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Immobilization of laccase onto micro-emulsified magnetic nanoparticles for enhanced degradation of a textile recalcitrant

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Abstract

The present investigation evaluates the efficiency of laccase binding onto the micro-emulsified magnetic nanoparticles (MNPs). The synthesized MNPs were characterized for its nature, particle size and functional moieties using transmission electron microscopy Fourier transform infrared spectroscopy and X-ray diffraction studies. It was found that the MNPs had a uniform and mean average diameter of 12.63±3.2 nm. The TEM analysis and XRD patterns also confirmed the presence of MNPs after laccase binding. The maximum activity of the immobilized laccase was obtained at its weight ratio of 19.55×10^{-3} mg bound laccase mg^{-1} MNPs. The stability and reusability analyses were also studied to evaluate the activity of the immobilized laccase with that of free laccase. Application of laccase immobilized MNPs were extended to degrade a reactive textile dye; reactive green 19A (RG19A).

Key words

Laccase, Micro-emulsion, Magnetic nanoparticles, Reactive green 19A

Introduction

Textile industry is the largest generator of coloured and voluminous effluents into the environment, and they pose a great threat to the abiotic and biotic components (Xu*et al*., 2012; Jadhav *et al*., 2007). Many conventional physico-chemical treatment strategies have been proposed to control the pollution, but have failed to eradicate the pollutants completely because of their durability and persistence (Karthikeyan *et al*., 2014; Vidhyadevi *et al*., 2014; Rekuc *et al*., 2010; Madhu *et al*., 2009; Robinson *et al*., 2001).

Bioremediation using microbial cells or enzymes are emerging technologies of pervasive importance over conventional treatment (Kumar *et al*., 2015; Biyik *et al*., 2012; Wang *et al*., 2010). Bioremediation using microorganisms are much preferred than the conventional techniques because of their eco-friendly and pollution free nature (Selvanaveen *et al*., 2015; Vinodhini *et al*., 2015). Laccase is an oxidoreductase enzyme isolated from fungal source is burgeoning and has drawn great attention during the recent years due to its efficient decolorizing ability (Balaji *et al*., 2014; Kumar *et al*., 2014a; Kumar *et al*., 2012a; Kunamneni *et al*., 2008).

The potential problem in employing laccase or any enzyme in bioremediation is difficulty in production and its relatively high production cost. The aforementioned disadvantages can be overcome by immobilizing the enzymes onto a support matrix serving as an effective mean

to ameliorate the stability, activity and reusability (Subhashini *et al*., 2013; Kumar *et al*., 2012b; Busca *et al*., 2008; Jiang *et al*., 2005). Immobilizing enzymes onto the magnetic nanoparticles (MNPs) renders high enzyme loading capacity, and separation is easier by simple magnetic decantation (Daniel *et al*., 2015; Seenuvasan *et al*., 2014a,&b; Seenuvasan *et al*., 2013a,&b; Yang *et al*., 2012; Bayramoglu and Arica 2008; Hong *et al*., 2007). There are many methods employed for the synthesis of MNPs like co-precipitation, thermal decomposition, hydrothermal synthesis and micro-emulsion (Gubin *et al*., 2005). Micro-emulsion is more advantageous over other techniques as they yield uniformly, narrow sized distributed MNPs (Santra *et al*., 2001).

The present investigation emphasizes on the *in-situ* synthesis of amino functionalized MNPs (AMNPs) by micro-emulsion method and to immobilize fungal laccase produced from the previous work of Balaji *et al*., (2014) after carboxyl group activation of AMNPs. The particles were characterized using TEM, XRD and FT-IR for studying their size, morphology and functional groups involved. The stability and activity analyzes were done for free and immobilized laccase by studying their pH, temperature, reusability and storage activity. The applicability of immobilized laccase was evaluated by employing them in decolorizing a textile dye; RG19A.

Materials and Methods

Synthesis of MNPs : The MNPs were synthesized by water-in-oil micro emulsion method.Two individual micro emulsions, one with an equal volume of 0.15 M $FeCl_3.6H_2O$ and 0.1 M $FeCl_2.4H_2O$ and the other with 25-30% $NaBH_4$ solutionwere prepared and stirred for 30 min after the addition of Triton X-100 at inert condition. The reaction was followed by dropwise addition of $NaBH_4$ in an ultra sonicator bath Shah (1998). The dark brown precipitate confirmed the formation of MNPs. Removal of surfactants was done by flocculation followed by washing several times with water and twice with ethanol. The MNPs thus obtained were magnetically decanted and dried in a vacuum oven.

