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Isolation and characterization of novel potent Cr(VI) reducing alkaliphilic *Amphibacillus* sp. KSUCr3 from hypersaline soda lakes

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Abstract A strain KSUCr3 with extremely high Cr(VI)-reducing ability under alkaline conditions was isolated from hypersaline soda lakes and identified as *Amphibacillus* sp. on the basis of 16S rRNA gene sequence analysis. The results showed that *Amphibacillus* sp. strain KSUCr3 was tolerance to very high Cr(VI) concentration (75 mM) in addition to high tolerance to other heavy metals including Ni^{2+} (100 mM), Mo^{2+} (75 mM), Co^{2+} (5 mM), Mn^{2+} (100 mM), Zn^{2+} (2 mM), Cu^{2+} (2 mM) and Pb (75 mM). Strain KSUCr3 was shown to be of a high efficiency in detoxifying chromate, as it could rapidly reduce 5 mM of Cr(VI) to a non detectable level over 24 hrs. In addition, strain KSUCr3 could reduce Cr(VI) efficiently over a wide range of initial Cr(VI) concentrations (1-10 mM) in alkaline medium under aerobic conditions without significant effect on the bacterial growth. Addition of glucose, NaCl and Na_2CO_3 to the culture medium caused a dramatic increase in Cr(VI)-reduction by *Amphibacillus* sp. strain KSUCr3. The maximum chromate removal was exhibited in alkaline medium containing 1.5% Na_2CO_3, 0.8% glucose, and 1.2% NaCl, at incubation temperature of 40ºC and shaking of 100 rpm. Under optimum Cr(VI) reduction conditions, Cr(VI) reduction rate reached 237 μMh^1 which is one of the highest Cr(VI) reduction rate, under alkaline conditions and high salt concentration, compared to other microorganisms that has been reported so far. Furthermore, the presence of other metals, such as Ni^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+} slightly stimulated Cr(VI)-reduction ability by the strain KSUCr3.The isolate, *Amphibacillus* sp. strain KSUCr3, 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 also revealed the possible isolation of potent heavy metals resistant bacteria from extreme environment such as hypersaline soda lakes.

Keywords: *Amphibacillus* sp., bioremediation, chromate reduction, heavy metals, soda lake

INTRODUCTION

Hexavalent chromium is a very dangerous carcinogen, oxidizing agent, mutagen, and teratogen and listed as class A human carcinogen by the US-EPA (Quievryn et al. 2003; Costa and Klein, 2006; Desai et al. 2008b). It is released into the environment from many industrial processes including electroplating, leather tanning, dye and pigment manufacturing, wood treatment, textile dyeing and the steel and alloy industries (Cheung and Gu, 2007). 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 formation of ternary adducts of intracellular Cr(III) with DNA, proteins and oxidative damage of DNA by Cr(V) and ROS (Ackerley et al. 2006; Desai et al. 2008a; Sarangi and Krishnan, 2008). According to the World Health Organization (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). Traditional methods for removing metals from industrial effluents 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, large-scale applications of these methods are consuming energy excessively and utilize huge amounts of reagents in addition of 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).

Chromium(III) is rather benign, less mobile, forms water insoluble compounds in aqueous solution and easily absorbed in soils and waters, whereas Cr(VI), which is the toxic form of chromium, is readily adsorbed and soluble (Zahoor and Rehman, 2009). Subsequently, 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 (Desai et al. 2008b), *Achromobacter* sp. (Wani et al. 2007) and others (Viti et al. 2003; Pal and Paul, 2004; Puzon et al. 2005; Thacker et al. 2006; Sultan and Hasnain, 2007; Sarangi and Krishnan, 2008). The application of bacteria to detoxify metals has been tested in a number of systems, but the viability and metabolic activity of cells are still major limiting factors affecting the bioremval 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 effluents released containing toxic metals are under 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. 2005; Amoozegar et al. 2007). Therefore, bacteria that can survive under highly alkaline and high salt conditions and can detoxify metals need to be identified. Hypersaline alkaline soda lakes are ecological niche for isolation of halophilic and halotolerant microorganisms that are suitable candidates for bioremediation processes under such harsh conditions (Horikoshi, 1999; Horikoshi et al. 2011). Soda lakes are widely distributed throughout the world; however, as a result of their inaccessibility, few of such lakes have been explored from the microbiological point of view. One of those environmental niches, which have not been studied in details, is the Wadi Natrun soda lakes in northern Egypt. Here we report isolation and characterization of extremely potent Cr(VI) reducing bacterium isolated from Wadi Natrun hypersaline soda lakes and investigation of the influence of various parameters on the detoxification 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 30º 15' north and longitude 30º 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: Hamara, Bani salama, Dawood, and Elbida lakes, kept in refrigerator and were transferred to the laboratory (King Saud University, Saudi Arabia) within two weeks. A chemical analysis of a soil sample from Wadi Natrun soda lakes was done in soil analysis laboratory (Faculty of Agriculture, KSU, Saudi Arabia), and indicated that the soil sample was rich in Na^+ (21.7 %) and CO_3^{+3} (7.29%) along with low content of P (0.14%), K (0.11%), Ca (0.83%), Mg (1.72%), Cd (0.04 mg/kg), Cr (4.5 mg/kg), Pb (2.6 mg/kg), Zn (7.1 mg/kg) and Hg (0.03 mg/kg).

