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ORIGINAL PAPER

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Effect of culture conditions on manganese peroxidase production and activity by some white rot fungi

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Abstract The ligninolytic system of white rot fungi is primarily composed of lignin peroxidase, manganese peroxidase (MnP) and laccase. The present work was carried out to determine the best culture conditions for production of MnP and its activity in the relatively little-explored cultures of *Dichomitus squalens*, *Irpex flavus* and *Polyporus sanguineus*, as compared with conditions for *Phanerochaete chrysosporium* and *Coriolus versicolor*. Studies on enzyme production under different nutritional conditions revealed veratryl alcohol, guaiacol, Reax 80 and Polyfon H to be excellent MnP inducers.

Keywords Manganese peroxidase · White rot fungi · Lignin · Veratryl alcohol

Introduction

Manganese peroxidase (MnP), first detected in the culture fluid of *Phanerochaete chrysosporium*, catalyzes the oxidation of Mn(II) to Mn(III), which in turn can oxidize phenolic structures in lignin [12,13]. Such reactions may lead to polymer fragmentation but the role of MnP is not as apparent as that of lignin peroxidase. MnP from *Coriolus versicolor* plays an important role in delignification of kraft pulp, decolorization of bleach plant effluents [23,26] and degradation of several dyes [11,27,30]. Crude MnP preparations from *Nematoloma forwardii* [17] and *Clitocybula dusenii* [38] are capable of degrading humic acids derived from coal. Keeping in mind the increasing interest in MnP, the present work was carried out on MnP production by *Coriolus versicolor*, *Irpex flavus*, *Dichomitus squalens* and *Polyporus sanguineus* and various media variables were tested in an attempt to improve enzyme production.

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Materials and methods

Organisms

Five white rot fungal cultures, *Coriolus versicolor* (MTCC 138), *Phanerochaete chrysosporium* (BKM-F-1767), *I. flavus* (MTCC 168), *D. squalens* (FP-105351-sp) and *Polyporus sanguineus* (MTCC 137) were selected. *C. versicolor*, *I. flavus* and *P. sanguineus* were procured from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The remaining cultures were received from Dr. C.R. Bergman, Forest Products Laboratory, Wisconsin, USA. The cultures were maintained on yeast extract glucose agar (YGA) slants and stored at 4°C. Orzan S was obtained from Crown Zellerbach (Camas, Wash.). Indulin AT, Polyfon H, and Reax 80 were obtained from Westvaco Chemical Division (North Charleston, S.C.).

Production profile of MnP

MnP production was studied in mineral salts broth (MSB) for a period of 20 days. Ten milliliters of MSB containing glucose (10 g), KH_2PO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), sodium acetate (20 mM), thiamine HCl (1 mg), ammonium tartrate (0.2 g), trace element solution (10 ml), in 1 l distilled water, was dispensed in 100-ml Erlenmeyer flasks and inoculated with two mycelial discs (each 7 mm in diameter) obtained from the periphery of 6 to 7-day-old fungal cultures grown on YGA plates. The flasks were incubated at 25 ± 1°C. Every 2 days the contents of triplicate flasks were filtered through Whatman filter paper no. 1, which was then dried at 90°C to constant weight to obtain the biomass; the filtrate was centrifuged at 10,000 *g* for 20 min and the supernatant was treated as the enzyme extract.

MnP production in different basal media and the effect of supplements

MnP production was studied in three basal media – MSB, 0.5% malt extract broth (MEB) and mineral salt malt extract broth (MSB-MEB). Among the different supplements, veratryl alcohol and guaiacol were added to all three basal media at a concentration of 0.4 mM. Lignin preparations that included Indulin AT, Polyfon H, Reax 80 and Orzan S were supplemented to MSB at a concentration of 0.1%. Indulin AT is a kraft pine lignin while the

others are lignosulphonates. The degree of sulphonation of Polyfon H is 0.5, whereas for Reax 80 it is 4.7. Agricultural residues (wheat straw, rice straw and sugarcane bagasse) were added to MSB and MEB at a concentration of 1% as described previously [2].

