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In Vitro Effect of Molasses Concentration, pH, and Time on Chromium Removal by *Trichoderma* spp. from the Effluents of a Peruvian Tannery

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Abstract: The effluents generated by the tannery industry have a high content of chromium and other toxic elements, representing a potential threat to ecosystems. An eco-friendly alternative to treat these effluents is the use of microorganisms, such as fungi, with the capacity to biosorb heavy metals. The present work aims to determine the effect of the molasses concentration, pH variation, and time on the removal of total chromium using the filamentous fungus *Trichoderma* spp. An experimental design was adopted using pH (4 and 6), concentrations of molasses (0.5 and 1%), and time (8 and 12 days) as independent variables. The *Trichoderma* inoculum was constant in all the treatments. The different treatments were evaluated after 0, 8, and 12 days by taking 50 mL of sample from each bioreactor. The chromium concentration was subsequently determined in each sample. The results show that treatment 3 (1% molasses and pH 4) showed higher chromium removal after both 8 and 12 days. The concentrations of total chromium decreased from 665 mg/mL to values of 568 mg/mL by day 8 and 486 mg/mL by day 12. These values are, however, still above the maximum threshold imposed by Peruvian law regarding the discharge of non-domestic effluents into the sewage system. The results show that *Trichoderma* spp. can increasingly remove chromium from the effluent with longer incubation periods. However, future studies are necessary to determine the mechanisms of chromium biosorption by the fungus and the influence of other physicochemical parameters.

Keywords: *Trichoderma*; pH; molasses; chromium removal; tannery effluents

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1. Introduction

Heavy metals are harmful environmental pollutants that cause negative effects on living organisms, even in minute concentrations. In addition, these can cause pollution in aquatic and terrestrial environments, leading to public health problems [1,2]. Within heavy metals, chromium (Cr) is a contaminant with carcinogenic and cytotoxic effects that can reach the environment through the discharge of untreated industrial or agricultural effluents [3,4]. Chromium has two oxidation states: trivalent (Cr-III) and hexavalent (Cr-VI) chromium. Cr-VI is the most toxic due to its solubility in water and high reactivity, particularly at acidic pH, and, thus, it can enter cells. It has been reported that Cr-VI can affect DNA causing various types of cancer. On the other hand, Cr (III) is less toxic to the environment [2]. It should be said that socio-economic development, especially tanning organizations, has brought several inconveniences of an environmental nature, such as air, water, and soil pollution. Based on a sustainability criterion, the tannery sectors

have been determined to be highly polluting, where their different production processes produce chemical compounds that contain heavy metals and organic waste and, therefore, cause toxicity in living organisms and ecosystems [5]. For example, different Cr species, in particular Cr (VI), result in severe toxicity in plants through the formation of reactive oxygen species (ROS) above the basal level, which affects the development of the plant, including its death [6].

The leather industry is an ancient industrial sector that produces a wide range of commodities, such as leather footwear, leather bags, leather clothes, and other products [7,8]. This sector contributes to the economy of some nations; however, its process generates large volumes of wastewater that pollute the environment. The waste generated by the leather industry can be categorized into two distinct kinds: solid waste and wastewater composed of volatile organic compounds (VOCs) and harmful chemicals [9–11]. The most important element in tanning is chrome, the use of which has become essential in the tanning industry owing to the high leather quality standards to be achieved. However, when wastewater with chromium content is discharged into the environment, it has consequences and is highly dangerous to living beings. The tannery effluents are complex mixtures, are highly toxic, contain significant amounts of chromium (Cr-III), acid, and alkaline liquors, are intensely colored, and have a bad odor [10]. Due to the high pollutant load, tannery effluents are far below the desired level of acceptability [12].

Chromium VI is used in leather tanning [13–15], while Cr III reacts to form insoluble compounds considered non-toxic [11]. Between 80 and 90% of the hides produced in the world are leather-tanned (chrome tanning), and for every 1000 kg of tanned hides, 0.5 kg of chrome can remain in the tanning solutions [10]. On the other hand, wastewater from tanneries is of environmental concern because in some countries it is used as irrigation water for crops [16]. During the tanning process, approximately 40% of the chrome remains in the liquid phase and ends up in wastewater, eventually becoming a potential source of pollution for the environment [17]. Various physicochemical and biological methods have been tested to assess the amount of chromium in tannery effluents, for example, adsorption, chemical precipitation, coagulation and flocculation, electrochemical treatment, electrocoagulation, electro-oxidation, electro-flotation, ion exchange, membrane filtration, electrodialysis, bioremediation, and phytoremediation [10,11]. Some physicochemical methods can additionally be used to examine sludge formation, and others are more expensive [18].

