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DETOXIFICATION OF WASTEWATER USING ISOLATED BACTERIAL STRAINS

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ABSTRACT

Detoxification or Bioremediation is an innovative and most promising technology in today's world to overcome the effect of pollutants and to neutralize the harmful pollutants from contaminated sites using microorganisms. The present study was based on the bioremediation of the wastes obtained from the sites situated nearby the banks of The River Chambal. The aim was to study the physico-chemical properties and the isolation of bacterial population from the various study sites and their bioremediation potential. The bacterial species were isolated, identified and characterized as per standard methods and their capability to reduce industrial waste contaminants was determined for the process of bioremediation. The physicochemical qualities of the effluent samples of one Wastewater sites (Raipura site) and one control site (Akelgarh site) in Kota district of Rajasthan, India were evaluated. The physicochemical parameters assessed included color, odor, pH, temperature, TDS, turbidity (NTU), BOD, COD, DO and Heavy metals. The results of the evaluation were as follows: the colour was found milky with very pungent odour and temp. (28 ºC-36ºC), COD (17-92mg/L), and turbidity (1.96-286 NTU), BOD (2-7mg/l), DO (1-7mg/l) and TDS (175-1890mg/l) and also these parameters were not found to be in the recommended limits. Some of the parameters exceeded from their normal ranges and some was found to be very low and considering the

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samples unhealthy for drinking and highly polluted. At Akelgarh site, *Streptococcus pyogene*, *Escherichia coli* and *Bacillus cereus* bacteria was found as prominent, At Raipura site, *Nitrosomonas*, *Nitrobacter* and *Pseudomonas aeruginosa* bacterial strains was found in high amount. To determine the adaptation and reducing capability of these bacteria various physio-chemical properties were studied and as a positive result was obtained in the form of decreased level of BOD, COD, and turbidity while an increase in the DO level of the water samples collected. The Raipura site was found highly contaminated. The heavy metals were concentrated in the research area, which suggests that the river is severely polluted. Zn was found highly (267ppm) accumulated in the sewage water of Raipura site followed by Cr. We conclude that these sewage dumping areas of water sources are polluted to their respective receiving watersheds and threats to public and environmental health.

Keywords: *Chambal River, Bioremediation, Bacteria, physicochemical, reduction potential*

Introduction

The shortage of water resources has become a globally serious problem. Sewage after appropriate treatment and the reuse of the recycled water have become the consensus of all over the world [1]. With growing population, advanced agricultural practices, industrialization, urbanization, and multiple use of water has increased the demand for water. According to the manual of the Central Public Health and Environmental Engineering Organization (CPHEEO), Ministry of Urban Development, in India the per capita per day water requirement is 100lpcd [2]. Domestic wastewater treatment has become a remarkable aquatic environmental problem for all over the world.

Bioremediation is a recent and promising technology using organisms or microbes to remove or neutralize harmful pollutants from various polluted sites which includes water and soil. It requires a set of techniques to select those organisms capable of uptake or release some enzymes which can degrade these pollutants. The biological materials are much more effective than the traditional strategies because of its direct action at the site of contaminant without the need of transferring contaminant materials [3]. In the bioremediation process, bacteria alone,

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consortia, or combination of bacteria and algae can be used for effective biodegradation [4,5]. Bacteria, Fungi, and algae are the most known organisms for bioremediation of toxic hazardous materials including heavy metals [6].

The current research was directed in the largest state of India that is Rajasthan and particularly of the Kota district. Due to arid and semiarid climate and insufficient surface water resources, Rajasthan depends heavily on groundwater for drinking and for irrigation [7]. In addition, Chambal River is the fundamental resource of water necessary for various thermal, fertilizers, chemical and glass industries in Kota. In Kota city, there are two water treatment plants in city, named as Akelgarh (270 MLD) and Mini Akelgarh (Sakatpura, 130 MLD) distributing about 390 MLD water to the public. Due to water treatments, contaminants are less at this site. Therefore, this site has been used as a control in this study. On the other hand, all the wastewater of big industrial of Kota city meets in Raypura Naala. Thus, these all effluents are being continuously added to water bodies and affect the physiochemical quality of water making them unfit for use of livestock and other organisms [8]. Additionally, this also affect the quality of soil health and ground water and plant tissues of the region [9]. Hence, Raypura naala have been used as polluted site in the present study for bioremediation. Keeping these points in view, the present study has been designed accordingly to remove the pollutants from the study area using microbial methods for Bioremediation.

