# Mycoremediation of Acetaminophen by the Fungi of *Dothideomycetes* sp. Isolate

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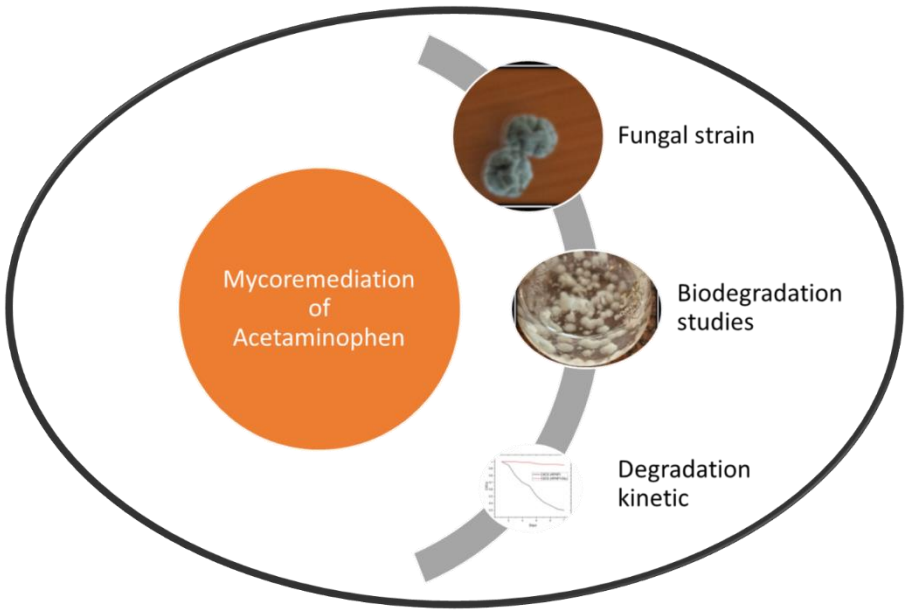


## Abstract

Resources like air, water, and land are found to be contaminated by a broad variety of pharmaceutical contaminants. The release of industrial effluents, hospital wastewater, and untreated domestic water, which contains medications, colours, pharmaceutical blisters, and heavy metals that are ecotoxic even at concentrations of nanograms to micrograms, is a significant cause of water pollution. Mycoremediation is an operative approach is to detoxify these contaminants. The fungal strain was isolated from pharma industrial wastewater. Identification of the fungi was founded on the morphological and 18s rRNA sequencing technique. Isolate shared 99.82 % similarity with *Zasmidium cellare* and *Cladosporium iridis* strain CBS 138.40, suggesting the new species of fungi. The strain has received Gen bank accession number of OR081620.1. The fungal strain was found to degrade 85% of acetaminophen (1000 ppm) in 10 days when grown in mineral salt medium with acetaminophen as only carbon source. Glucose was found to enhance sporulation potential but not the degradative potential of the fungi. Identification of biodegradative metabolite was done by using thin layer chromatography, high performance liquid chromatography and liquid chromatography-mass spectroscopy. Mycodegradative metabolite of 4 Aminophenol was identified as acetaminophen.

***Keywords:*** *Acetaminophen, Dothideomycetes, LCMS, Mycoremediation, Zasmidium*

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## 1.0 Introduction

Microorganisms are becoming more multidrug resistant, and several diseases are emerging, which increases medicine usage. Numerous pharmaceutical micropollutants are discharged into the aquatic ecosystem because of household, commercial, and healthcare activities (Ribeiro et al., 2015). The active components of pharmaceutical and personal care products (PCP) have also been found in the environment (Brausch and Rand, 2015; Montesdeoca-Esponda et al., 2018). Drugs, heavy metals, and microplastics are among these pollutants (Zhou et al., 2020; Waghmode et al., 2022). According to the microbial models of human metabolism, microorganisms have systems which are like cytochrome P450 and can detoxify pharmaceuticals. The classification of medications into distinct categories, such as expired, fake, or rejected, is crucial for researching microbial deterioration (Waghmode et al., 2023). According to studies, various microorganisms have the capacity to detoxify harmful chemicals by employing enzymes such oxidoreductase and exopolysaccharide aided mechanisms (Rao et al.,2010; Gadkari et al., 2022).

