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ORIGINAL ARTICLE

Chlorpyrifos degradation using binary fungal strains isolated from industrial waste soil

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Abstract

Chlorpyrifos is a broad-spectrum organophosphate pesticide and used frequently on different cropping systems to control phytopathogens. Being a hazardous substance, chlorpyrifos may affect human health as some unutilized pesticide percolates through soil and contaminates ground water. Present study was planned to establish an effective remediation method of chlorpyrifos using two fungal isolates (C1 and C3), recovered from the waste (soil) of a refrigeration industry. C1 and C3, identified as *Byssochlamys spectabilis* and *Aspergillus fumigates* respectively were able to tolerate 600 mg L^{-1} of chlorpyrifos. "The Half-life of chlorpyrifos (control) was 231 days which could be reduced to 13.6 days" under *in vitro* condition in the presence of mixture of two fungi in Czapek dox medium (CDM). Consortium of the fungi showed 98.4 % degradation of chlorpyrifos within 30 days in CDM. Degradation of the pesticide was related to specific laccase activity of the fungi and the level of enzyme activity was better in consortium 9.09 and 10.29 U mL^{-1} after 6 and 10 days respectively. Immobilized fungal enzymes also showed appreciable biodegradation of the pesticide by providing extra matrix for enzyme substrate reaction. Residual analysis of chlorpyrifos in the soil of onion (*Allium cepa*) in a phytoremediation experiment revealed a significant decrease in $t_{1/2}$ value in the presence of fungi. Hence, it is concluded that chlorpyrifos biodegradation ability of fungal consortium reveals the potential of these isolates in biodegradation of toxic compounds from contaminated water and soil. However, further studies are needed to access the biodegradation of the pesticide in different soil types, crops and under varied environmental conditions at molecular level.

Keywords Chlorpyrifos · Biodegradation · Half-life · Laccase · Consortium

Introduction

Pesticides, the integral part of pest management since decades have rescued the agriculture from devastating effects of pest damage. They occupy central place in the management of crop diseases and pests but at the same time they impose adverse effect on agricultural ecosystems, environment and living beings because of their toxicity and persistency. Organophosphates are broad spectrum pesticides and account approximately 38 % of total pesticides used in agriculture against many pests. Extensive use of these pesticides has generated severe ecological problems of ground water and air pollution (Zeinat et al. 2008). According to Thengodkar and Sivakami (2010), Chlorpyrifos (o, o-diethyl o-(3, 5, 6-trichloro-2-pyridyl phosphonothioate) is one of the widely used broad spectrum organophosphates which is used to manage a variety of chewing and sucking insect pests and mites on a broad range of economically vital fields, horticultural and plantation crops, such as potatoes, vegetables, citrus fruits, banana, coffee, tea, cocoa, cotton, wheat and rice etc. Due to board spectrum and persistent nature, chlorpyrifos causes severe damage to non-target insect pests and contaminates environment and ground water (Ruan et al. 2012). Evidences are available regarding the contamination of terrestrial and aquatic ecosystems by chlorpyrifos (Bouchard et al. 2011). Exposure to organophosphates may cause nervous breakdown and muscular diseases in human and animals due to their ability to suppress acetylcholine esterase activity (Zhang et al. 2008). Chlorpyrifos may also affect immune, endocrine,

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cardiovascular, respiratory, nervous and reproductive systems in humans (Anderson et al. 1994).

Most of the physical and chemical methods used to detoxify chlorpyrifos are costly and not much effective hence use of microbial methods to bioremediate chlorpyrifos from contaminated sites has attracted the attention of scientists (Horne et al. 2002; Rudakiya et al. 2021) have mentioned some advanced strategies to reduce pesticide contamination using fungi. According to Singh (2008), fungi are ubiquitously present in the nature and can be successfully used to remove a variety of xenobiotic compounds. Low specificity of fungal enzymes and their nature of utilizing organic chemicals as growth substrate and extensive mycelia formation by most fungi make them well suited for bioremediation processes (Harms et al. 2011). Fungi are potential candidates in nutrient recycling and biodegradation studies (Liang et al. 2005). Recently, Rudakiya et al. (2020) have investigated the role of fungal laccase in biodegradation of different pesticides, tolerance to toxic metals, organic solvents and potent inhibitors. According to Wang et al. (2020), immobilized *Phlebia* sp. (a fungi) by corn stover degraded chlorpyrifos (200 mg Kg^{-1}) in 7 days upto 74.35 %. Kulshrestha and Kumari (2011) and Fang et al. (2008) reported the ability of *Acremonium* sp. strain GFRC-1 and *Verticillium* sp. strain DSP to degrade chlorpyrifos. Silambarasan and Abraham (2013) reported degradation of chlorpyrifos and trichloro pyridinol (300 mg L^{-1}) in liquid broth and in soil @ 300 mg Kg^{-1} by *Aspergillus terreus* JAS after 24 h. Some fungal strains used in biodegradation/bioremediation of pesticides are *Fusarium proliferatum* strain CF2, *Aspergillus* sp. strain P4-7 and *Microsphaeropsis* sp. CBMAI 1675 (Cai et al. 2016; Birolli et al. 2018).

