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Articles

# Bioadsorption of Co(II) and Mn(II) in aqueous solutions by the *Rhodococcus opacus* bacterium

# Bioadsorción de Co(II) y Mn(II) en soluciones acuosas por la bacteria *Rhodococcus opacus*

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**Abstract**

The bioadsorption of Co(II) and Mn(II) in aqueous solutions was evaluated by *Rhodococcus opacus* inactivated and treated with NaOH, and the physicochemical characterization of the bioadsorbente. The physicochemical characterization of bacteria was performed by scanning electron microscopy coupled to energy dispersive X-ray analyses (SEM-EDS), Fourier transforms infrared spectroscopy (FTIR,) and Zeta Potential (ZP). Likewise, the operating conditions in the process were determined, evaluating biosorbent concentration, initial metal concentration ($C_i$), pH, and contact time. The experiments were performed at the laboratory, and the bacteria was previously treated with NaOH (0.1M). The interaction between *R. opacus* and metal ions was confirmed by the variation of isoelectric point (IEP) and functional groups. Removals and uptake of 87.8 % and 24.9 mg/g for Co(II) and 79.9 % and 6.1 mg/g for Mn(II) were achieved under test-operating conditions. These conditions were pH 7, biomass 4 mg/l, $C_i$ 50 mg/l, and 180 minutes of operation time. While for Mn(II): pH 5, biomass 3 mg/l, $C_i$ 5 mg/,l and 10 minutes operation time. The present study shows that *R. opacus* can be applied to treat effluents containing low concentrations of Co(II) and Mn(II).

**Keywords**: Bioadsorption, removal, cobalt, manganese, biotechnology, *Rhodococcus opacus*.

## Resumen

Se evaluó la bioadsorción de Co(II) y Mn(II) en soluciones acuosas por *Rhodococcus opacus* inactivado y tratado con NaOH, y se efectuó la caracterización fisicoquímica del bioadsorbente. La caracterización fisicoquímica de la bacteria se realizó mediante microscopía electrónica de barrido acoplada al análisis de dispersión de energía de rayos X (MEB-EDS), espectroscopía infrarroja por transformada de Fourier (FTIR) y potencial zeta (PZ). Asimismo, se determinaron las mejores condiciones de operación en el proceso, evaluando la concentración del biosorbente, concentración inicial del metal (Ci), pH y tiempo de contacto. Los experimentos se hicieron a escala de laboratorio y la bacteria fue previamente tratada con NaOH (0,1 M). La interacción entre *R. opacus* y los iones metálicos se confirmó por la variación del potencial isoeléctrico (PIE) y grupos funcionales. En las mejores condiciones de operación se alcanzaron remociones y adsorción de 87.8 % y 24.9 mg/g para Co(II), y 79.9 % y 6.1 mg/g para Mn(II). Estas condiciones fueron pH 7, biomasa 4 mg/l, Ci 50 mg/l y 180 minutos de tiempo de operación. Mientras que para el Mn(II): pH 5, biomasa 3 mg/l, $C_i$ 5 mg/l y 10 minutos de tiempo de operación. El presente estudio demuestra que el *R. opacus* puede ser aplicado en el tratamiento de efluentes conteniendo bajas concentraciones de Co(II) y Mn(II).

**Palabras clave**: bioadsorción, remoción, cobalto, manganeso, biotecnología, *Rhodococcus opacus*.

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# Introduction

In recent years, there is a great interest in studying the accumulation of heavy metals, from anthropogenic sources, in the environment (water, air, soil) due to their toxicity on humans and other life forms (Vishan, Saha, Sivaprakasa, & Kalamdhad, 2019). While in Europe the main anthropogenic sources of environmental pollution such as industry and intensive agriculture have been investigated ((Sedlakova-Kadukova, Kpcakova, Gresakova, Godany, & Pristas, 2019), in Latin America water pollution by heavy metals has also been the subject of research (Bundschuh *et al*., 2012; Guittard *et al*., 2017), this problem is related to practices that are not associated with a sustainable resource management.

In Peru, for example, the presence of manganese has been reported at levels above the environmental quality standard (ECA) in the Tambo, Rimac, Santa, Mantaro and Chamaya rivers (Minagri, 2013). While, in the Titicaca hydrographic region both metals exceed the ECA (OECD, 2017).

The presence of Co(II) and Mn(II) in effluents is due to mining activities, alloy production, catalysts and batteries among other sources (Barrio-Parra *et al*., 2017). Manganese can often be found in groundwater and is associated with water coloration, clothing staining and clogging of pipes, whereas exposure to this metal causes chronic poisoning of the human body (Rose, Fabbro, & Kinnear, 2017). Likewise, high exposures to Co(II) are associated with low blood pressure, nausea, vomiting, nervous system disorders and paralysis (Abbas, Kaddour, & Trari, 2014).

Over time, a series of treatments have been developed to eliminate or mitigate heavy metal contamination and restore a healthy balance in favor of human and environmental dynamics. The techniques conventionally used in the treatment of industrial effluents, containing metals, are based on physicochemical processes such as electro-flotation and membrane filtration (Nascimento-Do, De-Oliveira, Rizzo, & Leite, 2019); they are also characterized by high cost and difficult implementation. Furthermore, these processes have shown low removals when metal concentrations are less than 100 mg/l (Volesky, 2003). In response to this situation, technologies such as biotreatments, which are more environmentally friendly and less costly, have emerged (Ziagova *et al*., 2007; Hasan, Abdullah, Kofli, & Yeoh, 2016; Jobby, Jha, Yadav, & Desai, 2018).

