BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2024. Vol. **21**(2), p. 805-819

Comparative Study of Thermotolerant Hexavalent Cr Bioremediating Bacteria from Dharavi in India

Victoriya Manoranjitham[1] and Jayaprada Rao Chunduri[2]

[1]Department of Biotechnology, Ramniranjan Jhunjhunwala College, Mumbai, Maharashtra, India.
[2]Marine Biology and Biotechnology, 100 N Park Rd, Apt1230 Reading , PA, USA.

http://dx.doi.org/10.13005/bbra/3266

(Received: 27 February 2024; accepted: 21 June 2024)

The Indian leather industry, crucial for the economy, relies on chrome tanning, using 69,000 tons of chromium annually, with 39% ending up as hazardous waste. The non-biodegradable and toxic nature of released chromium poses health risks and contributes to soil contamination. Exploring extremophiles, especially thermophiles, for metal reduction shows promise for efficient bioremediation. The study aims to isolate and identify microorganisms efficient in hexavalent Cr (Cr6+) bioremediation, encountering two Cr6+ resistant thermotolerant isolates, MW50 and TJ100. The isolates MW50 and TJ100 could tolerate up to 700ppm and 600ppm of Cr6+ respectively. Atomic absorption spectroscopy (AAS) revealed MW50 to reduce 97.58% of 200ppm Cr6+, and TJ100 to reduce 90.26% 200ppm Cr6+. Also, the isolates were resistant to multiple heavy metals and antibiotics. The 16Sr RNA studies identified MW50 as Ochrobactrum anthropi and TJ100 as Bacillus aerius. MW50 showed extracellular chromate reductase activity. Crude form of the enzyme was extracted and studied for bioremediation. The enzyme was partially purified by ammonium sulphate precipitation, dialysis and, ion exchange chromatography, and its molecular weight was found to be 72 kDa by SDS PAGE. The DNA samples isolated from both the isolates showed the presence of chrA gene responsible for Cr bioremediation.

Keywords: AAS; chrA gene; Chromate reductase; ion exchange chromatography; SDS-PAGE; 16S rRNA.

Anthropogenic activities are a source of heavy metal pollution nefarious to life on earth[1]. About 5 million sites are contaminated by heavy metals and metalloids with concentrations higher than the regulatory limits in the world[2, 3]. Contamination of the soil with heavy metals affects the functions of the microbial communities of the soil that contribute to the degradation of organic pollutants and can lead to shifts in microbial population[4]. Hexavalent Cr pollution is of worldwide concern because of its long persistence and highly toxic effect on the environment and human life. It is carcinogenic, genotoxic, and mutagenic. Industries including leather tanning, chrome plating, alloying, textile, dye and, pigment manufacturing, aircraft, and wood preservation extensively utilize Cr[5, 6]**.** Tanning industries in India release an estimated 2000-3000 tons of Cr into the environment each year, with effluent containing up to 5000 mg/L, compared with a safe limit of 2 mg/L and 0.05 mg/L in drinking water[7, 8]. As per the reports of Vyawahare Malavika (2017)[9], there

*Corresponding author E-mail: manoranjithamvictoria344@gmail.com

are more than 300 hazardous waste dump sites across the country. Among these identified sites of pollutants, the most often detected heavy metal was Cr followed by lead, cadmium, mercury, and pesticides.

Bioremediation, a cost-effective technology that does not harm the ecosystem is the need of the hour to restore such sites[10, 11]. The use of potential microorganisms for this purpose is emerging as an effective technique[12].

Thermophiles grow at 55°C to 80°C, while Thermo-tolerant microorganisms grow better at 25°C to 40°C but can tolerate 60°C to 80°C, showing adaptability to extreme temperatures[13]. Thermophiles use mechanisms like horizontal gene transfer, gene reduction, higher Guanine and Cytosine (G and C) content, and heat shock protein synthesis to survive high temperatures[14]. Temperature significantly affects heavy metal adsorption by microorganisms, influencing their actions, metabolism, and enzyme activity for faster bioremediation[15]. However, the potential of thermophiles in reducing toxic metals remains underexplored[16]. Microorganisms thriving in high-temperature conditions emerge as promising candidates for bioremediation, offering increased process efficiency[17]**.** These organisms, with their extremozymes, are valuable biocatalysts for industrial processes, crucial for challenging conditions like high temperature, pH, or salinity. The past decade has seen an increased exploration of extremophiles revealing new potential for chemical activities in biocatalysis[18].

The current study focuses on isolating and identifying microorganisms capable of Cr^{+6} bioremediation, analyzing the effects of heavy metals, nutrient components, pH, and temperature on its growth pattern. Additionally, it examines the isolates' resistance to multiple metals and antibiotics. Furthermore, the study compares the bioremediation potential of hexavalent Cr^{6+}- bio-remediating bacteria and provides insights into the mechanisms underlying their bioremediation process.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from the surface (0 to 10 cm deep) at ML-Wadi (19.04553N 72.85353E) and T-Junction (19.04710N, 72.85144E) in Dharavi, Mumbai (Figure 1). Samples were obtained from three sites at each location, spanning distances of 100 to 200 meters, and subsequently combined.

Isolation of hexavalent Cr-resistant bacteria

The minimal medium comprised Solution A - (KH2PO4 - 0.3 grams, Na2HPO4 - 0.6gms, NH4Cl - 0.2 grams, NaCl - 0.5 grams in D/W - 80 mL) and Solution B - (glucose 0.8 grams, MgSO4.7H2O - 0.01 grams in D/W - 20 mL) with a pH - 7.2. A sterile 50 mL of minimal media was supplemented with 10ppm, 50ppm, 100ppm, 250ppm, and 500 Cr^{6+} individually. The collected soil sample of one gram was inoculated into a medium containing heavy metals and incubated at room temperature for three days under shaker conditions of 100 rpm. Cr^{6+} -resistant bacteria in the enriched broth were then serially diluted and spread plated on sterile minimal agar plates containing the corresponding Cr^{6+} concentrations the plates were incubated at RT for 24 to 48 hours to get well-isolated colonies[19].

Molecular characterization of the isolates

The isolates were subjected to 16S rRNA sequencing. The obtained sequence data was compared with sequences in the NCBI BLAST database. Subsequently, the evolutionary relationships were explored using the Neighbour-Joining and the Kimura 2-parameter method. A phylogenetic relationship was deduced through cluster algorithm analysis in MEGA X software, and phylogenetic trees were constructed.

Submitting the sequences and acquiring the accession number

The partial 16S rRNA sequences were submitted to NCBI Gene Bank, and their accession numbers were obtained.

Determination of Minimum inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC), defined as the concentration at which bacterial growth ceases, was determined for the isolates resistant to Cr^{6+}. A 0.5 mL culture suspension was inoculated into 10 mL of sterile minimal media with chromium concentrations ranging from 50 ppm to 1000 ppm. Following 72-hour incubation at room temperature, the growth from the tubes was streaked onto sterile minimal

agar plates with the corresponding chromium concentrations to confirm the MIC[20].

