

# Laccase Enzyme Embedded on Zinc Oxide/silver Doped Zinc Oxide Nanoparticle-chitosan-PVPP Composite Beads

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Laccase enzyme has gained popularity due to its wide range of substrates, use of only molecular oxygen as a co-factor and release of water as a by-product. In this study, laccase enzyme was immobilized on zinc oxide-chitosan/polyvinylpyrrolidone (Lac-Zn-CS-PVPP) composite beads and the beads explored for the degradation of Bismarck brown dye. The beads showed significant dye removal in batch studies (with 89 % and 94 %) after 24 hours and continuous packed bed (with 72 % and 86 %) for Lac enzyme free and Lac enzyme loaded beads respectively. FTIR showed that adsorption of the dyes onto the beads was due electrostatic interaction between the hydroxyl, carbonyl and amine functional groups on the beads and the dye molecules.

## 1. Introduction

The escalating production of consumer goods, encompassing textiles, paper, dyes, and agrochemicals (Sarkar et al., 2020, 2017), has led to a substantial release of pollutants, particularly polyphenols and their derivatives (Shanker et al., 2017). These compounds are notorious for their high toxicity, carcinogenicity, and resistance to degradation (Rafiq et al., 2021). When released into water bodies, they hinder sunlight penetration (Fatima et al., 2019), causing detrimental effects on aquatic ecosystems (Saeed et al., 2022). A myriad of methods, spanning physical, chemical, and biological approaches (Anwer et al., 2019), have been deployed to address this predicament, often yielding unwanted secondary waste products (Reza et al., 2017). Therefore, the quest for environmentally sustainable and economically viable solutions to combat pollutant degradation is of paramount importance.

In this context, the textile and paper industries have been notable contributors to environmental contamination, primarily due to the release of organic dyes (Varjani et al., 2020). Enzymatic degradation, as an eco-friendly approach, has garnered considerable attention (Saravanan et al., 2021). Laccase, a versatile enzyme found in various organisms, exhibits specificity toward a broad spectrum of substrates, including phenols (Iark et al., 2019) and polyphenols (Motamedi et al., 2021). Laccases are widely employed in pollutant degradation (Kyomuhimbo and Brink, 2023), particularly in dye removal, through one-electron oxidation followed by oxygen reduction (Yadav et al., 2021). Nevertheless, the inherent drawbacks of laccase, including low stability and limited reusability, necessitate the exploration of immobilization techniques to enhance its performance (Alsaiani et al., 2021).

In this study, we focus on composite beads (consisting of ZnONPs nanoparticles, chitosan and polyvinylpyrrolidone, PVPP) as the immobilization matrix for laccase enzymes. Chitosan, a natural polymer found in crustacean shells and fungal cell walls (Ribeiro et al., 2021), possesses unique characteristics such as biodegradability, biocompatibility, and adsorption capacity, making it an intriguing alternative to synthetic polymers (Verma et al., 2020). In this study, the application of laccase enzymes immobilized on metal-polymer composite beads for degradation of Bismarck brown dye was investigated in this study. This composite material showed advantages in terms of enzyme stability, reusability, and dye degradation. The finding highlighted the potential enhancement in dye removal efficiency using laccase in combination with metal-polymer composite beads, showcasing the environmental remediation capabilities of this innovative approach. Since various dye degradation studies have been carried out under batch conditions that do not depict actual industrial settings,

this study further explores the application of composite beads for dye removal in a packed bed reactor setting for possible application in continuous systems.

## 2. Materials and methods

Chitosan (medium molecular weight), PVPP, zinc sulphate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 99 %), acetic acid ( $\text{CH}_3\text{COOH}$ , 99.9 %) and sodium hydroxide (NaOH, 98 %) were obtained from Sigma-Aldrich, USA. All other reagents used in the experiment were also obtained from Sigma-Aldrich, South Africa and used without any further purification.