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_2CrO_7 (1-20 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_2CrO_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 agar media until single homogeneous colonies were obtained.

Table 1. Isolation of Cr(VI) resistance alkaliphilic bacteria from Wadi Natrun hypersaline soda lakes.

Isolate	Cr(VI) MIC (mM)
KSUCr1	80
KSUCr2	50
KSUCr3	80
KSUCr4	20
KSUCr5	55
KSUCr6	15
KSUCr7	80
KSUCr8	20
KSUCr9	30
KSUCr10	20
KSUCr11	30
KSUCr12	40

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, NY, USA) 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 100 µl, and the reaction mixture contained each primer (0.5 µM) at, each deoxynucleoside triphosphate (200 µM), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin and 2.5 U of *Taq* DNA polymerase. The PCR reaction was run for 35 cycles in a DNA thermal cycler under the following thermal profile: Initial denaturation at 95ºC for 5 min, denaturation at 95ºC for 1 min, primers annealing at 52ºC for 1 min and extension at 72ºC for 1.5 min. The final cycle included extension for 10 min at 72ºC to ensure full extension of the products. PCR products were analyzed using agarose gel electrophoresis and purified from the gel using a QIAquick Gel Extraction Kit (Qiagen, NY, USA,) and sequenced using an automated sequencer (Research Center, King Faisal Hospital, Riyadh, Saudi Arabia). Sequence was analyzed at NCBI server using (BLAST) algorithm (Altschul et al. 1997). The sequence was deposited at GenBank with accession no. JF690755.

Evaluation of resistance to chromium and other heavy metals

For investigation of the tolerance of the isolated strain to Cr (VI) and other heavy metals, the agar dilution method was used. Melted alkaline agar medium supplemented with various concentrations of chromate (1-100 mM), Ni^{2+} (1-100 mM), Mo^{2+} (1-100 mM), Co^{2+} (1-50 mM), Mn^{2+} (1-100 mM), Zn^{2+} (1-10 mM), Cu^{2+} (1-10 mM), and Pb (1-100 mM), were poured into plates. Then 50 µl of overnight culture of strain was inoculated on each plate and incubated at 30ºC for 7 days.

Table 2. Bioreduction of Cr(VI) by the isolated strains, KSUCr1, KSUCr3, and KSUCr7. Results represent the means of three separate experiments. Standard deviation was in range of 1.0-5.0 %.

Initial Cr (VI) conc. (mM)	Residual chromium (%)											
	KSUCr1				KSUCr3				KSUC7			
	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
0.5	20.1	0	0	0	0	0	0	0	34.6	0	0	0
1	25.6	0	0	0	0	0	0	0	52.3	21.9	0	0
2	43.5	26.2	0	0	0	0	0	0	68.3	42.6	23.9	6.4
3	65.5	47.7	21.3	13.6	0	0	0	0	77.1	55.6	39.5	23.7
5	72.3	56.9	41.2	33.7	0	0	0	0	83.5	61.4	55.6	48.7

Table 3. Chromate resistance and reduction rates in different organisms.

Organism	MIC of chromate (mM)	Chromate reduction rate (μMh^{-1})	Reference
Amphibacillus sp. KSUCr3	80	237	This study
Lysinibacillus fusiformis ZC1	60	80	He et al. (2011)
Ochrobactrum sp. strain CSCr-3	2.7	14.2	He et al. (2009)
Bacillus sp. JDM-2-1	1.6	1.4	Zahoor and Rehma (2009)
Intrasporangium sp. Q5-1	17	38	Yang et al. (2009)
Pseudomonas sp. G1DM21	7	39	Desai et al. (2008b)
Burkholderia cepacia MCMB-821	19.2	39	Wani et al. (2007)
Brucella sp. DM1	19.2	18	Thacker et al. (2007)
Ochrobactrum intermedium SDCr-5	288	53	Sultan and Hasnain (2007)
Nesterenkonia sp. strain MF2	600	8.3	Amoozegar et al. (2007)
Providencia sp. UTDM314	19.2	64	Thacker et al. 2006
Bacillus sphaericus AND303	15.4	5	Pal and Paul (2004)
Pseudomonad CRB5	10	37	McLean and Beveridge, (2001)

