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Reduction of Hexavalent Chromium by a Potent Novel Haloalkaliphilic *Nesterenkonia* sp strain NRC-Y Isolated from Hypersaline Soda Lakes

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Abstract

We reported a novel haloalkaliphilic bacterial strain NRC-Y with remarkable hexavalent chromium reduction efficiency and studied its detoxification mechanism. Strain NRC-Y was identified based on 16S-rDNA sequence analysis as *Nesterenkonia* sp. Strain NRC-Y exhibited high Cr^{6+} reduction efficiency under a wide range of sodium chloride concentration, pH, shaking velocity, and temperature, showing maximum Cr^{6+} reduction at 6% NaCl, pH 9, 150 rpm and 40ºC, respectively. In addition, strain NRC-Y could grow and reduce Cr^{6+} effectively in the presence of a variety of heavy metals and oxyanions. The co-existence of Cd^{2+} and Zn^{2+} caused a slight decrease in the Cr^{6+}reduction, while Fe^{3+} and Cu^{2+} ions significantly increased the Cr^{6+} reduction by 1.7- and 1.8-fold, respectively. Among the tested electron donors, maximum Cr^{6+} reduction was observed using glycerol. Cell fractionation experiments proved that Cr^{6+} bioremoval occurred because of chromate enzyme activity, which was detected mainly in the bacterial cell membranes (73.2%) and cytoplasm (22.6%). SEM–EDX analyses confirmed the biosorption of chromium species into the cells. Strain NRC-Y exhibited a potent Cr^{6+} bioreduction efficiency, which could reduce up to 800 mgL^{-1} within 24 h. Therefore, strain NRC-Y is a promising candidate in the bioremediation of Cr^{6+} contaminated sites, particularly under harsh conditions. Further investigation using chromium containing industrial waste is recommended

Keywords: Bioremediation; Extremophiles; Haloalkaliphiles; Hexavalent chromium

1. Introduction

Chromium (Cr) is one of the main toxic heavy metals, which has a wide range of industrial applications such as metal electroplating, textile dyeing, tanneries, pigment synthesis, fertilizers, and dye industries [1, 2, 3]. Cr can occur in different oxidation states, of which trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) are the most thermodynamically stable [4]. However, Cr^{6+} has about 100 and 1000 times more toxic and mutagenic effects on all living systems than Cr^{3+}, respectively [5, 6]. The high toxicity of Cr^{6+} is because of its high solubility and permeability, which can cross the cell membranes and generate intracellular reactive oxygen radicals that cause severe oxidative damage to cell proteins, DNA, and other metabolites [6, 7]. Thus, Cr^{6+} can cause severe health problems, including skin ulcers, allergies, respiratory tract disorders, irritation, and cancer [9]. Therefore, Cr^{6+} is one of the major sources of pollution that seriously affects the environment and biological systems and has been classified as a class A carcinogenic hazard by USEPA [3, 10].

In contrast, Cr^{3+} is much less toxic, mobility, and bioavailability, rapidly forming insoluble hydroxide/oxides in the environment [11, 12]. Hence, Cr^{6+} transformation to Cr^{3+} is considered an effective approach for the remediation of chromium-contaminated environments. Furthermore, compared to the physiochemical remediation approaches, bioremediation of Cr^{6+} using microorganisms is attracting more attention and is given priority because of its high efficiency, biomass reusability, cost-effectiveness, and environment-friendly approach generating no secondary pollution [13, 14].

Most of the reported Cr^{6+}reducing bacteria are neutrophiles, growing optimally in neutral conditions, such as *Acinetobacter*, *Stenotrophomonas*,

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Ochrobactrum, Bacillus, Vigribacillus, Cellulosimicrobium, Pseudochrobactrum, and others [3, 14, 15, 16]. However, the high salinity and alkalinity of some chromium-contaminated areas represent an additional problem that can adversely affect the growth and detoxification of the bacterial cells applied for chromium bioremediation [17]. Furthermore, only few Cr^{6+} reducing bacteria were reported that can grow and function in environments with high pH or high salt concentrations and rare reports for the combined harsh extreme environments. Hence, microorganisms that can reduce Cr^{6+} under high alkaline and high salt conditions are of great importance for effective Cr^{6+} bioremediation of industrial effluents and contaminated soil characterized by high pH and salt concentrations [18, 19]. Therefore, there is continuous research for Cr^{6+} reducing candidates that can function under such harsh conditions. Hypersaline alkaline soda lakes are usually enriched by haloalkaliphilic microorganisms that can be potential candidates for Cr^{6+} reduction under high alkaline/salt conditions. Wadi Al-Natrun lakes (Egypt) are characterized by very high salinity (up to 15%–25%) and alkalinity (pH 10–12). Therefore, they are a suitable environment for the isolation of haloalkaliphilic microorganisms. In the present study, we report isolation and characterization of a haloalkaliphilic bacterium with remarkable Cr^{6+} detoxification efficiency from hypersaline soda lakes, optimization of the reduction efficiency, the kinetics of the bacterial reduction process, and investigation of the detoxification mechanism.

2. Materials and Methods

2.1. Soil and water sample

Wadi Al-Natrun valley is characterized by several hypersaline soda lakes located about 100 km northern Cairo City (Egypt). This valley extends between latitudes 30º 15′N and longitude 30º 30′E east and is 23 m below sea level. Water and sediment samples were collected from different sites in sterile containers, maintained in an icebox, and transferred to the laboratory within 3–5 h.

