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Management of chromium induced oxidative stress by marine *Bacillus licheniformis*

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Abstract
The present study describes management of oxidative stress induced by heavy metal chromium in marine *Bacillus licheniformis* (NCBI GenBank Accession Number- HM194725). It is identified that the isolate reduces 10-1500 mg/L of Cr (VI) within 24-72 h. Existence of chromium reductase in growth medium implies the possibility of enzymatic reduction of Cr (VI) to Cr (III). Nevertheless, no correlation was observed between the real concentration of Cr (III) and the predicted concentration upon reduction of Cr (VI). Extracellular surface-active agent (biosurfactant) of the isolate was found responsible for the reduced level of Cr (III) in the solution which eventually provides tolerance to the cells towards hexavalent chromium and protects the cells from oxidative stress.

Keywords: Chromium reductase; tolerance; resistance; biosurfactants; oxidative stress.

Introduction

Studies on heavy metals and their environmental impact are always in the limelight of basic research. Although the requirement of heavy metal is at tracer level for various biochemical activities of all living organisms, generation of wastes with various oxidation levels of heavy metals poses severe hazard and toxicity. Some of the bacterial species exhibit high tolerance and resistance towards high concentration of heavy metals. The reason behind such resistance and tolerance behavior of microorganisms needs more elucidation because of the emergence of resistance species (Charyulu *et al*., 2009). Microorganisms of marine origin in general, thrive under extreme environmental conditions, which provide them enormous potential and tolerance (Das *et al.*, 2006).

Actually, most of the toxic compounds especially heavy metals, preferably follow the reduction pathway instead of oxidative pathway by native microbes, with a few exceptions, since the reduced forms are less toxic. Nies (1999) reported bacteria follow efflux system; accumulation; complexation and reduction mechanisms to tolerate heavy metal. Lawrence (2000) explained clustering of genes on a plasmid during stress conditions imparts resistance to organisms for their survival under multiple stresses. Baquero *et al.* (1998) reported mutation of genes is responsible for tolerance and multiplication of organisms. Bosa *et al.* (2007) reported relationship of heavy metal tolerance and antibiotic resistance of bacterial species.

Among heavy metals, environmental impact of chromium is at higher level, and the environmental behavior of chromium lies in its oxidation states (Losi *et al*., 1994). The higher oxidation state is more toxic (10-100 times) than lower oxidation state. According to the available reports, only 0.1 ppm of Cr (VI) is recommended for fresh water and marine life and 0.8 ppm for irrigation water, whereas for Cr (III), the level raised to 0.5-5.0 ppm regardless to fresh, marine, irrigation and drinking water.

Due to carcinogenic effect, attempts were made to remove Cr (VI) from soil, water and air (Ekenberg *et al*., 2005) and among various methods, *in situ* bacterial reduction attracts the scientific world and numbers of bacterial isolates were identified for their efficient reduction and detoxification. All the reports conclusively accepted the possibility and feasibility of bacterial species in reducing Cr (VI) to Cr (III) at varied concentration levels, which solely depend on genus and species levels. Most of the reports suggest more elucidation is still required for reduction as well as accumulation of Cr (III), nevertheless, all the species studied claims detoxification or reduction of Cr (VI). However, the major unknown factor of tolerance or resistance towards continuous accumulation of Cr (III) produced upon reduction of Cr (VI) is still unanswered. According to the reports, presence of chelating factors (Iyer *et al*., 2004) and

precipitations of Cr (III) complex (Fabiani *et al*., 1997) are the most possible reasons for the tolerance.

Microbes synthesize biosurfactants for interacting with neighboring cells and in most microbes these biosurfactants serve as cementing material, which further extends to develop into biofilm. The polysaccharides of biofilm contain uronic acid at high percentage level, which provides the polyanionic character, able to interact with heavy metals. Sutherland (2001) reported that ions specifically interact with exposed carboxylic acid groups of biosurfactant, which yield a network of macromolecules and further claims interaction of biosurfactant with proteins and micelles. However, the ionic radii of the cations are detrimental to their interactions with biosurfactant. In addition, interaction of metal ions provides rigidity to biosurfactant. Rickard *et al*. (2000) reported the binding of biosurfactant of aquatic organisms with metals and reports are available on accumulation of heavy metals by biosurfactant of microorganisms.

