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Research Paper

Impact of environmental stress on biochemical parameters of bacteria reducing chromium

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Abstract

Chromium pollution is produced in connection with industrial processes like in tanneries. It has been suggested that bioremediation could be a good option for clean up. The stress effect of variable chromate levels, pHs and growth temperatures on biochemical parameters of two Cr(VI) reducing bacterial strains *Pseudomonas aeruginosa* Rb-1 and *Ochrobactrum intermedium* Rb-2 was investigated. Transmission electrone microscopy (TEM) was performed to study the intracellular distribution of Cr(VI). It was observed that initial stress of 1000 μgmL^{-1} caused significant enhancement of all studied biochemical parameters at pH 7.0 and growth temperature of 37 °C showing great bioremediation potential of the strains. Transmission electron microscopy revealed that the distribution of chromium precipitates was not uniform as they were distributed in the cytoplasm as well as found associated with the periplasm and outer membrane. Fourier transform infrared spectroscopy showed the possible involvement of carboxyl, amino, sulpohonate and hydroxyl groups present on the bacterial cell surface for the binding of Cr(VI) ions. Cr(VI) stress brought about changes in the distridution of these functional groups. It can be concluded that the investigated bacterial strains adjust well to Cr(VI) stress in terms of biochemical parameters and along that exhibited alteration in morphology.

Key words: Cr(VI), pH, temperature, biochemical parameters, TEM, FTIR spectroscopy.

Introduction

Despite the fact that bacteria are resistant to a variety of compounds, they are at the same time sensitive to even minute changes in the surrounding environment (Weilharter *et al.*, 2011). Stressful environmental conditions lead to a wide range of responses at the morphological, physiological, cellular and biochemical levels (Gustavs *et al.*, 2009). The ability of bacterial strains to cope with sudden changes in the surrounding environment ensures their ecological dominance under stress conditions. Heavy metal pollution has turned into a major environmental problem and caused severe threats to environmental protection and human health (Järup, 2003). Chromium is among the most hazardous heavy metals (Xu *et al.*, 2009). Due to high solubility, Cr(VI) can easily pass across biological membranes and exhibit a range of toxic effects evident at cellular and molecular levels (Poljsak *et al.*, 2011). The much less soluble Cr(III) is less toxic. Bacteria adapt different strategies to combat high level stress along with transformation of Cr(VI) to Cr(III) either intra or extracellularlly (Cervantes and Campos-García, 2007). Chromium stress can induce many types of metabolic responses in living organisms such as (a): increased production of metabolites *e.g.*, peroxidase, auxin as a direct response to Cr stress, (b): alterations in the metabolism resulting in the production of new metabolites, *e.g.*, glutathione, proline which may be responsible for resistance or tolerance to chromium stress (Nagajyoti *et al.*, 2010). Heavy metal stress cause severe oxidative damage to biomolecules due to the production of reactive oxygen species (ROS). The high concentrations of ROS led to the disruption of the normal physiological and

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cellular functioning of the living cells. To combat with such stress, bacteria have developed certain enzymatic systems such as peroxidases (Panda and Choudhury, 2005). One of the major reasons of using the microbes for the control and remediation of metal polluted environment is their biochemical versatility, a result of their genetic plasticity and ability to modify physiology as to make them best competitor in a constantly changing environment (Murugesan and Maheswari, 2007). To understand the measures adapted by bacteria to cope with stress of hexavalent chromium, different biochemical parameters of chromium reducing bacterial strains *Pseudomonas aeruginosa* Rb-1 and *Ochrobacterum intermedium* Rb-2 were analyzed. Transmission electron microscopy (TEM) was performed to localize the distribution of chromium particles intra as well extra-cellularly.

Materials and Methods

Bacterial strains and growth conditions

Pseudomonas aeruginosa Rb-1 (FJ870126) and *Ochrobactrum intermedium* Rb-2 (FJ870125), Gram negative Cr(VI) reducing bacterial strains previously isolated from tannery effluent were obtained from bacterial stock cultures of Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. They were normally grown in Luria Bertani (LB) agar (pH 7.0) at 37 °C.

Biochemical analysis of bacteria reducing hexavalent chromium

Bacterial strains were aerobically grown in Luria Bertani (LB) broth supplemented with different hexavalent chromium concentrations (100, 500 and 1000 μg mL^{-1} of K_2CrO_4). Cultures were incubated at variable temperatures (28, 37 and 40 °C) and pHs (5, 7 and 9) for 24 - 48 hours. Under aseptic conditions, harvesting of cells was done by centrifugation (5,000 x g for 10 min) at 4 °C. All the experiments were done in triplicates. Following biochemical parameters of hexavalent chromium reducing bacteria were estimated.

Peroxidase activity and estimation of soluble protein

Peroxidase activity of bacterial strains was determined according to Davy and Murry (1965). Briefly, harvested bacterial cells were disrupted in cold 0.1 M phosphate buffer (pH 7.0) by sonication for 5 min (Heilscher Ultrasonic Processors UP 400, S) at 4 °C. The ratio of buffer to bacterial pellet was 4:1 (v/w). The homogenate was centrifuged at 14,000 x g for 10 min. The supernatant was used for the estimation of enzyme peroxidase.

Formula used for peroxidase activity is as follows:

$$\frac{\text{O.D of Test} - \text{O.D of Control}}{\text{O.D of Control}} \times \frac{\text{Weight of bacterial}}{\text{pellet (g)}}$$

where O.D = Optical density at 470 nm.

For extraction of soluble proteins, samples were prepared accordingly (Bhatti *et al.*, 1993) whereas for soluble protein analysis version of Lowry's method was adopted (Lowry *et al.*, 1951). Amount of soluble proteins was calculated from standard curve obtained by using Bovine serum albumin (BSA) as standard at wavelength of 750 nm on Beckman D-2 spectrophotometer.

Auxin biosynthesis

Auxin production by bacterial strains both in the presence and absence of K_2CrO_4 was determined by using Salkowski colorimetric technique (Glickmann and Dessaux, 1995). Auxin content was estimated by measuring the absorbance at 535 nm with Beckman D-2 spectrophotometer. Optical densities of various concentrations of indole-3-acetic acid (IAA) (standard) were also measured to construct a standard curve. From the standard curve the actual amount of auxin was measured and calculated as μg gm^{-1} fresh weight of bacteria.

