

Bioreduction of Cr (VI) by Biosurfactant Producing Marine Bacterium *Bacillus subtilis* SHB 13

T H Swapna, N K Papathoti, M Y Khan, G Reddy and B Hameeda*

Department of Microbiology, University College of Science, Osmania University, Hyderabad 500 007

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Heavy metal tolerant *Bacillus subtilis* SHB 13 was isolated from marine source and characterised for biosurfactant activity. Selected strain SHB 13 was able to tolerate 800 ppm nickel (Ni), 1000 ppm chromium (Cr), 3000 ppm lead (Pb). Biosurfactant produced by this strain was surfactin, as characterized by FT-IR spectrum and further confirmed by molecular characterization and gene length was found to be 675 base pairs. *B. subtilis* SHB 13 was able to reduce 98% of 100 ppm Cr within 72h at optimized conditions of pH 7, temperature 37°C and 4% NaCl concentration. Surfactin (10mg/ml) produced by SHB 13 was efficient in removing 74 % Cr (VI) within 72h when analysed with AAS and its role in chelation was elucidated by FTIR, XRD and SEM equipped with EDS.

Keywords: Biosurfactant, Bioremediation, *Bacillus subtilis*, Surfactin.

Introduction

Heavy metal pollution is observed as one of the most important environmental concerns, mostly due to anthropogenic activities like metal smelting, agriculture waste disposal, release of industrial effluents, e-waste etc¹. Chromium is chosen as one of the model hazardous heavy metals, considered to be second most toxic heavy metal, found mostly in tanneries². This heavy metal when consumed causes several health effects such as mental disorders, spasms, geno-toxicity etc³. In nature, Cr is commonly found in the form of Cr (III) and Cr (VI) and it is reported that Cr (III) is immobile essential micronutrient for human beings and animals for metabolism of macronutrients, whereas Cr (VI) is observed to be easily soluble, toxic and bio-available in nature⁴. Environment protection agency has recommended permissible Cr (VI) limit for drinking water and domestic usage to be less than 50µg/l. Due to high solubility and lower concentrations of Cr (VI) in soil, treatment of this metal ion has become tedious and indispensable⁵. Another important aspect of concern is removal of chromium in presence of high salt (NaCl) concentration, as observed in tanneries⁶. Physical and chemical treatment methods have been employed for Cr (VI) treatment, which pose disadvantages like

high operation cost and hazardous by product accumulation⁷. Biological methods practiced regularly are bioleaching, biosorption, immobilization, enzyme catalysed transformations, phytoremediation and application of microbial surfactants. Biosurfactants are surface active agents produced by varied group of microorganisms, with different structures such as glycolipids, lipopeptides, lipopolysaccharides and poly proteins. Microbial surfactants being non-toxic possess emulsification, foaming, dispersion properties and can be safely used for bioremediation of heavy metals, polyaromatic hydrocarbons and removal of oil spills etc⁸. Previous studies report the use of rhamnolipid produced by *Pseudomonas* sp. in Cr (VI) reduction from contaminated soil and water and there are other reports on use of surfactin for removal of Pb, Cu and Zn⁹. However there are no reports on surfactin and its role in Cr (VI) reduction. Hence, the present work was focussed to explicate the role of surfactin from marine *Bacillus* strain for reduction of Cr (VI).

Materials and methods

Chemicals used throughout the study were of analytical grade and metal salts used for Ni were NiSO₄.6H₂O, Cr were K₂Cr₂O₇, Pb were [Pb (CH₃COO)]₂ (Hi-Media).

Isolation and screening for biosurfactant producing bacteria

Soil, sludge and sewage samples were collected from different places of India such as Telangana, Kerala, Tamil Nadu and West Bengal and

*Author for correspondence
E-mail: drhami2009@gmail.com

enrichment cultured for a month in Bushnell-Hass (BH)¹⁰ mineral salts medium comprising (g/l): MgSO₄(0.2), CaCl₂(0.02), KH₂PO₄(1), K₂HPO₄(1), NH₄NO₃(1), FeCl₃ (0.05); trace element solution g/l [FeSO₄ (2), MnSO₄ (1.5), [(NH₄)₆Mo₇O₂₄] (0.6)], 1 ml/l, amended with 0.5 % each poly aromatic hydrocarbon (PAH) such as naphthalene (Naph), dibenzothiophene (Dbt), anthracene (Anth) and phenanthrene (Phe) as sole carbon source. Samples were collected from the enriched flasks, at an interval of one week and plated onto nutrient agar (NA) medium amended with 2 % PAH (Naph, Dbt, Anth, Phe) or 100 ppm of heavy metals (Cr, Pb and Ni). Bacterial colonies with biosurfactant production property were selected, purified and preserved as glycerol stocks with suitable label.

