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Efficient Recovery of Laccase Enzyme from Mushroom Spent and Its Application in Dye Biodegradation in Wastewater

Ekanayake EMRD[1], Kalamulla KWYR[1], Yapa PN[1]
[1]Department of Biological Sciences, Faculty of Applied Sciences, Rajarata University of Sri Lanka

Abstract

Environmental pollution caused by synthetic dyes, especially azo dyes, poses significant challenges due to their persistence and toxicity in wastewater. This study aimed to extract and evaluate the laccase enzyme from mushroom spent, an underutilized agricultural waste for its potential in dye biodegradation. Spent substrates from *Pleurotus ostreatus* (Oyster mushroom) and *Agaricus bisporus* (Button mushroom) were collected and processed to extract crude laccase enzyme using sodium acetate buffer under cold conditions. Qualitative screening confirmed the presence of laccase activity, and the extracted crude was tested for its efficiency in degrading bromothymol blue and methyl orange dyes. Among the tested samples, the oyster mushroom spent from Gannoruwa yielded the highest amount of crude enzyme (average 4.18 g per 20 g of spent), whereas the button mushroom spent showed significant dye degradation, achieving up to 48.6% decolorization of bromothymol blue. Notably, the oyster mushroom laccase from Gannoruwa showed promising activity against methyl orange (60.13% degradation), but not bromothymol blue. The study also highlighted the importance of parameters such as pH and temperature in maintaining enzymatic activity, with optimal degradation observed at pH 4.5. These findings suggest that mushroom spent is a viable and sustainable source of laccase for eco-friendly dye wastewater treatment. However, future studies should focus on enzyme purification, activity quantification, and testing with a broader range of dyes for industrial application.

Key Words: *Laccase, Mushroom Spent, Dye degradation, Bioremediation, Wastewater Treatment*

Introduction

With the rapid growth of global urbanization and industrialization, environmental pollution has become a major problem. The major pollutants which contaminate the environment are pesticides, pharmaceutical compounds, microplastics, endocrine disruptors, organic dyes, and heavy metals, all of which cause soil, water and air pollution. Among these, synthetic organic dyes are emerging as critical aquatic contaminants. Dyes consist of aromatic compounds with aryl rings and delocalized electron systems that enable them to absorb electromagnetic radiation at different wavelengths. Chromophores within the dye molecules facilitate energy transitions in these delocalized electrons, enhancing efficient light absorption (Singh et al., 2020).Consequently, these resilient compounds are extensively utilized across the textile, leather, paper, and cosmetics sectors. Among all organic dyes, azo dyes are widely used in the textile industry due to their cost-effectiveness and broad spectral range (Afrin et al., 2021). Concurrently, sulfonephthalein dyes are heavily exploited for specialized applications, including spectrophotometric determinations, optical sensors, clinical medicine, and industrial manufacturing (Magnaghi et al., 2023).

Despite their industrial utility, synthetic dyes present severe ecological threats. Globally, approximately 70 million tons of synthetic dyes are manufactured annually in the textile sector alone consuming over 10,000 tons (Al-Tohamy et al., 2022). It is estimated that approximately 300,000 tons of synthetic dyes are discharged into wastewater treatment facilities worldwide each year (Halepoto et al., 2022). Up to 15% of the initial chemical dye load is systematically lost during standard industrial processing and routinely released into natural water bodies (Ahlawat & Singh, 2009).Beyond causing severe aesthetic impairment, these highly colored effluents block sunlight penetration into aquatic ecosystems, which significantly diminishes the photosynthetic capacity of aquatic flora and rapidly depletes downstream dissolved oxygen levels (Banat et al., 1996).Furthermore, many of these discharged compounds generate highly toxic breakdown products that are well-documented as mutagenic and carcinogenic (Ahlawat & Singh, 2009).

Conventional remediation strategies primarily rely on physicochemical methodologies, such as electrokinetic coagulation, Fe (II)/Ca $(OH)_2$ electro-flotation, membrane filtration, precipitation, ozonation, and activated carbon-air mixtures (the Katox process). However, these engineering approaches are restricted by prohibitive operating costs, poor adaptability across diverse dye matrices, and the generation of substantial volumes of secondary sludge. Consequently, attention has shifted toward eco-friendly biological alternatives utilizing the natural biodegradation and biosorption capacities of specialized microorganisms (Khlifi et al., 2010). Spent mushroom compost (SMC) represents an abundant, underutilized lignocellulosic byproduct of the commercial mushroom sector that is traditionally discarded into landfills or used as raw garden fertilizer. This agricultural waste is heavily enriched with active fungal mycelia and robust extracellular enzymes secreted by basidiomycetes

to break down complex structural lignin, cellulose, and hemicellulose into low molecular-weight nutrients (Lim et al., 2013).

Among these extracellular enzymes, laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) stands out as a highly stable, multicopper glycoprotein oxidase (Rodríguez Couto & Toca Herrera, 2006). True laccases possess unique copper atoms in their catalytic centers that enable the enzyme to oxidize a wide range of substrates such as polyphenols, methoxy-substituted phenols, and aromatic diamines via a single-electron transfer process. At the same time, molecular oxygen is reduced directly to water through a four-electron reaction. These include the fact that laccase, unlike peroxidases, does not require the addition or synthesis of a low molecular weight cofactor like hydrogen peroxide, as its co-substrate, oxygen is usually present in its environment (Notes, 2006). Because these enzymes are primarily extracellular and display excellent structural stability, they serve as ideal candidates for the degradation of xenobiotics and persistent environmental pollutants (Rodríguez Couto & Toca Herrera, 2006).

This study aims to efficiently extract crude laccase enzymes from commercial *Pleurotus ostreatus* and *Agaricus bisporus* spent substrates, optimize their extraction dynamics, and systematically evaluate their comparative catalytic efficacy in the biodegradation of target synthetic azo and sulfonephthalein dye matrices in wastewater.

