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Mercury tolerance of *Penicillium* sp isolated from kefir grains

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Abstract

Inorganic contaminants contaminations poses one of the greatest threats to the environment and human health. It has been recently reported that probiotics protect the organism from inorganic contaminant damage by their bioabsorption capacity of its components. Kefir is a beverage obtained through the metabolized fermentation by Lactobacillus and yeasts in the forms of aggregate, creating the grains of kefir. In addition to hundreds of species of bacteria these grains also contain filamentous fungi living in symbiosis. In order to isolate inorganic contaminant resistant microorganism, the kefir grains were crushed and the supernatant obtained was inoculated in malt-agar medium (2%) and cultured for 120 hours. Occurred formation of halos of growth which were measured every 24 hours, in this way the growth inhibition was determined. The isolated microorganism was also cultured in liquid media for five days. The taxonomic analysis revealed to be, fungus of the genus Penicillium. The results obtained showed the capability of mercury bioabsorption by the colonies of Penicillium sp isolated from kefir. Modifying the conditions of the media (pH 4.0 and 9.0) the bioabsorption did not drastically modify the activity. Contrary to what was expected there was an increase in biomass when growth in liquid medium, which could suggest the formation of storage structures for the inorganic contaminants around the cell membrane. These results suggest that kefir contains in its composition microorganisms with potential to protect people who consume it from harmful damages caused by inorganic contaminants.

***Keywords**: Kefir grains; Bioabsorption; Inorganic contaminants; Probiotics*

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1 Introduction

Inorganic contaminants contaminations are one of the greatest threats to the environment and human health because of their toxicity aggravated by the fact that they contain non-biodegradable properties, which means they are bioaccumulated in the organism and soils (SHENG et al., 2016). Through the effect of biomagnification on the food chain, inorganic contaminants can threaten the health of humans (ZHAI et al., 2016).

In order to alleviate the effects of Inorganic contaminant toxicity, several methods for removal have been suggested. Among these are the use of algae and microorganisms (JAHROMI et al., 2017). The use of probiotics to remove Inorganic contaminants from the organism, is also well known (TONG et al., 2017; ZHAI et al., 2017). It has been reported that some bacteria from probiotics possess great potential to eliminate Inorganic contaminants from food and water (ELSANHOTY et al., 2016). For example, *Lactobacillus plantantarum*, a natural inhabitant of the intestine promotes several beneficial effects to health, among there is the Inorganic contaminant removal (ABU-BRAKA et al., 2017). Previously studies have been corroborated the bioabsorption and elimination of lead by the lactic acid bacteria *L. pentosus* and *L. acidipiscis* (JAHROMI et al. 2017).

Intestinal lumen contains millions of cells that are expelled and renewed every day. ZHAI et al. (2016) confirmed that a diet supplemented with probiotics might exert a protective effect against Inorganic contaminants ingested because when the digestive process expelled these microorganisms also eliminates the bio-absorbed inorganic contaminants.

The ability of probiotics to remove inorganic contaminants from the human body has been confirmed (THONG et al., 2017; ZHAI et al., 2017). For example, *Lactobacillus plantarum*, a component of probiotics and also a natural inhabitant of the intestine, promotes several beneficial health effects, including the removal of Inorganic contaminants (ABU-BRAKA et al., 2017).

Zhai et al. (2016) confirmed that a diet supplemented with probiotics might exert a protective effect against inorganic contaminant ingested. Elsanhoty et al. (2016) confirmed the ability of lactic acid bacteria and probiotic bacteria to remove Inorganic contaminants including cadmium (Cd), lead (Pb) and arsenic (As) as well as aflatoxin B1 (AFB1) from contaminated water, reducing the tolerance limit approved by World Organization of Health (WHO).

