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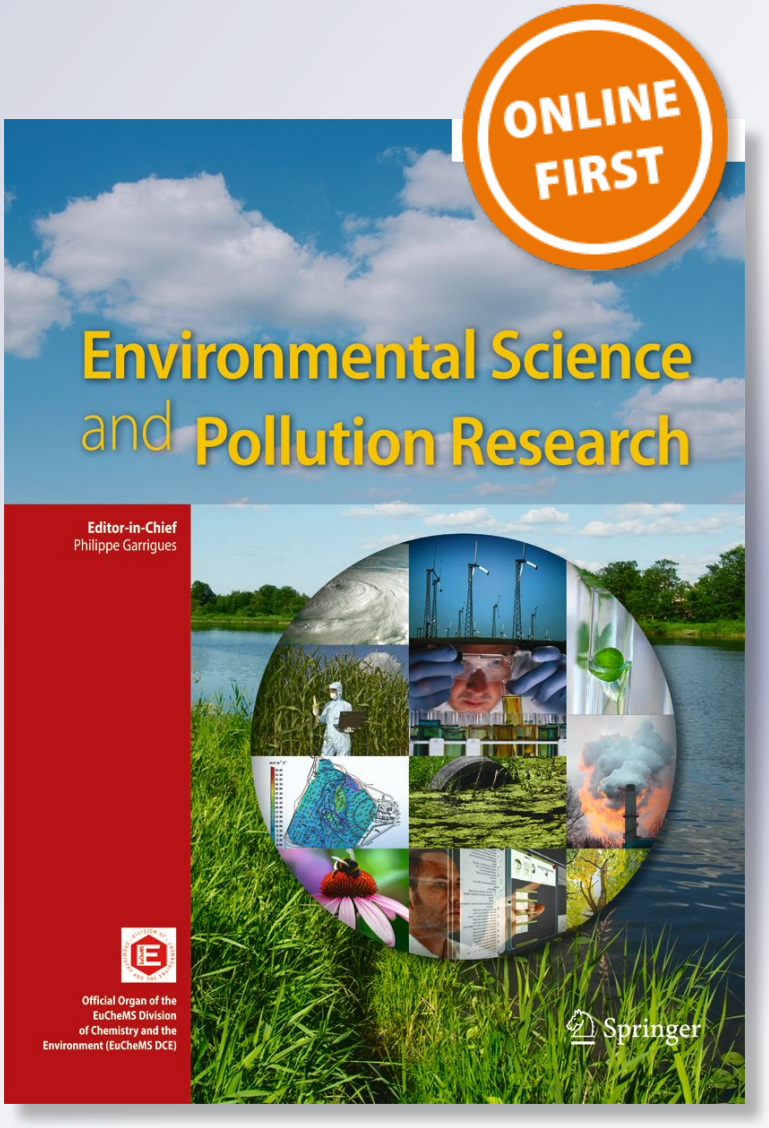

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RESEARCH ARTICLE

In vitro Cr(VI) reduction by cell-free extracts of chromate-reducing bacteria isolated from tannery effluent irrigated soil

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Abstract Four efficient Cr(VI)-reducing bacterial strains were isolated from rhizospheric soil of plants irrigated with tannery effluent and investigated for in vitro Cr(VI) reduction. Based on 16S rRNA gene sequencing, the isolated strains SUCR44, SUCR140, SUCR186, and SUCR188 were identified as *Bacillus* sp. (JN674188), *Microbacterium* sp. (JN674183), *Bacillus thuringiensis* (JN674184), and *Bacillus subtilis* (JN674195), respectively. All four isolates could completely reduce Cr(VI) in culture media at 0.2 mM concentration within a period of 24–120 h; SUCR140 completely reduced Cr(VI) within 24 h. Assay with the permeabilized cells (treated with Triton X-100 and Tween 80) and cell-free assay demonstrated that the Cr(VI) reduction activity was mainly associated with the soluble fraction of cells. Considering the major amount of chromium being reduced within 24–48 h, these fractions could have been released extracellularly also during their growth. At the temperature optima of 28 °C and pH7.0, the specific activity of Cr(VI) reduction was determined to be 0.32, 0.42, 0.34, and 0.28 μmol Cr(VI)$min^{-1}mg^{-1}$ protein for isolates SUCR44, SUCR140, SUCR186, and SUCR188, respectively. Addition of 0.1 mM NADH enhanced the Cr(VI) reduction in the cell-free extracts of all four strains. The Cr(VI) reduction activity in cell-free extracts of all the isolates was stable in presence of different metal ions tested except Hg^{2+}. Beside this, urea and thiourea also reduced the activity of chromate reduction to significant levels.

Keywords Chromate reduction · Specific activity · Cell-free extracts · Cr(VI) · Chromate-reducing bacteria

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Introduction

Hexavalent chromium is released as a by-product by several industrial activities like tanning, wood preservation, production of steel, paper, pigment, dye, welding, chrome plating, thermonuclear weapons, etc.(Patra et al. 2010). Tannery industries are one of the most polluting industries causing chromium pollution in the environment. In India, there are more than 2,500 tanneries, and most of them (nearly 80 %) are engaged in chrome tanning process (Chandra et al. 2010). Besides, several agronomic practices, including the use of organic biomass like sewage sludge or fertilizers that contain varying degree of chromium, contribute to environment contamination (Viti et al. 2003). Cr (VI) exists in solution as CrO_4^{2-}, and due to structural similarity with SO_4^{2-}, can overcome the cellular permeability barrier, entering via sulfate transport pathways (Patra et al. 2010), rapidly reducing to Cr(V) and generating free radicals (Mabbett and Macaskie 2001). Due to generation of free radicals, it is toxic (Wise et al. 2004) to all forms of living systems including microorganisms by causing oxidative stress (Ackerley et al. 2006), DNA damage (Mabbett and Macaskie 2001), and altered gene expression (Bagchi et al. 2002). Moreover, Cr(VI) is also mutagenic (Puzon et al. 2002), carcinogenic (Codd et al. 2003), and teratogenic (Asmatullah et al. 1998), and has been recognized as a priority pollutant (Cheung and Gu 2007). In view of the seriousness of Cr(VI) pollution and its alarming effects on human health, the US Environmental Protection Agency has listed it in class A human

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carcinogen categories (Costa and Klein 2006). The toxicity of Cr(VI) in plants is also observed at multiple levels, reduced overall growth, and inhibition of enzyme functions leading to lower yields (Shanker et al. 2005).

Although hexavalent chromium is highly toxic, its trivalent form is an essential micronutrient for animal and human being involved in glucose metabolism (Vincent 2000), stimulation of enzyme system (Karuppanapandian et al. 2009), stabilization of nucleic acids by increasing the processivity of DNA polymerase (Snow and Xu 1991), and is relatively inert and much less toxic than the hexavalent form (Krishna and Philip 2005).

Metal pollutants are nondegradable and can only be transformed to less toxic oxidation states or removed either by adsorption/accumulation or by physicochemical treatments. However, it has been observed that these processes are costly and unreliable (Malik 2004). On the other hand, microbial reclamation is safe, ecofriendly, is a cost-effective technology, and an alternative to the traditional physicochemical methods. Several microorganisms have the exceptional ability to survive noxious metal-polluted environments by developing mechanisms to avoid metal toxicity like metal resistance plasmids, metal efflux channels, adsorption uptake, DNA methylation, and metal biotransformation either directly by enzymatic reduction to less mobile and toxic forms or indirectly through making complexes with metabolites (such as H_2S) (Pei et al. 2009). A variety of Cr-resistant bacteria with high potential of Cr(VI)-reducing ability have been reported including *Bacillus*, *Pseudomonas*, *Deinococcus*, *Enterobacter*, *Agrobacterium*, *Escherichia*, *Shewanella*, *Thermus*, and other species (Opperman 2008a). However, Cr(VI) resistance and Cr(VI) reduction have been considered to be unrelated (Ohtake et al. 1987). The availability of selected strains able to resist and reduce chromate elevate the possibility of employing microorganisms for bioremediation of Cr(VI) contaminated site.

