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Biodegradation of the Ciprofloxacin Antibiotic by White Rot Fungus *Trametes hirsuta* D7

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Abstract

This study highlights the potential of fungal microorganisms, particularly *Trametes hirsuta* D7, in addressing antibiotic contaminants, such as ciprofloxacin, in the environment. The degradation process was conducted at room temperature over 7 days, and the results demonstrated that laccase was predominant in the degradation capacity; this was evidenced by the laccase enzyme activity levels obtained, namely 93 U/L, 120 U/L, and 130 U/L, compared to manganese peroxide activity of 7 U/L, 16 U/L, and 13 U/L at concentrations of 100, 300, and 500 mg/L, respectively. Notably, the laccase enzyme of *T. hirsuta* D7 exhibited significant degradation of ciprofloxacin, with high degradation rates of 64% at 100 mg/L, 48% at 300 mg/L, and 26% at 300 mg/L. This indicates that laccase from *T. hirsuta* D7 effectively degraded ciprofloxacin at various concentrations. Furthermore, this study revealed that ciprofloxacin did not significantly affect the growth of *T. hirsuta* D7. This suggests that microorganisms can survive and function effectively in the presence of antibiotic contamination without being impaired by these compounds. In conclusion, this study presents a potential solution for environmental antibiotic contamination by utilizing fungal microorganisms, particularly *T. hirsuta* D7, and their laccase enzymes. The findings of this study provide valuable insights for developing more environmentally sustainable and efficient degradation methods for antibiotic contamination in the ecosystem.

1. Introduction

The use of antibiotics in some countries continues to rise yearly, leading to a proportional increase in negative impacts on ecosystems. Antibiotics are widely prescribed to treat infectious diseases in humans and animals [1]. Ciprofloxacin (CIP, $C_{17}H_{18}FN_3O_3$) is a commonly broad-spectrum antibiotic used for bacterial infections. It belongs to the fluoroquinolone class and is effective against gram-positive and gram-negative bacteria. It has been used for over 20 years to treat various diseases and inhibit the growth of harmful bacteria in the body [2, 3]. However, CIP is one of the most common contaminants in aquatic environments and soil [4]. It typically enters the environment through wastewater discharge from pharmaceutical factories, hospitals, and households where unused or expired drugs are improperly discarded. This widespread contamination has contributed to the development of antibiotic-resistant bacteria, posing significant risks to environmental and human health [5].

Antibiotic contamination in the environment can be mitigated through several methods, including physical, chemical, and biological. Chemical methods, such as the study by Wagner *et al.* [6], demonstrated the use of Fe_2O_3 nanostructured particles for the photodegradation of CIP under terrestrial solar radiation, achieving nearly 20% removal of CIP from clinical wastewater. However, this method involves high system maintenance and expensive chemical reagents. Other studies have explored using

chlorinated compounds as disinfectants or oxidizing agents, but these reactions can generate carcinogenic byproducts [7]. Additionally, methods like ultraviolet irradiation, ozonation, photocatalytic oxidation, Fenton reactions, and electrocatalysis face significant challenges, including high costs and complex operational requirements [1].

Biological degradation methods provide an environmentally friendly alternative for degrading pollutants in situ [8]. Bacteria, algae, and fungi are some of the most common degraders—*Pseudomonas* sp. SF1 and A12 have demonstrated effective antibiotic degradation capabilities, remaining viable through three consecutive degradation cycles [9]. Algae-based degradation technologies are also effective but present significant challenges. These complex and time-consuming systems rely on genetic engineering and are influenced by numerous factors, such as pH, temperature, CO_2 enrichment levels, light intensity, algae species, substrate concentration, and the structure of the target antibiotic [10].

