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Copper-Contaminated Substrate Biosorption by *Penicillium* sp. Isolated from Kefir Grains

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Abstract: This work aimed to carry out a bioremediation study to evaluate the resistance of *Penicillium* sp. isolated from kefir grains for the treatment of copper. The fungal culture medium was prepared using a 2% malt-agar medium at pH 7.0 in which *Penicillium* sp. was inoculated. *Penicillium* sp. growing in a liquid medium showed a decrease in biomass in presence of $Cu(NO_3)_2$ (800 $mg.L^{-1}$), suggesting that the metal impacts the growth rate of the fungus. Moreover, the combined factors of pH and the presence of the inorganic contaminant impacted the radial growth of the fungus, causing inhibition of 73% at pH 4.0, 75% at pH 7.0, and 77% at pH 9.0 in liquid medium compared to control. However, images obtained with scanning electron microscopy showed the integrity of the fungus cell, even at high doses of copper in the medium. Therefore, it can be concluded that *Penicillium* sp. isolated from kefir grain can bioremediate the environment and that the harmful effects of heavy metals can be minimized as a result biosorption. Although the growth of *Penicillium* sp. is inhibited, such retardation requires high doses of copper nitrate, thus ensuring the use of this microorganism for protection against the harmful effects of non-essential copper in the environment.

Keywords: bioremediation; copper nitrate; *Penicillium* sp.; kefir; inorganic contaminant

1. Introduction

Owing to progressive industrial development and technological activities, contamination by heavy metals is increasing every year, constituting a serious risk to the environment and public health [1]. Anthropogenic activities, such as fungicide spraying in agriculture [2] and mining, have resulted in copper (Cu) contamination in environmental compartments like soil, water, and sediment at levels that sometimes exceed the allowable limit of toxicity [3,4]. Likewise, the high level of copper and other heavy metals coming from many industrial effluents has caused many serious environmental and health problems [5,6].

Copper can contaminate large areas; its main source is mining wastewater [7]. For human health, the consumption of contaminated water is life-threatening. Copper is one of the essential elements for all living beings, including humans. It acts as a cofactor in a series of metalloenzymes involved in the formation of hemoglobin and the metabolism of carbohydrates [8].

Fungi play an important role in biodegradation owing to the morphology of their mycelium. Fungus mycelium is highly reactive to the environment and has an extensive biological surface. In

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addition, fungus physiology is characterized by the high capacity for stress, enzyme production, and secondary metabolites [9].

Kefir is a microbial complex consisting of fungi with high biosynthetic capacity. It exists in symbiosis with *Lactobacillus* with high catabolic capacity [10]. ***Kefir*** is a fermented milk drink similar to a thin yogurt that is made from ***kefir*** grains, which are gelatinous masses, the color of which depends on the medium in which they are grown [11].

This work aimed to carry out a bioremediation study to evaluate the resistance of *Penicillium* sp. isolated from kefir grains to copper treatment. This step represents the initial phase in the selection of potentially promising microorganisms for use in the bioremediation of environments contaminated by copper. Moreover, it is an extension of our prior work demonstrating how the consumption of kefir can protect those living in areas contaminated by heavy metals such as copper.

2. Results

2.1. Effects of copper on the growth of Penicillium sp.

Copper affected the radial growth *Penicillium* sp. strains. The effects of different concentrations of copper nitrate on the radial growth of *Penicillium* sp. mycelium are shown in Table 1. The minimum inhibitory concentration (MIC) was 43% (51.05 ± 0.50 mm) when the copper concentration reached 500 mg.L-1, and it increased to 75% (22.17 ± 1.75 mm) at a concentration of 800 mg.L^{-1}. ANOVA indicated significant differences among concentrations ($F = 8.57$; $p < 0.05$).

Total inhibition of microorganismal growth was only achieved when copper nitrate at the concentration of 1000 mg.L^{-1} was used. Therefore, the data demonstrate that the microorganism has the capacity to tolerate and biosorb copper nitrate in high doses.