Several types of enzymatic Cr(VI) reduction have been reported in bacteria, which include Cr(VI) reductase, aldehyde oxidase, cytochrome P450, DT-diaphorase, etc. (Patra et al. 2010). Similarly, several oxidoreductases with different metabolic functions have also been reported to catalyse Cr(VI) reduction in bacteria, which include nitroreductase (Kwak et al. 2003), iron reductase, quinone reductases (Gonzalez et al. 2005), hydrogenases (Chardin et al. 2003), flavin reductases (Ackerley et al. 2004), as well as NADH/NADPH-dependent reductases (Puzon et al. 2002). The enzymatic chromate reduction occurs both in anaerobic and aerobic bacteria (Cervantes et al. 2001). In anaerobic bacteria, chromate reduction occurs in presence of membrane bound enzymes. In contrast, enzymes for chromate reduction have been localized as soluble cytosolic proteins in most of the aerobic bacteria (Puzon et al. 2002).

The present study was carried out to explore the localization and mechanisms associated with Cr(VI) reduction in four Cr(VI) tolerant bacteria, isolated from the soil irrigated with tannery effluents, with higher activities to reduce chromate. The optimal conditions (temperature and pH) for Cr (VI) reduction as well as the effects on Cr(VI) reduction rates due to the presence of metal ions, protein denaturants, and electron donors were also elucidated in this study.

Material and methods

Soil sampling

Soil samples were collected from a long-term tannery effluent irrigated site near Kanpur (26°28′ N and 80°24′ E), India. Well-growing plants were uprooted, and soil adhering loosely to the roots was removed by shaking the plants. The soil firmly adhering to the roots, designated as rhizospheric soil, was collected. The samples were passed through 2-mm sieve and well dispersed. The properties of the soils are shown in Table 1. A portion of the soil samples was air dried for chemical analyses. Soil pH was measured in distilled water with a ratio of soil/solution of 1:2.5. DTPA-extractable heavy metals (Lindsay and Norvell 1978) were detected by optical emission spectrophotometer (Perkin Elmer) (Table 2).

Isolation of bacterial strains

A large number of bacterial isolates (more than 200) were isolated from rhizospheric soil, using Luria agar (casein enzymic hydolysate, 10 gL^{-1}; yeast extract, 5.0 gL^{-1}; sodium chloride, 5.0 gL^{-1}; agar powder, 15 gL^{-1}; Himedia, India) medium supplemented with filter-sterilized 1,000 mg Cr(VI) L^{-1} as K_2CrO_4. The Cr(VI) stock solutions were filter sterilized using a 0.22-μm membrane filter. Plates were incubated at 28 °C, and actively growing strains were isolated after 1 week. These Cr-tolerant strains were further evaluated for chromate reduction at 0.2 mM concentration of Cr(VI).

On the basis of chromium reduction (data not provided), four strains (SUCR44, SUCR140, SUCR186, and SUCR188) were selected for further studies.

Table 1 The basic properties of rhizosphere soils

Soil sample number	Total N ($kgha^{-1}$)	Available P ($kgha^{-1}$)	Available K ($kgha^{-1}$)	pH	EC (μscm^{-1})
1	580	87	732	6.34	14,550
2	586	174	573	6.74	5,930
3	596	175	500	6.76	5,950
4	486	162	556	6.81	6,070

Table 2 Concentration (mg kg^{-1}) of heavy metals (DTPA-extractable) in rhizosphere soils contaminated with tannery effluents

Sample number	Corresponding plants	Cd	Cr	Cu	Fe	Mg	Mn	Pb	Zn
1	*Triticum aestivam*	0.10±0.03	107.95 ±0.24	0.76±0.08	87.56±0.68	275.45±33.87	12.93±0.61	1.83±0.159	0.50±0.070
2	*Brassica oleracea*	0.11±0.015	166.15±46.42	0.45±0.035	67.62±28.88	225.50±36.76	5.78±1.53	0.77±0.106	0.46±0.088
3	*Brassica campestris*	0.06±0.014	61.12±4.065	0.32±0.036	52.37±8.83	255.58±42.30	8.47±1.62	0.43±0.123	0.15±0.035
4	*Trifolium alexandrinum*	0.08±0.012	108.12±12.55	0.57±0.070	60.83±3.12	304.75±43.48	10.25±0.53	0.30±0.035	0.26±0.053

Quantification of bacterial growth and Cr(VI) reduction of selected strains at different concentration of Cr(VI)

The culture flasks (100 ml) containing 20 ml nutrient broth (sodium chloride, 5.0 gL^{-1}; beef extract, 1.5 gL^{-1}; yeast extract, 1.5 gL^{-1}; peptic digest of animal tissue, 5.0 gL^{-1}, pH7.0±0.2; Himedia, India) supplemented with different concentrations of Cr(VI), i.e., 0.2, 0.4, 0.6, 0.8, and 1.0 mM, were inoculated with 0.5 mL of logarithmic phase bacterial culture (OD 1.2±0.1 at 600 nm), grown for 18 h in nutrient broth. All the cultures including biotic [nutrient broth without Cr(VI)] and abiotic [nutrient broth with Cr(VI) but not inoculated with bacteria] controls in triplicate were incubated for 120 h at 28 °C temperature with shaking at 150 rpm. The density of the bacteria was monitored at definite time intervals by measuring optical density of the culture at 600 nm. To measure the Cr(VI) reduction, 1 mL culture from each of the above flasks was centrifuged (6,000×*g* for 10 min), and the Cr(VI) in the supernatant was analyzed spectrophotometrically at 540 nm, according to the 1,5-diphenylcarbazide method described by APHA (1995).

Effect of temperature and pH on Cr(VI) reduction

Chromium reduction was studied at different temperatures (20, 28, 35, and 42 °C) and pH (6.0, 7.0, and 8.0). The initial pH was adjusted using with 1 N HCl and 1 N NaOH. Appropriate buffers (0.05 M), phosphate buffer (pH6.0), and Tris–HCl buffer (pH7.0 and 8.0) were added for avoiding the shifting of pH (Olajuyigbe and Ajele 2005). Flasks containing 20 mL nutrient broth amended with K_2CrO_4 to final concentration of 0.2 mM Cr(VI) were inoculated with 0.5 mL of logarithmic phase bacterial culture (OD 1.2±0.1 at 600 nm), grown for 18 h in nutrient broth. All the cultures including biotic and abiotic controls, in triplicate, were incubated for 120 h with shaking at 150 rpm. Aliquots (2 mL) were withdrawn at regular time interval (every 24 h) from each replicated tube and centrifuged at 6,000×*g* for 10 min. The concentration of Cr(VI) in the supernatant was analyzed for Cr(VI) reduction. Experiments for all isolates were done in triplicates and were repeated twice.

Resting cell assay

Bacterial cultures in 100 mL of nutrient broth were grown overnight (18 h) at 28 °C with shaking at 150 rpm. Cells were harvested from aforesaid cultures (OD at 600 nm were 1.2±0.1) by centrifugation at 6,000×*g* at 4 °C for 10 min. Pellets of bacterial cultures were washed twice with 5 mL of 0.1 M potassium phosphate buffer, pH7.0 and resuspended in same buffer. These cell suspensions were spiked with 0.2 mM concentration of Cr(VI) as K_2CrO_4 and adjusted the final system volume to 10 mL. The tubes were vortexed

briefly for 2 min and incubated at 28 °C for 6 h. Aliquots (1 mL) were withdrawn at regular time interval and analyzed for Cr(VI) reduction. Cr(VI) spiked in heat-treated (100 °C for 30 min) resuspended cells served as controls. Experiments for all isolates were done in triplicates and were repeated twice.

Permeabilized cell assay

Overnight (18 h) grown cells of bacterial isolates were harvested and washed twice with potassium phosphate buffer pH7.0, as described above and resuspended in the same buffer. Suspended cells were treated with 0.2 % (*v/v*) Tween 80 and 0.2 % (*v/v*) Triton X-100 by vortexing for 20 min to achieve cell permeabilization. Cr(VI) as K_2CrO_4 (0.2 mM concentration) was added to suspended cells, and the final volume was adjusted to 10 mL, and the samples were incubated for 6 h at 28 °C. Aliquots (1 mL) were withdrawn at regular time intervals and analyzed for Cr(VI) reduction as described above. Permeabilized cells heated at 100 °C for 30 min served as controls. Experiments for all isolates were done in triplicates and were repeated twice.

