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ORIGINAL PAPER

Hexavalent chromate reduction by alkaliphilic *Amphibacillus* sp. KSUCr3 is mediated by copper-dependent membrane-associated Cr(VI) reductase

Abdelnasser S. S. Ibrahim · Mohamed A. El-Tayeb · Yahya B. Elbadawi · Ali A. Al-Salamah · Garabed Antranikian

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Abstract The present study was aimed to localize and characterize hexavalent chromate [Cr(VI)] reductase activity of the extreme alkaliphilic *Amphibacillus* sp. KSUCr3 (optimal growth pH 10.5). The resting cells were able to reduce about 62 % of the toxic heavy metal Cr(VI) at initial concentration of 200 μM within 30 min. Cell permeabilization resulted in decrease of Cr(VI) reduction in comparison to untreated cells. Enzymatic assays of different sub-cellular fractions of *Amphibacillus* sp. KSUCr3 demonstrated that the Cr(VI) reductase was mainly associated with the membranous fraction and expressed constitutively. In vitro studies of the crude enzyme indicated that copper ion was essential for Cr(VI) reductase activity. In addition, Ca^{2+} and Mn^{2+} slightly stimulated the chromate reductase activity. Glucose was the best external electron donor, showing enhancement of the enzyme activity by about 3.5-fold. The K_m and V_{max} determined for chromate reductase activity in the membranous fraction were 23.8 μM Cr(VI) and 72 μmol/min/mg of protein, respectively. Cr(VI) reductase activity was maximum at 40 °C and pH 7.0 and it was significantly inhibited in the presence of disulfide reducers (2-mercaptoethanol), ion chelating agent (EDTA), and respiratory inhibitors (CN and Azide). Complete reduction of 100 and 200 μM of Cr(VI) by membrane associated enzyme were observed within 40 and 180 min, respectively. However, it should be noted that biochemical characterization has been done with crude enzyme only, and that final conclusion can only be drawn with the purified enzyme.

Keywords Chromate reductase · *Amphibacillus* sp. KSUCr3 · Bioremediation · Membrane-bound enzyme · Alkaliphiles · Sub-cellular · Chromium · Soda lakes

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A. S. S. Ibrahim (✉) · M. A. El-Tayeb · Y. B. Elbadawi · A. A. Al-Salamah
Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
e-mail: ashebl@ksu.edu.sa

G. Antranikian
Institute of Technical Microbiology, Hamburg University of Technology, Kasernenstr. 12, 21073 Hamburg, Germany

Introduction

Hexavalent chromium is a toxic heavy metal that is widely used in a variety of industries, owing to its impressive corrosion resistance, including electroplating, leather tanning, textile dyeing, metal processing, wood treatment, and alloy industries (Cheung et al. 2006; Sarangi and Krishnan 2008). Hexavalent chromium [Cr(VI)] is produced as a by-product of theses industrial process, and Cr(VI) containing wastewater have become a well recognized hazard in water pollution control due to the extremely toxic, mutagenic, and carcinogenic effect of Cr(VI) on the biological systems (Mclean and Beveridge 2001; Elangovan et al. 2006; Xu et al. 2012). Moreover, Cr(VI) has been listed as one of the 17 chemicals posing greatest threat to humans by United States Environmental Protection Agency (USEPA), (McCullough et al. 1999; Kavita and Keharia 2012). Chromium exists mainly as two stable oxidation states, Cr(VI) and Cr(III), which differ completely in their physiochemical properties and toxicity (Ishibashi et al. 1990). The structure similarity of Chromate (CrO_4^{2-}) to sulfate (SO_4^{2-}) allows it, as analog substrate, to readily diffuse into the cell through the sulfate transport system in the cell membranes (Cheung et al. 2006). Within the cell, Cr(VI) is

partially reduced to highly unstable Cr(V) radical, leading to formation of reactive oxygen species attacking nucleic acids and damages cellular materials (Sarangi and Krishnan 2008; Opperman et al. 2008). In contrast, Cr(III) being sparingly soluble, and is also oxidatively more stable and thus less toxic in comparison to Cr(VI) (Rai et al. 1987; Ilias et al. 2011).

Conventional physicochemical methods for removing of toxic heavy metals from industrial effluents include chemical precipitation, oxidation or reduction, reverse osmosis, membrane technologies, filtration, ion exchange, and evaporation recovery (Cheung et al. 2006; Ahluwalia and Goyal, 2007 Zahoor and Rehman 2009; Ozturk et al. 2009; Sau et al. 2010). However, there are several shortcomings of these methods, including consumption of excessive energy, poor removal efficiency, production of large amounts of chemical sludge, in addition to the high cost of chemicals used for Cr(VI) reduction, particularly for the removal of relatively low concentrations of Cr(VI) (Sau et al. 2010; He et al. 2011). Due to its efficient, affordable, and environmentally friendly advantages, biotransformation of Cr(VI) to the less toxic Cr(III) by microorganisms is considered as an alternative promising approach for the remediation of Cr(VI) pollution (Cheung et al. 2006; Sarangi and Krishnan 2008; Xu et al. 2012; Ozturk et al. 2009; He et al. 2011). Bioreduction of toxic Cr(VI) to non-toxic Cr(III), through chromate reductase enzymes, has been demonstrated in several bacterial species including *Pseudomonas* sp. (Jimenez-Mejia et al. 2006), *Bacillus* sp., *Leucobacter* sp. and *Exiguobacterium* sp. (Sarangi and Krishnan 2008), *Ochrobactrum* sp. (He et al. 2009), *Achromobacter* sp. (Wani et al. 2007), *Shewanella oneidensis* MR-1 (Chourey et al. 2006) and other bacterial species (Viti et al. 2003; Pal et al. 2005; Puzon et al. 2005; Thacker et al. 2006; Sultan and Hasnain 2007; Okeke et al. 2008). The enzymatic reduction of Cr(VI) mostly involves a soluble cytosolic chromate reductases (Park et al. 2000; Megharaj et al. 2003; Camargo et al. 2003; Pal et al. 2005) or membrane-associated chromate reductases (Wang et al. 1990; Shen and Wang, 1993; Myers et al. 2000; Cheung et al. 2006; Ibrahim et al. 2011; Xu et al. 2012).