Kefir is a beverage obtained from the fermentation of milk, fruit juice or sugar being metabolized by *Lactobacillus* and yeast that together constitutes the “kefir grains”. The kefir grains are similar to cabbage blossom, sizing from 3 mm to 1 cm. They have irregular shape, white or yellow color, actually they assume the color of the media in which they grown (OLIVEIRA et al., 2017a) and contains firm texture (LA RIVIERE et al., 1967) known as “kefir grains”. Kefir is considered a probiotic composed basically of lactic acid bacteria and yeast. Other microorganisms eventually enter in their composition such as filamentous fungi and bacteria. Kefir is characterized by the presence of fungi with high biosynthetic capability (OLIVEIRA et al., 2017b).

What makes it interesting to use kefir grains to assess their capability for bioabsorption and the synergy between the microorganisms involved. Thus, in the present we study we isolate a microorganism of kefir responsible for the bioabsorption of inorganic contaminant, suggesting that the ingestion of it, can confer to the organism protection against damages caused by Inorganic contaminant ingestion.

2 Material and Methods

2.1 Isolation of *Penicillium sp* of kefir grains

10 g of kefir grains diluted in 10 ml of distilled water were crushed in a homogenizer ultra-turrax. A droplet of the obtained supernatant was inoculated into Petri dishes in which malt-agar medium (2%) previously poured. As a result, fungal colonies were formed with morphological structures (conidiophores and conidia) that were evaluated for identification at gender level, based on sections of the morphological characteristics. The resulting microorganism was identified as *Penicillium sp*.

2.2 Preparation of the culture medium

Culture medium for fungus was prepared using malt-agar medium (2%) at pH 7.0. In Petri dishes (90 mm), approximately 25 ml of culture medium was poured. In this medium isolate microorganism was inoculated with inoculation loop

2.3 Toxicological Prediction

In order to identify some undesirable properties of the compounds with mercury the server http://tox.charite.de/tox/ was used. The criteria used was similarity of the functional groups in the molecule, as well with the toxicological properties such as; toxicological class, toxic fragments generation and LC50.

2.4 Cultivation of *Penicillium* sp in solid medium and Minimum Inhibitory Concentration (MIC)

To analyze the radial growth, *Penicillium* sp was inoculated in malt-agar medium (2%) incubated for 72 h at room temperature (27±1°C) in Petri dish (90 mm) supplemented with different concentrations of the $Hg(NO_3)_2$ (0, 100, 150, 175 and 200 ppm).

Thereafter, the growth halo was measured using a digital caliper. Inhibition of growth was calculated using the following formula:

Where MIC is the Minimum Inhibitory Concentration, X is the mean of radial growth of the control without the inorganic contaminant ($Hg(NO_3)_2$ 0.0 ppm) and Y is the mean of radial growth obtained by culturing the fungus in medium with inorganic contaminant.

The Minimum Inhibitory Concentration (MIC) of the metal caused 50% of growth inhibition (MIC_{50}) for the selected microorganism *Penicillium* sp.

Growth was measured using the halo method using a digital caliper Digimess (Brazil). The first 24 hours were left free of measurement to enabling the growth of the fungus, and then the growth measures were checked every 24 hours until reaches the maximum growth in the disk of the 90 mm, 120 hours later.

The control was characterized as containing inorganic contaminant free culture medium. Meanwhile mercury was inserted into the culture medium at the concentrations of 100, 150, 175 and 200 mg/L of mercury nitrate $Hg(NO_3)_2$. After a period of 120 hours the halo of the isolate growing in the medium with different concentrations of mercury nitrate (0, 100, 150, 175 and 200 ppm) was measured and calculated.

2.5 *Penicillium* sp biomass and Minimum Inhibitory Concentration (MIC)

To obtain the total biomass, *Penicillium* sp as inoculated in malt-agar medium (2%) after that incubated at room temperature (27±1°C) using shaker with at 75 rpm of mixing speed. Small portions (0.5 mm) of the fungus was transferred to an Erlenmeyer flask (250 ml) containing 100 ml of the malt-agar medium (2%) supplemented with $Hg(NO_3)_2$ at different concentrations of (0, 100, 150, 175 and 200 ppm) and incubated at 27±1°C for 72 h. After this period the culture medium with biomass was filtered on whatman n° 1 filter paper and washed with deionized water in order to remove the excess of culture media.

