



Evaluation of the heavy metals tolerant UV rays treated bacteria isolated from anthropogenic sites of Chambal region, India

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Industrial waste is released into the environment and leads to various types of heavy metal, which are toxic, mutagenic and carcinogenic in nature. Heavy metals are not biodegradable but accumulated by living organisms and cause diseases at even low concentrations. In this study, we selected four anthropogenic sites from Chambal region, isolated bacteria and investigated its heavy metal removal capability. The bacteria was isolated and identified as *Escherichia coli* (Ag-5), on the basis of biochemical and 16S rRNA gene sequence. Among the five (cadmium, cobalt, lead, nickel and zinc) heavy metals studied, Ni²⁺ has been observed to be highly toxic with minimum inhibitory concentration score of 200 ppm. *E. coli* could tolerate Zn²⁺ (300 ppm), Cd²⁺ (400 ppm), Co²⁺ (400 ppm) and Pb⁺² (500 ppm). Heavy metal tolerance capability was also evaluated by UV rays treated *E. coli* (Ag-5) isolate and compared with wild strain Ag-5. The result indicated that the tolerance capability was enhanced by UV rays treated bacterial isolate as compared to wild strain with respect to all tested heavy metals. Atomic absorption spectroscopy results revealed that wild strain removed 78.2% cadmium nitrate, while UV rays 30 and 60 s. exposed strain removed 85.9 and 83% cadmium nitrate. Wild strain removed 64.4% nickel chloride, while UV rays 30 and 60 s exposed strain removed 66.9 and 74.5% nickel chloride. The results indicate that indigenous *E. coli* treated with UV rays could serve as heavy metal tolerant bacteria and utilized in bioremediation processes.

Keywords: Antibiotic profiling, Atomic absorption spectroscopy, Bioremediation, Heavy metal pollution, Metal tolerant bacteria

Environmental components viz. air, water and soil have been extremely polluted due to increased population, rapid urbanization, industrialization and expansion of the existing ones. These sources release various types of harmful substances that are toxic to the biological process. Industries discharged the heavy metals from the environment by various sources such as mining, smelting, electroplating, fertilizers, plastics manufacturing, and insecticides. Heavy metals are elements with a molecular weight greater than 53, a density greater than 6 g/cm³, and an atomic number greater than 20¹. Heavy metals, such as nickel, mercury, cadmium and lead are different from other pollutants and these are not biodegraded but accumulated by some living organisms and cause diseases at even low concentrations². Heavy metals are extremely poisonous for humans, animals, microbes and plants which can damage cell membranes, alter enzymes and destroy the structure of DNA³. The uncontrolled discharges of large quantities of wastes containing heavy metals develop

a large burden affecting economic and health care⁴. Chemical methods, such as oxidation or reduction and precipitation, have been widely used but are expensive and less effective compared to biological methods. Bioremediation is one of the techniques to decontaminate the environment with the help of microorganisms. This technique is cost-effective, simple and more importantly environment friendly. Some bacteria accumulate metals and have ability to survive at high concentrations⁵. Some microorganisms acquire resistance mechanisms which are plasmid encoded and tend to be specific for a particular metal⁶. Others are general conferring resistance to a variety of metals. The ability of these microorganisms in such an extreme environment makes them an attractive alternative for *in situ* bioremediation⁷. Laboratory investigation had shown that many bacteria which were resistant to the effect of high concentration of heavy metals were concomitantly resistant to several antibiotics⁸.

The bacterial community inhabits an extreme environment including high UV radiation habitats. Their survival in spite of higher UV dosage as well as resistance to salt and metal ions were some of the

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interesting characteristics of these microbes⁹. Therefore, in the present study, we tried to isolate and identify the heavy metal resistant bacteria from the polluted sites of Chambal region, In addition, we looked at the co-resistance to different antibiotics to determine the resistance mechanism and understand the resistance mechanism and biodegradability of the bacterial isolate.

Material and Methods

Study area and collection of soil

The study sites were selected on the basis of the extent of pollutants produced by various industries, waste treatment plants, etc. Soil samples were collected from four different anthropogenic sites in Chambal region. The four sites were Banmore (26.3553°N, 78.0947°E), Rairu (26.3001°N, 78.1294°E), Morena (26.4934°N, 77.9910°E) and the Malanpur (26.3670°N, 78.2875°E). The heavy metals contaminated soil samples were collected 4-6 inch depth from the surface of the contaminated site. Soil Samples were collected in sterile polyethylene plastic bags and stored at 4°C for further processing.

