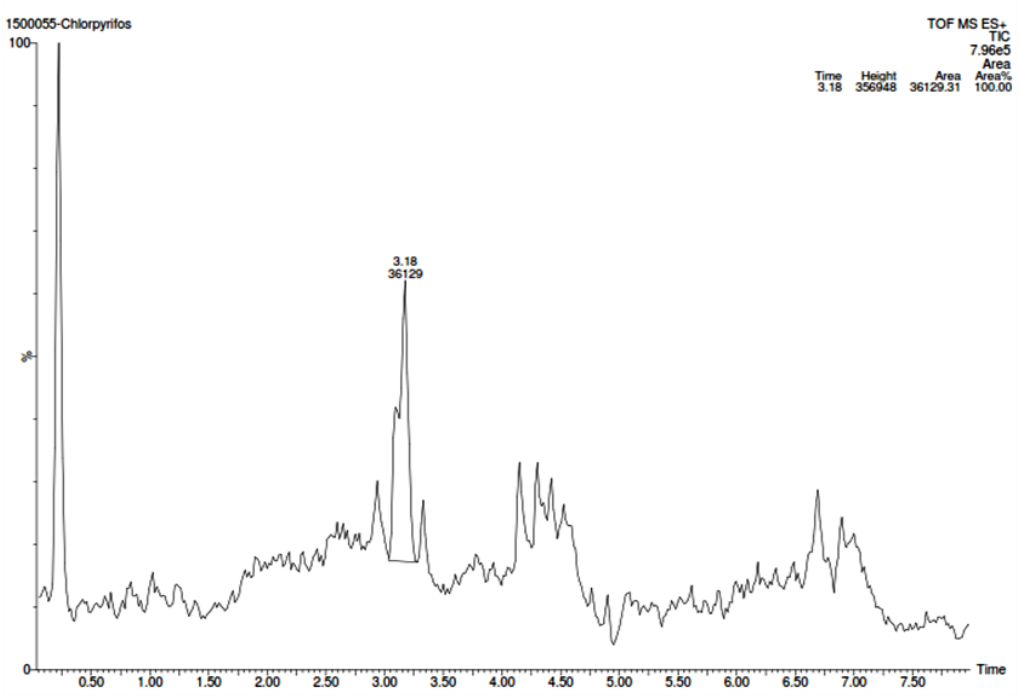

Figure-7: Total Ion Chromatograph of Internal Standard Chlorpyrifos.

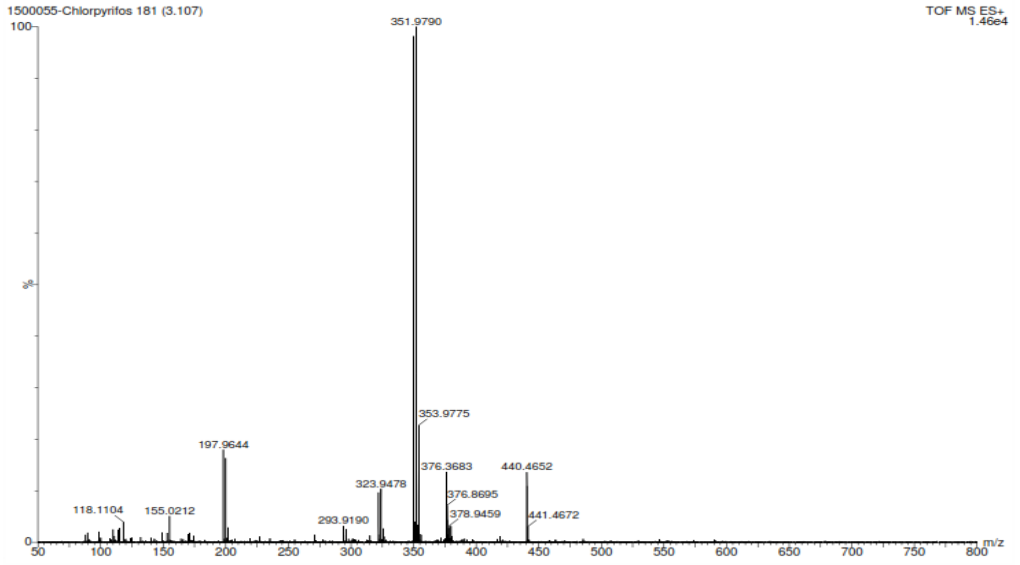

Figure-8: LC-MS fragmentation of Internal Standard Chlorpyrifos.

