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Improved Catalytic and Dye Decolorization Properties of Chitosan Beads Immobilized Manganese Peroxidase from *Ganoderma lucidum* IBL-05

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Abstract

A fungal manganese peroxidase (MnP) from *Ganoderma lucidum* IBL-05 was immobilized on glutaraldehyde activated chitosan beads by cross-linking method. Chitosan beads (2 mm size) activated with 2% glutaraldehyde for 3h demonstrated maximum immobilization yield (⊠82.5%). The immobilized MnP displayed optimum activity at pH 7.0 and 60°C temperature whereas the free MnP had pH 5.0 and 35°C temperature as its optima. The Km and Vmax values for immobilized MnP were 98 mM and 695 U/mL, respectively. The immobilization was confirmed by comparing the surface morphologies of free and MnP bound chitosan beads by Scanning Electron Microscope (SEM). The free and immobilized MnP were also used for decolorization of different textile dyes. More than 90% decolorization of all dyes was achieved with immobilized enzyme in 12 h time. The immobilized MnP retained 65% of its activity after seven successive dye-decolorizing batch operations and exhibited remarkable storage stability after 60 days at 4 °C. The acute toxicity assay with brine shrimp (*Artemia salina L.*) revealed the nontoxic nature of the dye solutions treated with immobilized MnP. The results suggested that immobilized MnP is an attractive option for use as industrial biocatalyst in larger scale bioremediation of textile industry effluents.

Key words: Manganese peroxidase, immobilization, cross-linking, chitosan beads, characterization, dye decolorization

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1. Introduction

In the past decade, ligninolytic enzymes including lignin peroxidases (LiPs E.C. 1.11.1.14), Mn+2 dependent peroxidases (MnPs E.C. 1.11.1.13) and laccases (Lac E.C. 1.10.3.2) along with some accessory enzymes and mediators have got prominent place in various industries including delignification of plant biomass for biofuel production, biopulping, biobleaching, bioremediation, oxidation of organic pollutants, stabilization of fruit juices, biosensors development, textile, beverage processing, animal feed, cosmetics, detergent manufacturing, paper and pulp, transformation of antibiotics and steroids (Asgher and Iqbal, 2011; Asgher *et al*., 2012; Fillat *et al*., 2012). MnP is an extracellular heme-containing glycoprotein that catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to highly reactive Mn^{3+}, the latter, stabilized by chelating dicarboxylic acids like malonate, oxalate, L-tartrate, oxaloacetate, L-malate and methylmalonate (Makela *et al*., 2005) acts as low-molecular-mass diffusible mediator, which nonspecifically oxidizes a variety of phenolic and non-phenolic dyes and other toxic pollutants. (Urek & Pazarlioglu, 2005). The aromatic structures are depolymerized via formation of phenoxy or aryl cation radicals, which finally result in the breakdown of the molecule (Rogalski, 2006).

Despite numerous biotechnological advantages, the use of native microbial enzymes on industrial scale encounters a number of practical problems like high cost isolation and purification, instability under process conditions, activity inhibition and reusability (Krajewska, 2004; Asgher *et al*., 2014b). Thermostable enzymes are desirable catalysts as these can withstand harsh industrial conditions of high temperatures and alkaline pH conditions (Shin *et al*., 2002). With the advancement in enzyme

modification techniques, it is now possible to tailor and modify the properties of enzymes. Thus, enzymes with desired properties can be constructed through mutations, genetic engineering and immobilization (Asgher *et al*., 2014).

Enzyme modification through different immobilization strategies is considered as most successful innovation in green biotechnology that could provide additional benefits, as facile recovery, reusability, longer half-lives, protection from shear damage and reduction in protease activity (Spahn and Minteer, 2008; Wang *et al*., 2012). Enzyme-based approaches with attributes like efficiency, equipped performance and multifarious use, are increasingly replacing conventional chemical methods in both laboratories and industries. However, commercialization of immobilized enzymes is still at lower pace because of their high cost and storage problems (Asgher *et al*., 2014).

A variety of new carriers supports and strategies have been developed in the recent past to improve traditional enzyme immobilization technology (Xie *et al*., 2009). Chitosan is considered as well-known support for enzyme immobilization owing to its unique characteristics such as good biocompatibility, low cost, physiological inertness and great affinity for proteins. Chitosan is glucosamine biopolymer derived from deacetylation of naturally occurring chitin, which exists in the cell wall of fungi and outer skeleton of insects, crustaceans, mushrooms and beetles (Juang *et al*., 2001; Dasha *et al*., 2011; Sionkowska, 2011). Reactive amino groups present in chitosan can readily serve as sites for chemical modification thus make chitosan one of the few natural cationic polyelectrolytes (pKa-6.5).

There is no previous report in literature on immobilization of MnP on chitosan microspheres to improve its catalytic properties for industrial applications. In the present work *Ganoderma lucidum* MnP immobilization was carried out using chitosan as support matrix and glutaraldehyde as cross-linking/activating agent. The effect of immobilization on activity, stability and reusability of MnP for dye decolorization was also investigate

2. Material and Methods

2.1. Chemicals and Substrate

Chitosan (degree of deacylation 95%), boric acid and glutaraldehyde were procured from Sigma-Aldrich (USA). Wheat bran collected from Qatar Flour Mill, Faisalabad, Pakistan was oven dried (60°C) and used as substrate for MnP production in solid state fermentation. For decolorization studies, different textile dyes including Sandal-fix Turq blue GWF (Reactive Blue 21), Sandal-fix Black CKF (Mixture), Sandal-fix Red C4BLN 150% (Reactive Red 195A), and Sandal-fix Golden yellow CRL 150% (Reactive Yellow 145A) were collected from Sandal dyestuff industries, Faisalabad, Pakistan.

