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# Cr (VI) resistant *Bacillus* and *Acinetobacter* isolated from soil of Narran valley

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## Abstract

*Narran valley is famous for its beauty however anthropogenic activities are not only destroying the beauty of this valley but also lead to the pollution. Cr (VI) is considered as a major environment pollutant as it is mutagenic, carcinogenic and teratogenic. Current study deals with an attempt to know the Cr (VI) reduction potential of the indigenous bacterial isolates of soil of Narran valley. Total ten bacterial strains (JM1, JM5, JM6, JM7, JM8, JM9, JM10, J11, JM12, and JM13) were isolated from Narran valley soil. The morphological and biochemical characterization of selected strains were done. Maximum tolerable concentration of $K_2Cr_2O_4$ was found to be 300 $mgL^{-1}$ for all of these strains. These bacteria were found to have multiple metal resistance. These strains could efficiently convert hexavalent chromium into trivalent form (96-98%) at an initial concentration of 300 µg $mL^{-1}$ of Cr (VI). In comparison with other purified isolates, (JM8) exhibited highest Cr (VI) reduction potential at all the preliminary concentrations (100, 300 and 900 µg $mL^{-1}$). Best carbon and nitrogen sources for Cr (VI) reduction were sodium acetate and yeast extract, respectively. 16S rRNA gene sequencing revealed that JM9 and JM13 showed 99% similarity with genus Bacillus whereas JM8 was found to be homologous to genus Acinetobacter. FTIR study showed the contribution of sulphonate, carboxyl, amino and S-H groups of bacterial cell surface in the metal binding process. These chromium resistant bacterial isolates can be appropriate candidate for the remediation of chromate contaminated areas.*

**Keywords:** Bioremediation, heavy metal pollution, FTIR, Cr (VI) toxicity

## Introduction

Soil pollution with toxic heavy metals is considered as a major environmental issue. This is because of the waste that is released by industrial, mining, and anthropogenic activities (Mohanty and Patra, 2013). As, Soil works as a basin for toxic heavy metals like chromium, copper, mercury and lead where they stay for longer period disturbing the nutritional value of soil (Tipayno *et al*., 2018). Annually, 107 tons of Cr is produced around the world, from which 60–70% is used in alloys, such as stainless steel, and approximately 15% is used in chemical industrial processes, including electroplating, leather tanning, wood preservation, metal processing industries and textile dyeing. In Pakistan, the concentrations of Cr reported in soils of areas adjacent to tanneries is up-to 630 mg $kg^{-1}$ and nearly 2700 mg $kg^{-1}$ was observed in India (Rai *et al*., 2016). Hexavalent chromium being strong, immensely poisonous carcinogenic agent has been accounted numerous folds more toxic than trivalent chromium because it can promptly cross mammalian cells membranes (Kanmani *et al*., 2012). Cr (VI) is readily available to plant for uptake and drain into groundwater because of its weak adsorption into soil (Benhammou *et al*., 2007). Numerous physicochemical techniques are currently employed to remove chromate from contaminated sites which include ion exchange, chemical reduction, electrolysis, and reverse osmosis. The traditional management strategies utilized for chromate removal from waste waters are costly, leading to the formation of poisonous by-product, as well as not effective at higher preliminary amount of Cr (VI) (Sen and Dastidar, 2010; Jacob *et al*., 2018). Amongst the organisms that are naturally found on earth, microbes having distinctive capabilities for instance metal accumulation, assimilation, or resistance could be easily recognized (Mejáre and Bülow, 2001). Bioremediation and biotransformation technologies carried out by the help of microorganisms are quite inexpensive as they need low energy as well as also there would be no formation of secondary pollutant (Sultan and Hasnain, 2007). Metal precipitation is reported to occur when a microorganism oxidizes or reduces metal species. Chromium is one of the metals that can be converted to a precipitated form by microbial action involving the Cr (VI) reduction into Cr (III), that can be precipitated to form chromium oxides, sulfides, or phosphates (National Research Council, 1993). Reduction of chromate can take place aerobically as well as anaerobically, but rate of aerobic reduction is comparatively high. Aerobic Cr (VI) reduction is generally associated to miscible proteins, where electron donor is NADH and it can act either as a

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prerequisite or for enhancement of activity. Anaerobic Cr (VI) reduction is by the involvement of either a soluble reductase, or a membrane-linked reductase, or both (Opperman and Van Heerden, 2007). Variety of bacteria can bring about the transformation of toxic Cr (VI) which include *Exigobacterium*, *Shewanella*, *Pseudomonas*, *Bacillus*, *Ochrobacterum* and *Achromobacter* sp. (Batool *et al*., 2012).

