



Microbial Reduction and Detoxification of Chromium from Tannery Effluent by Natural Inhabitants

Sawkat Ara Pinki^(**), Md. Reazul Karim^{*}, Dipankar Dewanjee^{*}, Habibur Rahman Bhuiyan^{***}, H. M. Abdullah Al Masud^{*} and Md. Imranul Hoq[†]

^{*}Department of Microbiology, Faculty of Biological Sciences, University of Chittagong, Chattogram-4331, Bangladesh

^{**}National University, Gazipur-1704, Bangladesh

^{***}Industrial Microbiology Research Division, Bangladesh Council of Scientific and Industrial Research, Chattogram-4220, Bangladesh

[†]Corresponding Author: Md. Imranul Hoq; imran.mbio@cu.ac.bd

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ABSTRACT

Chromium (Cr), a chemical agent, has long been used extensively in leather tanning. Hexavalent chromium (Cr-VI) found in tannery effluent is highly toxic, carcinogenic and mutagenic to humans. Transformation of Cr-VI to its trivalent counterpart, Cr-III, is the basic process in its detoxification, and microbial transformation of Cr-VI to Cr-III has been one of the most widely studied forms of Cr bioremediation. This study aims to explore the ability of naturally occurring bacteria in reducing and detoxifying Cr in vitro and also from tannery effluent. Five efficient Cr reducing and detoxifying bacteria were isolated from tannery effluent, their morphological, cultural, physiological and biochemical characteristics investigated. They were identified as *Aeromonas eucrenophila*, *Bacillus megaterium*, *B. carboniphilus*, *B. licheniformis* and *B. subtilis*. Coincubation of the isolates with varying concentrations of potassium dichromate ($K_2Cr_2O_7$), a Cr salt, in minimum salts medium, pH 7 revealed notable reduction and detoxification of Cr within 24-72 h as determined by 1,5-diphenylcarbazide colorimetric method and atomic absorption spectrophotometry, respectively. The isolates exhibited substantial resistance or tolerance to 125 to 500 ppm $K_2Cr_2O_7$. Bacterial detoxification or reduction of was increasingly increased as the incubation period increased from 24 to 48 or 72 h and substrate concentration increased from 125 to 250 or 500 ppm. Most of the isolates exhibited increased reduction and detoxification at 37°C compared to that at 30°C or 45°C, and at pH 7 or 8 compared to that at pH 5 or 6. Furthermore, all the isolates exhibited highest detoxification or reduction when peptone was used as carbon source instead of glucose or ammonium acetate. In a chosen or optimized condition of 37°C temperature, pH 7, 125 ppm $K_2Cr_2O_7$ concentration and 48 h incubation period, most isolates exhibited 85-99% Cr reduction and detoxification from tannery effluent. It was, therefore, inferred that the isolates have potential as biological agent in reducing and detoxifying Cr from industrial effluent.

INTRODUCTION

Industrial pollution is a major factor to cause significant degradation to the environment around us. Since industrialization is continuously growing worldwide, in the present time, industrial wastes play the most common source of water pollution (Ogedengbe & Akinbile 2004). In a developing country like Bangladesh, solid waste or effluent from a tannery is directly discharged into water bodies or sewers without treatment (Verheijen et al. 1996; Favazzi 2002). Hence, tannery wastewater is thus found extremely polluted in terms of total dissolved solids (TDS), total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), heavy metals, etc. (Mondal et al. 2005). In Bangladesh, tanning industries are one of the main economic activities. Bangladesh now has 165 leather and footwear factories. Furthermore, it has 161

tanning industries that process rawhides into finished leather. The vast majority of these industries are located in and around Dhaka, whilst a very few are scattered all over the country (Gain & Moral 1999). A very recent study has expressed an estimation that a total of 232-ton solid waste and 20-million-liter wastewater are produced every day from these industries (Saha & Azam 2021). In the tanning industries, extensive use of Cr in the chrome tanning process results in the release of Cr that contaminates soil and water at production sites (Turick et al. 1996). The solid waste or wastewater discharged from the tanneries contains substantial amount of Cr (Franco et al. 2005). Cr is a highly toxic heavy metal and is known to be mutagenic, carcinogenic and teratogenic to humans, animals and plants. However, Cr exists in nature in various oxidation states ranging from divalent to hexavalent ones. Cr-III and Cr-VI are the most stable states, and the biological, toxicological and geochemical properties of them

vary remarkably. Cr-VI is known to be much more toxic than Cr-III for multiple reasons. In nature, Cr-III exists at a narrow range of concentration, and is an essential element because it regulates the metabolism of glucose in human body (Srinath et al. 2001). In contrast, Cr-VI is highly soluble in water, rapidly permeable through biological membranes, and prone to subsequently interact with intracellular proteins and nucleic acids. Cr-VI interacts with DNA quite indirectly, and exhibits genotoxicity via reactive intermediates produced during its intracellular reduction to Cr-III. The types of DNA damage caused by Cr-VI is thereby grouped into two major categories: oxidative DNA damage and Cr-III-DNA interaction (Sobol & Schiestl 2012). Even at very low concentration, Cr-VI is highly toxic and hazardous to human health. Other than Cr, the most commonly occurring heavy metals in tannery wastewater include lead, manganese, cadmium and copper. Occurrence of these toxic substances in surface water and soil leads to serious public health threat and significant risk to the ecological system (Sundar et al. 2010). The present study thus aims to investigate the ability of naturally occurring bacteria in reducing and detoxifying Cr at privileged nutritional and environmental conditions, and evaluate their potentiality as candidate agent of bioremediation of the heavy metal.

MATERIALS AND METHODS

Collection of Sample

Tannery effluent samples were collected in sterile plastic bottles from RIFF Leather Industries, Kalurghat Industrial Area, Chattogram, Bangladesh. They were immediately placed in an insulated box with frozen refrigerant packs in an insulated box and transported to the laboratory. Other than instant analysis, the samples were preserved at 4°C for further analysis.

Isolation of Bacteria from Tannery Effluent

The nutrient agar medium was used for the isolation of bacteria from the effluent, where the pH of the medium was adjusted to the pH of the sample. Pour plate and streak plate techniques were applied for the isolation, and well discrete identical single colonies were picked up and transferred into nutrient agar slants. In case no single colony appeared onto any plate, repeated streaking of the culture was carried out from plate to plate until at least one single colony was appeared. The slants were sealed in polyethylene bags and preserved in a refrigerator at 4°C as stock culture for further study. Subculturing at 2-week interval was maintained to keep the physiology and metabolic activity of the isolates unimpaired.

