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Removal and Recovery of Mercury *in Vitro* Using Immobilized Live Biomass of *Chlorella* sp.

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Abstract

Objectives: This research was aimed at evaluating the capacity of Mercury removal and recovery using live biomass of *Chlorella* sp. immobilized in scourer (*Luffa cylindrica*). **Methods/Statistical Analysis:** The algal biomass was bio-augmented in photobioreactors with 4 mM Agrimins during 20 days in constant agitation. For the immobilization of the microalga, scourer fragments were used. The mercury removal and/or desorption capacity was determined during 24 h, and desorption was carried out by acid digestion. An ANOVA and the Tukey test for significant differences (p-value ≤ 0.05) were performed with the results in the InfoStat software. **Findings:** an average immobilization of *Chlorella* sp. of 1.69g of algal cells/scourer fragment after 20 days of incubation. The statistical analysis showed significant statistical differences between all removal times, presenting the highest averages at 24 h of exposure, with a Mercury removal of 98.58% and a desorption of 82.61%. Likewise, in the lowest concentrations the microalga showed greater capacity of Mercury sorption, while at the highest concentrations the desorption of said heavy metal was greater. **Improvements/Applications:** *Chlorella* in bioremediation techniques of heavy metals are positioned as a biotechnological alternative, which thanks to its high rate of removal and desorption allows the ecological disposition of the contaminant.

Keywords: Immobilization, Mercury, Microalga, Scourer

1. Introduction

Heavy metals cause environmental pollution, and their high toxicity is responsible for serious consequences for public health and the environment, which have led to a greater interest in the development of environmental biotechnology approaches[1]. Aquatic ecosystems are prone to heavy metal contamination[2] such as mercury (Hg), which can be found as mercury ion (Hg^{22+}), mercuric ion (Hg^{2+}) and elemental mercury (Hg^{0})[3]. Organic Hg is commonly found as organic methylmercury and dimethylmercury, considered as a highly toxic heavy metal and its availability creates several environmental problems[4], being methyl-mercury its most dangerous form, because it can alter the synthesis of proteins, damage the active sites of enzymes[5] affect the structural integrity of the membrane[6] alter photosynthesis and transpiration[7].

The Hg also has the ability to act as an environmental factor related to autoimmune diseases, presenting inorganic Hg which causes an autoimmune response much stronger than methylmercury[8]. At present the removal of heavy metals from wastewater is carried out through physicochemical methods among which are: chemical precipitation, electrokinetics, coagulation, flocculation, ion exchange, biosorption, membrane treatment, reverse osmosis, filtration, anaerobic degradation and aerobic[4,9-11].

However, the removal of metal ions from wastewater on an industrial scale represents a significant economic challenge due to the high costs of chemical products and the incomplete removal of heavy metal ions, identifying themselves as limiting factors in the application of these techniques[1]. In addition, the use of conventional heavy metal remediation methods is uneconomical and generates a large amount of secondary waste[12]. Therefore, the

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use of biomaterials as biosorbents is considered an innovative bioremediation technology in terms of profitability and environmental impacts[1,13].

Bioremediation involves the adsorption, reduction or elimination of pollutants from the environment with the help of biological resources, which offer economic and ecological ways for the removal of heavy metals; therefore, they are considered efficient and alternative tools for the elimination of said pollutants[12].

The use of immobilized microalgae is an excellent alternative for the efficient removal of environmental contaminants, but the type of substrate that contains the biosorbent is an important limitation in this biotechnology. For this reason, a key requirement for the development of the immobilization technique is the absence of adverse effects on the viability and photosynthetic activity of the cells[14] and the ideal support must preserve the metabolic activity of the microalgal cells for as long as possible, but should not prevent the transfer of mass and the propagation of light in the culture system[15].

The use of biological remediation techniques, propose an important potential in terms of caring for the environment, also presenting high efficiency and low cost for the removal of heavy metals from diluted solutions, and may also involve the regeneration of the sorbent, as well as the recovery of metals[16]. The Bioremediation processes involve mechanisms such as biosorption, bioaccumulation, biotransformation and biomineralization through the use of biological systems[12].