Test for biosurfactant activity

Biosurfactant activity was characterized based on below assays Biosurfactant production of the selected colonies was characterised by Haemolytic activity¹¹, Cetyl-trimethyl ammonium bromide (CTAB) agar plate assay¹², Lipase, esterase¹³, oil displacement¹⁴, drop collapse assay¹⁵ and surface tension reduction property¹⁶. Emulsification index (EI %) and emulsification activity (EA) was performed using standard methods described^{17, 18} and emulsification units calculated as below:

1 Emulsification unit = 0.01x dilution factor

Selection of heavy metals and PAHs tolerant bacteria

Nutrient agar (NA) plates and nutrient broth (NB) tubes were supplemented individually with heavy metal salt solution ranging from 100 to 3000 ppm concentrations and PAH ranging from 10mg/l to 500mg/l. Plates and tubes were inoculated with 100µl of overnight grown bacterial culture, incubated at 37 °C for 24h and observed for presence of growth.

Identification of efficient biosurfactant producing bacterial isolate

Based on biosurfactant production and metal tolerance, potential isolate SHB 13 was selected and identified by morphological, cultural and biochemical characteristics as per Bergey's manual of Systemic Bacteriology¹⁹. Molecular identification was done by 16S rRNA sequence and phylogenetic tree was constructed using Mega 4 software. 16S rRNA sequence was submitted to EMBL nucleotide sequence submission and accession number received.

Biosurfactant production and extraction

Overnight culture of SHB 13 at 2% (v/v) was inoculated into 1 litre BH medium amended with 2% sucrose weight/volume (w/v) as carbon source in a 2 litre Erlenmeyer flask and incubated at 37 °C for 48h²⁰. Culture broth was centrifuged at 10,000 rpm for 20 min and supernatant was used for extraction of biosurfactant using chloroform and methanol²¹.

Characterization of biosurfactant

Fourier Transform Infra red spectroscopy (FTIR)

FTIR was used to detect functional groups and chemical nature of the extracted compound²². Lyophilized active dry sample was crushed with KBr into a thin pellet with a pressure of 5-6 tons cm⁻² with the help of hydraulic press and analysed on Shimadzu UV FTIR spectrometer in the wave number range of 4000-400 cm⁻¹.

Molecular characterisation of surfactin synthesis gene

Characterization of surfactin gene in bacterial isolate SHB 13 was characterized using *srf* gene specific primers. *B. subtilis* MTCC 2423 was also used as reference strain for identification of surfactin gene. Genomic DNA of *B. subtilis* MTCC 2423 and SHB 13 was isolated using bacterial genomic DNA mini kit procured from Xcelris genomics, Ahmedabad. *Srf* gene specific primers were designed using primer3 program, primer sequence accomplished were FP: 5'TTTACTCATACTA CGTCAAC3', RP: 5'GTGTATTAAGAAATTCG AGC3'. *Srf* genes of SHB 13 were amplified using PCR (Bio-rad). PCR products were analysed on 0.8 % agarose gel along with 100 bp DNA marker and observed using gel doc.

Effect of pH, temperature and NaCl on bio-reduction of chromium

Chromium reduction by the selected *B. subtilis* SHB 13 was studied at various pH (6-10), temperature (25- 45 °C) and NaCl (2-10%) by inoculating 2% (v/v) overnight culture into NB medium (100ml) amended with chromium (100 ppm) and incubated for 48h. Samples were drawn at regular time intervals, centrifuged (10,000 rpm, 5 min) and supernatant was analysed for residual chromium concentration using diphenyl carbazide (DPC) assay²³.