Cr Salt Used for *In Vitro* Investigation

Chemical name: Potassium dichromate

Chemical formula: $K_2Cr_2O_7$

Molecular weight: 294

Atomic weight: 52

Screening of Chromium (Cr) Reducing or Detoxifying Bacteria

The isolates possessing the ability to detoxify or reduce Cr in vitro were screened by incubating mid logarithmic growing cells of 106/mL density in varied concentrations of $K_2Cr_2O_7$ supplemented minimal salts medium (MSM) broth base, pH 7 for a substantial period of time (Bibi et al. 2012), and measuring the concentration of residual Cr-VI or total Cr, respectively, in the culture supernatant.

Measurement of Cr-VI Concentration

The concentration of residual Cr-VI in $K_2Cr_2O_7$ supplemented MSM culture supernatant following centrifugation at 4000 rpm for 5 min was measured by diphenylcarbazide colorimetric method (APHA 1998). In this method, 1,5-diphenylcarbazide (DPC) reagent was used as a colorimetric indicator, which reacted with Cr-VI in strongly acidic solution and resulted in the formation of magenta colored carbazone inner complex salt of a chromous ion (Cr-II). The absorbance of the color was measured at 540 nm in a spectrophotometer (Shimadzu UV-VIS 1800, Japan). In the course of the measurement, a calibration curve was prepared where the standard solution was diluted ranging the concentrations of 0.2-1 ppm by an interval of 0.2 ppm. The possible slight loss of Cr during digestion or other operations of the analysis was compensated by adding no inoculum to the standard solutions but following the same treatment to be applied to the sample. The calibration curve was constructed by plotting the blank-subtracted absorbance of the standard solutions versus respective concentration.

Measurement of Total Cr Concentration

The concentration of total Cr in $K_2Cr_2O_7$ supplemented MSM culture supernatant following centrifugation at 4000 rpm for 5 min was measured by atomic absorption spectrophotometry (APHA, WEF, AWWA 1992). In this method, the supernatant was mixed to double volume of concentrated HNO_3 , and the mixture was heated to 100°C by a proper hollow cathode lamp of 10 mA current on a hot plate in an atomic absorption spectrophotometer (AAS) (Shimadzu AA-7000, Japan) to accomplish acid digestion until the final volume went down to initial supernatant volume. The extract was filtered through a Whatman 42 filter paper to remove any insoluble material, collected into a volumetric flask and diluted. The chrome

plating operation emitted total Cr, which was quantified by measuring the absorbance at 357.9 nm. In the course of the measurement, a calibration curve was prepared where the standard solution was diluted ranging the concentrations of 1-8 ppm by an interval of 2 ppm. The calibration curve was constructed by plotting the blank-subtracted absorbance of the standard solutions versus respective concentration.

Characterization of Cr Detoxifying or Reducing Bacteria

The bacterial isolates that possessed the ability to reduce or detoxify Cr following in vitro investigations were subjected to cultural, morphological, physiological and biochemical examinations. Cultural examinations include colony characteristics of the isolates onto nutrient agar plate, i.e., form, color, elevation, margin, surface and their growth pattern onto nutrient agar slant. Morphological examinations include Gram and acid-fast staining of the isolates and microscopic observation of their shape and arrangement. Physiological and biochemical examinations include motility, indole, Voges-Proskauer, methyl red, Simmons citrate, nitrate reduction, triple sugar iron, H₂S production, catalase, oxidase and urease tests.

Determination of Bacterial Resistance or Tolerance to Cr

Bacterial isolates exhibiting substantial ability to reduce or detoxify Cr were examined to determine their resistance or tolerance to Cr by a broth dilution method. In this method, mid logarithmic growing cells of 10⁶ cells/mL density were incubated in brain heart infusion broth supplemented with varied concentration of K₂Cr₂O₇ for 24 hours at 37°C with agitation at 100 rpm. The turbidity of the broth, proportionate to bacterial growth, was measured at 600 nm in a UV-visible spectrophotometer (PG Instruments-UK, Model: T 80+ UV-VIS). The isolates that exhibited good to moderate growth at a significant concentration of Cr salt were considered as resistant or tolerant to Cr.

Determination of Minimum Inhibitory Concentration of Cr Against Bacterial Isolates

Minimum inhibitory concentration (MIC) of Cr against the isolates was determined by the broth dilution method described above. In this case, the minimum concentration of K₂Cr₂O₇ that prevented visible growth of an isolate was considered as MIC of Cr against that particular organism.

Determination of Minimum Bactericidal Concentration of Cr Against Bacterial Isolates

The broth culture of MIC test was applied as inoculum to determine minimal bactericidal concentration (MBC), where 100 µL of 18-h old culture was inoculated by pour plate method to nutrient agar plate. The plate was incubated at

37°C and the appearance or absence of growth was observed at 24 h. The minimum concentration that resulted in complete absence of growth onto the plate was considered as MBC.

Determination of Optimum Conditions for Bacterial Detoxification or Reduction of Cr

The efficiency of bacterial isolates in detoxifying or reducing Cr in vitro were investigated further as described above, whereas variety of conditions applied to determine the optimum ones that include K₂Cr₂O₇ concentration in MSM base, incubation period, reaction temperature, medium pH, and additional supplementation of the medium with glucose, peptone or ammonium acetate as carbon source. In every case, a number of pilot experiments were conducted to reveal closely fitted parameters.

Evaluation of Bacterial Detoxification or Reduction of Cr from Tannery Effluent in Optimized Conditions

Fresh tannery effluent sample was collected as described above and sterilized by filtration using 0.22 µ filter. The initial concentration of Cr-VI and total Cr in the effluent was determined as described above. The pH of the sample was adjusted to the optimum for each isolate, inoculated by mid logarithmic growing cells of 10⁶ cells/mL density and incubated at optimized temperature for optimized time. The efficiency of the isolates was evaluated by measuring the residual Cr-VI or total Cr concentration as described above. The data were compared with that obtained under unoptimized conditions.

RESULTS

Cr Detoxifying or Reducing Ability of Bacterial Isolates from Tannery Effluent

A total of 16 pure cultures were isolated on nutrient agar slants from the nutrient agar plates following pour and streak plate inoculations of undiluted and diluted tannery effluent samples. The isolates were designated as E1-E16 and investigated for their ability to detoxify or reduce Cr within 24, 48 or 72 h in MSM base, pH 7 supplemented with 125, 250 or 500 ppm K₂Cr₂O₇ and incubated at 37°C. Interestingly, most of the isolates exhibited their ability to detoxify or reduce Cr in vitro at various time periods or Cr salt. However, both detoxification of Cr-VI and reduction of total Cr by the isolates designated as E3, E7, E9, E11 and E14 at 48 h and 250 ppm K₂Cr₂O₇ were remarkable, as plotted in Fig. 1. Hence, these five isolates were considered as potential Cr detoxifiers or reducers and chosen for further investigations.

