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Sodium Alginate/Polyvinyl Alcohol Immobilization of *Brevibacillus brevis* OZF6 Isolated from Waste Water and Its Role in the Removal of Toxic Chromate

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Authors' contributions

Author PAW designed and planned all the experiments and methods of the present study and also wrote this article, authors ANO, NR and OOS performed the tests under in vitro conditions, author IAW did the analytical analysis whereas author SW did statistical analysis, made the graphs as well as collected all the literature for performing the tests and for writing the paper. All the authors read and approved the final manuscript.

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ABSTRACT

Aim: In this study we wanted to determine bacteria for chromium (VI) removal under pH, chromium concentration, carbon source and immobilizing agents.
Place and Duration of Study: This study was carried out in the Department of Biological Sciences, College of Natural and Applied Sciences, Crescent University, Abeokuta, Nigeria in the year 2015.

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Methodology: Isolation of bacteria was done from industrial waste water of Abeokuta, Nigeria which is often released into water bodies and thus contaminates water during 2015. Nutrient agar added with 100 µg/ml Cr (VI) was used to isolate resistant bacterial strains. Resistance of the strains for Cr (VI) was evaluated on nutrient agar media. Natural material [sodium aliginate (SA)] and synthetic material (PVA)] immobilized bacterial cells for Cr (VI) removal experiment was done by 1, 5–diphenyl carbazide method.

Results: The strain OZF6 was characterized as *Brevibacillus brevis* using 16S rRNA gene sequence. All isolates (8 strains) were tolerant to chromium (VI). Among all strains, only *Brevibacillus brevis* OFZ6 reduced Chromium (VI). *Brevibacillus brevis* OZF6 reduced maximum Cr (VI) (72.5%) at pH 7. *Brevibacillus brevis* OFZ6 also reduced chromium (VI) significantly under various concentrations of chromium. *Brevibacillus brevis* OZF6 detoxified the metal 81% at 50 µg Cr/ ml, 75% at a concentration of 100 µg/ ml and 68% at 150 µg/ ml respectively. Among electron donors, maximum reduction was observed under influence of lactose. Among different matrices combinations for whole cell immobilization of OZF6, combination of 10% PVA, 10% sodium alginate (SA) proved to be best combination for Cr (VI) reduction.

Conclusion: Due to above properties, bacteria will be utilized for Cr (VI) detoxification in contaminated industrial waste water and thus will protect environment from contamination. There needs a proper regulation and treatment of these effluents prior their release into water bodies or into soil and thus ultimately protect population from carcinogenesis and other ill hazards.

Keywords: Chromium (VI) tolerance; Brevibacillus brevis; Chromium (VI) reduction; Immobilization; electron donors.

1. INTRODUCTION

Use of Cr (VI) from different industries contaminates our biosphere [1-3]. Chromium has mainly two forms which are Cr (III) and Cr (VI), decreases the number of the microbes and also their growth [4]. Toxic and carcinogenic effect of hexavalent chromium is through solubility of chromium (VI) and may damage proteins and nucleic acids because they have high affinity for proteins and nucleic acids [5,6]. When microbes convert the toxic chromium Cr (VI) to stable and less soluble Cr (III), and thus can be used for the detoxification of Cr (VI) [7]. The conversion of Cr (VI) to Cr (III) by bacteria is therefore less expensive and safe method to save our soil and water from the toxic effect of these metals. Detoxification of the metal is studied in *Bacillus* sp. [8,9] *Pseudomonas* sp., [10,11] *Escherichia coli*, [12] *Microbacterium*, [13] *Ochrobactrum intermedium* [14] and *Micrococcus* [15].

