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Cr(VI) reduction by *Cellulosimicrobium* sp. isolated from tannery effluent

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Abstract: Cr(VI) is a toxic metal that has carcinogenic and mutagenic effects on all living organisms. In order to study the contribution of microbes towards Cr(VI) reduction into the less toxic Cr(III) form, an indigenous chromium-resistant bacterial strain (A_8) was isolated from a tannery effluent. It was identified as *Cellulosimicrobium* sp. on the basis of morphological and biochemical characterization and 16S rRNA gene sequencing. It could tolerate up to 1800 µg mL^{-1} of K_2CrO_4 and showed an optimum reduction (98.6%) of Cr(VI) at the concentration of 900 µg mL^{-1} aerobically. Cell-free enzyme assay exhibited the reduction of Cr(VI) in vitro. A total of 4 synthetic materials, sand, PVC, stone, and rubber tubing, were used as solid support to evaluate the ability of the isolated chromium-resistant bacterial strain for biofilm formation. In column experiments among bacterial film-coated materials, sand exhibited an excellent Cr(VI) reduction (96%), while PVC pipe, rubber tubing, and stone showed 94.5%, 90%, and 88.4% reduction potential of Cr(VI), respectively, after 96 h of incubation. Fluorescent microscopy also revealed that bacterial biofilm was distributed thoroughly on the surface of the sand particles. FTIR spectroscopy showed mainly the involvement of the glycerol units, polysaccharides, and C-N (alkyl) functional groups of the bacterial strain (A_8) in the reduction of Cr(VI).

Key words: Biosorption, *Cellulosimicrobium* sp., Cr(VI) reduction, FTIR spectroscopy, bacterial columns

1. Introduction

Naturally chromium exists in many oxidation states, but Cr(III) and Cr(VI) are of significant concern biologically. Chromium is an essential metal that is involved in the metabolism of glucose in humans and animals, but its hexavalent form is very toxic and carcinogenic (1). Demand for Cr(VI) is increasing day by day due to its extensive use in many industrial and chemical processes such as film and photography, galvanometric and electrical procedures, metal cleaning, plating and electroplating, leather, and mining (2). These industrial processes generate toxic effluent in a large amount that contains hexavalent chromium with concentrations ranging from tens to hundreds of milligrams per liter along with other forms of chromium (3). According to United States Environmental Protection Agency, the maximum acceptable concentration of Cr(VI) in drinking water is 50 µg L^{-1} (4). The industrial waste water causes great threat to the environment, and it is irreversibly toxic (5). Effective metal recovery is required before the discharge of waste water containing heavy metals. Physical and chemical methods are available for removing these heavy metals from water, such as precipitation, coagulation, reduction, membrane processes, ion exchange, and adsorption; however, many of these are inefficient and costly (6). A number of studies have demonstrated the bioreduction of chromate by using immobilized cell-free chromate reductase. One unit of enzyme activity is defined as the amount of enzyme that is used to reduce 1 µmol of Cr(VI) per minute at 30 °C (7).

Biosorption has been defined as the property of certain biomolecules (or types of biomass) to bind and concentrate selected ions or other molecules from aqueous solutions (8). Wastes from agricultural and industrial activities, naturally available seaweeds, and especially propagated biomass of bacteria, yeast, and fungi are considered as useful materials for the biosorption process (9). More recently, biosorption of metals by immobilized cell systems has been used effectively for the removal of metals from industrial effluent. This technology exploits the natural tendency of cells to accumulate elements or their innate ability to degrade recalcitrant organic compounds. Traditionally, a very diverse range of materials such as rocks, sands, plastics, latex, paper, and steel have been used as biofilm supports. Polystyrene sheets, needle-punched polyester, and polyvinyl chloride (PVC) foils in various geometries have lately been in use with growing popularity (10). Metal ion removal from aqueous solution also takes

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place by complex formation on the cell surface after the interaction between metal ions and active groups (11).

Keeping all this in view, the aim of the present study was to isolate the chromium-resistant bacterial strain from the waste water of the tannery industry. This chromium-resistant bacterial strain would be a better choice in comparison to the conventional costly chemical techniques for bioremediation of a chromium-contaminated environment.

