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# BIOSYNTHESIS OF IRON NANOPARTICLES BY SULPHATE REDUCING BACTERIA AND ITS APPLICATION IN REMEDIATING CHROMIUM FROM WATER

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## ABSTRACT

Chromium is an industrially important metal, extensively used in metal plating, tanning, pigment and refractory industries; hence its discharge in to the environment is unavoidable. Due to its persistence, toxicity, bioaccumulation property it has become a threat to living organisms. Present study deals with isolation of a hypersaline sulphate reducing bacteria (SRB) strain WCA1 from Ribandar saltpan, Goa, India which produced Iron sulfide nanoparticle and studied for Cr remediation from water.The SRB isolate was closely related to *Desulfotomaculum acetoxidans* by biochemical and 16S rRNA gene sequence analysis. The SRB synthesizes iron sulfide nanoparticles in the growth media when supplied with 0.5M $FeSO_4.7H_2O$. From SEM-EDS and XRD results, the nanoparticles were characterized to be iron sulfide nanoparticle of 21nm size. These nanoparticles were found effective in Cr remediation from water. The maximum adsorption of Cr by the nanoparticle was achieved within 2 hours of reaction time. The optimum nanoparticle concentration for maximum removal was determined to be $0.25g.L^{-1}$. The efficiency of Cr remediation increases in acidic conditions.Ca-Alginate immobilized iron sulfide nanoparticle beads had an advantage over its bare form in terms of low mobility, less self-aggregation and easy separation from a solution. The bead form increased the overall Cr remediation capacity of nanoparticle and it contributed to 99% of Cr removal from the water.

**KEYWORDS:** ***Chromium, sulphate reducing bacteria, remediation, self-aggregation, iron sulfide nanoparticle***

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## INTRODUCTION

Microbes are the primary agents responsible for modification, degradation and detoxification of pollutants and contribute for natural attenuation of the environment. They carry out various biological and chemical processes like bioleaching, bioremediation, bioaccumulation, biodegradation and sometimes nanoparticle production to deal with detoxification of pollutants.[1] The biological methods for synthesizing nanoparticle has received a great attention of researchers worldwide as it diminishes the problems associated with physicochemical synthesis methods.[2]SRB carryout bioprecipitation of various metals by microbiologically produced sulphide, which precipitate them as highly insoluble metal sulfides.[3-8]Earlier synthesis of various metal sulphide nanoparticles by SRB has been reported.[6, 8, 9]SRB can sequester metals in the form of nanoparticles in anoxic water by producing reactive $H_2S$.[10]Chromium is one of the hazardous pollutant of the environment and it exist in eight different valance states, among which Cr(VI) is considered to be the most toxic form. Cr is highly mobile in the environment and possesses toxicity to living organism such as microbes, plants, animals and humans.[11]Cr(VI) has serious threat to human life as it can damage lungs, kidney liver, intestine etc and can interrupt the organ functions.[12]Chromium compounds viz.; Zinc chromate, calcium chromate, Lead chromate etc. are highly toxic and carcinogenic. Cr(VI) can bind to double stranded DNA and induce DNA damage.[13-14]The presence of Cr in a niche decreases the microbial population and also affects microbial respiration.[15] In recent years nanoparticles have received attention for treating toxic contaminants effectively even at low cost.[16]To minimize the negative impact of the synthetic procedure of nanoparticles, biological resources like bacteria, fungi, algae and plants have been successfully used to produce low cost, energy efficient and environment friendly nanoparticles. Bacterially produced FeS have served as an adsorbent for a wide range of heavy metals and known to play critical role in environmental decontamination.[8,17]In the present study, a hypersaline SRB from Ribandar saltern Goa,India was isolated and the biological route of iron nanoparticle synthesis was investigated. Involvement of a hypersaline SRB strain WCA1 in the synthesis of iron sulphide nanoparticles was observed, that could effectively remediate Chromium from water. These nanoparticles can be efficiently entrapped in the Ca-alginate matrix without changing their activity and enhancing the overall Cr remediation from water.