Study of Cr^{6+} bioremediation capability of the isolate

Atomic Absorption Spectroscopy (AAS) [Agilent 240FS – AA 112 M047] was employed to assess the isolates' capability to reduce chromium. A 1 mL sample of an actively growing culture, adjusted to an absorbance of 0.8 at 600 nm, was introduced into 30 mL of sterile minimal medium supplemented with 50 ppm, 100 ppm, 150 ppm, and 200 ppm Cr^{6+}. The flasks were incubated at 100 rpm speed on a rotary shaker for 72 hours at room temperature. After incubation, 10 mL of the culture was transferred to a sterile centrifuge tube, and centrifuged at 6000 rpm for 25 minutes. The remaining chromium concentration was subsequently analyzed. The chromium removal rate was calculated using the following formula:

$$\text{Percentage Metal Absorbed} = \frac{(Cr)i - (Cr)f}{(Cr)i} \times 100$$

Where, (Cr)i : Initial Cr ion concentration (ppm)
(Cr)f : Final Cr ion concentration (ppm)

Multiple metal resistance

At tannery waste-polluted sites, co-contamination with heavy metals other than Cr salts, and organic pollutants fosters the development of resistance of multiple metals in microorganisms. The isolates were examined for their resistance to additional metals, including cadmium, lead, copper, zinc, nickel, arsenic, silver, and mercury. The cultures were spot inoculated onto sterile minimal media agar plates containing a 10 ppm concentration of each metal and incubated for 48 hours at room temperature.

Susceptibility to antibiotics

The isolates' sensitivity and resistance to various antibiotics {Ampicillin (25mcg), Streptomycin (25mcg), Vancomycin (10mcg), Kanamycin (5mcg), Erythromycin (10mcg), Chloramphenicol (25mcg), Gentamicin (50mcg), Amoxicillin (25mcg)} were assessed using the Kirby-Bauer disc diffusion method. Additionally, the MAR (multiple antibiotic resistance) index was calculated for each isolate, expressed as a/b, where 'a' represents the number of antibiotics to which the isolates were resistant, and 'b' represents the total number of antibiotics used for the test[21].

Study of the isolates' growth in the presence and absence of Cr^{6+} and determination of their doubling time

To examine the impact of metal stress on the organism's growth, a growth curve was conducted in the presence and absence of Cr^{6+}. For the analysis, 30 mL of sterile minimal medium was inoculated with the actively growing culture, to attain an OD of 0.04–0.06 at 600 nm. Optical density readings were recorded using a colorimeter (Equiptronics – EQ-651A) at one-hour intervals until reaching the stationary phase, and a graph

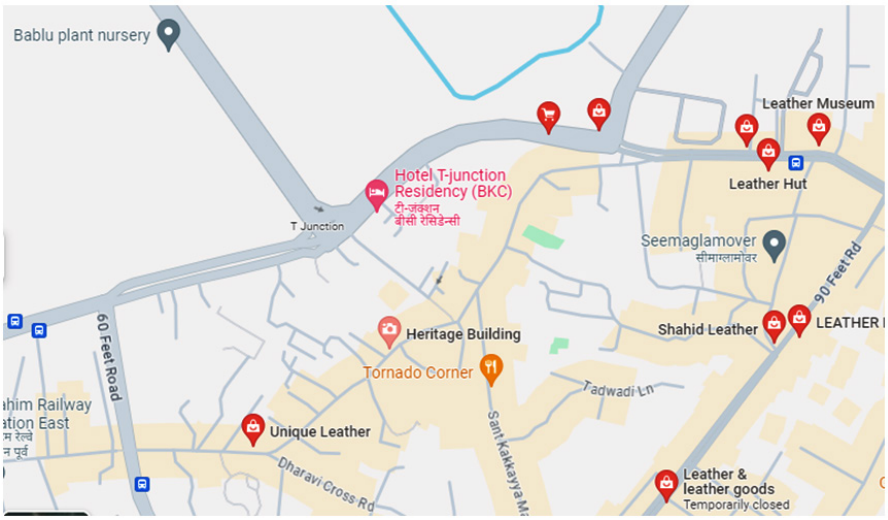


Fig. 1. Location of the sample collection site

illustrating time versus absorbance at 600 nm was generated. The doubling time (T) for the isolates was calculated in both stressed and unstressed conditions, with minimal media and minimal media supplemented with peptone providing an additional nitrogen source, using Microsoft Excel version 11.

Effect of media constituents (C, N) and physical parameters (pH, temperature) on the growth of the isolates

Medium optimization aims to enhance bacterial performance by adjusting concentrations or altering components like C and N, along with conditions such as pH and temperature. Nitrogen input is a critical nutritional factor influencing Cr-reductase potential, while pH and temperature impact Cr^{6+} removal[22, 23].

The basal media used for the isolation of Cr^{6+}-resistant organisms had only glucose as the C source. So the media was first amended by the addition of a nitrogen source (peptone - 0.5%) and its effect on their growth was noted. Then the further optimization of the media was done by varying the concentration of glucose (0.4%, 0.8%, and 1.2%) and peptone (0.5%, 0.8%, and 1.2%) and exposing the cultures to different pH (6, 7, and 8) and temperatures (RT, 37°C, 55°C, and 70°C).

Study of the bioremediation mechanism of the Cr^{6+} resistant isolates

Screening for enzyme mediated reduction of hexavalent Cr

Preparation of crude enzyme

To explore the enzymatic reduction of Cr^{6+} by the isolates, a 12 mL aliquot from a 72-hour-old culture was centrifuged using Remi centrifuge - C24 BL. The collected supernatant was treated as the crude enzyme and employed for subsequent analysis.

Chromate reductase assay

The Chromate reductase activity was assayed by measuring the decrease in the concentration of hexavalent Cr[24]**.** For assessing chromate reductase enzyme activity, reaction mixtures with varying $K_2Cr_2O_7$ concentrations (10 ìM to 200 ìM) were prepared. Each mixture contained 0.2 mL of the corresponding substrate, 0.2 mL of 50 mM Tris buffer (pH 7) with 0.1mM NADH and 0.2 mL of crude enzyme. Incubation at 37°C for 30 minutes followed. Afterward, 0.5 mL of 0.1N HCl and 1.5 mL of Diphenyl carbazide (DPC) reagent were added, standing for 10 minutes. Colour development was measured at 530 nm using a colorimeter, indicating the chromate reductase enzyme activity. A blank sample, replacing the enzyme with buffer, was prepared to account for non-enzymatic colour changes[25, 26].

