

Diversity of Chlorpyrifos-Degrading Fungi Isolated from Chlorpyrifos-Contaminated Agricultural Soil

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ABSTRACT

Chlorpyrifos is among the most extensively used organophosphorus pesticides in agriculture to control crop pests, reduce damage to insects, control mosquitoes, and reduce household pests. The extensive usage of chlorpyrifos has resulted in widespread contamination of the natural environment, affecting non-target organisms and causing potential environmental jeopardy. As for the toxicity of chlorpyrifos in the environment, the exploration of chlorpyrifos-contaminated soil fungus to remediate the contaminated area is of immense importance. This study isolated and identified fungi from chlorpyrifos-contaminated soil (6° 32' 30.5"N, 100° 15' 39.3"E) in pursuing our search to find a chlorpyrifos bioremediation agent. Five chlorpyrifos-surviving fungi were successfully isolated from the chlorpyrifos-contaminated soil and identified as *Fusarium oxysporum*, *Teleromyces verruculosus*, *Albifimbria terrestris*, *Aspergillus terreus* and *Purpureocillium lilacinum*. Their identification had been proven by molecular analysis based on 18S rRNA sequence homology and morphological characteristics. The identified strains had the potential to clean up chlorpyrifos pesticide-contaminated environment.

INTRODUCTION

Chlorpyrifos [O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphonothioate] is a globally widely used insecticide of chlorinated organophosphorus. It is a degradable compound. Thus, it has been used globally since its introduction in 1965 in the United States despite its high toxicity. Chlorpyrifos is a colourless to white crystalline solid [1] and is marketed as liquid, granular, pellets, wettable powder, dustable powder, and an emulsifiable concentrate.

It is promptly soluble in most organic solvents but has a very low water solubility (2 mg/L) [2]. Chlorpyrifos has a mild mercaptan odour, comparable to the smell of sulfur compounds in rotten eggs, garlic, and onions. Chlorpyrifos residues have been detected in freshwater and soil habitats, and due to their acute toxicity and persistence, they may cause serious human health problems [3]. According to the previous study, long environmental half-lives of chlorpyrifos can last up to four years, depending on the

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application rate, ecosystems, and pertinent habitats [4]. Microbes can degrade chlorpyrifos metabolically [5,6] or cometabolically [7]. The first indicates mineralization as a direct source of carbon and energy, while the second is the by-product metabolism in which the microbe transforms the compound with no advantages.

Numerous genera of bacteria can break down chlorpyrifos, including *Bacillus cereus* CP6, *Klebsiella pneumoniae* CP19 [8], and *Enterobacter* sp. SWLC2 [9], *Shewanella* BT05 [10], *Pseudomonas nitroreducens* AR-3 [11], *Pseudomonas putida* MB285 (12), *Ochrobactrum* sp. JAS2 (13), *Sphingobacterium* sp. JAS3 (14) and *Sphingomonas* sp. DSP2 (15). *Flavobacterium* sp. ATCC 27551, *Arthrobacter* sp. [16], and *Enterobacter* sp. [17] were reported to mineralize chlorpyrifos. A study has shown that the *Alcaligenes faecalis* strain could degrade chlorpyrifos and utilize it as the sole source of carbon and phosphorus [18]. Various types of bacteria, including *Sphingomonas*, *Stenotrophomonas*, *Bacillus* sp., *Brevundimonas* sp. and *Pseudomonas* sp., were capable of 100% degrading 100 mg/L of chlorpyrifos which were obtained from polluted water samples in China and enriched in mineral salts medium [19]. In 4 days, *Paracoccus* sp. isolated from an activated sludge sample and cultivated in TYC medium completely degrades 50 mg/L of chlorpyrifos and 3,5,6-trichloro-2-pyridinol [20]. Fungi are known to have the ability to form extended mycelial networks. Their catabolic enzymes' low specificity and independence in using organic chemicals as a growth substrate make fungi suitable for bioremediation. However, there is limited study regarding the degradation of chlorpyrifos by fungi.

Aspergillus sp. [21], *Trichoderma viride* [22], and *Fusarium* [23] were used in earlier studies of chlorpyrifos biodegradation with varying degrees of success. Besides that, *Aspergillus terreus*, *Phanerochaete chrysosporium*, and *Verticillium* sp. DSP has also been investigated regarding their ability to degrade chlorpyrifos [24,25,26]. In addition, 83.9% of chlorpyrifos was used as a source of C and N by *Acremonium* sp. [27]. This study aimed to identify the chlorpyrifos-degrading fungi from the agricultural soil using the enrichment culture medium. Besides, the diversity of soil fungi also was determined.

