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Research Paper

Decolorization of Synthetic Dyes by Laccase of *Phellinus noxius* BRB 11 Immobilized with Halloysite Nanotubes

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Abstract
Halloysite nanotubes (HNTs) have been proposed as promising supports for enzyme immobilization. Developing an effective technique for enzyme immobilization on HNTs is crucial for their practical application. In this study, we present a method for immobilizing laccase enzymes on HNTs using various amino acids as green cross-linkers for eco-friendly decolorization of synthetic dye. The modified HNTs were characterized using FT-IR, FE-SEM, and HR-TEM analyses. Furthermore, the effects of various parameters—including pH (ranging from 3 to 8), temperature (30-70°C), toxicity, and reusability—on the activity of both free and immobilized laccase were investigated. The results indicated that glycine (Gly) provided the most effective support for laccase immobilization on HNTs. The modified HNTs/Gly-Lac achieved an immobilization yield of 98.45% and successfully decolorized 94.60% of Acid Blue 129 (AB129) within 2 h. The successful immobilization of laccase onto functionalized HNTs was confirmed through FT-IR, FE-SEM, and HR-TEM analyses. Morphological observations of HNTs/Gly for laccase immobilization showed a thickened and roughened appearance with a thin, light gray layer on the outer surface. Additionally, the presence of an imine ($-C=N^-$) bond peak and a peak at 1410 cm^{-1}, corresponding to the ($-NH$) bond, suggested successful covalent immobilization. The toxicity analysis revealed a reduction in the toxicity of laccase after immobilization onto modified HNTs. A reusability assessment conducted over five cycles demonstrated that decolorization efficiency remained above 45%. These findings suggest that HNTs/Gly-Lac could serve as an effective material for textile dye wastewater treatment.

Keywords
Acid Blue 129, Crosslinker, Decolorization, Environmental, Glycine

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1. INTRODUCTION

Indonesia, as a major producer of synthetic dyes for the textile industry, plays a significant role in their consumption and utilization (Andriana and Puspitawati, 2022). Synthetic dyes are known for their toxicity and resistance to degradation, raising environmental concerns due to their widespread use in various industries such as textiles, color imaging, paper printing, pharmaceuticals, cosmetics, and food production (Sharma and Shirkot, 2019). These dyes primarily consist of stable and complex aromatic compounds that are naturally difficult to degrade. Various dyes are used in the textile industry, including anthraquinone and azo dyes. Approximately 30% of the used dye is absorbed by the material, while the remaining 70% is discharged as waste (Scholz and Yaseen, 2019). This high waste percentage complicates the remediation process and contributes to water body contamination (Moyo et al., 2022).

Acid Blue 129 (AB129) is a commonly used anthraquinone dye in the textile industry. Its strong chemical structure makes the decomposition difficult, leading to severe environmental and health issues, including endocrine disorders (Routoula and Patwardhan, 2019). Various remediation methods for textile dyes, both chemical and physical, have been developed and implemented in wastewater treatment to mitigate the negative impact of these dyes (Godoi et al., 2021; Rashid et al., 2021; Siregar et al., 2022; Priatna et al., 2023). However, these methods require careful consideration, and often involved high cost that are unsuitable for large-scale application (Singh et al., 2019).

Biological approaches including those utilizing fungal and bacteria (Ikram, 2020; Muharni et al., 2023), demonstrate

significant potential for remediation of synthetic dyes waste. Though the use of fungal-mediated remediation is considered more promising due to its practicality, safety, environmental friendliness, cost-effectiveness, and the highly valued characteristics it offers in waste management (Jankowska et al., 2019; Lellis et al., 2019). Numerous studies have reported the potential of various species of fungi, such as *Trametes hirsuta, Phanerochaete velutina, Aspergillus niger, and Trametes versicolor*, in the biodegradation of textile waste (Yanto et al., 2021; Zafiu C, 2021; Priyanka and Lens, 2022; Martínez-Castillo et al., 2023).

The use of enzymes is crucial in breaking down the dye molecules. Oxidative enzymes like manganese peroxidase, lignin peroxidase, laccase, and reductive enzymes such as azo reductase are commonly used for this purpose (Kumar and Chandra, 2020). Laccase, a multi-copper oxidase, is particularly effective in degrading various organic compounds, especially synthetic dyes. It is widely regarded as the most efficient enzyme for bioremediation due to its environmentally friendly nature and advantageous biochemical and catalytic properties (Unuofin et al., 2022; Alshiekheid et al., 2023). However, the direct use of pure laccase for the degradation process can be costly due to its inability to regenerate, instability in harsh environmental conditions, and frequent protein degradation Kyomuhimbo and Brink, 2023. The enzyme immobilization is one of the strategies that could be used to overcome these challenges.

Enzyme immobilization is a technique that involves binding the enzymes within a chemically or physically insoluble matrix. The immobilization of laccase can address the limitations of its pure form by increasing resistance to adverse external conditions, thus improving the reusability and stability of enzymes (Kujawa et al., 2021). Previous studies have extensively utilized immobilization techniques, including adsorption, entrapment, covalent bonding, and cross-linking (Adamian et al., 2021). The cross-linking immobilization technique is an efficient method, as the cross-linking agent forms strong covalent bonds, thereby enhancing the enzyme activity and facilitating its use in industrial applications. This method involves replacing the hydroxyl (−OH) functional group with another functional group to create cross-links between the enzyme molecule and the supporting material. The support materials and the occurred cross-linking can influence the performance, stability, and hyperactivation of the immobilized enzyme (Wahab et al., 2020). Various support materials have been explored and developed for the immobilization of cross-linked laccases, including chitosan, magnetic cross-linked dialdehyde cellulose, silica nanocarriers, Cu_2O@MOF, cellulose nanofibers (CNFs), and HNTs-CTS (Bansal et al., 2018; Bilal et al., 2019; Primozic et al., 2020; Aslam et al., 2021; Hürmüzlü et al., 2021; Yuan L, 2023; Behram et al., 2024).

Many studies are focused on developing potential support materials for cross-linking immobilization. Halloysite nanotubes ($Al_2Si_2O_5(OH)_4.2H_2O$) (HNTs) are promising support materials that are environmentally friendly. They possess unique nanotubular structures, are biocompatible, water-soluble, non-toxic, and highly porous (Cheng et al., 2020; Balci et al., 2023). Compared to other nanoscale materials, HNTs is more accessible and significantly more cost-effective (Wang et al., 2022). It shows great potential for applications across various fields. Additionally, it is highly promising as a color remover and is easy to process and utilize as a nanomaterial (Ukkund et al., 2021). Previous research demonstrated that laccase from Trametes versicolor immobilized on M-HNTs-CTS achieved a decolorization efficiency of 87% for Direct Red 80 (DR80) dye within 3 h (Kadam et al., 2018), while CTS-HNTs achieved a decolorization of Remazol Red RR dye ranging from 43% to 54% (Hürmüzlü et al., 2021). However, these studies had certain limitations, including a prolonged decolorization process and inadequate efficiency. The study was restricted to only two specific types of dyes, hindering the ability to generelized the findings to other textile dyes with varying chemical structures that may provide greater decolorization challenges.

