PROPERTIES OF FREE AND IMMOBILIZED LACCASE ON MAGNETIC IRON OXIDE NANOPARTICLES

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ABSTRACT

In this study, *Trametes versicolor* laccase (Lac) was immobilized on magnetic nanoparticles (MNPs) surface to improve potential continuous flow applications. The nanoparticles were functionally activated by APTES and glutaraldehyde, and the chemical bond between support and enzyme exhibited stability during leaching tests. The simple storage approach for the immobilized enzyme, i.e., dispersed in buffer and kept in fridge, showed stability for up to 14 days (activity of 1.35±0.25 U mg^{-1}). Compared to free enzyme, the Lac-MNPs showed superior resistance to UVC irradiation (42 W m^{-2}) and temperature changes (ranging from 25 to 65°C). The pH profiles were similar to free and immobilized Lac, resulting in higher activity at acid media. Overall, the properties of Lac-MNPs can be considered attractive when developing wastewater treatments to degrade environmental contaminants.

Keywords: Heterogeneous catalysis. Oxidoreductase enzyme. Reusability. Thermostability.

1 INTRODUCTION

The industrial application of free enzymes poses challenges, such as low operational stability and loss of biocatalysis after first use. The study of enzyme immobilization opens up exciting perspectives and provides an opportunity to design reactors operating in a continuous flow regime. Considering reactor operationality and recovery feasibility, the promising technique of binding enzyme on magnetic nanoparticle surfaces to degrade emerging contaminants has gained attention in recent years.[1,2]

Magnetic iron oxide nanoparticles (MNPs) have found extensive utility in biological and environmental applications, including as a support for laccase. The biocompatibility of these particles, coupled with the flexibility of surface modification and immobilization, makes it a suitable support for various industrial applications.[1] In this sense, this study aims to develop an approach for laccase immobilization on MNPs surface and characterize the enzyme stability and recovery, which can be particularly interesting for industrial continuous flow bioprocesses. Belonging to the oxidoreductase class of enzymes, laccase is a robust biocatalyst and requires only molecular oxygen to exert their redox action and oxidize persistent contaminants.[2]

2 MATERIAL & METHODS

The purified *Trametes versicolor* laccase enzyme (Lac, ≥0.5 U mg^{-1}, Sigma-Aldrich) was immobilized on magnetic nanoparticle (MNPs) surfaces. The MNPs were synthesized by alkaline coprecipitation method, resulting in specific surface area of 87 m^2 g^{-1} and pore size of 4 nm.[3] The immobilization procedure was adapted from literature[4,5], starting with the dispersion of MNPs in ethanol/water (at 1:1 proportion) by sonication. Dispersed MNPs were stirred vigorously, and APTES was gradually added, aiming to amino-functionalize their surfaces.

In sequence, aiming to provide aldehyde groups on MNPs surface, the cross-linking agent glutaraldehyde was mixed with the MNPs and sodium-phosphate buffer (pH 7). The enzyme was immobilized on MNPs by keeping it stirring for 24 h in citrate-phosphate buffer (pH 3.5). After immobilization, the Lac-MNPs were separated by centrifugation (supernatant collected for enzyme assay) and washed with citrate-phosphate buffer. The Lac-MNPs were dispersed in citrate-phosphate buffer and kept at 4°C for storage.

For the enzyme assay, 500 µL ABTS (1.5 mM) were mixed with 2450 µL citrate-phosphate buffer (pH 3.5) and 50 µL or 2 mg of enzyme sample. After incubation for 4 min at 45 °C, the substrate oxidation was monitored by absorbance read at 420 nm (ε_{420}= 36 000 L mol^{-1} cm^{-1}). The U unit of laccase was defined as the enzyme amount capable of oxidizing 1 µmol of substrate per minute.

Besides the Lac-MNPs storage stability in citrate-phosphate buffer at 4°C (monitored up to 14 days), the enzyme activity using this same buffer, prepared in a pH range from 2 to 9, was measured for free and immobilized laccase. The stability of enzyme incubation for 4 min in temperatures ranging from 25 to 65°C was also studied. In addition, free and immobilized laccase were subjected to UVC irradiation to explore their resistance to stress conditions. Enzyme samples were kept up to 60 min under the UVC lamp (λ_{max} = 254 nm), emitting a fixed irradiance of 42 W m^{-2} nm^{-1}.