EXPERIMENTAL

Soil Sample

Soil samples were collected as the inoculum from Ladang Harum Manis Bukit Reban Jalan Kaki Bukit, Perlis (6° 32' 30.5"N, 100° 15' 39.3") that had been contaminated by chlorpyrifos for years in Perlis, Malaysia. Soil samples were obtained from 0 to 15 cm depth and air dried at room temperature, where all the debris was removed using a 2 mm mesh screen. All the samples were kept at 4°C for further studies.

Morphological Identification of Isolated Fungi

The fungi isolates were identified by assessing their macroscopic and microscopic characteristics. Macroscopic is based on the characteristics that are visible by the naked eye, such as the presence and absence of mycelium, pigment production, length, and growth, as well as the colour of the fungi. On the other hand, for microscopic observation, an adhesive tape was gently pressed onto the surface of the fungal colony to attach mycelia to the adhesive tape. The tape was then placed onto a glass slide containing a drop of methylene blue. The observation was performed using a CX12 light microscope under a total magnification of 1000.

DNA Extraction

The isolates were grown on Potato Dextrose Agar (PDA) plates for five days, and their morphology was investigated by light microscope. Colony morphology was observed on PDA plates incubated at 27°C at 1,2,3,4 and 5 days. The fungal isolates were used for genomic DNA extraction. The mycelia were scraped and collected, and genomic DNA was extracted according to the Fungi/Yeast Genomic DNA Isolation Kit protocol from Norgen Biotek Corporation.

Molecular identification of isolated fungi

The primer pairs ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the DNA sample. The 25 µL reaction mixture included 50 ng of DNA template, 1 U of Taq polymerase, 10 mM dNTPs, and 10 µM of each primer. Amplifications were carried out using a thermocycler under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 15 minutes. The PCR products were sent to the third party for sequencing, and the similarity between biological sequences was determined using the Basic Local Alignment Search Tool (BLAST). Based on results from the GenBank BLAST search and considering the average weighted infraspecific ITS variability in the kingdom fungi, which was calculated to be 2.51% with a standard deviation of 4.57%, it is reasonable to start with 80% query coverage and 97-100% sequence similarity for assigning a species name.

Phylogenetic tree

The phylogenetic analysis was performed with ITS gene sequences using the neighbour-joining method in MEGA 4 software (version 4.0) with 1000 bootstrap replicates. The resulting tree was trimmed to save space, and the closest relatives were retained.

RESULTS AND DISCUSSION

A total of five potential degraders have been successfully isolated from the soil and were grown on Potato Dextrose Agar (PDA). All the fungi were observed under macroscopic and microscopic observation and identified molecularly.

Molecular Identification of Isolated Fungi

The molecular identification method was performed using the Fungi/Yeast Genomic DNA Isolation Kit. The genomic DNA of selected chlorpyrifos-degrading fungi was first extracted, and the presence of the genomic DNA was confirmed by running the gel electrophoresis. The results of the extracted DNA on 1% agarose gel were visualized under UV light. The genomic DNA of isolated fungi was subjected to amplification by using the ITS1/ITS4 primers. The 18S rRNA genes of the ITS region were amplified by PCR to determine the efficacy and reliability of this technique. (Fig. 1). The bands that appeared on agarose gel were identified as *Fusarium oxysporum* strain SHBV2, *Talaromyces verruculosus* isolate ATT281, *Albifimbria terrestris* strain SCAU180, *Aspergillus terreus* strain LPSC 1180 and *Purpureocillium lilacinum* isolate HT003 after sequence alignment performed in GenBank database by using BLAST.

Fusarium oxysporum strain SHBV2

Fusarium oxysporum was observed to produce a white cottonish colony with the aerial mycelia becoming tinged in purple on Potato Dextrose Agar (PDA). Macroscopic and microscopic images shown in Fig. 2 were taken after five days of incubation. Large and complex conidia (macroconidia) and more simple conidia (microconidia) were observed under the microscope. The microconidia were usually non-septate and slightly curved. They can be seen accumulating around the conidiophores. Besides, macroconidia were large and were cumulative over the conidiophores. They can be seen having three to five septa. This description matched the morphological traits that had previously been published for *F. oxysporum* [28]. Besides, similar macroscopic and microscopic images of *F. oxysporum* were also reported [29]. *F. oxysporum* can be found in every type of soil and can be considered a normal constituent of fungal species in the rhizosphere of plants. Based on a study [30], all *F. oxysporum* strains are saprophytic and can withstand organic matter in soil and the rhizosphere of various plant species. Certain strains of *F. oxysporum* can penetrate the roots and invade the vascular system, but some cannot.

