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Manganese Peroxidase from *Trichoderma harzianum* and Increasing Its Efficiency for Phenol Removal from Wastewater

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Authors' contributions

This work was carried out in collaboration between all the authors who read and approved the final manuscript.

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ABSTRACT

Manganese peroxidase (MnP) was isolated and purified from *T. harzianum*. The free and immobilized enzyme was activated by thioglycolate and reduced glutathione (GSH). After treatment with thioglycolate, the V_{max} values for the free, alginate-immobilized MnP (alginate-E) and chitosan-immobilized MnP (chitosan-E) were 24.9, 27.9 and 32.6 Umg^{-1} protein, respectively. While, the K_m values were 0.06, 0.053 and 0.071 mM. The V_{max} values for the three forms of MnP in presence of GSH were 25.6, 26.9 and 33.7 U mg^{-1}protein, respectively but K_m values were 0.077, 0.065 and 0.11 mM. Free and immobilized forms were inhibited by *o*-phenanthroline, *α,α*-dipyridyl and ethylene glycol tetraacetate (EGTA). IC_{50} values for the free, alginate-E and chitosan-E in presence of *o*-phenanthroline were 0.6, 0.94 and 1.7 mM. In case of *α,α*-dipyridyl the values were 0.63, 0.7 and 0.89 mM. However, with EGTA the values were 0.77, 0.9 and 1.4 mM, respectively. Free and immobilized MnP expressed potentiality to remove phenol with $T_{0.5}$ of 4.2, 2.95 and 1.5 h for free, alginate-E and chitosan-E, respectively. Our study reports an effective method to increase the

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catalytic efficiency of MnP using thiols. In addition, immobilized MnP from *Trichoderma harzianum* can be used for biotechnological application in removal of phenol from wastewater.

Keywords: Trichoderma harzianum; manganese peroxidase; immobilization; chitosan; alginate; thiols; phenol removal.

1. INTRODUCTION

MnP is an enzyme with great potential for removal industrial wastes. It is heme center-containing glycoprotein catalyzing H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+} [1-2]. Mn^{3+} is then complexed with dicarboxylic organic acids for the subsequent oxidation of different phenolic compounds which are derived from lignin [3].

The natural catalysts are selective, efficient and sustainable but they are not adapted perfectly for industrial applications. The reusability and stability should be considered for promotion of the enzyme application in the industry [4]. Enzyme immobilization represents the key for such purpose [5]. Enzyme immobilization on various solid carriers offers several advantages including greater stability to organic solvents and autolysis [4].

Wastewaters contain aromatic compounds such as phenols and these wastewaters are produced from different industries including textiles, coal conversion, petroleum refining, resins, plastics, dyes and organic chemical production [6]. Many of these compounds are toxic and others are carcinogens [6].

Peroxidases from different sources including *Coprinus macrorhizus* (CMP), horseradish (HRP), soybean (SBP), *Arthromyces ramosus* (ARP) and *Coprinus cinereus* (CIP) expressed an appreciable efficiency in removing various aromatic compounds from wastewaters [6-10]. Thus, peroxidases may replace biological and chemical treatments because of their ability to selectively remove a targeted class of contaminant; ability to remove substrate to low levels and reduced oxidant requirement. Peroxidases catalyze the oxidation of phenolic compounds and produce reactive free radicals which couple spontaneously forming water- insoluble polymers in the presence of hydrogen peroxide [11]. Fig. 1 shows the equation of the enzymatic removal of aromatic compounds.

Finding a new MnP with improved capacity to degrade various environmental pollutants could be helpful in the practical application of MnP in the area of environmental remediation. The goal of the present work was to investigate the influence of thiol compounds and chelating agents on free and immobilized manganese peroxidase (MnP) activity from *Trichoderma harzianum*. Also, it is aimed to investigate the potentiality of the free and immobilized enzyme to remove phenol from wastewater.

