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ROLE OF *PSEUDOMONAS PUTIDA* IN CADMIUM REMOVAL ISOLATED FROM THE AGRICULTURAL SOILS OF NORTHERN HIMALAYAS OF KASHMIR, INDIA

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Abstract– The effectiveness of bacteria to resist changes in the environment with respect to metal accumulation in the soil was the main aim of the study. Soil Samples obtained mainly from agricultural land uses of South and North district of Kashmir, J&K showed no alarming levels of Cadmium accumulation although with continuous and bulk use of chemicals mainly fertilizers and pesticides may become problematic in the years to come. Out of seven isolates (AXCd1, AXCd10, AXCd6, AXCd7, AXCd8, AXCd9 and PDYCd42) obtained from the soil samples, isolate AXCd8 significantly exhibited better performance in tolerating different doses of Cadmium metal in nutrient agar media amended with Chloride salt solution ($CdCl_3$). The bacterial population decreased with the increase in the concentration of the metal with isolates resisting concentration of 100 ppm were selected for further analysis. MIC of 150 ppm was exhibited by the isolate AXCd8 while isolates AXCd7, AXCd9, AXCd10, AXCd6, PDYCd 42 showed MIC of 120 ppm. However, during *in vitro* evaluation of these bacterial strains, percent bioaccumulation was calculated significantly maximum by isolate AXCd8 (48.9%)>AXCd7 (45.70%)>AXCd9 (42.15%)>AXCd10 (41.68%)>AXCd6(34.97%)>PDYCd42(30.8). Atomic Absorption spectrometry was used to estimate the presence of cadmium in soil samples as well as in the growing media to evaluate bioaccumulation (%). Molecular analysis of isolate AXCd8 showed the phylogenetic resemblance with Pseudomonads family and was identified as *Pseudomonas putida.*

INTRODUCTION

The term 'heavy metal' is arbitrary and precisely defined as, "any metal that has metallic properties (ductility, conductivity, density, stability as cations, ligand specificity, etc.) and an atomic number greater than 20. Several metals must be present in a specific concentration range in order for biological systems to function. In fact, they serve as crucial co-factors for enzymes and metalloproteins, and as a result, their low quantities reduce metabolic activity. They can be toxic to higher organisms and microorganisms. Non-essential metals are tolerable in very small amounts but interfere with metabolic activity at higher levels. The presence of heavy metals in polluted areas may impede remediation processes due to their toxicity. The gradual accumulation of metals may hinder the degradation of organic contaminants or humic of substances in the environment. This issue can be solved by an increase of heavy metal resistance of the bioremediating system. Metals are somewhat unique in that they do not undergo either chemically or biologically induced degradation that can alter or reduce their toxicity over time (Knox *et al.*, 2002). Microorganisms are not alchemists; regardless of how they interact with a poisonous metal, the metal remains intact (Lovley and Lloyd, 2000). Microbes are ubiquitous in nature and use a variety of substrates as a source of carbon as a result, they are found in unusual habitats where they can absorb a variety of contaminants (Kour *et al.*, 2022). Their

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ability for survival in such unusual conditions also enhances their effectiveness. For instance, psychrophiles flourish in cold regions, acidophiles may survive in acidic environments, and halophiles can live in salty areas (Perera and Hemamali, 2022). Many diverse mechanisms are used by microbes to remove contaminants from the environment. These mechanisms can be divided into two major types- mineralization and immobilisation (Mahmoud, 2021; Kumar *et al*., 2022). During the mineralization process, microbes aid in the transformation of pollutants into end products like carbon dioxide and water or other intermediate metabolic compounds. Immobilization is a process that involves transferring substances into unavailable form. Consider the transformation of organic nitrogen from nitrate nitrogen (Pratush *et al.,* 2018). This method is typically used to bioremediate heavy metals, particularly in extremely polluted areas.