Table 1. Growth of *Penicillium* sp. isolated from kefir grain (Pkg) in malt-agar medium (2%) with different concentrations of $Cu(N0_3)_2$.

	CTR mg/L	400,0 mg/L^{ns}		500 mg/L*		600 mg/L***		800 mg/L***		ANOVA
Penicillium sp.	D (mm)	D (mm)	IC (%)	D (mm)	IC (%)	D (mm)	IC (%)	D (mm)	IC (%)	F
	90^{a}	73 ± 1.67^{a}	19	51.50^{b} ± 0.50	43	40.33^{c} ± 1.89	55	22.17^{d} ± 1.75	75	8.57***

D = Diameter in millimeters; % IC = Inhibitory Concentrations Percent; ns= no significance compared to CTR (control); * = p>0.05; ** = p > 0.05; *** = p > 0.001; ^{a,b,c,d} = differences among treatments.

2.2. Growth in pH 4.0, 7.0 and 9.0

The effects of pH on the growth of *Penicillium* sp. were evaluated with the medium at pH 4.0, 7.0, and 9.0 (Table 2). No growth occurred at pH 2.0. The combined factors of pH and the presence of the inorganic contaminant did impact radial growth of the fungus, causing inhibition of 73% at pH 4.0, 75% at pH 7.0, and 77% at pH 9.0 compared to control.

Table 2. Growth in millimeters (mm) of *Penicillium* sp. isolated from kefir grain (Pkg) in 2% malt-agar medium with 800 mg.L^{-1} of copper nitrate at pH 4.0, 7.0, and 9.0.

	48 h	IC%	72 h	IC%	96 h	IC%	120 h	IC%	F
Pkg -CTR	20,00 ± 1.26		45,00 ± 6.33		18,00 ± 1.28		90,00		932.85**
Pkg –Cu pH 4.0	07.25 ± 1.18	6	12,00 ± 0.58	6	14,00 ± 1.76	5	24.40 ± 0.5	73	
Pkg -Cu pH 7.0	6.00 ± 1.06	28	13,00 ± 1.0	3	19,00 ± 1.83	2	22.50 ± 1.9	75	
Pkg -Cu pH 9.0	5,00 ± 0.65	24	14,00 ± 20.10	19	15,00 ± 1.16	18	20.66 ± 0.76	77	

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Pkg -CTR = control (*Penicillium* sp isolate of kefir grain (Pkg) in 2% malt-agar medium with 0 mg $Cu(NO_3)_2$ and Pkg -Cu= *Penicillium* sp isolate of kefir grain (Pkg) in 2% malt-agar medium with 800 mg $Cu(NO_3)_2$.

2.3. Toxicological prediction

Toxicological prediction was performed (Table 4) to compare the ability of microorganisms to tolerate the inorganic contaminant. The lethal concentration (LC_{50}) of mammals to copper nitrate is 25 $mg.kg^{-1}$. Considered Toxicological Class 2, this compound is not carcinogenic, but it is toxic when ingested comes in contact with the skin. Pulmonary toxicity is probably related to the oxidative effects of copper in the lung. Chronic exposure to copper can damage the liver and kidneys with symptoms like those found in Wilson's disease.

Table 3. Toxicological prediction of $Cu(NO_3)_2$.

Toxicological Prediction	
Toxicological Class	2
LC_{50}	25 $mg.kg^{-1}$
Molecular weight	187.56
Number of Hydrogen Acceptors	6
Number of atoms	9
Number of connections	6
Molecular polar surface area (PSA)	137.76

Using the toxicity prediction program (http://tox.charite.de/tox/), the molecular polar surface area (PSA) for $Cu(NO_3)_2$ was calculated. PSA is a very useful parameter for predicting drug transport. It also represents the sum of bridges formed by the polarity of the surface of atoms, which is related to intestinal absorption capacity and the ability to break through the blood-brain barrier (BBB).

