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# Bioremediation of Cr (VI) by Microbial consortium isolated from an Industrial waste water contaminated site for potential Industrial application

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## Abstract:

Present study involves isolation of bacterial strains with high tolerance to Chromium (Cr (VI)) from industrial waste water contaminated soils. Two isolates SBS1 & SBS3 were found to have Cr (VI) degrading capacity up to 2000 ppm, identified as *Bacillus cereus*, *Bacillus thuringiensis* by 16S rRNA sequencing. The strains when optimized for growth conditions showed optimal growth at 28 - 37 ºC and pH 5 - 7.5 respectively. The strains showed 90 % reduction of 2000 ppm Cr (VI) by 48 h. Bacterial consortium showed high degradation rate of 65 % in sporulation phase in industrial effluent with an initial Cr (VI) concentration of 1500 – 2000 ppm when compared to individual cultures. Upon immobilization of the sporulating consortia in sodium alginate beads, bio-reduction reached to 73 % in 24 h. ChrR encoding chromate reductase gene was detected in the plasmid and genomic DNA of both the cultures indicating its potential importance in bio-reduction. This study signifies the bio-reduction capacity of the bacterial consortia in sporulation phase at extreme Cr (VI) concentrations of up to 2000 ppm. The consortium may be applicable for biological treatment of Cr (VI) contaminated sites for bioremediation.

**Keywords:** Bioremediation, Cr (VI), industrial effluents, batch bioremediation, immobilization.

## 1. Introduction:

The environment faces an escalating burden due to ongoing industrialization and mining activities, leading heavy to metal pollution (Das *et al.,* 2009). The uncontrolled discharge of metals into the environment from these operations poses a significant menace to ecosystems and the health of organisms. Industries such as mining, electroplating, painting, tanning, and textile industries contribute to chromium contamination (Chibuike *et al.,2014*). Chromium exposure is associated with chronic bronchitis, skin irritations, liver problems, renal failure, and lung cancer (Wuana *et al.,*2011).

Tannery industries produce copious amounts chromium containing effluents (Thacker *et al.,*2006; Sayel *et al., 2012)*. Patancheru, located in Hyderabad, Telangana, India stands as a landmark for numerous manufacturing industries that release untreated waste water directly in to the environment (Dasaram *et al.,2011*). This untreated waste mixes up with all the sources of underground water and has detrimental effects on flora and fauna. Chromium (Cr) is known to exist in various oxidation states ranging from $^{+}2$ to $^{+}6$. Of all the oxidation states present Cr (III) & Cr (VI) are most extensively studied (Gupta *et al.,* 2009). Cr (VI) is the most toxic form and has higher O-R potential values and rapid permeability through biological membranes.

Traditional methods are cost effective. Hence biological reduction Cr (VI) using indigenous microorganisms offer a cost effective and environmentally compatible technology. This study proposes a remediation route for detoxification of Cr (VI) using indigenous microorganisms.

## 2. Review of Literature:

Chromium contamination of the environment is of concern because of the mobility and toxicity of Cr (VI). Cr (VI) species are extremely water soluble and mobile in the environment. Traditional methods like Reverse osmosis, Chemical precipitation and



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adsorption are effective for lower concentrations of Chromium (Ahuwalia *et al.*,2007). An efficient and sustainable way achieve metal bioremediation is with the aid of microorganisms such as bacteria present in the contaminated sites which are able to adapt to high concentrations of heavy metals and develop mechanisms for removing heavy metals from the environment (Ndeddy Aka *et al.*,2017). Microbial degradation of Chromium contamination is achieved by two methods. One of the mechanisms involves the efflux of Chromate ions from the cell cytosol while the other entails the direct reduction of Cr (VI) to Cr (III) (Ramirez-Diaz *et al.*,2008). Previous studies have shown that enzymes such as chromate reductase (gene: ChrR) from *Pseudomonas putida* and YieF protein from *Escherichia coli* exhibit the capability to convert Cr (VI) to Cr (III) (Mala *et al.*,2015).

The present study is an attempt to isolate and identify a group of bacteria with high tolerance to Cr (VI) (1000 – 2000 ppm) from industrial waste water contaminated soils of Patancheru & Bollarum in order to be applied in a promising bioremediation system. The outcome of this study will provide an effective solution to address the removal of Chromium from industrial waste water contaminated soils.

