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Research Note

Screening and Production of Manganese Peroxidase from *Fusarium* sp. on Residue Materials

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Abstract In this study, we report the manganese peroxidase production ability from a *Fusarium* sp. strain using an inexpensive medium of agriculture residues of either rice straw or wood chips as carbon source. The highest manganese peroxidase activity on rice straw medium and on wood chips was 1.76 U/mL by day 9 and 1.91 U/mL by day 12, respectively.

Keywords *Fusarium* sp., Hard wood chips, Manganese peroxidase, Rice traw

Lignocellulose biomass is a complex biopolymer consisting of cellulose, hemicellulose, pectin, and lignin; the first three components account for 70% dry weight. Lignocellulose biomass has been proposed as a potential carbon source for the production of biofuels. However, this potential has been limited by the high cost of pretreament processing to convert lignocelluloses to fermentable sugar needs to be overcome [1]. Lignocellulose biomass pretreatment is a process in which lignin links are broken to produce cellulose and hemicellulose which can be further degraded by cellulases, hemicellulases, respectively. Pretreatment methods comprise physical pretreatment, chemical pretreatment, solvent fractionation, and biological decomposition. Compare to other methods, biological decomposition using organisms or enzymes is preferred due to the environmentally friendly nature, less energy consume and high efficiency [2, 3].

Manganese peroxidases (MnPs) (EC 1.11.1.13) belong to a unique enzyme groups that hydrolyze lignin substrates in nature; other members are lignin peroxidases, laccases, and versatile peroxidases. MnPs are extracellular glycoproteins with an iron protoporphyrin IX (heme) prosthetic group. MnPs are assumed to be the first in the line of proteins expressed during fungal catabolism of lignin [4, 5]. Due to its lignin hydrolysis ability, there has been developing interest in MnPs. Potential application of MnPs has been extended in biopulping [6], biobleaching [7], and bioremediation [8].

Naturally, MnPs are found mainly in white-rot fungi, such as *Phanerochaete chrysosporium*, *Ganoderma* sp., *Pleurotus* sp., *Trametes* sp., and *Irpex lacteus* [4, 5]. More recently, the production of ligninolytic enzymes including MnP was demonstrated in *Phyllosticta*, *Aspergillus*, *Fusarium*, and *Penicillium* [9, 10]. However, knowledge of MnP secreted by *Fusarium* is limited.

This study identified biological production of MnP from tropical forest wood decay materials. MnP production was also investigated an inexpensive medium containing rice straw or wood chips as the carbon source.

Wood decay fungal mycelia were collected from Thua Thien Hue province, Vietnam forest bulk during the rainy season November and December, and stored at −20°C. One gram bulk material was dissolved in 50 mL of sterilized water. One hundred microliters of the supernatant was spread on peptone dextrose agar (PDA; 2% peptone, 20% potato, 1.5% agar) and incubated at 28°C until colonies form. Fungal mycelia were transferred to a fresh PDA plate for storage and determination of MnP activity.

MnP producing strains were detected by inoculating fungal mycelia on basal salts medium containing 0.01% guaiacol as described previously [11]. After 24 days culture, the MnP producing strains were confirmed by a halo to colony ratio of oxidized guacicol around the fungal colonies.

To investigate MnP production, the selected fungal strain was cultured on the rice straw or wood chips medium.

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Rice straw was collected from a farm in Hue City, Vietnam, and blender to yield material ≤ 2 mm in size. Wood chips were collected from local wood furniture factory. The selected strain was cultured in 5 mL PD liquid medium (2% peptone, 20% potato) at 30°C for 3 days with shaking at 180 rpm. One milliliter of the culture was transferred into 50 mL MnP production medium containing 5% (w/v) rice straw or wood chips, and 0.2 g/L KH_2PO_4, 0.05 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $CaCl_2{\cdot}2H_2O$, 0.08 g/L $CuSO_4{\cdot}5H_2O$, 0.05 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.033 g/L $MnSO_4{\cdot}H_2O$, 0.043 g/L $ZnSO_4{\cdot}7H_2O$, and 0.05 g/L $FeSO_4{\cdot}7H_2O$. The culture was maintained at 30°C for 24 days with shaking at 180 rpm. One milliliter of fluid was collected at intervals of 3-day and MnP activity was measured. All chemicals were of analytical quality.