In a previous work we have isolated potent Cr(VI) reducing alkaliphilic *Amphibacillus* sp. KSUCr3 from hypersaline soda lake located in Wadi Natrun valley, Egypt (Ibrahim et al. 2011). *Amphibacillus* sp. is facultatively anaerobic, moderately alkaliphilic, Gram-positive, spore-forming and rod-shaped bacterial strain. The genus *Amphibacillus* was first proposed by Niimura.et al. (1990) and the genus currently comprises four recognized species, *Amphibacillus xylanus* [Niimura.et al. 1990], *Amphibacillus fermentum*, *Amphibacillus tropicus* [Zhilina et al. 2001], and *Amphibacillus sediminis* [An et al. 2007]. To the best of our knowledge this the first report about chromate (VI) reductase characterization from *Amphibacillus* sp. Extremophiles, including halophiles and alkaliphiles, have adapted to thrive in ecological niches with harsh conditions (high pH, temperature, and high salts concentrations, etc.). As result, these microorganisms produce unique biocatalysts that function under harsh conditions in which their mesophilic counterparts could not survive, permitting the development of additional industrial and bioremediation processes (Ibrahim et al. 2011). *Amphibacillus* sp. KSUCr3 showed strong Cr(VI) reduction under alkaline condition and the nutritional and environmental factors were investigated for optimization of the detoxification process (Ibrahim et al. 2011). Elucidation of the enzymatic mechanisms involved in direct chromate reduction is crucial in designing an efficient Cr(VI) bioreduction process. Hence, the present study aimed to elucidate the sub-cellar localization and in vitro characterization of the enzymatic chromate reductase activity of the alkaliphilic *Amphibacillus* sp. KSUCr3.

Materials and methods

Strain and culture conditions

Alkaliphilic *Amphibacillus* sp. KSUCr3 used in this study, showing potent Cr(VI)-reducing ability under alkaline conditions and resistant to hexavalent chromium of up to 80 mM in alkaline medium, was previously isolated from hypersaline soda lakes (Wadi El-Natrun, Egypt) (Ibrahim et al. 2011). The strain was grown in alkaline medium (pH 10) under the optimum growth conditions determined previously, containing glucose (8 g/l; Sigma), yeast extract (5 g/l; Difco), casamino acids (5 g/l; Difco), peptone (5 g/l; Difco), NaCl (120 g/l), Na_2CO_3 (15 g/l), 300 µl trace elements solution, and K_2CrO_7 (1 mM). The trace element solution contained: $CaCl_2{\cdot}2H_2O$ (1.7 g/l), $FeSO4{\cdot}7H_2O$ (1.3 g/l), $MnCl_2{\cdot}4H_2O$ (15.4 g/l), $ZnSO_4{\cdot}7H_2O{\cdot}7H_2O$ (0.25 g/l), H_3BO_3 (2.5 g/l), $CuSO_4{\cdot}5H_2O$ (0.125 g/l), Na_2MoO_4 (0.125 g/l), $CoNO_3{\cdot}6H_2O$ (0.23 g/l) and 2.5 ml 95–97 % H_2SO_4. The sodium carbonate, trace elements solution and K_2CrO_7 were autoclaved separately (Ibrahim et al. 2011).

Estimation of hexavalent chromium

Hexavalent chromium [Cr(VI)] was determined calorimetrically using *S*-diphenylcarbazide (DPC) method (Bartlett and James, 1996). Sample of 300 µl was added to 10 ml of glass-distilled water in a test tube, followed by the addition of 1 ml 0.25 % (w/v) 1,5-diphenyl carbazide (Sigma, USA) solution, prepared in acetone (AR) to

minimize deterioration, and one drop of H_3PO_4. The mixture was kept at room temperature for 10 min for color development, and then the optical density was measured at 540 nm. Calibration curve was made using $K_2Cr_2O_7$ at concentration ranged from 10 to 300 μg/ml. All experiments were performed in triplicate and mean values were recorded.

Bacterial growth versus Cr(VI) reductase production

Amphibacillus sp. KSUCr3 was repeatedly sub-cultured several times in fresh alkaline agar containing no Cr(VI) to get rid of any chromium in the cell itself, in addition to subculture in chromium containing alkaline agar medium. Colonies from each plate, with and without chromium were transferred to 250-ml Erlenmeyer flasks containing 50 ml of liquid culture medium with and without Cr(VI), respectively, and incubated at 40 °C under orbital shaking (100 rpm). These cultures were used to inoculate 1-l flasks containing 250 ml of the same medium (with and without chromium) and cultivated under the same conditions. Samples (1 ml) were withdrawn at 2 h interval up to 60 h for measurement of growth and chromate reductase activity. Samples (1 ml) were centrifuged at 10000×*g* at 4 °C, and the pellets obtained were washed twice using Tris buffer (pH 7), and resuspended in 1 ml of the same buffer. Absorbance was measured at 600 nm against Tris Buffer (pH 7) as blank, and was reported as growth of the bacterium. Triplicate of each time period was taken to calculate growth. For assay of Cr(VI) reductase activity, the suspended cells were recentrifuged and the cells pellets were used for the enzyme assay. The cell pellets were resuspended in 1 ml of reaction mixture containing 50 mM phosphate buffer (pH 7), $K_2Cr_2O_7$ (0.2 mM), Cu^{2+} (1 mM) and glucose (1 %), and incubated at 40 °C in a shaking water bath for 30 min. At the end of incubation period, the reaction mixture was centrifuged at 10000×*g* for 5 min and the supernatant was analyzed for the residual Cr(VI) determination by DPC method as described above. Heat-killed cells were used as controls. All assays were performed in triplicates and mean values were recorded.

