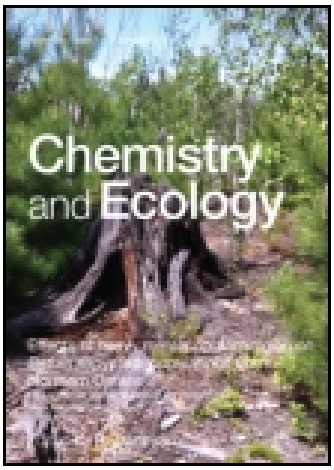

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Developed fungal–bacterial biofilms as a novel tool for bioremoval of hexavelant chromium from wastewater

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Remediation measures for hexavalent chromium [Cr(VI)] are required for a safe environment. As a recent development in microbiology, bacterial biofilms are being studied as effective bioremediation agents. When bacteria are in fungal surface-attached biofilm mode, they are called fungal–bacterial biofilms (FBBs). They have not been tested for bioremediation so far. Hence, this study was conducted to develop FBBs and glass-wool-attached bacterial biofilms (BBs), and to evaluate Cr(VI) tolerability and removal of bacterial monocultures, BBs and FBBs. FBBs showed a significantly high level of Cr(VI) tolerance and resistance compared with its BBs or monocultures. After 10 days, up to 90% of Cr(VI) had been removed, which was significantly higher than that of BBs or its monocultures. Thus, it is clear that FBBs can be used as a novel tool to decontaminate Cr(VI) both in situ and ex situ.

Keywords: Cr(VI); bioremediation; fungal–bacterial biofilms; tolerance; resistance

1. Introduction

Chromium (Cr) is an industrially important element that has been used in various chrome-based industries.[1] As a result of industrial revolution, anthropogenic usage of Cr has increased rapidly. Consequently, a large quantity of Cr is being discharged into environment and Cr contamination of the environment is extensive.[2,3] The hexavalent form of Cr [Cr(VI)] and trivalent form [Cr(III)] are abundant among contaminants. However, Cr has both beneficial and detrimental properties. Cr(III) is an essential trace element in mammalian metabolism. By contrast, Cr(VI) is hazardous by all exposure routes.[4] Cr(VI) compounds are more toxic than Cr(III) due to their high water solubility and mobility, whereas Cr(III) is insoluble and thus immobile under ambient conditions.[5] Hexavalent Cr prevails in particular in industrial effluents rich in organic matter which possess high anaerobic conditions.[6] Discharging such effluents directly into soil or water sources may lead to destruction of the ecosystem. Hence, reduction of highly toxic and mobile Cr(VI) to the less toxic, less mobile Cr(III), and/or removal of Cr(VI) from system is vital to refresh and remediate soil and water systems.

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Various techniques have been adopted for the remediation of Cr(VI).[7–9] Of these, chemical precipitation has been the most common method, even in industry. Ion exchange, electrocoagulation, electrochemical precipitation, phytoremediation, bioremediation, adsorption and filtration are the other methods that have been used in Cr(VI) remediation.[10] However, economical infeasibility and toxic residual and sludge production in downstream processes are major issues in conventional remediation techniques.[11] The incapability of removing low contaminant concentrations is another drawback in conventional remediation techniques.[12] Thus, environmental friendly, easy, economical and feasible remediation methods, strategically based on bioremoval may be better alternatives for chrome-based industries.

