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Laccase immobilized peroxidase mimicking magnetic metal organic frameworks for industrial dye degradation

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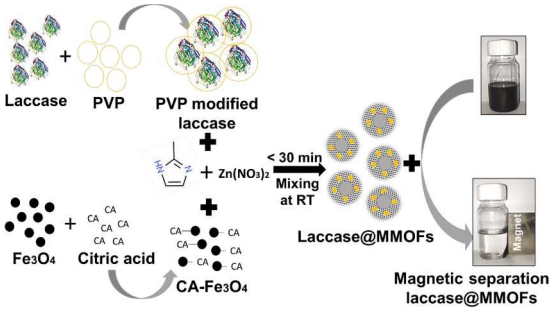
GRAPHICAL ABSTRACT



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ABSTRACT

In the present work, laccase was successfully immobilized in peroxidase mimicking magnetic metal organic frameworks (MMOFs) within 30 min using a facile approach. The integration of magnetic nanoparticles during synthesis significantly eases the separation of prepared biocatalyst using an external magnet. The immobilization of laccase was confirmed using different characterization techniques. The laccase@MMOFs found spherical in nature with an average particle size below 100 nm. The synthesized laccase embedded framework exhibits supermagnetic property with the saturation magnetization (Ms) of 34.12 emu/gm. The prepared bio-metallic frameworks maintain high surface area and thermal stability. The laccase@MMOFs was successfully exploited for the degradation of industrial dyes in batch and continuous mode with an average degradation efficiency of 95%. The prepared laccase structure had an excellent recyclability retaining upto 89% residual activity upto 10th cycle and can be stored at room temperature upto 30 days without any significant loss of activity.

1. Introduction

The rise in water pollution level is todays universal emergency. The intensified use of chemicals from variety of industries is one of the most serious issues. The extensive use of chemical dyes, such as brilliant green (BG), malachite green (MG), methylene blue (MB), crystal violet (CV) etc. in various industries like textile, leather, food, paper etc. has now been increased drastically. Due to resistance to the degradation; majority of dyes can cause severe damage to aquatic life and also manifest hazardous effects on human and plant life. Among this, the

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dye, crystal violet (CV) and methylene blue (MB) are used for a variety of applications. Both dyes are known for their recalcitrant properties as they remain in the surrounding environment for longer duration and pretense noxious effects on the surroundings (Chen et al., 2017; Navarro et al., 2017; Saber-Samandari et al., 2017). They are potent carcinogens, clastogens, mitotic poisons, enhances tumor augmentation in some species of fish and other aquatic moieties. They are also responsible to cause severe eye irritation, convulsions, cyanosis, skin sensitivity, methemoglobinemia and tachycardia in humans. Thus, both MB and CV are considered as biologically hazardous moieties. Moreover, because of their fine solubility in water, the discharge of wastewater containing these dyes directly into rivers and natural streams leads to severe environmental problems. Owing to the complex and aromatic structures and their chemical composition; these dyes are resistive against aerobic disintegration, heat, light and some oxidizing reagents. Thus, removal or degradation of these dyes is one of the major requirements during wastewater treatment.

Conventionally, these dyes were treated using chemical and physical methods including coagulation, adsorption, and oxidation. Recently, some advanced oxidation processes (AOPs) have also been studied such as ultrasonic irradiation, UV and ozone, photocatalytic degradation, separation using membranes, flocculation, biological treatment, photo-catalytic decomposition etc. (Asif et al., 2018; Saber-Samandari et al., 2017). However, the operational and raw material costs associated with these methods are high. Adsorption of dyes on solid supports such as bentonite, sawdust, chitosan, zeolite and clay are another methods which are simple, cost-effective and are easy to operate (Nguyen et al., 2016b). But these methods are only partial solution as these adsorbents requires subsequent disposal; hence it is yet another important task which needs to be considered.

Recently, the use of green biocatalysts from oxidase family such as peroxidase, laccase, glucose oxidase, phenol oxidase etc. for the degradation of variety of pollutants and dyes from wastewater has been studied (Rouhani et al., 2018). Considering the process efficacy of these biocatalyst, they have received a great attraction (Nguyen et al., 2016a,b). Amongst, the enzyme laccase (EC 1.10.3.2), a copper containing enzyme largely found in fungi, insects and plants has been studied extensively for the degradation of dyes and pulp. Laccase is also applied for the removal of lignin from biomass in textile and printing industries (Le et al., 2016; Li et al., 2018). Furthermore, it was explored for the oxidation of numerous phenolic and non-phenolics including variety of environmental pollutants and dyestuffs (Costa et al., 2019; Lonappan et al., 2018; Xu et al., 2013). However, the direct application of enzymes in its free form leads to not only generation of secondary waste but also can't be recycled once used in the reaction which increases the overall processing cost (Talekar et al., 2013b). Also, the activity of some of the enzymes is greatly inhibited by some operating parameters during waste water treatment.

The immobilization of enzymes on the solid support is one of the ways where the stability and performance of enzymes increases giving advantage of reusability (Liu et al., 2016). In recent years, the degradation of many industrially important dyes using immobilized laccase has been reported (Dai et al., 2016). Hence, the use of this biocatalyst in the immobilized form for dye degradation is a promising approach. Various immobilization methods such as adsorption, encapsulation, entrapment and cross-linking on solid support are reported (Ghodake et al., 2018; Le et al., 2016; Zhang et al., 2020). But, the conventional methods of enzyme immobilization undergoes several drawbacks (Ladole et al., 2017). In recent times, the use of magnetic nanoparticles (MNPs) in some modified adsorbents such as Chitosan-MNPs (Kadam and Lee, 2015), Zn-MNPs (Konicki et al., 2013), Graphene-MNPs (Yao et al., 2012) etc. are used for adsorbing pollutants and dyes from waste water. These adsorbents have strong absorptivity and easy separation from the aqueous solution due to their magnetic property (Hou et al., 2015). In recent time, immobilization of enzymes on nanomaterials such as nano-flowers (Santhosh et al., 2016), magnetic nanoparticles (MNPs) (Talekar et al., 2012), and metal-organic Frameworks (MOFs) (Qi et al., 2018), have shown very promising and fruitful results when applied in bio-catalysis. Mainly, enzyme embedded MOFs have received a wide acceptance owing to their ability of complete integration with the enzymes and hence expanded for various applications in bio-fields (Gascón et al., 2017).