With regard to surfactants, both ionic and non-ionic forms play a major role in each part of human life (Lawrence and Rees 2000; Sabah *et al*., 2002) and vital in fundamental and applied sciences. These surfactants also find application in adsorption of heavy metals as reported by Tibor *et al*. (2002). Reports are available on remediation of subsurface chromium contamination using surfactants and enhancement of chromium removal from soil in the presence of surfactant and Xia *et al*. (1992) studied the entrapment of hydrophobic substances in the surfactant micelles, which helps in isolating them from the aqueous solution. The nature of the hydrophobic group is detrimental for its biodegradability. The *in situ* production of biosurfactant by microbial species exemplifies enormous advantages. Gnanamani *et al*. (2008) reported the possibility of vesicle formation in hydrocarbons mediated by the *in situ* production of biosurfactants.

Thus, complex formation of dissolved metals/metal ions with microbial products (surfactants) is an attractive and an alternative research in the field of remediation of heavy metals. The present study emphasizes reduction of chromium by marine microbial isolate followed by remediation of chromium accumulation and finally elucidates the reasons for their tolerance property.

Materials and Methods

Collection, isolation and identification

Marine samples, viz., water, sediments, mussels, shells and sand, collected from coastal areas of Tamil Nadu, stored at 4 °C until use. Both Zobell marine broth (ZMB) and Zobell marine agar (Hi Media, Mumbai) used for isolation. All the obtained isolates were examined for growth, biosurfactant production and biosurfactant activity and the isolates were selected based on their biosurfactant production and biosurfactant activity. Identification of the isolates was made by morphological staining, biochemical analysis, carbohydrate utilization, antibiotic sensitivity test, SEM and 16s rDNA analyses using universal primers 518F and 800R. The sequence obtained blasted in MEGABLAST and the coincidence of maximum percentage obtained submitted to NCBI sequence submission with HM 194725 as accession number.

Inoculum preparation

Inoculum for the experiments prepared by growing the isolate in ZMB using 24 h stock culture of marine *B.licheniformis* (HM 194725) individually and the cell density of 100 µl cell suspension with 0.6 optical density (OD) at 600 nm, employed for batch and continuous studies.

Biosurfactant extraction and purification

Marine *B.licheniformis* cultured individually in ZMB (100 ml in 1000 ml capacity conical flask) at 37°C for 72 h under shaking (200 rpm) condition. Followed by incubation, cell free supernatant obtained upon centrifugation at 10,000 rpm at 4 °C, treated with equal volume of ice-cold ethanol. The residual pellet obtained after centrifugation, dissolved in water, lyophilized, and examined for physical, chemical and surfactant properties.

Surfactant activity measurements

Qualitative surfactant activity of biosurfactant of marine *B.licheniformis* measured, by drop-collapse test and oil displacement method and the surface tension measurements made with GBX-3S tensiometer (DM).

Carbohydrate and protein content of bio-surfactant

Total carbohydrate content of biosurfactant of marine *B.licheniformis* determined using phenol-sulfuric acid method (Dubois *et al*., 1956). Protein content of biosurfactant examined using

Bradford reagent and protein concentration quantified using bovine serum albumin as standard.

Spectral analyses
UV-visible spectral analysis of biosurfactant measured using Shimadzu UV-2450 UV-visible spectrophotometer with the spectral range of 200–800 nm. For FT-IR analysis, 100-200 µl of sample directly mixed with KBr and measured the spectrum-using Spectrum one (Perkin-Elmer Co., USA model).

Chromium reductase assay
Chromate reductase activity of marine *B.licheniformis* made according to the procedure of Camargo *et al*. (2003). To 1.0 mL of l mM K_2CrO_4 in 600 µL of 0.2 M phosphate buffer (pH 7.4), added 200 µL of culture supernatant and incubated for 15 minutes at 37 °C. One unit of Cr (VI) reductase activity defined as the amount of enzyme convert 1.0 µM Cr (VI) per minute at 37 ˚C.

Batch experiments
Growth and chromium reduction property of marine *B.licheniformis* assessed by growing the isolate in 100 ml of pre-sterilized Zobell marine broth containing Cr(VI) at increasing concentration (0-2000 mg/L) in 1000 ml Erlenmeyer flask with 100 µl inoculum at 200 rpm at 37˚ C for 2 to 3 days. Hexavalent chromium (Cr (VI)) analyzed using diphenylcarbazide according to APHA (1989). Trivalent chromium (Cr (III)) in culture supernatant analyzed using chromazurol S at 590 nm according to Ksheminska *et al*. (2006).

