A Practical Culture Technique for Enhanced Production of Manganese Peroxidase by *Anthracophyllum discolor* Sp4

Francisca Acevedo[1,4]**, Leticia Pizzul**[2]**, María del Pilar Castillo**[3]**, Olga Rubilar**[4]**, María Elena Lienqueo**[5]**, Gonzalo Tortella**[4] **and María Cristina Diez**[4*]

[1]*Universidad de La Frontera; P. O. Box 54-D; Temuco - Chile.* [2]*Uppsala BioCenter; Department of Microbiology; Swedish University of Agricultural Sciences; P. O. Box 7025; SE-750 07; Uppsala - Sweden.* [3]*JTI - Swedish Institute of Agricultural and Environmental Engineering; P. O. Box 7033; SE-750 07; Uppsala - Sweden.* [4]*Scientific and Technological Bioresource Nucleus; Department of Chemical Engineering; Universidad de La Frontera; PO Box 54-D; Temuco - Chile.* [5]*Department of Chemical and Biotechnology Engineering; Faculty of Physical and Mathematical Sciences; University of Chile; Beauchef, 850; Santiago - Chile*

ABSTRACT

In this study, different growth conditions of Anthracophyllum discolor *Sp4 including the effect of agitation, additions of lignocellulosic support, inducer and surfactant were evaluated on the MnP production in Kirk medium using a culture system made up of the tubes containing the glass bead . The highest MnP production (1,354 U/L on day 13) was obtained when the medium was supplemented with wheat grain and 0.25 mM MnSO₄ as inducer, under static conditions at 30°C. Two isoenzymes were purified (35 and 38 kDa respectively). MnP presented a maximal activity in the pH range between 4.5 and 5.5, a relatively high temperature tolerance (50ºC) and a high catalytic activity for 2,6-dimethoxyphenol and hydrogen peroxide.*

Key words: white-rot fungi; ligninolytic enzymes, purification, lignocellulosic material

INTRODUCTION

Global population growth, urbanization and industrialization have caused environmental contamination by the organic compounds. Microorganisms play an important role in removing, transforming, or mineralizing the xenobiotic compounds to less toxic form (Whiteley and Lee, 2006). White-rot fungi, which are responsible for the biodegradation of lignin present in the wood, offer valuable ecological and metabolic characteristics that make them useful in the bioremediation processes (Pointing, 2001). These microorganisms have a remarkable ability to transform and even mineralize a large spectrum of organic compounds (Rabinovich et al, 2003). This is due to their characteristic enzymatic system, which is comprised of non-specific extracellular oxidative enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (L) produced during the secondary metabolism (Pointing, 2001).

Optimal culture conditions are required for high enzyme yields because the efficient pollutant degradation is mainly correlated with the high enzymatic activities. Furthermore, practical and efficient culture systems for the fungal biodegradation tests are required. An efficient

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*Author for correspondence: mcdiez@ufro.cl

culture system depends on many factors that can contribute to high enzymatic yields in white-rot fungi. Fungal MnP production can be enhanced by the substrate/support, e.g., lignocellulosic materials such as poplar, or fir sawdust (Giardina et al, 2000), grape seeds, barley bran and wood shavings (Moredo et al, 2003). The enhancement of MnP production and stimulation of enzymatic activity by the white-rot fungi have been observed following the addition of inducers such as $MnSO_4$ (Gill and Arora, 2003) and cosubstrates such as Tween 80 (Watanabe et al, 2000; Garon et al, 2002). The expression of MnP in the fungal cultures is dependent on Mn, which regulates the *mnp* gene transcription (Ma et al, 2004). Tween 80 can transform the cell membrane structure and thereby promote the permeation of MnP from the cell into the medium and the solubility of the contaminants (Zheng and Obbard, 2002).

Ligninolytic enzymes have been purified, characterized and used as biocatalysts in the biodegradation of pollutants. Over the past few years, a great deal of attention has been paid to the potential use of MnP in biobleaching of pulp and paper and for xenobiotic compounds degradation because of the characteristics of its catalytic cycle (Eibes et al, 2002; Wariishi et al, 1992).

Anthracophyllum discolor Sp4 is a Chilean white-rot fungus isolated from the decayed wood in the rain forest of southern Chile (Tortella et al, 2008). This fungus has a potential for degradation of organic pollutants because of its high production of ligninolytic enzymes, especially MnP (Rabinovich et al, 2003; Rubilar et al, 2007), and can thus be used in bioremediation systems.

It is necessary to test the ability of *A. discolor* Sp4 to degrade various pollutants before designing the bioremediation systems. Biodegradation tests require the analyses of large numbers of samples over the time, which can be difficult to manage in terms of space, rational use of the chemicals, extraction efficiency, etc., especially using the Erlenmeyer flasks as culture system. In addition, the biodegradation tests normally require destructive sampling (complete destruction of sample for the analysis), particularly when the target compound is hydrophobic, or is applied at the concentrations where it is insoluble in the growth medium. A practical and simple culture technique using the tubes containing the glass beads has been used for the study of polycyclic aromatic hydrocarbon (PAHs) degradation by the

actinobacteria (Pizzul et al, 2006) and could be of interest in the fungal cultures.