## MATERIALS AND METHODS

### *Culture Isolation & Bacterial growth conditions*

SRB StrainWCA1 was isolated on modified Hatchikian's medium [18] at 50psu salinity from surface water of crystallizer pond of Ribander saltpan (15°29' 51"N 73°50' 44.8"E) Goa, India. The sulphate reduction was determined by analyzing the increase in sulphide concentration in the growth medium. Growth rate of SRB strain WCA1 was calculated by measuring the OD at 480nm and haemocytometer counting of SRB cells followed by simultaneous measurement of sulphide concentration.[19]

### *Biochemical and molecular Characterization of the SRB*

Utilization of electron donors and electron acceptors was tested in 25ml glass vials containing growth medium supplemented with a sterile stock solution of substrates (5mM final concentration) and growth was detected with an increase in sulphide concentration on the 7$^{th}$ day of incubation. Motility, catalase, oxidase, presence of desulfoviridin and cytochromes was carried out for physiological characterization. Cell structure was acquired with ZEISS EVO 18 Scanning Electron Microscope. For molecular characterization the genomic DNA was isolated followed by 16S rRNA gene amplification using universal primer 27F and 1492R and sequenced.[20] Further the sequence was searched for similarity match in NCBI Gen bank BLAST utility. Sequences with higher similarity match were selected and its evolutionary history was inferred using neighbour joining method. The bootstrap consensus 16S rRNA gene tree was inferred from 1000 replicates. The evolutionary analysis was conducted on Mega6 software tool.

### *Biosynthesis of Iron nanoparticle*

Biosynthesis of iron nanoparticles using SRB strain WCA1 was carried out by growing them anaerobically in 15ml anaerobic tubes containing 50psu salinity of liquid Hatchikian's media added with 0.5 M Ferrous sulphate and incubated at 30°C anaerobically in static conditions. After 21 days of incubation, the black colored precipitate settled at the bottom of the culture tube, were collected by centrifugation at 14000rpm for 15min and processed for their characterization.

### *Characterization of nanoparticle*

The Iron nanoparticle was characterized by scanning electron microscopy and energy-dispersive x-ray spectroscopy (SEM-EDS) and x-ray diffraction (XRD). SEM-EDS were performed with a JEOL -JSM-6360 LV SEM operated at 15–20 keV, equipped with OXFORD INCA 200 Energy Dispersive Spectrometer (EDS) to acquire the elemental composition of the nanoparticle. X-ray diffraction pattern of the powder samples was obtained using a RigakuMiniflex II desktop X-ray diffractometer.

### *Immobilization of nanoparticles*

The nano zero valent iron entrapment method[21]was adapted for immobilization of SRB synthesized nanoparticles. Deoxygenated MilliQ water was used to prepare 2% sodium alginate solution and 3.5% calcium chloride solution. The nanoparticles were mixed gently in sodium alginate solution followed by sonication and immediately dropped into calcium chloride solution and formed Ca-Alginate beads with entrapped nanoparticles. The beads were retained in the deoxygenated $CaCl_2$ solution for 12 hour to ensure hardening of the bead.

### *Chromium Remediation study*

### *Preparation of Cr solutions*

Chromium stock solution (1000ppm) was prepared by dissolving 2.829gram of AR grade $K_2Cr_2O_7$ in 1L MilliQ water. The desired working solutions were prepared by

diluting the stock solution. The remediation study was conducted with initial concentration of 10, 50, 100 mg.$L^{-1}$. Remediation efficiency was studied for bare and entrapped nanoparticles. Cr concentrations were analyzed by varian's AAS (AA240FS Fast Sequential Atomic Absorption Spectrophotometer). The percentage of Cr removal was calculated by the following equations:

$$\% \text{ Cr Remediation} = \frac{\text{Initial concentration of Cr -Final concentration of Cr}}{\text{Initial concentration of Cr}} \times 100$$

***Effect of reaction time***

Remediation efficiency of the nanoparticle was determined at different time intervals (0, 30, 60, 90, 120, 150, 180,210, 240, 270,300 minutes) with an initial concentration of 50mg/L Cr-Solution. Remediation percentage was calculated for bare and entrapped nanoparticles.

