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Purification and characterization of manganese peroxidase produced by *Fusarium equisetti*

By

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Abstract

Microbial ligninolytic enzymes including laccase, manganese peroxidase, and lignin peroxidase are increasingly valued across diverse industries, with manganese peroxidase standing out as a principal component of the microbial lignin-degrading arsenal. This study was aimed at isolating managanese producing fungi from environmental samples. The fungi was revealed as Fusarium equisetum, and was used for the production of managanese peroxidase using Sawdust as substrate. The enzyme was purified and characterized. Specific activity in the pooled fraction equals 148.4 µmol min⁻¹ ml⁻¹. The 23.3-fold increase was also comparable.). The enzyme retained more than 80 % of its initial activity across the 30 °C – 50 °C when held for two hours. The residual activity slowly declined with longer incubation hours. The Fusarium equisetum managanese peroxidase was an acidophilic one retaining one-third of its activity at pH 3.0. The enzyme was relatively stable in the presence of metal ions Magnesium, aluminium, copper, and mercury salts. This manganese peroxidase showed relative potential for further biotechnological uses.

Key words: *Manganese peroxidase, Fusarium, purification*

Introduction

Lignocellulose comprises of cellulose, hemicellulose, pectin, and lignin, lignocellulose biomass is a complex biopolymer that makes up 70% of its dry weight (Huy, 2017). One possible carbon source for the synthesis of biofuels is lignocellulose biomass. However, this potential has been limited by the high cost of pretreatment processing to convert lignocelluloses to fermentable sugar needs to be overcome (Nidhi et al., 2020).

Manganese peroxidase, also known as E.C.1.11.1.13. Mn^{2+}: H_2O_2 oxidoreductase, is a ligninolytic enzyme widely found in nature (X Qin, 2017). Owing to its numerous and potential uses in various biotechnological fields, this enzyme has become more in demand in recent years (Chowdhary et al., 2019; Asgher, 2014). The most promising, well-known, and well-researched enzymatic activity amongst the lignocellulosic enzymes is that of Manganese peroxidase (MnP), which is also quite versatile and has several industrial uses (Bilal, 2017). The MnP enzyme oxidizes the phenolic and non-phenolic substances when it converts Mn (II) to Mn(III). MnPs are the widely distributed extracellular potential peroxidases that fungi (white-rot fungus) generate or manufacture.

Several researchers have reported on the occurrence of MnPs in many microorganisms such as bacteria, fungi, and algae. One or more of the three extracellular enzymes—LiP, MnP, and laccase—that are required for the breakdown of lignin are produced or secreted by fungi (Chowdhary et al., 2019b). *Dichomeris squalens, P. radiata, P. rivulosu, P. sordida, P. chrysosporium, and C. subvermispora* all produce extracellular MnPs (Zhang, 2018). In an attempt to optimize the numerous potential applications of MnPs, there exists the need to research MnPs with increased resistance to organic solvents and inorganic ions to improve the capacity to degrade xenobiotics.

Because it is more cost-effective and environmentally benign than alternative methods, the biological pre-treatment method that uses microorganisms to break down the result of the Maillard reaction and optimizes the process in a lab setting is intriguing (Ravikumar et al., 2021).

Ligninolytic enzymes can be used in a wide range of organic and inorganic substrate specificities in a number of industrial and biotechnological applications. These applications include the removal of phenolic compounds from beer and wine in the food sector, the bleaching and delignification processes in the paper and pulp industries, and the detoxification of industrial effluent, primarily from the textile and petrochemical industries (Nidhi et al., 2020).

According to Huy et al. (2017) rice straw or wood chips can be utilized as the main source of carbon and are acknowledged as an affordable agricultural residual medium for the synthesis of MnP from *Fusarium* sp. Additionally,

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MnP with a 45 kDa molecular mass was isolated from the white-rot fungus C. unicolor BBP6 and given the designation MnP-BBP6 at the ideal pH and temperature (Zhang, 2018). Due to the accumulation of rice straw as agricultural waste that contributes to numerous environmental issues globally, the degradation of lignin and rice straw is currently receiving a lot of attention, particularly in Egypt (Kheti et al., 2023; Nidhi et al., 2020).

This current study focused on the screening and production of Manganese peroxidase by *Fusarium equiseti*.