2. MATERIALS AND METHODS

2.1 Experimental Microorganism and Growth Medium

Trichoderma harzianum was isolated from Damietta branch of the Nile River near the water treatment station at Mansoura City, Dakahliya Governorate, Egypt. The fungal isolate was maintained at 4°C on Czapek's yeast extract agar slants. Static cultivations were carried out in liquid medium using 250 ml Erlenmeyer flasks containing 50 ml of Czapek's broth medium. Flasks were then inoculated with two

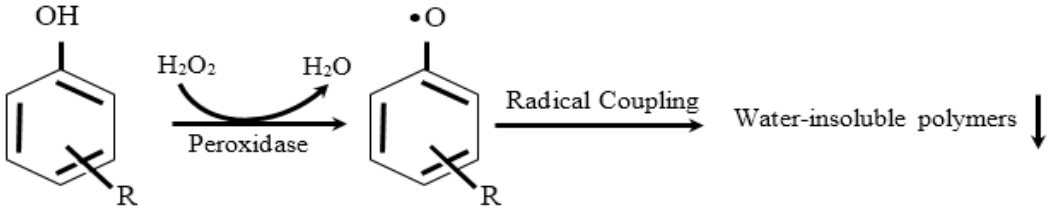

Fig. 1. Removal of phenolic compounds

7-mm agar plugs with mycelium and the cultivation proceeded at 30°C in the dark without aeration for peroxidase production [12].

2.2 Extraction of MnP

Both of pellet (mycelia mat) and supernatant were separated by the method of Absar [13] by filtration through a Whatman filter paper, grade 4. The resulting supernatant was removed and 1 g of the pellet was suspended in 100 ml of 50 mM sodium acetate buffer (pH 5.2) followed by homogenization with mortar and pestle then kept in an ice bath. The resulting extract was disrupted by ultrasonification (Ultra-Turax, T-2.5) at 4°C. The cell debris in the solution was removed through centrifugation (Thermo-Fisher centrifuge, USA) at 10,000 g for 10 min at 4°C and filtered. This obtained supernatant was used as crude enzyme preparation.

2.3 Assay of MnP

The reaction mixture contained in 3 ml the following components: 1 ml of 20 mM sodium succinate buffer (pH 4.5), 0.1 mg of phenol red ml^{-1}, 1 ml of 25 mM lactate, 1 mg of bovine serum albumin ml^{-1}, 0.5 ml of 0.1 mM $MnSO_4$, 0.5 ml of enzyme extract. The reaction was started by adding H_2O_2 to 0.1 mM as final concentration. The reaction was stopped with 100 µl of 10% w/v trichloroacetic acid followed by measuring the absorbance at 610 nm. Control assays were carried out by excluding $MnSO_4$ from the reaction mixture. The activity of MnP was calculated by subtracting the activity in the absence of Mn^{2+} from the activity in presence of Mn^{2+} [14].

2.4 Determination of Protein Content

The soluble protein content in each sample was estimated according to Bradford [15]. One ml of the extract was mixed with 5 ml diluted Coomassie Brilliant blue G-250 and kept in the dark for 1 min. The absorption was measured spectrophotometrically at 595 nm and the protein concentration was determined using standard curve of bovine serum albumin.

2.5 Immobilization of MnP

The MnP enzyme was immobilized on Ca-alginate and chitosan was carried out according to El-Shora et al. [16].

2.6 Effect of Thiol Compounds on free and Immobilized MnP Activity

The effect of thiol compounds such as thioglycolate and reduced glutathione (GSH) on free and immobilized MnP were investigated at 0.2, 0.4, 0.6, 0.8 and 1.0 mM in the reaction mixture, followed by enzyme assay as mentioned before.

2.7 Effect of Chelating Agents on free and Immobilized MnP Activity

The chelating agents *o*-phenanthroline, *α,α*-dipyridyl and EGTA were tested regarding their effect on free and immobilized MnP activity. They were tested at 0.2, 0.4, 0.6, 0.8 and 1.0 mM in the reaction medium followed by measuring enzyme activity.

