Enhanced Production of Manganese Peroxidase by *Phanerochaete chrysosporium*

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ABSTRACT

Production of manganese-dependent peroxidase (MnP) by the white-rot fungus Phanerochaete chrysosporium *BKM-F-1767 (ATCC 24725) was monitored during growth in different media and growth conditions. The effect of some activators of MnP production, Mn^{2+}, Tween 80, phenylmethylsulphonylfloride (PMSF), oxygen, temperature, pH, glycerol and nitrogen was studied. Supplementing the cultures with Tween 80 (0.05 %, v/v) and Mn^{2+} (174 μM) resulted a maximum MnP activity of 356 U/L which was approximately two times higher than that obtained in the control culture (without Tween 80). Decolourisation of Direct Blue 15 and Direct Green 6 (50 mg/L) was also achieved with MnP.*

Keywords: *Phanerochaete chrysosporium*; manganese dependent peroxidase; Tween 80; Mn^{2+}; phenylmethylsulphonylfloride

INTRODUCTION

White-rot fungi are primarily responsible for the initial decomposition of lignin in wood, which occurs via an oxidative and relatively nonspecific process (Hatakka, 1994; Hammel et al., 2002). *Phanerochaete chrysosporium*, a white-rot-wood decaying basidiomycete, produces a potent lignin degrading system that oxidizes lignin completely to CO_2 (Breen and Singleton, 1999). The major components of the lignin degrading system include lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), laccase and hydrogen peroxide producing enzymes (Silva et al.,, 2005). Based on enzyme production pattern, white-rot fungi have been classified in different categories (Tuor et al., 1995).
Manganese and nitrogen have strong regulating effects on the ligninolytic machinery (Hamman et al., 1999). In white-rot fungus, *P. chrysosporium*, ligninolytic enzymes are synthesized in response to nitrogen, carbon or sulfur limitation (Hamman et al., 1997). The mode of cultivation also influences production of the enzymes by basidiomycetes (Niku-Paavola et al.,1990).
MnP (EC. 1.11.1.13) is a ligninolytic enzyme produced by most white-rot fungi (Hatakka, 1994). The catalytic cycle is similar to that of other peroxidases. The principal role of Mn^{2+}-dependent peroxidase is related to oxidation of Mn^{2+} to Mn^{3+}, which then binds to an appropriate ligand, diffuses from the enzyme, and, in turn oxidizes phenolic substrates (Warrishi et al., 1989; Glenn et al., 1986). Among ligninolytic enzymes secreted by white rot fungi, MnP has been reported as the main enzyme involved in lignin depolymerization, effluent decolourization and biobleaching of pulp (Sahoo and Gupta, 2005; Hofrichter, 2002).

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In order to improve both production and stability of MnP secreted by *P. chrysosporium* culture conditions have been modified by adding different substances to the culture media. Among these additives, Mn^{2+}, Tween 80, PMSF, oxygen, glycerol, nitrogen and temperature, pH as physical conditions were selected, to study their influence on the MnP secreted by cultures of *P. chrysosporium*. The analysis of the obtained data in flask cultures will give insight into the main production variables, which would directly affect enzymatic regulation, fungal growth and properties of the growth medium.

MATERIALS AND METHODS

Microorganism and growth medium
P. chrysosporium BKM-F-1767 (ATCC 24725) was maintained at 37°C on 2% malt agar slants and plates. Spores were harvested, filtered through glass wool, and kept at -20°C before use (Jäger et al., 1985). The growth medium was prepared according to Tien and Kirk (1988) with 10 g glucose per litre as carbon source, except that dimethylsuccinate was replaced by 20 mM acetate buffer (pH 4.5) (Tien and Kirk, 1988; Dosoretz et al., 1990a).

Culture conditions of *P. chrysosporium*
Spores (6 days old) were harvested in sterile water, filtered through glass-wool and diluted to 0.5 at OD_{650}. 1 ml of this spore suspension was added to growth medium (10 ml) in 100 ml flasks. Incubation was carried out statically at $37^{o}C$. Enzyme production was carried out growth medium containing selected inducers.
The production medium composition was identical with the growth medium. Some flasks were supplemented with Mn^{2+} (100-354 μM), oxygen (1.2 MPa), Tween 80 (0.03-0.12 %, v/v), others with phenylmethylsulphonylfloride (PMSF, to a final concentration of 0.1 mM) and the rest with nitrogen (11-44 mM) and glycerol (0.665-10 g/L). These were added the beginning incubation whereas PMSF was introduced after 3 and 5 days of incubation in order to inhibit protease activity. Additionally, in the experiment with oxygen, the headspace was aseptically flushed with O_2 gas for 3 min at the time of inoculation and afterwards once a day.