Cr(VI) reduction by permeabilized cells of *Amphibacillus* sp. KSUCr3

Bacterial cell permeabilization was carried out using previously reported protocols (Desai et al. 2008a, b). *Amphibacillus* sp. KSUCr3 was grown for 48 h, harvested and washed with Tris buffer (pH 7.0) as described above. The suspended cells were treated with Tween 80, Triton X-100 and Toluene at final concentrations of 0.1 0.2, 0.5, 1, and 2 %, by vortexing for 20 min to achieve cell permeabilization. Cr(VI) reduction was initiated by adding 0.2 ml (after dilution 20 times) of these permeabilized cells suspensions to 0.8 ml 50 mM Tris buffer containing $K_2Cr_2O_7$ (0.2 mM), Cu^{2+} (1 mM) and glucose (1 %), and incubated at 40 °C in a shaking water bath for 30 min. At the end of incubation period, the reaction mixture was centrifuged and the supernatant was analyzed for residual Cr(VI) concentration by the DPC method as described above. Permeabilized cells heated at 100 °C for 10 min were used as controls. All experiments of permeabilization and assays were performed in triplicates and mean values were recorded.

Sub-cellular fractionation of *Amphibacillus* sp. KSUCr3 for localization of chromate reductase activity

Sub-cellular fractionation of *Amphibacillus* sp. KSUCr3 was performed using previously published protocols with some modifications (Pal et al. 2005; Desai et al. 2008b; Sau et al. 2010). Cells of *Amphibacillus* sp. KSUCr3 were grown in 1-l alkaline broth medium in a 5-l Erlenmeyer flask. After 48 h of incubation at 40 °C under orbital shaking (100 rpm), the cells were harvested by centrifugation at 1000×*g* at 4 °C, and the obtained cell pellets were washed twice with 50 mM Tris buffer (pH 7.0) and resuspended in 50 ml of the same buffer. These cell suspensions were placed in ice bath and disrupted using an Ultrasonic Probe (Omni 400, UK) with amplitude of 35 % at 50 W with 9 s pulses and 1 s off-mode for 20 min. The cell lysate (CL), thus obtained was then centrifuged at 30000×*g* for 30 min at 4 °C and the supernatant or the cytosolic fraction (cell-free extract) thus obtained was used as source of soluble enzyme. The sonicated cell pellets or the cell membranous fraction (CM) were washed twice with phosphate buffer and accordingly resuspended in same volume of Tris (pH 7.0) and kept for subsequent assays. Aliquots (200 μl) of culture filtrate, cell-free extracts or membranous fractions were used for enzyme assay in order to localize the chromate reductase activity of *Amphibacillus* sp. KSUCr3 either in extracellular, cystolic (soluble enzyme) or membrane-associated enzyme, respectively. All of the enzyme assays were conducted in triplicates with freshly prepared sub-cellular fractions and mean values were recorded.

Chromate reductase assay

Enzymatic hexavalent chromium reduction was estimated using modification from those described in previous studies (Park et al. 2000; Camargo et al. 2003; Pal et al. 2005). The reaction mixture (1 ml) contained Cr(VI) at final concentration of 0.2 mM, Cu^{2+} (1 mM), glucose (1 %) in 50 mM Tris buffer (pH 7) and 0.2 ml of enzyme source (resting cell, permeabilized cells, culture filtrate, CL, CFE, or CM).

Unless stated, the enzyme assay conditions were kept constant with a reaction time of 30 min at 40 °C in shaking water bath. At the end of incubation period, samples were centrifuged and the remaining Cr(VI) concentration was estimated from the supernatant following DPC method as described above. Abiotic control reaction mixtures without the addition of enzyme, and subjected to same assay conditions as those followed for experimental reactions was included. Unit enzyme activity for chromate reductase was derived as amount of enzyme that reduces 1 μmol Cr(VI) per minute at 40 °C, and specific enzyme activity was defined as unit chromate reductase activity per milligram protein. Protein concentrations were estimated using Bradford reagent (Bradford 1976) using bovine serum albumin as the standard protein.

Characterization of hexavalent Cr(VI) reductase of *Amphibacillus* sp. KSUCr3

Influence of temperature on Cr(VI) reductase activity

The influence of temperature on the catalytic activity of cell membrane-associated chromium reductase of *Amphibacillus* sp. KSUCr3 was determined by measuring the enzyme activity at reaction temperatures ranged from 25 to 60 °C under the standard assay conditions. Non-enzymatic Cr(VI) reduction was checked following inactivation of membrane fraction at 100 °C for 5 min. All assays were performed in triplicate and the mean values were recorded.

Influence of pH on Cr(VI) reductase activity

The effect of pH on the Cr(VI) reductase activity was determined by measuring the enzyme activity at varying pH values ranging from 4 to 11 at 40 °C. The membranous fractions were prepared in respective buffers of differential pH range and the same buffers were used for Cr(VI) reductase assays. The buffers used for Cr(VI) reductase assays were 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM sodium phosphate (pH 7.0 and 8.0), 50 mM glycine–NaOH buffer (9.0 and 10.0) and 50 mM carbonate–bicarbonate buffer (11.0 and 12), respectively. All assays were performed in triplicate and the mean values were reported.

