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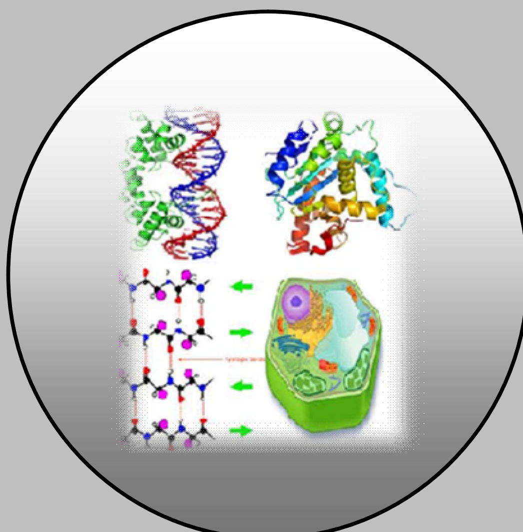

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Synergistic Chromium Bioremediation by Water Lettuce (*Pistia*) and Bacteria (*Bacillus cereus* GXBC-1) Interaction

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ABSTRACT

A laboratory investigation has been performed to examine the enhancement of uptake capacity of water lettuce (Pistia sp), an aquatic plant to decontaminate chromium (VI) laden waste water in conjunction with a bacterial strain isolated and identified as Bacillus cereus GXBC-1 following 16S r DNA genome sequencing. The strain was found in discharged outlet of a tannery treatment plant unit. The macrophyte alone was found to tolerate and remove Cr (VI) up to an initial concentration of 8 mg/L, whereas a maximum percent of removal (78%) was noted in correspondence to initial concentration of 3 mg/L after 4 days of contact period without any apparent inhibitory effect on living biomass. The plant root colonised with bacterial strain exhibits a lowering of uptake Cr (VI) but tolerates even up to 8 mg/L, however, a marked synergistic effect of plant and microbial strain could be observed in conducing more removal of Cr(VI) from the solution. The overall removal for non-colonised and colonised strains at the root zone of the living plant were found to be 78 and 94.67% respectively in corresponds to initial concentration of 3 mg/L whereas 24 and 58.75% removal were observed for an initial concentration of 8 mg/L after 4 days of contact period. Hence a noticeable improvement of overall removal of chromium was detected in Pistia-Bacillus cereus GXBC-1 interacting association.

Key words: Chromium (VI), Bioremediation, Pistia, Bacillus cereus GXBC-1, Plant-microbe interaction and Synergistic effect.

INTRODUCTION

Chromium contamination in fresh water ecosystem and other water bodies as a result of discharge from electroplating industries, tanneries, and metal processing units is considered to be as one of the serious water pollution. Chromium (VI) is a toxic metal and adversely affects human physiology and other aquatic planktons including fishes as genetic disorder, carcinogenic symptoms and changes in water quality for extinguishing flora and fauna. Conventional physico-chemical methods of chromium (VI) removal is of late not encouraged since there are many secondary problems encountered in the technology with high energy requirement, lower efficiency of removal at low concentration level and generation of chemical sludge as solid waste (Ahluwalia and Goyal 2007; Demir and Arisoy 2007; Mahamadi 2011). It is also recommended that low cost bioremediation technique would be the better option to alleviate metal contaminated sites including metal laden wastewater. Phytoremediation is recently proved as a promising biotechnical tool for removing heavy metals from water and lithosphere environment and reported in literatures as a potential method for metal removal process from polluted environment through rhizofiltration, phytoextraction etc. (Kamal et al. 2004 ;Maine et al. 2004). The bacteria play an important role to provide uptakeable ions to the plant root system. Plant based wastewater treatment systems using aquatic plants with high biomass like water hyacinth in many parts of the world have been implemented and are applied too either in a pilot or field scale, specially in wetland system (Sooknah and Wilkie 2004; Zimmels et al., 2006). However, the technology suffers some limiting applications, such as reduction of dissolved oxygen, hindrance of penetration of sunlight and final harvest. Comparatively lower sized plant species, such as *Pistia* (water lettuce)*, Lemna, Spirodela* (duckweed) etc. were further investigated as the candidate materials as living or dried biomass for biosorptive removal of heavy metals including chromium (Espinoza Quinones et al. 2006; Sinha et al. 2009; Mufarrege et al. 2010; Singh and Sinha 2011). It is mentioned in the literature that pH is one of the important controlling factors affecting biosorption of heavy metals (Onyancha et al. 2008). pH affects the availability of soluble metal ions in aquatic environment. Most of the earlier work in the relevant area was carried out with the assumptions that plant accumulates preferentially the metallic ions either as metabolic need or simple diffusional sorption process. However, the role of specific bacteria and other micro-organisms in the surrounding root zones of such aquatic weeds and floating plants should also be accounted in conjunction with bioaccumulation rate of metal ions by the plants as reported by few earlier scientists (Faisal and Hasnain 2005; Abou shanab et al. 2007). This plant-microbe phenomenon plays a vital role for enhancing uptake capacity of the plant. It has been mentioned in the literature that high amount of metallic ions are non-available to plant-root system and its transport to upper parts, for which success of phytoremediation depends is limited due to sequestration of cell body as toxicity, appears after a threshold value. Presence of rhizosphere bacteria can change the mobility of metallic ions and make the specific metals available to root system by catabolic pathway of metal tolerant behaviour of microbes.