Biofilm formation is a strategy used by microorganisms to survive under extreme environmental stresses, including high heavy metal concentrations.[13] Therefore, this strategy might be used as a potential bioremediation technique to remove heavy metal contaminants from the environment. Hence, biofilm-based bioremediation has been highlighted recently as a potential and feasible tool to remove hazardous metal contaminants such as Cr(VI).[14] Biofilm-based remediation methods are economical and easy to operate or use compared with physical and chemical methods. In the case of physiochemical methods, the regeneration of materials from their disposal again involves with a cost. However, most biofilm-based remediation studies have been based on monospecies cultures, whereas nearly all biofilm communities in nature that are associated with effective bioremediation comprise a variety of microorganisms.[15] Fungal–bacterial interactions that may serve as multispecies biofilms have been used more effectively in bioremediation.[16] When bacteria are in a fungal surface-attached biofilm mode, they are called fungal–bacterial biofilms (FBBs), which can be developed in vitro from microbial monocultures.[17] Thus, it is worthwhile studying FBB-based bioremediation methods for Cr(VI) bioremoval. This study was conducted to isolate Cr-tolerant bacterial and fungal species, to develop bacterial biofilms (BBs) and FBBs, and to evaluate the Cr tolerability and removal of bacterial monocultures, BBs and FBBs.

2. Materials and methods

2.1. *Sample collection site*

Sterile containers were used to collect soil from the wastewater channels (pH 7.8, 32°C) of an industrial tannery where chrome tanning is used (Mattakkuliya, Sri Lanka, 6°54′ N 79°52′ E), and transported to the laboratory of the Institute of Fundamental Studies (IFS), Sri Lanka for microbial isolation.

2.2. *Microbial isolation*

The soil samples were serially diluted (10-fold) before plating on sterile Nutrient Agar (NA, 20 g L^{-1} medium; Himedia™, India), Combined Carbon Medium (CCM),[18] Czapek Dox Agar (CZA, 49.01 g L^{-1} medium; Himedia™) plates. For selective isolation of Cr-tolerant bacteria, 50 μg mL^{-1} of Cr(VI) was incorporated into NA, CCM and CZA. All the inoculated plates were incubated for 24 h at 37°C. Several morphologically different colonies were selected and sequentially subcultured for purification in the above media.

2.3. *Isolate screening*

Isolated colonies on CCM was subjected to the acetylene reduction assay [19] using a gas chromatograph (Shimadzu GC 9AM), with hydrogen flame equipped with flame ionisation detector

(FID), a CARBOXEN™ 1010 PLOT capillary column and helium (99.99%) as the carrier gas at a flow rate of 0.625 mL s^{-1} for evaluation of nitrogenase activity. Isolated colonies on CCM and NA media were subjected to the microtiter plate biofilm assay [20] to evaluate the biofilm forming ability. Bacterial isolates were identified biochemically,[21] whereas fungal isolate was identified based on morphology.

2.4. *Study of the tolerance and resistance of Cr(VI)*

Thereafter, one FBB was developed using selected fungal and bacterial isolates following protocols developed by the IFS, Sri Lanka.[22] Similarly, one BB was formed on glass wool following the same biofilm-developing protocol. Light microscopy observations under an oil immersion lens were made to verify biofilm formation. Lactophenol Cotton Blue was used for staining. Morphological characterisation of the biofilms was also carried out using a scanning electron microscope (EI Quanta 200). Monocultures of all the bacterial isolates were incorporated into Nutrient Broth (NB, 13 g L^{-1} medium; Himedia™). Then, all the bacterial isolates, FFBs and BBs were subjected to different concentrations of Cr(VI) – 200, 250, 300, 400 and 500 μg mL^{-1} – in flasks for 96 h of incubation. Bacterial colony forming unit (CFU) counts were taken in every 24 h until the incubation was completed.

