BIOREMEDIATION OF HEXAVALENT CHROMIUM BY FREE AND IMMOBILIZED BIOMASS OF *CHLORELLA* AND *PSEUDOMONAS*

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ABSTRACT: Hexavalent chromium is a toxic heavy metal and carcinogen. The purpose of present study was to analyze Cr(VI) resistant bacterial strain S7 in combination with *Cholera* for observing the mutualistic relationship for remediation processes. By 16S rRNA sequencing technique, the strain S7 was identified to be *Pseudomonas* species (KR095629). It was able to tolerate Cr(VI) stress up to 600µg/mL and showed an optimum Cr(VI) reduction of 100µg/mL. Sodium alginate (2%) was used for immobilization of S7 and *Chlorella*. Chromium (VI) removal by immobilized cells of S7 and *Chlorella* was 4.5mg/g and 6.20mg/g, with reduction potential of 58% and 60%, respectively. A direct relation between Cr(VI) concentration and root length of *Chlorella* was observed. Maximum root length was observed for *Chlorella* and S7 *i.e.* 1.9cm at 500µg/ml Cr(VI) concentration. The presence of several absorption peaks in the Fourier transform infrared spectroscopy (FTIR) of the biomass indicated its complex nature. This technique revealed the involvement of hydroxyl group, N-H and C-O group. The Cr(VI) was effectively removed by bacterial strain when used with *Chlorella.*

Key Words: Symbiosis, *Pseudomonas, Chlorella*, Cr (VI) reduction and FTIR.

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INTRODUCTION

Heavy metals, environment pollutants are non-biodegradable and accumulate in the ecosystem (Batool *et al.,* 2012) and tissues of living organisms causing harmful effects (Pereira *et al.,* 2009). Heavy metals are environmental hazards (Karpagam and Nagalakshmi 2014) that have negative influence on human health and agriculture by decreasing the soil microbial activity (He *et al.,* 2009). Chromium is considered to be a toxic metal and its overuse in the past few decades made it a major contaminant of soil and water (Cervantes *et al.,* 2006). Hexavalent chromium is more toxic, mutagenic and carcinogenic in nature compared with trivalent chromium (He *et al.,* 2009). Therefore, hexavalent chromium is of major concern in water pollution control. Biosorption and immobilization are effective techniques for metal removal from industrial effluents. Immobilization methods involve the use of beads as matrix that act as barrier between the internal and external environment. This helps to retain the agents being used for treatment inside the bead, saving organisms residing in wastewater, and permitting uninterrupted tertiary wastewater treatment (Oliveira, 2012). The raw materials used for bioremediation of Cr(VI) is cheap, environment-friendly and highly efficient. The biosorbant readily adsorb the heavy metal and could effectively be recovered or recycled from it (Naja *et al.,* 2010).

Recent studies have reported improved pigment, lipid content, growth, cell size and population size of entrapped microalgae due to co-immobilization of bacteria and microalgae reported by (De-Bashan *et al.,* 2016). Present research was done to isolate the chromium resistant bacteria and determine its Cr (VI) reduction ability in combination with *Chlorella*.

MATERIALS AND METHODS

Sampling for isolation of bacteria: The soil sample was collected in sterile petri plate from a pond near Sheikhupoora, Pakistan. The temperature and pH of sample were recorded and transferred to the lab under aseptic conditions. Chromium (Cr) resistant bacterial strains from the soil sample were isolated by inoculating on supplementing LB-agar with K_2CrO_4 in different concentrations i.e. 100, 150, 250, 350, 450, 500 and 600µg/mL. The agar plates were incubated for 3-4 days at 37°C. Twenty-five morphologically different colonies were selected and strains were purified by streaking (Faisal *et al.* 2005).

Resistance level of isolates: The minimum inhibitory concentration of selected strains was determined by increasing concentrations of Cr(VI). The strains were grown in LB broth supplemented with increasing concentration (100 - 600µg/mL) of hexavalent chromium and incubated for 24h at 37˚C. UV-spectrometer (IRMECO UV-Vis Spectrophotometer Model U2020) used to measure the optical density of broth cultures at 600nm (Faisal and Hasnain, 2005).

