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Research Article

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Characterisation of Laccase Isolated from Pleurotus pulmonarius for Antibiotic Degradation Potential

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ABSTRACT

The study aimed to characterize a laccase enzyme isolated from the white rot fungi Pleurotus pulmonarius and to evaluate its potential in biodegradation of antibiotics. Laccase was produced by the isolated white rot fungi under solid-state fermentation supplemented with rice bran as the lignocellulosic support. A time-course study for enzyme production indicated that day ten (10) gave the maximum enzyme production. The crude Pleurotus pulmonarius laccase (Ppl) was precipitated by 80 % ammonium sulphate precipitation and purified using a Sephadex G-100 column, which was equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). The optimum pH and temperature of the laccase were 3.5 and 60°C, respectively. The stability of Pleurotus pulmonarius laccase to temperature was studied, at various temperatures of 50, 60, and 70 °C in which the enzyme activity was most stable at 50 °C after incubating for 120 min. Additionally, the enzyme was more stable at a pH of 6.0. The maximum velocity (Vmax) and Michaelis constant (K_m) of the enzyme are 3333.33 µmol/min and 0.6667 µM, respectively using 2,2 azino bis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) as substrate. Furthermore, metal ions significantly influenced laccase catalytic activity. The effect of some metal ions on the activation of enzyme activity was in the following order Cu2+> Mg^{2+}> Zn^{2+} > Mn^{2+} > Ba^{2+}. The enzyme was characterised and evaluated in vitro for its potential for antibiotic degradation. The spectral studies on the degradation of antibiotics revealed that Pleurotus pulmonarius laccase significantly decreased the absorbance of the antibiotics at their maximum absorption wavelength at different concentrations as the incubation time was increased. Hence, the findings highlight that Pleurotus pulmonarius laccase (Ppl) has the potential for antibiotic degradation, opening new avenues for biotechnological applications. The utilization of enzymatic bioremediation, particularly employing laccases from lignin-degrading fungi such as Pleurotus pulmonarius, offers a promising avenue for targeted degradation of pharmaceutical pollutants. The significance of this research lies in its potential to offer a sustainable and efficient approach to pharmaceutical wastewater treatment, promoting the removal of antibiotics and reducing the risk of antibiotic resistance in the environment. Furthermore, the outcome of this study can contribute to the development of environmentally friendly practices in pharmaceutical industries, fostering a more responsible and eco-conscious approach to wastewater management.

Keywords: Biodegradation, Laccase, Antibiotics, Pharmaceutical Wastewater, Pleurotus Pulmonarius, White Rot Fungi, Rice Bran

Introduction

The presence of antibiotics in wastewater streams has raised concerns about the emergence of antibiotic-resistant bacteria and their potential impact on human health and ecological systems (Santás-Miguel et al., 2020). The accumulation of antibiotics in wastewaters causes selective pressure on the bacterial strains present in that niche which forces them to develop various resistance mechanisms that act as defence strategies against antibiotics for them to survive in an antibiotic-loaded water environment and trigger the development of antibiotic resistance [1]. According to the United Kingdom Government-

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commissioned Review on Antimicrobial Resistance stated that by 2050, ABR might lead to the death of 10 million people each year [2]. Hence, there is an urgent need to develop efficient and sustainable treatment methods to mitigate the adverse effects of antibiotics in wastewater in the environment as conventional wastewater treatment processes are ineffective and not environmentally safe [3]. The utilization of enzymatic bioremediation, particularly employing laccase from lignin-degrading fungi such as Pleurotus pulmonarius, offers a promising avenue for targeted degradation of pharmaceutical pollutants. Laccases are blue multicopper oxidases, found in various species of bacteria, fungi, insects, and plants, they possess the capability to oxidize a wide range of organic compounds, including antibiotics, through non-specific mechanisms [4]. Laccase-based degradation offers a potential strategy to address the issue of environmental antibiotic pollution, a contributing factor to resistance development. By degrading antibiotics in wastewater and potentially other sources, laccase enzymes can help reduce the environmental load of antibiotics and potentially slow down the emergence of resistance [5]. Enzymatic processes have advantages over the traditional chemical processes which include mild reaction conditions, higher specificity and low waste generation (Mathur et al., 2021). Biocatalytic reactions mediated by these enzymes involve cleaving of chemical bonds and transfer of electrons in an oxidation-reduction reaction during which the contaminants are oxidized to harmless compounds (Varga et al., 2019).