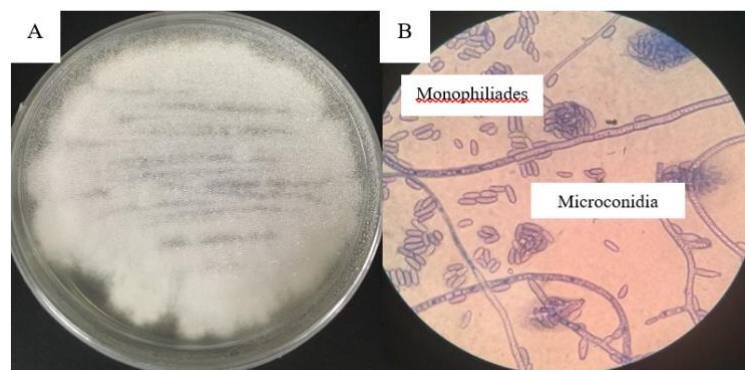


Fig. 1. Amplification of PCR of the ITS products obtained with ITS1/ITS4 primers. PCR products from several fungi are in 1% agarose gel and stained with Gel-Red. 1- 100bp ladder; 2- *Fusarium oxysporum* strain SHBV2; 3- *Talaromyces verruculosus* isolate ATT281; 4- : *Albifimbria terrestris* strain SCAU180; 5- *Aspergillus terreus* strain LPSC 1180; 6- *Purpureocillium lilacinum* isolate HT003.

***Talaromyces verruculosus* isolate ATT281**

Talaromyces verruculosus was observed as a white to greenish colony on Potato Dextrose Agar (PDA) (Fig. 3). It was considered a fast-growing fungus as the plate was fully covered after four days of incubation. Macroscopic and microscopic observations of this isolate after five days of incubation are shown in Figure 3. Globose to sub-globose in the shape of conidia was observed. The metulae and phialides were branched from the conidiophores. *Talaromyces verruculosus* is also known as *Penicillium verruculosum* [31]. Diverse strains of *T. verruculosus* were isolated primarily from the soils. For decades, *T. verruculosus* has been studied as a promising fungus for cellulase production and anti-microbial extrolites [32,33].

Fig. 2. *Fusarium oxysporum* strain SHBV2; 1000x magnification (A: Macroscopic view; B: Microscopic view)



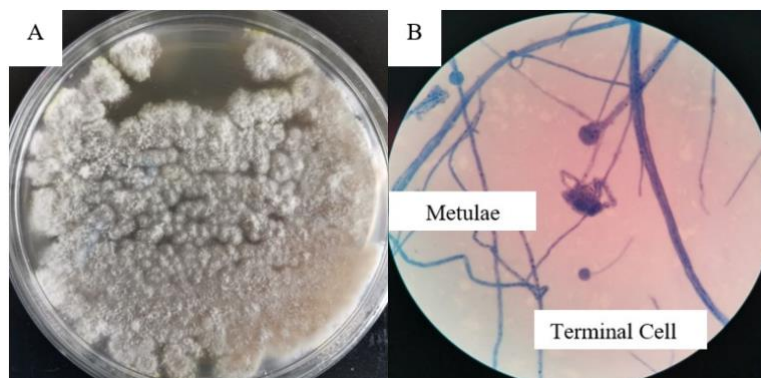


Fig. 3. *Teleromyces verruculosus* isolate ATT281; 1000x magnification (A: Macroscopic view; B: Microscopic view)

***Albifimbria terrestris* strain SCAU180**

A white cottonish colony of *Albifimbria terrestris* was seen on Potato Dextrose Agar (PDA) (Fig. 4). *A. terrestris* was a fast-growing isolate as it was fully covered on PDA after six days of incubation. Microscopic observation of this isolate showed the metulae and philiade attached to the conidiophore. The conidia were globose in shape. However, the impacts and functions of these isolates still need to be researched.

