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BIODEGRADATION AND IMPACT OF MUTATIONAL STUDIES, PLASMID CURING ON BIOTRANSFORMATION OF CHROMIUM

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ABSTRACT

The present study was performed to bioremediate chromium toxicity in the leather processing industrial effluents of Dindugal district. Based on the isolation, screening and biochemical characterization, the bacterial isolates were identified as Staphylococcus aureus and Bacillus subtilis based on the Bergey's manual of systematic bacteriology. The MIC study revealed that these two strains have been found potential and tolerant to increased chromium concentration of upto 800 ppb. The reduction of different concentration of chromium occurred was due to the enzymatic action of microorganisms monitored by FTIR. The resistant colonies to UV- irradiated and mutated colonies were confirmed by temperature sensitive method. Studies pertaining to curing of plasmids from the bacteria were monitored to analyze dissemination of genes conferring resistance to toxic pollutants. Both the species revealed single plasmid with 12 kb for Staphylococcus aureus and 15 kb for Bacillus subtilis. Transformation experiments were performed into competent cells of E.coli DH5α strains which suggested the plasmid of chromium tolerance strains were transformed. This paved the way that the plasmid encoding heavy metal resistance is an important aspect of the environmental research.

KEY WORDS

biodegradation, biotransformation, chromium, Fourier Transform Infrared Spectroscopy, mutation, Staphylococcus aureus

INTRODUCTION

Rapid industrialization and increased urbanization grossly contaminated the environment by discharging the heavy metals like chromium as effluent causes severe health hazards to the living beings [1]. Chromium is widely used in industries like tanneries, metal finishing, electroplating industry and petroleum refineries [2].

Tannery wastes contain 79.9-249.7 mg/l of chromium (VI), but the recommended discharge of chromium (VI) into the water is less than 0.05 mg/l. Above the recommended level of chromium is toxic, carcinogenic and mutagenic [3]. Cr (VI) is extremely water soluble and mobile in the environment but Cr (III) is less soluble and comparatively immobile. Moreover, Cr (VI) is recognized to be highly toxic, carcinogenic, mutagenic and teratogenic for mammal including humans, whereas Cr (III) is an essential trace element necessary for glucose, lipid and aminoacid metabolism as well as popular dietary supplement [4]. Cr (VI) is approximately 100 times more toxic and 1000 times more mutagenic than Cr (III). Cr (VI) causes severe diarrhoea, ulcers, eye and skin irritations, kidney dysfunction and probably lung carcinoma. It is also associated with decrease in plant growth and changes in

plant morphology. Hence, the removal of hexavalent chromium is mandatory.

The conventional methods to remove Cr (VI) are (a) reduction to Cr (III) followed by precipitation as chromium hydroxide (b) removal of ion exchange and (c) removal by adsorption. These methods are costly due to operational treatment and sludge disposal costs [5]. Microorganisms can play an important role in the removal of Cr (VI) from the polluted sites. A wide variety of bacteria has been reported to reduce Cr (III) and Cr (VI) under aerobic and anaerobic conditions.

Recently, research for new and innovative technologies has centered on the biological treatment methods [6]. Bacteria which possess extra chromosomal DNA, called plasmids, it possesses functional genes for the resistance of compounds (eg. Aromatic compound degrading agents) Plasmid curing is a process of completely removing plasmids of bacteria by means of chemical agents such as Acriflavin or acridine orange. The purpose of this technique was to introduce a foreign plasmid into bacteria and to use those bacteria to amplify the plasmid in order to make large quantities of it. This is based on the natural function of a plasmid to transfer genetic information vital to the survival of the bacteria [7].

Hence the present study was designated to isolate and to study the role of indigenous microbes in removing Cr^{6+} (hexavalent chromium) in laboratory conditions. In addition, an attempt was made to identify whether the plasmid or chromosome mediated determinants of the isolated chromium tolerant microorganisms to confer resistance to heavy metals (chromium).

MATERIAL AND METHODS

Isolation and Screening of Chromate-Resistant Microorganisms

The effluent sample was collected from a tannery industry situated at Dindigul in Tamil Nadu. The bacterial isolates of tannery effluents were screened on nutrient agar plates supplemented with potassium dichromate ($K_2Cr_2O_7$) by the standard spread plate method. The plates were incubated at 37°C for 24 to 48 hours. These bacterial isolate were selected and used for further studies.

