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Manganese (II) removal from aqueous solutions by *Cladosporium halotolerans* and *Hypocrea jecorina*

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ABSTRACT

Manganese (Mn) is toxic at higher concentrations requiring its removal before returning the wastewater to the environment. This article reported the Mn removal of two fungi strains isolated from mine wastewater. ITS rRNA region sequencing identified the fungi strains as *Cladosporium halotolerans* and *Hypocrea jecorina*. Mn^{2+} removal assays were performed in Sabouraud broth with 50 mg L^{-1} Mn^{2+} supplemented and bioleaching assays using MnO_2 instead of $MnSO_4$ at the same conditions. *C. halotolerans* removed 96 % of 50 mg L^{-1} Mn^{2+} at two weeks without MnO_2 bioleaching with 649.9 mg of biomass and *H. jecorina* removed about 50 % of Mn^{2+} in 21 days from initial 50 mg of Mn^{2+} L^{-1} with 316.8 mg of biomass. Extracellular laccases were present in *C. halotolerans* agar regardless of the Mn addition. Mn adsorbed was detected on *C. halotolerans* hyphae. Mn oxidation was positive to *H. jecorina* by reaction of its medium with Leucoberbelin blue.

1. Introduction

Manganese (Mn) is a toxic metal found in silicates, oxides, carbonates and others minerals. Extracted Mn is typically used for steel production [1]. Mn interacts with several other metals, participating in their cycles. For example, Mn oxides may oxidize As^{2+} to As^{4+} [2], Co^{2+} to Co^{3+} [3], and U^{4+} to U^{6+} [4]. Mn^{2+} is stable in environments with low E_h and pH, whereas Mn^{3+} and Mn^{4+} are stable in environments with higher E_h or pH [5].

Mining generates wastewater with elevated Mn concentrations that may cause environmental impacts if discharged without treatment. The usual treatment of wastewater relies on physico-chemical reactions. Superoxide, chloride, peroxide and other ions are used as oxidizing agents, and lime, sodium carbonate, and sodium hydroxide are used as alkalizing agents that also induce Mn precipitation; this treatment is commonly used around the world and generates mainly Mn carbonate [6–8]. Treatments which produce Mn oxides are more profitable because oxides have sorption capacity that recruits other Mn^{2+} ions to the growing oxide structure [9].

An alternative is the biological removal of Mn. Several studies have demonstrated the ability of microorganisms to clear Mn^{2+} from waters, such as *Phoma* sp., *Paraconiothyrium* sp. and *Acremonium* sp. [10–12]. There are several descriptions of the manner in which some fungi precipitate Mn, including the formation of Mn oxides in extracellular or intracellular deposits [13,14]. Multicopper oxidases (Mcos) are enzymes that are able to catalyze Mn oxidation and its consequent removal, and these enzymes can be found in bacterial manganese-oxidizing spores [15]. In fungi, there are several types of Mcos. One class of Mcos called laccases specifically mediate the Mn oxidation associated with organic acids and chelating compounds [16,17]. The aim of this study was to evaluate the potential of Mn sequestering or oxidation performed by a fungal strain isolated from wastewater for future application to the bioremediation of Mn contamination in waters. This study employed Mn assays using carbon-rich culture media and also tested whether the fungal strain had the capability to reduce inorganic Mn oxides.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); CTAB, Hexadecyltrimethylammonium bromide; EDX, Energy-dispersive spectroscopy; ITS, Intergenic Spacer; LAC1/2, Laccase genes 1/2; Mcos, Multicopper oxidases; nBLAST, nucleotide Basic Local Alignment Search Tool; PCR, Polymerase Chain Reaction; rRNA, ribosomal Ribonucleic Acid; SEM, Scanning electronic microscopy; LBB, Leucoberbelin blue or N.N'-Dimethylamino-β, β'-triphenylmethane-o-sulphonic acid.

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2. Materials and methods

2.1. Source and isolation of fungi strains

An acid mine water sample was collected from the Iron Quadrangle region (Minas Gerais, Brazil) 20° 39′ 36″ S, 43° 47′ 9″ W (-20.66, -43.785833), and inductively coupled plasma optical emission spectrometry (ICP-OES, Varian 725) was used to analyze the sample composition for metals. The complete characterization of this wastewater was described in [18]. One liter of mine water sample was filtered through a 0.22-μm membrane (Millex, Millipore), and the membrane was inoculated into Sabouraud (HiMedia), pH 5.6. Later, a blackish and a greenish fungal colony were isolated and preserved in 15 % glycerol.

2.2. Fungi identification

Genomic DNA was extracted from two sample of fungal mycelium (200 mg) by the CTAB (Hexadecyltrimethylammonium bromide) method [19]. The integrity of DNA was observed on a 0.6 % (wt/vol) agarose gel. The DNA concentration was measured (Nano Vue, GE). Amplification of the ITS ribosomal region was performed following the protocol [20]. The primers used for PCR were ITS4 (reverse) and ITS5 (forward) described in [21]. In this experiment, a negative control was created using water instead of genomic DNA. PCR products were run on a 1.2 % agarose gel and stained with ethidium bromide for visualization on a UV transilluminator (Vilber Lourmat). The negative control was visualized without amplicons. Three samples were sequenced using an automated Sanger sequencer (AB1 3100, Applied Biosystems) and a Big Dye cycle sequence kit (Applied Biosystems). A consensus sequence was subjected to nBLAST to retrieve type material sequences. The related sequences were aligned with the consensus sequence in CLUSTAL Omega; Phylip format and the sequences' global alignment were cut in Phylogeny FR, Gblocks. The Phylip archive was used to construct a distance tree using the Kimura algorithm (2000 replicates). The phylogenetic tree was visualized in FigTree 1.4.2.