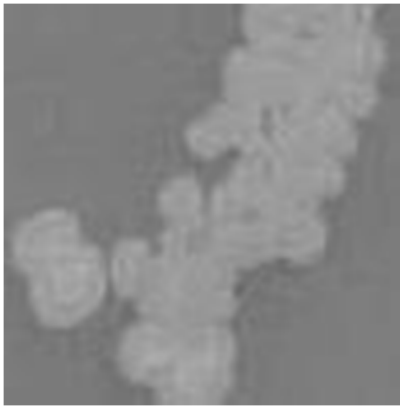
Continuous experiments
To 500 ml flask containing 50 ml of ZMB, added 20-mg/L concentration of filter sterilized Cr (VI) solution with 100 µl of inoculum and incubated at 37˚C under shaking condition (200 rpm) for 24 h. Followed by the determination of Cr (VI) and Cr (III) concentrations at 12 h interval, an additional 20mg/L concentration of Cr (VI) supplemented to the same medium and continues the incubation to next 24 h. The process of addition of Cr (VI) to the same medium continued at 24 h interval until we observe the complete reduction of applied Cr (VI). Once reduction of Cr (VI) is stopped, incubation proceeded without any further addition, but analyses of Cr (VI) and Cr (III) continued until the concentration of Cr (VI) approaches zero.

Reduction of Cr (VI) using chromate reductase
Different concentrations (0-500µl of 1mM) of Cr (VI) incubated at 37 ˚C at different time points (5 minutes to 2 h) with varied chromate reductase activity (2.0-17.5 U/ml) obtained as culture supernatant of 24 h culture of marine *B.licheniformis*. Analysis of Cr (VI) and Cr (III) carried out after inactivating the enzymes at respective time points.

Scanning electron microscope (SEM) and energy dispersive X-ray spectrometer (EDAX)
The existence of metal ion on the surface of the bacterial biomass determined using energy-dispersive X-ray (EDAX) spectroscopy coupled with SEM. Biomass obtained at the end of the continuous addition experiments, processed according to standard methods and surface morphology of the gold coated samples visualized and photographed.

Results
Out of 200 marine isolates obtained from coastal areas of Tamil Nadu, only 18 isolates showed growth in the presence of Cr (VI), nevertheless, marine *B.licheniformis* exhibits growth and tolerance at higher concentration of Cr (VI). Biochemical and molecular analyses of the isolate reveals that it belongs to *Bacillus* genera, ME-001ESS exhibits creamy white colony without any pigment production (Figure 1).

Figure 1: Growth of marine *B.licheniformis* in Zobell marine agar plate.



Analysis of 16s rDNA sequence of ME-001ESS showed 99% similarity with *Bacillus licheniformis*. Marine *B.licheniformis* grows well in the Zobell media with extended log and stationary phase with appreciable biosurfactant production. The physical and chemical characteristics of biosurfactants are summarized in Table 1.

Table 1

S. No.	Characteristics	*Bacillus licheniformis*
1	Physical appearance	Milky white semisolid
2	Phospholipids (µg / ml)	7.6
3	Protein (µg / ml)	80.28
4	Carbohydrate (µg / ml)	2.65
5	Surface tension (mN/m)	31.1

Interestingly, the obtained biosurfactant exhibits surfactant activity and reduces surface tension of water to 30 ± 4 mN/m. Antibiotic sensitivity assay reveals that marine *B.licheniformis* is resistant towards Penicillin, Methicillin, Erthromycin and Bacitracin.

Figure 2 (a & b) illustrates UV-visible and FT-IR spectral analyses of biosurfactant of marine *B.licheniformis* and suggests no distinct peak in the visible region but high absorption peak in the UV region (200-230nm) followed by a shoulder at 250- 280 nm. However, FT-IR spectrum showed number of characteristic peaks at 3500 – 3300, 2950 - 2850, 1750 – 1710, 1660 – 1600, 1470 – 1400, 1260 – 1200, 1100 – 1000, 860 – 800 cm^{-1} corresponding to -OH; -CH; -C=O; C=C; COO–; C –C–O; –C–O; any sugar moiety, respectively.

Figure 2: (a) UV-visible (b) FT-IR spectral analysis of biosurfactant of marine *B.licheniformis*.

