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Biosorption of heavy metals by *Talaromyces helicus*: a trained fungus for copper and biphenyl detoxification

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Abbreviations: BP: biphenyl
DBF: dibenzofuran
MM: mineral medium
RF-HPLC: reverse phase
SEM: scanning electron microscopic

At present, it is common to observe environments with organic and inorganic pollution, defined as co-contamination. Most industrial and urban effluents releases both pollutant types, leading to a complex environmental problem, as the biota must be tolerant to both xenobiotics. *T. helicus*, an efficient strain to degrade biphenyl, was trained with high copper levels, and became co tolerant to cobalt, lead and cadmium

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when was cultured in their presence. The copper adaptation was the result of physiological mechanisms, and the activated biochemical processes conferred resistance to Cu^{2+} as well as to other heavy metals. Furthermore, the Cu^{2+} adaptation of the mycelium was also transferred to the spores, that removed twice as much copper from solution than those of the no trained parentals. Interestingly, metals combinations were less toxic than single ones, and co tolerance development indicated that the cellular mechanisms that conferred resistance were non-specific, so the micobiota isolated from co contaminated environments often exhibited resistance to more than one ions. These results emphasized the detoxification abilities of *T. helicus* and the adaptation to heavy metals and biarylic compounds. This data is significant for the environmental biotechnology, suggesting that such tolerance and co tolerance could be acquired in natural environments. So a simple bioremediation strategy could enhance the detoxification of these polluted areas, as the degrader organisms could be present.

Remediation of co-contaminated sites with organic and metallic pollutants is a complex problem, as the components must be differently treated, yet 55% of the hazardous waste sites are co-contaminated (Roane et al. 2001; Sandrin and Maier, 2003). Alternatively, the area may be capped to prevent metal-transporting, or to reduce metal-mobilization by anaerobiosis (Roane et al. 1996; Kong, 1998; Kamashwaran and Crawford, 2001), or organic matter and clay additions (Malakul et al. 1998; Jonioh et al. 1999; Neilson and Maier, 2001), or environmental pH-changes (Majumdar et al. 1999; Sandrin and Maier, 2002) that determine ions bioavailability and affect the biota (Zoumis et al. 2001).

In either case, metal removal or stabilization is likely to be the first step to detoxificate co-contaminated sites (Atlas and Unterman, 1999; Liu et al. 2001), as inorganic pollutants in the ionic forms inhibit remediation through interaction with enzymes directly involved in biodegradation (*e.g.* specific oxygenases) or in general metabolism, by binding to the enzymes sulfhydryl-groups (Nies, 1999).

Unfortunately, few studies provide information on the metal levels that decreased the biotransformation due to the wide range of reported concentrations, and to different experimental protocols ions bioavailability and toxicity (Ricart et al. 2004). Alternatively, geo-chemical modelling software predicted metal speciation as a function of pH and ionic strength, and at least three computational models were developed to assess the ions impact on organics detoxification (Jin and Bhattacharya, 1996; Nakamura and Sawada, 2000; Amor et al. 2001).

Therefore, the aims of this study were to assess: a) the physiological adaptation to Cu^{2+} in *Talaromyces helicus* cultures in BP presence, b) the transference of Cu^{2+}-tolerance from the mycelium to the spores and to confer this resistance to other heavy metals and c) to quantify the inhibitory effects of metals on the fungal transformation BP.

MATERIALS AND METHODS

Fungal isolation and identification

T. helicus was isolated from co-contaminated sludge of the East Channel, near the YPF-oil Refinery, La Plata, Argentina. Diluted sediments were plated on MM-BP (Romero et al. 2001) for the presence of biarylic degrading fungi by standard spread-plate methods, by triplicate. The species were purified by streaking repeatedly on the same medium, and the fungus was identified by colonies showing typical yellow reverse, by ascospores that were only delicately spinulose, and the shaped ascogonia around which thin antheridia coil tightly, soon grew into a large terminal coil from which the ascogonous hyphae originated, and SEM observation.

Culture conditions and metabolites identification

One-hundred ml MM with 2% glucose was inoculated with mycelial plugs (4 mm ø) of the previous culture. After 3 days incubation at 30ºC, a sloop full was used as inoculum of 100 ml MM-BP, and incubated at 30ºC, 180 rpm. Controls with the fungus without BP and not inoculated flasks were made, by triplicate, and the standard deviations were no more than 10%.

At different sampling periods, 1 ml culture was centrifuged (5000 x g, 5 min), and metabolites were analyzed in 100 µl supernatant separated from the mycelium by centrifugation during 130 hrs, the supernatants were extracted twice with ethyl-acetate at pH 7, and once again at pH 2. The organic phases were dried over Na_2SO_4, and the solvent removed by evaporation. The obtained residues were dissolved in methanol. The mycelium was washed twice with 5 ml methanol; the methanolic extract was dried over Na_2SO_4 and the solvent reduced to 1 ml by evaporation. The three extracts were analyzed by RF-HPLC (Hammer and Schauer, 1997). The UV-visible absorption spectra of the degradation products were determined in a diode array detector HP 1040 (Hewlett Packard, Bad Homburg, Germany), and the BP-derivatives were identified by comparison with standard compounds.

