Water Air Soil Pollut (2016) 227: 175
DOI 10.1007/s11270-016-2872-5

A Comparison of Microbial Bioaugmentation and Biostimulation for Hexavalent Chromium Removal from Wastewater

Filipe Selau Carlos · Patricia Giovanella · Jovana Bavaresco · Clarissa de Souza Borges · Flavio Anastácio de Oliveira Camargo

Received: 8 January 2016 / Accepted: 29 April 2016 / Published online: 11 May 2016
© Springer International Publishing Switzerland 2016

Abstract Hexavalent chromium (VI) in wastewater is a great risk to human health and to the quality of water sources. However, adapted microorganisms can rapidly reduce this chemical species to the trivalent form (III) and make it less active. Our objective was to evaluate the capacity of bacterial isolates for Cr (VI) reduction in nutrient medium and in effluent and to compare indigenous microorganisms with those isolated from wastewater contaminated with Cr (VI). Cr (VI) reduction was also tested with different sources of carbon, nitrogen, and phosphorus at two temperatures (10 and 30 °C). Initially, the resistant microorganisms were isolated from the solution with 100 mg L^{-1} of Cr (VI). Subsequently, we evaluated the effectiveness of the isolates in reducing Cr (VI) I in culture medium under temperature-controlled conditions, with concentrations of 10 and 100 mg L^{-1} of Cr (VI). In the subsequent step, we studied the isolates and autochthonous microorganism efficiency to reduce Cr (VI) present in contaminated effluent, with the addition of nutrients and at different temperatures (10 and 30 °C). In the culture medium containing 10 mg L^{-1} of Cr (VI), isolates were reduced by 100 % in 48 h. When tested against 100 mg L^{-1} of Cr (VI), the decrease was 70 and 40 % at 120 h of incubation of the isolates 6 and 11, respectively. In the effluent, there was no significant reduction without nutritional biostimulation. When carbon and phosphorus were applied, isolates 6, 11, and indigenous microorganisms reduced 100 % of the Cr (VI) in 72 h. Nitrogen was not limited in terms of effluent characteristics. At 10 °C incubation temperature, Cr (VI) was completely reduced but slower compared to incubation at 30 °C. The results demonstrate that nutritional biostimulation aided by bioremediation is an excellent tool for reducing hexavalent chromium in wastewater.

Keywords Bioreduction · Hexavalent chromium · Chromium-resistant bacteria

1 Introduction

Chromium is a metal which occurs naturally in rocks, animals, plants, soil, volcanic ash, and gases (ATSDR 2008). In the environment, there are predominantly two oxidation states: trivalent chromium (III), which is an essential nutrient for glucose and protein metabolism (Shrivastava et al. 2002), and hexavalent chromium (VI), which is rarely found in nature and is therefore artificially produced for use in industrial processes (Ye et al. 2010; Mangaiyarkarasi et al. 2011). The hexavalent form of chromium has a high solubility and environmental mobility (USEPA 1998). Chromium (VI) has high permeability in cell membranes and is reduced inside the cell to Cr (III), being highly mutagenic, reacting directly with the DNA and proteins (Salnikow and Zhitkovich 2007; Mangaiyarkarasi et al. 2011). The characteristics of non-biodegradability and high water solubility of the Cr (VI) increase the toxicity and

F. S. Carlos (✉) · P. Giovanella · J. Bavaresco · C. d. S. Borges · F. A. d. O. Camargo
Federal University of Rio Grande do Sul, Porto Alegre, Brazil
e-mail: filipeselaucarlos@hotmail.com

environmental contamination capability of this heavy metal (Colin et al. 2012; Liu et al. 2012).

There are various technologies for the remediation of chromium-contaminated effluent. They include precipitation, coagulation, reverse osmosis (Ahluwalia and Goyal 2007), or redox chemical processes such as the use of Fe (II) (Franco et al. 2009). Conventional methods for the removal of metals from contaminated sites require large amounts of energy and of chemical reagents with the possible generation of secondary pollution (Liu et al. 2012). The use of microorganisms, on the other hand, is an alternative method of treatment of metal-contaminated effluents, which allows the application of large volume, low cost of operation, and energy efficiency of metal removal (Thacker et al. 2007; Liu et al. 2012).

The most common microbial mechanisms for reducing Cr (VI) are resistant to periplasmic biosorption, interplasmic bioaccumulation, and biotransformation directly caused by enzymes (Okeke et al. 2008; Ozturk et al. 2012; Sagar et al. 2012) or indirectly by metabolites (Camargo et al. 2003). The microorganism surface is negatively charged due to the presence of anionic molecules and structures and can adsorb cationic metal (Chen and Hao 1998). Recent studies demonstrated that the growth of cultures of bacteria, fungi, and microalgae has intracellular levels many times greater than the amounts biosorbed (Kader et al. 2007). Biosorption, however, is a process that depends on environmental conditions such as pH, ionic strength, and the presence of organic and inorganic binders (Kumar et al. 2008). Enzyme biotransformation along with interplasmic bioaccumulation mechanisms is more effective in reducing Cr (VI) due to its constant ability for regeneration and optimization potential from the isolation of resistant microorganisms (Malik 2004; Kathiravan et al. 2011).

During the reduction reaction, Cr (VI) can follow different routes, such as intracellular sequestration, organic-metal chelating, reactions with extracellular organelles, or precipitation (Ozturk et al. 2012). According to Cheung and Gu (2007), the reduction mechanism follows the following steps: first Cr (VI) accepts a molecule of NADH and generates Cr (V) as an intermediate, and then Cr (V) accepts a pair of electrons to form Cr (III). Cr (VI) reduction occurs naturally in the environment, but dietary imbalances, adverse temperature, and microorganisms not adapted to Cr (VI) present in the effluent may slow the reduction of the metal (Kumar et al. 2008), reducing the operational efficiency of wastewater treatment plants.

