

Isolation and Characterization of *Aspergillus* Species with Cypermethrin-Degrading Potential

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ABSTRACT

Cypermethrin is a widely used synthetic pyrethroid pesticide that enhances global agricultural productivity. Its massive use for improved agricultural yields, particularly in developing countries, is heading to its exponential peak in the near future. However, cypermethrin eco-accumulates and poses significant environmental risks due to its persistence in soil. In this study soil samples were collected and screened for fungal strains capable of utilizing cypermethrin as a sole carbon source. An indigenous fungal isolate that efficiently biodegrades cypermethrin was obtained from cypermethrin-contaminated soil. Morphological, biochemical and molecular analyses identified the efficient degrader as an Aspergillus species. Optimization of degradation conditions revealed that the fungal strain exhibited maximum biodegradation efficiency at pH 7.0, temperature 35°C, substrate concentration 6g/L, and an incubation period of 168 hours. Gas chromatography-mass spectrometry (GC-MS) analysis confirmed the breakdown of cypermethrin into less toxic metabolites, including 3-phenoxybenzoic acid (3-PBA) and cyclopropane carboxylic acid derivative; cis/trans-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (cis/trans-DCCA), with a degradation efficiency of 56.5% (that is greater than of most the previously known fungal strains) under optimal conditions. The findings highlight the bioremediation potential of Aspergillus species, offering an eco-friendly and cost-effective alternative to conventional pesticide removal methods. This research provides crucial insights into sustainable strategies for mitigating cypermethrin pollution in agricultural environments.

Keywords: Cypermethrin, *Aspergillus* species, Pesticide, 3-phenoxybenzoic acid, Biodegradation

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1. INTRODUCTION

Pesticides are extensively applied in modern agriculture to control weeds, insects and rodents [1]. But their persistence and accumulation in food, soil, and water [2-4] raise serious environmental and health concerns [5,6]. Pesticide residues can alter soil fertility, disrupt microbial activity and contaminate aquatic systems through runoff and leaching, ultimately reaching human populations via drinking water as well as food web [7,8].

Cypermethrin, a synthetic pyrethroid insecticide, is widely used in crop production, animal health, and domestic pest control [6,7]. Although considered safer than organophosphates [9], continuous and excessive use has been linked to bioaccumulation, neurotoxicity, endocrine disruption, and carcinogenic effects in humans, as well as high toxicity to bees, fish, and other non-target organisms

[10-13]. Its environmental persistence and long-range transport further amplify contamination risks [14]. Existing physicochemical removal methods are limited by high costs, process complexity, and incomplete degradation [15,16].

Biological degradation, particularly by fungi, presents a cost-effective and eco-friendly alternative. While several bacteria have been reported to degrade cypermethrin and other recalcitrant pesticides [17-19]. And in essence, some fungal isolates were reported to degrade other environmentally toxic chemicals [18], fungal-based approaches to cypermethrin degradation in particular, remain underexplored, especially with indigenous strains that may adapt better to local field conditions. Considering the heavy reliance on pesticides in Nigeria for food production, and the growing concern over soil and water contamination, there is an urgent need to investigate indigenous fungal species with cypermethrin biodegradation potential. Such studies will not only contribute to sustainable agricultural practices but also provide low-cost strategies for mitigating pesticide pollution in resource-limited settings. This study therefore aims to isolate and characterize cypermethrin-degrading fungi from the Kura irrigation site in Nigeria and analyze their degradation metabolites using GC-MS.

2. MATERIALS AND METHODS

All chemicals and reagents employed in this study were of analytical grade. Standard biosafety protocols were strictly observed during all microbiological experiments.

2.1 Sample Collection

Soil samples were obtained from farmland in Kura Local Government Area, Kano State, Nigeria, where cypermethrin and other pesticides have been applied for over a decade. Sampling was carried out using a sterile scoop after removal of the surface layer, at a depth of 15 cm, and transferred aseptically into sterile polyethylene bags [20].