Till now, exploitation of fungi in bioremediation of organophosphates has not gained major attention as it deserves. Present study was performed to explore the potential of two fungal isolates and their immobilized enzymes in biodegradation of chlorpyrifos under *in vitro* and *in situ* conditions under some objectives like (i) Isolation of chlorpyrifos degrading fungi, (ii) *In vitro* degradation kinetics and half-life of chlorpyrifos in liquid medium, (iii) Quantification of laccase, (iv) To analyze degradation kinetics of the pesticide with immobilized enzyme (v) Phytoremediation experiment using onion crop. Our results showed biodegradation of chlorpyrifos and minimization of its half-life in liquid and soil by *Aspergillus fumigates* and *Byssochlamys spectabilis.*

Materials and methods

Isolation and enrichment of fungal strains

To isolate chlorpyrifos degrading fungi, 5 g waste material (obtained from a refrigerator industry, Silvassa, Dadar Nagar Haveli) was mixed in 100 mL Potato Dextrose Broth, supplemented with 10 mg L^{-1} chlorpyrifos. Flasks were incubated overnight at 28 under shaking conditions at 150 rpm. Standard dilution method was used to isolate fastidious fungi from the broth. After dilution, recovered fungal isolates were purified by growing them on Czapek Dox agar supplemented with 10 mg L^{-1} chlorpyrifos. Inoculated plates were incubated for 5 to 7 days at 28 . Same medium was enriched with higher concentrations of chlorpyrifos and growth of fungal isolates was observed. Composition of Czapek-Dox medium was (g L^{-1}): Sucrose − 30, $NaNO_3$- 3, K_2HPO_4 -1, KCl − 0.5 and $FeSO_4$ − 0.01, $MgSO_4$ − 0.5, Agar- 20 g. The pH of the medium was maintained at 6. For chlorpyrifos biodegradation experiment, sucrose and phosphorus were removed from the medium and chlorpyrifos served as a source of carbon and phosphorus (Siddique et al. 2003).

DNA extraction, PCR amplification and sequencing of ITS region

Extraction of genomic DNA of the test fungal isolates was done using Nucleo - pore® gDNA Fungal Mini Kit. Internal transcribed spacer (ITS) region of 18S rDNA of extracted genomic DNA was subjected to amplification using polymerase chain reaction (PCR) in a thermal cycler (PTC-100™, MJ Research). Sequences of universal eukaryotic primers were: forward ITS 1: 5'-TCC GTA GGT GAA CCT GCG G -3', reverse ITS 4: 5'-TCC TCC GCT TAT TGA TAT GC -3' (Ferrer et al. 2001). Primers were purchased from GeNei™. PCR program followed initial denaturation temperature of 95 for 5 min, 35 cycles at 95 for 30 s each, annealing at 55 for 1 min and extension at 72 for 1 min. Final extension single cycle was maintained at 72 for 6 min. Amplified PCR products of ITS region were sequenced and phylogenetic analysis of the fungal strains was performed by retrieving DNA sequences through National Centre for Biotechnology Information (NCBI) GenBank database. Clustering was done using Neighbor-joining method after BLAST analysis. Phylogenetic tree was constructed using MEGA-7 software based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Obtained gene sequences were submitted to GenBank.

In vitro degradation of chlorpyrifos

Degradation of chlorpyrifos using fungal isolates in 50 mL Czapek-Dox medium (devoid of carbon and phosphorus sources) was studied in 100 mL flask. Chlorpyrifos was supplemented to all the flasks @ 20 mg L^{-1} followed by the addition of fungal agar discs (6 mm) from 5 days old fungal plates. Chlorpyrifos degradation was tested in the presence of individual fungal isolate (C1 and C3) and mixture of the two (C1 + C3). Inoculated flasks were incubated under shaking

condition at 150 rpm and 28 . On 15th and 30th day, 1 mL broth was taken from each flask separately to extract residual chlorpyrifos. Uninoculated flask spiked with chlorpyrifos acted as control.

Quantitative estimation of fungal laccase

Fungal culture(s) (agar discs of 6 mm diameter, cut from 5 days old fungal plates) were inoculated in 50 mL potato dextrose broth. Inoculated flasks were incubated at 28 under shaking condition at 150 rpm for 10 days. Laccase activity of the test fungi was checked after 3, 6 and 10 days of incubation according to Monseff et al. (2016). For laccase estimation, 1 mL fungal broth was centrifuged at 10,000 rpm in a refrigerated centrifuge. Obtained supernatant was then transferred to a test tube to which 3 mL Sodium Acetate buffer (10 mM) and 1 mL of guaiacol (2 mM) was added. Absorbance of the reaction mixture was taken at 450 nm after incubating the mixture for 15 min at 30 . A water blank with no supernatant served as control. Laccase activity (U mL^{-1}) was calculated using the equation E. A = AxV/txεxy, where, enzyme activity is denoted as E.A, absorbance as (A) at 450 nm, V is the total volume of the mixture in ml, γ is enzyme volume in mL, incubation time is t and ε is extinction coefficient of guaiacol (0.674 μm cm^{-1}).

Immobilization of fungal enzyme for chlorpyrifos degradation

To prepare immobilized alginate beads of laccase enzyme, fungal isolates were grown in 50 mL potato dextrose broth, supplemented with 5 mM guaiacol at 28 for 6 days. Then 1ml supernatant (obtained by centrifugation of fungal culture) was mixed with 5 mL sodium alginate solution (12 %) (Wang and Wang 2010). Mixture of free enzyme (supernatant) and sodium alginate was added dropwise into 2 % cold solution of calcium chloride (w/v) with the help of a syringe. Prepared beads were kept at 4 for 4 h for hardening. Biodegradation experiment using alginate beads of fungal enzyme was conducted in mineral salt medium (MSM). Composition of MSM in g L^{-1} was K_2HPO_4 (1.8), NH_4Cl (4.0), $MgSO_4.7H_2O$ (0.2), NaCl (0.1) and $FeSO_4.7H_2O$ (0.01). Chlorpyrifos @ 50 mg L^{-1} was added in 50 mL of MSM. Ten beads were added in each flask. To test chlorpyrifos biodegradation, activity of free enzyme was also estimated using the same method. Beads without enzyme served as a control.