***In-situ* synthesis of AMNPs and its activation :** In order to immobilize enzyme onto the MNPs, naked MNPs were amino-functionalized by *in-situ* microemulsion method.About 1.0 ml of APTES was added to $NaBH_4$ solution followed by its drop wise addition to the $FeCl_3$ emulsion under constant stirring in the presence of nitrogen gas for 24 hrs. The resulted AMNPs were washed several times with ethanol and water, magnetically decanted and stored in 10 ml of toluene.15A mg of AMNPs were suspended in glutaraldehyde solution (10% v/v), stirred for 4 hrs at 200 rpm in the presence of N_2. The AMNPs were activated by glutaraldehyde by imparting the carboxylic group over them. These activated AMNPs were washed with ethanol followed by magnetic decantation and then stored in toluene for further use.

Immobilization of laccase onto AMNPs : 500 µl of laccase solution was added to 50 mg of activated AMNPs at varying concentration (10-150 µlin acetate buffer, pH 6.0). The mixture was sonicated for 5 min to obtain uniform suspension. The sonication was performed repeatedly to ensure complete dispersion of mixtures after its storage at 4°C for 1 hrs. The contents were washed thrice with acetate buffer at room temperature and the supernatant from each wash were assayed for protein using BSA as standard (Lowry *et al*.,1951).

Laccase assay : The laccase activity was assayed spectrophotometrically by employing guaiacol as substrate. The assay mixture included: 4.8 ml of 0.1 M sodium acetate buffer (pH 6.0), 0.1 ml of 0.01 M guaiacol and 0.1 ml of enzyme extract. After incubation for 45 min at 37 °C, the absorbance was recorded at 450 nm. All the experiments were performed in triplicates, and the mean value was recorded. One unit of enzyme activity is defined as the amount of enzyme which oxidizes 1 µmol of substrate per min. The laccase activity was calculated using extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm by the following formula :

$$E.A = (A^* V) / (t^* e^* v)$$

where, E.A is the enzyme activity (U ml^{-1}); A is the absorbance at 450nm; V- is the total volume of reaction

Table 1 : Effect of initial amount of laccase on percentage immobilization, weight ratio and percentage relative activity: MNPs=5.0 mg, pH 6.0 at 25°C

Laccase added (µl)	Laccase immobilized (%)	Weight ratio (mg bound laccasemg $AMNPs^{-1}$) x10^{-3}	Relative activity (%)
13.56	97.6	9.80	65.32
27.13	77.1	15.48	83.60
47.72	52.3	18.47	100
67.78	37.9	19.01	92.47
87.97	28.4	18.49	86.29
108.83	24.2	19.49	82.26
135.48	19.5	19.55	75.02

