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Biosorption of Hexavalent Chromium by *Paenibacillus pabuli* and *Bacillus cereus* Isolated from Alkaline Industrial Contaminated Soil in Puducherry, India

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ABSTRACT

In the present study, we intended to remediate Cr(VI) with alkaliphilic bacteria *Paenibacillus pabuli* (JX561107) and *Bacillus cereus* (JX561108) isolated from alkaline industrial contaminated soil in Pondicherry. The isolated bacteria were tested for the removal efficiency of hexavalent chromium at different concentrations (50 mg/L, 200mg/L, 400mg/L). At 50 mg/L of hexavalent chromium concentration, *Paenibacillus pabuli* and *Bacillus cereus* were found to be highly efficient in removing Cr(VI) in 72 hrs at the inoculum rate of 1% of overnight grown bacterial cultures. The isolates could remove 98% and 74% of Cr(VI) within 72 hrs of treatment at 9.5 pH. When the concentration of the Cr(VI) was increased to 400 mg/L, there was a gradual decrease in Cr(VI) removal. SEM images were obtained from the tested bacteria to observe the bacterial cell surface for the changes in the morphology and EDX analysis were also carried out to confirm whether the adhered particles are of chromium.

INTRODUCTION

Alkaliphilic microorganisms attract increased attention during the last decades in the context of their great potential for biotechnological applications and research of ecological diversity (Rossi et al. 2003). Several alkaliphilic bacteria have been isolated from different environments, for example, deserts, soda lakes, and arid soils (Li et al. 2002). Most intense and extensive intervals of organisms have been observed generally in "moderate" environments. It would also have been known to have an "extreme" environment on the Earth are considered to avoid the endurance of existence. Habitats in these ecological environments, for instance, pH, temperature and salinity concentrations are exceedingly elevated or low. The extreme environment is populated by a group of organisms that have been particularly modified to these specific circumstances and these types of intense microbes are frequently referred to as alkaliphiles, halophiles, thermophiles and acidophiles, and reflect an exacting type extreme environment in which they inhabit (Horikoshi 1991).

Hexavalent chromium is a strong oxidizing agent, frequently present as hydrochromate ($HCrO_4$), chromate (CrO_4)) or dichromate (Cr_2O_7) oxyanions, depending on the pH (United States, Environmental Protection Agency, US EPA 1998). The widespread utilization of hexavalent chromium in industries such as stainless steel production and tanning, cause chromium contamination of soil and groundwater in and around the production site (Turick & Carmiol 1996). Microbes that can resist this stress are more likely to survive and influence chromium speculation (Francisco et al. 2002, Branco et al. 2005). The chromium resistant bacterial isolates showed removal efficiency of around 45.5% at 28°C ambient temperature (Sen et al. 2019). The rationale of this study was to examine the ability of alkaliphilic bacteria *Paenibacillus pabuli* and *Bacillus cereus*, isolated from alkaline industrial contaminated soil, to remediate hexavalent chromium at different concentrations.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from alkaline industry-contaminated soil from Pondicherry in the area of Thavalakuppam. Triplicate sampling at each station was collected in a plastic container during the post-monsoon months from October-December. Before the collection of the soil, the pH of the soil sample was measured at the collection site and recorded as 9.5 (alkaline). The soil samples were collected from five randomly selected spots from the locations with the help of a scooper from the topmost sediment layer, i.e. 0-15 cm layer of sediment samples (approximately 500 g) and transferred

into a sterile sample container and brought to the laboratory (Das et al. 2011).

Isolation of Alkaliphilic Bacteria

Isolation of alkaliphilic bacteria from the soil samples collected aseptically from contaminated soil using rich alkaline nutrient agar medium containing sodium sesquicarbonate solution (modified method of Horikoshi 1999) and was performed by making serial dilution of the samples. The dilution used for the study was 100µL of the 10^{-7} dilution spread in Alkaline Nutrient Agar. The pH of the soil was adjusted to 9.5. The plates were incubated at 37°C for 48 hrs. The isolated colonies were sub-cultured with alkaline nutrient agar to check the purity of the isolates. Identification of the bacterial isolates was carried out by bacteriological methods based on colony morphology, Gram staining, motility and biochemical tests. Barcoding of bacterial isolates was done by the method described by Marmur (1961).

