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The bioremediation potential of filamentous fungi in soil contaminated with lead

O potencial de biorremediação de fungos filamentosos em solo contaminado com chumbo

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ABSTRACT

Population increase resulting from industrial activities has worsened soil contamination with toxic metals. Given the complex dynamics of these pollutants and the complexity of soil matrices, one of the biggest challenges faced by the environmental field lies on developing effective technologies to remediate contaminated soils. Thus, bioremediation may be a decontamination alternative based on using microorganisms. The aims of the current study are to isolate and characterize filamentous fungi with bioremediation potential to be used in soils contaminated with lead. A soil sample was incubated in Sabouraud Caf Agar medium in BOD at 28ºC. CFUs were counted after 72h of incubation; the three most prominently grown colonies were isolated in new plates containing the same medium. Fungi were transferred to liquid submerged fermentation medium with 20 ppm of lead after 24 h of incubation; they remained in shaker incubator at 30°C, 120 rpm, for 120h. Next, the MP-AES analysis was performed to evaluate the final lead concentration. Isolated fungi such as Aspergillus, Penicillium and Trichoderma removed, 56.82%, 66.77% and 75.29% lead ions, respectively, in comparison to the control. Results confirmed the bioremediation potential of these fungi and their possible use in areas contaminated with the herein investigated metal.

Keywords: Fungus; Bioremediation; Toxic metals

RESUMO

O aumento populacional associado às atividades industriais tem elevado a contaminação de metais tóxicos no solo. Em razão da complexa dinâmica desses poluentes e da complexidade das matrizes de solo, um dos maiores desafios na área ambiental é o desenvolvimento de tecnologias eficazes para remediação solos contaminados por poluentes inorgânicos. Nesse sentido, a biorremediação pode ser uma alternativa para a descontaminação, pela utilização de microrganismos, como os fungos, capazes de remover contaminantes tóxicos inorgânicos do meio ambiente. O objetivo do presente estudo foi isolar e caracterizar fungos filamentosos com potencial biorremediador em solos contaminados com chumbo. Uma amostra desse solo foi incubada em meio Sabouraud Caf Agar em BOD a 28º C. Após

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72h, realizou-se a contagem das UFC e as três colônias de crescimento mais proeminente foram isoladas em novas placas com o mesmo meio. Após 24h de incubação, os fungos foram transferidos para meio líquido de fermentação submersa com 20 ppm de chumbo, onde permaneceram em incubadora shaker a 30°C, a 120 rpm, durante 120h Em seguida, foi realizada análise em MP-AES para avaliação da concentração final de chumbo. Os fungos isolados Aspergillus, Penicillium e Trichoderma removeram, respectivamente, 56.82%, 66.77% e 75.29% dos íons chumbo, quando comparados ao controle. Os resultados confirmam o potencial biorremediador desses fungos e a sua possível utilização em áreas contaminadas pelo metal.
Palavras chave: Fungo; Biorremediação; Metais tóxicos

1 INTRODUCTION

Population increase due to industrial activities has negative impacts on natural environments. Thus, soil pollution by toxic metals deriving from the development of industrial, agricultural and urbanization activities is outstanding (GIANNETTI et al., 2007; ANDRADE et al., 2009). The metallurgical industry stands out among industrial activities accounting for the production of large amounts of tailings rich in heavy metals. Vegetation cover destruction in mining areas worsens soil degradation and leads to water erosion and to contaminant leaching towards water tables (ANDRADE et al., 2009). The improper disposal of solid waste in urban areas can generate slurry containing heavy metals; this liquid can be percolated and reach water tables (MARIGA, 2005).

The State Environmental Foundation (FEAM - Fundação Estadual do Meio Ambiente) is the agency responsible for controlling and monitoring contaminated and rehabilitated areas in Minas Gerais State. The last FEAM inventory collected data about 175 counties in Minas Gerais State and recorded 662 contaminated and rehabilitated areas in them. According to the aforementioned inventory, metals accounted for 27.7% of the total contamination in the investigated area; it only lost position to contamination caused by hydrocarbons (69.3%). The incidence of metals in the soil is often associated with the leaching of improperly disposed industrial waste and of elements found in soil or rock matrices. Lead stands out as the main contaminant among metals; it accounts for 17% of metals and it is followed by arsenic, which accounts for 9% of them (FEAM, 2018).

