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Isolation of Alkaliphilic Bacterium *Citricoccus alkalitolerans* CSB1 : An Efficient Biosorbent for Bioremediation of Tannery Waste Water

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Abstract

The present study designed to isolate and characterize an alkaliphilic chromate-resistant bacterium from crude salt and was identified as *Citricoccus alkalitolerans* CSB1 by 16S rDNA sequence analysis. The isolate could tolerate up to 25% NaCl (w/v) at pH 8.0-10.0 and 210 µg mL^{-1} of hexavalent chromium. Removal of Cr (VI) at concentration of 120 µg mL^{-1} was found to be about 98% within a period of 72 hours, at pH 9.0. The Cr (VI) removal efficiency after 60 hours was found 84.6%. The results on EDX analysis demonstrated surface binding as well as intracellular uptake of chromium by the bacterial cell. Zeta potential measurement indicates that the cell surfaces display a net negative charge at pH 10.0 (–29.80 mV). This was supported by Fourier transform infrared spectroscopy analyses demonstrating that the cells are dominated by surface proton releasing ligands, including carboxyl, phosphoryl and amino functional groups. The negative zeta potential which might be facilitating Cr binding. Cr adsorption experiments further reveal that functional groups play a primary role in metal adsorption. Furthermore, the Cr (VI) sorption by bacterial cell surface fits well in Freundlich and Langmuir isotherm models with qmax value of 15.8 µgmg−1 fresh biomass. Results demonstrated that the *C. alkalitolerans* CSB1 is an efficient bacterium for bioremediation of Cr (VI) contaminated effluents particularly in saline and alkaline environments.

Keywords: Alkaliphiles; *C. alkalitolerans* CSB1; FTIR; Zeta potential; SEM- EDX; Tannery effluent

Introduction

India is the third largest leather producer in the world, behind China and Italy. The states of Tamil Nadu, West Bengal, and Uttar Pradesh together have 88% of the tannery units of the country. The river Ganga in Kanpur (India) is reportedly one of the most polluted places and is heavily contaminated with heavy metals. The nature and behavior of Cr in wastewater depends on the physicochemical conditions of the effluents originating from various industrial sources [1-10]. The tanning industry is highly water intensive industry and generates a huge quantity of wastewater which is characterized by high organic load, suspended solids, high salinity (1% to 10% NaCl by wt.) and presence of excess quantity of Cr (VI) which hinder the treatment of tannery waste water [11-19]. Although Cr (III) is an essential micronutrient, soluble Cr (VI) but it is as carcinogen and is found to be toxic to all living beings [20-33]. For removal of such toxic me-tals by the different Physico-chemical methods of waste water treatment includes reverse osmosis, solvent extraction, lime coagulation, ion exchange, chemical precipitation, and membrane separation process, filtration, and incineration are being used [22,34-40], but these processes are cost effective and require large area to dispose off the tannery sludge. Various microorganisms capable of reducing the level of toxic pollutants can be used for biological treatment of waste water which can minimize the recurring expenses, high energy requirement and generation of secondary sludge [41-69]. Detoxification of hexavalent chromium has been carried out by using variety of bacteria under both aerobic and anaerobic conditions e.g. *Pseudomonas fluorescens* LB 300 [70-76], Enterobacter cloacae HO1 [77], *Bacillus* sp. [78]. The ability of the cell wall of *Bacillus* subtilis to interact with different heavy metals has been much studied [71]. Several reports have shown that the cell walls of Gram-positive cocci such as those of *Staphylococcus xylosus* and *Micrococcus luteus* have an affinity for metal ions [50], Application of indigenous microbes with greater tolerance against high concentrations of heavy metals and may play an important role in detoxifying the contaminated water and soil. For example, Wei et al. [79-82] recently identified Agrobacterium (CCNWRS33-2) from the Taibai gold mining region in China as the bacterial strain exhibiting resistance against heavy metals and was able to grow in the presence of 2 mM of copper and lead. Various workers have reported that the chromate reduction by bacteria is restricted to acidic and near-neutral pH conditions [64]. Very few reports have described chromate detoxifying bacteria from alkaline conditions [15,72]. Since, chromium containing tannery effluents are haloalkaline solutions with a high organic loading, there is need to search for the biological agents exhibiting tolerance against salinity, chromium and alkaline pH conditions which contribute to characteristic feature of tannery waste water [56]. The chromium tolerant microbes adapted to both hypersaline and extreme alkaline pH conditions [83] can be useful to overcome the challenges faced by the tannery water treatment plants. [11]. The major aim of this study was to isolate and characterize the alkalitolerant and chromium resistant bacterium and assess its potential to remove the pollutants load in tannery waste water.

Material and Methods

Isolation, culture conditions and characterization of isolated strain

The bacterium was isolated from Sambhar Salt Lake, Rajasthan (India). The bacterium was allowed to grow in CM media used for halophiles. The medium containing Yeast extract-10, KCl -2, $FeCl_3$ -0.02, Casamino acid -7.5, tri-sodium citrate-3.0, $MgSO_4.7H_2O$-20, NaCl -100 (g L^{-1}) and supplemented with $K_2Cr_2O_7$ -20 µg mL^{-1} at pH

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8 ± 0.02 the flasks were incubated at room temperature for 24 h on an orbital shaker. After 24 h, the bacterial strain was isolated from salt containing chromium enriched agar plate. The bacterium CSB1 was isolated and identification was done by 16 S rRNA sequencing as described elsewhere [11].

16S rRNA based identification

The isolation of the CSB1 isolate, characterization and identification was done by biochemical tests and 16S rRNA sequencing as described in previous study [11]. The isolate was identified by 16S r DNA sequencing using Universal primers (forward primer; 27 F 5'- AGA GTTT GAT CMT GGC TCAG -3' and reverse primer with modification; 1492 R 5'- TA CGG YTA CCT TGT TAC GAC TT-3'). Multiple alignments with sequences of actinobacteria of the family Micrococcaceae and calculations of levels of sequence similarity were carried out using CLUSTAL_X [70]. A phylogenetic tree was reconstructed using the neighbour-joining method [58]. Later, nucleotide sequence data were deposited in the Gen-Bank sequence database. The online program BLASTn was used to find out the related sequences with known taxonomic information in the databank at NCBI website (http://www.ncbi.nlm.nih.gov/ BLAST) to accurately identify the strain CSB1.

