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# Isolation and Characterization of a Cr(VI) Reducing *Bacillus firmus* Strain from Industrial Effluents

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Abstract

A chromium resistant bacterial strain KUCr1 exhibiting potential Cr(VI) reducing ability under *in vitro* aerobic condition is reported. The bacterial strain showed varied degree of resistance to different heavy metals. The MIC of chromium to this strain was found to be 950 mM under aerobic culture condition in complex medium. The factors affecting Cr(VI) reduction by this strain under culture condition were evaluated. Maximal Cr(VI) reduction was observed at the pH 8 to 10 and at a temperature of 35°C. Higher concentration of Cr(VI) slowed down the reduction, eventually all the metal could be reduced with longer incubation time. Different toxic metals showed differential effect on reduction. Cadmium and zinc were found to inhibit reduction. Cr(VI) reduction and bioremediation were found to be related to the growth supportive condition in terms of carbon, phosphorous and nitrogen supply in wastewater fed with tannery effluent indicating cell mass dependency of Cr(VI) reduction. Through biochemical characterization and 16S rDNA sequence analysis, the strain KUCr1, as the name given to it, was identified as a strain of *Bacillus firmus*.

Key words: ambient factors, bioremediation, hexavalent chromium reduction

## Introduction

Chromium (Cr) has been identified by US Environmental Protection Agency (US EPA, 1998) as one of the 17 toxic metals/metalloids posing the major hazard in environment. This transition metal is widely found in nature in different valence states as Cr(VI) to Cr(III) forms. Among these Cr(VI) is highly soluble, thus mobile and biologically available in the ecosystems and thus exerts toxicity; whereas Cr(III) forms complexes that precipitate as amorphous hydroxide (Palmer and Wittebrodt, 1991; Sawyer *et al*., 1994).

The toxic form of Cr is released in environment with effluents from different industries (Bailar, 1997; US EPA, 1998; Abdel-Sabour, 2007). The technologies for Cr clean-up from the contaminated sites consist mostly of, (i) removing the maximum Cr(VI)-contaminated parts from the site; (ii) immobilizing the chromium to prevent further leaching; or (iii) reducing the Cr(VI) to its non-toxic species, *i.e.* Cr(III) state. Microbial reduction of toxic Cr(VI) has practical importance in this respect, because biological strategies provide cost-effective and ecofriendly technology (Eccles, 1995).

Bacteria detoxify chromium mainly by reducing Cr(VI) to Cr(III) *via* Cr(V) and Cr(IV) intermediates (Camargo *et al*., 2003; Xu *et al*., 2004, 2005; Pal *et al*., 2005; Cheung *et al*., 2006), therefore it is a potentially useful process for remediation of Cr(VI)-affected environments (Michel *et al*., 2001). Many aerobic and anaerobic microbes were reported to reduce Cr(VI) to Cr(III) while utilizing a wide range of electron donors (Bopp and Ehrlich, 1988; Ishibashi *et al*., 1990; Francis *et al*., 2000; Fredrickson *et al*., 2000; McLean and Beveridge, 2001).

The vision of our work was to isolate and characterize a chromium resistant bacterial strain that could be exploited for its ability to reduce Cr(VI) to non-toxic Cr(III). Biochemical and molecular characterization (16S rDNA sequence analysis) were carried out to identify the strain KUCr1.

## Experimental

### Materials and Methods

**Isolation of chromate resistant bacterial strain.** Chromate-resistant bacteria were isolated from soil samples fed with Cr containing effluents of electroplating industries nearby Kolkata, India. The sample was serially diluted and inoculated on PYG agar (peptone,

