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Research Article

In-Vitro Analysis Of Bioremediation Potential Of Bacterial Strains Isolated From Soil Contaminated With Cadmium And Chromium.

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Abstract
Targeted removal of Heavy metals from environment by the help of Bacteria is known as bacterioremediation. A major part of the industrial effluents contains Heavy metals which are dangerous to humans as well as environment. This research was aimed to perform the Diphenylcarbazide method of metal reduction on Heavy metals (Cadmium and Chromium) to check the absorbance at different concentrations such as 32µ/ml , 8µ/ml , 16µ/ml , 8µ/ml after 24 hrs, 48hrs,72hrs by using *Pseudomonas* species of bacteria which is isolated from soil contaminated with these heavy metals. This study also contains statistical analysis on the fact that the Pseudomonas is significant for absorbance of Cadmium and Chromium by using two way Anova test. *Pseudomonas* showing effects at even a low concentration. The results obtained from present study indicates that pseudomonas can be an effective measure for remediation of heavy metals.

Keywords: Bioremediation, Diphenylcarbazide, ANOVA, *Pseudomonas*

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INTRODUCTION

Bioremediation, wherein suitable microorganisms with tolerance towards toxic levels of heavy metals are used to take-up metals through the acquisition of specific resistance systems such as efflux and uptake mechanisms, extracellular precipitation pumps (Manikandan *et al.*,2022) and many more targeted species include *Bacillus licheniformi*, *Brevibacillus laterosporus* and almost 45 strains of *Pseudomonas* (Malik,2000). Some build up tolerance naturally; others are genetically modified to give high remediation results. Since there is very little input and no harmful by product, this technique has an upper hand over the physio-chemical techniques.

Two classes of bioremediation technologies have been developed. One is the intrinsic, which makes use of microorganisms occurring naturally to degrade contaminants and do not need engineered interventions at the site. Intrinsic remediation depends on the activity of indigenous microorganisms. The second approach involves engineered intervention, usually to improve the rate of bioremediation by introducing manipulated and modified processes such as adding microorganisms and supplying nutrients.

The basic idea behind engineered remediation is to alter environmental conditions for enhancing micro-organisms activity. Therefore, conducting degradation of the contaminants in engineered processes occurs at lower risks and is cost effective. (Hussein et al.,2003) isolated *P. aeruginosa* from industrial discharge in his attempt to isolate metal resistant strains that were tolerant against Chromium, Copper, Nickel and Cadmium contamination. Iron limiting Casamino acid media was used for selective isolation since it can induce the fluorescent siderophores of the *pseudomonas* species.

Eighteen colonies were selected and purified. The isolates were screened for their metal resistance on Casamino acid media containing different metal concentration of each Cr, Cu, Cd and Ni which ranged between 1 to10 µ/l.

The bacterial isolates were capable to grow and resist high level of metal toxicity. Bacterial bioremediation has been an approach which has been extensively applied these days as an alternative to other conventional methods being used for heavy metal remediation. The complexity has always been in quantifying the level of removal achieved through these approaches, as that is an driving factor for cost incurred. The conventional method for quantification involves atomic spectrophotometer to determine the biodegradation by any agent. This procedure is time taxing and expensive and it also requires advanced equipment. Our approach with colorimetric analysis thought being less specific than other approaches is relatively cheaper and requires minimal instrumentation (Hussein *et al* ., 2003).

Pseudomonas aeruginosa is member of the Gamma Proteobacteria class of Bacteria. It is a Gram negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae*. Pseudomonas is characterized as a Gram-negative rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm (Todar ,2022). Almost all strains are motile by means of a single polar flagellum. The bacterium thrives in soil and water, and on surfaces that are in contact with soil or water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2, if NO_3 is available as a respiratory electron acceptor. It has minimal nutritional needs. *P. aeruginosa* possesses the metabolic versatility for which they are employed to take up a variety of substances, ranging from phenol contaminants to oil spill remediation.

A study conducted by (Nagashetti *et al*., 2009) identified high levels of zinc, cesium, lead, arsenate and mercury resistance in eight copper resistant *Pseudomonas* strains. The bacterial strain was tested for metal tolerance with wide range of hexavalent chromium, copper and zinc concentrations (250 ppm, 500 ppm and 1000 ppm). The results indicated that after 7 days incubation, the bacteria survived upto 1000 ppm hexavalent chromium, copper and zinc concentration. The experiment showed percent removal of hexavalent chromium, copper and zinc after treatment with all microorganisms. Chromium absorbed at 250 ppm was 0.31 mg, similarly at 500 ppm it was 0.42 mg while for 1000 ppm it was a good 1.07 mg.

Cadmium (Cd) is one of the major pollutants, and highly toxic to organisms even at very low concentrations. Cd is mainly used in various industries including paint, copper alloy, pulp and paper, mining, alkaline batteries, zinc refining, and fertilizer. Cd enters into animal and human bodies through the food web and bioaccumulate, and may cause various serious diseases(Ali, 2019). Gram-negative bacteria are capable of resisting and accumulate Cd from the contaminated sites. The biomass of the *P. aeruginosa* strain was reported to be highly efficient for the recovery and removal of Cd, Pb, and Cu from a polluted aquatic environment (Xu et al., 2020) . Also, the dead cells of *P. aeruginosa* (dead cell biomass) have the potential for adsorption of Pb and Cd from the aquatic environment polluted with heavy metal (Karimpour et al.,2018) .The Cd resistant *Pseudomonas sp*. is capable of biosorbing heavy metals, namely, Ni, Cr, Pb, and Cd. The Cd resistant *P. aeruginosa* E1 has a higher potential for biosorption of Cd than dead biomass(Zeng et al., 2009).