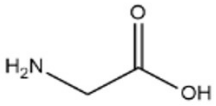
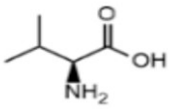
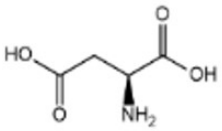
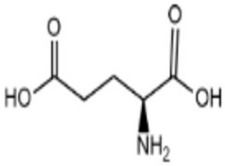
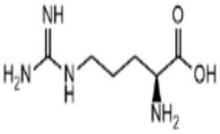
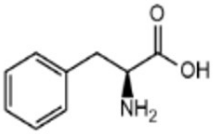
In the present study, laccase enzyme from *Phellinus noxius* BRB 11 was immobilized on halloysite nanotubes (HNTs) using various synthetic amino acids, including glycine, L-arginine, L-phenylalanine, L-valine, L-aspartate, and L-glutamic acid, with glutaraldehyde serving as a cross-linking agent to facilitate covalent immobilization. The novelty of this research lies in the strategic use of amino acids for covalent binding, selected for their exceptional enzymatic stability. This approach aims to enhance enzyme activity and stability, contributing to advancements in biocatalysis and materials science for the eco-friendly decolorization of synthetic dyes. To characterize the enzyme-loaded HNTs, we employed Fourier Transform Infrared Spectroscopy (FT-IR), Field Emission Scanning Electron Microscopy with Energy Dispersive Spectroscopy (FE-SEM), and High-Resolution Transmission Electron Microscopy (HR-TEM) to analyze both attached and unattached enzyme structures. Furthermore, we examined the effects of pH and temperature on the activity of both free and immobilized laccase. The AB129 dye removal study also included an evaluation of the reusability and toxicity of the immobilized enzyme in synthetic dye decolorization, aiming to develop an efficient method for environmentally and sustainable industrial bioremediation.

2. EXPERIMENTAL SECTION

2.1 Materials *Phellinus noxius*

Laccase (EC 1.10.3.2) Obtained from *Phellinus noxius* BRB 11 (NCBI GenBank accession number MT804574) was recently isolated from Berbak-Sembilang National Park, located in Jambi and South Sumatera Provinces, Indonesia. Oil Palm Empty Fruit Bunch (OPEFB) samples were collected from an oil palm plantation in Cikasungka, West Java, Indonesia. Malt extract agar, malt extract, glucose, hipolypeptone, and $CuSO_4$ were obtained from Merck, Germany. Halloysite nanotubes (HNTs) ($Al_2Si_2O_5(OH)_4 \cdot nH_2O$), 25% glutaraldehyde ($C_5H_8O_2$), and 2,2-azino-bis-[3-ethyl benzothiazoline-6-sul-

Table 1. Properties of Amino Acids

Amino Acids	Chemical Structure	Molecular Formula	Molecular Weight (g/mol)
Glycine		$C_2H_5NO_2$	75.07
L-Valine		$C_5H_{11}NO_2$	117.15
L-Aspartic Acid		$C_4H_7NO_4$	133.10
L-Glutamic Acid		$C_5H_9NO_4$	147.13
L-Arginine		$C_6H_{14}N_4O_4$	174.20
L-Phenylalanine		$C_9H_{11}NO_2$	165.19

phonic acid] (ABTS) were sourced from Wako Pure Chemical Ltd., Japan. Acid Blue 129 (AB129) was purchased from Sigma Aldrich (USA). The variations of amino acids used in this study, also procured from Sigma Aldrich (USA), are presented in Table 1.

The instruments used in this study include glassware (Corning Inc, USA), analytical balance (Mettler Toledo, Switzerland), hot plate stirrer (Thermo Fisher Scientific, USA), Eppendorf 5920 R Centrifuge (Eppendorf, Germany), Field Emission Scanning Electron Microscopy with Energy Dispersive Spectroscopy (FESEM-EDS) (Thermo Quatro, USA), High Resolution Transmission Electron Microscopy (HR-TEM) (Thermo Quarto, USA), Fourier Transform Infra-Red (FT-IR) (Perkin Elmer, USA), ACE AM-11 homogenizer (Nissei, Japan), Infinite 200 Pro Microplate Reader (Tecan, Swiss), Freeze-Dry (BUCHI Labortechnik, Swiss), Eppendorf 5428 R Centrifuge (Eppendorf, Germany), pH meter (Mettler Toledo, Germany), and Thermo-shaker TS-100 (Biosan, Russian).

2.2 Culture Preparation of *Phellinus noxius* BRB 11

The Preparation of isolate culture was conducted using the method described by Anita et al. (2019). The white-rot fungus Phellinus noxius BRB 11 was cultivated on malt extract agar (MEA) and incubated at a temperature range of 25-30°C for 7 days. The fungal culture was subsequently inoculated into a liquid medium containing 20 g/L malt extract, 20 g/L glucose, and 1 g/L hipolypeptone (MGP). The MGP medium was sterilized at 121°C for 15 minutes prior to inoculation. The inoculum was prepared by transferring four plugs (0.5 mm in diameter) of fungal mycelium into 100 mL of MGP medium, followed by incubation at 25-30°C for 7 days. After the incubation period, the fungal culture was homogenized using an ACE AM-11 homogenizer (Nissei, Japan). The homogenized culture was then utilized for enzyme production.

2.3 Laccase Enzyme Production and Extraction

Laccase production and extraction was conducted according to optimum conditions reported by Anita et al. (2020). Laccase enzyme was produced by using a solid-state fermentation (SSF) system. Oil palm empty fruit bunches (OPEFB) with a mesh size of 40-50 were selected as the substrate. The baglog was prepared by combining 150 g of OPEFB fibers, 200 mL of medium, 2 mM $CuSO_4$, and 100 mL of distilled water, followed by sterilization at 121°C for 15 minutes. Subsequently,

a 10% (w/v) of homogenized mycelium was inoculated into the baglog and incubated at 30°C for 30 days.

The baglogs were then harvested after the incubation period. The OPEFB fibers, fungal mycelium, and acetate buffer (pH 4.5) were homogenized at 10.000 rpm for 10 minutes using an ACE AM-11 homogenizer (Nissei, Japan). The homogenate was centrifuged at 10.600 × g and 4°C for 20 minutes using an Eppendorf 5920 R centrifuge (Eppendorf AG, Germany). The resulting supernatant was subjected to precipitation with 50% (w/v) ammonium sulfate, followed by an additional centrifugation at 8.000 rpm and 4°C for 20 minutes. The precipitated crude enzyme was dissolved in acetate buffer (pH 4.5) and subsequently freeze-dried using a BUCHI Labortechnik system (Switzerland) for 72 h.

2.4 Laccase Activity Assay

Laccase activity was assessed utilizing a TECAN Infinite 200 Pro microplate reader (Switzerland). The reaction mixture comprised 250 μL of 2 mM ABTS, 200 μL of 0.1 M acetate buffer at pH 4.5, and 50 μL of enzyme, measured at 420 nm over a duration of 60 seconds. The laccase activity (U/mL) was determined using Equation (1), while applying a molar absorptivity coefficient (ε) of 36.000 M^{-1} cm^{-1} (Anita et al., 2020).

- Enzyme activity (U/mL)

$$= \frac{(\text{Abs}(t) - \text{Abs}(0)) \times V_{\text{total}}(\text{mL}) \times 10^3}{\varepsilon \times V \times t \times d} \times 100\% \quad (1)$$

Where Abs (t) is the final absorbance, Abs (0) is the initial absorbance, (t) is the reaction time (1 min), (d) is the length of the cell, molar absorptivity (ε) is 36.000 M^{-1} cm^{-1}, and 10^3 is the correction factor (μmol/mol).