Cell-free assay and localization of chromate reduction activity

Cell-free extracts of bacterial isolates prepared by following previously published protocol (Desai et al. 2008a). Cells grown for 18 h in 250 mL nutrient broth were harvested (OD at 600 nm were 1.2±0.1) by centrifugation at 6,000×*g* for 10 min at 4 °C, washed and resuspended in 20 mL of 0.1 M potassium phosphate buffer pH7.0. These cell suspensions were placed in ice bath and disrupted using an Ultrasonic Probe (Rivotek, frequency 30 KHz±3 KHz) at 120 W with 15-s pulses at 15-s interval for 30 min. Sonicates thus obtained were then ultracentrifuged at 175,000×*g* (Beckman Coulter) for 90 min at 4 °C. The cytosolic fractions or supernatants thus obtained were filtered through 0.22 μm filters to yield the cell-free extracts devoid of membrane fractions and were immediately used for Cr(VI) reduction assay. The sonicated cell pellets were accordingly resuspended in same volume of phosphate buffer. Aliquots of 300 μL of cell-free extracts or cytosolic fractions and sonicated pellet or membrane fractions were used for chromate reduction assay in order to localize the chromate reduction activity in the cells of each isolate. Experiments for all isolates were done in triplicates with freshly prepared cell-free extracts.

Enzyme assays

Chromate reduction was estimated using a standard calibration curve of Cr(VI) as in the form of K_2CrO_4. The reaction system (of 1 mL) used contained Cr(VI) final concentrations (0.2 mM) in 0.7 mL of 0.1 M potassium phosphate buffer with 0.3 mL aliquots of cell-free extracts for chromate reduction. The system volume of 1 mL was kept constant for all experiments. Assay conditions were kept constant with a reaction time of 30 min at 28 °C. Abiotic control contained 0.2 mM Cr(VI) in 0.7 mL of phosphate buffer (0.1 M) with 0.3 mL of heat (100 °C for 30 min) treated cell-free extract. Experiments for all isolates were done in triplicates. Specific activity was defined as unit chromate reductase activity per milligram protein concentration in the cell-free extract. Protein concentrations of cell-free extract were estimated using Folin-phenol reagent by reading absorbance at 750 nm, following the principle of Lowry et al. (1951). Known concentrations of bovine serum albumin (BSA) prepared in phosphate buffer (pH7.0) were used for drawing the standard calibration curve.

Effect of metal ions, electron donors, and protein denaturants on Cr(VI) reduction by cell-free extracts

Hexavalent chromate reductase activity in the cell-free extract of bacterial isolates was also determined in the presence of (0.1 mM each) metal ions (Cd^{2+}, Pb^{2+}, Hg^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, and Zn^{+2}) supplemented as $CdCl_2$, $Pb(NO_3)_2$, $HgCl_2$, $NiCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$, electron donors (NADH, succinate, and citrate) protein denaturants (urea and thiourea) by incubating for 30 min at 28 °C.

Extraction of DNA from bacterial culture

Bacterial genomic DNA was isolated from overnight grown cells using standard procedures (Chachaty and Saulnier 2000).The extracted DNA was electrophoresed on 0.8 % agarose gel in TAE buffer and visualized under UV in Uvitec (Bangalore Genei, India) to check for integrity. The quantity of the extracted DNA was checked spectrophotometrically (Nanodrop ND1000)

Amplification of 16S rRNA

The universal primers (forward 5′-AGAGTTTGATCCTGG CTCAG-3′ and reverse 5′- ACGGCTACCTTGTTACGA CTT-3′) described earlier (Awasthi et al. 2011) were used for amplification of the 16S rRNA gene from the bacterial strain. Approximately 25 ng of bacterial genomic DNA and 10 pmol of forward and reverse primer, 0.6 U of Taq polymerase, and 2.5 μL of 10× buffer (Bangalore Genei, India) were used for amplification in a Mastercyler gradient (Eppendorf) programmed as 94 °C for 5 min; 34 cycles of 94 °C for 1 min, 57.4 °C for 1 min, 72 °C for 2 min; 72 °C for 10 min; and 4 °C for an infinite period. The amplification of PCR products were checked in 1.26 % agarose gels

in TAE buffer stained with ethidium bromide (0.5 mg mL^{-1}) and visualized under UV in Uvitec. The PCR product was purified using PCR Cleanup Kit (Genexy) according to the manufacturer's instructions and directly sequenced using the forward universal primer and Big Dye® Terminator *v*3.1 cycle sequencing kit (Applied Biosystems, USA) on a 3130xl Genetic Analyzer (Applied Biosystems, USA) using the manufacturer's protocol.

Molecular characterization and phylogenetic analysis of isolates

16S sequence analysis was carried out using the nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to identify and download the nearest neighbor sequences from the NCBI database. All the sequences were aligned using ClustalW alignment tool. ClustalW was accessed through the MEGA, version 5 software (Tamura et al. 2011). The phylogenetic tree was constructed using bootstrapped neighbor-joining tree method from MEGA5.

Statistical analysis

Data were subjected to two-way ANOVA to determine the main effects and interactions among factors. Differences among treatment means were compared using Tukey post hoc test with the help of ASSISTAT Version 7.6 beta software (2011).

Results

Screening and identification of bacterial isolates

Homology searching (Table 3) and Blast analysis using 16S rRNA gene sequencing revealed that the newly isolated strains SUCR44, SUCR186, and SUCR188 belong to *Bacillus* and exhibited 99 %, 98 and 100 % similarities with *Bacillus cereus* (EU162012), *Bacillus thuringiensis* (FJ236808), and *Bacillus subtilis* (JN641293), respectively, whereas SUCR140 was identified as *Microbacterium* and showed 99 % similarity with *Microbacterium paraoxydans* (HM235673). Phylogenetic positions in relation to other related organisms have been shown in Fig. 1a and b. 16S rRNA gene sequence of SUCR44, SUCR140, SUCR186, and SUCR188 have been submitted to the NCBI GenBank under the accession numbers JN674188, JN674183, JN674184, and JN674195, respectively.

Reduction of Cr(VI) and growth of bacterial strains

The growth of bacterial strains was affected with increase in Cr(VI) concentration (Fig. 2). The growth of bacterial strains and corresponding Cr(VI) reduction at different times intervals with graded concentration of Cr(VI) (0.2–1.0 mM), as potassium chromate, has been depicted in Fig. 2. All the four isolates completely (100 %) reduced the Cr(VI) at 0.2 mM concentration within a period of 24–120 h. However, strain SUCR140 could completely reduce the Cr(VI) within 24 h. There were negligible levels of chromate reductions in abiotic controls.