The purpose of this study was to study the effect of Mn^{2+}, Tween80 and lignocellulosic substrate on MnP production by *A. discolor* Sp4 and to evaluate a simple and practical culture system for MnP production using the tubes containing the glass beads as replacement of Erlenmeyer flasks.

MATERIALS AND METHODS

Chemicals

2,6-DMP (2,6-dimethoxyphenol) (> 99.0%) was supplied by the Aldrich Chemical Co., Germany. 3,4-dimethoxy-benzyl alcohol (> 95.0%) was supplied by the Fluka. All other chemicals were supplied by the Merck, Oxoid, Sigma and Duchefa (Netherlands).

Microorganism

The fungal strain used was *Anthracophyllum discolor* Sp4 isolated from decayed wood in the rain forest of southern Chile (culture collection of the Environmental Biotechnology Laboratory at the Universidad de La Frontera, Chile). The fungus was stored at 4ºC in glucose malt extract agar (G-MEA) slants containing (g/L) malt extract 30, agar 15 and glucose 10. The fungus was transferred from the slant culture tubes to Petri dishes with G-MEA medium and incubated at 30ºC for 5 days.

MnP production by A. discolor Sp4 in liquid medium supplemented with wheat grain

Five malt agar plugs (6 mm diameter) of active mycelia of *A. discolor* Sp4 from 5-day-old cultures on G-MEA medium were inoculated in 100 mL of Kirk medium (Tien and Kirk, 1988) in an 1-L Erlenmeyer flask. The Kirk medium contained the following (g/L): glucose 10 , peptone 2 , KH_2PO_4 2 , $MgSO_4$ 0.5 , $CaCl_2$ 0.1 and, thiamine 2 mg/L and mineral salts 10 mL/L (which contained in g/L KH_2PO_4 2 , $MgSO_4 \cdot 7H_2O$ 0.5 , $CaCl_2 \cdot 2H_2O$ 0.1 , and in mg/L, $MnSO_4 \cdot 5H_2O$ 50 , NaCl 10 , $FeSO_4 \cdot 7H_2O$ 1.0 , $CoCl_2 \cdot 6H_2O$ 1.0 , $ZnSO_4 \cdot 7H_2O$ 1.0 , $CuSO_4 \cdot 5H_2O$ 0.1 , $AlK(SO_4)_2$ 0.1 , H_3BO_3 0.1 , $NaMoO_4 \cdot 2H_2O$ 0.1). Sterile wheat grains (32 g) were added to the medium and the fungus was grown without agitation. Cultures containing *A. discolor* Sp4 grown in Kirk medium without wheat grain under then static conditions, or on a shaker at

100 rpm were used as the controls. The cultures were incubated at 30ºC for 28 days. Samples were taken periodically and MnP activity, proteolytic activity, total protein concentration and pH were ascertained.

Effect of MnSO$_4$ and/or Tween 80 on MnP production by A. discolor Sp4 grown in liquid medium supplemented with wheat grain

In order to evaluate the effect of MnSO$_4$ for MnP production, Kirk medium (100 mL) containing 0.002 mM MnSO$_4$ as trace element and supplemented with 32 g of sterile wheat grain was modified by adding various concentrations of monohydrated manganese sulfate (MnSO$_4$·H$_2$O) in order to obtain its 0.002, 0.25, 0.5, 1.0 and 2.0 mM concentration. The medium was inoculated as described above and the flasks were incubated at 30ºC under static conditions for 28 days. The MnP activity was determined periodically. In order to evaluate the effect of Tween 80 on the MnP production, 100 mL of Kirk medium supplemented with 32 g of sterile wheat grain and 0.25 mM of MnSO$_4$·H$_2$O, was modified by adding Tween 80 (0.05% v/v) and inoculated as described above. The flasks were incubated at 30ºC under static conditions for 28 days. A control flask without Tween 80 was run in parallel. The MnP activity was determined periodically.

Cultivation suitability of A. discolor Sp4 for MnP production in the tubes containing glass beads

A. discolor Sp4 was cultivated in three culture media using two culture techniques, 100-mL Erlenmeyer flasks and 50-mL tubes containing 20 g of sterile glass beads (5 mm diameter) in Kirk medium. The Erlenmeyer flasks and tubes contained 20 and 10 mL, respectively of the following media: a) modified Kirk medium containing 0.25 mM MnSO$_4$·H$_2$O, b) modified Kirk medium containing 0.25 mM MnSO$_4$·H$_2$O and sterile wheat grain (0.32 g/mL), and c) modified Kirk medium containing 0.25 mM MnSO$_4$·H$_2$O, sterile wheat grain (0.32 g/L) supplemented with 0.05% v/v of Tween 80. The media were inoculated with active mycelia of A. discolor Sp4 from 5-day-old cultures on G-MEA medium (one plug/10 mL medium). Incubation was carried out at 30ºC under static conditions for 29 days. The MnP activity in the supernatant of the fungal culture samples was monitored periodically by 2,6-DMP method. The values

reported are the average of three samples with their standard deviation.

