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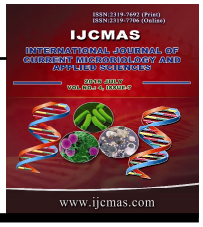

Original Research Article

Evaluation of the Potential Biotechnological Removal of the Ion Cd(II) by *Rhizopus arrhizus* UCP 402

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Keywords

Rhizopus arrhizus, Biomass, Chitin, Chitosan, Cadmium, Ultrastructure

A B S T R A C T

This study concerns the efficiency of cadmium removal from aqueous solution in different pH, concentrations and biosorbents biomass, chitin and chitosan obtained from *Rhizopus arrhizus* UCP 402. The cultivation resulted in tolerance of cadmium and it was observed during the growth in all conditions tested in response to the heavy metal. The strain exhibits potential for cadmium removal and its efficiency is related to the concentration of heavy metal. The exposure to cadmium showed variations in electrondensity of the biosorbents surfaces. The strain shows potential to remove high concentrations of cadmium revealing ability of cadmium removal in impacted environments.

Introduction

The heavy metal contamination is now one of the most concerns to environmental health; they are easily transported in solution, and can reach high concentrations in enclosed areas for their own provision or by biological amplification. Cadmium is widely used in the manufacture of batteries, in electroplating, pigments, alloys of low melting point, electrolytic coating of metals, enamels and textile dyes in control rods in nuclear fission, among others. The metal is discharged to the environment can contaminate air, water and soil (Gadd, 2013; Mani and Kumar, 2014).

The metal ions are responsible for protein denaturation and reduction of enzymatic activity. Several examples of this effect have

been described for enzymes of the Krebs cycle (Gadd, 2013). Currently there are physical and chemical processes of precipitation, flocculation, electrolysis, crystallization or adsorption for the decontamination of environments, however, these processes can be costly and/or contribute to the formation of new environmental contaminants thus becomes necessary to develop more economical and practical technologies for the removal of these elements, which are responsible for a high level of toxicity to living systems (Gadd, 2013; Fomina and Gadd, 2014).

The microorganisms are transformed metals through oxidation-reduction, methylation and demethylation reactions. Together, these processes affect the toxicity and mobility of metals in the environment (Hasunuma *et al.*, 2013; Fomina and Gadd, 2014; He and Chen, 2014; Jadhav and Hocheng, 2014). Using the natural ability of micro-organisms in dealing with heavy metals, environmental biotechnology emerges as an alternative tool of great prospect for the remediation of contaminants/pollutants in different ecological niches. Thus, they are considered highly efficient tools which can cause a feasible, efficient and low cost not generate toxic byproducts (Zeng *et al.*, 2010; Jadhav and Hocheng, 2014).

Among the microorganisms, fungi receive special attention because of its greater efficiency in respect of bacteria. Fungi of the class Zygomycetes exhibit great economic and environmental importance in the processes of biodeterioration, biodegradation and industry (Bilal *et al.*, 2013). In this perspective, the study had investigated the potential of biotechnology in the removal of cadmium and the effects of metal ion in the activity of enzymes from *Rhizopus arrhizus* UCP 402 isolated from mangrove.

Materials and Methods

Microorganism and culture conditions

The strain of *Rhizopus arrhizus* UCP 402 was obtained from the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology from Catholic University of Pernambuco - Brazil, included in the Northeast Network of Microorganisms of North and Northeast (RENEBRA) and registered in the World Federation Culture Collection (WFCC). The strain was maintained in Potato Dextrose Agar (PDA) at 5°C. The strain was cultivated in Synthetic Medium of Mucoralean in order to produce spores and incubated at 28°C for 6 days, for the production of pre-inoculum.

Growing Conditions - Analysis of radial growth and Enzymes activities

Pre-inoculum corresponding to cultures discs, with one centimeter in diameter, were inoculated in Petri dishes, containing Synthetic Medium for Mucoralean, containing cadmium chloride, prepared in distilled and deionized water, pH 6.0, at concentrations of 0.5, 1, 2, 3 and 4mM. The cultures were incubated at 28°C for 15 days. Control-samples were grown according to the procedures cited without the heavy metal. The growth was evaluated through the radial expansion, measured by the diameter of colonies, in millimeters, every twenty-four hours of incubation. The results are expressed as arithmetic average of triplicates. To measure enzymes activities, the pre-inoculum was inoculated in Nutrient Agar Medium with addition of the substrates: Galic Acid for phenoloxidases activities, Tanic Acid for tannase activity, Soluble Starch for amylase activity and Cellulose for cellulase. The measure of enzymatic halos has made after 15 days of incubation.

