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Utility of *Chromobacterium violaceum* SUK1a, an indigenous bacterial isolate for the bioremediation of Cr(VI)

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Abstract: The potential of an indigenous bacterial strain, *Chromobacterium violaceum* SUK1a, isolated from surface water samples collected from Sukinda Valley in Odisha, India, has been evaluated for the first time for the bioremediation of toxic hexavalent chromium (Cr(VI)) ions. The isolate was assessed for its Cr(VI) biosorption efficiency and the various parameters affecting the biosorption process were evaluated. A maximum Cr(VI) biosorption of about 50% was obtained, and the residual chromium was in the form of less toxic Cr(III). The Gibbs free energy of biosorption was determined to be -26.3 kJ/mol, suggestive of a chemisorption process. Additionally, the Cr(VI) biosorption by the isolate followed pseudo second order kinetics. FTIR spectral studies indicated that the surface functional groups present on the bacterial isolate such as, carboxyl, hydroxyl, amino, and phosphate groups were involved in the complexation of chromium ions with the bacterial cells. X-ray photoelectron spectroscopic studies on Cr(VI) interacted bacterial cells revealed an additional peak corresponding to Cr(III) in the Cr(2p) spectra. The surface charge of the bacterial cells subsequent to interaction with Cr(VI) were less negative compared to the pristine cells, which further substantiated the bioreduction of Cr(VI) to Cr(III). The bioremediation mechanism of Cr(VI) by the bacterial isolate is delineated to be governed by both biosorption and bioreduction processes under metabolism independent conditions. The results obtained indicate that the isolate can be a promising candidate for Cr(VI) bioremediation applications.

Keywords: bioremediation, chromium, *Chromobacterium violaceum* SUK1a, biosorption, bioreduction

1. Introduction

Chromite mining from the chromite ore, $FeCr_2O_4$, is an industrially important process, and chromium thus obtained is an indispensable element in steel making, leather tanning, chrome electroplating, and paint manufacturing industries (Jacobs and Testa, 2005). According to the Indian Minerals Yearbook 2017, chromite ore reserves (shipping grade) are spread across the world particularly in Kazakhstan, South Africa, India, Turkey, and USA. In India, the Sukinda Valley of Jajpur, Kendujar and Dhenkanal districts in Odisha, comprises of more than 96% of the country's chromite ore reserve (Anonymous, 2017). The natural oxidation of chromite in serpentine rocks facilitated by weathering processes by both chemical and microbial actions, could result in the formation of hexavalent chromium (Cr(VI)) species, which gets mobilized into the nearby water bodies (Dey and Paul, 2010; Mishra and Sahu, 2013). Additionally, run off from overburden dumps during monsoon also results in mobilization of chromium to surface water bodies. Thus, it becomes crucial to come up with a suitable method to complex the dissolved Cr(VI) ions.

There are certain conventional methods available for chromium remediation such as chemical precipitation, electrochemical separation, membrane filtration, and ion exchange. However, they are

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high on their operational costs and more importantly cause secondary pollution in the form of sludge (Hawley et al., 2004; Agrawal et al., 2006; Owlad et al., 2009; Oliveira et al., 2011). Bioremediation, on the other hand, is a more reliable and an ecofriendly alternative that can provide promising outcomes. Bioremediation using bacterial cells is more researched upon because of their ubiquity, ability to grow under controlled conditions and faster rates of pollution remediation owing to their small size (Mohan and Pittman, 2006; Gadd, 2009; Sen and Dastidar, 2010). Bacterial strains isolated from natural soil or water samples containing chromium could be expected to be tolerant to Cr(VI) ions and thus could exhibit better bioremediation efficiencies. Studies has been carried out using *Chromobacterium* sp. for its contaminant detoxification or degradation. Carepo et al. (2004), reported about the arsenic resistance, cyanate degradation and acid dehalogenation properties *of Chromobacterium violaceum*. *Chromobacterium* sp. along with other isolated native soil microbes was found to be beneficial for treating a mixture of polyaromatic hydrocarbons (PAH) which included, naphthalene, anthracene, phenanthrene, pyrene, dibenzoanthracene and benzopyrene (Silva et al., 2009). Faramarzi et al. (2004), have reported that *Chromobacterium violaceum* has the capability to form water-soluble metal cyanides under cyanide forming conditions thereby mobilising gold as dicyanaoaurate from electronic waste, copper as cyanide-complexed copper during biological treatment of shredded printed circuit boards scrap and nickel as tetracyanonickelate from fine-grained nickel powder. The detoxification of Cr(VI) from a tannery effluent by *Chromobacterium violaceum* has been reported by Priya et al., 2013. However, the ability of *Chromobacterium violaceum* for the bioremediation of Cr(VI) under metabolism-independent conditions has not been exploited yet.

In the present study, an indigenous bacterial strain has been isolated from the Sukinda Valley water samples. The strain has been characterised, and its efficacy for the bioremediation of toxic Cr(VI) has been investigated. Additionally, the mechanisms involved in the bioremediation of Cr(VI) by the bacterial isolate under metabolism independent conditions have been delineated.

2. Materials and methods

2.1. Analytical reagents

Potassium dichromate, $K_2Cr_2O_7$, of analytical grade, as source of Cr(VI) was obtained from Merck, Germany. NaOH and HCl used for the pH adjustments were of analytical grade. A Cr(VI) complexing agent, 1,5 diphenylcarbazide, of analytical grade was purchased from Merck, Germany for spectrophotometric analysis. Other reagents used for various experiments such as, H_2SO_4 and acetone were all of analytical grade. Deionised water of resistivity 18.2 MΩ cm from a MilliQ system was used in all the experiments.

2.2. Isolation and characterisation of a Cr(VI) tolerant bacterial strain from the Sukinda Valley water samples

2.2.1. Isolation of Cr(VI) tolerant bacterial strain

Water samples were collected from Sukinda Valley in Odisha, India, in sterile screw capped polypropylene bottles. The samples were then aseptically transported immediately and stored at 4^{o}C for the subsequent studies. The physical characteristics of and chromium concentrations in the samples were analysed.

Cr(VI) tolerant bacterial strains were isolated and identified by following the appropriate microbiological and molecular biological procedures, respectively. For the isolation procedure, 100 µL of the water samples collected from the Sukinda Valley were diluted 1000 times before being spread plated on to Nutrient Agar plates containing different concentrations of Cr(VI) to prevent formation of a mat. After a week of incubationat 30 ^{o}C, the plates contained well separated colonies of three different coloured bacterial colonies (white, yellow, and orange) (Fig. S1(a)).

2.2.2. Characterisation and identification of the isolated Cr(VI) tolerant bacterial strain

Among the many bacterial colonies that appeared on the chromium containing NA petriplates following incubation, a colony that differed in their morphology was chosen. The bacterial isolate

chosen, in order to obtain a pure culture was then streaked onto NA plates. The pure bacterial isolate was further characterized based on biochemical assays and Gram staining.