Statistical analysis was performed using a Student´s *t*-test for the independent paired data for comparing the culture techniques on the production of MnP by *A. discolor* Sp4 growing in various media. Differences between the mean values at p $\leq$ 0.05 were considered to be significant.

MnP purification

A. discolor Sp4 was grown under the optimized culture conditions from which MnP was subsequently purified. The mycelium was separated from the culture medium by filtration through a membrane filter Whatman (0.45 µm). The filtrate was concentrated by ultrafiltration with the Amicon tubes (10 kDa) at 4ºC, and the enzyme purification was carried out using FPLC (Fast Protein Liquid Chromatograph, Amersham Pharmacia Sweden). The concentrate was applied to a 1-mL column Q Sepharose, which had been equilibrated with 20 mM piperazine (pH 5.0). Elution of the proteins was achieved by a 1 mL/min linear gradient from 0 – 2 M NaCl with 20 mM piperazine pH 5.0. The fractions containing the enzyme were concentrated by the Amicon tubes (10 kDa) and then loaded to a GFC-100 gel filtration column. This column was equilibrated and eluted with 20 mM piperazine (pH 5.0) buffer with 100 mM NaCl. The gradient was isocratic and the flow was 1 mL/min. Specific activity was determined by the ratio of total activity to protein content. The purification yield was evaluated by the ratio between the total activity for each step and the initial one. The fold purification was calculated by the ratio between the specific activities at each purification step. All the steps during the purification were performed at 4ºC.

Enzyme characterization

The apparent Michaelis-Menten constant K$_M$ of the purified MnP was determined by Lineweaver-Burk plots of activity versus the substrate profiles for 2,6-DMP in the range of 10 - 2,000 µM concentration and for H$_2$O$_2$ in the range of 40 – 2,400 µM concentration. The enzymatic activity of the purified MnP was determined at various pH (pH 3 – 9) at 30ºC after 15 min of incubation. The buffer solutions used were 150 mM sodium tartrate solution pH 3.0, 150 mM sodium malonate solution pH 4.5 and 5.5, 150 mM Tris-HCl solution pH 6.5, 7.5, 8.0 and 9.0. The enzymatic

activity of the purified MnP was determined at various temperatures between 25 and 70ºC after 15 min of incubation. The activation energy (Ea) was calculated by plotting the log of activities of the purified manganese peroxidase versus 1/T (in K) according to the Arrhenius equation. The value of activation energy was obtained by a computed linear regression analysis of the experimental data (Rao and Gianfreda, 2000). Enzyme thermostability was determined by incubating the purified MnP at different temperatures (40, 50, 60 and 70ºC) in sodium malonate solution (pH 4.5) for 2 h.

The molecular mass of the purified MnP was determined using the SDS-PAGE electrophoresis (Laemmi, 1970) with the Protein III apparatus, Biorad. Protein bands were visualized with silver nitrate solution. The molecular mass marker used was the broad range protein molecular weight marker from the Promega. The presence of MnP bands were confirmed using a non-denaturing electrophoresis (zymogram analysis). The samples were applied in non-reducing denaturizing loading buffer without boiling. The gels were incubated in 50 mM sodium malonate (pH 4.5) and 1 mM $MnSO_4$ for 1 h and then stained for the MnP activity with a solution containing the same solution supplemented with 1 mM of 2,6-DMP and the oxidative agent 0.4 mM H_2O_2. Bands were stained orange, the characteristic color of MnP activity.

Analyses
Enzyme assays
The manganese peroxidase activity in the supernatant of the fungal culture sample (after centrifugation for 10 min at 5,000 rpm) was determined by monitoring the oxidation of 2,6-DMP spectrophotometrically at 30ºC. The purified MnP activity was also determined by the 2,6-DMP method.

2,6-DMP assay: the reaction mixture (1.0 mL) contained 200 μL of 250 mM sodium malonate (pH 4.5), 50 μL of 20 mM 2,6-DMP, 50 μL of 20 mM $MnSO_4$·H_2O, and 600 μL of supernatant. The reaction was initiated by adding 100 μL of 4 mM H_2O_2 and the absorbance of the colored product was measured at 468 nm and corrected for the laccase activity (Wariishi et al, 1992). One MnP activity unit (U) was defined as the amount of enzyme transforming 1 μmol 2,6-DMP per minute at pH 4.5 and 30ºC (Wariishi et al, 1992).