Narran valley is a medium sized town located 119 kilometers in Mansehra city in the upper valley of Kaghan, Pakistan. This area is renowned for the cultivation of many fruits particularly pear, apricot, peach and plum. Due to increasing trend of plantation of orchards, gardeners started to using waste water for the irrigation leading to accumulation of toxic metals. Other sources of heavy metal contamination include extensive use of chemical fertilizers, pesticides and some natural phenomenon for example weathering and erosion (Khan *et al*., 2010). The current study describes the purification and characterization of the indigenous Cr (VI) resistant bacterial isolates from the Narran valley soil. So, these strains could be utilized for the reclamation of land polluted with heavy metals.

## Materials and Methods

### Purification and characterization of indigenous Cr (VI) resistant bacterial isolates

For the isolation of Cr (VI) resistant bacteria, soil sample was collected from Narran valley, Pakistan. For the isolation of bacterial strains, serial dilution method was used. Soil sample was spread on LB-agar plate with added $K_2Cr_2O_7$ salt at 100 μg $mL^{-1}$ concentration by using spread plate technique. Incubation was done at 37°C for 24-48 hours and the growth of bacterial isolates was observed. Ten colonies were chosen due to distinct morphology for purification. The selected bacterial strains were characterized morphologically (colony morphology), physiologically (Gram and spore staining, motility). Biochemical characterization included catalase, cytochrome oxidase, starch hydrolysis, citrate utilization, glucose fermentation, MR-VP, indole production, nitrate reduction, and indole production test (Gerhardt, 1994).

### Maximum tolerance concentration (MTC) and minimum inhibitory concentration (MIC)

MIC and MTC of bacterial isolates were performed by the dilution plate technique. Bacterial isolates were grown on LB-agar plates supplemented with variable concentration of $K_2Cr_2O_7$ and incubated at 37°C for 24-48 hours. The lowermost concentration of chromium metal that retarded the growth of bacterial isolates was recorded as MIC whereas the maximum concentration of Cr (VI) that has no effect on bacterial growth was determined as the MTC.

### Cross heavy metal resistance profile

For the determination of cross heavy metal resistance profile, five metals $Cu^{2+}$ ($CuSO_4$), $Co^{2+}$ ($CoCl_2$), $Zn^{2+}$ ($ZnSO_4$), $Ni^{2+}$ ($NiCl_2$), $Mn^{2+}$ ($MnSO_4$) (Riedel-de Haën, Seelze, Germany) were used and MIC of all heavy metals was estimated using dilution plate technique.

### Genetic analysis of bacterial strains

For ribotyping, the purified colonies were sent to Macrogen Inc. Seoul, Korea. Analysis of both bacterial isolates sequences was done with the help of the Ribosomal Database Project. Phylogenetic trees were formed by means of a neighbor-joining tree-building algorithm (Saitou and Nei, 1987). Evolutionary investigation was performed in MEGA5 software (Tamura *et al*., 2011).

### Cr (VI) reduction

For estimation of Cr (VI) reduction, DeLeo and Ehrlich medium was used (DeLeo and Ehrlich, 1994). Cr (VI) resistant bacteria were cultured in broth at an initial concentration of 300, 600 and 900 μg $mL^{-1}$ of Cr (VI) at 37°C and 150 rpm for interval of 48 hours. Supernatant from the centrifuged cultures (10,000 rpm for 5 mins) was taken for the estimation of residual Cr (VI) by Diphenylcarbazide method (Clesceri *et al*., 1998).

### Optimization of environmental conditions for Cr (VI) reduction

Reduction of Cr (VI) is greatly affected by the environmental conditions. In order, to optimize the conditions, effect of different growth pH (5, 7, 9), carbon (acetate, sodium gluconate, glucose, fructose, lactose) and nitrogen (yeast extract, beef extract, $KNO_3$, $NH_4Cl$) sources on reduction of Cr (VI) was observed. In this case, minimal broth with slight modifications at an initial Cr (VI) concentration (300 μg $mL^{-1}$) was used (Batool *et al*., 2012). Bacterial isolates were cultured in minimal broth under respective conditions at 37°C for 96 hours. Samples were taken out after specific time intervals and supernatant was used for the evaluation of Cr (VI) (Clesceri *et al*., 1998).

