

In vitro* Studies on Biodegradation of Chlorpyrifos by *Trichoderma viride* and *T. harzianum

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Although a wide application of pesticides and herbicides are an essential part of augmenting crop yields, excessive use of these chemicals leads to the microbial imbalance of soil, environmental pollution and health hazards to human and animals. An ideal pesticide should have the ability to destroy target pests quickly and should get degrade the toxic and nontoxic substances as quickly as possible, but some pesticides leaves residues without complete degradation. Among the methods of degradation, soil microorganisms especially fungi play a vital role in degrading pesticides, thus preventing the environmental pollution and preserve the soil fertility. In the present study, the microbial degradation of chlorpyrifos a organophosphorus chemical by using *Trichoderma viride* and *T. harzianum* and its consortium was attempted *in vitro* and their performance was compared. *T. viride*, *T. harzianum* and its consortium were observed to be able to grow in fungal culture medium in the presence of added 20% chlorpyrifos (125µl/100ml of medium) and it showed increase level of biomass production and protein. The results of the study reports that *T. viride* and *T. harzianum* possess the ability to degrade the Chlorpyrifos remarkably. However the result shows the consortium of these fungi possess low ability to degrade chlorpyrifos comparing to their individualistic performance in the order *Trichoderma viride*, *T. harzianum* and Consortium. The results of the growth of fungi and degradation level of chlorpyrifos were discussed in detail.

Key words: Pesticide degradation, Biodegradation, Soil fungi,
Trichoderma viride, *Trichoderma harzianum*, Chlorpyrifos.

Pesticides are the chemical substances that kill pests and these are often referred to according to the type of pest they control. Even though the pesticides and herbicides are not advisable, it is widely used in agriculture for crop improvement and it yield to meet the population

requirement. Continuous and excessive use of pesticide chemicals leave residue in soil environment and have stay longer period which leads to the microbial imbalance of soil, environmental pollution and health hazards to human and animals. Several reports suggest that the contamination of soil by these pesticides as a result of their bulk handling at the farm yard or following application in the field or accidental release may lead to occasional contamination of a wide range of water and terrestrial ecosystems (Singh *et al.*, 2004).

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In India, the production of pesticides started in 1952 and there has been a steady increase in the production of technical grade pesticides from 5,000 metric tons in 1958 to 102,240 metric tons in 1998 (Saiyed *et al.*, 1999; Hundal *et al.*, 2006) and further increase is possible. The continuous usage of pesticides leads to be an environmental problem unless proper treatment technology is developed and transferred to the industry (Fulekar, 2005). As a result, pesticide residue remains in the soil-water environment causing toxicity to the biota and thereby entering into the food chain (CFTRI, 2003). The World Health Organization (WHO) data show that only 2 - 3% of applied chemical pesticides are effectively used for preventing, controlling and killing pests, while the rest remains in the soil (EPA, 2005). Pesticides reaching to the soil are acted upon by several physical, chemical, and biological forces. However, physical and chemical forces are acting upon/degrading the pesticides to some extent, microorganism's plays major role in the degradation of pesticides. Many soil microorganisms have the ability to act upon pesticides and convert them into simpler non-toxic compounds which is otherwise known as "biodegradation".

Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants by Harman, (2006) which are due to antibiosis, parasitism, inducing host-plant resistance, and competition. Most biocontrol agents are from the species of *T. harzianum*, *T. viride* and *T. hamatum*. *Trichoderma* strains isolated from rhizosphere soil samples show biocontrol potential against *Sclerotium rolfsii* and *Fusarium ciceri* (Anand and Jayarama Reddy, 2009). The degradation of [14C] photodieldrin by *T. viride* was studied by Tabet *et al.*, 1976 and found that it was associated with a continuous decline of hexane-soluble radiocarbon and a steady increase of water-soluble metabolites in the fungal media. Rochkind-Dubinsky *et al.*, (1987), revealed that some synthetic chemicals such as Pesticides, polycyclic aromatic hydrocarbons (PAHs), and dyes and found which are extremely resistant to biodegradation by native flora compared with the naturally occurring organic compounds that are readily degraded upon in natural environment. The utilization of phosphorus and sulfur of insecticide origin by fungi was reported by Omar (1998) which clearly indicates that the fungi able to grow and

assimilate organophosphorous in the media environment.