The use of algal biomass in bioremediation techniques in recent years has aroused interest for its outstanding potential for bioaccumulation and removal of heavy metals[17,18]. Among its mechanisms, ion exchange is the most important mechanism in the biosorption of heavy metal ions by algae biomass[19]. Specifically, *Chlorella* microalgae have been used in processes of bioremediation of metallic elements of wastewater, since it has an important affinity for polyvalent metals[15].

The choice of support material is a key step in the success of the technique. The support material can be natural or synthetic[14]. The advantages of natural supports are hydrophilicity, biocompatibility and ease of use, while synthetic materials have great mechanical, chemical and biological stability[15] although it can present high costs. That is why the applications of microalgae immobilized in environmental research have recently increased, proving to be a very promising topic in biotechnological research and the immobilization of the algal biomass can occur passively or actively[20], presenting in the passive immobilization the natural tendency of the microalgae to create biofilm on the surfaces, which gives the community algal protection and greater tolerance to contaminants, and this is reflected in an increase in removal efficiency. Therefore, the objective of the present investigation was to determine the capacity of mercury removal and recovery using live biomass of *Chlorella* sp. immobilized in scourer fragments (*Luffa cylindrica*) and analyze by atomic absorption spectroscopy Mercury levels in each treatment.

2. Materials and Methods

2.1 Culture Medium and Biomass Obtained from *Chlorella* sp.

Cells of *Chlorella* sp. they were bioaugmented in phycobioreactors with capacity for 2.5 L of culture medium at 4 mM of Agrimins that provides nutrients such as K, Mg, S, P, Fe, Cu, Zn, Mn, and B, necessary for a normal algal growth and each container will be inoculated with a concentration of *Chlorella* sp. of $1x10^6$UFC and optical density of 0.1 of Abs measured with a λ of 647 nm[21]. The phycobioreactors were incubated at 30±1°C with constant agitation to avoid sedimentation of the biomass and average luminosity of 2500 lux during the 12 h of light and the remaining 12 h were kept in darkness, the process took 24 days until the microalga reached stationary phase[22].

2.2 Growth Curve of *Chlorella* sp.

Every day for up to 24 days algal growth measurements were made using a spectrophotometer UV-vis Spectroquant Pharo 300 from Merck at a wavelength of 647 nm where the absorbance measured was in proportional to the concentration of the microalgae in the culture, starting on day 0 with a concentration of 0.1 Abs[21].

3. Immobilization of Biomass in *Luffa Cylindrica*

For the immobilization of the microalga, the dry fruit of *Luffa cylindrica* was used as support, which was cut at the ends to remove the seeds and impurities with successive washes with water and detergent for 30 min[23]. Then cut pieces of scouring pad 2.5±0.2 cm in diameter and

2.8±0.2 cm thick, again washed with distilled water and dried at 70±1°C to be sterilized and transferred to culture medium with 4 mM Agrimins for 24 h, after this time they were inoculated in the solution of microalga in stationary phase (19 days), after which the scourers were removed, washed to eliminate the excess microalgae, and the immobilized biomass was determined by the difference of the weight of the scourer before and after immobilization[24].

4. Preparation of Mercury Concentrations

The Mercury concentrations were prepared from a stock solution of 6 mg/L of $HgCl_2$ analytical grade, Merck brand, in sterile tap water, and subsequently from this, diluted solutions of 300 mL were made at concentrations of 3.0 mg/L, 3.5 mg/L, 4.0 mg/L and 4.5 mg/L. All the glass material used was washed in 30% nitric acid and rinsed three times in sterile distilled water before being used in the experiments.

5. Biosorption and Desorption Experiment

The ability to remove Mercury by *Chlorella* sp. immobilized in scourer was determined in the concentrations previously described, establishing three time of removal: 8 h, 16 h and 24 h, after each time the algal biomass was removed by centrifugation at a speed of 6000 rpm for 5 min and the supernatant solution was analyzed by atomic absorption spectrophotometry with cold vapor. The experiment was carried out in triplicate.