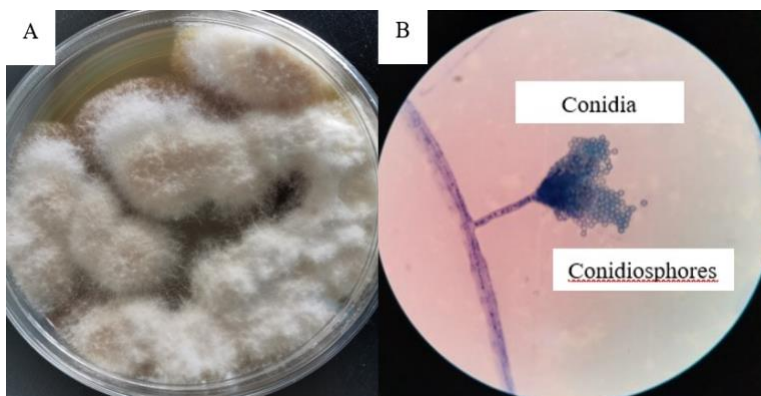


Fig. 4. *Albifimbria terrestris* strain SCAU180; 1000x magnification (A: Macroscopic view; B: Microscopic view)

***Aspergillus terreus* strain LPSC 1180**

Macroscopic observation of *Aspergillus terreus* showed bright orange-brownish colonies and appeared as various shades of cinnamon brown on Potato Dextrose Agar (PDA). Fig. 5 shows the colony formation after five days of incubation and is also considered a fast-growing isolate. Microscopically, the conidiophores attached to the foot cell were smooth, and the conidial head was compact. As shown in Figure 5, the metulae were as long as the philiade. *A. terreus* which belongs to the genus *Aspergillus*, is a natural soil saprophyte recovered from desert fields, grasslands, compost heaps, and stored contaminants of barley, peanuts, and corn [34]. *A. terreus* is a source of numerous bioactive substances with anticancer properties [35]. Lovastatin, an antihypercholesterolemic drug, was one of the secondary metabolites produced by *A. terreus* [36]. This species can cause infections, such as onychomycosis and invasive aspergillosis (IA), in severely immunocompromised hosts. Thus, *A. terreus* is clinically significant.

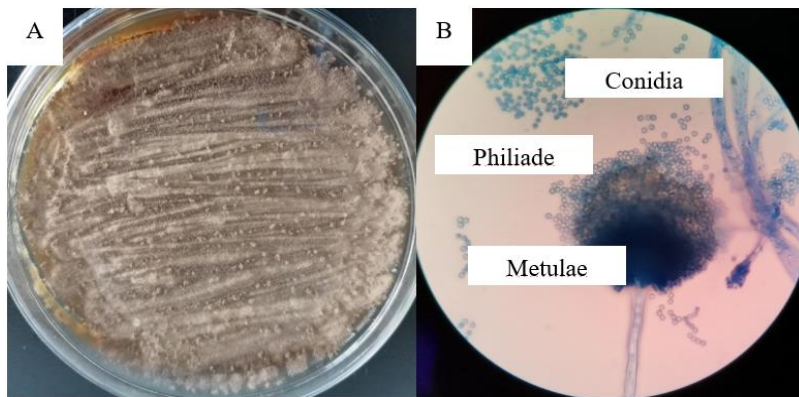


Fig. 5. *Aspergillus terreus* strain LPSC 1180; 1000x magnification. (A: Macroscopic view; B: Microscopic view)

***Purpureocillium lilacinum* isolate HT003**

Fig. 6 shows the image of the isolate captured after seven days of incubation on Potato Dextrose Agar (PDA). Thus, this verified that this isolate was a fast-growing fungus [37] and is known as *Purpureocillium lilacinum*. *P. lilacinum* was observed to have an irregular shape and a slightly white cottonish texture. No color changes in the isolate after 14 days of incubation. Figure 5 also shows the microscopic image of the incubated colony after three days of incubation. It was observed that a large vesicle was covered by metulae and phialide was attached to the metulae on the conidiophore. Conidia was seen in globose-ellipsoidal to fusiform in shape. *P. lilacinum* (known initially as *Paecilomyces lilacinus*) is a member of the Ascomycota phylum [38]. *P. lilacinum* is a saprobic species that can be isolated from soil, insect and insect larvae, nematodes, decaying vegetation, humans, animals, and air. Based on previous studies [39,40,41], this species can cause contamination of creams and lotions, sodium bicarbonate solutions, and colonizing materials. *P. lilacinum* forms a biofilm, together with *Aspergillus*, *Fusarium*, and *Acremonium*, as it can grow on wet and moist surfaces of water distribution systems [42].

