Isolation, and characterization of *Cladosporium alboflavescens* for Acetaminophen biodegradation

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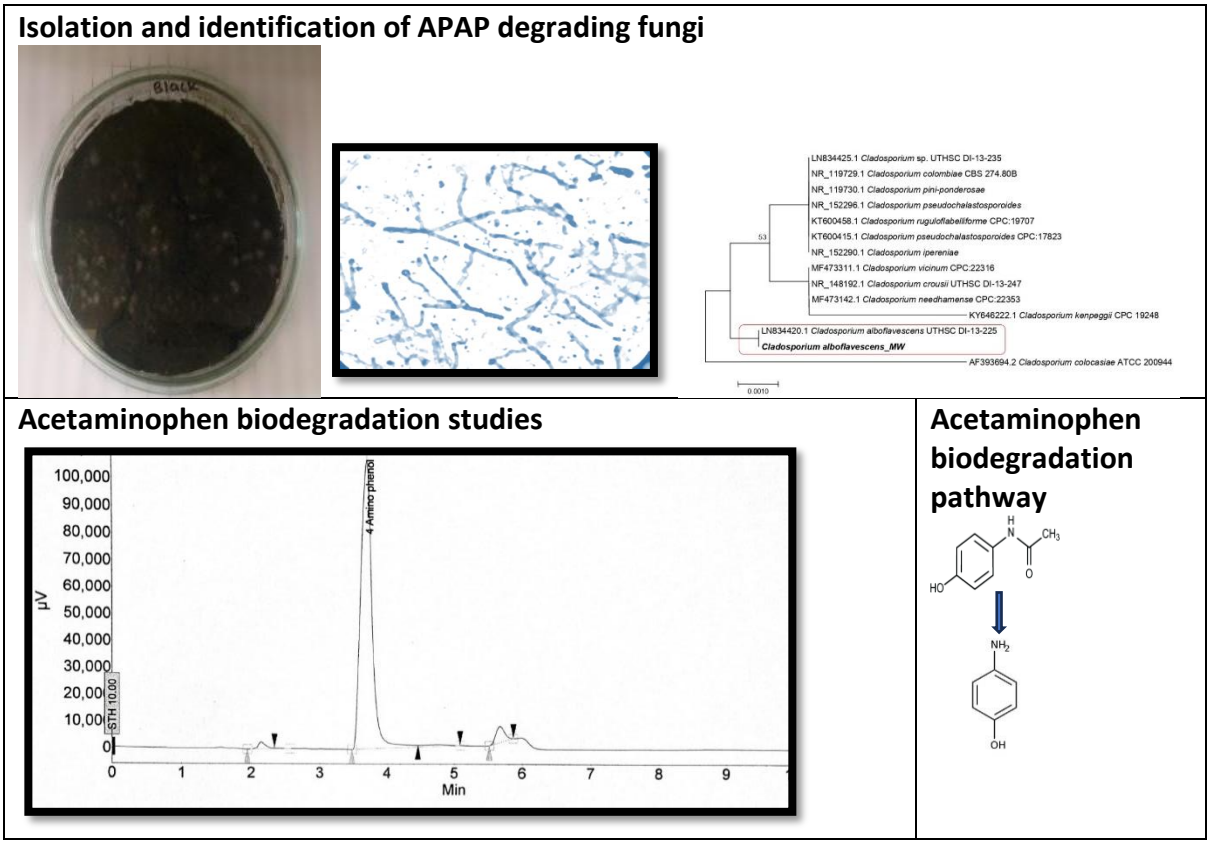

Abstract

One of the pharmaceutical micropollutants with a detrimental effect on the environment is paracetamol. Mycoremediation of the pollutants is a widely accepted concept based on cost and eco-friendly nature. Fungi isolated from pharmaceutical industry effluent have been used to target the biodegradation of paracetamol. The fungus's internal transcribed spacer (ITS 1) sequencing matched that of *Cladosporium alboflavescens* (GenBank accession number OQ977005) by 99.81%. The strain demonstrated 89% biodegradation of paracetamol (1000 ppm) after 96 hours of incubation with 4-aminophenol as the predominant biodegradative metabolite, according to the spectrophotometric and high-performance liquid chromatographic analysis. The half-life of 1.44 days and simple first order kinetics were proposed by the Computer Assisted Kinetic Evaluation (CAKE) tool used to examine the biodegradation kinetics.