For the desorption of the mercury, the removed algae biomass was mixed with a 10 mM HCl solution and stirred at 400 rpm for 30 min at 30±1°C. The final concentration of metal ions in the aqueous phase was determined using an atomic absorption spectrophotometer with cold vapor. The percentage of mercury removal was determined by the following formula:

$$Percentages\ of\ Mercury\ removal\ (\%) = \frac{C_i - C_f}{C_i} x100$$

Where C_i is the initial concentration of Mercury and C_f is the final concentration of Mercury after the treatment is applied[25].

The desorption ratio was calculated from the amount of mercury desorbed by the eluent and the amount of Mercury adsorbed by the immobilized microalga, using the following formula:

$$Percentages\ of\ Mercury\ desorption\ (\%) = \frac{Hg_d}{Hg_a} x100$$

Where Hg_d is the amount of Mercury desorbed and Hg_a is removed by the microalga *Chlorella* sp. Immobilized.

6. Analysis by Atomic Absorption Spectroscopy with Cold Steam

To the supernatants solutions of all the treatments were measured the Mercury levels by atomic absorption with cold vapor. The sample preparation protocol consisted of transferring the sample to a 500 mL Erlenmeyer flask, adding 100 mL of sample, 1.5 mL of HNO_3(65%), 1.5 mL of H_2SO_4 (97%) and volume of KmnO (5%) solution until the purple colour remained, 8 mL of potassium persulfate (5%) solution and heated for one hour in a water bath at 95±1°C. Then, the digest was collected in a cold steam generator glass and a hydroxylamine (10%) solution was added, maintaining the purple coloration.

7. Statistic Analysis

A completely random design was carried out with a 2x2x4 factorial arrangement, previously determining the normality criterion with the Shapiro-Wilks test. Significant statistical differences were established with the Tukey multiple range test (p-value ≤ 0.05). The data was processed in the free version InfoStat software. All treatments were performed in triplicate.

8. Results and Discussion

8.1 Growth Curve of the Microalga *Chlorella* sp.

The microalga *Chlorella* sp. it was incubated under conditions of mixotrophic growth, showing a logarithmic growth, in which, the first two days of the experiment started, the adaptation of the microalga to the environment was observed and experiencing an increase in growth that extended until day 19, and from this, until day 24 microalgae culture entered the stationary phase (Figure 1).

Microalgae are eukaryotic organisms with the capacity to perform photosynthetic processes by exposure to light and the biosequestration of nutrients such as Carbon,