Effect of temperature and pH on Cr(VI) reduction by bacterial strains

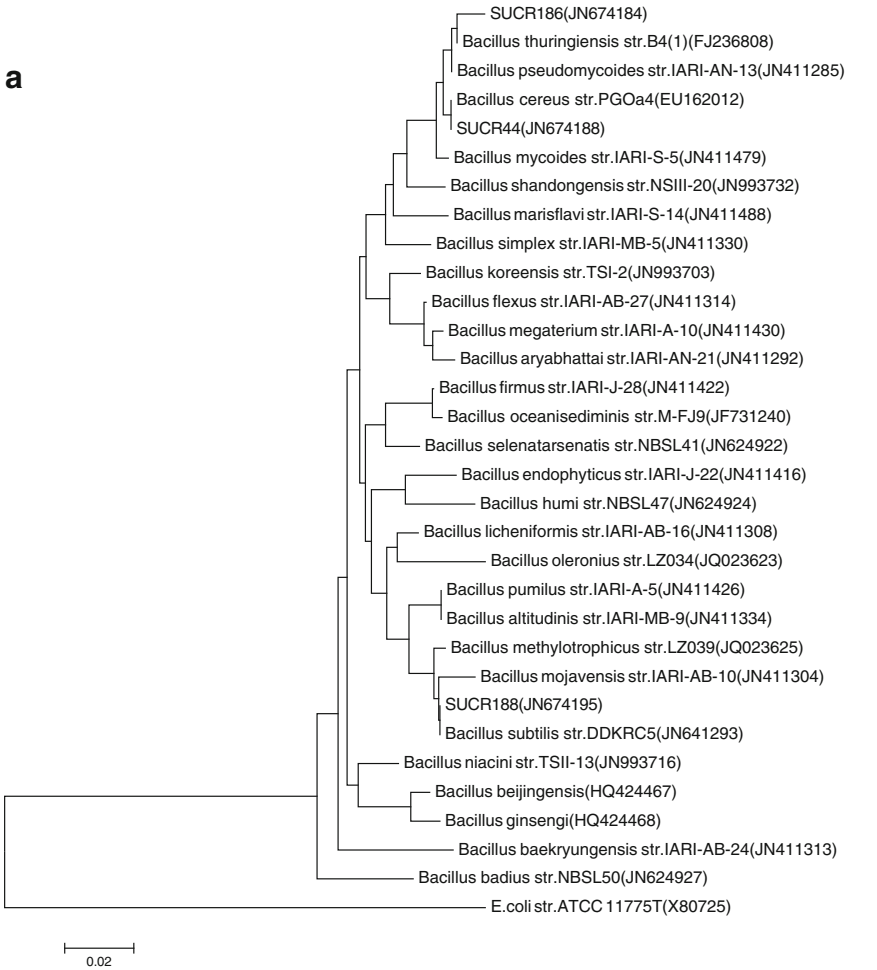
Over the strains, the microbial growth was higher, both in presence and absence of Cr(VI), at pH 7.0 at 28 °C (Fig. 3). However, the Cr(VI) reducing strength of bacterial strains was found to be affected by strain identity, temperature, pH, and time (Table 4). Significant interactions were noticed among these parameters. Chromate reduction by all four bacterial strains was investigated at regular time intervals at different temperatures (20–42 °C) and pH (6.0–8.0) (Fig. 4). Maximum Cr(VI) reduction activity of strains SUCR44 and SUCR140 at 0.2 mM Cr(VI) was established at 28 °C at pH 7.0, while SUCR186 and SUCR188 showed maximum Cr(VI) reduction activity at pH 6.0 at 28 °C. Over the pH, maximum reduction of chromate was observed at 28 °C, this activity of all the strains decreased at both lower (20 °C) and higher temperatures (35 and 42 °C). Reduction of chromate was negligible in case of abiotic control at all the temperature and pH (data not presented) after 120 h.

Localization of chromium reduction activity

For detecting the localization, chromate reduction assays were carried out using resting and permeabilized cells of all the four strains by exposing the cells to 0.2 mM Cr(VI) for 6 h at 28 °C. Figure 5 shows the concentration of residual Cr(VI) upon exposure of resting and permeabilized

Table 3 Homology search of bacterial isolates

Isolate	Identification	GenBank accession no.	Similar organism	Accession number	Sequence similarity (%)
SUCR44	*Bacillus* sp.	JN674188	*Bacillus cereus*	EU162012	99
SUCR140	*Microbacterium* sp.	JN674183	*Microbacterium paraoxydans*	HM235673	99
SUCR186	*Bacillus thuringiensis*	JN674184	*Bacillus thuringiensis*	FJ236808	98
SUCR188	*Bacillus subtilis*	JN674195	*Bacillus subtilis*	JN641293	100



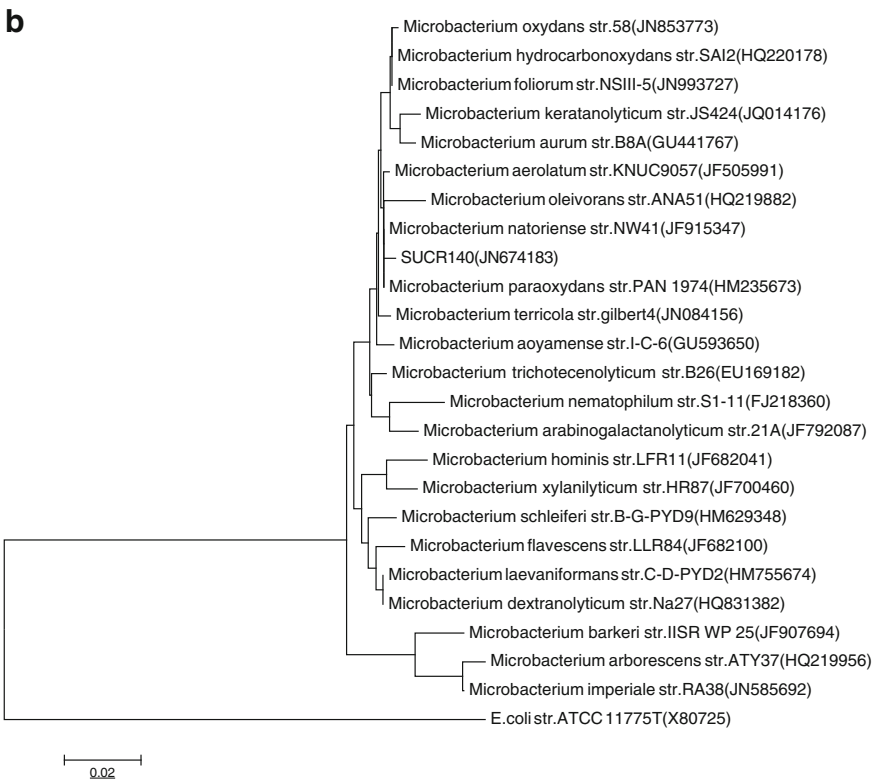


Fig. 1 **a** Phylogenetic tree constructed from the 16S rRNA gene of strains SUCR44, SUCR186, and SUCR188 and related organisms constructed using neighbor-joining algorithm from an alignment of 710 nucleotides. Accession numbers of corresponding sequences are given in *parentheses*, and *scale bar* represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications. *E. coli* str. ATCC 11775T was taken as an out-group. **b** Phylogenetic tree constructed from the 16S rRNA gene of strain SUCR140 and related organisms constructed using neighbor-joining algorithm from an alignment of 716 nucleotides. Accession numbers of corresponding sequences are given in *parentheses*, and *scale bar* represents 1 base substitution per 20 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications. *E. coli* str. ATCC 11775T was taken as an out-group

