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BIODEGRADATION OF CHLORPYRIFOS AND 3,5,6-TRICHLORO-2-PYRIDINOL BY FUNGAL CONSORTIUM ISOLATED FROM PADDY FIELD SOIL

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Abstract

Pesticides are considered among the most serious environmental pollutants, frequently used in the control of agricultural and domestic pests. Biodegradation of chlorpyrifos and its 3,5,6-trichloro-2-pyridinol (TCP) derivative was studied in mineral medium and soil with fungal consortium consisting of JAS1 and JAS4 strains which were isolated from paddy field soil. The fungal consortium was spiked with 300 mg l^{-1} chlorpyrifos which was degraded completely within 12 h of incubation in the mineral medium along with the major metabolite TCP. The course of the degradation process was studied using HPLC and FTIR analyses. Two experiments were carried out in soil which included the addition of nutrients (Carbon, Nitrogen and Phosphorous) with fungal consortium and fungal consortium without addition of nutrients. In both the experiments, chlorpyrifos (300 mg kg^{-1} soil) and its metabolite TCP were degraded within 24h and48 h, respectively. These results showed that the chlorpyrifos degrading fungal consortium had the potential to degrade the pesticide from contaminated soil.

Key words: biodegradation, chlorpyrifos, 3,5,6-trichloro-2-pyridinol

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1. Introduction

Large amount of pesticides reaches the soil through the direct application or from aerial spraying, or as plant and animal remains. Organophosphates are a group of highly toxic chemicals that exhibit broad spectrum activity against insects and are widely used against major agricultural pests. Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is one of the most widely used organophosphate insecticides in the developing countries like India. In the year 2000, it was the fourth highest consumed pesticide after monocrotophos, acephate and endosulfan (Thengodkar and Sivakami, 2010). It is a broad spectrum organophosphate insecticide and acaricide, and is widely used for pest control on grains, cotton, fruits, and vegetable crops, as well as lawns and ornamental plants (Fang et al., 2006). The physico-chemical properties of chlorpyrifos are given in Table 1.

Table 1. Physico-chemical properties of chlorpyrifos (Venkta Mohan et al., 2004)

Properties	***Chlorpyrifos***
Chemical name	O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl
Molecular weight	350.62
Formula	$C_9H_{11}Cl_3NO_3PS$
Vapour pressure at 25° C (MPa)	2.49
Activity	Insecticidal
Soil sorption coefficient (mg g^{-1})	849
Solubility at 25 ° C in water (mg L^{-1})	1.39

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It has moderate toxicity, low water solubility (1.39 mg L^{-1}) and high soil sorption (Singh and Walker, 2006). Its half-life in soil varies from 10 to 120 days, with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. This high half-life range is due to the fact that degradation of chlorpyrifos in soil is affected by its initial concentration, soil moisture, temperature, and pH (Awasthi and Prakash, 1997; Derbalah et al., 2016; Racke et al., 1994). TCP is moderately mobile due to its greater water solubility, leading to widespread contamination of soils, sediments and water (Li et al., 2007). The accumulation of TCP has antimicrobial effects on microorganisms which prevents its own degradation by microorganisms and also limits chlorpyrifos degradation (Singh and Walker, 2006).

Remediation of pesticide contaminated ecosystems is an important environmental issue in the reduction of detrimental side effects. The environmental fate of chlorpyrifos has been studied extensively and its degradation may involve a combination of photolysis, chemical hydrolysis and microbial degradation (Xu et al., 2008). Biodegradation using microorganisms is one of the safest and cheapest ways of reducing pesticide levels in the environment. Degradation of pesticides in soil and water can occur by biotic and abiotic pathways, but the main mechanism of degradation and detoxification is by microorganisms. Microbial consortium is more efficient in the removal of pollutants because they can readily avoid the toxic metabolites accumulation derived from microbial degradation (Kumar and Philip, 2006; Wang et al., 2011). Using the technique of enriched cultures from contaminated environments, consortium of microorganisms with a high ability to degrade persistent and toxic compounds can be obtained (Krishna and Philip, 2009).

