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ORIGINAL RESEARCH ARTICLE

Rhus vernicifera Laccase Immobilization on Magnetic Nanoparticles to Improve Stability and Its Potential Application in Bisphenol A Degradation

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Abstract In the present study, *Rhus vernicifera* laccase (*Rv*Lac) was immobilized through covalent methods on the magnetic nanoparticles. Fe_2O_3 and Fe_3O_4 nanoparticles activated by 3-aminopropyltriethoxysilane followed with glutaraldehyde showed maximum immobilization yields and relative activity up to 81.4 and 84.3% at optimum incubation and pH of 18 h and 5.8, respectively. The maximum *Rv*Lac loading of 156 mg/g of support was recorded on Fe_2O_3 nanoparticles. A higher optimum pH and temperature of 4.0 and 45 °C were noted for immobilized enzyme compared to values of 3.5 and 40 °C for free form, respectively. Immobilized *Rv*Lac exhibited better relative activity profiles at various pH and temperature ranges. The immobilized enzyme showed up to 16-fold improvement in the thermal stability, when incubated at 60 °C, and retained up to 82.9% of residual activity after ten cycles of reuses. Immobilized *Rv*Lac exhibited up to 1.9-fold higher bisphenol A degradation efficiency potential over free enzyme. Previous reports have demonstrated the immobilization of *Rv*Lac on non-magnetic supports. This study has demonstrated that immobilization of *Rv*Lac on magnetic nanoparticles is very efficient especially for achieving high loading, better pH and temperature profiles, and thermal- and solvents-stability, high reusability, and higher degradation of bisphenol A.

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Keywords Bisphenol A · Enzyme immobilization · Magnetic nanoparticle · Reusability · *Rhus vernicifera* laccase · Stability

Introduction

Biocatalysts have been widely used for biotransformation applications such as industrial and environmental sectors [1, 2]. The enzymes as cell-free biocatalysts are founded more desirable to use in bioconversion reactions due to their high specificity towards substrate and reaction rate, easy in product separation, and tolerance towards higher substrate concentration, and solvents [3–5]. Primarily, the uses of the enzyme are limited due to their high cost and low stability. Various strategies have been employed to improve enzyme properties through the selection of suitable microbial sources, protein engineering, and immobilization [6–9]. The immobilization of biocatalysts in the form of either whole-cells or enzymes has been widely demonstrated to enhance their stability during biotransformation [10–14]. The enzyme's properties such as activity and stability are significantly varied after immobilization on supports [11, 15]. Numerous methods have been evaluated for immobilization, including (1) adsorption on solid supports or polymers [16, 17], (2) encapsulation or entrapment within a polymeric matrix [10], or as metal-protein hybrids [18–20], (3) cross-linking through aggregation by cross-linker such as glutaraldehyde [21], and (4) covalent through functional groups binding [22, 23]. The extent of immobilization is largely dependent upon enzymes properties such as purity, size, surface charge, and their conformation, and the supports properties such as size, surface area, morphology, porous nature, and functional groups on their surface for the binding [21–24].

Additionally, the immobilization conditions such as a buffer (pH), temperature, and time of incubation are also proved effective in the course of immobilization [3, 25].

Various kinds of solid supports have been used for the immobilization of enzymes such as activated carbon [10], chitosan [26], rice straw [27], silica [23], tin oxide [3], and composite particles [17, 22]. For effective immobilize enzymes on supports, the loading of enzymes and high reusability are important aspects that are highly dependent on the structural properties of the supports such as surface area and their rigidity [11, 28]. A significant development in nanotechnology is leading to synthesize unique kinds of nanomaterials with controlled uniform morphology and high surface area that can positively influence the properties of the enzyme on immobilization [21, 23]. The magnetic nature of supports such as iron oxides (Fe_2O_3, and Fe_3O_4), and their composites have a beneficial influence of easy recovery or separation from the reaction by the simple use of the external magnetic field over non-magnetic supports [17, 21, 29].

Commercially available enzymes such as glucose oxidase [21], horseradish peroxidase [17], lipase [23], and laccase [29, 30] have been widely demonstrated for their immobilization on nanomaterials. Laccase is a multi-copper oxidase that catalyzes the oxidation of phenolics, and non-phenolics compounds [10, 31, 32]. Laccases are important enzymes due to their broad biotechnological applications: (1) biodegradation and bioremediation in (a) paper and pulp industry, (b) textile and food processing industry, (2) biosensor, and (3) biofuel production [8, 10, 21, 22, 33]. The immobilization of laccases is required for improving the process economy through enhanced activity, stability, and reusability [10, 28]. *Rus vernicifera* and bacterial laccases exhibit quite similar redox potential ~ 400 mV [34, 35]. In contrast, fungal laccases show higher redox potential between 470 and 810 [35]. Laccase immobilization on various kinds of supports has been established, including silica [24], kaolinites [10], membranes [36], and sepiolite [37]. Although numerous kinds of supports have been used to immobilize laccase, still there is a need for efficient supports to retain high loading, improved stability, and reusability for a potential application. Laccase from *Trametes versicolor* has been largely studied for immobilization [10, 24, 38]. A few reports have been noted for immobilization of *R. vernicifera* laccase (*Rv*Lac) on supports, including chitosan [26, 39], nylon membrane [16], sepiolite [37], and zirconium chloride [28]. In this study, *Rv*Lac immobilization on magnetic nanoparticles Fe_2O_3, and Fe_3O_4 functionally activated by 3-aminopropyltriethoxysilane (APTES) followed with glutaraldehyde was evaluated to improve loading and enzyme properties. The covalently immobilized *Rv*Lac on F_3O_4 magnetic nanoparticles showed improved stability and kinetic properties over free enzyme and exhibited high reusability. Further, the potential application of immobilized laccase was demonstrated for the degradation of bisphenol A.