### Fourier transform infrared (FTIR) spectroscopic analysis

In order to analyze the role of various functional groups in Cr (VI) binding, Fourier Transform Infrared (FTIR) spectroscopy of strain JM9 was performed. For that purpose, strain was grown overnight in LB-broth with and without supplemented Cr (VI). After 24 hours, cultures were centrifuged, and pellet was obtained and dried at 60°C. Then, FTIR spectrum was determined by using a spectrophotometer within the range of 500-4000 $cm^{-1}$.

## Statistical analysis

All work was done in triplicate and data was statistically analyzed (Steel and Torrie, 1980).

## Results

### Purification and characterization of indigenous Cr (VI) resistant bacterial isolates

The soil samples collected from Narran valley, Pakistan showed average concentration of Cr i.e. 0.0546 g $kg^{-1}$. Ten morphologically distinct indigenous bacterial colonies (JM1, JM5, JM6, JM7, JM8, JM9, JM10, J11, JM12, and JM13) were isolated and purified under $K_2Cr_2O_7$ stress (100 µg $mL^{-1}$). Selected bacterial strains were characterized morphologically and biochemically. Most of the strains showed moderate colony size, creamy texture and slightly raised with irregular margins. All the isolated strains were Gram positive except (JM6, JM8) spore former (except JM1, JM5, JM6) Bacilli (except JM5; JM6). These strains exhibited positive activities for catalase, oxidase (except JM8), starch hydrolysis (except JM6, JM8, JM12, and JM13), citrate utilization, glucose fermentation (except JM8), nitrate reduction (excluding JM6, JM11) and negative for indole production tests. Minimum inhibitory concentration (MIC) for all the strains was 500 µg $mL^{-1}$ whereas maximum tolerable concentration (MTC) was 300 µg $mL^{-1}$.

### Cross heavy metal resistance profile

These selected bacterial strains showed multiple metal resistance ability. These strains exhibited maximum resistance against copper and nickel (800 µg/ml) as presented in table 1.

### Cr (VI) reduction

Ability of Cr (VI) reduction of isolated strains was estimated at three variable initial Cr (VI) concentrations (300, 600 and 900 µg $mL^{-1}$). Maximum reduction of Cr (VI) was shown by almost all strains 96- 98% at 300 µg $mL^{-1}$. At 600 µg $mL^{-1}$, highest removal was done by JM5, JM6, and JM10 (65.8, 62.7, 69.1%) whereas at 900 µg $mL^{-1}$, maximum reduction of Cr (VI) was exhibited by JM6, JM7, JM8, JM9, JM11 (48.6, 43.2, 45.4, 44.5, 42.8%) after 48 hours of incubation (Table 2).

### Optimization of environmental conditions for

**Table 1: Heavy metal resistance profiling of isolated Cr (VI) resistant bacteria**

| Bacterial Strains | Heavy metals used (µg $mL^{-1}$) | | | | | Level of resistance |
|---|---|---|---|---|---|---|
| | $CuSO_4$ | $CoCl_2$ | $ZnSO_4$ | $NiCl_2$ | $MnSO_4$ | |
| JM1 | 900 | 400 | 400 | 900 | 900 | $Cu^{2+}$=$Ni^{2+}$=$Mn^{2+}$>$Co^{2+}$=$Zn^{2+}$ |
| JM5 | 500 | 400 | 500 | 900 | 900 | $Ni^{2+}$= $Mn^{2+}$> $Cu^{2+}$=$Zn^{2+}$> $Co^{2+}$ |
| JM6 | 900 | 700 | 400 | 900 | 900 | $Cu^{2+}$=$Ni^{2+}$=$Mn^{2+}$> $Co^{2+}$> $Zn^{2+}$ |
| JM7 | 900 | 700 | 800 | 900 | 900 | $Cu^{2+}$=$Ni^{2+}$=$Mn^{2+}$> $Zn^{2+}$> $Co^{2+}$ |
| JM8 | 900 | 700 | 600 | 900 | 900 | $Cu^{2+}$=$Ni^{2+}$=$Mn^{2+}$> $Co^{2+}$> $Zn^{2+}$ |
| JM9 | 400 | 400 | 400 | 400 | 400 | $Cu^{2+}$=$Ni^{2+}$=$Mn^{2+}$=$Co^{2+}$=$Zn^{2+}$ |
| JM10 | 500 | 400 | 400 | 900 | 900 | $Ni^{2+}$=$Mn^{2+}$>$Cu^{2+}$>$Co^{2+}$=$Zn^{2+}$ |
| JM11 | 900 | 700 | 600 | 900 | 900 | $Cu^{2+}$=$Ni^{2+}$=$Mn^{2+}$> $Co^{2+}$> $Zn^{2+}$ |
| JM12 | 500 | 400 | 400 | 900 | 900 | $Ni^{2+}$=$Mn^{2+}$>$Cu^{2+}$>$Co^{2+}$=$Zn^{2+}$ |
| JM13 | 400 | 700 | 400 | 900 | 900 | $Ni^{2+}$=$Mn^{2+}$>$Co^{2+}$>$Cu^{2+}$=$Zn^{2+}$ |