Preparation of biosorbents

The biomass was produced through Synthetic Medium for Mucoralean (SMM), using Erlenmeyer flasks with 1000 ml capacity, incubated at 150rpm at 28°C for 15 days. The biomass produced in SMM medium was collected by filtration and washed in distilled water. After washing of the biomass was tested in two ways: as Living biomass and inactivated biomass and chitin and chitosan. The living biomass was just washed and used in removal tests. For inactivation of the mycelium, it was incubated in a solution of formaldehyde (1%) for 2 hours under agitation (100rpm, 28°C). After this period the sample was washed in distilled water, filtered, resuspended and homogenized in 100ml of sterilized water for 2 hours (150 rpm and 28°C). Then the sample was filtered through a nylon filter (0.8µm). Chitin and Chitosan were obtained by deproteinization of the mycelium with 2% sodium hydroxide (w/v), followed by centrifugation, acid hydrolysis of 10% acetic acid (v/v) to obtain chitin and successive washings with acetone and ethanol for precipitation of polysaccharides. Chitosan was obtained by deacetylation of chitin (Synowiecki and Al-Khateeb, 1997).

Cadmium Removal Tests

Samples of living and inactivated biomasses corresponding to 200mg sample were added to Erlenmeyers flasks containing 125ml of solutions with different concentrations of cadmium (0.5, 1, 2, 3 and 4mM), at pH 2, 3, 4, 5 and 6. The flasks were incubated under orbital shaker at 150rpm at 28°C for 18 hours. Chitin and chitosan were tested under the following conditions: a solution of chitin 1% or chitosan (w/v) were added to 125ml of solutions of cadmium and tested with the same conditions as living and inactivated biomasses.

Ultrastructural analysis - scanning electronic microscopy

Samples collected within 15 days of cultivation, were washed twice in PBS, pH 7.2, for 10 minutes. Then they were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 hour at room temperature. After the stage-setting, all samples were washed again twice with phosphate buffer, for 10 minutes. This procedure was followed by the post-fixing with osmium tetroxide 1% in phosphate buffer, for 1 hour at room temperature, in absence of light. Then the samples were once again washed with 0.1 M phosphate buffer, and submitted to the process of dehydration. The dehydration of the samples was done with ethanol, in concentrations of 50%, 70%, 90% (5 minutes for each exchange) until the proportion of 100% (three times, 10 minutes each exchange). After this step, the samples were submitted to the critical point, followed by the assembly in support of aluminum and subsequent gold metallization. Once prepared, samples were examined and photographed in the Scanning Electronic Microscope, JEOL LV 5600, operating at 20KV.

Determination of Cadmium Removal Efficiency from Biomass, Chitin and Chitosan

Samples under the conditions mentioned above were subjected to atomic absorption spectrophotometry to determine the concentration of the heavy metal. All experiments were performed in triplicate. The efficiency of the metal removal - q (mg of metal ion/gram of biomass) was calculated using the following equation:

$$q = (C_0 - C_f)/m \qquad \textit{(Eq. 1)}$$

Where, C_0 and C_f corresponded to the initial and final concentrations of the metal (mg/l), respectively, and m is the biomass on dry weight (g), according to Göksungur *et al.* (2005).

Results and Discussion

Effects of cadmium in the growth and enzymes activities of *Rhizopus arrhizus* UCP 402

With the aim to study the effects of cadmium presence in the growth media, Figure 1 shows results for the radial growth obtained for the cultivation of *Rhizopus arrhizus* UCP 402 in Synthetic Medium for Mucoralean, in absence and presence of cadmium, at concentrations of 0.5, 1, 2, 3 and 4mM, respectively.

The results verifying that the presence of the metal, cadmium, the culture medium alters the pattern of radial expansion of the colonies in media without regard to the presence of heavy metal (control cultures), and the change is directly related to heavy metal concentration. However, it should be noted that although a reduction in radial expansion of the colonies there was no complete inhibition of the isolated, except during the first 24 and 48 hours for exposure to cadmium 3mM and 4mM, respectively. This fact points to the ability to grow even if exposed to high concentrations of heavy metal.

