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Full Length Research Paper

Bioreduction of Cr (VI) by potent novel chromate resistant alkaliphilic *Bacillus* sp. strain KSUCr5 isolated from hypersaline Soda lakes

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Isolation of Cr (VI) resistant alkaliphilic bacteria from sediment and water samples collected from Wadi Natrun hypersaline Soda lakes (located in northern Egypt), resulted in isolation of several alkaliphilic bacterial strains that can tolerate up to 2.94 g/l of Cr (VI) in alkaline medium. However, with increasing Cr (VI) concentration up to 29.4 g/l, only one strain, KSUCr5, was able to tolerate up to 22 g/l (75 mM) and with MIC value of 23.5 g/l (80 mM) in alkaline medium (pH 10.5) containing 10% NaCl. Based on the 16S rRNA gene analysis, strain KSUCr5 was identified as *Bacillus* sp. with 99% similarity and was referred to as *Bacillus* sp. KSUCr5. In addition, *Bacillus* sp. strain KSUCr5 showed high tolerance to several other heavy metals including Cd^{2+} (50 mM), Mo^{2+} (75 mM), Mn^{2+} (100 mM), Cu^{2+} (2 mM), Ni^{2+} (100 mM), Pb (75 mM), Co^{2+} (5 mM) and Zn^{2+} (2 mM). Strain KSUCr5 was shown to be of a high efficiency in detoxifying chromate, as it could rapidly reduce up to 40 mg/l of Cr(VI) to a non detectable level over 24 h. In addition, at initial Cr(VI) concentration of 60 to 80 and 100 mg/l, 100% of the chromate reduction was achieved within 48 and 72 h, respectively. Strain KSUCr5 could reduce Cr(VI) efficiently over a wide range of initial Cr(VI) concentrations (10 to 300 mg/l) in alkaline medium under aerobic conditions without significant effect on the bacterial growth. It was able to reduce Cr(VI) in a wide range of NaCl (0 to 20%) with a maximum reduction yield at concentration of 0 to 1.5%, indicating the halo tolerance nature of the bacterium. It was found that addition of glucose and Na_2CO_3 to the culture medium caused a dramatic increase in Cr(VI)-reduction by *Bacillus* sp. strain KSUCr5. The maximum chromate removal was exhibited in alkaline medium (pH 10) containing 1.2% Na_2CO_3, 1.5% glucose and 1% NaCl and at incubation temperature of 35°C and culture shaking of 150 rpm. Under optimum Cr (VI) reduction conditions, Cr(VI) concentration of 80 mg/l was completely reduced within 24 h, with reduction rate of 3.3 mg h^{-1} which is one of the highest Cr(VI) reduction rate under high alkaline conditions, compared with other microorganisms that has been reported so far. Furthermore, the presence of other metals such as Ni^{2+}, Mo^{2+}, Cu^{2+} and Mn^{2+} at concentration of 100 mg/l together with Cr(VI) in the culture medium slightly increased Cr(VI)-reduction by the strain KSUCr5. Moreover, the isolate, *Bacillus* sp. strain KSUCr5, exhibited an ability to repeatedly reduce hexavalent chromium without any amendment of nutrients, suggesting its potential application in continuous bioremediation of Cr(VI). The results reveal the possible isolation of potent heavy metals resistant bacteria from extreme environment such as hypersaline Soda lakes and their application in bioremediation of heavy metals.

Key words: Chromate reduction, bioremediation, heavy metals, *Bacillus* sp., Soda lakes.

INTRODUCTION

Hexavalent chromium Cr(VI) in ionic forms such as: $HCrO^{4-}$, CrO_4^{2-} and $Cr_2O_7^{2-}$ are ubiquitously employed in many industrial processes including electroplating, leather tanning, dye and pigment manufacturing, wood processing, textile dyeing, steel and alloy industries, photographic sensitizer manufacturing and others (Cheung and Gu, 2007). Uncontrolled disposal of these

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high levels of toxic chromates containing industrial wastes results in anthropogenic contamination of environments (Sarangi and Krishnan, 2008). Hexavalent chromium is a very dangerous carcinogen, oxidizing agent, mutagen and teratogen and listed as Class A human carcinogen by the US-EPA (Costa et al., 2006; Desai et al., 2008a). Chro-mate oxyanions can readily permeate through biological membranes and their intracellular reduction results in the dire consequences of the chromate induced toxicity (Desai et al., 2008a). Inside the cells, Cr (VI) is partially reduced to highly unstable Cr(V) radical, which leads to the formation of reactive oxygen species (ROS). The molecular mechanisms of mutagenesis involve the for-mation of ternary adducts of intracellular Cr(III) with DNA, proteins and oxidative damage of DNA by Cr(V) and ROS (Ackerley et al., 2006; Sarangi and Krishnan, 2008; Desai et al., 2008b).

According to the WHO, the allowable concentration of Cr(VI) in drinking water is 0.05 mg l^{-1}. Thus, it is essential to reduce Cr(VI) concentrations from water/wastewater to acceptable levels (WHO, 1993; Ozturk et al., 2009). Strategies for Cr(VI) removal from industrial effluents and contaminated environments include chemical oxidation or reduction, chemical precipitation, filtration, ion exchange, electrochemical treatment, reverse osmosis, evaporation recovery and membrane technologies (Ahluwalia and Goyal, 2007; Zahoor and Rehman, 2009). However, major limitations of these physicochemical processes include the high-energy inputs, different chemical treatments and generation of unnecessary sludge, reactive chemical species as secondary wastes, in addition to their high cost. Instead, bioremediation of toxic metals containing waste by bacteria is getting increased attention due to its efficient, affordable and environmentally friendly advantages (Ozturk et al., 2009; He et al., 2011).

Most persistent forms of chromium in the environment are the soluble, mobile and most toxic hexavalent species Cr(VI), usually found as oxyanions, whereas trivalent chromium Cr(III), which is hundred times less toxic, less soluble and less mobile, mostly found as oxides, hydroxides or sulfates, generally bound to organic matter in soils (Cheung and Gu, 2007). Therefore, bioreduction of Cr(VI) to Cr(III) is an effective way of combating Cr(VI) pollution and is the most promising practice with proved expediency in bioremediation (Sarangi and Krishnan, 2008). Diverse bacteria have developed several strategies to resist chromate mainly through chromate reduction and chromate efflux. The main role of these strategies is to depress chromate toxicity to cells. Hence, chromate-reducing bacteria are able to reduce bioavailable, highly soluble chromate [Cr(VI)] to thermodynamically stable and less toxic trivalent chromium [Cr(III)], (Cheung and Gu, 2007; He et al., 2011). Bioreduction of Cr(VI) has been demonstrated in several bacterial species including *Pseudomonas* sp. (Jimenez-Mejia et al., 2006), *Shewanella* sp (Guh et al., 2001), *Achromobacter* sp. (Wani et al., 2007) and others (Pal and Paul, 2004; Puzon et al., 2005; Thacker et al., 2006; Sarangi et al., 2008; Sultan and Hasnain, 2007). The application of bacteria to detoxifying metals has been tested in a number of systems, but the viability and metabolic activity of cells are still major limiting factors affecting the bioremoval efficiency of the cellular biomass and enzymes involved (Cheung and Gu, 2007).