Sarfraz Hussain & Muhammad Arshad (2007) isolated fungal strains by employing enrichment techniques while using endosulfan as a sole sulfur source, and tested for their potential to degrade endosulfan. The surface soil treatment technique used for bioremediation of pesticides using activated cow-dung and soil microflora would be an effective treatment technology reported by (Fulekar & Geetha, 2008). Arata Katayama *et al.*, (2009) reported as *Trichoderma harzianum*, was found to degrade DDT, dieldrin, endosulfan, pentachloronitrobenzene, and pentachlorophenol and concluded that the major enzyme system responsible in *Trichoderma harzianum* for degradation of endosulfan is an oxidative system. The phosphate solubilization potential and phosphatase activity of Rhizospheric *Trichoderma harzianum* was studied by Anil Kampri and Lakshmi Tewari, (2010) and found the high P-solubilizing potential of *Trichoderma sp.* and its ability to exploit for the solubilization of fixed phosphates present in the soil to enhance soil fertility and plant growth. Subashini. *et al.*, (2007) reports *Trichoderma viride* is combined with Traditional Plants with Medicinal Properties Viz., *Cipadessa baccifera*, *Clausena dentate*; *Dodonaea angustifolia* and *Melia dubia* able to degrade the commonly used pesticides namely, endosulfan, acephate and quinalphos under invitro conditions.

Further evidences from the literature show the role of many bacteria in the degradation of various pesticide components including soil bacteria by Poorva *et al.*, 2010; enterobacter by Singh *et al.*, 2004; lactic acid bacteria by

Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is one of the most widely used is a organophosphorus pesticide compound effective against a broad spectrum of insect pests of economically important crops. Chlorpyrifos is an effective by contact, ingestion and vapour action but is not systemically active. It is used for the control of mosquitoes (larvae and adults), flies, various soil and many foliar crop pests and household pests. It is also used for ectoparasite control on cattle and sheep. It has a high soil sorption co-efficient and is stable under normal storage conditions. Chlorpyrifos is

defined as a moderately toxic compound having acute oral LD₅₀; 135–163 mg kg⁻¹ for rat and 500 mg kg⁻¹ for guinea pig. The half-life of chlorpyrifos in soil varies from 10 to 120 days (Racke *et al.*, 1988) with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. Therefore the present study is carried out using commercial grade chlorpyrifos obtained from local market.

Therefore, it is important to investigate the technology advances in bioremediation for the treatment of pesticide wastes as well as effluent by using different treatment methods are essential for pesticide industry and in agriculture. The main aim of this study is to degrade chlorpyrifos because it is one of the most used and persisting organophosphorus pesticide in soil. *Trichoderma viride* and *Trichoderma harzianum* are found almost in all types of soil, well known Biocontrol agent and they are easy to maintain in laboratory conditions, therefore, it is felt to utilize these fungi for the study. Therefore the present study is mainly focused in degrading Chlorpyrifos (Organophosphorus pesticide) using the Fungi *Trichoderma viride* and *Trichoderma harzianum*.

MATERIALS AND METHODS

Cleaning of Glassware, sterilization and culture media preparation

All glassware were first soaked in chromic acid cleaning solution containing 10% potassium dichromate in 25% sulphuric acid for 3 hr and washed thoroughly in tap water. Then they were washed with detergent solution, and then washed thoroughly in tap water and finally rinsed in distilled water and dried in an oven. All the Glassware, culture media and accessories were sterilized in an autoclave at 121°C for 20 min at 15 psi.

To isolate and maintain the fungal cultures, Potato Dextrose Agar (PDA) with normal pH and modified Czepak-Dox medium (without phosphorus source) was used. The individual Nutrient ingredients were weighed and dissolved in distilled water well before mixing with agar powder. Then the media were kept for sterilization in Autoclave. After sterilization of the media were cooled to warm temperature and used for experimental studies.