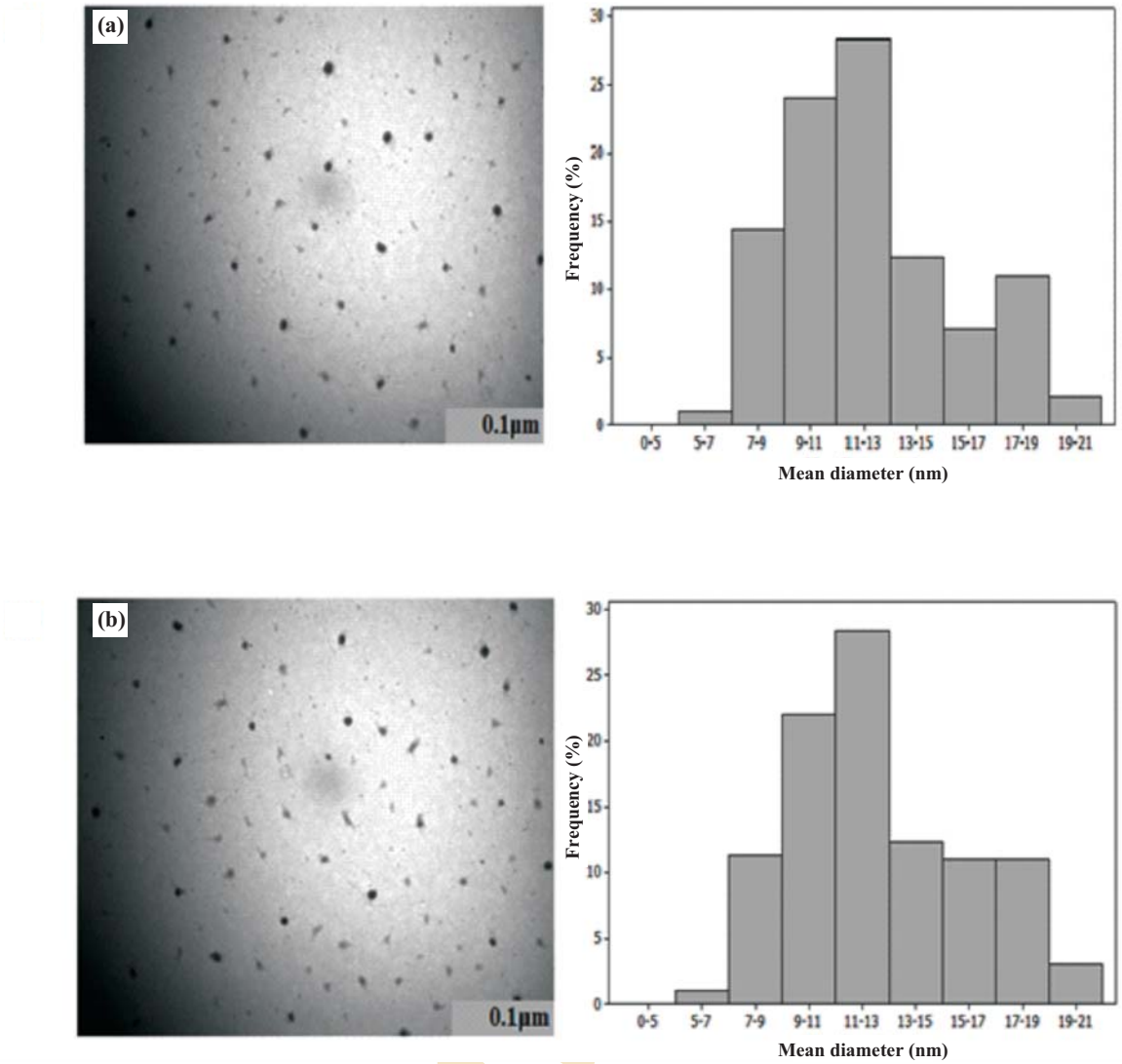

Fig. 1 : Micrographs and size distribution analysis of (a) naked and (b) laccase bound MNPs

mixture (ml); v enzyme (ml); t is the incubation time (min) and e is the extinction coefficient ($M^{-1}cm^{-1}$).

Stability and reusability assays : The pH and thermal stability of laccase activity were evaluated by measuring the activity of free and immobilized laccase at varying pH (3.0-8.0) and temperature (5-55°C) levels, respectively. The reusability of immobilized laccase were evaluated by collection of the supernatant followed by magnetic decantation at the end of each cycle. The fresh substrate (guaiacol) was added to the immobilized laccase, and the procedure was repeated until the enzyme activity reached half of its maximum.

Degradation of RG19A by laccase and laccase bound MNPs : To investigate the ability of free and immobilized laccase to degrade dye,100 (mg l^{-1}) RG19A in 0.1 M acetate buffer (pH 6.0) was incubated individually with equal and known quantity of free and immobilized la*ccase* at 25°C for 6 hrs. The heat-denatured laccase solution was used as the control and the reaction mixture, except RG19A, was used as control, RG19A decolorization and degradation was measured by recording the absorbance spectra of the initial and final laccase and laccase bound MNPs treated reaction

mixture between 190 and 900 nm.

Instrumental analysis : The absorbance was measured using Shimadzu UV-Vis spectrophotometer UV-1800, Tokyo, Japan. The average particle size, morphology and size distribution of nanoparticles were determined using TEM (Technai 10, Philips). The morphological and phase changes of the MNPs was determined using the XRD patterns, using Philips X'pert Pro Materials Research Diffractometer in the receiving slit operation mode with a single Cu-Kα radiation (λ=0.154 nm). The surface modification and enzyme loading were confirmed by FT-IR spectroscopy (Perkin Elmer Spectrum RX 1 using potassium bromide (KBr) pellet method in the range of 400-4000 cm^{-1}).

Statistical analysis : Student's t-test provided by statistical software Minitab 14 (Ver.14.0, U.S. Federal Government Commonwealth of Pennsylvania, USA) facilitates the analysis of size distribution of the particles before and after laccase binding.