The hazardous pollutant paracetamol is often introduced into aquatic environments because of high emission rates and high consumption from manufacturing facilities and hospitals (Waghmode et al., 2022). Untargeted animals including fish, germs, and algae are being negatively impacted by drugs that have been introduced into the environment. Paracetamol has been proven to produce adverse, long-lasting impacts on non-target organisms, even at low exposure levels (Piedade et al., 2020).

Due to its promiscuous nature and ability to convert environmental pollutants into innocuous compounds or less harmful forms, bioremediation offers a non-invasive, less costly, and environmentally friendly alternative to conventional treatments (Perelo, 2010). There are various biological remediation techniques, including phytoremediation, bioremediation, cyanoremediation, and Mycoremediation (Yaashikaa et al., 2022). Based on the concept of Green Liver Systems, macrophytes *(*species from the genera *Azolla, Elodea, Egeria, Myriophyllum, Pontederia, and Ceratophyllum)* have been reported to be useful to resolve water quality issues (Esterhuizen, and Pflugmacher, 2023). Report is available on the remediation of diclofenac using macrophytes (Esterhuizen and Pflugmacher, 2023). Acetaminophen remediation has been reported by *Armoracia rusticana* hairy root cultures; and ornamental plant (Alternanthera spp.) (Kotyza et al.,2010; Mohammed et al., 2021). The efficacy of phytoremediation of pharmaceutical pollutants could be enhanced by mycoremediation (Esterhuizen-Londt et al., 2016). Due to their vigorous growth, extensive hyphal network, synthesis of adaptable extracellular enzymes, and resistance to heavy metals, fungi are a top candidate for the cleanup of diverse contaminants (Akhtar and Mannan ,2020).

## 2.0 Materials and methods:

### 2.1 Isolation, and identification of APAP degrading fungi

A sample of industrial wastewater from pharma was used to isolate fungus that break down paracetamol. By spreading the wastewater sample over mineral salt, media that had been laced with acetaminophen (100–3000 ppm), acetaminophen-tolerating fungus were identified. For paracetamol Mycoremediation tests, the fungal isolate with the highest tolerance to the drug was employed. The morphological characterizations have been supported by 18s rRNA sequencing data.

Isolate was identified at the National Centre for Microbial Resource (NCMR) sequencing laboratory at the National Centre for Cell Science, Pune. After extracting genomic DNA using the traditional phenol/chloroform extraction method, the ITS sections were amplified in PCR at the facility using the universal primers ITS1 [5'-TCC GTA GGT GAACCT GCG G -3'] and ITS4 [5'-TCC TCC GCT TAT TGA TAT GC -3'] (Sanbrook, 1989). The amplified ITS PCR product was cleaned using the PEG-NaCl precipitation technique in

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accordance with the manufacturer's instructions. The ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) was then used to analyze it immediately. Essentially, sequencing was carried out from both ends to guarantee that each site was read a minimum of twice. A tentative identification was made after assembly using the Lasergene programmed and NCBI BLAST against sequences from type material (Boratyn et al., 2013). Isolate was screened for amidase enzyme using plate screening method with the help of Potato dextrose agar amended with acetamide (Rahim et al., 2003).

**2.2 Biodegradation Studies**

The isolate was inoculated in mineral salt (MS) broth ($p^H$ 6) spiked with 1000 ppm APAP as only carbon source based on the upper limit of acetaminophen tolerance. In another flask, MS broth was spiked with 100 mM glucose and 1000 ppm of acetaminophen. The flasks were kept in a dark, room temperature (26 °C) incubator with periodic measurements of the biomass and residual APAP concentration made using a spectrophotometric technique (Khaskheli et al., 2007).

For the computation, the standard dosage response curve of paracetamol 10-100 ug/mL was referred. The biodegradation of APAP was calculated by Eq. 1:

$$\text{Rate of degradation (\%)} = (C_0\text{-}C_t)/C_0 \times 100 \quad \text{------} \quad (1)$$

where $C_0$ is the primary concentration of APAP; $C_t$ is the concentration of APAP after incubation at time 't'. Kinetic parameters during biodegradation were assessed using Computer Assisted Kinetic Evaluation (CAKE) tool (22. https://cake-kinetics.org/)

**2.3 Characterization of Acetaminophen biodegradative metabolite**

The supernatant was separated from the fermented broth using centrifugation. Methanol was used to carry out the solvent extraction procedure. Sample processing for TLC and reverse phase HPLC was done after solvent evaporation. A solvent system consisting of ethyl acetate and hexane (1:1), a developing agent of UV 365 nm, and pure 4-aminophenol as standard were used to perform chromatography of the extracted biodegradative metabolite (Braimah et al., 2019). A general acidic technique was used for qualitative HPLC. The apparatus was a Waters Alliance 2695 HPLC with PDA detector 2996. There were two solvent systems used: Mobile phase A, which contained water, acetonitrile, and fumaric acid (95:05:0.1), and mobile phase B, which contained water, acetonitrile, and fumaric acid (10:90:08). One ml/min of flow rate was employed using a gradient elution scheme. The data analysis was performed with Empower2 software.