Leaching tests were performed by mixing the Lac-MNPs with sodium-phosphate buffer (pH 6.5) and keeping it in a shaker at 150 rpm and 24ºC. Supernatant was collected periodically (up to 24h), and laccase was measured. Finally, to understand the reusability of immobilized enzyme, 2 tubes containing 2 mg of Lac-MNPs were prepared to conduct up to 10 cycles of laccase activity measurement with the substrate ABTS. Between each cycle, the Lac-MNPs material was recovered with a magnet and washed to remove ABTS in excess.

3 RESULTS & DISCUSSION

By measuring the laccase activity in supernatant after Lac-MNPs washing step, it was found that the theoretical loss of enzyme was 25%, and the measured activity on nanoparticles was 1.87±0.08 U mg^{-1}. The leaching tests resulted in zero enzyme loss during the 24 hours of supernatant monitoring, indicating that the chemical bond between the functionalized magnetic nanoparticles surface and enzyme is strong. Further, as shown in Fig. 1(a), the Lac-MNPs have exhibited storage stability. At day 14, the activity was 1.35±0.25 U mg^{-1} (decrease of 28% from day 4).

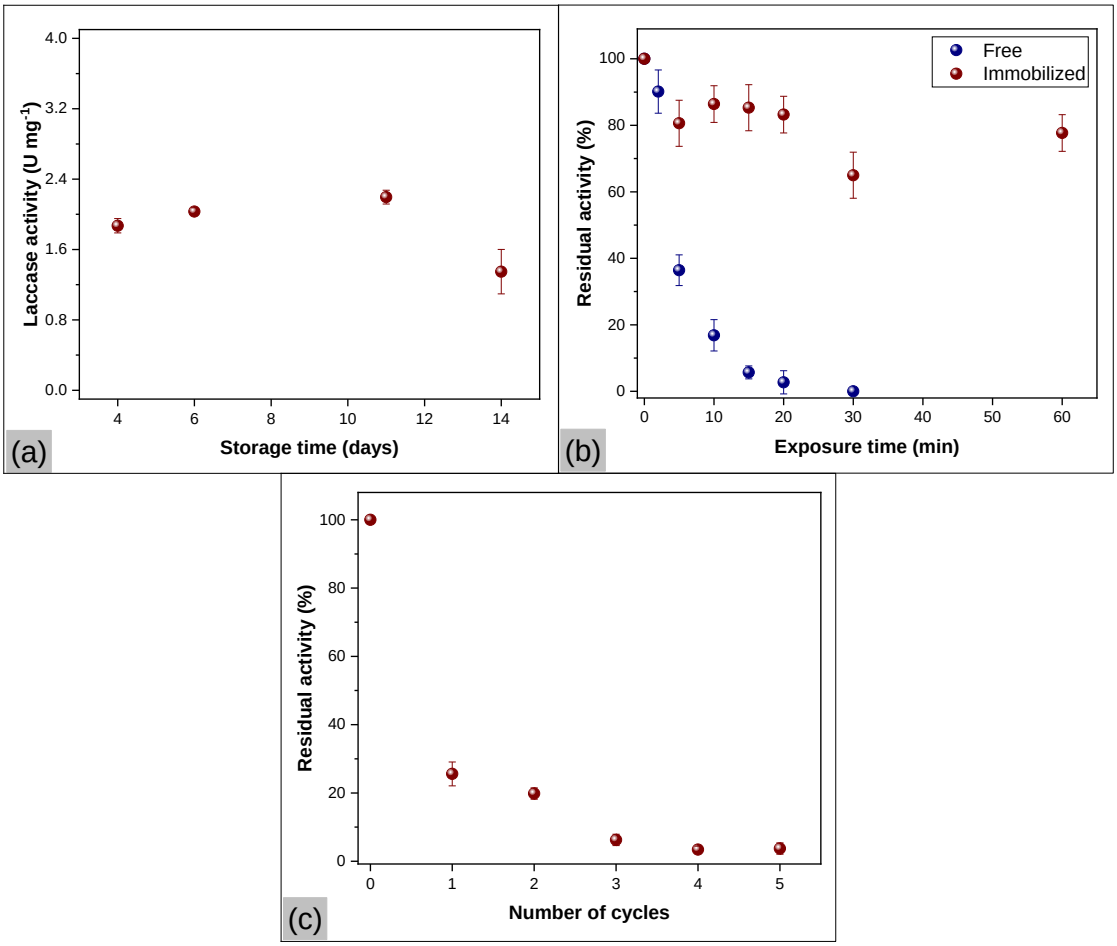

Figure 1 Results of laccase stability (a) stored at 4°C in citrate-phosphate buffer, (b) under UVC irradiation, and (c) during reuse cycles towards ABTS oxidation.

