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Evaluation of Enzyme Activities, Dye Degradation and Effect of Silver Nitrate on Bioremediation of Congo Red by Fungi Isolated from Kolli Hills

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ABSTRACT

Fungi are known to produce a number of compounds, including some industrially important enzymes. The aim of this study was to isolate and identify fungal species from leaf litter in Kolli hills region and evaluate their ability to degrade Congo red dye, a synthetic pollutant. In the current study, two fungal strains were isolated from the leaf litter of Kolli hills and identified as *Aspergillus niger* and *Colletotrichum acutatum*. The fungal strains were evaluated for their potential to produce cellulase and xylanase enzymes. The isolates were found to produce both the enzymes, with *Aspergillus niger* showing increased activity. *A. niger* and *C. acutatum* showed 95% and 92% of Congo red dye degradation respectively, which is highly efficient. The Decolourization of Congo red was found to be negatively influenced by the presence of silver nitrate. The optimum temperature for dye decolourization was found to be 30°C for *C. acutatum* and 35°C for *A. niger*. Fungal communities inhabiting leaf litter play crucial roles in nutrient cycling and ecosystem functioning. Additionally, their potential for degrading environmental pollutants presents promising avenues for bioremediation strategies.

Keywords: *Aspergillus niger*, *Colletotrichum acutatum*, Cellulase, Kolli hills, UV-spectrophotometer, Silver nitrate, Xylanase.

INTRODUCTION

Fungi, as nature's recyclers, possess a remarkable arsenal of enzymes capable of breaking down complex polysaccharides present in plant biomass. Among these enzymes, xylanases and cellulases play pivotal roles in the degradation of hemicellulose and cellulose, respectively, unlocking the energy and nutrients stored within these abundant renewable resources. Derived from fungal sources, xylanases and cellulases have garnered significant attention for their diverse applications in biotechnology, bioenergy, and environmental sustainability.

Xylanases are enzymes that catalyse the hydrolysis of Xylan, a major component of hemicellulose found in plant cell walls. Fungal xylanases exhibit remarkable efficiency in cleaving the β-1,4-glycosidic linkages of xylan molecules, leading to the release of xylooligosaccharides and xylose monomers. These enzymatic activities are pivotal for the degradation of hemicellulose-rich biomass, including hardwoods, softwoods, agricultural residues, and industrial by-products. Fungal xylanases are characterized by their broad substrate specificity, thermal stability, and pH tolerance, making them valuable biocatalysts for various industrial applications.

Cellulases are a group of enzymes responsible for the hydrolysis of cellulose, the most abundant polysaccharide in nature. Fungal cellulases encompass a suite of enzymes, including endoglucanases, exoglucanases (cellobiohydrolases), and β-glucosidases, which synergistically degrade cellulose into glucose monomers. Fungi secrete cellulases to break down cellulose-rich substrates, such as plant cell walls, wood, agricultural residues, and paper waste. (Tong and Wang *et al.* 2024). Notably, fungal cellulases exhibit high enzymatic activity, substrate specificity, and adaptability to diverse environmental conditions, rendering them indispensable tools for biorefinery processes, biofuel production, textile processing, and waste management.

Fungal xylanases and cellulases stand as exemplars of nature's biodegradative prowess, offering versatile enzymatic tools for unlocking the potential of renewable biomass resources. Their wide-ranging applications in biotechnology, bioenergy, and environmental management underscore their

significance in addressing global challenges related to resource utilization, waste valorisation, and sustainable development. Continued research efforts aimed at understanding the enzymatic mechanisms, substrate specificity, and biotechnological potential of fungal xylanases and cellulases hold promise for advancing green technologies and fostering a more sustainable future (Saini and Sharma *et al.* 2021).

The biodegradation of Congo Red dye by fungi follows multiple pathways, with various enzymatic reactions leading to the transformation of the dye molecule into simpler and less toxic intermediates (Wang, and Xu *et al.* 2017). These pathways involve sequential steps of oxidation, reduction, hydrolysis, and conjugation reactions, mediated by the concerted action of different enzymes present in the fungal metabolic machinery (Majumdar and Chowdhury *et al.* 2022).

