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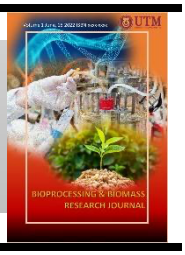

Research Article

Effects of Incubation Time and Laccase Concentration on Immobilization of Laccase on Magnetic Spent Tea: Adsorption and Cross-linking Method

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ARTICLE INFO

Article History:
Received 29 November 2024
Received in revised form 12 December 2024
Accepted 20 December 2024
Available online 31 December 2024

Keywords:
Magnetic Spent Tea,
Laccase,
Immobilization

ABSTRACT

This study explores the impact of incubation time and laccase concentration on the immobilization efficiency and activity of laccase using adsorption and crosslinking method, employing magnetic spent tea as a sustainable carrier. The results indicate that the highest immobilization yield occurred at an incubation time of 24 h, achieving 96.51% immobilization yield with only 0.11 U, while the optimal enzyme activity of 4.31 U was recorded at a shorter incubation duration of 6 hours with 74.16% immobilization yield. This finding suggests that extended incubation times may enhance covalent bonding between the enzyme and the carrier but can also lead to reduced enzymatic activity due to potential over-binding. The ideal laccase concentration was identified at 1.0 mg/mL, which resulted in a notable immobilization yield of 74.16% while preserving significant enzyme activity. Higher concentrations of laccase caused steric hindrance, adversely affecting performance. Furthermore, reusability assessments revealed that the immobilized laccase's relative activity increased from 23% in the first cycle to 100% by the fifth cycle, likely facilitated by ABTS as a mediator. Utilizing magnetic spent tea not only serves as an effective and economical method for enzyme immobilization but also aids in waste reduction by transforming tea byproducts into valuable resources. This dual approach underscores the potential for enhancing enzymatic processes while advancing environmentally responsible waste management practices in biotechnological applications.

INTRODUCTION

Over the past few decades, laccase has been the focus of numerous studies due to its potential applications in a variety of bioremediation disciplines. According to Ba et al. (2013), laccases have demonstrated a significant capacity to eliminate various pollutants that pose a threat to the environment from soil and water bodies. Enzymes have many benefits such as high specificity for their substrates, ability to operate in mild condition and environmentally friendly, but they are also limited by harsh environments such high acidity, which can damage free enzymes and lose their ability to function and lowering the substrate affinity (Franssen et al., 2013).

Therefore, to get around these restrictions, immobilization technologies have been applied to enhance the durability and catalytic traits of enzymes where it permits the optimization of interactions between substrates and enzymes while also minimizing non-specific interactions (Gao et al., 2022; Homaei et al., 2013). There are several methods for immobilizing enzymes, including entrapment, covalent bonding, adsorption, and cross-linking. Each of these methods have their own advantages and disadvantages (Syukri et al., 2023). Through immobilization, the enzyme becomes a heterogeneous mixture instead of

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remains as homogenous mixture. Enzyme immobilization offers several benefits, including the ability to easily extract the immobilized enzyme from the reaction mixture and to utilize it repeatedly, both of which will boost continuous process productivity. Moreover, compared to free enzymes, they typically have longer shelf lives, exhibit better activity, and are more resilient to harsh conditions like extreme pH and temperature (Zhang et al., 2017).

Nevertheless, while various carriers have been explored in previous studies such as nanomaterials, carbon-based material and polymers. Carrier from plant-based materials is more secure and easier to prepare, as they are environmentally friendly adsorbents that remove a range of pollutants quickly and affordably (Yaashikaa et al., 2022). Several studies conducted using plant-based material as carrier including coconut shell (Al-sareji et al., 2023a) rice straw biochar (Imam et al., 2021), raw wood chips (Li et al., 2018), spent grain (Girelli & Scuto, 2021a) and pomegranate peels (Al-sareji et al., 2023b)

According to Panneerselvam et al. (2011), the cell walls of the tea leaves are rich in cellulose, hemicellulose, tannins, lignin, and proteins which contain numerous functional groups including carboxyl and hydroxyl. The present of these functional groups contribute to their potential as adsorption (Humphrey & Yakubu, 2024). For instance, in a prior study, Hameed (2009) used spent tea to effectively remove the cationic dye methylene blue from an aqueous solution. However, the main drawback of this powder-like adsorbent, as stated by Yang et al. (2021), is that it is difficult to separate from the liquid state due to smaller particle size and evenly distributed in an aqueous solution. In order to resolve this problem, magnetic spent tea was used as the carrier for laccase immobilization in this study.

