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## Hexavalent Chromium (VI) Removal by *Penicillium* sp. IA-01

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### Abstract

The objective of this work was to study the removal of chromium (VI) in aqueous solution by the fungus *Penicillium* sp. IA-01, isolated from polluted air with industrial vapors. To obtain the fungal biomass, pre-inoculums were performed in thioglycolate broth from a strain isolated from vapours contaminated with Cr (VI). The fungus was incubated for four weeks at ambient temperature, filtered, and washed three times with trideionized water. In preparing cellular fractions, it was necessary to break the fungal cells with glass beads using a homogenizer being careful to keep the samples in frosty cold ice. To obtain the fungal biomass, the fungus was filtered and stored in an oven at 80°C, allowing it to dry for 48 h. Removal of Cr (VI) in vitro was evaluated using different cellular fractions and dead fungal biomass. We determine the optimal characteristics for metal removal in the reaction mixture. Concluding that the ideal conditions for the removal of Cr (VI) in the cell extracts were 37°C and pH 7.0, also we observe that the highest enzyme activity was in the mixed membrane fraction. In dead fungal biomass, the ideal conditions for removal of metal are 60°C and pH 1.0.

**Keywords:** *Penicillium*, Fungal biomass, Cellular fractions, Chromium (VI), Biosorption, Removal

## 1. Introduction

Environmental pollution with heavy metals is caused by anthropogenic and natural actions. Discharges of wastewater from various industrial activities such as electroplating, mining, paint factory, plastics, coating metal cables, and automotive radiators, and certain industries producing energy, metal engineering industry and producers of welding materials contain high concentrations of metals. Several heavy metals are highly toxic and ingestion of these metals by drinking contaminated water or breathing polluted air can cause serious health problems in human beings. Several metals are considered toxic at certain levels of concentrations in wastewater, such as arsenic, cadmium, cobalt, copper, chromium, nickel, lead and mercury. Unlike the organic compounds, heavy metals cannot be biodegraded or destroyed, therefore they must be removed. There are several methods for removal of heavy metals: ion exchange, membrane separation, and separation and electrochemical adsorption on various adsorbents. [1].

Chromium (Cr) is one of the major environmental pollutants coming from industrial effluents and tannery. It is considered the major pollutant cataloged by the United States Environmental Protection Agency (EPA: [www.epa.gov](http://www.epa.gov)), since it is stable in aqueous solution and hence high in mobility in different environments. Chromium is a metal element in the periodic table. It is odorless and tasteless; is found in rocks, plants, soil, and volcanic dust, humans and animals; and exists in the environment most commonly as the trivalent [(chromium (III))], hexavalent [(chromium (VI))] and metallic [(chromium (0))]. Chromium (III) is generally contained in many vegetables, fruits, meats, grains and yeast. Industrial processes generally produce chromium (VI) and chromium (0). The main sources of chromium (VI) in drinking water are discharges from steel and pulp, and erosion of natural deposits of chromium (III). In many places, chromium compounds have been scattered to the environment through leaks, poor storage or improper disposal practices. The chromium compounds are very persistent in water and sediment [2].

Chromium is regarded as an environmental pollutant due to its wide use in various industrial activities, such as electrolytic plating, leather tanning, explosives manufacturing etc. The stable forms of chromium in the environment are trivalent (Cr (III)) and hexavalent chromium (Cr (VI)). Further, Cr (VI) is highly soluble, making it mobile in soil and aquatic environments, with consequent toxicity ecosystems. Chromium in their different forms can be use in the production of steel alloys and other metals chromed, for dyes and pigments, and the preservation of leather and wood. It can also be find naturally in the soil. The primary forms of chromium found in nature are chromium (III) and chromium (VI) and these forms are converted to each other depending on environmental conditions [2]. Cr (VI) is consider the most toxic form of chromium, and is usually associated with oxygen as chromates ( $\text{CrO}_4^{-2}$ ) and dichromates ( $\text{Cr}_2\text{O}_7^{-2}$ ) [3], which due to its high solubility are highly mobile in soil environments and water [4]. Moreover, Cr (III) is in the form of oxides, hydroxides or poorly soluble sulfates, by which it is much less mobile, and there joined organic matter in the soil and aquatic environments [5, 6]. Cr (VI) is a strong oxidizing agent, and in the presence of organic matter is reduced to Cr (III); this transformation is faster in acidic environments [3]. However, high

levels of Cr (VI) may exceed the reducing capacity of the environment and thus can persist as a contaminant. It has been established now that various chromium compounds as oxides, chromates and dichromates, are environmental contaminants in water, soil, and industrial effluents, because this metal is widely used in various manufacturing, such as electrolytic plating, explosives manufacturing, leather tanning, metal alloy, dyes and pigments manufacturing, etc. [1, 5].

There are studies of many methods for removal of chromium ion present in water industrial waste, for example: ion exchange on resins, coagulation-flocculation, adsorption on activated carbon, reduction, chemical precipitation, sedimentation, etc., [7], which in most cases are expensive or inefficient, especially when the concentration of these ions is very low [8]. Therefore arise emerging technologies such as biosorption, the process of attracting various chemical species by biomass (live or dead), by physicochemical mechanisms as adsorption or ion exchange [9].

Fungal cells interact with chromium at different levels from the cell wall and, from the periplasm to the cytoplasm and cell organelles. These microorganisms require detecting and regulating intracellular levels of chromium through homeostasis systems that maintain a balance between the incorporation, expulsion, and arrest of metal [1]. It is common for native microorganisms of sites contaminated with chromate ion, show resistance because they have asset or liability mechanisms that allow them to remove from detoxification. In certain species these mechanisms are know in detail, some of which are of basic interest and biotechnological importance, the latter in the context of developing new technologies for the treatment of industrial effluents and for bioremediation of contaminated sites. These mechanisms generally include biotransformation of Cr (VI) reduced species (chemical reduction), which may be direct (enzymatic) or indirect (enzyme); incorporation and bioaccumulation; biosorption of Cr (III), and Cr (VI); and immobilization [1, 9]. Some filamentous fungi reduce Cr (VI) to Cr (III), by different mechanisms of Cr (VI) detoxification, like reducing power generated by carbon metabolism [10, 11, and 12]. *Aspergillus niger* var. *tubingensis* Ed8, has demonstrated the ability to both biotransform Cr (VI) and accumulate it in the biomass, by a reduction and a sorption processes, using electron microscopy techniques [13].

*Aspergillus niger* strains have been described as coping with chromium mainly via the biosorption of the metal into the cells, rather than via the use of reducing activity [14]. Extracellular reduction of Cr (VI) to Cr (III) was observed during the growth of *Candida utilis* by mechanisms independent from the intensity of culture growth or initial chromium concentration [15], and they hypothesized that Cr (VI) reduction in *C. utilis* could be partly dependent on pH changes of broth during the exponential phase or on exo-enzymatic activities during stationary phase. Also, the biosorption of this metal has has been investigated in different fungi and yeast: *Cyberlindnera fabianii*, *Wickerhamomyces anomalus* and *Candida tropicalis* in aqueous solutions at different pH conditions. Cr (VI), and pH range between 2 and 4 where the most effective for the three species [16]. Secondly, *Candida maltosa*, isolated from tanning liquors from a leather factory has the capacity to reduce Cr (VI) both in the presence of viable intact cells and in cell-free extracts [17]. This ability was related to NADH-dependent chromate reductase activity associated with soluble proteins and, to a lesser extent, with the membrane fraction [17].

Recently, the reduction of Cr (VI) to Cr (III) through an enzymatic mechanism has been observed in *Pichia*. Both in intact cells and in cell-free extracts of *P. jadinii* M9 and *P. anomala* M10 strains, chromate was reduced, suggesting the presence of a chromate reductase activity possibly associated with the cytosolic or membrane proteins [18]. In the bacteria *Pseudomonas putida* F1, challenged with Cr (VI) in minimal médium (instead of in the complex LB medium), an ATPase involved in DNA repair-like protein (Pput 2963) was overexpressed compared with untreated cultures, suggesting that DNA damage occurs [19], and a non-enzymatic mechanism of Cr (VI) reduction has been described for *A. niger* [20]. The purpose of this chapter is to elucidate the characteristics of removal of chromium (VI) by *Penicillium* sp. IA-01 cells.

## 2. Materials and methods

### 2.1. Screening of the microorganism showing the resistant to Chromium (VI) and chromate resistance test

We isolate a chromate resistant mycelial fungus from polluted air near the Faculty of Chemical Science, UASLP (San Luis Potosí, México), and this was used for the screening. The chromate resistant filamentous fungus contained in the air was grown on the Petri dish containing modified Lee's minimal medium (LMM) (with 0.25%  $\text{KH}_2\text{PO}_4$ , 0.20%  $\text{MgSO}_4$ , 0.50%  $(\text{NH}_4)_2\text{SO}_4$ , 0.50% NaCl, 0.25% glucose, and 2% agar) supplemented with 500 mg/L  $\text{K}_2\text{CrO}_4$ ; the pH of the medium was adjusted and maintained at 5.3 with 100 mmol/L citrate-phosphate buffer. The plates were incubated at 28°C for seven days. The strain was identified based on characteristic macroscopic and microscopic observation [21]. Fungal cultures grown in thioglycolate broth were used as primary inoculums. Chromate-resistant tests of the isolated strain, filamentous fungus *Penicillium* sp IA-01, were performed on liquid LMM containing the appropriate nutritional requirements and different concentrations of Cr (VI) (as potassium chromate), and the dry weight was determined.

### 2.2. Biosorption tests by using dry cells

The fungal cells were grown at 28°C in an stirred and aerated liquid media containing thioglycolate broth at a concentration of 8g/L (p/v). After five days of incubation, the cells were recovered by centrifugation (3000 rpm/10 min), and washed twice in the same conditions with deionized water, and subsequently it was dried (80°C/24 h) in an oven. Solutions of Cr (VI) for analysis, were prepared by diluting 71.86mg/L of stock metal solution. The concentration range of chromium (VI) solutions was 50-1000mg/L. The pH of each solution was adjusted to the required value by adding 1M  $\text{H}_2\text{SO}_4$  solution before mixing with the microorganism. The biosorption of the metal by fungal dry cells was determined at different concentrations (50–1,000mg/L) of 100 mL Cr (VI) solution, with 1g of fungal biomass, at 120 rpm, and the sample was filtered. The filtrate containing the residual concentration of Cr (VI) was determined spectrophotometrically. For the determination of rate of metal biosorption, 200, 400, 600, 800, and 1,000mg/L of Cr (VI) solution was used. The supernatant was analyzed for residual Cr (VI) after the contact period at different times. For determination of the effects of pH and



temperature, four solutions (pH 1, 2, 3, and 4) and temperatures (28, 40, 50, and 60°C) were respectively used.

