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Effect of Heavy Metals on the Degradative Activity by Wood-Rotting Fungi

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Polluted wastewaters often have recalcitrant aromatic compounds together with heavy metals (Babich et al. 1982; Gabriel et al. 1996). Before disposal into water courses, their removal and degradation is imperative; however this goal is complex and difficult to achieve (Dott et al.1995). One of the possible alternatives studied for degradation of these compounds is the use of ligninolytic fungi (Field et al. 1992; Kotterman et al. 1996). The process based on the use of white rot fungi depends on the activity of enzymatic complexes such as laccase, lignin peroxidase (LiP) and especially the most ubiquitous peroxidase in most of the white-rot fungi: manganese peroxidase (MnP) (Pelaez et al. 1995). Manganese peroxidase catalytic action enables the degradation of mixtures of refractory substances owing to their non-specific degradative ability.

The influence of heavy metals on mycelial growth of several basidiomycetes has been determined (Gabriel et al.1996; Falih 1997). These metals influence the fungal physiology in several ways, either by inhibition of the fungal growth (Babich and Stotzky 1977; Rogers and Li 1985) or by repression of the secretion of enzymes produced both in primary and secondary metabolism (Balbrian et al.1996). Both drawbacks may be overcome by the selection of fungal strains, which can develop their oxidative system in the presence of metal ions.

The objective of this work is the screening of fungal strains, which present neither growth inhibition nor ligninolytic activity suppression in the presence of different heavy metal ions. The strains selected for this study were reported to have a proved strong ligninolytic activity (Moreira et al., 1997). Not only the effect of one single metal ion was evaluated on growth and ligninolytic activity, but also combination of two and three different metal ions were considered with two objectives. These objectives are: i) to investigate the application of fungal strains in much more complex case studies, as for example, degradation of recalcitrant compounds in wastewaters with high concentrations of heavy metals, a common event in a great variety of industrial effluents; and ii) the synergist or antagonist effect obtained by the combination. The results obtained will provide further knowledge on the degradative capability of these microorganisms.

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MATERIAL AND METHODS

Polyporus ciliatus and *Stereum hirsutum* were obtained from the Culture Collection of Basidiomycetes, Department of Chemical Engineering, University of Santiago de Compostela, Spain. All strains were maintained at 4°C on peptone yeast extract slants from which it was transferred to glucose malt extract (ME) plates (Mester et al. 1996). The ME plates were incubated at 25°C for 5 to 6 days. Two agar plugs (5-mm diameter) were punched from the leading edge and used as inoculum for the experiments.

$CdCl_2$, $ZnCl_2$, $Pb(NO_3)_2$, $Ni(NO_3)_2$, $K_2Cr_2O_7$, $MnSO_4$ and sodium tartrate were obtained from Aldrich, phenol red was obtained from Merck and Poly R-478 was obtained from Sigma. All chemicals possess analytical grade.

To find out, in a qualitative way, the threshold concentration of metal ions for which the selected strains showed any kind of inhibition, both fungi were incubated in Petri plates. The culture medium used for Petri plates contained ME, different concentrations of one of the following metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+} or Cr^{6+}) and the polymeric dye Poly R-478 (0.02 %wt/v). All cultures assays were performed by quadruplicate.

