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Fungal Tolerance: An Alternative for the Selection of Fungi with Potential for the Biological Recovery of Precious Metals

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Abstract: The behavior of various filamentous fungi in the presence of metals such as Cu, Zn, Ni, Fe, Mn, and V has been widely reported. However, there is little information regarding metals such as Au, Ag and Pt that are not in the form of nanoparticles. The growth of eight filamentous fungi was evaluated at increasing doses of Au, Ag and Pt. The fungi were reactivated in Petri dishes with potato dextrose agar. Subsequently, individual mycelial disks from each strain were inoculated in PDA plates with the following doses of AuCl₃, Ag₂SO₄ and PtCl₄: 0, 50, 150 and 300 mg L⁻¹, respectively. The plates were then incubated for 20 days—a period in which the diameter of the colony was measured every 24 h. Au showed the highest toxicity for the tested fungi. All silver doses decreased the growth of most of the fungi, while platinum did not cause any inhibitory effect on the growth of the eight tested fungi. With a simple test, it was possible to observe the effect of precious metals (PMs) on the growth of filamentous fungi and consider their possible biotechnological applications in the recovery of PMs from primary or secondary sources.

Keywords: filamentous fungi; metals; secondary sources; primary sources

1. Introduction

Precious metals (PMs) are a group of elements (Au, Ag, Pt, Pd, Ir, Rh, Os and Ru) that have taken on great importance in recent years due to their diverse applications in the medical and high-technology industries; thus, their demand and price have increased [1–4]. The problem that surrounds the PMs is that they are quite diluted in the Earth's crust, and current extraction-refining methods are challenging, expensive and not environmentally friendly [3–6]. Considering the above-mentioned factors, it is necessary to develop new biotechnologies for the extraction/recovery of PMs from diverse materials. In the literature, there are a large number of reports on the interaction presented by metals such as K, Na, Mg, Ca, Mn, Fe, Cu, Zn, Co, Ni, Al, Cd, Hg and Pb with bacteria, fungi and algae primarily [7–15], but the studies are limited to a great extent to the interaction with PMs that are in the form of nanoparticles. For instance, it was found that some bacteria play an important role in the biogeochemical cycle of Au, since microbes have been mobilizing Au for more than hundreds of years [16–20]. This ability has been used to generate biotechnological leaching techniques that allow Au to be recovered from refractory minerals [21–28]. Furthermore, research on the recovery of Au from

secondary sources (electronic waste and jewelry waste) using microorganisms has gained importance in recent years. Studies have reported Au solubilization (20–90%) from printed circuit boards (PCBs) from cell phones and computers by cyanogenic bacteria (*Chromobacterium violaceum*, *Bacillus megaterium* and *Pseudomonas balearica*) under different culture conditions [29–35]. In the case of filamentous fungi, there is less information; however, for instance, it has been mentioned that *Aspergillus niger* is capable of recovering Au from secondary sources such as PCBs from cell phones and computers [36–38].

On the other hand, even though Ag has been reported as a highly toxic metal for most microorganisms, some bacteria are able to accumulate and reduce it [39–42]. Among these bacteria is the mixture of *Acidithiobacillus ferroxidans* and *Acidithiobacillus thiooxidans*, which is capable of leaching Ag from sulfurous minerals [43]. Research was also carried out to create biotechnological techniques with which Ag can be recovered from refractory minerals, but to a lesser extent than for Au [44–46]. Under other conditions, it had been shown that the recovery of Ag by microorganisms from secondary sources is poor. Brandl et al. [31] report that *Pseudomonas plecoglossicida* can mobilize around 5% of Ag found in jewelry waste dust in one-day incubation. Similarly, Kumar et al. [33] report that *Pseudomonas balearica* managed to recover 33.8% of Ag from a PCB pulp, with a density of 10 g L^{−1} and at pH 9.0.

Regarding the information about the interaction of Pt with microorganisms, it has been reported that cultures of the halophilic bacteria of the families Halomonasae, Bacillaceae and Idiomarinaceae have recovered 98% of Pt (II) and 97% of Pt (IV) at pH 2, in a time of 3 to 21 h, from solutions of K₂Pt(II)Cl₄ and K₂Pt(IV)Cl₆ at 100 mg L^{−1} [47]. In addition, for secondary sources of Pt, Brandl et al. have reported the recovery of Pt (0.2%) from waste from automobile catalytic converters using cyanogenic bacteria [31].