DNA Bar Coding

Genomic DNA isolation: Alkaline strains were grown in an alkaline nutrient broth of pH 9.5 at 37°C. After 12 hours of incubation, 1.5 mL of the cultured broth was taken and centrifuged at 8,000 rpm for 6 min. The pellets were re-suspended with 330 µL of GTE solution and incubated at 37°C for 30 minutes. The pinch amount of lysozyme was added to the same solution and incubated at 37°C for 1 h. Ten µL of 20% SDS was added and incubated at 37°C for overnight. RNase (0.1 mg/mL) was added to the solution to remove the RNA from the solution and kept at 37°C. After 3 h of incubation, 17 µL of EDTA (0.5M) was mixed and incubated at 50°C for 10 min. Proteinase K (10 µL) was added and incubated at 37°C for 3 h. After incubation, 200 µL of phenol:chloroform (24:1) was mixed slowly and centrifuged at 16,000 rpm for 15 min. After centrifugation, the aqueous phase layer was collected and mixed with an equal volume of isopropanol. It was slowly shaken up and down until to see the pool of DNA, and centrifuged at 16,000 rpm for 15 min. The DNA pellet was washed with 1 mL of 95% ethanol and centrifuged at 16,000 rpm for 15 min. After centrifugation, the pellet was air-dried and dissolved in 40 µL of 1X TE buffer. It was confirmed by running the agarose gel electrophoresis.

Agarose gel electrophoresis: The isolated DNA sample was separated on 0.8% agarose gel. 1X TAE buffer was prepared by an appropriate concentration of 1 mL of 50X TAE buffer and mixed with 49 mL distilled water. Further, 0.4 g of powdered agarose was added and mixed well. They were allowed to boil until agarose dissolved completely. Then 3 µL of ethidium bromide (0.5 µg/mL) was added from the stock solution of 10 mg/mL and mixed well. The warmed agarose solution was poured into the gel casting tray and allowed to set for 30-45 min at room temperature. The gel was mounted in the electrophoresis tank. Electrophoresis buffer was added to cover the gel to a depth of about 1mm. The isolated DNA sample was mixed with a loading buffer and loaded into the well of the submerged gel using a micropipette. A voltage of 50-60V was applied. After 1-2 h, the gel was taken out from the buffer and examined under a UV illuminator (UVI-TEC). The clear band was observed as red, orange fluorescence. The molecular weight was measured by using appropriate marker DNA.

Polymerase Chain Reaction (PCR)

The total genomic DNA from the isolates was done by 16SrDNA PCR assay by using the 16SrDNA universal primers (F- 5'AGA GTT TGA TCC TGG CTC AG-3' and R- 5'-CGG TTA CCT TGT TAC GAC TT-3'). They were amplified using the above mentioned PCR mixture. The PCR was run using Eppendorf Gradient thermocycler, the gradient PCR assay was done by using the various annealing temperatures. The PCR cycle used was initial denaturation at 95ºC; 4 min denaturation at 95ºC; 30 s. Annealing 55ºC; 30 s. Extension 72ºC; 30 s. Final extension 72ºC; 10 min cycles 30 cycles. The PCR products were analysed by 0.8% agarose gel electrophoresis. The PCR-amplified 16SrDNA's were purified using the quick PCR purification kit from Bangalore Genie. The sequencing was performed (Eurofins, Bangalore) and sequencing was deposited in the NCBI Genbank. The study of the arrangement and homology and the construction of a phylogenetic tree for the sequenced nucleotide were carried out by the bioinformatics tools.