Sample collection from tannery

The sample tannery effluent (waste water) was collected from Super House Tannery No-1 District Unnao (Uttar Pradesh) India, in the sterile glass bottles. Immediately, after collection of the sample, the temperature and pH of the wastewater were determined using a portable pH meter (Haunna HI96107), the effluent preserved at 4°C in refrigerator in the laboratory for further use. The raw tannery effluent was analyzed for some physicochemical parameters such as total chromium, chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), total dissolved solids (TDS), total solids (TS) and chloride concentration etc. within 72 h of the sample collection [13]. During bacterial treatment and water quality parameters were analyzed.

Growth measurement in NaCl

The flask of CM broth with different initial concentration of NaCl ranging from (0% to 25% w/v) at pH 8.0 were inoculated with overnight grown bacterial culture to obtain an absorbance of 0.05 and the flask were incubated at 30°C. Aliquots (3 ml) were withdrawn at regular time interval and were centrifuged at 8000 × g min^{-1} for 10 min at room temperature. The pellet was re-suspended in 3.0 ml of Millipore water and the growth was determined by measuring the absorbance of bacterial cells at 600 nm (UV-Vis Spectrophotometer, 1601 Shimadzu) against Millipore water as blank.

Growth measurement and chromium removal

The CM broth supplemented with 10% NaCl (w/v) and different concentrations of chromium (0 to 210 µg mL^{-1}) were inoculated with bacterial culture to obtain an optical density of 0.05 and incubated at 35°C under shaking condition (150 rpm). Aliquots (3 mL) were withdrawn at regular time interval and were centrifuged at 8000 × g min^{-1} for 10 min at room temperature. The supernatant was used to measure the residual chromium concentration [11]. The growth was determined by measuring the absorbance of bacterial culture at 600 nm against blank. During the growth, viability and contamination of culture was checked by plating the culture on agar plates.

Effect of pH

The Complex media supplemented with 10% NaCl (w/v) and 60 µg mL^{-1} concentration of Cr (VI) under several pH conditions i. e. 6 to 10, were incubated overnight with bacterial culture to obtain an absorbance of 0.05. Aliquots (3 ml) were withdrawn at regular time interval to monitor the growth as well as residual level of chromium in the medium.

Scanning electron microscope (SEM)

The mid-exponential phase culture were taken from both chromium treated and untreated cell suspension and was centrifuged at 6000 rpm for 10 min at 4°C. After washing with phosphate buffer the cells were fixed into a (2.5 mL) eppendorf tube containing of 2.5% of glutaraldehyde (Loba Chemi, INDIA) in Millipore water for 1-2 hours at room temperature. Fixed culture were washed twice with Millipore water, and post-fixed with 2% solution of Osmium tetra oxide stain (0.5 ml) for 1 hour. The culture was subsequently dehydrated using graded concentration of ethanol (10%, 30%, 50%, 70%, 905 and 100%) prepared with Millipore water for 5 minutes at each concentration. The final dehydration was carried in 100% ethanol out for 10 minutes. The dehydrated culture drop was fixed onto the cover slip and then dried overnight in an oven and desiccator till mounting. The specimens were mounted over stainless steel stab onto the sample holder with double-stick carbon adhesive tapes and coated with platinum using a sputter coater prior to viewing in a scanning electron microscope of JEOL (JSM 6490 LV) Japan. An energy dispersive X-ray spectrometer (EDXS) detector was used to acquire the X-ray spectra. X-ray plotting was done using EDXS in conjunction with scanning electron microscopy (SEM).

Fourier transforms infrared spectroscopy (FTIR)

The chemical characteristics of the biosorbent surface before and after Cr (VI) adsorption were analyzed by FTIR spectroscopy of the biomass of *C. alkalitolerans* CSB1. One milligram of finely crushed dried biomass was mixed with 100 mg of spectrometric grade potassium bromide (KBr). The mixture was ground into fine powder and translucent sample disks were prepared obtained by using a manual hydraulic press at a pressure of 100 kg cm^{-2}. The disk was then fixed in a Spectrometer (FTIR) of Thermo-Scientific (Nicole 6700). FTIR spectrum of the biomass without chromium as well as biomass treated with 30 ppm Cr (VI) was scanned in the range of 500 to 4000 cm^{-1}. The FTIR spectra were recorded at a resolution of 4 cm^{-1} with scanning speed of 0.5 cm/s for each FTIR spectrum a number of 32 scans were averaged and spectrum was corrected for absorbance by KBr.

Mesurement of cell surface of zeta potential

The cell suspensions of bacterium grown in broth were prepared after through washing 14,000 rpm (10 min) to remove all the residues and clean the bacterial cells surface before analysis. The cell pellets were washed five times with 0.5 mM potassium phosphate buffer solution (pH 7.4) to clean the bacterial cells before analysis. The bacterial cell suspension was prepared by re-suspending the cell pellet in 0.5 mM potassium phosphate buffer solution (pH 7.4). The Absorbance at wavelength 590 of the final dispersion varied between 0.12 and 0.15 [29]. For the electrophoretic measurements of Zeta potential, 0.5 mL of the stock bacterial suspension was added to 10 mL of the phoshate buffer and the mixture was vortexed prior to transferring it to the Malvern polystyrene U-shaped cell. The measurements were made in the phosphate buffer solutions (5 mM) of different pH (from pH 6.0 to 10.0). A measurement of Zeta potential was performed to assess the net electrical charge on the cell surface of *C. alkalitolerans* CSB1. The cell free buffer solution was used as control. Zeta potential analyses were performed using an Instruments Nano Plus Zeta/Nano Particle Analyzer (Otsuka, E.C.L., Japan).