Fungi, particularly white-rot fungi (WRF), degrade antibiotics more efficiently than bacteria and algae due to their powerful enzyme systems and flexibility. Unlike bacteria and algae, which have limited substrate specificity and are more susceptible to environmental changes, fungi may survive in a wide range of conditions, including high pH, temperature, and nutrient limitations. Their high effectiveness is due to extracellular enzymes, especially lignin, manganese peroxidase, and laccase, which may degrade the complicated aromatic compounds of many antibiotics [11]. Laccase and manganese peroxidase (MnP) break down antibiotics via oxidation. Laccase converts oxygen into radicals that damage the antibiotic's structure, breaking it into smaller, less toxic molecules. Meanwhile, MnP oxidizes manganese ions (Mn^{2+}) into Mn^{3+} using hydrogen peroxide (H_2O_2), breaking the chemical bonds of antibiotics. Both enzymes can digest various antibiotics, making them valuable for decreasing pollution in water and soil [12, 13, 14].

Several studies have demonstrated the ability of white-rot fungi and their enzymes to degrade antibiotics. Research by Cuprys *et al.* [12] showed that *Trametes versicolor* reduced ciprofloxacin concentration by up to 68% within 24 hours. Similarly, de Araujo *et al.* [15] observed the degradation of sulfamethoxazole and trimethoprim by 74% and 40%, respectively, within a 15- day incubation period using *Pleurotus ostreatus* and *Pleurotus pulmonarius*. The rapid decrease in ciprofloxacin levels by 95% over just 14 days, as reported by Singh *et al.* [16] and Bai *et al.* [4], underscores the potency of the isolates and enzymes. In previous studies by Roy *et al.* [3], laccase was demonstrated to degrade trimethoprim antibiotics by 95% successfully. On the other hand, a study by Lueangjaroenkit *et al.* [17] identified two manganese peroxidases and a laccase enzyme from the white-rot fungus *Trametes polyzona*, which degraded ciprofloxacin with a degradation activity of 73%. Moreover, Gao *et al.* [11] showed that white-rot fungi *Pycnoporus sanguineus* could remove 99% of CIP within 48 hours.

Based on the discussion above, white-rot fungi and their enzymes have shown strong potential for degrading environmental antibiotic pollutants. However, the ability of *T. hirsuta* D7, a recently discovered basidiomycete from decaying wood, to degrade ciprofloxacin has not been thoroughly studied. Due to its promising features, *T. hirsuta* D7 is an important subject for further research. Thus, this study aims to investigate the degradation potential of *T. hirsuta* D7 and its enzymes, including laccase and MnP.

2. Experimental

2.1. Chemicals and Reagents

Ciprofloxacin HCl monohydrate commercial tablets (500 mg) were purchased from the local drugstore. The following chemicals and reagents were used, malt extract agar (MEA) (Merck, Germany), malt extract broth (MEB) (Merck, Germany), glucose (Merck, Germany), peptone (Sigma Aldrich, USA), 0.1 M acetate buffer pH 5, ethanol (Merck, Germany, 95%), ethyl acetate (1:1, v/v) (Merck, Germany, 99%), 2,2'-azino-bis(3-ethylbenzothiazoline -6-sulfonic acid (ABTS) (Sigma Aldrich, USA), 50 mM malonic buffer pH 4.5 (Sigma Aldrich, USA), 20 mM dimethoxyphenol (DMP) (Sigma Aldrich, USA), manganese sulfat ($MnSO_4$) (Merck, Germany), and 2 mM hydrogen peroxide (H_2O_2) (Merck, Germany).

2.2. Tools and Instruments

The tools used in this study were Pyrex glassware (Corning Inc., USA), hot plate stirrer (Thermo Fisher Scientific, USA), stirrer bar, shaker incubator BioShaker BR-43 FM (Taitec, Japan), laminar airflow, analytical balance (Mettler Toledo, Switzerland), Autoclave LAC-510 5D, blender waring 8010BU SS 610, oven (Labtech, Italy), centrifuge Suprema 21 (Tomy Seiko Co., Ltd, Japan), homogenizer ACEAM-11 (Nissei, Japan), centrifuge tubes, spatula, microtube, micropipette, incubator, and Spectrophotometer UV-Vis 1800 (Shimadzu, Japan).

2.3. Fungal Strains and Inoculum Preparation

White-rot fungus *Trametes hirsuta* D7 (NCBI GenBank accession number KX444204; collection of Microbial Composite Research Group, Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN)) isolated from peat swamp forest in Bengkalis, Riau was used in this study. The stock culture of *T. hirsuta* D7 was maintained at MEA at 4°C. One plug (Ø 0.8 mm) of fungi from stock culture was inoculated on MEA and incubated for 7 days at room temperature (~25-30°C) to prepare the inoculum. After the incubation, *T. hirsuta* D7 was ready to be used for the degradation process [16].

