Biosorption and enzymatic reduction of hexavalent chromium by chromium resistant bacteria ameliorate phytotoxicity

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Research article

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Abstract

Background: Chromium (Cr) (VI) is one of the toxic heavy metals and environmental hazards. Alleviating the levels of contaminants in the environment is imperative, and studying the bioremediation of Cr via reduction or biosorption is an indispensable approach to this cause. In this study, we investigated the efficiency of reduction and biosorption of Cr(VI) by chromate resistant bacteria isolated from tannery wastewater. Results: From screening, 28 Cr resistant bacteria were selected, and only two isolates, SH-1 and SH-2, were found as potential candidates for the reduction of Cr(VI). Post 16s rRNA sequencing, SH-1 isolate was identified to be *Klebsiella* sp and SH-2 isolate as *Lysinibacillus* sp. SH-1 could tolerate up to 2000 mg/L of Cr(VI) whereas, SH-2 could tolerate up to 1500 mg/L of Cr(VI). In Luria-Bertani media containing 100 mg/L of Cr(VI), the relative reduction level was 95% (SH-1) and 88.77% (SH-2) but their reduction rate was 63.08% (SH-1) and 49.89% (SH-2) of Cr(VI), respectively in the tannery effluents after 72h period of incubation. In the presence of Cr(VI) at a concentration of 10 mg/L Cr(VI), the cell-free extracts of pre-grown SH-1 and SH-2 showed a reduction of 72.2% and 33%, respectively. This reduction indicates the production or the activity of Cr reducing enzyme being higher in these two isolates than that of control in the presence of Cr(VI). In biosorption study, live and dead biomass of SH-1 biosorbed 51.25 mg and 29.03 mg chromium per gram dry weight, respectively. However, 28.83 mg and 27.65 mg chromium per gram dry weight were biosorbed by live and dead biomass of SH-2, respectively. Both the Langmuir model -for monolayer adsorption- and Freundlich model -for adsorption characteristics for the heterogeneous surface- were suitable for describing biosorption of Cr(VI) by SH-1 live biomass. The chickpea seed germination study confirmed the beneficial environmental effect of Cr(VI) reduction by these two isolates. Conclusion: The bacterial isolates can be exploited for their potential for reduction and biosorption of toxic hexavalent chromium in biological treatment of hexavalent chromium-containing wastes.

Abstract

Background: Chromium (Cr) (VI) is one of the toxic heavy metals and environmental hazards. Alleviating the levels of contaminants in the environment is imperative, and studying the bioremediation of Cr via reduction or biosorption is an indispensable approach to this cause. In this study, we investigated the efficiency of reduction and biosorption of Cr(VI) by chromate resistant bacteria isolated from tannery wastewater.

Results: From screening, 28 Cr resistant bacteria were selected, and only two isolates, SH-1 and SH-2, were found as potential candidates for the reduction of Cr(VI). Post 16s rRNA sequencing, SH-1 isolate was identified to be *Klebsiella* sp and SH-2 isolate as *Lysinibacillus* sp. SH-1 could tolerate up to 2000 mg/L of Cr(VI) whereas, SH-2 could tolerate up to 1500 mg/L of Cr(VI). In Luria-Bertani media containing 100 mg/L of Cr(VI), the relative reduction level was 95% (SH-1) and 88.77% (SH-2) but their reduction rate was 63.08% (SH-1) and 49.89% (SH-2) of Cr(VI), respectively in the tannery effluents after 72h period of incubation. In the presence of Cr(VI) at a concentration of 10 mg/L Cr(VI), the cell-free extracts of pre-grown SH-1 and SH-2 showed a reduction of 72.2% and 33%, respectively. This reduction indicates the

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Conclusion: The bacterial isolates can be exploited for their potential for reduction and biosorption of toxic hexavalent chromium in biological treatment of hexavalent chromium-containing wastes.

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Background

Wastewater from industrial application contains heavy metals and anonymous organics, causing alarming environmental pollution worldwide. Although Chromium (Cr) is a toxic heavy metal, it is widely used in leather tanning, metal processing industries, electroplating, and textile dyeing. Chromium discharge in wastewater contaminates water bodies; endangers aquatic lives and poses a serious health hazard. Hence, it is a public health concern which needs to be addressed by reducing toxicity or load of the heavy metals from industrial effluents.

There are about 250 leather processing industries in Bangladesh at Hazaribagh and Hemayetpur of Dhaka city which is discharging both liquid and solid wastes into canals and rivers, thus increasing the amount of Cr in the surrounding water bodies [1]. Human Right Watch reported that tannery wastewater from 47 tanneries of Hazaribagh contained extremely elevated levels of chromium (4,043 mg/L). On the other hand, the tannery effluents in Chittagong contained hexavalent chromium of 70.33 mg/L [2]. Cr(VI) was also found in the deep tube wells from Hazaribagh, which exceeded the Bangladesh standard limit (0.05 mg/L) of chromium in drinking water [3].

Cr(VI) is toxic as it is highly soluble in water and can pass rapidly through cell membranes, subsequently interact with proteins and nucleic acids [4]. Thus, accumulation of toxic heavy metals in human leads to carcinogenesis, mental retardation, renal malfunction, and other abnormalities [5]. The prevalence of diseases among the tannery workers were reported that 73.9% worker had scabies, 71.7% had a gastrointestinal problem, 71.7% had diarrhea, 52.2% had blood pressure, 49.9% had asthma, and 46.7% had eye problem [6].

