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OPTIMIZATION OF LACCASE IMMOBILIZATION ON CHITOSAN BEADS USING THE CROSS-LINKING METHOD

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ABSTRAK

Limbah industri tekstil merupakan salah satu masalah utama lingkungan saat ini. Limbah tersebut mengandung residu pewarna dan berbagai senyawa kompleks. Lakase menjadi salah satu pilihan untuk degradasi biokatalitik zat warna karena biodegradabilitas dan biokompatibilitasnya. Pemanfaatan lakase untuk detoksifikasi polutan secara luas menjadi terbatas karena kerentanannya terhadap perubahan kondisi operasional. Untuk mengatasi masalah tersebut, lakase diimobilisasi pada kitosan dengan glutaraldehida sebagai agen pengikat silang. Penelitian ini dilakukan untuk menentukan kondisi optimum metode ikat silang dan imobilisasi lakase pada manik-manik kitosan. Pada penelitian ini, lakase dari *Aspergillus sp*. diimobilisasi pada butiran kitosan yang berikatan silang dengan glutaraldehida. Butiran kitosan dibuat dengan menggunakan kitosan 2% (b/v) dan diikat silang dengan glutaraldehida. Selanjutnya, butiran yang sudah teraktivasi glutaraldehida diinkubasi dengan lakase. Lakase yang terimobilisasi pada butiran kitosan diuji kadar protein dan aktivitasnya untuk memperoleh nilai efisiensi imobilisasi. Hasil penelitian menunjukkan bahwa konsentrasi glutaraldehida optimum adalah 0,8% dengan waktu ikat silang selama 6 jam, dosis lakase 0,4 mg/mL dan waktu imobilisasi selama 4 jam. Dari kondisi ini, didapatkan efisiensi imobilisasi sebesar 16,51% dengan aktivitas lakase sebesar 24,58 U/g. Berdasarkan karakterisasi gugus fungsi manik-manik kitosan terimobilisasi lakase, adanya puncak untuk gugus fungsi C=N mengkonfirmasi bahwa lakase telah terimobilisasi pada kitosan secara kovalen.

ABSTRACT

The textile industry is one of the significant environmental problems. The waste contains dye residues and various complex compounds. Laccase is a viable option for biocatalytic degradation due to its biodegradability and biocompatibility. The widespread use of laccase for pollutant detoxification is limited due to its susceptibility to changes in operational conditions. To overcome this problem, laccase was immobilized on chitosan with glutaraldehyde as a cross-linking agent. This research was conducted to determine the optimum conditions for the cross-linking method and laccase immobilization on chitosan beads. In this study, chitosan beads were developed using 2% (w/v) chitosan and cross-linked with glutaraldehyde. The activated chitosan beads were then incubated with laccase. The immobilized laccase was tested for its protein content and activity to obtain an immobilization efficiency value. The results showed that the optimum glutaraldehyde concentration was 0.8% with a cross-linking time of 6 hours, a laccase dose of 0,4 mg/mL, and an immobilization time of 4 hours. From these conditions, an immobilization efficiency of 16.51% was obtained with an enzyme activity of 24.58 U/g. Based on the functional group characterization of laccase-immobilized chitosan beads, the presence of the C=N functional group peak confirmed that laccase had been covalently immobilized on chitosan.

How to Cite

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INTRODUCTION

Environmental problems are a critical issue and a global challenge today. With the increasingly widespread development of industry, environmental problems are getting worse. One of the main ecological problems is water pollution caused by toxic chemicals from textile industry waste. The textile industry produces several liquid wastes containing poisonous materials, dye residues, and various complex compounds. Most dye residues hurt the environment and health because they are toxic, mutagenic, and carcinogenic (Aslam et al., 2021).

Various techniques have been used to degrade dye residues in waste using physical and chemical methods, such as membrane filtration, adsorption, ion exchange, flocculation-coagulation, and chemical oxidation (Adane et al., 2021). However, these methods are less preferred due to the high production of sludge and toxic products (Morsi et al., 2020). Therefore, biological methods are often considered a better alternative to degrading dyes. One agent that can be used to degrade dyes biologically is enzymes. The enzymatic degradation process of a pollutant can be an effective solution because it has low toxicity and high chemical selectivity in environmental conditions (Bilal & Iqbal, 2019).

Laccase is a prospective enzyme for biocatalytic degradation and detoxification of dye waste (Morsi et al., 2020). It catalyzes the oxidation reaction of phenolic substances using oxygen molecules as an electron acceptor (Deng et al., 2024). However, laccase tends to be sensitive to environmental influences, is less stable, requires high purification costs, and is difficult to separate if it is used repeatedly.

