

# The differential stress response of adapted chromite mine isolates *Bacillus subtilis* and *Escherichia coli* and its impact on bioremediation potential

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**Abstract** In the current study, indigenous bacterial isolates *Bacillus subtilis* VITSUKMW1 and *Escherichia coli* VITSUKMW3 from a chromite mine were adapted to 100 mg L<sup>-1</sup> of Cr(VI). The phase contrast and scanning electron microscopic images showed increase in the length of adapted *E. coli* cells and chain formation in case of adapted *B. subtilis*. The presence of chromium on the surface of the bacteria was confirmed by energy dispersive X-ray spectroscopy (EDX), which was also supported by the conspicuous Cr–O peaks in FTIR spectra. The transmission electron microscopic (TEM) images of adapted *E. coli* and *B. subtilis* showed the presence of intact cells with Cr accumulated inside the bacteria. The TEM–EDX confirmed the internalization of Cr(VI) in the adapted cells. The specific growth rate and Cr(VI) reduction capacity was significantly higher in adapted *B. subtilis* compared to that of adapted *E. coli*. To study the possible role of Cr(VI) toxicity affecting the Cr(VI) reduction capacity, the definite assays for the released reactive oxygen species (ROS) and ROS scavenging

enzymes (SOD and GSH) were carried out. The decreased ROS production as well as SOD and GSH release observed in adapted *B. subtilis* compared to the adapted *E. coli* corroborated well with its higher specific growth rate and increased Cr(VI) reduction capacity.

**Keywords** Cr(VI) · Isolate · Adaptation · Oxidative stress · Toxicity

## Introduction

Microorganisms used for bioremediation are continuously subjected to varied concentration of heavy metal (Martinez et al. 2009). The bacteria tend to adapt to stress by attaining homeostasis between microbial physiology and the heavy metal present in the environment (Bong et al. 2010; Jose et al. 2011). The resistance to metal ions has been proved to be an inherent property of microbes indigenous to the contaminated sites. The bacteria that could not tolerate the toxicant cannot be used as an efficient tool for bioremediation. However, when the concentration of metal ion increases to a level not tolerable to the microorganisms, they tend to adapt resulting in a change in morphology or cell alignment (Padmini et al. 2009). Bacteria indigenous to mine water system were reported to undergo cell elongation and chain formation during their adaptation to high Cr concentration (Samuel et al. 2012). Arrangement in chain was reported on a gram negative bacteria *Acidocella* sp.

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GS19h, on interaction with Cd or Ni (Chakravarty et al. 2007). Bacterial cell elongation was reported in a gram negative bacteria *Ochrobactrum tritici* 5bv11 (Francisco et al. 2010).

Heavy metal contamination is at a constant increase, owing to increase in the anthropogenic activities like mining. The Sukinda valley of Odisha contains 97 % of India's deposits of chromite ore and its one of the prime open cast chromite ore mines in the world (Das and Mishra 2009). In this area, hexavalent chromium pollution has caused major health hazard affecting around 2,600,000 people (Blacksmith Institute 2007). In environment, chromium exists in several forms of which Cr(III) and Cr(VI) have stable oxidation states. Of that hexavalent chromium is identified as a carcinogen and surface water, soil, ground water contaminant (Cervantes et al. 2001). Slight rise in Cr(VI) level might possibly lead to disastrous environmental and health hazards because of its high toxicity, mutagenicity and carcinogenicity (Sharma et al. 1995; Nishioka 1975; Venitt and Levy 1974).

Bio-reduction of highly toxic Cr(VI) to less toxic Cr(III) results in emission of free radicals as reactive oxygen species (ROS), which causes oxidative stress. It has been reported that adaptive and cross-protective responses play an important role in bacterial adaptation to oxidative stress (Monkolusk et al. 1997). Superoxide dismutase (SOD) acts as one of the key defense mechanism against oxidative stress, by catalyzing the unbalanced superoxide anion into hydrogen peroxide and molecular oxygen (Sies 1985). Glutathione *S*-transferase (GST) is another enzyme that reported to assist in microbial biotransformation of the metals (Wilhelmfilho et al. 2001).

However, the significance of the current study is to explore the ability of the adapted mine water isolates to grow in the presence of Cr(VI) and reduce Cr(VI) to Cr(III). Only the bacteria which have the ability to withstand toxic effect of the contaminant can be used to remediate the toxicant. In this context, it is very important to understand the adaptive changes of bacteria both morphologically and biochemically in response to toxic contaminants. The detailed morphological and ultra-structural changes in the cells (mine water isolates) owing to stress response has not been explored. The current work reports for the first time, the role of toxicity on differential growth and Cr(VI) reduction (and therefore bioremediation) capacity of

adapted gram negative and gram positive bacteria isolated from a chromite mine, through detailed growth, ultrastructural, and biochemical assays. The morphological changes were studied through light, phase contrast, scanning electron and transmission electron microscopy. FTIR analysis was carried out to study the presence of Cr on bacterial surface on interaction with surface functional groups. Biochemical assays analyzing ROS, SOD and GSH contents were carried out to study the possible role of Cr(VI) toxicity on specific growth rate and thus the Cr(VI) reduction capacity of isolates.

## Materials and methods

### Adaptation

The indigenous bacterial strains, *B. subtilis* VITS-UKMW1 and *E. coli* VITSUKMW3 (GenBank: JF309279 and JN393206 respectively) isolated from chromite mine site in Sukinda, India (Samuel et al. 2012). Henceforth these strains will be referred as *B. subtilis* and *E. coli* respectively. These isolates were serially adapted at 20, 40, 60, 80, 100 mg L<sup>-1</sup> of Cr(VI). The control (unadapted untreated indigenous) cells were taken as initial inoculum and inoculated in 20 mg L<sup>-1</sup> of Cr(VI) which thereafter was subsequently inoculated in the same concentration of Cr(VI) until the maximum microbial growth was as similar as that of the control. The cells adapted to 20 mg L<sup>-1</sup> of Cr(VI) were taken as primary inoculum to inoculate in nutrient broth amended with 40 mg L<sup>-1</sup> of Cr(VI). The cells were subsequently inoculated in 40 mg L<sup>-1</sup> of Cr(VI) concentration amended nutrient broth until the maximum microbial growth was as similar as that of the control. The cells adapted to 40 mg L<sup>-1</sup> of Cr(VI) were taken as primary inoculum to inoculate in nutrient broth amended with 60 mg L<sup>-1</sup> of Cr(VI). The cells were subsequently inoculated in 60 mg L<sup>-1</sup> of Cr(VI) concentration amended nutrient broth until the maximum microbial growth was as similar as that of the control. The cells adapted to 60 mg L<sup>-1</sup> of Cr(VI) were taken as primary inoculum to inoculate in nutrient broth amended with 80 mg L<sup>-1</sup> of Cr(VI). The cells were subsequently inoculated in 80 mg L<sup>-1</sup> of Cr(VI) concentration amended nutrient broth until the maximum microbial growth was as similar as that of the control. The cells adapted to 80 mg L<sup>-1</sup> of Cr(VI) were taken as primary

inoculum to inoculate in nutrient broth amended with  $100 \text{ mg L}^{-1}$  of Cr(VI). The cells were subsequently inoculated in  $100 \text{ mg L}^{-1}$  of Cr(VI) concentration amended nutrient broth until the maximum microbial growth was as similar as that of the control. The adaptation was carried out until the maximum microbial growth was as similar as the unadapted untreated indigenous ones (Fig. S1 provided with the online resource). The bacteria cultured in the nutrient broth medium without Cr(VI) are referred to as unadapted henceforth. The adapted isolates were maintained and further characterized at  $100 \text{ mg L}^{-1}$  of Cr(VI).

