CHARACTERIZATION OF A NOVEL MANGANESE PEROXIDASE PURIFIED FROM SOLID STATE CULTURE OF *Trametes versicolor* IBL-04

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A novel manganese peroxidase (MnP) produced by an indigenous white rot fungal strain *Trametes versicolor* IBL-04 in solid state medium of corncobs was purified and characterized. The fungus produced 964U/mL MnP in the presence of additional carbon (glucose) and nitrogen (yeast extract) supplements added at a C:N ratio of 25:1, 1mM Tween-80 (1mL), 1mM $MnSO_4$ (1mL), and 1mM $CuSO_4$ (1mL). The MnP was purified by ammonium sulfate fractionation (65% saturation) and dialysis, followed by Sephadex G-100 gel filtration chromatography. Purification procedures resulted in 2.4-fold purification with an overall yield and specific activity of 3.4% and 660 U/mg, respectively. The purified MnP was monomeric of molecular weight of 43 kDa, showing a single band on sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE). The enzyme was optimally active at pH 5 and 50°C and was stable for 1 h over a broad range of pH (4-7) and temperature (40-65°C). Kinetic constants K_M and V_{max} of purified MnP were 70 µM and 540 U/mL for $MnSO_4$ substrate. The effect of possible activators and inhibitors of enzyme were also investigated, and it was observed that EDTA, Cystein, and Ag^+ caused MnP inhibition and inactivation to different extents, whereas MnP was activated by 4 and 3 mM of Cu^{2+} and Fe^{2+}, respectively. High thermo-stability, low K_M and high V_{max} features of this novel MnP isolated from culture filtrate of *T. versicolor* IBL-04 suggests its suitability for various industrial and biotechnological applications.

Keywords: T. versicolor IBL-04; Manganese peroxidase; Purification; SDS-PAGE; Characterization

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INTRODUCTION

Microorganisms capable of degrading lignin include the wood-rotting fungi and, to a lesser extent, some actinomycetes and bacteria (Coll et al. 1993). These micro-organisms produce two major families of enzymes ligninolytic enzymes, peroxidases and laccases. *T. versicolor* is one of the most valued white-rot fungi in industrial and environmental biotechnology because of its ability to degrade lignin and phenolic compounds. Its potential simultaneously to produce extracellular (Tavares et al. 2006) ligninolytic and hydrogen peroxide-producing enzymes, which are essential for peroxidase activity, makes it an attractive candidate for a range of biotechnological applications (Levin et al. 2002). Lignin-modifying enzymes (LMEs) of white rot fungi (WRF) have numerous applications for various industries, including delignification of

lignocellulosic biomass for fuel ethanol production, food, brewery, animal feed, textile, laundry detergents, and pulp and paper (Bhat 2000; Rodríguez-Couto and Sanroman 2006; Papinutti and Forchiassin 2007).

Manganese-dependent peroxidases (MnP; EC 1.11.1.13) are heme-containing 30-65kDa glycoproteins secreted either as one entity or in multiple isoforms and catalyze the oxidation of Mn^{2+} to Mn^{3+} (Tien and Kirk 1984; Glenn and Gold 1985; Asgher et al. 2008). Many WRF secrete MnP as the main ligninolytic enzyme and a few even secrete it as the sole enzyme for lignin degradation. MnP require hydrogen peroxidase as well as Mn^{+2} for their activity (Bermek et al. 2004; Asgher et al. 2008). The natural function of MnP is the degradation of the complex lignin polymer of higher plants. The enzyme catalyzes the H_2O_2-dependent oxidation of Mn^{2+} into highly reactive Mn^{3+} that complexes with a dicarboxylic acids such as oxalate and malate produced by the fungus. The Mn^{3+}-organic acid complexes, in turn, oxidize phenolic structures in lignin and various lignin-related organic compounds (Tien and Kirk 1984; Glenn and Gold 1985; Arora and Gill 2000; Takano et al. 2010). MnP oxidizes a wide range of substrates, including several phenolic compounds, rendering it an interesting enzyme for potential applications in various industries (Bermek et al. 2004; Boer et al. 2006).