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10 g/l; yeast extract, 5 g/l; glucose, 3 g/l; agar, 20 g/l; pH 7.2) plates having different concentration of Cr (0.5, 1, 1.5, and 2 mM) as $K_2CrO_4$. The colonies that could tolerate the highest concentration of Cr (2 mM) were selected randomly and assessed for its Cr(VI) reductive ability. The isolate that showed highest Cr(VI) reductive ability was selected for further experiment in this study and the strain was designated as KUCr1. The isolate was then purified by cycles of single colony isolation and liquid culture transfers on minimal medium ($K_2HPO_4$, 3 g/l; $Na_2HPO_4$, 6 g/l; NaCl, 5 g/l; $NH_4Cl$, 2 g/l; $MgSO_4$, 0.1 g/l; glucose, 8 g/l; pH 7.2) supplemented with 2 mM Cr. A single culture was eventually chosen for further experiment on the basis of their Cr reductive ability and is being maintained.

**Characterization of the Cr-resistant bacterial isolate.** The isolate was identified based on the morphological and standard biochemical tests according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The strain was also tested for its resistance to different toxic metals in PYG medium. Furthermore, the genomic DNA was isolated from the pure culture pellet and ~1.4 kb rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer. The sequencing of the PCR product was done at Chromous Biotech Pvt. Ltd., Bangalore, India. The sequence data were aligned and analyzed to identify the bacterium and its closest neighbors using BLAST function (Altschul *et al*., 1990) at NCBI database and the Ribosomal Database Project (Maidack *et al*., 1997).

**Growth and Cr(VI) reduction in PYG and minimal media under chromium stress.** To assess the effect of Cr (VI) on cell growth under aerobic condition, KUCr1 were inoculated with same volume of young cell suspension (finally to have ~6.2 log CFU/ml) into both undefined PYG and minimal media supplemented with 2 mM Cr (VI) as $K_2CrO_4$ and incubated on a rotary shaker at 37°C, and compared with respective control set without Cr. The growth responses were determined by counting the colony forming units (CFU) on PYG agar plate. The chromate reduction was measured at different time intervals by measuring the residual Cr(VI) in the cell-free supernatant following centrifugation.

**Chromium(VI) reduction and its analysis.** Chromate reduction was assayed as the decrease of chromate with time using Cr(VI) specific colorimetric reagent S-diphenylcarbazide (DPCZ). One ml of 0.05% DPCZ (w/v in acetone) and 3 ml of 0.16 M sulfuric acid were added for minimizing the deterioration (Urone, 1955) to 1 ml sample of known dilution of Cr(VI). DPCZ reacts with chromate and forms a purple complex that absorbs light at 540 nm. The absorbance of the reaction mixture was taken immediately at 540 nm in a spectrophotometer (Cecil CE7200, England). Quantity of Cr(VI) was measured obtaining the standard curve using solutions of $K_2CrO_4$ as standard.

**Effect of pH and ambient temperature on chromium reduction.** The influence of pH on chromate reduction under aerobic culture condition was assessed in PYG broth having different pH values, inoculated with same volume of young cell suspension and incubated at 37°C. The influence of incubation temperature on chromate reduction under aerobic culture condition was assessed in PYG broth (pH 7.2), inoculated with same volume of young cell suspension and incubated on a rotary shaker at different temperature. Chromate reduction at different time intervals was measured.

**Effect of different metals/metalloid on Cr(VI) reduction.** Normally the Cr-contaminated sites show simultaneous presence of toxic metals/metalloids which effect the survival of the inoculant. In order to assess the exact effect of such metals/metalloid (Cd, Co, Ni, As and Zn) on its Cr(VI) reductive ability, the isolate was grown on PYG broth having 2 mM Cr with different test metals (0.1 mM in the media) separately. Different treatment regimes were inoculated with same volume of young cell suspension (finally to have ~5.7 log CFU/ml), incubated on a rotary shaker at 37°C and chromate reduction was measured.