Determination of MIC

Maximum resistance of the selected isolates against increasing concentration of Cr on LB agar plates was evaluated until the strains unable to give colonies on the agar plates. The initial concentration used (100 ppb) was added from 100mg/100ml stock solution. The stock solution of $K_2Cr_2O_7$ was prepared in distilled water and was sterilized. The colonies grown at a given concentration were subsequently transferred to the next higher concentration. Based on the evaluation minimum inhibitory concentration (MIC) was determined after 48 hrs of incubation at 37°C.

Identification and Characterization of Isolated Organisms

The bacterial isolates were biochemically characterized for the activities of starch hydrolysis, utilization of sugars, citrate utilization, nitrate reduction, indole test and gelatin hydrolysis. The biochemical tests were used to identify the isolates according to Bergey's Manual of Systematic Bacteriology.

Cr (VI) Reduction

The bacterial strain was cultured overnight in Luria Bertani (LB) broth. Culture flasks containing LB broth medium amended with Cr (VI) in different concentration. All the cultures, including control were incubated for 24 hrs at room temperature (30°C) with shaking speed of 100 rpm. Growth of the bacteria was monitored. The Cr (VI) reduction by growing cells was analyzed by taking a 1-ml culture from each of the above mentioned flasks and centrifuged at 8000 rpm for 10 min). The chromium Cr (VI) reduction was

analyzed by Atomic Absorption Spectrophotometer and Fourier Transform Infrared Spectroscopy.

Mutagenesis

About 5 ml culture was centrifuged at 10,000 rpm for 1min and the supernatant was discarded. The pellet was suspended with water and it was serially diluted upto 10^{-5} dilution. 0.1ml from 10^{-4} and 10^{-5} dilution was plated on yeast peptone agar plates and these plates were exposed to UV-radiation from 5 to 25 minutes. Non-irradiated plates served as a control [8]. To screen the temperature sensitive mutants, the irradiated and non-irradiated plates were incubated at 37°C and 23°C. If there was no growth in plates incubated at 37°C, then it is confirmed that they were temperature sensitive mutants [9].

Plasmid Profiles

In order to study the relationship of multi-resistance of *Bacillus* and *Staphylococcus* strains and their indigenous plasmids, plasmid profiles of the selected two strains were carried out using the alkaline lysis method [10].

Plasmid Transformation

To confirm the nature of isolated plasmids, these were transformed into non-resistant *E.coli* DH5α strains. For this purified plasmid DNA isolated from resistant strains were used for transformation into calcium chloride treated cells of *E.coli* DH5α strains.

Plasmid Curing

To determine and to ascertain the resistant gene is encoded by a plasmid, 10% sodium dodecylsulphate was used to eliminate the plasmids from the strains and simultaneously it was heated to serve as second control. The strains were grown with SDS (100µg/ml) and then spread on LB agar plates supplemented with chromium and without chromium. Replica plates for both were incubated at 37° C. Plasmids were considered to be eliminated from those colonies that grow on the metal free medium only. For heat treatment the strains were grown at 47°C and sub cultured into fresh medium.

RESULT AND DISCUSSION

Chromium (VI) is one of the major pollutants released from tannery industry; dyes and textile industry waste effluents and were highly toxic and carcinogenic in nature. Hexavalent chromium (Cr VI) is associated with various forms of cancer particularly pancreatic cancers [11] and respiratory tract [12]. The chemical methods for the bulk treatment of these effluents often fail to reduce the level to meet the environmental regulations. Bioremediation is considered a better eco-friendly alternative to chemical treatment. A wide variety of mechanisms exist for the removal of heavy metal from aqueous solution by bacteria, fungi, ciliates, algae, and higher plants [13, 14].

Chromium tolerant bacteria have been isolated from tannery effluent by several groups [15]. In the present investigation, five chromium tolerant strains were isolated and their chromium tolerance limits was determined. Out of these five strains TE-1 and TE-4 have been found potential tolerant to elevated chromium concentration of upto 800ppb. The chromium tolerant microorganisms were identified as *Staphylococcus aureus* and *Bacillus subtilis* in accordance with the Bergey's manual of determinative bacteriology.