In situ chlorpyrifos remediation with onion crop in sterile and unsterile soil

Soil used in the pot experiment was collected from the upper 15–30 cm layer of Breeding Research Centre (BRC), Pantnagar. Soil was air dried, sieved with < 5 mm mesh and sterilized for three days consequently at 121ºC for 60 min in an autoclave. Autoclaved soil was spiked with chlorpyrifos @ 50 mg Kg^{-1}, prepared in 25 ml acetonitrile and left for 16 h at room temperature for complete evaporation of acetonitrile. Fungal strains were grown in potato dextrose broth (50 mL) for 5 days at 28ºC under shaking condition. Five mL of homogenous fungal culture was mixed with half kilogram of spiked soil per pot. Onion (*Allium cepa*) seedlings of variety Pusa red were obtained from Vegetable Research Center (VRC), Pantnagar. Healthy seedlings with similar parameters (height 8–10 cm) were used in the pot experiment. Before plantation, roots of onion seedlings were surface sterilized with $HgCl_2$ (0.2 %) for 5 min. and then washed twice with sterile distilled water. Four onion seedlings were planted in each pot. Pots were regularly irrigated with sterilized water. The experiment was conducted in departmental net house. Ten pots containing 0.5 kg soil per pot were taken for study. Five pots were filled with sterile soil and five were filled with unsterile soil. Different treatments were prepared by using different combinations of fungal isolates. Treatments in sterile/unsterile soil were: (C1 + soil + onion, C3 + soil + onion, C1 + C3 + soil + onion). Soil containing chlorpyrifos, fungal isolates and seedlings acted as positive control while soil with chlorpyrifos, seedlings without fungal isolates acted as negative control. Observations on plant (height and chlorpyrifos uptake) and soil parameters (chlorpyrifos residue) were taken after 30 days of the experiment.

Analytical methods (Chlorpyrifos extraction and HPLC)

Aliquots of 1 ml from liquid medium or 5 g soil from each pot were taken separately for pesticide extraction. After adding 10 mL acetone to individual samples, flasks were shaken for 1 h. After shaking, 1 mL sample was withdrawn and centrifuged at 10,000 rpm for 10 min. Supernatant was then transferred in a separating funnel, to which 1 g sodium sulfate was added to maintain anhydrous condition and 1 ml acetonitrile was also added. After mixing thoroughly mixture was left for 1 h. Upper hydrophobic layer containing chlorpyrifos (extracted in acetonitrile) was collected and allowed to stand overnight to evaporate acetonitrile (Cook et al. 1999). Dried leftover of chlorpyrifos was solubilized in 2 mL acetonitrile and filter sterilized using 0.22 μm filter. To extract chlorpyrifos from the plant samples, 1 g anhydrous sodium sulphate was mixed with 5 g homogenized plant sample to which 5 ml acetone was added in a flask. Mixture was filtered and after keeping in a shaker for 1 h, acidified active carbon (0.1 g) was added to the filtrate to remove soluble plant pigments (Negi et al. 2014). Quantification of chlorpyrifos was done by HPLC at Department of Chemistry, Pantnagar. HPLC (Thermo Scientific Dionex UltiMate 3000) with support for pressure up to 620 bar at flow rate of 10 mL min^{-1} and injection cycle

time as low as 15 s was used. A mixture of acetonitrile and water (70:30, v/v) was used as mobile phase at a flow rate of 1 mL min^{-1} using a reverse phase C-18 column (Thermo Fisher, Scientific). Standard solutions of chlorpyrifos (0.25, 1, 5, 10, 15 and 20 mg L^{-1}) were prepared according to Alam (2013).

Degradation kinetics of chlorpyrifos

For kinetic analysis of chlorpyrifos biodegradation, first order kinetic equation ($Ct = C_0e^{-kt}$ Eq. 1) was followed to fit experimental data. Percent biodegradation of chlorpyrifos was calculated using the equation. Logarithm (C) of the residual value of chlorpyrifos against time t was used to find k value. Therefore, ln $Ct = C_0 - kt$ Eq. (2) where C_0 is the initial concentration of chlorpyrifos (20 mg L^{-1}) in Czapek Dox medium, 50 mg L^{-1} in immobilized minimal medium and 50 mg Kg^{-1} in soil. Ct is the concentration of chlorpyrifos at time t, i.e., final concentration, k is the degradation rate constant per day and t is the reaction time. Graph was plotted and half-life ($t_{1/2}$) of chlorpyrifos in control and treatments of liquid and soil was calculated by $t_{1/2} = \ln 2/k$ Eq. (3).

Statistical analysis

Completely Randomize Design (CRD) was used to conduct the experiments with three replicates per treatment. IBM SPSS Statistics software (Version 19) was used for statistical analysis. One-way analysis of variants (ANOVA) and Duncan's test was done for analyzing data. The data presented in graphs and tables are mean values ± SD (Standard Deviation).