Adsorption of Cr (VI) by isolated strain

Bacterial culture after 24 hour growth in CM broth were harvested and dried in hot air oven at 40°C overnight. The adsorption of Cr (VI) at different concentrations of chromium 15 -75 µg mL^{-1} was measured using 1 mg dry biomass mL^{-1} as adsorbent. The samples were withdrawn at regular intervals of 15 min up to 60 min. The cell suspension was centrifuged and the pellets were washed with (EDTA) 5 mM solution and centrifuged (8000 rpm, 10 min.). The EDTA washed fraction was used for measuring the centrifuged adsorbed chromium. Intracellular Cr (VI) concentrations was determined in the EDTA washed pellets after digesting it with 10-15 mL HNO_3 and $HClO_4$ (ratio of 3:1) on hot plate at 60°C to 80°C until mixture became colorless. The solution was filtered through Whatman No. 42 filter paper before analysis. The chromium concentrations was determined by Atomic absorption spectrophotometer (AA 240 FS: Fast Sequential AAS Varian, Netherland) at a wavelength 357.87 nm. The uptake rate was expressed as µg Cr (VI) mg^{-1} dry weight of biomass.

$$Metal\ concentration\ mg/L = \frac{A - B \times C}{D} \quad (1)$$

Where A = Metal conc. in sample, B = Sample without metal, C = Final volume of digested solution (ml), D = Volume taken for digestion (ml)

Adsorption isotherms

Equilibrium adsorption isotherm models (Langmuir and Freundlich) were applied to describe the experimental the biosorption of Cr onto biomass of CSB1. Langmuir adsorption isotherm model was applied to analyze the kinetics of the biosorption process [6,39]. The amount of Chromium adsorbed per unit biomass of CSB1 (µg Cr per mg biosorbent) was calculated by following equation [44]:

$$q_e = \frac{(C\,i - Ce)\,v}{m} \quad (1)$$

Where Ci is the initial metal concentration (µg mL^{-1}), Ce is the equilibrium metal concentration in solution (µg mL^{-1}), V is the volume of the solution (ml), m is the mass of the biosorbent (mg).

The Langmuir isotherm was obtained by plotting the graph between 1/qe vs 1/ce. Langmuir constants (qmax and b) were calculated from slope and intercept of the linear plot. The Langmuir isotherm equation is given in linear form as [39].

$$\frac{C_e}{q_e} = \frac{1}{q_{max} b} + \frac{C_e}{q_{max}} \quad (2)$$

Where Ce is the equilibrium concentration (µg mL^{-1}), qe is the amount of Cr adsorbed per gram of adsorbent at equilibrium (µg mg^{-1}), qmax (µg mg^{-1}) is the Langmuir constant related to the maximum adsorption capacity and b (L mg^{-1}) the energy of adsorption.

The Freundlich isotherm model was applicable to adsorption on heterogeneous surfaces and can be represented by linear equation in linear form as [6]:

$$log q_e = log K_F + 1/n\ log C_e \quad (3)$$

Where qe is the amount of Cr adsorbed (µg mg^{-1}) at equilibrium, Ce is the equilibrium concentration of Cr in solution (ug mL^{-1}), KF (µg mg^{-1}) is the adsorption capacity and n is the intensity of adsorption. Freundlich constants (KF and n) were calculated from the slope and intercept of the plot of log qe versus log Ce.

Results

Isolation and characterization of bacterial strain

The bacterium CSB1 was isolated from salt of Lake of Rajasthan, India. The strain was a Gram-positive, non-motile, non-spore- forming cocci shaped aerobic bacterium with approximately about 0.5–0.8 µm in diameter (Fig. 1A). Colonies were light yellow, circular, entire, somewhat convex, opaque and approximately 1.5 mm in diameter. Bacterial growth occurred between 10 and 37°C with an optimal growth at 28°C. Optimum pH condition for growth was between pH 8.0 - 9.0 and 10% w/v, respectively. The bacterium showed NaCl tolerance up to 10% (w/v) concentration.

Characterization and molecular identification of the strain CSB1

Based on 16S rDNA gene sequence, the isolate CSB1 was identified as *Citricoccus alkalitolerans*. The sequence was submitted to NCBI Gen-Bank database with assigned accession number: KF322100.1 The closest relative of the strain CSB1 was *Micrococcus xinjiangensis*. Sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool. Phylogenetic tree was constructed by the neighbor-joining method using the MEGA 6.06 software based on 16S rRNA sequence is presented in (Figure 1) Such a higher identical value confirmed the strain CSB1 to be *Citricoccus alkalitolerans.*

Effect of NaCl on growth of *C. alkalitolerans* CSB1

The growth of isolate was measured in the presence of varying NaCl concentrations (0-25% w/v). The results on the bacterial growth in the presence of exhibited over all concentration dependent reduction in the growth when compared with control (without NaCl). In general the bacterial isolate exhibited time dependent growth up to 25% NaCl,

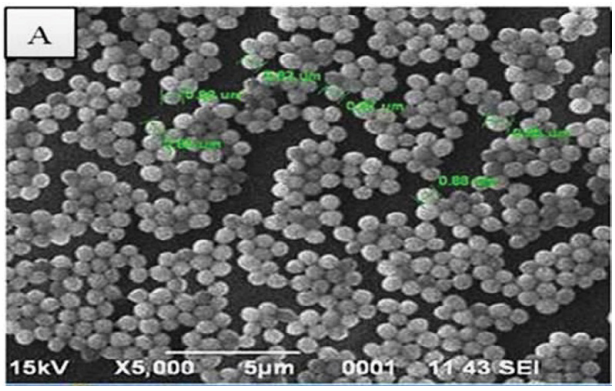

Figure 1: Scanning electron micrograph of the isolated bacterium CSB1 (A) Phylogenetic tree constructed for *Citricoccus alkalitolerans* CSB1 (GenBank accession no. KF 322100.1). Numbers in bracket represents Gen Bank accession numbers.

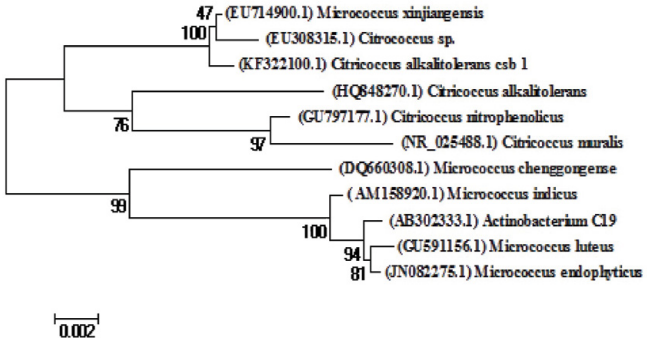

Figure 2: Growth of CSB1 at different concentrations of NaCl (0% to 25%, w/v). Results are expressed as mean ± SD of 3 replicates.

when compared with initial bacterial density on first day. However a sluggish growth was found beyond 20% NaCl concentration (Figure 2).