2.4. Fungal Growth Inhibition by Ciprofloxacin (CIP)

Fungal resistance to different concentrations of CIP was studied according to the method by Rodarte-Morales *et al.* [18]. One plug (Ø 0.8 mm) of *T. hirsuta* D7 was inoculated on MEA agar media containing various concentrations of the antibiotic ciprofloxacin at 100 mg/L, 200 mg/L, 300 mg/L, and 400 mg/L, respectively. The plates were incubated at room temperature (~25-

30°C) for 7 days. The growth of the fungus was monitored, and the hyphal extension of each fungus was measured from the center of the colony to the edge of the plate every two days.

2.5. In Vivo Degradation of Ciprofloxacin (CIP)

In vivo, degradation of CIP refers to the modified research of Gao *et al.* [11]. Three plugs of *T. hirsuta* D7 colony (Ø 0.8 mm) were inoculated into a 100 mL Erlenmeyer flask containing 20 mL of MGP medium. The culture was then incubated for 7 days at room temperature (~25-30°C). Following incubation, the antibiotic ciprofloxacin was added to the fungal culture at the final concentrations of 100 mg/L, 300 mg/L, and 500 mg/L, respectively. Each treatment was carried out in triplicate. The cultures were then re-incubated for 7 days at room temperature (~25-30°C).

Additionally, two types of controls were prepared: (1) fungal culture without the addition of antibiotics as biotic control and (2) antibiotics without fungal inoculation as abiotic control. After 7 days, the fungal culture was centrifuged at 10,000 rpm, 4°C, for 10 minutes to separate the solid (pellet) and supernatant. The solid was weighed to determine the wet weight of the biomass (mycelium). The absorbance and enzyme activity of the supernatant were measured using a UV-Vis spectrophotometer. The standard curve of CIP (5-40 mg/L) was used to comparatively assess and determine the concentration of CIP left in the samples after the degradation experiment. The degradation of ciprofloxacin was determined by calculating the percentage removal of CIP using Equation (1).

$$Degradation\ (\%) = \frac{C_0 - C_1}{C_0} \times 100\% \quad (1)$$

Where, C_o is the initial concentration of CIP before degradation, and C_1 is the concentration of CIP at the end of the degradation experiment.

2.6. Enzyme Activity Assay

Laccase activity was determined using 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate according to the method by Alam *et al.* [19]. The sample (0.1 mL) was mixed into a cuvette containing 0.4 mL of 0.1 M acetate buffer (pH 4.5) and 0.5 mL of 2 mM ABTS. Enzyme activity was monitored at a wavelength of 420 nm for 60 s using a UV-Vis spectrophotometer and calculated from the molar extinction coefficient (ε) of 36,000 $M^{-1}cm^{-1}$. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute. Manganese peroxidase (MnP) activity was determined using DMP as a substrate according to the method by Anita *et al.* [20]. Sample (0.1 mL) was mixed into a cuvette containing 0.175 mL of 50 mM malonic buffer (pH 4.5), 0.125 mL of 10 mM DMP, 0.125 mL of 20 mM $MnSO_4$, and 0.3 mL of 2 mM H_2O_2. The MnP activity was measured at a wavelength of 470 nm using a UV-Vis spectrophotometer and calculated from the molar extinction coefficient (ε) of 49,600 $M^{-1}cm^{-1}$. One unit of MnP was defined as the enzyme needed to oxidize 1 mmol of Mn (II) to Mn (III) per minute. Enzyme activity (U/L) was calculated using Equation (2).

$$\text{Enzyme activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{Abs(t) - Abs(0) \times V_{reaction}\,(mL) \times 10^3}{\varepsilon \times V_{enzyme}(mL) x\, t} \quad (2)$$

Where, Abs(t) is the final absorbance, Abs(0) is the initial absorbance, t is the reaction time (minute), 10^3 is the correction factor (µmol/mol), ε is the molar extinction coefficient, and V is the volume (mL).