The use of MOFs for enzyme immobilization are characterized by strong attraction with enzymes (to prevent it from leaching), large surface area and high enzyme loading efficiency (Hou et al., 2015). Taking advantage of magnetic property, the supermagnetic nano-particles (Fe_3O_4-MNPs) can also be incorporated in MOFs providing an ease in the separation and recycling of the prepared nanomaterials from the reaction mixture (Zhao et al., 2015). Recently, Nath et al., (2016) reported that certain chemical modifications in MOFs leads to exhibit or mimic natural enzymatic properties. Phosphotriesterase, hydrogenase, peroxidase, chymotrypsin, carbonic anhydrase etc. are some example of enzymes whose activities are mimicked by different MOFs (Nath et al., 2016).

Thus, in this work, an attempt to synthesize peroxidase mimicking MMOFs with slight modifications based on the previous work by Hou et al. (2015) has been made. In the synthesized MMOFs, laccase enzyme was embedded and has been reported for the first time. The prepared MMOFs and laccase immobilized MMOFs were characterized using various characterization techniques. The prepared catalyst was analyzed for the thermal and pH stability. The kinetics of the prepared enzyme MMOFs was also studied. Finally, the prepared magnetic biocatalyst framework has been utilized for the application in the degradation of industrial dyes such as methylene blue and crystal violet using batch and continuous mode of operation.

2. Materials and methods

2.1. Materials

The enzyme laccase was gifted by DBT-ICT-CEB, ICT, Mumbai, with an initial activity of 0.4 U/mg. Polyvinylpyrrolidone (PVP), 2-methylimidazole, o-phenylenediamine (OPD), $FeCl_3{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, methylene blue, crystal violet, fluorescein isothiocyanate (FITC), guaiacol and NaOH were purchased from Hi-media Pvt. Ltd., (India). Ethylene glycol, polyethylene glycol, sodium acetate, citric acid (CA), acetic acid, H_2O_2 and other chemicals and reagents were of analytical grade and procured from S.D. Fine Chemicals (India). All other chemicals used were of analytical grade with the highest purity.

2.2. Preparation of MNPs, MMOFs and laccase@MMOFs

The primary step while preparing MMOFs is synthesis of MNPs. As reported in our previous work, the synthesis of MNPs was carried out using chemical co-precipitation method (Ladole et al., 2015). The synthesized MNPs were washed and dried in vacuum oven and stored for further use. The peroxidase mimicking MMOFs were synthesized as per the method reported by Hou et al. (2015) with some modifications. The MNPs were first modified by citric acid. In brief, 500 mg of bare MNPs were mixed in 50 mL DI and 2.5 mL citric acid (CA, 2.0 M) was added drop wise for the surface modification. The reaction was performed under N_2 atmosphere at 90 °C. The CA modified MNPs were then dried and stored for further use. For MMOFs synthesis, 500 mg of CA modified MNPs were suspended in 30 mL ethanol:water (50–50%) solution containing HCL (0.05 mmol) and $Zn(NO_3)_2{\cdot}6H_2O$ (0.5 mmol). To ensure proper mixing, the mixture was stirred for 15 min in ultrasonic bath. After 15 min, the solution (10 mL, 50% ethanol) containing 2-methylimidazole (3 mM) and PVP (M_w 4000, 100 mg) was added drop-wise to the above mixture. The whole mixture was later stirred for 10 min, and the final product was decanted magnetically and washed with ethanol and deionized water (DI). Laccase embedded MMOFs were synthesized using the same method except the addition of laccase

(100 mg) in 2-methylimidazol and PVP solution. After reaction, the laccase@MMOFs were magnetically separated and washed with acetate buffer (0.1 M, pH 5.0) and stored for further use.

The percentage immobilization of laccase was calculated using the below equation:

$$\text{Percentage immobilization} = \frac{C_i V_i - C_0 V_0}{C_0 V_0} \times 100 \quad (1)$$

where, C_o & C_i = Final and initial and concentration of protein (mg/mL) and V_o & V_i = Final and initial volume of solution (mL).

The activity recovery of enzyme on chitosanMNPs was calculate using below equation:

$$\text{Activity recovery}(\%) = \frac{\text{Activity of immobilized enzyme (U)} \times 100}{\text{Activity of free enzyme used for immobilization(U)}} \quad (2)$$

2.3. Determination of laccase activity and peroxidase-like property of MMOFs

The laccase activity was calculated using guaiacol as the substrate (100 mM acetate buffer, pH 4.5, 10% acetone) (Shojaat et al., 2016). The absorption changes of the same was measured at 470 nm after 5 min of incubation. The unit activity of laccase (U/mg) is defined as the quantity of enzyme necessary to convert 1 mM of substrate in 1 min at ambient conditions. The activity of laccase@MMOFs was calculated by measuring the absorbance after magnetic decantation of the immobilized catalyst.

The peroxidase-like property of MMOFs was studied by dispersing MMOFs (10 mg) in sodium acetate buffer (0.2 M, pH 4.0) in the presence of OPD (0.5 mM, 400 µl) and H_2O_2. The reaction was carried out at 40 °C for 10 min. After 10 min, the MMOFs were separated from the mixture using an external magnet and the absorbance of the final product was measured at 454 nm. The changes in the absorbances of OPD oxidation was also measured at different pH (3.0 to 7.0) and temperature (30 to 70 °C) conditions.