Results and Discussion

The morphology as well as size distribution of the MNPs and laccase bound MNPs were studied by TEM analysis as shown in Fig. 1(a-b). The laccase bound MNPs (Fig.1b) had an overall mean diameter of 12.63±3.2 nm and did not exhibit any significant change in size when compared with the naked MNPs (12.23±3.5 nm).

The statistical analysis suggests that the binding process did not cause any significant change in size (α=0.05, p-value=1.00); and from the physical inspection of the images, it was noticeable that no additional aggregation occurred as a result.

The XRD patterns for MNPs and laccase bound MNPs had five characteristic peaks for Fe_3O_4 (2θ= 30.39, 35.43, 43.37, 57.22, 62.9) marked by their indices (220), (311), (400), (520) and (441) as seen in Fig. 2(a) was similar to the laccase bound MNPs. The XRD diagram showed series of Bragg's reflections corresponding to pure spinal structure of Fe_3O_4 and it was confirmed by the presence of a characteristic peak at 35.43 nm.

The particle size was measured from the broaden bands according to the Debye-Scherer formula for MNPs and laccase bound MNPs, and was found to be 12.21 and 12.62 respectively, which is in agreement with the TEM analysis.

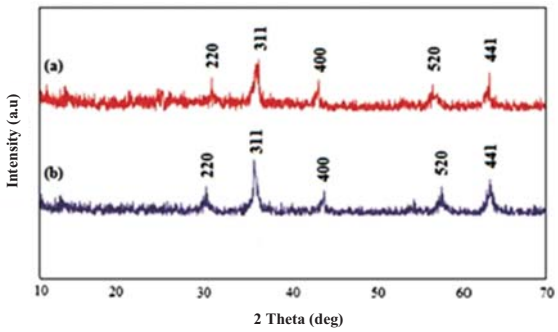

Fig. 2 : XRD patterns of (a) MNPs (b) laccase bound AMNPs

FT-IR spectra of the naked MNPS, AMNPs, and laccase bound MNPS and free laccase were scanned in the range of 400-4000 cm^{-1} is shown above in Fig.3. The FT-IR spectra confirmed the presence of various functional groups such as iron, amines, amides, alkanes and aldehydes in the naked MNPS, AMNPs, and laccase bound MNPS and free laccase. Simultaneous synthesis of MNPs by micro-emulsion method followed by amino functionalization and AMNPs by *in-situ* micro-emulsion synthesis was done to compare whether the characteristic features and functionalization obtained during the *in-situ* synthesis-cum-functionalization are similar to AMNPs functionalized after MNPs synthesized separately.

FT-IR spectrum of MNPs at 582 cm^{-1} confirmed the presence of Fe-O bond, 1608cm^{-1} represents the presence of primary amines (NH) as shown in Fig. 3(a). The shift in frequency from 1608 to 1594 cm^{-1} and formation of new peaks 829 and 1009 cm^{-1} indicated the formation of amide bond as shown in Fig. 3(b) and 3(c) of AMNPs. The C-O stretching vibration was found at 829 cm^{-1} The spectra, as shown in Fig. 3(d), exhibited peak at 1634 cm^{-1} indicating the presence of aldehyde, amide and primary amine groups, and also indicates C=O stretching vibrations according to Seenuvasan *et.al*, (2013) which showed similar peaks indicating 585cm^{-1} for Fe-O bond, 1626 cm^{-1} for amine stretching in comparison to the present work. The successful binding of laccase onto the activated AMNPs was confirmed by the appearance of peak at 1581 cm^{-1} in laccase bound MNPs and this might be attributed through the reaction between the amine group of laccase and carboxyl group of the activated AMNPs.

The relative activity of immobilized laccase at varying ratios of activated AMNPs is shown in Table 1, and their maximum weight ratio was determined to be 19.55 (mg