Among the biotreatments, biosorption, which involves the use of adsorbents of biological origin, stands out and has shown to have high removal, in addition to not generating solid residues or toxic substances during the process (Costas & Tavares, 2016). The use of live or inactive bacteria as biosorbents is a potential alternative in the adsorption of metals, some of the most studied correspond to the genera: *Citrobacter, Pseudomonas, Streptomyces,* and *Bacillus* (Jacob *et al*., 2018).

Likewise, several investigations have reported the application of *R. opacus* and the extracellular polymeric substances of this bacterium, in the bioadsorption of various metals, such as Al(III) (Cayllahua & Torem, 2010); Mn(II) (Silvas, Buzzi, Espinosa, & Tenório, 2011); Cd(II); Pb(II); Ni(II); Co(II), and Cr(VI) (Dobrowolski, Szcześ, Czemierska, & Jarosz-Wikołazka, 2017).

Bioadsorption, using living or inactivated cells, is a process that does not depend on cell metabolism since the metal is adsorbed on the surface of the bioadsorbent (Nascimento-Do *et al*., 2019) and is mostly a reversible process (Arivalagan, Singaraj, Haridass, & Kaliannan, 2014). Microbial cell wall structure has been identified as the main responsible for metal adsorption (Escudero, Quintas, Wuilloud, & Dotto, 2019). Regarding this, it has been reported that functional groups present in Gram-negative bacteria present a high availability in the removal of metal species (Quiton, Doma, Futalan, & Wan, 2018; Escudero *et al*., 2019; Wang *et al*., 2019).

However, other authors argue that Gram-positive bacteria, by presenting a thicker cell wall (and particular components) also present higher removals and adsorption of metal ions, when compared to Gram-negative bacteria (Gourdon, Bhende, Rus, & Sofer, 1990; Jacob *et al*., 2018). Escudero *et al*. (2019). Escudero *et al*. (2019) indicate that the behavior of the bacteria is dependent on the system in which the experiments are carried out.

The main functional groups, present in the bacterial cell wall and related to metal adsorption are: hydroxyl, carboxyl, sulfonate, amide, and phosphonate (Arivalagan *et al*., 2014); whereas, the physicochemical mechanisms related to this adsorption include electrostatic interaction, complex formation, ion exchange and micro precipitation (Anagnostopoulos, Vlachou, & Symeopoulos, 2015).

The use of inactivated bacterium as adsorbent is a treatment with much potential for the removal of this type of metal contaminants, being important to study the main physicochemical characteristics of the process, which will allow the approach to future applications in the field of water treatment; being the greatest challenges the identification of a readily available bioadsorbent, of simple application, which allows a more economical and efficient process (Barquilha, Cossich, Tavarese, & Silva, 2019; Bankar & Nagaraja, 2018).

The objective of this research is to evaluate the bioadsorption of Co(II) and Mn(II) in aqueous solutions by inactivated and NaOH-treated *Rhodococcus opacus*, as well as the physicochemical characterization of the bioadsorbent.

## Materials and methods

## Obtaining and preparation of *R. opacus*

The *R. opacus* bacterium was acquired from the microbial culture collection of the André Tóselo Tropical Research and Technology Foundation, Campinas, Sao Paulo, Brazil. Then, it was cultured in a solid medium composed of glucose (10 g/l), peptone (5 g/l), malt extract (3 g/l), yeast extract (3 g/l) and agar-agar (20 (g/l).

Afterwards, 200 ml of liquid culture medium (in 500 ml Erlenmeyer flasks) was prepared with the same composition described for the solid medium, except for the absence of agar-agar. The pH was adjusted to 7.2, with NaOH 0.1M or HCL 0.1M. The liquid medium was sterilized in a Prismatec vertical autoclave at 121 °C for 20 minutes. Then, in a biological safety cabinet (Filterflux class II A2) previously sterilized, the microorganism was transferred from the solid culture medium to the liquid medium with a platinum loop, previously flamed.

Incubation was performed at 28 °C, for 48 h and 125 rpm, in a horizontal rotating platform incubator (Cientec, CT-712). After cell growth in the liquid culture medium, the bacterial cell was collected and washed three times with deionized water by centrifugation at 3500 rpm for 10 minutes and then sterilized in a vertical autoclave under the same conditions described above, which made it possible to obtain the inactivated biomass (BSPT).

## Pre-treatment of bioadsorbent

Pre-treatment of the inactivated bacterium was performed by applying 30 ml of NaOH (0.1M) per 100 ml of biomass. The cell suspension was agitated on a horizontal rotating platform at 125 rpm at 25 °C for three hours. Finally, this product was washed with deionized water three times and the pH was adjusted to values close to 7, with HCl, 0.1M. The pre-treated biomass (BPT from the Spanish abbreviation) was characterized and used in the bioadsorption experiments.

## Concentration of bioadsorbent

BPT (pre-treated biomass) concentration was determined by applying the analytical method suggested by Standard Methods (2540 Solids, 2017) for total suspended solids (TSS), by gravimetric measurement to a constant dry weight of the cell mass. For this, 40 ml of cell concentrate was pipetted and filtered under vacuum (0.45 µm cellulose acetate filter paper), the retained solid was dried at 103-105 °C for two hours (in an oven); then measurements and calculations were made regarding dry weight. In this study, the concentration of bacterium was evaluated at values between 1 and 5 mg/l.

## Preparation of Co(II) and Mn(II) solutions

Standard solutions of each metal, at a concentration of 500 mg/l, were prepared using deionized water and Co(II) and Mn(II) salts ($CoCl_2.6H_2O$ at 98 % purity and $MnSO_4$.H2O at 98.01 % purity). By means of successive dilutions, solutions of both metals between 50-120 mg/l were obtained.

## Bioadsorption experiments

The experiments were performed in a batch system, on a horizontal rotating platform with 250 ml Erlenmeyer flasks (containing 50 ml of solution) at 25 °C and 125 rpm for all experiments.