2.4. Characterization of the prepared biocatalyst

The Fourier transform infrared (FT-IR) spectroscopy was used to confirm the successful binding of laccase in MMOFs (IRPrestige-21, Shimadzu) in the range of 400–4000 cm^{-1}. The field emission gun-scanning electron microscopy (FE-SEM) (S4800 Type-II, Hitachi High Technologies Corporation Tokyo, Japan) was used to study the surface morphology of MNPs and laccase@MMOFs. The crystal structure and phase changes of MNPs, MMOFs and laccase@MMOFs were studied by X-ray diffraction (XRD) analysis, (D-8 Advance, Bruker Axs GnbH, Berlin, Germany). The radiation source used was Cu-Kα and Ni as filter (k = 0.154 nm) and the scanning range was used between 20 and 80°. The Brunauer-Emmett-Teller (BET) method was applied to assess the specific surface area of MMOFs and laccase@MMOFs. The magnetic property of MNPs, MMOFs and laccase@MMOFs were measured at room temperature using vibrating sample magnetometer (VSM LakeShore-7407, USA). The thermogravimetric analysis study (TGA-DTG-60H EME) was also used to confirm enzyme immobilization on the synthesized support. In TGA, the MNPs and enzyme embedded MMOFs were allowed for percentage weight loss by heating over the range of 30 to 500 °C under N_2 atmosphere at heating rate of 10 °C/min. The UV-double beam spectrophotometer (UV-1800, Shimadzu) was used to measure all the absorbances of the liquid samples.

2.5. Preparation of fluorescence labelled immobilized laccase

Recently, the technique of enzyme tagging using fluorescence dye and observing the same under confocal microscopy has been used to confirm the successful enzyme immobilization (Kadam et al., 2017). In brief, the laccase embedded MMOFs (25 mg) was dispersed in phosphate buffer (1 mL, 0.1 M, pH 7.0). The whole mixture was then added to carbonate buffer (2 mL, 0.5 mM, pH 9.0) and stirred gently. After proper mixing, the freshly prepared FITC solution (0.5 mL, 1 mg/mL of 0.5 mM carbonate buffer, pH 9.0) was added and stirred at 28 ± 2 °C. Further, the unreacted FITC was removed by washing laccase@MMOFs (after separation using external magnet) with carbonate buffer (0.5 mM, pH 9.0). Finally, the laccase@MMOFs were observed under confocal laser scanning microscope.

2.6. Enzyme kinetics

The kinetics of laccase (free and laccase@MMOFs) *viz.* maximum velocity of reaction (V_{max}) and Michaelis constant (K_m) was estimated by calculating initial rates of the reaction at various substrate concentrations (guaiacol) at optimized pH and temperature, followed by plotting a double reciprocal Lineweaver-Burk plot to estimate the values (Muley et al., 2019).

2.7. Stability of laccase@MMOFs

The immobilized enzymes are known to exhibit an enhanced thermal and pH stability to carry out a variety of applications. To determine the extent of stability and activity, the immobilized enzymes were subjected to incubation over a wide range of pH (3.0–7.0) and temperature (40–80 °C) conditions for a known period of time. The activities of the enzyme were then measured using standard assay protocol (Xia et al., 2016).

2.8. Determination of thermal deactivation kinetics and thermodynamics constants

The stability of laccase (free and immobilized) at various temperature range (60, 70 and 80 °C) was studied by incubating them in acetate buffer (0.1 M, pH 4.5) for 60 min (Talekar et al., 2013a). The laccase@MMOFs were withdrawn after every 10 min, cooled and assayed for residual activity using standard enzyme assay. The deactivation rate constant (k_d) was determined by plotting a semi-log residual activity (%) versus time and their slope was taken as k_d. Using this rate constant, the half-life of enzyme was calculated as $0.693/k_d$. The enzyme half-life is the time necessary to reduce activity of enzyme to its half. Also, the enzyme inactivation energy (E_d) (free and immobilized) was estimated using Arrhenius plot, from the slope of ln(k) against 1/T. The thermodynamics parameters *viz.* enthalpy for activation (ΔH°), Gibbs free energy (ΔG°) and entropy (ΔS°) for the inactivation of native and immobilized laccase affected by heat treatment were calculated using Eyring's transition state theory.

2.9. Dye degradation study in batch and continuous mode

The degradation study of the selected dye was carried out using only MMOFs and then laccase@MMOFs. For this, a known concentration of CV and MB (50 mL) was taken separately in 250 mL Erlenmeyer flasks. To this, a specific amount of laccase@MMOFs and MMOFs were added separately and kept for continuous stirring using an overhead glass stirrer for 30 min. The supernatant was collected after each 5 min time interval for further analysis by separating MMOFs and laccase@MMOFs from the solution using an external magnet. The absorbances of the dye CV (590 nm) and MB (663 nm) before and after treatment using MMOFs and laccase@MMOFs were measured using UV–Vis spectrophotometer (Chen et al., 2017).

The percentage degradation was calculated using the following equation.

$$Degradation\,(\%) = \frac{A_0 - A_t}{A_t} \times 100 \quad (3)$$

where, A_0- Initial absorbance of the dye, A_t- Absorbance of the dye after 't' time interval.

Besides batch mode, the efficacy of laccase@MMOFs for dye degradation was also evaluated in continuous mode of operation. The prepared biocatalyst (known amount) was packed inside two separate glass columns and both the ends of columns were packed with filter papers which were pre-wetted in both CV and MB dye seperately to avoid leakage of the nano-biocatalyst (Wang et al., 2011). The columns were then connected with silicon tubing from both the ends and the dyes were passed from the bottom using peristaltic pumps separately. The other end of silicon tubing was kept in a beaker to collect the samples (Fig. 4). The concentrations of both dyes were measured and plotted after each interval of 5 min spectrophotometrically.

2.10. Reusability and storage stability

The main purpose of enzyme immobilization is to exploit the advantage of its reusability. From an economical and industrial point of view, the reusability of immobilized enzyme is very crucial. To study this, the enzyme embedded MMOFs were used in the reaction for several number of cycles in the batch mode. After completion of each reaction, the catalyst was decanted magnetically, washed twice with acetate buffer (pH 4.5, 0.1 M) and reused to carry out the next reaction with the fresh substrate (Sadeghzadeh et al., 2020).

Another important factor after enzyme immobilization is the storage stability study. Enzyme storage is one of the key factors which needs to be consider. To study the storage stability, laccase@MMOFs were kept in acetate buffer (pH 4.5, 0.1 M) for 30 days. After every five days, the immobilized biocatalyst was subjected for enzyme activity analysis (Ladole et al., 2018).

3. Results and discussion

3.1. Preparation and characterization of laccase@MMOFs

The laccase@MMOFs were synthesized as per the previous protocol given by Hou et al., (2015). In the given approach, MNPs were first modified with citric acid (CA) and then used for MMOFs synthesis. In comparison to other processes of MOFs preparation which usually takes around 2–3 days (hybrid nanoflowers, multi-enzymatic systems and co-embedded methods), the present method is a one step process which is significantly quick and can be done within 30 min and produces nano sized enzyme embedded MMOFs.