Microbial consortium is more efficient in the removal of pollutants as several organisms are involved in each step and they compete for the pesticide or breakdown products during mineralization and there by more easily avoid the accumulation of toxic compounds derived from initial microbial degradation. Therefore, biodegradation using native microorganisms for its removal from the environment is quite attractive. In the present study, a consortium comprising of fungal strains isolated from paddy field soil was employed in biodegradation of chlorpyrifos and its metabolite TCP in the mineral medium. The study aims at elucidating a possible application of isolated fungal strains for bioremediation of chlorpyrifos contaminated environment.

2. Material and methods

Certified standard of analytical grade chlorpyrifos (99% chemical purity) and TCP (99% chemical purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). Technical grade chlorpyrifos, a 20% emulsifiable concentrate used in this study was obtained from Isagro (Asia) Agrochemical Pvt. Ltd., Mumbai.

In order to isolate potential microorganisms to degrade chlorpyrifos and its primary metabolite TCP, a fungal consortium was developed from strains isolated from paddy field soil by selective enrichment technique where in providing chlorpyrifos as a sole carbon source. From the enrichment culture, morphologically different fungal strains were isolated. The number of isolates was lesser in the soil collected for the study, which might be due to the inhibitory action of pesticide on the soil microbial population. These strains were further purified and checked for their ability to tolerate chlorpyrifos by inoculating them in M1 medium containing chlorpyrifos as sole carbon and energy source. The minimum inhibitory concentration of chlorpyrifos was determined for the fungal isolates, JAS1 and JAS4 strains were capable of tolerating 400 mg l^{-1} and 500 mg l^{-1} of chlorpyrifos respectively, and both of the strains were found to grow well up to 300 mg l^{-1} of chlorpyrifos and hence, selected for further studies (Silambarasan and Abraham, 2013a; 2013b). The degradation studies of chlorpyrifos and its primary metabolite TCP by the bacterial consortium was carried out in orbital shaker. Seed culture of each isolated strains were grown in potato dextrose broth containing (g l^{-1}) potato infusion, 200; dextrose, 20; agar, 15 and pH 5.6 ± 0.2. Degradation of chlorpyrifos was performed in 250 mL Erlenmeyer flasks containing 100 mL of M1 medium composed of $NaNO_3$, 2 g KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g $FeCl_3$, 10 mg; $BaCl_2$, 0.2 g; $CaCl_2$, 0.05 g per liter at pH 6.8 supplemented with 300 mg l^{-1} chlorpyrifos as the sole carbon source and inoculated with 0.5 mL of each fungal culture. Flasks were incubated at 30 ± 2°C on a rotary shaker at 120 rpm. Samples were taken at 12h, 24h and 48h from culture flasks and analyzed the removal of chlorpyrifos and TCP residues by HPLC. For biodegradation studies of chlorpyrifos and its metabolite TCP, soil samples from which fungal strains were isolated was used. Soil samples was collected from the top layer (0-15 cm) were air-dried at room temperature, mixed thoroughly and sieved (2 mm) to remove stones and debris. The physico-chemical properties of the soil were determined at the Shri AMM Murugappa Chettiar Research Centre, Taramani, Chennai, India.

Before using for analysis, the soil was sterilized by autoclaving for 30 min at 121°C. 100 g of this soil was placed in 250 mL Erlenmeyer flask and treated with chlorpyrifos (300 mg kg^{-1} soil), under sterile conditions. After mixing, 30 mL of a solution containing consortium of fungal spore suspension, nitrogen, phosphorus and glucose were added, under sterile conditions. The amount of carbon, nitrogen and phosphorous were calculated using the relationship C/N/P 100:10:1. The sources of carbon, nitrogen and phosphorous were glucose, $(NH_4)_2SO_4$ and K_2HPO_4 respectively (Martin et al., 2007). In addition, the fungal consortium and chlorpyrifos (300 mg kg^{-1} soil) were mixed with soil without nutrients supplement. This experimental variant was used as a control for

comparison with chlorpyrifos biodegradation with nutrient supplement. All flasks were incubated in an incubator at 30 ± 2 °C. Samples of soil treatments were periodically removed aseptically for the determination of chlorpyrifos and TCP residues. Detailed physico-chemical properties of the soil are presented in Table 2.

