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Article

Purification and characterization of manganese peroxidases from native and mutant *Trametes versicolor* IBL-04

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ABSTRACT

Extracellular manganese peroxidases (MnPs) produced by native and mutant strains of *Trametes versicolor* IBL-04 (EB-60, EMS-90) were purified by ammonium sulphate precipitation and dialysis, followed by ion-exchange and gel-permeation chromatography. The purified enzymes elucidated a single band in the 43-kDa region on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The optimum pH and temperature of the purified enzymes were found to be 5.0 and 40 °C, respectively. Mutant strain MnPs exhibited a broader active pH range and higher thermal stability than native MnP. Purified MnPs from selected mutants showed almost identical properties to native MnP in electrophoresis, steady-state kinetics, and metal ion and endocrine-disrupting compound (EDC) degradation efficiency. Although the fastest reaction rates occurred with Mn²⁺, MnPs displayed the highest affinity for ABTS, methoxyhydroquinone, 4-aminophenol and reactive dyes. MnP activity was significantly enhanced by Mn²⁺ and Cu²⁺, and inhibited in the presence of Zn²⁺, Fe²⁺, ethylenediaminetetraacetic acid and cysteine to various extents, with Hg²⁺ as the most potent inhibitory agent. MnPs from all sources efficiently catalyzed the degradation of the EDCs, nonylphenol and triclosan, removing over 80% after 3 h of treatment, which was further increased up to 90% in the presence of MnP-mediator system. The properties of *T. versicolor* MnPs, such as high pH and thermal stability, as well as unique Michaelis-Menten kinetic parameters and high EDC elimination efficiency, render them promising candidates for industrial exploitation.

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1. Introduction

Enzymes are efficient and safe biocatalysts with outstanding potential for different industrial processes that require high activity, selectivity and specificity under mild environmental conditions [1–6]. Manganese-dependent peroxidases (MnPs; EC 1.11.1.13) exhibit significant prospects for biotechnological applications, including lignocellulose de-polymerization [7], polymer synthesis [8], bio-bleaching of paper pulp [9], decolorization of textile dyes [10], bio-sensing [11], green chemistry [12], and bio-transformation and detoxification of environmentally persistent aromatics [13]. However, because indige-

nous strains of white-rot fungi (WRF) have a long fermentation period, low productivity and poor enzymatic stability, they are inefficient for commercial purposes [12,14]. Strain improvement through mutagenesis is considered to be a reliable, short-term and highly developed technique, which incorporates the latest advances from a wide range of scientific and technical disciplines [12,15].

Studies on the isolation and purification of MnPs, and their specific properties, enable evaluation of their suitability for industrial uses, which represents 80% to 90% of the total production costs. Industrial processes require enzymes with high resistance to many physico-chemical environmental factors,

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including pH, temperature and metal ions, accompanied by a high catalytic potential [16]. Furthermore, the development of simplified but viable purification strategies has gained remarkable interest in modern enzyme biotechnology [17–19]. Because enzymes are produced in conjunction with various proteins, some of which have undesired catalytic activity, final volumetric activity may be negatively affected. In addition, contaminant proteins/enzymes with opposing catalytic activity may decrease the enantio- and/or regio-selectivity or specificity of the biocatalyst [6].

Bio-remediation, including wastewater treatment, is a field that uses the metabolic potential and enzymatic systems of WRF to clean the environment [20]. Over the last decades, the emergence of potentially toxic pollutants, known as endocrine-disrupting compounds (EDCs), has been identified and monitored in different water sources. Nonylphenol (NP), a non-ionic surfactant bio-degradation metabolite, and triclosan (TCS), an antibacterial agent, are the two most common EDCs [16] that cause feminization and carcinogenesis in living organisms [21]. Owing to their high tolerance to toxic substances, several approaches employing fungal ligninolytic enzymes have garnered interest for the mineralization of these EDCs [22]. This study aimed to isolate and purify a highly active MnP enzyme from native and mutant strains of *Trametes versicolor*, and to conduct biochemical characterization. The biotechnological potential for degradation of the EDCs NP and TCS was also assessed.

2. Experimental

All chemicals and reagents were of high-grade purity and used as received. Sugarcane bagasse was procured from a local fruit market in Faisalabad, Pakistan. The collected substrate was oven-dried at 60 °C, crushed in a commercial mill (Ashraf Herbal Laboratories limited, Faisalabad, Pakistan) and sieved to 40-mesh particle size.