2.2. Isolation of Cr^{6+} reducing haloalkaliphilic bacteria

A rich alkaline medium supplemented with K_2CrO_7 (Cr^{6+}) was used for enrichment and isolation of Cr^{6+} resistant haloalkaliphilic bacteria. The alkaline agar medium (pH 10.5) contained (gL^{-1}): 5 g casamino acids, 5 g peptone, 5 g yeast extract, 10 g glucose, 100 g NaCl, 10 g Na_2CO_3, 300 µL trace elements solution, K_2CrO_7 (100–1000 mgL^{-1}) and agar 15.0 [20]. Briefly, the sediment samples (5 g) were resuspended in 50 mL of 50 mM Tris buffer (pH 10) containing 10% (w/v) sodium chloride; and stirred the mixtures for 30 min using magnetic stirring. Then, the suspensions were serially diluted up to 10^{-5} and spread 200 µL of each dilution on the agar medium amended with different concentrations of Cr^{6+} ranging from 100 to 1000 mgL^{-1} and incubated aerobically for five days at 35°C. Pure isolates were obtained by repeatedly re-streaking the morphologically distinct colonies on fresh agar plates. The chromium bioremoval efficiency of the isolated bacterial strains was determined by growing the isolates in the same alkaline broth medium supplemented with 100 mgL^{-1} of $Cr^{6+}$and incubated for 48 h at 35 °C in a shaking incubator (100 rpm). After that, the remaining Cr^{6+} in the culture filtrate was determined after 24 and 48 h incubation period, as described below. One isolate (strain NRC-Y) showing the highest Cr^{6+} tolerance and reduction efficiency was selected for further investigation.

2.3. Bacterial identification

Strain NRC-Y was identified based on the 16S rDNA gene sequence analysis [20, 21]. Briefly, strain NRC-Y was propagated in an alkaline liquid medium (described above) for 24 h, and the cells biomass was collected by centrifugation and washed three times with sterile saline solution. Then, the total bacterial DNA was extracted and purified from the cell biomass using Quick-DNA™ Miniprep Plus Kit (ZYMO Research, USA), according to the manufacturer's procedures. Next, the 16S rDNA gene was amplified using Polymerase Chain Reaction (PCR) in a final reaction volume of 25 µL containing: 12.5 µL MyTaq Red Mix (Meridian Bioscience, USA), 4 µL DNA template, 1 µL of forward and reverse primers (10 Pico mol) and 6.5 µL nuclease-free water. The universal eubacterial primers included forward primer 16F27: 5′-AGAGTTTGATCC TGGCTCAG-3′) and reverse primer 1525R: (5′-AAG GAG GTG ATC CAG CCG CA-3′ [22]. PCR reaction was run in a DNA thermal cycler (GeneAmp® 9700) for 35 cycles under the following thermal profile: Initial denaturation at 94ºC (6 min), denaturation at 94ºC (45 sec), annealing temperature at 56ºC (45 sec), extension at 72ºC (1 min) and final extension at 72ºC (7 min). The PCR products was analyzed on 1.5% agarose gel electrophoresis and purified from the gel using a QIAquick Gel Extraction Kit (Qiagen, USA) following the manufacturer's procedures, and sequenced using an automated sequencer. The obtained 16S rDNA sequences were subjected to the online BLAST analysis www.ncbi.nlm.nih.gov/BLAST [23].

2.4. Optimization of parameters for Cr^{6+} reduction

For optimization of the bacterial growth and Cr^{6+} reduction efficiency of the isolated strain NRC-Y, investigation of several physiological factors were carried out, including NaCl concentration (0%–16%), incubation temperature (30–45°C), aeration level (0–200 rpm), and medium pH (5–12), co-existence of a variety of heavy metals (Fe^{3+}, Ni^{2+}, Co^{2+}, Pb^{+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, $^{+}Mo^{2+}$) and oxyanions (HCO_3^-, NO_3, SO_4^- and NO_3^-) at a final concentration of 50 mgL^{-1}. The experiments were carried out using the rich alkaline medium supplemented with 300 mgL^{-1} Cr^{6+}. Briefly, inoculum culture was prepared to the mid of log phase (1×10^8 cells mL^{-1}), then used to inoculate 50 mL of the liquid medium in 250 mL culture flasks and incubated for 24 h. An uninoculated medium was included to monitor any possible abiotic Cr^{6+} reduction. After that, the bacterial growth and residual Cr^{6+} concentrations were determined [24].

2.5. Effect of electron donors on Cr^{6+} reduction

The influence of different external electron donors (EDs) on the efficiency of Cr^{6+} reduction by strain NRC-Y was studied using whole resting cells [25]. Briefly, cell biomass was harvested from 100 mL bacterial culture prepared under the determined optimum conditions described above, washed three times with 50 mM Tris buffer (pH 7), and resuspended in 10 mL of Tris buffer. After that, 1.0 mL of cells suspensions was added to 4 mL of 50 mM Tris buffer containing 300 mgL^{-1} Cr^{6+}, 50 mgL^{-1} Cu^{+2}, and 1% of different EDs (nicotinamide adenine dinucleotide (NADH), glycerol, glucose, fructose, sodium acetate, lactose, and sucrose). In addition, a reaction mixture with no Eds was set up as a control. Then, the reaction mixtures were incubated at 40°C in a shaking water bath (150 rpm) for 6 h. After the incubation period, the samples (1.0 mL) were withdrawn from the reaction mixtures, centrifuged, and the remaining Cr^{6+} was determined using the DPC method described below.

