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The effect of sulphate and phosphate ions on Cr(VI) reduction by *Streptomyces* sp. MC1, including studies of growth and pleomorphism☆

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ABSTRACT

To help address conflicting opinions regarding the ability of chromate ions to use sulphate and phosphate membrane transporters to penetrate microbial cells, this work reports on an initial study related to the effect of these oxyanions on Cr(VI) removal by an actinobacterium, *Streptomyces* sp. MC1. Aspects related to growth and pleomorphism of this strain under Cr(VI) exposure are also presented. Although this strain was able to remove Cr(VI) from a liquid medium, significant decreases in both growth and filament branching were observed under metal exposure. The presence of sulphate and phosphate ions in the culture medium did not reverse the Cr(VI)-induced morphological transition. However, both ions mitigated the inhibitory effect of Cr(VI) on bacterial growth, and increased their removal from culture supernatants. Since total chromium concentration in the supernatant remained constant, this finding may indicate that sulphate and phosphate ions play a key role in the external reduction of Cr(VI) by *Streptomyces* sp. MC1, and that therefore Cr(VI) bioremoval could be optimized in terms of time and cost.

1. Introduction

Chromium is an element found in rocks, soil, plants, animals, volcanic dust, and gasses. It is the seventh most abundant element on earth, and it occurs in various oxidation states ranging from −2 to +6. Only trivalent chromium Cr(III) and hexavalent chromium Cr(VI) are ecologically important, since these are the most stable oxidation states in the natural environment (Cefalu and Hu, 2004). Cr(III) and Cr(VI) differ widely in their physicochemical properties and biological reactivity levels. Cr(III) is an essential trace element necessary for glucose, lipid, and amino acid metabolism, and is even a popular dietary supplement (Viamajala et al., 2004). However, at high concentrations, Cr(III) has been shown to have negative effects on cellular structures. Cr(VI) species are extremely water-soluble and mobile in the environment. They are recognized as being highly toxic, carcinogenic, mutagenic, and teratogenic for mammals, including humans (Flores and Perez, 1999). In-vivo studies have revealed that Cr(VI) is approximately 1000 times more cytotoxic and mutagenic than Cr(III) (Czakó-Vér et al., 1999).

Industrial effluents containing Cr(VI) are often released into natural water resources without proper treatment, resulting in anthropogenic contamination (Viti et al., 2003; Cefalu and Hu, 2004; Cheung and Gu, 2007). Conventional treatment processes for chromium detoxification generally involve aqueous reduction of Cr(VI) by a reductant and subsequent pH adjustment to neutral ranges in order to precipitate the less soluble Cr(III). However, this process requires large amounts of chemicals and energy, and therefore is often not economically feasible. As a result, it has become critical to search for new techniques that can reduce heavy metal concentrations to acceptable environmental levels, at manageable costs.

The potential of certain taxa to degrade and detoxify particular contaminants can be used in microorganism-based bioremediation techniques (Colin et al., 2012). Biotransformation of Cr(VI) to

☆ This work is dedicated in memoriam to Prof. Carlos M. Abate, excellent partner and great teacher.

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Cr(III) with chromium bioaccumulation using various species of bacteria is the most pragmatic approach that has been found to date for chromium removal at contaminated sites (Megharaj et al., 2003; Camargo et al., 2004; Bae et al., 2005; Liu et al., 2006). Numerous chromium homeostasis mechanisms under anaerobic and aerobic conditions have been described. These processes include, on the one hand, Cr(VI) reduction outside of the cell, generating Cr(III) that is unable to cross cellular membranes. On the other hand, Cr(VI) uptake and subsequent reduction inside the cell have also been reported (Ramírez-Díaz et al., 2008). In relation to this, some authors have suggested that the chromate is able to use sulphate and phosphate transport routes to penetrate microbial cells, due to the chromate's structural similarity to these oxyanions (Brown et al., 2006; Ramírez-Díaz et al., 2008; Ünal et al., 2010). However, other researchers have reported on studies in which sulphate and phosphate ions have had no effect on Cr(VI) bioremoval (Wang and Xiao, 1995; Liu et al., 2006; Zakaria et al., 2007).

It has to be mentioned that the sulphate ion is a very common pollutant from the mining, food, fermentation, paper, and tanneries industries (Jong and Parry, 2003; Zhao et al., 2011). Meanwhile phosphate ions can also be considered a contaminant if the concentrations are high; these reach the environment through sewage containing household detergents and cleaning preparations, through agricultural effluents, and through effluent from the fertilizer, detergent, and soap industries (Pradyot, 1997; cited by Krishnaswamy et al., 2011). Thus it is necessary to know whether the presence of sulphate or phosphate ions, which are often present in effluent or polluted sites, interferes with Cr(VI) removal processes of bacterial cells.

Streptomyces sp. MC1, an actinobacterium isolated from sugarcane from the province of Tucumán in Argentina (Polti et al., 2007), has shown ability to decrease Cr(VI) concentrations in a liquid medium, as well as in soil extracts and soil samples (Polti et al., 2009). In addition, effective chromate reductase activity has been detected in all cellular fractions of this strain (Polti et al., 2010). Although it has been demonstrated that Cr(VI) exposition may produce oxidative stress, and as a result, morphological changes linked to the stress level produced by metal exposure (Ackerley et al., 2006; Francisco et al., 2010; Ünal et al., 2010), there are no previous studies on pleomorphic changes of actinobacteria exposed to Cr(VI). Here we report on the first study on the effect of sulphate and phosphate ions on Cr(VI) removal by *Streptomyces* sp. MC1 into a liquid medium, as well as analyses of the macro and microscopic features of the growth, with the aim of further characterizing the physiological state of the organism under the study conditions.

2. Materials and methods

2.1. Microorganism, maintenance, and culture conditions

The microorganism used in this work was *Streptomyces* sp. MC1 (PROIMI Collection, NCBI accession number: AY741287), which is resistant to Cr(VI) (Polti et al., 2007). This strain was maintained on starch-casein agar slants (SC agar) containing (g l^{-1}): starch, 10.0; casein, 1.0; K_2HPO_4, 0.5; and agar, 12.0. The pH was adjusted to 7.0 prior to sterilization.

