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Heavy Metal Tolerance of *Aspergillus Piperis* Using the Agar Well Diffusion Method

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A lead resistant strain of *Aspergillus piperis*, a subgroup of *A. niger* with known heavy metal remediation properties, was tested for heavy metal resistance. The agar well diffusion method was used with metal solutions containing 2000 ppm of copper (Cu(II)), iron (Fe(II)), lead (Pb(II)), magnesium (Mg(II)), manganese (Mn(VI)), selenium (Se(VI)), cadmium (Cd(II)), and zinc (Zn(II)) ions, respectively. Of the metals tested, *A. piperis* only exhibited substantial growth inhibition in Cd(II) up to 23 mm from the well center, while simultaneously completely inhibiting spore formation. Lesser inhibition was observed in Se(IV), Pb(II), and Zn(II) of which the latter two exhibited evidence that additional metal resistance was developed during incubation. After five days the fungus had successfully grown in the presence of all the other metals, making *A. piperis* a promising candidate for heavy metal mycoremediation research.

Key words | Aspergillus piperis, bioremediation, agar well diffusion method, metal tolerance, lead remediation

1. Introduction

Heavy metal contamination remains a prevalent issue in South Africa (Tandon and Singh, 2016) and globally (Rehman et al., 2018), especially in the mining industry. Bioremediation is a method of removing xenobiotic chemicals from an environment using living organisms instead of other techniques like precipitation and stabilization (Brooks et al, 2010: 2). There are several benefits to bioremediation, namely low operating costs, high efficiency, and the possibility of metal recovery (Rathoure and Dhatwalia, 2016). Mycoremediation applications, such as fungal filters are particularly useful because they are cheap and readily available (Price et al., 2001). The first step to exploit fungi this way is to identify strains that hold metal resistant properties.

In 2017, a wild strain of fungus contaminated a set of agar plates containing 80 ppm of lead in the Water Utilization Engineering Laboratory at the University of Pretoria, South Africa. The fungus was isolated and preserved for further research (Peens, 2018). Preliminary tests suggested that the fungus survives in the presence of lead (II) while simultaneously lowering the metal ion concentrations in solution (de Wet, 2019). Genetic sequencing identified the fungus as *Aspergillus piperis*, one of eight species that fall under the *Aspergillus niger* fungal group (D'hooge et al., 2017). The fungus mycelium is white, and though it produces no fruiting bodies, it bears similar characteristics to the *A. niger* group, including distinctive black spores. *A. niger* has become an invaluable contributor to medical mycology (Perrone et al, 2011), biotechnology (Gomes et al, 2011), and other fields such as citric acid production (Lee, 2015).

The aim of this observational study was thus to gauge whether the fungus could be used in industrial remediation practices by establishing a preliminary list of metal environments in which it can survive. To identify these habitable environments for the fungus, metal solutions were introduced to the center of an inoculated agar plate, using the simple and cost-effective agar well diffusion method (Xie et al., 2005).

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2. Materials and methods

2.1 Materials and reagents used

A. piperis, like most *A. niger* fungi, had already exhibited the ability to thrive in Potato Dextrose Agar (PDA) (Dynowska et al., 2011). PDA from Merck KGaA (Darmstadt, Germany), was prepared according to the manufacturer prescribed method of 39 g/L. Pb(II), Cu(II) and Zn(II) were chosen for their occurrence in industrial effluents, especially in mining (Wei et al., 2019), and the remaining metals were selected according to laboratory availability. The dry reagents used were: 1.598 g $Pb(NO_3)_2$, 2.952 g $Cu(NO_3)_2$, 2.896 g $Zn(NO_3)_2$, 4.037 g $CoCl_2.6H_2O$, 2.720 g $FeSO_4$, 3.918 g $MgCl_2$, and 3.076 g $MnSO_4.H_2O$. The dry reagents were each dissolved in 100 mL distilled water, after which they were serially diluted to the desired concentration of 2 000 ppm. Stock solutions of Se(VI) and Cd(II) were readily available at a concentration of 10 000 ppm and were also diluted to a final concentration of 2 000 ppm metal ions. The metal concentrations were chosen so that the PDA would have a maximum concentration of 2 000 ppm and a minimum of 280 ppm metal ion concetration after five days. Although industrial contaminations vary (Manivasakam, 2016), for experimental purposes this range would be reasonably broad to indicate the fungus' minimum inhibiting concentrations (MIC) for the different pollutants.