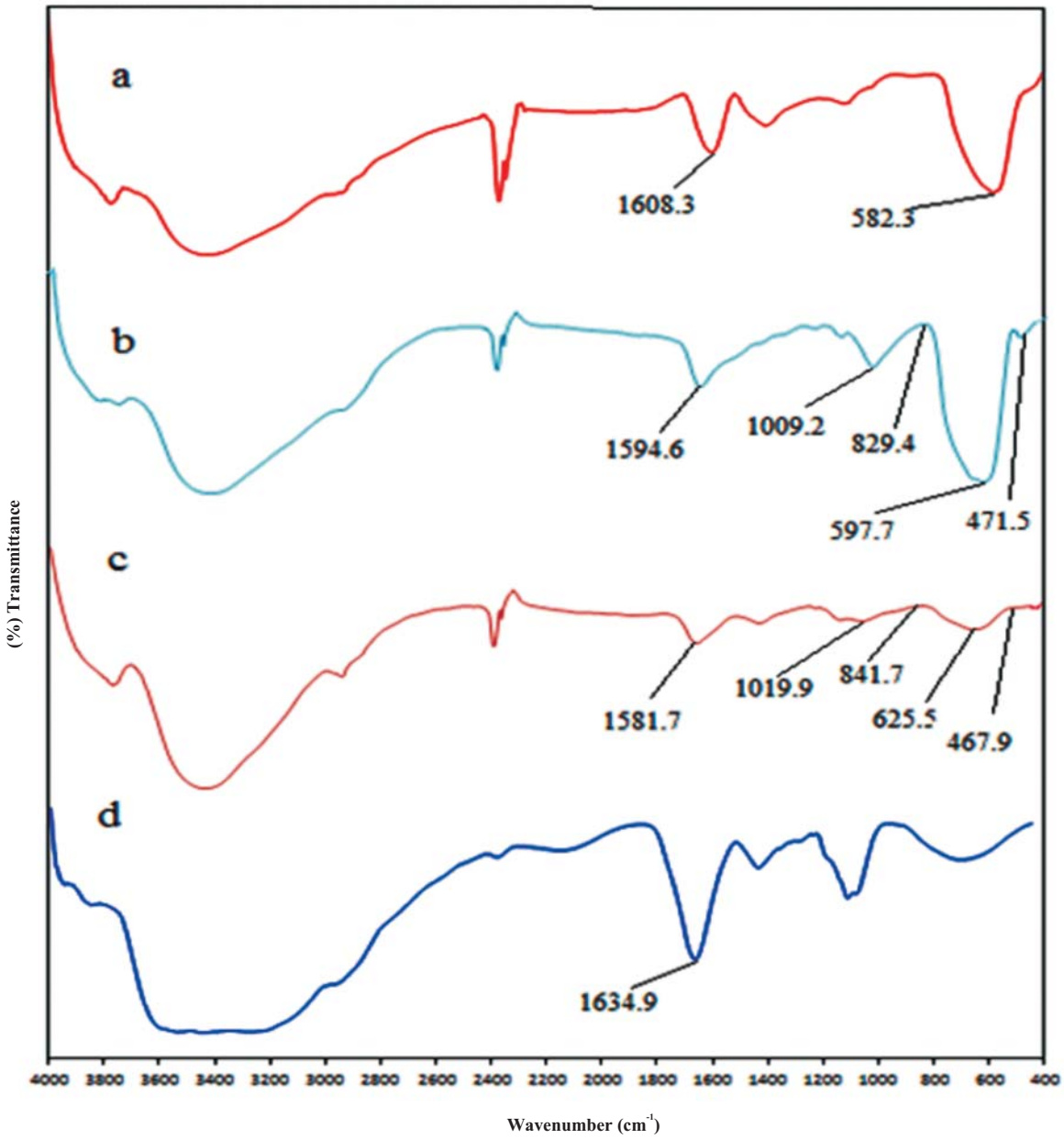

Fig. 3 : FT-IR spectra of (a) MNPs (b) AMNPs (c) laccase bound MNPs and (d) free laccase

bound laccasemg^{-1} AMNPs) x10^{-3}. The saturation of laccase loadings on the activated AMNPs surface was found to be 19.01 (mg bound laccase/mg AMNPs) x10^{-3}.

The weight ratio, relative percentage activity increased with increase in initial amount of laccase for immobilization onto AMNPs. The surface of AMNPs exerted a steric hindrance effect among the laccase molecules that ultimately blocked the active sites over AMNPs, which might result in non-availability of binding sites for the target substrates.