Growth and Cr(VI) removal assays were carried out in liquid minimal medium (MM) as formulated by Amoroso et al. (1998), modified for this study to contain (g l^{-1}): glucose, 10.0; L-asparagine, 0.5; K_2HPO_4, 0.5; $MgCl_2 \cdot 7H_2O$, 0.20; and $FeSO_4 \cdot 7H_2O$, 0.01. *Streptomyces* sp. MC1 spore suspensions (100 μl of 10^8 CFU ml^{-1}) from solid MM (1.2% agar) were inoculated in Erlenmeyer flasks with liquid MM (called control or C), and also supplemented with sulphate ions (called S) or phosphate ions (called P), to final concentrations of 5 mM. *Streptomyces* sp. MC1 cultivated under the same conditions, but with Cr(VI) to a final concentration of 20 mg l^{-1}, were labelled as CCr, SCr, and PCr, respectively. Sulphate, phosphate, and Cr(VI) were added to liquid MM as Na_2SO_4, K_2HPO_4, and $K_2Cr_2O_7$ from stock solutions. Is important to mention that at pH 7, chromate and dichromate ions coexist at a ratio of 1:1.

The cultures were incubated at 30 °C on an orbital shaker at 170 rpm. Samples were taken every 24 h. Cultures with Cr(VI) added but without the inoculums were included as abiotic controls, in order to verify that the components of the culture medium did not participate in the Cr(VI) reduction.

2.2. Determination of growth parameters

Samples were centrifuged at 10,000 g for 15 min at 4 °C, and cells were washed twice with bi-distilled water. Dry weight was determined using aluminum foil cups dried to constant weight at 80 °C (approximately 48 h). Supernatants were stored at −20 °C for analysis of residual glucose, Cr(VI), and total chromium concentration. Maximum specific growth rate (μ_{max}; h^{-1}) was calculated as the slope of the regression line of the natural logarithm of culture biomass versus time.

Residual glucose was measured using the dinitrosalicylic acid method as described by Miller (1959) and modified by Villegas et al. (2008). Readings were interpolated from a standard curve prepared using a series of glucose dilutions (0–1 g l^{-1}).

Specific glucose consumption (q_{glu}) was calculated based upon Eq. (1), where C_0 and C are the initial and residual glucose concentrations in the medium after a time interval, respectively, and X is the biomass concentration. This parameter was expressed as grams of glucose consumption per gram of biomass.

$$q_{glu} = [(C_0 - C)/X] \quad (1)$$

2.3. Measurement of chromium

Extracellular residual Cr(VI) was measured using a colorimetric reagent specific for Cr(VI), 1,5-diphenylcarbazide, dissolved in acetone at a final concentration of 0.05% (APHA, 1992). Absorbance was measured at 540 nm, and Cr(VI) concentration was calculated with a standard curve prepared using a series of Cr(VI) dilutions (1–25 mg l^{-1}).

Specific removal of Cr(VI) ($q_{Cr(VI)}$), expressed as milligrams of removed metal per gram of biomass, was calculated based on the equation (1) as previously described, where now C_0 and C are the initial and residual Cr(VI) concentrations after a time interval, respectively; and X is the biomass concentration. Total extracellular Cr concentration was also measured in the supernatants using an atomic absorption spectrophotometer.

2.4. Morphological characterization using digital image analysis

The morphology of *Streptomyces* MC1 sp. was characterized after 96 h of cultivation by using digital image analysis (Nikon Eclipse Net software, version 1.20). In order to analyse the macroscopic morphology, average diameters were determined for an appropriate number of bacterial floccules ($n \geq 100$). Cellular morphology was also analysed using differential interference contrast (DIC) images. Two microscopic parameters were determined according to Trinci (1974) for an appropriate number of hyphal elements ($n \geq 30$): hyphal diameter (D) and hyphal growth unit length (L/N), where L is total hyphal length and N is the

number of tips. Note that *L*/*N* is an inverse measurement of the degree of filament branching. Lengths of hyphal trees were obtained using a pre-set calibration in microns at a total magnification of 2500 ×.

2.5. Statistical analyses

Statistical analyses was performed using Infostat (version 2004) and Minitab (version 14) software for Windows, and results are presented here as mean ± standard deviation, with the assays carried out in triplicate. Statistical significance values for the means were evaluated using one-way analysis of variance. Subsequent comparisons were performed using Tukey's post-hoc test. Differences were accepted as significant when $p < 0.05$. Associations between variables were also assessed using Pearson's correlation coefficient.

3. Results

3.1. The effects of sulphate, phosphate, and chromium on growth parameters

Fig. 1 shows the growth kinetics of *Streptomyces* sp. MC1 during cultivation in MM, in the presence and absence of sulphate or phosphate, as well as in the presence and absence of Cr(VI). In the absence of Cr(VI), only after 72 h of cultivation was biomass concentration found to be significantly higher in S and P compared to C ($p < 0.05$) (Fig. 1A). At the end of each incubation period (120 h), bacterial growth had increased by 13% in S and 30% in P, when compared to the control (C).

The growth kinetics of *Streptomyces* sp. MC1 were also evaluated in parallel in media supplemented with 20 mg l^{-1} Cr(VI) (Fig. 1B).

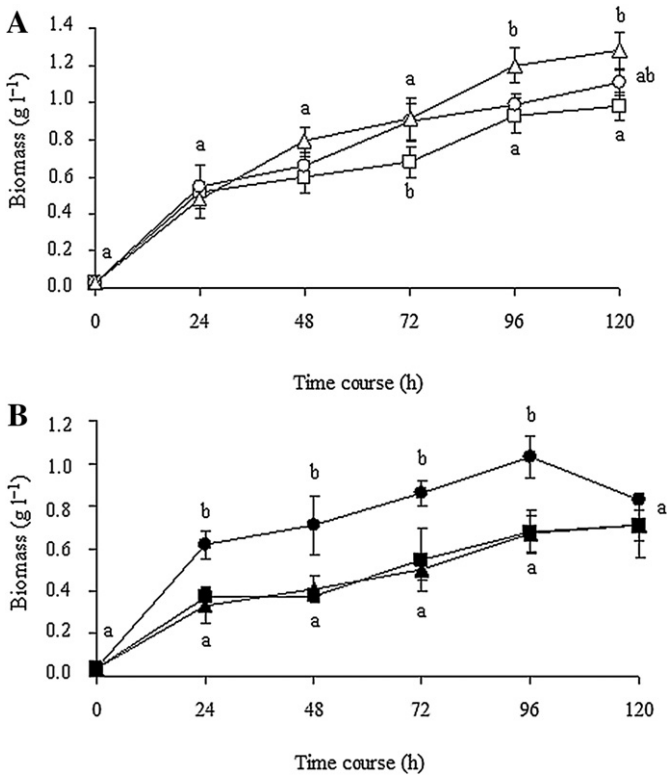

Fig. 1. Growth kinetics of *Streptomyces* sp. MC1 during cultivation in minimal medium either in the absence **(A)** or the presence **(B)** of 20 mg l^{-1} Cr(VI): (□/■) without sulphate and phosphate ions; (○/●) with 5 mM sulphate; (Δ/▲) with 5 mM phosphate. Error bars represent the standard deviation calculated from three independent experiments. The values with different letters are significantly different ($p < 0.05$).