MnP activity was measured as described previously with slight modification [12]. Briefly, 100 µL of culture medium was incubated with 900 µL of 50 mM sodium malonate buffer containing 1 mM $MnSO_4$. The reaction was initiated by adding hydrogen peroxide to a final concentration of 0.1 mM followed by incubation at 35°C for 30 min. The complex form of Mn^{3+}-malonate was qualified at 270 nm (ε_{270} = 11.59/mM/cm). One unit enzyme was defined as amount of required enzyme to produce 1 µmol product per minute under the experimental conditions.

The effect of pH on crude MnP was investigated at different pH levels ranging from 3.0 to 8.0. One hundred milliliters crude MnP was incubated with 900 µL of 50 mM sodium malonate buffer containing 1 mM $MnSO_4$ at 35°C for 1 hr. Enzyme activity was qualified as described above. The optimal temperature for crude MnP was determined by assaying for enzyme activity at optimum pH and temperature from 20°C to 50°C at intervals of 5°C. Enzyme was incubated with buffer for 1 hr at the appropriate temperatures before determination.

The effect of metal ions on enzyme were analyzed by incubating the enzyme solution in buffer containing 5 mM Mn^{2+}, Al^{3+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, or Zn^{2+} in 1 hr at the optimal pH and temperature. The residual enzyme activity was measured as described above.

Fungal mycelia were cultured on 5 mL PD medium and maintained for 3 days as described above. Mycelia were harvested by centrifuging at 14,000 rpm and 4°C for 5 min, and washed with autoclaved distilled water. The washed mycelia were resuspended in 500 µL cetyltrimethyl ammonium bromide (CTAB) buffer (100 mM Tris-HCl, pH 8; 1.4 M NaCl; 20 mM ethylenediaminetetraacetic acid; 2% CTAB; 0.2% β-mercaptoethanol). Cell walls were broken by sonication at 60 Hz in 6-sec interval for 5 min using a VC-130 sonicator (Sonics, Newton, CT, USA) and incubated at 65°C for 30 min. Supernatants were harvested by centrifugation 14,000 rpm and 4°C for 10 min. Fungal genomic DNA was purified by adding 500 µL of a 25 : 24 : 1 volume mixture of phenol : chloroform : isopropanol and mixed well by vortexing. The supernatant following centrifugation at 14,000 rpm for 10 min at 4°C was recovered and transferred to new 1.5 mL tube. Genomic DNA was precipitated with two volume of pure ethanol, follow by washing with 70% ethanol, and was dissolved in 50 µL distilled-water.

The basic morphology characterization of the isolation was observed using an Eclipse 55i microscope (Nikon, Tokyo, Japan), while molecular identification was performed by the internal transcribed spacer 1 and 4 (ITS 1-4) sequence [13]. Briefly, the ITS region was amplified by PCR using 40 ng genomic DNA, 10 pmol of each primer (ITS1, 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4, 5′-TCCTCC-GCTTATTGATATGC-3′) and 1× Master mix (Invitrogen, Carlsbad, CA, USA) in a 25 µL reaction volume and following standard protocol. PCR products were qualified by 1% agarose gel electrophoresis and visualized with 0.5% ethidium bromide. Finally, PCR products were purified and sequenced using ITS1 and ITS4 primers. The sequence was Blast-search against the NCBI nucleotide database and the fungal strain was defined.

