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Laccase Immobilized In-Situ Magnetic Cryogel for Dephenolization of Olive Mill Wastewater

Highlights:

- Laccase was immobilized onto in-situ magnetic cryogel.
- Immobilization improved its catalytic properties.
- Immobilized laccase dephenolized 79% of olive mill wastewater.

Keywords:

- Laccase
- Immobilization
- Magnetic cryogel
- Olive mill wastewater

ABSTRACT:

Olive mill wastewater (OMW) is one of the major environmental issues due to high amount of phenolic compounds and organic substances. Physical, chemical and biological processes are applied to decrease the concentrations of these compounds; however, more ecofriendly and cost-effective applications are needed. Among biological treatments, enzymes, especially laccase, come to the front for the reduction of phenolic content of OMW. Nevertheless, laccase is often inactivated in harsh conditions, therefore, immobilizing laccase offers an effective alternative. To this aim, a laccase-immobilized cryogel was prepared to improve its stability and to obtain a reusable catalyst. Laccase was covalently-immobilized onto a magnetic poly(HEMA) cryogel decorated with epoxy groups of epichlorohydrin. Insolubilization improved its stability in terms of pH, temperature, storage and reuse. K_m and V_{max} of free and immobilized laccase were determined as 1.248 µM and 23.75 µM, and 0.450 U/mg and 0.405 U/mg, respectively. Laccase-immobilized cryogel was evaluated for the potential to reduce phenolic compound amount of OMW and resulted in 79% reduction. Effects of enzyme concentration, time and OMW volume on dephenolization were also evaluated.

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INTRODUCTION

Enzymes are the biological catalysts of living things maintaining and controlling metabolic reactions to provide the balance of the organisms (Murakami et al., 1996). After verified that the enzymes were discovered to be active outside of vital cells by Buchner (1897), enzymes have been isolated and purified from different organisms for numerous applications (Linke and Berger, 2011). Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a glycosylated multicopper oxidase with great capability to oxidize a wide range of substrates including phenols, non-phenols, carboxylic acids, aromatic amines by four-electron reduction of molecular oxygen to water (Morozova et al., 2007; Fernandez-Fernandez et al., 2013; Mate and Alcalde, 2017). It has been one of the intensively utilized enzymes for several chemical processes and industrial areas such as biotechnology, bioremediation, food, wood, paper, textile and cosmetic industries and biosensors (Madhavi and Lele, 2009).

Especially in environmental sciences, laccase-assisted treatments of complex wastewaters have been reported. Laccase detoxifies pollutants/contaminants by oxidation and converts them into less hazardous polymers or precipitates and provides an alternative greener and cost-effective route compared to traditional treatments (Sutaoney et al., 2022). One of the phenolic-rich targets of laccase is the effluent of olive oil industry, olive mill wastewater (OMW). It is a highly-pollutant by-product which is both beneficial thanks to its valuable nutrients and also harmful because of its high salt content, low pH, and phytotoxicity caused by high levels of polyphenols that are toxic to plants and soil-borne microorganisms (Khdair and Abu-Rumman, 2020; Zerva et al., 2020; Shabir et al., 2023). Laccase can degrade phenolic compounds in OMW. However, for such a valuable enzyme, low stability, high cost, and non-reusability lead to limited applicability for industrial uses. The aforementioned drawbacks can be overcome with immobilization of the enzyme onto a matrix which can be performed by different methods classified as covalent bonding, adsorption, encapsulation and cross-linking (Wang et al., 2022). Properties of enzyme and support matrix, application of immobilized enzyme and the reaction medium should be considered for an efficient immobilization technique (Liu et al., 2021).

Cryogels are a subclass of hydrogels that are formed by polymerization of precursors at subzero temperatures where ice crystals behave as pore forming agents and precursors are concentrated. After incubation, cryogels are thawed and a polymeric macroporous interconnected network is obtained. Cryogels have gained attention due to their structural benefits such as macroporosity, shape-memory properties, mechanical strength, low-cost and easy-preparation (Eigel et al., 2021; Akin and Ozmen 2022). Various applications of cryogels have been demonstrated in literature including biomedicine, tissue engineering, enzyme immobilization, separation science, bioremediation (Tripathi and Melo, 2019).

In this study, covalent immobilization of laccase onto in-situ magnetized poly(HEMA) cryogels via epoxy chemistry was investigated. Enzymatic characteristics such as optimum pH, temperature, stability was examined for free and immobilized laccase. As an application, ability of immobilized laccase on removal of phenolic compounds present in olive mill wastewater was evaluated under different conditions such as time, OMW volume and number of laccase immobilized cryogels.

MATERIALS AND METHODS

Reagents

Epichlorohydrin (ECH), 2-hydroxyethyl methacrylate (HEMA), N,N′-methylenebisacrylamide (MBAAm), ammonium persulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), laccase (from *Trametes versicolor*, ≥ 0.5 U/mg), (3-aminopropyl)triethoxysilane (APTES), $FeCl_2 \cdot 4H_2O$,

$FeCl_3 \cdot 6H_2O$, NaOH, syringaldazine (SYR), Folin-Ciocalteu reagent (FCR) and Na_2CO_3 were obtained from Sigma (St. Louis, USA). Other solvents and reagents were of analytical grade and used without further purification. Ultrapure water obtained by a water purification equipment (Millipore, Simplicity UV, France) with a specific resistivity of 18.2 MΩ cm was used in all experiments. Olive mill wastewater was supplied from a unit that produces olive oil in Tire-İzmir, Türkiye.