### Phase contrast microscopy

Changes in adapted bacterial cells with increasing concentration of Cr(VI) in comparison to the un-interacted indigenous strain were studied through phase contrast microscopy (Ackereley et al. 2006). Thin smear of adapted isolates at varied concentration were prepared at room temperature and studied under phase contrast microscope at  $1,000\times$  magnification (Leica DM 2500, GMBH) and the images were captured by Lexica DFC 295 camera. The cell length was calculated using Leica Application Suite Ver. 3.8 for three samples in three positions for each isolate. For *E. coli*, an average of 15, 19, 15, 18, 15 and 15 cells were m at 0 (unadapted un-interacted), 20, 40, 60, 80 and  $100 \text{ mg L}^{-1}$  of Cr(VI) concentration respectively. For *B. subtilis* chains, an average of 29 measurements was carried out for unadapted un-interacted and 19 measurements at 20, 40, 60, 80,  $100 \text{ mg L}^{-1}$  of Cr(VI) concentration each respectively.

### Cell morphology and Cr characterization

The changes in surface elemental composition and morphology of *B. subtilis* and *E. coli* were analyzed using SEM–EDX. Samples were collected at 20 h of incubation, washed and fixed on glass slides using 2 % glutaraldehyde for 1.5 h. Then it was washed by a series of dehydration with ethanol and then air dried. With carbon tape, the glass slide was fixed on specimen mount. Gold sputtering was carried out in argon atmosphere. The surface morphology of bacteria and the surface elemental composition were observed under SEM–EDX (S-400, HITACHI, and Tokyo, Japan; JEOL JSM-5510) (Paul et al. 2012). The SEM images and EDX spectra of unadapted

bacterial isolates were obtained at  $40,000\times$  magnification and that of adapted isolates were taken at  $14,000\times$  magnification.

Cr(VI) adapted and indigenous un-interacted cells of *B. subtilis* and *E. coli* were collected at 20 h of incubation and centrifuged. The pellet was collected for TEM examination. Pellet were washed and fixed with 3 % glutaraldehyde in salined 0.1 M phosphate buffer. It was again washed and post fixed followed with staining 1 % osmium tetroxide in deionized water for 20 min. Samples were washed thrice in a continuous series of distilled water followed by staining in uranyl sulphate for 19 min in deionized water. It was then continuously washed in different alcohol gradation for 3 min. After 5 min propylene oxide was added. Epoxy resin and propylene oxide (1:1, 3:1) for 15 min and epoxy resin for 10 min only. The samples were then embedded in a fresh epoxy resin and polymerized at  $75^\circ\text{C}$  (45 min) and  $97^\circ\text{C}$  (45 min). Finally, the resin block were allowed to cool overnight before ultra-thin sectioning (70–80 nm thick) was made using ultra microtome. Thin sections were viewed under microscope (Bencosme and Tsutsumi 1970). The internal elemental analysis of unadapted and Cr(VI) interacted adapted bacteria was carried out by energy dispersive X-ray spectroscopy (EDX). The samples were analyzed and the spectra were recorded using JEOL JSM-5510 equipment.

### FT-IR spectroscopy

Surface chemical characteristics of the Cr(VI) adapted and indigenous un-interacted bacteria were studied by FT-IR Spectroscopy. It helps in providing the fingerprint spectra for rapid characterization of microbial strains (Schmitt and Flemming 1998). The bacterial samples were pelleted at 4,000 rpm, 15 min. The bacterial samples were washed with  $1\times$  PBS buffer to remove residues, these samples were then frozen and lyophilized. Sample was mixed with  $100 \text{ mg KBr}$  and was pressed to form a pellet; the tablets obtained were inserted in the instrument and the spectra is recorded (Samuel et al. 2012; Sannasi et al. 2009).

### Cr(VI) reduction kinetics

Aerobic Cr(VI) reduction kinetic studies were carried out using shake flask at  $37^\circ\text{C}$ . Different

Cr(VI) concentration (25, 50, 75, 100 mg L<sup>-1</sup>) in 100 mL nutrient broth medium was used for Cr(VI) reduction studies (pH 7.5). Unadapted and 100 mg L<sup>-1</sup> Cr(VI) adapted bacterial cells were used as inoculum with initial cell concentration of  $1.5 \times 10^8$  CFU mL<sup>-1</sup>. Samples were taken periodically to measure cell growth and Cr(VI) concentration. Cell free nutrient broth medium containing different Cr(VI) concentration (25, 50, 75, 100 mg L<sup>-1</sup>) was used as control. Cell growth was measured at 600 nm using UV–Vis spectroscopy. A colorimetric method using 1,5-diphenylcarbazide (DPC) was employed to analyze the concentration of Cr(VI) in the supernatant (details in Samuel et al. 2012). Total Cr concentration was analyzed using flame atomic adsorption spectrophotometer (AAAnalyst400/HGA 900, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) at a wavelength of 359.9 nm (Samuel et al. 2012). Cr(VI) reduced to Cr(III) was calculated from the difference between total Cr and Cr(VI) concentration. Cr(VI) reduction capacity of the unadapted and adapted bacterial cells was measured at the end of Cr(VI) reduction studies using following equation modified from Molokwane et al. (2008).

$$R_c = C_0 - C/X$$

where  $R_c$  Cr(VI) reduction capacity (mg Cr(VI) removed/mg cells),  $C_0$  initial Cr(VI) concentration (mg L<sup>-1</sup>),  $C$  Cr(VI) concentration at the time of incubation  $t$ , and  $X$  viable cell concentration (mg L<sup>-1</sup>) at time  $t$  (incubation).

The details of estimation for viable cell concentration and dry weight of biomass is discussed in our previous report (Samuel et al. 2012).

A set of experiment using heat killed (treated) cells (heat killed cells + Cr(VI) + nutrient broth) were carried out to find out the fraction of Cr(VI) adsorbed onto the adapted cells. The adapted cells of *E. coli* and *B. subtilis* were grown in nutrient broth with 100 mg L<sup>-1</sup> of Cr(VI). The grown cells were harvested and heat killed by autoclaving. The heat killed cells were inoculated into the different Cr(VI) concentration (25, 50, 75 and 100 mg L<sup>-1</sup>) spiked nutrient broth at 37 °C. The supernatant for analysis was collected at 20 h by centrifuging the nutrient broth. Cr(VI) adsorbed onto the cells was calculated from the difference between the initial and final Cr concentration studied by DPC assay and AAS.

## Oxidative stress

Generation of ROS was quantified using 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescence probe (Kuczynska-Wis'nik et al. 2009). DCFH-DA is membrane permeable and oxidizes in the presence of cellular esterases and ROS to form the green fluorescent DCF. Intracellular ROS generation was monitored in indigenous un-interacted and Cr(VI) adapted interacted bacterial cells. Five milliliter of cell suspension was incubated with DCFH-DA with a final concentration of 100 μM at 37 °C for 30 min (Wang and Joseph 1999). Fluorescence was measured using a spectrofluorometer (SL174, ELICO) with excitation and emission wavelengths of 490 and 519 nm, respectively. Experiments were performed in triplicates.