The parameters were evaluated considering a pH range of 4-8 for Co(II) and 3-7 for Mn(II); and for both metals: bioadsorbent concentration 1-5 mg/l; initial metal concentration 50-120 mg/l and contact time 10-180 min. For the pH evaluation, the bioadsorbent concentration (1 g/l), metal concentration (50 mg/l) and contact time (180 minutes) were set. Next, the pH value that allowed the highest removal and adsorption values to be achieved was identified, and this value was set in the following experiments.

Subsequently, the concentration of the bioadsorbent was evaluated, keeping the pH, metal concentration (50 mg/l) and contact time (180 minutes) constant; similarly, the initial metal concentration and contact time were evaluated. In other words, the value of the factor with which the highest percentage of metal removal and adsorption was achieved in each experiment was incorporated into the following experiment.

At the end of each experiment, the solution was centrifuged and sent for analysis by flame atomic absorption spectrophotometry (AAS, Model 1100DDD, Perkin Elmer); these analyses were carried out at the chemical analysis laboratory of the chemistry department of the Pontifical Catholic University of Rio de Janeiro.

The removal percentage (%R) and adsorption of each metal (q, mg/l), was calculated according to Equations 1 and 2 (Quiton *et al*., 2018):

$$\%R = \frac{C_i - C_f}{C_I} x100 \quad (1)$$

$$q = \frac{(C_i - C_f)V}{M} \quad (2)$$

Where, $C_i$ is the initial concentration of the metal in solution (mg/l); $C_f$ is the final concentration of the metal in solution (mg/l); V represents the volume of the solution (L), and M is the mass of the bioadsorbent (g).

## Statistical analyses

This study proposes the use of structural equation modeling (SEM), using Amos v.25; in order to determine the effect of the studied parameters or input parameters (pH, bioadsorbent concentration, initial metal concentration and contact time) on the removal and adsorption of metal ions (or output parameters); considering the significance value $p < 0.05$. The use of SEM has been extensively applied in ecology (Grace *et al*., 2012) and models cause and effect interrelationships, as well as the inclusion of dependencies between the parameters (Angelini, Heuvelink, & Kempen, 2017).

## Characterization of the *R. opacus* surface

The characterization of the bacterium, as well as the bioadsorptionn process, was performed by scanning electron microscopy, equipped with an energy dispersive spectroscopy (EDS) system in a Carl Zeiss-DSM 960 microscope, as well as by Fourier transform infrared spectroscopy (Nicolet 6700 FTIR spectrophotometer) in order to identify the functional groups responsible for bioadsorptionn for each metal. The surface electric charge measurements of *R. opacus* or ZP were carried out in the Zeta meter system 4.0 equipment. The pre-treated bacterial biomass (BPT) was used as a control in the characterization process.

# Results

## Bioadsorption

Figure 1 shows the influence of the evaluated parameters on the bioadsorptionn process of metals by the inactivated and pretreated bacterium. In Figure 1a, it is shown that the bioabsorption of Co(II) increased with increasing solution pH. This increase corresponds to an ascending slope up to pH 7, where the biomass achieved the maximum values of removal (59 %) and adsorption of this metal (29.5 mg/g). In the case of Mn(II) a similar behavior was observed, but up to pH 5.0, where 32.2 % removal and 3.8 mg/g adsorption were reached. From this value on, there was a marked change in the slope, which evidenced a decrease in the capacity of Mn(II) captured by the bacterium.

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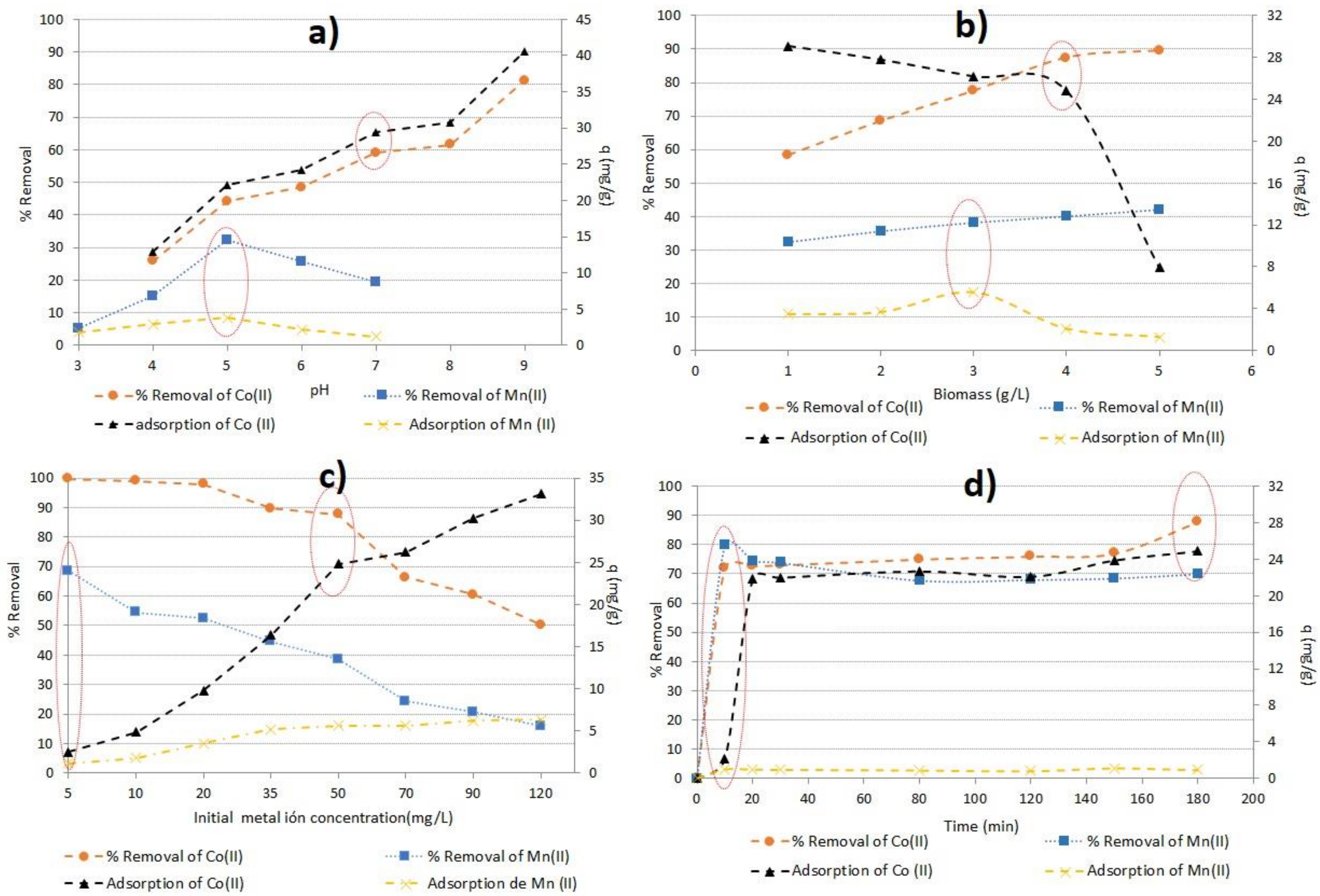