Results

Isolation and characterization of chlorpyrifos degrading fungus

A sum of 11 fungal isolates was recovered from the waste discharge sample of a refrigeration industry. Among the recovered fungi, two potent fungal isolates (C1 and C3) were selected as they grew on modified Czapek Dox medium, supplemented with 10 mg L^{-1} chlorpyrifos as a source of carbon and phosphorus (Fig. 1). Selected fungal isolates showed maximum tolerance (600 mg L^{-1}) for chlorpyrifos. Growth of both the fungi increased gradually with increase in chlorpyrifos concentration. On amplification of ITS region, presence of a distinct band of approximately 750 bp confirmed the preserved ITS region in both the fungal isolates (Supplementary material- SM1). On the basis of maximum homology (80–95 %) and BLAST match, C1 and C3 were characterized as *Byssochlamys spectabilis* and *Aspergillus fumigates* respectively (Fig. 2) and provided with accession numbers MH430256 and MH430257 respectively (web link https://www.ncbi.nlm.nih.gov/nuccore/MH430256 or MH430257).

Chlorpyrifos degradation in Czapek Dox medium

Percent degradation of chlorpyrifos in control was 3.3 and 6.0 on 15th and 30th day respectively. On zero day 100 % of chlorpyrifos was present in medium. Control only showed abiotic degradation. Abiotic degradation was taken into account to calculate total degradation of the pesticide. Chlorpyrifos degradation was high in the presence of fungal isolates as compared to the control (SM2). On 15th day, highest degradation of chlorpyrifos was recorded in the presence of fungal consortium (C1 + C3). After 30th day, again highest degradation (98.4 %) was observed with consortium however C1 and C3 individually showed 96.7 and 93.45 % degradation respectively (Fig. 3a). Degradation of chlorpyrifos was 9 times higher than the control. Maximum biotic degradation was observed in consortium and the degradation pattern was: consortium (92.02 %), C1 (90.75 %) and C3 (87.45 %) after 30 days. Our results showed that the fungal isolates utilized chlorpyrifos for their growth and metabolism. Half-life ($t_{1/2}$) of the pesticide in control was 231 days which showed significant reduction in different treatments. Minimum half life of chlorpyrifos was 13.58 days under C1 + C3 treatment while C1 and C3 treatments showed half life of 14.1 and 17.7 days respectively when used independently significantly better than the control (Table 1; SM3 (a-g)).

Presence of laccase enzyme

Enzyme assays are performed to analyze degradation pattern of complex compounds in microorganisms. Results presented in Table 2 indicated that both the fungal isolates produced laccase enzyme however the quantity of enzyme was low and ranged between 5.9 and 7.7 U mL^{-1} while consortium showed 9.09 U mL^{-1} after 6 days of incubation. An abrupt increase (7 and 6 times) in enzyme activity was observed by C1 and C3 respectively within initial 3 to 6 days, but after 10th day of growth, five times decrease in enzyme activity was observed in both the cultures while consortium showed increase in enzyme activity 10.29 U mL^{-1} after 10 days of incubation.

Immobilized and free fungal enzyme-based degradation

Immobilized beads of laccase enzyme from C1 and C3 and mixture of enzymes from both the fungal isolates were used separately to degrade chlorpyrifos. In control (only beads with no enzyme) degradation of chlorpyrifos was 2 and 3.3 %

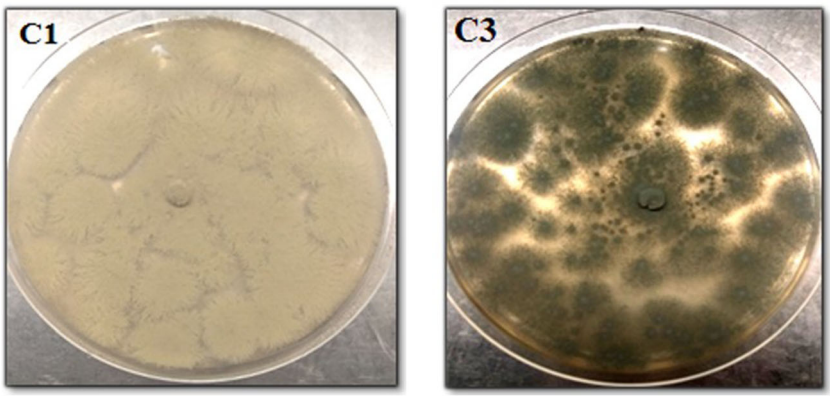

Fig. 1 Growth of fungal isolates on Potato Dextrose Agar C1. *Byssochlamys spectabilis* C3. *Aspergillus fumigates*

respectively on 5th and 10th day of incubation. Chlorpyrifos degradation was higher in the presence of immobilized fungal enzyme. Immobilized beads of C1 + C3 enzyme showed 61.55 and 63.75 % degradation of chlorpyrifos on fifth and tenth day respectively (Table 3). Treatments containing beads of individual enzyme(s) of C1 and C3 showed 58.25 and 53.85 % degradation respectively in comparison to control which showed only 1.5 % degradation of chlorpyrifos after five days (Fig. 3b). A reduction in the half-life of chlorpyrifos (from 346.5 days to 46.2 days) was observed in the presence of mixture of enzymes of C1 + C3 where as it was 53.3 and 63 days under the treatment of individual enzymes of C1 and C3 respectively.