The main anthropogenic sources of lead contamination in the environment are associated with mining activities, with the incorrect disposal of industrial and metallurgical waste, with the use of agricultural inputs and with atmospheric depositions (PIERANGELI et al., 1999; CÉSAR et al., 2011). Lead is a toxic, nonessential metal that accumulates in one's body and in food chains. Its presence in the environment can lead to reduced vegetation growth and even to its extinction, besides being toxic to microorganisms and animals, including humans (LANDMEYER, BRADLEY and CHAPELLE, 1993; ALVES et al., 2008). It has neurotoxic effects on humans; besides, it can damage individuals' cardiovascular system and virtually affect every human organ and system (XIE et al., 1998; BARBOSA et al., 2005).

Decontamination techniques are required due to lead's high toxicity and bioaccumulation capacity. Thus, bioremediation stands out when microorganisms capable of removing toxic contaminants from the environment are used. This technique has been the subject of several studies, since it is efficient, safe, inexpensive and less disturbing to the environment (CARNEIRO and GARIGLIO, 2010). Fungi have great advantage among microorganisms used in bioremediation processes, due to their resistance to high metal concentrations and to their metabolic potential to digest several compounds (WETLER-TONINI, REZENDE and GRATIVOL, 2010). The use of filamentous fungi, as well as of their metabolites, in bioremediation processes has increased due to their high degradative and biosorption potential, as well as to resistance mechanisms triggered by them under adverse environmental conditions (CONCEIÇÃO, ATTILI-ANGELIS and BIDOIA, 2005).

Given the importance of these fungi and their decontamination capacity, several studies have been conducted in order to identify new fungi species and to optimize bioremediation processes. Oladipo et al. (2016) have isolated five fungi species belonging to genus *Aspergillus* from mine soils and tested different concentrations of metals such as cadmium (0–100 ppm), copper (Cu) (0–1000 ppm), lead (Pb) (0–400 ppm), arsenic (As) (0 –500 ppm) and iron (Fe) (0–800 ppm). The authors observed that tolerances varied between species and metals, but overall all

species were tolerant to metal concentrations higher than the ones allowed in soils worldwide. Thus, these species proved to be potential candidates to be used in bioremediation processes.

Sim and Ting (2017) have analyzed *Trichoderma asperellum* potential to bioabsorb lead II, copper II, zinc II and cadmium II metals in multi-metal solutions in comparison to single-metal solutions. They observed reduced metal removal in multi-metal solutions, a fact that may be associated with antagonistic interactions between metals. The following removal preference was observed: Pb (II) > Cu (II) > Zn (II) ≥ Cd (II).

Wahab et al. (2017) have isolated lead-tolerant fungi in mangrove soils and found that *Penicillium citrinum* KR706304 was the most tolerant species. They analyzed the effects of parameters such as pH, temperature, initial metal concentration, biomass and age, stirring and lead contact time on metal removal efficiency. Results focused on parameters recording the highest absorption rates and indicated the bioremediation potential of the investigated species.

Given the difficulties found in soil decontamination processes, the use of bioremediation as auxiliary mechanism to accelerate soil decontamination is promising, mainly when it is necessary removing heavy metals from the environment. Thus, the main aim of the current study was to characterize filamentous fungi collected in soils contaminated with lead in order to evaluate their bioremediation potential.

2 MATERIALS AND METHODS

2.1 Soil sample incubation and fungi isolation

Table 1 presents the fertility analysis applied to soil samples used in the experiments.

Table 1- Parameters of soil fertility

Parameters	**Soil**
Organic matter (g dm^{-3})	16.4
pH	5.6
Phosphor (mg dm^{-3})	1.3
Potassium (mmolc dm^{-3})	2.4
Calcium (mmolc dm^{-3})	4.1
Magnesium (mmolc dm^{-3})	1.3
Aluminum (mmolc dm^{-3})	0.1
Nitrogen (g dm^{-3})	1.24
Total bases (mmolc dm^{-3})	7.6
% V (base saturation)	56
CTC (mmolc dm^{-3})	31.6
Clay (g dm^{-3})	390
Sand (g dm^{-3})	161
Silt (g dm^{-3})	449

Source: authors

A soil sample was contaminated with 1000 ppm of lead ion (Pb^{2+}); approximately 1g of this soil sample was subjected to the serial dilution technique at saline solution (0.9% w / v) concentrations of 10^{-1} g mL^{-1} and 10^{-2} g mL^{-1}. Next, 1mL of each dilution was incubated on Petri dishes containing Sabouraud Caf Agar culture medium, based on the Spread Plate technique (DA SILVA et al., 2010). The Sabouraud Caf Agar is a selective medium used to isolate yeasts and filamentous fungi. The plates were kept in BOD (Eletrolab, model 101/2) oven at 28ºC, for at least 72h.