The study justification lies in the urgent need to address the environmental and public health risks posed by the presence of antibiotics in pharmaceutical wastewater. Antibiotics in wastewater can lead to the development of antibiotic-resistant bacteria, which is a global concern. Enhancing the degradation of antibiotics using Pleurotus pulmonarius laccase, contributes to the development of effective and sustainable wastewater treatment strategies as laccase provides an eco-friendly strategy for the transformation of harmful pollutants to less or non-toxic compounds, as it acts oxidatively on the aromatic ring of a wide range of compounds including antibiotics, releasing water as the only by-product [6]. This approach has the potential to reduce the release of antibiotics into the environment, mitigate the emergence of antibiotic-resistant bacteria, and ultimately safeguard both ecosystems and human health, hence the justification for the study.

Materials and Methods

Chemicals and Reagents

All the chemicals used in the study were of analytical grade and were obtained directly from the manufacturers or purchased from a local commercial vendor. Major chemicals include; 2, 2′ azino-bis (3-ethylbenz-thiazoline-6- sulfonic acid) (Sigma-Aldrich, Germany), Bovine Serum Albumin (Sigma-Aldrich, Germany), Folin Ciocalteau (Sigma-Aldrich, Germany), Potato dextrose agar, ammonium sulphate, absolute ethanol, acetone, acetonitrile, propanol, isopropyl-alcohol, and sodium hydroxide (JHD, China), Glutaraldehyde solution (v/v 25%) (Burlington, MA, United States). The antibiotics amoxicillin (AMX) (Reyoung Pharmaceutical Company Ltd China), ciprofloxacin (CIP) (Globela Pharma Private Ltd. India) and tetracycline (TET) (Zhejiang Dongri Pharmaceutical Company Ltd. China).

Equipment

All the equipment used in the study were available at the Department of Biochemistry University of Nigeria Nsukka. They include: Visible-spectrophotometer (Model 721, China), Rotary Shaker (Mk V Orbital Shaker, Model 064108), Pressure cooker (Dixons ST3028), Magnetic stirrer (Model SSH, China), Centrifuge (Model 800, China), Digital Electronic Weighing Balance (Model Hx502T, China), Water bath (Model SSY-H (stainless-Steel, Thermostatic, China), pH meter (Model PHS-3c, China).

Sample Collection

The WRF used for the study was isolated from a dead wood within the University of Nigeria, Nsukka Campus. The lignocellulosic waste (rice bran) used in solid-state fermentation for laccase production was obtained from a local market commonly known as Ogige Main Market in Nsukka Local Government Area of Enugu State, Nigeria.

Molecular Identification of Fungi Strain

The white rot fungi were cultured to obtain a pure culture and identified by the amplification and sequencing of the ITS region of the fungal DNA followed by BLAST sequence search, multiple sequence alignment, and phylogenetic analysis to trace the evolutionary relationship with other organisms in GenBank.

Fungal Isolation and Culturing

This was carried out by the method described by with slight modifications [7]. This was done by dissolving 7.8 g of PDA (potato dextrose agar) in a small volume of distilled water and making up to 200 ml with distilled water. The dissolved agar was then transferred into a 250 ml Erlenmeyer flask and the bottle was corked with cotton wool and capped with aluminium foil. The agar in the bottle was sterilized by autoclaving for 15 min at 15 psi pressure (121 °C). After cooling to about 50 °C, the flask was swirled thoroughly to mix the agar and nutrients, the agar was then poured (20 ml each) into Petri dishes and allowed to gel at room temperature. Microbial samples from McCartney bottles were inoculated onto the media in Petri plates with a sterile wire loop under aseptic conditions. The inoculated plates were wrapped in aluminium foil and stored in an aseptic environment for five days for the preparation of inoculum volume.