3. Results and Discussion

3.1. Fungal Growth Inhibition by Ciprofloxacin (CIP)

The growth rate of *T. hirsuta* D7 isolates following the addition of CIP at concentrations of 100, 200, 300, and 400 mg/L exhibited a notable trend. Optimal growth was observed for 7 days until it reached the periphery of the petri dish (Table 1), indicating a distinct response to the CIP. The incubation duration and the medium selection were critical factors influencing mycelial growth. MEA was the medium that exhibited significant growth rates and the most favorable appearance with denser mycelia (Table 1), highlighting its significance in this research. MEA contains malt extract, a primary energy source for fungal growth.

The results of our fungal isolation from WRF colonies were noteworthy, as we obtained four pure isolates from 4 different concentrations (Table 1). The characteristics of the WRF isolate, as observed in terms of morphology, are defined by the distinctive color of its mycelia and spores. Mycelia, the multicellular fungal structures formed by an aggregation of hyphae, and spores, the fungal reproductive units, were meticulously examined—macroscopic identification involved comprehensively observing mycelia, spores, and colony surfaces. Macroscopically, the WRF fungus was identified by its characteristic white mycelia and spores. The surface of WRF fungal colonies appeared flat but exhibited a rough, fibrous texture and smooth margins.

The growth of D7 colonies in the control and media containing CIP was significant; more than 1 cm increased daily. However, on days 5 to 7, the growth of *T. hirsuta* isolates took an unexpected turn and became fixed (Figure 1). This indicates that the presence of CIP in MEA has no significant effect on the colony growth rate of isolate D7, even though the concentration has increased. MEA contains a high maltose concentration, making it suitable for yeast and mold growth.

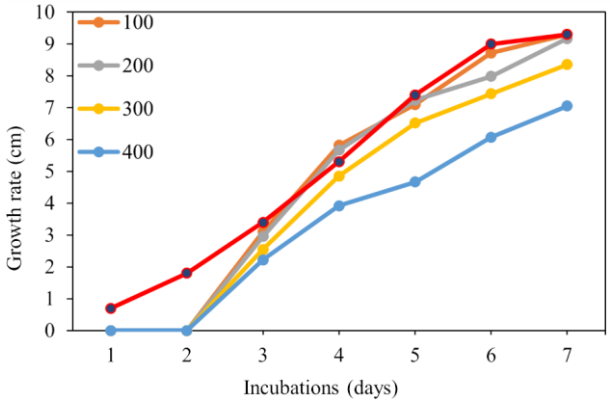
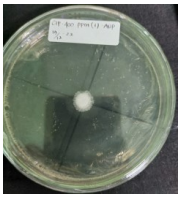
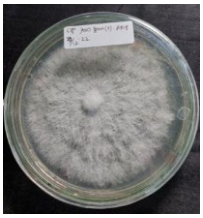
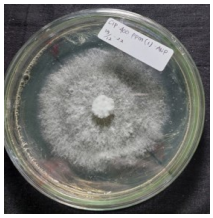


Figure 1. The growth rate of isolate *T. hirsuta* D7 against CIP degradation

Table 1. The optimum growth *T. hirsuta* D7

Day	Concentration of ciprofloxacin (mg/L)				
	0 (Control)	100	200	300	400
1					
7					

Our findings indicate a clear relationship between antibiotic concentration and fungal growth. At a concentration of 400 mg/L, the fungal isolate exhibited a significantly smaller diameter compared to the other concentrations on day 7. This observation suggests that the 400 mg/L antibiotic concentration may inhibit fungal growth and reduce the lignolytic capacity of fungi at elevated antibiotic concentrations. The fungus' secretion of enzymes such as lignin peroxidase (LiP), MnP, and laccase when adhered to the substrate (antibiotics) further corroborates this finding, as it facilitates the degradation of pollutants at varying concentrations.