2.5. *Study of Cr(VI) removal*

Cr(VI) removal by FBBs, BBs and their monoculture were studied using small-scale bioreactors. The reactors were made up of a plastic vessel (diameter 12 cm, height 18 cm) with an air-tight lid. Bioreactor air exchange was facilitated through air filters (Midisart® 2000 In-Line Air Filter, 0.2 μm). First, vessels were filled with 900 mL of modified nutrient medium (5 g peptone, 5 g NaCl, 2 g yeast extract, 1 g beef extract, 5 g sucrose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 1000 mL distilled water, pH 7.00). Then, 100 mL inoculant of 10^8CFU/mL isolates of screened bacterial isolates was aseptically inoculated to separate bioreactors. In vitro formation of FBB was performed on a glass slide (75 × 25 mm) wrapped with a piece of nylon mesh (30 μm, 0.2 × 0.2 mm pore size). Similarly, BB was formed on glass-wool-attached glass slides. The glass slides were inserted into the bioreactor setup. A reactor containing all material inputs except any microbial inoculation was maintained as the control. Every treatment was replicated three times. Bioreactors were incubated for one week at room temperature (30°C) with shaking (100 rpm). Thereafter, 100 ppm Cr(VI) stress was induced using $K_2Cr_2O_7$ (Sigma-Aldrich, Germany) to each reactor setup. Subsequently, 20 mL of the liquid portion was removed from each bioreactor setup on day 1, 2, 3, 5 and 10 and filtered (0.25 μm, Nylon, Flowpore, UK). Filtrate Cr(VI) was determined colorimetrically [23] using a UV/Vis spectrophotometer (Model, UV-160 A, Shimadzu). Total Cr was measured by direct flame atomisation using atomic absorption spectroscopy (Model GBC 933 AA, GNC Scientific Equipment, Braeside, Australia). Fourier transform infrared spectroscopy (FT-IR, Thermo Nicolet, Thermo Fisher Scientific, Waltham, MA, USA) analysis was performed on Cr-loaded and -unloaded biofilm biomass. Spectra were collected over a range 500–4000 cm^{-1} with a resolution of 4 cm^{-1}. Each spectrum was produced by 64 scans. Spectra were analysed using OMINIC® software.

2.6. *Statistical analysis*

The experiment was arranged according to a completely randomised design (CRD). The effects of the treatments on measured parameters were analysed by analysis of variance (ANOVA) and

the means were separated by LSD test at 5% probability level. SAS v. 9.0 for Windows was used for the data analysis.

3. Results and discussion

3.1. *Cr(VI) tolerance and resistance of biofilms*

Based on the tolerance of Cr(VI) at 50 $\mu g\,mL^{-1}$, eight bacterial isolates were chosen from the plates and purified by subculturing. They were then screened for nitrogenase activity, because N_2 fixers play a key role in the growth and persistence of effective microbial communities by supplying N through biological nitrogen fixation. Their biofilm-forming ability was also considered. The screening procedures reduced the number to four bacterial isolates and they were identified as *Pseudomonas* sp. (CRB 1), *Bacillus* sp. (CRB 5), *Azotobacter* sp. 1 (CRD 6) and *Azotobacter* sp. 2 (CRB 8). One fungal isolate that was tolerant to 50 $\mu g\,mL^{-1}$ Cr(VI) was also picked from the CZA plates and identified as *Acremonium* sp. The FBBs were formed by the colonisation of different bacteria on fungal mycelium, whereas the BBs were developed on glass wool by colonisation of the same bacteria (Figure 1). Both biofilm modes showed significantly high Cr(VI) tolerance compared with their monocultures at 200 $\mu g\,mL^{-1}$ Cr(VI) and from 300 to 500 $\mu g\,mL^{-1}$ Cr(VI) (Figure 2). This is because biofilms maintained a higher cell density ($\sim 10^7$) than the monocultures ($\sim 10^6$). Further, a metabolic gradient generally developed within the biofilm structure,[24] which leads to the non-uniform distribution of heavy metal ions [25] and enables them to survive at extreme heavy metal levels. At 500 $\mu g\,mL^{-1}$ Cr(VI), the FBBs showed higher tolerance than the BBs, possibly due to complex interactions between the fungal mycelium and colonised bacteria, as described previously.[16] At 250 $\mu g\,mL^{-1}$ Cr(VI), there was no difference in the Cr(VI) tolerance of both biofilm modes and the monocultures. The reason for this is not yet known.