For enhancing more metallic ion uptake by the plant root system, microbes release chelators and supplementing change of redox potential. In this context, some chromate tolerant bacteria were explored by some earlier researchers to accomplish their Cr (VI) tolerant capacity up to a certain threshold limit (Shakoori et al. 1999; Verma et al. 2001; Camargo et al. 2003; Sundar et al. 2010; Ezaka and Anyanwu 2011). Plant-microbes interacting venture is perhaps to be considered as a potential tool for enhancing metal removal from wastewater media. A group of researchers [Abou shanab et al. (2007)] carried out an earlier investigation pertaining to the chromium removal with chromate tolerant bacteria isolated from rhizospheric zone of water hyacinth. They observed seven to thirty five times more Cr accumulation in roots of water hyacinth inoculated with bacterial strains in comparison to non-inoculated plants (control).
However, the investigation of the inoculation of chromate tolerant bacteria in other aquatic floating and submerged plants like water lettuce, duckweeds, *Hydrilla* etc. are not reported much in literatures. The present study was undertaken to examine any scope of elevating the overall removal ability of aquatic plant *Pistia* in conjunction with *Bacillus cereus* GXBC-1 for removal of chromium (VI) from water environment. The bacterial species have been found chromium tolerant as isolated from tannery effluent treatment plant outlet in a separate investigation done by the authors (Chakraborty et al. 2012,). The secondary aim of the present study was to explore the low cost plant based bioremediation technology as a tertiary treatment of chromium contaminated tannery wastewater.

MATERIAL AND METHODS

Characterization of micro-organism

The bacterial isolate was characterized and identified as *Bacillus cereus* GXBC-1 following 16S r DNA genome sequencing in a separate study performed by the authors (Chakraborty et al. 2012].

Collection of plants and acclimatization

The young plants (*Pistia*) were collected from a nearby pond and acclimatized hydroponically in a flat bottom HDPE tray. The plant did not possess any prehistorical exposure in chromium contaminated environment. Plants harvested from the tray were subsequently washed in single distilled water and finally in deionised water. They were grown hydroponically in 10% Hoagland solution (Hoagland and Arnon 1950) as a nutritional supplement at pH 7 ± 0.2 in approximately 10,000 lux/m^2 light intensity under controlled environment provided with fluorescent tubes. The experiment was carried out at ambient temperature (25 ± 5 ^{0}C) with a 8h photoperiod. Approximate 60 g of equal sized plants were placed in the aquarium and allowed to retain in 1.5 litre volume of Hoagland solution. Volume of solution was adjusted with distilled water after aliquots of solution were taken for analysis and to make up the loss due to evaporation.

The present experiments were carried out in multicells aquarium made of perspex sheet in the Environmental Engineering laboratory, Department of Civil Engineering, Jadavpur University, Kolkata, West Bengal, India.

Preparation of spiked Cr (VI) sample

Stock potassium dichromate solution of the strength of 1000 mg of Cr (VI)/L was prepared by dissolving 2.82 g $K_2Cr_2O_7$ (oven dried) in 1 L of double distilled water. A set of intermediate stock chromium (VI) of different diluted strength solution was made by adding deionized distilled water to stock in such quantity that finally solution volume in each container became one litre.