Therefore, it is of utmost importance to treat the effluents before discharging into the environment for the reduction of environmental pollution and the disease burden. Numerous technologies are available to mitigate Cr contaminated wastewater, which includes redox chemical processes [7], reverse osmosis, coagulation, and precipitation [8, 9]. However, these methods, as mentioned above, are expensive and

energy consuming as well as has the risk of secondary chemical contamination [7, 10]. Wastewater and effluents with heavy metal contamination can alternatively be treated with potential microorganisms. Use of such microorganisms sanctions large volume of effluent treatments, all at once with a low operational cost and low energy demand with higher efficiency of metal removal [10–12]. Microorganisms provide higher surface area to the volume due to the small size and can assimilate metals from surrounding settings [13]. Various microorganisms are found the industrial effluents discharging area and are capable of protecting themselves from the toxicity of existing heavy metals in the effluents [14–16]. These microorganisms including bacteria, fungi, algae, and protozoa use diverse systems of survival against heavy metal toxicity such as uptake of heavy metal, adsorption, oxidation, methylation, and reduction of heavy metals to its nontoxic forms [14, 17]. Toxic Cr(VI) reduction to its nontoxic Cr (III) form is shown to be one of the mechanisms as mentioned above, followed by many organisms to survive in the Cr VI) contaminated effluents. These microorganisms include *Acinetobacter* and *Ochrobactrum* [18]; *Arthrobacter* [19]; *Serratia marcescens* [20]; *Bacillus* spp. [21]; *Cellulomonas* spp. [22]; and *Pseudomonas fluorescens* LB300, *Intrasporangium* sp. Q5-1, *Enterobacter cloacae*, *Bacillus* sp. ES29, and *E. coli* [23–27] and also a mixture of different microbes [28] can reduce the highly soluble and toxic Cr(VI) to the less soluble and less toxic Cr (III).

The application of microbes for bioremediation of heavy metals and other contaminants has been studied in other countries [29] but, yet to be initiated in the developing country like Bangladesh. There is no concrete study on the isolation and application of chromium resistant bacteria for the treatment of wastewater. Therefore, the present study was conducted for the isolation and identification of Cr(VI) resistant bacteria from tannery effluents and their application in the removal of Cr(VI) from effluent water.

Results

Physicochemical characteristics of tannery effluents

Collected raw tannery effluent samples were preliminarily tested for different physicochemical parameters. Several important physicochemical characteristics of fourteen tannery effluents were analyzed. The recorded temperature of the collected samples were ranged from 18.4 to 26.1 °C; pH was found in the range of 3.61 to 9.51; conductivity from 752.0 to 106300.0 $\mu\text{S}/\text{cm}$ and total dissolved solids (TDS) ranged from 376.0 to 68000.0 mg/l; chloride ranged from 68.86 to 4366.25 mg/L (Table S1). The salinity and turbidity ranged from 0.4 to 74.5 ppt and 36.1 to 519.0 NTU, respectively. The COD of the collected samples ranged from 361.0 to 7788.0 mg/L (Table S1). The total chromium load of the tannery effluents varied from 0.005 to 1423.05 mg/L (Table S1). Guidelines for Bangladesh standard for wastewater disposal for these parameters are listed in Table S2. From the result, we observed that total chromium concentration of 50% (n=7) of the collected effluent water crossed the Bangladesh standard for wastewater disposal.

Screening of Cr(VI) reducing bacteria from tannery effluents

We hypothesized that Cr(VI) resistant organism might be found in the tannery effluents as these effluents contain a high amount of chromium (Table S1). We have screened for Cr(VI) resistant as well as reducing bacteria of which the procedure is shown in Figure 1A. At first, all 14 samples were tested for the presence of Cr(VI) resistant bacteria using 100 mg/L Cr(VI) and 28 discrete colonies were found based on morphological observation (Table S3). As shown in Figure 1B, nine isolates were found to be resistant to more than 500 mg/L Cr(VI). We have also screened for Cr(VI) sensitive bacteria from different species that were preserved in the Environmental Microbiology Laboratory to use as a negative control. An environmental isolate of *Vibrio cholerae* was found most sensitive (100 mg/L Cr(VI), Figure 1B). Two of the isolates (SH-1 and SH-2 can tolerate up to 2000 mg/L and 1500 mg/L, respectively) showed very high resistance to Cr(VI). Representative plates of two isolates from Cr(VI) resistance experiment are shown in Figure 1C. Next, we tested for the Cr(VI) reduction capacity of these isolates and interestingly two isolates that showed the highest resistance also showed the highest rate of reduction (SH-1 showed 97% reduction whereas, SH-2 showed 88% reduction; Figure 1D). After performing ANOVA followed by post-hoc Tukey's Test, all the isolates showed statistically significant differences for the reduction of Cr(VI) as compared to the negative control (*Vibrio cholerae*). This experiment proceeded with the two isolates (SH-1 and SH-2) that showed a maximum reduction. Besides, we have tested these two isolates for their resistance against different antibiotics and other heavy metals. Isolates SH-1 was resistant only to penicillin and erythromycin whereas sensitive to chloramphenicol, ciprofloxacin, gentamycin, and intermediately resistance to cefotaxime, meropenem, streptomycin (Table S4). Isolates SH-2 was found to be sensitive to all eleven (11) antibiotics except intermediately resistance to amoxicillin and erythromycin (Table S5). Results also revealed the resistance pattern for heavy metals by SH-1 was $\text{Cr}^{6+} > \text{Pb}^{2+} > \text{Mn}^{2+} > \text{Ni}^{2+}, \text{Cu}^{2+}, \text{Co}^{2+} > \text{Zn}^{2+}, \text{Cd}^{2+}$ and for SH-2 was $\text{Cr}^{6+} > \text{Pb}^{2+} > \text{Co}^{2+}, \text{Cu}^{2+}, \text{Ni}^{2+}, \text{Zn}^{2+} > \text{Cd}^{2+}$ (Figure 1E). The data from primary screening for Cr(VI) resistant bacteria showed that organism with higher resistance has a higher capacity of reduction. This intriguing finding leads us to further study the mechanism of the reduction and possible application for bioremediation.

Identification of the isolates by 16s rRNA sequencing

The analysis of 16S rRNA sequencing identified the two bacterial isolates that were capable of efficiently reducing hexavalent chromium as *Klebsiella* sp (SH-1) and *Lysinibacillus* sp (SH-2) (Table 1). These two bacteria have not been reported in other studies as resistant as well as chromium-reducing. The submitted sequences can be found in the NCBI database, of which accession numbers are MF465571.1 and MF465572.1 (Table 1).

