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Bio-reduction of Cr(VI) by exopolysaccharides (EPS) from indigenous bacterial species of Sukinda chromite mine, India

R. Harish · Jastin Samuel · R. Mishra · N. Chandrasekaran · A. Mukherjee

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Abstract Chrome mining activity has contributed intensively towards pollution of hexavalent chromium around Sukinda Valley, Orissa, India. In an attempt to study the specific contribution of exopolysaccharides (EPS) extracted from indigenous isolates towards Cr(VI) reduction, three chromium (VI) tolerant strains were isolated from the effluent mining sludge. Based on the tolerance towards Cr(VI) and EPS production capacity, one of them was selected for further work. The taxonomic identity of the selected strain was confirmed to be *Enterobacter cloacae* (showing 98% similarity in BLAST search to *E. cloacae*) through 16S rRNA analysis. The EPS production was observed to increase with increasing Cr(VI) concentration in the growth medium, highest being 0.078 at 100 mg/l Cr(VI). The extracted EPS from *Enterobacter cloacae* SUKCr1D was able to reduce 31.7% of Cr(VI) at 10 mg/l concentration, which was relevant to the prevailing natural concentrations at Sukinda mine effluent sludge. The FT-IR spectral studies confirmed the surface chemical interactions of hexavalent chromium with EPS.

Keywords *Enterobacter cloacae* SUKCr1D · Bioremediation · Sukinda Valley · Exopolysaccharides (EPS)

Introduction

Chromium is one of the most widely used heavy metal that exists in various oxidation states of which the hexavalent and trivalent are predominant. Hexavalent chromium exerts a detrimental effect on the environment (Sayari et al. 2005) and also causes various health hazards to human beings (Kaufaman 1970; Snow 1994; Kowalski 1994). Chromium pollution is caused by various industrial activities such as metallurgy, leather tanning, electroplating, textile manufacturing, petroleum refining and mining. Among them chrome mining activity has contributed significantly towards hexavalent chromium pollution. One of the largest open cast chrome ore mines in the world is located in Sukinda Valley, Jajpur district, Orissa, India. The place has been listed as the fourth most polluted places in the world by the Blacksmith Institute, USA (Blacksmith Institute 2007).

Chromium remediation is usually achieved employing various physical and chemical processes including coagulation, ion exchange, precipitation and adsorption (Pagnanelli et al. 2001). These

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R. Harish · J. Samuel · R. Mishra · N. Chandrasekaran · A. Mukherjee (✉)
Centre for Nanobiotechnology, School of Biosciences and Technology, VIT University, Vellore 632014, India
e-mail: amit.mookerjea@gmail.com

conventional processes are often expensive and the disposal of the waste after the treatment remains as a prime concern (Fendorf and Li 1996). Thus numerous attempts have been made to develop a bio-based remediation approach using bacteria which efficiently and effectively removes contaminant heavy metals (Tsezos and Volesky 1981; Costa et al. 2001; Sahi et al. 2006). Most of the microorganisms produce exopolysaccharides (EPS) in natural environment (Decho 2000; Dupraz et al. 2009). There have been reports of utilizing EPS secreted by bacteria for attachment, tolerance and reduction of hexavalent chromium (Jorand et al. 1998; Sutherland 2001; Dupraz et al. 2009; Ozturk et al. 2009).

EPS is a comprehensive terminology used to represent the high molecular weight secretions from the microorganisms and the product of cellular lysis and hydrolysis of macromolecules (Decho 2000; Sheng et al. 2010; Pacwa-Plociniczak et al. 2011). Anionic property of EPS enables it to trap the positively charged inorganic as well as organic pollutants by electrostatic interaction and hence playing a key role as biosorbents for metal remediation and recovery (Sheng et al. 2010). There are several negatively charged functional groups such as hydroxyl, carboxyl, phenolic and sulfhydryl present in the EPS which help in the adsorption of chromium (Sethuraman and Balasubramanian 2010).

Studies relating to reduction of hexavalent chromium to trivalent form by native microbial species isolated from different sources have been reported in the past (Rege et al. 1997; Malik 2004; Pal and Paul 2008; Wei-hua et al. 2009; Raicevic et al. 2010; Sethuraman and Balasubramanian 2010). The vital role of EPS along with the cell in the adsorption and reduction of chromium has been noted recently (Ozturk et al. 2009; Sheng et al. 2010; Quintelas et al. 2011). A detailed study on bio-removal of chromium by indigenous bacterial isolates from tannery polluted places has also been reported (Sundar et al. 2011).

