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Bioreduction of hexavalent chromium by *Hypocrea tawa* in a concentric draft-tube airlift bioreactor

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ORIGINAL RESEARCH ARTICLE

ABSTRACT

A fungal strain (*Hypocrea tawa*) was batch-cultivated in a concentric draft-tube airlift bioreactor to evaluate its potential to reduce highly toxic and water-soluble hexavalent chromium [Cr(VI)] to much less toxic and less mobile trivalent chromium [Cr(III)]. *H. tawa* exhibited a remarkable capacity to reduce very high Cr(VI) concentrations (4.54 mM) (overall efficiency of Cr(VI) reduction = 100%) completely under aerobic conditions. Furthermore, high volumetric and specific rates of Cr(VI) reduction were attained in the airlift bioreactor. The performance characteristics of *H. tawa* for Cr(VI) reduction in a concentric draft-tube airlift bioreactor indicate that this fungal strain may have potential applications related to the detoxification of Cr(VI)-contaminated wastewaters.

KEYWORDS

airlift bioreactor; Cr(VI) reduction; hexavalent chromium; *Hypocrea tawa*

1. INTRODUCTION

An increase in industrial and technological anthropogenic activities has resulted in the release of heavy metal contaminated wastewaters into the environment, which has caused serious damage to terrestrial and aquatic ecosystems, as well as to human health (Netzahuatl-Muñoz et al., 2012). Among different heavy metals, chromium [Cr] is one of the most widely used in industrial processes and is therefore the most common pollutant in industrial wastes (Wang, 2000).

Chromium exists in nature in nine oxidation states, from -2 to +6 (Jacobs and Testa, 2005). Of these, the most stable, common and ecologically important states are the trivalent [Cr(III)] and hexavalent [Cr(VI)] forms (Francisco et al., 2002). Both of these oxidation states differ widely in biological, physicochemical, geochemical and toxicological properties (Morales-Barrera et al., 2008a; Joutey et al., 2015). Cr(VI) is of the most concern because it is highly soluble in water, mobile in the environment, toxic, mutagenic, genotoxic, carcinogenic and teratogenic (Guertin, 2005; Stanin, 2005). In addition, it is the widely used in industrial processes including leather tanning, textile dyeing, metal cleaning, mining, plating and electroplating, corrosion inhibition, wood preservation, as well as the manufacture of dyes, pigments, photographic film, electronics and electronic equipment (Testa, 2005; Aranda-García et al., 2014). In contrast, Cr(III) is less soluble in water, less mobile in the environment, less bioavailable, 100 times less toxic and 1000 times less mutagenic and cytotoxic than Cr(VI) (Netzahuatl-Muñoz et al., 2010). It is considered an essential trace element in the diet of animals and humans (Lopez-Nuñez et al., 2014; Parveen et al., 2015). Due to these distinguishing toxicity and water-solubility properties of Cr(III) and Cr(VI), a reduction of Cr(VI) to Cr(III) has been proposed as a key remediation technology

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for Cr(VI)-contaminated water and wastewater.

The reduction of Cr(VI) to Cr(III) can be achieved by chemical or biological means. However, the biological form is a better option because it is cost-effective, environmentally friendly and produces less sludge (Camargo et al., 2003; Liu et al., 2006; Joutey et al., 2015).

Microorganisms can reduce Cr(VI) to Cr(III) via several mechanisms, including enzymatic reactions, indirectly with the production of organic or inorganic reducing metabolites, reducing reactions that occur on microbial surfaces and other methods (Hawley et al., 2005; Juvera-Espinosa et al., 2006). The microbial transformation of Cr(VI) to Cr(III) has gained increasing interest among scientists and engineers because this process may not only mitigate Cr(VI) toxicity in living organisms but also facilitate the precipitation of chromium at near-neutral pH in the form of $Cr(OH)_3$ for further physical removal (Morales-Barrera et al., 2008a). A microbial Cr(VI) reduction process has been considered as an economically feasible treatment method for the detoxification of Cr(VI)-contaminated water and wastewater (Cheung and Gu, 2003).