Protease activity was measured using the Rowley and Bull method (1977). The supernatant of the samples was incubated with azocasein (2.5 g/L) at 37ºC for 20 minutes. The protease activity was stopped by the precipitation of proteins with 2.0 mL of 2 M trichloroacetic acid. The precipitated proteins were eliminated by centrifugation and the change in color produced by the protease activity on the azocasein in the supernatant was measured at 400 nm. The activity was expressed in units (U) where one unit equals 1% azocasein hydrolysis in 20 min.

Protein concentration
Protein concentration in the supernatant of the fungal culture sample (after centrifugation for 10 min at 5,000 rpm) was determined using the Bradford method (1976) with crystalline bovine serum albumin (BSA) as the protein standard. Protein content in the purified MnP also was determined using the Bradford method. The supernatant (1.0 mL) was added to 3.0 mL of Bradford reagent (Biorad) and mixed by gentle vortex.The absorbance of the sample was measured at 595 nm using an ultraviolet-visible spectrophotometer against the Bradford reagent blank between 2 min and 1 h after the mixing. The amount of protein was quantified by comparing the absorbance with a standard curve. The values reported are the average of three samples with their standard deviation.

RESULTS AND DISCUSSION

MnP production by A. discolor Sp4 in liquid medium supplemented with wheat grain
The addition of wheat grain to the static culture of *A. discolo*r Sp4 produced an increase in MnP activity (Fig. 1) compared to the controls (without wheat grain, with or without agitation). MnP activity reached the maximum value of 1,100 U/L after 13 days of growth, which was 3.8-fold higher than the control under static conditions and 8.6 times higher than the control with agitation (Fig. 1). The results were in agreement with those reports in which higher ligninolytic activities were detected when the lignocellulosic material was used as a substrate for fungal growth. The activity of MnP in *Pleurotus ostreatus* cultures with poplar sawdust was eight times higher than in the cultures with fir sawdust (Giardina et al, 2000). The

production of ligninolytic enzymes by *Phanerochaete chrysosporium* and *Trametes versicolor* grown with different lignocellulosic materials increased in relation to the control (without lignocellulosic material), particularly with barley and grape residues (Moredo et al, 2003). White-rot fungi normally grow on the decaying wood and forest litter. They degrade the cellulose and lignin by the means of their extracellular enzymatic system, which suggests that the lignocellulosic substrates as energy source are suitable for their growth. The immobilization of the mycelium on the surface of wheat grains provided a better surface area and increased the mass transfer, thus improving the production of the enzymes (Herpoël et al, 1999). Moreover, the fungal mycelium may penetrate the lignocellulose support, releasing additional water-soluble aromatic/phenolic substances, which in turn may induce the secretion of the ligninolytic enzymes (Kapich et al, 2004).
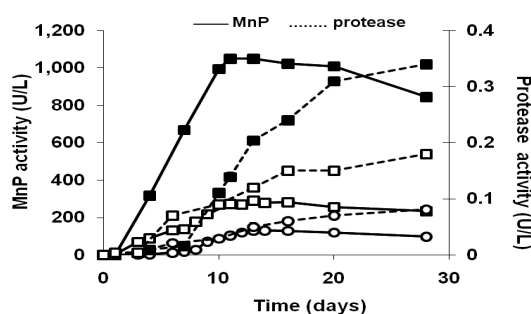

Figure 1 - Manganese peroxidase (─) and protease (.....) activity (U/L) in *A. discolor* Sp4 cultures growing in modified Kirk medium (■) supplemented with wheat grain and static conditions, (□) without wheat grain, under static conditions, (○) without wheat grain, on a shaker at 100 rpm.

Low MnP activity was observed in the agitated cultures. Venkatadri and Irvine (1990) reported that the formation of mycelia pellets and O_2 limitation, shear stress of mycelia and denaturation of enzymes at high agitation speeds (100 to 200 rpm) were some explanations to this loss of activity. The loss in the enzyme activity has been well-correlated to the degree of agitation, as described by Venkatadri and Irvine (1990) for ligninases. Therefore, static cell cultures are desirable for the MnP production due to the increase in the contact area between the cells and oxygen without shear stress (Ürek and Pazarlioğlu, 2007).

With the addition of wheat grain, the proteases activity was higher than the controls (without wheat grain, under static conditions, or on a shaker at 100 rpm) and reached 0.20 U/L after 13 days. Under the static conditions, the protease activity in the control without agitation increased 2-fold on the 13th day in comparison with the agitated control, reaching a maximum value of 0.12 U/L (Fig. 1).

Some authors have reported that the total glucose consumption by the white-rot fungi in Kirk medium leads to protease production as a consequence of cell lysis, which affects the ligninolytic enzyme production (Staszczak et al, 2000). In the presentstudy, the MnP reached a maximum activity on day 13, followed by a significant increase in the protease activity when using wheat grain (Fig. 1), suggesting a metabolic shift from lignin and cellulose to protein and carbohydrate degradation. In the absence of wheat grain, *A. discolor* Sp4 produced the proteases (for peptone degradation) without showing a substantial MnP activity (Fig. 1).