A thorough literature survey revealed that there have been limited detailed attempts to look at the specific role of EPS in bioremediation of hexavalent chromium respect to indigenous bacterial species from a mine site. Therefore the major objective of the present study was to examine the specific contribution of extracted EPS from indigenous bacterial species isolated from mining sludge of Sukinda valley in the reduction of hexavalent chromium.

Materials and methods

Sample collection and isolation of Cr(VI) tolerant bacteria

Sludge sample from the contaminated chromite mine site of Sukinda Valley, India was acquired and stored in polypropylene bottles at 4°C. Serial dilution of the sludge sample was carried out with sterile distilled water. This suspension was added to M9 minimal medium (Megharaj et al. 2003) agar amended with 10 mg/l of Cr(VI) in the form of $K_2Cr_2O_7$ and was kept in incubator at 37°C. After 24 h, the observed bacterial colonies with different colony morphology were isolated and labelled as 1A, 1C and 1D. This was then plated on nutrient agar medium to obtain pure cultures. Morphological observations, Gram staining and biochemical tests were performed to ensure the genus level difference between the isolates.

Maximum tolerance concentration (MTC) of isolates to Cr(VI)

Maximum tolerance concentration (MTC) of isolates to Cr(VI) was carried out in Peptone-Yeast-Glucose medium by plate and broth assay with varying Cr(VI) concentrations ranging from 10 to 1000 mg/l. Maximum concentration of Cr(VI) which supports the bacterial growth in the broth was defined as its MTC (Calomoris et al. 1984).

Extraction and quantification of exopolysaccharides (EPS)

To select the highest EPS producing isolate, a loop full culture of each isolate was inoculated into 100 ml of Nutrient Broth (NB) respectively, and was allowed to grow for 48 h with temperature maintained at 30°C, shaking at 180 rpm. The cultures were then centrifuged at 10,000×*g* for 10 min and the supernatant of each culture was harvested. To the collected supernatant, equal volume of ethanol was added and was kept at 4°C overnight to precipitate the EPS. The obtained mixture was then centrifuged at 10,000×*g* for 30 min and the pellets were collected. The pellets were then washed twice with ethanol and were dialyzed against de-ionized water. The EPS obtained was estimated for each sample by phenol–sulphuric acid method (Dubois et al. 1956).

Characterization of the selected isolate

16S rRNA sequencing was performed for the selected isolate to confirm its identity. Phenol–chloroform method (Sambrook et al. 1989) was employed for genomic DNA isolation from the bacterial strain grown in NB medium. The 16S rRNA nucleotide sequencing was performed by fluorescent dye terminator method (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). The sequence obtained was then analysed using BLAST. The nearest neighbouring sequences were downloaded and aligned using Clustal W version 1.6. Phylogenetic tree was constructed using aligned sequences by the neighbour joining algorithm using CLC free workbench 3.2 software. On the basis of different morphological, biochemical characteristics and 16S rRNA gene analysis the taxonomic identity of the strain was confirmed.

Growth profile in presence of Cr(VI)

Growth kinetics of the selected bacterial isolate was carried out in the presence of different concentrations of Cr(VI) i.e. 10, 50 and 100 mg/l, where the absence of Cr(VI) was treated as control. The Cr(VI) stock solution was prepared using sterilized distilled water and was then filter sterilized with 0.45 μm Whatman syringe filter. Flasks containing 100 ml NB supplemented with different Cr(VI) ($K_2Cr_2O_7$) concentration were added with 1.5×10^8 cells as initial inoculum and growth was measured at 600 nm.

Quantification of EPS at different chromium concentration

The selected isolate was inoculated into 100 ml nutrient broth at varying Cr(VI) concentrations (0, 5, 10, 20, 40, 50 and 100 mg/l). The EPS was then extracted after 3 days and was quantified using phenol–sulphuric acid method (Dubois et al. 1956).

A kinetic study at 2-h intervals on EPS production at three different Cr(VI) concentrations (i.e. 10, 50, 100 mg/l) was also carried out and quantified using phenol–sulphuric acid.