Estimation of proline content

Proline was determined by the modified ninhydrin method (Derminal and Turkan, 2006). Harvested bacterial cells were suspended in 1 mL sterilized distilled water and placed in boiling water bath for 20 min to extract all water soluble compounds in hot water and cooled at room temperature. The bacterial suspension was then centrifuged at 13,000 x g for 5 min. The supernatant (200 μL) was taken and 150 μL distilled water and 1 mL of ninhydrin reagent was added in a test tube and placed in boiling water bath for one hour. The test tubes were cooled on ice to stop the reaction. Toluene (6 mL) was added by vigorous shaking and tubes vortexed for 20 seconds. The optical density of resulting inorganic layer was measured at 520 nm with Beckman D-2 spectrophotometer. The amount of proline produced by bacteria was calculated from standard curve.

Estimation of nitrate reductase activity

Weighed bacterial pellet (1 g) was homogenized with 10 mL ice cold extraction buffer (0.1 M phosphate buffer, pH 7.5 containing 0.5 mM EDTA). The extract was filtered and stored on ice. 1 mM KNO_3 , 0.1 mM NADH, 100 mM Phosphate buffer (pH 7.5) and 0.5 mM EDTA were added to the enzyme extract (1 mL) followed by incubation at 25 °C for 20 min. The reaction was terminated by the addition of 0.25 mL saturated zinc acetate and 0.5 mL 80% ethanol. After centrifuging, 0.5 mL of 1% (w/v) solution of sulfanilamide in 3 M HCl and 0.5 mL of 0.02% (w/v) solution of N-l-naphthyl-ethylene-diamine was added and left at room temperature for 30 min for color de-

velopment. Optical density was measured at 540 nm with the Beckman D-2 spectrophotometer.

Analysis of nonprotein thiols and estimation of cysteine

Total GSH (Gamma-L-glutamyl-L-cysteninyl-glycine) and GSSG [Bis (gamma-Glutamyl-L-cysteinyl-glycine) Disulfide] were measured by the GSSG recycling method, with GSSG as the standard (Satoh *et al.*, 2002).

Estimation of cysteine and cystine content (*mM*) of both the strains was done according to Gaitonde (1967) (Gaitonde, 1967).

Fourier Transform infrared spectral analysis

For the FTIR study, bacterial cell pellets were centrifuged and lyophilized, followed by weighing. Then 20 mg of finely ground biomass was encapsulated in 200 mg of KBr (Sigma) in order to prepare translucent sample disks. The spectra of the lyopholized bacterial cell pellets were obtained by using PerkinElmer spectrum BX FTIR system (Beacon field Buckinghamshire HP9 1QA) equipped with diffuse reflectance accessory with the range of 500-4000 cm^{-1}. All spectra were acquired in transmission mode, by the KBr disc method to get the information specific to the functional groups.

Electron microscopy

The samples for thin-sectioning were prepared as described (Lounatmaa, 1985). Briefly, the samples were prefixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.2) with or without tannic acid for 2 hours at room temperature. The fixed cells were washed three times with phosphate buffer. All samples were post-fixed with phosphate-buffered 1% osmium tetra oxide and dehydrated in acetone series and embedded in Taab resin. The thin-sectioned cells were post-stained with uranyl acetate and lead citrate. The samples were viewed using a transmission electron microscope (JEM-1200EX), operated at 60 kV.

Statistical analysis

Data was statistically analyzed using SPSS personal computer statistical package (version 16, SPSS Inc, Chicago). Analysis of variance (ANOVA) was performed and then means were separated using Duncan's multiple range test ($p = 0.05$).

Results

Effect of chromate

There was a significant increase in all the studied biochemical parameters (except nitrate reductase) of both the strains with the increase in initial Cr(VI) concentration (Table 1). At low level of chromate (100 and 500 $\mu g\ mL^{-1}$), the soluble protein content of *O. intermedium* Rb-2 (42.56 and 55.63 $mg\ g^{-1}$ cells fresh weight, respectively) was higher

Table 1 - Effect of variable initial chromate concentration (100, 500 and 1000 $\mu g\ mL^{-1}$) on biochemical parameters of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2.

Strain/stress ($\mu g\ mL^{-1}$)		Biochemical parameters								
		Soluble protein ($mg\ g^{-1}$ fresh weight)	Peroxidase (units g^{-1} fresh weight)	Auxin ($\mu g\ g^{-1}$ fresh weight)	Proline ($\mu g\ g^{-1}$ fresh weight)	Nitrate reductase ($\mu g\ g^{-1}$ fresh weight)	Cystine (mM)	Cysteine (mM)	GSH (nM)	GSSG (nM)
Rb-1	Control	15.44 ± 0.46(a)	29.12 ± 1.30(a)	13.18 ± 0.59(a)	410.52 ± 1.51(a)	1.93 ± 0.02(d)	0.04 ± 0.00(a)	0.83 ± 0.01(a)	2.87 ± 0.04(a)	4.33 ± 0.21(a)
	100	29.2 ± 0.55(b)	54.6 ± 1.48(b)	19.27 ± 0.39(b)	546.52 ± 0.74(b)	1.26 ± 0.02(c)	1.57 ± 0.14(b)	1.86 ± 0.06(b)	7.7 ± 0.29(b)	9.95 ± 0.24(b)
	500	44.37 ± 1.06(c)	65.64 ± 1.78(c)	58.55 ± 0.95(c)	881.94 ± 0.66(c)	0.72 ± 0.03(b)	3.66 ± 0.04(c)	2.97 ± 0.14(c)	13.04 ± 0.32(c)	25.37 ± 1.20(c)
	1000	52.82 ± 1.47(d)	95.34 ± 1.43(d)	84.59 ± 0.73(d)	988.12 ± 0.64(d)	0.36 ± 0.02(a)	5.12 ± 0.05(d)	4.53 ± 0.12(d)	22.28 ± 0.54(d)	46.00 ± 0.85(d)
Rb-2	Control	19.73 ± 0.50(a)	23.35 ± 0.91(a)	16.99 ± 0.66(a)	428.65 ± 1.78(a)	2.82 ± 0.05(d)	0.05 ± 0.00(a)	1.00 ± 0.01(a)	3.76 ± 0.05(a)	6.56 ± 0.13(a)
	100	42.56 ± 0.48(b)	50.02 ± 0.28(b)	22.62 ± 0.48(a)	681.38 ± 1.27(b)	2.16 ± 0.01(c)	1.16 ± 0.09(b)	2.83 ± 0.03(b)	10.85 ± 0.13(b)	25.08 ± 0.28(b)
	500	55.63 ± 0.75(c)	49.13 ± 0.27(b)	65.39 ± 1.19(b)	908.73 ± 1.67(c)	0.87 ± 0.01(b)	1.38 ± 0.02(b)	4.14 ± 0.06(c)	17.95 ± 0.24(c)	42.59 ± 0.44(c)
	1000	67.25 ± 0.75(d)	98.24 ± 0.55(c)	120.36 ± 1.83(c)	999.51 ± 9.88(d)	0.58 ± 0.01(a)	4.36 ± 0.11(c)	6.22 ± 0.03(d)	59.18 ± 0.97(d)	70.55 ± 0.96(d)