Under the three conditions (CCr, SCr, and PCr), biomass concentration decreased significantly ($p < 0.05$) compared to the respective controls without added Cr (C, S, and P). Bacterial growth inhibition was substantially lower in SCr (7%–12%) than in CCr (27%–29%) and in PCr (31%–44%), when compared to their respective levels of growth without the added metal.

The μ_{max} values for *Streptomyces* sp. MC1 cultivated in MM, both in the presence and absence of Cr(VI), are showed in Table 1. According to statistical analysis, the μ_{max}, which was reached between 24 and 48 h of cultivation, showed no significant differences in the reference cultures C, S, and P ($p > 0.05$). However, in the presence of Cr(VI), the kinetic parameter decreased by 25% for CCr and 18% for PCr, compared to C and P, respectively, while in SCr, μ_{max} decreased by only 8% compared to S. Finally, after 48 h of cultivation, the growth rate values decreased significantly in all cases, reaching stationary phase after approximately 96 h of cultivation.

The q_{glu} by *Streptomyces* sp. MC1 in the presence and absence of Cr(VI) was estimated by measurement of residual glucose in the culture supernatants (Fig. 2). Glucose consumption by cells grown in CCr, SCr, and PCr (Fig. 2B) was markedly lower than in the reference cultures (Fig. 2A). After 48 h, q_{glu} decreased between 37 and 53% for CCr, 12–45% for SCr, and 20–80% for PCr. At the end of each incubation period, only 17%–28% of the total glucose was consumed by this strain in the presence of Cr(VI). Finally, the decrease in q_{glu} observed under conditions of exposure to Cr(VI) was correlated with a drop-off in the biomass concentration ($r = 0.806$; $p < 0.001$).

3.2. The effects of sulphate and phosphate oxyanions on chromium removal

The extracellular Cr(VI) concentration was determined in each supernatant during 120 h of cultivation (Fig. 3). In the three conditions studied (CCr, SCr, and PCr), Cr(VI) concentration decreased in a time-dependent manner, reaching the minimum values of residual Cr(VI) after 96 h of incubation (Fig. 3A). At the end of each incubation period, Cr(VI) removal was 20% for CCr, 50% for PCr, and 70% for SCr. It is important to point out that while in SCr residual Cr(VI) decreased from the beginning of cultivation, in PCr a significant level of Cr(VI) removal compared to CCr was observed only after 96 h of cultivation.

The $q_{Cr(VI)}$ increased in a time-dependent manner in CCr, SCr, and PCr, with maximum values reached at 96 h, after which the values remained constant (Fig. 3B). However, while CCr and PCr showed no significant differences between them until 120 h, in SCr this parameter was three to four times higher than for CCr throughout the entire assay. Surprisingly, total chromium concentration in the supernatant was only slightly reduced (about 5–7%)

Table 1
Maximum specific growth rate (μ_{max}) of *Streptomyces* sp. MC1 incubated in minimal medium.

Culture conditions	(μ_{max}; h^{-1})
C	0.12 ± 0.01^{a}
CCr	0.09 ± 0.004^{b}
S	0.12 ± 0.02^{a}
SCr	0.11 ± 0.04^{a}
P	0.11 ± 0.02^{a}
PCr	0.09 ± 0.01^{b}

C (minimal medium), S (minimal medium with sulphate added), P (minimal medium with phosphate added), CCr (minimal medium with chromium added), SCr (minimal medium with sulphate and chromium added), PCr (minimal medium with phosphate and chromium added). Results are presented as the mean ± standard deviation calculated from three independent experiments. The values with different letters are significantly different ($p < 0.05$).

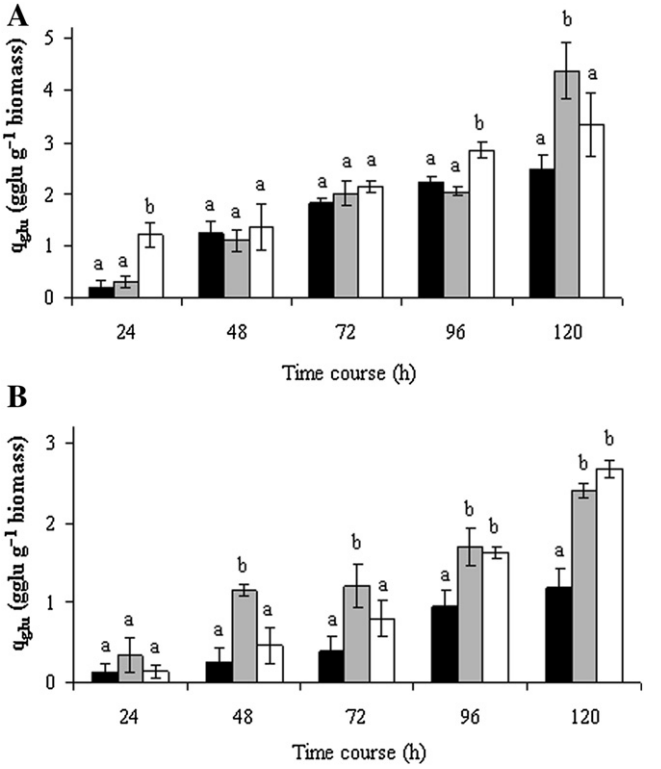

Fig. 2. Specific glucose consumption by *Streptomyces* sp. MC1 during cultivation in minimal medium either in the absence **(A)** or the presence **(B)** of 20 mg l^{-1} Cr(VI): (■) without sulphate and phosphate ions; (▨) with 5 mM sulphate; (□) with 5 mM phosphate. Error bars represent the standard deviation calculated from three independent experiments. The values with different letters are significantly different ($p < 0.05$).