The obtained pure bacterial strain was identified by carrying out 16S rRNA sequence based molecular technique. For this technique, genomic DNA of the pure strain was isolated, and the agarose gel electrophoresis was run to check its purity and quantity. Subsequent to this, the 16S rRNA gene primers were used to amplify about 1.4 kb gene in a thermal cycler, from the isolated genomic DNA. The forward and reverse primer sequences used were 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-ACGGCTACCTTGTTACGACTT-3′ respectively. The PCR product thus obtained was then gel purified and quantified prior to performing the nucleotide sequencing. The nucleotide sequence thus obtained was then analysed and the consensus sequence was generated from the forward and reverse sequences using the Aligner software. This sequence was then aligned and compared with those stored in GenBank nucleotide sequence database (NCBI) by using the BLAST alignment software and finally the phylogenetic tree was constructed.

2.3. Growth curve analysis of the isolated indigenous bacterial strain

The pure strain of the bacteria isolated from the water samples collected from the Sukinda Valley was grown in nutrient broth (NB) medium of pH 7, the constituents of which are 5 $g \cdot dm^{-3}$ peptone, 2 $g \cdot dm^{-3}$ yeast extract, 1 $g \cdot dm^{-3}$ beef extract and 5 $g \cdot dm^{-3}$ NaCl. For preparing Nutrient Agar (NA) plates, 2 % of agar was added to the NB medium. The NB medium containing 10% of the freshly inoculated bacterial isolate was agitated in a Orbitek shaker at a temperature of 30 ^{o}C for 24 h at 200 rpm. The growth of the isolate was then monitored by bacterial cell counting using a Petroff Hausser counter in conjunction with a Leitz phase contrast microbiological microscope (Laborlux K Wild MPS12).

2.4. Estimation of chromium

Total chromium present in the samples was estimated using Thermo Electron Corporation M Series Atomic Absorption Spectrometer (AAS). 1, 5 diphenylcarbazide (DPC) method was used to determine the Cr(VI) concentration in the samples using a Labomed Inc. UV-Vis spectrophotometer at the wavelength of 540 nm. The concentration of Cr(III) present in the sample was obtained by subtracting Cr(VI) concentration determined using DPC method from the total Cr concentration.

2.5. Biomass preparation

An appropriate volume of 24 h bacterial culture grown in NB medium ranging from 30 cm^3 to 270 cm^3 was pelleted at 10,000 rpm for 10 min using a Remi refrigerated centrifuge for the biosorption experiments. The obtained pellet was then washed thoroughly with deionised water and used for the experiments. The bacterial cell concentration was obtained using a Petroff-Hausser counter in conjunction with Leitz phase contrast microscope (Laborlux K Wild MPS12). The dry weight of the bacterial cells was obtained by drying the biomass in an air oven at 60^{o}C for 3 h. After cooling to room temperature (28±1 ^{o}C) the weight of the dried biomass was measured.

2.6. Biosorption test procedure

The biosorption test procedure adopted is detailed below: The concentration of Cr(VI) used was 4 mg dm^{-3} in the bioremediation experiments carried out as function of time, pH and biomass loading. In the biosorption isotherm experiments, the initial concentration of Cr(VI) was varied from 2 to 10 mg dm^{-3}. The cell concentration was maintained at $8\pm0.5 \times 10^{10}$ cells/cm^3 for all the experiments except in the case of the biomass loading wherein the cell count ranged from 2.5×10^{8} cells/cm^3 to 1.25×10^{11} cells/cm^3. The pH was adjusted to 1 using a Systronics digital pH meter in all experiments, expect in the case of the biosorption study carried out as a function of pH, wherein the pH was varied from 1 to 6. The chromium interacted cell suspension was made up to a final volume of 100 cm^3 and transferred to 250 cm^3 Erlenmeyer conical flasks and then agitated in a Orbitek rotary shaker at 200 rpm and at 30 ^{o}C for 2 h, except in the case of the kinetics study, wherein the time was varied from 0.17 h to 2.5 h. The suspensions were then centrifuged in a Remi refrigerated centrifuge at 10,000 rpm for 10 min. The chromium

concentration in the supernatant was determined by the analytical methods described earlier and the amount biosorbed by the bacterial cells was estimated by difference.

2.7. Desorption studies

For the desorption studies, chromium loaded bacterial cells obtained after the biosorption experiment were centrifuged at 10,000 rpm for 10 min and the pellet was redispersed in deionised water adjusted to the respective pH used for the corresponding biosorption experiments. These samples were then agitated in an orbital shaker at 30 ^{o}C for 2 h at 200 rpm. The Cr(VI) and total chromium concentrations were determined as per the procedures detailed earlier.

2.8. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy

The biomass samples of the isolate both before and after the bioremediation experiments were lyophilised for 12 h. The samples were then loaded onto to the diamond crystal of the Bruker ALPHA FTIR spectrometer and the spectra were recorded in the wavenumber range of 4000 to 400 cm^{-1}.

2.9. X-ray Photoelectron Spectroscopy (XPS)

For the analysis, the freeze dried bacterial samples before and after the biosorption studies of Cr were pressed into pellets in order to obtain a thin disc. The analysis was carried out using Kratos Axis DLD X-ray Photoelectron Spectrometer with monochromatic Al Kα X-ray radiation.

2.10. Electrokinetic studies

The surface charge of the bacterial cells both before and after the Cr(VI) biosorption studies were recorded using Zetasizer Nanoseries (Nano-ZS90) manufactured by Malvern Instruments Ltd. Worcestershire, UK. For this study, 30 cm^3 of a fully grown bacterial cell culture (24 h) was centrifuged and washed with MilliQ water. The bacterial cells subsequent to washing were then interacted with Cr(VI) solution over a range of pH from 1 to 12 and made up to 100 cm^3 in a standard volumetric flask such that the final concentration of Cr(VI) was 4 $mg{\cdot}dm^{-3}$ and the bacterial cell concentration was $2.5x10^8$ cells/cm^3. The suspension was then equilibrated for 2 h (equilibrium time) at 30 ^{o}C in a Orbitek rotary shaker at 200 rpm. The indifferent electrolyte concentration was maintained at 10^{-3} M NaCl.

All the above experiments were carried out in duplicate and the standard deviation was determined.

3. Results and discussion

3.1. Isolation, characterisation, and identification of the Cr(VI) tolerant bacterial strain from Sukinda Valley water samples

The water samples collected from the Sukinda Valley were found to contain 2.96 $mg{\cdot}dm^{-3}$ total Cr, 2.46 $mg{\cdot}dm^{-3}$ of Cr(VI) and 0.5 $mg{\cdot}dm^{-3}$ of Cr(III). The pH of the water samples collected was 7.3. On performing the isolation procedure of bacteria from the water samples, three different coloured colonies of bacteria namely, white, yellow and orange appeared on the Cr(VI) containing NA plates (Fig. S1(a)). From the bacterial colonies that appeared on incubation subsequent to the inoculation of the water samples onto Cr(VI) containing NA plates, one white colony was chosen for further characterisation and identification procedures. The streak plate of the pure isolate (white colony) is shown in Supplementary Fig. S1(b). The selected bacterial colony was found to be circular, opaque and to have a smooth and buttery texture. A biochemical kit (KB003 Hi25™) was used to carry out the biochemical characterisation of the bacterial isolate and the results are given in Table 1. The results denoted as +ve or –ve indicate that the particular strain or isolate does or does not respond to the corresponding biochemical test respectively and V denotes that the biochemical test is 11-89 % positive. The bacterial isolate is found to be negative towards lysine and certain carbohydrate utilisation tests and positive towards citrate utilisation, nitrate reduction and urease activity.