2.2 Media Preparation

Mineral salt medium (MSM) was prepared based on the following composition: per 1000 cm³ of distilled water, 1.5 g K₂HPO₄, 0.5 g KH₂PO₄, 1.0 g (NH₄)₂SO₄, 0.5 g NaCl, 0.2 g MgSO₄, and 0.02 g FeSO₄. The pH was adjusted to 7.2 with NaOH prior to sterilization at 121 °C for 15 min. Cypermethrin was added as the sole carbon source immediately before inoculation [21].

2.3 Enrichment, Isolation, and Screening of Cypermethrin-Degrading Fungi

Fungal enrichment was performed as described by [22]. Soil sample (1 g) was inoculated into 100 cm³ of sterile MSM in a 250 cm³ Erlenmeyer flask containing cypermethrin (50 ppm) as the sole carbon source. Cultures were incubated at 37 °C on a rotary shaker (150 rpm) for 7 days. After incubation, 1 cm³ of culture was transferred into fresh MSM with the same cypermethrin concentration and re-incubated for another 7 days. Aliquots from the second enrichment were spread onto MSM agar plates supplemented with cypermethrin (10 ppm). Distinct colonies were isolated, purified by repeated streaking on Potato Dextrose Agar (PDA), and preserved for further characterization.

2.4 Preservation of Fungal Isolates

Pure cultures were maintained on PDA slants and incubated at 25 °C for 72 h. Slants were labeled appropriately, stored at 4 °C, and sub-cultured at 4-week intervals to ensure viability [23].

2.5 Identification of Cypermethrin-Degrading Fungi

2.5.1 Morphological Identification

Fungal isolates were identified based on macroscopic and microscopic features. For microscopic examination, smears were prepared from fresh cultures, stained with lactophenol cotton blue, and examined under the microscope. Structures were documented photographically and compared with standard taxonomic keys [21].

2.5.2 Biochemical Characterization

2.5.1 Amylolytic activity

Amylolytic activity was assessed using starch agar as described by Castro et al. [24]. Following incubation, plates were flooded with iodine solution. The presence of clear zones indicated starch hydrolysis and positive enzyme activity.

2.5.2 Proteolytic activity

Proteolytic activity was determined using PDA supplemented with 1% skimmed milk. After 7 days of incubation at 30 °C, protease activity was confirmed by the formation of clear halos around fungal colonies [25].

2.5.3 Lipolytic/Esterase activity

Lipolytic/esterase activity was evaluated on basal medium containing 1% olive oil as the sole carbon source. The appearance of calcium crystal precipitation around colonies after 7 days at 30 °C indicated lipase/esterase production [25].

2.6 Molecular Identification and Phylogenetic Analysis

Genomic DNA was extracted using the phenol–chloroform method [26]. The internal transcribed spacer (ITS) region of the 18S rRNA gene was amplified using ITS1 (forward: TCCGTAGGTGAACCTGCGG) and ITS4 (reverse: TCCTCCGCTTATTGATATGC) primers. PCR amplification was performed using 2× Taq Master Mix. Products of the expected size were purified (GDSBio, China) and sequenced via Sanger sequencing (Inqaba Biotechnology, South Africa). Sequence data were edited, aligned in MEGA 11, and compared against the NCBI GenBank database using BLAST for taxonomic identification.

2.7 Optimization of Fungal Growth and Degradation Conditions

The effect of different parameters on fungal growth and cypermethrin degradation was evaluated using the one-factor-at-a-time (OFAT) approach [23].

2.7.1 Incubation time

MSM inoculated with fungal suspension (100 μ L) and cypermethrin (100 μ g/ml) was incubated at 28 °C with shaking. Optical density (OD₆₀₀ nm) was measured at 24 h intervals up to 168 h. Controls lacked fungal inoculum [27].

2.7.2 pH

MSM was adjusted to pH 5.0–8.0 using 1 M HCl or NaOH prior to sterilization. Flasks were inoculated with fungal suspension and supplemented with cypermethrin (100 μ g/ml). Growth was monitored spectrophotometrically after 7 days [20,28].