Moreover, biosorption to the contaminated soil and water was examined. Four Erlenmeyer glass flasks containing 5g of fungal biomass and 20g of contaminated soil and 20 mL of water (297mg Cr (VI)/g soil or 155mg Cr(VI)/L water), of tannery (Celaya, Guanajuato, México), was completed to 100 mL with trideionized water, were incubated during seven days at 120 rpm, and filtered in Whatman filter paper No. 1, and the concentration of Cr (VI) of the filtrate analyzed with 1, 5 diphenylcarbazide [22].

### 2.3. Reduction of Cr (VI) by living cells

Reduction efficiency of Cr (VI) by living, resting, and permeabilized cells was examined. To examine the living cells, cultures in 100 mL of LMM were inoculated with  $5 \times 10^5$  spores/mL (28°C, and 48 h), the cells were centrifugated (2000 rpm, at 4°C/10 min), and washed twice with sterile trideionized water and the pellet was resuspended in 3 mL of the same solution, and was transferred at a fresh LMM (100 mL with 50mg/L Cr (VI)). At different times, 1 mL aliquots were removed and centrifuged (5000 rpm/10 min), and we determine the concentration of Cr (VI) or total Cr in the supernatant.

Reduction efficiency of Cr (VI) was examined by the resting cells.  $5 \times 10^5$  spores/mL of *Penicillium* sp. was inoculated and incubated in 100 mL thioglycolate broth (pH 7.0) for five days, and was harvested ( $3000 \times g$  at 4°C); cell pellets obtained were washed by centrifugation twice with 100 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. The suspended cell pellets were added in 2-10mg/100 mL of Cr (VI) solution, mixed for 30 min, and incubated at 30°C for 6 h. Heat-killed culture pellets (2 mL), which were treated at 100°C for 10 min were used as control. After the incubation, the tubes were centrifuged, and 100  $\mu$ L aliquots were withdrawn from each sample to estimate the remaining Cr (VI).

Reduction efficiency of Cr (VI) was also examined by the permeable fungal cells. Culture of *Penicillium* sp. IA-01 was grown for five days, harvested, and washed with potassium phosphate buffer (pH 7.0) as described above. The suspended culture pellets were treated with 0.2% (w/v) sodium dodecyl sulphate, 0.2% Tween 80, (v/v), 0.2% Triton X-100 (v/v), and 0.2% toluene (v/v), by vortexing for 30 min to achieve cell permeabilization. Permeabilized cell suspensions (0.5 mL) were then added with 2–10mg/100 mL of Cr (VI) as final concentrations and incubated for 6 h at 30°C.

### 2.4. Activity of chromate reductase

Cell-free extracts (CFE) of *Penicillium* sp IA-01 were prepared by modifying the previously published protocols. The pellets were resuspended in 5% (v/v) of the original culture volume in 100 mM potassium phosphate buffer (pH 7.0). These cell suspensions were placed to an ice bath and disrupted using an Ultrasonic Mini Bead Beater (Densply) with 15 cycles of 60 sec for each one. The sonicate thus obtained was then centrifuged at  $3000 \times g$  for 10 min at 4°C. The pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.0), and this is the CFE.

Enzymatic chromate reduction was estimated as described previously using a standard curve of Cr (VI) 0–30 mM. The assay was as follows: The reaction system (1.0 mL) was made up of varying Cr (VI) final concentrations (5–30 mM) in 700  $\mu$ L of 100 mM potassium phosphate buffer (pH 7.0) added with 250  $\mu$ L aliquots of CFE for chromate reduction and 50  $\mu$ L of NADH. The system volume of 1.0 mL was kept constant for all experiments. Chromate reductase activity was measured at 37°C at different pH values using several buffers (100 mM phosphate citrate, pH 5.0; 50 mM phosphate, pH 6.0–8.0, and 50 mM Tris-HCl, pH 8–9). The effect of temperature was studied by measuring chromate reductase activity at different incubation temperatures between 20 and 60°C, at optimum pH. The CFE samples were also treated with several metal ions to a final concentration of 1mM at optimal pH and temperature; Na<sup>+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup>, Hg<sup>2+</sup>, Mg<sup>2+</sup>, Cd<sup>2+</sup>, and Fe<sup>3+</sup> were tested by using 10 mM solutions of Na<sub>2</sub>SO<sub>4</sub>, CaCl<sub>2</sub>, CuCl<sub>2</sub>, HgCl<sub>2</sub>, MgCl<sub>2</sub>, CdCl<sub>2</sub>, and FeCl<sub>3</sub>. The electron donors tested were NADH, glucose, sodium acetate, formic acid, citrate, cystin, lactic acid, and ascorbic acid in a final concentration of 1mM, and the inhibitors were EDTA, KCN, NaN<sub>3</sub>, and  $\beta$ -mercaptoethanol at the same concentration. For chromate reductase activity, one unit was defined as enzyme that reduces 1mmol of Cr (VI)/min/37°C, and the specific activity was defined as unit chromate reductase activity/min/mg protein in the CFE. Protein concentrations were determined by the Lowry method [23].

## 2.5. Determination of hexavalent, trivalent, and total amount of chromium

Hexavalent and trivalent chromium were quantified employing diphenylcarbazide [22] and chromazurol S [24], respectively, the total amount of Chromium was determined by electro-thermal atomic absorption spectroscopy [22]. Three dependent experiments were carried out and the mean value was shown

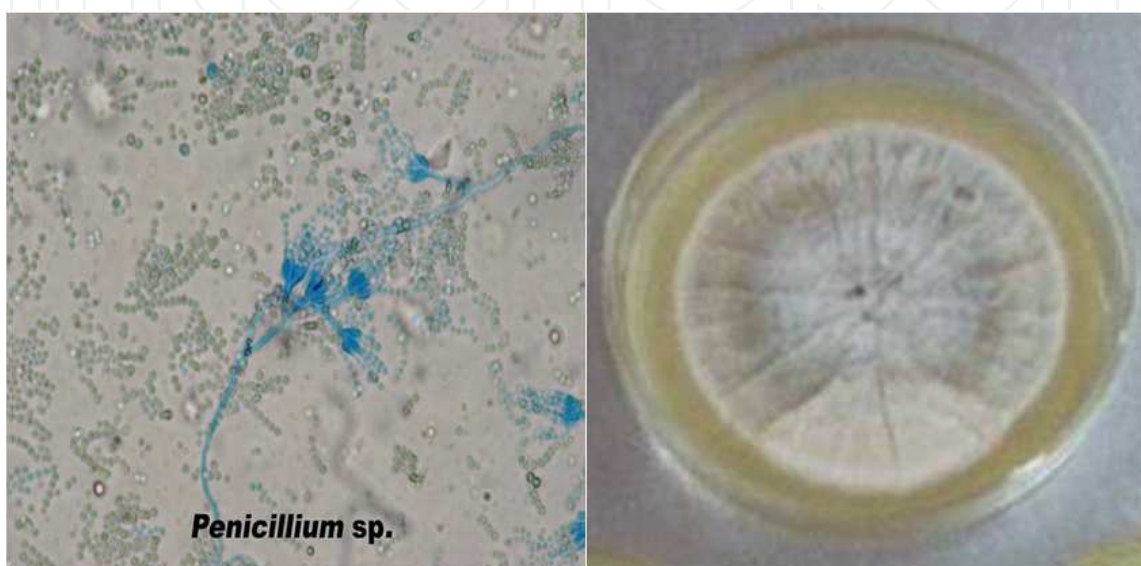
## 3. Results and discussion

### 3.1. Isolation and identification of a fungal strain tolerant to Cr (VI)

Microorganism was grown on the LMM agar plates containing 500 mg/L of K<sub>2</sub>CrO<sub>4</sub>, and the largest colony of fungi was isolated. Colonies isolated grew rapidly within three days. Colonies are usually fast growing, in shades of green, sometimes white, mostly consisting a dense conidiophores. Microscopically, chains of single-celled conidia (ameroconidia) are produced in basipetal succession from a specialized conidiogenous cell called phialide. In *Penicillium*, the phialides may be produced singly, in groups or from branched metulae, giving a brush-like appearance known as a penicillus (Figure 1) [25].

The cells of the isolated strain grew on LMM supplemented with 2 g/L of Cr (VI) about 50% of growth relative to control (85mg of dry weight without metal) was obtained (Figure 2) and, therefore probably is resistant to the metal. Different microorganisms that are Cr (VI) resistant have been isolated from different contaminated sites [1, 16, 26, and 27], and Chromate tolerance has been described in the mutants of stocked culture, and in native isolates of contaminated sites, as in this work; in several cases, both yeast and filamentous fungi showed that tolerance

to Cr (VI) is due to transport of sulfate disturbance that leads to reduced incorporation of chromate [28] in other cases, phenotypes of hypersensitivity to Cr (VI) are produced as a result of alteration of the vacuolar ATPase and vacuolar structures [29] or by alteration of proteins that protect the oxidative effect of Cr (VI) as the alkyl hydroperoxide reductase [30] or Cu-Zn-superoxide dismutase and methionine sulfoxide peptide reductase [31]. However, the mechanism of tolerance in *Penicillium* sp IA-01 fungus are not investigated. Thus, we precisely examined the characteristics of the *Penicillium* strain to estimate the mechanism in the following experiments.



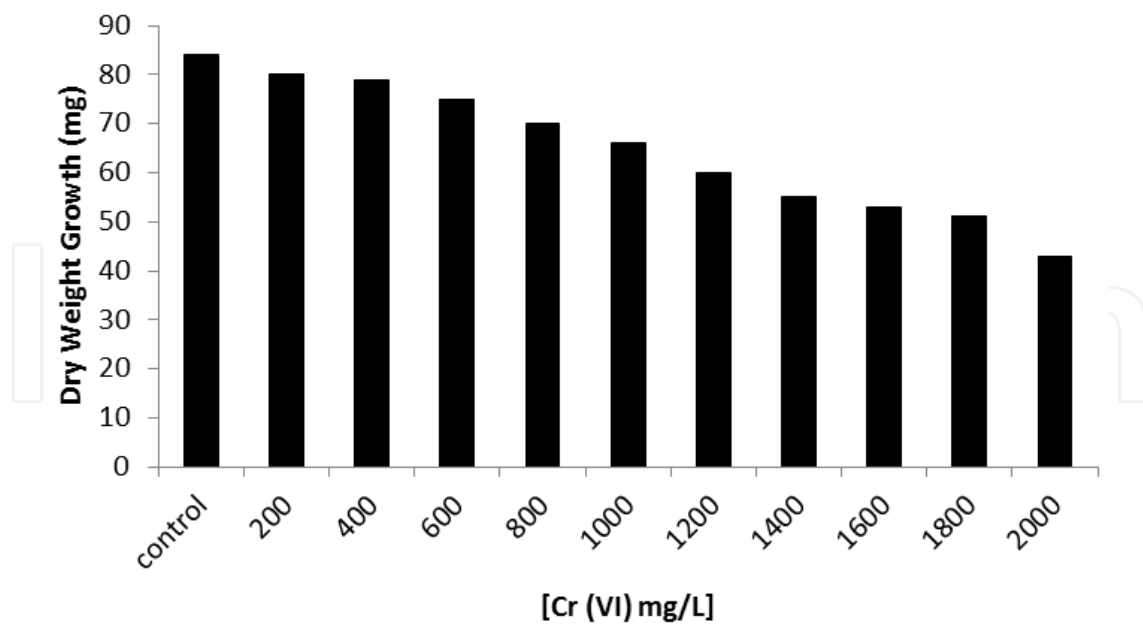
**Figure 1.** Macroscopic and microscopic morphology of the fungus *Penicillium* sp IA-01

