



Enzymatic treatment of phenolic pollutants by a small laccase immobilized on APTES-functionalised magnetic nanoparticles

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Abstract

In this study, we have successfully synthesized magnetic nanoparticles (MNPs), functionalised them by silanization and used them for the covalent immobilization of a recombinant small laccase (rSLAC) from *Streptomyces coelicolor*. The immobilized recombinant laccase (MNP-rSLAC) was subsequently used for the treatment of phenol, 4-chlorophenol (4-CP) and 4-fluorophenol (4-FP). The enzyme completely degraded 80 µg/mL of the selected phenolic compounds within 2 h in the presence of a natural mediator, acetosyringone. The MNP-rSLAC retained > 73% of initial activity (2,6-dimethoxyphenol as substrate) after 10 catalytic cycles and could be easily recovered from the reaction mixture by the application of magnetic field. Furthermore, immobilised rSLAC exhibited better storage stability than its free counterpart. The Michaelis constant (K_m) value for the immobilised rSLAC was higher than free rSLAC, however the maximum velocity (V_{max}) of the immobilised SLAC was similar to that of the free rSLAC. Growth inhibition studies using *Escherichia coli* showed that rSLAC-mediated treatment of phenolic compounds reduced the toxicity of phenol, 4-CP and 4-FP by 90, 60 and 55%, respectively. Interestingly, the presence of selected metal ions (Co^{2+} , Cu^{2+} , Mn^{2+}) greatly enhanced the catalytic activity of rSLAC and MNP-rSLAC. This study indicates that immobilized small laccase (MNP-rSLAC) has potential for treating wastewater contaminated with phenolic compounds.

Keyword Bioremediation · Immobilization · Magnetic nanoparticles · Phenolic pollutants · Small laccase

Introduction

The extensive use of phenolic compounds mainly as herbicides, flame retardants, pesticides, biocides and wood preservatives (Dai et al. 2015) has led to their detection in surface water. Removal of these compounds from the contaminated water is necessary due to their low biodegradability (Pera-Titus et al. 2004) and toxicity (Liu et al. 2012)

as well as the pressure to comply with stringent standards against their disposal (Karam and Nicell 1997). Laccases have been extensively investigated as potential candidates for the remediation of several pollutants (Srinivasan et al. 2019, 2020). Laccases are multi-copper oxidases which have been gaining a lot of attention mainly due to their ability to catalyse the oxidation of a broad range of substrates without the aid of any cofactors (Upadhyay et al. 2016). They use molecular oxygen as an electron acceptor during substrate oxidation, leading to the formation of reactive radicals. The radicals may polymerise and precipitate out of the solution which facilitates the removal of the pollutant. Alternatively, they can cleave covalent bonds such as C–C, C–O, sometimes assisted by oxidised mediators, leading to the breakdown of pollutants (Huber et al. 2018; Kudanga et al. 2011). In this sense, laccases, due to their relative promiscuity, can be applied in the bioremediation of several pollutants (Zdarta et al. 2018), including phenolic pollutants. However, there are a number of limitations associated with their use as free enzymes which include large consumption, difficulty in separation from

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the reaction mixture and low recycling capability (Fernandes et al. 2017; Ranjan et al. 2018). Furthermore, low stability and potential contamination of the free enzyme in wastewater (Rotková et al. 2009) restricts the industrial use of free laccase in wastewater treatment plants (WWTPs).

Alternatively, enzyme immobilisation can solve the challenges associated with free enzymes and enables enzyme recovery, reusability and tolerance towards extreme conditions (Gianolini et al. 2020). Enzyme immobilization can be achieved by physical adsorption or covalent bonding (Mohamad et al. 2015). Adsorption is a relatively simple method, however, leaching of the enzyme from the immobilized matrix after a few cycles limits its application (Sheldon and Woodley 2018). In contrast, covalent binding provides an advantage over the adsorption method by stabilising strong bonding between the enzyme and the matrix via a chemical reaction, thereby minimising enzyme leaching (Faridi et al. 2017). Among the several immobilization matrices that have been studied, magnetic nanoparticles (MNPs) have proven to be a promising candidate for the immobilization of enzymes (Ranjan et al. 2018). They possess unique properties such as superparamagnetism, low toxicity, large surface area-to-volume ratio, biocompatibility, and ease of separation by the application of an external magnetic field (Fortes et al. 2017; Mohamed et al. 2017; Pereira et al. 2017; Selvam et al. 2016; Srinivasan et al. 2020). However, formation of agglomerates due to magnetic dipole interactions (Villa et al. 2016) and the unstable nature (in acidic medium) of naked iron magnetic particles, could greatly affect their longevity and reusability.

Functionalisation via chemicals and biological materials could enhance and optimise the surface properties of MNPs such as dispersibility, biocompatibility and biodegradability (Xu et al. 2014). In addition, surface functionalisation provides the anchoring surface which mediates the attachment of functional groups, leading to an engineered surface with a selective affinity towards diverse bio-compounds (Seyed et al. 2015). In one recent study, polydopamine (PDA) coating was used to functionalize Fe_3O_4 nanoparticles for immobilization of a fungal laccase (Zhang et al. 2017). However, the high cost of dopamine could potentially impact its usage in industrial catalysis (Wang et al. 2016). Silanization is a viable alternative owing to its responsivity, high stability, low cytotoxicity as well as the ease of surface chemical transformation. In addition, no specialized equipment is required, making silanization an ideal method for functionalization (Faridi et al. 2017). Despite these favourable properties, silanization has not been used in the immobilization of laccase.

Therefore, the aim of this study was to investigate silanization for surface functionalization of MNPs followed by immobilization of rSLAC onto the functionalized MNPs.

The immobilized rSLAC was then applied in the treatment of selected phenolic pollutants.