The effects of heavy metals, especially cadmium, on the growth of microorganisms are reported. It is believed that once in contact with the cells, the metal ions could be located in organelles, or could be linked to proteins by displacing the ions suitable for cell function from their original positions, thereby causing metabolic functions (Göksungur *et al.,* 2005; Abdel-Aty *et al.,* 2013).

Thus the studies indicate the inhibitory effect, which however vary with the species isolated strain, strain, the metal concentration, exposure time to the metal ion and cell-cycle phase (Kelly-Vargas *et al.,* 2012; Mudhoo *et al.,* 2012; Bhatti and Amin, 2013). The sensitivity cadmium occurs even at low concentrations. In this study, although high concentrations were used growth occurred, which could be determined as a tolerance to the metal form.

In response to exposure to an environment unfavorable living systems are able to alter its physiological conditions, biochemical, molecular and also the expression of genes to survive. This ability is associated with response and non-enzymatic antioxidant enzyme. The response includes the variation of the enzymatic activity of enzymes such as phenol, tannase, among others (Chakraborty *et al.,* 2013). Furthermore, metals also influence the activity of extracellular enzymes. Figure 2 shows results for enzymes activities revealing the influence of cadmium on its expression.

The isolate shows activity for all enzymes tested in the absence of cadmium. It is found that the enzyme activity is variable depending on the presence of the metal and its concentration. Additionally, it is clear that the cellulase appears more influenced by the heavy metal.

One of the consequences of exposure to heavy metals is the inhibition of enzymatic reactions. Considering the relevance and industrial biotechnology, numerous enzymes, oxidative and extracellular, have been evaluated in the presence of metals. Studies show that once reaching the cell, the metal ions influence their production, either positively or negatively (Mudhoo *et al.,* 2012; Chakraborty *et al.,* 2013).

Evaluation of the potential of cadmium removal by the biosorbents

The figure 3A and 3B shows the relationship between the amounts of metal biosorbed per unit of biomass. The efficiency of removal was carried out according to the equation $q = (C_0 - C_f)/m$. The data demonstrates that the efficiency of removing the metal of culture increases by the increase of the initial concentration of metal ion and pH, resulting in 66.44 mg/g/biomass, 135.95 mg/g/biomass, 168.23 mg/g/biomass, 135.59 mg/g/biomass and 188.23 mg/g/biomass for concentrations of 0.5mM, 1mM, 2mM, 3mM and 4mM, respectively for the sample with living biomass at pH 6; as like 76.59 mg/g/biomass, 153.64 mg/g/biomass, 207.56 mg/g/biomass, 155.49 mg/g/biomass and 277.56 mg/g/biomass for concentrations of 0.5mM, 1mM, 2mM, 3mM and 4mM, respectively for the sample with inactivated biomass at pH 6.

The figure 4A and 4B shows the relationship between the amounts of metal biosorbed per unit of biopolymer according to the equation $q = (C_0 - C_f)/m$. The data demonstrates that the efficiency of removing the metal of culture increases by the increase of the initial concentration of metal ion and pH, resulting in 80.53 mg/g/biopolymer, 162.84 mg/g/biopolymer, 256.54 mg/g/biopolymer, 267.76 mg/g/biopolymer and 308.94 mg/g/biopolymer for concentrations of 0.5mM, 1mM, 2mM, 3mM and 4mM, respectively for the sample with chitin at pH 6; as like 92.45 mg/g/biopolymer, 186.89 mg/g/biopolymer, 305.45 mg/g/biopolymer, 325.78 mg/g/biopolymer and 438.45 mg/g/biopolymer for concentrations of 0.5mM, 1mM, 2mM, 3mM and 4mM, respectively for the sample with chitosan at pH 6.

Bioremediation is the use of biomass, cultivated or obtained as by-product of fermentation, after drying, and immobilization, as well as their derivatives, generating lower costs, reduced waste and tailings and high efficiency in removing metals in waste very dilute. Numerous systems have been evaluated in biological removal of heavy metals from aqueous solutions in order to control the environment (Kelly-Vargas *et al.*, 2012; Mudhoo *et al.*, 2012; Bhatti and Amin, 2013).