2.2. Organism and Inoculum development

For inoculum development *Ganoderma lucidum* IBL-05, available in culture collection of Industrial Biotechnology laboratory, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan was grown in Kirk's basal medium of pH 4.5 (additionally containing 1% (w/v) milli-pore filtered glucose solution) in shaking incubator (Sanyo-Gallenkamp, UK) at 120 rpm and 35 °C for 5 days in an orbital shaker to obtain homogenous spore suspension having 1×10^6 to 1×10^8 spore/ml (Kay-Shoemake and Watwood, 1996).

2.3. MnP production under pre-optimized conditions

MnP was produced by *G. lucidum* in pre-optimized solid state fermentation medium of wheat bran (moisture, 50% w/w; substrate, 5 g; pH, 5.5; temperature, 30 °C; carbon source, 2% glucose; nitrogen source, 0.02% yeast extract; C: N ratio, 25:1; inoculum size, 5 ml; fermentation time, 5 days) (Asgher *et al*., 2013a). After 5 days, 100 mL of distilled water was added to the fermented biomass that was shaken (150 rpm) for 30 min. The contents were filtered and filtrates were centrifuged (Eppendorf 5415C) at 3000xg. The supernatants were collected as crude enzyme extracts that were used for enzyme assay and purification.

2.4. Purification of MnP

A four step purification procedure involving ammonium sulphate fractionation, dialysis, DEAE-cellulose ion exchange and G-100 Sephadex gel permeation chromatography was gradually employed for the purification of MnP. The purity of the enzyme was confirmed by running it on 12% native and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. MnP Assay and protein content determination

MnP was assayed by following the reported method of Wariishi *et al*. (1992) using manganese sulphate as a substrate. Assay mixture (3.5 mL) contained 1 mL of 1 m M $MnSO_4$, 1 mL of 0.5 mM sodium malonate buffer, 0.5 mL of 0.1M H_2O_2 and 1 mL of enzyme solution. The activity of the reaction mixture was measured against a reagent blank at the wavelength of 270 nm (Є270 =11570). Total protein contents of the crude and purified enzymes were determined according to Bradford, (1976) methodology using the Bradford reagent calibrated with serum bovine albumin.

2.6. Immobilization of MnP using Chitosan beads

2.6.1 Preparation of chitosan beads

Immobilization matrix was developed by previously described method of Krajewska *et al*. (1990) with slight modifications. Chitosan powder (1%) was thoroughly dissolved in 1% acetic acid solution by mild heating at 50 °C with continuous stirring. This chitosan solution was extruded drop wise into 1 M KOH solution with the help of 5 ml syringe and resulting beads were allowed to harden for 4 h at room temperature. Beads of uniform shape and size were collected, filtered through Whatman filter paper, washed thrice and stored in phosphate buffer at 4°C. The beads were activated by treating with varying concentrations of glutaraldehyde solution (1-4%, v/v) overnight at room temperature. The activated chitosan beads were thoroughly washed with 50 mM Na-malonate buffer of pH 5.0 to remove any unattached glutaraldehyde from the beads surface. The beads were incubated with purified MnP (0.2-0.6 mg/mL) for 24 h at 4 °C for immobilization. The beads were washed thrice with Na-malonate buffer (pH 5.0) and used for measurement of MnP activity at 280 nm. MnP immobilization efficiency of the beads was evaluated as follows:

2.7. Confirmation of MnP immobilization by scanning electron microscopy (SEM)

The surface morphologies of free and MnP bound chitosan beads were envisaged using scanning electron microscopy (SEM) to confirm the presence of MnP on these beads. The free and MnP immobilized beads were washed with distilled water and immediately fixed in a 2% (w/v) glutaraldehyde solution for 2 h at room temperature. The fixed beads were washed again with distilled water to remove excess glutaraldehyde. The resulting beads were immersed in a 30% ethanol solution for 5 min. The procedure was repeated sequentially in 50, 70 and 90% ethanol solutions for 5 min. The beads were finally treated with absolute ethanol for 15 min to remove the last traces of water and dried in a CO_2 atmosphere under critical conditions. The dried beads were subjected to scanning electron microscopy (S 2380- Hitachi) available in Central Hi-tech Laboratory, University of Agriculture, Faisalabad, Pakistan for surface characterization.

2.8. Steady state kinetics

To determine pH and temperature optima, the free and immobilized enzymes were assayed by incubating the enzymes in at varying pH buffers ranging from 3-10 and at different temperatures (30-70 °C), respectively. Varying pH buffers used were: tartarate-buffer, pH 3.0; sodium-malonate buffer, pH 4.0; citrate-phosphate, pH 5.0, pH 6.0; sodium-phosphate, pH 7.0, pH 8.0 and carbonate-buffer of pH 9.0 and 10.0. The effect of substrate concentration on free and immobilized MnP activity was studied using varying concentrations of $MnSO_4$ (0.1-1 m M) keeping enzyme concentration constant. Lineweaver-Burk plots were constructed between reciprocals of the initial reaction rates (1/Vo) and varying substrate concentrations [1/S].