Identification of Cr Detoxifying or Reducing Bacteria

The cultural, morphological, physiochemical and biochemical

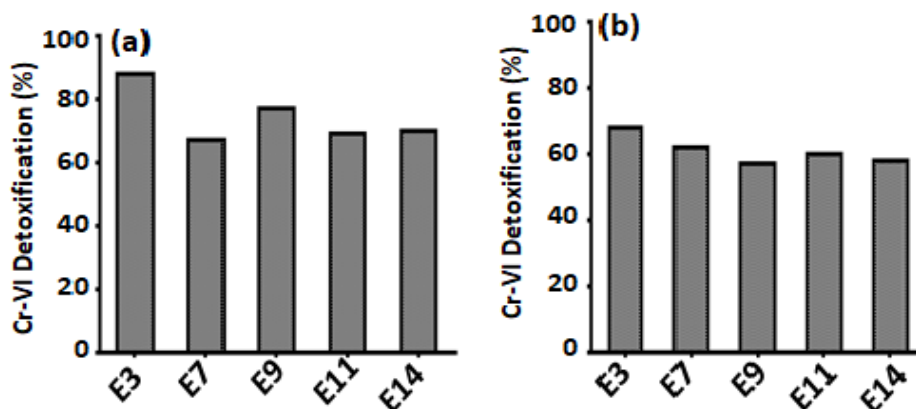


Fig. 1: Detoxification of Cr-VI and reduction of total Cr by selected isolates from tannery effluent. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 250 ppm of $K_2Cr_2O_7$ and incubation at $37^\circ C$ for 48h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

characteristics of the chosen 5 bacterial isolates that exhibited prominence in detoxifying or reducing Cr, as visualized in Table 1, were compared with the standard descriptions of wild bacteria according to Bergey's manual of determinative bacteriology, 8th (Buchanan & Gibbons 1974) and 9th (Goodfellow et al. 1994) editions. Comparative study revealed that the characteristics of the isolate designated as E3 closely resembled with that of *Aeromonas eucrenophila*. The isolate E3 was, therefore, identified as *Aeromonas eucrenophila*. Likewise, through comparative analysis, the isolate E7 was identified as *Bacillus megaterium*, E9 as *Bacillus carboniphilus*, E11 as *Bacillus licheniformis* and E14 as *Bacillus subtilis*.

Bacterial Resistance or Tolerance to Cr

In order to evaluate the candidacy of Cr detoxifying or reducing bacteria as biological agent, we examined their level of resistance or tolerance to Cr by culturing the isolates in brain heart infusion broth supplemented with various concentrations of $K_2Cr_2O_7$. The isolates exhibited diverse patterns of resistance or tolerance to Cr as revealed by their level of growth (Fig. 2). For example, the isolate *A. eucrenophila* exhibited tremendous resistance up to 125 ppm $K_2Cr_2O_7$ concentration, which dramatically reduced when the concentration increased to 250 ppm. On the other hand, the other isolates exhibited a dose dependent pattern of resistance with little fluctuations.

MIC and MBC of Cr Against Bacterial Isolates

The MIC of Cr against *A. eucrenophila* and *B. megaterium* was found 125 ppm, whereas against *B. carboniphilus*, *B. licheniformis* and *B. subtilis*, it was found 500 ppm. The MBC of Cr against all the isolates were found 2000 ppm

(Table 2). Fig. 3 visualizes MBC values of *A. eucrenophila* and *B. carboniphilus*.

Concentration and Time Dependent Bacterial Detoxification or Reduction of Cr

The prevailing conditions with regard to Cr detoxification and reduction by the isolates were investigated in this study. The first attempt was made for Cr concentration and incubation period. Since the isolates exhibited substantial degree of resistance or tolerance to 125-500 ppm $K_2Cr_2O_7$ and also the MIC values of $K_2Cr_2O_7$ against the isolates ranged from 125-500 ppm at 24 h, we investigated the efficiency of the isolates at 125, 250 and 500 ppm $K_2Cr_2O_7$ at 24, 48 and 72 h. As illustrated in Figs. 4-8, most of the isolates were remarkably efficient in detoxifying or reducing Cr at all the concentrations applied. Notably, a 2-fold increase in concentration of $K_2Cr_2O_7$ from 125 to 250 did not cause massive variation in their efficiency. The same was observed when the concentration was increased from 250 to 500 ppm. In contrast, the efficiency of the isolates was significantly increased when the incubation period was increased from 24 to 48 h, or from 48 to 72 h at any concentration applied.

Temperature Dependent Bacterial Detoxification or Reduction of Cr

The effect of temperature on Cr detoxification or reduction by the bacterial isolates was evaluated at 30, 37 and $45^\circ C$ while keeping the concentration of $K_2Cr_2O_7$ as 125 ppm and incubation period as 48 h. Most of the isolates appeared to exhibit maximum level of Cr detoxification or reduction at $37^\circ C$, and with few exceptions the efficiency of their detoxification or reduction was reduced when the temperature

Table 1: Cultural, morphological, physiological and biochemical characteristics of Cr detoxifying or reducing bacteria.

Characteristics	Observations				
	E3	E7	E9	E11	E14
Cultural					
Colony Form	Circular	irregular	irregular	circular	punctiform
Colony Color	Off-white	Off-white	Off-white	cream	cream
Colony Elevation	Raised	Flat	Flat	Flat	Convex
Colony Margin	Serrate	serrate	erose	entire	entire
Colony Surface	Smooth	smooth	smooth	smooth	smooth
Slant Character	Echinulate	Echinulate	Echinulate	Filiform	Filiform
Staining and Morphology					
Gram Staining	-	+	+	+	+
Acid Fast Staining	-	-	-	-	-
Shape	Short rod	Short rod	Short rod	Short rod	Short rod
Arrangement	Single	Single or pair	Single	Single	Single
Physiological and Biochemical tests					
Motility test	+	+	-	+	+
Indole	+	-	-	-	-
Voges-Proskauer	-	-	-	+	-
Methyl red	+	+	-	-	-
Simmons' citrate	-	+	-	-	+
Nitrate Reduction	+	+	-	+	+
Catalase	+	+	+	+	+
Oxidase	-	+	+	-	+
Urease	-	-	-	-	-
TSI	a/k	a/nc	a/k	a/k	a/a
H ₂ S production test	-	-	+	-	-