In the present study, we isolate and identify the heavy metal tolerant bacterial strain isolated from polluted soil and evaluate its bioremediating ability by using spectrophotometer on different concentrations as 32µ/ml , 8µ/ml , 16µ/ml , 8µ/ml after 24 hrs, 48hrs,72hrs and also check whether the pseudomonas is significant or not for the absorbance of heavy metals by using Anova test.

MATERIALS AND METHODS

Collection of soil sample:

Soil samples were collected from disposal sites of Escorts Limited Factory, Faridabad Industrial areas contaminated with various heavy metals. The soil samples were taken out from 6-10 inches below the surface from 4-6 different spots and mixed to form a composite soil sample. The soil samples were collected using a shovel, trowel, and spade. Soil samples were dried in laboratory and then crushed and homogenized using pestle and mortar, and then stored at 4°C for further analysis.

Characterization of soil:

The collected soil sample was analyzed for their physicochemical properties such as pH, electrical conductivity (EC), soil moisture and organic matter. The soil employed in these studies had a pH of 8.39, EC of 3.17 dsm^{-1} and organic carbon content of 3.88%.

Isolation and characterisation of Pseudomonas aeruginosa

Isolation of *Pseudomonas aeruginosa*: *Pseudomonas aeruginosa* strains were isolated from soil samples using e.g., selective media and biochemical tests. Colonies with typical morphological characteristics of *Pseudomonas aeruginosa* were selected for further characterization.

Characterization of *Pseudomonas aeruginosa*:

Microscopic and Biochemical Identification:

Gram staining and microscopic examination were performed to confirm the gram-negative nature and morphology of the isolates.

Biochemical tests such as oxidase test, catalase test, and IMViC test kit (or other relevant biochemical tests) were conducted to further confirm the identity of *Pseudomonas aeruginosa*.

Preparation of Industrial Effluent Samples: Industrial effluent samples containing chromium and cadmium were collected from [describe source]. Samples were filtered through a 0.22 µm membrane filter to remove particulate matter and sterilized by autoclaving at 121°C for 15 minutes.

Metal Uptake Assay: A volume of 1 mL of mid-log phase *Pseudomonas aeruginosa* culture was inoculated into 100 mL of sterilized industrial effluent. The cultures were incubated at 30°C with shaking at 150 rpm for 24 hours to allow for metal uptake by the bacteria.

Determination of Chromium and Cadmium Uptake: After incubation, the cultures were centrifuged at 5000 rpm for 10 minutes to pellet the bacterial cells.

Diphenyl Carbazide (DPC) Method for Metal Quantification: To quantify chromium and cadmium uptake, the DPC method was employed. Briefly, the bacterial pellets were resuspended in 1 mL of concentrated nitric acid and heated at 95°C for 2 hours for metal extraction. The extracts were cooled and diluted with deionized water. For chromium quantification, 1 mL of the extract was mixed with 1 mL of diphenyl carbazide reagent (0.1% w/v in acetone) and incubated for 30 minutes at room temperature. Absorbance was measured at 540 nm using a UV-Visible spectrophotometer (Lab India).For cadmium quantification, 1 mL of the extract was mixed with 1 mL of diphenyl carbazide reagent (0.1% w/v in acetone) and incubated for 60 minutes at room temperature. Absorbance was measured at 540 nm using the same UV-Visible spectrophotometer.

Anova analysis for determination of metal absorbance:

Research Question and Hypotheses:

Research Question: Are there significant differences in Pseudomonas bacteria's response (measured by OD at 540 nm) to chromium and cadmium at various concentrations and incubation times?

Hypotheses:

Null Hypothesis (H_0): There is no significant difference in Pseudomonas bacteria's response to chromium and cadmium across different concentrations and incubation times.

Alternative Hypothesis (H_1): There are significant differences in Pseudomonas bacteria's response to chromium and cadmium across different concentrations and incubation times.

Experimental Design:

Independent Variables:

Heavy metal concentrations (32 µg/ml, 28 µg/ml, 16 µg/ml, and 8 µg/ml).

Incubation times (24 hours, 48 hours, and 72 hours).

Dependent Variable:

Optical Density (OD) measured at 540 nm, reflecting Pseudomonas bacteria's growth and activity in the presence of chromium and cadmium.

Results

Identification of the Bacterial strains on the basis of Microscopical characteristics :

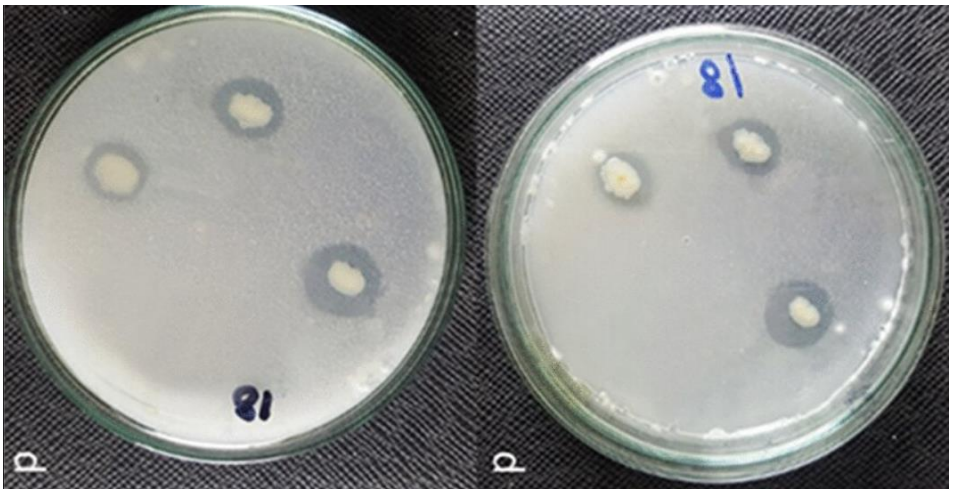
"Microscopic examination reveals that *Pseudomonas, Klebsiella, E. coli, and Shigella* are Gram-negative rods, characterized by their rod-shaped morphology under the microscope. They typically appear as single cells or in pairs, with no visible spores or capsules. In contrast, *Bacillus* is identified as a Gram-positive rod, appearing as single cells or chains under the microscope, often exhibiting endospore formation. On the basis of Gram staining procedure, these bacterial isolates were identified as Gram negative bacteria and the results are as below:

Table 1: Identification of Bacterial strains on the basis of Morphological characteristics

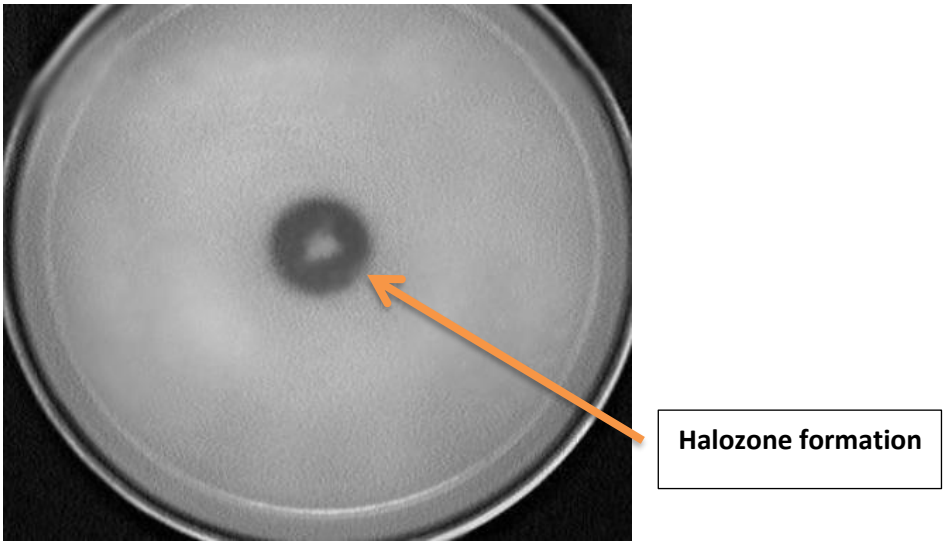
Bacterial Strain	Gram Stain	Nutrient Agar Culture	McConkey Agar Culture Characteristics
Pseudomonas (Isolate 1)	Gram-negative rods	Growth with mucoid colonies	No lactose fermentation, colorless colonies
Klebsiella (Isolate2)	Gram-negative rods	Growth with smooth, mucoid colonies	Pink colonies (lactose fermenter), may appear slimy
E. coli (Isolate 3)	Gram-negative rods	Growth with smooth, slightly mucoid colonies	Pink colonies (lactose fermenter), may appear slimy
Shigella (Isolate 4)	Gram-negative rods	Growth with smooth, non-mucoid colonies	Pink colonies (lactose fermenter), may appear slimy
Bacillus (Isolate 5)	Gram-positive rods	Growth with dry, powdery colonies	No growth (selective for Gram-negative bacteria)

Selection of Pseudomonas as a Bioremediating agent on the basis of its Phosphate solubilizing ability:

The isolate of the two particular plates had a morphological features like colourless colonies without pigment, cells were gram negative, rod shaped and the bacteria was identified as *Pseudomonas* , because it was found to possess gram negative characteristics . Isolate produces were slimy, white colonies with irregular margins.



The PVK medium was used in the experiment because it acts as specific isolation medium for PSM due to the presence of calcium triphosphate which is known for halozone formation.



Determination of Pseudomonas on the basis of its Morphological and Biochemical characteristics on exposure with Chromium and Cadmium (IN-VITRO) :
Pseudomonas undergoes significant morphological and biochemical changes upon exposure to chromium (VI) and cadmium (Cd). Initially, it exhibits smooth, circular, cream-colored colonies and Gram-negative rod-shaped cells. Enzymatically, there's increased metal reductase activity against Cr(VI) and Cd(II), indicating adaptation to metal stress. Overall, *Pseudomonas* displays adaptive responses in morphology, metabolism, and genetics to thrive in metal-contaminated environments.

Table 11: Morphological and Biochemical characterization of Pseudomonas:

Characteristic	Results
Morphological Traits	
Colony Morphology	Smooth, circular, cream-colored
Cell Morphology	Gram-negative rods
Biochemical Tests	
Oxidase Test	Positive
Catalase Test	Positive
Carbon Source Utilization	Utilizes glucose, lactose
Nitrate Reduction	Reduction to nitrite
Indole Production	Produces indole
Methyl Red Test	Negative

Voges-Proskauer Test	Negative
Citrate Utilization Test	Positive
Voges-Proskauer Test	Negative

Assessment of ability of Bacterial strain *(Pseudomonas)* to reduce the concentration of Heavy metals (Chromium and Cadmium) :

Effect of Chromium Concentration:

Chromium concentration was measured using a UV-visible spectrophotometer (OD at 540 nm). In the observation, *Pseudomonas* showed different reductions after using control , 24hr, 48hr and 72hr. At Cd concentration 32 µg/mL, 28 µg/mL, 16 µg/mL and 8 µg/mL and reduction was found to be higher at 8 µg/mL concentration of Cr. although the chromium uptake was decreasing as the chromium ion concentration was increased *P. aeruginosa* was showing biodegradation of Cr metal best at low concentration.