Synthesis of Epoxy-Modified Magnetic Poly(HEMA) Cryogel

Epoxy-modified magnetic poly(HEMA) cryogel was synthesized in two steps. First, 1.3 mL HEMA and 0.283 g MBAAm were dissolved separately in 5 mL and 10 mL of water, respectively. Solutions were cooled to +4°C and then mixed. Upon stirring the solution at 200 rpm, APS (20 mg) and TEMED (25 µL) were added. Following 20s of vigorous stirring, 150 µL of aliquots from the polymerization solution were pipetted into the wells of a 96-well plate. The plate was covered and placed into the freezer immediately, and kept at -12°C for 24h. At the end of polymerization, ice crystals were thawed at room temperature and water was added into each well (Bakhshpour et al., 2020). Swelled cylindrical cryogels were removed carefully using a laboratory forceps and transferred into a beaker containing water and washed for three times. Secondly, 50 cryogels were immersed in a 100 mL ethanolic solution of Fe(II):Fe(III) (1:2 mole ratio) for 3h. Then, cryogels were washed with ethanol twice and unbound metal salts were removed. Metal-ion loaded cryogels were transferred into 0.75 M NaOH solution and stirred at 200 rpm for 3h. Finally, cryogels were washed until the pH of the water was neutral (Şahiner et al., 2015). To insert epoxy moieties, cryogels were incubated in 1.0% APTES at room temperature for 24h (Kuru et al., 2016) and ethanol:NaOH:ECH (15:15:5, pH 10) solution for 6h at 48°C, respectively. Then, cryogels were washed with water and dried in an oven at 50°C.

Characterization of Epoxy-Modified Magnetic Poly(HEMA) Cryogel

Structural properties and elemental compositions of plain and laccase-immobilized cryogels were examined using a ZEISS EVO LS10 scanning electron microscope (SEM) equipped with an energy dispersive X-ray (EDX) detector. Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy was employed to evaluate the chemical structures of the plain cryogel and laccase immobilized cryogel using a Perkin Elmer Spectrum Two (Llantrisant, UK) spectrometer. Water uptake (WU) of the cryogel was determined by immersing the dried cryogel (0.013 g) in water at 25°C. After 5 min, wiping the excess water on the cryogel surface, the weight of swollen cryogel was recorded. WU was calculated from Equation 1:

$$WU = \left[\frac{m_{\text{swollen cryogel}} - m_{\text{dried cryogel}}}{m_{\text{dried cryogel}}}\right] * 100 \quad (1)$$

Covalent Immobilization of Laccase

Laccase was immobilized onto magnetic poly(HEMA) cryogel through the epoxy groups of epichlorohydrin. For this process, 50 cryogels were added into a 50 mL solution of laccase (10 mg/mL) in pH 7.0 PBS and incubated by gentle stirring at +4°C for 24h. Initial and final laccase concentrations were calculated using the decrease in absorbance at 280 nm using a Shimadzu UV-1900i (Japan) UV-Vis spectrophotometer (Doğan et al., 2015) and immobilized amount of laccase was calculated from Equation 2.

$$Q = (C_0 - C)\, V/m \quad (2)$$

where, Q is the immobilized laccase amount on one gram of cryogel (mg/g); C_0 and C are the initial and final laccase concentrations (mg/mL), respectively; V is the volume of the solution (mL); and m is the mass of the dried cryogel.

Activity Assay for Free and Immobilized Laccase

Laccase activity was measured according to Leonowicz and Grzywnowicz (1981) and Bayramoğlu et al., 2010) using syringaldazine (SYR) as the substrate (ε_{530} = 65,000 M^{-1} cm^{-1}) with slight modifications. For free laccase activity, 90 µL 1.0 mM SYR (final concentration 30 µM), 2.82 mL 0.1 M citrate-phosphate buffer (pH 5.0) and 90 µL laccase solution (0.5 mg/mL) were reacted at 55°C for 10 min and increase in absorbance at 530 nm occurred due to the oxidation of syringaldazine into a quinone was recorded using the kinetic mode. For immobilized laccase activity, three pieces of laccase-immobilized cryogels were immersed into the substrate solution (10 mL, 30 µM SYR, pH 5.0) and allowed to react for 10 min at 55°C. The color of the reaction medium was turned into pink as a result of oxidation of SYR by laccase. The reaction was terminated by removing cryogels and the absorbance value was recorded at 530 nm. One unit of enzyme was expressed as the amount of laccase that was required to oxidize one micromole of SYR in a minute under optimum conditions. Optimum pH and optimum temperature were determined by measuring activities of free and immobilized laccase in 0.1 M citrate-phosphate buffer for pHs 3.5-4.0-4.5-5.0-5.5-6.0-6.5 and 4-15-25-35-45-55-65-75°C, respectively. Kinetic parameters, Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}), of free and immobilized laccase were determined at optimum pH and temperature, calculating the initial reaction rates of the enzymatic reaction at varying concentrations of SYR (0−15 µM) in 0.1 M citrate-phosphate buffer from Lineweaver-Burk plots that fit the Equation 3, where [S] and V were the substrate concentration and the initial enzymatic velocity, respectively :

$$\frac{1}{V} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax} \quad (3)$$

Storage stabilities were determined by keeping free laccase solution and laccase-immobilized cryogels in 0.1 M citrate-phosphate buffers of pH 5.0 and 5.5, respectively, at +4°C for 30 days and their activities were measured at certain time intervals. Maximum activity was expressed as 100% and relative activities were calculated for each triplicate experiments (Arica et al., 2017).