Analytical measurement of Cr (VI)

Dissolved hexavalent chromium was estimated spectrophotometrically with the development of red purple colour (chromium 1, 5-diphenyl-carbohydrazine complex) by reaction with diphenylcarbazide in acid solution. The adsorption of colour was measured at 540 nm with UV-VIS Spectrophotometer (Varian, USA make, model Cary 50) [Pattanapipitpaisal et al. 2001]. Liquid samples were pipetted out from each cell at regular intervals and analyzed for residual chromium (VI) as described above. All the analytical values were done in triplicate and average values were taken as experimental data.

Removal of chromium (VI) by Pistia

Cr (VI) removal assay by *Pistia* was conducted by exposing young plants of same size to the aqueous solution of Cr (VI) containing different initial concentrations viz. 3, 5 and 8 mg/L along with 10%Hoagland solution in. Amount of residual Cr (VI) left in the solution was determined at a regular interval as stated earlier. The experiment was conducted for a batch period of 4 days of exposure.

Chromium uptake by the bacterial isolates

Bacterial strain was inoculated (1% inoculum) in 5 ml nutrient broth supplemented with 10 mg/L of Cr (VI) using $K_2Cr_2O_7$ solution. The selected culture was placed in the incubator at 37 ^{0}C. After 24 h, the culture was centrifuged (5000 rpm for 10 min) and Cr (VI) concentration was estimated in the supernatant as described above to observe the amount of Cr (VI) reduced in the solution.

Effect of pH on Cr (VI) removal

Effect of pH on Cr (VI) removal by *Pistia* plant was observed for 4 days with 60g/L biomass at different pH values i.e. pH 4, 5, 6, 7 and 8. Phosphate buffer was used for maintaining different pH values. The pH of the solution was measured analytically by a digital pH meter. Necessary calibration and standardization were done with standard buffering solutions of pH 4, 7 and 9.

Plant-microbe interaction study

Acclimatization of bacterial culture in Hoagland solution

The isolated bacterial strain was allowed to grow in Hoagland solution supplemented with 1.5% peptone as the organic nitrogen source. Modified Hoagland solutions after necessary sterilisation were inoculated with 2% overnight grown culture of bacterial isolate. These were incubated at 37 ^{0}C for 48 h at pH 7.0 ± 0.1.

Colonization of bacteria in root zone of *Pistia* 60 g of living biomass comprises of freshly grown *Pistia* plants were taken in and its roots were suspended in 50 ml of previously grown bacterial culture .The bacterial strain was allowed to adsorb to the root surface of the *Pistia* for overnight. Then, the bacteria along with the plants were transferred to plastic aquaria containing 10% Hoagland solution with the adjustment of pH to 7. Since it was observed that chromate tolerant bacteria performs well at pH 7.0 ± 0.2 (Chakraborty et al. 2012). Final volume was adjusted to 1.5 L. One set was kept only with bacteria without any plants as control 1. Another set was kept there only with plants but without any bacteria as control 2. All these experimental sets were tested with 3 mg/L of initial Cr (VI) concentration. At this level of concentration, plant alone exhibited a reasonable amount of removal. The above study was also carried out with 8 mg/L of initial concentration at the same time because at this initial concentration level, plant shows lesser uptake capacity and considerable inhibition. At the end of 4 days contact period, removal of Cr (VI) was detected by measuring the remaining Cr (VI) concentrations at regular intervals following the method as stated earlier. The experiments were performed in triplicates and mean values were considered only.

RESULTS AND DISCUSSION

Removal kinetics of chromium (VI) by Pistia

The batch kinetic results for Cr (VI) uptake by *Pistia* alone in presence of 10% Hoagland solution for initial concentration ranging from 3 to 8 mg/L are shown in Figure 1.