Influence of metal cations, electron donors and inhibitors on Cr(VI) reductase activity

Hexavalent chromium reductase of *Amphibacillus* sp. KSUCr3 was determined in the presence of 1 mM of various metals ions including, Cu^{2+}, Na^{+}, Zn^{2+}, Mg^{2+}, Ni^{2+}, Ca^{2+}, Mn^{2+}, Cd^{2+}, and Co^{2+}, electrons donors (NADH, glucose, lactose, sucrose, fructose, sodium carbonate, glycine, potassium acetate, sodium format, trisodium citrate, ethanol, and methanol), in addition to the effect of various enzyme inhibitors including 2-mercaptoethanol, EDTA, Hg, and respiratory inhibitors (KCN, NaN_3).

Cr(VI) reduction by membrane-associated enzyme as a function of time

Cr(VI) reductase activity in the cells membrane fraction was measured at initial Cr(VI) concentrations range from 100 to 600 μM under the standard assay conditions described above. Aliquots were withdrawn at various reaction period intervals (0–180 min) and the residual Cr(VI) concentration was estimated using DPC method described above. Furthermore, effect of initial Cr(VI) concentration on the enzyme activity was investigated and kinetic parameters (K_m and V_{max}) were estimated.

Results and discussion

Initial Cr(VI) reductase activity assay screening indicated the essential of glucose, as external electron donor, and copper ion for the enzyme activity in *Amphibacillus* sp. KSUCr3 (see below), and hence, they were included in enzymatic reaction mixture. The growth pattern and the time course of Cr(VI) reductase production by *Amphibacillus* sp. KSUCr3 was carried out in the presence and absence of Cr(VI) in alkaline medium (pH 10) at 40 °C under orbital shaking (100 rpm). The stationary phase of growth was reached after about 44 h of cultivation. Interestingly, the growth of *Amphibacillus* sp. KSUCr3 was slightly higher in the presence of Cr(VI) (Fig. 1a). Chromium is an essential micronutrient required for the growth of many organisms, however, at high concentrations, Cr(VI) is toxic, mutagenic, carcinogenic and teratogenic (Costa and Klein 2006). The production of Cr(VI) reductase was increased with increasing of the incubation period, showing maximal production of 5.7 U/ml and 4.8 U/ml after about 42 h of cultivation, in the presence and absence of Cr(VI), respectively. These results suggested the constitutive nature of chromate reductase in *Amphibacillus* sp. KSUCr, with slight enzyme induction in the presence of Cr(VI) (Fig. 1b). Similar enzyme behavior was also detected in *Pseudomonas* CRB5 (Mclean and Beveridge 2001) and *Bacillus sphaericus* AND 303 (Pal et al. 2005), *Pseudomonas* sp. G1DM21 (Desai et al. 2008b) and *Bacillus firmus* KUCr1 (Sau et al. 2010). However, chromate reductase has been reported to be an induced enzyme in some other bacteria including *Pseudomonas putida* MK1 (Park et al. 2000), *Bacillus megaterium* TKW3 (Cheung

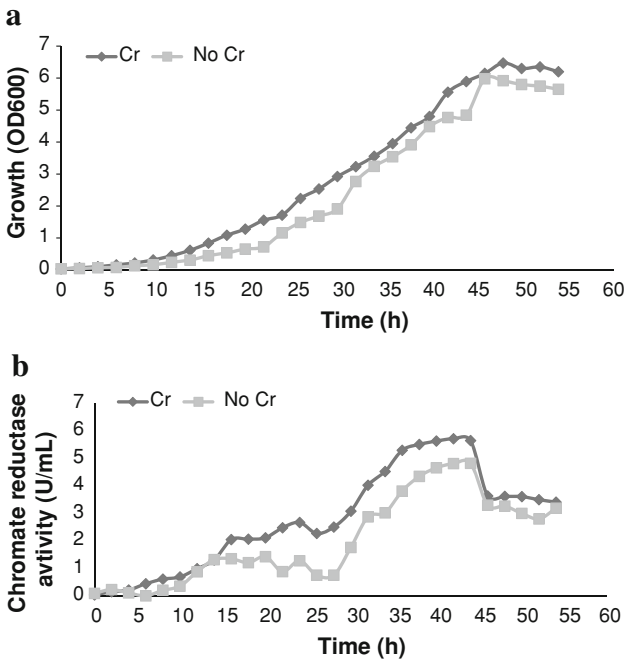

Fig. 1 Growth (**a**) and Cr(VI) reductase activity (**b**) by *Amphibacillus* sp. KSUCr3 in the absence and presence of Cr(VI) at final of 1 mM at 40 °C under orbital shaking (100 rpm)

et al. 2006) and *Bacillus* sp. JDM-2-1 and *Staphylococcus capitis* enzyme (Zahoor and Rehman, 2009).

Chromium reduction by permeabilized cells of *Amphibacillus* sp. KSUCr3

The resting *Amphibacillus* sp. KSUCr3 cells were able to reduce about 62 % of Cr(VI) at initial concentration of 200 μM within 30 min (Table 1). The inability of the heat-killed cells to reduce Cr(VI) showed that the reduction of Cr(VI) was mediated by cell-associated proteins. Permeabilization of *Amphibacillus* sp. KSUCr3 with various concentrations of Toluene, Triton X-100 and Tween, not only did not increase Cr(VI) reduction, but also resulted in decrease of Cr(VI) reduction in comparison to untreated cells (Table 1). Cells permeabilization with 1 % Triton-X100, Tween 80, or Toluene led to decrease of Cr(VI) reduction by 24.4, 48.7 and 6.3 % of that using untreated cells. These results are in agreement with results reported by Mangaiyarkaras et al. (2011), where permeabilization of alkaliphilic *Bacillus subtilis* cells resulted in significant decrease of Cr(VI) reduction. However, cell permeabilization was found to increase Cr(VI) reductase activity in other bacteria including *Bacillus* sp. (Desai et al. 2008b) and *Pseudomonas* sp. G1DM21(Desai et al. 2008a). Toluene and Triton X are known to permeabilize cell by dissolving the membrane phospholipid and solubilizing inner membrane proteins, respectively (Asenjo, 1990). Thus, less