The effect of pH on the activity of free and immobilized laccase was investigated at varying pH from 2.0

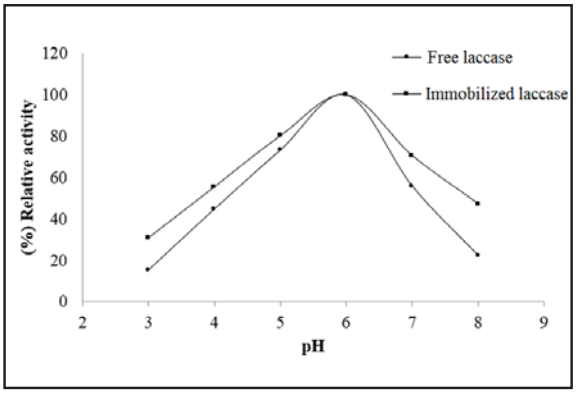

Fig. 4 : Effect of pH on the relative activity of free and immobilized laccase

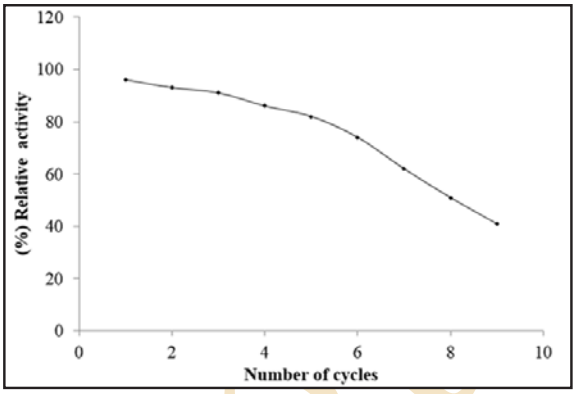

Fig. 6 : Residual activity of laccase bound AMNPs

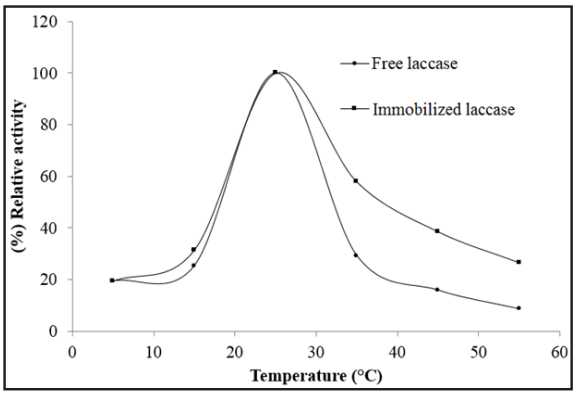

Fig. 5 : Effect of temperature on the relative activity of free and immobilized laccase

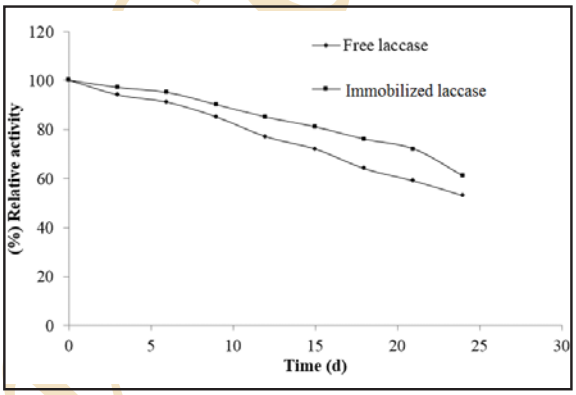

Fig. 7 : Storage stability of free laccase and immobilized laccase

to 8.0 as shown in Fig. 4. Free and immobilized laccase showed a maximum activity at pH 6.0, and the relative percentage activity of immobilized laccase remarkably increased over the free laccase.

In the acidic region, the AMNPs provide a favorable environment for laccase to catalyze guaiacol. In case of free laccase, the change in pH may not affect the shape of laccase but may alter the configuration or properties of guaiacol so that either guaiacol could not be able to undergo catalysis. But, immobilization of laccase onto AMNPs provides a larger surface area so that the aforementioned effect is nullified.

Thermal stability of free laccase and immobilized laccase were analyzed by incubating them at varying temperature (0-55°C) (Fig. 5). The optimum relative activity for free laccase and immobilized laccase was obtained at 25°C. As temperature increased, relative activity of free laccase decreased steeply due to denaturation of free laccase, and this was prevented with the coating on MNPs.

The operating stability of the immobilized laccase was compared with the free laccase is shown in Fig.6. The free laccase retained 53% of its residual activity after 24 hrs incubation, whereas the immobilized laccase retained 61% of its activity.