Factors affecting Cr(VI)-reduction

The Cr(VI)-reduction efficiency of strain KSUCr3 was characterized by investigation the effects of initial Cr(VI) concentration (1-10 mM), incubation temperature (25-45ºC), Na_2CO_3 (0-2%), aeration level (shaking with 0-300 rpm), glucose concentration (0-2%), and NaCl concentration (0-25%). Cr(VI)-reduction was studied in aerobic batch cultures. Sterile alkaline medium (100 mL) in 250 mL culture flasks was supplemented with Cr(VI), inoculated from exponential phase bacterial culture and incubated at the appropriate temperature with shaking. Cell-free controls were also used for each Cr(VI)-reduction assay to monitor any abiotic Cr(VI)-reduction. Samples were aseptically drawn at defined times, centrifuged at 7000 x *g* for 10 min and the supernatant analyzed for residual Cr(VI) by using the standard diphenyl carbazide method (Thacker et al. 2007). Furthermore, the effects of other heavy metals including Ni^{2+}, Mo^{2+}, Co^{2+}, Mn^{2}, Zn^{2+}, Cu^{2+} and Pb with final concentration of 1 mM on Cr(VI)- reduction by strain KSUCr3 were also investigated. Alkaline medium (100 mL) in culture flasks was supplemented with Cr(VI) to a final concentration of 8 mM, together with other metals (1 mM) and incubated for 24 hrs at optimum temperature and shaking level. The experiments were performed in triplicate as described above and the mean values were reported.

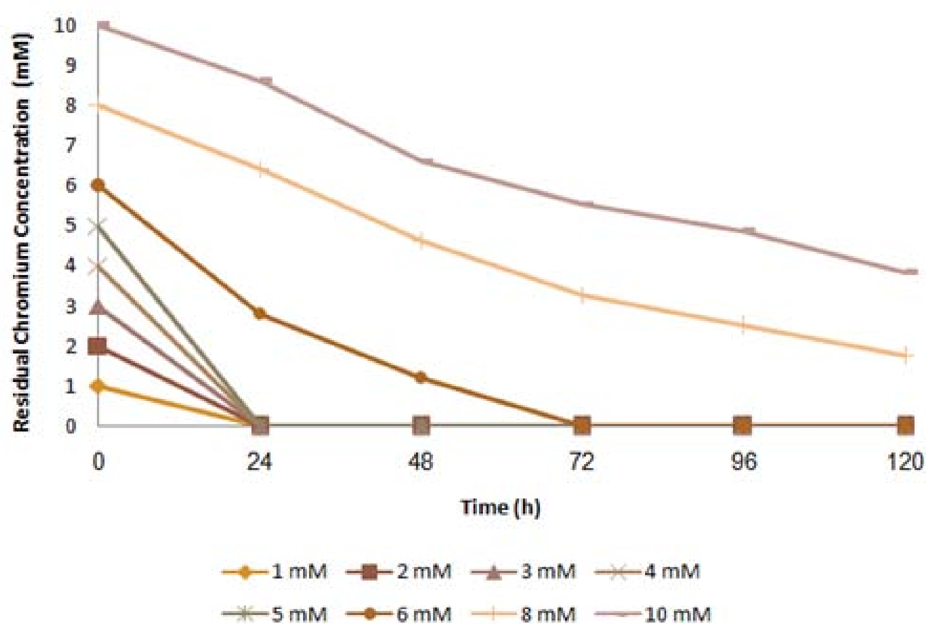

Fig. 1 Chromium reduction by *Amphibacillus* sp. KSUCr3 with different initial chromium concentration (1-10 mM). Results represent the means of three separate experiments. Standard deviation was in range of 1-5.0%.

Repeated detoxification of Cr(VI)

Bacterial culture grown for overnight to an A660 of 1.0 in 100 ml sterile alkaline broth was amended with 1 mM Cr(VI) as final concentration and incubated at 40ºC under gyratory shaking of 100 rpm. Two ml culture suspensions were withdrawn after every 12 hrs of the incubation to measure Cr(VI) remaining as described below and the culture flasks were repeatedly added with increments of 1 mM 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 determination of the bacterial growth, samples (2 ml) were drawn and centrifuged at 7000 g for 10 min at 8ºC. The obtained pellet was resuspended in 2 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) S-diphenyl carbazide (DPC) prepared in acetone (AR) to minimize deterioration. The reaction mixture was set up in 5 ml volumetric flask as follows: 100 µl or 200 µl sample volume was made to 1 ml using distilled water followed by addition of 330 µl of 6M H_2SO_4 and 400 µl of DPC and final volume was made to 5 ml using glass distilled water. Optical density were measured immediately at 540 nm. Calibration curve was made using $K_2Cr_2O_7$ ranged from 0.1 to 1 mM. All experiments were performed in triplicate and the mean values were reported.