Up to four days of inducing 500 μg Cr(VI) mL^{-1} of stress, the developed FBBs and BBs showed significantly high resistance compared with their monoculture isolates (Figure 3), because biofilm bioinorganic reactions and their products help to transform toxic oxidation stages into non-toxic stages of heavy metal ions, resulting in high persistence of biofilmed microorganisms.[26] It

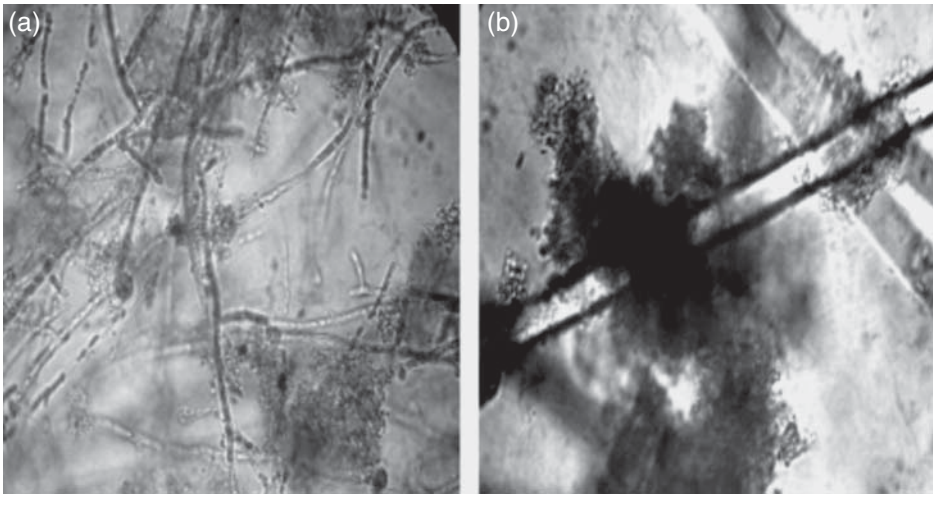

Figure 1. (A) Fungal mycelium colonised by different bacterial strains forming FBB at × 1000 magnification. (B) Glass wool surface colonised by different bacterial strains at × 3000 magnification. Darkness is due to stain absorbed by the EPS produced by the biofilms.

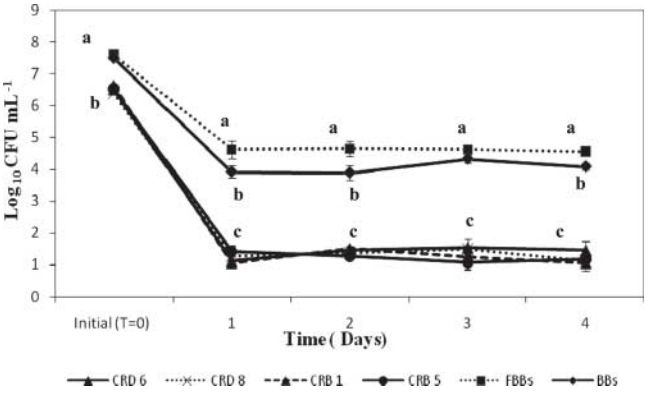

Figure 2. Change in CFU of bacterial isolates (CRD 6, CRD 8, CRB 1, CRB 5) and biofilms (FBBs and BBs) against different Cr(VI) concentrations after 24 h incubation. Different letters in each concentration show significant differences at 5% probability, whereas the absence of letters indicates the absence of significant differences at the same probability level. Vertical bars show standard errors.