2.4. Culture of Penicillium sp. in liquid medium

The fungal culture medium was prepared using a 2% malt-agar medium at pH 7.0 in which *Penicillium* sp. was inoculated. *Penicillium* sp. growing in a liquid medium showed a decrease in biomass in presence of $Cu(NO_3)_2$ (800 $mg.L^{-1}$), suggesting that the metal impacts the growth rate of the fungus. Decreasing pH value in the liquid medium also played a role in decreasing the microorganism's growth rate (Table 4).

Table 4. Biomass *of Penicillium sp*. grown in liquid media with 0 to 800 $mg.L^{-1}$ of $Cu(NO_3)_2$.

Treatment	$Cu(NO_3)_2$	Biomass (µg)	Inhibition (%)	pH
1	Pkg (control group)	578 ± 2.75		7.00
2	Cu^{2+} 400 mg	257 ± 3.00	56%	4.33 ± 0.032
3	Cu^{2+} 500 mg	156.67 ± 2.52	73%	4.19 ± 0.025
4	Cu^{2+} 600 mg	55.33 ± 0.58	90%	4.08 ± 0.003
5	Cu^{2+} 800 mg	41.67 ± 2.08	93%	3.37 ± 0.078

2.5. Biomass analysis of Penicillium sp. isolated from kefir grains by scanning electron microscopy (SEM)

SEM analysis showed the changes in fungal mycelium with increasing concentrations of copper nitrate in the liquid medium. In particular, cracks in mycelium owing to the accumulation of heavy metal inside could be observed (Figure 2d).

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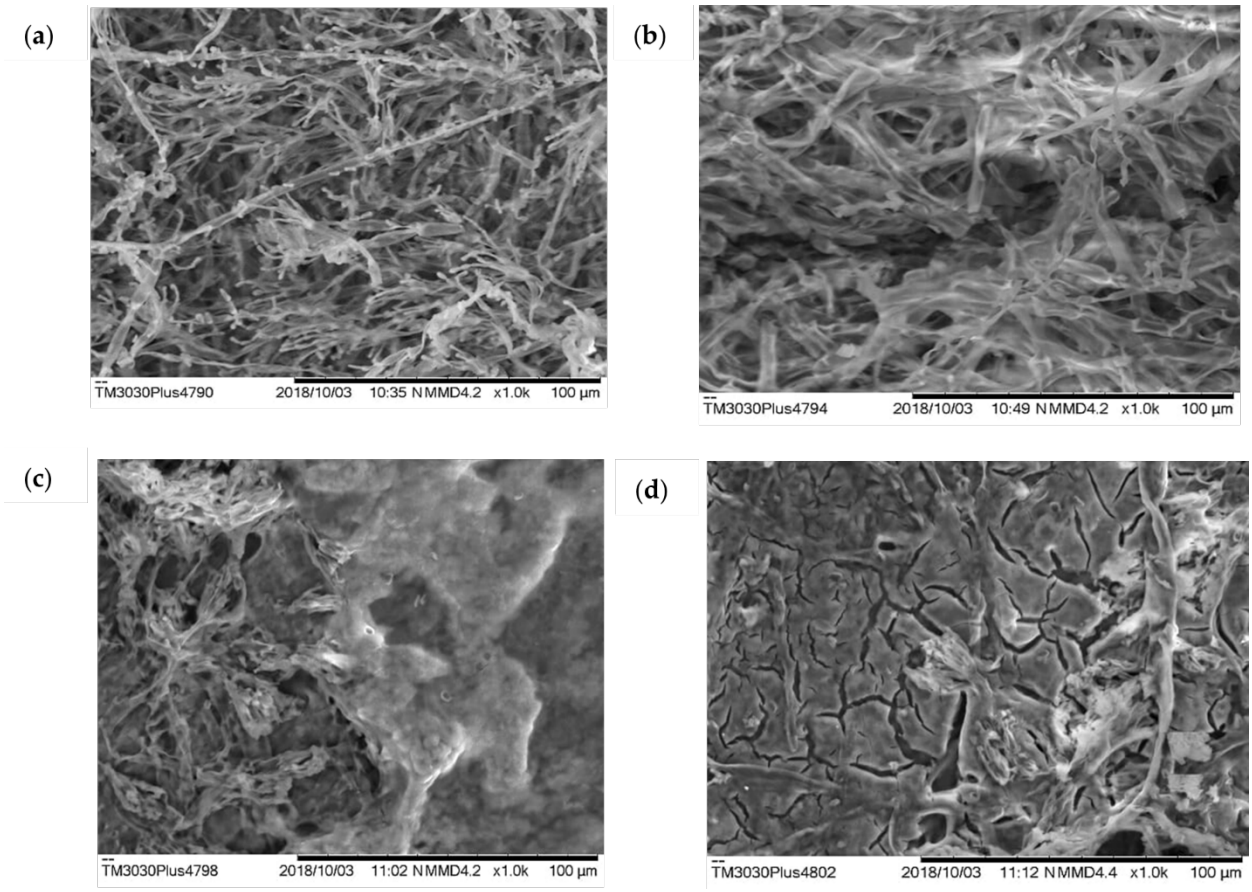