Effect of chromium under optimum growth conditions

The optimum growth was determined for both isolates. At 37°C, the growth rate of both isolates was greater than that of room temperature, although the growth rate started to decline when the temperature was increased up to 44°C. The bacterial isolates SH-1 and SH-2 can also grow in a wide range of pH (5.5 to 10.0). Interestingly, in the present study, pH 9.0 was found to be optimum for the growth of both isolates. The ability to grow in the presence of Cr(VI) was also determined, in which isolates SH-1 showed

similar growth curve as its control, whereas isolates SH-2 showed slightly lower curve pattern than the control. As compared to the negative control (*V. cholerae*), both of the isolates showed greater growth efficiency in the presence of Cr(VI) (Figure 2A and 2B).

Isolates showed different Chromium (VI) reduction capacity in different media

This study has found that these isolates have very high chromium (Cr) resistance; hence, we hypothesized that these isolates might have Cr reducing ability. Afterward, we tried to confirm this hypothesis by conducting a time course study of Cr(VI) reduction by these isolates in LB broth containing 100 mg/L of Cr(VI) at the optimum temperature (37°C) and pH (9.0). It has been reported that the optimal Cr(VI) reduction capacity is directly associated with the optimum pH condition (8.0) for the growth of *Bacillus* isolates [30]. In the present study, we observed that both isolates could reduce Cr(VI) rapidly at 37°C and pH 9. Isolates SH-1 could reduce Cr(VI) approximately 81.12%, 91.66%, and 95% from the medium after 24h, 48h, and 72h, respectively (Figure 2C). On the other hand, SH-2 was also able to reduce 69.14%, 77.28%, and 88.77% from the medium after 24h, 48h, and 72h, respectively (Figure 2C). Statistical significance was determined by paired two-tailed student's t-test. SH-1 was found to be more effective for chromate reduction compared to SH-2. Furthermore, we have found that Cr(VI) reduction also occurred in the controls, which were cell-free (Figure 2C).

To determine the reduction efficiency of both isolates in surface water, they were inoculated in pond water and tannery effluents containing 100 mg/L of Cr(VI) without the addition of any nutrients. Their reduction efficiency was also compared with the indigenous microorganism present in the water samples during these periods. After 72h of incubation in pond water with initial Cr(VI) concentration of 100 mg/L, SH-1 and SH-2 showed 45% and 38.37% reduction, respectively, whereas the control (with indigenous organisms) showed only 13.52% reduction (Figure 2D). Therefore after subtracting the reduction percentage (13.52%) by the indigenous organism, the increase in reduction percentage via the addition of our isolates was calculated to be 31.48% and 24.85% (SH-1 and SH-2, respectively). Using one way ANOVA, we obtained a statistically significant difference ($p < 0.05$) for different concentration reduction of both isolates followed by Tukey's Post Hoc Test. Additionally, in tannery effluents, SH-1 showed 63.08% reduction after 72h of incubation; likewise, SH-2 reduced 49.89%, (Figure 2E). However, the indigenous organisms present in the effluents exhibited only 36.65% reduction after 72h (Figure 2E). The reduction by indigenous microorganisms was observed, but the reduction was much lower compared to the isolates. Since SH-1 can itself reduce 26.34% Cr(VI) in the presence of indigenous organisms after 72h of incubation, it indicates that higher Cr(VI) resistance may have helped SH-1 to outgrow the indigenous organisms in the presence of 100 mg/L Cr(VI).

Isolates showed enhanced chromate reductase activity in the presence of Cr(VI)

The chromate reductase activity of crude cell extracts was obtained by using proteins collected from cells pre-grown; both in the presence and absence of Cr(VI). The soluble proteins from SH-1 and SH-2, in presence and absence of Cr(VI), could reduce Cr(VI) as indicated in Table 2, whereas protein sample boiled at 100°C and negative control (*V. cholerae*) failed to show any significant chromate reduction. The

reduction caused by protein sample boiled at 100°C was used as blank control and was subtracted from the other reduction data. Protein samples from isolates that were treated with Cr(VI) showed 72.2% (SH-1) and 33% (SH-2) reduction, but protein samples from isolates that were not treated with Cr(VI) showed 52% (SH-1) and 26.6% (SH-2) reduction. Therefore, reduction caused by Cr(VI) treated proteins was higher than the reduction of untreated proteins of the isolates (Table 2). As we have used the same amount of protein for every sample, we can deduce that chromate reductase activity increased with Cr(VI) reduction ability (Table 2).

SH-1 live biomass showed better biosorption capacity in response to different conditions

The biosorption capacities of the bacterial biomass (SH-1 live, SH-2 live, SH-1 dead, SH-2 dead) for the removal of Cr(VI) were found as a function of initial metal ion concentration, pH and time.

(i) Low pH showed better biosorption. As wastewater containing metal ion have different pH values, that's why biosorption study of Cr(VI) was performed at different pH. Initial pH values were set from 1 to 8 of 100 mg/L Cr(VI) solution. As illustrated in Figure 3A, the capacity of adsorption increased from pH 1.0 to pH 2.0, and then decreased with the increase of pH. We observed a negative correlation of Cr(VI) adsorption individually between the variables (SH-1 live, SH-1 dead, SH-2 live and SH-2 dead) and the increasing pH ($r_1 = -0.89$, $r_2 = -0.94$, $r_3 = -0.88$ and $r_4 = -0.96$ respectively); which were statistically significant ($p < 0.01$). The maximum rates of biosorption were 33.28 mg/g of live biomass (SH-1 live) at pH 2 and 16.77 mg/g of dead biomass (SH-2 dead) at pH 1 (Figure 3A).

(ii) Biosorption increased with the increasing initial concentration of Cr(VI). The biosorption process was conducted at optimized pH (pH 2 for live and pH 1 for dead) at different initial concentrations (20 mg/L to 200mg/L). When the initial Cr(VI) concentration was increased from 20 to 200 mg/L, the absorbing capacity was also increased from 8.76 to 51.25 mg/g (SH-1 live), 3.46 to 28.83 mg/g (SH-2 live), 6.21 to 29.03 mg/g (SH-1 dead), 1.8 to 27.65 mg/g (SH-2 dead) biomass (Figure 3B). We observed a positive correlation of Cr(VI) adsorption individually between the variables (SH-1 live, SH-1 dead, SH-2 live and SH-2 dead) and the increasing Cr(VI) concentration ($r_1 = 0.99$, $r_2 = 0.96$, $r_3 = 0.95$ and $r_4 = 0.93$ respectively); which were statistically significant ($p < 0.01$). The maximum biosorption capacity was found in the SH-1 live biomass at 200 mg/L.