Materials and Methods

Chemicals and Materials

Laccase (*R. vemicifera*), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), APTES, glutaraldehyde, and fluorescein isothiocyanate (FITC) were procured from Sigma-Aldrich, USA. Fe_2O_3 (average size of 30–50 nm and surface area of 25–35 m^2/g) and F_3O_4 (average size of 50–100 nm and surface area of 6–8 m^2/g) particles were obtained from Nanostructured and Amorphous Materials, Inc., USA. All other chemicals and reagents from commercial suppliers were used of analytical grade.

Nanoparticles Functional Activation and Enzyme Immobilization

The functional modification of magnetic particles by APTES (2%, v/v) in toluene followed with glutaraldehyde (2%, v/v) in phosphate buffer (50 mM, pH 7) was carried out at room temperature as described previously [8, 40]. The immobilization of *Rv*Lac was performed through covalent methods on supports at an enzyme loading of 50 mg/g of supports at 4 °C for incubation up to 30 h with the agitation of 90 rpm. After enzyme immobilization, particles were recovered through centrifugation (Gyrozen 1580R, Republic of Korea) at 10,000 rpm for 10 min [41], and washed twice by a buffer. The concentration of protein was measured through the Bradford method in the supernatant [24]. Further, the influence of enzyme loading (50–300 mg/g of supports) was performed to improve immobilization. The immobilization yield (IY) and relative efficiency (RE) were calculated as follows: equation 1 [17], and equation 2 [22].

$$\text{IY}\ (\%) = \text{ratio of the amount of immobilized and initially added } Rv\text{Lac} \times 100 \quad (1)$$

$$\text{RE}(\%) = \text{ratio of immobilized and free } Rv\text{Lac activity} \times 100 \quad (2)$$

RvLac Activity Measurements

Enzyme activity was measured using the oxidation of ABTS through spectrophotometrically at 420 nm

(ε_{max} = 3.6 × 10^4/M × cm) [21]. Unit (U) of activity represents the amount of enzyme required to oxidize one µmol of ABTS per minute under standard conditions.

Characterization of Immobilized *Rv*Lac

The influence of process parameters pH [2.5–6.0: 2.5 (glycine–HCl), 3.0–3.5 (sodium-citrate), and 4.0–6.0 (sodium-acetate)] and temperature (25–70 °C) on the activity of free and immobilized *Rv*Lac was evaluated using ABTS. Kinetic parameters (K_m and V_{max}) were measured by varying ABTS concentrations 0.01–10.0 mM at optimum pH for free and immobilized *Rv*Lac in 50 mM buffer at 25 °C [17].

Stability and Reusability

The stability of the free or immobilized *Rv*Lac was evaluated at a higher temperature of 60 °C by measuring residual enzyme activity over different time intervals at optimum pH. Further, the reusability of immobilized *Rv*Lac was assessed up to ten cycles using ABTS. The immobilized enzyme was recovered through centrifugation at 10,000 rpm for 10 min and used to the next cycle of reaction. The initial *Rv*Lac activity was considered as 100%.

Solvents Stability and Degradation of Bisphenol A

The stability of free and immobilized *Rv*Lac on Fe_2O_3 nanoparticles was compared in various solvents (25%, v/v) based buffer reaction at optimum pH, including methanol, ethanol, propanol, acetone, and benzene for 4 h incubation at 25 °C. The bisphenol A degradation was evaluated in the flask with a working volume of 10 ml containing bisphenol A (50–125 µM) and free (pH 3.5, sodium-citrate) or immobilized *Rv*Lac (pH 4.0, sodium-acetate) on Fe_2O_3 (1 U/ml) in the buffer (50 mM) for 12 h of incubation at room temperature (25 °C) under shaking of 100 rpm. Thereafter, the reaction was halted by adding a few drops of concentrated hydrochloric acid. The residual bisphenol A amount was measured via 4-AAP coupled reaction spectrophotometrically (506 nm). The bisphenol A degradation efficiency was calculated as follows (Eq. 3) [42]:

$$\text{Degradation efficiency } (\%) = \text{ratio of degraded to initially added bisphenol A} \times 100 \quad (3)$$

Instrumental Measurements

All reactions assay absorption spectra were measured spectrophotometrically (Varian Cary 100 Bio UV–Vis spectrophotometer, USA) [43, 44]. The decomposition analysis of particles was measured using thermogravimetric analysis (TGA) [45]. Confocal laser scanning microscopy (CLSM) analysis was performed using FITC-labeled laccase immobilize on Fe_2O_3 nanoparticles by FV-1000 Olympus confocal microscope, Olympus, Japan [21]. All experiments were carried out in triplicate.