Chromium (VI) removal by microorganisms may occur by direct or indirect means and is influenced by pH, concentration, incubation period and different types of microbes. In the direct mode it is the chromium reductases which may remove chromium (VI) from the medium while as in case of indirect method, it is the reductants or oxidant, such as H_2S, which may remove chromium from the medium [16]. Chromium (VI) conversion is either aerobic or anaerobic when electron donors are supplemented in the medium or in the presence of cell extracts but not both. Conversion of Cr (VI) to Cr (III) by reductases is anaerobic [17], aerobic [18], also by both anaerobically and aerobically [19]. Detoxification of Cr (VI) is through reductases found attaced to the membrane as studied in *Pseudomonas fluorescens* and *Enterobacter cloacae* [20]. Insoluble precipitate is formed due to detoxification of the metal from higher form (Cr (VI)) to lower form (Cr (III)), which will be washed away easily from wastewater [7]. Many researchers have purified and characterized reductase in *P. ambigua* [21] and *Bacillus* sp. [22]. In a study the researchers purified and characterized soluble chromate reductase from a bacterium *P. putida* [23]. Reduction depends upon NADH- or NADPH. H_2S produced Cr (VI) reduction is done under anaerobic condition by the microbes found in soils containing abundant sulfate [24]. Microbes convert sulfur to hydrogen sulfide in soils and effluents which are rich in sulfate and is precipitated easily as FeS [25]. Microbial production of Fe (II) and H_2S, are good for the detoxification of Cr (VI) [26].

Generally free cells are used for reduction of chromium in laboratories from the industrial effluents [27,28], but in industries these free cells can not achieve the goal as they will not be able to separate biomass/effluent. The above difficulty will be overcome by using immobilized cells as they can be used again and again and

regenerate with solid-liquid separation [29,30]. The high toxic effect of Cr (VI) is neutralized when cells are immobilized using different immobilizing agents and thus improve their cellular activities compared to free cells. Immobilized cells can be used in continuous or stirred bioreactors for converting higher form of chromium to lower and less toxic chromium [13,31]. Bacteria can be immobilized on various matrixes such as agar, alginate, polyacrylamide etc [32] and varies with their choice with the type of microorganisms. Combined supporting materials are an important factor for immobilization [33]. Present study was thus designed to (1) check the resistance of bacteria to Cr (VI), (2) to study the detoxification of Cr (VI) under the influence of pH, chromium and donors of electrons (3) check sodium aliginate/polyvinyl as an immobilizing matrix for Cr (VI) removal (4) check the reduction in both batch and fed batch conditions.

2. MATERIALS AND METHODS

2.1 Waste Sample Collection

In this study waste water was collected from the contaminated waste water of alloy manufacturing industry of the industrial areas of Abeokuta, Ogun state, Nigeria which is generally released into the river water and results in accumulation of the metals into water.

2.2 Isolation of Bacteria

Bacteria were isolated on nutrient agar plates from the industrial waste water containing mixture of metals including chromium of industrial area of Abeokuta amended with100 µg/ml Cr (VI) by spread plate method. Dilution of waste water was done by adding 10 ml into 90 ml NSS (normal saline solution). 0.1 ml of dilution factor was spreaded on agar plates and the media was incubated at a temperature of 28±2 ℃ for a period of about 24hrs. The bacteria which growed on the nutrient agar were streaked on the same medium so that the colonies will get purified and they were then maintained on the same medium for other studies. Bacterial strain was identified by morphological, cultural and biochemical methods [34].

2.3 16S rRNA Identification of Bacteria

Strain OZF6 was further identified by 16S rRNA.16S rRNA sequencing of strain OZF6 was performed by Macrogen Inc., Amsterdam, Netherlands using 785F (5′CCAGCAGCCGCGGTAATACG3′) and 907R (5′TACCAGGGTATCTAATCC3′) primers. Similar sequences were recognized by nBLAST present at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) for the identification of strain OZF6.

2.4 Assay of Tolerance to Chromium (VI)

Tolerance of resistant strains against different concentrations of chromium (VI) (0-1000 µg/ml) was carried out on nutrient agar plates [34] inoculated with 10^8 cells/ml and were kept at 28±2℃ for a period of three days. The maximum Cr (VI) concentration which was supporting growth of the bacterial isolates was known as maximum resistance level (MRL). Experiments were performed three times.