Effect of Cr (VI) on growth and chromium removal by bacterium *C. alkalitolerans* CSB1

The isolate was grown in the presence of different concentrations of hexavalent chromium (0 -210 µg mL^{-1}). The results (Figure 3A) revealed a concentration dependent decline in the growth after 150 µg mL^{-1} of concentration of chromium. Time dependent growth of the isolate in the presence of hexavalent chromium showed that the bacterial isolate reached to maximum growth after 60 hours. The I50 value of CSB1 was found to be at 1165 µg mL^{-1} of chromium (Figure 3B). The bacterial culture was incubated in a broth medium supplemented with 30-210 µg mL^{-1} Cr (VI) for 84 hours. The result showed that percent removal of chromium by the bacterial isolates increased with increase in incubation time (up to 84 hour). Result (Figure 4) revealed maximum rate of Cr

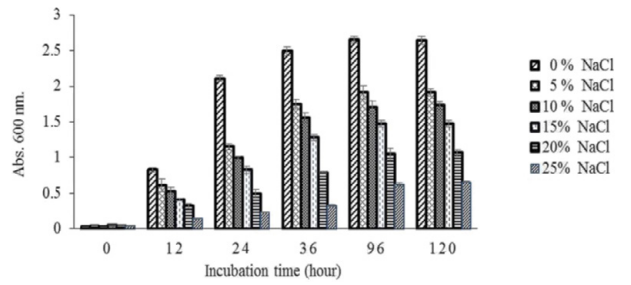

Figure 3: Time dependent (0-84 h) growth of *C. alkalitolerans* CSB1 measured at different concentrations of chromium (0-210 µg mL^{-1}) containing 10 (%) NaCl at pH 8.0. Percent inhibition of growth at each concentration of Cr (VI) (0-150 µg mL^{-1}) after 72 hours was plotted to calculate the I-50 value (50% growth inhibitory concentration of chromium). Results are expressed as mean ± SD of 3 replicates.

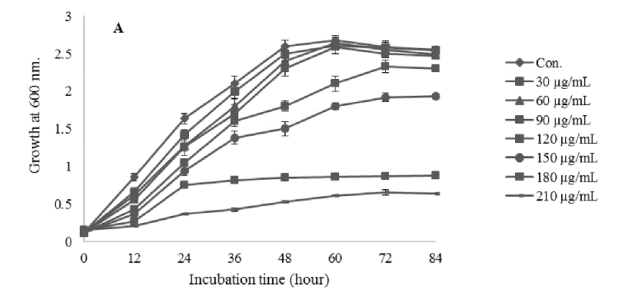

Figure 4: Percent residual concentration of Chromium in the medium at different time interval in the presence of varying concentrations of Cr (VI) (30-150 µg mL^{-1}) at pH 8.0. Results are expressed as mean ± SD of 3 replicates.

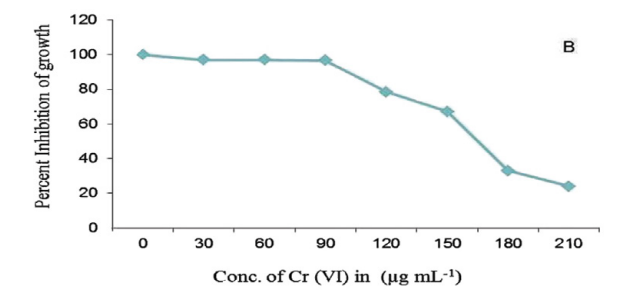

Figure 5: Growth and percent removal of Cr (VI) at fixed concentration of Cr (60 µg mL^{-1}) under different pH (6.0–10) condition. Results are expressed as mean ± SD of 3 replicates.

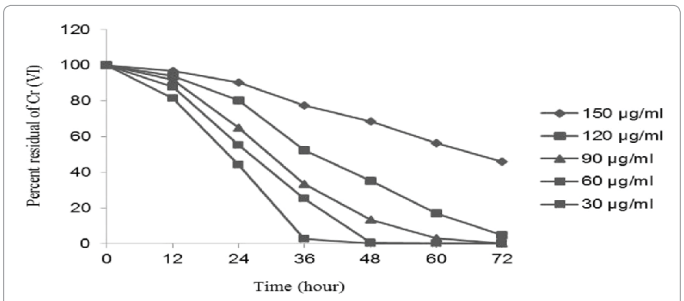

Figure 6: EDX analysis of chromium in the bacterium *C. alkalitolerans* CSB1 cultivated in the presence of $K_2Cr_2O_7$.

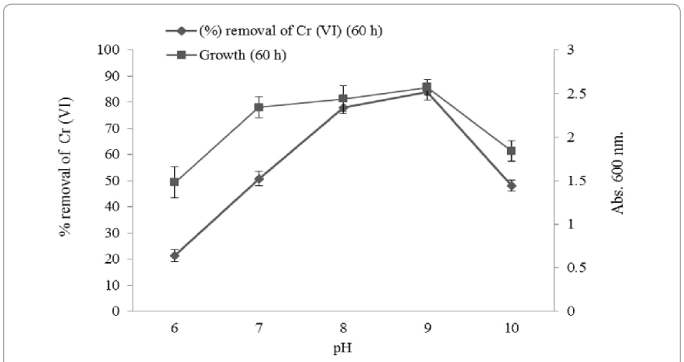

Figure 7: FTIR spectra of *C. alkalitolerans* CSB1 (A) treated with Cr (VI) and (B) without chromium.

removal after 72 hours. In general, 150 µg mL^{-1} concentration of Cr (VI) showed an adverse effect on Cr removal efficiency of bacterial isolate perhaps due to toxic effect of Cr (VI) on the growth.

Effect of pH on growth and chromium removal efficiency of *C. alkalitolerans* CSB1

Different pH (6.0-10) conditions were used for growth and chromium removal of efficiency of bacterial isolate. The bacterial broth cultures were supplemented with 60 µg mL^{-1} of chromium and incubated for 60 hour under different pH conditions. The results obtained with *C. alkalitolerans* CSB1 on growth as well as percent removal of chromium showed optimum pH between 8.0-9.0 (Figure 5).

Observation of metal accumulation within bacterial biomass

The *C. alkalitolerans* CSB1 were observed by SEM after 24 hour of incubation culture in the presence of $K_2Cr_2O_7$, and EDX was used to characterize their elemental composition (Figure 6). EDX clearly showed the uptake and adsorption of chromium, through the characteristic K and L emission peaks with in the cells of bacterium (0.8, 5.5 and 6.0 keV, respectively). The EDX spectrum demonstrated a Cr peak in the Cr (VI) (60 µg mL^{-1}) treated sample that confirmed the uptake Cr by the cell at the cell surface which has washed out during the PBS washing steps and acetone dehydration.