2.1. Organism, culture conditions and inoculum development

Locally isolated culture of *Trametes versicolor* IBL-04 (available in the culture stock at IBL-UAF) was refreshed and multiplied by transfer onto freshly prepared potato dextrose agar (PDA) slants at 28 ± 2 °C, pH = 4.5 for 6 to 8 d, and then stored at 4 °C. Inoculum was developed by growing the fungus in separately labeled triplicate Erlenmeyer flasks (500 mL) containing Kirk's basal nutrient medium [23] with the following composition (in g/L): glucose, 10.0; ammonium tartrate, 0.2; KH_2PO_4 , 0.21; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; CaCl_2 , 0.01; thiamine, 0.001; Tween 80 (10%), 10 mL; 100 mmol/L veratryl alcohol, 10 mL; and 10 mL trace mineral solution. Before sterilization, the medium was adjusted to pH = 4.5 using $M(\text{HCl})/M(\text{NaOH})$. The sterilized medium was supplemented with 1% Millipore-filtered sterile glucose solution. A loop with *T. versicolor* culture from the PDA slants was aseptically transferred to the sterile basal medium and incubated (Sanyo Gallenkamp, UK) at 30 °C for 5 to 7 d. The number of spores was counted with a hemocytometer, and the suspension was adjusted to a spore

concentration of $1 \times (10^7\text{--}10^8)/\text{mL}$ for use as the inoculum [12].

2.2. Chemical mutagenesis

2.2.1. Ethidium bromide (EB)

A stock solution containing 1.0 mg/mL of EB was used to create four further dilutions (25, 50, 75 and 100 $\mu\text{g}/\text{mL}$). In a vial, 1.0 mL of EB solution was added to 9.0 mL of Vogel's medium containing *T. versicolor* spores (1×10^7 spores/mL). After pre-designated EB treatment intervals (30, 60, 90, 120 and 150 min), spores were harvested by washing three times with sterile biological saline solution (0.89% NaCl and 0.1% yeast extract), followed by centrifugation at 12000 r/min for 15 min. The mutant spores were spread on agar plates at 30 °C for colony formation.

2.2.2. Ethyl methanesulfonate (EMS)

A stock solution of 500 $\mu\text{g}/\text{mL}$ (v/v) was used to prepare different EMS concentrations to treat fungal spores. After specific time intervals (30, 60, 90, 120 and 150 min), the EMS-treated spores were washed, centrifuged (12000 r/min for 15 min) and plated on nutrient agar plates to give 30 colonies or less per plate.

2.2.3. Selection and evaluation of mutants

To identify potentially high yield mutants, the selective marker 2-deoxy-D-glucose was used [15]. Spores exhibiting the best growth in the presence of 2-deoxy-D-glucose were designated as mutant and selected. Five colonies in each case were picked from the plates and subsequently tested for MnP production.

2.3. MnP production

MnP was produced in cotton-plugged triplicate shake flasks containing 5.0 g sugarcane bagasse substrate pre-moistened with Kirk's basal salts solution (60% moisture (w/w)). After sterilization (121 °C for 15 min at 15 psi), the flasks were inoculated with 5 mL of homogenized *T. versicolor* spore suspension and left undisturbed in an incubator (Sanyo, Japan) at 30 °C for 5 d. After fermentation, the spores were harvested by adding 100 mL of Na-malonate buffer (pH = 4.5), shaking (120 r/min for 30 min), and filtration through Whatman filter paper. The filtrates were centrifuged at 12000 r/min for 10 min, and the supernatants were analyzed for MnP activity [10].

2.4. MnP activity assay and protein estimation

After selection of the mutant strains, MnP activity was analyzed, as described previously [24]. A typical assay mixture comprised MnSO_4 (1 mL; 1 mmol/L), 1 mL of 50 mmol/L Na-malonate buffer at pH = 4.5, 500 μL of H_2O_2 at 25 °C, with 100 μL aliquots of appropriately diluted culture supernatant or purified enzyme. Oxidation of Mn^{2+} to Mn^{3+} was monitored by spectrophotometry (HALO DB 20) according to the increase in absorbance at 238 ($\epsilon = 6.5 \times 10^3 \text{ mol}^{-1} \text{ L cm}^{-1}$). Enzyme activity

was expressed as international units (IU). One unit corresponds to the amount of enzyme, which oxidizes 1 $\mu\text{mol/L}$ Mn^{2+} to Mn^{3+} per min (at pH = 4.5 and 25 °C). The control cuvette comprised 100 μL of distilled water, instead of enzyme extract. Total protein contents were determined by the Bradford protein assay using bovine serum albumin (BSA) as a calibration standard.

