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NOVEL METHODOLOGY FOR WASHING OF CHROMIUM CONTAMINATED SOIL USING BIOSURFACTANT IN PAKISTAN

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Abstract

Chromium is acutely toxic, mutagenic, and carcinogenic for all forms of life. Industrial sources release chromium thus contaminating water and soil. As biosurfactants are natural, easily degradable, non-toxic, environment-friendly, and cost-effective products, we have applied them here for bioremediation and prepared rhamnolipids as biosurfactants. Rhamnolipids were prepared by using Pseudomonas aeruginosa in mineral salt (MS) media at pH 7 and kept in an incubating shaker for 48 hours at 37 °C. Centrifugation was used to extract the cells from the culture broth and cell-free broth was later used for washing the soil. $K_2Cr_2O_7$ was used for soil spiking. By optimizing different variables like pH, time, concentration of chromium, dilution of rhamnolipids, and percentage removal of chromium studied, the best removal (89.25 %) of chromium was obtained in 6 hours using 3:1 rhamnolipid dilution for 500 ppm of $K_2Cr_2O_7$ at pH 4. However in some cases, after 24 hours, as rhamnolipid started releasing chromium, a decrease in chromium removal percentage has been observed which represents the mechanism shift from micelle remediation to reduction one and causes reduction of Cr^{+6} to insoluble Cr^{+3}. Results of the present research suggested that microbial metabolites such as biosurfactants are found to be significantly efficient for the removal of chromium (89.25 %) from the contaminated soil within 6 hours using 3:1 rhamnolipid dilution for 500 ppm of $K_2Cr_2O_7$ at pH 4. These biosurfactants can be used for the reduction of Cr^{+6} from wastewater.

Keywords: *Bioremediation, Biosurfactant, Chromium, Heavy metals, Optimization, Pseudomonas aeruginosa, Rhamnolipid, Washing of soil, Waste treatment*

INTRODUCTION

Contamination of water and soil because of the excess use of chemicals and industrial waste has become a major environmental issue all over the world (1). During the past two decades' industrial sector in Pakistan has expanded exponentially. A large number of industries functioning in Pakistan like leather, textile, metal processing units, pulp, paper, etc., are considered a major source of pollution by releasing huge amounts of heavy metals especially chromium into the environment (2). The most common pollutants are petroleum hydrocarbons, solvents, pesticides, lead, chromium, and other heavy metals. Chromium is found to be the second most common heavy metal at these contaminated sites (3). Its wide diversity of applications and discharge of untreated industrial effluents have made it a serious pollutant to the environment. Waste is directly discharged into water bodies as most industrial units do not have waste

treatment mechanisms leading to the emergence of serious health concerns. Weak execution of environmental laws and the expensive cost of water treatment plants are the major reasons for the current situation.

Tanning of leather, electroplating plant effluents, textile manufacturing, etc., are observed to be the major sources of chromium-based contaminated soils and groundwater. It is reported that in an acidic medium, chromium-III is oxidized to chromium-VI which is highly toxic, mutagenic, and carcinogenic (4). Literature reported that the high mobility of chromium is the major cause of groundwater and soil contamination (5).