2.5 Synthesis of HNTs-Amino Acids

The synthesis was carried out using amino acids (AAs), including glycine, L-arginine, L-phenylalanine, L-valine, L-aspartic acid, and L-glutamic acid (Table 1), which were dissolved in distilled water. A glass beaker containing water was heated to 90°C in a water bath. HNTs (2 g) were weighed into a 100 mL Erlenmeyer flask, followed by the addition of 50 mL of amino acid solution and 2 mL of concentrated H_2SO_4, and dissolved in a water bath with continuous stirring for 7 h at 450 rpm and 90°C. The synthesis process was maintained at 90°C. The water in the tub was ensured that it did not dry out. After the synthesis, the mixture was thoroughly washed with distilled water and centrifuged using an Eppendorf 5920 R Centrifuge for 10 minutes at 10.000 rpm, 25°C and dried at 50-60°C in an oven for 24 h. The obtained powder of HNTs/AAs was used for further experiment.

2.6 Immobilization of Laccase

One gram of synthesized HNTs-AAs, consisting of glycine, L-arginine, L-phenylalanine, L-valine, L-aspartic acid, and L-glutamic acid, was weighed in a beaker. Subsequently, 10 mL of 5% glutaraldehyde was added as a binder. The resulting mixture was stirred for 4 h at 400 rpm. After homogenization, the mixture was washed twice with distilled water and centrifuged at 10,000 rpm for 10 minutes. The precipitate was then transferred into a 50 mL beaker, followed by the addition of 5 mL of laccase enzyme. The suspension was stirred until homogeneous, sealed with plastic wrap, and incubated at 9°C in a controlled cooling environment for 24 h. The mixture was then subjected to centrifugation at 10.000 rpm, 4°C for 20 minutes. The supernatant was analyzed to determine enzyme activity and quantify the level of the free laccase. Meanwhile, the precipitate was dried and prepared for application in textile dye decolorization.

The activity of immobilized laccase was determined by subtracting the total initial laccase activity from the free laccase activity remaining in the solution post-immobilization. The immobilization yield was calculated using Equation (2), while the specific activity of immobilized laccase per mg of HNTs-AAs was determined by following the Equation (3) (Anita et al., 2020; Hürmüzlü et al., 2021).

- Immobilized laccase yield (%)

$$= \frac{\text{Immobilized lac activity } U/mL}{\text{Total initial lac activity } U/mL} \times 100\% \quad (2)$$

- Enzyme loading (U/mg)

$$= \frac{\text{Immobilized lac activity } U/mL \times V \times \text{lac solution (mL)}}{\text{Weight of HNTs (mg)}} \quad (3)$$

2.7 Decolorization of Dye AB129

The effectiveness of free and immobilized laccase in dye decolorization was evaluated using the following reaction. A total of 5 mL Acid Blue 129 (AB129) dye solution at 100 mg/L (100 ppm) concentration dissolved in 0.1 M acetate buffer was added to 60 mg of immobilized laccase HNTs/AAs with an enzyme loading of 0.1 U/g in a 3 mL vial. The decolorization process was carried out at 450 rpm and maintained at 30°C for 2 h. Samples were collected at 1 h intervals to measure the residual dye concentration. To terminate the enzymatic reaction, the samples were heated at 80°C for 20 minutes (Anita et al., 2020).

The extent of decolorization was quantified at a wavelength of 629 nm. The decolorization efficiency was determined using Equation (4), where A_o represents the initial absorbance and A_1 denotes the absorbance after decolorization (Anita et al., 2020).

- Decolorization (%)

$$= \frac{\text{Initial Abs of Dye } (A_0) - \text{Final Abs of Dye } (A_t)}{\text{Initial Abs of Dye } (A_0)} \times 100\% \quad (4)$$

The effects of pH and temperature on dye removal were assessed to determine the optimal conditions for HNTs/GlyLac activity. Experiments were conducted using 5 mL of a 100

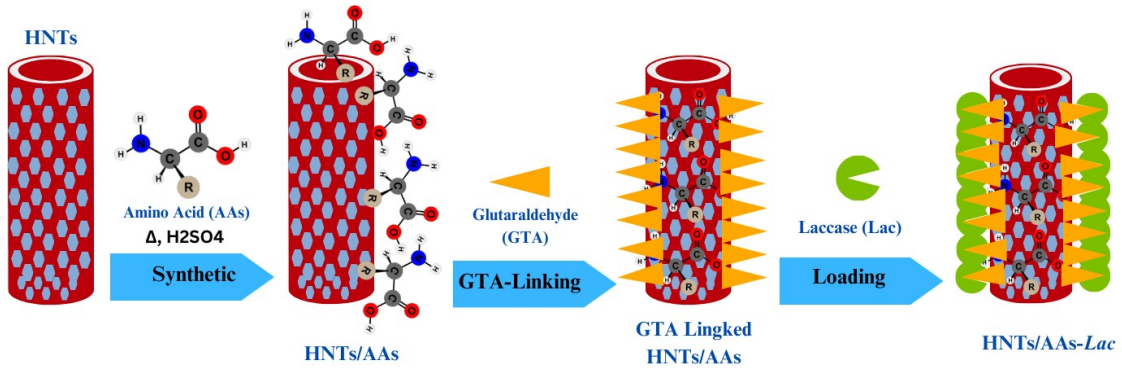


Figure 1. Schematic Illustration of Laccase Immobilization Mechanism

mg/L AB129 dye solution with a 60 mg sample and a 2 h of incubation period. The effect of pH was evaluated using acetate buffer at pH 3, 4, 4.5, and 5, while phosphate buffer was used for pH 6, 7, and 8. Meanwhile, the effect of temperature was examined within a range of 30-70°C at the optimal pH.

2.8 Characterization of HNTs, laccase, and HNTs/Gly-Lac

The FTIR, FESEM-EDX, and HR-TEM analyses were conducted to examine the morphology and crystal structure of enzyme-containing beads as well as those without enzymes. The structural characteristics of HNTs, laccase, and modified HNTs/Gly-Lac were investigated using Field Emission Scanning Electron Microscopy with Energy Dispersive Spectroscopy (FESEM-EDX) and High-Resolution Transmission Electron Microscopy (HR-TEM). Additionally, the functional group composition of the modified materials was characterized through Fourier Transform Infrared (FTIR) spectroscopy within the wavelength range of 400 to 4000 nm.

2.9 Toxicity Assays

The Brine Shrimp Lethality Test (BSLT) is a common method used to asses the cytotoxicity a substance. It is a simple and cost-effective technique that requires only a small amount of test material (Sarah et al., 2017). The process involves suspending *Artemia salina* larvae in natural seawater and incubating them under a 25 W lamp at room temperature for 48 h. Subsequently, ten larvae are transferred to microplate wells with increasing concentrations of dye (0.05, 0.10, 0.15, 0.20, and 0.25%) in a total volume of 1 mL, negative controls using seawater are also included. All the experiments are carried out in triplicate. Mortality rate was calculated according to Equation (5).

- Mortality (%)

$$= \frac{\text{Mortality Before Treatment} - \text{Mortality After Treatment}}{\text{Mortality Before Treatment}} \times 100\% \quad (5)$$

y = ax+b, where y = constant probit 50, a = variable x, and b = constant value of intercept.