FTIR analysis of bacterium *C. alkalitolerans* CSB1

There are outstanding differences in the overall IR spectrum between the Cr (VI) treated and untreated cells (Figures 7 and 8, Tables 1 and 2). The infrared spectra of Gram-positive bacterium C. alkalitolerans CSB1. The infrared spectra exhibited distinct peaks corresponding to carboxyls, amides, phosphates, and carbohydrates in specific regions of the IR spectrum (4000 to 500 cm^{-1}). The wavenumber 3410.9 cm^{-1} of

hydroxyl and secondary amide shifted to 3295.8 cm^{-1} due to the mainly N-H stretching in the sec-amide proteins indicating the involvement of membrane proteins in chromium binding. The IR peak 2927.0 cm^{-1} depicted the lipids, proteins and polysaccharides, the shifting of these bands to 2931.4 indicated the involvement of N-H and C-H asymmetric stretching of phospholipids and polysaccharides in the adsorption process. A most prominent feature of the metal treated cells was the appearance of a relatively strong and well-resolved peak of C=O group or esters groups stretching vibration, at 1735.7 cm^{-1} was due to changes in lipids after chromium treatment. The wavenumber of 1652.1 cm^{-1} of amide-I band of proteins indicating asymmetric stretching vibrations C=O group proteins, the peak corresponding of protonated carboxyl groups. And the deprotonated carboxylate anion, COO−, was expected to appear around 1542 cm^{-1} in alkaline cell suspensions. It was obscured by the intense amide bands in the range of 1500-1650 cm^{-1}. However, the symmetric stretching of COO− vibration appeared around 1396 cm^{-1}. A characteristic peak of -COOH group of side chains of Amino and Fatty acids observed around 1401.0 cm^{-1} in untreated cells shifted to lower wavenumbers 1386.9 cm^{-1} due to interaction of chromium, indicating symmetric stretching vibrations of -COO- free fatty acids. The involvement of absorption peak at wavenumber 1077.8 cm^{-1}, mainly contributed by phosphodiester group of nucleic acid and membrane phospholipids, shifted to 1059.7 cm^{-1} due to Asymmetric and Symmetric of stretching PO_2^-, $P(OH)_2$ in phosphodiester band of phosphate suggesting major role of these functional groups in the surface binding of Cr (VI). Shifting in absorption positions may be caused by many factors. These includes; the physical states, electronic and mass effects of neighboring substitutes, conjugations, intra-molecular and intermolecular hydrogen bonding and ring strain [44]. The FTIR spectra showed the presence of the electronegative functional groups (i.e., carboxyl, hydroxyl, phosphate, amide and amino groups) on the surface of bacterium, which facilitated the binding of Cr (VI).

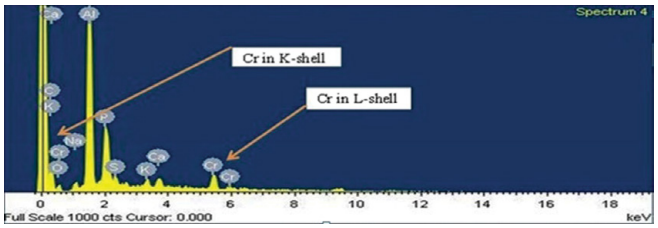

Figure 8: FTIR spectra of *C. alkalitolerans* CSB1 (A) before treatment with tannery wastewater and (B) after treatment with tannery wastewater.

Zeta Potential

Characterization of bacterial cell charge

Bacterial elctrophoretic mobilities are often measured as a function of pH at constant ionic strength. The pH dependence of the Zeta potential (Figure 9) was calculated from the electrophoretic mobility

Observed Wavenumbers (cm^{-1}) at different concentration of Cr [VI]			
Representing frequency range in cm^{-1}	Without treatment Control	Treated with Chromium	Band Assignment
3500-3200	3410.9	3295.8	N-H and O-H stretching of Sec-amide and hydroxyl group [11]
2938-2926	2927	2931.4	CH_3 asymmetric stretching from lipids, proteins, polysaccharides [40]
2960-2850	2361	2362.7	Asymmetric stretching band of CO_2 hydrates [12]
1790-1720	0	1735.7	C=O stretching of esters/ carboxylic group in protonated form of lipids [11, 34]
1680-1630	1652.1	1653.7	C=O stretching of proteins in Amide -I of α - helical structures [26]
1550-1520	0	1542.8	N-H bending and C-N stretching in amide (Amide –II) [34]
1460-1425	0	1453.3	Scissoring motion of CH_2 groups of peptidoglycan and phospholipids [34]
	1401	0	–COO– group of Amino acids side chains and Fatty acids [21]
	0	1386.9	Symmetric stretching of fatty acids [25]
1310-1240	1314.4	0	C-N stretching, N-H (sym.) bending of Amide-III band of proteins [21]
	0	1256.6	P=O stretching (asymmetric0 of phosphodiesters [21]
1150- 950	1077.8	1059.7	Asymmetric PO_2^- (sym) and Symmetric P $(OH)_2$ stretching in phosphate [17,34]
	992.8	984.9	Vibrations of C-OH and C-C bonds in polysaccharides [34,63]

Table 1: Assignment of functional groups associated with major vibration bands in mid-IR spectra of *C. alkalitolerans* CSB1.

Observed frequency (cm^{-1}) of *C. alkalitolerans* CSB1			
Representing frequency range in cm^{-1}	Control (Without Treatment)	Treated with T.E.	Band Assignment
3500-3200	3409.8	3435.1	N-H and O-H stretching of Sec-amide and hydroxyl group [12]
2950-2850	2962.1	2960.9	Asymmetric C-H stretching of CH_3 lipids and proteins [11]
	0	2930	C-H stretching asymmetric bands of CH_2 [70]
1670-1630	1654.7	1661.9	C=O stretching of proteins in Amide -I [24]
1550-1510	1544.1	1538.2	Amide -II bands and C-N stretching [34]
1500-1350	1416.1	1447	C-N stretching, N-H (bend), CH_3 deformation (asymmetric) [23]
	0	1393.9	Symmetric stretching of fatty acids [25]
1310-1240	1309.7	1311.8	Amide III band components of proteins [24]
1250-1220	1236.6	1236.8	P=O stretching (asymmetric) of phosphodiester band [21]
1200-900	1154.7	1121.9	PO_2^- symmetric stretching of phosphodiester [20]
1080-1010	0	1074.4	PO^{2-} (Asym and Symmetric) P $(OH)_2$ stretching in phosphate [17]
1000-700	0	860.6	P-OH and P-O-P vibrations, Protonation phosphate oligomers [34]

Table 2: (A) FTIR Wavenumber cm^{-1} of *C. alkalitolerans* CSB1 control (B) treated with tannery effluent wavenumber cm^{-1} of *C. alkalitolerans* CSB1 treated with tannery effluent and without tannery effluent (control).

