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Toxic Heavy Metals Tolerance in Bacterial Isolates Based On Their Inducible Mechanism

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Abstract: **The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Removal of heavy metals from contaminated sites using microorganisms is a cheaper alternative to chemical technologies. The present study was carried out to isolate microbial strains from the battery manufactured polluted soil and to test their metal tolerance to cadmium, lead and nickel (Cd, Pb, Ni). In the primary screening were isolated 30 bacterial strains that were tested on higher concentrations of Cd, Pb and Ni (up to 2000 ppm), the widespread hazardous chemicals used by battery industry. It has been noticed that the intracellular accumulation of Pb changes the color of colonies grown on media with lead. The selected 7 strains were morphologically and physiochemically characterized and the growth kinetics studied on the basis of two types of developed inoculum. Data indicated that the isolated bacteria have a great potential in heavy metals bioremediation.**

Keywords: **Battery manufactured polluted soil, Heavy metal, Metal tolerance, Bioremediation, MTC.**

1. INTRODUCTION

Heavy metal soil contamination is a significant environmental problem due to the increased release of metals to the environment. Methods for the removal of heavy metals from the environment can be divided into two groups: 1) biotic methods, which are based on the accumulation of heavy metals by plants or microorganisms and 2) abiotic methods, which are based on the removal of heavy metals using physiochemical processes such as precipitation, co-precipitation, ion exchange, and adsorption of heavy metals by suitable adsorbent (Celis et al., 2000; Vijayaraghavan et al., 2008). In the present study we are checking tolerance of three heavy metals cadmium, lead and nickel.

Cadmium is well known for its toxicity, bioaccumulation and biomagnification through the food chain. Cadmium has no essential biological function and is extremely toxic to humans. In chronic exposure, it also accumulates in the body, particularly in kidney and the liver (Clarkson, 1997; Williams et al., 2008). These properties, along with its common usage in Ni-Cd batteries, make cadmium one of the commonest environmental metal poisonings. Acute poisoning from inhalation of fumes and ingestion of cadmium salts can also occur and at least one death has been reported from self-poisoning with cadmium chloride (Baldwin & Marshall, 1999). Cadmium has a wide variety of sources in the environment and from industry. One source is from ingestion of grown foodstuffs, especially grain and leafy vegetables, which readily absorb cadmium from the soil. It may also contaminate fish (Hu et al., 1998; Williams et al., 2008). In addition, being a constituent of alloys, pigments, batteries, metal coatings, plastics and fertilizers, it may occur naturally or as a contaminant. Contaminants such as sewage sludge, fertilizers, polluted groundwater and mining effluents are important sources of Cd. Occupational exposure may occur from the manufacture of these products and from welding, and smelting of lead, zinc and copper as these occur in mixed ores with cadmium. It is also found in cigarette (0.007 to 0.35 µg per cigarette) and vehicular fumes. Residential sites may be contaminated by municipal waste or leaks from hazardous waste sites (Taylor & Francis, 1995; Hu, 1998). Cadmium (Cd) is nonessential but poisonous for plants, animals, and humans (Gupta and Gupta, 1998). Cadmium is one of the most toxic pollutants of the surface soil layer,

released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels (Tang et al., 2010).

Lead has no known biological function, is highly toxic and accumulates in humans. People have known about the toxicity of lead for centuries, at least since 200BC, when Dioscorides wrote, "lead makes the mind give way". Despite this, lead has been used extensively for both industrial and domestic applications for hundreds of years (Baldwin & Marshall, 1999). The major source of lead in the environment is earth's crust. Lead enters the food and water supply quite naturally and is absorbed by foodstuffs (particularly green leafy vegetables) growing on soil where lead is present. Contamination from vehicle exhausts or wastes or from the areas naturally high in lead (Hu, 1998) is substantial sources of Pb. Previously; tetraethyl lead was an additive in petrol and lead was used in plumbing (Hu, 1998; Williams et al., 2008). In many countries the use of leaded petrol is being phased out, and lead piping in households is gradually being replaced, due to health concerns. Lead also used to be used in paints and some cases of lead poisoning are due to small children eating flakes of this paint (Taylor &Francis, 1995). Today, lead is still used in batteries, some insecticides, and is found in cigarette smoke, where there is between 0.017 and 0.98 micrograms per cigarette (Taylor & Francis, 1995). Lead (Pb), a major pollutant that is found in soil, water and air is a hazardous waste and is highly toxic to human, animals, plants and microbes.