Optimization of production and activity of MnP

Enzyme production and activity were studied at various pH (2.5–9.5) and temperature (10–50°C) values. Enzyme activity was studied at different concentrations of substrate, H_2O_2, and in the presence of organic acid chelators. Thermostability studies were carried out by pre-incubating the enzyme extract at 50–100°C for 1 h.

MnP assay

MnP was assayed at 25°C according to Orth et al. [25]. The reaction mixture contained 50 mM sodium succinate buffer (pH 4.5), 50 mM sodium lactate (pH 4.5), 0.1 mM $MnSO_4$, 5 mg gelatin, 0.1 mM phenol red, 50 mM H_2O_2 and 0.5 ml enzyme extract. The reaction was initiated by adding H_2O_2. At intervals of 1 min up to 4 min, 1 ml of the reaction mixture was sampled and to it was added 40 µl 5 N NaOH; absorbance was read at 610 nm. One unit of MnP was defined as equivalent to an absorbance increase of 0.1 OD units per minute per milliliter of the culture supernatant.

Results

Production of MnP

D. squalens began enzyme production earlier than the other strains; it was detected on the second day and peaked on day 4; a second peak was observed on day 10 (Fig. 1a). However, *Phanerochaete chrysosporium* was the best producer and gave an activity of 0.6 U on day 6 (Fig. 1c). Enzyme production started later with *I. flavus* and *Polyporus sanguineus*, being detected on days 6–8, and reached an optimal value in 12–18 days (Fig. 1b, d).

Effect of basal media

Enzyme production was studied in different media – MSB, MEB and MSB-MEB – after 8 days of incubation. MEB supported maximum enzyme production by *D. squalens* and *Polyporus sanguineus*. *Phanerochaete chrysosporium* gave better enzyme yields in MSB-MEB, while MEB and MSB-MEB were equally effective with *C. versicolor*. *I. flavus* produced the enzyme only in MSB medium. (Table 1).

Effect of veratryl alcohol and guaiacol

Supplementation of veratryl alcohol and guaiacol in different media gave different results for MnP production by different fungi. Veratryl alcohol enhanced enzyme production by *Phanerochaete chrysosporium* and *C. versicolor* in all media but the response was pronounced only with MEB and MSB. With the other fungi, the inductive response occurred only in MSB-MEB. MnP was produced by *I. flavus* and *Polyporus sanguineus* in MSB-MEB only with supplementation with veratryl alcohol. However, its supplementation to MSB and MEB suppressed enzyme production in *I. flavus*, *D. squalens* and *P. sanguineus* (Table 1).

Supplementation with guaiacol in MSB and MSB-MEB enhanced enzyme production 12-fold with *P. sanguineus* and *C. versicolor*. However, its supplementation suppressed enzyme production in *D. squalens* in MSB and MEB (Table 1) while *I. flavus* gave negligible enzyme activity only in MSB.

Effect of lignin preparations

Adding different lignin preparations to MSB resulted in enhancement or suppression of MnP production to variable levels in different fungi. Reax 80 was the best inducer for *Phanerochaete chrysosporium*, giving a 6-fold increase. It was closely followed by Polyfon H and Orzan S, which increased enzyme production by 2- to 6-fold in *P. chrysosporium* and *C. versicolor*. Indulin AT was not an efficient inducer since it increased enzyme production (3.5-fold) only in *C. versicolor*. All lignin preparations partially or completely repressed MnP production in *D. squalens* and *Polyporus sanguineus* (Table 2).

Effect of agricultural residues

Of the various residues tested, supplementation of wheat straw to MEB and MSB enhanced enzyme production by 2- to 3- fold with *C. versicolor*, while with *D. squalens* and *Polyporus sanguineus*, an enhancement of 2- and 8-fold, respectively was noted only in MSB. *I. flavus* produced the enzyme in MEB medium only upon addition of either sugarcane bagasse or wheat straw (Table 3).

Optimization of production and activity of MnP

MnP production was higher at higher temperatures and the optimal yield was obtained at 30°C with all the fungi. There was no enzyme production at 35–40°C (Fig. 2). Activity of the enzyme was optimally expressed at 25–30°C and was completely lost above 35°C, except with *Polyporus sanguineus*, where no activity was observed above 30°C. The enzyme extract obtained from all the fungi when treated at 60°C for 1 h lost all activity and retained only 8–13% of its activity at 50°C for 1 h. Optimum enzyme production occurred at pH 5.5 with all the fungi (Fig. 3) while activity was best expressed at pH 4.5–5.5. Enzyme production was completely lost at and above pH 7.5.