2.3. Manganese removal assay for the fungi strains

The fungi strains were introduced separately into flasks containing 150 mL of sterilized Sabouraud broth plus 100 μg mL^{-1} ampicillin and 50 mg L^{-1} Mn^{2+} (using $MnSO_4$), with a pH of 6.5 and a temperature of 28 °C. Sabouraud broth supernatants aliquots were collected at seven-day intervals, recovered by centrifugation at 12,000 g_n for 10 min, preserved in 2 % HCl, diluted ten-fold and then stored until measurement of Mn using an emission spectrometer (ICP-OES, Varian725). Control flasks had the same components, except for the fungi strains. Each flask was incubated for five weeks and pH was measured weekly. The biomass of both fungi were dried by incubation at 70 °C and weighted daily for three days until they reach a constant value.

2.4. Manganese bioleaching by fungi strains

To evaluate the Mn bioleaching ability developed by the two fungi strains isolated from wastewater, a reduction experiment was conducted in parallel. Fungi strains was introduced separately to flasks containing 150 mL of sterilized Sabouraud broth plus 100 μg mL^{-1} ampicillin and 50 mg L^{-1} Mn^{4+} (using MnO_2 powder) pH 6.5 at 28 °C; [22]. Sabouraud broth supernatants aliquots were collected at seven-day intervals and recovered by centrifugation at 12,000-g_n for 10-min, preserved in HCl 2 %, diluted tenfold and stored until measurement of Mn using an emission spectrometer (ICP-OES, Varian 725). Control flasks had the same components except for the fungi strains, and each flask was incubated for five weeks.

2.5. Detection of Mn oxidized - leucobbercaine blue (LBB) assay

Stock solution of leucobberellin blue (N.N'-Dimethylamino-β, β'-triphenylmethane-o-sulphonic acid) and reaction solution was made according to [23]. LBB solutions added to MnO_2 and $MnCO_3$ were used as positive and negative controls respectively. Samples of 1.5 ml volume of medium were collected weekly of *C. halotolerans* and *H. jecorina* to LBB reaction.

2.6. Multicopper oxidase plate assay

The presence of extracellular Mcos (laccases and others) was determined with the oxidation of ABTS (Sigma-Aldrich, USA) in Petri dish plates inoculated with the fungal strain containing Sabouraud agar medium plus 100 μg mL^{-1} ampicillin, which were incubated for two weeks in the presence of 140 mg L^{-1} Mn^{2+} (using $MnSO_4$), and without Mn^{2+}, pH 7.0; 28 °C [24]. The reduction of Mcos with consequent substrate oxidation was indicated by blue-green coloration formed in the agar plate.

2.7. Scanning electronic microscopy (SEM) and energy-dispersive spectroscopy (EDX) analysis

Fungi strains were grown in slide culture using Sabouraud *agar* medium for five days. In a plastic Petri dish, a Sabouraud agar block cast from Sabouraud agar medium plus 100 μg mL^{-1} ampicillin adjusted to pH 7.0 was sandwiched between two sheets of slide glass. The slide culture was kept moist with watered cotton and inoculated with fungus. After two weeks of incubation the two slide glasses were fixed according to [25], one sample with 140 mg L^{-1} Mn^{2+} (using $MnSO_4$) and other without any Mn^{2+}. Each slide glass was overlaid with gold using a rotary-pumped sputter coater (Q150R ES, Quorum). EDX analysis was performed by mapping sections in the sample area, and the elemental composition was detected by spectroscopy and visualized by attached software (Oxford coupled to Vega3 Tescan SEM).

3. Results and discussion

3.1. Isolation and molecular identification of fungi of waste water

Once preserved, the "31" isolated cultures had a greenish-black coloration. Fig. 1a. Shows the phylogenetic tree based on the consensus ITS rRNA gene sequence, 531 bp in length, derived from 31 fungal strain. The only sequences returned by BLASTn with above 90–95 % identity to the consensus sequence were from species of genus *Cladosporium.* In the phylogenetic tree, the 31 isolates were placed alongside *Cladosporium halotolerans* (NR 119605.1), which presented 100 percent sequence identity with the consensus sequence (data not shown). Isolate 7 had a white greenish coloration and its phylogenetic analysis showed greater proximity in the branch of *Hypocrea jecorina*, Fig. 1b, with bootstrap of 1000 replicates. A consensus of 638 bp was obtained for isolate "7", of ITS rRNA gene sequence, were recovered sequences above 97–100 % of identity and an e-value equal to 0. Phylogenetic analysis was performed using sequences retrieved from GenBank using a type filter [26].

