academicJournals

Vol. 10(14), pp. xxx-xxx, 30 July, 2015
DOI: 10.5897/SRE2015.6268
Article Number:xxxxxx
ISSN 1992-2248

http://www.academicjournals.org/SRE

Scientific Research and Essays

Full Length Research Paper

Purification and biochemical characterization of extracellular manganese peroxidase from *Ganoderma lucidum* IBL-05 and its application

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Received 28 May, 2015; Accepted 10 July, 2015

In this study an extracellular manganese peroxidase (MnP) was isolated from culture filtrate of an indigenous fungal strain *Ganoderma lucidum* IBL-05 under static conditions using wheat bran as substrate. The enzyme was purified by applying successively ammonium sulphate precipitation, dialysis, ion exchange and gel filtration chromatographic techniques. Purification procedure resulted in 3.43-fold purification with corresponding specific activity of 539.59 Umg-^{1}. The purified MnP elucidated single band in 43 kDa region on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The purified MnP showed optimum activity at pH 5 and 40°C temperature. The K_m and V_{max} for MnP toward $MnSO_4$ as a substrate were found to be 65.5 mM and 640 UmL^{-1}, respectively. It was observed that MnP activity enhanced by Mn^{2+} and Cu^{2+} and inhibited in the presence of Zn^{2+}, Fe^{2+}, EDTA and Cysteine to various extents with Hg^{2+} (most inhibitory). The purified MnP efficiently catalyzed the transformation of different synthetic textile dyes (Sandal-reactive dyes). Characterization revealed that MnP isolated from *G. lucidum* have potential to be used for myriad industrial and biotechnological applications.

Key words: Manganese peroxidase, *Ganoderma lucidum* IBL-05, purification, characterization, kinetics, dye decolorization.

INTRODUCTION

Significant efforts have been made on filamentous fungal biotechnology in recent years in order to obtain value added products such as enzymes, chemicals, liquid biofuel, secondary metabolites and spores (Reina et al., 2013). White-rot basidiomycetes are the principal organisms that secrete a unique set of extracellular oxidoreductases comprising lignin peroxidase (LiP; Ec1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13) and laccase (Lac; EC 1.10.3.2). These catalysts are known with remarkable potential to depolymerize lignin and various environmental pollutants such as polycyclic aromatic hydrocarbons, synthetic dyes and cholorophenols (Du et al., 2015). Different white rot fungi (WRF) secrete different set of lignin mineralizing

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enzymes (LMEs) and each fungus secretes one or more of the three enzymes essential for lignin degradation (Levin et al., 2008). Besides, some versatile peroxidases (VP; EC.1.11.1.16) with combined LiP and MnP catalytic properties have also been reported (Morgenstern et al., 2008).

Due to their interesting catalytic properties, MnPs have gained considerable interest in various industrial areas. The most intensively studied applications have included bioremediation, biomass delignification, oxidation of organic pollutants, bio sensing, textile, animal feed, cosmetics, detergent manufacturing, paper and pulp, transformation of antibiotics and steroids etc (Sylvia et al., 2015). MnP (EC 1.11.1.7) is a lignin-modifying glycoprotein synthesized by wood-colonizing basidiomycetes during secondary metabolism. It catalyzes phenolic compounds to phenoxy radicals by oxidation of Mn^{2+} to reactive Mn^{3+} in H_2O_2 dependent enzymatic reaction (Ferhan et al., 2012). However, some low molecular weight mediators can increase the substrate range of MnPs to non-phenolic structures (Giardina et al., 2000). Further, many proteins acting synergistically with MnPs, has expanded the role of these enzymes in fungal lignolysis (Hilden et al., 2000).

Among WRF, *Ganoderma lucidum* is considered as the most commonly used organism in biodegradation studies due to its good ligninolytic properties, fast growth potentials, and environmental-friendly nature (Batool et al., 2013). Keeping in view the extensive industrial applications of MnP, the present study was accomplished with an objective to purify and characterize the extracellular MnP from an indigenous WRF strain *G. lucidum* IBL-05 and then tested it for its ability to decolorize different textile dyes.