Experimental Study

Degradation of Cr(VI): The experimental study involving three different hexavalent chromium concentrations, i.e. 50 mg/L, 200 mg/L and 400 mg/L was carried out in 250 mL conical flasks (100 mL in each) at 9.5 pH. One per cent of sucrose was added to all the conical flasks and 1 % of overnight grown bacterial cultures were seeded in each conical flask. The flasks were kept on a rotary shaker at 180 rpm at 37°C. Hexavalent chromium depletion was estimated at an interval of 24 h till 72 h to depletion by the isolated bacterial strains with different chromium concentrations. After absorption, the mixture was centrifuged at 4000 rpm for 15 min. The remaining metal concentration in the solution was measured. Quantity of metals bound was taken to be the distinction among the initial and final concentrations of metal (Gardea-Torresdey et al. 1998). The concentration of metal in the solution was estimated with an atomic absorption spectrophotometer. The experiment was done thrice and the average value was taken for discussion.

Scanning Electron Microscopic Study and EDX Analysis

Scanning Electron Microscopic (SEM) studies were performed to examine the changes in surface morphology before and after treatment and elemental composition of the biosorbent along with EDX study using a scanning electron microscope. Pellets of bacterial culture obtained through centrifugation were used for these spectral studies. The pellets were washed twofold with 0.1 M phosphate-buffered saline (PBS; 15 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 at 25ºC) and fixed overnight in 2% glutaraldehyde (prepared in 0.1 M PBS). The cells were washed with PBS and distilled water earlier to dehydration throughout an ethanol series (10% in absolute), held at each concentration for 30 minutes. Samples were positioned on a brass stub, sputter-coated with gold and examined by Scanning Electron Microscope (De et al. 2008). SEM and EDX studies were performed with a Hitachi S-3400N Scanning electron microscope, available at Central Instrumentation Facility (CIF) Pondicherry University.

RESULTS

Identification of Isolated Alkaliphilic Bacteria from Alkaline Industry-Contaminated Soil

Alkaliphilic bacteria were isolated from alkaline industrial-contaminated soil. They exhibited most favourable growth at pH 9.5. *Paenibacillus pabuli* and *Bacillus cereus* were isolated from the alkaline industry-contaminated soil, and the molecular characterizations were done. Based on phylogenetic analyses, 16S rDNA gene sequence of alkaliphilic bacteria was compared with sequence existing in the Genbank catalogue by BLAST software and sequence were submitted to NCBI Genbank.

The phylogenetic tree derived from 16S rRNA gene sequence of *Paenibacillus pabuli* and *Bacillus cereus* and sequences of the closest phylogenetic neighbours obtained by NCBI BLAST analysis confirmed the relationships among the selected isolates (Fig. 1 and Fig. 2)

Removal of Hexavalent Chromium from Tannery Effluent by Isolated Alkaliphilic Bacteria

Chromium was removed from the tannery effluent by alkaliphilic bacterial strains isolated from alkaline industrial soil at pH 9.5 (pH of the alkaline industry effluent contaminated soil from which bacterium was isolated). The removal efficiencies of hexavalent chromium in solution were calculated from the differences between the initial and the residual concentration after 72 hrs of treatment at 37°C at pH 9.5. The concentration of total Cr(VI) was reduced to 1.1 mg/L from 50 mg/L in 72 hrs of treatment with *Paenibacillus pabuli* showed 98 % efficiency and *Bacillus cereus* removed Cr(VI) to 12.92 mg/L from 50 mg/L in 72 hrs with 74 % efficiency (Fig. 3). When the concentration of Cr(VI) was increased to 200 mg/L there was a gradual decrease in the Cr(VI) concentration to 57% from 200 mg/L in 72 hrs of treatment with *Paenibacillus pabuli* and *Bacillus cereus* reduced Cr(VI) to 33% from 200 mg/L in 72 hrs of