Table 1 Hexavalent chromium reduction by resting and permeabilized cells of *Amphibacillus* sp. KSUCr3 performed at initial Cr(VI) concentrations of 200 μM at pH 7.0 and 40 °C

Treatment	Remaining Cr(VI) concentration (μM)
Control	200
Resting cells	75.1 ± 0.8
Triton X-100 (%)	
0.1	129.7 ± 0.8
0.2	131.6 ± 0.8
0.5	131.7 ± 0.87
1	134.0 ± 1.0
2	136.2 ± 1.1
Tween 80 (%)	
0.1	131.6 ± 0.85
0.2	144.5 ± 1.2
0.5	154.6 ± 1.5
1	155.2 ± 1.1
2	161.0 ± 1.8
Toluene (%)	
0.1	114.6 ± 1.1
0.2	121.1 ± 1.3
0.5	123.9 ± 0.85
1	118.4 ± 2.2
2	135.0 ± 1.6

Cr(VI) reduction observed with Triton X-100 treatment was due to the solubilization and inactivation of membrane-bound proteins, suggesting that Cr(VI) reductase of *Amphibacillus* sp. KSUCr3 may be membrane-bound enzyme (Mangaiyarkaras et al. 2011).

Localization of chromate reductase activity in *Amphibacillus* sp. KSUCr3

For localization of Cr(VI) reductase activity in *Amphibacillus* sp. KSUCr3, various sub-cellular fractions were prepared, and then enzyme activity assay was measured using culture supernatant, cell-free extract (CFE), membranous fraction (CM), in order to localize the chromate reductase activity either in extracellular, cystolic (soluble enzyme) or membrane-associated enzyme, respectively. Chromate reductase activity was detected mainly in the membranous fractions (CM) and negligible activity in CFE but no enzyme activity was detected in culture supernatant with specific activity of 71.5, 0.9 and 0.0 U/mg protein, respectively. In addition, the heat treated enzyme (boiling for 5 min) was not able to reduce Cr(VI). We, therefore, suggest that chromate reductase of *Amphibacillus* sp. KSUCr3 is a membrane-bound enzyme. Bacterial chromate reductases have been previously localized either in

cytosolic (CFE) (Park et al. 2000; Megharaj et al. 2003; Camargo et al. 2003; Sau et al. 2010; Pal et al. 2005; Desai et al. 2008b; Kavita and Keharia 2012; Xu et al. 2012) or membrane-bound proteins (Shen and Wang 1993; Cheung et al. 2006; Opperman et al. 2008). However, Ilias et al. (2011) have recently reported detection of chromate reductase in both culture supernatant and membrane fraction of *Staphylococcus aureus* and *Pediococcus pentosaceus*.

Characterization of hexavalent Cr(VI) reductase of *Amphibacillus* sp. KSUCr3

Influence of metals and electron donors

Initially, chromate reductase of *Amphibacillus* sp. KSUCr3 could not be detected even after long reaction incubation period (up to 20 h), without addition of external additives and addition of various electron donors to the reaction mixture did not result in significant increase of enzyme activity (data not shown). Therefore, the influence of addition of external metals ions (Cu^{2+}, Na^{+}, Zn^{2+}, Mg^{2+}, Ni^{2+}, Ca^{2+}, Mn^{2+}, Cd^{2+}, and Co^{2+}) on the enzyme activity was investigated. Amongst the metal ions tested Cu^{2+} ion markedly stimulated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3 (Fig. 2). Moreover, in the absence of Cu^{2+}, the enzyme activity was almost negligible, suggesting that Cu^{2+} is essential for the Cr(VI) reductase of *Amphibacillus* sp. KSUCr3. Furthermore, the enzyme activity was increased with increasing of Cu^{2+} ion concentration up to 1 mM, and then reached plateau up to 2 mM. Stimulation of chromate reductase activity by Cu^{2+} ion has been also observed in *Bacillus* sp. (Elangovan et al. 2006; Camargo et al. 2003; Desai et al. 2008b), *Pannonibacter phragmitetus* (Xu et al. 2012) and *Pseudomonas* sp (Desai et al. 2008a). However, the extent of stimulation of chromate reductase activity by Cu^{2+} in *Amphibacillus* sp. KSUCr3 was much higher than that reported in those strains. Hence we presume that Cr(VI) reductase activity in *Amphibacillus* sp. KSUCr3 is dependent on Cu^{2+} ion. The role of Cu^{2+} in stimulation of Cr(VI) reductase has been attributed to its action as a protective agent for electron transport, as a single electron redox center and as a shuttle for electrons between protein subunits (Camargo et al. 2003; Desai et al. 2008b). In addition, for oxygen-sensitive chromate reductase, it is also possible that Cu^{2+} is indirectly involved in the protection of chromate reductase from O_2 (Xu et al. 2012). On the other hand, Cu^{2+} has also been reported to inhibit the membrane-associated chromate reductase activity of *Enterobacter cloacae* (Wang et al. 1990), soluble chromate reductase activity in *Pseudomonas putida* (Park et al. 2000) and *B. sphaericus* AND 303 (Pal et al. 2005). Ca^{2+} and Mn^{2+} slightly stimulated the chromate reductase activity of *Amphibacillus* sp. KSUCr3, whereas, other divalent cations such as Zn^{2+}, Na^{+}, Mg^{2+}, Co^{3+}, Ni^{2+}, and Cd^{2+}, did not exhibit significant effect on the enzyme activity.