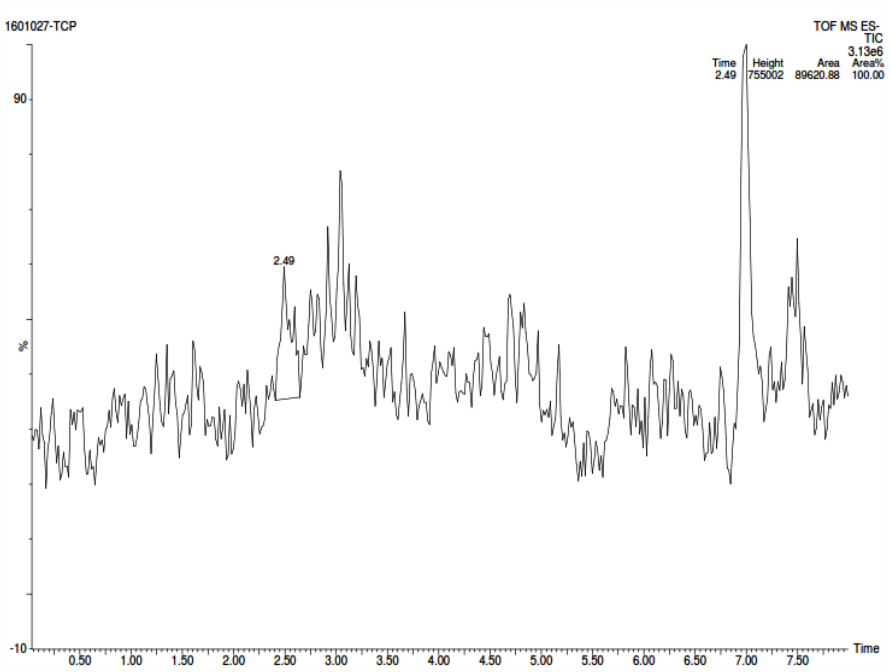

Figure-9: Total Ion Chromatograph of Internal Standard TCP.

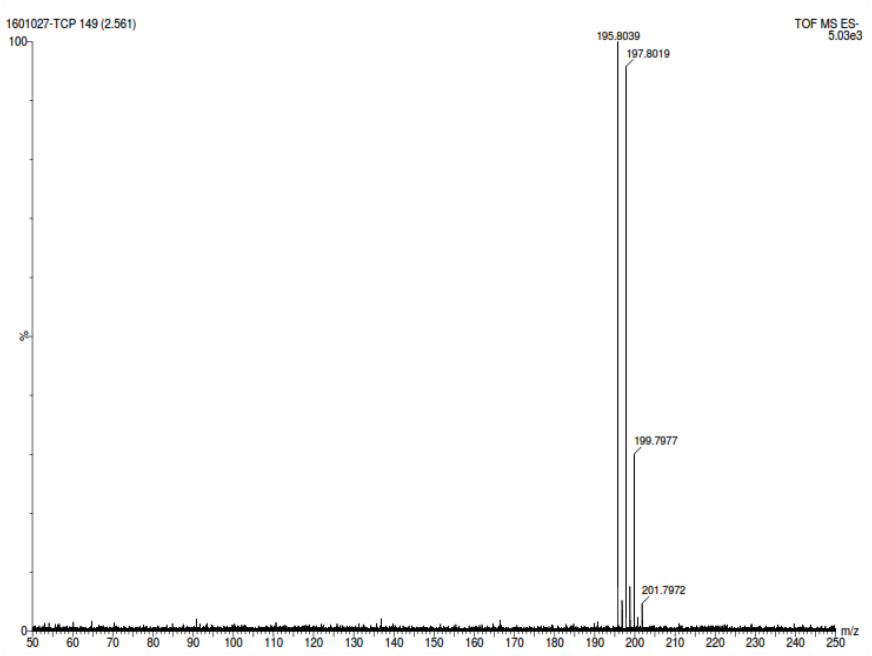

Figure-10: LC-MS fragmentation of Internal Standard TCP.

