

Research Paper

Aflatoxin detoxification by manganese peroxidase purified from *Pleurotus ostreatus*

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Abstract

Manganese peroxidase (MnP) was produced from white rot edible mushroom *Pleurotus ostreatus* on the culture filtrate. The enzyme was purified to homogeneity using $(\text{NH}_4)_2\text{SO}_4$ precipitation, DEAE-Sephadex and Sephadex G-100 column chromatography. The final enzyme activity achieved 81 U mL^{-1} , specific activity 78 U mg^{-1} with purification fold of 130 and recovery 1.2% of the crude enzyme. SDS-PAGE indicated that the pure enzyme have a molecular mass of approximately 42 kDa. The optimum pH was between 4-5 and the optimum temperature was 25°C . The pure MnP activity was enhanced by Mn^{2+} , Cu^{2+} , Ca^{2+} and K^+ and inhibited by Hg^{+2} and Cd^{+2} . H_2O_2 at 5 mM enhanced MnP activity while at 10 mM inhibited it significantly. The *MnP*-cDNA encoding gene was sequenced and determined (GenBank accession no. AB698450.1). The *MnP*-cDNA was found to consist of 497 bp in an Open Reading Frame (ORF) encoding 165 amino acids. MnP from *P. ostreatus* could detoxify aflatoxin B1 (AFB1) depending on enzyme concentration and incubation period. The highest detoxification power (90%) was observed after 48 h incubation at 1.5 U mL^{-1} enzyme activities.

Key words: manganese peroxidase, aflatoxin B1, *Pleurotus ostreatus*, purification.

Introduction

Manganese peroxidase (MnP) (Ec 1.11.1.13) catalyses the H_2O_2 dependent oxidation of Mn II to Mn III which is a non-specific oxidant and seems to play an important role in initiating lignin attack (Kuwahara *et al.*, 1984; Paszcynski *et al.*, 1986; Perez and Jeffries, 1992). The enzyme is an extracellular heme protein produced by wood rotting and little degrading basidiomycetes. It is small enough to diffuse into the compact lignocelluloses complex, and oxidizes the aromatic structures via formation of radicals, leading finally to the breakdown of covalent bonds (Evans *et al.*, 1994). The purified MnP differentiated by different column chromatography into two isoenzymes, MnP1 and MnP2 with very close molecular masses of 42 kDa and 44 kDa, respectively (Schneegaß *et al.*, 1997). The main and more active isoenzyme was MnP2 from *Nematoloma frowardii* (Schneegaß *et al.*, 1997), *Lentinula edodes* (Boer *et al.*, 2006), *Pleurotus eryngii* (Martinez *et al.*, 1996) and *Phanerochate flavido-alba* (de la Rubia *et al.*, 2002).

The mushroom genus *Pleurotus* has the capacity to degrade lignin with degradation of cellulose (Kamra and

Zadrazil, 1986); this phenomenon is of relevant biotechnological importance in delignification process for feed production and paper-pulp industry (Kamra and Zadrazil, 1986; Martinez *et al.*, 1994). Appreciable levels of peroxidases involved in lignin biodegradation by *Pleurotus* species such as lignin peroxidase (LP) and MnP (Kirk and Farrell, 1987). It has been reported that MnP can oxidize Mn^{+2} to Mn^{+3} which act as a better substrate than veratryle alcohol at physiological pH 4.5. It was suggested also that the generation of chelated Mn^{+3} could be the main role of MnP in lignin degradation (Khindaria *et al.*, 1995).

The increasing interest in this enzyme is due to its production by most white rot fungi including lignin degrading species that lack LP (Lobos *et al.*, 1994; Périé and Gold, 1991), its capacity to depolymerise synthetic lignin *in vitro* (Wariishi *et al.*, 1991), its oxidation to phenolic and non-phenolic compounds (Bao *et al.*, 1994), its biodegradation of polycyclic aromatic hydrocarbon (PAH) (Pickard *et al.*, 1999; Wariishi *et al.*, 1992), humic acid (Ziegenhagen and Hofrichter, 1998), synthetic dyes (Heinfling *et al.*, 1998) and more recently detoxification of mycotoxins (Motomura *et al.*, 2003; Wang *et al.*, 2011).