***Effect of nanoparticle concentration***

To determine the optimum nanoparticle concentration for maximum Cr remediation, various concentration (0.01- 5 g.$L^{-1}$) of nanoparticle in bare form and bead form were added to an initial concentration of 50mg/L with pH7 and allowed to react for 3hours. After the time period, the nanoparticles were separated from the solution and wereanalyzed for chromium concentration.

***Effect of pH***

A known amount of nanoparticle concentration (0.5g) in bare and bead form were added to 50 mg.$L^{-1}$ Cr solution of different pH value(pH 2 - pH14). The Cr solution was prepared with de-ionized water and pH was adjusted using 0.1M $HNO_3$ and 0.1M NaOH. The pH of Cr solution was measured using a Thermo Orion pH electrode. The reaction was allowed for 3 hours before removing the nanoparticles from the solution followed by Cr concentration analysis.

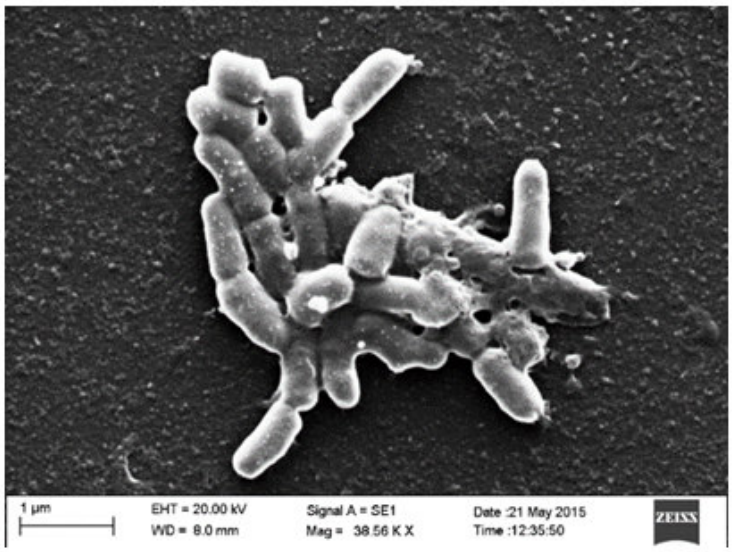
## RESULTS

***Culture Characteristics***

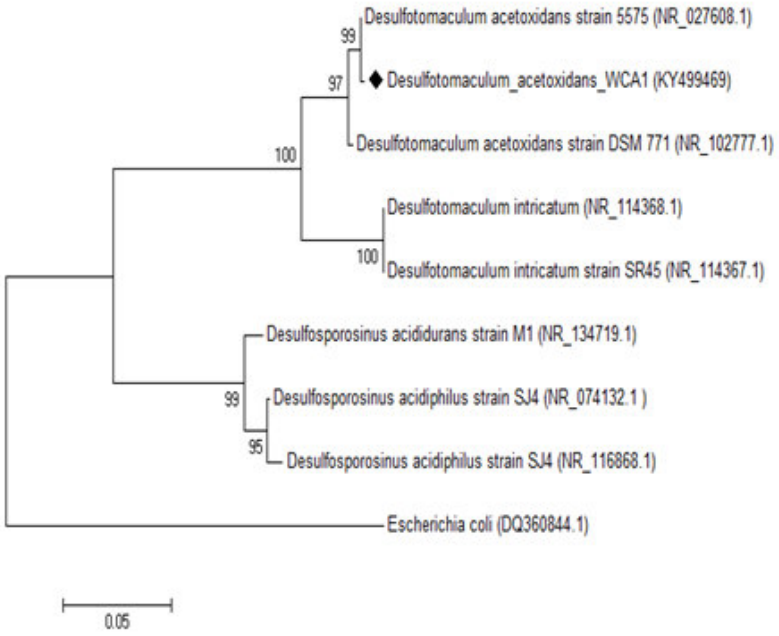
SRB strain WCA1 were Gram positive, rod shaped cells and size varied from 0.6 to 1.2 µm in length and 0.4 to 0.6 µm in width (Figure 1a).The SRB strain was found to utilize acetate, lactate, butyrate and benzoate as an electron donor. It used sulphate and thiosulfate as electron acceptor. SRB strain WCA1 could grow on a broad salinity range (10- 300psu) with an optimum growth at 50psu. The optimum pH for growth was found to be pH 8. Its growth favors, alkaline pH and its tolerance to higher salinity of 300psu indicates the culture to be halophilic SRB. Cell density reaches a maximum (1.9 x $10^8$cells/ml) by the 21$^{st}$ day in the medium with acetate and sulphate. The sulphide production by WCA1 was assessed by measuring dissolved sulphide concentration of the media, which attained a maximum value of 27.5mM by the 21$^{st}$ day, after which dissolved sulphide concentration decreased to 17.1mM by the 35$^{th}$ day.