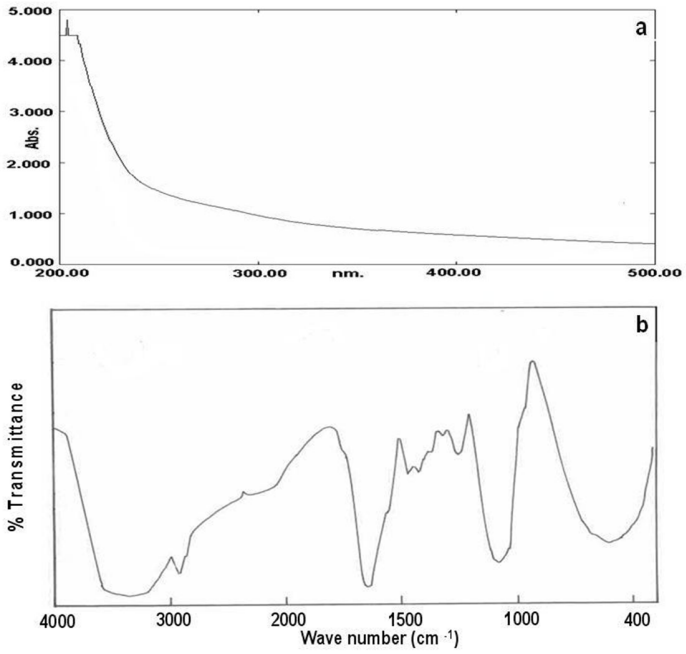


Figure 3 depicts growth of marine *B.licheniformis* with and without hexavalent chromium at 200-mg/L concentrations at different time intervals of exposure. No significant change was observed in the growth of marine *B.licheniformis* in the presence of Cr (VI) compared to without Cr (VI).

Figure 3: Growth of marine *B.licheniformis* with increasing concentration of Cr (VI) after 24 h of incubation at 37° C.

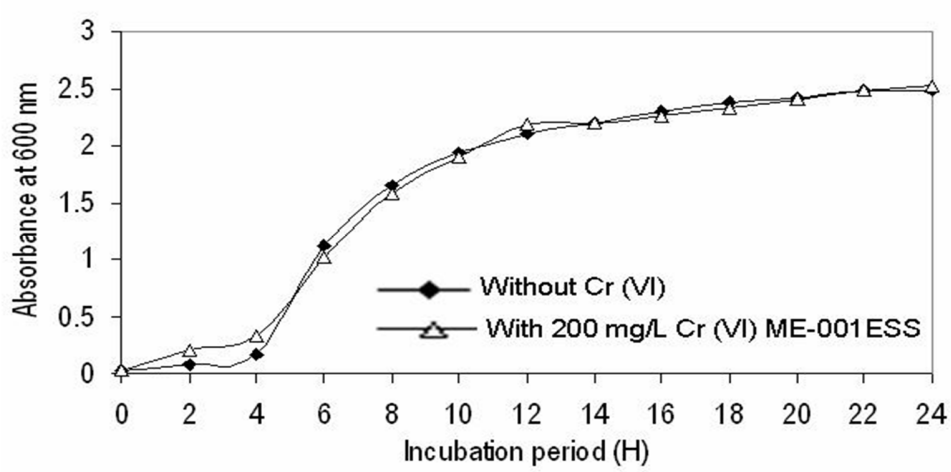
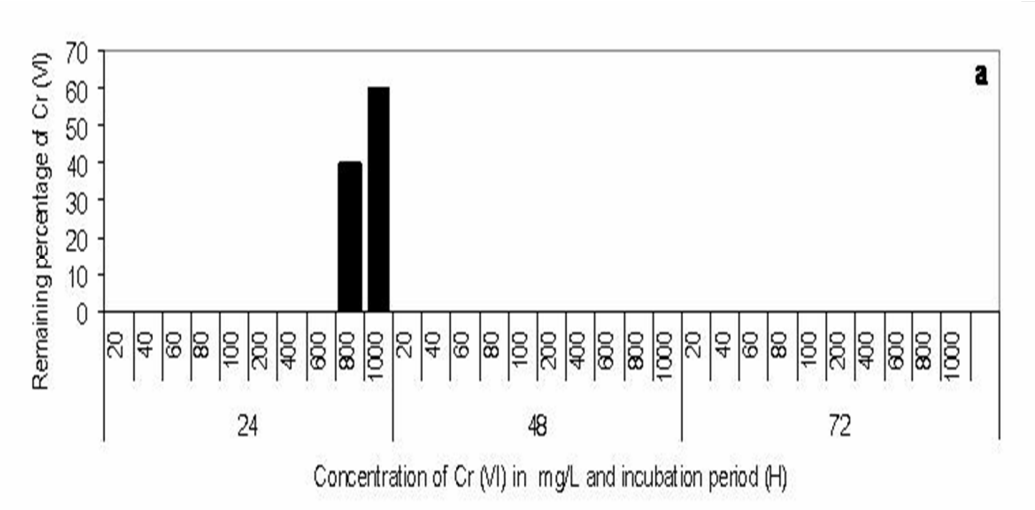