The influence of various external electron donors on the activity of the membrane-associated Cr(VI) reductase of *Amphibacillus* sp. KSUCr3 was investigated in the presence of copper ion, including NADH, glucose lactose, sucrose, fructose, sodium carbonate, glycine, potassium acetate, sodium format, tri-sodium citrate, ethanol, and methanol at final concentration of 1 %. As shown in Table 2, Cr(VI) reductase activity was increased on supplementation in the reaction mixtures with most tested electron donors, suggesting that exogenous electron donors were essential to improve the reductase activity of *Amphibacillus* sp. KSUCr3. Glucose and fructose showed highest stimulation effect, by an increase of up to 3.5-fold. Further enhancement of activity by 1.6- to 2.0-fold was observed in presence of electron donors such as NADH,

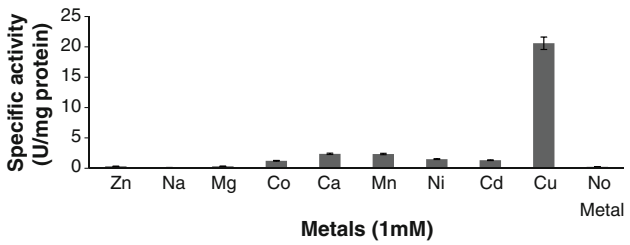

Fig. 2 Effect of different metal ions on membrane-associated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3 at pH 7.0 and 40 °C for 30 min. Results represent the means of three separate experiments, and *error bars* indicated

Table 2 Effect of electron donors on membrane-associated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3 in the presence of cupper ion (1 mM) at pH 7.0 and 40 °C for 30 min

Electron donors	Specific activity (U/mg protein)
Without ED	20.0 ± 0.8
NADH	37.3 ± 1.6
Glucose	70.4 ± 1.5
Lactose	13.7 ± 0.6
Sucrose	32.3 ± 1.4
Fructose	67.2 ± 2.0
Sodium carbonate	15.3 ± 0.9
Glycine	17.7 ± 0.4
Potassium acetate	34.8 ± 0.8
Sodium format	36.1 ± 0.7
Trisodium citrate	38.5 ± 1.2
Ethanol	19.4 ± 0.9
Methanol	20.5 ± 0.7

Results represent the means of three separate experiments

sucrose, potassium acetate, sodium formate and tri-sodium citrate. The influences of glucose and fructose, showing the highest stimulation effect, were further investigated at various concentrations and it was found that glucose at concentration of 1 % showed the highest stimulation effect as electron donor for membrane-associated Cr(VI) reductase of *Amphibacillus* sp. KSUCr3. Glucose has been reported to act as an electron donor to increase Cr(VI) reduction rate by *Bacillus sphaericus* AND 303 (Pal et al. 2005), also formate-dependent Cr(VI) reductases have been reported in *Shewanella putrefaciens* MR-1 (Myers et al. 2000). NADH has been also reported to enhance Cr(VI) reductase activity in several bacteria (Desai et al. 2008a, b; Xu et al. 2012).

Effect of pH and temperature on chromate reductase activity

For determination of the optimal pH of the membrane-bound Cr(VI) reductase of *Amphibacillus* sp. KSUCr3, enzyme assays were performed at varying pH values ranging from 5.0 to 11.0 using suitable buffer at 35 °C under the standard assay conditions. The activity showed a characteristic pH curve for the enzymatic activity with an optimum pH of 7 as depicted from Fig. 3. 50 and 10 % of activity was detected at pH 9.0 and 10.0, respectively. Similar observations of the influence of pH on bacterial Cr(VI) reduction have been made by others (Mclean and Beveridge, 2001; Sultan and Hasnain 2007; Desai et al. 2008a, b; Kavita and Keharia, 2012). Although *Amphibacillus* sp. KSUCr3 is an alkaliphilic bacterium, being isolated from hypersaline soda lake (Egypt) with an optimum growth of pH 10 (Ibrahim et al. 2011), the optimal pH for chromate reductase activity was around neutral. These results suggests the possibility of application of the crude enzyme in detoxification of Cr(VI) containing wastewater with pH around neutral, whereas resting cell of *Amphibacillus* sp. KSUCr3 is more suitable for Cr(VI) containing wastewater with alkaline pH. Most alkaliphiles have an optimal growth pH at around 9–10, however, the internal pH is maintained at around 8, despite a high external pH of 8–11 (Horikoshi 1999). Therefore, one of the key features in alkaliphily is associated with the cell surface, which discriminates and maintains the intracellular neutral environment separate from the extracellular alkaline environment. The cells have two barriers to reduce pH values from 10.5 to 8. Cell walls containing acidic polymers function as a negatively charged matrix and may reduce the pH value at the cell surface. Plasma membranes may also maintain pH homeostasis by using the Na^+/H^+ antiporter system, K^+/H^+ antiporter, and ATPase-driven H^+ expulsion (Horikoshi 1999; Horikoshi et al. 2011).

The temperature profile of the membrane-associated Cr(VI) reductase of *Amphibacillus* sp. KSUCr3 was estimated by measurement of the enzyme activity at various temperatures ranged from 25 to 60 °C at pH 7. The enzyme showed maximal activity at 40 °C, and lost about 50 % of it its activity at 45 °C. The enzyme was denatured at 60 °C (Fig. 4). It has been reported the optimal temperature of Cr(VI) reduction to be in the range of 30–40 °C (Megharaj et al. 2003; Desai et al. 2008a, b; Park et al. 2000; Pal et al. 2005; Kavita and Keharia, 2012 Xu et al. 2012). However, chromate reductases from thermophilic *Thermus scotoductus* SA-01 (Opperman et al. 2008) and *Bacillus firmus* KUCr1 (Sau et al. 2010) have been recently identified with an optimum temperature of Cr(VI)-reduction at 65 and 70 °C, respectively.