Biostimulation and bioaugmentation are specific methods which can be used to accelerate the industrial effluent decontamination process. Biostimulation involves an adjustment in the chemical and physical conditions of the environment, including pH, temperature, adjustments of nutrient availability (carbon, phosphorus, nitrogen, and others), and electron acceptors (Chambers et al. 1991). Bioaugmentation, in turn, is based on the acceleration and increase in pollutant decontamination efficiency by the insertion of specialized populations in the biotransformation/degradation and resistance to the contaminant (Das and Mishra 2010). According to Bento et al. (2005), the best performance by bioaugmentation can be achieved using previously selected microorganisms thus increasing the population and microbial diversity. Hence, our objectives were to evaluate in culture medium the reduction of Cr (VI) by two bacteria resistant to this metal and analyze the Cr (VI) reduction capability (VI) in effluent contaminated by the same bacterial isolates and autochthonous microorganisms with the addition of carbon sources, nitrogen, and phosphorus at different temperatures.

2 Materials and Methods

2.1 Collection and Characterization of the Effluent

The effluent used in this work is generated by the effluent treatment system of soil analysis laboratory effluents (SSALE). The hexavalent chromium contents present in this effluent are above 0.05 mg L^{-1}, which is the limit allowed by the US Environmental Protection Agency (USEPA) (Sharma and Adholeya 2011). The total volume of effluent necessary to conduct the experiments was collected, filtered through a 76-μm mesh, pH adjusted to 6.5 with NaOH, and stored under refrigeration at 4 °C. The physical and chemical characteristics of the effluent SSALE are shown in Table 1 and were determined according to the methodology described by the American Public Health Association (APHA) (1998). The characterization of industrial landfill effluent is also shown in Table 1. From this, contaminated effluents were isolated and adapted microorganisms resistant to high levels of Cr (VI).

Table 1 Physicochemical characteristics of the SSALE effluent and contaminated effluent

Parameter	Ssale effluent	Industrial effluent
$N\text{-}NH_4^+$ (mg L^{-1})	78.8 ± 2.2	452.8 ± 24.2
$N\text{-}NO_3^-$ (mg L^{-1})	4.8 ± 0.2	2.4 ± 0.2
BOD (mg L^{-1})	27.8 ± 1.2	1302.0 ± 76.0
COD (mg L^{-1})	48.1 ± 2.3	380.6 ± 14.2
Total Kjeldahl N (mg L^{-1})	84.5 ± 4.7	110.6 ± 6.7
Total P (mg L^{-1})	1.0 ± 0.05	4.0 ± 0.2
Total S (mg L^{-1})	44.1 ± 2.1	134.2 ± 15.2
Cr (VI) (mg L^{-1})	4.0 ± 0.01	1.0 ± 0.1
Cr (III) (mg L^{-1})	5.0 ± 0.01	44.0 ± 6.2
Total Cr (mg L^{-1})	9.1 ± 0.3	45.1 ± 4.3
pH	5.5	7.4

Mean ± SD

2.2 Experimental Unit

In all experimental tests, flask-type bottles were used with a volume of 300 ml with three replications.

2.3 Selection of Cr (VI)-Resistant Isolates

Ten bacteria previously isolated from the contaminated effluent (Table 1) originated from an industrial landfill, characterized by high dissolved organic carbon, nitrogen, and heavy metals (Table 1). The isolates were inoculated into Luria Bertani (LB) culture medium plus 100 mg L^{-1} Cr (VI). Isolates that showed growth capacity within 48 h of incubation at 30 ± 2 °C were selected for further studies.

2.4 Molecular Identification of Chromium-Resistant Bacteria

The bacterial genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, WI). The 16S rDNA was amplified in a polymerase chain reaction (PCR) using universal primers for bacteria: 27F (5′-AGATTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTT ACGACTT-3′) for amplification of 16S rDNA in PCR. The amplification reaction was based on Sambrook and Russell (2001): buffer (50 mM of Tris–HCl, pH 9.0, 50 mM of KCl, 2.5 % of Triton X-100), deoxynucleotide triphosphates (dNTPs) (200 μM from each), 0.2 μM of $MgCl_2$, 0.25 pmol of each primer, 0.8 ng/μL of DNA sample, and 0.02 U of Taq DNA polymerase. The amplification was performed in a thermal cycler (MJ Research Inc., Watertown, MA, USA) and the program consisted of 35 cycles (initial denaturation at 95 °C for 5 min, subsequent denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 90 s, and final extension for 5 min at 72 °C). The PCR products were purified by the standard method of precipitation with PEG 8000 (polyethylene glycol). Kits of labeled terminators (GE Healthcare) with primer 19r (5′-GWATTACCGCGGCKGCTG-3′) were used for the sequencing reactions of PCR fragments. All generated sequences were submitted to the GenBank/National Center for Biotechnology Information (NCBI) database.

2.5 Minimal Inhibitory Concentrations

The bacterial isolates were evaluated for their ability to grow in LB medium containing different concentrations of $K_2Cr_2O_7$. All tests were performed in 96-multiwell plates, each well filled with 200 μL of the growth medium. Each well was inoculated with 5 μL of overnight culture following incubation at 30 °C for 72 h. The lowest concentration of chromium at which no visible growth was observed was considered as the minimal inhibitory concentration (MIC) (Ball 2007). Uninoculated LB medium served as negative controls and inoculated LB medium without mercury served as positive controls.

2.6 Minimal Biocide Concentration

The isolates were tested for minimal biocide concentration of chromium in 96-well polystyrene plates on LB medium containing different concentrations of $K_2Cr_2O_7$. Each well was inoculated with 5 μL of overnight cultures following incubation at 30 °C incubated for 72 h at 30 °C under agitation at 100 rpm. After the incubation period, 25 μL of culture was inoculated in Petri dishes containing Agar nutrient. The biocide concentration was determined after 72 h of incubation at 30 °C by the presence or absence of microbial growth.

2.7 Preparation of Microbial Cells

Each chromium-resistant isolate was inoculated onto LB medium (bacteriological tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}) pH 6.7, containing $K_2Cr_2O_7$ (10 mg L^{-1}) and incubated for 24 h at 30 °C

under agitation at 180 rpm. Initial bacterial counts of 10^7 colony forming units (CFU)/mL were standardized by turbidimetry at 600 nm and the corresponding CFU/mL in Petri plates. The uninoculated culture medium was used as negative control.