### 3.2. Absorption of Cr (VI) by the dry cells of *Penicillium* sp. IA-01

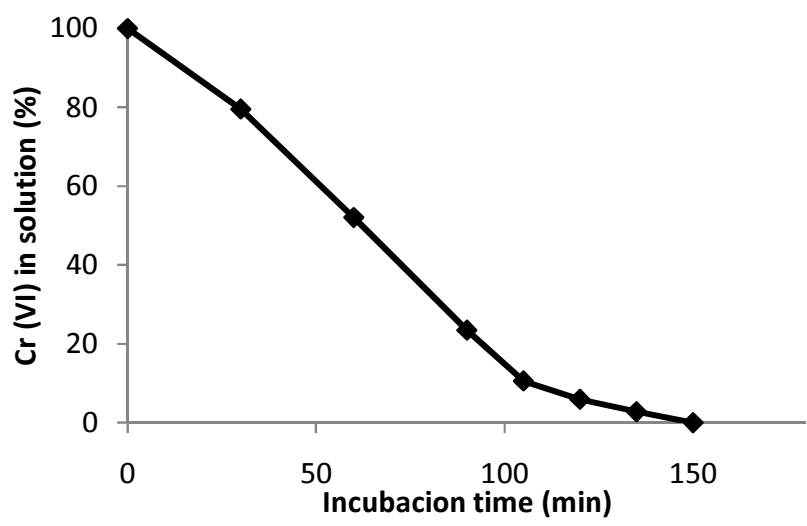
First, the ability of absorption was examined by using *Penicillium* sp. IA-01 cells to clarify the mechanism of Cr (VI) tolerance. Figure 3 shows the effect of the incubation time on Cr (VI) removal by *Penicillium* sp biomass. The optimum time for Cr (VI) removal was 150 min at constant values of pH (1.0), biosorbent dosage (1g 100/mL), initial metal concentration (50mg/L) and temperature (28°C). Some studies [32], report an incubation time of 48 h at pH 1.0 by fruiting bodies of the jelly fungus *Auricularia polytricha*, 24 h for *C. fabianii*, *W. anomalus* and *C. tropicalis*, at pH range between 2 and 4 for the three species [16], to pH of 2.0 to five days for *Aspergillus niger* [20], the latter with 10 g/L of biomass, and at the same pH of 2.0. Changes in the permeability of the fungal cell wall, of unknown origin, could partly explain the differences found in the incubation time, providing greater or lesser exposure of the functional groups of the cell wall of the biomass tested [33].

With respect to the influence of initial pH on removal efficiency, it was found that the highest activity was evident at pH 1.0, at 150 min the metal is removed, while at pHs 2, 3, and 4, the authors did not observe significant differences (20% of removal), and at neutral or alkaline pH's, there was no removal (Figure 4). A pH optimum has been reported of 1.0 to removal Cr (VI)





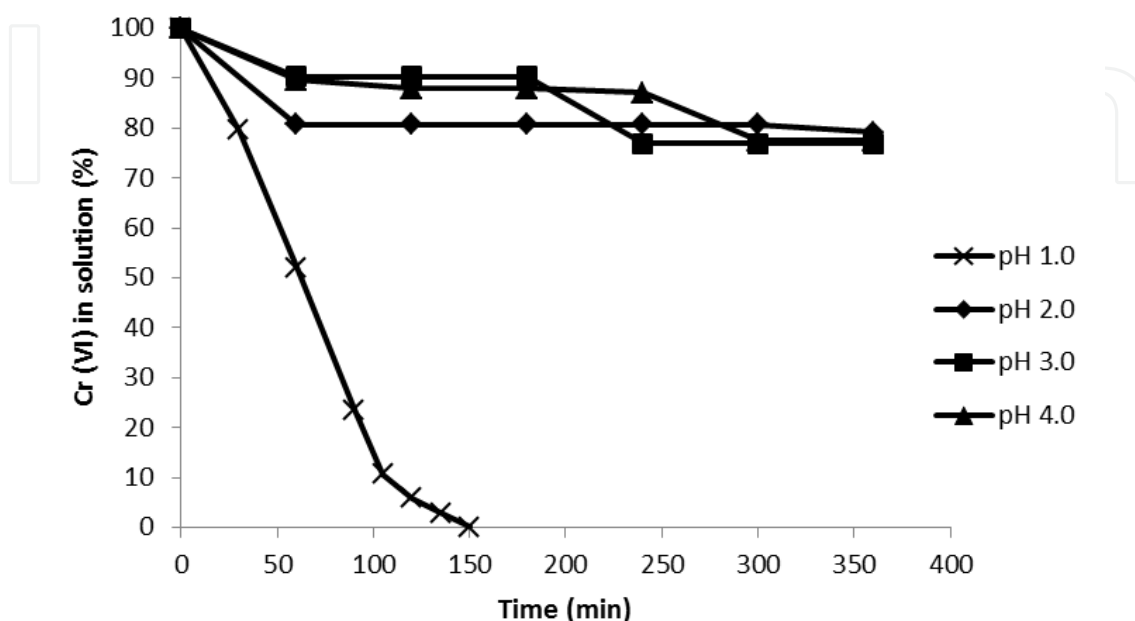
**Figure 2.** Growth in dry weight of *Penicillium* sp., IA-01 with different concentrations of Cr (VI),  $1 \times 10^5$  spores/mL, 28°C, seven days of incubation, 100 rpm.



**Figure 3.** Effect of incubation time on Cr (VI) removal by *Penicillium* sp. 50mg/L Cr (VI), 100 rpm, 28°C, pH 1.0, and 1.0 g of fungal biomass.

by fruiting bodies of the jelly fungus *A. polytricha* [32] and for the yeast *Saccharomyces cerevisiae* and the fungi *Rhizopus arrhizus* a pH range of 1.5-2.5, at 4 h [34], although most indicate a pH optimum range of 2.0 to 4.0 with the yeasts *C. fabianii*, *W. anomalus* and *C. tropicalis*, isolated from sediments in Morocco [16], both *Mucor hiemalis* [35] and *R. arrhizus* [36], at 24 h, *Rhizopus nigricans* in 8 h [37]. The Cr (VI) is found as  $\text{HCrO}_4^-$ ,  $\text{Cr}_2\text{O}_7^{2-}$ ,  $\text{CrO}_4^{2-}$ ,  $\text{Cr}_4\text{O}_{13}^{2-}$ , and  $\text{Cr}_3\text{O}_{10}^{2-}$  [12]. A decrease in pH causes protonation of the surface of the adsorbent, which induces a strong attraction for the Cr (VI) ions from solution negatively charged, so that the biosorption increases with increasing acidity of the solution. However, as the pH increases, the concen-

tration of OH<sup>-</sup> ions increases, causing changes in the surface of the adsorbent and preventing the biosorption negatively charged of Cr (VI) ions, thereby decreasing the adsorption of metal to high pH values. It has been reported [11] that while Cr (VI) is obtain by eliminating indigenous strains of filamentous fungi, most of the cations may be reduced to Cr (III).

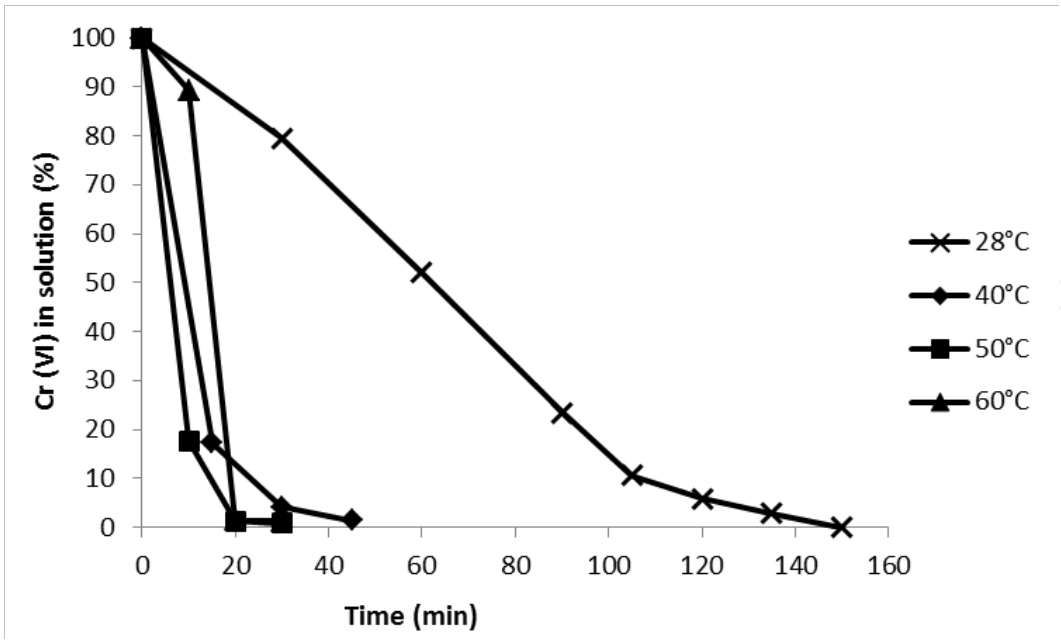


**Figure 4.** Effect of pH on Cr (VI) removal by *Penicillium* sp. 50mg/L Cr (VI), 100 rpm, 28°C, and 1g of fungal biomass.