High-Resolution Mass Spectrometry (HR-MS) was used to analyze the biodegradative metabolite of APAP. For the mass analysis of the acetaminophen biodegradable metabolite, a Bruker Impact HD analyzer was used. HR MS was performed under the following conditions: a double electrospray ionization source operating in positive ion mode; a scanning range of 50 to 1200 m/z; a scan rate of 4.0 spectra/sec; a source gas temperature of 200 °C; a gas flow rate of 7.01 l.min-1; and a sample injection volume of 10 µl with 1.7 bar pressure to the nebulizer. Bruker Compass Data Analysis 4.2 Software was used to analyze the data (Waghmode et al., 2023).

**3.0 Results**

**3.1 Isolation, and identification of APAP degrading fungi**

Wastewater samples from pharmaceutical industries were used for the isolation of acetaminophen tolerating strains. Five fungal strains were found to have the potential to use acetaminophen as a carbon source, when grown on mineral medium with acetaminophen. This

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strain was found to tolerate 1500 ppm of acetaminophen. Morphological culture characters were recorded after 72 hours of growth on mineral salt medium spiked with acetaminophen. Light microscopy observations were done using Magus model no. SM100 at 400X magnification. Colonies on MS medium with acetaminophen, were of 12-16 mm diameter with bottle green color margin (Figure 1). Mycelium was found to be hyaline, slight green, and septate. Small, unbranched as well as branched ovate shaped conidia were observed (Figure 2). The plate screening method was adopted to check the extracellular amidase production by fungal strain, the strain was found to be amidase negative (no pink color zone) as shown in Figure 3.

**Figure 1:** Growth of fungal strain on mineral salt medium spiked with acetaminophen.

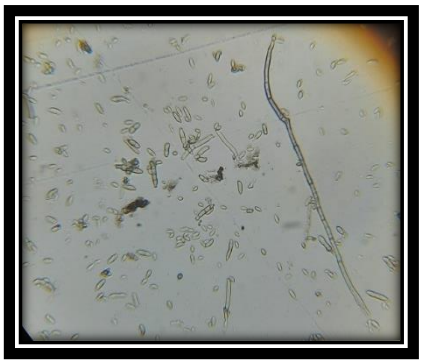

**Figure 2:** Microscopic observation of fungi

**Figure 3.** Amidase negative fungal strain

The fungal sample identification was based on a single gene sequence for a total of 1137 bp length of the 18S rRNA gene with its closest type strains in the database. The closest phylogenetic neighbours found for the sequence are *Cladosporium iridis* strain (CBS 138.40) and *Zasmidium cellare*. A comparative ITS gene based phylogenetic analysis placed strain (C Mar 22 215) in a clade with the species *Cladosporium iridis* and *Zasmidium cellare* strain and revealed pairwise similarities ranging from 100 to 99% (Figure 4). Based on the results from the phylogenetic tree using 1137 bp sequence of ITS gene and pairwise similarity results using GenBank database, it can be inferred that this might be another genus of the *Cladosporium* and *Zasmidium*.