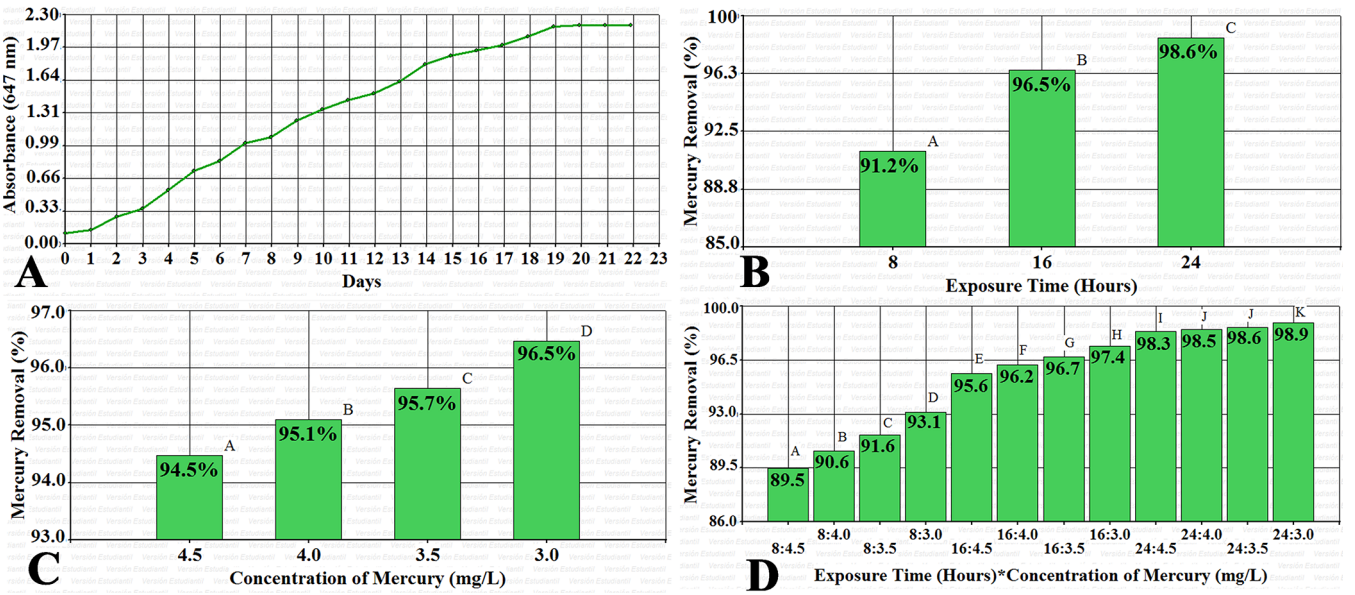

Figure 1. A. Growth curve of *Chlorella* sp., B. Mercury removal test by exposure time (Hours), C. by concentrations of Mercury (mg/L), and D. Interaction between exposure time (Hours) and Mercury concentration (mg/L).

Nitrogen, Phosphorus, etc., subsequently generating biomass, with Nitrogen and Phosphorus being necessary elements in the cultivation of microalgae *Chlorella*, which determine yield and productivity, in a 15:1 ratio of N:P microalgal biomass yields of 3568±158 mg/L and sequestered 40% of CO_2 are generated[26].

Microalgae have been the subject of research, not only because of the high rate of growth, but also because of the low costs for their production[27].

8.2 Immobilization of *Chlorella* sp. in Scourer Fragments (*Luffa Cylindrica*)

Chlorella sp. was immobilized in dry scotch fruit at an average of 1.69 g at 18 days by 2.5x2.8 cm scourer fragments (Figure 2). Immobilized microalgae have many advantages compared to microalgae in suspension, so applying this biotechnology has shown an increasing trend because it provides a support for higher cell den-

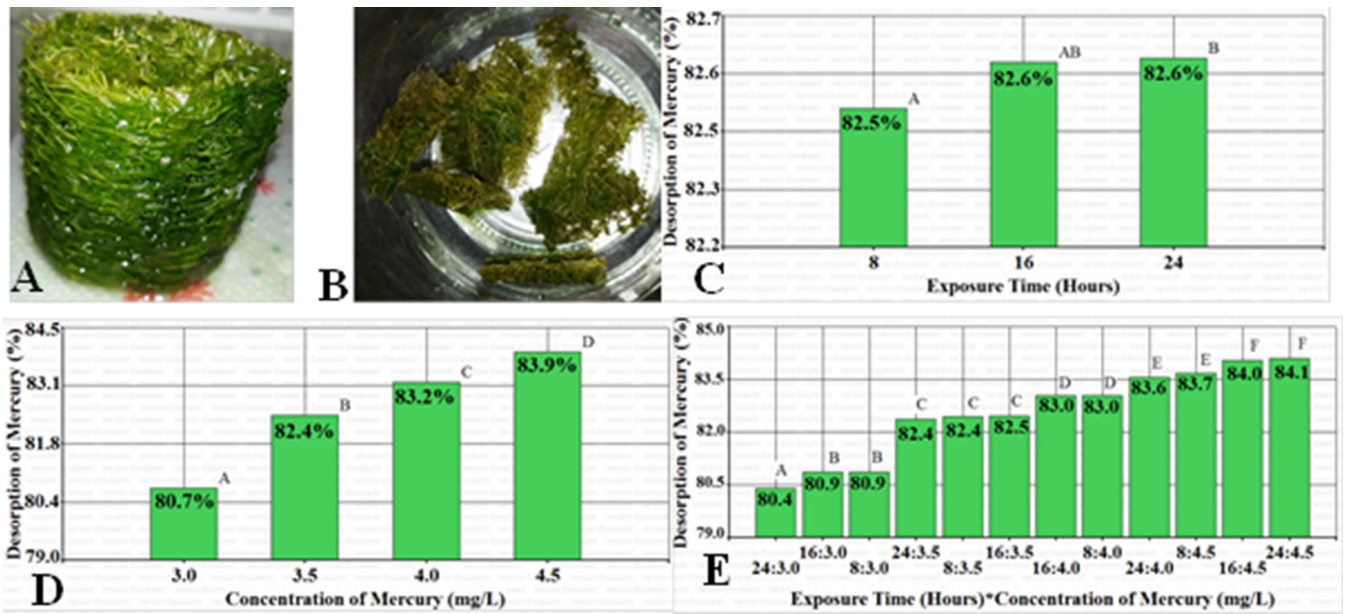

Figure 2. A. Immobilized microalgae, B. Mercury desorption process, C. Mercury desorption tests by exposure time (Hours), and D. by concentrations of Mercury (mg/L). E. Interaction between exposure time (Hours) and Mercury concentration (mg/L).