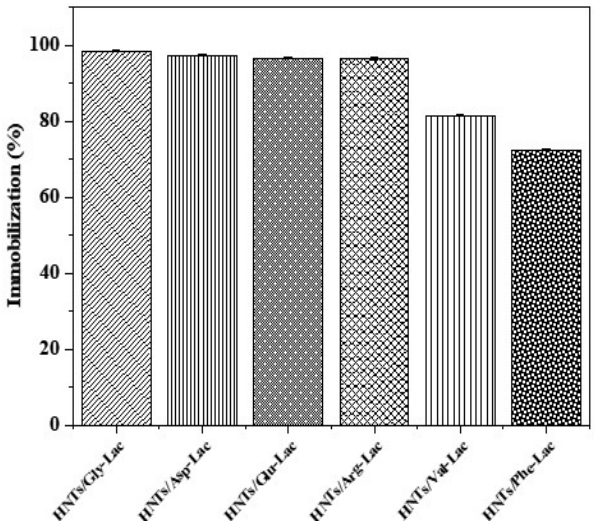


Figure 2. Immobilization Yield of Laccase on HNTs Using Various Amino Acids

2.10 Reusability of HNTs/Gly-Lac

The reusability test was conducted using a modified method from Anita et al. (2020). A total of 60 mg of HNTs/Gly-Lac was used with 5 mL of 100 ppm AB129 dye for decolorization. The test involved removing the dye in several sequential reactions. All experiments were conducted in triplicate using a shaker set at 450 rpm and maintained at room temperature for 2 h. Following the reaction, samples were collected, transferred into microtubes, heated at 80°C for 15 minutes to terminate enzymatic activity, and centrifuged at 10.000 rpm for 10 minutes. Absorbance measurements were recorded every 2 h, and the decolorization process was performed over three cycles, with each cycle lasting 2 h. The immobilized HNTs/Gly-Lac decolorization was determined as a 100% in the first cycle.

Table 2. Results of Immobilization and Enzyme Loading

Sample	Total Laccase Activity (U/mL)	Free Laccase Activity (U/mL)	Immobilized Laccase Activity (U/mL)	Immobilized Yield (%)	Enzyme Loading (U/g)
HNTs/Gly-Lac	1.33 ± 0.10	0.02 ± 0.00[a]	1.31 ± 0.00[f]	98.45 ± 0.19[e]	0.11
HNTs/Asp-Lac		0.04 ± 0.00[b]	1.29 ± 0.00[e]	97.25 ± 0.19[d]	0.11
HNTs/Glu-Lac		0.05 ± 0.00[c]	1.28 ± 0.00[a]	96.59 ± 0.22[c]	0.11
HNTs/Arg-Lac		0.05 ± 0.01[c]	1.28 ± 0.01[d]	96.09 ± 0.38[c]	0.11
HNTs/Val-Lac		0.24 ± 0.00[d]	1.09 ± 0.00[c]	81.58 ± 0.19[b]	0.09
HNTs/Phe-Lac		0.37 ± 0.00[e]	0.96 ± 0.00[b]	72.46 ± 0.14[a]	0.08

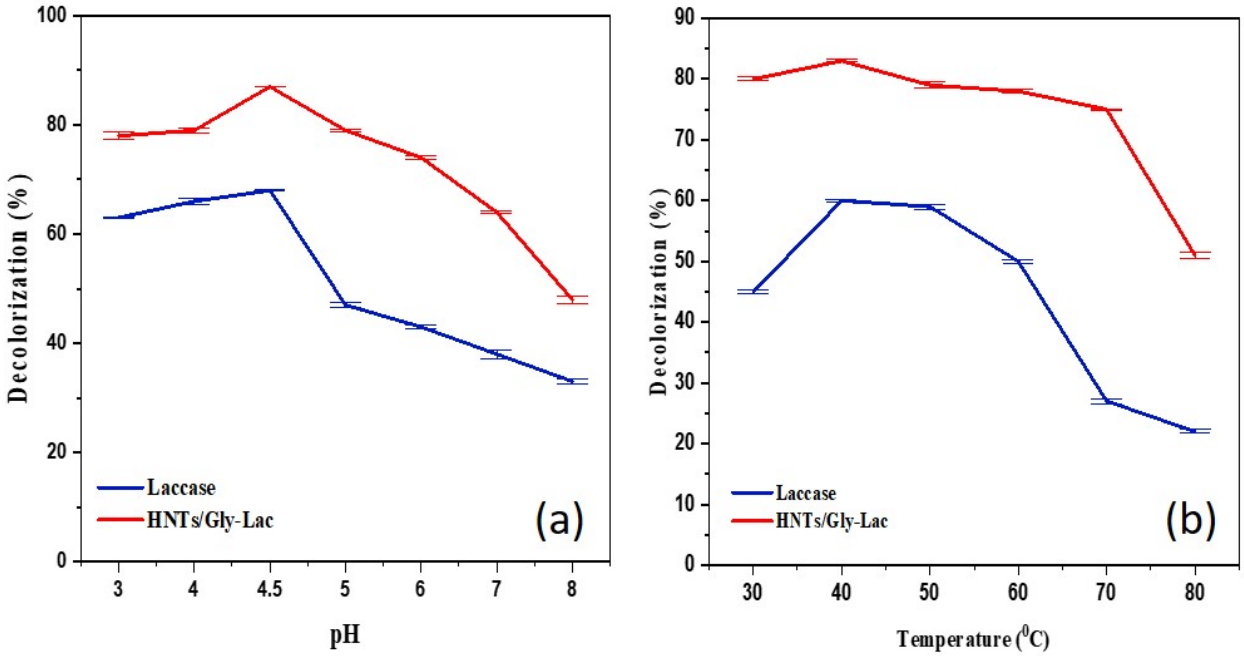


Figure 3. Effects of (a) pH, and (b) Temperature on Both HNTs/Gly-Lac, and Free Laccase on Decolorization of AB129 Dye

2.11 Data Analysis

The numerical data are presented as mean ± standard deviation, as the experiments were performed at least in triplicate. Statistical analyses were carried out using IBM SPSS software version 23.0. Comparisons between treatments were conducted through analysis of variance (ANOVA), followed by Duncan's post-hoc test ($p \leq 0.05$) to determine significant differences

3. RESULTS AND DISCUSSION

3.1 Strategy for Immobilization Laccase on HNTs/AAs-Lac

The process of modifying HNTs with amino acids (AAs) and immobilizing laccase is illustrated in Figure 1. Initially, 2 g of HNTs were combined with 50 mL of amino acid solution, with H_2SO_4 acting as an acid catalyst to initiate intermediate reactions during the synthesis (Salami et al., 2022). Functionalizing HNTs with amino acids enhances their strength and durability (Dogahe et al., 2021), improving the bonding between amino groups on the surface of the HNTs. Additionally, amino acids improve the thermal stability of HNTs composites. For example, hybrid nanoparticles created by combining amino-functionalized HNTs with other materials, such as graphene oxide, have shown enhanced thermal properties.

As shown in Figure 1, the amino groups on the modified HNTs' surface also contributed to improved dispersion in the epoxy matrix, promoting thermal conductivity and stability (Zhao X, 2022). In the next step, glutaraldehyde (GLU) is used as a cross-linking agent to link the amine groups of the amino acids and the laccase enzyme, forming a covalent bond between the amino acid, GLU, and laccase. Glutaraldehyde creates stable covalent bonds with the amino groups of laccase, preserving the enzyme's structural integrity during operation (Kadam et al., 2017). This modification greatly improves its interaction with organic compounds, including in dye removal (Saif et al., 2018; Delyanee et al., 2021). As a result, a stable

composite that can be used for textile dye decolorization is successfully produced.