Figure 2. Scanning electron microscopy (SEM) analysis shows the difference in hyphal growth in Penicillium sp. grown in liquid medium a) without copper; b) with 400 mg.L^{-1}; c) with 600 mg.L^{-1}, d) and with 800 mg.L^{-1} of $Cu(NO_3)_2$.

3. Discussion

Heavy metal pollution is a growing problem resulting from anthropogenic activities [4,12]. Of the two types of inorganic contaminants, one includes those that are not essential to our body. However, since ions of non-essential inorganic contaminants have the same valence as necessary ions, the contaminants are absorbed by the body with damaging effects. Among these are lead (Pb), cadmium (Cd), and mercury (Hg). Another group of contaminants are considered essential for the functioning of essential biochemical activities in the body in small doses, but in large concentrations, they become toxic, such as zinc (Zn), iron (Fe), and copper (Cu) [8,13–15].

Copper is part of this second class of contaminants, and its presence is essential in tiny amounts in the human body. It plays an essential role in energy production in cells. Copper is one of the components of myelin which is the lipid layer that protects neurons. It also ensures the maintenance of blood and the functioning of various enzymes in the body [16]. Copper helps in the formation of some blood cells, hormones, and antioxidant enzymes that contribute to the synthesis of neurotransmitters, formation of the myelin sheath, and regulation of gene expression. It also regulates the formation of iron in the body and the formation of connective tissues [16].

Higher concentrations of copper can, however, cause several deleterious effects, e.g., Alzheimer's disease and liver damage [17]. In this study, it was observed that the growth of *Penicillium* sp. was reduced when using 800 mg.L^{-1} of copper nitrate, whereas the reduction in fungal growth was only 19% at 400 mg.L^{-1}. The inhibition of growth at 800 mg.L^{-1} was 75%, but the inhibition of growth at 1000 mg,L^{-1} was 100%.

Therefore, the remediation of environments overloaded with copper becomes necessary. Several chemical methods have been attempted for the remediation of inorganic contaminants, but in

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addition to the high cost, they can be harmful to the environment [18]. Bioremediation is a biotechnological method with reduced cost avoiding damage to the environment [19]. The use of microorganisms in the bioremediation of soils contaminated with heavy metals has gained the attention of researchers, as it can be considered effective, economical, and environmentally sustainable [11,12]. As Sahu [20] rightly stated, bioremediation is the most promising technology for the remediation of contaminated environments.

In general, it can be said that bioremediation can occur in two ways. In one, the contaminant adheres to the cell membrane of the living organism, but in this case, the microorganism may be dead. When bioremediation takes place inside the cell, causing it to metabolize the inorganic contaminant, it is called biosorption. The term biosorption is used when the sorbent material is of biological origin, as well as when the bioremediation process takes place in living cells [21].

Removal by absorption is a metabolic process involving the transport of metal ions across the cell membrane barrier and subsequent accumulation within the cell, which is a slow and irreversible process and occurs only in living cells. During absorption, the contaminant is metabolized inside the cell and usually accumulates in vacuoles [22]. Recently, it was discovered that several strains of filamentous fungi can be successfully used in bioremediation owing to their high capacity for biosorption of heavy metals, notably copper and cobalt [23].