**Table 2: Cr (VI) reduction potential by selected bacterial strains at variable initial Cr (VI) concentration (µg/ml)**

| Bacterial Strains | Cr (VI) reduction potential (%) | | |
|---|---|---|---|
| | 300 | 600 | 900 |
| JM1 | 97.86±0.37 | 47.9±0.47 | 41.1±0.37 |
| JM5 | 97±0.43 | 65.8±0.33 | 41.4±0.47 |
| JM6 | 96.9±0.33 | 62.7±0.51 | 48.6±0.33 |
| JM7 | 98.22±0.35 | 59.8±0.23 | 43.2±0.51 |
| JM8 | 96.52±0.48 | 50.18±0.42 | 45.2±0.23 |
| JM9 | 96.9±0.39 | 49.75±0.33 | 44.5±0.42 |
| JM10 | 96.5±0.23 | 69.1±0.48 | 40.9±0.33 |
| JM11 | 97.16±0.51 | 49.8±0.39 | 42.8±0.48 |
| JM12 | 97.25±0.33 | 50.9±0.29 | 39.5±0.39 |
| JM13 | 96.8±0.47 | 57.37±0.37 | 40.6±0.29 |

Mean of three replicates ± standard error of the mean.

## Cr (VI) reduction

Cr (VI) reduction was determined at variable growth temperatures i.e., 28, 37 and 42°C at 300 µg $mL^{-1}$ as a preliminary Cr (VI) concentration. It was found that all selected bacterial strains showed maximum Cr (VI) reduction at 37°C whereas lowest reduction at 42°C (Figure 1 A). The impact of different pH levels on the Cr (VI) reduction was observed at pH 5, 7 and 9 with a preliminary Cr (VI) concentration of 300 µg $mL^{-1}$. All the strains showed maximum chromium reduction (67.3 - 98.2%) at pH 9 whereas lowest removal (39.4 - 73.5%) was found at pH 5 after 48 hours (Figure 1 B). Five different carbon sources i.e. acetate, gluconate, glucose, fructose and lactose were used to observe their effect on Cr (VI) reduction. Maximum chromium reduction (49.3 - 74.7%) was shown by using acetate (52.9 -79.3%) as carbon source whereas all the strains showed lowest removal (22.7 - 43.1%) when lactose