2.8 Phenol Removal by MnP

Mixing of MnP with hydrogen peroxide and phenol for a specific time causes oxidation of phenol to less toxic form. The concentration of total phenol was measured using a colorimetric method. Phenolic materials react with 4-aminoantipyrine in the presence of potassium ferricyanide at pH 10 to form a stable reddish-brown colored antipyrine dye. The amount of color produced is a function of the concentration of phenolic material [17].

3. RESULTS AND DISCUSSION

3.1 Effect of Thioglycolate on Free and Immobilized MnP Activity

Thioglycolate as sulfur-containing compound was tested regarding its effect on MnP from *T. harzianum*. The results in Fig. 2a show that increasing thioglycolate concentration resulted in an increase in the activity of free, alginate-E and chitosan-E.

The V_{max} for the three forms of MnP was calculated from Figs. 2b, 2c and 2d and its values were 24.9, 27.9 and 32.6 Umg^{-1}protein, respectively. The K_m values were 0.06, 0.053 and 0.071 mM. The increase in MnP by thioglycolate is consistent with the results reported for protease by El-Shora et al. [18]. Thioglycolate can activate the enzyme through maintaining the sulfhydryl group in reduced state [19].

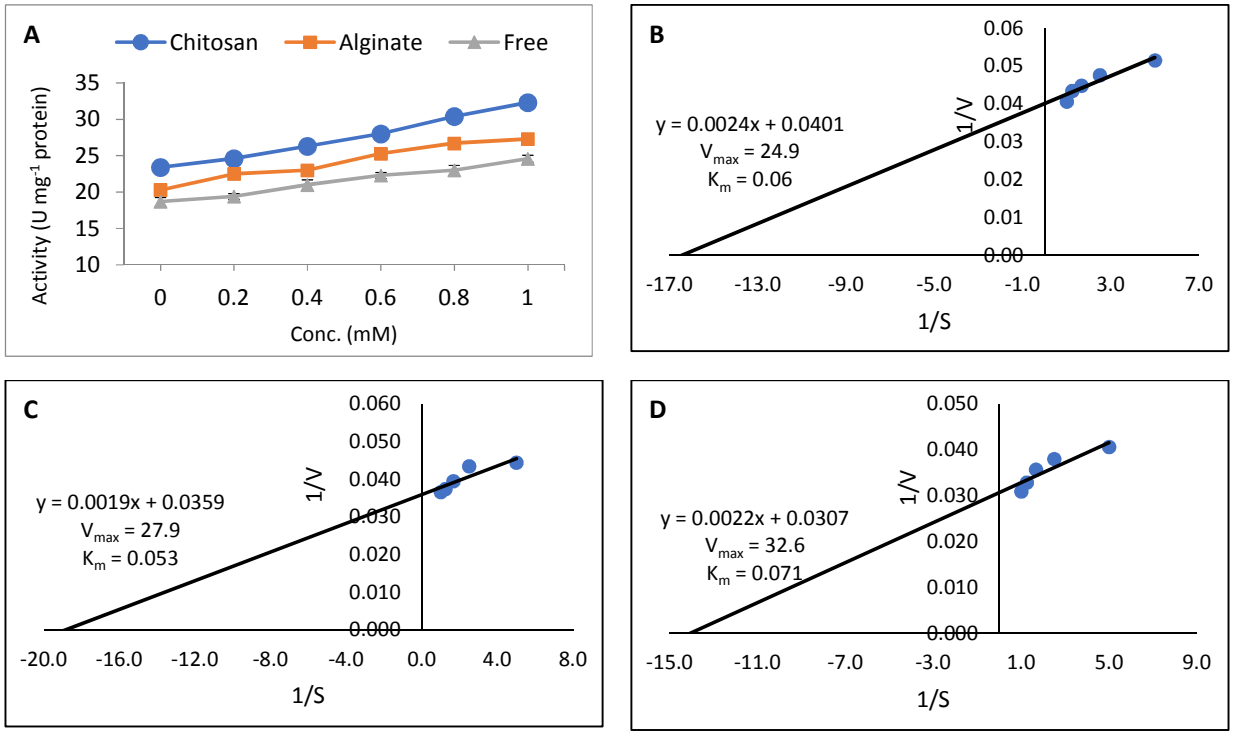

Fig. 2. The effect of thioglycolate on free and immobilized MnP activity. A: the relation between concentration and enzyme activity. B, C and D: Lineweaver-Burk plot for free, alginate-E and chitosan-E.