Extracellular protein (ECP)

A 2-h periodic study was done on ECP production of the selected isolate at three different Cr(VI) concentrations (10, 50 and 100 mg/l). NB without Cr(VI) was treated as control. A loop full culture was inoculated into 100 ml nutrient broth incubated at 30°C, shaking at 180 rpm. At every 2 h interval, 20 ml of samples were taken from different flasks containing varying Cr(VI) concentrations. The samples were then centrifuged at $10{,}000 \times g$ for 10 min. To the supernatant 100% tricholoro acetic acid (TCA) was added in the ratio 1:4 and was incubated at 4°C for 15 min. After incubation centrifugation was done at $10{,}000 \times g$ for 40 min and the pellet obtained was washed twice with cold acetone. The pellet was then dried and was dissolved in 1 ml of deionised water. The protein precipitated was estimated by Bradford assay (Ozturk et al. 2008).

Reduction of Cr(VI) by the selected isolate

A loop full of selected isolate was inoculated into 100 ml NB supplemented with 10 mg/l of Cr(VI) concentration. To measure the reduction of Cr(VI) by bacterial cells, the culture from each flask was harvested after 24 h of incubation at 30°C, shaking at 180 rpm and was then centrifuged at $10{,}000 \times g$ for 10 min. The supernatant was analysed for Cr(VI) concentration. The initial and final Cr(VI) concentrations were calculated using the colorimetric technique of 1-5-diphenylcarbazide (Clesceri et al. 2005).

Reduction of Cr(VI) by extracted EPS from selected isolate

A loop full of selected isolate was inoculated into 100 ml NB. 20 ml samples were taken after 18 h of incubation at 30°C, shaking at 180 rpm. The EPS was then extracted from the pooled samples and then interacted with 5 ml of 10 mg/l Cr(VI) concentrations for 24 h, shaking at 180 rpm. The initial and final Cr(VI) concentrations were computed (Clesceri et al. 2005).

Estimation of chromium

Hexavalent chromium concentration was analysed using a 1,5-diphenylcarbazide colorimetric method (Clesceri et al. 2005). Samples were acidified with 0.2 N H_2SO_4 and absorbance was read at 540 nm. Total chromium was estimated at 359.9 nm using a flame atomic adsorption spectrophotometer (Analyst400/HGA 900, Perkin Elmer, USA) equipped with

a 35 mA chromium hollow cathode lamp. Prior to analysing, the samples were acidified using 1 N HNO_3 in order to extract adsorbed Cr(VI).

FT-IR analysis

To understand the surface characteristic and chemical bonding properties of the EPS, FT-IR analysis was carried out. Infrared spectra of the Cr(VI) interacted and un-interacted with extracted EPS were studied. Both the samples were lyophilized and 10 μg of each sample were mixed with 100 mg KBr and pressed to form transparent tablets. The infrared spectra were obtained using a Fourier transform infrared spectrometer (Thermo Nicolet Model: 6700). The spectra were collected within a scanning range of 4,000–400 cm^{-1}.

Statistical analysis

Each set of experiments was carried out in triplicate. Experiments were repeated separately to ensure reproducibility. Statistical analysis was performed for "EPS quantification at various Cr(VI) concentrations after 3 days" using one-way ANOVA followed by Bonferroni's Multiple Comparison Test (Post Test) in Graph Pad *Prism* version 5.01 software. Similarly Student's *t* test was performed for "EPS production for every 2 h at various Cr(VI) concentrations" in Graph Pad *Prism* version 5.01 software. The results obtained were considered statistically significant if, $P < 0.05$.

Results and discussion

Isolation of Cr(VI) tolerant bacteria

The genus level difference between isolates 1A, 1C and 1D were affirmed based on the microscopic observation of colony morphology, Gram staining and biochemical tests (data provided in Online Resource 1). Chromium tolerant bacteria capable of reduction of hexavalent chromium have been reported from chromium polluted Sukinda environment (Das and Mishra 2010; Dhal et al. 2010). Isolation of indigenous bacteria from the sludge of Sukinda mine would be apt to perform in situ remediation as they are site specific and tolerant to Cr(VI).

Maximum tolerance concentration (MTC) of isolates to Cr(VI)

The tolerance test was done based on the growth of the isolates in the nutrient media amended with various concentrations of Cr(VI) ranging from 10 to 1000 mg/l. Out of the three isolates, two showed tolerance up to 500 mg/l whereas one namely 1D showed maximum tolerance till 1000 mg/l.