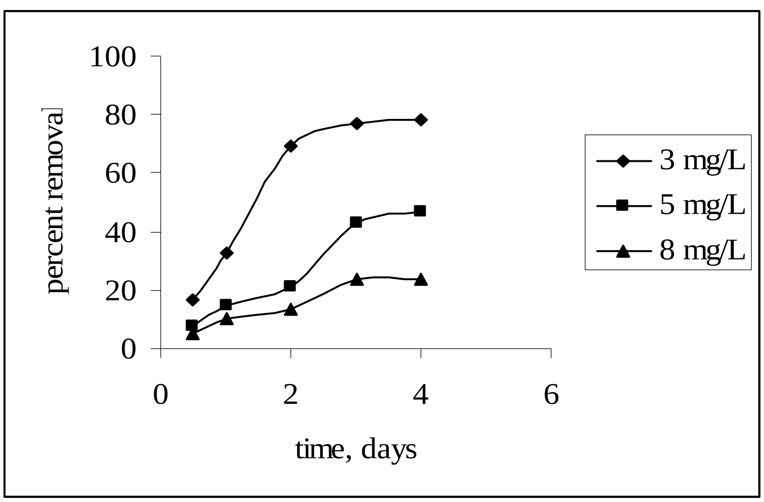

Figure 1. Removal of Cr (VI) by *Pistia*.

From Figure 1, it is observed that the plant could remove 78 % in correspondence to 3 mg/L of initial concentration after an equilibrium time of 4 days of contact period. It is revealed that the sorption by the plants was maximum at lower initial concentration (3 mg/L) and started declining at the higher initial concentrations (5 and 8 mg/L) which could be due to the imposition of toxic effects to the plant metabolism and subsequent damage of plant protoplasm. Figure 1 also entails that beyond 3 days of contact period, the uptake rate was increased marginally for any initial level of concentrations. However, at higher level of chromium (VI) concentration (8 mg/L), the plant showed its reducing capacity of uptake which clearly evidenced by its limiting root adsorptive capacity per unit biomass of the plant.

Effect of pH

It is shown from the result (Figure 2) that the maximum removal of 65 and 58.4% took place at a pH value of 4 for both initial concentration of 3 and 5mg/L of Cr (VI) respectively. At lower pH value, the removal is favourable for Cr (VI) adsorption and as the pH is increased the uptake capacity gradually descended for less availability of chromate ions in Cr (VI) forms. Between the pH ranges 5-6, substantial decrease of Cr (VI) uptake was noticed for initial concentration of 3 mg/L where as beyond pH value 7, the removal rate fallen further for initial concentration of 5 mg/L.

In a particular study with *Hydrilla* , pH 4 was highly conducive to remove a large amount (99.70%) of Cr (VI) after a contact period of 7 days (Shaikh and Bhosle 2011). The reason for this reduction at higher pH perhaps due to the precipitation effect of Cr (VI) on the root surface because of change in pH in the root area caused by the exudates as observed by others [Sune et al. (2007)].

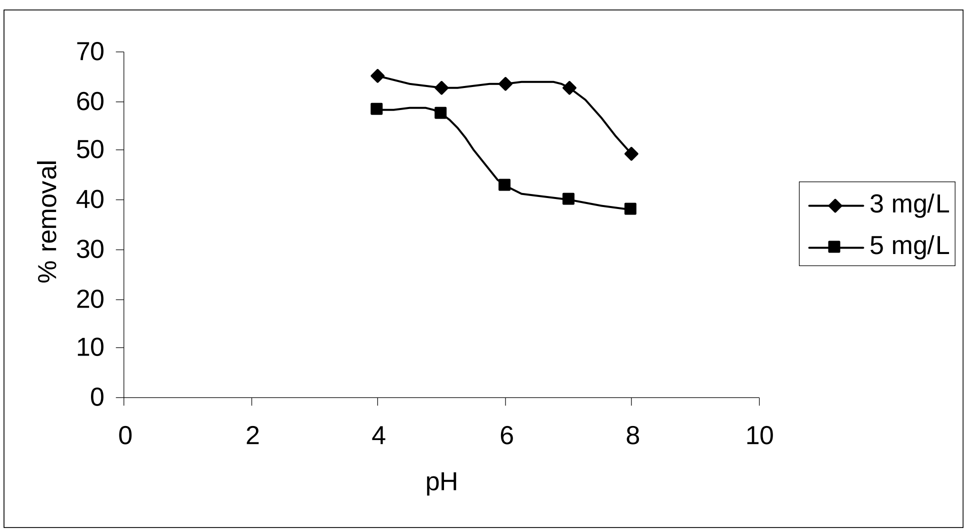

Figure 2. Effect of pH on percent removal of Cr (VI) by *Pistia.*

The experimental results of pH influence on uptake capacity of *Pistia* corroborated the results as obtained by others (Faisal and Hasnain 2005) who used a different plant species i.e. *Eicchornia crassipes* (water hyacinth) in conjunction with chromate resistance bacterial strains *Ochrobactrum intermedium* CrT-1, *Brevibacterium* CrT-13, and CrM-1 .They have also reported that in slightly acidic medium (pH 5.0), maximum removal is possible. However, they conducted the study at much higher level of initial chromium concentration (500 mg/ L).