Table 2. Physico-chemical properties of the soil used in the experiment

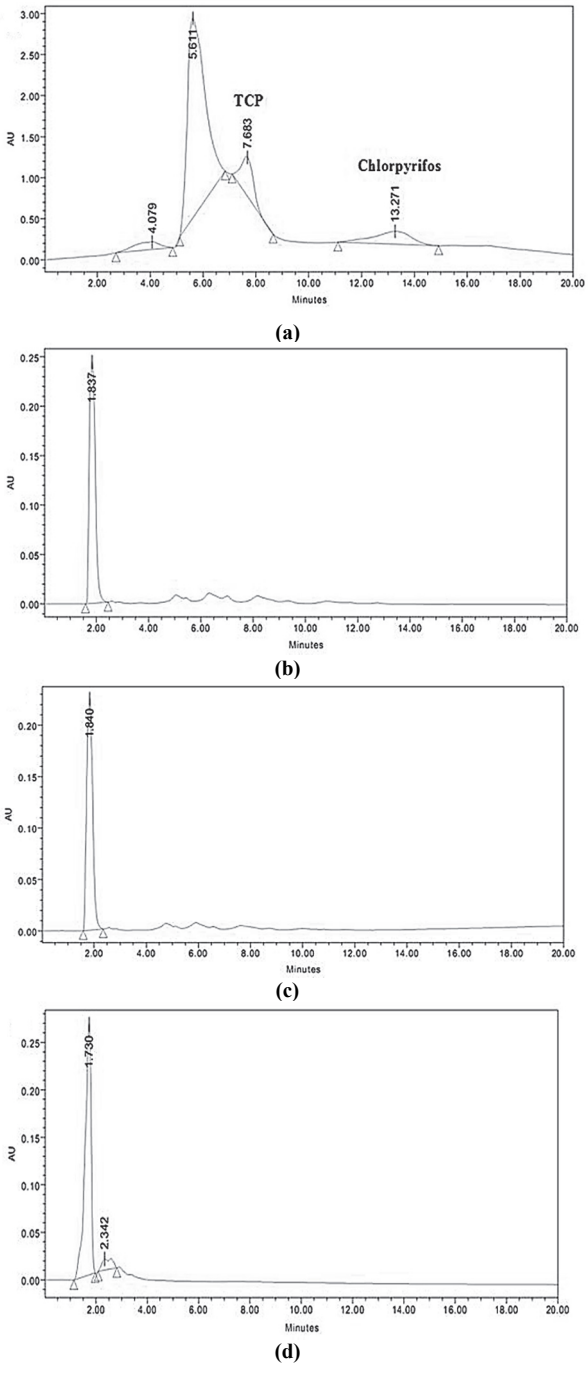
Properties	***Paddy field Soil***
pH	7.32
EC	0.21
Organic Carbon	0.43 kg acre^{-1}
Organic Carbon	0.47 %
Nitrogen	108.03 kg acre^{-1}
Phosphorus	12.61 kg acre^{-1}
Potassium	99.3 kg acre^{-1}
Calcium	478.04 mg kg^{-1}
Magnesium	154.82 mg kg^{-1}
Sodium	111.39 mg kg^{-1}
Iron	8.52 mg kg^{-1}
Manganese	9.87 mg kg^{-1}
Copper	1.74 mg kg^{-1}
Zinc	0.83 mg kg^{-1}
Sulfate	20.46 mg kg^{-1}
Humus	98.61 kg acre^{-1}
Total Minerals	219.94 kg acre^{-1}

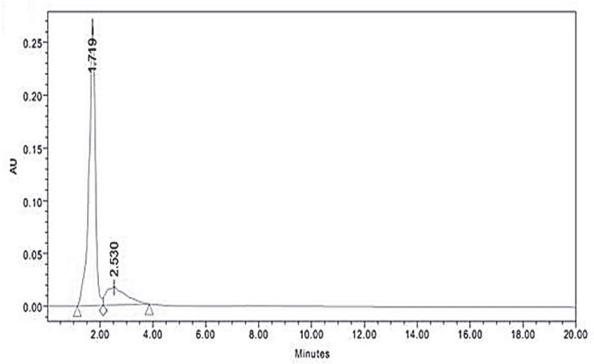
The chlorpyrifos and TCP were extracted from mineral medium and soil using dichloromethane and acetonitrile as described earlier by Silambarasan and Abraham (2013a). On the day of extraction 5-10 mL of aqueous samples were recovered from culture flasks and centrifuged at 7200 × g for 10 min to obtain a cell-free medium. Chlorpyrifos and TCP residues were extracted from supernatant using equal volume of dichloromethane (DCM) twice. Organic layer of DCM was aspirated, pooled and evaporated at room temperature under nitrogen.