2.9. Dye decolorization by free and immobilized MnP

To investigate the decolorization capability of free and chitosan immobilized MnP, 0.01% solutions of four synthetic textile dyes including Sandal-fix Turq blue GWF (λmax: 664 nm), Sandal-fix Black CKF (λmax: 598 nm), Sandal-fix Red C4BLN 150% (λmax: 540 nm), and Sandal-fix Golden yellow CRL 150% (λmax: 414 nm) were used in triplicate. Control flaks for each dye contained un-inoculated dye solutions. The 10 ml soluble and 5 g of immobilized MnP were transferred to 250-mL Erlenmeyer flasks containing 100 mL of individual dye solutions (0.01 g/100 mL) prepared in Na-malonate buffer (50 mM; pH 4.5). The decolorization flasks were incubated at 30 °C temperature for 12 h on rotary shaker (150 rpm). After stipulated time period, the samples were taken and centrifuged (8,000×g, 10 min) to eliminate the suspended particles. Residual dye

concentrations in the supernatants were determined by measuring absorbencies at corresponding wavelengths (λmax) of original dye solution in 50 m M Na-malonate buffer of pH 4.5).

2.10 Total organic carbon (TOC) measurement

Measurement of the TOC in dye samples was carried out before and after treatment with free and immobilized MnP. 1 mL of 2 N $K_2Cr_2O_7$ solution and 1.6 mL of H2SO4 (98%) were taken in digestion vials, containing 4 mL filtered dye samples. The solution in digestion vials were digested in oven for 90 min at 110 °C. After digestion and cooling, the absorbance was monitored at 590 nm (Iqbal *et al*., 2014).

2.11. Reusability and storage stability studies

The beads used in one dye decolorization batch were filtered, washed three times with Na-malonate buffer and reused in the next batch for treatment of dye solutions in triplicate. For storage stability studies, free and immobilized MnP were stored for 60 days in minimal volume of 50 mM Na-malonate buffer (pH 5.0) at 4 °C in order to maintain semi-dry condition. The percent residual activities of free and immobilized enzymes were determined at regular intervals using the standard assay procedure as mentioned earlier.

2.12. Toxicity studies

The maximally decolorized dye samples were subjected to acute toxicity tests by brine shrimp (*Artemia salina* L.) as reported previously (Iqbal *et al*., 2014). The free and immobilized MnP treated dye solutions were centrifuged at 8000 x g for 10 min under cold condition, supernatants were collected and sterilized by passing through 0.45 µm pore size filter. Sensitivity test was performed for esulting filtrate using 6 to 24 h old neonates. The tests were carried out at 20±0.1 ºC for 48 h in the absence of light and number of immovable organisms were counted after exposing to light for 20 sec. Tests were carried out in triplicate using distilled water as control.

2.13 Statistical Analysis

Mean and standard deviation (SD) of the results from triplicate runs were calculated using Microsoft Excel-software (Microsoft) and the standard error (SE) values have been displayed as Y-error bars in figures.

3. Results and Discussion

3.1. Production and purification of MnP

G. lucidum produced 717±2.3 U mL^{-1} MnP in solid-state culture of wheat bran under previously optimized fermentation conditions. The crude extract thus produced was purified to homogeneity to get 3.43 fold purified MnP with 539.59 U/mg specific activity.

3.2. Immobilization of MnP on chitosan beads

The purified MnP was immobilized onto gluter-aldehyde activated chitosan beads. The activated chitosan beads provide an excellent biocompatible surface with immobilization efficiency (IE) of 82.5%. In order to get beads with desired mechanical strength, varying concentrations of chitosan solution ranging from 2 to 5% were used to develop beads of different sizes and strengths. Beads prepared with 2.5% (w/v) chitosan solution and of 2 mm size (Fig. 1) were found to be most suitable for immobilization of *G. lucidum* MnP. In a second step chitosan beads were treated with varying concentrations of glutaraldehyde to assess the degree of enzyme immobilization, followed by coupling of enzyme to the active sites. The highest MnP entrapment efficiency was achieved using 2% gluteraldehyde at pH 7 after 3 h of activation time and 40 °C temperature (Table 1).

It was demonstrated that some aldehyde groups are generated on the surface of glutaraldehyde (used as cross-linking agent) treated chitosan beads that may form Schiff's base with amino group of the enzyme leading to enhanced immobilization efficiency. As a matter of fact, the presence of NH_2 groups on the chitosan backbone greatly improves its potential during the entrapment process. With lower concentration of glutaraldehyde, lesser attachment points i.e., aldehyde groups, were generated which caused lower immobilization yield (IY) (Srivastava and Anand, 2014). Initially increasing glutaraldehyde concentration furnished better immobilization efficiency but at elevated concentration beyond 2%, the immobilization was decreased presumably due to spatial hindrance caused by multiple cross-linking points on the chitosan surface between enzyme molecules.

3.3. SEM of chitosan beads surface with and without MnP

The external morphology of dried chitosan microspheres was evaluated using this technique. Results shown in scanning electron micrographs of chitosan beads, beads surface without attached MnP and beads surface with bound MnP (Fig. 2 A, B) confirmed that the MnP enzyme was commendably immobilized on the surface of chitosan beads due to high surface area provided by beads. The prepared chitosan beads were spherical in shape with a diameter of about 2 mm (Fig.2 C, D). Further, it was observed that the surface morphology of chitosan bead was comparably different depending on the concentration of chitosan solution. The chitosan concentration had more effect on immobilization yield; therefore it is necessary to standardize chitosan concentrations to maximize the performance of entrapped enzyme.

