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Studies on development of Cr^{6+} resistant strain of *Pseudomonas aeruginosa* for improved Cr^{6+} biosorption: Scanning Electron Microscopic and spectrophotometric studies

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Hexavalent chromium (Cr^{6+}) is one of the most potent heavy metal pollutants released from various industries into water bodies. Most commonly used physico-chemical methods for removal of Cr^{6+} from wastewater are inefficient, expensive and produce unmanageable toxic sludge. Recent studies showed that biosorption based on metal-biomass interaction can be an alternative to commonly employed methods for Cr^{6+} removal from wastewater. In this study four types of bacteria such as *Escherichia coli, Bacillus pumilus, Bacillus subtilis* and *Pseudomonas aeruginosa* were screened for high Cr^{6+} concentration tolerant strain isolation. It was found that *Escherichia coli* and *Bacillus pumilus* are resistant to 250 ppm and *Bacillus subtilis* resistant to 350 ppm and *Pseudomonas aeruginosa* can resist upto 500 ppm of initial Cr^{6+} concentration.

Scanning Electron Microscope studies of mycelia of parent cells of *Pseudomonas aeruginosa*, and Cr^{6+} resistant cells of *Pseudomonas aeruginosa* revealed that high Cr^{6+} concentration of resistant strain induces some morphological changes. Metal-biomass interaction was confirmed by Fourier Transform Infra-Red (FTIR) spectroscopic study which also indicates the presence of hydroxyl amine, amide, carbonyl and carboxyl groups in cells wall of *Pseudomonas aeruginosa.* EDX analysis also showed that the parent *Pseudomonas aeruginosa* does not contain Cr^{6+} but the Cr^{6+} resistant *Pseudomonas aeruginosa* contains chromium along with carbon, oxygen and platinum.

Keywords: Hexavalent chromium, biosorption, resistance, *Pseudomonas aeruginosa*, EDX spectra.

Introduction

Heavy metals contamination is a growing issue throughout the World posing threats to humans, animals and plants, and affecting the stability of overall ecosystem. Due to rapid industrial development, direct and indirect discharges of heavy metals to the environment through wastewater have tremendously been increased. In case of treatment of industrial wastewater containing chromium, copper, cadmium, lead, zinc and nickel are considered as more toxic pollutants and getting more attention of researchers. Among them chromium(VI) is one of the most hazardous heavy metals with a high carcinogenic and recalcitrant nature.

A variety of physico-chemical methods e.g. chemical precipitation, evaporation, coagulation, ion-exchange, membrane filtration, electrolytic and adsorption techniques are commonly used for the removal of toxic heavy metals from wastewater. These methods were found to be either inefficient or expensive and may also be associated with the generation of unmanageable toxic sludge[1–5]. In recent years, biosorption have gained much importance as an alternative eco-friendly, cost-effective method for heavy metal removal from industrial wastewater. It is a metabolic independent process which can be performed by living and dead cells. Recently microbial systems like bacteria[6,7], yeast[5] and algae[8] have been used as biosorbent for heavy metal removal, as they can adopt quickly to toxic concentrations of heavy metals and become metal resistant[9,10]. The permissible limit of Cr^{6+} to heterotrophic organisms is 50 ppb[11–14]. It is observed that the Cr^{6+} resistant *Pseudomonas aeruginosa* AB200 is much efficient than the parent strain of the organism, in withstanding Cr^{6+} toxicity. Scanning Electron microscopy (SEM) has high resolution to study the spatial relationship between cells and absorbed metal. Fourier Transform Infra-Red spectroscopy (FTIR) was used to determine the functional groups present on the surface of *Pseudomonas aeruginosa*, which may be involved in Cr^{6+} biosorption. In addition, Energy Dis-

persive X-ray spectroscopy (EDX) is an analytic technique used for the elemental analysis for chemical characterization of samples to determine the surface morphology of cell surface of bacteria before and after biosorption of Cr^{6+}.