Materials and Methods

Materials

Mushroom spent samples of oyster mushroom (*Pleurotus ostreatus*) and button mushroom (*Agaricus bisporus*) were chosen as the samples for this study. Spent samples were collected from Gannoruwa agricultural department in Kandy and Greenu agricultural and Organic mushroom farm in Kurunegala district in November -December 2024, from the growers within 14 days after the mushroom harvest. Three subsamples were collected from each species that was grown on the same substrate. From each sample, 250 g of mushroom spent was collected into zip lock bags with proper labeling until transport to the laboratory, Faculty of Applied Sciences, Rajarata University of Sri Lanka.

Methods

Qualitative Screening of Laccase Activity using α-Naphthol test

To preliminarily detect laccase activity, a qualitative plate assay was performed using α naphthol as a chromogenic substrate. A total of 120 mL of Potato Dextrose Agar (PDA) was supplemented with 5 mL of α-naphthol before autoclaving. After sterilization, 20 mL of the medium was poured into each plate. Once the medium solidified, a small mycelial piece (approximately 5 mm in diameter) of the oyster mushroom (*Pleurotus ostreatus*) and button mushroom (*Agaricus bisporus*) was aseptically inoculated at the center of the respective plates(Malini, 2011). These Petri plates were incubated at 28–30 °C for 72 hours and then screened for the formation of colored zones around the fungal colonies (Technology, 2025).

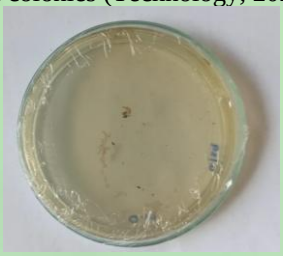


Figure 1. Inoculated plate – *Pleurotus ostreatus*

Figure 2. Inoculated plate – *Agaricus bisporus*

Extraction of laccase enzyme from mushroom spent

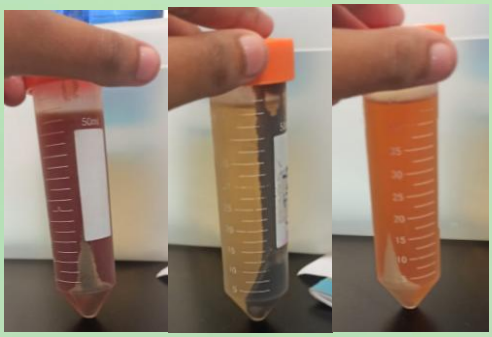
For enzyme extraction, the spent mushroom compost (SMC) was first dried at 40 °C for 4 hours to effectively reduce its moisture content while preserving the integrity of heat-sensitive enzymes such as laccase. Once fully dried, the material was finely grounded using a grinder and then accurately weighed for extraction. Laccase

activity, similar to that of other extracellular ligninolytic enzymes, typically remains stable within an optimal temperature range of 50–70 °C(Notes, 2006),ensuring its functionality is not compromised during the drying process. For the extraction of laccase, 20 grams of the dried SMC was mixed with sodium acetate buffer at pH 4.5, maintaining a solid-to-liquid ratio of 1:5. The mixture was gently agitated and incubated at 4 °C for a duration of 3 hours to facilitate efficient enzyme extraction while minimizing enzymatic degradation (Lim et al., 2013).

Figure 3. Dried mushroom spent samples.
a. Oyster mushroom spent sample- Gannoruwa
b. Button mushroom spent sample- Kurunegala
c. Oyster mushroom spent sample- Kurunegala

a b c

Figure 4. Comparative Visual Analysis of Spent Substrate mixed with Buffer Samples.
a. Oyster mushroom- Gannoruwa
b. Button mushroom- Kurunegala
c. Oyster mushroom- Kurunegala



a b c

Figure 5. Visual Comparison of Supernatant and Pellet After First Centrifugation of solutions.
a. Oyster mushroom- Gannoruwa
b. Button mushroom- Kurunegala
c. Oyster mushroom- Kurunegala

Purification of laccase

protein precipitation by ammonium sulphate is much more efficient method use in purification of laccase (Shraddha et al., 2011). The remaining purification steps were carried out at 4°C (Chefetz et al., 1998). Centrifugation was done at 10,000 rpm at 4ºC for 15 minutes (Supplied et al., 2021) and supernatant was then subjected to ammonium sulfate precipitation (Malini, 2011). Ammonium sulfate saturation to 80% was used for the precipitation process (Asgher et al., 2012). The amounts of solid ammonium sulfate required to reach an 80% saturation were determined using an online calculator (http://www.encorbio.com/protocols/AM-SO4.htm)(Wingfield, 2016). After keeping it overnight, the solution was centrifuged again at 6000 rpm for 20 minutes at 4°C. The upper phase of the centrifuged sample was filtered and pellet was dissolved in 5 mL distilled water.

Figure 6. Collected supernatant after first centrifugation
a. Oyster mushroom- Gannoruwa
b. Button mushroom- Kurunegala
c. Oyster mushroom- Kandy

a. Button mushroom b. Oyster mushroom
Figure 7. The supernatants were saturated with 80% ammonium sulfate for protein precipitation

Biodegradation of Azo and sulfonephthalein dyes using extracted laccase enzyme

To test the biodegradation ability of the extracted crude laccase enzyme, calibration plots of bromothymol blue and methyl orange dyes were constructed for quantitative measurements. As an initial step, a 100 mg/L stock solution of each dye were prepared by dissolving 10 mg of the dye in 100 mL of distilled water. From this stock solution, a concentration series of 2.5 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, and 25 mg/L was prepared. For each concentration, 20 mL of solution was prepared in small test tubes in duplicate, and the absorbance of each solution was measured at 420 nm.

A calibration plot of mean absorbance versus dye concentration was constructed, and the standard deviation for each data point was calculated and included in the same plot. Subsequently, 0.2 g of crude laccase enzyme extract was added to each duplicate vial containing the dye solutions. After 48 hours of incubation, the absorbance of each solution was measured again at 420 nm using distilled water as the blank. Finally, data reporting and analysis were carried out based on the measured absorbance values and the corresponding dye concentrations.

