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Effects of manganese, 2,5-xylidine, veratryl alcohol and tween 80 on the production of ligninolytic enzymes by *Ceriporiopsis subvermispora*

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The effects of adding manganese, 2,5-xylidine, veratryl alcohol and Tween 80 in a culture medium used for the production of ligninolytic enzymes by polyurethane foam-immobilized *Ceriporiopsis subvermispora* were studied. While 11 ppm Mn^{2+} promoted the highest maximum activity of manganese peroxidase (108.0 ± 43.3 U/L, in the 6th day of cultivation), the medium without manganese led to the highest maximum activity of laccase (15.5 ± 2.1 U/L, in the 12th day of cultivation). By supplementing the medium containing 11 ppm Mn^{2+} with 1.0 mM 2,5-xylidine, it was possible to improve the maximum activity of laccase to 21.5 ± 4.9 U/L. The supplementation of the medium containing 11 ppm Mn^{2+} with 1.0 mM veratryl alcohol, in turn, led to an apparent second peak of MnP activity (110.0 ± 1.4 U/L, in the 24th day of cultivation; compared to 147.5 ± 60.1 U/L, in the 9th day of cultivation). When the medium containing 11 ppm Mn^{2+} and 1.0 mM 2,5-xylidine was supplemented with 0.05% (v/v) Tween 80, the maximum activities of Lac and MnP reached 53.3 ± 17.7 U/L (21st day of cultivation) and 174.8 ± 1.4 U/L (9th day of cultivation), respectively. During the cultivations, the exhaustion of glucose in the medium promoted nutritional stress, which, in turn, led to cell autolysis; reflected by an apparent reduction in the concentration of mycelium, and by an increase in the concentration of ammonium. The concentrations of extracellular proteins increased throughout the cultivations; such concentrations, however, did not generally exhibit good correlations with the measured enzyme activities.

Key words: *Ceriporiopsis subvermispora*, manganese, 2,5-xylidine, veratryl alcohol, Tween 80; manganese peroxidase, laccase.

INTRODUCTION

Ceriporiopsis subvermispora, a fungus that has already been used for biopulping in industrial scale (Akhtar et al., 2000), is known for its selectivity in lignin degradation, due to its inefficiency in degrading wood polysaccharides, mainly cellulose, and to its efficiency in degrading lignin (Fernandez-Fueyo et al., 2012a; Fernandez-Fueyo et al.,

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Abbreviations: Lac, Laccase; **MnP,** manganese peroxidase; **DNS,** 3,5-dinitrosalicylic acid.

2012b). The degradation of lignin by *C. subsermispora* is related to its non-specific extracellular enzymatic system composed by laccases (Lac) and manganese peroxidases (MnP) (Carvalho et al., 2008), which can perform their action in association with low molecular weight compounds (Aguiar and Ferraz, 2008).

The production of ligninolytic enzymes by filamentous fungi is regulated by several factors. In this context, the sources of carbon, nitrogen and inorganic ions available in the cultivation medium, as well as the presence of inducing compounds, like aromatics, are mostly important (Bonnarme and Jeffries, 1990; Buswell et al., 1995; Arora and Gill, 2000). For example, the addition of veratryl alcohol in cultures of *Botryospheria sp.* increased the production of constitutive Lac (PPO-I and PPO-II) in 100 and 25-fold (Dekker and Barbosa, 2001). The supplementation with 2.5-xylidine, in turn, has proved very effective in inducing the activity of Lac in cultures of *Trametes versicolor* (Rancaño et al., 2003). Moreover, Mn^{2+} plays a central role in ligninolysis by many fungi, including *C. subvermispora*; stimulating the secretion of and acting as a substrate for the manganese peroxidases (Hofrichter, 2002).

With regard to the non-ionic surfactant Tween 80, many authors, including Ürek and Pazarlioglu (2005) and Dekker et al. (2007), have observed an increase in the excretion of enzymes when cultures of ligninolytic fungi were performed in the presence of this surfactant. Previously, Asther et al. (1987) have suggested that Tween 80 transforms the structure of the cell membrane, favoring the excretion of enzymes into the medium.

To evaluate the effects of such additives in the production of MnP and Lac by *C. subvermispora* immobilized in polyurethane foam, a culture medium with defined composition, supplemented or not with manganese, 2,5-xylidine, veratryl alcohol and Tween 80, was used for cultivations under fixed environmental conditions. By following the concentrations of biomass, reducing sugars, ammonium and proteins, as well as the values of pH and conductivity, during the cultivations, it was possible to establish the metabolic behavior exhibited by the fungus in selected media.

MATERIALS AND METHODS

Fungus and inoculum preparation

C. subvermispora, from a stock culture, was initially activated in agar plates, using a medium composed by 2% (m/v) malt extract and 2% (m/v) agar, at 27°C for 7 days. Then, the inoculum was prepared in 2 L Erlenmeyer flasks containing 200 mL of liquid medium composed by 2.4% (m/v) potato/dextrose extract and 0.7% (m/v) yeast extract; sterilized at 121°C for 15 min, and inoculated with 20 pellets (8 mm diameter) taken from a recently activated culture. After 12 days of static incubation at 27°C, the grown mycelium was recovered by filtration, washed (300 mL sterile water), and macerated (100 mL sterile water) using an aluminum blender. An aliquot of 20 mL was taken from this suspension and used to determine the dry mass of mycelium contained in the suspension, by oven-drying at 105°C until constant mass. Based on this determination, the volume of suspension needed to inoculate each cultivation flask with an initial concentration of 500 mg of mycelium per liter of medium was determined.

Basal medium

The basal medium (Ruttiman-Johnson et al., 1993) was composed, per liter of solution, by: 10.0 g glucose, 10.0 mmol ammonium tartrate, 10.0 mmol trans-aconitic acid, 2.0 g KH_2PO_4, 0.5 g $MgSO_4$ x 7 H_2O, 0.1 g $CaCl_2$ x 2 H_2O, 1.0 mg thiamine chlorhydrate, and 7.0 mL of a solution of trace elements. The solution of trace elements was composed, per liter of solution, by: 15.0 g nitrilotriacetic acid, 1.0 g $FeSO_4$ x $7H_2O$, 1.8 g CoCl x $6H_2O$, 1.0 g $ZnSO_4$ x $7H_2O$, 0.07 g $Al_2(SO_4)_3$ x 18 H_2O, 1.0 g $CuSO_4$ x 5 H_2O, 0.1 g H_3BO_3, 0.1 g $NaMoO_4$ x 2 H_2O, 30.0 g $MgSO_4$ x 7 H_2O, 10.0 g NaCl, 0.82 g $CaCl_2$. The component solutions were autoclaved separately, at 121°C for 15 min, prior to the formulation of the culture medium; with exception of the thiamine chlorhydrate solution, which was sterilized by filtration (syringe filter, membrane with 22 µm).