## 3. Materials & Methods:

### 3.1 Sample Collection:

Crude soil samples were collected from industrial waste water contaminated sites at Patancheru (17.5396° N, 78.2562° E) and Bollarum (17.5218° N, 78.5110° E) Hyderabad city, Telangana state, India respectively. Soil samples were collected in sterile capped containers, enclosed in sterile bags, brought to the laboratory and stored at 4 ºC.

### 3.2 Enrichment Culturing & isolation of chromium reducing bacteria:

The samples from industrial waste water contaminated sites were enriched in to minimal salt medium containing 100 ppm of Cr (VI) in the form of $K_2Cr_2O_7$ for 24 h at 37 ºC. Samples from the flask were serially diluted and inoculated onto the Nutrient agar medium supplemented with 500 ppm, incubated at 37 ºC for 24 h. Isolates that could resist 500 ppm were selected for further study.

### 3.3: Evaluation of Cr (VI) tolerance & concentration:

To evaluate the tolerance of selected isolates to varying concentrations of Cr (VI), bacterial growth was monitored. The selected isolates were inoculated into 5ml of Luria Bertani broth containing 100 - 2500 ppm of Cr (VI). The tubes were incubated at 37 ºC for 24 - 48 h and growth was monitored at 600 nm. The left-over Cr (VI) in the media was measured using Diphenyl carbazide method (DPC) (Fulladosa *et al.*,2006). The percentage of Cr (VI) reduction was calculated using the following formula

$$\text{Cr (VI) reduction} = C - \frac{S}{C} x\ 100$$

C = Absorbance of Control; S = Absorbance of Sample
The residual chromium in culture supernatants were sent for analysis by ICP-MS.

### 3.4 Identification of Potential isolate by 16sRNA sequencing:

The genomic DNA from the SBS1, SBS3 was isolated by Phenol Chloroform method (Sambrook *et al.*,2006). The isolated DNA samples were resuspended in 200 µl of TE (TRIS- EDTA) buffer and used for amplification of 16s rRNA sequence by PCR. The 16s rRNA gene was amplified using Universal primers: forward primer sequence 5’ ACCGCATAACGT3’ and reverse primer sequence 3’ TTCATACAC 5’. The PCR products were sent for nucleotide sequence analysis at Qzene laboratories, Hyderabad, Telangana, India. The sequences of bacterial isolates were identified by submitting the sequences to NCBI NT Blast & phylogenetic tree is constructed using Mega XI software.

### 3.5 Optimization Studies for pH and temperature:

The Bio - reduction of Cr (VI) is governed by factors like pH & temperature for effective degradation. To investigate this optimization studies were carried out LB appended with 100 ppm of Cr (VI) maintained at pH 5- 9 and inoculated the isolates (SBS1 & SBS3). Similar to pH, optimization of temperature was carried using the above process maintained at 28 - 45º C. Culture was sampled at 0 & 24 h. OD values were recorded at 600 nm.

### 3.6 Batch Bioremediation of Cr (VI) using Industrial effluent:

Batch bioremediation study was conducted in 90 ml industrial effluent. To each flask 10 ml of bacterial cultures SBS1 and SBS3, taken from both exponential growth and sporulation phases were inoculated. Three different treatments were administered: Industrial Effluent with SBS1 Culture (exponential phase), Industrial Effluent with SBS1 Culture (Sporulation phase) and Industrial Effluent with consortium (exponential phase one set and another set in sporulation phase). The flasks were then incubated at 37 ºC for 24 h. Cr (VI) reduction was measured using the DPC method as described.