**Biosorption of chromium.** In order to assess whether the bacterial strain has Cr biosorbing ability apart from its Cr(VI) reductive activity, the dead cell mass (both dry and wet) were put into $K_2Cr0_4$ solution in saline buffer finally having 2 mM Cr(VI). Bacterial cell mass of 72 h old was harvested by centrifugation at 7000 × g for 10 min and the cell pellet was dried at 60°C for 24 h to have dry dead cell or the cell pellet was autoclaved to have wet dead cell. The removal of Cr by the dead cell was measured at different time intervals by measuring the residual Cr(VI) in the supernatant following centrifugation at 7000 × g for 10 min.

**Survival of KUCr1 and Cr(VI) reduction in tannery effluent.** Wastewater having 0.272 mM Cr(VI) collected from an effluent fed canal nearby a tannery industry, pH was adjusted to 7.5. 100 ml of 12 h grown cell suspension culture grown in PYG medium was added to the experimental sets containing sterilized 20 ml wastewater supplemented with 2 mM Cr(VI), and to a control set without added Cr. The ambient temperature (35°C) was adjusted as per respective optimal value for Cr(VI) reduction obtained in PYG medium.

Anticipating the poor nutritional support in tannery wastewater, in another experiment it was assessed whether addition of extra nutrient has any positive role on Cr reduction or not. For this study wastewater was supplemented with equal amount (0.8% w/v) of $NH_4Cl$, glucose and $KH_2PO_4$ as N, C and P sources respectively. 100 ml of 12 h grown cell suspension was added to 20 ml of extra nutrient added wastewater

with or without additional Cr (2 mM) and the cultures were kept at 35°C on shaking incubator. The viable cell numbers were determined for each set by dilution plate technique on PYG plates having 2 mM Cr. Chromate reduction was compared between the sets having extra nutrient and sets without extra nutrient.

## Results and Discussion

The selected KUCr1 strain showed varied degree of resistance to different heavy metals/metalloid and the order of toxicity of the test elements to the bacterium in PYG broth was Cd > As = Co = Ni > Zn > Cr. The minimum inhibitory concentration (MIC) of the test metals for the bacterium are 3 mM, 10 mM, 22 mM and 950 mM, respectively. Based on the biochemical tests and analysis of the 16S rDNA sequence (1196 bp) using BLAST function at NCBI database and Ribosomal Database Project, the isolate KUCr1 was identified as a strain of *Bacillus firmus* (NCBI GenBank Accession No. EU784699).

The growth of the isolate and course of the Cr(VI) reduction in PYG and minimal media (MM) supplemented with 2 mM Cr were compared (Fig. 1 and 2). Because of less cell mass yield by the strain at the maximum tolerance level of Cr as per MIC data, in this experiment 2 mM, which is even much higher than that in the industrial effluents, was used to have a substantial bacterial cell mass. The bacterial strain showed steady growth in the complex medium (PYG) accompanied with maximum Cr reduction capacity. However, when the strain was grown in minimal medium the growth rate as well as Cr reduction ability was declined considerably. The differences in growth and Cr removal in those media may be explained by the fact that bioavailable metal content was reduced due to complexation with undefined components in the PYG medium. Almost 100% removal of Cr(VI) occurs in PYG medium whereas about 80% removal occurs in MM after 6 days. The level of Cr tolerance of this strain under aerobic conditions is significant in comparison to that of the earlier reported bacilli strains (Shakoori *et al*., 2000; Camargo *et al*., 2003). There are many available other reports on Cr reduction in *Bacillus* (Campos *et al*., 1995; Garbisu *et al*., 1998; Nurbap Nourbakhsh *et al*., 2002; Cheung and Gu, 2005, 2007; Pal *et al*., 2005), however, reports on *B. firmus* are scanty in particular, and this article reports a highly Cr resistant and Cr(VI) reducing *B. firmus* strain. Removal of metal ions (Pb, Cu and Zn) from aqueous solution by extracellular polysaccharide produced by *B. firmus* was only reported earlier (Salehizadeh and Shojaosadati, 2003).