Isolation and screening of heavy metal resistant bacteria

One gram of soil from each site were homogenized and bacteria were isolated by serial dilution¹⁰ on nutrient agar plates containing various heavy metals like-cadmium nitrate [Cd(NO₃)₂], lead nitrate [Pb(NO₃)₂], cobalt chloride (CoCl₂) and nickel chloride (NiCl₂). After preliminary screening, pour culture were inoculated in Luria Bertani (LB) broth containing 50 ppm cadmium nitrate, lead nitrate, cobalt chloride and nickel chloride, respectively in separated flasks and incubated at 30°C and 120 rpm and growth was observed in the term of optical density at A₆₀₀.

Identification & characterization of selected isolate

The isolate was identified by cell morphology, biochemical characteristics, and molecular analysis. The cell morphology such as shape, size and biochemical characteristics were studied¹⁰.

For molecular study, bacterial genomic DNA was isolated from isolate and 16S rRNA sequencing was carry out using universal primers, (5'-CCGAATTCGT CGACAACAGAGTTTGATCCTGGCTCAG-3') & reverse primer (5'-CCC GGGATCCA ZAGCTTAC GGCTACCTTGTTACGACTT-3'). Polymerase chain reaction was used to amplify DNA products¹¹. The amplified product was sequenced in both directions by an automatic sequencer. In phylogenetic analysis

16S rRNA gene sequence was compared with available 16S rRNA gene sequence data in NCBI, Genbank database. A multiple sequence alignment of nine closely related 16S rRNA gene sequences were retrieved from NCBI, Gene Bank data and were aligned using Clustal W 2.1 with PHYLIP output option. Phylogenetic tree was drawn using PHYLIP version 3.695. Evolutionary distance matrix was computed using neighbour-joining/UPGMA method and DNADIST algorithm program¹².

Inoculum preparation

A fresh bacterial culture was added to conical flask having 100 mL of LB broth medium then incubation was held at 30°C until achieving the optical density (OD) 1 at 600 nm (OD₆₀₀ of ca.1)¹³. Then 1 mL aliquot of bacteria (1×10⁷) was prepared to use for further study.

Optimization of growth parameters (temperature and pH)

The optimal growth conditions with range of temperature and pH were studied. For studying the effect of pH, 1ml of overnight broth culture (OD₆₀₀ of ca.1) of isolate was inoculated into LB broth medium with different pH values of 3, 5, 7, 9 and 11 and incubated under shaker (120 rpm) for 24 h. The effect of temperature was studied by inoculation of isolates in LB broth at different temperature ranges from 25, 30, 35 and 40°C and growth was observed in the term of optical density at A₆₀₀. The experiments were conducted in triplicate¹⁴.

Antibiotic profiling

The antibiotic resistance of the bacterial isolate was determined by the disc diffusion method in Muller-Hinton agar using the following antibiotic tetracycline, chloramphenicol, erythromycin, kanamycin, rifampicin, streptomycin, gentamicin and penicillin. The inhibition zone's diameters around the disc were measured. The following concentration were used, tetracycline (30 µg/disc), kanamycin (30 µg/disc), chloramphenicol (30 µg/disc), erythromycin (15 µg/disc), rifampicin (5 ppm), streptomycin (25 µg/disc), gentamicin (10 µg/disc) and penicillin (2 unit). The clearing zones around the discs were measured¹⁵.

Determination of minimum inhibitory concentration (MIC)

Heavy metals resistance study was done by determining the minimum inhibitory concentration of the metals in LB broth. The concentrations of the different metals were varied depending on the inhibitory concentration of the respective metals and bacterial strain. Initial concentration was 100 ppm.

The growth was studied in successively higher concentrations of metals until the bacterial growth was inhibited¹⁶. The experiment was carried out in triplicate. The conical flasks filled with the metal salts of different concentrations dissolved in LB broth and inoculated with prepared inoculum at 35°C, 150 rpm for five days and OD was observed 600 nm and mean value were calculated.

Effect of UV irradiation on selected bacterial isolates

To determine the effect of UV irradiation on bioremediation potential of selected isolate, 100 µL of bacterial isolate (OD_{600 nm} = 0.8) was spread over a nutrient agar plate, then the plates were exposed under UV lamp (20W) for different time intervals 30, 60 and 90 s, respectively and incubated at 35°C for 24 h. The plates were exposed to the UV irradiation under the UV chamber supplied with a 20W and 280 nm UV B light source (germicidal lamp) for a specified time i.e. 30, 60 and 90 s subsequently incubated at 35°C. The surviving fraction was calculated after 24 h by determining the titer of culture after irradiation divided by UV radiated control⁹.

