Isolation and Characterization of Novel Chlorpyrifos Degrading Fungus *Isaria Farinosa*

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Abstract: Chlorpyrifos is a well known organophosphorus pesticide used worldwide. Microorganisms including bacteria, fungi and actinomycetes have been reported to be efficient degraders of chlorpyrifos. The present study was successful in isolating a novel fungus that could degrade chlorpyrifos effectively upto 800 ppm concentration. Morphological and molecular characterization studies revealed the identity of the fungus as *Isaria farinosa*.

Key words: Chlorpyrifos, bioremediation, fungus, concentrations, intervals, degradation.

1. Introduction

Chlorpyrifos is one of the dominated broad spectrum organophosphorus insecticides inhibiting the neuron function of sucking, chewing and boring insects both in crop and soil [1]. It is reported to have a moderately high persistence in soil. The wide use of these pesticides especially chlorpyrifos over the years have resulted in environmental pollution, human and animal health hazards [2]. Considering the inherent toxic nature of these pesticides it is essential to remove them from the environment employing suitable remedial measures. Bioremediation exploiting microbial technology is one of the recent techniques for environmental clean-up. In the process, heterotrophic microorganisms break down hazardous compounds to obtain carbon and energy [3]. Generally bacteria are reported to be efficient degraders of chlorpyrifos. The first bacteria, that could degrade organophosphorus compounds was isolated in 1973 [4]

and identified as *Flavobacterium sp.* Since then several bacteria and a few fungal species have been isolated which can degrade a wide range of organophosphorus compounds in liquid cultures and soil systems. There are also many reports of chlorpyrifos degradation by fungal species. Fungi like *Trichoderma*, *Phanerochaete*, *Aspergillus*, *Penicillium* [5], *Phanerochaete chrysosporium* [6] *Hypholoma fasciculare* and *Coriolus versicolor* [7] are some of the examples of fungal degraders of chlorpyrifos. The present study could identify the fungi *Isaria farinosa* as an efficient degrader of the pesticide chlorpyrifos.

2. Experimental Sections

Soil samples were collected from cardamom plantations of Idukki district where chlorpyrifos drenching was regularly practiced by the farmers. Microorganisms capable of degrading chlorpyrifos were isolated from these samples by enrichment culture technique. For the purpose soil samples collected was enriched continuously with 100 ppm

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chlorpyrifos for a period of 8 to 10 weeks. After enrichment the soil was mixed uniformly and one gram of enriched soil was added to mineral salts medium [3, 8], supplemented with 100 ppm chlorpyrifos and incubated at room temperature. After one week, 1 mL from the above medium was transferred to fresh MS (mineral salts) medium containing chlorpyrifos and this was repeated 2-3 times for further purification. Finally 1 mL was plated on MS media containing 100 ppm chlorpyrifos. A fungal isolate (M5) was obtained which was identified in the later part of the study as *Isaria farinosa*. The growth of the isolate (M5) and degradation of chlorpyrifos in different concentrations of chlorpyrifos—100, 200, 400 and 800 ppm was evaluated. Significant growth and degradation of the isolate was recorded. An important observation was that mycelial mat production was significantly reduced but the isolate could produce significant colony count in MSM (mineral salts medium) medium.

3. Results and Discussion

3.1 Results

On 7th day of inoculation, in 100 ppm chlorpyrifos *Isaria farinosa* recorded a population build up of 72.50×10^3 cfu·mL⁻¹ and this increased to 100×10^3 cfu·mL⁻¹ and 112.50×10^3 cfu·mL⁻¹ after 15 and 20 days after inoculation, respectively. Later the population slightly decreased to 110×10^3 cfu·mL⁻¹ and 72.50×10^3 cfu·mL⁻¹ during 25th and 30th day after inoculation respectively.

In 200 ppm concentration the fungus—*Isaria farinosa* showed a population build up of 112.50×10^3 cfu·mL⁻¹, 113.50×10^3 cfu·mL⁻¹ and 122.50×10^3 cfu·mL⁻¹ after 7, 15 and 20 days after inoculation respectively. During the 25th and 30th day, *Isaria farinosa* showed slight decrease in population build up which recorded 119×10^3 cfu·mL⁻¹ and 70×10^3 cfu·mL⁻¹, respectively.

On 7th day of inoculation, in 400 ppm chlorpyrifos,

Isaria farinosa recorded a population build up of 128.50×10^3 cfu·mL⁻¹ and this increased to 130×10^3 cfu·mL⁻¹ and 146×10^3 cfu·mL⁻¹ on 15th and 20th day after inoculation, respectively. On 25th day, *Isaria farinosa* recorded a population of 144×10^3 cfu·mL⁻¹ and the population declined to 77.50×10^3 cfu·mL⁻¹ on 30th day after inoculation.