Material and Methods

I) Collection of water samples

Sewage sample was collected according to standard procedures from APHA [10] (American Public Health Association) [11,12].

II) Physicochemical analysis of water

Selected physicochemical parameters such as color, odor, pH, temperature, turbidity, TDS, BOD, COD in the water samples were analyzed according to **APHA, 1995** [11,13]. The DO parameter was analyzed according to Romanian Standard **(SR ISO 5814, 1990)** [14,15,16].

III) Isolation and Identification of micro-organisms from water samples

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A) Isolation of Bacteria

The samples for bacterial isolation were processed in the laboratory and characterized based on Cultural characterization, biochemical identification and Microscopical observations. [17].

B) Identification of bacteria [17].

- **Gram's staining:** The cultures were differentiated into Gram positive and Gram negative based on the stains retained by the bacterial cells and characterized under the microscope.
- **Endospore staining:** The Schaeffer-Fulton`s method was used in the study.
- **Biochemical Characterization:** The Biochemical identification of all the isolates was further obtained by performing various biochemical tests (Indole test, Citrate Utilization, Urease test, Nitrate Reduction, Catalase test, Oxidase test, Motility testing and Haemolysis was performed on Blood agar) according to Bergey's manual of systematic Bacteriology (1994). [17].

IV) Bacterial adaptation (Kumar *et al.*, 2013)

Bacterial adaptation studies were carried out using bacteria grown in Nutrient broth in increasing concentration of sterilized sewage. The various concentration of sterilized sewage included 5 %, 10 %, 20 %, 30 %, 40% 50% 60%, 75%, 90% and 100%. The transfer of culture from one concentration to the next was made when the respective bacteria was in the exponential phase of growth [18].

V) Estimation of bioremediation potential of bacteria

A) Characterization of wastewater samples- Sterilized wastewater samples were characterized before and after the treatment. All parameter such as pH, BOD, COD, TDS, Dissolved Oxygen, Turbidity, Temperature and Heavy metal were analysed by using Standard Methods for the Examination of Water and Wastewater (APHA, 2005) [12]. Removal efficiencies of all parameters were calculated according to the following equation:

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Removal Efficiency (RE %) = C0-RC/C0 × 100 Where, C0 =Initial Concentration before Treatment, RC= Final Concentration after Treatment.

B) Bioremediation potential of bacteria (Merugu *et al.*, 2014; Sonune and Garode 2015)- Sewage water samples were filtered through a Whatman filter No. 42 and sterilized. Each bacterial cultures were inoculated individually in pre-sterilized 50ml wastewater broth (WWB) and kept in shaker at 120 rpm for 72 h. After treatment, all samples were centrifuged at 10,000 rpm for 20 minutes at 10°C and supernatants were used for further analysis [19,20].

VI) Statistical analysis

All experimental data obtained and analysed by using analysis of variance (ANOVA). The real effect data then continued with Multiple Range Test (Duncan) with a significance level of 5% [21].