**Figure 1**. Influence of parameters on Co(II) and Mn(II) bioadsorption by inactivated and pretreated *R. opacus:* a) pH; b) biomass. Concentration; c) initial metal ion concentration; d) bacterium-metal contact time.

Figure 1 also shows the removal and adsorption of Co(II) and Mn(II) according to the concentration of the bioadsorbent. Regarding Co(II) in Figure 1b, a removal of 87.4 % and adsorption of 24.8 mg/g is observed for a biomass concentration of 4 g/l, which is the point where the slope of the removal and adsorption changes. Similar behavior is observed for Mn(II), when the bioadsorbent concentration is 3 g/l, reaching a removal of 38.2 % and adsorption of 5.6 mg/g, then a remarkable adsorption decay occurs; similar to what was observed for Co(II).

Figure 1c shows the removal and adsorption behavior as a function of the initial concentration of each metal. An increase in the initial metal concentration leads to a decrease in metal removal. Regarding the adsorption of Co(II), an increase of this metal is observed with the increase of Ci; and no effect of Ci on the adsorption of Mn(II). The maximum removal and adsorption values were 99.8 % and 33.1 mg/g for Co(II) and 68.5 % and 5.33 mg/g for Mn(II), respectively.

According to Figure 1d a rapid removal of Co(II) and Mn(II) of more than 60 % is observed in the first 10 minutes. The maximum removal value of 79.9% for Mn(II) was reached in this time. However, 180 minutes of contact time was necessary for the maximum removal of Co(II). Table 1 presents the results obtained in this study and in the literature for both metals, where the high adsorption values obtained are highlighted.

**Table 1**. Removal efficiency (%R) and adsorption (q) of Co(II) and Mn(II) reported in the literature.

| Bioadsorbent | Metal | q (mg/g) | %R | Operating conditions | Reference |
|---|---|---|---|---|---|
| *R. opacus* | Ca (II)<br>Fe (II)<br>Al (II)<br>Mg (II)<br>Mn(II)<br>Zn (II) | 48.2 | 11,7 | pH = 2.6; temperature = 25 °C, biomass = 1 g/l | Silvas *et al*. (2011) |
| *SPE of *R. opacus* | Co(II) | 88.3 | 100 | pH = 3-4; Ci (II) = 150-200 mg/l; biomass = 300 mg/l; temperature = 25 °C; 120 rpm; contact time = 30 min | Dobrowolski, *et al*. (2017) |
| SPE de *R. opacus* | | | 50 | | |
| Magnetotactic bacterium | Mn(II) | - | 15.26 | Induction of magnetic field, | Diaz-Alarcón, Alfonso-Pérez, Vergara- |

| | | | | | |
|---|---|---|---|---|---|
| | | | | temperature (18 ± 0,1 °C) and pH (6.87 ± 0.01); contact time = 3-5 min | Gómez, Díaz-Lagos and Martínez-Ovalle (2019)) |
| *Bacillus. cereus I6* | Pb (II) + Mn(II) | 19.9 | 56,3 | pH = 7; Mn(II) + Pb (II) = 50 mg/l; biomass = 0.3-1.5 mg/l | Hasan *et al.* (2016) |
| *Ralstonia pickettii* | Mn(II) | - | 46.02 | pH = 6; Ci = 400 mg/l; contact time = 72 h; temperature = 40 °C | Huang *et al.*(2018) |
| *Geobacillus thermantarcticus* | Co(II) | 11.95 | 43.6 | pH = 5 for Co(II) and 6 for Mn(II), Ci = 50 mg/l; contact time = | Özdemir, Kılınç, Poli and Nicolaus (2013) |
| *Anoxybacillus amylolyticus* | Co(II) | 8.83 | 35.1 | | |

| | | | | | |
|---|---|---|---|---|---|
| *Geobacillus thermantarcticus* | Mn(II) | 22.44 | 65.1 | 60 min; biomass = 50 mg; contact of temperature = 60 °C; 120 rpm | |
| *Anoxybacillus amylolyticus* | Mn(II) | 9.36 | 36.6 | | |
| | | | | | |
| R.opacus | Co(II) | 24.9 | 87.8 | pH = 7; biomass = 4 mg/l; Ci = 50 mg/l; contact time = 180 min | Present study |
| | Mn(II) | 6.1 | 79.9 | pH = 5; biomass = 3 mg/l; Ci = 5 mg/l; contact time = 10 min | |

*SPE= extracellular polymeric substance.