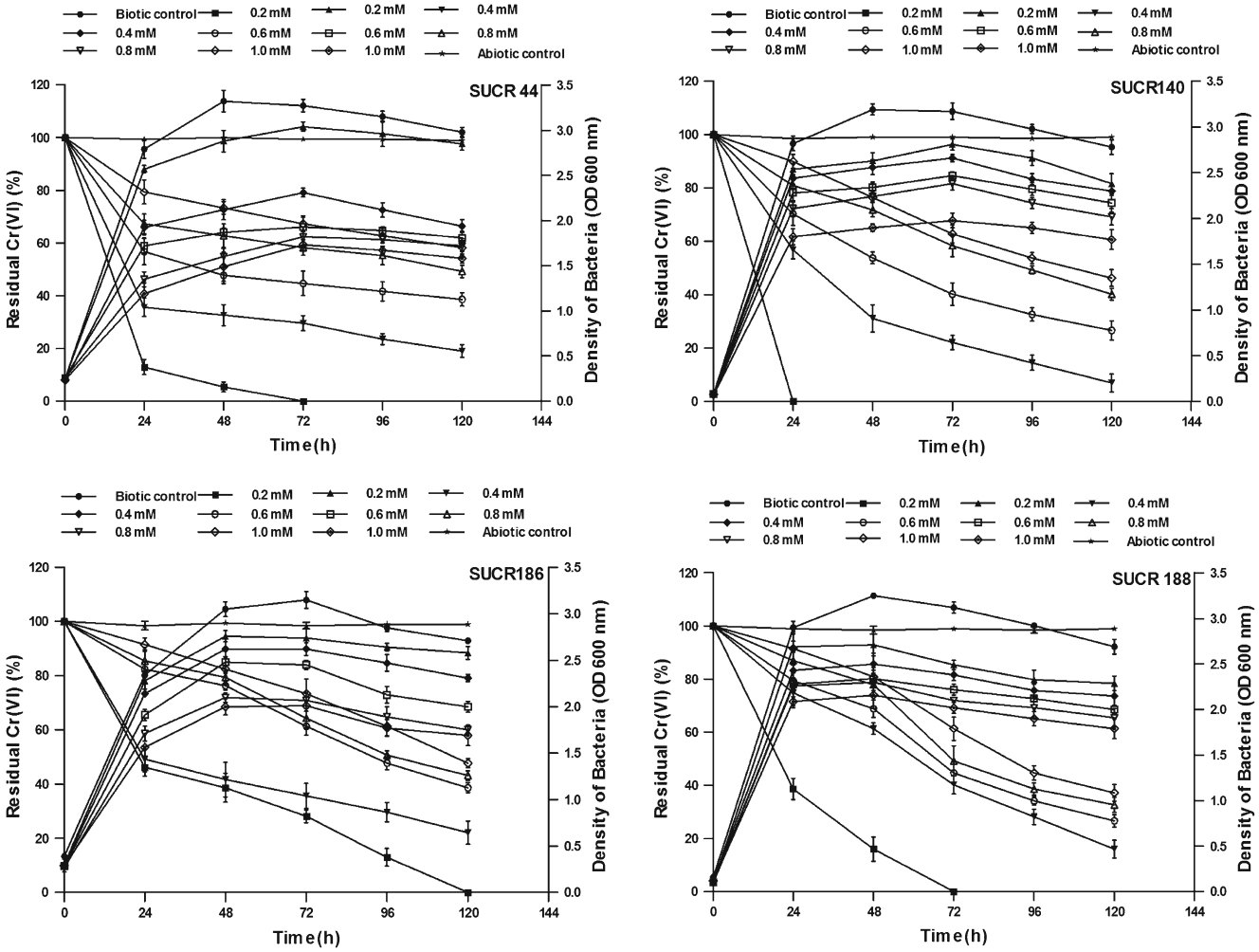


Fig. 2 Kinetics of growth and Cr(VI) reduction. Bacterial isolates were cultured with Cr(VI) 0.2, 0.4, 0.6, 0.8, and 1.0 mM Cr(VI) as K_2CrO_4 and % Cr (VI) reduction in nutrient broth medium at 28 °C and pH7.0

cells of all the four strains. As observed from the figure, cell permeabilization significantly increased the Cr(VI) reduction in all four strains. Among the two reagents used for permeabilization namely Tween 80 and Triton X-100, Tween 80 brought about higher permeabilization of bacterial cells, which resulted in increased Cr(VI) reduction. On treatment with Tween 80, complete reduction of Cr(VI) was observed at 0.2 mM Cr(VI) concentration by permeabilized cells of SUCR44, whereas 58, 62, and 57 % of Cr (VI) could be reduced by SUCR140, SUCR186, and SUCR188 respectively. On the other hand, permeabilization through Triton X100 resulted in reduction of 97, 48, 59, and 51 % by the strains SUCR44, SUCR140, SUCR186, and SUCR188, respectively. Considering Cr(VI) reduction by resting cell as 100 %, the Cr(VI) reduction by SUCR44, SUCR140, SUCR186, and SUCR188 was increased by 35, 56, 49, and 57 %, respectively, on treatment with Tween 80. Similarly, 12, 51, 41, and 30 % enhancements in Cr(VI) reduction were observed on treatment with Triton X100 of respective aforesaid strains. Chromate reduction assays were followed using initial concentration of 0.2 mM Cr(VI) with ultrasonicated cytosolic fraction or cell-free extract and membrane fraction (ultrasonicated pellet). As observed from Table 5, reduction of Cr(VI) is mainly associated with soluble fraction (cell-free extracts) in all the four strains, indicating the presence of hexavalent chromate-reducing principle in the cytoplasm (cytosolic fraction). No significant activity of chromate reduction was noticed in membrane fraction derived from ultrasonicated cells of all the isolates. Heated (100 °C for 30 min) cell-free extracts acting as control failed to reduce Cr(VI). These results confirm the presence of soluble enzymatic mechanism in the cytoplasmic fraction (crude cell-free extracts) of all the four strains. At the temperature optima of 28 °C and pH7.0, the specific activity of Cr(VI) reduction was determined to be 0.32 (0.16 %), 0.42 (0.21 %), 0.34 (0.17 %), and 0.28 (0.14 %) μmol Cr(VI) $min^{-1} mg^{-1}$ for isolates SUCR44, SUCR140, SUCR186, and SUCR188, respectively (Table 5).

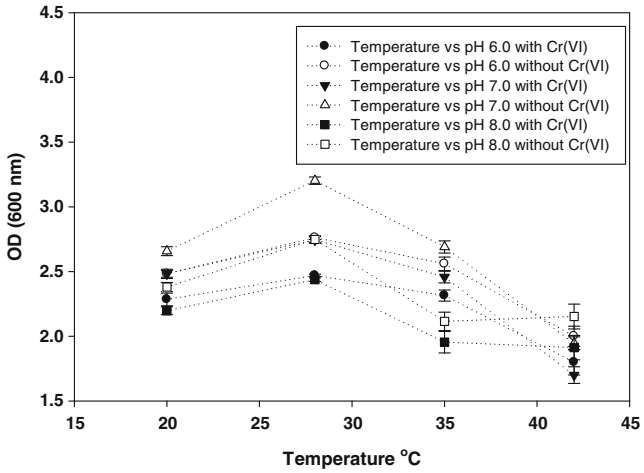


Fig. 3 Bacterial growth (over the strains) after 48 h in the presence and absence of Cr(VI) at different temperature and pH

Effect of metal ions, protein denaturants, and electron donors on chromium reduction activity of cell-free extract

Chromium reduction activity of the cell-free extract of isolated strains was estimated in presence of (0.1 mM) metal ions, protein denaturants, and electron donors at initial Cr(VI) concentrations of 0.2 mM upon incubation at 28 °C and pH7.0 for 30 min in 0.1 M potassium phosphate buffer. Among the metal ions tested, 0.1 mM of Cd^{2+} inhibited the reduction of Cr(VI) by the cell-free extracts of SUCR140, SUCR186, and SUCR188 by 17, 21, and 32 %, respectively, whereas no significant inhibition in the reduction of Cr(VI) was noticed in SUCR44. Cr(VI) reduction activity of cell-free extract of all the strains was not affected by Pb^{2+}, whereas other divalent cations such as Ni^{2+} and Zn^{2+} could influence the Cr (VI) reduction activity of some strains. Hg^{2+} strongly inhibited the Cr(VI) reduction in all the four isolates by 86–93 %. On the other hand, reduction of Cr(VI) by the cell-free extract of SUCR44, SUCR140, SUCR186, and SUCR188 was enhanced by Cu^{2+}, increase of 37, 33, 44, and 28 %, respectively, as observed from Table 6. Co^{2+} also stimulated the Cr(VI) reduction in crude cell-free extract of SUCR44, SUCR140, SUCR186, and SUCR188, an increase of 15, 16, 35, and 18 %, respectively. Urea, a protein denaturant, inhibited the Cr(VI) reduction by 78, 88, 94, and 89 % in SUCR44, SUCR140 SUCR186, and SUCR188, respectively, while another protein denaturant thiourea inhibited the Cr(VI) by 88, 86, 91, and 86 %, respectively, indicating the denaturation of protein(s) responsible for inhibition of Cr(VI) reduction in all four strains. The specific activity of Cr(VI) reduction in the cell-free extracts of all the strains showed an increase with the addition of 0.1 mM NADH; addition of 0.1 mM NADH in the reaction mixture containing cell-free extract increased the reduction of Cr(VI) by 141, 148, 159, and 150 % in the strains SUCR44, SUCR140, SUCR186, and SUCR188, respectively. Citrate, a possible electron donor, during the reduction of Cr (VI) increased Cr(VI) reduction by 30, 21, 32, and 32 % in SUCR44, SUCR140, SUCR186, and SUCR188, respectively, as observed from data presented in Table 6. Succinate had no significant effect on the reduction of Cr(VI) by the cell-free extract of all the four isolates.