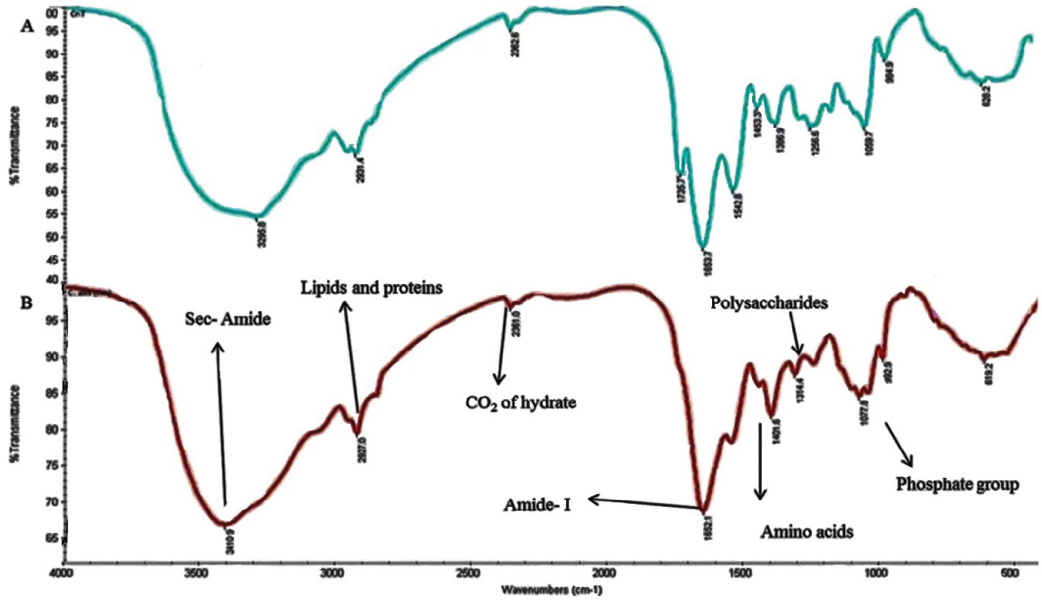

Figure 9: Zeta potential (mV) measurement of *C. alkalitolerans* CSB1 under different pH (4-10) condition.

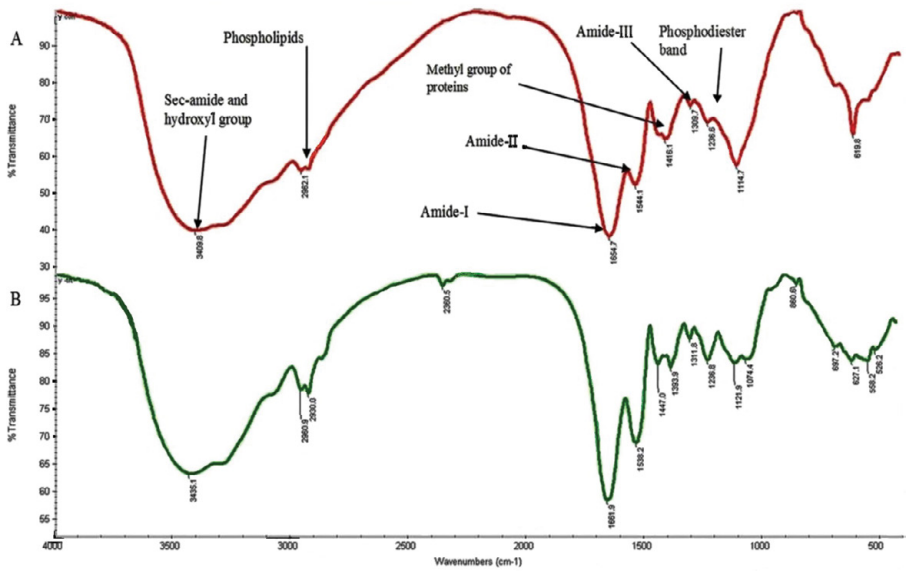

Figure 10: Langmuir isotherm plot of Cr (VI) adsorption on *C. alkalitolerans* CSB1 biomass.

Langmuir constants			Freundlich constants
q_m (µg mg^{-1})	b (L /mg)	R^2	n
15.8	0.052	0.998	1.25

Table 3: Isotherm parameters for Cr [VI] adsorption on *C. alkalitolerans* CSB1 biomass.

using the Helmholtz-Von Smoluchowski for selected isolate in 10 mM phosphate buffer. The charge at the cell surface of *C. alkalitolerans* CSB1 was determined as a function of pH. The zeta potential remained below –29.80 mV (at upper surface of cell - 48.55 mV and lower surface - 20.16) at pH 10, indicating that the cells constantly possessed a net negative surface charge in the alkaline pH range. In particular, the surface of the cells displayed of a sharp increase in electronegativity from pH 6 to 10, which was found to be the maximum at pH 10.0. The electrophoretic charge on the cell surface and the cationic charge on metal like Cr (VI) facilitated the metal binding on the bacterial surface. At lower ionic strength, the zeta potential of living cells increased with increase in pH > 9–10. It can be seen from these figures that the magnitude of zeta potential decreases yielding less negative values with increasing ionic strength, from pH 4 to 10. The is close or below - pH of isoelectric point 2 for *C. alkalitolerans* CSB1.