MATERIALS AND METHODS

Collection of samples

The soil samples were collected to obtain the indigenous soil bacteria from apple orchards and paddy fields of north and south districts of Kashmir, J&K. Samples were collected in zip lock plastic bags and stored at refrigerated conditions at Laboratory of Basic Sciences and Humanities, SKUAST-K, Wadura Sopore for isolation and further analysis of identification.

Determination of heavy metals from the soil sample

The calibration plot method was used for the analysis of heavy metal concentration with Atomic Absorption Spectroscopy (AAS). For element under study, the instrument was auto-zeroed using the blank (distilled water) followed by standard aspirates into the flame from the lowest to highest concentration. The corresponding absorbance was obtained by the instrument and the graph was plotted. The samples were analysed in triplicate with the concentration of the metals present being displayed in ppm after extrapolation from the standard curve.

Isolation and Screening of bacteria from soil

After labelling the soil samples according to the site, the bacteria were isolated on nutrient agar media amended with different concentrations of $CdCl_3$ solution starting from 25 ppm, 50 ppm, 75 ppm and 100 ppm. The 1000 ppm stock solution of $CdCl_3$ was prepared in double distilled water using $CdCl_3$ salt. The stock solution of heavy metal was sterilized separately through bacteriological filters before adding to sterilized nutrient agar medium to prepare the concentrations 25 ppm, 50 ppm, 75 ppm and 100 ppm of Cadmium. The bacterial isolates at highest concentration (100 ppm) of $CdCl_3$ in nutrient agar were selected for further evaluations.

Minimum Inhibitory Concentration (MIC)

The metal salt of $CdCl_3$was used for heavy metal tolerance. The stock solution of Cadmium was prepared on the basis of molar concentrations as per formula 'M= Moles of solute/Volume of solution'. One per cent culture from an overnight grown culture of a single colony was transferred to 3 ml of media supplemented with Cadmium. The initial metal concentration was taken as ppm. The tolerance was measured on the basis of growth observed (turbidimetry method) within 24, 36 and 48 hrs. If the growth was observed, inoculum was added in the media with increasing concentrations of the metal. This step was repeated in all the seven strains till minimum inhibitory concentration (MIC) was obtained by the cessation of growth (Yilmaz, 2003).

In vitro evaluation of bacterial isolates

Bacterial isolates grown in nutrient broth containing different concentrations of heavy metals for 48h was taken into consideration. The bacterial cells were harvested by centrifugation and suspended in 1 ml of distilled water. The metal content of bacterial cells was determined after acid dissolution of bacterial cells according the method of Ganje and Page (1974). Metal concentrations were measured by atomic absorption spectrophotometry.

Percentage bioaccumulation was calculated by using formula;

$$\%\ bioaccumulation = \frac{Initial\ metal\ concentration - Final\ metal\ concentration}{Final\ metal\ concentration}$$

Molecular identification of most effective Cadmium accumulating microorganism

The genomic DNA was isolated using microbial DNA isolation kit (MOBIO) according to manufacturer's protocol. The PCR amplification of 16S rDNA extracted from the bacterial isolates were used in PCR with universal primer set (27F and 1492R). The reaction each of 50 µl containing 2 µl of the DNA template, 5 µl 10X Taq buffer (Qiagen,

Hilden, Germany), 2.5 U Taq DNA polymerase (Hot-Start; Qiagen, Germany), 1.5 mM $MgCl_2$, 200 ìM deoxynucleotide triphosphates and 0.5 µM of each primer. PCRs were performed in a thermal cycler (Eppendorf) with the following cycling conditions- 4 mins of denaturation at 95 ^{0}C, 25 cycles of 1 min at 94 ^{0}C, 1 min at 53 ^{0}C, 1.5 min at 72 ^{0}C and a final cycle of extension of 72 ^{0}C for 5 mins. This PCR reaction was carried out using 2.5 µl of crude DNA as a template following the program given by Hussain *et al.* (2015). PCR products were analysed by electrophoresis in a 1% (w/v) agarose gel and purified using the MOBIO Gel Extraction Kit according to the manufacturer's manual guide. The PCR products were sequenced with Terminator v 3.1 cycle sequencing kit (Applied Bio systems, Foster City, USA) in accordance with the manufacture's protocol followed by sequencing using ABI 16S rDNA sequencing kit.