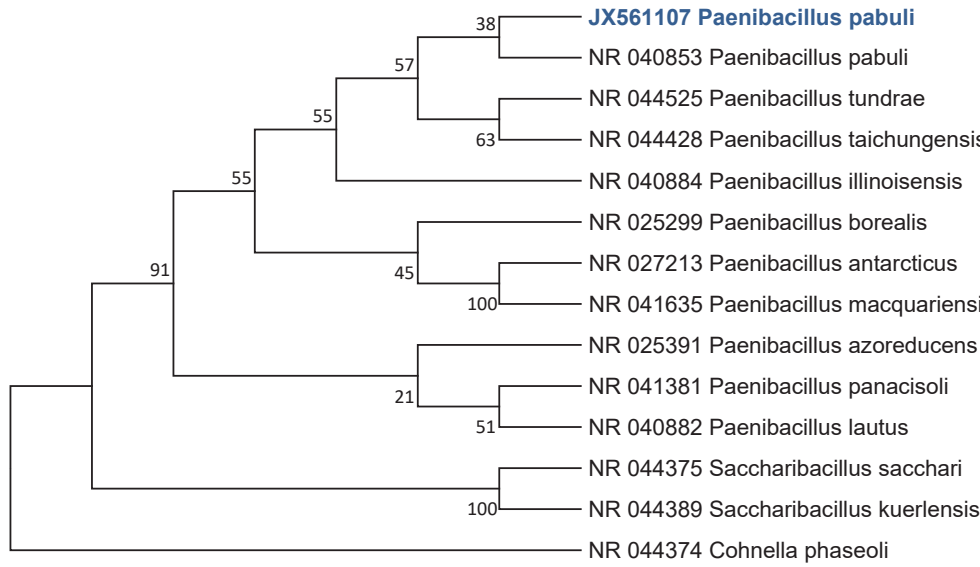


Fig. 1: Phylogenetic tree of *Paenibacillus pabuli* and NCBI accession number - JX561107.

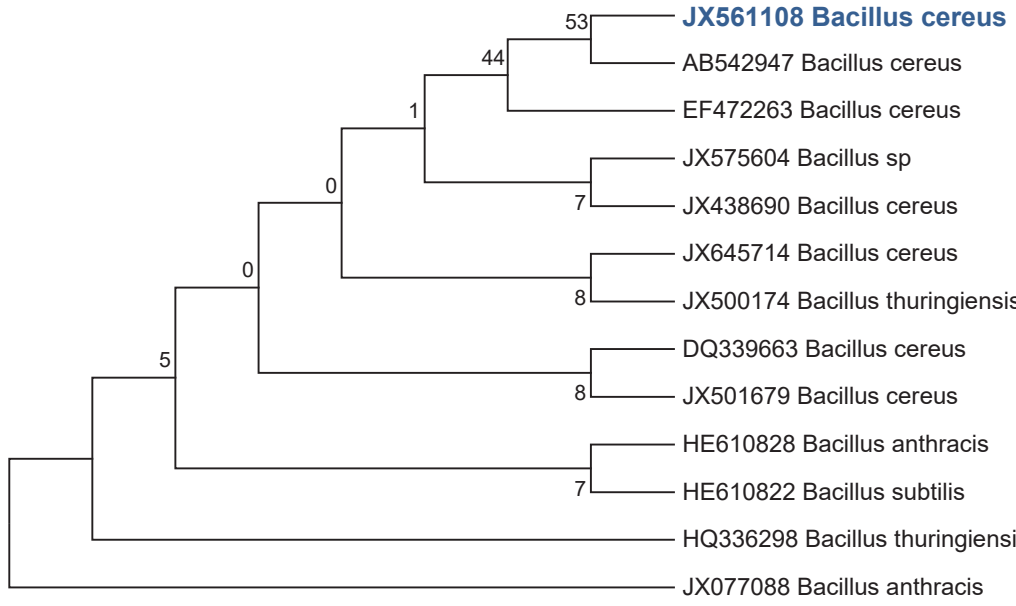


Fig. 2: Phylogenetic tree of *Bacillus cereus* and NCBI accession number - JX561108.

treatment (Fig. 4). Whereas, when the Cr(VI) concentration is increased to 400 mg/L, there was no absorption indicating the intolerance of bacterial biomass in a higher concentration of chromium due to high stress exerted by the higher level of metal ions in the medium.