Cultivation media and conditions

The cultivation of the immobilized cells was performed as follows: 12 cubes of 1.5 cm^3 of polyurethane foam, previously washed, dried and weighted, were added in 125 mL Erlenmeyer flasks. The flasks were autoclaved at 121°C for 15 min. After cooling, 30 mL of basal medium and 15 mg (dry basis) of homogenized mycelium were added in each flask. The cultures were incubated statically at 27°C for 30 days, with samples (1 flask = 1 sample) taken every three days. Four sets of cultivations were performed: In the first, the concentration of manganese ions (Mn^{2+}) was adjusted to 0, 11 or 40 ppm of Mn^{2+}; added or not as $MnSO_4$ x H_2O. In the second, the medium containing 11 ppm of Mn^{2+} was supplemented with either 0.5 or 1.0 mM of 2,5-xylidine. In the third, the medium containing 11 ppm of Mn^{2+} was supplemented or not with either 1.0 or 2.0 mM of veratryl alcohol. In the last, the medium containing 11 ppm of Mn^{2+} and 1.0 mM of 2,5-xylidine was supplemented or not with either 0.05 or 0.50% (v/v) of Tween 80. The additives were sterilized by filtration (syringe filter, membrane with 22 µm) and added into the culture medium at the beginning of the cultivations, prior to inoculation; with exception of the manganese sulfate, which was added or not in the solution of trace elements. The cultivation using the basal medium containing 11 ppm Mn^{+2} was carried out in sextuplicates; the others, in duplicates.

Recovery of mycelium and determination of biomass concentration

The mycelium was recovered by filtration. The content of each Erlenmeyer flask was quantitatively transferred into a stainless steel funnel adapted to a glass filter (AP40, Millipore), previously dried and weighted, and coupled to a Kitasato flask maintained under vacuum. The material retained in the filter (mycelia and sponge cubes) was washed with 30 mL of distilled water, transferred into weighing bottles, and oven-dried at 105°C until constant mass; which was used to determine biomass concentrations, after discounting the foam dry weights.

Determination of glucose concentration

The concentration of glucose in the samples was determined by using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). In a

Table 1. Maximum activities of manganese peroxidase and laccase (average ± standard deviation) determined during the cultivations performed in the different media.

Cultivation medium	MnP (U/L)	Lac (U/L)
Mn 0 ppm	37.0 ± 2.8$^{a\ (p < 0.20)}$	15.5 ± 2.1$^{a\ (p < 0.05)}$
Mn 11 ppm	108.0 ± 43.3^{b}	5.3 ± 2.9^{b}
Mn 40 ppm	55.5 ± 26.2^{a,b}	7.0 ± 1.4^{b}
Mn 11 ppm	108.0 ± 43.3$^{a(p < 0.20)}$	5.3 ± 2.9$^{a(p < 0.05)}$
Mn 11 ppm + X 0.5 mM	91.0 ± 15.6^{a}	14.0 ± 1.4^{b}
Mn 11 ppm + X 1.0 mM	99.5 ± 19.1^{a}	21.5 ± 4.9^{b}
Mn 11 ppm	108.0 ± 43.3$^{a(p < 0.20)}$	5.3 ± 2.9$^{a(p < 0.20)}$
Mn 11 ppm + VA 1.0 mM	147.5 ± 60.1^{a}	4.0 ± 0.0^{a}
Mn 11 ppm + VA 2.0 mM	100.0 ± 19.8^{a}	8.5 ± 2.1^{a}
Mn 11 ppm + X 1.0 mM	99.5 ± 19.1$^{a(p < 0.20)}$	21.5 ± 4.9$^{a(p < 0.05)}$
Mn 11 ppm + X 1.0 mM + T80 0.05% v/v	174.8 ± 1.4^{b}	53.3 ± 17.7^{b}
Mn 11 ppm + X 1.0 mM + T80 0.50% v/v	181.8 ± 23.3^{b}	60.6 ± 5.7^{b}

For each set of cultivations, maximum enzyme activities denoted with different superscript letters represent statistically different values.

tube, 0.5 mL of sample, 1.0 mL of deionized water and 3.0 mL of DNS were added. After heating the mixture at 100°C for 5 min in a water bath, and cooling, 20 mL of deionized water was added. After mixing, the absorbance at 540 nm was measured. The value was converted in glucose concentration, by using an appropriate calibration curve.

Determination of ammonium concentration

The concentration of ammonium in the samples was determined by using the phenol-hypochlorite method (Weatherburn, 1967). Two reagent solutions were prepared before each analysis: Solution A, composed by 5.0 g of phenol and 25.0 mg of sodium nitroprusside, dissolved in 500 mL of deionized water, and Solution B, composed by 2.5 g of NaOH and 4.2 mL of sodium hypochlorite (5% active chlorine), dissolved in 500 mL of deionized water. In a tube, 20 µL of sample, 5.0 mL of solution A and 5.0 mL of solution B were added. The mixture was homogenized and left under room temperature for 30 min, then, had its absorbance at 625 nm determined. The value was converted in ammonium concentration, by using a calibration curve prepared with ammonium sulfate.

Determination of protein concentration

The concentration of extracellular proteins in the samples was determined by using the Bradford's micro-method (Bradford, 1976). In a tube, 0.1 mL of sample and 1.0 mL of Bradford's reagent were added. After homogenization, the mixture was kept under room temperature for 15 min. Afterwards, the absorbance at 595 nm was measured. The value was converted in protein concentration, by using a calibration curve prepared with bovine serum albumin.

Determination of enzyme activities

The activity of Lac was determined using ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] as substrate. The oxidation reaction was carried out in 0.3 mL of 50 mM citrate-phosphate buffer pH 5.0, 0.1 mL of deionized water, 0.5 mL of sample and 0.1 mL of 1 mM ABTS. The substrate oxidation was monitored at 420 nm, considering the value of 36,000 M^{-1} cm^{-1} (Bourbonnais and Paice, 1990) as the absoptivity of the oxidized product.

The activity of MnP was determined using phenol red as substrate. The reaction mixture was composed by 1.5 mL of sodium succinate buffer (50 mM, pH 3.2), 1.5 mL of 50 mM sodium lactate, 0.5 mL of 0.1% phenol red, 0.5 mL of 1 mM manganese sulfate, 0.25 mL of 1.8% bovine albumin, 0.25 mL of 2 mM hydrogen peroxide, and 0.5 mL of sample. After the beginning of the reaction, fractions of 1.0 mL were removed from the tube containing the reaction mixture every 1 min and transferred into cuvettes containing 65 µL of 6.5 M sodium hydroxide, prior to reading the absorbance at 610 nm. The value of 22.000 M^{-1} cm^{-1} (Khindaria et al., 1994) was considered as the absoptivity of the oxidized product.

Determination of pH and conductivity

The pH was determined by using a pH meter; the conductivity, by using a conductivimeter. The equipments were calibrated before the determinations.

Statistical analysis

One way analysis of variance was utilized for multiple sample comparisons with respect to maximum enzyme activities determined during each set of cultivations. The mean values were then compared to each other, by means of Tukey's or Kruskal-Wallis's tests, in order to identify statistically significant differences.