Effect of plant-microbe interaction on Cr (VI) uptake

The plant-microbe interaction study was carried out to examine if there is any change in Cr (VI) removal by *Pistia* plants alone and in combination with bacterial isolate colonized at its root zone area. The plots of experimental data (Figure 3 and Figure 4) shows a descended trend in the concentration of Cr (VI) accumulated by *Pistia* due to the reduction of more soluble and permeable Cr (VI) to less permeable Cr (III).The retarding effect in Cr (VI) uptake in corresponding to both concentrations 3 and 8 mg/L were found in case of inoculation of isolated strain because the chromate tolerant bacterial strain is more readily and efficient Cr (VI) reducer as compared to plant alone.

In case of initial concentration of 3 mg/L of Cr (VI), after 4 days of contact period, the concentration level of Cr (VI) was reduced to 0.67 mg/L (78% removal) by the plants alone (Figure 3). Figure 3 also depicts that when the strain was in direct contact with 3 mg/L of Cr (VI) concentration level, after 4 days of contact period about 24.62% removal of Cr (VI) was observed. Figure 4 indicates a marginal removal (24%) by the plant alone also for an initial concentration of 8 mg/L, though there was marked sign of wilting effect on root and leaf in appearance. The bacterial isolate was efficient to remove Cr (VI) even at 8 mg/L of initial concentration (23.82 %). In an earlier investigation, in continuation of the present study, it was observed that the above strain could tolerate up to a value of 40mg/L (Chakraborty et al. 2012) However, the isolate imparted a reduction of the uptake capacity of the plants but the conjunctive effect of the *Pistia* and bacterial strain effecting enhancement of percent removal of Cr (VI) from the solution for same initial concentration (8 mg/L). The cumulative separation of Cr (VI) from the spiked sample was accomplished by the sum of Cr (VI) accumulated, loosely bound to plant roots and bioadsorptive reduction by the bacterial strain. At an initial concentration of 3 mg/L after 4 days of contact time, 94.67% removal was observed by plant – microbe interaction (Figure 3). For the same combination but at an initial concentration of 8 mg/L of Cr(VI), 58.75% removal was exhibited as compared to 24 % by the plant alone (Figure 4) which evidence a conducive effect of elevation of uptake of Cr(VI) due to plant-microbes interacting phenomenon.

The presence of bacterial strain descended the efficacy of the plant for the reduction mechanism. Due to a high degree uptake capacity of bacterial strain, the availability of Cr (VI) within the root clusters is not much. In the present study, bacterial strain was found to be efficient both as Cr (VI) tolerant and uptake for which Cr (VI) was attenuated from the solution.

Their removal capacity was also extenuated successfully in conjunction with *Pistia* plants. *Pistia* provides necessary carbon and energy by releasing exudates for sustaining bacterial strains. The fact is also supported by Cunnigham et al. (1996); Faisal and Hasnain (2005). The overall removal in *Bacillus cereus* GXBC-*Pistia sp.* was promoted as compared to non-inoculated control.