The optimal H_2O_2 concentration for phenol red oxidation was 75–100 µM. The K_m value for phenol red varied from 0.028 to 0.04 mM for the different fungi.

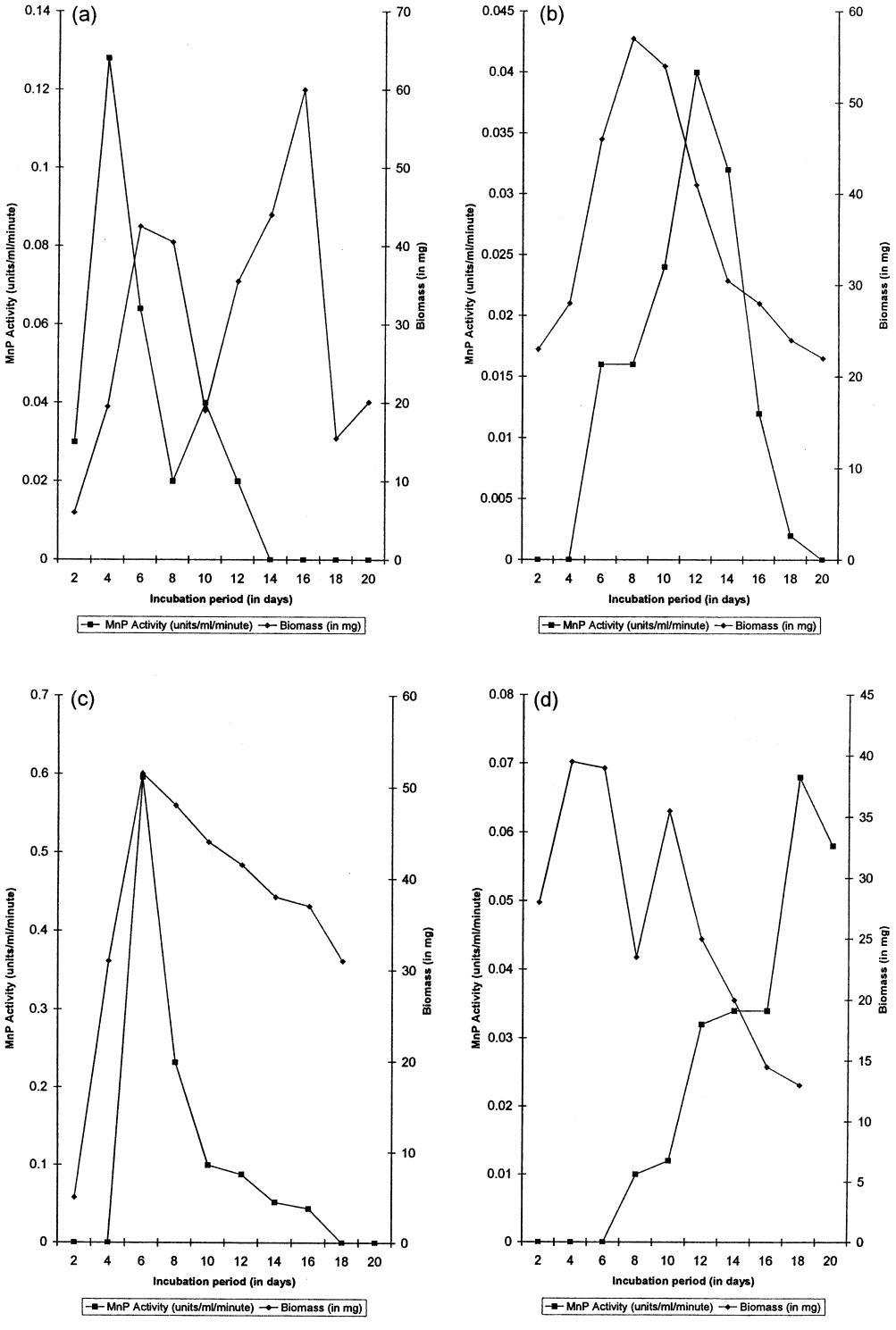

Fig. 1 Effect of incubation period on manganese peroxidase (MnP) production by **a** *Dichomitus squalens*, **b** *Irpex flavus*, **c** *Phanerochaete chrysosporium* and **d** *Polyporus sanguineus* at 25°C in mineral salts broth (MSB) medium. *Squares* MnP activity (U/ml), *diamonds* biomass (mg). Standard deviations were less than 10%

Among the various organic acids tested, tartrate served as the best chelating agent for MnP activity.

