Nature Environment and Pollution Technology
An International Quarterly Scientific Journal
p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454
Vol. 24 | No. 1 | Article ID D1670 | 2025

Original Research Paper
https://doi.org/10.46488/NEPT.2025.v24i01.D1670
Open Access Journal

Using *Azospirillum* Bacteria Isolated from Soil as Bioremediation Agent in Wastewater Contaminated with Cadmium in Iraq

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Abbreviation: Nat. Env. & Poll. Technol.
Website: www.neptjournal.com

Received: 12-05-2024
Revised: 15-06-2024
Accepted: 21-06-2024

Key Words:

Azospirillum
Cadmium
Bioremediation
Biosorption
Wastewater

Citation for the Paper:

Abbas, Z. R., Al-Ezee, A. M., Al-Shandah, B. T. and Shafeeq, M. A., 2025. Using Azospirillum bacteria isolated from soil as bioremediation agent in wastewater contaminated with cadmium in Iraq. *Nature Environment and Pollution Technology*, 24(1), D1670. https://doi.org/10.46488/NEPT.2025.v24i01.D1670

Note: From year 2025, the journal uses Article ID instead of page numbers in citation of the published articles.

ABSTRACT

Bioremediation is an important technique to remove heavy metals from wastewater. The current research aimed to use *Azospirillum* bacteria in removing cadmium ions from wastewater. The source of *Azospirillum* bacteria was the soil of Al-Mishkhab in Al-Najaf province, Iraq (rice fields), while the source of wastewater was taken from the Al-Rustamia wastewater treatment plant, in Baghdad in October 2020. All the experiments were carried out in Soil and Water Research Center, Ministry of Science and Technology. After collecting the soil, the microorganisms were isolated through the Immunomagnetic beads (IMB) process and were incubated on a certain synthesized medium. The concentration of cadmium ion was determined through the Absorption Spectrophotometer (AAS) technique. The *Azospirillum* colonies were identified and characterized as white colonies while the concentration of cadmium ion ranged from 0.03-1.6 mg/L and applying the microorganism on the wastewater will decrease the concentration up to 99.9% in a process called biosorption. Treatment time was also studied for 24, 48, 72, and 168 hours. The statistical analysis shows that increasing time will enhance the removal of cadmium. Cadmium is one of the heavy metals responsible for soil contamination; bacteria play a crucial role in bioremediation, demonstrating stability in decomposing various compounds and materials. *Azospirillum* is employed for soil decontamination purposes; increasing incubation time will enhance the removal of the trace element; also further investigate the effect of other factors such as temperature, pH, and the effect of using other microorganisms.

INTRODUCTION

Bioremediation is a method used to take out pollutants and remove them using microorganisms such as bacteria. However, due to the development of civilization and industry, as well as the huge increase in the activities of human beings, consequently, it has a bad reflection on the environment and increased the rate of pollution which mostly ends and accumulates in water as water pollutants (Manisalidis et al. 2020).

Bacteria and sometimes fungi are used in bioremediation due to their ability to decompose compounds and materials and devour them as well which ultimately detoxifies the surroundings such as water or soil and makes it not hazardous to the environment and all living creatures (Bala et al. 2022), this indicates its microbiological activity. In this, the microorganism can behave as an agent that can store the pollutants after devouring them in a very accurate procedure (Kraemer et al. 2019). Bacteria are the most common microorganisms that are used as a bioremediation agent that performed as a considerable agents to remove pollutants from sediments, water, and soil (Quintella et al. 2019).

Heavy metals, which are non-biodegradable contaminants, pose severe threats to the environment, particularly impacting water and soil (Al-Shammari et al. 2022, Al-Shammari et al. 2023). These metals originate from various sources, including automobile exhaust, storage batteries, industrial activities, mining, pesticides, fertilizers, and more. Some of the most common and highly toxic heavy metals that adversely affect human health and the well-being of flora and fauna include nickel, gold, silver, cadmium, arsenic, selenium, mercury, and chromium. Numerous approaches and methods have been proposed to eliminate these metals from the environment (Priya et al. 2023).