The ability of fungi to degrade Congo Red dye has significant environmental implications, offering a sustainable and eco-friendly approach to remediate dye-contaminated environments. By harnessing the enzymatic capabilities of fungi, it is possible to mitigate the environmental impact of synthetic dyes and reduce their persistence in soil, water, and sediments. Moreover, fungal-mediated biodegradation of dyes can contribute to the restoration of ecosystems and the preservation of biodiversity by eliminating toxicants and restoring natural balance (Asses and Hamdi *et al.* 2018).

Leaf litter serves as a reservoir of organic matter in forest ecosystems, supporting diverse microbial communities crucial for nutrient recycling and soil health. Among these microorganisms, fungi play pivotal roles in decomposing complex organic compounds, including recalcitrant pollutants such as synthetic dyes. Congo red dye, a xenobiotic compound used extensively in various industries, poses environmental risks due to its persistence and potential toxicity (Mohammed and Khan *et al.* 2024). Fungi possess enzymatic machinery capable of degrading such pollutants, making them valuable assets in bioremediation strategies. This study aims to isolate and investigate the degradation of Congo red dye by fungal strains isolated from leaf litter in Kolli hills (Singh *et al.* 2014).

MATERIALS AND METHODS

Sample Collection

Leaf litter sample was collected near Semmedu-Solakkadu region coordinates are about [$11^0$17’21.0” N $78^0$21’56.1” E] in Kolli hills, located in the Eastern Ghats of India. Sampling was conducted in the month of September. Leaf litter was collected from the forest floor using sterile gloves and tools to minimize contamination. Samples were placed in a sterile collection bag and transported to the laboratory for further processing and stored in a bread box at 4°C.

Isolation of Fungal Strains

Upon arrival at the laboratory, leaf litter sample was processed for fungal isolation using a modified serial dilution plating technique (Bisby *et al.* 1933). Briefly, the leaf litter was homogenized using a sterile mortar and pestle, and the suspension was prepared by mixing 25 g of the homogenate with 225 ml of sterile distilled water. Serial dilutions of the suspension (10^{-3} to 10^{-5}) were then plated onto potato dextrose agar (five replicates) supplemented with 200 µg/ml chloramphenicol to inhibit bacterial growth and incubated at room temperature for 7 days.

Identification of Fungi

Following incubation, the dominant species were selected for further analysis. Pure cultures were obtained by streaking individual colonies onto fresh Czapek dox agar (CDA) plates and incubated at 35°C. Morphological characterization of fungal isolates was conducted using microscopy, with emphasis on colony morphology, hyphal characteristics, and spores. Representative specimens were photographed to document morphological features. The isolated fungal mycelia were placed on a glass slide and then stained with lactophenol cotton blue (1% v/v) and examined under a light microscope. Fungi isolated were identified using standard reference manuals (Gilman, 2001).

Qualitative analysis of enzyme activities

Cellulase and xylanase activity was screened based on clear zone formation on modified Czapek Dox Agar (CDA) media. 1% carboxy-methylcellulose for cellulase assay and birchwood xylan for xylanase assay was used as the sole carbon source in CDA (Teather and Wood 1982). Fungal isolates were inoculated on modified CDA and incubated at 35°C for 5 days. To visualize the clear zone, the plates were flooded with 0.5% (w/v) solution of Congo red, incubated for 15 minutes and counterstained with 1 M NaCl for 15 minutes. The

presence of clear yellow zones around the colonies of fungi was indicative of enzyme activity (Sari *et al.* 2017).

Quantitative Analysis of enzyme activities

The filtrate of 7 days old fungal isolates was obtained by passing the culture through Whatman No.1 filter paper and centrifuged at 2800 g for 10 mins to obtain the supernatant (Siva *et al.* 2021). 0.05 M citrate buffer was mixed with 1 ml of supernatant and 0.5 ml of 1% of CMC or Birchwood Xylan individually. It was incubated at 50°C for 30 mins followed by addition of 3 ml of 3, 5-dinitro salicylic acid (DNS), which was incubated in a water bath at 100 °C for 10 mins. Reaction mixture without the enzyme supernatant served as the control. Optical density of the samples was read at 540 nm in UV-spectrophotometer. One unit of cellulase or xylanase is expressed as the amount of enzyme producing 1 g of reducing sugar equivalent to glucose per minute (Miller, 1959; Bairagi, 2016).

