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EFFECT OF HEXAVALENT CHROMIUM ON BIOSYNTHESIS OF BIOFILM BY *PSEUDOMONAS PUTIDA* KI ON DIFFERENT CARRIER MATERIALS

Sania Kiran • Shahid Mahmood* • Muzammil Anjum • Azeem Khalid

AUTHOR'S AFFILIATION

Department of Environmental Sciences, PMAS-Arid Agriculture University, Rawalpindi 46300, Pakistan
*Corresponding author's email: shmahmood@uaar.edu.pk

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ABSTRACT

Background Hexavalent chromium Cr(VI) is highly toxic pollutant that released from tannery and other industrial effluents. Present study was aimed to use ecofriendly method for Cr(VI) removal from industrial effluents.

Methodology Four different agricultural materials i.e. rice husk biochar, coal, corn cob, corn cob biochar were used as adsorbents to evaluate their potential for reduction of Cr(VI). A set of experiments was conducted to check the removal efficiency of four different adsorbents including rice husk biochar, coal, corn cob, corn cob biochar by treating it with varying concentrations (5, 10, 15, 20 and 25 ppm) of Cr(VI) in shaking incubator of 180 rpm for 180 minutes at 30°C. Cr(VI) concentration was analyzed with UV-visible spectrophotometer at 540 nm.

Results Rice husk biochar exhibited maximum adsorption i.e. 62.4% at 15 ppm Cr (VI) concentration after 180 minutes. So, rice husk biochar was selected as a support matrix for development of *Pseudomonas putida* KI biofilm. Biofilm synthesis was observed after 21 days of biofilm formation. In biosorption experiment, increasing trend was observed for biosorption from 5 ppm to 15 ppm (71.6% to 73.2%) Cr(VI) concentration. However, further increase in Cr(VI) concentration had negative impact on biosorption efficiency of carrier materials. During biofilm formation, growth and viability of bacterial cells was also determined by optical density and colony forming unit (cfu) per mL. Isothermal models were also applied to observe the biosorption kinetics and Langmuir and Redlich-Peterson models were found to be best fitted model for this experiment.

Conclusion The results obtained clearly indicated the potential of biofilm-coated support materials to be used on industrial scale for the removal of Cr (VI) from wastewater.

INTRODUCTION

Chromium (Cr) has been used in various industrial applications, especially in tanning industry that resulting in releases of huge quantities of Cr metal ions in its wastewater (Ashraf et al. 2020). This is affecting the water quality of both surface and underground waters in the surrounding areas. It also directly influences the living organisms (Fu and Wang 2011). Chromium has many oxidation forms but Cr(VI) and Cr(III) are more dominant. Cr(VI) is highly toxic in nature because of its high mobility, high water solubility and potentially more hazardous for entire life forms because it completely changes the structure of many proteins (Singh et al. 2011). While Cr (III) has less mobility and solubility and acts as important micronutrient for several organisms. Chromium has been released from various industries like tanning, textile, metal cleaning, dye and steel making industries and directly discharged into aquatic system. Compounds of Cr are also naturally found in many rocks and soils. Cr(VI) is second frequently found metal that mainly existing in state of dichromats and oxides (Kang et al. 2014).

High concentration of Cr(VI) in biological system has become a serious threat to all life because it is considered as a persistent and bio-accumulative. Due to its toxicity, Cr(VI) is also associated in causing

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irritable reactions when contact with skin and severe damage of kidneys and liver. Moreover, it also causes severe damage in DNA when it contacts with enzyme DNA-polymerase (Chhikara et al. 2010). Its consumption also causes cancer in stomach and lungs because of high permeability and oxidizing power (Frois et al. 2011).

Different methods like chemical reduction by the process of precipitation, ion exchange, bioremediation process and other membrane technologies are mostly used for the Cr(VI) contamination removal (Ramana et al. 2012). The process of bioremediation is considered as most desirable technique due to its economic viability, high efficiency, involve less operational time, less production of intermediate toxic products and produced biomass is reusable (Shinde et al. 2012). In this process of bioremediation, various micro-organisms that mainly include specific bacteria, fungi and yeast can be used because of their capability to concentrate heavy metal ions and store them in their cell structure from the contaminated solutions (Thatoi et al. 2014). Recently, the bio-sorption method has been carried out extensively using microbial biomass (Song et al. 2006). Biosorption methods using microbes is considered as most effective process because of its efficient binding ability with heavy metals. Normally, biosorption method has capacity to decrease the working costs up to 36% compared to other commonly used conventional techniques (Loukidou et al. 2004).

The specific bacterial usage is extremely required as a biosorbent due to its small size that provide more surface area per unit mass for binding of heavy metal, its growth capacity in poor conditions and high flexibility towards widespread environmental conditions compared to other microorganisms (Carvalho et al. 2008). Various structural polymers are also present in the bacterial cell that show phosphoryl, amino and carboxyl groups which involves in fast reactivity with other bacterial cells (Kulczycki et al. 2002). Furthermore, bacteria continuously grow and able to develop a biofilm that has capacity towards the adsorption of heavy metal and help to reduce it into less toxic form. Biofilms of the bacteria are the microbial communities that attached to the particular and surface attached microbes completely entrenched in matrix of Extracellular Polymeric Substances (EPS) (Van Houdt et al. 2006).