Results and discussion

Screening of four different bacteria for Cr^{6+} tolerance study:

Effect of increasing concentrations (ranging from 50 ppm to 500 ppm) of Cr^{6+} in the fermentation media of *Escherichia coli, Bacillus pumilus, Bacillus subtilis* and *Pseudomonas aeruginosa* are given in Fig. 1. The figure indicates that among four categories of bacteria, *Pseudomonas aeruginosa* can resist higher concentration of Cr^{6+} (500 ppm) in the media. Therefore, this bacteria (*Pseudomonas aeruginosa*) then used for further studies to the development of Cr^{6+} resistant strain by using proper concentration of Cr^{6+} biosorption potency of the resistant colonies were studied for the selection of suitable strain for Cr^{6+} removal.

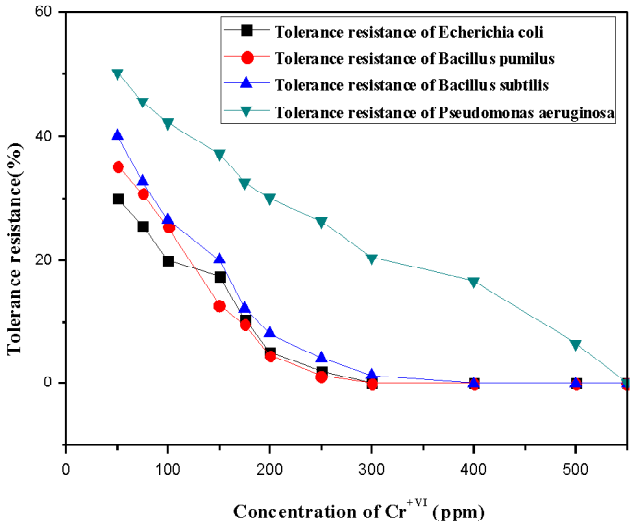

Fig. 1. Cr^{6+} tolerance of *Escherichia coli, Bacillus pumilus, Bacillus subtilis* and *Pseudomonas aeruginosa*.

Scanning Electron Microscopic analysis:

There are some reports[15] that the morphology of microorganism may change during the presence of some metal cations. SEM photo-micrographs of the mycelia with Cr^{6+} unexposed *Pseudomonas aeruginosa* was given in Fig. 2 and Cr^{6+} exposed *Pseudomonas aeruginosa* AB200 were

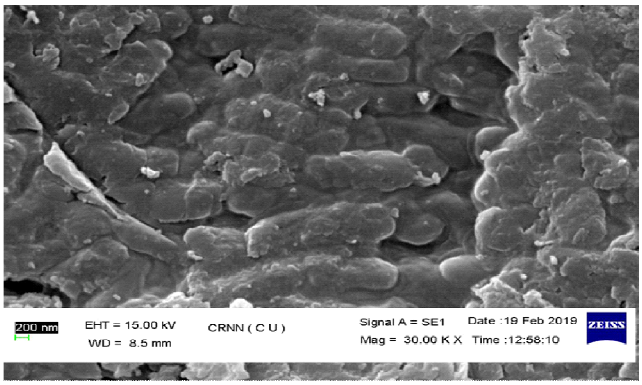

Fig. 2. Surface morphology of parent *Pseudomonas aeruginosa* strain.

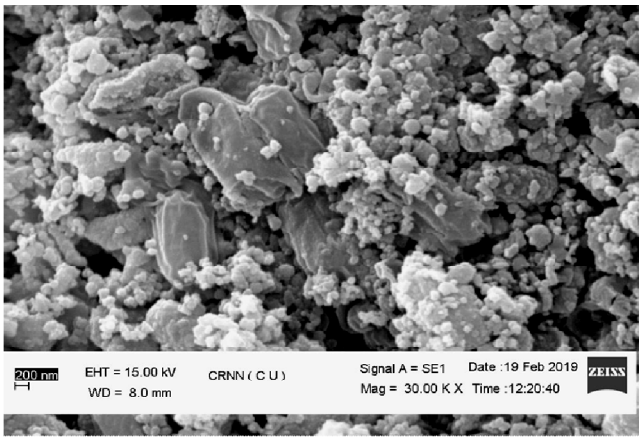

Fig. 3. Surface morphology of Cr(VI) resistance *Pseudomonas aeruginosa* strain.

given in Fig. 3 (500 ppm Cr^{6+} resistant). Fig. 2 shows smooth surface area and longer bacteria, whereas Fig. 3 indicates comparatively shorter bacterial cell and also indicates the rough and altered surface structural facilitates higher biosorption of Cr^{6+}.