Thereafter, both fungi were cultured in Erlenmeyer flasks to evaluate the effect of the metal ions on Poly R-478 decolorization and MnP production (assays carried out in the absence of the dye). Erlenmeyer flasks containing 10 mL of culture medium were inoculated with two agar plugs of both fungi and incubated statically under an air atmosphere at 25°C during 14 days. The culture medium used contained 10 g/L glucose, 0.2 g/L diammonium tartrate, 16 g/L sodium acetate (pH 4.5), 100 mL/L BIII mineral medium (Tien and Kirk 1988) with and without the polymeric dye Poly R-478 (0.02 %wt/v). Metal ions were added as $CdCl_2$, $ZnCl_2$, $Pb(NO_3)_2$, $Ni(NO_3)_2$, $K_2Cr_2O_7$, $MnSO_4$ in concentrations ranging from 5 ppm to 20 ppm. Controls with no metal addition were run in parallel. The medium was autoclaved (120°C for 20 min) and then a filter-sterilized thiamine solution (400 mg/L) was added (5 mL/L). In the case of *S. hirsutum*, the combination of the addition of several heavy metal was analysed for their effect on Poly R-478 decolorization, mycelial growth and enzymatic secretion. The composition of the culture medium was the one described previously with the combination of the different metal ions detailed in Tables 2 and 3. The mycelium was removed from the culture medium by filtration through Whatman GF/C (Cat No 1822 047). After filtration, the mycelium was thoroughly washed with deionised water. The weight of fungal mycelium retained by the filter paper was determined after drying to constant weight in an oven at 80°C for 24 hr as a measure of fungal mycelium dry weight. The activity of MnP was measured by a modified method previously described by Kuwahara (1984). This method uses 0.01 %wt/v phenol red as substrate in the presence of 0.1 mM H_2O_2 and 0.1 mM $MnSO_4$ in 100 mM sodium tartrate (pH 5.0). The mixture was incubated at 30°C for 5 min and the reaction was stopped by the addition of NaOH (0.2 mM final concentration). The absorbance increase was measured (ε_{610}= 4460 $M^{-1}cm^{-1}$) in a Bausch & Lomb, Spectronic 21

spectrometer. One unit (U) of peroxidase oxidizes 1μmol of substrate/min. The decolorization of the polymeric dye (Poly R-478) in the liquid culture was expressed as the rate of decolorization (decrease in the absorbance ratio A_{520}/A_{350} versus days). When harvested, the cultures were centrifuged at 2000 rpm during 15 min and absorbance at 520 and 350 nm was measured in the culture fluid with a Hach DR 4000/V, UV-VIS spectrometer. All assays were performed by quadruplicate.

The polymeric dye, Poly R-478, was selected as degradation substrate due to the lack of evidence in chemical interaction such as: no precipitates formation, no spectral alterations and no redox reactions with metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+} or Cr^{6+}).

RESULTS AND DISCUSSION

Initially, metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+} or Cr^{6+}) were assayed in different concentrations of 5, 10, 15 or 20 ppm to evaluate if their presence had any significant effect on fungal growth and dye decolorization in *Polyporus ciliatus* and *Stereum hirsutum*. This analysis was performed in fungal cultures growing on Petri plates at 25°C with or without the addition of Poly R-478, which was selected as an indicator of the peroxidase production (Glenn and Gold 1983). During 14 days, the growing diameter of the mycelium was measured and the decolorization of the dye checked.

The results showed no notable difference in fungal growth for any metal ion at concentrations lower than 5 ppm (Table 1). Higher concentrations of Cd^{2+}, Ni^{2+}, Pb^{2+} and Cr^{6+} produced a remarkably negative effect on the mycelial growth. No growth was observed for 15 ppm Pb^{2+} and 20 ppm of Ni^{2+} and Cr^{6+}. Other authors indicate that only at concentrations as high as 1 mM Pb^{2+} and Cr^{6+} (Baldrían and Gabriel, 1997), the mycelial growth was affected for *S. hirsutum.*

On the basis of these results, experiments were performed in Erlenmeyer flasks with culture medium containing 0, 1, 5, or 10 ppm of Zn^{2+}, Cd^{2+}, Cr^{6+}, Pb^{2+} or Ni^{2+}. The decolorization of Poly R-478 was quantified during 16 days in parallel experiments in the presence of the dye.

Figure 1 shows that *S. hirsutum* was the strain with the highest decolorization rate. For all the concentrations assayed with Zn^{2+} and Cd^{2+}, no significant decrease of decolorization rate was observed. However, for increasing concentrations of Pb^{2+}, Cr^{6+} and Ni^{2+} a progressive inhibitory effect on the decolorization rate was obtained, this effect being exhibited at 5 ppm of Pb^{2+} or Ni^{2+} and 10 ppm of Cr^{6+}.

