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Immobilization of Laccase Enzyme on Zinc Oxide and 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 order to improve its reusability in harsh conditions, various supports have been explored for its immobilization. In this study, laccase was immobilized on zinc oxide-chitosan/polyvinylpolypyrolidone (ZnONPs/CS/PVPP) and silver doped zinc oxide-chitosan/polyvinylpolypyrolidone (Ag@ZnONPs/CS/PVPP) beads. The Ag@ZnONPs/CS/PVPP beads showed a higher immobilization yield and retained enzyme activity (76.0% and 50.0% respectively) compared to the ZnONPs/CS/PVPP beads (50.4% and 41.7% respectively). The biocatalysts demonstrated improved enzyme stability at higher temperatures and longer storage stability compared to the free enzyme hence show potential for further biotechnological applications.

1. Introduction

Laccases are polyphenol oxidase enzymes produced by fungi, plants, bacteria and insects. They catalyze a wide range of organic substrates including phenols, polymethoxybenzenes, cresols and aromatic amines by direct reduction of molecular oxygen to water without formation of hydrogen peroxide (Cao et al., 2021; Fortes et al., 2017). Since laccases use only oxygen as the co-substrate and release only water as a by-product, they have been referred to as green catalyst (Ji et al., 2017). Due to their ability to oxidize a wide range of organic compounds under mild conditions, they have found application in organic synthesis, food industry, paper and pulp industry, wastewater treatment and biosensors (Drozd et al., 2018; Mohammadi et al., 2018).
However, like any other enzyme, use of soluble laccase is associated with denaturation under harsh conditions, non-reusability and low storage stability which affect their practical application in industrial processes (Liu et al., 2020). The immobilization of enzymes has therefore been explored to improve their operational stability in industrial conditions and design biocatalytic reactors with easier enzyme reuse and process control (Zhou et al., 2021). In this technique, the enzyme is confined in a specific space or linked to an insoluble support to allow reuse by easier product separation which prevents carry-through of enzyme to subsequent process steps and thus permit continuous processes (Haro-Mares et al., 2022). During immobilization, aspects such as the chemical properties and structure of the enzyme, the physiochemical properties of the support and the immobilization method have to be considered (Kohori et al., 2018). All these affect the ultimate performance of the biocatalyst in terms of immobilization yield, residual activity, kinetic parameters and substrate specificity (Adamian et al., 2021). Supports that are cost effective, inert with high thermal and mechanical resistance and eco-friendly are preferred during enzyme immobilization (Daronch et al., 2020).
In this study, laccase enzyme was immobilized on zinc oxide and silver doped zinc oxide-chitosan-PVPP composite beads. Chitosan is a natural polymer and is a promising enzyme's immobilization carrier due to its non-toxicity, biocompatibility, biodegradability, good physiochemical stability and bio-adhesive properties (Rafiee and Rezaee, 2021). It contains cationic amino groups that can either form ionic bridges with anionic residues of enzymes for immobilization through adsorption or can be derivatized by a cross linker to facilitate

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covalent immobilization (Nunes et al., 2021). PVPP is an inert and insoluble cross-linked synthetic polymer of polyvinylpyrrolidone (PVP) with high phenolic binding affinities due to presence of carbonyl groups. It has gained popularity in clarification and stabilization of wines and juice by reduction of color and phenolic compounds (Gil et al., 2019). The use of nanoparticles for immobilization of enzymes is advantageous because nanoparticles provide a high surface area for enzyme attachment thus allowing high enzyme loading, provide high mechanical resistance and preserve the biological activity of the enzyme due low mass transfer resistance (Fernandes et al., 2017). Incorporating nanoparticles in polymers for enzyme immobilization allows free expression of the enzyme with maximum protection from the polymer hence prolonged reusability of the catalyst (Kyomuhimbo and Brink, 2023). The biocatalyst beads can be used in various processes such as dye degradation in industrial effluents, juice processing and wine stabilization. To our knowledge, no investigation has been reported on the immobilization of laccase enzyme on ZnONPs/CS/PVPP and Ag@ZnONPs/CS/PVPP composite beads.