Initially, the peroxidase mimicking MMOFs were prepared and laccase was embedded into them. Thus, the prepared laccase@MMOFs exhibits both peroxidase and laccase activity together. Compared to other processes where adsorption of enzymes is carried out, in this protocol a de-novo approach was utilized in which the enzymes get immobilized in MMOFs (Hou et al., 2015). This process not only prevents the leaching of enzymes (as enzyme gets entrapped inside the matrix) but also provides a greater stability and protection for the enzyme over a wider range of pH and temperature. This process delivers a huge scope of enzyme and MMOFs for many other applications. Alongside, the MNPs help for the easier separation of laccase@MMOFs from the reaction mixture using an external magnetic field which is remarkable separation process rather than filtration or centrifugation techniques (Talekar et al., 2017).

The synthesis of MMOFs starts with the preparation of MNPs and then modifications of the synthesized MNPs. The modifications of MNPs was confirmed by FTIR analysis. The characteristic peaks at 2856 and 2925 cm^{-1} of CA-MNPs is attributed to citric acid and band at 580 cm^{-1} is assigned to stretching of Fe-O bond. The appearance of absorption bands at 2926 and 3062 cm^{-1} corresponds to aliphatic and aromatic stretch, also bands at 900–1300 cm^{-1} and 1396 cm^{-1} attributed to the plane bending and stretching of imidazole ring, respectively (Hu et al., 2011). The Zn-N stretch was confirmed from the appearance of band at 421 cm^{-1}. The presence of band at 997 cm^{-1} and 1450 cm^{-1} confirms the formation of laccase@MMOFs. From all the spectra, it was confirmed that all the functional groups were present which were associated to MMOFs and laccase@MMOFs. The XRD analysis was carried out to study the crystallinity and purity of MMOFs and laccase@MMOFs. From XRD patterns it can be seen that there was no significance difference considering the crystallinity and crystal structure of both materials. This can be assigned to the super-positioning of the face centered cubic Fe_3O_4 nanoparticles and the prepared MOFs structure (Hou et al., 2015). Also, the prepared MNPs were found in the pure phase (Ladole et al., 2017).

The surface morphology and changes in the structure of MNPs after modification was observed using scanning electron microscope. Results indicated that MNPs before modification were spherical and well distributed with an average particle size of upto 100 nm (Sahu et al., 2016). From the SEM images of MMOFs, it was also confirmed that the MNPs were uniformly coated with a very thin grey layer shell of the prepared framework. Similar result was reported by Hou et al., (2015) in which they have also observed an appearance of a thin grey layer shell on the prepared MMOFs structure. In contrast to MOFs, the significance of MMOFs for being magnetic in nature make these composite particles quite attractive due to an ease in the separation from the reaction mixture using an external magnet. The presence of elements while preparing MMOFs was confirmed using EDS. The occurrence of C, N, Zn, and O peaks confirmed the successful synthesis of MMOFs (Hou et al., 2015). Also, the peaks of Fe and part of O is attributed to the presence of Fe_3O_4 nanoparticles. Moreover, the porosity of MMOFs and laccase@MMOFs was estimated using BET analysis. Results indicated that the surface area (423.72 m^2g^{-1}) of MMOFs was higher than laccase@MMOFs (343.27 m^2g^{-1}). The decrease in BET surface area after laccase immobilization confirmed the successful loading of laccase in MMOFs (Hou et al., 2015).

The magnetic nature of bare MNPs, MMOFs and laccase@MMOFs were studied using VSM analysis. From results one can see that the MNPs possess highest saturation of magnetization (Ms) of 60.12 emu/g followed by MMOFs 43.23 emu/g and laccase embedded MMOFs 35.43 emu/g respectively. The reduced value of Ms of MMOFs and laccase@MMOFs is attributed to successful loading of laccase and other components on MNPs during preparation (Qiu et al., 2019). Moreover, from the present results, it was also depicted that the magnetic hysteresis curves of the synthesized material did not show coercivity, remanence and hysteresis which specifies the supermagnetic behavior at room temperature (Ladole et al., 2018). Also, it can be seen that the laccase@MMOFs were quickly attracted towards the external magnet; thus, the immobilized laccase can be easily separated from the reaction mixture using an external magnetic force.

Thermogravimetric analysis (TGA) is one of the known methods to confirm the successful loading of enzymes. From results it was observed that the total weight loss of bare MNPs is around 2.9%. In which, the initial weight loss of 1.8% was observed below 130 °C. This might be due to the loss of adsorbed water from sample. But, after this, an unpredicted weight gain was observed in the temperature range of 140 to 230 °C. The reason behind the weight gain can be ascribed to the oxidation of Fe_3O_4 to Fe_2O_3 (Caruntu et al., 2004). After 230 °C, the weight loss again started and reduced to 2.9% which was due to the dehydration of –OH groups from the surface and loss of adsorbed water. Further, the TGA curves for MMOFs and laccase@MMOFs showed the weight reduction of 9.13 and 12.98% respectively. This weight loss was observed in the temperature range of 200 to 500 °C. From results, it can be seen that the weight loss occurred below 200 °C could be due to the loss of water adhered on the surface of the synthesized composite (Badruddoza et al., 2010). Further reduction in the weight at high temperature can be occurred because of thermal composition of various

compounds such as PVP, 2-methyl imidazole and citric acid in MMOFs and an additional weight loss of enzyme was observed in laccase@MMOFs. The extra weight reduction of laccase@MMOFs implies successful loading of laccase in MMOFs. This data gives an additional confirmation towards the immobilization of laccase@MMOFs.

Along with these techniques, recently, the fluorescein isothiocyanate (FITC) labelling method is used to ensure successful enzyme loading and surface functionalization of enzymes after immobilization. The property of FITC dye to bind on the amino groups of enzymes helps to identify enzyme immobilization on the support. The FITC labelled material was analysed under excitation (488 nm) and emission (535 nm) wavelengths using confocal laser scanning electron microscopy (CLSM) (Kadam et al., 2017). Results showed that, bare MNPs showed no fluorescence however, a vibrant green fluorescence emission was observed for laccase@MMOFs. This indicates an effective loading of laccase in MMOFs (Ladole et al., 2017). Also, some of the images showed the porous nature of the laccase embedded MMOFs which again confirmed porous nature of the prepared laccase@MMOFs.