The reduction of hexavalent chromium to trivalent chromium showed significant advantages over physicochemical treatment techniques. In the present study the chromium reducing capability of *Bacillus subtilis* and *Staphylococcus aureus* were studied with different concentrations of chromium i.e., 200ppb, 500ppb and 750ppb. Chromium reduction is monitored by FTIR studies which exhibit functional group changes due to degradation. The control treated samples

exhibited a peak of 1647.41 cm^{-1} and 1629.85 cm^{-1} indicated amide 1 components of proteins. Another peak at1120.64 cm^{-1} corresponds to C-O-C, C-O dominated by ring vibrations in various polysaccharides. On treatment with *Staphylococcus aureus* amended with 200 ppb of chromium exhibited a peak of 2929 cm^{-1} indicated the presence of CH groups. Similarly another peak of 1654.92cm^{-1} indicated the amide 1 of α-helical structures of proteins. Another similar peak at 3431.36cm^{-1} is due to the vibration of hydrogen bonded –OH group (Fig. 1).

Table: 1 Atomic Absorption Spectroscopy analysis of total chromium treated with *Staphylococcus* and *Bacillus* sp

S.No	Chromium concentration	Total Chromium Concentration (in ppb)
1	Control 200ppb	0.016
2	Control 500ppb	0.023
3	Control 750ppb	0.040
4	*Staphylococcus* sp 200ppb	0.012
5	*Staphylococcus* sp500ppb	0.420
6	*Staphylococcus* sp 750ppb	0.422
7	*Bacillus* sp 200ppm	0.020
8	*Bacillus* sp 500ppm	0.422
9	*Bacillus* sp 750ppm	0.424

Figure: 1. FTIR spectral analysis of the 200 ppb chromium treated with *Staphylococcus aureus*

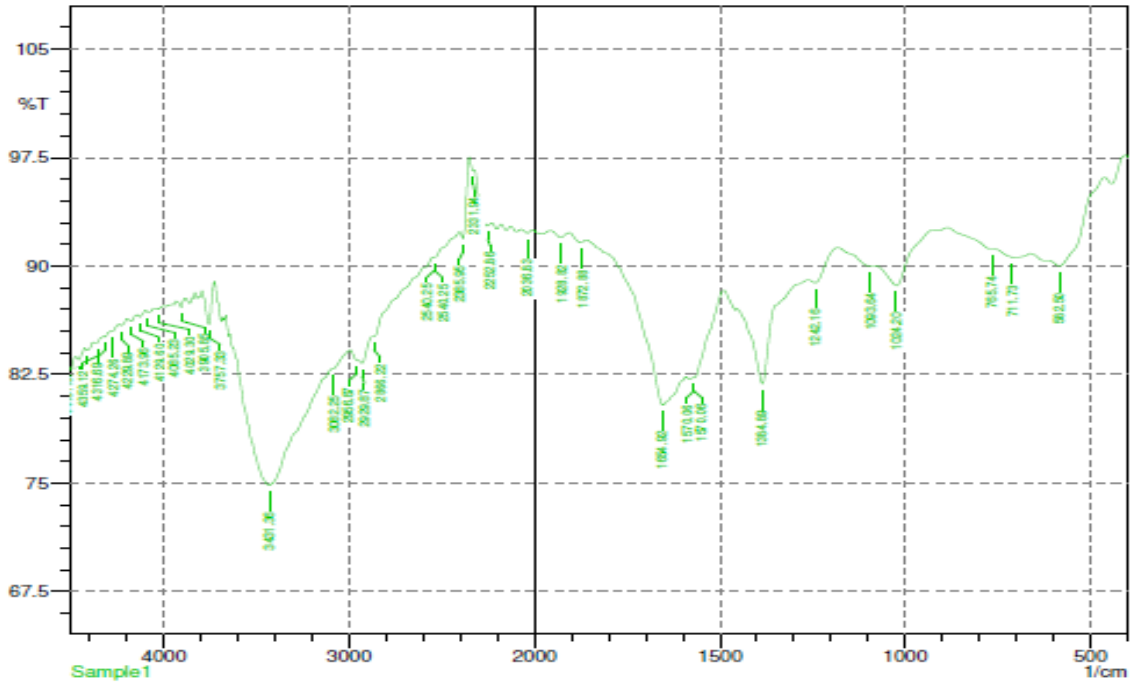

Figure: 2. FTIR spectral analysis of the 200 ppb chromium treated with *Bacillus subtilis*

