Environment Protection Engineering

Vol. 47 2021 No. 1

DOI:10.37190/epe210107

SHAMILA ROUHANI[1], SHOHREH AZIZI[2, 3], MALIK MAAZA[2, 3],
BHEKIE MAMBA[1, 4], TITUS A.M. MSAGATI[1, 5]

COVALENT IMMOBILIZATION OF LACCASE ON Fe_3O_4–GRAPHENE OXIDE NANOCOMPOSITE FOR BIODEGRADATION OF PHENOLIC COMPOUNDS

Laccase from *Trametes Versicolor* (E.C. 1.10.3.2) was immobilized on the Fe_3O_4–graphene hybrid nanocomposite through the covalent attachment method (Lac/Fe_3O_4/GO). The effect of immobilization conditions on the activity and recovered activities such as contact time, the concentration of glutaraldehyde and enzyme was evaluated. The recovered activity of the immobilized laccase on the Fe_3O_4–graphene oxide nanocomposite was ca. 86%. Immobilized laccase unlike free laccase retained the activity and exhibited higher resistance to temperature and pH changes and also improved storage and thermal stability. Approximately 70% of relative activity for immobilized laccase was remained after being incubated for 2 h at 55 °C, but free laccase only remained 48%. Immobilized laccase retained 88% of initial activity after storage for 20 days, however, the free laccase only 32%. Finally, Lac/Fe_3O_4/GO capability was evaluated by the oxidation of phenol, *p*-chlorophenol, and 2,4-dichlorophenol. Lac/Fe_3O_4/GO was characterized by SEM, EDX, FT-IR, and AGFM.

[1]Institute for Nanotechnology and Water Sustainability (iNanoWS) Unit, College of Science, Engineering and Technology, University of South Africa, Johannesburg, 1709, South Africa, corresponding author S. Rouhani, email address rouhas@unisa.ac.za

[2]UNESCO-UNISA Africa Chair in Nanoscience and Nanotechnology College of Graduates Studies, University of South Africa, Muckleneuk Ridge, Pretoria, South Africa, 392.

[3]Nanosciences African Network (NANOAFNET), iThemba LABS-National Research Foundation, 1 Old Faure Road, Somerset West 7129, PO Box 722, Somerset West, Western Cape, South Africa.

[4]State Key Laboratory of Separation Membranes and Membrane Process, National Center for International Joint Research on Membrane Science and Technology, Tianjin 300387, China.

[5]School of Life Sciences and Bio-Engineering, The Nelson Mandela African Institution of Science and Technology, Tengeru 447, Arusha, Tanzania.

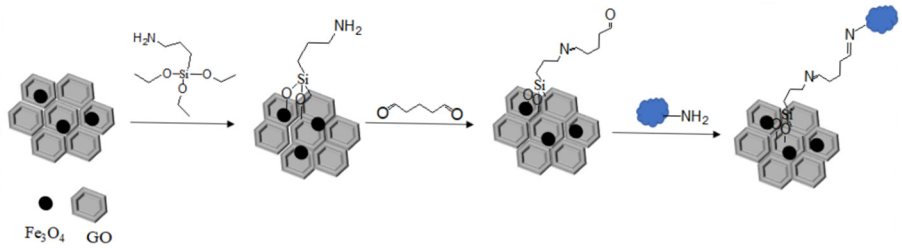
1. INTRODUCTION

In the past decades, the release of organic effluents from industries such as textile, paper and pulp, plastic, and petrochemicals have become one of the major environmental concerns owing to their carcinogenic, genotoxic, and/or mutagenic nature [1]. Among these organic pollutants, phenols are well known for their high toxicity in humans and animals. Poor solubility and biodegradability, long term ecological damage, and high toxicity of phenol and its derivatives led to calls by Environmental Protection Agency (EPA) for lowering phenol concentration in the wastewater to less than 1 $mg \cdot cm^{-3}$ before discharging them into the water reservoirs [2]. Currently, many physical and chemical methods such as adsorption, zonation, and filtration are available for decontamination of phenols. However, more attention has been focused on enzymatic degradation methods because they are often cheaper and more environmentally friendly. In recent years, the applications of laccase in environmental protection, especially in the degradation of phenols, were increasingly reported [3, 4]. Laccase (EC 1.10.3.2), a multi-copper oxidase, catalyzes the oxidation of a wide range of aromatic substrates including phenol derivatives, benzenethiols, polyphenols, and polycyclic aromatic hydrocarbons (PAHs) by reducing molecular oxygen to water. However, laccase applications are hindered by their sensitivity to denaturing agents and non-reusability [5].

One way to overcome these limiting factors is to immobilize laccases on various carriers by different mechanisms such as entrapment/encapsulation, adsorption, covalent binding, and self-immobilization, which can protect them from denaturation, improve their stability, maintain good catalytic efficiency, and lead to more economical processes [6]. Therefore, these procedures are widely followed to synthesize efficient, eco-friendly, and selective biocatalysts [7]. Nevertheless, the immobilization process is associated with the significant loss of enzyme activity that makes it a remaining major challenge [8]. In recent years, great efforts have been focusing on the achievement of high enzyme stability and reusability after the immobilization process, moreover its loading improvement via various immobilization methods. Recently, nanostructured composites and hybrid materials are gaining remarkable attention as suitable immobilization support, due to their great potential for improvement of immobilization efficiency [9]. The prominent properties of nanoparticles chosen for immobilization are low synthesis cost, stability, high binding capacity, high available surface area, the feasibility of functionalization, maintaining the biological function of the enzyme, and above all, minimizing diffusional limitation [10].

