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Chromate reductase activity in *Streptomyces* sp. MC1

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Biological transformation of Cr(VI) to Cr(III) by enzymatic reduction may provide a less costly and more environmentally friendly approach to remediation. In a previous report a Cr(VI) resistant actinomycete strain, *Streptomyces* sp. MC1, was able to reduce Cr(VI) present in a synthetic medium, soil extract and soil samples. This is the first time optimal conditions such as pH, temperature, growth phase and electron donor have been elucidated in vitro for Cr(VI) reduction by a streptomycete. Chromate reductase of *Streptomyces* sp. MC1 is a constitutive enzyme which was mainly associated with biomass and required NAD(P)H as an electron donor. It was active over a broad temperature (19–39°C) and pH (5–8) range, and optimum conditions were 30°C and pH 7. The enzyme was present in supernatant, pellet and cell free extract. Bioremediation with the enzyme was observed in non-compatible cell reproduction systems, conditions frequently found in contaminated environments.

Key Words——bioremediation; chromate reductase; *Streptomyces*

Introduction

Chromium is one of the most widely used metals in a variety of industrial processes, such as steel production, wood preservation, leather tanning, metal corrosion inhibition, paints and pigments, and it is mainly used as chromate or dichromate (Baldi et al., 1990). Industrial effluents containing chromium compounds are released directly or indirectly into natural water resources, mostly without proper effluent treatment, resulting in anthropogenic contamination of pristine environments (Cefalu and Hu, 2004; Cheung and Gu, 2007; Ryan et al., 2002; Shakoori et al., 2000; Viti et al., 2003).

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Chromium can exist in oxidation states ranging from 2^- to 6^+. Hexavalent chromium, Cr(VI), and trivalent chromium, Cr(III), are ecologically important because they are the most stable oxidation states in a natural environment (Cefalu and Hu, 2004; Megharaj et al., 2003). Cr(III) is an essential micronutrient for proper glucose metabolism, because it stimulates the enzyme system and stabilizes nucleic acids (Viti et al., 2003). Cr(VI) is more mobile and soluble in water than Cr(III), which is relatively inert, chemically more stable and less bioavailable due to its negligible permeability to biomembranes (Megharaj et al., 2003). Besides, Cr(VI) is toxic and mutagenic to most organisms (Ganguli and Tripathi, 2002).

In view of the seriousness of Cr(VI) and its alarming effects on human health, it has been listed as a priority pollutant and classified as a class A human carcinogen by the US Environmental Protection Agency (USEPA) (Costa and Klein, 2006).

Due to the ubiquity and toxicity of this environmental

contaminant, considerable interest exists in innovative, low cost methods for the remediation of Cr(VI) from contaminated environments (Smith et al., 2002). Studies have revealed that Cr(VI) is approximately 100 times more toxic (Beleza et al., 2001) and 1,000 times more mutagenic than Cr(III) (Czakó-Vér et al., 1999). Reduction alters the valence and may consequently alter the toxicity and environmental mobility of a metal. Therefore conversion of Cr(VI) to Cr(III) could be an effective method of combating Cr(VI) pollution. The existing treatment processes for chromium detoxification generally involve aqueous reduction of Cr(VI) by a reductant and subsequent pH adjustment to neutral ranges so as to precipitate the less soluble Cr(III). This process requires large amounts of chemicals and energy and hence is not economically feasible, and besides it can be a potential source of metal pollution through the resultant metal-containing chemical sludge (Shakoori et al., 2000).

Microbial reduction of Cr(VI) is considered to be an effective alternative method to chemical processes, and have a potential use in bioremediation.

Biotransformation of Cr(VI) to Cr(III) using bacteria is the most pragmatic approach with a well-established feasibility in bioremediation. Reduction of Cr(VI) has been demonstrated in various bacterial species including *Bacillus* sp. (Camargo et al., 2004; Liu et al., 2006), *Pseudomonas* sp. (Ganguli and Tripathi, 2002; Park et al., 2000), *Escherichia coli* (Bae et al., 2005), *Desulfovibrio* sp. (Mabbett and Macaskie, 2001), *Microbacterium* sp. (Pattanapipitpaisal et al., 2001), *Shewanella* sp. (Myers et al., 2000; Vaimajala et al., 2002) and *Arthrobacter* sp. (Asatiani et al., 2004; Megharaj et al., 2003). Bacterial enzymes such as hydrogenases (Chardin et al., 2003), nitroreductases (Kwak et al., 2003) and quinone reductases (González et al., 2005) have been reported to exhibit chromate reductase activity. However, bacterial enzymes responsible for direct reduction of chromate have not been thoroughly characterized to date.