Cost-effective production of ligninases is a key for successful exploitation of lignocellulosic resources as renewable energy source. In previous studies it has been reported that the production and activity of MnP is enhanced by the addition of manganese ($MnSO_4$) in lower concentrations (0.1-1mM), as Mn is a specific inducer of MnP production and mediator in its catalytic mechanism (Rajan et al. 2010). The variability in ligninolytic enzyme production by different WRF has been reported due to genetic variations as well as variation of fermentation process conditions. The ligninases production process using many different strains of WRF have been optimized at laboratory scale by the addition of carbon and nitrogen sources, mediators, surfactants, and minerals to the medium. Nutritional variables including carbon, nitrogen and manganese significantly influence the production of ligninolytic enzymes by white rot fungi (Pascal et al. 1991). Different strains of *Trametes spp., Trametes versicolor, T. cingulata, T. elegans* and *T. pocas* have been found to produce LiP, MnP, and laccase in different ratios (Tekere et al. 2001). The ligninase production by *Trametes versicolor* is affected by C, N and Mn^{2+} concentration in the medium (Maceiras et al. 2001).

T. versicolor is one of the most potent lignin-degrading microorganisms that produce extracellular peroxidases with high catalytic potentials. Keeping in view the wide range of industrial applications of MnP, the present study was focused on purification and characterization of a novel heat-stable acidic MnP isolated from solid-state culture filtrates of an indigenous strain *T. versicolor* IBL-04 grown on inexpensive and easily available lignocellulosic solid waste Corncobs through SSF.

EXPERIMENTAL

Chemicals and Reagents

Sephadex G-100, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Coomassie Brilliant Blue G-250, sodium dodecylsulphate (SDS), N,N,N′,N′-tetra-

methylethylenediamine (TEMED), ß-mercaptoethanol (BME), trizma base and standard protein markers were purchased from Aldrich-Sigma (USA). All other chemicals were of analytical grade and purchased from Merck (Germany) and Scharlau (Spain).

Lignocellulosic Substrate

Corncobs used as growth substrate was collected from CPC-Rafhan, Faisalabad, Pakistan. The substrate was chopped, oven dried (60°C), ground to 40 mm mesh particle size, and stored in air tight plastic jars to avoid humidity.

Microorganism & Inoculum Preparation

The MnP producing culture of indigenous strain *T. versicolor* IBL-04 available in Industrial Biotechnology Laboratory, UAF, Pakistan was used. Inoculum was prepared by growing the fungus in Kirk's basal nutrient medium (Tien and Kirk 1988) additionally supplemented with 1% Millipore filtered sterile glucose solution. The medium was sterilized (121°C) in a laboratory-scale autoclave (Sanyo, Japan) for 15 min. After cooling to room temperature, a loopful culture of *T. versicolor* IBL-04 from PDA slant was transferred into the broth in laminar air flow (Dalton, Japan). The inoculated flask was incubated for 5 days at 30°C in an orbital shaker (Sanyo-Gallemkemp, UK) to obtain a homogenous fungal spore suspension.

Production of MnP

Production of MnP from *T. versicolor* IBL-04 was carried out in 250 mL Erlenmeyer flasks under some pre-optimized growth conditions. Triplicate flasks containing 5 g corncobs were moistened with Kirk's basal nutrient medium (60% w/w); autoclaved at 121°C for 15 min and inoculated with 5 mL of freshly prepared homogeneous inoculum under sterilized conditions using a sterilized pipette. The inoculated flasks were allowed to ferment at 30°C in a temperature controlled still culture incubator (EYLA SLI-600ND, Japan) for five days. After five days, the MnP was extracted by adding distilled water (100mL) to the fermented biomass, and the flasks were shaken at 120 rpm for 30 min (Gomes et al. 2009). The contents were filtered (Whatman No.1 filter paper) and washed thrice with distilled water. The filtrates were centrifuged at 3,000×g for 10 min to get clear supernatant that was used as crude enzyme extract for MnP assay and purification purposes.

Estimation of MnP Activity and Protein Contents

MnP activity of supernatants was determined by the method of Wariishi et al. (1992) using a UV/Visible spectrophotometer (T60, PG Instruments, UK). The reaction mixture contained 1mL of 1 mM $MnSO_4$, 1mL of 50 mM sodium malonate buffer of pH 4.5, 0.5 mL of 0.1M H_2O_2, and 0.1 mL of enzyme solution. The activity of reaction mixture was measured against a reagent blank at 270 nm. The recorded activities were expressed as U/mL, while one unit activity was defined as the amount of enzyme required to produce a unit increase in absorbance at specific wavelength (nm) per mL of reaction mixture. Protein contents of the crude and purified enzyme extracts were determined by the method of Bradford (1976) using BSA as standard.