Results and Discussion

Immobilization of *Rv*Lac on Magnetic Nanoparticles

The illustration of the enzyme immobilization process on the magnetic particle is presented in Fig. 1. Immobilization of *Rv*Lac on magnetic supports occurs through covalent bonding between the basic amino acids such as lysine of enzyme and glutaraldehyde groups on the surface of the particles [22]. Initially, the immobilization profile was evaluated up to 30 h to measure the desirable incubation for the efficient immobilization of *Rv*Lac on functionally activated magnetic nanoparticles (Fig. 2a, b). The IY was increased with an increase in the incubation up to 18 h on nanoparticles. Thereafter, immobilization was stabilized up to 30 h of incubation with a maximum IY of 81.8 and 81.2% on Fe_2O_3 and Fe_3O_4 nanoparticles, respectively. The maximum RE of 84.3 (22.3 U/mg of protein) and 71.2% (18.8 U/mg of protein) were noted at the incubation of 18 h for Fe_2O_3 and Fe_3O_4 nanoparticles, respectively. A higher optimum IY and RE of 81.4 and 84.3% were observed on Fe_2O_3 for *Rv*Lac over the values of 77.2 and 71.2% on Fe_3O_4, respectively. Previously, up to ~ 200-fold lower IY (0.4%) was noted on chitosan for *Rv*Lac immobilization [39]. Similarly, *Rv*Lac immobilization on sepiolite and sepiolite modified with chitosan and copper showed significantly lower IY of 52, 55, and 62%, respectively [37]. A lower IY and RE of 56 and 36% were recorded on the chitosan microsphere [26]. These results suggested that the immobilization of *Rv*Lac is more efficient on magnetic particles over previous reports on sepiolite and chitosan [26, 39]. Previously, *Rv*Lac immobilization of magnetic (Fe_2O_3 or Fe_3O_4) particles has not been reported. A lower IY of 49.0 and 68.7% were shown on Fe_3O_4 chemically modified with glutaraldehyde and silica coating for laccase from *T. versicolor* [17, 38]. The loading of enzymes is an important criterion to improve process efficiency [5, 28]. Therefore, the concentration of enzyme was increased up to 300 mg/g of support for immobilization (Fig. 2c). The amount of enzyme was increased from 40.7 to 156 mg/g of support on Fe_2O_3 as compared from 38.6 to 128 mg/g of support on F_3O_4 nanoparticles on increasing concentration from 50 to 300 mg/g of supports. In contrast, the higher loading led to

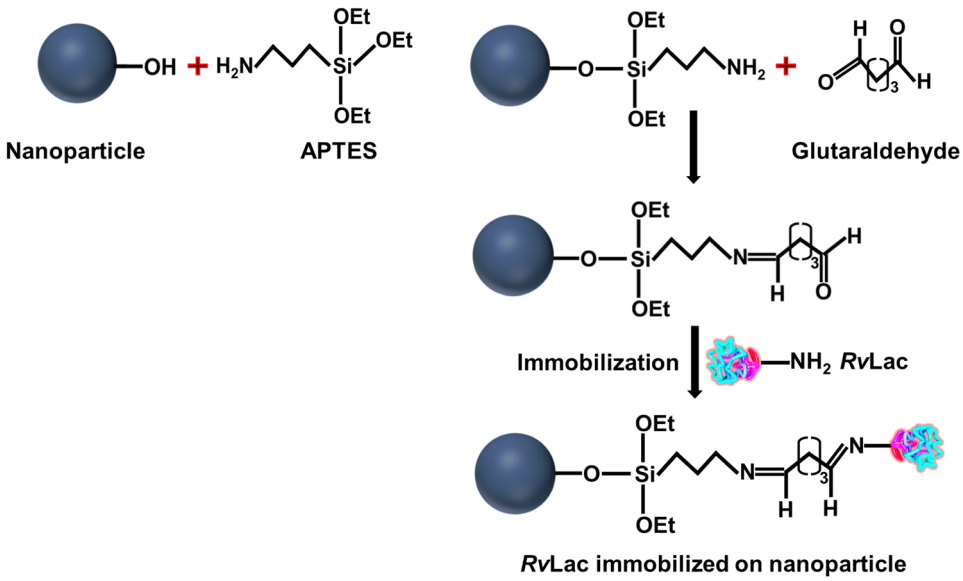

Fig. 1 Illustration of activation of magnetic nanoparticles by 3-aminopropyltriethoxysilane (APTE) followed with glutaraldehyde and immobilization of *Rv*Lac