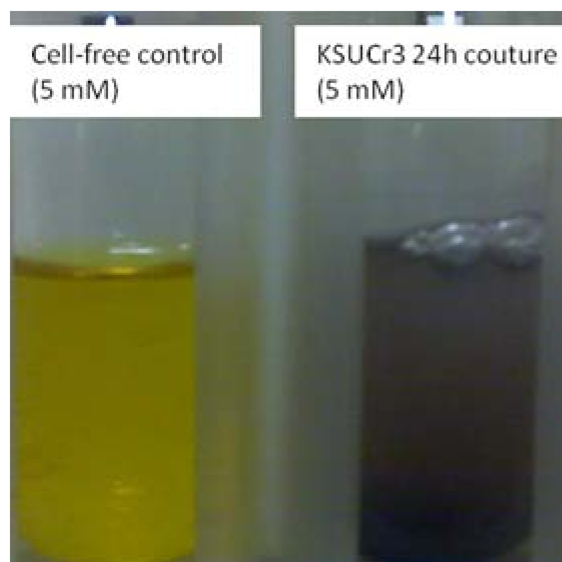

Fig. 2 Chromium reduction by *Amphibacillus* sp. KSUCr3. The strain was grown in alkaline medium containing 1% Na_2CO_3, 10% NaCl, 1% glucose and with chromium concentration of 5 mM and incubated for 24 hrs at 30ºC with shaking (150 rpm). Complete Cr(VI) reduction was achieved within 24 hrs, and white-grayish precipitate visible at the bottom of the bottle (Right). Cell-free control was used to monitor any abiotic Cr(VI)-reduction (Left).

RESULTS AND DISCUSSION

Isolation and identification of Cr(VI) resistant alkaliphilic bacteria

Enrichment and isolation of Cr(VI) resistant halo- alkaliphilic bacteria from Wadi Natrun hypersaline soda lakes resulted in isolation of 12 alkaliphilic-moderately halophilic strains representing morphologically different bacterial colonies (Table 1). All strains were able to tolerate up to 10 mM Cr(VI). With increasing the Cr(VI) concentration up to 100 mM, only three strains (KSUCr1, KSUCr3 and KSUC37) were able to tolerate up to 75 mM Cr(VI) and with MIC value of 80 mM in alkaline medium (pH 10.5) containing 10% NaCl (Table 1). The alkaliphilic strains KSUCr1, KSUCr3 and KSUC37, showing the highest MIC values were further investigated for bioreduction of Cr(VI) in alkaline liquid media. Strain KSUCr3 was shown to be of the highest efficiency in detoxifying chromate, as it could rapidly reduce 5 mM Cr(VI) to a non detectable level over 24 hrs (Table 2). Strain KSUCr3 was 99% identical to *Amphibacillus* sp. based on the 16S rRNA gene analysis and was referred to as *Amphibacillus* sp. KSUCr3, and the sequence was deposited at GenBank with accession no. JF690755. The resistance of *Amphibacillus* sp strain KSUCr3 to K_2CrO_4 is on a very high level, perhaps the highest recorded so far in alkaline medium (pH 10.5), compared to other microorganisms. More importantly, upon optimization (as described below) of the Cr(VI) reduction by *Amphibacillus* sp. strain KSUCr3, Cr(VI) reduction rate reached 237 μMh^{1} which is one of the highest Cr(VI) reduction rates compared to other microorganisms that has been reported so far (Table 3) and thus makes it a suitable candidate for bioremediation. 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 and thereby enhance bacterial tolerance to toxic metals (Margesin and Schinner, 2001; Amoozegar et al. 2007; Horikoshi et al. 2011). Furthermore, *Amphibacillus* sp. strain KSUCr3 showed high tolerance to several other heavy metals including Ni^{2+} (100 mM), Mo^{2+} (75 mM), Co^{2+} (5 mM), Mn^{2+} (100 mM), Zn^{2+} (2 mM), Cu^{2+} (2 mM), and Pb (75 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).

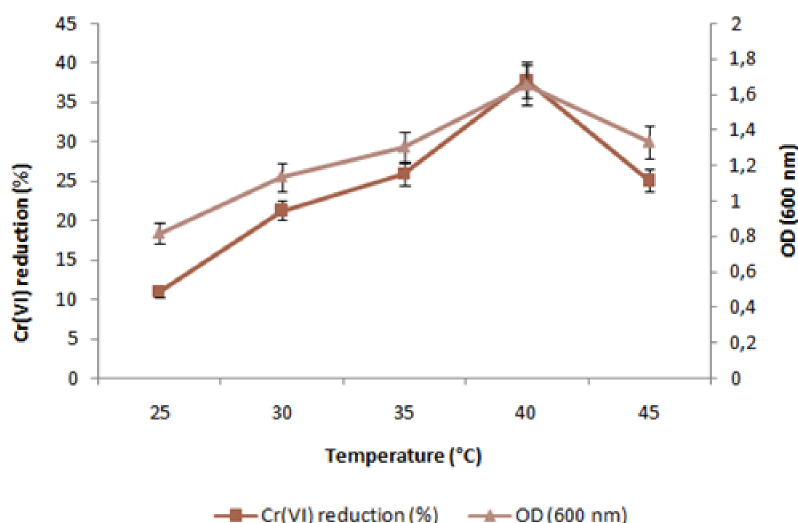

Fig. 3 Effect of temperature on growth and Cr(VI) reduction by the *Amphibacillus* sp. strain KSUCr3. The strain was grown in alkaline medium containing 1% Na_2CO_3, 10% NaCl, 1% glucose and with chromium concentration of 8 mM and incubated for 24 hrs at various temperatures (25-45ºC) with shaking (150 rpm). Results represent the means of three separate experiments, and deviated bars indicated.