Table 1 — Biochemical characterization of bacterial isolate Ag-5

Characteristics	Ag-5
Morphology	Rod
Diameter (µm)	0.6×1.3
Gram staining	—
Capsule Staining	—
Spore forming	—
Pigmentation on Nutrient Agar	No
Indole production	+
Methyl red	+
VogesProskauer	—
Citrate utilization	—
Phenylalanine diamine test	+
Carbohydrate fermentation	
Glucose	—
Lactose	—
Sucrose	—
Nitrate reductase	+
Casein hydrolysis	—
Starch hydrolysis	—
Gelatin hydrolysis	—
Catalase	+
Urease	—
H ₂ S	—
Lipid hydrolysis	—
Oxidase	—
TSI test	—
Lipase	—
Amylase	+
Cellulose activity	—

Bioaccumulation of heavy metal by wild and U. V. irradiated bacterial strain

Bioaccumulation is the amount of heavy metal uptake by bacteria. For this purpose 1ml each isolate (wild and UV irradiated) was inoculated in 100 mL of LB broth containing various heavy metals concentration and incubated at 35°C, 150 rpm for 48 h, after incubation the broth was centrifuged at 6000 rpm for 10 min. Then the supernatant was digesting, residual concentration of metal was analyzed by an atomic absorption spectrophotometer. (Perkin Elmer, USA model 800A Analyst)⁵. Bioaccumulation of heavy metal (ppm) = Initial concentration – residual concentration.

Results and Discussion

This study revealed that isolation and purification of forty bacteria, among the 40 bacteria isolates, only isolate Ag-5 was selected as the heavy metal accumulator, on the basis of the optimum growth under different heavy metals concentrations. Isolate Ag-5 was characterized and identified as *Escherichia coli*. Table 1 showed the biochemical characteristics of *Escherichia coli* (Ag-5). The 16S rRNA gene sequencing analysis represented that bacterial isolate Ag-5 species closely related to *Escherichia coli* strain NF3 accession no KP771990.1 based on 99%, similarity index in 16S rRNA gene sequences (Fig. 1). The 16S rRNA genes have been submitted with accession numbers MH478349.1 to the NCBI Genbank database.

The bacterial resistance to heavy metal ions was affected not only by the surface properties of the microorganism, but also by environmental conditions like temperature and pH. The bacterial isolate was able to grow over a wide range of temperatures with a high growth rate. The growth temperatures are important to characterize bacterial isolates for use in the bioremediation process. In the bioremediation process, temperature control is an important aspect and may be possible to control in some bioremediation processes¹⁷.

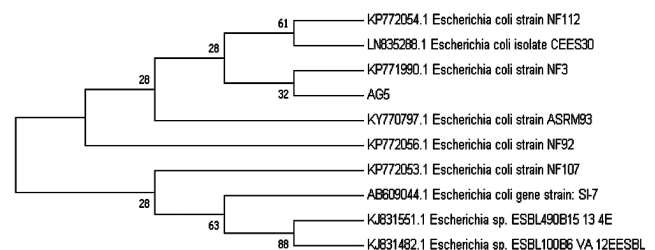


Fig. 1 — Phylogenetic spectrum of isolate *E. coli* (Ag-5)

The present results showed that maximum growth of isolate *E. coli* (Ag-5) was observed at 35°C (Fig. 2A). *E. coli* was grown at a temperature range 30-35°C as reported by other researchers also^{18,19}. The pH is an important factor because it affects the growth of bacteria. pH was affected at cellular surface sites as well as metal speciation²⁰. Most bacteria favor a neutral pH²¹. The value of pH was 6-7 that supported the maximum growth and degradation which was to be found higher at pH 7.0²². In this study, the maximum growth of bacterial isolate *E. coli* (Ag-5) was found at pH 7 (Fig. 2B).