3.2. Enzyme Activity Assay

The white-rot fungi *T. hirsuta* D7 is known for breaking down lignin in wood, leaving behind lighter-colored cellulose by producing oxidative enzymes [21]. These enzymes include laccase, MnP, and LiP. This study, however, focuses specifically on measuring laccase and MnP activity since they often work simultaneously in degrading antibiotics. During the degradation assays, laccase and MnP activities were measured. It was observed that with the increase in antibiotic concentration, the enzymatic activity of the fungus also increased (Table 2). The greater concentration of antibiotics creates stronger selective pressure, causing fungi to make more laccase to deal with the higher toxic environment [22].

Table 2. Enzyme activity of *T. hirsuta* D7 during CIP degradation

Concentration of ciprofloxacin (mg/L)	Activity enzyme (U/L)	
	Laccase	MnP
100	93	7
300	120	16
500	130	13

The laccase activity of the *T. hirsuta* D7 was higher than that of MnP. The maximum laccase activity observed in this study was 130 U/L at a CIP concentration of 500 mg/L. However, the maximum MnP activity observed in this study was 16 U/L at a CIP concentration of 300 mg/L (Table 2). Laccase activity was generally higher than MnP activity during antibiotic degradation by fungi due to its broader substrate specificity, lower catalytic requirements, and greater environmental stability. Unlike MnP, laccase can degrade phenolic and non-phenolic compounds without additional cofactors such as Mn^{2+} or H_2O_2. Laccase has a low redox potential, enabling it to catalyze only the single-electron oxidation of lignin phenolic constituents that are easily oxidized, coupled with the simultaneous reduction of O_2 to water.

Nonetheless, many antibiotics have non-phenolic structures that are challenging to degrade. Using mediators like ABTS, laccase activity can be enhanced to target and oxidize these non-phenolic components of antibiotics through an electron transfer mechanism, improving the overall efficiency of the degradation process [23, 24, 25, 26]. The maximum activity of laccase obtained in this study was higher than that of laccase from the WRF strain *T. versicolor* obtained by other researchers, which is <100 U/L [27].

3.3. In Vivo Degradation of Ciprofloxacin

Determination of CIP concentration starts with preparing a standard curve, which establishes the relationship between the antibiotic concentration in a sample and its absorbance value. This standard curve was generated using a UV-Vis spectrophotometer, with a blank sample prepared using distilled water as the solvent. A 1000 mg/L CIP stock solution was diluted to concentrations ranging from 10 mg/L to 40 mg/L, and the absorbance values of these solutions were measured. The standard curve equation, y = bx ± ay, was then used to calculate the remaining concentration of antibiotics in the sample after degradation based on the absorbance values obtained.

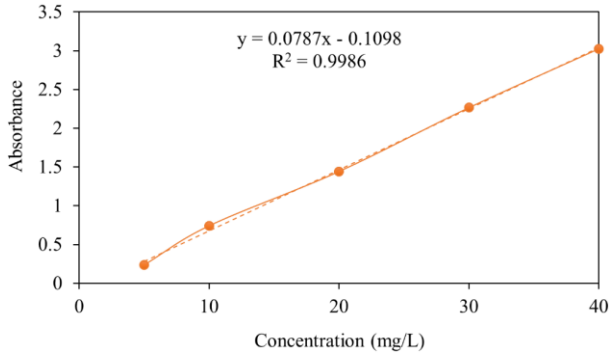


Figure 2. Standard curve of CIP

The standard solution test results showed a linear regression coefficient (R^2) of 0.9986, indicating a strong linear relationship between concentration and absorbance, with most data points closely aligning along a straight line. An R^2 value within the range of $0.9<R^2<10$ is considered excellent, demonstrating that in this study, the correlation between the antibiotic solution concentration and the instrument response is highly accurate (Figure 2). This standard curve plays a crucial role in determining the percent degradation of the antibiotic.

The ability of the laccase enzyme to degrade phenolic and non-phenolic compounds by reducing molecular oxygen followed by one-electron oxidation of the reducing substrate shows the potential for enzymatic degradation to be used in degrading fluoroquinolone antibiotic contaminants in the environment [28]. The *T. hirsuta* D7, a specific strain known for its high laccase enzyme production, was utilized in this study. Laccase activity significantly influenced the extent of ciprofloxacin degradation. The results showed that the highest degradation percentage of ciprofloxacin occurred at a ciprofloxacin concentration of 100 mg/L (64%), followed by 300 mg/L (48%) and 500 mg/L (29%) (Figure 3).