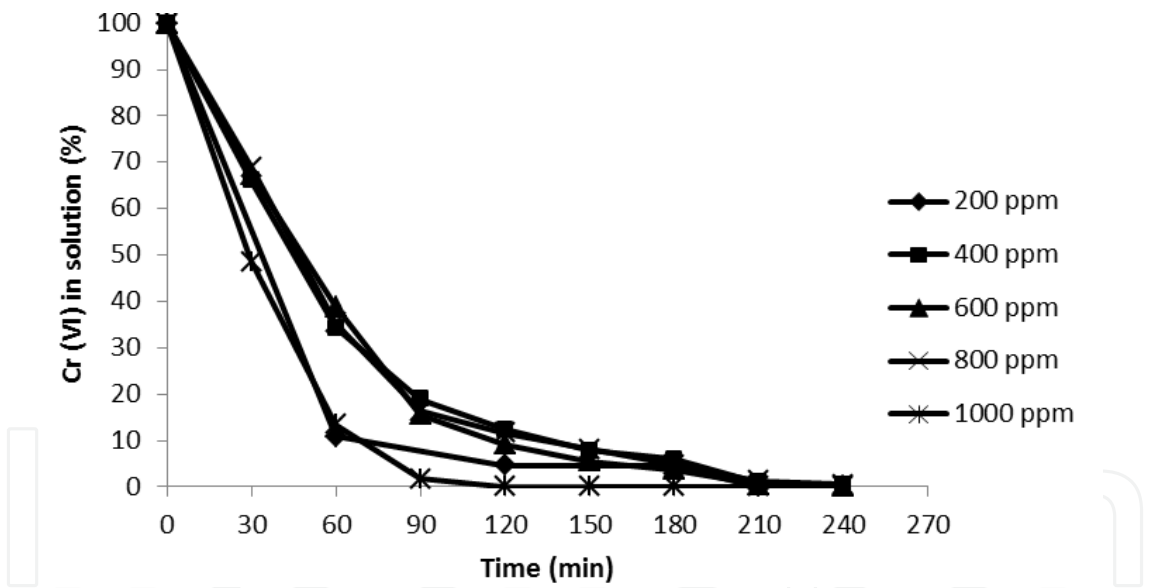
Temperature is found to be a critical parameter in the bioadsorption of Cr (VI) (Figure 5). The highest removal was observed at 50°C and 60°C. At this point the total removal of the metal is carried out at 100%, at 40 min. These results are likely for *Paecilomyces* sp [10] and *A. niger* [20] at 45°C and 24 h, but are different for *R. arrhizus* [36]. The increase in temperature increases the rate of removal of Cr (VI) and decreases the contact time required for complete removal of the metal, to increase the redox reaction rate [38].

At different metal concentrations (200, 400, 600, 800, and 1000mg/L), biomass studied, shows the same results for removal, adsorbing 100% between 210 and 240 min while 1000mg/L of metal is removed 100% up to 90 min of incubation (Figure 6) with respect to other fungal biomasses, some reports argue that the amount of metal increases in direct proportion with the increase in concentration of the metal ion in solution [35, 37], and others author report lower removal efficiencies of metal, for example 25 and 250mg/L of chitin and chitosan [36], and 1mg/L for cellulose acetate [39]. This was due probably to the increase in the number of ions found competing for the available functions groups on the surface of biomass [38].

The influence of the biomass on the removal capacity of Cr (VI) was depicted in Figure 7. If we increase the amount of biomass, we also increase the removal of Cr (VI) in the solution (although there is a 100% of remotion, with 3, 4, and 5g of biomass, 60 min), perhaps due to increased of biosorption sites of the same, because the amount of added biosorbent determines the number of binding sites available for metal biosorption [30]. Similar results have been



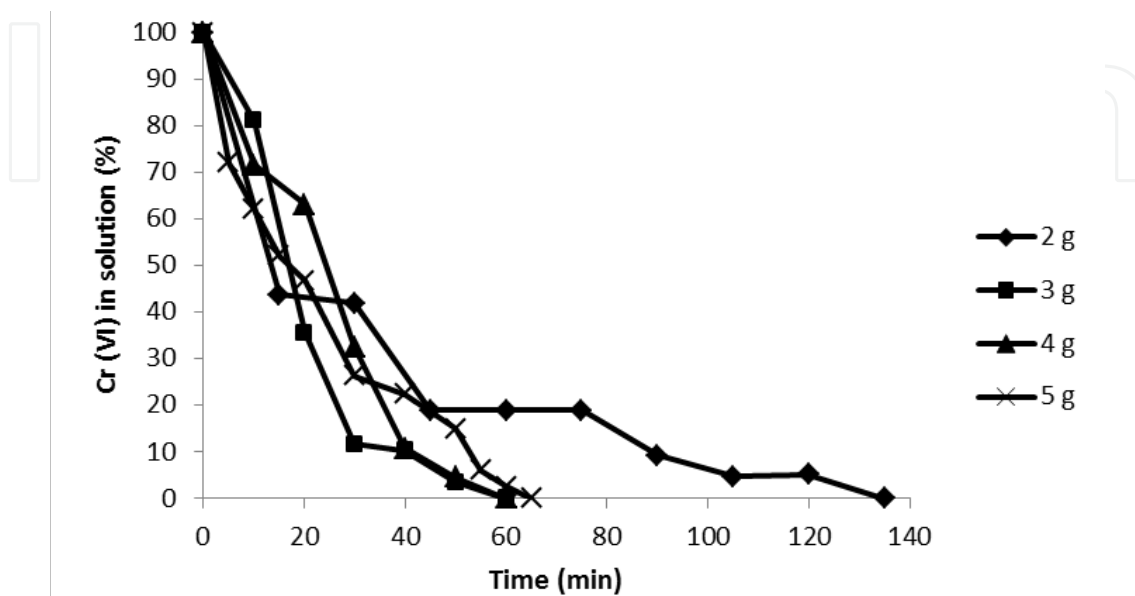
**Figure 5.** Effect of temperature on Cr (VI) removal by *Penicillium* sp. IA-01. 50mg/L Cr (VI), 100 rpm, pH 1.0, and 1g of fungal biomass.



**Figure 6.** Effect of initial metal concentration on chromium (VI) removal by *Penicillium* sp. IA-01. 28°C. pH 1.0, 100 rpm, and 1g of fungal biomass.

reported for *M. hiemalis* and *R. nigricans*, although the latter has 10g of biomass [35, 37], but different from those reported for biomass wastes from the mandarin (gabassa), whit an optimal concentration of biomass of 100mg/L [40]. Consequently, we found out the following results: *Penicillium* sp. IA-01 has the ability of absorption to Cr (VI) and the value of adsorption is as high as in the fruiting bodies of the jelly fungus *A. polytricha* [32], *C. fabianii*, *W. anomalus* and *C. tropicalis* [16], *A. niger* [20], *M. hiemalis* and *R. nigricans* [35, 37], and *Paecilomyces* sp. [10]. The

adsorption rate was affected by pH, temperature, initial concentration of Cr (VI) and dry cells. In the case of polluted soil and water, around 40% of Cr (VI) could not be removed. Therefore, absorption to the organic compounds contained in polluted soil and water may occur. The results suggest that the reduction of Cr (VI) is necessary to the bioremediation of soil and air.

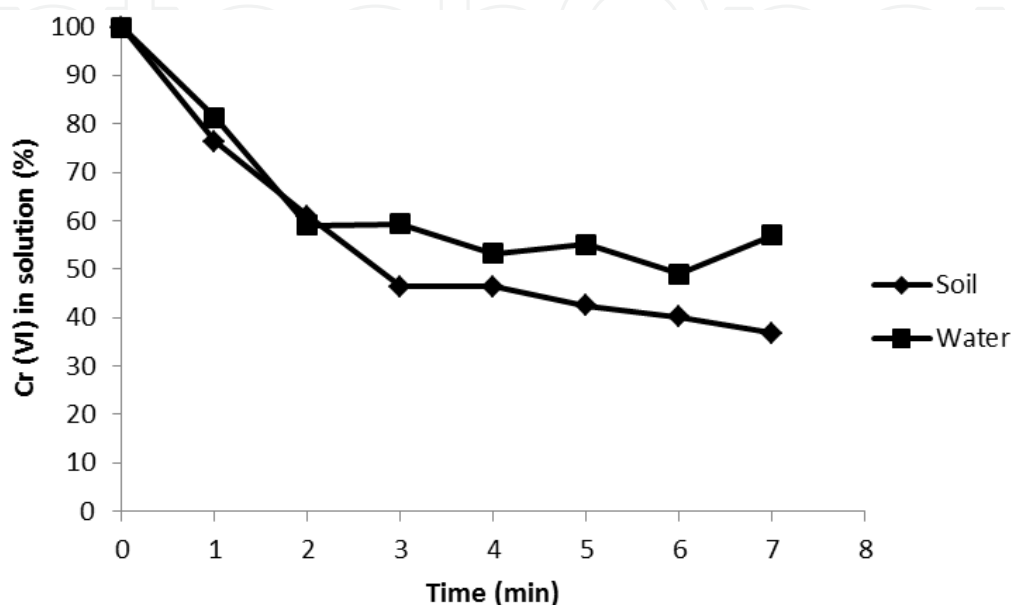


**Figure 7.** Effect of biomass concentration on chromium (VI) removal by *Penicillium* sp. IA-01. 50mg/L Cr (VI). 28°C, and pH 1.0, 100 rpm.

### 3.3. Removal of Cr (VI) in industrial wastes with fungal biomass

For the removal of the metal from industrial wastes, we incubate the fungal biomass (5g) with non-sterile oil and contaminated water (297mg Cr (VI)/g, and 155mg Cr (VI)/L), suspended in trideionized water. It was observed that after seven days of incubation with the biomass, the Cr (VI) concentration from soil and water samples decreased to 63.24% and 43%, respectively (Figure 8), without significant change in total chromium (not shown). In the absence of the biomass, the metal concentration of the soil samples decreased slightly (18%, not shown), maybe caused by indigenous microflora and (or) reducing components present in the soil [10, 11, and 18]. The capacity of *Penicillium* sp., biomass to remove the metal are lower to those reported for other biomasses, like *Litchi chinensis*, [41] tamarind shell [42], *Mammea americana* [43], and *C. reticulata* [44], and equal or better than that of *C. maltose* RR1 [17]. The *Penicillium* biomass was more efficient for the chromium removal under acidic conditions. Some studies were carried out at neutral pH [45]. *A. niger* mycelium removal 8.9mg/g dry weight at seven days of incubation (500ppm of Cr (VI)). Otherwise, *Paecilomyces* sp. biomass was incubate with non-sterilized contaminated soil containing 50mg Cr (VI)/g, suspended in LMM, pH 4.0, and it was observed that after eight days of incubation with the biomass, the Cr (VI) concentration of soil sample decreased fully [46]. We found out the following results: *Penicillium* sp, IA-01, has the ability to absorb Cr (VI) and the value of adsorption is as high as the fruiting bodies of the jelly fungus *A. polytricha* [32]; *C. fabianii*, *W. anomalus* and *C. tropicalis* [16]; *A. niger* [20], *M.*

*hiemalis* and *R. nigricans* [35, 37] and *Paecilomyces* sp. [10]. The adsorption rate was affected by pH, temperature, initial concentration of Cr (VI) and dry cells. In the case of the polluted soil and water, around 40% of Cr (VI) could not be removed. Therefore, absorption to the organic compounds contained in polluted soil and water may occur. The results suggest that the reduction of Cr (VI) is necessary to the bioremediation of soil and air.