Materials and methods

Materials

Phenol, 4-chlorophenol (4-CP), 4-fluorophenol (4-FP), 4-aminoantipyrine (4-AAP), potassium ferricyanide, 2,6-dimethoxyphenol (2,6-DMP) and acetosyringone (AS) were purchased from Sigma-Aldrich (South Africa). Orthophosphoric acid and acetonitrile were purchased from Merck (South Africa). All chemicals were of analytical grade and were used without any further purification. The rSLAC was produced and purified as previously described (Yadav et al. 2018).

Determination of laccase activity and protein concentration

Laccase activity was determined using 2,6-DMP as a substrate, as previously described (Yadav et al. 2018). The oxidation of 2,6-DMP (2 mM) was analysed by measuring the increase in absorbance at 477 nm ($\epsilon = 14,800 \text{ M}^{-1} \text{ cm}^{-1}$) in Tris-HCl buffer (20 mM, pH 8.0). One unit of laccase activity was defined as the amount of enzyme required for the oxidation of 1 μmol of substrate per minute. Protein concentration was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

Synthesis and silanization of MNPs

MNPs were synthesized by a chemical co-precipitation method as previously described (Ranjan et al. 2017). Synthesized MNPs were collected by the application of a magnetic field and washed with ethanol followed by Milli-Q water. Finally, MNPs were dried under vacuum overnight at 60 °C. Surface modification of the dried MNPs was carried out using (3-aminopropyl)-triethoxysilane (APTES) as described by Faridi et al. (2017). One gram of MNPs were dispersed in a mixture of 20% (w/v) APTES and 4 mL glycerol, and the resulting mixture was heated at 90 °C for 2 h under nitrogen sparging and stirring. The mixture was then cooled, sonicated for 10 min and the modified MNPs collected using a magnet followed by washing with ethanol and Milli-Q water and dried overnight under vacuum at 60 °C.

Immobilization of rSLAC on modified MNPs

The carboxyl groups of rSLAC were treated with 3-(3-dimethylaminopropyl) N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) as described by Faridi et al.

(2017). Briefly, 3 mg of EDC was added to a 5 mL solution of rSLAC and the mixture was incubated at room temperature (22 ± 3 °C) for 1 h with gentle shaking followed by the addition of 3 mg of NHS. Thereafter, 10 mg of MNPs were added to the mixture and the incubation was continued for a further 3 h. The binding efficiency of rSLAC onto silanized MNPs was determined by the difference in concentration of the free enzyme after immobilization against the initial enzyme concentration (Faridi et al. 2017).

Characterization of MNPs

The presence of functional groups on the surface of MNPs was assessed by FT-IR spectroscopy (Perkin Elmer, USA). The morphology of MNPs was studied by field emission scanning electron microscopy (FE-SEM) using a Zeiss Gemini instrument (Germany).

Biochemical and kinetic characterization of free and immobilized rSLAC

The optimum pH for the activity of free and immobilized rSLAC was determined by conducting enzyme assays in the pH range 5.0–9.0, in 1.0 pH value increments [20 mM sodium acetate buffer (pH 5.0), 20 mM Tris–HCl buffer (pH 6.0–8.0) and 20 mM glycine–NaOH buffer (pH 9.0)] at 80 °C. Optimum temperature for free and immobilized rSLAC was determined by performing enzyme assays at various temperatures (40 to 100 °C) at pH 8.0. The enzyme activity was presented in terms of relative activity, with the highest catalytic activity set at 100%.

Kinetic parameters (K_m and k_{cat}) were determined using 2,6-DMP (50–2000 μ M) as a substrate for the immobilized rSLAC and compared with the free rSLAC (Yadav et al. 2018). The reactions were carried out in Tris–HCl buffer (20 mM, pH 8.0) and oxidation of 2,6-DMP was determined at 477 nm ($\epsilon = 14,800 \text{ M}^{-1} \text{ cm}^{-1}$). The experiments were performed in triplicate.

Effect of metal ions and organic solvents on the catalytic activity of free and immobilized rSLAC

The effect of different metal ions (Li^+ , Co^{2+} , Cu^{2+} , Mn^{2+} , Ca^{2+} , Mo^{6+} , Al^{3+} , Mg^{2+}) on the catalytic activity of free and immobilized rSLAC was evaluated. Initially, enzymes were pre-incubated in presence of 10 and 30 mM of individual metal ions for 30 min (Sondhi et al. 2014) and thereafter enzyme activity was measured using 2,6-DMP as substrate as previously described. The enzyme activities of free and immobilized rSLAC without metal ions were considered as controls (100% activity). Similarly, the enzyme (free or immobilized) was pre-incubated for 30 min in organic

solvents (20 and 50% of methanol, ethanol, acetone, dioxane and DMSO) and the residual activity determined using 2,6 DMP as substrate. All experiments were performed in triplicate.

Storage stability and reusability of the immobilized rSLAC

The storage stability of the free and immobilized rSLAC was evaluated by storing the enzyme in Tris–HCl buffer (20 mM, pH 8) at 4 °C for 30 days. Sampling was done after every 6 d and the enzyme activity was determined using 2,6-DMP as a substrate. Enzyme activity at time zero was taken as 100% relative activity.

The reusability of the immobilized rSLAC was evaluated by recovering the MNP-rSLAC with a magnet after each cycle and washing three times with Tris–HCl buffer (20 mM, pH 8.0) to prepare for the subsequent cycle. Enzyme activity was determined using 2,6-DMP as the substrate. The enzyme activity for the first cycle was considered as 100% and relative activities of the subsequent cycles were calculated with respect to the activity of the first cycle.

Treatment of phenol, 4-CP and 4-FP by free and immobilized rSLAC

The rSLAC (2 U) was added to a reaction mixture (1 mL total volume) consisting of Tris–HCl buffer (50 mM, pH 8.0), phenols or halogenated phenols (80 μ g/mL each), and AS (8 μ g/mL) as a mediator, and the mixture was incubated for 4 h at 37 °C in the dark with gentle shaking (50 rpm). A negative control was also set in parallel where a denatured enzyme (in case of free rSLAC) and bare MNPs (in case of immobilized rSLAC), were used and incubated under identical conditions. Samples were collected every 10 min to analyse the residual concentrations of phenolic compounds by colorimetric and HPLC analysis. Furthermore, the effects of pH (4.0–8.0), mediator concentrations (2–10 μ g/mL) and reaction time, on the degradation of phenolic compounds (80 μ g/mL each), were studied.