In another experiment, free enzyme (1ml supernatant) was added in the flask containing 50 mg L^{-1} chlorpyrifos in 50 ml minimal medium. Control (no enzyme/beads) showed 1.5 and 2.2 % degradation of chlorpyrifos on 5th and 10th days respectively. On fifth day free enzyme of C1 + C3 showed 45.1 % degradation of chlorpyrifos but it was 39.4 and 37.41 % when treated with free enzymes of C1 and C3 respectively. Half-life in the presence of free enzyme also reduced to 77 days. Free enzyme in the medium does not maintain its stability so its activity was lost vigorously in comparison to immobilized beads. We observed higher degradation ability of immobilized enzyme with sodium alginate beads than the free

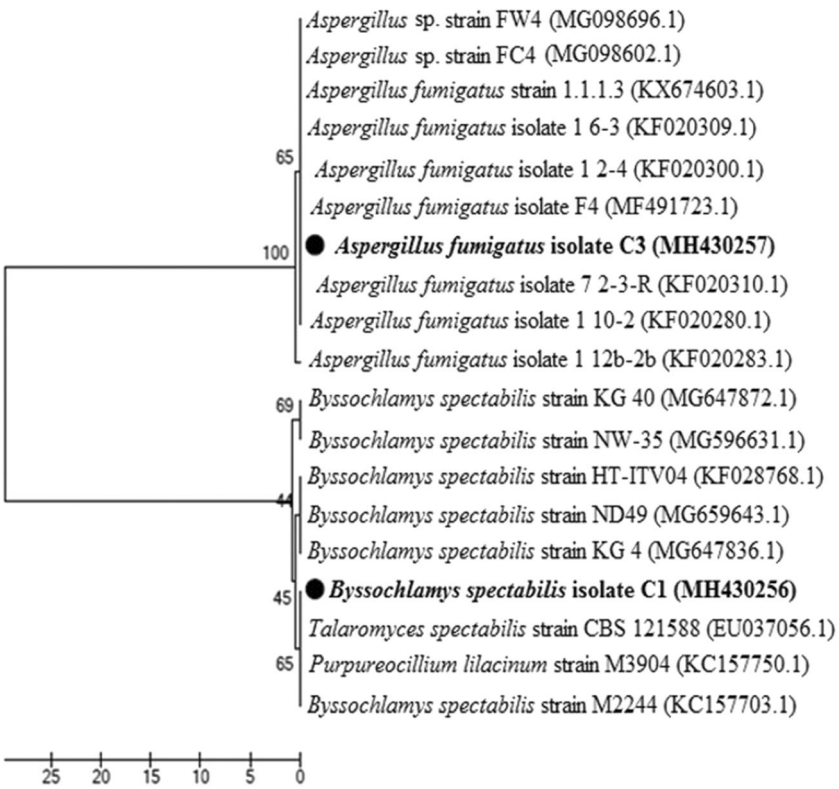

Fig. 2 Phylogenetic tree construction of C1 and C3 fungal isolates

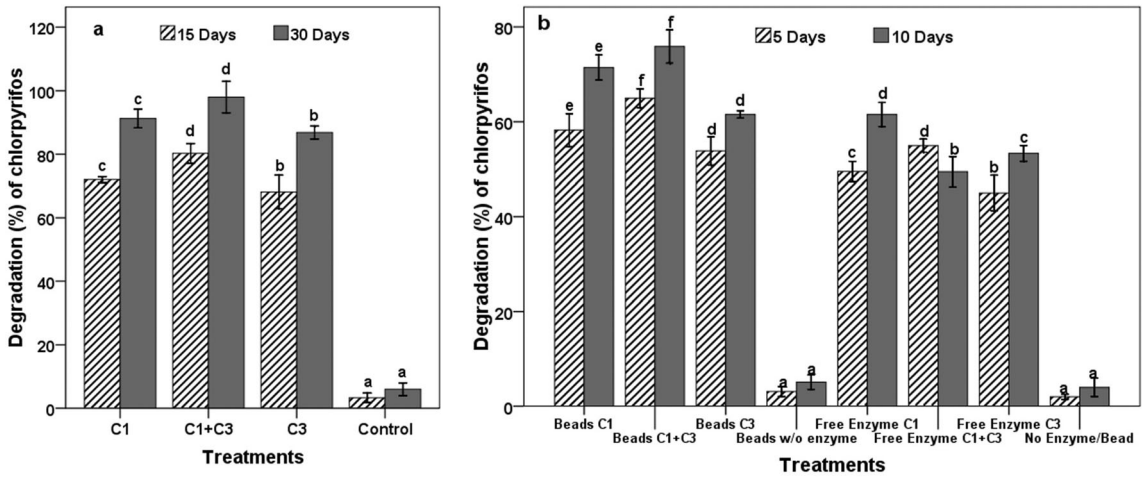

Fig. 3 Percent degradation of chlorpyrifos (a) by fungal isolates and consortium (b) by immobilized beads and free enzyme. Bars followed by different letters mean significant differences *(P < 0.05)* among the treatments

enzyme. These results indicated that the immobilized enzyme had better affinity with chlorpyrifos molecule.