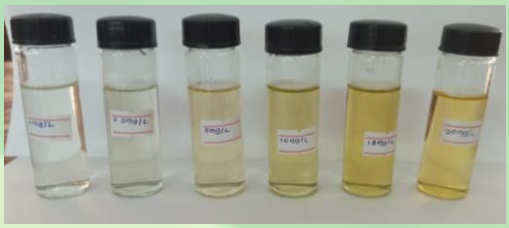


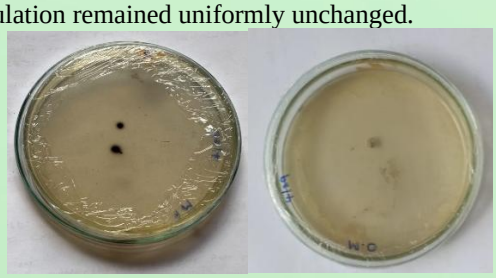
Figure 8. Serial Dilution of Bromothymol Blue for Absorbance/Indicator Studies

Figure 9. Serial Dilution of Methyl Orange for Absorbance/Indicator Studies

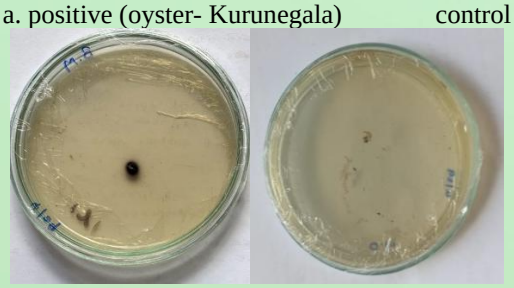
Results

Qualitative Screening of Laccase Activity using α-Naphthol

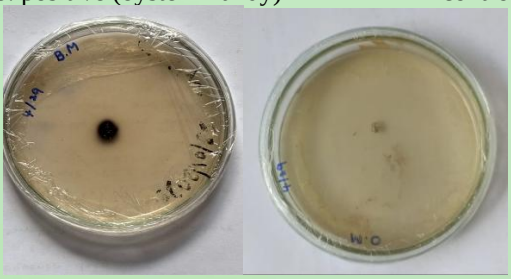
Upon incubation of the inoculated plates containing Potato Dextrose Agar (PDA) supplemented with α-naphthol, a distinct dark purple coloration developed around the mycelial growth zones of the mushroom samples. Control plates without fungal inoculation remained uniformly unchanged.



a. positive (oyster- Kurunegala) control



b. positive (oyster- Kandy) control



c. positive (Button mushroom) control

Figure 10. positive and control results of Qualitative Screening of Laccase Activity using α Naphthol

Extraction of laccase enzyme from mushroom spent.

Agaricus bisporus (Button mushroom) from Kurunegala

Table 1 – Yield of the laccase enzyme extracted from *Agaricus bisporus* (Button mushroom) spent- Trial 1

Weight of the empty falcon tube	*Falcon tube+ crude*	*crude*
12.084g	12.966 g	0.882g
12.044g	12.715 g	0.671g
12.072g	12.370 g	0.298g

Mass of the crude extracted using 20g of mushroom spent = (0.882g+0.671g+0.298g) =1.851g

Table 2 – Yield of the laccase enzyme extracted from *Agaricus bisporus* (Button mushroom) spent- Trial 2

Weight of the empty falcon tube	*Falcon tube+ crude*	*crude*
12.084g	12.292 g	0.208g
12.044g	12.478 g	0.434g
12.072g	12.745 g	0.673g

Mass of the crude extracted using 20g of mushroom spent =(0.208g+0.434g+0.673) =1.315g

$$Average\ yield = \frac{crude\ mass\ of\ trial\ 1 + crude\ mass\ of\ trial\ 2}{2}$$

Equation no:1

Average yield $= \frac{1.851g + 1.315g}{2} = 1.583g$

Therefore, the average yield of extract obtained from 20 g of button mushroom compost was 1.583 g.

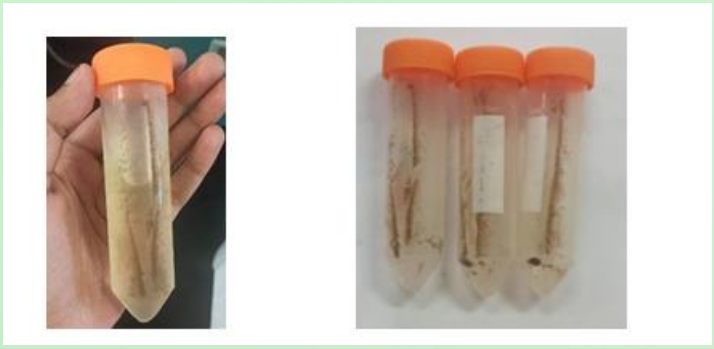


Figure:11. Enzyme crude extracted by button mushroom spent

Pleurotus ostreatus (Oyster mushroom) from Gannoruwa

Table 3 – Yield of the laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent from Gannoruwa- Trial 1.

Weight of the empty falcon tube	*Falcon tube+ crude*	*crude*
12.129g	13.187 g	1.058g
12.102g	13.791 g	1.689g
12.044g	13.523g	1.479g

Mass of the crude extracted using 20g of mushroom spent =(1.058g+1.689g+1.479g) = 4.226g

Table 4 – Yield of the laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent from Gannoruwa- Trial 2

Weight of the empty falcon tube	*Falcon tube+ crude*	*crude*
12.138g	13.132 g	0.994g
12.067g	13.691 g	1.624g
12.033g	13.548g	1.515g

Mass of the crude extracted using 20g of mushroom spent =(0.994g+1.624g+1.515g)= 4.133g

$$Average\ yield = \frac{crude\ mass\ of\ trial\ 1 + crude\ mass\ of\ trial\ 2}{2}$$

Average yield $= \frac{4.266g + 4.133g}{2} = 4.1795g$

Therefore, the average yield of extract obtained from 20 g of Oyster mushroom (Gannoruwa) compost was 4.1795.