Graphene oxide (GO), as a novel nanomaterial, has been attracting more, and more attention in recent decades, due to its novel properties, and wide range of potential applications [11]. The reason for its attractiveness includes its morphology, large accessible surface area, plentiful oxygenated functional groups along with good biocompatibility, and the ability for further functionalization to fulfill the criteria for other nano-

particles and further high enzyme loading on support, hence it can be appropriate support for enzyme immobilization. But like other nanomaterials with high aspect ratios, graphene oxide sheets tend to generate irreversible agglomerates due to the powerful π-π interactions, making immobilization processing difficult [12]. For these purposes, metallic nanoparticles are generally incorporated into GO or reduce GO nanosheets. Some of them cause an increase in catalytic effects of GO. Previous studies have indicated that anchoring nanoparticles to the graphene oxide could inhibit the aggregation of GO nanosheets and result in exfoliated graphene oxide with the maintenance of the functionalities as well as the high surface area. The great potential of GO-based magnetic composites for enzyme immobilization objective is known widely and due to the magnetic response property, large surface area, two-dimensional structure, easy surface modification, large enzyme immobilization capacity, simple preparation, and satisfactory reusability of magnetic graphene oxide (MGO) [13].



Scheme 1. The preparation of Fe_3O_4/GO and its chemical modifications for the covalent laccase immobilization

Nevertheless, the immobilization of various enzymes on MGO without any surface modification or any coupling reagent demonstrated that the loading capacity, stability, and reusability of immobilized enzymes are not appropriate for bio- and industrial applications. Direct attachment of the enzyme to MGO nanosheets constrains its molecular motion, in turn slowing down its reactivity. The results of previous studies represent the activity enhancement of the immobilized enzyme in the presence of spacer [14]. Concerning the above-mentioned reasons, in this work, the functionalization of magnetic graphene oxide (Fe_3O_4/GO) has been done with APTES and then its post-modification with glutaraldehyde as a spacer. In continuation of our studies on preparation and application of magnetically recyclable nanobiocatalysts [15,16], Herein, we have, for the first time, prepared the Fe_3O_4–graphene oxide nanocomposite by a facile in-situ co-precipitation method and was successfully utilized as a support for the immobilization of laccase (Lac/Fe_3O_4/GO) (Scheme 1). The effects of pH and temperature on the enzymatic property of Lac/Fe_3O_4/GO and the catalytic capacity of the Lac/Fe_3O_4/GO for phenol, *p*-chlorophenol, and 2,4-dichlorophenol degradation were also evaluated.

2. EXPERIMENTAL

Materials. Laccase (E.C. 1.10.3.2) from Trametes Versicolor, 2,2-azinobis(3-ethylbenzthiazolin-6-sulfonate) (ABTS), 3-aminopropyltriethoxysilane (APTES), glutaraldehyde (GA), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Ammoniumhydroxide (25wt. %), 2,4-dichlorophenol, *p*-chlorophenol, phenol, graphite, potassium permanganate, concentrated sulfuric acid, sodium nitrate, sodium hydroxide, hydrogen peroxide, (30 wt. %), toluene, ethanol were purchased from Sigma-Aldrich Co. Llc. (St. Louis, America). All other materials were also bought from Sigma and were used without any purification.

Preparation of graphene oxide. Graphene oxide (GO) was prepared using the modified Hummers method [17]. 3 g of graphite was added to 150 cm^3 of concentrated sulfuric acid under continuous stirring and ice water batch conditions. Then, 3 g of sodium nitrate was added and stirred. Afterward, 18 g of $KMnO_4$ was slowly added into the aforementioned system and continually stirred for 2 h. After that, the reaction mixture was heated to 35 °C and stirred for 30 min. After that 200 cm^3 of distilled water was continuously and slowly added into the mixture, and the temperature was raised to 90 °C reacting for 20 min. The reaction mixture was then cooled down naturally to room temperature. Subsequently, 18 cm^3 of hydrogen peroxide was added to eliminate the redundant $KMnO_4$. By now, a bright yellow solution was obtained, a precipitate was collected by centrifugation and washed several times with hydrochloric acid (5 vol. %). Then, the precipitate was washed with distilled water until the pH of the supernatant reached 7. Finally, the obtained sample was dried in a vacuum oven at 60 °C overnight.

Preparation of Fe_3O_4/GO nanocomposite. Fe_3O_4 nanoparticles were deposited over the surface of graphene oxide by a simple co-precipitation method based on the hydrolysis of a mixture of Fe^{2+} and Fe^{3+} ions (molar ratio 1:2). First, 3 g of GO was dispersed in 150 cm^3 of distilled water by sonication for 1 h. A solution of 3 g of $FeCl_3 \cdot 6H_2O$ and 1.10 g of $FeCl_2 \cdot 4H_2O$ in 100 cm^3 of distilled water was added dropwise to the suspension and mechanically stirred for 30 min under nitrogen protection at room temperature. Afterward, the temperature of the mixture was increased to 80 °C and then 60 cm^3 of NH_4OH solution (25%) was added dropwise until pH reached ca. 11. After 60 min, the temperature was decreased and the precipitate was filtered, washed with distilled water five times, and dried in a vacuum oven at 50 °C for 12 h to obtain Fe_3O_4/GO.

2.4. Preparation of NH_2/Fe_3O_4/GO, GA/Fe_3O_4/GO, and immobilization of laccase onto Fe_3O_4/GO nanocomposite. Before covalent bonding of laccase enzyme, Fe_3O_4/GO should be subjected to the surface modification for grafting essential functional groups. Primarily, Fe_3O_4/GO is treated with APTES for introducing the amine group. Herein,

3 g of Fe_3O_4/GO nanocomposite were added to the 60 cm^3 of anhydrous toluene, followed by adding 60 cm^3 of APTES. Then the solution was transferred to the reflux setup at 80 °C for 12 h. Finally, the product was separated by an external magnetic field using a magnet and washed several times with ethanol and dried in a vacuum oven at 60 °C overnight. Then NH_2/Fe_3O_4/GO was sonicated in PBS buffer (60 cm^3, 100 mM, pH = 7) for two hours, next it was crosslinked with glutaraldehyde solution (under the optimal GA concentration) at 25 °C under stirring for 2, 4, 6 and 8 h. The concentration of glutaraldehyde solution was set from 2 to 8%. Then the excess glutaraldehyde was washed with PBS buffer in the presence of an external magnet. This product, which contains aldehyde groups, is labeled as GA/Fe_3O_4/GO.