Mean of 04 replicates ± standard error of the mean. In each column within strains, figures followed by different letter (s) in parenthesis indicate significant difference by Duncan's multiple range test ($p < 0.05$).

than *P. aeruginosa* Rb-1 (29.2 and 44.37 mg g^{-1} cells fresh weight, respectively) and maximum soluble protein content was also recorded with *O. intermedium* Rb-2 (67.25 mg g^{-1} cells fresh weight) at 1000 μg mL^{-1} of Cr(VI). Hexavalent chromium stress lead to increase in peroxidase activity in both strains. The enzyme activity increased gradually with increasing Cr(VI) concentration for *P. aeruginosa* Rb-1 that exhibited peroxidase activity of 54.6; 65.64 and 95.34 μg g^{-1} cells fresh weight at 100, 500 and 1000 μg mL^{-1} of chromate, respectively compared to control. On the other hand, *O. intermedium* Rb-2 did not show the same pattern of peroxidase activity. It was 50.02; 49.13 and 98.24 μg g^{-1} cells fresh weight at increasing concentration of Cr(VI). Auxin content increased with the increasing chromate levels in both strains. *O. intermedium* Rb-2 was more efficient in auxin production than *P. aeruginosa* Rb-1. At 1000 μg mL^{-1} of chromate, *O. intermedium* Rb-2 produced 120.36 μg g^{-1} cells fresh weight auxin whereas *P. aeruginosa* Rb-1 produced 84.59 μg g^{-1} cells fresh weight. Proline content also increased with higher Cr(VI) concentration in both strains relative to control. At 1000 μg mL^{-1} of Cr(VI), *P. aeruginosa* Rb-1 exhibited a maximum proline content of 988.12 μg g^{-1} cells fresh weight and *O. intermedium* Rb-2 revealed proline content of 999.51 μg g^{-1} when compared to respective chromate free control. Nitrate reductase activity of both strains decreased with rising K_2CrO_4 in contrast to the other measured biochemical parameters. *O. intermedium* Rb-2 had higher nitrate reductase activity than *P. aeruginosa* Rb-1 at 1000 μg mL^{-1} of chromate (Table 1).

The content of non-protein thiols (cystine, cysteine, GSH and GSSG) in *O. intermedium* Rb-2 was generally higher than in *P. aeruginosa* Rb-1 under chromate stress with the exception of cystine. At 1000 μg mL^{-1} of chromate, *O. intermedium* Rb-2 exhibited enhanced production of cystine, cysteine, GSH and GSSG content, 4.36 mM, 6.22 mM, 59.18 nM and 70.55 nM, respectively. *P. aeruginosa* Rb-1 also showed enhanced cystine, cysteine, GSH and GSSG content (5.12 mM, 4.53 mM, 22.28 nM and 46.00 nM, respectively) under 1000 μg mL^{-1} of K_2CrO_4 over respective chromate free control (Table 1).

Effect of growth pHs

Generally, pH 7 was optimum for causing increment in biochemical parameters of both strains in chromate supplemented conditions compared to chromate free conditions. Cr(VI) stress manifested a maximum increase for all the studied biochemical parameters at all growth pHs by both bacterial strains revealed by t- test at $p = 0.05$. The soluble protein content of strains *i.e. P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 was highest at pH 7 under both chromate free and chromate supplemented conditions. Soluble protein content of *P. aeruginosa* Rb-1 was found to be 15.51 to 52.82 mg g^{-1} cells fresh weight whereas *O. intermedium* Rb-2 had a protein content of 18.38 and 67.25 mg g^{-1} cells fresh weight under chromate supplemented conditions. Peroxidase content of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 was recorded maximum at pH 7 in Cr(VI) solution exhibiting 95.34 μg g^{-1} and 98.24 μg g^{-1} cells fresh weight peroxidase content respectively. Under Cr(VI) stress, *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 manifested a maximum increase in auxin content at pH 7 under chromate supplemented conditions. Auxin content of *P. aeruginosa* Rb-1 was in the range of 70.36 to 84.59 μg g^{-1} cells fresh weight while *O. intermedium* Rb-2 exhibited a higher auxin content, 79.58 to 120.36 μg g^{-1} cells fresh weight, at the studied pHs (Table 2).

Proline content of *O. intermedium* Rb-2 was found to be higher than proline content of *P. aeruginosa* Rb-1 at all the studied pHs with maximum at pH 7 under chromate free as well as chromate supplemented conditions. At pH 7, the proline content of *O. intermedium* Rb-2 was 999.51 μg g^{-1} cells fresh weight whereas *P. aeruginosa* Rb-1 showed a proline content of 988.12 μg g^{-1} cells fresh weight under Cr(VI) stress. Nitrate reductase activity of both strains *i.e. P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 were maximum at pH 7 when assayed through *in vitro* system under chromate free as well as chromate supplemented conditions, but chromate lowered the activity. Nitrate reductase activity of *O. intermedium* Rb-2 was relatively higher (0.58 μg g^{-1} fresh weight) than *P. aeruginosa* Rb-1 (0.36 μg g^{-1} fresh weight) at pH 7 under Cr(VI) stress (Table 2).