2.6 Analysis by Scanning Electron Microscopy

The mycelium of *Penicillium* sp was scrutinized by Scanning Electron Microscopy (SEM) (TM3030Plus, Hitachi, Japan). The acceleration voltage used was set at 15 kV. The magnification used was x 25 K. It was attempted to analyze the morphological changes made by the mercury exposition.

2.7 Statistical Analysis

Experiments were conducted with three replicates considering the isolates as factor A and metal concentration as factor B. The analysis of variance (one-way ANOVA) followed by a Tukey test was conducted using software R 3.4.3 (R Core Team, 2017). Differences were considered significant when $p \leq 0.05$.

3 Results

3.1 Effects of mercury on the growth of *Penicillium sp*

Table 1 shows the effects of the different concentrations of mercury on the mycelia growth of *Penicillium* sp isolated from kefir grains. When the concentration of 175 mg/L was used, inhibition (MIC) was only 17% (70.0±1.04 mm).

The analyzes indicated that the concentrations were different ($F= 36.6$, $p<0.05$). Only on the concentration of 200 mg/L of mercury, total inhibited of the growth of the microorganism occur. Therefore, the data demonstrate that the microorganism has a high capacity of tolerance in the absorption of mercury

Table 1. Growth of *Penicillium* sp isolated from kefir grain in malt agar medium 2% with different concentrations of mercury

	0,0 mg/L	100,0 mg/L^{ns}		150,0 mg/L*		175,0 mg/L***		ANOVA
Strain	**D mm**	**D mm**	**% IC**	**D mm**	**% IC**	**D mm**	**% IC**	**F**
GK –1	90	87 ±0,86	3	78 ±0.29	13	70 ±1.04	17	36,6**

D (mm) = Diameter in millimeters; % IC = Inhibitory Concentrations Percent; ns = there was no significance in

Table 2 shows the effect of low pH on the growth of *Penicillium* sp. It can be seen that the combination of factors such as pH and presence of inorganic contaminant caused impact on the growth of the microorganism with inhibition of 19% in relation to the control.

Interestingly. But, maturation of the fungus was observed same under inhospitable conditions.

Table 2 – Growth of Penicillium sp isolated from kefir grains grown on malt-agar medium (2%) with 175 mg/L mercury nitrate $Hg(NO_3)_2$ at pH 4.0

Time (hs)	**24 mm**	**36 mm**	**% IC**	**48 mm**	**% IC**	**60 mm**	**% IC**	**72 mm**	**% IC**	**96 mm**	**% IC**	**120 mm**	**% IC**	F
Ctr	0	8,67 ±0,29		22 ±0,50		23,50 ±0,50		34,17 ±1,26		51,00 ±0,50		62,17 ±1,26		984,5**
Hg 175 mg/L	0	3,67± 0,29	58	12.33 ±0,76	44	14,83 ±0,29	37	27,67 ±1,04	19	35,83 ±0,58	30	50,50 ±0,50	17	

% IC = Inhibitory Concentrations Percent; *=p>0,05 **=p>0,05 ***= p>0,001

Figure 1: Growth of Penicillium sp isolated from the kefir grain cultured in malt agar medium (2%), showing A) control and B) medium malt-agar with 175 mg/L $Hg(NO_3)_2$

A)

B)

3.2 Toxicological prediction

In order to compare the tolerance capability of the microorganism to the inorganic contaminant with the mammals, the toxicological prediction was carried out (Table 3). It is noted that the lethal concentration (LC_{50}) of mercury nitrate $Hg(NO_3)_2$ for mammals is 25 mg/kg. It's considered as toxicological class 2 and this compound is not carcinogenic, but is toxic both ingested as in contact with the skin.

Table 3 – Toxicological prediction of mercury nitrate Hg $(NO_3)_2$

Toxicological Class	2
CL_{50}	25 mg/kg
Molecular weight	324,6
Number of Hydrogen Acceptors	6
Number of atoms	9
Number of connections	6
Polar Molecular Surface (MPSA)	137,76

Using the toxicity predictor used was http://tox.charite.de/tox/. We calculated the Polar Molecular Surface (MPSA) for the $Hg(NO_3)_2$. The MPSA is a very useful parameter for the prediction of drug transport; it represents the sum of the bridges formed by the polarity of surface atoms, which it's related to the intestinal absorption capacity as well as the capability to penetrate the blood brain barrier.