For laccase immobilization, an ultrasonicated GA/Fe_3O_4/GO solution in PBS solution (100 mM, pH = 5) for 30 min was mixed with 5 cm^3 solution of laccase in PBS solution (100 mM, pH = 5) with optimal laccase concentration (15.2 U, 5 $mg \cdot cm^{-3}$) and the reaction mixture was shaken at 4 °C for 24 h. The concentration of laccase solution was set from 1 to 7 $mg \cdot cm^{-3}$. Afterward, the Lac/Fe_3O_4/GO was separated with a magnet and then washed with PBS solution (100 mM, pH = 5). The washing process was repeated several times until no free laccase was detected in the rinsing solution. The residual concentration of laccase in the rinsing solution was determined by the Bradford method [18]. Finally, the product was dried under reduced pressure and stored at 4 °C for future use.

Activity assay of free laccase and Lac/Fe_3O_4/GO. The activities of free laccase and Lac/Fe_3O_4/GO were measured using ABTS as a standard in an acetate buffer (100 mM, pH = 5) as described previously. Reaction solution with 0.3 cm^3 of ABTS in 2.6 cm^3 Na-acetate buffer was incubated for 10 min at 25 °C. Then a suitable amount of free or immobilized enzyme in 0.1 cm^3 of acetate buffer was added and after that analyzed by measuring the absorbance at 420 nm (ε_{420} = 36 000 $M^{-1} \cdot cm^{-1}$) with a visible spectrophotometer. Enzyme activities are expressed as international units (U), where 1 U represents the amount of enzyme that forms 1 $\mu mol \cdot min^{-1}$ of products under standard assay conditions. The activity recovery (*AR*) of the enzyme was calculated using the equation:

$$AR = \frac{A_i}{A_f} \times 100\% \tag{1}$$

where A_i is the activity of Lac/Fe_3O_4/GO, and A_f is the activity of free laccase under similar experimental.

Effect of temperature and pH on the activity of free laccase and Lac/Fe_3O_4/GO. The effect of pH on the activity of free laccase and Lac/Fe_3O_4/GO was determined at a pH range from 3 to 8 at room temperature for 2 h using the standard ABTS assay. The buffers used were acetate buffer for pH 3–5 and phosphate buffer for pH 6–8. The effect

of temperature on the biocatalyst activity was assayed at 20–80 °C in PBS solution at pH = 5. Laccase activity was determined using the standard ABTS assay. Relative activities were normalized to the highest activity which was taken as 100%. All measurements were performed in triplicate.

Determination of kinetic parameters of free laccase and Lac/Fe_3O_4/GO. Change in kinetic parameters upon immobilization is a critical point for checking the success of an enzyme immobilization process. The Michaelis–Menten constant (K_m) and apparent maximum velocity (V_{max}) were determined with varying concentrations of ABTS (0.01 –1 mM) in PBS solution (100 mM, pH = 5) at 25 °C. The kinetic parameters values for the substrate (ABTS) were obtained according to Lineweaver–Burk plot and K_m and V_{max} were determined from the intercepts at x and y axes, respectively.

Thermal and storage stability of free laccase and Lac/Fe_3O_4/GO. Thermal stability was investigated by incubating the free laccase and Lac/Fe_3O_4/GO at 15–85 °C in PBS solution (100 mM, pH = 5) for 2 h; thereafter, the residual enzymatic activity was measured using the standard ABTS assay. Storage stability of the free laccase and Lac/Fe_3O_4/GO was ensured upon calculating the residual activity of immobilized and free laccase using the standard ABTS assay after storing at 4 °C in PBS solution (100 mM, pH = 5) for 20 days. The storage efficiency was defined as the ratio of the activity of the free laccase or Lac/Fe_3O_4/GO to the initial activity after stored for some time. In all stability experiments, the initial activity of Lac/Fe_3O_4/GO was assumed as 100%, while other activities were the relative values in comparison with the initial activity. All measurements were performed in triplicate.

Catalytic degradation of phenolic compounds by Lac/Fe_3O_4/GO. Phenol, *p*-chlorophenol, and 2,4-dichlorophenol degradations were studied with Lac/Fe_3O_4/GO in PBS solution (100 mM, pH = 5) at room temperature with continuous stirring. In each set of the experiment, 45 mg of the Lac/Fe_3O_4/GO was added into 10 cm^3 of phenolic compound solution (200 mM) with a reaction time lasting 8 h. Before reaction initiation, mixtures of buffer and phenol were saturated with oxygen by vigorously stirring the reaction mixture for 10 min. The percentages of phenol, *p*-chlorophenol, and 2,4-dichlorophenol degradation were determined at various time intervals by the HPLC technique.

The concentration of the phenolic compounds was analyzed using a Dionex Ultimate 3000 UHPLC system (Dionex Softron GmbH, Dornierstr. 4, Germany) equipped with a reversed-phase C18 analytical column of 100×2.1 mm and 1.7 μm particle size (Acquity UPLC® BEH, Waters, Ireland). The column temperature was maintained at 35 °C. The injected sample volume was 0.005 cm^3. Mobile phases A and B were water and methanol with 0.1% formic acid, respectively. The chromatographic determination was performed as follows: the initial mobile phase composition (2% B) constant for 1 min, followed by a linear gradient from 2% B to 100% B for 9 min, kept 100% B for 2 min

and then dropped back to 2% B 12.1 min and kept constant at 2% B for 2 min. The flowrate was 0.3 $cm^3 \cdot min^{-1}$ and the total run time was 14 min. Eluted compounds were detected by UV at 280 nm. The amount of enzymatic degradation of phenols was calculated as the difference between initial and final phenols concentrations. A control that contained the same phenol concentration without immobilized enzyme was operated under the same experimental conditions.