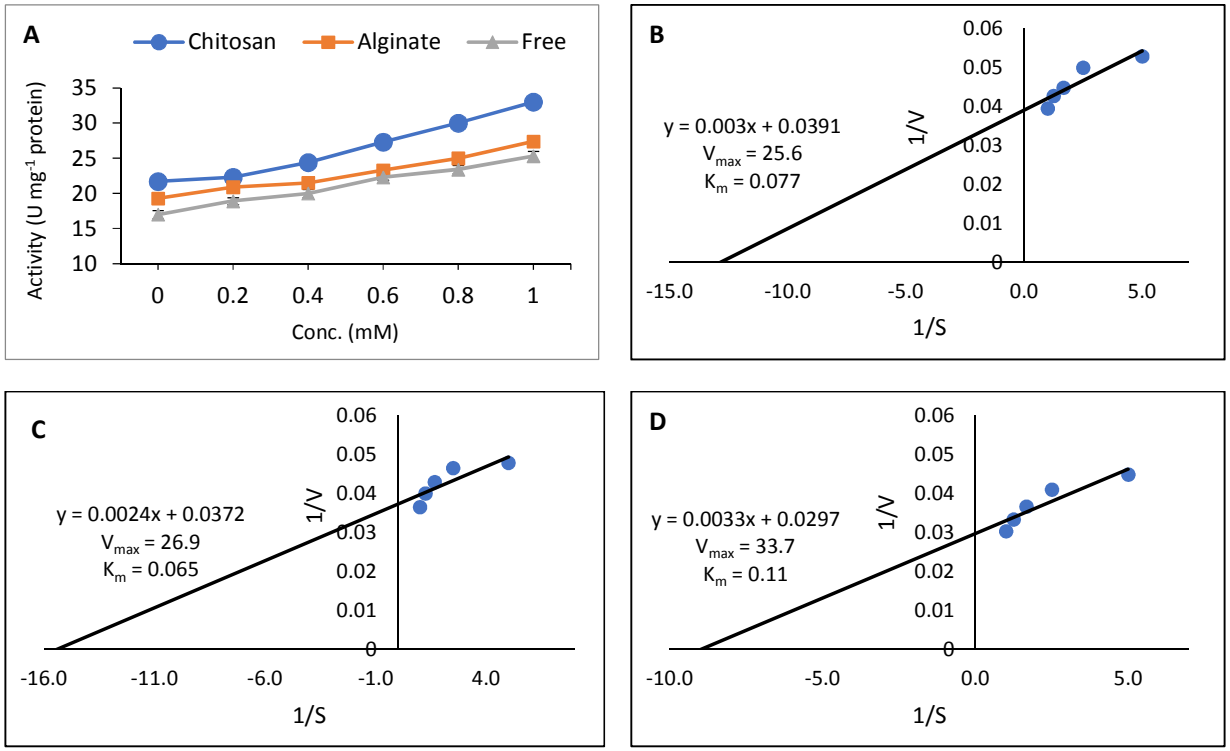

Fig. 3. The effect of GSH on free and immobilized MnP activity. A: the relation between concentration and enzyme activity. B, C and D: Lineweaver-Burk plot for free, alginate-E and chitosan-E