Results and Discussion

I. Analysis of Physicochemical parameters

In the present investigation, water samples were collected from 2 sites (one was the control site and 1 was taken as test site) of the Kota district to determine various physiochemical parameters. The results revealed that at Akelgarh site (control site) all the parameters were obtained within or around the standard range depicting the pH range from 7.0-8.5 with no odour, Temperature from 28°C to 36°C, BOD 2-2.7 mg/l whereas the content in COD was observed from 5-8.12mg/l. The level of DO was found 5mg/l to 7mg/l. The quantity of TDS varied between 175 mg/l to 193 mg/l. The visualization in terms of turbidity varied from 1-4 in terms of NTU (Nephelometric Turbidity Unit). All parameters were showed variations according to the months. At Raipura, pH range was observed in between 7.7-8.4, temperature 29°C to 33°C. The odour was very pungent, BOD was recorded in range 4.93 mg/l to 6.54 mg/l whereas, COD was observed from 77 mg/l to 99 mg/l which was very high as compared to standard values, resulting from the elevated organic discharge in water body. The level of DO was 1.28 mg/l to 2.70 mg/l and quantity of TDS varied between 530 mg/l to 1890 mg/l. The visualization in terms of turbidity varied from 122-286 in terms of NTU.

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II. Analysis for the presence of heavy metals

At Akelgarh site (Control), The amount of Pb was found maximum in March months (0.548ppm), and Cd was found to be maximum in September (0.688ppm), Zn in December (208ppm) while Cr in March (1.256 ppm). At Raipura site, the amount of Pb was observed maximum in September (4.321ppm), similarly Cd was found to be maximum in September (0.778ppm). Zn was found to be maximum in December (267.97ppm) while Cr was maximum in September (6.484 ppm). 3/4 heavy metals resulted to be maximum in the month of September.

III. Isolation and Identification of bacteria

The samples were plated on Nutrient media using serial dilution and pour plate methods. Furthermore, the subculturing was performed using streak plate method. The isolates were characterized on the basis of their characteristic colony morphology and appearance on the media and identified by the biochemical characterization on the basis of their metabolic activities (Fig. 1,2 and Table-1,2).

On the basis of different biochemical tests performed for the identification it was revealed that At Akelgarh site, three bacterial strains namely *Streptococcus pyogene, Escherichia coli* and *Bacillus cereus* were isolated which was taken as control site of water sample. Accordingly, nine bacteria were isolated from Raipura site: *Nitrosomonas, Nitrobacter, Pseudomonas aeruginosa, Staphylococcus aureus, Nitrobacteria windogradskyi, Bacillus licheniformis, Enterobacter* spp., *Pseudomonas denitrificans, Rhodococcus terrae* and *Thiobacillus ferrooxidans*. Prominent bacteria such as *Nitrosomonas, Nitrobacter* and *Pseudomonas aeruginosa* were taken for further studies.

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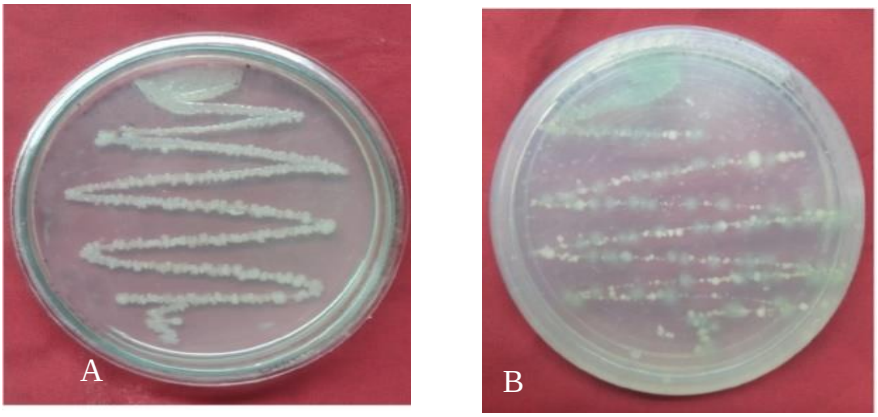

Figure 1: Bacterial isolates from water sample of A. Akelgarh site (AZ-III**), B. Raipura site (**RP- III).