Numerous adsorbents like granular carbon (Jung et al. 2013), inactive nanomaterials (Rajput et al. 2014), metallic oxides (Jiang et al. 2013) and organic sludge produced during agricultural wastewater treatment (Chen et al. 2015) also used along with microorganisms that increases the heavy metals elimination. There are several factors including pH, temperature, growth medium chemical composition and other stress factors such as occurrence of harmful heavy metals that highly affect EPS and microbial metabolic activity (Quintelas et al. 2011; Qiang et al. 2013). Bacterial biofilm are more effective for Cr(VI) removal because it shows more tolerance against stress factors and have proper settlement as compared to planktonic cells of the bacteria (Nancharaiah et al. 2010).

Pseudomonas putida strains are widely used in large number of environmental studies because it shows broad tolerance towards trace metals (Martínez-García et al. 2014; Maes et al. 2020). In the present study, previously isolated bacterial strain *Pseudomonas putida* KI was used for development of biofilm and effect of varying concentration of Cr(VI) on biofilm supported on specific carbon material was investigated. The present study would helpful to find out suitable carbon material for the development of biofilms for the treatment of Cr(VI) contaminated wastewater .

MATERIALS AND METHODS

Chemicals and culture medium

In this study, all chemical solutions were prepared in distilled water. For stock solution preparation of Cr(VI), $K_2Cr_2O_7$ was used as source of Cr(VI). Diphenyl carbazide reagent was used as color developing agent to analyze Cr(VI) concentration by spectrophotometer. Previously isolated bacterial strain *P. putida* KI was used for biofilm development. Bacterial strain was grown on minimal salt media (MSM). MSM was first prepared by dissolving 1.0 g of Na_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 1.0 g of KH_2PO_4, 1.0 g of NaCl, 0.1 g of $CaCl_2$, 4.0 g of Yeast and 16 g of agar in 1.0 liter distilled water. All solutions were autoclaved for 15 to 20 minutes at 121°C. In addition, ethanol was used as disinfectant to prevent the solutions from contamination.

Biochar preparation

Rice husk and corncob were used to prepare biochar. Materials were washed using distilled water to remove impurities and placed under sunlight for about 2 to 3 days to remove moisture. Dried materials were placed in furnace. The furnace was made air tight by filling the gaps with the help of mud. Nitrogen gas was flushed to remove oxygen from furnace chamber. Biochar was prepared in oxygen limited environment at the temperature of 400-500°C. After an hour, the furnace was turned off and material was left within the furnace for some time, so that it may not catch fire. Biochar was then kept in open air by spraying some water on it and then allowed to cool. Biochar ground in powdered form to enhance its surface area, and was stored in air tight plastic bags.

Cr(VI) adsorption experiment

Preparation of stock and its working solution

To prepare the 500 ppm stock solution of Cr(VI), 1.41 g $K_2Cr_2O_7$ was dissolved in 1 liter distilled water and 100 mL working solution of 5, 10, 15, 20 and 25 ppm were prepared by diluting the stock solution of Cr(VI).

Spectrophotometric analysis of Cr(VI)

Cr(VI) concentration was analyzed by UV-visible spectrophotometer at 540 nm. For identification of Cr(VI) concentration, diphenyl carbazide reagent was added that acted as color developer. 0.25 g of 1, 5-diphenyl carbazide was added in 50 mL of methanol that was again poured in 0.2 N sulphuric acid (H_2SO_4) to prepare this reagent. To prepare 0.2 N H_2SO_4, 1.0 mL concentrated H_2SO_4 was added into distilled water and diluted it to 100 mL volume. Potassium permanganate was also added in excess to completely convert Cr(III) into Cr(VI) before treating samples with one or two drops of 1,5-diphenylcarbohydrazide reagent. After taking readings of Cr(VI) standards, calibration curve was prepared in excel and its equation of linear regression was $y = 0.0231x + 0.2513$ ($R^2 = 0.99$)

Batch sorption operation

Batch sorption operation was conducted for Cr(VI) removal by four different carbon materials. Rice husk biochar, corn cob biochar, corn cob and coal were used as adsorbents. Experiment was conducted in 250 mL conical flask by adding 2.0 g of various carbon materials with 100 mL of 5, 10, 15, 20 and 25 ppm concentrations of Cr(VI) solution. Appropriate amount of 0.1 mol dm^{-3} HCl or NaOH was added in samples to adjust the pH. Each experimental step was carried out in three replicates and mean values were recorded. All experiments were carried out for 180 minutes at room temperature in 250 ml conical flasks, which were placed in shaking incubator with constant stirring speed of 180 rpm. The mixture was stirred continuously to facilitate better mass transfer with high interfacial surface area. After every 30 minutes interval, sample was taken and Cr(VI) concentration was examined by spectrophotometer at 540 nm until it reaches to its steady state. The treatments applied were as follows: T_1- Coal, T_2- Rice husk (biochar), T_3- Corn cob (biochar), T_4- Corn cob. Formula for removal percentage of Cr (VI) is:

Removal percentage = $C_i – C_f / C_i$

Desorption studies

Batch desorption experiment was carried out to determine that the adsorption of Cr(VI) on these adsorbents (carbon materials) were reversible or not. 1.0 g of each already used adsorbent was treated individually with 100 ml of two different desorbents i.e. 0.5 N NaOH and 0.5 N HCl in 250 mL conical flasks that were placed in shaking incubator with constant shaking of 125 rpm for 180 minutes at 30°C temperature. The sample solutions were filtered properly after specific time interval, and desorbed Cr(VI) was determined with Spectrophotometer at wavelength 540 nm.