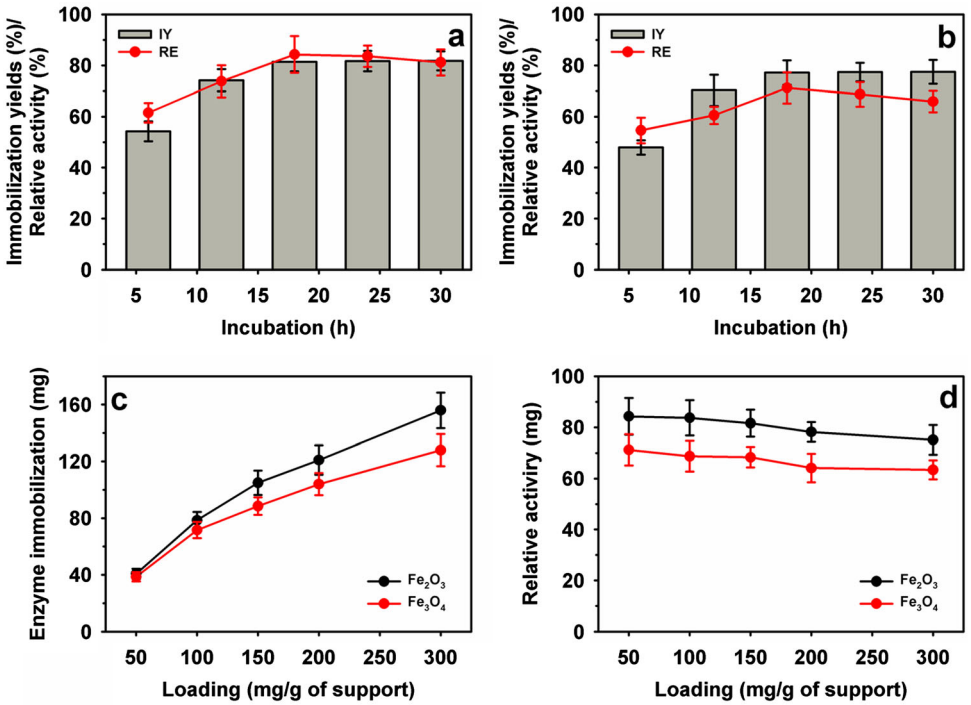

Fig. 2 Immobilization of *Rv*Lac on the magnetic nanoparticles: time profile on **a** Fe_2O_3 and **b** Fe_3O_4 nanoparticles, and **c**–**d** enzyme loading

lower RE of 75.1 and 63.4% on Fe_2O_3 and F_3O_4 nanoparticles, respectively (Fig. 2d). Previously, a significantly lower loading of 32.3 mg/g of support was used for the immobilization of *Rv*Lac on zirconium chloride nanoparticles [28]. *Rv*Lac after immobilization on the nylon membrane exhibited much lower activity of ~ 2.8% over free enzyme (9.6 µmol/min) as a control [16]. Similarly, laccase immobilization on Fe_3O_4 and Fe_3O_4@MoS_2 core–shell composite nanoparticles chemically modified with polyethyleneimine (PEI) showed lower 70 and 120 mg/g of supports, respectively [29]. In contrast, a significate lower loading up to 14.2 g/g of supports for *T. versicolor* laccase reported on graphene oxide/$CuFe_2O_4$ nanocomposites [46]. Similarly, *Rv*Lac immobilization noted more efficient over maximum loading of 17.3 and 82.6 mg/g of supports for *Bacillus subtilis*-derived laccase on Fe_3O_4 and magnetic carbon chemicals (MLC) particles, respectively [5]. Immobilization of enzymes on nanomaterials is highly influenced by particle size as well as surface area [17, 22, 24]. The higher immobilization of *Rv*Lac on Fe_2O_3 nanoparticles can be correlated with smaller size and ~ 4.4-fold higher surface area over Fe_3O_4

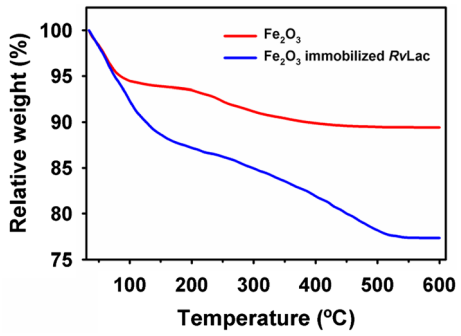

Fig. 3 Thermogravimetric profiles pure and *Rv*Lac immobilized Fe_2O_3 nanoparticles

