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(RESEARCH ARTICLE)

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Biochemical analysis of nitrogen fixing organism azotobacter for removal of toxic hexavalent chromium from groundwater resources causing ill-effects on the health of human population

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Abstract

Hexavalent chromium Cr(VI) is a common environmental contaminant that poses serious threats to ecosystems and human health, especially in groundwater. Conventional remediation techniques are frequently expensive and ineffective. The potential of the nitrogen-fixing bacteria Azotobacter in the bioremediation of groundwater contaminated with Cr(VI) is investigated in this work. We assessed Azotobacter's ability to remove Cr(VI) using a range of growth studies and biochemical tests. The findings show that, depending on variables like pH, temperature, and starting chromium concentration, Azotobacter has considerable Cr(VI) reduction capabilities. According to the results, Azotobacter may be a practical and long-term solution for the bioremediation of water sources contaminated with chromium.

Keywords: Azotobacter; Hexavalent Chromium; Bioremediation; Groundwater; Biochemical Analysis; Cr (VI) Reduction

1. Introduction

A highly hazardous and carcinogenic pollutant, hexavalent chromium (Cr(VI)) is frequently found in industrial effluents from processes like textile manufacture, leather tanning, and electroplating. There are serious health dangers associated with its presence in groundwater, such as skin irritation, respiratory problems, and an elevated risk of cancer among impacted populations [1]. Although successful, traditional cleanup methods including chemical precipitation, ion exchange, and reverse osmosis are frequently costly and produce secondary waste, hence sustainable alternatives are required[2].Sampling Area Of this study are Industrial Areas of Unnao district having large number of tannery Industries in its vicinity. All the Effluents Are discharged in the natural sources of water thereby contaminating the water resources causing severe ill effects on the health of human residing there. Rural Population are dependent on the contaminated water resources like Tube well Hand pumps for Drinking Purpose.

1.1. Health issues due to Chromium contaminated water consumption

It is concluded from analyzing the ground data that human civilization has been exposed to a significant quantity of harmful metals as a result of industrial operations, which has resulted in poisonings[3]. The race between them is led by arsenic and chromium. The water, food, and air that we come into touch with these metals through are the origins of toxicity. Bioaccumulation is one biological process that raises the amount in the human body, interfering with growth, proliferation, damage healing, and other bodily functions[4].These metals produces reactive oxygen species. It results in oxidative stress, inactivates enzymes, and impairs the body's antioxidant defense system. These metals bind to particular macro and micro-molecules and cause genetic instability. Because these harmful metals alter the structure of

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DNA, DNA repair is hampered[5]. They produce unwinding, which is commonly referred to as carcinogenecity, by binding with the hydrogen bonds of DNA. We currently know enough about metal carcinogenicity to warrant prompt, preventative, and efficient therapy. This is how bacteria suppress heavy metals through their chelation mechanism[6].

Various health surveys were conducted to study the harmful effects of hexavalent chromium on the health of people living in Unnao and adjoining industrial areas leading to Kanpur District. It was concluded that residents living in areas have higher complaints of Gasto-intestinal, dermatological and hematological abnormalities. Males and Females have higher number of Red Blood Cells with Low Number of Blood Platelets and MCV[7].

Figure 1 This Picture taken from Contaminated Water Supply in Unnao shows presence of neon colour water from handpump

Figure 2 Soil Field selected for Collection Of identification and isolation of Azotobacter for Bioremediation

1.2. Azotobacter study

Azotobacter which are free living nitrogen fixing are renowned for their adaptability to different ranges of environmental circumstances. Because of their capacity to detoxify heavy metals through enzymatic reduction, biosorption, and bioaccumulation processes, recent research has emphasized their potential in bioremediation . In particular, Azotobacter's reduction of Cr(VI) to Cr(III) not only purifies the environment but also changes chromium into a form that is less mobile and bioavailable [8]