The Erlenmeyer flasks were loosely capped with cellulose stoppers, which permitted a passive aeration, and incubated statically at 37°C and 90% humidity.

Enzymatic decolourization
The reaction mixture contained enzyme and dye (50 mg/L; Direct Blue 15 and Direct Green 6) in a total volume of 1 ml. The decolourization of dyes was determined as the relative decrease of absorbance at their maximum wavelengths by using Na-acetate buffer, pH 4.5 at 30°C.

Analytical methods
Mn (II)-dependent peroxidase activity was assayed spectrophotometrically by the method of Kuwahara et al. (1984). One unit was defined as the amount of enzyme that oxidised 1 μmol of dimethoxyphenol per minute and the activities at $30 ^{o}C$ were expressed in U/L.
Protease activity was measured with azocoll as substrate (Dosoretz et al.,1990a). One unit was defined as the amount of enzyme, which releases azodye causing an absorbance change of 0.001 per min, measured at 520 nm.
Mycelial dry weight was measured daily by filtering triplicate cultures and drying for 24 h at $105^{o}C$.
All results were the mean of the least three replicates.

RESULTS AND DISCUSSION

Since, media composition and growth conditions are important factors affecting the production of extracellular ligninolytic enzymes and, consequently lignin degradation (Niku-Paavola et al., 1990), in the present work, different supplementations were added to the culture medium in order to study the influence of such substances on the Mn (II)-dependent peroxidase production of this fungus.

Effect of Tween 80 on MnP activity
Several authors have shown an improvement in enzyme excretion in the presence of certain surfactants such as Tween 80 in cultures of *P. chrysosporium* (Jäger et al., 1985; Asther et al., 1987; Lestan et al., 1990 and 1993). Apparently, Tween 80 transformed the cell membrane structure and promoted the permeation of MnP from the cell

into the medium. The highest MnP activity was obtained with 0.05 % Tween 80 (v/v) (Fig.1).
When the culture was supplemented with 0.05 % Tween 80, MnP values were approximately 2-fold higher than those obtained in the control culture (180.2 U/L) (without Tween 80). The mechanism by which surfactants (i.e., Tween 80) enhance

extracellular enzyme production in filamentous fungi has not been elucidated. The mechanism possibly consists of modification of the cytoplasmic membrane (Jäger et al*.,* 1985) or in a change in lipid metabolism of the fungus (Asther et al.,1988a-b).
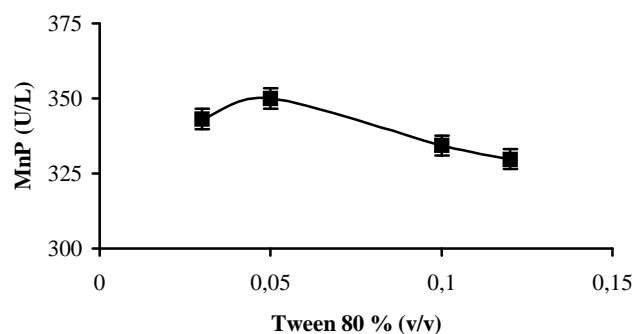

Figure 1 - MnP activity variation depends on Tween 80 concentration (%, v/v) in 6th day cultures of *P. chrysosporium* at pH 4.5 and 37°C
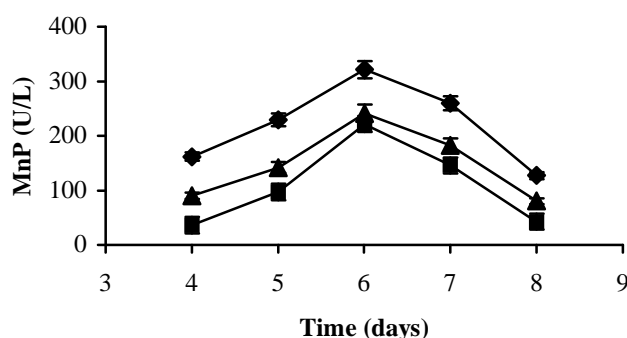

Figure 2 - MnP activity variation depends on concentrations Mn^{2+} (-▲-), 100 μM; (-♦-), 174 μM; (-■-), 354 μM with respect to incubation time at pH 4.5 and 37°C

Effect of Mn^{2+} on enzyme activity
An accumulation of manganese, deposited as MnO_2, can be observed in areas of intense lignin degradation by several white-rot fungi (Blanchette, 1984). The effect of adding various concentrations of Mn^{2+} on *P. chrysosporium* is shown in Fig. 2.
MnP activity increased steadily with increasing Mn^{2+}concentration. Mn^{2+} is implicated in MnP production and has a triple role: (i) an essential cofactor for functioning of the MnP protein (Glenn et al*.,* 1986); (ii) activates transcription of the *mnp* gene (Brown *et.al.,* 1990) and (iii) inhibits the

production of veratryl alcohol, which is highly correlated with LiP titers (Mester et al.,1995). A regulatory role in biosynthesis of MnP is ascribed to manganese ions (Perez and Jeffriez, 1992). In the present study, the highest enzyme activity of 321.1 U/L was obtained at 174 μM Mn^{2+}. The presence of high Mn^{2+} concentration (174 μM Mn^{2+}) in growth medium also elevated the production of cell mass. There might be interactions between high Mn^{2+} concentration and the nutrients that accounted for the differences in dry weight. Higher concentrations of Mn^{2+} than

174 μM did not produce a further increase in MnP level.