SEM and EDX Analysis

Bacterial species *paenibacillus pabuli* and *Bacillus cereus* showed high efficiency in the removal of chromium in 72h of treatment. SEM analysis was conducted to differentiate the morphological changes due to adsorption of Cr(VI). Before biosorption, the cell surface was smooth and even. After the biosorption, there was a significant change on the surfaces of the bacterial cell. The figures clearly show that the bacterial cell surfaces before the treatment were smooth without any adhesion of Cr(VI), while after the treatment the cell surface was rough and wrinkled due to the adhesion of metal on the bacterium (Fig. 5 and Fig. 7). The EDX spectrum confirms the existence of Cr(VI) in the pellets of bacteria used in chromium treatment (Fig. 6 and Fig. 8).

DISCUSSION

Current awareness has been drawn to the expansion of the absorption technique as a method of bioremediation. It has been focused on a relatively inexpensive and easier than using a chemical treatment to overcome the contamination of heavy metals (Nies 2000). Moreover, countless researchers have reported the biosorption of heavy metal into pure cultures of bacteria and algae and onto the natural microbial population, which is possible through efficient and environmentally friendly remediation technologies (Gutnick & Bach 2000).

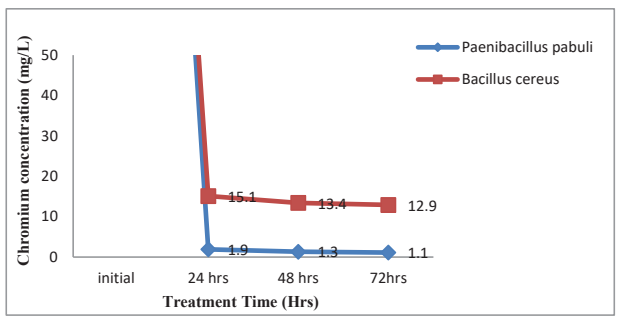


Fig. 3: Chromium sorption by isolated strain at 50 mg/L of chromium concentration in 72 hrs treatment at 9.5 pH.

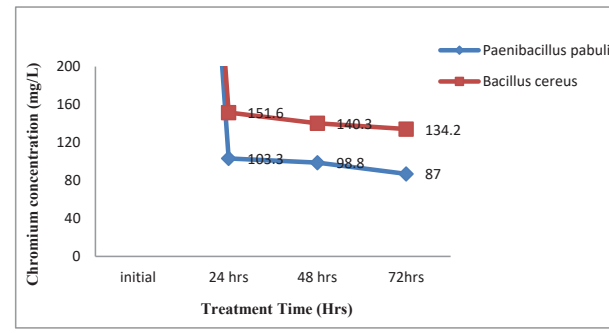
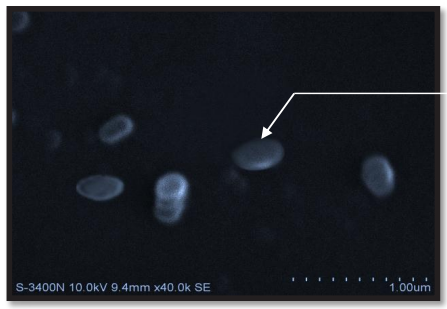
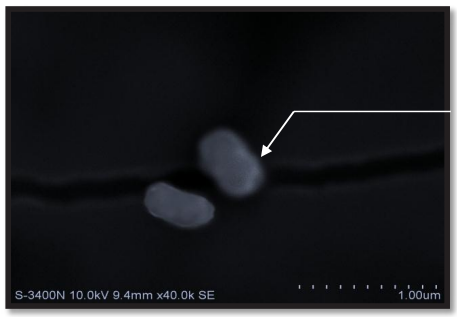


Fig. 4: Chromium sorption by isolated strain at 200 mg/L of chromium concentration in 72 hrs treatment at 9.5 pH.