For the removal of toxic chromium, different conventional methods like chemical reduction followed by precipitation (6), ion exchange (7), adsorption (8), soil flushing/enhanced extraction electrokinetics, phytoremediation, bioreduction, bioaccumulation, biomineralization, and bioprecipitation (9) are used. Jean Soro et al., used citric acid (biodegradable) and EDTA (non-biodegradable) to leach chromium whereas, Gitipour et al., used acetic acid, EDTA and 0.3 M HCl solution for the removal of chromium from refinery sludge samples and reported that HCl had the highest (82.69 %) removal rate (10, 11). Zhao et al., used EDDS and $FeCl_3$ separately and collectively and found that solution of EDDS and $FeCl_3$ had better leaching efficiency (21.29 %) (12). Wu et al., removed 80.46 % of chromium by using SA-FeSSi gel beads coated with Na-alginate (SA) (13). However, these methods require high energy or large quantities of chemical reagents. In contrast, these toxic Cr-VI can be converted to Cr-III by using microbes. These microbes can remediate this toxic Cr-VI by eating up, catalyzing reduction into Cr-III or releasing metabolites (like biosurfactants) (14, 15) and flushed out of contaminated soil and play a vital and cost-effective role in the field of green technology (16, 17). Surface active agents like biosurfactants are preferably used for bioremediation as they are highly tolerable under extreme conditions, biodegradable, least toxic, and highly specific. Biosurfactants are produced by bacteria, fungi and yeasts as part of cell membrane or extracellularly (18). In terms of surfactant characteristics rhamnolipids, produced aerobically by *Pseudomonas* species, are the most researched biosurfactants (19, 20). Rhamnolipids produced by *Pseudomonas aeruginosa* are more preferred because of their strong emulsifying action and low Critical Micelle Concentration. Lipid micelles help in extracting metal from the soil by transferring it into solution and making it simpler to flush out (21). Rhamnolipid is a carbohydrate that is composed of rhamnose sugar and β-hydroxyalkanoic acid. As compared to a bacterial cell, rhamnolipid molecules may readily migrate through soil due to their small size (<5 nm). Moreover, it contains one polar head and a non-polar tail in its structure. When this rhamnolipid comes in contact with metal-contaminated soil, its anionic part forms an ionic bond with cationic metal such as chromium. This bonding is stronger than the bonding between metal and soil and once it is developed it becomes easy to flush out the contaminates as water is pumped through the soil (22).

In the present research, Chromium VI was used as the pollutant, and for this purpose, soil was artificially polluted by using $K_2Cr_2O_7$. The pollutant chromium in the form of $K_2Cr_2O_7$ (with various concentrations as 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm) was analyzed by using an atomic absorption Spectrophotometer at a wavelength of 357 nm. For the removal of chromium from chromium-contaminated soil, the mixture of soil and rhamnolipids, produced by *P. aeruginosa*, was used as biosurfactant. According to our survey, no such simple procedure has been adopted for the removal of chromium in past and it is also important to mention here that a very small number of reports are available for the removal of chromium from soil and water under different dilutions of rhamnolipids (15, 23, 24).

MATERIALS AND METHODS

COLLECTION OF CONTAMINATED SOIL SAMPLES

Soil samples, that contain the desired microorganisms, were collected from Massa Kaswal oil wells (an industrial area) in sterile screw caped bottles. These samples were taken aseptically 4-5cm deep from soil surface and stored at 4°C till further use.

ISOLATION OF MICROORGANISMS

Oil enrichment technique was used for the isolation of bacterial strains from the contaminated industrial soil. An amount of 1 g of soil sample was added in 100 ml media of sterile mineral salt with 1g of glycerol. By using an orbital shaker (at 120 rpm), this mixture was incubated for 96 hours at 37°C. Once enriched, 1 ml of the cell suspension was taken and spread on to a nutrient agar plate and kept in incubation for 48 hours at 30°C. After incubation, colonies were appeared on this plate. These colonies were selected randomly and further sub-cultured to obtain the pure isolates (25).

IDENTIFICATION OF MICROORGANISMS

P. aeruginosa was isolated from the contaminated industrial soil of Massa Kaswal oil wells and was identified by the microbiologist at Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Pakistan.

PREPARATION OF BIOSURFACTANT

Freshly isolated *P. aeruginosa* stored at 4°C, was used for the preparation of biosurfactants by using mineral salt media (composition is given in Table I) as a fermentation medium in a shaking incubator (at 120 rpm) for four days at 37°C. This fermentation media was divided into tiny parts of 100 ml in 10 different conical flasks and its pH was maintained at 7 by using buffer before being placed in incubating shaker.

Table I. Amount of different chemicals used for the composition of salt media in 1 litre of distilled water

Chemical	Amount
Glucose (Sterilized)	1 g
KH_2PO_4	1.5 g
Na_2HPO_4	4 g
$NaNO_3$	1 g
$MgSO_4$	1 g
$CaCl_2$	0.004 g
$FeSO_4$	0.01 g

ACTIVITY DETECTION OF BIOSURFACTANT

Biosurfactants possess hemolytic activity and this was first time discovered when surfactant (biosurfactant), produced by Bacillus subtilis, was used to lyse red blood cells. Rhamnolipids were used for screening biosurfactants generated by fresh isolates. The main technique used for the screening of biosurfactant activity is blood agar lysis. In this technique microorganisms were allowed to grow on blood agar and lysis of red blood cells was observed after 24 hours (26).