***Phylogenetic analysis***

Phylogeny of partial 16S rRNA gene sequence (1189 bases) of SRB strain WCA1 showed a distinct clade in the genus *Desulfotomaculum.* It had highest sequence similarity with *Desulfotomaculum acetoxidans.* Biochemical characterization and phylogenetic analysis based on partial 16S rRNA sequence shows that the stain WCA1 belongs to dissimilatory sulfate reducing bacteria within the δ-proteobateria and belongs to the genus *Desulfotomaculum* (Figure.1b). The closest phylogenetic relatives of strain WCA1 was *Desulfotomaculum acetoxidans*, with the highest similarity of 99% in BLAST search. The 16S rRNA gene sequenceof the strain WCA1 was deposited in GenBank under accession number: KY499470




***(a)***
***SEM image of SRB strain WCA1***

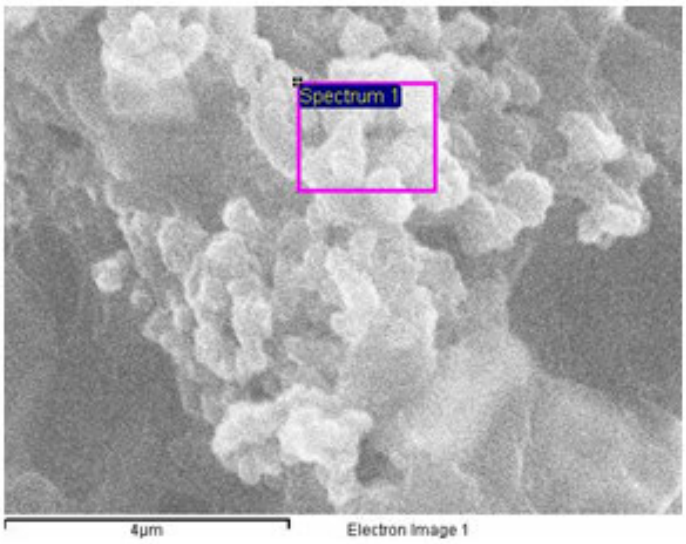



***(b)***
***Phylogenetic tree based on 16S rRNA gene sequence***

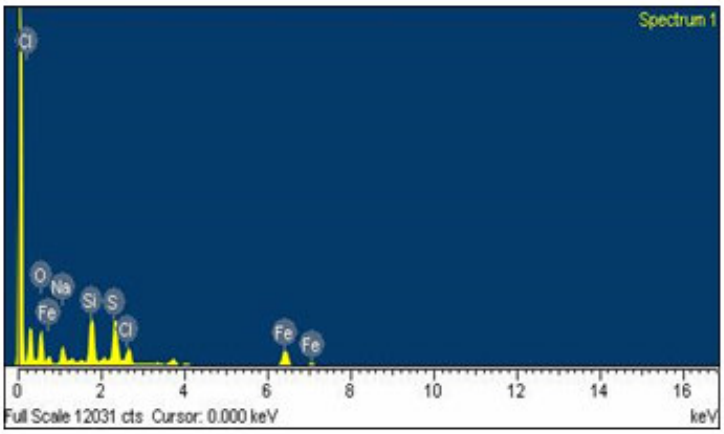
**Figure 1**
***Characteristic of SRB strain WCA1***