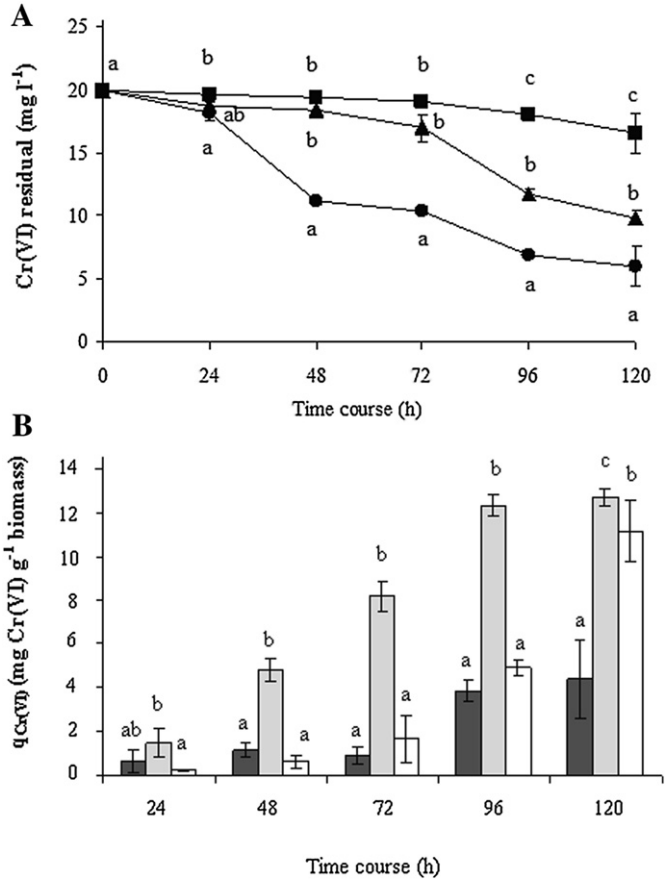

Fig. 3. Cr(VI) removal by *Streptomyces* sp. MC1 during cultivation in minimal medium either in the presence or the absence of sulphate or phosphate. **(A)** Residual Cr(VI): without sulphate and phosphate (■); with 5 mM sulphate (●); with 5 mM phosphate (▲). **(B)** Cr(VI) specific removal: Without sulphate and phosphate (■); with 5 mM sulphate (■); with 5 mM phosphate (□). Error bars represent the standard deviation calculated from three independent experiments. The values with different letters are significantly different ($p < 0.05$).

in the presence of sulphate ions. In the abiotic controls, concentration of Cr(VI) was not reduced, indicating that the bacteria was the factor responsible for the reduction activity. These results suggest that the reduction of Cr(VI) to Cr(III) occurs mainly outside of the microbial cell.

3.3. Characterization of pleomorphism upon chromium exposure

Although qualitative analysis of the morphological changes seen in *Streptomyces* sp. MC1 exposed to Cr(VI) has been performed in previous studies (Polti et al., 2011), this works presents the first quantitative morphological characterization of this strain after metal exposure. In terms of macroscopic analysis, the reference cultures (C, S, and P) did not exhibit significant differences between them. Under all three conditions, bacterial flocculation was observed, creating structures that ranged from "compact pellets" to loose mycelia aggregates that could best be described as "clumps" (Fig. 4A). In contrast, only compact pellets of a homogeneous size were observed in the cultures with Cr(VI) added (Fig. 4B).

In order to quantitatively characterize the macroscopic morphologies developed in *Streptomyces* sp. MC1, in both the presence and absence of Cr(VI), histograms were designed based upon the frequency distributions of the mean largest diameter of bacterial floccules (Fig. 5). In the absence of metal, a mixture of small pellets and prominent clumps was observed. These structures had mean diameters of 0.042 ± 0.006 μm in pellets and 0.084 ± 0.006 μm in clumps. In the presence of Cr(VI), the degree of bacterial flocculation increased, and only compact pellets of a homogeneous size (0.042 ± 0.006) were observed. On the basis of these results, bacterial floccules obtained in the absence of metal were looser and larger than those developed in the presence of Cr(VI).

In terms of the cellular morphology of *Streptomyces* sp. MC1, the presence of Cr(VI) in the MM encouraged unavoidable morphological changes (Fig. 6). Filaments not exposed to the metal (Fig. 6A) were more branched than those exposed to Cr(VI) (Fig. 6B). This was reflected in the *L/N* values, which increased by 41–50% in the presence of Cr(VI), compared to the cultures without Cr(VI) added (Table 2). In this connection, the increase in the *L/N* values observed in Cr(VI)-exposed filaments was correlated with the decrease in the q_{glu} ($r = -0.824$; $p < 0.001$). Finally, *D* values were not significantly modified by the presence of Cr(VI) in the MM.

4. Discussion

During the past 30 years, bioremediation has emerged from the laboratory to become a fully commercialized technology in many industrialized countries. Since the discovery of the first microbe capable of reducing Cr(VI) (Romanenko and Korenkov, 1977), the search for new routes of biological detoxification using both aerobic and anaerobic microorganisms has been enthusiastically pursued (Shanker et al., 2005; Zakaria et al., 2007). In previous studies, Polti et al. (2009, 2010, 2011) reported on the ability of *Streptomyces* sp. MC1 to decrease Cr(VI) concentrations in a variety of systems. In order to improve the efficiency of a potential biological approach to Cr(VI) detoxification using this strain, we have studied the effects of sulphate

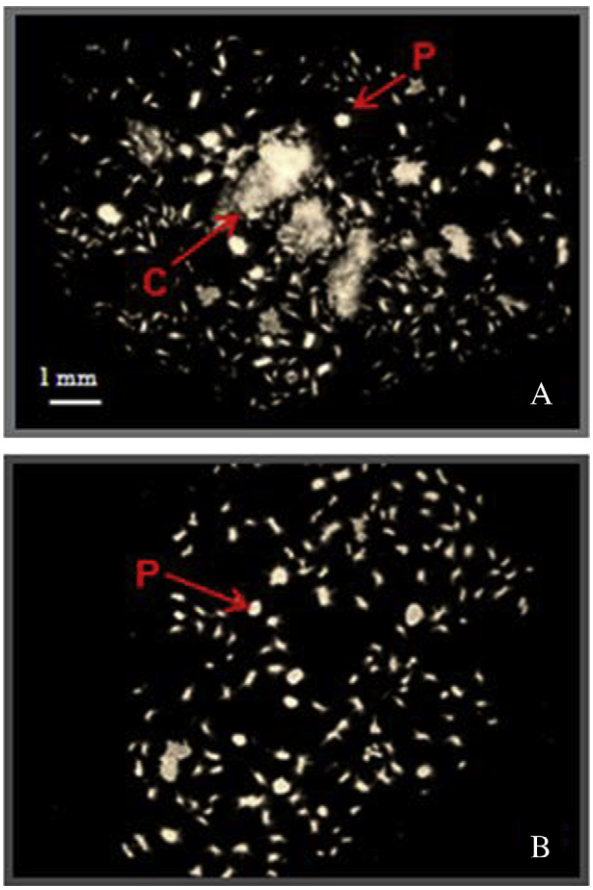

Fig. 4. Macroscopic morphology of *Streptomyces* sp. MC1 cultivated in minimal medium either in the absence **(A)** or the presence **(B)** of 20 mg l^{-1} Cr(VI). (C): Clumps; (P): Pellets. Both panels are at the same magnification, with the bar in **(A)** representing 1 mm.