Analysis of the residual phenolic compounds by a colorimetric method

Residual phenolic compound concentrations were measured using a simple colorimetric assay as described by Modaressi et al. (2005). The assay is based on the electrophilic attack of the phenolic compound by a primary amine (4-AAP) under alkaline conditions, and its subsequent oxidation by potassium ferricyanide to a red quinone-type dye (Abdollahi et al. 2018). The reaction mixture consisted of 700 μ L of Tris–HCl buffer (100 mM, pH 8), 300 μ L laccase treated phenolic compounds, 10 μ L 4-AAP (0.1 M) and 10 μ L potassium

ferricyanide solution (0.2 M) and was incubated at 25 °C for 15 min, with gentle shaking (50 rpm). The extent of degradation of the compounds was determined spectrophotometrically as the relative decrease of the maximum absorbance at 510 nm. Percentage degradation of the phenolic compounds was calculated according to Singh et al. (2017) as follows:

$$\text{Degradation efficiency (\%)} = (A_i - A_f)/A_i \times 100\%$$

where A_i is the initial absorbance of the phenolic compounds and A_f is the final absorbance of the phenolic compounds (after rSLAC treatment).

Analysis of the residual phenolic compounds by HPLC

HPLC analysis was performed using a Shimadzu HPLC system from Dionex (Massachusetts, USA) equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector. Chromatographic separation was achieved on C18 column (Sunfire, Waters, Ireland) using 0.1% orthophosphoric acid (A) and acetonitrile (B) as mobile phase, flow rate 1.0 mL/min, injection volume of 10 μ L and an oven temperature of 40 °C. The gradient was set up as follows: 2% B to 100% B (0–20 min), 100% B to 2% B (20–22 min), 2% B (22–30 min). The peaks for phenolic compounds as well as the transformation products were observed at 254 nm.

Toxicity analysis

Reduction in toxicity of phenolic compounds after rSLAC treatment was monitored by bacterial growth inhibition studies as described by Murugesan et al. (2010) using *Escherichia coli* ATCC 25922 as a test organism. Individual phenolic compounds (80 μ g, total volume of 900 μ L) and their degradation products (total volume of 900 μ L) were incubated separately in sterile nutrient broth (9 mL) with 100 μ L of 0.1 OD₆₀₀ overnight culture of *E. coli* ATCC 25922. The test tubes were incubated with shaking (200 rpm) in the dark at 37 °C and bacterial growth was monitored spectrophotometrically at 600 nm. Results were presented as percentage growth inhibition relative to the control sample (containing 9 mL nutrient broth 100 μ L of 0.1 OD₆₀₀ overnight culture of *E. coli* ATCC 25922 and 900 μ L saline).

Results and discussion

Characterization of MNPs and immobilized MNPs

FE-SEM images of bare and silanized MNPs showed no evidence of morphological changes after silanization. Further,

FE-SEM images of MNPs also revealed that their diameter was in the range of 50–200 nm (Fig. 1). Fig. S4 shows the typical High-Resolution Transmission Electron Microscopy (HR-TEM) image of MNPs, whereby MNPs appear to be nearly spherical in shape and exhibits a homogenous distribution, along with a noticeable agglomeration owing to the attractive forces between them. FT-IR spectra confirmed the presence of characteristic peaks for MNP, MNP/APTES and MNP/APTES/rSLAC (Fig. 2). A high-intensity peak at 589 cm^{-1} was observed in uncoated nanoparticles, which is a characteristic peak for MNPs (Zhang et al. 2007). Interestingly, we also observed that the intensity of MNP peaks (589 cm^{-1}) decreased after subsequent grafting of APTES and APTES/rSLAC, confirming the successful grafting of APTES and rSLAC onto the MNPs (Fig. 2). The appearance of a peak in the region of 2360 cm^{-1} is due to Si–H silane (Fig. 2b, c), which further confirmed the grafting of APTES onto MNPs. The appearance of a peak at 1648 cm^{-1} in