Table 1. Optimization of immobilization conditions for *G. lucidum* MnP immobilization on chitosan beads

Glutaraldehyde (%)	Activation time (h)	Immobilization (%)
0	3	62
0	6	59.2
1	3	69
1	6	67
2	3	81
2	6	78.4
3	3	74
3	6	70
4	3	72
4	6	68

Scanning electron microscopy (SEM) is a characteristically non-destructive type of electron microscopy technique, in which a beam of electrons interact with the experimental sample to generate signals. The detection of secondary and backscattered electrons emitted from the surface is used to envisage the 3-dimensional topology, particle size and surface composition of meso-porous materials at very high magnifications in the nm range (Zang *et al*., 2012)

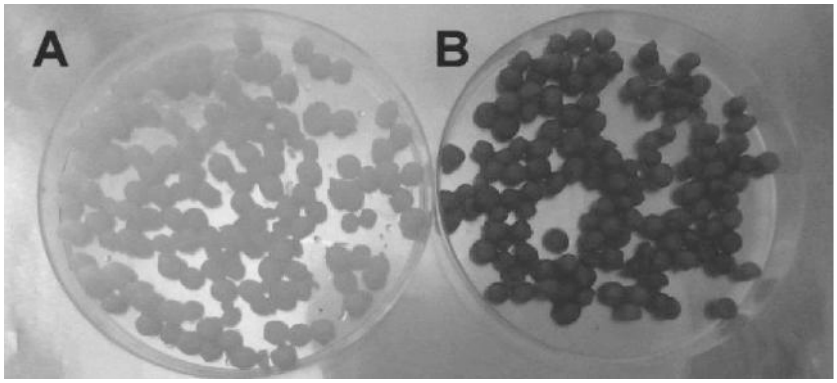

Fig. 1. Chitosan beads: (A) Free chitosan beads and (B) MnP enzyme bound chitosan beads

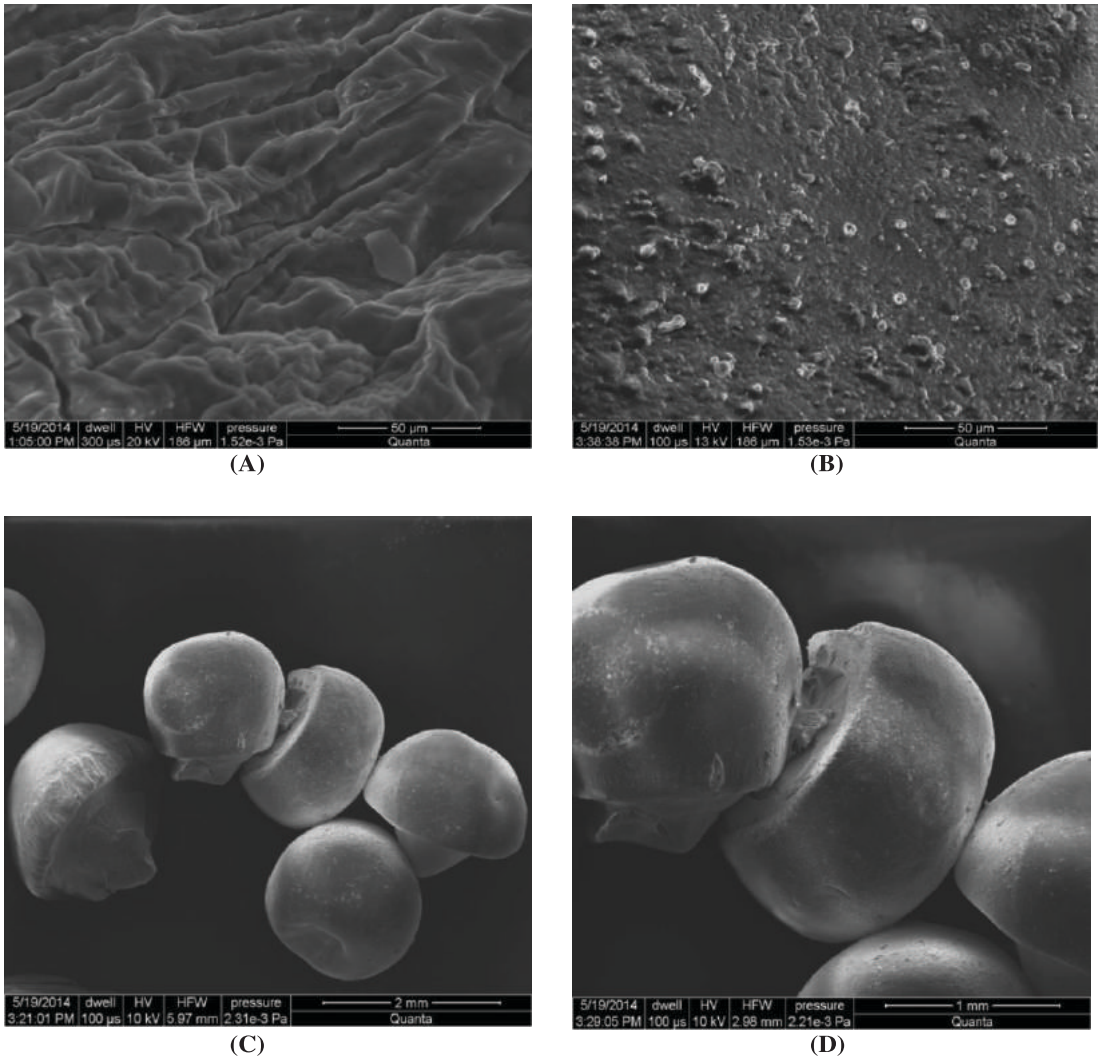

Fig.2. Scanning electron micrographs (surface view) of chitosan beads (A) Free chitosan beads, (B) Chitosan beads with immobilized MnP and (C & D) Over all Shape of chitosan beads