Partial purification of the enzyme by ammonium sulphate precipitation and Dialysis

The enzyme in the supernatant was treated with ammonium sulphate and then subjected to dialysis. To 50 mL of enzyme samples, different amounts of ammonium salts (ranging from 5.35 to 6.55 grams) as per the standard nomogram for ammonium sulphate precipitation were added to get specific saturation levels (0-20%, 20-40%, 40-60%, and 60-80%). After stirring for 10 minutes and equilibrating at 4°C for at least 30 minutes, the samples were centrifuged using Remi centrifuge - C24 BL at 6000 rpm for 10 minutes. The collected pellets were dialyzed overnight at 4°C in 10mM Tris (pH 8) using a membrane with a 3 to 10 kDa cut-off (Sigma, 10 kDa MWCO).

Ion Exchange Chromatography

1 mL of DEAE Sepharose resin (GE healthcare, now Cytiva) was packed into a glass column (10cm X 1.5 cm) and sequentially washed with de-ionized distilled water and a solution of 50 mM NaCl and Tris at pH 8.0. After equilibrating with 5 CV of 20 mM Tris at pH 8.0, a 10 to 12 mL protein sample with a concentration of 0.056 mg/mL (determined by Bradford assay) was loaded, allowed to bind, and washed with 3 CV of 20 mM Tris at pH 8.0. A step gradient elution with 2 CV each of increasing concentrations of NaCl (50 mM to 500 mM) was used, and eluted fractions were collected for analysis. Subsequently, the collected fractions underwent SDS-PAGE gel electrophoresis to assess purity and molecular weight of the target protein.

Molecular weight determination of the protein by SDS-PAGE

To determine the molecular weight of the partially purified protein, 10% SDS-PAGE under denaturing conditions was conducted. Protein samples and a standard molecular weight marker (Purgene) were loaded onto the gel. Following electrophoresis, proteins in the gel were visualized using the silver staining method, providing high sensitivity. The approximate molecular weight of the enzymatic protein was determined by comparing the migration pattern of protein bands

in the sample with the standard molecular weight marker.

Effect of pH and temperature on the enzyme activity

To optimize enzymatic activity, the impact of temperature and pH on the enzyme was explored. The reaction mixture was incubated at different temperatures (27°C, 37°C, and 50°C), and pH (6, 7, and 9).

Identification of the probable genes involved in Cr^{6+} bioremediation of the isolates

This study investigated the presence of

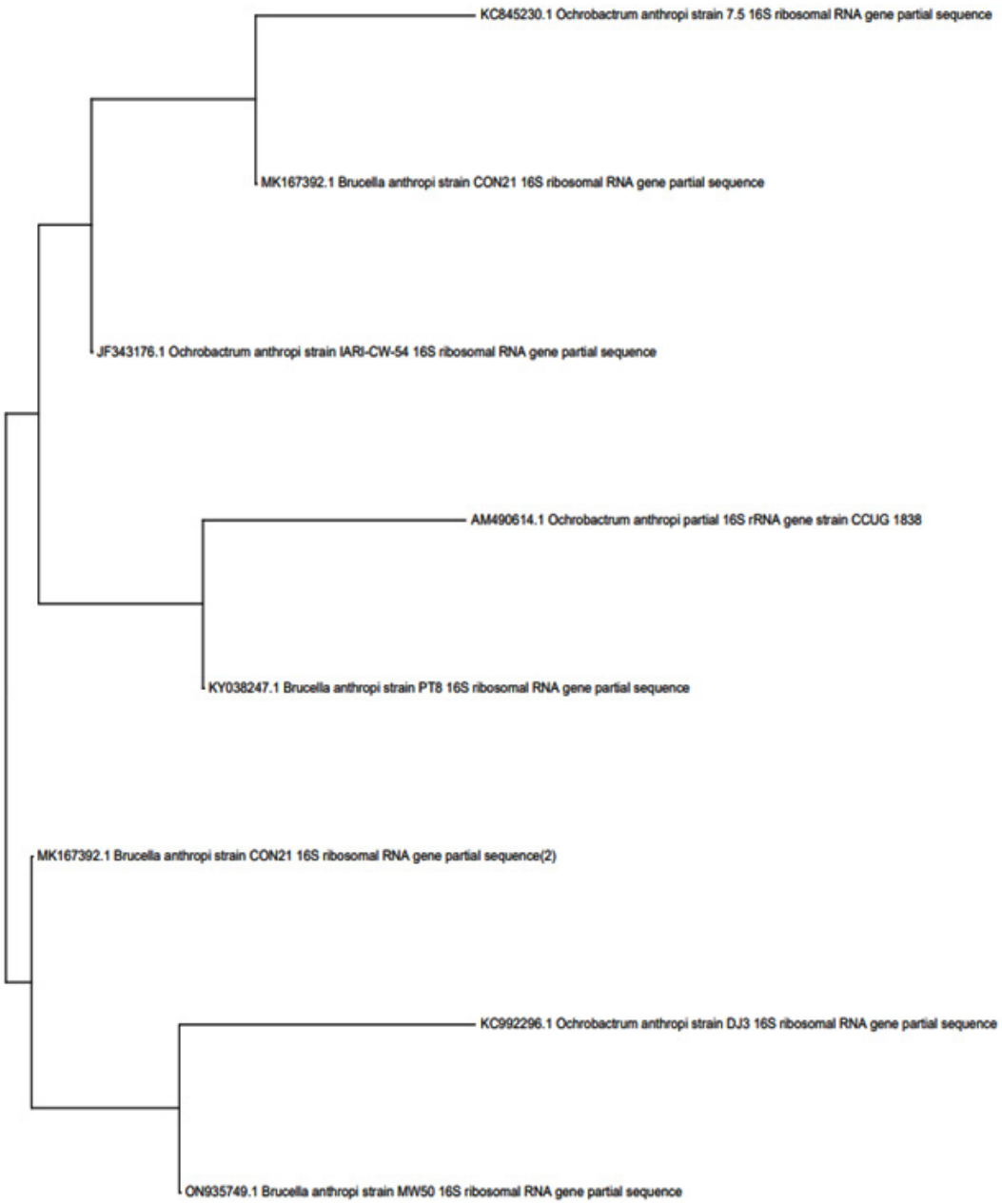


Fig. 2. The Phylogenetic tree of the isolate MW50 (*Ochrobactrum anthropi*)

specific genes (*chr*BAC and *chr*BACF) within the chromium resistance operon (*chr*), as reported by Aminur R in 2017[27], in the isolates. Genomic DNA extraction was done by the CTAB (cetyl trimethyl ammonium bromide) method, followed by purification through Lithium Chloride treatment. PCR was employed to amplify conserved regions related to chromium resistance gene (*chr*A). The