2.8 Determination of Cr (VI)

Chromium (VI) quantification was performed in a spectrophotometer according to the USEPA 7196A method (USEPA 1992). For analysis of Cr (VI), a sample of 500 μL of the different treatments was centrifuged for 10 min at 10,000 rpm and the supernatant (200 μL) was added to 3.8 ml of distilled water and 200 μL of 1,5-diphenylcarbazide and read in a spectrophotometer at 540 nm.

2.9 Reduction of Cr (VI) by bacterial isolates in culture medium

To assess the capacity to reduce Cr (VI) and the growth curve of isolates 6 and 11, the number of CFU/mL was prestandardized as previously described. In the LB culture medium containing 10 and 100 mg L^{-1} of Cr ($K_2Cr_2O_7$), the isolates were kept for 120 h at 30 ±2 °C under 180 rpm agitation. Readings of the remaining chromium, pH, and optical density were performed at times 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 48, 72, 96, and 120 h.

2.10 Nutritional Biostimulation

Different types of nutrients were added to the effluent under study to define the best strategy to reduce hexavalent chromium present in the SSALE effluent. We evaluated the level of analytical nutrients NH_4NO_3 (1 g L^{-1}), KH_2PO_4 (2 g L^{-1}), $Na_2H_2PO_4$ (3 g L^{-1}), and 0.5 % glucose. Subsequently, tests were performed with the addition of a carbon source (C), nitrogen (N), and phosphorus (P) which are low-cost sources and can be utilized in industries to treat effluents. The tested concentrations and inputs were sucrose, triple super phosphate (TSP), and urea. Different combinations of these nutrients and inoculation of isolates 6 and 11 were evaluated.

2.11 Temperature

Effluent with different nutritional combinations and controls (without the addition of nutrients) was incubated at 10 and 30 °C under agitation at 180 rpm. The remaining Cr (VI) was quantified at 0, 24, 48, 72, 96, 120, and 144 h of incubation. Temperatures of 10 and 30 °C were evaluated with nutritional combinations (CP and CNP) and controls (no added nutrients) under agitation at 180 rpm. The remaining Cr (VI) was quantified at 0, 24, 48, 72, 96, 120, and 144 h of incubation.

2.12 Statistical Analysis

Cr (VI) reduction by the isolates during the incubation time was evaluated with descriptive statistics data using the standard error of the mean. Cr (VI) reducing values for the indigenous microorganisms and pH values were submitted to ANOVA and, when significant, were submitted to Tukey test at a 5 % significance level. The SAS program was used for statistical analysis (SAS, 2008).

3 Results and Discussion

3.1 Selection of Chromium-Resistant Isolates

Ten Gram-negative bacteria were evaluated, but only two isolates (6 and 11) grew at a concentration of 100 mg L^{-1} of Cr (VI). Two bacteria (6 and 11) were able to grow in this concentration in just 24 h of incubation, while the other eight isolates did not grow within 48 h of incubation (data not shown).

3.2 Identification of the Isolates, Minimal Inhibitory Concentrations and Minimal Biocide Concentration

The 16S rRNA sequencing analysis showed two bacterial isolates capable of reducing hexavalent chromium. Both are Gram-negative bacteria and belong to the species *Alcaligenes faecalis* and *Pseudochrobactrum saccharolyticum* (Table 2). The *Alcaligenes* genus of bacteria has been reported in other studies as resistant and chrome-reducing (Fakruddin et al. 2009; Kathiravan et al. 2011) and *P. saccharolyticum* was first described by Long et al. (2013) as reducing chromium.

Isolates 6 and 11 showed high resistance to chromium (VI) at inhibitory concentrations between 145 and 150 mg L^{-1} Cr^{6+}. Similar values were observed by

Table 2 Identification of isolated, inhibitory, and biocide concentration of the isolates

Isolate Code	Geneticsimilarity index (%)	Accession number	Sequencelength (bp)	MIC (mg L^{-1})	MBC
U6	*Alcaligenes faecalis* (99 %)	AF155147.1	1312	145	155
U11	*Pseudochrobactrum saccharolyticum* (99 %)	NR0402473.1	1088	150	290

Okeke et al. (2008) using actinomycetes (*Cellulomonas* sp., *Mycobacterium* sp.) and *Bacillus* sp. The biocide concentration for isolate 6 was 155 mg L^{-1}, very close to MIC. For isolate 11, the biocide dose was 290 mg L^{-1} (Table 2). Thus, isolate 11 is more resistant to high concentrations of Cr (VI), although inhibited at the same concentration of isolate 6. These results indicate that the detoxification capacity of Cr (VI) is different among microorganisms. Isolate 6 presented the lowest biocide concentrations but a better performance in growth rate and reduction at high concentrations of Cr (VI) (100 mg L^{-1}). Thacker et al. (2006) reported that isolate *Providencia* sp. reduced 100 % of Cr (VI) L^{-1} (range 100–300 mg in LB medium). The same authors found an MIC of 1000 mg L^{-1} of Cr^{6+} for this isolate. The results indicate that isolates 6 and 11 could be used for the remediation of contaminated sites up to 150 mg L^{-1} Cr^{6+}.

3.3 Cr (VI) reduction by bacterial isolates in culture medium

The evaluated isolates (6 and 11) showed cell growth and the ability to reduce Cr (VI) present in the culture medium (Fig. 1a, b). At the concentration of 10 mg L^{-1}, isolate 6 had a lower latency phase than isolate 11 did, with the greatest initial reduction of Cr^{6+}. After 24 h of incubation, isolates showed the same capacity for Cr (VI) reduction, reaching 100 % in 48 h (Fig. 1a), with similar cell densities and initial concentration of 10 mg L^{-1}. Wani et al. (2007) reported the complete reduction of Cr (VI) by *Burkholderia* after 36 h of incubation in alkaline nutrient medium with an initial content of 75 mg L^{-1} of Cr^{6+}. Microorganisms operate as catalysts of Cr (VI) reduction reactions by a combination of mechanisms such as extracellular enzymatic reduction, non-metabolic reduction on bacterial surfaces, and intracellular reduction and precipitation (Hawley 2005). The enzymatic reduction is the primary reduction mechanism of Cr (VI) to Cr (III) (Camargo et al. 2003; Dermou et al. 2005).

