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Article

Novel catalytic and effluent decolorization functionalities of sol-gel immobilized *Pleurotus ostreatus* IBL-02 manganese peroxidase produced from bio-processing of wheat straw

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ABSTRACT

Solid state bio-processing of wheat straw was carried out through an indigenous fungal strain *Pleurotus ostreatus* IBL-02 under pre-optimized fermentation conditions. The maximum activity, 692 ± 12 U/mL, of the industrially important manganese peroxidase (MnP) enzyme was recorded after five days of still culture incubation. The crude MnP was 2.1-fold purified with a specific activity of 860 U/mg after purification on a Sephadex-G-100 gel column. On native and SDS-PAGE electrophoresis gels, the purified MnP fraction was a single homogenous band of 45 kDa. An active fraction of MnP was immobilized using hydrophobic sol-gel entrapment comprising tetramethoxysilane (T) and propyltrimethoxysilane (P) at different T:P molar ratios. Characterization revealed that after 24 h incubation at varying pH and temperatures, the MnP fraction immobilized at a T:P ratio of 1:2 in the sol-gel retained 82% and 75% of its original activity at pH 4 and 70 °C, respectively. The optimally active fraction at a 1:2 T:P ratio was tested against MnSO_4 as a substrate to determine the kinetic catalytic constants K_M and V_{max} . To explore the industrial applicability of *P. ostreatus* IBL-02 MnP, both the free and immobilized MnP were used for the decolorization of four different textile industrial effluents. A maximum of 100% decolorization was achieved for the different textile effluents within the shortest time period. A lower K_M , higher V_{max} , hyper-activation, and enhanced acidic and thermal resistance up to 70 °C were the novel catalytic features of the sol-gel immobilized MnP, suggesting that it may be a potential candidate for biotechnological applications particularly for textile bioremediation purposes.

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1. Introduction

Lignocellulosic biomass is a major renewable resource of potentially fermentable carbohydrates but is mostly discarded in the form of pre-harvest and post-harvest agricultural losses, and waste from food processing industries. The lignocellulosic wastes are present in abundance and therefore, are of great value as raw materials for the production of industrially important enzymes. It has been established that white rot fungi (WRF), a group of generally robust microbes, can produce ex-

tracellular ligninolytic enzymes comprising lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase that are involved in the degradation of lignin as well as in bio-remediation processes [1–3]. During recent years, there has been a great interest in the potential applications of MnP in bio-pulping and bio-bleaching as well as in bio-remediation processes [1–4].

MnPs are extracellular glycoproteins with an iron protoporphyrin IX (heme) prosthetic group mostly secreted in multiple forms during secondary metabolism [5]. Mn^{2+} functions as a mediator for MnP where MnP catalyzes the H_2O_2 dependent

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oxidation of Mn^{2+} to Mn^{3+} that subsequently forms complexes with different chelators like pyrophosphate malonate, oxalate, L-tartrate, oxaloacetate, L-malate, and methylmalonate, low-molecular-mass diffusible mediators, which non-specifically oxidize a variety of phenolic and non-phenolic substances including lignin and toxic pollutants [2].

Textile effluents contain several types of chemicals including real dye residues that are toxic, carcinogenic, mutagenic, and pose serious health hazards to the entire living ecosystem. An eco-friendly treatment method of textile waste effluents is still a major environmental concern of the modern world [6]. In spite of the existing physical and chemical technologies, which are usually expensive and commercially unattractive, biological processes provide an alternative cost effective and eco-friendly approach that can be applied to wide range of dye containing industrial effluents [4,6]. Industrial applications of enzymes in most cases involve the use of continuous reactors that require immobilized bio-catalysts in order to improve process efficiency through improvements in enzyme stability, reusability and controlled product release. A variety of immobilization techniques have been investigated to develop immobilized bio-catalysts [5]. However, among the different immobilization methods, gel entrapment is preferred because gel polymers are non-toxic and do not swell in aqueous or organic solvents, thus preventing the leaching of the entrapped enzyme and denaturation of its native conformation [3,7,8].

WRF has the ability to degrade contaminants by virtue of its extracellular ligninolytic enzymes [3–6,9]. During the last few years, we have demonstrated considerable improvement in many processes related to lignocellulose biotechnology and triggered in-depth studies of lignocellulosic materials, ligninolytic microorganisms, and their enzymes. Therefore, in this context, the capability of the indigenously isolated MnP to degrade/decolorize different textile dyes containing industrial waste effluents was investigated for its use in biotechnological applications particularly for textile bioremediation purposes.