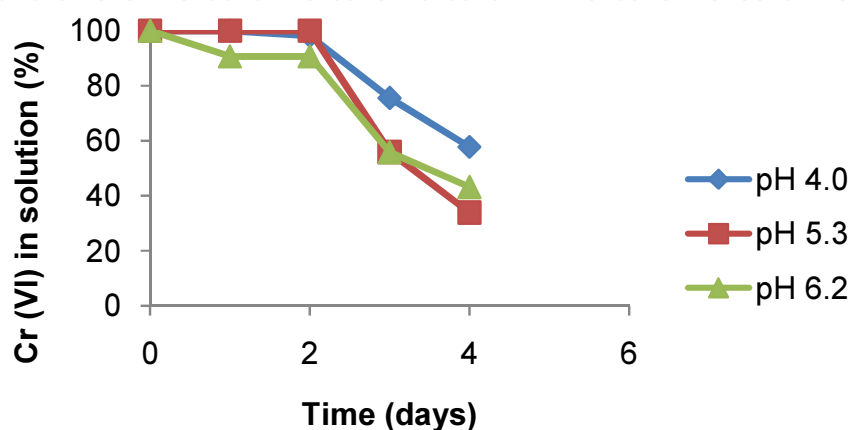
**Figure 8.** Removal of Cr (VI) in industrial wastes incubated with 5g of fungal biomass. 100 rpm, 28°C, 20g and 100 mL of contaminated soil and water, respectively (297mg Cr (VI)/g soil and 155mg Cr (VI)/L).

### 3.4. Removal of Cr (VI) by living cells of *Penicillium* sp. IA-01

Next, the reduction of Cr (VI) by *Penicillium* sp. IA-0 were examined by using living cells. The fungal cells, which were cultured in 100 mL LMM containing 50mg/L Cr (VI), under various pH, inoculated amount, Cr (VI) concentration and carbon sources. The amount of Cr (VI) was determined and the percentage of decreased amount to total amount was calculated. The effect of different pHs (4.0, 5.3, and 6.2), show a pH optimum of 5.5 (77% at four days, 28°C, and 100 rpm), while at pH of 4.0 and 6.2 were of 43% and 65%, respectively (Figure 9). About, Coreño-Alonso et. al. [13], reported a 95% of removal at pH of 5.3 and 24 h with *A. niger* var *tubingen-sis* strain Ed8, and also, at pH 5.0 for Cr (VI) removal with *A. niger* MTCC 2594 [14] and at pH 3.0–5.0 for  $Pb^{+2}$ ,  $Cd^{+2}$  and  $Cr^{+3}$  with the yeast *Saccharomyces cerevisiae* [47]. In contrast to our observations, the maximum adsorption capacities by both living yeasts were found at pH 4.0 for *C. fabianii* HE650139 and *W. anomalus* HE648168, and 3.0 for *C. tropicalis* HE650140, with a percentage of removal of 100%, by all living microorganisms [16], also, a maximum uptake of Cr (VI) at pH 7.0 with *Aspergillus foetidus* [48]. On the other hand, using a *Citrobacter* strain, it has been reported that an enhanced uptake of different heavy metals, is increased if pH is from 2.0 to 7.0 and also a decrease in the removal at higher pH values [49]. Al-Asheh and Duvnjak



[50] also reported most removal increasing pH in the range 4.0–7.0 on Cr (III) uptake using *Aspergillus carbonarius*. At low values of pH, the low efficiency of removal of the metal, may be due to the competition between hydrogen ( $H^+$ ) and metal ions [36], and at higher pH values (7.0), the efficient metal removal may be due to the ionization of functional groups and the increase in the negative charge density on the cell surface. At alkaline pH values (8.0 or higher), a reduction in the solubility of metals may contribute to lower uptake rates [1].

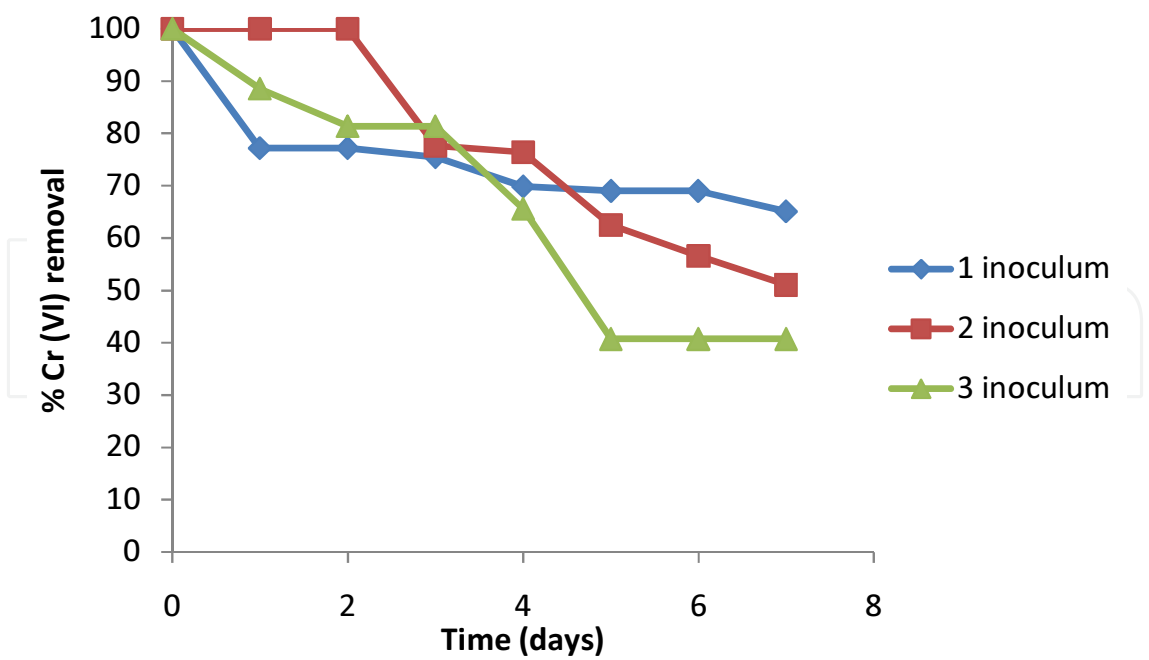


**Figure 9.** Effect of pH on chromium (VI) removal by biomass of *Penicillium* sp IA-01 50mg/L Cr (VI), 100 rpm, 28°C

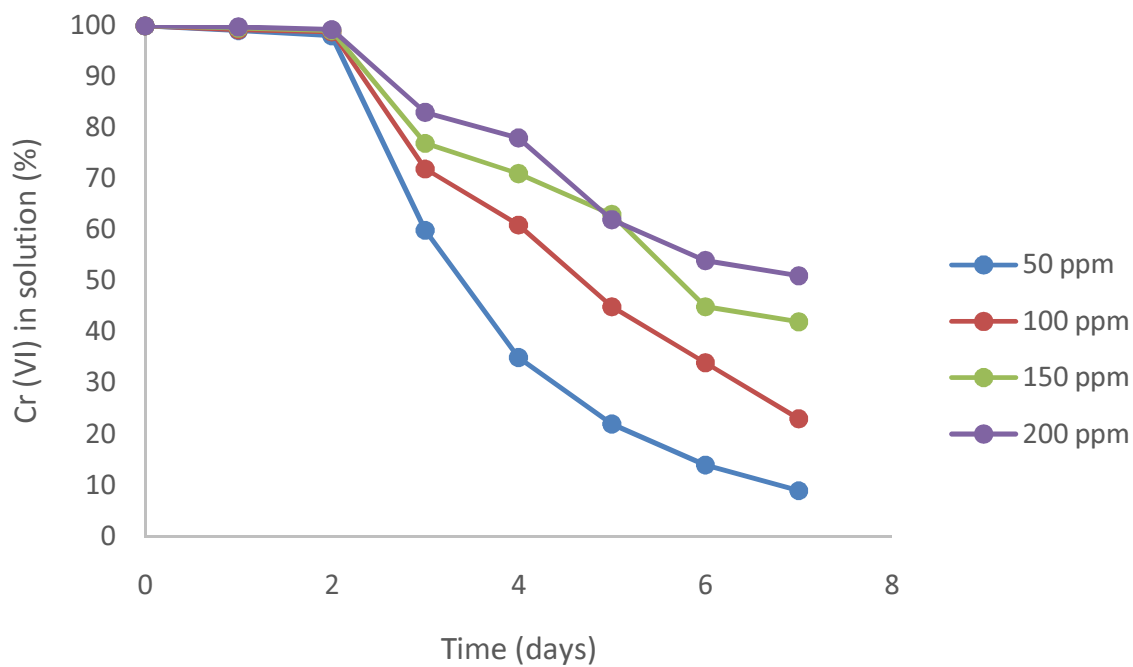
In Figure 10, the effect of the biomass concentration (72, 141, and 169 mg of dry weight) on Cr (VI) removal, with percentages of removal of 35%, 49%, and 60%, respectively, is shown. Similarly, most of the reports in the literature observe at higher biomass dose resulting in an increase in the percentage removal [3, 7, 8, 13, 16, 47, and 52]. With higher biomass dose, there are more binding sites for complex of Cr (VI) (e.g.,  $HCrO_4^-$  and  $Cr_2O_7^{2-}$  ions) [3, 28].

Figure 11, shows the effect of Cr (VI) concentration (50 to 200mg/L) on the removal of the same. If we increase the concentration of the metal, the removal of metal decreases (60%, 50%, 28%, and 11%, respectively). This is probably because, if we increase initial metal concentration, we increase the number of ions competing for the available functions group on the surface of biomass. Our observations are like most of the reports in the literature [3, 7, 8, 37, 47, 48, 53].