2.2 Methods

Collection of samples

Traditionally bioprospecting for metal resistant organisms involves samples taken from metal contaminated sites followed by microbe isolation and testing (Khan et al., 2019). However, in this instance a Pb(II) agar plate which was prepared for bacteria-related studies was infected by what appeared to be black mold that originated from the air. Spores from the fungus were preserved in 1.5 mL glycerol in a 2 mL vial and stored at −77 °C. For this study, one vial was thawed at room temperature and the spores were deposited onto a PDA plate. The plate was then incubated for five days at 35 °C. After five days a thick mass of spores had formed on the plate which was used as a spore repository for the inoculations.

Inoculation of microorganism

For plate inoculation, 1 mL sterile water was added to the spore repository plate and swirled to collect spores in the suspension. The 1 mL suspension was added to 9 mL of sterile distilled water so that a ten times spore dilution is achieved. It was not possible to enumerate the spore concentration due to a lack of equipment, although the presence of black spores in suspension was confirmed visually. 0.5 mL of the spore-infused serum was added to each of the set PDA plates, after which the plates were agitated to distribute the spores evenly.

Agar well diffusion method

The agar well diffusion method has been used, with various adaptations, since 1964 (Taylor, 2015), and can be an easy, quick, and cost-effective method of testing MIC in many microorganisms (Kumar et al., 2000). In this process, the surface of a set PDA plate is evenly inoculated, and then a 5 mm hole is bored into the center under aseptic conditions. The antimicrobe is then deposited into the well. Based on localized concentration phenomena the concentration of metal ions would be much higher in the center and diffuse radially outwards, with low concentrations around the edges, resulting in healthy microbial growth at these lower concentrations and less growth near the center where the concentration is uninhabitably hostile (Chalad et al., 2018).

Using 2 000 ppm stock solutions, 0.5 mL was deposited into the well in the middle of a set PDA plate so that 1 mg metal ions are present in the center. Doing so, the MIC of *A. piperis* in the presence of a specific metal can be inferred. An incubation period of five days at 35 °C provided the fungus ample time to establish a mycelial network and develop spores, after which photographs were taken of the plates.

3. Results and discussion

After incubation, there was spore growth on all the plates, except Cd(II). On this plate a white mat was observed (Figure 1a). To establish the nature of the white mat, and ensure that it is indeed mycelium, a sample was observed under 40 x microscopic magnification where characteristic mycelial hyphae (Yates et al., 2016) are visible (Figure 1b). There are numerous explanations for this observation: Either the hostile environment has a spore-suppressant effect on the fungi whereby resources are not allocated to propagation but rather to maintenance; or a genetic mutation occurred whereby the dominant fungal growth does not have the ability to produce spores. The last case would be particularly interesting because there are several benefits to non-propagating fungi, such as human safety from *Aspergillus* respiratory infection

(Shah et al., 2004), as well as biological containment in industry. To establish whether this is the case, subsequent experiments would require propagating clones from this plate and observing the fungal behaviour in the absence of heavy metals.