(iii) Effect of time on biosorption process. Figure 3C illustrates the contact time of Cr(VI) by the live and dead biomass at a concentration of 200 mg/L at the optimized pH mentioned above. Higher adsorption capacity was found in live biomass than the dead biomass (Fig 3C). Adsorption by the biomass (SH-1 live, SH-1 dead, SH-2 live and SH-2 dead) was increased rapidly throughout the first 60 min and continued to be constant until 90 min. Here, a positive correlation of Cr(VI) adsorption individually between the variables (SH-1 live, SH-1 dead, SH-2 live and SH-2 dead) and the time up to 90 min was found ($r_1 = 0.94$, $r_2 = 0.86$, $r_3 = 0.91$ and $r_4 = 0.88$ respectively); which were statistically significant ($p < 0.01$). Subsequently, after acquiring the equilibrium, no significant differences of Cr(VI) adsorption with time were observed, though the experiments were performed until 360 min.

(iv) Langmuir and Freundlich adsorption isotherm. To illustrate the mathematical expression of the biosorption, Freundlich and Langmuir adsorption models were used for live biomass of both isolates (SH-1 & SH-2). The constants of isotherms were assessed to compare the capacity of Cr(VI) biosorption by this biomass.

The values of Q_o and K_L were calculated from the slope and intercept of the Langmuir plot of $1/Q_e$ versus $1/C_e$ (Figure 3D). From the data showed in Figure 3E, the Langmuir Isotherm model generated the maximum monolayer coverage capacity (Q_o) of 72.99 mg/g while our live biomass of SH-1 showed 51.25 mg/g biosorption at 200 mg/L concentration, the constant of Langmuir isotherm (K_L) was found 0.01 L/mg and the equilibrium parameter (R_L) was found 0.23 which indicates that the equilibrium sorption was validated (favorable if $0 < R_L < 1$, linear if $R_L = 1$, and irreversible if $R_L = 0$) and the R^2 value of 0.94 provides well-fitted equation to the Langmuir Isotherm model (Figure 3D).

In the Freundlich model, the value of $1/n$ is 0.77 and n is 1.30 which reveals that the sorption of Cr(VI) ion unto live SH-1 biomass is favorable and the R^2 value is 0.994 (Figure 3E and 3F). So the value of K_f and n is the indication of easy uptake of Cr(VI) ion with a high adsorptive ability of live biomass. The isotherm models for SH-2 live biomass is added as a supplementary figure (Figure S6).

Phytotoxicity assay using chickpea seed germination

Toxicity of hexavalent chromium on seed germination along with the growth of roots and shoots was examined in this study. As shown in Figure 4A and 4B growth of shoot and root length have gradually decreased with increasing concentration of Cr(VI). Calculating correlation of Cr(VI) concentration individually with the shoot and root length we observed a negative correlation ($r_1 = -0.74$ and $r_2 = -0.91$ respectively), which indicates that Cr(VI) affected the growth of shoots and roots of chickpea seeds. Although Cr(VI) did not affect the rate of seed germination. Above 50 mg/L seedling growth was greatly hampered, and no seedling was observed at 100 mg/L (Figure 4A and 4B). A similar test was conducted to assess the mitigation of toxicity of bacterial treated and untreated Cr(VI) samples (Figure 4C). In control, the mean shoot and root length was observed as 14.9 cm and 7.7 cm, respectively (Figure 4D); while the mean shoot and root length of the seeds with Cr(VI) sample that was treated with SH-1 bacteria were 10.1 cm and 3.2 cm and Cr(VI) sample that were treated with SH-2 bacteria were 6.7 cm and 3.6 cm, respectively. On the other hand, the mean shoot and root lengths were 0.7 cm and 0.8 cm, respectively in case of 50 mg/L Cr(VI). These results indicate that 50 mg/L Cr(VI) treatment significantly reduces the growth of shoot and root (95.3% and 89.6%, respectively). Of note, Cr(VI) sample that was treated with SH-1 bacteria significantly recovered 93% and 75% shoot and root length respectively as compared to the 50mg/L Cr(VI) sample. Similarly, SH-2 treated Cr(VI) sample also significantly recovered 89% and 77% shoot and root length respectively as compared to the 50mg/L Cr(VI) (Figure 4C and 4D).

Discussion

Prokaryotes can tolerate toxic Cr better than the eukaryotes, and thus, bacteria can survive in a higher concentration of toxic Cr(VI) [31]. Cr(III) sulfate ($[\text{Cr}(\text{H}_2\text{O})_6]_2(\text{SO}_4)_3$) has long been used as a tanning agent and being leached into the effluents [32]. Though Cr(III) sulfate is being used, the transformation of Cr(III) of tanning agent into Cr(VI) is occurred through oxidation due to the repeated exposure of skin to direct sunlight and during other leather tanning processes [33, 34]. In this study, we investigated the screening of 28 Cr(VI)-resistant bacteria from tannery effluent, followed by Cr(VI) reduction and absorption by the two selected isolates.

The potentiality of efficient remediation of toxic metals by utilizing living microorganisms is a key factor which may enhance the application of heavy metals bioremediation [35]. In some previous studies, the bacteria which were tested showed a lower reduction of Cr(VI) over a relatively long period of time. For example, it was reported that with 120h of incubation, *Alcaligenes faecalis* reduced 70%, while *Pseudochromobacterium saccharolyticum* reduced 40% of Cr(VI) [36]. Another study by Sharma et al. revealed that *Bacillus* sp (Accession number FM334108.1), *Bacillus subtilis*, and *Bacillus* sp (Accession number FJ3480004.1) reduced 73.41%, 42.15% and 60% of 100 mg/L of Cr(VI) after 120h of incubation, respectively [37]. After 72h incubation, SH-1 showed 95%, 63% and 45% reduction in LB media, tannery effluent, and pond water, respectively. Although, we have found that Cr(VI) reduction also occurred in the control, which was cell-free (Figure 2C). It can be suggested that components may have caused the reduction in the LB medium since no cells were inoculated into the medium. A study by Shanab et al. found that medium components along with carbon sources are able to interact with toxic metals such as arsenate, cadmium, chromium, cobalt, copper, lead, nickel, mercury and zinc to give misleading results [38]. Indigenous organism present in the pond water and tannery effluent has also reduced 8% and 36% in pond water and tannery effluent, respectively. We assume that such high reduction of Cr(VI) in the tannery effluent probably due to the presence of organic and inorganic compounds as well as the probable presence of Cr resistant and reducing organism [14].