Table 4 Summary of statistical analysis: the main effect and interaction of bacterial strains, pH, time, and temperature on Cr(VI) reduction were analyzed by factorial ANOVA

Treatments[a]	*df*	SS	*F*
Strains	3	7,005.77162	850.5864*
Temperature	3	143,058.06921	17,369.0009*
pH	2	5,936.39659	1,081.1268*
Time	4	63,171.67061	5,752.3606*
Strains × temperature	9	42,549.09603	1,721.9937*
Strains × pH	6	11,458.98900	695.6308*
Strains × time	12	1,144.97787	34.7536*
Temperature × pH	6	36,251.26776	2,200.6738*
Temperature × time	12	8,463.93102	256.9062*
pH × time	8	248.30293	11.3051*
Strains × temperature × pH	18	43,614.25402	882.5507*
Strains × temperature × time	36	4,505.75202	45.5878*
Strains × pH × time	24	453.06145	6.8759*
Temperature × pH × time	24	1,407.86789	21.3665*
Strains × temperature × pH × time	72	1,921.25134	565.6957*
Error	240	658.91214	

*$p<0.01$)

[a] Strains (SUCR44, SUCR140, SUCR186, and SUCR188); temperature (20, 28, 35, and 42 °C); pH (6.0, 7.0, 8.0); times [(24, 48, 72, 96, and 120) in hours]

Discussion

The irrigation water contaminated with heavy metals is known to cause disturbance in microbial communities with emergence of bacterial species having elevated metal tolerance (Stepanauskas et al. 2005). Chromium irrigation exerts a strong selective pressure on microbial flora of tannery soils (Viti et al. 2003). In the present investigation, bacterial strains tolerating and reducing Cr(VI) were isolated from rhizospheric soil receiving long-term augmentations of chromate from tanneries. The isolates belonged to the genera *Bacillus* and *Microbacterium*. Strains of the genus *Bacillus* are known to tolerate and reduce Cr(VI) (Campos et al. 1995; Liu et al. 2006). Unlike the genus *Bacillus*, the bacteria belonging to genera *Microbacterium* are rarely known for Cr(VI) reduction under aerobic condition. Pattanapipitpaisal et al. (2001) earlier reported that *Microbacterium* sp. MP30 can reduce the Cr(VI) under anaerobic condition. In our experiments, higher Cr(VI) concentrations caused decrease in growth rate in all four strains tested when compared to growth at lower Cr(VI)

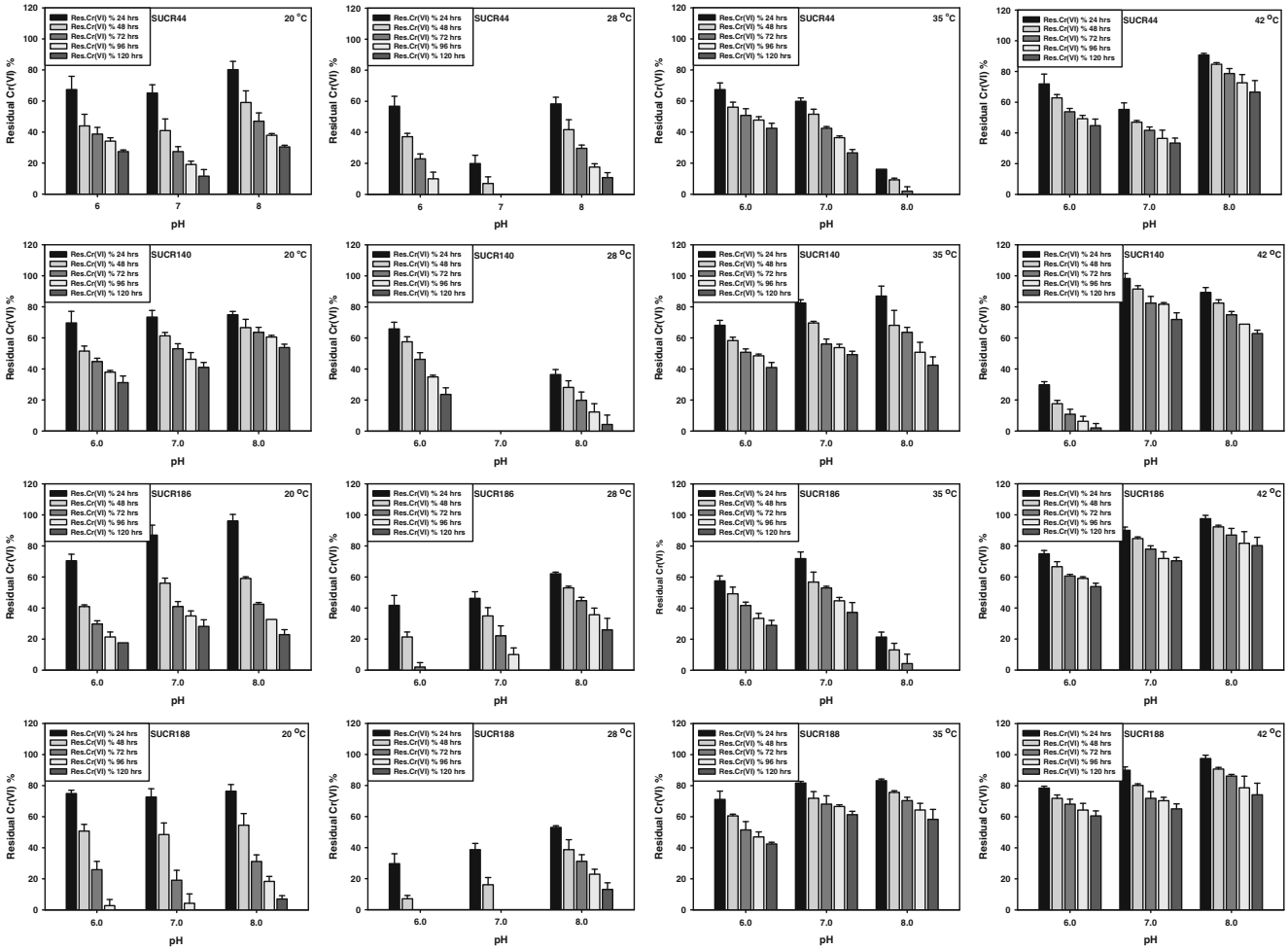


Fig. 4 Effect of pH and temperature on Cr(VI) reduction by bacterial strains supplemented with 0.2 mM Cr(VI) as K_2CrO_4

concentrations. It is also clear from our results (Fig. 2) that increase in chromate reduction was growth dependent; higher reduction were noticed during the first 48 h corresponding to log phase of the microbial growth. Most likely, bacterial growth and Cr(VI)-induced damage are competing processes, and bacteria can cope with Cr(VI) exposure only as long as metabolizable C-sources are available. Liu et al. (2006) noticed that this phenomenon might be explained as an increased time period for adaptation or repair during the exposure of high level of Cr(VI) in the medium. The high level of Cr(VI) in the medium induces frameshift errors and, to a greater extent, base pair substitution both in G–C and A–T base pairs (DeFlora et al. 1990). It has also been proposed that bacterial SOS function can repair the DNA damage caused by Cr(VI) (Oh and Choi 1997). Hexavalent chromate reduction by all the four strains was investigated at different temperatures (20–42 °C), an important factor affecting microbial Cr(VI) reduction. Maximum Cr(VI) reduction of all the four strains was established at 28 °C, which also corresponds to maximum growth of the bacterial strains (Fig. 3) again, indicating that Cr(VI) reduction was growth dependent. Such growth-dependent chromate reduction has also been earlier reported by Desai et al. (2008a). It has been reported that the optimal temperature of Cr(VI) reduction could be in the range of 25–37 °C (Cheung and Gu 2007; Ibrahim et al. 2012). However, optimum temperature of Cr(VI) reduction of thermophilic *Thermus scotoductus* SA-01 (Opperman et al. 2008b) and *Bacillus firmus* KUCr1 (Sau et al. 2010) have been reported at 65 and 70 °C, respectively. Cr(VI) reduction was found to be influenced by pH. Maximum Cr(VI) reduction activity of strains SUCR44 and SUCR140 at 0.2 mM Cr(VI) was noticed at pH7.0, while SUCR186 and SUCR188 showed maximum Cr(VI) reduction activity at pH6.0. Wang et al. (1990) reported that reduction of Cr(VI) in bacterial strain occurred at pH6.0–8.0 and was strongly inhibited at pH5.0 and 9.0. (Bopp et al. 1983). Our results clearly indicated that chromate reduction was dependent on pH, temperature, strain identity (Table 4), and the significant interaction observed among them, suggesting that different strains perform differently under different temperature and pH. Negligible reduction was noticed in abiotic control at all temperature and pH, indicating the direct interaction of microbes in Cr(VI)