Dephenolization of Olive Mill Wastewater

Total phenolic contents of initial and laccase-treated OMW solutions were determined according to Singleton et al. (1999) and Daâssi et al. (2013). Briefly, 1.0 mL of OMW sample diluted with water (1:1, v/v) was mixed with 47.0 mL of water and 0.5 mL of FCR was added. After 3 min, 1.5 mL of 2.0% Na_2CO_3 was added. Solutions were shaken at 25°C in darkness at 100 rpm for 2h with a benchtop shaker (Promax 2020, Heidolph Instruments GmbH & Co KG, Kelheim, Germany). Absorbance at 760 nm was measured against the blank solution. Results were expressed as removal percent calculated using the initial and final concentrations of phenolic compounds equivalent to gallic acid (y=0.00189x). The analysis was performed three times. Additionally, effects of time, OMW volume and number of laccase immobilized cryogels on removal of phenolic compounds from OMW were evaluated. Reusability of cryogels were also determined.

RESULTS AND DISCUSSION

Synthesis and Characterization of Epoxy-Modified Magnetic Poly(HEMA) Cryogel

Poly(HEMA) cryogel was synthesized via radicalic polymerization of HEMA monomer crosslinked with MBAAm in presence of APS/TEMED (initiator/activator) under freezing conditions. Magnetic responsive property was introduced by in-situ method where the mixture of Fe(II) and Fe(III) ions were loaded into the cryogel and treated with a concentrated base solution (Sahiner, 2013). The hydroxyl groups on the surface allowed to decorate the cryogel with -NH_2 groups of APTES and epoxy groups of ECH, respectively. Laccase was immobilized covalently through the epoxy functionalities of

cryogel and -NH_2 groups of the enzyme molecule and immobilized amount of laccase was calculated to be 283 mg/g cryogel (Figure 1).

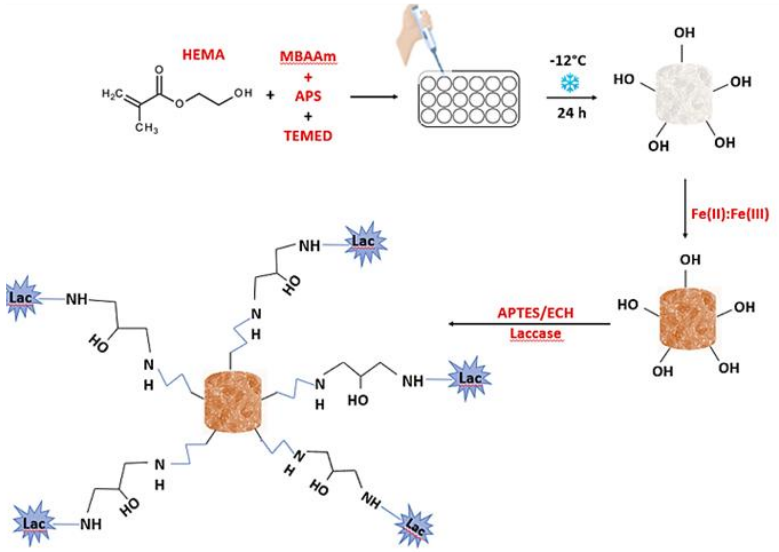

Figure 1. Schematic illustration for preparation of laccase-immobilized epoxy-modified magnetic poly(HEMA) cryogel

ATR-FTIR spectra of epoxy-modified magnetic poly(HEMA) cryogel and laccase immobilized epoxy-modified magnetic poly(HEMA) cryogel were demonstrated in Figure 2. The characteristic peaks for poly(HEMA) were O–H stretching at 3310 cm^{-1}, C-H stretching vibration at 2946 cm^{-1}, C=O stretching at 1715 cm^{-1}. The band at 900 cm^{-1} was the Si-O stretching vibration of APTES confirming the modification of the surface. Additionally, at 1072 cm^{-1} ether stretching vibration of epichlorohydrin was observed. The spectrum of laccase immobilized cryogel support laccase immobilization with C=O stretching (amide I) at 1650 cm^{-1} and N–H bending (amide II) at 1528 cm^{-1} (Kashefi et al., 2019). Additional peaks, observed after immobilization, at 967 and 964 cm^{-1} may be attributed to the interactions of epoxy groups and laccase.