The residues were dissolved in HPLC grade acetonitrile (1 mL) and then filtered through filter membrane (0.22 µm FH) to remove any particles and determine the pesticide concentration by HPLC. Soil analysis was performed with addition of nutrients and devoid of nutrients. In a 250mL Erlenmeyer flask 10g of soil sample was added and to this 20mL of acetonitrile (HPLC grade) was introduced and placed in a shaker for 30mins at 120rpm. After the soil had settled the supernatant was removed for pesticide residue analysis by HPLC. The isocratic mobile phase comprised of methanol:water (85:15, V:V), which was pumped through the column at a flow rate of 1 mL min^{-1}. Chlorpyrifos and its metabolite TCP was detected at 230 nm. Infrared spectra of the chlorpyrifos, before and after degradation with fungal consortium were recorded at room temperature in the frequency range of 4000-400 cm^{-1} with a Fourier transform infrared (FTIR) spectrophotometer (8400 Shimadzu, Japan, with Hyper IR-1.7 software for windows) with a helium neon laser lamp as a source of infrared radiation. The degradation rate constant (*k)* was determined using the zero-order kinetic $Ct–Co=kt$, where *Ct* is the amount of pesticide at time *t*, *Co* is the amount of pesticide at time zero, and *k* and *t* are the rate constant and degradation and degradation period in days, respectively. The time in which the pesticide concentration was reduced by 50% (DT_{50} values) was also calculated.

3. Results and discussion

The biodegradation of chlorpyrifos in 300 mg L^{-1} concentration in the M1 medium was assessed using fungal consortium. The extracted samples were analyzed by HPLC, to monitor the removal of chlorpyrifos and its major degradation product of 3,5,6-trichloro-2-pyridinol. The results of the HPLC analyses of chlorpyrifos degradation are presented in Fig. 1.





(e)

Fig. 1. HPLC chromatograms of chlorpyrifos and its metabolite at: (a) standard condition; (b) 12h, (c) 24h; (d) 48h in mineral medium; (e) with nutrients

The HPLC analysis showed that the consortium was able to efficiently degrade chlorpyrifos and TCP within 12h of incubation, which suggests that the fungi used these compounds as carbon and energy sources for growth. The degradation of chlorpyrifos and its primary metabolite TCP in the soil with addition of nutrients and soil without addition of nutrients revealed that microbial degradation is considered to be one of the main mechanisms of chlorpyrifos (300 mg kg^{-1} soil) dissipation in soil (Fig. 2). In soil with addition of nutrients inoculated with fungal consortium, the complete degradation of chlorpyrifos and TCP occurred within 24h and 48h of incubation respectively; however, there was no marked difference in soil inoculated with fungal consortium in the absence of nutrients which showed a similar trend of 100% degradation of chlorpyrifos and TCP within 24 h and 48 h of incubation respectively.

In the sterile soil with nutrients, the degradation of pesticide by consortium was characterized by a rate constant of 149 d^{-1} and DT_{50} was 1 d, following zero order kinetic model. The degradation of pesticide in soil without addition of nutrients was characterized by a rate constant of 146 d^{-1} and DT_{50} was 1.02 d, following zero order kinetic. Pino and Penuela (2011) reported the supply of glucose as external carbon source in the degradation of pesticide in soil which increased the efficiency of degradation of the pesticide to 100%. However, the study revealed that JAS1 and JAS4 strains released chlorpyrifos degrading enzymes even in the absence of readily available nutrient sources (Silambarasan and Abraham, 2013a, 2013b).