Cr(VI) reduction at high pH conditions is important for many bioremediation efforts as many toxic metals containing industrial effluents are in alkaline pH (Ye et al., 2004; Stewart et al., 2007). In addition, high concentration of salts in wastewater treatment systems can be a major problem for conventional biological treatments (Amoozegar et al., 2007). Therefore, bacteria that can survive under highly alkaline and high salt conditions and can detoxify metals need to be identified. Halophilic and alkaliphilic microorganisms are suitable candidates for bioremediation processes, since high concentrations of anions and cations are needed for their growth (Horikoshi, 1999, 2011). Hypersaline alkaline Soda lakes are ecological niche for isolation of halophilic and alkaliphilic microorganisms. Extremophiles, including halophiles and alkaliphiles, have adapted to thrive in ecological niches with harsh conditions that is high pH, temperature, high salts concentrations, etc. As a 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 (Amoozegar et al., 2007; Horikoshi, 1999, 2011). In this study, we reported isolation of potent chromate reducing alkaliphilic halotolerant bacterium from hypersaline Soda lakes (Wadi Natrun valley, Egypt) and investigation of the effect of various parameters on the bioreduction process.

MATERIALS AND METHODS

Soil and water samples

Sediment, soil and water samples were collected from hypersaline Soda lakes located in Wadi Natrun valley in northern Egypt. Wadi Natrun valley extends in a northwest by southeast direction between latitudes 30°15´ north and longitude 30°30´ east. The bottom of the Wadi Natrun area is 23 m below sea-level and 38 m below the water-level of Rosetta branch of the Nile. The lowest part of the depression, encircled by contour zero, covers an area of about 272 km^2 (Taher, 1999). Samples were collected in sterile tubes from the different locations of Wadi Natrun Soda lakes, kept in refrigerator and were transferred to the laboratory (King Saud University, Saudi Arabia) within two weeks.

Isolation of Cr (VI) resistant alkaliphilic bacteria

Isolation of Cr (VI) resistant alkaliphilic bacteria were carried out using rich alkaline agar medium supplemented with different concentrations of Cr (VI). The alkaline agar medium (pH 10.5) contained glucose (10 g/l; Sigma), yeast extract (5 g/l; Difco), casamino acids (5 g/l; Difco), peptone (5 g/l; Difco), NaCl (100 g/l), Na_2CO_3 (15 g/l), agar (15 g/l), 300 µl trace elements solution and $K_2Cr_2O_7$ (1 to 20 mM). The trace element solution contained: $CaCl_2.2H_2O$ (1.7 g/l), $FeSO_4.7H_2O$ (1.3 g/l), $MnCl_2.4H_2O$ (15.4 g/l), $ZnSO_4.7H_2O.7H_2O$ (0.25 g/l), H_3BO_3 (2.5 g/l), $CuSO_4.5H_2O$ (0.125 g/l), Na_2MoO_4 (0.125 g/l), $CoNO_3.6H_2O$ (0.23 g/l)

and 2.5 ml 95-97 H_2SO_4. The sodium carbonate, trace elements solution and $K_2Cr_2O_7$ were autoclaved separately before addition to the medium. Sediment and soil samples were suspended in 50 mM glycine-NaOH buffer (pH 10) containing 10% NaCl and serially diluted up to 10^{-5}. Aliquots (100 µl) of different dilutions were plated on the alkaline agar medium and incubated at different incubation temperatures for several days. The obtained colonies were sub-cultured several times in fresh Cr (VI) containing agar media until single homogeneous colonies were obtained.

Identification of the isolated strains

The selected strain was identified by 16S rRNA gene sequence analysis. The bacterial isolate was grown overnight in 5 ml alkaline broth medium and total DNA was extracted using DNeasy blood and tissue kits (Qiagen) according to the manufacturer's instructions. Eubacterial-specific forward primer 16F27 (5´-AGA GTT TGA TCC TGG CTC AG-3´) and reverse primer 16R1525 (5´-AAG GAG GTG ATC CAG CCG CA-3´) were used to amplify 16S rDNA gene (Lane, 1991). Polymerase Chain Reaction (PCR) amplification was performed in a final reaction volume of 50 µl and the reaction mixture contained 25 µl of GoTaq® Green Master Mix (2X), (Promega, cat no. 7122), 1 µl of upstream primer (10 µM), 1 µl of downstream primer (10 µM), 5 µl DNA template (200 ng) and 18 µl of nuclease-free water. The PCR reaction was run for 35 cycles in a DNA thermal cycler under the following thermal profile: Initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, primers annealing at 52°C for 1 min and extension at 72°C for 1.5 min. The final cycle included extension for 10 min at 72°C to ensure full extension of the products. PCR products were purified using a QIAquick gel extraction kit (Qiagen) and sequenced using an automated sequencer (Research center, King Faisal Hospital, Riyadh, Saudi Arabia). Sequence was analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST algorithm (Altschul et al., 1997). The sequence was deposited at GenBank with accession no. JF748751.

Resistance to other heavy metal ions

The resistance of the bacterial isolate to other heavy metals, in addition to Cr(VI) was determined by using stock solutions of different metal salts including Cr^{6+}, Pb^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Mo^{2+}, Ni^{2+} and Cd^{2+}. The cross metal resistance was checked by increasing the concentration of respective metal in a stepwise manner in the same alkaline agar medium (pH 10.5). Melted alkaline agar medium supplemented with various concentrations of the metals was inoculated with 50 µl of overnight culture of strain incubated at 30°C for 7 days.

Determination of optimum growth and Cr(VI)-reduction conditions

For determination of the optimum growth and Cr(VI)-reduction conditions by the selected strain, the influences of some parameters were considered. These included the effect of initial Cr(VI) concentration (10 to 300 mg/l), incubation temperature (25, 30, 35, 40, 45 and 50°C), pH (5 to 12), Na_2CO_3 concentration (0 to 2%), aeration level (shaking with 0 to 300 rpm), glucose concentration (0 to 2%), NaCl concentration (0 to 20%) and effect of different cell densities. Sterile alkaline medium (100 ml) in culture flasks (250 ml) was supplemented with appropriate concentration of Cr(VI), inoculated from exponential phase bacterial culture and incubated at the appropriate temperature and shaking. Cell-free controls were also used in each experiment to monitor any abiotic Cr(VI)-reduction. Samples were aseptically drawn at defined time intervals, centrifuged at 7000×*g* for 10 min and the supernatant analyzed for residual Cr(VI) by using the standard diphenyl carbazide method. In addition, the effects of other heavy metals including Pb^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Mo^{2+}, Ni^{2+} and Cd^{2+} with final concentration of 50 mg/l on Cr(VI)- reduction by strain KSUCr5 were also investigated. Alkaline medium (100 ml) in culture flasks was supplemented with Cr(VI) to a final concentration of 100 mg/l, together with other metals (50 mg/l) and incubated for 24 h at optimum conditions. The experiments were performed in triplicate as described earlier and the mean values were reported.

Repeated detoxification of Cr(VI)

Bacterial culture grown for overnight to an A600 of 1.0 in 100 ml sterile alkaline broth was amended with Cr(VI) to a final concentration of 50 mg/l and incubated at 35°C under gyratory shaking of 150 rpm. Two ml culture suspensions were withdrawn after every 12 h of the incubation to measure Cr(VI) remaining as described earlier and the culture flasks were repeatedly added with increments of 80 mg/l Cr(VI) until saturation in Cr(VI) reduction was observed.