Fungus culture

Trichoderma viride and *Trichoderma harzianum* were used in the present study for degradation of chlorpyrifos. The standard strains of *T. viride* and *T. harzianum* maintained in ARMATS BIOTEK Private Ltd., Chennai were received for the experiments. The Strains were inoculated in PDA slants and agar plates and maintained throughout the Study. The pure culture plates of *T. viride* and *T. harzianum* were shown in Fig. 2a-b.

Pesticide Used

Chlorpyrifos 20% E.C (commercial grade), an Organophosphate pesticide was obtained from the local market of Chennai and used throughout study. This pesticide is used commonly in agricultural fields of South India.

Screening for Pesticide Tolerance of *T. viride* and *T. harzianum*

The Preliminary screening is performed to identify the pesticide (chlorpyrifos) tolerance of *Trichoderma viride* and *Trichoderma harzianum*. Czepak-dox agar medium were distributed in 100 ml conical flasks and autoclaved by standard method. To the cooled sterilized medium, different concentration of chlorpyrifos 20% E.C as 25, 50, 75, 100, 125, 250, 500 µl/100ml is added as a phosphorus source. The chlorpyrifos amended medium was plated (approx. 20ml in each) and allowed to solidify. Mycelial discs of (10mm diameter) *T. viride* and *T. harzianum* were cut out from actively growing culture of PDA, and placed at the center of the each petriplates containing different concentration of chlorpyrifos. Two agar plates were kept as Control (without chlorpyrifos) for *T. viride* and *T. harzianum* respectively. At every 24 hrs interval the Radial Growth of the Fungi was measured.

Experiment on Liquid Culture

The effect of growth and degradation of *T. viride* and *T. harzianum* in Liquid culture medium of Czepak Dox medium was carried out in 2 Sets as with and without Chlorpyrifos amendment. In the presence of chlorpyrifos, 6 X 50 ml of Czepak-Dox medium (Without Phosphorus Source) was prepared and autoclaved. To the cooled sterilized medium 62.5µl (i.e. 125µl/100ml) of 20%chlorpyrifos as Phosphorus source added to each flask. To each flask approximately three discs (10mm each) of *T. viride* is inoculated and incubated for 10 days at

RT in dark kept as static culture. Similarly Experiment is repeated for *T. harzianum* and consortium. For consortium two discs (10mm each) of *T. viride* and *T. harzianum* was inoculated to each flask. Similar experiment was done in the absence of chlorpyrifos and carried out.

Determination of mycelial dry weight and extracellular protien

Samples from both sets of culture flasks were harvested periodically in 2 day interval and analyzed for mycelium dry weight determination and protein estimation by Bradford's method. After incubation, the flasks were filtered through pre-weighed Whatman No. 1 filter paper to separate the Mycelium. Mycelium was washed with double distilled water, dried at 65° C for 1 hr and weighed. The dry wieght values were recorded as mg/50 ml of medium. The Extracellular protein in the culture filtrate was estimated according to the Dye binding method by Bradford (1976). One ml of culture filtrate was added with 5ml of Bradford's reagent (Coomassie Brilliant Blue) and the intensity of blue colour that developed was read at 595 nm in a spectrophotometer. The amount of protein was determined using bovine serum albumin as the standard.

Extraction and analysis of pesticide residues

The culture flasks of *T. viride*, *T.*

harzianum and consortium were collected and filtered through Whatman No.1 filter paper. Extraction of chlorpyrifos from culture filtrate was done after Irani mukherjee and Gopal (1996). It was performed after 2 days and up to 10 days incubation period. The filtrate was taken in a separating funnel to which 20ml of 2% saline water is added. Then 40ml of hexane is added. The funnel is shaken vigorously and allowed to stand for 10 min, so that two phase as organic and aqueous phase get separated. The step is repeated thrice to recover the pesticide completely. Finally the funnel is allowed to stand for 15 minutes for complete separation of phases. The organic phase (Top layer) containing chlorpyrifos is collected separately and kept for further studies (Irani mukherjee and Gopal 1996). Then the samples containing the residues of chlorpyrifos were analyzed for both qualitative and quantitative pattern by standard GC-MS procedure by using Shimadzu QP 2010 Plus equipment.