As can be seen, most of the mentioned studies are oriented towards bacteria and, in a lesser extent, towards filamentous fungi. Thus, gathering additional information about the interaction of filamentous fungi with Ag and Pt is relevant to find new biotechnological applications.

The present investigation focused on evaluating the effect of increasing doses of Au, Ag and Pt on the growth of *A. niger*, *Trichoderma harzianum*, *Trichoderma koningiopsis*, *Trichoderma viridescens*, *Trichoderma* sp., *Hypocrea lixii*, *Fusarium oxysporum* and *Fusarium solani* in order to use fungal tolerance as a simple tool for the selection of the fungi with the potential to be used in the recovery of Au, Ag and Pt from primary or secondary sources.

2. Materials and Methods

2.1. Fungal Strains

A. niger MX5, *T. harzianum* MX2 and *F. oxysporum* MX17 were isolated from soil contaminated with metals, and *H. lixii* MXPE12 and *F. solani* MXPE15 were taken from an electronic board, with both samples coming from the surroundings of the El Tronconal sanitary landfill, Xalapa-Veracruz, Mexico. Additionally, *T. koningiopsis* MX11 and *T. sp.* MX13 were isolated from the rhizosphere of Liquidambar sp. at the Natura Park and *T. viridescens* MX1 was isolated from rhizospheric fern soil at the Library and Information Services Unit of the Universidad Veracruzana in Xalapa-Veracruz. The standard method for the isolation of filamentous fungi is described in greater detail in the work of Madrigal-Arias [48].

2.2. Identification of Fungi

DNA extraction from fungal strains was carried out using the method of Wilson et al. [49]. The PCR products were purified with the phenol-chloroform technique [50], and the purified products (40 ng) were sent for sequencing to the Microbial Genetic Resources Laboratory (CNRG-INIFAP) in Guadalajara, Mexico. The sequences were processed with different bioinformatic tools (BioEdit, ClustalX, Seaview, MEGA6) and on the BLAST platform for the allocation of identities, where the percentages for the species were considered to be greater than 97%, with values of 95% to 96% for the genus.

2.3. Culture of Filamentous Fungi in a Solid Medium with Au, Ag and Pt

For the activation of the growth of the eight filamentous fungi, they were cultivated in Petri dishes with potato dextrose agar (PDA Baker®) at 28 °C for 5 and 10 days. Afterwards, individual PDA disks (7 mm diameter) with each fungal strain were extracted and placed on new Petri dishes with PDA. Au, Ag and Pt were supplied in the culture medium by the addition of 0, 50, 150 or 300 mg L⁻¹ at pH 4.0 (AuCl₃, Ag₂SO₄, and PtCl₄, Sigma-Aldrich®). The Petri dishes were incubated at 28 °C for 20 days, and the fungal growth rate was assessed by measuring the diameter of each fungal colony every 24 h. Petri dishes without Au, Ag and Pt were used as controls.

2.4. Statistic Analysis

A completely randomized experimental design was used for the growth evaluation of the fungal strains using an 8 × 3 × 4 factorial (eight fungal strains, three metals and four doses). Each treatment had three repetitions. The obtained data were analyzed using the analysis of variance and the mean comparison test (Tukey, $\alpha = 0.05$) with the Statistical Analysis System (SAS) program [51].

3. Results

3.1. Fungal Tolerance to Au

The results show that the growth rate of treatments with doses of 0 and 50 mg L⁻¹ did not differ statistically, from the third day onwards, for the *Trichoderma* and *Hypocrea* strains (Figures 1 and 2). Besides, no growth was observed at the 150 mg L⁻¹ dose for *T. harzianum* MX2, *T. sp.* MX13 and *H. lixii*. However, at this gold concentration (150 mg L⁻¹), *A. niger* MX5 showed the highest growth, and a recovery in the growth of *T. viridescens* MX1, *T. koningiopsis* MX11, and *F. oxysporum* MX17 was observed during the experiment time (Figures 1 and 2). For the 300 mg L⁻¹ dose, no growth was found in any of the tested fungi.

Figure 1. Fungal growth rate of filamentous fungi strains exposed to four doses of gold (mg L⁻¹) (n = 3, means ± standard error).