Metal chelating activity of biosurfactant

Biosurfactant (surfactin) was extracted from 48h fermented BH medium and 10mg/ml (10mM Tris

buffer; pH 7.2) concentration solution was prepared, amended with 100 ppm Cr solution and incubated in shaker incubator for 72h. Samples were drawn every 12h and these solutions were centrifuged to separate metal biosurfactant complex (precipitate) formed and supernatant containing unbound metal ions and analysed using AAS.

X-Ray diffraction (XRD) study

Surfactin and Cr complexed sample was dried in speed vacuum and metal analysis was carried out with a Bruker AXS D4 endeavor diffractometer operating with Cu K α incident radiation at 40 KV and 30 mA. XRD scan was done from 10° to 80° and the diffraction patterns were recorded over a 2 θ range.

FTIR study for metal biosurfactant interaction

In 250 ml Erlenmeyer flask, 100 ml BH medium amended with 100 ppm of (Cr) (v/v) was inoculated with 2% (v/v) bacterial culture and incubated for 72h, 37 °C at 150 rpm. Surfactin was extracted from fermented broth using solvent extraction method (as mentioned above) and analysed by FTIR spectrometer to observe for change in chemical nature of the biosurfactant upon metal treatment. Surfactin extracted from BH medium without amendment of Cr served as control

Scanning electron microscopic and energy dispersive spectroscopy (SEM-EDS) studies

Over-night grown culture pellet of *B. subtilis* SHB 13 was suspended in Tris buffer amended with Cr (100ppm) and kept in shaker incubator for 72h, centrifuged to get cells with Cr. Cell pellet without Cr treatment were also processed which served as control. Surfactin (10mg/ml) metal binding

activity was also observed using this technique to detect interaction of biosurfactant with chromium. Each sample was processed for SEM-EDS observation²⁴. Note: *Bacillus subtilis* MTCC 2423 was used as reference strain for comparative studies for screening and surfactin characterization. All experiments were conducted in triplicates, thrice on different occasion. Values were calculated using two way ANOVA.

Results and Discussion

Analysis for biosurfactant production

A total of 270 bacterial isolates obtained from different source samples were screened for biosurfactant production and seven isolates were selected for preliminary study using qualitative and quantitative tests (Table 1). Beta haemolysis on blood agar medium was observed for the isolates SHB 13, 70 and 101 indicating production of surface active molecules. In CTAB test, SHB 70 and SHB 101 bacterial isolates were positive showing dark blue halo zone—indicative of glycolipid production. It was observed that SHB 13, 70, 86, and SHB 101 isolates showed highest EI value of 80-81 % within 48 h of incubation, which is comparable with earlier reports²⁵. Isolate SHB 13 was found to be having highest emulsification activity of 245 EUs which is significantly more than the reported marine bacteria having emulsification activity in the range of 1.4-213 EUs¹⁸. The bacterial isolates in our study were able to reduce surface tension from 71 to 25mN/m. Among the 7 bacterial isolates, SHB 13 isolated from Kovalam beach, Kerala showed highest reduction in surface tension (25mN/m) (Table 1). Reduced surface tension indirectly indicates production of biosurfactant by the studied isolates²⁶.

Table 1—Qualitative and semi quantitative assays to test biosurfactant activity and metal tolerance shown by isolated bacteria

Isolate	BA	CTAB	TBA	DC	OD	EA (EU)	EI %	ST mN/m	Maximum tolerance of metal ion (ppm)		
									Ni	Cr	Pb
SHB 13	++	-	+	++	++	245±0.76	80±0.76	25±0.28	800	1000	3000
SHB 70	++	+	+	+	+	227±3.2	80±1.3	30±1.5	600	500	2800
SHB 86	+	-	+	+	+	227±0.76	80±1.32	30±0.76	500	500	2500
SHB 89	+	-	+	++	+	230±1.25	81±1.60	32±0.28	500	500	2000
SHB 95	+	-	+	++	+	231±1	80±0.5	33±0.76	500	500	2000
SHB 98	+	-	+	+	+	227±1.15	73±1	35±0	600	500	2000
SHB 101	++	+	+	++	+	233±0.5	80±0.76	27±0.57	700	800	3000
<i>B. subtilis</i> MTCC 2423	+	-	+	++	+	222±0.76	80±0.28	30±0.18	100	50	500

Tolerance to heavy metals and PAH

All seven selected bacterial isolates showed metal ion tolerance in the range of 100 to 3000ppm (Table 1). Simultaneously, all the bacterial isolates with heavy metal tolerance were characterized for PAH tolerance and it was observed that bacterial isolates SHB 13, 70 and 101 showed tolerance to high concentrations of insoluble PAH (Table 1). Appreciable tolerance to heavy metals and MIC values were observed to be higher by isolates SHB 13, 70 and 101 when compared to previous studies²⁷.