Non-protein thiols (cystine, cysteine, GSH and GSSG) of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 were maximum at pH 7 under chromate free as well as chromate supplemented conditions. Non-protein thiol content (cysteine, GSH and GSSG) of *O. intermedium* Rb-2 was significantly higher than for *P. aeruginosa* Rb-1 except in case of cystine content which was higher in *P. aeruginosa* Rb-1 at all studied growth pHs under Cr(VI) stress (Table 2).

Effect of growth temperatures

For both bacterial strains, 37 °C was found to be optimal for all studied biochemical parameters under chromate free as well as chromate supplemented conditions giving highest values. All biochemical parameters of both the strains showed an increase at all growth temperatures when compared with chromate free control (revealed by t- test at $p = 0.05$). Soluble protein content at 37 °C under chromate supplemented conditions was higher for *O. intermedium* Rb-2 (67.25 mg g^{-1} cells fresh weight) than for *P. aeruginosa* Rb-1 (52.82 mg g^{-1} cells fresh weight). Peroxidase activity of Rb-1 and Rb-2 was highest at 37 °C. Under chromate supplemented conditions, peroxidase activity of *O. intermedium* Rb-2 varied in the range of 50.74 to 98.24 μg g^{-1} cells fresh weight at tested growth tempera-

Table 2 - Effect of variable growth pHs (5, 7 and 9) on biochemical parameters of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 under chromate free and chromate supplemented conditions (1000 μg mL^{-1} of K_2CrO_4).

Strain	Parameters	Chromate free			Chromate supplemented		
		5	7	9	5	7	9
Rb-1	Soluble protein (mg g^{-1} fresh weight)	5.11 ± 0.06	19.50 ± 0.97	15.44 ± 0.46	15.51 ± 0.17*	52.82 ± 1.47*	23.52 ± 0.38*
	Peroxidase (μg g^{-1} fresh weight)	14.78 ± 0.08	33.62 ± 0.39	21.09 ± 0.26	38.7 ± 1.32*	95.34 ± 1.43*	56.84 ± 0.32*
	Auxin (μg g^{-1} fresh weight)	2.73 ± 0.15	13.18 ± 0.59	6.36 ± 0.21	70.36 ± 1.12*	84.59 ± 0.73*	80.12 ± 1.22*
	Proline (μg g^{-1} fresh weight)	264.68 ± 0.17	410.52 ± 1.51	337.14 ± 1.20	672.35 ± 0.43*	988.12 ± 0.64*	808.07 ± 0.52*
	Nitrate reductase(μg g^{-1} fresh weight)	0.87 ± 0.02	1.93 ± 0.02	1.03 ± 0.02	0.16 ± 0.03*	0.36 ± 0.02*	0.23 ± 0.02*
	Cystine (mM)	0.02 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	1.70 ± 0.12*	5.12 ± 0.05*	2.35 ± 0.01*
	Cysteine (mM)	0.48 ± 0.00	0.83 ± 0.01	0.38 ± 0.01	2.5 ± 0.04*	4.53 ± 0.12*	3.18 ± 0.01*
	GSH (nM)	0.23 ± 0.01	2.87 ± 0.04	1.90 ± 0.26	11.76 ± 0.56*	22.28 ± 0.54*	5.08 ± 0.20*
	GSSG (nM)	0.96 ± 0.02	4.33 ± 0.21	4.03 ± 0.35	10.93 ± 0.27*	46.00 ± 0.85*	12.42 ± 0.30*
Rb-2	Soluble protein (mg g^{-1} fresh weight)	6.83 ± 0.08	19.73 ± 0.50	4.30 ± 0.05	18.38 ± 0.70*	67.25 ± 0.75*	46.83 ± 0.64*
	Peroxidase (μg g^{-1} fresh weight)	21.53 ± 0.49	23.35 ± 0.91	23.19 ± 1.03	31.2 ± 0.17*	98.24 ± 0.55*	51.98 ± 0.29*
	Auxin (μg g^{-1} fresh weight)	10.09 ± 0.14	16.99 ± 0.66	11.34 ± 0.15	79.58 ± 0.69*	120.36 ± 1.83*	86.00 ± 1.34*
	Proline (μg g^{-1} fresh weight)	270.85 ± 0.18	428.65 ± 1.78	352.49 ± 4.11	707.95 ± 0.46*	999.51 ± 9.88*	815.22 ± 0.53*
	Nitrate reductase(μg g^{-1} fresh weight)	0.63 ± 0.01	2.82 ± 0.05	0.88 ± 0.01	0.13 ± 0.00*	0.58 ± 0.01*	0.18 ± 0.00*
	Cystine (mM)	0.03 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	1.38 ± 0.02*	6.22 ± 0.03*	1.73 ± 0.15*
	Cysteine (mM)	0.26 ± 0.00	1.00 ± 0.01	0.21 ± 0.00	2.64 ± 0.10*	7.36 ± 0.11*	3.95 ± 0.06*
	GSH (nM)	0.53 ± 0.01	3.76 ± 0.13	0.68 ± 0.07	12.07 ± 0.31*	59.18 ± 0.96*	21.75 ± 0.43*
	GSSG (nM)	1.53 ± 0.03	6.56 ± 0.05	3.91 ± 0.02	29.02 ± 0.24*	70.55 ± 0.97*	32.68 ± 0.37*

Mean of 04 replicates ± standard error of the mean. "*" indicates significant difference between chromate free and chromate supplemented treatments by Student's t- test at p = 0.05. GSH (Gamma-L-glutamyl-L-cysteninyl glycine) and GSSG [Bis (gamma-Glutamyl-L-cysteinyl glycine) Disulfide].

tures whereas *P. aeruginosa* Rb-1 exhibited 16.74 to 23.35 μg g^{-1} cells fresh weight peroxidase activity. Auxin content of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 was recorded highest at growth temperature of 37 °C under chromate free as well as chromate supplemented conditions. Relatively higher auxin content (69.33 to 120.36 μg g^{-1} cells fresh weight) was shown for *O. intermedium* Rb-2 than for *P. aeruginosa* Rb-1 (65.45 to 84.59 μg g^{-1} cells fresh weight) at the studied growth temperatures under Cr(VI) stress (Table 3).