2. Experimental

2.1. Chemicals and lignocellulosic substrate

Propyltrimethoxysilane (P), tetramethoxysilane (T), polyvinyl alcohol, Sephadex-G-100, and standard protein markers were purchased from Sigma-Aldrich (USA). Agricultural waste wheat straw was collected from the Student Research Farm, University of Agriculture Faisalabad, Pakistan. The substrate was chopped, dried (60 °C), ground to a fine particle size and stored in polyethylene plastic bags to avoid free moisture until further use. For decolorization studies, four different dyes containing textile industry effluents, i.e., Nishat textile (NIT), K&N textile (KIT), Sitara textile (SIT), and Crescent textile (CRT) were collected on-site from local textile industries in Faisalabad, Pakistan.

2.2. Microorganism and preparation of inoculum

A pure culture of the indigenous fungal strain *P. ostreatus*

IBL-02 was raised on potato dextrose agar (PDA) slants at pH 4.5 and 28 °C. Homogenous spore inoculum was prepared by growing the fungus for 5 d in the Kirk's basal nutrient medium in the presence of an additional 1% (w/v) Millipore filtered sterile glucose solution to develop inoculums with 1×10^6 – 1×10^8 spore/mL. The main constituents of the medium were ammonium tartrate (0.22 g/L), KH_2PO_4 (0.21 g/L), $MgSO_4 \cdot 7H_2O$ (0.05 g/L), $CaCl_2 \cdot H_2O$ (0.01 g/L), thiamine (0.001 g/L), and Tween-80 (10 mL/L).

2.3. Production and extraction of MnP

P. ostreatus IBL-02 was grown in solid state fermentation (SSF) using a wheat straw based fermentation medium under pre-optimized growth conditions. The culture flasks (500 mL) contained 5 g wheat straw, moistened (66% w/w moisture) with Kirk's salt medium that was supplemented with glycerol and urea as an inexpensive carbon and nitrogen source in a ratio of 25:1 (C:N) and 1 mmol/L $MnSO_4$ (1 mL) as optimal nutritional factors. The SSF medium was autoclaved, inoculated (5 mL fresh fungal spores) and allowed to ferment at 30 °C in a still culture incubator (Sanyo, Japan). After 7 d of fermentation, 100 mL of distilled water was added to all of the fermented cultures and the flasks were shaken at 120 r/min for 30 min [10]. The contents were filtered and the filtrates were centrifuged at 3000 g for 10 min (Sanyo, Japan). The supernatants were pooled and used as a crude extract for the MnP activity assay, purification, and immobilization purposes.

2.4. Determination of MnP activity and protein content

MnP activity was measured by adopting the methodology previously described [2,6]. The MnP activity was measured against a reagent blank at 270 nm ($\epsilon_{270} = 11590 \text{ mol}^{-1} \text{ L cm}^{-1}$, UV 2000, Hitachi, Germany). The recorded activities were expressed as U/mL, where one unit of activity was defined as the amount of enzyme required to produce a unit increase in the absorbance at a specific wavelength (nm) per mL of the reaction mixture. To determine the protein content of the sample, the Bradford microassay was used with bovine serum albumin as the standard [11].

2.5. Purification of MnP

Purification of MnP was carried out by ammonium sulfate fractionation (85% saturation), followed by dialysis and gel filtration chromatography using a Sephadex-G-100 column (2×25 cm) as described previously [2]. Phosphate buffer (100 mmol/L) with 0.15 mol/L NaCl was used as the elution buffer and the flow rate was maintained at 0.5 mL/min. Up to 20 pooled fractions were collected, concentrated by ultra-filtration and used to determine molecular mass, enzyme activity as well as the protein content as described in section 2.4.

2.6. Native and SDS-PAGE

The purified and lyophilized sample was dissolved in a

minimum amount of 50 mmol/L malonate buffer, and subjected to 12% native PAGE and 10% SDS-PAGE using a vertical minigel electrophoresis apparatus (V-GES, Wealtec Corporation, USA) to determine sample purity and approximate mass of MnP according to the method of Laemmli [12].