With the generation of free radicals, SOD enzymes able to detoxify superoxide radicals. To study the release of anti-oxidant radical, SOD activity was analyzed in Cr(VI) adapted and indigenous un-interacted bacterial cells. Activity of SOD was determined by the method of Marklund and Marklund 1974. 0.1 mL of cell suspension was prepared to if 50 μL of cold absolute ethanol along with 30 μL of chloroform were added to it. After 15 min of shaking, the suspension was centrifuged for 15 min at 13,000 rpm and 0.1 mL of supernatant was used for the assay. The reaction mixture for auto-oxidation consisted of 2 mL Tris–HCl buffer (pH 8.2), 0.5 mL of 2 mM pyrogallol, 0.1 mL aliquots of the enzyme preparation and water to give a final volume of 4.5 mL. Blank containing 2 ml of Tris–HCl buffer (pH 8.2) and 2.5 mL of distilled water was prepared. The color developed was read at 470 nm in UV–Vis double beam spectrophotometer (Systronics, India Ltd).

The reduction of oxidized glutathione (GSSH) to reduced glutathione (GSH) is catalysed by glutathione reductase. This enzyme enables the cell to sustain adequate levels of cellular GSH, which acts as an antioxidant, reacting with free radicals and organic peroxides. Activity of glutathione reductase in Cr(VI) adapted and indigenous un-interacted bacterial cells were monitored spectrophotometrically. GSH assay was carried out as described by Moron. 0.2 mL of the cell suspension was precipitated with 5 % TCA and mixed well for the complete precipitation of protein and centrifuged. To an aliquot of the clear supernatant, 2.0 mL of DTNB was added and 0.2 M of phosphate buffer to make a final volume of 4 mL. The

**Table 1** Average length observed for *E. coli* and *B. subtilis* cells as well as for chains of *B. subtilis*, before and after adaptation to different Cr(VI) concentrations [20, 40, 60, 80 and 100 mg L<sup>-1</sup>]

Adapted/unadapted to Cr(VI)	<i>E. coli</i>	<i>B. subtilis</i>	
	Average cell length $\pm$ SD ( $\mu\text{m}$ )	Average chain length $\pm$ SD ( $\mu\text{m}$ )	Average cell length $\pm$ SD ( $\mu\text{m}$ )
Unadapted	4.11 $\pm$ 0.44	–	4.42 $\pm$ 0.27
Adapted at 20 mg L <sup>-1</sup>	6.66 $\pm$ 0.32*	9.55 $\pm$ 0.42*	4.13 $\pm$ 0.4
Adapted at 40 mg L <sup>-1</sup>	7.01 $\pm$ 0.07*	11.96 $\pm$ 0.12*	4.5 $\pm$ 0.33
Adapted at 60 mg L <sup>-1</sup>	7.22 $\pm$ 0.53*	12.55 $\pm$ 0.34*	4.46 $\pm$ 0.15
Adapted at 80 mg L <sup>-1</sup>	7.22 $\pm$ 0.12	14.05 $\pm$ 0.18*	4.71 $\pm$ 0.56
Adapted at 100 mg L <sup>-1</sup>	8.55 $\pm$ 0.64*	14.88 $\pm$ 0.23*	5.7 $\pm$ 1.42

absorbance was read at 412 nm against a blank (Moron et al. 1979). Data were reported as percentage release of GSH compared to unadapted.

Fluorescent microscopy was used to observe the oxidative stress in cells using DCFH-DA. Bacterial cultures (1 mL/A<sub>600</sub>) were collected and 4  $\mu\text{L}$  of dichlorofluorescein dye was added followed by incubation in dark for 5 min at 37 °C. The bacterial samples were pelleted and washed with 1 $\times$  PBS buffer. 4  $\mu\text{L}$  of sample was dropped on top of slide covered with glass coverslip and observed with 1,000 $\times$  magnification in an upright microscope (Ackereley et al. 2006).

### Statistical analysis

The data corresponding to growth study and Cr(VI) reduction are presented as mean  $\pm$  standard error. The data corresponding to cell length are presented as mean  $\pm$  standard deviation. The significance of reduction capacity between the isolates was validated using Student's *t* test (one tailed). The data for the ROS, SOD and GSH assays were carried out in triplicates, and the data are presented as mean  $\pm$  standard error and were processed using one-way ANOVA, followed by Dunnett's post hoc test at  $p < 0.05$ . Significant data points are denoted with the symbol asterisk.

## Results and discussion

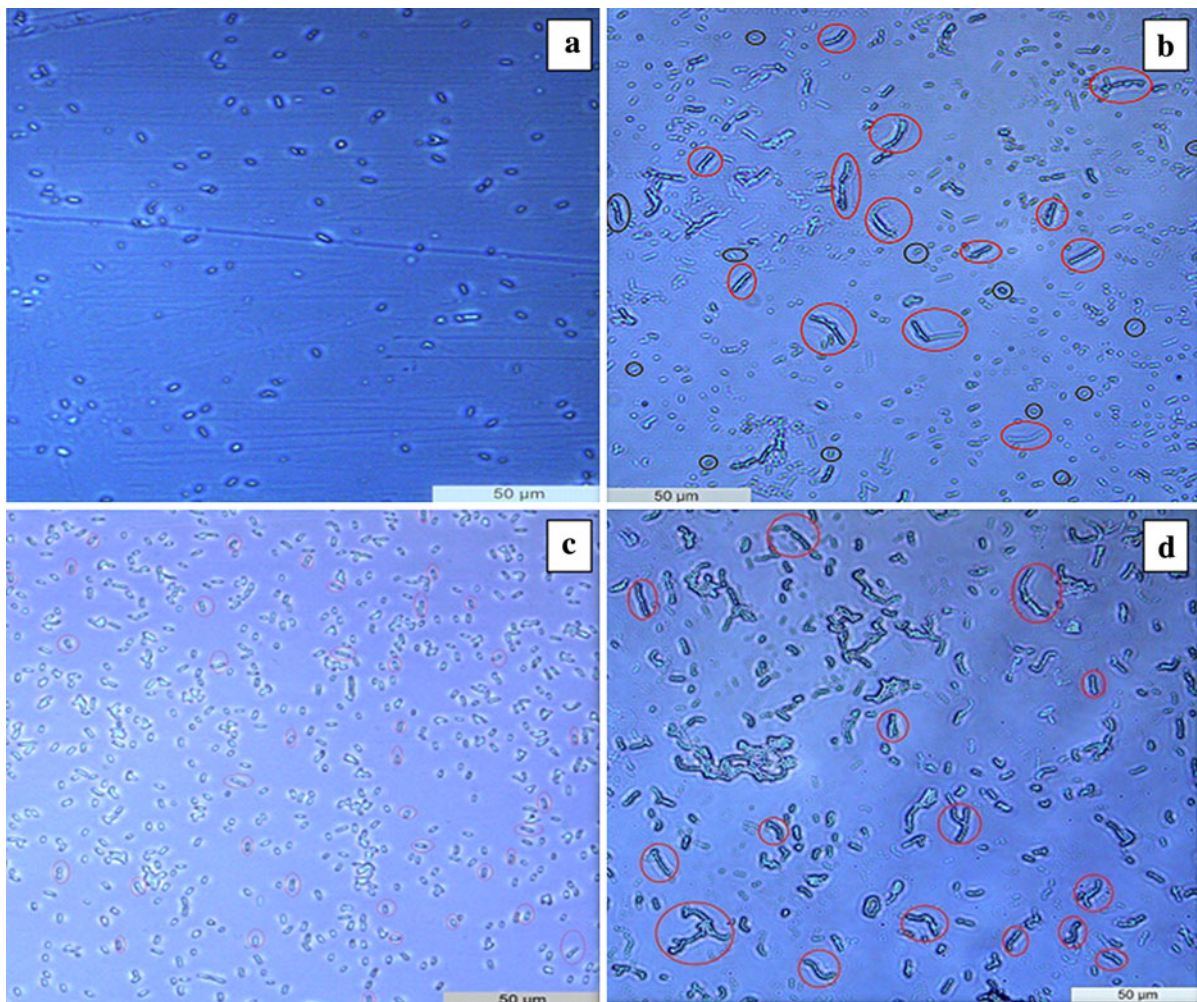
### Morphological and ultrastructural studies