Figure:12. Enzyme crude extracted by Oyster mushroom (Gannoruwa) spent

Pleurotus ostreatus (Oyster mushroom) from Kurunegala

Table 5 – Yield of the laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent from Kurunegala- Trial 2

Weight of the empty falcon tube	*Falcon tube+ crude*	*crude*
12.249g	13.309g	1.060g
12.741g	13.502g	0.761g
12.514g	13.381g	0.867g

Mass of the crude extracted using 20g of mushroom spent =(1.060g+0.761g+0.867g) = 2.688g

Table 6 – Yield of the laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent from Kurunegala- Trial 2

Weight of the empty falcon tube	*Falcon tube+ crude*	*crude*
12.084g	13.637 g	1.553g
12.044g	12.992 g	0.948g
12.072g	13.741g	1.269g

Mass of the crude extracted using 20g of mushroom spent =(1.553g+0.948g+1.269g) = 3.77g

$$Average\ yield = \frac{crude\ mass\ of\ trial\ 1 + crude\ mass\ of\ trial\ 2}{2}$$

Average yield $= \frac{2.688g + 3.77g}{2} = 3.299g$

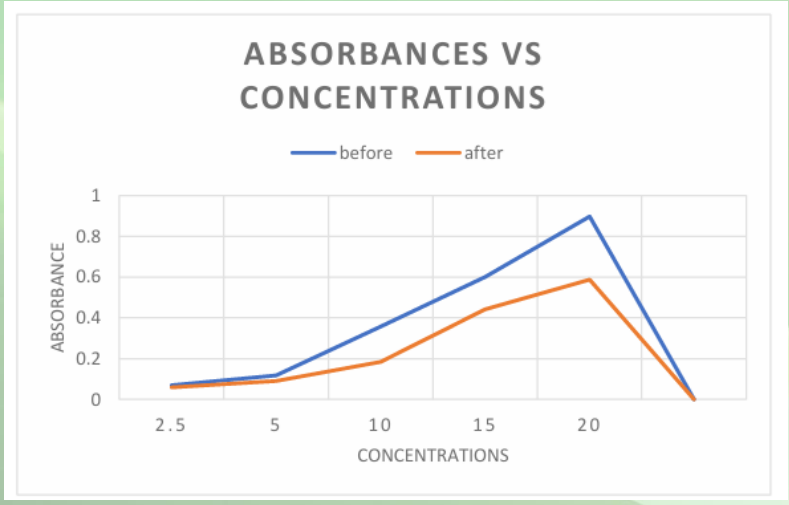
Therefore, the average yield of extract obtained from 20 g of Oyster mushroom (Kurunegala) compost was 3.229g.

Figure:13. Enzyme crude extracted by Oyster mushroom (Kurunegala) spent

Dye degradation ability of extracted laccase enzymes

Table 7-Testing the dye degradation ability of laccase enzyme extracted from *Agaricus bisporus* (Button mushroom) spent- Dye- Bromothymol blue-Absorbances(420nm)

Concentration (mg/dm3)	*Absorbance before adding the crude*	*Absorbance after adding the crude*	*Dye degradation percentage*
1.0	0.053	0.049	07.54%
2.5	0.070	0.059	15.71%
5.0	0.117	0.090	23.07%
10.0	0.358	0.184	48.60%
15.0	0.601	0.442	26.45%
20.0	0.898	0.588	34.52%



Graph 1-Absorbances vs Concentrations Before and After adding of Laccase enzyme crude extracted from Button mushroom spent- Dye- Bromothymol blue-Absorbances(420nm)

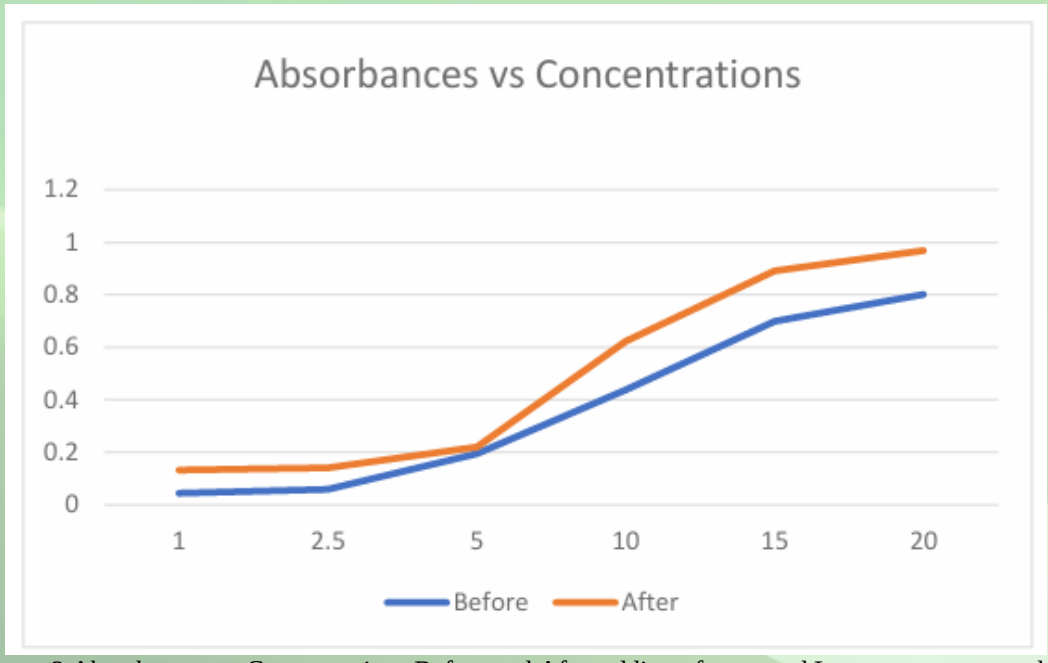
a. 2.5 mg/l solutions b. 5 mg/l solutions c. 10 mg/l solutions

d. 15 mg/l solutions e. 20 mg/l solutions

Figure:14. Visual Change in Bromothymol Blue Before and After Laccase Treatment.