With different carbon sources, like fermentable: glucose, sucrose, and citrate, and oxidable (glycerol). With glucose, sucrose, and citrate, the decrease in Cr (VI) levels occurred at a different rate, at six days of incubation (52%, 47%, and 27%, respectively), and the other carbon sugars were less effective (glycerol 7% of removal). With another inexpensive commercial carbon sources like unrefined sugar and brown sugar, the decrease in Cr (VI) levels occurred at a similar rate (96% and 100%, respectively) (Figures 12(a), (b)). If we incubate the fungal biomass without a carbon source, there are no changes in the initial Cr (VI) concentration during the experiment (data not shown), suggesting that a carbon source is required to decrease Cr (VI) concentration in the growth medium. Our results are similar to some reports: how in chromate-resistant strains of filamentous fungi indigenous to contaminated wastes,



**Figure 10.** The effect of cell concentration on the removal of Cr (VI). 50mg/L Cr (VI), 100 rpm, 28°C, and pH 5.3

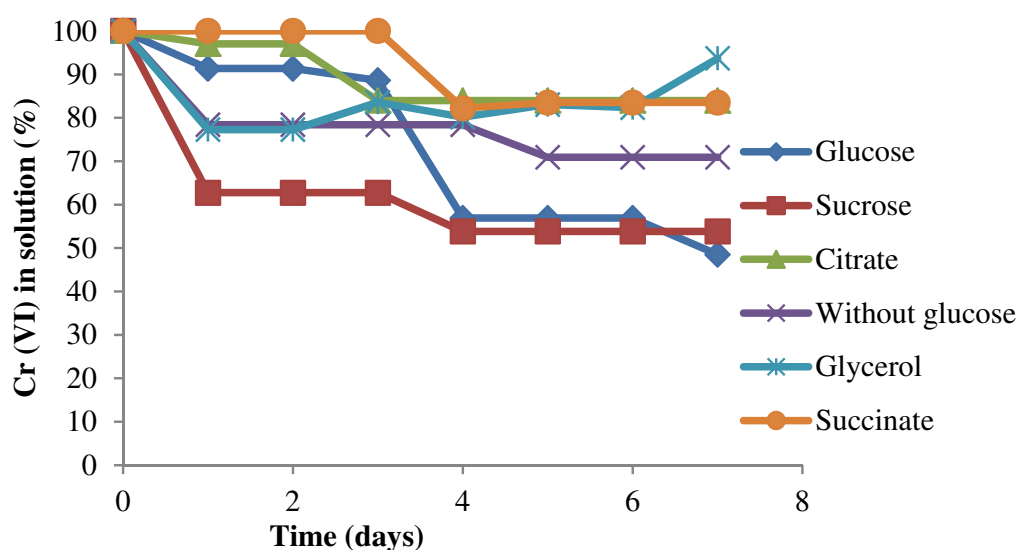


**Figure 11.** The effect of the concentration of Cr (VI) in the solution on the removal. 100 rpm, 28°C, pH 5.3

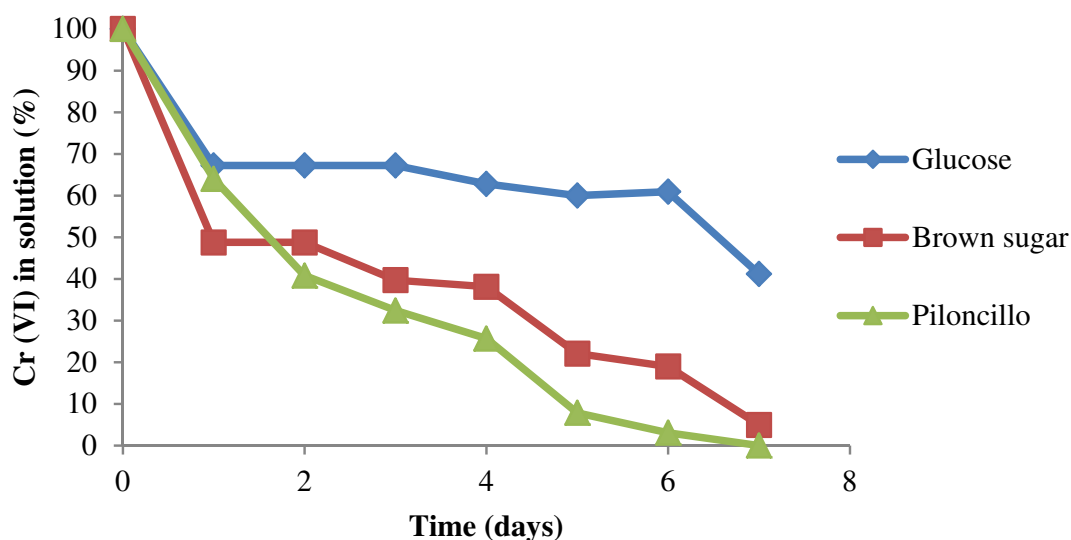
with *A. foetidus*, *A. niger* and *A. parasiticus* [11, 48, and 54] with glucose like carbon source, and other carbon sources like sucrose and citrate by a *Paecilomyces* sp fungal strain isolated from environment. [10], but are different from the observations of Srivasta and Thakur [55] with *Aspergillus* sp and *Acinetobacter* sp, who observed that the main carbon source is the sodium

acetate. Consequently, we found out the following results in this section: As shown in Figure 4, the difference between dry cells and living cells were clear. *Penicillium* sp. IA-01 cells could remove Cr (VI) at pH 4.0-6.2, although combining by absorption did not occur (Figure 4). The result suggests that *Penicillium* sp. cells can absorb and/or reduce Cr (VI) as well as adsorption. Additionally, as shown in Figure 12, the removal of Cr (VI) by adding glucose was higher than that of water (without glucose), and some carbon sources such as brown sugar and piloncillo enhanced the removal. There results suggest that the carbon sources induce the absorption rate of Cr (VI) or increase the amount of chromate reductase activity.

A



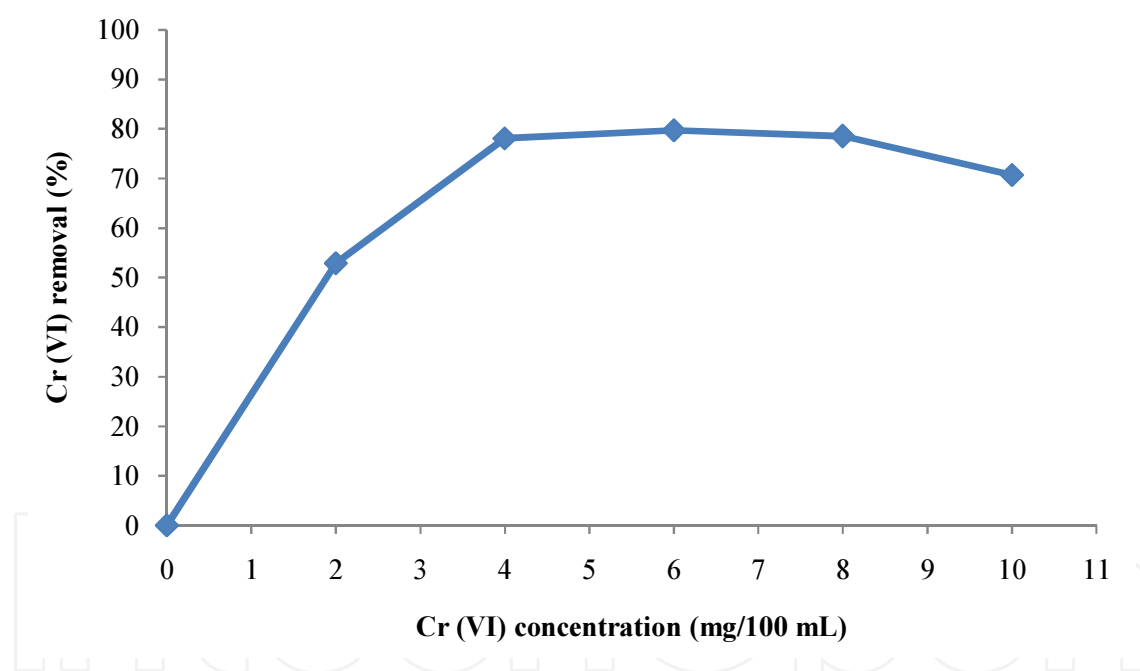
B



**Figure 12.** Influence of carbon source on the capability of *Penicillium* sp., IA-01 to decrease Cr (VI) levels in the growth medium. 100 rpm, 28°C, pH 5.3

### 3.5. Adsorption and reduction by resting and permeable cells

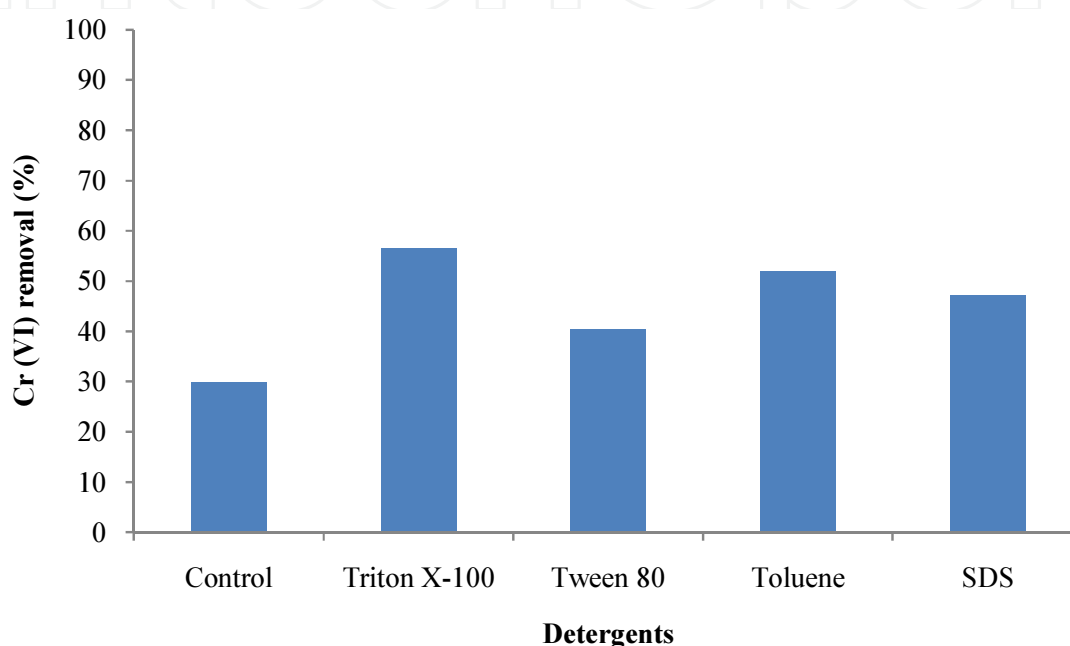
We also estimated the ratio of absorption and/or reduction to adsorption, as we found that the fungi *Penicillium* sp. IA-01, has these abilities as well as adsorption from the results in Section in 3.3. The resting cells and permeabilized cells were used for the examination, and heat-killed cells were used to examine the amount of adsorption. The removal was calculated as value of Cr (VI) in resting cells to the value from total value minus the value of Cr (VI) in heat-killed cells (0% of removal). First, the removal of the metal by resting cells was examined. The cell pellets of *Penicillium* sp, which were cultured in 100 mL thioglycolate broth, were incubated in 100 mM potassium phosphate buffer (pH 7.0) for 6h at 30°C. The resting cells of the fungus were expedient in reducing 0–10mg/100 mL Cr (VI) concentrations in 8 h as shown in Figure 13. The fungus removal was between 53% and 70% (2–10 mg/100/mL) of the metal, and these results resemble those reported by *A. niger* and *A. parasiticus* [54], *Fusarium solani* [56], *Paecilomyces lilacinus* [57], and the bacteria *Pseudomonas* sp. [58] and *Paecilomyces* sp. [46]. Structural properties of the biosorbent including the cellular support and other several factors are known to affect the biosorption rate [59].