Analytical methods

Growth and residual chromium was measured according to Thacker et al. (2007) with some modification. For the determination of the bacterial growth, samples (1 ml) were drawn and centrifuged at 7000×*g* for 10 min at 8°C. The obtained pellets were resuspended in 1 ml of distilled water and absorbance was measured at 600 nm against distilled water as blank and was reported as growth of the bacterium. The supernatant obtained after centrifugation was used to measure residual chromium concentration. The residual Cr (VI) was estimated as the decrease in chromium concentration with time using hexavalent chromium specific colorimetric reagent, 0.25% (w/v) 1,5-diphenyl carbazide (DPC) prepared in acetone (AR) to minimize deterioration. Supernatant (100 µl) was added to 10 ml of glass-distilled water in a test tube, followed by the addition of 1 ml of 1,5- diphenylcarbazide solution and 1 drop of H_3PO_4. The mixture was kept at room temperature for 10 min for color development and then optical density was measured at 540 nm. Calibration curve was made using various concentration of $K_2Cr_2O_7$ ranged from 10 to 300 µg/ml. All experiments were performed in triplicate and the mean values were reported.

RESULTS AND DISCUSSION

Isolation and identification of Cr (VI) resistant alkaliphilic bacteria

Isolation of Cr (VI) resistant alkaliphilic bacteria from sediment and water samples collected from Wadi Natrun hypersaline Soda lakes resulted in isolation of several strains that can tolerate up to 2.94 g/l of toxic Cr (VI). However, with increasing Cr (VI) concentration up to 29.4 g/l only one strain, KSUCr5, was able to tolerate up to 22 g/l (75 mM) and with MIC value of 23.5 g/l (80 mM) in alkaline medium (pH 10.5) containing 10% NaCl. In addition, KSUCr5 showed the highest reduction yield of Cr (VI) in alkaline liquid medium (data not shown). Based on the 16S rRNA gene analysis, strain KSUCr5 was 99% identical to *Bacillus* sp. and was referred to as *Bacillus* sp. KSUCr5 and the gene sequence was deposited at GenBank with accession no. JF748751. The resistance of *Bacillus* sp. strain KSUCr5 is on a very high level, perhaps the highest recorded so far in alkaline medium (pH 10.5), compared to other microorganisms (Table 1). It has been reported that the presence of Na and K in chemical structure of this oxyanion seems to be one of the reasons for such a high tolerance to oxyanions. Sodium and potassium are essential elements for ionic pumps and the enzymes activity in alkaliphiles and halophiles

Table 1. Chromate resistance of different organisms.

Organism	MIC of chromate (mM)	Reference
Bacillus sp. KSUCr5	80	This study
Lysinibacillus fusiformis ZC1	60	He et al. (2011)
Alkaliphilic *Bacillus subtilis*		Mangaiyarkaras et al. (2011)
Ochrobactrum sp. strain CSCr-3	2.7	He et al. (2009)
Bacillus sp. JDM-2-1	1.6	Zahoor and Rehman (2009)
Intrasporangium sp. Q5-1	17	Yang et al. (2009)
Pseudomonas sp. G1DM21	7	Desai et al. (2008b)
Bacillus sp. KCH2	2	Sarangi and Krishnan, 2008
Burkholderia cepacia MCMB-821	19.2	Wani et al. (2007)
Brucella sp. DM1	3.4	Thacker et al. (2007)
Ochrobactrum intermedium SDCr-5	288	Sultan et al. (2007)
Nesterenkonia sp. strain MF2	600	Amoozegar et al. (2007)
Providencia sp. UTDM314	3.4	Thacker et al. (2006
Bacillus sphaericus AND303	15.4	Pal and Paul, (2004)
Pseudomonad CRB5	10	McLean and Beveridge (2001)

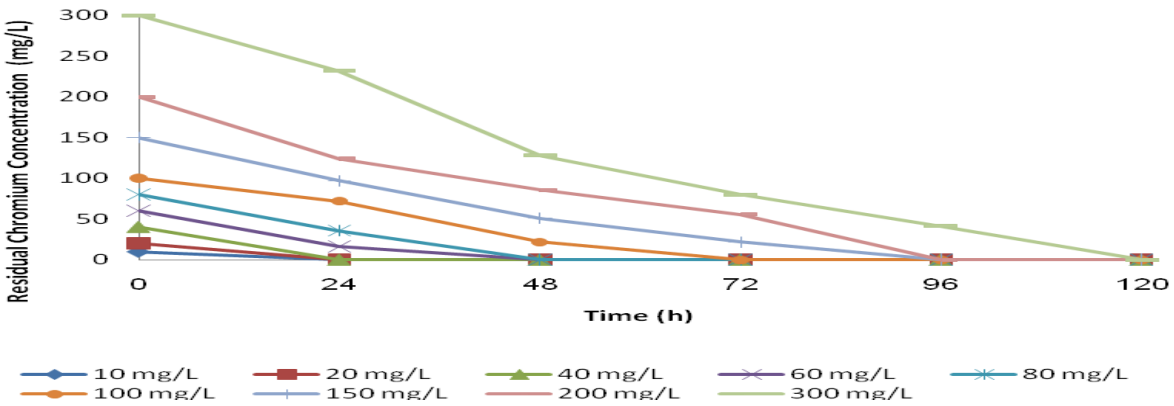

Figure 1. Chromium reduction by *Bacillus* sp. KSUCr5 with different initial chromium concentration (10 to 300 mg/l). Results represent the means of three separate experiments. Standard deviation was in range of 2 to 6.5%.

and thereby, enhance bacterial tolerance to toxic metals (Margesin and Schinner, 2001; Amoozegar et al., 2007; Horikoshi, 2011). Furthermore, *Bacillus* sp. strain KSU-Cr5 showed high tolerance to several other heavy metals including Cd^{2+} (50 mM), Mo^{2+} (75 mM), Mn^{2+} (100 mM), Cu^{2+} (2 mM), Ni^{2+} (100 mM), Pb (75mM), Co^{2+} (5 mM) and Zn^{2+} (2 mM). Since most polluted environments contain mixed waste, individual bacterial strain with enhanced capacities for remediating multiple pollutants is highly desirable (Ackerley et al., 2006).

Optimization of growth and Cr (VI) reduction

Effect of initial Cr (VI) concentration

Hexavalent chromate reduction by *Bacillus* sp. KSUCr5 was investigated at different initial chromium concentrations ranging from 10 to 300 mg/l as potassium dichromate ($K_2Cr_2O_4$). This bacterium was able to reduce Cr(VI) rapidly. As shown in Figure 1, complete Cr(VI) reduction was achieved within 24 h for initial Cr(VI) concentrations of up to 40 mg/l with chromate reduction rate of 1.7 mg h^{-1}. In addition, at initial Cr(VI) concentrations of 60 to 80 and 100 mg/l, 100% of the chromate reduction was achieved within 48 and 72 h, respectively. Furthermore, 78.2 and 44.2% of initial Cr(VI) concentration of 150 and 200 mg/l was reduced within 72 h, respectively, with white precipitate of Cr (III) visible at the bottom of the culture bottle (Figure 2). These results clearly indicate the potency of *Bacillus* sp. strain KSUCr5 in Cr(VI) reduction in comparison to previously isolated strains. *Microbacterium* sp. completely reduced 20 mg/l of Cr(VI) within 72 h (Pattanapipitpaisal et al., 2001). The *Pseudomonad* strain CRB5 showed complete reduction of 20 mg/l of chromate only after 120 h (McLean and

Figure 2. Chromium reduction by *Bacillus* sp. KSUCr5. The strain was grown in alkaline medium containing The strain was grown in alkaline medium containing 1% Na_2CO_3, 10% NaCl, 1% glucose and with chromium concentration of 100 mg/L and incubated for 24 h at and incubated for 24 h at 30°C with shaking (150 rpm). Complete Cr(VI) reduction was achieved within 72 h and white-grayish precipitate visible at the bottom of the bottle (Right). Cell-free control was used to monitor any abiotic Cr(VI)-reduction (Left).