+, positive; -, negative; k/a, alkaline slant, acidic butt; a/nc, acidic slant, no change; a/a; acidic slant, acidic butt

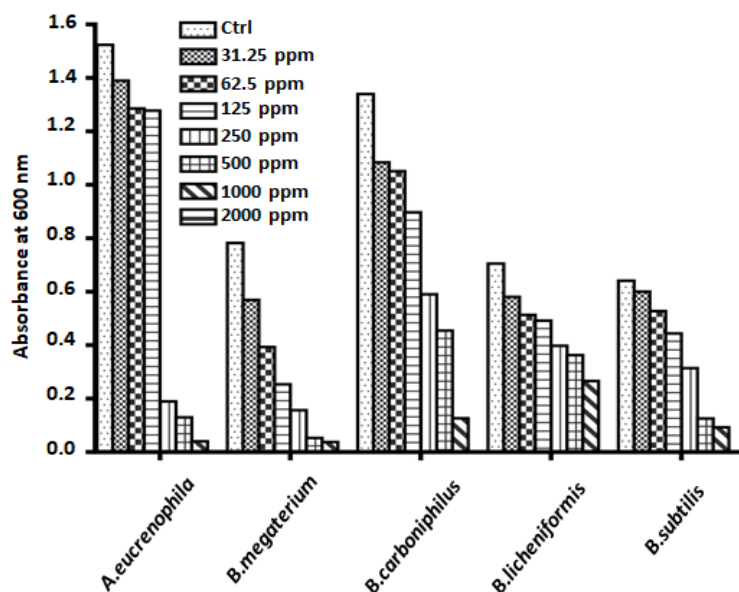


Fig. 2: Resistance or tolerance pattern of bacterial isolates from tannery effluent against Cr. The experiment was conducted by inoculation of brain heart infusion broth supplemented with 31.25, 62.5, 125, 250, 500, 1000, 2000 ppm of K₂Cr₂O₇ and incubation at 37° C for 24 h followed by the measurement of turbidity as bacterial growth at 600 nm in a UV-visible spectrophotometer.

Table 2: Growth of bacteria from broth dilution minimum inhibitory concentration (MIC) tests onto a nutrient agar plate

Bacteria	Concentration of $K_2Cr_2O_7$ (ppm)		
	500	1000	2000
<i>A. eucrenophila</i>	++	+	-
<i>B. megaterium</i>	++	+	-
<i>B. carboniphilus</i>	+++	+	-
<i>B. licheniformis</i>	+++	++	-
<i>B. subtilis</i>	++	+	-

Note: Positive (+ = scanty, ++ = moderate, +++ = good), Negative (-)

was either increased to 45°C or decreased to 30°C (Fig. 9).

pH Dependent Bacterial Detoxification or Reduction of Cr

In order to figure out the favorable pH of the isolates in detoxifying or reducing Cr, we incubated the isolates in 125 ppm $K_2Cr_2O_7$ supplemented minimal salt medium base adjusted to pH 5, 6, 7 and 8 at 37°C for 48 h and examined their efficiency. In case of detoxification of Cr-VI, all the isolates exhibited maximum efficiency at

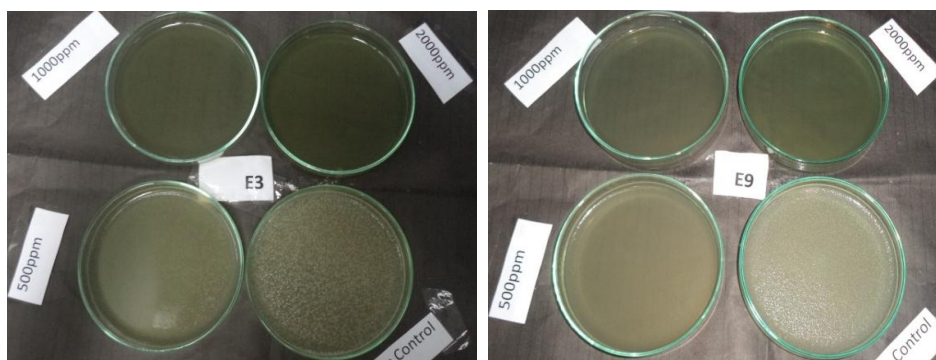


Fig. 3: Growth of (a) *A. eucrenophila* and (b) *B. carboniphilus* onto nutrient agar plate inoculated by overnight culture of brain heart infusion broth supplemented with varied concentration of $K_2Cr_2O_7$. The experiment was conducted by inoculation of nutrient agar plate by 18 h old culture of brain heart infusion broth supplemented with 500, 1000 and 2000 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 24 h.

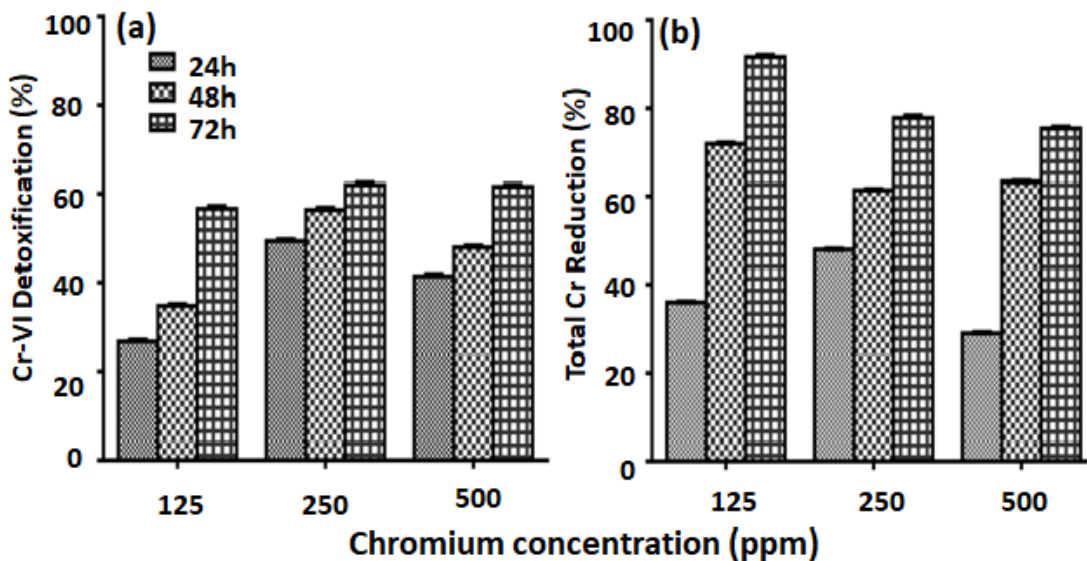


Fig. 4: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by *A. eucrenophila*. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125, 250 and 500 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 24, 48 and 72 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