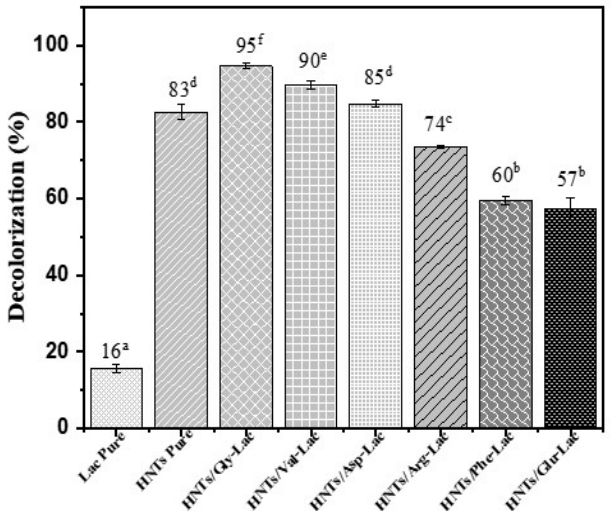


Figure 4. Decolorization Result of Dye Removal with HNTs Pure, Laccase Pure, and HNTs/AAs-Laccase

3.2 Enzyme Immobilization and Loading

The immobilization capacity of HNTs/AAs-Lac was evaluated based on laccase loading, with an immobilized enzyme concentration of 200 mg/mL. In this study, laccase was immobilized using six different amino acids-glycine (Gly), valine (Val), aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), and phenylalanine (Phe)-through covalent bonding as shown in Table 2. This approach enhances enzyme stability and minimizes enzyme leakage from the HNT support material. All laccase activity calculations were performed using Equation 1. After immobilization, the enzymatic activity of immobilized laccase was greater than that of free laccase, indicating improved accessibility, stability, efficiency, and enzyme degradation (Patel et al., 2016; Taheran et al., 2017). Previous study had reported the ability of glycine to improve the stability of immobilized penicillin G acylase, where the improvement may be resulted from the inhibition of unwanted enzyme-immobilizer interactions (da Rocha et al., 2022; Wahba, 2023). This inhibition helps blocked the reactive site on immobilizer as well as neuterelizing the reactive residues of Glutaraldehyde, therefore improving the stability of the immobilized enzyme (Chen and Roberts, 2002; Torres and Batista-Viera, 2012; Mihailovic et al., 2014). The proccess adding the amino acids is effectively supports the attachment of the enzyme to the surface of functionalized HNTs, producing novel composite materials as shown in Table 2.

The optimal immobilization yield for HNTs/Gly-Lac was 98.45 ± 0.19% (Figure 2), which is significantly higher than that of other amino acids. This superior performance is attributed to glycine's simple structure and minimal steric hindrance, which facilitates the formation of hydrogen bonds with HNTs as well as the Schiff base reaction with glutaraldehyde. This reaction covalently links glycine to the laccase enzyme, resulting in stable enzyme immobilization and enhanced catalytic activity in the decolorization process. Furthermore, electrostatic interactions during the synthesis of HNTs and glycine also influence enzyme immobilization, thereby contributing to improved stability (Katana et al., 2020). In addition, the functionalization of HNTs effectively maintains catalytic activity of laccase (Malmir et al., 2018). Covalent cross-linking also plays a dominant role in the interaction between the enzyme and HNTs/Gly, as it creates stable interactions between the carrier and the enzyme, thereby reducing the rate of enzyme deactivation (Salehizadeh et al., 2023). Analysis of variance (ANOVA) results confirmed that HNTs, in combination with various amino acids and immobilized with laccase, significantly influenced free laccase activity, immobilized laccase activity, and immobilization yield. Therefore, the immobilization process significantly enhanced the laccase enzyme characteristics on HNTs/Gly, enabling broader applications under various environmental conditions. Characterization through enzyme activity measurement is essential to ensure the effectiveness of the immobilized enzyme and to determine its potential for applications in textile dye wastewater treatment.The displayed values represent the mean ± standard deviation (SD). Differences in letters within the same column indicate significant differences based on the Duncan test at α = 0.05.

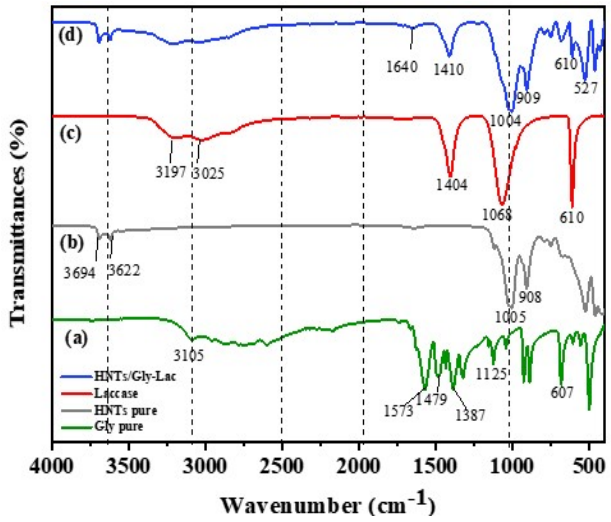


Figure 5. FTIR Spectra of (a)Gly Pure (b)HNTs Pure, (c) Laccase, and (d) HNTs/Gly-Lac

3.3 Optimization of pH and Temperature Decolorization

The effects of pH during decolorization of AB129 dye was examined using both free laccase and immobilized HNTs/Gly-Lac. For the free laccase, 60 mg of free laccase was added to AB129 dye (100 ppm) in a total volume of 5 mL, maintained at 25°C and 450 rpm. Similarly, for immobilized laccase, 60 mg of HNTs/Gly-Lac powder was added to 5 mL of AB129