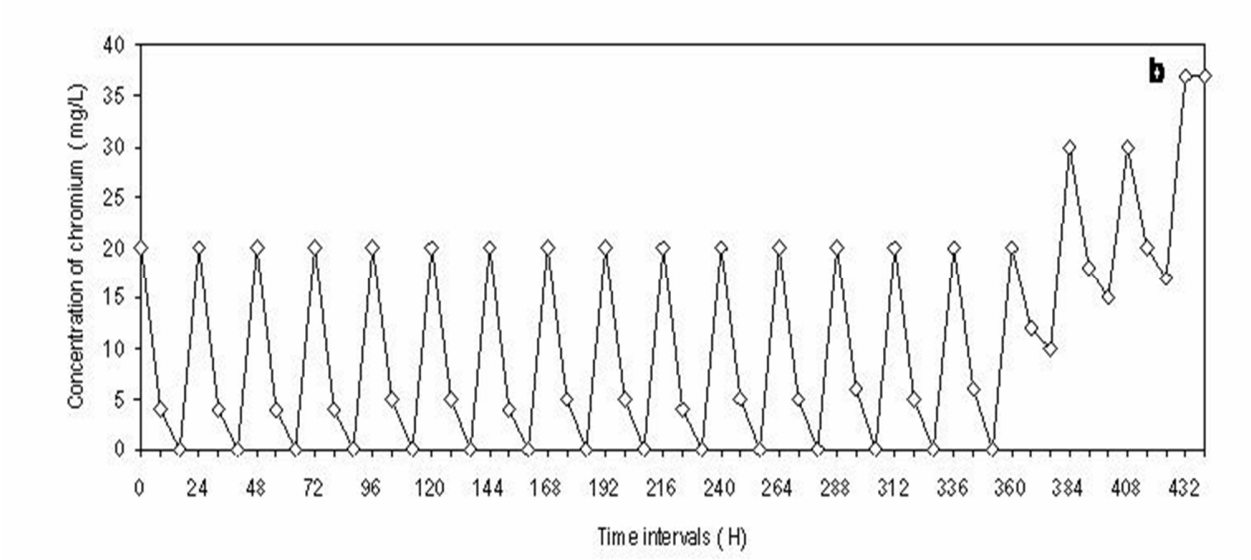
Figure 4 (a) illustrates Cr (VI) removal pattern of chosen isolate with increasing concentration (10-2000 mg/L) of Cr(VI) at 24, 48 and 72 h of incubation under batch mode. Marine *B.licheniformis* reduces more than 80% of the applied Cr (VI) of 400-mg/L concentrations with in 24 h of incubation and more than 85% with respect to 800 and 1000 mg/L concentration after 48 h respectively. Nevertheless, after 72 h of incubation the reduction is only less than 85% with 1500-2000 mg/L concentrations.

Figure 4a: Reduction of Cr (VI) by marine *B.licheniformis* at varying concentrations of Cr (VI) and at different time points under *in vivo* conditions.



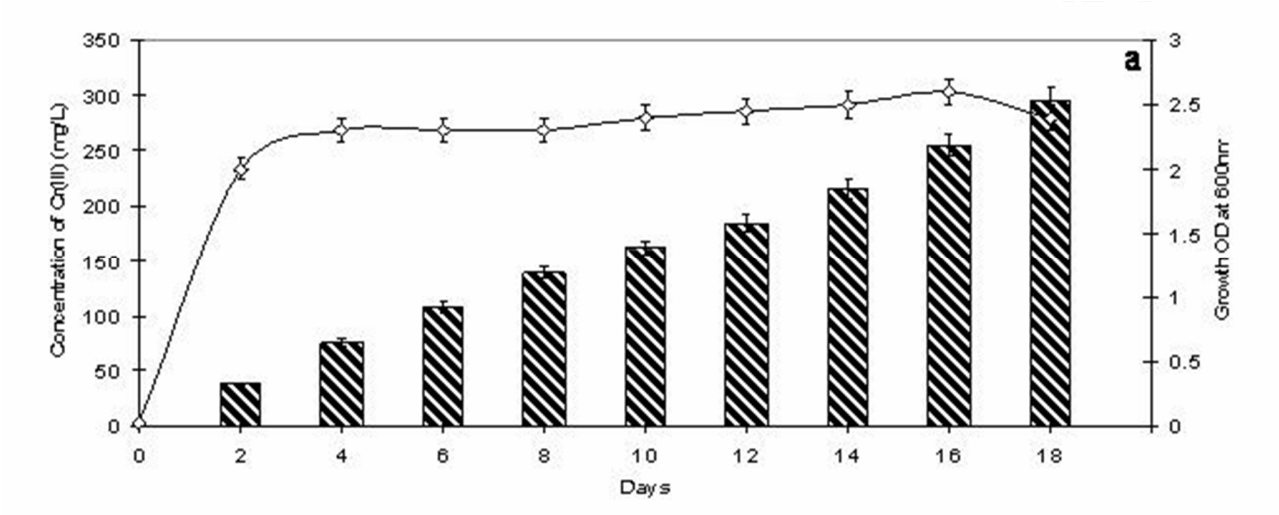
Based on the results obtained from the batch studies, we carried out a continuous study in which each flask receives 20-mg/L concentration of Cr (VI) at 24 h intervals till 384 h for the marine *B.licheniformis* and reduction of Cr (VI) observed only up to 360 h of incubation and nil reduction observed even if the addition of Cr (VI) stopped Figure 4(b).