Purification of MnP

Crude extract obtained from solid state cultures of *T. versicolor* IBL-04 was centrifuged at 3,000×g for 15 min to increase clarity using Eppendorfs centrifuge machine (Centrifuge 5415 C, Germany). The cell-free supernatant/filtrate was first brought to 35% saturation by the gradual addition of solid crystals of ammonium sulfate and kept for overnight at 4°C. The resulting precipitate was collected by centrifugation (3,000×g) for 15 min at 4°C. The pellets of precipitated proteins were discarded and in the supernatant, and more crystals of ammonium sulfate were added in order to achieve 65 % saturation. It was again kept for overnight at 4°C and centrifuged as described previously. After centrifugation the supernatants were discarded and sediments were dissolved in a minimal volume of 50 mM Sodium Malonate buffer of pH 4.5. The solution was kept in a dialysis bag and, after sealing securely, it was dialyzed against the same buffer. The dialyzate was concentrated by ultrafiltration and applied to a Sephadex G-100 2×25 cm glass column (Sigma, USA). Phosphate buffer (100 mM) with 0.15 M NaCl was used as elution buffer and the flow rate was maintained at 0.5 mL min^{-1}. Up to 25 positive fractions were collected, pooled, and concentrated by ultrafiltration. The concentrate was used to determine the enzyme activity as well as the protein content as described earlier.

Molecular Mass Estimation by Electrophoresis

The purity of MnP was analyzed by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was performed on 5% stacking and 12% resolving gel according to the method of Laemmli (1970) using Minigel electrophoresis apparatus (V-GES, Wealtec Corp. U.S.A). The molecular mass of the purified MnP was estimated in comparison to standard molecular weight markers (standard protein markers, 21-116kDa; Sigma, USA). Electrophoresis was performed at room temperature for 3 h with 120 volts, and the gel was placed in fixing solution (Methanol, 30 mL; Acetic acid, 10 mL; and H_2O, 60 mL) for 20 min, followed by washing with distilled water. The protein bands were visualized by staining with Coomassie Brilliant Blue G-250 (Sigma, USA).

Characterization of MnP

Effect of pH

The catalytic parameters of MnP were studied through characterization by studying the effect of pH (3-10) on MnP activity and stability with following buffers (0.2 M): sodium melonate buffer, pH 3.0 and 4.0; citrate phosphate, pH 5.0 and pH 6.0; sodium phosphate, pH 7.0 and pH 8.0; and potassium carbonate buffer, pH 9.0 and pH 10.0.

Effect of Temperature

The effect of different temperatures (30-80ºC) on MnP activity and stability was also studied. The enzyme was incubated for 15 min at varying temperatures before running the enzyme assay. For stability studies, the enzyme was incubated at varying temperatures for 1 h in the absence of substrate before running the routine assay.

Effect of Substrate Concentration: Determination of K_M and V_{max}

The Michalis-Menten kinetic constants K_M and V_{max} for MnP were calculated from Lineweaver–Burk plots using $MnSO_4$ with varying concentrations (0.1-1.0 mM) as substrate.

Effect of Various Modulators

The effect of various modulators (Cu^{2+}, Fe^{2+}, Ag^{+}, EDTA and Cystein) as possible activators and inhibitors on purified Mn-Peroxidase produced from *Trametes versicolor* IBL-04 was studied under optimum conditions. The enzyme activities for each case were determined under standard assay conditions as described earlier.

Statistical Analysis

All enzyme assays were performed in triplicates, and the data were statistically analyzed according to Steel et al. (1997). The means and standard errors of means (Mean ± S.E) were calculated for each treatment, and S.E values have been displayed as Y-error bars in figures.