MATERIALS AND METHODS

Fungal culture and inoculum development

For inoculum development, the indigenously isolated *G. lucidum* IBL-05 strain was grown in Kirk's basal nutrient medium (Tien and Kirk, 1988) in Erlenmeyer flask (250 ml) that was supplemented with Millipore filtered 1% glucose. Prior to sterilization, the medium was adjusted at pH 4.5 with 1 M NaOH/1 M HCl and inoculated with spores of *G. lucidum* IBL-05 from slant culture. The inoculated flask was incubated (120 rpm) at 30°C for 5 to 7 days to get homogenous spore suspension of the fungus (1×10^6-10^8 spores/ml) and used as inoculum.

Production and extraction of MnP

Triplicate conical flasks containing 5 g semi-solid wheat bran were autoclaved and inoculated with 5 ml homogenous inoculum (Ramzan et al., 2013). The inoculated flasks were allowed to ferment at 30°C in a temperature controlled incubator for 5 to 7 days at pH 4.5. After growth, sterile samples were taken after every 24 h and MnP activity was monitored. When MnP activity was peaked to a maximum level, the fermented biomass was harvested by adding 100 ml distilled water, filtered, centrifuged (Ependorf 5415C, Germany) and clear supernatant was assayed for MnP enzyme and stored at 4°C in refrigerator for further characterization. All experiments were carried in triplicate to avoid the discrepancy in results.

Determination of MnP activity and protein contents

The MnP activity was determined by monitoring the formation of Mn^{3+}-malonate complexes at 270 nm (ε_{270} = 11570 M cm^{-1}, Wariishi et al., 1992). Assay mixture contained 1 ml of $1x10^{-3}$M $MnSO_4$, 1 ml of $50x10^{-3}$M sodium malonate buffer (pH 4.5) and 0.5 ml of H_2O_2 in combination with 0.1 ml enzyme solution. Absorbance of each sample was measured Spectrophotometrically (HALO DB-20). The protein concentration was estimated according to Bradford method (1976) using bovine serum albumin (BSA) as a standard.

Purification of MnP

All the purification steps were conducted below 4°C. Briefly, crude MnP extract obtained from 5 days old culture of *G. lucidum* IBL-05 was centrifuged at 3,000 × g for 15 min. The cell-free supernatant was saturated (up to 35%) by gradual addition of ammonium sulphate and kept for overnight at 4°C. The resulting precipitate, thus obtained was recovered (3,000 × g for 20 min at 4°C) and supernatant was again saturated by adding ammonium sulfate (up to 65%), allowed to stand overnight at 4°C, centrifuged and pellets were dissolved in 50 mM Sodium Malonate buffer (pH 4.5). The solution was kept in dialysis bag and dialyzed against the same buffer, after sealing it securely and finally, dialyzate was freeze dried. The dialyzate obtained was submitted to ion-exchange chromatography using diethyl amino ethyl (DEAE) cellulose column, equilibrated with phosphate buffer (100 mM; pH 6.5) for 24 h and eluted with 0 to 1.0 M linear gradient of NaCl at a flow rate of 0.5 ml/min. The MnP active fractions were pooled and loaded onto Sephadex-G-100 column (10×300 mm). Up to 30 positive fractions (1 ml) with flow rate of 0.3 ml/min were collected and absorbance was measured at 280 nm (Zeng et al., 2013).

Gel electrophoresis and staining

The MnP purification was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). The molecular mass of MnP was approximated after gel staining with Coomassie Brilliant Blue G-250 followed by calibration against standard protein markers (Sigma, USA), ranging from 17-170 kDa.

Characterization of purified MnP

Effect of pH on MnP activity was investigated by incubating enzyme for 15 min at varying pH values (3 to 10). For stability studies the enzyme was pre-incubated at varying pH for 1 h. MnP activities were also determined at various temperatures between 30 and 70°C under optimal pH values. The enzyme was incubated for 15 min at varying temperatures before running the enzyme assay. For thermal-stability the enzyme was incubated at different temperatures for 1 h without substrate before carrying out MnP assay. K_m and V_{max} for the purified enzyme were calculated using the Lineweaver Burk transformation of Michaelis-Menten equation. The standard quartz cuvettes of 1 mm path length were used to calculate the values of kinetic parameters. The enzyme was incubated for 15 min at 30°C in sodium malonate buffer of pH 4.5 before carrying out standard enzyme assay protocol. Lineweaver-Burk (Double reciprocal) plot was generated with

Microsoft Excel Windows updated version 7 via nonlinear regression analysis using different concentrations (0.1 to 1.0 mM) of manganese sulphate as substrate at optimum pH 5 and 40°C temperature. The effects of various modulators (Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^{+}, Ethylene diamine tetra acetic acid (EDTA) and cysteine) on enzyme activities were tested in the concentration range of 5 to 20 mM. Enzyme activities measured without any modulator was considered as 100% (Zeng et al., 2013).