3.2. Manganese removal by C. halotolerans and H. jecorina

It is known that high redox potentials and pH greater than 11 cause physicochemical oxidation and consequent Mn precipitation. Any Mn oxide previously formed may sequester other Mn^{2+}

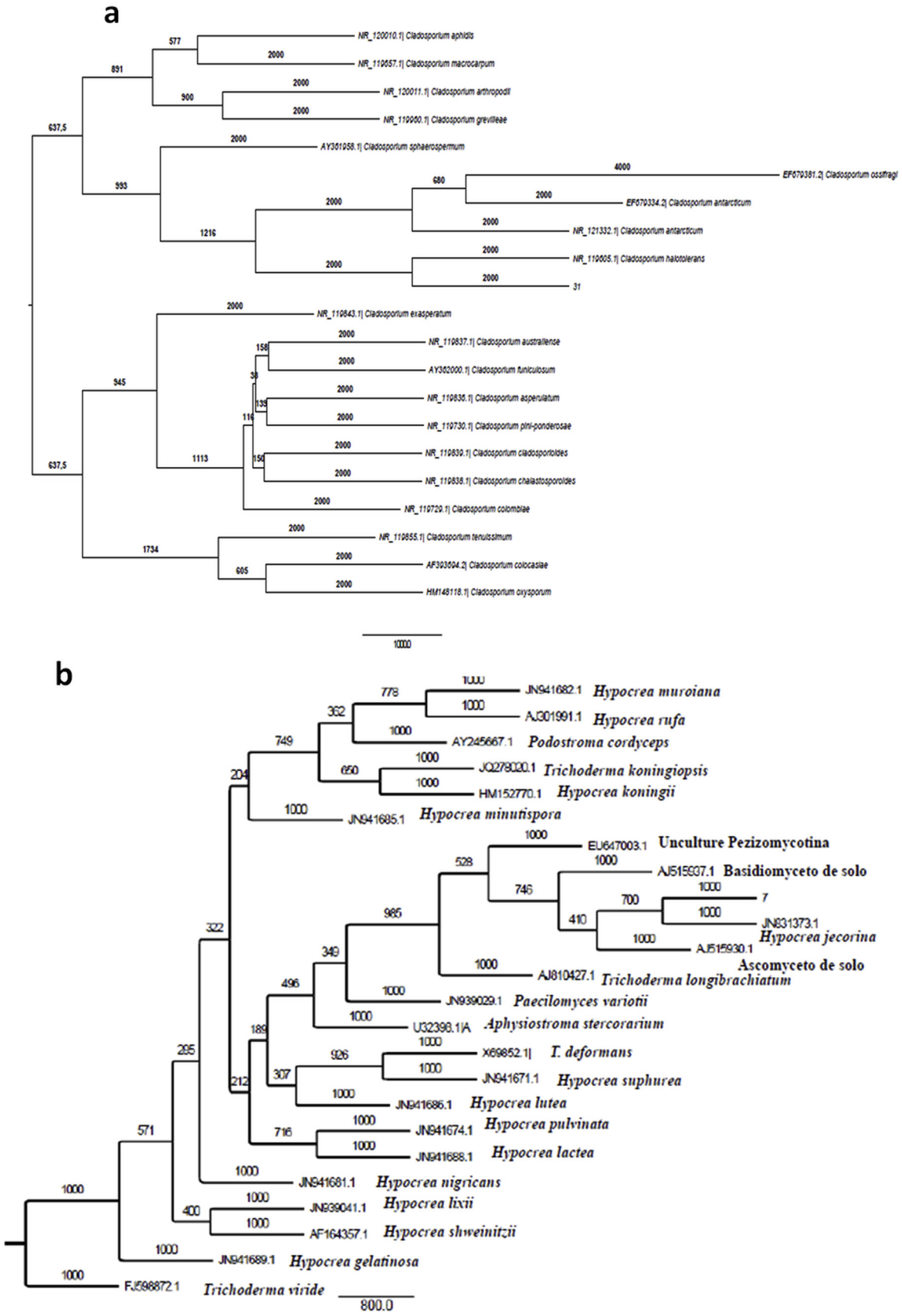

Fig. 1. Phylogenetic tree based on the sequence of the ribosomal ITS region: The phylogenetic tree was constructed using the Kimura neighbor-joining algorithm implemented in FigTree 1.4.2. A) *C. halotolerans*. B) *H. jecorina*.

ions because the oxide precipitation process is autocatalytic [27]. At pHs higher than 7.5–11, Mn^{2+} may be precipitated as $MnCO_3$, a compound that does not have an autocatalytic formation process, but that can be removed from water due to its insolubility [27]. According to Figs. 2a and 2b, in this assay, the pH varied from 5.0–7.0, indicating that chemical removal of Mn as $MnCO_3$ did not occur. After 14 days, the *C. halotolerans* removed 48 mg L^{-1} of Mn^{2+} of approximately 50 mg L^{-1} of Mn^{2+} initial concentration added in 150 mL of Sabouraud broth in acidic to neutral pHs, the assay performed lasted 5 weeks (Fig. 2a).