Furthermore, the Au doses induced morphological changes in the fungi and pigmentations in the culture medium, as observed in Figure 3. For instance, in the case of the *F. solani* MXPE15 fungus at a dose of 50 mg L⁻¹ Au, red pigmentation in the culture medium and purple and cottony mycelium were observed. Similarly, at the 50 and 150 mg L⁻¹ doses, the mycelium of *F. oxysporum* MX17 turned purple. Additionally, *A. niger* MX5 showed greater sporulation as the Au dose was increased, in addition to yellow mycelium and yellow pigmentation in the culture medium at the 150 mg L⁻¹ dose (Figure 3A).

Figure 2. Fungal growth rate of four filamentous fungi strains exposed to four doses of gold (mg L⁻¹).

Figure 3. Morphological changes and pigment production by three strains of filamentous fungi exposed to three doses of gold for 20 days. (A) *Aspergillus niger* MX5, (B) *Fusarium solani* MXPE15 and (C) *Fusarium oxysporum* MX17.

3.2. Ag Fungal Tolerance

In the case of Ag, statistical differences in fungal growth rates were found on the eight tested fungi. The fungi with the highest tolerance to Ag were *T. koningiopsis* MX11, *T. sp.* MX13, and *T. viridescens* MX1, while *F. oxysporum* MX17 and *F. solani* MXPE15 were the least tolerant (Figures 4 and 5). The different doses played a determining role in the behavior of each fungus, since the higher the Ag dose, the lower the growth for all the treatments. Thus, for the *Trichoderma* species, changes in the morphology, appearance and tonality of the mycelium were observed: the yellow mycelium turned green with a powder appearance—characteristics not observed in the controls (see Figure 6A). Furthermore, the *Fusarium* strains showed a white mycelium when the Ag dose was increased (Figure 6C,D).

Figure 4. Fungal growth rate of four filamentous fungi strains exposed to four doses of silver (mg L^{-1}). *T. viridescens* MX1 and *T. sp.* MX13 were more tolerant than other strains ($n = 3$, means $\pm$ standard error).

Figure 5. Fungal growth rate of four filamentous fungi strains exposed to four doses of silver (mg L^{-1}). *T. koningiopsis* MX11 was more tolerant than other strains ($n = 3$, means $\pm$ standard error).

Figure 6. Morphological changes of four filamentous fungi exposed to four doses of silver for 20 days. (A) *Trichoderma viridescens* MX1, (B) *Hypocrea lixii* MXPE12, (C) *Fusarium solani* MXPE15 and (D) *Fusarium oxysporum* MX17.

3.3. Fungal Tolerance to Pt

Platinum did not cause an inhibition of the growth of filamentous fungi tested with the three used doses. Furthermore, no statistical differences were found in the fungal growth rates between the treatments and their respective controls from the third day of evaluation onwards for the *Trichoderma* and *Hypocrea* strains (Figure 7). However, there were statistical differences in the fungal growth rates between the controls and the treatments for the fungus *F. solani* MXPE15 (Figure 8). It is worth mentioning that there were no morphological changes to consider due to the presence of Pt, as shown in Figure 9.

Figure 7. Fungal growth rates of four filamentous fungi strains exposed to four doses of platinum (mg L⁻¹) (n = 3, means ± standard error).

Figure 8. Fungal growth rates of four filamentous fungi strains exposed to four doses of platinum (mg L⁻¹) (n = 3, means ± standard error).

Figure 9. Morphological characteristics of four filamentous fungi exposed to four doses of platinum for 20 days. (A) *Trichoderma harzianum* MX2, (B) *Aspergillus niger* MX5, (C) *Hypocrea lixii* MXPE12 and (D) *Fusarium oxysporum* MX17.

4. Discussion

Morphological changes and the production of pigments in some fungi in contact with PMs, in a solid culture medium, have been reported for other metals such as Cd, Cu, Zn, Ni and Pb, showing that some fungal species reduce their growth due to metallic stress [52,53]. Such is the case of the fungi *Trametes versicolor* and *Stereum hirsutum* which, when grown in a solid medium with Cd (1 mM), showed several morphological changes in their mycelium and produced a brown pigment [52]. In the case of Cu (6 and 10 mM), the fungi *Wolfiporia cocos*, *Laetiporus sulfureus* and *T. versicolor* showed significant modifications in the intensity of the color (from white to brown) and the texture (from cottony to downy) of the mycelium [54]. In this sense, pigment production has been related to the survival mechanisms used by fungi to alleviate metallic stress; it has been observed that higher concentrations of metals lead to a greater number of produced pigments [55,56].