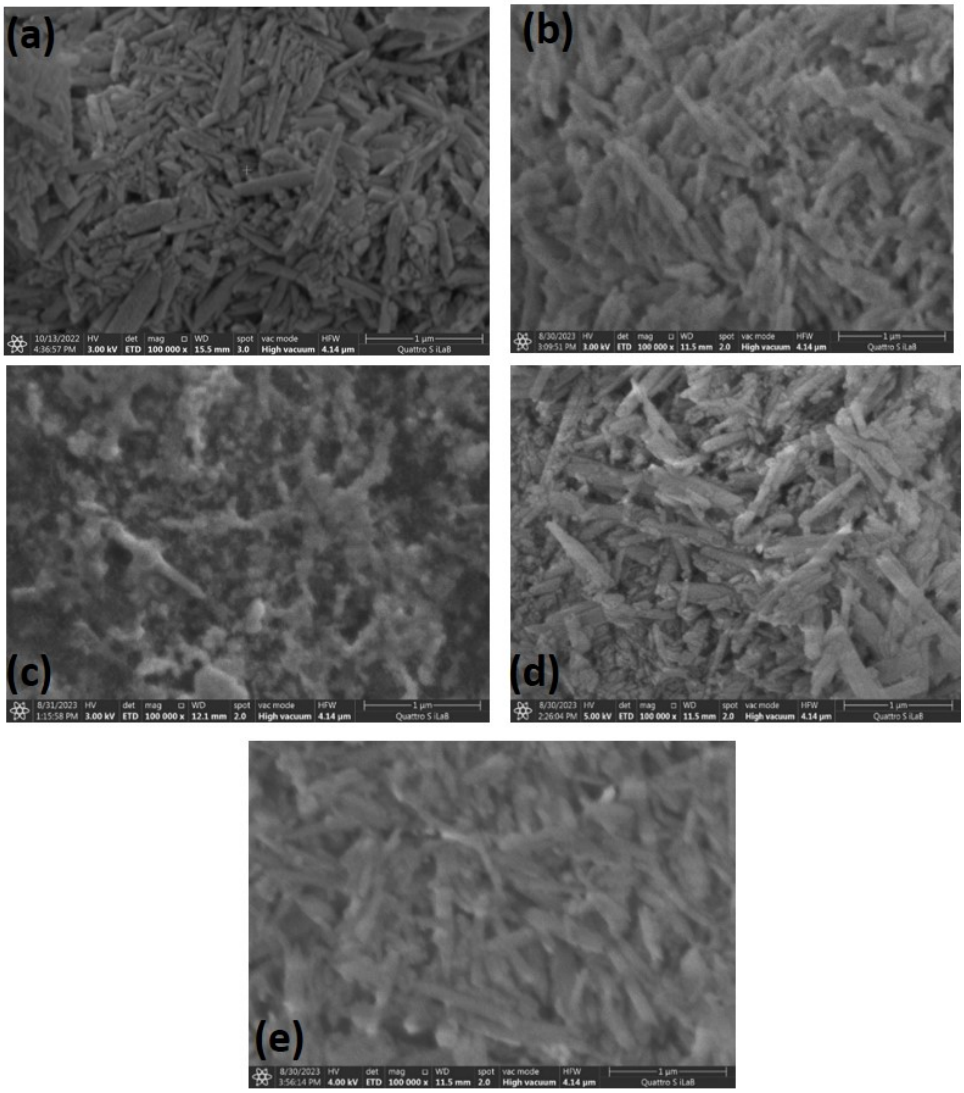


Figure 6. FE-SEM analysis of (a) HNTs (b) HNTs/AB129 (c) HNTs/Gly (d) HNTs/Gly-Lac (e) HNTs/Gly-Lac/AB129

dye (100 ppm) under the same conditions. The pH range was studied from 3 to 8 for both methods.

The free laccase method achieved an optimal AB129 decolorization of 68% at pH 4.5 (Figure 3a), but its efficiency dropped below 47% at pH levels between 5 and 8. This is likely because acidic pH provides the right environment for laccase, as most white rot fungus laccases has an optimum pH ranging from 2 to 5 (Ling et al., 2015; Zheng F, 2017; Wang et al., 2018a;Aftab and Ahmad, 2023). The slight acidic nature of the reaction medium resulting in higher activity and greater efficiency in removing the dye. As for HNTs/Gly-Lac, about 97% of wells within test plate was showed a decolorization when reacted at pH 4.5. HNTs/Gly-Lac decolorization is higher than free laccase at various pKa, which can be attributed to enzyme centers activated due to changes in the HNTs/Gly-Lac structure. The reaction medium pH may also affect the substrate solubility and availability, thereby affecting the decolorization rate (Yincan Z, 2017). HNTs/Gly-Lac behave better in a wide of pH as shown in Figure 3a, where it able to decolorize AB129 in a more neutral pH at similar rate to free laccase.

Temperature significantly affects enzyme activity, as excessive heat can lead to denaturation, altering the active site and preventing substrate binding. The effect of temperature to decolorization rate of AB129 dye by free laccase and HNTs/Gly-Lac shows that temperature plays a crucial role in decolorization. The optimal temperature for free laccase and HNTs/Gly-Lac is 40°C. This temperature is found to be ideal for both free and immobilized laccase as it enhances the enzyme's activity, which aids in decolorization of AB129 dye. HNTs/Gly-Lac showed a higher stability and decolorization rate than free laccase at high temperature, where it able to decolorize more than 70% of AB129 dyes at 70°C, as oppose to bellows 30% by free laccase. These results were in accordance with study by Ikram (2020), where the temperature above its optimum was causing

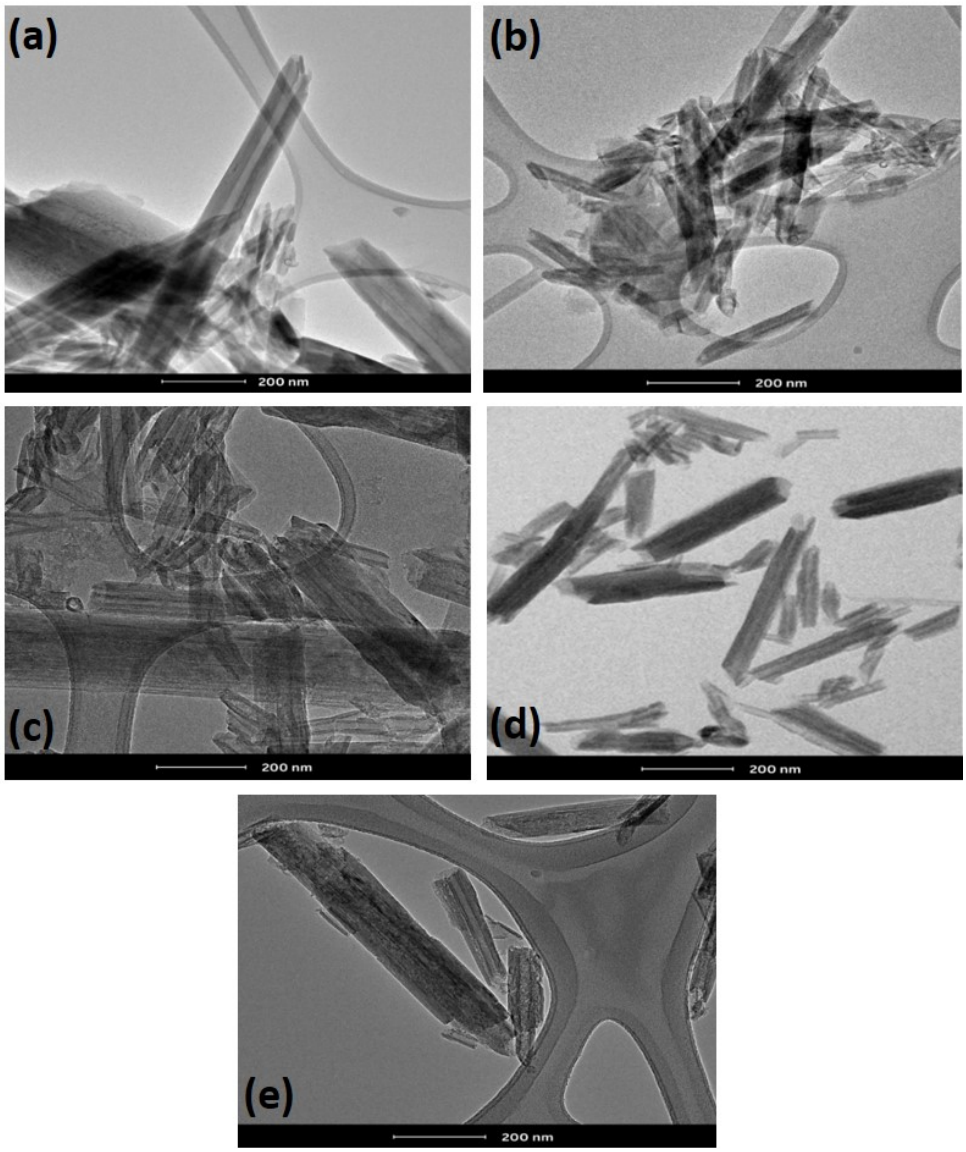


Figure 7. HR-TEM analysis of (a) HNTs (b) HNTs/AB129 (c) HNTs/Gly. (d) HNTs/Gly-Lac (e) HNTs/Gly-Lac/AB129

a persistent decline in decolorization rate. Therefore, the immobilization of laccase onto HNTs with the addition of glycine successfully improved the bioremediation properties of laccase, thus broaden its use for future application.