Colony forming units (CFUs) were counted after the incubation period was over. Next, three CFUs presenting the most significant growth were selected and transferred to new plates containing Sabouraud Caf Agar medium by using an inoculation loop. The new plates were incubated again in BOD oven at 28ºC, for 24h, until they were transferred to submerged fermentation medium containing Pb^{2+} ion at 20 ppm.

All procedures were performed near the Bunsen burner flame and used sterile materials and media.

2.2 Incubation in submerged fermentation medium containing lead

The submerged fermentation medium was prepared based on the methodology suggested by Colla, Hemkemeier and Gil (2012). The used media were transferred to 125 mL Erlenmeyer flasks and added with Pb^{2+} ions (20ppm). Next, the flasks were sterilized based on autoclaving (autoclave Prismatec – model CS) at 121ºC, fort 15 minutes, at pressure of 1 kgf/cm^2.

The three filamentous fungi that had been previously isolated in Sabouraud Caf Agar medium were transferred to Erlenmeyer flasks filled with the submerged fermentation medium added with lead. Transfer was performed near the Bunsen burner flame by using an inoculation loop. A fourth Erlenmeyer flask containing medium and metal was used as a control (the only difference between this flask and the other ones lied on the fact that the microorganism was not inoculated). Subsequently, incubation at 30°C was performed for 5 days, under constant stirring in shaker incubator (Nova Técnica laboratory equipment, model NT 712), at intensity of 120 rpm. Qualitative medium pH measurement was performed after 5 incubation days by using pH indicator paper (Nova Técnica laboratory equipment, model NT 712).

2.3 Lead content quantification

Aliquots of the hyphae-free submerged fermentation medium and of the control group were collected. Samples were examined in stereoscope (Nova Optical System, Nova XTX-5C model), at 40X magnification, to confirm the absence of hyphae or fungal fragments in the samples.

Sample opening was performed through wet processing; 0.5 g of each sample was added with 4 mL of digestion solution, as well as with mixtures of PA, nitric (HNO_3) and perchloric ($HClO_4$) acids at ratio of 3: 1 (v / v), respectively. The tubes were placed in Dry Block Digester (Tubo Macro – Thoth equipamentos) set to run at 150°C, for 1 hour. The entire procedure was carried out inside a fume hood (Lucadema).

In total, 25 mL of distilled water was added to the sample after the cooling process. Next, it was subjected to lead quantification analysis in microwave-induced plasma atomic emission spectrometry (MIP-AES 4200, Agilent Technologies). The analysis was conducted in triplicate.

2.4 Fungi identification

Fungi isolated in Sabouraud Caf Agar and, subsequently, incubated in the submerged fermentation medium added with lead, were identified through macroscopic and microscopic analysis. Macroscopic analysis was performed by keeping the Petri dish closed and by examining it at naked eye. This analysis is of paramount importance to identify filamentous fungi, since certain colony characteristics, such as color, texture, as well as the presence or absence of ridge and elevation, can be essential to their identification. Macroscopic analyses of the assessed fungi were based on Mesquita Filho (2012).

Samples of colonies deriving from the primary media were taken from the plates with a “L” platinum loop and placed between slides and coverslips added with 2 drops of lactophenol cotton blue for microscopic analysis under optical microscope (Bioval, model L2000A) at 400X magnification. Subsequently, microcultures of colonies of interest were performed in potato agar (incubated at room temperature, in the dark, for seven days) for microstructure analysis. Hyphae types and colors were observed during the microscopic analysis, as well as their reproduction structures.