2. Materials and methods

2.1. Strain isolation and growth conditions

The sampling for the isolation of the chromium-resistant bacterial strain was carried out from the waste water of the tannery industry of Kasur, Pakistan. The aseptically collected sample was serially diluted and plated on L-agar supplemented with 100 μg mL^{-1} to 500 μg mL^{-1} of $K_2Cr_2O_4$. The plates were then incubated at 37 °C for 24–48 h under aerobic conditions. Morphologically different bacterial colonies from each plate were picked and purified by repetitive streaking until a single colony was obtained.

2.2. Determination of minimum inhibitory concentration

To determine the minimum inhibitory concentration (MIC) of the isolated bacterial strains, they were inoculated in a series of LB broth supplemented with increasing concentrations of Cr(VI) and incubated for 24 h at 37 °C. The optical density of the broth culture was observed by UV-spectrophotometer at 600 nm.

2.3. Morphological and biochemical characterization of bacterial strain

For morphological characterization, Gram staining, spore staining, and motility test were performed. Following the methods of Gerhardt et al. (12), the colony morphology was examined and the shape, color, elevation, margins, texture, and light transparency of bacterial colonies were observed under the microscope. Biochemical characterization of chromium-resistant isolates was done by performing catalase, oxidase, starch hydrolysis, nitrate reduction, O-F, and MR-VP tests. To check the resistance of the isolate to other heavy metals, the following heavy metals were used: zinc ($ZnCl_2$), copper ($CuSO_4$), cobalt ($CoCl_2$), lead ($PbCl_2$), and mercury ($HgCl_2$). Five different antibiotics (ampicillin, erythromycin, tetracycline, chloramphenicol, and gentamicin) were used to check the antibiotic resistance profile, and then observations were made.

2.4. Identification of bacterial strain

The bacterial strain was sent to Macrogen Inc. (Seoul, Korea) for 16S rRNA sequencing. The 16S rRNA sequencing was done by Macrogen using the genomic DNA provided and primers chosen for their wide coverage of both Eubacteria and Archaea, and encompasses variable regions V5 and V6 of the 16S rRNA gene (13,14), as detailed on the Macrogen 16S rRNA service website (http://www.macrogen.com/eng/sequencing/16s.jsp). Sequences were analyzed using the Ribosomal Database Project. The evolutionary history was inferred using the neighbor-joining method (15). An evolutionary analysis was conducted with MEGA5 (16).

2.5. Estimation of Cr(VI) reduction potential by stationary vs. shaken culture

The chromate reduction ability of the strain was investigated under shaken and stationary conditions. One set of flasks having L-agar with varying concentrations of Cr(VI) such as 400 μg mL^{-1}, 900 μg mL^{-1}, and 1800 μg mL^{-1} was incubated under aerobic conditions with shaking at 200 rpm while the other set was incubated under stationary conditions (0 rpm) at 37 °C. Samples were withdrawn after 24 h and analyzed for reduction in Cr(VI). Samples were centrifuged at 10,000 rpm for 5 min and the supernatant was then used to estimate the remaining hexavalent chromium levels by using the diphenylcarbazide method (17). Reduction potential of Cr(VI) at different concentrations of chromium was calculated using following formula:

$$\text{Reduction potential \%} = \frac{\text{Initial concentration of Cr} - \text{Observed concentration of Cr} \times 100}{\text{Initial concentration of Cr}}$$

2.6. Chromate reductase assay

To prepare the crude cell-free extract (CFE), the bacterial cultures were grown in 200 mL of LB broth for 24 h at 37 °C both in the absence and presence of 1000 μg mL^{-1} of K_2CrO_4 (18). Following harvesting by centrifugation (10,000 × *g* for 30 min) and washing in 0.1 M phosphate buffer, they were kept on ice for 5–10 min. The cells were suspended in 100 mL of phosphate buffer and disrupted by sonication for 5 min (Hielscher Ultrasonic Processors UP 400, S) in cold conditions. The lysate was centrifuged (13,500 × *g* for 30 min) to remove unbroken cells and debris, resulting in crude extract. This extract was further centrifuged (150,000 × *g* for 90 min) to remove membrane-associated material. Reaction mixture was prepared by mixing 1000 μg mL^{-1} of K_2CrO_4 into 5 mL of 0.1 M phosphate buffer and 1 mL of CFE. Decrease in the concentration of Cr(VI) was estimated by using diphenylcarbazide in the reaction mixture after incubations of 30 min, 60 min, and 90 min. An assay mixture without crude extracts was used as the control. Chromium reduction was assessed according to the diphenylcarbazide method.