The chromate transport, resistance, and bacterial cell reduction mechanisms that may occur are (i) chromosome-encoded sulfate uptake pathway which is also used by chromate to enter the cell; (ii) extracellular reduction of Cr (VI) to Cr (III) which does not cross the membrane; (iii) intracellular Cr (VI) to Cr (III) reduction may generate oxidative stress, as well as protein and DNA damage; (iv) detoxifying enzymes are involved in protection against oxidative stress, minimizing the toxic effects of chromate; (v) plasmid-encoded transporters may efflux chromate from the cytoplasm; and (vi) DNA repair systems participate in protection from the damage generated by Cr derivatives (Ramirez-Diaz et al. 2008).

Isolates 6 and 11 showed a 24-h latency period in 100 mg L^{-1} of Cr^{6+}. Between 24 and 48 h, isolate 6 showed an exponential growth followed by a stationary phase with a 70 % reduction from Cr (VI) at 120 h of incubation (Fig. 1a). Isolate 11 presented high cell concentration after 48 h of incubation, but no reduction of Cr (VI). Only after 72 h of incubation and increasing cell concentration was a significant reduction of 45 % of Cr (VI) (Fig. 1a) obtained. At the concentration of 100 mg mg L^{-1} of Cr (VI), the isolates had a 24-h adaptation period, with low growth.

In the control (without isolates), the pH was kept constant and increased to 8.8 in treatment with isolates. In these treatments with isolates and Cr (VI) (10 mg L^{-1}), pH values were close to 8.0 after 24 h of incubation. In treatments with 100 mg L^{-1} of Cr (VI), the same result was observed after 48 and 72 h of incubation for isolates 6 and 11, respectively (Fig. 2). The increase of pH in the culture medium can be related to metabolites secreted by isolates during cell growth or due to consumption of H^+ in the reduction of Cr^{6+}, since 1 mol of Cr (VI) requires 8 mol of H^+ (Park et al. 2004).

3.4 Reduction of Cr (VI) in Effluent with Nutrient Biostimulation

There was no reduction of Cr (VI) in the SSALE effluent after inoculation of isolates 6 and 11 without the addition of nutrients (Fig. 3). Carbon content in the

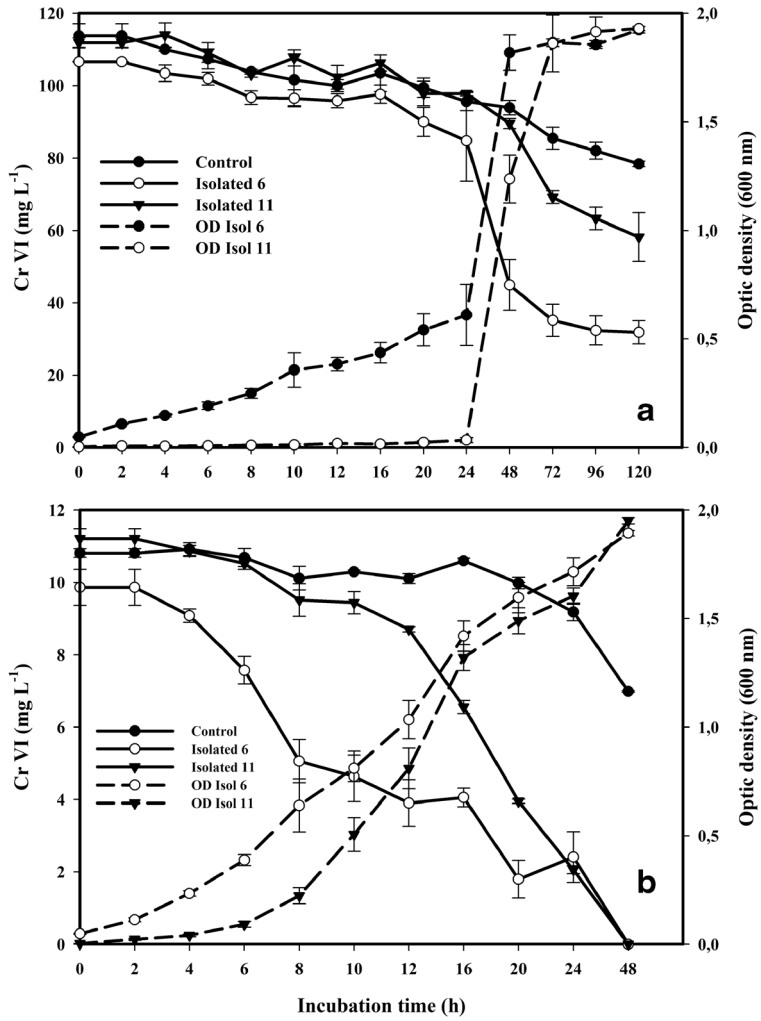

Fig. 1 Cr (VI) reduction and growth of isolates 6 and 11 in LB medium with 10 mg L^{-1} (**a**) and 100 mg L^{-1} (**b**) Cr (VI). Data are means of three replicates and the *bars* represent standard deviation