Identification and isolation of specific chromate reductase is of interest for development of bioremediation technology strategies and contribute to the knowledge of bacterial Cr(VI) resistance.

Actinomycetes represent an important constituent of the microbial population in most soils. Their metabolic diversity and specific growth characteristics, like mycelium formation and relatively rapid colonization of selective substrates, make them well-suited agents for bioremediation (Albarracín et al., 2005). However, there are only a few studies on Cr(VI) bioreduction by actinomycetes and their potential for bioremediation processes. The first report on Cr(VI) reduction by *Streptomyces* was by Das and Chandra (1990). Amoroso et al. (2001) reported on Cr(VI) bioaccumulation by *Streptomyces* strains, whereas Laxman and More (2002) determined Cr(VI) reduction by *Streptomyces griseus*.

In a previous report, Polti et al. (2009) determined Cr(VI) reduction by *Streptomyces* sp. MC1, isolated from sugar cane, in liquid minimal medium, soil extract and soil samples, as well as its potential use in bioremediation processes.

The present study evaluated the chromate reductase activity of *Streptomyces* sp. MC1, determined the nature of the chromium reducing activity and localized it. Furthermore, this is the first time optimal conditions for Cr(VI) reduction under different in vitro conditions such as pH, temperature, growth phase and electron donor have been elucidated.

Materials and Methods

Bacterial strains, culture media and growth conditions. The following strains were used: *Streptomyces* sp. MC1, resistant to Cr(VI) (Polti et al., 2007, 2009), *Streptomyces* sp. C35, resistant to Cr(VI), but not able to reduce it and *Streptomyces* sp. C21, sensitive to Cr(VI) (Polti et al., 2009). The strains were 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.

Strains were grown in minimal medium (MM) containing (g L^{-1}): glucose, 10.0; L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.20 and $FeSO_4 \cdot 7H_2O$, 0.01 and incubated at 30°C for 3 days for chromate reductase activity assays. Alternatively, *Streptomyces* sp. MC1 was grown in glycerol minimal medium (MMY) containing (g L^{-1}): glycerol, 3.0; L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.20; $FeSO_4 \cdot 7H_2O$, 0.01 (Polti et al., 2007).

A Cr(VI) stock solution (50 g L^{-1} $K_2Cr_2O_7$) was prepared by dissolving $K_2Cr_2O_7$ in distilled water and the solution was filter-sterilized before use.

Spore suspensions (100 µl of 1×10^9 CFU ml^{-1}) from solid MM (1.2% agar) were inoculated in flasks with MM or MMY. Cultures were incubated on an orbital

shaker (220 rpm) at 30°C for 72, 108 or 168 h. Assays were carried out in triplicate.

Chromate reductase activity assay. The reaction mixture (200 µl) contained 80 µl of sample, 0.1 mM NADH and 12.5 mg L^{-1} of Cr(VI) in 50 mM potassium phosphate buffer of pH 7.0. Reaction mixtures without sample or NADH were used as controls. The mixtures were incubated at 30°C for 30 min and residual Cr(VI) was quantified with DPC reagent using a standard curve of Cr(VI) (APHA,1992; Polti et al., 2009). One unit of chromate reductase activity was defined as the amount of enzyme that reduced 1 mmol of Cr(VI) per min under the assay conditions. Specific activity was defined as chromate reductase activity (units) per mg of sample protein.

Protein concentrations were calculated using a Bio-Rad protein assay kit by reading absorbance at 595 nm, following the principle of Bradford (1976).

Non-enzymatic Cr(VI) reduction was checked after inactivation of samples at 100°C for 5 min or by the addition of 1 mg ml^{-1} of proteinase K at 50°C during 2 h.

Characterization of chromate reductase activity. Cellular localization: *Streptomyces* sp. MC1 was grown in MM as described above. After 72 h, cells were harvested at 3,000 × *g* for 10 min at 4°C, washed and suspended in distilled water (30% v/v). Supernatant (S) was filter-sterilized with sterile 0.22 µm Millipore filters. The cell suspension was broken in a French pressure cell press at 20,000 psi (1.38×10^5 KN m^{-2}) and then centrifuged at 30,000 × *g* for 15 min at 4°C. Sample fractions belonging to whole cells (WC), S, cell-free extract (CFE) and cell wall (CW) were assayed for chromate reductase activity as described above.