For the determination of surfactant activity of each strain, microwell plate analysis is used. Sample strain of 1mL, from each 100 mL flask, was added in an Eppendorf and vortexed for 1 minute. An amount of µl of the supernatant was added to a 96-microwell plate (Cliniplate, labsystem). This plate was kept under observation by using a paper backing sheet with a black and white grid. Optical distortion of the grid confirms the presence of the biosurfactant (27). For the separation of bacterial cells, remaining contents of the apendrof were further centrifuged (1000 rpm) for 10 minutes and then for the broth standardization, it was heated for 30 minutes at 60 °C. The supernatant of this solution was then acidified to pH 2 by using concentrated HCl. Later, 750 µl of this solution and 750 µl of a (2:1) mixture of chloroform and methanol were vortexed for 1 minute. Lower organic phase was collected after centrifugation for 10 minutes. Extraction was repeated and the collected organic phases were dried by evaporation. It was resuspended in 1 ml of methanol, then filtered by using 0.45 µm membrane and dried. For TLC analysis, sample was resuspended in 20 µl methanol and a mixture of 18 % methanol, 2 % acetic acid and 8 % chloroform was used as mobile phase. Anthrone reagent dissolved in concentrated H_2SO_4 at a final concentration of 2 % was

used for the visualization of rhamnolipid spots. Different dilutions (1:1, 2:1 and 3:1) of this standardized rhamnolipids were prepared for studying its bioremediation.

SPIKING OF SOIL

For spiking of soil 300 ml of 100 ppm potassium dichromate solution was added in 100 g of soil. In order to obtain the chromium contaminated soil with level of 100 ppm potassium dichromate and 1.05 mg/g of chromium, this mixture was added incubating shaker for 96 hours and then oven dried at 80 °C for 24 hours.

This mixture of soil was put in incubating shaker for 96 hours and then dried in an oven at 80 °C for 24 hours to obtain chromium contaminated soil with level of 100 ppm potassium dichromate and 1.05 mg/g of chromium. In the same way, 2.10 mg/g, 3.00 mg/g, 4.03 mg/g and 5.25 mg/g concentrations of chromium were obtained by contaminating the soils with 200 ppm, 300 ppm, 400 ppm and 500 ppm of potassium dichromate, respectively.

WASHING OF SOIL

Rhamnolipid mixture in different dilutions (1:1, 1:2 and 3:1) were used for the washing soil. For this purpose, a mixture of rhamnolipid and soil (3:1) was made in a flask by adding a total of 18 ml of rhamnolipid solution, from each dilution, to 6 g of contaminated soil. For specific time, this mixture was kept in an incubating shaker. Later, these samples were filtered through Whatman No. 40-41 filter paper and oven dried (overnight) at 50 °C in a petri dish until a constant weight was achieved. Finally, fine grains of soil were obtained by grinding these samples.

OPTIMIZING CONDITIONS

Literature shows that the parameters like pH, time and concentrations of rhamnolipid have significant effects on the removal of heavy metals as compared to the ion strength (28). Therefore, to achieve the maximum removal of chromium, pH and time for different stages of chromium contamination were optimized. It is also reported that an increase in temperature converts the rhamnolipid micelles into vesicles and vesicles are not conductive for the desorption of metal from the soil (29), therefore, present research was done at room temperature that is, 25 °C. Values of pH were maintained between 4 to 8 by using HCl or KOH. For time optimization, rhamnolipid dilutions (1:1, 2:1 and 3:1) were added in various soil contamination, i.e. 1.05 mg/g, 2.10 mg/g, 3.00 mg/g, 4.30 mg/g and 5.25 mg/g of chromium for 6 hours and 24 hours.