In spite of higher protease production when wheat grain was used as a substrate, maximal MnP activity increased when compared with the results for the Kirk medium. This led to consider the use of wheat grains as substrate to be beneficial for enhanced MnP production, usable for the future studies of organic pollutants degradation by *A. discolor* Sp4.

The protein concentration was higher than the controls in the presence of wheat grain, reaching 225 mg/L after 13 days of growth (Fig. 2). In the control (without wheat grains, under static conditions), the total protein concentration increased from 6 mg/L on day 6 to 40 mg/L on day

13. Under agitation, the maximum total protein concentration was 6 mg/L (Fig. 2).

The increase in the total protein concentration (Fig. 2) correlated to the MnP activity (Fig. 1) as a response to the growth of *A. discolor* Sp4. Sato et al (2007) have identified a large number of proteins from *P. chrysosporium* grown on different liquid and solid substrates involved in the carbohydrate, lignin and protein metabolism. Van den Wymelenberg et al (2006) analyzed the *P. chrysosporium* genome, identifying 769 proteins within the secretosome, including the cell wall-bound and endoplasmic reticulum (ER)-related proteins.
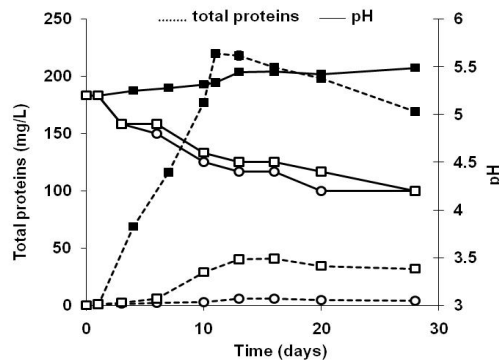

Figure 2 - Total proteins (……) and pH (—) in *A. discolor* Sp4 cultures growing in modified Kirk medium (■) supplemented with wheat grain, under static conditions, (□) without wheat grain, under static conditions, (○) without wheat grain, on a shaker at 100 rpm.

The presence of wheat grain slightly increased the pH in the culture medium from 5.2 to 5.5 (Fig. 2). This could be attributed to the presence of ammonium in the medium, which was produced by the use of amino acids and proteins of wheat grain. The pH in the culture medium without wheat grains (with and without agitation) decreased from 5.2 to 4.2 after 28 days (Fig. 2). This was probably due to the secretion of organic acids, such as oxalic, formic and glyoxylic acids (Dutton et al, 1996; 1996; Mäkelä et al, 2002). Physiological concentrations of oxalic acid have been shown to stimulate the MnP activity by chelating the unstable Mn^{3+} (Kuan and Tien, 1993). MnP can generate H_2O_2 by the oxidation of oxalic and glyoxylic acids, thus providing an endogenous source for extracellular H_2O_2 (Urzúa et al, 1998).

The pH ranges obtained in this study were adequate for the growth of white-rot fungi since they generally grew at pH 4.0-6.0, with an optimum for enzyme production between 4.5 and 5.0 (Ürek and Pazarlioğlu, 2007).

Effect of MnSO$_4$ and Tween 80 on MnP production by A. discolor Sp4 in liquid medium supplemented with wheat grain

The addition of 0.25 to 1 mM $MnSO_4·H_2O$ increased the production of MnP by *A. discolor* Sp4 in the culture medium in the presence of wheat grain (Fig. 3). The maximum MnP activity (1,400 U/L) was obtained after 8 days of incubation with the addition of 0.25 mM of $MnSO_4·H_2O$. The highest concentration of $MnSO_4·H_2O$ (2.0 mM) added showed an inhibitory effect on the fungus growth and MnP production. In this treatment, a small mycelium growth was observed as compared to the other cultures supplemented with lower amounts of $MnSO_4·H_2O$. Moreover, the supernatant of this culture appeared turbid and darkened to brown after 8 days. This color could be attributed to the formation of a MnO_2 precipitate when Mn^{3+} was oxidized in the presence of an insufficient organic acid level in the medium (Rogalski et al, 2006). The enzymatic activity of MnP requires manganese to function. However, high concentrations of manganese can inhibit the enzyme due to the generation of large quantities of hydrogen peroxide that inhibit the catabolic cycle of the enzyme (Wariishi et al, 1988; Bermek et al, 2002). In fact, hydrogen peroxide is known to be a product of numerous enzymatic reactions by the means of aryl alcohol oxidase, glyoxal oxidase action and ligninolytic enzymes (Gómez-Toribio et al, 2001). In addition, H_2O_2 is produced by the non-enzymatic reactions involving certain metals and metal complexes that have been shown to catalyze the reaction for the