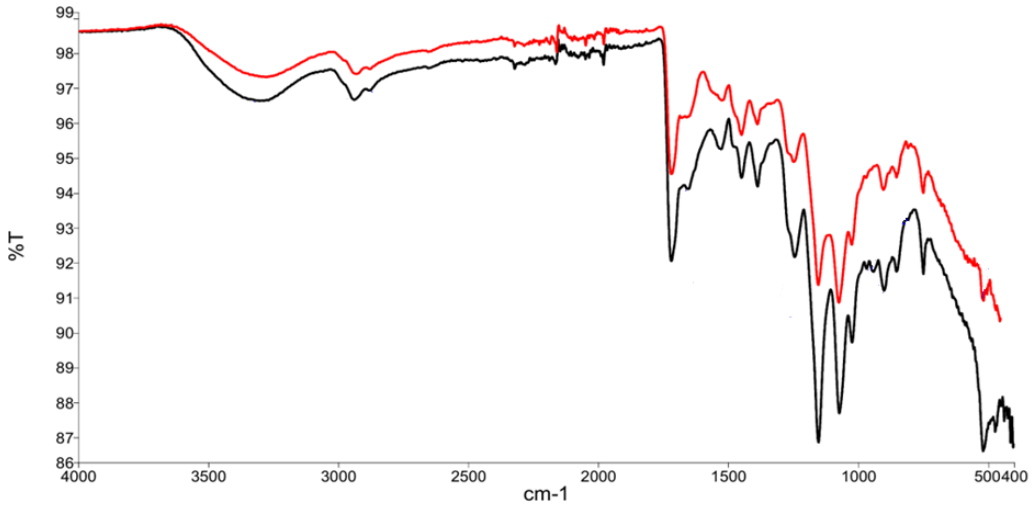

Figure 2. ATR-FTIR spectra of plain epoxy-modified magnetic poly(HEMA) cryogel (red line) and laccase immobilized epoxy-modified magnetic poly(HEMA) cryogel (black line)

Cryogels are highly elastic and porous materials with macropores ranging from 1 µm to 100 µm size. They can be dried and reswollen in a very short time restoring initial state (Ertürk and Mattiason, 2014). Water uptake or swelling properties of cryogels give idea about their porous structures. Photos of swollen poly(HEMA) [a], swollen Fe(II) and Fe(III) adsorbed poly(HEMA) [b], swollen [c] and

squeezed [d] epoxy-modified magnetic poly(HEMA) cryogel were presented in Figure 3. Water uptake (swelling degree) of magnetic cryogel was calculated to be 776.2%. Magnetic behaviour of epoxy-modified magnetic poly(HEMA) cryogel can be observed at Figure 3c when an external magnet was placed.

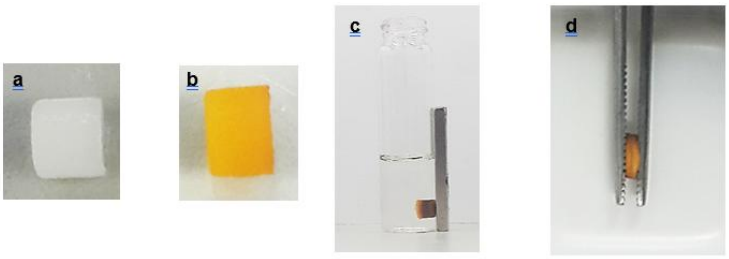

Figure 3. Photos of swollen poly(HEMA) [a], swollen Fe(II) and Fe(III) adsorbed poly(HEMA) [b], swollen [c] and squeezed [d] epoxy-modified magnetic poly(HEMA) cryogel

Porosity is one of the outstanding features of cryogels. Interconnected pores in cryogels are formed during initial freezing period, where solvent crystals grow until they contact with adjacent crystals (Rodrigues et al., 2013). The labyrinth-like channels of plain cryogel can be seen from SEM image in Figure 4A. This texture provided it a spongy structure. The roughness was assigned to the in-situ magnetization of the cryogel with Fe ions. A slight change on the pores and pore walls was observed after immobilization, however, it did not affect the macroporous structure of the cryogel (Figure 4B). The macroporous interconnected structure allows the migration of biomolecules and/or mobile phase easily (Bakhshpour et al., 2019).