Discussion

Most of the earlier studies on MnP have centered around *Phanerochaete chrysosporium*. The present work explored enzyme production of some other white rot fungi and compared it with that of *P. chrysosporium*. The latter was the best enzyme producer, though the rate of enzyme production was faster in *D. squalens* (Fig. 1). On comparing enzyme production by different fungi on various media, *Polyporus sanguineus* gave the highest titers (0.43 U) in MEB medium. No correlation could be established between enzyme production and fungal

Table 1 Effect of medium, veratryl alcohol (*VA*) and guaiacol (*G*) on manganese peroxidase (*MnP*) production at 25°C (8 day incubation). *MSB* Mineral salts broth, *MEB* malt extract broth, *MSB-MEB* mineral salts malt extract broth

Medium	MnP activity (U/ml) ± standard deviation				
	Phanerochaete chrysosporium	*Coriolus versicolor*	*Irpex flavus*	*Dichomitus squalens*	*Polyporus sanguineus*
MSB	0.020 ± 0.002	0.020 ± 0.001	0.015 ± 0.00	0.084 ± 0.008	0.010 ± 0.001
MEB	0.023 ± 0.000	0.045 ± 0.002	0	0.290 ± 0.03	0.430 ± 0.016
MSB-MEB	0.033 ± 0.002	0.045 ± 0.002	0	0.066 ± 0.007	0
MSB + VA	0.032 ± 0.00	0.080 ± 0.004	0	0.070 ± 0.004	0
MSB + G	0.026 ± 0.001	0.040 ± 0.002	0	0.034 ± 0.000	0.120 ± 0.012
MEB + VA	0.059 ± 0.002	0.070 ± 0.005	0	0.250 ± 0.01	0
MEB + G	0.038 ± 0.004	0.020 ± 0.001	0	0.050 ± 0.003	0.070 ± 0.007
MSB-MEB + VA	0.040 ± 0.00	0.050 ± 0.003	0.037 ± 0.003	0.080 ± 0.006	0.140 ± 0.012
MSB-MEB + G	0.033 ± 0.002	0.530 ± 0.02	0	0.030 ± 0.002	0

Table 2 Effect of lignin preparations on MnP production at 25°C (8 day incubation)

Medium	MnP activity (units/ml) ± standard deviation				
	Phanerochaete chrysosporium	*C. versicolor*	*I. flavus*	*D. squalens*	*Polyporus sanguineus*
MSB	0.020 ± 0.002	0.020 ± 0.001	0.015 ± 0.00	0.084 ± 0.008	0.010 ± 0.001
MSB + Indulin AT	0.026 ± 0.002	0.070 ± 0.004	0	0.080 ± 0.004	0
MSB + Polyfon H	0.110 ± 0.008	0.060 ± 0.004	0.080 ± 0.006	0.020 ± 0.001	0.010 ± 0.001
MSB + Reax 80	0.130 ± 0.007	0	0.070 ± 0.003	0.010 ± 0.001	0
MSB + Orzan S	0.100 ± 0.005	0.050 ± 0.002	0	0	0

Table 3 Effect of agricultural residues on MnP production at 25°C (8 day incubation). *WS* Wheat straw, *RS* rice straw, *SB* sugar bagasse