When a suitable micro-environment is provided by the carrier, the enzyme can maintain its maximum enzymatic activity. Further, reusability of immobilized laccase is shown in Fig.7. Immobilized laccase retained 80% of its initial activity after 5 cycles of oxidation of guaiacol and over 45% after 10 cycles.

Decolorization by biodegradation of dyes catalyzed by oxidoreductive laccase was determined by UV-vis spectral analysis. This analysis was performed after 24 hrs of complete decolorization in the range of 190 and 900 nm is shown in Fig.8. Degradation of dye was observed and a

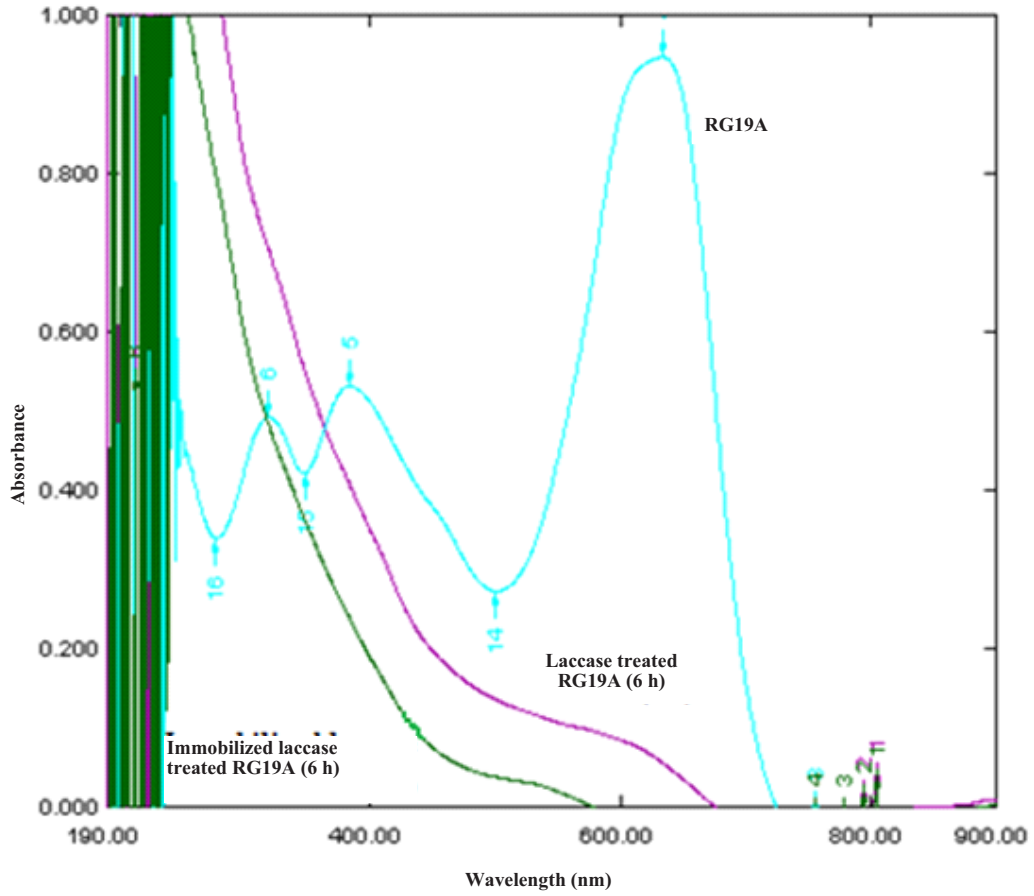

Fig. 8 : UV-vis spectral of initial RG19A and laccase treated solution of RG19A

decrease in the major peak or formation of new peaks was found (Kolekar *et al.*, 2012; Kumar *et al.*, 2016).

Decrease in the absorbance values or shift in the absorbance peak indicated that laccase had completely degraded RG19A into simpler compounds through oxidoreductive cleavage of azo bonds. Similar results were obtained when bacterial cells were used in decolorizing reactive azo dyes which showed the absorbance at 505 nm decreased, indicating the formation of new metabolites (Kumar *et al*., 2015; 2014b; 2012c).

Laccase was successfully immobilized on to the activated AMNPs synthesized by *in-situ* micro-emulsion method and was confirmed by the FT-IR spectrum. TEM and XRD analyses described that the immobilization did not cause any significant change in the size of MNPs. Maximum relative activity was observed in immobilized laccase through stability studies. Hence, these new findings will lead to the development of a more sophisticated system based on MNPs for the immobilization of laccase.

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