Cells of unadapted *B. subtilis* were single and had an average length of 4.42  $\pm$  0.27  $\mu\text{m}$  (Table 1). On

adaptation to Cr(VI), clusters formation were observed which were seen mostly in the form of chains (Fig. 1). With increasing Cr(VI) concentration, the length of chains increased significantly from 9.55  $\pm$  0.42  $\mu\text{m}$  at 20 mg L<sup>-1</sup> to 14.88  $\pm$  0.23  $\mu\text{m}$  at 100 mg L<sup>-1</sup> of Cr(VI) concentration. There was no significant increase in the length of individual cells of adapted *B. subtilis*. Unadapted cells of *E. coli* had an average length of 4.11  $\pm$  0.44  $\mu\text{m}$  (Table 1). A significant increase in average cell length was observed compared to unadapted with 6.66  $\pm$  0.32  $\mu\text{m}$  at 20 mg L<sup>-1</sup> to 7.22  $\pm$  0.53  $\mu\text{m}$  at 60 mg L<sup>-1</sup> of Cr(VI) (Table 1). There was no significant increase in 7.22  $\pm$  0.53  $\mu\text{m}$  of cell length adapted at 60 mg L<sup>-1</sup> to 7.22  $\pm$  0.12  $\mu\text{m}$  at 80 mg L<sup>-1</sup> of Cr(VI) (Table 1). A significant increase in average cell length was observed from 7.22  $\pm$  0.12  $\mu\text{m}$  at 80 mg L<sup>-1</sup> to 8.55  $\pm$  0.64  $\mu\text{m}$  at 100 mg L<sup>-1</sup> of Cr(VI).

In scanning electron micrograph, adapted *B. subtilis* was found to be swollen in shape with rough surface compared to the unadapted. The chain arrangement of *B. subtilis* cells was observed in adapted interacted cells (Fig. 2a, b). Surface roughness in response to Cr(VI) interaction was reported in gram positive bacteria *Arthrobacter* bacteria K-4 (Lin et al. 2006). However, chain arrangement in gram positive bacteria as an adaptive response to Cr(VI) has not been reported. Bacterial cells may arrange themselves together to protect from exposure to chemicals (Schembri et al. 2003). This may be the possible mechanism of adaptation followed by *B. subtilis* as observed by SEM. Surface elemental analyses by EDX shows the presence of Cr on the surface of adapted *B. subtilis* cells compared to unadapted (Fig. 3c, d). In adapted interacted *E. coli*, rough surface were observed in SEM image. A significant increase in length of *E. coli* cells was observed as a response to





**Fig. 1** Phase contrast microscopy. At  $\times 1,000$  magnification, **a** individual cells observed in unadapted *B. subtilis*. **b** Chains encircled in red and young cells encircled in black in the image of  $[100 \text{ mg L}^{-1} \text{ Cr(VI)}]$  adapted *B. subtilis*. **c** Individual rods

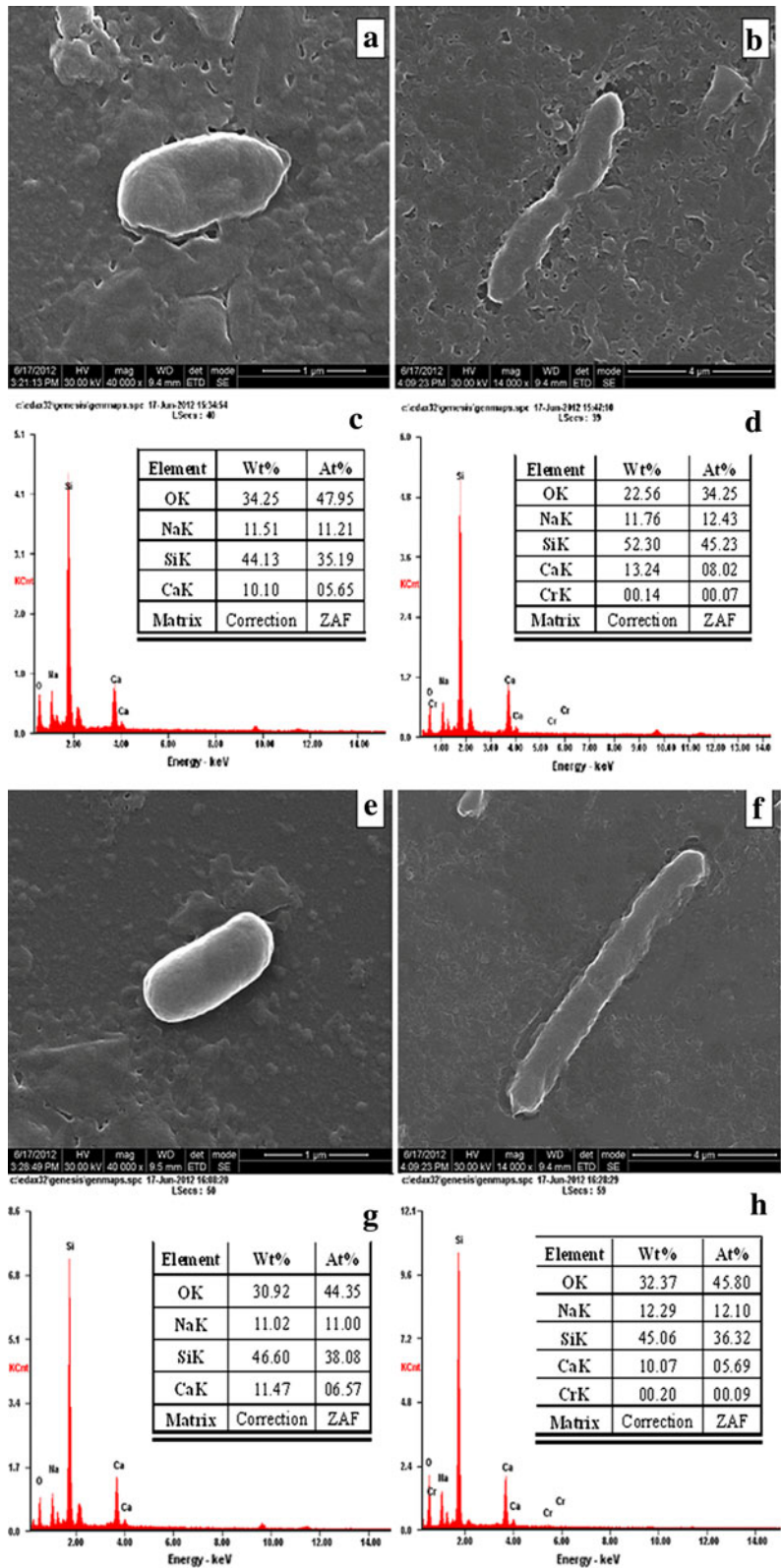
chromium stress compared to unadapted un-interacted cells (Fig. 2e, f). The average bacterial cell length increased from 1.5 to 2.55  $\mu\text{m}$  in un-interacted unadapted to 6.83–8.54  $\mu\text{m}$  in adapted *E. coli* after interaction with hexavalent chromium. This corroborates with the previous observations reported in *E. coli* K-12 exhibiting extreme filamentous morphology due to increase in length from 4.0 to 6.0  $\mu\text{m}$  on Cr(VI) challenge (Ackereley et al. 2006). Increase in the length of bacteria due to stress by Cr(VI) was reported for a gram negative bacteria *Ochrobactrum tritici* 5bv11 (Francisco et al. 2010). Surface roughness may be an adaptive mechanism observed in *E. coli* after Cr(VI) interaction which is similar to