2.7. Immobilization of purified MnP

Sol-gels of different hydrophobic characters were prepared using varying T:P ratios (1:1, 1:2, 1:5, 1:10) in ascending order by adopting the methodology as described previously [4], with minor modifications. Specific activity and protein content of entrapped enzymes (in gels of different hydrophobicities) were determined. The entrapped enzyme having the highest specific activity and immobilization efficiency was selected for further characterization in comparison to the purified free MnP extract.

2.8. Characterization of free and immobilized MnP

2.8.1. Effect of pH

To determine the effect of pH, the pH-activity profile of MnP was determined over a range of pH 3 to 9 on free and gel entrapped MnP. Both the free and gel entrapped MnPs were incubated separately at 30 °C without substrate using the following buffers: sodium malonate, pH 3.0 and 4.0; citrate phosphate, pH 5.0 and 6.0; sodium phosphate, pH 7.0 and 8.0, potassium carbonate buffer, pH 9.0.

2.8.2. Effect of temperature

Effect of varying temperatures on the free and immobilized MnP was also studied by adopting the methodology as described previously [8]. The free and gel entrapped MnPs were incubated in sodium malonate buffer at pH 4.0 for 24 h at temperatures ranging from 25–75 °C.

2.8.3. Determination of K_M and V_{max}

The effect of varying substrate concentrations on free and immobilized MnPs was investigated. Activities of free and immobilized enzymes were determined using varying concentrations (0.1–1.0 mmol/L) of $MnSO_4$ as the substrate. The data were then used to construct reciprocal plots using a Lineweaver-Burk transformation of the Michaelis-Menten equation by plotting the reciprocals of the initial reaction rates ($1/V_0$) against the reciprocals of the respective substrate concentrations [$1/S$].

2.9. Decolorization of real textile industry effluents

Decolorization of individual effluents was determined by a

reduction in optical density at the wavelength of maximum absorbance 590 nm (λ_{max}). The working conditions of a single continuous operation included two parallel batches of triplicate flasks containing either 10 mL of free or 5 g of sol-gel entrapped MnP with 10 mL of 50 mmol/L sodium malonate buffer and 100 mL of individual textile effluents. The flasks were incubated at 30 °C in a shaking incubator (120 r/min) for 5 h.

2.10. Statistical analysis

All enzyme assays were performed in triplicate, and the data were statistically analyzed. The means and standard errors of the means (mean $\pm$ SE) were calculated for each treatment and the SE values have been displayed as Y-error bars in figures.

3. Results and discussion

3.1. MnP production and purification

MnP was produced from an indigenous strain of *P. ostreatus* IBL-02 on wheat straw under pre-optimized fermentation conditions. A maximum MnP activity of 612 ± 12 U/mL was recorded when 5 mL of the fungal inoculum was inoculated into medium nutritionally supplemented with glycerol and urea at a 25:1 C/N ratio, 1 mL of 1 mmol/L tween-80 as a surface active agent, and 1 mL of 1 mmol/L $MnSO_4$ as a mediator. Ligninolytic enzyme production is strongly affected by the nature and amount of the nutrients and physical elements of the growth substrate. Different fungal species have particular responses to various nutrients and show different growth and enzyme activity patterns depending on the substrate [8,10]. The purification summary for *P. ostreatus* IBL-02 MnP is shown in Table 1. After Sephadex-G-100 filtration, the specific activity of MnP increased to 860 U/mg with 2.1-fold purification. Previously we have reported the successful use of Sephadex G-100 gel filtration column chromatography for the purification of various fungal enzymes, i.e., cellulases, protease, and various ligninolytic enzymes from different fungal cultures including *Trichoderma harzianum*, *Aspergillus niger*, *Trametes versicolor* IBL-04, and *Schizophyllum commune* IBL-06 [2–4,6,8,10,13]. In another study, MnP from *Phanerochaete sp.* was salted out at 65% $(NH_4)_2SO_4$ saturation with 2.6-fold MnP purification [14].

3.2. Native and SDS-PAGE

The purified MnP was resolved on native and SDS-PAGE with a molecular weight of 45 kDa relative to the standard molecular weight markers (Fig. 1). The molecular mass of *P. ostreatus* IBL-02 MnP was within the range of molecular weights

Table 1

Purification summary for *P. ostreatus* IBL-02 MnP.