2.0 Materials and methods

2.1 Isolation and screening of manganese peroxidase-producing fungi

The fungal strains were isolated from several soil samples gathered from various locations in Ekiti State University, on Potato Dextrose Agar (Oxoid). The fungal strains were periodically subcultured on potato dextrose agar and preserved at 4°C. According to Järvinen et al. (2012), fungal strains were inoculated onto potato dextrose agar medium supplemented with 0.2% tannic acid and incubated at 28°C for five days to screen for fungi that produced manganese peroxidase

2.2 Identification of manganese peroxidase-producing fungi

Manganese peroxidase-producing fungi was identified microscopically using lactophenol blue stain at 40x magnification as described by Geethanjali & Jayashankar (2017).

The isolate was characterized by sequencing the Internal Transcribed Spacer (ITS) region of the nuclear DNA (rDNA). The universal primers ITS1 and ITS4 were used to amplify the ITS target region(White et al., 1990).

Genomic DNA was extracted from the samples received using the Quick-DNA™ Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented below. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific. DNASTAR was used to analyse the. ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI) (Altschul et al., 1997).

ITS Primers sequences

Name of Primer	Target	Sequence (5' to 3')
ITS-1	ITS rDNA	sequence TCCGTAGGTGAACCTGCGG
ITS-4	ITS rDNA	sequence TCCTCCGCTTATTGATATGC

2.3. Production of manganese peroxidase

A solid state fermentation medium containing 10g of sterilized and inoculated material (Saw dust) was used to produce manganese peroxidase. The medium was then incubated for five days, and 200mL of distilled water was added. The fermentation medium was then filtered through Whatmann No. 1 filter paper, and the filtrate was centrifuged at 5000 rpm for fifteen minutes to obtain crude enzyme extract and for further research (Nidhi et al., 2020).

All of the cultivations were performed in duplicate and the presented results are average values.

2.4 Inoculum preparation

A 4-day-old culture cultivated on potato dextrose agar slants was used to make the fungal spore suspension. To suspend the spores, 10 milliliters of a 20% tween-80 solution was added and scraped with a loop. For later usage, the spore suspension was kept in screw-cap vials.

2.5 Assay of manganese peroxidase activity

The oxidation of manganic malonic complex was used to measure manganese peroxidase (MnP) activity. Assay mixtures (1.3 ml) included 0.05 ml of MnP sample, $MnSO_4$ (50 mM), H_2O_2 (0.1 mM), and sodium malonate buffer (pH 4.5, 50 mM). One mol of product generated per minute was considered to be one unit of MnP activity. Every enzyme test was conducted using a UV-Vis spectrophotometer (Bourbonnais et al., 1995).

2.6 Protein content determination

Protein concentration was assessed using the Bradford method with the Bio-Rad Protein Assay dye reagent (Kielkopf et al., 2020). In this procedure, 200 µl of the diluted dye reagent was added to 10 µl of the sample solution. The mixture was incubated at room temperature for 15 minutes to allow for color development. Absorbance was then recorded at 595 nm using a blank as the control, which contained all assay components except the sample—replaced with an equal volume of distilled water.

To generate a standard protein curve, a stock solution of bovine serum albumin (BSA) at 0.2 mg/ml was prepared and serially diluted to produce concentrations ranging from 0.02 to 0.01 mg/ml. For each standard, 200 µl of Bradford reagent was added, followed by a 15-minute incubation. Absorbance was subsequently measured at 595 nm to establish the standard curve.

2.7 Purification of managanese peroxidase

Solid crystalline ammonium sulphate was gradually introduced into the clarified enzyme extract until 40% saturation was achieved. The required quantity of ammonium sulphate was determined using standard reference tablets, as described by Jarvinen et al. (2012). The salt was dissolved at 4°C, and the resulting mixture was centrifuged at 6000 rpm for 20 minutes at the same temperature. The resulting precipitate was collected, dissolved in 50 mM sodium acetate buffer (pH 5.0), and stored under refrigeration. To eliminate residual ammonium sulphate, the enzyme solution underwent dialysis against 50 mM sodium acetate buffer (pH 5.0), using

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a dialysis membrane. The buffer was replaced every six hours, and the process was maintained at 4°C throughout (Järvinen et al., 2012).

A 100 mL portion of the dialyzed enzyme solution was subjected to further purification using gel filtration chromatography. This step was performed using a 2.5 × 82 cm column pre-equilibrated with three bed volumes of 50 mM sodium acetate buffer (pH 5.0). Elution was carried out with the same buffer at a flow rate of 25 ml/hr. All collected fractions were assayed for manganese peroxidase activity, and those corresponding to the activity peak were pooled, cooled, and prepared for further characterization (Järvinen et al., 2012).