Experiment set up for biofilm formation

Preparation of agar plates

For the preparation of agar plates, MSM was first prepared by dissolving 1.0 g of Na_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 1.0 g of KH_2PO_4, 1.0 g of NaCl, 0.1 g of $CaCl_2$, 4.0 g of Yeast and 16 g of agar in 1.0 liter distilled water and mixed completely by placing it in shaking incubator for few minutes and autoclaved it for 15 to 20 minutes at 121°C. After autoclaved, MSM was transferred into disposable petri plates that were stored at 4°C.

Enrichment of cell culture

Single colony was picked with the help of needle and transferred into 100 mL liquid media and placed in shaking incubator at 30°C with 150 rpm for 24 hours. Meanwhile, bacterial growth was spectrophoto-metrically analyzed by at 600 nm.

Biofilm formation

For biofilm development, batch experimental operations were conducted using 2.0 g rice husk (biochar) with 20 mL media and 100 mL of various concentrations (5, 10, 15, 20, 25 ppm) of Cr(VI) solution in conical flasks. pH of the samples was determined by pH meter (ORION 720A) and pH of samples were between 4.6 and 5.1 for Cr. The Erlenmeyer flasks were placed in shaking incubator at 35°C with 150 rpm for 21 days to increase bacterial biofilm contact with Cr(VI) solution. All experimental operations were carried out in triplicate. During 21 days of bacterial biofilm formation, samples of 1 mL were taken after every 3 days and centrifuged. After that, concentration of Cr(VI) was determined after analyzing samples with Spectrophotometer at 540 nm.

Bacterial viability and growth

During biofilm formation, growth of bacteria was spectrophotometrically analyzed at 600 nm. Viability of bacteria was determined using pour plate technique to calculate the bacterial colonies. In this method, dilutions were prepared from 1.0 mL of sample and spread on agar plate by taking 150 µL sample quantity. These plates were incubated at 35°C for 24 hours. Colonies of bacteria on agar plate was counted by colony counter. Following formula was used to calculate cfu.

$$CFU\ mL-1 = \frac{Number\ of\ CFU}{\text{Volume of plated (mL)}x\ diluations}$$

Statistical analysis

Results were presented in percent degradation with mean and standard deviation using Microsoft Excel. Graphic method was used for result presentation marked with standard deviation bars. Equilibrium isotherms for Cr(VI) adsorption by rice husk biochar and bacterial biofilm were showed by Redlich–Peterson and Langmuir model using Origin software.

RESULTS

In present study, effect of Cr(VI) on biosynthesis of biofilm by *Pseudomonas putida* strain KI supported on specific carbon material was determined. All the experiments were performed in the laboratory of Department of Environmental Sciences, PMAS Arid Agriculture University Rawalpindi, Pakistan.

Potential of selected agricultural materials as adsorbent for Cr(VI)

Percentage adsorption of 5 ppm Cr(VI) by different adsorbents (rice husk biochar, corn cob, corn cob biochar and coal) was showed in Figure 1. Results presented that overall process of adsorption of different adsorbents increased with passage of time but remained constant after 180 minutes. Rice husk biochar showed the maximum removal potential of 56.8% with standard deviation of 0.20 after 180 minutes, and corn cob biochar showed minimum removal of 36.6% after 180 minutes with standard deviation of 0.25.

Figure 2 showed that percentage adsorption of 10 ppm Cr(VI) by different adsorbents (rice husk biochar, corn cob, corn cob biochar and coal). Results revealed that adsorbance increased with the passage of time but remained constant after 180 minutes. Rice husk biochar showed the maximum removal potential of 60.6% with standard deviation of 0.26 and corn cob biochar showed minimum removal potential of 39.1% with standard deviation of 0.15% after 180 minutes.

As presented in Figure 3, percentage adsorption of 15 ppm Cr(VI) by different adsorbents (rice husk biochar, corn cob, corn cob biochar and coal) increased with the passage of time but remained constant after 180 minutes. Rice husk biochar showed the maximum removal potential of 62.4% with standard deviation of 0.10 and corn cob biochar showed minimum removal potential of 40.4% with standard deviation of 0.18 after 150 minutes.

Percentage adsorption of 20 ppm of Cr(VI) by different adsorbents (rice husk biochar, corn cob, corn cob biochar and coal) was presented in Figure 4 that revealed that after 180 minutes, adsorption rate of rice husk biochar was increased up to 60.5% with standard deviation of 0.18 and minimum adsorption rate 40.1% showed by corn cob biochar with 0.10 standard deviation after 150 minutes.

Percentage removal of 25 ppm of Cr(VI) by different adsorbents (rice husk biochar, corn cob, corn cob biochar and coal) was presented in Figure 5. Rice husk biochar exhibited the maximum adsorption potential of 55.7% with standard deviation of 0.18 after 180 minutes and corn cob had minimum adsoption potential of 34.6% with standard deviation of 0.06 after 150 minutes.