Before treatment with Cr effluent



After treatment with Cr effluent

Fig. 5: SEM images of *Paenibacillus pabuli* before and after the treatment.

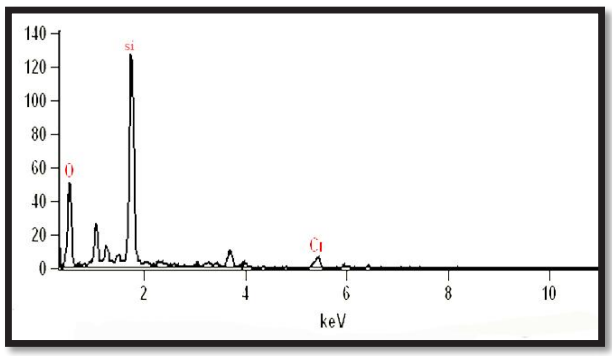
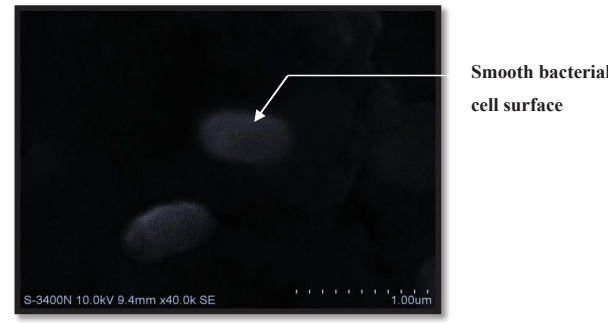
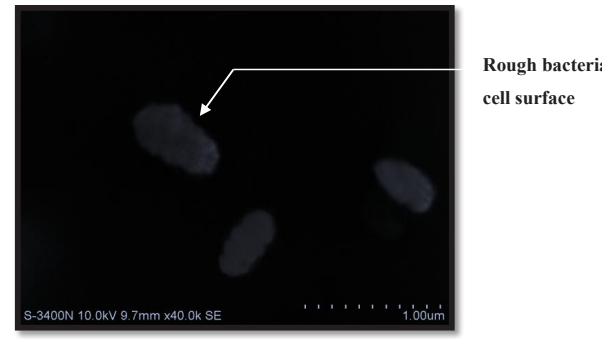


Fig. 6: EDX spectrum of *Paenibacillus pabuli.*



Before treatment with Cr effluent



After treatment with Cr effluent

Fig. 7: SEM images of *Bacillus cereus* before and after treatment.

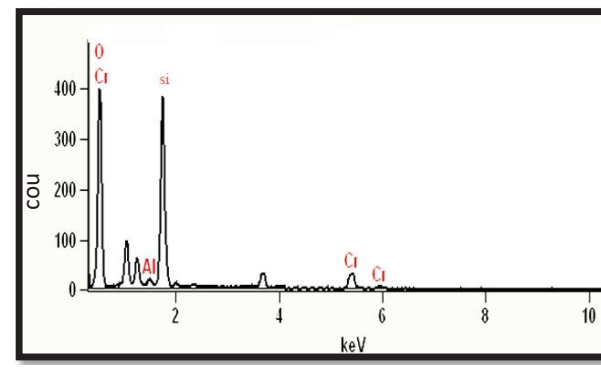


Fig. 8: EDX spectrum of *Bacillus cereus.*

In the present study, *Paenibacillus pabuli* and *Bacillus cereus* from alkaline industry-contaminated soil were evaluated for their chromium removal efficiency at 9.5 pH (pH of alkaline industry contaminated soil from which alkaliphilic bacteria were isolated). The results are quite encouraging, recording higher adsorption capacity at higher pH. The chromium(VI) efficiency of bacteria isolated from alkaline industrial contaminated soil, namely *Paenibacillus pabuli* and *Bacillus cereus* revealed 98% and 74% efficiency.