RESULTS AND DISCUSSION

Effects of the manganese concentration on the productions of MnP and Lac

In the first set of experiments, the effect of manganese concentration on the production of MnP and Lac was

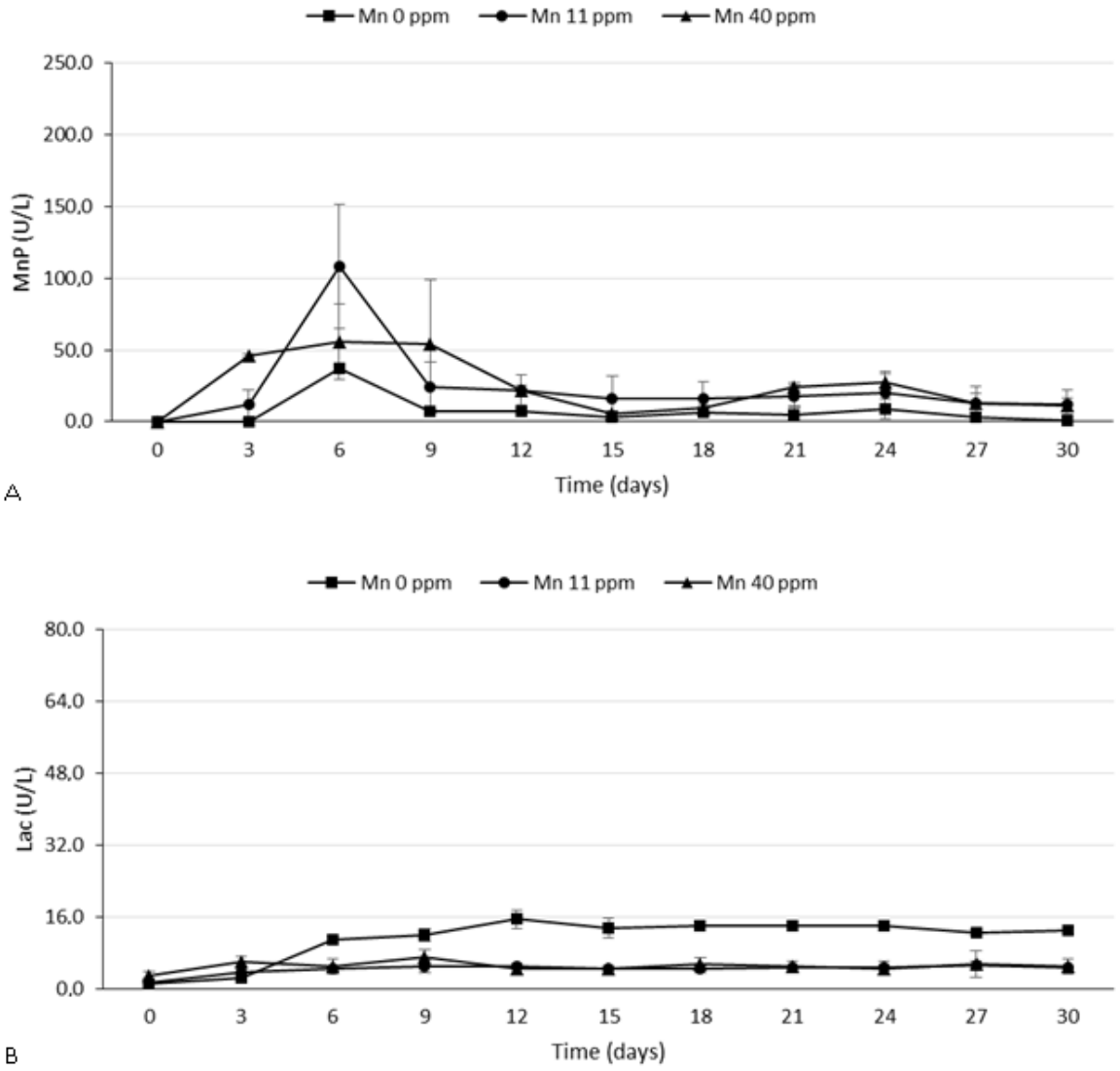


Figure 1. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of Mn^{2+}. Error bars represent standard deviations, calculated from sextuplicates.

evaluated by varying the concentration of Mn^{2+} ions in the culture medium, from 0 to 40 ppm.

As can be seen in Table 1, the medium absent in Mn^{2+} promoted a maximum MnP activity of 37.0 ± 2.8 U/L. As shown in Figure 1, this peak of activity was determined in the 6^{th} day of cultivation; a decrease of activity being observed thereafter. On the other hand, two peaks of MnP activity, 55.5 ± 26.2 and 27.5 ± 7.8 U/L, in the 6^{th} and 24^{th} days of cultivation, respectively, were observed when supple-menting the medium with 40 ppm of Mn^{2+}. As presented in Table 1, the statistical analysis of the data indicated that the supplementation of the medium with Mn^{2+} led to higher maximum MnP activities.

Ruttimann et al. (1992) reported that the addition of 11 ppm Mn^{2+} into the culture medium led to the highest production of MnP in submerged cultures of *C. subvermispora*. Manubens et al. (2003), in a further study, demonstrated that the addition of manganese into the medium not only affected the levels of transcription of *mnp* genes, but also was essential for the detection of extracellular MnP activity. Later, Gutierrez et al. (2008) proposed the existence of a robust homeostatic machinery to deal with the regulation of Mn^{2+} metabolism in this fungus; although upregulation in the expression of *mnp* genes had not been observed after the submerged cultivations in media containing from 0 to 5 mM Mn^{2+}.

Table 1 also shows that the media supplemented with Mn^{2+} promoted maximum Lac activities of 5.3 ± 2.9 (11 ppm) and 7.0 ± 1.4 U/L (40 ppm). The cultivation not supplemented with $MnSO_4$, however, led to the highest