2.5 Reduction of Hexavalent Chromium by Free Cells

This experiment of reduction of Cr (VI) was performed under the influence of pH. In this experiment we adjusted the pH of the media here in this case the nutrient broth (NB) with 5, 6, 7, 8 and 9 which was supplemented with 100 with µg/ml of chromium (VI) and the medium was incubated at a temperature of 28±2℃ for a period of 120 h. In second experiment we checked the performance of the bacterial strain OZF6 for chromium (VI) reduction under the influence of 0, 50, 100 and 150 µg/ml of chromium (VI) in nutrient broth and samples were incubated for a period of 120 hours at a temperature of 28±2℃. Strain was centrifuged at 6000 rpm for 10 min at 10°C for chromium reduction and remaining amount of Cr (VI) was detected by 1, 5 – diphenyl carbazide method [35] up to 120 h.

2.6 Effects of Carbon Sources on Hexavalent Cr (VI) Reduction

In order to study the role of carbon sources on reduction by the resistant bacterial strains, 100 ml NB was added with100 µg/ml of Cr (VI)) and the medium was grown upto a period of18 hours. Over night grown bacteria were then centrifuged at 6,000 rpm min^{-1} for 20 min at 4℃, which was then washed two times with 10 mMTris–HCl (pH 7.0), then this medium was again suspended in 100 ml of Tris–HCl buffer and then were added with 0.2 mM K2Cr_2O_7. Carbon sources were then added to the bacteria culture (10 ml) which were incubated at a temperature of 37℃ up to a period of 6 h. Nutrient broth acted as control whereas negative control were the bacteria

which were killed at a temperature of 100℃ for 10 min. Solutions were centrifuged at 6,000 rpm min^{-1} for 20 min at 4℃ and Cr (VI) remaining was calculated as above.

2.7 Effect of Bacterial Immobilization on Chromium Reduction

Various natural materials like sodium aliginate and synthetic material such as polyvinyl alcohol concentrations immobilized bacterial cells to see their effect on Cr (VI) reduction. Reduction was checked as per the above method. Combinations (natural and synthetic materials) were designed as follows: 0.5 g of sodium aliginate (2.5%) in 0.5 g of PVA (2.5%), 1 gm of sodium aliginate (5%) in 1 g of PVA (5%) and 1.5 g of sodium aliginate (10%) in 1.5 g of PVA (10%). Preparation of beads was performed as follows: (1) Both synthetic and natural materials such as PVA and sodium alginate (in combination) were added into 20 ml of deionized water which was heated at a temperature of 80^0 C in order to dissolve the SA and PVA; (2) After dissolution solution was cooled to 40^0 C; (3) Cooling was followed by the addition of about 1 g (fresh weight) of bacterial cells and was mixed throughly; (4) In order to get cell beads, prepared solution was added into the solution of degassed boric acid (50 ml) which is added with calcium chloride as drops at a concentration of 2% (w/v), and then kept immersed upto a period of 24 h. We then wash the beads with 100 ml sterile distilled water (three times) and add these beads to 100 ml NB medium containing 100 µg/ml $K_2Cr_2O_7$. The above solution was then incubated at a temperature of 37℃. Chromium (VI) content was detected as per the above method.

2.8 Reduction of Chromium (VI) in fed Batch Experiments

For fed-batch experiments, bottles containing NB (100 ml) having Cr (VI) at a concentration of100 µg/ml was added with free and immobilized OZF6 cells (1 g wet weight). The bottles were kept a temperature of 30℃. From bottles sample solution taken and checked for Cr (VI) reduction. When Cr (VI) almost finished in the medium, it was again added with fresh NB broth which was sterilized (100% exchange) and was containing Cr (VI). The Cr (VI) remaining in the samples were determined as mentioned above.