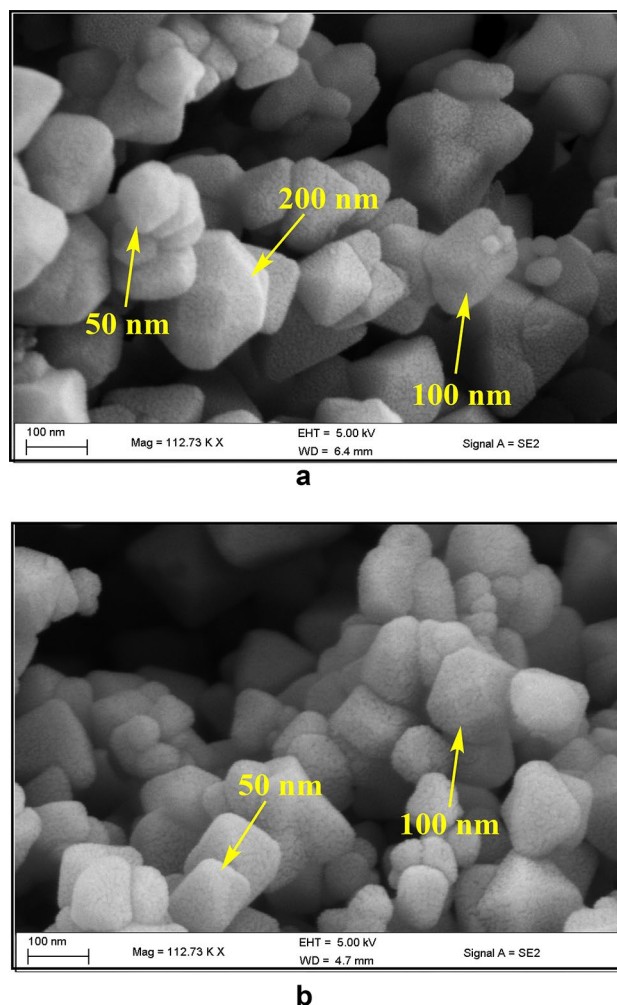
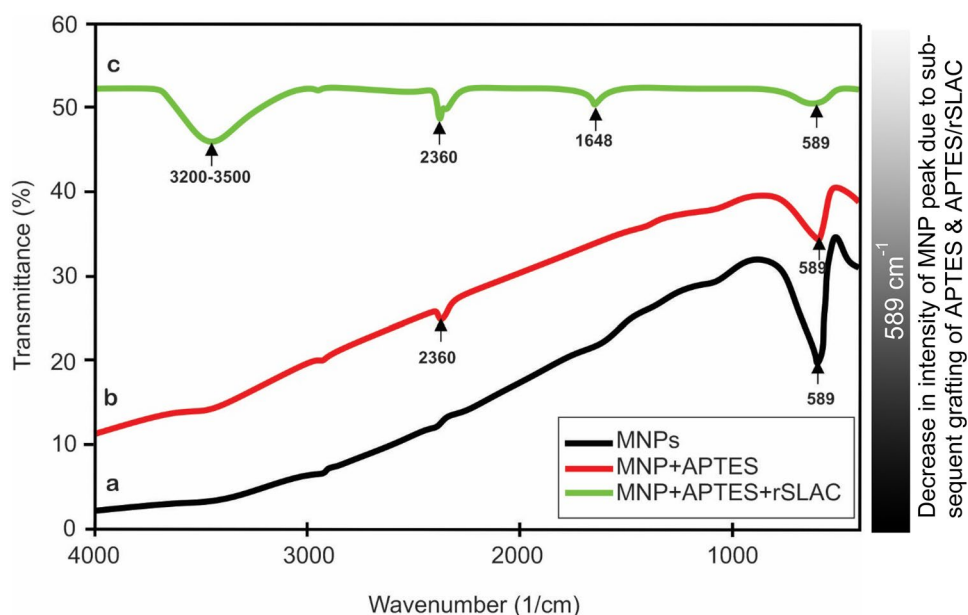


Fig. 1 FE-SEM images showing surface morphology of **a** bare and **b** functionalized MNPs

Fig. 2 FTIR spectra of MNPs (black), MNP + APTES (red) and MNP + APTES + rSLAC (green)



MNP/APTES/rSLAC (Fig. 2c) could be due to the CO-NH₂ group, which confirmed the formation of bond between enzyme and Si-MNP (Faridi et al. 2017). The appearance of a broad peak obtained at 3200–3500 cm⁻¹ in all three spectra depicts the presence of hydroxyl groups on the surface of nanoparticles (Ma et al. 2003). Furthermore, we also noticed that the peak intensity at 3200–3500 cm⁻¹ has subsequently increased after grafting of APTES and APTES/rSLAC onto MNPs, which could be due to the physical and chemical adsorption of water molecules after each coating. Thus, peaks shown by FT-IR confirmed the successful synthesis of MNPs and grafting of APTES and rSLAC onto the surface of nanoparticles.

Effect of pH and temperature on the catalytic activity of free and immobilized rSLAC

The catalytic activity of free and immobilized rSLAC was evaluated in the pH range 5.0–9.0 and maximum activity was observed at pH 8.0 for both free and immobilized enzyme (Fig. 3a). However, the immobilized enzyme showed higher catalytic activity at pH 5.0 and 6.0 compared to the free enzyme. The enhanced catalytic activity over a broad pH range could be attributed to the MNP support. Similar results have been observed by Zhang et al. (2017), who also reported that the immobilized enzyme showed a broader pH-activity profile. The optimum temperature for both enzymes was 80 °C (Fig. 3b). It was also observed that the immobilized rSLAC retained higher catalytic activity (~20%) at 100 °C compared to the free enzyme, which suggests enhanced thermostability after immobilization. Elevated temperature could change the conformation and enzyme–substrate interaction of free enzymes, causing

a negative effect on catalytic performance. On the other hand, immobilization can provide a protective effect against increase in temperature by reducing the conformational flexibility of enzymes. Therefore, enhanced thermostability of rSLAC immobilized on modified MNP could be attributed to the enzyme rigidity owing to the anchorage between the support and protein, promoted by covalent bond formation (Costa et al. 2019).

Determination of kinetic parameters for free and immobilized rSLAC

The Michaelis constant (K_m) for the free and immobilized enzymes were 54.05 and 54.17 μM , respectively while the maximum velocities (V_{max}) were 25.88 and 26.26 $\mu\text{M mg}^{-1} \text{min}^{-1}$, respectively. The slight increase in K_m value for the immobilized rSLAC could be due to the diffusional limitation, which resulted in a lower enzyme–substrate affinity. An increase in K_m values after immobilization of laccase has also been reported in several studies (Wang et al. 2010, 2013). Comparable V_{max} values of free and immobilized rSLAC suggest that the immobilization process did not compromise catalytic activity.