H_2O_2 production. Mn^{2+} reduces the superoxide to H_2O_2 and is oxidized to Mn^{3+} (Schlosser et al, 2002). At high concentrations of hydrogen peroxide, MnP II is converted to an inactive form (MnP III), not involved in the normal catalytic cycle (Mester and Tien, 2000). As a result, the amount of fungal biomass and the mycelia growth of the fungi are affected as well (Rothschild et al, 1999).
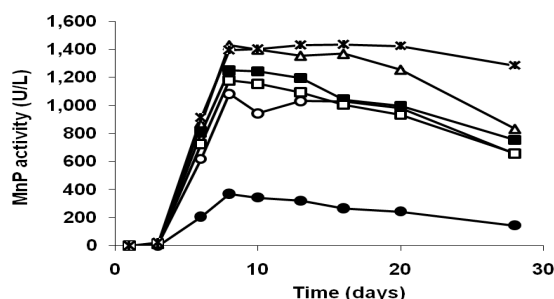

Figure 3 - Manganese peroxidase (MnP) activity (U/L) in modified Kirk medium inoculated with *A. discolor* Sp4 supplemented with wheat grain and (○) 0.002 (Δ) 0.25 (■) 0.5 (□) 1.0 (●) 2.0 mM of $MnSO_4$, (*) 0.25 mM of $MnSO_4$ and 0.05% v/v Tween 80, at 30°C under static conditions.

In the present study, the addition of 0.05% (v/v) Tween 80 maintained a high and constant level of the enzyme for 28 days (Fig. 3). Ürek and Pazarlioğlu (2005) observed that Tween 80 at 0.05% (v/v) would act as an inducer of MnP production from *P. chrysosporium*. The addition of surfactants such as Tween 80 would have a regulatory effect on the production of ligninolytic enzymes, especially for MnP (Garon et al, 2002; Watanabe et al, 2000). Surfactants also may modify the plasma membrane, altering the transport of compounds in and out of the cell, or protect against the mechanical inactivation of the enzymes (Venkatadri and Irvine, 1990). However, a high concentration of surfactants could have an inhibiting effect on the fungal growth and ligninolytic enzymes production (Zhou et al, 2007). The mechanism by which the surfactants such as Tween 80 increase the extracellular enzyme production has not been established.

Cultivation suitability of A. discolor Sp4 for MnP production in tubes containing glass beads
A. discolor Sp4 showed significant growth in the tubes containing the glass beads coating the hyphae at the bottom of the tube and favoring interaction between the fungus and the medium for enhancing the MnP production. Using this culture system, the volume of the samples was considerably reduced and each tube was used to perform the corresponding analysis without altering the assay (destructive sampling). This could be relevant for biodegradation assays over the time, when it is important to carry out several experiments in parallel managing a large number of samples in terms of space.

Table 1 shows the MnP production from *A. discolor* Sp4 grown in Erlenmeyer flasks, or tubes containing the glass beads in different culture media. There was an increase in MnP activity using Kirk medium supplemented with $MnSO_4 \cdot H_2O$ and wheat grain compared to the results obtained using Kirk medium supplemented with $MnSO_4 \cdot H_2O$ (without wheat grain). As shown above, the addition of wheat grain as lignocellulosic material favored the fungal growth and MnP production.

Using the tubes containing the glass beads, MnP activity reached the maximum value of 15.8 U/L day. The maximal activity using the Erlenmeyer flasks was 16.1 U/L day. The use of the tubes containing the glass beads for the cultivation of *A. discolor* Sp4 did not affect the MnP activity when using Kirk medium and $MnSO_4$, as the values obtained did not differ largely from those in the Erlenmeyer flasks. When wheat grain was used as the substrate, MnP activity in the tubes was even higher than that in Erlenmeyer flasks (p ≤ 0.05), particularly for the days 12, 15 and 19 after the inoculation. The MnP activity reached the maximum (956 U/L) after 19 days, 1.7-fold higher than that observed in the Erlenmeyer flasks after the same incubation period. In this case, *A. discolor* Sp4 covered all of the glass beads and

wheat grains, which resulted in a larger contact surface area. When Tween 80 was used, no significant differences in the MnP activity were

observed in the tubes containing glass beads or Erlenmeyer flaks.

Table 1 - Manganese peroxidase (MnP) activity (U/L) in *A. discolor* Sp4 cultures grown in Erlenmeyer flasks or tubes in different culture conditions. Values represent MnP activity mean N = 3. Asterisks (*) indicate a statistically significant difference between culture techniques at the same time (p < 0.05, Student's t-test).