observed in unadapted *E. coli* isolate. **d** Adapted  $[100 \text{ mg L}^{-1} \text{ Cr(VI)}]$  *E. coli* isolate and the red circles represent the increase in length and young cells

the reports in the case of *Vibrio fisheri*, a gram-negative bacteria subjected to Cr(VI) stress (Fulladosa et al. 2005). EDX studies revealed the presence of Cr on adapted *E. coli* surface which played a major role in cell elongation compared to unadapted cells (Fig. 2g, h).

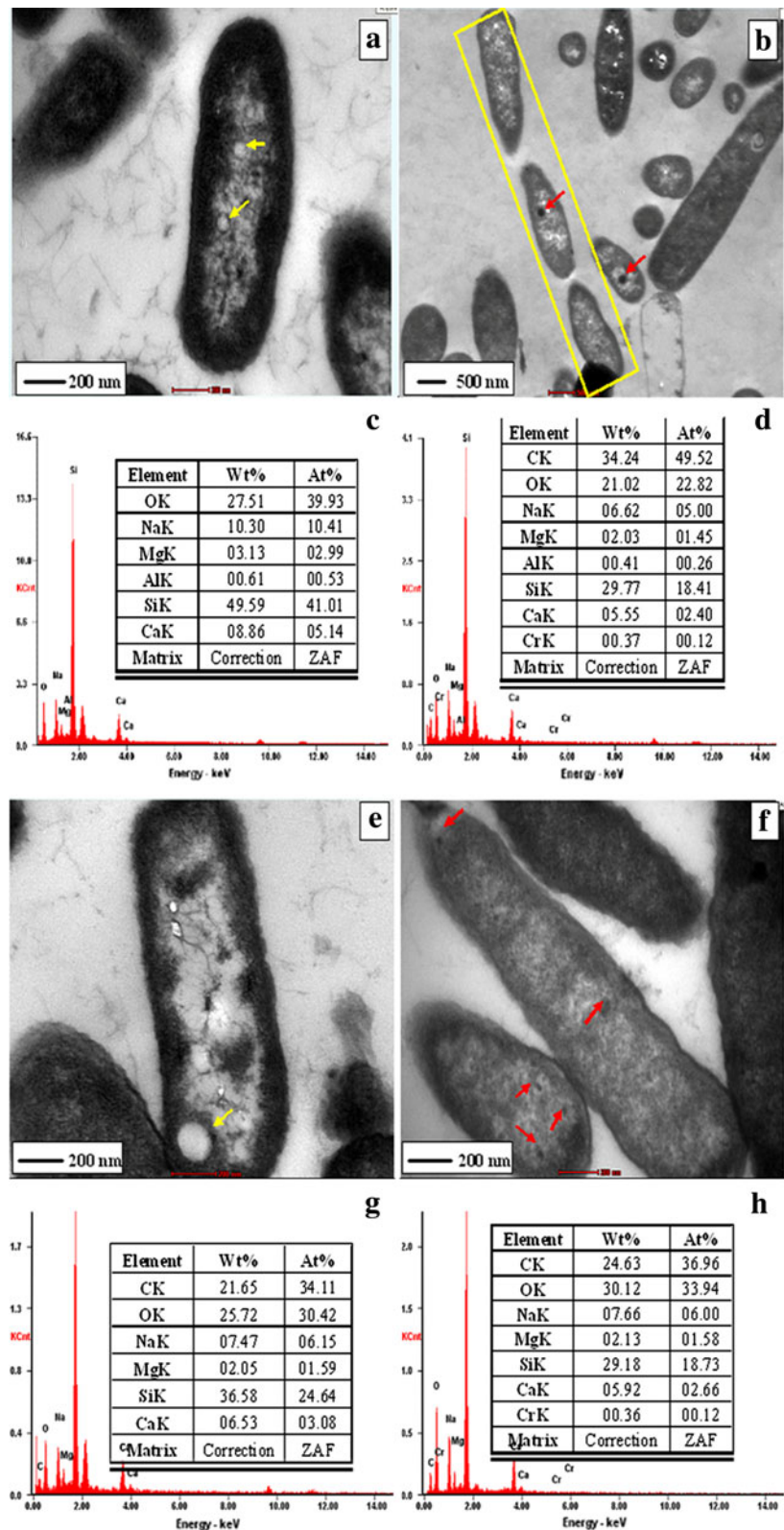
Transmission electron micrographs showed that the unadapted cells of *B. subtilis* were intact, food vacuole was present and the cell wall was thick (Fig. 3a). Whereas, the adapted cells of *B. subtilis* were arranged in chain. Chromium accumulates were present inside the cells which was confirmed by EDX (Fig. 3b–d). In the case of *E. coli*, unadapted cell wall was intact and food vacuole was present devoid of Cr which is

**Fig. 2** Scanning electron micrograph of **a** unadapted *B. subtilis* (unadapted) showing intact cell ( $\times 40,000$  magnification), **b** adapted [ $100 \text{ mg L}^{-1}$  Cr(VI)] *B. subtilis* showing cells arranged in chain ( $\times 14,000$  magnification), EDX spectrum of **c** unadapted *B. subtilis*, **d** adapted [ $100 \text{ mg L}^{-1}$  Cr(VI)] *B. subtilis* having Cr peak, **e** unadapted *E. coli* having smooth surface ( $\times 40,000$  magnification); **f** increase in cell length and roughening of cell surface observed in adapted *E. coli* ( $\times 40,000$  magnification); EDX spectrum of **g** unadapted *E. coli*, **h** adapted *E. coli* having Cr peak





**Fig. 3** Transmission electron microscopy. TEM image of **a** unadapted *B. subtilis* and **b** adapted [ $100 \text{ mg L}^{-1} \text{ Cr(VI)}$ ] interacted isolate of *B. subtilis* at  $100 \text{ mg L}^{-1} \text{ Cr(VI)}$ , EDX spectrum of **c** unadapted *B. subtilis*, **d** adapted *B. subtilis* having Cr peak, **e** unadapted *E. coli* and **f** adapted [ $100 \text{ mg L}^{-1} \text{ Cr(VI)}$ ] interacted isolate of *E. coli* at  $100 \text{ mg L}^{-1} \text{ Cr(VI)}$ , EDX spectrum of **g** unadapted *E. coli*, **h** adapted *E. coli* having Cr peak [notations used: *Yellow arrow* indicates vacuoles; *red arrow* indicates internalized Cr inside the cell; *Yellow box* shows the cells arranged in chain]