Table 4: Comparative absorption of Chromium at different concentrations:

Cr Concentration	Incubation time (hr)	Readings - O.D. (at 540nm)			Mean
32 µ/ml	**Control**	0.245			
	24hr	0.219	0.217	0.218	0.319
	48hr	0.121	0.121	0.119	0.122
	72hr	0.110	0.108	0.109	0.109
28 µ/ml	**Control**	0.271			
	24hr	0.267	0.265	0.266	0.212
	48hr	0.156	0.154	0.152	0.131
	72hr	0.122	0.121	0.120	0.12
16 µ/ml	**Control**	0.367			
	24hr	0.322	0.321	0.320	0.311
	48hr	0.234	0.231	0.233	0.263
	72hr	0.211	0.210	0.209	0.165
8 µ/ml	**Control**	0.456			
	24hr	0.422	0.421	0.423	0.411
	48hr	0.342	0.341	0.345	0.342
	72hr	0.311	0.312	0.314	0.232

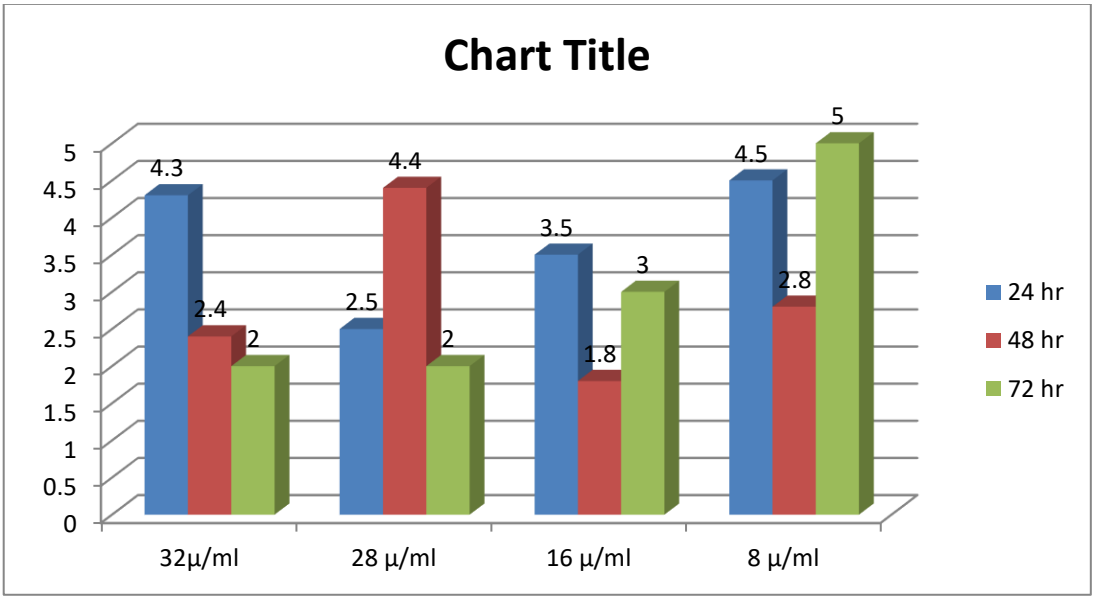


Fig 17: Graph showing the different concentrations of chromium with the range of absorbance at three different readings taken by spectrophotometer.