Degradation of Congo red dye by fungi

The maximum absorption of Congo red was determined spectrophotometrically by reading the optical density of 100 mg/L of Congo red dye at a wavelength range of 340 to 640 nm. Mineral Salts Medium (MSM) with composition of KH_2PO_4 (0.5 g/L); K_2HPO_4 (0.5 g/L); $(NH_4)_2SO_4$ (1.5 g/L); $CaCl_2$ (0.1 g/L); $FeSO_4$ (0.07 g/L); $MgSO_4.7H_2O$ (1.0 g/L); KCl (0.5 g/L) and $FeSO_4.7H_2O$ (0.01 g/L) was used as the culture medium (Ameen *et al.* 2021). MSM broth (pH 7) containing 100 mg/L of Congo red dye as sole carbon source was inoculated with fungal isolates individually and incubated at room temperature with constant agitation to promote fungal growth and dye degradation. Modified medium without fungal inoculum served as the control (Khelifi *et al.* 2009). Aliquots of culture supernatant were withdrawn at regular intervals and centrifuged to remove fungal biomass. Absorbance of the supernatant was measured at 498 nm, and the degradation was expressed as the decolourization percentage (D) which is calculated as given below.

$$D = [(C1-C2) *100] / C1$$

Where C1 is the OD value of Congo red in control.

C2 is the OD value of Congo red with fungal inoculum drawn at a particular time.

Parameters affecting Congo red degradation:

The effect of incubation temperature on dye degradation was determined by storing the culture at 25, 30, 35, 40, 45 and 50 ºC. On the 6th day the culture was filtered, and the absorbance was measured at 498 nm. Similarly, the effect of silver nitrate solution was studied by adding 1mM/L of Silver nitrate to the culture on the 4th day and recording the absorbance on day 4, day 6 and day 8. Inoculum lacking silver nitrate served as the control for comparison. The decolourization percentage (D) was calculated as mentioned above.

Statistical Analysis:

ANOVA analysis was performed to evaluate the effect of culture time, temperature and silver nitrate on degradation of Congo red dye by fungal isolates. The mean of the factors with significant differences were compared at $p < 0.05$.

RESULTS AND DISCUSSION

Fungal isolation:

A total of 42 colonies were isolated from the leaf litter sample. There were totally 6 distinct fungal species observed out of which two dominant species were selected for further studies. From the morphological characters of the spores, the two dominant isolates were identified as *Aspergillus niger* and *Colletotrichum acutatum*. The fungal biodiversity in Kolli hills is quite underexplored. In a previous study, arbuscular mycorrhizal and endophytic fungi were reported to be present in plant taxa collected from Kolli hills (Muthuraja *et al.* 2014). Similarly, three laccase producing fungal species were identified out of 25 carpophores collected from Kolli hills (Rathinasamy and Thayumanavan 2018). In the current study, *Aspergillus* was found to be the most dominant species followed by *Colletotrichum* in the leaf litter collected from Kolli hills. The two dominant strains were selected for evaluating enzyme activities and dye degradation capacity.

Qualitative analysis of enzyme activities

Both the strains were subjected to cellulase and xylanase plate assay using congo red. The formation of a clear yellow zone (Fig. 1) confirmed the ability of both the isolates to produce cellulase and xylanase enzymes. The fungal isolates were able to degrade CMC and xylan substrate by producing the corresponding enzymes, which led to

the decolorization of congo red causing the clear zone. The diameter of the clear zone is given in table 1, suggesting higher enzyme activities in *A. niger* when compared to *C. acutatum.* Both cellulase activity and xylanase activity were high in *A. niger* with 4.7 cm and 4.2 cm of zone diameters respectively.