CFE was put into a 10 kDa Millipore kit and ultra-filtration (UF) was carried out at 4°C and 5,000 × *g* for 20 min.

Induction of chromate reductase: Chromate reductase activity assays were carried out with CFE of *Streptomyces* sp. MC1 grown in MM at 30°C for 72 h without chromium or supplemented with 50 mg L^{-1} of Cr(VI) at the beginning of growth (Induced 0) or after 24 h of growth (Induced 24).

Effect of carbon source on chromate reductase activity: Chromate reductase activity was measured in CFE of *Streptomyces* sp. MC1 grown in MM and in MMY at 30°C for 72 h.

Effect of the growth phase on chromate reductase activity: To study the levels of chromate reductase activity during different stages of growth, CFE samples of *Streptomyces* sp. MC1 grown in MM at 30°C were used. Samples were taken after 72, 108 and 168 h, which belonged to exponential, early and late stationary growth phase, respectively.

Effect of pH and temperature on chromate reductase activity: To determine optimum pH, activity of CFE chromate reductase was measured at different pH using 50 mM potassium phosphate (pH 6.0–8.0) and 50 mM citrate phosphate (pH 4.0–5.0) at 30°C. In order to determine optimum temperature, chromate reductase activity of CFE was measured in a temperature range between 19 and 41°C at pH 7.0.

Effect of electron donors on chromate reductase activity: CFE chromate reductase activity was calculated in the presence of 1 mM NADH and NADPH electron donors. NADH oxidation during the enzymatic reaction was measured at 340 nm for 24 h at pH 6.0, 7.0 and 8.0. Non-specific NADH oxidation was also determined in the presence of Cr(VI) without CFE.

Results and Discussion

Detection of chromate reductase activity

Reduction of Cr(VI) is also obtained by NADH action without microorganisms (Table 1). This non-specific reduction was considered when calculating chromate reductase activity.

Cellular localization: Chromate reductase activity of *Streptomyces* sp. MC1 was detected in all four cell fractions assayed: CFE, WC, CW and S. Activity was highest in CFE and WC fractions (3.20 and 2.17 nmol min^{-1} mg of $protein^{-1}$, respectively), whereas values in CW and S were 0.7 and 0.28 nmol min^{-1} mg of $protein^{-1}$, respectively (Fig. 1). Consequently, chromate reductase activity in *Streptomyces* sp. MC1 was mainly associated with the CFE and not with the CW fraction. This agrees with results obtained for other bacterial

Table 1. Non-specific Cr(VI) reduction in the absence of *Streptomyces* sp. MC1, at 30°C, pH 7 during 30 min.

Mixture components	Cr(VI) reduction (%)[e]
Cr(VI)[a]	0.68 ± 0.01
Buffer[b] + Cr(VI)	5.16 ± 2.80
NADH[c] + Cr(VI)	25.64 ± 5.33
NADH + Buffer + Cr(VI)	21.15 ± 6.86
50% NADH[d] + Buffer + Cr(VI)	11.57 ± 5.84

[a] 20 mg L^{-1} Cr(VI); [b] 50 mM phosphate buffer, pH 7.0; [c] 0.1 mM NADH; [d] 0.05 mM NADH; [e] mean ± standard deviation.

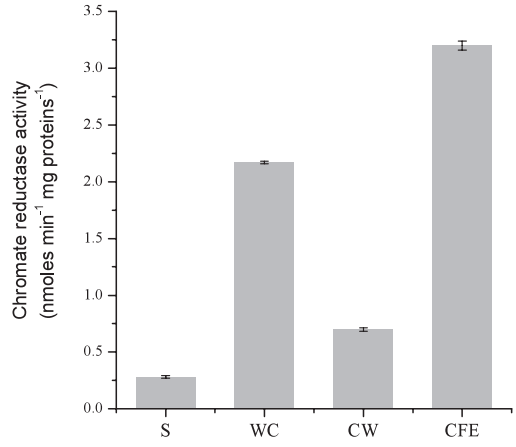

Fig. 1. Chromate reductase activity in different cell fractions of *Streptomyces* sp. MC1.