The molecular characterisation of the isolate revealed that the bacterial strain was 99 % homologous to *Chromobacterium violaceum* (Accession No. HM449690.1). The isolate was also found to be 96 % similar to the whole genome of *Chromobacterium violaceum* (Accession No. AE016825). The full genome of

Chromobacterium violaceum was sequenced by the Brazilian National Genome Project Consortium (Vasconcelos et al., 2003). The isolate was thus identified as *Chromobacterium violaceum* SUK1a and the consensus nucleotide sequence obtained for this has been deposited in GenBank under the accession number, MF599412.1.

The phylogenetic tree constructed for the bacterial isolate between their similar sequences found in the GenBank is shown in Supplementary Fig. S1(c). It is quite evident from the phylogenetic tree that the isolate is *Chromobacterium violaceum*.

Table 1. Characterisation of the isolate by performing different biochemical tests

S. No	Biochemical tests	Isolate	S. No	Biochemical tests	Isolate
1.	Lysine utilisation	-ve	14.	Arabinose utilisation	-ve
2.	β-galactosidase activity	-ve	15.	Xylose utilisation	-ve
3.	Ornithine utilisation	V	16.	Adonitol utilisation	-ve
4.	Urease activity	+ve	17.	Rhamnose utilisation	-ve
5.	Phenylalanine deamination	-ve	18.	Cellobiose utilisation	-ve
6.	Nitrate reduction	+ve	19.	Melibiose utilisation	-ve
7.	H_2S production	-ve	20.	Sucrose utilisation	-ve
8.	Citrate utilisation	+ve	21.	Raffinose utilisation	-ve
9.	Voges Proskauer's test	-ve	22.	Trehalose utilisation	V
10.	Methyl red	-ve	23.	Glucose utilisation	V
11.	Indole	-ve	24.	Lactose utilisation	-ve
12.	Malonate utilisation	-ve	25.	Oxidase test	-ve
13.	Esculin hydrolysis	-ve			

3.2. Growth curve analysis of the isolate, *C. violaceum* SUK1a

The growth curve obtained for the isolate, *Chromobacterium violaceum* SUK1a (Cb), is portrayed in Fig. 1(a). It is evident from Fig. 1(a) that after an initial lag phase to about 1 h, the bacterial cell count increases exponentially up to 14 h from $1.2x10^8$ cells/cm^3 to $6.6x10^9$ cells/cm^3 after which the stationary phase was attained. The generation time calculated from the growth curve for *C. violaceum* SUK 1a is found to be 1.9 h/gen. pH was also monitored over the period of bacterial growth which is found to increase from 7.0 to about 7.9 during the growth of the isolate. This can be attributed to the deamination reaction of the NB media components as a result of the metabolic activity of the bacteria during its growth period.

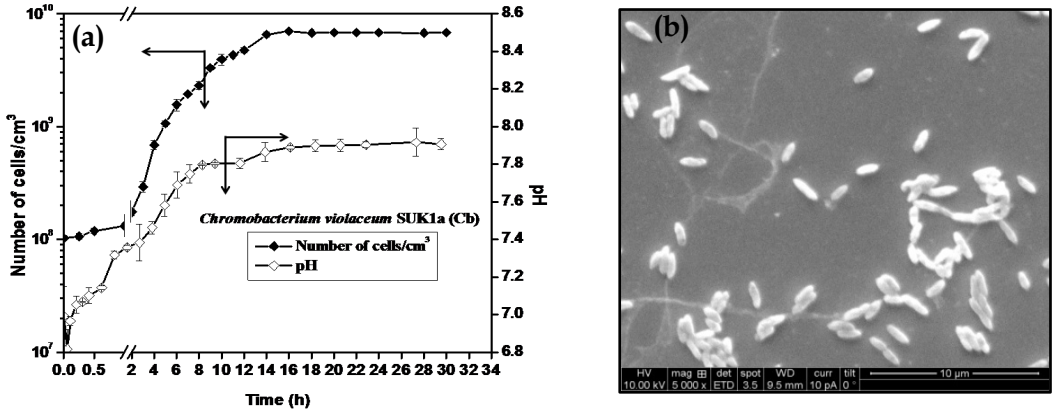

Fig. 1. (a) Growth curve of *Chromobacterium violaceum* SUK1a (Cb) bacterial cells and change in pH with time, (b) Scanning Electron Micrograph of *C. violaceum* SUK1a

The morphology and the type of cell wall possessed by the bacterial cells were analysed using SEM and Gram's differential staining technique respectively. The scanning electron micrograph obtained for *C. violaceum* SUK1a is depicted in Fig. 1(b). From the micrograph it is evident that the bacterial strain is rod shaped. On Gram staining, the bacterial isolate was found to be Gram negative.

3.3. Investigation of various factors affecting biosorption using the isolate as a biosorbent for Cr(VI) ions

3.3.1. Biosorption kinetics

Among the various factors affecting biosorption, the contact time of Cr(VI) ions with the bacterial cell surface was initially examined. For this, *C. violaceum* SUK1a of cell concentration $8x10^{10}$ cells/cm^3 was interacted with 4 mg·dm^{-3} of Cr(VI). It can be observed from Fig. 2 that, the Cr biosorption increases with increase in contact time of Cr(VI) ions with the bacterial cells up to 1.5 h after which a plateau is attained, attesting to attainment of sorption equilibrium between chromium in solution and on the cell surface. An equilibrium time of 2 h was thus chosen for the subsequent biosorption studies. Additionally, residual Cr concentration was also monitored. It is interesting to note the presence of Cr(III) ions in the residual solution formed by the bioreduction of Cr(VI), and occurs in conjunction with the biosorption process (Fig. 2). The co-occurrence of bioreduction of Cr(VI) ions to Cr(III) with biosorption, in the presence of bacterial cells under metabolism independent condition was also reported in our previous studies (Divyasree et al., 2014; Prabhakaran and Subramanian, 2017).

The biosorption kinetics data presented in Fig. 2 for the isolate, *C. violaceum* SUK1a, was also fitted to suitable kinetic models to evaluate the kinetics of Cr(VI) uptake using the linearised forms of the pseudo first order and pseudo second order kinetic expressions represented in equations 1 and 2 respectively as shown below:

$$\log(q_e-q_t) = -k_1t/2.303 + \log(q_e) \quad (1)$$

$$t/q_t = 1/(k_2q_e^2) + t/q_e \quad (2)$$

where, q_e is the Cr biosorbed at equilibrium per gram of cells, q_t is Cr biosorbed at any time t, k_1 and k_2 are the velocity constants for pseudo first order and pseudo second order respectively (Lesmana et al., 2009; Vijayaraghavan and Yun, 2008).