## Statistical analysis

The results of the effects of the various parameters analyzed on removal and adsorption are presented in Table 2.

**Table 2**. Standardized estimates of the effect of the various parameters on the bioadsorption process, as a function of removal. (%R) and adsorption (q).

| **Metal** | ***I** | ****S** | **β** | **Sig** |
|---|---|---|---|---|
| Co | pH | %R | 0.267 | 0.06 |
| | | *q* | 0.164 | 0.259 |
| | Biomass concentration | %R | 0.32 | 0.022 |
| | | *q* | -0.747 | 0.000 |
| | Metal concentration | %R | -0.252 | 0.077 |
| | | *q* | 0.263 | 0.064 |
| | Contact time | %R | 0.226 | 0.115 |
| | | *q* | 0.395 | 0.004 |
| Mn | pH | %R | 0.073 | 0.628 |
| | | *q* | 0.264 | 0.069 |
| | | %R | 0.315 | 0.028 |

| | | | | |
|---|---|---|---|---|
| | Biomass concentration | $q$ | -0.564 | 0.180 |
| | Metal concentration | %R | -0.442 | 0.001 |
| | | $q$ | 0.685 | 0.195 |
| | Contact time | %R | -0.14 | 0.348 |
| | | $q$ | 0.525 | 0.000 |

*I = input parameter, **S = output parameter

## Characterization of R. *opacus* before and after adsorption

### Zeta potential (ZP)

The ZP measurement provides information regarding the surface charge of *R. opacus* in solution at different pH values. The results before and after bioadsorption are shown in Figure 2. Increases in the negativity of the surface charge of the evaluated samples are observed with the increase in pH (pH > 3).

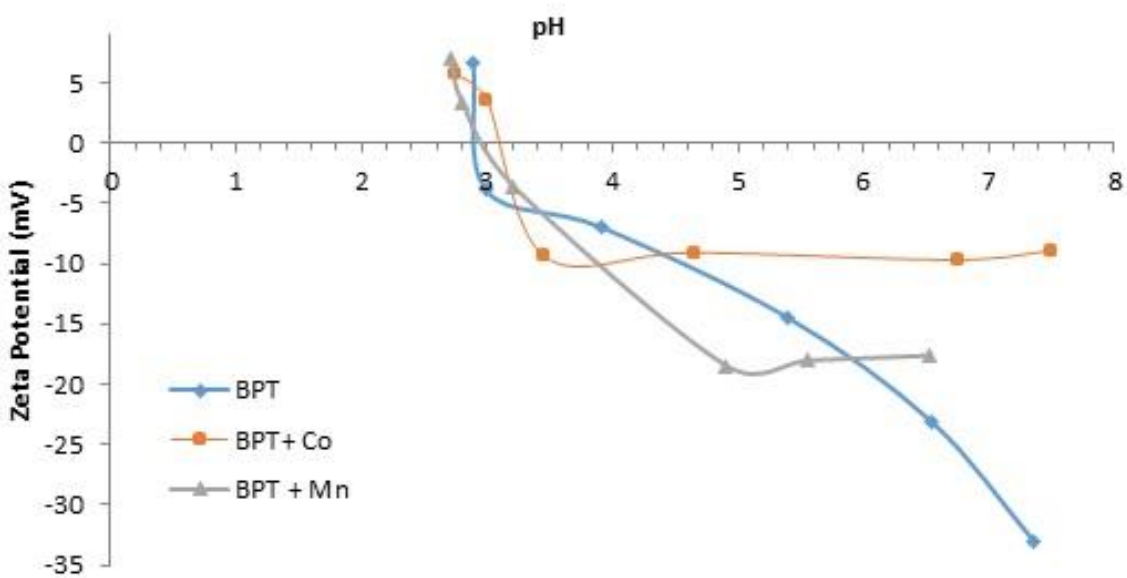


**Figure 2**. Variation of the Zeta Potential of the pre-treated bacterium before and after contact with metals (BPT, BPT + Co and BPT + Mn); concentration of NaCl 0.001M and 0.2 g/l of bioadsorbent.

## MEB-EDS analysis

Figure 3 (a-c) show the micrographs (SEM) as well as the micro-composition analysis of the elements of the bacterium in the form of EDS spectrum, before and after contact with Co(II) and Mn(II). The micrographs do not indicate alterations in the morphology of the bacterial cells, while the EDS spectra confirm the bioadsorption of the metals evaluated (Figure 3b and 3c).