Assays were carried out in potassium phosphate buffer (pH 7) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH at 30°C. S, supernatant; WC, whole cells; CW, cell wall; CFE, cell-free extract.

chromate reductases, which were localized either in the membrane or cytosol fractions (Bae et al., 2005; Camargo et al., 2004; Desai et al., 2007).

Streptomyces sp. MC1 CFE was purified by UF using a 10 kDa Millipore kit. This process eliminated the salts, glycosides, other substances of small molecular weight (below of 10 kDa) and part of the water content. Thus, the total volume was reduced by 3 times after UF process, but activity was 100% recovered. Further *Streptomyces* sp. MC1 CFE pre-treated with heat (100°C for 5 min) or proteinase K (1 mg ml^{-1} at 50°C during 2 h) did not show chromate reductase activity. These results suggest that the activity expressed by *Streptomyces* sp. MC1 is entirely enzymatic (Table 2).

Study of the effect of carbon source: Assays carried out in MM and MMY supplemented with 50 mg L^{-1} Cr(VI) revealed a reduction of Cr(VI) by *Streptomyces* sp. MC1 from 55 to 57% in both culture media. However, chromate reductase activity of CFE was 80% higher when glucose was used as the carbon source (3.20 nmol min^{-1} mg of $protein^{-1}$) compared to glycerol (0.58 nmol min^{-1} mg of $protein^{-1}$) (Fig. 2). These results suggest that Cr(VI) reduction in *Streptomyces* sp. MC1 could be an enzymatic activity regulated by glucose. Production of several enzymes and secondary metabolites in *Streptomyces* species are under glucose control (Desjardin et al., 2002).

Induction of chromate reductase: Chromate reductase activity of *Streptomyces* sp. MC1 (3.20 nmol min^{-1} mg of $protein^{-1}$) was present in CFE obtained

Table 2. Chromate reductase activity in *Streptomyces* sp. MC1 CFE, at 30°C, pH 7 with 20 mg L^{-1} Cr(VI) and 0.1 mM NADH.

Treatment	Total amount of protein (mg)[b]	Specific activity (nmol $min\ mg^{-1}$)[b]
CFE	0.169 ± 0.023	3.20 ± 0.04
CFE + UF[a]	0.251 ± 0.009	4.70 ± 0.18
CFE + 55°C[a]	0.136 ± 0.001	ND[c]
CFE + Proteinase K[a]	0.032 ± 0.002	ND

[a] CFE after ultra-filtration, heat or proteinase K treatment, respectively; [b] mean ± standard deviation; [c] ND: not detected.

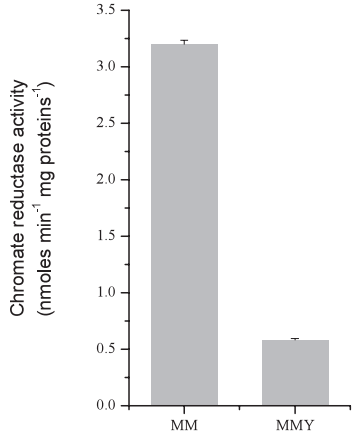

Fig. 2. Effect of glucose (MM) and glycerol (MMY) on chromate reductase activity in CFE of *Streptomyces* sp. MC1.

Assays were carried out in potassium phosphate buffer (pH 7) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH at 30°C.

from cultures without Cr(VI), suggesting the constitutive nature of this soluble enzyme. Nevertheless, an increase of more than 100% in the activity of induced cultures was observed, independently of the moment of induction: 6.45 and 7.68 nmol min^{-1} mg of $protein^{-1}$ for Induced 0 and Induced 24, respectively (Fig. 3). Das and Chandra (1990) determined similar results in *Streptomyces* sp. M3 and constitutive enzymes with Cr(VI) reducing activity were also found in *Bacillus* (Desai et al., 2007; Pal et al., 2005).