3. RESULTS AND DISCUSSION

3.1. OPTIMUM CONDITIONS FOR PREPARATION OF Lac/Fe_3O_4/GO

The effects of activation time and concentration of glutaraldehyde and enzyme for laccase immobilization were studied and optimized (Fig. 1). The optimal activity was observed at 5 vol. % glutaraldehyde and 4 h activation time (Figs. 1a, b). A higher concentration of glutaraldehyde could lead to aggregation, precipitation, loss of enzyme activity, and distortion of enzyme structure [19]. The activity of Lac/Fe_3O_4/GO and activity recovery of Lac/Fe_3O_4/GO achieved 65.3 U and 86%, respectively.

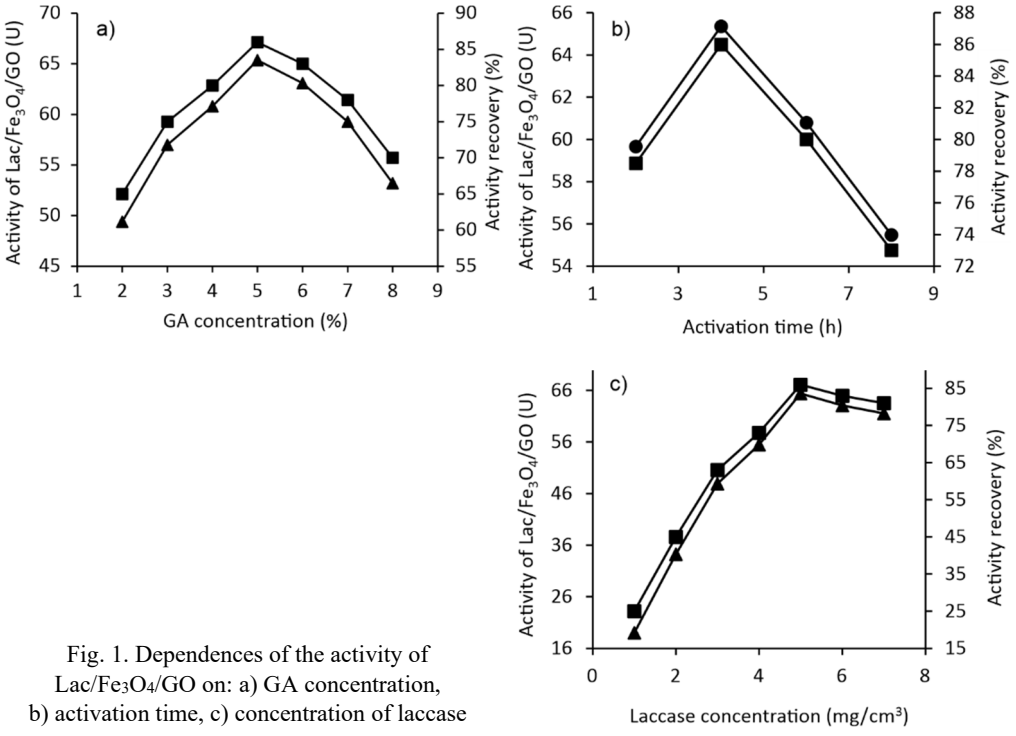


Fig. 1. Dependences of the activity of Lac/Fe_3O_4/GO on: a) GA concentration, b) activation time, c) concentration of laccase

To obtain the optimal amount of laccase, the amount of support was kept constant and different concentrations of laccase solution were used, from 1 to 7 mg·cm^{-3}. Although the immobilized amounts of laccase on support increased with the amount of the laccase concentration, the trends of activity recovery decreased when the laccase concentration was above 5 mg·cm^{-3} (Fig. 1c). Therefore, the activation time, glutaraldehyde concentration, and amount of laccase for further studies were selected as 4 h, 5 vol. %, and 5 mg/cm^3, respectively.

3.2. CHARACTERISATION OF Lac/Fe_3O_4/GO

Scanning electron microscopy (SEM) images of Fe_3O_4/GO and Lac/Fe_3O_4/GO are shown in Fig. 2. The SEM images confirm that Fe_3O_4 nanoparticles and laccase were located on the surface of GO layers.