Aflatoxin B1 (AFB1) is a potent mycotoxin with mutagenic, carcinogenic, hepatogenic and immunosuppressive properties. AFB1 treated with MnP from the white rot fungus *Phanerochaete sordida* Yk-624 was detoxified by 86% of the original value after 48 h (Wang *et al.*, 2011). Nuclear Magnetic Resonance ($^1\text{H-NMR}$) and High Resolution Electrospray Mass Spectrometry (HR-ESI-MS) analysis suggested that AFB1 is first oxidized to AFB1-8, 9-epoxide by MnP and then hydrolyzed to AFB1-8, 9-dihydrodiol (Wang *et al.*, 2011).

In the present study the detoxification of AFB1 by purified MnP extracted from the edible mushroom *Pleurotus ostreatus* was demonstrated. The active enzyme was characterized biochemically and in molecular basis.

Materials and Methods

Organism and culture condition

Pleurotus ostreatus was kindly supplied by Academy of Science and Technology, Alexandria, Egypt. It maintained on Potato Dextrose Agar media (PDA) at 4 °C. For the production of MnP, the fungal strain was grown in a medium (Grgic *et al.*, 2001) containing (g L⁻¹) glucose (10.0), KH₂PO₄ (0.2), CaCl₂ (0.11), (NH₄)₂HPO₄ (0.264), MgSO₄·7H₂O (0.05), ZnSO₄·7H₂O (0.0425), MnSO₄·H₂O (0.175), CoCl₂·6H₂O (0.007), CuCl₂·2H₂O (0.007), FeCl₃·6H₂O (0.0009), NaCl (0.0009), yeast extract (0.2), veratryl alcohol (0.07), tartaric acid (3.0) and 1 g of Tween 80 (pH was adjusted at 4.5). Sterilized flasks containing 100 mL each of liquid medium were inoculated with 5.0 inoculums of 0.5 mm diameter. The flasks were incubated under stationary condition at 28 °C. The cultures were filtered and the filtrates considered as crude MnP.

MnP activity assay

The activity of MnP was determined spectrophotometrically (Gold and Glenn, 1988) by monitoring the absorbance change at $\lambda = 240$ nm due to the formation of Mn⁺³ lactate and using the molar extinction coefficient value of 65.00 M⁻¹cm⁻¹. The reaction mixture 1 mL consisted of 50 μM MnSO₄, 50 μM H₂O₂ and 0.5 mL of the enzyme solution in 50 mM sodium lactate/lactic acid buffer pH 4.5 at 30 °C. One enzyme activity unit was defined as enzyme transformed 1 μmole of the Mn⁺² into the Mn⁺³ under the specified assay condition.

Enzyme purification

All procedures were performed at 4 °C. The culture filtrate containing crude MnP was centrifuged at 15,000 g for 30 min to remove particulate material then resuspended in bi-distilled water. One liter of culture supernatant was precipitated with solid ammonium sulfate to 80% saturation under constant stirring. The solution was centrifuged at 20000 g for 30 min and the precipitates were dissolved in 50 mM sodium acetate buffer (pH 5.0) followed by over-

night dialysis against buffer A (50 mM sodium acetate, pH 5.0, supplemented with 0.1 mM phenylmethanesulfonyl fluoride (PMSF)). The dialyzed sample was applied to a DEAE-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) pre-equilibrated with buffer A. The proteins were eluted with buffer A containing linear gradient of 0.0-0.6 M NaCl. The soluble proteins were applied to Sephadex G-100 column (80 cm x 2.0 cm) previously equilibrated with the same buffer A. The protein fractions (2.0 mL) were eluted at a flow rate of 1 mL min⁻¹. Each fraction was assayed for protein (A_{280nm}) and MnP activity. The pooled active fractions were dialyzed against water, lyophilized and stored at -20 °C.