Purification step	Total volume (mL)	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Purification fold
Crude enzyme	300	188700	468.0	403	1
$(NH_4)_2SO_4$ precipitation	20	10240	19.60	522	1.3
Dialysis	20	9960	16.2	615	1.5
Sephadex-G-100	14	3612	4.2	860	2.1

Fig. 1. SDS (a) and native (b) PAGE of MnP produced from *P. ostreatus* IBL-02. The molecular mass of the purified MnP was estimated in comparison to standard weight markers (standard protein markers, 21–116 kDa; Sigma, USA). The protein bands were visualized by staining with Coomassie Brilliant Blue G-250 (Sigma, USA) after documentation.

varying between 32 and 75 kDa commonly found for the MnP family [2,15]. The molecular weights for MnPs from *B. pumilus* and *Paenibacillus* sp. as determined by SDS-PAGE were 25 and 40 kDa, respectively, whereas MnP from *Irpex lacteus* was a single polypeptide of 53.2 kDa on SDS-PAGE [16]. As shown in our previous study, MnP from *T. versicolor* IBL-04 was a homogenous monomeric protein as shown by its single band of 43 kDa on SDS-PAGE [2].

3.3. Immobilization of *P. ostreatus* IBL-02 MnP

Specific activities of free and immobilized MnP and the percent immobilization efficiency in different gels are shown in Table 2. The specific activity of immobilized MnP was higher than that of the free enzyme and it increased as the T:P ratio hydrophobicity of the sol-gels increased. However, with increasing hydrophobic character (higher T:P ratios, i.e., 1:5 and 1:10), the specific activity of immobilized MnP significantly decreased, possibly because of the diffusion limitation barrier created by increasing the hydrophobic nature of the gel. Maximum specific activity (975 U/mg) was observed for the MnP fraction entrapped in the sol-gel matrix at a 1:2 of T:P ratio. Thus, it was selected for textile waste effluent decolorization studies. Entrapment of enzyme in sol-gels involves an adsorption phenomenon that has been reported as the best method for immobilization of laccase [7]. Previously, we reported that entrapment of LiP from *P. chrysosporium* and *Trametes versicolor* IBL-04 in sol-gels caused hyper-activation but an increase

Fig. 2. Effect of pH on free and sol-gel immobilized MnPs.

in hydrophobic character above certain optimum limits caused a decrease in LiP activity [3,17]. In addition, the results obtained by the sol-gel entrapment for the presently reported MnP immobilization are in the range of those attained by other methods such as covalent bonding of the enzyme on siliceous cellular foams, sepa beads and amine-terminated magnetic nano-composite by a glutaraldehyde cross-linking method [18,19].

3.4. Characterization of free and immobilized MnPs

3.4.1. Effect of pH on free and immobilized MnP

Characterization revealed that after 24 h incubation at varying pHs, the MnP fraction immobilized in the sol-gel at a 1:2 (T:P) ratio retained 82% of its original activity at pH 4 (Fig. 2). All four MnP fractions entrapped in varying T:P ratio gels showed broader pH ranges than that of the free MnP. The method of immobilization and ionic or hydrogen bonding and secondary interactions between enzyme and gel particles may be responsible for this broader pH stability profile under an acidic environment. Earlier studies reported the maximum activities of MnPs from different WRF were observed at pHs from 5.5 to 6.5 [15,16]. At a pH value more or less than 4, the MnP activity decreased. Our results are in accordance with immobilization process of D'Annibale et al. [20] who showed that the ligninase enzymes of *L. edodes* are stable over a pH range of 2.5–4.0. In support of our findings, Qiu et al. [7] found that immobilized as well as free laccase showed similar pH-activity profiles because of the clear surface of the nanoporous gold immobilization support used.

3.4.2. Effect of temperature on free and immobilized MnP

The MnP immobilized in the sol-gel at a 1:2 (T:P) ratio retained 75% of its original activity at 70 °C as compared to the free and other immobilized fractions (Fig. 3). The order of thermostability was 1:2 MnP > 1:1 MnP > 1:5 MnP > 1:10 MnP. In an earlier study, Asgher et al. [17] reported that the optimum temperature for gel entrapped lignin degrading enzymes ranged in 40–55 °C. Most of the previously reported MnPs were found to lose their activities at temperatures around 60 °C [21].