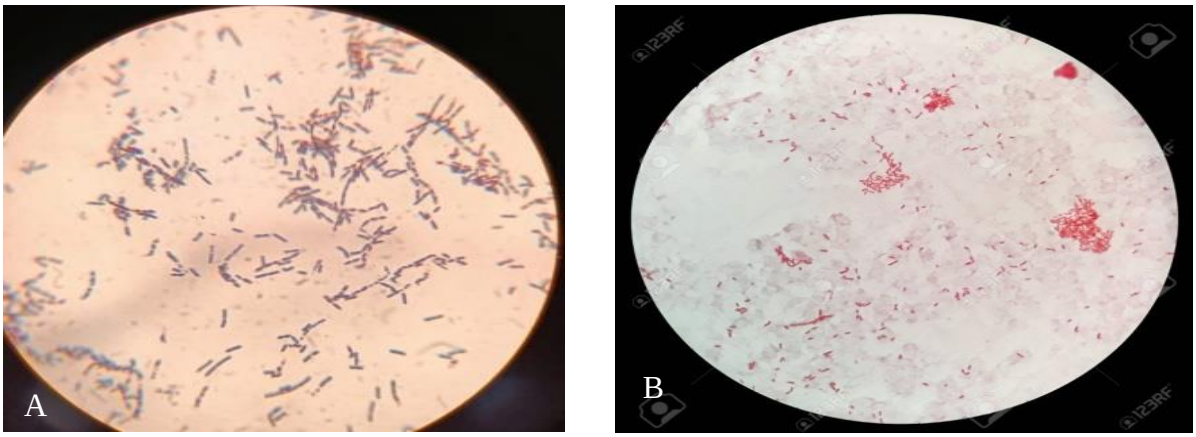

Figure 2: Gram staining of bacterial isolates from water sample of A. Akelgarh site (AK-III**), B. Raipura site (**RP-III).

Table-1 Cultural and Microscopic Characterization of various isolates obtained from water samples

S.	Isolated Bacterial	Cultural Characterization	Microscopic

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No.	Species		Observation
1.	*Streptococcus pyogenes*	Dome shaped with Gray to whitish and usually glisten colonies with clear margins	Gram-positive cocci, arranged in chains
2.	*Escherichia coli*	Large, thick, greyish white, moist, smooth, opaque, or translucent discs	Gram-negative, straight, rod-shaped, non-sporing,
3.	*Bacillus cereus*	Vegetative cells, singly or in short chains, granular or wrinkled colonies	Gram-positive aerobic or facultatively anaerobic, motile, spore-forming, rod-shaped
4.	*Nitrosomonas*	Change in the colour of the media from red to yellow	ellipsoidal or rod-shaped cells
5.	*Nitrobacter*	The media changes colour from clear to cloudy	rod-shaped, pear-shaped, or pleomorphic
6.	*Pseudomonas aeruginosa*	large, opaque, flat colonies with irregular margins, fruity odour	Gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium

Table-2 Biochemical Characterization of various isolates obtained from water samples

S. No.	Name of the test	Akelgarh site (Control site)			Raipura site		
		AZ- I	AZ-II	AZ- III	RP- I	RP-II	RP- III
1	Gram's Staining	Positive	Negative	Positive	Negative	Negative	Negative

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2	Shape	Cocci	Rod	Rod	Rod	Rod	Rod
3	Motility	Non- motile	Motile	Motile	Motile	Motile	Motile
4	Spore	No spore	No spore	Spore forming	No spore	No spore	No spore
5	Catalase	Negative	Positive	Positive	Positive	Positive	Positive
6	Oxidase	Negative	Negative	Negative	Positive	Positive	Positive
7	Citrate	Negative	Negative	Positive	Negative	Negative	Positive
8	Nitrate reduction	Negative	Positive	Variable	Positive	Positive	Positive
9	Urease	Negative	Negative	Negative	Positive	Positive	Positive
10	Indole	Negative	Positive	Negative	Negative	Negative	Negative
11	Hemolysis	Beta-hemolytic	Some strains show	Beta-haemolytic	Non-hemolytic	Non-hemolytic	Beta-hemolytic
Isolated Bacterial Species		*Streptococcus pyogenes*	*Escherichia coli*	*Bacillus cereus*	*Nitrosomonas*	*Nitrobacter*	*Pseudomonas aeruginosa*