Initial decay in pH flask from 7 to 5 of *H. jecorina* (Fig. 2b) caused no significant removal of Mn in the first 7 days; this period was also a period of hyphae growth. Stabilization in the pH occurred in the period of 7–14 days, at which time the concentration of Mn fell by half and also the hypha was already occupying the entire surface of the flask. At the end the Mn concentration was 15.91 mg L^{-1}, *H. jecorina* removed approximately 15.68 mg of Mn in all. After 14 days there was a mild decline in Mn concentration and the pH rose to 7 again. Pure chemical removal is caused in pHs above 7, then we can see that this fungus must have some Mn^{2+} removal mechanism. The period of greatest removal coincided with the period in which the mass of the hypha had grown over all surface of culture medium, 316.8 mg; SD = 0.04284 of biomass. When the pH was acid there was no detectable removal, but we may not say that the removal was chemical, since the pH remained from acid to neutral. *H. jecorina* is known for its biotechnological potential in producing cellulases and other enzymes that degrade various compounds [28].

In this present work, *C. halotolerans* removed over two weeks 22.08 mg of Mn of 23 mg added in the media, with a mean biomass of 649.9 mg; SD = 0.002779, the hyphae grew over 17 days, this is the time of greatest decrease in the concentration of Mn. While there were nutrients, space and Mn, *C. halotolerans* removed this metal from the media (Fig. 2a). After two and a half weeks, *C. halotolerans* stopped removing Mn because it was also depleted from the media; *C. halotolerans* had a greater biomass than *H. jecorina* and removed more Mn.

According to literature the ascomycete *Phoma* sp. removed 80 mg L^{-1} of Mn within 7 days in a water treatment using a carbon fiber [11], and *Paraconiothyrium* sp. removed approximately 300 mg L^{-1} in PY buffer over 12.5 days [10], but that experiment required the use of buffers to maintain neutral pH for multiple weeks. In this present experiment, the pH was oscillatory for *H. jecorina*; this factor negatively affected the quantity of the Mn removed because lower pH induces solubilization [27]. Nevertheless, in this present assay, similar rates of removal occurred even in acidic conditions. Here was demonstrated a Mn decay during the mycelium growth, Mn oxidation by *Cladosporium cladosporioides* but in enzymatic assays was performed by incubation of media culture with mycelium and smaller quantities of $MnSO_4$ [29].

3.3. Manganese bioleaching by C. halotolerans and H. jecorina

To treat waters is desired to use fungal strains that remove soluble Mn^{2+} without bioleach Mn ores concomitantly. Thus, reduction assays were conducted to detect if *C. halotolerans* promotes Mn bioleaching while it is removing Mn. When MnO_2 is added to Sabouraud broth, a slow bioleach of the oxide occurs, we can see at time 0 a concentration of Mn^{2+}. According to Fig. 3a, *C. halotolerans* continuously removed even the low amount of Mn^{2+} solubilized when MnO_2 was added to Sabouraud broth, the initial concentration. This fact is noted by control flasks Mn^{2+} levels that were lower than *C. halotolerans* flasks Mn^{2+} levels, suggesting that this fungus did not biologically bioleach MnO_2. A slightly bioleach of MnO_2 is visualized in the control rates of the experiment; the

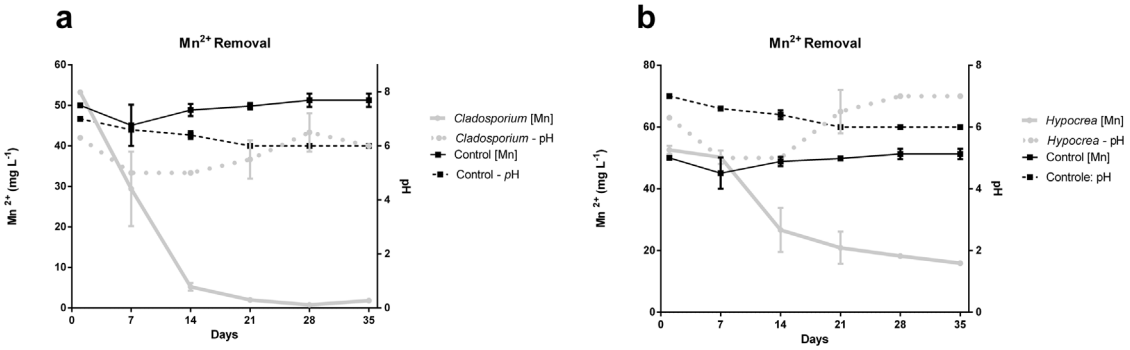

Fig. 2. Bio-removal of Mn^{2+} ions associated to pH dynamics (GraphPad Prism 6). A) *C. halotolerans* bio-removal of 50 mg L^{-1} Mn^{2+}. B) *H. jecorina* bio-removal of 50 mg L^{-1} Mn^{2+}. Error bars indicate the standard deviation of triplicate values. The assay was extended to a period 5 weeks long.