2.6. Kinetics studies

Under the optimized conditions, the bacterial growth and Cr^{6+} bioreduction were investigated at different Cr^{6+} initial concentrations (200–800 mgL^{-1}). First, the bacterial strain was sub-cultured several times in Cr^{6+} free agar medium to abstract any endogenous Cr. Then, inoculum cultures were prepared by transferring few colonies from agar plates into 50 mL liquid media in 250 ml flasks with and without Cr^{6+}, respectively, and incubated them overnight at 40°C and 100 rpm. These cultures were used to inoculate (2.0%) 100 mL of the liquid media (in 500 mL culture flasks) containing different Cr^{6+} concentrations, including 0.0, 200, 300, 400, 600, and 800 mgL^{-1}. A culture medium without bacterial inoculation was included to monitor any abiotic Cr^{6+} reduction. After that, samples (2.0 mL) were withdrawn from each culture at 2 h intervals up to 48 h. The collected samples were centrifuged at 15000 rpm for 10 min; and the remaining Cr^{6+} concentrations in supernatants were determined. To measure bacterial growth, the cells pellets were resuspended in 2.0 mL of sterile distilled water, and the optical density (600 nm) was measured against distilled water as blank [24].

2.7. Micro characterization of the bacterium

A SEM (QUANTA FEG250) with EDS (scanning electron microscopy-energy dispersive X-ray) available at the central laboratory facility (National Research Centre, Cairo, Egypt) was used to observe the strain NRC-Y surface morphology features and elemental composition before and after bacterial Cr^{6+} reduction [26].

2.8. Determination of the chromate reductase site

In order to investigate the exact site of chromate reductase produced by strain NRC-Y, different cells fractions were prepared [16, 20, 27]. First, the strain NRC-Y was propagated in 200 mL of the alkaline liquid medium using the optimum culture conditions. After that, the culture supernatant (CS) was collected by centrifugation, and washed the cell pellets were washed thrice with Tris buffer and resuspended in 20 mL of buffer, giving a final cell concentration of 0.1 g/mL (fresh wet weight). The cell suspension was subjected to ultrasonic disruption for 10 min (Ultrasonic Probe, Cole-Parmer) using the amplitude of 35%, 50 W with 10 seconds pulses, and 10 seconds off-mode. Then, the broken cells representing cells lysate (CL) was centrifuged at 15000 rpm for 10 min, and the cell-free extract, was collected representing the cytoplasmic fraction (CP). The membranous cells fraction (MB) was collected and resuspended in 20 ml Tris buffer. The reductase activity of all fractions was determined, including CS, CL, cytoplasm (CP), a membranous fraction (MB) besides whole cells (WC) suspension.

2.9. Assay of chromate reductase activity

The chromate reductase activity was measured as previously reported with some modification [20, 27]. The reaction substrate was prepared by dissolving 100 mgL^{-1} Cr^{6+}, 50 mgL^{-1} Cu^{2+} and 1.0 % glycerol in Tris buffer (50 mM, pH 7). Then, 1.0 ml enzyme preparations (CS, CL, CP, MB, and WC) were added to 4 mL substrate and incubated them for 6 h at 40°C in a shaking water bath (150 rpm). Boiled enzyme preparations were set up in the experiment as a control.

After the incubation period, 2 mL of the reaction mixtures were withdrawn, centrifuged, and the remaining Cr^{6+} concentrations were measured.

2.10. Determination of Cr^{6+} concentration

The concentration of Cr^{6+} was measured calorimetrically, according to Bartlett and James [28], using 1,5-diphenylcarbazide (DPC). Briefly, 300 µL samples were added to 5 ml of distilled water in glass test tubes. Then, 1.0 mL of DPC solution (0.25% [w/v], prepared in acetone), was added followed by addition of 50 µL phosphoric acid (85%). After incubating the reaction mixtures for 10 min at room temperature, the developed color absorbance was measured at 540 nm. The calibration curve was developed using $K_2Cr_2O_7$ at concentrations between 10 to 300 µgmL^{-1}.

2.11. Statistical analysis

The experiments and assays were carried out in triplicate. Microsoft Excel program were applied to illustrate the figures and estimate the standard deviations for each experiment, shown as error bars in the figures.

3. Results and Discussion

3.1. Isolation and identification of Cr^{6+} reducing haloalkaliphilic bacterium

Chromate-reducing haloalkaliphilic bacteria were isolated from hypersaline soda lakes in Wadi Al-Natrun valley (Egypt) by aerobic cultivation in an alkaline medium amended with different concentrations of Cr^{6+}. In addition, the capability of the isolated Cr^{6+} tolerating bacterial strains for Cr^{6+} reduction in broth medium supplemented with Cr^{6+} was investigated. Among the isolated bacteria, strain NRC-Y showed the highest Cr^{6+} resistant level with a MIC of 60 mM and highest reduction efficiency and therefore was selected for further investigation. Strain NRC-Y was identified by sequencing and analysis of the 16S rDNA, showing the highest similarity (97.6%) with *Nesterenkonia* sp., therefore it was designed as *Nesterenkonia* sp. strain NRC-Y, and the sequence was deposited in the GenBank (accession no. **MZ054157**).