Thakur *et al.*, 2016 observed the potential of indigenous bacteria from river Chambal flowing through Kota (Rajasthan) to degrade oil under in vitro conditions. The isolated namely *Pseudomonas* sp., *Bacillus* sp. and *Staphylococcus* sp. were obtained from Chambal water and then subjected to various biochemical tests for identification. Results revealed that these strains from river Chambal had better biodegradation potential for oil and can be used as potent hydrocarbon degraders [22]. Kumar *et al.*, 2016 investigated the bioremediation potential conducted on effluent discharge collected from textile industries of Bhilwara, Rajasthan-India [23]. Based on the results obtained from morphological, biochemical, and molecular datasets he identified potential bacterial strains belonging to species *Bacillus cereus, Exiguobacterium* sp. and *Acinetobacter* sp. All selected bacterial isolates showed decolourization of the dye effluent.

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Therefore, the study reveals that textile effluents consisting of potential bacteria that utilize the dyes as the source of energy and exhibit the property of treating of industrial effluents [23]. Verma *et al.*, 2017, studied the isolation and characterization of lead resistant bacteria isolated from two water bodies Udaisagar lake and Gadwa Pond of Berach river system, Udaipur, Rajasthan, India. Initially, among 13 of the total isolates were screened by him from water samples, 2 isolates were selected for study based on high level of heavy metal resistances [24]. Based on morphological, biochemical, and molecular characterization using 16S rDNA sequencing his isolates were identified as *Pseudomonas stutzeri* (KX692284) and *Pseudomonas stutzeri* (KX692285). The isolates exhibited high resistance to lead (Pb). His work furthermore stated that the *Pseudomonas stutzeri* showed highest MIC value for lead up to 1300 mg/l concentration. He concluded that lead resistant bacteria could be useful for the bioremediation of lead contaminated sewage and wastewater from various industries [24].

IV. Bacterial Adaptation

The bacterial isolates were inoculated in different percentage of sterile sewage water to identify the growth on the basis of turbidity at the 12 hrs. interval. The turbidity was increased up to 60% sterile sewage water. More than 60% sterile sewage waters i.e., 75%, 90% and 100% no turbidity was recognized. As shown, the bacterial isolates from each site had turbidity in increasing order till 36 hrs. with 60% sterile sewage water. Consequently, each bacterial isolate showed bacterial adaptation till 60% concentration of sewage water (Fig. 3).

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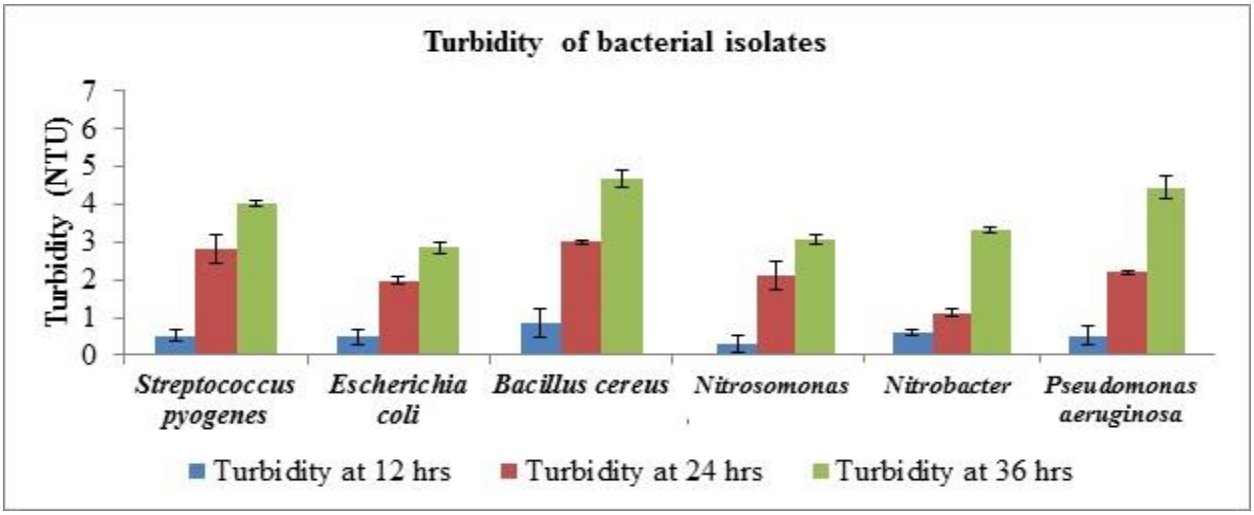