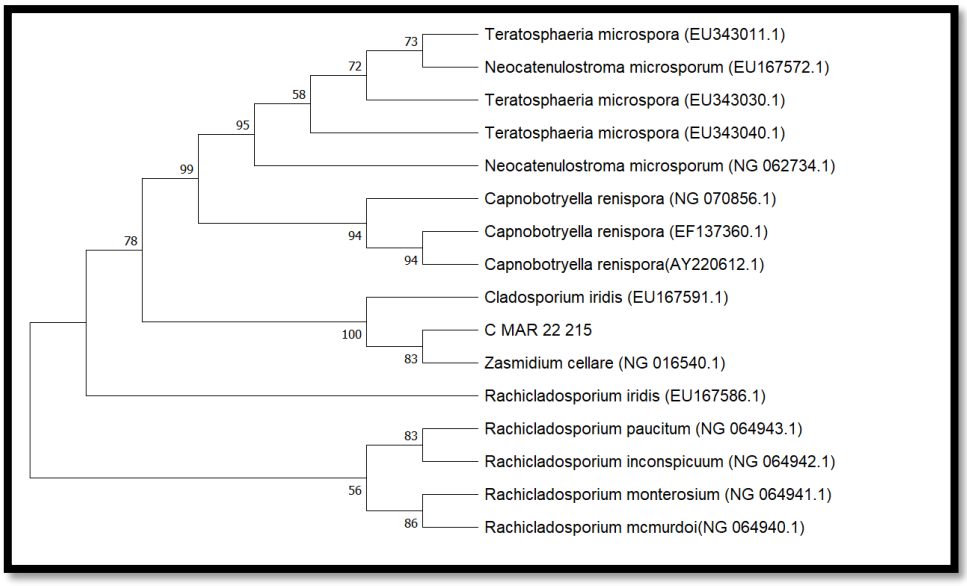


**Figure 4:** Phylogenetic tree of the *Dothideomycetes* sp.isolate

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### 3.2 Biodegradation studies

Mycoremediation of acetaminophen was done with the *Invitro* studies using *Dothideomycetes* sp. isolate. Mineral salt broth spiked with acetaminophen as sole carbon source found to have good fungal mycelial growth without sporulation as shown in Figure 5.

**Figure 5:** Mycelial growth of fungal strain in mineral broth spiked with 1000 ppm of acetaminophen.

**Figure 6:** Fungal sporulation in mineral broth spiked with 1000 ppm of acetaminophen and Glucose (100 mM).

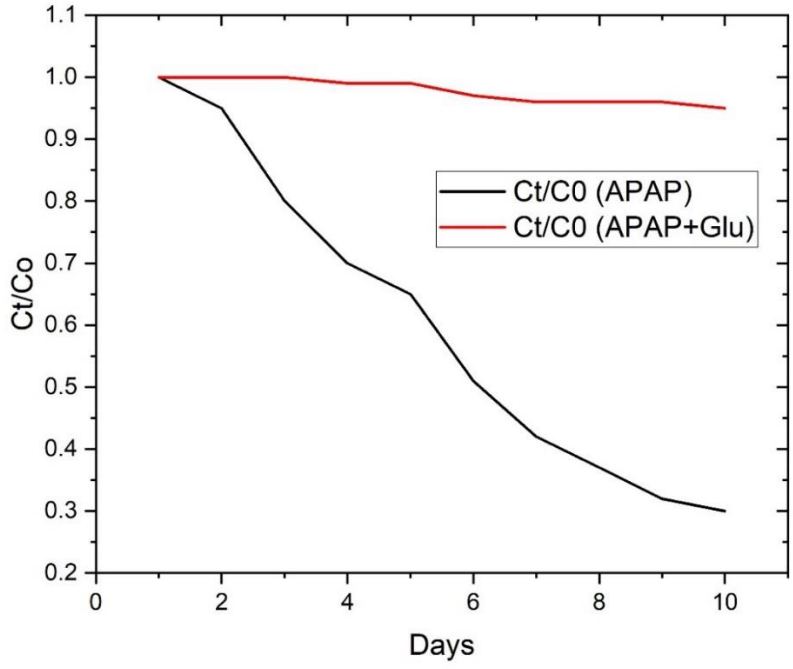


**Figure 7:** Plot of Ct/Co Vs Time during acetaminophen biodegradation by fungal strain

As shown in Figure 6, glucose enhances the sporulation of the fungi. The strain showed 85% biodegradation of acetaminophen (1000 ppm) after 10 days of incubation when acetaminophen was used as only carbon source as shown in Figure 7, whereas only 5% of degradation was observed when strain was grown in a medium spiked with 1000 ppm of

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acetaminophen and 100 mM glucose. The biodegradation kinetics were examined using the Computer Assisted Kinetic Evaluation (CAKE) tool, which suggested a half-life of 3.59 days and simple first order kinetics. The results are shown in Table 1.