2.7.3 Inoculum size

Fungal suspension volumes ranging from 50 μ L to 1000 μ L were inoculated into MSM containing cypermethrin (100 μ g/ cm³). Cultures were incubated at 28 °C for 7 days and OD₆₀₀ nm was measured [20].

2.7.4 Substrate concentration

MSM was supplemented with cypermethrin at 50–1000 ppm. Cultures were incubated at 28 °C for 7 days, and growth was measured as OD₆₀₀ nm.

2.7.5 Temperature

Cultures were incubated at different temperatures (25, 30, 35, 40, 45, and 50 °C) under shaking conditions (150 rpm). Growth was assessed after 8 days. For each optimization experiment, uninoculated MSM served as blank, while controls lacked fungal inoculum.

2.8 GC–MS Analysis of Biodegraded Products

Biodegradation products were analyzed using a Shimadzu GCMS-QP2010 PLUS system equipped with a Perkin Elmer Elite-5 capillary column (30 m $\times$ 0.25 mm, 0.25 μ m film thickness). Helium was used as the carrier gas at 0.5 cm³/min. The injection volume was 1 μ L, with the injector temperature maintained at 250 °C. The oven program was: initial 80 °C (4 min), ramped to 200 °C, then to 280 °C at 20 °C/min, and held for 5 min, giving a total run time of 25 min. The MS transfer line was maintained at 200 °C and the ion source at 180 °C. Electron impact ionization was performed at 70 eV. Compounds were identified by comparing mass spectra with the NIST GC–MS library [29].

2.8 Statistical Analysis

Statistical analyses were conducted using GraphPad InStat 3.10. Results are presented as mean $\pm$ standard deviation. One-way ANOVA was employed to compare group means, followed by Tukey's post hoc test, with statistical significance set at $P < 0.05$ [23].

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Cypermethrin-Degrading Fungus

A fungal isolate was obtained from cypermethrin-contaminated soils. Recovery of the isolate indicates its ability to withstand and adapt to pesticide-polluted environment, where the susceptible species were eliminated by the toxic conditions [30]. The first emerging colony was selected based on its apparent ability to utilize cypermethrin as an energy source [22]. The purified isolate was subsequently maintained on Potato Dextrose Agar (PDA) slants for further analysis.

3.2 Identification of Cypermethrin-Degrading Fungus

The selected isolate displayed colonies with a radial margin and raised elevation. Microscopically, it possessed long conidiophores extending from the base to the vesicle, smooth-walled hyaline structures, globose conidial heads, and septate hyphae (Table 1). Biochemical profiling revealed positive activities for amylase and protease, but no detectable lipase activity (Table 2). Both microscopic and biochemical characteristics of the isolate resemble those of genus *Aspergillus*. Molecular identification based on ITS1–ITS4 rRNA gene sequencing showed more than 99% sequence similarity with known *Aspergillus* species in the NCBI database. Phylogenetic analysis using the neighbor-joining method (MEGA 7) confirmed the isolate's close affiliation with *Aspergillus sclerotioniger*, suggesting a common ancestry (Figure 1).

Table 1. Microscopic characteristics of the fungal isolate

Isolate Code	Macroscopic Appearance	Microscopic features	Expected Organism
S1	Black colour, radial margin with a raised elevation	Long conidiophores, possesses hyaline with globes conidial head and septate hyphae	<i>Aspergillus</i>

Table 2: Biochemical characteristics of the fungal isolate

Isolate code	Amylase	Protease	Lipase	Expected Organism
S1	+	+	-	<i>Aspergillus</i>

Key: + = positive, - = negative

3.3 Characterization of Fungal Growth

3.3.1 Effect of Incubation Time

Growth of the isolate in MSM increased steadily with incubation time, reaching a maximum at 168 h (7 days) with no significant difference ($p > 0.05$) between 168 h and 192 h (Figure 2 (a)). Prolonged incubation beyond the optimum resulted in reduced growth, likely due to cell senescence and enzyme denaturation, which decreased the degradation potential. In contrast, earlier studies reported longer optimal incubation periods for *Aspergillus spp.* (15–21 days) [31,32]. The relatively

shorter incubation period observed in this study suggests that the isolate is well adapted to rapid utilization of cypermethrin as a carbon source.