Antibiotic resistance might be associated with a higher level of heavy metals tolerance capability because antibiotic resistance gene and heavy metal uptake gene are found in bacterial plasmid²³. The isolate *E. coli* (Ag-5) that was resistant to heavy

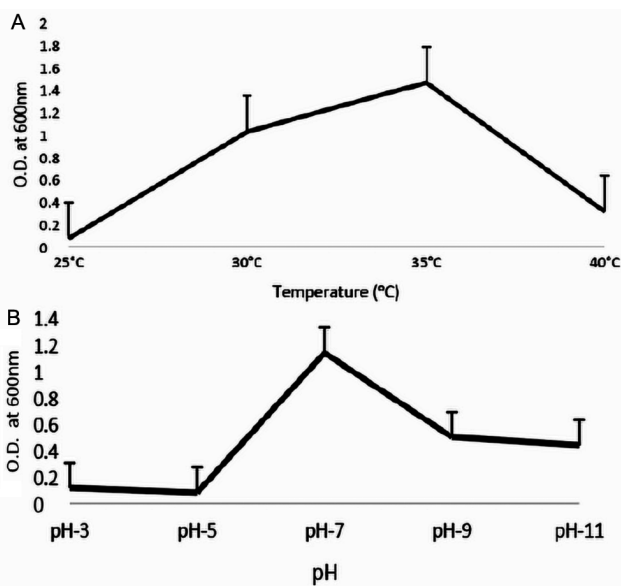


Fig. 2 — Effect of different (A) temperature; and (B) pH on the growth of isolate *E. coli* (Ag-5)

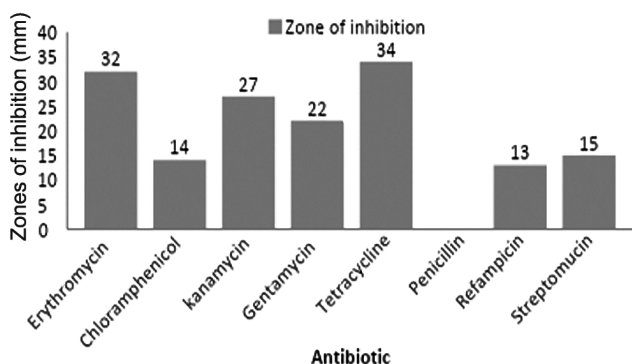


Fig. 3 — Antibiotic profiling of isolate *E. coli* (Ag-5) against different antibiotic

metals such as lead, nickel, cadmium, etc. was usually found resistant to various antibiotics. Isolate *E. coli* (Ag-5) showed complete resistance against penicillin (Fig. 3).

The lowest concentration of a substance that inhibits the growth of an organism is known as the minimum inhibitory concentration (MIC)²⁴. The growth of isolate *E. coli* (Ag-5) was dependent on heavy metal concentration. if metals concentration was increasing the bacterial growth was decreased. Nickel is highly toxic to bacterial isolate Ag-5. Minimum inhibitory concentration of different heavy metals viz. Cd²⁺, Ni²⁺, Co²⁺, Pb²⁺ and Zn²⁺ for bacterial *E. coli* was 400, 200, 400, 500 and 300 ppm, respectively. Isolate *E. coli* (Ag-5) showed maximum resistance against Pb²⁺ (500 ppm), and Cd²⁺ and the order of resistance regarding the metal concentration was therefore Pb²⁺ > Cd²⁺ > Co²⁺ > Zn²⁺ > Ni²⁺. *Enterobacter cloacae* and *Klebsiella sp.* found resistant against Cd Cr and Pb. *Enterobacter cloacae* resisted Cd (220 mg/L), Cr (800 mg/L) and Pb (1400 mg/L). *Klebsiella* strain (CMBL- Cd-2 and CMBL- Cd-3) showed resistance to Cd at the concentration of 110 mg/L and 100 mg/L, Cr 600 mg/L and 500 mg/L, Pb 1200 mg/L and 900 mg/L supplemented in the medium, respectively²⁵.

A gradual decrease in CFU was observed upon an increase in UV dose up to a certain extent, a rapid decline in individual populations was observed⁹. Table 2 showed the result of UV irradiation on the growth of isolate *E. coli* (Ag-5). The bacterial isolate *E. coli* (Ag-5), the number of colonies were decreased with increased exposure time. The number of colonies was 182, 60 and 11 when exposure time was 30, 60 and 90 s, respectively. Morphological changed bacteria were selected for further study.