The effect of the different metal ion concentrations on the activity of MnP was also evaluated for both fungal strains. Figure 2 shows the maximum MnP activity at each culture condition. For all of the concentrations assayed for Zn^{2+} and Cd^{2+}, no significant diminution of MnP activity was observed. However for both strains the

Table 1. Mycelial growth in solid culture media in the presence of metal ions.

Specie	Concentrations tested	Zn^{+2}	Cd^{+2}	Ni^{+2}	Pb^{+2}	Cr^{+6}
Polyporus Ciliatus	5 ppm	++++	++++	++++	++++	++++
	10 ppm	++++	+++	++	++	++
	15 ppm	++++	++	+	—	++
	20 ppm	++++	+	—	—	—
Stereum Hirsutum	5 ppm	++++	++++	++++	++++	++++
	10 ppm	++++	+++	++	++	++
	15 ppm	++++	+++	++	—	++
	20 ppm	++++	++	—	—	—

Ref.: 100% of covered plate ++++ ; 75% of covered plate +++; 62,5% of covered plate ++; 25% of covered plate + ; without mycelial growth — .

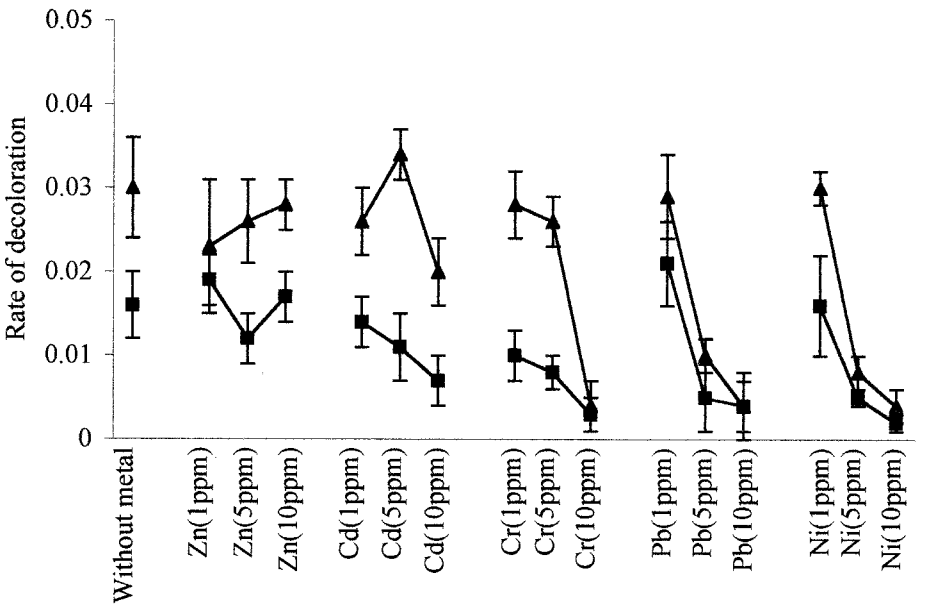

Figure 1. Mean values ± standard desviation for the decoloration rate (decrease in the absorbance ratio A_{520}/A_{350} versus days) of Poly R-478 in presence of metal ions on *P.ciliatus* (-□-) and *S. hirsutum* (-□-).