Extraction and quantification of EPS

The extracted EPS was quantified using phenol–sulphuric acid after 48 h. It was found that strain 1A, 1C and 1D produced 0.050 ± 0.0028, 0.0728 ± 0.0021, 0.0683 ± 0.002 mg/ml, respectively. Cr(VI) tolerant strains producing EPS of concentrations 356 and 548 mg/l by *Synechocystis* sp. BAS0672 and *Synechocystis* sp. BAS0670 have been reported by Ozturk et al. (2009). Quintelas et al. (2007) has also reported the polysaccharide production of three different strains namely *Bacillus coagulans* (CECT 12), *Streptococcus equisimilis* (CECT 926) and *Escherichia coli* (CECT 515) to be 9.19, 7.24 and 4.77 mg/g of biosorbent. A recent study (Sundar et al. 2011) shows the production of EPS with concentrations ranging from 0.059 to 0.063 mg/ml by chromium resistant strains isolated from chromium polluted tannery site in Vellore, India.

Characterization of the effective strain

The effective strain (1D) selected based on the MTC assay and maximum EPS production was used for further studies. The taxonomic identity of the selected strain was found out to be *Enterobacter cloacae* (showing 98% similarity in BLAST search to *E. cloacae*). The sequence size was 1425 bp. The phylogenetic tree of *Enterobacter cloacae* SUKCr1D (Genebank Accession Number: JF412828) is shown in Fig. 1. Previous reports (Rege et al. 1997; Sethuraman and Balasubramanian 2010) also emphasized the potential role of *E. cloacae* in the reduction of hexavalent chromium.

Growth profile of *Enterobacter cloacae* SUKCr1D

Figure 2 shows the growth profile of the selected strain *E. cloacae* SUKCr1D at different Cr(VI) concentrations

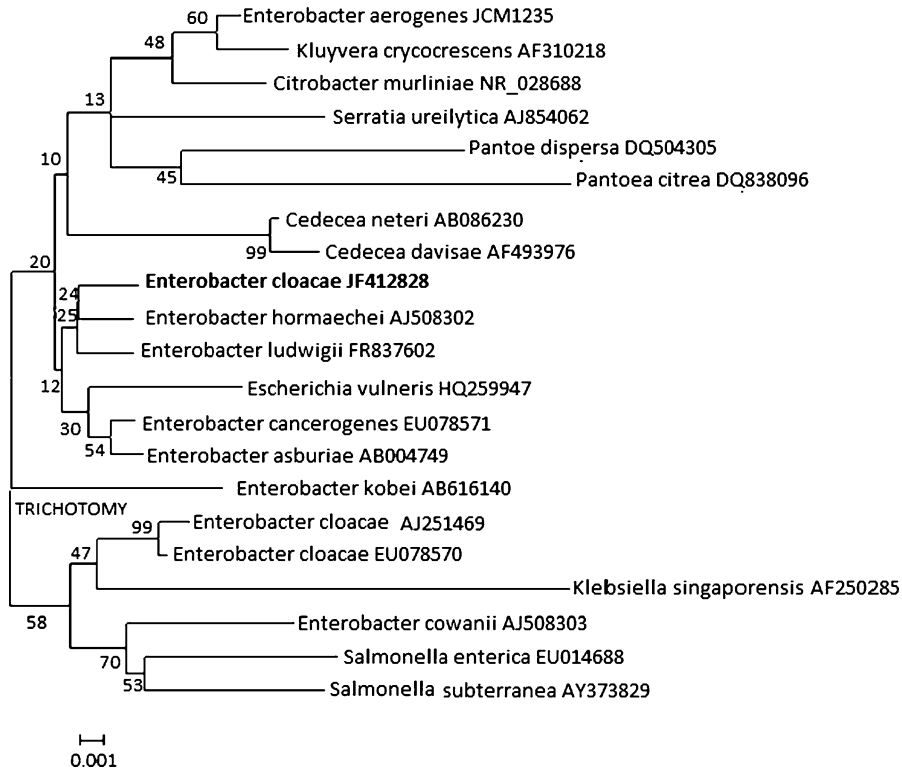

Fig. 1 Phylogenetic tree of *E. cloacae* SUKCr1D constructed by neighbor joining algorithm