Dyes and decolorization studies

Three dyes namely Sandal-fix Red C_4BLN (λmax: 540 nm), Sandal-fix Turq Blue GWF (λmax: 664 nm) and Sandal-fix Black CKF (λmax: 598 nm) were used to investigate the decolorization potential of purified MnP. For this, MnP solution was transferred to triplicate Erlenmeyer flasks (500 ml) containing 100 ml of individual dye solution (0.1 mg/ml) in combination with Na-malonate buffer (50 mM; pH 4.5). Flasks were incubated in rotary shaker (Sanyo-Gallenkamp, UK) at 40°C for 12 h. The flasks content were filtered, centrifuged (8,000 × g, 10 min) and dye removal was monitored spectrophotometrically (HALO DB-20) at respective wavelengths. The decolorization efficiency was calculated using relation (Equation 1).

$$\text{Decolorization } (\%) = \frac{Ai - At}{Ai} \times 100 \quad (1)$$

Where, A*i* and A*t* are representing absorbance at zero and time *t*.

Statistical analysis

Mean and standard deviation (SD) of the results based on three independent experiments were calculated using Microsoft Excel-software (Microsoft) and the standard error (SE) values were displayed as Y-error bars in figures.

RESULTS AND DISCUSSION

Production of MnP

In this study, a locally isolated fungal strain, *G. lucidum* IBL -5was exploited for ligninolytic enzyme (Lip, MnP and laccase) production potential in solid state medium of wheat bran under pre-optimized growth conditions such as moisture, 50%; substrate, 5 g; pH, 5.5; temperature, 30°C; carbon source, 2% glucose; nitrogen source, 0.02% yeast extract; C:N ratio, 25:1; fungal spore suspension, 5 ml and fermentation time period, 5 days (Ramzan et al., 2013). The enzyme extract contained 576.3, 717.7 and 323.2 UmL^{-1} of LiP, MnP and laccase, respectively.

Purification of MnP

A sequential four-step purification procedure involving ammonium sulphate fractionation, dialysis, DEAE-cellulose ion exchange and G-100 sephadex gel permeation chromatography was employed for the purification of MnP. A mixture of crude ligninolytic extract obtained from five days incubated culture (300 ml) of *G. lucidum* on wheat bran was purified to homogeneity as summarized in Table 1. The MnP was completely salted out at 65% saturation with $(NH_4)_2SO_4$ to 1.73 fold purification with specific activity of 273.01 U/mg. After ammonium sulphate precipitation, the crude extract was applied to ion exchange chromatography. The elution pattern on the DEAE-cellulose column showed two protein peaks at 280 nm (Figure 1). The fractions with high MnP activities were pooled, concentrated and loaded onto a Sephadex G-100 column. In Figure 2 it was clearly indicated that the MnP eluted in a single prominent peak on gel filtration chromatography. At the end of fourth purification step, the enzyme had been 3.43-fold purified with corresponding specific activity of 539.59 U/mg. The purified MnP appeared as a single band in 43 KDa region on SDS-PAGE analysis (Figure 3), suggesting that the enzyme was a monomeric protein. The MnPs also secreted in a multiple isoform with distinct structural configuration and molecular mass. The molecular masses of MnP vary from 32 to75kDa (Asgher et al., 2013).

Characterization of purified MnP

Effect of pH on MnP activity and stability

The pH profile for purified MnP has been illustrated in Figure 4. The MnP displayed maximum activity at optimum pH 5; beyond this pH value a marked decreasing trend in the activity was observed. Moreover, the purified MnP was fairly stable over a wide range of pH (4-6) at incubated time of 1 h, pH above 6 caused inactivation of the enzyme irrespective of incubation time. In previous studies, maximum activity of MnP from different WRF has been reported in the pH range of 4.5 to 6.5 (de Oliveira et al., 2009). MnP isolated from solid-state culture of corncobs by *Lentinula edodes* exhibited optimum activity at pH 4.5 (Boer et al., 2006); whereas, MnP from *S. commune* IBL-06 was optimally active at pH 5 (Asgher et al., 2013).