**Table 1.** Kinetic parameters for APAP biodegradation by using ***Dothideomycetes*** **sp. isolate**

| Parameter | Model Kinetic | APAP concentration | K ($d^{-1}$) | Chi-sq error | 50% Degradation time DT 50 (days) | 90% Degradation time DT90 (days) |
|---|---|---|---|---|---|---|
| Acetaminophen as only carbon source | Simple first order | 1000 ppm | 0.193 | 3.96 | 3.59 | 11.9 |
| Acetaminophen and Glucose | Simple first order | 1000 ppm | 0.00626 | 0.286 | 111 | 368 |

### 3.3 Characterization of Acetaminophen biodegradative metabolite

Depiction of the acetaminophen fungal acetaminophen biodegradative metabolite was done with the help of thin layer chromatography (solvent system- ethyl acetate: hexane in equal proportion) along with standards (Figure 8). Rf value of standard acetaminophen, and standard 4 aminophenol was found to be 0.31 and 0.57. The Rf values of degradative metabolites extracted from broth spiked with acetaminophen as carbon source were found to be 0.34 and 0.6, suggesting 4-aminophenol as product. The Rf value of degradative metabolites extracted from broth spiked with acetaminophen and glucose, was found to be 0.31, suggesting no degradation of acetaminophen. The acquired result was further validated using the HPLC method, which showed three peaks (Figure 9): oxalic acid (1.543 min retention time), 4-aminophenol (9.904 min retention time), and acetaminophen (parent drug, 8.067 min retention time). Further confirmation of acetaminophen degradative product was made with HRMS where peaks of acetaminophen (MW 151) and 4-aminophenol (MW 109) were detected (Figure 10).

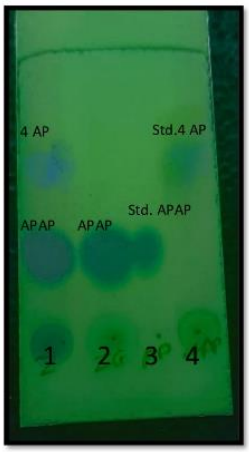


**Figure 8:** Thin layer chromatography of acetaminophen fungal biodegradative product Solvent system- ethyl acetate: hexane (1:1) visualized under UV365 nm. 1. Extracted acetaminophen biodegradative metabolite from MS broth spiked with acetaminophen, 2. Extracted acetaminophen biodegradative metabolite from MS broth spiked with

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acetaminophen and 100 mM glucose, 3. Standard acetaminophen, 4. Standard 4-aminophenol.