Identification of bacterial isolates: The morphology of bacterial strains was noted (Starr *et al.* 2013). Strains were biochemically characterized by performing catalase, oxidase, MR-VP, OF, TSI and citrate tests. Heavy metal profiles of strains were determined for the following metals: Cu^{2+} ($CuSO_4$), Cd^{+2} ($CdCl_2$), Mn^{2+} ($MnCl_2$), Co^{2+} [Co $(NO_3)_2$], Pb^{2+} [Pb $(NO_3)_2$], Zn^{2+} ($ZnCl_2$) (Faisal and Hasnain, 2005). Antibiotic resistance profiles against five antibiotics including erythromycin, tetracycline, ampicillin, gentamycin and chloramphenicol was performed (Faisal *et al.* 2005).

Strains were identified by 16SrRNA sequencing at Macrogen Inc (Seoul Korea) using primers 518F (CCAGCAGCCGCGGTAATACG) and 800F (TACCAGGGTATCTAATCC). Phylogenetic analysis was done in MEGA 5 and tree generated by neighbor joining method considering the two main domains eubacteria and archaea for bootstrap analysis (Katoh and Standley 2013).

Determination of hexavalent chromium reduction potential: Chromate reducing potential was determined in LB broth containing 100, 350 and 500µg/mL of K_2CrO_4 respectively incubated 150rpm and 37^{o}C for 120 hours. Bacterial cultures were cultivated in 20mL test tubes containing 7mL of media (absorbance of bacterial cultures was adjusted to 10^8-10^9 at 600nm). Bacterial strains having three different concentrations of chromate were incubated at shaking incubator with 150 rpm speed for 120 hours at 37°C. Samples (20ml) were drawn at intervals of 24 hours for 120 hours. These were centrifuged at 14000rpm for 5 minutes and residual Cr(VI) concentration in supernatant was determined by diphenyl-carbazide method (Singh *et al*., 2014).

Estimation of Cr (VI) reduction potential of immobilized chromium resistant bacterial strains with *Chlorella:* Sodium alginate (2%) was used for the immobilization of the bacterial strains and *Chlorella* as described by Batool *et al.* (2012). The Cr(VI) reduction potential estimation was determined over the intervals of 0 to 120 hours. Cr(VI) stress was induced with Cr(VI) concentration of 350µg/mL.

Estimation of hexavalent Cr uptake of immobilized cells: Hexavalent chromium uptake by immobilized bacterial cells and *Chlorella* were estimated as described by Humphires *et al.* (2005). Immobilized cells (0.5g) were drawn out every 12 hours over a period of 96 hours and optical density was measured at 540nm. Cr(VI) concentration was estimated by following (Faisal *et al*., 2005):

$$\text{Hexavalent chromium (mg/L)} = \frac{Ax100}{BxC}$$

Where A = hexavalent chromium (mg)
B = original sample (mL)
C = portion (mL) from 100mL digested sample

Fourier transform infrared spectroscopy: Luria-Bertani broth supplemented with and without 500µg/mL K_2CrO_4 was used to culture bacterial cells for overnight. Inoculated broth (10mL) was centrifuged at 10,000 rpm for 5 min and pellets were dried at 60^{o}C in a hot air oven and the biomass was further ground to powder. Samples were pressed into spectroscopic quality KBr pellet in a sample/KBr ratio of 1/100 following (Mangaiyarkarasi *et al.*, 2010). The Fourier Transform Infrared (FTIR) spectroscopy [Perkin Elmer spectrum BX FTIR system (Beacon field Buckinghamshire HP9 1QA)] was used to record FTIR spectra of dried cells. Samples were analyzed within the range of 500-4000cm^{-1}. Transmission mode was operated for all the spectra, by using KBr disc method for obtaining the specific information related to functional groups attached to dry cells.

Statistical analysis: All experiments were performed in triplicate. Data were analyzed using SPSS 16 One-way ANOVA, Duncan test was applied (SPSS Inc.) and considered significant if the p value was ≤ 0.05.