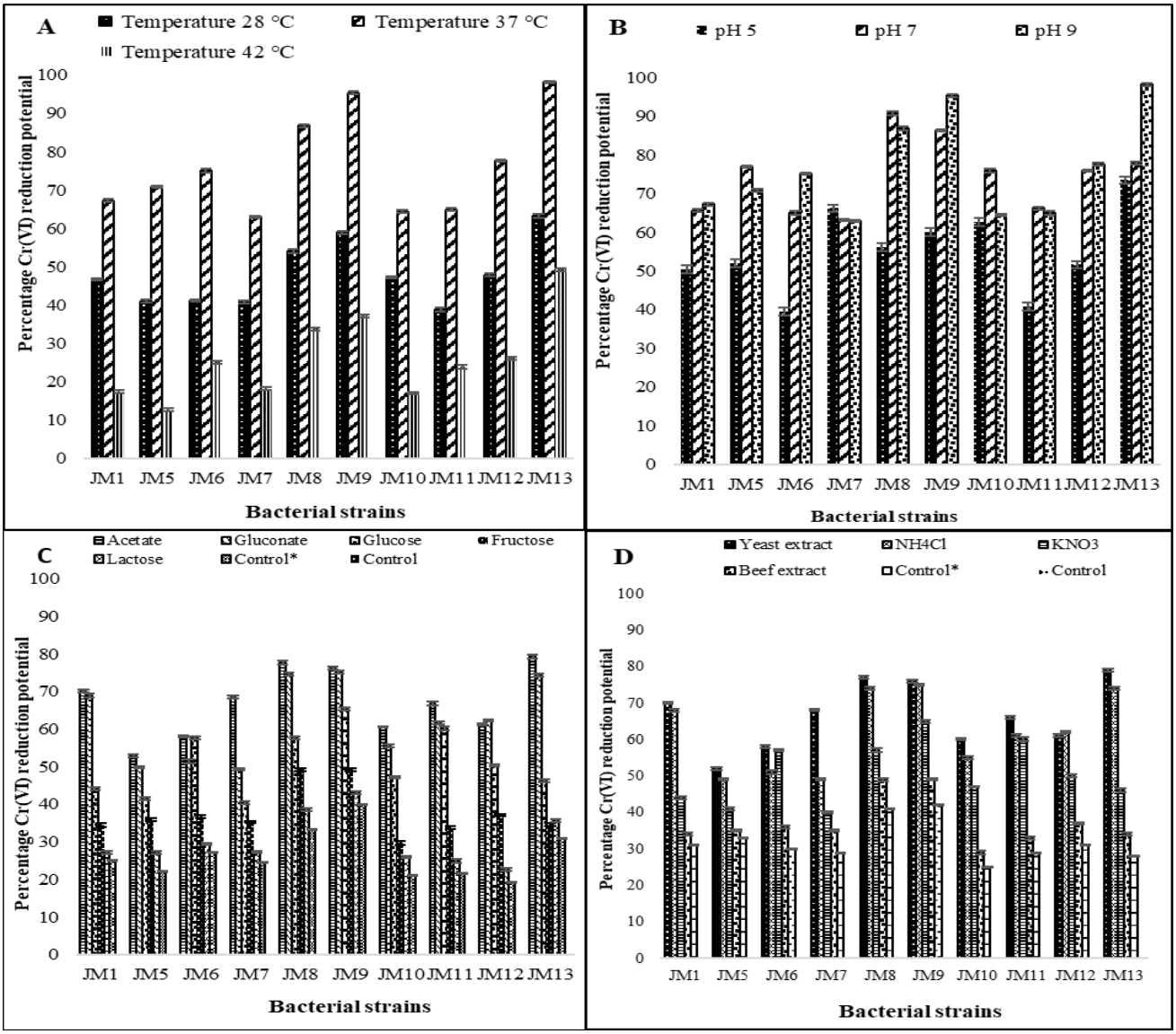


**Figure 1: Cr (VI) reduction potential (%) of bacterial isolates at different environmental conditions. Control* represents media without respective carbon source. Control represents un-inoculated treatment. Mean of 3 replicates ± standard error of the mean. A: temperatures (28, 37 and 42°C), B: pH (5, 7 and 9), C: carbon sources (acetate, sodium gluconate, glucose, fructose and lactose) and D: nitrogen sources (yeast extract, beef extract, $KNO_3$, $NH_4Cl$).**

was supplemented as carbon source, after 48 hours (Figure 1 C). Yeast extract was found to be best nitrogen source as all the strains showed maximum chromium reduction (52 - 79%) while beef extract caused lowest reduction (29 - 49%) of Cr (VI) after 48 hours of incubation at an initial Cr (VI) concentration of 300 μg $mL^{-1}$ (Figure 1 D).

## Genetic analysis of bacterial strains

Ribotyping was used to identify the three Cr (VI) resistant bacterial strains JM8, JM9 and JM13. These strains were selected because of their significant Cr (VI) reducing