ANALYSIS OF CHROMIUM

For the determination of the concentration of chromium, this soil was digested and analyzed by using atomic absorption spectrophotometer at wavelength of 357.9 nm. For digestion, 3 g of soil and 12 ml of Aqua Regia were added in 250 ml beaker. This mixture was covered with watch glass and heated on hot plate for 3 hours at 110°C. Later, 20 ml of 2 % HNO_3 was added in this mixture and kept on further heating for 15 minutes. This mixture was diluted up to 100 ml with distilled water and filtered in order to take absorbance by using atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

A separation technique, known as soil washing, is used for the removal of contaminants from the soil. In this technique, soil clods and large objects are removed by pretreating the excavated soil and then washed with fluids. To obtain the clean soil, the liquid solution (containing metal) is pumped out from this soil mixture. Recently, researchers are focused on biological wash fluid and one of them is rhamnolipid (biosurfactant), which has significant potential for the removal of metals from the soil and can easily be recycled and reused. Rhamnolipids are one of the major types of biosurfactants produced by *P. aeruginosa*, and these have several advantageous properties that make them suitable for various applications. Rhamnolipid, known as environmental benign washing agent, is preferred in present research due to its higher conditional stability constants with heavy metals (like Pb, Cr, Cu, and Cd) as compared to other

organic acids, due to its ability to cultivate with bacterial strains on large scale fermentation processes, allowing for the production and better complexation with toxic metals as compared to normal soil metals like calcium and magnesium (30). The optimization parameters studied were pH, chromium concentration and time against the chromium removal amongst different biosurfactant solutions i.e. stock, 1:1, 2:1 and 3:1. Thin Layer Chromatography (TLC) was performed in order to verify removal of Cr-VI by rhamnolipid. Visualization of rhamnolipid by above mentioned locating agent confirmed adsorption of Cr-VI by rhamnolipid. The percentage removal of chromium i.e. 89.25 % was significantly high after 6 hours as given in Table II.

Table II. Percentage removal of Cr after 6 hours at different pH and various rhamnolipid Dilutions

$K_2Cr_2O_7$ Conc. (ppm)	Cr Conc. per 6 g soil sample (ppm)	Rhamnolipids (RLs) mixture and water Ratio	% Removal of Cr after 6 hours at				
			pH 4	pH 5	pH 6	pH 7	pH 8
100	6.3	RL stock soln.	51.57	49.20	48.19	40.93	27.158
		1:1	51.78	48.56	44.62	43.95	43.17
		2:1	56.35	54.79	49.89	49.24	22.43
		3:1	40.38	37.73	37.73	31.74	4.33
200	12.6	RL stock soln.	51.55	49.17	47.86	47.69	40.99
		1:1	70.407	62.36	51.41	44.65	44.40
		2:1	52.079	50.12	48.36	45.21	44.23
		3:1	67.62	56.33	50.167	47.59	43.56
300	18.9	RL stock soln.	75.12	72.68	72.25	71.128	63.27
		1:1	78.87	72.25	70.883	68.86	67.517
		2:1	81.29	74.15	66.80	35.11	15.861
		3:1	76.64	70.44	66.911	66.64	65.32
400	25.2	RL stock soln.	86.63	83.11	76.430	70.32	68.63
		1:1	82.71	81.37	80.511	75.46	75.03
		2:1	82.39	77.87	74.68	73.73	71.15
		3:1	81.83	73.07	72.21	70.38	64.35
500	31.5	RL stock soln.	88.68	88.37	78.69	76.53	69.47
		1:1	85.58	73.29	72.26	72.12	70.13
		2:1	87.15	79.70	78.10	75.29	61.23
		3:1	89.25	76.43	70.23	76.01	68.93

It decreased after 24 hours because biosurfactant started releasing chromium as shown in Table III. After performing series of experiments, it was observed that the removal efficiency of Cr-VI by rhamnolipid is more significant in acidic medium than in basic one as it influences the solubility of contaminates and its interaction with soil particles.