1.3. Availability of Azotobacteria in the nature

Because of its high metabolic activity, the nitrogen-fixing, diazotrophic, nonparasitic bacterium Azotobacter is essential to the nitrogen cycle. Vitamins like thiamine and riboflavin can be produced by it[9]. Non-leguminous crops like cotton, rice, vegetables, sugarcane, sweet potatoes, and sweet sorghum are fertilized with it. It belongs to the family

Azotobacteriaceae. Azotobacter inoculation boosts mustard and rapeseed seed yield. It fixes about 30 kg of nitrogen per year and is commonly used in the sugarcane industry because it increases cane output by 25–50 t per hectare and sugar content by 10%–15%.Both acidic and alkaline soils contain Azotobacter[10]. Although A. chroococcum is the most common species in the soil, other species have also been found, including A. vinelandii, A. macrocytogenes, A. insignis, and A. beijerinckii. By preventing the growth of a number of dangerous fungi in the rhizosphere, Azotobacter creates antibiotics and antifungal compounds that reduce seedling mortality [11].

1.4. Isolation and identification of Azotobacteria from soil samples

From April to October 2023, soil samples were collected from the rhizosphere of plantations and agricultural crops in Unnao village as part of the study. Numerous media types, including nutrient agar, nutrient broth, and media specifically designed for Azotobacter (Ashby), were utilized in this work[12]. Additionally, a spectrophotometer and autoclave were employed, along with Bunsen, petri-dish, test tube, electric scales, pipette, and other tools. In this investigation, microorganisms were analyzed and evaluated using gram staining, catalase tests, and microscopic tests. The Verstraete method is used in soil respiration, along with the pour cup method to count colonies, the halo zone to assess Azotobacter's capacity to break down cellulose using CMC media, and a spectrophotometer to measure optical density[13].

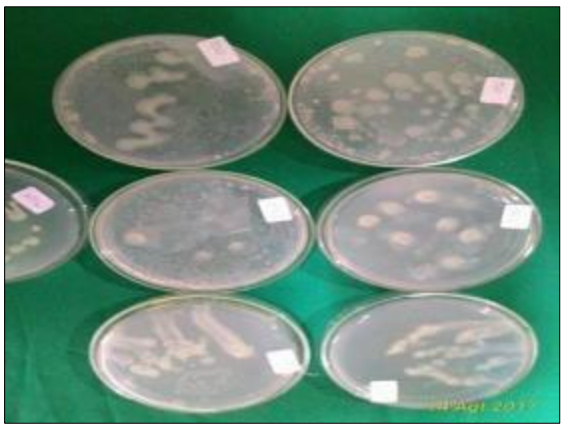


Figure 3 Above Petri dish shows the presence of Azotobacter isolated from the soil sample of district Unnao India following the isolation and identification protocol of azotobacter

Figure 4 Azotobacter in slant(Slant Culture) while in broth solution

2. Biochemical tests for identification of Azotobacteria

2.1. Catalase Test

Observation on bacterial ability to produce catalase enzyme was done by catalase test

Every culture sample cultivated on Ashby media has the capacity to create O2 and exhibits the property of releasing bubbles when 3% H2O2 is spilled. These results lend credence to the idea that Azotobacter also produces the enzymes catalase and reductase. The enzyme catalase has the ability to catalyze the conversion of hydrogen peroxide (H2O2) into oxygen and water[14]. When the colony is dumped into H2O2, the following chemical processes take place:

Catalase

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2} O_2 \text{ (air bubble) Peroxidase}$$

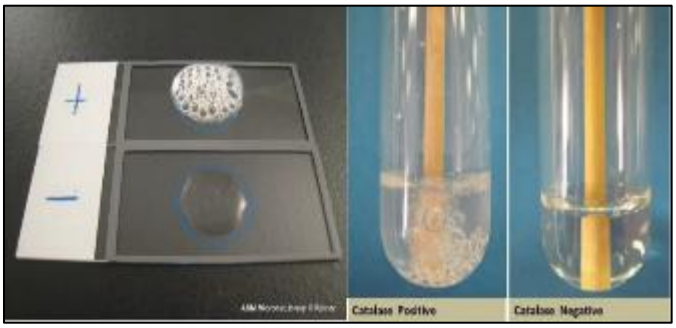


Figure 5 Slide and Test tube test For Azotobacter shows presence of bubble which is directly related to catalase positive strain of azotobacter