Protein determination

Total protein was determined by using bovine serum albumin as protein standard (Bradford, 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 12.5% polyacrylamide gels according to the method of Laemmli (1970). The separated proteins were stained with Coomassie Brilliant Blue R-250 (Fluka, Switzerland), and their molecular weights were determined by comparison with low range molecular weight markers (Bio-Rad) was used.

Enzyme characterization

The effects of temperature, pH and metals on MnP activity were tested successively. The optimum temperature for enzyme activity was determined in the range of 20 °C to 45 °C at 5 °C intervals. The optimum pH was tested in the range of 4.0 to 10.0 using 50 mM sodium acetate, sodium phosphate or glycine sodium hydroxide buffers. The effects of Ca⁺², Ni⁺², Cu⁺², Hg⁺², Mn⁺², Cd⁺², K⁺ and H₂O₂ on MnP activity were also assayed.

Assay of AFB1 detoxification by MnP

Reaction mixture containing 500 μL of the finally purified MnP, 10 mL of 1 mM AFB1 in 10% dimethylsulfoxide, 1 mM MnSO₄, 0.1% Tween 80, 200 μL glucose oxidase, and 2.5 mM glucose in 50 mM sodium lactate/lactic acid buffer, pH 4.5. Reactions were performed for 48 h at 30 °C and mixing at 150 r.p.m. The residual amount of AFB1 was determined by HPLC (Shimadzu) under the following conditions: column, RP18 (ODS) (4.6 mm x 150 mm, Wako Pure Chemical Industries); mobile phase, 40% aqueous methanol; flow rate, 0.5 mL min⁻¹; and detection wavelength, 365 nm.

Molecular characterization of MnP

Isolation of RNA was carried out according to the method outlined by Huang *et al.* (2007) by using Biozol reagent (BioFlux, Tokyo, Japan), and then purified using tri-reagent RNA kit (Sigma). cDNA synthesis was carried

out by using 1st strand cDNA kit (BIO BASIC INC.) according to the manufacturer instructions, in a reaction mixture (20 μ L final volume). Specific products were amplified by PCR, (using *T-GRADIENT* thermal cycler from Biometra) in 25 μ L volume containing 2.5 μ L of cDNA; 25 pmol of each primer forward primer AAT ACCAGGGTGAAGTTGAATCG and reverse AAT GCGTCTTGGGAACAGGAATA; 10 mM of each dNTP; 1U of *Taq* DNA polymerase; 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1 mM MgCl₂. Conditions for PCR were, denaturation at 94 °C for 3 min was followed by 35 cycles of 1 min at 94 °C; 2 min at 60 °C and 1 min at 72 °C with a final extension step at 72 °C for 7 min. Two oligonucleotide primers were synthesized according to the published se-

quence. The amplified fragment was separated, purified and sequenced.

Results and Discussion

It was found that when the dialyzed protein solution, obtained by ammonium sulphate precipitation of the culture supernatant was loaded on DEAE-Sepharose column and three major peaks with 6.8 purification fold and 20% recovery were detected (Table 1 and Figure 1A). As the eluted peaks of high MnP activity were subsequently submitted to further purification in Sephadex G-100, single activity peak was observed with final enzyme activity of 81 U mL⁻¹, specific activity of 78 U mg⁻¹ protein, purification fold 130 and recovery 1.2% of the crude preparation (Figure 1B).