It was observed that the biosorption of Cr(VI) by the isolate follows pseudo second kinetics with R^2 of 0.99. The parameters associated with the kinetic equations obtained for Cr(VI) biosorption such as k_2 and q_e are found to be 0.2 g·mg^{-1} min^{-1} and 1.1 mg·g^{-1} respectively (Supplementary Fig. S2).

3.3.2 Effect of pH on biosorption

The effect of pH on biosorption was studied by pelleting the fully grown bacterial isolate and dispersing it in Cr(VI) solutions of concentration 4 mg·dm^{-3} at varying pH from 1 to 6 for an equilibrium time of 2 h. It is evident from Fig. 3 that, Cr(VI) in the absence of bacterial cells is stable over the pH range chosen for the study. The percentage of total Cr biosorbed is found to be higher at lower pH (~50 %) and biosorption decreases as a function of pH. The higher uptake of Cr(VI) ions at lower pH is due to electrostatic attraction of negatively charged oxyanion of Cr(VI) with the positively charged functional groups present on the bacterial cell surface. From Fig. 3 it can also be noticed that the bioreduction process is found to be higher at acidic pH and decreases with increase in pH.

3.3.3 Effect of biomass loading on biosorption

The biosorption performance of the isolate, *C. violaceum* SUK1a, for Cr(VI) ions was studied as a function of biomass loading with the cell concentration varying from $2.5x10^8$ cells/cm^3 to $1.3x10^{11}$ cells/cm^3. The Cr(VI) biosorption experiments were performed at pH 1, for an equilibration time of 2 h, and an initial Cr(VI) concentration of 4 mg·dm^{-3}. From Fig. 4(a) it can be observed that biosorption of Cr increases with increase in bacterial cell concentration and a maximum biosorption of about 50 % using a bacterial cell concentration of $8x10^{10}$ cells/cm^3.

The specific uptake of Cr per cell for the isolate was also computed, and is found to decrease as a function of biomass loading, owing to the screening effect (Fig. 4(a)). Screening effect is observed under high biomass concentration wherein crowding of the cells results in the masking of potential chromium binding sites exposed by a bacterial cell, thereby decreasing the specific Cr uptake. In addition to analysis of Cr concentration on the bacterial cell surface, the changes in the Cr concentration as a function of biomass loading in the bulk solution was also monitored. From Fig. 4(b) it can be observed that, the Cr(VI) residual concentration is found to decrease with increase in biomass loading and at

higher bacterial cell concentrations, complete removal of Cr(VI) could be achieved resulting in a nil concentration of Cr(VI) in the residual solution. The bioreduction process occurring in conjunction with biosorption process was also monitored and it can be observed that Cr(III) concentration is found to increase as a function of biomass loading and a maximum bioreduction of about 65 % could be achieved for *C. violaceum* SUK1a.

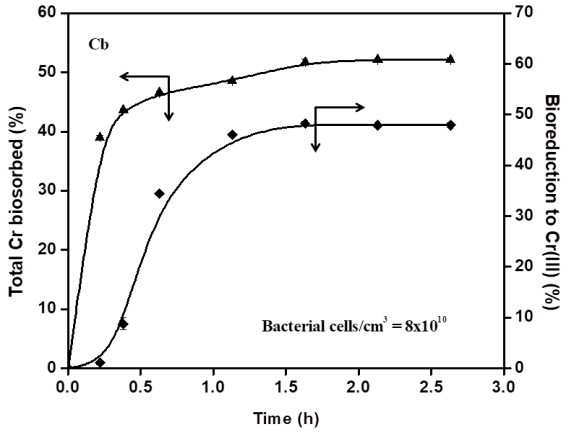

Fig. 2. Effect of contact time on Cr(VI) biosorption and bioreduction using *C. violaceum* SUK1a [Cr(VI) = 4 mg dm^{-3},pH 1, 8x10^{10} cells/cm^3 and 30 ^{o}C]

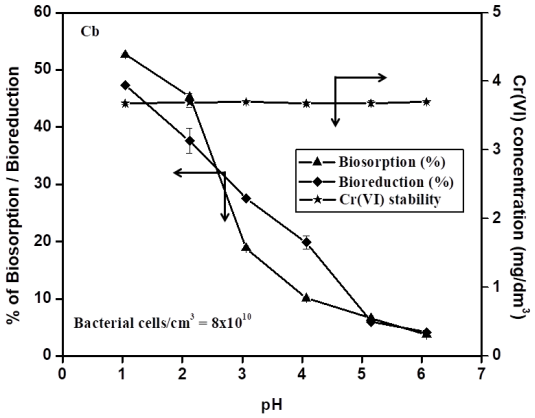

Fig. 3. Effect of pH on Cr biosorption: Cr(VI) stability and % of biosorption and bioreduction using *C. violaceum* SUK1a (8x10^{10} cells/cm^3) [Cr(VI) = 4 mg dm^{-3}, equilibrium time (t_e) = 2 h and 30 ^{o}C]

3.3.4. Biosorption isotherm

For the Cr(VI) biosorption isotherm experiments, *C. violaceum* SUK1a bacterial cells (8x10^{10} cells/cm^3 corresponding to 0.18 g dry weight) were allowed to interact with varying initial Cr concentrations from 2 mg dm^{-3} to 10 mg dm^{-3}, maintaining optimum conditions for the biosorption process. From the Cr(VI) biosorption isotherm obtained for the isolate as shown Fig. 5, the Cr uptake is found to increase initially up to about 5 mg·dm^{-3} equilibrium Cr concentration and thereafter tends to attain a saturation value, indicative of Langmuirian behaviour.

The results of the biosorption isotherm experiments discussed in Fig. 5, were fitted to the Langmuir isotherm model using the linearised equation 3, which is mostly used to describe biosorption equilibrium of biomass with the Cr(VI) sorbate ions and is represented below:

$$C_e/q_e = 1/(q_{max}\, b) + C_e/q_{max} \quad (3)$$

where, C_e (mg·dm^{-3}) is the equilibrium or residual concentration of Cr, q_e (mg·g^{-1}) is the Cr biosorbed at equilibrium per gram of cells, q_{max} (mg·g^{-1}) is the maximum Cr biosorbed per gram of cells, b is the constant related to the free energy of biosorption. The free energy for biosorption can be calculated using the formula:

$$\Delta G^{o} = -RT \ln(b) \quad (4)$$

where, R is gas constant with value 8.314 J·mol^{-1}·K^{-1} and T is temperature in Kelvin (K) (Volesky, 2003; Vijayaraghavan and Yun, 2008; Lesmana et al., 2009).