RESULTS AND DISCUSSION

Heavy metal pollution poses a dangerous effect on human health and on other living beings. As heavy metals accumulate in the water bodies due to immense industrialization, may cause serious environmental hazards. Microbes and hydrophytes living in heavy metal stress environment, developed mechanisms to reduce highly toxic Cr(VI) to Cr(III) which was less harmful (He *et al.*, 2009). Hydrophytes have the ability to adapt and survive under extreme environmental pressures by balancing the heavy metals concentrations in rivers and lakes by converting the toxic heavy metals into less harmful substances or to accumulate within the cells (Bhakta, 2017). This is how living organisms grow in polluted ponds and is the rational of selecting *Chlorella* in the present work. Bacterial strains were isolated under different Cr(VI) concentrations and only seven were selected due to their high chromium resistivity and Cr(VI) removal. The MIC exhibited that bacterial strains SA3, SA4, S6 and S7 were capable of tolerating 600µg/mL of K_2CrO_4 stress in the growth medium. Among these strains S7 was the best Cr(VI) removal. The chromium resistant strains have been reported by Sen *et al*. (2014) to tolerate 15mg/mL concentration of K_2CrO_4 and 1.8mg/mL concentration of Cr(VI) by Naeem *et al*. (2013). The isolated bacterial strain S7 was gram negative rod and non-motile as mentioned by Naeem *et al.* (2010) which had same biochemical properties as strain S7. *Pseudomonas* sp. was first reported for the conversion of Cr(VI) to Cr(III) by (Mishra and Bharagava, 2016).

Specific nutritional and growth conditions were required for optimum growth of microorganisms to study the growth curves. The stationary phase for S7 strain was started almost after 72 hours. Strain S7 and *Chlorella* were analyzed under Cr(VI) stress and non-stress conditions. The growth of strain S7 was very effective and remained stable under stress conditions for reasonable duration. A study showed the reduction potential of *Chlorella* and revealed that it could grow unaffected at concentration of 45–100 ppm of Cr(VI) reported by (Mishra and Bharagava, 2016 and Kumar *et al.,* 2015). Under stress and non-stress conditions, S7 strain showed increased resistance with an increase in the optical densities. Heavy metal profile of S7 strain indicated maximum growth under lead (Pb^{+2}) stress upto 550 µg/mL concentration. Isolated strain S7 was resistant to chloramphenicol with concentration of 25µg /mL.

After biochemical and molecular characterization, the organism was revealed as *Pseudomonas* sp. designated as S7 (Figure-1). Molecular phylogenetic was used for classifying organisms according to their similarities and differences related to other organisms (Brown, 2002). Phylogenetic trees were very helpful to carry out the evolutionary relationship among species (Horiike, 2016).

The reduction potential of Cr(VI) was performed which it showed that Cr(VI) reduction started quickly by all the strains SA3, SA4, S6 and S7 during initial stages of growth. S7 bacterial strain showed reduction potential of 94.34% at 500 µg/mL after 120 hours and was analyzed by one-way ANOVA (Table.1). Statistical analyses indicated that time and reduction potential are in direct relationship i.e. with increase in time the reduction potential was also increased to significant level by S7 strains. The maximal removal of Cr(VI) and biomass growth by *Pseudomonas* sp was recorded during 21 hours of incubation reported by Jayalakshmi *et al.* (2013). Reduction potential for free and immobilized *Chlorella* and bacterial strains was estimated to study the progresses in remediation mechanisms. The cells could be entrapped in alginate and degraded the toxic substances effectively (Wasi *et al.,* 2013). In immobilized solid assemblies a material with right size, rigidity, mechanical strength and porosity was provided which was essential for metal accumulation. By comparing reduction potential of both immobilized and free cells it was concluded that bacterial strain S7 and *Chlorella* in immobilized state could reduce Cr(VI) more efficiently (Figure-2). The high resistance of substrates could be achieved by immobilization, whereas the use of immobilized cells has been reported for reduction of harmful substances (Wasi *et al.,* 2013).

Maximum reduction potential of Cr(VI) for S7 bacterial strain in bounded form was 87.17 % and for *Chlorella* with S7 was 96.16%. The chromium resistant bacterial strains S7 that presented proficient Cr(VI) reducing properties can be used for treating industrial effluents. Wasi *et al*. (2011a, b) also described the effectiveness of Cr(VI) removal by immobilized cells of *P. fluorescence* SM1 strain as compared to free cells.

Table 1. Cr(VI) reduction by S7 crude extract with respect to time.