A wide variety of microbial species have been reported to reduce Cr(VI) to Cr(III) under aerobic and/or anaerobic conditions (Wang, 2000; Camargo et al., 2003; Cheung and Gu, 2003; Villegas et al., 2013). However, most previously studied microbial strains are capable of reducing only low Cr(VI) concentrations (commonly ≤ 0.5 mM) (Pal, 1997; Pattanapipitpaisal et al., 2001; QuiIntana et al., 2001; Cheung and Gu, 2003; Dmitrenko et al., 2003; Konovalova et al., 2003; Stasinakis et al., 2003 & 2004; Pazouki et al., 2007), which makes the microbial treatment of Cr(VI)-polluted wastewater difficult and inefficient (Pattanapipitpaisal et al., 2001). The efficient microbial bioremediation of Cr(VI)-contaminated soil and water requires that the chosen microorganisms can tolerate and reduce high Cr(VI) levels because this would keep treatment costs and metal toxicity low (Morales-Barrera et al., 2010).

Recently, a novel Cr(VI)-reducing fungal strain, identified as *Hypocrea tawa*, was isolated and studied in our laboratory for its ability to reduce Cr(VI) to Cr(III) in flasks. The fungal strain showed a remarkable ability to reduce high Cr(VI) concentrations when it is grown in flasks under aerobic conditions (Morales-Barrera et al., 2008a). However, the growth and Cr(VI) reduction ability of this fungal strain are adversely affected when it is cultured in mechanically agitated bioreactors, which could be because mycelial cells are very sensitive to the shear stress created by the mechanical stirrers. Therefore, the use of pneumatically agitated bioreactors is proposed for the culture of the *H. tawa* fungal strain in this study to minimize physical damage to the fungal cells and ensure an efficient rate of Cr(VI) reduction.

The primary goal of this work is to assess Cr(VI) bioreduction potential of *H. tawa* in a concentric draft-tube airlift bioreactor.

2. MATERIALS AND METHODS

2.1. Microorganism

A fungal strain that was isolated from a contaminated surface water source (Los Remedios River, in the Mexico City area) and molecularly identified as *Hypocrea tawa* was used throughout this work. This fungal strain was obtained from the Culture Collection of the Biochemical Engineering Department of the National School of Biological Sciences, National Polytechnic Institute, Mexico City, Mexico. The *H. tawa* strain was maintained on potato dextrose agar (PDA; BD Bioxon, Mexico) plates and routinely sub-cultured every 4 weeks. Inoculated plates were incubated at 28 ºC for 4 - 6 days and then stored at 4 ºC.

2.2. Culture medium

The liquid culture growth medium of *H. tawa* contained 10 g of glucose, 3 g of $(NH4)_2SO_4$, 1 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl, 0.05 g of $CaCl_2$, 0.01 g of yeast extract and 1 mg of $FeCl_3$ per liter of distilled water (Morales-Barrera et al., 2008a). The culture medium was sterilized using autoclave at 121 ºC for 20 min, and subsequently allowed to cool to room temperature. Later, a predetermined volume of sterile stock solution containing 20 g/L K_2CrO_4 (99.9% purity) was added to obtain the desired Cr(VI) concentration. The initial pH of the culture media was 6.0 ± 0.1. All chemicals used for the preparation of the culture media were of analytical grade and supplied by J.T. Baker (Mexico).

2.3. Development of the inoculum

The *H. tawa* inoculum was grown in 1000 mL Erlenmeyer flasks containing 200 mL of culture medium with an initial Cr(VI) concentration of 1 mM (≈ 52 mg/L). The flasks were shaken continuously

at 54 rpm and 28 ºC for 50 h. The obtained fungal biomass was aseptically separated by filtration through a nylon cloth. It was then washed twice with sterile distilled water to eliminate medium components and cell debris. The biomass was then re-suspended in a small volume of culture medium without Cr(VI), and the resulting suspension was homogenized. A sample of the biomass suspension was used as inoculum for every Cr(VI) reduction experiment performed in this study.

2.4. Cr(VI) reduction experiments in a concentric draft-tube airlift bioreactor

Cr(VI) reduction experiments were performed in a concentric draft-tube airlift bioreactor, with a working volume of 1.4 L. A diagram of the reactor is shown in Figure 1. The lid, base and column of the bioreactor were made of Pyrex glass. The cylindrical reactor column has an internal diameter of 95 mm and a total height of 270 mm. There was a concentric glass tube inside the column, which has an internal diameter of 78 mm and a total height of 120 mm. The top and bottom clearance were 62.5 and 15 mm, respectively.

The lid has ports for the addition of the inoculant, acid and alkali, for the supply of culture medium and to introduce oxygen and pH electrodes. The column also includes ports, through which culture samples were taken.