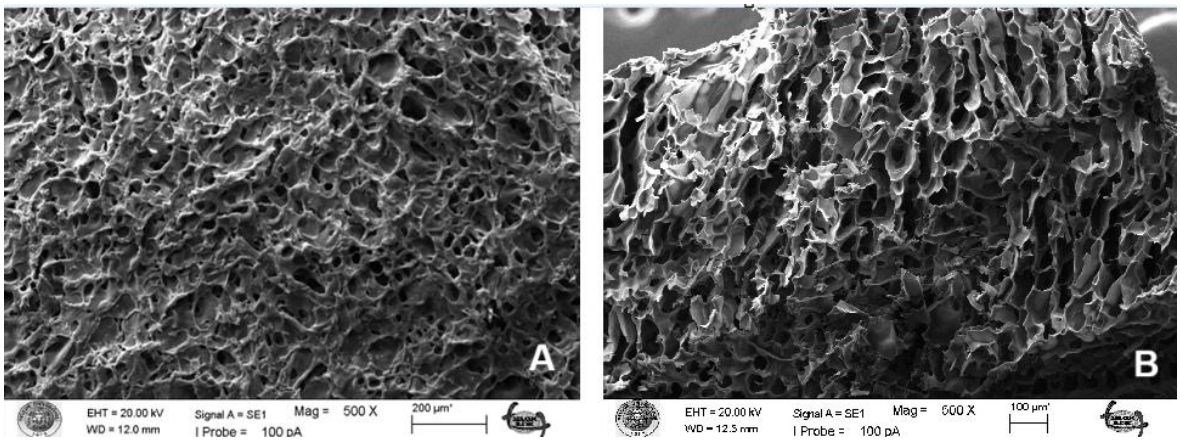

Figure 4. SEM images of plain (A) and laccase immobilized (B) epoxy-modified magnetic poly(HEMA) cryogel

EDX spectrum shown in Figure 5A reveals the elemental composition of epoxy-modified magnetic poly(HEMA) cryogel. The presence of Fe and Si atoms, additional to C, O and N, confirm incorporation of magnetic property and modification with APTES, respectively. In Figure 5B, the EDX spectrum of laccase immobilized cryogel indicated sulfur and copper incorporation which proved binding of laccase onto the cryogel.

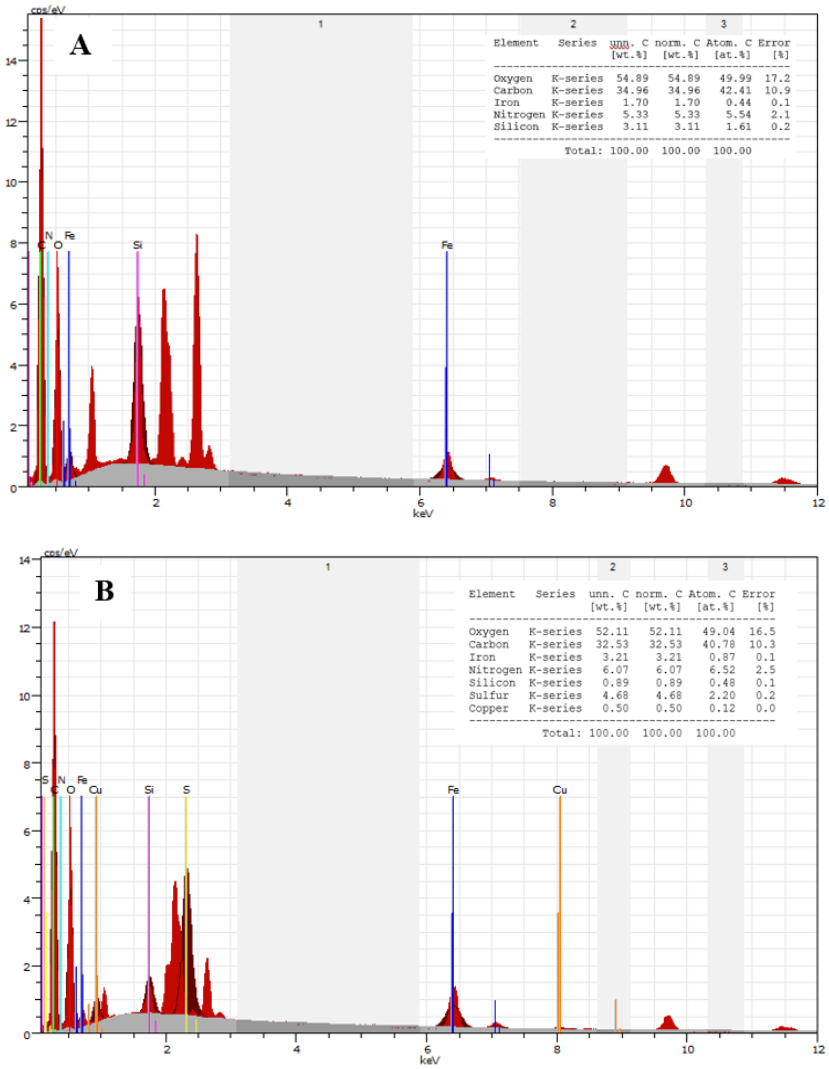

Figure 5. EDX spectra of plain (A) and laccase immobilized (B) epoxy-modified magnetic poly(HEMA) cryogel

Activities and Storage Stabilities of Free and Immobilized Laccase

Optimum pH range of fungal laccases are generally reported as 2-6. At higher pH values, due to the occupation of T2/T3 site by hydroxide ion, electron transfer from T1 to T2/T3 site is inhibited (Zofair et al., 2022). Figure 6A illustrates the pH-activity profile of free and immobilized laccase. Optimum pH of laccase shifted from 5.0 to 5.5 after immobilization. Similar shifts in optimum pH after immobilization of laccase have been reported by some researchers (Bayramoğlu and Arıca, 2009; Makas et al., 2010). For instance, Çelikbıçak et al. (2014) determined the optimum pHs for free and immobilized laccase from *T. versicolor*, using syringaldazine as the substrate, as 5.0 and 6.5, respectively. The slight shift of the optimum pH may be explained by the conformational changes related to the covalent attachment of enzyme to the cryogel. Considering the fact that, the microenvironment of enzyme was different from the soluble counterpart, the ionic states of the amino acid residues of active site affect the laccase's optimum pH. Additionally, in the light of these findings, it may also be concluded that the immobilized laccase system can be efficient in phenolic compound removal from OMW which had a pH of 4.8 used in this study.