Effect of the growth phase on chromate reductase activity: The influence of the growth phase on chromate reductase activity of *Streptomyces* sp. MC1 was remarkable. Maximum activity was obtained in CFE samples from a 72 h culture (3.20 nmol min^{-1} mg of $protein^{-1}$) (Fig. 4), coinciding with maximum reduction values in culture medium previously observed by Polti et al. (2009). Activity decreased with about 40% (1.92 nmol min^{-1} mg of $protein^{-1}$) and 60% (1.02 nmol

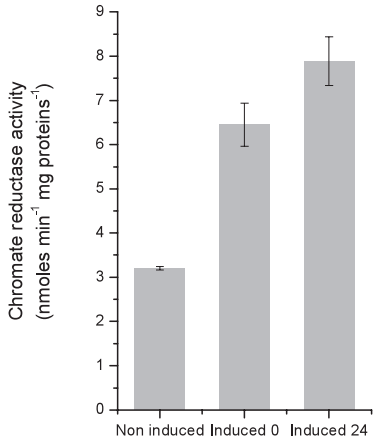

Fig. 3. Induction effect of Cr(VI) on chromate reductase activity in CFE of *Streptomyces* sp. MC1.

Assays were carried out in potassium phosphate buffer (pH 7) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH at 30°C.

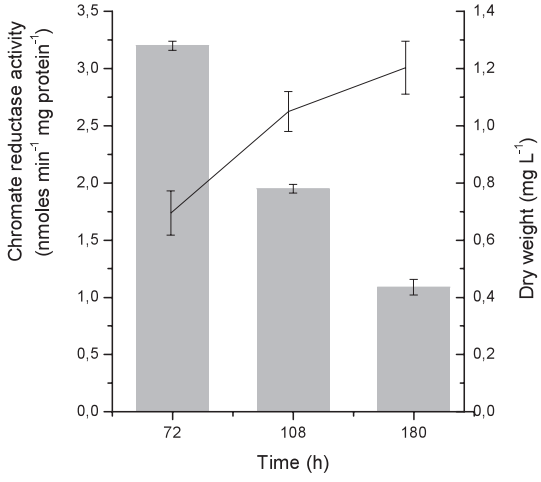

Fig. 4. Effect of growth phase on chromate reductase activity in CFE of *Streptomyces* sp. MC1.

Assays were carried out in potassium phosphate buffer (pH 7) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH at 30°C.

min^{-1} mg of $protein^{-1}$) after 108 and 180 h of incubation, respectively (Fig. 4). These results could be due to different expression levels of the protein during bacterial growth.

Effect of pH on chromate reductase activity: Chromate reductase activity, under different pH values, was evaluated in CFE of *Streptomyces* sp. MC1, *Streptomyces* sp. C21 and *Streptomyces* sp. C35.

Under the assay conditions, *Streptomyces* sp. C35 did not show chromate reductase activity, whereas *Streptomyces* sp. C21 showed activity only at pH 6 and 7 (0.47 and 0.39 nmol min^{-1} mg of $protein^{-1}$, respectively), these values were only 12 and 16% of activity reached by *Streptomyces* sp. MC1 at the same pH values. In addition, *Streptomyces* sp. MC1 showed activity under all pH assayed (Fig. 5). This suggests that enzymatic Cr(VI) reduction by *Streptomyces* sp. MC1 is specific.

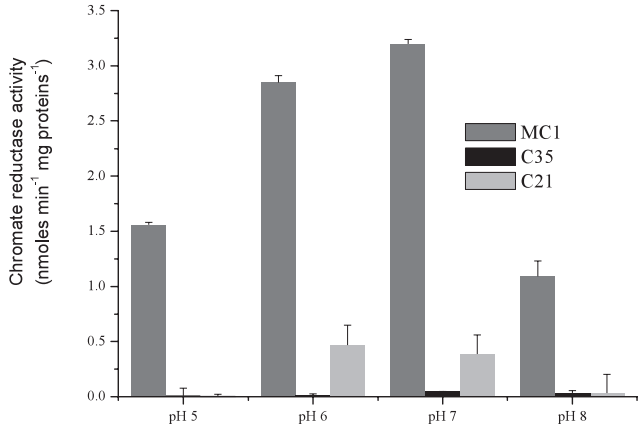

Fig. 5. Effect of pH on chromate reductase activity in CFE of *Streptomyces* sp. MC1, *Streptomyces* sp. C35 and *Streptomyces* sp. C21.

Assays were carried out in citrate phosphate buffer (pH 5) or potassium phosphate buffer (pH 6–8) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH at 30°C.