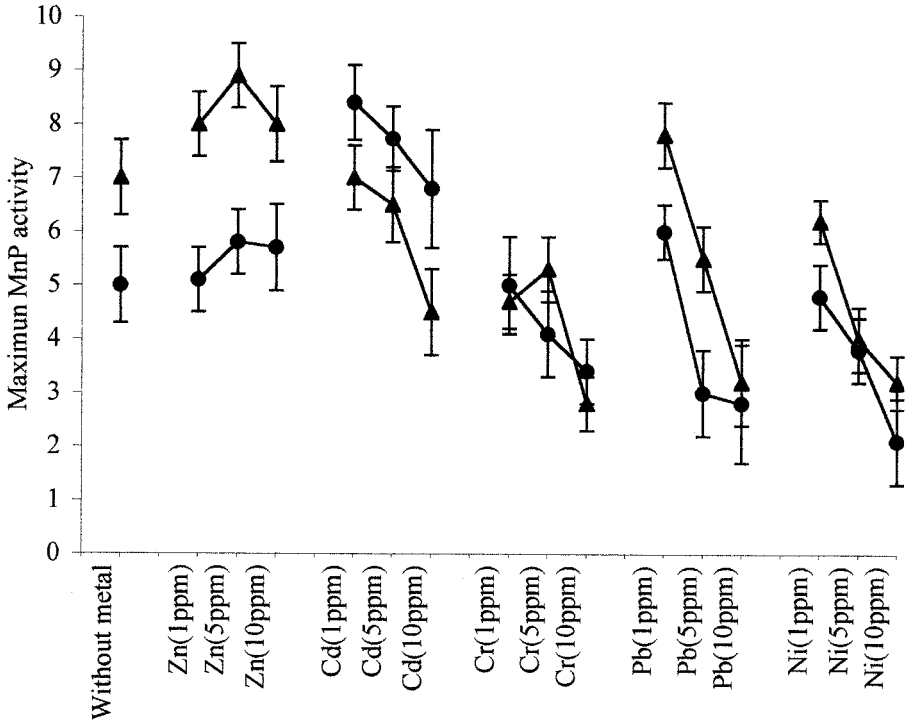

Figure 2. Mean values ± standard desviation for Maximun MnP activity (in U) in presence of metal ions on *P. ciliatus* (-□-) and S. *hirsutum* (-□-).

MnP activity decreased at higher concentration for Cr^{6+}(10 ppm), Pb^{2+} (5 and 10 ppm) and Ni^{2+} (5 and 10 ppm). The comparison between MnP activity and decolorization rate of Poly R-478 at different concentrations of the metal ions evidenced a parallel diminution for both parameters (Figures 1 and 2).

As *S. hirsutum* showed the highest growth and degradation rates, this fungus was selected to study the effect of the presence of two or three heavy metal ions in the culture media.

These results would be of significance in order to determinate the synergist or antagonist effects caused by the simultaneous presence of toxic metal ions. For this purpose, all possible dual combinations of Zn^{2+}, Ni^{2+}, Cd^{2+}, Pb^{2+} and Cr^{6+} were assayed at concentrations that had previously no inhibitory effect on decolorization rate and MnP production for the single ion considered.

For this assay *S. hirsutum* was inoculated in 10 mL of culture solution with and without Poly R-478 at different concentrations of two or three toxic metal ions. The system was cultivated at 25°C during 14 days. Under these conditions, neither

Table 2. Effect of ionic metal pairs on *S hirsutum.* Mean values ± standard desviation for this analyses .

Ionic metal pairs	Concentrations tested	Rate of decoloration for Poly R-478	Maximum MnP activity
Without metal		0.0730 ± 0.0092	5.78 ± 1.13
$Zn^{+2} + Cd^{+2}$	(10+10) ppm	0.0748 ± 0.0133	5.44 ± 0.80
$Zn^{+2} + Cr^{+6}$	(10+5) ppm	0.0847 ± 0.0081	4.08 ± 0.98
$Zn^{+2} + Pb^{+2}$	(10+1) ppm	0.0595 ± 0.0119	4.77 ± 0.69
$Zn^{+2} + Ni^{+2}$	(10+1) ppm	0.0668 ± 0.0123	5.71 ± 1.05
$Cd^{+2} + Cr^{+6}$	(10+5) ppm	0.0738 ± 0.0024	4.85 ± 0.89
$Cd^{+2} + Pb^{+2}$	(10+1) ppm	0.0662 ± 0.0051	4.02 ± 0.74
$Cd^{+2} + Ni^{+2}$	(10+1) ppm	0.0585 ± 0.0098	4.34 ± 0.61
$Cr^{+6} + Pb^{+2}$	(5+1) ppm	0.0581 ± 0.0064	4.09 ± 1.21
$Cr^{+6} + Ni^{+2}$	(5+1) ppm	0.0705 ± 0.0081	3.67 ± 0.72
$Pb^{+2} + Ni^{+2}$	(1+1) ppm	0.0731 ± 0.0097	3.50 ± 0.88

Table 3. Efects of three metal ions on *S hirsutum* . Mean values ± standard desviation for these analyces.