The activities of free and immobilized laccases were assayed at various temperatures between 4-75°C. Optimum temperatures were found to be 65°C and 55°C for free and immobilized laccase, respectively, as seen in Figure 6B. Activity of immobilized laccase showed a sharp decrease at temperatures higher than 55°C. The activity of immobilized laccase decreased after 50°C similarly using

SYR as substrate in the study of Arıca et al. (2009), where optimum temperature for free laccase was about 40 °C while for immobilized enzyme onto poly(GMA/EGDMA)-DAH-GA was between 40 and 50 °C. Herein, immobilized laccase showed higher activity compared to the free form up to 55°C and this phenomenon can be considered as advantageous since it can oxidize its substrates at lower temperatures. However, the structural/conformational changes may be effective on the restricted stability of immobilized laccase at elevated temperatures.

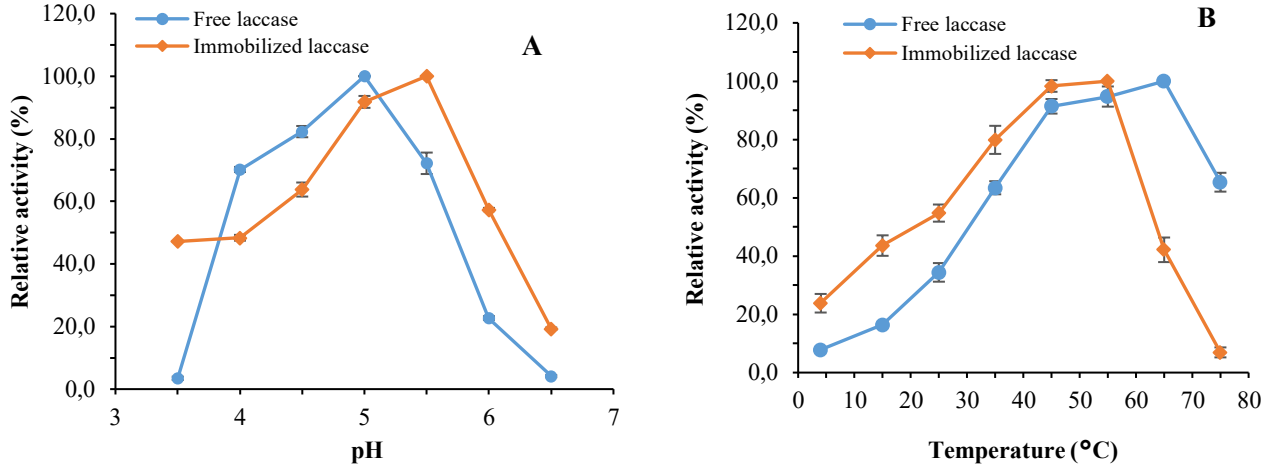

Figure 6. Optimum pH (A) and optimum temperature (B) profiles of free and immobilized laccase

Kinetic data obtained by measuring the initial velocities of oxidation at different concentrations of syringaldazine using free and immobilized laccase was fitted to Michaelis-Menten equation and plotted according to Lineweaver-Burk double-reciprocal model. Michaelis-Menten constants (K_m) for free and immobilized laccase were 1.248 µM and 23.75 µM and maximum rates (V_{max}) were 0.450 U/mg and 0.405 U/mg, respectively. K_m increased, whereas, V_{max} decreased slightly upon immobilization. Lower K_m is assigned to higher affinity of the enzyme towards its substrate, therefore, in present study, immobilized laccase showed lower affinity to SYR compared to its free counterpart. These findings align with results reported in literature (Othman et al., 2023; Hou et al., 2024) which state that diffusional limitations, steric hindrances, structural changes due to the attachment between support and enzyme and reduced flexibility are generally responsible for this statement. A 1.1-fold decrease in V_{max} was observed for immobilized laccase compared to its free form similar to the study of Arıca et al. (2009), where V_{max} of *Trametes versicolor* laccase towards syringaldazine decreased about 1.24-fold after covalent immobilization.