RESULTS

Screening for Pesticide Tolerance

The Growth of *T. viride* and *T. harzianum* in various concentrations of chlorpyrifos after 5 days of incubation are shown in the following

Table 1. Growth of *Trichoderma viride* in chlorpyrifos amended medium

Radial growth of <i>T. viride</i> measured in mm in Various concentration of 20% chlorpyrifos								
Days	Control	25ul	50ul	75ul	100ul	125ul	250ul	500ul
1 st day	20.25	14.5	12.5	11.5	12	12.5	0	0
2 nd day	40.75	29.25	15.75	15.5	15	15	11.7	0
3 rd day	48.75	49.75	41.37	30.75	25.5	18.25	12.6	0
4 th day	61.75	68	53	46.5	36.75	26	16.6	0
5 th day	84.2	80.27	63.5	57.5	47.25	33.25	19.4	0

Table 2. Growth of *Trichoderma harzianum* in chlorpyrifos amended medium

Radial growth of <i>T. harzianum</i> measured in mm in various conc of 20% chlorpyrifos								
Days	Control	25ul	50ul	75ul	100ul	125ul	250ul	500ul
1 st day	19.25	17	14.5	14	12	13.5	0	0
2 nd day	47	38.8	34.6	32.5	24	22	13.75	0
3 rd day	69.5	60.75	54	48	36.25	31.25	17.75	0
4 th day	90	75.75	71.5	63.1	48.5	40	18.2	0
5 th day	90	90	84	73.2	63.5	55.7	21.5	0

Table 3. Details of mycelia dry weight (mg/50ml) from culture filtrate of *T. viride*, *T. harzianum* and its consortium in presence and absence of chlorpyrifos at different incubation period

Days	Absence of chlorpyrifos			Presence of chlorpyrifos		
	<i>T.v</i>	<i>T. h</i>	Consortium	<i>T. v</i>	<i>T. h</i>	Consortium
2	49	56	152	69	116	130
4	140	153	245	219	288	457
6	194	250	490	242	315	531
8	210	331	518	412	496	671
10	297	379	574	443	524	721

Table 4. Extracellular protein (mg/ml of culture filtrate) production from culture filtrate of *T. viride*, *T. harzianum* and its consortium in presence and absence of chlorpyrifos at different incubation period

Days	Absence of chlorpyrifos			Presence of chlorpyrifos		
	<i>T.viride</i>	<i>T. harzianum</i>	Consortium	<i>T.viride</i>	<i>T. harzianum</i>	Consortium
2	0.0009	0.003	0.0006	0.003	0.007	0.007
4	0.001	0.004	0.004	0.007	0.009	0.010
6	0.003	0.010	0.006	0.012	0.036	0.011
8	0.004	0.020	0.015	0.018	0.022	0.026
10	0.006	0.030	0.007	0.017	0.019	0.020

Table 5. Area% of Peak Values for chlorpyrifos (Dursban)

S. No.	Species	Days of Incubation	Retention Time(min)	Area% for Dursban
1	<i>T.viride (T.v)</i>	2 days (initial)	7.573	2.13
		10 days (final)	7.575	0.02
2	<i>T. harzianum (T.h)</i>	2 days (initial)	7.567	2.06
		10 days (final)	7.577	0.05
3	Consortium(<i>T.v and T.h</i>)	2 days (initial)	7.567	2.06
		10 days (final)	7.569	0.83