The column was joined to the lid and base by stainless steel screws that connect segmented Nylamid flanges, so that the glass lips of the column are fastened between two flanges. The internal surfaces of the flanges were grooved to accommodate sterilizable silicon rubber O-rings.

At the center of the base of the reactor, there was a porous glass diffuser with a 72-mm diameter and a pore size of 30-40 μm. The bioreactor was sterilized by autoclaving at 121 ºC for 20 min. The air input was controlled at 4.0 kg/cm² through a pressure-regulating valve, and the airflow rate was measured with a Carboloy-float rotameter. An airflow rate of 5.0-5.3 L/min was used.

The experiments in the concentric draft-tube airlift bioreactor were conducted at room temperature (22 ± 2 ºC), and the pH of the culture medium was not controlled throughout the course of the experiments. The initial biomass concentration of all batch cultures was 0.9-1.2 mg (dry weight)/mL. Culture samples were collected at different incubation times and filtered to determine residual Cr(VI) and total chromium concentrations. Batch cultures were analyzed until no measurable Cr(VI) concentration could be detected in the culture medium. Moreover, fungal cells were cultured in a medium lacking glucose and yeast extract to determine if Cr(VI) reduction required metabolic activity.

All batch experiments were performed in triplicate and the mean values were reported.

Cr(VI) reduction performance of *H. tawa* in the concentric draft-tube airlift bioreactor was evaluated according to the following three criteria:

$$\text{Cr(VI reduction efficiency (E\%)}: \frac{C_0 - C_t}{C_0} \times 100 \quad (1)$$

$$\text{Overall volumetric rate of Cr(VI) reduction}: \frac{C_0 - C_F}{t_F - t_0} \quad (2)$$

$$\text{Overall specific rate of Cr(VI) reduction}: \frac{C_0 - C_F}{X_0 (t_F - t_0)} \quad (3)$$

where C_o is the initial Cr(VI) concentration [mg/L] at time t_o = 0 h, C_t is the residual Cr(VI) concentration [mg/L] at time t, C_F is the residual Cr(VI) concentration [mg/L] at time t_F, X_o is the initial biomass concentration [g/L] at time t_o = 0 h and t_F is the cultivation time [h] at which the residual Cr(VI) concentration was undetectable (less than 0.01 mg/L).

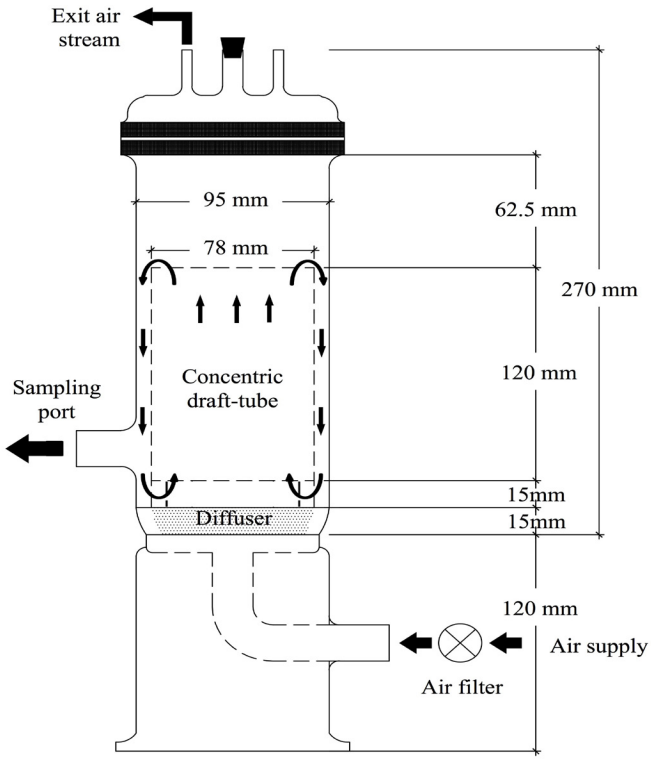

Figure 1. Schematic drawing of the concentric draft-tube airlift bioreactor.