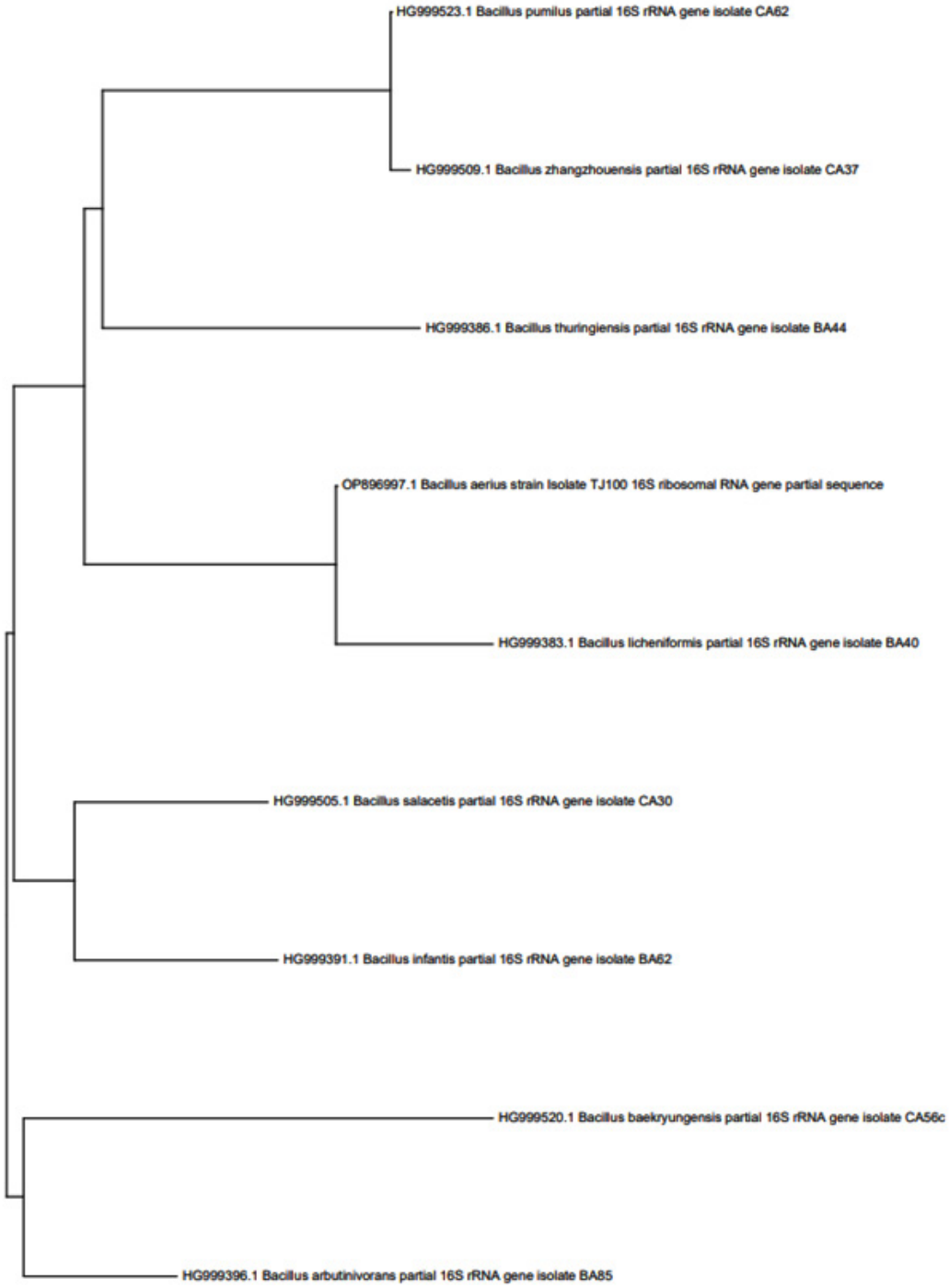


Fig. 3. The Phylogenetic tree of the isolate TJ100 (*Bacillus aerius)*

PCR reaction was conducted for 35 cycles in a GeneAmp 9700 thermocycler, and the products were visualized through gel electrophoresis under a gel documentation system. The forward and reverse primers used for amplification are - {*chrA*-F:TGAAAAGCTGTTTACCCCACT; *chrA-R*:TTACAGTGAAGGGTAGTCGGTATAA} and the temperature settings are as follows : initial denaturation - 96°C for 5 minutes, Denaturation - 95°C for 1 minute, Annealing - 52°C for 1 minute, Extension - 72°C for 1 minute, 72°C Final extension for 10 minutes[28, 27].

RESULTS AND DISCUSSION

Enrichment and isolation of Cr^{6+} resistant microorganisms

The collected soil samples from ML-Wadi and T-Junction were individually enriched in sterile minimal media with concentrations of 10ppm, 50ppm, 100ppm, 250ppm, and 500ppm for 3 days. Based on the growth and morphology, one colony each from the 50ppm Cr^{6+} plate of ML-Wadi (labeled as MW50) and the 100ppm Cr^{6+} plate of T-Junction (labeled as TJ100) was selected. The colonies on the 250ppm Cr^{6+} plate exhibited minimal growth and could not survive the subsequent subcultures, while the 500ppm Cr^{6+} plate displayed virtually no growth.Top of Form

Molecular identification by 16S rRNA method

Partial 16S rRNA sequences were obtained using PCR. These sequences were aligned and compared with 16S rRNA sequences in GenBank via the NCBI BLAST tool, determining percent identity with 100% query coverage for assessing similarity.

For investigating the evolutionary relationships of MW50 and TJ100, the Neighbour-Joining method and Kimura 2-parameter method were employed. Evolutionary distances between isolates were calculated, and a phylogenetic tree was constructed using a clustering algorithm with

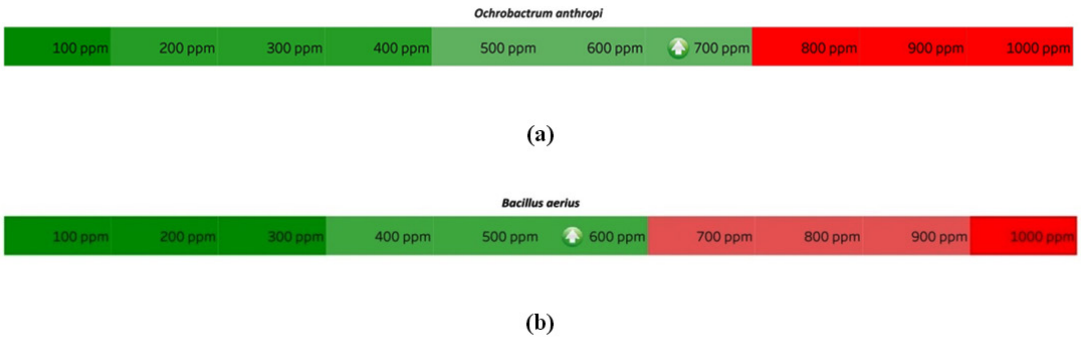


Fig. 4. MIC of the Cr^{6+} resistant isolates MW50 (*Ochrobactrum anthropic*) and TJ100 *(Bacillus aerius*)