Table 8-Testing the dye degradation ability of laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent- Gannoruwa -Dye- Bromothymol blue-Absorbances(420nm)

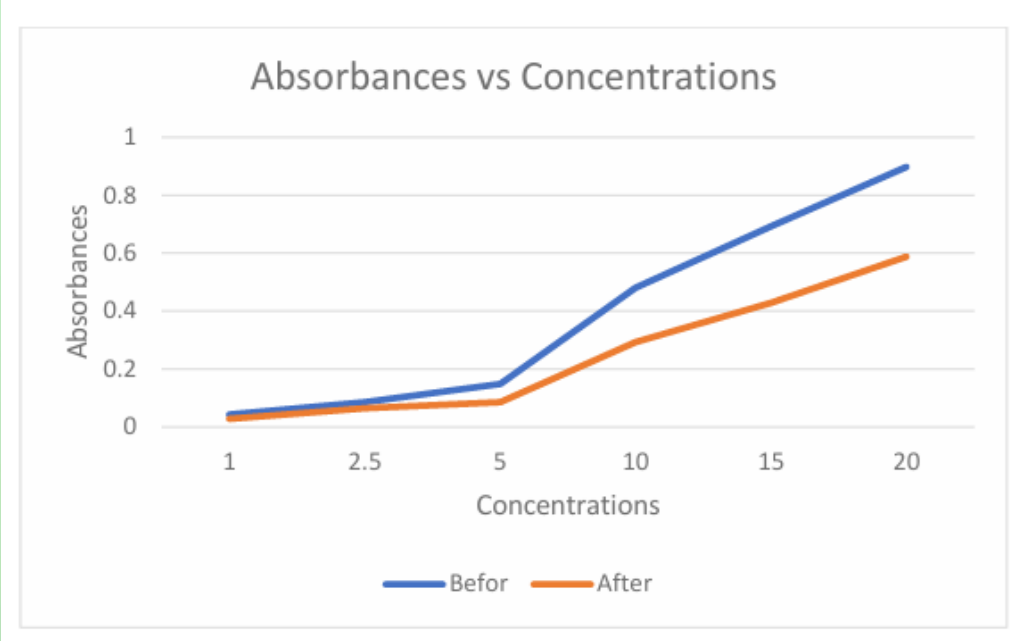
Concentration (mg/dm3)	*Absorbance before adding the crude*	*Absorbance after adding the crude*
1.0	0.044	0.132
2.5	0.059	0.141
5.0	0.194	0.220
10.0	0.437	0.622
15.0	0.699	0.891
20.0	0.801	0.968



Graph no: 2 Absorbances vs Concentrations Before and After adding of extracted Laccase enzyme crude from Oyster Mushroom spent (Gannoruwa)- Dye- Bromothymol blue-Absorbances(420nm)

Table 9-Testing the dye degradation ability of laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent- Gannoruwa -Dye – Methyl Orange- Absorbances(420nm)

Concentration (mg/dm3)	*Absorbance before adding the crude*	*Absorbance after adding the crude*	*Dye degradation percentage*
1.0	0.043	0.028	34.88%
2.5	0.085	0.064	24.70%
5.0	0.149	0.086	42.28%
10.0	0.481	0.293	39.08%
15.0	0.693	0.429	38.09%
20.0	0.898	0.588	60.13%

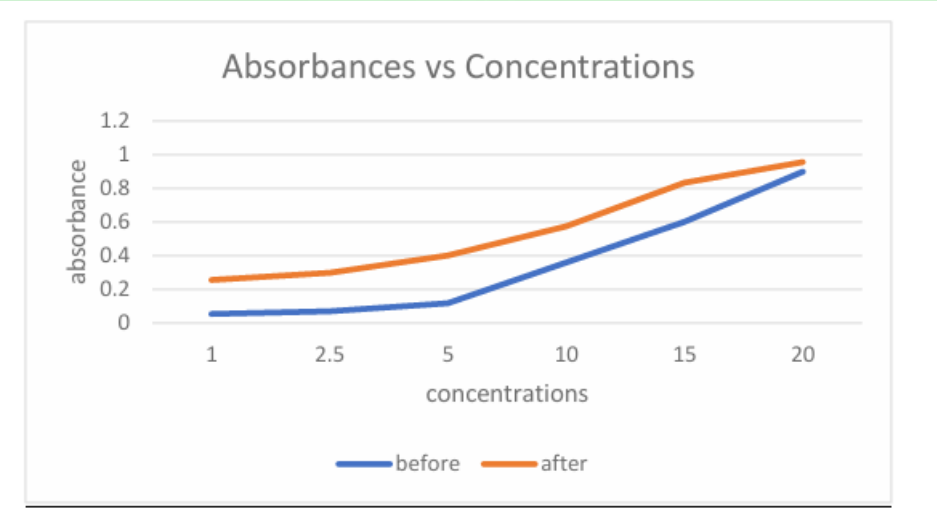


Graph no: 3 Absorbances vs Concentrations Before and After adding of extracted Laccase enzyme crude from Oyster Mushroom spent (Gannoruwa)- Dye- Methyl orange

Figure:12. Visual Change in Methyl Orange Before and After Laccase Treatment

Table 10 - Testing the dye degradation ability of laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent- Kurunegala. Dye- Bromothymol blue Absorbances(420nm)