Proline content of *O. intermedium* Rb-2 was higher than for *P. aeruginosa* Rb-1 at all the growth temperatures under chromate free as well as chromate supplemented conditions. Maximum proline content was recorded at 37 °C for both strains, *i.e.* 988.12 and 999.51 μg g^{-1} cells fresh weight by Rb-1 and Rb-2, respectively under stress of hexavalent chromium. The growth temperature of 37 °C was found to be conducive for the maximum nitrate reductase activity for both strains when assayed through *in vitro* system under chromate free conditions. *O. intermedium* Rb-2 exhibited relatively higher nitrate reductase activity (0.58 μg g^{-1} fresh weight) than *P. aeruginosa* Rb-1 (0.36 μg g^{-1} fresh weight) under Cr(VI) stress. Non-protein thiols contents of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 was maximum at growth temperature of 37 °C. Cystine, cysteine, GSH and GSSG contents of *O. intermedium* Rb-2 were found higher than for *P. aeruginosa* Rb-1 at all the studied growth temperatures under chromate supplemented conditions (Table 3).

Transmission electron microscopy

TEM analysis was performed to locate the intracellular distribution of Cr(VI). In the thin sections of both strains, cells were having smooth cell surface in the absence of chromium stress (Figure 1 A and C). Upon exposure to Cr(VI), cells of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 showed the increment in size and became irregular in shape. Cr(VI) stress caused lysis of bacterial cells of both bacterial strains (Figure 1 B and D). The thin sections of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 showed that precipitates of chromium were distributed in the cytoplasm as well associated with the periplasm and outer membrane (Figure 1 B and D). The cells of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 showed deposition of chromium precipitates at the cell periphery, even when grown in the absence of hexavalent chromium (Figure 1 A and C).

Fourier Transform Infrared (FTIR) spectroscopy

The FTIR spectra of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 grown in L-broth in the presence and absence of 1000 μg mL^{-1} of K_2CrO_4 were taken in the range of 500-4500 cm^{-1} (wave number) in order to determine the role of various functional groups present on the bacterial cell surface and were involved in the uptake of Cr(VI). The FTIR spectrum pattern of cells of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 grown in L-broth without Cr(VI) showed the presence of number of functional groups on their cell surface. The prominent absorption peaks in the region of 4000-3500 cm^{-1} were due to OH- symmetric stretch vibration. The absorption peaks in the region of 3500-3200 cm^{-1} were indicative of -OH group and -NH groups; 3000-2500 cm^{-1} showed existence of the carboxylic group; 2600-2500 cm^{-1} exhibited the presence of S-H group and the peaks in the region of 2400-2300 cm^{-1} specified the existence of amines. Absorption peaks at 2260-2100 cm^{-1} were due to C≡C whereas the peak at 1690-1640 cm^{-1} and 1640-1500 cm^{-1} showed the existence of primary and secondary amines and amides, (N-H bending) respectively. Carboxylate ions usually displayed the absorption peaks in the region of 1300-1420 cm^{-1}. Absorption peak in the region of 1239.99 cm^{-1} was due to presence of sulphonate (SO_2O^-) groups whereas absorption peaks in the region of 1300-1000 cm^{-1} corresponded to C-O stretching of COOH. Absorption peaks in the region of 750-1000 cm^{-1} showed the existence of S = O, -C-C- and C-Cl functional groups (Figure 2 A and C).

In the FTIR spectra of cells of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 grown in L-broth with 1000 μg mL^{-1} of K_2CrO_4, shifts were observed in the absorption peaks at different regions. Major changes were observed in the region of 2500-500 cm^{-1} under Cr(VI) stress shown by both strains (Figure 2 B and D).

Discussion

Chromate stress brought about changes not only in the bacterial morphology but it also affected the biochemical parameters of both investigated bacteria. Chromate stress resulted in stimulation of all the biochemical parameters of the two strains. Increased synthesis of various enzymes is one of the mechanisms to alleviate stress. The observed increment of all the biochemical parameters with the increase in initial concentration of Cr(VI) may be due to increased synthesis of metal binding proteins. Due to highly mutagenic nature, Cr(VI) induce responses in living organism at molecular level by causing damage to DNA. To prevent the cells from cellular oxidative damage during stress conditions, increased synthesis of various enzymes such as peroxidase and non protein thiols by metal resistant bacteria has previously been reported (Ramírez-Díaz *et al.*, 2008). Higher soluble protein content was recorded under chromate stress in both strains which may be due to increased synthesis of metal binding proteins under heavy metal stress. Increment in soluble protein content by *Pseudomonas* under Cr(VI) stress has been reported due to over expression of metal binding proteins (Murugesan and Maheswari, 2007). Similarly, enhanced protein content at higher levels of lead is reported due to increase of the synthesis of metal binding proteins (Andreoni *et al.*, 1997; Pant

Table 3 - Effect of variable growth temperatures (28, 37 and 42 °C) on biochemical parameters of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 under chromate free and chromate supplemented conditions (1000 µg mL^{-1} of K_2CrO_4).

