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Research Article

Chromate Tolerance and Extracellular Chromate Reductase Expression in *Bacillus* sp. Isolates -

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Abstract

Introduction: Chromium (Cr) is widely used in tanning of skins and hides, textile dyeing, stainless steel production and is discharged largely in industrial effluents. In leather industry, measures for chromium removal are of significant concern due to chrome tanning processes resulting in discharge of toxic Cr(VI) species. Hence, it is essential to study the responses of *Bacillus* sp. in chromium-induced stress that can lead to elimination/reduction of the pollution load.

Methods: *Bacillus* sp. and *Bacillus methylotrophicus* strains have been isolated from tannery sludge and their tolerance levels were analysed at different Cr concentrations. Chromate reductase expression, Cr(VI), Cr(III) levels and free and chelated Cr(III) concentrations were evaluated. Biosorption capacity of Cr(VI) in spent chrome liquor was also determined. The enzyme-mediated reduction was studied by Electron Paramagnetic Resonance (EPR) spectroscopy.

Result: A 0.25 mM Cr(VI) concentration was found to be tolerated by both strains in Luria-Bertani (LB) medium. Chromate Reductase (ChrR) expression was in the extracellular supernatant in both *Bacillus* sp. Intracellular accumulation of Cr(III) in *B. methylotrophicus* was observed due to transport of Cr(VI) into the cell mileu and subsequent reduction to Cr(III). Free and chelated Cr(III) increased in extracellular supernatant in low concentrations. Maximum biosorption of Cr(VI) occurred at 72h with 89.6% adsorption by *B. methylotrophicus*. Electron Paramagnetic Resonance (EPR) spectroscopy showed the formation of Cr(V) radical. Ser (S), His (H), sulfhydryl groups and the presence of S-S bonds were observed to be essential for ChrR activity.

Conclusion: The study provides a 'green' approach for Cr tolerance by *Bacillus* sp. and chromate reduction mediated by an extracellular ChrR with formation of a Cr(V) intermediate Cr species.

Keywords: *Bacillus* sp; Biosorption; Chromate reductase; Chromate tolerance; Chrome tanning.

INTRODUCTION

Global industrialization has been in a rapid pace till date to contribute to the welfare and societal impact of mankind through various industrial sectors. Tannery industry is a major sector to produce good quality leather products and chrome tanning is more efficient than vegetable tanning in terms of production of soft and pliable leather goods as accessories for personal and cultural benefits. In terms of export of leather goods, India is a potential market and is one of the largest distributors worldwide. Chrome tanning requires basic chromium sulfate or chrome as Cr(III) species in large amounts to the order of 10-12% of float and is carried out in large roller drums along with the enzymatically treated hides and skins (after beamhouse operations) for about 3-4h and is discharged as spent chrome liquor along with fleshings and other solid wastes into the mainstream effluent. This is now of primary concern as Cr(III) compounds after tanning process become oxidized to toxic Cr(VI) due to boiling and sun drying (Source-Wikipedia). This mainstream effluent with toxic Cr(VI) species and other leather process pollutants is allowed to merge with waterways into the industrial waste waters. Mala, et al. [1] have determined the different concentrations of Cr(III) and Cr(VI) in spent chrome liquor obtained from CLRI tannery with 1.5 g/ml and 383.7 µg/ml respectively, while the tannery effluent contained 127 mg/ml Cr(III) and 64.7 µg/ml Cr(VI) which are highly above the permissible limits of 0.05 mg/L Cr(VI) in drinking waters according to the World Health Organization [2].

The element chromium (Cr) belongs to the transition metal group (VIB) in the Modern Periodic table. Chromium exists in various valence states of -2 to +6 with the stable forms of Cr(III) and Cr(VI). Cr(VI) is a soluble toxic species and is readily absorbed, while Cr(III) is benign, less mobile and toxic at higher concentrations [3]. Cr is listed as priority pollutant and a Class A human carcinogen by the US-Environmental Protection Agency [4]. Cr in water bodies presents an environmental threat to plants, animals and through human consumption. Microorganisms encounter metals/metal ions during growth as trace elements for structural/catalytic functions or to tolerate metal species up to threshold limits or to employ detoxification mechanisms to form non-toxic products. Recently, microorganisms have been studied for detoxification and removal of Cr by Tang, et al. [5] and for chromate reduction by Lyu, et al. [6]. Bacteria, fungi, and yeasts can facilitate Cr uptake by bioaccumulation, biosorption, chromate efflux and chromate reduction [7]. Chromate tolerance activities of microorganisms are diverse and ubiquitous according to their habitual existence with other colonies.

Mala, et al. [7] have produced an extracellular chromate reductase by *B. methylotrophicus* in a defined medium. The chromate reductase enzyme was inducible and yielded a specific activity of 212.84 U/mg protein at 48h and required reduced glutathione as electron donor for maximal activity. Chromate reductases have been produced intracellularly [8-15] as well as extracellularly [7,16-19] or membrane-bound [20,21] and are promising candidates for chromate bioremediation in waste waters and metal-contaminated soils. Non-enzymatic chromate tolerance also contribute to bioremediation of Cr [22,23]. There have been many reports on the mechanisms of chromate reduction in different microorganisms explaining the intracellular transport of Cr(VI) into the cell milieu, formation of Reactive Oxygen Species (ROS) by unstable Cr(IV) and Cr(V) radicals and/or intracellular reduction mediated by soluble chromate reductases [24]; formation of nontoxic Cr(III) via one-electron transfer of an unstable Cr(V) intermediate radical [25,26] and by formation of Cr(IV) and Cr(V) intermediates [15]. Mechanisms of chromate tolerance include biosorption [1,15,27] and bioaccumulation [1,28], chromate efflux and chromate reduction [7,29] and is also related to growth parameters of the microorganisms [7,15,17,30]. Therefore there exists a need to develop efficient natural or engineered microbial strains that can show significant chromate tolerance and/or chromate bioremediation capacities in order to combat the overgrowing pollution load in land and water.