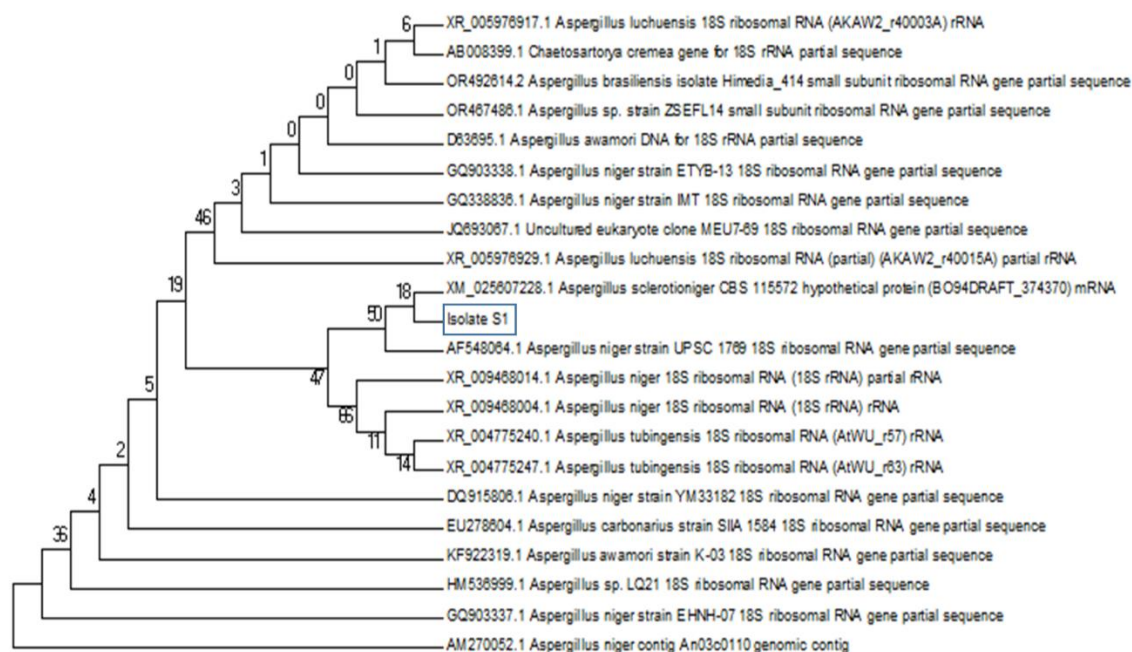


Figure 1. Cladogram indicating the genetic relationship between isolate S1 (as shown in a rectangular box) and related fungi based on ITS1–ITS2 sequence analysis.

3.3.2 Effect of pH

The isolate exhibited maximum growth at pH 7.0, with growth decreasing both above and below this value (Figure 2 (d)). Acidic (<5) and alkaline (>8) conditions significantly suppressed fungal growth, most likely due to protein denaturation and loss of enzymatic activity. The finding aligns with earlier reports showing that *Aspergillus spp.* achieves optimal degradation of cypermethrin at neutral pH [33,34].

3.3.3 Effect of Inoculum Size

Fungal growth and degradation activity increased with inoculum size until an optimum was reached, after which further increases led to reduced growth (Figure 2 (b)). Lower inoculum sizes delayed the degradation process due to insufficient biomass, while excessively high inoculum sizes caused nutrient and oxygen limitation within the medium [20]. The optimum inoculum size observed here differs from previous reports [35], suggesting that strain-specific genetic and ecological factors may influence inoculum requirements.