Microorganisms used in bioremediation can be classified into two categories: aerobic and anaerobic. Aerobic microorganisms, such as *Mycobacterium*, *Rhodococcus*, and *Pseudomonas*, are reported to be effective in degrading hydrocarbons and pesticides. On the other hand, anaerobic microorganisms are utilized for the bioremediation of polychlorinated biphenyls (PCBs) and the dechlorination of river sediments, as evidenced by various bacterial species (Sayqal & Ahmed, 2021). Additionally, *Trichoderma* has been used by Ali et al. (2023) for the removal of toxic dyes from aqueous mediums.

Many researchers studied how to involve microorganisms like; algae, yeast, fungi, and bacteria in the process of bioremediation they used *P. aeruginosa*, *Streptomyces*, and *Pseudomonas* (Azadi & Shojaei 2020, Al-Thukair et al. 2020, Qutob et al. 2022), it was noticed that certain types of bacteria have the ability to bind with the heavy metals through their thick lipo-wall forming a coordination bonds with the toxic heavy metal in a process called biosorption, among these microorganisms are *Saccharomyces*, *Streptoverticullum, Aspergillus*, and the genera of *Rhizopus* (Abdel-Raouf et al. 2022). Furthermore, *Azospirillumsp.* is renowned for its role as a promoter of plant growth across diverse cultures. Yet, its adaptive traits in challenging environments position it as a promising candidate for investigating remediation processes in environments contaminated with hydrocarbons and heavy metals (Cruz-Hernández et al. 2022).

In 2023, Zulfiqar et al. mentioned in their review article that microorganisms, such as bacteria, fungi, and algae, are known for their remarkable metal tolerance, exhibiting an impressive 98% capacity for bioremediation.

The current study, aimed to use *Azospirillum* bacteria isolated from soil in wastewater treatment via removing heavy metals, in particular, cadmium.

MATERIALS AND METHODS

Collection!of!Soil!Samples

The samples of soil were collected from a rice field in Al-Mishkhab/Al-Najafprovince, Iraq, this place was chosen as a source for the soil in the current research. The samples of the soil were collected in October 2020 because this month the humidity percentage increased. However, the samples of the soil were gathered at a depth of 35 cm, mixed, and then stored in the lab at room temperature (around 21 °C), the procedure was previously explained by (Dickman et al. 1984).

All the tests of soil and wastewater and the experiments were carried out in Soil and Water Research Center, Ministry of Science and Technology, Baghdad-Iraq.

Wastewater Samples

The wastewater samples were collected from the Al-Rustamia wastewater treatment plant in Baghdad City, Iraq. This location was chosen because it is expected it contain a lot of heavy metals due to the presence of a lot of industrial and chemical activities in the area which increases the presence of pollutants, especially, heavy metals.

Determination of Cadmium

The determination of the cadmium concentration in wastewater was done on both 7850ICP-MS according to SFS-EN ISO 17294-2 standard (Gray & Cunningham 2019), and Shimadzu AA - 6650 Atomic Absorption Spectrophotometer, the protocols were explained by (Costley & Wallis 2001, Sarker et al. 2015).

Serial Dilution Method

The method of serial dilution was carried out according to (Kanimozhi & Panneerselvam 2017, Damo et al. 2022), five grams of the soil were placed in a test tube, and 45 milliliters of SW (sterilized water) was added and mixed carefully, and thoroughly. After good mixing, the mixture was subjected to a process called Immunomagnetic beads [IMB] as explained by (Han & New 1998) after the IMB, only one milliliter of the solution was taken and placed in another test tube, and 9 mL of SW were added to complete the volume to 10 mL (dilution process DP). The DP was repeated for 10 times of dilution (10 folds in a process called serial dilution), however, the solutions that came out from the serial dilution were plated on culture repopulation ability (CRA) and incubated for 5 days at 32°C on NFb agar as described by (Rathore 2014).