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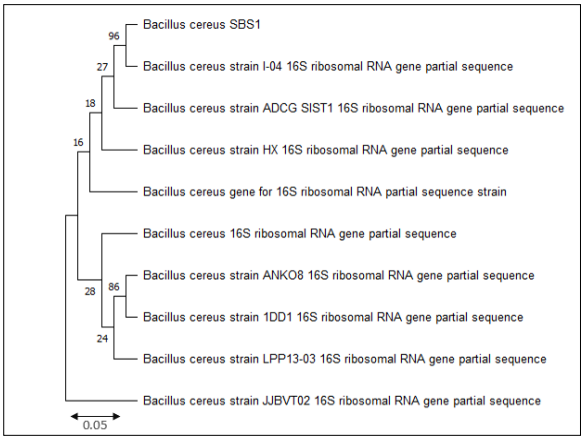
### 3.7 Bioremediation of Cr (VI) using Immobilized Sodium alginate beads of SBS1 & SBS3:

50 ml of 4 % sodium alginate solution was mixed with 50 ml of bacterial culture containing SBS1 & SBS3. The mixture was stirred vigorously for 15 min and droplets of this mixture were then added to a beaker containing 0.05 M $CaCl_2$ $2H_2O$. From this, 25 ml of immobilized beads were added to 100 ml of Cr (VI) incubated at 37ºC. Samples were collected at 3 h intervals for 24 h and analyzed for left over Cr (VI) using DPC method (Kumar *et al.*,2006).

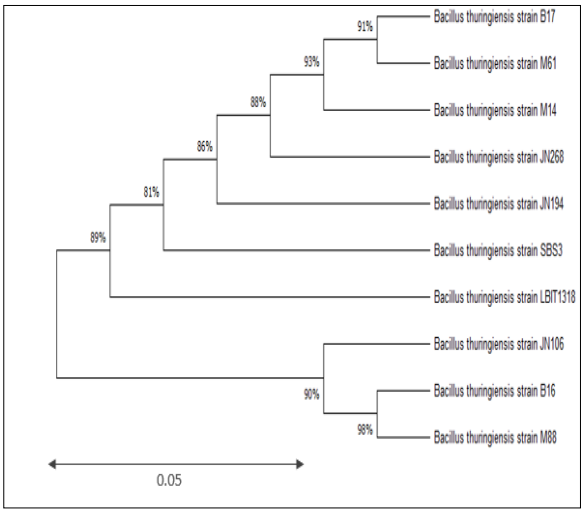
## 4. Results:

### 4.1 Isolation and identification of potential Cr (VI) reducing Bacteria:

Ten isolates were selected from LB plates inoculated with industrial waste water collected from local industrial sites. These isolates were inoculated in to the medium containing Cr (VI) in the concentration range of 500 - 2000 ppm.Two isolates namely SBS1 & SBS3 showed appreciable growth at high concentrations of 2000 ppm and hence were selected for further studies. Bacterial isolates were amplified and identified by 16srRNA sequencing. The DNA sequences of two isolates were submitted to NCBI database. Isolate SBS1 showed 99 % identity with *Bacillus cereus* and isolate SBS3 showed 99 % identity with *Bacillus thuringiensis*. The isolates were named as *Bacillus cereus* SBS1 (Accession no. PP922329) and *Bacillus thuringiensis* SBS3 (Accession no. PP922349). The sequence resemblance & phylogenetic tree were constructed and depicted in fig 1.




***a) Bacillus cereus SBS1***




**b) *Bacillus thuringiensis SBS3***

**Fig 1: Identification of Cr (VI) degrading bacterial strains by Neighborhood joining method**

### 4.2 Evaluation of Cr (VI) tolerance & concentration:

Two strains showed staggering Cr (VI) tolerance and were able to degrade even at 2000 ppm. SBS3 showed better growth at 2000 ppm and SBS1 could tolerate up to 1500 ppm. (fig 2). The concentration of Cr (VI) in LB broth after incubation was determined using DPC method. The reduction of Cr (VI) by two isolates was evaluated at concentrations of 1000 - 2500 ppm after 120 h of incubation using LB broth. Isolate SBS1 demonstrated the ability to reduce 1500 ppm of Cr (VI) to 86 % within 48 h, while isolate SBS3 achieved a reduction of 2000 ppm of Cr (VI) to 90 % within the identical time span (fig 3). ICPMS analysis confirmed these findings (Table 1).