3.4. Kinetic Characterization of free and immobilized MnP

3.4.1. Effect of pH on free and immobilized MnP

The pH-activity profiles for soluble and immobilized MnP (Fig. 3) showed that pH 7.0 was the optimum pH for immobilized MnP, while free MnP was optimally active at pH 5.0. In a variation to these pH optima, the activities of both free and immobilized MnP were significantly reduced. The immobilized enzyme displayed good activity in wider pH ranges, while soluble enzyme started losing its activity above pH 5.0. The development of secondary interaction between MnP and immobilization matrix could be responsible for broader pH stability profile. Previously, Hung *et al*. (2003) found similar results for chitosan immobilized lipase, pepsin and other enzymes also showed maximum activity in broader pH ranges with enhanced stabilities (Singh *et al*., 2011; Wentworth *et al*., 2004).

3.4.2. Effect of temperature on free and immobilized MnP

Chitosan entrapped MnP showed optimum activity at 65 °C while the free enzyme loosed activity above its optimum temperature of 35 °C (Fig. 4). The activity of free enzyme gradually decreased at higher temperatures and it lost much of its activity at 70 °C. However, immobilized MnP retained high activity up to 70 °C. From the results, it was inferred that supporting material

absorbs heat and develops conformational changes between matrix and biocatalyst through covalent linkages, thus significantly increasing the thermal tolerance of the enzyme.

An increase in optimum temperature of different enzymes by chitosan beads immobilization has also previously been described (Bissett and Sternberg, 1978). As compared to free enzyme, the immobilized MnP from *Trametes versicolor* exhibited more than 75% of its original activity at 70 °C (Asgher *et al*., 2013b). Contrary to this, Kumari and Kayastha (2011) observed no change in optimum temperature of free and chitosan beads bound α-amylase. Cetinus and Oztop (2003) reported same optimum temperature (35 °C) for both free and chitosan bound catalase enzyme. Hung *et al*., (2003) reported that soluble as well as chitosan attached lipase showed optimal activity at the same temperature but immobilized lipase was more vigorous than its free counterpart.

3.4.3. Effect of substrate concentration: Determination of Km and Vmax

The kinetic properties of purified free and immobilized MnP were studied with varying concentrations (0.1-1.0 mM) of $MnSO_4$. MnP was assayed by incubating the MnP with varying concentrations of MnSO4 substrate and the results thus obtained were plotted as graph of enzyme activity (U/mL) against concentration of substrate [S] (Fig.5). The Km and Vmax values for chitosan immobilized MnP were found to be 98 mM and 695 U/mL, respectively. While the Km (65.6 mM) and Vmax values (638 U/ml) for the soluble form was lower, compared to immobilized enzyme.

The variation in kinetic parameters of enzyme during immobilization process is a common manifestation. The occurrence of steric hindrance and conformational changes in enzyme molecule due to immobilization limits the accessibility of substrate to the enzyme, thus contributing to higher Km. Immobilization process may also results in the modification of some functional groups present on catalytic site of enzymes resulting in higher Km, enhanced catalytic efficiency and increasing thermal stability (Asgher *et al*., 2014a). Previously, (Moreira *et al*., 2000; Chiou and Wen-Teng, 2004; Kumari and Kayastha, 2011; Srivastava and Anand, 2014) recorded the higher kinetic constant (Km) of chitosan immobilized enzymes than that of free counterpart.

3.5. Decolorization of textile dyes by immobilized MnP and TOC reduction

The dye-decolorizing efficiency of free and immobilized MnP from *G. lucidum* was investigated for decolorization of four different reactive textile dyes and, the results are shown in Table 1 (% decolorization), Fig. 6 (Visual comparison) and Fig. 7A-D (UV-Vis absorption spectra). The free and entrapped MnP displayed different decolorization potential toward different industrial dyestuffs. It was observed that chitosan immobilized MnP appeared more efficient decolorizer of all the reactive dyes used in this study as compared to its free counterpart. The immobilized enzyme was found to maximally decolorize the S. F. Black CKF (95.7%) followed by S. F. Red C4BLN (95.53%), S. F. Golden Yellow CRL (94.4%) and S. F. Turq Blue GWF to 92.1% after 12 h of incubation with MnSO4 as redox mediator.