From the photo-morphographs it can be stated that Cr^{6+} influenced the mycelia morphology of *Pseudomonas aeruginosa* AB200. This might be due to the some modification of the branching enzyme activity or other enzymes of the cell wall required for tip growth of mycelium. The observation may also conclude the influence of Cr^{6+} on integrity of the cell wall structure of the bacteria.

FTIR analysis:

FTIR spectra of parent strain of *Pseudomonas aeruginosa*

without Cr^{6+} exposure shows the broad absorption peak at 3412 cm^{-1} which corresponds both O-H stretching vibration due to presence of hydrogen bonded polymeric compound such as primary, secondary and tertiary phenolic compounds indicating possibility of presence of alcoholic phenolic groups. Peak at 2934 and 2855 cm^{-1} indicate presence of methyl group where peak at 2934 cm^{-1} is for asymmetric stretching and 2855 cm^{-1} corresponds to asymmetric stretching of C-H bond. Peak obtained at 2348 cm^{-1} indicates presence of acid group. Peak at 2283 cm^{-1} corresponds to nitrile group. Peak at 1763 cm^{-1} is corresponding to C=O bond stretching. Two peaks at 1816 and 1763 cm^{-1} indicates presence of anhydrides where 1816 cm^{-1} obtained due to asymmetric stretch of anhydride functional group and 1763 cm^{-1} is due to symmetric mode stretching. There are two strong N=O stretch peaks for nitro group with the asymmetric stretch at 1584 cm^{-1} and the symmetric stretch at 1381 cm^{-1}. Peak at 1067 cm^{-1} is assigned at C-O bond stretching which indicates presence of alcoholic and esters groups. C-Cl bond stretching obtained at 779 cm^{-1}. Peak at 592 cm^{-1} corresponds to C-Br bond and peak at 479 cm^{-1}, 430 cm^{-1} are corresponds to C-I bond stretching.

FTIR analyses of cultured *Pseudomonas aeruginosa* resistant with Cr^{6+} (500 ppm) shows shift of absorption band of O-H stretching from 3412 to 3393 cm^{-1}. Peak at 2964 and 2928 cm^{-1} are due to stretching of sp^3 C-H bond. Peak at 1461 cm^{-1} corresponds to $-CH_2$ bonding. Peak at 2348 cm^{-1} indicates presence of acid group. Peak at 2290 cm^{-1} corresponds nitrile group. There are two C=O absorption peak at 1805 cm^{-1} (asymmetric stretch) and 1753 cm^{-1} (symmetric stretch) confirms presence of anhydride. Peak at 1658 cm^{-1} is due to stretching of C=C bond of alkene group. There are two strong N=O stretch peaks of nitro group, with the asymmetric at 1551 cm^{-1} and the symmetric stretch at 1383 cm^{-1}. Peak at 1461 cm^{-1} is due to bonding of C-H bond of $-CH_2$ group. Peak at 1055 cm^{-1} is assigned to C-O bond stretching which indicates presence of alcoholic ester group. Peak at 805 cm^{-1} corresponds to out-of-plane bonding of aromatics. Peaks at 671 cm^{-1}, 587 cm^{-1} and 448 cm^{-1} are obtained from alkyl halides.

So it can be seen that some of the absolute bond values of parent *Pseudomonas aeruginosa* is differing from the Cr^{6+} resistant (500 ppm) *Pseudomonas aeruginosa*. This change in bond pattern that occurred may be due to the exposure of the biosorption surface to the more toxic concentration of Cr^{6+} which leads to make the microorganism more potent to tolerate higher concentration of Cr^{6+}. In this regard it may be assumed that interaction of biosorbent surface with higher concentration of Cr^{6+} shifted the biosorption bands. Cr^{6+} resistant *Pseudomonas aeruginosa* has higher physical stability and surface activity due to the presence of these negative radicals which are responsible for the biosorption of Cr^{6+} as compared to the parent strain of *Pseudomonas aeruginosa*.