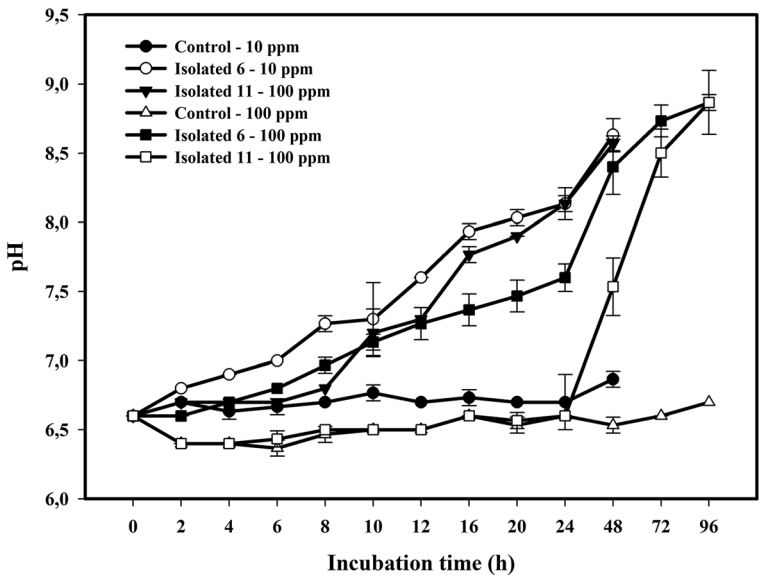

Fig. 2 pH variation of LB medium inoculated with isolates 6 and 10 with 10 and 100 mg L^{-1} Cr (VI) and 96 h of incubation at 30 °C. Data are means of three replicates and *error bars* represent the standard

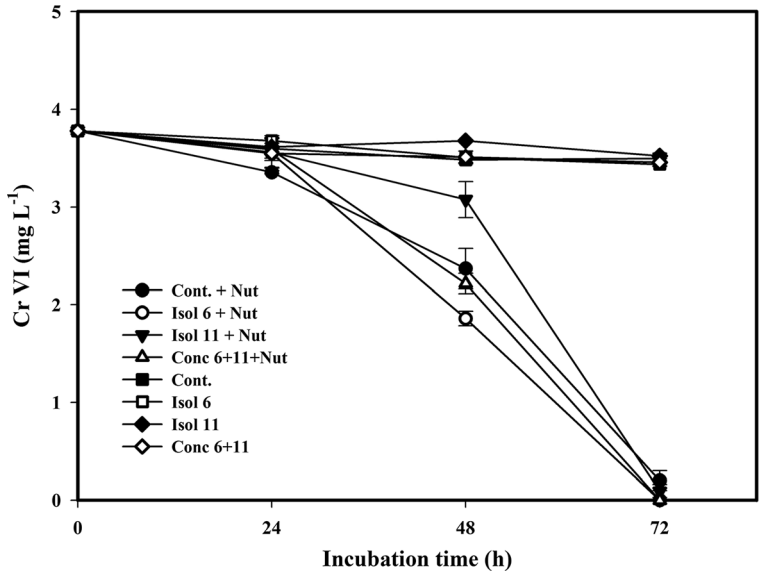

Fig. 3 Reduction of Cr (VI) by isolates 6, 11, consortium (6 + 11) and native microorganisms in effluent in 24, 48, and 72 h of incubation at 30 °C with the addition of analytical nutrients (Nut)

effluent (48 mg L^{-1}) is low as well as the biochemical oxygen demand (BOD; 27.8 mg L^{-1}) (Table 1). Phosphorus is also low (1 mg L^{-1}) (Table 1). According to Liebeg and Cutright (1999) C, N, and P are the most commonly used nutrients to increase microbial growth. These nutrients are limiting factors for the microbial degradation and appropriate concentrations stimulate biodegradation in soil (Zhou and Crawford 1995). Phosphorus is essential for the production of nucleic acids (DNA and RNA), sugars, phosphates, and molecules of energy transfer as ATP, ADP, and AMP (Atlas and Bartha 1998). Nitrogen is a constituent element of amino acids and nucleic acids. The N concentration in the effluent is suitable for the development of microorganisms, this being more NH_4^+ fraction (95 % mineral N) (Table 1).

However, Cr (VI) was reduced by 100 % in the effluent after 72 h of incubation regardless of the inoculation with the addition of C, N, and P. The total reduction of Cr (VI) with the addition of C, N, and P shows that these nutrients are a limiting factor for the reduction of Cr^{6+}. The biostimulation of indigenous microorganisms with CNP in the control was sufficient to reduce 100 % of Cr (VI) after 72 h of incubation (Fig. 3). Sundar et al. (2011) found that native isolates are more adaptable and more efficient in the remediation of tannery wastewater with high concentrations of chromium. In addition to nutrition, the effluent pH is a key variable for successful bioremediation.

Table 3 Reduction of Cr (VI) by native microorganisms in effluents in 24, 48, and 72 h of incubation at 30 °C with addition of nutrients of low-cost CNP

Treatment	Cr (VI) (mg L^{-1})		
	Incubation		
	24 h	48 h	72 h
Cont	4.90 ± 0.07*Aa	4.85 ± 0.05Aa	4.82 ± 0.03Aa
CNP	3.50 ± 0.19Aa	0.01± 0.01Bb	0.01 ± 0.01Bc
C	4.24 ± 0.13Aa	3.92 ± 0.16Aa	3.90 ± 0.03Ab
N	4.36 ± 0.19Aa	4.05 ± 0.07Aa	4.02 ± 0.02Aab
P	3.95 ± 0.07Aa	3.73 ± 0.10Aa	3.56 ± 0.06Ab
CN	4.42 ± 0.10Aa	4.26 ± 0.09Aa	4.15 ± 0.04Aab
CP	4.00 ± 0.06Aa	0.01 ± 0.01Bb	0.01 ± 0.01Bc
NP	4.30 ± 0.03Aa	3.93 ± 0.08Aa	3.84 ± 0.07 Ab
	CV% = 20.82		
	pH		
Cont	7.27Aa	7.47Aa	7.63Aa
CNP	4.42Abc	3.66Bc	3.70Bc
C	7.59Aa	7.56Aa	7.51Aa
N	7.61Aa	7.72Aa	7.66Aa
P	3.99Ac	4.52Ab	4.50Ab
CN	7.40Aa	7.72Aa	7.93Aa
CP	4.63Abc	3.44Bc	3.16Bc
NP	4.96Ab	4.93Ab	4.93Ab
	CV% = 5.15		

Values followed by the same uppercase letters in the line and lowercase letters in the column do not differ statistically (Tukey, $p < 0.05$)