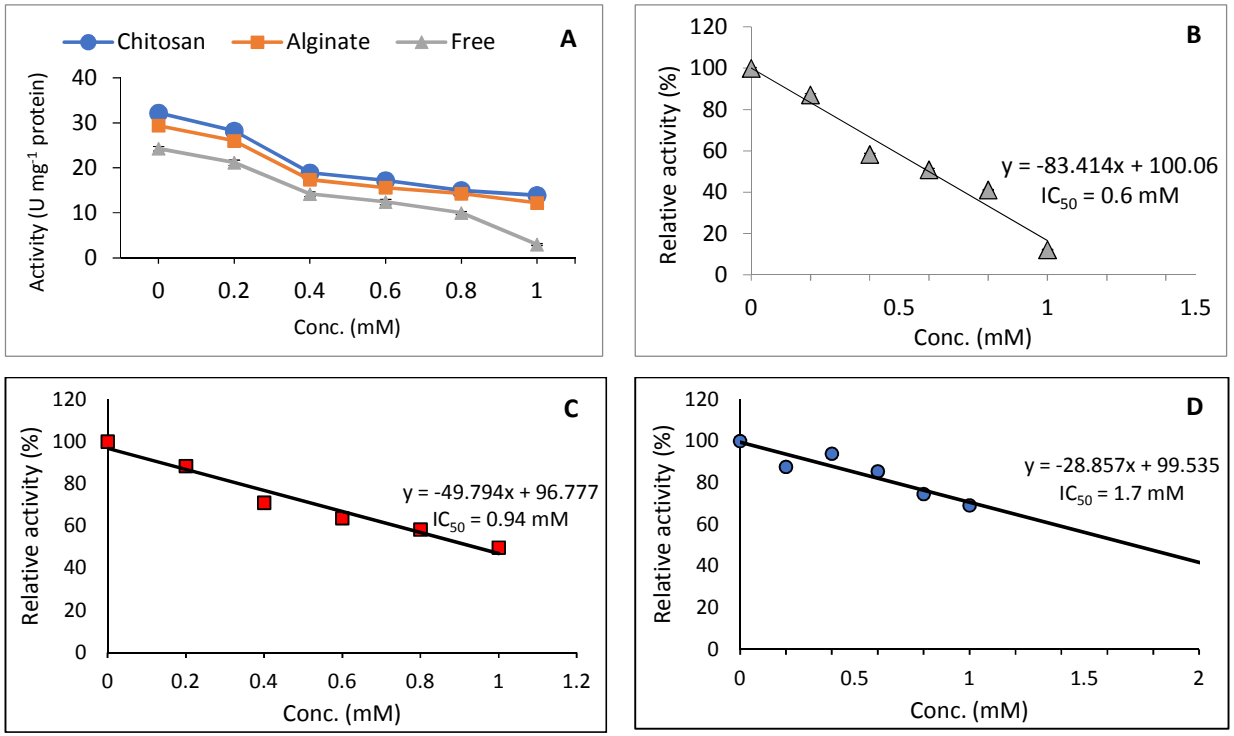

Fig. 4. The effect of *o*-phenanthroline on free and immobilized MnP activity. A: the relation between concentration and enzyme activity. B, C and D: IC_{50} for free, alginate-E and chitosan-E