Tolerance of microorganisms to copper can be attributed to their capacity for biosorption and chelation [24]. In this assay, *Penicillium* sp. showed a high capacity to live in an environment contaminated with copper nitrate, resisting up to 1000 $mg.L^{-1}$ of copper nitrate in the environment through biosorption. Whether bioremediation is best accomplished via adsorption or biosorption is a matter of debate. In this article, we advocate the use of biosorption as the most efficient method of protection. In support of this, we noticed no genetic improvement by adsorption, whereas in biosorption, through natural selection mechanisms, organisms become increasingly capable of managing inorganic contaminants.

Also, contaminants can accumulate in organisms in two ways. Bioaccumulation occurs when chemical substances from the environment are absorbed and retained by organisms, mainly through predation [25]. While the route of substance absorption by the exposed organism can occur orally through the consumption of contaminated food, absorption is also possible by the dermal or respiratory route [26]. It should also be noted that contamination by inorganic contaminants can occur through direct absorption from the environment or indirectly through the consumption of contaminated substrate [27].

Biomagnification, on the other hand, occurs by the accumulation of contaminants at different trophic levels, that is, by the transfer of the contaminant along the food chain when predation of one organism by another accumulates in its body the inorganic contaminant previously absorbed by its prey [27].

Fungi can live under a high concentration of inorganic contaminants and therefore have been widely used to adsorb heavy metal ions. They exhibit a high capacity for absorbing inorganic contaminants [28]. Such capacity is maintained through structures that are external or internal to the fungus. One mechanism of resistance to heavy metals developed by microorganisms is the formation of metallophores by accumulating heavy metals in vacuoles, such as lead, cobalt, and copper, among others [12].

Jakovljević et al. [29] studied the relationship between microorganisms and their ability to form biofilms to biosorb zinc, lead, cadmium, copper, and nickel. Inès, Mekki, and Ghribi [30] demonstrated the potential use of newly identified lipopeptides produced by *B. mojavensis* BI2 for the bioremediation of heavy metals in contaminated water.

Penicillium chrysogenum employs multiple stress mechanisms to withstand the effects of both copper and salinity [24]. Also, *Cupriavidus basilensis* SRS is a rod-shaped, gram-negative bacterium that has been shown to have predatory tendencies. The isolate displayed resistance to antibiotics as well as Cu [31]. Lacerda et al. studied the absorption capacity of the dead biomass of *Penicillium ochrochloron* and found an ability to act as a biosorbent to remove copper from an aqueous solution.

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They concluded that the dead biomass of *P. ochrochloron* could be successfully used in the bioremediation of copper in an aquatic environment [32].

It is well known that the regular ingestion of kefir produces a beneficial effect on health. Kefir microorganisms absorb inorganic contaminants and are expelled in the bowel movement. Penicillium sp. it is a component of kefir and, when ingested, can absorb inorganic contaminants such as copper and then be expelled from the body through the bowel movement. This is why the ingestion of kefir only has beneficial effects for health when done continuously, as it continuously absorbs the inorganic contaminants that are expelled along with the kefir in the bowel movement [10]

A predictive toxicological analysis shows that humans resist up to 25 $mg.kg^{-1}$ of copper in the environment and that *Penicillium* sp. resists up to 1000 $mg.kg^{-1}$. These statistics suggest that the consumption of kefir of which *Penicillium* sp. is a component may protect its consumers from excessive contact with copper-contaminated foods. Also, while humans only resist 25 $mg.kg^{-1}$ of copper in the environment, *Penicillium* sp. presented 75% inhibition when 800 $mg.L^{-1}$ of copper nitrate was used, and it was fully inhibited only when 1000 $mg.L^{-1}$ of copper nitrate was used in the culture medium, evidencing that the microorganismal components of kefir "take the heavy metal bullet", thus evidencing the efficient reduction of damaging effects to human through the ingestion of kefir.