In this regard, we have attempted to use *Bacillus* strains isolated in the laboratory for understanding the chromate reduction potential and have evaluated cell density, chromate tolerance, chromate reductase expression, identification of Cr in free and chelated forms and have identified the Cr species during chromate reduction by

Electron Paramagnetic Resonance (EPR) spectroscopy. We further propose a possible mechanism of chromate reduction in the bacterial cells based on our observations.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Bacterial strains isolated from tannery sludge were identified as *Bacillus* sp. and *Bacillus methylotrophicus* from CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India, which showed chromate reduction activities in the presence of $K_2Cr_2O_7$. *Bacillus* sp. and *B. methylotrophicus* strains were maintained on Luria-Bertani (LB) agar (% w/v: Bacteriological peptone-1.0; Yeast extract-0.5; Sodium chloride-0.5; Agar-2.5). The culture media used for bacterial growth for all experiments in our study was LB media without agar (broth), sterilized at 121°C and 15 lbs pressure for 20 min. The bacterial strains were first cultured in LB broth as preinoculum and incubated O/N at 30°C in a rotary shaker at 15rpm. A 1.0 ml of preinoculum of each strain was added to 50 ml of sterile culture media in 250 ml Erlenmeyer flasks. A 10 mM concentration of $K_2Cr_2O_7$ stock was prepared in LB medium and sterilized. The various concentrations of chromate used for experimental studies were taken as aliquots from this stock and added to culture media in the laminar flow chamber, then inoculated with the bacterial strains appropriately and allowed for growth in a rotary shaker at 150rpm at 30°C. All LB media were prepared in deionized water. At 24 h intervals, samples were withdrawn in sterile conditions. The samples were centrifuged at 8000 rpm for 15 min. to obtain the cell pellet and the extracellular supernatant.

Tolerance to chromate

About 25 ml of sterilized LB agar containing 0 mM, 0.1 mM, 0.25 mM, 0.5 mM $K_2Cr_2O_7$ concentrations were poured into culture dishes and spread plated with 0.1ml of 10^5 diluted *Bacillus* sp. and *B. methylotrophicus* strains from preinoculum. After 48h incubation at room temperature, colonies were counted to evaluate the growth tolerance of the strains to the various concentrations of Cr(VI) and expressed as $\log_{10}$ Colony Forming Units (CFU)/ml.

Determination of Minimal Inhibitory Concentration (MIC)

LB media prepared with 0.1 to 1.0 mM and 0.25 mM concentrations of chromate were inoculated with each *Bacillus* strain and incubated in a rotary shaker at 150 rpm at 30° C. After 24 h, the Optical Density (OD) at 600 nm of 3 ml of each sample was measured in a UV-Vis spectrophotometer (Shimadzu, Japan). The MIC of both the strains in chromate were estimated from the graph obtained.

Preparation of intracellular and extracellular samples

The culture was centrifuged at 8000 rpm for 15 minutes at 4°C and the supernatant collected was used as the extracellular sample for the subsequent experiments. The cell pellet after centrifugation of the culture was re-suspended in 25 ml of 0.2 M phosphate buffer, pH 7.0 and the cells were homogenized under ice and centrifuged at 8000rpm for 15 minutes at 4°C. The supernatant collected was used as intracellular sample for the subsequent experiments.

Chromate reductase enzyme assay

The chromate reductase activity was determined according to the method of Sau, et al. [31] with some modifications [7]. To state briefly, the assay mixture contained 0.2 ml of 0.2 mM $K_2Cr_2O_7$ substrate solution; 0.2 ml of 0.2 M phosphate buffer, pH 7.2 to give an effective chromate concentration of 0.1 mM; 0.2 ml of 0.2 mM NADH and 0.4 ml of the enzyme, which was incubated at 30°C. After 30 min., 0.5 ml of 20% Trichloroacetic Acid (TCA) was added to stop the enzyme reaction and 2.0 ml of 0.5% (w/v) of 1,5-diphenylcarbazide in acetone was added to develop a pink color. Cr(VI) was estimated by the measurement of absorbance at 540 nm. A set of controls were prepared in a similar manner except that the enzyme was added after addition of TCA. Initial chromate concentration was determined by abiotic chromate reduction (without enzyme) compensated by 0.2 ml of 0.2 M phosphate buffer, pH 7.2. Residual Cr(VI) concentration was determined from a standard graph with 10-100 µM $K_2Cr_2O_7$. The enzyme activity was determined from the reduced Cr(VI) concentration. One unit of chromate reductase activity is defined as the amount of enzyme which reduced 1 µM of chromate per min. at 30°C. Protein content was determined by Bradford's method [32] using 10-100 µg/ml Bovine Serum Albumin (BSA) as standard. Specific activity of chromate reductase is expressed as U/mg protein.

Estimation of chromium (VI)

Cr(VI) was estimated according to Clesceri, et al. [33] with modification of the acidification procedure. Instead of 0.2 N H_2SO_4 to bring the pH to 1.0 ± 0.3, TCA an alternate strong acid was used in our protocol to acidify the reaction mixture. About 1.0 ml of the extracellular or intracellular samples were taken and added 0.5 ml of 20% (w/v) TCA followed by 2.0 ml of 0.5% (w/v) 1,5-diphenylcarbazide solution in acetone. The absorbance was measured at 540nm along with a reagent blank. The concentration of Cr(VI) was estimated from the $K_2Cr_2O_7$ standard graph.