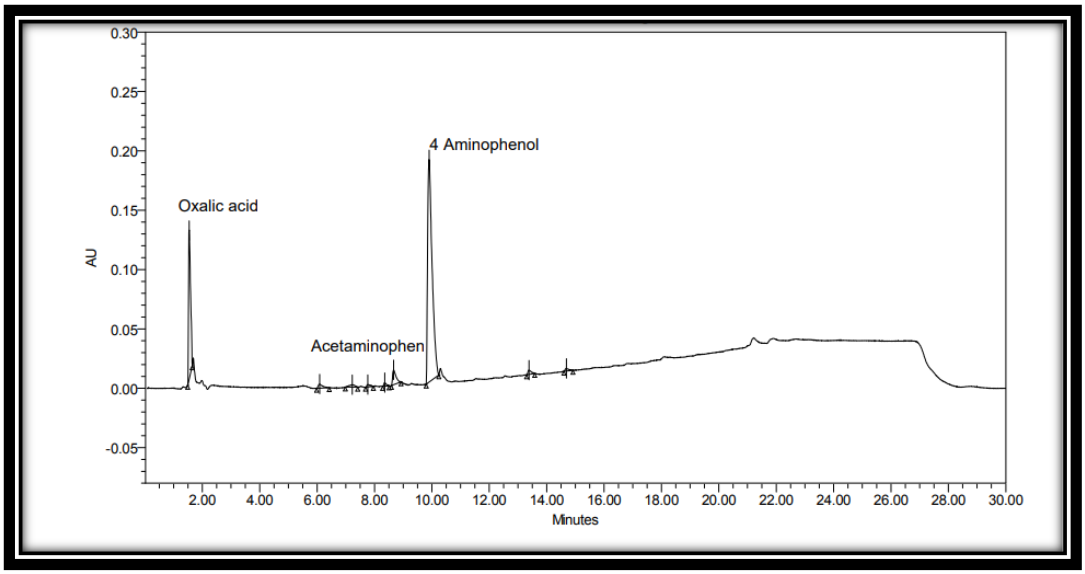


**Figure 9:** HPLC chromatogram of acetaminophen fungal biodegradative product

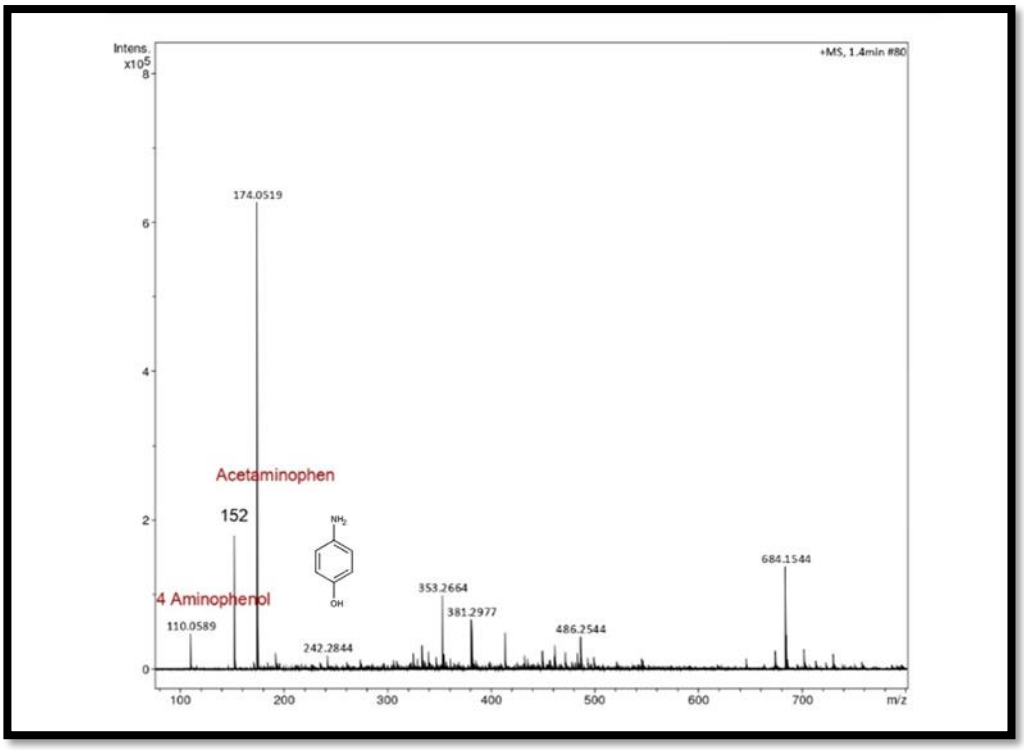


**Figure 10:** High resolution mass spectrum of acetaminophen fungal biodegradative product

## 4.0 Discussion

The already dreadful environmental conditions are being made worse by pharmaceutical chemicals emitted from the effluents of drug manufacturing facilities. The release of toxic chemicals affects the health of people and animals, disturbs the natural equilibrium, and depletes the already scarce supplies of potable water. Hazardous threshold levels of pharmaceuticals have reportedly occasionally been detected in environmental discharges (Larsson, 2014). Aquatic biota can be harmed by pharmaceutical effluents as per

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the study on microalgae (Waghmode et al., 2023). The environment becomes more and more contaminated with dangerous pharma micropollutants because of the failure of the widely utilised water treatment technologies employed in developing countries to remove these xenobiotics (Dhanalakshmi et al., 2022; Tiwari et al., 2017; Menon et al., 2020; Khan et al., 2020).

Due to fungi's widespread ability to degrade compounds using exoenzymes and their ability to absorb substances as sustenance, mycoremediation has great promise for the elimination of xenobiotics like paracetamol (Esterhuizen et al., 2021). Acetaminophen mycoremediation has been studied using *Penicillium chrysogenum* var. *halophenolicum* (Enguita et al.,2023). Based on the transcriptomic studies, it was concluded that acetaminophen mycoremediation was attributed to the role of intracellular and extracellular enzymes, viz., cytochrome P450, amidases, extradiol-dioxygenases, and, laccases (Enguita et al.,2023). Nonspecific nature of fungi to degrade xenobiotics through absorption, and enzymatic treatment, makes Mycoremediation as a promising technology (Gunjal et al., 2019; Vaksmaa et al., 2023; Sośnicka et al., 2022). Acetaminophen remediation efficiency (exposure concentration 250 µg/L) was found to be good in white rot fungi *Phanerochaete chrysosporium* as compared to fungi *Mucor hiemalis* (Esterhuizen et al., 2021; Sośnicka et al., 2022).