In agitated medium supporting the growth of *Penicillium* sp., the pH dropped from 7.0 to 4.33 with 400 $mg.L^{-1}$ copper nitrate. With increasing concentration from 400 to 800 $mg.L^{-1}$ of $Cu(NO_3)_2$, the pH dropped again to 3.37, demonstrating intense activity of the microorganism. This is shown in Figure 2 where adherent cells of the organism suggests intense activity. Although no perceptible differences were observed between the treatments and the different pH values in the solid medium, (Table 2), pH dropped to 3.37 in the liquid medium with agitation (Table 4).

Water pollution by inorganic contaminants affects the most varied organisms in this ecosystem, causing negative effects from the first levels of the food chain, reaching the top of this chain through biomagnification [27]. Thus, not only the exposure of human beings to potentially polluting activities, but, for example, the consumption of fish from contaminated environments also poses risks to human health.

Kefir consumption is protective since its ingestion provides for kefir microorganisms like *Penicillium* sp. to absorb inorganic contaminants which are then expelled through evacuation. Therefore, bioremediation would occur inside the organism and not the human body, thus bringing protection to populations who are exposed to contaminated water sources or ingest copper accumulated in fish through biomagnification.

As shown in SEM, increases in the concentration of $Cu(NO_3)_2$ result in changes to biomass. In the agitated medium, the pH declined from 7.0 to 3.37 with the use of copper nitrate (Figure 2). Environmental factors, such as pH, temperature, and ionic strength, affect the effectiveness of bioremediation processes [21]. The pH changes the charge of surface groups on the cell surface, which influences the efficiency of biosorption of heavy metal ions [33–35].

Lau et al. [36] indicate that temperature has a broad-spectrum effect on metal biosorption, but its influence is smaller than the impact of pH. Absorption of inorganic contaminants depends on pH and is closely related to structural chemistry in the solution, as well as acid-based properties of various functional groups on the cell surface [18].

The best pH for growth in a solid medium was 4.0, but even at pH 9.0, activity and sporulation occurred. As pH increases, these functional sites become deprotonated [37]; therefore, their negative charges increase, which facilitates the binding to cations. In general, as pH decreases, the cell surface becomes positively charged, reducing the attraction between biomass and metal ions. Thus, a higher pH facilitates the bonding with metal ions because the cell surface becomes more negatively charged. For example, with an increase in pH from 1.0 to 7.0, copper biosorption by Spirogyra sp. biomass is reported to have increased from 31% to 86% [38]. In this experiment, an increase in activity occurred with pH 4.0 (Table 2). This occurred both in radial growth and in a liquid medium with agitation. Therefore, in this work, contrary to what was expected, a decrease in the pH of the medium caused better growth (Table 2).

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Scanning electron microscopy (SEM) was used on samples of biomass cultivated in shaking for 120 hours. In the stirred medium, the pH dropped from 7.0 to 4.33 with the use of copper nitrate at 800 mg.L^{-1}. Thus, with the increasing concentration of $Cu(NO_3)_2$, SEM analysis shows that the biomass of *Penicillium* sp. reflected changes that show increasing bioactivity. Accordingly, biomass can serve as an important environmental cleanup agent for toxic heavy metal ions, instead of resorting to chemical processes that are not environmentally friendly. Thus, bacterial agents were identified that can serve as potential in situ bioremediation agents [39].

Through SEM, Martinelli & Santos [40] analyzed the morphological structures relevant to the identification of the main species of nematophagous fungi, while Juříková et al. [41] used SEM to monitor fungal hyphae cells, subsequently mapping molecular biomarkers. In the present study, SEM analysis showed the effects of increasing copper concentration on the culture medium in *Penicillium* sp. as a decrease in sporulation compared to that observed in the culture medium without presence of copper (Figure 2a).