2. Materials and methods

All chemicals were sourced from Sigma-Aldrich South Africa and used as purchased without any further purification. The zinc oxide nanoparticles (ZnONPs) and silver doped zinc oxide nanoparticles (Ag@ZnONPs) were synthesized by reduction of zinc sulphate ($ZnSO_4.7H_2O$, 99%) and a mixture of zinc sulphate and silver nitrate ($AgNO_3$, 99.8%) respectively using sodium borohydride ($NaBH_4$, 98%). To immobilize the enzyme a mixture solution of 2% chitosan, PVPP, nanoparticles and laccase was prepared in 1.5% acetic acid (CH_3COOH, 99.9%) 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. The immobilization yield was determined using the Bradford reagent assay and enzyme activity was determined by monitoring the oxidation of potassium ferrocyanide as a substrate on a spectrophotometer at 320 nm absorbance. The enzyme activity was calculated using the equation adopted from Baltierra-Trejo et al., (2015);

$$U = \frac{(\Delta A)(V_t)}{(t)(\varepsilon)(d)V_s)} \quad (1)$$

Where, U is enzyme activity (mM min^{-1}), ΔA is the change in absorbance (final-initial), ε is the molar extinction coefficient of the substrate (M^{-1} cm^{-1}), V_t is the total volume of the reaction, V_s is the volume of enzyme used, d is the optical trajectory (cm) and t is the reaction time.
The temperature and pH stability as well as the kinetics of the free and immobilized enzyme were explored. The mechanical stability of the free and immobilized enzyme was also determined.

3. Results and Discussion

3.1 Synthesis of CS-PVPP and NP/CS-PVPP polymer beads

Varying concentrations of PVPP (2-5%) were added to 2% chitosan (CS) solution (in 1.5% acetic acid) to form CS-PVPP beads. Increasing the concentration of PVPP beyond 5% made the solution very viscous that it was impossible to form beads through the syringe.
The mechanical stability of the CS-PVPP beads was determined for the different percentage compositions of PVPP by determining the retained percentage mass after sonication with glass beads for 2 min. The retained percentage mass increased with increasing percentage composition of PVPP as shown in Figure 1. The CS-PVPP polymer beads were then modified further by adding nanoparticles (NPs) (0.5-1.0% composition) to form NPs/CS-PVPP beads with ZnONPs as the representative NPs. Although the 5% CS-PVPP beads displayed the highest mechanical stability, addition of 0.5% NPs made the solution very viscous which hindered the formation of the beads. For the 4% PVPP beads, the composition of the NPs could not be increased beyond 0.5% while for 3% PVPP, the composition of the NPs could be increased up to 1%. Increasing the composition of the NPs to 1.5% destroyed the binding ability of CS hence polymer beads could not be formed. This could be due to the fact that the NPs were reduced and stabilized by sodium borohydride thus obtaining an overall negative surface charge (Khatoon et al., 2023). Increase in the concentration of NPs in the solution increases the adhesive properties between polycationic chitosan and the NPs at the expense of the cohesive (binding) properties (Shikha et al., 2021). The beads composed of 0.5% NPs, 2% CS and 3% PVPP were further used for immobilization of the enzyme as they showed the highest mechanical stability with a mass loss of 4.55%.

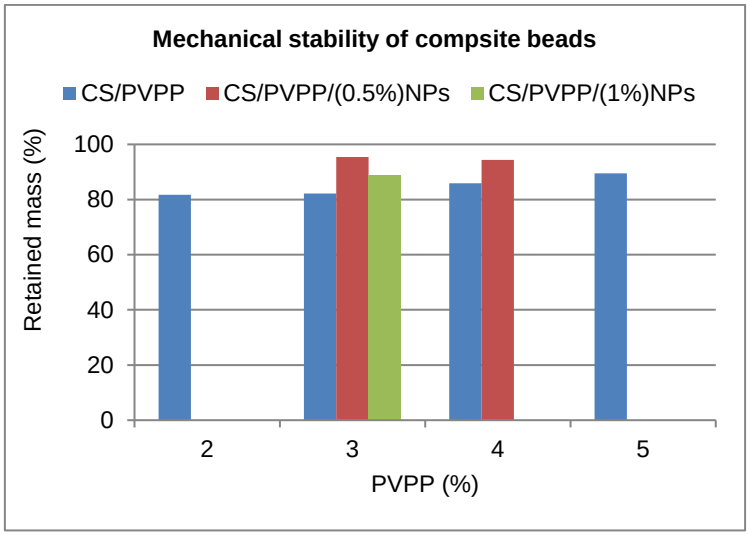

Figure 1: Retained percentage mass of beads at different concentrations of NPs, CS and PVPP.