3.4 Decolorization of AB129 Dye

The decolorization of AB129 dye at a concentration of 100 ppm using free laccase, HNTs and HNTS/AA-lac was shown in Figure 4. Among the tested amino acids, the addition of glycine (HNTs/Gly-Lac) resulted in a higher decolorization rate compared to other amino acids. This is attributed to enzyme immobilization and the addition glycine's, which enhances enzyme activity and stability, facilitating the breakdown of chemical bonds in the AB129 synthetic dye molecule.

The decolorization efficiency of HNTs/Gly-Lac reached approximately 95.30% of AB129 dye within 2 h, while pure laccase decolorization achieved only about 17.2%. Similar findings have been reported in previous studies, where HNTs/CTS-Lac-mediated decolorization of Remazol RR dye achieved efficiencies ranging from approximately 86.89% to 93.00% over 5 h (Hürmüzlü et al., 2021). Meanwhile, the decolorization of DR 80 dye reached around 90.00% within 3 h (Kadam et al., 2018). These results suggest that enzyme immobilization with a combination of specific components, influences the decolorization duration.

The concentration of AB129 dye has a highly significant impact on decolorization efficiency. Higher dye concentrations tend to reduce the decolorization percentage due to enzyme saturation and competitive inhibition by dye molecules. Additionally, it may be toxic to microorganisms or enzymes, thereby

reducing degradation efficiency. Falah et al. (2018) reported that laccase from *Leiotrametes flavida* was able to decolorize RBBR dye at 100 ppm concentration up to 62% within 24 h at but its efficiency decreased to 17% at 1000 ppm. This indicates that higher dye concentrations can hinder the decolorization process. Therefore, determining the optimal dye concentration is crucial to achieving maximum efficiency.

In the present study, HNTs/Gly-Lac exhibits high efficiency in the decolorization of Acid Blue 129 (AB129) compared to other methods. This composite material is significantly more efficient in terms of decolorization ability and reaction time than free laccase or immobilized enzyme using similar methods reported from previous studies, which require 3-5 h to achieve 90% efficiency (Hürmüzlü et al., 2021; Kadam et al., 2018). HNTs/Gly-Lac demonstrated higher decolorization efficiency in a shorter time of 2 h reaction. Furthermore, HNTs/Gly-Lac is more stable at higher dye concentrations than laccase from *Leiotrametes flavida*. The synergistic mechanism of adsorption by HNTs and degradation by laccase *Phellinus noxius* BRB 11 enhances enzyme effectiveness and stability, overcoming the limitations of conventional methods. Therefore, HNTs/Gly-Lac emerges as a promising, faster, more efficient, and stable solution for AB129 dye decolorization.

The structure of Acid Blue 129 (AB129) undergoes significant alterations after the decolorization process. This is affected by multiple factors, including the type of microorganism or enzyme employed, the presence of redox mediators, and the specific conditions maintained throughout the process (Bhavsar et al., 2018; Ramadhan et al., 2021; Nurhayat et al., 2022). The decolorization analyses result as shown in Figure 4 revealed the ability of free enzyme, HNTs and HNTs/AA-Lacc to potentially degrade AB129 dye. The use of laccase enzymes produced by *Phellinus noxius* BRB 11 can effectively remove the color of AB129. These laccases facilitate the oxidation of phenolic compounds, resulting in the breakdown of the dye's molecular structure (Sun et al., 2017; Krawczyk et al., 2020). The enzyme typically breaks down the chromophore groups responsible for the dye's color, resulting in smaller and simpler molecules. In addition to the loss of color, these structural changes may also involve modifications to functional groups within the dye molecule, which can impact the efficiency of the decolorization process (Afreen et al., 2018).

The statistical analysis using analysis of variance (ANOVA) revealed that sample variation had a significant effect on the decolorization rate of synthetic dyes (Figure 4). These results align with previous studies, which emphasize that factors such as enzyme type, substrate concentration, and reaction conditions, including pH and temperature, can substantially influence decolorization efficiency (Li et al., 2023). This study supports the importance of these parameters in determining the effectiveness of synthetic dye decolorization, whether through enzymatic reactions or adsorption methods. Thus, variations in sample characteristics are crucial factors in optimizing the decolorization process, requiring further consideration for industrial-scale applications.

Table 3. Toxicity Test of *Artemia salina* Shrimp Larvae

Sample	Concentration (%)	LC50
HNTs	0.05	
	0.1	
	0.15	1.21%
	0.2	
	0.25	
Laccase	0.05	
	0.1	
	0.15	0.90%
	0.2	
	0.25	
HNTs/Gly-Lac	0.05	
	0.1	
	0.15	1.57%
	0.2	
	0.25	

3.5 Characterization of Laccase and HNTs/Gly-Lac

3.5.1 Fourier transform infrared (FTIR) Analysis

Functional group analysis was conducted using FTIR spectroscopy on glycine, HNTs, free laccase, and HNTs/Gly-Lac within a wavelength range of 400-4000 cm^{-1}. Based on the FTIR analysis results (Figure 5a), the glycine peaks observed between 2000 and 3500 cm^{-1} overlapped with absorption peaks corresponding to the O−H stretching of the −COOH group and the N−H stretching of the NH_3^+ group from the glycine amino acid molecule (Damodaran et al., 2017). The FTIR spectrum of HNTs exhibited a peak at 90 cm^{-1}, attributed to the O−H deformation of the hydroxyl group. A strong absorption peak at 1005 cm^{-1} was detected, indicating the presence of the O−Si−O siloxane surface group. The peak at 1651 cm^{-1} corresponded to the O−H deformation of water, while peaks at 3622 cm^{-1} and 3694 cm^{-1} were associated with the Al−OH stretching. Additionally, the peak at 1117 cm^{-1} was characteristic of the Si−O vibration in HNTs (Figure 5b) (Najwa et al., 2019). The FTIR spectrum of laccase revealed a peak at 1404 cm^{-1}, attributed to amide III due to C−N stretching and N−H bending. A peak at 1068 cm^{-1} confirmed the presence of the characteristic C−O−C bond in laccase, while the peak at 610 cm^{-1} was associated with amide V and VI, corresponding to N−H bending and out of plane C=O vibrations (Figure 5c) (Fortes et al., 2017; Samui and Sahu, 2018). Furthermore, the appearance of a medium peak at 1640 cm^{-1}, attributed to the imine ($-C=N^-$) bond, and a peak at 1410 cm^{-1}, corresponding to the (−NH) bond, confirmed the successful covalent immobilization of HNTs/Gly-Lac through the bonding of glutaraldehyde with the laccase enzyme (Figure 5d). The study also suggested that peak broadening indicated the successful immobilization of laccase onto the HNTs structure (Samui and Sahu, 2018).