3.2. Optimization of enzyme loading on MMOFs

In order to get good enzyme loading with activity recovery during the process of immobilization, it is important to optimize enzyme concentration and thus the optimization of MMOFs to enzyme ratio was studied (Ladole et al., 2018). The amount of enzyme loaded or immobilized is directly proportional to the activity recovery (Talekar et al., 2017). From Fig. 1, it has been found that highest recovery of laccase activity was achieved at 1:3 MMOFs to laccase ratio. The reduction in the activity of laccase below this ratio was due to less loading of enzyme in MMOFs during immobilization process. The enzyme loading was increased significantly above 1:3 ratio. From results, it has been also observed that with an increase in enzyme loading; the activity retained got reduced. The most probable reason for this could be the excess enzyme loading which leads to the formation of enzyme aggregates that hinders enzymes active sites and hence reduction in the activity recovery (Talekar et al., 2017).

3.3. Peroxidase-like property of MMOFs

The main purpose of synthesizing these MMOFs was that they actually mimic peroxidase-like property. The enzyme peroxidase has been used for the degradation of variety of dyes. Hence, by immobilizing laccase in this peroxidase mimicking MMOFs, it is possible to generate a synergistic effect of peroxidase and laccase enzyme together for various applications. In order to confirm the peroxidase-like activity of the synthesized MMOFs, the oxidation of OPD in the presence of H_2O_2 was carried out and colourimetrically tested at 454 nm (Hou et al., 2015). The reaction of MMOFs with OPD produces an orange color product. The absorbance of the reaction increases with respect to time and within 10 min, a distinct absorbance was obtained which clearly indicates the peroxidase-like activity of the prepared MMOFs. Additionally, the stability of MMOFs over a range of pH and temperature was studied. From result it was found that the maximum absorbance was observed in the range of pH 3.5 to 4.5 which can be attributed to the influence of acidic pH towards the oxidation of OPD. Besides, the absorbance was increased with an increase in temperature upto 55 °C and above which it starts declining gradually. Similar trend of result was obtained and reported by Hou et al., (2015). Hence from all these results, MMOFs appeared to show peroxidase-like activity and the synergistic effect of peroxidase-mimicking MMOFs and laccase can be used for dye degradation study.

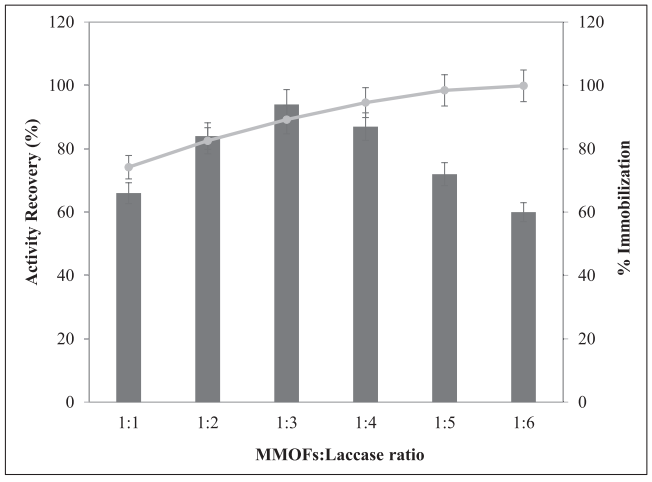

Fig. 1. Optimization of laccase loading during immobilization process. In the double y-axis graph, the bar repesents the activity recovery and the line represent the amount of enzyme immobilized in MMOFs during the immobilization process. The measurements were performed in triplicate and the error bar represents the percentage error in each set of readings.

3.4. Stability of laccase@MMOFs

It is necessary to carry out each enzyme catalyzed reaction at optimum processing parameters as they show maximum activity at their optimized pH and temperature conditions (Xia et al., 2016). From the present results, it has been found that the optimum pH of laccase@MMOFs was at pH 4.5. However, the optimum activity of free laccase was found at pH 5.0. Results indicated that there was a slight alteration in the optimum pH value of laccase towards acidic side after immobilization (Fig. 2A). The reason for the shifting of pH value was might be due to the buffering action on the surface of the prepared

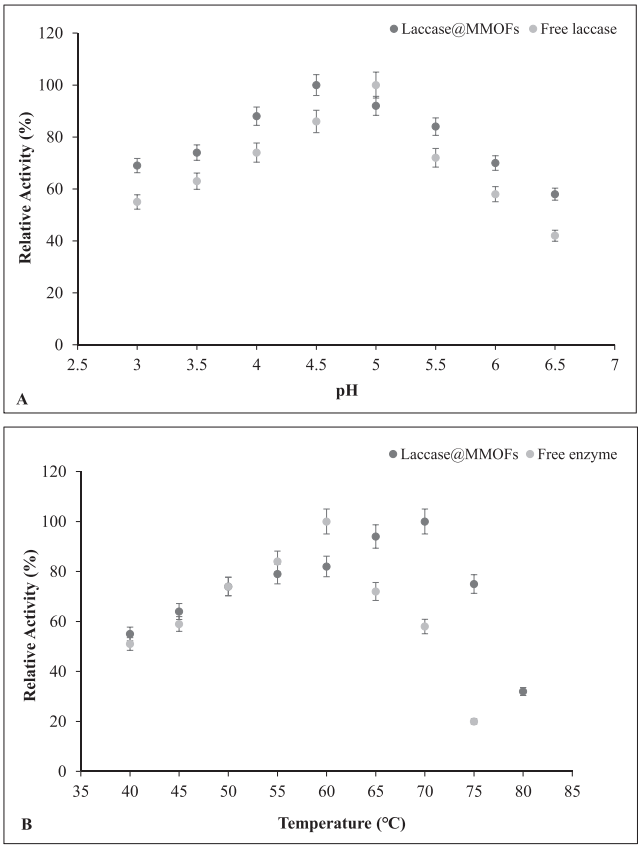

Fig. 2. Optimization of pH (A) and temperature (B) of free laccase and laccase@MMOFs. The measurements were performed in triplicate and the error bar represents the percentage error in each set of readings.