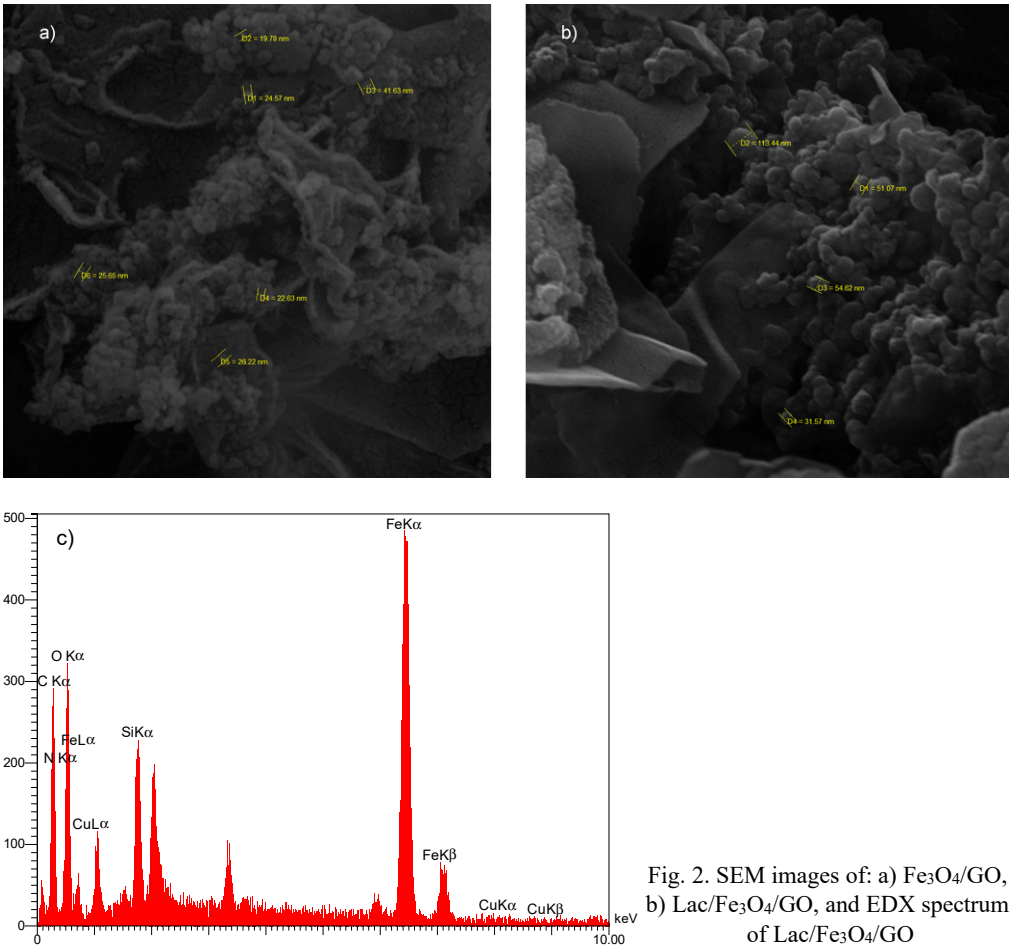


Fig. 2. SEM images of: a) Fe_3O_4/GO, b) Lac/Fe_3O_4/GO, and EDX spectrum of Lac/Fe_3O_4/GO

The average nanoparticle sizes of the Fe_3O_4/GO and Lac/Fe_3O_4/GO were about 16 nm and 98 nm, respectively, that showed the laccase immobilization has been done on Fe_3O_4/GO hybrid. The structure of the Fe_3O_4/GO hybrid materials was shifted from flake-like to a more crumpled shape, which could be attributed to chemical functionalization and laccase immobilization [20]. The preparation of nanocomposite and the presence of laccase on Fe_3O_4/GO were also confirmed using the energy-dispersive X-ray spectroscopy (EDX) analysis (Fig. 2c). The presence of the expected elements in the structure of the catalyst, namely iron, nitrogen, oxygen, silicon, and copper, has been verified.

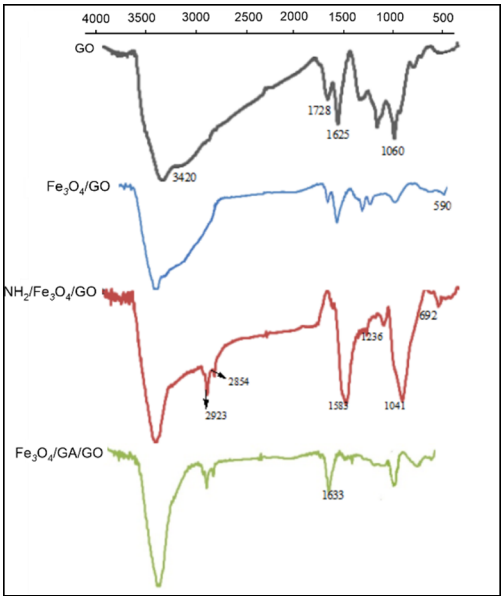


Fig. 3. FT-IR spectra of GO, Fe_3O_4/GO, NH_2/Fe_3O_4/GO, and GA/Fe_3O_4/GO

GO, Fe_3O_4/GO, NH_2/Fe_3O_4/GO and GA/Fe_3O_4/GO were characterized using Fourier transform infrared (FT-IR) spectroscopy (Fig. 3). The FT-IR spectrum of GO is in good agreement with that in previous work [18]. The broad, intense band at 3420 cm^{-1} is attributed to the stretching of O–H. The band at 1728 cm^{-1} is associated with stretching of the C–O bond of carboxyl groups. The peak at 1625 cm^{-1} can be assigned to the aromatic C=C groups. Formation of the C–O band of the epoxy group is observed at 1060 cm^{-1}. The Fe–O stretching vibration peak was seen at 590 cm^{-1}, which confirmed that Fe_3O_4 nanoparticles were successfully anchored onto the graphene sheet. After surface modification with APTES, the representative NH_2/Fe_3O_4/GO exhibited two additional peaks at 2923 and 2854 cm^{-1}, indicative of the asymmetric and symmetric stretching modes of C−H bonds from CH_2−CH_2 groups connecting with the NH_2 group. The other additional peaks at 692 and 1583 cm^{-1} could be ascribed to the symmetric N−H

out-of-plane bending vibration from the NH_2 group. Besides, the appearance of two new peaks at 1236 and 1041 cm^{-1} was assigned to the Si–O–Si asymmetric stretching and Si–O–C stretching vibration [21]. The intensity of the absorption peak around 3420 cm^{-1}, characteristic of the surface OH groups, significantly decreased, suggesting that the amino-silylation mainly occurred via reaction with surface OH. The successive step involved the modification with glutaraldehyde. A band at 1633 cm^{-1} (–C=N– bonds), due to the reaction between the free –NH_2 group of NH_2/Fe_3O_4/GO and –CHO of glutaraldehyde, appears.

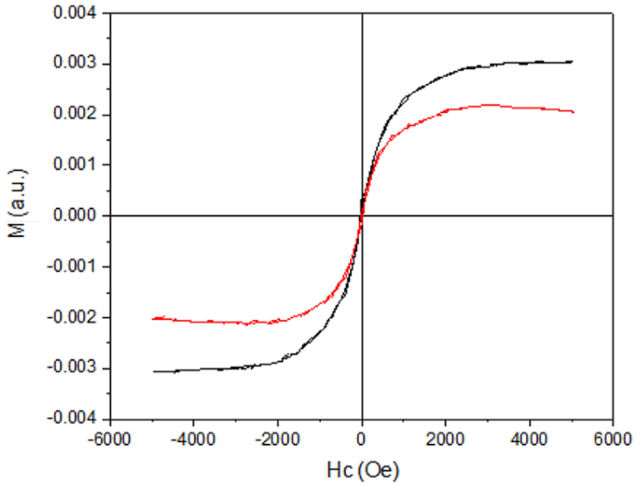


Fig. 4. Magnetic curves of Fe_3O_4/GO and Lac/Fe_3O_4/GO at room temperature

The magnetic properties of the Fe_3O_4/GO and Lac/Fe_3O_4/GO were measured using the AGFM technique. The room temperature magnetization curves of the Fe_3O_4/GO and the Lac/Fe_3O_4/GO are shown in Fig. 4. The Fe_3O_4/GO and Lac/Fe_3O_4/GO showed the saturation magnetization (M_s) value 0.002 Oe and 0.003 Oe, respectively. The Fe_3O_4/GO and Lac/Fe_3O_4/GO have a coercivity (Hc) of 27.24 and 8.89 Oe, respectively, and the remanent magnetization (M_r) of ca. 0.00011 and 0.00048 Oe, respectively. As a result, Lac/Fe_3O_4/GO has a typical superparamagnetic behavior and can be efficiently attracted with an external magnet.