Further experimental study on evaluation of concentration based efficiency, 200 mg/L of Cr(VI) concentration and the tested bacteria recorded about 57% removal; but at higher concentration of chromium (400 mg/L), there was no removal by isolated bacterial strain tested. Such observation indicates the intolerance of bacterial biomass in a higher concentration of chromium due to high stress exerted by the higher level of metal ions in the medium. Up to 200 mg/L of chromium, the bacteria were able to remove more than 57% of Cr(VI) but when the concentration was doubled (i.e., 400 mg/L), there was no removal. It has been also reported that the high chromium concentration prohibited the development of bacteria (Bopp & Ehrlich 1988).

Such intolerance of a higher concentration of chromium by the bacteria reveals that the capability of a given amount of bacterial biomass could have played a significant role in maintaining its effectiveness. In the present study, a loop full of overnight grown pure culture biomass was inoculated in different Cr(VI) concentrations. The amount of biomass given cannot be tolerated due to the high concentration of metal in the medium and it is assumed that the actual threshold level of each bacterium might be scaled out to maintain the high efficiency of the bacterium for future research. The present findings are in close conformity with the observation made by Wang & Xiao (1995) in *Bacillus* sp. Further, Basu et al. (2014) and Yilmaz (2003) reported that the removal percentage was decreased with increasing chromium concentration. This is because the amount of inoculum was constant, relatively less biomass was available for chromium (VI) removal from the media. It is proving to be reported at low concentrations that all the metal ions in the solution will interact with the binding sites and thus facilitate 100% adsorption. The higher the concentration, the sites available for adsorption are less compared to the molecules of the solute present. Thus, observations made in the experimental studies are in close conformity with previous reports.

On examining the mechanism behind the adsorption of chromium to the bacterial cell surface, it has been reported that to endure under metal-stressed circumstances, bacteria have emerged numerous types of mechanisms to endure and survive through biomechanism of the uptake of heavy metal ions. These mechanisms comprise the discharge of metal ions outer the surface of the cell, aggregation and complexation of the metal ions within the cell (Nies 1999). Further, the adhesion of metal to the bacterial cell surface might be due to the presence of an exopolysaccharide production (Loaëc et al. 1997).

To examine the current findings regarding the adsorption of hexavalent chromium on the surface of bacterial cells, SEM images of the bacterial cell surface of treated and untreated bacteria show the metal adhered on the surface of the bacterial strains, giving a wrinkled and rough surface morphology in *Paenibacillus pabuli* and *Bacillus cereus.* Similar images were also recorded by Jun Guo et al. (2012) in *Pseudomonas plecoglossicida* showing that before the absorption the cells were rod-shaped with a smooth surface, but after the absorption, there were changes on the cell surfaces. Similarly, Dhal et al. (2010) also obtained SEM-EDX images indicating adherence of hexavalent chromium on the surface of bacterial cells. Besides, to substantiate whether the adsorbed material is made of chromium, the EDX spectrum was obtained for *Paenibacillus pabuli* and *Bacillus cereus* used in treatment studies. The EDX spectrum established the occurrence of Cr(VI) in the pellets of bacteria treated in Cr(VI) remediation. Silica (Si) peak in the EDX spectrum may be due to the preparation of smear on glass slides and the peak of Al might have originated from the sample holder.

CONCLUSION

Concludingly, it is reported that for bioremediation, having less than 200 mg/L of chromium, the alkaliphilic bacteria *Paenibacillus pabuli* and *Bacillus cereus* were able to tolerate and adsorb high Cr(VI) concentrations and could be the potential species as bioadsorbents. The suitability of these alkaliphilic isolates, particularly for chromium removal, would open up new lines of application through suitable biotechnological tools and techniques.

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