and phosphate ions, frequent co-contaminants of Cr(VI), on the growth of *Streptomyces* sp. MC1 and on its ability to remove Cr(VI).

Our results revealed that in the absence of Cr(VI), both ions slightly increased the biomass production of *Streptomyces* sp. MC1, although they had no effect on growth rate (Table 1). As expected, the presence of Cr(VI) in the culture medium negatively affected the growth parameters of *Streptomyces* sp. MC1 (biomass concentration, μ_{max}, and q_{glu}), as was similarly reported for several other bacterial genera such as *Azotobacter*, *Bacillus*, *Pseudomonas*, and *Escherichia* (Kong et al., 2009; Parameswari et al., 2009), and filamentous fungi and yeasts (Dursun et al., 2003; Fernández et al., 2010).

In terms of chromium homeostasis, the presence of phosphate and, to a greater degree, sulphate ions in the MM, the growth of *Streptomyces* MC1 was accompanied by a decrease in Cr(VI) concentrations in the culture supernatants; however, extracellular total chromium concentrations were not significantly modified. This finding may indicate that, under the current assay conditions, the drop-off in Cr(VI) concentration is mainly due to extracellular reduction to Cr(III). Aerobic Cr(VI) reduction is normally associated with a soluble protein fraction utilizing NADH or NADPH as the electron donor. This aerobic reduction, which takes place either internally or externally to the plasma membrane, is considered to be a detoxification mechanism for Cr(VI), and was reported for first time in *Escherichia coli* (Shen and Wang, 1993).

Several authors have assumed that active transport of chromate in bacteria is performed across membranes using the sulphate uptake route, and have pointed out that the chromate is a competitive inhibitor of sulphate transport in a variety of bacterial species studied. Ohtake et al. (1987) noted the ability of sulphate to protect growing *Pseudomonas fluorescens* cells from the inhibitory effects of chromate, suggesting a direct competition between both oxyanions for the same transport carrier; however, the chromate resistance in *P. fluorescens* was related to a reduced uptake of Cr(VI). On the other hand, Branco et al. (2004) showed that growth of

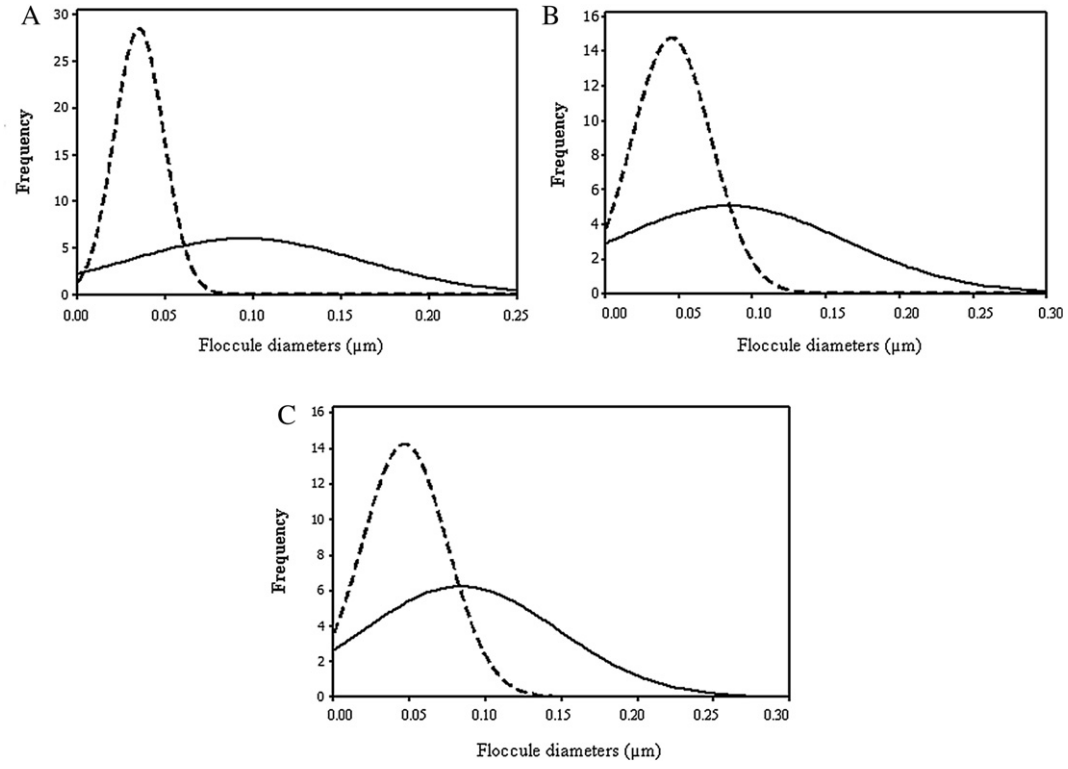

Fig. 5. Histograms showing the distribution of the mean largest diameter of bacterial floccules ($n = 100$) from *Streptomyces* sp. MC1, obtained in minimal medium either in the absence (—) or the presence (– –) of 20 mg l^{-1} Cr(VI). **(A)** Without sulphate and phosphate ions. **(B)** With 5 mM sulphate. **(C)** With 5 mM phosphate.