Phytoremediation of chlorpyrifos

Effect of fungal cultures, inoculated individually (C1/ C3) or as consortium (C1 + C3) in soil was observed on chlorpyrifos biodegradation using onion plants in a pot experiment. Highest plant height (22.8 cm) was observed in consortium treatment in unsterile soil, spiked with chlorpyrifos (50 mg Kg^{-1}) after 30 days. Performance of single isolate was less as compared to consortium as plant height in the presence of C1 and C3 was 21.0 and 20.5 cm respectively after 30 days. In control, average plant height was only 19 cm as compared to the plants treated with fungal isolates. In chlorpyrifos treated sterile soil, consortium performed best and showed plant height of 21 cm. In the presence of single fungal isolate (C1 and C3) average plant height was 20.0 and 19.9 cm respectively which was greater than the control (18.3 cm). Plant height was higher in all the treatments under unsterile soil. Consortium always performed better in comparison to individual fungal isolates on plant height parameter under sterile or unsterile condition (Table 4). Both the fungal treatments performed significantly better in relation to plant height and removal of chlorpyrifos in comparison to control in sterile or unsterile soil. Our results showed that the maximum chlorpyrifos (1.318 μg Kg^{-1}) was up taken by control plants grown in sterile soil. Minimum residue (2.637 μg Kg^{-1}) of chlorpyrifos was found in unsterile soil in the presence of fungal consortium. Half-life of chlorpyrifos in the soil inoculated with fungal consortium was 13.32 days (Table 5).

Discussion

Chlorpyrifos is one among the widely used organophosphate pesticides to control the pests of vegetables and cotton crops. Increased usage of this insecticide has adversely and severely affected the environment (Smegal 2000). Hence reclamation of highly contaminated arable land with insecticide is the need of hour. Physical and chemical methods are being used to decontaminate various sites globally but these methods are expensive and may contaminate the same sites again with other chemicals. In contrast to these methods, microbial methods are cheap, less expensive and sustainable ecologically and economically. Earlier studies of microbial remediation of chlorpyrifos have shown that fungi are most promising bioagents because of production of extracellular enzymes which

Table 1 Degradation kinetics of chlorpyrifos and half-life in presence and absence of fungal isolates in Czapek Dox medium (CDM)

Treatments	Regression equation	k (day^{-1})	$R2$	$t_{1/2}$ (day)
CDM+chlorpyrifos	$\ln(Ct/C_0) = -0.0025x+1.998$	0.003 ± 0.001^{a}	0.964 ± 0.005^{c}	231.00 ± 1.73^{c}
CDM+chlorpyrifos+C1	$\ln(Ct/C_0) = -0.049x+1.878$	0.049 ± 0.014^{b}	0.945 ± 0.003^{b}	14.10 ± 1.36^{a}
CDM+chlorpyrifos+C3	$\ln(Ct/C_0) = -0.039x+1.858$	0.039 ± 0.003^{b}	0.885 ± 0.001^{a}	17.70 ± 1.35^{b}
CDM+chlorpyrifos+C1+C3	$\ln(Ct/C_0) = -0.051x+1.823$	0.051 ± 0.002^{b}	0.887 ± 0.002^{a}	13.58 ± 1.00^{a}

Values in each column followed by the same letter were not significantly different ($P < 0.05$)

Table 2 Laccase activity at different time intervals

Fungal Cultures	Enzyme activity (U mL^{-1})		
	Day 3	Day 6	Day 10
C1	0.96±0.26[a]	7.77±1.10[a]	2.23±0.55[a]
C3	0.92±0.21[a]	5.92±1.05[a]	1.74±0.90[a]
C1+C3	4.39±1.02[b]	9.09±2.61[b]	10.29±1.52[b]

Values in each column followed by the same letter were not significantly different ($P < 0.05$)

can attack different recalcitrant/xenobiotic compounds and support survival of fungi by providing them essential nutrients (Chandra 2019). In this study, eleven chlorpyrifos utilizing/tolerant fungal isolates were recovered from the waste material of a refrigeration industry. Two fungal isolates (C1 and C3) showed their survival at 600 mg L^{-1} chlorpyrifos supplemented in Czapek Dox and also indicated biodegradation of chlorpyrifos as both the fungi were able to grow in the chlorpyrifos supplemented medium devoid of phosphate and carbon sources. Growth pattern of the individual microorganism indicates the dominance of particular fungal isolate on a particular substrate. Microorganisms like bacteria and fungi were capable to degrade chlorpyrifos by utilizing it as a source of carbon, nitrogen and phosphorus (Singh et al. 2004; Awad et al. 2011). Rapidly growing species of bacteria and fungi have extra benefit over slow growing species as they follow fast growth pattern and utilize the available resource faster (Marin et al. 1998). Improved growth of a fungus and its mycelium could be antagonistic to indigenous microorganisms of the soil (Singleton 2001). Chlorpyrifos utilization/degradation by a fungal strain of *Acremonium* sp. and by mixed fungal cultures was reported by Kulshrestha and Kumari (2011). Hussaini et al. (2013) reported four fungal isolates *Aspergillus niger, Ganoderma austral, Trichospora, Verticillium dahaliae* with biodegradation properties of Chlorpyrifos, Endosulfan, Malathion and Lindane. Organophosphorus insectiside degradation and finding of different metabolite recently proposed by using *Aspergillus sydowii* CBMAI 935 and its role in biotransformation reaction (Soares et al. 2021).

In the present study two fungal strains (C1 and C3) were characterized as *Byssochlamys spectabilis* and *Aspergillus fumigates* respectively on the basis of BLAST analysis of ITS region of 18S rDNA sequences. According to Schoch et al. (2012), ITS sequences constitute a conserved region in (ITS1) 18S and show highest likelihood of successful characterization and identification of a wide range of fungi. Present isolates showed 80–95 % homology with respective fungi in phylogenetic tree. *Byssochlamys spectabilis* is a heat-resistant fungus (Houbraken et al. 2010).