Estimation of chromium (III)

Cr(III) in the extracellular and intracellular samples were estimated according to the method of Ksheminska, et al. [16]. About 1.0 ml of the samples were treated with 1.0 ml of 1% (w/v) Sodium Dodecyl Sulfate (SDS), 1.0 ml of 0.5 M acetate buffer, pH 3.5 and 1.0 ml of 0.015% (w/v) of Chromazurol S. The solution was incubated in a boiling water bath between 100°C-110°C for 30 min. The solution was cooled and 1.0 ml of 1M H_2SO_4 was added. The absorbance of the samples were measured at 590 nm against a reagent blank. The concentration of Cr(III) in the samples were calculated from the standard graph of 10-100 µM chromium chloride with Chromazurol S assay protocol.

Estimation of chelated Cr(III)

By the method of Ksheminska, et al. [16], free Cr(III) can be estimated by the Chromazurol S assay. In order to determine the chelated Cr(III) that is complexed with proteins in the cytosol or extracellular samples, the complexed or chelated Cr(III) has to be digested and the resulting total free Cr(III) concentrations can be determined. We have therefore followed the procedure of Haupt [34] and suitably modified according to Mala, et al. [1] to carry out the digestion process. To state briefly, 20 ml of 72 h extracellular and intracellular samples were neutralised with 1M NaOH to pH 9.0 and added 1.0 ml of 30% (v/v) hydrogen peroxide. The solution was digested over a hot plate until excess hydrogen peroxide was removed. The solution was cooled and made up to a known volume. The total free Cr(III) in this solution was then estimated by Chromazurol S assay [16] and the concentrations were calculated from the standard graph of chromium chloride as mentioned previously.

Biosorption of chromium

Biosorption protocol was carried out by the method of Mala, et al. [1]. *Bacillus* sp. and *B. methylotrophicus* were grown in LB media for 24h and the biomass was harvested by centrifugation at 8000rpm for 15min at 4°C. About 1.0g of viable, wet biomass from each culture was added to 10ml of spent chrome liquor obtained after chrome tanning and incubated for 24, 48 and 72h in batches and the Cr(VI) concentration was estimated. The initial Cr(VI) content in the spent chrome liquor were estimated with *Bacillus* sp. and *B. methyltrophicus* biomass at 0h.

Identification of Cr intermediates by Electron Paramagnetic Resonance (EPR) spectroscopy

Extracellular samples of *Bacillus* sp. and *B. methylotrophicus* after 48h of culture were analysed by EPR spectroscopy in X-band CW EPR (BRUKER) to identify the reaction intermediates of Cr sp. during chromate reduction.

Chemical inhibition of extracellular chromate reductase

The extracellular chromate reductase activities of both *Bacillus* strains were estimated at 24h in the presence of different chemical inhibitors each specific for an essential amino acid or metal ion. The reaction mixture contained 0.2 ml of 0.2 mM potassium dichromate as substrate, 0.2 ml of 0.2 M phosphate buffer and 0.2 ml of 0.2 mM NADH. To this, 0.4 ml of enzyme preparation and 0.1ml of 1mM chemical inhibitor was added and incubated at 30°C for 30 minutes. After incubation, 0.5 ml of 20% (w/v) TCA and 2ml of 0.5% (w/v) 1,5-diphenylcarbazide solution in acetone were added and the absorbance was measured at 540 nm. The data were expressed as percent relative activity compared to control without inhibitor addition. The control chromate reductase activity was assumed to be 100%.

Statistical analysis

The experiments were carried out in triplicates and the data were reported as Mean ± S.D, using MS-Excel software 2013.

RESULTS

Bacillus isolates exhibit tolerance to chromate as colonies as well as in culture

In LB agar plates, after 48h, *Bacillus* sp. grew as colonies containing $1x10^8$ CFU/ml with 0.5 mM $K_2Cr_2O_7$ as equal to that in control plate in the absence of chromate, while *B. methylotrophicus* colonies were $8x10^7$ CFU/ml at 0.25 mM chromate. However, at 0.5 mM chromate concentration, *B. methylotrophicus* colonies were completely nil in the agar plates resulting in toxicity of this strain at 0.5 mM chromate. The $\log_{10}$CFU/ml values plotted against $K_2Cr_2O_7$ concentrations are shown in figure 1. For determination of MIC of the two *Bacillus* strains, chromate concentrations of 0.1 to 1.0 mM of chromate was used in LB broth prior to inoculation. The pattern of culture growth determined by absorbance at 600 nm at 24h showed that *Bacillus* sp. indicated increased tolerance to chromate even up to 1.0 mM with a high MIC of 0.9 mM retaining almost 50% of cell viability (Figure 2). In contrast, *B. methylotrophicus* showed increased cell density at 0.25 mM more than *Bacillus* sp. at the same concentration. The MIC for *B. methylotrophicus* was 0.3 mM chromate concentration.

Chromate reductase is extracellular in both isolates

Both *Bacillus* isolates exhibited extracellular production of chromate reductase than intracellular production in LB medium (Figure 3). The intracellular production of chromate reductase was very low and increased linearly from 24h to 72h with maximum activities of 5.25 U/mg protein at 72h by *Bacillus sp* and 3.97 U/mg protein at 72h by *B. methylotrophicus*. However, extracellular chromate reductase activities were maximum at 24h with 57.81 U/mg and 47.98 U/mg protein by *Bacillus* sp. and *B. methylotrophicus* respectively and decreased linearly with time.

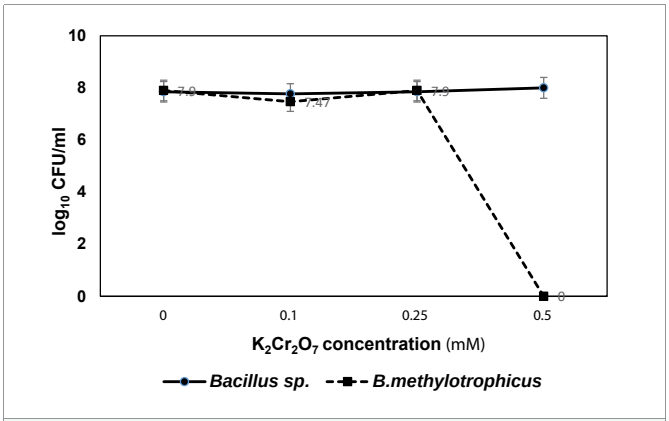

Figure 1: Tolerance of *Bacillus* sp. and *B. methylotrophicus* in the presence of chromate.