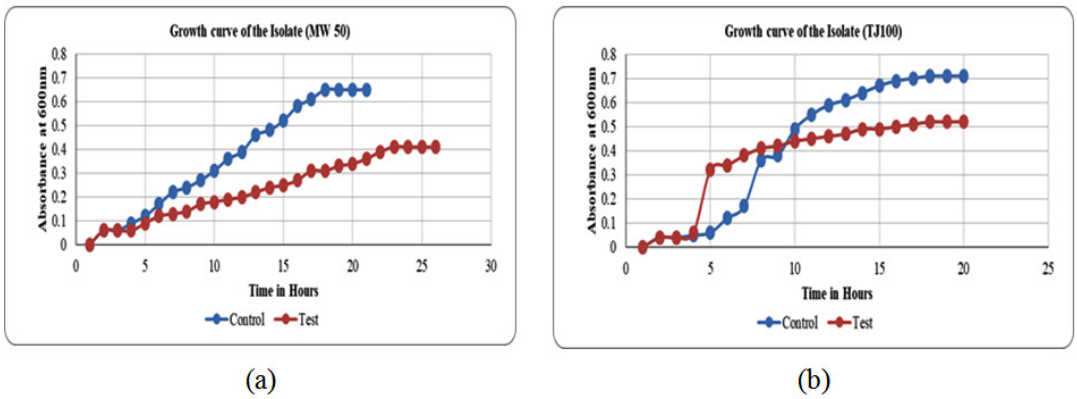


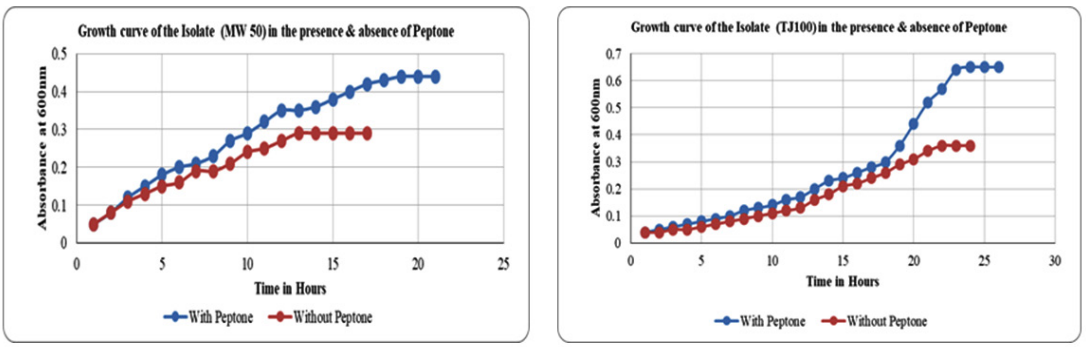
Fig. 5. Growth curve of (a) MW50 and (b) TJ100 in basal minimal media with glucose as the C source in the presence and absence of Cr^{6+}.

the top 10 hits from the NCBI nucleotide sequence database. MEGA X software was used for this analysis. Phylogenetic trees for the identified isolates are depicted in Figures 2 and 3. The isolate MW50 was identified to be *Ochrobactrum anthropi* and the isolate TJ100 was found to be *Bacillus aerius*.

The partial sequences of both organisms were submitted at the NCBI and the accession numbers OP896994 and OP896997 were obtained for MW50 and TJ100, respectively.

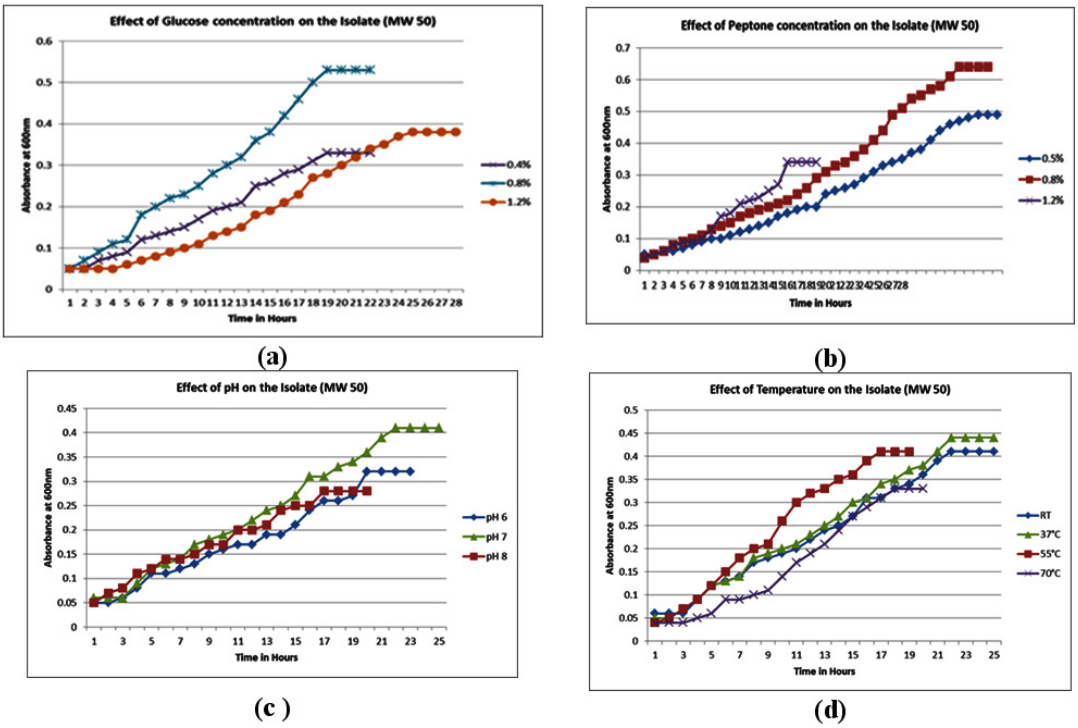
MIC of the Cr^{6+} tolerant isolates

MW50 and TJ100 demonstrated a higher tolerance to Cr^{6+} with concentrations of 700



(a) **(b)**

Fig. 6. Growth curve of (a) MW50 and (b) TJ100 in the presence and absence of peptone in basal minimal media



(a) **(b)**

(c) **(d)**

Fig. 7. Effect of a) glucose b) peptone c) pH and d) temperature on the growth of the isolate MW50 in the presence of Cr^{6+}

ppm and 600 ppm, respectively (Figure 4). They exhibited visible growth within 48 hours and, therefore, can be considered promising candidates for bioremediation.