Effect of temperature on MnP activity and stability

The MnP activity against temperature curve shown in Figure 5, the plot indicated that initial rise of temperature up to 40°C increased the activity as well as stability of the MnP. Further rise of temperature caused decrease of activity and stability as well due to denaturation of enzyme at elevated heat. The optimum temperature for purified MnP was found to be 40°C. The purified MnP showed fascinating thermal-stability up to 50°C without dropping much of its activity, which would be an attractive and desirable feature for a variety of industrial processes. However, MnP lost almost 50% of its activity when

Table 1. Purification summary of MnP produced by *G. lucidum* IBL-05 in solid state fermentation.

Step	Purification steps	Total volume (ml)	Enzyme activity (U ml^{-1})	Protein contents (mg ml^{-1})	Specific activity (U mg^{-1})	Purification fold
1	Crude enzyme	300	717.4	4.34	132.32	1
2	Ammonium Sulphate ppt.	22	613.7	2.45	273.01	1.73
3	Dialysis	21	598.09	2.13	390.91	1.95
4	DEAE-cellulose	12	584.3	1.68	420.34	2.78
5	Sephadex G-100	9	569.6	0.9	539.59	3.43

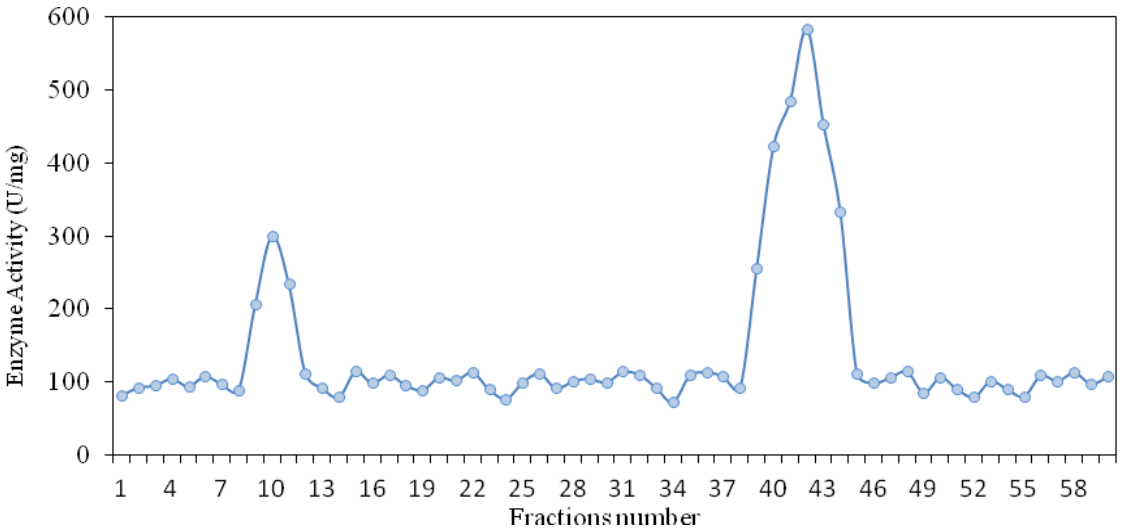

Figure 1. Ion exchange chromatographic purification profile for MnP from *G. lucidum*.