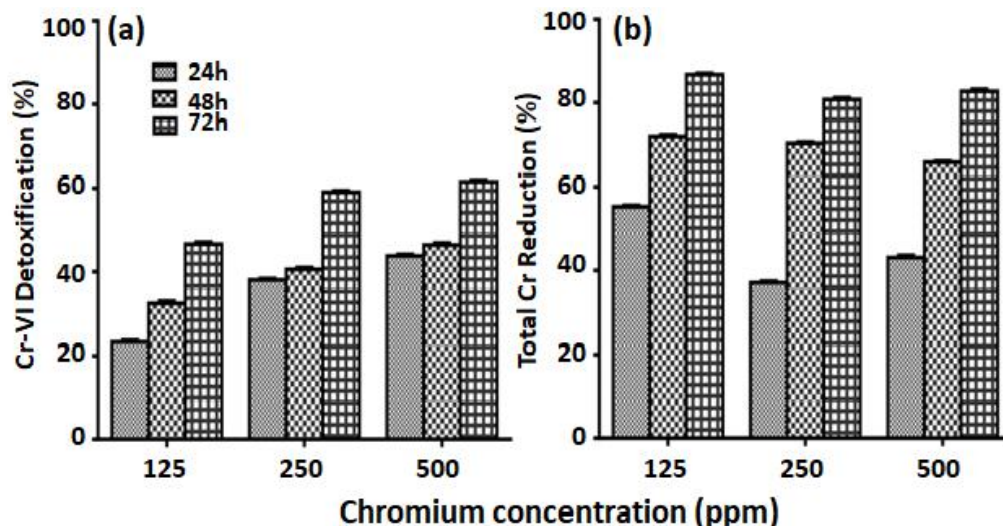


Fig. 5: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by *B. megaterium*. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125, 250 and 500 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 24, 48 and 72 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

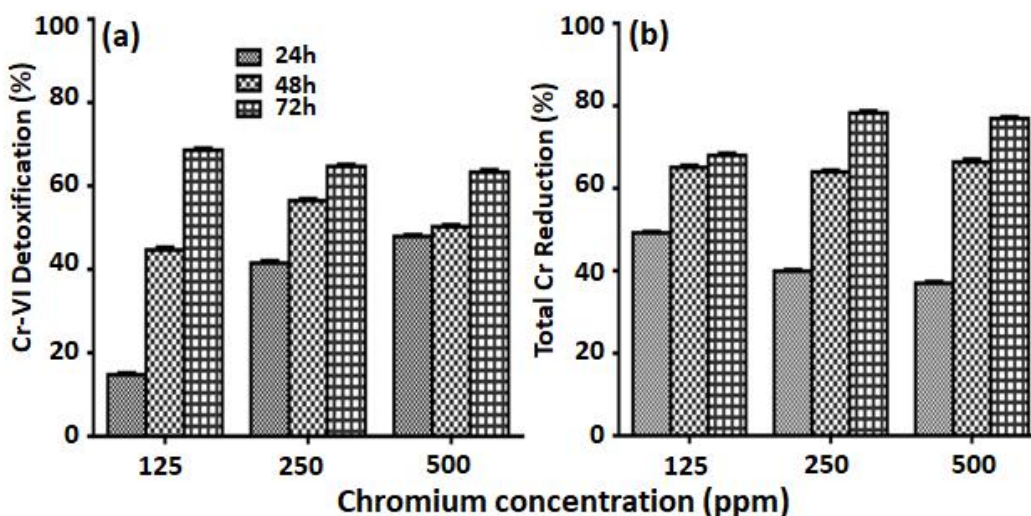


Fig. 6: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by *B. carboniphilus*. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125, 250 and 500 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 24, 48 and 72 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

both pH 7 and 8 (Fig. 10a). The efficiency of detoxification was decreasingly decreased when the pH of medium was decreased to 6 or 5. On the other hand, 4 out of 5 isolates exhibited maximum reduction of total Cr at pH 7 (Fig. 10b). *B. subtilis* is the only exception that exhibited maximum efficiency of reduction at pH 8. Likewise, with lowering of pH to 6 or 5, the efficiency of reduction of decreasingly decreased.

Carbon Source Dependent Bacterial Detoxification or Reduction of Cr

The effect of carbon source or co-substrate on Cr detoxification or reduction by the bacterial isolates was evaluated by using glucose, peptone or ammonium acetate in the test medium while keeping the concentration of $K_2Cr_2O_7$ as 125 ppm, incubation period as 48 h and temperature at 37°C.

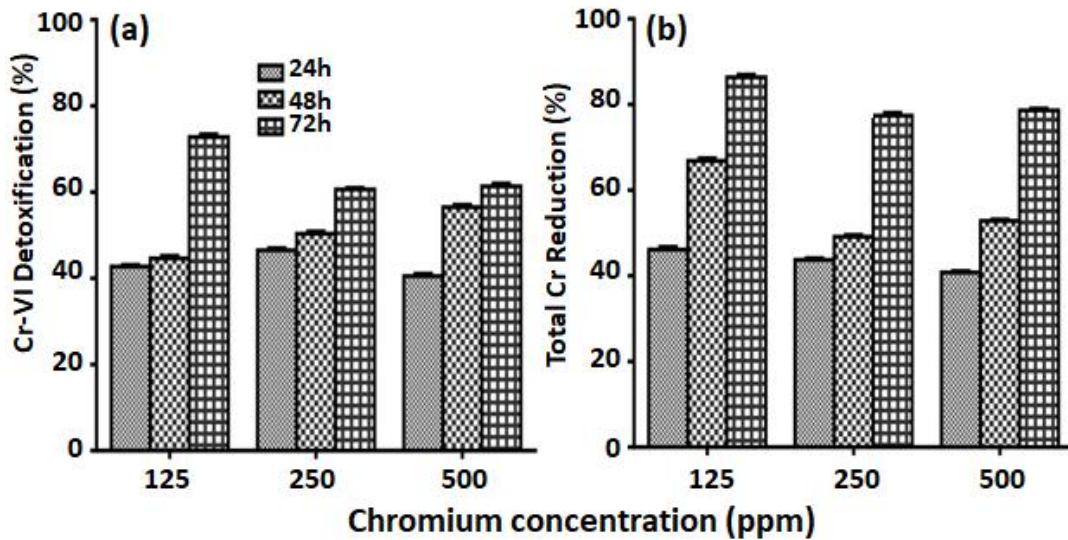


Fig. 7: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by *B. licheniformis*. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125, 250 and 500 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 24, 48 and 72 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

