

Bioaccumulation of Cadmium by *Pseudomonas Sp.* Isolated from Metal Polluted Industrial Region

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In the study bacterial strains were isolated from soil, sediment and water samples taken from the metal contaminated industrial area, and heavy metal resistance and bioaccumulation potential of the isolates were investigated. Cadmium analysis of the samples revealed that Cd concentration varied from $2.31 \mu g L^{-1}$ to $8.96 \mu g L^{-1}$ in water, from $0.55 \mu g g^{-1}$ to $25.44 \mu g g^{-1}$ in soil and from $0.45 \mu g g^{-1}$ to $22.90 \mu g g^{-1}$ in sediment. Cadmium resistance studies of the bacterial isolates showed that out of 164 collected isolates the majority demonstrated low resistance ($<500 \mu g/ml$), while the rest showed high resistance of $>1500 \mu g/ml$. 10 bacterial genera were recorded from soil, 11 from water and only 5 bacterial genera from sediment samples. *Bacillus, Pseudomonas* and *Enterobacter* were found in soil, sediment and water samples. Results of the cadmium removal study revealed that with an increase in time the biomass of selected *Pseudomonas sp.* increased. Correspondingly, with an increase in biomass the cadmium bioaccumulation also increased. Relatively, an increased removal of cadmium was observed in the first day of the experiment. About 40% of cadmium decreased in an experimental flask, while in the control flasks reduction was only 5% till the end of the experiment (74 hr). Comparatively, cadmium showed higher reduction at pH 7. The results allowed to conclude that the selected bacterial isolates possessed a potential for bioaccumulation activity and thus appeared to be an appropriate nominee for bioremediation processes.

Keywords: Industrial area, metal pollution, cadmium, metal resistance, Pseudomonas, bioaccumulation.

1. Introduction

Manufacture of heavy metal has greatly increased since the industrial revolution (Ayres 1992). Heavy metal represents a significant hazard for the human health and the ecosystem (Boopathy 2000), and it usually forms compounds that can be toxic, carcinogenic or mutagenic even at very low concentrations (Ruiz-Manriquez et al. 1997). Some heavy metal, including iron, zinc, copper and manganese, is micronutrient used in the redox processes, in regulation of osmotic pressure as enzyme cofactor, and it has special importance to maintenance of the protein structure (Vallee and Auld 1990). However, even essential metals, for example zinc and copper, are toxic at their elevated level. On the other hand, metals, including lead, cadmium etc., do not play any known physiological role and are, in

fact, toxic. Scientific literature indicates that cadmium is one of heavy metal that has been recognized as a potent human toxin with reports of many diseases. The kidney, especially the renal tract, is a critical organ of intoxication after the exposure to cadmium.

Conventional methods of removing metals such as precipitation, oxidation or reduction have been commonly used to remove heavy metal from industrial waste water. They are ineffective or expensive, though. Substitute methods of metal removal and revival based on biological resources have been considered. Certain types of microbial biomass can keep comparatively high quantities of metal by means of passive processes known as biosorption (Raras 1995). Such processes are of industrial interest, since the removal of potentially hazardous heavy metal from industrial effluents by microbial biomass can lead to detoxification and also to recovery of valuable elements (Brierley et al. 1985). The use of bacterial biomass for the metal removal process of effluents is a viewpoint suggested by many researchers dealing with metal-bacteria interactions (Gadd 1993).

The aim of the present research was to isolate and characterize bacteria from soil, sediment and water of the Eloor-Edayar industrial zone, Kerala, South India, to study the cadmium resistance pattern, the Cd bioaccumulation potential of the selected bacteria and Cd contamination in that industrial area.

2. Materials and Methods

Study area: the Eloor-Edayar industrial area – the largest industrial belts of Kerala are parts of Ernakulam districts of Kerala, south India, located in 76° 17' 32.9" - 076° 18' 31.8" E longitudes and 10° 04' 51.6" - 10° 04' 38" N latitudes, and it is a chronically polluted area, one of the biggest exporting centers of fertilizers and chemicals. Eloor is a of FACT, TCC, IRE, HIL and many other small and big industries situated on the lower flood plains of the river Periyar and it is an island of 14.21 km² formed between two distributaries of the Periyar.

Collection of Samples: Soil, sediment and water samples were collected from abandoned paddy fields, canals and river of the selected industrial area. Totally, seven sampling sites were identified and from each of them soil, sediment and water samples were collected. Soil samples were collected at the depth of 15 to 20 cm from the surface after removing the top layer. For each of the sampling sites, sub-samples of soil were collected from different locations, pooled together and homogenized in a way to obtain a representative sample. Samples were collected by means of spades which were thoroughly cleaned and disinfected between sampling to prevent crosscontamination. Sediment samples were collected using a Grab sampler and inserted into sterilized plastic bags. Water samples were collected using sterilized plastic bottles. Soil, sediment and water samples were transferred to an ice box and transported to the laboratory.