Time (days)	MnP activity (U/L)					
	Kirk medium Supplemented with MnSO4.H2O		Kirk medium supplemented with $MnSO_4\cdot H_2O$ and wheat grain		Kirk medium supplemented with $MnSO_4\cdot H_2O$, wheat grain and Tween 80	
	Flask	**Tube**	**Flask**	**Tube**	**Flask**	**Tube**
4	2.4 ± 0.2	3.4 ± 0.2	4.5 ± 1.6	6.1 ± 1.1	3.4 ± 1.9	0.4 ± 0.2
9	170* ± 19	26* ± 1	391 ± 52	357 ± 76	537 ± 85	320 ± 130
12	57* ± 7	191* ± 2	425* ± 92	779* ± 16	630 ± 157	815 ± 118
15	242* ± 27	143* ± 26	615* ± 134	935* ± 111	747 ± 225	769 ± 29
19	101 ± 11	140 ± 5	547* ± 156	956* ± 54	696* ± 38	969* ± 84
29	115* ± 17	41* ± 19	495 ± 128	276 ± 81	413 ± 12	96 ± 0.4

Pizzul et al (2006) used the glass-beads tube technique to test the PAHs degradation by actinomycetes. The system allowed for both good microbial growth confined to the liquid medium and glass beads matrix and suitable and rapid PAH quantification with a PAHs recovery yield of up to 95%. Glass beads also have been used to simulate the soil particles and monitor the growth of the mycorrhizal fungus *Glomus intraradices* (Rilling and Steinberg, 2002) and the actinobacterium *Streptomyces* (Nguyen et al, 2005). Ehlers and Rose (2005) evaluated the immobilization of three white-rot fungal cultures in trickling packed-bed reactors employing the glass beads as support in the biodegradation of phenol and 2,4,6-trichlorophenol-contaminated liquid. Microscopic examination showed that the glass beads were colonized by the fungal cultures. Furthermore, rapid uptake, removal and biodegradation of the compounds were obtained as a result of the large contact surface area and long exposure to lignin-degrading enzymes and mycelia (Ehlers and Rose, 2005).

MnP purification

MnP purification from *A. discolor* Sp4 cultivated under optimized culture conditions was carried out through two chromatographic steps: an anion-exchange chromatography using a Q-Sepharose column coupled with further gel filtration chromatography using a GFC-100 column. Table 2 summarizes the results obtained from the MnP purification process. The MnP specific activity increased after each purification step, which

suggested an increase in the MnP purity. In this study, a purification yield of approximately 48% was obtained after the anion-exchange chromatography but was reduced to 5.7% after the gel filtration chromatography step (Table 2). The purification factor increased from 4.35 to 4.70 after the second chromatographic step. This indicated that a single step with anion exchange chromatography was sufficient to obtain the purified MnP. After the first chromatographic step, fractions of elution volumes - 10, 10.5 and 11 mL - contained MnP activity (data not shown) and were collected and analyzed by the electrophoresis to determine the molecular mass of the protein. In the case of the second chromatographic step, two fractions (elution volumes of 5.5 and 6 mL) contained MnP activity (data not shown), which also were collected and analyzed by the electrophoresis.

MnP characterization

The K_M value, pH and optimum temperature values were determined in order to characterize the MnP produced by *A. discolor* Sp4. The apparent K_M values of the purified MnP for 2,6-DMP and H_2O_2 were 25 µM and 37 µM, respectively, indicating high affinity for both the substrates. Previous studies on kinetic parameters from *Phanerochaete chrysosporium* using 2,6-DMP as substrate have shown that K_M values ranged from 7 to 29 µM (Palma et al, 2000; Ürek and Pazarlioğlu, 2004) depending on the operational conditions and the isoenzymes produced. They were comparable and in the same magnitude order

as the results obtained for the MnP enzyme from *A. discolor*. The K_M values for *Bjerkandera* sp. obtained using the same substrate showed a variation of between 11 and 121 µM, which could be considered a higher degradation rate than the MnP from *A. discolor* Sp4. Regarding kinetic parameters considering H_2O_2 as a substrate, earlier works have shownthat the K_M values for *Phanerochaete chrysosporium* ranged from 5 to 71 µM (Palma et al, 2000; Ürek and Pazarlioğlu, 2004) and were comparable to the results obtained for MnP from *A. discolor* Sp4. The K_M values for *Bjerkandera sp*. obtained using the H_2O_2 showed a variation of between 3 and 5 µM (Palma et al, 2000) which could be considered lower as compared with the MnP from *A. discolor* Sp4.

Table 2 - Purification process of MnP from *A. discolor* Sp4 in Kirk medium supplemented with wheat grain and 0.25 mM $MnSO_4$ as inducer, incubated under static conditions at 30°C. The MnP activity in the crude extracellular fluid and after the different steps were estimated by monitoring the oxidation of 2,6-DMP spectrophotometrically. Protein content was estimated by means of the Bradford method.

Purification step	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extracellular fluid	109	18	6	100	1.0
Ultrafiltration 10 kDa	102	9	11	94	1.8
Q Sepharose	53	2	26	48	4.4
Gel filtration GFC100	6	0.2	28	6	4.7

The optimum pH range was between 4.5 and 5.5, with the relative activity dropping by 20% at pH 6.5 and being inactivated at pH 9.0 (data not shown). This pH range was in agreement with the earlier reports (Ürek and Pazarlioğlu, 2004); nevertheless, a specific white-rot fungus (*Aspergillus terreus* LD-1) has been found to have an alkaline optimum pH range of between 11.0 and 12.5 (Kanayama et al, 2002).