Table III. Percentage removal of Cr after 24 hours at different pH and various rhamnolipid dilutions

$K_2Cr_2O_7$ Conc. (ppm)	Cr Conc. per 6 g soil sample (ppm)	Rhamnolipids (RLs) mixture and water Ratio	% Removal of Cr after 24 hours at				
			pH 4	pH 5	pH 6	pH 7	pH 8
100	6.3	RL stock soln.	49.127	47.92	40.746	28.17	18.825
		1:1	46.49	43.62	41.98	39.46	29.08
		2:1	45.97	45.21	35.78	33.32	0.6825
		3:1	54.19	54.026	49.79	43.56	41.365
200	12.6	RL stock soln.	53.08	52.5	49.28	48.86	40.89
		1:1	70.407	45.24	44.93	41.10	32.428
		2:1	69.04	53.42	49.22	45.76	45.53
		3:1	65.65	59.95	47.68	47.08	39.11
300	18.9	RL stock soln.	77.23	75.93	73.1	72.717	63.27
		1:1	71.161	70.53	68.85	64.739	64.69
		2:1	75.344	73.65	71.84	72.87	68.33
		3:1	74.71	73.03	70.54	64.35	63.37
400	25.2	RL stock soln.	81.05	80.91	79.56	77.9	76.84
		1:1	83.36	79.68	72.43	69.92	68.04
		2:1	84.08	76.97	70.12	68.83	68.52
		3:1	76.40	72.29	63.66	62.1	48.58
500	31.5	RL stock soln.	85.96	79.37	75.47	72.28	52.66
		1:1	88.75	86.07	74.98	70.78	55.11
		2:1	88.19	83.52	78.76	77.64	72.99
		3:1	85.62	80.196	73.93	70.83	49.81

Zhang et al., worked on the remediation of co-contaminated soil by cadmium and phenanthrene with rhamnolipid. They showed that the formation of micelle and ionization of carboxyl and hydroxy group are increased as the pH value of rhamnolipid solution is increased. Furthermore, they also suggested that washing of metal by rhamnolipid depends on the complex formation between cadmium ions and carboxyl groups or hydroxyl groups of rhamnolipid and under strong alkaline or acidic conditions chelation ability of rhamnolipid is inhibited (28).