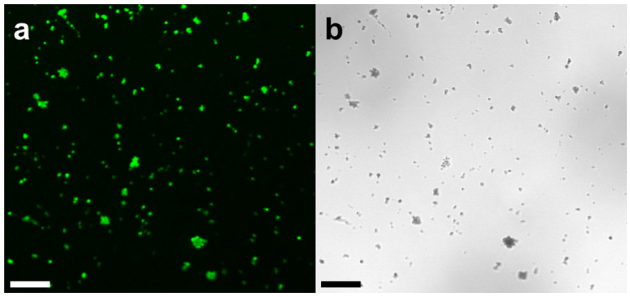

Fig. 4 Confocal laser scanning microscopy images of immobilized RvLac on Fe_2O_3 nanoparticles in **a** green and **b** bright-field (scale bar is 0.5 μm)

nanoparticles. The efficient immobilization of *Rv*Lac on Fe_2O_3 nanoparticles was confirmed by TGA measurements (Fig. 3). The high loading of *Rv*Lac on Fe_2O_3 nanoparticles correlated with a significant reduction of relative weight loss to 77.3% as compared to 89.4% for control particles. Further, the visualization of green fluorescence of FITC labeled laccase immobilized on Fe_2O_3 nanoparticles under CLSM analysis confirmed immobilization (Fig. 4).

Characterization of Immobilized *Rv*Lac

The physiological properties such as pH and temperature profiles of the enzymes are highly influenced after immobilization on the nanoparticles [3, 5, 37]. Therefore, the activity profiles of free and immobilized *Rv*Lac on magnetic nanoparticles were compared (Fig. 5a). The maximum activity of free *Rv*Lac of 26.4 U/mg of protein (100%) was noted at pH 3.5. At lower and higher pH of 2.5 and 6.0 free enzyme retained relative activity of 54.3 and 4.2%, respectively. After immobilization, a shift in pH optimum from 3.5 to 4.0 was recorded on both the nanoparticles. *Rv*Lac showed intermediate pH optima of 3.5 as compared to 3.0 for *T. versicolor* and 5.0 for *B. subtilis* towards ABTS [5, 17]. Immobilized *Rv*Lac exhibited a broader pH profile with a higher pH above 4.0 as compared to free form. At pH 6.0, immobilized *Rv*Lac on Fe_2O_3 and Fe_3O_4 particles revealed 3.9- and 3.3-fold higher relative activities to free enzyme (4.2%). In contrast, a similar pH optimum value of 7.0, 7.5, 7.5, and 7.5 noted for free and immobilized *Rv*Lac on chitosan [39], polypropylene membrane [36], nylon membrane [16], and zirconium chloride supports [28], respectively. Previously, a quite similar shift in pH profile was noted for Fe_3O_4@-MoS_2@PEI-facilitated laccase [29]. In contrast, *T. versicolor* laccase immobilized on Fe_3O_4 derived nanoparticles such as poly(amidoisophthalicacid)-coated (Fe@PA), cyclodextrin-anchored (Fe@-PA-CD), and chitosan-coated (Fe@PA-CD-Cs) supports exhibited quite similar pH profiles to free enzyme [47]. Similarly, *B. subtilis*-based laccase showed similar pH optima after immobilization on MLC [5].

*Rv*Lac showed an optimum temperature of 40 °C with an activity of 61.8 U/mg of protein (100%) (Fig. 5b). At higher temperatures, a significant decrease in the relative activity to 12.6% and 6.1% noted at 60 and 70 °C, respectively. After immobilization, a shift in temperature from 40 to 45 °C was recorded on magnetic nanoparticles. At a temperature of 70 °C, immobilized *Rv*Lac on Fe_2O_3 and Fe_3O_4 particles retained a much higher relative activity of 38.4 and 27.9% as compared to a free enzyme (6.1%), respectively. A quite similar shift in temperature profile was noted after the immobilization of *B. subtilis* laccase on MLC supports [5]. In contract, immobilized *Rv*Lac on chitosan, and Zirconium chloride supports showed optimum temperature 45 and 40 °C, respectively, which were similar to their free forms of the enzyme [26, 28]. Also, no shift in the temperature optima was noted for *T. versicolor* laccase immobilized on various magnetic supports, including Fe@PA, Fe@-PA-CD, and Fe@PA-CD-Cs [47]. Overall, a shift in pH and temperature optima of immobilized *Rv*Lac might be associated with a strong binding of the enzyme to supports that can lead to desirable changes in confirmation of the enzyme [23, 32, 48].

The K_m and V_{max} (apparent) values of *Rv*Lac observed 1.69 mM and 68.1 μmol/min/mg protein for ABTS, respectively (Table 1). After immobilization, *Rv*Lac showed quite a similar substrate affinity (K_m) value of 1.84 mM, and a V_{max} value of 61.7 μmol/min/mg protein compared to free enzyme. Previously, *T. versicolor* laccase immobilized on Fe_3O_4 nanoparticles was showed 2.2-and 1.6-fold lower K_m (0.06 mM) and V_{max} (1140 μmol/min/mg protein) as compared to free enzyme towards ABTS, respectively [17]. In contrast, laccase was exhibited a 2.2-fold lower V_{max} (26 mM/min) towered ABTS without a significant change in K_m (1.8 mM) after immobilization on GO-$CuFe_2O_4$ particles [46]. *Rv*Lac immobilized on the nylon membrane was showed a 35.5-fold lower V_{max} for quinone over free enzyme (9.58 μmol/min) [16]. In