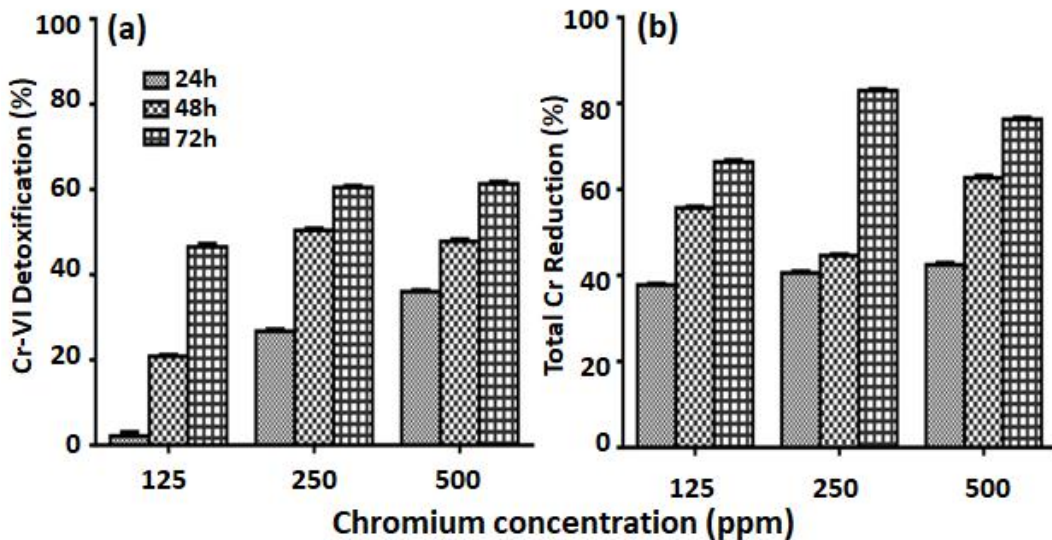


Fig. 8: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by *B. subtilis*. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125, 250 and 500 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 24, 48 and 72 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

Notably, 4 among 5 isolates detoxified or reduced Cr much better in peptone rich medium compared to in glucose or ammonium acetate rich ones. The only exception lied for *B. megaterium*, which exhibited better detoxification of Cr-VI in ammonium acetate rich medium but reduction of total Cr in glucose rich medium (Fig. 11).

Potential of the Isolates in Detoxifying or Reducing Cr from Tannery Effluent

In order to evaluate the candidacy of the isolates in

detoxifying or reducing Cr from tannery effluent, freshly collected sample was subjected to bacterial treatment in optimized conditions. Before inoculation, the sample was first sterilized by filtration to nullify the effect of existing microbial population. The pH of the effluent was adjusted to 7 while inoculated with *A. eicrenophila*, *B. megaterium*, *B. carboniphilus* or *B. licheniformis*. In case of *B. subtilis*, the pH was adjusted to 8. Co-incubation of effluent with bacterial isolates was performed at 37°C for 48 h. It is noteworthy that all the isolates exhibited significantly enhanced

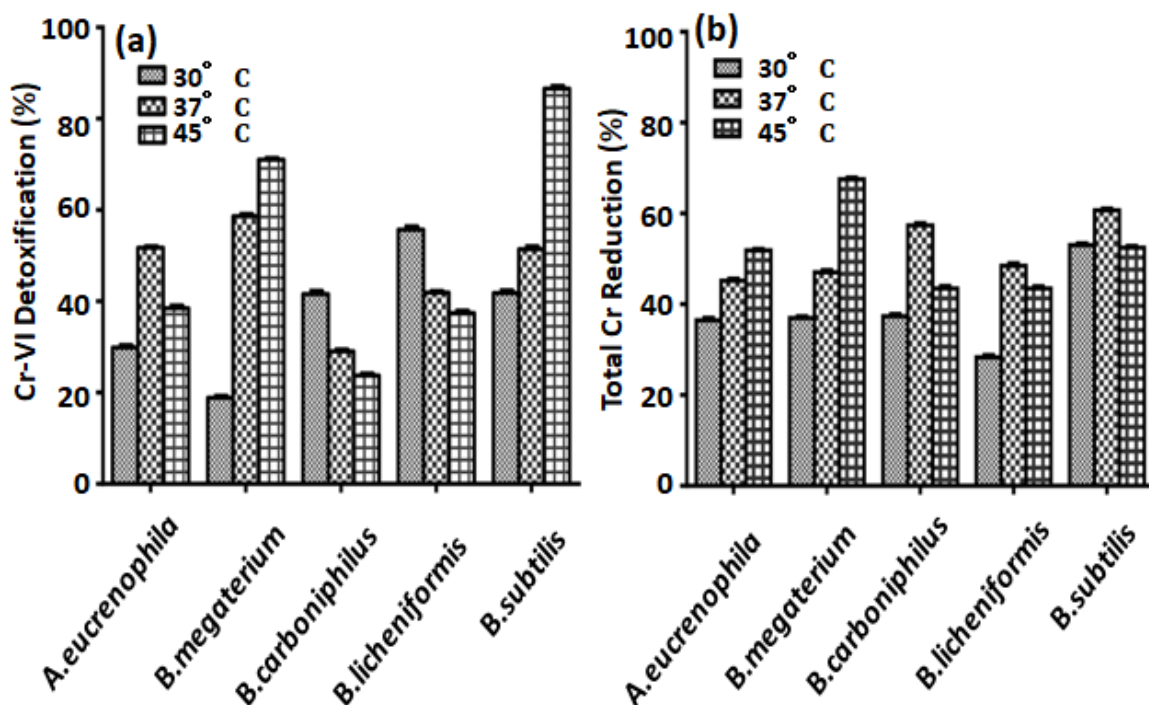


Fig. 9: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by bacterial isolates at 30, 37 and 45°C. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125 ppm of $K_2Cr_2O_7$ and incubation at 37, 37 or 45°C for 48 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