A wide variety of microorganisms can bind heavy metals; however, there are large differences in the responses of microbial species when exposed to solutions of metal. Thus the elucidation and understanding of processes involved in the accumulation of metals by microorganisms is essential for the development of processes of concentration, removal and recovery of metals from aqueous solutions. The literature shows that the application of dead biomass has advantages over the living biomass as regards the elimination of toxic metals (Göksungur *et al.*, 2005; Abdel-Aty *et al.*, 2013; Jadhav and Hocheng, 2014). An interesting aspect of the biological treatment process for removal of metals, including cadmium, is that it is most effective when the concentration of contaminants is above and below 100 mg/l (Chakraborty *et al.*, 2013).

The literature reveals the removal of metals such as cadmium, zinc, copper, cobalt, uranium, nickel chromium by the biomass, non-viable or dead filamentous fungi, with special emphasis on species of *Aspergillus* and *Rhizopus* (Zinadini *et al.*, 2014). The papers point out that the concentrations of metals are variable and depend on the responses of its concentration and the type of biomass. Additionally, it is possible to increase the removal of heavy metals as a result of physical pre-treatments and/or chemical means.

Numerous studies have shown an increase in efficiency of removal of heavy metals in aqueous solutions, after the submission of biomass the different types of physical and chemical treatments. For example, the biomass of *P. florida* subjected to drying, autoclaving, cold drying treatments with NaOH, $NaHCO_3$, Na_2CO_3, glacial acetic acid, oxalic acid, ortho-phosphoric acid, metal, dimethyl sulfoxide and formaldehyde exhibited increase in cadmium removal in relation to living biomass (3, 7, 21 mg/g 13mg/g of biomass). Biomass submitted to cold drying exhibited increased efficiency. Increasing the efficiency of removal after the treatment of biomass with drying and autoclaving can be attributed to exposure to a greater number of binding sites for cadmium on cell surface (Das *et al.*, 2007; Ge *et al.*, 2012; Bhatti and Amin, 2013; Haldorai and Shim, 2014). The results showed that the living biomass presents the lowest potential of metal removal at all concentrations tested as compared to inactivated biomass.

The biomass may be subject to modification processes that result in higher efficiency removal in the aqueous solution of heavy metals. After being inactivated with chemical agents (formaldehyde) the cells increase the access of the metal ion binding sites. Furthermore, the use of biopolymers, chitin and chitosan showed greater skills than the biomass, being chitosan the best remover system. The absence of cellular and extracellular materials, both chitin and chitosan have high affinity to metal and therefore have a great capacity of removal. The results of this study showed great removal efficiencies in all conditions. The reduction of the potential removal decreases for higher concentrations of cadmium, this fact is positive, since the effluents containing cadmium have low concentrations of it.

Furthermore, the effect of pH removal system was also evaluated. pH values below 4.0 induce precipitation of the metal in solution. The data show that increasing values of pH in the removal was more effective. Thus, the greater removal was obtained at pH 6.0.

As the pH increases, more functional groups are separated and are available for the binding of cadmium. In this way, the effect of pH on removal of cadmium was also investigated by diverse resins and similar results were obtained. For example, Lo *et al.* (1999) have noted a sharp increase in removal of cadmium and copper by increasing the pH of 2.5 to 6. Mishra *et al.* (2009, 2010) conducting study associated with removal of cadmium observed a significant reduction of adsorbed concentration, lowering the pH of 10.2 to 3.1. Kakoaba and Akcin (2005) showed that the removal of cadmium and lead in resins increased when the pH varies from 1 to 8. Similar results were also obtained by El-Kamash *et al.* (2005) for uranium and thorium with the use of zeolites.

Ultrastructural analysis

Through the ultrastructural study it was possible to verify variations in the electron density of the biosorbents surfaces submitted to presence of cadmium. The results are shown in figure 5A–H.

Ultrastructural changes are also reported as a result of exposure to metal ions. Variations in electron density and heterogeneity were visualized by scanning electron microscopy. After exposing the biomass and the skeletons of chitin and chitosan, we observed heterogeneity of the same, but the electron density as a factor that increases the adsorption.