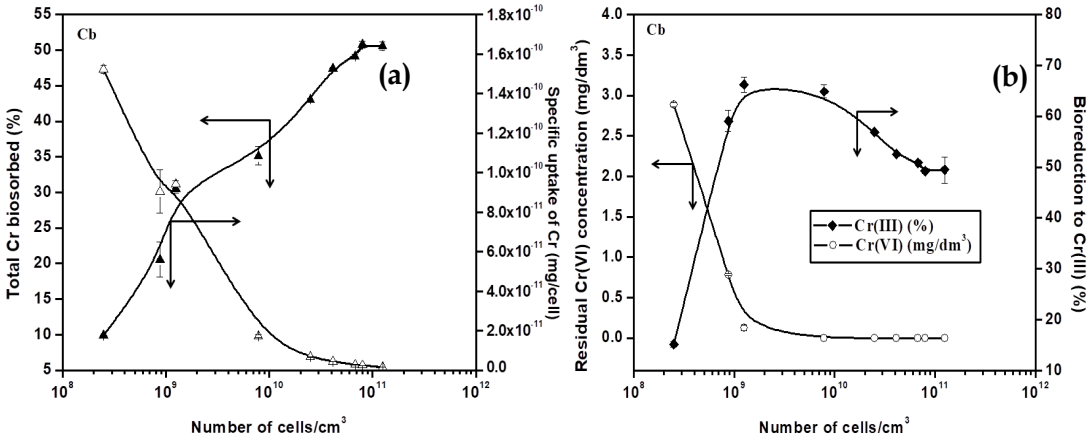

Fig. 4. (a) Effect of biomass loading on Cr biosorption [Cr(VI) = 4 mg·dm^{-3}, t_e = 2 h, pH = 1 and 30 ^{o}C]: Total Cr biosorption (%) and specific uptake (mg/cell) using *C. violaceum* SUK1a; (b) Residual Cr(VI) concentration and % bioreduction as function of concentration of *C. violaceum* SUK1a

It can be observed from Supplementary Fig. S3, that the isotherm for Cr(VI) for the isolate adheres to the Langmuir model with a good correlation with R^2 = 0.99 (Langmuir, 1918). The shape of the isotherm may also be categorized as L-type of the Giles classification (Giles et al., 1960). The parameters associated with the Langmuir model such as, b and q_o obtained from the fit are found to be 0.7 dm^3/mg and 2 mg/g respectively. The free energy (ΔG) value of chromium biosorption by the bacterial isolate is found to be -26.3 kJ/mol, which is suggestive of chemisorption.

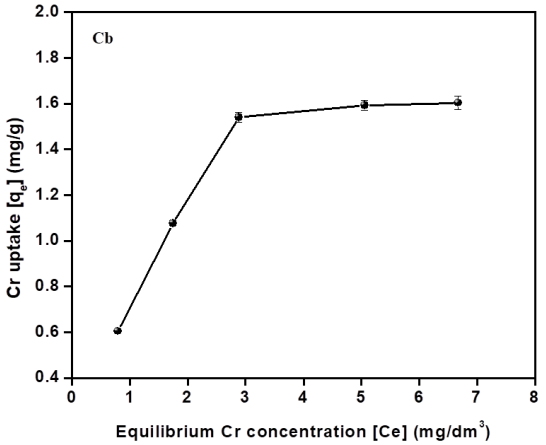

Fig. 5. Cr biosorption isotherm [t_e = 2 h, pH = 1 and 30 ^{o}C] for *C. violaceum* SUK1a bacterial cells interacted with Cr(VI) ions [8x10^{10} cells/cm^3 (dry wt. = 0.18 g)]

3.4. Elucidation of the mechanisms involved in the bioremediation of Cr(VI) by the isolate, *C. violaceum* SUK1a

3.4.1. Desorption studies

The nature of interaction between the bacterial isolate and Cr(VI) ions can be ascertained by carrying out desorption tests on the Cr(VI) biosorbed bacterial cells by maintaining the same conditions as that optimised for the biosorption process, namely, pH 1, bacterial isolate cell count at which a maximum biosorption was achieved and at 30 ^{o}C. From Fig. 6 it is evident that, only a maximum of about 4 % of Cr could be desorbed from the biosorbed cells indicating the irreversible nature of the biosorption

process. This further attests to a chemisorption process for the interaction of Cr(VI) ion with the isolate, *C. violaceum* SUK1a.

3.4.2. ATR-FTIR spectroscopy

FTIR spectral studies enabled to identify the different chromium binding groups present on the bacterial cells. The results obtained are summarised in Table S1. On comparing the spectra obtained for *C. violaceum* SUK1a before and after biosorption of Cr(VI) as shown in Figs. 7(a) and 7(b), it is evident that there are apparent shifts in wavenumber for many of the identified Cr binding groups present on the bacterial isolate cell surface. The wavenumber 3276 cm^{-1} obtained for hydroxyl (-OH) and amino (-NH) group stretching in the case of the bacterial cells alone is shifted to 3270 cm^{-1} for Cr(VI) interacted bacterial cells. A wavenumber shift for (-OH) vibration of carboxyl and (-CO) stretching of aldehyde can be observed from 2928 cm^{-1} and 1742 cm^{-1} to 2923 cm^{-1} and 1737 cm^{-1} respectively. Similarly, a shift in the amide I and amide II bands can also be observed from 1634 cm^{-1} to 1639 cm^{-1} and 1537 cm^{-1} to 1532 cm^{-1}. The (-CO) stretching of carboxyl group (-COOH) and (P=O) stretching are shifted from 1396 cm^{-1} and 1243 cm^{-1} to 1386 cm^{-1} and 1247 cm^{-1} respectively. Therefore, the shifts in wavenumber obtained for the functional groups such as (-OH), (-NH), (-COOH) and (P=O) present on the bacterial isolate cell surface interacted with Cr(VI) compared to the cells alone, confirm the involvement of these functional groups in the bioremediation process.

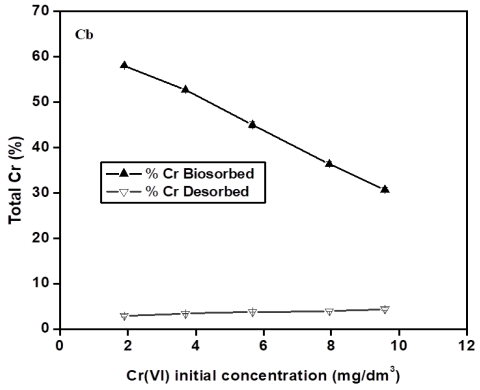

Fig. 6. Biosorption-Desorption (%) studies of Cr(VI) using *C. violaceum* SUK1a

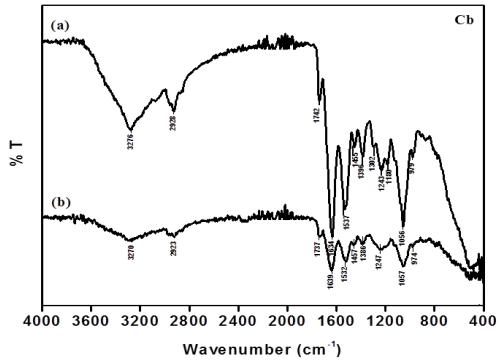

Fig. 7. ATR-FTIR spectra of: (a) *C. violaceum* SUK1a and (b) Cr(VI) interacted *C. violaceum* SUK1a

3.4.3. X-ray Photoelectron Spectroscopic studies

The Cr(2p) X-ray photoelectron spectrum for *C. violaceum* SUK1a was recorded before and after interaction with Cr(VI) ions (Fig. 8(a)). It can be observed from Fig. 8(a) that, Cr(III) ions formed by the bioreduction of Cr(VI) are not only released into the bulk solution but also get bound onto the surface of the bacterial cells. The two doublet peak obtained correspond to Cr(VI) and Cr(III) species. The binding energies of 580.0 eV and 590.0 eV correspond to $2p_{3/2}$ and $2p_{1/2}$ of Cr(VI), while 576.0 eV and

586.0 eV correspond to $2p_{3/2}$ and $2p_{1/2}$ of Cr(III) (Fig. 8(a)). These binding energy values are in close agreement with the values obtained by other researchers (Dambies et al., 2001; Park et al., 2007; Bhatt et al., 2015; Li et al., 2015; Li et al., 2015; Prabhakaran et al., 2016; Prabhakaran and Subramanian, 2017).