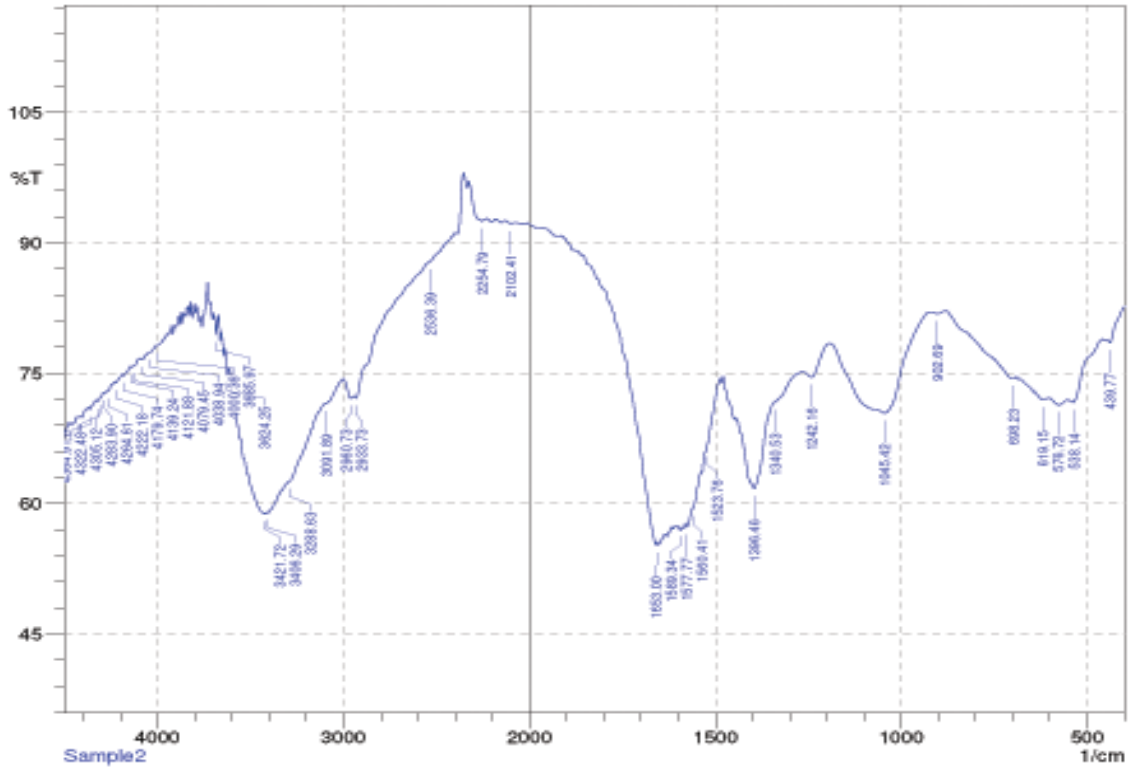

Figure: 3. FTIR spectral analysis of the 500 ppb chromium treated with *Staphylococcus aureus*