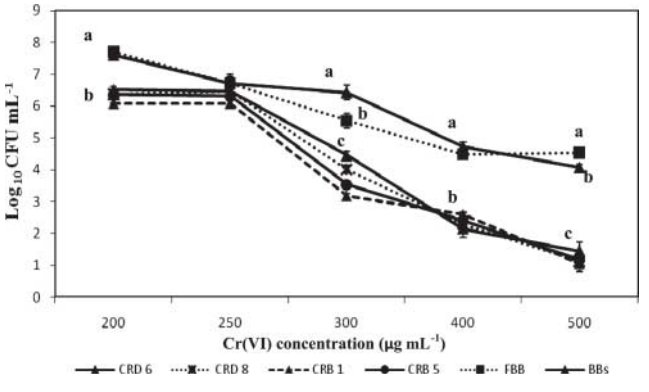

Figure 3. Change in CFU of bacterial isolates and biofilms over time in medium containing 500 mg mL^{-1} Cr(VI). CRD 6, CRD 8, CRB 1 and CRB 5 are bacterial isolates. Different letters at each time show significant differences at 5% probability, whereas the absence of letters indicates the absence of significant differences at the same probability level. Vertical bars show standard errors.

has also been observed that phenotypically varied subpopulations of different cells can be seen in biofilms which aid in resisting heavy metal stress compared with their planktonic stage.[13] Except on day 3 of the stress, FBBs showed significantly higher Cr tolerance than BBs (Figure 3), due to the high potential for biotransformation in multispecies biofilms.[15] Furthermore, the protective environment provided by biofilm extracellular polymeric substances (EPS) reduces the toxicity of pollutants to microorganisms.[14] In general, EPS buffers the biofilm microbial community from external toxic chemicals so that relatively small concentrations of pollutants reach the viable cells, facilitating enhanced tolerance and resistance.

3.2. *Cr(VI) biosorption and biotransformation of biofilms*

About 43% of the added Cr(VI) had been removed by the bioreactors containing FBBs, even after day 1 of Cr(VI) spiking (Figure 4), whereas all other treatments had removed only 10–16% Cr(VI). On day 10, ~ 90% Cr(VI) had been removed in comparison with ~ 60% by the BBs and monocultures. Biofilm-based Cr(VI) removal takes place through two phases.[27] First, Cr(VI) is accumulated on the external cell surface and then passes through cellular membranes for

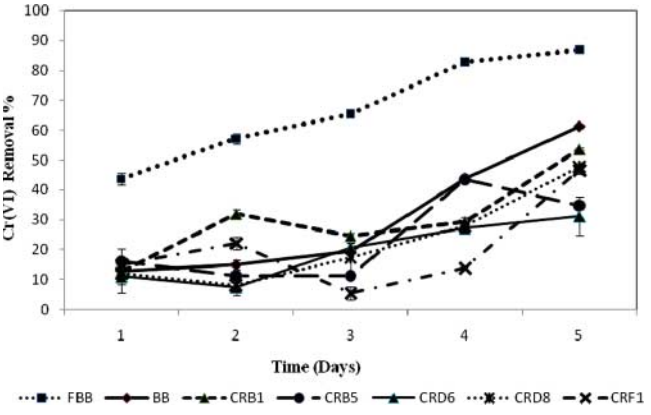

Figure 4. Cr(VI) removal over time by FBB, BBs and bacterial isolates (CRD 6, CRD 8, CRB 1, CRB 5). The initial concentration was 100 $\mu g\,mL^{-1}$ Cr(VI). Different letters at each time show significant differences at 5% probability, whereas the absence of letters indicates absence of significant differences at the same probability level. Vertical bars show standard errors.