Effect of PMSF on MnP activity

PMSF, inhibitor of serine protease was applied to a final concentration of 0.1 mM after 3 and 5 days of incubation and the effect on the MnP activity was measured. The loss of ligninolytic activity and its decay have been investigated in *P. chrysosporium*, in relation to protease production (Bonnarme and Asther, 1993). The protease activity is generally induced by starvation in nitrogen limited medium and is characteristic for *P. chrysosporium* metabolism. Therefore, the protease takes part in the regulation of MnP activity in the culture. PMSF has been reported to be non-inhibitory to ligninase activity in crude extracellular fluid (Tien and Kirk, 1984). The incubation of PMSF with *P. chrysosporium* did not increase MnP activity. Therefore, protease activity in 6th day culture of *P. chrysosporium* was determined as low as 0.6 U/L.

Effect of temperature and pH on MnP activity

In liquid cultures, it was demonstrated that incubation temperature influenced differently the activity pattern of ligninolytic enzymes over time. The change in temperature may affect fatty acid synthesis and membrane fluidity (Vyas et al., 1994). The action of temperature on secondary metabolism occurs at some point in the transitional period between the cessation of vegetative growth and the formation of secondary end products (Asther et al.,1988a). MnP production in medium containing 0.05 % Tween 80 and 174 μM Mn^{2+} was investigated at different temperatures (20-45°C) and at pH 4.5 or at different pH values (3-5.5) at 37°C (Figs. 3, 4).
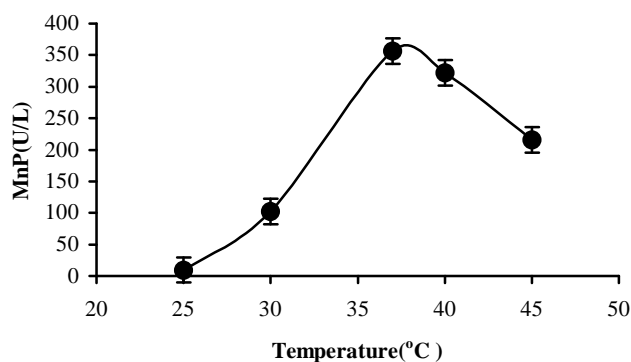
Figure 3 - Effect of varying temperature on the MnP activity of *P. chrysosporium* at pH 4.5
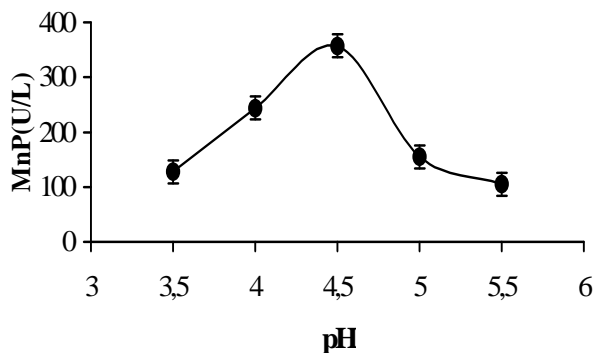
Figure 4 - Effect of varying pH on the MnP activity of *P. chrysosporium* at 37°C

MnP production was dependent on the cultivation temperature and no activity was detected at 25°C. Increasing the temperature to 30°C resulted in the production of small amounts of MnP. A decrease of enzyme production was observed at 40-45°C. The optimum temperature of 37°C reported for enzyme production for the fungus support its ecological adaptation in nature (Heinzkill et al., 1998). The MnP production rate and the maximum MnP activity varied with temperature and pH. For MnP production by *P. chrysosporium* the optimal value of medium pH was 4.5. The maximum MnP activity was obtained at 37°C and pH 4.5 after 6 days incubation.