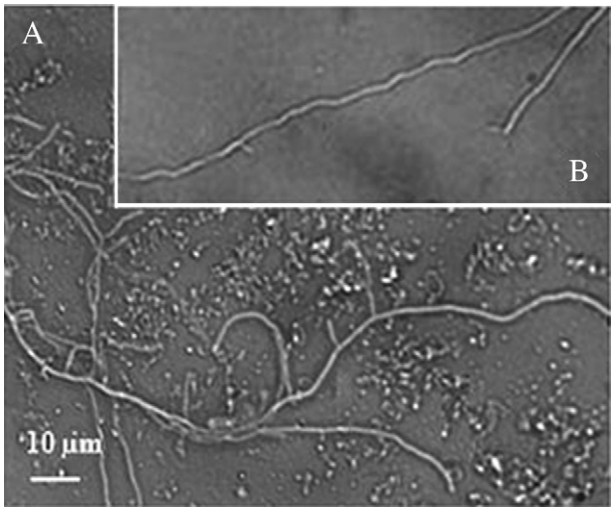

Fig. 6. Differential interference contrast images of *Streptomyces* MC1 sp. cells cultivated in minimal medium either in the absence **(A)** or the presence **(B)** of 20 mg l^{-1} Cr(VI). Both panels are at the same magnification, with the bar in **(A)** representing 10 µm.

Ochrobactrum tritici 5bvI1 was independent of concentrations of sulphate in the culture medium and the presence of this oxyanion did not produce any protective effect to the cells exposed to Cr(VI), as was suggested. Neither the presence of sodium azide (metabolic inhibitor), PO_4^{3-}, SO_4^{2-}, SO_3^{2-}, NO_3^-, or Pb(II), Zn(II), or Cd(II) ions resulted in a significant decrease in Cr(VI) reduction by *Acinetobacter haemolyticus* (Zakaria et al., 2007). Meanwhile, Guillén-Jiménez et al. (2008) reported that despite the fact that the presence of sulphate had no stimulatory or inhibitory effect on *Candida* sp. FGSFEP growth, higher volumetric efficiencies and rates of Cr(VI) reduction exhibited by this strain were obtained at higher concentrations of sulphate. These results are in good agreement with ours in relation to Cr(VI) reduction.

Regarding the *Streptomyces* genus, there is little background found in the literature about the influence of sulphate or phosphate ion in the removal of Cr(VI), with the exception of two studies on *Streptomyces griseus*. Laxman and More (2002) studied the deletion of some constituents of the medium, reporting that $MgSO_4$ did not significantly influence the growth of the bacterium or the reduction of Cr(VI). Similarly, it was later found that sulphate, nitrate, chloride, and carbonate had no effect on chromate reduction during growth, while the presence of Cd, Ni, Co, and Cu were inhibitory to various degrees (Poopal and Laxman, 2009).

Although the putative competition for the membrane transporters between sulphate, phosphate, and Cr(VI) species still needs to be demonstrated at the molecular level in most microbial systems, our results led us to conclude that the presence of both sulphate and, to a minor degree, phosphate, allow the species of Cr(VI) to remain free, and therefore available for enzymatic reduction by *Streptomyces* sp. MC1.

Laxman and More (2002) have also proposed that *S. griseus* was able to reduce Cr(VI) using glucose as the energy and/or electron donor. Likewise, Orozco et al. (2007) reported that the microbial consortium of an activated sludge was capable of removing chromium via its reduction to Cr(III) only if a suitable electron donor (such as lactose) was available. It was also observed that the specific glucose consumption of three indigenous yeast strains increased with the addition of Cr(VI) to the culture medium (Villegas et al., 2008). However, no correlation between specific glucose consumption and Cr(VI) specific removal was detected in this study.

Therefore, in the system analysed here, the results obtained are in agreement with those performed in soil where were reported study that the oxyanionic species of Cr(VI) compete with sulphate by the same adsorption sites (Zachara et al., 1989; Zayed and Terry, 2003). This supports our hypothesis that no active transport of Cr(VI) occurred and that free Cr(VI) is reduced enzymatically by *Streptomyces* sp. MC1 in the extracellular medium.

We have also quantitatively analysed the morphological changes undergone by *Streptomyces* sp. MC1 during Cr(VI) removal, in both the presence and absence of sulphate or phosphate ions. Gram-positive, soil-dwelling actinobacteria such as those from the genus *Streptomyces* are a typical example of microorganisms with an interesting life cycle, resembling in certain aspects that of lower eukaryotes such as filamentous fungi. Maturation and later spore germination will result in a new mycelium (Worrall and Vijgenboom, 2010). In liquid media, filamentous microorganisms can flocculate to form mycelial structures that range from “compact pellets” to mycelia aggregates or “clumps” (Papagianni, 2006). The macroscopic growth of *Streptomyces* sp. MC1 resulted in a remarkably increased bacterial flocculation in the presence of Cr(VI) in the culture media with the formation of small and compact pellets. The morphological pattern can affect the rheological properties or physical properties of the culture, and consequently the productivity of the process (Grimm et al., 2005). According to Ünal et al. (2010), such pelleted systems could potentially be useful in wastewater treatment for removal of polluting metals.

In terms of cellular morphology, there are reports in the literature of inevitable microscopic alterations resulting from chromium toxicity in microorganisms. Ackerley et al. (2006), for example, reported on morphological alteration of *E. coli* K-12 exposed to chromate, which exhibited extreme filamentous morphology. Chourey et al. (2006) studied the cellular morphology of *Shewanella oneidensis* MR-1 exposed to Cr(VI), as compared to non-exposed control cells. In contrast to the untreated control cells, Cr(VI)-exposed cells formed apparently aseptate, non-motile filaments that tended to aggregate. Francisco et al. (2010) found that the cells of *Ochrobactrum tritici* exposed to dichromate also showed morphological alterations compared to the morphology of control cells. In filamentous microorganisms such as the *Streptomyces* sp. MC1 actinobacterium, cellular morphology can vary from linear filaments to highly branched structures. Under our assay conditions, a significant decrease in filament branching was observed in Cr(VI)-exposed cells. The branching process is a dynamic and complex mechanism that involves the coordinated actions of numerous enzymes and cytoplasm precursors (Barreteau et al., 2008; Bouhss et al., 2008), where the initiation of new branching could become challenging for the cells under inhospitable conditions. Therefore, the decrease in the branching degree of Cr(VI)-exposed filaments could be an adaptive response by stressed

Table 2
Microscopic parameters for *Streptomyces* sp. MC1 developed in minimal medium after 96 h of cultivation.