Characterisation of biosurfactant producing bacteria

Based on biosurfactant activity and tolerance of the isolates to both organic and inorganic pollutants SHB 13 bacterial isolate was chosen to carry out further studies. SHB 13 was identified based on standard morphological and biochemical test. When all characteristics were compared with those listed in Bergey's manual of determinative bacteriology, SHB 13 isolate was identified as *Bacillus* sp. 16S rRNA sequence analysis was initially analyzed at NCBI using BLAST (n) tool. The phylogenetic tree was constructed using MEGA version 4.0. Based on nucleotide homology and phylogenetic analysis, SHB 13 isolate had 99% similarity with *Bacillus subtilis*. Therefore the isolate SHB 13 was identified as *Bacillus subtilis* SHB 13. 16S rRNA sequence was submitted to EMBL nucleotide sequence submission with accession number LK391829.

Characterization of extracted biosurfactant using Fourier transformed infra red spectrophotometer

Nature of biosurfactant as lipopeptide was further confirmed by stretches observed with FTIR. Intense stretches of $2956\text{--}1151\text{cm}^{-1}$ indicates C-H stretch of fatty acid of lipopeptides. $2956, 2856\text{cm}^{-1}$ is due to presence of CH_3 , 2928 stretches were of $\text{CH}, \text{CH}_2, \text{CH}_3$, 2360 was due to OH group, $1734, 1541$ of C=O, N-H group, 1697 peptide group, 1151 of C-H group. Stretches in the range of 1730cm^{-1} was considered to be due to presence of lactone carbonyl absorption specific for surfactin families of lipopeptides. These results were found to be compatible with the stretches of functional groups for surfactin²⁸.

Molecular characterisation of surfactin producing gene

Biosynthesis of surfactin is observed to be non-ribosomal by activity of multi enzyme surfactin synthetases and it is reported that *srf A-C* and *sfp* genes are responsible for production of surfactin biosurfactant. Genomic DNA of *B. subtilis* SHB 13 and *B. subtilis* MTCC 2423 was successfully extracted and single bands were observed in gel documentation of agarose gels. Amplified fragments of *B. subtilis* SHB 13 and reference strain *B. subtilis* MTCC 2423 were observed in between 650-700bp regions. And this study confirmed *B. subtilis* SHB 13 contains surfactin specific gene. According to previous reports, *B. subtilis* 168 and *B. subtilis* strain ATCC 21332 showed 675bp amplified fragments which corresponded to surfactin specific gene²⁹.