2.7. Column experiments

Four different synthetic materials, PVC pipe, rubber tubing, sand, and small stones (3–4 mm in size), were selected as adsorbents for column experiments. They were

autoclaved, and 2 g of each material was added separately into the flasks having autoclaved Cr(VI)-supplemented (500 μg mL^{-1}) LB broth. Into each respective flask, 1 mL of the bacterial strain (10^8 CFU mL^{-1}) was inoculated. All the flasks were kept on a shaker at 150 rpm for 1 week to get the coating of the bacterial strain into the form of biofilm on the materials. Columns were made manually with the help of sterile disposable syringes. Each of the adsorbent materials was filled into columns; a metal solution (1%) was added to each column. After specific time intervals, the metal solution was withdrawn from each column and the residual amount of Cr(VI) was estimated by using the diphenylcarbazide method.

2.8. Fluorescent microscopy

Synthetic materials coated with bacteria were stained with acridine orange. A small piece of bacterial-coated material was aseptically transferred to a clean glass slide, flooded with acridine orange solution, and kept at room temperature for 5 min. Later, washing was done with autoclaved distilled water and the slides containing adsorbent materials coated with bacteria were oven-dried. Visualization of the bacterial coating on different adsorbent materials was performed under a Leica DMLS fluorescent microscope (Leica Wetzlar GmbH) with a blue filter.

2.9. Fourier transform infrared spectroscopy

The bacterial cells were grown overnight in LB broth with and without supplementation of 1000 μg mL^{-1} of K_2CrO_4. Cultures were harvested by centrifugation, and the pellets were dried in a hot air oven at 60 °C. The dried biomass was ground to a fine powder using a mortar and pestle. The powdered sample was pressed into a spectroscopic quality KBr pellet with a sample/KBr ratio of 1/100 (19). The Fourier transform infrared (FTIR) spectra of dried cells were recorded using a FTIR spectrometer.

2.10. Statistical analysis

All experiments were performed in triplicate. Data were statistically analyzed using SPSS 16 (SPSS Inc.).

3. Results and discussion

Hexavalent chromium is posing a significant threat to the environment and public health because of its toxicity and persistence in nature. A large number of microorganisms are capable of growth in the presence of heavy metal ions and tolerate high concentrations (20). In the present study, a total of 8 bacterial strains were isolated from the waste water of the tannery industry. Among all the strains, A_8 grew well in LB broth amended with 1800 μg mL^{-1} of Cr(VI) and was found to have a MIC of 1800 μg mL^{-1} of Cr(VI). The MIC for Cr(VI) was reported as 40 mg/L in 2007 by Sultan and Hasnain (21) and 100 mg/L in 2006 by Horton et al. (22).

Morphological characterization showed that the isolated bacterial strain (A_8) was nonspore-forming, gram-negative, coccal, and nonmotile. The chromium-resistant bacterial strain isolated in 2009 by Mistry et al. (23) was also a gram-negative coccobacillus and nonspore-forming. The colony was pale in color, mucoid in texture with smooth margins, and round in shape while the elevation was convex. It was catalase- and MR-positive, can reduce nitrate, hydrolyses starch, and showed negative results for oxidase and VP tests. It showed acid production in an oxidation fermentation test while there was no gas production.

The bacterial isolate (A_8) showed resistance to all the tested heavy metals (Cu, Zn, Co, Pb, and Hg) while it was sensitive for all the used antibiotics (gentamicin, chloramphenicol, tetracycline, ampicillin and erythromycin) except for ampicillin at low concentrations. Through BLAST the 16S rRNA gene sequences were compared with those in the NCBI sequence database (GenBank; www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA sequence of strain A_8 was found to have 99% homology with that of *Cellulosimicrobium* sp., as can be seen from Figure 1.