(10, 50 and 100 mg/l) with 0 mg/l as control. The bacterial growth decreased with increase in Cr(VI) concentration in the media. It was found that 10 mg/l of Cr(VI) had a slight effect on the growth, but presence of 50 and 100 mg/l of Cr(VI) had considerable effect on bacterial growth. The lag phase duration was observed to be 2 h for 0 and 10 mg/l, 6 h for 50 mg/l and 8 h for 100 mg/l of Cr(VI) concentrations. The difference in lag phase durations at different Cr(VI) concentration is probably due to the time required for the organism to adapt to Cr(VI) present in the media. The specific growth rate was found to be 1.13 h^{-1}, 0.7157 h^{-1}, 0.2362 h^{-1} and 0.2013 h^{-1} for 0, 10, 50 and 100 mg/l, respectively. It has been elucidated that increase in Cr(VI) concentration in the media results in decreased specific growth rate and increased lag phase (Bae et al. 2000; Wei-hua et al. 2009).

Quantification of EPS at different chromium concentration

The kinetic study of EPS production carried out at various Cr(VI) concentrations (10, 50 and 100 mg/l) is shown in Fig. 3. At the end of the 12 h the highest amount of EPS (0.078 mg/ml) was produced at 100 mg/l Cr(VI) concentration by *E. cloacae* SUKCr1D in the range of concentrations tested. The control (devoid of Cr(VI)) produced only 0.0643 mg/ml. This concentration dependence shows that *E. cloacae* SUKCr1D may have produced more EPS in order to adapt itself to elevated concentrations of Cr(VI). However for particular time points the difference between EPS produced with respect to Cr(VI) concentrations was found to be statistically non-significant by Student's *t* test ($P > 0.05$).

The increased EPS production may play an important role in chromium tolerance. Ozturk et al. (2008) reported that there was significant toxic effect by Cr(VI) and reduced EPS production by *M. mesophilicum* up to 12 h. In this study, an increase in EPS production was observed due to the high tolerance of the indigenous mine isolate to Cr(VI). In a related work with tanning industry isolates, a similar increase in EPS production with increase in chromium concentration has been reported (Sundar et al. 2011).

The EPS production of *E. cloacae* SUKCr1D at various Cr(VI) concentrations (5, 10, 20, 40, 50 and 100 mg/l) were quantified after 3 days. It was found that EPS production saturates with increased chromium concentration and attains a constant value at the

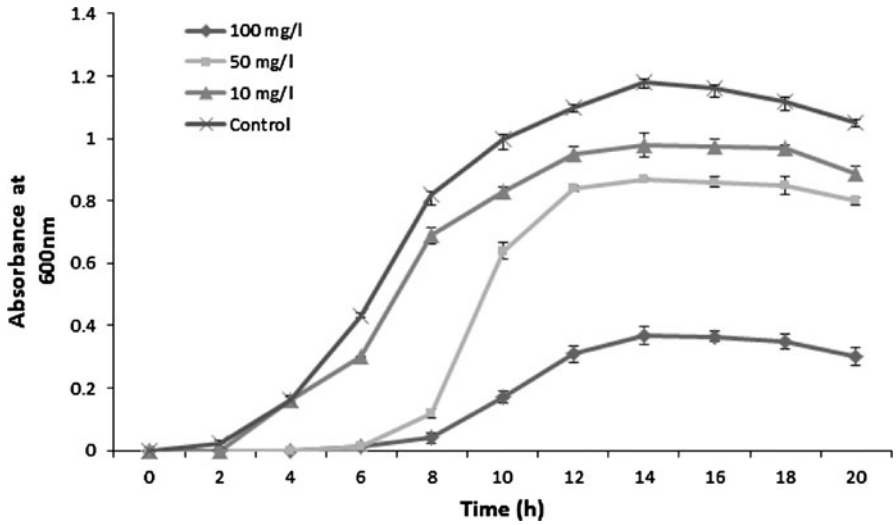

Fig. 2 Growth curve of *E. cloacae* SUKCr1D with different concentrations of Cr(VI), where the absence of Cr(VI) was taken as control. The standard error ranges from 0 to 0.029