Nickel (Ni) is the 24th most abundant element in the earth crust and has been detected in different media in all parts of the biosphere. Ni is classified as the borderline metal ion because it has both soft and hard metal properties and can bind to sulfur, nitrogen and oxygen groups (Costa and Klein, 1999). Ni has been implicated as an embryotoxin and teratogen (Chen and Lin, 1998).

The objective of this study is to determine heavy metals and antibiotic resistance of bacteria, MTC, growth studies, and biochemical and molecular characteristics were used to exploit these isolates for clean-up of industrial waste water and sewage.

2. MATERIALS AND METHODS

Sample collection and isolation of heavy metal tolerance bacteria:

The soil samples were collected from four different locations of the battery manufactured contaminated environment, at the depth of 0 - 18 cm below surface. All samples (named A1/2/3) were kept in clean sterile bags, labeled accordingly and stored at 4°C.

Isolation of cultures from the soil samples was carried out using enrichment isolation procedure. In the first step, in the case of each experimental set (T1A/B/C, T2A/B/C, T3A/B/C) 1.5 g of soil sample was incubated on LB medium, at the temperature of 37°C for 24 hours and the microbial density determined spectrophotometrically (550nm). To isolate metal resistant bacteria, 1 ml of microbial suspension was spread on 100ppm, 500ppm and 2000ppm metal (Cd, Ni and Pb) media. The growth of the bacterial colonies was measured after 16 - 24 hours of incubation at 37°C. Morphologically dissimilar colonies were randomly selected and maintained at 4°C for bacterial characterization. LA media were prepared by dissolving 1 g of bactotryptone, 0.5 g of yeast extract, 1 g of NaCl and 1.5 g of agar in 100 ml distilled water, pH 7.2 and the medium was autoclaved at 121°C for 15 minutes. Sterile stock solutions of metal ($NiCl_2$, $Pb(NO3)_2$, $CdCl_2$,) were added to LA media after sterilization to obtain the desired concentration of metal.

Preparation of Metal Solutions:

We have checked the tolerance of three heavy metals Ni, Cd & Pb at 500ppm, 500ppm & 2000ppm concentration respectively on thirty bacterial isolates that we have initially isolated from soil. The aqueous stock solutions of all the three heavy metals Ni (14000 ppm), Pb(100000 ppm) & Cd(14000 ppm) were prepared by using $NiCl_2$, $Pb(NO3)_2$, $CdCl_2$ respectively and double distilled water. All the metal solutions prepared as above were sterilized by autoclaving at 15psi for 15min. at 121°C.

We have examined the growth rates of all seven isolates in the presence of the above said heavy metals. Growth rates of all selected seven isolates were determined by growth curves. To start the growth curve for each culture we have developed two types of inoculums.

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Screening of the bacterial strains with metal tolerance:

In order to test the bacterial tolerance to metals, isolated colonies were picked up and streaked on LA agar medium with successively higher concentrations (500ppm, 750ppm and 2000ppm) of metal (Cd, Ni, Pb, and Zn). The strains capable of growing under these conditions were selected for further experiments.

Plasmid profile screening:

Plasmid DNA was isolated from selected strains using Plasmid Minipreparation System (Sambrook and Russel vol. 1) and visualized on 1 % agarose gel, in the presence of 3kb DNA ladder as a marker (Genei, Bangalore).

Reproducibility of the data:

The coefficient of variation (CV) was calculated from the ratio of standard deviation (SDV) and arithmetic mean in three independent experiments. The CV (%) < 18% shows there is not significant biological variation within the probe in experiments where the isolation of metal tolerant bacteria was performed.