Biodegradation of chlorpyrifos was tested on 15th and 30th day in modified Czapek Dox medium using both the fungal isolates. Maximum degradation of chlorpyrifos was observed in consortium treatment because of the cometabolism, where combination of enzymes produced by individual isolate(s) may be resposible for faster and better biodegradation. This might be due to their prolific growth during initial incubation period of 15 days and/or they carry out a relatively more efficient enzymatic system responsible for biodegradation. Higher biodegradation in 15 to 30 days could be most likely due to induction /activation of particular enzymes in the culture. A minimum degradation of chlorpyrifos in control could be related to autolysis, photodegradation and hydrolysis. Ishag et al. (2016) studied biodegradation of chlorpyrifos, dimethoate and malathion using bacterial consortium consisting of *Bacillus safensis* and *Bacillus cereus* and *Bacillus subtilis*. They observed highest percent degradation of chlorpyrifos. Peter et al. (2015) reported 98.4 % biodegradation of malathion (400 mg L^{-1}) by *Fusarium oxysporum* strain JASA 1 in minimal medium. Fungi have great potential for the biodegradation of pesticide like difenoconazole and pendimethalin, terbuthylazine (Pinto et al. 2012; Gao et al. 2012) studied complete metabolism of chlorpyrifos (50 mg L^{-1}) into TCP (a major metabolite of chlorpyrifos) within 5 days by *Cladosporium cladosporioides* Hu 01.

Table 3 Degradation kinetics of chlorpyrifos and its half-life in Mineral Salt Medium (MSM) by immobilized and free fungal enzyme

Treatments	Regression equation	k (day^{-1})	$R2$	$t_{1/2}$ (day)
MSM (w/o enzyme & beads)	$\ln(Ct/C_0)$ = -0.0025x+1.998	0.002±0.001[a]	0.996±0.003[d]	346.5±0.23[g]
MSM+Chlorpyrifos+Beads C1	$\ln(Ct/C_0)$ = -0.013x+1.941	0.013±0.001[d]	0.804±0.005[c]	53.30±1.04[b]
MSM+Chlorpyrifos+Beads C3	$\ln(Ct/C_0)$ = -0.011x+1.947	0.011±0.002[c]	0.795±0.006[a]	63.00±1.12[c]
MSM+Chlorpyrifos+Beads C1+C3	$\ln(Ct/C_0)$ = -0.015x+1.935	0.015±0.001[e]	0.799±0.004[a]	46.20±1.66[a]
MSM+Chlorpyrifos+Free enzyme C1	$\ln(Ct/C_0)$ = -0.008x+1.968	0.008±0.003[b]	0.828±0.003[b]	86.62±1.36[e]
MSM+Chlorpyrifos+Free enzyme C3	$\ln(Ct/C_0)$ = -0.007x+1.971	0.007±0.002[b]	0.849±0.005[a]	99.00±0.73[f]
MSM+Chlorpyrifos+Free enzyme C1+C3	$\ln(Ct/C_0)$ = -0.009x+1.959	0.009±0.002[b]	0.798±0.006[a]	77.00±0.25[d]

Values in each column followed by the same letter were not significantly different ($P < 0.05$)

Table 4 Plant growth parameters and residual analysis of chlorpyrifos in onion plants and rhizosphere soil after 30 days of fungal treatment

Treatments	Plant height (cm)		Pesticide uptake by onion plants (μg Kg^{-1})		Pesticide residue in soil (μg Kg^{-1})	
	Sterile soil	Unsterile soil	Sterile soil	Unsterile soil	Sterile soil	Unsterile soil
Control	18.30±0.56^{a}	18.53±0.41^{a}	1.31±0.35^{b}	1.207±0.30^{b}	14.28±2.23^{c}	11.64±1.90^{c}
C1	20.00±1.17^{b}	20.53±0.90^{b}	0.30±0.18^{a}	0.109±0.11^{a}	8.57±0.64^{b}	4.39±1.32^{b}
C3	19.90±1.05^{ab}	21.03±0.49^{b}	0.19±0.10^{a}	0.064±0.15^{a}	9.89±1.10^{b}	5.71±0.36^{b}
C1+C3	21.00±0.49^{b}	22.83±1.00^{c}	0.26±0.30^{a}	0.013±0.10^{a}	4.39±1.32^{a}	2.63±0.64^{a}

Values in each column followed by the same letter were not significantly different ($P < 0.05$)

Laccase, a polyphenol oxidase enzyme can break complex aromatic compounds and the presence of this enzyme is reported in different microorganisms showing the property of pesticide biodegradation. Enzyme assays are performed to analyze the degradation of complex compounds in microorganisms. Amount of laccase produced by the test fungal isolates in the present study was more in consortium as compared to single fungal isolates which show the potential of pesticide degradation or dye decolorization. Stoilova et al. (2010) isolated two potent dye decolorizing fungi that produced 1600–2000 U mL^{-1} of laccase enzyme. Studies on bacterial laccases were also performed by various researchers. Production of laccase was reported in bacteria namely *Bacillus halodurans, Azospirillum lipoferum, Pseudomonas desmolyticum*, *Bacillus pumilus, Bacillus subtilis* and *Pseudomonas putida* (Muthukumarasamy and Murugan 2014; Gangola et al. 2018) reported production of esterase and laccase by *Bacillus subtilis* and confirmed their production by amplification of respective genes by PCR. These enzymes facilitate biodegradation and detoxification of pesticides (Bhatt et al. 2019). Diamantidis et al. (2000) isolated *Azospirillum lipoferum* from rice rhizosphere which showed 124 U mL^{-1} laccase activity.