Adsorption isotherms

Experimental data of highly effective adsorbent (rice husk biochar) were represented by Langmuir and Redlich-Peterson model (Figure 6 and 7). Adsorption isotherms were plotted between Qe and Ce that represented an adsorption capacity and Cr(VI) concentration at equilibrium. Constants and parameters of models were calculated and also presented (Table 1 and 2). All equations fit the data reasonably well. The following formula was used for determination of Q_e .

$$Q_e = (C_o - C_e)\ V/\ W.$$

Where C_o and Ce is initial and equilibrium concentration of Cr(VI). V is metal solution volume in liters and W adsorbent mass in grams. Equations of Langmuir and Reddlich-Peterson model are :

Langmuir isotherm: $Q_e = Q_{max}\, bC_e / (1 + bC_e)$

Reddlich-Peterson isotherm: $Q_e = K_R C_e / (1 + a_R C_e^{\beta})$

Reductionl potential of Pseudomonas putida KI

Data presented in Figure 8 showed the Cr(VI) percentage removal of 5 ppm concentration during 21 days of biofilm formation. It was indicated from the results that Cr(VI) reduction was constant after 16 days of biofilm development and maximum reduction potential of 71.6% by bacterial biofilm with standard deviation of 1.2 at 5 ppm concentration after 21 days. Biofilm had 11.6% more efficiency to reduce Cr(VI) compared to the samples having no biofilm.

Results revealed that maximum Cr(VI) reduction potential of 73.1% at 10 ppm concentration was found by bacterial biofilm with standard deviation of 0.47 after 21 days (Figure 9). Biofilm had 10% more efficiency to reduce Cr(VI) as compared to samples having no biofilm. Maximum Cr(VI) reduction potential of 73.2% was shown by bacterial biofilm of *P. putida* KI using rice husk biochar as a support material at 15 ppm concentration with standard deviation of 0.31 after 21 days (Figure 10). Biofilm had 5% more efficiency to reduce Cr(VI) as compared

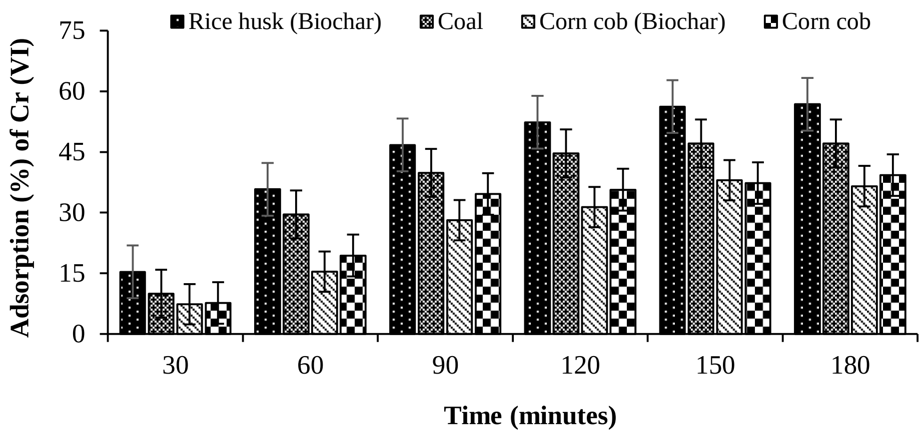

Figure 1 Adsorption of Cr(VI) on different carbon materials as adsorbents at 5 ppm Cr(VI) concentration

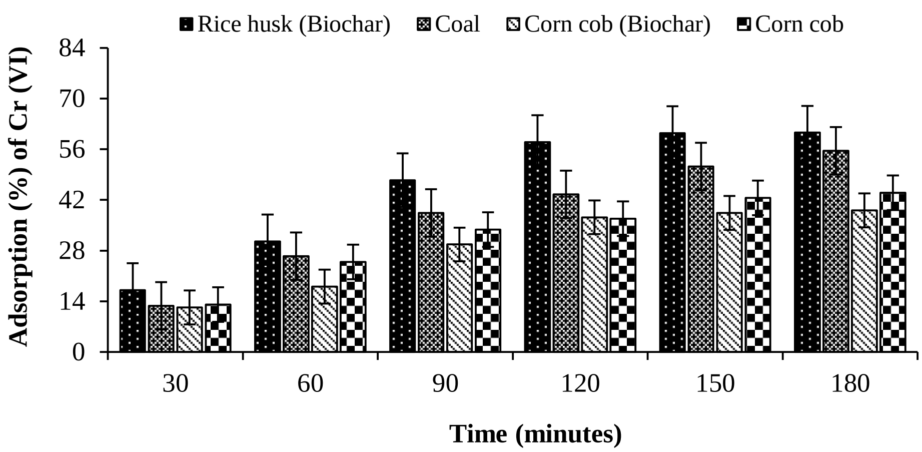

Figure 2 Adsorption of Cr(VI) on different carbon materials as adsorbents at 10 ppm Cr(VI) concentration

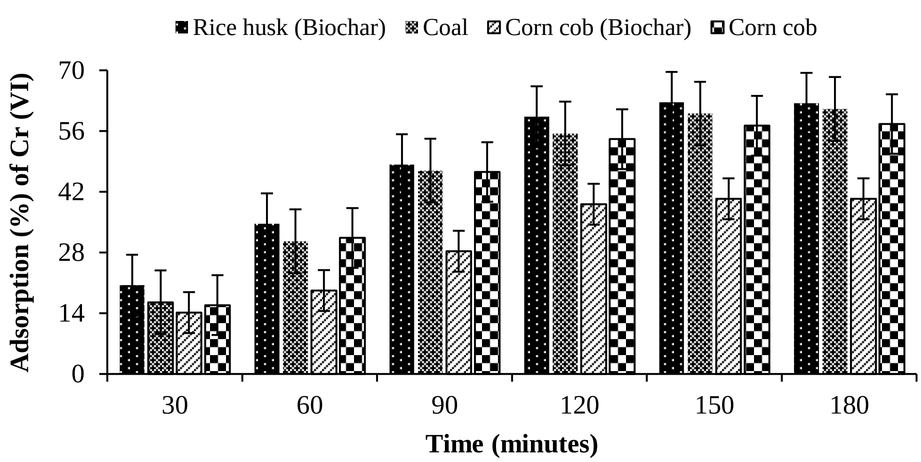

Figure 3 Adsorption of Cr(VI) on different carbon materials as adsorbents at 15 ppm Cr(VI) concentration