Enzymatic reduction of Cr(VI) has been described as one of the main mechanism that microorganism uses to survive in the chrome-polluted wastewater [27]. Zahoor *et al.* reported that cell-free extracts of *Bacillus* sp. JDM-2-1 reduced 83%, and *S. capitis* reduced 70% of 10 mg/L of toxic hexavalent chromium [14]. Previous study also showed the reduction of Cr(VI) caused by soluble type enzymes present in the cell-free extracts collected from *Bacillus* sp. [39]. In the present study, the cell-free extracts of SH-1 and SH-2 showed the reduction of Cr(VI) which indicates the production or the activity of the Cr reducing enzyme in the presence of Cr(VI).

Biosorption of heavy metals is considered an eco-friendly technique for the removal of toxic metals from the environment [13]. The current study revealed high sorption capacity of live biomass in response to different experimental conditions. At low pH, the biomass of isolates showed high biosorption rate as compared to high pH. Similar results were observed for the Cr(VI) biosorption by *Ochrobactrum anthropic* and green algae of which the maximal biosorption capacity was found at pH 2.0 [40, 41]. These results suggest that the negatively charged Cr species ($\text{Cr}_2\text{O}_7^{2-}$) bind through electrostatic attraction force to the positively charged ions on the biosorbents' surface. So, functional groups of biosorbent at low pH

become protonated and thus negatively charged Cr is attracted and binds in the surface. On the other hand, within pH 3.0 to 8.0 biosorption showed no statistically significant differences since at high pH deprotonation of biosorbent takes place and functional groups become negatively charged which prevents negatively charged Cr to bind with it [42].

The biosorption capacities of both the live and dead biomass were found to have increased with the increasing concentration of Cr ion. Similar trends were found in the biosorption study of the biomass of *Pantoea* sp. TEM18 [43]. This might be due to the increase in the competition for the close-fitted binding sites amount on the surface of biosorbents [44]; which indicates the properties of biosorbents (e.g., functional groups, surface area, etc.) and the properties of metal sorbates (e.g., atomic weight, ionic size, etc.) may play an important role in the biosorption process [43].

Biosorption rate increased during the first 60 minutes and slowed down after 90 minutes due to reaching the equilibrium phase. Biosorption rate might be depended on initial metal ion concentration as reported by others [44]. Adsorption isotherm models suggested by Freundlich and Langmuir were found suitable for describing the short-term biosorption of Cr(VI) in this study. The Langmuir isotherm describes monolayer sorption where the finite number of identical sites is present onto a surface and assumes constant energies of adsorption on the surface of the adsorbent. To represent the experimental data, the Freundlich model is appropriate where it is assumed that if the absorbing surface is heterogeneous the metal biosorbs to form monolayers [45].

The previous study showed that pea seeds germination was not significantly affected at high concentration of Cr, though the growth of radicle and plumule was suppressed at concentrations of 147 mg/L Cr(VI) [46]. Similar trends were also observed during our study of the effect of Cr(VI) on the seed germination. The data suggest that seed germination and successive seedling growth were interrupted by high levels of chromium in which more toxic effect was observed on roots rather than on shoots. But after using the bacterial treated chrome water, we observed the amelioration of the phytotoxicity. This evidence carries significant environmental benefits of using eco-friendly bacteria to reduce the toxicity of Cr(VI) by either reduction or biosorption.

In a nutshell, Figure 5 illustrates the overview of the work. The schematic diagram shows how toxic Cr is being leached from tannery to the effluent and then to the natural water body such as rivers and canals. Next, Cr resistant bacteria were isolated from that tannery effluent, and then significant biosorption and enzymatic reduction were found. Furthermore, a beneficial environmental effect was found by the chickpea seed germination and seedlings growth test.

Conclusion

Both the isolates studied here showed strong potential for the removal of hexavalent chromium in LB broth media, in tannery effluents as well as in pond water. Moreover, the present results illustrated the effective sorption capacity of live biomass of SH-1 with a high recapture of Cr(VI) within a short time. So, the outcomes considering their beneficial effect on chickpea seedlings support the possibility of

successful application of these chromium resistant bacteria for efficient bioremediation of tannery effluents containing toxic Cr(VI). The findings can be translated into technology by small-scale piloting and subsequently, in large scale to clean up chrome-polluted wastes including tannery effluents before discharging the wastes into the environment.

Abbreviations

ACS- American chemical society

TDS- Total dissolved solids

COD- Chemical oxygen demand

NTU- Nephelometric turbidity unit

ANOVA- Analysis of variance

NCBI- National center for biotechnology information

LB- Luria Bertani

NADH- Nicotinamide adenine dinucleotide

AAS- Atomic absorption spectrometry

PCR- Polymerase chain reaction

SPSS- Statistical package for the social sciences

Methods

Materials

Chemicals and reagents used in this study were ACS analytical grade unless stated otherwise. Double deionized water for all experiments was supplied from Mafco (MAR-100, Mirpur, Bangladesh) and Barnstead E-Pure Ultrapure Water Purification Systems (Thermofisher, USA). 1,5-diphenyl carbazide, NADH, $K_2Cr_2O_7$, $NiCl_2 \cdot 6H_2O$, $Pb(NO_3)_2$, $Co(NO_3)_2 \cdot 6H_2O$, $ZnCl_2$, $CdCl_2$, and $CuSO_4 \cdot 5H_2O$ were obtained from Sigma-Aldrich (St. Louis, MO, USA). $MnCl_2$ was purchased from Fisher Scientific, USA. All stock solutions were stored in amber glass bottles in the dark. Chick-pea seeds were obtained commercially from the retail market in the Dhaka city, Bangladesh and formally identified by one of the co-author Md. Shafiqul Islam, Ph.D.