Figure 3: Turbidity of different bacterial isolates from Akelgarh and Raipura site

V. The potential of bacterial species to improve the level of contaminants

The reduction potential of contaminants level by isolated bacterial species based on BOD, COD, DO, TDS, and turbidity revealed that BOD of water sample at the Raipura site found reduced, and turbidity and the level of dissolved oxygen increased significantly. The dissolved oxygen increased by 14.47%, 32.32% and 17.51%, respectively by *Nitosomonas* species, *Nitrobacter* species and *Pseudomonas* species in comparison to untreated sample at Raipura site. The highest bioremediation potential was shown by *Nitrobacter* species (Table-3).

Table 3: The potential of bacterial species to improve the level of contaminants at Raipura site

Raipura site					
S. No.	**Water Parameters**	**Sterile Sewage water**	***Nitrosomonas* species**	***Nitrobacter* species**	***Pseudomonas* species**

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1.	BOD (mg/l)	6.19±0.16	5.28±0.344	5.9±0.116	`5.0±0.123
2.	COD (mg/l)	78 ±2.05	69±4.99	68±3.41	74±2.89
3.	DO (mg/l)	2.97±0.18	3.4±3.12	3.93±2.74	3.49±1.87
4.	TDS (mg/l)	854 ±3.21	794±4.91	763±3.70	786±3.87
5.	Turbidity (NTU)	171 ±1.23	182±1.44	180±1.29	191±2.19

Min Jin *et al.*, (2005), reported that 91.7% COD removal and 99% ammonical-nitrogen removal efficiency can be obtained using *Nitrosomonas europea*, *Nitrobacteria windogradskyi*, *Bacillus licheniformis, Bacillus megatherium, Bacillus sphaericus* for the treatment of domestic sewage [25]. Behera *et al.*, 2020 reported the presence of a microbial community of bioremediation potential in terms of relative abundance and taxonomic biodiversity in sediment samples of river Ganga and Yamuna, India at nine different sites [26]. Shrestha *et al.*, 2020 study illustrated the efficiency of microalgae-based treatment system for the bioremediation of Yamuna water. They checked all the physicochemical parameters like pH, dissolved oxygen (DO), biological oxygen demand (BOD) and chemical oxygen demand (COD) using microalgae during the 20 days of study and observed a significant change in all these parameters, specifically ~37% reduction in both biological oxygen demand and Chemical oxygen demand was achieved while the DO was increased by 116% during study [27].

Pushkar *et al.*, 2019 analysed the physicochemical characteristics of the Mithi river (Mumbai) water samples has confirmed the deterioration of river water. In the study, mercury-resistant bacteria were isolated from Mithi river and identified by biochemical and molecular analysis [28].

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Conclusions

The present research focuses on the potential of bacterial species to effectively reduce the industrial waste contaminants present in wastewater of Chambal River of Kota district, Rajasthan. The adaptation capability and the potential of the isolated individual bacteria to improve the level of water contaminants was determined and as a positive result was obtained. The isolated bacteria were found to decrease the level of BOD, COD, and turbidity while increased the DO level in the sample water over a course time. Therefore, it can be concluded from the present study that bacteria can be best candidates to improve the water quality and reduce the level of contamination from the Chambal River. The use of bacteria for reduction of water contaminants is chemical free, non-toxic, cheap, and eco-friendly. The study was conducted *in vitro* and needs to be taken up to molecular level for the future use of microorganisms for bioremediation and reduction of water pollution needs to be studied at a commercial scale.

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Conflict of Interest: The authors have no conflicts of interest.

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