2.2. Indole test

Bacteria that express the tryptophanase enzyme are able to hydrolyze and deaminate the amino acid tryptophan. Tryptophan undergoes reductive deamination to produce indole through the intermediary molecule indole pyruvic acid. The amine (-NH2) group of the tryptophan molecule is eliminated during the deamination reaction, which is catalyzed by tryptophanase. Indiole, pyruvic acid, ammonium (NH4+), and energy are the reaction's end products. As a coenzyme, pyridoxal phosphate is necessary. The solution changes from yellow to cherry red when indole is mixed with Kovac's Reagent, which contains hydrochloric acid and p-dimethyl-amino-benzaldehyde in amyl alcohol. Since amyl alcohol is insoluble in water, it will develop an oily film on top of the soup to give it a crimson tint.**[15]**

2.3. Indole test results

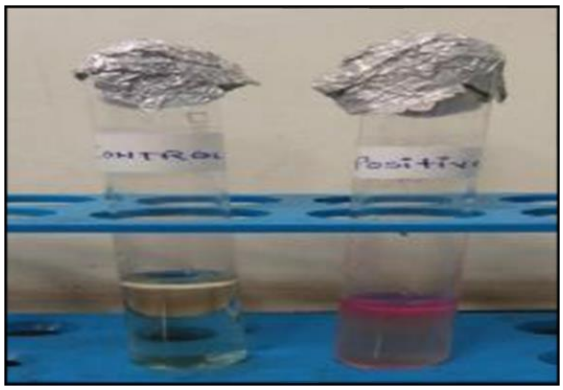


Figure 6 Presence of cherry red ring in the reagent layer shows Indole test positive for Azotobacter

3. Gram staining and optical density tests for identification of Azotobacteria

3.1. Bacterial gram staining

Gram staining is a crucial step in determining the type of bacterium, including both gram-positive and gram-negative ones [16]. Gram staining results revealed that the recovered isolates were rod-shaped and completely gram-negative in red or pink, suggesting that Azotobacter spp. gave them distinct properties[17].

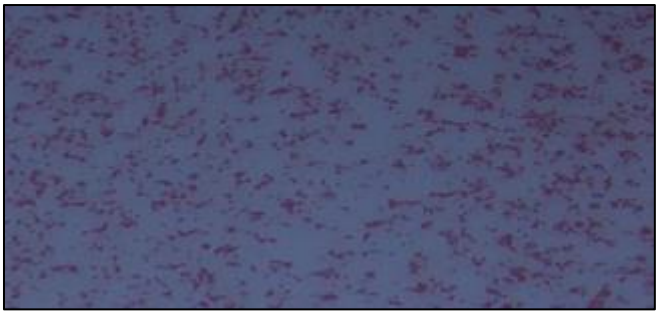


Figure 7 Appearance of Azotobacter

3.2. Optical Density

The optic density, as determined by the spectrophotometer with a wavelength of 550, reflects the growth rate of Azotobacter species in liquid media (nutrient broth). A spectrophotometer can be used to evaluate the population density of Azotobacter species in the nutritional broth; the more hazy the medium, the higher the optical density and denser population. Organic wetland rhizosphere samples (TSO) rank highest in this measurement, indicating that Azotobacter spp. proliferate and develop quickly in the natural organic atmosphere[18]. A number of potential isolates undergo biochemical testing in an effort to identify traits that are consistent with those of Azotobacter. These traits are then evaluated and measured in terms of their capacity to proliferate by measuring optical density and their capacity to break down cellulose by examining the size of the halo zone. Before being used in the field, all of the aforementioned test sets are helpful for the preliminary selection of isolates with benefits as biofertilizers and decomposers[19]. It turns out that in practically every metric, isolates from the organic rice rhizosphere have benefits in terms of both quality and quantity[20].