Beveridge, 2001). *Bacillus sphaericus* AND303 failed to completely reduce 10 mg/l of Cr(VI), (Pal and Paul, 2004). 50 mg/l were reduced to zero in 54 h by *Brucella* sp. (Thacker et al., 2007). Halophilic *Nesterenkonia* sp. strain MF2 showing the highest tolerance to the chromate (600 mM), for Cr(VI) concentration of 117.6 mg/l, it took 72 h for complete reduction and beyond this Cr(VI) concentration, the complete Cr(VI) reduction was not observed even after 120 h (Amoozegar et al., 2007). Mangaiyarkarasi et al. (2011) has reported Cr (VI) reduction by an akalipihilic *B. subtilis* and showed that 50 mg/l of Cr(VI) was reduced to near zero in 65 h, whereas 100, 150 and 200 mg/l were reduced by 71, 62 and 27% in 144 h, respectively.

However, (He et al., 2011) recently reported isolation of highly Cr(VI) reducing *Lysinibacillus fusiformis* strain. In that study, the bacterium was able to reduce 1 mM Cr(VI) within 12 h.

In all the following experiments, culture medium with initial Cr (VI) concentration of 100 mg/l was used as a basis for comparison.

Effect of temperature

Temperature is one of the most important parameters affecting microbial Cr(VI)-reduction. Bacterial growth and Cr(VI) reduction by the strain KSUCr5 were studied at various temperatures (25 to 50°C). As shown in Figure 3, chromate reduction was increased with temperature up to 35°C, which appear to be the optimal temperature for growth of the strain KSUCr5. At 40°C, chromate reduction was about 56.3% of the reduction yield at the optimum temperature (35°C). However, above 40°C, the bacterial growth and chromate reduction were dramatically decreased (Figure 3). Generally, the optimal Cr(VI) reduction depend mostly on the optimum growth temperature of the organism. However, It has been reported that the optimal temperature of Cr(VI) reduction is in the range of 30 to 37°C (Cheung and Gu, 2007). Maximum Cr(VI)-reduction by *Nesterenkonia* sp. strain MF2 (Amoozegar et al., 2007) and *Ochrobactrum* sp. CSCr-3 (He et al., 2009) was found to be 35°C, whereas for *Bacillus* sp., (Wang and Xiao, 1995) and *Pseudomonas* strain CRB5 (McLean et al., 2000), it was reported to be 30°C. However, chromate reductase from thermophhilic *Thermus scotoductus* SA-01 has been recently identified with an optimum temperature of Cr(VI)-reduction at 65°C (Opperman et al., 2008).

Effect of pH

Figure 4 shows the influence of initial pH (5 to 11) on Cr(VI)-reduction yield and growth of strain KSUCr5 in 24 h. The strain KSUCr5 was able to reduce Cr(VI) in a wide pH range (7 to 12) with an optimum growth and reduction yield at pH 10, indicating the alkaliphilic nature of *Bacillus* sp. strain KSUCr5 (Horikoshi, 1999, 2011). There was slight and significant decrease in the reduction yield when pH increased to pH 11 and 12, respectively, but Cr(VI)-reduction was completely ceased at acidic conditions (pH 5 to 6). This result is similar to that reported by He et al. (2009) that the optimum pH was 10 for Cr(VI) reduction by *Ochrobactrum* sp. CSCr-3. In addition, Shakoori et al. (2000) and Mangaiyarkaras et al. (2011) reported that the optimum pH was 9 for Cr(VI) reduction by a Gram-positive bacterium and alkaliphilic *B. subtilis*, respectively. However, optimum pH of 7 was also reported for Cr(VI) reduction by *Pseudomonas aeruginosa* (Liu et al., 2004).

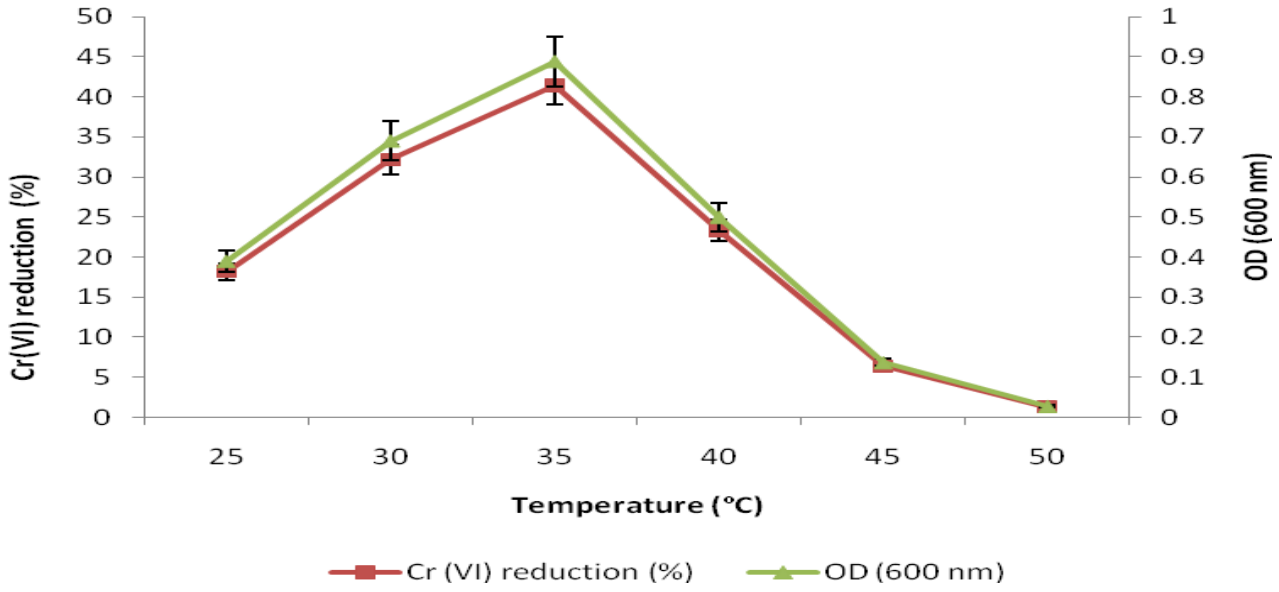

Figure 3. Effect of temperature on growth and Cr (VI) reduction by the *Bacillus* sp. strain KSUCr5. The strain was grown in alkaline medium containing 1% Na_2CO_3, 10% NaCl, 1% glucose and with chromium concentration of 100 mg/l and incubated for 24 h at various temperatures (25 to 50°C) with shaking (100 rpm). Results represent the means of three separate experiments and deviated bars indicated.