Media	MnP activity (units/ ml) ± standard deviation				
	Phanerochaete chrysosporium	*C. versicolor*	*I. flavus*	*D. squalens*	*Polyporus sanguineus*
MSB	0.020 ± 0.002	0.020 ± 0.001	0.015 ± 0.00	0.084 ± 0.008	0.010 ± 0.001
MSB + WS	0	0.065 ± 0.006	0	0.140 ± 0.012	0.080
MSB + RS	0	0	0.340 ± 0.03	0.112 ± 0.008	0
MSB + SB	0	0.095 ± 0.007	0	0.092 ± 0.007	0
MEB	0.023 ± 0.00	0.045 ± 0.002	0	0.290 ± 0.03	0.430 ± 0.016
MEB + WS	0	0.075 ± 0.004	0.020 ± 0.001	0.200 ± 0.01	0
MEB + RS	0	0.160 ± 0.01	0	0.082 ± 0.008	0
MEB + SB	0	0	0.026 ± 0.02	0.080 ± 0.005	0

growth except for *Phanerochaete chrysosporium*, where the optimal period for enzyme production almost coincided with that of maximum biomass production.

Best enzyme production in MEB can be attributed to the fact that it provides the complete pool of amino acids required for enzyme synthesis [1,9]. Moreover, malt extract is rich in the aromatic amino acids tryptophan and tyrosine. Tryptophan is a precursor for the synthesis of a large number of *N*-substituted aromatic secondary metabolites of fungi [33], many of which are substrates for MnP production [34]. These may then act as inducers for MnP in a way similar to veratryl alcohol and guaiacol, which are substrates as well as inducers for ligninase and laccase. This observation gains further support from earlier studies where the addition of tryptophan to cultures of some white rot fungi increases the production of lignin peroxidase [8]. In contrast to ligninase, which in general is best produced under nitrogen starvation conditions [2], better production of MnP in nitrogen-rich media was reported in peptone and albumin media for *Bjerkandera* spp. Strain BOS55, and in soybean medium for *Coprinus friesii* [16,22].

The inductive effect of lignin preparations on MnP production is in line with earlier studies on laccase and ligninase, though no such previous reports are available for their effect on MnP [1,2,4,5,10,15,28,29]. Some degradation products of lignin might impart a stimulatory effect on MnP production. The variability observed with different lignins may be attributed to their variable degree of sulphonation. Few studies have reported the effect of veratryl alcohol on MnP production [21,24], though such reports are available for lignin peroxidase [10,19]. Higher levels of induction were observed in the present study in comparison to those reported earlier

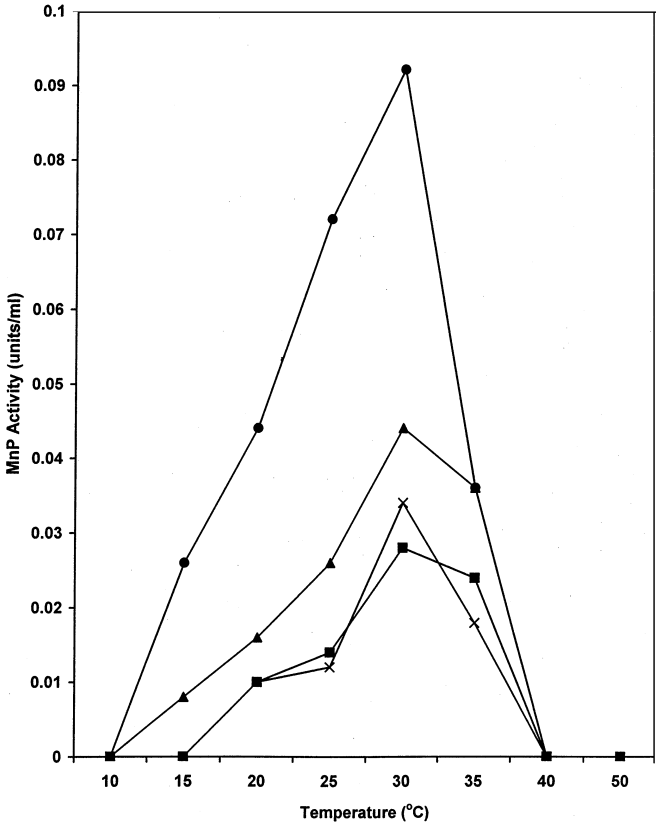

Fig. 2 Effect of temperature on MnP production by *D. squalens* (*circles*), *I. flavus* (*squares*), *Phanerochaete chrysosporium* (*triangles*) and *Polyporus sanguineus* (*crosses*) in MSB medium (8 day incubation)