Isolation of *Azospirillum*

NFb agar is a suitable medium was used for isolation of *Azospirillum* from the following components (gm/L):

Part A: Agar (1.75) g, $CaCl_2$ (0.02) g, Na Molybdate (0.002) g, Bromothymol blue (0.002) g, NaCl (0.1) g, $MgSO_4$ (0.2) g, $MnSO_4$ (0.01) g, $FeSO_4$ (0.5) g, Dipotassium phosphate (0.5) g (as a buffering reagent), and Malic acid (5.0) g (which is used particularly to grow *Azospirillum* only and not used by other NFb).

Part B. (4.0) g/L of KOH

Part **A** and Part **B** were mixed well together, and the pH was adjusted finally to around (6.9 ± 0.1), the well-mixed result in the medium was placed in an autoclave for 20 minutes and the temperature should be fixed at 121°C. The incubation is carried out for 8 days at 30°C.

Identification!of!*Azospirillum*!Bacteria

Bacterial isolates have been submitted to cultural, microscopical, and biochemical examination as mentioned by (Sulaiman et al. 2019).

Laboratory!Experiment

The experiment was carried out by inoculating a test tube containing well-mixed crude wastewater samples with a known concentration of cadmium in it, for the 10 samples that were used in the experiment. They were inoculated by loop via taking a swab of pure bacteria that were grown on NFb agar medium. The tubes were incubated in the incubator at 30°C for a period of specified time (Table 3).

Statistical!Analysis

The Statistical Analysis System - SAS (2018) program was used to detect the effect of different factors on study parameters. The least significant difference (LSD) test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study.

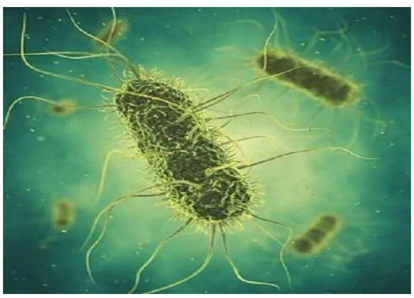

Fig. 1: *Azospirillum bacteria*.

Fig. 2: *Azospirillum* colonies in NFb Agar medium after 8 days of incubation.

RESULTS AND DISCUSSION

Isolation!and!Identification!of!*Azospirillum* Bacteria

According to Sulaiman et al. (2019), the isolates were identified as members of the genus *Azospirillum* based on their colony shape, Gram staining, and biochemical assays, as detailed in Table 1. On the medium surface, *Azospirillum* colonies appear as dense, white, undulating pellicles approximately 1 mm thick (Fig. 1). The characteristics of *Azospirillum* are listed in Table 1. The observed appearance and identification of *Azospirillum* align with the findings of Mallé et al. (2020) and Sulaiman et al. (2019).

Wastewater samples were collected at different times over ten days. The date, time, and cadmium concentration of these samples are shown in Table 2. It is crucial to specify the time and date accurately, as industrial activities affect the concentration of Cd ions. Nonetheless, the Cd concentration was observed to range from 0.03 to 1.6 mg/L.

According to the World Health Organization (WHO), the concentration of cadmium ions in drinking water should not exceed 0.003 mg/L (Steiner et al. 2011). However, the

Table 1: Biochemical and Morphological characterization of the *Azospirillum* isolates.

No.	Examination	Isolate
1	Characteristic of colonies	Mucous. Large, convex with full edge
2	Microscopic characters	Inflate rods with polymorphic cells from straight to curved
3	Production of acid	Positive
4	Liquefaction of Gelatin	Negative
5	Oxidase	Positive
6	Catalase	Positive
7	Production of Urease	Positive
8	Production of H_2S	Negative
9	Utilization of Citrate	Positive
10	Voges-Proskauer test	Negative
11	Production of Indole	Negative
12	Mannitol	Positive
13	Xylose	Negative
14	Dextrose	Positive
15	Maltose	Positive
16	Lactose	Positive
17	Gram's staining	Gram Negative
18	fermentation of Sucrose	Negative
19	fermentation ofGlucose	Positive
20	fermentation of fructose	Positive
21	Pigmentation	Nil

Table 2: Concentration of Cadmium ions in the samples.