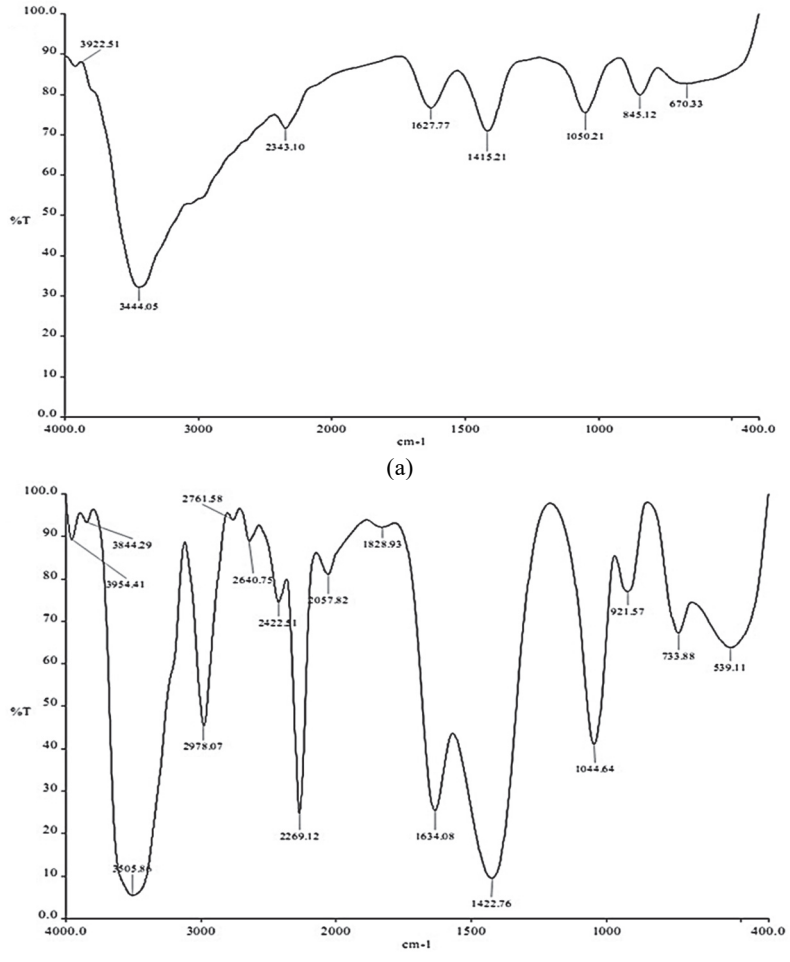


Fig. 2. (a) HPLC chromatogram of biodegradation of chlorpyrifos and its primary metabolite TCP in soil with nutrients at 48h, (b) HPLC chromatogram of biodegradation of chlorpyrifos and TCP in soil without nutrients at 48h

Several studies have revealed that TCP has been found to be a major metabolite of chlorpyrifos degradation as mediated by microorganisms. However, only a few numbers of studies have showed the degradation of TCP.

The primary metabolite TCP has been reported to be more toxic than chlorpyrifos and its accumulation to be inhibitory to cell growth and to chlorpyriofos degradation (Feng et al., 1997). Yang et al. (2005) reported *Alcaligenes faecalis* DSP3 bacterium capable of degrading chlorpyrifos and the primary metabolite was found to be TCP. Enterobacter strain B-14 biotransformed chlorpyrifos up to TCP only (Singh et al., 2004).

Chungjatupornchai and Fa-Aroonsawat (2008) isolated a gene for organophosphorus hydrolase from *Flavobacterium* sp. and expressed it in *Synechococcus* PCC 7942. Further they showed that this enzyme is located in both surface as well as intracellularly. It is also reported that phosphatases play an important role in the biodegradation of chlorpyrifos (Thengodkar and Sivakami, 2010; Madhuri and Rangaswamy, 2002). To the best of our knowledge, this is the first report on biodegradation of chlorpyrifos and its major degradation product (TCP) by fungal consortium consisting of *Aspergillus terreus* JAS1 and *Ganoderma* sp. JAS4 within 12h in mineral medium incubation. The mechanism of chlorpyrifos degradation in bacteria and fungi is fairly understood and a number of degradation products such as diethylthiophosphoric acid, 3,5,6-trichloro-2-pyridinol, chlorodihydro-2-pyridone, dihydroxypyridine, tetrahydro-2-pyridone, and maleamide semialdehyde have been identified (Singh and Walker, 2006).