Adsorption isotherms

The Langmuir and Freundlich equations were applied to describe the experimental data for the sorption of Cr (VI) onto biomass of CSB1 over the entire concentration range studied (15 to 75 µg mL^{-1} Cr). The Langmuir constants (qmax and b) were calculated from the slope and intercept of the linear plot of 1/qe vs. 1/Ce (Figure 10) and listed in (Table 3) along with their correlation coefficient R2. The maximum monolayer uptake (qmax) of chromium calculated from Langmuir

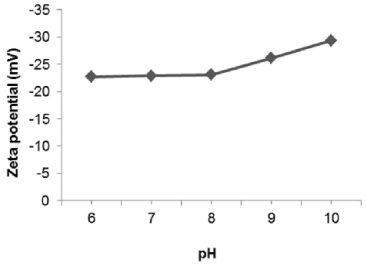

Figure 11: Fruendlich isotherm plot of Cr (VI) adsorption on *C. alkalitolerans* CSB1 biomass.

Parameters	Untreated tannery waste water	After treatment by *C. alkalitolerans* CSB 1
Color	Black	Grey
pH	10.02 ± 0.05	8.16 ± 0.03
TDS (mg L^{-1})	10886 ± 642	9361 ± 165
TS (mg L^{-1})	12335 ± 842	12149 ± 205
Total Hardness (mg L^{-1})	2817 ± 11.6	1342 ± 23
Chloride (mg L^{-1})	4931 ± 45	1126.8 ± 18
Alkalinity (mg L^{-1})	2080 ± 30	224 ± 15
Total Chromium (mg L^{-1})	114.6 ± 3.5	5.5 ± 2.8
COD (mg L^{-1})	3249 ± 13	1092 ± 19.8
BOD (mg L^{-1})	1025 ± 5	202 ± 12

Table 4: Physicochemical parameters of tannery wastewater before and after treatment by *C. alaklitolerans* CSB1.

adsorption isotherm model was 15.8 µg mg^{-1} at room temperature and it found close to experimental value. The Langmuir equation was applied for monolayer sorption onto a surface with a finite number of identical sites which are homogeneously distributed over the adsorbent surface [39]. Further, analysis of the R2 value (0.998) suggested that the equilibrium adsorption data fitted well with Langmuir isotherm model.

The Freundlich (KF and n) constants were calculated from the slope and intercept of the plot of log qe vs. log Ce (Figure 11) and presented in Table 4 along with their correlation coefficient R2. The Kf value was found to be 0.323 (Table 2), indicated that adsorption coefficient for Cr binding on to CSB2 cell surfaces. The value of constant n was found to be 1.25, indicated beneficial adsorption process [6]. The value of constant n represented bonding strength between the Cr and biosorbent and n value between 1 to 10 indicated favorable adsorption process [6]. Comparatively low value of correlation coefficient R2 (0.952) suggested that Freundlich adsorption isotherm model did not fit well to the experimental data for the biosorption of Cr (VI) onto biomass of CSB1.

Physicochemical Properties of Tannery Wastewater After remediation

Results of the analysis of the physicochemical parameters of untreated tannery effluent are depicted in Table 4. The results revealed that color of the untreated industrial effluent were blackish with unpleasant odor. This color may be contributed by undecomposed organic and inorganic matter. The Chromium concentration was about 114.2 (mg L^{-1}). This was 56 times higher than recommended by CPCB (India) for irrigation water. There was a significant change in different parameters like hardness, chloride, BOD, COD etc. The isolates showed more than 80% reduction in BOD, 70% reduction in COD and more than 95.2% remediation of total chromium was observed in the batch cultures inoculated *C. alkalitolerans* CSB 1 after 96 hour of incubation. The total hardness of tannery effluent after bacterial treatment was reduced by more than 50% as compared to control (untreated).

Discussion

The salt tolerant nature of alkaliphilic bacterium allows them to thrive in hypersaline environments. *Citricoccus alkalitolerans* CSB1 bacterium could grow in the range of 5% to 20% of NaCl. It has been reported that Halomonas GTW does not grow in the absence of sodium chloride [28]. Similarly, H. meridiana was capable of growth in saline media containing 0.5% to 17.5% salts with an optimum concentration between 3% to 10% salts [14]. Alkanivorax dieselolei Qtet3 is a halotolerant strain and could grow over a wide range (0% to 20%) of NaCl concentration with the highest growth occurring between at 2% to 5% [16]. The optimal growth of members of *Salini vibrio* occurred between 2.5% to 10% NaCl [45].

The *C. alkalitolerans* grew well in the presence of Cr (VI) showing tolerance up to 210 µg mL^{-1} concentration of Cr (VI) in the presence of 10% NaCl. Similar results are reported by Megharaj et al. [46] which showed that *Pseudomonas* strain isolated from Chromium contaminated foundry could tolerate up to 194 mg L^{-1} chromate on acetate minimal plates [7]. The *B. licheniform* grown in the presence of Cr (VI) showed a minimum growth inhibitory concentration at 225 mg L^{-1} of Cr (VI) [35]. Further, the Cr (VI) tolerance limit of (1500 mg L^{-1}) of a marine bacterium was found to be higher than *B. licheniform*, *Bacillus* sp. and *Cornynebacterium hoagie* strains [9, 75]. The *Ochrobactrum* sp. strain CSCr-3 isolated from chromium landfill could tolerate up to 800 mg L^{-1} Cr (VI) and was able to remove about 100-200 mg L^{-1} Cr (VI) within 96 h of incubation [30,37].

Results on pH dependent growth and Cr (VI) removal have shown an optimum pH 8.0 to 9.0 for growth and optimum removal of Cr (VI). Similar results were found in *Ochrobactrum* sp. strain CSCr-3 [84,85]. Shakoori et al., [66] reported that the optimum pH for removal of Cr (VI) in a Gram-positive bacterium Intrasporangium sp. Q5-1 was pH 9.0. Mangaiyarkaras et al. [40] also reported that the optimum pH for Cr (VI) removal by a Gram positive and alkaliphilic bacterium *B. subtilis* was 9.0. Earlier, it has been observed that the affinity of cationic species towards the functional groups present on the bacterial cellular surface was strongly pH dependent [76].

Zeta potentials are the physico-chemical corollaries of the capacity of bacterial cell surface to express those forces involved in the surface adsorption. There are many examples of the use of zeta potential measurements to probe bacterial surfaces [48,53,73]. The genus alkaliphilus may represent a novel group of bacteria that can utilize proteinaceous material and reduce the metals and sulfur compounds [81]. Cells surface of *C. alkalitolerans* CSB1 displayed strikingly different zeta potential profile in the pH range between 6.0 and 10.0. It is assumed that the negatively charged groups on the cell surface became deprotonated with increasing pH or decreasing H+ concentrations in the ambient electrolyte solution, with the presence of di- and trivalent anions consequently contribute to the absolute negative charge on the cell surface [62].