3.2. Optimization of Cr^{6+} reduction

Optimization of Cr^{6+} reduction by *Nesterenkonia* sp. strain NRC-Y was carried out by investigating the effect of some physiological factors, including sodium chloride concentration, medium pH, aeration level, incubation temperature, co-existence of different metals and oxyanions, and variety of external EDs.

3.2.1. Influence of sodium chloride

Investigation of the effect of NaCl concentration on growth and Cr^{6+} reduction by *Nesterenkonia* sp. strain NRC-Y (**Fig. 1)** indicated that the bacterium gave optimal growth and Cr^{6+} reduction at 10.0% and 8.0%, respectively, revealing that *Nesterenkonia* sp. NRC-Y is a moderately halophilic bacterium. In addition, strain NRC-Y grew well and reduced Cr^{6+} effectively in a wide range of NaCl concentrations (2.0%–12%). This finding is relatively similar to the results reported for *Nestetenkonia* sp. strain MF2 [29] and *Vigribacillus* sp. [30], where maximum Cr^{6+} was observed at 6.0% NaCl. However, the optimal NaCl for Cr^{6+} reduction by strain NRC-Y is much higher than *P. saccharolyticum* LY10 [19] and *Cellulosimicrobium funkei* strain AR8 [17], which showed maximum Cr^{6+} reduction at 2.0 and 3.0% NaCl, respectively.

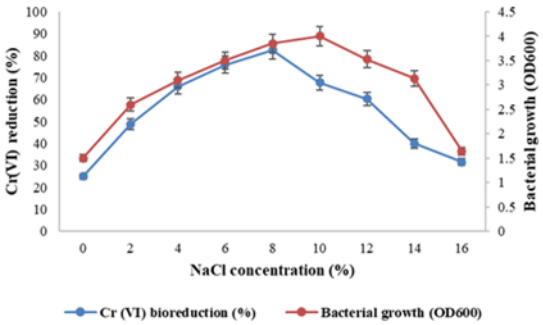

Fig. 1 Influence of different sodium chloride concentrations on bacterial growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y. Error bars represented standard deviation.

Na^+ and K^+ play an essential role in ionic pumps and the activity of several enzymes in halophilic microorganisms thus enhancing the microbial resistance/reduction efficiency of heavy metals [17]. This finding is in contrast to the usually observed adverse effect of high sodium chloride concentrations on microbial growth of most microorganisms owing to high ionic stress [30, 31]. The Cr^{6+} reduction efficiency of strain NRC-Y in the presence of high NaCl concentrations is a unique characteristic, and hence it is a promising candidate for Cr^{6+} bioremediation under saline conditions.

3.2.2. Influence of pH

The pH is a vital factor for efficient Cr^{6+} bioreduction in bioremediation, as it significantly affects the availability of metal ions and the ionic state of the enzymes' active sites, and chromate reductase activity [3]. The influence of the initial pH of the

culture medium on growth and Cr^{6+} reduction by *Nesterenkonia* sp. strain NRC-Y was conducted in media with pH ranging from 5 to 12. As shown in **Fig. 2**, strain NRC-Y showed good cell growth and high Cr^{6+} reduction efficiency in a wide pH range, from pH 7 to pH 10. However, optimal growth and Cr^{6+} reduction was observed at pH 9.0, showing a 66.2% reduction of the initial chromium concentration (100 mgL^{-1}). The optimum pH of cell growth of strain NRC-Y in an alkaline medium showed that this strain is an alkaliphilic bacterium. At pH 5 and 11 the Cr^{6+} bioremoval was drastically inhibited to 14.4% and 13.4%, respectively, which is mostly because of the unsuitable pH that adversely influenced chromate reductase ionization and restraining the cell metabolism and bacterial growth [32]. Moreover, the bacterial sulfate transport system, which plays a significant role in Cr^{6+} transportation into the cells, is inhibited at a low pH value [33]. It has been reported that most bacteria exhibited maximum Cr^{6+} reduction under neutral conditions because of the fact that optimal pH for most enzymatic activities and cell growth are usually close to pH 7 [34]. The microbial adaptability for Cr^{6+} detoxification under alkaline conditions is important because of the fact that several chromium-containing industrial effluents and contaminated sites have high alkalinity [17]. Hence, the high efficiency of Cr^{6+} reduction in a wide pH range and alkaline conditions of *Nesterenkonia* sp. strain NRC-Y is a unique feature and shows that strain NRC-R can be applied for Cr^{6+}bioremediation of alkaline contaminated sites besides neutral one.

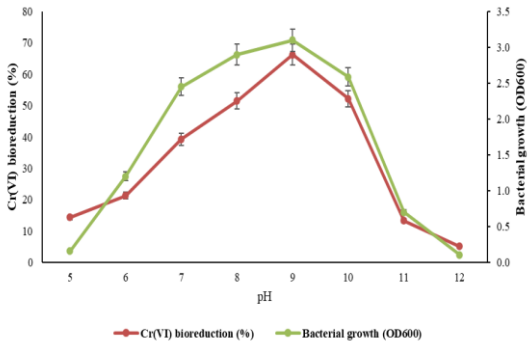

Fig. 2 Influence of medium initial pH on bacterial growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y. Error bars represented standard deviation.