In our studies, the complete degradation of chlorpyrifos and its metabolite TCP in the aqueous medium with fungal consortium was obtained. Feng et al. (1998) suggested the degradation of TCP was primarily converted to chlorodihydro-2-pyridone by reductive dechlorination followed by its degradation into tetrahydro-2-pyridone and then to maleamide semialdehyde, which was mineralized ultimately to water, CO_2 and ammonium.

The FTIR analysis clearly indicated the structural changes of chlorpyrifos. The IR spectra of chlorpyrifos control and extracted samples from mineral medium were shown in Fig. 3. Comparison of FTIR spectrum of control with extracted metabolites after complete degradation clearly indicated the biodegradation of chlorpyrifos.

The infrared spectrum of control sample showed band at 3444 cm^{-1} and 1627 cm^{-1} which are the characteristics of N-H and C=O amide groups, respectively. The peak positions at 1415 cm^{-1}, 1050 cm^{-1}, 845 cm^{-1}, and 670 cm^{-1} which are C=C stretching, C-O stretching, C-H bending, C-Cl stretching, respectively.

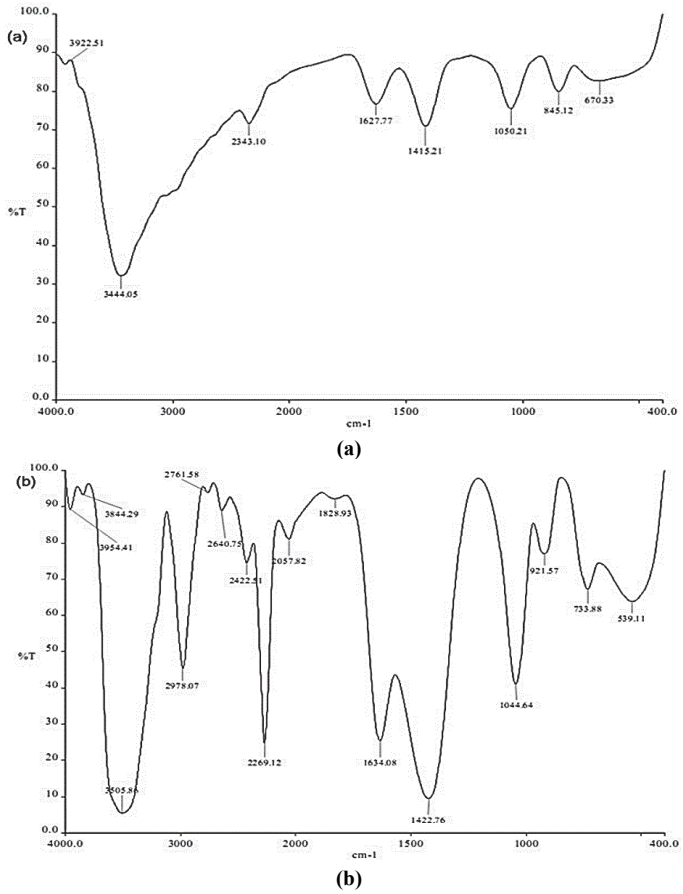


Fig. 3. FTIR spectrum of biodegradation of chlorpyrifos and its metabolite at:
(a) standard condition, (b) in mineral medium

The band at 1422 cm^{-1} in the degraded sample indicated the presence of formic acid as intermediate product of chlorpyrifos and TCP degradation. Previously, Bhalerao and Puranik (2009) reported the band at 1420 cm^{-1} corresponds to the formic acid formation during the degradation of organophosphorus pesticide. Emergence of the intense bands at 1242 cm^{-1} and 1047 cm^{-1} supports the hydrolytic cleavage of monocrotophos by *Aspergillus oryzae* ARIFCC 1054, but in our study the hydrolytic cleavage was presented at 1044 cm^{-1}.

4. Conclusions

It is concluded that microbial consortium composed of *A. terreus* JAS1 and *Ganoderma* sp. JAS4 is a novel approach for bioremediation of chlorpyrifos contaminated soil. The important feature is that these particular strains could also degrade TCP. Degradation of this compound by the same strain that degrades chlorpyrifos is very important because TCP is more toxic than its parent compound with antimicrobial activity.

These results highlight potential application of this consortium for the cleanup of pesticide contaminated environment.

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