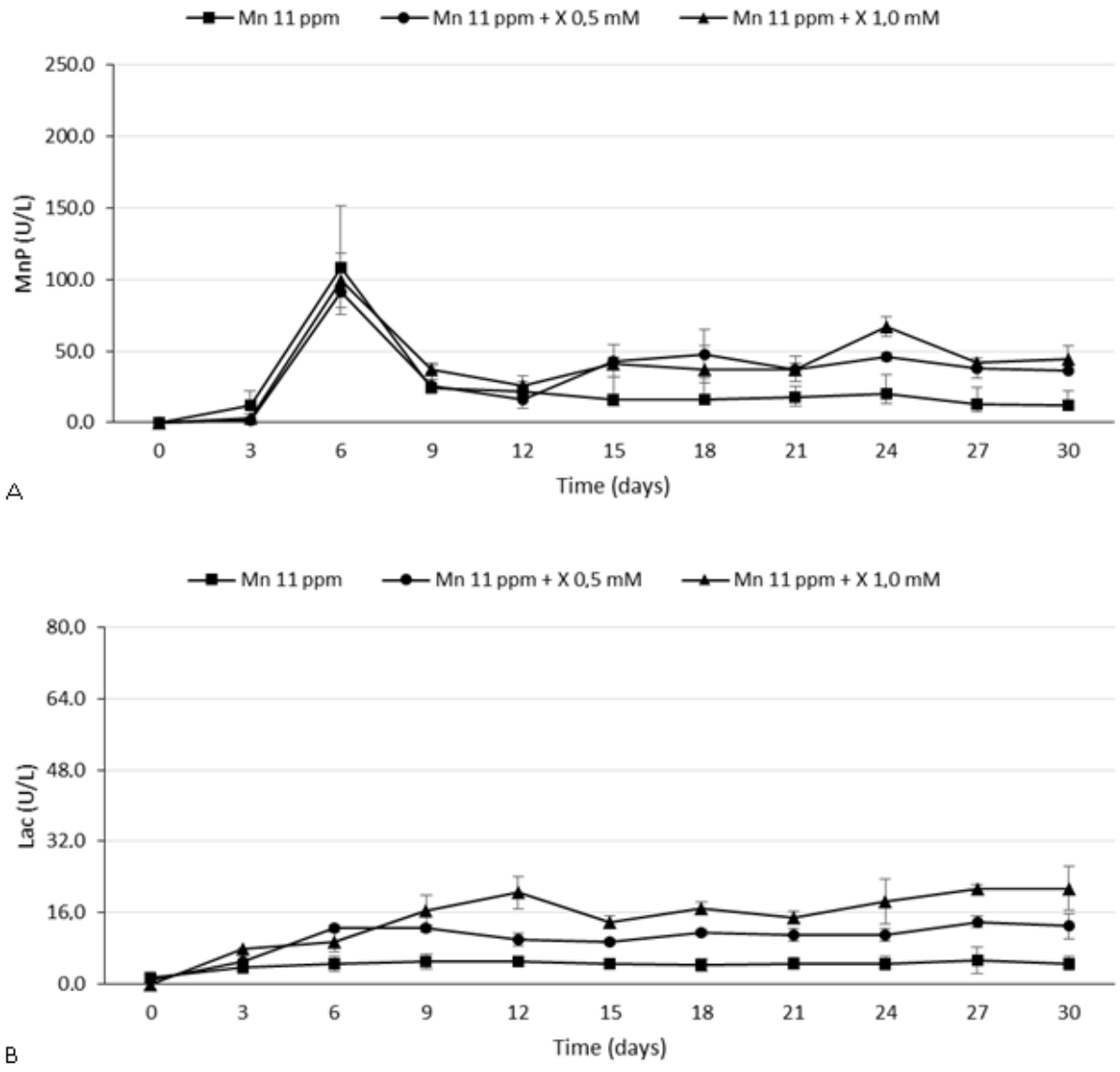


Figure 2. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of 2,5-xylidine, in presence of Mn^{2+} (11 ppm). Error bars represent standard deviations, calculated from duplicates.

maximum activity (15.5 ± 2.1U/L); in the 12th day of cultivation (Figure 1).

Daina et al. (2002), studying the degradation of β-5 lignin model dimers by *C. subvermispora*, however, did not observe variation in Lac titers when the fungus was grown in the absence or presence of 0.2 mM Mn^{2+}; although the production of MnP had been stimulated in the presence of this metal.

Effects of the addition of inducers on the productions of MnP and Lac

To evaluate a possible stimulation in the production of Lac and MnP, *C. subvermispora* was grown in the presence of 2,5-xylidine (0.5 and 1.0 mM) or veratryl alcohol (1.0 and 2.0 mM), using the basal medium supplemented with 11 ppm Mn^{2+}. The maximum activities of MnP and Lac are presented in Table 1; the time profiles of enzyme activities are shown in Figures 2 and 3.

As can be seen in Table 1, the maximum activity of MnP was not favored by the addition of veratryl alcohol nor by the addition of 2,5-xylidine, independently of the concentrations added into the medium. In spite of this, second peaks of MnP activity were apparently observed in the presence, but not in the absence, of the inducers. In the presence of 1.0 mM 2,5-xylidine, a MnP activity of

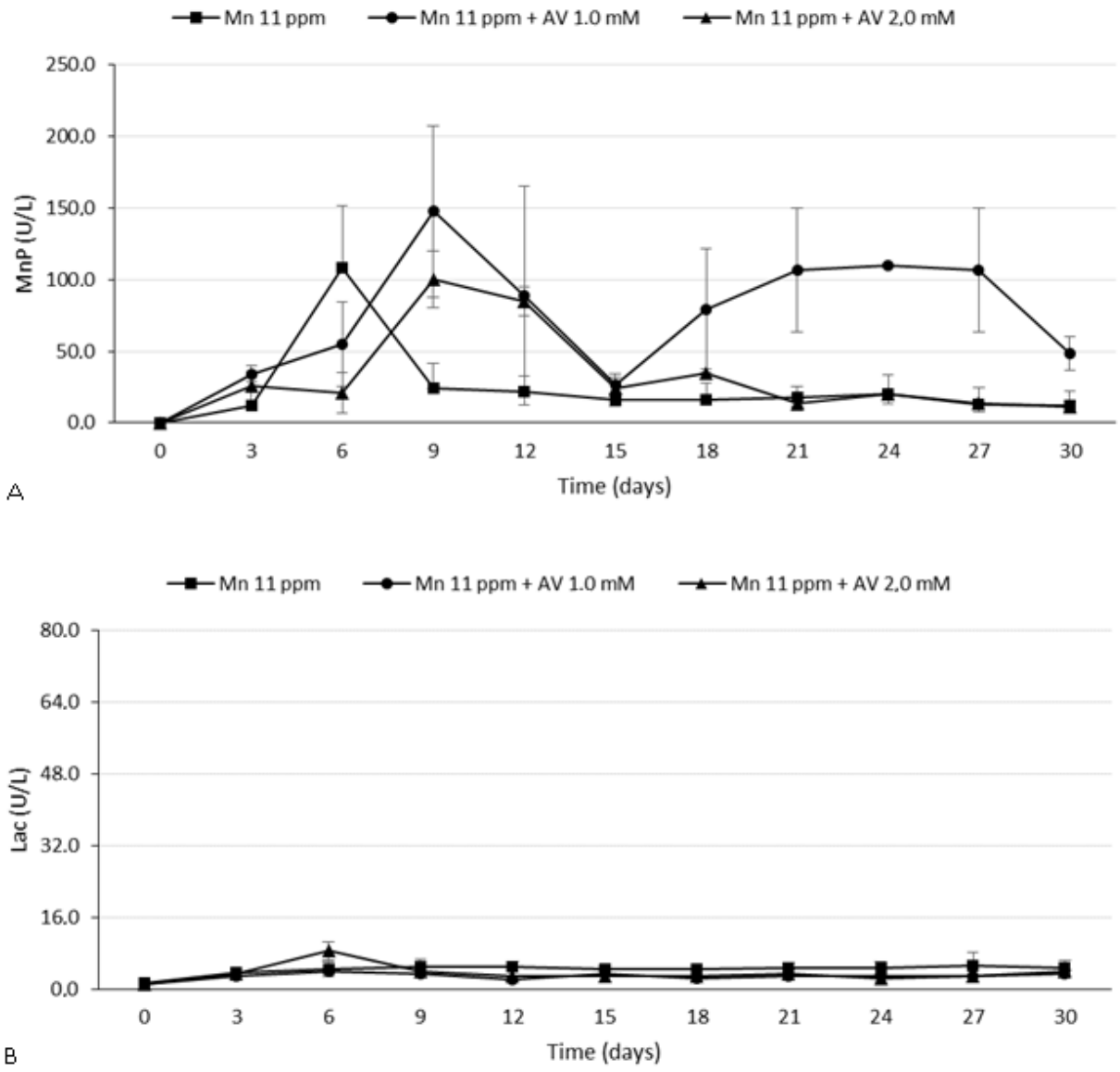


Figure 3. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of veratryl alcohol, in presence of Mn^{2+} (11 ppm). Error bars represent standard deviations, calculated from duplicates.

67.0 ± 7.1 U/L was determined in the 24th day of cultivation, compared to 99.5 ± 19.1 U/L (6th day of cultivation) (Figure 2). In the presence of 1.0 mM veratryl alcohol, a MnP activity of 110.0 ± 1.4 U/L was determined in the 24th day of cultivation, compared to 147.5 ± 60.1 U/L (9th day of cultivation) (Figure 3).

With regard to Lac production, the addition of veratryl alcohol into the cultivation medium did not significantly improve the maximum activity of this enzyme. The supplementation with increasing concentrations of 2,5-xylidine, on the other hand, did; from 5.3 ± 2.9 U/L, in the medium without inducer, to 21.5 ± 4.9 U/L, in the medium supplemented with 1.0 mM of inducer (Table 1).