Figure.1 Radial growth of *Rhizopus arrhizus* UCP402 in synthetic medium for mucoralean in absence and presence of cadmium concentrations

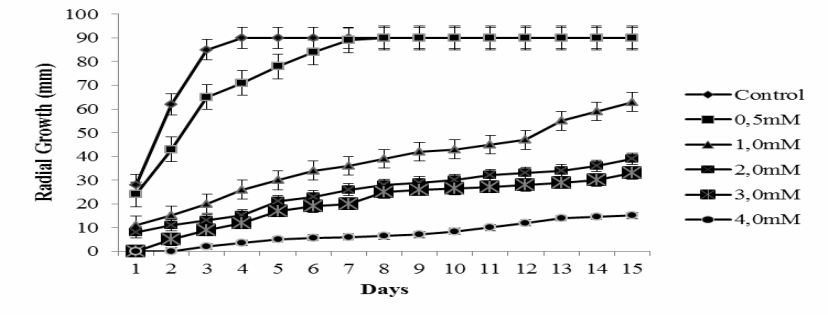


Figure.2 Enzymatic activity of *Rhizopus arrhizus* UCP402 in the absence and presence of cadmium

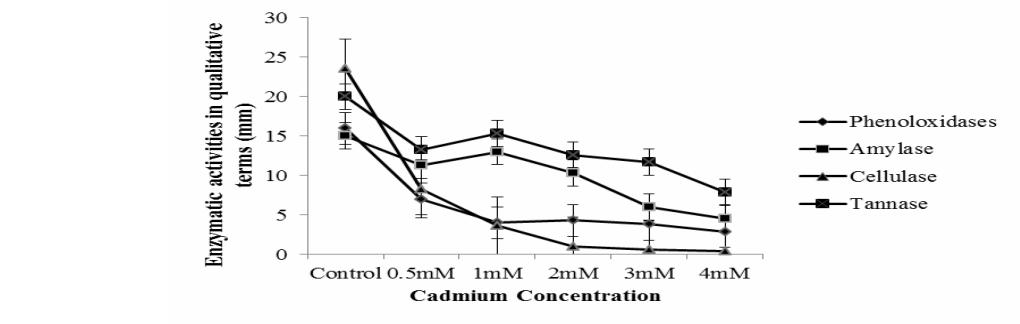


Figure.3 Cadmium removed per gram biomass from *Rhizopus arrhizus* UCP402. (A) living biomass; (B) inactivated biomass

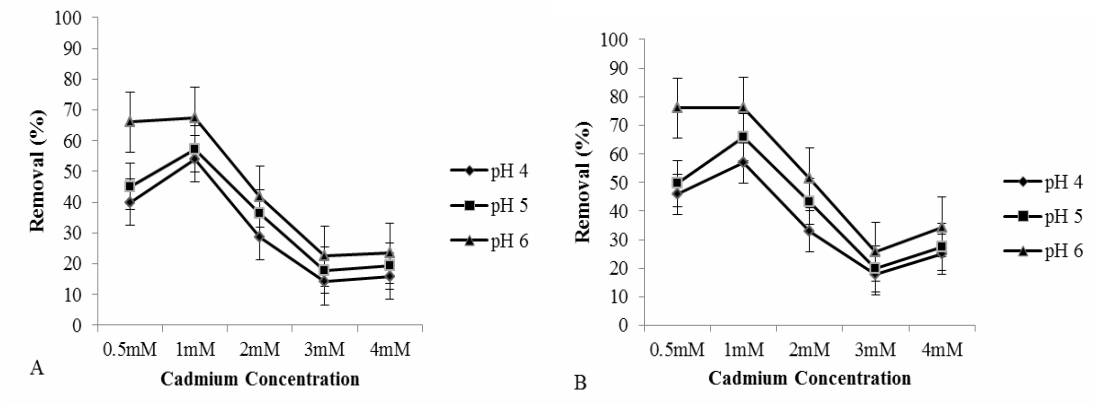


Figure.4 A-Cadmium removed per gram of chitin from *Rhizopus arrhizus* UCP402, B-Cadmium removed per gram of chitosan from *Rhizopus arrhizus* UCP402.