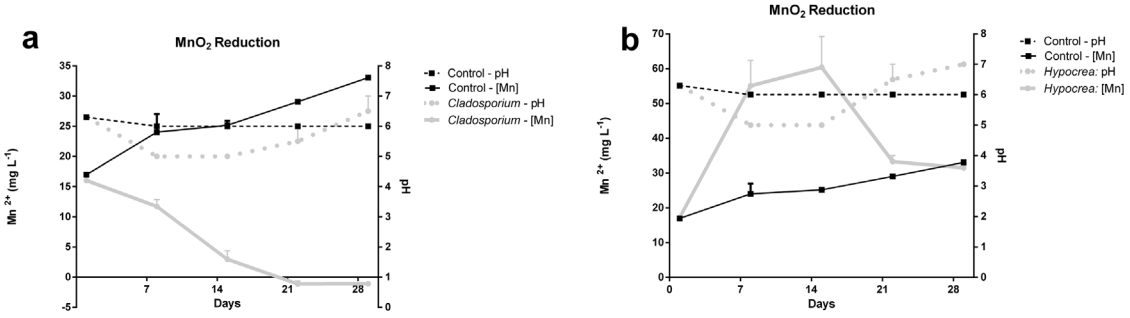

Fig. 3. Bioleaching of manganese dioxide in Sabouraud broth containing 50 mg L^{-1} Mn^{4+} (GraphPad Prism 6). This experiment was performed in triplicate and lasted 5 weeks. A slightly reduction in control flasks of Mn^{4+} is due to initial pH = 6.5. A) *C. halotolerans*. B) *H. jecorina*.

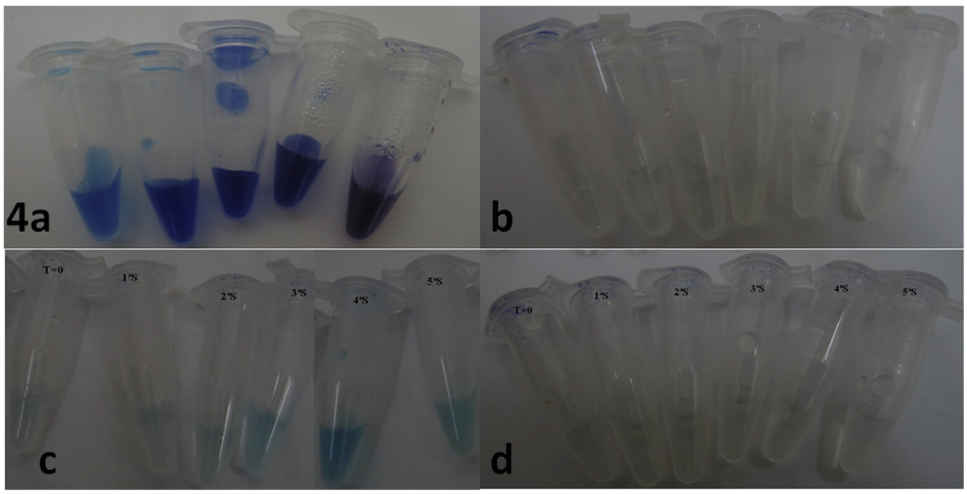

Fig. 4. LBB Assay: a) Positive controls performed concentrations of Mn dioxide (0.0001 mM, 0.0005 mM, 0.001 mM, 0.01 mM and 0.05 mM PA, Sigma). b) Negative controls carried out concentrations of Mn carbonate (0.0001 mM, 0.0005 mM, 0.001 mM, 0.01 mM and 0.05 mM PA, Sigma). c) Reaction of Mn^{3+} or Mn^{4+} oxides present in the culture medium of isolate 7(*H. jecorina*). We can see in the left to right the tube corresponding to time zero (T = 0), 1 st week of cultivation - 7 days (1 st S), 14 days of cultivation (2ªS), 21 days of cultivation (3ªS), 28 days of cultivation (4ªS) and 35 days of cultivation (5ªS). d) The *C. halotolerans* isolate does not present Mn oxides suspended in the culture medium.

initial pH of experiment was 6.5, physic-chemical studies reported that Eh, presence of other molecules and acid pH favors the bioleach of Mn oxides [27]. During the assay, the pH varied from 5.0–7.0, and no ore reduction occurred at any pH level. Lower pHs favor chemical reduction and consequent bioleaching [27], but *C. halotolerans* demonstrated decrease the soluble manganese concentrations even though the pH values were acid.

The *C. halotolerans* isolated in this study did not present any ability to bioleach oxides of Mn in Sabouraud broth according to Fig. 3a, while *Cladosporium oxysporum* has the ability to bioleach uranium [30], and strains of *C. cladosporioides* are known reducers of chromium (VI) to chromium (III) [31], but there are no reports of Mn reduction mediated by *C. halotolerans*. These results are desirable for water treatment.

Same experiments made with *H. jecorina* appointed an increase in MnO_2 bioleaching, the pH dynamics were practically the same as that observed in the removal test. In the period between 0 and 7 days, there was reduction of Mn oxide to Mn^{2+}. In pH 5-7of Mn removal experiment, we have a slight decrease in the concentration of Mn^{2+} (Fig. 2b) and an increase in the concentration of this ion in the bioleaching experiment (Fig. 3b). Therefore, we can suggest that the small removal of Mn^{2+} observed in Fig. 2b may be due to the fact that at this acid pH value, there is a prevalence of the reduction of the Mn oxide, and therefore, the removal of Mn^{2+} is practically annulled by this process. At pH 7 there is an inversion, the removal of Mn^{2+} became prevalent and occurred decay in bioleaching, therefore, we see a decrease in the concentration of Mn in the two last weeks of the experiment, fact perceived by the superposition of the removal and bioleaching graphs. Bioleaching performed by *H. jecorina* is probably due to low pH of acids produced during mycelium growth.