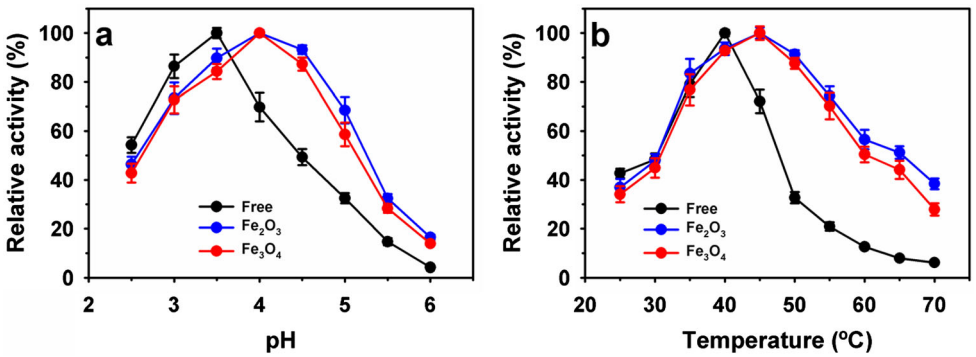

Fig. 5 Activity profiles of free and immobilized *Rv*Lac on the magnetic nanoparticles: **a** pH at 25 °C and **b** temperatures at optimum pH values

Table 1 Kinetic parameters of free and immobilized *Rv*Lac

*Rv*Lac	K_m (mM)	V_{max} (μmol/min/mg protein)
Free	1.69 ± 0.41	68.1 ± 6.6
Fe_2O_3 immobilized	1.84 ± 0.40	61.7 ± 5.8

contrast, a quite similar kinetic parameter (K_m and V_{max}) was reported for free and immobilized *B. subtilis* laccase on MLC [5]. These reductions in kinetic parameters might be associated with the strong attachment between enzyme and support that results in diffusion limitation or undesirable conformational changes within enzyme after immobilization on these supports [16, 17, 46].

Thermal stability and Reusability of Immobilized *Rv*Lac

Immobilization of the enzymes on supports is primarily carried out to improve their stability over the free form of the enzyme. The gain in the stability might be not directly dependent on the immobilization methods or a kind of supports [21, 36, 39, 48]. Therefore, the thermal stability of covalently immobilized *Rv*Lac was compared to free enzyme at a high temperature of 60 °C for incubation up to 60 min (Fig. 6a). Free enzyme lost ∼ 80% of residual activity within 30 min of incubation, whereas the residual activity decreased to 3.1% at a higher incubation of 60 min. On the other hand, immobilized *Rv*Lac showed more than 80% of the activity at 30 min of incubation at 60 °C. After 60 min of incubation, immobilized *Rv*Lac on Fe_2O_3 and Fe_3O_4 particles exhibited high residual activity of 61.3 and 55.8%, respectively. Previously, under similar conditions, *Rv*Lac immobilized on chitosan showed lower residual activity of 40.1% [39]. After immobilization on Fe_2O_3 and Fe_3O_4 particles, the thermal stability enhancement of 19.8- and 18.0-fold was noted at 60 °C as compared to the free enzyme. Previously, a significant lower enhancement of stability of ∼ 2.7-fold was noted for immobilized *Rv*Lac on polypropylene membrane chemically modified with chromic acid at 60 °C for incubation of 150 min [36]. In contrast, *B. subtilis*-derived laccase exhibited a much lower improvement of 1.6-fold at a similar temperature [5]. *Rv*Lac showed half-life ($t_{1/2}$) of ∼ 13 min at 60 °C as compared to $t_{1/2}$ values of 12 min for *T. versicolor* [17], and ∼ 20 min for bacterial laccase from γ-Proteobacterium JB [49]. The immobilized laccase on the chicken feather showed $t_{1/2}$ activity of 134 min over free enzyme (109 min) at 60 °C [30]. In contrast, free and immobilized bacterial laccase from γ-Proteobacterium JB on nitrocellulose membrane showed similar temperature stability at 60 °C [49].