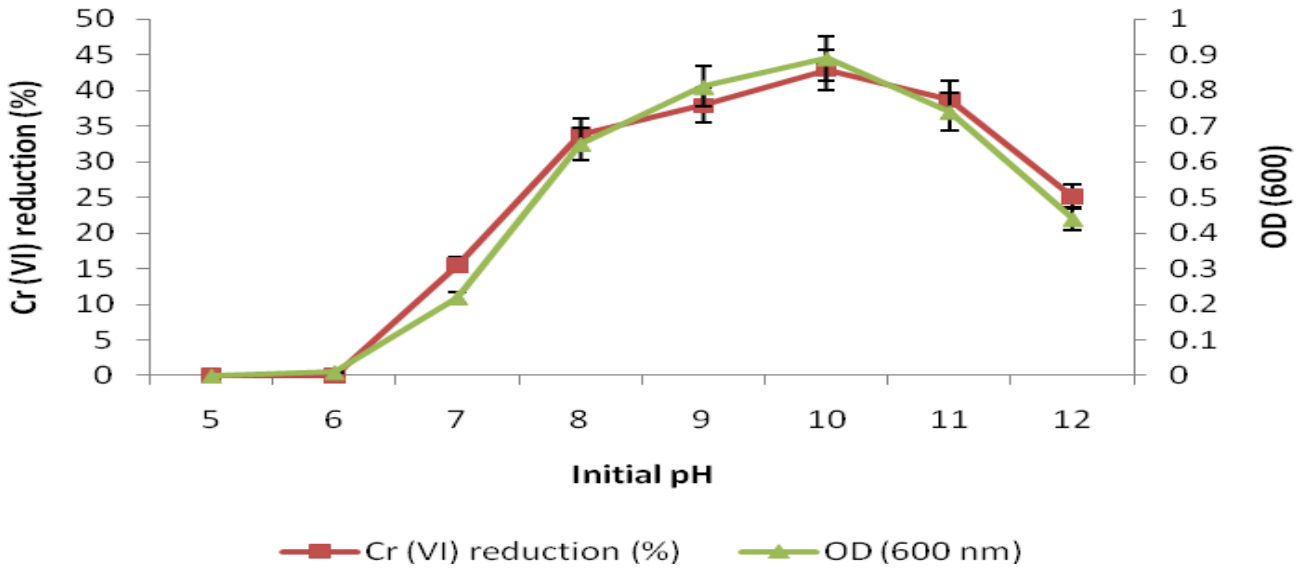

Figure 4. Effect of pH on growth and Cr (VI) reduction by the *Bacillus* sp. strain KSUCr5. The strain was grown in media with different initial pH (5 to 12), containing 1% Na_2CO_3, 10% NaCl, 1% glucose and with chromium concentration of 100 mg/l and incubated for 24 h at 35°C with shaking (100 rpm). Results represent the means of three separate experiments and deviated bars indicated.

Effect of sodium carbonate

It has been proved that the presence of sodium ions in the surrounding environment is essential for effective solute transport through the membranes of alkaliphilic bacteria (Horikoshi, 1999, 2011). Therefore, the effect of Na_2CO_3 concentration on hexavalent chromate reduction as well as growth of *Bacillus* sp. strain KSUCr5 was studied. In absence of Na_2Co_3 (pH around neutral), *Bacillus* sp KSUCr5 growth and Cr (VI) reduction drastically decreased, indicating the alkaliphilic nature of the organism. While maximum bacterial growth was seen at

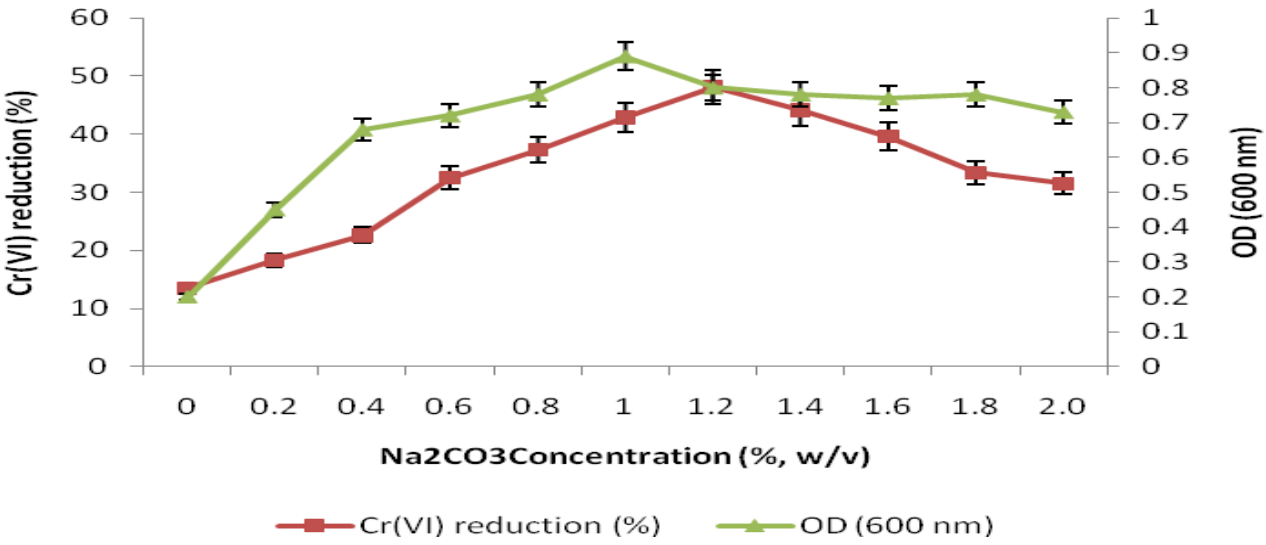

Figure 5. Effect of Na_2CO_3 concentration on growth and Cr (VI) reduction by *Bacillus* sp. KSUCr5. The strain was grown in medium containing different concentration of Na_2CO_3 (0 to 2), 10% NaCl, 1% glucose and with Cr (VI) concentration of 100 mg/l and incubated for 24 h at 35°C with shaking (100 rpm). Results represent the means of three separate experiments and deviated bars indicated.

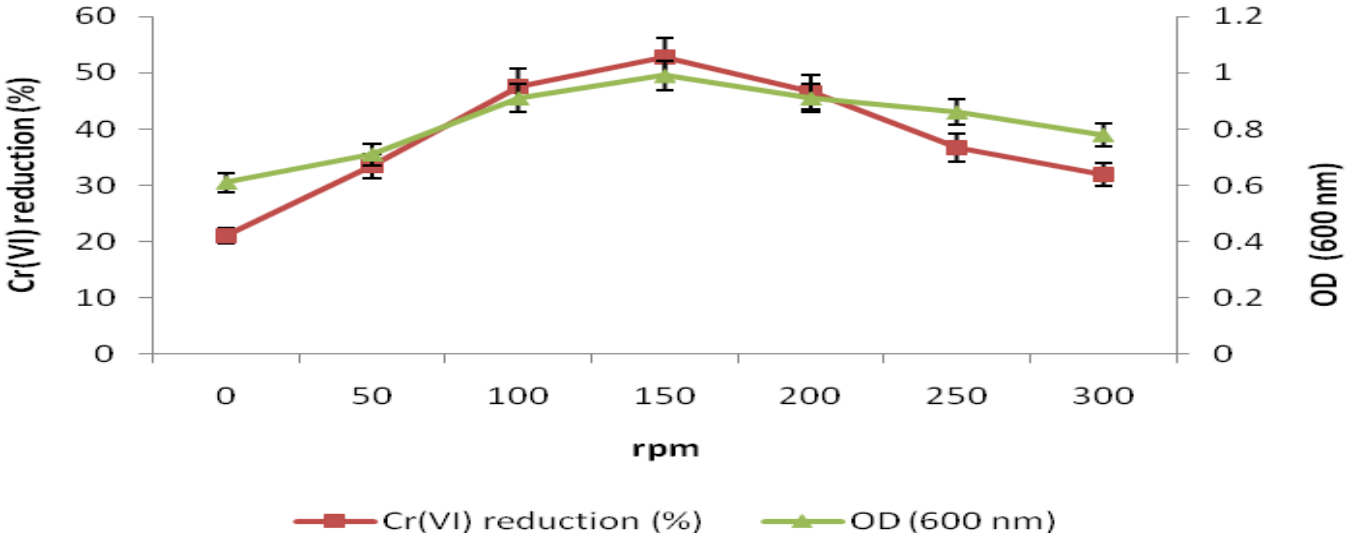

Figure 6. Effect of aeration level (shaking rpm) on the growth and Cr (VI) reduction by *Bacillus* sp. KSUCr5. The strain was grown in alkaline medium containing 1.2% Na_2CO_3, 10% NaCl and with Cr (VI) concentration of 100 mg/l and incubated for 24 h at 35°C with shaking (0 to 300 rpm). Results represent the means of three separate experiments, and deviated bars indicated.