Sample No	Date and time	Cd (mg/L)
1.	Saturday 3 Oct. 2020 at 10 am	0.9
2.	Monday 5 Oct. 2020 at 1:30 pm	0.07
3.	Sunday 11 Oct. 2020 at 9:35 am	0.85
4.	Monday 12 Oct. 2020 at 11:45 am	1.02
5.	Wednesday 14 Oct. 2020 at 4:05 pm	1.3
6.	Thursday 15 Oct. 2020 at 10:37 am	1.5
7.	Monday 19 Oct. 2020 at 9:48 am	1.6
8.	Tuesday 20 Oct. 2020 at 11:54 am	0.07
9.	Thursday 22 Oct. 2020 at 2:18 pm	0.06
10.	Friday 23 Oct. 2020 at 3:51 pm	0.03
P-value	-	0.0017 **

** ($P \leq 0.01$).

observed levels significantly exceed this recommended threshold.

Statistical analysis of the values in Table 2 shows significant differences in the cadmium content among the ten samples collected at different times, with a p-value of 0.0017 ($P \leq 0.01$).

Azospirillum was used to treat wastewater samples for different durations, as shown in Table 3. Statistical analysis results are also presented in Table 3 and Fig. 2.

Using *Azospirillum* on the contaminated samples revealed the complete removal of Cd traces. Furthermore, a notable correlation was observed between Cd concentrations and time, with the trace element ion being removed by 65% after 24 hours, 99% after 48 hours, and completely after 72 hours. This percentage remains unchanged even after a week, as depicted in Fig. 3.

Research has explored the capability of *Azospirillum*, a bacterium renowned for promoting plant growth, to remove heavy metals such as cadmium from wastewater. Studies indicate that certain strains of *Azospirillum* can enhance the absorption of heavy metals in the soil while preventing cadmium uptake by plants, suggesting a potential role for these microorganisms in bioremediation (Cruz-Hernández et al. 2022). Additionally, another study investigated the use of *Azospirillum* in a mixotrophic culture to eliminate cadmium from wastewater, highlighting its suitability and effectiveness in achieving a 98% bioremediation capacity (Zulfiqar et al. 2023).

Another study indicates that adding *Azospirillum* to plants treated with Cd can positively impact their biomass and root length. It has also been demonstrated to enhance nutrient uptake, stimulate overall plant growth, and mitigate the adverse effects of heavy metals such as Pb and Cd (Cruz-Hernández et al. 2022).

A study on *Pantoea agglomerans* UCP1320's ability to remove cadmium from aqueous solutions found that longer incubation times resulted in higher removal of cadmium (II). The study (Acioly et al. 2018) noted that desorption probabilities typically ranged from 6 to 24 hours during extended incubation times, a conclusion consistent with current research. This finding aligns with the results of Limcharoensuk et al. (2015). It can be concluded, however, that bioremediation cannot eliminate Cd; the maximum removal achieved was 94%, observed after 48 hours. This finding is consistent with the research of Kumar et al. (2021), suggesting that while 48 hours may not suffice for complete removal, longer durations (e.g., 72 hours) may be more effective.

Lipids present in *Azospirillum* membranes may assist in bioremediation by forming organometallic complexes

Table 3: The relationship between the concentration of Cd and treatment time.