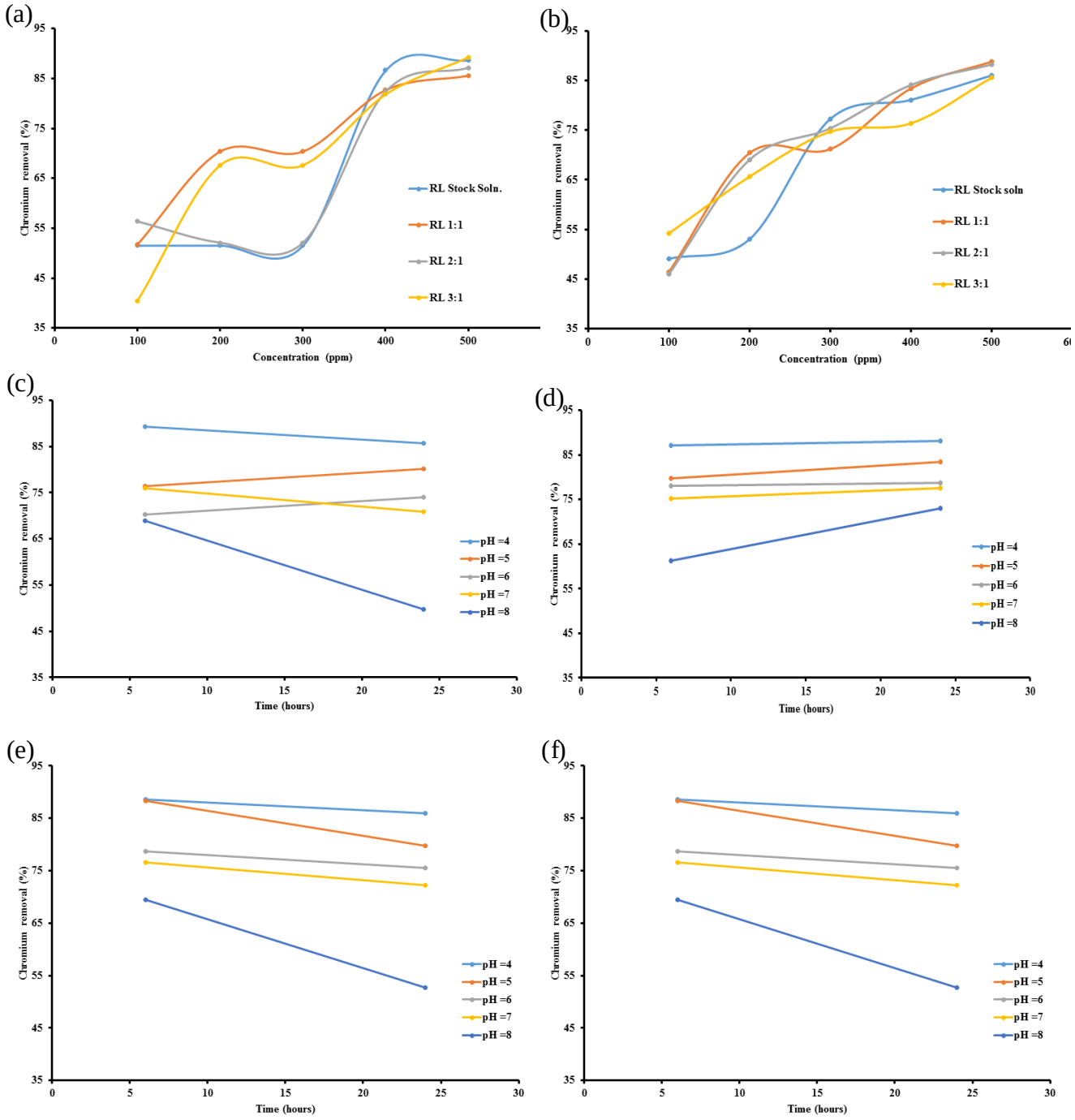

Fig. 1 (a) Comparison of Cr removal (%) by rhamnolipid dilutions at various salt concentrations for 6 hours and pH 4. **(b)** Comparison of Cr removal (%) by rhamnolipid dilutions at various salt concentrations for 24 hours and pH 4. **(c)** Effect of pH on chromium reduction (%) at different times by stock 3:1 dilution of RL for 500 ppm Cr salt concentration. **(d)** Effect of pH on chromium reduction (%) at different times by stock 2:1 dilution of RL for 500 ppm Cr salt concentration. **(e)** Effect of pH on chromium reduction (%) at different times by stock 1:1 dilution of RL for 500 ppm Cr salt concentration. **(f)** Effect of pH on chromium reduction (%) at different times by stock RL for 500 ppm Cr salt concentration

In present study, results also showed an inverse relationship between the percentage removal of Cr-VI and pH, that is, as the value of pH was increasing (becoming more alkaline), the percentage removal of Cr-VI was decreasing. It is found that maximum percentage removal of Cr-VI is obtained at pH 4 (as shown in Fig. 1c) which depicts the fact that effective micelle is formed at pH 4. Washing rate of metal ion is increased as the concentration of rhamnolipid is increased. Three facts are involved in this washing (i)

Carboxyl group of rhamnolipid binds with metal ions via chelation; (ii) desorption of metal ions is promoted by the interfacial interaction between rhamnolipid and soil; (iii) electrostatic repulsion between soil and rhamnolipid solution results in the dispersion of soil (29). After studying the effect of time (6 hours and 24 hours) on the activity of rhamnolipid and its dilutions, it was observed that all the dilution of rhamnolipids removed Cr-VI after 24 hours of treatment under optimal conditions in shake flask incubator as shown in Fig. 1 (a) and (b).

It is also noticed that maximum removal of chromium has been observed for 500 ppm chromium salt concentrations for all rhamnolipid dilutions at 6 hours as given in Table 1. Results for the percentage removal of chromium, as shown in Fig. 1 (c) to (f), for 24 hours showed a nearly constant trend line for most of the samples, however, a decline is observed in few cases.