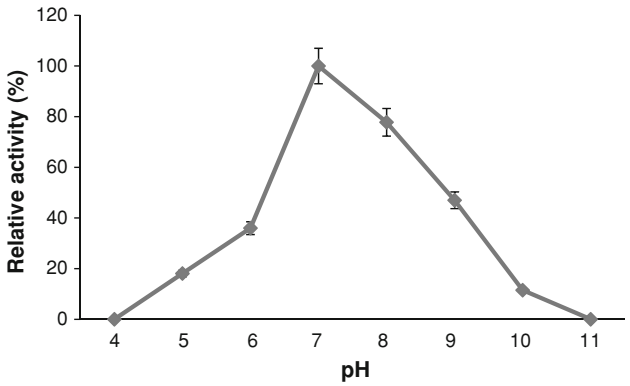

Fig. 3 Effect of pH on in membrane-associated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3, determined in suitable buffers (pH 4–11.0) with initial Cr(VI) concentration of 200 μM, at 35 °C. Results represent the means of three separate experiments, and *error bars* indicated

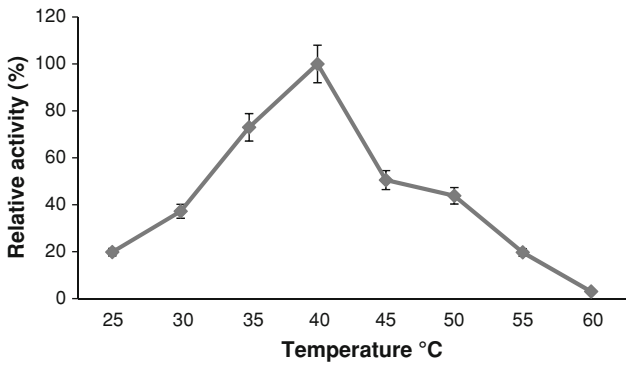

Fig. 4 Effect of temperature on membrane-associated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3, determined in potassium phosphate buffers (pH 7) with initial concentration of 200 μM Cr(VI), at various temperature (25–60 °C). Results represent the means of three separate experiments, and *error bars* indicated

Effect of different inhibitors on Cr(VI) reductase activity

The influence of some inhibitors including respiratory inhibitors on membrane-associated Cr(VI) reductase of *Amphibacillus* sp. KSUCr3 was investigated at final concentration of 1 mM (Fig. 5). Hg^{2+} strongly inhibited the Cr(VI) reductase activity by more than 95 % of the initial enzyme activity. Mercurial salts have been also reported to decrease the chromate reductase activity in *Pseudomonas* sp. G1DM21 (Desai et al. 2008a), *P. putida* (Park et al. 2000), *Bacillus* sp. (Camargo et al. 2003; Elangovan et al. 2006; Desai et al. 2008b) and *E. coli* ATCC 33456 (Bae et al. 2005). The inhibitory effect of mercury ions is owing mainly to their action as disulfide reducers causing denaturation of the reductase protein (Desai et al. 2008a, b), and these results were further confirmed by using 2-mercaptoethanol (2-ME) which again decreased the Cr(VI) reductase activity by about 55 % of the initial activity. EDTA (ion chelating agent) significantly reduced the enzyme activity by about 30 % suggesting that Cr(VI) reductase of *Amphibacillus* sp. KSUCr3 is an metalloenzyme which is consistent with the dependence of the enzyme activity on the presence of Cu^{2+} (Opperman et al. 2008). Furthermore, the effects of electron transport inhibitors, including azide and cyanide, on the CM-associated Cr(VI) reductase was assessed. Cyanide and azide markedly inhibited the enzyme activity by about 95 and 72 %, respectively. These results corroborate with those obtained in previous studies which reported that the inhibitory effect of azide and cyanide are more significant in membrane-associated chromate reductase than soluble enzyme (Myers et al. 2000; Cheung et al. 2006; Horikoshi et al. 2011). Respiratory inhibitors affect the respiratory chain intermediates responsible for Cr(VI) reduction, wherein Cr(VI) serves as a terminal electron acceptor. Therefore, it can be assumed that the chromate reductase of *Amphibacillus* sp. KSUCr3 is involved in Cr(VI) reduction which catalyzes initial one electron transfer to Cr(VI) to form an intermediate Cr(V), followed by two electron transfer and formation of Cr(III) as derived for *Pseudomonas putida* (Park et al. 2000) and *Pseudomonas* sp. G1DM21(Desai et al. 2008a).

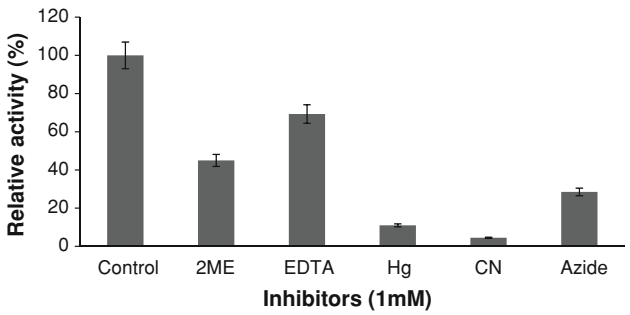

Fig. 5 Effect of different inhibitors on membrane-associated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3 at pH 7.0 and 40 °C. Results represent the means of three separate experiments, and *error bars* indicated