3.3 Culture of *Penicillium sp* in liquid medium

Correlating the physical characteristics of *Penicillium* sp grown in liquid medium, it can be observed increase of the amount of biomass and also pH, in the presence of $Hg(NO_3)_2$ (175 mg/L) suggesting the presence of the metal increases the grown rate of the fungus (Table 4).

Table 4 – Biomass grown of *Penicillium* sp in liquid media with 150 mg/L of $Hg(NO_3)_2$.

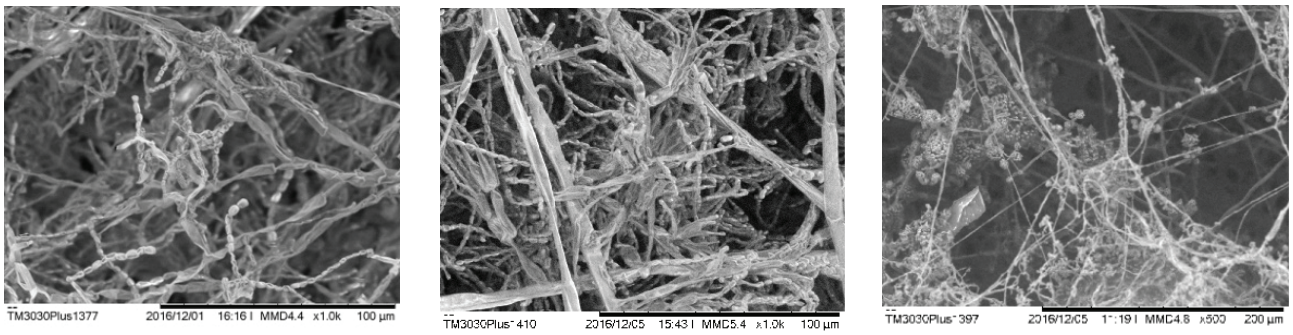
Treatment		Biomass	pH
1	GK-CTR	642±9,54	4,54
2	GK Hg	958,33±7,51	5,95

GK-CTR =control e GK-Hg= media with $Hg(NO_3)_2$

3.4 Analysis of sporulation of *Penicillium* sp isolated from kefir grains by Scanning Electron Microscopy (SEM)

The effects by increasing the concentration of mercury in the culture medium on *Penicillium* sp were perceptible on the sporulation of the fungus, decreasing the thick an the number of hyphae's per field (Figure 2B and 2C) rather than the observed on the mercury-free culture medium (Figure 2A).

Figure 2 - Scanning electron microscopy (SEM) image shows the sporulation difference in *Penicillium* sp cultivated in A) medium without mercury, B) with 100 mg/L; C) with 200 mg/L $Hg(NO_3)_2$



A) B) C)

4 Discussion

In the present study, it has been observed that the growth of *Penicillium* sp was influenced by the presence of mercury in the medium (Table 1). The lethal dose (LD_{50}) of mercury intake for a mammal is 25 mg / L. It was found in this study that the fungus resists up to 175 mg / L of mercury nitrate, that is, seven times as much as human can tolerate.

There is evidence of the correlation between the amount of microorganisms and the amount of metals in liquid media. The high initial amount of the microorganism provides an increase in forces to overcome any metal ion transfer resistance, besides to increase in the probability of collision between the ions of the metal and the cell wall of the bioabsorbent (TANWAAR et al., 2012, EL-GENDY et al, 2017). Also, there is evidence that several microorganisms develop a tolerance due to increase of metal concentration that results on the modification of their bioabsorb capability (TANWAAR et al., 2012, EL-GENDY et al, 2017). Therefore, there is a correlation between the biomass concentration and the absorbed metal.