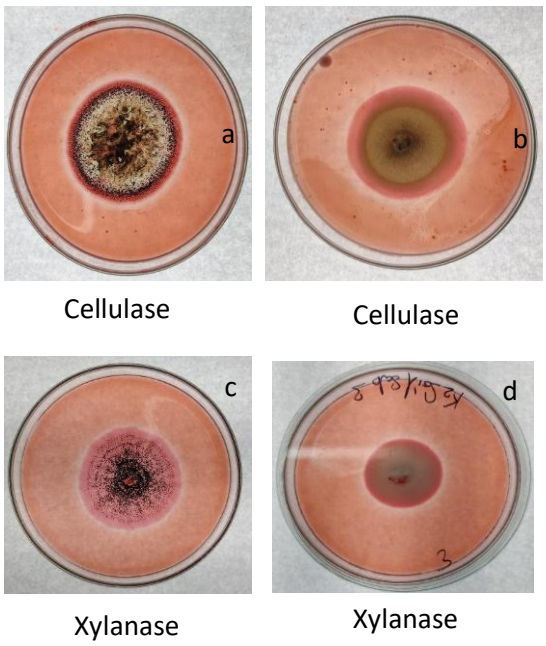

Figure 1: Screening of fungal isolate for enzyme activities

Table 1: Cellulase and xylanase activity exhibited by fungal isolates

Enzyme	Diameter of clear zone (in cm)	
	Aspergillus niger	***Colletotrichum acutatum***
Cellulase	4.7	4.1
Xylanase	4.2	3.6

Clear zone formation representing cellulase activity in (a) *Aspergillus niger* and (b) *Colletotrichum acutatum*. Zone formation representing xylanase activity in (c) *Aspergillus niger* and (d) *Colletotrichum acutatum*

Quantitative Analysis of enzyme activities

The enzyme activity was evaluated quantitatively by the DNS method. In case of cellulase activity *A. niger* showed marginally higher activity than *C. acutatum. A. niger* showed 15 U/ml of xylanase activity whereas *C. acutatum* showed 11.1 U/ml activity (Fig. 2). Many previous studies have reported cellulolytic activity in *Aspergillus* sp. Sivaramanan (2014) isolated fungal species from saw dust, sprinkled soil and decaying leaves from various locations. Isolated a total of 21 fungal species, of which *Helminthosporium* sp. was reported to show maximum cellulolytic activity, with *Aspergillus* sp. also showing significant activity. Kumar et al. (2014) isolated 11 fungal species from forest soil and leaf litter, of which *Trichoderma viride* and *Aspergillus niger* were able to produce cellulase. Martínez-Pacheco et al. (2009) utilized the lignocellulose from tequila industry waste as a carbon source to produce sugar by enzymatic degradation of cellulose waste. They reported high production of cellulase enzyme by the Colletotrichum species. In the current study cellulase production was slightly higher in *A. niger* than *C. acutatum.*

Number of researchers have reported the ability of various *Colletotrichum* species to produce xylanase enzyme (Ramanjaneyulu *et al.* 2015; Carli *et al.* 2016; Conejo-Saucedo *et al.* 2017 and Flores *et al.* 2023). But there is no report on the *C. acutatum* species in particular. Here we have reported the positive xylanase activity of *C. acutatum.* Previous reports have highlighted the ability of *A. niger* to produce xylanase enzyme (Tallapragada *et al.* 2011; Sarangi *et al.* 2024). In the present study, cellulase activity was higher in both the species when compared to xylanase activity.

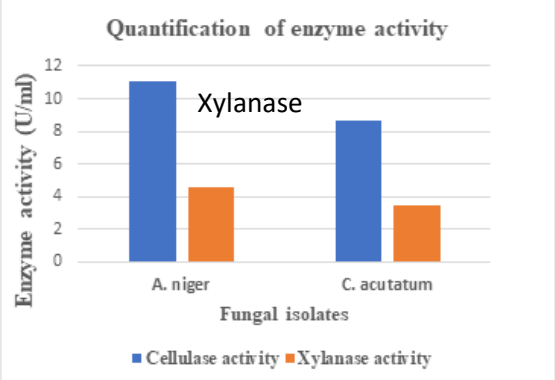

Figure 2: Quantitative analysis of enzyme activities in fungal isolates

Absorbance maxima of Congo red

In order to assess the degradation capacity of fungi, initially the maximum absorption of Congo red was measured between 370 and 610 nm wavelength. The maximum absorption was detected at a wavelength of 498 nm. The spectrum is represented

in (fig. 3). Further spectrophotometric studies were carried out at 498 nm wavelength.