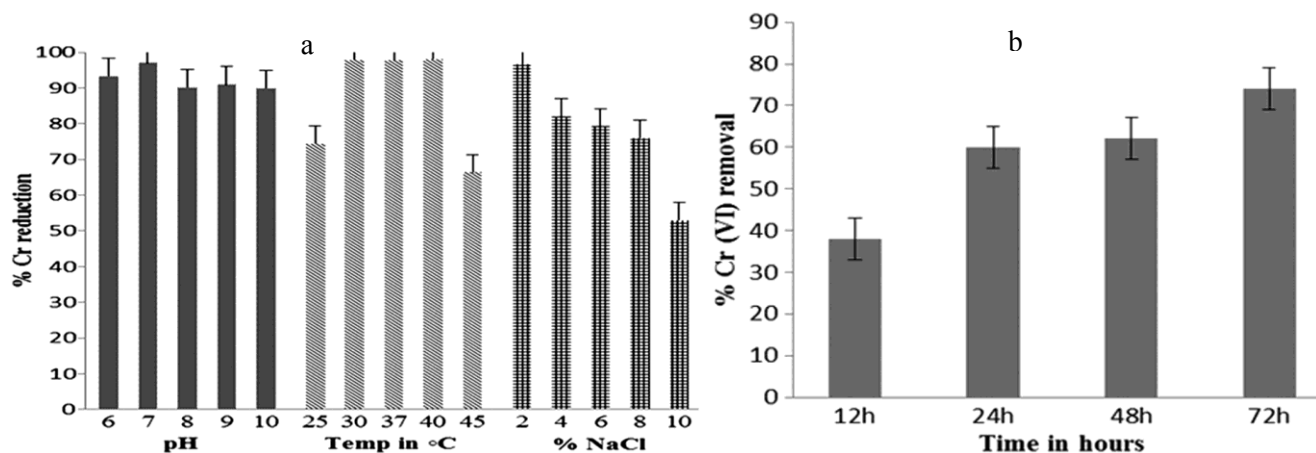


Fig. 1a—Effect of pH, temperature and %NaCl on 100 ppm Cr (VI) reduction by *B. subtilis* SHB 13; Fig. 1b—Removal of Cr (VI) using surfactin at varied time intervals

Effect of pH, temperature and NaCl on Cr (VI) reduction by *B. subtilis* SHB 13

Reduction of Cr (VI) by *B. subtilis* SHB 13 was studied under pH range of 6-10 and maximum reduction of Cr (VI) occurred at pH 7 (97%) followed by pH 8-10 (95-96%) (Fig.1). There are very few reports on Cr (VI) reduction in alkaline conditions. Previous studies report that bacteria such as *Bacillus* spp. and *Pseudomonas aeruginosa* were able to reduce Cr (VI) at pH 7³⁰. Present observation is significant as the studied bacterium is alkalophilic in nature and alkaline pH is apt for treatment of sewage and sludge for precipitation of chromium. Temperature is an important factor in Cr (VI) reduction and *B. subtilis* SHB 13 was able to give highest reduction (98%) of Cr (VI) at 40 °C followed by 37 °C and 30 °C (97%) as shown in Fig.1. Optimum temperatures for Cr (VI) reduction by alkalophilic *Bacillus* sp. KSUCr5 was reported to be at 37 °C³¹. Sodium chloride was found to be influencing reduction of Cr (VI) by *B. subtilis* SHB 13 (Fig.1). Maximum reduction of Cr (VI) by this bacterium was found to be 97% at 2% NaCl. Increasing concentrations of NaCl (4-8%) was negatively influencing the Cr (VI) reduction. Similar to this, previous report showed effective reduction of Cr (VI) by a sulphur reducing bacterium, at 4% NaCl concentration and increase in NaCl concentration decreased Cr (VI) reduction³². Hence, *B. subtilis* SHB 13 of this study can be considered as potential candidate for bioremediation studies of tanneries and industrial sludge which need bacteria tolerating extreme pH, salinity and temperature variations.

Metal precipitating activity by surfactin

Biosurfactant mediated metal chelation was observed by treating surfactin (10mg/ml) with chromium solution (100 ppm) over a period of 72h. Cr (VI) removal by surfactin was 38% at 12h and increased to 74 % on incubation up to 72h (Fig. 1b), indicating its possible role for effective removal of Cr from contaminated sites. Rhamnolipid produced by *Pseudomonas* sp. was used for removal of cadmium, lead and zinc³³. There are other reports too in which it was explained that apart from extracellular enzyme and biosurfactant, amino acids released also contribute in the reduction process of Cr (VI)³⁴. Similarly, in our study we may also expect possibility of Cr (VI) removal is due to the presence of amino acids and peptides in the extracted surfactin of

B. subtilis SHB 13. Presence of amino acids in the extracted surfactin was further confirmed by HPLC analysis (unpublished data).

X-Ray diffraction study

Chelation of Cr by surfactin was further confirmed by XRD analysis. Metal surfactin (M-S) complexed sample was incubated for 72h, dried and scanned under XRD. Intensity of Cr peaks increased with increasing time up to 72h. The peaks obtained with 2θ values were 23.92-28.02, 30.36-33.62, 40.84-44.04, 64.43 and 77.55 which corresponds the presence of Cr metal ions in the surfactin sample³⁵. This study indicated the ability of surfactin to form complex with Cr (VI) and reducing its toxicity in vicinity.