3.3. Sampling

For the Purpose of Testing Collection and Preparation of water samples from contaminated sites District Unnao, Uttar Pradesh, India is done . Samples Are collected from from Underground Water resources such as Handpumps , Wells And Tube wells. After Grab Sampling water samples were thoroughly Mixed and transferred to Laboratory following Specific Water Sampling Protocols

3.4. Sampling stations

Table 1 For the Purpose of sample collection from contaminated sites of district Unnao,India sample are collected and labelled as U1 U2 till U8 for easy labelling and testing

Sr. No.	Sampling station	Sampling code
1	Asoha	U-1
2	Sikanderpur Karan	U-2
3	Hasanganj	U-3
4	Sikanderpur Sirausi	U-4
5	Miyanganj	U-5
6	Purwa	U-6
7	Hilauli	U-7
8	Dharamkata(Before Bichia)	U-8

40 litres of Water samples are collected from 8 sampling station 5 litres each for the performance of 12 physico-chemical parameter test of water quality assessment on water samples[21].

3.5. Chromium Content

Chr (VI) is used in many industries as chromic acid and in various forms. Chromium acid is used in the electroplating process by the chrome plating business. This industry's wastewater may contain Cr (VI) ions. In order to prevent metal

corrosion, chromatic acid is also added to the water used in industrial cooling towers [22]. The use of Cr (VI) in 37,500 building roof cooling towers that leaked coolant into the air, resulting in an estimated 20 cancer fatalities, was recently prohibited by the EPA. Additionally, paints, pigments, tanning treatments, inks, fungicides, and wood preservatives include hexavalent chromium.The highest amount of Cr (VI) that can be discharged into waterways is 50 μg/L. Drinking water has a far lower level. Industrial contamination is thought to be suggested by a level more than 3 μg/L [23].This project will help you create a calibration curve for the detection of Cr (VI), solve a mystery surrounding Cr (VI) pollution, and look at various ways that industry might reduce the concentration of Cr (VI) before releasing their waste water.[24]

3.6. Contaminated water collected from Sampling stations of District Unnao, India

Water sample collected from different underground sources of water supply in Unnao region of Uttar Pradesh India shows Different types of characteristics such as

- Slimy Nature
- Froth Appearances
- Yellow to Green Colour
- Foul Odor
- Bad Taste

It can be easily seen that water supply From Underground water resources such as Handpump tubewells and wells that water is not fit for Drinking but rural people in the Area are forced to use it for their daily purpose. Below here are some Pictures taken from contaminated water supply Areas of District Unnao , Uttar Pradesh India.

Figure 8 Contaminated Water Supply From Sampling Area Of Bichia Unnao shows presence of green to neon color from the handpump which is consumed by the rural population for daily purpose

Figure 9 Froth like Appearance in water Supply of Sampling Area Bichia Unnao shows that the water supply is unfit for drinking

Figure 10 Presence Of Slimy Substance In water Supply Of Unnao which can be easily seen and felt while using the water from the contaminated sites of Bichia Unnao India Region

4. Procedure

4.1. Methods of Sampling and Preservation

4.1.1. Preparation of Standards

Prepare six standard solutions in large test tubes which can accommodate 15-20 mL of solution. Mix the quantities of Cr (VI). Mix the quantities of Cr (VI) solution and 0.18 M sulfuric acid given in the table below to make solutions 1-6.

Solution #	**1**	**2**	**3**	**4**	**5**	**6**
Cr (VI), mL	0.0	0.4	1.0	2.0	4.0	10.0
H2SO4, mL	10.0	9.6	9.0	8.0	6.0	0.0

Into each test tube pipet 0.5 mL of dipenylcarbazide solution. Mix the contents of the test tubes and let them stand for 5 minutes for color development.