Enzyme immobilization is an approach that can be used to increase the operational stability and reuse of enzymes in their use on an industrial scale (Jaiswal et al., 2016). In addition, enzyme modification through an immobilization strategy is the most effective method in biotechnological applications based on green chemistry principles , which allow the reuse of laccase, easy recovery, longer half-life, and stabilization of enzyme activity and structure (Bilal et al., 2017).

The selection of appropriate supporting materials and immobilization methods is are important thing to study in the immobilization process. This is because the use of cheap supporting materials and optimal processing conditions can improve the properties of the immobilized enzyme. The supporting materials used can be inorganic materials or organic polymers (Younes & Rinaudo, 2015). One natural polymer that is often used is chitosan. Chitosan was chosen as a supporting material for enzyme immobilization because of its good biocompatibility, biodegradability, easy modification, film-forming ability, and high affinity for proteins, as well as its high abundance. (Bilal et al., 2019; Bilal & Iqbal, 2019; Bilal M, Rasheed T, Zhao Y, Iqbal HMN, 2018)

Various immobilization methods have been developed to improve enzyme stability and reusability. The cross-linking method was chosen in this research because it has advantages compared to other methods, namely that it can form a stable bond between the enzyme and the supporting material so that the enzyme does not come off easily and the substrate can interact optimally. Glutaraldehyde is a cross-linking agent that is widely used because of its easy preparation and affordable price. This compound has two aldehyde groups, which can act as a link between the enzyme and the immobilization support material (Mahargyani et al., 2017).

Thus, this research was conducted to test the optimum conditions for the crosslinking method on chitosan beads using the crosslinking agent glutaraldehyde and the immobilization of laccase on chitosan beads. The success of enzyme immobilization

was proven by testing the catalytic activity of the enzyme before and after immobilization.

METHODS

The research began by making chitosan beads, cross-linking the chitosan beads with glutaraldehyde, and immobilizing laccase on activated chitosan beads. Laccase before and after immobilization was tested for protein content and activity. The chitosan beads before and after immobilization were analyzed for their functional groups using FTIR spectroscopy.

Materials

The material used in this research was the laccase enzyme from *Aspergillus sp.* produced by Sigma Aldrich®, chitosan powder from shrimp shells (deacetylation degree 88%, medium molecular weight) produced by Sigma Aldrich®, 25% glutaraldehyde from Merck®, sodium hydroxide (NaOH), glacial acetic acid, sodium acetate buffer, distilled water, Bradford reagent, ABTS (Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)) from Merck®, BSA (Bovine Serum Albumin) Himedia®.

Tools

The tools used in this research were beakers, measuring flasks, measuring cups, pipettes, syringes, stirring rods, iron spatulas, thermometers, pH meters, analytical balances, magnetic stirrers, funnels, filter paper, petri dishes, UV-Vis spectrophotometers Shimadzu® and Fourier Transform Infrared (FTIR) Spectrometer.

Procedure

Chitosan Beads Preparation

This stage is carried out through a coagulation process of chitosan dissolved in 1% acetic acid. The resulting solution was added dropwise into the 2 M NaOH coagulant using a syringe (10-12 drops per minute). The beads are harvested by filtration and washing until neutral. Next, the chitosan beads obtained are soaked in distilled water and stored at 4°C for use in the next stage.

Activation using Glutaraldehyde

Cross-linking was carried out using glutaraldehyde as a cross-linking agent. Chitosan beads (2.0 g) were mixed with 2.0 mL of glutaraldehyde solution with varying concentrations of 0.1%; 0.25%; 0.5%; 0.8%; 1%; and 2% (v/v) as well as time variations of 3 hours and 6 hours. The cross-linked chitosan granules were washed several times with deionized water to remove excess glutaraldehyde.

Laccase Cross-linking

Chitosan-glutaraldehyde beads were incubated with varying laccase doses of 0.2 mg/mL - 1 mg/mL (1:2). The incubation process was carried out at room temperature for 4-6 hours. After immobilization, the beads were washed with sodium acetate buffer (pH 5.5). Meanwhile, the remaining laccase solution from the incubation was separated for testing protein levels using the Bradford method. Then the laccase-immobilized chitosan beads were stored at 4°C in sodium acetate buffer (pH 5.5).