### 2.1 Synthesis and characterization of composite beads

The synthesis of Lac-ZnONPs-CS-PVPP beads was extensively reported in Kyomuhimbo et al., (2024). Briefly, a mixture solution containing 2 % chitosan, 3 % PVPP, 0.5 % ZnONPs and 2 % laccase was prepared in 1.5 % acetic acid while stirring at 400 rpm. The mixture was then dropped in 2 % sodium hydroxide solution using a syringe pump at a distance of 10 cm from the solution. The beads were allowed to cure for 4 h washed with deionized water and stored at 4 °C for further use. Control beads without laccase were also prepared.

### 2.2 Degradation of Bismarck brown using ZnONPs-CS/PVPP and Lac/ZnONPs-CS/PVPP beads

A 1000 mg/L stock solution of Bismarck brown (BB) dye was prepared in DIW and diluted to required concentrations for degradation in DIW. For batch studies at room temperature, 1 g of one of the beads (Lac-ZnONPs-CS-PVPP or ZnONPs-CS-PVPP) was suspended in 15 mL of 50 mg/L dye solution in 250 mL Erlenmeyer flasks while shaking at 150 rpm for 24 h. The concentrations of the dye over degradation time were measured using UV-Vis spectroscopy at wavelengths 465 nm. The percentage dye removal was calculated using Eq(1).

$$R\% = \left(1 - \frac{C_t}{C_0}\right) \times 100 \quad (1)$$

Where,  $C_0$  and  $C_t$  are the dye concentration initially and at a given time respectively.

## 3. Results and Discussion

This section discusses the characterization of the composite beads before and after BB degradation using FTIR as well the adsorption isotherms and kinetics of BB. The application of the beads in BB removal in a continuous setup using a packed bed reactor are also discussed.

### 3.1 Batch studies

Chitosan and PVPP polymers are rich in amine and hydroxyl and carbonyl functional groups respectively that allow effective adsorption of phenolic compounds through hydrogen bonding and hydrophobic interactions (Aranaz et al., 2021; Aziz et al., 2020). As observed in Figure 1a, there is significant absorption of the dyes onto the enzyme free beads of up to 89 % which could be attributed to the high number of primary amine groups on BB that easily form hydrogen bonds with the carbonyl groups on PVPP and amine and hydroxyl groups on chitosan (Folch-Cano et al., 2013). On addition of laccase enzyme to the beads, the rate of dye removal from the solution drastically increases with the greatest percentage removal happening within the first two hours as compared to six hours with the enzyme free beads. The increase in the rate of dye removal with laccase enzyme could be due to the reaction of laccase with BB to form highly reactive radicals that facilitate breakdown of the dye molecule into smaller compounds (Jayakumar et al., 2023) hence causing a concentration gradient of BB between the dye solution and beads surface thus leading to increased dye removal (Jiang et al., 2022).

Lagergren's pseudo first order (PFO) (Lagergren, 1898) and Ho and McKay's pseudo second-order (PSO) (Ho and McKay, 1999) Eq(2) and Eq(3) respectively) were used to predict the rate of BB removal from the solution by non-linear curve fitting as shown in Figure 1 b-c

$$Q_t = Q_e(1 - e^{-k_1 t}) \quad (2)$$

$$Q_t = \frac{k_2 Q_e^2 t}{1 + k_2 Q_e t} \quad (3)$$

Where  $k_1$  (L/h) is the PFO rate constant,  $k_2$  (g/mg/h) is the PFO rate constant and  $Q_e$  (mg/g) is the amount of dye removed at equilibrium.

The PFO model suitably described the kinetics for BB as demonstrated by the high  $R^2$  values (0.9949 and 0.9854 for Zn and Lac/Zn) compared to PSO (0.9841 and 0.9791 for Zn and Lac/Zn respectively). This suggests

that the sorption of BB dye molecule from the solution takes place on one active site and the sorption rate fluctuates with the availability of the vacant sites (Fatima et al., 2019). It was also worth noting that addition of laccase enzyme to the beads increases the rate constant 0.2454 to 0.7967 implying an increased rate of BB removal (Khan et al., 2015).