High resolution spectra for elements such as C, O, N, P and S were recorded to find out the involvement of other functional groups present on the bacterial cells in the bioremediation process as shown in Figs. 8(b-f) and the results obtained are summarised in Supplementary Table S2. Certain binding energy shifts for carbon containing functional groups such as C-O/C-N and C=O; oxygen containing functional groups such as O-C/P-O-C/C-O-C; nitrogen containing functional group -NH and also groups sulphur, for the bacterial cells after interaction with Cr(VI) as compared to the cells alone can be observed (Figs. 8(b-f) and Table S2). These shifts in binding energy for these functional groups consequent to interaction with Cr(VI) ions can be attributed to the involvement of these groups either in the chemical interaction with Cr ions and/or in the donation of electrons to bring about the bioreduction of Cr(VI) ions to the less toxic Cr(III) species.

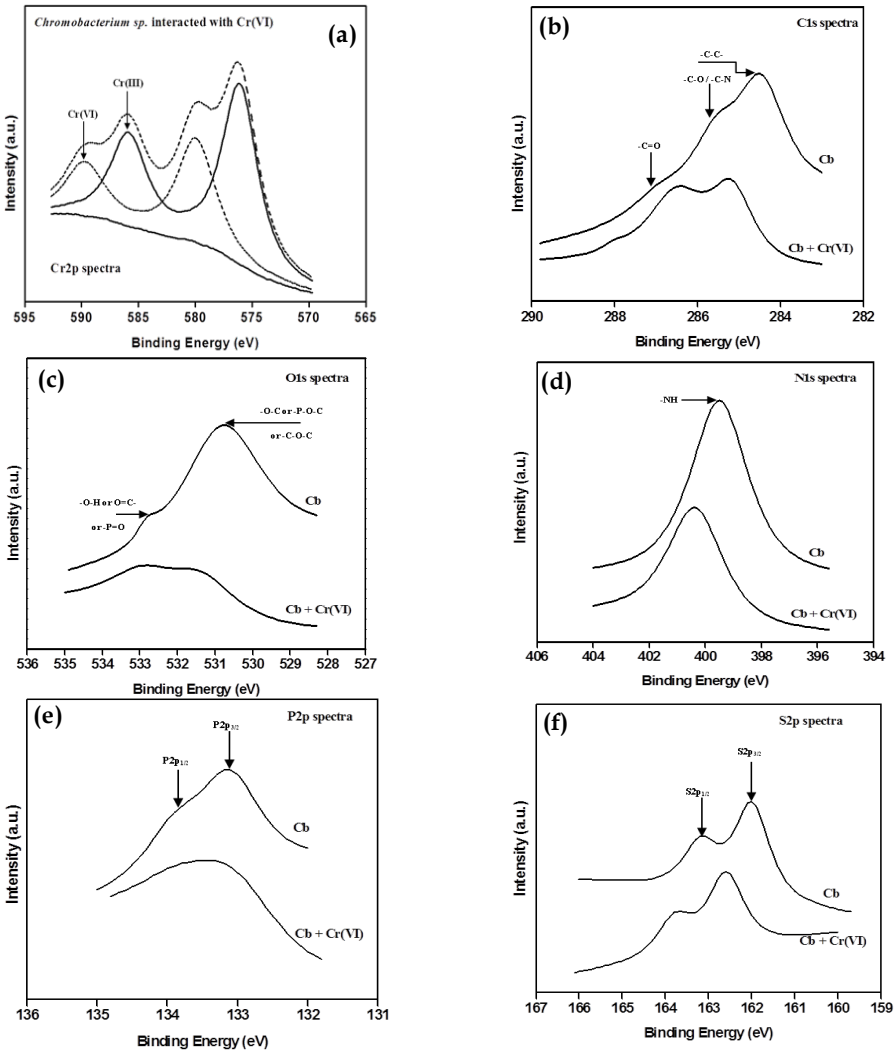

Fig. 8. (a) High resolution X-ray photoelectron spectra of Cr(2p) obtained for *C. violaceum* SUK1a; High resolution X-ray photoelectron spectra obtained for *C. violaceum* SUK1a interacted with Cr(VI) for elements: (b) C(1s), (c) O(1s), (d) N(1s), (e) P(2p) and (f) S(2p)

3.4.4. Electrokinetics study

Biosorption of chromium ions is dependent on the surface charge of the bacterial cells. The indigenous bacterial isolate, *C. violaceum* SUK1a, exposes functional groups such as hydroxyl, carboxyl, carboxyl, amino and phoshate for chromium binding and has been delineated using FTIR spectroscopic studies.

Thus, the bacterial cell surface is charge-regulated and the surface charge is a function of the ionisation of these Cr binding functional groups at the interfacial pH immediately adjacent to the bacterial cell surface, which itself is a function of the cell surface electrostatic potential and pH of the bulk solution. Hence, the bacterial cell surface charge vary as a function of the number and type of these cell surface functional groups, bulk solution pH and composition of the electrolyte. The protonation and deprotonation of these exposed functional groups controls the interaction of chromium ions with these functional groups at the interfacial pH immediately adjacent to the bacterial cell surface, which itself is a function of the cell surface electrostatic potential and pH of the bulk solution. Hence, the bacterial cell surface charge vary as a function of the number and type of these cell surface functional groups, bulk solution pH and composition of the electrolyte. The protonation and deprotonation of these exposed functional groups controls the interaction of chromium ions with the bacterial cell surface. The surface charge of bacterial cells can be analysed using zeta potential measurements. Zeta potential is the potential or charge at the slip plane and is considered as the surface charge of bacteria. For bacterial cells, it is assumed that the slip plane is located near the outer polymeric layer of the cell wall composed of the various cell wall components that exposes a myriad of the functional groups that interact with the chromium ions present in the bulk aqueous solution (Hong and Brown, 2008). In the present study, in order to find out the changes in the surface charge of *C. violaceum* SUK1a subsequent to the interaction with Cr(VI) ions, zeta potential measurements were carried out as a function of pH as shown in Fig. 9. It can be observed from Fig. 9 that, the surface charge of the bacterial isolate in the absence of Cr(VI) ions is negative over the pH range chosen for the study. However, subsequent to the interaction with Cr(VI) ions, the surface charge of the bacterial cells is found to be less negative as compared to the cells alone. The less negative surface charge for the bacterial cells interacted with Cr(VI) is due to the binding of positively charged Cr(III) ions formed by the bioreduction of Cr(VI) and this has been unequivocally confirmed from the X-ray photoelectron spectroscopic results. Additionally, a shift in IEP can be observed for the bacterial cells after interaction with Cr(VI) ions, which reinforces the involvement of chemical binding forces in the bioremediation process of Cr(VI) by the bacterial isolate. Specific ion adsorption processes involving chemical interactions brings about a shift in IEP (Parks, 1967).