Protein Content Measurement

Protein content measurements were carried out using the Bradford method. This method uses Coomassie Brilliant Blue as a dye to bind protein in the sample. The result is then compared to a standard curve from known quantities of a standard protein. The standard protein used in this study is Bovine Serum Albumin (BSA). The protein assay in immobilized laccase is estimated from the difference in protein in the solution remaining from incubation of the beads in the immobilization process with the total amount of protein in the enzyme solution used for immobilization.

Laccase Activity Test

Laccase activity was tested by measuring the oxidation speed using ABTS (2,2-azino-bis-(ethylbenzothiazoline-6-sulfonic acid)) substrate at room temperature. One unit of

enzyme activity is defined as the amount of laccase required to convert 1 µmol of substrate per minute into the product (Taha et al., 2020).

Free laccase activity testing was carried out by mixing 250 µL of 1 mM ABTS, 2500 µL of sodium acetate buffer (pH 4.5), and 250 µL of enzyme (0.4 mg/mL). Next, absorbance was measured at time intervals of 0 and 10 minutes of incubation. Measurements were carried out using a UV-Vis spectrophotometer at λ 420 nm (ε_{420} nm = 36,000 M^{-1} cm^{-1}).

Immobilized laccase activity testing was carried out by mixing 0.1 grams of wet chitosan-laccase beads with 250 µL mM ABTS (1 mM) in 2750 µL sodium acetate buffer solution (pH 4.5). The absorbance and wavelength measurements used were the same as those carried out in the free laccase activity assay.

$$Laccase\ activity\ = \frac{(A_t - A_0) \times Vtot \times 10^6}{\varepsilon\, maks \times Wenzyme \times t} \quad \text{(Eq.1)}$$

Laccase activity as the number of units of enzyme per mL or gram of protein (U/mL or U/g) with A_t as absorbance at 10 minutes, A_0 as absorbance at 0 minute, V_{tot} as mixture total volume (mL), ε_{maks} as ABTS molar absorptivity (36.000 $M^{-1}cm^{-1}$), W enzyme as enzyme volume (mL) or mass (g), and t as incubation time (minute).

Immobilization Yield

The immobilization yield value was obtained from the difference between the concentration of free laccase protein and the concentration of immobilized laccase protein compared to the concentration of free laccase. P_1 is the protein content in laccase before immobilization (ppm), and P_2 is the protein content in laccase after immobilization (ppm).

$$Y_i\% = \frac{P_1 - P_2}{P_1} \times 100\% \quad \text{(Eq. 2)}$$

Immobilization Efficiency

Determination of the immobilization efficiency (E_i%) of laccase on chitosan was obtained from the comparison of the total activity of immobilized laccase with the total activity of free laccase.

Total Activity (U)

Total activity of free laccase (U)= Enzyme activity ($\frac{U}{mL}$) × Enzyme volume (mL) (Eq. 4)

Total activity of immobilized laccase(U)= Enzyme activity ($\frac{U}{g}$) × Enzyme mass (g) (Eq. 5)

Immobilization Efficiency (E_i%)

$$E_i(\%) = \frac{\text{Total activity of immobilized laccase (U)}}{\text{Total activity of free laccase (U)}} \times 100\% \quad \text{(Eq. 6)}$$

Functional Group Characterization

FTIR spectroscopic analysis was carried out to identify the functional groups contained in chitosan beads, glutaraldehyde cross-linked chitosan beads, and glutaraldehyde cross-linked chitosan beads after immobilization of laccase.

RESULT AND DISCUSSION

Chitosan Beads

In this research, chitosan beads have been successfully developed to be used as a supporting material for laccase immobilization **(Figure 1)**. The advantages of supporting materials in the form of solid beads are their good shape, size, and stability. The laccase enzyme immobilized on chitosan beads forms a solid biocatalyst which will make it easier to separate the enzyme at the end of the reaction. Therefore, the immobilization process can increase the possibility of enzyme reuse, according to the purpose of enzyme immobilization. In addition, chitosan in the form of beads has good stability and helps maintain enzymes in the supporting material, thereby avoiding enzyme leakage due to the use of several reaction cycles (Jaiswal et al., 2016).

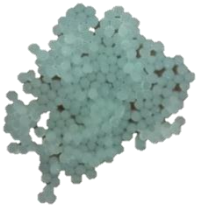

Figure 1. Chitosan beads

The process of chitosan bead formation is carried out by dripping the chitosan solution into the NaOH solution. Chitosan beads can be formed due to the influence of electrostatic forces. This force occurs between the chitosan solution which is acidic and the NaOH solution which is basic. Soaking in NaOH makes the beads form a hydrophobic surface, thereby avoiding chitosan dispersion in the medium (alkaline solution). In addition, chitosan beads are insoluble in high pH (alkaline) due to the presence of amine groups. This is what causes the beads in the NaOH solution to harden over time (Aricov et al., 2020). **Figure 2** shows the mechanism for the deposition of chitosan beads in NaOH solution.