Strain	Parameters	Chromate free			Chromate supplemented		
		28 °C	37 °C	42 °C	28 °C	37 °C	42 °C
Rb-1	Soluble protein (mg g^{-1} fresh weight)	2.34 ± 0.03	19.50 ± 0.97	5.81 ± 0.07	26.40 ± 0.18*	52.82 ± 1.47*	24.55 ± 0.16*
	Peroxidase (µg g^{-1} fresh weight)	23.56 ± 0.75	33.62 ± 0.39	24.87 ± 1.20	31.08 ± 0.83*	92.84 ± 1.41*	83.00 ± 2.32*
	Auxin (µg g^{-1} fresh weight)	5.09 ± 0.19	13.18 ± 0.59	8.18 ± 0.23	65.45 ± 1.05*	84.59 ± 0.73*	66.73 ± 1.07*
	Proline (µg g^{-1} fresh weight)	362.79 ± 0.36	410.52 ± 1.51	383.01 ± 0.25	820.4 ± 0.59*	988.12 ± 0.64*	911.07 ± 0.59*
	Nitrate reductase(µg g^{-1} fresh weight)	1.11 ± 0.02	1.93 ± 0.02	1.93 ± 0.02	0.24 ± 0.01*	0.36 ± 0.02*	0.26 ± 0.00*
	Cystine (mM)	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	1.4 ± 0.02*	5.12 ± 0.05*	2.36 ± 0.04*
	Cysteine (mM)	0.15 ± 0.00	0.83 ± 0.01	0.15 ± 0.00	1.28 ± 0.02*	4.53 ± 0.12*	0.87 ± 0.01*
	GSH (nM)	1.76 ± 0.07	2.87 ± 0.07	1.62 ± 0.04	13.07 ± 0.48*	22.28 ± 0.54*	7.50 ± 0.38*
	GSSG (nM)	2.92 ± 0.04	4.33 ± 0.21	4.02 ± 0.16	19.69 ± 0.61*	46 ± 0.85*	37.13 ± 0.91*
Rb-2	Soluble protein (mg g^{-1} fresh weight)	5.57 ± 0.06	19.73 ± 0.50	9.09 ± 0.10	31.31 ± 0.35*	67.25 ± 0.75*	62.38 ± 0.70*
	Peroxidase (µg g^{-1} fresh weight)	16.74 ± 1.08	23.35 ± 0.91	17.32 ± 0.49	50.74 ± 1.08*	98.24 ± 0.55*	80.63 ± 0.45*
	Auxin (µg g^{-1} fresh weight)	6.15 ± 0.11	16.99 ± 0.66	10.45 ± 0.15	69.33 ± 0.45*	120.36 ± 1.83*	75.41 ± 2.79*
	Proline (µg g^{-1} fresh weight)	363.44 ± 3.73	428.65 ± 1.78	410.79 ± 2.60	827.67 ± 0.53*	999.51 ± 9.88*	945.05 ± 5.54*
	Nitrate reductase(µg g^{-1} fresh weight)	1.31 ± 0.01	2.82 ± 0.05	1.81 ± 0.02	0.39 ± 0.03*	0.58 ± 0.01*	0.26 ± 0.01*
	Cystine (mM)	0.09 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	3.91 ± 0.03*	6.22 ± 0.03*	3.28 ± 0.05*
	Cysteine (mM)	0.27 ± 0.00	1.00 ± 0.01	0.18 ± 0.00	5.18 ± 0.07*	7.36 ± 0.11*	3.83 ± 0.03*
	GSH (nM)	1.96 ± 0.05	3.76 ± 0.13	1.53 ± 0.09	10.69 ± 0.42*	59.18 ± 0.96*	13.16 ± 0.24*
	GSSG (nM)	4.79 ± 0.02	6.56 ± 0.05	5.34 ± 0.06	17.23 ± 0.26*	70.55 ± 0.97*	23.84 ± 0.39*

Mean of 04 replicates ± standard error of the mean. "*" indicates significant difference between chromate free and chromate supplemented treatments by Student's t- test at p = 0.05. GSH (Gamma-L-glutamyl-L-cysteninylglycine) and GSSG [Bis (gamma-Glutamyl-L-cysteinylglycine) Disulfide].