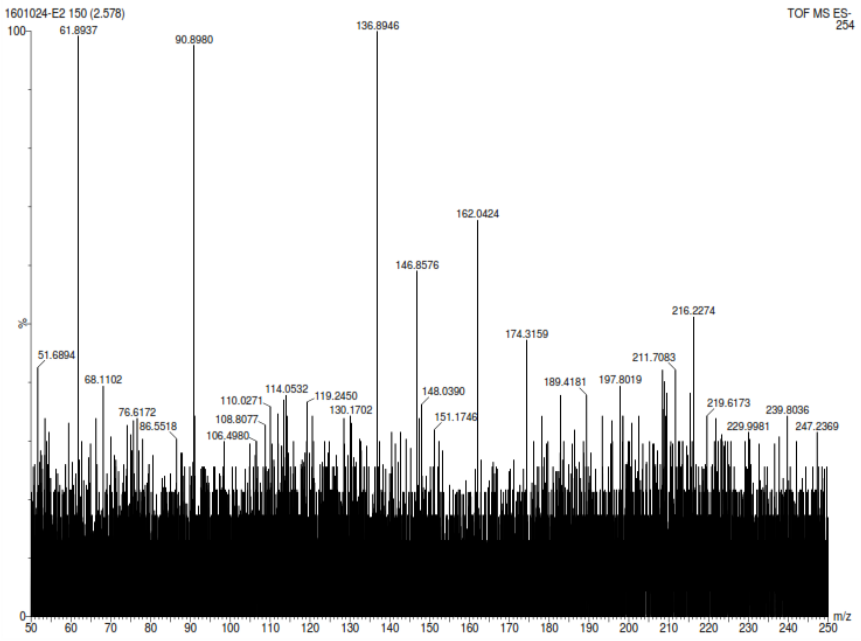

Figure-11: *m/z* values of LC-MS analysis of sample S1 showing the presence of TCP.

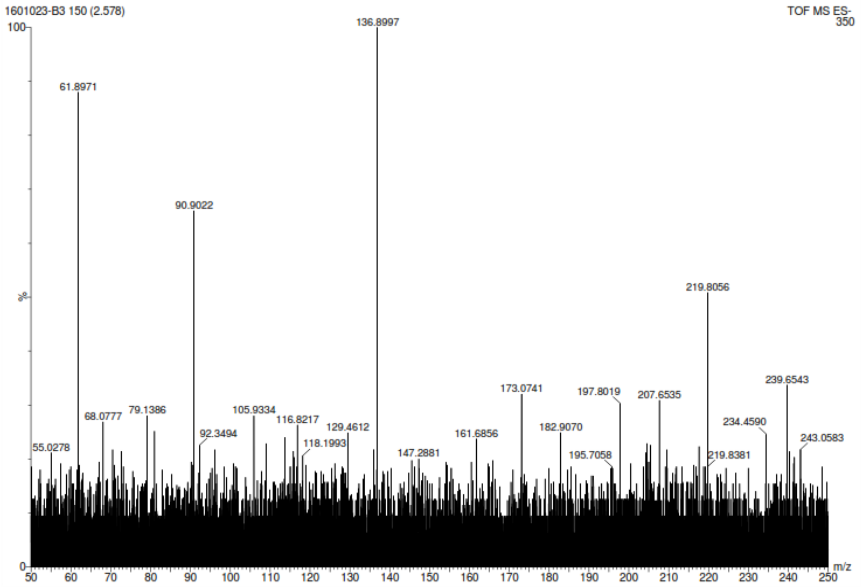

Figure-12: *m/z* values of LC-MS analysis of sample S2 showing the presence of TCP.

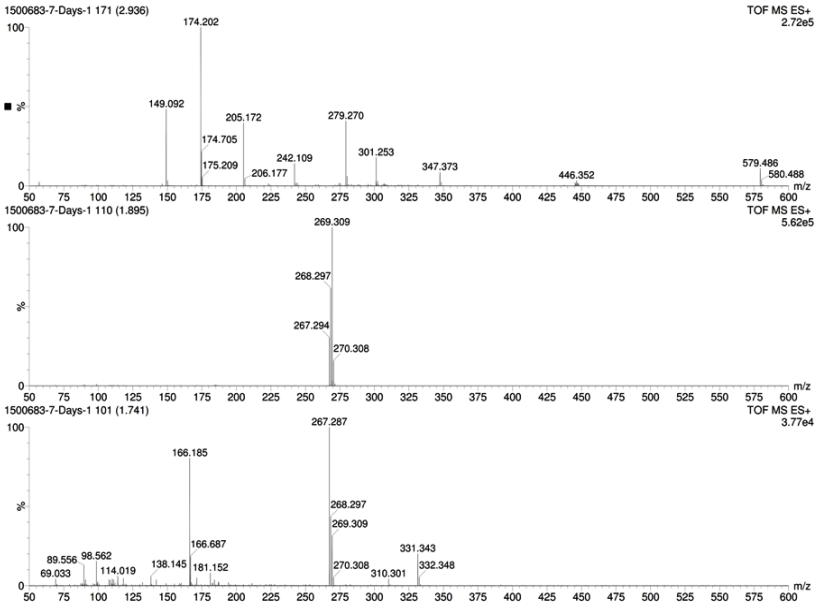

Figure-13: *m/z* values of LC-MS analysis of Chlorpyrifos and its metabolites obtained from C1 culture grown in the Mineral medium after 7 days of incubation.