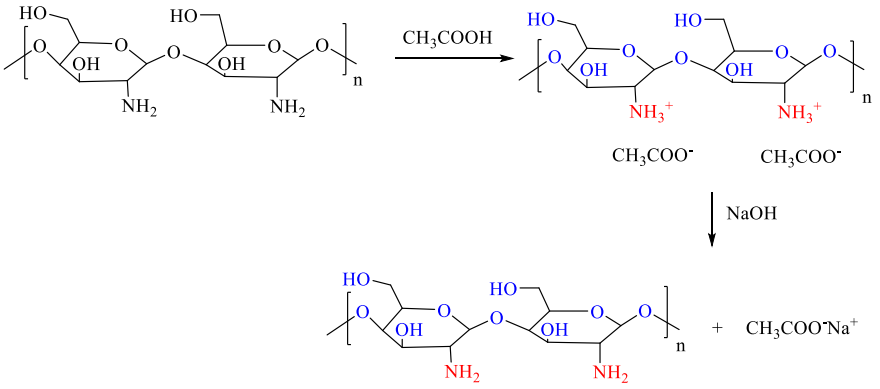

Figure 2. Mechanism of chitosan beads deposition

Chitosan-glutaraldehyde Cross-linking

Chitosan beads were activated with glutaraldehyde. Glutaraldehyde-activated chitosan beads provide a biocompatible bead surface leading to optimum immobilization efficiency. Without glutaraldehyde treatment, the structure of chitosan beads would be very weak.

Structurally, glutaraldehyde reacts adequately with chitosan beads to form a Schiff base so that the enzyme molecule attaches to the support with glutaraldehyde acting as a bridge. The aldehyde group in glutaraldehyde will bond with the amine group in chitosan and the amino group in the enzyme forms the chitosan-glutaraldehyde-laccase (Zdarta et al., 2018).

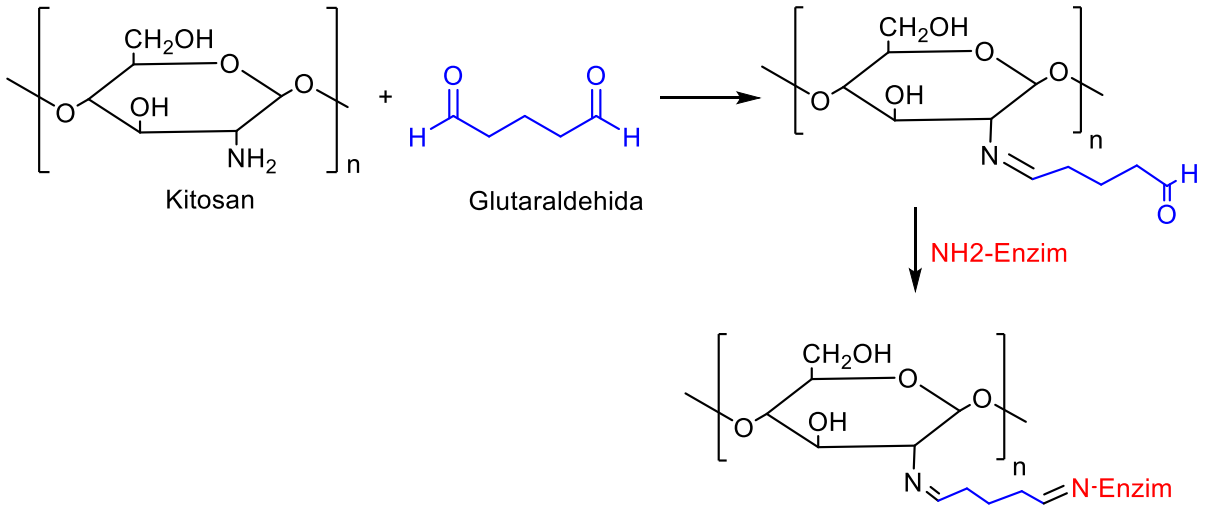

Figure 3. Chemical bond in chitosan-glutaraldehyde-laccase

The reaction between glutaraldehyde and the amine groups of chitosan facilitates the cross-linking of the polymer chains, leading to an increase in the mechanical resistance of the support and avoiding its solubility in acidic environments due to its cationic nature (Younes & Rinaudo, 2015).