The effect of Cr(VI) concentration in the medium on the overall rate of reduction was studied at varied

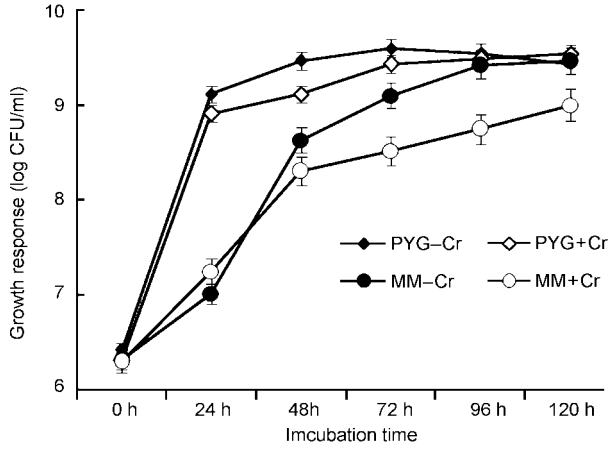


Fig. 1. Growth response of the test strain grown in PYG and minimal media (MM) supplemented with 2 mM Cr as $K_2CrO_4$ (+ Cr) or without chromium (– Cr). Data are the mean of three replications with error bars.

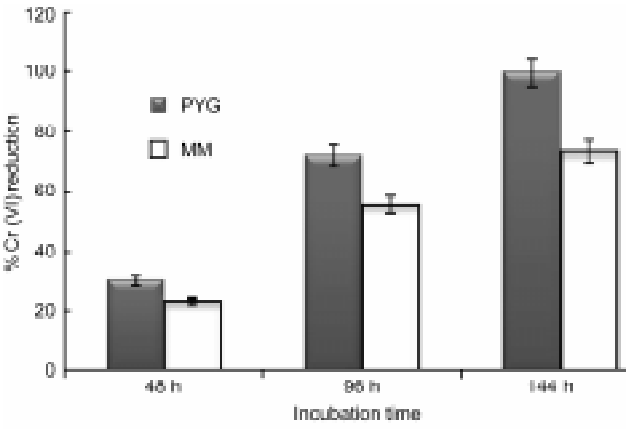


Fig. 2. Cr (VI) reduction by the test strain in PYG and minimal media (MM) supplemented with 2 mM Cr as $K_2CrO_4$. Data are the mean of three replications with error bars.

concentrations (0.5 mM to 2 mM). Cr(VI) was almost completely reduced at all concentrations after 120 h (Fig. 3). At lower concentrations (0.5 mM and 1 mM) reduction rate increased sharply by 24 h and the metal was completely reduced after 48 h. The reaction rate was slowed in the first 24 h at concentrations of 1.5 mM and 2 mM, then it took 72 h to reduce Cr(VI) completely at 1.5 mM and 120 h at 2 mM concentration. It seems higher concentration posed a selection pressure due to Cr toxicity and thus lengthened the growth phase, and for having substantial cell mass it took longer period for complete reduction. Similar observation was also reported earlier by McLean *et al*. (2000). Cr(VI) concentration dependence on reduction kinetics in *Bacillus* was also reported (Wang and Xiao, 1995; Camargo *et al*., 2003).

Bacterial Cr(VI) reduction was found to be dependent on ambient pH and temperature which affect enzymatic reactions necessary for Cr(VI) reduction. For most of the isolates so far reported, the optimal pH