Key words*: Acetaminophen, 4-aminophenol, Cladosporium, Mycoremediation*

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1. Introduction

Acetaminophen (APAP) is one of the xenobiotic compounds detected in water in concentrations of 5 µg/L, suggesting employment of phytoremediation and mycoremediation techniques (Esterhuizen-Londt et al., 2016). Acetaminophen (APAP), regarded as a developing pharmaceutical contaminant, is regularly introduced into aquatic ecosystems due to its high consumption and emission rates from manufacturing facilities and hospitals. Drugs released into the environment negatively affect non-target animals like fish, bacteria, and algae. Even at modest exposure levels, paracetamol has been shown to have negative, permanent effects on non-target organisms (Piedade et al. 2020). Acetaminophen naturally degrades into hydroquinone and 4-aminophenol (Gusseme et al., 2011; de Souza et al., 2020).

Bioremediation is a permanent solution that can transform environmental contaminants into innocuous chemicals, or less harmful forms. It is an environmentally benign, non-invasive, and less expensive alternative to conventional physico-chemical methods (Perelo, 2010). Fungi are a prime candidate for the remediation of various pollutants due to their robust growth, vast hyphal network, production of versatile extracellular enzymes, and heavy metals resistance (Akhtar, and Mannan, 2020).

Due to the general ability of fungi to break down substances through exoenzymes and the assimilation of substances as nutrition, fungi based mycoremediation is a promising strategy for the removal of xenobiotics like acetaminophen (Esterhuizen et al., 2021). Mycoremediation of acetaminophen is a poorly studied topic (Enguita et al., 2023). Fungi have been extensively studied for electrochemical degradation using the dried fungal biomass as the biofuel (Mbokou et al., 2016; Shabani et al., 2021). Use of the fungal biomass in the biofuel cell is carried out using a single strain or in combination with biofilm forming bacteria strains (Shabani et al., 2021).

Considering the importance of microbial solution for the removal of pharmaceutical micropollutants viz., dyes, drugs, heavy metals, microplastics, this research was aimed to explore the potential of *Cladosporium* sp., for the remediation of acetaminophen (Waghmode et al., 2022). Fungi can be used in both forms i.e., live, and dead biomass for detoxifying of the pollutants.

2. Materials and methods:

2.1 Isolation, and identification of APAP-degrading fungi

Acetaminophen degrading fungi was isolated following the protocol developed by Chopra and Kumar, with slight modifications (Chopra and Kumar, 2020). Acetaminophen tolerating fungi were isolated by culturing on mineral salt medium with acetaminophen (100 -3000 ppm). The fungal isolate showing maximum tolerance to acetaminophen, was used for further studies. The characterization was confirmed by internal transcribed sequence-1 (ITS -1) sequencing and phylogenetic analysis.

2.2 Biodegradation Studies

Mineral salt broth (pH 6.5) was inoculated with the isolate and spiked with 1000 ppm APAP. Uninoculated mineral salt broth was kept as control. The flask was incubated at room temperature in dark conditions for four days with an intermittent determination of biomass, and the residual concentration of APAP using a spectrophotometric method (Khaskheli et al., 2007). The dry biomass of fungi was calculated gravimetrically in duplicate. Whatman filter paper was used to filter each 10 mL sample. The fungal pellets were dried for 48 hours at 60 °C. The samples were weighed after 60 minutes in the oven at 50 ^{0}C till constant weight. The amount of dry biomass in 10 millilitre of medium was the biomass concentration (mg/ml). The standard dose response curve of paracetamol 10-100 ug/mL was used as a reference for the calculation. The biodegradation of APAP was calculated by Eq. 1:

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

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

2.3 TLC and Reverse-phase HPLC based analysis of Acetaminophen biodegradative metabolite

The fermented broth was centrifuged, and the supernatant was collected. The supernatant was extracted with methanol. The solvent was evaporated and analysed using TLC and reverse phase HPLC. Thin layer chromatographic analysis of extracted biodegradative metabolites was done using ethyl acetate and hexane (1:1) solvent system, UV lamp at 365 nm was used for visualizing and pure 4-aminophenol used as the standard. The elution of paracetamol was done by reverse phase HPLC with isocratic mode, JASCO (C-18 column 250 × 4.6 mm, packed with 5 µ). The solvent system used was Acetate buffer: Methanol (80:20) with 1.2 ml/min flow rate and monitored at 245 nm.