Measure the absorbance of each sample at 540 nm and plot a standard curve. For the blank, use test tube 0. The absorptivity for the diphenylcarbazide-Cr (VI) complex is 40,000 Lg-1cm-1 a 540 nm.4[25]

4.1.2. Determination of Chromium Concentration in Water Samples

- Prepare a water sample for testing by placing 10.0 mL of water in a test tube. Test a sample of tap water and the sample labeled polluted water.
- Add 12 drops pf 3 M sulfuric acid to the sample.
- Pipet 0.5 mL of diphenyl-carbazide solution into the test tube and allow 5 minutes for color development.
- Determine the amount of Cr (VI) present by measuring the absorbance of the sample at 540 nm.of Cr (VI)

4.1.3. Reducing Cr (VI) Levels for Disposal

Dilution Method

The maximum permissible level of Cr (VI) allowed to be released in 50 μg/L. Assume that an industry has 100 L of Cr (VI) polluted water containing 0.127 mg/L of Cr (VI). Calculate how many liters of chromium-free water must be mixed with the polluted water so that it can be released.

Reduction Method

Cr (VI) can be easily reduced to Cr (III). A maximum permissible level of Cr (III) of 1,000 μg/L may be released into natural waters.

Take a sample of the polluted water and add 5 drps of ascorbic acid solution (a mild reducing agent). Swirl the mixture and determine the Cr (VI) spectrophotometrically[26].

5. Determination of Heavy Metal Concentration through Atomic Absorption Spectrometer

5.1. Digestion of sample

The APHA Standard technique (APHA 2005) was used to digest the water sample.

A 100 ml sample of water was collected in a beaker. HNO3 (5 ml) was added. Under fume hood conditions, the solution was put on the hot plate. Following the vaporization of the remaining 10 ml of the solution, 100 ml of deionized water was added to the volumetric flask (APHA 2005)

5.2. Results obtained from chromium contaminated water samples from different sampling stations

Table 2 This table records the various parameters tested of the water samples from the contaminated sites of District Unnao. It clearly shows that U8 sampling station Bichia contains hexavalent chromium far above the permissible level(0.05 mg/l)

S. No.	Code	pH	EC	TDS	TH	Alkalinity	Cl	F	NO_3	Fe	SO_4	Mg	Cr
1	U-1	7.92	701	1129	255	496	240	0.79	5.9	0.56	386	19.43	0.01
2	U-2	7.90	526	426	206.2	269	28.8	0.79	4.52	0.69	7.01	37.23	0
3	U-3	6.9	462.3	289	201.9	399	42.43	0.40	9.92	0.07	31.9	35	0.21
4	U-4	7.79	647	459	179	408.9	223.7	2.03	3.42	2.31	72.23	32.33	0.17
5	U-5	7.97	567	412.3	216	253	38.02	0.29	28.29	0.27	46.03	36.29	0.023
6	U-6	7.69	963	511.02	247.63	436.28	173.97	1.032	31.02	13	43.82	27	0.0112
7	U-7	7.82	857	392	198.03	273.79	43.27	0.81	3.72	0.39	29.37	41	0.0102
8	U-8	7.8	578.9	459.37	247	382	69.23	1.03	23.36	0.79	28.29	41.02	2.10

Parameters	WHO	EPA	EU	Egyptian
PH	6.5-8.5	6.5-8.5	6.5-8.5	6-9
Cond., µS/cm	-	-	400 GV*	-
Colour	-	15 units	-	-
TDS, mg/L	1000	500	500	1500
PO_4^{3-}, mg/L	0.3	-	-	0.3
SO_4^{2-}, mg/L	400	500	250	400
Cl^-, mg/L	250	250	250	600
NO_3^-, mg/L	45	45	-	45
Ca, mg/L	200	-	100 GV	200
Mg, mg/L	150	-	50	150
Na, mg/L	200	-	150	-
K, mg/L	-	-	12	-
Al, mg/L	-	0.05-0.2	0.2	-
B, mg/L	0.3	-	1 GV	-
Fe, mg/L	0.3	0.3	0.2	1
Mn, mg/L	0.05	0.05	0.05	0.5
Cu, mg/L	1	1	0.1	1
Zn, mg/L	5	5	0.1 GV	5
As, mg/L	0.01	0.01	0.05	0.05
Cd, mg/L	0.005	0.01	0.005	0.01
$Cr_{(total)}$, mg/L	0.05	-	0.05	0.05
Hg, mg/L	0.001	-	0.001	0.001
Ni, mg/L	-	0.1	0.05	0.1
Pb, mg/L	0.05	0.005	0.05	0.05
Se, mg/L	0.02	0.05	0.01	0.01