Effect of oxygenation conditions
Since O$_2$ is necessary to support the ligninolytic system, two methods of oxygen supply (headspace and 1.2 MPa) were tested. Oxygenation at 1.2 MPa did not increase enzyme activity, because the polysaccharide production might increase (Dosoretz et al., 1990a-b). An effect was found with the oxygenation supply, which presented no regulatory effect directly on enzyme synthesis. The growth-limiting factor could not be attributed to oxygen because increasing it's concentration in the media had no effect on the growth and

production of enzyme. Similar result was observed in *Bjerkandera sp.* strain BOS55 (Mester et al.,1996). Mechanically agitated cultures are known to have an inhibitory effect on ligninolytic enzyme production due to the shear stress for mycelia (Kirk et al.,1978). Therefore, the static cell culture is desirable for the production of MnP because it increases the contacting area between cells and oxygen without shear stress.

Effect of nitrogen concentration
Ligninolytic enzymes are produced by carbon, nitrogen or sulfur limitation (Faison and Kirk, 1985). It was demonstrated that the regulatory role of nitrogen concentration affected lignin degradation and the biosynthesis of MnP. To test the effect of nitrogen source levels on *P. chrysosporium* (NH$_4$)$_2$SO$_4$ (11-44 mM) was added to growth medium (Fig. 5). Results showed that increased nitrogen concentration decreased enzyme activity. The appearance of MnP activity with high nitrogen conditions could be due to nutrient limitation into the mycelial mat (Mester et al., 1996). The effect of nitrogen concentration on MnP activity could be related with the C/N ratio. The highest enzyme activity was obtained at 22 mM concentration of nitrogen.
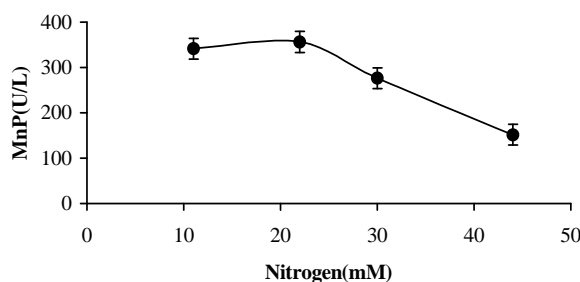

Figure 5 - MnP activities obtained during the 6th day in cultures of *P. chrysosporium* with different nitrogen concentrations at pH 4.5 and 37°C

Effect of glycerol concentration
It is assumed that the metabolized substrate is essential for fungi not only for the synthesis of lignin degrading enzymes, but also for the production of peroxide and synthesis of effectors of the ligninolytic system (Faison and Kirk, 1985). Therefore, experiments were conducted to determine the effect of glycerol concentration on MnP production. The addition of various glycerol

concentrations (0.665-10 g/L) did not increase the MnP activity than obtained with glucose (Table 1). Thus, glycerol was a poorer carbon source compared to glucose (Kern, 1990). Growth on glycerol leads to carbon limitation which affects the onset of secondary metabolism (Buswell et al., 1984). Therefore, glucose (10 g/L) was selected as carbon source.

Table 1 - MnP activities obtained in 6th day cultures of *P. chrysosporium* with different glycerol concentrations at pH 4.5 and 37°C.

Glycerol concentration (g/L)	MnP activity (U/L)
0.665	80.1
1.00	102.9
6.65	226.3
10.0	45.5

CONCLUSION

Highest MnP activity was achieved as 356 U/L at optimized conditions: 174 μM Mn^{2+}, 0.05 % Tween 80, 22 mM nitrogen, 10 g/L glucose, 37°C

and pH 4.5 (Fig.6). Under these conditions, growth of the microorganism was achieved with a maximum value of 3.1 g/L biomass.
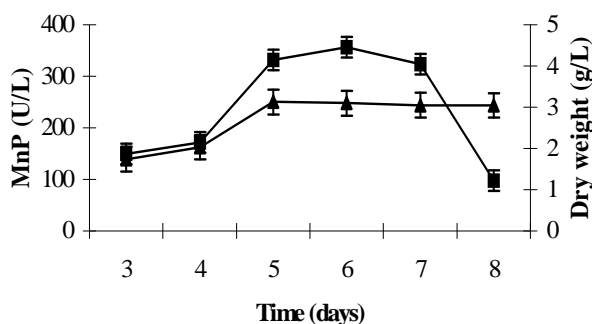

Figure 6 - MnP production in 6th day cultures of *P. chrysosporium* at optimized conditions, pH 4.5 and 37°C: (-■-) MnP activity; (-▲-) dry weight

Decolourisation studies by enzyme

Synthetic dyes such as Direct Blue 15 (50 mg/L; λ=610 nm) and Direct Green 6 (50 mg/L; λ= 620 nm) were treated with MnP. The decolourization of these dyes was performed for Direct Blue 15 as 12 % and Direct Green 6 as 5.4 % after 5 min of treatment. This was an extremely short period to achieve azo dye degradation. According to the results, MnP produced by *P.chrysosporium* was able to play a role for the decolourization of these direct azo dyes (Kasikara Pazarlioglu et al.*,* 2005; Harazono et al.*,* 2003).

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