2.8 Characterization of purified enzymes

Characterization of manganese peroxidase was performed following modification of methods described by (Zhang et al., 2016).

2.8.1 Effect of pH on enzyme activity and stability of purified enzymes

To determine the optimum pH of the purified enzyme, enzyme assays were performed across a pH range of 3.0 to 11.0. The following 50 mM buffers were used depending on the pH range: citrate buffer (pH 3.0), sodium acetate buffer (pH 4.0–6.0), Tris-HCl buffer (pH 7.0–8.0), and glycine-NaOH buffer (pH 9.0–11.0) (Wakil & Osesusi, 2017). To evaluate the pH stability of crude manganese peroxidase, enzyme solutions were incubated with buffers of varying pH (3.0–11.0). Each mixture contained 2 ml of buffer and 1 ml of enzyme solution, and was maintained at 4°C for 24 hours. The residual enzyme activity was then assessed using the standard assay method earlier described.

2.8.2 Effect of temperature on enzyme activity and stability

The optimal temperature for activity of the purified enzyme was determined by conducting the standard assay at eight different temperatures: 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C.

To assess thermal stability, enzyme solutions were incubated at various temperatures (50°C to 100°C). Enzyme half-life ($t_{1/2}$) and residual activity were measured over time and plotted to evaluate thermal inactivation kinetics.

2.8.3 Effect of Metal Ions on enzyme activity

The influence of selected divalent metal ions and EDTA on enzyme activity was studied by mixing equal volumes of enzyme solution with five different concentrations (1, 5, 10, 20, and 30 mM) of each additive. The metal ions tested included Cu^{2+}, Mg^{2+}, Hg^{2+}, Ca^{2+}, Mn^{2+}, and Fe^{2+}—all in chloride form(Osesusi et al., 2021).

A control sample containing only purified enzymes in 50 mM Tris-HCl buffer (pH 9.0) was prepared, while a blank consisted of buffer alone. After 30 minutes of incubation, enzyme activity was determined using the standard assay procedure.

Results

In this current study, five fungal isolates were isolated and screened from different soil locations (environmentally-stressed soils) around Ekiti State University. The ecologically stressed soil were selected for the isolation of Manganese peroxidase producing fungi.

All the five isolates were inoculated on PDA media supplemented with 0.2% tannic acid. From amongst the five fungi, the isolate MG1 showed better potential for managenese production by the formation of a clear halo zone around the colony.

The fungi MG1 was identified as *Fusarium equiseti* with accession number PP319671.1, this was based on the similarity between the sequence queried, and compared to the biological sequences within the NCBI database.

Table 1 and Figure 1 summarizes the purification of manganese peroxidase extracted from *Fusarium equisetum.* Gel-filtration on Sephadex G-100 yielded a 23.3-fold purification and a 26.3 % recovery of activity, giving a total specific activity of 148.4 µmol min^{-1} ml^{-1}

Figure 2 shows that the enzyme maintained > 80 % of its original activity after 120 min at 30–50 °C. Prolonged incubation or higher temperatures led to progressive loss of activity, with faster inactivation as the temperature increased.

The enzyme was relatively stable at 30, 40 and 50 °C and retained more than 80 % of its initial activity after 120 min of incubation. The residual activity of manganese peroxidase decreased with the increase in the time of incubation. Also increase in temperature showed reduction in the activity of the enzyme (Figure 3).

Manganese peroxidase from *Fusarium equisetum* exhibited 35.1 % of its maximal activity at pH 3.0. Activity climbed steadily to 96.1 % at pH 4.0 and reached its peak at pH 5.0. Beyond this point, further alkalinization progressively diminished activity (Figure 4)

As depicted in Figure 5, the enzyme remained highly stable in acidic conditions. After 120 minutes, residual activity was 100 % at pH 3, 96.4 % at pH 4, and 84.6 % at pH 5. Stability declined sharply in neutral conditions, with only 44.7 % activity retained at both pH 6.0 and pH 7.0 after the same incubation period.

Figure 6 demonstrates that $MgCl_2$ and $AlCl_3$ enhanced manganese-peroxidase activity, whereas Cu^{2+}, Fe^{2+}, and especially Hg^{2+} (≈ 91.5 % inhibition) exerted strong inhibitory effects. Among the activators, Mg^{2+} produced the greatest stimulation relative to the control.