Description of the sampling sites

The sampling site was selected based on the presence of heavy tannery industries where approximately 250 tannery industries are located [1]. In this study, tannery effluent samples were collected from different points of Hazaribagh and Hemayetpur tannery industrial area of Dhaka metropolitan area, Bangladesh, after obtaining appropriate permission from the authority (Figure S7).

Sample collection and physicochemical analysis

Tannery effluents were collected in wide-mouth (Nalgene, USA) sterilized bottles from a different point of Hazaribagh and Hemayetpur, Dhaka, Bangladesh from October to November 2015. Tannery effluents from fourteen tannery industries were taken from the nearby drain, close to the discharging outlet where it was thoroughly mixed with water from different sources. Five hundred (500) mL of effluent sample was collected for isolation and characterization of Cr(VI) resistant and reducing bacteria. Physicochemical parameters (temperature, °C; pH; salinity; electrical conductivity; total dissolved solids) were measured by portable pH meter (Orion- 2 STAR, Thermo Scientific, USA) and HACH conductivity meter (SensION5, USA) at the sampling points. Standard methods were used for the collection, processing, and measurement of chemical and physicochemical parameters [47]. Total chromium in the samples was determined by Atomic Absorption Spectrophotometer (AAS, Thermo Scientific, USA).

Screening of potential chromium reducing bacteria

For the isolation of chromium resistant bacteria, 50 µl of fourteen tannery effluents were separately spread on Luria-Bertani (LB) agar plates containing Cr(VI) concentration of 100 mg/L and incubated at 37°C for 24 h. After this incubation period, the presence of microbial growth was observed, and colonies were selected based on discrete morphological characteristics. Next, selected colonies were inoculated in LB broth media supplemented with increasing concentration of Cr(VI) from 500 mg/L to 2500 mg/L and incubated for 24h at 37°C in a rotary shaker incubator. Then, 50 µL of the cultures was dropped on nutrient agar (NA) media and incubated at 37°C for 24h. Colonies that survived at concentrations above 500 mg/L of Cr(VI) were selected from the NA plates and preserved for further study. Chromium reduction study of the selected isolates was carried out in LB broth medium containing 100 mg/L of Cr(VI). After 72 hours of incubation at 37°C in a rotary shaker (120 rpm), 1 mL from the respective cultures was taken, spun down at 1100 g for 5 min and supernatants were used to estimate remaining Cr(VI) concentration in the medium. The modified diphenylcarbazide method was used to determine the initial and final concentration of Cr(VI) according to the method used by Zahoor and Rehman [14]. Another screening was performed using laboratory isolates to find out the lowest resistant bacteria to use as a negative control.

16S rRNA identification of isolates

The bacterial genomic DNA was extracted according to the manufacturer's instruction using Maxwell 16 automated DNA extractor (Promega, USA). The 16S rRNA was amplified by conventional PCR using universal primer set for bacteria: 27F (5'-AGATTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTT ACGACTT-3') [36]. The amplification was performed in a thermal cycler (G2 Gene Atlas, Astec, Japan) and the program consisted of 30 cycles (initial denaturation at 95 °C for 5 min,

subsequent denaturation at 95 °C for 30 s, annealing at 48 °C for 30s, extension at 72 °C for 90 s, and final extension for 5 min at 72 °C). The PCR products were then purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and were used for Sanger Dideoxy Sequencing. The sequencing was performed in 1st Base Laboratories in Malaysia, and all generated sequences were submitted to the Genbank/National Center for Biotechnology Information (NCBI) database.

Determination of optimum growth condition of the isolates

Determination of the optimum temperature and pH for the growth of two isolates were performed according to the procedure described elsewhere [14]. The effect of Cr(VI) on the growth of bacteria was also investigated according to Zahoor and Rehman, 2009, with slight modifications [14]. Growth curves of bacterial isolates SH-1 & SH-2 were determined from LB broth medium with 50 mg/L concentration of Cr(VI) and without chromium as control. Here, an environmental isolate of *Vibrio cholerae* was used as a Cr(VI) sensitive organism (negative control). For each bacterial isolate, 10 ml medium was taken in sterilized falcon tube and then inoculated with 20 µL of overnight grown inoculum. The cultures were incubated at 37°C in a shaker at 120rpm. An aliquot of culture was taken out at regular intervals from 0h to 24h. Absorbance was measured at 600 nm.

Resistance to antibiotics and other heavy metals

Disk diffusion method was used to determine the antibiotic resistance properties of the isolated bacteria SH-1 and SH-2 [48]. After 24h of growth period, the inhibition zones were measured following the standard antibiotic disc sensitivity testing method [49]. Further, stock solutions of 5000 mg/L of different metal salts (lead nitrate, cadmium chloride, copper sulfate, zinc chloride, cobalt nitrate, manganese chloride, and nickel chloride) were prepared, and 50 mg/L were used to determine the heavy metal resistance of bacterial isolates (SH-1 & SH-2). Sterilized falcon tubes containing 10 ml LB medium with increasing concentration of respective metal ions were inoculated with 20 µL young bacterial cultures subsequently incubated in a rotary shaker incubator at 37°C for 48 hrs. Then droplets of 100 µL from the respective test tubes were inoculated onto nutrient agar (NA) medium and incubated at 37°C for 24 hrs. After this incubation period, the presence or absence of microbial growth concerning their heavy metal resistance ability was determined.

Analysis of reduction capacity of Cr(VI) by the isolates in the culture medium, pond water, and tannery effluents

The ability of bacterial isolates SH-1 & SH-2 to reduce Cr(VI) was examined in sterilized 125 mL Erlenmeyer conical flask. LB broth media (25 mL) with added Cr(VI) concentration of 100 mg/L as $K_2Cr_2O_7$ was inoculated with 20 µL of the selected young bacterial suspension. Cultures were incubated at 37°C with 120 rpm. Then 1 ml of the culture was taken after 24, 48 and 72 h, centrifuged at 1100 g for 5 min and the supernatant was analyzed for the residual Cr(VI) using 1,5-diphenyl carbazide method [14].