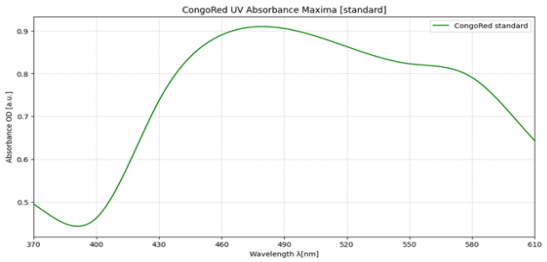

Figure 3: Absorbance of Congo red dye

Absorbance maxima of Congo red dye by isolated fungi

There are several mechanisms by which fungi degrade the Congo red dye. Potential amount of the dye gets removed by adsorption onto the mycelium (Binupriya *et al.* 2008). The filamentous nature gives an edge to the fungi over bacteria as a bioremediation agent. Both the fungal isolates showed significant degradation of the dye (fig. 4,5,6). Almost 50% of the dye was degraded within 5 days of culture growth. Up to 95% of degradation was achieved in 8 days of culturing. The rate of degradation was marginally high in *A. niger* than *C. acutatum*. Similar observations were reported by Singh and Dwivedi (2022). They reported a 93.94% decolorization by *A. niger*. Mohan et al. (2015) showed 93.21% of decolorization by *A. niger*. There are not many reports available on the Congo red degradation by *C. acutatum*, however other *Colletotrichum* species have been found to degrade the dye effectively (Bharti *et al.* 2023; Sheik *et al.* 2020). In the present study *C. acutatum* was found to degrade 92% of the dye in 8 days. This report suggests that both the leaf litter isolates can be considered as good biodegradation agents for toxic industrial wastes.