EDX analysis:

Energy Dispersive X-ray spectroscopy is an analytical technique used for the elemental analysis or chemical characterization of samples. It relies on the interaction of some source of X-ray excitation and a sample. Its characterization capabilities are due in large to the fundamental atomic structure allowing unique set of peaks on its electromagnetic emission spectrum.

Parent and Cr^{6+} resistant strain of *Pseudomonas aeruginosa* were analysed under SEM combined with EDX analysis to determine the surface morphology and elemental composition of cell surface of the used *Pseudomonas aeruginosa*, before and after biosorption of Cr^{6+} (500 ppm). Fig. 4 shows that the parent *Pseudomonas aeruginosa* does not contain hexavalent chromium. It contains carbon, oxygen and platinum.

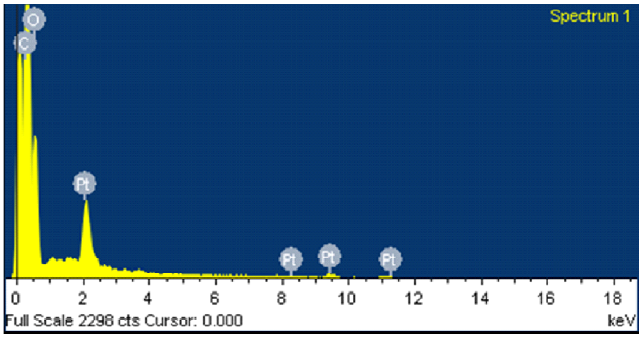

Fig. 4. EDX spectra of *Pseudomonas aeruginosa* before biosorption of Cr(VI).

Fig. 5 given below shows that *Pseudomonas aeruginosa* after Cr^{6+} resistance (500 ppm) contains chromium along with carbon, oxygen and platinum.

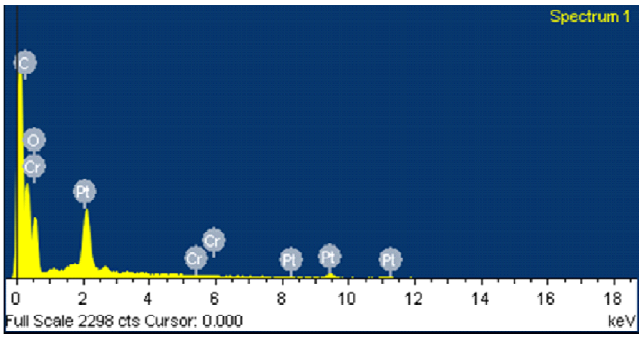

Fig. 5. EDX spectra of *Pseudomonas aeruginosa* after resistance of Cr(VI).

This analysis provides a strong evidence of Cr^{6+} biosorption to cells surface and this is the reason behind the higher biosorption of the resistance strain of *Pseudomonas aeruginosa* in reference to the parent strain of *Pseudomonas aeruginosa*.

Experimental

Microorganism and culture conditions:

Four different microorganisms were used primarily in the present study and they were *Escherichia coli, Bacillus pumilus, Bacillus subtilis* and *Pseudomonas aeruginosa*. These four bacteria was maintained by regular transfer to nutrient agar slant test tubes containing (g/L) dextrose 10.0, yeast extract 2.0, peptone 5.0, beaf extract 1.0, sodium chloride 5.0 and Agar 40.0; pH was adjusted at 7.0 with 1 (*N*) HCl or 1 (*N*) NaOH. Slants were incubated at 37°C for 48 h.

Preparation of biosorbent:

10 ml of sterilized demineralised double distilled water was poured into each slant containing each of the microorganisms. Then using separate inoculation loop for different microorganisms, growth on the surface of different media containing Agar slant were scraped off carefully. Each type of microorganisms was harvested in separate sterilized cotton plugged conical flasks shaken properly in order to minimize clumping of microorganisms.