sities, greater tolerance of microalgae for example to stresses, to the salinity, at extreme pH, to heavy metals[15].

However, the type of substrate for algal immobilization is a limiting factor in this biotechnology, because synthetic materials can inhibit cell division, photosynthetic rate and other key factors of cell integrity. The natural support materials such as the dry fruit of the genus *Luffa* alter the repeatability of the processes, since the structure of the fiber is not homogeneous in all cases, and this depends on the conditions of the crop[28]. In any case, immobilized microalgae have shown higher percentages of removal of contaminants than free cells.

Chlorella sorokiniana microalga immobilized in scouring fibers was used for the removal of Nickel (II) from aqueous solutions, removing 25% more of said heavy metal than free cells[20]. It has been reported that the *Chlorella marine* microalgae immobilized in alginate beads removed 90% of nitrate and 60% of phosphate in 24 hours[29]. While *Tetraselmis* sp. immobilized removed 41% nitrate and 18% silicate at 3 hours of tannery wastewater[30].

It has been used the polyethyleneimine polymer for the immobilization of the microalga *Chlorella vulgaris*, achieving an efficiency in the immobilization, but impaired the cell viability by inhibiting the photosynthetic rate of the microalga, and in comparison to the polymers polyethyleneimine and diglycidyl ether of diethylene glycol, hepiclorohidrina demonstrated higher binding speed of algal cells[14].

8.3 Mercury Removal/Desorption Test by *Chlorella* sp. Immobilized in Scourer

With the results of the sorption/desorption and/or mercury recovery tests, the normality criterion was met with the Shapiro-Wilks test, finding a p-value of 0.0635 for the variable mercury removal and a p-value of 0.0782 for the Mercury desorption variable. Established the normality criteria the ANOVA was performed by means of a design with a factorial arrangement, determining as influencing factors of the response variable, the exposure times of the immobilized microalga whose levels were 8 hours, 16 hours and 24 hours, and as a second factor mercury concentrations like mercury chloride whose levels were 3.0 mg/L, 3.5 mg/L, 4.0 mg/L and 4.5 mg/L, as well as the interaction between these two factors, finding significant statistical differences (p-value < 0.05) among all the factors as well as between interactions. Therefore, the Tukey range multiple test was applied, finding significant statistical differences (p-value < 0.05) between all the exposure times, showing the highest averages of removal with 98.58% at 24 h of exposing the immobilized algal biomass to the different concentrations of Mercury (Figure 1B). However, from the 8th hour the removal of Mercury was higher than 91%.

Likewise, for the different concentrations of Mercury, the Tukey test shows significant statistical differences (p-value < 0.05) with the highest averages of removal of said heavy metal at the lowest concentrations, this was 3.0 mg/L with 96.47% and as the concentration of Mercury increases, the biosorbent removal capacity decreases, reaching a removal of 94.48% to 4.5 mg/L of mercury (Figure 1C).

When performing the interaction between the time factor of exposure and mercury concentrations, the Tukey test shows significant statistical differences (p-value < 0.05) showing the highest averages of removal in the longest exposure time and the lowest concentrations of Mercury, which indicates that the immobilized biomass of *Chlorella* sp. It is efficient in the removal of mercury in diluted solutions this is at concentrations of 3.0 mg/L of Mercury during 24 h of exposure (Figure 1D).

The use of microalgae in biotechnological applications has increased, but the small size of individual cells implies a problem in their application, which is why cell immobilization techniques have been developed to solve these problems[20].