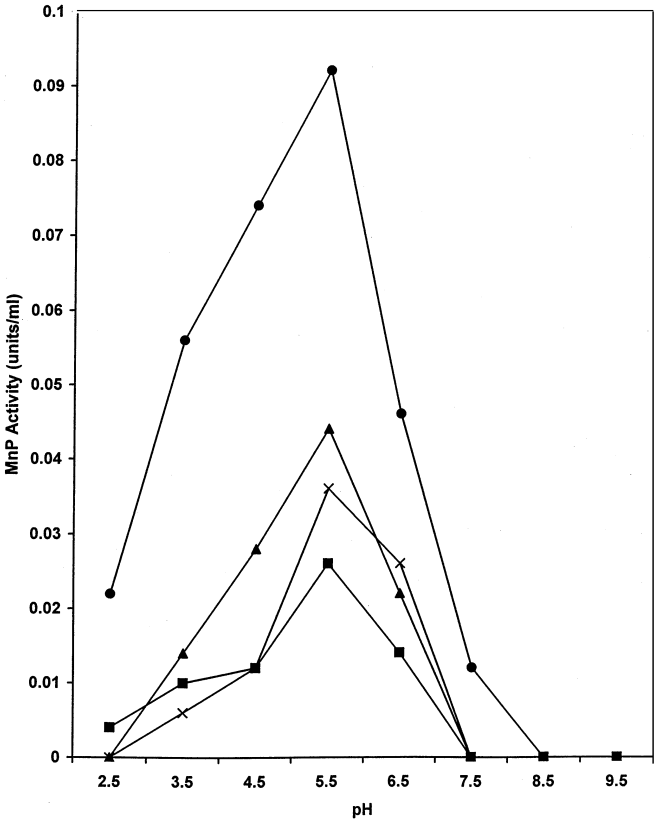

Fig. 3 Effect of pH on MnP production by *D. squalens* (*circles*), *I. flavus* (*squares*), *Phanerochaete chrysosporium* (*triangles*) and *Polyporus sanguineus* (*crosses*) in MSB medium (8 day incubation)

[24]. Veratryl alcohol differed in its inductive or suppressive effect on different fungi as a function of the basal media employed (Table 1). No such media-dependent effect of veratryl alcohol was reported by earlier workers. Further, in most previous studies, veratryl alcohol was added on day 3–4 when fungal growth was already initiated; in the present work, it was added at the time of inoculation. Thus, it might have interfered with fungal metabolism, accounting for the observed repression in some cases. Veratryl alcohol is also produced de novo by some white rot fungi and its exogenous addition may further raise the concentration to levels toxic for enzyme production. Similar media-dependent inductive and repressive effects of guaiacol have been observed on MnP production. However, its inductive effect was reported earlier on laccase and lignin peroxidase [1,2,3].

Increases in enzyme yield with *Coriolus versicolor* and *I. flavus* in the presence of agricultural residues are in consonance with earlier studies by Schlosser et al. [29] wherein wheat straw and beechwood induced MnP production in *T. versicolor*, though the enzyme was not produced in glucose-based basal media. *Pleurotus* spp. produce high levels of MnP on wheat straw [7]. MnP is the major protein produced during growth of some white rot fungi on wheat straw [14,17,35]. An inductive effect of these residues was also reported for laccase [1,2,27].

Low temperature optima reported for enzyme production and activity for these white rot fungi support their ecological adaptation in nature. The heat sensitive nature of MnP is in consonance with the results of earlier studies [16]. As reported for *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus*, the pH optima lie between 4.5 and 5.5 [6,12,31], favoring the release of extracellular enzymes as suggested by Lindeberg and Fahraeus [21]. Inhibition of enzyme activity upon increasing the H_2O_2 concentration can be attributed to the fact that hydroxyl radical and superoxide anion radical, which can be formed from H_2O_2, cause protein degradation [36]. Excess H_2O_2 has also been shown to inactivate other enzymes, viz. ligninase, horseradish peroxidase and cholesterol oxidase [18,32,37], though the exact mechanism is not known.

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