FTIR study for metal biosurfactant interaction

Extracted crude biosurfactant of *B. subtilis* SHB 13 and metal linked surfactin FTIR spectral analysis showed that there occurred a shift in vibration frequencies from 1444 to 1222 cm⁻¹. Shift of peaks from higher to lower frequency indicated ionic bonding between metal ions and the biosurfactant. There was variation in shifts in bonds from 1734-1635 cm⁻¹ and 1834-1633 cm⁻¹. Similar observation is reported in case of study performed with crude biosurfactant and Pb and Cd metal ions who have reported shift in frequencies from 1453-1358 cm⁻¹³⁶. These frequencies were also related to OH stretches of carboxyl groups which are found to affect during metal interactions.

SEM-EDS study

B. subtilis SHB 13 grown in nutrient broth without Cr (VI) appeared slender, whereas cell pellet directly treated with Cr (VI) solution (100 ppm) appeared bulged as observed by SEM-EDS (Fig. 2a and 2c). Cells grown in nutrient broth with Cr (VI) appeared normal (Fig. 2b). This variation in cell morphologies may be due to production of surfactin having chelating property of metal ion, which in turn protecting the cells from direct contact with Cr (VI). Presence of peaks in EDS study indicates that Cr (VI) is immobilised by surfactin released by *B. subtilis* SHB 13. Thus SEM –EDS reports showed biosurfactant involvement in protection of cells in spite of repeated exposure of *B. subtilis* SHB 13 to increasing concentrations of Cr metal ions. Similar observation was reported in case of *B. licheniformis* who have explained role of biosurfactant released by *B. licheniformis* in Cr (VI) tolerance and removal³⁷.

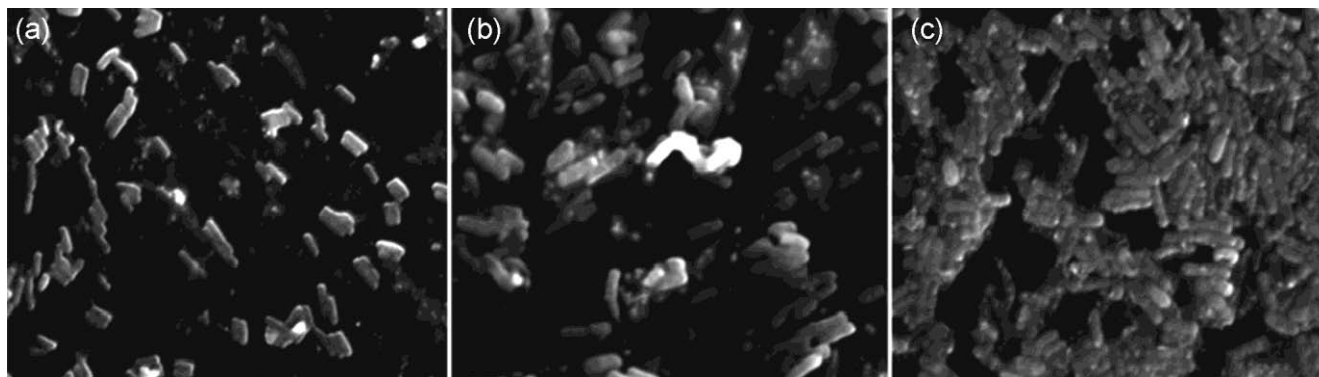


Fig. 2—Metal surfactin interaction studies; (a) Scanning electron microscopic photomicrograph of *B. subtilis* SHB 13 without Cr treatment (b) *Bacillus subtilis* SHB13 bacterium grown in NB medium amended with 100 ppm Cr (c) Scanning electron microscopic image of *Bacillus subtilis* SHB 13 after direct treatment of biomass with 100 ppm of chromium metal ion.

Conclusion

Cr (VI) removal by surfactin from metal containing solutions was studied and found to be due to desorption. Observations made in this study show positive perspectives for the use of the biosurfactant, surfactin, produced by a novel isolate *B. subtilis* SHB 13 in bioremediation of chromium in industrial effluents and its recycling.

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