Factors affecting Cr(VI) reduction

Effect of initial Cr(VI) concentration. Hexavalent chromate reduction by *Amphibacillus* sp KSUCr3 was monitored at different initial chromium concentrations ranging from 1 to 10 mM as potassium dichromate ($K_2Cr_2O_4$). This bacterium was able to reduce Cr(VI) extremely rapidly. As shown in Figure 1, complete Cr(VI) reduction was achieved within 24 hrs for initial Cr(VI) concentration of up to 5 mM, with white precipitate visible at the bottom of the bottle (Figure 2). In addition, at initial Cr(VI) concentration of 6, 8, and 10 mM, 100%, 51.4% and 44.5% of the chromate was reduced within 72 hrs, respectively. These results clearly indicated the extreme potency of *Amphibacillus* sp. strain KSUCr3 in Cr(VI) reduction in comparison to previously isolated strains. *Microbacterium* sp. completely reduced 20 mg/L of Cr(VI) within 72 hrs (Pattanapipitpaisal et al. 2001). In addition, the *Pseudomonad* strain CRB5 showed complete reduction of 20 mg/L of chromate only after 120 hrs (McLean and Beveridge, 2001). Even recently isolated halophilic *Nesterenkonia* sp. strain MF2 showing the highest tolerance to the chromate (600 mM), for Cr(VI) concentration of 0.4 mM, it took 72 hrs for complete reduction and beyond this Cr(VI) concentration, the complete Cr(VI) reduction was not observed even over 120 hrs (Amoozegar et al. 2007). Furthermore, He et al. (2011) reported isolation of highly Cr(VI) reducing *Lysinibacillus fusiformis* strain. In that study, while the bacterium was able to reduce 1 mM Cr(VI) within 12 hrs, at the initial Cr(VI) concentration of 5 mM Cr(VI), it was reduced to 2.46 mM in 84 hrs.

In all the following experiments, culture medium with initial Cr(VI) concentration of 8 mM was used as a basis for comparison.

Effect of temperature. Temperature is an important parameter that has an significant effect on microbial Cr(VI)-reduction. Bacterial growth and Cr(VI) reduction by the strain KSUCr3 werestudied at various temperatures (25 to 45ºC), (Figure 3).

Chromate reduction was increased with temperature up to 40ºC, which appear to be the optimal temperature for growth of the strain KSUCr3. However, at 45ºC the bacterial growth and chromate reduction were dramatically decreased (Figure 3). It should be noticed that the optimal Cr(VI) reduction depend mostly on the optimum growth temperature. It has been reported the optimal temperature of Cr(VI) reduction to be in the range of 30 to 37ºC (Cheung and Gu, 2007). Maximum Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3 (He et al. 2009) and *Nesterenkonia* sp. strain MF2 (Amoozegar et al. 2007) was found to be 35ºC, whereas reported to be 30ºC for *Bacillus* sp (Wang and Xiao, 1995) and *Pseudomonas* strain CRB5 (McLean et al. 2000). Chromate reductase from thermophhilic *Thermus scotoductus* SA-01 has been recently identified with an optimum temperature of Cr(VI)-reduction at 65ºC (Opperman et al. 2008).

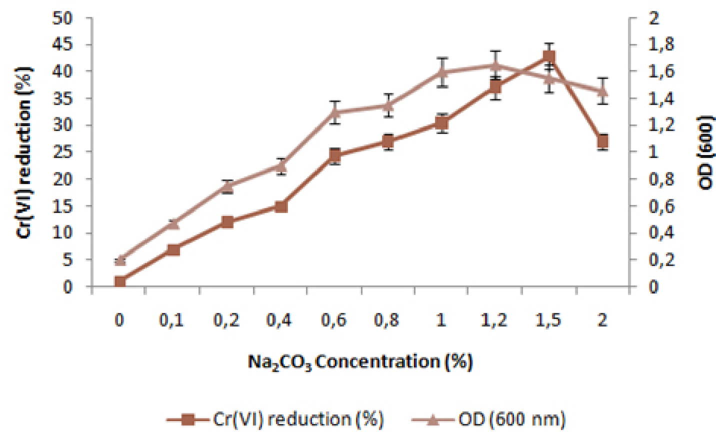

Fig. 4 Effect of Na_2CO_3 concentration on growth and Cr(VI) reduction by *Amphibacillus* sp. KSUCr3. The strain was grown in medium containing different concentration of Na_2CO_3 (0-2), 10% NaCl, 1% glucose and with Cr(VI) concentration of 8 mM, and incubated for 24 hrs at 40ºC with shaking (150 rpm). Results represent the means of three separate experiments**,** and deviated bars indicated.