3.4. Detection of Mn oxidized - leucobbercaine blue (LBB) assay

Fig. 4 shows the reaction of LBB with aliquots of culture medium from five weeks of culture. We can see that there was formation of oxidized LBB in every week for medium culture of *H. jecorina* (Fig. 4c). So we can say that *H. jecorina* oxidizes Mn^{2+} to Mn^{4+} or Mn^{3+}. The removal performed by this isolate is not simply Mn^{2+} precipitation in $MnCO_3$ (Mn^{2+} insoluble). No detection of oxidized Mn suspended in the culture medium of *C. halotolerans* was visualized in any of the 5 weeks samples (Fig. 4d).

3.5. Participation of Mco in manganese removal mediated by C. halotolerans

To evaluate the possibility of manganese removal performed by Mco activity, ABTS plate assays were made using *C. halotolerans* cultures. *C. halotolerans* grew and secreted Mcos in both presence and absence of 140 mg L^{-1} of Mn^{2+} from 2 days onwards in the same color intensities. Some laccase genes have metal-activated enhancers [32], but *C. halotolerans* formed blue-green halos in the Sabouraud agar independent of the Mn presence or absence (Fig. 5a–d). The colored zone covered the entire plate after five days' culture. This result suggests that Mcos activity could be related to decrease in Mn concentrations of culture medium. The present results show that the fungal secretion of Mcos into the Sabouraud agar is not activated nor increased by presence of environmental manganese, and the secretion of this enzyme may be associated with the observed manganese removal because the relationship of Mcos and manganese removal is well described in Mn-oxidizing bacteria [15]. *C. halotolerans* demonstrated laccase activity on the 2nd day of culture at 28 °C in Sabouraud agar.

Laccases produced by *Trametes hirsuta* have ability to oxidize Mn^{2+} in the presence of organic acids to Mn^{3+} complexes, and kinetics experiments were performed using ABTS as substrate to demonstrate the substrate oxidation and consequent production of water from peroxide and superoxide radicals by that laccase [17]. *Cladosporium tenuissimum* demonstrated laccase activity on the 4th day of culture at 25 °C in potato dextrose agar [24]; *C. halotolerans* demonstrated laccase activity on the 2nd day of culture at 28 °C in Sabouraud agar (Figs. 5a, 5b, 5c and 5d), showing that this fungal strain has a higher affinity for its substrate than *C. cladosporioides*. Other studies with *C. cladosporoides* indicate that Mn oxidation is mediated by an extracellular enzyme [29]. Many researches attribute Mn^{2+} oxidation to Mcos activity [17,33] and these results show a Mn removal and a Mco secretion by *C. halotolerans*, suggesting that here the Mn^{2+} removal was associate with Mcos enzymes.

H. jecorina specie suffered loss of laccases within the genus *Hypocrea* [34], so as expected *H. jecorina* grew in presence of 140 mg L^{-1} of Mn^{2+} and without Mn^{2+} (the control plate), presented no evidence of laccase secretion in ABTS assay (Fig. 5e and f).