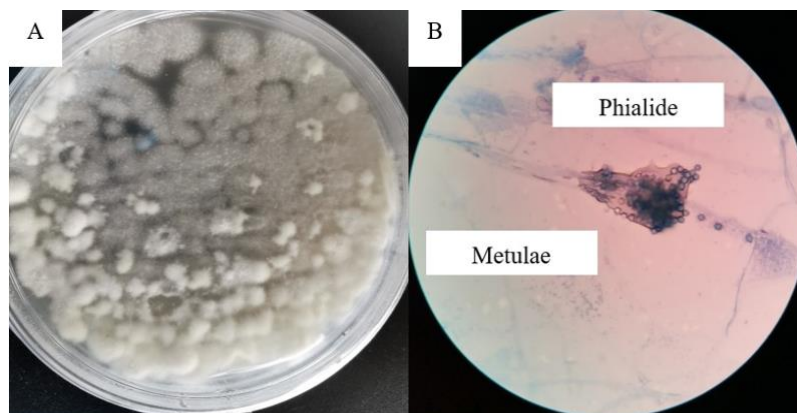


Fig. 6. *Purpureocillium lilacinum* isolate HT003; 1000x magnification. (A: Macroscopic view; B: Microscopic View)

Phylogenetic tree of isolated fungi

The phylogenetic tree was constructed to study the evolutionary relationship or histories among the fungal isolates. The branching pattern in a phylogenetic tree determines how species and other groups evolved from a series of the same ancestor. The phylogram in Fig. 7 was constructed using MEGA 5.10 software. The root of the tree represents the ancestral lineage, and the tips of the branches represent the descendants of the ancestors. The horizontal line bar represents evolutionary lineage change over time. The clade is commonly known as a monophyletic group where the group shares the same ancestor and all the descendants of the ancestor. A clade was formed between *Talaromyces verruculosus* isolate ATT281 and *Purpureocillium lilacinum* isolate HT003, showing that these species shared the same ancestor and *Aspergillus terreus* strain LPSC 1180 is also closely related. Moreover, a clade formation of *Fusarium oxysporum* strain SHBV2 and *Albifimbria terrestris* strain SCAU180 showed they also share a common ancestor. All five isolated fungi diverged from a common ancestor a long time ago. A neighbor joining tree method was constructed and shown in Figure 7.

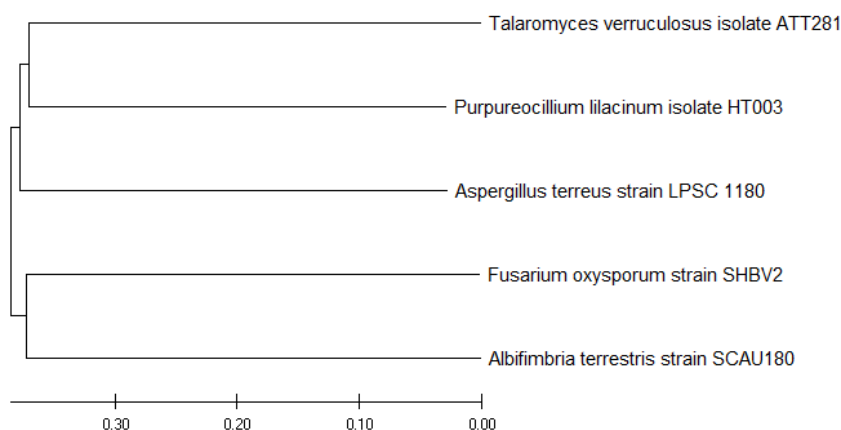


Fig.7. Phylogenetic tree of the isolated and identified soil fungi.

CONCLUSION

Five fungi have been successfully isolated and identified in this study which were *Fusarium oxysporus*, *Talaromyces verruculosus*, *Albifimbria terrestris*, *Aspergillus terreus* and *Purpureocillium lilacinum*. These fungi can be used as microbial inoculants with their properties to degrade pesticides to reduce contamination. In this study, all those isolated fungi were identified as CP-degrading fungi, and further research should be done to determine the ability of the degrader before successful bioremediation of the organisms can be implemented.

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AUTHOR'S CONTRIBUTION

The article was written and revised by Nur Fatin Nabihah Hassan and Zaidah Zainal Ariffin, who also did the research. Zaidah Zainal Ariffin, Muhd Fauzi Safian and Wan Nurhayati Wan Hanafi conceptualized the main idea for the research, provided the theoretical foundation, designed the study, oversaw its progress, anchored the review, approved corrections, and approved the publication submission.

CONFLICT OF INTEREST STATEMENT

The authors affirm that there are no competing interests in the publication of this paper.

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