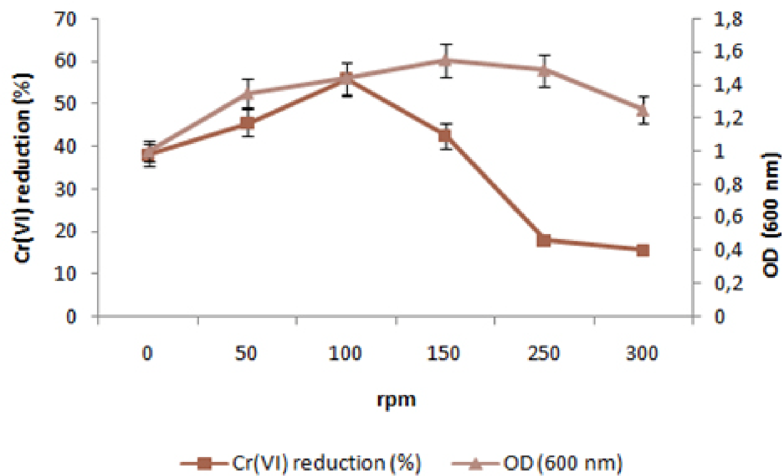

Fig. 5 Effect of aeration level (shaking rpm) on the growth and Cr(VI) reduction by *Amphibacillus* sp. KSUCr3. The strain was grown in alkaline medium containing 1.5% Na_2CO_3, 10% NaCl and with Cr(VI) concentration of 8 mM, and incubated for 24 hrs at 40ºC with shaking (0-300 rpm). Results represent the means of three separate experiments, and deviated bars indicated.

Effect of sodium carbonate. *Amphibacillus* sp. KSUCr3 is an alkaliphilic bacterium showing optimum growth at pH of 9.5 to 10 (data not shown). The effect of Na_2CO_3 concentration on hexavalent chromate reduction as well as growth of *Amphibacillus* sp. KSUCr3 was studied. In absence of Na_2Co_3 (pH around neutral), *Amphibacillus* sp KSUCr3 growth and Cr(VI) reduction drastically decreased, indicating the alkaliphilic nature of the organism. Maximum bacterial growth was seen at 1.2% Na_2CO_3, whereas maximum Cr(VI) reduction (42.9%) was found to be at Na_2CO_3 concentration of 1.5% (Figure 4). It has proved that the presence of sodium ions in the surrounding environment to be essential for effective solute transport through the membranes of alkaliphilic bacteria. 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; Horikoshi et al. 2011).

Effect of aeration level. The effect of aeration level on bacterial growth and Cr(VI) reduction was investigated by incubating the cultures at various shaking level ranged from 0 to 300 rpm. The results presented in Figure 5 revealed that aeration level has a significant effect on the growth and Cr(VI) reduction by *Amphibacillus* sp. KSUCr3. At shaking of 100 rpm the bioreduction was about 1.5 fold higher than that at static conditions, indicating that Cr(VI) reduction by the strain KSUCr3 is occurred under aerobic conditions. However, at higher aeration level the Cr(VI) reduction started to decline. Chromate reduction has been reported by both aerobic (Desai et al. 2008a; Poopal and Laxman, 2009) and anaerobic bacteria (Michel et al. 2001; Chardin et al. 2002). In the presence of oxygen, bacterial Cr^{6+} reduction commonly 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).Cr^{3+} (Cheung and Gu, 2007).

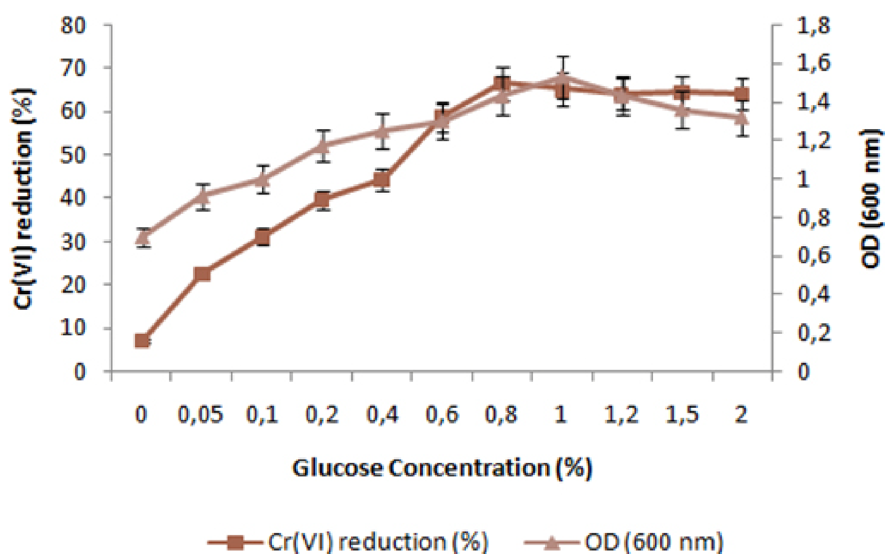

Fig. 6 Effect of glucose concentration on growth and Cr(VI)-reduction by *Amphibacillus* sp KSUCr3. The strain was grown in alkaline medium containing 1.5% Na_2CO_3, 10% NaCl, glucose (0-2%), 10% NaCl and with Cr(VI) concentration of 8 mM, and incubated for 24 hrs at 40ºC with shaking (100 rpm). Results represent the means of three separate experiments, and deviated bars indicated.