MMOFs and the force between MMOFs and enzyme molecules. The other reason could be the changes in the ionization of basic and acidic amino acids side chains in the surrounding environment near the prepared MMOFs or at the active sites of laccase which might be responsible for the shift in the optimal pH towards acidic side. Owing to the strong affinity between prepared framework (MMOFs) and laccase, it provides much robust interface with MMOFs and free laccase. Also, the microenvironment provided by MMOFs decreases the drastic conformational changes than free laccase, thus resulted in resistance towards increased pH (Zheng et al., 2012). Besides, the activity laccase@MMOFs was found to be on the higher side at all pH range compared to the free laccase.

Another important parameter evaluated was the temperature optimization. From Fig. (2B), one can see that laccase@MMOFs showed excellent stability over the entire temperature range and especially at elevated temperature and showed maximum activity at 70 °C compared to the free laccase which was at 60 °C (Fig. 2B). The activity of laccase@MMOFs and free laccase starts decreasing beyond 70 °C and 60 °C respectively which might be due to the denaturation of enzyme at higher temperature (Munde et al., 2017). The difference in the activity values of laccase@MMOFs and free enzyme might be attributed to the affinity property of the synthesized MMOFs towards enzyme laccase and hence more stability of the enzyme in the surrounding environment. Also, the MMOFs protects enzyme embedded in it thereby preventing enzyme denaturation at elevated temperature.

3.5. Enzyme kinetics

To investigate the enzyme activity in the free and immobilized form, the enzyme kinetics was studied by estimating Michaelis constant (K_m) and maximum velocity of reaction (V_{max}). These values for laccase (free and immobilized) were estimated by measuring the initial reaction rates with varying amounts of the substrate (Table 1). The K_m value of laccase@MMOFs was found slightly more compared to free laccase. Lower the K_m value; higher is the affinity of enzyme towards substrate molecule. Similar results were reported by Xia et. al., (2016) in which they have obtained slightly high K_m value of laccase after immobilization. However, some study reported very high K_m values compared to free enzymes (Liu et al., 2016). In the present study, the greater surface area provided by MMOFs to interact with the substrate molecules deliver strong affinity and interaction between enzyme and substrate molecules; hence, marginal increased in the K_m value compared to the free form.

Along with this, the V_{max} of laccase@MMOFs were recorded higher compared to the free form. Hence, it specifies a higher rate of reaction using immobilized enzymes at similar substrate concentration compared to the free form. It also indicates an enhanced catalytic efficiency as given by the V_{max}/K_m ratio. This shows an increased interaction efficiency between substrates and enzymes after immobilization. The increased value of V_{max} could be due to the high surface area to volume ratio and higher accessibility of substrate to interact with the enzyme molecules.

3.6. Thermal inactivation kinetics of free laccase and laccase@MMOFs

It is well known that the use of enzymes at high temperature lowers the catalytic activity. This reduced activity is irreversible as there is disruption and alteration in the active sites of enzymes at elevated temperature. Hence, it is very important to check the activity and stability of enzymes at different temperatures and this can be done by studying the thermal inactivation kinetics of the enzymes. For this, the enzymes (free and immobilized) ability to attain the denaturation process under the influence of heat treatment in buffer (without substrate) at different intervals of time was observed and studied. The inactivation kinetics profile of free laccase and laccase@MMOFs indicates a steady decrease in the activity at different studied temperatures. Results indicated that k_d values of laccase in the immobilized form is less as compared to its native form (Table 2). Hence, the $t_{1/2}$ values of laccase@MMOFs were higher at all studied temperature range compared to free form. The average half-life of laccase@MMOFs was 2.3-folds higher compared to its native form (Talekar et al., 2017). This indicates that, the entrapment of laccase in the core of MMOFs protects the enzyme to maintain the active tertiary structure form even at high temperature conditions; hence, exhibiting high thermal stability.

Table 1
Enzyme kinetics constant of free laccase and laccase@MMOFs.

Enzyme form	K_m (mg/mL)	V_{max} (μmol/min)
Free laccase	0.49 ± 0.13	29.85 ± 0.59
Laccase@MMOFs	0.74 ± 0.21	43.48 ± 0.46

3.7. Different thermodynamic parameters of free and immobilized laccase

The enzyme inactivation energy (E_d) is the lowest energy necessary to start the enzyme denaturation. Higher the E_d value; higher is the thermal stability (Muley et al., 2019). The E_d of enzyme in free and immobilized form was measured using a linear fit of Arrhenius plot. The temperature range selected was 60, 70 and 80 °C. The E_d value of laccase@MMOFs was found to be 41.19 kJ/mol which is 1.2-fold high compared to the free laccase. This indicates that an additional energy was needed to break the immobilized form of laccase compared to its native form. This can be attributed to the affinity of the synthesized MMOFs towards laccase which provides an additional shield and hence prevent it from the denaturation. The enzyme stability during storage or during reaction is depend on the equilibrium between the steady and non-steady force which are dependent on several bonds or linkages and folding or unfolding state of unit molecules (de Castro et al., 2015; Muley et al., 2019). Different thermodynamics parameters such as ΔH° (Enthalpy of activation), ΔG° (Gibbs free energy) and ΔS° (Entropy of activation) gives a comparative impression about the stability and functionality of enzymes at different operational environments when assessed with the thermal inactivation kinetics parameters (Table 3). By using the thermal inactivation kinetic parameters, these parameters were calculated based on the Eyring's transition state theory which helps to determine the interaction of enzymes after immobilization. The ΔH° values of both free and immobilized laccase declined steadily with an increment in the temperature. This indicates the requirement of small amount of energy for enzyme denaturation at elevated temperature. In view of the deactivation process, the positive E_d values and higher ΔH° of laccase@MMOFs indicates the high thermal stability. Also, ΔH° is linked to a degree of breaking the linkages related to transition state stabilization *viz.* from an active stable state to denatured inactive state (Muley et al., 2018).