**Table 1. ICP MS analysis of Cr (VI) concentration:**

| S. No. | Bacterial isolates | Cr (VI) Concentration in ppm | Left over Cr (VI) concentration in ppm | % of degradation |
|---|---|---|---|---|
| 1. | *Bacillus cereus SBS1* | 1500 (Control) | 1158.123 | 79.9% |
| | | 1500 (Test Sample) | 232.243 | |
| 2. | *Bacillus thuringiensis SBS3* | 2000 (Control) | 1710.50225 | 72% |
| | | 2000 (Test Sample) | 473.031 | |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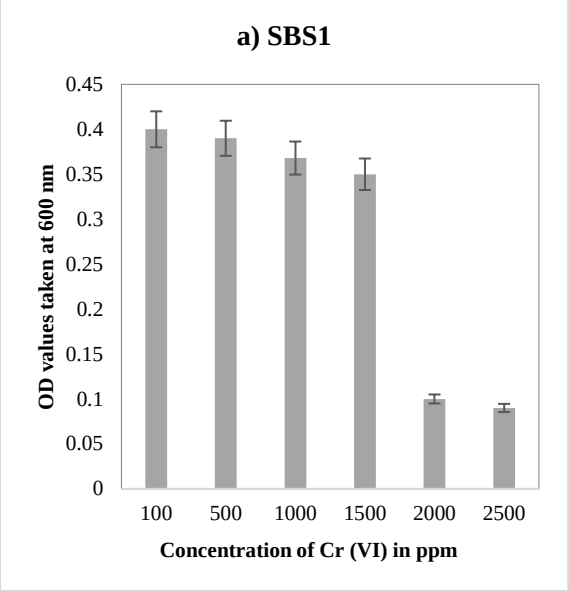


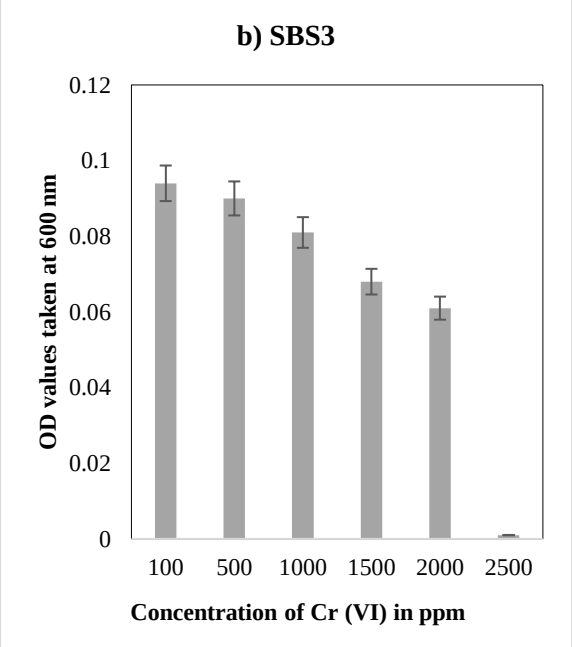
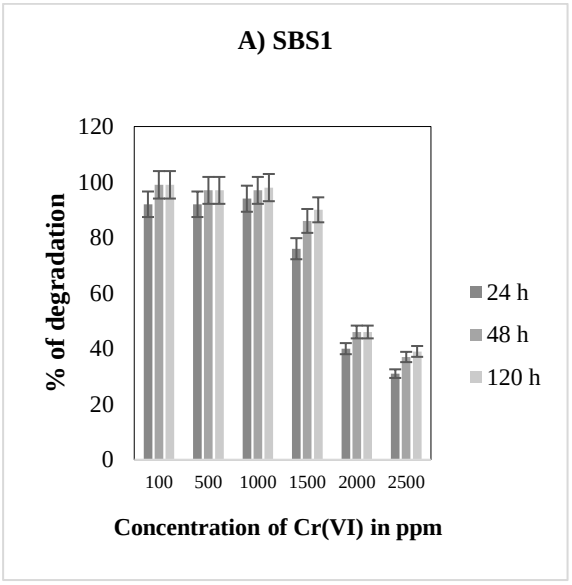




**Fig 2: Estimation of tolerance of bacterial strains SBS1 and SBS3 to Cr (VI)**

Bacterial strains SBS1 and SBS3 were incubated at varying concentrations of Cr (VI) overnight and absorbance of the cultures was measured at 600 nm.