RESULTS AND DISCUSSION

Production of MnP

T. versicolor IBL-04 was cultured in solid substrate fermentation medium of corncobs moistened with Kirk's medium of pH 4.0 (60% w/w) and incubated at 30°C for five days under pre-optimized conditions of growth (Iqbal et al. 2011). Maximum MnP activity of 964±12U/mL was harvested when corncobs supplemented with glucose and yeast extract in 25:1 C:N ratio, 1 mL of 1 mM Tween-80 as surface active agent, 1 mL of 1 mM $MnSO_4$ as mediator, and $CuSO_4$ as metal ion source was inoculated with 5 mL freshly prepared spore suspension of *T. versicolor* IBL-04. Different white rot fungi show different growth and enzyme activity patterns on different substrates in the presence of additional supplements due to genetic diversity among WRF cultures (Selvam et al. 2006). Generally, at low C:N ratio, the WRF are carbon starved and do not show efficient growth and MnP formation (Xiong et al. 2008), while at a high C:N ratio (nitrogen limitation), WRF cultures produce large amounts of polysaccharides (Rothschild at el. 1995). It has also been reported that the addition of $MnSO_4$ to the culture medium of white-rot fungi have a regulatory affect to boost up the production of MnP in high amount (Have and Teunissen 2001; Khiyami et al. 2006).

The locally isolated indigenous strain used in this study had an extraordinary capability to produce higher amounts of novel MnP (964 U/mL) than those described in the literature: 0.229 U/mL from *P. radiata* 79 (Vares et al. 1995), 30 U/mL from *P. ostreatus* (Palmieri et al. 2000), 1.28 U/mL from *Trametes trogii* (Levin et al. 2002), 214.5 U/mL from *Trametes trogii* (Levin et al. 2005), 0.148 U/mL from *T. versicolor* (Mikiashvili et al. 2005), 0.2 U/mL from *Schizophyllum sp.* F17 (Xiaobin et al. 2007), 25 U/mL from *Flavodon flavus* (Mtui and Nakamura 2008), and 4.48 U/mL from *M. racemosus* CBMAI 847 (Bonugli-Santos et al. 2010).

Purification MnP

The cell-free supernatant with MnP activity of 192800 U/200mL and specific activity of 276 U/mg was used as crude enzyme solution and subjected to partial purification by ammonium sulfate precipitation. The crude enzyme extract was maximally precipitated at 65% saturation with specific activity of 375 U/mg and 1.4-fold purification.

The best yielded fraction was loaded on Sephadex G-100 column, and after gel filtration the enzyme was purified up to 2.4-fold with specific activity of 660U/mg (Table 1). A-el-Gammal et al. (2001) and Mtui and Nakamura (2008) achieved 50 to 80% $(NH_4)_2SO_4$ saturation followed by chromatographic purification techniques for the recovery of pure ligninolytic enzymes.

Table 1. Purification Summary of MnP Produced by *T. versicolor* IBL-04 Grown on Corncobs under Optimum Conditions

Sr. No.	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
1	Crude Enzyme	200	192800	698	276	1	100
2	$(NH_4)_2SO_4$ Precipitation	30	27360	73	375	1.4	14.2
3	Dialysis	25	22200	51	435	1.6	11.5
4	Sephadex-G-100	12	6600	10	660	2.4	3.4

SDS-PAGE

To confirm its purity, the purified MnP was resolved on 5% stacking and 12% running gel and found to be a homogenous monomeric protein, as evident by a single band corresponding to 43kDa on SDS-PAGE (Fig. 1). The narrow range of molecular masses of MnP family varies between 37 and 60 kDa (Glenn and Gold 1985; Forrester et al. 1990; Martinez et al. 1996; Boer et al. 2006). The *T. versicolor* IBL-04 MnP was evidently different in molecular mass from other reported WRF MnPs like from *Irpex lacteus* (37kDa) (Baborova et al. 2006), *Schizophyllum sp.* F17 (48.7 kDa) (Cheng et al. 2007) *Irpex lacteus* (53 kDa) (Shin et al. 2005; Sklenar et al. 2010); and *Rhizoctonia* sp. SYBC-M3 (40.4kDa) (Cai et al. 2010).

Characterization of Purified Mn-Peroxidase

Effect of pH on MnP activity & stability

The pH-activity profile showed that MnP from *T. versicolor* IBL-04 was very active in a broad pH range of 3 to 8, and it displayed optimum activity at a pH 5 (Fig. 2). A further pH hike showed a sharp decreasing trend. The purified MnP was absolutely stable in a large pH range (4.0 to 7.0) for 1 h, but was inactivated at higher pH values.