Microorganisms play an important role in the bioremediation of polluted soils, water, and effluents. Bacteria, fungi, and microalgae can adapt and grow in adverse environments, thus becoming excellent chromium biosorption agents. Biosorption is a metabolic process carried out by active or inactive microorganisms and is passive, fast, reversible, and independent. It presents advantages over conventional methods, such as low cost, high removal capacity and efficiency, reduced generation of chemicals and biological waste, and minimal nutritional requirements. Phosphoryl, carbonyl, sulfhydryl, and hydroxyl groups have a role in microorganism biosorption [19].

The microbial remediation of Cr (VI) from the environment is one of the most viable and sustainable methods for reducing excess Cr (VI) levels in the environment [12]. Fungi are good candidates for heavy metal bioremoval. In addition to extracellular enzyme production, fungal biomass has been identified as the most effective biosorbent for the removal of toxic metals, such as chromium, copper, mercury, nickel, cadmium, and lead, from wastewater due to the presence of functional groups in their cell walls, giving them excellent biosorbent capacity [18]. Fungi cell walls, particularly filamentous ones, are composed of polysaccharides, such as glucan, chitin, chitosan, glycoproteins, lipids, melanins, D-galactosamine polymers, and polyuronides, and are considered places with an elevated number of metal binding sites [19]. Some fungal species have been tested for chromium removal, such as *Cladosporeum perangustum*, *Penicillium commune*, *Paecilomyces lilacinus*, *Fusarium equiseti* [20], *Penicillium citrinum* and *Trichoderma viride* [18], and *Fusarium chlamydosporium* SPFS2-g [21].

Trichoderma spp. are microorganisms that belong to the fungi kingdom, that is, they are fungi used in various fields. For example, they are used as bioinsecticides in soil bioremediation, but different studies have also been carried out with these microorganisms to treat tannery chrome [22]. Filamentous fungi of the genus *Trichoderma* are capable of colonizing different environments. *Trichoderma* contributes to ecology by decomposing plant waste, biodegrading synthetic compounds, and bioaccumulating large quantities of different metals from wastewater and soil [23]. These fungi have physiological characteristics that make them candidates for use in different biotechnological fields [24]. Some species have shown the potential to remove heavy metals [25,26]. Different studies show that *Trichoderma* can tolerate and reduce chromium concentrations by biosorption mechanisms [27–29]. *Trichoderma* spp. are versatile fungi that can survive in adverse environments, which makes them one of the most important biosorbents examined for Cr (VI) biosorption since they are able to tolerate Cr (VI) values above 10,000 mg/L [19].

In this sense, the aim of the investigation was to determine the effect of time, molasses concentration at 0.5 and 1%, and pH (4 and 6) on chromium removal by the filamentous fungus *Trichoderma* spp. to improve the chromium removal process through the use of this fungus in such a way that the chromium concentration is reduced to acceptable levels, thus reducing its impact on the environment when it is released into wastewater from the tanneries. The importance of this study contributes to the right of society, which is to live in a quality environment. So, the researchers investigated the filamentous fungus *Trichoderma* spp. as a potential agent of chromium biosorption in an effluent sample from a tannery. This could reduce the purchase of chemicals used to treat chromium in the tannery industry.

2. Materials and Methods

2.1. Research Design

The research design was trifactorial, having time, molasses concentration, and pH as independent variables. This type of design allows for the study of the effects of independent variables on a given response (chromium total removal). Eight treatments (T1–8) were carried out among the three independent variables (Table 1). Three replicates were made for each treatment.

Table 1. Experimental design of the independent variables.

Treatments	Independent Variables *			
	pH	Molasses Concentration	Time (Days)	Combinations
1	P1	M1	T1	P1M1 T1
2	P1	M1	T2	P1M1 T2
3	P1	M2	T1	P1M2 T1
4	P1	M2	T2	P1M2 T2
5	P2	M1	T1	P2M1 T1
6	P2	M1	T2	P2M1 T2
7	P2	M2	T1	P2M2 T1
8	P2	M2	T2	P2M2 T2
Total combinations	2(P)	2(M)	2(T)	2X2X2

* P: pH level (P1:6, P2:4); M: Molasses concentration (MA:1%; M2:0.5%); T: time (T1:12; T2:8).