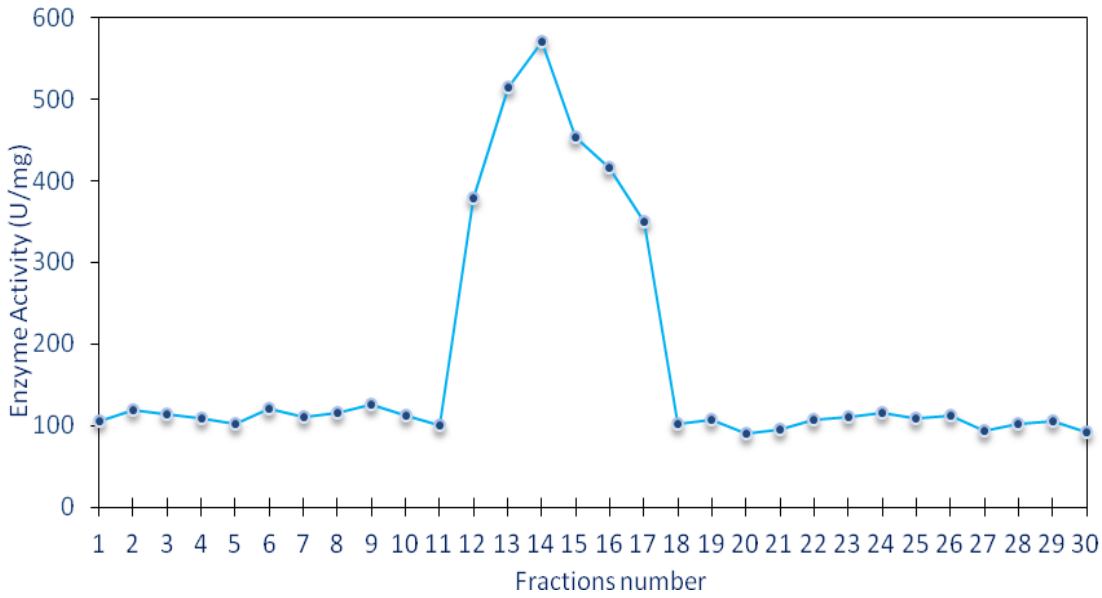

Figure 2. Gel filtration chromatographic purification profile for MnP from *G. lucidum*.

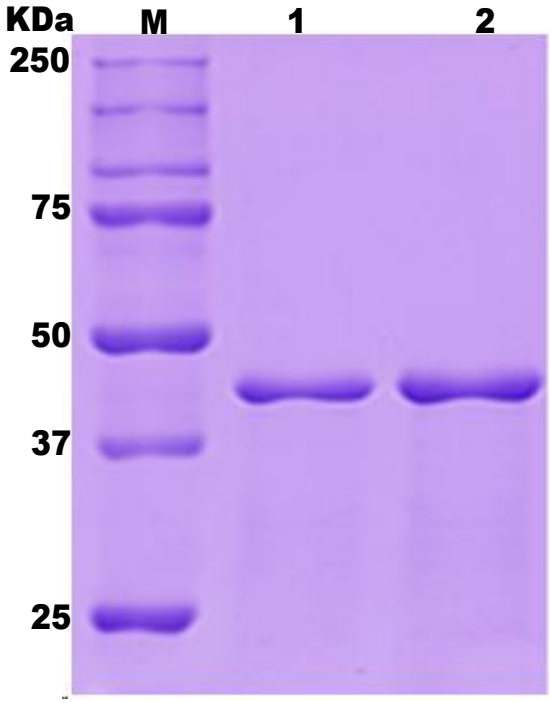

Figure 3. SDS-PAGE of MnP produced from *G. lucidum* IBL-05. The molecular mass of the purified MnP was estimated in comparison to standard protein marker, 25-250 kDa; (Sigma, USA). The protein bands were visualized by staining with Coomassie Brilliant Blue G-250.