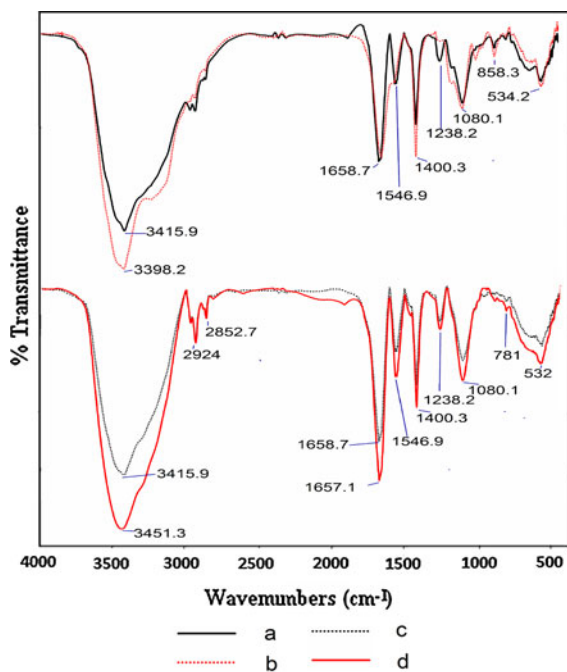




understood from the EDX data (Fig. 3e, g). The elongation of cells evident in the adapted interacted *E. coli* (Fig. 3f) might have resulted due to the Cr interaction evident from the peak observed in EDX spectra. In adapted cells of *E. coli* the cell surface was rough and the cell wall organization was disturbed. But, still the bacteria were intact with internalized Cr present, which is evident from the EDX analyses (Fig. 3g, h). TEM study on internalization of Cr was reported in the case of *Pannonibacter phragmitetus* LSSE-09 confirmed by EDX (Xu et al. 2011).

#### Surface chemical studies by FT-IR spectroscopy

The FT-IR analysis was carried out to identify the possible interactions between Cr(VI) and the adapted bacteria compared to unadapted. Unadapted cells of *B. subtilis* and *E. coli* (Fig. 4a, c) showed the presence of a broad band at  $3,415.9\text{ cm}^{-1}$  corresponding to OH vibrations (Lin et al. 2006). Peaks at  $1,658.7$  and  $1,546.9\text{ cm}^{-1}$  correspond to amide I and amide II proteins in bacterial cell wall. C=O stretching was



**Fig. 4** FTIR analysis. Spectra of **a** unadapted *B. subtilis*, **b** adapted [ $100\text{ mg L}^{-1}$  Cr(VI)] *B. subtilis* interacted at  $100\text{ mg L}^{-1}$  Cr(VI), **c** unadapted *E. coli* and **d** adapted [ $100\text{ mg L}^{-1}$  Cr(VI)] interacted isolate of *E. coli* at  $100\text{ mg L}^{-1}$  Cr(VI)

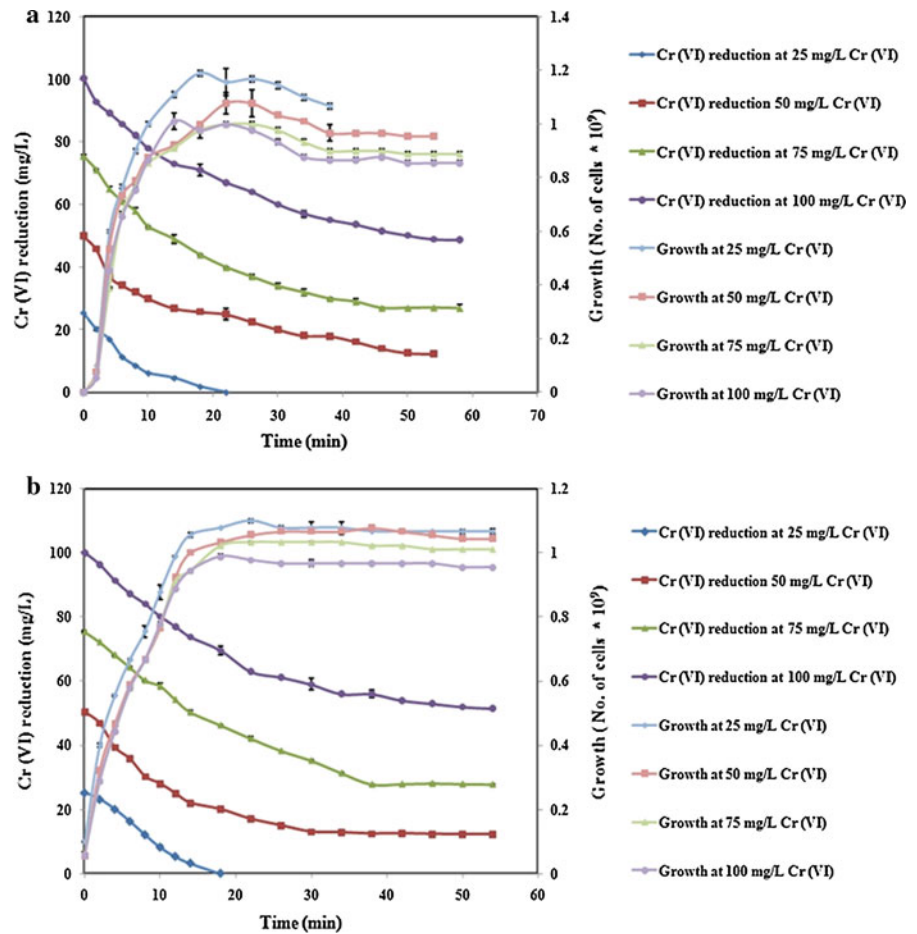
present at  $1,400.3\text{ cm}^{-1}$ . A characteristic peak attributed to  $\text{PO}_2^-$  vibrations present in phospholipids of cell wall was evident at  $1,238.2\text{ cm}^{-1}$  (Kamnev 2008). Peak at  $1,080.1\text{ cm}^{-1}$  corresponding to the stretching of polysaccharides in bacterial cells were observed (Harish et al. 2012). A broad peak corresponding to glycogen units and  $\text{CH}_2$  vibrations of polysaccharides in cell wall was noted at the finger print region with peak intensity  $534.2$  and  $532\text{ cm}^{-1}$  (Parikh and Chrover 2005; Nakamoto 1963).

After interaction of adapted bacteria with Cr(VI), considerable changes in peak intensity and peak shift were observed. In adapted *B. subtilis* and *E. coli*, peak corresponding to N–H stretching increased with a peak shift at  $3,398.2$  and  $3,451.3\text{ cm}^{-1}$  respectively (Fig. 4b, d). In *E. coli*, there was no change in peak corresponding to NH stretch at  $2,924$  and  $2,852.7\text{ cm}^{-1}$ . There was no significant increase in amide I, II peak of adapted *B. subtilis*, whereas in adapted *E. coli* increase in amide I stretching at  $1,657.1\text{ cm}^{-1}$  and amide II at  $1,546.9\text{ cm}^{-1}$  were noted. A slight increase in C=O stretching at  $1,400.3\text{ cm}^{-1}$  attributed to cell wall,  $\text{PO}_2^-$  vibrations corresponding to phospholipids of cell wall at  $1,238.2\text{ cm}^{-1}$  and polysaccharide stretching at  $1,080.1\text{ cm}^{-1}$  was observed for adapted *B. subtilis* and *E. coli*. The presence of Cr(VI) on the cell wall of adapted interacted *Bacillus subtilis* and *E. coli* is confirmed by the Cr–O peak observed at  $858.3$  and  $781\text{ cm}^{-1}$  respectively (Samuel et al. 2012). A reasonable increase in peak corresponding to glycogen units and  $\text{CH}_2$  vibrations of polysaccharides in cell wall was evident at  $534.2$  and  $532\text{ cm}^{-1}$  of finger print region.