The ΔG° values for both native and immobilized laccase increases with an increase in temperature. But, compared to free laccase, the ΔG° values of laccase@MMOFs are higher for each studied temperature. This gives an idea about the additional energy requirement for the denaturation of immobilized laccase at higher temperature. The ΔS° values were found negative which was due to an increase in the values of ΔG°, this might be because of the formation of enzyme aggregates during immobilization process. This enhanced entropy value shows more enzymes in the transition activated state (Maisuria and Nerurkar, 2012). The negative values of ΔS° are related with decrease in the disorder or randomness of the enzyme system (Mohapatra, 2017).

3.8. Dye degradation study

The synthesized laccase@MMOFs were evaluated for the degradation of selective industrial dyes *viz.* crystal violet and methylene blue. The degradation of each dye was carried out in two approaches i.e. a

Table 2
Thermal inactivation kinetics of free laccase and laccase@MMOFs.

Enzyme form	Thermal inactivation kinetics parameters	Temperature (°C)			E_d (kJ/mol)
		60 °C	70 °C	80 °C	
Free Laccase	K_d (min^{-1})	0.037 ± 0.0005	0.054 ± 0.0014	0.075 ± 0.0002	
	$t_{1/2}$ (min)	18.73 ± 4.91	12.91 ± 4.31	9.29 ± 7.11	34.28 ± 3.42
	D-value	62.23 ± 2.55	42.88 ± 12.15	30.87 ± 10.61	
Laccase@MMOFs	K_d (min^{-1})	0.0153 ± 0.0002	0.026 ± 0.0001	0.036 ± 0.0042	
	$t_{1/2}$ (min)	45.29 ± 5.62	27.28 ± 1.89	19.5 ± 4.18	41.19 ± 3.12
	D-value	150.50 ± 3.75	90.65 ± 10.21	64.86 ± 6.39	

batch and continuous mode. For batch mode, a known amount of the as prepared MMOFs and laccase@MMOFs were mixed in both dyes separately. Under all optimized conditions, the degradation was carried out for the period of 30 min with continuous stirring. The highest degradation of methylene blue (91%) and crystal violet (93%) was obtained in 20 min. The degradation was steady beyond 20 min of treatment time. However, with an individual application of MMOFs, the degradation of MB and CV was only 44% and 53% even though incubated for 30 min (Fig. 4A). This indicates that the enzyme laccase was responsible for the additional degradation of the dyes and hence more degradation (%) was achieved with the application laccase@MMOFs rather than native MMOFs.

For continuous treatment mode, the laccase@MMOFs were packed in the glass column and each dye was passed from the bottom of the column using silicon tube separately. The schematics of the continuous mode of operation is represented in Fig. 3. The absorbance of samples collected on the other side was then analyzed after each interval of 5 min. The flow rate of the supply of dye was adjusted using a peristaltic pump. From results, it was found that the highest degradation for both MB (96%) and CV (98%) was achieved in 15 min and was steady after that. The degradation with an individual approach of MMOFs obtained was 47% for MB and 56% for CV (Fig. 4B). The increase in the degradation of both dyes using continuous mode might be due to high mass transfer rate and superior interaction of dyes with laccase@MMOFs and MMOFs. This indicates that the laccase enzyme was accountable for further degradation of both dyes in laccase@MMOFs and showed good degradation efficacy. Similar trend of results was obtained by Chen et al., (2017) where the laccase immobilized graphene oxide magnetic nanoparticles were used for the degradation of certain dyes but the time required for degradation was almost 9 times more compared to laccase@MMOFs driven degradation used in the present work. The reduction in the time requirement for degradation using laccase@MMOFs could be attributable to the high surface area of the prepared laccase@MMOFs and affinity of enzymes towards substrate molecule which might have enhanced the interaction of substrate with the enzyme molecules. Also, the prepared MMOFs possesses peroxidase-like property which helped to build a synergistic effect of peroxidase and laccase together to give higher degradation in less time.

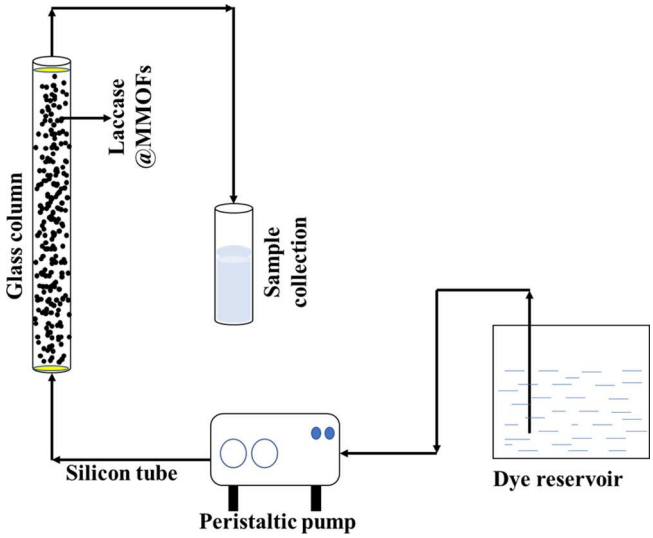

Fig. 3. Schematics of the set-up for continuous dye degradation using laccase@MMOFs.

3.9. Reusability and storage stability

Enzyme reusability is one of the main advantages of immobilized enzymes. This property of reusing enzymes several times is an important factor and is helpful for many industrial applications from an economic point. Hence, the extent of reusability for laccase@MMOFs was also evaluated. Results indicated that the laccase@MMOFs can be used upto 10^{th} cycle of reusability retaining upto 89% remaining activity (Fig. 5). Also, the residual activity was maintained as original upto 4^{th} cycle. The excellent reusability of this biocatalyst indicated that the MMOFs help to retain the original activity of the enzyme and protects the enzymes from deactivation hence can be used multiple times. This also confirms the laccase@MMOFs are robust in nature and can be used for multienzyme catalytic system simultaneously (Chen et al., 2017; Liu et al., 2016).

Storage of enzymes is yet another important factor as enzymes are sensitive towards surrounding environment and can get deactivated after certain time when stored at room temperature or above; hence, it

Table 3
Thermodynamic parameters of free laccase and laccase@MMOFs.