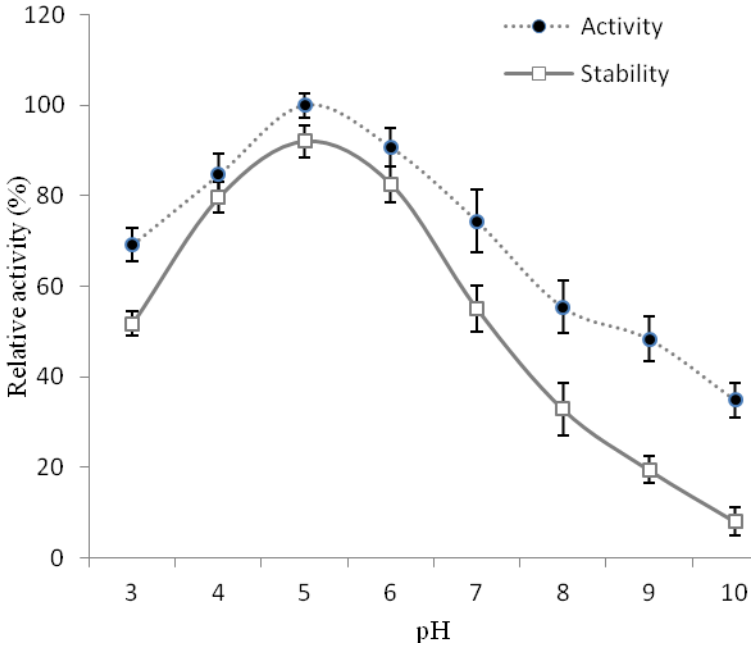

Figure 4. Effect of pH on the activity and stability of MnP. The results presented arethe means of three independent experiments and the bars represent the standard deviation of the means.

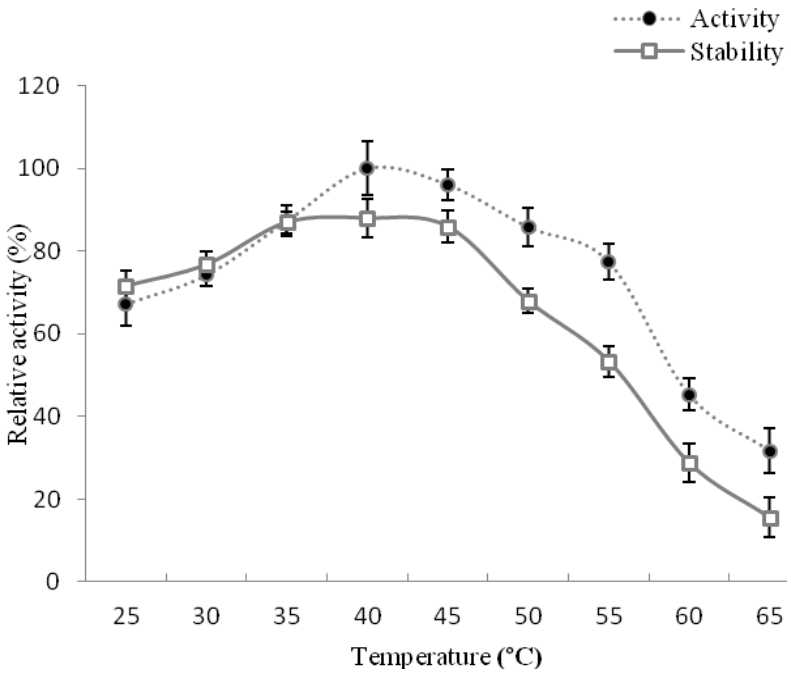

Figure 5. Effect of temperature on the activity and stability of MnP. The results are the means of three replicates and the bars represent the standard deviation of the means.

incubated at 55°C for 1 h. The MnP isolated from different WRF demonstrated optimum temperature around 40 to 60°C (Hakala et al., 2005). The MnP from WRF strain, *Irpex lacteus* was stable in the temperature range of 30 to 40°C (Shin et al., 2005), while *Rhizoctonia* sp. isolated MnP was deactivated over 55°C (Cai et al., 2010). The MnP2 isozyme from *Lentinula edodes* showed thermal-stability up to 40°C (Boer et al., 2006).

Determination of kinetic constants *Km* and *Vmax*

The K_m and V_{max} values were calculated by intercepting line on X-axis and Y-axis of the reciprocal plot, respectively, using different concentration (0.1 to 1.0 mM) of $MnSO_4$ as assay substrate (Figure 6). At 0.5 mM $MnSO_4$ concentration, the maximum MnP activity (859 UmL^{-1}) was furnished with K_m 65.64 µM and V_{max} 640 UmL^{-1} using non-linear regression analysis at optimum pH and temperature. The difference in K_m values of MnP from different reported fungal species might be due to the genetic variations and substrate specificities among various species. The interaction of enzyme with its substrate was indicated through K_m values and a lower K_m value reflect high affinity of enzyme for its substrate and higher V_{max} indicated that small amount of enzyme can convert substrate into the product (Asgher et al., 2014).