3.2 Immobilization of laccase enzyme on ZnONPs/CS/PVPP beads

Different concentrations of enzyme were added to the NP/CS/PVPP solution and the immobilization yield, retained enzyme activity and mechanical stability of the beads were obtained as summarized in Figure 2. The immobilization yield decreased with increasing enzyme concentration as a result of leaching of the enzyme from the beads during the curing stage (Naghdi et al., 2017). The beads containing 2% laccase solution were selected for further studies as they demonstrated fair immobilization yield and reasonable retained enzyme activity and mechanical stability. Composite beads containing 0.5% Ag@ZnONPs were also used and their beads showed generally better performance (yield, enzyme activity and mechanical stability) compared to the ZnONPs composite beads.

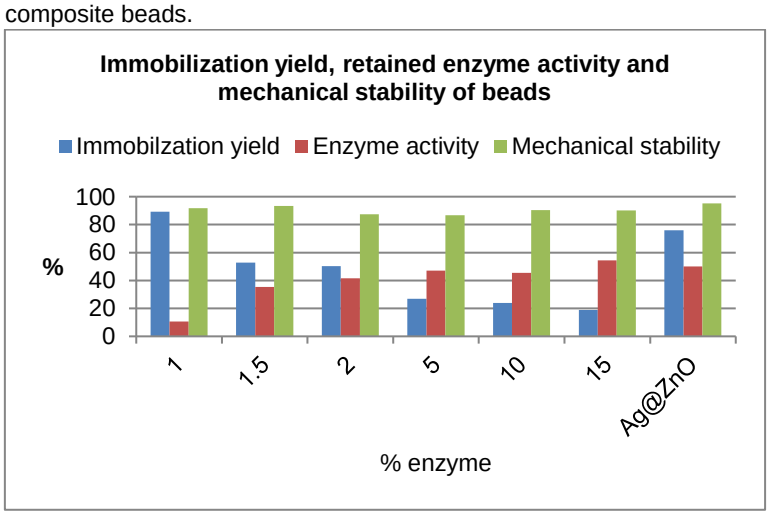

Figure 2: Immobilization yield, retained enzyme activity and mechanical stability of ZnONPs/CS/PVPP composite beads at varying concentration of laccase and Ag@ZnONPs/CS/PVPP composite beads at 2% laccase concentration.

3.3 Temperature and pH stability of free and immobilized enzyme

Laccase activities observed at optimum pH and temperature were defined as 100%. As observed in Figure 3, free and immobilized laccase displayed the optimum pH at 4.5 hence immobilization didn't affect the enzyme's pH activity. This is common with adsorption/encapsulation since the enzyme structure is unaltered (Datta et al., 2021; Durán et al., 2002; Makas et al., 2010; Yamak et al., 2009). At optimum pH value, free laccase showed maximum activity at 30 ˚C, Lac/ZnONPs/CS/PVPP at 50 ˚C and Lac/Ag@ZnONPs/CS/PVPP at 35 ˚C as shown in Figure 4. The activities of both free and immobilized laccase gradually decreased above their optimum

temperature but immobilized laccase retained over 40% of its maximum activity at 70 ˚C while free laccase could only maintain 8.6% at 70 ˚C. This shows that encapsulation of the enzyme in the beads improved its stability towards heat denaturation. The thermal stability is provided by a net of the NPs/polymer beads which preserve the tertiary structure of the enzyme and prevent disassembling of the active site at high temperatures (Naghdi et al., 2019; Zhang et al., 2018). The storage stability of the free and immobilized enzyme was also determined by storage at 4 ˚C for two months. The Lac/ZnONPs/CS/PVPP and Lac/Ag@ZnONPs/CS/PVPP retained 88.7% and 76.8%, respectively, of the original enzyme activity after two months storage while the free enzyme only retained 18.2% of its original activity.