Figure 4b: Reduction of Cr (VI) in continuous (repeated) addition of Cr (VI) at 20 mg/L at 24 h by the marine *B.licheniformis*.



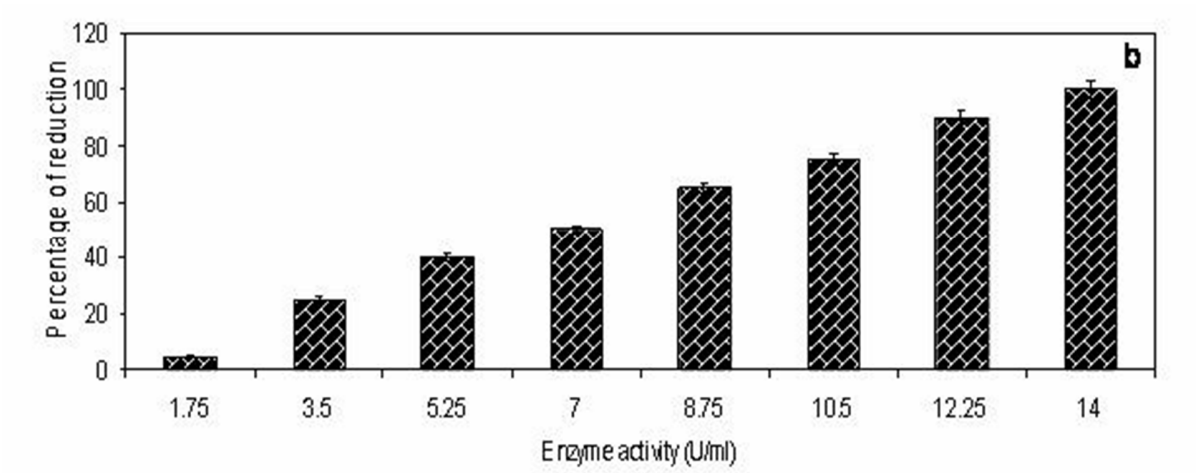
To confirm Cr (VI) reduction in continuous addition, analyses of Cr (III) and growth of organisms was made at 24 h intervals. As shown in figure 5(a), Cr (III) concentration increases with respect to incubation period and the maximum Cr (III) concentration and increase in Cr (III) concentration was observed until day 18. However, growth pattern showed a difference with an alternative log and stationary phase of growth and showed a decline from day 18 onwards.

Figure 5a: Concentration of Cr (III) and growth of marine *B.licheniformis* during continuous addition of Cr (VI) with respect to increase in incubation period.