To check the reduction potential of isolated strains a stationary vs. shaken culture experiment was performed. The isolated bacterial strain was incubated at room temperature under stationary as well as shaken conditions with 3 different concentrations of Cr(VI) at 400 μg mL^{-1}, 900 μg mL^{-1}, and 1800 μg mL^{-1}. Maximum reduction was observed to be 98.6% at 900 μg mL^{-1} under the shaken culture while minimum reduction potential was 97.1% at the same concentrations under stationary culture. In comparison of the stationary and shaking conditions, the shaking condition was found to be more effective than the stationary condition because the shaking condition improved the rate of aeration, which facilitated the growth of the bacterial strain and, consequently, the reduction process.

Cr(VI) reduction experiments were also conducted using the CFE of the A_8 bacterial strain to understand the component responsible for the biotransformation of Cr(VI). After sonication of the bacterial pellet, CFE was obtained and incubated at room temperature along with K_2CrO_4 and 0.1 M phosphate buffer. Estimation of chromium reduction by using diphenylcarbazide showed 82.4%, 84.3%, and 81.6% reduction of Cr(VI) into Cr(III) after the incubations of 30 min, 60 min, and 90 min, respectively, as shown in Table 1. The results reflected that the process of reduction was enzymatic and chromate reductase activity was present in the soluble fraction of the cell. According to Megharaj et al. (24), the Cr(VI) reduction ability of *Arthrobacter* sp. is also associated with soluble fraction of enzyme.

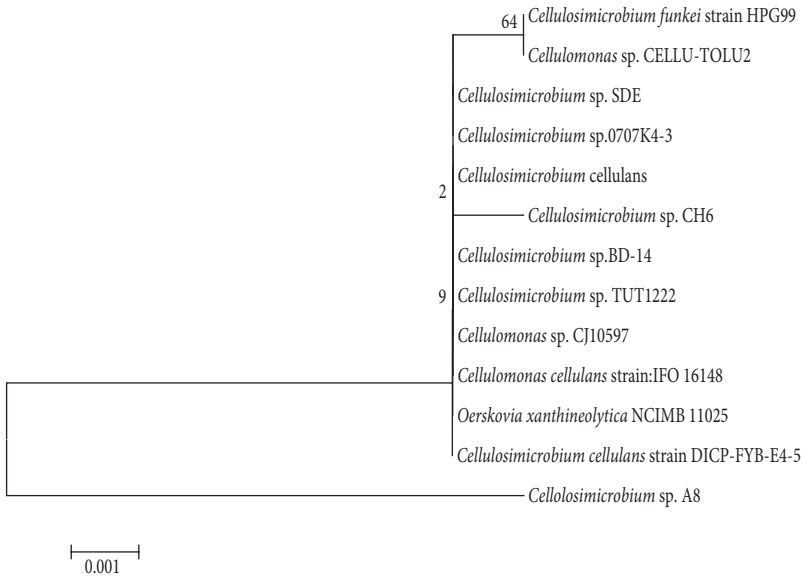

Figure 1. The evolutionary history was inferred using the neighbor-joining method. Evolutionary analyses were conducted with MEGA5.

Table 1. Cr(VI) reduction by crude extract of A_8 in the presence and absence of 1000 µg mL^{-1} of K_2CrO_4.

Time (min)	Reduction potential (%)	
	Experimental [with Cr(VI)]	Control [without Cr(VI)]
30	82.4 ± 0.06	76.3 ± 0.06
60	84.3 ± 0.06	74.1 ± 0.03
90	81.6 ± 0.08	73.3 ± 0.017

The bacterial strain (A_8) was incubated for 1 week with sand, PVC, stone, and rubber tubing. After the formation of biofilms on these synthetic materials, they were filled into the columns. A 1% solution of hexavalent chromium in the form of K_2CrO_4 was passed from this column. The percentage reduction was estimated by using diphenylcarbazide at different intervals of time. Percentage reduction potential increased with the increment in time (Figure 2). The highest reduction was observed in the case of sand, which showed 96% reduction of Cr(VI) after 96 h. However, minimum reduction potential was exhibited by the stone, which was 88.4% (Tables 2 and 3). For detecting the process of biosorption, different synthetic materials were used, i.e. sand, stone, PVC, and rubber tubing. The bacterial strain was grown on these materials by incubating them with bacterial culture in shaking conditions for 1 week. Reduction of Cr(VI) was estimated by passing the solution of Cr(VI) through the columns filled with these biofilm-coated materials.