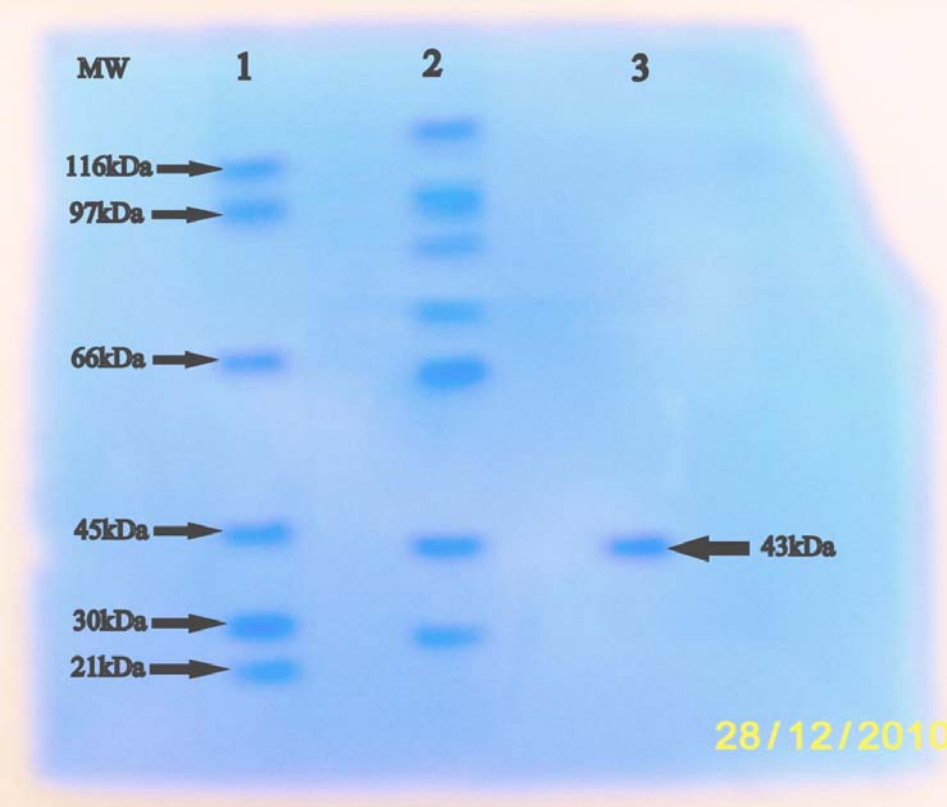

Fig. 1. SDS-PAGE of MnP isolated from *T. versicolor* IBL-04

[Lane MW, Molecular weights in kDa of standard marker; lane 1, standard protein markers (β-Galactosidase, 116kDa; Phosphorylase B, 97kDa; albumin, 66kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa and trypsin inhibitor, 21kDa); lane 2, Crude extract; lane 3, Purified MnP]

The purified enzyme was reasonably stable over a wide pH range. Earlier studies reported optimum activities of MnPs from different WRF in the pH range 5.5 to 6.5 (Shin et al. 2005; Baborova et al. 2006; Boer et al. 2006). Chefetz et al. (1998) reported that the enzyme retained 65 % of its optimum activity at pH 8, while according to Shin et al. (2005) MnP from *Irpex lacteus* was optimally active at pH 6.

Effect of temperature on MnP activity and stability

The temperature optimum for purified MnP was 50°C. The MnP from *T. versicolor* IBL-04 was heat-stable at temperatures up to 65°C without loosing much of its activity (Fig. 3). For a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme (Beg, and Gupta 2003; Joo et al. 2003; Haddar et al. 2009). Most of the earlier reported MnPs were found to lose their activities at temperatures around 60°C. In this regard, the MnP of *T. versicolor* IBL-04 was more thermo-stable, as it retained almost 90% of its activity at 65°C for 1 h. MnP from *Irpex lacteus* was stable in the 30 to 40°C range (Shin et al. 2005), whereas MnP from another *Irpex lacteus* strain showed better thermo-stability at 50 to 60°C (Baborova et al. 2006) and the from *Rhizoctonia* sp. SYBC-M3 was stable at 55°C (Cai et al. 2010).