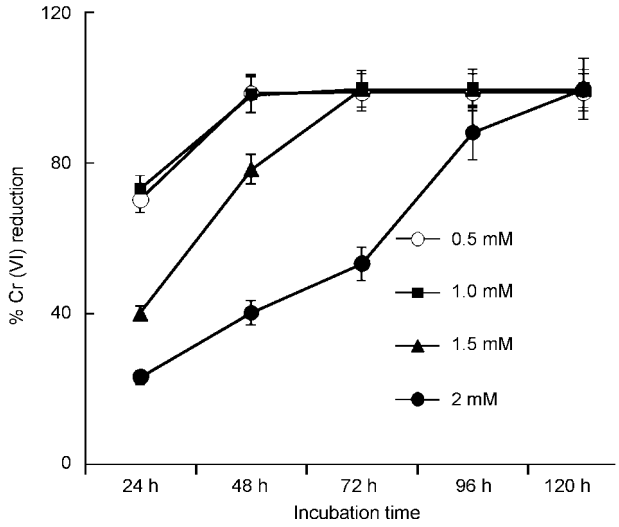


Fig. 3. Cr(VI) reductive ability of KUCr1 grown in PYG broth (pH 7.2) having different concentration of chromium. Data are the mean of three replications with error bars.

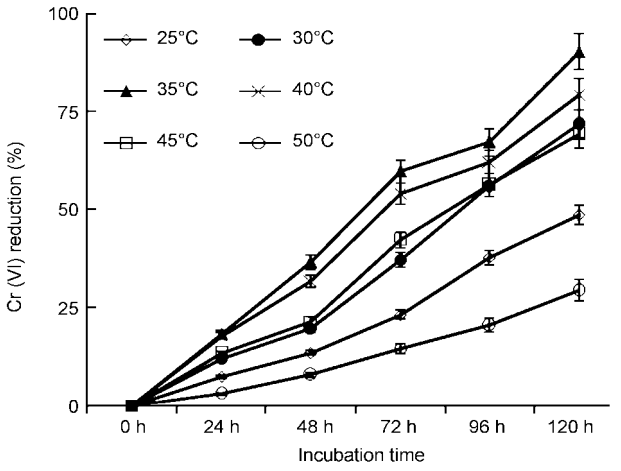


Fig. 5. Effect of different temperature on Cr (VI) reduction by the test strain under aerobic culture condition grown in PYG medium supplemented with 2 mM Cr as $K_2CrO_4$. Data are the mean of three replications with error bars.

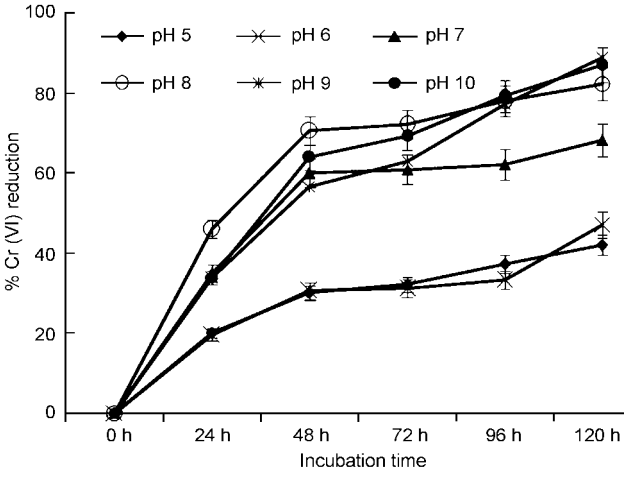


Fig. 4. Effect of different pH on Cr (VI) reduction by the test strain under aerobic culture condition grown in PYG medium supplemented with 2 mM Cr as $K_2CrO_4$. Data are the mean of three replications with error bars.

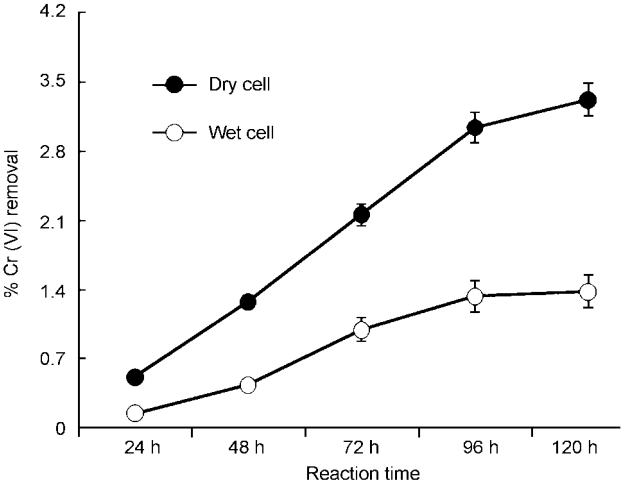


Fig. 6. Chromium removal (%) by the dead cellmass (wet and dry) from chromate solution having 2 mM Cr(VI). Data are the mean of three replications with error bars.