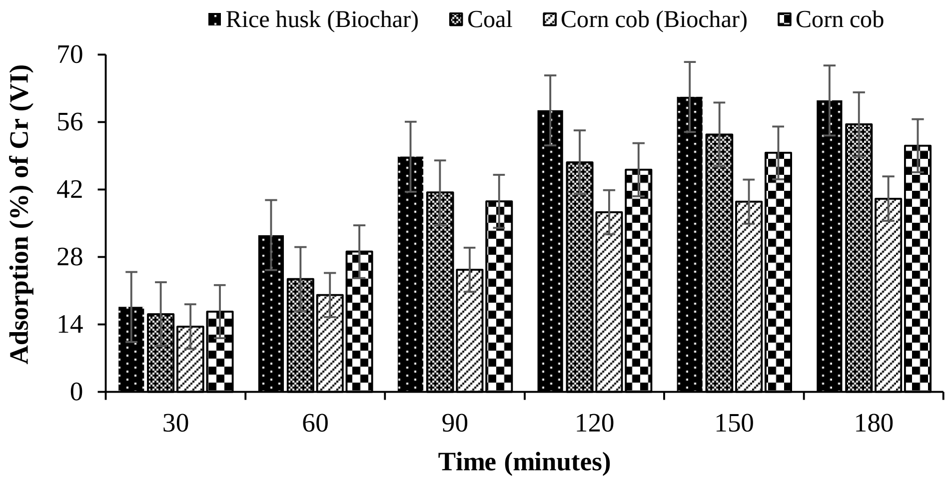

Figure 4 Adsorption of Cr(VI) on different carbon materials as adsorbans at 20 ppm Cr(VI) concentration

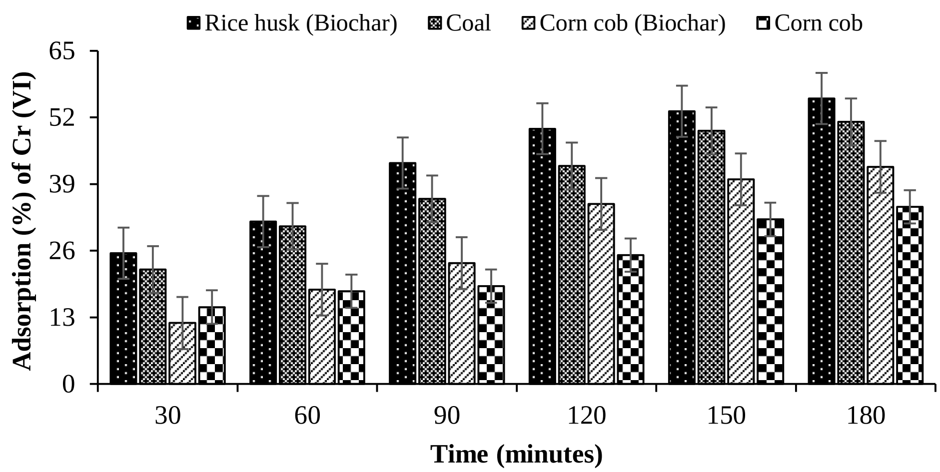

Figure 5 Adsorption of Cr(VI) on different carbon materials as adsorbents at 25 ppm Cr(VI) concentration

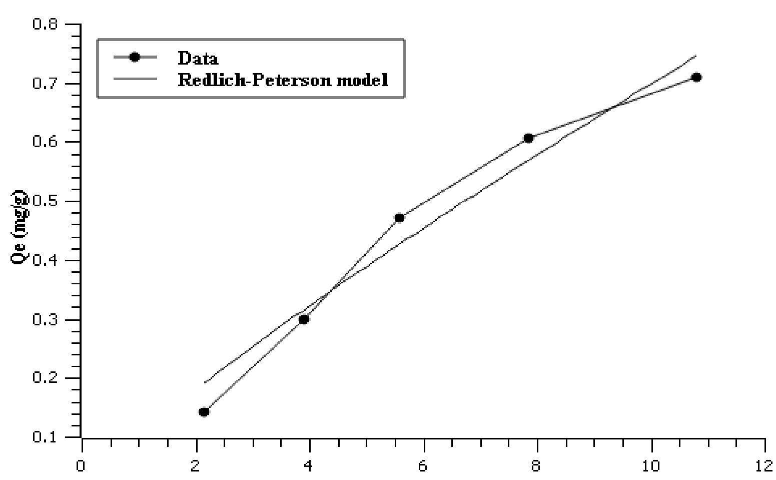

Figure 6 Cr(VI) sorption isotherm by rice husk (biochar) using Redlich-Peterson model