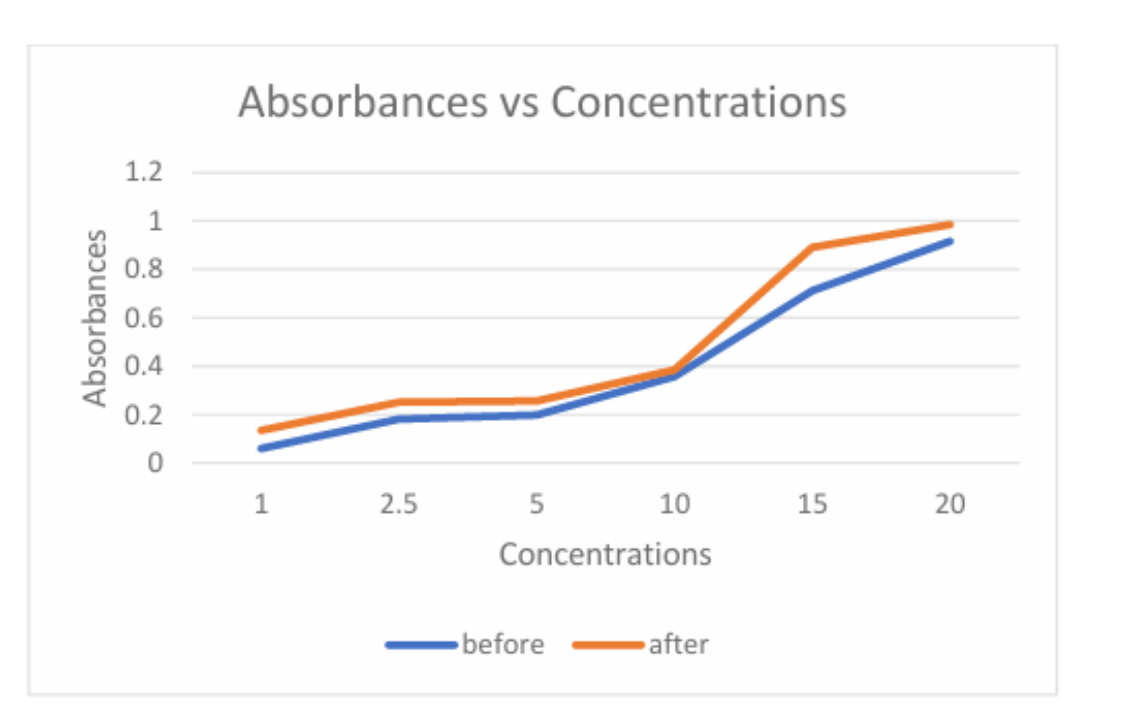
Concentration (mg/dm3)	*Absorbance before adding the crude*	*Absorbance after adding the crude*
1.0	0.053	0.255
2.5	0.070	0.298
5.0	0.117	0.401
10.0	0.358	0.574
15.0	0.601	0.832
20.0	0.898	0.955



Graph no: 4 Absorbances vs Concentrations Before and After adding of extracted Laccase enzyme crude from Oyster Mushroom spent (Kurunegala)- Dye- Bromothymol blue Absorbances(420nm)

Table 11 - Testing the dye degradation ability of laccase enzyme extracted from *Pleurotus ostreatus* (Oyster mushroom) spent- Kurunegala -Dye- Methyl Orange Absorbances(420nm)

Concentration (mg/dm3)	*Absorbance before adding the crude*	*Absorbance after adding the crude*
1.0	0.060	0.135
2.5	0.181	0.252
5.0	0.199	0.257
10.0	0.358	0.384
15.0	0.712	0.891
20.0	0.916	0.985



Graph no: 4 Absorbances vs Concentrations Before and After adding of extracted Laccase enzyme crude from Oyster Mushroom spent (Kurunegala)- Dye- Methyl Orange Absorbances(420nm)

Discussion

Qualitative Screening of Laccase Activity using α-Naphthol

1-Naphthol, or α-naphthol, is an organic compound with the formula C10H7OH. It is a fluorescent white solid. 1-Naphthol differs from its isomer 2-naphthol by the location of the hydroxyl group on the naphthalene ring. The naphthols are naphthalene homologues of phenol. This compound is commonly used in biochemical assays and as a precursor in dye synthesis(Kulys et al., 2003). Laccase catalyzes the oxidation of α-naphthol (1-naphthol), a phenolic compound, resulting in the formation of a distinct dark purple color. This color change occurs due to the enzymatic removal of an electron from the hydroxyl group of α-naphthol, producing phenoxy radicals. These radicals are unstable and spontaneously undergo coupling reactions, forming dimers, oligomers, and eventually high-molecular-weight polymeric compounds. Some of these products contain conjugated structures such as quinonoid derivatives, which have strong absorbance in the visible light spectrum, particularly around 520–560 nm, giving rise to the purple coloration. The intensity and hue of the color depend on several factors, including pH, substrate concentration, and reaction time.