2.9 Statistical Analysis

The results of my study were analyzed by analysis of variance (ANOVA) and the calculation of LSD was performed at 5% level of probability. Means of the results obtained were compared byTukey test.

3. RESULTS

3.1 Morphological, Biochemical and Molecular Characterization of Bacteria

Strain OZF6 was found to be G +ve, rod shaped, aerobic, mortile and produces glossy, butyrous, cream coloured colonies on nutrient agar medium. Strain OZF was found to be catalase +Ve, oxidase +Ve, triple sugar iron agar +Ve and was able to hydrolyze gelatin. The bacterial strain OZF6 utilized D-glucose, D-fructose, maltose, glycerol, mannitol and rhibose. Bacterial strain OZF6 did not ferment D-mannose, L-arabinose and erythritol. The strain was negative for H_2S production, urea, starch hydrolysis, indole production, Voges- Proskauer and could utilize citrate. On the basis of the above properties the bacterial strain OZF6 was found to be *Brevibacillus* and 16S rRNA sequence confirmed that the strain OZF6 belongs to *Brevibacillus brevis* and exhibited 99% similarity with *Brevibacillus brevis* (DZBY12). 16S rRNA gene sequencing of OZF6 is deposited inNCBI and was given the accession number as KX276151. The other strains (OZF 1, 2, 3, 4, 7 and 8) were identified using physiological, morphological and biochemical tests.

3.2 Bacterial assay for Cr (VI) Tolerance

Tolerance was studied for bacterial isolates against Cr (VI) on nutrient agar medium (Fig. 1). Bacterial strains varied with regard to their tolerance. It was OZF5 and OZF6 who showed maximum tolerance to Chromium (VI) (1000 µg/ml).

3.3 Effects of pH and Chromium (VI) Concentration on Cr (VI) Removal

This study was designed to determine whether the bacterial strain OZF will detoxify the most toxic Cr (VI) or not. This study confirmed that *Brevibacillus brevis* OZF6 significantly reduced chromium (VI). This study was conducted to (i) show whether there was reduction in the presence of pH and (ii) check effect of various concentrations of Cr (VI) on the removal of Cr (VI).

Detoxification of chromium (VI) under the influence of pH is shown in (Fig. 2). *Brevibacillus brevis* OZF6 significantly removed Cr (VI) at different pH and it was found that highest reduction was found to be at pH 7.0 (72.5). Strain OZF6 similarly removed C(VI) significantly at pH 5 (27.5), pH 6 (55%), pH 8 (65%) and at pH 9 (32.5%) respectively in nutrient agar medium amended with 100 µg Cr/ ml after five days.

Next in this study we performed whether there is any effect of concentrations of the metal [Cr (VI] on the removal of chromium in nutrient broth by the bacterial strain OZF6 (Fig. 3). As the concentration of the metal increased chromium (VI) removal decreased. Strain *Brevibacillus brevis* OZF6 reduced Cr (VI) significantly and maximum reduction occurred at 120 hour of incubation (Fig. 3) at 50 µg/ml of chromium. OZF6 reduced chromium (VI) 81% 50 µg Cr/ ml, 75% at100 µg/ ml chromium (VI) and 68% at 150 µg Cr/ ml respectively.

3.4 Role of Electron Donors for Reduction

In this study we checked chromium (V) removal by *Brevibacillus brevis* OZF 6 under the influence of different electron donors which significantly reduced Cr (VI) (Fig. 4). Bacterial strain OZF 6 reduced maximum amount of the metal when sucrose was added to the solution, which was followed by methanol thus confirmed that chromium (VI) is maximally reduced when solution was containing sucrose.