**Figure 13.** Resting cell assays for Cr (VI) reduction by *Penicillium* sp. IA-01 performed at initial concentrations of 0–10mg/100 mL of Cr (VI), pH 7.0, and 37°C in 8 h

The cell permeabilization increased the Cr (VI) reduction by the resting cells, as the permeabilized cells with Triton X-100 which could reduce 57%, toluene 52%, SDS 47.4%, and Tween 80 40.4% (Figure 14) of 30 mM Cr (VI) within 6 h, suggesting an efficient intracellular mechanism of chromate reduction. The Cr (VI) reductase activity in CFE of cells grown in the absence of Cr (VI) was 94.07  $\mu\text{moles/min/mg protein}$ . These results indicate that the Cr (VI) reductase was associated with the CFE. Fungal, yeast, and bacteria chromate reductases have been localized either in CFE of *A. niger* and *A. parasiticus* [54], *Pichia jadinii* M9, *Pichia anomala* M10

[60], *Pichia* sp. [61], and *Bacillus* sp. [62], cytosolic fraction of *C.maltosa* [17], *Pichia* sp. [62], and *Pannonibacter phragmitetus* [63] of membrane fraction of *Pseudomonas* sp. G1DM21 [58], *Bacillus megaterium* [64], and *Enterobacter cloacae* [65]. The results by resting and permeable cells suggest as follows: As shown in Figure 13, 70-80% of Cr (VI) could be removed by resting cells, and the result suggests that absorption of Cr (VI) occurs without energy of carbon sources or ATP. Additionally, as shown in Figure 14, the ratios of the removal of Cr (VI) in case of the pre-treatment by Triton X-100, toluene and SDS of glucose were 2-2.5 times higher. Therefore, the transport through cell membrane is the rate-limiting steps.

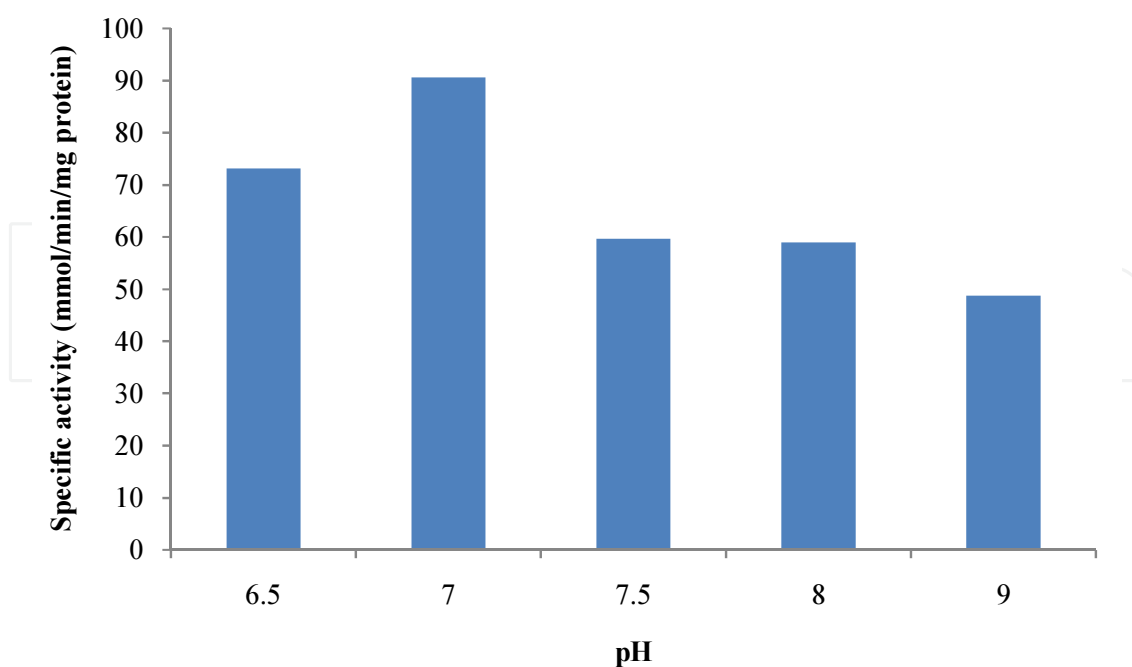


**Figure 14.** Permeabilized cell assays for Cr (VI) reduction by *Penicillium* sp. IA-01 performed at initial concentrations of 28mM of Cr (VI), pH 7.0, and 37°C

### 3.6. Chromate reductase activity

The result of permeable cells (Figure 14) suggests that *Penicillium* sp. IA-01 has the enzymatic ability of reduction to Cr (VI). Thus, we investigated the reduction of Cr (VI) by *Penicillium* sp. IA-01. The activity of chromate reductase is examined in the cell-free extract. The function of the chromate reductase of *Penicillium* sp., was characterized in different *in vitro* conditions. In determining the optimal pH for the chromate reductase activity, we used the following buffers at different pH ranges: potassium phosphate, citrate phosphate, and Tris-HCl; and we found the maximum enzymatic activity to be at an optimum pH of 7.0, with potassium phosphate buffer, as depicted in Figure 15, and these results resemble those reported by the fungi *A. niger* and *A. parasiticus* [54], the yeast *P. jadinii* M9 [60], and cell-free extract of *Arthrobacter* sp. SUK 1201 [66]. Other authors reported stability between 7.0 and 7.4 for the bacteria *Pseudomonas* sp. G1DM21 [58], 6.5 and 7.5 for *E. coli* CFE [67], and in the range of 5.0 to 8.0 for *Bacillus* sp. [68].

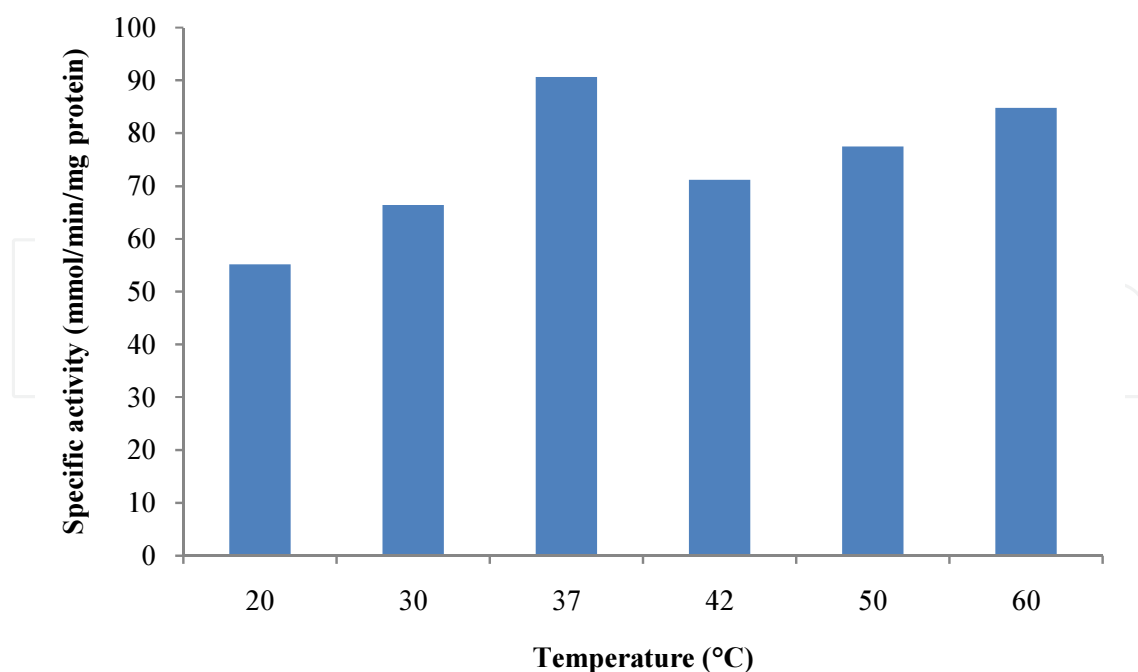




**Figure 15.** Effect of pH on Cr (VI) reductase activity in cell-free-extracts of *Penicillium* sp. IA-01 determined in different buffers (pH 6.5–9.0) with an initial concentration of 5.6mM Cr (VI), at 37°C

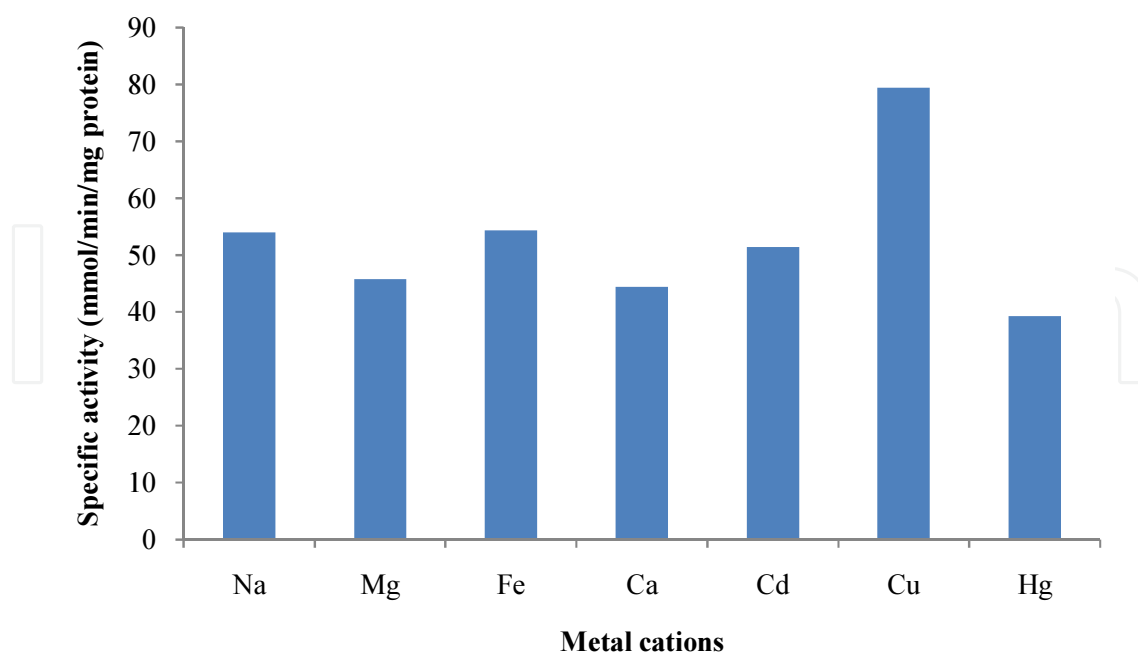
The optimal temperature for the Cr (VI) reductase activity was 37°C, but the reductase activity was altered significantly at 20°C (39% of inhibition); but when the assays were performed at 50°C the reductase activity showed 14.2% of inhibition (Figure 16). For *P. jadinii* M9, incubation at 55°C produced a reduction in activity of 55% [60]. In *P. anomala* M10, when incubated at 8°C, a decrease in activity of 25% was observed, and at 50°C the activity was at 50%. [69]. For *A. niger* and *A. parasiticus* [54], *Pseudomonas* sp. G1DM21 [58], *E. coli* [66], and *Bacillus* sp. CFEs [67], the thermal stability was of 30°C [66, 67], and for the activity in cell-free extract of *Arthrobacter* sp. SUK 1201, it was 32°C [66]. On the contrary, *Pseudomonas putida* CFE probed to be more resistant, keeping its stability up to 50°C [69].