Effect of metal ions and organic solvents on free and immobilized rSLAC

Heavy metal ions are common environmental pollutants and are often found in industrial wastewater (Zeng et al. 2017). These metal ions could activate or inhibit the catalytic activity of enzymes (Couto et al. 2005), which can affect the enzymatic remediation process. Therefore, the effects of different metal ions on the catalytic activity of free and

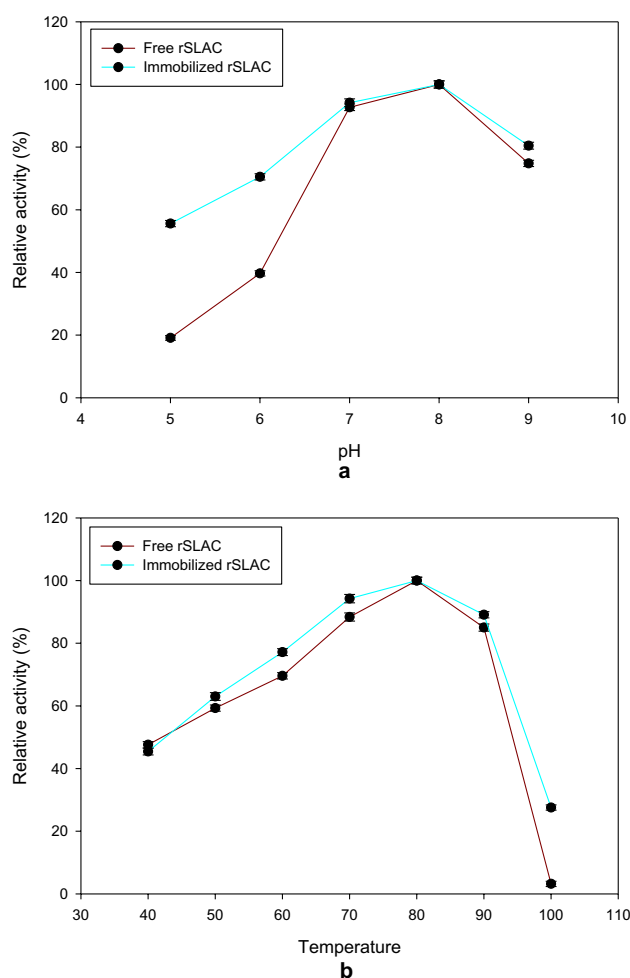


Fig. 3 Effect of (a) pH and (b) temperature on the catalytic activity of free (—●—) and immobilized (—●—) rSLAC. The effect of pH was assessed using different buffers [20 mM sodium acetate buffer (pH 5.0), 20 mM Tris–HCl buffer (pH 6.0–8.0) and 20 mM glycine–NaOH buffer (pH 9.0)] at 80 °C. Temperature optimisation was carried out by performing laccase assays at different temperatures (40 to 100 °C) at pH 8.0 using 2,6 DMP as a substrate. Values are means $\pm$ standard deviation ($n=3$)

immobilized rSLAC were evaluated. Interestingly, it was observed that Co^{2+} , Cu^{2+} and Mn^{2+} ions greatly stimulated the catalytic activity of free and immobilized rSLAC (Table 1), where the order of enhancement in rSLAC activity was $\text{Co}^{2+} > \text{Mn}^{2+} > \text{Cu}^{2+}$. Activation of rSLAC by Cu^{2+} is not surprising as copper is an essential component of the active site (Chakroun et al. 2010; Lu et al. 2012a, b). The results are consistent with those obtained using a recombinant polyphenol oxidase, where Co^{2+} stimulated a higher catalytic efficiency compared to Cu^{2+} (Moon et al. 2018). In addition, apart from copper, manganese also has a known role in inducing the transcription and activity of laccases (Baldrian et al. 2000). However, the other metal ions only slightly improved the activity of the enzymes, with Al^{3+}

Table 1 Effect of different metal ions on catalytic activity of free and immobilized rSLAC

Metal ions	Concentrations (mM)	Relative activity (%)	
		Free rSLAC	MNP-rSLAC
Control	—	100 $\pm$ 0.16	100 $\pm$ 1.62
Li^+	10	104 $\pm$ 0.58	103 $\pm$ 1.53
	30	114 $\pm$ 2.08	112 $\pm$ 1.17
Co^{2+}	10	563 $\pm$ 2.00	556 $\pm$ 2.52
	30	604 $\pm$ 2.08	598 $\pm$ 1.54
Cu^{2+}	10	179 $\pm$ 2.07	175 $\pm$ 2.06
	30	253 $\pm$ 2.59	250 $\pm$ 3.04
Mn^{2+}	10	315 $\pm$ 2.04	311 $\pm$ 3.52
	30	333 $\pm$ 2.53	329 $\pm$ 1.50
Ca^{2+}	10	120 $\pm$ 3.08	116 $\pm$ 2.40
	30	141 $\pm$ 2.02	138 $\pm$ 2.50
Mo^{6+}	10	126 $\pm$ 2.48	128 $\pm$ 1.24
	30	137 $\pm$ 2.10	136 $\pm$ 1.56
Al^{3+}	10	117 $\pm$ 0.82	119 $\pm$ 2.08
	30	94 $\pm$ 1.57	96 $\pm$ 1.58
Mg^{2+}	10	114 $\pm$ 2.59	112 $\pm$ 1.54
	30	119 $\pm$ 1.55	121 $\pm$ 1.18

All values are means $\pm$ SD ($n=3$)

Laccase activity in the absence of metal ions was considered as 100%

reducing activity at 30 mM (Table 1). Enhanced catalytic activity of laccase by several metal ions viz., Mn^{2+} , Mg^{2+} , Mo^{6+} , Ca^{2+} has also been reported in a previous study (Zhao et al. 2012). The negative effect of Al^{3+} observed here is consistent with a previous report where the suppressive effect of this trivalent metal ion towards laccase- Cu^{2+} inhibited the degradation of 4-nitrophenol (Lu et al. 2012a, b). Overall, our results suggest that the presence of some metal ions in wastewater could facilitate the remediation of phenolic compounds.

