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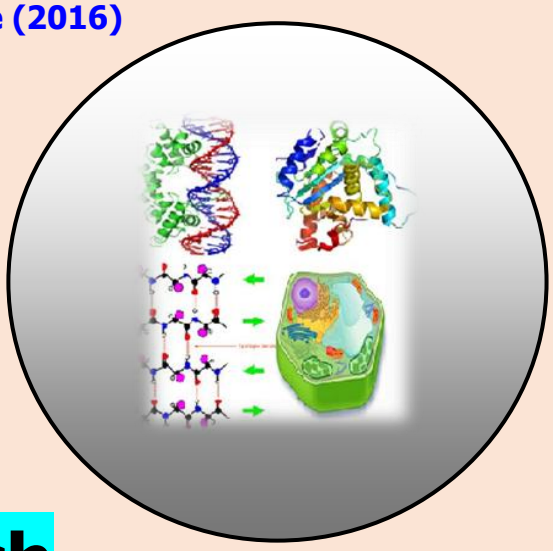

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RESEARCH PAPER

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Chromium Reduction by *Acinetobacter pittii* Isolated from Chromite Mine Area of Pauni Region of Maharashtra, India

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ABSTRACT

Ability to reduce chromium by the bacterium isolated from the chromite mine located in the Pauni region of Bhandara district of Maharashtra state of India was carried out in the laboratory conditions. Based on the biochemical, morphological and 16S rRNA sequence information isolate was identified as Acinetobacter pittii strain NA+LB. Firstly A. pittii (NA+LB)was tested at the tolerance level with chromium added in the medium ranging from (100, 300, 500, 700, 900, 1100, and 1300 ppm concentration). Later on, its reducing ability was checked at the concentrations viz., 100, 300, 500 and 700 ppm of Cr (VI). In a result, firstly maximum tolerance level of Cr (V) by A. pittii was found to be 1100 ppm and up to 700 ppm it was able to reduce it. As per SEM and EDX analysis results, chromium reaction with A. Pittii (NA+LB) showcased defined morphological changes in cell structure and possibly because of accumulated chromium on to its surface and hence we represent another promising isolate possibly be useful in Cr bioremediation in coming time in number of industry especially in waste management sector.

Key Words: Chromium tolerant bacterium, Acinetobacter pittii, Chromium reduction, SEM-EDX analysis, bioremediation.

INTRODUCTION

World remains affected by the metal pollution and especially heavy metals represents the major danger which can lead to number of diseases (Marilena and Castanas, 2008).Similar to other metals, Chromium (Cr) is naturally occurring element present in earth's crust and mined as chromite. In nature, it represents different oxidation states and the most stable are hexavalent and trivalent states. Trivalent chromium is less toxic, remains in trace amount and required by plants and animals (Chowdhury et al., 2003). Where Cr (VI) and its compounds are mutagenic as well as carcinogenic in nature (Langard, 1982; Costa and Catherine, 2006). Due to its soluble nature, hexavalent chromium generally persists in water for a longer period of time and that is the reason, why it gets easily spread in the environment and has adverse effects on plants, animals, and microbial flora. Since chromium is an industrially important metal and majorly used in industries for manufacturing of stainless steel, wood preservation, dyes etc. it can easily reach to the environment (Lunk, 2015).

Environment has a great impact on the quality of every creature. Here problems arise due to polluted environment which is not only experienced by plants and animals, but it also affects the microbial habitat. As

microorganisms are ubiquitous and abundant in biosphere, they are always getting exposed to different kinds of environments. In one of the aspects, interaction of metals with microbes certainly affects their growth and development (Bharagava and Mishra, 2015; Shi et al., 2002; Fernando etal., 1977). Soil is the main reservoir for pollutants and trace elements discharged through various anthropogenic activities. Prolong exposure of heavy metals to the microbesis the main cause for the development of resistant strains by bringing number of genetic changes (Clausen, 2000).Microbes, especially bacteria developed various mechanisms like oxidation, adsorption, accumulation, uptake, and reduction to adjust themselves in these unfavorable conditions (Carlo et al., 2014; Nezha et al., 2015; Das et al., 2015).