The effect of different metal cations on the chromate reductase activity of *Penicillium* sp. was determined in Figure 17. All the metal ions tested inhibit the Cr (VI) reductase activity of the CFE of 12% with  $\text{Cu}^{2+}$  and 40.2% with  $\text{Na}^+$ , and these results are different than those reported for the yeast *P. jadinii* M9 chromate reductase because only  $\text{Cu}^{2+}$  and  $\text{Na}^+$  produced an augmentation in the activity of 63 and 30%, respectively [60]; in *Arthrobacter* sp. SUK 1201,  $\text{Cu}^{2+}$  also increases the activity [66], and all other ions tested had an inhibitory effect but in different levels. A decrease of 56.5% was observed with  $\text{Hg}^{2+}$ , while addition of  $\text{Mg}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Cd}^{2+}$  resulted in a decrease of activity between 40% and 51%. In *P. anomala* M10 chromate reductase, only  $\text{Cu}^{2+}$  produced a rise in activity of 31% [69]. Inhibition by  $\text{Hg}^{2+}$  was higher in *P. anomala* and *Pseudomonas* sp., than in *Penicillium* sp., with a decrease in activity of 85% and 90%, respectively [58, 60]. Inhibition by  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  was approximately 50%, while  $\text{Fe}^{3+}$  reduced the activity to 32%. These results agree with those reported for *Arthrobacter crystallopoietes* [44] and *Bacillus* sp. [67]. On the other hand, inhibition by  $\text{Hg}^{2+}$  can be related



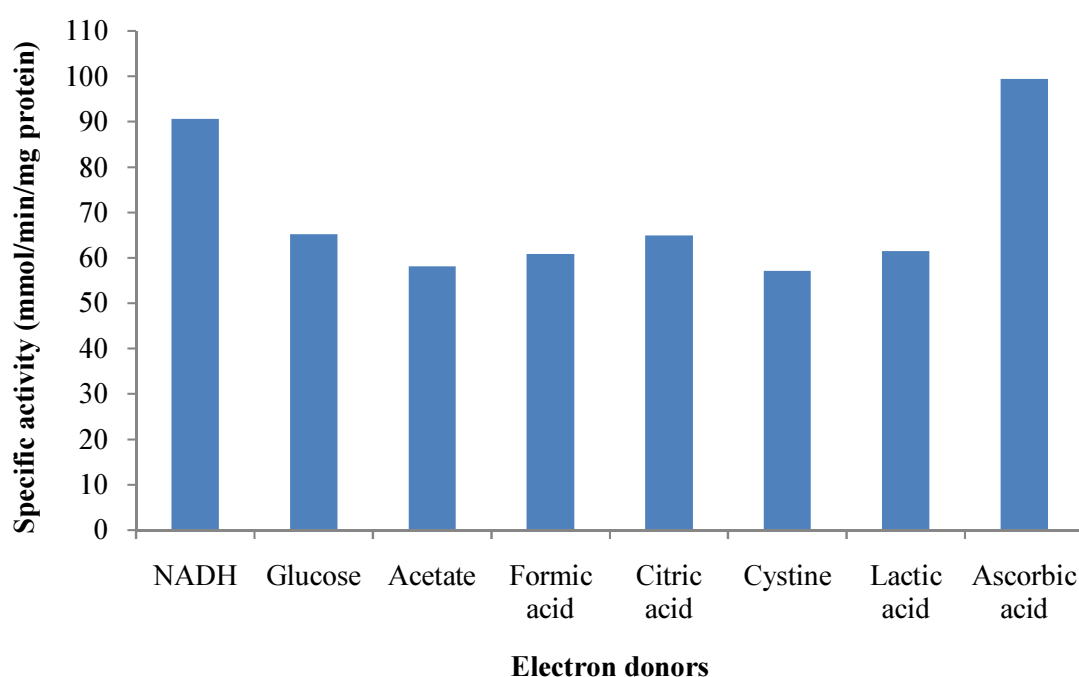
**Figure 16.** Effect of temperature on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 with initial concentrations of 28mM Cr (VI) at pH 7.0

to its affinity to –SH ligands, then suspecting the presence of this chemical group in the active site of the enzyme related to chromate reductase activity [70].



**Figure 17.** Effect of different metal cations on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 at pH 7.0 and 37°C

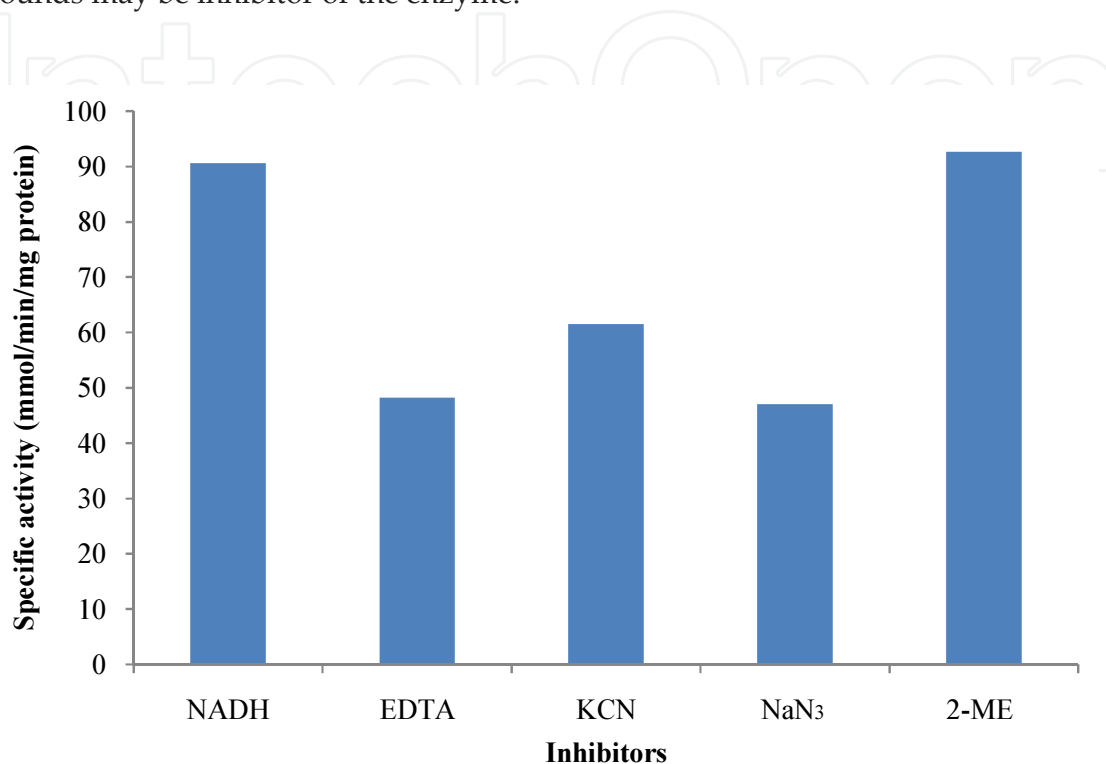
The reductase activity increased on supplementation in the reaction mixtures with electron donors. All the electron donors analyzed increased the activity, and the most efficient were ascorbic acid, NADH, glucose, and citrate by 4.4, 4.0, 2.9, and 2.87 times, respectively (Figure 18), and these results are like those reported for the yeasts *P. jadinii* M9 and *P. anomala* chromate reductase with NADH [60] and *Pseudomonas* sp. with citrate, acetate, glucose, and formate [58]. In previous reports of *Bacillus* sp., glucose has been reported to act as an electron donor and has been demonstrated to increase Cr (VI) reduction [72, 73], and also formate-dependent Cr (VI) reductases have been reported in *Shewanella putrefaciens* MR-1 [74]. Our work supports other studies reporting NADH or NADPH-dependent enzymatic reduction of Cr (VI) under aerobic conditions [58, 60, 67, 69, and 70]. Ramirez-Díaz et al. [75], report the oxidation of NADH donates an electron to the chromate reductase enzyme, and then the electron is transferred to Cr (VI) reducing it to the intermediate form Cr (V) which finally accepts two electrons from other organic substances to produce Cr (III).



**Figure 18.** Effect of different electron donors on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 at pH 7.0 and 37°C

Respiratory inhibitors like azide (1mM), EDTA (1mM), and cyanide (1mM) caused inhibitions of 48%, 47%, and 32%, respectively (Figure 19), in the Cr (VI) reductase activity; these results agree with those obtained in previous studies [66], and it has been observed that cyanide and azide partially inhibited purified chromate reductase of *E. coli* ATCC 33456 19 [67] and aerobic chromate reduction by *Bacillus subtilis* [71] and inhibited more than 50% of membrane-associated chromate reductase activity of *S. putrefaciens* MR-1 [74], while no inhibition was observed in CFE of *Bacillus* sp. ES29 [70]. Respiratory inhibitors act on de novo protein synthesis or affect the respiratory chain intermediates responsible for Cr (VI) reduction,

wherein Cr (VI) serves as a terminal electron acceptor [69]. As shown in Figures 15 and 16, the optimal pH and temperature of chromate reductase in *Penicillium* sp. IA-01 were pH 7 and 37°C, and the results were supported by the results of living cells. Therefore, the reduction is mainly occurred to remove Cr (VI) and to show resistance to high concentration of Cr (VI). Whereas, addition of electron donors caused the decrease of the activity, and therefore, these compounds may be inhibitor of the enzyme.



**Figure 19.** Effect of different inhibitors on Cr (VI) reductase activity in cell-free extracts of *Penicillium* sp. IA-01 at pH 7.0 and 37°C

#### 4. Conclusion

We isolated a *Penicillium* sp. IA-01 strain, which grow about 50% relative to control (85 mg of dry weight without metal) in LMM, probably is resistant to the metal, and also removes efficiently 1g/100 mL of Cr (VI) after 90 min of incubation, and removes 63.2% and 43% from soil and water samples contaminated, respectively. This strain showed an efficient capacity of reduction (91%) of 50mg/L Cr (VI) in the growth medium after seven days of incubation, at 28°C, pH 5.3, 100 rpm and with an inoculum of 169mg of dry weight. The Cr (VI) reduction potential of the resting cells was increased by cell permeabilization. The optimum temperature and pH of chromate reductase activity of the CFE, were found to be 37°C and 7.0, respectively, and activity was enhanced in the presence of 0.1mM NADH and other electron donors. 1mMol of metal ions like Cu<sup>2+</sup>, Na<sup>+</sup>, Hg<sup>2+</sup>, Mg<sup>2+</sup>, Fe<sup>3+</sup>, Ca<sup>2+</sup>, and Cd<sup>2+</sup> and respiratory inhibitors resulted in a decrease of the activity. Finally, these results suggest the potential applicability of *Penicillium* sp for the remediation of Cr (VI) from polluted soils and waters.

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