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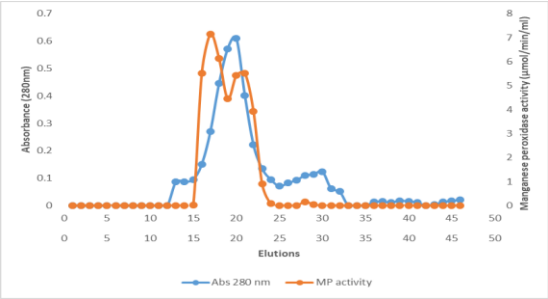


Figure 1: Elution profile of manganese peroxidase from Sephadex G-100 column chromatography

Table 1: Summary of Purified Manganese Peroxidase from *Fusarium equisetum*

Purification step	Manganese Peroxidase activity (µmol/min/ml)	Protein concentration	Total activity (µmol/min/ml)	Total protein	Specific activity	Yield (%)	Fold
Crude Enzyme	1.13	33.46	565	1673	0.034	100	1
Ammonium Ppt	2.72	23.4	108.8	936	0.116	19.25	3.44
Gel	5.19	6.59	148.43	188.47	0.787	26.27	23.31

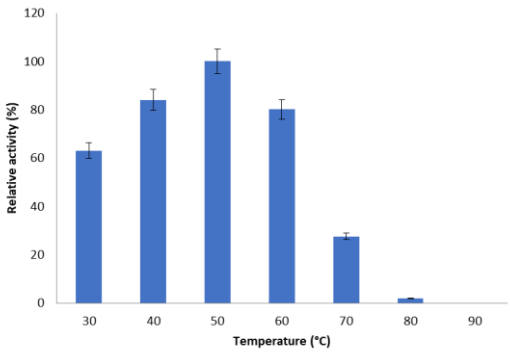


Figure 2: The effect of temperature on *Fusarium equisetum* Manganese Peroxidase Activity

Figure 3: The effect of temperature on *Fusarium equisetum* Manganese Peroxidase Stability

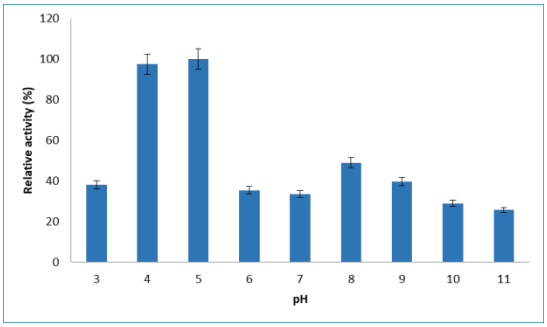
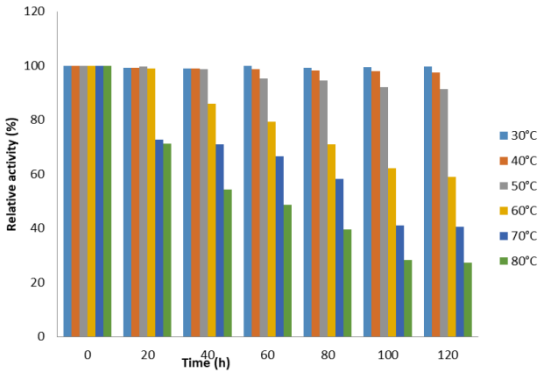


Figure 4: The effect of pH on *Fusarium equisetum* Manganese Peroxidase Activity.



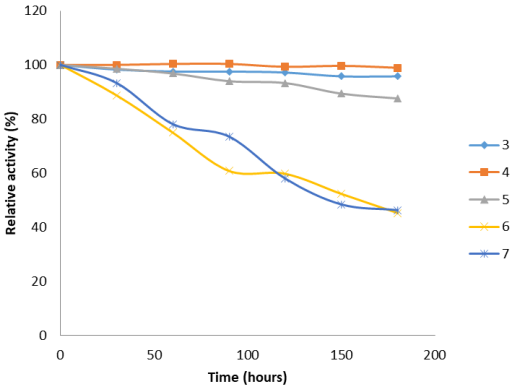


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Figure 5: The effect of pH on the Stability of *Fusarium equisetum* Manganese Peroxidase

