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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 Mn^{2+}, MnPs displayed the highest affinity for ABTS, methoxyhydroquinone, 4-aminophenol and reactive dyes. MnP activity was significantly enhanced by Mn^{2+} and Cu^{2+}, and inhibited in the presence of Zn^{2+}, Fe^{2+}, ethylenediaminetetraacetic acid and cysteine to various extents, with Hg^{2+} 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.

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 indigenous 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 *Tremetes 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; $MgSO_4{\cdot}7H_2O$, 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*(HCl)/*M*(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 Gallenkemp, 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 × (10^7–10^8)/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 μg/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 μg/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 (ε = 6.5 × 10^3 mol^{-1} L cm^{-1}). Enzyme activity