4. Materials and Methods

4.1. Isolation Methodology of Penicillium sp. of kefir

Kefir samples were collected at the Research Laboratory of Drugs of the Federal University of Amapá (UNIFAP). The isolated microorganism used in the study was obtained from kefir grains. To obtain the isolate, malt-agar extract (2%) with antibiotic insertion (chloraphenicol) was used. Approximately 10 g of kefir grains were ground with a vortex device (Instrucamp/Ika Vortex /Brazil). After a brief rest of two minutes, the supernatant was inoculated into Petri dishes containing the culture medium. After this isolation procedure, the Petri dishes containing the isolates were transferred to a Biochemical Oxygen Demand incubator (B.O.D) (Being/ Bio-RWP/China). The experiment was maintained under conditions of a photoperiod of 12 hours and a temperature of 26 ± 1°C.

After seven days of incubation, the colonies and the morphological structures (conidiophores and conidia) of the isolates were evaluated for the identification of Penicillium sp. at the genus level based on morphological keys of sections and species developed by Gams and Bisset [42].

4.2. Culture Medium Preparation and Radial Growth Measurement

The fungal culture medium was prepared using a 2% malt-agar medium at pH 7.0. Approximately 25 ml of culture medium were poured into Petri dishes (90 mm), in which the Penicillium sp. strains were inoculated in triplicate. The Minimum Inhibition Concentration (MIC) was calculated to analyze the influence of copper (Cu^{2+}) concentration on the growth of the microorganism.

4.3. Toxicological Forecast

To identify some undesirable properties of copper compounds, the http://tox.charite.de/tox server was used to predict toxicity. It was based on the similarity between the functional groups and the molecules of interest, *in vitro* and *in vivo*, and on toxicological properties, such as Toxicological Class, generation of toxic fragments, and LD_{50} values to achieve this goal.

4.4. Biomass production of Penicillium sp. and Determination of Minimum Inhibitory Concentration (MIC)

The selected strains of Penicillium sp. were inoculated on malt-agar medium incubated at room temperature (27 ± 1°C) for the preparation of biomass. After 5 days, a small portion (0.5 mm) of fungal mass was transferred to an Erlenmeyer flask (250 mL) containing 100 ml of malt-agar medium (2%) supplemented with different concentrations of the target metal (400, 600, 800, and 1000 mg.L^{-1} of $Cu(NO_3)_2$) and incubated in triplicate at a temperature of 27 ± 1 °C for 120 hours. After this period, the biomass was filtered on Whatman No. 1 filter paper and washed with deionized water to remove the culture medium.

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The collected mycelium was dehydrated in an oven (Carbolite Gero/AX 60, Brazil) at 60 °C for 48 hours, and the dry weight was measured using a digital scale accurate to 0.1 m. Inhibition of biomass production was calculated on a dry weight basis using the following Formula 1, where PI is the inhibition percentage, X is the control biomass with 0.0 ppm of the target metal, and Y is the sample biomass with the respective heavy metal

$$PI = \left(\frac{X(X-Y)}{X}\right) x\ 100 \quad (1)$$

The minimum inhibitory concentration (MIC) of each metal, causing 50% growth inhibition (MIC_{50}) of selected Penicillium sp. isolates, was calculated from the growth inhibition. Growth was measured using the halo method with a Digimess digital caliper (DIGIMESS World Tools, Product No: 006797/Brazil). The first 24 hours were left free, after which the halos were formed every 12 hours up to 120 hours when growth of the control experiment covered the entire surface of the Petri dish. The control was characterized by containing only the culture, while the target samples were placed in 400, 600, 800, 1000, and 1200 $mg.L^{-1}$ copper $Cu(NO_3)_2$.

4.5. Preparation of the culture medium

The culture medium for fungal culture was prepared using malt-agar medium 2% at pH 7.0. In the Petri dishes (90 mm), 25 ml of culture medium were poured in which the Penicillium sp. strains were inoculated in triplicate.

4.6. Cultivation of Penicillium sp. in solid medium and Minimum Inhibitory Concentration (MIC)

To analyze radial growth, selected strains of Penicillium sp. were inoculated in malt-agar medium (2%), incubated for 120 hours at room temperature (27 ± 1°C) in Petri dishes (90 mm), and supplemented with different concentrations of the $Cu(NO_3)_2$ (0, 200, 400, 600, 800, and 1000 $mg.L^{-1}$).