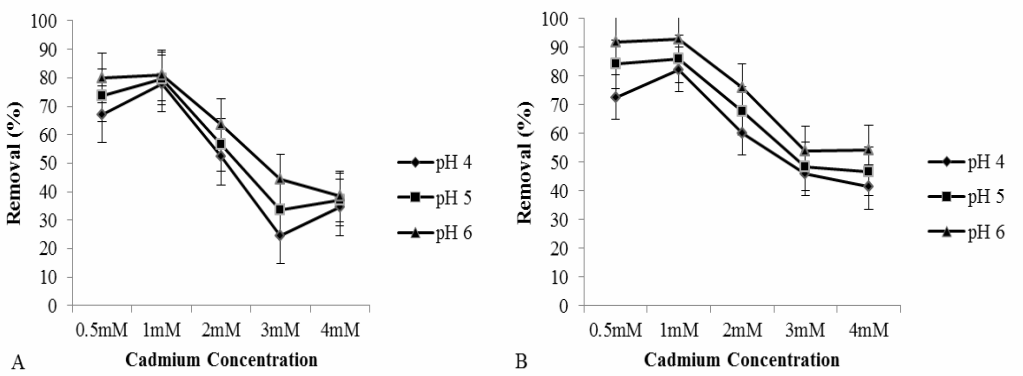
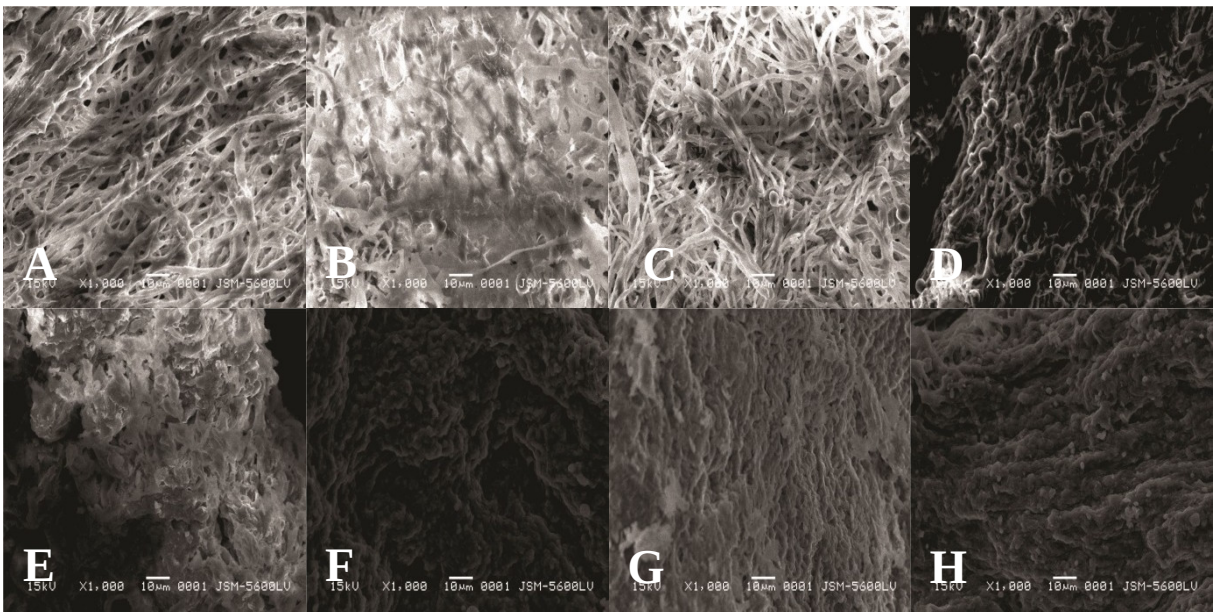


Figure.5 Electronmicrographs of *Rhizopus arrhizus* UCP204. A- Living biomass (control); B- Living biomass submitted to cadmium on 4mM; C- Inactivated biomass (control); D- Inactivated biomass submitted to cadmium on 4mM; E- Chitin (control); F- Chitin submitted to cadmium on 4mM; G- Chitosan (control); H- Chitosan submitted to cadmium on 4mM



For the chitosan was higher adsorption capacity but also higher electron density than chitin, since the presence of ionic groups confer unique properties to this biopolymer.

For the control samples it was verified the presence of homogeneous mycelium or biopolymer exhibiting low electron density. Moreover, samples submitted to 4mM of cadmium showed heterogeneity on hyphae and biopolymers, increased electron density. The observed changes were directly related to the concentration of metal.

In this study, the presence of cadmium altered the growth of the colonies of *Rhizopus arrhizus* UCP 402, but the strain exhibited tolerance in response to metal exposure. The strain showed good activity

for all enzymes tested, but its activities were reduced due to the presence and concentration of metal. The ability of removal of the strain has evaluated where the living biomass has showed the lower potential for metal removal at all concentrations tested. The polymers extracted from the strain exhibited the highest rates of removal of heavy metal, and chitosan was the best remover system than chitin. Additionally the results for the efficiency of the metal removal point the isolated for studies of remediation of metals.

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