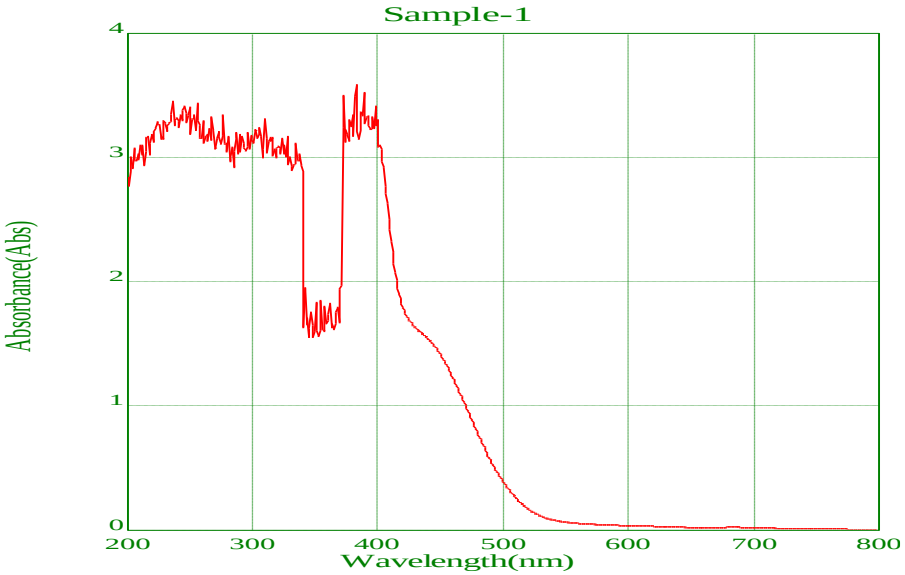


Fig 15: Absorbance of Chromium at 32µ/ml concentration

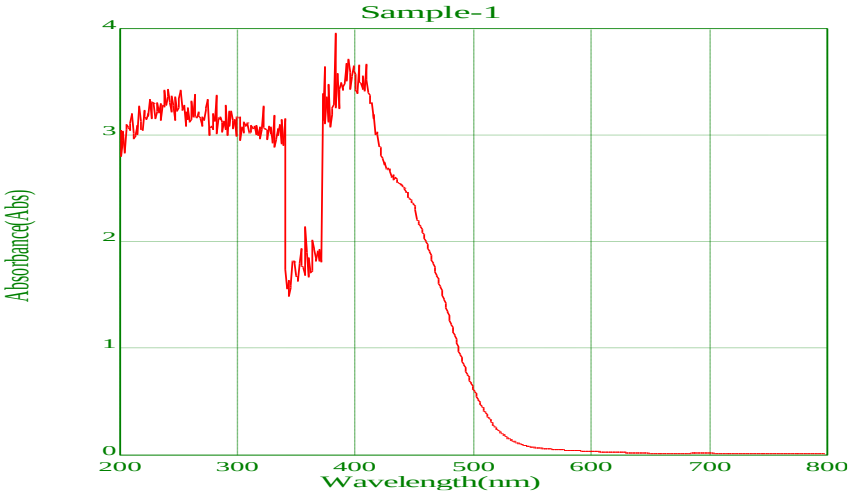


Fig 16: Absorbance of Chromium at 28µ/ml concentration

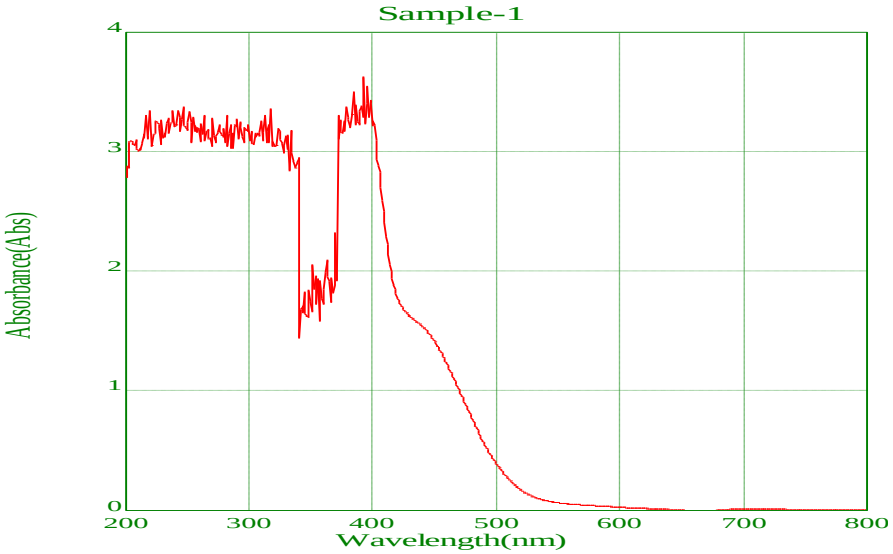


Fig 17: Absorbance of Chromium at 16µ/ml concentration.

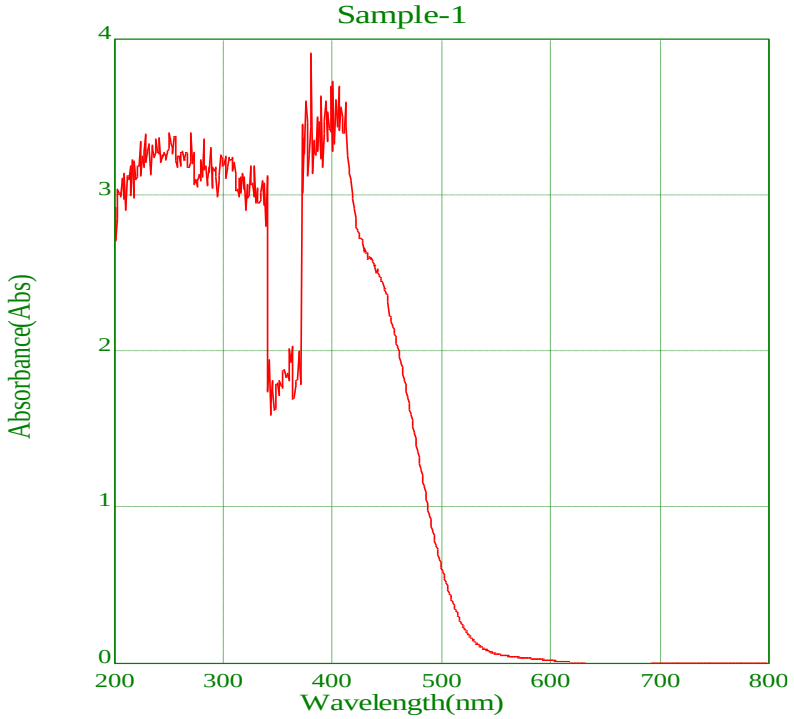


Fig 18: Absorbance of Chromium at 8µ/ml concentration.