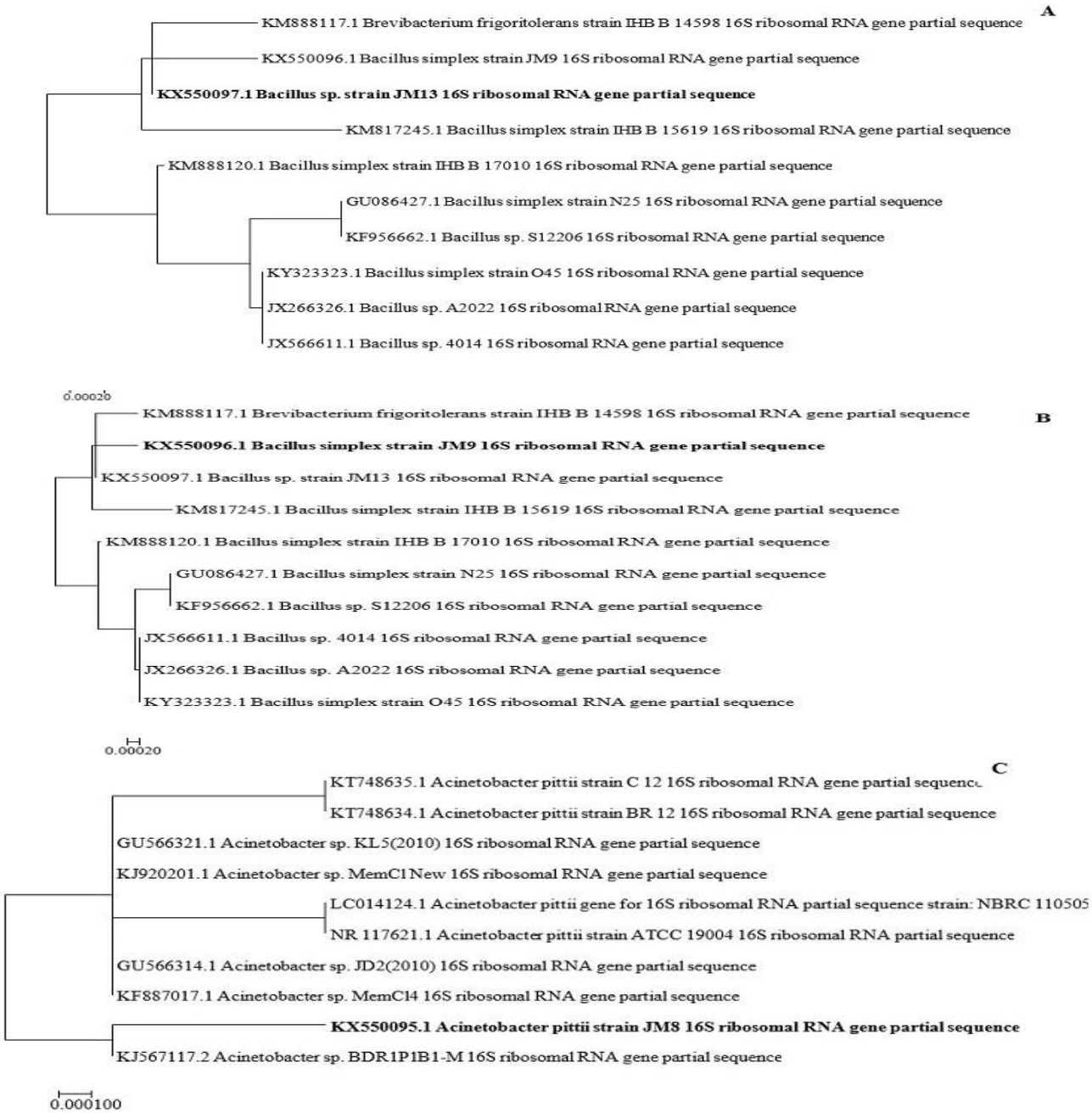


**Figure 2: Phylogenetic analysis by Neighbor-Joining method. Evolutionary analysis was carried out by MEGA5 (Tamura *et al.*, 2011). A: Strain JM13; B: Strain JM9; C: Strain JM8.**

properties. Blast analysis revealed that bacterial strains JM9 (KX550097) and JM13 (KX550096) exhibited 99% similarity with Genus *Bacillus* however JM8 (KX550095) was found to be homologous to Genus *Acinetobacter pitti* (Figure 2).

## Fourier transform infrared (FTIR) spectroscopic analysis

Fourier Transform Infrared (FTIR) spectroscopy describes the role of various functional groups in binding with chromate ions. For this purpose, FTIR spectrum of bacterial strain JM9 which exhibited excellent chromate reduction grown with and without Cr (VI) stress was determined in the range of 550- 4000 $cm^{-1}$. FTIR spectrum of cells of JM9 without Cr (VI) stress, showed numerous prominent absorption peaks revealing the complex nature of biomass. The absorption peaks in the region of 500-1000 $cm^{-1}$ indicated the presence of S = O, -C-C- and C-Cl, 1200-1400 $cm^{-1}$ showed sulphonate and carboxyl groups.