Immobilization of enzymes on magnetic supports exhibits the benefits of easy separation using magnet over non-magnetic based supports [29]. The better reusability of immobilized enzymes primarily demonstrates the beneficial influence contribution toward the process economy [47, 48]. The reusability of *Rv*Lac immobilized on magnetic nanoparticles was evaluated for the oxidation of ABTS (Fig. 6b). After five- and ten-cycles of reuses, Fe_2O_3 immobilized *Rv*Lac retained residual activity of 93.2, and 82.9%, respectively. Under similar conditions, *Rv*Lac immobilized Fe_3O_4 showed slightly lower residual activity of 85.2 and 75.4%, respectively. The decline in residual activity towards successive cycles might be associated with partial inactivation of enzyme or leaching [19, 21]. Previously, Fe_3O_4@MoS_2@PEI immobilized laccase showed lower residual activity of 62.0% after ten cycles of reuses [29]. Magnetic nanoparticles Fe@PA, Fe@-PA-CD, and Fe@PA-CD-Cs immobilized *T. versicolor* laccase retained significantly lower residual activity up to ∼ 40.0% [47]. In contrast, metal-protein hybrids using cooper and zinc metal ions and laccase showed much lower reusability of 16.5 and 43.7% after ten cycles of reuses, respectively [42]. Here, the lower reusability associated with structural instability of metal-protein hybrids during the process over better structural rigidity maintained by nanoparticles that led to high reusability to magnetic nanoparticles based enzyme immobilization system. The cumulative relative

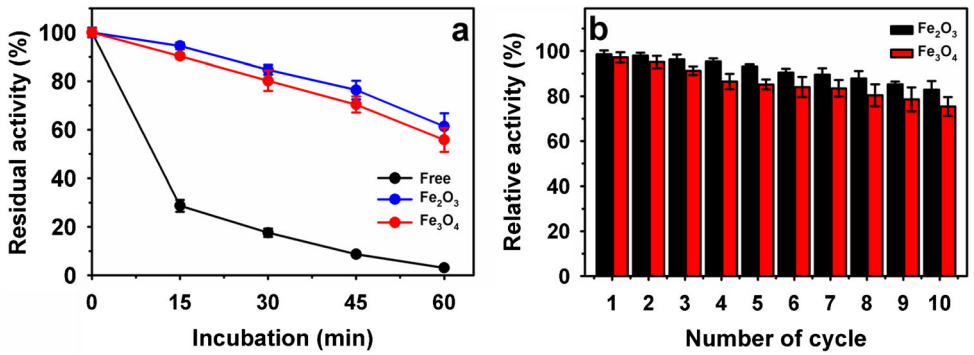

Fig. 6 **a** Thermal stability of free and immobilized *Rv*Lac on the magnetic nanoparticles at 60 °C and **b** reusability

activity of 918% retained by covalently immobilized *Rv*Lac on Fe_2O_3 after ten cycles of reuses, these results indicated that immobilization will be 7.7-fold cost-effective compared to uses of free enzyme activity. Under similar conditions, 2.4-fold cost–benefit was estimated for Fe_3O_4 nanoparticles immobilized laccase from *T. versicolor* through adsorption [17]. Overall, *Rv*Lac immobilized on Fe_2O_3 nanoparticles exhibited better IY, RE, and reusability than previous reports of laccase immobilization on various matrices (Table 2).

Solvent Stability and Bisphenol A degradation

The phenolics compounds exhibit low solubility in water and require a solvent system in a few cases. Laccase shows a wide range of specificity towards phenolics substrate [17, 50]. After incubation in various solvents, *Rv*Lac exhibited a significant decline in the residual activity to the ranges of 8.9–68.7% with the order propanol > ethanol > methanol > benzene > acetone (Fig. 7a). Immobilized *Rv*Lac on Fe_2O_3 nanoparticles retained superior residual activity in the presence of solvents and showed higher solvents tolerance up to 8.5-fold as compared with the free enzyme. This result suggested that immobilized *Rv*Lac can be potentially applied for a solvent-based reaction system [21, 39]. Bisphenol A is a commonly used substrate in the industrial process, including plastics and resins [21]. The generation of a large quantum of bisphenol A as waste material is leading to environmental problems due to their toxicity towards aquatic fauna [5, 42]. The *Rv*Lac resulted in the degradation efficiency of 63.8% at bisphenol A concentration of 50 µM (Fig. 7b). Thereafter, an increase in the concentration up to 125 µM led to a decline in degradation efficiency to 38.1%. In contrast, immobilized *Rv*Lac on Fe_2O_3 nanoparticles showed significantly higher bisphenol A degradation efficiency of 84.9 and 72.2% at a concentration of 50 and 125 µM, respectively. After the immobilization of *Rv*Lac on Fe_2O_3 nanoparticles, bisphenol A degradation efficiency improved up to 1.9-fold. Previously, lower bisphenol A degradation of 53.0 and 68% reported for *T. versicolor* laccase immobilized on magnetic nanoparticles Fe@PA and Fe@-PA-CD, respectively [47]. Similarly, a lower enhancement of 50% was noted for *B. subtilis*-derived laccase immobilized on magnetic carbon nanocarriers [5]. In contrast, 1.3- and 1.5-fold higher degradation of bisphenol A over copper and