To identify the MnP producing microorganisms, we first isolated fungi from wood decal material grown on PDA

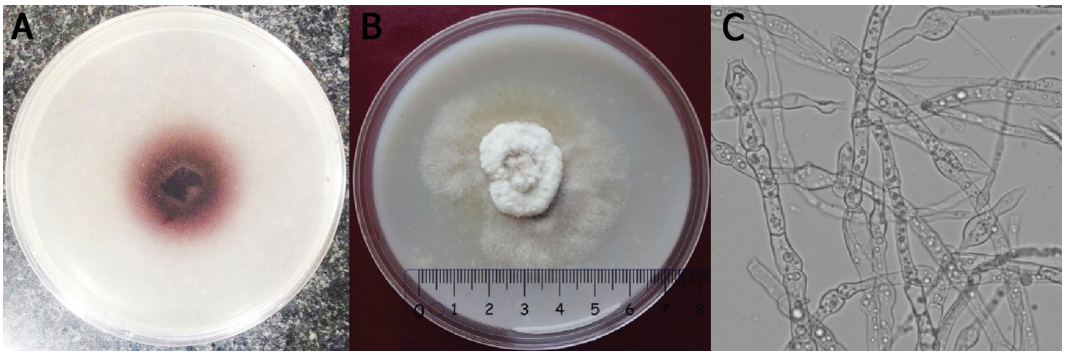

Fig. 1. Growth and morphology of *Fusarium* sp. HUIB01. A, Growth of *Fusarium* sp. on peroxidase selected medium containing 0.1% guaciacol; B, Growth of *Fusarium* sp. on peptone dextrose agar medium; C, Morphology of *Fusarium* sp. The fungus was grown on peroxidase medium containing 0.1% guaciacol; an oxidized zone confirmed peroxidase activity of the fungus. The morphology of *Fusarium* sp. was ascertained by light microscopy with a zoom of 40× using an Eclipse 55i microscope (Nikon, Japan).

medium. Approximately after 3-day inoculation, the fungal mycelia were observed. Total of eleven fungal mycelia which showed different morphotypes such as color, diameter of mycelia, and microscopic observation of spore formation were isolated and purified by placing of each specimen to new PDA medium. The pure cultures were stored on PDA plates at 4°C and sub-cultured on PDA plates at 28°C for 7 days for further studies. To screening potential peroxidase production, the mycelia were transferred into a new Petri dish containing PDA supplemented with 0.01% guaiacol. The potential secretion of peroxidase into medium was indicated by a guaiacol oxidation zone (Fig. 1A). The isolation mycelia had a light white-yellow color with a white cap in central zones. The diameter of mycelia at the 3-day incubation was 6 cm (Fig. 1B).

MnP production from the selected fungal strain was examined by growth on medium containing rice straw or wood chips as the carbon source. On 3-day of incubation, MnP activity was 1.1 and 0.81 U/mL on the medium containing rice straw or wood chips, respectively. MnP activity was highest after 9 days culture on rice straw medium, reaching 1.76 U/mL, and after 12 days culture of wood chips medium, reaching 1.91 U/mL (Fig. 2A). Enzyme accumulation decreased to ≤ 50% of maximal activity after 18 days cultivation in both media.

A study of *Fusarium concolor* ligninolytic enzyme reported that on wheat straw medium, the fungus was able to produce laccase, lignin peroxidase, and MnP under liquid fermentation; MnP activity was 0.9 U/g biomass as an intracellular protein [10]. A certain level of MnP was found on extracellular enzyme of *Fusarium verticillioides* [9] and *Fusarium solani* [14]. Similarly, we observed MnP in supernatant with a level of expression in accordance with *F. verticillioides*.

The effect of pH on MnP was studied (Fig. 2B). Similar to other MnPs, the MnP displayed high activity in an acidic medium with a pH around 4.0. The results also indicated MnP is very sensitive to pH. Enzyme activity decreased rapidly when pH was ≥ 5.0 and ≤ 3.0. The retained activity was ≤ 60% maximum activity, especially above pH 6. *Phanerochaete chrysosporium* is one of the most well studied fungus model for MnP production. The optimal pH of *P.*