Thermodynamic parameter	Enzyme form	Temperature (°C)		
		60 °C	70 °C	80 °C
ΔH° (kJ/mol)	Free Laccase	31.50 ± 1.12	31.42 ± 1.41	31.34 ± 0.51
	Laccase@MMOFs	38.41 ± 0.41	38.33 ± 0.11	38.24 ± 0.71
ΔG° (kJ/mol)	Free Laccase	89.57 ± 1.41	91.29 ± 0.29	93.07 ± 1.32
	Laccase@MMOFs	92.02 ± 0.41	93.42 ± 0.45	95.25 ± 1.31
ΔS° (J/mol/K)	Free Laccase	−174.39 ± 1.12	−174.54 ± 0.24	−174.88 ± 1.88
	Laccase@MMOFs	−160.99 ± 0.41	−160.62 ± 0.11	−161.48 ± 0.91

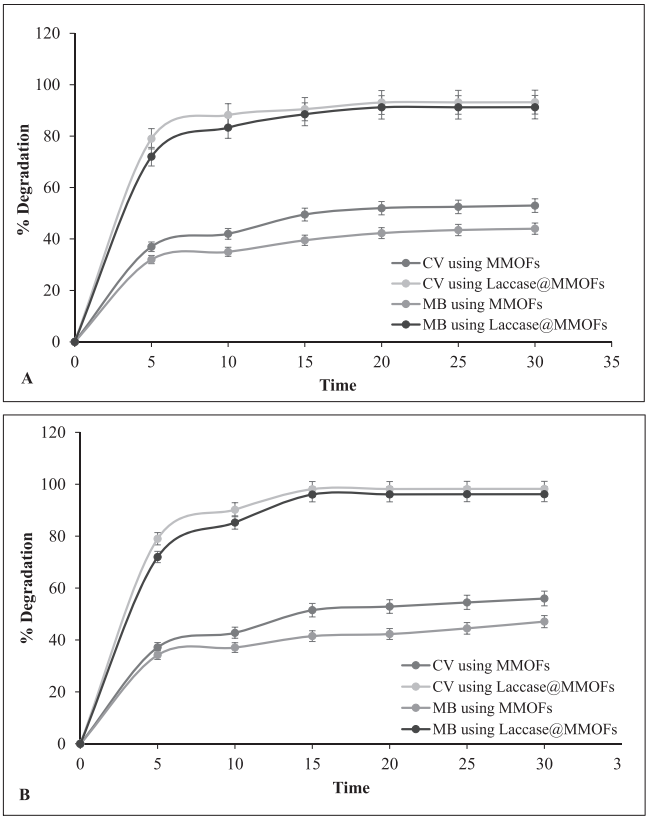

Fig. 4. Dye degradation MB and CV using MMOFs and laccase@MMOFs in batch mode (A) and continuous mode (B) The measurements were performed in triplicate and the error bar represents the percentage error in each set of readings.

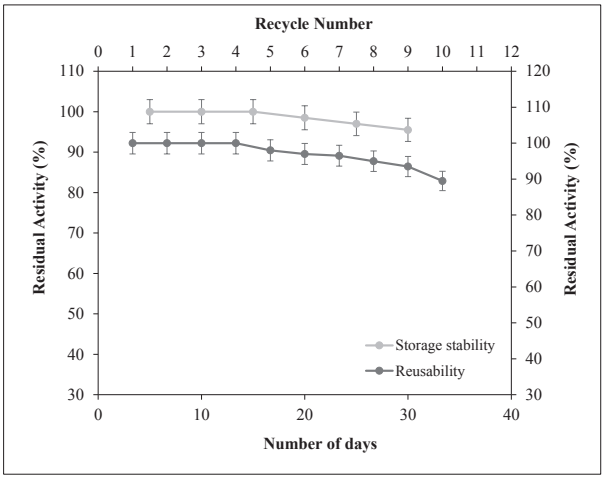

Fig. 5. Reusability study upto 10 cycles and storage stability study upto 30 days of laccase@MMOFs. The measurements were performed in triplicate and the error bar represents the percentage error in each set of readings.

is recommended to be stored at refrigerated conditions. Enzyme immobilization can help to protect enzymes from such external environment and can preserve the activity to some extent. Thus, the stability of immobilized laccase@MMOFs by storing at room temperature for the period of 30 days has been studied (Fortes et al., 2017). Results indicated that laccase@MMOFs can retain its original activity upto 25th day and there was slight reduction in the activity till 30th day (Fig. 5). This proves that the MMOFs structure is capable of providing a good environment to the enzymes hence retaining the enzyme's original activity from the buffering action (Rouhani et al., 2018; Xu et al., 2013). From these studies, we can say that the enzyme embedded MMOFs can be widely used for variety of industrial applications.

4. Conclusion

In the current work, peroxidase mimicking MMOFs were synthesized using a simple approach. In the synthesized MMOFs, enzyme laccase was successfully immobilized. The occurrence of enzyme in the synthesized bio-metallic framework was confirmed using various characterization techniques. The prepared biocatalyst possesses high temperature and pH stability. The thermal stability and catalytic activity of the prepared biocatalyst showed remarkable improvement. The prepared laccase@MMOFs structure was found very efficient for the degradation of industrial dyes compared to peroxidase mimicking MMOFs. The laccase@MMOFs showed an excellent reusability and storage stability. This catalyst can be explored for the potential applications in various industries.

CRediT authorship contribution statement

Conceptualization: Mayur R. Ladole, Pravin B. Pokale, Sujata S. Patil, Aniruddha B. Pandit. Data curation: Mayur R. Ladole, Pravin B. Pokale, Sujata S. Patil, Prasad G. Belokar. Formal analysis: Mayur R. Ladole, Sujata S. Patil. Investigation: Prof. Aniruddha B. Pandit. Methodology: Mayur R. Ladole, Pravin B. Pokale, Sujata S. Patil, Prasad G. Belokar. Supervision: Prof. Aniruddha B. Pandit. Validation: Mayur R. Ladole, Sujata S. Patil, Pravin B. Pokale, Prasad G. Belokar. Writing - original draft: Mayur R. Ladole, Pravin B. Pokale, Sujata S. Patil, Prasad G. Belokar, Aniruddha B. Pandit. Writing - review & editing: Mayur R. Ladole, Pravin B. Pokale, Sujata S. Patil, Aniruddha B. Pandit.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.124035.

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