It was observed that with the increase in CIP concentration, the degradation percentage decreased. This result is in line with the findings of Mathur *et al.* [28], who reported that the percent degradation of levofloxacin antibiotics using crude laccase from *Pleurotus eryngii* decreased along with increasing levofloxacin concentration. This may be due to the greater strength of the laccase enzyme required to degrade ciprofloxacin antibiotics in large concentrations. Meanwhile, research by Navada and Kulal [29] suggests that this trend can be explained by Michaelis-Menten degradation kinetics. According to this model, the initial degradation rate follows a first-order reaction but eventually becomes independent of the substrate (antibiotic) concentration, transitioning to zero-order kinetics. In other words, while the degradation rate initially increases with higher antibiotic concentrations, it ultimately decreases because the enzyme cannot effectively oxidize antibiotics at excessively high concentrations.

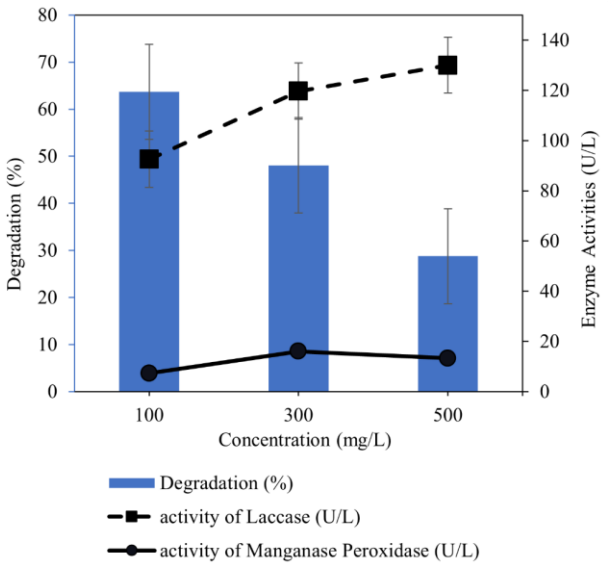


Figure 3. The degradation percentage of CIP and enzyme activity during CIP degradation by *T. hirsuta* D7

CIP is very difficult to degrade; therefore, it may take more than 7 days. This study showed that the maximum degradation of CIP (64%) by *T. hirsuta* D7 occurred at a CIP concentration of 100 mg/L after 7 days of incubation. Gao *et al.* [11] demonstrated the degradation of CIP by white-rot fungi *Phanerochaete chrysosporium*, which removed 65% of 10 mg/L of CIP after eight days of incubation. Another previous study on CIP degradation by other fungi demonstrated that the maximum degradation of CIP (10 mg/L) by the white-rot fungi *Irpex lacteus*, *Dichomitus squalens*, and *Pleurotus ostreatus* reached 60%, 23%, and 37%, respectively, after 14 days of incubation [30]. A study by Manasfi *et al.* [31] demonstrated that *Trichoderma asperellum* achieved an 81% degradation rate of 200 mg/L of CIP after 13 days of incubation. By comparing these data, it can be concluded that *T. hirsuta* D7 is capable of degrading the antibiotic ciprofloxacin and shows potential for use in degradation applications.

4. Conclusion

This study highlights the significant potential of white-rot fungi *T. hirsuta* D7 and its laccase enzyme in degrading the antibiotic ciprofloxacin, achieving notable efficiency in just 7 days. Significant degradation of ciprofloxacin at concentrations of 100, 300, and 500 mg/L (64%, 48%, and 29%, respectively) further confirms the ability of this enzyme to overcome antibiotic contamination in pharmaceutical waste. These findings confirm the effectiveness of laccase enzyme *T. hirsuta* D7 in the degradation of these chemicals and open promising prospects for its application in pharmaceutical waste treatment. Future research could focus on improving the degradation efficiency, developing broader enzyme application techniques, and evaluating the sustainability of using this enzyme at an industrial scale for more environmentally friendly pharmaceutical waste management.

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