***Biosynthesis of Iron nanoparticle***

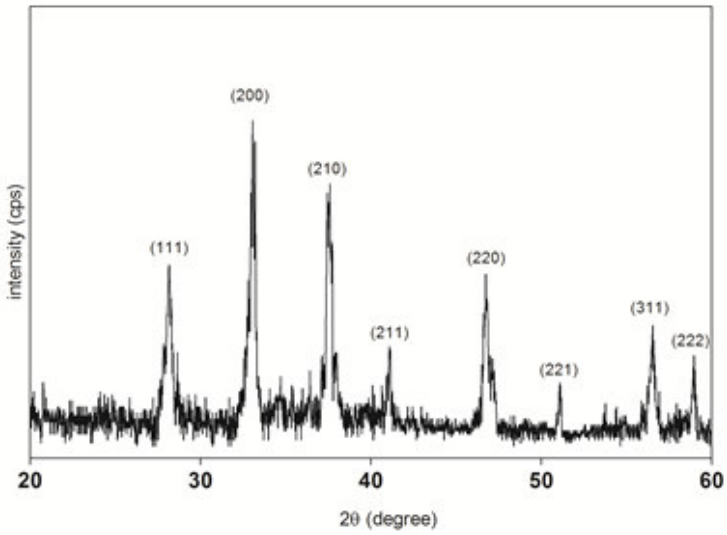
When Strain WCA1 was challenged with 0.5M ferrous sulphate in the growth media, it produced intense black colour precipitate after $7^{th}$ days of incubation and the precipitation quantity increases with prolonged incubation duration. The Black precipitates were formed due to the reaction between $H_2S$ produced by strain WCA1 and the ionic iron present in the media. It was characterized to benano sized iron sulphide particles. These particleswere found in aggregated form when the nanoparticle cluster was viewed by SEM (Figure 2 a).From the EDS analysis iron and sulphide was found to be present in a dominant proportion (Figure 2b). The formation of iron sulphide nanoparticle does not occur in the media with iron salt solution; it formed only in presence of strain WCA1 in the media. SEM image of SRB synthesized nanoparticles (Figure 2a) showed clusters of nanoparticles with an average diameter of 21nm. The XRD pattern obtained from the nanoparticle is shown in figure 2c. X-ray diffractogram contained eight prominent peaks that were clearly distinguishable. Peaks with 2θ values of 28.16, 33.06, 37.6, 41.08, 46.74, 51.08, 56.54 and 58.96 corresponded to crystal planes of (111), (200), (210), (211), (220), (221), (311) and (222) of $FeS_2$ nanoparticle. Based on XRD results, an approximate crystallite size was calculated (as per Debye-Scherrer's equation) to be 21.88 nm.




***(a)***
***SEM image of Iron Nanoparticles***




***(b)***
***EDS showing elemental composition***




***(c)***
***XRD pattern showing Crystallinity of iron nanoparticle***

**Figure 2**
**Characterization of nanoparticle**

***Immobilization of Iron sulfide nanoparticle***

The $FeS_2$ nanoparticles were successfully entrapped in Ca-alginate beads and had an average diameter of 2.6±0.1.The beads had a spherical shape and 24±2 beads per mL of Na-alginate solution were prepared. The nanoparticle containing beads appeared black in colour, while the normal beads were transparent. Upon entrapment the aggregation of nanoparticle is limited; this provides a solution for decreasing the property of self-aggregation in bare nanoparticles and enhances its reactivity.


***(A)***
***Ca-Alginate bead***

***(B)***
***Iron nanoparticle entrapped in Ca-Alginate bead***

**Figure 3**
***Entrapment of nanoparticle in Ca-alginate bead***

***Remediation of Chromium***

Experiments were conducted for remediation of Cr from water using the bare iron sulfide nanoparticle and entrapped iron sulfide nanoparticle at 3 initial concentrations of 10, 50 and 100 mg/L. The bare form reduced the Cr concentrations to 2.4mg/L(76% reduction), 8.8 mg/L (82.4% reduction) and 14.3 mg/L (85.7% reduction) respectively. The bead form reduced the Cr concentration to 0.3mg/L (97.2% reduction), 1.85mg/L (96.3% reduction) and 5.4mg/L (94.6%) respectively. The Cr remediation efficiency was found to be higher in the bead form of iron sulfide nanoparticle.