Screening for Laccase Producing Fungi

The qualitative screening for laccase production by the WRF was conducted using the method proposed by using indicator compounds such as ABTS, 2, 6- DMP, and α-naphthol [8].

Laccase Production

Laccase production was carried out under solid-state fermentation system using basal medium as described by [9].

Assay for Laccase Activity

Laccase activity was assayed by the modified method of [10]. The assay was carried out by monitoring the rate of oxidation of 2, 2′ azino-bis (3-ethylbenz-thiazoline-6- sulfonic acid) (ABTS) to a cation radical (ABTS-azine) by the enzyme at 25 oC in 0.1 M sodium acetate buffer, pH 4.5. The concentration of the cation radical responsible for the intense blue-green colour was correlated to enzyme activity.

Determination of Protein Concentration
Protein content of the enzyme was determined by the method of using bovine serum albumin as standard [11].

Purification of Laccase
Laccase was purified via ammonium sulphate precipitation, as reported by Ezike et al. and the enzyme obtained was further purified using gel filtration (Sephadex G-100) as described by [12,13].

Characterisation of Laccase
Effect of pH on Laccase Activity
The optimum pH of laccase was carried out by a method described by [14]. Laccase was assayed at different pH ranging from 3.0-7.5 using 0.1 M sodium acetate buffer (pH 3.0-5.5) and 0.1 M phosphate buffer (pH 6.0-7.5) at pH interval of 0.5 to determine the optimum pH.

pH Stability of Laccase
The stability of the enzyme was tested in a pH range of 3.0 - 6.5 by introducing the enzyme to different pH for 120 min at 25 °C and the residual enzyme activity was assayed at regular time interval using ABTS as a substrate.

Effect of Temperature on Laccase Activity
The effect of temperature on laccase activity was investigated at different temperatures ranging from 30 to 100 oC (10 oC intervals) at the optimum pH. A mixture of 0.1 ml substrate (ABTS) and buffer (0.1 M sodium acetate at pH 4.5) was incubated in a water bath for 10 min at different temperatures for equilibration before the enzyme was added to start the reaction and laccase activity was assayed.

Thermal Stability of Laccase
The thermal stability of the enzyme was done using the method reported by Ezike et al. This was investigated by incubating the enzyme at different temperature ranges of 50 to 70 oC at the optimum pH for regular time intervals over 120 min in a temperature-controlled water bath. Test tubes containing the enzyme solution were sealed to prevent change of volume due to evaporation, which might affect the concentration. Following heating, aliquots of enzyme were withdrawn at regular time intervals, cooled on ice and the activity was assayed [12].

Effect of Substrate Concentration on Laccase Activity
The effect of substrate concentration on the activity of laccase was investigated by incubating the enzyme with different concentrations of ABTS (10 – 200 µM). The reaction mixture contained different concentrations of ABTS in different volumes of sodium acetate buffer (0.1 M, pH 4.5) with a constant vol. (0.1 ml) of the partially purified laccase in a total volume of 3 ml each in different test tubes. Thereafter, laccase activity was determined at different concentrations of the substrate keeping the enzyme concentration constant. The values obtained were used to plot against substrate concentrations. The inverses (of activity and substrate concentration) were used to obtain the Lineweaver-Burk plot that was later used to determine The Vmax and Km values of the enzyme.

Effect of Metal Ions on Laccase Activity
The effect of some metals such as Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Ba^{2+} on laccase activity was investigated according to [15]. The metal ions were incubated with 0.1 ml of the partially purified enzyme in 0.1 M sodium acetate buffer pH at 4.5. The reaction started with the addition of the substrate (ABTS) and the residual activity was determined. A reaction mixture containing all except the metal ion was used as the control.