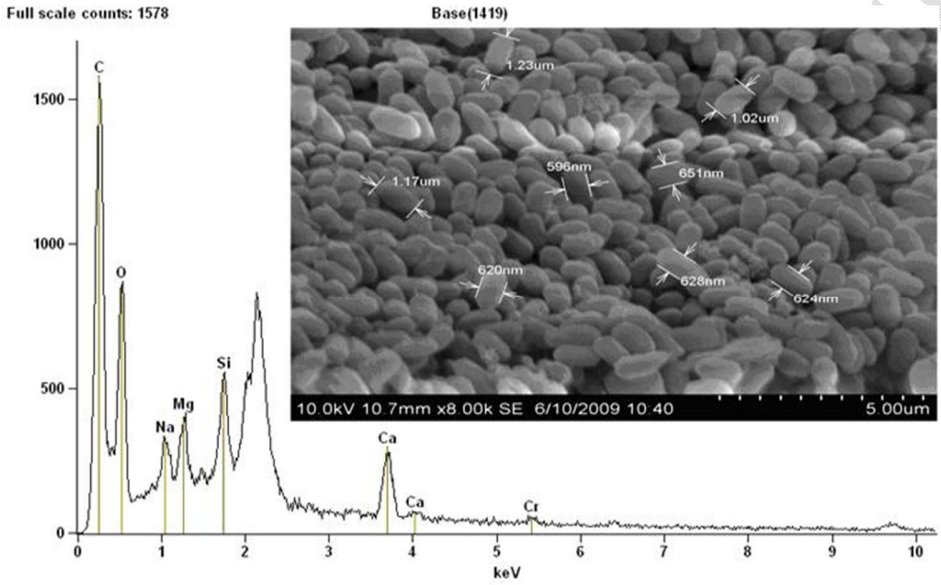
In order to ascertain the reduction capacity of the isolate and to evaluate the reduction hypotheses, an *in vitro* study was carried out using extracellular enzyme and biosurfactant obtained after 24 h of growth. Analyses of extracellular fluid exhibit maximum chromium reductase activity of 17.5 U/ml ME-001ESS. Chromate reductase activity of 12 – 14 U/ml completely reduces 20mg/L of Cr (VI) (Figure 5b) and suggests, reduction of Cr (VI) mediated through extracellular enzyme of the chosen isolate.

Figure 5b: Percentage reduction pattern of Cr (VI) in the presence of chromium reductase at different activity levels with respect to the marine *B.licheniformis*.



Since the isolate exhibits appreciable biosurfactant production, there may be a possibility of Cr (VI) reduction through biosurfactant also. Hence, we conducted separate experiments using biosurfactant of marine *B.licheniformis* on Cr (VI) reduction. Results reveal that biosurfactant (25 mg) of the species completely reduces 200 mg/L Cr (VI) within 30 minutes of exposure. Scanning Electron micrograph and EDAX analyses of isolate marine *B.licheniformis* after 28 and 18 days of Cr (VI) exposure reveal the magnitude of exocellular components (Figure 6) without any aggregation of cells.

Figure 6: Scanning Electron Micrograph and EDAX analyses of marine *B.licheniformis* after continuous exposure to Cr (VI) for the period of 28 days.



Discussion

Microorganisms in general, play a significant role in bioremediation of heavy metal contaminated soil and wastewater. So far, some of the terrestrial microorganisms (*Bacillus*, *Enterobacter*, *Escherichia*, *Pseudomonas,* yeasts and fungi) transform Cr (VI) effectively. Seng and Bielefeldt (2002) reported chromium (VI) biotransformed enzymatically to Cr (III). Some bacterial isolates are resistant to chromium and can be exploited to bioremediate toxic Cr (VI) contaminated environment. According to Polisak *et al*. (2009), chromium tolerance or resistance mechanism of microbes is of particular importance for bioremediation and wastewater treatment technology. Nevertheless, no detailed experimental evidence for the reasons behind the resistance or tolerance is available. Till date, (i) plasmid mediated (Nies *et al*., 1998); (ii) genetically mutated (Miranda *et al*., 2005); (iii) bioadsorbtion of the cells (Ozdemir *et al*., 2004); (iv) bioaccumulation (Preetha and Viruthagiri, 2007); and finally (v) biotransformation (Guha *et al*., 2001) are the rationale suggested for the tolerance exhibited by the organisms.

It is well known that, whenever there is a stress, most of the microorganisms exhibit an explicitly different mechanisms, which provide them high tolerance and resistance. In general, the tolerant species may transform the toxic components to non-toxic via enzymatic or non-enzymatic pathway and finally utilize the transformed components as nutrients or ignored them after complexing with other components. Nevertheless, this kind of transformations could not be expected with all microbes and the contaminants. As shown in the results, marine

isolate *B.licheniformis* grows well in the presence of Cr (VI) and the minimum inhibitory concentration 225 mg/L observed in the present study is high compared to other microbial species (Srinath *et al*., 2002). Further, the tolerance limit of 1500 mg/L of Cr (VI) observed for marine *B.licheniformis* is also high compared to *Bacillus* sp (Camargo *et al*., 2003; Viti *et al*., 2003) and *Cornynebacterium hoagie* (Viti *et al*., 2003).

When chromium as Cr (VI) is applied continuously at the rate of 20 mg/L concentration at 24 h intervals up to 18 – 28 days, it immediately reduced to Cr (III) with out affecting the growth. Thacker and Madamwar (2005) and Desai *et al*. (2008) reported reduction of Cr (VI) by *Ochrobactrum* sp and *Bacillus* sp upto 3- 5 days respectively. Further, results on Cr (VI) reduction in the presence of extracted chromate reductase reveal that Cr (VI) reduced to Cr (III) at 12-14 U/L of chromate reductase activity. Thacker *et al.* (2006) reported reduction of Cr (VI) by chromium reductase of *Providencia* sp. In addition, reduction is also encountered by the presence of biosurfactant.