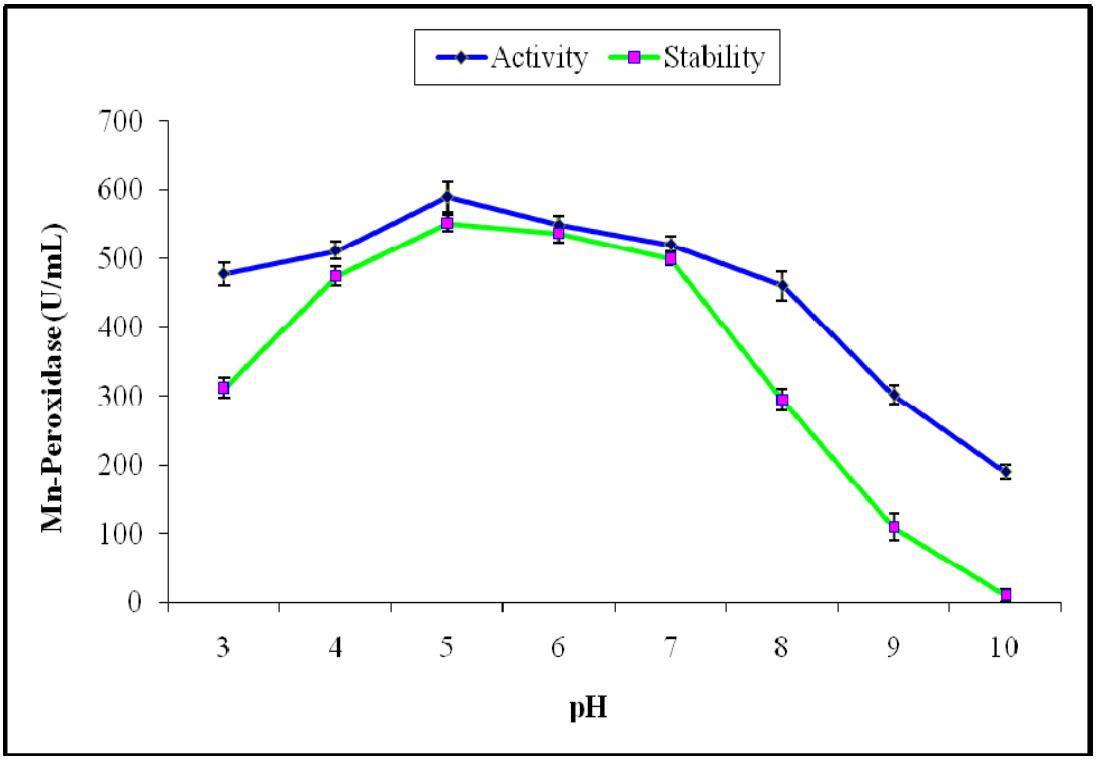

Fig. 2. Effect of pH on activity and stability of MnP from *T. versicolor* IBL-04