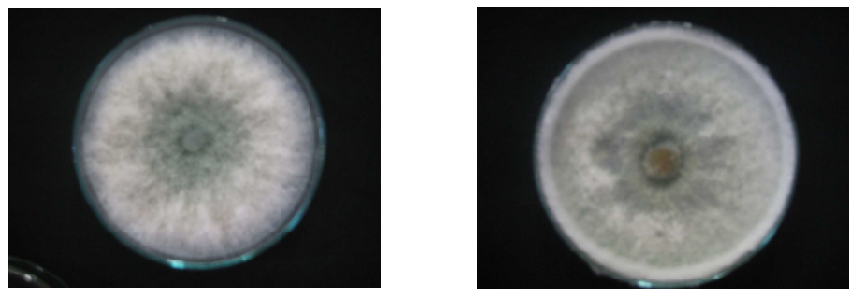
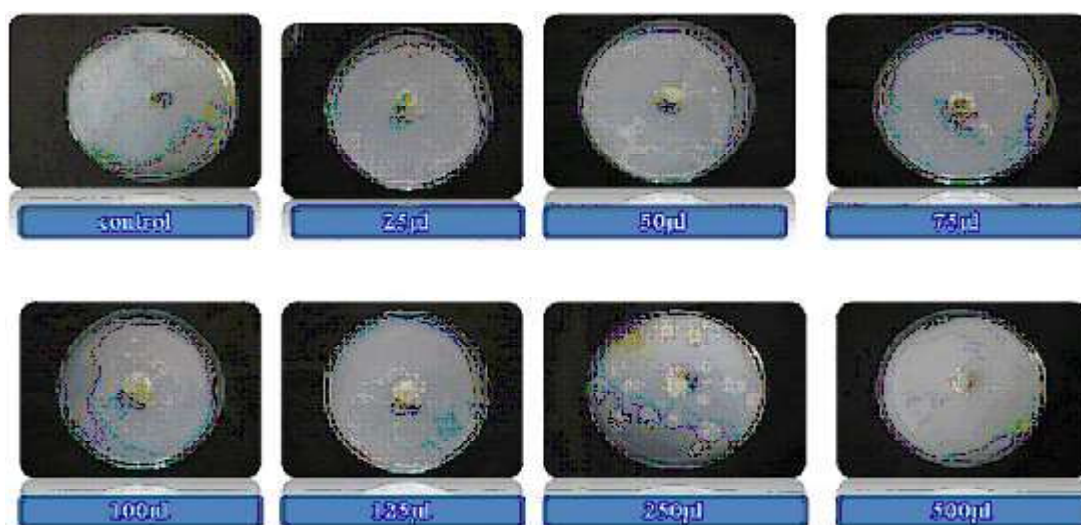
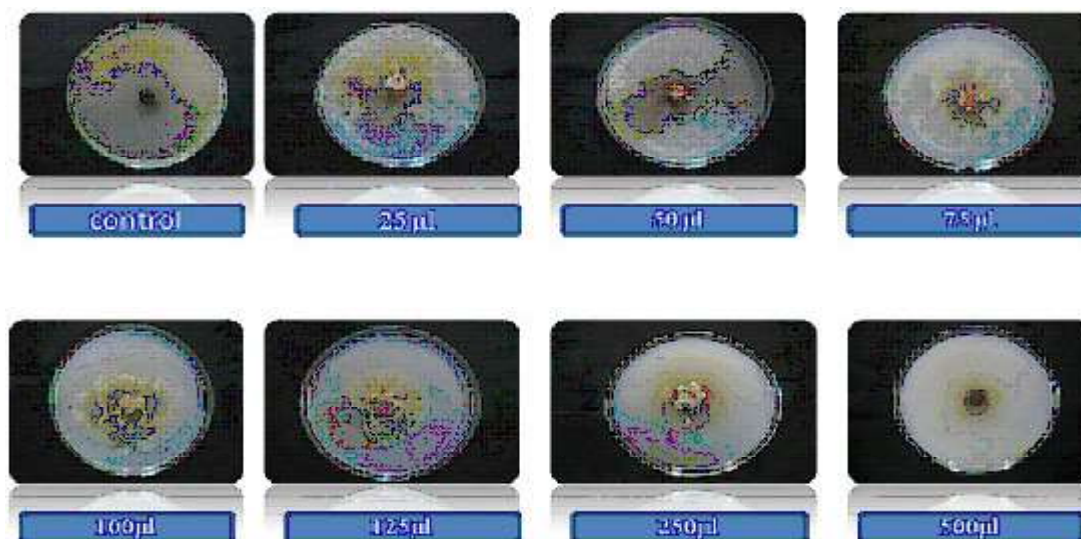
Fig. 3 & Fig.4 respectively, which show the growth of fungus increased their growth at prolonged period of incubation. However, in the increased concentrations of chlorpyrifos amendment after 100 µl and up to 500 µl, there is a decrease in the growth of both the organisms.