The population was comparatively less in 800 ppm concentration of chlorpyrifos and it recorded 36×10^3 cfu·mL⁻¹, 41.50×10^3 cfu·mL⁻¹, 50×10^3 cfu·mL⁻¹, 48.50×10^3 cfu·mL⁻¹ and 49.50×10^3 cfu·mL⁻¹ after 7, 15, 20, 25 and 30 days after inoculation, respectively (shown in Table 1).

The degradation potential of *Isaria farinosa* was assessed by analyzing the residue of chlorpyrifos in the medium in GC-MS (gas chromatography-mass spectrometry). After 7 days of incubation the isolate *Isaria farinosa* showed 53.7% degradation of 100 ppm chlorpyrifos and this increased to 59.7, 59.5, 62.4 and 64.2 percent on 15th, 20th, 25th and 30 days after inoculation respectively (Table 2). Similarly the isolate could degrade 200 ppm chlorpyrifos which recorded 34.3, 46.3, 58.4, 73.6 and 77.3 percent on 7, 15, 20, 25 and 30 day after inoculation, respectively. In 400 ppm chlorpyrifos *Isaria farinosa* showed 62.4, 62.9, 62.8, 63.5 and 80.8 percent reduction of residue of chlorpyrifos on 7th, 15th, 20th, 25th and 30th days after inoculation respectively. The fungus was also efficient in degrading 800 ppm concentration of chlorpyrifos and recorded 60.4, 61.2, 61.5, 61.3 and 61.0 percent reduction of chlorpyrifos under in vitro conditions (Table 2).

The fungal isolate was grown on plates containing mineral salts medium for 6 days for studying the colony morphology. The fungus has white flat cottony growth. The upper side of colony appeared pure white and lower side showed pale colour in MSM. Lemon shaped transparent conidia are another characteristic feature of this fungi.

Total genomic DNA was isolated and the 18 SrRNA gene was amplified by PCR (polymerase

Table 1 The population buildup of *Isaria farinosa* in different concentrations of chlorpyrifos in MSM.

Concentrations (ppm)	Viable count (cfu $\times 10^3$ cfu·mL ⁻¹)				
	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
100	72.50	100.0	112.50	110.00	72.50
200	112.50	113.50	122.50	119.00	70.00
400	128.50	130.00	146.00	144.00	77.50
800	36.00	41.50	50.00	48.50	49.50

DAI: days after inoculation.

Table 2 Degradation of chlorpyrifos in MSM by *Isaria farinosa* at different intervals.

Concentrations (ppm)	7th DAI		15th DAI		20th DAI		25th DAI		30th DAI	
	Residue (ppm)	% Reduction	Residue (ppm)	% Reduction	Residue (ppm)	% Reduction	Residue (ppm)	% Reduction	Residue (ppm)	% Reduction
100	42.3	53.7	37.1	59.7	37.05	59.5	34.05	62.4	32.1	64.2
200	130	34.3	104.0	46.3	79.35	58.4	50.20	73.6	43.0	77.3
400	137.1	62.4	135.2	62.9	135.1	62.8	132.1	63.5	69.3	80.8
800	293.1	60.4	287.3	61.2	284.3	61.5	282.3	61.3	279.0	61.0

Table 3 Residue of chlorpyrifos (ppm) in control at different intervals.

Concentrations (ppm)	7th DAI	15th DAI	20th DAI	25th DAI	30th DAI
	Residue of chlorpyrifos (ppm)	Residue of chlorpyrifos (ppm)	Residue of chlorpyrifos (ppm)	Residue of chlorpyrifos (ppm)	Residue of chlorpyrifos (ppm)
100	93.000	92.400	91.700	90.800	90.100
200	198.00	194.00	192.00	191.00	190.00
400	365.00	364.00	363.00	362.00	361.00
800	741.40	740.20	737.40	729.60	724.30

chain reaction) with the universal primers NS1 (GTAGTCATATGCTTGTCTC) and NS4 (CTTCCGTCAATTCTTTAAG).

Molecular characterization the fungi showed similarity towards *Isaria farinosa* (Fig. 1). The amplification of genomic DNA of fungi showed the amplicons of size between 1 KB and 1.5 KB were observed. The rDNA regions of isolate were sequenced by using the Big-Dye Terminator v3.1 Cycle Sequencing Kit. The quality was checked using Sequence scanner software v1 (Applied Biosystems). Sequence alignment and editing of the obtained sequence were carried out using Geneious Pro v5.1. Sequencing of the isolate was done using universal primers of rDNA (18 SrRNA). The sequences obtained from isolate were as follows:

>M5-18S

TTATACAGCGAACTGCGAATGGCTCATTA

TATAAGTTATCGTTTATTTGATAGTACCTTACT
 ACTTGGATAACCGTGGTAATTCTAGAGCTAAT
 ACATGCTGAAAATCCCGACTTCGGAAGGGAG
 GTATTTATTAGATTAAAAACCAATGCCCTCTG
 GGCTCCTTGGTGATTCATGATAACTGTTTCGAA
 TCGCACGGCCTTGCGCCGGCGATGGTTCATTC
 AAATTTCTTCCCTATCAACTTTCGATGTTTGGG
 TATTGGCCAAACATGGTCGCAACGGGTAACG
 GAGGGTTAGGGCTCGACCCCGGAGAAGGAGC
 CTGAGAAACGGCTACTACATCCAAGGAAGGC
 AGCAGGCGCGCAAATTACCCAATCCCGATTC
 GGGGAGGTAGTGACAATAAATACTGATACAG
 GGCTCTTTTGGGTCTTGTAATTGGAATGAGTA
 CAATTTAAATCTCTTAACGAGGAACAATTGGA
 GGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT
 TCCAGCTCCAATAGCGTATATTAAAGTTGTTG
 TGGTTAAAAAGCTCGTAGTTGAACCTTGGGCC
 TGGCTGGCCGGTCCGCCTCACCGCGTGTACTG

Fig. 1 Growth (a) and microscopic view (b) of *Isaria farinosa*.

GTCCGGCCGGGCCTTTCCCTCTGTGGAACCTC
 ATGCCCTTCACTGGGTGTGGCGGGGAAACAG
 GACTTTTACTTTGAAAAAATTAGAGTGCTCCA
 GGCAGGCCTATGCTCGAATACATTAGCATGG
 AATAATAAAATAGGACGCGTGGTTCTATTTTG
 TTGGTTTCTAGGACCGCCGTAATGATTAATAG
 GGACAGTCGGGGGCATCAGTATTCAATTGTCA
 GAGGTGAAATTCTTAGATTTATTGAAGACTAA
 CTACTGCGAAAGCATTTGCCAAGGATGTTTTT
 ATTAATCAGGAACGAAAGTTAGGGGATCGAA
 GACGATCAGATACCGTCGTAGTCTTAACCATA
 AACTATGCCGACTAGGGATCGGACGATGTTAT
 TTTTGTACGCGTTCGGCACCTTACGAGAAATC
 AAAGTGCTT

The sequences obtained were analyzed using bioinformatics tool viz. BLASTN and tBLASTx i.e., nucleotide query is used to search in amino acid database. The fungi showed 100% similarity towards

Isaria farinosa with 1,023 bp and query sequence of 101 blast hits.

3.2 Discussion

An increase in growth with increase in concentration of chlorpyrifos was observed with *Isaria farinosa*. Even though the population buildup gradually increased with increase in concentration of chlorpyrifos up to 400 ppm, a decrease was observed at 800 ppm concentration. The population buildup of *Isaria farinosa* was less in 800 ppm concentration compared to lower concentrations. The growth of *Isaria farinosa* was significantly reduced in 800 ppm concentration of chlorpyrifos. A very high concentration usually leads to failure of biodegradation because certain microorganisms are not resistant to such high concentrations. The significant number of active microbial population depends on resistant level of microbial strain and also the chemical nature of material to be degraded [9]. Another significant observation was that even though there was significant colony count; the mycelial mat formation was comparatively less in chlorpyrifos amended medium which is often advantageous for development of liquid formulations. Similar results were obtained by Bhalariao and Puranik [10], who noticed inhibition of mycelial growth of *Aspergillus oryzae* in flask with increasing concentration of chlorpyrifos. Similarly, mycelial mat formation was found to be reduced in *Ganoderma sp.* [11] and in *Aspergillus terreus* [12]. The isolate *Isaria farinosa* gave significant reduction of chlorpyrifos residue of 64.2 percent on 30 DAI in 100 ppm concentration. A similar trend was also observed in 200, 400 and 800 ppm concentrations. Experiments conducted by Jones and Hastings [5], who could obtain 95 to 98 percent degradation of 50 ppm chlorpyrifos by a group of forest fungi namely *Trichoderma harzianum*, *Penicillium vermiculatum* and *Mucor sp.* after 28 days of incubation. Even though growth and degradation of chlorpyrifos by *Isaria farinosa* was comparatively less in 800 ppm

concentrations about 60.4 percent degradation was observed on 7th day after inoculation and this trend continued even up to 30 days after inoculation which recorded 61.0 percent. In 800 ppm, maximum degradation was observed in 20-30 days. Hua [13] *et al.*, also obtained similar results wherein maximum degradation of chlorpyrifos was observed on 35th day after inoculation. There are many earlier reports of other degrading chlorpyrifos but the degradation efficiency of *Isaria farinosa* has not been reported earlier.

4. Conclusions

The present study vividly established the efficiency of the novel fungi *Isaria farinosa* to degrade chlorpyrifos even up to 800 ppm concentration.

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