In this experiment, this effect is seen along with the growth of the microorganism, allowing the gradual adaptation to the contaminated environment. It was observed gradual adaptation to heavy metal with increase of the capability to survive in a contaminated environment (Table 2). These same conclusions were obtained when *Penicillium* (IANIS et al, 2006) and *Trichoderma* (CHEW et al., 2012) were cultivated in similar conditions.

The higher the dose of adsorbent in the solution, the greater the availability of exchangeable sites for the ions.

The increase of the mycelial mass does not allow the effective contact between the adsorbent and the metal. Only a partial cell aggregation occurs with high concentration of biomass leading to the decrease of active sites (EL-GENDY et al, 2017).

There is evidence showing that an increase in pH causes a decrease in the number of protons in solution affecting the possibility of interactions with the proteins of the cellular membrane (EL-GENDY et al, 2017). Therefore, in some cases the increase in pH enables bioabsorption.

Studies have shown that maximum absorption by *Aspergillus versicolor*, *Rhizopus oligosporus, Penicillium purpurogenum*, was achieved at pH 6.0 (MARTÍNEZ-JUÁREZ et al.). The maximum value of ions removal of the metal by the fungus *Penicillium resedanum*, *Aspergillus wentii, Alternaria alternata*, and *Eupenicillium katangense* was reached in the pH range of 5-8.

In this experiment, the initial pH of the culture medium was 7.0, which decreased after the 5 day culture to 4.6. However, when cultured in liquid medium, the pH of the culture medium was 7.7, so an increase in pH was observed

The effects of the toxicity of different concentrations of mercury on the mycelial growth of *Penicillium* sp isolated from kefir grain allowed to observe that as the concentration of mercury increases there is slight decrease of the growth of the microorganism. There was also difference in sporulation that was less crowded and more dispersed in relation to the control (see Figure 1). Therefore, *Penicillium* sp growth was influenced by doses of mercury up to 175 mg/L, which shows that the microorganism has the capacity to survive in environments loaded with mercury. However, the most notable effect of mercury was on the sporulation that was delayed in relation to the control (Figure 1)

When mercury was used at a concentration of 200 mg/L, almost total inhibition of fungus growth occurred and the spores became more dispersed (Figure 2C). As well, the behavior of *Penicillium* sp isolated from kefir grain in liquid medium showed that tolerance to mercury was higher in liquid medium than in solid medium.

Regarding the pH in liquid medium for the control was 4.54 while the pH of the medium with mercury was 5.95 which shows that the strategy of the microorganism perceptibly resulted in increased pH. This strategy of the microorganism allowing tolerance in the environment results in increased pH

When mercury is inserted in solid malt agar medium, a decrease in pH occurs, but in liquid media an increase in pH occurs, suggesting a defense mechanism of the microorganism whose biochemical reactions have not yet been fully delineated. It is known that the fungus grows in a relatively extensive pH range and adapts to extracellular pH through a genetic regulatory system mediated by a key PacC component, which is the pH transcription gene (RASPANTI et al., 2009).

As shown in Figure 2 in photos obtained by scanning electron microscopy (MEV), there is a change in the medium with mercury. Sporulation is detected in the medium with and without mercury but appears smaller in the medium with mercury. This is in agreement with the study by Raspanti et al. (2009) that concluded that mercury in this way affects sporulation in the concentration used. The physicochemical properties of the environment influence the toxicity of the contaminant to the biota (BAGY et al., 1991). The toxicity of a pollutant can be reduced in some ecosystem, while the same dose in another environment with different physico-chemical characteristics may have increased potential (BABICH, STOTZKY 1980), making this environment subject to high risk condition (BABICH, STOTZKY 1980). (BABICH, STOTZKY 1980). These latter environments should be considered high risk environments (BABICH et al 1981).

5 Conclusion

The results showed the capacity of mercury bioabsorption by *Penicillium* sp isolated from kefir. At pH 4.0 this activity did not suffer strong inhibition. Contrary to what was expected there was an increase in biomass when grown in liquid medium which suggests the formation of storage structures of the inorganic contaminant around the cell membrane. These results

suggest that kefir contains in its composition microorganisms with potential to protect people who consume it from harmful damages caused by inorganic contaminants

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