Organic solvents have been known to inhibit enzyme activity via protein unfolding (Afreen et al. 2017). Results on the effect of different organic solvents showed that catalytic activities of free and immobilized rSLAC were not affected by the solvents tested (at 20 and 50%) except for DMSO (Table 2). The activities of both the free and immobilized enzymes were significantly increased in the presence of dioxane (Table 2). To the best of our knowledge, the stimulatory effect of dioxane has not been reported, which could be a novel feature of rSLAC. The rSLAC retained full activity at 20 and 50% methanol, ethanol and acetone whilst a minor effect was observed with DMSO (2% inhibition at 20% DMSO and 8% inhibition at 50% DMSO). In a previous study with rCotA laccase, however, DMSO significantly inhibited the enzyme (62% inhibition with 10% DMSO) (Wang et al. 2015) which indicates that the rSLAC has a much better tolerance to DMSO. As some of the pollutants, such as the phenolics studied here, polycyclic aromatic

Table 2 Effect of different organic solvents on catalytic activity of free and immobilized rSLAC

Organic solvent	Concentration (%, v/v)	Relative activity	
		Free rSLAC	MNP-rSLAC
Control	—	100 ± 1.16	100 ± 1.62
Methanol	20	110 ± 3.08	109 ± 1.56
	50	104 ± 1.58	106 ± 1.54
Ethanol	20	115 ± 2.06	113 ± 1.57
	50	109 ± 2.56	110 ± 3.62
Acetone	20	109 ± 2.54	107 ± 2.07
	50	101 ± 2.06	103 ± 2.53
Dioxane	20	123 ± 1.74	122 ± 2.48
	50	198 ± 3.05	194 ± 2.58
DMSO	20	98 ± 2.00	99 ± 3.06
	50	92 ± 1.18	98 ± 1.16

All values are means ± SD ($n=3$)

Laccase activity in the absence of organic solvents was considered as 100%

hydrocarbons (PAHs), organophosphorus pesticides and azo dyes, are only soluble in organic solvents, the use of immobilized rSLAC could be useful for their detoxification.

Storage stability and recycling efficiency of immobilized rSLAC

The use of enzymes in an industrial application is greatly affected by the cost of their production, thus, enzymes with long-term storage stability and high recycling efficiency are preferred (Alex et al. 2014). To this end, storage stability and reusability of immobilized rSLAC were assessed. The free and immobilized enzymes were stored in Tris–HCl buffer (20 mM, pH 8) at 4 °C and their activities monitored every 6 days over a period of 30 days. Both enzymes retained ≥ 95% of initial activity (Fig. 4a).

The recycling efficiency of immobilized rSLAC was evaluated in a batch operation mode for 10 cycles, using 2,6-DMP as a substrate. The MNP-rSLAC retained > 73% of its initial activity after 10 consecutive cycles (Fig. 4b). The decrease in laccase activity after repeated cycles could be due to the leaching of the enzyme from the immobilized support or accumulation of reaction products that may reduce mass transfer efficiency and make the enzyme less accessible by its products. A fungal laccase immobilized on MNPs functionalised by PDA retained 70% of its initial activity after 10 catalytic cycles (Zhang et al. 2017). This implies that silanization, with favourable properties outlined earlier, could be a viable alternative functionalisation approach. The remarkable storage stability along with the reusability of the MNP-rSLAC during 10 successive cycles, emphasises its potential for large-scale application.

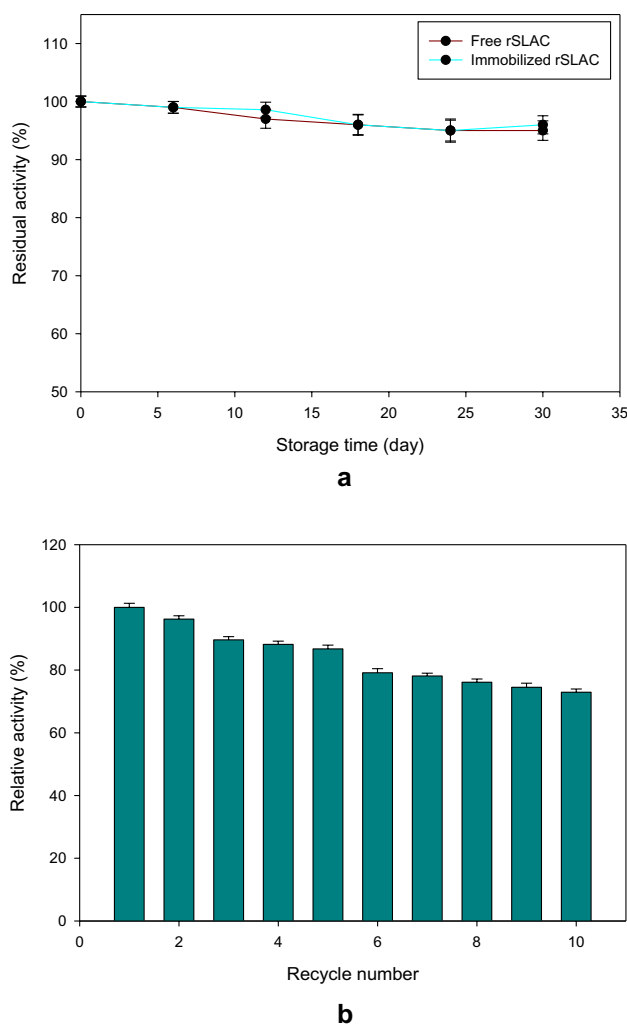


Fig. 4 **a** Storage stability of free (—●—) and immobilized (—●—) rSLAC. This was evaluated by keeping both free and MNP-rSLAC in 20 mM Tris–HCl buffer (pH 8.0) at 4 °C for a period of 30 days. **b** Reusability of immobilized rSLAC (MNP-rSLAC) was monitored over 10 reaction cycles. Values are means ± standard deviation ($n=3$)

Transformation of phenol, 4-CP and 4-FP by rSLAC

To enhance transformation, the effects of various parameters, such as pH, mediator concentrations and time, on the degradation of the phenolic compounds, were investigated.