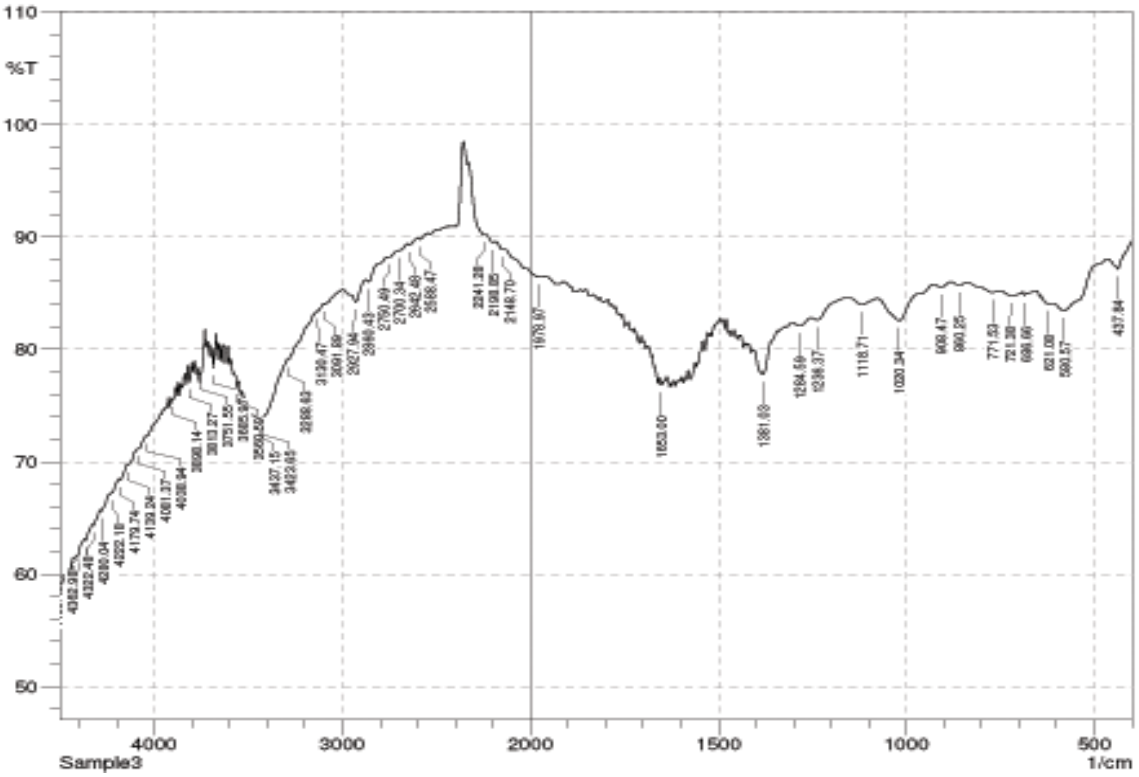

Figure: 4. FTIR spectral analysis of the 500 ppb chromium treated with *Bacillus subtilis*

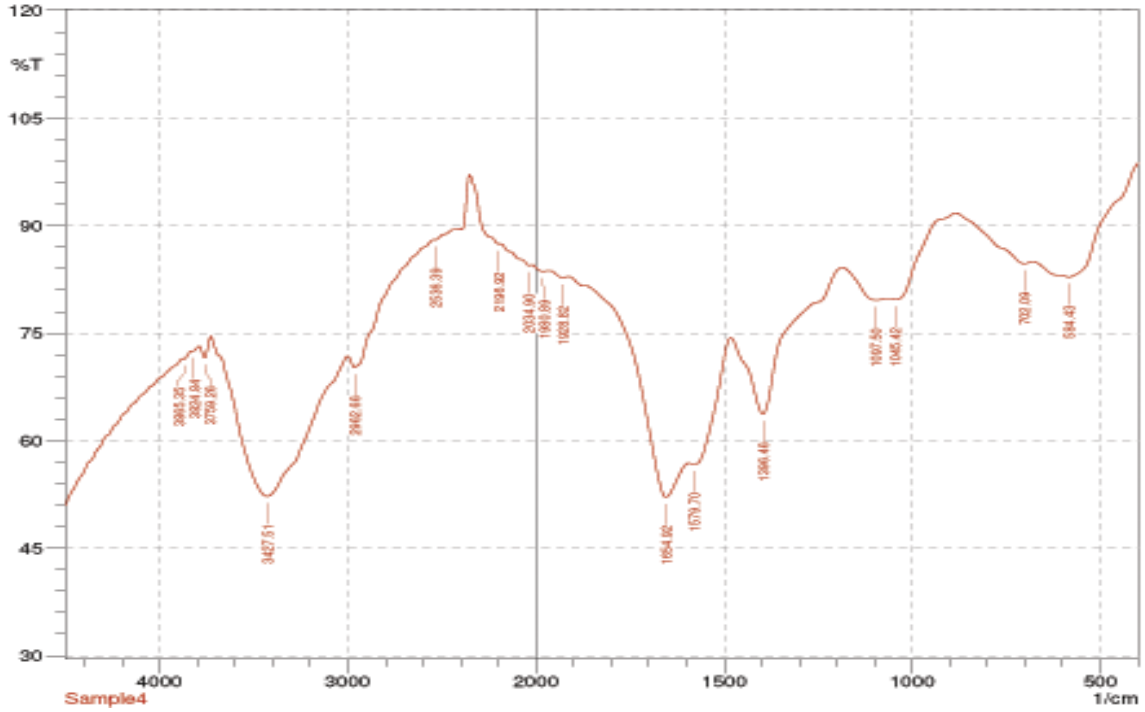

Figure: 5. FTIR spectral analysis of the 750 ppb chromium treated with *Staphylococcus aureus*