Effect of various modulators on activity of MnP

In order to identify the nature of enzyme, the effects of various organic compounds and metal ions as possible inhibitors and activators on MnP activities were studied (Table 2). The results revealed that Mn^{2+} and Cu^{2+} enhanced the activity of MnP at all tested concentrations. The metal ion Mn^{2+} showed the most significant role to activate *G. lucidum* MnP, which was consistent with the findings of Boer et al. (2006). In addition, low concentrations of Co^{2+} (5 mM) drive up MnP activity but at higher concentration of Co^{2+} (20 mM) a slight inhibition was observed (91%). Elevated concentrations of K^+ enhanced the MnP activity (117%) but lower K^+ concentrations (5 mM) did not exert any effect on MnP activity. On the other hand, $Zn^{2+}$and Fe^{2+} partially inhibited the MnP, whereas MnP activity was strongly inhibited by Ethylene diamine tetra acetic acid (EDTA) and cysteine and even Hg^{2+} fully inactivated MnP enzyme. The MnP from *P. chrysosporium* was inhibited by NaN_3, *β*-mercaptoethanol and dithreitol, whereas co-oxidants such as glutathione, un-saturated fatty acids and Tween 80, significantly enhanced the MnP activity (Urek and Pazarlioglu, 2004). *Trichophyton rubrum* LSK-27 MnP was entirely inhibited by Hg^{2+}, while Fe^{3+}, Ca^{2+} and Ni^{2+} did not show any inhibitory effect on enzyme activity (Boer et al., 2006).

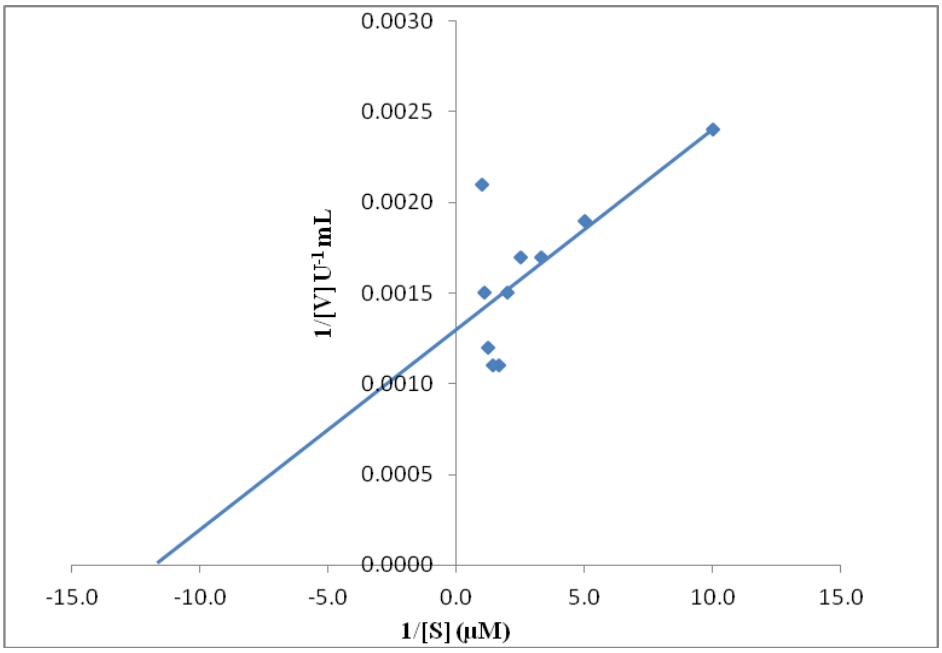

Figure 6. Lineweaver Burk Plot for determination of K_m and V_{max} for purified MnP.

Table 2. Effects of various modulators on the activity of purified MnP from *G. lucidum*.

Modulators	Concentration (mM)	Relative activity of MnP (%)
Mn^{2+}	5	119±6.4
	10	139±5.3
	20	147±3.7
Zn^{2+}	5	89±2.8
	10	71±2.5
	20	58±4.2
Co^{2+}	5	109±5.1
	10	103±4.6
	20	91±4.7
Cu^{2+}	5	114±4.5
	10	117±3.9
	20	128±4.9
Fe^{2+}	5	82±1.4
	10	57±2.4
	20	42±4.2
Hg^{2+}	5	14±0.9
	10	7.6±0.13
	20	3.3±0.29
K^{2+}	5	101±4.0
	10	109±3.9
	20	117±5.3

Table 2. Contd.

EDTA	5	45±1.9
	10	32±2.3
	20	27±2.4
Cysteine	5	38±1.7
	10	21±2.1
	20	9±0.73

All data are expressed in mean value ±standard deviation.