2.5. MnP Purification

Briefly, crude MnP extracts obtained from 5-d cultures of native and selected mutant strains of *T. versicolor* IBL-04 were centrifuged at 3000 r/min for 15 min to increase clarity using an Eppendorf centrifuge machine (Centrifuge 5415 C, Germany). The cell-free supernatant was saturated (up to 35%) by gradual addition of ammonium sulphate and stored overnight at 4 °C. The resulting precipitate was recovered (3000 r/min for 20 min at 4 °C) and the supernatant was saturated by adding ammonium sulfate (up to 65%), allowed to stand overnight at 4 °C, centrifuged, and then the pellets were dissolved in the minimum volume of 50 mmol/L Na-malonate buffer (pH = 4.5). The solution was dispersed in a dialysis bag and after sealing securely, dialyzed against the same buffer for 72 h with buffer changes at different intervals for salt removal. The dialysate was submitted to ion-exchange chromatography using a diethylaminoethyl cellulose column. The column was equilibrated with 100 mmol/L phosphate buffer (pH = 6.5) for 24 h, and eluted with a 0 to 1.0 mol/L linear gradient of NaCl in 50 mmol/L malonate buffer at a flow rate of 0.5 mL/min. A total of 60 fractions, each 1.5 mL, were collected, and analyzed for enzyme activity and protein content. The MnP active fractions were pooled and loaded onto a Sephadex G-100 column (10 mm $\times$ 300 mm). A 50 mmol/L malonate buffer was used for elution at a flow rate of 0.3 mL/min. Up to 30 positive fractions (1.0 mL) were collected and absorbance was measured at 280 nm (CE Cecil 7200, Germany). The purified and concentrated enzyme was stored at -20 °C [10].

2.6. Molecular mass determination by SDS-PAGE

The molecular mass of purified MnPs was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after gel staining with Coomassie Brilliant Blue G-250, followed by calibration against broad-range molecular mass standard protein markers ranging from 17 to 170 kDa (Sigma, USA).

2.7. Effect of pH and temperature on the stability and activity of MnPs

The effect of pH on enzyme activity was investigated by exposing purified MnPs from the native and mutated strains to a pH range of 3.0 to 10.0. The pH stability was determined by pre-incubation of the purified MnPs in buffers of pH = 5.0 and pH = 8.0 at standard assay temperature for 90 min. To examine the effect of temperature on enzyme activity, the purified enzymes were incubated at temperatures ranging from 20 to

70 °C using MnSO_4 as an assay substrate at optimized pH. Thermo-stability was evaluated at the selected temperatures of 40 and 60 °C for 210 min. The appropriate controls were maintained wherever required during the above studies.

2.8. Steady-state kinetics

Steady-state kinetic constants for all substrates were determined in 100 mmol/L sodium tartrate buffer at pH = 3.5, except for Mn^{2+} (pH = 5.0), and veratryl alcohol and syringol (pH = 3.0). Each substrate was individually added to the assay mixture and enzymatic activities were calculated from the molar absorbance of the reaction products of 2,6-dimethoxyhydroquinone ($\epsilon_{397} = 562$), veratryl alcohol ($\epsilon_{310} = 9300$), ABTS ($\epsilon_{420} = 36000$), guaiacol (o-methoxyphenol) ($\epsilon_{456} = 12100$), 4-methoxyphenol ($\epsilon_{253} = 4990$), 4-aminophenol ($\epsilon_{246} = 15627$), methoxyhydroquinone ($\epsilon_{360} = 1252$), syringol ($\epsilon_{468} = 27500$), catechol (1,2-benzenediol) ($\epsilon_{238} = 6500$) and Mn^{2+} ($\epsilon_{238} = 6500$). In the case of dyes, enzyme activities were monitored from substrate decolorization using the molar absorbance of Reactive Red C₄BLN ($\epsilon_{540} = 30000$) and Reactive Black CKF ($\epsilon_{598} = 50000$). Enzyme activities were measured under standard assay conditions and results were used to construct Lineweaver-Burk reciprocal plots (Graph Pad Prism 6 software).

2.9. Effect of modulators (activators and/or inhibitors)

The effect of various modulators, including Mn^{2+} , Zn^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , K^+ , ethylenediaminetetraacetic acid (EDTA) and cysteine, on enzyme activities were tested at a concentration range of 5 to 20 mmol/L. MnP activity in the absence of modulators was considered to be the control (100%). Residual activities were determined according to the standard assay protocol described earlier.

2.10. Enzymatic degradation of NP and TCS

The enzymatic removal of NP and TCS was performed according to Cabana et al. [25]. The reaction mixture contained EDCs (NP or TCS; 10 ppm), purified MnPs, Na-malonate buffer (50 mmol/L; pH = 4.5), MnSO_4 (50 $\mu\text{mol/L}$), and 0.4 mmol/L H_2O_2 . During NP treatment, 0.5% methanol and 0.075% Tween-80 were added to the reaction mixture to enhance the solubility of NP. The reaction was performed at 30 °C with stirring at 150 r/min. For the MnP-mediator system, 0.2 mmol/L MnSO_4 was added to the reaction mixture as MnP treatment. To verify that degradation was specifically caused by enzymatic oxidation, a control reaction was conducted using distilled water instead of enzyme solution. Samples were periodically collected to investigate the remaining enzymatic activities and EDC concentration.

2.11. Statistics analysis

All experiments were conducted independently in triplicates, and the data presented are the mean of three replicates along with the standard deviation ($\pm$ SD). Results were sub-

jected to analysis of variance (ANOVA) using statistical analysis system software (Minitab 15.1, version year 2007). *P*-values < 0.05 were considered to denote statistically significant differences.