2.2. Samples

The collected sample was from the Ecological Tannery of the North E.I.R.L in Trujillo. The sampling was random, and 20 L of tannery effluents was obtained. This sample was analyzed at the Institute of Scientific and Technological Research of Cesar Vallejo

University (Trujillo, Peru). The sample was vacuum filtered to remove organic matter or suspended solids.

2.3. Trichoderma Culture and Preparation of Inoculum

The strain of *Trichoderma* spp. used is part of the collection of strains of the Institute for Research of Science and Technology—UCV Trujillo. This fungus has the characteristics of tolerating the presence of metals during its growth in solid and liquid culture media. The strain of *Trichoderma* spp. was reactivated in Sabouraud agar and incubated at 25 °C for 5 days. After a time, the macroscopic cultural characteristics of the colony were verified (presence of concentric rings and green coloration). In addition, a microculture was carried out in blocks of Sabouraud agar to observe the microscopic characteristics by lactophenol blue. The shape of the conidia and pyramidal-shaped phialides was observed by a microscope at 40×.

To prepare the inoculum, the *Trichoderma* fungus was seeded on five Sabouraud agar plates using the puncture technique and immediately incubated at 25 °C for 7 days until the presence of green spores was observed on the surface of the colony. Once the incubation was finished, the spores were harvested by adding 3 mL of sterile 0.8% physiological saline solution (SSFE) on the surface of the colonies, and with the help of a Drigalski spatula, the spores were detached, and a sterile pipette was used to collect and transfer them to a tube with 10 mL of 0.8% SSFE. Finally, the initial concentration of spores (spores/mL) present in the tubes with 0.8% SSFE was determined by counting in a Neubauer chamber. The standardization of the spores was carried out by adding 5 mL of the initial spore solution to a flask containing 45 mL of 0.8% SSFE; the obtained final concentration of the inoculum was 5×10^5 spores/mL.

2.4. Obtaining Molasses

The molasses was obtained from the Laredo Agro-Industrial Company at a concentration of 80%. The company provided its physicochemical characteristics, which are detailed in Table 2.

Table 2. Physicochemical characteristics of molasses.

Parameter	Value
Degree °Brix	88.40
Specific weight (g/cm)	1.47
Sucrose %	36.41
Reducing sugars %	9.09
Total sugars %	45.50
Do not sugar %	42.90
Water %	11.60

2.5. Treatments

Five 1-L Erlenmeyer flasks were used, and the air was pumped into them using an air pump (T0). A total of 500 mL of tannery effluent was used. One of the 1-L flasks was used as the control group. Each flask received a 10% *Trihocherma* inoculum (5×10^5 spores/mL) and one of two molasses concentrations (0.5 and 1%). Two of the four flasks had a pH of 4, while the other two had a pH of 6. They were put into action right away and incubated at room temperature. The total chromium levels were determined on days 8 and 12, as shown in Table 3.

Table 3. Conformation of the treatments.

Treatments	Effluent 20% (mL)	Molasses (%) [a]	*Trichoderma* Inoculum (%) [b]	pH	Evaluation Time
T0	500	---	---	---	---
T1	500	1.0	10	6	8
					12
T2	500	0.5	10	6	8
					12
T3	500	1.0	10	4	8
					12
T4	500	0.5	10	4	8
					12

[a] The concentration of molasses 1%: 5 g; 0.5%: 2.5 g. [b] The concentration of spores was 5×10^5 spores/mL.

2.6. Total Chromium and Chromium Removal

The amount of total chromium was measured by the colorimetric method (3500-Cr B) according to the Standard Methods for the Examination of Water and Wastewater [30]. The determination of the total chromium is based on the reaction of Cr (VI) with 1,5-diphenycarbazide in an acid medium to form an intense colored red-violet complex. The absorbance is measured at 546 nm. Prior to the total chromium analysis, the sample is digested using a persulfate and an acid solution. For this, the solutions were filtered with Whatman N°1 filter paper (150 mm Ø) to separate the hyphae and spores. To report the values of the amount of total chromium removal, the following formula is needed:

$$\text{Total chromium removal (\%)} = C_0 - C_1/C_0 \times 100 \quad (1)$$

where:

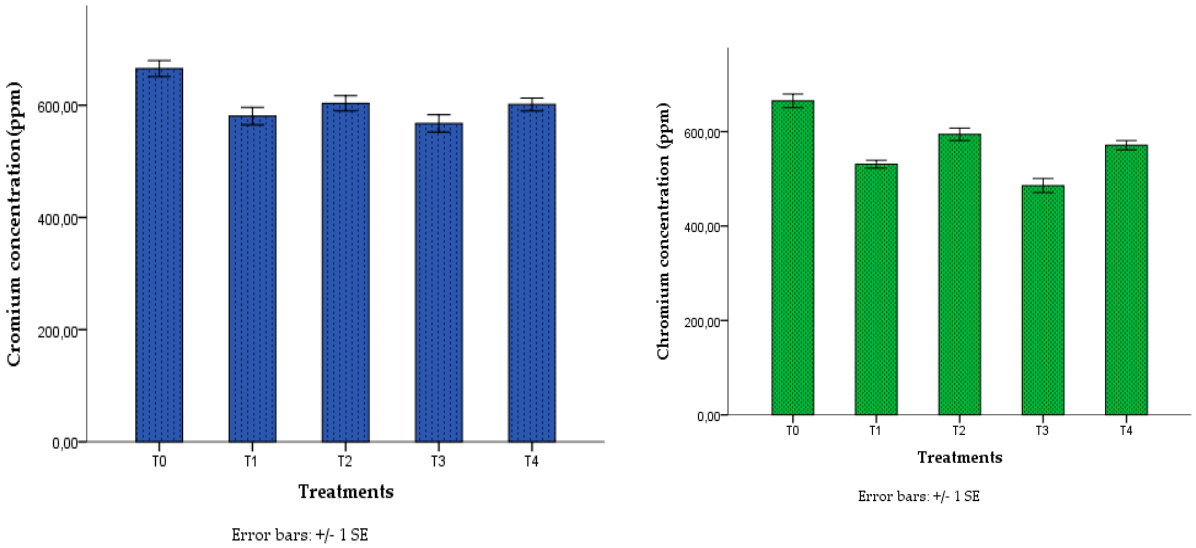
- C_0: Initial concentration (mg/mL)
- C_1: Final concentration (mg/mL)

2.7. Analysis of Data

Three repetitions were conducted when measuring the total chromium concentration on days 8 and 12. The statistical program IBM SPSS v25 was used for the data analysis. The data used were the means of the values. A one-factor ANOVA was used for the mean analysis. Likewise, to compare the different treatments with the control group, Tukey's test was used. To generate bar graphs with error bars +/− 1 SE, the IBM SPSS program was used. Law No. 30224 was considered, which is a law that allows the National Quality System and the National Quality Institute to carry out experimentation and analyses, which are subject to INACAL, which, in turn, is in charge of the Peruvian Metrological Standards. At the same time, data collection instruments (technical sheets) were used, which were validated by specialists in the field in the same way as the equipment and materials.

3. Results

Figure 1 shows the mean values of the total chromium in the tannery effluents after treatment with two molasses concentrations (1 and 0.5%) and two pH variations (4 and 6 pH), compared to the control group (treatment 0, T0).



(**a**) (**b**)

Figure 1. Graphs of the final total chromium concentration in Peruvian tannery effluent samples measured at eight days (**a**) and twelve days (**b**), previously treated with different molasses concentrations (0.5 and 1%) and the *Trichoderma* fungus and two pH variations (4 and 6).

The values in Figure 1a show the mean values of the total chromium measured on the eighth day of treatment. Only treatments 1 (1% molasses, 10% *Trichoderma* inoculum, and pH 6) and 3 (1% molasses, 10% *Trichoderma* inoculum, and pH 4) showed statistically different mean values concerning the control ($p \leq 0.05$), and they were the lowest values reached on the eighth day of measurement. Tukey's statistical test showed two subgroups, where the mean values of Treatment 2 (603 mg/mL), Treatment 4 (601 mg/mL), and the control (665 mg/mL) were statistically equal ($p = 0.059$), and the mean values of Treatment 1 (581 mg/mL), Treatment 2 (603 mg/mL), Treatment 3 (568 mg/mL), and Treatment 4 (601 mg/mL) ($p = 0.438$) were statistically equal.

Figure 1b shows the mean total chromium on day twelve of measurement. The lowest values of total chromium achieved for each treatment were the following: treatment 3 (486 mg/mL), Treatment 1 (531 mg/mL), Treatment 4 (571 mg/mL), and Treatment 2 (594 mg/mL). There was a significant difference in all the values of the means of the total chromium in all the treatments concerning the control (665 mg/mL) on day 12 ($p > 0.05$). On the other hand, Tukey's statistical test showed four subsets: subset 1 (Treatments 1 and 3; $p = 0.147$), subset 2 (Treatments 1 and 4; $p = 0.220$), subset 3 (Treatments 2 and 4: $p = 0.691$) and subset 4 (control group, T0).