Extraction of laccase enzyme from mushroom spent

The extraction results revealed notable differences in the crude enzyme yield obtained from various mushroom spents. The Oyster mushroom spent from Gannoruwa produced the highest average yield (4.1795 g per 20 g of spent), followed by the Oyster mushroom spent from Kurunegala (3.229 g). In contrast, the Button mushroom (*Agaricus bisporus*) spent resulted in the lowest crude yield (1.583 g). These yield variations may be influenced by differences in mushroom species, cultivation substrates, and regional growing conditions. The extraction was carried out following a laccase-specific methodology, designed to isolate extracellular laccase enzymes from lignocellulosic spent mushroom compost. However, it is important to note that the extract obtained is still considered crude, and although it is expected to be enriched in laccase, some level of non-target substances or inactive protein residues may still be present. Therefore, while the extraction method targets laccase, the yield values presented here reflect total crude mass, not pure laccase content. Interestingly, despite the higher crude yield observed from oyster mushroom spent, the quantitative yield does not necessarily indicate functional enzyme effectiveness or purity. These aspects are better evaluated through enzyme activity assays and functional tests, which are addressed in subsequent sections. In previous studies, extracted crude laccase was typically quantified and compared by assessing its enzymatic activity using standard substrates such as ABTS. However, due to limited resources, the present study estimated laccase quantity based on biomass measurements instead of activity-based assays. In a previous study by (Chefetz et al., 1998), further reported in the work on *Pleurotus sp.* (PMC3184503), laccase was purified through a multi-step process that included ammonium sulfate precipitation, DEAE-cellulose anion exchange chromatography, and gel filtration using a Sephadex G-100 column. The enzyme was first extracted from the culture filtrate, followed by salting out with ammonium sulfate, dialysis, and lyophilization. Ion exchange chromatography was performed using sodium acetate buffer (pH 4.5) with a NaCl gradient, and active fractions were pooled, concentrated, and further purified by gel filtration. This approach yielded a 72.2-fold purification with a final yield of 22.4%, and the purified laccase showed a single band on SDS-PAGE, indicating homogeneity, with a molecular weight 18 of ~40 kDa. Specific activity increased from 36 U/mg to 2600 U/mg across the purification steps (More et al., 2011). In the study by(Lim et al., 2013), laccase activity was quantified using a standard colorimetric assay based on the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). For this assay, 10 µL of enzyme extract obtained from spent mushroom compost (SMC) was mixed with 90 µL of 50 mM sodium acetate buffer (pH 4.5) containing 1 mM ABTS. The mixture was incubated at room temperature, and the reaction was terminated by adding 100 µL of 20% trichloroacetic acid (TCA). Oxidation of ABTS was monitored by measuring absorbance at 420 nm using a spectrophotometer. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute under assay conditions. Among the three *Pleurotus* species tested, the highest laccase activity was observed in extracts from *P. eryngii* SMC, with a recorded value of 8.01 U/g. This was followed by *P. cornucopiae* (3.77 U/g) and *P. ostreatus* (2.97 U/g). These results highlight significant species-specific differences in enzyme productivity and demonstrate that *P. eryngii* spent compost is particularly rich in ligninolytic enzymes such as laccase. The assay findings further confirmed the potential of SMC as a viable and economical source of industrially relevant enzymes, especially for applications such as dye decolorization and bioremediation. In summary, oyster mushroom spent appears to be a more productive substrate for crude enzyme extraction, but further analysis is necessary to evaluate the specific activity and practical applicability of the extracted enzyme.

Dye degradation ability of extracted laccase enzymes

The dye degradation assays provided critical insights into the functional capability of the laccase enzyme extracts obtained from different mushroom spents. Notably, only the crude enzyme extracted from *Agaricus bisporus* (button mushroom) spent demonstrated significant dye degradation activity against Bromothymol Blue, with degradation percentages ranging from 7.54% to 48.60% depending on the dye concentration. The highest

degradation occurred at 10.0 mg/dm³, suggesting an optimal concentration range for effective enzymatic action. Interestingly, dye degradation percentages were lowest at the 1.0 and 2.5 mg/dm³ concentrations despite using the same volume of crude enzyme. This may be attributed to reduced substrate availability, which limits enzyme-substrate interactions, and to lower measurement sensitivity at very low absorbance values. However, beyond a certain concentration (10 mg/dm³), the trend began to plateau or decline, possibly due to enzyme saturation or feedback inhibition. A previous study reported that after 5 days of spent mushroom substrate (SMS) mixing, dye decolorization was inversely proportional to the initial dye concentration, with nearly 100% decolorization at 25 ppm and only 90% at 150 ppm .The same study noted that in many cases where fungi were used as decolorizing agents, decolorization efficiency tended to be higher at lower dye concentrations, particularly in treatments involving *Pleurotus florida* and *Pleurotus ostreatus* (Singh et al., 2013).However, the results of the present study differ notably from this trend. This opposite pattern may be due to differences in fungal strain efficiency, the composition or activity of enzymes present in the SMS, the specific dye used, or environmental conditions such as pH and temperature. These findings suggest that decolorization behavior can vary significantly depending on the system and highlight the potential of SMS to perform effectively even at higher contaminant loads. While the yield is moderate, the extract demonstrated strong dye degradation ability, confirming that button mushroom spent is a viable and cost-effective substrate for laccase extraction. Optimizing extraction parameters may further improve the yield without compromising activity. In contrast, the crude extracts obtained from *Pleurotus ostreatus* (oyster mushroom) spent from both Gannoruwa and Kurunegala did not demonstrate meaningful dye degradation. Instead of decreasing, absorbance values increased after adding the enzyme extract, suggesting no degradation and possibly even interactions that intensified the dye's color. The observed increase in absorbance could be due to the presence of interfering compounds such as pigments 24 or phenolic residues in the crude extract that absorb light at the same wavelength (420 nm) as the dye, leading to an overestimation of dye concentration. Additionally, the crude extract may contain a high proportion of non-enzymatic substances, dilute the actual enzyme concentration and thereby reduce the dye degradation efficiency. Suboptimal assay conditions such as inappropriate pH, absence of mediators, or unfavorable temperatures may also have contributed to the poor enzymatic activity. These factors highlight the importance of not only assessing crude yield but also evaluating the specific enzymatic activity and optimizing the degradation conditions to accurately determine the effectiveness of the laccase enzyme in dye degradation. Interestingly, while the oyster mushroom spent samples yielded the highest amounts of crude extract (as discussed previously), the functional effectiveness of these extracts in degrading dyes was poor. This disparity underscores a key point: higher crude yield does not necessarily equate to higher enzyme activity. Since the extraction was performed using a laccase-targeted methodology, the presence of other enzymes is unlikely, but the crude nature of the extract means it could still contain non-functional proteins, polysaccharides, or inhibitory compounds that impair laccase function. When testing the dye degradation ability of the same crude extract from Oyster Mushroom spent (Gannoruwa) using Methyl Orange, the absorbance decreased indicating a dye degradation. This positive result suggests that the laccase enzyme present in the oyster mushroom crude extract was effective in breaking down Methyl Orange under the given conditions. Unlike Bromothymol Blue, Methyl Orange has a simpler molecular structure and is more susceptible to oxidative degradation by laccase. This contrast highlights that the enzyme's efficiency can vary depending on the chemical nature of the dye and that Methyl Orange is likely more compatible with the oxidative mechanism of the laccase enzyme extracted from oyster mushroom spent. It has also been stressed that different fungi have varied decolorization potential against chemically different dyes, as supported by findings from previous studies(Ahlawat & Rajor, 2017).It is emphasized that dye decolorization efficiency can vary significantly depending on the chemical structure of the dye. In this study, *Schizophyllum commune* and *Pezizomycotina sp*. were screened on solid agar, both fungi exhibited complete decolorization of Rhodamine B and Methyl Violet 2B, with S. commune achieving 100% decolorization on the entire agar plate. However, differences were observed in their decolorization abilities, which were attributed to the structural differences between the 25 two dyes. This highlights that the interaction between fungal strains and dyes is not uniform and that the effectiveness of fungal decolorization is often dye-specific. In the case of the oyster mushroom sample from Kurunegala, the crude extract showed no dye degradation activity, as evidenced by the increase in absorbance values after treatment for both Bromothymol Blue and Methyl Orange dyes. Despite obtaining a moderate crude yield (average of 3.229 g from 20 g of spent mushroom), the enzyme extract failed to reduce the dye concentrations in the solution. This unexpected outcome can be explained by several factors. Firstly, the laccase activity in this particular crude extract may have been very low or completely inactive, possibly due to differences in mushroom strain, growth conditions, or substrate composition in Kurunegala. A higher mass of crude does not guarantee higher enzymatic effectiveness; it could still be rich in non-enzymatic proteins, polysaccharides, or other substances that dilute or interfere with enzyme function. Secondly, the increase in absorbance suggests that the crude extract may contain compounds that absorb at the same wavelength (420 nm), interfering with spectrophotometric measurements and giving the false impression of increased dye concentration. Another possibility is that compounds in the extract interacted with the dyes, altering their structure or causing partial aggregation, which can lead to shifts or increases in absorbance. Finally, suboptimal conditions such as inappropriate pH, temperature, or absence of necessary mediators may have