The temperature required for maximize MnP production at pH 4.5 was 50°C after 15 min of incubation, and the activity was completely lost at 70°C (data not shown). Temperatures for maximizing the MnP activity have been described from 22 (Paszczynski et al, 1988) to 60°C (Wang et al, 2002), depending on the fungus species. Activation energy (Ea) of 16.5 kJ/mol was determined in the temperature range between 30 and 50°C. This value was according to the Ea obtained for free enzymes such as invertase (20 kJ/mol) (Gianfreda et al, 1991) and urease from jack bean (13-15 kJ/mol) (Gianfreda et al, 1995).

Regarding thermal stability, the MnP showed high stability between 40 and 50°C (Fig. 4). More than 95% activity was retained after 120 min of incubation at 40°C. At 50°C, MnP lost 30% of its activity after 2 h of incubation, reaching a value of 70% of its maximal activity (Fig. 4). In contrast, fast inactivation occurred at 60°C where the residual activity was 20% after 2 h of incubation and a sudden loss of activity was observed at 70°C where the activity decreased to zero after only 30 min of incubation. This indicated that the enzyme could be used in a broad temperature range due to its relatively high temperature tolerance.

Molecular mass

Two major bands were observed in the SDS-PAGE gel after the purification, which suggested the presence of MnP isoenzymes (MnP_1 and MnP_2) (Fig. 5a). The molecular mass of MnP isoenzymes was estimated to be 35 and 38 kDa (Fig. 5b) by SDS-PAGE electrophoresis. The zymogram analysis of the purified enzyme sample confirmed the presence of two isoenzymes (Fig. 5b). The molecular mass of the two isoenzymes was lower than those reported in the literature. The molecular mass range of the MnP in white-rot fungi is quite broad. Hofrichter (2002) reported a molecular mass from 38 kDa for *Trametes troggi* up to 52.5 kDa for *Ceriporiopsis subvermispora*.

The existence of multiple MnP isoenzymes in different ligninolytic fungi has been described (Lobos et al., 1994). The differences in the fungal species and strains and growth conditions (composition of the culture medium, incubation time, with or without agitation) strongly affect the isoenzyme patterns produced by the different fungi (Boer et al., 2006). Purification methods and storage also can affect the relative isoenzymatic levels (Cullen and Kersten, 1996).
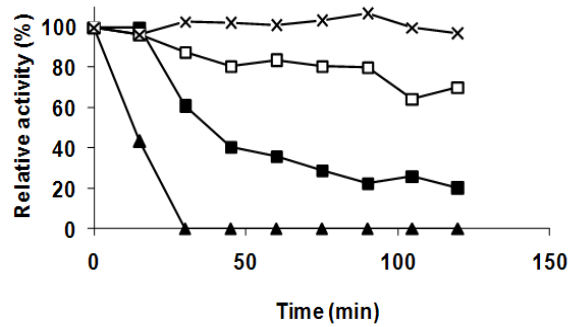
Figure 4 - Effect of temperature on MnP activity stability during 2 h of preincubation at 40°C (x), 50°C (□), 60°C (■), 70°C (▲) at pH 4.5.
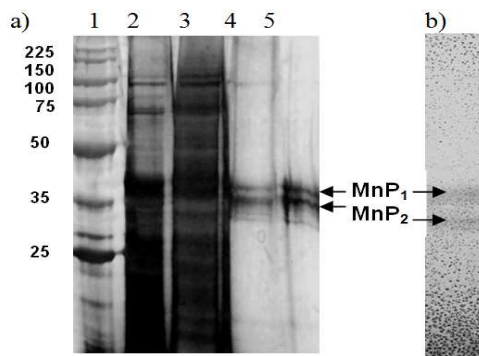

Figure 5 - a) Electrophoresis SDS-PAGE of MnP from *A. discolor* Sp4 with silver nitrate staining (1) proteins standards (Promega) (2) extracellular fluid (3) concentrate (4) after Q-Sepharose column chromatogrphy (5) after gel filtration column chromatography b) Zymogram of MnP isoenzymes.

CONCLUSIONS

The results showed that the addition of wheat grain, 0.25 mM $MnSO_4$ and 0.05% (v/v) Tween 80 in Kirk medium significantly increased the MnP production from *A. discolor* Sp4. The enzyme produced by *A. discolor* Sp4 presented biochemical and kinetic properties, which could be quite advantagenous for the biodegradation purposes. The simple glass-bead technique used in this study in liquid medium allowed the growth of the fungal culture and enzyme production at the same levels compared to the standard Erlenmeyer flasks technique. This technique could be more practical in terms of space, resulted high MnP yields and simultaneously allowed a destructive sampling. The culture system in the tubes containing the glass beads could be considered a promising and suitable method for testing the bioremediation assays using the white-rot fungi.

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