Bacterial strain identification and phylogenetic analysis

Specific 16S rDNA sequences were edited with Chromas Lite software (version 2.01) for further DNA analysis. A Blast search (Altschul *et al.*, 1990; Zhang *et al.*, 2000) at the NCBI server was conducted to identify the nearest neighbours. Multiple alignments were performed with the ClustalW2 program at the EBI according to Larkin and his associates (2007).

A phylogenetic tree was generated with the MEGA5 software (Tamura *et al.*, 2011) on the basis of evolutionary distances calculated by the neighbour-joining method described by Saitou and Neil, 1987 using Maximum Composite Likelihood Model (Tamura *et al.*, 2004).

Statistical Analysis

Critical difference (CD value) in the experimental

Table 1. Location of soil samples in the study area

Sample Name	Sample area	Location	Cultivation	Latitude	Longitude	No. of samples
AP1	Haigam	North	Apple orchard	34.2411	74.50431	2
AP2	Sangrama	North	Apple orchard	34.2538	74.47563	2
AP3	Choora	North	Apple orchard	34.372	74.468	2
AP4	Hamray	North	Apple orchard	34.132	74.317	2
AP5	Goshbug	North	Apple orchard	34.228	74.549	2
AP6	Andragam	North	Apple orchard	34.206	74.549	2
AP7	Syedpora	North	Apple orchard	34.312	74.528	2
AP8	Palhallan	North	Apple orchard	34.192	74.455	2
AP9	Gondhbal	North	Apple orchard	34.142	74.553	2
AP10	Wanigam	North	Apple orchard	34.174	74.325	2
AP11	Athoora	North	Apple orchard	34.087	74.556	2
AP12	Tarzoo	North	Apple orchard	34.279	74.775	2
AP13	Singhpora	North	Apple orchard	34.385	74.490	2
AP14	Wadura	North	Apple orchard	34.340	74.375	2
AP15	Seelu	North	Apple orchard	34.332	74.408	2
AP16	Achabal	North	Apple orchard	34.681	74.423	2
AP17	Amberpora	North	Apple orchard	34.263	74.504	2
AP18	Dhangerpora	North	Apple orchard	34.326	74.456	2
AP19	Bhuna topper	North	Apple orchard	34.196	74.543	2
AP20	Goripora	North	Apple orchard	34.357	74.440	2
PDY21	Soibugh	Center	Paddy	33.075	74.713	2
PDY22	Haripora	Center	Paddy	33.051	74.3423	2
PDY23	Goshbug	North	Paddy	34.113	74.563	2
PDY24	Haigam	North	Paddy	34.1526	74.3034	2
PDY25	Goshan	North	Paddy	34.228	74.557	2
PDY26	Bangil	North	Paddy	34.134	74.563	2
PDY27	Sangrama	North	Paddy	34.294	74.461	2
PDY28	Athoora	North	Paddy	34.096	74.782	2
PDY29	Hamray	North	Paddy	34.135	74.319	2
PDY30	Chadoora	North	Paddy	33.942	74.780	2

groups were calculated by Two way ANOVA using R-software.

RESULTS AND DISCUSSION

Concentration of Cadmium in soil samples

The concentration of Cadmium present$_3$ in soil samples collected from the orchard and paddy fields were considerably low (Table 3). It is safe to say that in these regions, Cadmium is present in mostly in traces and contamination has no yet taken place. However, in coming years, with the advent of not only industrialization but injudicious applications of chemical fertilizers and pesticides containing Cadmium may cause serious concerns in our lands. This is because everything that's being applied to the soil, gets eventually accumulated in the system unless remediated. In this study the bacterial isolates from the samples were generally low in occurrence. It aligns with the work done by Ahmad *et al.*, 2016; Chen *et al.*, 2016; Siyahambal *et al.*, 2017, who concluded that the heavy metal tolerant plant growth promoting rhizobacteria (PGPRs) are abundantly found in soils with high contamination of heavy metals.