***Effect of contact time***

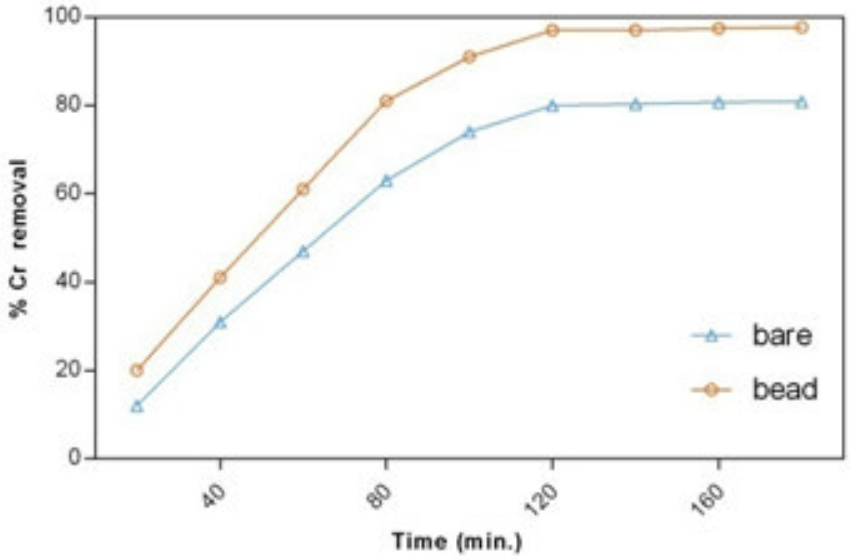
Cr remediation was studied by varying the contact time of nanoparticles with Cr solution (50mg/L) from 0-300 min using 5mg of iron sulfide nanoparticle. The Cr removal rate of bare form and the bead form are shown in Graph 1a. Though we studied for 300min but it was observed that with the increase in contact time the adsorption increases and attains a maximum adsorption of 80% (with Bare form), and 97% (with bead form) by 120minutes (2Hours) and further it remains constant. Thus in Figure 1a we showed the adsorption till 180 min.

***Effect of Nanoparticle concentration***

Graph 1b showing the effect of initial nanoparticle concentration on Cr remediation, was examined by varying the nanoparticle concentration from 0.01 – 5 $g.L^{-1}$ in Cr water (50mg/L). The removal efficiency increased from 67% to 89% with increasing bare nanoparticle concentration while the immobilized nanoparticle removed 69% to 99% of Cr from the water. With the increase in nanoparticle concentration the Cr removal percentage increased due to increase in overall surface area for Cr adsorption. At 0.5 $g.L^{-1}$ concentration of nanoparticle, Cr removal percentage reaches to a maximum of 89% for bare form and 99% for bead form but beyond this concentration, the remediation percentage showed a decreasing trend. Thus the optimum nanoparticle concentration was found to be 0.5$g.L^{-1}$ for both bare and bead form of $FeS_2$ nanoparticles. The immobilized form had an advantage over the bare form as the Cr adsorption was higher and this occurs probably due to the increased aggregation occurred in the bare form of nanoparticle, thus resulting in a less reactive surface area being available for adsorption.