Table 2. Decolorization of different synthetic dyes using chitosan beads immobilized MnP from *G. lucidum*

Dyes	Classification	λmax (nm)	Dye removal (%)
S. F. Turq Blue GWF	Reactive dye	664	92.1±0.5
S. F. Black CKF	Reactive dye	598	95.7±2.5
S. F. Red C_4BL	Reactive dye	540	95.3±1.3
S. F. Golden Yellow CRL	Reactive dye	414	94.4±2.1

Mean ±SD (n= 3); Dye removal percentage was performed by monitoring the loss in absorbance at max

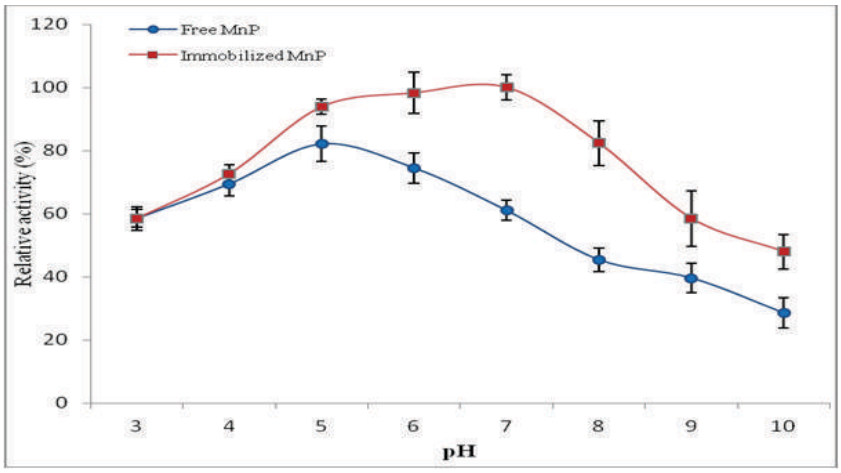

Fig.3. Effect of pH on the activities of free and chitosan beads immobilized MnP from G. lucidum IBL-05 (Values are means of replicate runs ± SD).

Several investigators have elaborated the role of white-rot fungal enzymes in the decolorization of dyes (Vishwakarma *et al*., 2012; Ramsay and Nguyen, 2002). Asgher *et al*., (2013b) efficiently decolorized (⊠ 90%) textile industrial effluent, after 5 h of incubation with sol-gel immobilized MnP than that of free enzyme. Peralta-Zamora *et al*. (2003) found decolorization of four synthetic dyes including Remazol Brilliant Blue R, Remazol Black B, Reactive Orange 122 and Reactive Red 251 dyes in the range of 35-45%, 10%, 10-30%, and 5-55%, respectively. Due to chemical structure variations in textile dyes and substitution of various functional groups on aromatic bases, all dyes are not susceptible to similar degradation by MnPs. The MnPs play a major role in complete biotransformation of dyes by acting as initiators of a chain reaction, leading to dye mineralization by generating highly active free radicals like Mn3+ and lipid, and hydroxyl radicals (Lade *et al.,* 2015).

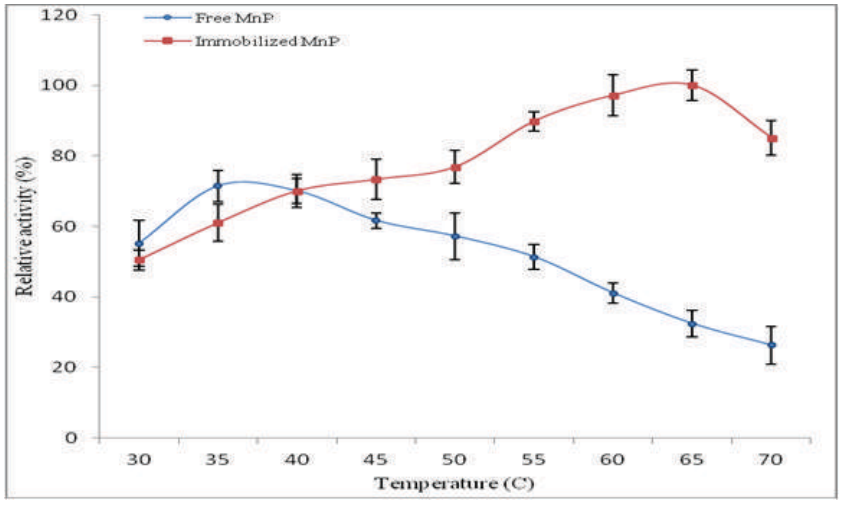

Fig.4 Effect of temperature on the activities of free and chitosan beads immobilized MnP from *G. lucidum* IBL-05 (Values are mean of triplicate runs ± SD).

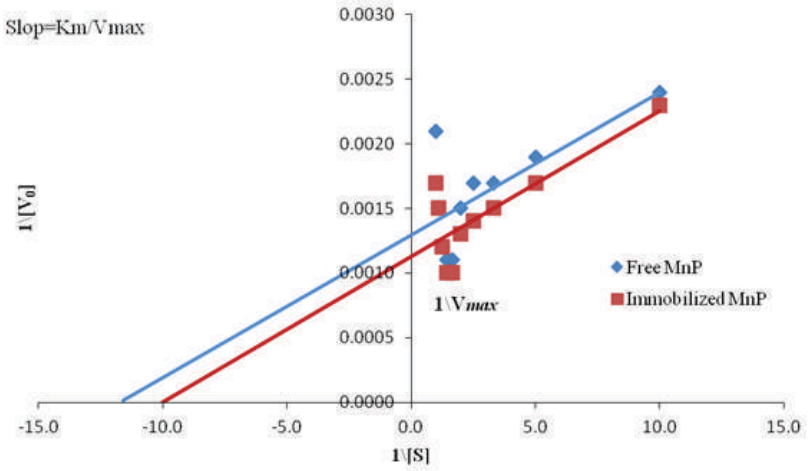

Fig.5. Determination of Km and Vmax for free and chitosan beads immobilized MnP by Line-weaver Burk Plot using MnSO4 as substrate

The degradation of reactive dyes by free and chitosan immobilized MnP was confirmed by total organic carbon (TOC) analysis. The maximally decolorized dye samples showed 62, 77, 68, and 71% reduction in TOC level (free MnP), and 89, 94, 92.4, and 93% (immobilized MnP) for Sandal-fix Turq Blue GWF, Sandal-fix Black CKF, Sandal-fix Red C4BLN, and Sandal-fix Golden Yellow CRL, respectively. This significant reduction in TOC values using free and immobilized MnP supported the efficient mineralization of dyes.