Isolation and screening of the bacteria

The bacteria were isolated from the soil samples on nutrient agar medium with different concentrations of $CdCl_3$ starting from 25 ppm up to 100 ppm. The mean bacterial population (cfug^{-1}) at 25 ppm was observed highest as 116 cfug^{-1} of soil and low of 43.33 cfug^{-1} of soil. For the nutrient agar media with the concentration of 50 ppm $CdCl_3$, the mean bacterial population was 118.33 cfug^{-1} (maximum) of soil with lowest of 43 cfug^{-1}. Similarly, with the concentration of 75 ppm of $CdCl_3$, the highest population of 108 cfug^{-1} of soil was observed while 38.67 cfug^{-1} of soil was lowest mean of the bacterial population at the concentration of 75 ppm. In case of media having 100 ppm of $CdCl_{3,}$ the highest of

Table 2. Location of soil samples in the study area

Sample Name	Sample area	Location	Cultivation	Latitude	Longitude	No. of samples
AP31	Khamri	South	Apple orchard	33.876	74.8961	2
AP32	Aglar	South	Apple orchard	33.810	75.0267	2
AP33	Keegam	South	Apple orchard	33.794	74.8612	2
AP34	Gaberpora	South	Apple orchard	33.773	74.8474	2
AP35	Ganopora	South	Apple orchard	33.846	74.9782	2
AP36	Trichel	South	Apple orchard	33.781	74.9430	2
AP37	Sheermal	South	Apple orchard	33.785	74.9421	2
AP38	Seloom	South	Apple orchard	33.827	74.8401	2
AP39	Pachahar	South	Apple orchard	33.532	73.0617	2
AP40	Gulshanabad	South	Apple orchard	33.924	75.0968	2
PDY41	Dompora	South	Paddy	33.920	74.9432	2
PDY42	Ratni pora	South	Paddy	33.946	74.9215	2
PDY43	Kakapora	South	Paddy	33.810	75.0263	2
PDY44	Pinglena	South	Paddy	33.813	75.0271	2
PDY45	Aglar	South	Paddy	33.834	74.9803	2
PDY46	Angam	South	Paddy	33.776	74.9112	2
PDY47	Wasoora	South	Paddy	33.873	74.8851	2
PDY48	Chitragam	South	Paddy	33.776	74.9112	2
PDY49	Babgund	South	Paddy	33.873	74.885	2
PDY50	Zainapora	South	Paddy	33.776	75.0053	2
AP51	Dompora	South	Apple orchard	33.926	75.0971	2
AP52	Arigam	South	Apple orchard	33.934	75.0976	2
AP53	Babgund	South	Apple orchard	33.872	74.8834	2
AP54	Boonora	South	Apple orchard	33.873	74.8927	2
AP55	Nikas	South	Apple orchard	33.874	74.7961	2
AP56	Drabgam	South	Apple orchard	33.835	74.0472	2
AP57	Tengweni	South	Apple orchard	38.873	74.8976	2
AP58	Tikenbatapora	South	Apple orchard	38.846	74.8697	2
AP59	Mitrigam	South	Apple orchard	33.857	74.8493	2
AP60	C.B. Nath	South	Apple orchard	33.961	74.8574	2