Thereafter, the growth halo was measured using a digital caliper. Inhibition of growth was calculated using the following formula 2, where MIC is the Minimum Inhibitory Concentration, X is the media of radial growth of the control free of inorganic contaminant, Cu $(NO_3)_2$ at 0.0 ppm, and Y is radial growth obtained by culturing the fungus in a medium with the inorganic contaminant.

$$MIC = \left(\frac{X-Y}{X}\right) x100 \quad (2)$$

The Minimum Inhibitory Concentration of the metal causing 50% inhibition of growth (MIC_{50}) of the selected microorganism Penicillium sp. was calculated from inhibition of growth unique to copper $Cu(NO_3)_2$. Growth was measured using the halo method using a Digimess digital caliper. The first 24 hours were not measured to enable fungal growth; then growth measures were checked every 12 hours until total growth in the 90mm disk was completed, which occurred within 72 hours in the control experiment.

The control was characterized by containing the inorganic contaminant-free culture medium. Meanwhile, copper nitrate, $Cu(NO_3)_2$ was inserted into the culture medium at concentrations of 200, 400, 600, 800, and 1000 mg.L-1.

4.7. Penicillium sp. biomass and quantification of copper

Penicillium sp. was inoculated in agar-malt medium (2%) and incubated at room temperature (27 ± 1°C) using a shaker at 75 rpm (LabFriend/ VKS 75A/Brazil) to obtain biomass. A small portion (0.5 mm) of the fungus was transferred to an Erlenmeyer flask (250 mL) containing 100 mL of the malt medium (2%), supplemented with different concentrations of the target metal (0, 200, 400, 600, 800, and 1000 $mg.L^{-1}$), followed by incubation in triplicate at a temperature of 27 ± 1 °C for 120 hours. After this period, the culture medium with biomass was filtered on Whatman No.1 filter paper and washed with deionized water to remove the culture medium. The collected mycelium was dehydrated in an oven at 60 °C for 48 hours, and the dry weight was measured using a digital scale.

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After gauging the pH of the filtered, was quantified copper in the supernatant using an Atomic Absorption Spectrophotometer (AAS Shimadzu model 6300).

The biomass of the microorganism cultivated for 120 hours in the shaken medium in shaker at 75 rpm (LabFriend/ VKS 75A/Brazil) was dehydrated and submitted to analysis by SEM) (TM3030Plus, Hitachi, Japan).

4.8. Estimation of Residual Metals in the Culture Medium

After a period of 72 hours, the biomass of each isolate grown in a modified medium with different concentrations of $Cu(NO_3)_2$ (0, 400, 600, 800, and 1000 $mg.L^{-1}$) was measured, and both concentration and growth calculations were performed.

4.9. Scanning Electron Microscopy (SEM) Analysis

Penicillium sp. mycelium isolated from kefir was analyzed by SEM. We tried to analyze the possible differences between the control and those samples inoculated in medium with different concentrations of Cu(NO3)2 in a liquid medium with agitation or shaking.

4.10. Statistical Analysis

The experiments were conducted using a completely randomized factorial design with three replications, considering the isolates as factor A and the metal concentrations as factor B. The Analysis of Variance (ANOVA) was conducted using software R 3.4.3 (R Core Team, 2017). Differences were considered significant when $p \leq 0.05$.

5. Conclusions

The bioremediation study to evaluate the resistance of *Penicillium* sp. isolated from kefir grain was carried out by submitting it to a culture medium with different concentrations of copper. This constitutes the initial phase for the selection of potentially promising microorganisms to be used in the bioremediation of environments contaminated by copper. We conclude that *Penicillium* sp. isolated from kefir grain can successfully bioremediate the environment, which also suggests that its consumption can minimize the harmful effects of ingesting heavy metal because it contains microorganisms, in this case, *Penicillium* sp., that absorb copper in the medium.

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