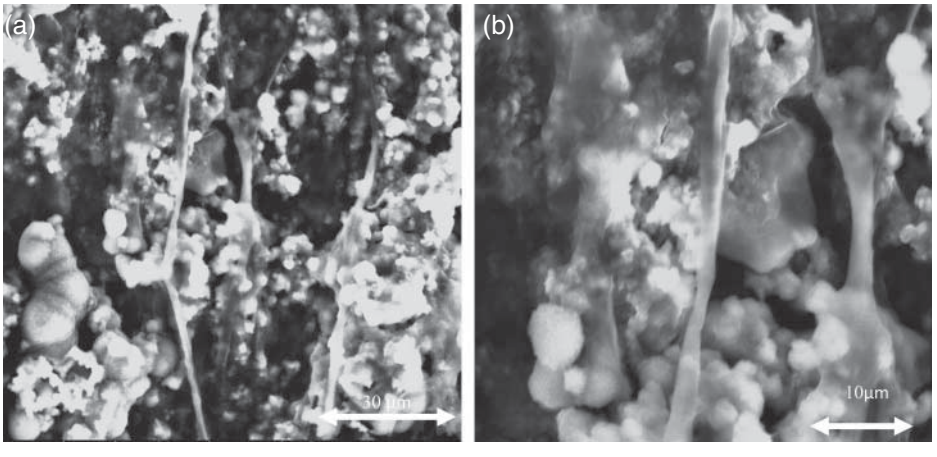

Figure 5. SEM images of FBBs. Extensive EPS covering of FBBs can be seen. The scale bar represents 30 and 10 μm, respectively.

intracellular accumulation.[27] In addition, biofilms produce significantly higher amounts of EPS than their planktonic stage.[28] The EPS greatly facilitate Cr immobilisation through biosorption, and it is reported that they also act as the central element in bioremediation.[29] Fungal mycelium itself produces a high amount of EPS in addition to bacterial EPS production, which may result in higher EPS production in the FBBs than the BBs.[30] Scanning electron microscopy also indicated high EPS secretion by FBBs (Figure 5). As a result, higher Cr immobilisation was observed in the FBBs than the BBs.

In bioreactors containing FBBs, pH fluctuated between 5.6 and 6.1, whereas in reactors containing BBs it changed from 6.8 to 7.1 (Figure 6). It has been reported that the adsorption of Cr(VI) metal ions greatly depends on solution pH, the higher the acidity, the higher the adsorption.[31] The acidic nature of the medium influences electrostatic binding of ions to corresponding functional groups. The EPS contain a complex mixture of polysaccharides, proteins, nucleic acid and various other organic compounds, which can serve as a matrix of hydroxyl, carboxyl, amino, ester, sulfhydryl, carbonyl and phosphate groups, and can take part in binding of metal ions.[28,32] Interaction of the EPS with ions like Cr is determined by the degree of protonation of the functional

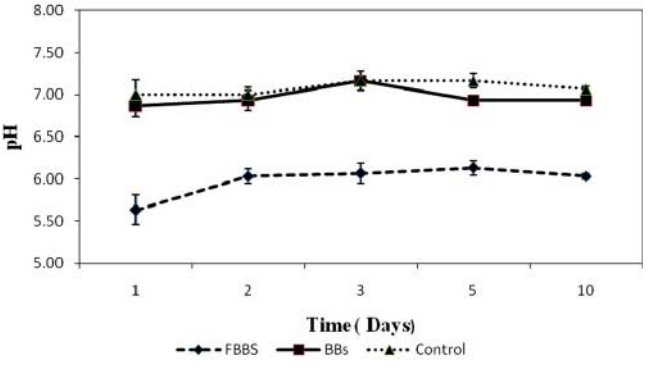

Figure 6. Change in pH in bioreactors containing FBBs and glass-wool-attached BBs over time. Vertical bars show standard errors.

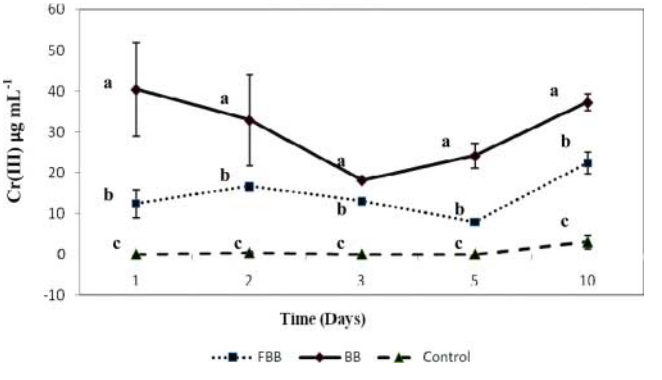

Figure 7. Cr(III) concentration in the bioreactor over time by FBBs and glass-wool-attached BBs. Different letters at each time show significant differences at 5% probability. Vertical bars show standard errors.

groups, which in turn depends upon the solution pH.[33] Thus, the increased binding of Cr(VI) ions at low pH can be explained by the electrostatic binding to positively charged groups in the EPS.[34] As such, significantly low pH of the FBBs showed higher Cr(VI) removal than BBs.