The results presented in Table 1 and Table 2 indicates that both the strains have the ability to grow in the pesticide amended media to some extent. As the incubation days increases, both the strains increased their growth. But in the increased concentrations of chlorpyrifos after 100 µl, there is

a decrease in the growth of organisms. Both strains showed Maximum tolerance up to 250ul concentration of 20 % chlorpyrifos and limiting tolerance in 500ul concentration.

Mycelial Biomass determination

The growth of mycelium and its turbid appearance in the liquid culture medium is shown in Fig. 5. The mycelium dry weight of the fungi found to be varied with individual species amended with chlorpyrifos 20% in the liquid culture medium. The growth of *T. viride*, *T. harzianum* and its consortium with and without addition of

a. *Trichoderma viride*b. *Trichoderma harzianum***Fig. 1.** Growth of fungal strains**Fig. 2.** Growth of *T. viride* in various concentrations of chlorpyrifos after 5 days**Fig. 3.** Growth of *T. harzianum* in various concentrations of chlorpyrifos after 5 days

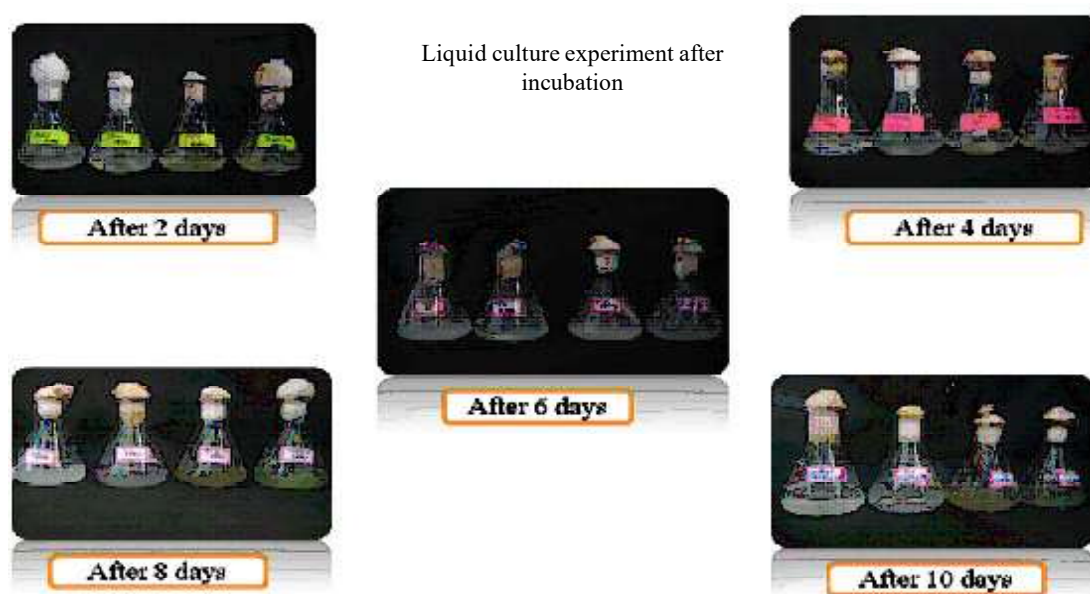


Fig. 4. The culture broth showing after Incubation

chlorpyrifos 20% E.C. (125 μ l/100ml) is given in Table 3.

Estimation of Extracellular protein

The amount of extracellular protein produced in the medium was also varied with organism at different concentrations of chlorpyrifos added. The extracellular protein secretion by *T. viride*, *T. harzianum* and its consortium in the presence of Chlorpyrifos were shown Table 4; Graph1, which is found to be 0.018 mg/ml of protein in 8th day, 0.036 mg/ml of protein 6th day, 0.026 mg/ml of protein in 8th day respectively. But the production of extracellular protein by *T. viride*, *T. harzianum* and its consortium were 0.006 mg/ml on 10th day, 0.0360mg/ml on 10th day, 0.015 mg/ml on 8th day respectively in the absence of Chlorpyrifos which were shown in Table 4; Graph 2.