WHO 1993; US.EPA 2001; EU 1997; ECS, 1994; GV: Guide value*

Figure 11 Permissible values of various physicochemical parameters fixed by various regulatory agencies

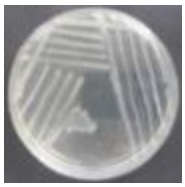
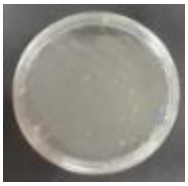
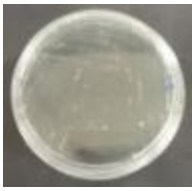
5.3. Instrument and Material Sterilization

All glassware was sterilized using an autoclave (ASC, Jerman) set to 121°C for approximately two hours. Non-glassware was sterilized by soaking it in a solution of chlorine at a specific concentration for half an hour, then washing it with Aquadest and allowing it to dry. An autoclave was used to sterilize the stock solution, physiological solution, nutrient broth (NB), nutrient agar (NA), and other materials[27]. For approximately sixty minutes, the medium with autoclaving.

6. Biodegradation Tests

6.1. Minimum Inhibitory Concentration (MIC) test of chromium – Azotobacter S8 bacteria

At 24 and 48 hours, as well as continuing for up to 168 hours, the Minimum Inhibitory Concentration (MIC) test was carried out. The agar medium containing chromium in different quantities that were determined in the preliminary test was inoculated with the Azotobacter bacterium. Five milliliters of growth medium, five milliliters of pollutant, and seventy parts salinity were combined to create the agar medium. A chromium stock solution was diluted with saltwater to yield the pollutant with an initial salinity of 70 ppt. NaCl was then added to achieve the desired salinity. This was done in order to produce a growth medium that was 35 ppt salinized, distinct from the previous dilutions. This 35 ppt salinity promotes the growth of azotobacteria. To each testing samples 0.5 ml of peptone water was added . This Peptone water boosts the bacterial growth providing additional nourishment to the bacterial cells The following stage involved identifying the concentration to be employed in the chromium removal test using a screening method [28]. Table displayed the results of the screening test and the 24- to 168-hour incubation period. Table indicates that bacterial growth is reduced in the media with a greater chromium concentration. Azotobacter exhibits the best growth on the media devoid of chromium. The bacteria thrive well when exposed to a chromium concentration of 20 mg/L. This circumstance demonstrates that the bacterium can withstand both concentrations. After 72 hours of incubation, Azotobacter exhibits good growth at the 45 mg/L concentration. This circumstance shows that the growing exposure to chromium concentrations is beginning to limit the growth of the Azotobacter bacterium. Azotobacter has a high tolerance to chromium concentrations ranging from 0 to 50 mg/L[14].The findings were scored using the MIC test. On the petri dish the area of growth of bacteria and corresponding change in color when compared to control media were used to calculate the MIC scoring. The biggest worry was the region where bacteria were growing on the medium surface. The proliferation of germs on the medium indicates that the bacteria are able to endure in the contaminated media[28]. The following grading criteria were applied in this investigation for the MIC observation:

Time(hrs/days)	Chromium concentration(mg/L upto)		
	0	20	45
24/ 1 day			
48/ 2 days			
72/ 3 days			
96/ 4 days			