Precipitation of Laccase
To determine the best solvent for laccase precipitation before crosslinking, free laccase enzyme was prepared by mixing chilled organic solvents (n-propanol, acetone, ethanol, acetonitrile, isopropyl alcohol 10 ml each), saturated ammonium sulphate solution (10 ml) was added dropwise separately to samples of free enzyme mixture. The laccase activity was assayed to ascertain the compound which gave the highest activity.

Production of Crosslinked Laccase Aggregates (CLA)
A larger quantity of the free enzyme mixture was used to study the cross-linking step. 80% saturated ammonium sulphate solution (10 mL) was added to free enzyme mixture (500 mL) in capped centrifuge tube. After keeping the mixture for 30 min at 4°C for complete precipitation of the enzymes, glutaraldehyde was added to the final concentration of 40 mM. The mixture was kept at 30°C for 4.5 h with constant shaking at 150 rpm. Then the suspension was centrifuged

at 10,000×g for 10 min. The supernatant was collected and checked for laccase activity. The pellet was washed three times with 0.1M sodium phosphate buffer, pH 7 to remove unreacted glutaraldehyde and unbound proteins. The final enzyme preparation was kept in the same buffer (2 mL) at 4°C.

Degradation of Antibiotics by CLA
Degradation of antibiotics by crosslinked laccase aggregates was carried out by preparing three different concentrations of the antibiotics amoxicillin (AMX), ciprofloxacin (CIP), and tetracycline (TET) from a stock concentration of 500 mg/ ml. The stock solution was prepared by dissolving 500 mg in 100 ml of sodium acetate buffer, pH 4.5 to achieve a stock concentration of 500 mg/ml. The three test concentrations were 10 µl, 100 µl, and 1000 µl. The three concentrations per antibiotic were prepared by taking 0.01 ml of the stock solution labelled (low), 4.79 ml of buffer, and 0.2 ml of crosslinked laccase, for the tube with 0.1ml of the stock solution labelled (moderate), 4.7 buffer, and 0.2 ml of crosslinked laccase was added and for the tube labelled high, 1ml of stock solution, 3.8 buffer and 0.2 of the cross-linked enzyme was added to make a total of 5ml working volume. This was carried out for the three antibiotics. The reaction mixture was incubated for zero time (T0), 5 minutes (T5), 15 minutes(T15), 30 minutes (T30) and 1hr (T60). The aliquot of the reaction mixture was spectrophotometrically analysed for the antibiotic's degradation potential using a UV spectrophotometer. A plot of absorbance against wavelength for three antibiotics was prepared and presented.

Results
Laccase was produced using the isolated white rot fungi under solid-state fermentation using rice bran as the lignocellulosic support. A time-course study for enzyme production indicated that day ten (10) gave the maximum enzyme production. The crude Pleurotus pulmonarius laccase (Ppl) was precipitated by

80 % ammonium sulphate precipitation and purified using a Sephadex G-100 column, which was equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). The partially purified enzyme was characterised and evaluated in vitro for its potential in antibiotic degradation. The optimum pH and temperature of the laccase were 3.5 and 60°C, respectively. The stability of Pleurotus pulmonarius laccase to temperature was studied, at various temperatures of 50, 60, and 70 °C in which the enzyme activity was most stable at 50 °C after incubating for 120 min. Additionally, the enzyme was more stable at a pH of 6.0. The maximum velocity (Vmax) and Michaelis constant (Km) of the enzyme are 3333.33 µmol/min and 0.6667 µM, respectively using 2,2 azino bis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) as substrate. Furthermore, metal ions significantly influenced laccase catalytic activity. The effect of some metal ions on the activation of enzyme activity was in the following order Cu^{2+}> Mg^{2+}> Zn^{2+} > Mn^{2+} > Ba^{2+}. The spectral studies on the degradation of antibiotics revealed that Pleurotus pulmonarius crosslinked laccase aggregates significantly decreased the absorbance of the antibiotics at their maximum absorption wavelength at different concentrations as the incubation time was increased. Hence, the findings highlight that Pleurotus pulmonarius laccase (Ppl) have the potential for antibiotic degradation, opening new avenues for biotechnological applications. The utilization of enzymatic bioremediation, particularly employing laccases from lignin-degrading fungi such as Pleurotus pulmonarius, offers a promising avenue for targeted degradation of pharmaceutical pollutants.