Cadmium analysis: Cadmium in water was determined with Anodic Stripping Voltammetry (797 VA Computrace, Metrohm) after acid digestion of the sample as per the method described in APHA 1998. For sediment and soil, the samples were firstly air dried and then ground to fine powder using a pestle and mortar. Then the samples were separated into two different granulometric fractions, < 200 μ m and < 63 μ m, using stainless steel sieves. An aliquot of 0.25g of powdered sediment of < 63 μ m was digested with Selectipur Nitric acid using a microwave digester (MARS X PRESS, CEM, USA) as per USEPA 3051a for heavy metal (Kingston & Jassie 1988; Kingston et al. 1997). The digested solution was filtered through Whatman No: 1 filter paper and finally the volume amounted to 25 ml with ultrapure water (Elga ultrapure water system UK). Cadmium was then determined by Voltammetric Trace Metal Analyzer-797 VA Computrace, Metrohm (Ireland- Ripert et al. 1982; Lo & Lee. 1994).

Isolation and identification of bacteria: Isolation and enumeration of bacteria were carried out by the standard serial dilution plate technique. Serially diluted samples were sown on nutrient agar and incubated at 37°C for 24-48 hr. Bacterial colonies from nutrient agar were isolated, purified and maintained as pure culture for a further study. Bacterial isolates maintained as pure culture on nutrient agar were characterized and identified up to a genus level by morphological tests as per Bergey's Manual of Determinative Bacteriology: 9th edition (Holt *et al.* 1994) and 8th edition (Buchanan and Gibbons 1974). Morphological tests carried out for identification of the isolates were Gram's staining, cell shape and arrangement, pigment production, O/F glucose tests, Endospore staining, Motility, Catalase, Oxidase etc.

Bacterial cadmium resistance test: Resistance of the bacterial isolates to varying concentrations of cadmium was determined by an agar dilution method (Luli et al. 1983). Fresh overnight cultures of the isolates grown in peptone water were aseptically inoculated into the nutrient agar plates which were supplemented with increasing concentrations of cadmium metal ions (500 μ g ml⁻¹ to 1.5mg ml⁻¹). The plates were incubated at the room temperature and observed for bacterial growth. The lowest concentration of cadmium at which no growth occurred when compared to the control plates was considered as the Minimal Inhibitory Concentration (MIC). Metal salt was added to the medium after autoclaving and cooling to 45-50°C, from the filter sterilized stock solutions. Metal salt used for that study was Cadmium nitrate (Cd(NO₃)₂.4H₂O).

Bioaccumulation of Cadmium: The bacterium (strain TCC50) growing in the largest cadmium concentration was selected for the study and it was identified as Pseudomonas. The bioaccumulation method at different pH (5, 7 and 9) with living bacterial cells was used for cadmium removal. To study heavy metal removal with live cells, nutrient broth amended with initial concentration of cadmium (20mg L⁻¹) was inoculated from overnight grown cultures of the selected bacterial isolates. The inoculated flasks were incubated in shaking conditions at the room temperature for 72 hr. An aliquot of 5mL sample was taken daily (with a 24 hr interval) from each flask. The control flasks without bacterial biomass were running simultaneously with the experiment flasks. The samples were centrifuged to remove suspended biomass, and the supernatant heavy metal concentration was determined.

3. Results

A total of 18 bacterial genera were recorded from the selected industrial area. 10 bacterial genera were represented from soil, 11 from water and only 5 were recorded from sediment samples (Table 2).

Table 1.	Heavy metal concentration in soil,		
	water and sediment samples from the		
	selected industrial area		

Sample	Metal concentration				
Name	Soil (µg g	Water (µg L ⁻	Sediment		
	1)	1)	$(\mu g g^{-1})$		
TCC	1.22	3.465	0.45		
BPM	25.44	5.774	21.48		
KDM	0.55	2.316	2.78		
BR	2.47	nd	1.72		
WMH	1.34	nd	0.60		
RFH	nd	5.086	22.90		
WB	5.95	8.962	12.73		
Mean/SD	5.28/9.1	3.6/3.2	8.95/9.9		

Wetland near Binanipuram (WB), Wetland near Merchaam and HIL (WMH), Kuzhikandam canal (KDM), Canal near Binanipuram (BPM), the Binananipuram river (BR), the Pathalam bund river (TCC), River near FACT and HIL (RFH), Standard deviation (SD).