3.5 Role of Immobilization for Metal Detoxification

In this study, *Brevibacillus brevis* OFZ6 was studied for chromium (Cr) reduction when the strain was immobilized by sodium aliginate and PVA compared to free cells after 120 hours of incubation (Fig. 5). All the combinations of sodium aliginate and PVA, significantly removed the metal in comparision to the bacterial cells which were in free state (control cells) (Table 1). Among different matrices combinations, the combination of 1.5 g PVA, 1.5 g sodium alginate was the best combination for Cr (VI) removal in nutrient broth. Bacterial strain reduced a lot of Cr (VI) when OZF6 was immobilized by 1. 5 g PVA, 1.5 g sodium aliginate compared to the other combinations of 0.5 and 1.0 g PVA and SA. Concentration of 1.5 g PVA, 1.5 g SA, showed an increase of 12.5% in Cr (VI) reduction by *Brevibacillus brevis* OZF 6, compared to free cells after 120 hours of incubation.

3.6 Reduction of Cr (VI) in Fed Batch

In this study we saw the effect of free and immobilized OZF6 for removal of Cr (VI) (Fig. 6) which was added to the medium after 5 days of interval upto a period of 15 days. There was almost complete reduction of chromium (VI) in each batch. *Brevibacillus brevis* OZF 6 reduced more than 80% of Cr (VI) when the strain was immobilized by 1.5 g of PVA, 1.5 g SA in first batch compared to free cells. In the second cycle i.e after ten days of incubation, Cr (VI) reduction decreased compared to the first cycle, but the decrease was very less, but reduction was sustained in the second cycle. Same trend was observed in the third cycle (after 15 days of incubation), but there was little more decrease in reduction compared to first and second cycle. This study confirmed that there was sustained removal chromium.

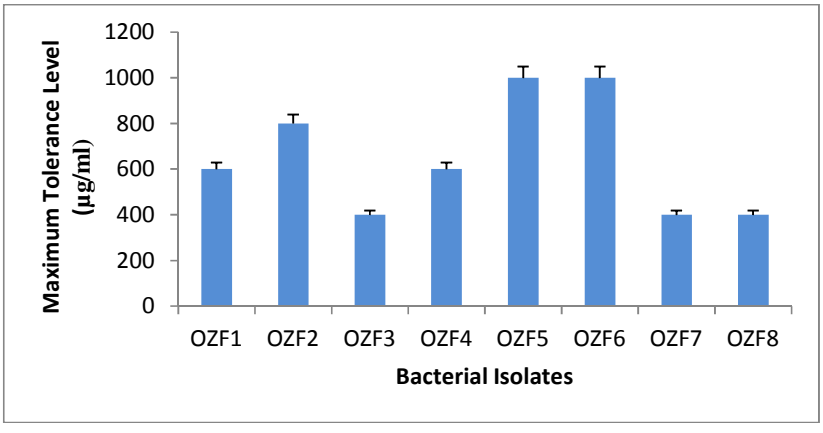

Fig. 1. Tolerance of bacterial strains to chromium (VI)