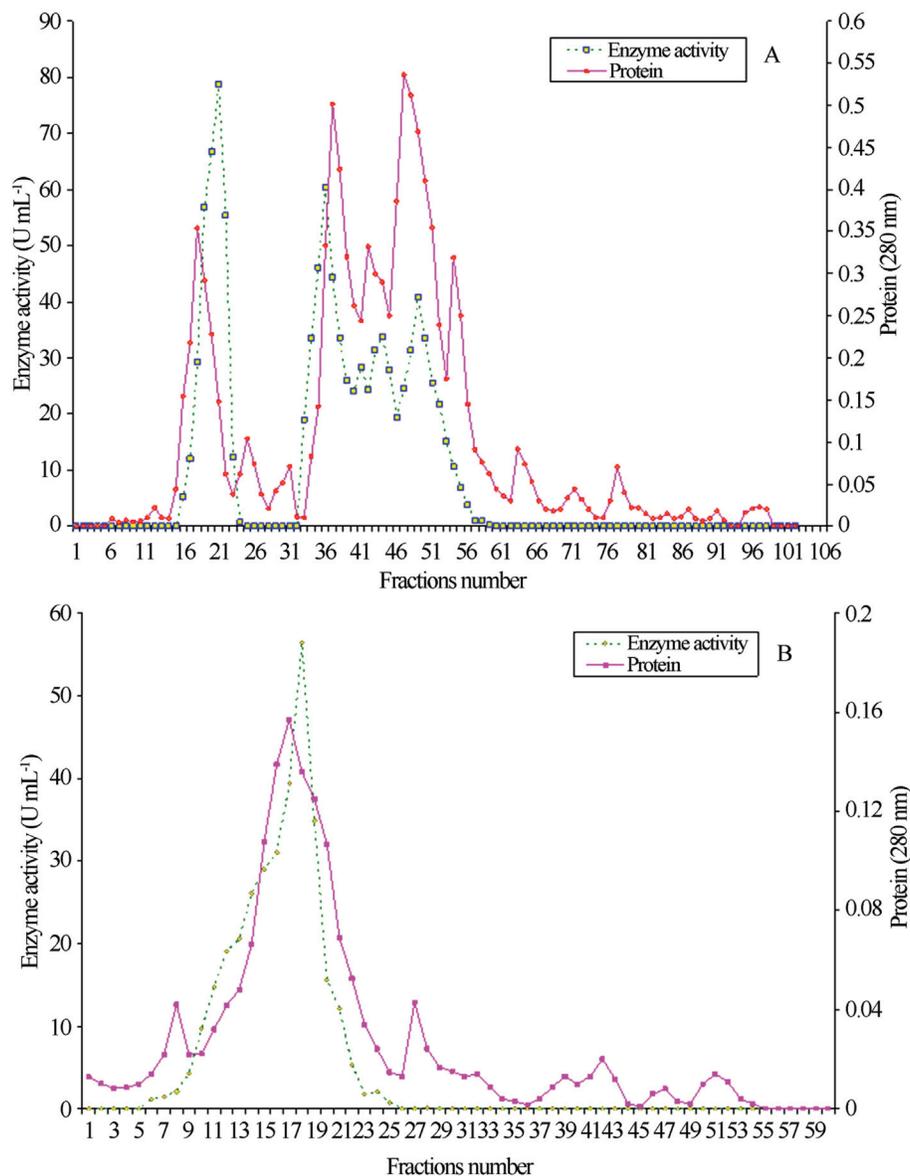


Figure 1 - Typical elution profile for the behavior of *P. ostreatus* MnP on (A) DEAE-Sepharose, (B) Sephadex G-100.

Table 1 - Purification steps of Mn-peroxidase from culture filtrate of edible mushroom *P. ostreatus*.

Purification step	Protein (mg)	Enzyme activity (U)	Specific activity (U mg ⁻¹)	Purification fold	Recovery (%)
Crude filtrate	90.2	56	0.6	1	100
NH ₄ (SO ₄) ₂	78.1	62	0.8%	1.3	86.6
Dialysis	75	65	0.86	1.4	83
DEAE-Sepharose	18	74	4.1	6.8	20
Sephadex G100	1.04	81	78	130	1.2

The apparent molecular mass of the purified MnP enzyme was estimated to be 42 kDa by SDS-PAGE (Figure 2). Similar molecular mass to that of *P. ostreatus* MnP was recorded in *Phlebia radiate* (47-48 kDa), *Ridigo lignosus* (42 kDa) (Hatakka, 1994) and *Phanerochaete Flavido-alba* (45 kDa) (de la Rubia *et al.*, 2002).