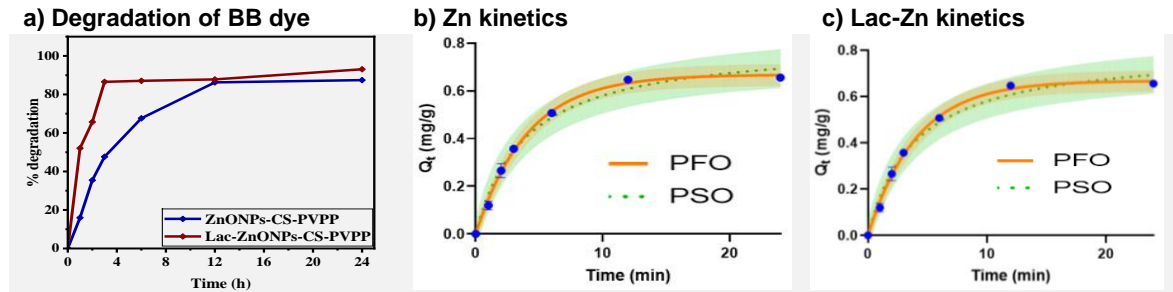


Figure 1: (a) Time dependent degradation of Bismarck brown in deionized water and PFO and PSO graphs for removal of Bismarck brown using (b) Zn-CS-PVPP and (c) Lac-Zn-CS-PVPP beads

To analyse the equilibrium data and adsorptive capacity of the beads, three isotherm models namely, Langmuir, Freundlich and dual-site Langmuir were used in their non-linear forms in Eqs(4-6) respectively.

$$Q_e = \frac{Q_{m1}K_{L1}C_e}{1 + K_{L1}C_e} \quad (4)$$

$$Q_e = K_F C_e^{1/n} \quad (5)$$

$$Q_e = \frac{Q_{m1}K_{L1}C_e}{1 + K_{L1}C_e} + \frac{Q_{m2}K_{L2}C_e}{1 + K_{L2}C_e} \quad (6)$$

Where,  $q_{m1}$  and  $q_{m2}$  are the monolayer adsorption capacities of adsorption site 1 and 2 (mg/g)  $K_{L1}$  and  $K_F$  ((mg/g) (L/mg)<sup>1/n</sup>) and  $n$  the adsorption potential and strength constants of the Freundlich isotherm model.

Various concentrations (2.5 mg/L to 100 mg/L) of BB were contacted with 1 g of beads at ambient temperature for 24 hours and  $Q_e$  was plotted as a function of the equilibrium concentration,  $C_e$  (mg/L) Figure 2.

As earlier observed in the kinetic models, addition of the enzyme increased the maximum adsorption capacity of BB and the dual-site Langmuir and Freundlich demonstrated the best fit for BB adsorption implying that there are two or more adsorption sites available on the beads surfaces for a single dye molecule (Al-Ghouti and Da'ana, 2020; Kumar et al., 2019).

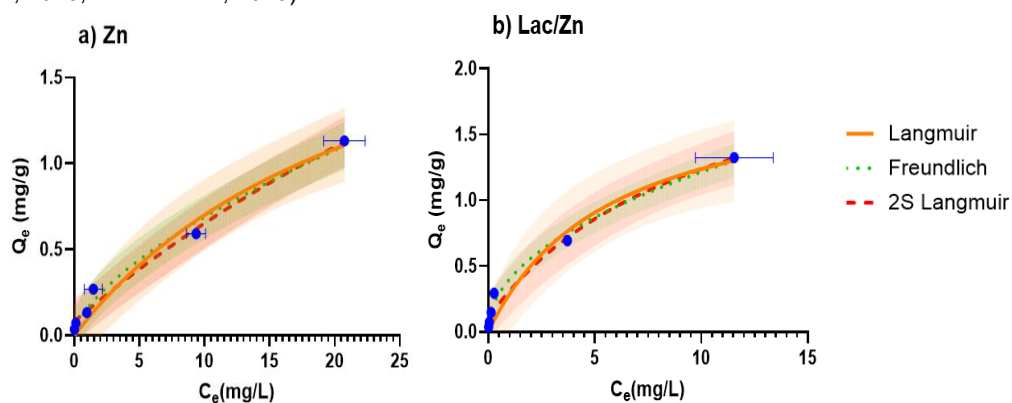


Figure 2: single-site Langmuir, dual-site Langmuir and Freundlich isotherms for degradation of Bismarck brown on (a) Zn-CS-PVPP and (b) Lac-Zn-CS-PVPP beads