2.5. Analytical techniques

Culture samples were filtered through previously weighed Whatman GF/A filters (1.6 μm). The filtrates were used to determine residual Cr(VI) and total chromium concentrations. Cr(VI) and total chromium concentrations in aqueous solutions were quantified via photocolorimetric methods using a Genesys™ 10 UV-Visible spectrophotometer (Thermo Electron Scientific Instruments Corporation), following the procedures outlined in the Hach Water Analysis Handbook (Hach Company, 2008). Cr(VI) concentrations in the filtrates were measured at 540 nm using the 1,5-diphenylcarbohydrazide method and a single dry powder formulation called ChromaVer3™ Chromium Reagent. This reagent contains an acidic buffer combined with 1,5-diphenylcarbohydrazide, which turns purple when Cr(VI) is present (Hach Company, 2008). Color intensity was directly proportional to the amount of chromium present in the samples.

Total chromium concentration in the filtrates (essentially, the sum of the trivalent [Cr(III)] and hexavalent [Cr(VI)] chromium) was determined using the alkaline hypobromite oxidation method (Hach Company, 2008). In this method, the Cr(III) present in the samples was oxidized to Cr(VI) by the hypobromite ion at boiling temperature under strong alkaline conditions. Then, the samples were acidified and the Cr(VI) concentration, which equals the total chromium concentration, was determined using the 1,5-diphenylcarbohydrazide method. Total chromium levels were then compared to those of non-inoculated controls.

Cr(VI) and total chromium concentrations were proportional to their optical absorbance and quantified by external standards, with a ten-point calibration curve.

3. RESULTS AND DISCUSSION

Batch cultures of *H. tawa* were performed in a concentric draft-tube airlift bioreactor containing culture media amended with increasing Cr(VI) concentrations, ranging from 1.57 to 4.54 mM (81.56 to 236.1 mg/L).

The *H. tawa* strain was capable of growing at all the initial Cr(VI) concentrations analyzed and formed large and heavy masses of aggregated mycelium. This caused a heterogeneous concentration of fungal biomass in the concentric draft-tube airlift bioreactor. Consequently, the *H. tawa* growth could not be suitably measured. These results indicate that the environmental conditions in the concentric draft-tube airlift bioreactor favored the formation of large mycelial aggregates, thereby affecting the growth patterns of *H. tawa*. These findings agree with previous reports that describe morphological changes in the fungal hyphae and formation of mycelial aggregates as a result of the hydrodynamic conditions in bioreactors (Znidarsic and Pavko, 2001; Morales-Barrera and Cristiani-Urbina, 2006).

Figure 2 depicts the Cr(VI) reduction curves obtained at different initial Cr(VI) concentrations. Note that as the experimental incubation time increased, the residual Cr(VI) concentration progressively decreased until no measurable Cr(VI) concentration could be detected in the culture media. Conversely, no significant changes were detected in the total chromium concentration in solution throughout the *H. tawa* incubation period, regardless of the initial Cr(VI) concentration. In fact, more than 97% of the total chromium initially added to the culture media was always present in solution (data not shown).

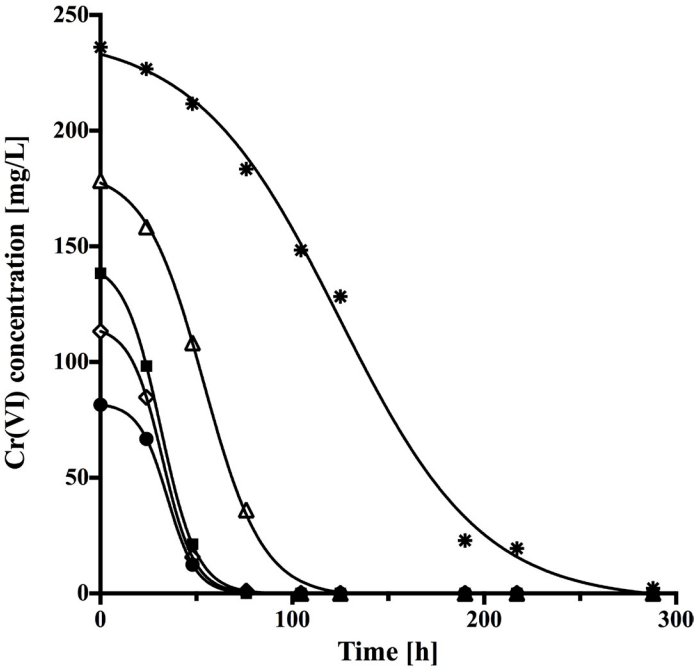

Figure 2. Cr(VI) variation profiles for batch cultures of *Hypocrea tawa* in a concentric draft-tube airlift bioreactor [Initial Cr(VI) concentration (mM): ●, 1.57; ◇, 2.18; ▪, 2.66; Δ, 3.43; *, 4.54].