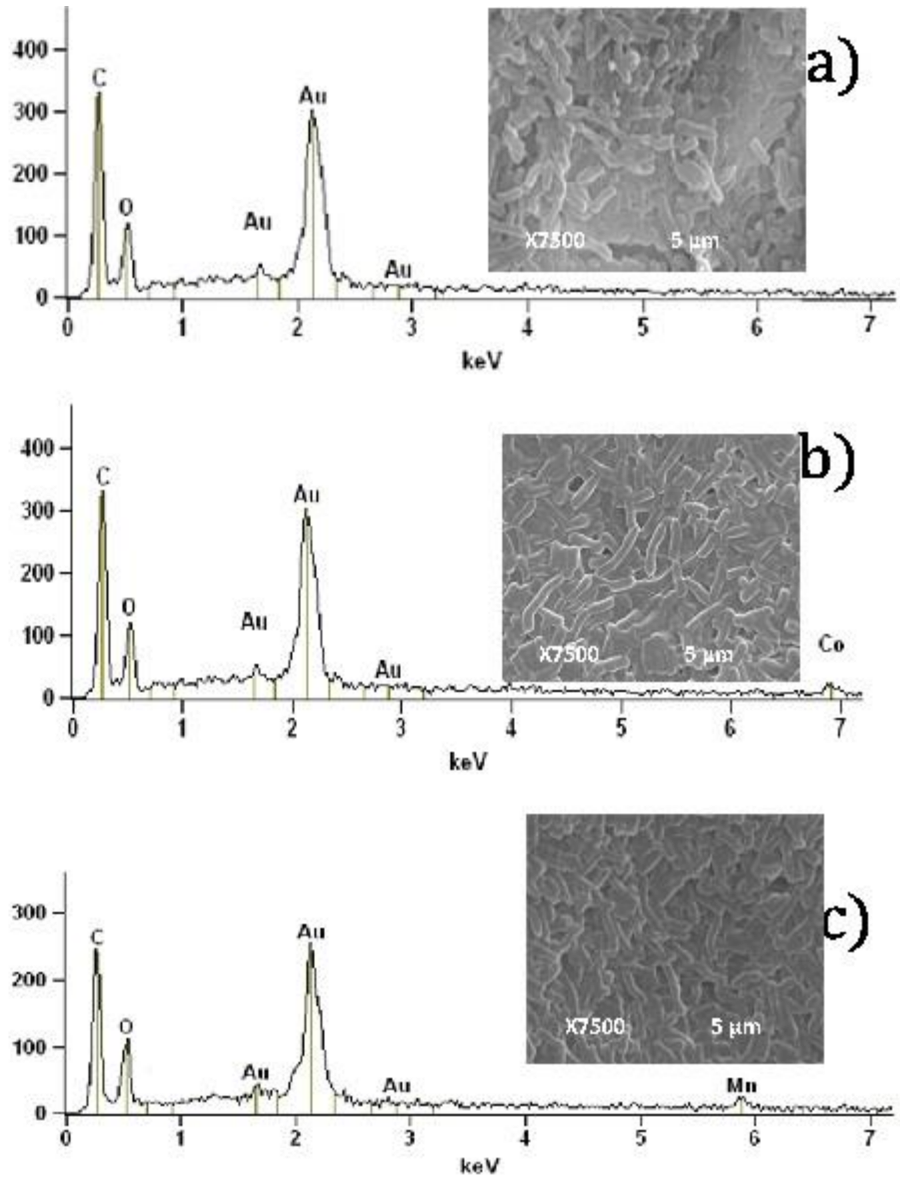


**Figure 3**. MEB-EDS analysis (Spectra) of *R. opacus* before and after bioadsorption a) Pre-treated biomass (BPT: Spanish abbreviation) b)

BPT after contact with Cobalt (BPT + Co) c) BPT after contact with manganese, (BPT + Mn).

## Characterization with FTIR

Biomass was analyzed without pretreatment (BSPT), with pretreatment (BPT), and after contact with metals (BPT + Co and BPT + Mn). The results are presented in Figure 4. In this Figure, differences are observed in the intensities of the absorption bands between BSPT, BPT, BPT+ Mn and BPT + Co; indicating changes in the chemical composition of the inactive cells by the pre-treatment and by the bioadsorption. The greatest differences in band intensity are observed in BPT-Co.

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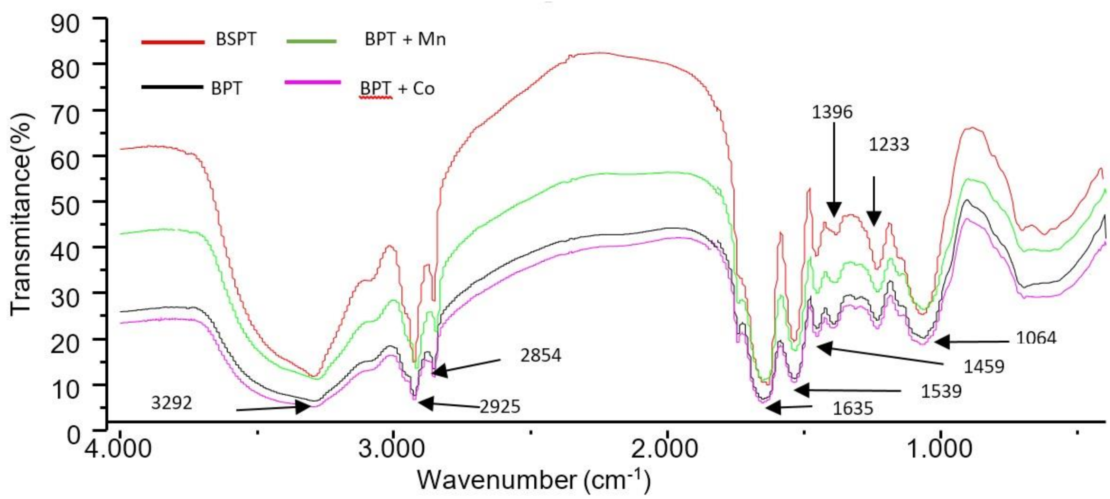


**Figure 4**. FTIR analysis of the bioadsorbent in the control stages and after contact with Mn(II) and Co(II).

# Discussion

## pH influence

The pH has a strong influence on the chemical characteristics of metal-containing solutions. In addition, it affects the activity of functional groups present in the cell wall, as well as the competition of metal ions for the active sites present in the bioadsorbent (Zhenggang *et al*., 2018). The lower Co(II) removals observed at low pH values can be attributed to the competition of $H^+$ and metal ions for the active sites of the bacterium.