3. Results

3.1 Isolation, and identification of APAP-degrading fungi

Acetaminophen tolerating fungi was isolated from pharma polluted wastewater. The strain was identified based on the morphological, and internal transcribed spacer sequencing. Conidiophores were found to be erect, straight, cylindrical, septate, and branched (Fig.1). The strain was identified based on the ITS sequencing as *Cladosporium alboflavescens* with 99.81% matching (Fig.2). ITS-1 sequence was deposited to GenBank with accession number as OQ977005. The strain belongs to Ascomycota division, Class Dothideomycetes, Capnodiales order, Davidiellaceae family and *Cladosporium* genus (Ogórek et al., 2012).

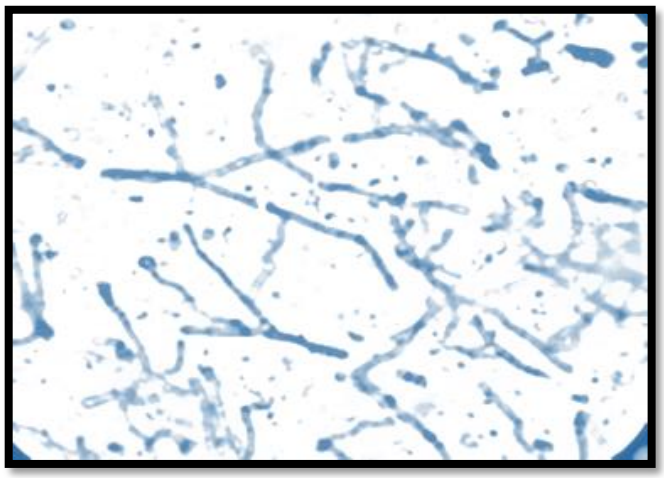

Figure 1: Micro morphology of the fungi

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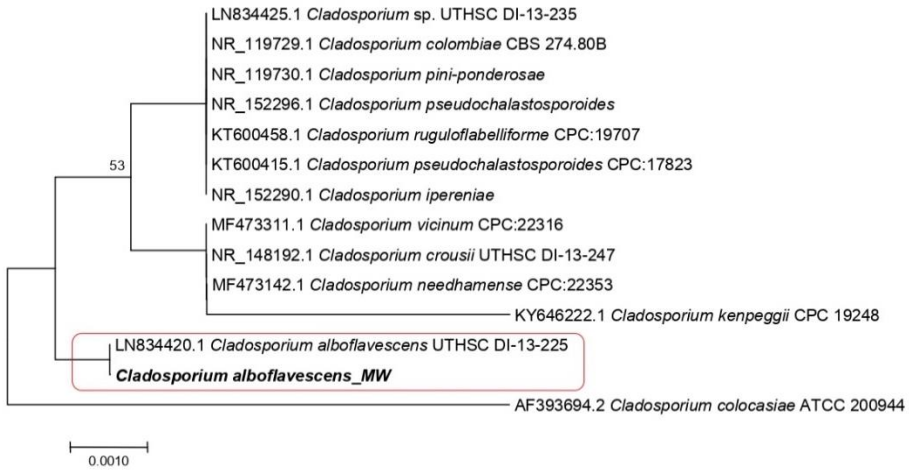

Figure. 2 Phylogenetic tree of *Cladosporium alboflavescens*

3.2 Biodegradation Studies

In-vitro mycoremediation of acetaminophen was done using isolate *Cladosporium*. The strain showed 89% biodegradation of paracetamol (1000 ppm) after 96 hours of incubation, with the increase in fungal biomass as shown in Fig.3. The half-life of 1.44 days and simple first order kinetics were proposed by the Computer Assisted Kinetic Evaluation (CAKE) tool used to examine the biodegradation kinetics; data is given in Table.1.