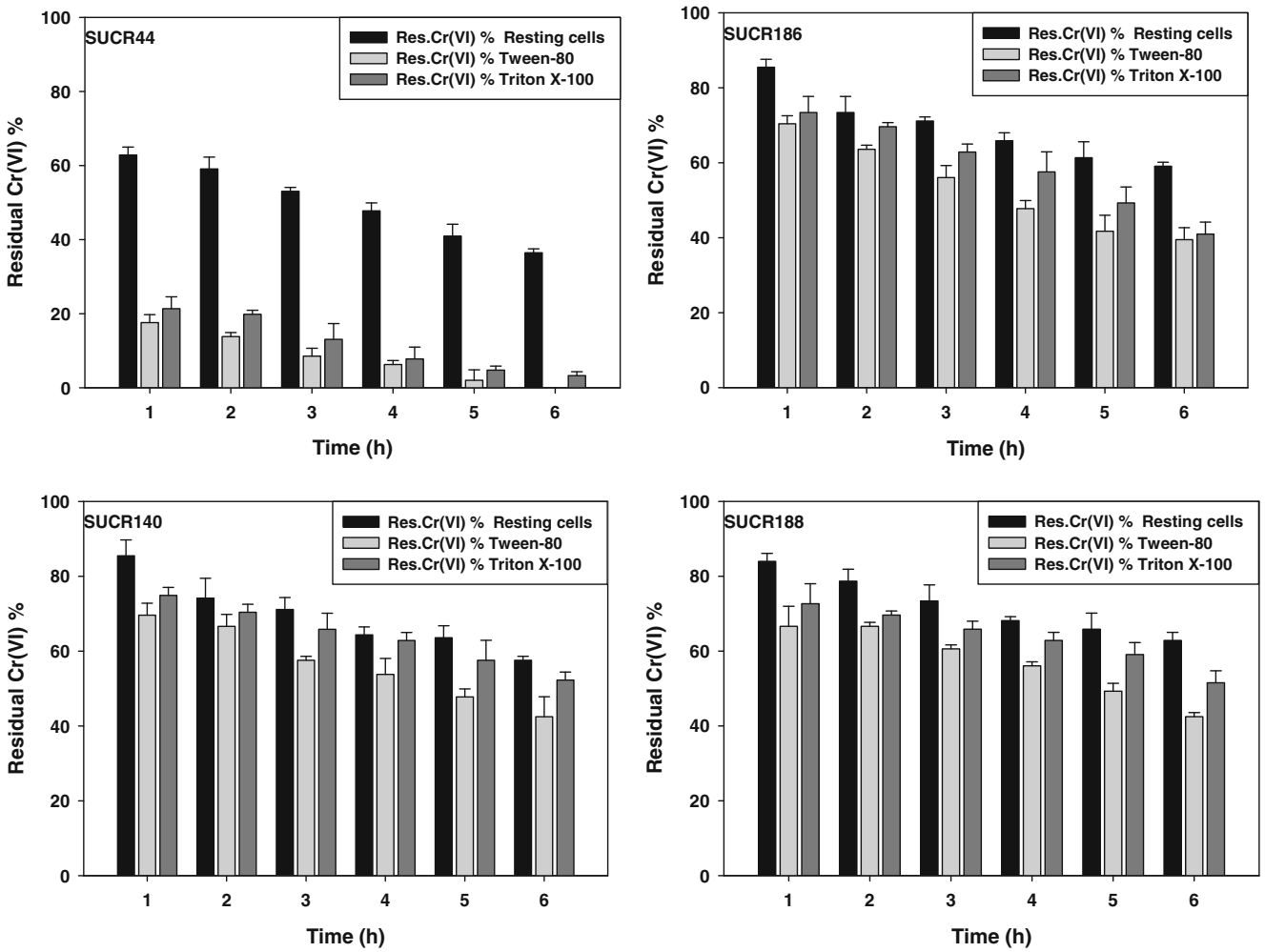


Fig. 5 Hexavalent chromium reduction by resting and permeabilized cells of isolates SUCR44, SUCR140, SUCR186, and SUCR188 in 0.1 M potassium phosphate buffer supplemented with 0.2 mM Cr(VI) as K_2CrO_4 at 28 °C and pH7.0

reduction. Similar results indicating negligible reductions in abiotic control have been earlier reported by Masood and Malik (2011) and He et al. (2010).

Trivalent form of chromium readily precipitates as Cr $(OH)_3$ at pH7.0 (Bopp et al. 1983). However, a number of recent studies suggest that Cr(VI) reduction by some bacteria strains such as *Pseudomonas* (Dogan et al. 2011) and *Bacillus* sp. (Desai et al. 2008a) led to the production of soluble Cr(III) end products instead of $Cr(OH)_3$. Dogan et al. (2011) found that the release of exopolymeric substances (EPS) during microbial Cr(VI) reduction with *Pseudomonas* bacteria leads to enhanced solubility of Cr(III) in solution. Puzon et al. (2002) suggested that an intracellularly located *E. coli* enzyme system converts Cr(VI) to a soluble and stable NAD^+–Cr(III) complex, and cytochrome c-mediated Cr(VI) reduction produces cytochrome c–Cr(III) adducts. Similarly, Priester et al. (2006) found that *P. putida* cell lysis releases constitutive reductases that catalyzes the extracellular reduction of Cr (VI) to Cr (III). Their findings provide evidence that chromium exposure to cells produced elevated concentration of microbial EPS. Puzon et al. (2005) suggested that Cr(VI) intracellularly reduced in the cytoplasm by a bacterial enzyme, using NADH as the reductant, and, after cell lysis, forms soluble Cr (III) end products in solution. Wang et al. (1990) found that bacteria with membrane bound reductases can reduce Cr(VI) to Cr(III) by extracellular processes., e.g., using electron-shuttling compounds coupled to membrane

Table 5 Subcellular localization of chromate reduction activity in SUCR44, SUCR140, SUCR186, and SUCR188 at 0.2 mM Cr(VI) concentration

Chromate reduction activity ($\mu mol\,min^{-1}\,mg^{-1}$ protein)				
	SUCR44	SUCR140	SUCR186	SUCR188
Cytosolic fraction (cell-free extracts)	0.32±0.109	0.42±0.098	0.34±0.080	0.28±0.150
Membrane fraction (sonicated pellets)	0.07±0.046	0.03±0.006	0.02±0.006	0.05±0.040

Table 6 Effect of 0.1 mM metal ions

Protein denaturants and electron donors on hexavalent chromate reductase activity in the crude cell-free extracts of SUCR44, SUCR140, SUCR186, and SUCR188 in 0.1 M potassium phosphate buffer of pH7, on incubation of 30 min at 28 °C