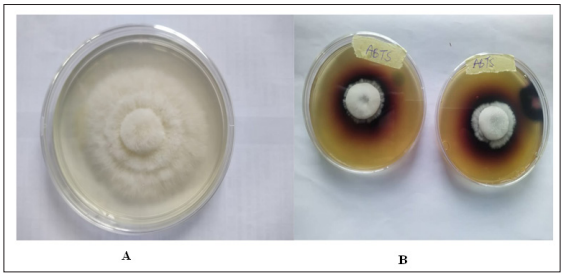

Plate 1: (A) Pure culture of Pleurotus pulmonarius used for laccase production
(B) Qualitative screening for laccase production showing ABTS oxidation

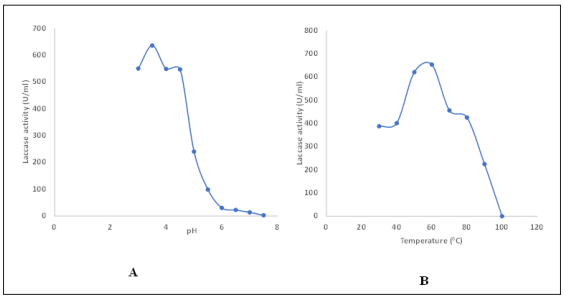

Figure 1: (A) Effect of pH on Pleurotus pulmonarius laccase activity
(B) Effect of temperature on Pleurotus pulmonarius laccase activity

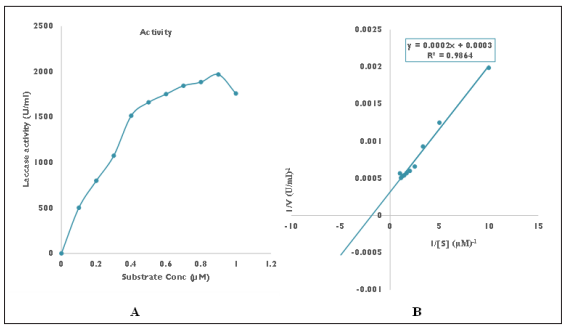

Figure 2: (A) Michaelis-Menten's plot on the effect of substrate concentration on the activity of Pleurotus pulmonarius laccase. (B) Lineweaver-Burk plot the effect of substrate concentration on the activity of Pleurotus pulmonarius laccase

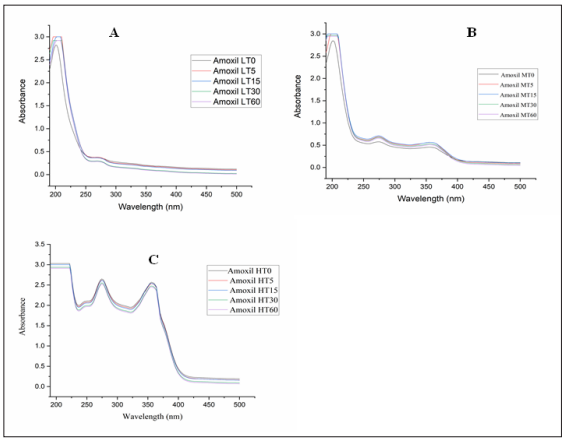

Figure 3: The degradation plot of absorbance against wavelength at different incubation time and different concentrations of Amoxil (A) low 10 µl (B)moderate 100 µl (C) high 1000 µl