Na_2CO_3 concentration of 1%, maximum Cr (VI) reduction (48.1%) was found to be at 1.2% (Figure 5). According to the chemiosmotic theory, the proton motive force in the cells is generated by excreted H^+ derived from ATP metabolism by ATPase or by the electron transport chain. H^+ is then reincorporated into the cells with co-transport of various substrates. In Na^+-dependent transport systems, the H^+ is exchanged with Na^+ by Na^+/H^+ antiporter systems, thus, generating a sodium motive force, which drives substrates accompanied by Na^+ into the cells (Horikoshi, 1999, 2011).

Effect of aeration level

The influence of aeration level on bacterial growth and Cr(VI) reduction by strain KSUCr5 was investigated by incubating the cultures at various shaking level from 0 to 300 rpm. The results shown in Figure 6 indicated that aeration level has a significant effect on the growth and Cr(CVI) reduction by *Bacillus* sp. Strain KSUCr5. At

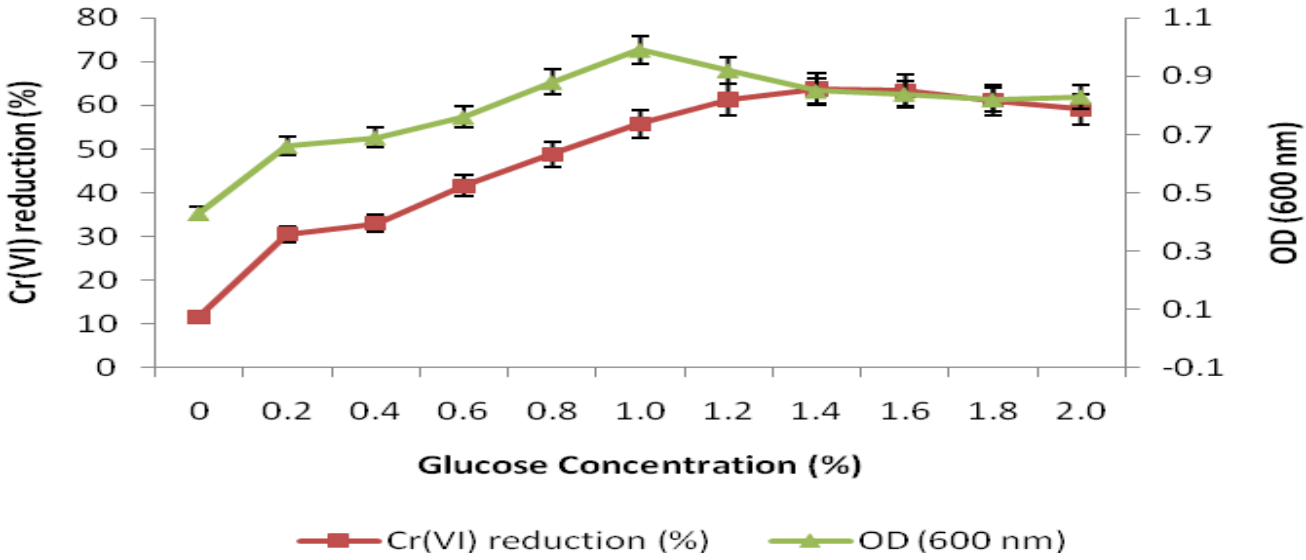

Figure 7. Effect of glucose concentration on growth and Cr(VI)-reduction by *Bacillus* sp KSUCr5. The strain was grown in alkaline medium containing 1.2% Na_2CO_3, 10% NaCl, glucose (0 to 2%), 10% NaCl and with Cr (VI) concentration of 100 mg/l and incubated for 24 h at 35°C with shaking (150 rpm). Results represent the means of three separate experiments and deviated bars indicated.

shaking of 150 rpm the Cr(CVI) bioreduction was about 2.5 fold higher than that at static conditions, indicating that Cr(VI) reduction by the strain KSUCr5 occurred under aerobic conditions. However, at higher aeration level the Cr(VI) reduction started to decline. Chromate reduction has been reported to occur under both aerobic (Pal and Paul 2004; Poopal et al., 2009) and anaerobic conditions (Michel et al., 2001; Chardin et al., 2002). In the presence of oxygen, bacterial Cr^{6+} reduction com-monly occurs as a two- or three-step process with Cr^{6+} initially reduced to the short-lived intermediates Cr^{5+} and/or Cr^{4+} before further reduction to the thermodynamically stable end product, Cr^{3+} (Cheung and Gu, 2007).

Effect of glucose concentration

It has been previously reported that chromate-reducing bacteria may utilize a variety of organic compounds as electron donors for Cr(VI) reduction (Guh et al., 2001; Liu et al., 2004; He et al., 2009). In this study, the influence of glucose on Cr(VI)-reduction and bacterial growth was studied. As shown in Figure 7, Cr(VI) reduction was increased dramatically by addition of glucose to the culture medium. Furthermore, bacterial growth and Cr (VI) reduction increased with increasing glucose concentration, showing maximum growth and bioreduction yield (65.7%) at initial glucose concentrations of 1 and about 1.5%, respectively. At higher concentration, there was no further increase of Cr(VI)-reduction (Figure 7). These results are in consistence with other reports indicating requirement of glucose as electron donor for Cr(VI)-reduction. Glucose has been reported to act as an electron donor and demonstrated to significantly increase Cr(VI) reduction by *Bacillus* sp. (Liu et al., 2006; Pal et al., 2005), *Ochrobactrum* sp. CSCr-3 (He et al., 2009) and *Streptomyces griseus* (Poopal et al., 2009). However, other electron donors like formate, fructose, and carbonate have also been reported to increase Cr(VI) reduction (Myers et al., 2000; Desai et al., 2008b; He et al., 2011).

Effect of NaCl concentration

Figure 8 shows the influence of NaCl concentrations on growth and Cr(VI) reduction by *Bacillus* sp. KSUCr5. The bacterium was able to grow and reduce Cr(VI) in a wide range of NaCl concentration from 0 to 20%, with maximum growth and reduction yield (81.7%) at concentration of 0 to 1.5%. Beyond this concentration, both bacterial growth and reduction level started to decline. However, at high NaCl concentrations of 4, 10 and 20%, *Bacillus* sp. strain KSUCr5 was able to reduce 44.1 35.3and 23.5% of the initial chromate concentration (100 mg/l), respectively, indicating the halotolerance nature of the strain KSUCr5. Amoozegar et al. (2007) reported that complete reduction of 0.2 mM Cr(VI) after 24 h by halophilic *Nesterenkonia* sp. strain MF2 was achieved only when the concentration of NaCl increased from 0.1 to 1 M.