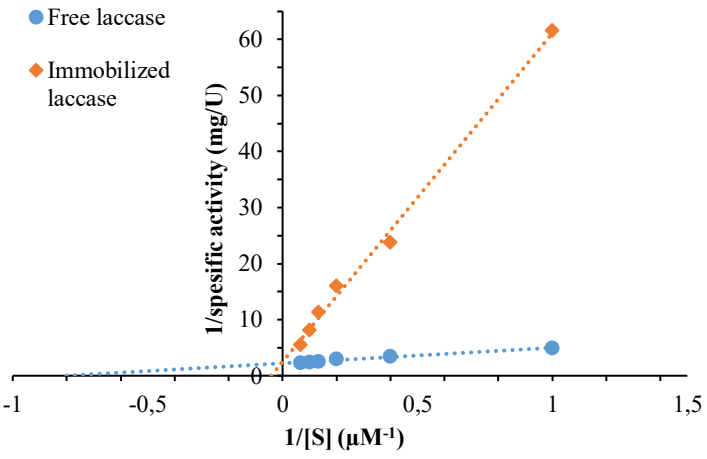

Figure 7. Lineweaver-Burk plots for free and immobilized laccase

Immobilization affects the stability since the enzyme becomes susceptible to different microenvironments and support-induced alterations (Chen et al., 2022). The long-term stability of enzymes is important for promoting its industrial application (Zhang et al., 2020). Activities of free and immobilized laccase were monitored periodically. As shown in Figure 8, after 30 days of storage, free enzyme lost about 80%, while immobilized laccase only lost 35% of its initial activity. Activity retention of immobilized enzyme was higher than the free one, probably linked to the protection of the enzyme conformation and active site due to immobilization (Zayed et al., 2024). Enhanced storage stabilities for immobilized laccase have been demonstrated by Lin et al. (2017) and Qiu et al. (2020) preserving 60% and 80%, respectively, after 30 days of storage.

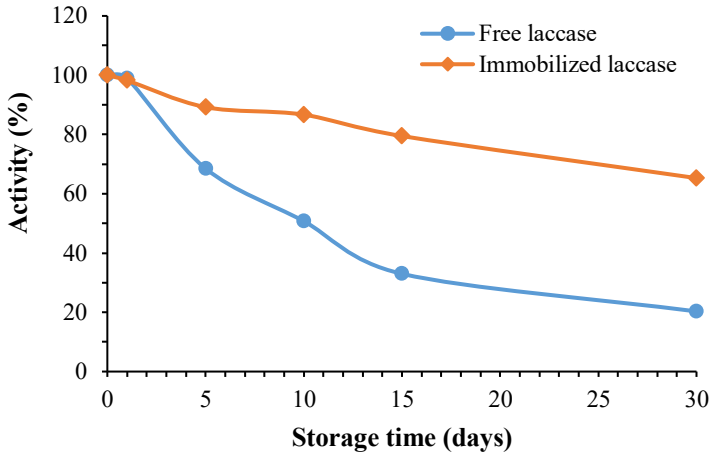

Figure 8. Storage stabilities of free and immobilized laccase at +4°C

Dephenolization of Olive Mill Wastewater by Immobilized Laccase

Industrial effluents have been serious threats for the environment and ecosystems. Researchers have been searching for suitable adsorbents to remove pollutants from industrial water. Olive mill wastewater generally bears 0.5-24 mg/L phenolic compounds, and if it is released without any treatment, it may cause toxic effects (Hamimed et al., 2021; Girelli et al., 2021). Although conventional techniques such as distillation, adsorption, extraction, and chemical oxidation have been applied to remove or remediate OMW, sustainable and green approaches are in demand. The use of oxidoreductases in free, crude or immobilized forms have been studied for treatment of OMW. Laccase is one of the key enzymes for removal of phenolic compounds. Herein, laccase immobilized-epoxy-modified magnetic poly(HEMA) cryogels were applied to remove phenolic compounds from olive mill wastewater. Porous composite materials such as cryogels can be bound with enzymes and may achieve pollutant removal with high efficiency. Incorporation of magnetic property into such adsorbents improve their targeting and adsorption capacity, porosity, resistance and biological activity (Atta et al., 2018).

In OMW dephenolization studies, effects of number of laccase immobilized cryogels, OMW volume and time on the removal of phenolic compounds were detected by the spectrophotometric method of Folin-Ciocalteu. Figure 9A depicts that as the number of laccase-loaded cryogels increased, the dephenolization capacity also scaled up but also a plateau was observed when 3, 4 and 5 cryogels were used. This phenomenon may be resulted from the accumulation of reaction products. Therefore, use of three cryogels loaded with laccase was enough to evaluate the phenolic removal in further studies. To investigate the effect of OMW volume on dephenolization efficiency, 1.0, 2.0, 4.0, 7.0 and 10 mL of 1:1 diluted OMW were used and the final volume was adjusted to 30 mL with water. Phenolic contents of the solutions were determined at the beginning and after 24h. As seen from Figure 9B, the increment in OMW volume resulted a decrease about 8% from 1.0 to 10.0 mL, which is probably due to reaching

maximum capacity and no further improvement was obtained. Laccase has been reported to lose its activity in different percentages, for instance, when encountered with effluents containing increasing dye concentrations (Yadav et al., 2021; Zhang et al., 2024).