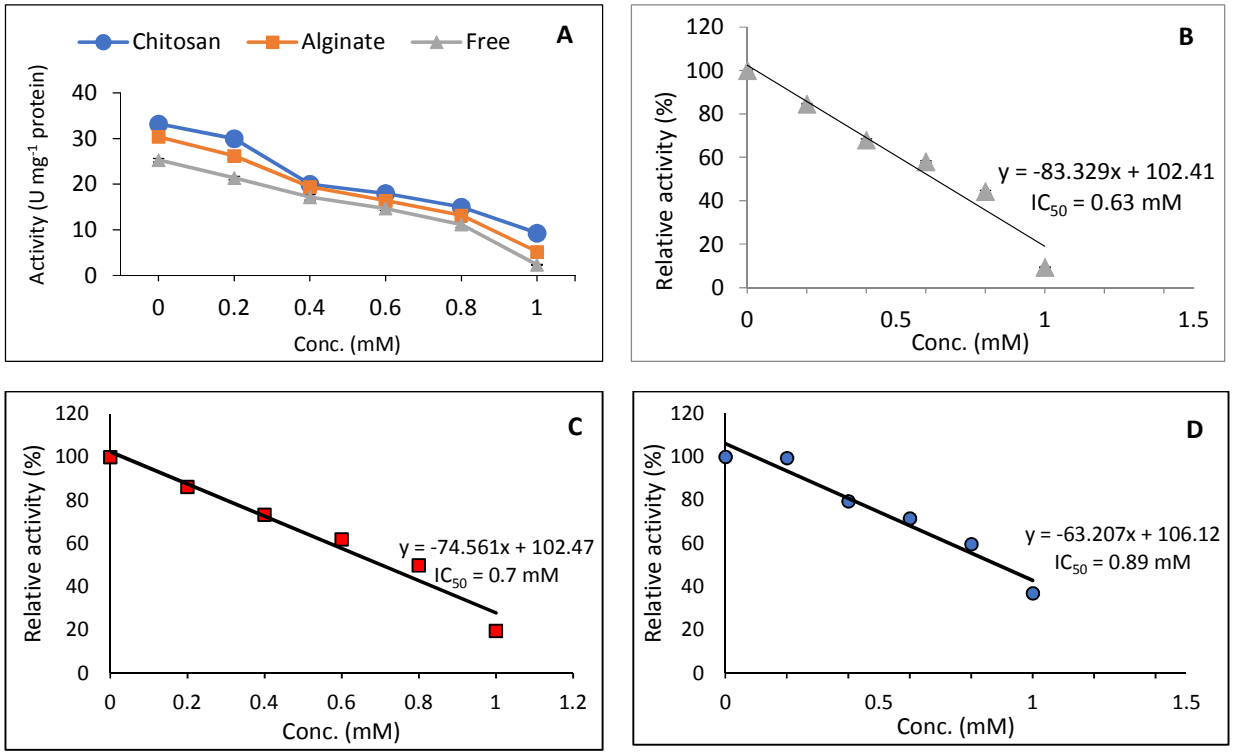

Fig. 5. The effect of *α,α*-dipyridyl on free and immobilized MnP activity. A: the relation between concentration and enzyme activity. B, C and D: IC_{50} for free, alginate-E and chitosan-E.