#### Cr(VI) reduction studies

Cr(VI) reduction by *B. subtilis* and *E. coli* was growth dependent (Fig. 5a, b). The Cr(VI) reduction capacity of *B. subtilis* and *E. coli* were  $5.74$ ,  $10.07$ ,  $12.54$ ,  $13.96$  and  $4.66$ ,  $5.04$ ,  $7.57$ ,  $8.61\text{ mg Cr(VI)}$  per mg of viable cell at  $25$ ,  $50$ ,  $75$  and  $100\text{ mg L}^{-1}$  of initial Cr(VI) concentration which was high compared to the unadapted (control) cells respectively (Table S1 provided with the online resource). Cr(VI) reduction capacity and specific growth rate of adapted *B. subtilis* were significantly high ( $p < 0.05$ ) compared to that of *E. coli*. This affirms that Cr(VI) reduction was related to bacterial growth. The lag phase of both *B. subtilis*

**Fig. 5** Growth and reduction study. Growth and reduction at 25, 50, 75, 100 mg L<sup>-1</sup> of initial Cr(VI) concentration by **a** *E. coli*, **b** *B. subtilis*



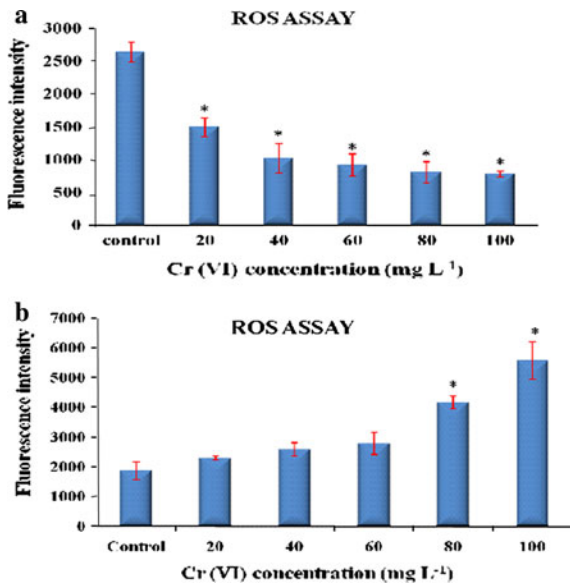
and *E. coli* decreased as they were sequentially adapted to increasing Cr(VI) concentration from 20 to 100 mg L<sup>-1</sup> compared to unadapted un-interacted (control) of each (data not shown). In the Cr(VI) reduction study, specific growth rate of *B. subtilis* and *E. coli* were decreased with increase in initial Cr(VI) concentration of 25, 50, 75 and 100 mg L<sup>-1</sup> respectively (Table S1 provided with the online resource). The metabolites, enzymes and membrane proteins of bacterial cells are reported to play major role in growth based Cr(VI) reduction (Megharaj et al. 2003).

The Cr(VI) adsorbed onto the heat killed cells of *E. coli* and *B. subtilis* is tabulated (Table S2 provided with the online resource). Since, there was no difference between the Cr(VI) concentration and total Cr concentration in the supernatant; it shows that only adsorption has taken place and not the reduction of Cr(VI) by the dead cells. Hence, the reduction of Cr(VI) to Cr(III) by live cells of adapted *E. coli* and *B. subtilis* is confirmed.

#### Oxidative stress assessment

The toxicity of heavy metals on interaction with bacteria is determined by the release of ROS (Kachur et al. 1998). To study the role of Cr(VI) toxicity affecting the specific growth rate and thus Cr(VI) reduction capacity, the definite assays for the released ROS and ROS scavenging enzymes (SOD and GSH) were carried out.

Adapted *B. subtilis* showed a significant ( $p < 0.05$ ) concentration dependent decrease [20 > 40 > 60 > 80 > 100 mg L<sup>-1</sup> of Cr(VI)] in intracellular ROS generation as compared to unadapted *B. subtilis* (Fig. 6a), which may be due to the adaptation effect of bacteria to Cr(VI). ROS generated by adapted *B. subtilis* at 100 mg L<sup>-1</sup> of Cr(VI) was 3 times less compared to the unadapted. Adapted *E. coli* showed a significant ( $p < 0.05$ ) increase in ROS production at 80 and 100 mg L<sup>-1</sup> of Cr(VI) compared to unadapted *E. coli* (Fig. 6b). There was no significant increase in

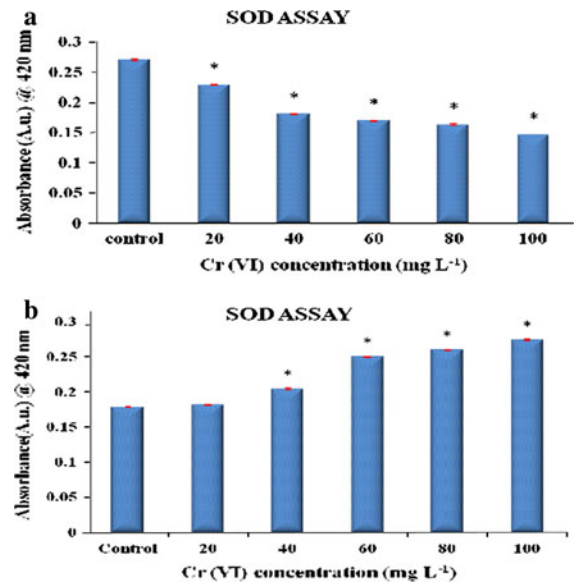


**Fig. 6** Oxidative stress analysis. **a** ROS production in *B. subtilis* as a result of oxidative stress with varying concentration of Cr(VI) ranging between 20 and 100 mg L<sup>-1</sup>. **b** Production of ROS in *E. coli* with the increase in Cr(VI) concentration from 20 to 100 mg L<sup>-1</sup>

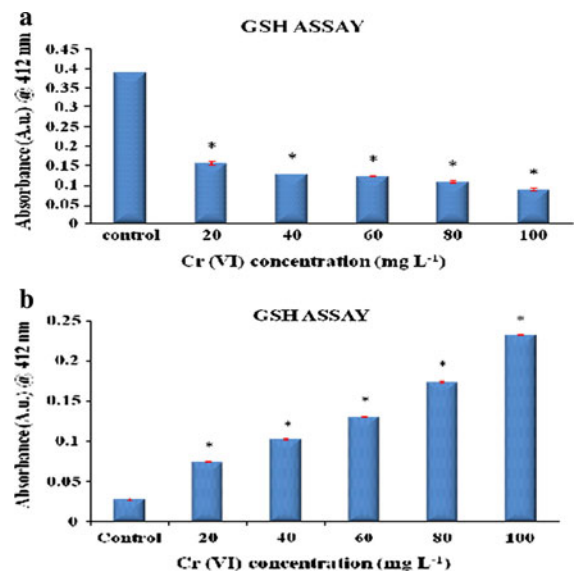
ROS production at 20, 40 and 60 mg L<sup>-1</sup> of Cr(VI) by adapted *E. coli* compared to unadapted. The less ROS production shows that Cr(VI) is less toxic to *B. subtilis* and this result corroborates with high specific growth rate and Cr(VI) reduction capacity compared to unadapted *B. subtilis*. Cr(VI) is more toxic to *E. coli* due to which the specific growth rate and Cr(VI) reduction capacity were less compared to *B. subtilis*. Existing literature has established the role of oxidative stress on bacterial cells, due to ROS formation, in the presence of heavy metal in *E. coli* and *Salmonella typhimurium* (Lushchak 2001).