On the other hand, the high removals obtained at pH ≥ 8 are attributed to the formation of Co(II) complexes and their subsequent precipitation. Considering these values would induce precision errors in adsorption removal calculations (Yüzer, Kara, Sabah, & Çelik, 2008; Negm, El-Sheikh, El-Farargy, Hefni, & Bekhit, 2015; Djoudi, Le-Page-Mostefa, & Muhr, 2019). The strongest Co(II) ion bonds are made at pH values close to neutrality; this is due to the affinity to carboxylic groups (Mack, Wilhelmi, Duncan, & Burgess 2007) among others. Maximum Co(II) removals at pH 7 have been reported by Tajer-Mohammad-Ghazvini *et al*. (2016), who reached a removal value of 88.55 %, with *Alphaproteobacterium*, MTB-KTN90, (Gram negative bacterium grown in modified liquid medium).

Likewise, Dharanguttikar (2018) reported a maximum removal of 43.5 % using *Pseudomonas aeruginosa* (Gram-negative bacterium) at this same pH value. However, Özdemir, Kılınç, Poli and Nicolaus (2013) observed maximum Co(II) removal values of 43.6 and 35.1 % with

*Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus* (Gram-positive bacteria) at pH 5.

The low Mn(II) removal and adsorption values, despite the high electronegativity of BPT+Mn(II) reported in Figure 2, may indicate that electrostatic attraction is not the main mechanism in the adsorption of this metal. In addition, the high solubility of Mn(II), could cause desorption of ions previously attached to the cell wall (Silva, Rodríguez, Gómez, & Cantero, 2009). A 41.1 % removal of Mn(II) at pH 6 values was reported by Huang *et al*. (2018).

The differences reported in the bibliography with respect to pH value and maximum removal of the metals evaluated are due to different cell wall compositions, culture medium, bioadsorbent pre-treatment and bacterium-metal affinity.

## Influence of biomass concentration

The increase of the inactive microbial dose in the system generates an increase of active sites, available to adsorb a greater amount of the dissolved metal, increasing the percentage of removal. Likewise, this increase in mass reduces the adsorption of the metal, which is an intensive property (mg of metal adsorbed/gram of biomass). Table 2 confirms an opposite behavior between removal and adsorption for both metals, expressed in the β factor, thus the positive sign of β for removal contrasts with the negative sign of the adsorption factor, confirming its effect ($p < 0.05$).

Previous studies report the existence of a margin where excess bacterial cells can form aggregates inhibiting the effective adsorption of the metal on the cell wall (Colagar, Azizi, & Hafeziyan, 2011; Esmaeili & Beni, 2015). This explains the change in slope observed in Figure 1b, for the removal of Co(II) and Mn(II).

Maximum Co(II) adsorption values were obtained with 80 g/l biomass of *Oscillatoria* sp. (Colagar *et al*., 2011). Likewise, Özdemir *et al*. (2013) reported removals of 43.6 % Co(II) and 65.16% Mn(II) with *Geobacillus thermantarcticus*. The same authors reached removal values 35.1 % of Co(II) and 36.6 % of Mn(II) with 50 mg of dry biomass of *Anoxybacillus amylolyticus*.

In this study, values of 4 and 3 mg/l for Co(II) and Mn(II) were established as the best biomass concentration for Co(II) and Mn(II), respectively, for presenting the best removal and adsorption ratio for these metals.

## Influence of the initial metal concentration ($C_i$)

The decrease in removal for both metals, with the increase in $C_i$ can be explained by the scarcity of free active sites in the adsorbent; while the increase in adsorption would be due to the interparticle diffusion of the metal ion into the bioadsorbent and/or the binding of the ions to the biopolymers released by cell damage (Mathivanan, Rajaram, & Annadurai, 2018).

Considering that the highest efficiencies are obtained at low metal concentrations, bioadsorption could be applied in the final stages of effluent treatment containing Co(II) or Mn(II). In this study, Ci values of 50 and 5 mg/l were set for Co(II) and Mn(II), with the best removal and adsorption ratio of these metals for further experiments.

## Influence of contact time

More than one stage in ion transfer to the binding sites has been reported (Özdemir *et al*., 2013). In Figure 1d, two phases in the removal of Co(II) are observed. The initial phase, where rapid adsorption occurs, involves mechanisms of a physical adsorption or ion exchange, whereas the latter phase of slow adsorption could involve complex formation, micro precipitation or saturation of active sites (Esmaeili & Beni 2015).

Díaz-Alarcón, Alfonso-Pérez, Vergara-Gómez, Díaz-Lagos and Martínez-Ovalle (2019) achieved a maximum Mn(II) removal of 15.26 % by applying a magetotactic bacterium with contact times between 3 and 5 min. While Özdemir *et al*. (2013) reported a longer time of 120 min to achieve maximum adsorption of Co(II) and Mn(II) with the inactive bacterium *Anoxybacillus amylolyticus.*

According to the results, the best Co(II) and Mn(II) removal and adsorption values were achieved at 180 and 10 minutes of contact time.

## Statistical analysis

The values presented in Table 2 allow us to identify the statistical significance of the effect of the evaluated parameters (input parameters, E) on removal and adsorption (output parameters, S), and also indicate whether this effect is positive (β) or negative (-β).

The pH has a positive effect (β positive), but not significant ($p>0.05$), on the removal and adsorption of both metals. This positive effect is also evident in Figure 1a for Co(II) and Mn(II) (up to pH 5). Likewise, the concentration of R. *opacus* has a positive and significant effect on the percentage of removal for both metals, but a negative and significant effect on Co(II) adsorption.