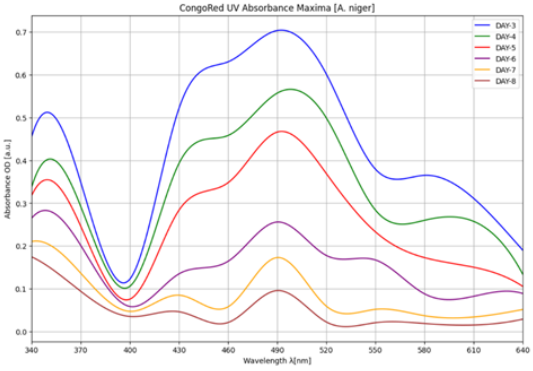

Figure 4: Degradation of Congo red by the fungal isolate A.niger

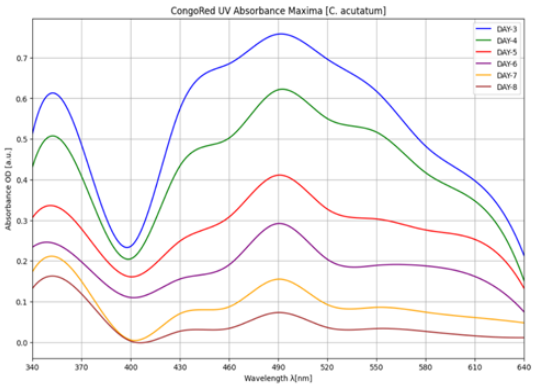

Figure 5: Degradation of Congo red by the fungal isolate C. acutatum

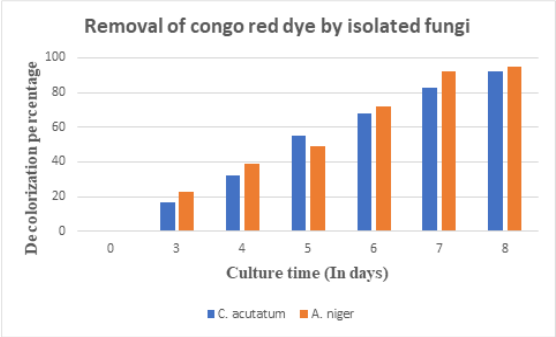

Figure 6: Degradation of congo red by the fungal isolates

Effect of temperature on biodegradation

The influence of temperature on the degradation capacity of the isolates was studied by maintaining the dye added cultures at 6 different temperatures. In case of *A. niger,* maximum activity was observed at 35°C (77%) on day 6. *C. acutatum* showed a maximum of 67% degradation at 30°C (Fig. 4,5,6). Further increase in temperature caused a steady decline in the degradation activity. Singh and Dwivedi (2022) reported an optimum temperature between 25–35°C for maximum dye degradation, which synchronizes with the current report. The temperature mainly impacts the enzyme activities, which is one of the important mechanisms of dye degradation. Chatterjee et al. (2020) stated that the manganese (II) peroxidase activity was optimum at 40°C whereas laccase activity was optimum at 30°C. Asses et al. (2018) correlated the dye degradation with manganese peroxidase and lignin peroxidase production. They observed that 30°C was optimum for the degradation and stated that there was an inactivation of the enzymes and decline in the cell viability of increasing the temperature further.

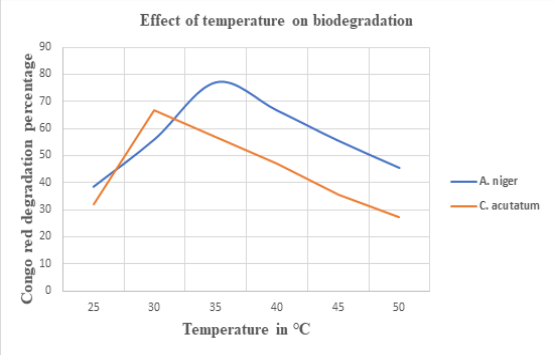

Figure 5: Temperature dependant degradation of Congo red dye

Effect of Sliver nitrate on biodegradation

The effect of silver nitrate on degradation was done by adding $AgNO_3$ on the 4th day of culture period. On $AgNO_3$ addition, there was an instant spike in the OD value observed, which led to a decrease in the degradation percent (Fig. 6). Precipitation was observed due to $AgNO_3$ addition thereby causing a sudden spike in the OD values, which was otherwise not observed in the culture without $AgNO_3$ (control). While 41% degradation was observed in *C. acutatum* control culture, only 15% degradation was obtained in the culture with $AgNO_3$. Similarly, *A. niger* control culture showed 43% degradation whereas $AgNO_3$ added culture showed only 27% degradation. Subsequently on day 6 and day 8 there was further decline in the degradation rate rather than increase. This depicts the distress imposed on both the fungal isolates by $AgNO_3$ causing a decline in the growth of the fungi. Previous report suggests that the silver ions tend to inactivate the enzymes and disrupt the plasma membrane increasing the permeability, thereby causing death of the microbes (El Kadi *et al.* 2018). In the current study it is apparent that silver nitrate has a negative impact on the cell growth, thereby the dye degradation activity making it an unsuitable agent.

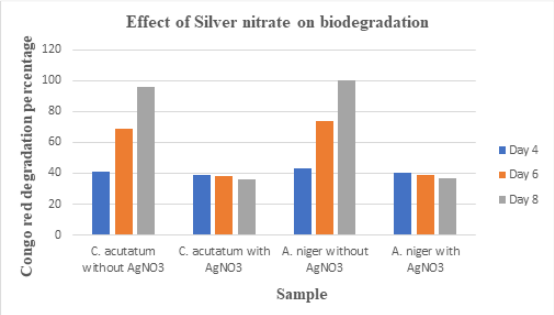

Figure 6: Silver nitrate affecting the dye degradation ability of fungal isolates

CONCLUSION

This report throws light on the biodegradation capacity of fungal isolates from leaf litter samples of Kolli hills. About 95% and 92% of degradation was achieved by *A. niger* and *C. acutatum* isolates respectively in 8 days of culture. The effect of temperature and silver nitrate on degradation activity was studied and reported. The cellulase and xylanase enzyme activities were evaluated and found to be significantly high in both the isolates. The dye degradation capacity was found to be highly efficient making the source leaf litter and the isolates, potential bioagents for industrial wastewater treatment.

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