Study of the bioremediation potentials of the isolates

The bioremediation potential of isolates MW50 and TJ100 was studied using Atomic Absorption Spectroscopy. MW50 demonstrated a reduction of 92.57% for 100ppm and 97.582% for 200ppm, while TJ100 showed reductions of 79.87% for 100ppm and 90.26% for 200ppm Cr^{6+}. Both the isolates exhibited increased Cr^{6+} reduction with higher concentrations, i.e. 200ppm, indicating their promise for bioremediation of toxic hexavalent Cr.

Growth pattern of the isolates MW50 and TJ100

The graph (Figure 5) depicts the substantial impact of Cr^{6+} on the growth of both MW50 and TJ100 compared to their growth in the absence of Cr^{6+}. MW50 shows a lag and reduced log phase in the presence of Cr^{6+}, while TJ100 exhibits an immediate, brief growth phase in response to Cr^{6+}. The unstressed isolates display smoother, prolonged log phases. The addition of peptone to the minimal media resulted in faster growth of the cultures and an extended log phase, which is advantageous for the bioremediation process (Figure 6).

Calculation of Doubling time 'T' to study the effect of the Cr^{6+} stress and additional nitrogen source on the growth of the isolates

The doubling time ('T') for isolates MW50 and TJ100 was calculated using Microsoft Excel under stressed and non-stressed conditions. The presence of the heavy metal Cr^{6+} in minimal media (with only glucose as the carbon source and no nitrogen source), prolonged the doubling time for both isolates, exceeding twice the time compared to the absence of Cr^{6+}. Yet, with peptone added as a nitrogen source to the minimal media, MW50's generation time decreased, while TJ100's remained mostly unaffected, implying MW50's need for nitrogen for its metabolic activity (Table 1).

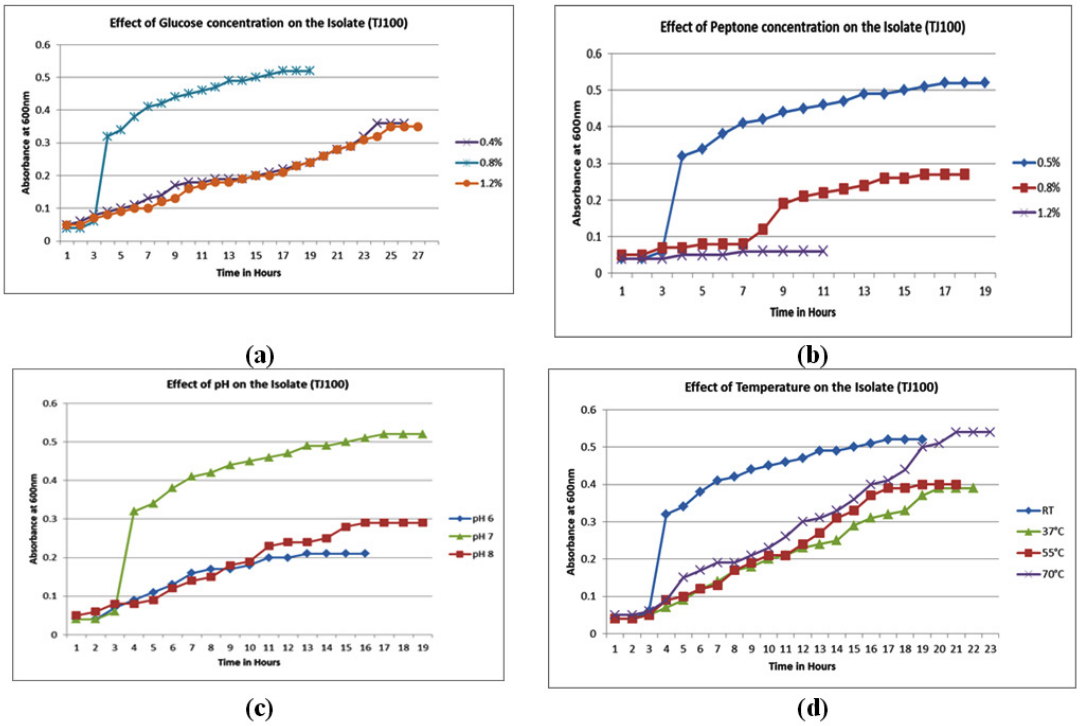


Fig. 8. Effect of a) glucose b) peptone c) pH and d) temperature on the growth of the isolate TJ100 in the presence of Cr^{6+}

Effect of media constituents (C, N) and physical parameters (pH and temperature) on the growth of the isolates

Isolate MW50 displayed optimal growth with 0.8% glucose, experiencing compromised growth at lower (0.4%) and higher (1.2%) concentrations. At 0.8% peptone concentration, MW50 exhibited optimal growth and an extended log phase, with growth also observed at 0.5% and 1.2% peptone concentrations. MW50 displayed broad pH tolerance but achieved its best growth at pH 7. Moreover, it exhibited notable survival and growth across various temperatures, including room temperature (RT), 37°C, 55°C, and 70°C (Figure 7). The isolate showed optimal, sustained growth at 37°C but exhibited better and faster growth at 55°C, reaching an early stationary phase. It also grew at 70°C, demonstrating its Thermo-tolerance.

Isolate TJ100 exhibited comparable growth at 0.4% and 1.2% glucose concentrations, with a notable increase at 0.8% glucose. Optimal growth for TJ100 occurred at 0.5% peptone concentration, while higher concentrations (0.8%) led to a lag phase and a shorter log phase, indicating a slight decrease in growth rate. At 1.2% peptone concentration, TJ100 failed to grow. The isolate preferred pH 7, displaying rapid growth with an early entry into the log phase. At pH 8 and 6, a similar growth pattern