3. RESULTS

Isolation of heavy metal tolerance bacteria:

Investigations were focused on the isolation and characterization of the selected bacterial strains with metal tolerance to identify potential candidates for heavy metals bioremediation. Soil samples were collected from the battery manufactured polluted environment, where lead, zinc, nickel and cadmium are the widespread hazardous chemicals used by battery industry. Similarly, Fagade et al. (1999) described the isolation of two *Pseudomonas* strains that effectively accumulated lead from a battery manufactured effluent. The enrichment isolation technique was mostly used to isolate metal resistant bacteria. The microbial density of the experimental sets (T 1/2/3 A/B/C) kept at 37°C in enrichment media for 20 hours were determined by spectrophotometer and the biological variation calculated. The heterotrophic bacterial counts range from 10^6 - 10^8 CFU/ml. The coefficient of variation (CV) per sample was less than 18 % (12.2345, 17.6811 and 4.5019), showing that there is no significant variation within the experimental sets, regardless of the date when the analysis was done. However, a value of 11.3674% for CV total proved that samples isolated from three different locations had different microbial densities (Table 1)

Table 1. Bacterial density in samples isolated from battery manufactured contaminated soil

Experimental set	**OD 550 nm**	**OD 550 nm**	**OD 550 nm**	**Average**	**Average/ sample**	**SDV/ sample**	**CV/ sample (%)**
Date	**10.04. 2011**	**14.04. 2011**	**20.04. 2011**				
T1a	0.921	0.720	0.679	0.7733	0.8288	0.1014	12.2345
T1b	0.821	0.567	0.914	0.7673			
T1c	0.935	0.845	1.058	0.946			
T2a	0.899	0.994	1.022	0.9716	0.8280	0.1464	17.6811
T2b	0.578	0.677	0.782	0.679			
T2c	0.639	1.029	0.832	0.8333			
T3a	0.749	0.849	1.019	0.8723	0.8663	0.0390	4.5019
T3b	0.839	0.712	0.923	0.8246			
T3c	0.698	0.921	1.087	0.9020			
Control	0.032	0.065	0.022				
CV total (%)					0.8410	0.0956	**11.3674**

Determination of MTC:

Maximum tolerance of the selected isolates against increasing concentrations of Cd, Pb and Ni on LA plates was evaluated until the strains unable to grow colonies on the agar plates. The initial metal concentration used 50ppm was

prepared from 1 M stock solution. We checked tolerance upto maximum 2000ppm metal concentration. The culture grow at a given concentration were subsequently transferred to the next concentration. Based on the evaluation, maximum tolerance concentration (MTC) was determined at 37°C for 7 days. We also studied MTC in enriched medium LB broth.

Determination of antibiotic resistance:

Resistance to antibiotics was determined on Mueller Hinton agar plates (Hi Media, India). Inhibition zone was noted after 38 h incubation, resistance was recorded as positive. Strains were considered susceptible when the inhibition zone was 14 mm or more in diameter. Tests were performed in triplicate. The following antibiotics were tested, ampicillin (100µg/ml), chloramphenicol (50 µg/ml), erythromycin (50 µg/ml), Kanamycin (25 µg/ml), neomycin (30 µg/ml), nalidixicacid (15 µg/ml), streptomycin (30 µg/ml) and tetracycline (20 µg/ml).

Table 2- MTC of heavy metals and antibiotics against isolated bacteria

Heavy metals/antibiotics	AV1	AV2	AV3	AV4	AV5	AV6	AV7
Cadmium	400	400	400	500	200	400	500
Lead	2000	2000	2000	2000	2000	2000	2000
Nickel	400	400	750	750	750	500	500
Ampicillin	15 (R)	12 (R)	10 (R)	14 (R)	NZ	15 (R)	10 (R)
Chloramphenicol	25 (R)	20 (R)	15 (R)	25 (S)	10 (R)	12 (R)	10 (R)
Erythromycin	15 (R)	10 (R)	12 (S)	14 (S)	12 (S)	15 (R)	12 (R)
Kanamycin	20 (R)	15 (R)	12 (R)	10 (S)	12 (R)	10 (R)	15 (R)
Nalidixic acid	15 (R)	5 (R)	10 (S)	NZ	10 (S)	12 (R)	10 (R)
Streptomycin	18 (R)	12 (R)	18 (S)	12 (S)	15 (R)	10 (R)	15 (R)
Tetracycline	25 (R)	10 (R)	22 (S)	10 (R)	12 (S)	15 (R)	12 (R)