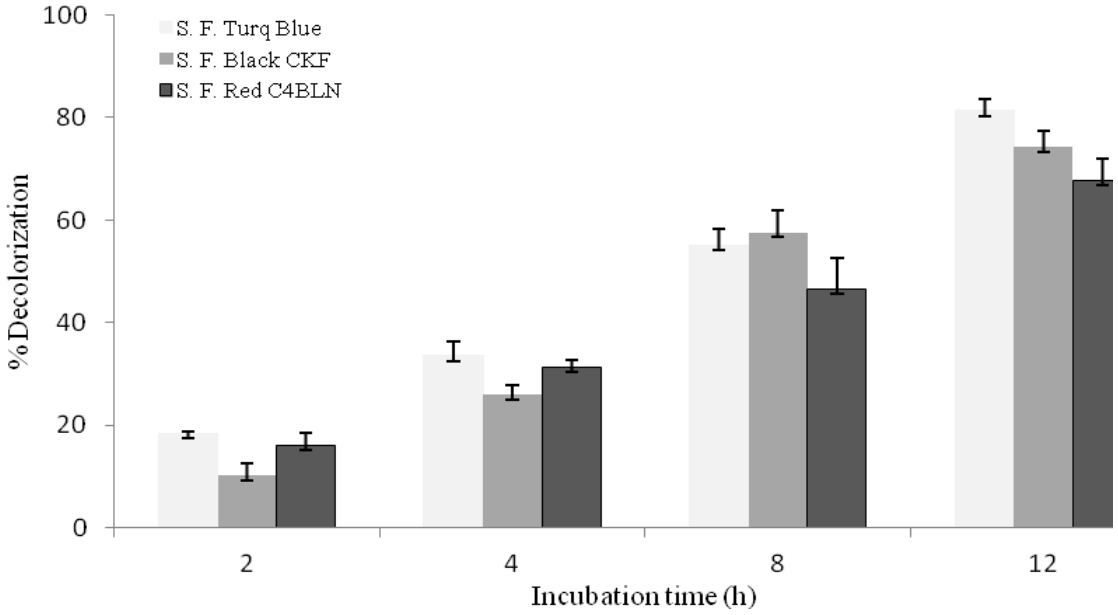

Figure 7. Percentage decolorization of textile dyes by purified MnP from *G. lucidum* IBL-05. The results are the means of three replicates and the bars represent the standard deviation of the means.

Decolorization of different textile (synthetic) dyes by purified MnP

The dye-decolorizing potential of purified MnP from *G. lucidum* was demonstrated for different synthetic dyes (Sandal-reactive dyes) at different time periods. From data in Figure 7 it can be seen that MnP caused maximum decolorization of S.F. Turq Blue dye to 81.3%, followed by S.F. Black CKF to 74.2% and S. F. Red C_4BLN dye to 67.8% within 12 h of incubation period. The MnP was more effective for decolorization of different textile dyes including Remazol brilliant blue R (RBBR), Congo red, methylene blue and ethyl violet (Bazanella et al., 2013). The findings correlated with previous investigations (Cheng et al., 2007), which confirmed that MnP has remarkable catalytic potential to degrade and mineralize dyes and colored effluents. The peroxidases caused dye degradation by generating highly active free radicals such as Mn^{3+}, lipid, hydroxyl, and peroxy-radicals (Hofrichter, 2002). However, the dyes are not uniformly susceptible to biodegradation because of the structural diversity (Murugesan et al., 2006).

Conclusion

The ion exchange and gel filtration column chromatography techniques were used to purify MnP enzyme from *G. lucidum* up to 3.43-fold. The molecular weight of purified MnP was determined to be 43 kDa from SDS-PAGE analysis. Purified MnP showed encouraging activity and stability at their optimal pH and temperature. Further, the purified MnP possesses effective dye decolorization capability, indicating a useful tool for

bioremediation purposes. The high level MnP production and its novel catalytic features suggest its suitability for industrial and biotechnological applications. Nevertheless, further molecular approaches are needed for improving its catalytic and thermal-stability characteristics that will be the focus of future research.

Conflict of Interest

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENT

This study was supported by Higher Education Commission, Islamabad, Pakistan.

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