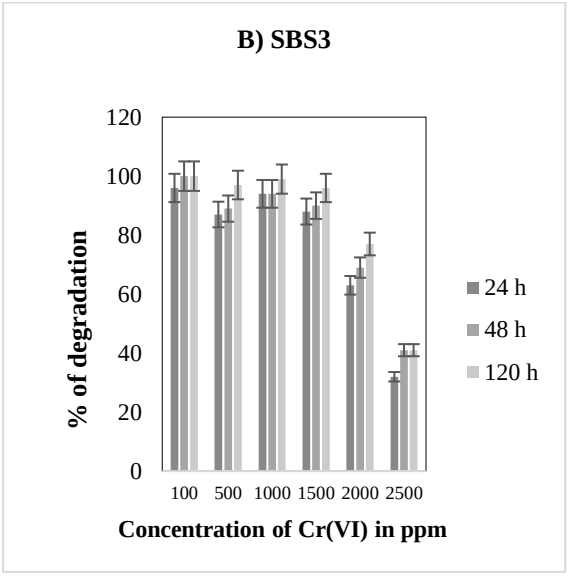




**Fig 3: Estimation of Cr (VI) concentration**

Bacterial strains SBS1 and SBS3 were incubated at varying concentrations of Cr (VI) for 24 - 120 h and percentage of Cr (VI) degradation was estimated by DPC method.

## 4.3 Optimization of growth parameters:

Optimum growth conditions for SBS1 & SBS3 were studied by cultivating them at temperatures ranging from 28 - 45º C and pH range of 5 – 9. Both the isolates showed appreciable growth in the range of 28 - 37º C and pH 5- 7 (Fig 4,5).



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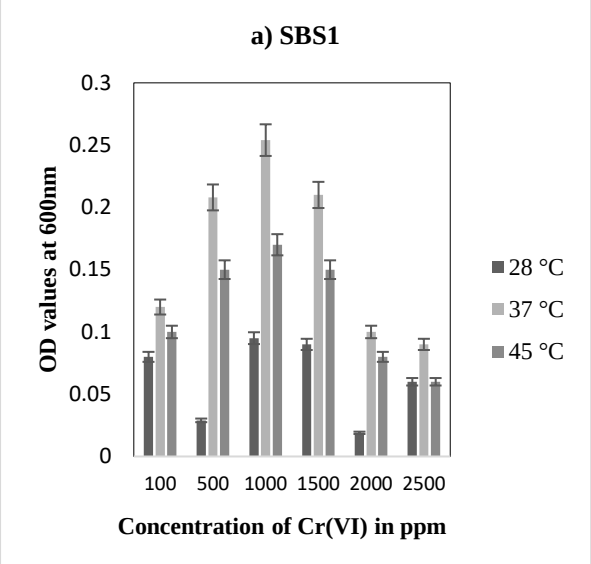


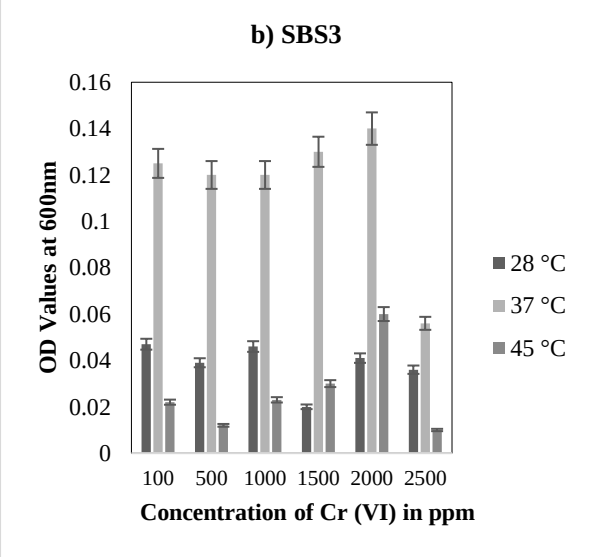
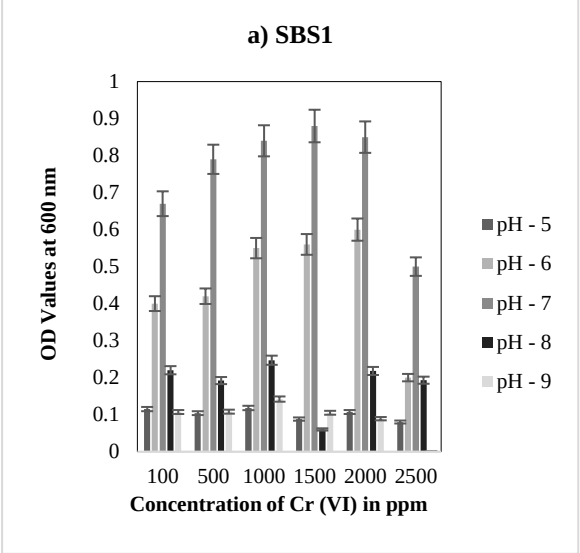