3.3.4 Effect of Substrate Concentration

Substrate concentration had a significant impact on fungal growth, with maximum growth observed at 6 g/L of cypermethrin (Figure (2e)). Concentrations above this optimum inhibited fungal proliferation, likely due to substrate toxicity [20]. Previous studies reported slightly lower optimal concentrations [31], suggesting that the present isolate may possess higher tolerance to cypermethrin, potentially reflecting local adaptation or strain variation.

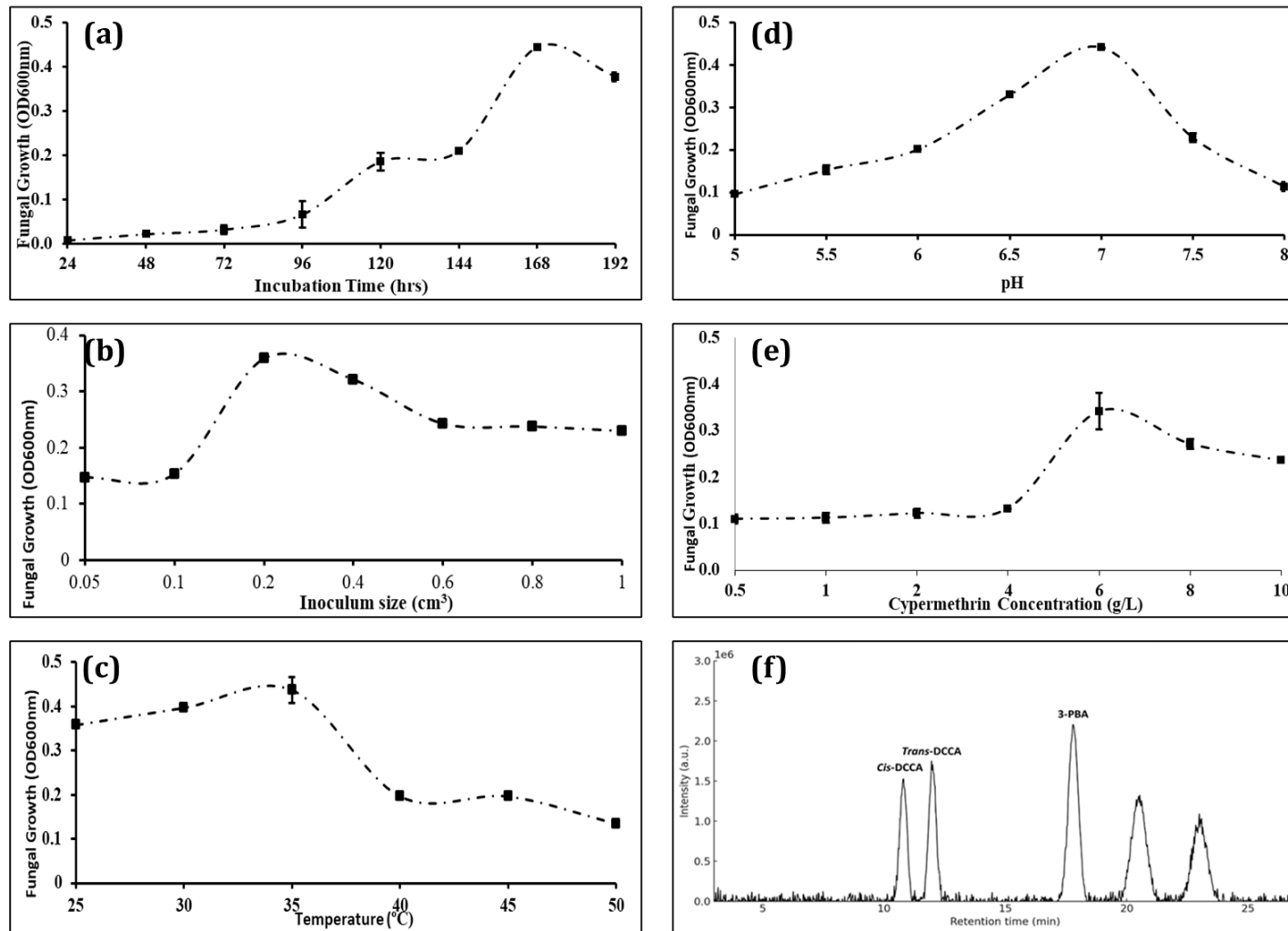


Figure 2: Effect of (a) Incubation Time, (b) Inoculum Size, (c) Temperature, (d) pH, and (e) Cypermethrin Concentration on the growth of the fungal isolate in cypermethrin-containing medium. (f) MS chromatogram of degraded cypermethrin (6g/L) inoculated with 0.2 ml inoculum and incubated for 168 hours at 35 °C.

3.3.5 Effect of Temperature

The isolate grew across a temperature range of 25–50 °C, with optimum growth observed at 35 °C (Figure 2(c)). Growth declined at higher temperatures, likely due to enzyme denaturation, while lower temperatures slowed metabolic activity. Earlier reports indicated optimal growth of *Aspergillus* species between 25–30 °C [36,37]. The slightly higher optimum temperature observed here suggests strain-specific thermotolerance, possibly reflecting adaptation to the local environmental conditions of northern Nigeria.

3.3.6 Cypermethrin Degradation Products

The GC–MS analysis of the culture filtrates obtained after incubation of the fungal isolate in cypermethrin-supplemented medium revealed distinct chromatographic peaks corresponding to known degradation products (Fig. 2(f); Table 3). The presence of *cis*- and *trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-/*trans*-DCCA) was observed at retention times of approximately 10.8 and 12.0 minutes, respectively. These metabolites represent the cyclopropane carboxylic acid moiety of cypermethrin and are widely recognized as primary hydrolysis products of pyrethroid pesticides [38,38]. Their detection in the fungal culture confirms the cleavage of the ester bond of cypermethrin, which is a critical first step in microbial degradation [40,41,42].