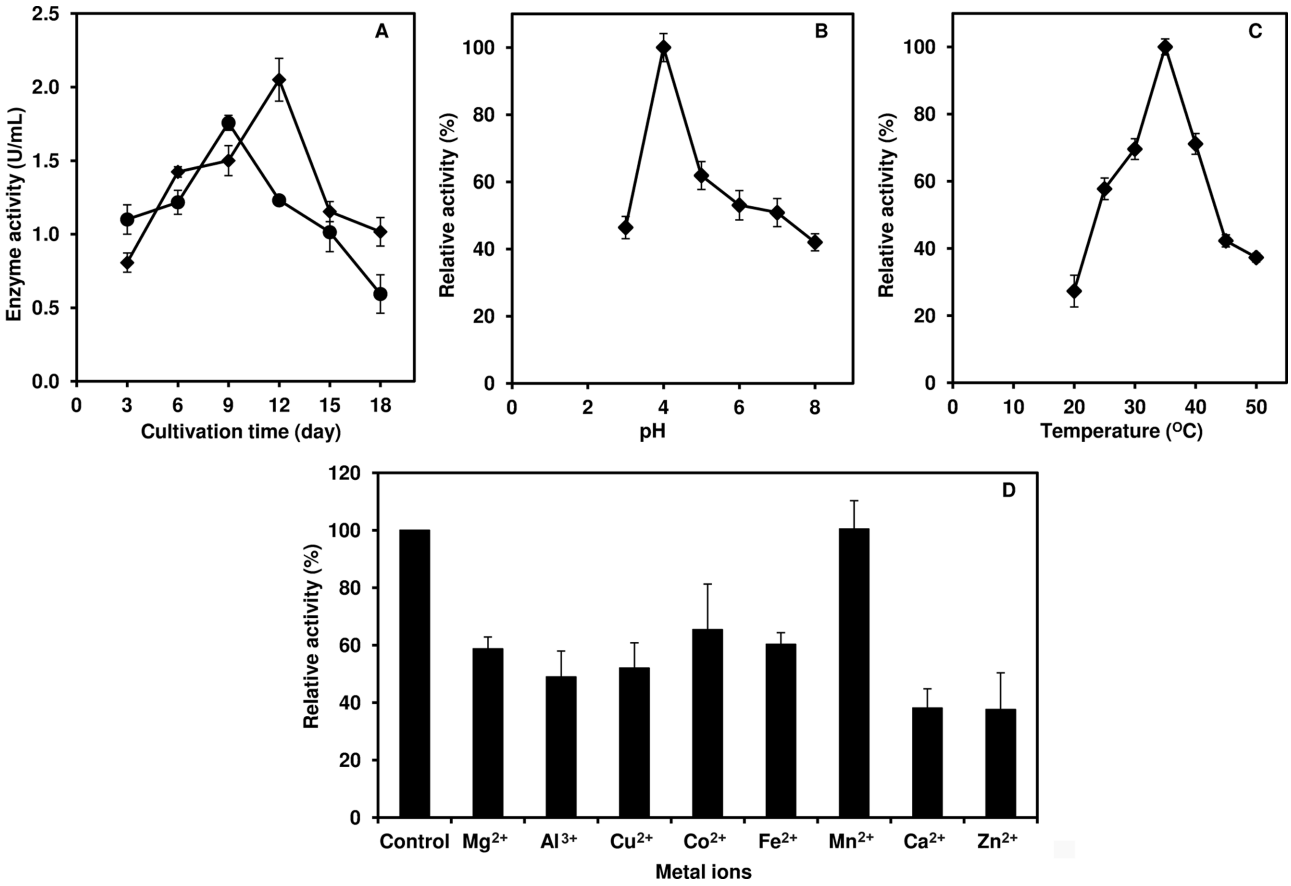

Fig. 2. A, Manganese peroxidase (MnP) production from *Fusarium* sp. HUIB01 on rice straw (closed circle) and wood chips medium (closed diamond); B, Effect of pH on crude MnP activity; C, Effect of temperature on crude MnP activity; D, Effect of metal ion on crude MnP activity. The fungus was cultured on MnP production medium containing rice straw or wood chips as carbon source. The medium fluid was taken every 3 days and MnP activity was determined. Data represent the means of three experiments, and error bars represent means ± standard errors.