3.3. ACTIVITY AND PROPERTIES OF FREE LACCASE AND LAC/FE_3O_4/GO

Free laccase and Lac/Fe_3O_4/GO have similar optimum pH at 5 (Fig. 5a). 60% of the initial activity of free laccase was lost when pH was decreased to 3, while 65% of the relative activity of Lac/Fe_3O_4/GO remained at pH 3. At an elevated pH (8), the relative activity of Lac/Fe_3O_4/GO and free laccase was found to be 60% and 25.3%, respectively (Fig. 5b). The results show that the resistance of laccase to acid and basic denaturation was considerably increased after immobilization.

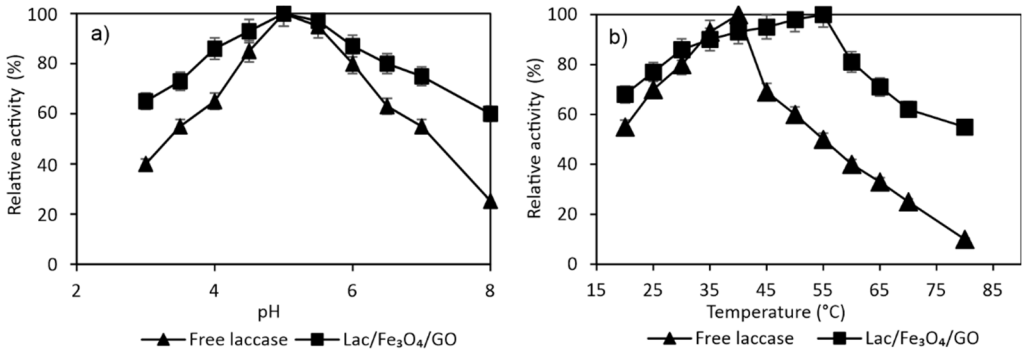


Fig. 5. Effect of pH on the activity of free laccase and Lac/Fe_3O_4/GO (a), acetate buffer for pH 3–5 and phosphate buffer for pH 6–8 by the ABTS aerobic oxidation reaction at room temperature for 2h; effect of temperature on free laccase and Lac/Fe_3O_4/GO (b), the relative activity determined at various temperatures at pH = 5 by ABTS aerobic oxidation. All Experiments performed in triplicate. The error bars calculated (5%)

The effect of temperature on the relative activity of free laccase and Lac/Fe_3O_4/GO is shown in Fig. 5b. The maximal activity of free laccase and Lac/Fe_3O_4/GO was observed at 40 and 55 °C, respectively. This was followed by a stepwise decrease in activity with increasing temperature. Lac/Fe_3O_4/GO was stable at a broader range of temperatures (40–65 °C, Fig. 5b) compared to free enzyme. At 80 °C, the activity of free laccase was almost completely lost (90%) and for Lac/Fe_3O_4/GO it was 45%. The immobilization causes higher resistance of the enzyme to thermal inactivation. This might be attributed to the increased structural stability of immobilized laccase molecules due to the existence of multilayer graphene oxide-enzyme nanosheets, while the multipoint covalent interactions between laccase molecules and functionalized graphene oxide–Fe_3O_4 nanocomposite might provide further protection against inactivation at a higher temperature and pH changes [22].

Table 1

Kinetic parameters of free laccase and Lac/Fe_3O_4/GO

Entry	Enzyme	K_m [mM]	V_{max} [mM·min^{-1}]
1	free laccase	0.045	2.9
2	Lac/Fe_3O_4/GO	0.073	1.4

Kinetic parameters such as the Michaelis constant (K_m) and V_{max} determine the enzyme catalytic efficiency. K_m is the substrate concentration at which the reaction rate is at half its maximum, while V_{max} represents the maximum reaction rate attained when all of the enzyme molecules are bound to the substrate. The Lineweaver–Burk plots for the free laccase and Lac/Fe_3O_4/GO on oxidation of ABTS (0.01–1 mM) were used to obtain kinetic parameters. The Michaelis–Menten constants for free laccase and Lac/Fe_3O_4/GO

are presented in Table 1. The K_m of Lac/Fe_3O_4/GO is about 1.62 times higher than that of free enzyme and the V_{max} of Lac/Fe_3O_4/GO is 2.07 times lower than that of a free enzyme (Table 1). The higher K_m value for Lac/Fe_3O_4/GO laccase indicates a lower affinity for the substrate after immobilization which may be ascribed to enzyme conformational changes or less accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitation [23]. These values indicate how the immobilization process affects the enzyme reaction rate. Average errors in kinetic parameters were ±4% for K_m and ±3% for V_{max}.