Bacillus, Pseudomonas and *Enterobacter* were found in soil, sediment and water samples. Cadmium

analysis of the samples revealed that Cd concentration was varying from 2.31 μ g L⁻¹ to 8.96 μ g L⁻¹ in water (Mean/SD - 5.28/9.1), 0.55 μ g g⁻¹ to 25.44 μ g g⁻¹ in soil (Mean/SD - 3.6/3.2) and 0.45 μ g g⁻¹ to 22.90 μ g g⁻¹ in sediment (Mean/SD - 8.95/9.9). Cadmium levels in soil, sediment and water samples of the study area are represented in Table 1. Cadmium resistance studies of the bacterial isolates showed that out of 164 collected isolates the majority showed low resistance of >1500 μ g/ml.

Table 2.Bacterial genera found in soil, sediment andwater samples from the Eloor- Edayar Industrial belt

Sl	Sample				
No.	Soil	Sediment	Water		
1	Bacillus	Kurthia	Staphylococcus		
2	Caryophanon	Pseudomonas	Acinetobacter		
3	Listeria	Bacillus	Azotobacter		
4	Kurthia	Enterobacter	Bacillus		
5	Agromyces	Escherichia	Pseudomonas		
6	Arthrobacter		Xanthobacter		
7	Cellulomonas		Enterobacter		
8	Deinococcus		Escherichia		
9	Psedomonas		Klebsiella		
10	Enterobacter		Aeromonas		
11			Thiobacillus		

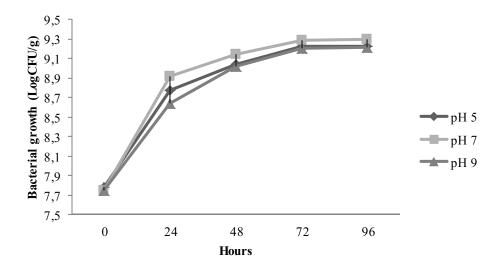


Fig. 1. Growth kinetics of Pseudomonas sp. at different p^H with 20mg/L initial concentration of cadmium

Results of the cadmium removal study revealed that with an increase in time the biomass of selected *Pseudomonas sp.* increased (Fig. 1). Correspondingly, with an increase in biomass the cadmium bioaccumulation also increased. Generally, 40% of cadmium in the experimental flask decreased, while only a 5% reduction occurred in the control flasks till the end of the experiment (74 hr) (Figs 2-4) Comparatively, the cadmium removal showed higher reduction at pH 7 (Fig. 5). Relatively, an increased

removal of cadmium was observed in the first day of the experiment.

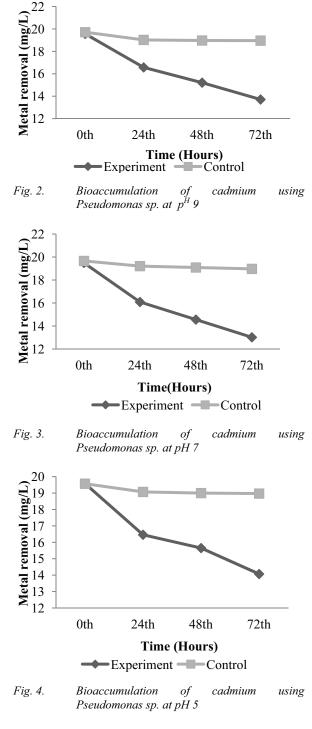
4. Discussion

Amounts $(3\mu g L^3)$ of cadmium in the water samples obtained from the selected industrial area were higher than permitted by the WHO 1993 standard. Typically, fresh water contains the level of

cadmium below 1 microgram per liter, but the concentrations up to 10µgL1 may occur on rare occasions due to the environmental disturbances such as acid rain. In the present work in water samples a 2-3 times higher content of cadmium was found which may be caused by the leakage of waste water from the industrial area. The cadmium level in soil of the selected industrial area was generally higher than the WHO 1993 standard value of 2.0mgkg⁻¹. The level of cadmium in sediment far more exceeded the acceptable limit of 0.6mgkg⁻¹. In the case of soil samples, cadmium showed the enrichment level exceeding the normally expected distribution, giving rise to concern over suitability of soil in the study area (Srinivasa et al. 2005; Yefang et al. 2006; Govil et al. 2008). High levels of cadmium were observed in several pockets, closer to the industries which specified that the source of those elements could be industrial effluents.

Cadmium resistance studies of the bacterial isolates showed that out of 164 collected isolates the majority showed low resistance (<500µg/ml) and many isolates showed high resistance of >1500µg/ml. High level of resistance and widespread tolerance that was found among the isolates were probably attributed to the cadmium contents in soil (Abou-Shanab et al. 2007). The varying level of tolerance among 164 cultures may be due to the difference in the concentration of cadmium in the environment. The site from which the samples were taken had been polluted with high level of heavy metal for many years, perhaps giving a diverse range of bacteria a chance to adapt to the environment either by convergent evolution of resistance mechanisms or by transferring the resistance genes via a plasmid. Resistance to heavy metal, including cadmium, zinc, copper, chromate, cobalt, arsenic and nickel is most often carried out by bacteria on plasmids or transposons and it has been theorized that this allows for lateral transfer in the environment (Silver 1992).