To observe the coating of the bacterial cells on different synthetic materials, fluorescent microscopy was performed, and acridine orange was used for staining purposes. Fluorescent microscopy revealed that sand had the best coating of bacterial biofilms. Stone and PVC also showed good bacterial biofilm formation while rubber tubing exhibited little coating of the bacteria, as shown in Figure 3. Fluorescent microscopy was performed to evaluate the best material coated with biofilms. All of the materials having bacterial coating were stained with acridine orange, which was used to detect the presence of live as well as dead bacterial cells. Acridine orange gives orange fluorescence when it binds with live bacterial cells having both DNA and RNA, while it fluoresces with a green color when it binds with dead cells of bacteria. Other

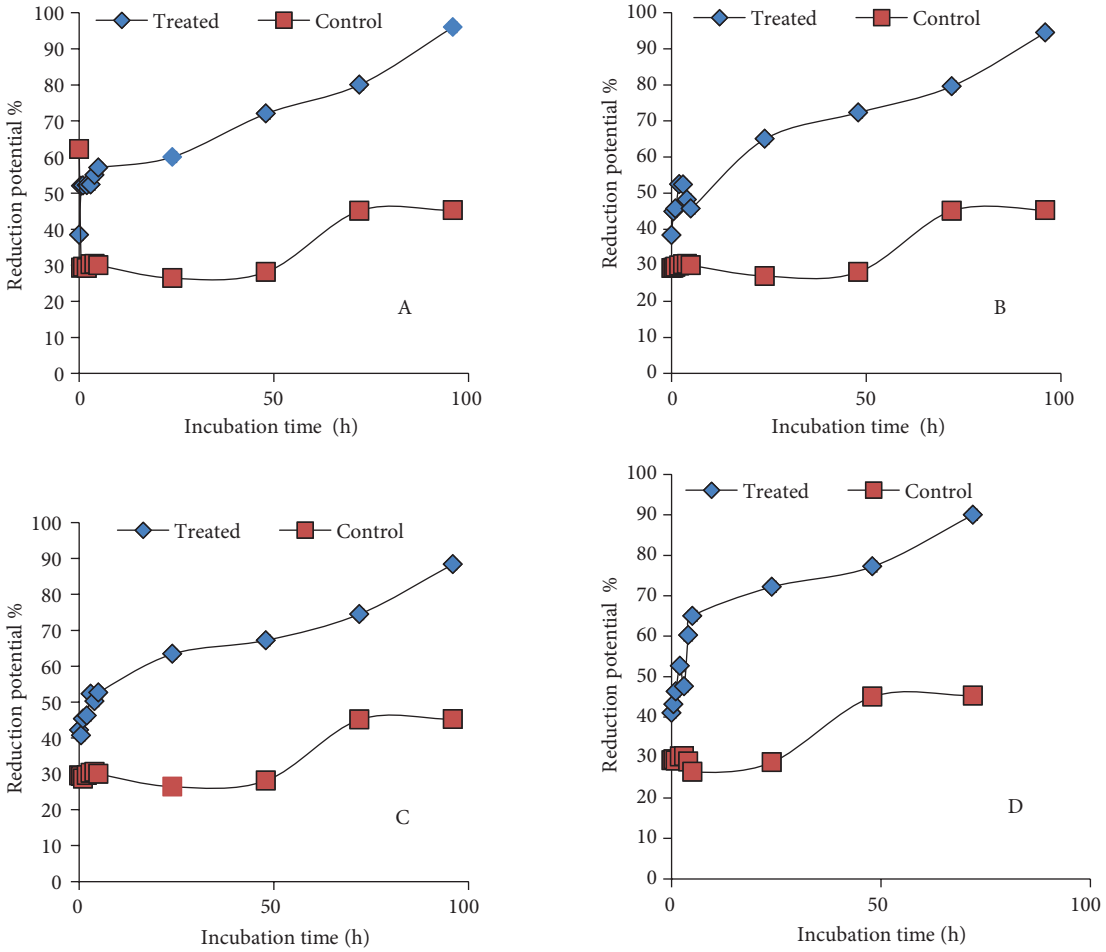

Figure 2. Estimation of Cr(VI) reduction potential of bacterial strain (A_8) at different time intervals coated on A) sand, B) PVC pipe, C) rubber tubing, and D) stone.