Such results confirm the inductive effect of 2,5-xylidine in stimulating the production of Lac by different fungi. For instance: *C. subvermispora* (Fukushima and Kirk, 1995), *Dichomitus squalens* (Perie et al., 1998), *Panus tigrinus* (Quaratino et al., 2008), *Pleurotus dryinus* (Elisashvili et al., 2006) and *T. versicolor* (Rancaño et al., 2003). According to Eggert et al. (1996), one of the functions of Lac is the detoxification of aromatic compounds that are highly reactive, promoting the formation of free radicals and their concomitant polymerization; being postuladed that the induction of Lac by 2,5-xylidine is due to a mechanism of defense developed by fungi to eliminate the toxic effects of this compound.

Effects of the addition of surfactant on the productions of MnP and Lac

Tween 80 is a surfactant that favors the excretion of extracellular enzymes by filamentous fungi. In order to evaluate the effect of this compound in the production of MnP and Lac, cultivations of *C. subvermispora* in medium supplemented with 11 ppm of Mn^{2+} and 1.0 mM of 2.5-xylidine, in the presence (0.05 or 0.50% v/v) or absence of Tween 80, were performed.

As can be seen in Table 1, the supplementation of the medium with Tween 80, in both concentrations, significantly improved the activities of both MnP and Lac. The maximum activities of MnP determined in the cultures supplemented with 0.05 and 0.50% (v/v) of Tween 80 were 174.8 ± 1.4 and 181.8 ± 23.3 U/L, respectively, compared to 99.5 ±19.1 U/L (medium not supplemented with surfactant). The maximum activities of Lac determined in the cultures supplemented with 0.05 and 0.50% (v/v) of Tween 80, on the other hand, were 53.3 ± 17.7 and 60.6 ± 5.7 U/L, respectively, compared to 21.5 ± 4.9 U/L (medium not supplemented with surfactant).

While, in the medium supplemented with 11 ppm Mn^{2+} and 1.0 mM 2,5-xylidine, the activity of MnP was relatively small and unstable, decreasing sharply after the 6^{th} day of cultivation, in the media supplemented with Tween 80, the activities of MnP reached considerably higher values that were maintained for relatively longer periods. Qualitatively, the same behavior was observed for Lac activities (Figure 4).

Couto et al. (2001), for example, also reported that the supplementation of a defined medium with 0.05% (v/v) Tween 80, in addition to 2 mM veratryl alcohol, improved the production of extracellular ligninolytic enzymes by immobilized *Phanerochaete chrysosporium*.

Metabolic behavior exhibited by the fungus during selected cultivations

As can be seen in Figure 5, in all the three selected cultivations, namely in the media supplemented with 11 ppm Mn^{2+}, with 11 ppm Mn^{2+} and 1.0 mM 2,5 xylidine, and with 11 ppm Mn^{2+}, 1.0 mM 2,5 xylidine and 0.05% v/v Tween 80, the fungus completely consumed the available glucose in the first 6 to 9 days of cultivation. Although there was considerable variation in the measurement of biomass concentrations, the growth of mycelium appears to have occurred mainly during this time, together with the consumption of ammonium ions. Afterwards, increases in the concentrations of ammonium were detected in all the three cultivations, coinciding with increases in the conductivities and pHs of the culture media.

From the above mentioned results, it can be inferred that the exhaustion of glucose in the medium promoted nutritional stress, which, in turn, led to cell autolysis; reflected by an apparent reduction in the concentration of mycelium, and by an increase in the concentration of ammonium in the medium. According to White et al. (2002), the phenomenon of cell autolysis is frequent during cultivations of filamentous fungi. Bainbridge et al. (1971), for example, reported the occurrence of cell autolysis, simultaneous to an increase in the concentration of ammonium in the medium, when continuous cultures of *Aspergilus nidulans* were submitted to limitation in the carbon source; but not when the fungus was grown in excess of this nutrient.

According to Eden and Eden (1984), the conductivity of fermentation media is related to the production and consumption of electrolytes due to the microbial metabolism. Colombie et al. (2007), for example, observed that the assimilation of ammoniacal nitrogen during cultivations of a wine making yeast led to the decrease in the conductivity of the fermentation medium. In the present study, as already mentioned, the initial decreases in the conductivities (and also in the pHs) of the fermentation media were followed by a period of increases in these parameters; which is compatible with an initial consumption of the ammonium available in the media, followed by the occurrence of cell autolysis due to glucose limitation.

It is worth to mention, as well, that the basal medium used in the present study was buffered, by the addition of trans-aconitic acid. This, however, did not prevent variations in the pH of the medium during the cultivations.

Last, it was observed that the concentrations of extracellular proteins increased throughout the cultivations (Figure 5). Maximum contents of 137.4 ± 12.2, 89.2 ± 13.3 and 52.9 ± 31.1 mg/L were achieved for the media supplemented with Tween 80, 2,5-xylidine and manganese, respectively. Such concentrations, however, did not generally exhibit good correlations with the measured enzyme activities.

No activities of lignin peroxidase (LiP) and cellobiose dehydrogenase (CDH) were detected during the cultivations (data not shown); in spite of the existence of studies reporting the production of LiP (Tanaka et al., 2009) and CDH (Harreither et al., 2009) by *C. subvermispora*. Moreover, it was not possible to stablish a correlation between the concentrations of extracellular proteins determined by Bradford's method with those determined by direct light absorption at 280 nm (data not shown); probably due to the presence of interfering compounds (Zaia et al., 1998). Similarly, it was not possible to stablish a correlation between the absorbances determined at 405 and 610 nm with the respective activities of MnP and Lac (data not shown); in spite of the existence of studies reporting the use of such spectral analyses in both qualitative and quantitative characterizations (Rubia et al., 2002; Cambria et al., 2000).