Table 3. Estimation of Cadmium metal concentration in soil samples

Sample name	Cadmium (mg^{-1}/kg^{-1})	Sample name	Cadmium (mg^{-1}/kg^{-1})
AP1	0.045	AP31	0.063
AP2	0.098	AP32	0.074
AP3	0.069	AP33	0.045
AP4	0.051	AP34	0.076
AP5	0.059	AP35	0.065
AP6	0.050	AP36	0.047
AP7	0.045	AP37	0.053
AP8	0.055	AP38	0.048
AP9	0.050	AP39	0.055
AP10	0.055	AP40	0.071
AP11	0.045	PDY41	0.076
AP12	0.059	PDY42	0.053
AP13	0.045	PDY43	0.041
AP14	0.055	PDY44	0.055
AP15	0.045	PDY45	0.054
AP16	0.050	PDY46	0.059
AP17	0.059	PDY47	0.061
AP18	0.050	PDY48	0.067
AP19	0.050	PDY49	0.054
AP20	0.055	PDY50	0.055
PDY21	0.045	AP51	0.051
PDY22	0.045	AP52	0.068
PDY23	0.045	AP53	0.074
PDY24	0.045	AP54	0.076
PDY25	0.050	AP55	0.065
PDY26	0.045	AP56	0.055
PDY27	0.045	AP57	0.045
PDY28	0.045	AP58	0.071
PDY29	0.045	AP59	0.054
PDY30	0.055	AP60	0.063

bacterial population was 98.33 $cfug^{-1}$ of soil and 37.33 $cfug^{-1}$ was observed to be lowest bacterial population (Table 4).

The results of the study show decrease in the bacterial population with the increase in the concentration of heavy metal in the medium. The results are in accordance the study of Syed and his associates (2021) concluding that high levels of heavy metals may reduce the bacterial population likely due to metal ion surface binding and degradation of membrane functions.

Minimum Inhibitory concentration (MIC)

Seven isolates obtained were found resisting highest concentration (100 ppm) of $CdCl_3$salt added in the nutrient agar. The isolates were named as AXCd1, AXCd10, AXCd6, AXCd7, AXCd8 (*Pseudomonas putida)*, AXCd9 and PDYCd42. These isolates were further examined for their bioaccumulation capacity followed by the identification of the most prominent isolate (Table 5).

Table 4. Bacterial population after isolation of soil samples at different concentrations of Cd

Sample	$Cfug^{-1}$ soil			
	25 ppm	50 ppm	75 ppm	100 ppm
AP1	116.00	104.00	93.00	89.33
AP10	121.33	118.33	108.00	98.33
AP6	100.67	92.67	105.33	81.00
AP7	88.00	104.33	95.00	95.67
AP8	92.67	101.67	98.00	98.00
AP9	87.00	101.67	102.67	82.67
PD21	55.33	45.67	40.67	37.33
PD23	66.33	58.67	52.00	46.00
PD25	49.33	40.33	38.67	38.00
PD41	46.33	43.00	47.67	43.67
PD42	58.67	55.33	51.00	42.00
PD48	68.67	62.67	57.33	56.33
Mean	79.19	77.36	74.11	67.36
CD ($p \leq 0.05$)	Factor 1=0.697	Factor 2=1.065	Factor 3=1.845	

Factor 1=conc., factor 2=isolates, factor 3=conc: isolates

Out of seven isolated resisting 100 ppm of $CdCl_3$ concentration, the highest MIC of 150 ppm was shown by isolate AXCd8 (*Pseudomonas putida)*, while the MIC of AXCd1, AXCd10,AXCd6, AXCd7, AXCd8 AXCd9 and PDYCd42 was observed to be 120 ppm. The study clearly goes well with the study of Pal and Sengupta (2019) conducted an experiment that showed some of the isolates of *Pseudomonas putida* had MIC ranging from 150 to 200 ppm.