The efficiency of the bacterial isolates SH-1 and SH-2 to reduce Cr(VI) in LB broth as well as in surface water containing Cr(VI) were tested according to the method described by Zahoor and Rehman [14]. In these experiments, 25 mL pond water and tannery effluents were taken separately in sterilized 125 mL Erlenmeyer conical flask with added Cr(VI) concentration of 100 mg/L. Then, 20 µL of an overnight culture of the selected isolates were inoculated into the respective pond water and effluents. After incubation of 24, 48 and 72 h in an orbital shaker at 37°C, samples (1mL) were taken for the measurement of residual Cr(VI) in the solution using above method [14].

Enzyme assay

The bacterial isolates (SH-1, SH-2, and *V. cholerae*) were grown in 10 mL LB broth medium for 48 h at 37°C in a rotary shaker incubator with chromium (10 mg/L) and without chromium. Cells harvesting were performed by centrifugation at 1100 g for 5 min. Pellets were washed twice with 10 mM phosphate buffer (pH 7.2) and stored at -20°C. Then B-PER bacterial protein extraction reagent with lysozyme and DNase 1 (Thermo Scientific, Rockford, IL, USA) were used for cell lysis on ice. The remaining homogenate was centrifuged at 11000 g for 5 min at 4°C; the supernatants were used as a crude cell extract. Protein concentrations of the crude extracts were measured using the modified Lowry Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Chromate reductase activity of this soluble proteins was examined as described elsewhere [30]. A reaction mixture of 1 mL was prepared containing 10 mg/L chromate in 0.85 mL of 10 mM phosphate buffer (pH 7.2). Then 0.15 mL protein samples with NADH (14.3 µl from 140 µM) were added to the reaction mixture. In this experiment, protein samples, heated at 100°C for 30 min, were used as blank control and protein samples of *V. cholerae* acted as a negative control. After 12h of incubation at 37°C, Cr(VI) reduction was assessed according to the diphenylcarbazide method [14].

Batch biosorption experiments

The preparation of live and dead cell biomass was performed as described in other studies [43, 50]. A batch equilibrium method [51] was used to determine the sorption of Cr(VI) by the biomass of the isolates (SH-1 live, SH-2 live, SH-1 dead, and SH-2 dead). All sets of experiments were done with 10 mg of the dried biomass in fixed volume (10 ml) of Cr(VI) solution in sterilized falcon tube (15 ml). Bacterial biomass was exposed to Cr(VI) solution separately for 6h on a rotary shaker (140 rpm) at 30°C. The effects of pH, contact time, and initial Cr(VI) concentration on biosorption were recorded. All pH values of the solutions were adjusted from 1.0 to 8.0 using 1N HCl and 1N NaOH. The samples were taken at different time intervals and centrifuged at 1100 g for 5 minutes afterward, the supernatant was analysed for Cr(VI) concentration by 1,5 diphenylcarbazide method [14].

Measurement of metal uptake

The formulae described by Vanderborght and Van Griekenm was used to calculate the amount of adsorbed Cr(VI) (mg/g) [52], which is as follows:

$$Q = V (C_i - C_f) / M \dots\dots\dots (1)$$

Where, Q = capacity of Cr ion uptake (mg/g), V = adsorbate volume (L), C_i = initial concentration of Cr in solution before the sorption analysis (mg/L), C_f = final concentration of Cr in solution after the sorption analysis (mg/L), M = Dry weight of biosorbent (g).

Adsorption isotherm analysis

The biosorption equilibrium study was carried out according to Langmuir and Freundlich isotherm models described by Dada *et al.* [45]. The Langmuir adsorption equation (i)

$$Q_o = \frac{Q_o K_L C_e}{1 + K_L C_e} \quad (i)$$

The parameters of the Langmuir adsorption model were transformed into linear form as follows.

$$\frac{C_e}{Q_o} = \frac{1}{Q_o K_L} + C_e \quad (ii)$$

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$$Q_o K_L C_e$$

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Where: Q_e = the amount of Cr(VI) adsorbed per gram of biomass at equilibrium (mg/g), C_e = the equilibrium concentration of Cr(VI) (mg/L), K_L = Langmuir isotherm constant (L/mg), Q_0 = maximum monolayer coverage capacity (mg/g).

The equilibrium parameter R_L , the separation factor, is a dimensionless constant in Langmuir isotherm that is used to describe the adsorption nature of Cr(VI) [53].

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$1 + (1 + K_L C_0)$

00

$1 + (1 + K_L C_0)$

$R_L =$

Freundlich model is suitable for the heterogeneous surface of sorbents to define the adsorption characteristics [45]:

$Q_e = K_f C_e^{1/n}$ (iv)

Where: C_e = the equilibrium concentration of Cr(VI) (mg/L), K_f = Freundlich isotherm constant (mg/g), Q_e = the amount of Cr(VI) adsorbed per gram of the adsorbent at equilibrium (mg/g), n = adsorption

intensity. Linear form of the above equation (iv) as follows:

$$\log Q_e = \log K_f + 1/n \log C_e \dots\dots\dots (v)$$

K_f indicates the approximate adsorption capacity, $1/n$ is a function of the strength of adsorption [54]. When $n = 1$, the partition between phases are not dependent of the concentration, $1/n < 1$ indicates a normal adsorption, and $1/n > 1$ designates cooperative adsorption [55]. A favorable sorption process is attained if n is in between 1 to 10 [56].

Assessment of toxicity of Cr(VI) on Chick Pea seed germination

For each group, three chickpea seeds were used, and fungal contamination was avoided by immersing seeds into 3% (v/v) formaldehyde solution for 5 min. Seeds were then washed with double deionized water followed by placing in a petri dish containing filter-sterile pond water with different concentration of Cr(VI) (12.5, 25, 50 and 100 mg/L) and without Cr(VI) as control. Seeds were set under 12/12 h light/dark cycle and temperature of around 25°C during the day & around 18°C during the night. Growth of seedlings was observed and measured after one week.