(a) (b)

Figure 1: (a) Cadmium supresses mycelium growth up to 23 mm from the centre where 1 mg metal was deposited, (b) White mycelial hyphae on Cd (II) plate at 40 x magnification

In estimating the growth threshold in cadmium (II), twelve distance measurements were taken from the edge of the well to the start of the mycelium and an arithmetic mean of the measurements of 23 mm was obtained. Using the solution of the partial differential equation (1), and describing free diffusion in one dimension (equation 2), (Bonev et al., 2008), an MIC of 1320 ppm Cd(II) was estimated.

$$c(x,t) = c(0,0)\exp\left(-\frac{x^2}{4Dt}\right) \tag{1}$$

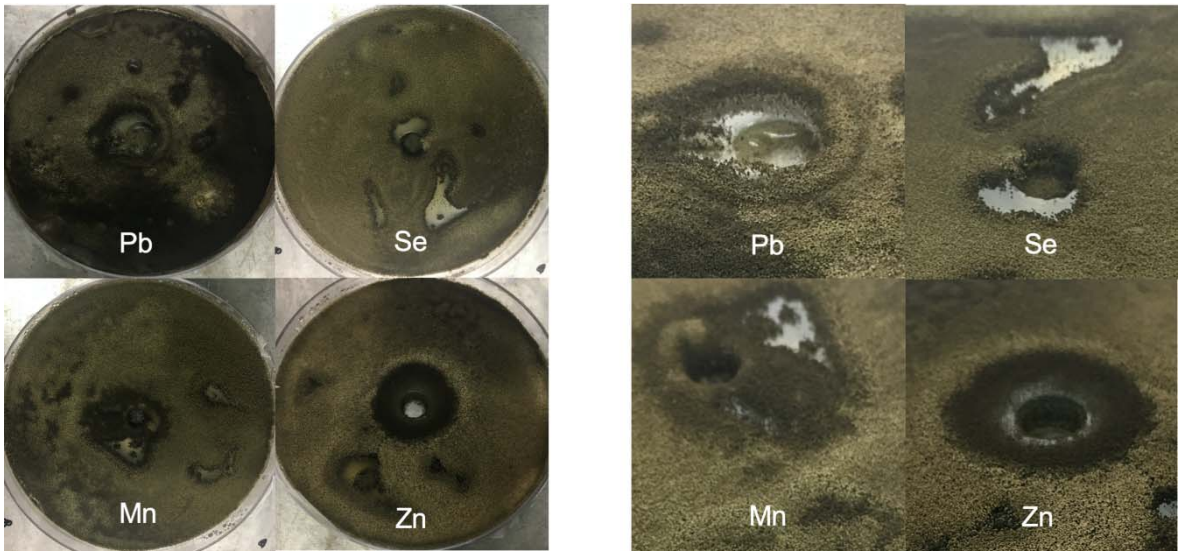
$$D\frac{\partial^2 c(x,t)}{\partial x^2} = \frac{\partial c(x,t)}{\partial t} \tag{2}$$

where $c(x,t)$ describes the metal concentration as a function of distance from the source and time, x is the distance from the edge of the well, D is the diffusion coefficient of the metal ion, and t is the time of metal ion diffusion.

Other interesting observations were made for Pb(II), Mn(II), Se(IV) and Zn(II), (Figure 2a), where there was either clearly uninhabitable areas, or a change in spore texture. Only selenium (IV) proved to have areas that are completely hostile for the fungi, with little or no growth in the center (Figure 2b). In subsequent experiments it may be useful to increase the metal concentration by at least a factor of 10 in all the metals so that clear thresholds can be established.

Another observation is the difference between spore growth close to the well and on the edges. Although denser, the spores around the sides do not appear to be as vibrant and vividly black as the ones in the center. Zinc (II), for example (Figure 2b), shows a clear deviation from normal spore growth.

At a first glance an incorrect assumption was made — that the center ring may simply be covered with a layer of spores that became airborne, thus accounting for the change in texture. However, Upon microscopic inspection, it became clear that at 30 mm from the hole the spores have a very different texture, with numerous empty and collapsed conidiophores which would imply that the spores have already drifted off and that that area was populated earlier (Figure 3a), whereas there are healthy conidiophores and spore clusters present (Figure 3b) directly adjacent from the center.