Metal ions	Concentrations tested	Rate of decoloration for Poly R-478	Maximum MnP activity
Without metal		0.0645 ± 0.0072	5.05 ± 0.55
$Zn^{+2} + Cd^{+2} + Cr^{+6}$	(10+10+5) ppm	0.0640 ± 0.0171	4.58 ± 0.70
$Pb^{+2} + Zn^{+2} + Cd^{+2}$	(1+10+10) ppm	0.0730 ± 0.0234	5.57 ± 1.02
$Pb^{+2} + Zn^{+2} + Cr^{+6}$	(1+10+5) ppm	0.0640 ± 0.0046	6.19 ± 0.77
$Pb^{+2} + Cd^{+2} + Cr^{+6}$	(1+10+5) ppm	0.0648 ± 0.0070	4.55 ± 0.63
$Cr^{+6} + Cd^{+2} + Ni^{+2}$	(5+10+1) ppm	0.0710 ± 0.0143	5.68 ± 1.22
$Cr^{+6} + Zn^{+2} + Ni^{+2}$	(5+10+1) ppm	0.0809 ± 0.0154	5.45 ± 0.62
$Cd^{+2} + Zn^{+2} + Ni^{+2}$	(10+10+1)ppm	0.0785 ± 0.0120	6.23 ± 0.75
$Cr^{+6} + Pb^{+2} + Ni^{+2}$	(5+1+1) ppm	0.0843 ± 0.0122	4.18 ± 1.10
$Zn^{+2} + Pb^{+2} + Ni^{+2}$	(10+1+1) ppm	0.0665 ± 0.0170	6.22 ± 0.97
$Cd^{+2} + Pb^{+2} + Ni^{+2}$	(10+1+1) ppm	0.0818 ± 0.0140	5.24 ± 0.31

mycelial growth nor decolorization of the dye was observed (data not shown). Taking into account these results, it was concluded that the presence of several ions strengthen the hypothesis that toxic effects of heavy metals against any organism include inhibition of growth, induction of morphological changes and a variety of metabolic changes such as the inhibition of enzymes (Gadd 1992).

To overcome the fact that *S. hirsutum* was unable to grow and decolorize Poly R-478 in the presence of two or three heavy metal ions, another assay considered the degradation of Poly R-478 in a liquid culture media once the lag stage of the fungal growth was over. *S. hirsutum* was inoculated in 10 mL of culture medium at 25°C during 6 days and allowed to grow in the absence of metal ions. Thereafter, the culture medium was extracted and new media containing 0.02 %wt/v of Poly R-478 and two or three metal ions were supplemented. The values obtained are shown in Table 2 and 3. The toxic effect in all of the cases was negligible. No significant decrease in the decolorization rate and the MnP activity was observed in experiments using 6-day free-metal ions fungal inoculum.

The results obtained from this study suggest that the toxic effect caused by Cd^{2+}, Cr^{6+}, Zn^{2+}, Pb^{2+} and Ni^{2+} and the antagonist effect caused by the presence of two or three heavy metal ions in the culture media was overcomed in *S. hirsutum* strain, provided that the addition was performed once the lag stage of growth of the mycelium was over. Under these conditions the culture presented a normal growth (regarding the growth without heavy metal ions) and its enzymatic system decolourised efficiently the polymeric dye Poly R-478.

The toxic effect of the metal ions that inhibits the degradative activity of *S. hirsutum*, and at the same time involves certain mechanisms that act on the lag stage (Buswell et al., 1987) can be avoided. Therefore, the white rot fungus *S. hirsutum* can be considered as a potential biodegrading fungus in a liquid media polluted with heavy metal ions.

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