The Lac-MNPs material showed superior resistance to UVC exposure compared to free laccase, as presented in Fig 1(b). From the initial activity of free enzyme, set as 100%, the residual activity after 5 and 15 min of exposure were, respectively, 36.4±4.6% and 5.7±1.9%. The immobilized enzyme maintained a residual activity of 77.7±5.5% after 60 min of exposure.

Regarding reusability, the Lac-MNPs residual activity in the first cycle was 25.6±3.5% and continued to decrease, reaching 3.7±1.0% in the fifth cycle (see Fig 1(c)). From cycles 6 to 10, the activity in the reused Lac-MNPs material was zero. As the leaching test showed that the bond between enzyme and support is stable, this fast decrease in enzyme activity can be attributed to catalytic site saturation or denaturation, similar to results reported elsewhere.[4,6]

Regarding buffer pH during incubation, both free and immobilized Lac showed similar behavior, as presented in Fig. 2(a). Higher activities were reported at acid media (pH 2 to 4), and for neutral and alkaline media (pH 6 to 9) it decreased to zero. The thermostability for free laccase showed higher activity at 45°C and 55°C, while the immobilized enzyme was stable through the studied range, maintaining a residual activity >60%, as shown in Fig. 2(b).

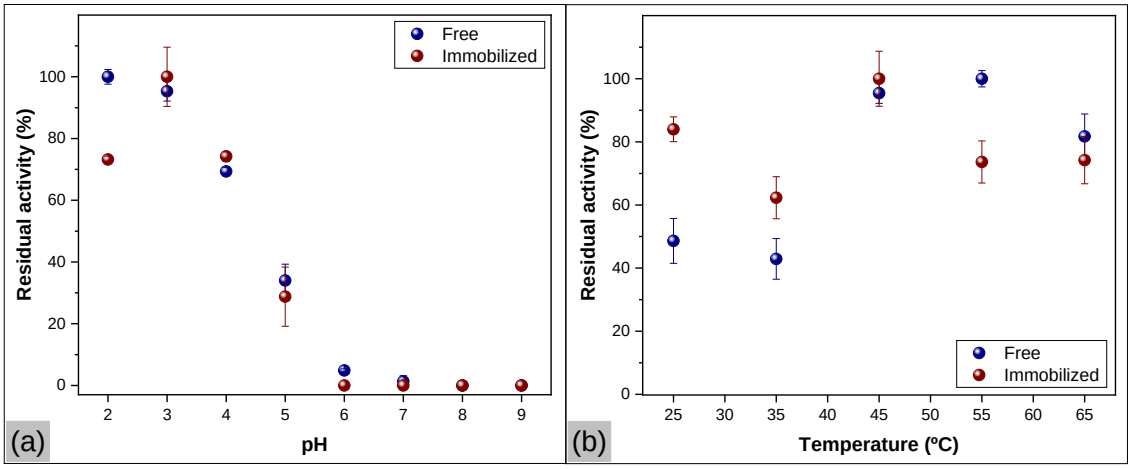

Figure 2 Behavior of free and immobilized laccase when incubated at varying (a) pH and (b) temperature conditions. Note: the 100% residual activity was set for the highest activity value of each set of results.

4 CONCLUSION

The immobilization of laccase on functionalized magnetic iron oxide nanoparticle surfaces can be considered a promising alternative to improve the application of enzymes to industrial reactions, mainly operated in continuous-flow regime, given the feasibility of fixing the Lac-MNPs in magnets. The immobilized enzyme showed superior resistance to UVC irradiation and temperature changes compared to the free form. In addition, the simple storage approach for the material, i.e., dispersed in buffer and kept in fridge, showed stability for up to 14 days. These properties can be considered attractive when developing wastewater treatments to degrade environmental contaminants.

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