Reduction potential (%) Mean ± SE	
Time (hrs)	Experimental with Cr(VI)
24	54.45±0.06[a]
48	67.17±0.02[b]
72	75.55±0.01[c]
96	89.02±0.03[d]
120_i	94.34±0.04[e]

with increase in time the Cr(VI) reduction increased to significant level with p value ≤0.05.

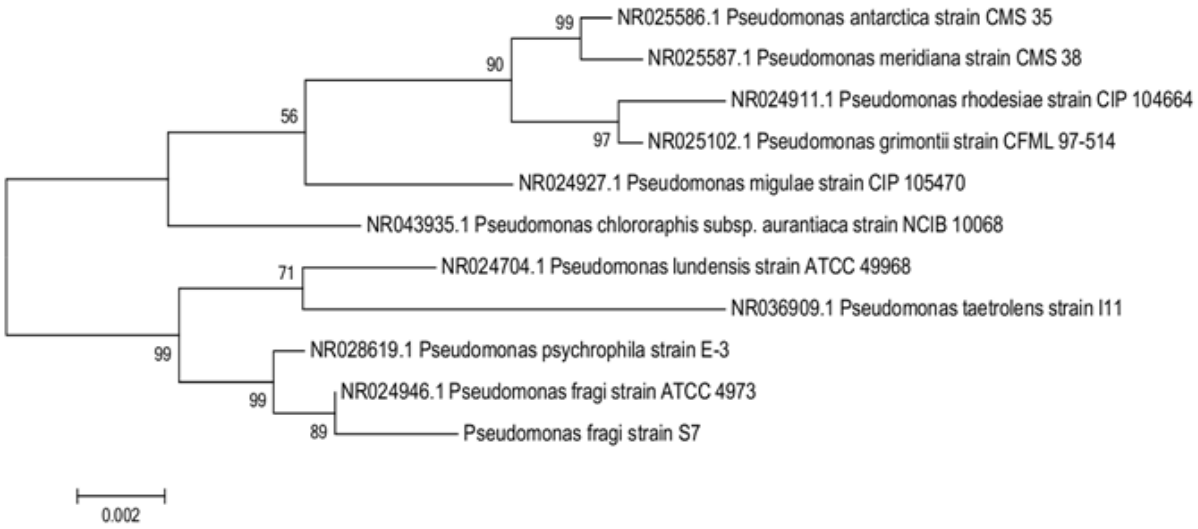
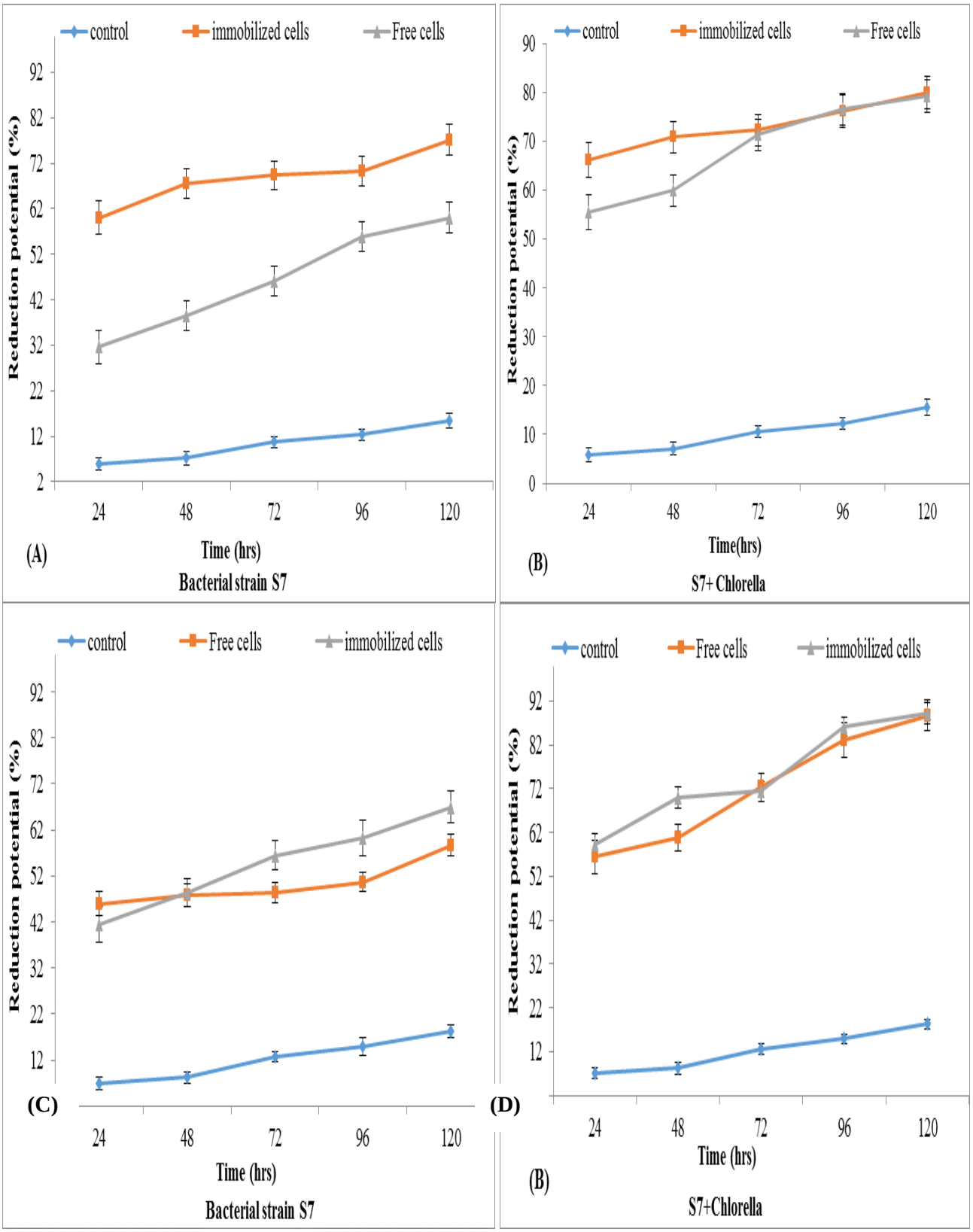