As shown in Figure 2, the percentages of the total chromium removal in the tannery effluents reached on day 8 (blue bars) were 14.70% (T3), 12.74% (T1), 9.60% (T4), and 9.32% (T2). Treatment 3 (1% molasses, 10% *Trichoderma* inoculum, and pH 4.0) was the one that was most effective at removing the total chromium. All the removal percentages were statistically different from the control ($p \leq 0.05$); however, only Treatments 2 and 4 were statistically equal ($p = 0.976$). In the same way, on day twelve of the evaluation (green bars), the highest value of total chromium removal was 26.97%, corresponding to Treatment 3. Likewise, all the treatments were statistically different concerning the control ($p \leq 0.05$). Tukey's test showed four subsets of treatment with statistical differences between them ($p = 1.000$). Treatments 1, 4, and 2 achieved removal values of 18.85%, 14.11%, and 10.67%, respectively.

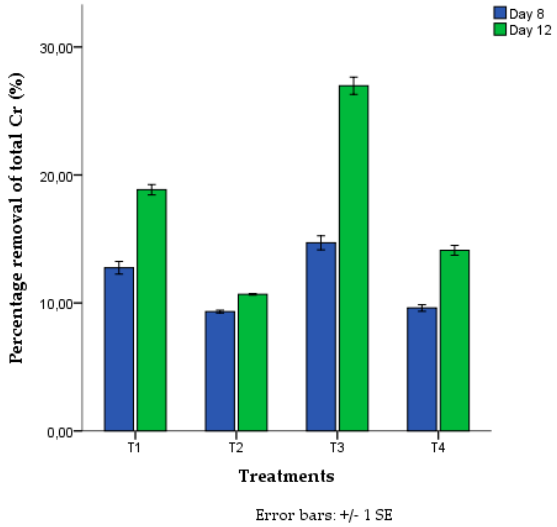


Figure 2. Total chromium percentage in Peruvian tannery effluent samples previously treated with different molasses concentrations (0.5 and 1%), *Trichoderma*, and the variation of two pH values (4 and 6).

4. Discussion

The maximum value of total chromium found in the Peruvian tannery effluents was 691 mg/mL, which decreased to 486 mg/mL 12 days after being treated with the *Trichoderma* fungus plus 10% molasses at pH 4. However, the Regulation of Maximum Admissible Values (VMA)—No. 010-2019—VIVIENDA of Peru establishes that tannery effluents must have 10 mg/mL of total chromium [31]. Likewise, the results show that there was no prior treatment to lower the levels of chromium in these effluents, which becomes a risk to the environment because sometimes this effluent ends up contaminating nearby crops in the areas where it flows. However, the problem with heavy metals, such as chromium, is their bioaccumulation potential and the associated chronic toxicity since heavy metals are known to accumulate within biological systems [32].

The *Trichoderma* treatments may have influenced the reduction of total chromium due to the fungi's ability to develop in media with recalcitrant compounds, showing biosorbent capabilities [33–35]. The fact that *Trichoderma* grows in a chromium-contaminated environment could be due to several factors, such as its ability to produce extracellular enzymes, its ability to take up intracellular organic matter, and a higher intracellular Cr discharge rate from the cell, which could prevent Cr (VI) from getting into the cells of the fungus, thus avoiding damage by chromium [36]. On the other hand, it is known that anionic chromate ions bind to positively charged groups on the fungal biomass [19]. In one study, FTIR analysis suggested that chromium hexavalent binds to *Trichoderma* surface binding sites where carboxyl and amine groups are present [37].

Molasses, used at two concentrations (0.5% and 1%), was used as a substrate for the fungus, as it can be used as a source of carbon and nitrogen for the growth of *Trichoderma* [38]. Treatment 3 produced lower total chromium values at both eight and twelve days of evaluation. In both cases, the concentration of molasses was 1% (Figure 1). Subsequently, with this same treatment (T3), it was possible to observe a decrease in the amount of total chromium by *Trichoderma* at pH 4, which may influence the growth rate of *Trichoderma* since it has better development in acidic conditions with a pH range of 4 to 6 [39,40]. On the other hand, Aquise and Kent (2019) showed that under optimal pH conditions (4.5 and 5.5), *Trichoderma* reduced hexavalent chromium by up to 47% [41]. However, the final total chromium values on days 8 and 12 in Treatments 3 and 1, with pH values of 4 and 6, respectively, showed some statistical similarities. In another study, better sorption of hexavalent chromium occurred at a pH of 5.5–5.8 [42]. These studies, including the current data, indicate that total chromium removal is more effective at an acidic pH. Thus,