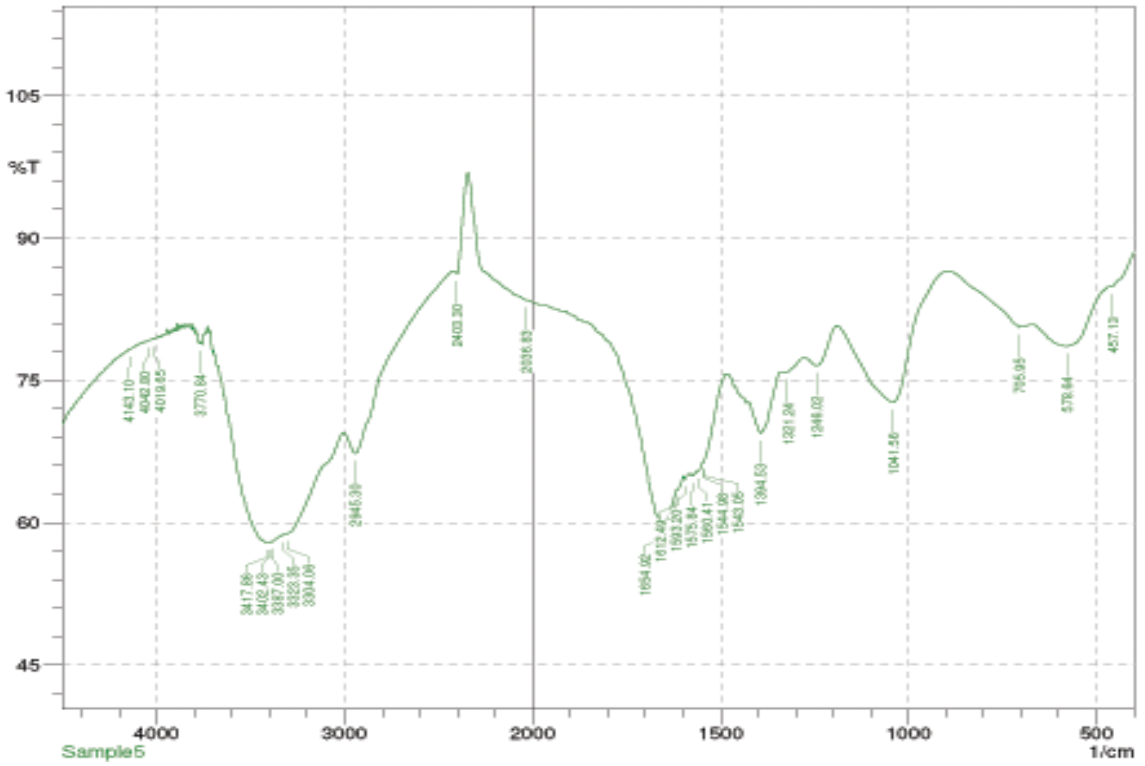

Figure: 6. FTIR spectral analysis of the 750 ppb chromium treated with *Bacillus subtilis*