Optimization of Glutaraldehyde Concentration

The efficiency of enzyme immobilization depends on the degree of glutaraldehyde cross-linking with chitosan beads (Cheng et al., 2016). Within a certain concentration range, cross-linkers can increase the level of cross-linking between the supporting material and the enzyme, thereby stimulating enzyme immobilization. Higher concentrations of cross-linkers, however, may reduce the activity of immobilized laccase because of enzyme denaturation, blockage of the enzyme's active site by the cross-linker, and an inadequate amount of laccase immobilization.

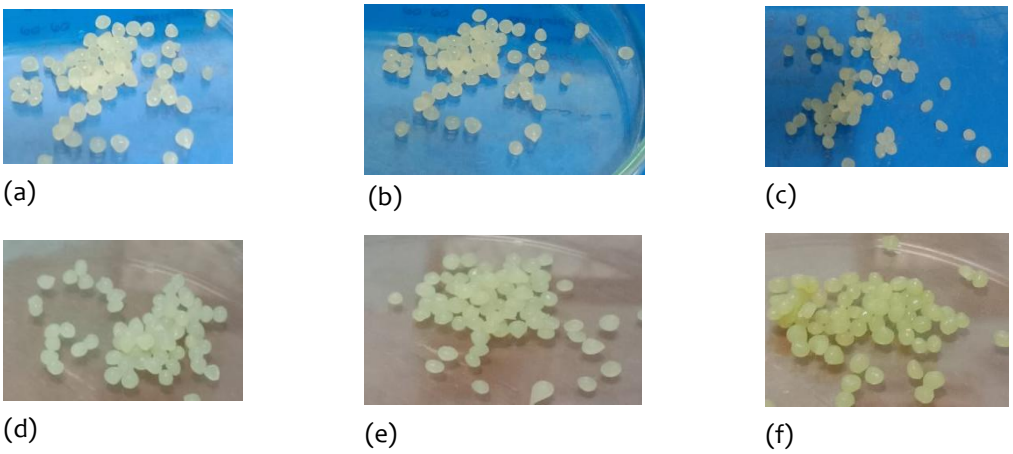

Figure 4. Chitosan beads with varying glutaraldehyde concentration: (a) 0,1% (b) 0,25% (c) 0,5% (d) 0,8% (e) 1% (f) 2%.

In this research, the beads were incubated with a concentration of glutaraldehyde (0.1%-2%) to evaluate the optimum conditions for laccase immobilization. **Figure 4** shows the chitosan beads resulting from different glutaraldehyde concentrations. Cross-linking treatment with different concentrations of glutaraldehyde gave different physical appearances, namely different bead colors. The chitosan beads cross-linked with glutaraldehyde changed from transparent white to yellowish white. However, as the concentration of

glutaraldehyde increases, the color of the chitosan beads becomes increasingly yellow. This could be caused by the loading of glutaraldehyde into the beads, causing a color change.

Table 1. Immobilization yield, laccase activity, and immobilization efficiency

No.	Glutaraldehyde concentration (%)	Immobilization yield (%)	Laccase activity (U/g)	Immobilization efficiency (%)
1	0,1	12,7	13,71	9,2
2	0,25	33,6	16,83	11,3
3	0,5	82,8	17,42	11,7
4	0,8	93,5	21,67	14,5
5	1	98,5	12,5	8,4
6	2	53,0	9,67	6,5

Table 1 shows that beads cross-linked with 0.8% glutaraldehyde produced the highest immobilization efficiency (14.5%). From these, it can be observed that increasing the concentration of glutaraldehyde increases the activity of the laccase enzyme initially, but at concentrations greater than 0.8%, the laccase activity decreases, which can be caused by the presence of steric hindrance due to the many cross-linking points between the enzyme molecule and the chitosan surface.

At the optimum concentration (0.8%), glutaraldehyde can react with the enzyme sufficiently to form a Schiff base so that more enzyme molecules are bound to the support with glutaraldehyde acting as a bridge (Younes & Rinaudo, 2015). Another study also reported that the activity of immobilized laccase (from the fungus *Trametes pubescens*) increased along with increasing glutaraldehyde concentration, namely from 0.1% to 0.8% (v/v) (Zheng et al., 2016). Optimum conditions were obtained at a glutaraldehyde concentration of 0.8%. After that, laccase activity decreased sharply at a glutaraldehyde concentration of 1%.

The glutaraldehyde concentration lower than 0.8% results in lower immobilization efficiency. This can be caused by inefficient activation of chitosan beads for enzyme attachment. Lower glutaraldehyde concentrations result in fewer enzyme attachment points resulting in lower immobilization efficiency.