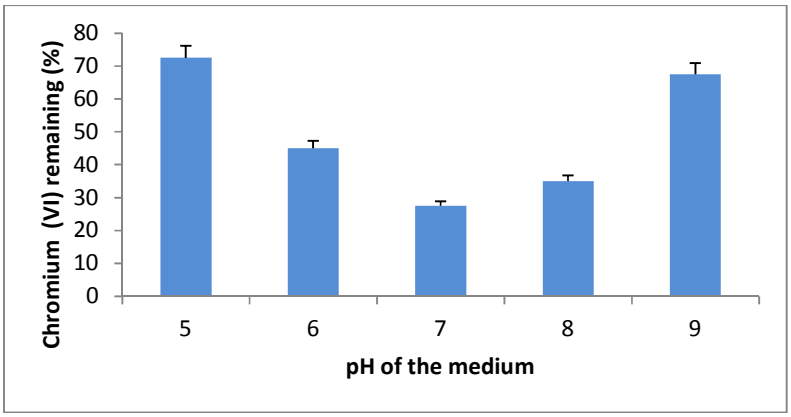

Fig. 2. Cr (VI) reduction by OZF6 at different pH after 120h of growth

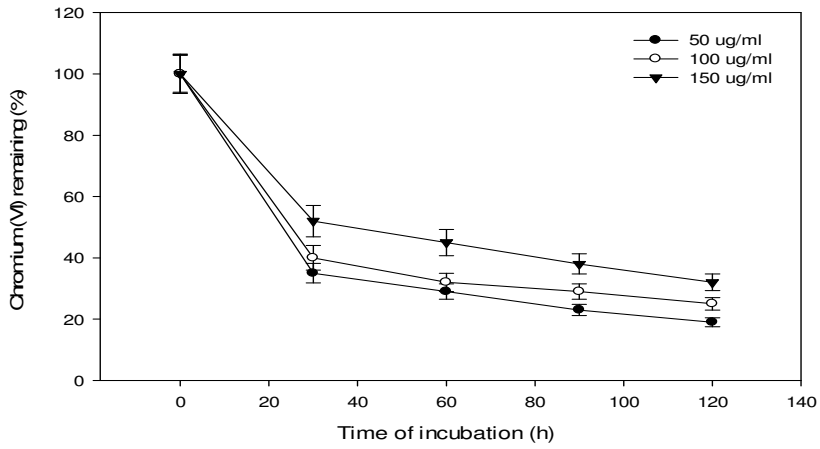

Fig. 3. Reduction of chromium (VI) by the bacterial isolate OZF6 under the influence of chromium

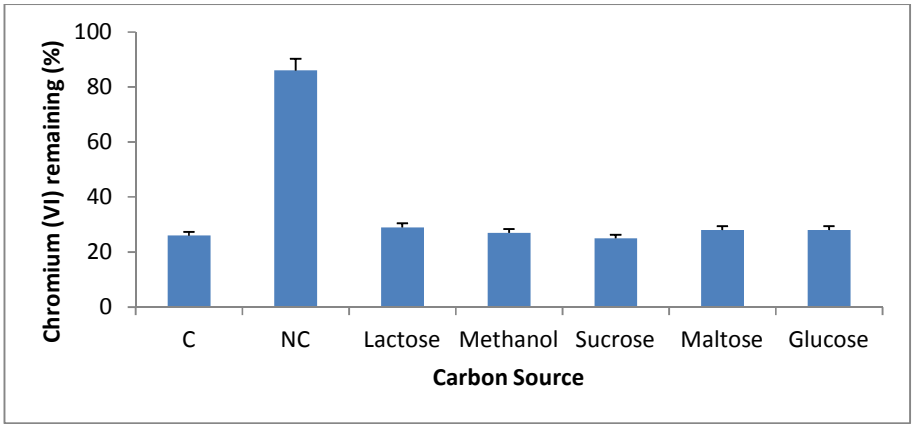

Fig. 4. Chromium (VI) reduction by bacterial strain OZF6 in the presence of carbon sources

4. DISCUSSION

Morphological, biochemical and 16S rRNA studies confirmed bacterial strain OZF6 belongs to *Brevibacillus brevis*. In the present study bacterial strains showed different tolerance to Cr (VI) and *Brevibacillus brevis* OZF6 was tolerant upto 1000 µg/ml. Many studies showed the