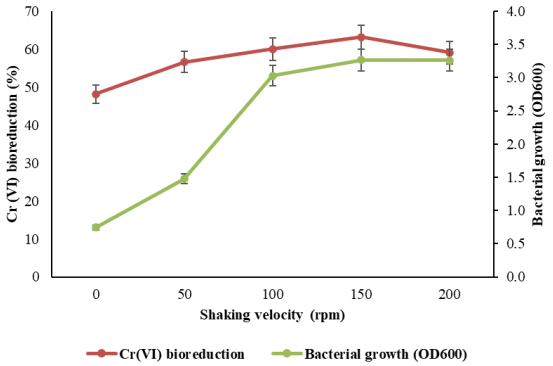

Fig. 3. Influence of the culture shaking velocity on bacterial growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y. Error bars represented standard deviation.

3.2.3. Influence of aeration level

Variation in the agitation level of the liquid culture has been reported to affect the efficiency of microbial Cr^{6+} bioreduction [35, 36]. The effect of aeration level on bacterial growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y was studied by incubating the cultures in shaking incubators with different agitation velocities. The results showed that the agitation speed of the cultures had a substantial effect on cell growth and Cr^{6+} bioreduction by strain NRC-Y, exhibiting optimal bacterial growth and Cr^{6+} reduction (63.2%) at 150 rpm (**Fig. 3**). There was about 1.3-fold increase in the Cr^{6+}bioreduction efficiency compared to static conditions, revealing that aerobic conditions were favorable for Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y. A relatively similar influence of varied shaking levels on Cr^{6+} reduction has also been reported for other microorganisms [16, 36, 37]. It has been reported that increasing the agitation speed of the liquid cultures leads to an increase of the oxygen pressure and reduction of the partial pressure of the dissolved carbon dioxide, thus affecting the metabolic activities and cell growth [36]. In the presence of oxygen, Cr^{6+} is reduced to unstable Cr^{5+} or Cr^{4+} intermediates, which are further reduced to the thermodynamically stable Cr^{3+} [3, 37].

3.2.4. influence of incubation temperature

The incubation temperature is one of the major factors affecting microbial metabolic activities, growth, and enzymes production. The optimal temperature for Cr^{6+} reduction is varied and depends on the microorganisms [3, 39]. The influence of different incubation temperatures ranging from 30 to 45ºC, on bacterial growth and Cr^{6+} bioreduction by

Nesterenkonia sp. strain NRC-Y was investigated at a Cr^{6+} concentration of 100 mg/L and pH 9.0 in a shaking incubator (150 rpm). As shown in **Fig. 4**, it was observed that both *Nesterenkonia* sp. strain NRC-Y growth and Cr^{6+} reduction were increased with increasing the incubation temperature up to 40ºC. Further increase of the temperature to 45ºC led to a severe decline of growth and Cr^{6+} reduction to 18.4%, which is mostly because of the loss of cell viability and metabolic activity at higher incubation temperatures [30, 35, 36]. In addition, at low incubation temperature, the fluidity of the bacterial cell membranes is significantly decreased, which adversely affect the efficiency of the cellular transport systems, and hence the nutrients enter the bacterial cell at a slower rate than that required to support the cell growth [37, 39].

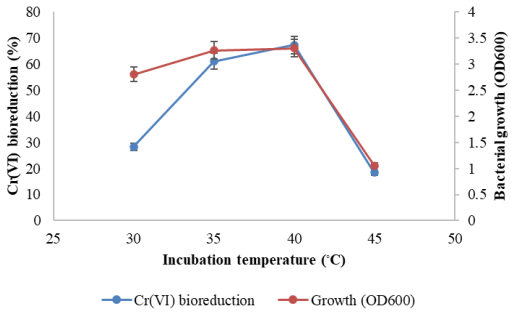

Fig. 4 Influence of incubation temperature on the bacterial growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y. Error bars represented standard deviation.

3.2.5. Influence of co-existing ions

The chromium-containing industrial effluents may contain other heavy metals, therefore, in the present work, the co-existence of different metals and oxyanions on bacterial growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y was studied (**Fig. 5**). The results showed that strain NRC-Y showed good bacterial growth and Cr^{6+} reduction in most tested heavy metals. Compared to the control, Co^{2+} and Ni^{2+} caused a slight enhancement of the Cr^{6+} reduction from 50.6% to 60.2% and 61.8%, respectively. In addition, Fe^{3+} and Cu^{2+} ions significantly increased the Cr^{6+} reduction by about 1.7- and 1.8-fold, respectively, which showed that they might act as co-factors for chromate reductase of *Nesterenkonia* sp. strain NRC-Y [32]. However, in Cd^{2+} and Zn^{2+}, the Cr^{6+} reduction was slightly decreased from 50% (control) to 39.1% and 41.2%, respectively. The decrease in Cr^{6+} reduction in the presence of Zn and Cd presumably because of the high toxic effect of these metals on the bacterial cells and inhibition of the chromate reductase enzyme responsible for Cr^{6+} bioreduction [16, 40]. A similar pattern of cadmium effect on Cr^{6+} reduction was previously reported for *Bacillus* sp. CRB-B1 [16] and *Exiguobacterium mexicanum* [36].