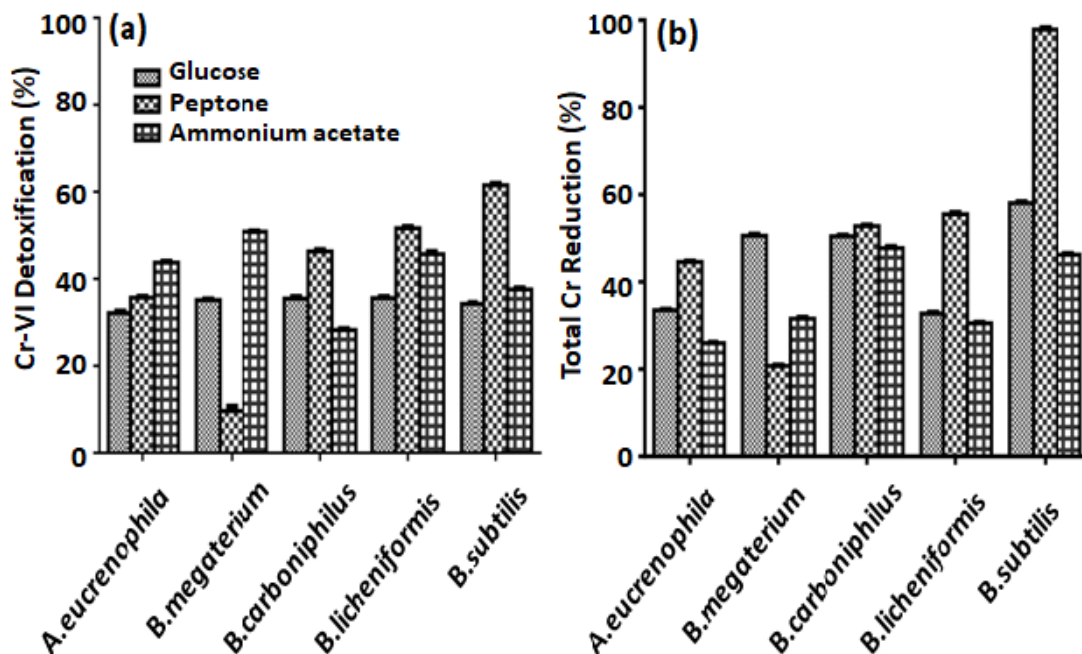


Fig. 10: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by bacterial isolates at pH 5, 6 and 8. The experiments were conducted by inoculation of minimal salts medium base, pH 5, 6 or 8 supplemented with 125 ppm of $K_2Cr_2O_7$ and incubation at 37°C for 48 h followed by measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

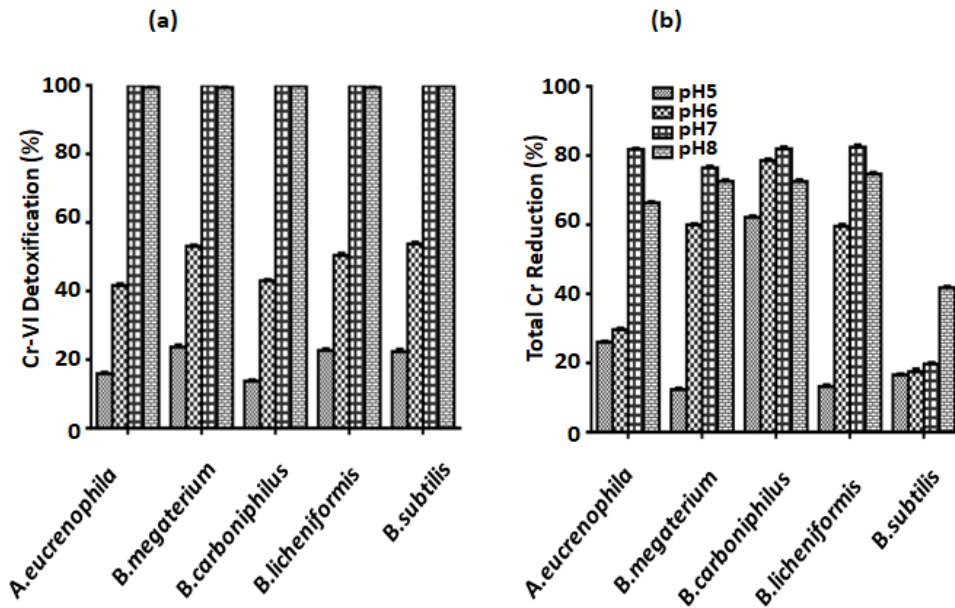


Fig. 11: Detoxification of Cr-VI and reduction of total Cr as $K_2Cr_2O_7$ by bacterial isolates using glucose, peptone or ammonium acetate as carbon source. The experiments were conducted by inoculation of minimal salts medium base, pH 7 supplemented with 125 ppm of $K_2Cr_2O_7$ and 400 ppm of glucose, peptone or ammonium acetate followed by incubation at 37°C for 48 h and measurement of Cr-VI detoxification (a) by 1,5-diphenylcarbazide colorimetric method and reduction of total Cr (b) by atomic absorption spectrophotometry.

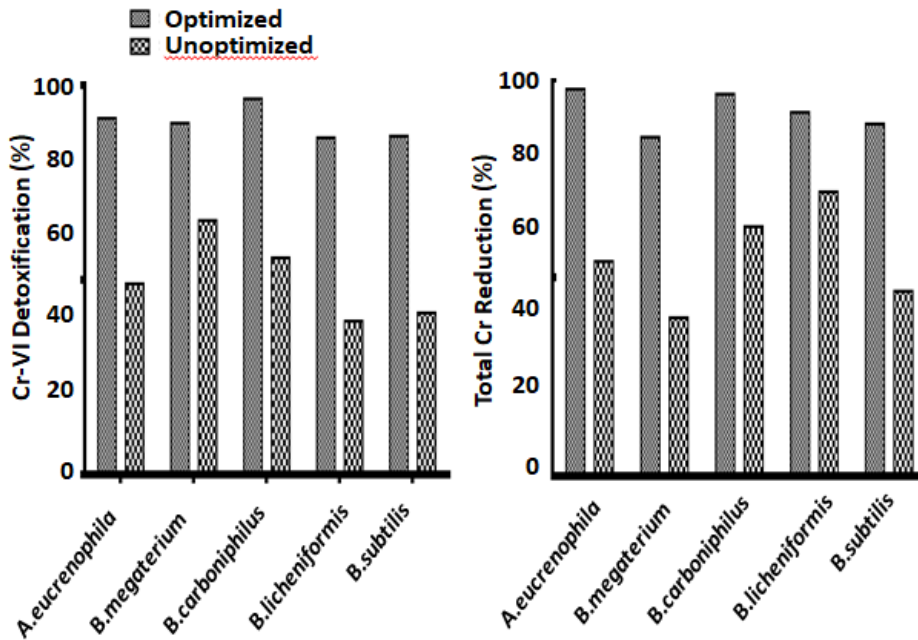


Fig. 12: Detoxification of Cr-VI and reduction of total Cr from tannery effluent by the isolates in optimized conditions. Freshly collected sample was preconditioned by filtration sterilization followed by pH adjustment to 7 for *A. eicrenophila*, *B. megaterium*, *B. carboniphilus* or *B. licheniformis*, and to 8 for *B. subtilis*. The treatment was conducted by inoculation of the preconditioned sample followed by incubation at 37°C for 48 h. Initial and final concentration of Cr-VI and total Cr in the effluent was measured (a) by 1,5-diphenylcarbazide colorimetric method and (b) by atomic absorption spectrophotometry, respectively. The rate of detoxification of Cr-VI and reduction of total Cr was calculated by subtracting final concentration from initial one, dividing by initial concentration, and multiplying by 100. The result was expressed as percent (%) of Cr-VI detoxification or total Cr reduction.

detoxification or reduction of Cr in optimized conditions compared to their unoptimized counterparts (Fig. 12).