The effects of pHs on purified MnP enzyme activity showed a pH optima in the range of 4.0 to 5.0 (Figure 3A). The enzyme activity then attenuated as the pH increased. This optimum pH is lower than the pH optima reported for MnP of *P. chrysosporium* (Kuan *et al.*, 1993) and comparable to MnP obtained from *Lentinula edodes* range (4.0-6.0) (Boer *et al.*, 2006). In this study the optimum temperature was found to be 25 °C (Figure 3B) which is near to value of 28 °C for MnP of *P. chrysosporium* and similar to that of

Fomes durissimus (Singh *et al.*, 2011) whereas it is much lower than that obtained from *Lentinula edodes* (45 °C) (Boer *et al.*, 2006).

The purified enzyme was inhibited by Cd²⁺ and Hg²⁺ whereas it enhanced by Mn²⁺ > K⁺ > Cu²⁺ > Ca²⁺ > Ni²⁺ (Table 2). MnP was also resistant to presence of up to 5 mM H₂O₂, but it was inhibited in the presence of 10 mM H₂O₂. In terms of industrial applications, the stability of MnP in the presence of high concentration of H₂O₂ is very important property. MnP requires H₂O₂ for its activity. However, H₂O₂ could also inactivate MnP quickly when it was applied in high concentrations (Bermek *et al.*, 2002; Wariishi *et al.*, 1988).

The isolated MnP enzyme gene from *P. ostreatus* mycelia has been sequenced. The nucleotide sequence of the

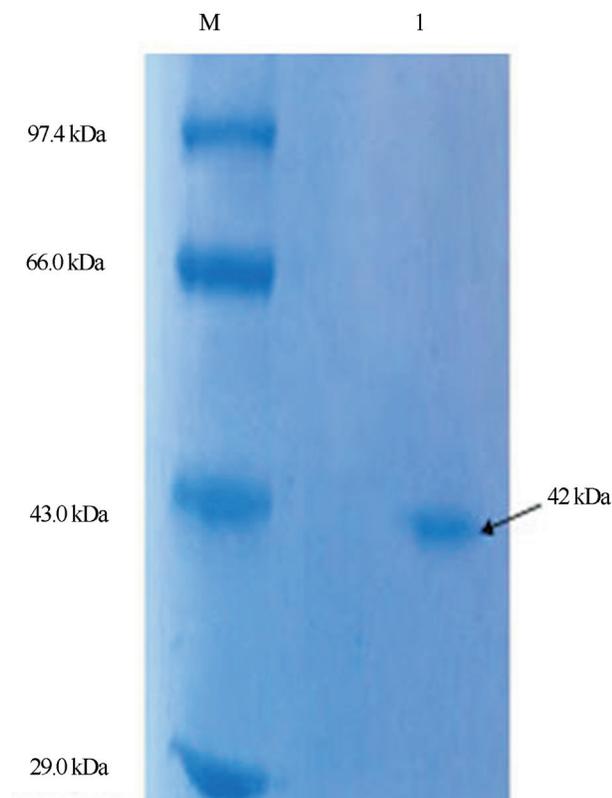


Figure 2 - SDS-PAGE for purified MnP from *Pleurotus ostreatus*; lane (M) Bangalore Genei Protein marker (kDa). Phosphorylase b 97.4. Bovine Serum Albumin 66.0. Ovalbumin 43.0. Carbonic anhydrase 29.0. Soybean Trypsin Inhibitor 20.1 and lane (1) purified enzyme.