Earlier, it has been shown that the surfaces of *Bacillus* sp. cells were negatively charged under broader pH 2–8 range [6]; 2.4 –10 [81]. The zeta potential in all bacterial cells become negative with increasing pH [38]. Similar results were obtained for *E. coli* and *S. aureus* (– 44.2 and −35.6 mV, respectively), and their surface charges were more electronegative due to the Gram negative nature of the bacteria [2,18]. The present observations are in conformity with the earlier reports, where it has been demonstrated that surface charge neutralization

leads to altered membrane permeability [3]. Another finding showed a decrease in the negativity of the Zeta potential of *S. aureus* than *E. coli,* when studied at identical concentration [29]. *P. aeruginosa* PAO1 cells had a negative surface charge (zeta potential) due to carboxylate groups present in the B-band LPS [62].

As determined by SEM EDS analyses, the selected bacterium grown in the presence of 60 µg mL-1 ($K_2Cr_2O_7$) displayed intracellular chromium deposits within the cell, together with diffuse deposits at the bacterial surface (Figure 6). Similar results were found for *Shewanella oneidensis* and *Acinetobacter haemolyticus* [15,84] in which Cr (III) precipitates assumed to be Cr (III), restricted to the outer surface due to the inability of chromate anions to bind with electronegative surface functional groups. The carboxyl, amide, glycopyranosyl glycerol phosphate, and teichuronic acid, phosphatel and hydroxyl groups are commonly found on cell wall of the gram-positive bacteria [8, 34]. Similar results were found in cyanobacteria and *Micrococcus luteus* DE2008 [41,42,68], where peptidoglycans played important role in metal binding. Cell walls of bacterial biomass offer particularly abundant metal-binding functional groups, such as carboxylate, hydroxyl, sulfate, phosphate, amino groups and slimes [4,11,74]. The IR spectra of *C. alkalitolerans* CSB1 biomass indicated the presence of ionizable functional groups i.e., amine R–NH_2 (amino acids, proteins, glycoproteins, etc.), carboxylic acid (fatty acids, lipopolysaccharides, etc.), hydroxyls and phosphates are able to interact with metal ions (Table 1) [49].

The in the wavenumber of 2361 cm^{-1} to 2400 cm^{-1} was influenced by Cr binding observed in treated cells might be attributed to CO_2 hydrates and induced the trans-gauche ratio in acyl chains [11,69]. The shifting of wavenumber 1652.1 cm^{-1} with the increasing and decreasing frequency 1653.7, 1542.8 and 1453.3 cm^{-1} which was the typical complexation and coordination due to the N-H, C=O stretching of proteins [27,69]. The spectrum of Cr induced shifting of the frequency 1401.0 cm^{-1} with a decrease in intensity corresponding to O–H bending and strong interaction of the –O–C=O group was assigned to binding of with the carboxylate ions. A shift in the wavenumber at 1256.6 cm^{-1} suggested P=O stretching (asymmetric) stretching of phosphodiesters band was due to the adsorption of Cr (VI) on to the cell surface [20]. The wavenumber shifted at 1077.8 to 1059.7 cm^{-1} after interaction with the Cr (III) bio sorption due to the Symmetric stretching of P (OH)2 in phosphate. The alteration was takes place at the peak 992.8 cm^{-1} in the chromium (VI) treated biomass, and it could be attributed to the presence of chromium (VI)–C-OH of polysaccarides [34]. The *C. alkalitolerans* CSB1 exhibited a broad absorption band in the hydroxyl groups of 3500 - 3200 cm^{-1} due to complexation of – OH groups with chromium metal and other pollution load. Similar studies are reported in Azospirillum brasilense SP245 after treatment of bacterial cells with Cu^{2+} [36], in chromium treated *P. aeruginosa* [10]. Similar results were reported in *E. coli* after treatment of cells with Cd (II), Cr (VI), Fe (III) and Ni (II) [55,65].

The pollutants from tannery effluent interacted with the isolated bacterium indicating important role in tannery waste water treatment. The values obtained in the present investigation clearly indicated that they were much above the permissible limit. But after treatment by bacterium *C. alkalitolerans* CSB1 resulted in to reduce COD (66%), BOD (80.15%) and total chromium 95% after 96 hour of treatment. Similar results were observed in *P. aeruginosa* and E. homiense which removed about 80% COD from the saline waste water [67]. The BOD of tannery waste was removed by 76%. [51]. The chromate reduction and chromium tolerance are known to be independent processes [52]. In previous studies, an *Ochrobactrum* sp. strain CSCr-3 completely reduced 100 -200 mg L^{-1} of Cr (VI) within 96 h of incubation [31]. A *Pseudomonas* strain CRB5 had completely reduced 20 mg L^{-1} Cr (VI) following 120 h incubation [43]. *Leucobacter* sp. G161 strain performed better and completely reduced 400 mg L^{-1} Cr (VI) within 96 hours of incubation. *Serratia* sp. C8 could reduce 80% of 20 mg L^{-1} of Cr (VI) [54]. Comamon asacidovorans MTCC 3364 removed 99% of 100 mg L^{-1} hexavalent chromium in the presence of 10% salt concentration [57].

Conclusion

The *C. alkalitolerans* CSB1 is alkaliphilic and salt tolerant bacterium which can easily grow in the tannery waste water. Due to electronegative charge on the bacterial surface it has the ability to bind with cationic species. The dominant functional groups on bacterial surfaces like carboxyl, amide, phosphate, hydroxyl, and carbohydrate related moieties contribute to metal binding characteristic of the bacterium as evident from the results of FTIR. The equilibrium data on adsorption of Chromium supported the monolayer sorption of metal by the bacterium as revealed by the Freundlich and Langmuir isotherm model. The results on tannery waste water treatment by this bacterium clearly demonstrated significant reduction in the pollution load of the waste water in terms of reduced level of BOD, COD, Chromium, total hardness, chloride and alkalinity. Thus, the isolated bacterium *C. alkalitolerans* CSB1 is a good adsorbent for removal of Cr (VI) and other pollutants due its ability to growth at alkaline pH and, also its ability of salt tolerance, which are associated attributes of the tannery effluent.

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