The differences found between the residual Cr(VI) concentration and residual total chromium concentration of culture media indicate that *H. tawa* was able to reduce Cr(VI) to lower oxidation states.

Taking into consideration that the stable forms of chromium in the environment are trivalent and hexavalent, it seems more likely that *H. tawa* was able to reduce the highly soluble and toxic Cr(VI) to the much less soluble, mobile, toxic, mutagenic and cytotoxic Cr(III) (Cheung and Gu, 2003; Stanin, 2005; Villegas et al., 2013; Joutey et al., 2015). These results clearly demonstrate that this fungal strain is capable of transforming Cr(VI) to Cr(III), were in agreement with previous studies (Morales-Barrera et al., 2008a).

The experiments showed that the pH of the culture media dropped from 6.0 to about 3.8 as a result of the metabolic activity of *H. tawa* cells. As it is known that low pH values favor Cr(VI) reduction by organic matter (electron donors) and chromium adsorption by microbial cells, experiments with two different abiotic controls (biomass-free and heat-killed cell controls) were conducted previously at pH values ranging from 3.5 to 6.5 and initial Cr(VI) concentrations from 0.59 to 4.13 mM, in order to determine whether the pH affects Cr(VI) reduction or adsorption in the absence of living fungal cells. Results showed that neither Cr(VI) reduction by medium constituents nor adsorptive removal of Cr(VI) by fungal cells were significant, which indicates that the Cr(VI) reduction observed in the experiments conducted with viable *H. tawa* cells was not due to the pH changes that occurred during fungus growth and that the fungal cells did not adsorb chromium to any extent (Morales-Barrera et al., 2008a).

Furthermore, the incubation of *H. tawa* cells in culture media without any carbon or energy source (culture media without glucose and yeast extract), but in the presence of Cr(VI), exhibited no measurable changes in the Cr(VI) and total chromium concentrations. This suggests that carbon and energy sources were needed in the fungal growth media to provide the reducing power required for transforming Cr(VI) to Cr(III). This finding indicates that the observed Cr(VI) reduction was only due to the metabolic activity of *H. tawa* cells.

The Cr(VI) reduction efficiency obtained at different experimental incubation times is depicted in Figure 3. The Cr(VI) reduction efficiency increased as the experimental period progressed, reaching a level of 100% at all of the initial Cr(VI) concentrations analyzed. However, the time required to reach 100% Cr(VI) reduction efficiency was dependent on the initial Cr(VI) concentration. For initial Cr(VI) concentrations ranging from 1.57 to 2.66 mM, approximately 76 h were required to achieve 100% Cr(VI) reduction. Conversely, longer incubation times were needed for higher initial Cr(VI) concentrations as illustrated in Figure 3. An increase in the incubation time required for Cr(VI) reduction with increasing initial Cr(VI) concentration has also been reported in experiments with pure cultures of *Trichoderma inhamatum* (Morales-Barrera and Cristiani-Urbina, 2008b), *Trichoderma viride* (Morales-Barrera and Cristiani-Urbina, 2006), *Bacillus* sp., *Pseudomonas fluorescens* (Wang and Xiao, 1995), *Escherichia coli* (Shen and Wang, 1993) and activated sludge (Stasinakis et al., 2003).

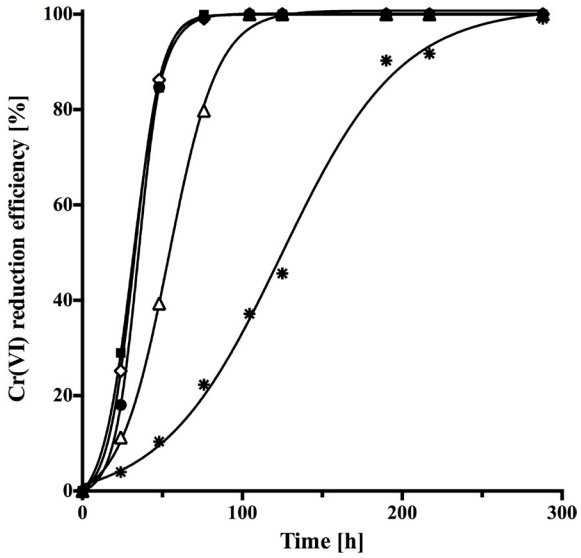

Figure 3. Profiles of Cr(VI) reduction efficiency [Initial Cr(VI) concentration (mM): ●, 1.57; ◇, 2.18; ▪, 2.66; Δ, 3.43; *, 4.54].