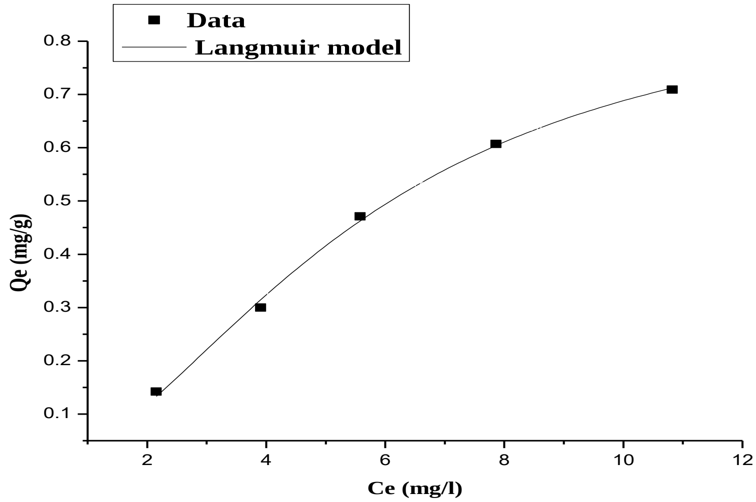

Figure 7 Cr(VI) sorption isotherm by rice husk (biochar) using Langmuir model

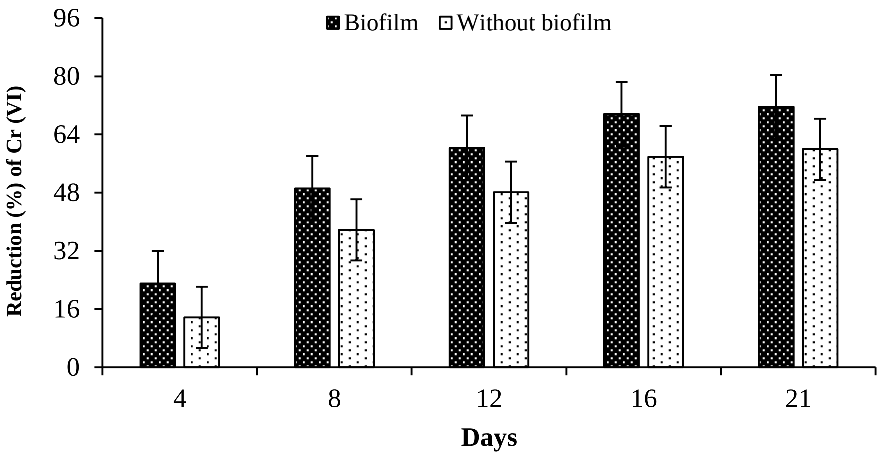

Figure 8 Reduction of Cr(VI) by biofilm of *Pseudomonas putida* KI using rice husk biochar as a support material at 5 ppm concentration

to samples having no biofilm. Figure 12 showed Cr(VI) percent removal at 25 ppm concentration during 21 days of biofilm formation. Results revealed that Cr(VI) maximum reduction potential of 65.1% by bacterial biofilm with standard deviation of 0.15 after 21 days. Biofilm had 9% more efficiency to reduce Cr(VI) as compared to samples having no biofilm.

Bacterial viability

Viability of bacterial cells was decreased with the increase of Cr(VI) concentration (Figure 13). It was found that as Cr(VI) concentration raised, cfu decreased from 2.61×10^7 to 1.27×10^7 after biofilm development.

Growth P. putida during biofilm development

The optical density of bacteria at diferent Cr(VI) concentrations of 5, 10, 15, 20 and 25 ppm increased till 12 days and then started to decrease till the end of biofilm development.

Isotherms onto P. putida biofilm

Results indicated that optical density was increased up to 12 days of biofilm formation and then decreased at the end of biofilm development. It was found in literature that decrease in rate of bacterial growth specified that bacterial culture had entered into another phase of its growth due to bacterial cell aggregation during the development of biofilm. Langmuir and Redlich–Peterson models were also fitted with data that described sorbent (rice husk biochar) capability to fix with adsorbate (Figure 14, 15). This research study was confirmed that the biofilm played the significant role in important role in bio-sorption process.

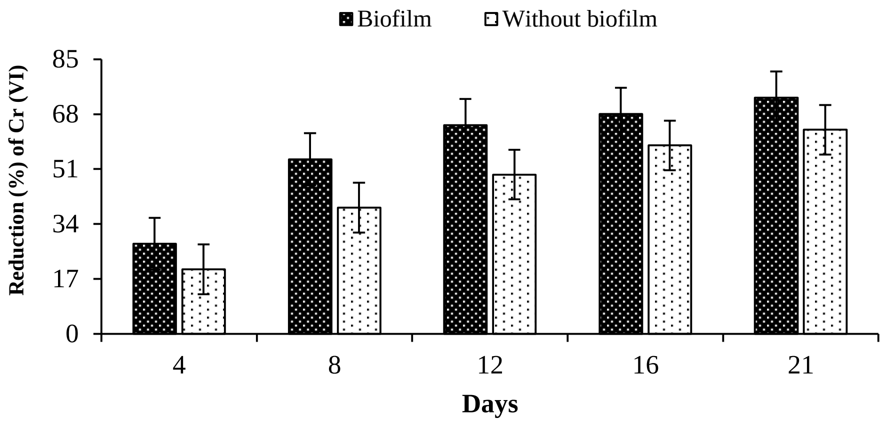

Figure 9 Reduction of Cr(VI) by biofilm of *Pseudomonas putida* KI using rice husk biochar as a support material at 10 ppm concentration