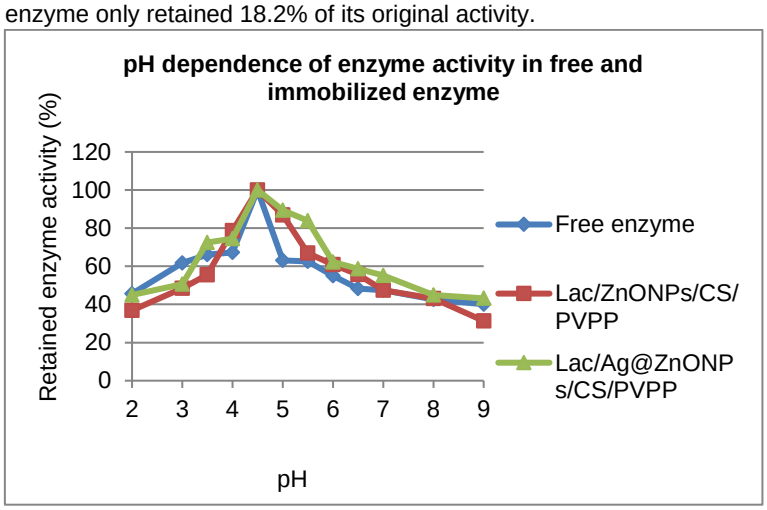

Figure 3: Activity of free and immobilized laccase enzyme at varying pH of 2 to 9

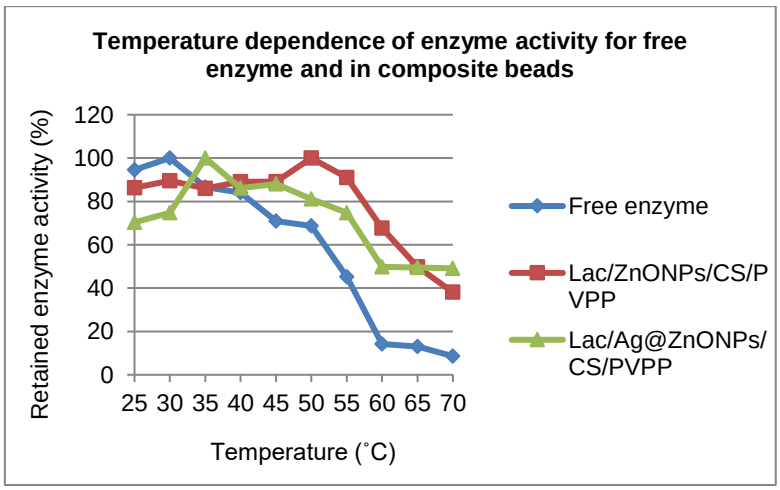

Figure 4: Activity of free and immobilized enzyme at varying temperatures from 25 to 70 ˚C

3.4 Kinetic properties

To determine the kinetic parameters, K_m (Michaelis constant) and V_{max} (maximum reaction velocity), of free and immobilized laccase, enzyme activity was measured for substrate concentrations ranging from 0.2 to 10 mM at room temperature and pH 4.5. The Lineweaver-Burk plot of 1/*v* (reaction velocity) versus 1/[S] (concentration of the substrate) displayed linear relationships for free and immobilized laccase. The K_m of the free enzyme changed from 0.546 mM to 0.266 mM and 0.765 mM for Lac/ZnONPs/CS/PVPP and Lac/Ag@ZnONPs/CS/PVPP respectively while the V_{max} changed from 2.939 mM to 0.714 mM and 0.839 mM for Lac/ZnONPs/CS/PVPP and Lac/Ag@ZnONPs/CS/PVPP respectively. Immobilization increases the K_m value indicating a lower affinity for the substrate due to reduced protein flexibility and diffusional limitations (Latif et al., 2022; Piao et al., 2019). The decrease in K_m value for Lac/ZnONPs/CS/PVPP could be due to the reaction between zinc oxide and potassium ferrocyanide to produce zinc ferrocyanide thus increasing the affinity of the substrate (Murov and Stedjee, 2001).

4. Conclusion

ZnONPs/CS/PVPP and Ag@ZnONPs/CS/PVPP beads were used for immobilization of laccase and immobilization process optimized. Although the immobilized laccase displays lower substrate affinity than the free enzyme, it demonstrated improved stability at high temperatures and longer storage stability. The improved stability of the enzyme in the biocatalyst shows potential for application in various biological processes.

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