Among number of metals, ability to tolerate the chromium presence and its further reduction was recorded to be prominent in different bacterial genus reported till date viz., *Pseudomonas spp* (Konovalova et al., 2003; Garbisu et al., 1998; Ishibashi et al., 1990), *Enterobacter spp*, (Wang et al., 1990), *Escherichia coli*(Shen and Wang 1993), *Bacillus spp* (Chaturvedi, 2012) *Ochrobactrum intermedium* (Batool et al., 2012), *Lysinibacillus spp,*(Montenegro et al., 2015; Kipkurui et al., 2016,*Acinetobacter spp*., (Méndez et al., 2017), *Microbacterium spp* (Panneerselvam et al., 2013; Sarkar et al., 2016)and *Rhodococcus* spp (Banerjee et al., 2017).It is also understood that chromium reduction and resistance are independent processes among different species (Verma et al., 2009).Here microbial chromium reduction was first reported in *Pseudomonas* speciesby Romanenko and Koren' Kov (1977).

Current study focused on the chromium tolerant bacterium isolation from chromite mine area of Bhandara district, Maharashtra, India which has been further evaluated for chromium reducing ability to be the promising agents in future chromium bioremediation.

MATERIALS AND METHODS

Soil Sampling

As per the report of Directorate of Geology and Mining, Government of Maharashtra Nagpur, chromite reserves from 20°47'00" N, 79°39'00" E at Pauni, Bhandara district was selected for sampling. Soil Samples were subjected to X- ray fluorescence analysis at Mineral testing Lab, Indian Bureau of Mines, Nagpur for determination of chromium concentration in the given soil. In addition, electrical conductivity, organic carbon content, total nitrogen (Kjeldhal method), phosphorus and potassium were also subjected to investigation.

Isolation of Chromium Tolerant Strains

1g chromium rich soil samples from the given site was serially diluted and processed further by enrichment culture technique followed by plating of every dilution. Luria Bertani medium before adding the diluent of soil inoculum was supplemented with 50 ppm of potassium dichromate ($K_2Cr_2O_7$) and all plates were incubated at 37°C ± 2 for 24-48 hrs. The plates were then examined for morphologically distinct colonies and sub cultured to maintain the isolates and identification carried out using conventional biochemical tests (Cappuccino and Sherman, 1987) and by16S rRNA sequencing.

Evaluation of Chromium Tolerance:

Ability of chromium tolerant bacteria was evaluated when 1 O.D. fresh culture of the isolate was inoculated in 30 ml of LB Broth containing different concentration of potassium dichromate ($K_2Cr_2O_7$) ranging from 100-1300 ppm and allowed to incubate at 37ºC ± 2 for 24-48 hrs. The growth of bacteria was determined by measuring the optical density on spectrophotometer at 600 nm.

Molecular Characterization

Total DNA isolation and extraction from bacterial cells for PCR analysis was done by Genomic CTAB protocol. PCR amplification of the 16S rRNA gene fragment was done by using 27forward (AGAGTTTGATCMTGGCTCAG) and1492reverse (ACGGYTACCTTGTTACGACTT) primers. The amplification mixture contains of 32.0 µl nuclease free water, 5.0 µl PCR buffer 10x, 2.0 µl dNTP (10 mM), 4.0 µl forward primer (10 µM), 4.0 µl reverse primer (10 µM), 1.0µl Taq DNA polymerase enzyme (1U/ µl) and 200ng DNA template. PCR reaction was programmed as: Initial denaturation of 3 min. at 94 ºC, denaturation of 1 min. at 94 ºC, primer annealing for 1 min. at 54 ºC, extension of 2 min. at 72 °C, final extension for 5 min. at 72 °C; total 30 cycles and stored at 4 °C. Amplicon was sequenced and analyzed by BLAST to find the best scored close homolog and further accession number of isolate was obtained through NCBI genbank database. In a phylogenetic analysis, top five best homologs werealigned in CLUSTALW and later on designed for phylogram in MEGA5 software.

SEM-EDX Analysis

To study the effect of chromium on bacterial cell surface SEM-EDX analysis were carried out. The cells were grown with and without Cr (VI) and then washed with 0.1 M phosphate buffer saline and fixed overnight in 2% gluteraldehyde followed by rinsing in distilled water. Later it was dehydrated in a series of ethanol concentrations (20%, 40%, 60%, 80%, 95% and 100%), dried and kept in a desiccator until the use. The samples were mounted on brass-stubs and sputter coated with gold and then examined by Scanning Electron Microscope equipped with an Energy Dispersive X-ray spectrophotometer (EDX) from Dept. of Metallurgy, Vishweshwarya National Institute of Technology, Nagpur, Maharashtra.