**Fig 4: Optimization of incubation temperature for Cr (VI) degrading strains SBS1 and SBS3**
SBS1 and SBS3 were grown in LB broth overnight at varying temperatures. After incubation, absorbance of the cultures was measured at 600 nm.



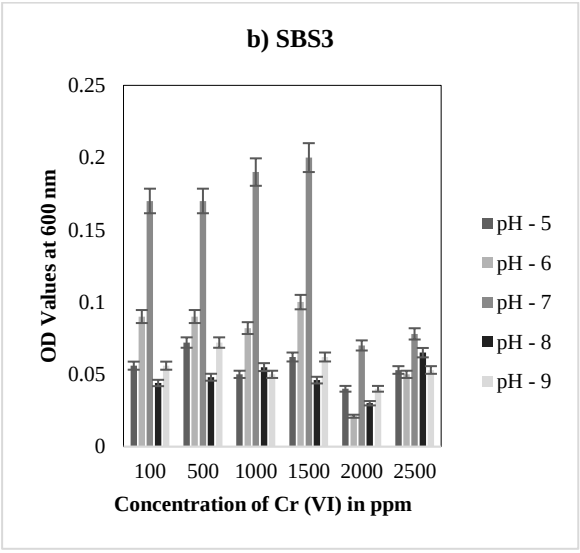




**Fig 5: Optimization of pH for Cr (VI) degrading strains SBS1 and SBS3**
SBS1 and SBS3 were grown in LB broth overnight at varying pH and absorbance of the cultures was measured at 600 nm.

## 4.4 Identification of chromium degrading genes:

The reduction of Cr (VI) can be carried out either by enzymes or metal transporters. ChrR gene in particular, is known to be responsible for chromium degradation. After extraction of plasmid and genomic DNA from SBS1 & SBS3, PCR products of the plasmids showed amplification of ChrR gene at 500 bp (fig 6). The sequence of the ChrR of SBS1 showed 78.9 % homology with ChrR of *Shigella flexneri* while of SBS3 showed 68 % homology with ChrR of *Escherichia coli* (Sanjay and Mohamed *et al.*,2020).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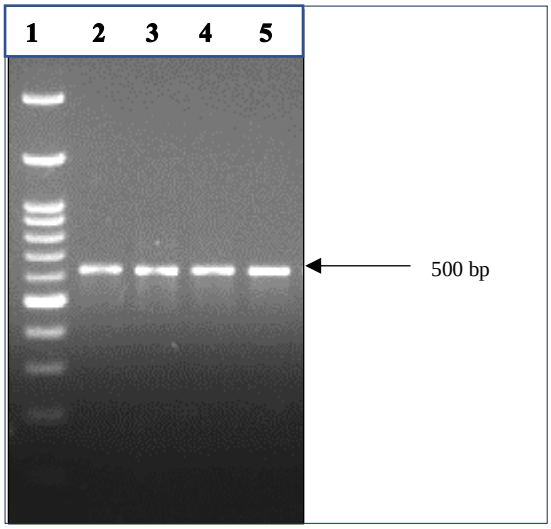


**Fig 6: Amplification of ChrR gene from plasmid and genomic DNA of SBS1 and SBS3**
Overnight cultures of SBS1 and SBS3 grown in LB broth were processed for isolation of genomic and plasmid DNA. 100 ng of DNA was used to amplify ChrR gene by polymerase chain reaction. The amplified products were electrophoresed on 1 % agarose gel and visualized by Ethidium bromide stain in a UV transilluminator. Image shows a 500 bp DNA fragment corresponding to ChrR gene. Lane 1: 100 bp ladder; Lane 2: SBS1 Plasmid DNA; Lane 3: SBS3 Plasmid DNA; Lane 4: SBS1 Genomic DNA; Lane 5: SBS3 Genomic DNA