(a) (b)

Figure 2: (a) Fungal spore inhibition demonstarated in Pb(II), Se(IV), Mn (IV) and Zn(II), (b) Se(IV) closeup shows areas where the metal concentration was too high for the fungi to survive, while Pb(II), Mn(IV) and Zn(II) exhibit a ring around the center

(a) (b)

Figure 3: (a) Older spores on Zn (II) plate 30 mm from centre, (b): New spores on Zn (II) plate 8 mm from centre

The remainder of the plates showed no obvious evidence that the fungus had reached its maximum concentration limit (Figure 4), although higher concentrations of these metals could result in the emergence of a clear threshold.

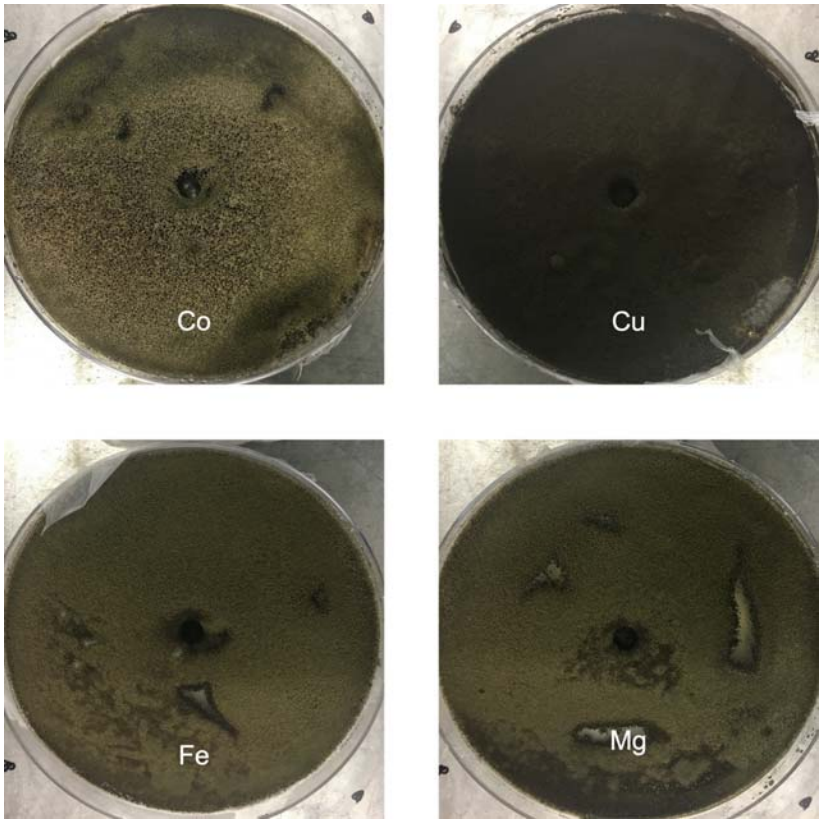


Figure 4: Metal solution plates after five days: Little visual evidence that metal tolerance threshold has been reached

4. Conclusions and recommendations

This study provided evidence that *A. piperis* can thrive in the presence of Cu(II), Fe(II), Pb(II), Mg(II), Mn(II) within concentrations that can reasonably expected in industrial effluent. At concentrations approaching 2 000 ppm of Pb(II), Se(IV), and Zn(II) the fungus battles to grow, but quickly starts developing some resistance. Cd(II) poses an interesting case study whereby spore formation is completely suppressed at all tested concentrations and exhibits a clear survivability threshold.

While these findings are promising, it is recommended that the experiments be repeated with higher concentrations of all metals, barring cadmium, so that clear thresholds can be established for fungal growth. In addition to this, the plates could be incubated for shorter time periods and monitored closely to determine when metal resistance starts and at what rate it develops.

The *A. niger* group is notoriously robust (Ponizovskaya et al., 2017), and extremely ubiquitous (Blackburn, 2006) and *A. piperis* appears to be no exception. Both traits make it an easily accessible candidate for industrial applications, and now that heavy metal tolerance is established, metal-targeted remediation research would be a valuable endeavor.

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