3. Results and discussion

3.1. MnP production by selected mutants

To obtain mutant strains of *T. versicolor* IBL-04 displaying more efficient MnP production, chemical mutagenesis was performed using solid-substrate fermentation with sugarcane bagasse as a growth substrate. Two mutagenic chemicals, EB and EMS, were applied independently at optimized concentrations of 50 and 100 µg/mL, respectively, for different time periods [14]. After exposure for 60 min (EB-60) and 90 min (EMS-90), MnP activity was increased by 29.6% and 32.5%, respectively, compared with the parent strain (Fig. 1). The high MnP production in culture filtrates of mutated strains may be attributed to overexpression of the *mnp-1* gene caused by the chemical mutagens [26].

In general, strain modification for hyper-production of extracellular enzymes using filamentous fungi is achieved by inducing mutagenesis with various agents, including UV light, N-methyl-N-nitro-N nitrosoguanidine (MNNG), nitrous acid (HNO₂), EB and EMS, applied individually or in combination [27–30]. EB is an aromatic agent that causes frame-shift mutations by intercalating with double-stranded DNA. In contrast, EMS is thought to have both mutagenic and carcinogenic effects, and it causes random point mutations by promoting nucleotide substitution, in particular, guanine alkylation [14].

Several investigators have documented an increase in enzyme production after exposure of fungi to various mutagens at

Fig. 1. Production of MnPs by native and mutant strains of *T. versicolor* at different exposure time. WT, wild type; EB, Ethidium bromide; EMS, Ethyl methanesulfonate.

sub-lethal concentrations. The effectiveness of the induced mutagenesis depends on the type and concentration of the mutagenic agent, and the duration of exposure [28]. However, treatment of fungi with high concentrations of EB or EMS (> 100 µg/mL) reduced MnP activity, probably owing to the detrimental effect of mutagens on cell growth [15]. Similarly, Dhawan et al. [28] also highlighted the lethal effects of high EB concentrations on viability of the WRF *Cyathus bulleri*. Notably, in the present study, the mutagenic agents primarily caused young and actively growing fungal cells to release higher amounts of MnPs than native cultures of *T. versicolor*, which displayed much lower MnP activity. Previously, Arunkumar and Abdulla [26] demonstrated that young and metabolically active cells were more susceptible to EB than cells in the stationary phase.

Table 1

Purification of MnP from wild type and mutant strains of *T. versicolor* IBL-04.

Enzyme source	Purification step	Total protein (mg)	Total activity ^a (UI/mL)	Specific activity ^b (UI/mg)	Purification ^c (fold)	Recovery ^d (%)
WT strain	Culture supernatant	2193	580.18	132.27	1	100
	(NH ₄) ₂ SO ₄ precipitation	82.3	497.23	241.67	1.83	85.70
	Dialysis	73.4	473.01	257.77	1.95	81.53
	DEAE-anion exchange chromatography	5.58	413.42	740.98	5.60	71.26
	Gel permeation chromatography	1.03	164.32	957.21	7.24	28.32
EB-60 strain	Culture supernatant	1765.5	824	140.01	1	100
	(NH ₄) ₂ SO ₄ precipitation	63.6	709.13	334.5	2.39	86.06
	Dialysis	57.4	694.17	362.01	2.59	84.24
	DEAE-anion exchange chromatography	8.7	589.23	609.55	4.35	71.51
	Gel permeation chromatography	0.856	317.04	1481.49	10.58	38.48
EMS-90 strain	Culture supernatant	1267.36	859	203.34	1	100
	(NH ₄) ₂ SO ₄ precipitation	43.96	773.6	387.15	1.9	90.05
	Dialysis	39.31	759.3	405.63	1.99	88.39
	DEAE-anion exchange chromatography	4.97	534.21	1289.84	6.34	62.19
	Gel permeation chromatography	1.36	293.13	1939.83	9.54	34.12

^a UI is defined as the amount of enzyme oxidizing 1 µmol of substrate per min.

^b Specific activity = UI per mg protein.

^c Purification fold = specific activity at given step/specific activity of initial extract.

^d Recovery = (total activity at given step/total activity of initial extract) × 100.

The selection of an appropriate plant residue for fungal growth may play a significant role in the development of an efficient technology for enzyme production. Sugarcane bagasse is a major by-product of sugar cane and the alcohol industry. Owing to its high cellulose (50%), hemicellulose (25%) and lignin content, sugarcane bagasse offers advantages over other crop residues such as rice straw and wheat straw. Peksen et al. [31] used various substrates for *Ganoderma lucidum* cultivation, and reported the suitability of rice bran, rice husks, coconut fiber, peanut hulls and sugarcane bagasse as substrates for ligninase production. Similarly, Karp and colleagues [32] reported that sugarcane bagasse was the best substrate for the production and characterization of laccase enzyme by solid-state fermentation of *Pleurotus ostreatus*.