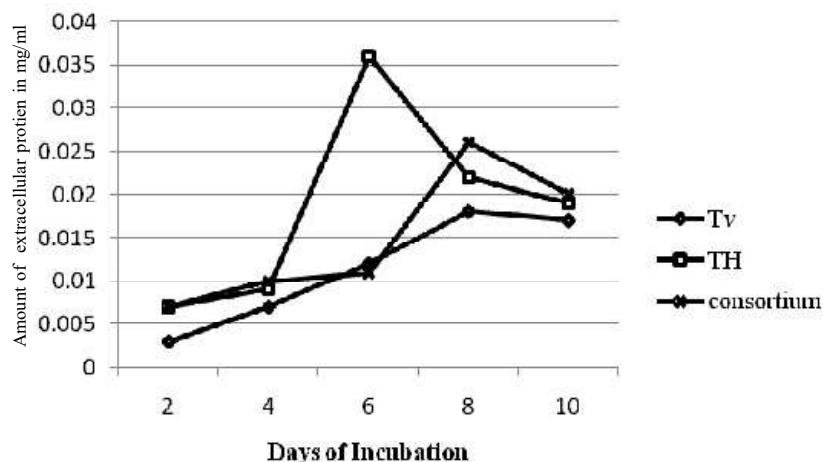
Degradation ability of *Trichoderma viride* and *Trichoderma harzianum*

The percentage of pesticide residue present in the medium supplemented with chlorpyrifos after two days incubation was 2.13% which decreased to 0.02% after 10th day of incubation by the fungus inoculated with *T. viride*. Similar results also were obtained in the medium inoculated with *T. harzianum* which showed 2.06% pesticide after 2 days incubation and 0.05% after

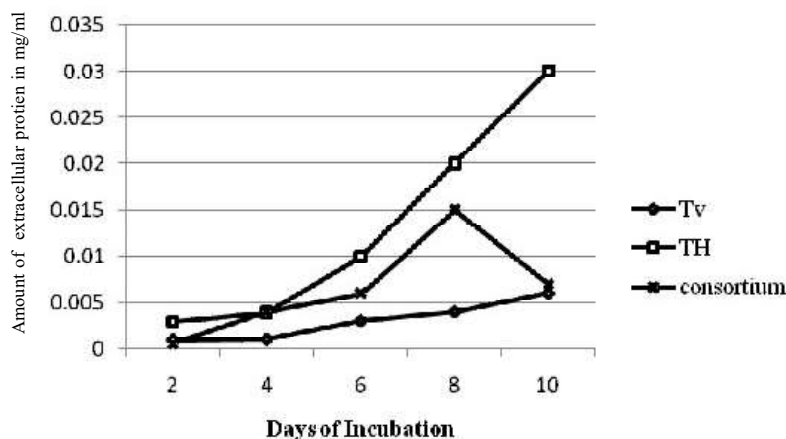
10th day of incubation (Table 5). However, the consortium showed 2.06% after 2nd day and 0.83% after 10th day which slightly lesser than the ability of individual species (Table 5). The retention time (RT) in mins and the area% of Peak Values for chlorpyrifos (Dursban) in the test samples after the growth of *T. viride*, *T. harzianum* and consortium on various days of Incubation is shown in Table 5.

DISCUSSION

The present study demonstrate the degradation of chlorpyrifos by *T. viride* and *T. harzianum* in culture medium amended with various concentrations of pesticide show increased growth and degradation of chlorpyrifos up to 125 μ l/50 ml of medium after 10 days of incubation period. Similar results were reported by Omar (1998), as the degradation of different pesticide chemicals including chlorpyrifos by different species of fungi which proves as the fungi can utilize phosphorus and sulfur of insecticide origin for their growth by mineralizing the insecticide which clearly indicates that the fungi able to grow and assimilate organophosphorous in the media environment. The ability of *Trichoderma viride* on degradation of some commonly used pesticides