Chromate reductase activity in CFE of *Streptomyces* sp. MC1 was found to be influenced by pH. Optimum enzyme activity (3.20 nmol min^{-1} mg of $protein^{-1}$) was at pH 7, but activity at pH 6 was almost 90% (2.85 nmol min^{-1} mg of $protein^{-1}$) (Fig. 5). At pH 5 and 8 activity was 51% (1.56 nmol min^{-1} mg of $protein^{-1}$) and 70% (1.09 nmol min^{-1} mg of $protein^{-1}$) lower, respectively. The chromate reductase enzymes previously reported showed different optimum pH values: 5.0 for *Pseudomonas putida* (Park et al., 2000); 6.0 for *Bacillus* (Desai et al., 2007; Pal et al., 2005); 6.3 for *Thermus scotoductus* LTD-01 (Opperman et al., 2008) and 6.5 for *E. coli* ATCC 33456 (Bae et al., 2005). Other strains demonstrated neutral to alkaline pH values: 7 to 9 for *Bacillus* (Camargo et al., 2003; Liu et al., 2006) and 9 for *Achromobacter* sp. Ch-1 (Ma et al., 2007).

Similar observations about the influence of pH on bacterial Cr(VI) reduction have been found by other researchers (Pal et al., 2005; Sultan and Hasnain, 2007).

Effect of temperature: The *Streptomyces* sp. MC1 enzyme kept activity within an extensive temperature range (20 to 40°C), with optimum activity between 28 and 30°C (3.10–3.20 nmol min^{-1} mg of $protein^{-1}$) (Fig.

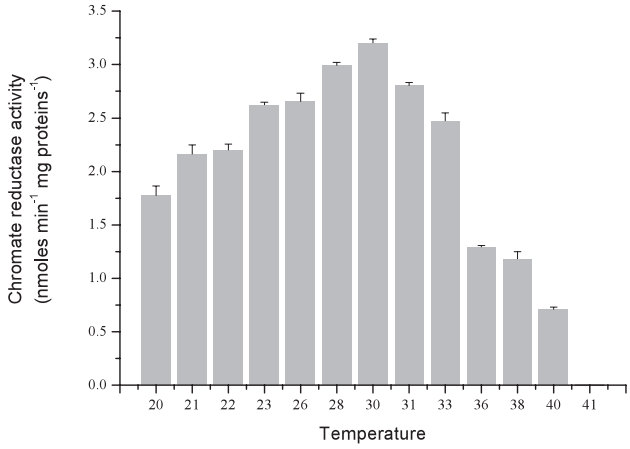

Fig. 6. Temperature effect (20–41°C) on chromate reductase activity in CFE of *Streptomyces* sp. MC1.

Assays were carried out in potassium phosphate buffer (pH 7) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH.

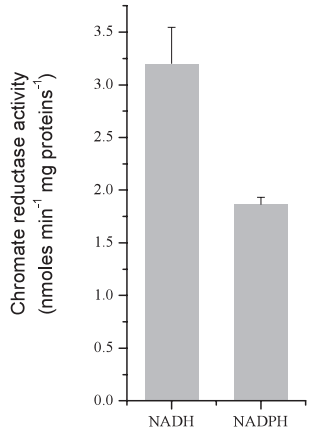

Fig. 7. Effect of electron donor (NADH or NADPH) on chromate reductase activity in CFE of *Streptomyces* sp. MC1.

Assays were carried out in potassium phosphate buffer (pH 7) with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NAD(P)H at 30°C.

6). This coincides with the optimum growth temperature for *Streptomyces* sp. MC1. A similar behavior with equal optimum temperatures for growth and activity was observed previously in other bacteria: several *Bacillus* species had optimum temperatures of 30°C (Camargo et al., 2003; Desai et al., 2007; Pal et al., 2005), *E. coli* ATCC 33456 and *Bacillus* sp. presented an optimum growth and activity temperature of 37°C (Bae et al., 2005; Liu et al., 2006), and *Thermus scotoductus*, a thermophilic bacterium, optimum temperatures of 65°C (Opperman et al., 2008). In contrast to this, a chromate reductase of *Pseudomonas putida* was reported to have an optimum temperature of 80°C, whereas the optimum growth temperature was 30°C (Park et al., 2000).

Effect of electron donors on chromate reductase activity: Bacterial chromate reductase activity is attributed to enzymes with diverse characteristics, although they all have in common the utilization of an electron donor for optimum activity. Chromate reductase activity of *Streptomyces* sp. MC1 was assayed in the presence of NADPH and NADH as electron donors. NADH was the more efficient cofactor because activity was 40% higher than with NADPH (3.20 nmol against 1.86 nmol min^{-1} mg of $protein^{-1}$, respectively)(Fig. 7).