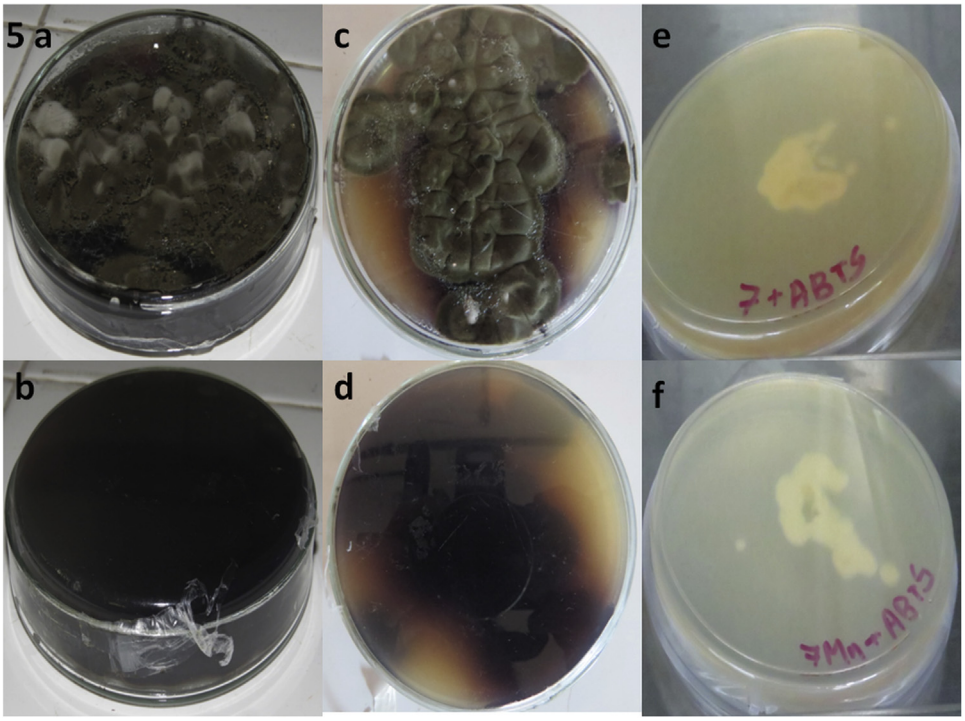

Fig. 5. Laccase activity – ABTS. a) *C. halotolerans* strain: Qualitative plate laccase activity in Sabouraud agar with 140 mgL^{-1} of Mn^{2+}, with the blue color showing Mco activity indicated by the arrow - front and b) *C. halotolerans* strain: 140 mgL^{-1} of Mn^{2+} - Back. c) *C. halotolerans* strain: Qualitative plate laccase activity in Sabouraud agar without Mn, with the blue color showing Mco activity indicated by the arrow - front. and d) *C. halotolerans* strain: Without any Mn - Back. e and f) *H. jecorina* strain: without and with 140 mgL^{-1} of Mn^{2+}, presenting no evidence of laccase secretion in ABTS assay.

3.6. SEM and EDX evidence of mechanisms of manganese removal

The EDX analysis reveals 1.5 % Mn on the *C. halotolerans* hyphal surface in 2 weeks of growth (Fig. 6a and b). Of the elements found on the slide glass, K, Mg, Na and O are present in the hyphae, and Si is a slide glass component. Mn found on the surface of conidia indicated which *C. halotolerans* isolate from waste waters has developed a mechanism of Mn removal by chelating whereas LBB detected no Mn oxidation to this fungal strain.

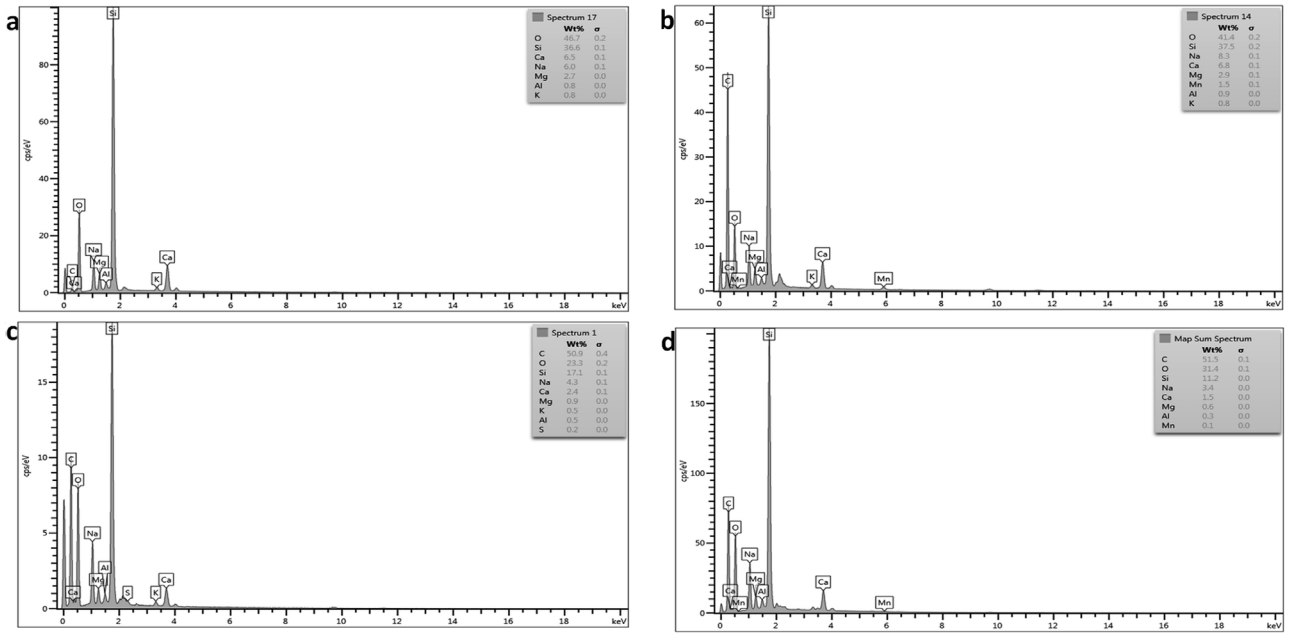

Fig. 6. Composition Analysis of surface hyphae: a) *C. halotolerans* EDX - without Mn: *C. halotolerans* slide glass preparation; the elements described in the EDX graphics show the composition of hyphae and glass. b) *C. halotolerans* EDX - plus 140 mgL^{-1} of Mn^{2+}: 1.5 % of Mn adsorbed on the surface of the hyphae. The Mn percentage is the major difference between the two slide glasses. c) *H. jecorina* EDX – without Mn: slide glass preparation; the elements described in the EDX graphics show the composition of hyphae and glass. d) *H. jecorina* EDX - plus 140 mgL^{-1} of Mn^{2+}: 0.1 % of Mn adsorbed on the surface of the hyphae.