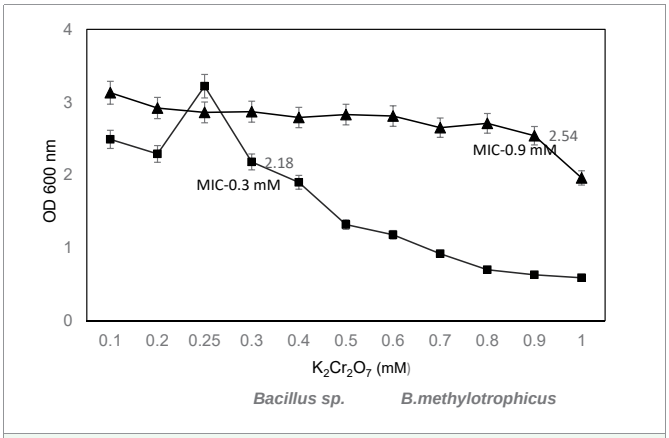

Figure 2: MIC of *Bacillus* sp. and *B. methylotrophicus* in the presence of chromate.

Intracellular and Extracellular Cr(III) production is time-dependent

Intracellular and extracellular Cr(VI) and Cr(III) levels of *Bacillus* sp. is shown in figure 4. Intracellular Cr(III) production was maximum at 72h for *Bacillus* sp. while in the extracellular fraction also, Cr(III) was maximum at 72h with 9.5 and 12.1 µM respectively. There was a linear increase of Cr(III) intracellularly and extracellularly with time up to 72h.

Intracellular accumulation of Cr(III)

In *B. methylotrophicus*, intracellular fraction exhibited highest level at 48h with 16.6 µM Cr(III) (Figure 5). This is reflected by the transport of Cr(VI) into the cell milieu with concomitant intracellular reduction to Cr(III), while in the extracellular environment also, Cr(VI) was reduced to Cr(III) but in the order of 4.5-5.5 µM and residual Cr(VI) was only about 2.98 µM.

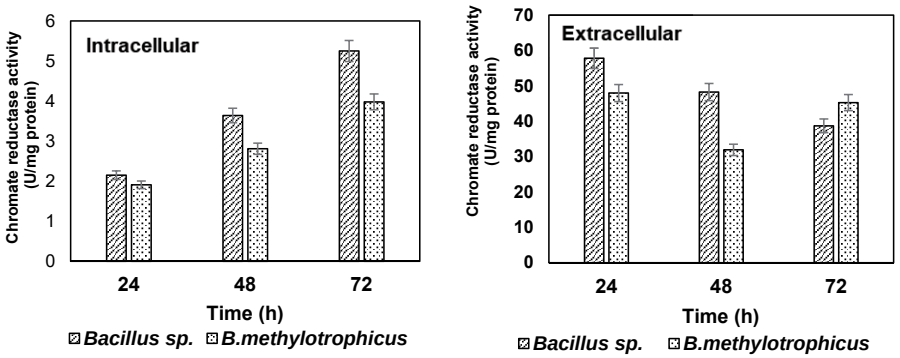

Figure 3: Expression of intracellular and extracellular Chromate Reductase (ChrR) in *Bacillus* sp. and *B. methylotrophicus.*

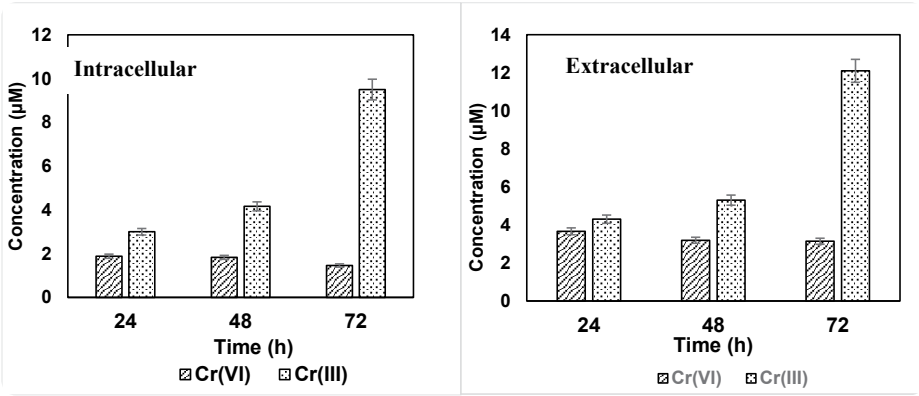

Figure 4: Cr(VI) and Cr(III) levels in intracellular and extracellular fractions of *Bacillus* sp.

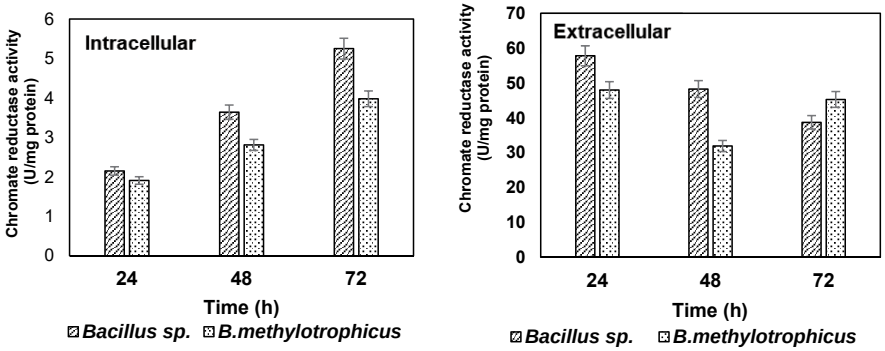

Figure 5: Cr(VI) and Cr(III) levels in intracellular and extracellular fractions of *B. methylotrophicus*.