However, there is limited understanding regarding the interaction between laccase and magnetic spent tea. A weak interaction between the enzyme and the carrier will result in enzyme leaching during the immobilization process. Therefore, to improve the use of magnetic spent tea as a carrier for laccase immobilization, this study investigated the effects of incubation time and laccase concentration on enhancing the immobilization yield and activity of the immobilized laccase.

MATERIALS AND METHOD

Materials

Laccase from *Trametes Versicolor,* tris(hydroxymethyl)aminomethane, hydrochloric acid (HCl), was obtained from Sigma-Aldrich, US. Iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) was obtained from Merck, Germany. Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) from VWR International, US, ammonium hydroxide solution (25%) from Acros Organics, US. Ammonium sulphate from Carl Roth, Germany. glutaraldehyde from Alfa Aesar, US and the 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution was obtained from Bio-Rad Laboratories, US.

Preparation of Magnetic Spent Tea

The spent tea was collected and fully washed using distilled water to remove all dirt substances. Subsequently, spent tea were boiled repeatedly in a tap water with intervening washes until the boiling water remains visually clear. Then, the washed spent tea was dried under the sunlight. After the spent tea was fully dried, they were treated using HCl/NaOH treatment following method by Girelli & Scuto (2021b). Then, the magnetic spent tea was synthesized using co-precipitation method as conducted by (Mozaffari et al., 2023).

Immobilization of Laccase on Magnetic Spent Tea

The immobilization of laccase was conducted with two-step immobilization using laccase pH 4 (acetate buffer), which started with physical adsorption and followed by cross-linking using 0.05% glutaraldehyde incubated for 0.5 hr at room temperature under mild agitation. Two parameters were varied for the laccase immobilization which are incubation time and laccase concentration. The incubation time were varied at 6, 12, 18 and 24 hr followed by testing different laccase concentration at 0.5, 1.0, 1.5 and 2.0 mg/ml to investigate the effect of both parameter on immobilization yield and laccase activity. All measurements were conducted in triplicates

Laccase Assay

The assessment of both free laccase and immobilized laccase activities was conducted using the ABTS oxidation at 420 nm for 3 min as described on our previous research Mohd Syukri et al. (2020). The laccase activity was calculated using Equation **(1)**.

$$U = \frac{\Delta A \times V \times 10^6}{\varepsilon \times L \times t} \quad \textbf{(1)}$$

where, ΔA is the change in absorbance after reaction at 420 nm, V is the reaction volume, ε is molar extinction coefficient (36000 $M^{-1}cm^{-1}$), L is the optical path (1 cm), and t is the reaction time (3 min). One unit of enzyme activity (U) is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute. While the immobilization yield (IY) was then calculated using the formula shown in Equation **(2).**

$$IY\ (\%) = \frac{U_I - U_F}{U_I} \times 100\ \% \quad \textbf{(2)}$$

where, U_I is the laccase activity before immobilization and U_F is the laccase activity after immobilization.

Reusability

The reusability test was conducted by five repetitive cycles of ABTS oxidation. The immobilized laccase was rinsed and washed using acetate buffer at pH 4 after each cycle. Then the relative activity was calculated using Equation **(3).**

$$RA\ (\%) = \frac{U_A}{U_{max}} \times 100\ \% \quad \textbf{(3)}$$

where U_A is the immobilized activity at each cycle and U_{max} is the maximum activity of immobilized laccase in the cycle.

RESULTS AND DISCUSSION

Effect of Incubation Time

Figure 1 shows that the immobilization yield was highest at an incubation time of 24 h, reaching 96.51%. This was followed by an 18-h incubation time, which achieved a yield of 79.25%. Although significantly high, it is still lower than the 24-h mark. Incubation times of 6 and 12 h both showed the same immobilization yield of 74.15%. From the results, it was observed that the immobilization yield increases with incubation time, peaking at 24 h. This suggests that longer

incubation times might allow more extensive covalent bonding between the enzyme and the support, leading to higher immobilization yields (Bayramoglu et al., 2010). The big difference in yield between 18 and 24 h indicates a significant improvement in the binding process during the extended incubation period. Immobilization typically follows a time-dependent process where initially, binding sites on the carrier material interact with the enzyme molecules. Over time, as more binding sites are occupied, the rate of enzyme binding may slow down. The extra incubation time from 18 to 24 h might allow more enzyme molecules to find available binding sites, maximizing yield (Deng et al., 2022).