Note: Heavy metal concentration in ppm; R- Resistant; S- Sensitive; NZ- No Zone; Antibiotic concentration in µg

Biochemical characteristics of the isolated bacteria:

Selected bacterial isolates were grown on LA agar media (HiMedia, India). The shape and colours of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of oxidase, catalase, V-P test, MR-VP test, starch hydrolysis and gelatin hydrolysis, motility, indole production and citrate utilization. The tests were used to identify the isolates according to Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986).

Determination of optimal growth conditions:

The optimal growth conditions with reference to pH and temperature were determined. The isolates were grown in LB medium with different pH values (5, 6, 7, 8, and 9) and incubation was carried out at temperature 25°C, 30°C, 35°C, 37°C and 40°C. The optical density of the log phase growing cultures (6-12 h) conditions was noted at 550 nm to determine the growth.

Growth studies:

Growth studies of selected bacterial isolates was studied in 250 ml flasks containing 100 ml LB medium supplemented with above said different concentration of Cd, Pb and Ni. Flasks were inoculated with 0.5 ml of overnight culture and agitated on a rotary shaker (150 rev/min) at 37°C. Growth was monitored as a function of biomass by measuring the absorbance at 550 nm using spectrophotometer (Hitachi, Japan).

Table 3- Biochemical analysis of selected bacteria

Selected Bacteria	AV1	AV2	AV3	AV4	AV5	AV6	AV7
Morphological							
Gram nature	+	+	+	+	+	+	+
Shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Structure	Chain	Chain	Chain	Chain	Chain	Chain	Chain

International Journal of Novel Research in Life Sciences
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Biochemical							
Arginine	+	+	-	-	-	+	+
Casein	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
Gelatin	+	+	+	+	-	+	+
Indole	+	+	-	-	-	-	-
MRVP	+	-	+	-	-	+	-
Oxidase	-	+	+	-	-	+	+
Starch	-	+	-	+	+	-	-
VP	-	-	-	-	-	-	-
Utilization of							
Glucose	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-
Fructose	-	+	-	+	-	+	+
Lactose	-	-	-	-	-	-	-
Mannose	-	-	-	-	-	-	-
Sucrose	+	-	+	-	+	-	+
Growth at							
4°C	-	-	-	-	-	-	-
25°C	-	+	+	+	-	+	+
30°C	+	+	+	+	+	+	+
35°C	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+
42°C	-	-	-	-	-	-	-

Note: + positive; - negative

Inoculum Development:

Inoculum with metal (WM)- it was prepared using 3 ml. LB medium & 1% culture along with above said metal concentration at incubation temperature of 37°C at 150rpm on a rotating shaker. So for each culture we have developed the inoculums for Ni, Cd & Pb. We have repeated this procedure and conditions for inoculum development with metals all three metals four times for all cultures to increase the adaptability of each culture to resist the presence of heavy metals (here in this case Ni, Pb, Cd) in LB medium. During the course of development of inoculums for all selected seven cultures with each of the three heavy metals we have observed that the inoculum WM in case of Ni & Pb for all cultures have reached to its log phase within 16-20 hrs. in subsequent four transfers while in case of Cd it was not so. The time period for each culture required to reach in the log phase has decreased in the subsequent transfers.

On the other hand, Inoculums without metal (W/OM) was prepared using 3 ml. LB medium & 1% culture. In this case the inoculums for all cultures have developed without metals. All the cultures in the absence of heavy metals reached to their exponential phase within 8-12 hrs.

After developing both kind of inoculum WM & W/OM, the growth curve of each culture was started using 1 % inoculum WM & W/OM (OD at 550 nm.≈ 0.050-0.080) in 100ml LB medium having the desired metal concentration (500 ppm in case of Ni & Cd, & 2000 ppm in case of Pb) against which we had to check the tolerance of each culture. After starting of growth curve for each culture OD values at 550 nm. were determined at different time durations. Thus for each metal we

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have got two growth curves for each of the seven cultures one started with inoculum developed WM and other inoculum W/OM.