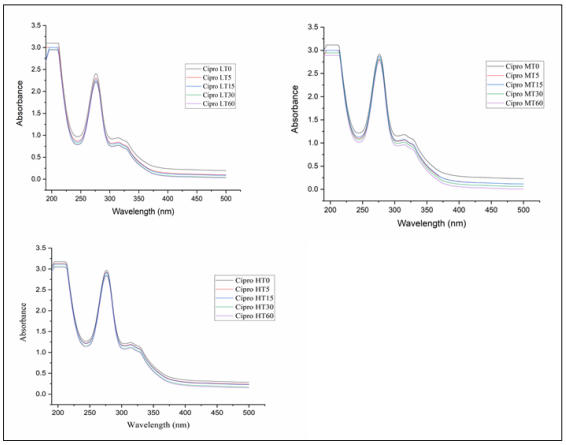

Figure 4: The degradation plot of absorbance against wavelength at different incubation time and different concentrations of Ciprofloxacin (A) low 10 µl (B)moderate 100 µl (C) high 1000 µl

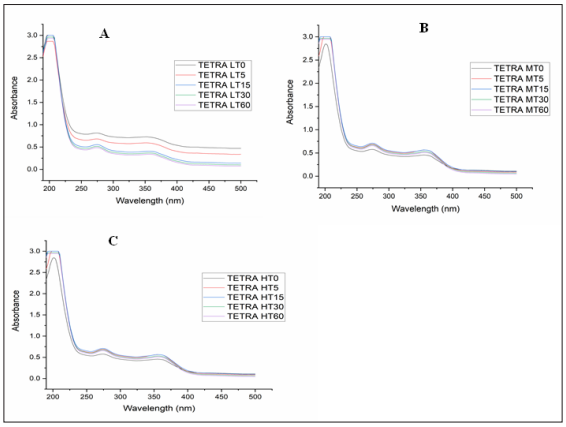

Figure 5: The degradation plot of absorbance against wavelength at different incubation time and different concentrations of Ciprofloxacin (A) low 10 µl (B)moderate 100 µl (C) high 1000 µl

Conclusion

The present study on the characterisation of a thermostable laccase isolated from the fungi Pleurotus pulmonarius is a promising potential for the degradation of antibiotics in wastewater. Laccases are versatile oxidoreductase enzymes that can catalyse the oxidation of a wide range of phenolic and non-phenolic compounds, including various classes of antibiotics. The thermostable nature of the laccase from Pleurotus pulmonarius is a desirable trait since it can withstand the elevated temperatures often encountered in industrial wastewater treatment processes. The ability of this laccase to effectively degrade different antibiotics, such as tetracyclines, fluoroquinones, and β-lactams, demonstrates its broad substrate specificity and suitability for targeted removal of these persistent pharmaceutical pollutants. The findings from this study suggest that the utilization of this thermostable laccase can be a sustainable and environmentally friendly approach in promoting the removal of antibiotics and reducing the risk of antibiotic resistance in the environment.

Recommendation

Further research is recommended to explore laccase's ability to degrade a wider range of antibiotics commonly found in wastewater. For example, the catalytic properties of laccases can be further optimized through protein engineering techniques to enhance their efficiency and specificity toward antibiotic degradation. Additionally, investigations into optimizing reaction conditions and enzyme immobilization techniques for enhanced stability and reusability are crucial for industrial-scale application. Furthermore, studies on the integration of laccase with other treatment methods could be explored to develop a comprehensive strategy for efficient antibiotic removal from wastewater streams. By employing laccase as a suitable means of degrading antibiotics, we can significantly reduce impact of these contaminants and contribute to the preservation of antibiotic efficacy. The future prospects of laccase-based antibiotic degradation is promising, with the potential to remodel wastewater treatment practices and promote a more sustainable future.

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