As for the enzyme activity, **Figure 1** shows the highest result was obtained with an incubation time of 6 h, showing 4.31 U. The activity shows a decreasing trend as the incubation time increased. More than half of the activity was reduced at 12 h incubation time which was 1.74 U and at 18 hours showed a slightly higher activity at 1.92 U. The lowest enzyme activity was observed at 24 h, with only 0.11 U. In contrast to immobilization yield, the highest activity recovery was at the shortest incubation time of 6 hours. This suggests that while fewer laccase molecules were attached, those that did attach remained very effective and active. As incubation time increased, activity recovery sharply decreased, with the lowest activity recovery at 24 h. According to Arica et al. (2017), immobilization can later the natural state of an enzyme. Extensive immobilization at longer times might lead to over-binding, restricting the conformational flexibility of the enzyme, thus reducing its activity.

Based on the findings, the optimum incubation time chosen for immobilizing laccase is 6 h. This duration resulted in the highest enzyme activity of 4.31 U and a significant immobilization yield of 74.15%. Longer incubation times, such as 18 and 24 h, showed higher immobilization yields but significantly lower activity recoveries, suggesting potential enzyme denaturation or structural changes due to prolonged exposure. Therefore, a shorter incubation time of 6 hours strikes a balance between achieving adequate enzyme binding efficiency and preserving enzymatic activity, making it optimal for practical applications requiring active immobilized enzymes.

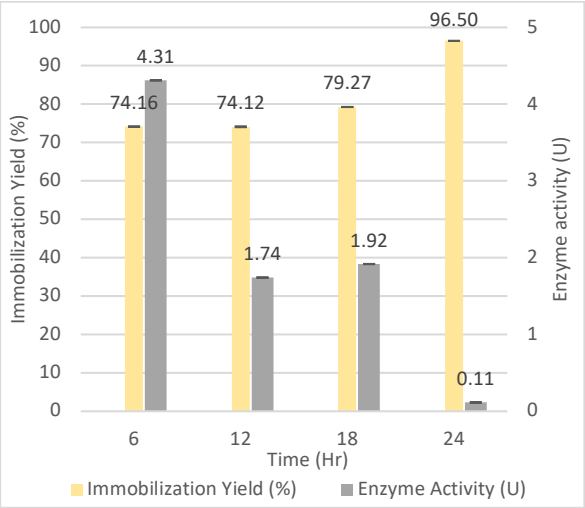

Figure 1 Effect of incubation time on the immobilization yield and enzyme activity of immobilized laccase

Effect of Laccase Concentration

The immobilization yield for laccase concentration was shown in **Figure 2**, where the immobilization yield was highest at a laccase concentration of 1.0 mg/mL with 74.16%, followed by a concentration of 0.5 mg/mL with 31.81%. At concentration of 1.5 mg/mL, the yield dropped to 20.04% and for 2.0 mg/mL, the yield was slightly lower at 19.80%. This pattern is similar to the findings of Deng et al. (2022). Higher concentrations (1.5 and 2.0 mg/mL) lead to overcrowding on the carrier surface, causing steric hindrance and reducing yield. In contrast, a lower concentration (0.5 mg/mL) does not provide sufficient enzyme molecules to maximize interaction with the carrier.

As for enzyme activity, in **Figure 2,** the highest activity was observed at 1.0 mg/mL with 4.31 U, followed by 2.0 mg/mL with 2.22 U, 1.5 mg/mL at 1.23 U, and lastly 0.5 mg/mL at 0.19 U. The peak activity at 1.0 mg/mL suggests that this concentration allows for optimal binding while maintaining enzyme activity, indicating that the immobilized laccase functions best at this concentration. Although the enzyme activity at 2.0 mg/mL is relatively high, it is lower than at 1.0 mg/mL. Similarly, at 1.5 mg/mL, the trend shows a reduced enzyme activity compared to 1.0 mg/mL. This might be due to excessive concentration leading to decreased activity because of the blockage of active sites. The lowest activity was observed at 0.5 mg/mL, suggesting that lower concentrations may not provide enough enzyme molecules to maximize potential interactions with the carrier, leading to lower immobilization efficiency and activity Deng et al. (2022).

Based on the results obtained, the optimum laccase concentration was determined to be 1.0 mg/mL. This concentration showed the highest immobilization yield and enzyme activity, indicating effective binding to the carrier and retention of significant enzymatic activity. The high yield and activity recovery at 1.0 mg/mL make it practical and cost-effective, ensuring substantial enzyme immobilization per unit volume. Therefore, 1.0 mg/mL is the ideal concentration for maximizing both immobilization efficiency and enzymatic functionality, crucial for applications like bioremediation.