Immobilized microalgae cells, compared to free-living cells, provide greater advantages, in that they simplify the treatment of wastewater, because the entrapment of living cells can increase the life time and maintain their metabolic activities for long periods[31] which makes them the most efficient method. The immobilized algae has been used to obtain biomass and the removal of nutrients[20], as reported for the microalgae *Chlorella marina* immobilized in pearls of alginate, who removed 90% of nitrate and 60% of phosphate in 24 hours[29]. While *Tetraselmis* sp. immobilized removed 41% nitrate and 18% silicate at 3 hours of tannery wastewater[30].

The use of immobilized microalgae represents several advantages for the treatment of wastewater, as well as several challenges[31], such as proper pH control, pearl stacking density, concentration and leakage of algae cells[30,32]. The use of a scourer for the immobilization of *Chlorella sorokiniana* algal biomass for Nickel (II) removal[24] has been reported, demonstrating 25% more

accumulation of Nickel than free cells after an exposure of 20 min[20].

With respect to mercury desorption tests for the immobilized biomass of *Chlorella* sp. in scourer fragments (Figure 2. B), the statistical analyzes show significant statistical differences (p-value < 0.05) between the factors exposure times and Mercury concentrations, as well as between their interactions. Finding significant differences (p-value < 0.05) between the exposure times at 8 h and 24 h, although between 16 h and 24 h did not present significant differences (p-value > 0.05) showing the highest average desorption of Mercury when the alga was exposed to this last period, with an average desorption of 82.61% (Figure 2. C). While for mercury concentrations, the Tukey test shows significant statistical differences (p-value < 0.05), showing the highest averages of desorption in the concentration 4.5 mg/L with 83.93%, and this decreases as the concentration of this metal (Figure 2D).

The Tukey test for the desorption percentages shows significant differences (p-value < 0.05) between exposure times and Mercury concentrations, presenting the highest desorption averages at the highest concentrations of this heavy metal, at 24 h of exposure with average of 84.10%, while the lowest averages of desorption were presented by the concentrations of 3.0 mg/L (Figure 2E).

Microalgae are unicellular organisms with photosynthetic pigments capable of sequestering heavy metals by producing peptides[33], thereby neutralizing their toxicity by creating organometallic complexes that are stored in vacuoles for cytoplasmic control[34].

Chlorella is a microalga with a high potential as a biosorbent for the removal of heavy metals from wastewater, due to the high affinity for polyvalent metals[15]. These microalgae have molecular mechanisms that allow them to discriminate non-essential heavy metals from essential ones and use them for their growth[33]. On the contrary, non-living algal biomass captures metal ions on the surface of the cell membrane, because it acts as a set of polymers (sugars, cellulose, pectins, glycoproteins, etc.) that are capable of binding to cautions of heavy metals, considered a low-cost wastewater treatment technique[35-37]. The algal cell has different functional binding groups, such as hydroxyl, phosphorylation, carboxyl, sulfuryl, amine, imidazole, sulfate, phosphate, carbohydrate, etc., on which it has been inferred that they are responsible for the capacity of ion biosorption of heavy metals[38].

However, the heavy metal sorption potential of the algal biomass can be affected by factors such as: the amount of functional groups in the cell, the chemical status of the binding sites, the coordination number of the metal ion to be absorbed. The accessibility of the metal to the binding groups and the speed of formation of the metalion complex with the functional group[1]. As well as the physicochemical and environmental parameters such as pH, temperature, ionic strength, contact time, biomass concentration, atomic weight and valence of the metal, and the nature of the biosorbent, such as the species, age, physiological state and growth conditions can determine differences in selectivity and affinity to metal ions[39,40].

With respect to chemical parameters, changes in pH affect live algae to a greater extent than non-living algae since most algae grow in neutral or slightly alkaline media[41] whereas acidic media can affect the growth rate of algae and the basic ones cause the precipitation of metal ions[42] so their elimination at extreme pH favors mainly non-living algae[1].

9. Conclusions

The removal of Mercury by *Chlorella* sp. immobilized was close to 99%, demonstrating its potential as bioremediation techniques, positioning itself as a biotechnological alternative for the elimination of mercury from aquatic systems.

The use of microalgae immobilized in scouring pads allows higher rates of mercury removal than microalgae in free cells, as well as the ecological disposition of the contaminant.

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