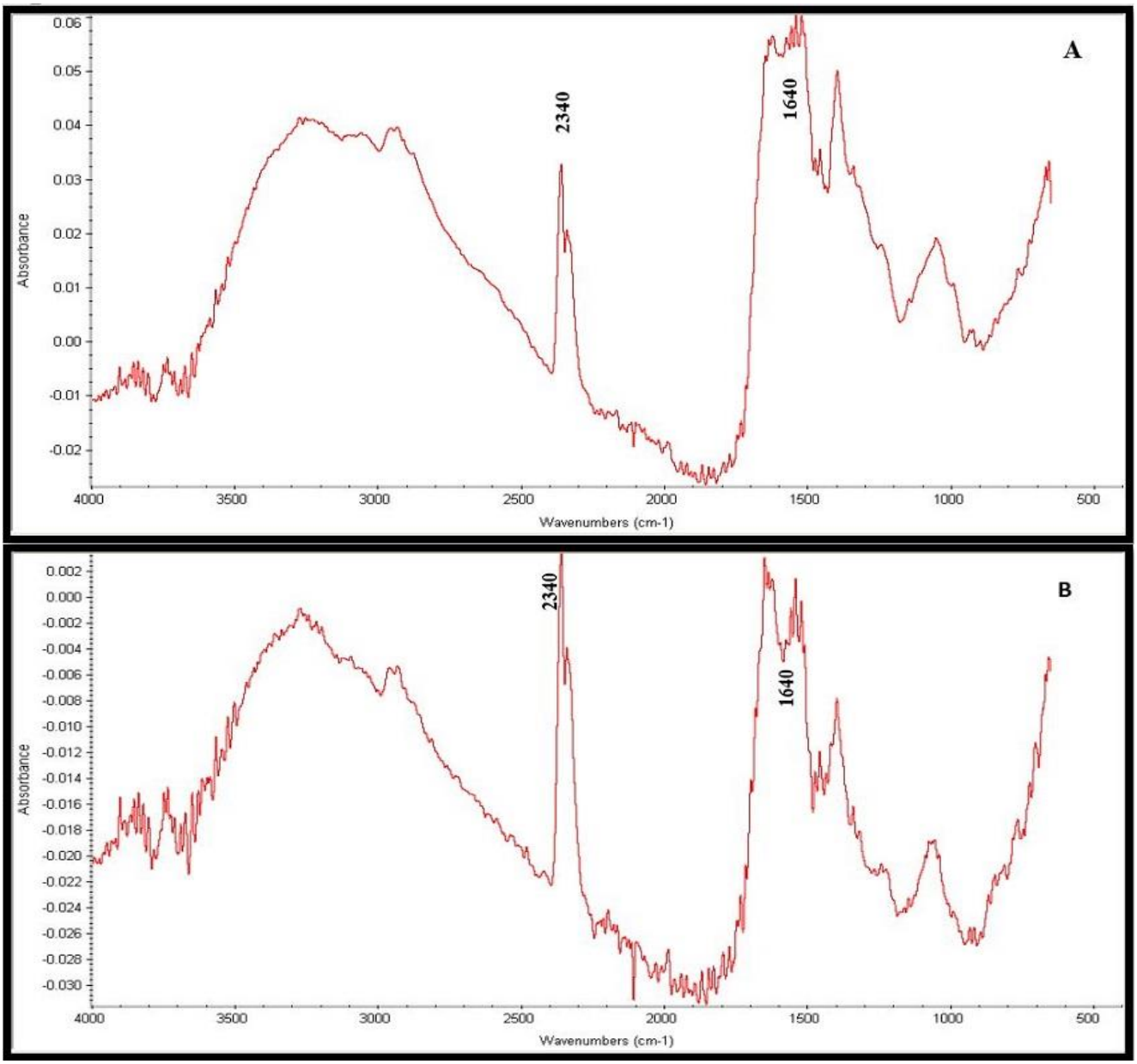


**Figure 3: FTIR analysis of bacterial strain JM9 grown A: with Cr (VI) stress B: without Cr (VI) stress.**

Absorption peaks at 1500-1640; 1640-1690 and 2300-2400 revealed the presence of primary and secondary amines, amides and amines, respectively. Characteristic absorption peak around 2100 $cm^{-1}$ was due to CC triple bond stretch. S-H groups showed the absorption peak in the region of 2500-2600 $cm^{-1}$. The absorption peaks in the region of 2500-3000 $cm^{-1}$; 3200-3500 $cm^{-1}$ and 3500-4000 $cm^{-1}$ corresponded to carboxylic group, OH and NH groups and OH- symmetric stretch vibration. In the FTIR spectra of cells of bacterial strain JM9 in the presence of Cr (VI) stress, shifts were detected in the absorption peaks at various regions. Main variations were found in the region of 1640 and 2340 $cm^{-1}$ indicating the involvement of primary and secondary amines and O–H (Carboxylic acids) group in the metal binding process (Figure 3 A, B).