The ability of accumulating considerable amounts of

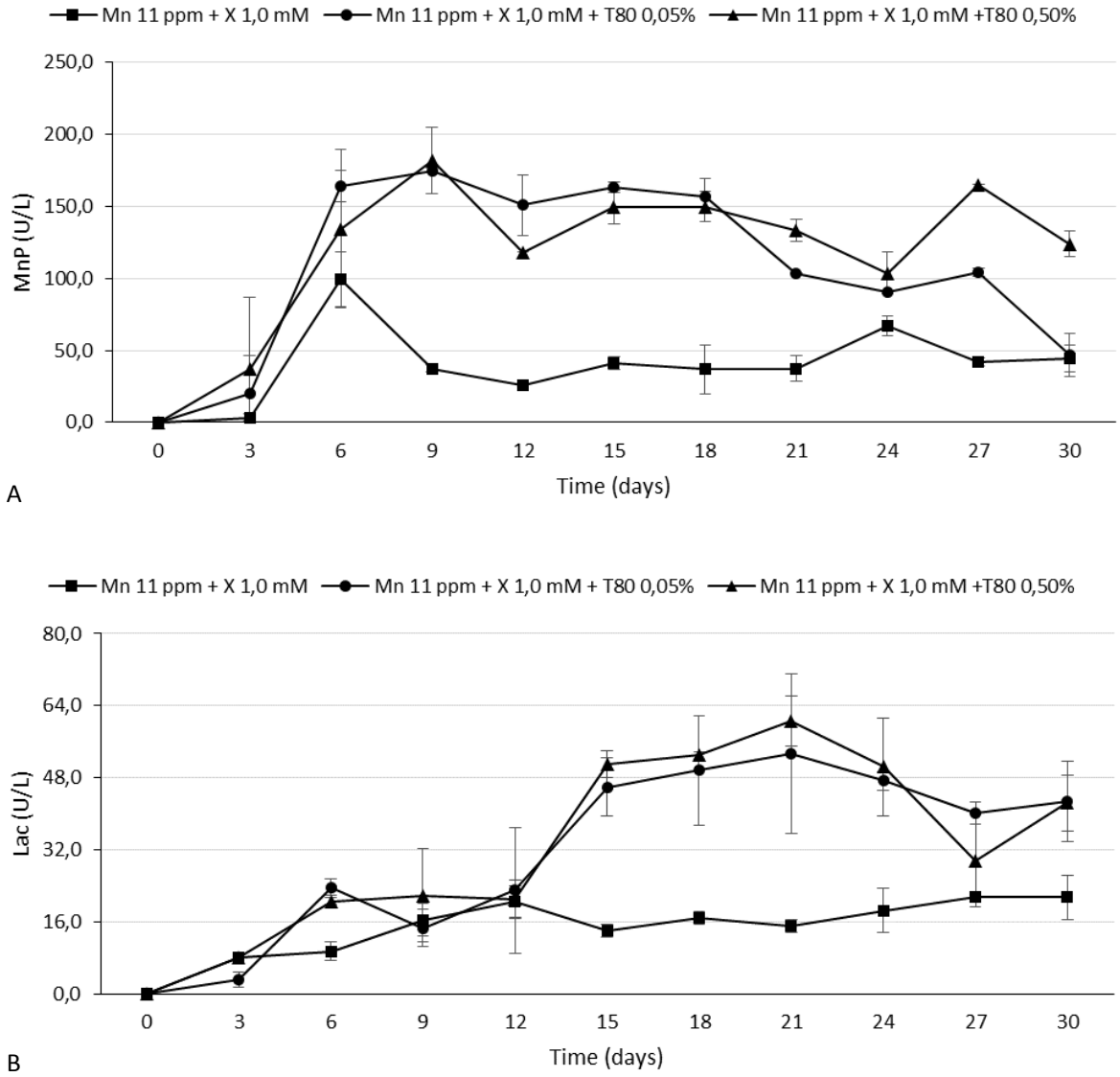


Figure 4. Activities of manganese peroxidase (A) and laccase (B) determined during the cultivations supplemented or not with different concentrations of Tween 80, in presence of Mn2+ (11 ppm) and 2,5-xylidine (1,0 mM). Error bars represent standard deviations, calculated from duplicates.

extracellular proteins, exhibited by ligninolytic fungi, is well reported in the literature. Galhaup et al. (2002), for example, reported that the concentration of extracellular proteins reached 700 mg/L at the end of a fed-batch cultivation of *Trametes pubescens* in a reactor of 20 L. The medium used for the submerged cultivation was composed by glucose (40 g/L), meat peptone (10 g/L), $MgSO_4 \cdot H_2O$ (1 g/L) and $CuSO_4 \cdot 5\ H_2O$ (2 mM); the feed, by a solution of glucose (320 g/L).

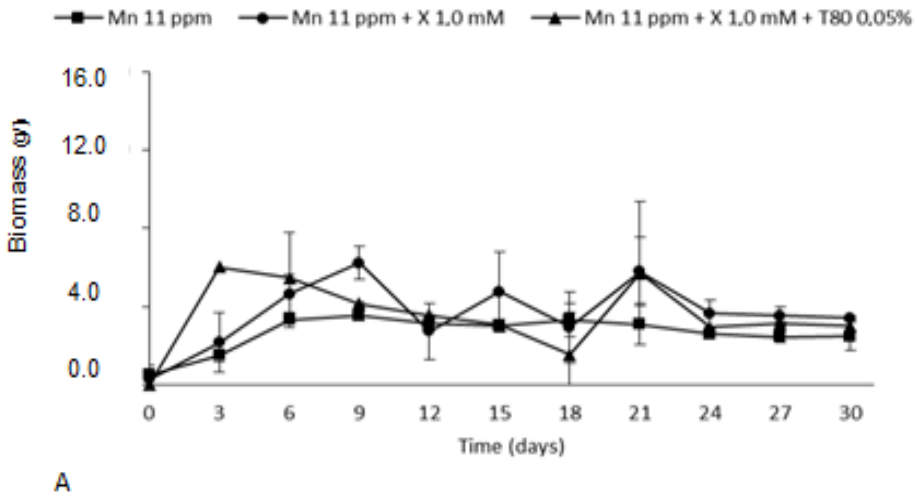
Conclusions

While the supplementation of the basal medium with manganese stimulated the production of MnP, the suppression of this metal in the medium led to a higher production of Lac.

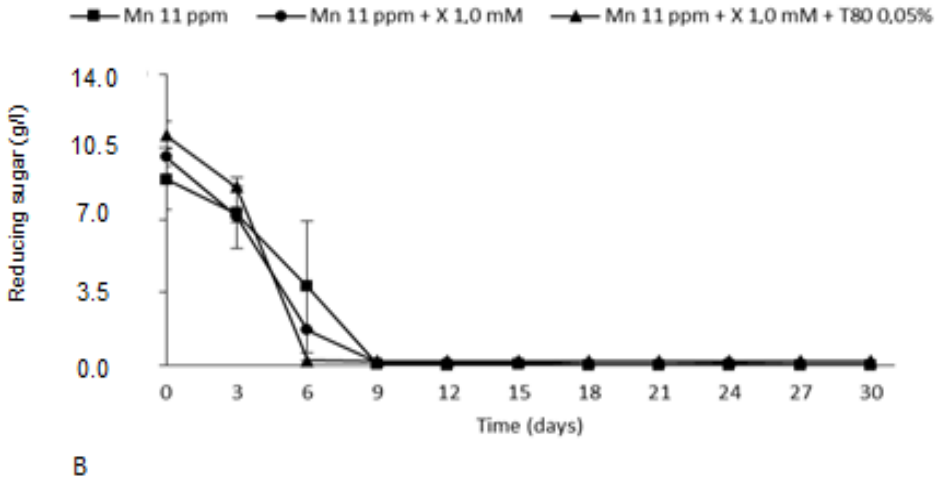
The supplementation of the medium containing 11 ppm Mn^{2+} with 1.0 mM 2,5-xylidine stimulated a higher production of Lac; the supplementation of the same medium with 1.0 mM veratryl alcohol, generated a second peak of MnP activity.

Tween 80 improved the activities of both MnP and Lac determined during the cultivations; the maximum values were significantly higher than that determined in the medium without surfactant, and were also maintained for relatively longer periods.