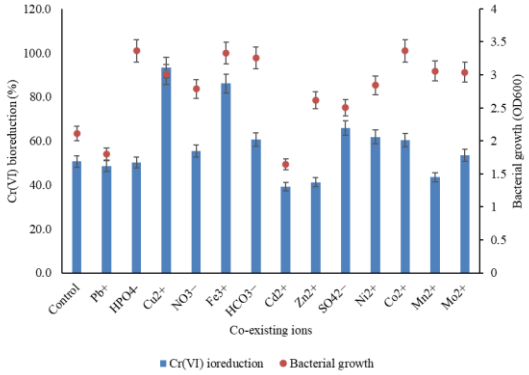

Fig. 5. Effect of co-existing ions on growth and Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y. Error bars represented standard deviation.

Oxyanions such as HPO_4^-, NO_3^-, SO_4^{2-}, and HCO_3^- are widely present in the groundwater and various industrial wastes. Such oxyanions can act as competing electron acceptors, adversely affecting the Cr^{6+} reduction process [16, 41]. The results shown in **Figure 5** revealed that *Nesterenkonia* sp. strain NRC-Y grew and reduced Cr^{6+} effectively in the presence of the tested oxyanions, including NO_3^-, SO_4^{2-}, and HCO_3^-. In previous reports, Cr^{6+} reduction by several bacteria was inhibited by NO_3^- such as in *Pannonibacter phragmitetus* LSSE-09 [42], *P. aeruginosa* PCN-2 [41], and *Bacillus* sp. CRB-B1 [16].

3.2.6. Influence of EDs

Different EDs influence on Cr^{6+} bioreduction by *Nesterenkonia* sp. strain NRC-Y was investigated using resting cells at 300 mgL^{-1} initial Cr^{6+} concentration for 8 h. The results showed that EDs played an essential role in Cr^{6+} reduction by strain NRC-R which confirmed that the Cr^{6+} removal by *Nesterenkonia* sp strain NRC-Y occurred because of enzymatic activity rather than an activity absorption bioprocess [25]. Maximum Cr^{6+} reduction (100%) was observed using glycerol as ED followed by fructose (95.1%), NADH (76.3%), glucose (67.6%), sucrose (34.8%), lactose (35.3%) and sodium acetate (32.4%), respectively (**Fig. 6**). Thus, glycerol was the best ED

among the tested carbon source and was included in the culture medium in the next investigations. Similarly, glycerol was reported to be the best ED for Cr^{6+} bioreduction by *Bacillus* sp. M6 [25] and *O. oncorhynchi* W4 [15]. However, other carbon sources were also reported as the best EDs for other bacteria, such as glucose for *B. amyloliquefaciens* [37], sodium lactate for *P. umsongensis* CY-1 [43], and fructose for Cr^{6+} reduction by *E. mexicanum* [36].

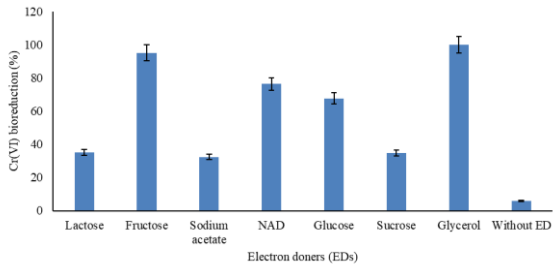

Fig. 6. Effect of different electron donors (EDs) on the Cr^{6+}reduction by *Nesterenkonia* sp. strain NRC-Y. Error bars represented standard deviation.

3.2.6. Kinetics of growth and Cr^{6+} bioreduction

Kinetics of growth and Cr^{6+} reduction by *Nesterenkonia* sp. strain NRC-Y was studied at various initial Cr^{6+} concentrations (0.0–800 mg/L) under the determined optimum conditions, including incubation temperature at 40°C, pH 9, 8.0% NaCl, 50 mg/L $CuSO_4$, 1.0% glycerol and shaking velocity of 150 rpm. The results shown in **Fig. 7a** indicated that Cr^{6+} had no significant effect on the bacterial growth, which increased exponentially in all Cr^{6+} concentrations (0–800 mg/mL) up to 16 h followed by a stationary growth phase up to 24 h. In addition, the results shown in **Fig. 7b** revealed that *Nesterenkonia* sp. strain NRC-Y reduced Cr^{6+} effectively in a short time, even at high Cr^{6+} concentrations. After 12 h of incubation, the remaining Cr^{6+} concentrations were 41.0, 59.3%, 66.1.%, 77.8%, and 80.5% for initial Cr^{6+} concentrations of 200, 300, 400, 600, and 800 mgL^{-1}, respectively. After 20 h, 200–300 mg/L Cr^{6+} was completely reduced, and the remaining Cr^{6+} for 400, 600, and 800 mgL^{-1} were 10.5%, 14.5%, and 33.6%, respectively, which were completely reduced after 24 h.

The results clearly showed the remarkable Cr^{6+} reduction efficiency of *Nesterenkonia* sp. strain NRC-Y in compared to previously reported microorganisms. For instance, *Cellulosimicrobium* sp. needed 96 h to achieve 84.62% and 62.28% reduction at 200 and 300 mgL^{-1} Cr^{6+} concentrations, respectively [44].