Reduction of Cr (VI)

Chromium reduction ability was analyzed by 1, 5-diphenylcarbazide method by measuring the decrease in the concentration of hexavalent chromium in supernatant. The LB broth containing different concentrations of Cr (VI) in the form of $K_2Cr_2O_7$ (100 to 700 mg/L) was inoculated with 1ml of O.D culture and incubated under 37°C for 120 hours. Un-inoculated medium containing Cr (VI) used as a control. Samples were collected at different time intervals (24, 48, 72, 96, and 120 hrs.) and centrifuged at 10,000 rpm for 10 min. The supernatant obtained after centrifugation was used to measure Cr (VI) concentration. The Cr (VI) was determined by 1, 5-diphenylcarbazide method by measuring absorbance of the purple complex of Cr (VI) at 540nm using spectrophotometer (Zahoorand Rehman 2009).

Results and Discussion

Soil Characteristics:

As per laboratory testing Pauni soil sample was having electronic conductivity as 0.21deci/m, organic carbon content 0.50%, total nitrogen 100 Kg/ha, phosphorus 65.69 kg/ha, potassium 40.32 kg/ha. As per XRF analysis about 23.82 % of chromium was recorded in the Pauni soil sample.

Morphological and Biochemical characterization:

As per dilution and enrichment method very few isolates were retrieved from the Pauni soil samples when allowed to grow in presence of chromium containing medium and isolate NA+LB was able to survive with continuous sub culturing in the given medium having stress of the chromium. Morphological and biochemical features of the Isolate NA+LB are showcased in Table 1.

Table No. 1. Morphological and Biochemical Characteristics Isolate.

Sr. No	Tests	NA+LB
1	**Gram Staining**	Gram Negative
2	**Motility test**	Non-motile
3	**Catalase test**	Positive
4	**Oxidase Test**	Negative
5	**Indole Test**	Positive
6	**Methyl Red Test**	Negative
7	**Voges Proskauer Test**	Negative
8	**Citrate Utilization Test**	Positive
9	**H_2S Production Test**	Positive
10	**Sugar FermentationDextrose**	Positive
11	**Sucrose**	Positive
12	**Mannitol**	Negative
13	**Maltose**	Positive

Evaluation of Chromium Tolerance

Isolate NA + LB was selected on the basis of their ability to grow and tolerate variable concentrations of chromium (100, 300, 500, 700, 900, 1100, 1300ppm) in LB broth medium and recorded the tolerance at different time intervals. The bacterial growth was recorded by measuring the optical density at 600 nm at 24 hours' time intervals using a spectrophotometer. Growth response of isolate NA+LB to different concentrations of chromium up to 120 hours showed in Figure 1.

In chromium tolerance study it was observed that as the concentration of chromium increases bacterial growth rate decreased significantly. At the initial 24 hour, expected lag phase was observed and then it entered into the log phase up to 120 hours which was the last recording. Isolate NA+LB found to be promising in growth up to 1100 ppm concentration as in Fig. 1.

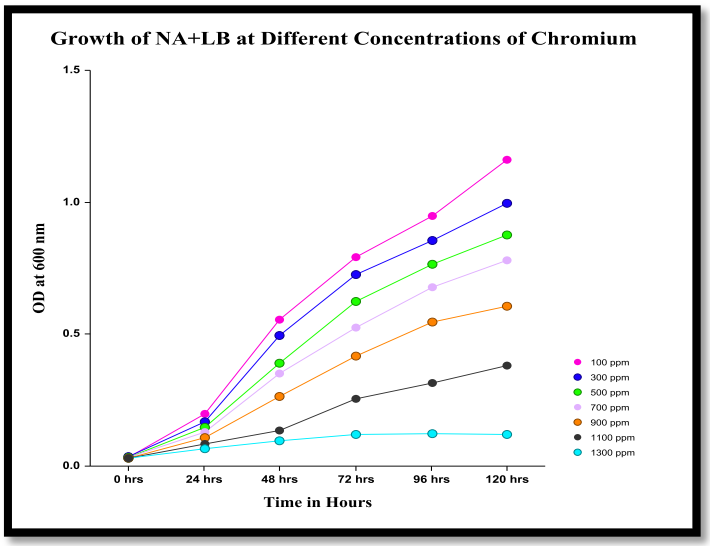


Figure 1. Growth response of isolate NA+LB to differentconcentrations of Chromium with respect to time period.

Identification of Isolate

Isolate NA+LB which was appeared as creamish, slightly dull yellow color colony with irregular margins on Luria Bertani medium was identified by targeting 16S rRNA gene sequence and found to be better homolog with *Acinetobacter pittii* (NR_117621.1) with 83% identity of 96% conserved sequence as per BLASTN and phylogram designed with five top scorer sequences was also highlighting the same output as shown in Figure 2. The NA+LB sequence was further submitted to Gen Bank NCBI and assigned with accession numberLC155825.