The atomic absorption spectrophotometer was done to evaluate residual heavy metals concentrations. Resistance can be accomplished by using a biosorption and bioaccumulation mechanism²⁶. In bioaccumulation mechanisms in bacterial cells were associated with the availability of the operon gene in accordance with the accumulated metal²⁷. Other work reported that *E. coli* had also been attributed with

Table 2 — Effect of UV irradiation on the growth of *E. coli* (Ag-5)

Time of UV exposure (s)	No. of colonies
30	182
60	60
90	11

Table 3 — Concentration of heavy metals (ppm) in samples by atomic absorption spectroscopy

<i>E. coli</i> (Ag-5)	Cd(NO ₃) ₂			
	I	R	A	%
Wild	400	87.1±0.024	312.9±0.020	78.22
30 s	400	56.1±0.021	343.9±0.027	85.9
60 s	400	67.25±0.021	332.75±0.023	83
		NiCl ₂		
Wild	200	71.2±0.031	128.8±0.022	64.4
30 s	200	66.2±0.025	133.8±0.028	66.9
60 s	200	51±0.020	149±0.030	74.5
<i>E</i>		CoCl ₂		
Wild	400	89.2±0.042	310.8±0.021	77.7
30 s	400	75.5±0.022	324.5±0.034	81.12
60 s	400	76±0.039	324±0.022	81
<i>E</i>		Pb(NO ₃) ₂		
Wild	400	135±0.033	265±0.023	66.25
30 s	400	136±0.030	310.98±0.019	77.7
60 s	400	137±0.031	293.78±0.024	73.44
<i>E</i>		ZnSo ₄		
Wild	300	97.1±0.031	202.9±0.040	67.6
30 s	300	23.21±0.034	276.79±0.036	92.2
60 s	300	42±0.027	258±0.033	86

[I, initial concentration (ppm); R, residual concentration(ppm); A, accumulation(ppm); %, removing percentage. Measurements were done triplicate. Error bar indicates ±SD]

biosorption properties of metals like copper, nickel and cobalt²⁸. Other researchers⁵ also reported that the isolate belongs to *Pseudomonas stutzeri* removal ability was 57.1 and 52.9% of Pb and Cd. Degrading potentiality was assessed of *Gemella* sp. and *Micrococcus* sp. showed 55.16±0.06% and 36.55±0.01% reduction of Pb, respectively. On the other hand, moderate degradation of Cd was shown by *Gemella* sp. (50.99±0.01%) and *Micrococcus* sp. (38.64±0.06%). Atomic absorption spectroscopy results showed in Table 3. Atomic absorption spectroscopy revealed that wild isolate *E. coli* (Ag-5) removed 78.2% cadmium nitrate, 64.4% nickel chloride, 77.7% cobalt chloride, 66.25% lead nitrate, and 67.7% zinc sulphate, Many researcher reported that β -Proteobacteria and Firmicutes have maximum survival rate at high UV dosage. Metals like Mn⁺², Cu⁺², Zn⁺² and Co⁺² enhance the survivability of UVR resistant microbes because these metals block the Fenton reactions that indirectly prevent formation of several toxic oxides and by-products which can alter the cell membrane^{29,30}. The ability of these microbes in such an extreme environment make them an attractive choice for *in-situ* bioremediation of radioactive wastes. After exposure of UV (30-180 s) high capacity of Cu+2 ion sequestration was detected in strain *Bacillus subtilis* and *Kocuria turfanensis*⁹. In this study, UV rays exposed 30 and 60 s. isolates Ag-5

removed 85.9 and 83% cadmium nitrate, 66.9 and 74.5% nickel chloride, 81.12 and 81% cobalt chloride, respectively, for lead nitrate and zinc sulphate isolate Ag-5 *E. coli* exposed to 30 and 60 s UV rays removed 77.7,73.44, 92.20 and 86.0%, respectively.

Conclusion

In the present study, we investigated the heavy metal tolerance ability of *E. coli* collected from four industrial polluted sites (Banmore, Rairu, Morena and Malanpur) in Chambal region. The phylogenetic tree suggested that this isolate was *Escherichia coli*. This bacterial isolate was subjected to five toxic heavy metals (cadmium, cobalt, lead, nickel and zinc), to study its tolerance capability. Nickel is highly toxic to bacterial isolates. To augment the resistance capability of heavy metal and biodegradability of bacterial isolates, the bacterial isolate was exposed to UV irradiation. The results revealed that UV rays exposed bacterial isolate *Escherichia coli* had a significantly increased up to 7 fold compared to the wild *E. coli* indicating its potential use as a bioremediation agent and detoxify the surrounding environment of the industrial sites.

Conflict of Interest

Authors declare no competing interests.

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