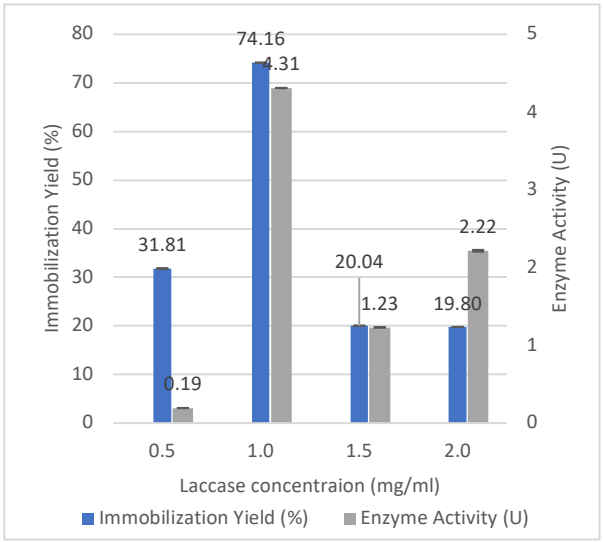

Figure 2 Effect of laccase concentration on the immobilization yield and enzyme activity of immobilized laccase

Reusability

Figure 3 shows the reusability test of immobilized laccase on magnetic spent tea. Usually, the relative activity of reusability test for immobilized laccase will decrease after repetitive cycle due to structural damage to the enzyme during the reusability process (Oraby et al., 2025). Contrary, in this study, it starts with initial activity of 23% at the first cycle, then it increases to 58% at the second cycle, 68% at the third cycle, 70% at the fourth cycle and reach 100% at the fifth cycle. This trend shows an increase in relative activity from 20% at the first cycle to 100% at the fifth cycle. This might be due to the presence of ABTS, which is a mediator known to enhance laccase activity. According to Cañas & Camarero (2010), laccase from *Trametes versicolor* has a redox potential approximately ~760 mV, which is crucial for enhancement its catalytic efficiency. The presence of ABTS in the current study might have further increased the redox potential, enhancing the enzyme activity with each reuse cycle. ABTS accelerates the electron transfer or radical hydrogen atom transfer, which is likely also enhances the overall activity of laccase-mediator system (Deng et al., 2022). Currently, there is also an increasing research trend in immobilizing mediator or co-immobilization of mediator in expanding the range of laccase substrate and broadening the application field of laccase (Gu et al., 2021). The ABTS might have been adsorbed by the magnetic spent tea and becoming co-immobilized with laccase in the the first cycles. Subsequently. the ABTS acted as the mediator for the oxidation of ABTS on the following cycles. As a result, the relative activity in this study improved with each successive cycle.

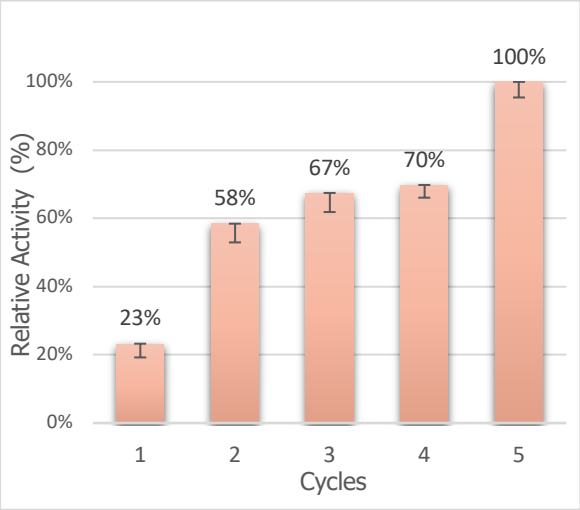

Figure 3 Reusability test of immobilized laccase on magnetic spent tea

CONCLUSION

This study highlights the effectiveness of magnetic spent tea as an environmentally friendly carrier for laccase immobilization, demonstrating its potential to reduce agricultural waste and promote sustainability. The optimal incubation time of 6 hours achieved a significant immobilization yield of 74.15% while preserving peak enzyme activity, indicating that shorter incubation times can enhance enzymatic function without compromising binding efficiency. Furthermore, the ideal laccase concentration of 1.0 mg/mL maximized both immobilization yield and enzymatic activity, showcasing the importance of balancing these parameters for practical applications. The reusability tests revealed that immobilized laccase exhibited a remarkable increase in relative activity from 23% to 100% by the fifth cycle, attributed to the presence of ABTS as a mediator. Utilizing magnetic spent tea not only provides an effective and low-cost solution for enzyme immobilization but also contributes to waste reduction by repurposing tea byproducts. This approach supports environmentally sustainable practices in biotechnological applications, making it a promising strategy for addressing environmental challenges while minimizing waste.

Acknowledgement

The authors acknowledge the financial support from Ministry of Education Malaysia through Fundamental Research Grant Scheme (FRGS/1/2024/TK08/UMS/02/4) and Universiti Malaysia Sabah for giving cooperation and full of support in this research activity.

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