[a] Standard deviation

Several studies in artificial medium or industrial effluents show that it is necessary to adjust the nutrient content and pH for effective reduction of Cr (VI) (Camargo et al. 2003; Thacker et al. 2007). A study of fungus *Paecilomyces lilacinus* by Sharma and Adholeya (2011) found that addition of 2 % (by volume) of sugar was necessary to microbial growth and reduce 1.24 mg L^{-1} of Cr (VI) after 18 h, totaling a reduction of 7.91 mg L^{-1} after 36 h. Nutritional composition in industrial wastewater varies widely. Besides the evaluation of elements such as N and P, it is also important to evaluate the dissolved organic carbon (DOC) and BOD which indirectly indicates the levels of organic matter and supports the growth of microorganisms and reduction of Cr (VI) (Sharma and Adholeya 2011; Gong et al. 2015). In a pilot experiment, Pan et al. (2014) found that supplementation of 1 % glucose in industrial wastewater helped to reduce microbial of 100.8 mg L^{-1} of Cr (VI) in 72 h.

3.5 Cr (VI) Reduction in Effluent with Biostimulation of Low-Cost Nutrients

In treatments with the addition of CNP (sucrose, urea, and TSP) and CP (sucrose and TSP), 100 % of Cr (VI) of the effluent was decreased after 72 h of incubation at

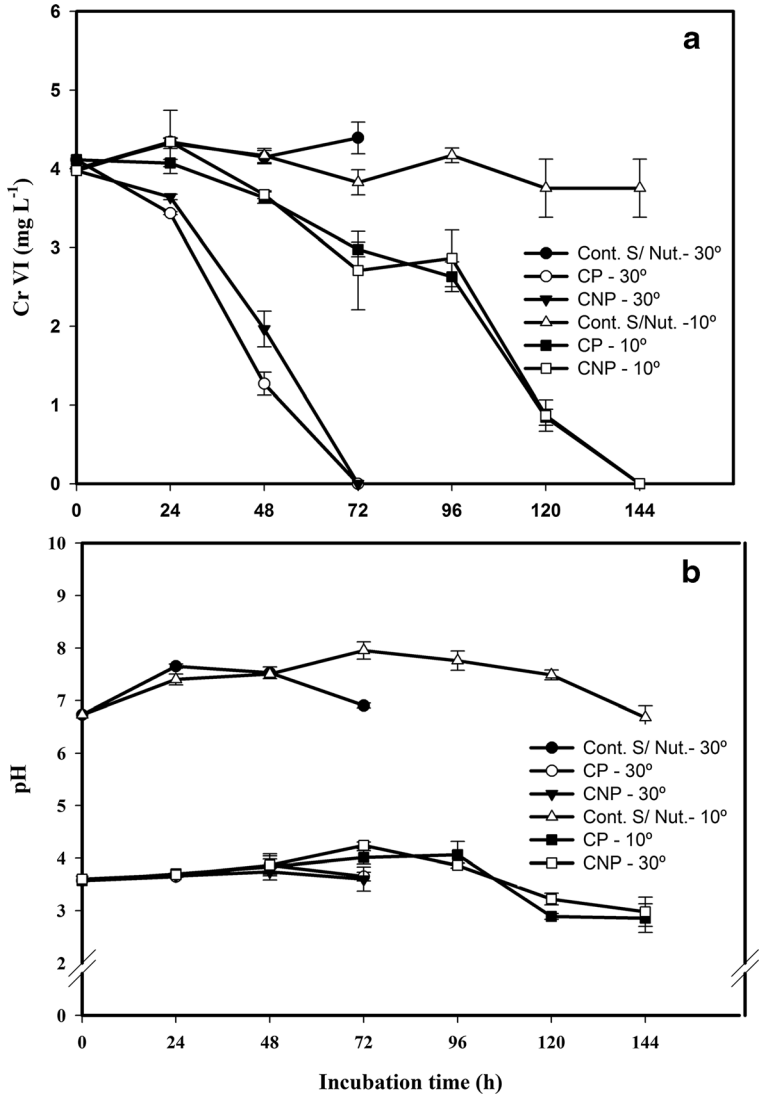

Fig. 4 Reduction of Cr (VI) (**a**) and pH (**b**) by the native microorganisms in the effluent in 72 h of incubation at 10 and 30 °C with the addition of low cost nutrients (Nut). Data are means of three replicates and *error bars* represent the standard.

30 °C and pH also decreased (Table 3). This demonstrates that the N effluent (84 mg L^{-1}) was sufficient for growth and reduction of Cr (VI) by the native biota. In treatments with NP and P, there was a decrease in pH (3.5–4.5) and no reduction of Cr (VI) (Table 3). Thus, it can be inferred that the Cr (VI) reduction was not caused by the low pH of the effluent but by nutritional stimulation of C and P to microorganisms. The low pH values were found in all treatments with phosphorus addition (Table 3, Fig. 4b). TSP ($Ca(H_2PO_4)_2\ H_2O$), when it reacts with water, dissociates phosphate ions (PO_4^{2-}) which are available to the microorganisms and H^+ responsible for the acidification of the environment. The Cr (VI) reduction occurs in a wide pH range (5–9). However, it is important that the pH of the intercellular medium is near 7.5. The microorganisms can achieve this adjustment of the external pH by absorption or extrusion of protons (Conceição et al. 2007).

3.6 Effect of Temperature

The indigenous microorganisms reduced 100 % of Cr (VI) present in the effluent after 72 h of incubation at 30 °C with the application of C and P. A similar reduction occurred at a temperature of 10 °C after 144 h of incubation (Fig. 4a). Camargo et al. (2003) observed that microbial isolates from contaminated soil changed rates of Cr (VI) reduction in a liquid growth medium when subjected to low temperatures. The reduction of Cr (VI) in the effluent was slower at 10 °C compared with a 30 °C temperature with 72 h (Fig. 4a). Temperatures below 25 °C cause a substantial reduction in the microbial metabolism, since there are small conformational changes of the proteins and reduction in the fluidity of membranes which result in a lower reaction rate (Dijkstra et al. 2011). Okeke et al. (2008) using *Bacillus* sp. to reduce Cr (VI) concluded that the optimum temperature reduction is 35 °C. The reduction of pH by the addition of P also occurred at 10 °C (Fig. 2b).