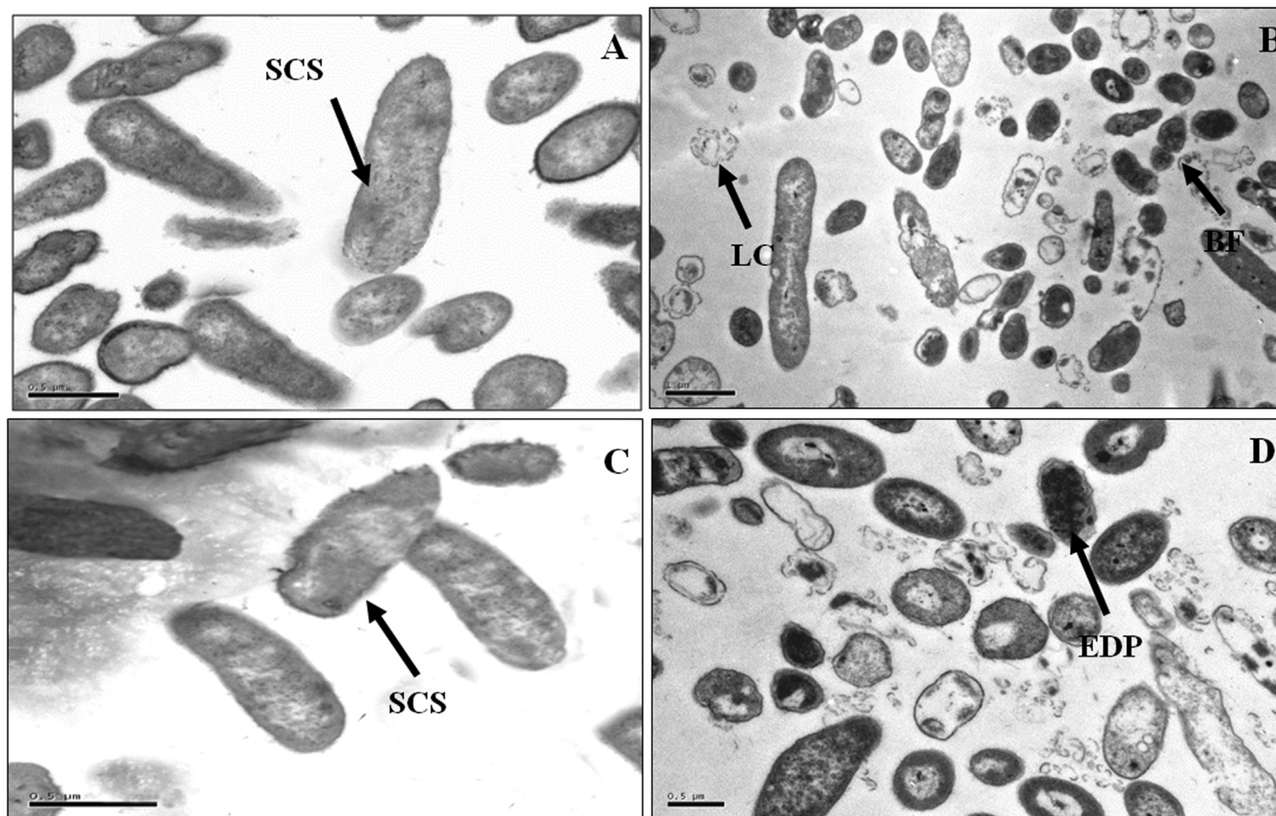

Figure 1 - TEM images of cross sectioned *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 cells. A: Cells of *P. aeruginosa* Rb-1 grown without Cr(VI) for 48 hours. B: *P. aeruginosa* Rb-1 cells grown with 1000 μg mL^{-1} of Cr(VI) C: Cells of *O. intermedium* Rb-2 grown without Cr(VI) for 48 hours D: *O. intermedium* Rb-2 cells grown with 1000 μg mL^{-1} Cr(VI). Arrows indicate SCS=smooth cell surface; BF= cell showing binary fission; EDP=electron dense particles; LC = lysed cells.

et al., 2011). Chromate stress induced in both investigated strains a close to three time higher peroxidase activity at pH7. The higher peroxidase activity under chromate stress can be related to the fact that Cr(VI) causes oxidative damage and peroxidases produced by metal resistant bacteria have the ability to protect cellular proteins and DNA from oxidation during stress conditions (Ramírez-Díaz *et al.*, 2008; Pant *et al.*, 2011). Indole acetic acid (IAA) is a common natural auxin and is a common secondary metabolite of most of the rhizospheric microorganisms (Yurekli *et al.*, 2003; Khamna *et al.*, 2010). Growth temperature of 37 °C and pH 7 was found to be optimal for maximum production of auxin by both bacterial strains. Proline is known to be an indicator of stress tolerance and functions as metal chelator. Under stress conditions, intracellular accumulation of proline in microbes is a well-documented fact (Köcher *et al.*, 2011). Both strains, *i.e. P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 exhibited significant enhanced production of proline under Cr(VI) stress compared to chromate free conditions. Nitrate reductase activity was the only biochemical parameter found to be inhibited under Cr(VI) stress treatments in both strains. Inhibition of nitrate reductase activity due to heavy metal stress has previously been reported (Awasthi, 2005; Srivastava and Thakur, 2007). One of the reasons for the inhibition of nitrate reductase activity is interference of heavy metal ions with sulphydryl (-SH) groups in enzymes which are involved in determining the secondary and tertiary structure of proteins (Awasthi, 2005). This can lead to lowered enzyme activity.

Intracellular concentration of GSSG increases at the cost of GSH under intense stress conditions (Ackerley *et al.*, 2006). We observed non-protein thiol production was enhanced under Cr(VI) stress in both studied strains. GSH (Gamma-L-glutamyl-L-cysteninyl glycine) and GSSG [Bis (gamma-Glutamyl-L-cysteinyl glycine) Disulfide] content was peaking at pH 7, 1000 μg mL^{-1} of K_2CrO_4 and 37 °C. GSH concentration was lower than GSSG content. GSH and GSG content of *O. intermedium* Rb-2 was remarkably higher than for *P. aeruginosa* Rb-1. Cellular exposure to oxidants resulted in reduction in the level of GSH and increment in the level of its oxidation product (GSSG). Cysteine and cystine content of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 was highest at pH 7, 1000 μg mL^{-1} of K_2CrO_4 and 37 °C, but *O. intermedium* Rb-2 produced more cysteine and cystine. This difference in the ability to induce the non-protein thiols among these strains might be due to variation in tolerance level to chromate. Although, there is very little information available about intracellular