Heavy-metals training

T. helicus grew on MM-BP and was trained to increasing Cu^{2+} -levels by serial transfer every 3 days to medium with 200, 300, 400, 500 and 600 ppm Cu^{2+}. The media were adjusted to pH 5, as metals occurs as divalent cations at this pH. Not-exposed strains grew on the same medium without Cu^{2+} (non-trained strain), all the assays were done by triplicate.

Table 1. Copper removed by non- and trained *T. helicus* at different Cu levels.

Cu added	Cu removed (%, respect to added Cu)	
(ppm)	non trained strains	Cu-trained strains
200	4.7	8.9
300	9.4	17.9
400	9.8	28.0
500	8.5	39.5
600	7.5	52.0

Then, a second assay were performed, and non- and Cu^{2+} -training biotypes (plugs: 4 mm ø) were plated in MM-BP amended with 50 to 700 ppm Cd (as $CdCl_2$. 5 H_2O), or 10 to 225 ppm Co (as $CoCl_2$. $6H_2O$) or 50 to 800 ppm Pb (as $Pb(NO_3)_2$). Metal tolerance was assessed by the mycelial extension and computing radial growth rates (mm x day^{-1}).

Single-spore cultures

To determine if the Cu^{2+} -resistant of the mycelia could be transferred to their spores, one sporangium from non- and Cu-trained fungus were suspended in distilled water and agitated to release the spores. The spore suspensions were serially diluted in distilled water, and the spores from no training biotypes were plated on agar-medium without Cu^{2+}, and those from Cu^{2+}-adapted strain on 50 ppm Cu^{2+}, a no inhibitory levels.

After incubation at 27ºC for 24 hrs, spores germinated and formed an hyphal mass; hyphal plugs were first transferred to fresh media with no inhibitory Cu^{2+} -level, and then 100 to 600 ppm Cu, and mycelial extensions were measured. The Cu^{2+}-sorbed by mycelia in both treatments were determined by measuring the level of Cu^{2+} -free in solution with a selective electrode.

Statistics

Three replicates were done for each assay and metal concentrations, data are expressed as arithmetic mean ± standard error. The Student's two-tailed t-test was used to evaluate the differences between non-trained and trained experimental means, with $P < 0.5$ being considered significant. In the single-spore cultures, the variation coefficients were determined to assess the variability degree among spores isolated from a common sporangium.

RESULTS AND DISCUSSION

The ascomycete *Talaromyces helicus* was isolated within a number of yeast and filamentous fungi from co-contaminated sludge (Romero et al. 2001, Romero et al. 2002). Although, *T. helicus* did not use biarylics like BP or DBF for growth, the fungus was able to co-metabolized these compounds. Three product types were formed, such as hydroxylated products in high amounts, more hydrophilic sugar-conjugates as well as a ring-cleavage product in lower amounts (Romero et al. 2005). This isolated *T. helicus* accumulated 6 BP-derivatives; in contrast, four of six other *Talaromyces* strains studied produced 4-hydroxy-BP as the only metabolite. Therefore, this strain was selected to develop copper and other heavy metals tolerance.

The growth rates of non- and trained fungus were initially inhibited at 150 ppm, being the no treated strains more sensitive. With 400 ppm Cu^{2}+, the no training biotypes did not develop, whereas the treated ones exhibited growth even at 600 ppm Cu^{2}+ (Figure 1).

Further assays evaluated the sensitivity of both *T. helicus* cultures to Co^{2+}, Pb^{2+} and Cd^{2+}, being the treated biotypes more tolerant to the heavy metals than the no trained parentals (Figure 2). No trained *T. helicus* cultures exhibited slight development with 350 ppm Cd, whereas

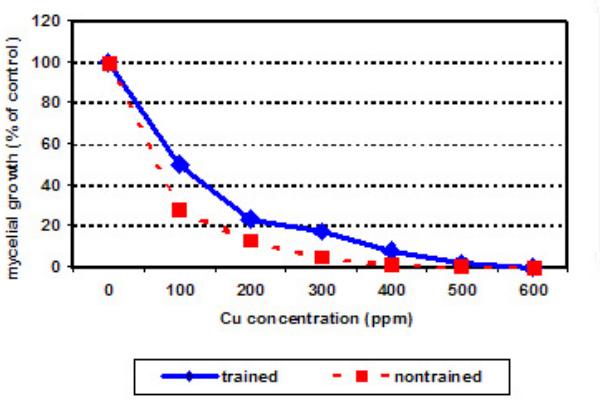

Figure 1. Cu effects on the mycelial growth on non- and trained *Talaromyces helicus*.