Optimization of Cross-linking Time

Optimization of chitosan cross-linking time with glutaraldehyde was carried out. Chitosan beads were incubated with 0.8% glutaraldehyde for varying times of 3 hours and 6 hours to obtain the highest immobilized laccase activity. **Figure 5** shows that the optimum crosslinking time was 6 hours with an immobilization efficiency of 16.51%. This result is in line with previous research (Aricov et al., 2020), namely that the maximum relative activity of immobilized laccase was obtained after cross-linking glutaraldehyde for 6 hours. This indicates that this reaction time is suitable for the binding of glutaraldehyde to chitosan beads for enzyme attachment.

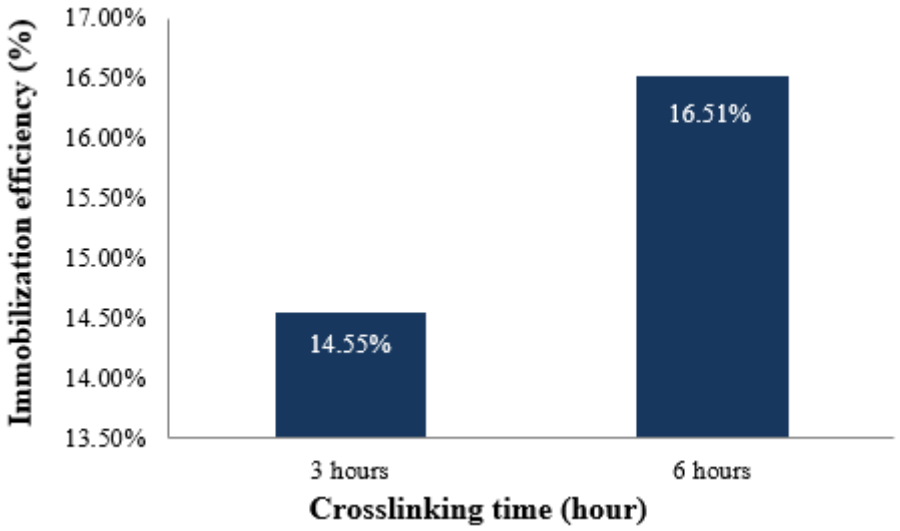

Figure 5. Immobilization efficiency with varying crosslinking time

Optimization of Laccase Dose

Laccase dose optimization was carried out to determine laccase activity. the beads were incubated with laccase solution at a dose of (0.2 mg/ml – 1 mg/ml). The result is shown in **Figure 6.**

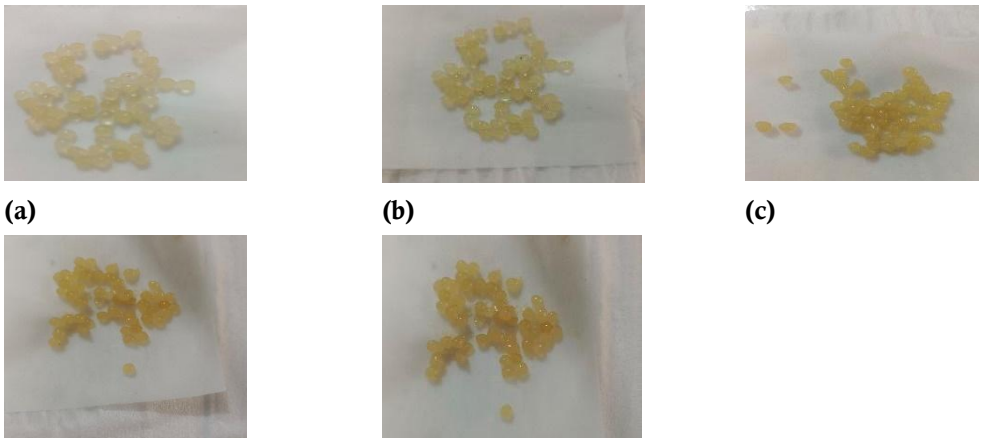

Figure 6. Laccase immobilized-chitosan beads with varying laccase dose (a) 0.2 mg/ml (b) 0.4 mg/ml (c) 0.6 mg/ml (d) 0.8 mg/ml (e) 1 mg/ml)