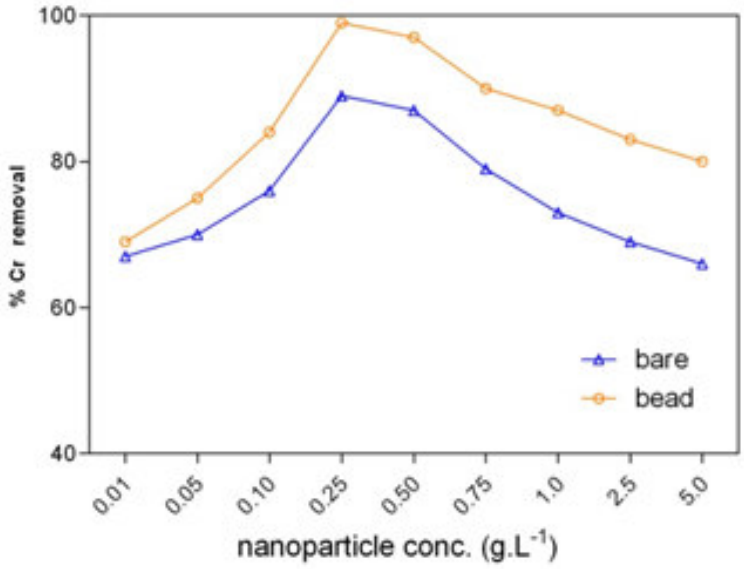
***Effect of pH***

The pH plays a major role in Cr removal as it influences the adsorption of Cr on the iron nanoparticle. As depicted in Graph 1c, the adsorption increases at acidic pH and a maximum adsorption of 99.7% was attained at pH4 while beyond pH 10 the adsorption was nullified. Thus it can be inferred that these bio-nanoparticles work better at acidic to neutral pH and alkaline pH reduces the adsorption. The bead form of nanoparticle provides a better sorption capacity than the bare form.

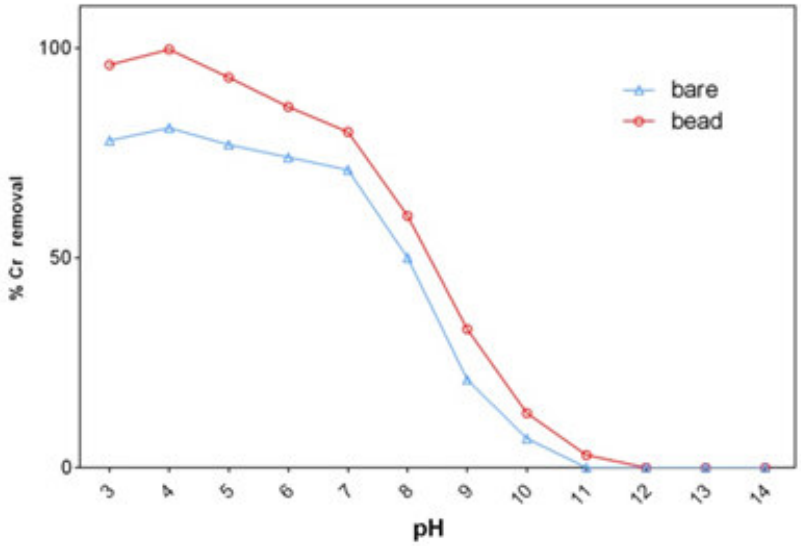



***(a)***

***Effect of contact time on Cr remediation***




***(b)***
***Effect of nanoparticle concentration on Cr remediation***




***(c)***
***Effect of pH on Cr remediation***

**Graph 1**
***Cr remediation by the SRB synthesized nanoparticles***

## DISCUSSION

Solar salterns are man-made extreme saline environment and a niche for halophilic and halotolerant microorganisms. In the saltpans with the evaporation of water metals get concentrated in the saltpans, thus the organisms residing in such environments are generally exposed to high salinity and high metal concentrations, resulting into the evolution of various survival strategies to counterbalance the environmental stress conditions. The water fed into the Ribandar saltern of Goa is containing high iron content in the water due to anthropogenic activities of iron ore barge transport through Mandovi River. In the saltpan, the iron gets concentrated in different ponds and the bacteria from Ribandarsaltern is expected to tolerate iron. Thus in this study we tried to explore the iron tolerance of SRB and unravel the formation of iron nanoparticle by these SRB. SRB are known to synthesize various metals nanoparticle viz. Cd, Au, Ni, Pd and Ptin the form of metal sulfides.[4,22,23]We could successfully isolate a halophilic SRB from the Ribandar saltern and it was identified to be a representative of genus *Desulfotomaculum*. Based on its physiological and morphological characteristics along with its 16S rRNA gene sequence analysis, the strain WCA1 was found to be closely related to *Desulfotomaculum acetoxidans.* On challenging the SRB with iron, it could sequester it into its nanoform by producing iron sulfide nanoparticles. The electron microscopic observations of the nanoparticle confirmed the nano size with an average diameter of 21nm. The XRD analysis revealed the particle to be crystalline and matching with the $FeS_2$