In vitro evaluation of bacterial isolates

The nutrient broth was prepared in Erlenmeyer flasks of 250 ml, cotton plugged and autoclaved at 121°C for 30 mins. When the nutrient broth cooled down salt solution $CdCl_3$was added separately using bacteriological filter paper with concentrations 50 ppm, 75 ppm and 100 ppm. The broth was transferred to sterilized test tubes of 30 ml and all the seven isolates were inoculated inrespective concentrations of $CdCl_3$ nutrient broth for about 48 hrs in incubation shaker at 150 rpm. After 48 hrs, the growth was observed in each tube except the control. The nutrient broth with three different concentrations of $CdCl_3$ was transferred to 10 ml sterilized tubes and plugged followed by centrifugation at 2000 rpm. This step separates the liquid supernatant from the bacterial biomass. The acid dissolution technique given by Ganje and Page (1974) was carried out in harvested cells and

microbial cell mass were separately treated to estimate the per cent bioaccumulation. The readings were taken using Atomic Absorption Spectrometry (AAS). The calculation of % bioaccumulation of Cadmium was done using a formula.

Out of all the seven isolates, isolate AXCd8 (*Pseudomonas putida)* exhibited significantly higher per cent bioaccumulation of 48.9% followed by 45.7% by AXCd1, 42.57% by AXCd7, 42.15% by AXCd9, 41.68% by AXCd10, 34.97% by AXCd6 and lowest of 30.84% by isolate PDYCd42 (Table 6). The results showed the with the increase in time, there is increase in bacterial biomass. Therefore, with the increase in biomass, the cadmium accumulation also increased. According to Bai *et al.* (2002), increase in the surface area can be due to increase in the bacterial biomass thereby improving absorptive nature or increases active binding sites on cell surfaces. In the initial stages, the Cadmium removal efficiency of all the isolated increased but gradually became less with increase in concentration and less occupancy of binding sites. According to Pramanik *et al.* (2016), *Klebsiella pneumonia* had very promising cadmium removal ability. But the lower initial concentration may be implied as low heavy metal availability for bacteria, therefore, the heavy metal removal per cent can be enhanced with the higher concentration of supplemented heavy metal in medium although the growth of bacteria reduces with increased metalconcentration.

Chojnacka *et al.* (2005) explainedthe fact that the negatively charged groups (carboxyl, hydroxyl and phosphoryl) of the bacterial cell walls adsorb metal cations through various mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or the combination of such processes.

The results of our study are in accordance with the study of Li *et al.* (2013) whose study suggests the suitability of *Pseudomonas putida* for heavy metal removal. Moreover, the metal removal by *Pseudomonas putida* (Choi and his associates, 2009) as well as *Pseudomonasveronii* (Vullo and his team, 2008) has been likewise proved. The study of Wang *et al.* (2000) on *Pseudomonas florescence* Pf-5 showed similar results for metal removal capacity.

Molecular identification of most effective Cadmium accumulating microorganism

Isolate AXCd8 was selected for molecular identification. The DNA of this bacterial isolate was extracted using microbial DNA isolation kit

Table 5. Turbidity of isolates at different concentrations of Cd during three-time intervals

Concentration	24 hours			Sub	36 hours			Sub	48 hours			Sub	Factor Means	
Isolates	50	75	100	Mean	50	75	100	Mean	50	75	100	Mean	Isolates	Concentration
AXCd1	0.065	0.043	0.015	0.041	0.144	0.185	0.216	0.182	0.834	0.854	0.841	0.843	0.355	0.352
AXCd10	0.093	0.101	0.054	0.083	0.257	0.333	0.368	0.319	0.825	0.854	0.866	0.848	0.417	0.375
AXCd6	0.085	0.065	0.025	0.058	0.193	0.203	0.224	0.207	0.786	0.810	0.815	0.804	0.356	0.378
AXCd7	0.067	0.056	0.012	0.045	0.155	0.174	0.208	0.179	0.852	0.874	0.865	0.864	0.363	
AXCd8	0.075	0.037	0.035	0.049	0.184	0.236	0.254	0.225	0.845	0.892	0.874	0.870	0.381	
AXCd9	0.084	0.093	0.045	0.074	0.245	0.315	0.354	0.305	0.914	0.954	1.024	0.964	0.448	
PDYCd42	0.025	0.015	0.025	0.022	0.131	0.154	0.205	0.163	0.533	0.626	0.613	0.591	0.259	
Mean	0.071	0.059	0.030	0.053	0.187	0.229	0.261	0.226	0.798	0.838	0.843	0.826		