These results suggests, Cr (VI) reduced completely by the chosen isolate at concentration more than 1000 mg /L under batch conditions after 48 h and at cumulative concentration 320 – 460 mg /L under continuous additions for 16 to 25 days. Chromium reduction by the isolate may be mediated through (i) exocellular chromium reductase; (ii) biosurfactant; (iii) persistence of biosurfactant activity and finally (iv) unspent nutrients or the oxidized form of nutrients or minerals in the growth medium. However, it is uncertain which one took part in the reduction process of Cr (VI) as far as the present study is concerned.

Followed by reduction, we expected the reduced form of chromium (VI) as insoluble Cr (III) complexes. However, we found presence of Cr (III) in the solution and in free form as evidenced through EPR analysis (Figure not shown) and only a meager percentage of Cr (III) in the cells as evidenced through EDAX (Figure 6). These observations further emphasize the insoluble Cr (III) may interact with biosurfactants accessible in the aqueous medium. The hydrophobic groups in the biosurfactant have opposite charges to that of Cr (III), which makes the association to develop into a micelle or reverse micelle. If the biosurfactant produced is in ionic form in the aqueous environment, we may expect that the hydrophobic tail phasing outside and the hydrophilic head orient towards inside, resulting with the charged environment, and when Cr (III) starts accumulating as a result of reduction of Cr (VI), there may be a possibility of ionic exchange which leads to fully charged micelle formation. The charged micelle further interacts with biosurfactant and immobilizes the Cr (III) in the matrix. This hypothesis further authenticated with the results of SEM and EDAX analysis of the cells after exposed to Cr (VI) environment for the period of 18 – 28 days. Thus, we like to conclude the reduced Cr (III) initially interact with biosurfactant followed by binding with biosurfactant and this nature of binding of Cr (III) protects the bacterial cells upon direct contact with the metals and thus ignored by the cells. In other words, when Cr (III) immobilized by biosurfactant, the biosurfactants activity of biosurfactant, further solubilizes the Cr (III) and makes it available as nutrients. This could be the reason why in our study we observed restoration of growth followed by repression.

However, the present hypothesis raises some questions viz., (i) whether we can expect the similar kind of bindings with respect to all the heavy metals; (ii) If the bacterial species are not exhibiting biosurfactant production, whether any external addition of biosurfactants will bring the same kind of protection to the cells; (iii) what is the level of interactions of biosurfactants with Cr (III); (iv) whether the binding of Cr (III) is mediated by biosurfactants followed by interaction with biosurfactants.

Further, in the present study, biosurfactant production reduces the rigidity in microbial matrix, results with the sloughing off the film, and thus we have observed only scarce level of very thin flexible film in the SEM pictures (Figure 6). Available reports suggest the biosurfactants are involved in the horizontal transfer of biosurfactant from one bacterial species to another and its production enables the cell components with in the biofilms to solubilize and make it available. Further, the interaction of biosurfactants with Cr(III) may be explained as initial interaction of Cr (III) with the hydrophobic head group of biosurfactants followed by further chelation with biosurfactant. In other words, the initial bindings of Cr (III) with biosurfactant further interact with biosurfactant results with normal micelle or reverse micelle. Otherwise, the interacted chromium (III) and binds with biosurfactant as monolayer followed by monolayer formation.

The above-summarized illustration emphasizes biosurfactant and the biosurfactants play a major role in protecting the marine bacterial cells from the toxic heavy metal load. However, it has been explained that heavy metal tolerance of bacterial species is proportional to the existence of antibiotic resistance (Bosa *et al.,* 2007) and in our study, we found the chosen isolate though originates from marine sediments, exhibits resistance towards penicillin, methicillin, erythromycin and bacitracin.

Conclusion

Remediation of heavy metal chromium by biological methods is always an attractive process, however, limited with microbial species. The present study illustrates bioremediation of chromium (hexavalent) by potential marine isolate. The remediation process carried out by the isolate is identified: (i) through exocellular enzymatic reduction; (ii) interaction with biosurfactant and finally (iii) through transformation of the reduced chromium as available nutrients by biosurfactants. These three agents mediate the remediation process, keep the cells active all the time, and provide tolerance and resistance towards high concentration of hexavalent chromium.

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