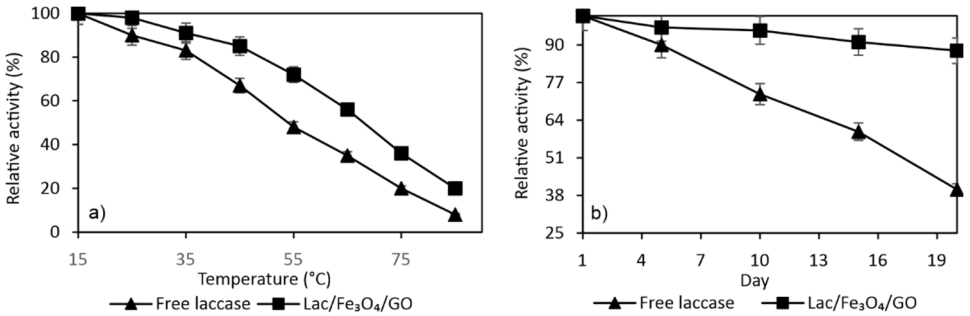


Fig. 6. Temperature dependences of thermal stability of free laccase and Lac/Fe_3O_4/GO (a) in Phosphate buffer (100 mM, pH = 5) by the ABTS aerobic oxidation reaction for 2 h; storage stability of free laccase and Lac/Fe_3O_4/GO (b) in Phosphate buffer (100 mM, pH = 5) determined by the ABTS aerobic oxidation reaction at 4 °C. Experiments were performed in triplicate. The error bars were calculated (5%)

The activity of Lac/Fe_3O_4/GO decreased more slowly than that of free laccase (Fig. 6a). At 55 °C, the residual activities of free laccase and Lac/Fe_3O_4/GO were 48% and 72%, respectively. If an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. The free laccase and Lac/Fe_3O_4/GO were stored in phosphate buffer (100 mM, pH = 5) at 4 °C for up to 20 days, and their activities were determined periodically to evaluate their storage stability. The activity of the free enzyme decreased gradually with time and reduced to 40% of its initial activity over a period of 20 days, whereas Lac/Fe_3O_4/GO had 88% residual activity during the same period (Fig. 6b). There was a significant increase in the storage stability of Lac/Fe_3O_4 probably due to the covalent interactions between the multi-layers magnetic graphene oxide nanosheet and laccase, which can reduce enzyme mobility and the conformational changes; thus, resulting in increased stability towards thermal denaturation.

3.4. THE APPLICATION OF Lac/Fe_3O_4/GO FOR CATALYTIC DEGRADATION OF PHENOLS AND REUSABILITY

In batch systems, about, 74, 83, and 88%, of phenol, *p*-chlorophenol, and 2,4-dichlorophenol were degraded by Lac/Fe_3O_4/GO in 8 h (Fig. 7a). The degradation rates

for the oxidation of phenols, measured in terms of time required to oxidize phenols decreased in the following order: 2,4-dichlorophenol > *p*-chlorophenol > phenol. The degradation rate of phenolic compounds decreases after 7 h (Fig. 7a) probably due to the possible accumulation of the degradation products causing inhibition of the degradation of phenols as previously reported [24]. Another reason for the decrease in degradation rates could be related to the decreasing concentration gradient of phenols in the aqueous medium.

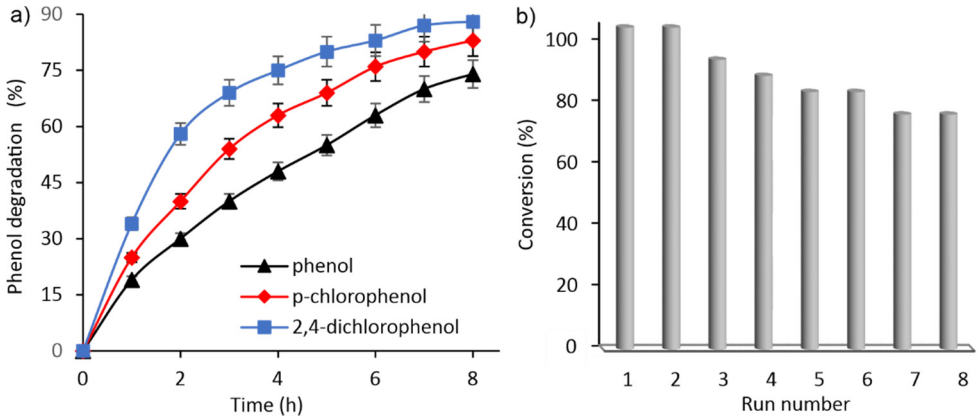


Fig. 7. Degradation rate of phenolic compounds by Lac/Fe_3O_4/GO, and reusability of Lac/Fe_3O_4/GO in degradation of 2,4-dichlorophenol in phosphate buffer (100 mM, pH = 5)

The ability to preserve the catalytic activity of Lac/Fe_3O_4/GO is highly desirable in practical applications. The immobilized laccase on Fe_3O_4-graphene oxide nanocomposite was repeatedly used 8 times for the degradation of 2,4-dichlorophenol (general procedure). After each cycle, Lac/Fe_3O_4/GO was separated with a magnet and washed with PBS solution (100 mM, pH = 5) and the solution was replaced with fresh phenol solution. The activity of Lac/Fe_3O_4/GO decreased by about 12% after the third use in the reactor. Finally, the immobilized enzyme retained more than 70% of its initial activity after 8 consecutive repeated uses (Fig. 7b). The good reusability of Lac/Fe_3O_4/GO indicated that the existence of multilayer graphene oxide–enzyme nanosheets can preserve the laccase activity in a real application case. Based on previously reported mechanisms for the catalytic application of laccase [25], it is proposed that this reaction includes aerobic oxidation of the phenols to the corresponding ketone or acid in the presence of Lac/Fe_3O_4/GO.

4. CONCLUSIONS

One of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme. The extent of stabilization depends on the enzyme structure, the immobilization methods, and the type of support. The laccase from *Trametes*

Versicolor was successfully immobilized onto Fe_3O_4–graphene oxide nanocomposite using glutaraldehyde as a cross-linking reagent and then characterized by various techniques. The optimum conditions for laccase immobilization on the functionalized Fe_3O_4/GO were determined. Compared to free laccase, the stability of Lac/Fe_3O_4/GO in terms of tolerating high temperature, low pH, and long-term storage was improved by up to 37, 25, and 48%, respectively. The catalytic performance of Lac/Fe_3O_4/GO was evaluated by the degradation of phenols. The removal efficiency of phenols by Lac/Fe_3O_4/GO has been achieved up to 88% after 8 h. Therefore, besides possessing the advantage of external magnetic separation, the reuse of immobilized laccase on Fe_3O_4–graphene oxide nanocomposite proposed in this work shows a promising potential application in wastewater treatment.