a decreased pH promotes the protonation of the bond sites on the fungal surface, meaning a positive charge on the microbe surface, which contributes to the metal bond [32,43]. In another similar study, chromium removal by *Trichoderma viride* was tested at pH 1 and 2, suggesting a mechanism for the bioremoval of hexavalent chromium. This mechanism consists of protonation (H^+) on the surface of *T. viride* due to acidic pH, which leads to a significantly strong electrostatic charge between the positively loaded surface of the biomass and the negatively loaded chromate ions [44].

The values of the means of the percentage of total chromium removal (Figure 2) presented significant differences ($p \leq 0.05$) with respect to the values of the control group on day 12 of measurement. However, these percentages are low compared with other studies. Shukla and Vankar used a species of *Trichoderma* and managed to reduce 97.39% of the hexavalent chromium in a chromium solution [42]. Narolkar and Mishra observed that other *Aspergillus* species had more chromium VI sorbet power than *Trichoderma* [34]. In addition, the heavy metals and total carbon decreased with the contact time. However, this would not have an effect if the fungus reached its saturation with the metal. One study showed that *Trichoderma harzianum* might remove up to 90.2% of chromium VI in seven days of incubation at a pH range of 4–5 and 30 °C. In addition, concentrations of 40 mg/L and 30 mg/L could inhibit the mycelium growth [38]. This result indicates that high chromium concentrations could exceed the chromium tolerance level of *Trichoderma*. Therefore, the amount of total chromium in the effluent did not decrease because the concentrations were very high, exceeding the adsorption capacity of *Trichoderma*, which generated low values of removal percentages on day 8 (14.70%) and day 12 (26.97%). Both cases correspond to Treatment 3. In addition, the results suggested that the inoculum concentration should be increased because the fungus is considered to play an important role in heavy metal biosorption. For improved chromium biosorption, the concentration of fungal biosorbent should be increased. A rise in biosorbent concentration promotes heavy metal biosorption due to the increased surface area [33]. Hlihor et al. [44] reported that the Cr(VI) level decreased by increasing the biomass dosage from 3 to 10 g/L. On the other hand, by increasing the concentration of the biosorbent, the contact time is reduced, which can be from 20 to 4 days. This leads to proposing research regarding the form of presentation of the biosorbent since, in our study, a concentration of spores was used; however, fungal mycelium (biomass) could be used alive or dead.

There are still challenges, such as the influence of physicochemical factors that could optimize the biosorption of chromium by *Trichoderma*. Vendruscolo et al. [19] highlighted that in order to optimize the biosorption of hexavalent chromium, some of the independent variables must be modified, for example, the concentration of the inoculum of the pure or mixed culture, pH, temperature, or contact time. Another necessary factor to study is the effect of chromium removal using dead biomass, which was analyzed in another study and showed promising results [33]. Temperature is an important factor necessary for the biosorption of chromium because if the temperature increases, the biosorption of chromium also increases [44]. However, this was not taken into consideration, since room temperature was used throughout the process. Although total chromium removal was not considerable or close to permissible levels (10 mg/mL), possibly due to extrinsic and intrinsic aspects of *Trichoderma*, this study serves as a reference to address additional differences in the methods of chromium removal by the *Trichoderma* fungus.

5. Conclusions

Treatment 3 (1% molasses, 5×10^5 spores/mL, and pH of 4 and 6) was the one that presented the best results concerning the removal of total chromium in tannery effluents, both at days 8 and 12 of evaluation. The results showed a reduction in the values from a mean initial concentration of 665 mg/mL to 568 mg/mL (day 8) and 486 mg/mL (day 12), with total chromium removal percentages of 14.70% and 26.97% at pH 4 and 6, respectively. On the other hand, it is necessary to carry out future studies on the mechanisms of tolerance and biosorption of chromium by *Trichoderma* and the influence of the other parameters. For

future work, it is recommended to carry out investigations where the study time is longer in order to identify the desorption stage of microorganisms with chromium. In addition, other concentrations of molasses, different pH values, and other variables, such as temperature, inoculum, and agitation, were used to optimize the 100% chromium reduction process in the wastewater. The number of replicates per treatment was increased as a function of time to improve the accuracy and precision of the results. This will provide an optimal product for tanneries, greatly reducing the economic costs that are currently used for a more environmentally friendly product.

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