3 RESULTS AND DISCUSSION

3.1 Counting the fungi isolated in Sabouraud Caf Agar medium

Colony forming unit counting in Sabouraud Caf Agar medium has shown $3.3x10^3$ CFU g^{-1} of soil contaminated with 1000 ppm of lead ion. This value was lower than the one recorded for non-contaminated tropical soils - from 10^4 to 10^6 CFU g^{-1} (ALEXANDER, 1977). It happened due to the toxic effects of Pb^{2+} ion on microbiota.

Andrade and Silveira (2004) have evaluated the effect of lead addition to the soil on soil biomass and microbial activity under the influence of mycorrhizal fungi in the soybean rhizosphere. They observed that lead has negatively changed the carbon in the biomass and the activity of rhizosphere microbiota, as well as found stress symptoms resulting from metal addition to the soil.

3.2 Lead absorption and fungi identification

The three fungi isolated in the soil have grown in the submerged fermentation medium with lead, a fact that evidenced their tolerance to this metal at concentrations up to 20 ppm.

Iram et al. (2013) have tested different fungi species belonging to genus *Aspergillus* and found sensitive, moderate and high tolerance rates at lead concentration of 1000 ppm. Paria, Mandal and Chakroborty (2018) have shown that fungal species *Aspergillus penicilluodes* was highly capable of absorbing metals (Cd^{2+} and Pb^{2+}) and presented relatively high tolerance to metal concentrations of 1000 ppm.

The pH measurement conducted in submerged fermentation media added with lead, after five incubation days, recorded values ranging from 5.0 to 5.5. Lead precipitation happens at pH range from 6 to 8, a fact that makes it insoluble and hinders its absorption by microorganisms found in the liquid medium (PIERANGELI et al., 2001). Thus, it was possible seeing that lead found in the samples did not precipitate; it was dispersed in the medium and such dispersion facilitated its absorption by fungi.

Table 2 shows the concentration of Pb^{2+} ions (mg L^{-1}) in submerged fermentation media after five incubation days, by taking into consideration four samples - three containing the isolated fungi (samples 1 to 3) and the control group (without the fungus) -, as well as the removal rate. It also presents fungal identification at genus level.

Table 2 – Concentration Pb^{2+} ions after 5 incubation days in submerged fermentation media, removal rate and e fungi genera

Samples	**[Pb^{2+}] (mg L^{-1})**	**Removal rate (%)**	**Fungi genera**
1	8.11 ± 0.13	56.82	*Aspergillus*
2	6.24 ± 0.10	66.77	*Penicillium*
3	4.64 ± 0.15	75.29	*Trichoderma*
Control group	18.78 ± 0.14	0	

Source: authors

All fungi were capable of removing the lead ion from the medium; the removal rate ranged from 56.82% to 75.29% (Table 2).

According to Barros et al. (2005), microorganisms have metal biosorption features and can remove metals from contaminated media. Biosorption may happen by means of complexation, ion exchange and inorganic adsorption or microprecipitation mechanisms that may act simultaneously. Biosorption properties depend on the cell coating features of the species in contact with the metal (MELO and AZEVEDO, 2008). According to Iram et al. (2013), biosorption processes can change from species to species and can be influenced by several factors such as metal concentration, pH and solution temperature, time in contact with the microorganism and ionic composition of the medium.

Aspergillus was one of the fungal genera isolated in the soil contaminated with lead (Table 2). Representatives of this genus, which are easily found in non-contaminated soils (STAMFORD et al., 2005), have been identified in soils subjected to mining activity, herbicide and hydrocarbon applications, metallurgical activities, as well as in wastewater, among others (PRICE, CLASSEN and PAYNE, 2001; SILVA JUNIOR and PEREIRA, 2007; COLLA et al., 2008; OLADIPO et al., 2016). It has shown efficiency in degrading industrial waste containing metals, dyes and refined oil (RAO and VIRARAGHAVAN, 2002; KOTSOU et al., 2004; SANTOS and LINARDI, 2004; FREITAS NETO et al., 2007).

Aspergillus recorded approximately 57% lead absorption (Table 2). Khamesy, Hamidian and Atghia (2016) have found similar results at similar pH (5.0) in two species belonging to this genus, which were isolated from tailings generated in zinc factories. The aforementioned researchers observed lead and cadmium ion removal rates ranging from 40% to 70%. In addition to these metals, representatives of genus *Aspergillus* were able to remove other metals such as mercury, chrome, nickel, gold, silver, zinc, copper and uranium (LEMOS et al., 2008; TASTAN, ERTUĞRUL and DÖNMEZ, 2010, IRAN et al., 2013).