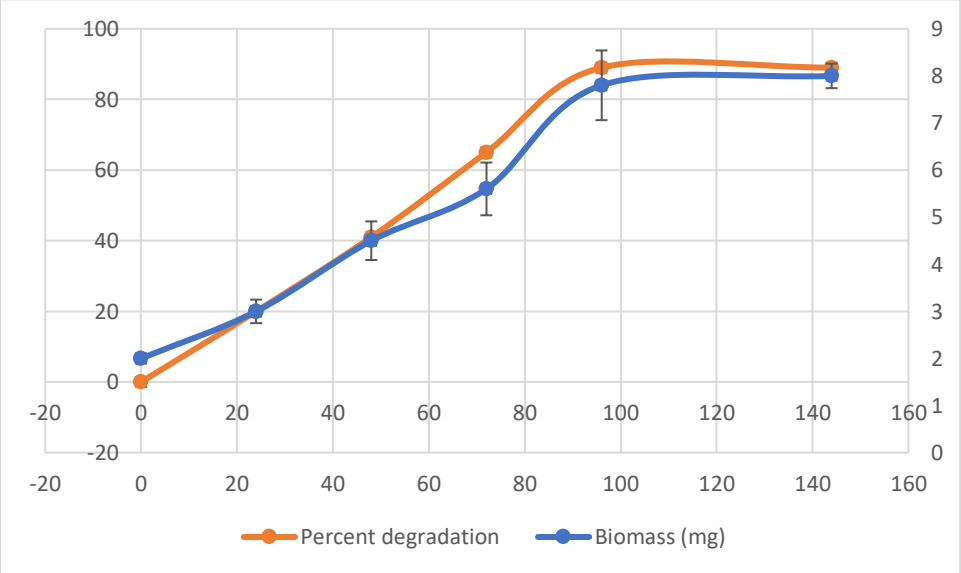

Figure 3: Acetaminophen biodegradation studies mediated by *Cladosporium alboflavescens*

Table 1: Kinetic parameters for APAP biodegradation by *Cladosporium alboflavescens*

Model Kinetic	APAP concentration	K (d^{-1})	**Chi-sq error**	**50% Degradation time DT 50 (days)**	**90% Degradation time DT90 (days)**
Simple First Order	1000 ppm	0.481	11.3	1.4	4.79

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3.3 TLC and Reverse-phase HPLC based analysis of Acetaminophen biodegradative metabolite

Characterization of the acetaminophen biodegradative metabolite was done using thin layer chromatography (ethyl acetate and hexane (1:1) solvent system) where fungal degradative metabolite showed the same Rf value (0.78) of 4 aminophenol. The obtained result was further confirmed by using HPLC method where 2 peaks, one of 4 aminophenol (3.658 min retention time) and other of paracetamol (5.874 min retention time, parent compound) were observed.

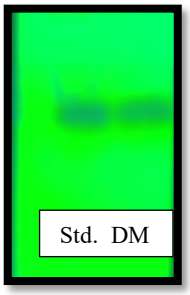

Figure 4: TLC of Standard 4 aminophenol and acetaminophen degradative metabolite.