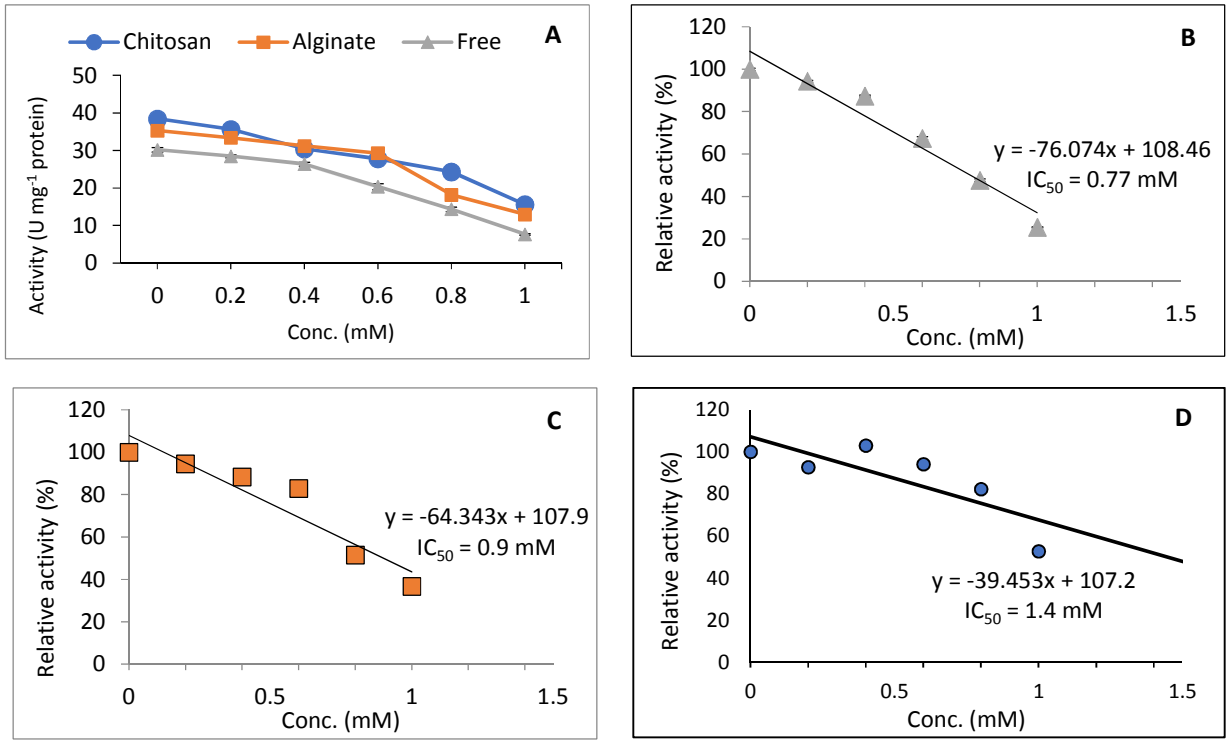

Fig. 6. The effect of EGTA on free and immobilized MnP activity. A: the relation between concentration and enzyme activity. B, C and D: IC_{50} for free, alginate-E and chitosan-E.

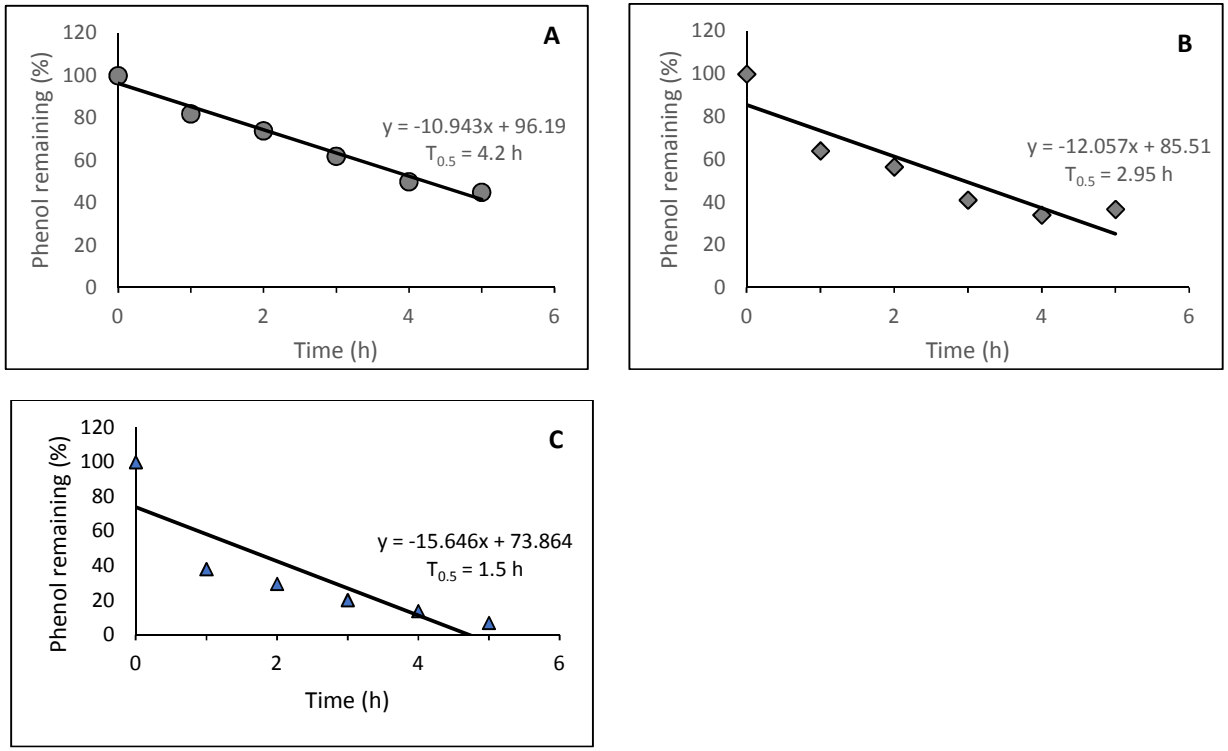

Fig. 7. Removal of phenol by MnP from *T. harzianum.* A: Free MnP, B: alginate-E and C: chitosan-E.