Biodegradation of chlorpyrifos by immobilized beads using supernatant of both the fungal isolates (C1 and C3) showed maximum degradation (61.55 %) of chlorpyrifos because of the enzyme activity of both the fungi. It is presumed that combination of enzymes may be resposible for better biodegradation (6 times) of the pesticide than the control. It can be concluded from the results that the activity of enzyme was maximum up to 10th day as chlorpyrifos concentration gradually decreased which might have affected degradation rate. It is also proposed that the activity and stability of enzymes play key role in degradation of complex compounds. Fang et al. (2021) used immobilized technique for bioremediation of organophosphorus insecticides in industrial wastewater applying fungus *Cupriavidus nantongensis* X1T. Xie et al. (2010) reported chlorpyrifos biodegradation by immobilized enzyme from *Fusarium* strain WZ-1.

Phytoremediation experiment was conducted on onion (*Allium cepa*) to analyze the efficiency of selected fungal isolates to remove of chlorpyrifos (50 mg Kg^{-1}) from the soil under natural conditions in sterile and unsterile soil. Plant growth and chlorpyrifos residue in onion plants and rhizospheric soil were tested after 30 days. Plant height was maximum in consortium treatment. All the treatments performed better under unsterile condition. Fungal consortium always performed better on plant height parameter under sterile or unsterile condition in comparison to individual fungal treatments. Negi et al. (2014) reported residual amount of carbendazim (1.33 and 3.33 mg Kg^{-1}), α-endosulfan (2.43 and 7.23 mg Kg^{-1}) and β-endosulfan (3.10 and 8.83 mg Kg^{-1}), imidacloprid (4.5 and 9.43 mg Kg^{-1}) in the soil after 30th day of a pot experiment in the presence of bacterial consortium where initial concentration of pesticides was 50 and 100 mg Kg^{-1} soil. Zabermawi and Bestawy (2017) reported presence of residual Atrazine (0.232 and 0.14 mg Kg^{-1}) after 25 and 50 days respectively as initial concentration was applied in outdoor (field) condition was 750 g/600 L water. Ong et al. (2019) observed > 80 % degradation of chlorpyrifos and cypermethrin by *Metarhizium anisopliae* if provided @ 500 mg Kg^{-1} after 21 days in soil. After treatment with *Metarhizium anisopliae* the residual concentrations of chlorpyrifos and cypermethrin were 19.39 and

Table 5 Degradation kinetics and half-life of chlorpyrifos in soil

Treatment	Regression equation	k (day^{-1})	$R2$	$t_{1/2}$ (day)
Control	$\ln(Ct/C_0)$ = -0.0025x+1.998	0.006±0.002^{a}	0.949±0.15^{c}	693.00±1.00^{d}
C1	$\ln(Ct/C_0)$= -0.045x+1.870	0.045±0.005^{b}	0.901±0.04^{b}	15.40±1.24^{b}
C3	$\ln(Ct/C_0)$= -0.041x+1.872	0.041±0.003^{b}	0.887±0.24^{b}	16.90±0.36^{c}
C1+C3	$\ln(Ct/C_0)$= -0.052x+1.810	0.052±0.001^{c}	0.852±0.12^{a}	13.32±0.45^{a}

Values in each column followed by the same letter were not significantly different ($P < 0.05$)

19.68 mg Kg^{-1} in soil respectively. Our results showed that chlorpyrifos was completely mineralized into low molecular weight compounds in CDM, MSM and soil when treated with fungal isolates and/or with their enzyme(s).

Conclusions

The fungal isolates (C1 and C3) recovered from the waste of a refrigeration industry were capable to grow on chlorpyrifos supplemented @ 600 mg L^{-1} in Czapek Dox agar. They efficiently degraded chlorpyrifos 20 mg L^{-1} under *in vitro* conditions (liquid medium). Both the isolates produced extracellular laccase enzyme which helped in biodegradation of the pesticide. Immobilized enzymes and/or fungal isolates were more effective in biodegradation of chlorpyrifos in comparison to free enzyme or cultures. Consortium of the fungal cultures and their enzymes showed best results as 61.55 % of chlorpyrifos (50 mg L^{-1}) was degraded by C1 + C3 enzyme in 5 days. *In situ* pot experiment on onion with chlorpyrifos used @ 50 mg Kg^{-1} of soil showed minimal residue of the pesticide with consortium. HPLC analysis revealed the conversion of chlorpyrifos into different metabolites. This is the first study on chlorpyrifos biodegradation using *Byssochlamys spectabilis* which is a most ubiquitous fungus having high potential for biodegradation. *Aspergillus fumigates* is also a potent fungus and used in various bioremediation studies. Consortium of both the fungal strains can be used in degradation of toxic chemicals from different sites. In future, further study can be done by using *Byssochlamys spectabilis* on large scale using different concentrations of the pesticide and crops at different locations.

Abbreviations CDM, Czapek Dox Medium; MSM, Mineral salt medium; $t_{1/2}$, Half life; ITS, Internal transcribed spacer; PCR, Polymerase chain reaction; $FeSO_4$, Ferrous sulfate; K_2SO_4, Potassium sulfate; E.A, Enzyme activity; HPLC, High Performance Liquid Chromatography; ANOVA, Analysis of Variance; BLAST, Basic Local Alignment Search Tool

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Author contributions Ashish Kumar wrote the manuscript; Anita Sharma designed the experiments, provided experimental materials and guidance; Parul Chaudhary help in data analysis and editing of the manuscript; Saurabh Gangola conducted the experiment. All the authors read and approved the manuscript.

Declarations

Conflicts of interest Authors declare no conflict of interest.

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