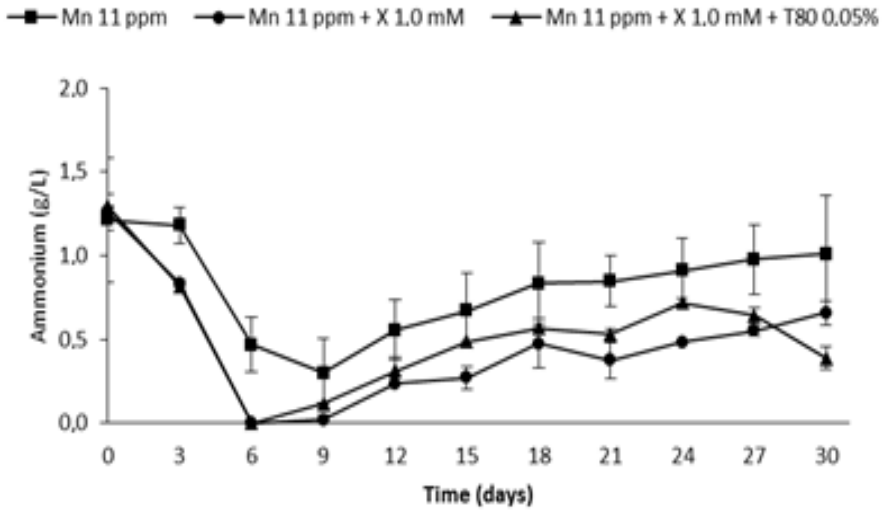
The exhaustion of the glucose available in the culture


Mn 11 ppm
Mn 11 ppm + X 1,0 mM
Mn 11 ppm + X 1,0 mM + T80 0,05%
Biomass (g)
16.0
12.0
8.0
4.0
0.0
0 3 6 9 12 15 18 21 24 27 30
Time (days)

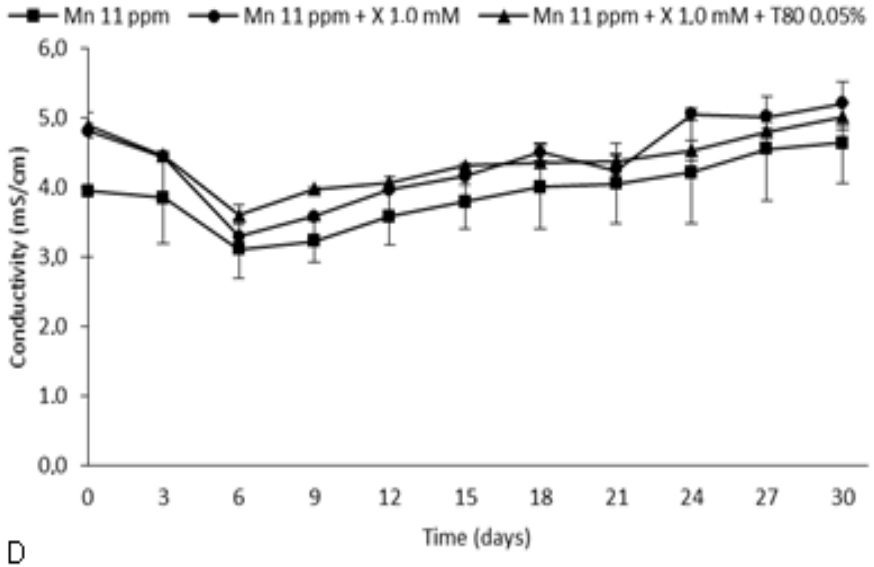
A


Mn 11 ppm
Mn 11 ppm + X 1,0 mM
Mn 11 ppm + X 1,0 mM + T80 0,05%
Reducing sugar (g/l)
14.0
10.5
7.0
3.5
0.0
0 3 6 9 12 15 18 21 24 27 30
Time (days)

B


Mn 11 ppm
Mn 11 ppm + X 1,0 mM
Mn 11 ppm + X 1,0 mM + T80 0,05%
Ammonium (g/L)
2,0
1,5
1,0
0,5
0,0
0 3 6 9 12 15 18 21 24 27 30
Time (days)

C


Mn 11 ppm
Mn 11 ppm + X 1,0 mM
Mn 11 ppm + X 1,0 mM + T80 0,05%
Conductivity (mS/cm)
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0 3 6 9 12 15 18 21 24 27 30
Time (days)

D

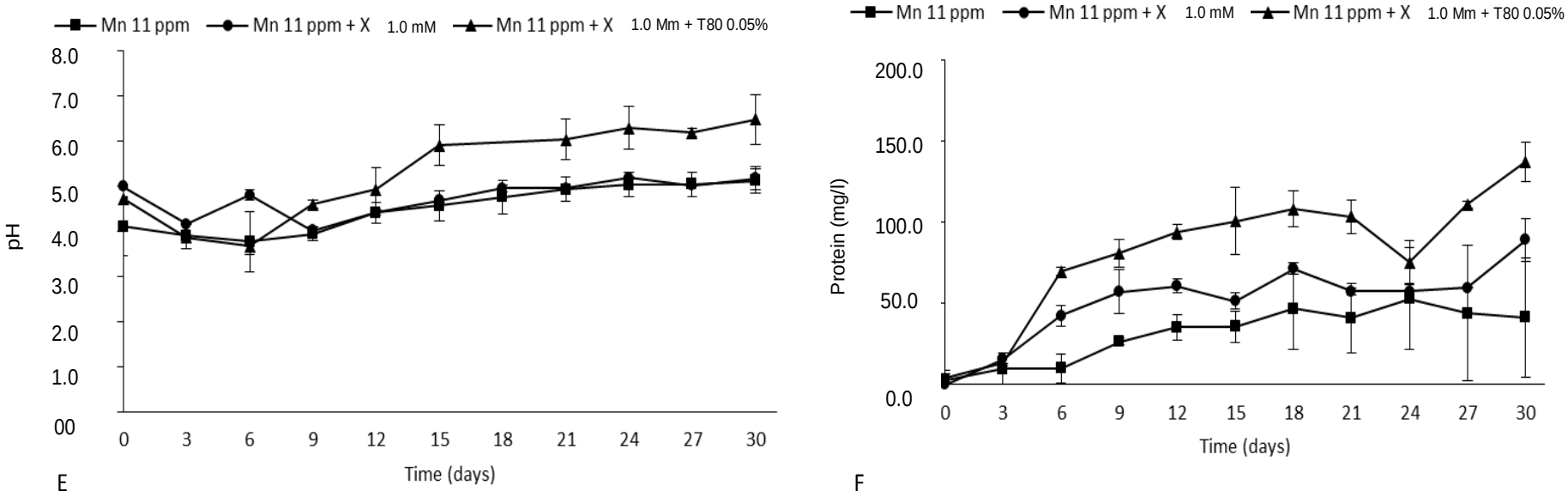


Figure 5. Concentrations of biomass (A), reducing sugars (B), ammonium ions (C) and proteins (F), and values of conductivity (D) and pH (E) determined during the cultivations in selected media. Error bars represent standard deviations, as described in Figures 1, 2 and 4. Mn – Mn^{2+}; X – 2,5-xylidine; T80 – Tween 80.

medium led to cell autolysis; reflected by an apparent reduction in the concentration of mycelium, and by an increase in the concentration of ammonium in the medium.

The concentrations of extracellular proteins increased throughout the cultivations; such concentrations, however, did not generally exhibit good correlations with the measured enzyme activities.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Aguiar A, Ferraz A (2008). Relevance of extractives and wood transformation products on the biodegradation of *Pinus taeda* by *Ceriporiopsis subvermispora*. Int. Biodeterior. Biodegr. 61:182-188.

Akhtar M, Scott GM, Swaney RE, Shipley DF (2000). Biomechanical pulping: a mill-scale evaluation. Resour. Conserv. Recy. 28:241-252.

Arora DS, Gill PK (2000). Laccase production by some white rot fungi under different nutritional conditions. Bioresour. Technol. 73:283-285.