## Discussion

In the current study, three bacterial strains from the soil of Narran valley were recognized as *Bacillus* (JM9; JM13) and *Acinetobacter pitti* (JM8). These strains could remove significant amount of Cr (VI). Isolation of strains was done from a site that is not significantly reported to be polluted with chromium because of anthropogenic activities. Microbes can be considered as a suitable candidate for the remediation of chromium polluted environment because of their ability to carry out chromate reduction and tolerance (Camargo *et al*., 2003). Utilization of metal resistant bacteria for the remediation of chromium polluted environment had been previously reported (Sultan, and Hasnain, 2007; Batool *et al*., 2012).

Chromium is reported as one of the toxic metals towards living organisms. Increase in initial Cr (VI) concentration cause alteration in cellular and morphological characters leading to less microbial growth (Upadhyay *et al*., 2017). The soil sample contained 0.054 g $kg^{-1}$ Cr concentration and similar results were reported by Khan *et al*., (2016). Present findings showed the decrease in the percentage of chromate reduction with a rise in Cr (VI) concentration that can be related to decrease in rate of bacterial growth. These results are in agreement with the reports of former investigators (Ghalib *et al*., 2014; Upadhyay *et al*., 2017). Chromate reduction is greatly dependent upon the environmental conditions as these environmental factors will determine the rate of bacterial growth. Impact of different environmental factors on Cr (VI) reduction capacity of the isolated strains was studied. Most appropriate temperature and pH for significant chromate reduction was 37˚C and 9. Previously, optimal chromate reduction by *Bacillus* sp. strain KSUCr5 at 37˚C and 9 was also reported (Ibrahim *et al*., 2011). Metal resistant bacteria may use various compounds as electron donors (Liu *et al*., 2004). All the selected strains preferred acetate to be used as carbon source. In the previous study, *P. aeruginosa*, *B. circulans* and *B. coagulans* are reported to choose acetate as electron donor. *B. coagulans* preferred to use the intermediate products such as acetate formed during the Krebs's cycle as compared to glucose which need catabolization into pyruvate before entering the Krebs's cycle (Zakaria *et al*., 2007). Two species of *Bacillus* were reported to show increased chromate reduction when acetate was supplemented as the carbon source (Desai *et al*., 2008). Cr (VI) resistant microbes transformed the hexavalent chromium into trivalent chromium by a reduction reaction, which involved the transmission of electrons to Cr (VI). The carbon source performed the role of an electron donor during this procedure, hence microbes exhibited higher Cr (VI) reduction (Das *et al*., 2014). Among the nitrogen sources tested, yeast extract was found to be best. Yeast extract was described as one of the best nitrogen sources for the Cr (VI) reduction by *Aspergillus* FK1 strain (Srivastava and Thakur, 2006).

As contaminated environments are loaded with a variety of other toxic compounds, so these selected strains were found to have multiple metal resistances. Previously chromate resistant bacteria with tolerance to other heavy metals have already been reported (Sultan and Hasnain 2005; Sayel *et al*., 2012). Due to multiple metal resistance capability, these bacteria can survive under harsh polluted environments and can bring about the remediation of toxic chromium compounds (Faisal and Hasnain, 2004). FTIR analysis was done to study the role of functional groups in sequestration of Cr (VI). FTIR analysis of the bacterial cells grown with and without chromium stress specified the existence of sulphonate, carboxyl, amino and S-H groups. These functional groups can be ionized and bind with Cr (VI) ions (Bueno *et al*., 2008). Major shift under stress condition showed the involvement of carboxyl group in metal binding process. In cyanobacteria, binding of chromium ions with protein molecules had been previously reported (Pandi *et al*., 2009).

## Conclusion

Present findings revealed that these indigenous chromium resistant *Bacillus* and *Acinetobacter* isolated from Narran soil can be used for the remediation of metal polluted sites. These chromium resistant bacterial isolates can be appropriate candidate for the remediation of chromate contaminated areas.

## Acknowledgements

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