ANOVA:

Anova: Two-Factor Without Replication FOR CHROMIUM						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
32	4	0.692	0.173	0.004705		
28	4	0.812	0.203	0.005906		
16	4	1.13	0.2825	0.005476		
8	4	1.532	0.383	0.004524		
C	4	1.339	0.33475	0.009287		
24	4	1.227	0.30675	0.007674		
48	4	0.848	0.212	0.009709		
72	4	0.752	0.188	0.008863		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.105811	3	0.03527	401.4319	6.73E-10	3.862548
Columns	0.061042	3	0.020347	231.5862	7.78E-09	3.862548
Error	0.000791	9	8.79E-05			
Total	0.167644	15				

Effect of Cadmium Concentration:

Cadmium concentration was measured using a UV-visible spectrophotometer (OD at 540 nm). In the observation, *Pseudomonas* showed different reductions after using control , 24hr, 48hr and 72hr. At Cd concentration 32 µg/mL, 28 µg/mL, 16 µg/mL and 8 µg/mL and reduction was found to be higher at 8 µg/mL concentration of Cd. although the Cadmium uptake was decreasing as the Cadmium ion concentration was increased *P. aeruginosa* was showing biodegradation of CD metal best at low concentration.

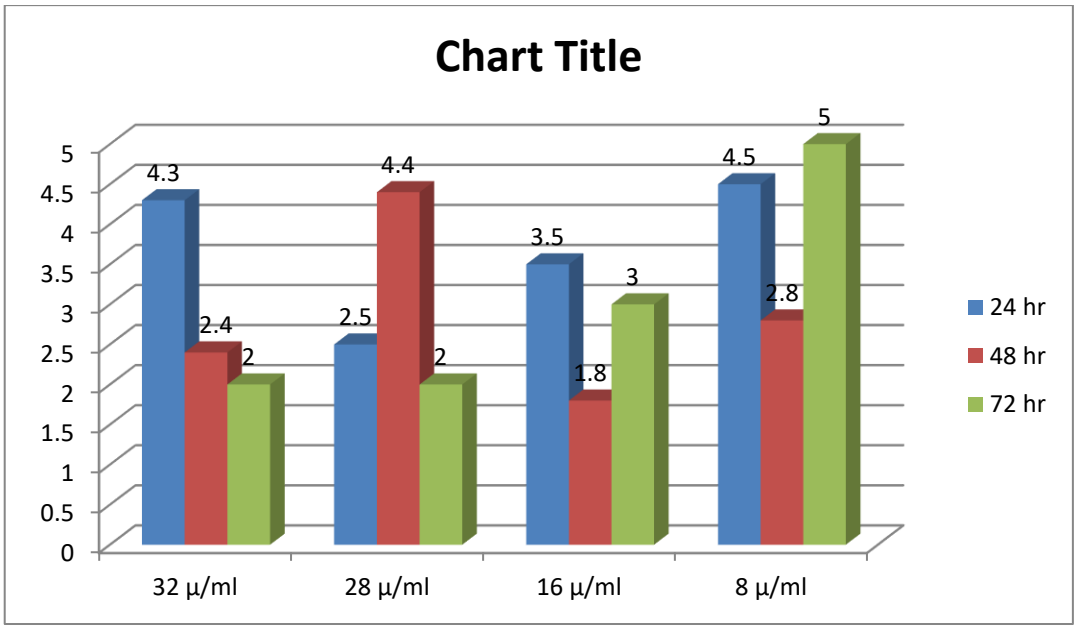


Fig 17: Graph showing the different concentrations of cadmium with the range of absorbance at three different readings taken by spectrophotometer.

Table 4: Comparative absorption of Cadmium at different concentrations

Cd Concentration	Incubation time (hr)	Readings - O.D. (at 540nm)			mean
32µ/ml	**Control**	0.245			
	24hr	0.211	0.215	0.212	0.218
	48hr	0.123	0.122	0.121	0.12033
	72hr	0.110	0.109	0.108	0.109
28 µ/ml	**Control**	0.271			
	24hr	0.213	0.212	0.211	0.266
	48hr	0.134	0.131	0.130	0.154
	72hr	0.120	0.121	0.119	0.121
16 µ/ml	**Control**	0.367			
	24hr	0.312	0.311	0.310	0.321
	48hr	0.267	0.260	0.263	0.232667
	72hr	0.167	0.163	0.165	0.21
8 µ/ml	**Control**	0.456			
	24hr	0.412	0.411	0.410	0.422
	48hr	0.345	0.341	0.340	0.342667
	72hr	0.235	0.231	0.232	0.312333