Effect of glucose concentration. It has previously been reported that chromate-reducing bacteria may utilize a variety of organic compounds as electron donors for Cr(VI) reduction (Guha 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 6 Cr(VI) reduction was increased dramatically by addition of glucose to the culture medium. Furthermore, Cr(VI) reduction increased with increasing glucose concentration with maximum bioreduction (66.6%) at final glucose concentration of 0.8%. At higher concentration there was no further increase of Cr(VI)-reduction. However, while optimum reduction was

seen at 0.8% glucose, maximum *Amphibacillus* sp KSUCr3 growth was seen at glucose concentration of 1% (Figure 6). These results are in consistent with other reports indicating requirement of glucose as electron donor for Cr(VI)-reduction. For example, Poopal and Laxman, (2009) reported maximum Cr(VI)-reduction by *Streptomyces griseus* in the presence of glucose as electron donor. Glucose has also been reported to act as an electron donor and has been demonstrated to significantly increase Cr(VI) reduction by *Bacillus* sp (Pal et al. 2005; Liu et al. 2006) and *Ochrobactrum* sp. CSCr-3 (He et al. 2009). However, other electron donors like formate, fructose, carbonate and have been also reported to increase Cr(VI) reduction (Myers et al. 2000; Desai et al. 2008b; He et al. 2011).

Effect of NaCl concentration. The results presented in Figure 7 shows the influence of NaCl concentrations on *Amphibacillus* sp. KSUCr3 growth and Cr(VI) reduction. Both bacterial growth and Cr(VI) reduction was increased dramatically by addition of NaCl to the culture medium, with maximum growth and chromium reduction (71.1%) at 10% and 12%, respectively. Hence, the presence of salts in culture medium appeared to be a prerequisite for strain KSUCr3 growth and chromate removal, indicating the halophilic nature of the strain KSUCr3. Amoozegar et al. (2007) reported that complete reduction of 0.2 mM Cr(VI) after 24 hrs by halophilic *Nesterenkonia* sp. strain MF2 was achieved only when the concentration of NaCl increased from 0.1 to 1 M.

Effects of other metals on Cr(VI)-reduction. As other heavy metals can also be present in industrial effluents, effects of other heavy metals on Cr(VI)-reduction by the strain KSUCr3 was also studied in this work. As shown in Figure 8, the presence of 1 mM of Ni^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+} together with Cr(VI) in the culture medium slightly increased Cr(VI)-reduction, whereas Zn^{2+}, Mo^{2+}, and Pb^{+} had no effect on Cr(VI)-reduction by strain KSUCr3. Stimulatory effect of Cu^{2+}, Co^{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+} to be related to electron transport protection or acting as electron redox center and, in some cases, as a shuttle for electrons between protein subunits (Abe et al. 2001; Camargo et al. 2003; He et al. 2009).

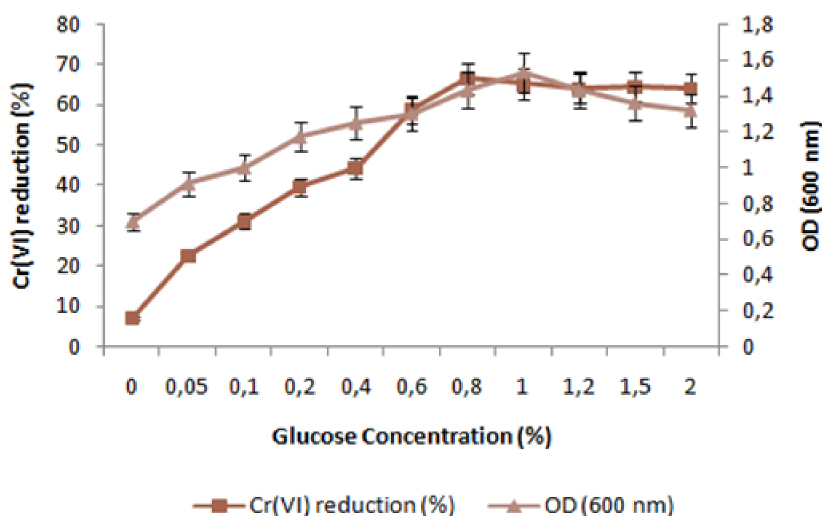

Fig. 7 Effect of NaCl concentration on growth and Cr(VI)-reduction by *Amphibacillus* sp. KSUCr3. The strain was grown in alkaline medium containing 1.5% $NaCO_3$, 0.8% glucose, NaCl (0-2%), and with Cr(VI) concentration of 8 mM and incubated for 24 hrs at 40ºC with shaking (100 rpm). Results represent the means of three separate experiments, and deviated bars indicated.

Repeated detoxification of Cr(VI) by *Amphibacillus* sp KSUCr3

The chromate reducing ability of *Amphibacillus* sp KSUCr3 was tested by six repeated additions of 1 mM K_2CrO_4 every 12 hrs. *Amphibacillus* sp. KSUCr3 exhibited complete reduction of 1 mM Cr(VI) up to five consecutive inputs as observed from Figure 9 andcould still reduce about 67% of the sixth addition of 1 mM Cr(VI) in 24 hrs. The ability of *Amphibacillus* sp KSUCr3 to repeatedly reduce hexavalent chromium without any amendment of nutrients, suggests its potential application in continuous bioremediation of Cr(VI).