Isolation of Cr^{6+} resistant colonies of different microorganisms:

From Cr^{6+} tolerance study it was evident that microorganisms can resist different concentrations of Cr^{6+} which can be assumed by comparing the dry cell weight of the experiment of different microorganisms. Each microorganism found to be resistant upto a certain concentration of Cr^{6+} (Fig. 1). Firstly, each of the microorganisms was inoculated into corresponding fermentation broth media amended with maximum Cr^{6+} concentrations which they can resist. After proper incubation period, standard serial dilution and pour plate method was performed for each microorganism. After incubation period, larger identical colonies from each plate for each microorganism were isolated. They were transferred to nutrient Agar slant test tubes and after proper incubation period they were stored at 4°C temperature. Six resistant colonies of *Escherichia coli* were obtained at 200 ppm of Cr^{6+} concentration containing fermentation broths. Seven resistant colonies of *Bacillus pumilus* were obtained at 250 ppm Cr^{6+} concentration containing fermentation broths. Five resistant colonies of *Bacillus subtilis* were obtained at 350 ppm Cr^{6+} concentration containing fermentation broths. Six resistant colonies of *Pseudomonas aeruginosa* were obtained at 500 ppm Cr^{6+} concentration containing fermentation broths.

Comparative study of biosorption potency of isolated Cr^{6+} resistant strains:

Each of the high Cr^{6+} concentration resistant colonies of each organism were tested for their biosorption potency study amended with increasing concentration of Cr^{6+}, in the suitable fermentation broth. After proper incubation period, using the filtrate, after filtrating off the biomass from the broth by Whatman filter paper, residual Cr^{6+} concentration in the filtrate was determined. Residual Cr^{6+} concentrations were determined by Atomic Absorption Spectrophotometer (Varian, Model Number: AA240, GTA120 fitted). From this residual Cr^{6+} concentrations present in the filtrate, percentage of Cr^{6+} biosorption can be calculated by:

$$\text{Percentage of } Cr^{6+} \text{ biosorption} = [(C_i - C_f)/C_i] \times 100$$

where C_i = initial Cr^{6+} concentration present in the fermentation media, C_f = residual Cr^{6+} concentration in the filtrate.

Scanning Electron Microscopic study of the parent and resistant Pseudomonas aeruginosa:

Nutrient broth media was used both for parent and Cr^{6+} resistant *Pseudomonas aeruginosa*. After proper incubation mycelia mat were removed carefully from each set and standard dehydration process was followed. Dehydrated parent strain and Cr^{6+} resistant cells were attached to 10 mm metal mount using double site adhesive tape. Chamber pressure

was maintained at 110 Pa which is generated by injecting the water vapour. Surface morphology of the samples was visualized by LED detectors of the FEL QUANTA Scanning Electron Microscope (Model number: 200 MK2). SEM allowed the identification of any interesting structural changes on the cell surface before and after Cr^{6+} biosorption.

Fourier Transform Infra-Red (FTIR) spectroscopic studies of both the parent and Cr(VI) resistant strain of Pseudomonas aeruginosa:

In order to determine the cell surface functional groups responsible for Cr^{6+} biosorption, FTIR spectroscopy was carried out using a Perkin-Elmer RXI FTIR System (Model number: 54350). Nutrient broth media was used both for parent and Cr^{6+} (500 ppm) resistant culture of *Pseudomonas aeruginosa* AB200. After proper incubation mycelia mat were removed carefully from each set and samples were dried in hot air oven to remove the water vapour. Then dried cells were crushed into powdery form, using mortar-pestle and prepared for FTIR analysis as KBr mixed dices.

Conclusion

Biosorption is being demonstrated as useful alternative solution to conventional systems for cleaning the environment, contaminated by toxic metals. The development of biosorption process requires further investigation in the direction of large scale set up to remove the toxic metals from industrial effluents after optimization of physical and chemical parameters. Current investigation was a laboratory scale development of high Cr^{6+} concentration resistant strains for removing more and more Cr^{6+} solution. To obtain a successful technological process the above work may be extended with actual industrial effluents.

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