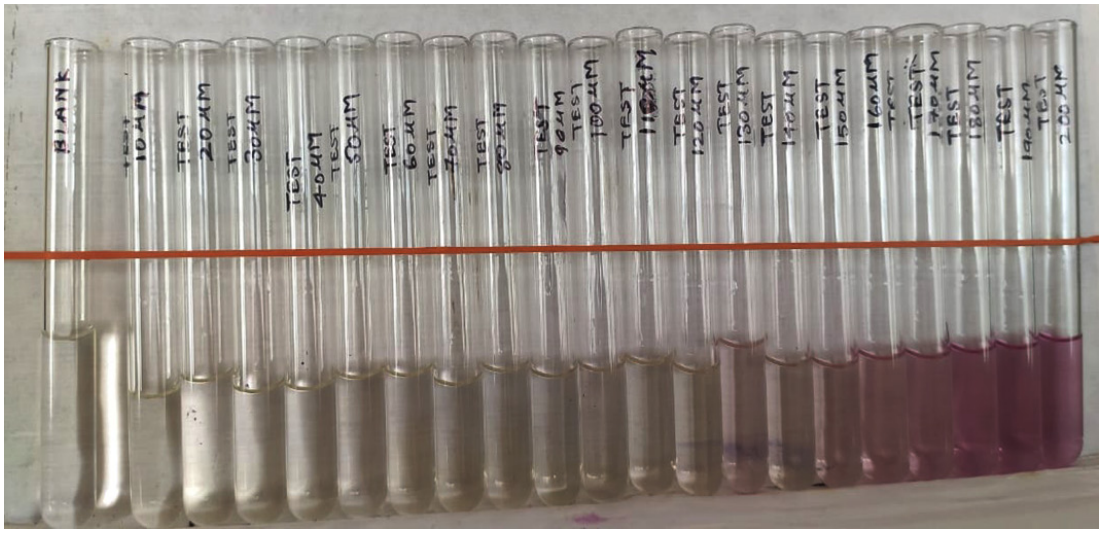
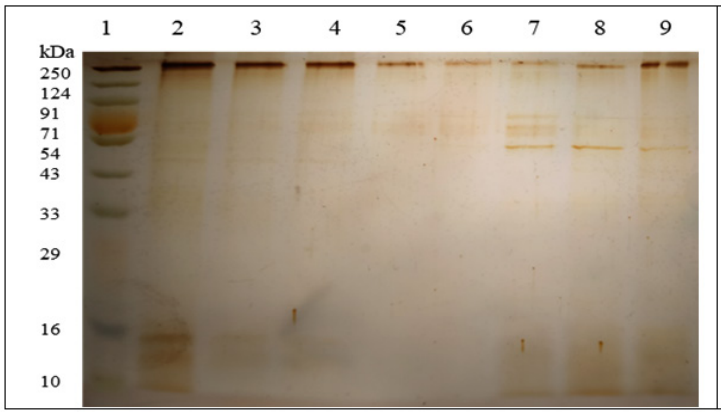


Fig. 9. Enzyme substrate reaction



Lane No.	Sample ID
Lane 1	Marker
Lane 2	Loading sample
Lane 3	Flow through
Lane 4	Flow through binding buffer
Lane 5	Elution 1
Lane 6	Elution 2
Lane 7	Elution 3
Lane 8	Elution 4
Lane 9	Elution 5

Fig. 10. 10% SDS PAGE, stained with silver staining, under reducing condition showing analysis of the partially purified fraction of the enzyme

was observed, but the stationary phase occurred earlier. TJ100 demonstrated excellent growth at room temperature, entering the log phase early. At 37°C, 55°C, and 70°C, it showed similar early growth with a gradually prolonged log phase. At 70°C, it exhibited better and longer growth with a delayed entry into the stationary phase, indicating high-temperature tolerance (Figure 8).

Thus, the isolates MW50 and TJ100 exhibit robust growth at extreme temperatures of 55°C and 70°C, highlighting their thermotolerant nature. This ability to thrive in high-temperature environments makes them promising candidates for bioremediation of Cr^{6+} contamination, especially in challenging settings like high-temperature radioactive waste sites[29].

Multiple metal resistance and susceptibility to antibiotics

The isolates were tested against 10ppm concentrations of Cd, Pb, Cu, Ni, As, Zn, Ag and