To determine the Cr(VI) reducing effect, isolates (SH-1 & SH-2) were incubated with Cr(VI) and then added into the seed germination plate with or without 50 mg/L Cr(VI) as described above. The filter-sterile pond water was used as control and pond water with 50 mg/L Cr(VI) was used as a negative control. Seeds were placed under the same conditions described above. Germination rate, as well as root and shoot length, were observed and measured after one week.

Statistical analysis

The Statistical package for the social sciences (SPSS) and Microsoft Excel were used for the analysis of experimental data (SPSS version 20.0, IBM). All the experiments were run in triplicates unless otherwise stated. One way Analysis of Variance (ANOVA) and students t-test was used as statistical analysis to validate the results and to ensure the variability of data as well as Tukey's (Post-Hoc) Test ($p < 0.05$) was performed to compare the mean values as described in the specific figure legends. All data in the figure and table are expressed as mean $\pm$ standard deviation (SD).

Declarations

Ethics approval and consent to participate

The Ethical Review Committee of International Centre for Diarrhoeal Disease Research, Bangladesh approved the study.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SH1, SH2, MRI, KMI, MSI, ZHM conceived and designed the experiments. SH1, SH2, MRI, SA, MHK performed the experiments. SH1, SH2, MRI analyzed the data. MHK, SA, MSI, TJM, AKP, NA, ZHM contributed reagents/materials/analysis tools. SH1, SH2, KMI, NA, ZHM wrote the paper. All authors read and approved the final manuscript.

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Figure Legends

Figure 1 Screening of Cr(VI) reducing bacteria from tannery effluent. (A) Schematic diagram of Cr(VI) resistant organism isolation from tannery effluent. (B) Resistance of the isolates at different concentration of Cr(VI). (C) Representative growth plates of SH-1 (Top image) and SH-2 (Bottom image) isolates from Cr(VI) resistance experiment. (D) Reduction of Cr(VI) by the isolates in LB broth with an initial concentration of 100 mg/L after 72 h of incubation at 37°C. One way ANOVA followed by Tukey's Post-Hoc Test was performed to find statistical significance. “*”denotes significant difference between

negative control (Vc) and other isolates (*P <0.05, **P < 0.01, ***P < 0.001). (E) Resistance to different heavy metals (This experiment was done once).

Figure 2 Cr(VI) reduction ability of the isolates (SH-1 and SH-2) in different media. (A) Effect of Cr(VI) on the growth of SH-1. (B) Effect of Cr(VI) on the growth of SH-2. (C) Reduction of Cr(VI) by the isolates in LB broth with an initial concentration of 100 mg/L after 24, 48 and 72 h of incubation at 37°C. (D) Reduction of Cr(VI) by the isolates in pond water with an initial concentration of 100 mg/L after 24, 48 and 72 h of incubation at 37°C. (E) Reduction of Cr(VI) by the isolates in tannery effluents with an initial concentration of 100 mg/L after 24, 48 and 72 h of incubation at 37°C. Here, LB, PW, and TE denote experiments conducted in Luria Bertani media, Pond Water and Tannery Effluent, respectively. In figure 2C, D and E, paired two-tailed students t-test was performed between control and the study group and data are expressed as mean ± standard deviation (SD) (*P <0.05, **P < 0.01, ***P < 0.001).

Figure 3 Biosorption assay of isolates and Isotherm Model of the SH-1 live biomass. (A) Effect of pH on biosorption process (B) Effect of initial concentration of Cr(VI) on biosorption process (C) Effect of contact time on biosorption process (D) Langmuir isotherm model for SH-1 live biomass (E) Freundlich isotherm model for SH-1 live biomass (F) Isotherm model constants for adsorption of Cr(VI) by SH-1 live biomass.

Figure 4 Phytotoxicity test using chickpea seeds. (A) Determination of toxicity of different concentration of Cr(VI) on seed germination and growth. (B) The mean shoot and root length of the seeds of figure 4A. (C) Images of reduction of the toxicity of Cr(VI) by bacterial treated samples. (D) The mean shoot and root length of the treated seeds of figure 4C. In figure B and D paired two-tailed students t-test was performed between control and the study group and data are expressed as mean ± standard deviation (SD). “*” denotes significant differences between control group and study group (*P <0.05, **P < 0.01, ***P < 0.001). “#” indicates significant differences between Cr(VI) 50mg/L and study group (#P <0.05, ##P < 0.01).

Figure 5 The schematic diagram shows an overview of the work.

Table legends

Table 2

^a The amount of enzyme that converts 1.0 µM Cr(VI) per min at 37°C was defined as one unit of Cr(VI) reductase activity.

^b Cr(VI) reduction was measured after 12 h of incubation.

All data in the table are expressed as mean ± standard deviation (SD).

Additional files

Supplementary data 1: Table S1: Analysis of Physicochemical properties of Tannery effluents.

Supplementary data 2: Table S2: Bangladesh National Standards – Waste Discharge Quality Standards for Industrial Units and Projects: Quality at Discharge Point. Notes: (1) Land Surface Water refers to any pond, tank, water body, water hole, canal, river, spring or estuary Public Sewer refers to any sewer connected with fully combined processing plant including primary and secondary treatment Irrigated Land refers to an appropriately irrigated plantation area of specified crops based on quantity and quality of wastewater.

Source: http://old.doe.gov.bd/publication_images/15_etp_assessment_guide.pdf

Supplementary data 3: Table S3: Morphological characteristics of Cr(VI) resistant isolates from tannery effluents. Bold lines indicate selected isolates for further study. Selected isolates were renamed as SH-1 and Sh-2 as indicated in the table S3.

Supplementary data 4: Table S4: Antibiotics sensitivity of SH-1.

Supplementary data 5: Table S5: Antibiotics sensitivity of SH-2.

Supplementary data 6: Fig S6. Biosorption assay of isolates and Isotherm Model of the SH-2 live biomass.

Supplementary data 7: Fig S7: Locations from where the 14 samples were collected. 12 samples were collected from Hazaribagh area of Dhaka, Bangladesh (A) and 2 samples from Hemayetpur, Dhaka, Bangladesh (B).

Supplementary Files

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