Metals ions	SUCR44 Specific activity ($\mu mol\,min^{-1}\,mg^{-1}$ protein)	SUCR140 Specific activity ($\mu mol\,min^{-1}\,mg^{-1}$ protein)	SUCR186 Specific activity ($\mu mol\,min^{-1}\,mg^{-1}$ protein)	SUCR188 Specific activity ($\mu mol\,min^{-1}\,mg^{-1}$ protein)
CFE (control)	0.32±0.109	0.42±0.098	0.34±0.080	0.28±0.150
Cd^{2+}	0.29±0.057	0.35±0.075	0.27±0.092	0.19 ±0.063
Pb^{2+}	0.35±0.086	0.40±0.103	0.32±0.069	0.30±0.051
Hg^{2+}	0.03±0.005	0.06±0.023	0.04±0.017	0.02±0.005
Ni^{2+}	0.25±0.121	0.44±0.080	0.32±0.063	0.22±0.080
Cu^{2+}	0.44±0.098	0.56±0.127	0.49±0.075	0.36±0.127
Co^{2+}	0.37±0.109	0.49±0.167	0.42±0.063	0.33±0.092
Zn^{+2}	0.33±0.121	0.36±0.040	0.30±0.086	0.23±0.069
Protein denaturants				
Urea	0.07±0.034	0.05±0.023	0.02±0.011	0.03±0.011
Thiourea	0.04±0.011	0.06±0.017	0.03±0.011	0.04±0.017
Electron donors				
NADH	0.77±0.032	1.04±0.248	0.88±0.178	0.70±0.138
Succinate	0.31±0.080	0.45±0.069	0.33±0.103	0.30±0.075
Citrate	0.40±0.196	0.51±0.127	0.45±0.161	0.37±0.086

reductases. The metal reduction can also be mediated by the surfaces of bacterial spores (Junier et al. 2009) and such mechanism may be relevant for spore forming bacteria like *Bacillus*.

Resting and permeabilized cell assays provided the better evidence of the presence of an Cr(VI) reduction mechanism in cytosol fraction as observed in previous findings of Megharaj et al. (2003). Permeabilization with Tween 80 and Triton X-100 resulted in increased Cr(VI) reduction, indicating that cytoplasmic proteins were released and all the four strains reduced Cr(VI) through soluble cytosolic reductases and not through membrane associated reductases. The inability to reduce hexavalent chromium by boiled cell-free extract, which served as control, showed that reduction process is enzymatic and not due to absorption or chemical reaction. The cell lysis played an important role in Cr(VI) reduction. Ishibashi et al. (1990), Pal et al. (2005), and Elangovan et al. (2006) observed that chromate reductase activity was associated with soluble protein and not with the membrane fraction. Desai et al. (2008b) suggested that a soluble chromate reductase associated with the cytoplasmic membrane catalyzed Cr(VI) reduction by *Pseudomonas* sp. G1DM21 by transferring initial one electron to Cr(VI) to form an intermediate Cr(V), followed by two electron transfer for Cr(III) formation. Priester et al. (2006) reported that the chromate reductases originated in the cytoplasm left cells by cell lysis and reduces Cr(VI) extracellularly. Our results indicate that, although the chromate-reducing fraction is located in cytosol, these fractions might be released extracellularly considering major amount of chromium being reduced by all strains during initial 48 h of their growth. McLean and Beveridge (2001) reported that the extracellular reductase activity in cell filtrates from 48-h-old cultures was due to either secretion or cell lysis.

Metal ions have been known to affect chromate reductase activity. Reduction of Cr(VI) by the cell-free extract was enhanced by Cu^{2+}. Camargo et al. (2003) reported the stimulation of Cr(VI) reduction in *Bacillus* sp. ES 29 on addition of 1 mM of Cu^{2+}. Elangovan et al. (2006) also found that on addition of 1 mM Cu^{2+} reduction of Cr(VI) was stimulated in *Bacillus* sp. The stimulation of enzyme activity by Cu^{2+} might be due to its nature as a prosthetic group of many reductase enzymes (Sau et al. 2010). Camargo et al. (2003) reported that the increase in the reduction of Cr(VI) in presence of Cu^{2+} has been attributed to its action as an electron-transport protector or its action as a single electron redox center. On the contrary, Cu^{2+} has also been reported to inhibit the membrane associated chromate reductase activity of *Enterobacter cloacae* (Ohtake et al. 1990) and soluble chromate reductase activity in *Pseudomonas putida* (Park et al. 2000) and *B. sphaericus* AND 303 (Pal et al. 2005). Addition of Pb^{2+} showed a diminutive inhibitory effect on Cr(VI) reduction, while Hg^{2+} strongly inhibited the Cr(VI) reduction activity, whereas other divalent cation such as Ni^{2+}, Cd^{2+}, and Zn^{2+} inhibited the Cr (VI) reduction to a variable degree. These variations seem to be due to the different functional nature of the reductase enzymes, which warrants further investigation. Metal ions may affect microbial Cr(VI) reduction in two ways: destruction of cells (decrease in cell growth) and inhibition of enzymes responsible for Cr(VI) reduction. Metal ions may absorb on to cell walls or complex with enzymes responsible for Cr(VI) reduction. The absorption of metal ions onto cell

walls or the formation of metal–enzyme complexes may lead to inactivation of chromate reductase enzymes or sites responsible for Cr reduction (Mabbett et al. 2002; Dogan et al. 2011). The reduction of Cr(VI) by cell-free extract was stimulated by Co^{2+}. Similar results have been observed in *Bacillus firmus* strain KUCr1 (Sau et al. 2010) at 0.2 mM, though Pal et al. (2005) reported the inhibition of Cr(VI) reduction activity of *Bacillus sphaericus* AND 303 by Co^{2+} at 100 μM concentration. On the contrary, Desai et al. (2008a) reported the enhancement of Cr(VI) reduction activity even at 1,000 μM concentration. Mercury is the most commonly reported inhibitor of reductase, which suggests the role of thiol group in catalysis (Park et al. 2000); Cr(VI) reduction by all the four strains was highly inhibited by mercuric ion. The electron donor such as citrate had significant stimulatory effect on Cr (VI) reduction. Studies by Mabbett et al. (2002) and Desai et al. (2008a) show that the presence of low molecular organic molecules such as citrate protected the chromate reductase enzymes from inactivation by removing toxic products of microbial reduction. Mabbett et al. (2002) also found a close connection between the amount of Cr(VI) reduced and the equilibrium constants of Cr-ligand complexes with more Cr (VI) being reduced with much stronger complexes. Succinate, on the other hand, did not show any noteworthy effect on Cr (VI) reduction. Desai et al. (2008b) observed improved chromate reductase activity by *Pseudomonas* sp. G1DM21 in the presence of electron donors such as citrate, acetate, and succinate. The Cr(VI) reduction of *Bacillus* sp. and *Microbacterium* sp. belongs to the NADH-dependent type. An addition of NADH improved Cr(VI) reduction both in *Bacillus* and *Microbacterium.* There have been reports supporting NADH-dependent Cr(VI) reduction from *Pseudomonas* sp., *Escherichia coli*, and *Bacillus* sp.; probably, these can use NADH as the electron donor (Bae et al. 2005). Previous reports have demonstrated that intracellular Cr(VI) accepts a single electron from an NADH molecule forming a Cr(V) intermediate, which in turn accepts two electrons from two molecules of NADH to form stable Cr(III) (Suzuki et al. 1992). Urea and thiourea are well known protein denaturizing agents. In our study, both inhibited the Cr(VI) reduction.

To conclude, all the four strains were tolerant to Cr(VI) and completely reduced Cr(VI) through soluble reductases within a period of 24–120 h. SUCR140 could completely reduce the Cr(VI) within 24 h at 28 °C at pH7.0 but failed to reduce it completely at pH6.0 even after 120 h where SUCR 188 performed the best. Interestingly, SUCR 140 at higher temperatures worked well even at pH6.0 whereas SUCR 188 could perform well only at low temperatures (≤28 °C). At 35 °C, SUCR44 and SUCR186 showed relatively higher chromate reductase activity, which improved at pH8.0. Therefore, SUCR140 and SUCR188 could be more useful for subtropical areas, while strain SUCR140 might also be useful for the moderate acidic regions experiencing high temperature. On the other hand, strains SUCR44 and SUCR186 might be useful under neutral and moderate alkaline soil environment for tropical regions. Our studies clearly suggest a strong interaction among strains, pH, and temperature. Therefore, this study provides useful information for identifying the strains of Cr(VI) reducing bacteria, which would perform better under different soil environments and may be employed into sustainable microphytoremediation.

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