Effect of cell concentration on Cr(VI)-reduction

The effect of initial cell densities from 1×10^7 to 3.12×10^9 cells/ml on Cr(VI) reduction is shown in Figure 9. Cr(VI)-

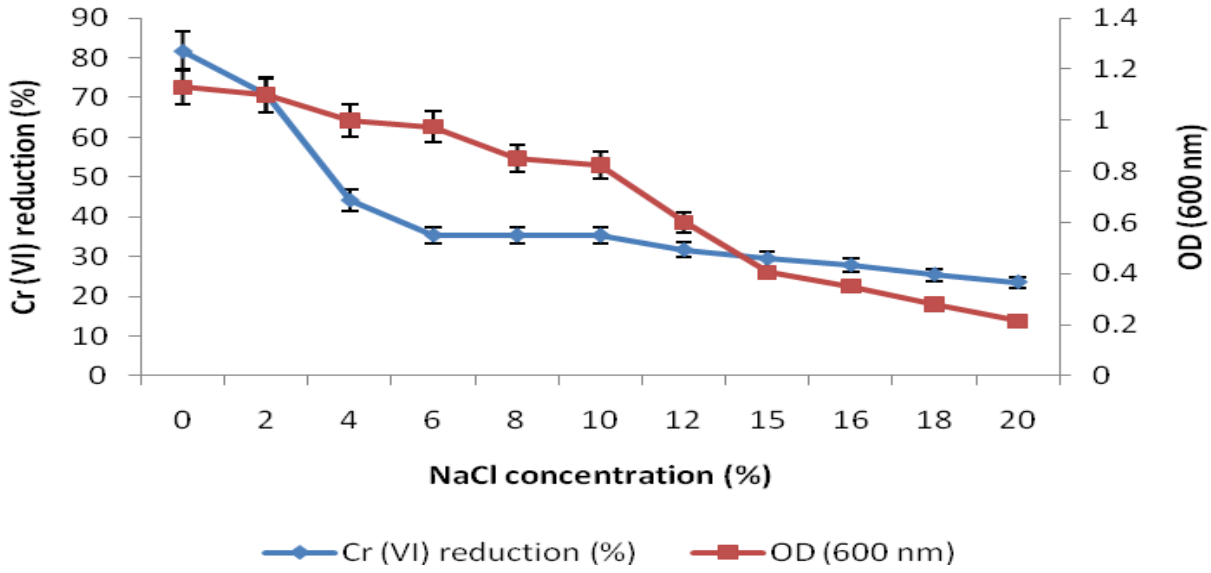

Figure 8. Effect of NaCl concentration on growth and Cr(VI)-reduction by *Bacillus* sp. KSUCr3. The strain was grown in alkaline medium containing 1.2% $NaCO_3$, 1.5% glucose, NaCl (0 to 2%) and with Cr (VI) concentration of 100 mg/l and incubated for 24 h at 35°C with shaking (150 rpm). Results represent the means of three separate experiments and deviated bars indicated.

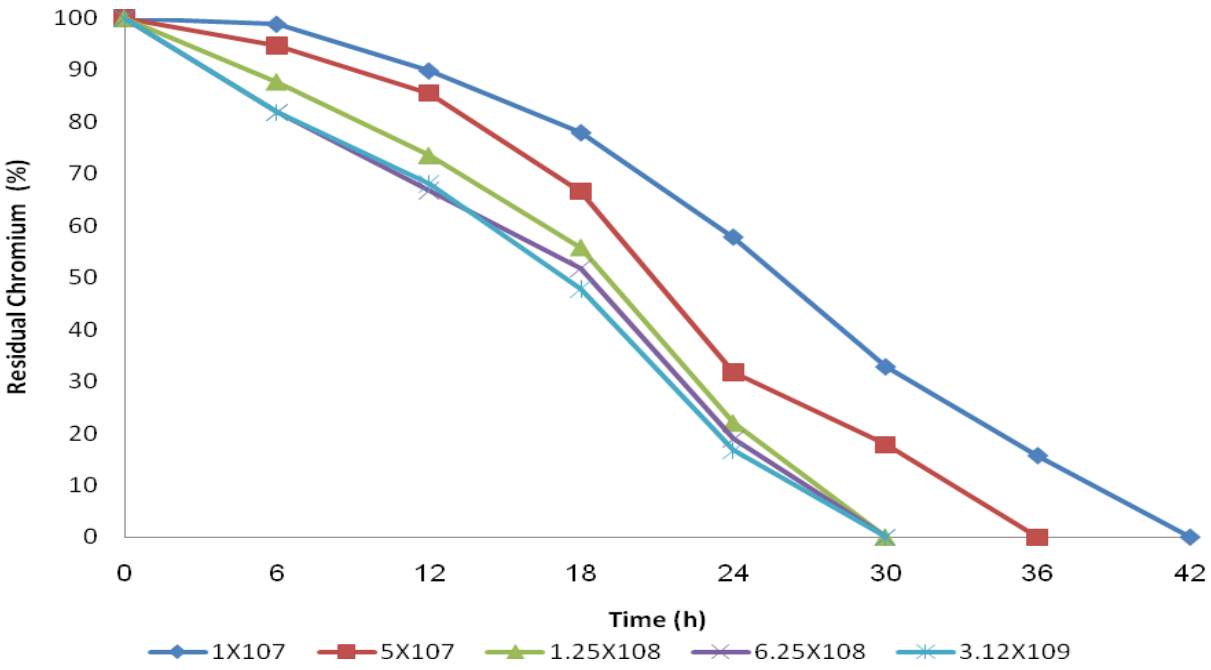

Figure 9. Effect of cells destinies on Cr(VI)-reduction by *Bacillus* sp. KSUCr5. The strain was grown in alkaline medium containing 1.2% $NaCO_3$, 1.5% glucose, 1 % NaCl and with Cr (VI) concentration of 100 mg/l and incubated for 24 h at 35°C with shaking (150 rpm). Results represent the means of three separate experiments**.** Standard deviation was in range of 2 to 6.5%.

reduction by strain KSUCr5 increased with an increase in an initial cell concentration from 1×10^7 to 1.25×10^8. However, higher cell concentrations had no further effect on the Cr(VI) reduction. At the initial cell concentration of 1.25×10^8 cells/ml, 100% of the initial Cr(VI) was reduced within 30 h, but with lower cell concentration the same yield was obtained within 42 h. A similar trend was also observed with *Pseudomonas* CRB5 (McLean et al.,

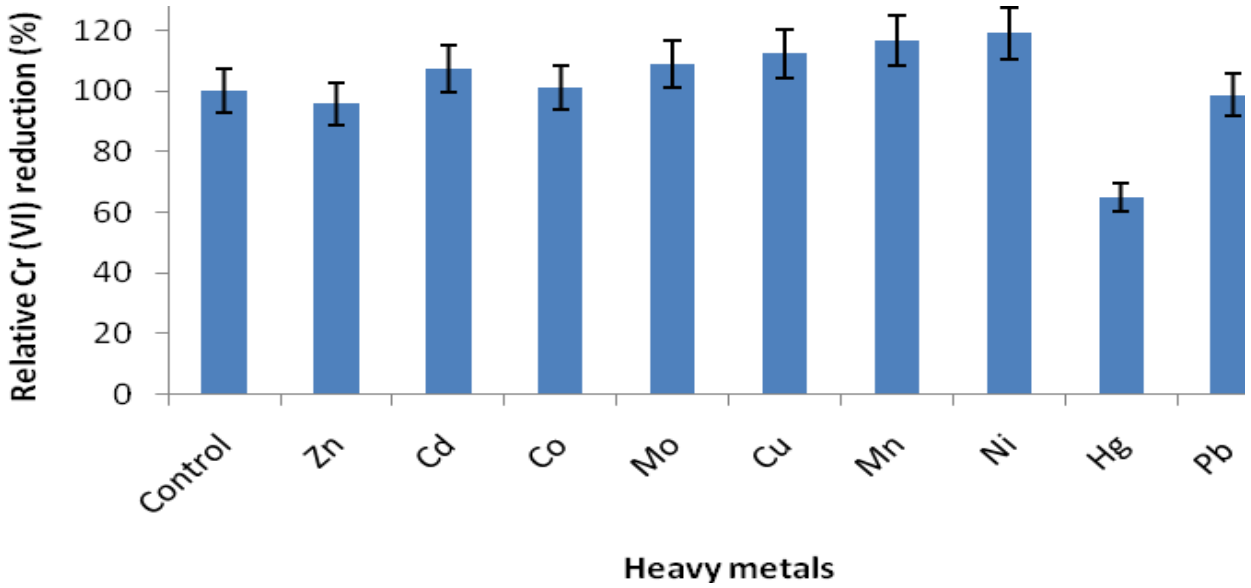

Figure 10. Effects of heavy metals supplementation on Cr(VI)-reduction yield by *Bacillus* sp. KSUCr3. The strain was grown in alkaline medium containing 1.2% $NaCO_3$, 1.5% glucose, 1% NaCl, and with Cr (VI) concentration of 100 mg/l together 50 mg/l of different metals and incubated for 24 h at 35°C with shaking (150 rpm). Results represent the means of three separate experiments and deviated bars indicated.