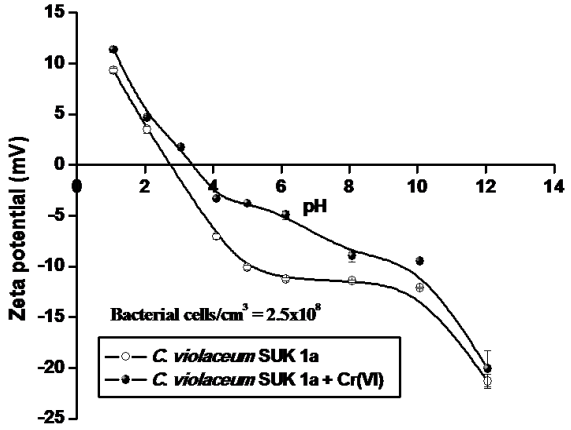

Fig. 9. Zeta potential of *C. violaceum* SUK1a before and after interaction with Cr(VI) for 2 h as a function of pH

3.4.5. Mechanisms of bioremediation involved at the C. violaceum SUK1a-Cr(VI) solution interface

Based on the results obtained for biosorption and the different characterisation techniques, the mechanisms involved in the bioremediation of toxic Cr(VI) ions by the Gram negative Cr(VI) tolerant isolate, *C. violaceum* SUK1a, can be elucidated. The bioremediation of Cr(VI) by the bacterial isolate, being a metabolically independent process, will be profoundly governed by the surface interactions of Cr(VI) ions with the Cr binding groups exposed by the isolate cell surface. The cell wall surface of the Gram negative isolate is composed of two major layers, namely, a thin peptidoglycan layer followed by an outer membrane composed of lipopolysaccharides (LPS) and surface proteins (Willey *et al.*, 2008). The outer membrane layer of a Gram negative bacterium is made up of major and minor polypeptides that pass through the membrane with LPS of variable chain length emerging from the membrane suface (Beveridge, 1989). Salton (1960) has found out that strains of bacteria belonging to the genus

Chromobacterium possess monosaccharides such as galactose, glucose, mannose, and rhamnose in their cell wall. Additionally, presence of aminoarabinose, an amino-pentose, in the lipopolysaccharides of the *Chromobacterium violaceum* cell wall has been reported by Hase and Rietschel (1977). Furthermore, D-glycero-D-galacto-heptose has been found to be a constituent of bacterial cell wall polysaccharide of *Chromobacterium violaceum* (Maclennan and Davies, 1957). Therefore, these cell wall components expose a myriad of chromium binding groups that play a key role in the biosorption and bioreduction processes and the same has been confirmed from FTIR and XPS results discussed earlier. Bioreduction of Cr(VI) to Cr(III) is brought about by the interaction of Cr(VI) ions with the hydroxyl and sulphydryl groups, -OH and –SH respectively and subsequent formation of chromate esters. The chromate esters thus formed, being unstable, decomposes immediately, consequently transferring electron from oxygen and sulphur electron donor centers to Cr(VI) electron acceptor center resulting in the reduction of Cr(VI) to Cr(III) (Prabhakaran et al., 2016; Prabhakaran and Subramanian, 2017). The functional groups containing oxygen and sulphur that are involved in the bioreduction of Cr(VI) to Cr(III) are contributed by lipopolysaccharides and surface proteins present on the bacterial cell surface (Volesky, 2003). The reduction of Cr(VI) to Cr(III) in the presence of organic compound has been reported and chromate being a strong oxidising reagent is being used for the oxidation of organic compounds such as alcohol and sulphur containing amino acid derivative glutathione (Lanes and Lee, 1968; Westheimer, 1949; McAuley and Olatunji, 1977). Cr(III) thus formed via bioreduction of Cr(VI) is either released to the bulk solution or forms chemical complexes with functional groups such as hydroxyl, carboxyl, amino and phosphorus containing groups present on the bacterial cell surface. Bioremediation of Cr(VI) by *C. violaceum* SUK1a is thus brought about by two processes, namely, biosorption of Cr, that involves both electrostatic and chemical interactions and bioreduction of Cr(VI) to the less toxic Cr(III) species.

4. Conclusions

Bioremediation studies of Cr(VI) was carried out using a Cr(VI) tolerant bacterial strain isolated from water samples collected from the Sukinda Valley, Odisha, India. The strain was identified to be *C. violaceum* SUK1a on performing the molecular biological 16S rRNA based sequencing technique. The Gram negative bacterial isolate was then used as Cr(VI) biosorbent wherein various parameters affecting biosorption were evaluated. A maximum chromium biosorption of about 50 % was achieved using *C. violaceum* SUK1a, for a biomass loading of $8x10^{10}$ cells/cm^3, Cr(VI) concentration of 4 $mg \cdot dm^{-3}$ and pH 1. Chromium in the residual solution after the treatment with the bacterial strain was found to be in the less toxic Cr(III) form, indicating the concomitant occurrence of bioreduction process with biosorption. Thus, a complete bioremediation of toxic Cr(VI) ions in terms of both biosorption and bioreduction processes could be achieved using the isolate, thereby meeting the USEPA regulatory limits for safe effluent discharge. The biosorption process was found to follow pseudo second order kinetics and the Cr(VI) biosorption isotherm was found to adhere to a typical Langmuirian behaviour. The Gibbs free energy (ΔG) value of Cr(VI) biosorption by *C. violaceum* SUK1a was found to be -26.3 kJ/mol, suggestive of involvement of chemical binding forces between the Cr binding groups present on the bacterial surface and Cr. The irreversible nature of biosorption was indicated by only a marginal desorption of Cr(VI) from the bacterial cells, initially interacted with the Cr(VI) species, into the bulk solution. The mechanisms involved at the bacterial cell surface-chromium solution interface occurring during the bioremediation of Cr(VI) were further validated using different characterisation techniques. FTIR spectroscopic studies revealed the involvement of hydroxyl, carboxyl, amino and phosphate groups in the biosorption of Cr(VI). Electrokinetic and X-ray photoelectron spectroscopic studies provided evidence in support of biosorption and bioreduction mechanisms of Cr remediation. Based on the results obtained from the bioremediation and characterisation studies, the mechanisms of bioremediation of Cr(VI) by the isolate, *C. violaceum* SUK1a involved both biosorption and bioreduction processes.