Table 2

Specific activities of free and immobilized MnPs with immobilization efficiency.

Gel precursors (T:P) molar ratio	Specific activity ^a (U/mg)		Immobilization efficiency ^b (%)
	Free MnP	Immobilized MnP	
1:1	660	722	88.3
1:2	860	975	93.6
1:5	735	885	72.1
1:10	522	748	58.4

^a Calculated in U/mg of protein.

^b Difference between the enzyme loaded and the enzyme in supernatant after washing.

Fig. 3. Effect of temperature on free and sol-gel immobilized MnP.

MnP from *Irpex lacteus* was stable over 30–40 °C [16], whereas MnP from *P. chrysosporium* was completely inactivated within 15 min at 60 °C [22].

3.4.3. Effect of substrate concentration: Determination of K_M and V_{max}

Sol-gel matrix immobilization enhanced the substrate affinity and catalytic efficiency of MnP as confirmed by its lower K_M (27 mmol/L) and higher V_{max} values (227 U/mg) for the immobilized enzyme compared to the free MnP that had values of K_M (54 mmol/L) and V_{max} (182 U/mg) (Fig. 4). Results shown in Fig. 4 indicated that MnP entrapped at a 1:2 of T:P ratio had more affinity for its substrate, but its interaction decreased with a further increase in gel hydrophobicity. In previous studies, different K_M values for MnP from different WRF strains such as *Bjerkandera* sp. and *Phanerochaete chrysosporium* (45–51.9 μ mol/L) [23], and *Irpex lacteus* (46.7 μ mol/L) [24],

Fig. 4. Lineweaver-Burk reciprocal plot: K_M and V_{max} for free and immobilized MnP. Standard quartz cuvettes having path length of 1 mm with exterior width, length, and height ($W \times L \times H$) dimensions of 12.5 mm $\times$ 3.5 mm $\times$ 45 mm were used to determine the values of kinetic parameters for free and immobilized MnP. Both free and immobilized MnP was incubated for 15 min at 30 °C in sodium malonate buffer of pH 4.5 before carrying out standard enzyme activity assays. Lineweaver-Burk plot created with Microsoft Excel Windows updated version 7 via non-linear curve fitting, using varying concentrations (0.1–1.0 mmol/L) of $MnSO_4$ as a substrate.

Fig. 5. UV-Vis absorption spectra of textile effluents obtained after 5 h treatment with free MnP (a) and 1:2 of T:P immobilized MnP (b).

have been reported. The difference in K_M values of MnPs from *P. ostreatus* IBL-02 and other reported fungal species can be explained on the basis of genetic variability and substrate specificities among different species.

3.5. Decolorization of real textile industry effluents

CRT, a textile industrial effluent, was 100% decolorized, followed by SIT (98.5%) after 5 h of incubation with an immobilized MnP as compared to its free counterpart (Fig. 5). In a previous study, *Coriolus versicolor* was found to decolorize Arzoo textile industry effluents to 84% in 3 d [25]. The variation in effluent composition results in the differences in the decolorization by enzyme extracts from different fungi [17,26]. In this context, the present sol-gel matrix-entrapped MnP seems to be suitable as an enzyme for bioremediation in the textile industry.

4. Conclusions

The catalytic features of the *P. ostreatus* IBL-02 MnP makes it a multipurpose enzyme suitable for a variety of biotechnological applications. Characterization results revealed that the MnP fraction immobilized in the sol-gel at a 1:2 (T:P) ratio retained 82% and 75% of its original activity at pH 4 and 70 °C, respectively. The novel catalytic features of the sol-gel immobilized MnP included a lower K_M , higher V_{max} , hyper-activation, and enhanced acidic and thermal stability up to 70 °C, suggesting that it is a potential candidate for biotechnological applications particularly for textile bioremediation purposes.

Graphical Abstract

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Novel catalytic and effluent decolorization functionalities of sol-gel immobilized *Pleurotus ostreatus* IBL-02 manganese peroxidase produced from bio-processing of wheat straw

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Agro-industrial based lignocellulosic waste material i.e. wheat straw was used as a fermentation feed stuff for MnP production. A promising and eco-friendly approach was adopted to immobilize the purified MnP into the sol-gel matrix network. To explore the textile industrial applicability, the immobilized MnP was tested against real dye containing textile effluents.

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