H. tawa was able to completely reduce Cr(VI) concentrations up to 4.54 mM. This concentration was similar to the highest Cr(VI) concentration (4.13 mM) reduced by this fungal strain in flasks (Morales-Barrera et al., 2008a). The concentration was also higher than that reduced by other fungi such as *T. viride* (Morales-Barrera and Cristiani-Urbina, 2006), *T. inhamatum* (Morales-Barrera and Cristiani-Urbina, 2008b), *Fusarium lichenicola* (Morales-Barrera et al., 2010), *Aspergillus* sp., *Penicillium* sp. (Acevedo-Aguilar et al., 2006), *Penicillium chrysogenum* (Pazouki et al., 2007), *Phanerochaete chrysosporium* (Pal, 1997), *Candida maltosa* (Ramírez-Ramírez et al., 2004), *Candida* sp. LMB2 (Juvera-Espinosa et al., 2006), *Candida* sp. FGSFEP (Guillén-Jiménez et al., 2009) and by bacterial consortia (Cheung and Gu, 2003; Francisco et al., 2002; Smith et al., 2002; Stasinakis et al., 2003) and pure bacterial cultures (Pattanapipitpaisal

et al., 2001; QuiIntana et al., 2001; Dmitrenko et al., 2003; Konovalova et al., 2003; Asatiani et al., 2004; Villegas et al., 2013). Nevertheless, it should be taken into account that the microbial capacity for Cr(VI) resistance and Cr(VI) reduction was dependent on the culture medium composition and cell density (Wang, 2000; Muter et al., 2001).

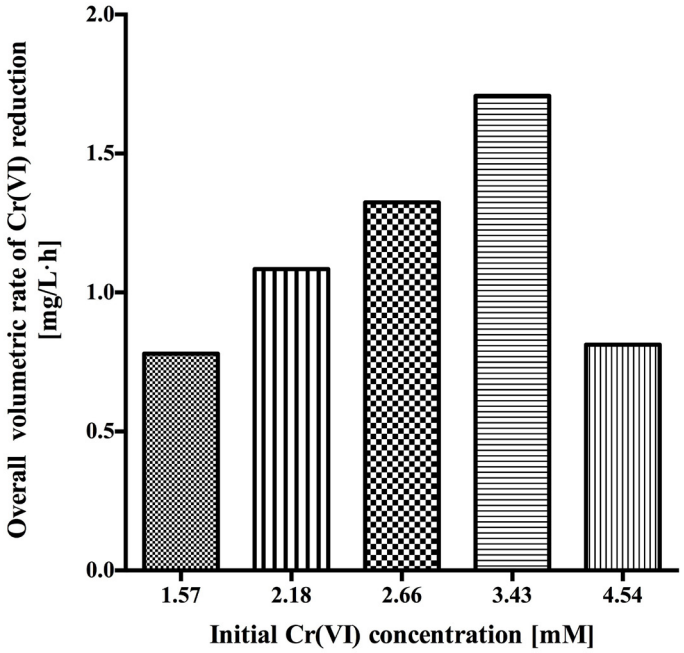

Figure 4. Effect of initial Cr(VI) concentration on overall volumetric rate of Cr(VI) reduction by *H. tawa* in a concentric draft-tube airlift bioreactor.