Fig.6 Visual comparison of Sandal-fix Turq blue GWF (A), Sandal-fix Black CKF (B), Sandal-fix Red C4BLN (C) and Sandal-fix Golden yellow CRL (D) dyes decolorization with free and chitosan beads immobilized MnP

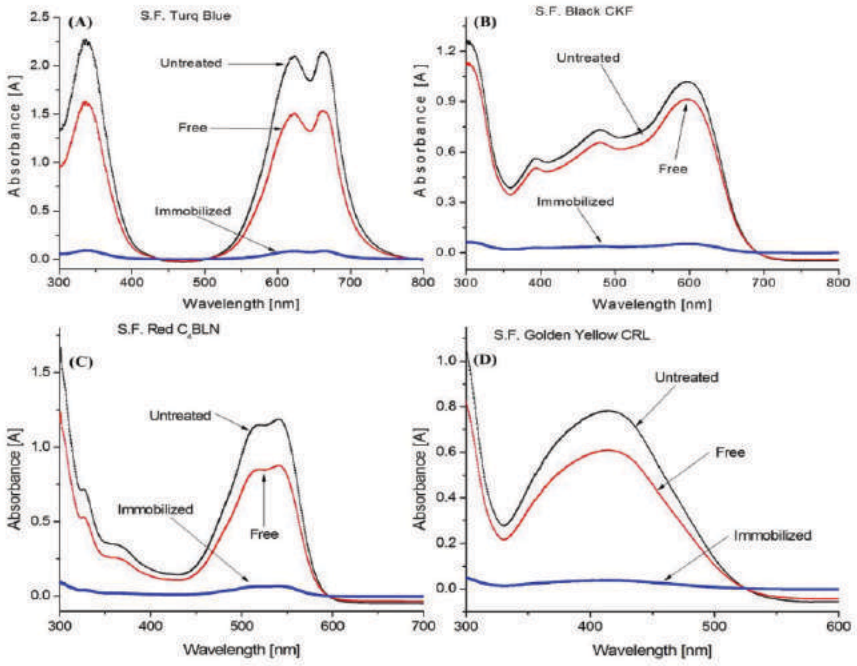

Fig.7. Comparative UV-Vis absorption spectra of textile S. F. Turq Blue GWF (A), S. F. Black CKF (B), S. F. Red C4BL (C) and S. F. Golden Yellow CRL (D) dyes treated with free and chitosan beads immobilized MnP

Immobilization phenomenon modifies the catalytic activities, selectivities and equipped permanence of the coupled enzymes. The present chitosan matrix-bound MnP appears to have potential usage to encounter the challenges of biotechnology sector, especially for bioremediation in the textile industry. Our results indicated that immobilized MnP on chitosan beads is a better choice with greater efficiency for elimination of toxic pollutants/dyestuffs. The degradation of dye into smaller fragments, including the breaking of the azo bond, can ultimately lead to a declined in the absorbance of the visible ranges and in a colorless solution (Jamal *et al.,* 2013).

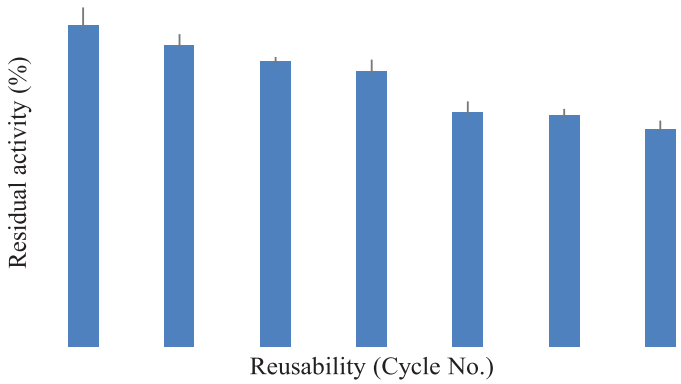

Fig.8. Residual activity of chitosan immobilized MnP after repeated use for S. F. Black CKF dye decolorization in 7 cycles (Values are mean of triplicate runs ± SD).

3.6. Reusability of enzymes

The chitosan coupled enzyme could be easily separated from the dyes solution and assessed for its residual catalytic efficiency. In order to determine the repeated cycles of chitosan entrapped enzyme, beads were removed after 12 h of reaction time and then thoroughly washed with distilled water. After 7 times repeated use, the chitosan bound MnP preserved 64.9% dye removal efficiency for S.F. Black CKF (Fig. 8). It was observed that with increasing in cycle numbers, the catalytic efficiency gradually declined. This gradual decline in activity might be attributed to the result of plugging of the membrane pore and accumulation of high active free radicals e.g., Mn^{3+}, hydroxyl, and peroxy-radicals) and dimmer in the interior environment of each microsphere which entangled the active site of enzyme leading to enzyme inactivation (Zille *et al*., 2003).