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Supplementary Figures and Tables

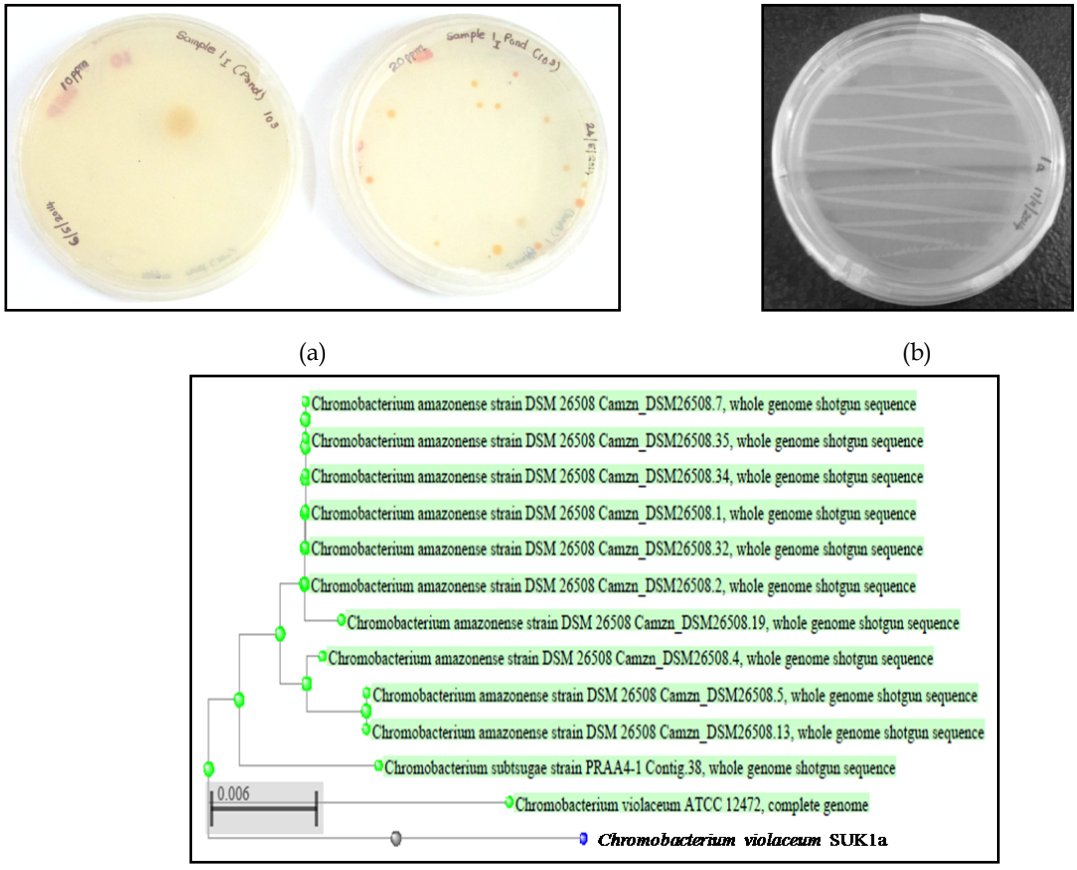

Fig. S1. (a) The different bacterial colonies obtained after spread plating of the water samples collected from Sukinda Valley, (b) Streak plate of the pure strain of the isolate (white colony), (c) Phylogenetic tree obtained for the isolate (white colony) = *Chromobacterium violaceum* SUK1a

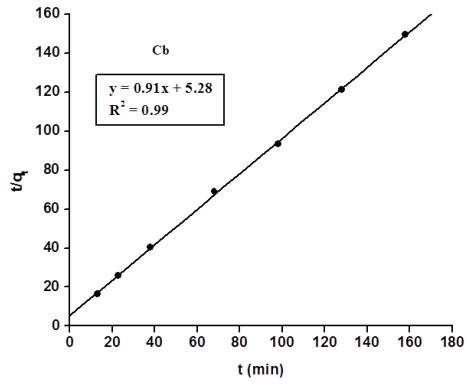

Fig. S2. Pseudo second order kinetics plots for Cr(VI) biosorption using *C. violaceum* SUK1a [Cr(VI) = 4 mg dm^{-3},pH 1, $8x10^{10}$ cells/$cm^3$and 30 ^oC]

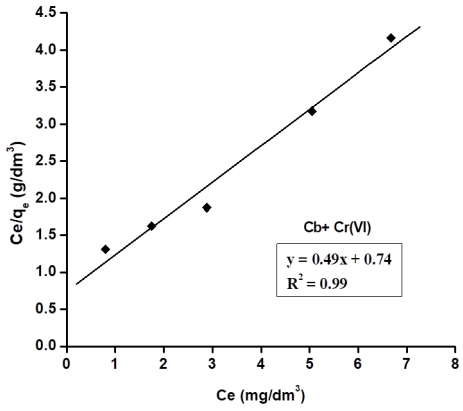

Fig. S3. Langmuir fit obtained for Cr(VI) biosorption studies using *C. violaceum* SUK1a [t_e = 2 h, pH = 1, 30 ^oC and $8x10^{10}$ cells/cm^3(dry wt. = 0.18 g)]

Table S1. Characteristic wavenumbers of typical functional groups for *C. violaceum* SUK1a before and after interaction with Cr(VI) ions obtained by ATR-FTIR spectroscopy

Functional groups identified	**Wavenumber for *C. violaceum* SUK1a (cm^{-1}) Fig. 7(a)**	**Wavenumber for Cr(VI) interacted *C. violaceum* SUK1a (cm^{-1}) Fig. 7(b)**
Hydroxyl (-OH) + (-NH) stretching	3276	3270
(-OH) vibration of carboxyl	2928	2923
(-C=O) stretching in amide [Amide I band]	1634	1639
(-NH) bending and (-CN) stretching in amide [Amide II band]	1537	1532
-CH_2 scissoring	1455	1457
(-CO) stretching of (-COOH)	1396	1386
P=O stretching	1243	1247
Polysaccharide related	1056, 976	1057, 974

Table S2. Binding Energy (eV) values obtained for elements present in the isolate, *C. violaceum* SUK1a before and after interaction with Cr(VI) ions using XPS

Samples	**Elements present in the bacterial samples analysed using XPS and their corresponding Binding Energy (eV)**													
	Cr(2p)				**C(1s)**			**O(1s)**		**N(1s)**	**P(2p)**		**S(2p)**	
	Cr(VI)		**Cr(III)**											
	$2p_{3/2}$	**$2p_{1/2}$**	**$2p_{3/2}$**	**$2p_{1/2}$**	**C-C**	**C-O or C-N**	**C=O**	**O-C or P-O-C or C-O-C**	**-OH or O=C or P=O**	**-NH**	**$2p_{3/2}$**	**$2p_{1/2}$**	**$2p_{3/2}$**	**$2p_{1/2}$**
***C. violaceum* SUK1a**	-				284.5	285.5	287.2	530.8	532.8	399.5	133.2	133.9	162.0	163.2
***C. violaceum* SUK1a + Cr(VI)**	580.0	590.0	576.0	586.0	285.2	286.5	288.0	531.5	532.9	400.4	133.3	134.0	162.6	163.8