Table 2. Estimation of Cr(VI) reduction potential of chromium-resistant bacterial strain (A_8) after column experiments.

Time Period (h)	Reduction potential (%)			
	Sand		PVC	
	Treated	Control	Treated	Control
0.0	38.4 ± 0.1	62.2 ± 0.08	38 ± 0.06	29.2 ± 0.14
0.5	52 ± 0.06	29.3 ± 0.09	44.9 ± 0.05	29.3 ± 0.09
01	52.1 ± 0.05	29.5 ± 0.01	45.7 ± 0.1	29.5 ± 0.01
02	52.2 ± 0.1	29.2 ± 0.08	52.4 ± 0.06	29.9 ± 0.08
03	52.3 ± 0.06	30.2 ± 0.08	52.3 ± 0.05	30.2 ± 0.05
04	55 ± 0.05	30.3 ± 0.05	48.2 ± 0.1	30.3 ± 0.15
05	57 ± 0.1	30.0 ± 0.01	45.7 ± 0.03	30.0 ± 0.03
24	60 ± 0.03	26.4 ± 0.17	65 ± 0.08	27 ± 0.17
48	72 ± 0.08	28.1 ± 0.09	72.3 ± 0.42	28.1 ± 0.09
72	80 ± 0.4	45.1 ± 0.03	79.6 ± 0.03	45.1 ± 0.03
96	96 ± 0.03	45.2 ± 0.09	94.5 ± 0.16	45.2 ± 0.06

Table 3. Estimation of Cr(VI) reduction potential of chromium-resistant bacterial strain (A_8) after column experiments.

Time Period (h)	Reduction potential (%)			
	Stone		Rubber tubing	
	Treated	Control	Treated	Control
0.0	42 ± 0.1	29.5 ± 0.09	38.8 ± 0.08	29.6 ± 0.05
0.5	40.7 ± 0.2	29.3 ± 0.05	41 ± 0.2	29.3 ± 0.09
01	45.3 ± 0.1	28.6 ± 0.14	43.1 ± 0.2	29.5 ± 0.01
02	46.2 ± 0.1	29.6 ± 0.05	46.3 ± 0.1	29.2 ± 0.08
03	52.3 ± 0.08	30.2 ± 0.08	52.6 ± 0.08	30.2 ± 0.03
04	50.2 ± 0.5	30.5 ± 0.3	47.6 ± 0.05	30.3 ± 0.04
05	52.6 ± 0.08	30.0 ± 0.02	60.2 ± 0.14	29.0 ± 0.01
24	63.4 ± 0.05	26.4 ± 0.17	65 ± 0.08	26.4 ± 0.17
48	67.2 ± 0.14	28.1 ± 0.08	72.2 ± 0.1	28.8 ± 0.08
72	74.5 ± 0.08	45.1 ± 0.02	77.2 ± 0.1	45 ± 0.15
96	88.4 ± 0.13	45.2 ± 0.09	90 ± 0.08	45.3 ± 0.08