Chelated Cr(III) is present in low concentrations intracellularly and extracellularly

The free and chelated Cr(III) concentrations of *Bacillus* sp. and *B. methylotrophicus* are represented in figure 6. In the extracellular fraction, free Cr(III) of *Bacillus* sp. and chelated Cr(III) of *B.methylotrophicus* were higher in both isolates in the order of 4.33 and 5.31 µM respectively. Chelated Cr(III) of *Bacillus* sp. was 46% of the free Cr(III) present in the intracellular extract, while free and chelated Cr(III) of *B. methylotrophicus* were almost the same in the

order of 1.3 µM.

Bacillus strains demonstrate biosorption efficiency of Cr(VI) in spent chrome liquor

Bacillus strains were evaluated for biosorption of Cr(VI) in Spent chrome liquor with 24h biomass and 0.25% (w/v) $K_2Cr_2O_7$ solution at intervals of 24h up to 72h (Table 1). The metal uptake at equilibrium qe was comparable in both strains as normalized from their initial concentrations of Cr(VI). *Bacillus* sp. and *B. methylotrophicus* exhibited 82.5% and 89.6 % maximal biosorption at 72h of incubation. The metal uptake qe was 2.07 by *B. methylotrophicus* and 4.01 by *Bacillus* sp.

Identification of intermediate Cr(V) radical by Electron Paramagnetic Resonance spectroscopy

The Electron Paramagnetic Resonance (EPR) spectra of *Bacillus* sp. and *B.methylotrophicus* 48h culture supernatant is depicted in figure 7. Both strains revealed a dip in intensity peak due to the unstable Cr(V) reaction intermediate and a plateau of Cr(III) with increase in time of measurement. However, *Bacillus* sp. exhibited a strong intensity peak about 3 times that of *B. methylotrophicus* suggesting increase in concentration of the reaction intermediate Cr(V) by Cr(VI) reduction proceeding to the final reduction product, Cr(III) in the extracellular supernatant. *B. methylotrophicus* displayed similar peaks with low concentration of Cr(V) intermediate due to low amounts of Cr(VI) reduction in the extracellular fraction with a rugged line graph due to interference with other proteins or chemical entities outside the cell mileu. The probable mechanisms by which *Bacillus* sp. and *B. methylotrophicus* exerted chromate reduction is represented in figures 8,9.

Chemical inhibition of chromate reductase activity reveals essential amino acid residues and disulfide bridges

Chemical inhibition study to identify essential amino acid residues is presented in figure 10. Inhibition of chromate reductase activity by DEPC and PMSF indicated that His and Ser residues were essential for ChrR activity and were present in the active site of the enzyme. Sulfhydryl groups of S-containing amino acid residues and disulfide bridges were present in the protein and were required for chromate reduction by inhibition of ChrR activity by IAA (-SH) and DTT (S-S). Metallic cofactors such as Ca^{2+} or Mg^{2+} were also required for enzyme activity due to inhibition of ChrR in the presence of EDTA.

DISCUSSION

Chemical reduction of Cr(VI) to nontoxic Cr(III) requires high energy, high costs, safe disposal of toxic sludge, and results in incomplete reduction with residual Cr(VI) in the system. Hence, biological methods for Cr(VI) reduction are better alternatives than conventional methods due to nontoxicity, less energy requirements, low costs of reduction systems and are 'green' approaches for maintenance of the ecological niche. Several researchers have studied chromate reduction by microorganisms which are potent resources in production of nontoxic Cr(III) by different mechanisms as part of their metabolic activities. Sanjay, et al. [34] have studied the isolation and identification of chromate reducing bacteria from tannery effluent. Cr(III) cannot traverse the cell membrane of microbial cells and hence are generally reduced in the extracellular mileu or

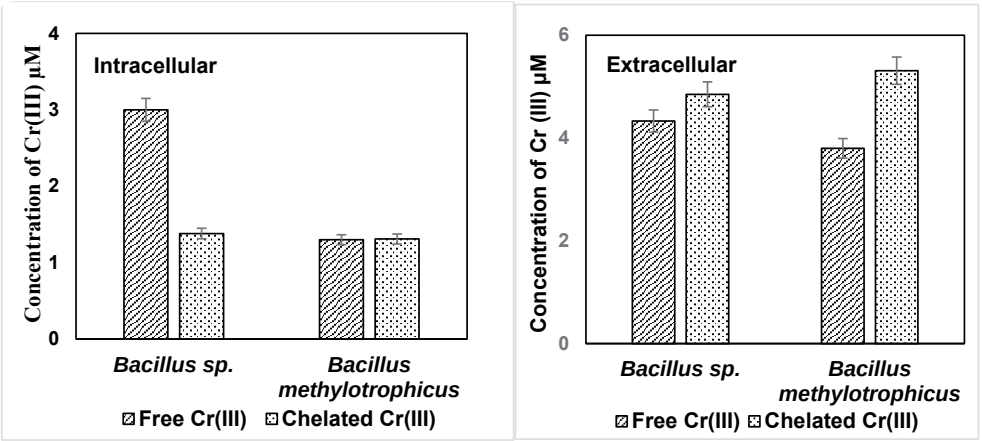

Figure 6: Free and chelated Cr(III) levels in intracellular and extracellular fractions of *Bacillus* sp. and *B. methylotrophicus*.