Figure-1: Molecular phylogenetic analysis by Neighbor joining method.


control
immobilized cells
Free cells
Reduction potential (%)
Time (hrs)
(A)
Bacterial strain S7
Time(hrs)
(B)
S7+ Chlorella
(C)
Bacterial strain S7
(D)
S7+Chlorella

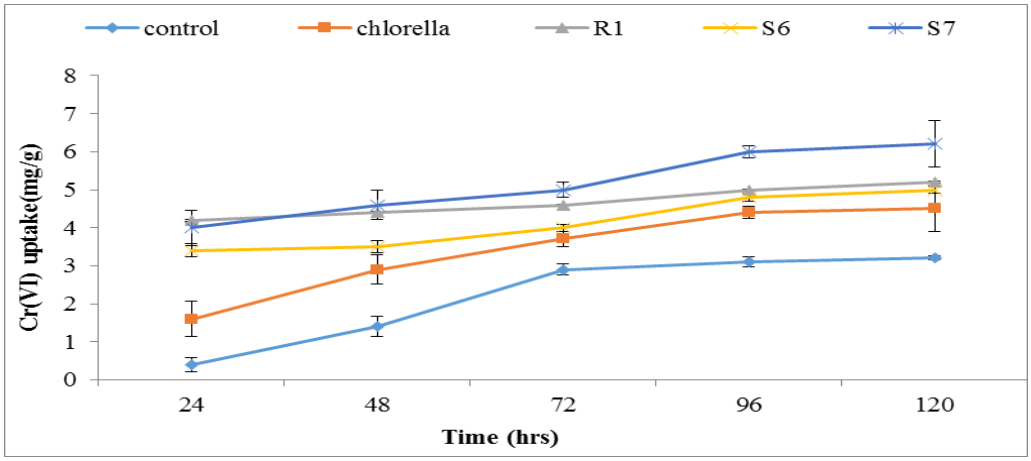


Figure-2: Cr(VI) reduction potential estimation using immobilized and free bacteria in sterile effluent (A) bacterial [illegible]7 (B) S7 with *Chlorella*. (C) bacterial strain S7 and (D) S7 with *Chlorella***.** (E) Cr(VI) uptake by immobil **(E)** mium resistant bacterial strains.