In the State of Rio Grande do Sul, large volumes of wastewater contaminated with Cr (VI) are still routinely generated in the industry, especially the footwear industry. As seen in this work, the chemical composition of the effluent directly influences the microbial activity and reduction of chromium. The addition of essential nutrients, such as C, N, and P from inexpensive sources such as agricultural fertilizers, accelerates the reduction of Cr (VI) and can improve the effluent treatment systems. With the addition of nutritional supplies, the total reduction of Cr (VI) occurred in 24 and 48 h in some cases. This is a very important result for higher operability of wastewater treatment plants. Indigenous effluent microorganisms, already adapted to Cr (VI), showed a similar performance to these isolates at high concentrations of Cr (VI). In most cases, the major benefits of the effluent remediation effluent using microorganisms are low-cost and efficient technology and little or no use of additional substances is present that can indirectly cause damage to the environment and human health.

4 Conclusions

The bacterial isolates studied here reduce 100 % of the Cr (VI) in the LB medium and low concentrations at 30 °C. The tested isolates are effective in reducing Cr (VI) and isolate 6 provided the best response to high concentrations of Cr (VI) in LB medium. Biostimulation is necessary to reduce hexavalent chromium in an effluent with a low concentration of N, P, and C, and bioaugmentation is not always the best strategy for the bioremediation of contaminated wastewater. Low temperatures slow down the reduction of Cr (VI) but do not preclude the process. This study demonstrates that the bacterial isolates and indigenous microorganisms, when stimulated nutritionally, can effectively be used in bioremediation of industrial effluents with Cr (VI).

Acknowledgements We thank CNPq for the financial support and the scholarships granted.

References

Ahluwalia, S. S., & Goyal, D. (2007). Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresource Technology, 98*, 2243–2257.

APHA, AWWA, & WPCF (Eds.). (1998). Standard methods for the examination of water and wastewater, Washington.

Atlas, R. M., & Bartha, R. (1998). *Microbial ecology: fundamentals and applications* (4th ed.). Redwood: Cummings Science, Menlo Park.

ATSDR - Agency for toxic substances and disease. (2008). *Registry. Toxicological profile for chromium. U.S. Public Health Service*. Atlanta, GA: U.S. Department of Health and Human Services.

Ball, M. M. (2007). Mercury resistance in bacterial strains isolated from tailing ponds in a gold mining area near el callao (Bolivar State, Venezuela). *Current Microbiology, 54*, 149–154.

Bento, F. M., Carmargo, F. A. O., Okeke, B. C., & Frankenberger, W. T. (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. *Bioresource Technology, 9*(96), 1049–1055.

Camargo, F. A. O., Okeke, B. C., Bento, F. M., & Frankenberger, W. T. (2003). In vitro reduction of hexavalent chromium by a cell-free extracts of Bacillus sp. ES 29 stimulated by Cu^{2+}. Ap. *Microbial Biotechnology, 62*, 569–573.

Chambers, D. C., Willis, J., Giti-Pour, S., Zieleniewiski, L. J., Rickabaugh, J. F., Mecca, M. I., Pasin, B., Sims, C. R., Sorensen, L. D., Sims, L. J., Mclean, E. J., Mahmood, R., Dupont, R. R., & Wagner, K. (1991). *In-situ treatment of hazardous waste contaminated soil* (2nd ed., p. 44-64, 176-209, 299-327). New Jersey: Noyes Data Corporation.

Chen, J. M., & Hao, O. J. (1998). Microbial chromium (VI) reduction. *Critical Reviews in Environmental Science and Technology, 28*, 219–225.

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

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.

Conceição, D. C., Jacques, R., Bento, F., Simonetti, A., Selbach, P., & Camargo, F. (2007). Chromium hexavalent reduction by bacteria isolated from soil contaminated with chromium. *Ciência Rural, 6*(37), 1661–1667.

Das, A. P., & Mishra, S. (2010). Biodegradation of the metallic carcinogen hexavalent chromium Cr (VI) by an indigenously isolated bacterial strain. *Journal Carcinogenesis., 9*, 1–6.

Dermou, E., Velissariou, A., Xenos, D., & Vayenas, D. V. (2005). Biological chromium (VI) reduction using a trickling filter. *Journal Hazardous Materials., 126*, 78–85.

Dijkstra, P., Thomas, S. C., Heinrich, P. L., Koch, G. W., Schwartz, E., & Hungate, B. A. (2011). Effect of temperature on metabolic activity of intact microbial communities: Evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency. *Soil Biology and Biochemistry, 10*(43), 2023–2031.

Fakruddin, M., Mazumdar, R. M., Tania, T. K., Islam, S., Nipa, M. N., Iqball, A., & Bhuiyan, H. R. (2009). Isolation and characterization of chromate resistant and reducing bacteria from tannery effluent of Chittagong, Bangladesh. *Journal Biosciences., 17*, 71–76.

Franco, D. V., Da Silva, L. M., & Jardim, W. F. (2009). Reduction of hexavalent chromium in soil and ground water using zero-valent iron under batch and semi-batch conditions. *Water, Air, & Soil Pollution, 197*, 49–60.

Gong, Y. F., Song, J., Ren, H. T., & Han, X. (2015). Comparison of Cr(VI) removal by activated sludge and dissolved organic matter (DOM): importance of UV light. *Environmental Science and Pollution Research, 22*, 18487–18494.

Hawley, E. L., Deeb, R. A., Kavanaugh, N. C., & Gabocbs, J. (2005). Treatment technologies for chromium. In J. Guertin, J. Jacobs, & C. P. Avakian (Eds.), *Chromium (VI) handbook* (pp. 273–308). Boca Raton: CRC, Taylor and Francis Group.