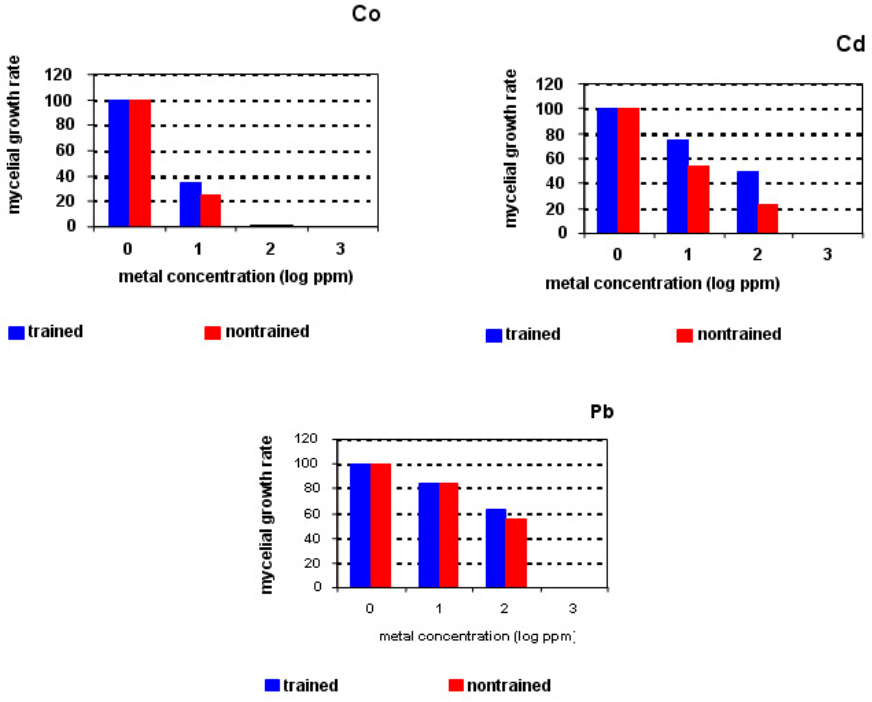

Figure 2. Co-tolerance to Cd, Co and Pb of non- and trained *T. helicus*.

some growth occurred in Cu-treated strains with 600 ppm Cd. In the cobalt assays, the no treated biotypes grew till 100 ppm Co; while the treated strains were tolerant to 200 ppm Co. The lead exhibited a significant lower toxicity for both culture types.

The hyphae from single-spore plates of Cu-trained *T. helicus* grew better on Cu-amended media, and the variation coefficients of the hyphae growth obtained from single-spore of the same sporangium of non- and Cu-trained fungus were significantly different. Furthermore, trained mycelia removed twice as much Cu from solution than those of the no trained parentals, and the amount of Cu-removed by both cultures were correlated with the Cu-solution levels (Table 1).

The comparative toxicity of the studied metals to non- and trained *T. helicus* followed the sequence $Co^{2+} > Cd^{2+} > Cu^{2+} > Pb^{2+}$, but their relative toxicities were independent of the ion sizes and membrane penetration, as their no hydrated ionic radii were 0.069, 0.072, 0.114 and 0.072, respectively (Atlas and Unterman, 1999). Therefore, other mechanisms were acquired by the fungus, as a variety of interactions between fungi and ions had been proposed (Ariff et al. 1999; Pascoal and Cássio, 2004).

This fungus produced large amounts of BP-metabolites, hydroxylated ones, sugar conjugates and a cleavage product, similarly to *Penicillium canescens* (Hammer et al. 2001), *Debaryomyces vanrijiae* (Lange et al. 1998), *Paecilomyces lilacinus* (Gesell et al. 2001) and *Trichosporon* strains (Sietmann et al. 2002). This conspicuous potential was considered to select the strain for further assays.

The Cu-adaptation and the co-tolerance to other heavy metals in *T. helicus* is a remarkable data, as biosorption of other ions but not co-tolerance had been reported among filamentous fungi (Yan and Viraraghavan, 2000; Borghouts et al. 2001; Massaccesi et al. 2002). A noteworthy result was also the mycelia responses from Cu-trained spores derived from the same sporangium that tolerated higher copper levels.

The impact of metals on wild soil communities had been examined on phenanthrene (Maslin and Maier, 2000), naphthalene (Sandrin et al. 2000) and phenol biotransformation (Nakamura and Sawada, 2000). These studies pointed out those metals affected adversely to no degrading members of the consortium that play a vital but indirect role in the biodegradation processes.

Some studies investigated the impact of various metals on the bioremediation of a single organic compound. Interestingly, metals combinations were less toxic than single ones, and co tolerance development indicated that the cellular mechanisms that conferred resistance were non-specific, so the microbiota isolated from co-contaminated environments could exhibited resistance to more than one ions, consequently co-tolerance may be a common natural response (Gadd and Sayer, 2000; Amor et al. 2001).

The mechanisms by which metals inhibit the biodegradation vary with the composition and complexity

of the system under study and include both physiological and ecological components.

In conclusion, the *T. helicus* resistance to heavy metals suggested that the co-tolerance and efficient biarilic transformation were acquired in nature. The issue of co-contamination is a serious one, therefore, the training of fungal strains to degradate both pollutants are a valuable environmental technology for the detoxification of co-contaminated habitats, by bioaugmentation strategy (Roane et al. 2001).

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