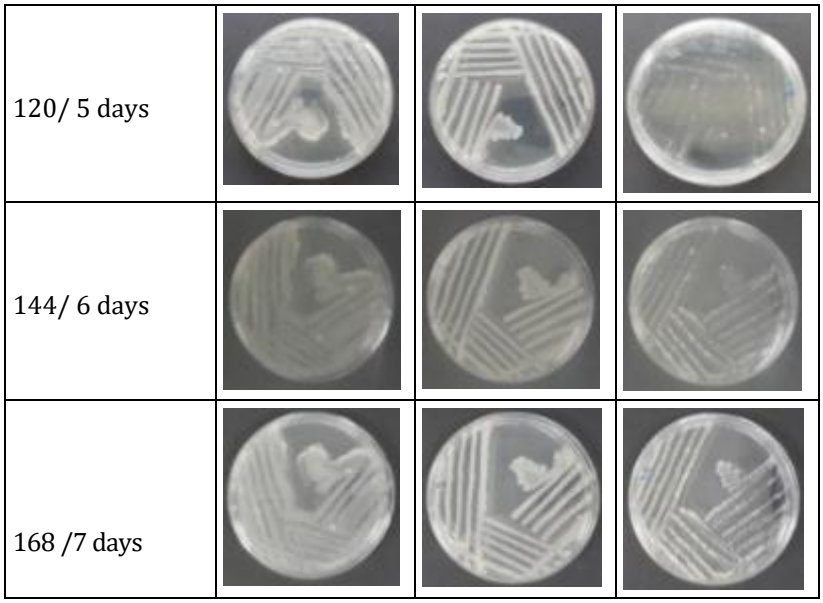


Figure 12 Minimum Inhibitory concentration Test shows the interaction of Azotobacter colonies with hexavalent chromium in an inverse relation

There was no growth for the concentrations at:

- 80 mg/L
- 150 mg/L
- 300 mg/L

7. Determination of Heavy Metal Concentration through Atomic Absorption Spectrometer

7.1. Digestion of sample

The APHA Standard technique (APHA 2005) was used to digest the water sample that had the calculated amount of azotobacter concentration up to 5mg/l, 20mg/l, 45mg/l and 70mg/l . A 100 ml sample of water was collected in a beaker. HNO3 (5 ml) was added. Under fume hood conditions, the solution was put on the hot plate. Following the vaporization of the remaining 10 ml of the solution, 100 ml of deionized water was added to the volumetric flask (APHA 2005)[29-32].For higher concentration testing potassium dichromate was added in order to check the efficiency of Azotobacter on the biodegradation of hexavalent chromium.

Table 3 Result Obtained of Azotobacter treated and inoculated water sample.

Absorbance	**After 24 hrs**	**% reduction**	**After 48 hrs**	**% reduction**	**After 72 hrs**	**% reduction**	**After 96 hrs**	**% reduction**
Absorbance at 358 nm	5mg/l	15	20mg/l	30	45mg/l	65	70mg/l	75

Above table shows the reduction of hexavalent chromium from azotobacter treated water sample and corresponding time needed for reduction.

Figure 13 After bioremediation of water sample using azotobacter as chelating agent Change in color is observed

8. Conclusion

The present study concludes that azotobacterial strains isolated from soil of unnao have the ability to resist higher concentrations of hexavalent chromium. In this work hexavalent chromium was found up to 2.1mg/l which is removed easily by the azotobacter strains. However higher concentration of hexavalent chromium was also tested by performing specific experiment which was also removed successfully. Hexavalent Chromium is released into the environment by a large number of processes such as electroplating, leather tanning, wood preservation, pulp processing, and steel manufacturing. This metal is of major concern because of its larger usage in developing countries and their non-degradable nature. This toxic heavy metal is very dangerous for human health as it causes severe diseases in Humans including cancer. The physical parameter studied in the water samples collected from under ground water resources namely hexavalent chromium was at higher level. From this study, it may be concluded that potential strains of Azotobacteria having the capacity of effective biosorption of heavy metals which is chromium and can be utilized for bioremediation of heavy metal as an eco-friendly measure.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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