3.2 Effect of GSH on Free and Immobilized MnP Activity

Reduced glutathione is a tripeptide contains cysteine, glycine and glutamate. In the present work, treatment of MnP with various concentrations of GSH caused activation of MnP in a concentration-dependent manner (Fig. 3a). V_{max} values were 25.6, 26.9 and 33.7 U mg^{-1} protein for free, alginate-E and chitosan-E. K_m values were 0.077, 0.065 and 0.11 mM for the forms of MnP in the same order (Figs. 3b, c and d). Thus, immobilization increased V_{max} and it was apparent in case of chitosan-E. GSH activated other enzymes such as urease [20] and protease [18]. The activation of MnP by GSH may be due to protection of sulfhydryl group of the enzyme during the incubation time. Increasing the affinity of MnP for its substrate by GSH cannot be ruled out.

3.3 Effect of *o*-Phenanthroline on Free and Immobilized MnP Activity

Treatment of MnP with various concentrations of *o*-phenanthroline resulted in the inhibition of the enzyme activity in a concentration-dependent manner (Fig. 4a). IC_{50} for free, alginate-E and chitosan-E were 0.6, 0.94 and 1.7 mM, respectively (Figs. 4b, c and d). *o*-phenanthroline inhibited other microbial enzymes such as protease from *Aspergillus niger* and *Aspergillus terreus* [21] and *α*-glucosidase from *Penicillium notatum* [22]. The inhibition of MnP by *o*-phenanthroline reveals that the enzyme is metalloenzyme.

3.4 Influence of *α,α*-dipyridyl on Free and Immobilized MnP Activity

Treatment of the three forms of the enzyme with *α,α*-dipyridyl reduced the enzyme activity depending on the concentration (Fig. 5a). IC_{50} values were 0.63, 0.7 and 0.89 mM for free, alginate-E and chitosan-E (Figs. 5b, c and d). Thus, the free enzyme was strongly inhibited by *α,α*-dipyridyl. The later compound inhibited other enzymes including fungal *α*-glucosidase [22], bacterial myrosinase [23] and plant glutaminase [24]. The inhibition of MnP by *α,α*-dipyridyl confirms that it is metalloenzyme.

3.5 Effect of EGTA on Free and Immobilized MnP Activity

EGTA treatment for MnP resulted in the inhibition of the free and immobilized enzyme in a concentration-dependent manner (Fig. 6a). IC_{50} values were 0.77, 0.9 and 1.4 mM for free, alginate-E and chitosan-E (Fig. 6b, c and d). These results indicate that immobilization protected the enzyme against chelating effect of EGTA. In support, EGTA inhibited MnP from wood-rotting fungi [25]. Furthermore, MnP produced by *Phanerochaete chrysosporium* was inhibited by EGTA [26]. EGTA inhibited other enzymes such as acid phosphatase from *Cladosporium cladosporioides* [27], urease [20] and bacterial myrosinase [23]. The inhibition of MnP by EGTA gives a third piece of evidence that it is a metalloenzyme.

3.6 Removal of Phenol by MnP from *T. harzianum*

The free and immobilized MnP were tested for removing phenol from wastewater. The results in Fig. 7 indicate that immobilizing enzyme showed greater efficiency to remove phenol compared to the free enzyme, particularly chitosan-immobilized enzyme. $T_{0.5}$ values were 4.2, 2.95 and 1.5 h for the three forms of MnP mentioned above in the same sequence. Xu et al. [28] reported that MnP of white rot basidiomycete *Ganoderma lucidum* was capable to degrade various phenolic compounds.

4. CONCLUSION

MnP in presence of thiol compounds will be more useful to improve a potential biotechnological purpose and to increase its catalytic efficiency. Also, the immobilized MnP on alginate or chitosan expressed appreciable potentiality to remove phenol from wastewater and exhibited more resistance against the chelating agents. This is a privilege in that the industrial wastewater may contain some chelating agents which can suppress the enzyme activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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