Table 1: Biosorption efficiency of *Bacillus* isolates in spent chrome liquor.

Spent chrome liquor	*Bacillus* isolates.	Initial chromium (C_i mg/ml)	Residual chromium (mg/ml)			Final chromium (C_f mg/ml)	Time (h)	[a]qe	Adsorption (%)
			24 h	48 h	72 h				
C_r(VI) (mg/ml)	*B. methylotrophicus*	231.37	54.43	29.94	23.96	23.96	72	2.07	89.65
	Bacillus sp.	487.19	204.15	136.12	85.47	85.47	72	4.01	82.46

$$aqe = \frac{V(C_i - C_f)}{S}$$

qe = Metal uptake at equilibrium; V = Volume of spent chrome liquor; C_i = Initial Cr(VI) concentration, per ml; C_f = Final Cr(VI) concentration, per ml; S = *Bacillus* isolate biomass.

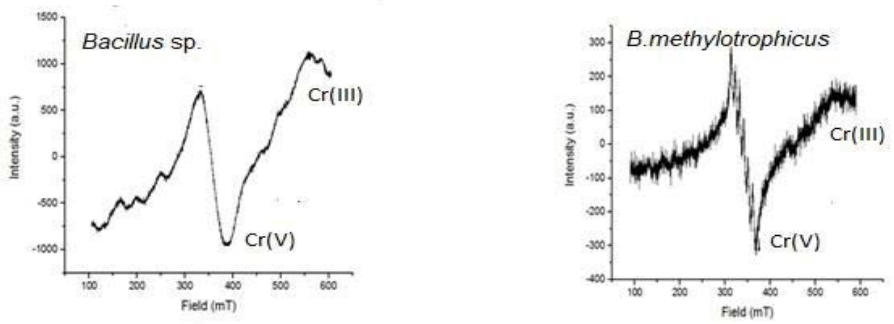

Figure 7: Electron Paramagnetic Resonance (EPR) spectra of extracellular Cr in *Bacillus* sp. and *B. methylotrophicus*.
EPR spectra of the extracellular supernatants of the *Bacillus* isolates at 48h shows extracellular chromate reduction mediated by extracellular ChrR in *Bacillus* sp. while in the extracellular supernatant of *B. methylotrophicus* showed low levels of Cr(III) about 3 times lower than Cr(III) in *Bacillus* sp. The rugged graph is due to extracellular Cr(III) complexation with proteins or metabolites..

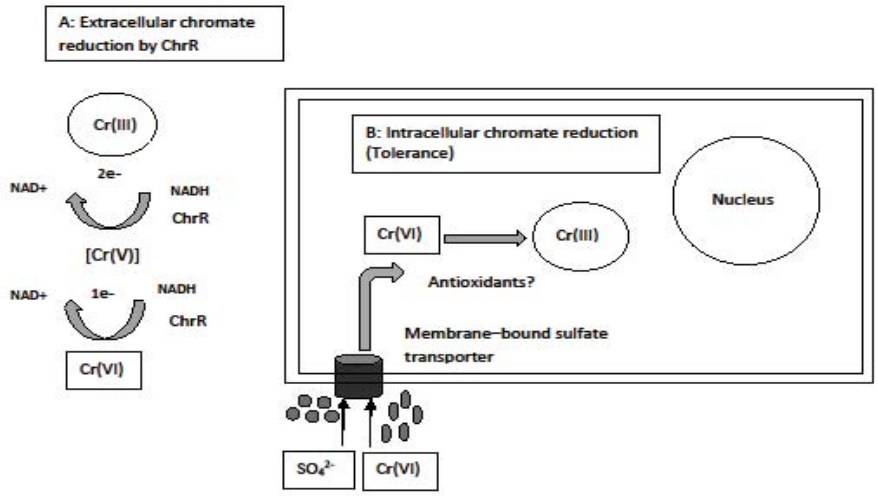

Figure 8: Mechanism of chromate reduction in *Bacillus* sp.
Chromate reduction in *Bacillus* sp. occurs both in the intracellular mileu and in the extracellular medium. Cr(VI) traverses the cell membrane via the membrane -bound sulfate transporter into the cell mileu and gets reduced to Cr(III) in the absence of intracellular ChrR enzyme. This reduction then could be due to cellular reductants such as antioxidants. Extracellularly, Cr(VI) is reduced by the extracelluar ChrR enzyme by a one-electron transfer from NADH to the unstable intermediate Cr(V) radical which is subsequently reduced by a two-electron transfer from NADH to reduce to Cr(III) mediated by ChrR.

Cr(VI) may be transported into the cells via sulfate-receptors and intracellular reduction to Cr(III) may occur. Accumulation of Cr(III) may be nontoxic; however, in most cases, when the intracellular accumulation reaches to much higher levels, Cr(III) can lead to DNA damage and production of reactive oxygen species which are harmful to the microbial cells itself. Hence, microorganisms can adapt under such circumstances to tolerate increased Cr levels up to certain concentrations which are varied between different microorganisms. In this context, bioremediation of toxic Cr(VI) by reduction to Cr(III) by microorganisms are very much desired. Hence, we have studied the tolerance levels of two *Bacillus* sp. isolated from tannery sludge and studied their capacities to reduce Cr(VI) in $K_2Cr_2O_7$ as well as their biosorptive capacities in spent chrome liquor and have derived at a mechanism by investigations of Cr(VI) and Cr(III) contents and free and chelated Cr levels in the bacterial isolates cultured in LB media and by EPR spectroscopy.