2000), *Microbacterium liquefacient* (Pattanapipitpaisal et al., 2001) *Ochrobactrum* sp. strain CSCr-3 (He et al., 2009) and *L. fusiformis* ZC1 (He et al., 2011).

Effects of other metals on Cr(VI)-reduction

Chromium containing industrial effluents and metal contaminated soils can also contain other heavy metals. Therefore, the influence of other heavy metals on Cr(VI)-reduction by the strain KSUCr5 was also studied in this work. As shown in Figure 10, the presence of 50 mg/l of Ni^{2+}, Mo^{2+}, Cu^{2+} and Mn^{2+} together with Cr(VI) in the culture medium slightly increased Cr(VI)-reduction, whereas Co^{2+}, Cd^{2+} and Pb^{+} had no effect on Cr(VI)-reduction by strain KSUCr5. However, Zn^{2+} and Hg^{2+} showed slight and significant inhibition of Cr(VI) reduction by KSUCr5, respectively. Stimulatory effect of Cu^{2+}, $Ni^{2+}$and Mn^{2+} on Cr(VI) reduction activity has been also reported for Cr(VI)-reduction by *Bacillus* sp. ES 29 (Camargo et al., 2003), *Ochrobactrum intermedium* strain SDCr-5 (Sultan and Hasnain, 2007) and *Ochrobactrum* sp. strain CSCr-3 (He et al., 2009), respectively. However, many other studies, have reported an inhibitory effect of Cu^{2+} on Cr(VI) reduction. Chromate reduction by *B. sphaericus* was inhibited by the presence of Ni^{2+}, Co^{2+} and Pb^{2+}, even at low concentration (20 mg/l), (Pal and Paul, 2004). The stimulatory mechanism of Cr(VI) reduction activity by Cu^{2+} and other metals is not clear. However, Cu^{2+} is a prosthetic group for several reductase enzymes. In addition, it has been reported that function of Cu^{2+} is related to electron transport protection or acting as electron redox center (Abe et al., 2001; Camargo et al., 2003b; He et al., 2009).

Repeated detoxification of Cr(VI) by *Bacillus* sp KSUCr5

The chromate reducing ability of *Bacillus* sp. KSUCr5 was tested by five repeated additions of 80 mg/L K_2CrO_4 every 12 h under optimum reduction conditions. *Bacillus* sp. KSUCr5 exhibited complete reduction of 80 mg/l Cr(VI) up to four consecutive inputs as observed from Figure 11. In addition, it could still reduce about 68.3% of the fifth addition of 80 mg/l Cr(VI) within further 24 h. *Bacillus* sp. KSUCr5 was able to repeatedly reduce hexavalent chromium without any amendment of nutrients, which suggests its potential application in continuous bioremediation of Cr(VI).

Conclusion

This study reports isolation of potent Cr (VI) reducing alkaliphilic *Bacillus* sp. strain KSUCr5 from hypersaline Soda Lake located in Wadi Natrun valley, Egypt. Strain KSUCr5 can effectively reduce Cr(VI) to Cr(III) under alkaline condition, high sodium chloride concentration, wide range of temperatures and high Cr(VI) concentrations (10 to 300 mg/l mM). Under optimum Cr (VI) reduction conditions, Cr(VI) concentration of 80 mg/l was completely reduced within 24 h, with reduction rate of 3.3 mg h^{-1}, which is one of the highest Cr(VI) reduction rate

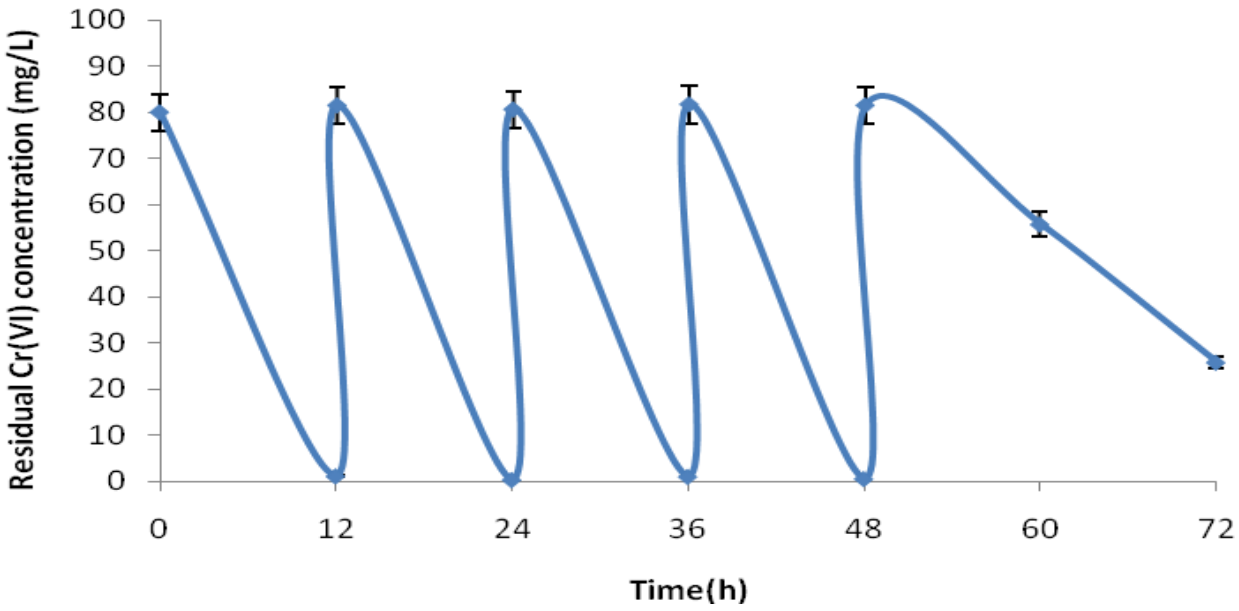

Figure 11. Repeated detoxification of 80 mg/l by *Bacillus* sp. KSUCr5 under the optimum conditions of Cr(VI) reduction.

under high salt concentration and high alkaline conditions, compared with other microorganisms that has been reported so far. In addition, KSUCr5 showed resistance to several other heavy metals including Cd^{2+}, Mo^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+} and Zn^{2+} and since most polluted environments contain mixed waste, individual bacterial strain with enhanced capacities for remediating multiple pollutants is highly desirable. Furthermore, the isolate, *Bacillus* sp. strain KSUCr5, exhibited an ability to repeatedly reduce hexavalent chromium without any amendment of nutrients, suggesting its potential application in continuous bioremediation of Cr(VI). In addition, the results also revealed the possible isolation of potent heavy metal resistant bacteria from extreme environment such as hypersaline Soda lakes and its possible application in bioremediation process. Purification and characterization of chromium reductase of *Bacillus* sp. strain KSUCr5 are in progress and to be published elsewhere.

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