3.2. Enzyme purification and gel electrophoresis

The crude MnP enzyme extracts, which were obtained from parent and mutant strains of *T. versicolor* IBL-04 (EB-60 and EMS-90) under optimized culture conditions, were purified separately to homogeneity after being subjected to ammonium sulfate fractionation, dialysis, ion-exchange and gel-permeation chromatography. A list of the purified MnPs is provided in Table 1. MnPs from the parent, EB-60 and EMS-90 strains were optimally salted-out with 60% ammonium sulfate precipitation to achieve 1.95-, 2.59- and 1.99-fold purification, respectively, after dialysis. The de-salted enzyme extracts were first applied to ion-exchange chromatography. The fractions showing high MnP activity were pooled, concentrated, and further purified by gel-permeation chromatography using a Sephadex G-100 column. After the final purification step, the MnPs were between 7.24- and 10.58-fold purified, with the recovery yield ranging from 28% to 39%. A variety of MnPs from different fungal cultures have been purified using the ammonium sulphate fractionation method followed by multiple column chromatographic steps [10,33–36]. The specific activity (UI/mg), recovery (%) and fold-purification of the MnPs in this study are compared with those of previously reported fungal sources in Table 2.

The purified enzyme from the native and selected mutant strains elucidated a single band on SDS-PAGE with an estimated molecular mass of 43.0 ± 1.5 kDa (Fig. 2). Molecular masses of fungal MnPs usually range from 32.0 to 75.0 kDa [37–42]. The molecular mass of the *T. versicolor* MnPs isolated in this study was similar to that reported previously for other WRF [10,43].

3.3. Enzyme characterization

3.3.1. Effect of pH on the activity and stability of the purified MnPs

Both pH and temperature play an important role in determining the activity and stability of enzymes. MnP activity was studied at varying pH values (from 3.0 to 9.0) using buffers. The optimum pH of the purified MnPs secreted by native and mutant strains of *T. versicolor* was 5.0 (Fig. 3(a)). Further increases in pH caused a rapid decrease in activity, indicative of enzy-

Table 2

Comparison of specific activity, recovery and purification factor achieved after the last purification step.

Organism	Specific activity (UI/mg)	Recovery (%)	Purification fold (%)	Ref.
<i>Phanerochaete crassa</i>	260.7	28.9	432	[38]
<i>Irpex lacteus</i>	4.2	24.3	11	[39]
<i>Schizophyllum</i> sp. F17	9.2	3.8	—	[52]
<i>B. pumilus</i>	31.36	802.23	5.84	[42]
<i>Paenibacillus</i> sp.	8.45	685.71	1.28	—
<i>Tremetes versicolor</i>	660	3.4	2.4	[43]
<i>Pleurotus ostreatus</i>	297.34	—	3.07	[35]
<i>Pleurotus ostreatus</i>	318	—	3.22	[44]
<i>Bjerkandera</i> sp.	32.3	40.2	2.4	[53]
<i>Phanerochaete chrysosporium</i>	33	1.1	1.2	—
<i>Physisporinus rivulosus</i>	86.6	6.2	6.5	—
<i>Phlebia radiata</i>	30.8	13.8	2.8	—
<i>Phlebia</i> sp. Nf b19	55.2	9.3	5.2	—
<i>Schizophyllum commune</i>	506	22	1.8	[36]
<i>Pleurotus ostreatus</i>	78	1.2	130	[54]
<i>Ganoderma lucidum</i>	539.59	—	3.43	[10]
<i>Tremetes versicolor</i>	957.21	28.32	7.24	Present study
EB-60	1481.49	38.48	10.58	Present study
EMS-90	1939.83	34.12	9.54	Present study

matic deactivation at non-optimal pH values. At pH = 8.0, the activity of MnP from the native strain decreased to 47.9%, whereas MnPs from the mutants (EB-60 and EMS-90) retained 73.7% and 73.2%, respectively, of their maximum activities. Studies on MnPs derived from *Irpex lacteus*, *Phanerochaete crassa* WD1694, *Pleurotus ostreatus*, *Ganoderma lucidum* and *Schizophyllum commune* showed that the optimal pH for fungal MnPs ranges from 4.5 to 6.5 [36,41,42]. Generation of novel MnPs with activity across a wider pH range than that obtained by chemical mutagenesis is believed to be beneficial for industrial applications.

Fig. 2. Electrophoresis of MnPs from *T. versicolor* IBL-04 stained with Coomassie brilliant blue. Lane M, protein standard marker; Lane 1, MnP from native *T. versicolor*; Lane 2, MnP from EB-60 strain; Lane 3, MnP from EMS-90 strain.

Fig. 3. Effect of pH on MnPs activity (a) and pH stability for MnPs (b).