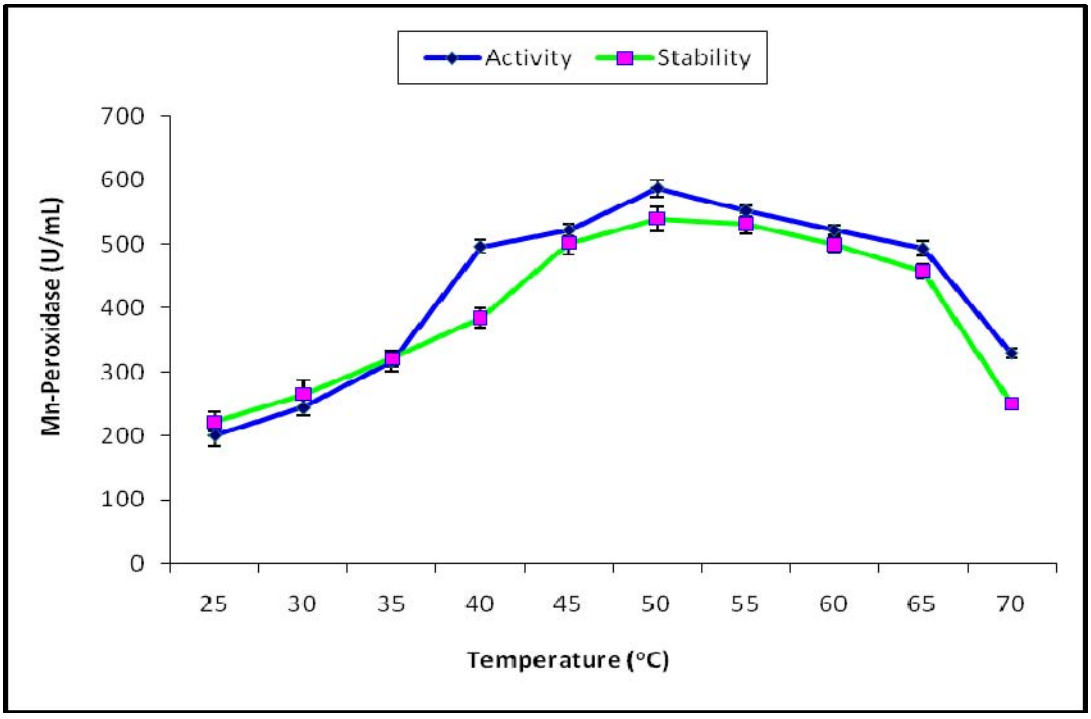

Fig. 3 Effect of temperature on activity and stability of MnP from *T. versicolor* IBL-04

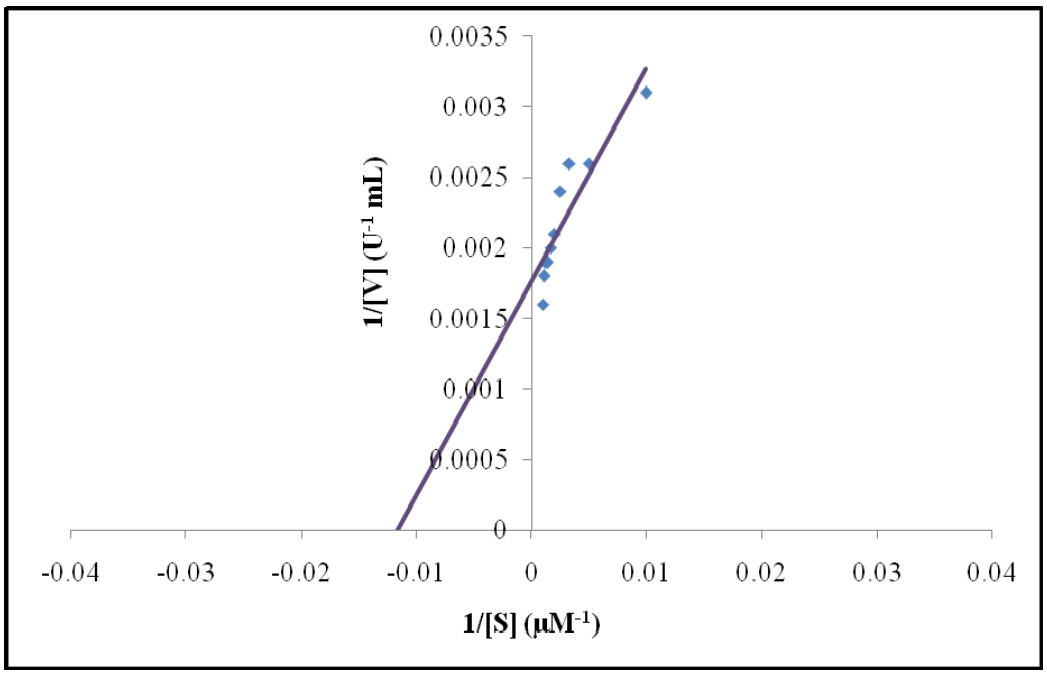

Fig. 4. Lineweaver-Burk reciprocal plot: Determination of K_M and V_{max} for purified MnP from *T. versicolor* IBL-04