At all sampling times, it was observed that reactors containing BBs had significantly higher Cr(III) concentrations than reactors with FBBs (Figure 7). This was simply due to higher EPS production by FBBs than BBs, which contributes to high Cr(III) retention, thus reducing Cr(III) in the reactor solution. The reduction takes place at adjacent electron-donor groups of the EPS.[35] Also, due to the repulsion between the transformed Cr(III) and positively charged groups of EPS, Cr(III) can again be released into the aqueous phase.[36] It is also reported that transformed Cr(III) can be immobilised with phosphate on the biomass EPS.[37] In addition, complexation of Cr(III) with EPS is also possible.

The constituents of the bioreactor medium can be replaced by low-cost inputs when reactor is scaled up to an industrial level. Molasses or malt extract can be used as a carbon source for microbial growth.[38,39] The nitrogen source can be replaced by whole soy flour or dried spent yeast.[40,41] Table salt, wood ash and top soil can be used as alternative and inexpensive mineral inputs. However, further experiments are essential to optimise the fermenter conditions according to the low-cost inputs.

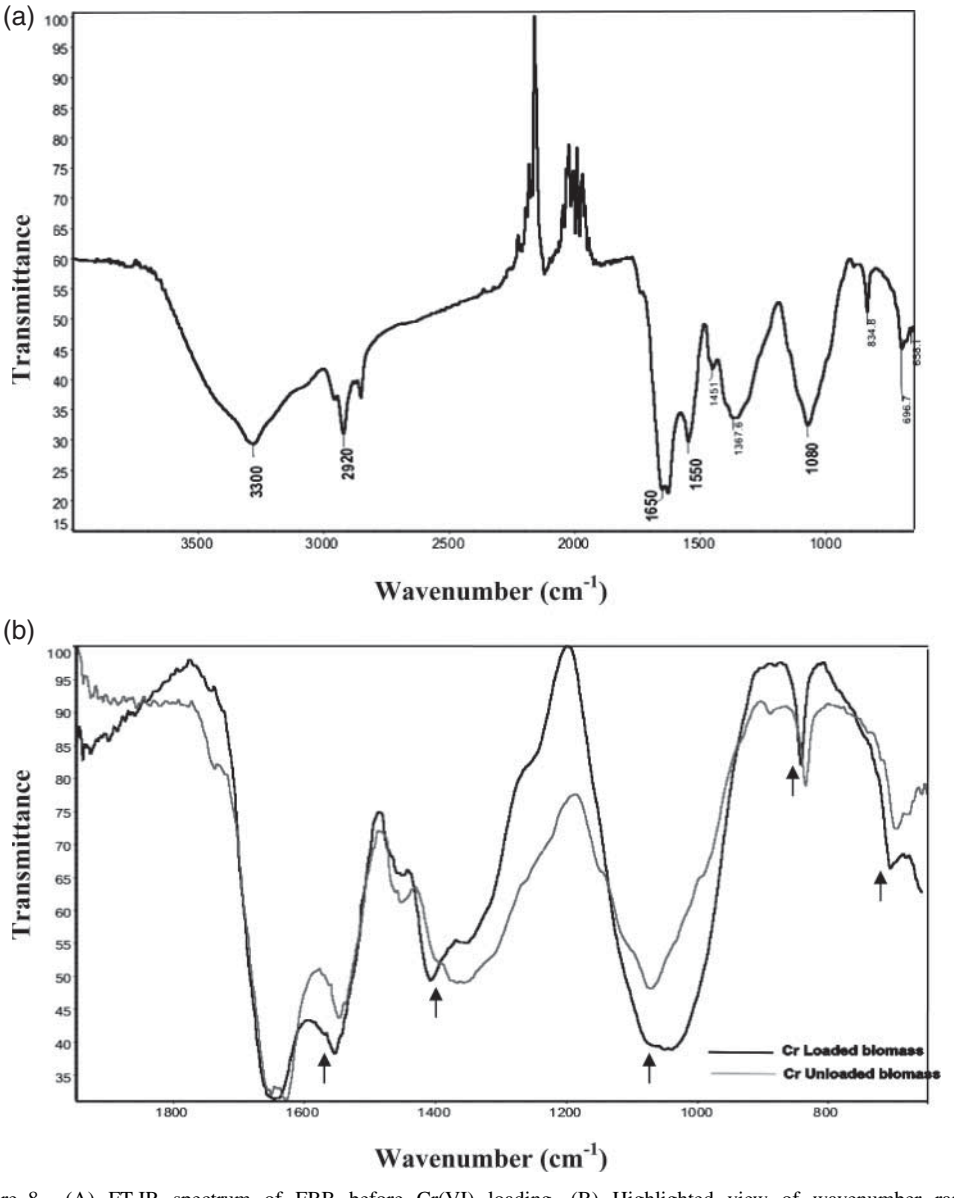

Figure 8. (A) FT-IR spectrum of FBB before Cr(VI) loading. (B) Highlighted view of wavenumber range 800–1800 cm^{-1} of FBB before and after Cr(VI) loading. Black arrows indicate spectral pattern shifting of certain bands observed in the spectrum.

3.3. *FT-IR spectroscopy*

Spectrocopy studies were conducted to observe the sorption of Cr(VI) on biofilms. In the FT-IR spectrum of the FBBs, a number of absorption peaks were observed, indicating the complex nature of the FBBs biomass and EPS (Figure 8A). The broad absorption peak around 3300 cm^{-1} was indicative of the existence of O–H and N–H stretching, which represent the hydroxyl and amine groups. The strong absorption peak at 2920 cm^{-1} can be assigned to C–H stretching, those at 1740 cm^{-1} are due to C = O stretching of carboxyl groups, and absorption bands at 1650 cm^{-1} represent C = O chelate stretching of primary amides and 1550 cm^{-1} can be attributed to N–H