The NADH oxidation rate was evaluated at the same time (Fig. 8). The level of NADH oxidation coincided with chromate reductase activity at pH 7, whereas the oxidation NADH rate at pH 8 was higher than chromate reductase activity. This would indicate a secondary NADH oxidation in the presence of CFE. Finally,

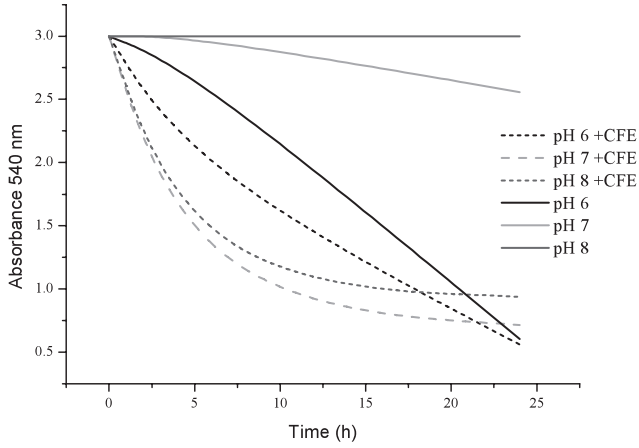

Fig. 8. NADH oxidation at different pH, in the presence or absence of CFE of *Streptomyces* sp. MC1 and with an initial concentration of 12.5 mg L^{-1} Cr(VI) and 0.1 mM NADH at 30°C.

non-specific NADH oxidation was observed in the absence of CFE at pH 6.

Chromate reductase purified from *P. ambiguous* G-1 was dependent on NAD(P)H (Suzuki et al., 1992), just like the activity found for *P. putida* (Park et al., 2000) and *E. coli* ATCC 33456 (Bae et al., 2005). However, in the case of chromate reductase from *Thermus scotoductus* LTD-01, NADPH was found to be a better electron donor (Opperman et al., 2008), and this enzyme also required Ca^{2+} or Mg^{2+} for its activity. On the other hand, chromate reductase activity of *Shewanella putrefaciens* MR-1 only occurred in the presence of formate or NADH as electron donors, but not with lactate or NADPH (Myers et al., 2000). Chromate reductase present in CFE from *Bacillus* IS 29, *Arthrobacter crys-*

tallopoietes IS 32 and *Bacillus* sp. was promoted in the presence of NADH (Camargo et al., 2003, 2004; Desai et al., 2007). Other chromate reductases are flavoproteins with associated flavins to their structure and have been described in *E. coli* and *P. putida* (Puzon et al., 2002; Vaimajala et al., 2002).

Wang et al. (1990) determined chromate reductase activity in membranes of *Enterobacter cloacae* H01. This enzyme utilized ascorbate-reduced phenazine methosulfate as an electron donor.

Streptomyces sp. MC1 was screened for the presence of plasmids, including very large plasmids, by pulsed field gel electrophoresis (PFGE). These techniques, under the assayed conditions, did not allow the isolation of any kind of plasmids from *Streptomyces* sp. MC1 (data not shown). The chromate reductase genes are localized at the chromosomal level, characteristic of this resistance system. A fragment of 222 bp amplified from *Streptomyces* sp. MC1 (accession number FJ911900) related to FMN oxidoreductases, suggests that Cr(VI) resistance and reduction by *Streptomyces* sp. MC1 could be related to this protein family (data not shown).

Streptomyces sp. MC1 previously showed ability to reduce Cr(VI) in liquid culture medium and soil samples. The results presented in this study confirm the enzymatic nature of this reduction and for the first time a chromate reductase has been characterized in a streptomycete. To our knowledge this report presents the first characterization of a *Streptomyces* chromate reductase.

This partial characterization suggests the presence of a neutral chromate reductase enzyme produced by *Streptomyces* sp. MC1 because activity was totally inhibited by protease or heat treatment. The enzyme is intracellular or linked to the membrane and showed activity within a broad pH (5.0 to 9.0) and temperature range (20 and 40˚C). Activity increased significantly in the presence of NAD(P)H and the studies also revealed the constitutive nature of the enzyme.

This chromate reductase enzyme is active in the cell free extract; and it could be utilized in bioremediation processes at conditions incompatible with the cell reproduction.

Acknowledgments

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