Graph 1. Extracellular protein secretion in presence of chlorpyrifos



Graph 2. Extracellular protein secretion in absence of chlorpyrifos

like endosulfan, acephate and quinalphos under invitro conditions have been reported by Subashini. *et al.*, (2007) in which the fungi *Trichoderma viride* is combined with Traditional Plants with Medicinal Properties viz., *Cipadessa baccifera*, *Clausena dentate*, *Dodonaea angustifolia* and *Melia dubia* in the experiment. Sarfraz Hussain & Muhammad Arshad (2007) isolated fungal strains by employing enrichment techniques while using endosulfan as a sole sulfur source, and tested for their potential to degrade endosulfan. Cow-dung and soil microflora would be an effective treatment technology for other group of pesticides and its effects reported by Fulekar & Geetha (2008) and further reported as the higher concentration of nutrients in cow dung

slurry and soil further enhanced the microbial activities and degradation of pesticides. Arata Katayama *et al.*, (2009) reported as *Trichoderma harzianum*, was found to degrade DDT, dieldrin, endosulfan, pentachloronitrobenzene, and pentachlorophenol and concluded that the major enzyme system responsible in *T. harzianum* for degradation of endosulfan is an oxidative system which may be responsible for the metabolism.

Further evidences from the literature show the involvement of many bacteria in the degradation of various pesticide components including soil bacteria and marine bacterium *Bacillus firmus* by Lakshmi *et al.*, (2009); enterobacter by Singh *et al.*, (2004); lactic acid bacteria by Kye Man Cho *et al.*, (2009) and aerobic

bacteria by Ghosh *et al.*, (2010), which indicates the ability of bacteria on degradation of pesticides on various substrates.

Therefore, the findings of the present study in line with several reports by various authors in different parts of the world which encourage the treatment of pesticide residues by using fungi especially soil living fungi as *T. viride* and *T. harzianum*. From the present study, it is found that as the incubation day's increases, both strains *T. viride*, *T. harzianum* increased in their Radial growth. But when the concentration of chlorpyrifos increases in the medium, there is a decrease in the growth of organisms as in the order of 25ul > 50ul > 75ul > 100ul > 125ul > 250ul > 500ul of 20% chlorpyrifos. However, in liquid culture experiments, when comparing the results of (Table 3) mycelial growth (Table 3) in the presence and absence of chlorpyrifos of *T. viride* *T. harzianum* and its consortium after different days of incubation states bio mass production of *T. viride*, *T. harzianum* and its consortium is poor in absence of chlorpyrifos than the growth in chlorpyrifos amended media. These findings match the results obtained by Omar (1998), who reported as the addition of insecticide resulted in greater fungal growth in the media which may be due to assimilation organic and inorganic compounds by fungi for their effective growth. The secretion of extracellular protein also was higher in the medium with chlorpyrifos by *T. viride* *T. harzianum* and its consortium after different days of incubation when compared to the medium without addition of chlorpyrifos. This infers *T. viride* *T. harzianum* and its consortium are capable up taking Chlorpyrifos present in the medium as the report of Omar (1998), where the extracellular Protein Secretion is enhanced in presence of chlorpyrifos as a Phosphorus source. But Abdel-Basset *et al.* (1992) stated that the inhibition of extracellular protein production in the presence of Insecticide. However, the GC-MS results confirms that *T. viride*, *T. harzianum*, and its consortium are capable of degrading chlorpyrifos and it is observed *T. viride*, *T. harzianum*, and its consortium dissipated Chlorpyrifos 99.06%, 97.57%, 59.70% respectively after 10 days of Incubation.

Utilization of xenobiotic compounds by soil microorganisms is a crucial phenomenon by which the adverse effect of these compounds can

be removed from the environment and the environmental pollution may be minimized. The results from the present study suggest that *T. viride*, *T. harzianum* and its consortium is able to grow in medium in the presence of added 20% chlorpyrifos (125µl/100ml) and may therefore be used for bioremediation of chlorpyrifos-contaminated soil. However the result shows consortium of these Fungi possess low ability to degrade chlorpyrifos comparing to their individualistic action and it is observed in the order of *Trichoderma viride* > *Trichoderma harzianum* > Consortium.

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