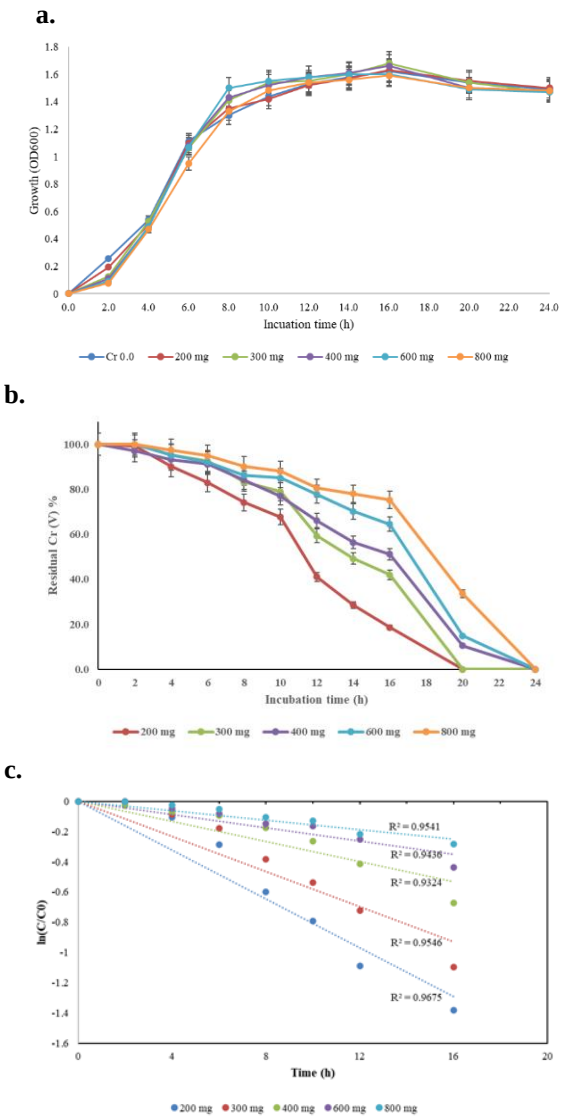

Fig. 7 The growth curves of *Nesterenkonia* sp. strain NRC-Y (**a**), Cr^{6+} reduction (**b**), and the Kinetics of Cr^{6+}reduction (**c**), at different initial Cr^{6+}concentrations.

Bacillus sp. M6 reduced 45.9% of Cr^{6+} concentration of 200 mg/L within 60–72 h [25]. Tan et al reported that *Bacillus* sp. CRB-B1 remove 43.1% of 300 mgL^{-1} Cr^{6+} after 48 h [25]. *Stenotrophomonas maltophilia* required 120 h to reduce 92% of Cr^{6+} at a concentration of 500 mgL^{-1} [45]. *E. mexicanum* reduced 100 mgL^{-1} Cr^{6+} only after 96 h of the incubation period [36]. *Stenotrophomonas acidaminiphila* 4-1 required seven days to reduce 75.7% of the initial Cr^{6+} concentration of 15 mg L^{-1} [14].

The exponential decay equation investigated the kinetics of the Cr^{6+} reduction by *Nesterenkonia* sp. strain NRC-Y at different initial Cr^{6+} concentrations [37, 46]. The results shown in **Figure 7c** revealed that

the Cr^{6+} bioreduction process fitted well with the exponential decay equation over time ($R^2 > 0.9$), with the highest reduction rates of 113.3 uM/h. Furthermore, the reduction rate of Cr^{6+} is considered one of the highest reduction rates reported so far [3, 16, 36].

3.3. Micro characterization of the bacterial cells.

The cell morphology and elemental composition of *Nesterenkonia* sp. strain NRC-Y cells grown in the absence and presence of Cr^{6+} were investigated using SEM–EDX analysis. As shown in **Fig. 8a & c**, the strain grown in the absence of Cr^{6+} was spherical cocci with a diameter of about 0.49–0.57 nm, showing a regular shape and smooth surface cells with no Cr detection. In contrast, cells grown in the presence of 400 $mgmL^{-1}$ Cr^{6+} appeared irregular with wrinkled morphology (**Fig. 8b**). These findings agreed with previous studies, where similar changes in cells morphology for other bacteria grown in the presence of Cr were reported [16, 26]. EDX spectra proved the accumulation of a little of Cr in strain NRC-Y cells (**Fig. 8d**).

The weight percentage of carbon, nitrogen, oxygen, and Cr were 53.83%, 13.52%, 32.18%, and 0.47% (**Table 1**), respectively. These findings were similar to those reported for *Bacillus* sp. CRB-B7, where the weight of the accumulated chromium was 0.49% [26]. The Cr weight for *Cellulosimicrobium* sp. was 0.71% [44], and for *P. umsongensis*, CY-1 was 0.22% [43]. However, Tan et al reported a much higher weight of accumulated chromium (3.45%) within *Bacillus* sp. CRB-B1 cells [16].

Table 1: Elemental composition using EDX analysis of *Nesterenkonia* sp. strain NRC-Y cells grown at 400 mgL^{-1} Cr^{6+}.

Element	Weight (%)	Atomic (%)	Net Int
C	53.83	60.02	67.62
N	13.52	12.93	2.75
O	32.18	26.94	19.45
Cr	0.47	0.12	1.01

3.4. Localization of the active Cr^{6+} reductase

For determination of the location of the chromate reductase produced by *Nesterenkonia* sp. strain NRC-Y, different cell fractions were prepared and assayed for chromate reductase activity, including culture supernatant (CS), cells lysate (CL), cytoplasm (CP), membranous fraction (MB) besides whole resting cells suspension (WC).