DISCUSSION

The breakthrough towards the sustainable mitigation of overwhelming pollution caused by the tannery wastewater is installing an efficient, cost-effective, simple and environmentally friendly effluent treatment plant in each industry outlet. From time to time, diverse chemical and biological treatment systems, either independently, subsequently one after another, or in concert, have long been practiced as effective measures in dealing the hazardous effluents. Noticeably, the noble, operative and enduring abilities of naturally occurring bacteria to tolerate and reduce or detoxify heavy metals look the ever-expected and most reliable mean. This concept was the driving force in designing the present study to isolate, identify and optimize environmental conditions of natural bacteria to reduce or detoxify toxic Cr residue in tannery effluents.

The findings of this study reflect potential candidacy of indigenous and intrinsic bacteria of *Aeromonas* and *Bacillus* species in bioremediation of toxic Cr discharged into the wastewater from tanning industries. Our data clarify that an increased rate of detoxification or reduction was dependent on bacterial growth and exposure time. Our data also delineated the efficiency of the isolates was merely concentration dependent. Noticeably, the efficiency of the isolates increased when the incubation time was increased from 24 to 48 or 72 h. However, the elevated efficiency of most of the isolates over time was predominant up to 48 h. This observation also corresponds to the growth dependent efficiency of the isolates inferring the logarithmic phase of bacterial growth. It is likely that growth of bacteria and damage induced by Cr-VI are competing phenomena, where bacteria can adjust with the exposure of Cr-VI as long as the sources of metabolizable nutrients are available in the medium. Liu et al. (2006) explained the phenomenon as the bacteria required an increased incubation period for repair or adaptation when exposed to high level of Cr-VI in the medium. It has also been suggested that Cr-VI induced DNA damage can be repaired by the bacterial SOS function (Oh & Choi 1997). Growth dependent detoxification or reduction of Cr was also evidenced by Desai et al. (2008). Moreover, the efficiency of most of the isolates was found the utmost at 37°C compared to that observed at 30 or 45°C, which also corresponds to the growth dependent pattern of Cr detoxification or reduction. Reportedly, the optimal temperature of Cr-VI detoxification or total Cr reduction ranged between 25 and 37°C (Cheung & Gu 2007, Ibrahim et al. 2012). In this study, we also revealed detoxification or reduction of Cr to be influenced by pH. All the bacterial isolates exhibited their

efficiency at neutral or higher pH. In contrast, the efficiency was reversed when the pH of the medium was lowered to 6 or 5. The efficiency of total Cr reduction but not of Cr-VI detoxification by *B. carboniphilus* was not much influenced by pH range tested. Bacterial detoxification of Cr-VI was reported to be significant at pH 6-8 (Wang et al. 1990) and insignificant at pH 5 or 9 (Bopp et al. 1983). Our data thus imply bacterial detoxification or reduction of Cr is enzyme mediated, since variation of pH affects the degree of ionization of most of the enzymes, thereby changes conformation of the proteins, and eventually the activity of the enzymes (Farrell & Ranallo 2000). The growth of the isolates as well as the efficiency of them in detoxifying or reducing Cr was found greatly influenced when glucose, peptone or ammonium acetate was additionally supplemented in the medium as a direct source of carbon or co-substrate. The co-substrate dependent bacterial efficiency lied on the property of any of the co-substrates as an electron donor for Cr detoxification or reduction. Our data clearly revealed the utmost detoxification or reduction of Cr by peptone as co-substrate compared to that by glucose or ammonium acetate. Being expensive, the potential of peptone to be used in an effluent treatment plant is considerably difficult. Ammonium acetate exhibited inconsistent pattern of potency as a co-substrate for the isolates investigated. Glucose, in contrast, exhibiting an optimum range of influence on bacterial detoxification or reduction of Cr, around 60%, seems reliable as a co-substrate. Horitsu et al. (1987) revealed the effect of glucose on the activity of Cr-VI reducing enzyme in *Pseudomonas ambigua* G-1, which was indeed exerted by completely protecting the enzyme from inactivation on dialysis but not by inducing the activity of the enzyme. Hence, glucose can be considered as co-substrate in Cr bioremediation because of its strong enzyme stabilizing ability.

The present study strongly configured that bacterial detoxification or reduction of Cr was highly dependent on exposure time and incubating temperature of the reaction as well as the pH of the medium. Negligible degree of detoxification or reduction was noticed in abiotic control at any time period, temperature and pH applied, indicating direct interaction of the bacteria in Cr-VI detoxification or total Cr reduction. When the optimized time, temperature and pH revealed by in vitro examinations were implemented in detoxifying or reducing Cr from tannery effluent, the efficiency of the isolates were found significantly increased. Hence, each of the bacterial isolates investigated in this study seems to possess potential as biological agent in Cr remediation from industrial effluent or contaminated soil or wastewater.

CONCLUSION

It is the pivotal findings of the present study that the naturally

occurring bacteria of tannery effluent, namely, *Aeromonas eucrenophila*, *Bacillus megaterium*, *B. carboniphilus*, *B. licheniformis* and *B. subtilis* exhibited identical efficiency of detoxifying or reducing Cr. The bacteria also exhibited significant level of resistance or tolerance to greatly increased concentration of Cr. It is noteworthy that the bacteria exerted maximum level of potency at 37°C and pH 7 following 48 h of incubation. These environmental or physicochemical parameters closely resemble to the most common or natural conditions of the biological reactor of an effluent treatment plant. Hence, the bacteria isolated, identified and investigated in details in this study possess tremendous potential to be used as biological agents in Cr bioremediation from tannery effluent or Cr contaminated soil or wastewater of industrial premises.

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