The pH stability of the MnPs was studied by incubation at pH = 5.0 and pH = 8.0 for 90 min, and the results are depicted in Fig. 3(b). At pH = 5.0, the MnPs from EB-60 and EMS-90 retained 92% and 96%, respectively, of their initial activity after 60 min, falling to 81% and 86%, respectively, after 90 min. In contrast, MnP from the native strain displayed a 22% and 45% reduction in activity at pH = 5.0 after 60 min and 90 min, respectively. At pH = 8.0, the activity of MnPs from the native, EB-60 and EMS-90 strains declined by 76%, 71% and 68% respectively, after 90 min. The loss of enzymatic activity observed at a basic pH may be explained by irreversible denaturation. The majority of purified fungal MnPs exhibit moderate-to-high activity and reasonable stability within the pH range of 4.0 to 7.0 [10,44].

3.3.2. Effect of temperature on the activity and stability of the purified MnPs

MnPs from both the native and mutant strains had an optimum temperature of 40 °C, with enzyme activity increasing from 25 to 40 °C and dropping gradually at temperatures above 40 °C (Fig. 4(a)). MnPs of the native EB-60 and EMS-90 strains of *T. versicolor* retained up to 44%, 53% and 62% of their respective activities at 60 °C. The EMS-90 mutant MnP displayed the highest activity level over the widest temperature range,

followed by EB-60 and then the parental strain. Most MnPs isolated from different fungal cultures retain their original activities within a temperature range of 30 to 50 °C [39,41,45].

The stability of the enzymes at different temperatures is shown in Fig. 4(b). Residual enzyme activities were 78%, 87% and 89% for the native, EB-60 and EMS-90 strains of *T. versicolor*, respectively, after 3 h at 40 °C. At temperatures above 50 °C, enzyme stability gradually declined. The native strain MnP lost 50% of its original activity at 60 °C after 120 min, whereas the EB-60 and EMS-90 strains retained 62% and 63% of their respective activities even after 180 min. Many industrial processes occur at extreme pH levels (either acidic or alkaline) and high temperatures, making it necessary to identify enzymes with superior thermal stability [46]. Enzymes must also be capable of withstanding harsh conditions for longer processing time. The use of high temperatures (50 °C and above) has the advantage of reducing microbial contamination of the substrate being processed. Owing to their high thermal tolerance and activity across a wide pH range, the *T. versicolor* MnPs represent valuable enzymes to be explored for industrial uses.

3.3.3. Substrate specificity and kinetic parameters (K_m and V_{max})

Fig. 4. Effect of temperature on MnPs activity (a) and thermal stability of MnPs from native and mutant strains of *T. versicolor* (b).

The steady-state kinetic constants for the oxidation of veratryl alcohol, syringol, Mn^{2+} , reactive dyes, different hydroquinones, substituted phenols and other aromatic compounds by native and mutant *T. versicolor* MnPs were calculated, and the respective apparent K_m and V_{max} values are listed in Table 3. The substrates, including 2,6-dimethoxyhydroquinone, veratryl alcohol, guaiacol, 4-methoxyphenol, syringol and catechol, displayed apparent K_m values that were greater than those for Mn^{2+} oxidation, suggesting lower affinity for enzymes. Similar observations regarding the higher affinity of MnP for Mn^{2+} have been made previously [10,35,36]. Notably, with respect to Mn^{2+} , the MnPs from all sources showed higher affinities for 4-aminophenol, methoxyhydroquinone, ABTS and the reactive dyes tested. Catalytic efficiency (V_{max}/K_m) was highest for ABTS and Mn^{2+} , followed by reactive dyes and other hydroquinones. The substrate oxidizing potential by MnPs from native and mutant strains was very similar, revealing that they belong to the same group of class II peroxidases [47].

3.3.4. Effect of metal ion supplementation on the activity of purified MnPs

During catalysis, metal ions form complexes with enzymes that either maintain or disrupt the three-dimensional structure and configuration [48]. Therefore, the potential stimulatory or inhibitory effects of various organic compounds and metal ions on the activity of MnPs from the native and mutant strains were investigated (Table 4). The data revealed that Mn^{2+} and Cu^{2+} substantially enhanced the activity of MnPs (by 13% to 53%) at all concentrations tested. The metal ion Mn^{2+} was the most potent inducer of *T. versicolor* MnP, which is in accordance with the findings of Boer et al. [41]. In addition, Co^{2+} at a low concentration (5 mmol/L) moderately increased MnP activity, while at a high concentration of Co^{2+} (20 mmol/L), slight inhibition (7% to 10%) was observed. Elevated K^+ concentrations improved MnP activity (117%), whereas a low concentration (5 mmol/L) did not exert any effect on MnP activity. Zn^{2+} and Fe^{2+} had the smallest impact on MnP activity. In contrast, EDTA and cysteine strongly inhibited MnP activity, and even

Table 4

Effects of various modulators on the activity of purified MnPs from native and mutant *T. versicolor*.