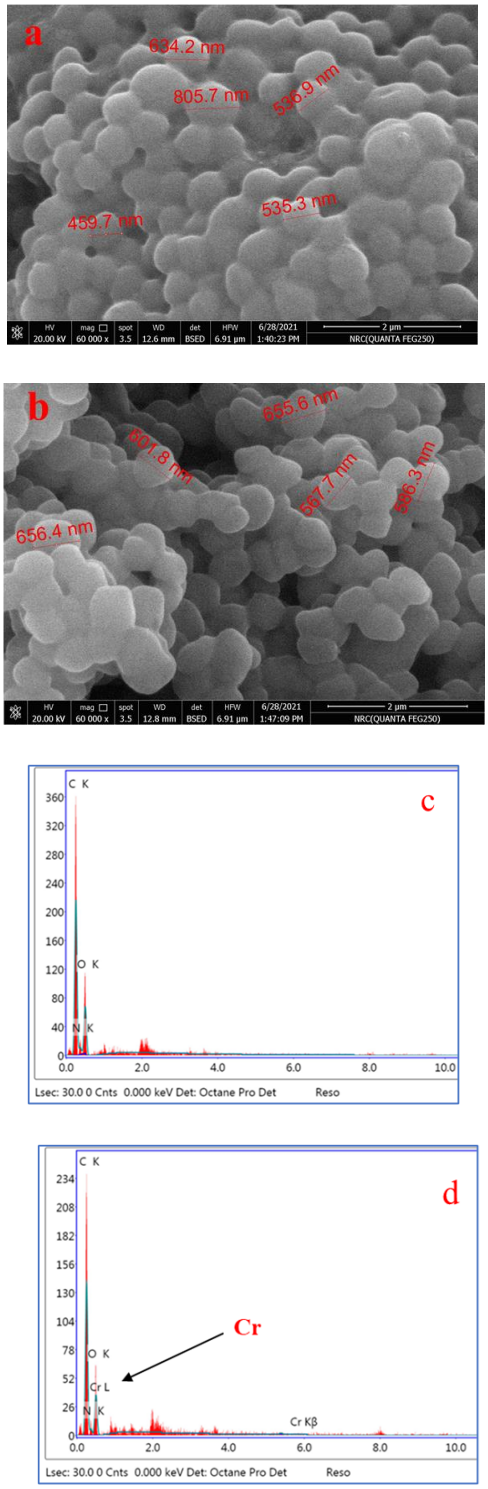

Fig. 8 SEM–EDX images of *Nesterenkonia* sp. strain NRC-Y. (**a**) Cells grown in the absence of Cr^{6+}, (**b**) Bacterial cells grown in 400 mg/mL of Cr^{6+}, (**c**) EDX analysis of cells grown in the absence of Cr(VI, and (**d**) EDX analysis of cells grown in 400 mg/L Cr^{6+}. Analysis carried out at the Central Laboratory (National Research Centre, Egypt).

The results presented in **Fig. 9** revealed that most of the chromate reductase was associated with the cell

membranes showing a 73.2% reduction of Cr^{6+} initial concentration of 300 mgL^{-1}. In addition, little reductase activity was detected in the cytoplasmic fraction (CP), showing a 22.6% reduction. However, almost no extracellular reductase activity was detected. The main sites of bacterial chromate reductases could be in the cytoplasm, such as in *Bacillus* sp. M6 [25], cell envelope such as in *O. oncorhynchi* W4 [15], extracellularly such as *Bacillus* sp. CRB-B1 [16], or both extracellularly and in the cytoplasm as in *Bacillus* sp. strain CRB-7 [26].

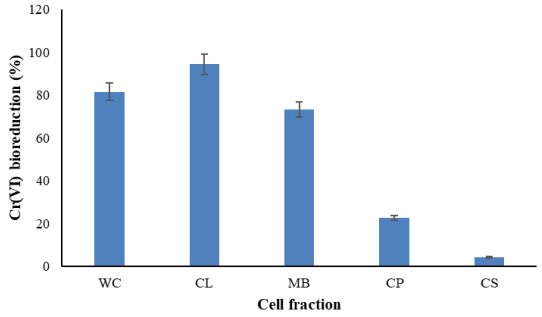

Fig. 9. Chromate reductase activity of *Nesterenkonia* sp. strain NRC-Y cell components, including culture supernatant (CS), cells lysate (CL), cytoplasm (CP), a membranous fraction (MB), and whole cells suspension (WC). Error bars represented standard deviation.

Conclusion

A potent novel Cr^{6+} reducing haloalkaliphilic strain NRC-Y, identified as *Nesterenkonia* sp., was isolated from Wadi El-Natrun valley hypersaline soda lakes. Strain NRC-Y exhibited remarkable Cr^{6+} reduction efficiency under combined harsh conditions, including high alkalinity, ionic stress, and co-existence of different toxic heavy metals and oxyanions. The mechanism of *Nesterenkonia* sp. strain NRC-Y removal by strain NRC-Y involved the production of chromate reductase enzyme, which was mainly associated with the cell membranes besides the little amount in the cell cytoplasm. As a result, strain NRC-Y exhibited a potent Cr^{6+} bioreduction efficiency that could completely reduce up to 800 mgL^{-1} within 24 h. In conclusion, strain NRC-Y is a highly efficient Cr^{6+} reducing haloalkaliphilic bacterium with significant potential in bioremediation of Cr^{6+} contaminated sites, particularly under harsh alkaline and saline conditions. Further investigation using chromium containing industrial waste is recommended.

Conflicts of interest

There are no conflicts to declare

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