Figure 4 confirms that the overall volumetric rate of Cr(VI) reduction was highly dependent on the initial Cr(VI) concentration. As the initial Cr(VI) concentration increased from 1.57 mM to 3.43 mM, the volumetric rate of Cr(VI) reduction increased from 0.78 to 1.71 mg Cr(VI)/L.h. However, at the highest Cr(VI) concentration (4.54 mM), the volumetric rate decreased to 0.812 mg/L.h. This may be attributed to the toxic effects of Cr(VI) on fungal cells. Similarly, in a study conducted with a bacterial consortium, Chirwa and Wang (2000) found that the volumetric Cr(VI) reduction rate of 0.95 mg/L.h observed at 0.385 mM decreased at initial Cr(VI) concentrations higher than 0.385 mM. Likewise, *F. lichenicola* was capable of reducing up to 2.06 mM at an average volumetric rate of approximately 1 mg/L.h. However, at higher initial Cr(VI) concentrations, the volumetric rate decreased (Morales-Barrera et al., 2010). In contrast, the volumetric rate of Cr(VI) reduction exhibited by *P. chrysosporium* decreased as the initial Cr(VI) concentration increased from 0.01 to 0.45 mM, where the maximum rate of Cr(VI) reduction was approximately 0.11 mg Cr(VI)/L.h which was obtained at 0.01 mM initial Cr(VI) concentration (Pal, 1997). Likewise, the volumetric Cr(VI) reduction rate by *T. inhamatum* (Morales-Barrera and Cristiani-Urbina, 2008b) and *T. viride* (Morales-Barrera and Cristiani-Urbina, 2006) decreased as the initial Cr(VI) concentration increased, which was attributed to metal toxicity. It has been proposed that Cr(VI) is toxic to microbial cells because it is capable of inducing several cellular stress responses, as well as damaging different cellular components, such as nucleic acids, proteins and membranes (Labra et al., 2004; Ksheminska et al., 2005). The above results clearly show that *H. tawa* has the ability to reduce high Cr(VI) concentrations at a high rate, which makes this fungal strain potentially attractive for the bioremediation of Cr(VI)-polluted wastewater.

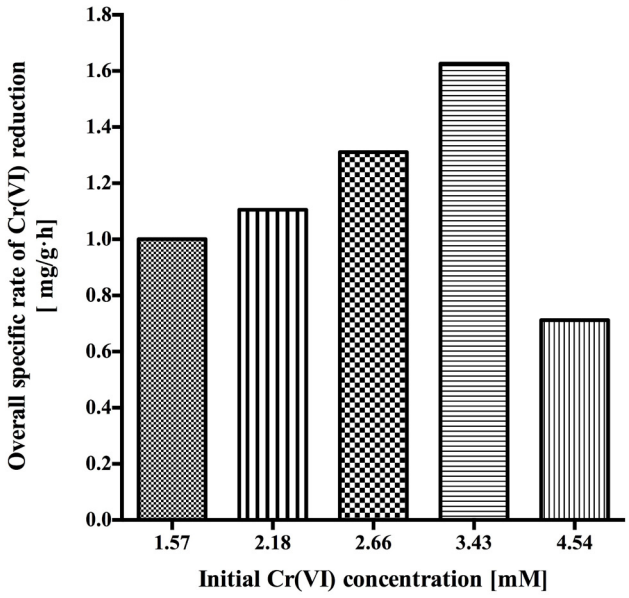

Figure 5. Dependence of the specific rate of Cr(VI) reduction of *H. tawa* in a concentric draft-tube airlift bioreactor on initial Cr(VI) concentration.

The specific rate of Cr(VI) reduction was calculated on the basis of the initial biomass concentration (Eq. (3)) and was found to reach a maximum at an initial Cr(VI) concentration of 3.43 mM (Figure 5). In contrast, a linear correlation between the specific rate of Cr(VI) reduction and initial Cr(VI) concentrations was reported for activated sludge, showing values for specific Cr(VI) reduction rate from 0.005 to 0.08 mg Cr(VI)/g.h, with initial Cr(VI) concentrations ranging from 0.0096 to 0.096

mM (Stasinakis et al., 2004). Likewise, the specific rate of Cr(VI) reduction exhibited by *T. inhamatum* decreased as the initial Cr(VI) concentration rose from 0.83 to 2.43 mM (Morales-Barrera et al., 2008b).

These results show that *H. tawa* exhibits a high capacity to tolerate and reduce very high Cr(VI) concentrations at a high rate when cultivated in a concentric draft-tube airlift bioreactor. Therefore, it could potentially be used for the detoxification of Cr(VI)-laden wastewaters.

4. CONCLUSIONS

The Cr(VI) reduction process, which was catalyzed by *H. tawa*, was characterized in batch cultures in a concentric draft-tube airlift bioreactor at initial Cr(VI) concentrations ranging from 1.57 to 4.54 mM. *H. tawa* was capable of reducing 100% of the Cr(VI) initially present in the culture media with high volumetric and specific Cr(VI) reduction rates obtained at high initial Cr(VI) concentrations. The results suggest that culturing *H. tawa* in a concentric draft-tube airlift bioreactor could be an attractive technological alternative for the aerobic detoxification of Cr(VI)-contaminated wastewater.

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