The possible reason for this drop may be as the rhamnolipid started releasing chromium so the percentage removal of chromium starts decreasing and thus shifted the mechanism of removal from micelle remediation (Cr-VI) to reduction remediation (insoluble Cr-III). A direct relationship is observed between the chromium concentration and its percentage removal under optimized conditions. It means that the percentage removal of chromium (Cr-VI) is increased as the concentration of Cr-VI is increased; however, the rate of reduction is varied with the amount of Cr-VI present initially. As a whole, the optimum recovery of chromium is obtained for 500 ppm chromium contaminated soil, as shown in Fig. 1 (a), and justified that the micelle formation is also accelerated at higher concentration of chromium. Overall, maximum removal (89.25 %) of Cr-VI is obtained, after 6 hours, by 3:1 rhamnolipid dilution for 500 ppm of Cr-VI salt concentration at pH 4 (Fig. 1c). Whereas, the results obtained by the stock solution of rhamnolipid were also interesting and showed the next best removal rate (88.68 %) for 500 ppm Cr-VI salt concentration, after 6 hours' time duration. Comparison of %age removal of Cr-VI at various pH values after 6 hours using different dilution ratios of rhamnolipids to water has been compared in the form of bar chart as shown in Fig. 1 (b). It was observed that best removal of Cr-VI from water took place at 4 pH values within 6 hours only.

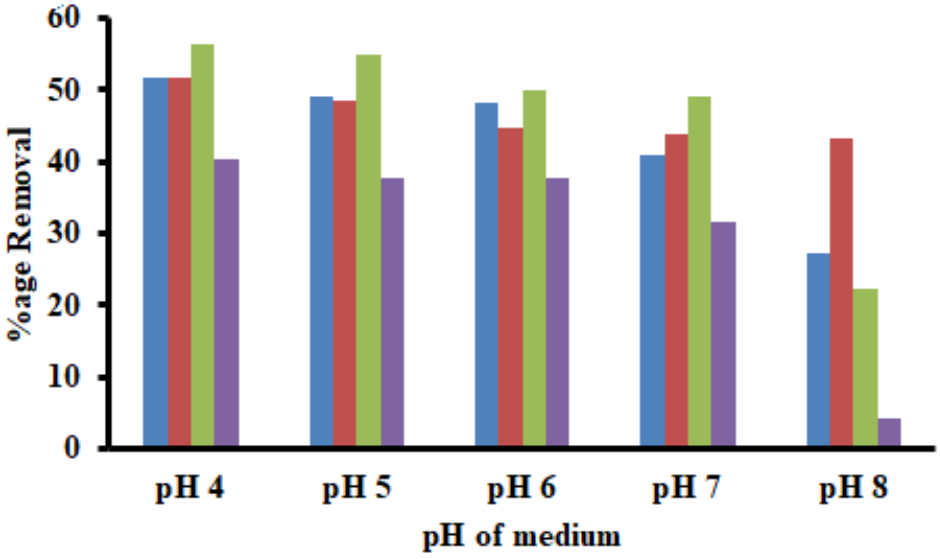

Fig. 2. Bar chart showing effect of pH of the medium on the %age removal using various rhamnolipid to water dilutions after 6 hours of start of process

Findings obtained from present research proves that biosurfactants such as rhamnolipids can also be used as a remediation method for washing of soil. The aim of the study was to determine the viability of chromium removal by using biosurfactant and it is successfully accomplished. It is further recommended that with the help of these biosurfactants, other heavy metals and toxic organic compounds can also be eliminated.

Overall, removal of Cr-VI by using biosurfactant (rhamnolipid) is proved to be of great potential and applicability because of its safe, environment friendly and economically cheap methodology. However, this method was done at laboratory scale and could be applied widely to the chromium polluted areas in order to control the seriously increasing rate of soil pollution.

CONCLUSION

Removal of chromium form water and soil has gained much attention from waste water and soil due to industrialization and urbanization. Among several methods available for removal of chromium, use of biosurfactant is found to be a very important one because it is nontoxic and cost effective. In this work we have produced rhamnolipids, in an incubator, using *Pseudomonas aeruginosa* in MS media under optimized conditions of pH, temperature and time. Then this biosurfactant was used for the removal of chromium from soil samples which was spiked by using $K_2Cr_2O_7$ salt. Removal of chromium under different conditions of pH and dilution of rhamnolipids was done. It was found that maximum removal of chromium (89.25 %) was achieved at pH 4 with 3:1 rhamnolipids dilution for 500 ppm $K_2Cr_2O_7$ contaminated soil. It was noticed that maximum removal was achieved within 6 hours and in most of the experiments %age removal started decreasing after 24 hours because biosurfactant started releasing chromium. These results confirmed that rhamnolipids served as an excellent biosurfactants for efficient removal of chromium form soil samples.

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