nanoparticle. This confirmed that the formed nanoparticle is a crystalline $FeS_2$ nanoparticle synthesized by strain WCA1.Ca- alginate bead entrapment is one of the most common method used for immobilization of living cells, bacteria, fungi, also a cost effective techniques.[24-30] The porosity in the bead allows the solute to diffuse into the bead depending on cross linking of Ca-ion and to come in contact with the entrapped materials.[25,30]The SRB synthesized $FeS_2$ nanoparticles were successfully entrapped in Ca-Alginate beads. This reduces the mobility of iron nanoparticle and their self-aggregation. The entrapment doesnotchange the characteristics of the nanoparticle, thus it could be effectively used in remediation of contaminants from water. Cr is an industrially important metal but possess threat to human health and environment due to its toxicity, bioaccumulation properties.In the present study the iron sulfide nanoparticle was used in the uptake of Cr from water withintent to develop an efficient method for ground water remediation. While assessing the Cr-remediation efficiency of the SRB synthesized$FeS_2$ nanoparticle, it was observed that the bead form of the nanoparticle had an overall increase in remediation than the bare form of $FeS_2$ nanoparticle. The bead form reduced the mobility and limits the nanoparticle aggregation within theCa-alginate. The bare form tends to agglomerate which results in decrease in thereactive surface for Cr adsorption. From the study it was found that 96% -99% of Cr could be removed with ease by the entrapped iron sulfide nanoparticle.The Cr removal by the nanoparticle with respect to its contact time showed that the adsorption equilibrium was achieved in 2hours of reaction. In the initial 60 minutesthe metal uptake occurred at a higher rate and later the adsorption rate slowed down and almost approached equilibrium. The increase in Cr removal efficiency from 67% to 99 % was observed with increased nanoparticle concentration from 0.01 $g.L^{-1}$ to 0.25$g.L^{-1}$ but beyond this concentration the Cr adsorption is decreased. Thus the optimum nanoparticle concentration was 0.25$g.L^{-1}$. The higher nanoparticle concentration, increases self-aggregation thus, decreasing the available surface area for Cr adsorption.The optimum Cr removal was observed in acidic pH and at alkaline pH the Cr removal efficiency decreased due to the presence of higher $OH^-$ ions in the reaction mixture. The comparative study of bare and bead form of nanoparticle showed the efficiency of bead form is higher than the bare form for Cr removal, even though their optimum pH remains the same. These bionanoparticles showed promising characteristics in Cr removal, even at pH6-8, it could remediate 80-70% of Cr from the solution. This suggests that bionanoparticles could be ideal candidate for Cr remediation from ground water (pH range 6.5-8.5). There are several reports on chromate adsorption on ferric oxide, hematite, magnetite etc.[31]The SRB strain WCA1 synthesized nanoparticles had a higher Cr removal capacity of 99% when its immobilized form was used, even when compared to previously reported cases.[31-32]As observed in Graph1, the immobilized iron sulfide nanoparticles had a slight advantage over the bare form of iron sulfide nanoparticle in Cr removal efficiency approaching 99%.The bead form provides a promising tool for Cr remediation from ground water.

## CONCLUSION

This study has demonstrated that, hypersaline SRB, *Desulfotomaculum acetoxidans* strain WCA1 isolated from Goan saltpan could synthesize Iron nanoparticles. These nanoparticles were characterized to be $FeS_2$ Nanoparticles of 20nm size. These bionanoparticles were successfully entrapped in Ca-alginate beads without significant reduction in their reactivity. These Bio-nanoparticles could effectively remediate Cr from water while the bead form had an advantage over the bare form in remediating Cr. The alginate entrapped $FeS_2$ nanoparticle provides an easier method for separation and reduces the nanoparticle mobility and enhances remediation efficiency.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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