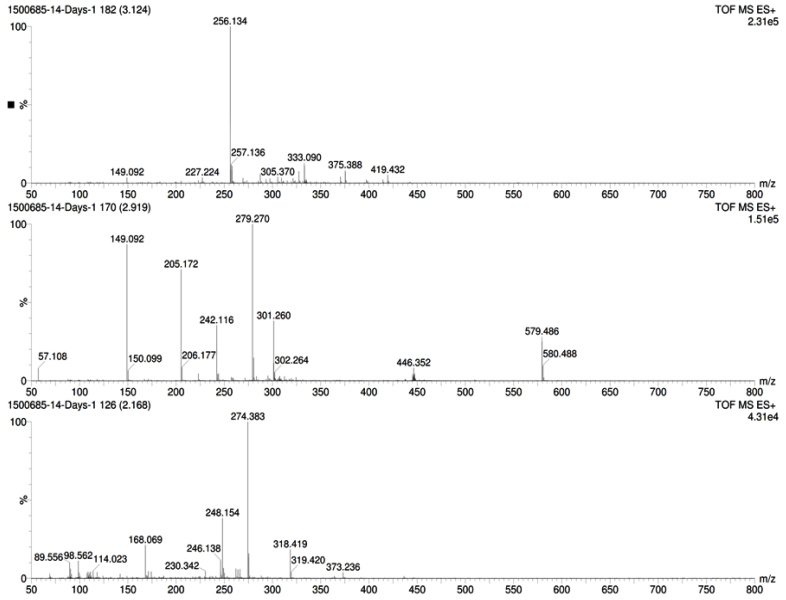

Figure-14: *m/z* values of LC-MS analysis of Chlorpyrifos and its metabolites obtained from C2 culture grown in the Mineral medium after 7 days of incubation.

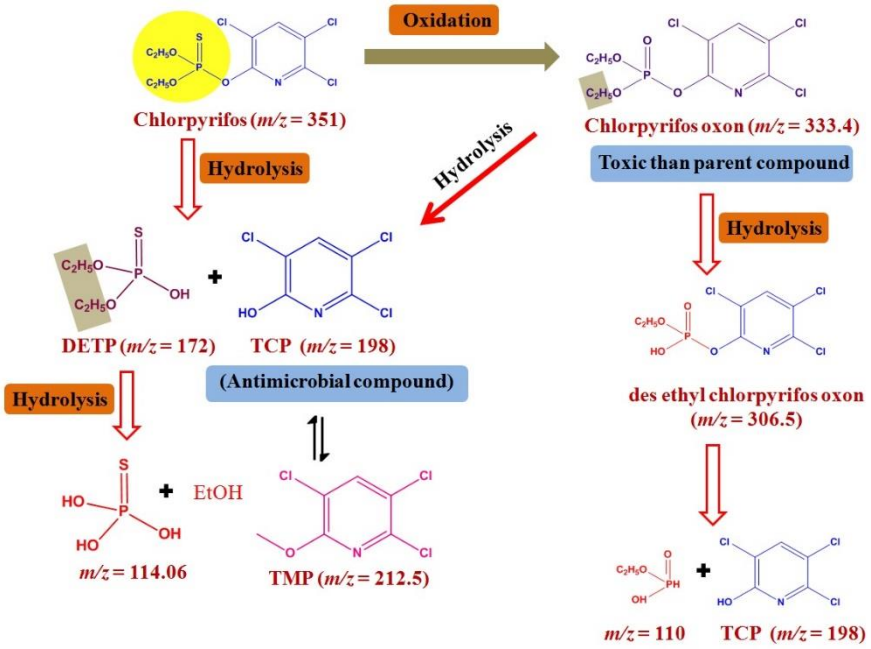

Figure-15: Possible biodegradation pathway.

CONCLUSION

According to a fair amount of research, microorganisms may be used in the biodegradation of chlorpyrifos-contaminated soils. Utilizing microbial capacity for in-situ and ex-situ detoxification of Chlorpyrifos is an efficient technique for biodegradation. In the current investigation, a novel chlorpyrifos-degrading fungus with the unusual capacity to break down Chlorpyrifos and TCP in soil and culture media was isolated and identified as Aspergillus and Fusarium. Numerous investigations have shown that Chlorpyrifos completely degrades in soil and liquid cultures. The current study shows that Aspergillus and Fusarium can totally mineralise Chlorpyrifos and its metabolite TCP in material medium and soil without producing any recognized intermediates. This study demonstrates that chlorpyrifos-degrading fungi can be effectively employed to remediate soils that have been contaminated with the chemical.

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Conflict of interest

There was no potential conflict of interest by the author.

Author contribution

Soil sample analysis was carried out by Yogesha P and isolation of microorganisms was conducted by Bharath M and Santhosh M Sosale and LC MS analysis was done by Ashashree N R.

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