The present investigation found that Au was more toxic to the tested filamentous fungi than Ag and Pt. Some studies with other microorganisms have found that the presence of Au^0 and Au^{3+} ions

in trace amounts may not affect the growth of the microorganisms due to the fact that Au ions are usually deposited in the cell wall and the periplasmic membrane [57]. Therefore, this could explain why the used fungi did not show any growth inhibition for the concentration of 50 mg L^{-1} . However, Karamushka and Gadd [58] indicate that the growth of *Saccharomyces cerevisiae* is inhibited in culture media with Au at doses higher than 39.4 mg L^{-1} , suggesting that the used fungi are more tolerant than the mentioned yeast. As in the present study, Novelli et al. [59] found that the fungus *A. niger* seems to be quite tolerant to five organometallic compounds of Au, which could indicate that, within the *Aspergillus* genus, the *niger* species are capable of tolerating a variety of Au compounds. Regarding the utility of tolerance tests for the selection of filamentous fungi in the recovery of Au from secondary or primary sources, Madrigal-Arias et al. [38] carried out previous tolerance tests for Au (0, 50, 100, 300 mg L^{-1}) from several species of the gender *Aspergillus* for their Au bioleaching experiments from electronic residues, finding that the consortium of the most tolerant fungi managed to leach 87% of this metal. Another example of the usefulness of these tests is the pigmentation that *F. solani* MXPE15 presented before 50 mg L^{-1} of Au; in this situation, the fungus was tested in a liquid medium with 30 and 37 mg L^{-1} Au, showing that it is capable of precipitating this metal [60]. Furthermore, Díaz-Martínez et al. [61] reported that *A. niger* MX5 in combination with *Sphingomonas* sp. is capable of bioleaching approximately 1% of Au from the printed circuit boards (PCB) of cell phones.

Regarding Ag, it has been reported that fungi isolated from nearby silver mine soils can tolerate concentrations above 170 mg L^{-1} without any problem, but there has been no report on whether the fungi show morphological changes in their growth [62]. This information could only be compared with more Ag-tolerant fungi such as *T. koningiopsis* MX11, *T. sp.* MX13 and *T. viridescens* MX1. Concerning the usefulness of tolerance tests with silver, Díaz-Martínez [63] reported that the fungi *H. lixii* MXPE12 and *F. oxysporum* MX17 are capable of bioleaching 0.02% of Ag from printed circuit boards from computers. Besides, the same work showed that *F. solani* MXPE15 was able to leach 1% of Pd, another precious metal.

On the other hand, due to the scarce information on the effect that Pt has on filamentous fungi, it is not possible to fully compare the results obtained from the effect that this precious metal has on its growth. However, in bacteria, the presence of platinum ($\text{Cl}_6\text{Pt}^{2-}$, 75 mg L^{-1}) inhibits growth in *Escherichia coli* B, *E. coli* C, *E. coli* K12, *Aerobacter aerogenes*, *Alcaligenes faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Lactobacillus* sp. and *Serratia marcescens*, which contrasts with the results that we observed in the fungal genera *Trichoderma*, *Aspergillus*, *Hypocrea* and *Fusarium*. This implies that the chemical form in which platinum is found is a factor to consider for microbial tolerance [64,65]. It is not ruled out that, in future research, the filamentous fungi with a higher tolerance to precious metals found in this research could be used in other studies to recover some of the precious metals contained in primary or secondary sources, reaffirming the usefulness of the fungal tolerance test as a tool for the selection of fungal strains with biotechnological potential.

Finally, it is important to mention that the costs for the implementation of this trial were not estimated. However, we assume that by using cheaper culture media (for instance, media based on agri-food residues) and perhaps recovering the precious metals from the media already used (by chemical and biological techniques), the testing costs could be considerably reduced.

5. Conclusions

The study shows that PM tolerance tests in a solid medium are useful to observe the effect that PMs have on the growth of filamentous fungi (morphological changes) and on the selection of fungi with the potential to recover PMs from primary or secondary sources. It was found that the most tolerant fungal genera for Au are *Aspergillus* and *Fusarium*, while those for Ag are *Trichoderma* and *Fusarium*. Lastly, we found that the chemical form of Pt does not have an inhibitory effect on the growth of most of the used fungi.

Finally, it is important to highlight that the results reported here could be complemented with new experiments that allow the development of possible biotechnological applications. For instance,

the capacity of the most resistant fungi to extract suitable precious metals should be evaluated. Likewise, the morphological and coloration changes observed for the different fungi could help to develop low-cost sensors for the assessment of toxicity in contaminated water samples.

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