Bacillus sp. extremely tolerated Cr levels up to 1.0 mM suggesting the utilization of Cr as a trace element for its metabolic growth, while *B. methylotrophicus* gradually declined to exhibit tolerance from 0.3 mM to 1.0 mM. As common to many of the trace elements, higher chromate from 0.3 mM indicated gradual toxicity up to 1.0 mM for this strain. The nil growth as colonies in plates and about 50% of cell density in culture at 0.5 mM chromate could be explained that this cell density was not able to be grown in plates at 48h or the colonies could have been observed if kept after 48h of incubation. We have also obtained higher growth of the bacterial cultures in LB growth media supplemented with different $K_2Cr_2O_7$ concentrations with maximum absorbance values of 3.13 for *Bacillus* sp. at 0.1 mM and

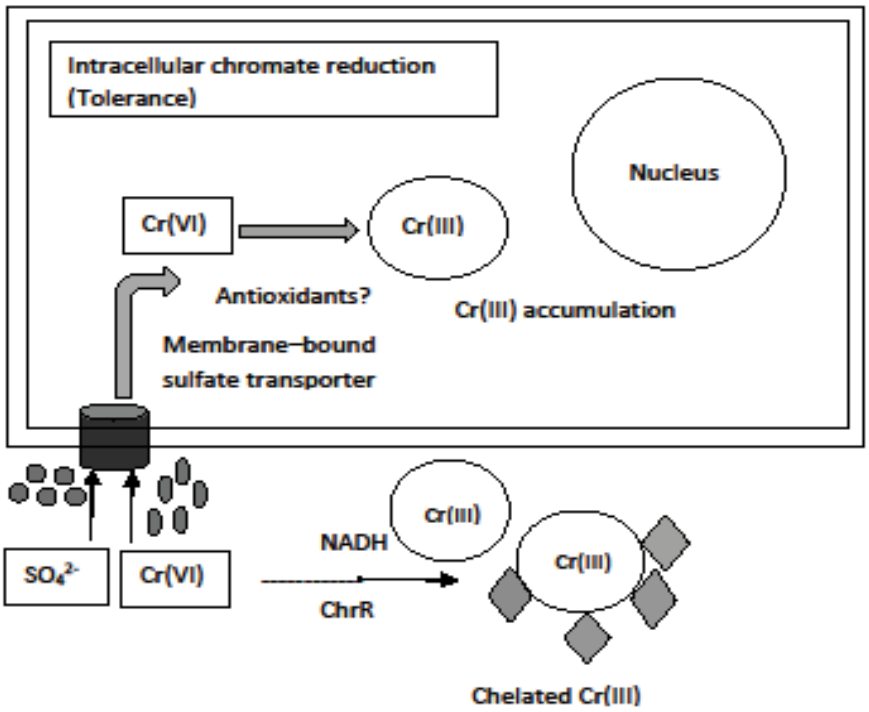

Figure 9: Mechanism of chromate reduction in *B. methylotrophicus.*
Reduction of Cr(VI) occurs mainly in the intracellular compartment and is not mediated by intracellular ChrR enzyme or by intracellular ChrR in very low levels. Cr(VI) traverses the cell membrane via the membrane -bound sulfate transporter into the cell mileu and gets reduced to Cr(III) which could be due to cellular reductants such as antioxidants. As most of the Cr(VI) is transported into the cell, Cr(III) accumulation takes place intracellularly. Extracellular Cr(VI) reduction occurs in very low amounts of Cr(III) mediated by extracellular ChrR enzyme. Chelated Cr(III) complexed with proteins or other metabolites is present in the extracellular medium in very low amounts.

3.22 for *B. methylotrophicus* at 0.25 mM. This shows that both *Bacillus* isolates can exhibit very high chromate tolerance levels.

An increase in chromate reductase activity at 72h observed in *B.methylotrophicus* indicates the enzyme is inducible as reported previously [7]. There has been reports of lower ChrR activities with 3.67 ± 0.014 U/ml [17] and 14.83 U/mg [13]. Thereby, we have obtained higher production in LB medium without any optimization in a complex medium formulation at a shorter duration of 24h. These results indicate that the *Bacillus* isolates have the potential for chromate reduction in a very simple and efficient process.

The *Bacillus* isolates showed maximum chromate reduction capacities of 0.25 mM Cr(VI) at 72h while, *Klebsiella* sp. PB6 isolated from contaminated soil showed maximum chromate reduction of 0.1 mM Cr(VI) at 120h [22]. Our study showed a feasible and low cost approach for bioremediation of high chromium contaminated soils or industrial effluents. Time-dependent increase in chromate reducing capacity is also observed in dichromate resistant bacteria where chromate reduction increased with time up to 96h [35].

The difference between free and chelated chromium is that free Cr(III) is accessible in the cells and extracellular supernatant but chelated Cr(III) is inaccessible in the form of Cr(III)-stable complexes with proteins or other chemical entities. Hence, we have digested the extracellular and intracellular samples with H_2O_2 and estimated the

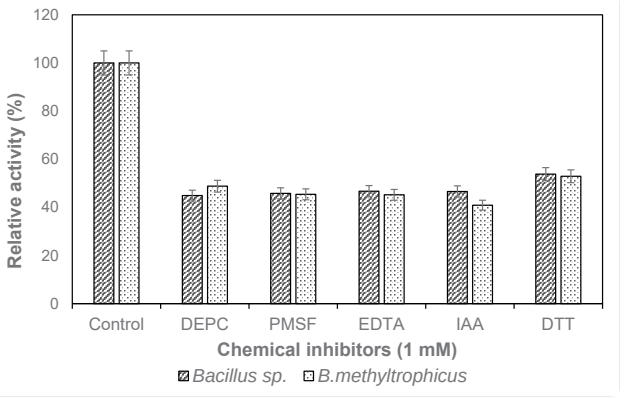

Figure 10: Chemical inhibition studies of chromate reductase in *Bacillus* isolates in extracellular medium at 24h.

total Cr(III) by Chromazurol S assay from which chelated Cr(III) was calculated [16]. We have observed higher chelated Cr(III) in the extracellular fraction than in the intracellular fraction due to the presence of proteins and other metabolites in the extracellular medium which can complex with extracellular free Cr(III). However, when considering the magnitude of extracellular and intracellular Cr(III), the chelated Cr(III) is in very low amounts both intracellularly

and extracellularly. This shows that most of the Cr(VI) is reduced either extracellularly or intracellularly and very low amounts of Cr(III) is chelated in the extracellular medium. Some reports indicate DNA damage by Cr(III) complexing with ds DNA forming Cr-DNA adducts in the cell nucleus [36]. In our study, chelation of Cr(III) is mainly extracellular and hence renders lower toxic effects to the bacterial cells.