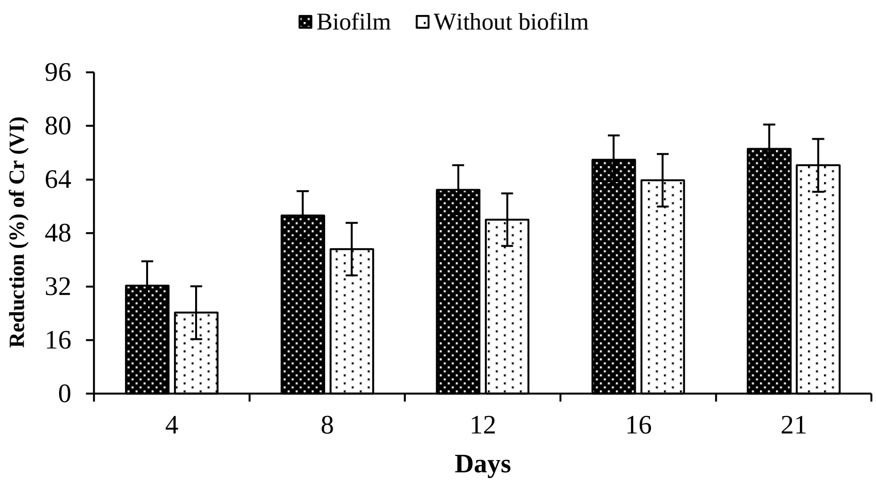

Figure 10 Reduction of Cr(VI) by biofilm of *Pseudomonas putida* KI using rice husk biochar as a support material at 15 ppm concentration

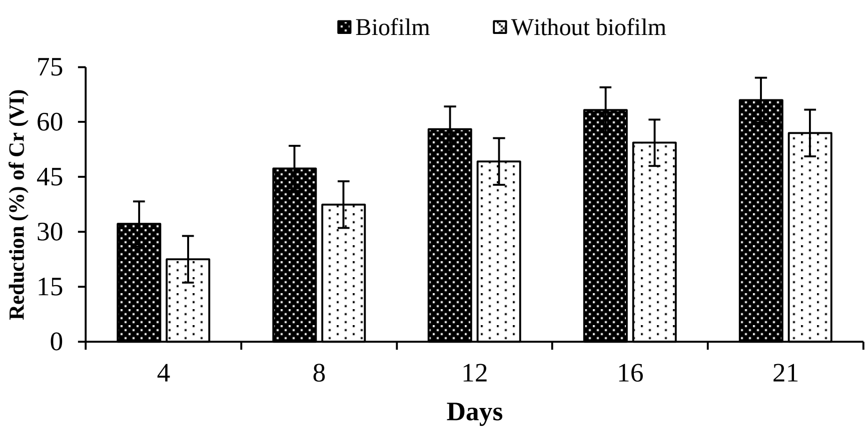

Figure 11 Reduction of Cr(VI) by biofilm of *Pseudomonas putida* KI using rice husk biochar as a support material at

20 ppm concentration

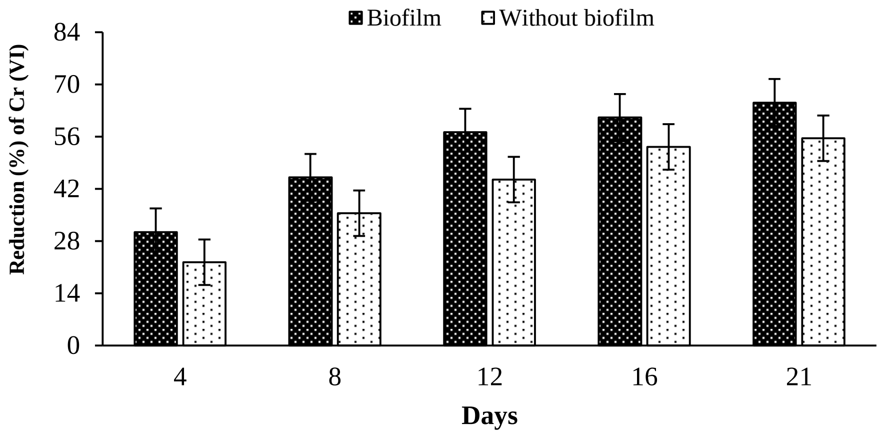

Figure 12 Reduction of Cr(VI) by biofilm of *Pseudomonas putida* KI using rice husk biochar as a support material at 25 ppm concentration

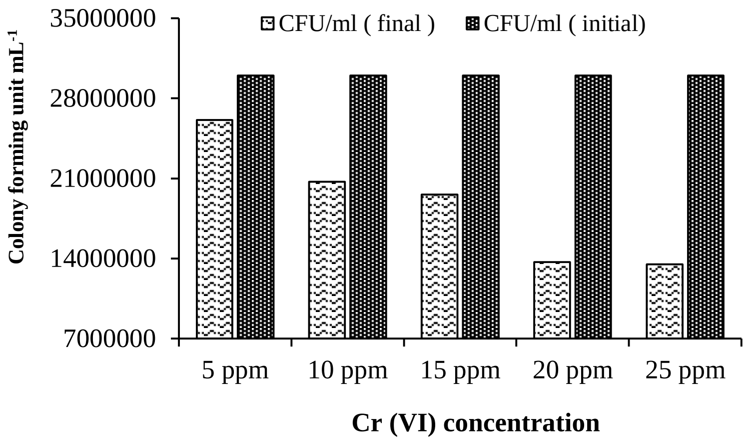

Figure 13 Colony forming unit (CFU mL^{-1}) of biofilm samples having different concentrations (5, 10, 15, 20 and 25 ppm) of Cr(VI) at start and end of biofilm development