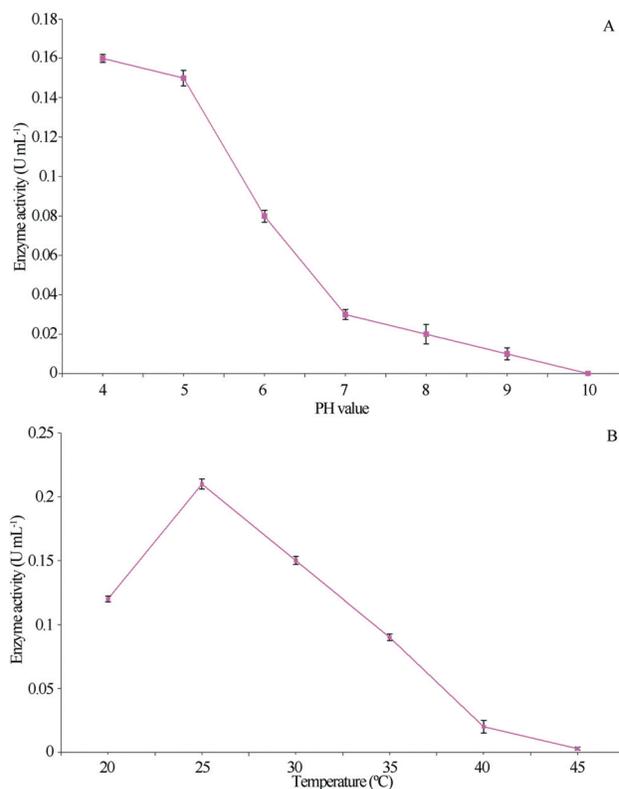


Figure 3 - Effect of some factors on the activity of purified MnP. (A) Effect of pHs, (B) Effect of different temperatures.

Table 2 - Effect of some metals and H₂O₂ on purified MnP activity.

Reagent	Concentration	Activity % of control
Control	-	100
Cd ⁺²	1 mM	25
Ni ⁺²	1 mM	93
Ca ⁺²	1 mM	101
Mn ⁺²	1 mM	130
Hg ⁺²	1 mM	15
Cu ⁺²	1 mM	105
Cu ⁺²	5 mM	75
K ⁺	1 mM	109
H ₂ O ₂	1 mM	82
H ₂ O ₂	5 mM	79
H ₂ O ₂	10 mM	17

gene revealed a single open reading frame (ORF) of 497 bp encoding 165 amino acids (Figure 4). These sequence data has been submitted to the GenBank under accession number AB698450.1. The *MnP*-cDNA gene (AB698450.1) exhibited high sequence homology (100%) with manganese peroxidase isolated from *P. ostreatus* isolate 5145 (GenBank Accession No. JN020144.1), (97%) of (GenBank Accession No. AJ243977.1), (95%) of Mn-peroxidase isolated from *P. pulmonarius* (GenBank Accession No. AY916529.1). The deduced amino acid sequence showed high homology (97%) with *P. ostreatus* isolate 5145 (GenBank Accession No. AER35423.1). This enzyme gene belong to a group of peroxidases containing a heme prosthetic group (ferritytoporphyrin IX), (plant heme-dependent peroxidase superfamily) which catalyzes a multistep oxidative reaction involving hydrogen peroxide as the electron acceptor. The plant peroxidase-like superfamily is found in all three kingdoms of life and carries out a variety of biosynthetic and degradative fractions (Marchler-Bauer *et al.*, 2011).

The detoxification potentiality of MnP to aflatoxin B1 showed that after 24 h the level of AFB1 was reduced to 67% in the presence of 1.5 U mL⁻¹ enzyme while the maximum detoxification (90%) was attained after 48 h at enzyme concentration of 1.5 U mL⁻¹ (Table 3). MnP from *Phanerochaete sordida* Yk-624 removed approximately 70% of AFB1 after 24 h and reach to complete detoxification by multitreatment with MnP (Wang *et al.*, 2011). Culture supernatant of *Myxococcus fulvus* was able to degrade AFB1, AFG1 and AFM1 effectively in solution. Significant reduction of AFB1 (71.89%), AFG1 (68.13%) and AFM1 (63.82%) were observed after 48 h treatment with the culture supernatant from strain ANSM068 (Zhao *et al.*, 2010). These findings are in agreement with those obtained by Alberts *et al.* (2006) who reported that AFB1 was effectively degraded by extracellular extracts from *Rhodococcus erythropolis* liquid cultures, with only 33.2% residual

Table 3 - Detoxification of AFB1 by different concentrations of purified MnP at different incubation periods.