stretching of secondary amides bond due to the presence of protein peptide bond. The moderately strong bands at 1080 cm^{-1} can be assigned to –CN stretching vibration of the protein fractions on the EPS.[34,42]

The FT-IR spectrum of Cr(VI) unloaded biomass showed slight changes in the region of 1650–700 cm^{-1} compared with Cr(VI) loaded biomass. The peak around 1740 cm^{-1} (C = O stretching) disappeared from the chromium-loaded biomass, whereas that around 1650 cm^{-1} (C = O chelate stretching) became larger. This implies that the carboxyl groups are involved in the binding of chromium in Cr(VI) biosorption.[35] Meanwhile, the peak around 1550 cm^{-1} shifted slightly after Cr(VI) biosorption. Because the absorption peaks in the range 1550 to 1650 cm^{-1} correspond to –NH bending, the amino groups might also be involved in Cr(VI) biosorption.[35] The change in the band at 1080 cm^{-1} might be due to Cr(VI) adsorption to protein fractions available on EPS.[34] Therefore, the peak shifts observed in the spectrum indicated the possible involvement of those functional groups on the surface of the biomass in biosorption process.

4. Conclusion

It is clear that well-developed highly Cr-tolerant FBBs can be used as a potential tool for the bioremediation of Cr(VI) contaminants in situ and ex situ. FBBs can be used ex situ in biobarrier columns and bioreactors. Thus, it is important to investigate the potential of FBBs as a novel Cr bioremediation tool, and further research is necessary to improve the efficacy and understanding of the fundamentals behind the process.

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