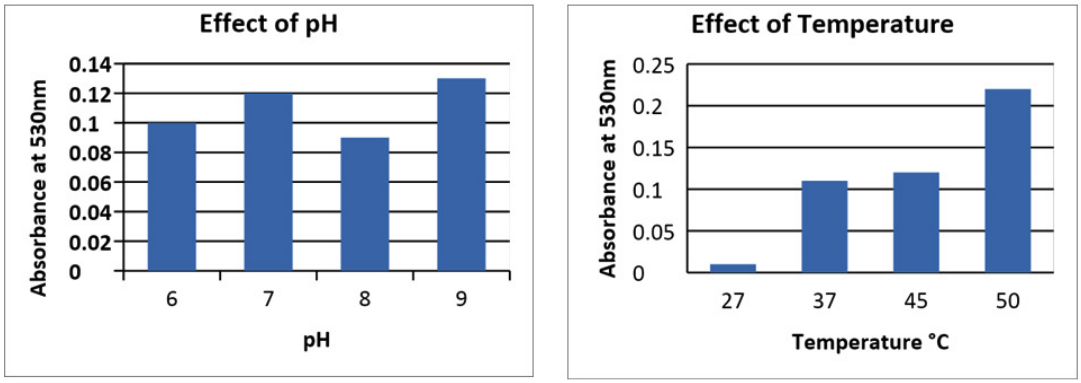


Fig. 11. Effect of temperature and pH on the enzyme activity

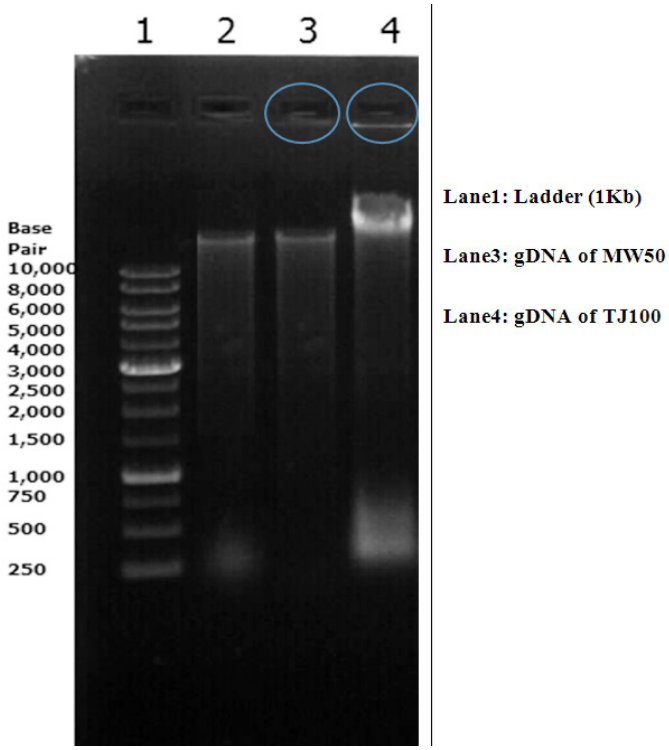


Fig. 12. Genomic DNA of the isolates –MW50 and TJ100

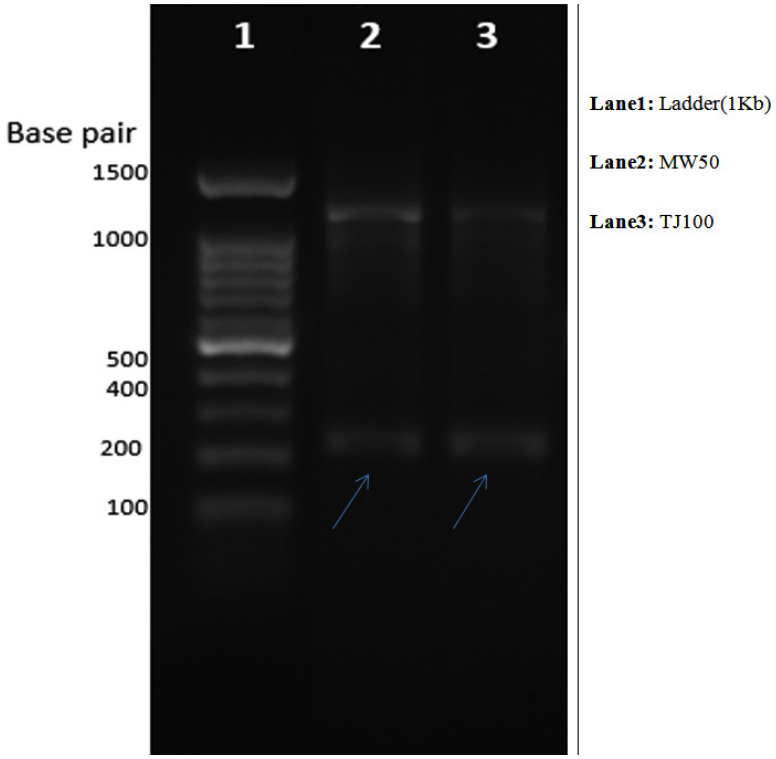


Fig. 13. Amplification of *chrA* gene from the isolates MW50 and TJ100

Table 1. Doubling time of MW50 and TJ100 under different conditions: with and without Cr^{6+} in minimal media, and with peptone supplementation to minimal media with Cr^{6+}

Name of the isolate	Doubling time (T) in the absence of Cr^{6+} in minimal media (hours)	Doubling time (T) in the presence of Cr^{6+} in minimal media (hours)	Doubling time (T) in the absence of peptone in minimal media with Cr^{6+} (hours)	Doubling time (T) in the presence of peptone in minimal media with Cr^{6+} (hours)
MW50	5.20	9.55	7.45	6.80
TJ100	3.96	7.31	6.41	6.08

Hg. MW50 exhibited resistance to Cd, Pb, Cu, Ni, As, and Zn but showed sensitivity to Ag and Hg. Meanwhile, TJ100 demonstrated resistance to all tested metals. Thus, both the isolates exhibit resistance to multiple metals.

Isolate MW50 exhibited resistance to all tested antibiotics [Ampicillin (25mcg), Streptomycin (25mcg), Vancomycin (10mcg), Kanamycin (5mcg), Erythromycin (10mcg), Chloramphenicol (25mcg), Gentamicin (50mcg), and Amoxicillin (10mcg)], except for gentamicin. Conversely, TJ100 was sensitive to erythromycin, chloramphenicol, and gentamicin, while showing resistance to the remaining tested antibiotics. Also, the MAR index for MW50 and TJ100 was found to be 0.85 and 0.65 respectively which are higher than the accepted value 0.25, thus indicating their multiple drug resistance.

Mechanism for bioremediation potential of the isolates

Preparation of crude enzyme - Chromate reductase

A cell-free extract was obtained by centrifuging 72-hour-old stationary phase cultures

of MW50 and TJ100. The resulting supernatant was considered as the crude enzyme, and utilized for subsequent analysis.

Chromate reductase assay and enzyme kinetics

Enzyme activity was assessed using different concentrations of the substrate $K_2Cr_2O_7$ (10iM - 200iM) with the cell-free supernatants of isolates TJ100 and MW50. The isolate MW50 showed a complete reduction up to 120µM, whereas the isolate TJ100 showed no hexavalent chromium reduction (Figure 9)**.**

Partial purification, Ion exchange chromatography and SDS PAGE for determination of molecular weight of the enzyme – chromate reductase

The crude enzyme in the cell-free extract obtained by centrifugation of the stationary phase culture was precipitated using 40% of ammonium sulphate and then subjected to ion-exchange chromatography using DEAE-Sepharose Column. All the eluted fractions were collected and subjected to SDS PAGE. Silver staining of the gel revealed a protein of 70kDa (Figure 10).

Effect of pH and temperature on the enzyme activity

The enzyme exhibited activity at elevated temperatures, actively degrading metals even at 50°C. Additionally, the enzyme demonstrated activity across a range of pH levels (pH 6, 7, 8, and 9) remaining effective at both slightly acidic and alkaline pH conditions (Figure 11).

Identification of the probable gene (*chr*A) involved in the bioremediation of the isolates

The genomic DNA of isolates MW50 and TJ100 was extracted using the CTAB method and confirmed by agarose gel electrophoresis (Figure 12). The DNA was then screened for the presence of the gene *chrA* using the primers (*chrA*-F: TGAAAAGCTGTTTACCCCACT and *chrA*-R: TTACAGTGAAGGGTAGTCGGTATAA) through PCR analysis that revealed the presence of *chrA* gene in the DNA samples of MW50 & TJ100. This was demonstrated by subjecting the PCR products to electrophoresis on 1.5% Agarose (Figure 13). Amplification of the gene for *chr*A gene has given similar results as that reported by Baldiris, R et al. (2018)[30].

CONCLUSION

Bacterial strains MW50 and TJ100, resistant to hexavalent chromium with MICs of 700ppm and 600ppm, exhibited substantial Cr^{6+} reduction through AAS analysis. Identified as *Ochrobactrum anthropi* and *Bacillus aerius* via 16S rRNA sequencing, MW50 synthesized chromate reductase, contributing to Cr^{6+} reduction and carrying the *chR*A gene. In contrast, TJ100 lacked chromate reductase activity but still carried the gene. Further investigation is needed to understand TJ100's potential for Cr^{6+} reduction and survival under stress mediated by the *chr*A gene. Resistant to multiple metals and antibiotics, these isolates hold promise for bioremediation in sites co-polluted with other metals and organic compounds. Their thermotolerance, survival at varying pH, and MW50's chromate reductase activity make them promising tools for hexavalent Cr^{6+} bioremediation.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Rohan Gavankar and Dr. Deepa Verma from VIVA Centre for Advanced Research and Development (VCARD), VIVA college of Arts, commerceand Science, Virar (W), Maharashtra and National Facility for Biopharmaceuticals, Matunga, Mumbai, Maharashtra for the facilities provided for this work.

Conflict of Interest

The authors do not have any conflict of interest.

Funding Sources

The authors did not receive any funds for this research.

Authors' Contribution

The author Dr. Victoriya Manoranjitham contributed for the conceptualization, sample collection. Methodology, analysis and interpretation of the results and manuscript preparation. The author Dr. Jayaprada Rao contributed for the conceptualization, analysis and interpretation and manuscript preparation

Data Availability Statement

Yes the manuscript incorporates all data sets produced or examined throughout this research study

Ethics Approval Statement

Not applicable.

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