prevented laccase from acting effectively. These observations highlight the variability in enzyme quality from different mushroom sources and emphasize the need to assess actual enzyme activity, not just crude mass, before using the extract for dye degradation studies.

Effect of Extraction Temperature on Enzyme Activity

In this study, the enzyme extraction was performed at room temperature instead of the commonly recommended 4 °C. The resulting crude extract did not exhibit any observable dye biodegradation activity. This outcome strongly suggests that extraction temperature plays a critical role in maintaining enzyme activity, particularly for sensitive enzymes such as laccase. Enzymes are highly susceptible to denaturation and degradation when exposed to elevated temperatures during extraction and handling. Cold conditions (typically 4 °C) are known to stabilize enzyme structure and reduce the rate of degradation, thereby its preserving activity throughout the extraction process. The lack of dye biodegradation observed in this case emphasizes the importance of maintaining low temperatures during laccase extraction to ensure the enzyme remains active.

Influence of pH on Dye Degradation Activity

The crude enzyme extracted from button mushroom spent was tested for dye decolorization across a range of pH values. Notably, color degradation was observed only at pH 4.5, while no significant decolorization occurred at other tested pH levels. This indicates that the enzymatic activity of the extract is strongly pH-dependent. These findings highlight the importance of optimizing environmental conditions, especially pH, when assessing the dye-degrading potential of crude enzyme extracts. For future studies, maintaining acidic conditions close to the optimal pH is crucial to accurately evaluate the biodegradation efficiency of fungal-derived enzymes. Findings from previous studies have demonstrated that laccase enzymes from the white-rot fungus *Trametes versicolor* typically exhibit optimal activity under mildly acidic conditions and moderate temperatures. For instance, in a study involving partially purified laccase, the enzyme showed maximum activity at pH 5.0 and an optimal temperature of 42.5 °C. Furthermore, the enzyme remained stable within pH range of 4.0–7.0 for the first five hours, retaining over 80% of its activity. Temperature stability tests revealed that the enzyme maintained more than 90% of its activity between 20–40 °C and over 70% even at 50 °C after 48 hours of incubation (Brazkova et al., 2016). Importantly, the optimum pH value can vary depending on the substrate used, as different substrates induce different oxidation reactions for laccase enzymes, which may shift the enzyme's apparent pH optimum in different assays(Shraddha et al., 2011).

Conclusion

The study successfully demonstrated the potential of laccase enzyme extracted from mushroom spent, particularly from *Pleurotus ostreatus* and *Agaricus bisporus*, in the biodegradation of synthetic dyes. Qualitative screening using α-naphthol confirmed laccase activity in both mushroom types. Among the tested samples, oyster mushroom spent from Gannoruwa yielded the highest amount of crude enzyme (4.226g and 4.133g in two trials), suggesting its suitability for enzyme extraction. The dye degradation assays showed promising results, with the button mushroom laccase achieving up to 48.6% degradation of bromothymol blue, and the oyster mushroom laccase from Gannoruwa showing up to 60.13% degradation of methyl orange concentration. However, unexpected increases in absorbance after enzyme treatment were observed in some samples, indicating possible experimental errors, dye-enzyme interactions, or limitations in crude extract purity. These results highlight the importance of standardizing purification and activity assessment protocols. Looking ahead, to improve the scientific validity and practical applicability of the current findings, several key areas should be addressed in future studies. Firstly, the number of experimental replicates should be increased to enhance the statistical reliability and reproducibility of results. Secondly, the crude enzyme extracts should be further purified using methods such as dialysis, chromatography to isolate active laccase and eliminate interfering substances. Additionally, enzyme activity should be quantitatively measured using standard substrates like ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), which is commonly used for laccase assays. This would allow for precise comparisons of enzyme activity across different samples and conditions. Future research should also explore the enzyme's performance on a broader range of dyes, including structurally diverse compounds such as azo, anthraquinone, and reactive dyes, to better understand the substrate specificity and potential industrial applications. These improvements will help establish a more comprehensive understanding of laccase-based dye degradation and support its development for effective, eco-friendly wastewater treatment.

Author Contribution Statement

Ekanayake EMRD: Data collection, investigation, methodology, formal analysis, and writing the original draft

Kalamulla KWYR: Project administration, sample collection, supervision, conceptualization, methodology, review and editing

Yapa PN: Project administration, supervision, conceptualization, and review and editing

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