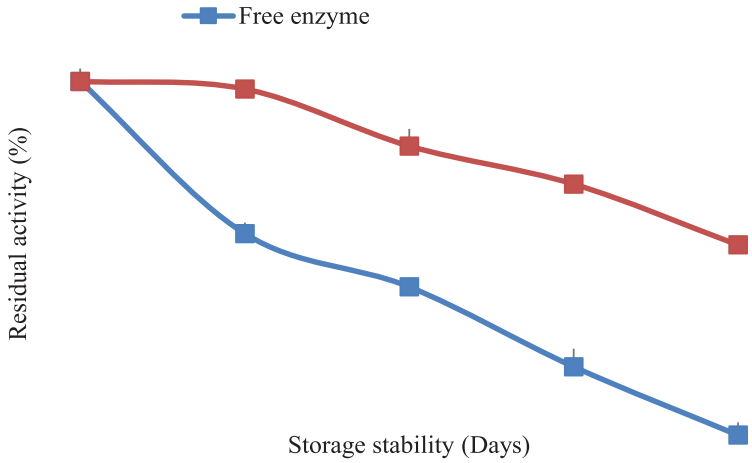

Fig. 9. Storage stabilities of free and chitosan beads immobilized MnP

3.7. Storage stability studies

After immobilization the storage stability of chitosan immobilized MnP significantly increased than that of free enzyme (Fig. 9). The carrier bound enzyme retained more than 50% of its original activity even after 60 days of storage. This considerably increased storage stability due to immobilization could be valuable feature of this enzyme to be exploited for longer storage periods in industrial sectors. As soon as the enzyme molecule is separated from its natural catalytic system, it readily starts losing its activity and stability. Immobilization of enzyme seems most reliable method to retain the higher catalytic efficiency of the enzymes for longer period of time (Cetinus and Oztop, 2003). Enhanced storage stability has also been reported for lipase, catalase and pepsin immobilized on activated chitosan beads compared to their free forms (Cetinus and Oztop, 2003).

3.8. Acute toxicity studies

Dyes as well as their degradation metabolites are known to be deleterious for human health and aquatic biota. The acute tests with brine shrimp have been widely documented as screening method for the evaluation of toxicity to mammals and humans (Iqbal *et al*., 2014). Thus, the relative toxicities of four reactive dyes, treated with free and immobilized MnP, were assessed by acute toxicity test with brine shrimp. The results of the toxicity analysis are represented as percent mortality of brine shrimp (*Artemia salina* L.) occurred with control and enzyme treated dye samples. Before treatment, the brine shrimp mortality rate was found in the range of 32.4-38.3%.

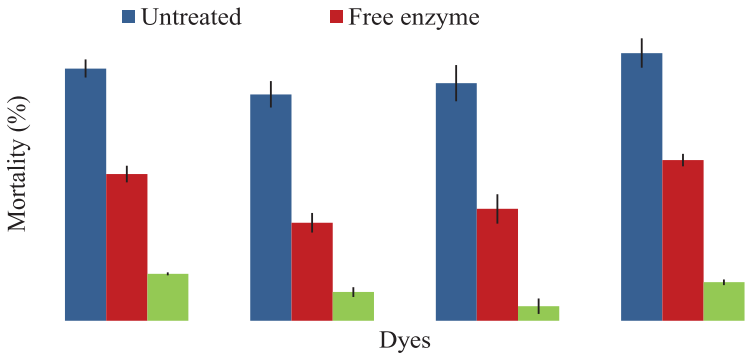

Fig.10. Toxicity assay of industrial dyes after treatment with free and Chitosan beads immobilized MnPs

(1) Sandal-fix Turq Blue GWF, (2) Sandal-fix Black CKF, (3) Sandal-fix Red C4BLN and (4) Sandal-fix Golden Yellow CRL

After MnP treatment, toxicity reduced significantly and percent death was recorded to be 16-23% in case of free MnP, whereas it decreased to only 2.03-6.7% when treated with immobilized MnP (Fig. 10). Therefore, it can be concluded that degradation using free MnP was not sufficient to eliminate the complete toxicity of dyes, as 16-23 (%) mortality of brine shrimp was observed in these treated samples. In contrast, the immobilized MnP treated dyes sample showed very little mortality rate suggesting its effectiveness for removal of toxicity from decolorized samples. Recently, Lade and coworkers (2015) found 44-52% mortality of Daphnia magna exposed to culture supernatants containing azo dyes treated with bacteria consortium. While complete death of Daphnia magna by azo dyes has also been reported (Franciscon *et al*., 2009).

4. CONCLUSIONS

Ganoderma lucidum MnP was immobilized on glutaraldehyde activated chitosan microspheres with an overall immobilization efficiency of 82.5%. The immobilized enzyme showed optimum activity at pH 7.0 and 60°C temperature. The immobilized enzyme showed better catalytic properties including Km and Vmax values 98 mM and 695 U/ml, respectively as compared to free MnP. The immobilized MnP showed 90% dye decolorization in 12 h and retained 65% of its activity after seven dye-decolorizing cycles. The acute toxicity assay with brine shrimp revealed the detoxification MnP treated dyes. The novel catalytic, stability, reusability and dye degrading capabilities of the chitosan immobilized MnP suggests its potential for diverse biotechnological applications particularly for industrial scale textile industry effluent bioremediation.

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