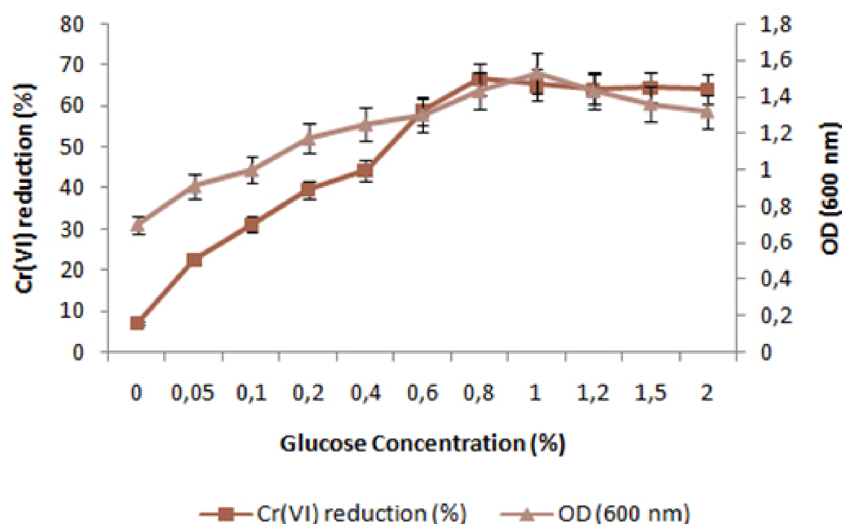

Fig. 8 Effects of heavy metals supplementation on Cr(VI)-reduction yield by *Amphibacillus* sp. KSUCr3. The strain was grown in alkaline medium containing 15% $NaCO_3$, 0.8% glucose, 1.2% NaCl, and with Cr(VI) concentration of 8 mM together with different heavy metals (1 mM). The cultures were grown at 40ºC for 24 hrs with shaking at 100 rpm. Results represent the means of three separate experiments, and deviated bars indicated.

CONCLUDING REMARKS

The present study demonstrates isolation of extremely potent Cr(VI) reducing halo-alkaliphilic *Amphibacillus* sp. strain KSUCr3 from hypersalin soda lake located in Wadi Natrun valley, Egypt. KSUCr3 can effectively reduce Cr(VI) to Cr(III) under alkaline condition, high sodium chloride concentration, wide range of temperatures and high Cr(VI) concentrations (1-10 mM). Under optimum Cr(VI) reduction conditions, *Amphibacillus* sp. strain KSUCr3, Cr(VI) reduction rate reached 237 μMh[1] which is one of the highest Cr(VI) reduction rate, particularly under alkaline conditions and high salt concentration, compared to other microorganisms that has been reported so far. In addition, KSUCr3 showed resistance to several other heavy metals including Ni^{2+}, Mo^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb, and since most polluted environments contain mixed waste, individual bacterial strain with enhanced capacities for remediating multiple pollutants is highly desirable. Furthermore, the isolate, *Amphibacillus* sp. strain KSUCr3, 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 also revealed the possible isolation of potent heavy metal resistant bacteria from extreme environment such as hypersaline soda lakes. Purification and characterization of chromium reductase of *Amphibacillus* sp. strain KSUCr3 are on progress and to be published elsewhere.

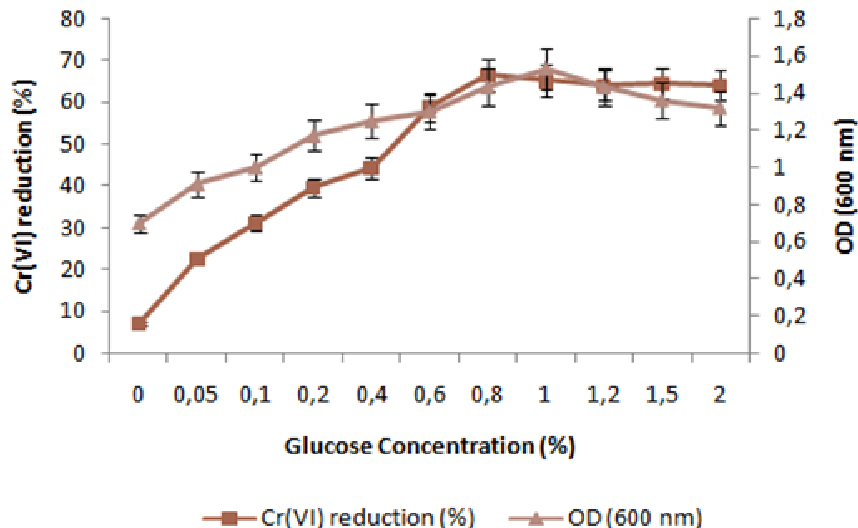

Fig. 9 Repeated detoxification of 1 mM Cr(VI) by *Amphibacillus* sp. KSUCr3 under the optimum conditions of Cr(VI) reduction.

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