The *Penicillium* genus was the second to be isolated (Table 2). In addition to being common in non-contaminated soils (STAMFORD et al., 2005; CAVALCANTI et al., 2006), representatives of this genus have been found in soils contaminated with herbicides such as triazine or sulfentrazone, with petroleum, in copper mine soils and in areas subjected to the deposition of industrial waste contaminated by heavy metals, among others (SANTOS et al., 2007; SILVA JUNIOR and PEREIRA, 2007; COLLA et al., 2008; MARTINEZ, et al., 2010; LIMA, OLIVEIRA and CRUZ, 2011).

Penicillium recorded lead ion removal rate of approximately 67% in the current study (Table 2). This genus have shown to be a relevant to remove metals from soils and aquatic environments (PAL, GHOSH and PAUL, 2006). Martins et al. (2016) have tested the ability of eight species belonging to genus *Penicillium* to remove several metals (cadmium, cobalt, copper, lithium, lead and nickel) found in aqueous waste. The highest absorption rates were observed for lead, whose values were similar to the ones reported in the present study (60%), but within 1 experimental hour. Species *Penicillium brasilianum* recorded 74.4% lead removal rate in a binary mixture with lithium. In addition to the metals tested by the aforementioned authors, representatives of the investigated genus have shown the ability to remove zinc, chromium copper, gold, mercury and manganese (LEMOS et al, 2008; OLIVEIRA et al., 2018).

Tian et al. (2019) have recently suggested the mechanisms used by species *Aspergillus niger* and *Penicillium oxalicum* to reduce Pb toxicity. Fungi naturally secrete oxalic acid, which reacts to Pb^{2+} and forms insoluble metal minerals, mainly lead

oxalate. Then, biosorption is stimulated by the formation of a new membrane in cell wall, which prevents Pb^{2+} transport in hyphae.

Genus *Trichoderma* was the third to be isolated. It is a rhizosphere fungi used in biopesticide production in agriculture, as the source of enzymes in industrial activities and in the clinical field. The genus is also important bioremediation agent used to remove metals, xenobiotic compounds, toxins, as well as soil and water contaminants (MUKHERJEE et al., 2013; TANSENGCO et al., 2018).

Representatives of genus *Trichoderma* recorded the highest lead removal rate (75%), as shown in Table 2. Similar study was developed by Tansengco et al. (2018), who isolated resistant fungi from Mine Tailings in Itogon, Benguet. Representatives of genus *Trichoderma* were also microorganisms presenting the highest bioremediation potential after incubation in culture medium containing different metals (copper, chromium, lead, nickel and zinc), with emphasis on their ability to tolerate high chromium and lead levels.

Similar removal rate was observed for species *Trichoderma asperellum* (HOSEINZADEH, SHAHABIVAND and ALILOO, 2017), which was able to remove 68.4% of lead, at pH 5.6, 100 mg L-1 Pb^{2+} using (commercial) PDB culture medium.

Thus, the current study isolated filamentous fungi from lead-contaminated soil and showed the metal removal ability of three genera (*Aspergillus*, *Penicillium* and *Trichoderma*) by using submerged fermentation medium, which was easily produced in laboratory environment.

4 CONCLUSIONS

Techniques adopted in the current study allowed isolating and analyzing the bioremediation potential of filamentous fungi in soils contaminated with lead ion (Pb^{2+}). Sabouraud culture medium added with chloramphenicol was effective in exclusively isolating fungi (i.e., without bacterial contamination). The submerged fermentation medium was efficient in enabling microbial growth; besides surviving in this medium, the molds were able to remove Pb^{2+} ions from the substrate. Therefore,

it is an efficient and inexpensive technique that can be easily produced at large scale in laboratory environment based on accessible ingredients. Species belonging to genera *Aspergillus*, *Penicillium* and *Trichoderma* removed 56.82%, 66.77% and 75.29% of lead ions, respectively, in comparison to the control. Results have confirmed the bioremediation potential of these fungi and their possible use in metal-contaminated areas.

Thus, in addition to showing the presence of fungi with bioremediation potential in the soil, the use of a simple and low-cost technique enables conducting further tests with other fungi species and metals, either together or in separate.

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