The effects of the bacterium concentration on both parameters are confirmed in Figure 1b. Regarding Ci, a negative effect on removal and a positive effect on adsorption is observed. However, the statistical significance of the effect is only for Mn(II) removal. The negative effect of Ci on the removal of both metals is confirmed in Figure 1c. Bioadsorbent-metal contact time significantly affects adsorption but not removal. The non-significant effect of contact time on removal is also evidenced in Figure 1d.

Few studies report a statistical analysis of heavy metal adsorption by applying inactive microorganisms. However, they highlight what was reported by Yuan *et al*. (2019), who evaluated the effect of contact time, pH and initial metal concentration on Cd adsorption; with *Shewanella putrefaciens*, all the evaluated parameters presented statistical significance in the removal of this metal. Likewise, Hu *et al*. (2020) evaluated the effects of contact time, pH and temperature of Pb adsorption with *Rhodococcus sp*., it was determined that only the adsorbent concentration is the statistically significant parameter in the process.

These differences observed in the different studies are related to the different characteristics of the microorganism and affinity of the metal evaluated.

## Characterization of *R. opacus*

The variation of the ZP of the evaluated samples, presented in Figure 2, is consistent if we assume an electrostatic attraction between the metal cations and the bacterial surface. In this Figure, it is observed that the bioadsorption of Co(II) and Mn(II) alters the behavior of the ZP curve of the BPT. Likewise, a slight shift of the isoelectric point (IEP) is noticeable with an initial pH of 2.95 towards pH values higher than 3.15 for Co(II) bioadsorption and higher than 3.0 for Mn(II).

According to the literature, adsorption affects both the functional groups bound to the metal and the electrical charge of the bioadsorbent, while the increase in pH is related to the increase in the negative charge of the microbial surface (Prithviraj *et al*., 2014).

The reduction of the negative superficial charge, mainly after Co(II) bioadsorption and slightly in case of Mn(II) would indicate a stronger interaction of Co(II) ions with the bacterium. Moreover, as observed, the cations present in the solution do not totally cancel the bacterial charge, i.e., not all the active sites of the bacterium were occupied.

After the bioadsorption of Co(II) and Mn(II), almost constant ZP values (~ -9.5 mV) were produced at pH values higher than 3.5 for BPT + Co, while at values higher than pH 4.9 it generated values of charge close to -18.0 mV, for BPT + Mn. This behavior suggests that electrostatic attraction and chemical adhesion are the main mechanisms of the bioadsorption process.

The predominant negative charge on the bacterial surface is attributed to the presence of functional groups, their deprotonation, the increase in pH and the pre-treatment of the bacterium. In the latter case, it was verified that BPT improves the bioadsorption of heavy metals, due to the elimination of lipids and proteins that obstruct the active sites of the bacterial wall (Abdoun-Ouallouche, Djefal-Kerrar, Amrani, & Zerrouki, 2014).

According to the results of MEB-EDS analysis, in Figure 3 (a-c), the pre-treatment performed on the bacterium, as well as the subsequent bioadsorption do not alter the morphology of the bacterium. The C and O present in the EDS are representative elements of bacterial cells (Rahman, Thomas, & Singh, 2019). These elements are part of the proteins and carbohydrates present in *R. opacus.* Whereas the presence of gold detected is peculiar to the method applied in the sample preparation for SEM/EDS analysis as indicated by Rahman, Thomas and Singh (2019). Figures 3b and 3c confirm the adsorption of Co(II) and Mn(II) on the bacterial surface.

Regarding FTIR characterization, Figure 4 shows the spectra of the microorganism before and after bioadsorption. In the case of BPT+Co, no alterations in the absorption peak distributions were observed indicating that the bacterium-metal bonds are not covalent in nature (Ferraz, 2007). Regarding BPT+Mn, the intensities were slightly higher than BPT, showing no alterations in the absorption bands, confirming the interaction of functional groups with metal cations (Dmytryk, Saeid, & Chojnacka, 2014).

The presence of absorption bands in the region close to 3 292 $cm^{-1}$ is characteristic of the hydroxyl group (-OH). Likewise, the region close to 2 925 and 2 854 $cm^{-1}$ shows the presence of C-H groups, especially alkyl (2 925 $cm^{-1}$) and aldehyde (2 854 $cm^{-1}$) groups that result from proteins or carbohydrates from cell walls (Li, Yang, Zhao, & Xu, 2007). Amides I and II (e.g., O = CN-H) are detected in the regions close to 1635 and 1 539 $cm^{-1}$, respectively. The 1 459 band is related to C-N groups, and the peaks close to 1 064 $cm^{-1}$ may correspond to C-O bonds (primary alcohols) as well as C-N bonds (amines). Likewise, carboxylic groups are detected near 1 396 $cm^{-1}$ and phosphate groups near 1 233 $cm^{-1}$, as sugars (Huang *et al*., 2018).

The bioadsorbent samples after pre-treatment show more defined absorption bands, characteristic of carboxylic groups (1 396 $cm^{-1}$). This variation is due to the incorporation of OH- in the bioadsorbent and/or the emergence of functional groups that potentiate the adsorption of the bacterium.

## Conclusions

The application of the inactive bacterium *R. opacus* is an efficient adsorbent material for the removal of Co(II) and Mn(II) metals. The increase in pH and concentration of the bacterium are related to increases in the removal percentages of both metals. Whereas increases in the initial metal concentration have an opposite effect with respect to removal. Regarding adsorption, the parameters that have a statistically significant effect are the contact time, for both metals, and *R. opacus* concentration for Co(II).

The adsorption of metal ions on the bacterial surface was characterized and confirmed by MEB-EDS, ZP and FTIR analysis.

Kinetic and thermodynamic studies are necessary to gain a deeper understanding of the mechanisms involved in bioadsorption.

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