No.	Concentration of Cd (mg/L)					
	Crude wastewater (Not treated)	After (24) hours	After (48) hours	After (72) hours	After one week (168) hours	P-value
1	0.900	0.315 (65%)	0.009 (99%)	0.000 (100%)	0.000 (100%)	0.0001 **
2	0.070	0.025 (65%)	0.000 (99%)	0.000 (100%)	0.000 (100%)	0.0424 *
3	0.850	0.553 (65%)	0.008 (99%)	0.000 (100%)	0.000 (100%)	0.0001 **
4	1.020	0.663 (65%)	0.01 (99%)	0.000 (100%)	0.000 (100%)	0.0001 **
5	1.300	0.631 (65%)	0.013 (99%)	0.000 (100%)	0.000 (100%)	0.0001 **
6	1.500	0.53 (65%)	0.015 (99%)	0.000 (100%)	0.000 (100%)	0.0001 **
7	1.600	0.56 (65%)	0.016 (99%)	0.000 (100%)	0.000 (100%)	0.0001 **
8	0.07	0.024 (65%)	0.000 (99%)	0.000 (100%)	0.000 (100%)	0.0488 *
9	0.06	0.021 (65%)	0.000 (99%)	0.000 (100%)	0.000 (100%)	0.074 NS
10	0.03	0.011 (65%)	0.000 (99%)	0.000 (100%)	0.000 (100%)	0.316 NS

* ($P \leq 0.05$), ** ($P \leq 0.01$).

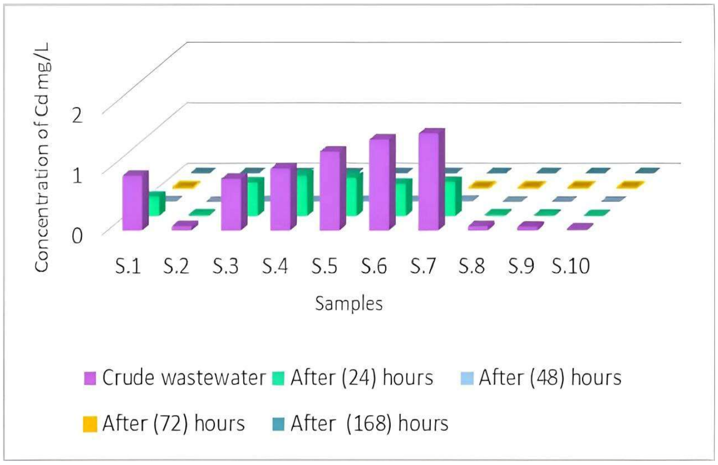

Fig. 3: The relationship between the concentration of Cd and treatment time.

with cadmium ions via biosorption (Haque et al. 2021). The removal process is pH-sensitive. Increasing the pH to alkaline levels (pH 7.8) enhances the release of Cd ions from the medium and strengthens their binding to the microorganisms' cell walls (Javanbakht et al. 2014).

Table 3 reveals notable variations in cadmium concentrations between crude wastewater samples and those treated with bacteria at different time intervals. Statistical analysis indicated significant differences for samples No. 1, 3, 4, 5, 6, and 7 at a significance level of 0.001 ($P \leq 0.01$), while samples No. 2 and 8 showed significant differences at a significance level of 0.005 ($P \leq 0.05$). However, samples No. 9 and 10 did not exhibit significant differences for the same variables.

CONCLUSION

Cadmium is among the heavy metals commonly found in domestic and commercial wastewater. Bacteria, due to their ability to degrade various substances and materials, can be employed in biological treatments to mitigate hazardous levels of this substance in aquatic environments. *Azospirillum* bacteria effectively remove these contaminants from soil. Results indicated that extending the incubation period enhanced the rate of cadmium removal from contaminated water; after 72 hours of incubation, *Azospirillum* bacteria biosynthesized 100% of the cadmium ions. While the study demonstrates the effectiveness of *Azospirillum* bacteria in cadmium removal, further investigation and specific experimental data may be necessary to comprehensively evaluate their efficacy in wastewater treatment. Factors such as temperature, pH, bacterial strain, and their interplay with biological efficiency should also be considered.

ACKNOWLEDGMENT

The present work was supported by Mustansiriyah University (www.uomustansiriyah.edu.iq / Baghdad, Iraq), for which the authors are grateful.

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