Hexavalent chromium uptake was assessed by acid digestion reported by Batool *et al.* (2012) of beads drawn at specific time intervals (24, 48, 72, 96, 120) form the Cr(VI) stress solution and tannery effluent (Figure-2E). The uptake was reduced when *Chlorella* and bacterial strain were used together. Some microbes like *Alcaligenes eutrophus, Pseudomonas aeruginosa, Enterobacter cloacae* and *Pseudomonas fluorescens* had lesser chromate ions toxicity reported by (Chakraborty *et al.*, 2017) that might be due to an effective efflux pump that helped them to export chromate ions into the extracellular environment, thus preventing cells from toxicity. The Cr(VI) uptake in *Chlorella* was observed by increase in root length under different concentrations (0, 350 and 500 µg/mL) Cr(VI) concentrations in solution that also contained other essential nutrients necessary for growth of *Chlorella*. A direct relationship was observed between Cr(VI) concentration and algal root length. The IR spectra for bacterial strain S7 identified as *Pseudomonas* sp. and *Chlorella* treated with Cr(VI) was done for surface analysis and localization of functional groups responsible for reduction of the $(CrO4)^{2-}$. Fourier transform infrared spectra in 500–4000 cm^{-1} range were examined so as to determine the functional groups responsible for the process of bio-sorption (Figure-3). This technique provided standard peaks through which the experimental data is compared. Different functional groups were associated with different wavelength ranges such as existence of glycerol and polysaccharides (750-600 cm^{-1}) reported by (Sun *et al.*, 2009) amine group (1550-1650 cm^{-1}) mentioned by (Wenning and Scherer, 2013), aromatic compounds (1500-1400 cm^{-1}) by (Zhu *et al*., 2012), presence of hydroxyl group (3600-2800cm^{-1}) by (Wang *et al.*, 2009) and alcohols and phenols (970-1250 cm^{-1}) by (Wei *et al.*, 2011). Different functional groups such as hydroxyl group, N-H and C-O groups were reported to be attached on surface of dried S7 bacterial cells.

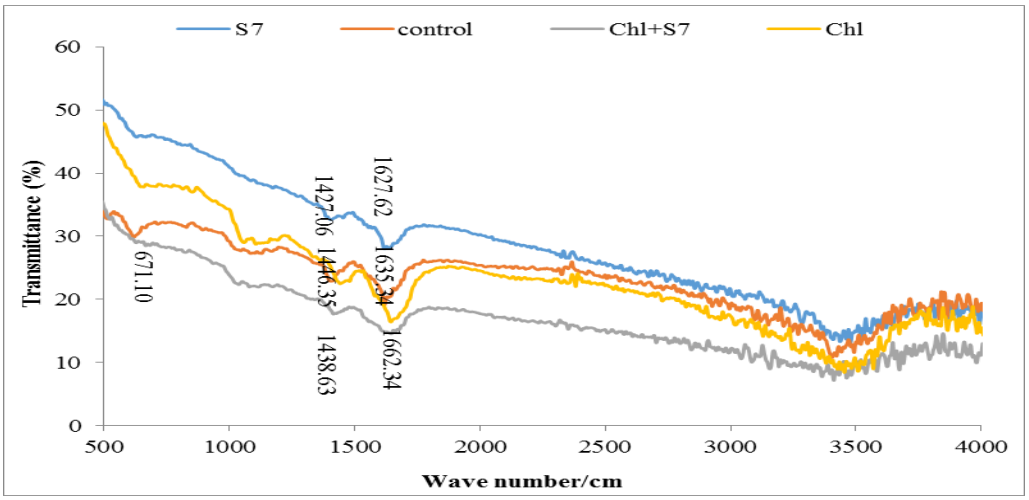


Figure-3: FTIR spectroscopy of isolated Cr(VI) resistant s7 bacterial strain and *Chlorella.*

Conclusion: The Cr(VI) resistant bacterial strain has shown to be highly significant to reduce hexavalent chromium to less toxic trivalent chromium. It also indicated that if used along with *Chlorella* had the ability for remediation of soils contaminated with hexavalent chromium.

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