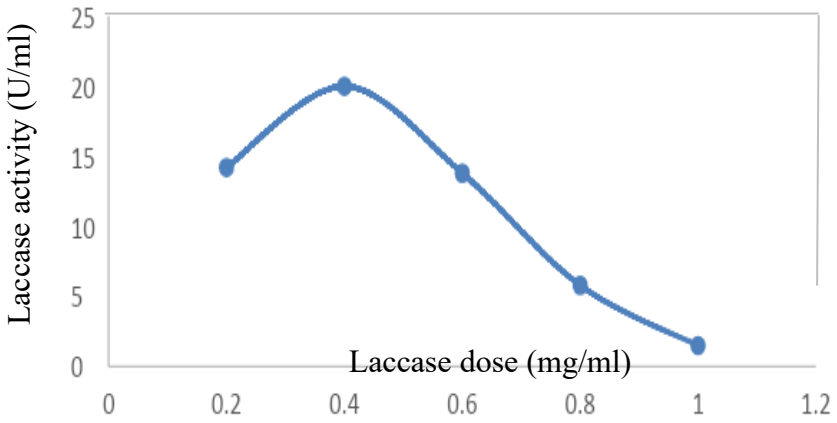

Figure 7. Laccase activity with varying laccase dose

From **Figure 7**, the optimum laccase dose was obtained at 0.4 mg/mL with an activity value of 20 U/ml. Laccase activity decreased at a dose of 0.6 mg/mL and continued to decrease up to a dose of 1 mg/ml. This decrease in activity is caused by excessive doses of laccase which can cause a lack of space between molecules which results in mass transfer, and can also limit the dispersion between the substrate and the product so that enzyme activity is small.

Optimization of Immobilization Time

Optimization of immobilization time was carried out by incubating chitosan beads in laccase for a certain time. The immobilization time is 4-5 hours with 0.4 mg/ml laccase solution. The result is shown in **Figure 8.**

(a) (b) (c)

Figure 8. Laccase immobilized-chitosan beads with varying immobilization time: (a) 4 hours, (b) 5 hours, (c) 6 hours

Figure 9 shows that the optimum immobilization time is 4 hours. This research shows that at an immobilization time of 4 hours, immobilized laccase has the highest activity, namely 20 U/g. Laccase activity continued to decrease at 5 and 6 hours of immobilization.

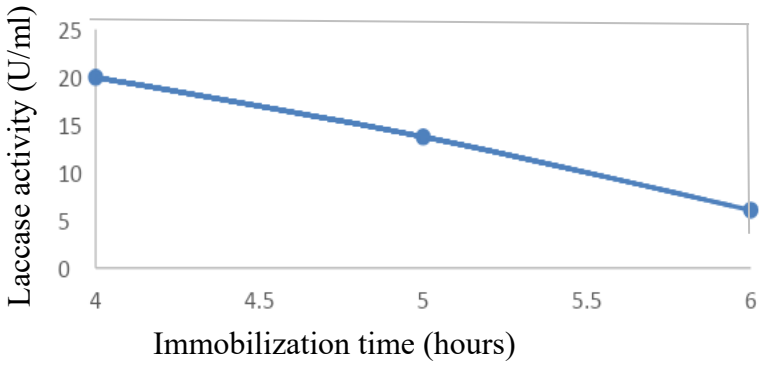

Figure 9. Laccase activity with varying immobilization time

As reported in other studies (Zheng et al., 2016), the highest laccase activity was obtained at an immobilization time of 4 hours. Increasing the immobilization time results in the laccase molecules in the buffer being too full to make optimal contact with the substrate and resulting in a decrease in enzyme activity. Additionally, if too much laccase is bound to the support material, the temperature will rise, as a result of the chemical reaction catalyzed by the solid laccase (Flores et al., 2019).

In this study, functional group analysis was carried out using an FTIR spectrophotometer on chitosan beads, glutaraldehyde cross-linked chitosan beads, and glutaraldehyde cross-linked chitosan beads after immobilization of laccase. This analysis was carried out to confirm whether there were cross-links formed between chitosan, glutaraldehyde, and the laccase enzyme. **Figure 10** showed the FTIR spectra of chitosan beads, chitosan beads-glutaraldehyde, and laccase immobilized-chitosan beads-glutaraldehyde.