Table 4. Reusability Decolorization with Other Materials

Material	Initial Decolorization Efficiency	Reusability Efficiency (After 5 Cycles)	Optimal pH	Optimal Temperature	Study Conclusion	References
Halloysite nanotube	80-90%	50-68%	5.0	30 °C	Effective initial decolorization, reduced efficiency after 5 cycles (gradual loss)	This study (AB129 Decolorization Research using HNTs)
Metal-Organic Frameworks (MOFS)	~93%	80%	7.5	40 °C	High efficiency and good reusability, excellent for dye adsorption applications	(Wang et al., 2018b)
Chitosan Beads	~85%	60%	4.5	35 °C	Efficient at lower pH, but moderate reusability	(Liu et al., 2014)
Graphene Oxide (GO)	~98%	85%	6.0	30 °C	Exceptional initial and reusability, showing strong adsorption capacity for dyes	(Jiao et al., 2020)
Silica Gel	~75%	45-50%	5.5	30°C	Lower initial efficiency, with a significant drop in efficiency after reuse	(Deng et al., 2012)
Activated Carbon	~95%	70%	7.0	25°C	High initial efficiency and moderate reusability, good for organic dye removal	(Albadarin et al., 2011)
Zeolite Nanoparticles	~92%	65%	6.5	40°C	High efficiency and moderate reusability, suitable for wastewater treatment	(Feng et al., 2010)

3.5.2 Field Emission Scanning Electron Microscopy with Energy Dispersive Spectroscopy (FESEM-EDX) Analysis

The surface morphology of HNTs before and after immobilization was observed using FESEM at 100.000× Magnification. The pure HNTs are about 1 μm in size and have a visible tubular shape. The original HNTs are cylindrical and have a relatively smooth surface (Figure 6a) (Zhai M, 2022). However, after immobilization the surface of HNTs showed thickening and roughness, indicating successful immobilization between HNTs and laccase enzyme (Figure 6d). EDX data revealed changes in the elemental composition of silicon (Si), aluminium (Al), and oxygen (O), supporting the successful immobilization of the enzyme. Thickening and surface roughness after immobilization may result from the addition of laccase enzyme layer on the HNTs surface, which is detected as a change in the morphological structure. These changes are often indicated by a shift or increase in peaks in the EDX spectra that correlate with specific elements (Zhimiao Z, 2019; Wang et al., 2021). Additionally, a decrease in the clarity of the HNTs after immobilization suggests the occurrence of a physical or chemical interaction between the HNTs and the laccase enzyme. These interactions may involve covalent or non-covalent bonds, such as hydrogen bonds and electrostatic interactions, which indicates that the immobilization process successfully facilitated the attachment of the enzyme on the HNTs surface. Therefore, the FESEM observation and EDX analysis prove that the immobilization of laccase enzyme on HNTs has been successfully carried out.

3.5.3 High-Resolution Transmission Electron Microscopy (HR-TEM) Analysis

The surface morphology of natural HNTs and HNTs immobilized with laccase is depicted in images taken by HR-TEM at 390.000× Magnification. The pristine HNTs display a cylindrical morphology with a hollow interior and smooth, clean walls. The internal and external diameters of the HNTs ranged from 20-30 nm and 100-1000 nm, respectively (Figure 7a) (Zandi-Mehri E, 2022). Following laccase immobilization, a thick grey layer is observed, and the walls of the HNTs become rough, indicating that the enzyme is trapped inside the hollow interior and attached to the surface (Figure 7d). This demonstrates that the laccase was successfully immobilized on

the HNTs/Gly surface and remained functionally active

3.6 Toxicity Activity

The toxicity assay was used to determine the mortality rate of Artemia salina larvae across varying substance concentrations. This assay compares test results against a control and analyzes data to determine the LC_{50} value. The LC_{50} value represents the substance concentration required to achieve 50% mortality of the tested organisms within a specified time frame. The LC_{50} values were lower for pure HNTs in the control group than for HNTs/Gly-Lac. As shown in Table 3, the LC_{50} values of HNTs/Gly-Lac were higher than those of free laccase and pure HNTs (1.57% or 157 ppm) > (1.21% or 121 ppm) > (0.90% or 90 ppm). These results suggest that free laccase is highly toxic before immobilization, while its immobilized form (HNTs/Gly-Lac) demonstrates moderate toxicity to Artemia salina larvae, in which the LC_{50} value falls within the range of 100 to 500 ppm (Hamidi et al., 2014).

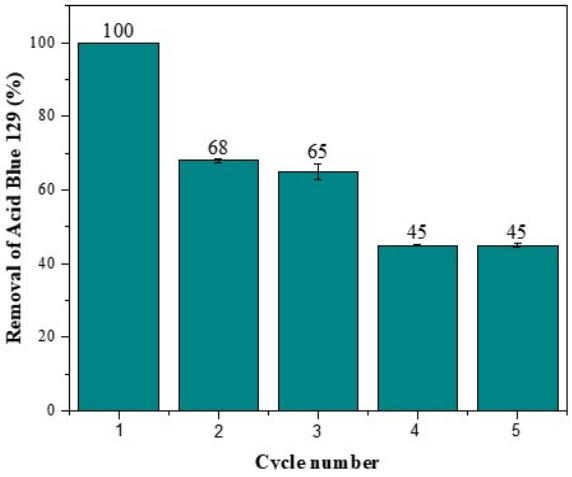


Figure 8. Reusability of HNTs/Gly-Lac up to Four Reuse Cycles

3.7 Reusability of Immobilized Laccase

The decolorization ability and reusability of various materials against AB129 dye showed significant performance variations (Table 4). The results indicate that Halloysite Nanotubes (HNTs), while effective at initial decolorization with 80-90% efficiency, the HNTs/Gly-Lac experienced a 50-60% drop after 5 cycles of use, limiting their long-term applicability (Figure 8). This decline is likely due to enzyme leakage during repeated cycles (Anita et al., 2020). The saturation of inner and outer surface of HNTs with laccase may be also contributed the lower decolorization rate after repeated cycles, as adsorption of dye molecule will be lower compare do that of pure HNTs. Activated Carbon and Chitosan Beads also showed decreased reusability, although both maintained moderate efficiency after multiple uses. Zeolite Nanoparticles and Metal-Organic Frameworks (MOFs) exhibited good overall performance, with MOFs showing high decolorization efficiency and strong reusability. Graphene Oxide (GO) stood out as the best-performing material, with an initial decolorization efficiency of 98% and excellent reusability, maintaining 85% efficiency even after 5 cycles. Materials such as GO and MOFs are suitable for long-term applications, while HNTs and Silica Gel are more effective for initial decolorization but have limitations in terms of reusability.

4. CONCLUSIONS

The covalent immobilization of laccase onto functionalized HNTs was successfully achieved using glycine as the cross-linker. Analysis via FTIR, FESEM, and TEM revealed significant changes in the functional groups and morphological characteristics of HNTs/Gly post-immobilization. The laccase immobilized on HNTs/Gly exhibited excellent performance in the decolorization of AB129 dye at high concentrations. Additionally, HNTs/Gly-Lac demonstrated superior enzymatic properties at high temperatures and pH levels compared to free laccase. This effective decolorization, facilitated by both adsorption and degradation mechanisms, suggests that HNTs/Gly-Lac could be a promising new material for treating textile dye wastewater.

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