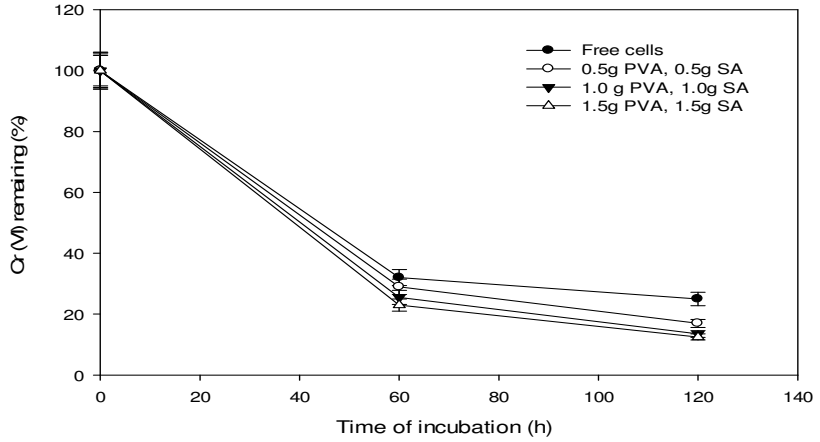

Fig. 5. Reduction by strain OZF6 under both free and immobilized state

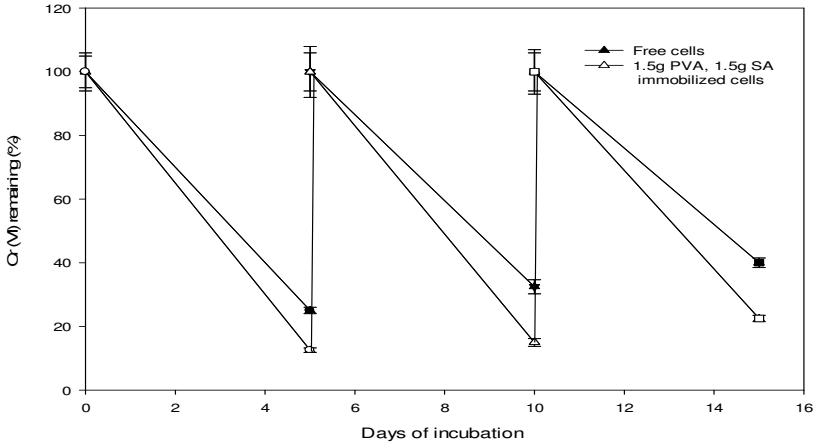

Fig. 6. Chromium (VI) reduction by bacterial strain OZF6 in immobilized and free state using repeated spiking of 100 µg /ml Cr (VI) in nutrient broth (pH 7.0) after every five days

Table 1. Summary of statistical analysis: Effect of pH, chromium concentration, carbon sources and polyvinyl alcohol with sodium aliginate on Cr (VI) reduction were analyzed using Analysis of Variance (ANOVA *P<0.05)

Treatments	df	SS	F
pH	4	3524.7165	90.694*
Chromium concentration	2	280.25	32.21*
Carbon sources	6	8357.452	153.465*
PVA+SA	3	70.063	43.84*

tolerance of bacteria to Cr (VI) [36]. Many studies confirmed tolerance of bacteria to chromium (VI) which is due to different growth conditions [37]. For example, *Intrasporangium* sp. Q5-1 was tolerant to 17 mM of Cr(VI) [38] comparison to *Bacillus spp*. PZ3 and *Streptococcus* PZ4 which tolerated chromium (VI) upto 700 µg/ml [39].

Many industries release a lot of chromium into the water bodies and thus can contaminate the water and thus population will be at high risk and may result into health hazard. Solubility, permeability and the reaction of the metal (chromium (VI)) with the proteins and nucleic acids enhances the carcinogenicity of this element [5]. When Cr (VI) is detoxified, it is converted to less soluble, stable and least toxic form of the metal which is Cr (III) and is an important technique for remedy of metal polluted soil [40]. Thus, bacterial detoxification of chromium is a useful process to free the soil from the metal pollution. Thus this study we planned to check bacteria strain for its chromium (VI) reducing ability. Due to its high resistance *Brevibacillus brevis* OZF6 reduced chromium (VI) significantly. Strain *Brevibacillus brevis* OZF6 showed significant reduction in Cr (VI) at all the pH and concentrations of the metal. As the

amount of the metal increased, time of reduction also increased. Here we observed that *Brevibacillus brevis* OZF6 at 50 µg/ml significantly reduced the metal, which occurred at 120 h. OZF6 also significantly reduced chromium (VI) at higher concentrations of the metal (100 and 150 µg Cr/ ml). Present study is in correlation with Yang et al. [38] who study that there was significant chromium removal. This was also observed by Wani et al. [39] who observed significant removal of Cr (VI) at pH 7.0 and 50 µg/ml of chromium.