Modulator	Concentration (mmol/L)	Relative activity (%)		
		WT	EB-60	EMS-90
Mn^{2+}	5	113±6.4	118±2.4	114±3.5
	10	137±5.3	140±1.9	149±2.9
	20	146±3.7	153±2.3	145±2.6
Zn^{2+}	5	87±2.8	91±1.4	89±2.4
	10	74±2.5	74±1.9	75±2.6
	20	56±4.2	64±1.3	63±1.2
Co^{2+}	5	108±5.1	117±4.2	121±5.6
	10	104±4.6	119±1.7	123±3.1
	20	90±4.7	93±1.4	91±3.4
Cu^{2+}	5	115±4.5	123±2.4	114±4.0
	10	118±3.9	124±3.8	123±1.1
	20	129±4.9	129±1.2	130±1.4
Fe^{2+}	5	83±1.4	87±1.8	88±2.1
	10	58±2.4	59±1.6	63±1.9
	20	41±4.2	49±1.8	49±1.3
Hg^{2+}	5	14±0.9	19±0.29	20±0.41
	10	7.6±0.13	10.6±0.31	10.9±0.27
	20	3.3±0.29	5.3±0.91	5.1±0.43
K^+	5	102±4.0	101±3.1	101±4.8
	10	108±3.9	109±2.2	112±2.5
	20	116±5.3	117±1.4	121±2.1
EDTA	5	47±1.9	45±1.6	51±2.5
	10	34±2.3	39±1.3	40±2.1
	20	28±2.4	33±1.4	33.2±1.6
Cysteine	5	33±1.7	38±1.2	38.9±1.3
	10	21±2.1	21±3.5	24±2.4
	20	8±0.73	9±0.6	14±0.52

Data are expressed as the mean ± SD ($n = 3$).

Hg^{2+} fully de-activated the MnP enzyme. Hg^{2+} is highly toxic because it disrupts the tertiary protein structure by oxidizing -SH groups found on cysteine residues [49]. It has a potent inhibitory effect on MnPs from different fungal strains, including *Phanerochaete crassa* WD1694 [38], *Trichophyton rubrum*

Table 3

Steady-state kinetics constants for oxidation of different substrates by MnPs from native and mutant *T. versicolor*.

Substrate	<i>Tremetes versicolor</i>		EB-60		EMS-90	
	K_m (μmol/L)	V_{max} (UI/mg)	K_m (μmol/L)	V_{max} (UI/mg)	K_m (μmol/L)	V_{max} (UI/mg)
2,6-Dimethoxyhydroquinone	47.0±1.8	51±1.7	42±1.2	59±2.1	54±0.7	33±0.4
Veratryl alcohol	2378±5.6	38±1.1	2145±4.7	46±1.4	2432±6.3	29±0.6
ABTS	7.0±0.04	57±2.6	9.0±0.06	56±2.6	14.0±0.4	62±1.3
Guaiacol	82.0±1.2	19.0±0.5	67±2.5	79±1.9	68±0.9	37±1.2
4-Methoxyphenol	127±1.7	43±1.4	143±3.8	34±1.1	132±1.7	47±2.3
4-Aminophenol	32.0±0.9	21±0.8	21.0±1.2	22±0.8	32±0.6	29±0.3
Methoxy hydroquinone	12.0±0.3	42±2.1	9.0±0.2	44±1.0	10.0±0.04	28±0.5
Syringol	297±3.8	22±1.2	282±4.2	26±1.3	300±3.5	26±0.4
Catechol	123±2.6	49±1.3	104±1.9	38±1.3	112±1.4	46±1.1
Reactive Red C ₄ BLN	38.0±0.06	142±3.3	38.0±0.04	148±3.4	41.0±0.02	149±1.3
Reactive Black CKF	37.0±0.08	129±2.1	36.0±0.06	141±1.9	34.0±0.2	142±2.0
Mn^{2+}	39±0.8	213±3.7	37±1.2	228±2.7	53±2.1	252±3.1

Data are expressed as the mean ± SD ($n = 3$).

LSK-27 [41], *Pleurotus ostreatus* [35], *Schizophyllum commune* [36] and *Ganoderma lucidum* [10]. MnP from *P. chrysosporium* was inhibited by NaN_3 , β -mercaptoethanol and dithreitol, whereas co-oxidants, including glutathione, un-saturated fatty acids and Tween-80, significantly improved MnP activity [33]. In another study, Hg^{2+} abolished MnP activity, while Fe^{3+} , Ca^{2+} and Ni^{2+} did not affect enzyme activity [41].