Effect of pH on degradation of phenolic compounds

pH is one of the critical factors for any enzyme-catalysed reaction. Therefore, the effect of pH on rSLAC-mediated degradation of phenol, 4-CP and 4-FP was evaluated in the pH range of 4.0–8.0. Interestingly, it was observed that ≥ 98% degradation was achieved over a wide pH range of pH 6.0–8.0 (Fig. 5a). However, the degradation of phenolic compounds was relatively low at acidic pH; it increased

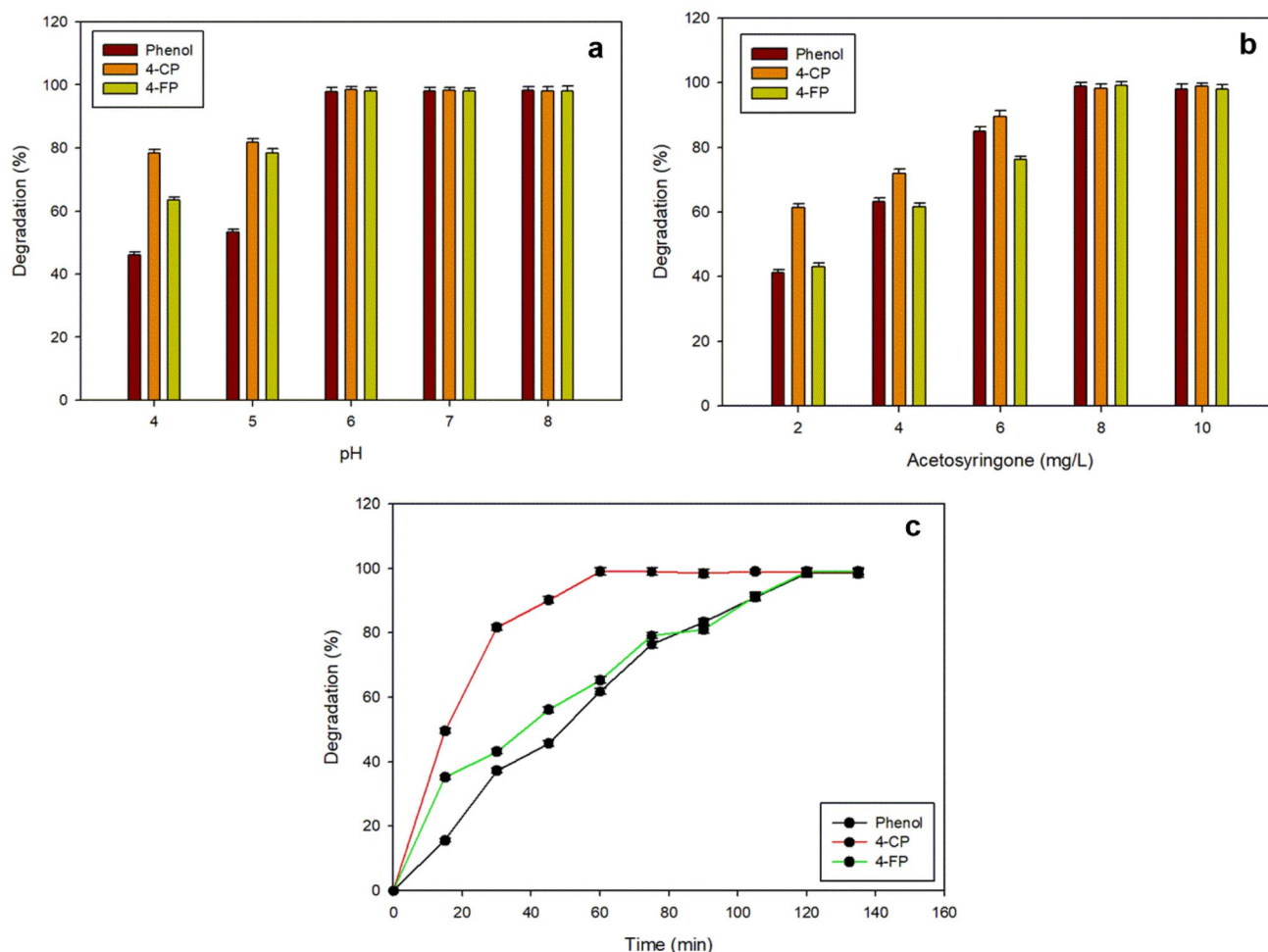


Fig. 5 Effect of pH (**a**), acetosyringone concentration (**b**) and time (**c**) on the transformation of phenol, 4-CP and 4-FP. **a** Effect of pH on the transformation of phenol, 4-CP and 4-FP. The reaction mixture contained 80 mg/L of each phenolic compound in different pH buffers and laccase (2 U/mL), and was incubated at 37 °C for 4 h. **b** Effect of mediator (acetosyringone) concentration (2–10 mg/L) on the transformation of phenol, 4-CP and 4-FP using rSLAC. Reaction was carried out in Tris–HCl buffer (50 mM, pH 8.0) with 80 mg/L of phe-

nol, 4-CP, 4-FP and laccase 2 U/mL. **c** Time dependent degradation profile of phenol, 4-CP and 4-FP using rSLAC in the presence of a redox mediator, acetosyringone. Reaction conditions: 80 mg/L phenol, 4-CP or 4-FP, 2U rSLAC, 8 µg/mL acetosyringone, 37 °C, pH 8. The residual concentrations of phenol, 4-CP and 4-FP were determined colorimetrically (510 nm). Data is presented as the mean $\pm$ SD ($n = 3$)

substantially in the alkaline range (Fig. 5a). Since the pH of wastewater effluents are in the range of 5.5–9.8 (Gaitan et al. 2011; Nair et al. 2013), the application of rSLAC in real wastewater conditions could be feasible.

Effect of mediator concentration on degradation of phenolic compounds

The inclusion of mediators has been the general approach in enhancing laccase-mediated transformation of recalcitrant compounds (Murugesan et al. 2010). Therefore, the effect of different mediators such as acetosyringone (natural) and 1-hydroxybenzotriazole (synthetic), on the degradation of phenolic compounds was evaluated. Both mediators showed

similar effects (data not shown), therefore further experiments were carried out with the natural mediator due to its eco-friendly properties (Kunamneni et al. 2008). It was observed that complete degradation was achieved at 8 mg/L concentration of acetosyringone (Fig. 5b). A previous study used a much higher concentration of artificial mediator (ABTS; 642.5 mg/L) to achieve the complete degradation of 4-CP (64.28 mg/L) (Chakraborty et al. 2010).