and temperature for growth correlated with highest rate of Cr(VI) reduction (Wang *et al*., 1990; Wang and Xiao, 1995; Shakoori *et al*., 2000; Camargo *et al*., 2003). The KUCr1 showed significant growth at wide range of pH, however, pH 7 to 10 was found to be growth yield supportive for significant Cr(VI) reduction (data not shown). Accordingly, Cr(VI) reduction increased with elevated ambient pH value above 7 to pH 10 (Fig. 4). However, the optimal pH for the growth of KUCr1 ranges from 7.5 to 9. The relationship between pH and Cr(VI) reduction was not surprising because chromate ($CrO_4^{2-}$) offers the dominant Cr(VI) species in an aqueous environment at pH 6.5 to 9 (Mc Lean and Beveridge, 2001).

The optimum temperature for better growth yield in KUCr1 was found to range from 35°C to 40°C, and Cr(VI) reduction achieved its highest value at 35°C (Fig. 5). Earlier Losi *et al*. (1994) reported an optimum temperature of 30 to 37°C required for Cr(VI) reduction under culture condition. Wang *et al*. (1990) also reported interference of temperature on chromate reduction. Requirement of an ambient temperature of 30°C for highest Cr(VI) reduction in *Bacillus* sp. was also reported (Wang and Xiao, 1995; Camargo *et al*., 2003).

Cr(VI) reduction was variedly affected by the addition of different heavy metals (0.1 mM) under culture condition. Cadmium and zinc significantly inhibited Cr(VI) reduction whereas arsenic, cobalt and nickel salt did not show any notable effect on Cr(VI) reduction (Table I) by the bacterial isolate, even though all treatment regimes showed similar cell mass yield (data

Table I
Effect of different metals/metalloids on Cr (VI) reduction (%)[a] by the test strain[b]

| Control[c] | Metal/Metalloid used | | | | |
|---|---|---|---|---|---|
| | Zn | Cd | As | Ni | Co |
| 99.00 (±0.577) | 44.37 (±0.595) | 26.23 (±0.606) | 87.93 (±0.520) | 85.65 (±0.693) | 80.27 (±0.589) |

[a] Data are the mean of 3 replications with standard error and were obtained after 120 h of incubation.
[b] For metal effect study PYG medium supplemented with 2 mM Cr additionally with different test metals (0.1 mM) separately.
[c] Bacteria were inoculated to PYG without any test metal except Cr (2 mM).

not shown), as the strain has inherent resistance to that metals/metalloid. It seems Cd and Zn have some interference on the biochemistry of Cr(VI) reduction. Faisal and Hasnain (2004) reported both Cr(VI) reduction enhancement and inhibition by Zn (as 200 ppm $ZnSO_4$) and Co (as 50 ppm $CoCl_2$) respectively in one strain of *Brevibacterium* under culture condition. McLean and Beveridge (2001) observed concentration dependent inhibition in Cr(VI) reduction by a pseudomonad strain. Desjardin *et al*. (2003) reported Ni and Cd induced Cr(VI) reduction during first 72 h and thereafter did not affect the reduction in *Streptomyces thermocarboxydus*. The mechanism of Cr(VI) reduction inhibition by Cd and Zn in this strain needs further investigation.