3.4. Bio-degradation of NP and TCS by MnP-catalyzed oxidation

The most common EDCs, NP and TCS, were treated with MnPs from native and mutant strains (EB-60 and EMS-90) of *T. versicolor* IBL-04 at pH = 5.0 and 40 °C. Fig. 5 illustrates the time-course elimination profile of NP and TCS. The removal efficiency of NP was determined to be 45% and 70% after 30 min and 1 h of treatment, respectively. Over 95% of NP was eliminated from the solution after 3 h of treatment (Fig. 5(a)). Similarly, the MnP enzyme preparation removed 23% of TCS from the solution after 30 min, 37% after 60 min, and 82% after 3 h (Fig. 5(c)). Further, the ability of the natural mediator (MnSO_4 ; 0.2 mmol/L) to improve the bio-degradation of NP and TCS was assessed. Mediators are proposed to broaden the substrate range over MnPs [25]. The remaining efficiency (%) after 1 h of mediator treatment is compared in Fig. 5(b) and (d). It was noted that the use of MnSO_4 substantially enhanced the elimination efficiency of both toxic chemicals tested ($\alpha = 0.05$).

Using MnSO_4 , over 90% removal of both chemicals was achieved after 1 h. However, the mediator alone did not eliminate any NP and TCS.

In a previous study, 8-h treatment of NP and TCS (10 ppm) with purified *Pycnoporus sanguineus* laccases (Lac I and Lac II) yielded over 90% degradation efficiency [16]. After 4 h of treatment, more than 65% of NP, BPA and TCS (5 ppm) were eliminated [25]. The estrogenic activities of NP and BPA (5 ppm) were completely abolished within 12 h of treatment with MnP and laccase from *Phanerochaete chrysosporium* ME-446 and *Trametes versicolor* IFO70473, respectively [50]. In a preliminary experiment, the same research group treated NP and BPA with *P. chrysosporium* and *T. versicolor*, demonstrating that the majority of both compounds were mineralized after 7 d. Compared with whole cultures, the ligninolytic enzyme-based technology led to more rapid disappearance of NP and bisphenol A (BPA). In addition, several authors highlighted the efficiencies of ligninolytic enzymes obtained from different fungal cultures, with bio-degradation of NP and TCS (50 ppm) in the range of 60% to 95% [51].

4. Conclusions

The yield of MnPs from *T. versicolor* IBL-04 was considerably increased by chemical mutagenesis. Compared with the native strain, the activity of MnPs from two genetically modi-

Fig. 5. EDCs bio-degradation by MnPs from native and mutants *T. versicolor* strains, Elimination profile of NP (a, b) and TCS (c, d).

fied mutants, EB-60 and EMS-90, was increased by 29.6% and 32.5%, respectively. The mutant MnPs were tolerant to a wider pH and temperature range than the native MnP. They showed remarkable potential to eliminate toxic EDCs with good degradation efficiency at 40 °C in aqueous solution. Immobilization strategies to improve the activity and thermo-stability characteristics of the purified enzymes may be future options for developing economically viable and highly sustainable industrial processes.

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Graphical Abstract

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Purification and characterization of manganese peroxidases from native and mutant *Trametes versicolor* IBL-04

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Manganese peroxidases (MnPs) from native and chemically mutant strains of *Trametes versicolor* IBL-04 were purified and characterized. The mutant MnPs were active and tolerant over broader pH and temperature ranges, and exhibited remarkable potential to eliminate toxic EDCs.

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从野生与突变株云芝 IBL-04 提纯锰过氧化物酶及其表征

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摘要: 由野生及突变株云芝 IBL-04 制得细胞外锰过氧化物酶(MnPs), 并经过硫酸铵沉淀、透析、离子交换和凝胶渗透层析法等步骤提纯。纯化的酶在十二烷基硫酸钠-聚丙烯酰胺凝胶电泳(SDS-PAGE)上于 43 kDa 区域呈现单一谱带, 它适宜的 pH 值和温度分别为 5.0 和 40 °C。突变株 MnPs 表现出比野生株 MnPs 更宽的活性 pH 值范围和更高的热稳定性。从所选突变株所得纯化的 MnPs 表现出与野生株 MnP 几乎相同的电泳性质、稳态动力学、金属离子和 EDCs 降解效率。该生物酶与 Mn^{2+} 一起催化的反应速率最快, 但最高的亲和性对应于 ABTS、甲氧基羟苯基乙二醇、4-氨基苯酚和活性染料。 Mn^{2+} 和 Cu^{2+} 可显著提高 MnPs 的活性, 但 Zn^{2+} , Fe^{2+} , EDTA 和半胱氨酸则会不同程度地抑制其活性, Hg^{2+} 是最强的活性抑制剂。所有来源的 MnPs 均可有效催化 EDCs、壬基苯酚和二氯苯氧氯酚降解, 处理 3 h 可除去 80% 以上, 在 MnPs-介质体系中可进一步提高到 90%。综上, 云芝 MnPs 生物酶具有较高的 pH 适用性和热稳定性、独特的 Michaelis-Menten 动力学参数和高的 EDCs 去除效率等特点, 因而有望工业化应用。

关键词: 锰过氧化物酶; 云芝 IBL-04; 提纯; 表征; 热稳定性; 生物降解

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