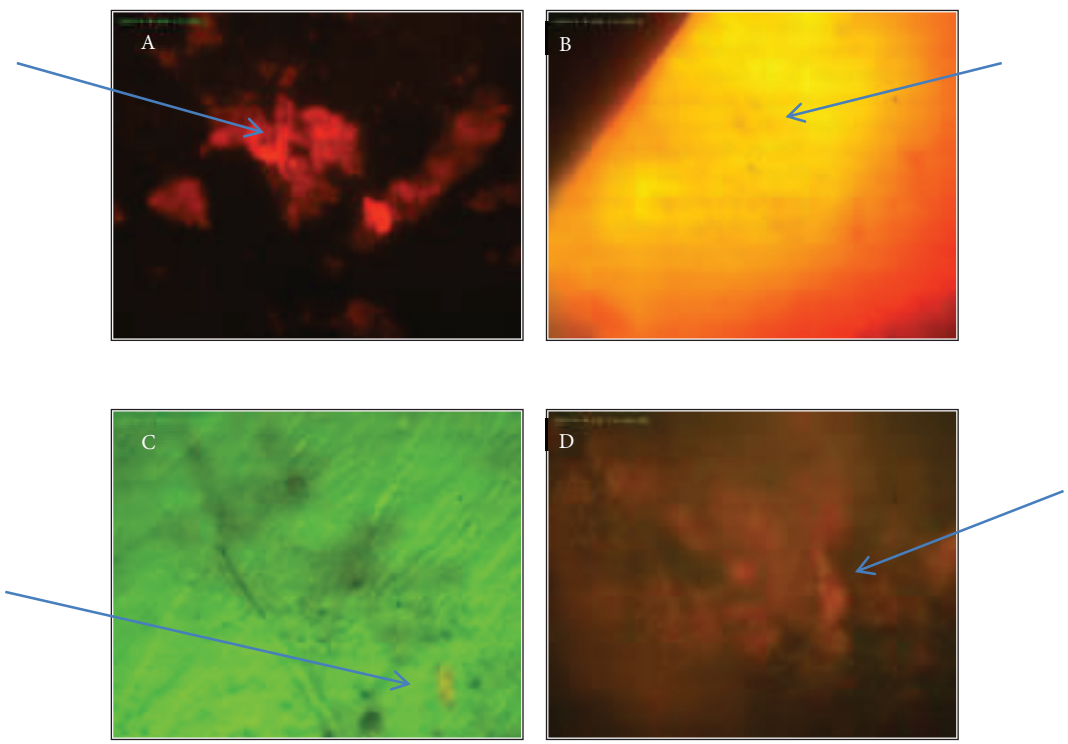

Figure 3. Fluorescent microscopy of different synthetic materials coated with bacterial biofilm: A) sand, B) PVC pipe, C) rubber tubing, and D) stone. Arrowheads represent the distribution of bacterial cells on the surface of the solid support.

fluorescent stains such as ethidium bromide bind with both living and dead cells and do not differentiate between living and nonliving cells.

In column experiments and in the case of fluorescent microscopy, sand proved to be the best material among all the used materials as it exhibited maximum reduction of Cr(VI), and its particle showed the best bacterial coating. Bacterial-coated PVC pipe and rubber tubing also exhibited significant reduction of Cr(VI), while the least Cr(VI) reduction potential was shown by stone having biofilm of bacteria. According to the studies in 2005 by Chen and Zhu (25) regarding bacterial and silica sand surface, free energies can be reflected by their interfacial surface tensions with the liquid. These interfacial surface tensions are initiated by interactions of bacteria and silica sand with the liquid, which include van der Waals interactions and Lewis acid/base interactions. This can be a reason for the best bacterial coating being on sand.

The FTIR spectra of the A_8 strain with Cr(VI) and without Cr(VI) were taken in the range of 400–4000 cm^{-1}. This represents the involvement of different functional groups in the reduction of Cr(VI) into Cr(III). The spectra of the biomass displayed a number of adsorption peaks, reflecting the complex nature of the biomass. Changes were observed in the spectra of FTIR; in particular, the peak at 617 cm^{-1} was missing in the case of stress conditions, which represented the glycerol units, polysaccharides. The P-OH stretching band shifts from 995.3 cm^{-1} to 995.87 cm^{-1} while the C-N (alkyl) stretching band shifts from 1043.52 cm^{-1} to 1048.10 cm^{-1}. These differences indicated that there was a binding process taking place on the surface of the cells with certain functional groups (26). Changes in certain characteristic bands between the cell samples treated with or without chromium could be observed in the spectra. It is likely that the oxidation of biomass during Cr(VI) biosorption had resulted in changes in the absorption frequencies of various functional groups present in the biomass (27).

In conclusion, the findings of the present study demonstrated that the isolated indigenous microbial strain *Cellulosimicrobium* can efficiently reduce the toxic and soluble Cr(VI) into the nontoxic and insoluble Cr(III) form aerobically. As the isolated bacterial strain is nonpathogenic and has an excellent reduction potential of Cr(VI), it can be exploited at the commercial level for bioremediation of Cr(VI)-contaminated environments.

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