Table 3: Retention times of biodegraded cypermethrin metabolites by the isolate S1

Peak No.	Detected metabolite	Retention time	Note
1	<i>cis</i> -DCCA (<i>cis</i> -3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid)	~10.8	Early eluting metabolite; polar, short-chain acid
2	<i>trans</i> -DCCA (trans-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid)	~12.0	Stereoisomer of DCCA; slightly longer retention
3	3-Phenoxybenzoic acid (3-PBA)	~17.8	Aromatic metabolite; major biomarker of pyrethroid degradation

Another prominent peak was identified at a retention time of ~17.8 minutes, corresponding to 3-phenoxybenzoic acid (3-PBA). This compound originates from the aromatic portion of the cypermethrin molecule and is a major biomarker of pyrethroid biodegradation [10,12]. The accumulation of 3-PBA, along with *cis*-/*trans*-DCCA, strongly indicates that the fungal isolate possesses enzymatic machinery capable of breaking down both the ester and aromatic structures of

cypermethrin, consistent with earlier reports on *Aspergillus spp.* involvement in pyrethroid degradation [58,59].

Conclusion

A fungal isolate belonging to a genus *Aspergillus* was isolated from cypermethrin-contaminated soil. The isolate was found to be an efficient degrader of cypermethrin as a sole carbon source. Optimization of degradation conditions revealed that the fungal strain exhibited maximum biodegradation efficiency at pH 7.0, temperature 35°C, substrate concentration 6g/L, and an incubation period of 168 hours. Gas chromatography-mass spectrometry (GC-MS) analysis confirmed the breakdown of cypermethrin into less toxic metabolites, including 3-phenoxybenzoic acid (3-PBA) and cyclopropane carboxylic acid derivative; cis/trans-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (cis/trans-DCCA), with a reasonable degradation efficiency under optimal conditions. The findings highlight the bioremediation potential of *Aspergillus* species, offering an eco-friendly and cost-effective alternative to conventional pesticide removal strategies. This research provides crucial insights into sustainable strategies for mitigating cypermethrin pollution in agricultural environments.

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