Table 2 Immobilization of laccase on different matrices

Matrix	Method	Immobilization yield (%)	Relative efficiency (%)	Reusability (%)	References
Chitosan microsphere	Adsorption	70.0	45.0	–	[26]
Graphene oxide/$CuFe_2O_4$	Covalent	14.2[a]	88.0	80.0	[46]
Nylon membrane	Covalent	–[b]	2.8	–	[16]
Water–soluble chitosan	Adsorption	56.0	30.0	80.0	[26]
Zirconium chloride	Adsorption	32.3[b]	–	–	[28]
Fe_3O_4	Adsorption	49.0	45.3	21.3	[17]
	Adsorption	4.7	80.0	–	[29]
Fe_3O_4@MoS_2	Adsorption	8.0	90.0	62.0	[29]
$SrFe_{12}O_{19}$	Covalent	66.5	42.7	–	[22]
$Y_3Fe_5O_{12}$	Covalent	68.7	46.9	–	
Fe_2O_3	Covalent	81.4	84.3	82.9	This study

[a]Amount of enzyme immobilization in mg/g of support

[b]Not available or applicable

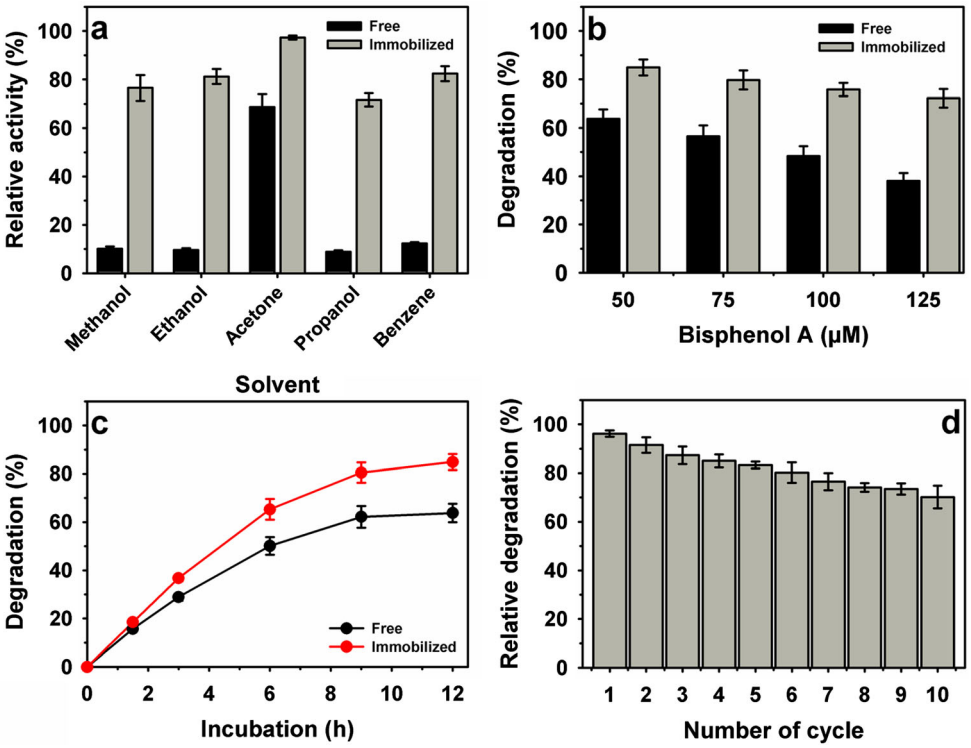

Fig. 7 Free and immobilized *Rv*Lac on Fe_2O_3 nanoparticles: **a** solvent stability (25%, v/v), **b** bisphenol A degradation, 50 μM bisphenol A degradation **c** time profile, and **d** immobilized enzyme reusability for incubation of 6 h each cycle

zinc-based metal-protein hybrids, respectively [42]. Remarkably, a low enhancement of ~ 3.0% was recorded by Fe_3O_4@MoS_2@PEI immobilized laccase over free enzyme [29]. The degradation of bisphenol A increased sharply up to 9 h of incubation followed by stabilization at 12 h (Fig. 7c). After ten cycles of reuses, immobilized *Rv*Lac retained a high relative degradation efficiency of 70.2% (Fig. 7d). In contrast, a low bisphenol A degradation efficiency of 13.0% was maintained for fungal laccase from *T. versicolor* immobilized on Fe@-PA-CD [47]. On the other hand, ~ 60% bisphenol A degradation was recorded for magnetic carbon nanocarriers immobilized bacterial laccase from *B. subtilis* under similar recycling conditions [5].

Conclusion

In conclusion, this study reports covalent immobilization of *Rv*Lac on magnetic nanoparticles Fe_2O_3 and Fe_3O_4 modified with APTES followed by glutaraldehyde. The *Rv*Lac immobilization was established more efficiently on F_2O_3 nanoparticles due to smaller size and high surface area compared to Fe_3O_4 nanoparticles. After immobilization, the enzyme exhibited better relative activity profiles at high pH and temperatures, and significantly improved thermostability as compared to free form. Immobilized *Rv*Lac on Fe_2O_3 particles retained high reusability and showed higher bisphenol A degradation. Previous studies have reported *Rv*Lac immobilization on non-magnetic supports. This developed magnetic nanoparticles-based biocatalyst can be used efficiently for other kinds of potential biotechnological applications.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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