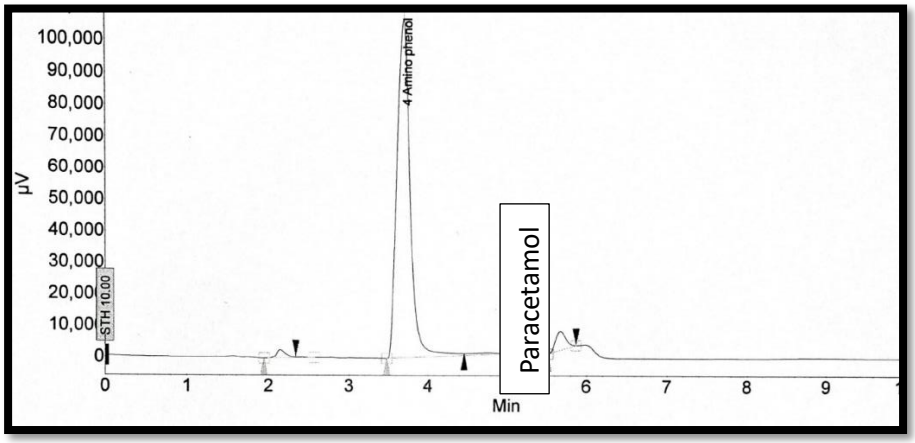

Figure 5: Reverse HPLC chromatograph of solvent extracted Acetaminophen degradative metabolite mediated by Cladosporium fungi

4. Discussion

The hazardous threshold values of pharmaceuticals are present in the environmental discharges which can poison aquatic biota were reported in literature (Alimba et al., 2019). The environment becomes increasingly contaminated with harmful pharmaceutical substances due to the failures associated with widely employed water treatment methods (Esterhuizen-Londt et al., 2016).
Mycoremediation can be a cost-efficient, environmentally responsible, and successful technique to address the issue of soil and water pollution caused by anthropogenic activities. Currently, limited scientific reports are available on the biodegradation of acetaminophen in liquid media. Data is available on the use of dried fungal biomass for biosorption studies or in electrochemical biodegradation of acetaminophen. Compared to the other reported fungal strains, *Cladosporium alboflavescens could* tolerate high acetaminophen concentration with remarkable degradation as given in Table 2.

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Table 2: Mycoremediation of Acetaminophen using fungal isolates

Name of fungi	Fungal cultivation/usage condition	Concentration of APAP	Mechanism	Reference
Mucor hiemalis	Dried Biomass	5- 100 ng mL−1	Biosorption	Esterhuizen-Londt et al., 2016
Scedosporium dehoogii	Growth in liquid media	900 ppm	Electrochemical biodegradation	Mbokou et al., 2016
Penicillium chrysogenum var. halophenolicum	Growth in liquid media	151.2 ppm, 90 % degradation in 96 Hours	Enzyme assisted biodegradation	Enguita et al., 2023
Trichoderma harzianum and *Pseudomonas fluorescens*	Dried Biomass	-	Electrochemical biodegradation	Shabani et al., 2021
Cladosporium alboflavescens	Growth in liquid media	1000 ppm, 89 % degradation in 96 Hours	Biodegradation	Current study

Transcriptomic study of *P. chrysogenum* var. *halophenolicum* mediated acetaminophen degradation was conducted for the elucidation of genes involved in biodegradation pathway (Enguita et al.,2023). As per the study, acetaminophen act as inducer for EN45-065010, EN45-051840 and EN45-110750 genes encoding amidase enzyme, and EN45-044880 genes encoding laccase and EN45-053090 genes encoding for cytochrome P450-like enzymes (Enguita et al.,2023). For the bioconversion of acetaminophen into 4 aminophenol, amidase enzymes are mainly reported. However, the generation of more ecotoxic product 4 aminophenol, suggests further research with biosorption approach using *Cladosporium* fungal dead biomass. Biosorption will be advantageous to remove the acetaminophen in short period without generation of toxic product. Bioremediation of acetaminophen and its degradative product 4 aminophenol has been reported with mixed biofilm of *Trichoderma harzianum*/*Pseudomonas fluorescens* (Shabani et al., 2021). Similarly, mixed consortia of *Cladosporium alboflavescens* and 4 aminophenol degrading strains can be formulated to have safe degradative product from acetaminophen.

5. Conclusion

Pharma derived micropollutants must be removed from the occurrence site for environmental sustainability. Compared to physico chemical methods, biological methods are less expensive and promising. The current study concluded that Mycoremediation can be achieved with the selection of proper fungal strains and remediation approach. Lab scale studies can be further explored at the wastewater treatment plants to find the feasibility of the studies. In future, *in situ* and *ex situ* bioremediation of the soil and water contaminated with the pharmaceutical micropollutants can be achieved with fungal biomass. Further research is needed on the development of microbial consortia for the conversion of acetaminophen into less ecotoxic products.

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