The results of heavy metal removal studies showed that with an increase in time, the biomass of the bacterial strains increased. Likewise, with an increase in the biomass, the cadmium bioaccumulation also increased. An increase in the surface area that can be due to an increase in biomass improves the adsorptive nature or increases the number of active binding sites on cell surface (Bai et al. 2002). The selected metal resistant strains showed that their growth was only slightly affected with different pH. Therefore, it is clear that growth of our isolates is not inhibited with different pH and this fact makes them strong candidates for future application to metal bioremediation.



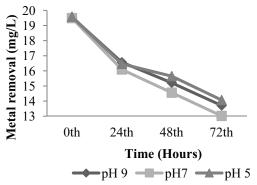


Fig. 5. Effect of pH on cadmium removal using Pseudomonas sp.

The active mode of metal accumulation by living cells is usually designated as bioaccumulation. This process is dependent on the metabolic activity of the cell referred to its intrinsic biochemical and structural properties, physiological and/or genetic adaptation, environmental modification of metal specification, availability and toxicity (Cha and Cooksey 1991). The capacity of living cells to remove metal ions from aqueous solutions is also significantly influenced by environmental growth conditions, as the temperature, pH and biomass concentrations (Chen and Ting 1995).

Many researchers have reported on efficiency and mechanisms of bacteria to remove different metal ions, many of their statements are comparable to the present study. Richard *et al.*, (2002) reported that Cu^{+2} and Pb⁺² seemed to bind to materials on their cell surface. Lead is precipitated in an insoluble form that is localized to the cell membrane or cell surface (Aiking *et al.* 1985; Levinson *et al*, 1996; Roane 1999). This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl and phosophryl) of the bacterial cell wall adsorb metal cations through various mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or the combination of such processes (Chojnacka *et al*, 2005).

As pH values in metal-containing water and wastewater can vary, to examine the effect of heavy metal removal the solutions of different pH values are to be used. In the present work, the initial pH has been adjusted in the range of 5, 7 and 9 before the addition of biosorbent. The medium pH affects solubility of metals and ionization state of the functional groups such as carboxylate, phosphate and amino ones of the cell wall. Inconsistency in the literature regarding the influence of pH on biosorption seems to indicate that the way pH would alter the adsorption of metal ions to biomass varies with both the type of adsorbents (biomass) and the type of adsorbates (metal ions). Obtained results have shown that the selected Pseudomonas sp. is a good bioaccumulation medium for cadmium ions and has a high adsorption yield for the treatment of wastewater containing cadmium ions. Consequently, bacteria bioaccumulation technologies are being developed and much more work in this field is to be done.

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Kadmiu užteršto pramoninio regiono išvalymo analizė naudojant biologiškai sukauptas bakterijas *Pseudomonas Sp*

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Atliekant tyrimą, buvo tirtas bakterijų atsparumas sunkiųjų metalų poveikiui, taip pat įvertintas biologinės akumuliacijos potencialas. Iš sunkiaisiais metalais užteršto pramoninio regiono buvo paimta dirvožemio, nuosėdų ir vandens pavyzdžių. Analizė parodė, jog kadmio (Cd) koncentracija kito nuo 2,31µg L–1 iki 8,96 µg L–1 vandenyje, nuo 0,55µg g–1 iki 25,44µg g–1 dirvožemyje ir 0,45µg g–1 iki 22,90µg g–1 nuosėdose. Bakterijų atsparumo kadmiui tyrimas parodė, kad iš 164 surinktų bakterijų rūšių dauguma buvo neatsparios (<500 µg/ml), tik nedidelė dalis buvo labai atsparios (>1500 µg/ml). 10 bakterijų rūšių buvo aptiktos dirvožemio pavyzdyje, 11 – vandenyje ir 5 – nuosėdų bandiniyje. Bacillus, Pseudomonas ir Enterobacter buvo rastos dirvožemio, vandens ir nuosėdų bandiniuose. Kadmio pašalinimo tyrimai atskleidė, jog padidėjus Pseudomonas sp. biologinei masei padidėjo metalų atranka, taip pat padidėjus biologinės masės kiekiui daugiau buvo sukaupta kadmio. Santykinai daugiau kadmio buvo pašalinta pirmąją eksperimento dieną, nes apie 40 proc. esančio bandinyje kadmio buvo įsisavinta. Paskutinę bandymo dieną (74 h) metalo buvo įsisavinta tik 5 proc. Didesnis akumuliacinis potencialas buvo esant optimaliam pH (apie 7). Atliktas tyrimas parodė, jog bakterijos labai geba kaupti kadmį ir yra tinkamos teritorijoms, užterštoms sunkiaisiais metalais, valyti.