In an effort to ascertain the interactions between BB and the beads' surfaces, Fourier transform infrared spectroscopy was carried out on BB and enzyme free as well enzyme loaded beads before and after dyedegradation. BB displayed bands at 3320 cm<sup>-1</sup>, 3147 cm<sup>-1</sup>, 1604 cm<sup>-1</sup>, 1518 cm<sup>-1</sup>, 1384 cm<sup>-1</sup>, 1248 and 1112 cm<sup>-1</sup>, and 1018, 876 and 705 cm<sup>-1</sup> for N-H stretching (primary amine), C-H stretching (alkene), C=C stretching

(conjugated alkene), C=C stretching (cyclic alkene), C-N stretching (aromatic amine), C-N stretching (amine) and C=C bending (alkene) respectively (Sudatta et al., 2020). The composite beads displayed functional groups with 2 overlapping broad bands at 3359 for N-H stretching (secondary amine) and 3287 for O-H stretching (intermolecular bonded alcohol). 2898 was due to O-H stretching (intramolecular alcohol), 1631 due to C=O stretching (amide), 1421 for O-H and C-H bending carboxylic acid and aromatic compound respectively, 1374 for S=O stretching (sulfate), 1289 for C-O stretching (aromatic ester), and 1075 and 1030 for C-N stretching for amine (Zhang et al., 2021). A shift and increase in intensity in the bands associated with N-H and O-H stretching is observed, 2898 disappeared while the C-N, C=O and O-H bands for amine, amide and carboxylic acid, respectively, significantly decreased in intensity (Figure 3 a-b). The change in the band intensities suggests that adsorption of BB on the beads mainly occurs by intermolecular forces of attraction between the functional groups on the beads surface and BB molecule (Yang et al., 2021; Zhu et al., 2018).

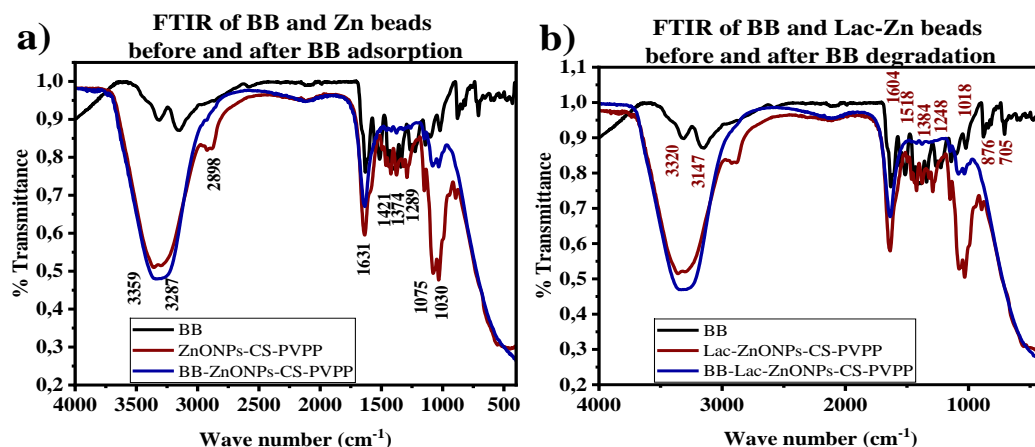


Figure 3: FTIR spectra for BB, A) enzyme free beads and B) enzyme loaded beads before and after BB degradation

### 3.2 Continuous dye removal experiments

A packed bed apparatus was used in this study consisting of glass column with a height of 80 mm and an internal diameter of 15 mm and an influent glass column of 18 mL volume. The influent glass column was placed on top of the packed bed column and the flow of the dye solution was facilitated by gravity. The influent concentration ( $C_0$ ) was fixed at 50 mg/L and the flow rate at 0.2 ml/min. during the experiment, effluent samples were collected and analyzed using the same method described in the batch process.