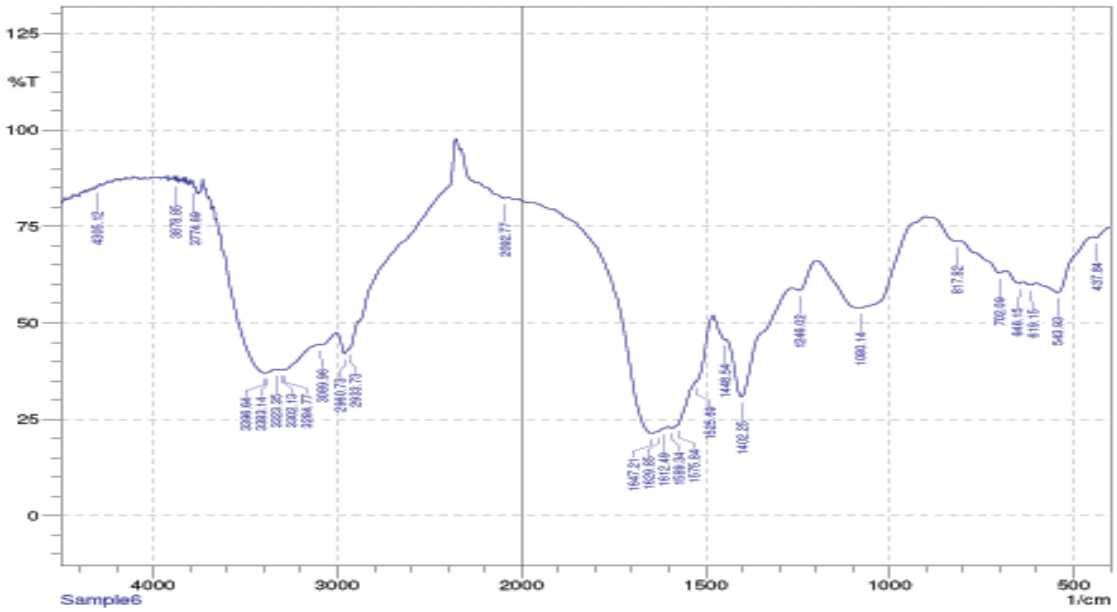

Plate. 1

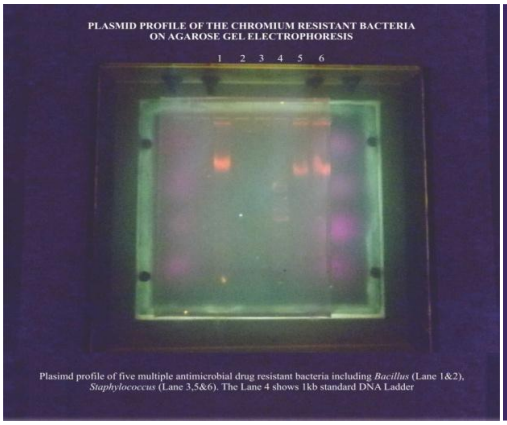

Plate: 2

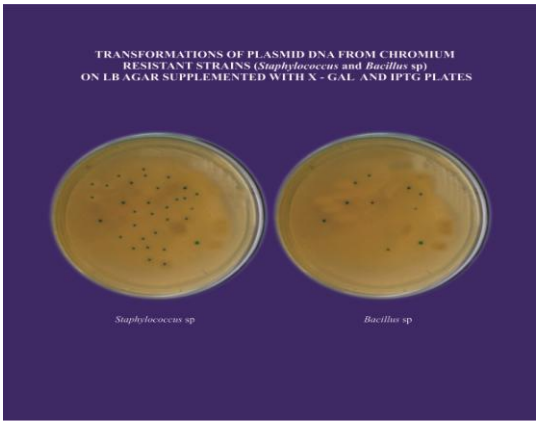

Plate. 3

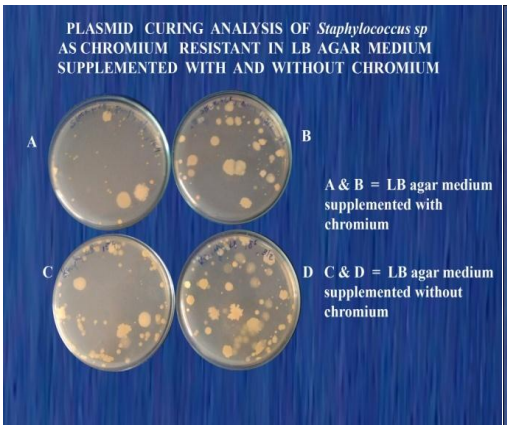

Plate. 4

FTIR analysis of hexavalent chromium degradation exposed over 200ppb of chromium inoculated with *Bacillus subtilis* exhibited a peak of 3421.72cm^{-1} indicated surface –OH group was one of the functional group responsible for adsorption. Simultaneously another peak exhibited a spectral wave length of 1526.76 cm^{-1} indicated aromatic C-NO_2 stretching for the adsorption of chromium. The peak at 1045.42 cm^{-1} indicated the Si-O stretching (Fig.2).

On analysis with a 500 ppb concentration of chromium with *Staphylococcus aureus* the peak values at 3431.15 cm^{-1} indicated a broad peak which was usually found due to the vibration of hydrogen bonded OH groups. Similarly, on monitoring further peak in the same concentration revealed a wavelength of 1653 cm^{-1} indicating amide 1 bonds suggested the presence of α-helical structure (Fig.3). On analysis with *Bacillus subtilis* amended with 500ppb of hexavalent chromium indicated a pronounced peak of 2962.06 cm^{-1} indicated the C-H asymmetric stretching of –CH_3 in fatty acids. Similar peak of 1091 cm^{-1} indicated the Si-O stretching which is responsible for adsorption (Fig.4). On analysis with degradation of chromium at 750 ppb treated with *Staphylococcus aureus* revealed a peak of 3417.86 cm^{-1} denoted a surface OH group responsible for functional group changes leading to adsorptiom. Another FTIR spectrum exhibited an intense band around 1321.24 cm^{-1} suggested an anion carboxylate which are responsible for adsorption of chromium by microorganisms. Similarly the FTIR spectrum also revealed a band value of 1544.98cm^{-1} exhibited aromatic C-NO_2 stretching (Fig.5). The FTIR spectrum for biotransformation of chromium with 750 ppb inoculated with *Bacillus subtilis* indicated broad peaks around 3396.64 cm^{-1} to 3284.77 cm^{-1} usually found due to vibration of hydrogen bonded OH groups. Similarly a prominent peak of 2092 revealed the presence of CH groups. A peak at 1525.69cm^{-1} indicated aromatic C-NO_2 stretching which was quite interesting to note intermediate compound appeared due to degradation (Fig.6).