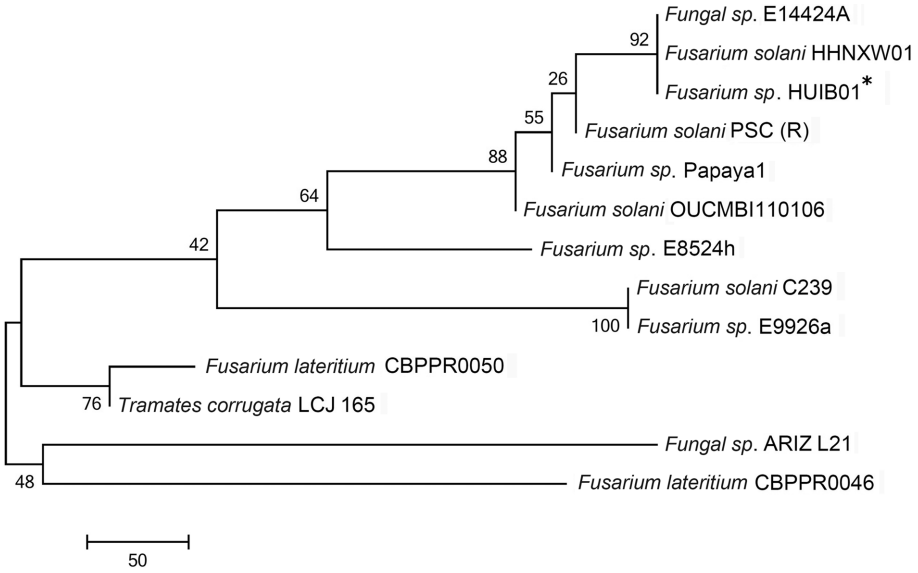

Fig. 3. Phylogenetic relationship tree among *Fusarium* sp. HUIB01 and other *Fusarium* strains based on the internal transcribed spacer rDNA gene sequences. The *Fusarium* sp. HUIB01 was marked as asterisk. The tree was generated using MEGA 6.0 software.

chrysosporium MnP is reported 2.5–4.0 [15, 16]. However, MnP produced from *Trametes* has a higher pH optimal with a range of 4.0–5.5 [17].

MnP activity dramatically increased when the temperature was increased from 20°C and reached its highest activity at 35°C. Enzyme activity diminished with increasing temperature. The retained MnP activity was ≤ 30% when the reaction temperature was ≥ 50°C (Fig. 2C). The optimal temperature of the presently studied is in accordance with MnP from *P. chrysosporium* [18] but differs from *Trametes* MnP, which displayed an optimal temperature of 70°C [17].

We also examined the effect of various metal ions on MnP activity. Metal ions inhibited MnP activity (Fig. 2D) except for Mn^{2+}. Mn^{2+} did not affect MnP activity from *Trametes*, but Zn^{2+}, Na^{+}, Mg^{2+}, and Al^{3+} reduced enzyme activity, similar to the present observation. *Trametes* MnP was strongly against Ni^{2+}, Li^{+}, Ca^{2+}, and K^{+} [17].

The morphology characterization revealed common shape of *Fusarium* species such as straight elongation of microconidia, barely notched of basal cells, papillate curved of apical cells [19, 20]. We also performed molecular identification of the strain through the sequence of ITS1-4 region. We obtained a PCR product with size of 550 bp. The PCR product was then sequenced and the sequence was searched against the NCBI database using BLAST function. The strain was highly relevant to *Fusarium* species such as *Fusarium* sp. E8524h (HQ117863), *F. solani* C239 (KU377471), *F. solani* OUCMBI110106 (KP269017), and *F. lateritium* CBPPR0046 (KT211538) (Fig. 3). Hence, it was designated as *Fusarium* sp. HUIB01. The ITS sequence was also deposited on NCBI with accession number KX388183. In this study, we isolated a potential MnP producing fungal strain isolated from a forest area. The strain produced MnP under fermentation separately using rice straw and wood chips as the carbon source. Highest MnP activity on rice straw medium was 1.76 U/mL and 1.91 U/mL on wood chips medium. The crude enzyme had optimal pH and temperature of 4.0 and 35°C, respectively. Genetic comparison of the strain to others revealed it to be *Fusarium* sp. Our findings point the way to a strategy capable of achieving the enzymatic conversion of biomass residual to a valuable product.

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