CD ($p \leq 0.05$) Factor 1 = 0.0013 Factor 2=0.0008 Factor 3=0.0008 Factor 4=0.0023 Factor 5=0.0023 Factor 6=0.0015Factor 7=0.004

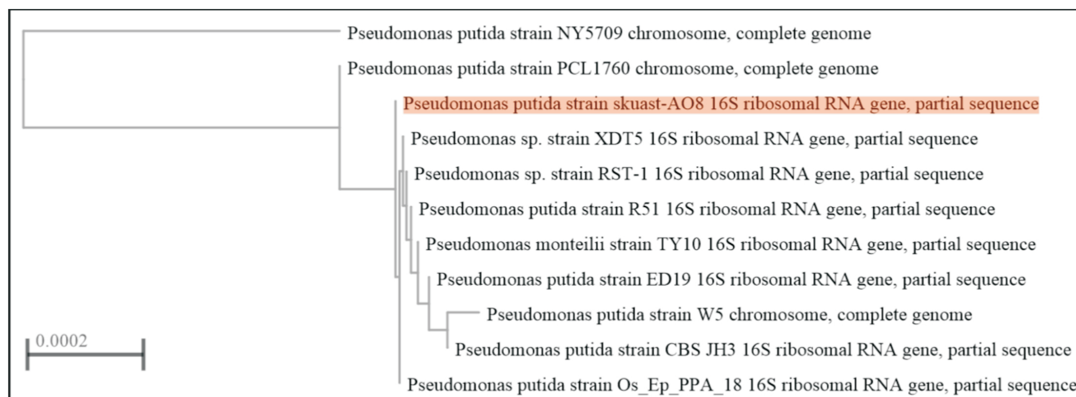

Fig. 1. Phylogenetic relationship of PDYCd8 and resemblance with *Pseudomonas putida*

Table 6. % Bioaccumulation by bacterial isolates after 48 hrs

Concentration Isolates	48 hrs 50	75	100	Mean
AXCd1	45.12	46.16	45.82	45.70
AXCd10	42.78	40.51	41.75	41.68
AXCd6	34.51	34.82	35.57	34.97
AXCd7	40.89	42.15	44.68	42.57
AXCd8	48.18	49.97	48.54	48.90
AXCd9	41.47	41.64	43.35	42.15
PDYCd42	29.07	27.20	36.24	30.84
Means	40.29	40.35	42.28	40.97
CD ($P \leq 0.05$)	Factor 1=0.697	Factor 2=1.065	Factor 3=1.845	

Factor 1= conc., factor 2 = isolates, factor 3 = conc: isolates

(MOBIO) according to manufacturer's protocol.The extracted DNA was amplified by PCR kit. The presence of DNA was verified using gel electrophoresis technique. It was followed by the sequencing of the extracted DNA and blast analysis showed 99.33% resemblance with *Pseudomonas putida*. The isolate AXCd8 is registered in NCBI database *as Pseudomonas putida* strain skuast-AO8 (Fig. 1).

CONCLUSION

In this study bacterial isolates obtained from agricultural lands can be used for bioremediation of heavy metal affected soils. Pseudomonas putida (PDYCd8) can growth effectively in nutrient broth with metal ions of Cadmium exhibiting metal accumulation in an aqueous system. Furthermore, the study suggests that with the increase in concentration of metal, the growth of bacteria increases up to a certain concentration. Molecular identification shows 99.33% of resemblance of isolate PDYCd8 with *Pseudomonas putida.*

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Conflict of Interest- None

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