Increase in Cr (VI) detoxification depends on the most suitable electron donor like amino acid, NADH or carbohydrate [41]. The role of electron donors is to increase the hydrogen ion concentration which will eventually stimulate the bacterial cells thus will detoxify chromate ions. These electron donors release hydrogen ions which results in chromium (VI) reduction [19]. In this study Chromium (VI) reduction by *Brevibacillus brevis* OZF 6 was observed under the influence of different electron donors which showed a significant reduction in Cr (VI). There is a lot of removal of Cr (VI) under the influence sucrose, followed by methanol. Similar results were also studied by Pal et al. [42] who observed most removal of Cr (VI) under the influence of glycerol and glucose. This study showed that sucrose is the best for Cr (VI) removal. In another experiment Pal et al. [42] saw that glucose and glycerol were effective for Cr (VI) reduction. In another study, Desai et al. [28] observed that when *Bacillus* species G1DM20 and G1DM64 in acetate medium there was an increase in Cr (VI) reduction. In other reports, it is NADH which is good for the removal of the metal [27,3,28]. However, this study showed that there was less Cr (VI) removal when bacteria were grown in the presence of other carbon sources except sucrose and methanol, possibly because Cr(VI) reductase enzyme were not dependent upon the above mentioned carbon sources.

In the present study, we checked the co-effect of immobilizing agents like sodium aliginate and PVA on Cr (VI) reduction by *Brevibacillus brevis* OFZ6 compared to free cells after 120 hours of incubation. All the combinations of sodium aliginate and PVA, significantly reduced Cr (VI) compared to the control cells. Among various matrices combinations for immobilization of strain OZF6, the combination of 1.5 g PVA, 1.5 g SA was best for detoxification. This experiment is in agreement with (Humphries et al. and Poopal and Laxman) [29,33] who observed that when *Desulfovibrio vulgaris* was immobilized by agar, reduced 0.5 mM Cr (VI) in 22 hours whereas when *Microbacterium* sp. NCIMB 13776 was immobilized by agar, reduced 0.5 mM Cr (VI) within 65 hours of incubation [29]; while *Streptomyces griseus* immobilized by PVA in combination with alginat, removed 0.48 mM Cr(VI) in a period of 24 h [33]. In another study, Pang et al. [43], also observed 50% Cr (VI) reduction in 84 hours when the polyvinyl alcohol/sodium aliginate immobilized bacterium (*Pseudomonas aeruginosa)*. In another study Batool et al. [44], observed chromium (VI) in bacteria immobilized by 2% sodium aliginate and 2.5% agar. Maximum reduction of chromium (VI) of 89% was achieved by the bacterial isolate E1 and 93% by E4 when the strains were immobilized by sodium aliginate whereas reduction was 39% by E1 and 48% by E4 when the strains were immobilized by the beads of agar.

There was full removal of the metal in each cycle when the bacterial strain OZF6 was immobilized, compared to control cells. This study has demonstrated that Cr (VI) removal was depended on the starting bacterial culture, this was also observed by other researchers [45]. Furthermore, the negative impact of the metal is avoided if already grown bacteria are used for removal of the metal. This study concluded that for successful bioremediation, it is not necessary to pre-expose the bacterial cells to chromium for subsequent microbial enrichment. This could be due to the role of reductases, thus correlating the earlier study of high and quick removal of the metal by *Pseudomonas putida* biofilms [46].

5. CONCLUSIONS

This study concludes that polyvinyl alcohol and sodium alginate immobilized cells can remove chromium (VI) more efficiently and in high concentration than free cells and thus, these bacteria can be used for detoxification of chromium (VI) from industrial waste water and thus can protect the water bodies from metal contamination and ultimately the people from high risk of metal exposure.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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cultures of bacteria. Water Res. 1995;29: 2467–2474.

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