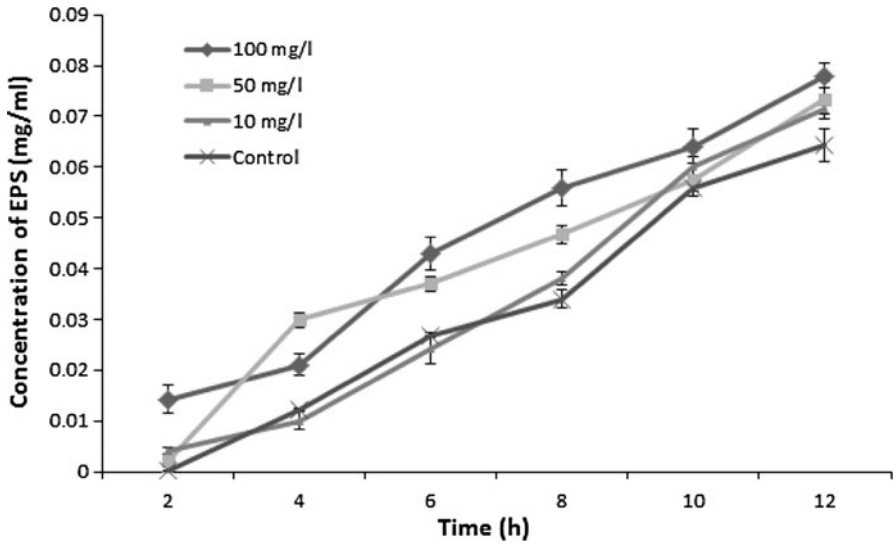

Fig. 3 EPS production for every 2 h at various Cr(VI) concentrations (10, 50 and 100 mg/l) with 0 mg/l as control. The standard error ranges from 0.00023 to 0.0036

end of 3 day (Fig. 4). Bonferroni's Multiple Comparison Test (Post Test) was performed after one-way ANOVA in order to compare the significance of EPS production at various Cr(VI) concentrations and was found to be non-significant.

Periodic study of ECP production

To study the role of ECP in chromium reduction the ECP production of *E. cloacae* SUKCr1D at various Cr(VI) concentrations (10, 50 and 100 mg/l) were quantified using Bradford assay (Ozturk et al. 2008). A decrease in ECP production was observed for different Cr(VI) concentrations as compared to control (Fig. 5). The decreased ECP production for different Cr(VI) concentrations significantly decreases the probability of its involvement in bioreduction of chromium.

Cr(VI) reduction by extracted EPS

Cr(VI) reduction by microorganisms occurs mainly by intracellular and extracellular mechanisms. In this study, the extracted EPS from the indigenous isolate was interacted with 10 mg/l Cr(VI) with an aim to find out the contribution of EPS alone in reduction of Cr(VI). The concentration of total chromium and hexavalent chromium in Sukinda mine sludge was found to be 9.64 and 1.56 mg/l, respectively. Das and Mishra (2010) has reported a similar total chromium

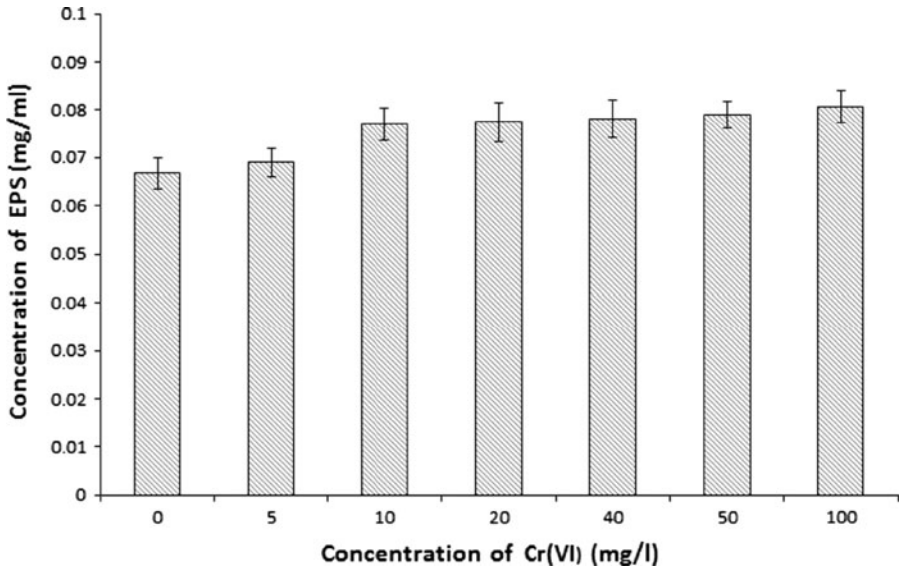

Fig. 4 EPS production of *E. cloacae* SUKCr1D at various Cr(VI) concentrations (5, 10, 20, 40, 50 and 100 mg/l) were calculated using phenol–sulphuric acid after 3 days. The standard error ranges from 0.0028 to 0.0041