Among bacteria, *Bacillus* sp. has been identified as having a high potential for metal sequestration and has been used in commercial biosorbent preparation. In order to assess whether this *Bacillus* strain has Cr biosorbing ability apart from its Cr(VI) reduction, dead cell mass (wet and dried) was exposed to $K_2CrO_4$ solution. To avoid the artifacts might arise due to the presence of insoluble Cr(III) in the precipitate coming from reduction by active cells, we could not perform the experiment of bioaccumulation by viable cells. A significant cellular accumulation of chromium in *Brevibacterium* (Faisal and Hasnain, 2004) and in *Bacillus* (Nurbap Nourbakhsh *et al*., 2002) was reported earlier. The amount of chromium adsorbed by the dry and wet dead cell mass of KUCr1 increased with increasing contact time with metal solution (Fig. 6). Dried cell mass adsorbed a bit higher amount of chromium than the dead wet cell mass did, which contradicts the earlier observation (Faisal and Hasnain, 2004). Overall chromium removal by dead cell mass in this study was found to be very insignificant in comparison to the Cr(VI) reduction by the active cell mass of this strain under culture condition.

An experiment was conducted to determine Cr-reducing activity of the isolate with a number of different carbon and energy sources. The purpose was to enrich for potential Cr(VI)-reducing strain by carbon amendment and its possible optimization (Smith *et al*., 2002). The results showed that the isolate grew significantly better in glucose containing medium, thus the Cr(VI) reduction was also enhanced (result not shown). From this finding the wastewater was supplemented with glucose and other nutrients (N and P). The bacteria grew significantly better when additional nutrients were provided and Cr(VI) reduction was found to be doubled (Fig. 7A and 7B). It was observed that under nutrient supportive condition the bacterial strain reduced 64.4% of the Cr from the wastewater after 6 days. But when the strain was grown in nutrient deficient condition it reduced 32.5%. This finding signifies the cell mass dependency of Cr(VI) reduction.

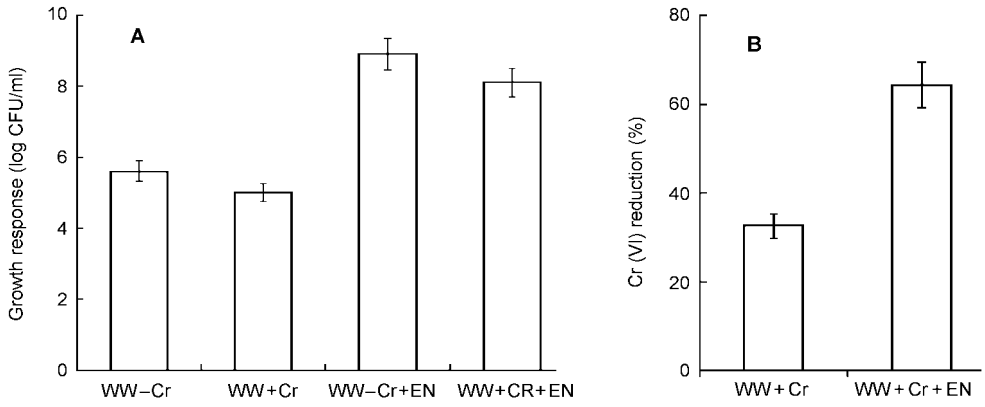


Fig. 7. Growth response (A) and Cr(VI) reduction ability (B) of KUCr1 in wastewater from tannery industry after 6 days initially having 5.7 log CFU/ml. WW = wastewater; –Cr/+Cr = without/with added Cr; –EN/+EN = without/with extra nutrients as carbon, phosphorous and nitrogen source (*vide* Experimental part). Data are the mean of three replications with error bars.

However, potentiality of using cost effective natural or non-conventional carbon or other mineral sources to support *in situ* bioremediation of Cr(VI) by this bacterial candidate warrants further investigation.

The studied ambient parameters that affect chromate reduction are important physico-chemical factors regulating bioremediation strategies for sites contaminated with toxic species of chromium. This strain shows promise for Cr(VI) reduction and its features would be useful for successful microbiological detoxification of chromate for both *in situ* or *ex situ* bioremediation of Cr-contaminated sites with appropriate growth and reduction supportive conditions.

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