## 4.5 Batch Bioremediation of Cr (VI) in industrial effluents

Local industrial effluents were collected to study remediation of Cr (VI) using SBS1 and SBS3. To test the efficacy of the strains, 1500 – 2000 pm of Cr (VI) was added externally to industrial effluents. It was observed that the strains in sporulating phase showed enhanced Cr (VI) degradation compared to log phase cultures. SBS1 strain exhibited a degradation of 37 % and 45.7 % in log and sporulation phases, respectively. SBS3 strain showed degradation rates of 42 % and 45.7 % in log phase and sporulation phase respectively. The mixed consortium of isolates exhibited slightly higher degradation rates of 58 % in log phase and 65 % in sporulation phase. These results emphasize the significance of sporulation phase and synergistic activity of the mixed consortium in achieving higher levels of degradation (Table 2).

**Table 2: Percentage of Cr (VI) reduction in Industrial effluents with various treatments**

| Treatments | % Cr (VI) reduction |
|---|---|
| Industrial Effluent | 48 ppm |
| Industrial Effluent + Log phase culture (SBS1) | 97 |
| Industrial Effluent + Sporulation phase culture (SBS1) | 98 |
| Industrial Effluent + Log phase culture (SBS3) | 99 |
| Industrial Effluent + Sporulation phase culture (SBS3) | 100 |
| Industrial Effluent + Cr (VI) 1500 ppm + Log phase culture (SBS1) | 37 |
| Industrial Effluent + Cr (VI) 1500 ppm + Sporulation phase culture (SBS1) | 45.7 |
| Industrial Effluent + Cr (VI) 2000 ppm + Log phase culture (SBS3) | 42 |
| Industrial Effluent + Cr (VI) 1500 ppm + Sporulation phase culture (SBS3) | 45.7 |
| Industrial Effluent + Cr (VI) 1500 ppm + Log phase culture (SBS1 & SBS3) | 58 |
| Industrial Effluent + Cr (VI) 1500 ppm + Sporulation phase culture (SBS1 & SBS3) | 65 |

## 4.6 Bioremediation of Cr (VI) using Immobilized Sodium alginate beads of SBS1 & SBS3

Upon testing the efficacy of SBS1 and SBS3, further analysis was carried to design a recyclable method of Cr (VI) bioremediation. A formulation for entrapment of the cells was carried out. SBS1 and SBS3 in sporulating phase were collected and immobilized in sodium alginate beads, exposed to 1500 – 2000 ppm Cr (VI) in saline. The bio-reduction efficiencies of immobilized sodium alginate beads of bacterial isolates are summarized in Table 3. A consistent decline in the Cr (VI) concentration over a period of 3 – 24 h was observed. Interestingly, bio-reduction efficiency was slightly elevated during sporulation phase and in mixed consortium. Mixed consortium exhibited 60 % of bio reduction in 18 h during sporulation phase which reached 73 % by 24 h.


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**Table 3: Percentage of Cr (VI) reduction by immobilized sodium alginate beads of strains**

| | % Cr (VI) reduction | | | | | |
|---|---|---|---|---|---|---|
| **Time (h)** | **T1** | **T2** | **T3** | **T4** | **T5** | **T6** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 2.5 | 15 | 4 | .9.6 | 8 | 10 |
| 6 | 7.5 | 28 | 7 | 13 | 18 | 24 |
| 9 | 15 | 30 | 7.5 | 23 | 21 | 29 |
| 12 | 25 | 40 | 20 | 28 | 28 | 36 |
| 15 | 40 | 42.5 | 26 | 33 | 38 | 49 |
| 18 | 43 | 43 | 35 | 35 | 54 | 60 |
| 21 | 52 | 47.5 | 42 | 39 | 58 | 66 |
| 24 | 55 | 62.5 | 46 | 53 | 64.5 | 73 |

T1: *B. cereus (*log phase); T2: *B. cereus* (sporulation phase); T3: *B. thuringiensis* (log phase); T4: *B. thuringiensis* (sporulation phase); T5: Consortium (log phase); T6: Consortium (Sporulation phase).