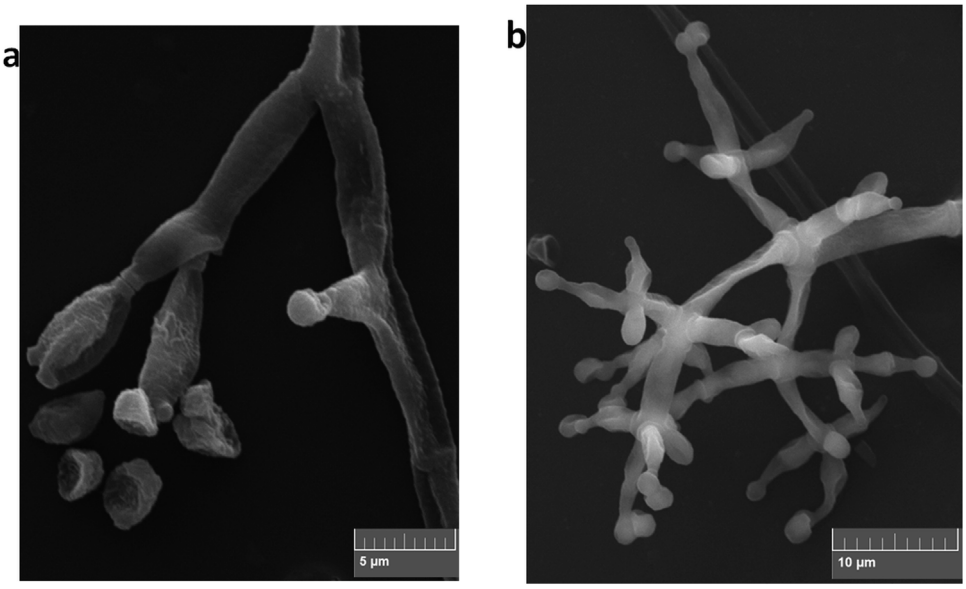

Fig. 7. SEM analysis showing morphological aspects of hyphae. Prominent conidia corroborate the molecular identification. A) *C. halotolerans*. B) *H. jecorina*.

As shown in Figs. 7a and b, the presence of Mn^{2+} did not diminished the fungal growth; hyphae did not alter microscopic aspects of conidia either in the presence of 140 mg L^{-1} Mn^{2+} or without Mn^{2+}. Furthermore, macroscopic aspects of *C. halotolerans* colonies which grew in presence of Mn were different than in absence. Hyphal were darker and stiffer when grown in Mn presence (data not shown). Additionally, the hyphal features corroborate the molecular analysis, suggesting a successful identification of the isolate to genus *Cladosporium* (Fig. 7a).

There are several studies which showed that fungal species may remove Mn by adsorption beside Mco activity. *Cladosporium* species may adsorb or uptake metals corroborating the results showed in this study (Fig. 6a and b). *C. cladosporioides* isolate already showed the ability to precipitate Mn in intracellular crystals [14]; Adsorption of Au ions in acid conditions due to electrostatic interactions in *C. cladosporioides* have been demonstrated [35]; other abilities of *Cladosporium* sp. remove Cd^{2+} [36], Zn and Cu [37] and to accumulate Fe^{3+} [38] have also been documented.

Melanin has properties of UV protection, chelating metals as Cu, Hg, Pb, Cr and etc, avoiding oxidative stress, widely described [39]. Melanin is present in fungal cell walls and two laccase genes, LAC1 and LAC2, participate of melanin synthesis in some fungi as *Cryptococcus neoformans*. LAC1 is transmembrane, LAC2 is cytoplasmatic and in its biosynthesis occurs a transportation of melanin to cell wall [40,41]. The *C. halotolerans* demonstrated a high affinity in ABTS assays suggesting a high laccase expression which can be linked to its black color indicating a high melanin production. *C. halotolerans* would sequestrate Mn^{2+} present in Sabouraud broth and hold it by chelating. This mechanism is supported by EDX evidence where we may observe Mn^{2+} in cell wall fungal surface considering that melanin has properties of chelating other metals as mentioned above.

H. jecorina EDX did not present significant Mn rates on hyphal surface considering the isolate which grew in Mn presence, Fig. 6c and d. SEM of *H. jecorina* in the presence of Mn did not cause visible stress on the external appearance of the hypha and corroborated with usual appearance of genus *Hypocrea* species (Fig. 7b). The possible mechanism of Mn removal by this fungus is the formation of oxides in the culture medium; these oxides were detected in the test with LBB.

4. Conclusion

C. halotolerans has high potential for Mn removal removing 48 mg L^{-1} of Mn^{2+} from Sabouraud broth initially containing 50 mg of Mn^{2+} L^{-1}. This amount corresponds to 96 % of the initial concentration in two weeks, with a dry biomass of 649.9 mg; SD = 0.002779. Mn removal occurred due to chelating mechanisms. Notably, it was unnecessary to use buffers to maintain pH, and the pH was acidic to neutral throughout the experiment, confirming biological removal. *H. jecorina* oxidizes and bioleaches Mn from culture medium; Mn^{2+} removal rates compensate its Mn^{4+} reduction, presenting an overbalance of 50 % with a dry biomass of 316.8 mg; SD = 0.04284. These fungi were isolated from the same place and present different Mn removal mechanisms.

CRediT authorship contribution statement

Ester Alves Mota: Writing - original draft, Writing - review & editing, Investigation, Validation, Conceptualization, Methodology. **Érica Barbosa Felestrino:** Investigation. **Versiane Albis Leão:** Supervision. **Renata Guerra-Sá:** Funding acquisition, Project administration, Supervision, Resources.

Declaration of Competing Interest

We confirm that it is not any financial or interest conflicts.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00431.

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