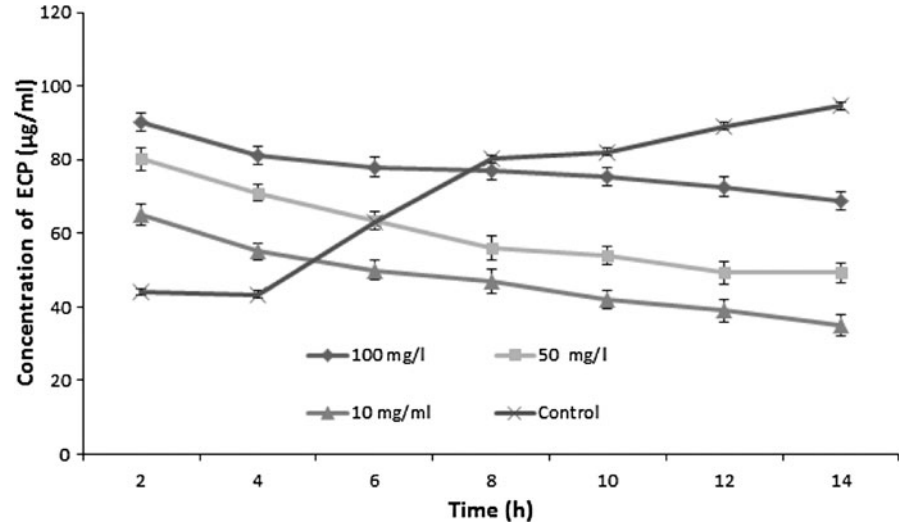

Fig. 5 ECP production of *E. cloacae* SUKCr1D at various Cr(VI) concentrations (10, 50 and 100 mg/l) were calculated using Bradford assay. The standard error ranges from 2.1 to 3.2

concentration range of 9 ± 12 mg/g of soil. In order to emphasize the application potential of this Bio-reduction study with respect to natural conditions prevailing at Sukinda mine site, a related concentration of 10 mg/l was selected for this study. After 24 h the final Cr(VI) concentration was observed to be 6.83 mg/l indicating a total reduction of 31.7%. Similarly the Cr(VI) reduction by *E. cloacae* SUK-Cr1D with growing whole cells was also studied. The initial and final concentration of Cr(VI) was 9.42 and 2.65 mg/l, corroborating the net reduction of 71.9%. The whole cells showed greater reduction as compared to the extracted EPS, indicating the involvement of chromium reductase enzyme playing a substantial role in the bio-reduction process (Cheung and Gu 2007).

In a similar study Xu et al. (2010) reported a removal percentage of Cr(VI) and total Cr by EPS to be 18.58 and 21.12%, respectively. Ozturk et al. (2009) has also reported a reduction percentage of 89% by whole cells of *Synechocystis* sp. BAS0672 after 7 days interaction. These results show that EPS played a specific role in Cr(VI) reduction. There are a few reports stating the reduction of Cr(VI) by various indigenous isolates stressing the role of biosorption (Rege et al. 1997; Wei-hua et al. 2009; Sethuraman and Balasubramanian 2010; Sundar et al. 2011). This