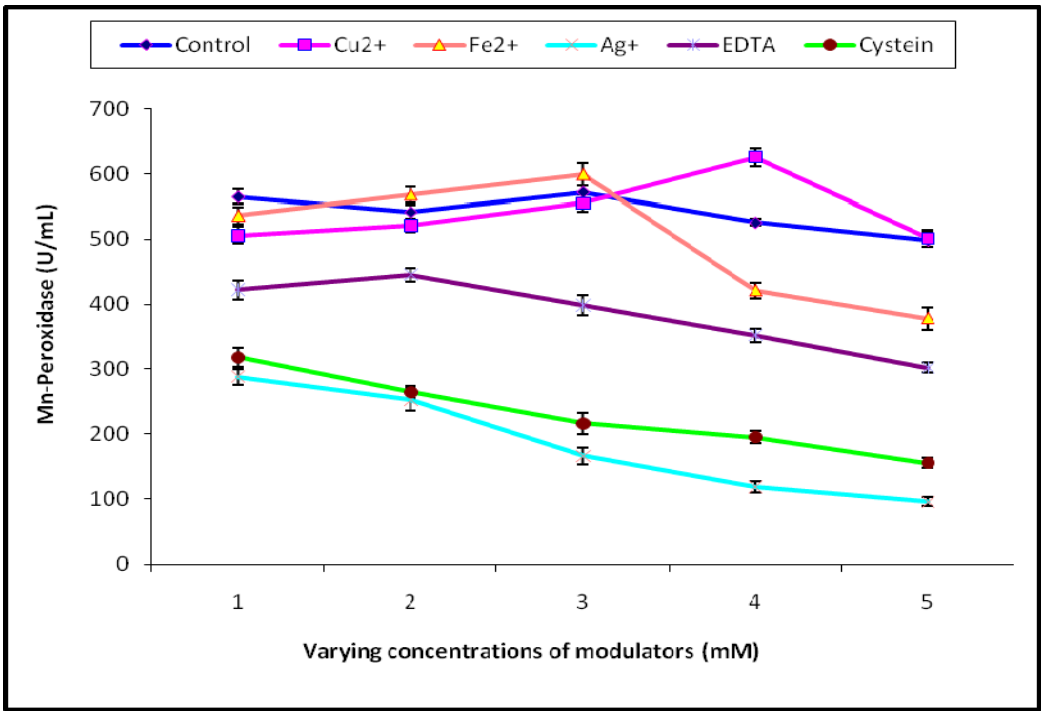

Fig. 5. Effect of varying concentrations of different activators/inhibitors on MnP from *T. versicolor* IBL-04

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

Using $MnSO_4$ as substrate, the K_M and V_{max} values of purified MnP from *T. versicolor* IBL-04 were 70 µM and 540 U/mL, respectively (Fig. 4), reflecting high substrate affinity and catalytic efficiency of MnP at pH 5.0 and 50°C temperature. The K_M value for MnP was slightly higher than reported for MnP proteins from *Phanerochaete flavidoalba* (53.7 µM) (Matsubara et al. 1996), *Bjerkandera sp*. (51.0µM) (Mester and Field 1998), *Phanerochaete chrysosporium* (45-51.9µM) (Palma et al. 2000; De la Rubia et al. 2002), *Rhizoctonia* sp. SYBC-M3 (25.3 and 53.9 µM) (Cai et al. 2010), and *Irpex lacteus* (46.7 µM) (Sklenar et al. 2010). The difference in K_M values of MnPs from *T. versicolor* IBL-04 and other reported fungal species can be explained on the basis of genetic variability and substrate specificities among different species.

Effect of various modulators

The effect of various organic compounds and metal ions (Cu^{2+}, Fe^{2+}, Ag^{+}, EDTA and Cystein) as possible activators / inhibitors on MnP was investigated. The purified MnP was incubated for 10 min at 50°C in 0.2 M citrate phosphate buffer of pH 5.0 and 100 µL of varying concentrations solutions of each activator/inhibitor. Partial inhibition of MnP was observed with EDTA, while the enzyme was strongly inhibited by cystein. Among the metal ions used, only Ag+ caused complete enzyme inhibition, whereas MnP was activated by 4 and 3 mM Cu^{2+} and Fe^{2+}, respectively. Higher concentrations of all metal ions were inhibitory (Fig. 5). EDTA is a metal chelating agent that has the ability to complex with inorganic cofactors and prosthetic groups of enzymes and therefore, was found inhibitory to the purified MnP when applied in high concentrations. The Mn-binding site of MnP is more flexible and allows a broad range of metal ions to bind to its active site (Sundaramoorthy et al. 2005). Metal ions like Hg^{2+} and Ag^{+} have also previously been reported as stronger inhibitors of WRF MnPs, while Fe^{3+}, Ca^{2+} and Ni^{2+} do not cause any alteration in the activity (Boer et al. 2006; Cheng et al. 2007).

CONCLUSIONS

T. versicolor IBL-04 produces high titers of MnP in solid state cultures of corncobs. The pH and temperature optima, thermo-stability features, and kinetic characteristics of this novel MnP suggest that this MnP is valuable enzyme for various industrial and biotechnological applications.

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