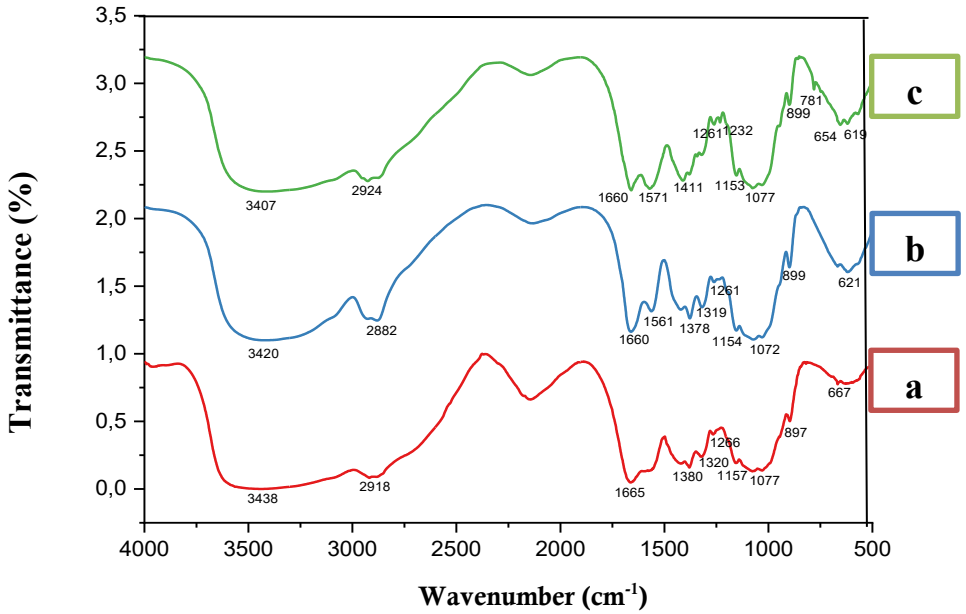

Figure 10. FTIR Spectra of: a) chitosan beads; b) chitosan beads-glutaraldehyde; c) laccase immobilized-chitosan beads-glutaraldehyde

In its role as an immobilization supporter, chitosan has a group that plays an important role in immobilization, namely the amino group. The glutaraldehyde cross-linker interacts with the amino group of chitosan to form a Schiff base. Generally, the reaction mechanism between the amino group and the aldehyde group is caused by the electrophilicity effect of the carbonyl group in the aldehyde.

In the figure of the spectrum of chitosan beads, it can be seen that there is an absorption band at wave number 3438 cm-1 as a result of the stretching vibration of the –OH group. The wide absorption and shift in the wave number of the –OH group is due to the overlap with the –NH group of the amine. The absorption at wave number 2918 cm-1 indicates the C–H stretching vibration of alkanes. Absorption at a wave number of 1665 cm-1 confirms the presence of a C=O group originating from the amide group (–NH–CO–CH3) which shows that in chitosan there is still an amide group that has not been deacetylated to become an amine group. Then the absorption at wave number 1320 cm-1 shows the stretching vibration of the C-N amide III group. Meanwhile, absorption at wave number 897 cm-1 indicates the presence of β-1→4 glycosides. These peaks are in line with the previous study (Edokpayi et al., 2015), which showed that the band observed at 3322 cm^{-1} could be attributed to $–NH_2$ or -OH groups stretching vibration, 2915 cm^{-1} to –CH stretching vibration, and 1644 cm^{-1} to –NH characteristic of chitosan.

The spectrum results of glutaraldehyde cross-linked chitosan beads show a wavelength range similar to the spectrum of uncross-linked chitosan beads. Identification of cross-linking was obtained by the presence of an absorption band at wave number 1562 cm-1 which indicated the presence of an imine bond (C=N). These results confirm the existence of cross-linking between chitosan and glutaraldehyde through the formation of Schiff bases. Akakuru's (OU & BO, 2017) study showed a reduced intensity of –NH bending vibration at 1635 cm^{-1}. And shift to a lower wavenumber when compared with the peak of uncrosslinked chitosan.

The spectrum of laccase-immobilized chitosan beads was not significantly different when compared with glutaraldehyde cross-linked chitosan beads. The laccase immobilization treatment caused an absorption shift in the chitosan beads spectrum. Immobilization of laccase leads to the formation of an absorption band visible at 1571 cm-1 which is an imine group with a stronger intensity than the absorption produced by the imine group on glutaraldehyde-chitosan beads.

This is due to an overlap due to the formation of a new Schiff base as a result of the reaction between the functional group on glutaraldehyde and the amine group of the laccase enzyme (Taha et al., 2020). These results confirm the presence of the laccase enzyme covalently bound to the chitosan beads.

CONCLUSION

Based on the research that has been carried out, it can be concluded that the optimum concentration of glutaraldehyde in crosslinking chitosan beads is 0.8% with a crosslinking time of 6 hours and a laccase dose of 0.4 mg/mL with an immobilization time of 4 hours. The results of FTIR spectrum analysis show the presence of C=N groups on the laccase-immobilized chitosan beads, which indicates that the laccase has been immobilized on the chitosan covalently.

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