Metal accumulation of chromium or biotransformation is an alternative mechanism for metal detoxification in bacteria. Atomic Absorption spectroscopy analysis revealed a drastic difference between control and experimentally treated sample. Among the organisms, *Bacillus subtilis* reduced Cr (VI) under aerobic conditions. This may be due to the presence of chromium reductase. The total chromium concentration between control and experimental treated *Staphylococcus aureus* revealed the reduction in total chromium of 0.021 ppb for 200 ppb. On exposure to 750 ppb of chromium, it was found out that much variation was observed at 750 ppb within control and experiment. Simultaneously, when chromium was treated with *Bacillus subtilis* at 200 ppb revealed an accumulation of 0.020 ppb at 200 ppb whereas at 750 ppb, the result was found to be 0.024 ppb respectively (Table. 1). Our finding coincides with the work of James [16].

Mutation was carried out in order to analyze whether DNA damage could alter the chromium remedial activity of the organism. Mutagenesis was carried out by exposing the organisms to UV radiation. The distinguishable characteristics of non-radiated and radiated plates (UV irradiated for 20 min) were studied. The colonies which tolerated the UV-radiation were mutated and those mutated colonies were confirmed by temperature sensitive methods. Both the plates were incubated at 37^{0}C for 24 hrs [17].

In order to study the genetic changes and mechanism of chromate resistance in these bacteria, curing of plasmids in the bacteria as well as the effect on chromium uptake are being investigated. Horizontal dissemination of genes conferring resistance to toxic pollutants may play

an important role in the adaptation of bacteria to toxic contaminants in the environment [18]. In the present study, the plasmid DNA was isolated from *Bacillus subtilis* and *Staphylococcus aureus*. Both the microorganisms revealed the presence of single plasmid with the size of approximately 12 kb for *Staphylococcus aureus* and 15 kb for *Bacillus subtilis* (Plate. 1). The present results were in total conformity with the findings of Edward Raja and Selvam [19], wherein they isolated single plasmid from *Pseudomonas* sp with the molecular weight of approximately 23Kb.

To determine whether the resistance markers were actually on plasmid DNA, transformation experiments was performed by employing plasmid DNA isolated from *Staphylococcus aureus* and *Bacillus subtilis*. Plasmid DNA of *Staphylococcus* and *Bacillus subtilis* was transformed into competent *E.coli* DH5α strain. Growth was observed as blue coloured transformed colonies on the plates. These observations suggested that, plasmids of the chromium tolerant strains were transformed (Plate.2).

Plasmid DNA elimination or curing from bacterial cell is the best test to locate a genetic trait in specific plasmid. Plasmid can be eliminated by agents that interfere with its replication or its membrane attachment sites. Among the resistant strains, about 20% of the strains that grow on the metal free medium only after curing were found to have plasmid encoded resistance to chromium (Plate.3 and 4). The present observations were similar to the findings of Coral *et al* [20]. Moreover transformation and curing results of the study suggested the chromium resistance were conferred by plasmid DNA. Some heavy metal resistant determinants move from plasmid to chromosome (or in the reverse direction). This makes plasmid encoding heavy metal resistance as an important aspect of environmental research. The plasmid can be the source of resistance genes for cloning purpose which have potential use in the bioremediation process [21].

CONCLUSION

In the present study on biodegradation of persistent heavy metal chromium was carried out using the bacteria Staphylococcus aureus and Bacillus subtilis. MIC has proved that these two strains were found versatile organism since it degrade chromium upto 800 ppb. It was confirmed by analyzing through FTIR. The FTIR study was performed to analyze intermediate compounds and functional group changes such as appearance of OH groups, CH groups and exhibition of spectral wavelength of 1526.76 cm^{-1} an indication of aromatic C-NO_2 stretching for absorption of chromium. Moreover to study the role of bacteria and to introduce the resistant plasmid and to amplify for transfer of genetic information to produce tolerant microorganisms. Further, the resistant colonies were UV irradiated and these mutated colonies were reconfirmed by temperature sensitive method. Besides, studies pertaining to curing of plasmids from bacteria were performed to analyze dissemination of genes conferring resistance to toxic pollutants like chromium.

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