Kader, J., Sannasi, P., Othman, O., Ismail, L. B. S., & Salmijah, S. (2007). Removal of Cr (VI) from aqueous solutions by growing and non-growing populations of environmental bacterial consortia. *Global Journal Environmental Research., 1*, 12–17.

Kathiravan, M. N., Karthick, R., & Muthukumar, K. (2011). Ex situ bioremediation of Cr (VI) contaminated soil by Bacillus sp.: batch and continuous studies. *Chemical Engineering Journal, 169*, 107–115.

Kumar, P., Prasad, B., Mishra, I. M., & Chand, S. (2008). Decolorization and COD reduction of dyeing wastewater from a cotton textile mill using thermolysis and coagulation. *Journal of Hazardous Materials, 153*, 635–645.

Liebeg, E. W., & Cutright, T. J. (1999). The investigation of enhanced bioremediation through the addition of macro and micro nutrients in a PAH contaminated soil. *International Biodeterioration & Biodegradation, 44*, 55–64.

Liu, Z., Wu, Y., Lei, C., Liu, P., & Gao, M. (2012). Chromate reduction by a chromate-resistant bacterium, Microbacterium sp. *World Journal of Microbiology and Biotechnology, 28*, 1585–1592.

Long, D., Tang, X., Cai, K., Chen, G., Chen, L., Duan, D., Zhu, J., & Chen, Y. (2013). Cr (VI) reduction bey a potent novel alkaliphilic halotolerant strain Pseudochrobactrum saccharolyticum LY10. *Journal Hazardous Materials, 256*(257), 24–32.

Malik, A. (2004). Metal bioremediation through growing cells. *Environmental International, 30*, 261–278.

Mangaiyarkarasi, M., Vincent, S., Janarthanan, S., Rao, T. S., & Tata, B. (2011). Bioreduction of Cr(VI) by alkaliphilic Bacillus subtilis and interaction of the membrane groups. *Saudi Journal of Biological Sciences, 2*(18), 157–167.

Okeke, B. C., Layomon, J., Crenshaw, S., & Oji, C. (2008). Environmental and kinetic parameters for Cr(VI) bioreduction by a bacterial monoculture purified from Cr(VI)-resistant consortium. *Biology Trace Element Research., 123*, 229–241.

Ozturk, S., Kaya, T., Aslim, B., & Tan, S. (2012). Removal and reduction of chromium by pseudomonas spp. and their correlation to rhamnolipid production. *Journal of Hazardous Materials, 231*, 64–69.

Pan, X., Liu, Z., Chen, Z., Cheng, Y., Pan, D., Shao, J., Lin, Z., & Gan, X. (2014). Investigation of Cr(VI) reduction and Cr(III) immobilization mechanism by planktonic cells and biofilms of Bacillus subtilis ATCC-6633. *Water Research, 55*, 21–29.

Park, D., Yun, Y. S., & Park, J. M. (2004). Reduction of hexavalent chromium with the brown seaweed Ecklonia biomass. *Environmental Science & Technology, 38*, 4860–4864.

Ramirez-Diaz, M. I., Dıaz-Perez, E. C., Vargas, E. E., Riveros-Rosas, E. H., Campos-Garcia, E. J., & Cervantes, E. C. (2008). Mechanisms of bacterial resistance to chromium compounds. *Biometals, 21*, 321–332.

Sagar, S., Dwivedi, A., Yadav, S., Tripathi, M., & Kaistha, S. D. (2012). Hexavalent chromium reduction and plant growth promotion by Staphylococcus arlettae Strain Cr11. *Chemosphere, 86*, 847–852.

Salnikow, K., & Zhitkovich, A. (2007). Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chemical Research in Toxicology, 21*, 28–44.

Sambrook, J., & Russell, D. (2001). *Molecular cloning: a laboratory manual* (3rd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

SAS-Statistical Analyses System. (2008). *Statistical analysis system user's guide. Version 9.2.*. Cary: Statistical Analyses System Institute.

Sharma, S., & Adholeya, A. (2011). Detoxification and accumulation of chromium from tannery effluent and spent chrome effluent by Paecilomyces lilacinus fungi. *International Biodeterioration & Biodegradation, 65*, 309–317.

Shrivastava, R., Upreti, R. K., Seth, P. K., & Chaturvedi, U. C. (2002). Effects of chromium on the immune system. *FEMS Immunology & Medical Microbiology., 34*, 1–7.

Sundar, K., Mukherjee, A., Sadiq, M., & Chandrasekaran, N. (2011). Cr (III) bioremoval capacities of indigenous and adapted bacterial strains from Palar river basin. *Journal of Hazardous Materials, 187*, 553–561.

Thacker, U., Parikh, R., Shouche, Y., & Madamwar, D. (2006). Hexavalent chromium reduction by Providencia sp. *Process Biochemistry, 41*, 1332–1337.

Thacker, U., Parikh, R., Shouche, Y., & Datta, M. (2007). Reduction of chromate by cell free extract of Brucella sp. isolated from Cr(VI) contaminated sites. *Bioresource Technology, 98*, 1541–1547.

USEPA. (1998). *Toxicological review of hexavalent chromium*. Washington: U.S. Government Printing Office.

USEPA. (1992). Method 7196A: chromium, hexavalent (colorimetric). In: TEST methods for evaluating solid waste, physical/chemical methods – SW-846. Washington: U.S. Government Printing Office.

Wani, R., Kodam, K. M., Gawai, K. R., & Dhakephalkar, P. K. (2007). Chromate reduction by Burkholderia cepacia MCMB-821, isolated from the pristine habitat of alkaline Crater Lake. *Applied Microbiology and Biotechnology, 3*(75), 627–632.

Ye, J., Yin, H., Mai, B., Peng, H., Qin, H., He, B., & Zhang, N. (2010). Biosorption of chromium from aqueous solution and electroplating wastewater using mixture of Candida lipolytica and dewatered sewage sludge. *Bioresource Technology, 101*, 3893–3902.

Zhou, E., & Crawford, R. L. (1995). Effects of oxygen, nitrogen and temperature on gasoline biodegradation in soil. *Biodegradation, 6*, 127–140.