Mala, et al. [1] have studied the biosorption capacity of the fungus, *Aspergillus niger* MTCC 2594 in spent chrome liquor. In the previous study, Cr(VI) was biosorbed by the fungal biomass to 79% at 36 h which is comparatively lower than the bacterial isolates used in our study. Though *Bacillus* sp. showed lower biosorption capacity than *B. methylotrophicus* its qe was higher not only than *B. methylotrophicus* but also higher than *A. niger* studied earlier [1]. Thereby, we have obtained good biosorption efficiency and higher metal uptake by both *Bacillus* isolates and in a shorter time duration.

There exists several biological methods by which Cr is sequestered by microorganisms such as chromate reduction, bioaccumulation, biosorption and biotransformation. The inherent mechanisms proposed so far are resistance to toxic Cr levels, tolerance to Cr using cell defense machinery such as antioxidants in response to increase in ROS, chromate reductase expression and DNA repair. Several factors have been considered for Cr removal such as pH of the media, temperature, initial Cr concentration, inoculum size, electron donors and effect of co-contaminants [37]. Chromate reduction is a viable process in the bioremediation of Cr. Chromate reduction can occur extracellularly by extracellular and membrane-bound ChrRs or intracellularly by intracellular ChrRs. Intracellularly, Cr(VI) is reduced by one-electron transfer to intermediate unstable Cr(V) species and subsequent 2-electron transfer to Cr(III) mediated by ChrR and with the electron donor NADPH. Identification of NADPH as electron donor in chromate reduction has been reported by Suzuki, et al. [26] and Ray, et al. [38] Identification of Cr(V) as intermediate radical has been reported by Rao, et al. [25] and Suzuki, et al. [26] during enzyme-mediated chromate reduction. Chromate tolerance in the presence of antioxidant enzymes can lead to intermediate Cr(V) and Cr(IV) radicals during reduction to Cr(III) [14]. This is not mediated by chromate reductase enzyme. Intracellular transport of chromate occurs via membrane-bound sulfate transporter and accumulation of Cr(VI) may lead to efflux by a $ChrA^{+}$ transporter encoded by plasmid DNA [24]. This mechanism is responsible for the resistance to Cr(VI).

In the case of *Aspergillus niger*, Cr removal was mainly by Cr(VI) reduction, surface immobilization and intracellular Cr(III) accumulation [39]. *Pseudomonas brenneri* isolated from coalmine waste water accumulated Cr metal ions in the exponential growth phase both by adsorption on the cell and intracellularly within the cell mileu, the cytoplasm [30]. In our study, to elucidate the underlying mechanism of chromate reduction by the *Bacillus* isolates we have considered the chromate expression, Cr(VI) and Cr(III) levels in both *Bacillus* isolates and chelated Cr(III) levels and EPR spectra. Chromate reductase was mainly expressed extracellularly and intracellular expression was negligible in both isolates. This reveals that the chromate reduction of Cr(VI) to Cr(III) by enzyme should be mainly in the extracellular supernatant. It is known that Cr(III) cannot traverse into the cytoplasm as the cell membrane is impermeable to Cr(III). Hence, it should be considered that Cr(VI) must be transported into the cell for reduction to occur intracellularly, and this is not mediated by the enzyme. The levels of Cr(III) with *Bacillus* sp. was 9.5 µM intracellularly and 12.1 µM extracellularly, both at 72 h showing that Cr(VI) was reduced in both fractions. In *B.methylotrophicus*, intracellular Cr(III) was accumulated to 16.6 µM at 48h while the extracellular Cr(III) level was not appreciable up to 72h. This showed that Cr(VI) was reduced intracellularly at a rapid rate and hence does not depend on ChrR expression in the cytoplasm. Chelated Cr(III) in both isolates was in the order of 1.31 µM in the intracellular fraction, while it was 5.31 µM in the extracellular fraction in *B. methylotrophicus* suggesting complexation of extracellular Cr(III) with proteins and other components in the media. The EPR spectra of extracellular supernatants revealed the formation of Cr(V) radical in the *Bacillus* sp. with high intensity and in *B. methylotrophicus* erratically which could be due to interference of proteins.

Reduction of ChrR activity in the presence of IAA was higher than inhibition by DTT to an order of 60% and 47% suggesting that many S-containing amino acid residues were present which were required for enzyme activity and formed S-S bridges between two or more oligopeptides of the protein. Sulfhydryl groups have been known to be present in the active site of the reductase enzyme [40].

CONCLUSION

The *Bacillus* isolates used in our study showed tolerance to Cr and expressed chromate reductase enzyme mainly extracellularly. The Cr(VI) and Cr(III) contents showed good chromate reduction efficacy and accumulation of intracellular Cr(III). Chelated Cr(III) was present in low concentrations. Both isolates demonstrated good biosorption efficiency of Cr(VI) in spent chrome liquor. Cr(V) was identified in the extracellular fraction as the intermediate species in chromate reductase mediated reduction by EPR spectroscopy. The essential amino acid residues were identified by chemical inhibition studies. The research study provides insights of the mechanisms of chromate reduction by *Bacillus* isolates which could be exploited for chromium bioremediation in contaminated soils and water bodies.

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