A significant ( $p < 0.05$ ) concentration dependent decrease in SOD level was detected [20 > 40 > 60 > 80 > 100 mg L<sup>-1</sup> of Cr(VI)] for adapted *B. subtilis* compared to un-interacted unadapted (Fig. 7a) which correlates with ROS production. Adapted *E. coli* had no significant increase at 20 mg L<sup>-1</sup> of Cr(VI) but showed a significant ( $p < 0.05$ ) increase at 40 < 60 < 80 < 100 mg L<sup>-1</sup> of Cr(VI) compared to un-interacted unadapted (Fig. 7b). Adapted *E. coli* showed elevated level of SOD on interaction with Cr(VI) correlating with ROS results.

Reduced glutathione was observed to deplete significantly ( $p < 0.05$ ) with increase in Cr(VI)

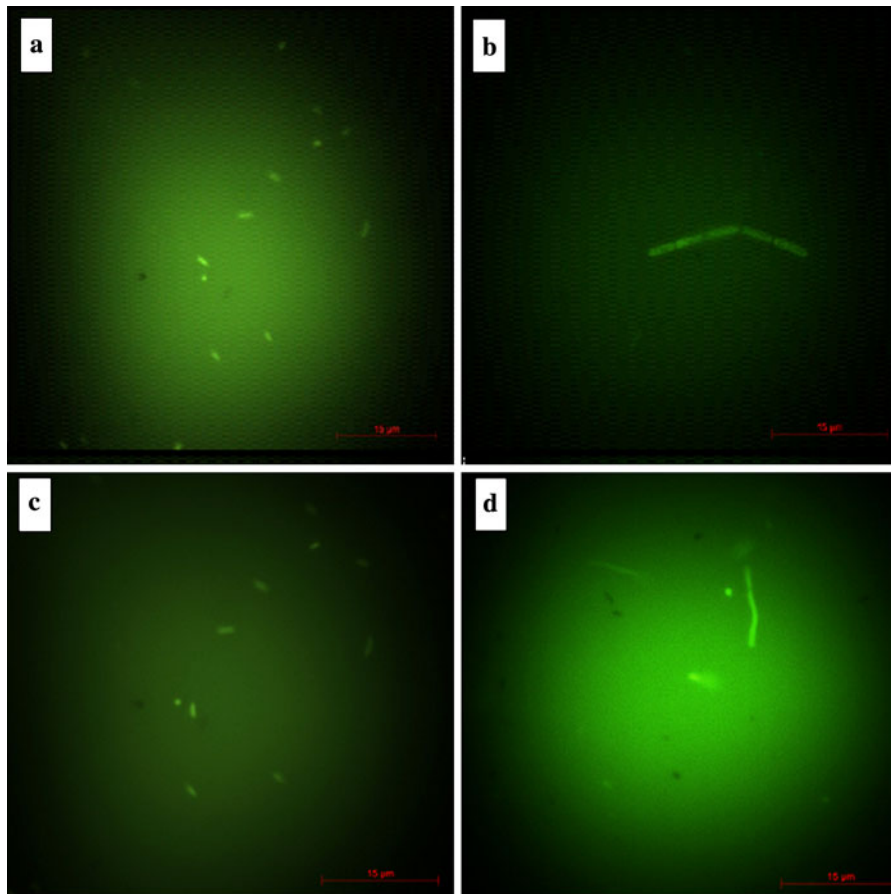


**Fig. 7** Superoxide dismutase activity (SOD). **a** The SOD activity with increasing Cr(VI) concentration from 20 to 100 mg L<sup>-1</sup> in *a B. subtilis*, **b** *E. coli*



**Fig. 8** Total reduced glutathione assay (GSH). The total glutathione reductase activity at varied Cr(VI) concentration from 20 to 100 mg L<sup>-1</sup> in **a** *B. subtilis*, **b** *E. coli*

concentration [20 > 40 > 60 > 80 > 100 mg L<sup>-1</sup> of Cr(VI)] in adapted compared to unadapted *B. subtilis* (Fig. 8a). The decrease in reduced glutathione correlates with decrease in ROS and SOD production in the presence of Cr(VI). Reduced glutathione increased significantly ( $p < 0.05$ ) with increase in Cr(VI)



**Fig. 9** Fluorescent micrographs of **a** unadapted *B. subtilis*, **b** adapted [ $100 \text{ mg L}^{-1} \text{ Cr(VI)}$ ] *B. subtilis* and **c** unadapted *E. coli*, **d** adapted [ $100 \text{ mg L}^{-1} \text{ Cr(VI)}$ ] *E. coli* isolates interacted with the  $\text{H}_2\text{O}_2$ -activated green fluorescent dye

DCFH-DA. The increased green fluorescence observed in the unadapted *B. subtilis* and Cr(VI) challenged adapted *E. coli* isolates is indicative of high oxidative stress

concentration ( $20 < 40 < 60 < 80 < 100 \text{ mg L}^{-1}$ ) in adapted *E. coli* cells compared to unadapted (Fig. 8b). The increase in reduced glutathione due to increase in ROS in the presence of Cr(VI) shows that the bacteria has the ability to scavenge ROS which is produced in response to Cr(VI) stress due to toxicity.

The oxidative stress was observed in fluorescence microscope using DCFH-DA dye. The intensity of green fluorescence when incubated with DCFH-DA showed the level of oxidative stress in bacteria. The adapted *B. subtilis* had very less fluorescence compared to unadapted symbolizing less oxidative stress (Fig. 9a, b). Adapted *E. coli* isolates interacted with the  $\text{H}_2\text{O}_2$ -activated green fluorescent dye DCFH-DA showed enhanced green fluorescence, which is indicative of high oxidative stress due to ROS produced in response to Cr(VI) interaction (Fig. 9c, d). These

results corroborate with the high fluorescence intensity observed for *E. coli* K-12 with high oxidative stress reported (Ackereley et al. 2006).

## Conclusion

In the current study, differential specific growth rate, high Cr(VI) reduction capacity (*B. subtilis*:  $13.96 \text{ mg g}^{-1}$ ; *E. coli*:  $8.61 \text{ mg g}^{-1}$ ) and toxicity were observed between *B. subtilis* and *E. coli* isolated from the same mine site and adapted to similar Cr(VI) concentration. Therefore, proof of concept studies using single bacterial isolates from heavy metal contaminated sites may pose conflicting scenario with respect to bioremediation. Further step in this regard is to form a consortium of adapted isolates which may iron out the differences improving the overall



reduction capacity and in situ bioremediation potential suitable for specific contaminated sites. The ability of adapted isolates to reduce Cr(VI) in the absence of any external electron donor at a comparatively high reduction capacity suggests the potential application prospect in scale-up of this batch process for large scale, effective and long-term Cr(VI) remediation to develop a lab scale reactor to monitor the efficiency of adapted bacterial consortia in treatment of Cr contaminated water from the mine site. Different reports on remediation of metals by ex-situ (pump and treat, bio-filter) and in-situ (biostimulation, bioaugmentation) strategy suggest the feasibility of the process (Nyman et al. 2006; Khan et al. 2004).

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