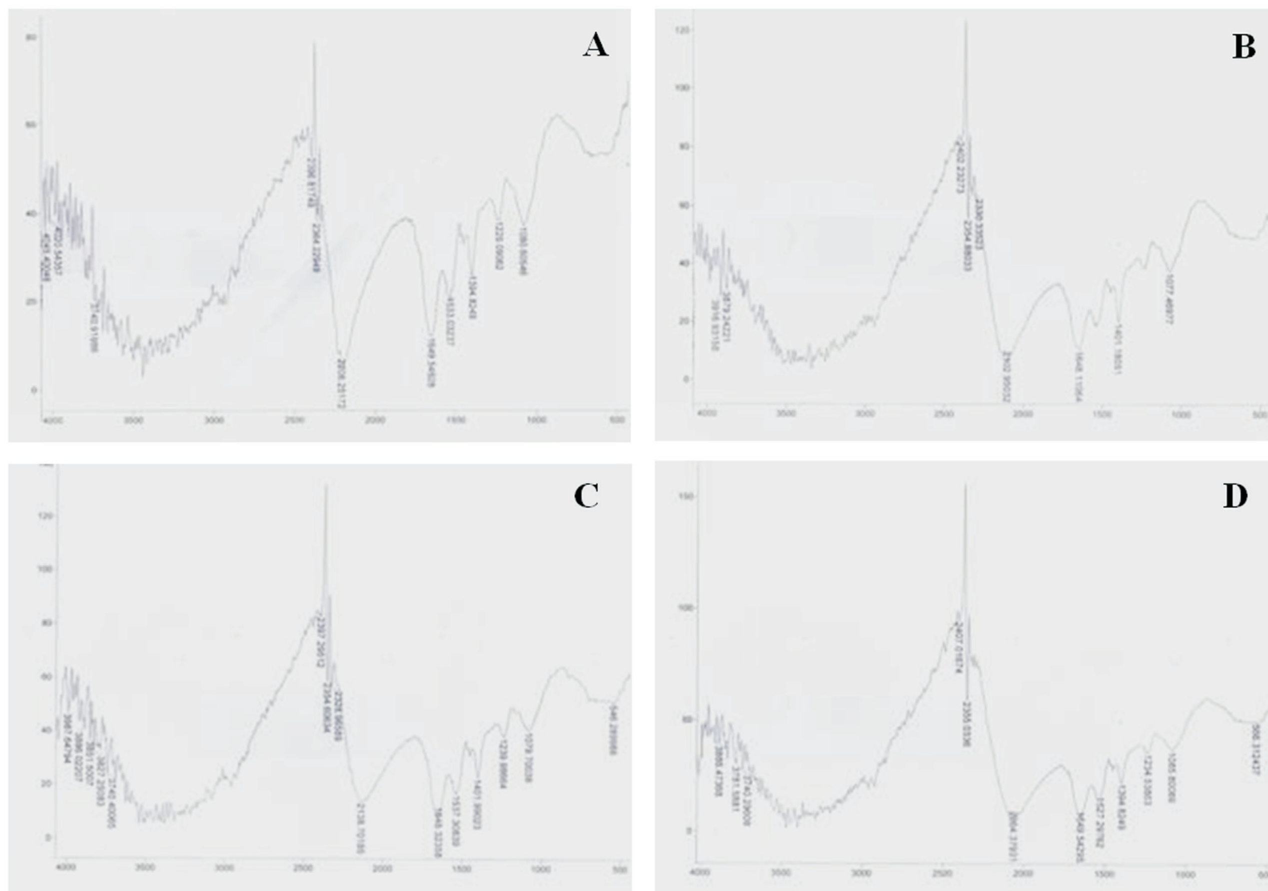

Figure 2 - FTIR spectra of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 cells. A: Cells of *P. aeruginosa* Rb-1 grown without Cr(VI) for 48 hours. B: *P. aeruginosa* Rb-1 cells grown with 1000 μg mL^{-1} of Cr(VI) C: Cells of *O. intermedium* Rb-2 grown without Cr(VI) for 48 hours D: *O. intermedium* Rb-2 cells grown with 1000 μg mL^{-1} Cr(VI).

concentration of cysteine in living organisms, it is reported that in several species of eukaryotic algae, cysteine content ranges from 0.6 to 12 mM (Satoh *et al.*, 2002). Cysteine concentrations of both *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 were also within this range. Increase in content of non-protein thiols under Cr(VI) stress suggests their possible involvement in chromate detoxification.

Electron microscopy gives the possibility to study the cell physiology and especially changes in cell structure as a result of exposure to pollutants. Transmission electron microscopic examination of *P. aeruginosa* Rb-1 and *O. intermedium* Rb-2 exhibited the distribution of electron dense precipitates intra as well as extra-cellularlly as a result of exposure to chromium. The distribution of precipitates was not uniform in case of both bacterial strains and this up take of metals by individual cells within a culture may vary because of physiological reasons. Differential distribution of uranium by the cells of *P. aeruginosa* and *S. cervisae* has already been reported (Mullen *et al.*, 1989). Intracellular localization of electron dense precipitates indicated the intracellular reduction of Cr(VI) as shown in figure 1 (B) and (D). The intracellular reduction pathway for *Shewanella oneidensis* was previously reported and *Acinetobacter* sp. strain, PCP3 also showed intracellular localization of electron dense precipitates (Daulton *et al.*, 2007; Srivastava and Thakur, 2007). These precipitates are mainly supposed to be Cr(III) in the form of hydroxyl and carboxyl groups (Bruins *et al.*, 2000; Bencheikh-Latmani *et al.*, 2007). Routinely both bacterial strains were maintained on Cr(VI) supplemented media, and they accumulate Cr(VI) intracellularly. When the cells were grown in chromate free media, they exhibited the deposition of chromium particles at their boundary even in the absence of Cr(VI). This was due to the gradual release of intracellularly accumulated Cr(VI) as indicated in figure 1 (A) and (C).

Electron microscopic results showed the distribution of chromium on the bacterial cell surface. Thus, FTIR analysis was performed to investigate the role of functional groups present on the bacterial cell surface in sequestration of chromium. FTIR analysis of the bacterial cells grown with and without Cr(VI) indicated the presence of amino, carboxyl, hydroxyl and sulphonate groups. Cr(VI) stress brought shifts in the absorption peaks. Major shifts in absorption peaks were observed in the region of 4000-

3500 cm^{-1}, 3500-3200 cm^{-1}, 1300-1450 cm^{-1} and 1200-1250 cm^{-1} under Cr(VI) stress conditions. These shifts indicated binding of the metal ions with certain specific functional groups namely; hydroxyl, amino and carboxyl and sulphonate groups, respectively. These functional groups are ionizable and reported to bind with the metal ions (Bueno *et al.*, 2008). Involvement of the carboxyl group in sequestration of chromium with the protein molecules in cyanobacteria under Cr(VI) stress has been described (Pandi *et al.*, 2009). Bacterial cell walls are mainly composed of carbohydrates, lipids and proteins thus proposing the possible involvement of above said functional groups in complexation of chromium with the bacterial cell surfaces (Mungasavalli *et al.*, 2007; Lameiras *et al.*, 2008).

Conclusion

It can be concluded that Cr(VI) stress severely alters the bacterial morphology in terms of the shape and size. Cr(VI) stress led to enhancement in production of certain polysaccharides and formation of cell protrusions. These polysaccharides entrapped metal ions present in the surrounding environment thus reducing the availability to the bacterial cells. Significant increase of proteins and enzyme activities was exhibited by chromium addition for both *Pseudomonas aeruginosa* Rb-1 and *Ochrobacterum intermedium* Rb-2 highlighting their potential for bioremediation. *Ochrobacterum* Rb-2 showed a stronger response in measured biochemical parameters than Rb-1. Variation in the biochemical parameters under Cr(VI) stress may be one of the major reasons of their ecological dominance in metal contaminated environment. Entrapment of Cr(VI) by these two strains evident by electron micrographs proved them as a good candidates for the remediation of metal contaminated environments.

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