MnP conc. (U mL ⁻¹)	Incubation period (h)													
	0		8		16		24		32		40		48	
	AFB1 (mM)	%DT	AFB1 (mM)	%DT	AFB1 (mM)	%DT	AFB1 (mM)	%DT	AFB1 (mM)	%DT	AFB1 (mM)	%DT	AFB1 (mM)	%DT
0.1	1 ^a	0	0.94 ^a	6	0.88 ^a	12	0.81 ^a	19	0.79 ^a	21	0.65 ^a	35	0.53 ^a	47
0.2	1 ^a	0	0.89 ^b	11	0.76 ^b	24	0.69 ^b	31	0.63 ^b	37	0.52 ^b	48	0.41 ^b	59
0.4	1 ^a	0	0.77 ^c	23	0.63 ^c	37	0.55 ^c	45	0.52 ^c	48	0.41 ^c	59	0.32 ^c	68
0.8	1 ^a	0	0.62 ^d	38	0.49 ^d	51	0.42 ^d	58	0.40 ^d	60	0.33 ^d	67	0.19 ^d	81
1.5	1 ^a	0	0.54 ^e	46	0.42 ^e	58	0.33 ^e	67	0.29 ^e	71	0.22 ^e	78	0.18 ^e	90

Different superscripts are significant difference ($p < 0.05$) according to Duncan test.
%DT (percentage of detoxification) = $(\text{AFB1 (control)} - \text{AFB1 (sample)}) / \text{AFB1 (control)} \times 100$.

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ATGAAATTGTTGAAGCCCAGAAGCCGTTTCATCGCTCGTCACAACATCTGTGCTGCCGACTTTATTCAATTCGCCG
M K L L K P R S R S S L V T T S V L P T L F N S P
GTGCCATTGGCGGATGCCTCCAGACCCACCTGACGGCCTCGTCCCGGAGCCTTTCGATGATGTGACTAAGATT
V P L A D A S Q T P P D G L V P E P F D D V T K I
CTTAGCAGGATGGGTGACGCTGGATTGAGCACTGTGCAAGTCGCTGGCTGCTTTCCTCCACACGATCGCTGCA
L S R M G D A G F S T V E V V W L L S S H T I A A
GCCGATCTAGTTGACCCCTCCATTTCCGGAACTCCCTTCGACTCAACTCCATCTACCTTCGATTCTCAATTCTTC
A D L V D P S I S G T P F D S T P S T F D S Q F F
TTGGAGACAATGTTACAAGGGACTGCCTTCCAGGAACCCAGGTAACCAGGGTGAAGTTGAATCGCCACTTGCG
L E T M L Q G T A F P G T P G N Q G E V E S P L A
GGTGAATGAGACTCCAATCCGACTTCTTGCTTGCCCGAGACTCGAGGAGTGCCTGTGAATGGCAGTCCATGGTC
G E M R L Q S D F L L A R D S R S A C E W Q S M V
AACACATGCCGAAGAACCAGAACAGATTCACTCAAGTCATGAAG
N N M P K N Q N R F T Q V M K

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Figure 4 - Nucleotides sequence analysis of manganese peroxidase gene (*MnP*-cDNA) with accession number (AB698450.1) and deduced amino acid sequence from the mycelium of *P. ostreatus*, the sequence extends, 497 nucleotides in length. The translated amino acids of the gene are shown below the nucleotide sequence.

AFB1 after 72 h. These results also went parallel with those obtained by Lillehoj *et al.* (1971) who reported that AFM1 ($8 \mu\text{g mL}^{-1}$) is completely removed from the liquid medium of *Flavobacterium aurantiacum* by incubation with 5×10^{10} resting cells per milliliter for 4 h.

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