Time course of Cr(VI)-reduction by membrane-associated Cr(VI) reductase

Membrane-bound chromate reductase assay was performed as a function of time by estimating the remaining Cr(VI) at regular 20 min intervals reaction incubation period at 40 °C up to 180 min. As shown in Fig. 6, complete reduction of 200, 400, and 500 μM of Cr(VI) were observed within 20, 80 and 120 min of incubation at 40 °C in Tris buffer (pH 7), respectively. Furthermore, within 180 min of reaction time, 69.5 and 57.6 % of 600 and 800 μM Cr(VI) were reduced, respectively. Heat-killed control of membrane-associated Cr(VI) reductase failed to reduce Cr(VI). These results further confirm the presence of enzymatic mechanism in the membrane fraction *Amphibacillus* sp. KSUCr3. In addition, it indicated the efficiency of chromate reductase of *Amphibacillus* sp. KSUCr3 in reduction of high Cr(VI) concentrations. Moreover, Fig. 7a indicates the effect of initial Cr(VI) concentration on the enzyme activity. Specific activity increased with increasing initial concentration up to 100 μM, after that it slowed down but reduction continued. The saturation kinetics of Cr(VI) reduction by membrane-bound Cr(VI) reductase fit with the linearized Lineweaver–Burk plot, and the apparent Michaelis–Mentent constant (K_m) and maximum velocity (V_{max}) were found to be 23.8 μM chromate and 72 μmol/min/mg protein, respectively (Fig. 7b). The V_{max} for the chromate reductase activity of the *Amphibacillus* sp. KSUCr3 is much higher than that previously reported for Cr(VI) reductase of other bacterial strains (Ishibashi et al. 1990; Mclean and Beveridge 2001; Bae et al. 2005; Opperman et al. 2008; Desai et al. 2008a;), suggesting the presence of an efficient

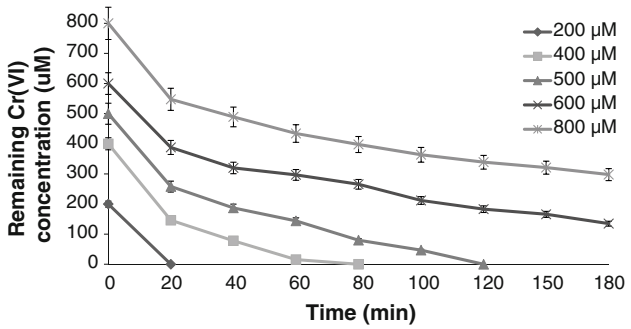

Fig. 6 Cr(VI) reduction by membrane-associated Cr(VI) reductase of *Amphibacillus* sp. KSUCr3 as a function of time determined with 100–600 μM Cr(VI) as initial concentrations at pH 7.0 and 40 °C. Results represent the means of three separate experiments, and *error bars* indicated

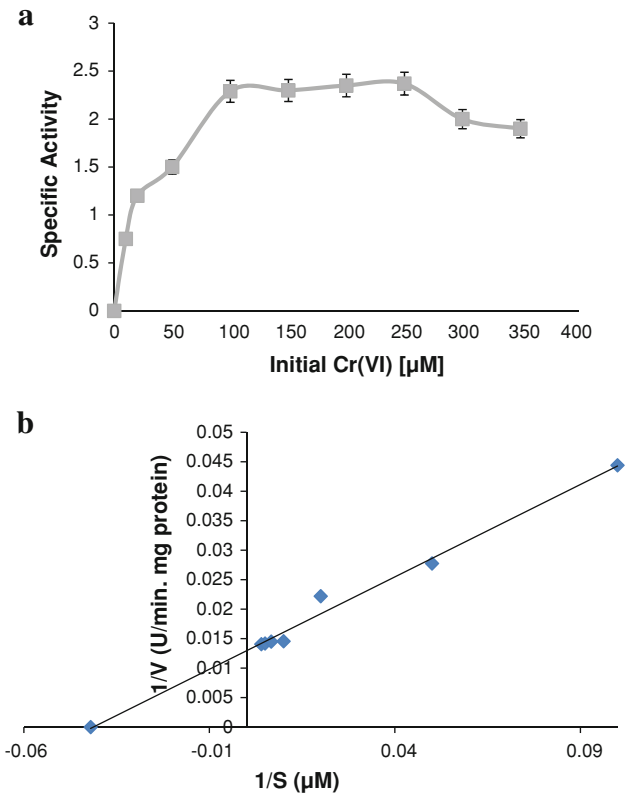

Fig. 7 Effect of initial Cr(VI) concentration on membrane-associated Cr(VI) reductase activity of *Amphibacillus* sp. KSUCr3 at pH 7.0 and 40 °C (**a**) and linearized Lineweaver–Burk plot (**b**)

chromate reductase in this strain. Furthermore, lower K_m value of Cr(VI) reductase suggests high affinity for the substrate (Sau et al. 2010). However, we should mention that these kinetic properties are approximate values due to the fact that crude enzymes *Amphibacillus* sp. KSUCr3 was used.

Conclusion

The present study demonstrates localization and preliminary characterization of a potent Cr(VI) reductase of alkaliphilic *Amphibacillus* sp. KSUCr3. Chromate reductase assay of the membranous fraction have shown a high Cr(VI) reductase activity, implicating the localization of enzyme in the cell membrane. The Cr(VI) reduction potential of the resting cells was decreased by cell permeabilization. Cu^{2+} ion was essential to the Cr(VI) reductase activity, presumably because of its electron-transport protection, acting as a single electron redox center and as a shuttle for electrons between protein subunits. Cu^{2+} could also be indirectly involved in the protection of chromate reductase from O_2 for oxygen-sensitive reductase of alkaliphilic *Amphibacillus* sp. KSUCr3. External electron donors were essential to improve the Cr(VI) reductase activity, and glucose showed the most significant increase in activity. Optimum temperature and pH of chromate reductase activity of the bacterium was found to be 40 °C and pH 7.0, respectively. Respiratory inhibitors significantly inhibited the enzyme activity, confirming the association of the enzyme with the cell membrane. The enzyme could rapidly reduce 100 % of 200, 400, and 500 μM of Cr(VI) within 20, 80 and 120 min, respectively. The results of higher rates of Cr(VI) reduction by the membrane fraction, functionality of the Cr(VI) reductase at ambient temperatures, pH and in presence of metals indicates a potential application of *Amphibacillus* sp. KSUCr3 for Cr(VI) bioremediation process. However, it should be noted that biochemical characterization has been done with crude enzyme only, and purification of chromate reductase of *Amphibacillus* sp. KSUCr3 to homogeneity is on progress for further analysis.

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