Culture conditions	Microscopic morphological parameters			
	D (µm)	*N*	*L* (µm)	*L*/*N* (µm)
C	0.77 ± 0.04[a]	6–7	366.5 ± 30.9	43.0 ± 3.5[a]
CCr	0.76 ± 0.10[a]	0–1	163.9 ± 28.7	60.5 ± 7.8[b]
S	0.79 ± 0.06[a]	6–7	323.8 ± 49.7	40.5 ± 6.2[a]
SCr	0.82 ± 0.07[a]	0–1	174.9 ± 24.5	58.3 ± 8.2[b]
P	0.76 ± 0.10[a]	6–7	360.6 ± 30.0	40.1 ± 3.3[a]
PCr	0.77 ± 0.11[a]	0–1	174.6 ± 25.8	60.0 ± 8.9[b]

C (minimal medium), S (minimal medium with sulphate added), P (minimal medium with phosphate added), CCr (minimal medium with chromium added), SCr (minimal medium with sulphate and chromium added), PCr (minimal medium with phosphate and chromium added), *D* (hyphal diameter), N (number of tips), *L* (total hyphal length), *L*/*N* (hyphal growth unit length). Results are presented as the mean ± standard deviation calculated from thirty hyphal elements. The values with different letters are significantly different ($p < 0.05$).

cells. Surprisingly, the presence of sulphate or phosphate ions did not modify the macro and micromorphological parameters of this strain in the presence of Cr(VI). Accordingly and in contrast of what was suggested, our results did not suggest a protective effect of sulphate or phosphate since the pleomorphism of *Streptomyces* sp. MC1 was consistent with a stressed culture.

We propose that the supplementation with phosphate ions, or to a greater degree with sulphate ions, might increase the biological potential for *Streptomyces* sp. MC1 to perform Cr(VI) removal, optimizing both the time and costs involved with treatment of industrial effluents contaminated with chromium. Moreover, further studies are needed to clearly understand the possible mechanisms that this strain employs for Cr(VI) reduction and their relation with sulphate and phosphate transporter systems. Interestingly, a recent study revealed that the presence of Cr(VI) at concentrations more than 5 mg l^{-1} significantly inhibited the P removal by bacteria in the system called "enhanced biological phosphorus removal" (EBPR), widely implemented in wastewater treatment plants (Fang et al., 2012). Considering these factors, new perspectives have emerged and require attention with regard to Cr(VI) removal as well as the removal of common co-contaminants such as sulphate and phosphate.

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References

A.P.H.A, 1992. Standard Methods for the Examination of Water and Wastewater, eighteenth ed. American Public Health Association, Washington DC.

Ackerley, D.F., Barak, Y., Lynch, S.V., Curtin, J., Martin, A., 2006. Effect of chromate stress on *Escherichia coli* K-12. Journal of Bacteriology 188, 3371–3381.

Amoroso, M.J., Castro, G., Carlino, F.J., Romero, N.C., Hill, R., Oliver, G., 1998. Screening of heavy metal-tolerant actinomycetes isolated from the Sali River. The Journal of General and Applied Microbiology 44, 29–32.

Bae, W.C., Lee, H.K., Choe, Y.C., Jahng, D.J., Lee, S.H., Kim, S.J., Lee, J.H., Jeong, B.C., 2005. Purification and characterization of NADPH-dependent Cr(VI) reductase from *Escherichia coli* ATCC 33456. Journal of Microbiology 43, 21–27.

Barreteau, H., Kovac, A., Boniface, A., Sova, M., Gobec, S., Blanot, D., 2008. Cytoplasmic step of pectidoglycan biosynthesis. FEMS Microbiology Reviews 32, 168–207.

Bouhss, A., Trunkfield, A.E., Bugg, D.T., Mengin-Lecreulx, D., 2008. The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiology Reviews 32, 208–233.

Branco, R., Alpoim, M.C., Morais, P.V., 2004. *Ochrobactrum tritici* strain 5bvI1—-characterization of a Cr(VI)-resistant and Cr(VI)- reducing strain. Canadian Journal Of Microbiology 50, 697–703.

Brown, S.D., Thompson, M.R., VerBerkmoes, N.C., Chourey, K., Shah, M., Zhou, J., Hettich, R.L., Thompson, D.K., 2006. Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. Molecular and Cellular Proteomics 5, 1054–1107.

Camargo, F.A.O., Bento, F.M., Okeke, B.C., Frankenberger, W.T., 2004. Hexavalent chromium reduction by an actinomycete, *Arthrobacter crystallopoietes* ES 32. Biological Trace Element Research 97, 183–194.

Cefalu, W.T., Hu, F.B., 2004. Role of chromium in human health and in diabetes. Diabetes Care 27, 2741–2751.

Cheung, K.H., Gu, J.D., 2007. Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. International Biodeterioration and Biodegradation 59, 8–15.

Chourey, K., Thompson, M.R., Morrell-Falvey, J., VerBerkmoes, N.C., Brown, S.D., Shah, M., Zhou, J., Doktycz, M., Hettich, R.L., Thompson, D.K., 2006. Global molecular and morphological effects of 24-Hour Chromium(VI) exposure on *Shewanella oneidensis* MR-1. Applied and Environmental Microbiology 72, 6331–6344.

Colin, V.L., Villegas, L.B., Abate, C.M., 2012. Indigenous microorganisms as potential Bioremediators for environments contaminated with heavy metals. International Biodeterioration and Biodegradation 69, 28–37.

Czakó-Vér, K., Batic, M., Raspor, P., Sipicki, M., Pesti, M., 1999. Hexavalent chromium uptake by sensitive and tolerant mutants of *Schizosacchoromyces pombe*. FEMS Microbiology Letters 178, 109–115.

Dursun, A.Y., Uslu, G., Cuci, Y., Aksu, Z., 2003. Bioaccumulation of copper (II), lead (II) and chromium (VI) by growing *Aspergillus niger*. Process Biochemistry 38, 1647–1651.

Fang, J., Sun, P.D., Xu, S.J., Luo, T., Lou, J.Q., Han, J.Y., Song, Y.Q., 2012. Impact of Cr(VI) on P removal performance in enhanced biological phosphorus removal (EBPR) system based on the anaerobic and aerobic metabolism. Bioresource Technology 121, 379–385.

Fernández, P.M., Figueroa, L.I.C., Fariña, J.I., 2010. Critical influence of culture medium and Cr(III) quantification protocols on the interpretation of Cr(VI) bioremediation by environmental fungal isolates. Water Air Soil Pollution 206, 283–293.

Flores, A., Perez, J.M., 1999. Cytotoxicity, apoptosis, and in vitro DNA damage induced by potassium chromate. Toxicology and Applied Pharmacology 161, 75–81.