Asther M, Corrieu G, Drapron R, Odier E (1987). Effect of Tween80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-12. Enzyme Microb. Technol. 9:245-249.

Bainbridge BW, Bull AT, Pirt SJ, Rowley BI, Trinci APJ (1971). Biochemical and structural changes in non-growing maintained and autolysing cultures of *Aspergillus nidulans*. Mycol. Res. 56:371-385.

Bonnarme P, Jeffries TW (1990). Mn (II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. Appl. Environ. Microbiol. 56:210-217.

Bourbonnais R, Paice MG (1990). Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. 267: 99-102.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Buswell JA, Cai Y, Chang S (1995). Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Lentinula (Lentinus) edodes*. FEMS Microbiol. Lett. 128:81-88.

Cambria MT, Cambria A, Ragusa S, Rizzareli E (2000). Production, purification, and properties of an extracellular laccase from *Rigidoporus lignosus*. Protein Expr. Purif. 18:141-147.

Carvalho W, Ferraz A, Milagres AMF (2008). Clean-up and concentration of manganese peroxidases recovered during

the biodegradation of *Eucalyptus grandis* by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. 43:193-198.
Colombie S, Latrille E, Sablayrolles JM (2007). Online estimation of assimilable nitrogen by electrical conductivity measurement during alcoholic fermentation in enological conditions. J. Biosci. Bioeng. 103:229-235.
Couto SR, Ratto M, Dominguez A, Sanroman A (2001). Strategies for improving ligninolytic enzyme activities in semi-solid state bioreactors. Process Biochem. 36:995-999.
Daina S, Orlandi M, Bestetti G, Wiik C, Elegir G (2002). Degradation of β-5 lignin model dimmers by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. 30:499-505.
Dekker RFH, Barbosa AM (2001). The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete *Botryosphaeria* sp. Enzyme Microb. Technol. 28:81-88.
Dekker RFH, Barbosa AM, Giese EC, Godoy SDS, Covizzi LG (2007). Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05. Int. Microbiol. 10:177-185.
Eden R, Eden G (1984). Impedance microbiology. Research Studies Press Ltd. pp. 93-98.
Eggert C, Temp U, Eriksson KE (1996). The ligninolytic system of the white-rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl. Environ. Microbiol. 62:1151-1158.
Elisashvili V, Penninckx M, Kachlishvili E, Asatiani M, Kvesitadze G (2006). Use of *Pleurotus dryinus* for lignocellulolytic enzymes production in submerged fermentation of mandarin peels and tree leaves. Enzyme Microb. Technol. 38:998-1004.
Fernandez-Fueyo E, Ruiz-Dueñas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P, Larrondo LF, James TY, Seelenfreund D, Lobos S, Polanco R, Tello M, Honda Y, Watanabe T, San RJ, Kubicek CP, Schmoll M, Gaskell J, Hammel KE, John FJS, Wymelenberg AV, Sabat G, Bondurant SS, Syed K, Yadav JS, Doddapaneni H, Subramanian V, Lavin JL, Oguiza JA, Perez G, Pisabarro AG, Ramirez L, Santoyo F, Master E, Coutinho PM, Henrissat B, Lombard V, Magnuson JK, Kues U, Hori C, Igarashi K, Samejima M, Held BW, Barry KW, Labutti KM, Lapidus A, Lindquist EA, Lucas SM, Riley R, Salamov AA, Hoffmeister D, Schwenk D, Hadar Y, Yarden O, Vries RP, Wiebenga A, Stenlid J, Eastwood D, Grigoriev IV, Berka RM, Blanchette RA, Kersten P, Martinez AT, Vicuña R, Cullen D (2012a). Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc. Natl. Acad. Sci. U.S.A. 109: 5458-5463.
Fernandez-Fueyo E, Ruiz-Dueñas FJ, Miki Y, Martinez MJ, Hammel KE, Martinez AT (2012b). Lignin-degrading peroxidases from the genome of the selective ligninolytic fungus *Ceriporiopsis subvermispora.* J. Biol. Chem. 287: 16903-16916.
Fukushima Y, Kirk T K (1995). Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. Appl. Environ. Microbiol. 61:872-876.
Galhaup C, Wagner H, Hinterstoisser B, Haltrich D (2002). Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme Microb. Technol. 30:529-536.
Gutierrez M, Rojas LA, Mancilla-Villalobos R, Seelenfreund D, Vicuña R, Lobos S (2008). Analysis of manganese-regulated gene expression in the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Curr. Genet. 54:163-173.
Harreither W, Sygmund C, Dünhofen E, Vicuña R, Haltrich D, Ludwig R (2009). Cellobiose dehydrogenase from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Appl. Environ. Microbiol. 75(9):2750-2757.
Hofrichter M (2002). Review: Lignin conversion by manganese peroxidase (MnP). Enzyme Microb. Technol. 30:454-466.
Khindaria A, Grover TA, Aust SD (1994). Oxalate-dependent reductive activity of manganese peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. 314: 301-306.
Manubens A, Avila M, Canessa P, Vicuña R (2003). Differential regulation of genes encoding manganese peroxidase (MnP) in the basidiomycete *Ceriporiopsis subvermispora*. Curr. Genet. 43:433-438.
Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.
Perie FH, Reddy GVB, Blackburn NJ, Gold MG (1998). Purification and characterization of laccases from the white-rot basidiomycete *Dichomitus squalens*. Arch. Biochem. Biophys. 353: 349-355.
Quaratino D, Ciaffi M, Federici E, D'Annibale A (2008). Response surface methodology study of laccase production in *Panus tigrinus* liquid cultures. Biochem. Eng. J. 39:236-245.
Rancaño G, Lorenzo M, Molares N, Couto SR, Sanromán A (2003). Production of laccase by *Trametes versicolor* in an airlift fermentor. Process Biochem. 39:467-473.
Rubia T, Linares A, Perez J, Muñoz-Dorado J, Romera J, Martinez J (2002). Characterization of manganese dependent peroxidase isoenzymes from the ligninolytic fungus *Phanerochaete flavido-alba*. Res. Microbiol. 153:547-554.
Rüttimann C, Schwember E, Salas L, Cullen D, Vicuña R (1992). Ligninolytic enzymes of the white-rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Biotechnol. Appl. Biochem. 16:64-76.
Rüttimann-Johnsson C, Salas L, Vicuña R, Kirk TK (1993). Extracellular enzyme production and synthetic lignin mineralization by *Ceriporiopsis subvermispora.* Appl. Environ. Microbiol. 59:1792-1797.
Tanaka H, Koike K, Itakura S, Enoki A (2009). Degradation of wood and enzyme production by *Ceriporiopsis subvermispora*. Enzyme Microb. Technol. 45: 384-390.
Ürek RÖ, Parzalioglu NK (2005). Production and stimulation of manganese peroxidase by immobilized *Phanerochaete chrysosporium*. Process Biochem. 40:83-87.
Weatherburn MW (1967). Phenol-hypoclorite reaction for determination of ammonia. Anal. Chem. 38: 971-974.
White S, McIntyre M, Berry DR, McNeil B (2002). The autolysis of industrial filamentous fungi. Crit. Rev. Biotechnol. 22:1-14.
Zaia DAM, Zaia CTBV, Lichtig J (1998). Determinação de proteínas totais via espectrofotometria: vantagens e desvantagens dos métodos existentes. Quim. Nova. 21:787-793.