Effect of time on degradation of phenolic compounds

The transformation of phenol, 4-CP and 4-FP were performed using the purified rSLAC (2 U or 40 µg/mL) at

optimal pH and mediator concentration. Remarkably, $\geq 98\%$ degradation of all the phenolic compounds was achieved within 2 h (Fig. 5c). Moreover, degradation of 61, 98 and 65% was observed for phenol, 4-CP and 4-FP, respectively, in only 1 h of reaction time. However, in a previous study, 100% degradation of 2-chlorophenol was achieved in 4 h using 10 U of laccase (Gaitan et al. 2011). Similarly, Zhang et al. (2017) achieved 86% degradation of 4-CP in 2 h using 250 mg/L of immobilized laccase. The requirement of the low amount of laccase for the complete degradation of phenolic compounds, in this study, could be attributed to the high catalytic efficiency of the rSLAC, as previously reported (Yadav et al. 2018). In addition, we also noticed that the degradation of halogenated phenols was attained in less time compared to the non-halogenated phenol (Fig. 5c). This is consistent with previous reports where 4-chlorophenol was degraded much faster than phenol (Arica et al. 2009; Liu et al. 2012).

HPLC analysis of transformation products

The transformation of phenol, 4-CP and 4-FP by rSLAC was further analysed by HPLC. The retention time for phenol was $t_R = 8.7$ min; the peak completely disappeared after laccase-catalysed transformation and several transformation products were formed which eluted at longer retention times (Fig. S1c). The retention times for the elution of 4-CP and 4-FP were observed at $t_R = 10.6$ and $t_R = 9.6$ min, respectively, in control samples (Fig. S2a and S3a). Similarly, the peaks for 4-CP and 4-FP disappeared in test samples and multiple peaks appeared after delayed retention times (Fig. S2c and S3c). The delayed retention times observed in all test samples could be due to the formation of oligomeric products (Cabana et al. 2007). Similar findings were observed in laccase-mediated transformation of triclosan (Murugesan et al. 2010).

Toxicity analysis

Toxicity analysis of phenolic compounds and their products were assessed by performing the bacterial growth inhibition studies using *E. coli* ATCC 25922 as a test organism. It is well known that phenolic compounds increase bacterial cell membrane permeability which leads to the release of their cell wall components and ultimately cell death (Denyer 1995; McDonnell and Russell 1999). In the present study, 80 $\mu\text{g/mL}$ of the phenolic compounds used (phenol, 4-CP and 4-FP) completely inhibited the growth of *E. coli* ATCC 25922 (Fig. 6). However, the transformation products inhibited the bacterial growth by 10, 40 and 45% for phenol, 4-CP and 4-FP, respectively. This suggests that the toxicity of phenol, 4-CP and 4-FP transformation products were reduced by 90, 60 and 55%, respectively. The halogenated compounds

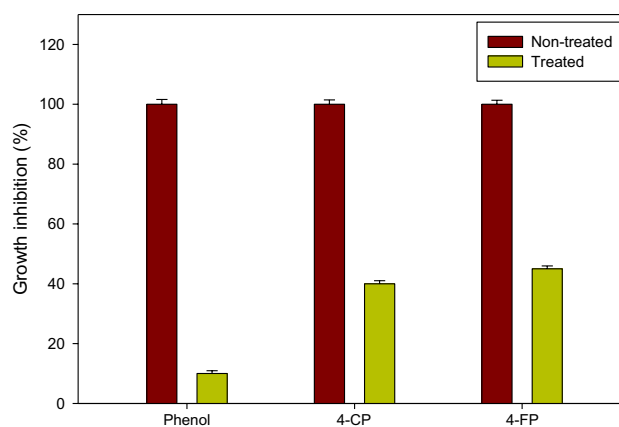


Fig. 6 Effect of rSLAC treatment of phenolic compounds on bacterial growth inhibition. Toxicity analyses of the degradation products of phenol, 4-chlorophenol (4-CP) and 4-fluorophenol (4-FP) were tested using *E. coli* ATCC 25922 as reference organism (■) denotes untreated samples (no enzyme) and (■) shows rSLAC-treated samples. Data is presented as the mean $\pm$ SD ($n = 3$)

viz., 4-CP and 4-FP showed higher toxicity compared to the phenol. Higher toxicity of halogenated phenolic compounds was also observed in a previous study, where it was reported that an increase in the number of chlorine atoms was associated with an increase in bacterial growth inhibition (Wang et al. 2008). On the other hand, it was observed in a previous study that degradation products were more toxic than the parental compounds (Lu et al. 2015). In this study, we observed that after the rSLAC treatment of phenolic compounds the products were precipitated out of the reaction mixture, which could be due to the polymerisation of the products. This could provide an additional advantage for the physical separation of the polymerized products from a wastewater sample using filtration methods. Separation of precipitates by filtration methods has also been previously reported (Abdollahi et al. 2018).

Conclusion

In this study, rSLAC was for the first time successfully immobilized onto MNPs functionalised by silanization. Enzyme immobilisation was confirmed by SEM and FTIR. The immobilised enzyme retained 73% of initial activity after 10 cycles, exhibited improved storage stability, and showed enhanced activity when exposed to selected metal ions. The enzyme showed great potential for the removal of phenolic compounds within a wide pH range, significantly reducing the toxicity of the compounds. In the presence of a natural mediator, acetosyringone, $\geq 98\%$ transformation of phenol and halogenated phenols was achieved within 2 h. These findings suggest that immobilized rSLAC could be

a candidate for the sustainable bioremediation of phenolic compounds in wastewater.

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Availability of data and material Data will be made available on request.

Code availability N/A

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Ethics approval: This article does not contain any studies with human participants or animals.

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