Francisco, R., Moreno, A., Morais, P.V., 2010. Different physiological responses to chromate and dichromate in the chromium resistant and reducing strain *Ochrobactrum tritici* 5bvl1. Biometals 23, 713–725.

Grimm, L.H., Kelly, S., Krull, R., Hempel, D.C., 2005. Morphology and productivity of filamentous fungi. Applied Microbiology and Biotechnology 69, 375–384.

Guillén-Jiménez, F., Barrera, L., Morales-Jiménez, J., Hernández-Rodríguez, C., Cristiani-Urbina, E., 2008. Modulation of tolerance to Cr(VI) and Cr(VI) reduction by sulphate ion in a Candida yeast strain isolated from tannery wastewater. Journal of Industrial Microbiology and Biotechnology 35, 1277–1287.

Jong, T., Parry, D., 2003. Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale upflow anaerobic packed bed reactor runs. Water Research 37, 3379–3389.

Kong, B., Zeng, X., Liu, X., Li, X., Li, J., Luo, S., Wei, W., 2009. Kinetic study and mathematical modeling of chromium(VI). Reduction and microorganism growth under mixed culture. Current Microbiology 59, 565–571.

Krishnaswamy, U., Muthuchamy, M., Perumalsamy, L., 2011. Biological removal of phosphate from synthetic wastewater using bacterial, consortium. Iranian Journal of Biotechnology 9, 37–49.

Laxman, R.S., More, S., 2002. Reduction of hexavalent chromium by *Streptomyces griseus*. Minerals Engineering 15, 831–837.

Liu, Y.G., Xu, W.H., Zeng, G.M., Li, X., Gao, H., 2006. Cr(VI) reduction by *Bacillus* sp. isolated from chromium landfill. Process Biochemistry 41, 1981–1986.

Megharaj, M., Avudainayagam, S., Naidu, R., 2003. Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Current Microbiology 47, 51–54.

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for determination of reducing sugar. Analytical Chemistry 31, 426–428.

Ohtake, H., Cervantes, C., Silver, S., 1987. Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid. Journal of Bacteriology 169, 3853–3856.

Orozco, A.M., Contreras, E.M., Bertola, N., Zrotzky, N.E., 2007. Hexavalente chormoium removal using arobic activated sludge batch systems added with powdered activated carbon. Water SA 33, 239–244.

Papagianni, M., 2006. Quantification of the fractal nature of mycelial aggregation in *Aspergillus niger* submerged cultures. Microbial Cell Factories 5, 5.

Parameswari, E., Lakshmanan, A., Thilagavathi, T., 2009. Biosorption of chromium (VI) and nickel (II) by bacterial isolates from an aqueous solution. EJEAFChe 8, 150–156.

Polti, M.A., Amoroso, M.J., Abate, C.M., 2007. Chromium(VI) resistance and removal by actinomycete strains isolated from sediments. Chemosphere 67, 660–667.

Polti, M.A., Amoroso, M.J., Abate, C.M., 2010. Chromate reductase activity in *Streptomyces* sp. MC1. The Journal of General and Applied Microbiology 56, 11–18.

Polti, M.A., Amoroso, M.J., Abate, C.M., 2011. Intracellular chromium accumulation by *Streptomyces* sp. MC1. Water Air Soil Pollution 214, 49–57.

Polti, M.A., García, R.O., Amoroso, M.J., Abate, C.M., 2009. Bioremediation of chromium (VI) contaminated soil by *Streptomyces* sp. MC1. Journal Basic of Microbiology 49, 285–292.

Poopal, A.C., Laxman, R.S., 2009. Studies on biological reduction of chromate by *Streptomyces griseus*. Journal of Hazardous Materials 169, 539–545.

Pradyot, P., 1997. Hand Book of Environmental Analysis, Chemical Pollutants in Air, Water, Soil and Solid Wastes. Lewis Publishers, CRC Press. Inc. p. 233.

Ramírez-Díaz, M.I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., Cervantes, C., 2008. Mechanisms of bacterial resistance to chromium compounds. Biometals 21, 321–332.

Romanenko, V.I., Korenkov, V.N., 1977. A pure culture of bacterial cells assimilating chromates and bichromates as hydrogen acceptors when grown under anaerobic conditions. Mikrobiologiya 46, 414–417.

Shanker, A.K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S., 2005. Chromium toxicity in plants. Environment International 31, 739–753.

Shen, H., Wang, Y.T., 1993. Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Applied and Environmental Microbiology 53, 3771–3777.

Trinci, A.P.J., 1974. A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. Journal of General Microbiology 81, 225–236.

Ünal, D., Isik, N.O., Sukatar, A., 2010. Effects of Chromium VI stress on green alga *Ulva lactuca* (L.). Turkish Journal of Biology 34, 119–124.

Viamajala, S., Peyton, B.M., Sani, R.K., Apel, W.A., Petersen, J.N., 2004. Toxic effects of chromium (VI) on anaerobic and aerobic growth *of Shewanella oneidensis* MR-1. Biotechnology Progress 20, 87–95.

Villegas, L.B., Fernández, P.M., Amoroso, M.J., Castellanos de Figueroa, L.I., 2008. Chromate removal by yeasts isolated from sediments of a tanning factory and a mine site in Argentina. Biometals 21, 591–600.

Viti, C., Pace, A., Giovannetti, L., 2003. Characterization of Cr (VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. Current Microbiology 46, 1–5.

Wang, Y.T., Xiao, C., 1995. Factors affecting hexavalent chromium reduction in pure cultures of bacteria. Water Research 29, 2467–2474.
Worrall, J.A.R., Vijgenboom, E., 2010. Copper mining in *Streptomyces*: enzymes, natural products and development. Natural Product Reports 27, 742–756.
Zachara, J.M., Ainsworth, C.C., Cowan, C.E., Resch, C.T., 1989. Adsorption of chromate by subsurface soil horizons. Soil Science Society of America Journal 53, 418–428.
Zakaria, Z.A., Zakaria, Z., Surif, S., Ahmad, W.A., 2007. Hexavalent chromium reduction by *Acinetobacter haemolyticus* isolated from heavy-metal contaminated wastewater. Journal of Hazardous Materials 146, 30–38.
Zayed, A.M., Terry, N., 2003. Chromium in the environment: factors affecting biological remediation. Plant and Soil 249, 139–156.
Zhao, C., Yang, Q., Chen, W., Li, H., Zhang, H., 2011. Isolation of a sulfate reducing bacterium and its application in sulfate removal from tannery wastewater. African Journal of Biotechnology 10, 11966–11971.