REFERENCES

[1] SOTO A.M., JUSTICIA H., WRAY J.W., SONNENSCHEIN C., *p-Nonylphenol. An estrogenic xenobiotic released from modified polystyrene*, Environ. Health Perspect., 1991, 92, 167–173.

[2] BUSCA G., BERARDINELLI S., RESINI C., ARRIGHI L., *Technologies for the removal of phenol from fluid streams: a short review of recent developments*, J. Hazard. Mater., 2008, 160, 265–288.

[3] GIANFREDA L., SANNINO F., RAO M.A., BOLLAG J.M., *Oxidative transformation of phenols in aqueous mixtures*, Water Res., 2003, 37, 3205–3215.

[4] KO C.H., CHEN S.S., *Enhanced removal of three phenols by laccase polymerization with MF/UF membranes*, Biores. Techn., 2008, 99, 2293–2298.

[5] STANESCU M.D., FOGORASI M., SHASKOLSKIV B.L., GAVRILAS S., LOZINSKY V.I., *New potential biocatalysts by laccase immobilization in PVA cryogel type carrier*, Appl. Biochem. Biotechn., 2010, 160, 1947–1954.

[6] SAOUDI O., GHAOUAR N., BENSALAH S., OTHMAN T., *Denaturation process of laccase in various media by refractive index measurements*, Biochem. Biophys. Rep., 2017, 11, 19–26.

[7] TRAN D.N., BALKUS K.J., *Perspective of recent progress in immobilization of enzymes*, ACS Catal., 2011, 1, 956–968.

[8] SINGH R.K., TIWARI M.K., SINGH R., HAW J.R., LEE J.K., *Immobilization of Larabinitol dehydrogenase on aldehyde-functionalized silicon oxide nanoparticles for Lxylulose production*, Appl. Microbiol. Biotechn., 2014, 98, 1095–1104.

[9] GAO Z., ZHAROV I., *Large pore mesoporous silica nanoparticles by templating with a nonsurfactant molecule, tannic acid*, Chem. Mater., 2014, 26, 2030–2037.

[10] ZDARTA J., MEYER A.S., JESIONOWSKI T., PINELO M., *A general overview of support materials for enzyme immobilization. Characteristics, properties, practical utility*, Catalysts, 2018, 8, 92–119.

[11] GEIM K., NOVOSELOV K.S., *The rise of graphene*, Nat. Mater., 2007, 6, 183–191.

[12] LI J., ZHANG S., CHEN C., ZHAO G., YANG X., LI J., WANG X., *Removal of Cu(II) and fulvic acid by graphene oxide nanosheets decorated with Fe_3O_4 nanoparticles*, ACS Appl. Mater. Interf., 2012, 4, 4991–5000.

[13] CHANG Q., HUANG J., DING Y., TANG H., *Catalytic oxidation of phenol and 2,4-dichlorophenol by using horseradish peroxidase immobilized on graphene oxide/Fe_3O_4*, Molecules, 2016, 21, 1044–1054.

[14] OZYILMAZ G., *The effect of spacer arm on hydrolytic and synthetic activity of Candida rugosa lipase immobilized on silica gel*, J. Mol. Catal. B, Enzym., 2009, 56, 231–236.

[15] ROUHANI S., ROSTAMI A., SALIMI A., POURSHIANI O., *Graphene oxide/$CuFe_2O_4$ nanocomposite as a novel scaffold for the immobilization of laccase and its application as a recyclable nanobiocatalyst for the green synthesis of arylsulfonyl benzenediols*, Biochem. Eng. J., 2018, 133, 1–11.
[16] ROUHANI S., ROSTAMI A., SALIMI A., *Preparation and characterization of laccases immobilized on magnetic nanoparticles and their application as a recyclable nanobiocatalyst for the aerobic oxidation of alcohols in the presence of TEMPO*, RSC Adv., 2016, 6, 26709–26718.
[17] HUMMERS W.S., OFFEMAN R.E., *Preparation of graphitic oxide*, J. Am. Chem. Soc., 1958, 80, 1339.
[18] BRADFORD M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*, Anal. Biochem. 1976, 72, 248–254.
[19] CHANDRIKA G.P., *Crosslinking of enzymes for improved stability and performance*, Cur. Opin. Biotechn., 1999, 10, 331–335.
[20] PATEL S.K., CHOI S.H., KANG Y.C., LEE J.K., *Eco-friendly composite of Fe_3O_4-reduced graphene oxide particles for efficient enzyme immobilization*, ACS Appl. Mater. Interf., 2017, 9, 2213–2222.
[21] MARIA-CHONG A.S., ZHAO X.S., *Functionalization of SBA-15 with APTES and characterization of functionalized materials*, J. Phys. Chem. B, 2003, 107, 12650–12657.
[22] PATILA M., KOULOUMPIS A., GOURNIS D., RUDOLF P., STAMATIS H., *Laccase-functionalized graphene oxide assemblies as efficient nanobiocatalysts for oxidation reactions*, Sensors, 2016, 16, 287–301.
[23] KUMAR S., HAQ I., PRAKASH J., RAJ A., *Improved enzyme properties upon glutaraldehyde cross-linking of alginate entrapped xylanase from Bacillus licheniformis*, Int. J. Biol. Macromol., 2017, 98, 24–33.
[24] RUSSO M.E., GIARDINA P., MARZOCCHELLA A., SALATINO P., SANNIA G., *Assessment of anthraquinone-dye conversion by free and immobilized crude laccase mixtures*, Enzyme Microb. Techn., 2008, 42, 521–530.
[25] KUNAMNENI A., PLOU F.J., BALLESTEROS A., ALCALDE M., *Laccases and their applications. A patent review*, Rec. Pat. Biotechn., 2008, 2, 10–24.