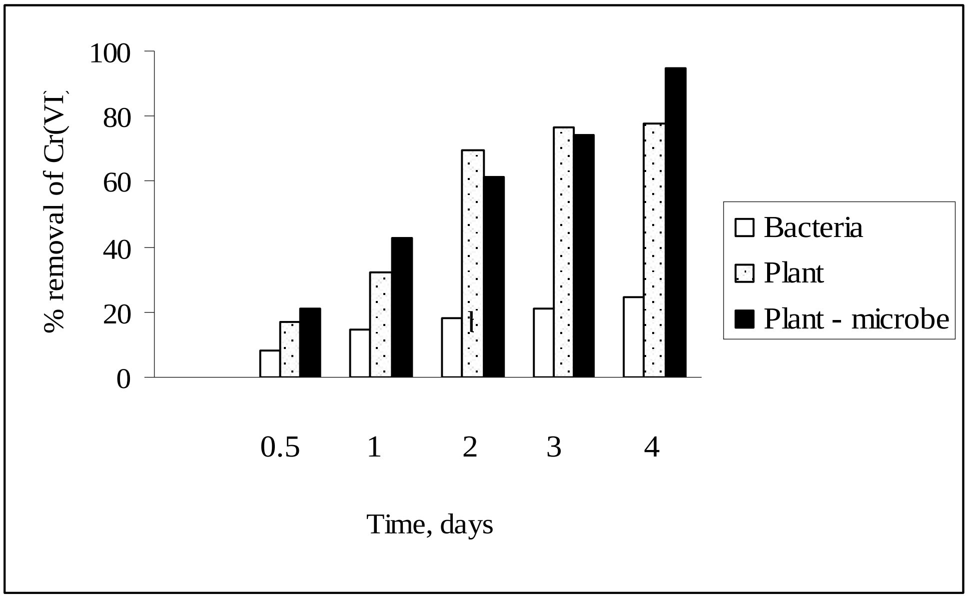

Figure 3. Effect of plant-microbe interaction on Cr (VI) removal at an initial Cr (VI) concentration of 3 mg/L

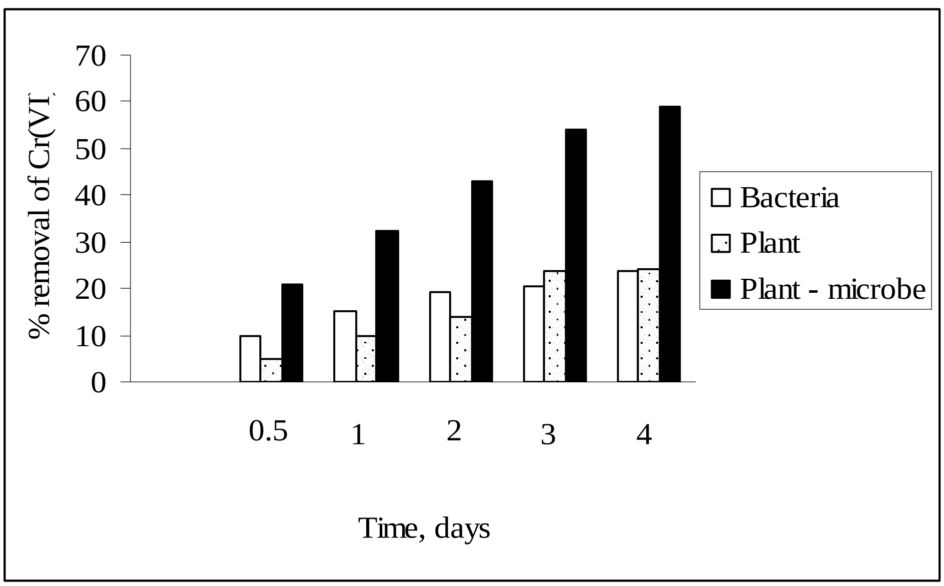

Figure 4. Effect of plant-microbe interaction on Cr (VI) removal at an initial Cr (VI) concentration of 8 mg/L.

CONCLUSION

Plant – microbe interaction activity have received considerable attention in recent times as clean up technology specially on advanced phytoremediation method for heavy metal contaminated wastewater by exploiting metal adsorbing capacity of bacterial strain and aquatic wet land plant species in conjunction as a pseudo-symbiotic roles. The present investigation holds a potential promise of phytodecontamination tool in association with metal tolerant bacterial strain (Bacillus *cereus* GXBC) inoculating in root zone. The experimental results showed that Cr (VI) uptake by *Pistia* plant depends on initial concentration. The removal percentage decreases as the initial Cr (VI) concentration of solution increases. The results exhibited that pH has significant effect on Cr (VI) uptake by the plant and indicated that at lower pH the uptake rate has been enhanced. The bacterial strain (Bacillus *cereus* GXBC) as isolated from treated tannery effluent was found to be very efficient in the removal of Cr (VI) from the solution and the activity was extenuated along with *Pistia* in conjunction with the solution. The overall removal of Cr (VI) by synergistic effect of *Pistia* in association with *Bacillus cereus* GXBC was found to be enhanced as compared to non- inoculated control plants. The removal was inhibited by the plant alone at an initial concentration of 8 mg/L, on the other hand, 58.75% removal took place for colonised strain in the root zone of the plant which clearly advocates the benefit of potential application of plant-microbes interaction to alleviate Cr (VI) uptake by wetland plant system.

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