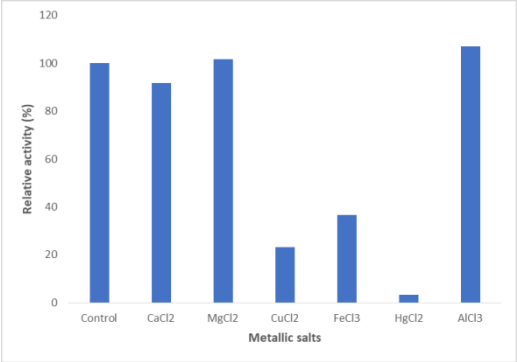

Figure 6: The effect of metallic salts on the activity of Manganese Peroxidase

Discussion

In this study *Fusarium equisetum* isolated from stressed environments was used to produce manganese peroxidase. This study showed a simplified purification process with the ability to produce comparative purification yields and specific activities. Culture supernatant and gel chromatography were able to purify the manganese peroxidase to a reasonable level of yield. This closely agrees with findings of Nidhi et al. (2020) which produced managanese peroxidase from nut husks. Fungal MnPs typically weigh 40–50 kDa, so they migrate through the column at an intermediate elution volume, whereas larger polysaccharidases elute early and smaller peptides elute late. The 23.3 fold increase also showed that more than 95 % of non-MnP proteins were removed. A single gel-filtration pass achieving $>20\times$ purification is considered highly efficient, suggesting that MnP is one of the dominant mid-sized proteins in the crude filtrate (H Zhang, 2018). The specific activity when expressed per milligram protein (38.2 µmol min^{-1} mg^{-1}), this value approaches the 30–50 µmol min^{-1} mg^{-1} range reported for near-homogeneous fungal MnPs in the literature, indicating >90 % purity by activity (Vrsanska et al., 2016).

When the enzyme was held for two hours, it retained more than 80 % of its initial activity across the 30 °C – 50 °C range, an excellent stability range for most mesophilic or moderately thermophilic industrial processes (e.g., pulp-bleaching, dye-decolourisation, or biomass pretreatment carried out below 50 °C). This is similar to earlier works (Zhang et al., 2016). Above 50 °C the residual activity declined steadily, indicating that heat-induced unfolding or heme-centre disruption outpaced the enzyme's natural refolding capacity.

The thermal-stability profile observed for the *Fusarium equisetum* manganese-peroxidase (MnP) mirrors patterns reported for many fungal MnPs. At 30 – 50 °C the enzyme retained ≥ 80 % of its initial activity after 2 h, indicating that its tertiary structure and the heme-Mn^{2+} catalytic site can tolerate mesophilic temperatures for extended periods. Comparable half-lives have been documented for *Phanerochaete chrysosporium* MnP (approximately 85 % activity after 2 h at 45 °C) (Glenn & Gold, 1985) and *Pleurotus ostreatus* MnP (close to 80 % after 1.5 h at 50 °C) (Eichlerová et al., 2015).

The pH profile indicates that *Fusarium equisetum* manganese peroxidase (MnP) is an acidophilic enzyme**;** typical of fungal MnPs that operate in the acidic micro-environment of decaying lignocellulose(Eichlerová & Baldrian, 2020). Even at pH 3.0 the enzyme remains one-third active, showing that protonation of surface and catalytic residues has not yet disrupted the heme-centre or Mn^{2+}-binding site. Further alkalinisation reduces activity because deprotonation of key acidic residues alters the redox potential of compound I and weakens Mn^{2+} binding. Similar downturns are reported for *Phanerochaete chrysosporium* MnP (optimum pH 4.5–5.0) and *Pleurotus ostreatus* MnP (pH 5.0) (Eichlerová et al., 2015).

The differential effects of metal ions on *Fusarium equisetum* manganese-peroxidase (MnP) activity shed light on how the enzyme's catalytic machinery and structure respond to divalent cations. Similar enhancements have been reported for *Phanerochaete chrysosporium* MnP, where Mg^{2+} raised activity by ~30 %. (Zhang et al., 2016). Xu et al. also reported that Cu^{2+} and Fe^{2+} acted as potent inhibitors of *Ganoderma applanatum* MnP, consistent with oxidative heme-degradation mechanisms (Xu et al., 2017).

Conclusion

This study screened and characterized manganese peroxidase produced by *Fusarium* equisetum isolated from stressed environments. Findings from the study revelaed the stability of the enzyme at extremes of pH and temperatures. This further bolsters the biotechnological potential of the enzyme. Subsequent optimization could focus either on boosting yield (for large-scale production) or on adding a polishing step (for applications demanding near-homogeneity), but the present result already positions this MnP as a viable biocatalyst candidate.

Declarations

Competing interests: "The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article".

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