## Discussion:

Cr (VI) is a highly toxic heavy metal frequently found as an environmental contaminant (Zayed *et al.,*2003). Application of biological systems offers a safer alternative method for bio-reduction of Cr (VI) to conventional chemical methods (Mukherjee *et al.,*2015). In the present study optimization of two bacterial strains *Bacillus cereus SBS1* & *Bacillus thuringiensis SBS3* for Cr (VI) bio reduction was carried out. A large number of Cr (VI) tolerant strains have been previously reported. Among these *Bacillus sphaericus* is known to tolerate up to 800 ppm of Cr (VI) concentrations (Sinha *et al.,*2012). In this study, strains SBS1 & SBS3 were observed to be tolerant to up to 1500 - 2000 ppm Cr (VI) concentrations. *Bacillus spp.* is known to reduce 50 ppm and 100 ppm of Cr (VI) to 100% within 12 to 48 h, while *S. capitis* reduced 100 ppm of Cr (VI) by 29% in 24 h of incubation. (Masood *et al.,*2011; Zahoor. A *et al.,*2009). Isolates SBS1 & SBS3 in this study exhibited higher Cr (VI) efficiency at extreme Cr (VI) concentrations with reduction times of up to 48 h.

SBS1 & SBS3 in this study showed optimum growth at pH 5 - 7.5. and temperature range of 28 – 37 $^{o}$C. A previous study reported *Bacillus cereus IST 105* with highest growth and reduction at pH 7 (Naik *et al.,*2012). SBS1 & SBS3 showed the presence of ChrR gene. Similarly, presence of ChrR gene in *Phormidesmis molle* and *Bacillus cereus* strains has been reported by other groups (Rathnayake *et al.,*2013; Sundar *et al.,*2010).

Intensive research has been carried out for evaluation of Cr (VI) reduction by potential bacterial strains in LB broth (Das *et al.,*2014). Although, few strains are known to be successful in bio-reduction trials in industrial effluents. In the current study ability of the bacterial isolates SBS1 & SBS3 was tested at pilot scale using the local industrial effluent appended with excess Cr (VI). Interestingly, when used individually, spore phase of the both SBS1 and SBS3 reduced Cr (VI) by 45 % in 24 h and when used in consortium, 65 % reduction of Cr (VI) was observed. Comparatively, when used in vegetative state, the consortium could show 58 % reduction, indicating that utilizing the consortium in spore phase has slightly higher efficiency. Since the strains were able to tolerate the effluent conditions well in spore form, the strains hold promise for utilization in environmental scenarios where water bodies may be contaminated with industrial effluents. Similar findings have been observed in studies involving *Staphylococcus capitis and Bacillus spp JDM-2-1* in industrial wastewater, where up to 89% reduction of Cr (VI) was seen with initial concentrations of 100 ppm (Zahoor *et al.,*2009). This is a first report of degradation of Cr (VI) using mixed sporulated consortium (Sporulation phase) of bacterial strains with extreme concentration of up to 2000 ppm in effluents. This indicates that the strains have potential tolerance to high concentrations of Cr (VI) in sporulation phase and therefore may be utilizable in natural water bodies contaminated with effluents. Also, sporulation of the bacteria may help them survive for longer duration in the water bodies while also reducing toxic concentration of Cr (VI).

Biosorption using various adsorbents have been proven effective for bioremediation. One of the most widely studied naturally occurring biopolymers is polysaccharide-based bio sorbents such as sodium alginate. In this study, entrapment and biosorption of SBS1 and SBS3 in sporulation stage resulted in effective remediation of Cr (VI).

## 5. Conclusion:

Two Cr (VI) resistant bacteria *Bacillus cereus SBS1 & Bacillus thuringiensis SBS3 isolated* from industrial


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effluents and waste water contaminated sites were shown to have highest tolerance of up to 1500 & 2000 ppm Cr (VI). The remediation of Cr (VI) was observed to be due to the presence of cytosolic chromate reductase in the strains. Batch bioremediation and immobilization of the consortium in sodium alginate beads with industrial effluent samples showed efficient Cr (VI) degradation even at high initial concentrations of 2000 ppm. In conclusion, high efficiency of the two strains could serve as potential source to eradicate the toxic chromate from metal contaminated water bodies.

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