DISCUSSION

Untreated wastewater contaminated with heavy metals has been discharged into environment from the industrial effluents. Some of these metals are Cr, copper (Cu), cadmium (Cd), zinc (Zn), nickel (Ni), cobalt (Co) and mercury (Hg). Amongst these, Cr is considered as extremely harmful and noxious that is highly discharged by leather and tanning industries (Dehghani et al. 2015). Chromium has been existing in various forms. Some oxidation states +3 and +6 are more frequently exist in nature. There is a need of proper treatment strategy for the elimination of Cr(VI) from wastewater before it releases into aquatic bodies and causes harm to aquatic forms of life. Various treatment methods that include process of reduction by membrane separation, electrocoagulation, bio-chemical precipitation, ion exchange method, nano-particles, electrodialysis, and filtration process can involve in Cr(VI) eradication from polluted water (Akbal and Camci 2012). Use of these techniques are often minimum due to its large economical costs. Mainly, when small quantity of heavy metals present in huge quantity of industrial runoffs.

Adsorption method might be considered a promising approach because of its simple strategy and cost effectiveness for elimination of harmful metals of polluted water. Various agricultural materials can be used as suitable alternatives of other expensive sorbents like activated carbon. Some cheap materials like sawdust, rice polish, fly ash, tea waste etc. have been used in many research studies for this purpose. Widespread applicability of Cr in industries causes release into environment. Biological metal reduction through microbes involves numerous mechanisms that include chelation, complexation, adsorption and metal

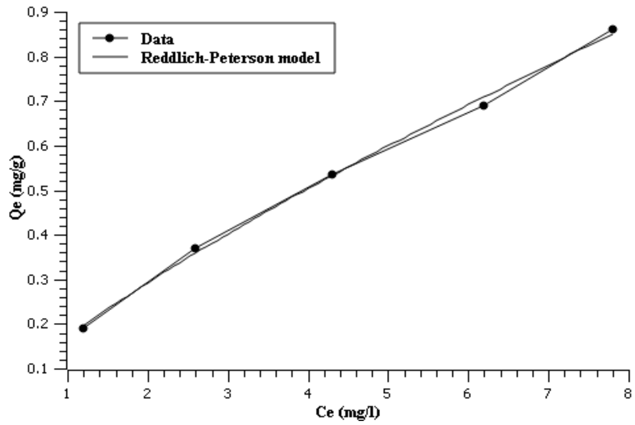

Figure 14 Cr(VI) sorption isotherm of bacterial biofilm adjusted by Redlich-Peterson model

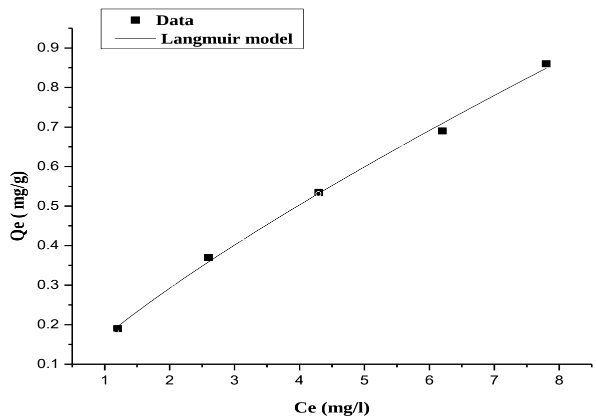

Figure 15 Cr(VI) sorption isotherm of bacterial biofilm adjusted by Langmuir model

entrapment process (Reddy et al. 2012).

It was clearly observed that efficacy of different adsorbents towards Cr(VI) removal was enhanced with corresponding rise in Cr(VI) concentration from 5 to 15 ppm. At 5 ppm initial concentration of Cr(VI), adsorption occurred with maximum removal of 56.8% by rice husk biochar, 47.1% by coal, 39.3% by corn cob and 36.6% by corn cob biochar. At concentration of 10 ppm, maximum Cr removal was 60.6% by rice husk biochar, 55.6% by coal, 44% by corn cob and 39.1% by corn cob biochar. At 15 ppm concentration, removal efficiency of rice husk biochar was increased up to 62.4% by coal, 57.6% by corn cob and 40.4% by its biochar. At 20 and 25 ppm initial Cr(VI) concentration, substantial decrease of 58.5% and 55.7% in Cr(VI) removal by rice husk biochar, 55.5% and 51.2% by coal, 51.1% and 42.4% by corn cob and its biochar removed 37.2% and 34.6% of Cr(VI). Similar results were reported by Ashraf et al. (2020), Maes et al. (2020). According to pervious literature, this evidence of reduction was happened because of inhibitory effect of high Cr concentration (Thatoi et al. 2014). It was obtained from the results that samples having bacterial biofilm supported on rice husk biochar showed more effective results as compared to samples without biofilm.

CONCLUSION

The sequence of test adsorbents in terms of removal efficiencies for Cr(VI) was rice husk biochar > coal > corn cob > corn cob. A decreasing trend in percent adsorption of Cr has been observed with rise in Cr initial concentration.

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