The percentage removal of BB dye over time is represented in Figure 4a and the column could remove up to 72 % and 86 % for Zn-CS-PVPP and Lac-Zn-CS-PVPP beads respectively after 30 days. The residence distribution time was recorded as 7.798 minutes and using the Ergun Eq(7), the pressure drop was calculated and a plot of pressure along the column length is presented in Figure 4b. From these results it is clear that the system has significant potential for industrial application, exhibiting remarkable removal of Bismarck Brown over an extended period of operation, while limited pressure drop is observed and therefore reducing pumping costs, and therefore operational costs, in the system.

$$\frac{\Delta P}{\Delta z} = \frac{G}{\rho g_c D_p} \left( \frac{1 - \phi}{\phi^3} \right) \left[ \frac{150 (1 - \phi) \mu}{D_p} + 1.75 G \right] \quad (7)$$

## 4. Conclusion

Enzyme free and enzyme loaded Zn-CS-PVPP beads were successfully used for the removal of Bismarck brown dye from water. The beads demonstrated superior catalytic activity in the removal of the dye from water in batch (with 89 % and 94 % removal for enzyme free and enzyme loaded beads respectively) and continuous studies (with 72 % and 86 % dye removal for enzyme free and enzyme loaded beads after 30 days run). The proposed technology therefore show potential for application in removal of dye in various water treatment processes. However, the study is carried out in deionized water which is not representative of industrial effluents hence the need to test the beads on real industrial dye effluents. Also, more studies need to be carried out concerning the mechanical properties of the beads such as mechanical stability under various conditions of pressure and temperature as well as the reusability of the beads.

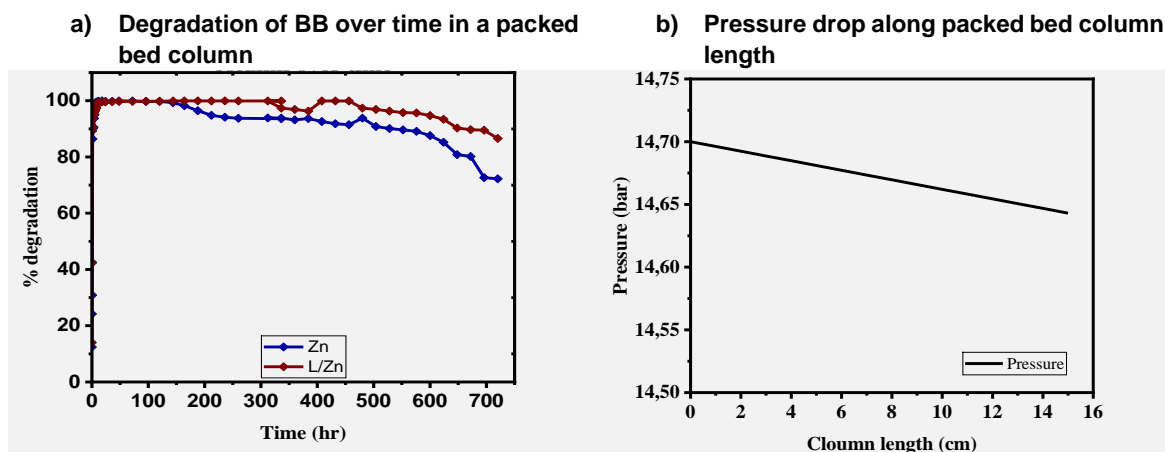


Figure 4: a) Time dependent removal of BB dye from water and b) pressure drop along a packed bed column

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