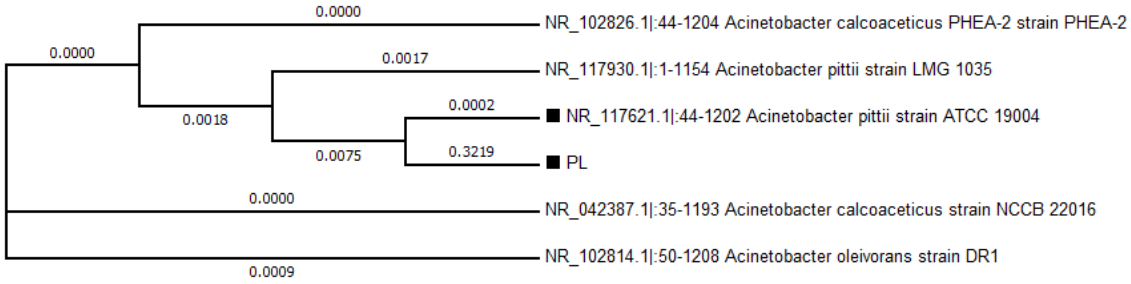
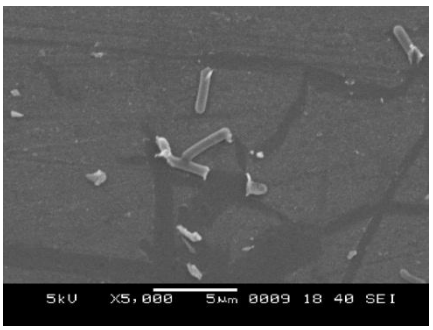
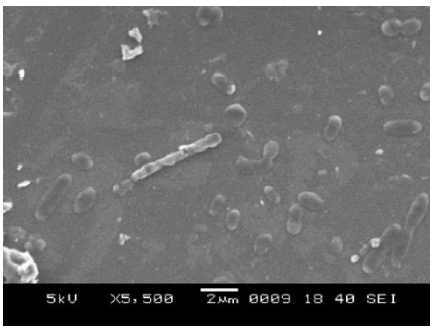


Figure No. 2. Phylogenetic Tree of NA+LB i.e. PL

SEM-EDX Analysis



(a)



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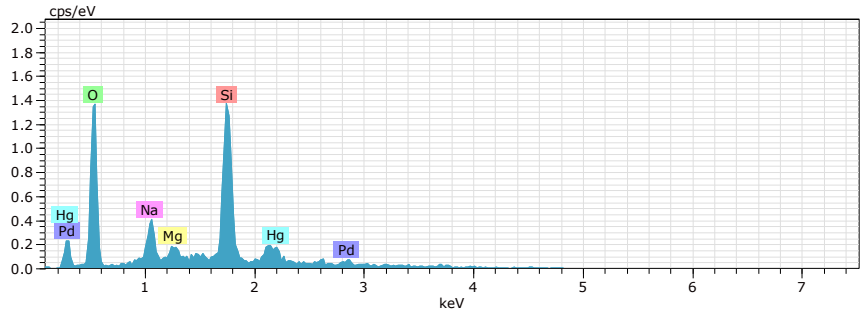

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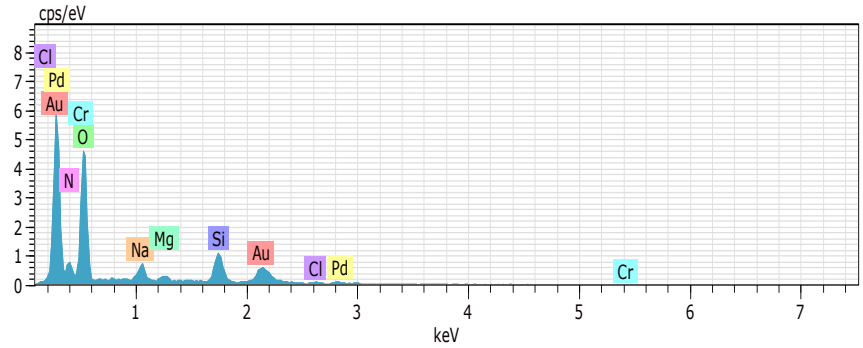

(d)

Figure 3. a) SEM in absence of Chromium, (b) SEM in Presence of Chromium, (c) EDS in absence of Chromium, (d) EDS in Presence of Chromium of *Acinetobacter pittii* (NA+LB).