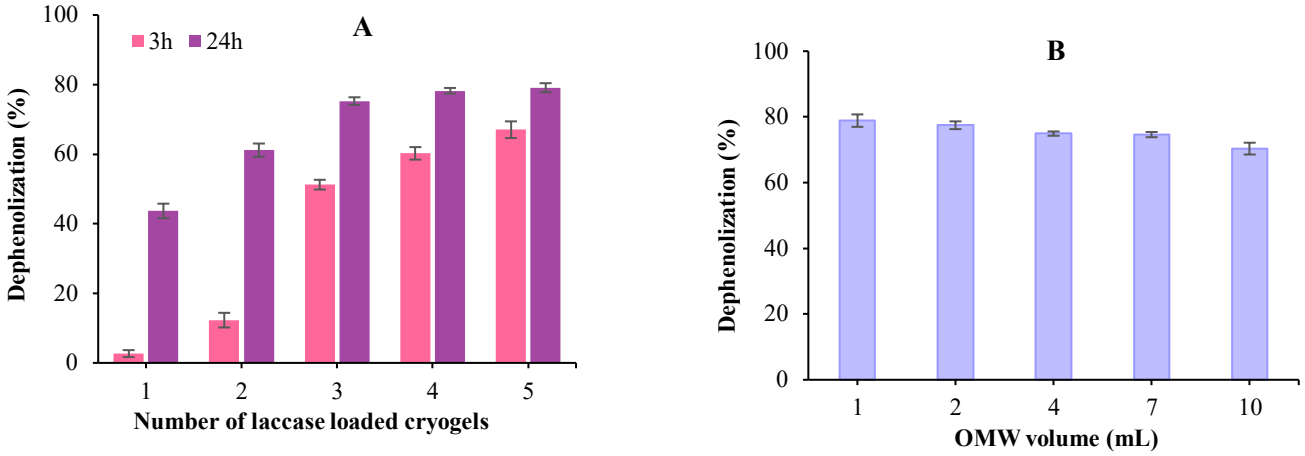

Figure 9. Effect of number of immobilized laccase concentration (A) and volume of OMW (B) on removal of phenolic compounds at 25°C using laccase immobilized cryogels

It can be seen from Figure 10A that in 24h, about 75% dephenolization was achieved and a plateau was observed after 300 min. Dephenolization experiments were conducted using plain cryogels (without laccase) and the removal was negligible with a percentage of 1.3%. Girelli et al. (2021) have reported 90% of phenol removal by immobilized laccase on silica-chitosan support in 21h.

Operational stability, in other words reusability of immobilized enzymes is a significant feature considering its commercial use. Herein, laccase-immobilized cryogel was used repeatedly and its performance to remove phenolic compounds was determined in each cycle. As depicted in Figure 10B, immobilized laccase maintained its activity by a decrease of only 3% compared to initial activity after three consecutive reuses. Increasing the number of cycles caused activity reduction, as expected, probably due to denaturation of laccase or inefficient removal of products (Atiroğlu et al., 2024; Bani et al., 2024).

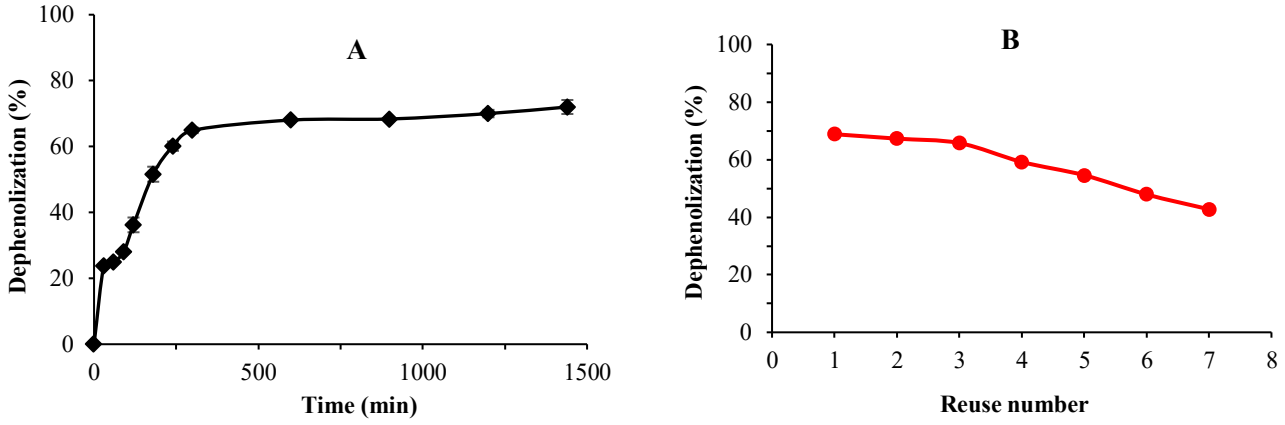

Figure 10. Effect of time on removal of phenolic compounds by immobilized laccase from OMW at 25°C (A) and reusability of immobilized laccase (B)

CONCLUSION

Enzyme-assisted remediation of effluents has become a replacement for conventional techniques. Among various enzymes potential of laccase for treatment of olive mill wastewater has been extensively explored in order to reduce the amount of its phenolic compounds. To circumvent drawbacks of using

free enzymes involving low stability, high cost and non-reusability in such operations, immobilized laccase provides an efficient way. Focusing on these issues, in this study, laccase was immobilized through epoxy moieties onto an in-situ prepared magnetic cryogel. Results suggest that immobilization provided superior catalytic activity and storability. It was observed that immobilized laccase was successfully used for dephenolization of OMW by 79% removal with a remarkable recyclability. The results of this study underlined that further research should focus on developing novel immobilization matrices for various enzymes and investigating the utilizations of these bio-tools for greener processes.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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