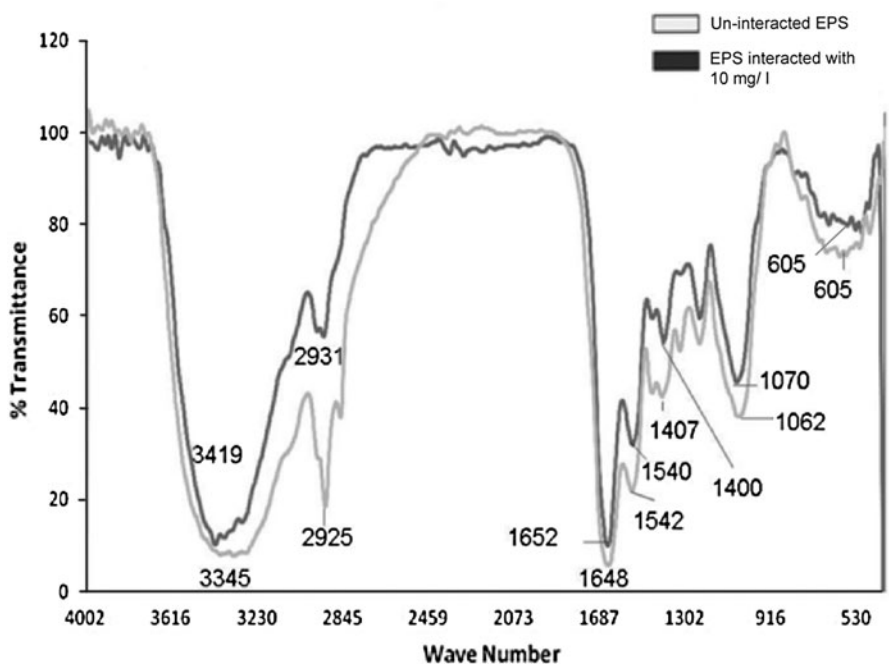

Fig. 6 FT-IR image of un-interacted and interacted EPS with 10 mg/l Cr(VI)

adsorption is mainly due to the presence of EPS which are secreted by the isolates. A recent study directly correlated the metal adsorption characteristics with the composition of the isolated bacterial EPS (Ozturk et al. 2009).

FT-IR analysis

The FT-IR spectra (Fig. 6) of Cr(VI) interacted and un-interacted EPS were performed to understand the possible EPS–metal ion interactions. Table 1 shows the corresponding assignments of IR frequencies for both pristine and Cr(VI) interacted EPS. The FT-IR spectra mostly consisted of hydroxyl, amino and carboxyl group owing to presence of carbohydrates and proteins from the EPS (Tapia et al. 2009).

At 3345 cm^{-1} N–H and O–H stretching vibrations from polysaccharides and proteins were observed in un-interacted EPS, which on interaction with Cr(VI) shifted to 3419 cm^{-1}. The bands specific to protein secondary structure: (C=O) stretching at 1648 cm^{-1} [amide (I)] and the N–H bending at 1542 cm^{-1} (amide (II)) showed a marginal decrease in intensity after interaction ruing out their active participation. A sharp

Table 1 FT-IR peaks of un-interacted and interacted EPS with Cr(VI)

Wave numbers (cm^{-1})		Assignment	Reference
Un-interacted	Interaction with Cr(VI)		
3345	3419	N–H and O–H stretching vibrations from polysaccharides and proteins	Das and Guha (2007)
2925	2931	CH_3 asymmetric stretching from lipids, proteins, polysaccharides	Kang et al. (2007)
1648	1652	Amide I (protein C=O stretching)	Das and Guha (2007)
1542	1540	Amide II (protein N–H bending and C–N stretching)	Kang et al. (2007
1407	1400	C=O stretching	Doshi et al. (2007)
1062	1070	C–O bond of polysaccharides	Dhal et al. (2010)
605	605	CH_2 vibrations of polysaccharides	Parikh and Crover (2005)

decrease in the band 1407 cm^{-1} corresponding to C=O stretching was observed in the interacted EPS. Doshi et al. (2007) also reported as role of carboxyl groups in chelating the Cr(III) thus helping in biosorption. The C–O bond of polysaccharide at 1062 cm^{-1} was found to decrease drastically indicating its involvement in interaction of Cr(VI) with EPS. The peak at 605 cm^{-1} corresponding to the CH_2 vibrations of the polysaccharide observed in the Cr(VI) un-interacted EPS underwent a nominal decrease in the intensity after the interaction of Cr(VI) with EPS. The difference in intensity of the peaks and a noticeable peak shift in the Cr(VI) interacted EPS (Table 1) may be due to surface chemical interactions of hexavalent chromium with the EPS.

Conclusion

Based on the MTC assay and maximum EPS production capacity, *E. cloacae* SUKCr1D was selected among three bacterial isolates from mining effluent sludge sample of Sukinda valley, Orissa, India. This indigenous isolate showed increase in EPS production in response to increased Cr(VI) concentrations in the growth medium. Possible surface interactions of EPS with chromium were proved through FT-IR results. In conclusion, this study brings out the specific contribution of EPS from chromium mine sludge isolate *E. cloacae* SUKCr1D towards bio-reduction of Cr(VI) employing chromium concentrations range relevant to the Sukinda mine site.

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