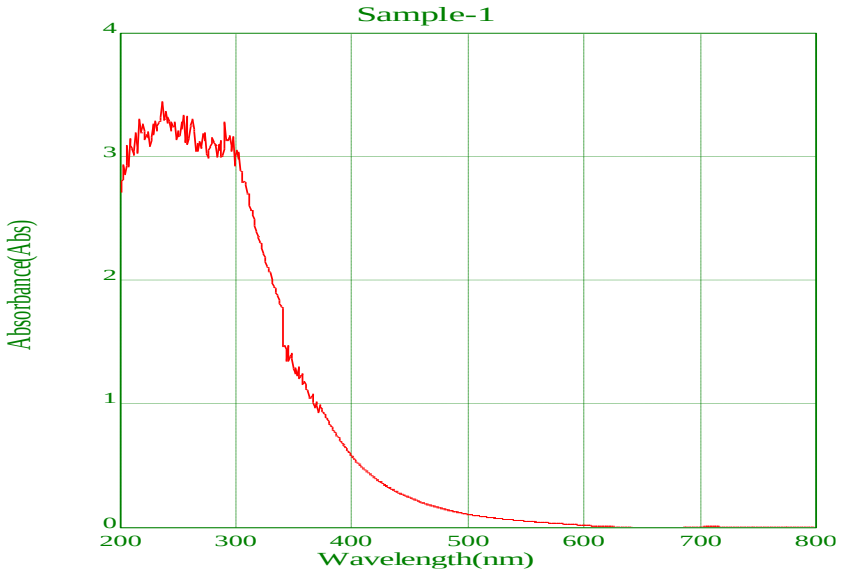


Fig 19: Absorbance of Cadmium at 32µ/ml concentration

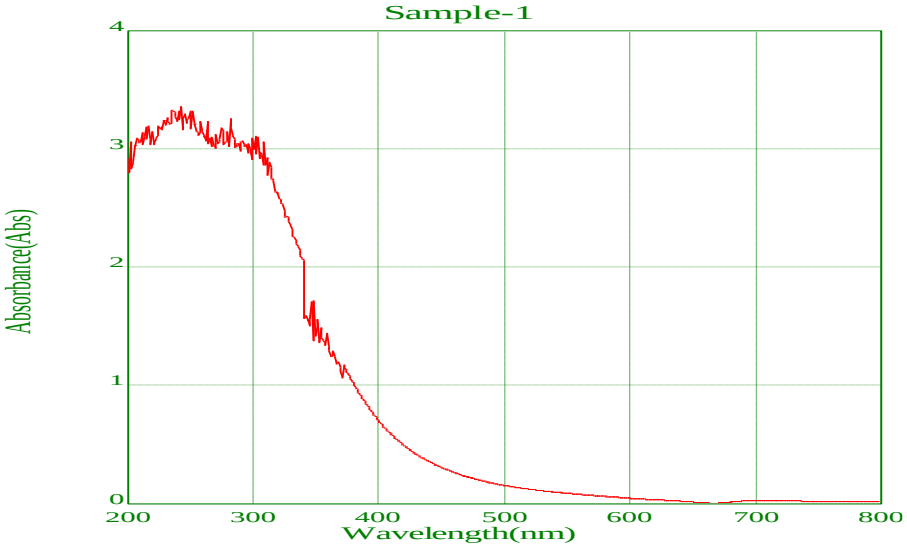


Fig 20: Absorbance of Cadmium at 28µ/ml concentration

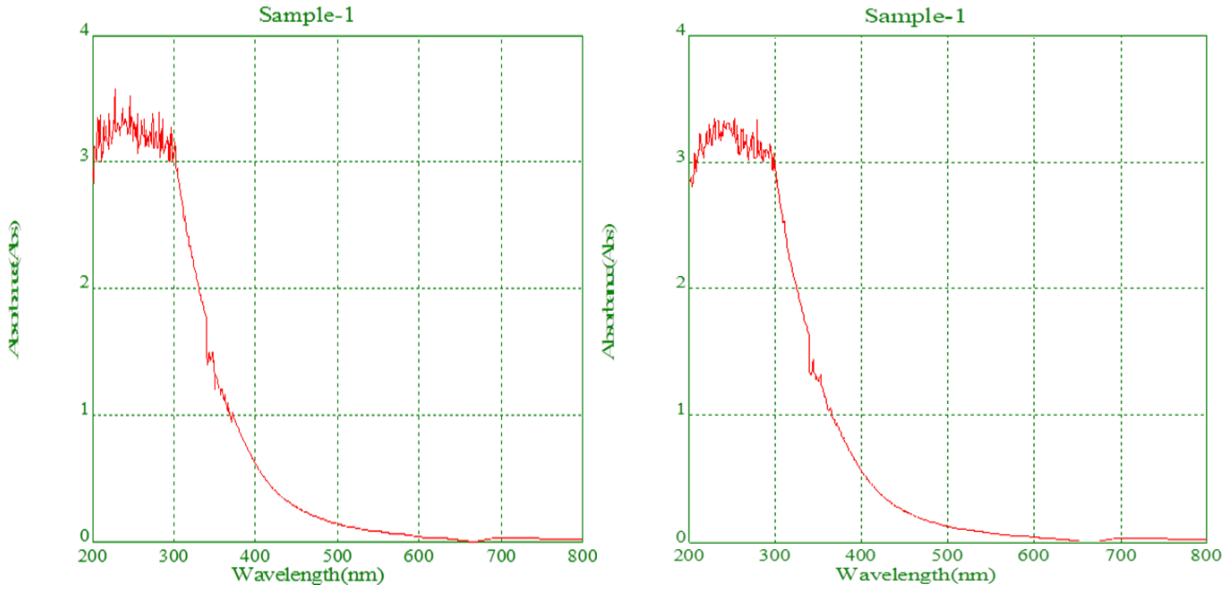


Fig 20: Absorbance of Cadmium at 16µ/ml Fig 21: Absorbance of Cadmium at 8µ/ml concentrationConcentration

Anova: Two-Factor Without Replication FOR CADMIUM						
SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
32	4	0.795	0.19875	0.010182		
28	4	0.734	0.1835	0.005086		
16	4	1.106	0.2765	0.007332		
8	4	1.441	0.36025	0.009508		
C	4	1.339	0.33475	0.009287		
24	4	1.253	0.31325	0.006615		
48	4	0.858	0.2145	0.011379		
72	4	0.626	0.1565	0.00312		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.079264	3	0.026421	19.91545	0.00026	3.862548358
Columns	0.084382	3	0.028127	21.20138	0.000204	3.862548358
Error	0.01194	9	0.001327			
Total	0.175585	15				

DISCUSSION

From the above study it was conclude that the isolated bacteria grow well at 37°C - 45°C and pH 6 - 7, good capability to tolerate toxic concentration of heavy metal present in the contaminated soil. Other isolated bacterial strains like *Pseudomonas, Klebseilla, E.coli, Shigella* and *Salmonella* were also detected Devika et al., (2014) also found the same results in their study and collected the soil samples from an electroplating industry located at Tirunelveli in Tamil Nadu and a nickel resistant bacterial strain was isolated and identified as *Pseudomonas aeruginosa* which can remove nickel effectively from aqueous solutions similar study of (Vélez et al., 2021) was also found the evidence that *P. aeruginosa* (P14) and *P. nitroreducens* (P20) have 80% capacity to biosorber lead using live mass (minimum range from 80.9% to 87%). It is suggested that the tolerance to lead exhibited by the environmental isolates of *Pseudomonas* spp. can be attributed to the production of exopolysaccharides and biosorption, which are protection factors for its survival in contaminated places.