Mycoremediation of the pharma pollutants with the use of fungal biomass or their metabolites, is a cost effective and efficient method for the detoxification of the environment. Among the fungal strains, aquatic fungi *Mucor hiemalis*, white-rot fungus *Trametes versicolor*, *Phanerochaete chrysosporium*, *Lentinula edodes* have been reported to have the potential to degrade pharmaceuticals (Akhtar, and Mannan, 2020). Dried fungal biomass of *Mucor hiemalis* could internalize between 1 and 2 µg APAP when exposed to 5-100 ng $mL^{-1}$ APAP for 24–48 h, suggesting its efficacy for low concentration of acetaminophen polluted site with daily fungal pellet replacement strategy (Esterhuizen et al., 2016) .

In the current study, fungal strains resembling *Cladosporium* sp. and *Zasmidium* sp., were isolated and checked for the remediation of acetaminophen. Mycoremediation of pharma pollutants will give new insights for the exploration of fungal biomass and its metabolites in environmental protection. Identification was done with the help of the morphological, and 18s rRNA sequencing method but still there is need of whole genome sequencing. The data based on the 18s rRNA report is unclear to name the organism. As the sole carbon source, the strain may be able to withstand and break down high paracetamol concentrations (1000 ppm). In the presence of glucose, the strain found to have more sporulation with negligible acetaminophen degradation. Glucose was found to play the role as inducer for the fungal growth instead of bioremediation of APAP. *Penicillium chrysogenum* var. *halophenolicum* has been reported to degrade acetaminophen in the concentration of 0.661 mM (approx.100 ppm) to 2.00 mM (approx.300 ppm). More than 90% degradation was observed for the 1 mM concentration of APAP (Enguita et al.,2023). Following 96 hours of incubation, *Cladosporium alboflavescens* (GenBank accession number OQ977005) showed 89% biodegradation of paracetamol (1000 ppm), with 4-aminophenol serving as the main biodegradative metabolite (Waghmode and Patil, 2023). In this study, 1000 ppm concentration was found to be optimum to serve as the carbon source for mycelial growth of fungi. Compared to the reported strain, this strain could tolerate and degrade high concentration of acetaminophen with the 4 aminophenol as main degradative metabolite. As this fungal strain is slow grower in the absence of glucose, the time required for the degradation is more compared to the reported strains. This amidase negative strain might be using different strategy for the tolerance and degradation of acetaminophen. Whole genome sequencing and transcriptomic studies will reveal the underlying mechanism in the degradation of acetaminophen by these fungi.

This experimental data suggests that this strain has the potential to degrade 85% of APAP but it will not be useful in the wastewater treatment facilities due to slow growth and

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ecotoxic 4-aminophenol as a product. To enhance the biodegradation potential of microorganisms at sewage treatment sites, some strategies must be adopted. $H_2O_2$-stimulated upflow fixed-bed bioreactor (UFBR) has been reported to accelerate the acetaminophen biodegradation potential of bacteria (Baratpour, and Moussavi, 2018). Laccase produced by the white-rot fungus *Trametes* sp showed 76% removal of acetaminophen in aqueous solution (Sybuia et al., 2024). Optimization of the culture combination, and growth conditions ($_pH$, temperature, nitrogen, acetaminophen concentration) could be beneficial for improving the performance of fungi (Tormo-Budowski et al., 2021; Utami et al., 2024). A safe degradative product from acetaminophen can be obtained by using mixed consortia of acetaminophen and 4-aminophenol degrading strains (Shabani et al., 2021; Ahmed et al., 2001).

## 5.0 Conclusions

Pharma micropollutants are leading imposers of negative consequences on the environment due to their short term and long-term effects. Biological approaches show promise and are less expensive than physical-chemical procedures. Mycoremediation of pharma pollutants is one of the interested research topics among researchers. Drugs and heavy metals can be effectively removed using both dead and living fungal biomass. The current study found that the new fungus strain and remediation strategy can be used to achieve mycoremediation. To determine whether this research is feasible, lab scale investigations at wastewater treatment plants can be investigated further. Pharmaceutical waste treatment using these fungal strains in bioreactors seems to be a promising approach for the future. This work is the initial assessment of Dothideomycetes sp. isolate's acetaminophen-degrading capacity.

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