As per SEM analysis, *Acinetobacter pittii*(NA+LB) exposed to 100 ppm chromium when tested for the cell response, result revealed that NA+LB accumulated the chromium content onto the cell surface and resultant cell morphology brought about a rough appearance and in number of cells swelling also been observed as in Figure 3 a and b. In an evidence by performing the EDX analysis of the same set of isolates exposed to chromium (1100 ppm), prominent presence of the chromium onto the cell surface was evident which was not there in control set as in Figure 3 b and c. Similar kind of analysis were performed to study the effects of cadmium and nickel (Bhagat et al., 2016; Kazy et al., 2006; Yang et al. (2017), in case of bio removal of copper from CMP waste water and in case of chromium removal reported with *Acinetobacter haemolyticus* by Pei et al., (2009).

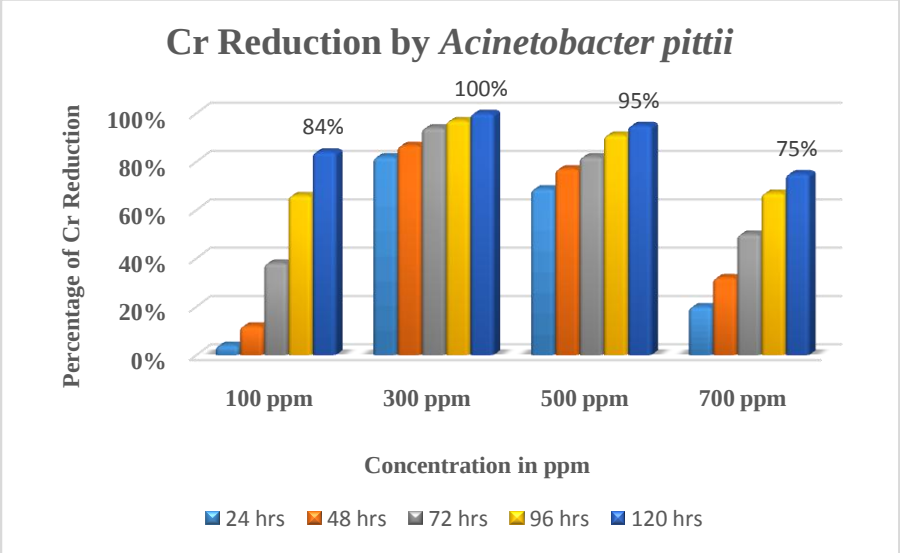

Figure 4. Chromium (VI) reduction at different concentrations and time by *Acinetobacter pittii.*

Chromium Reduction Assay

In the percent reduction assay of chromium by the *A. pittii*carried out by Diphenyl Carbazide method, result highlighted that at 100 ppm concentration of chromium probably with more dilution factor,it has affected the interaction of the isolate with the content of chromium and resultant even though concentration was low only 80% of maximum reduction was recorded. In case of 300 ppm it has been observed that 100% Cr reduction was observed in presence of *A. pittii*(NA+LB) and which was 95% at 500ppm and lowered down again to 75% at 700 ppm as in Fig. 4.

An interesting fact observed that, bacteria can tolerate up to 1100 ppm but can reduce only 700 ppm conc. of chromium. Thus, it could be said that Cr (VI) reduction capability of *A. pitti i*(NA+LB) was not related to Cr (VI) tolerance ability. Similar results were observed by Dongyanet al., 2013.*Acinetobacter* is wider group of bacteria; some members of these genera have been reported for their chromium tolerance as well as reduction ability. Bhattacharya et al. and others shown efficiency of *Acinetobacter sp. B9* in bio removal of chromium (Pei et al., 2009; Bhattacharya et al., 2014; Essahale et al., 2012; Pandaand Sarkar, 2012). Pattnaik et al.(2017) and Šipošováa, et al., (2017) reported Acinetobacter spp. tolerating up to 2500 ppm conc. of chromium. In this study we have reported *Acinetobacter spp.* tolerating chromium conc up to 1100 ppm.

Bacteria from such metal enriched area recorded to have unique abilities which may be useful in the field of bioremediation. In this study isolated *Acinetobacter pittii*from chromite mine area certainly been useful to control chromium pollution by restricting the spread in the region where that metal is found to be above tolerance level. This is the first report of isolation of bacterium capable of chromite reduction and bioaccumulation from chromite mine area of Pauni region of Bhandara districts of Maharashtra, India.

CONCLUSION

Study put forward once again that by selecting proper sampling site for candidates isolation involved in metal bioremediation as in this case 'Pauni mine soil', chances of receiving promising isolates rises many folds. Study effectively identified the *A. pittii*(NA+LB) isolate found to be tolerating 1100 ppm of chromium existence around them and also been able to reduce it up to 75% with concentration of 700 ppm. Study reported another isolate which is been capable of chromium reduction from the nature by adsorbing it onto its cell surface and may be useful in coming time for effective bioaccumulation.

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