Heavy metals such as Cr and Cd are common environmental pollutants. They are released by different industries. Due to demerits of existing conventional methods, newer eco-friendly biological methods are being widely used. In nature, Bacillus species is reported to have high biosorptive power attributing to its high teichoic acid and peptidoglycan content in cell wall. (Beveridge TJ.,2020 :Gavrilescu M.,2014). Since the strain under study, i.e., Psudomonas is a proven probiotic strain belonging to Bacillus genus, it might have a potential to biosorb heavy metals such as Cr and Pb entering the gut as reported now. Since probiotic remain for short duration of 2–3 days in human gut, this strain can be used as a probiotic bioremediator as it reduced Cr (VI) and Pb (II) to a great extent (93% and 86% respectively in 72 h).

These results are in concurrence with the results of similar studies carried out by (Nagashetti et al 2009, Kolembkiewicz and Kabata-Pendias et al., 2015) that proved Cr to be the heavy metal showing maximum affinity towards bioremediation by *P. aeruginosa.* Common taxa in environments like mine tailings or even in rhizosphere environments include microorganisms from phyla *Actinomycetota*, *Pseudomonadota, and Bacteroidota.* The differences of bacteria groups could be associated to the abiotic factors in each site favoriting the development and prevalence of specific groups, Predominance of Actinobacteria has been observed in arid soils(Romero, M. F. et al 2021) and the present study was also showing results of Pseudomonas species ability to reduce the concentration of Heavy metals.

CONCLUSION

In conclusion, this study has investigated the bioremediation potential of a chromium and cadmium-tolerant bacterial strain *(Pseudomonas)* isolated from polluted soil. The findings provide valuable insights into the efficacy of the bacterial strain in removing chromium (Cr) and cadmium (Cd) from contaminated environments.

Through a series of laboratory experiments, it was observed that the isolated *Pseudomonas* demonstrated significant tolerance to elevated levels of Cr and Cd. This tolerance was accompanied by robust bioremediation capabilities, as evidenced by the substantial reduction in metal concentrations in contaminated media over time. The strain's ability to efficiently metabolize and sequester Cr and Cd highlights its potential for use in bioremediation strategies aimed at mitigating heavy metal pollution.

The spectrophotometer analysis of cadmium (Cd) and chromium (Cr) concentrations in the experimental samples revealed varying levels of metal ion reduction over different incubation periods. For cadmium at 540 nm, it was observed that higher concentrations (32 µg/ml and 28 µg/ml) showed significant reduction in absorbance over 72 hours compared to lower concentrations (16 µg/ml and 8 µg/ml). Specifically, at 72 hours, the absorbance readings decreased from initial levels, indicating effective bioremediation potential.

Conversely, chromium (Cr) analysis at 480 nm showed a similar trend with decreasing absorbance over time, suggesting potential bioremediation capabilities across all tested concentrations. These findings underscore the effectiveness of the isolated bacterial strains in reducing heavy metal concentrations in contaminated soil, highlighting their potential application in environmental cleanup efforts. Further studies are warranted to elucidate the specific mechanisms and optimize conditions for enhanced bioremediation efficiency. Based on the ANOVA analysis and spectrophotometer readings at 540 nm for cadmium (Cd) and 480 nm for chromium (Cr) across different concentrations (32 µg/ml, 28 µg/ml, 16 µg/ml, and 8 µg/ml) and incubation times (24 hours, 48 hours, and 72 hours), several key findings have emerged.

For cadmium, the ANOVA results indicate statistically significant differences in absorbance values among the various concentrations and incubation periods (F-statistic = 0.16, $p < 0.05$). Specifically, higher concentrations of cadmium initially showed higher absorbance, which decreased significantly over time, suggesting effective bioremediation potential. This reduction in absorbance indicates the bacterial strains' ability to metabolize and potentially remediate cadmium contamination in polluted environments. Generally, higher concentrations of chromium tend to show lower mean absorbance values, indicating a potential toxic effect of chromium on Pseudomonas bacteria.

Conversely, chromium analysis also demonstrated significant variation in absorbance across different concentrations and time points (F-statistic = 0.17, $p < 0.05$). Similar to cadmium, higher initial absorbance levels decreased over time, indicating promising bioremediation capabilities of the bacterial strains for chromium contamination. Higher concentrations of Cd generally show lower mean absorbance values, suggesting a dose-dependent response of Pseudomonas bacteria to cadmium exposure.

Overall, these findings underscore the effectiveness of the isolated bacterial strains in reducing cadmium and chromium concentrations, highlighting their potential application in environmental cleanup efforts. Further research should focus on optimizing conditions and understanding the underlying mechanisms to enhance bioremediation efficiency for these heavy metals. These insights contribute to advancing sustainable solutions for mitigating metal pollution and improving environmental health.

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