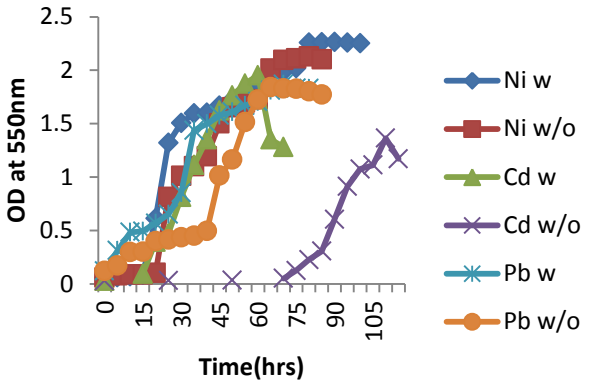

Fig. 1 Growth curve of Culture AV1

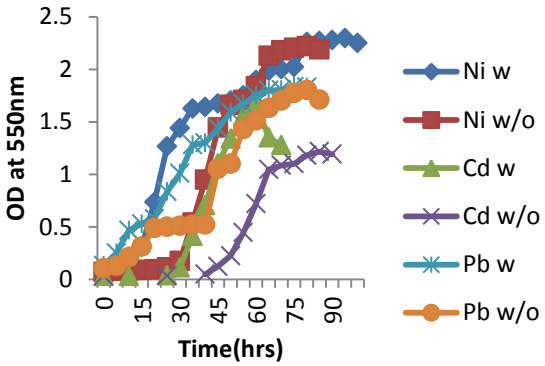

Fig. 2 Growth curve of Culture AV2

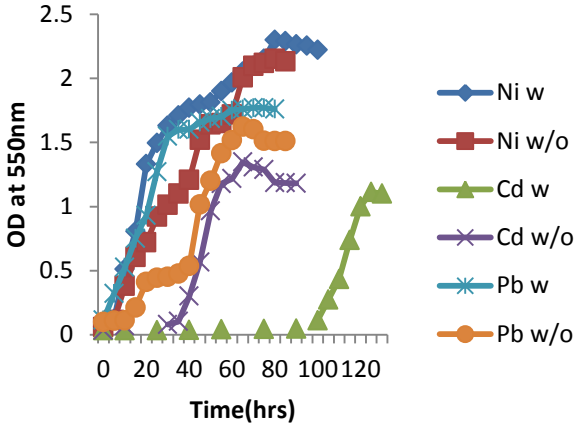

Fig. 3 Growth curve of Culture AV3

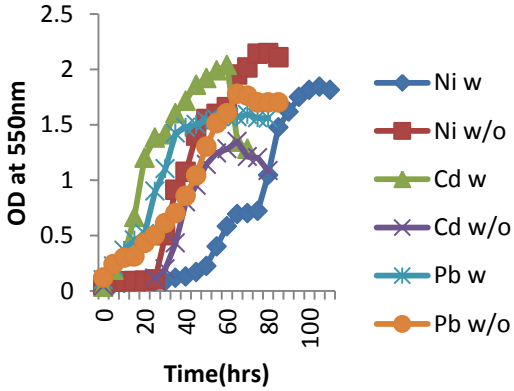

Fig. 4 Growth curve of Culture AV4

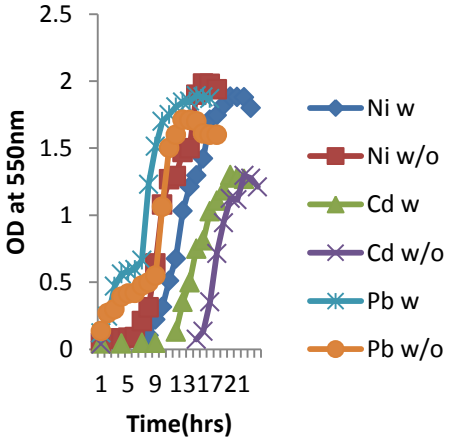

Fig. 5 Growth curve of Culture AV5

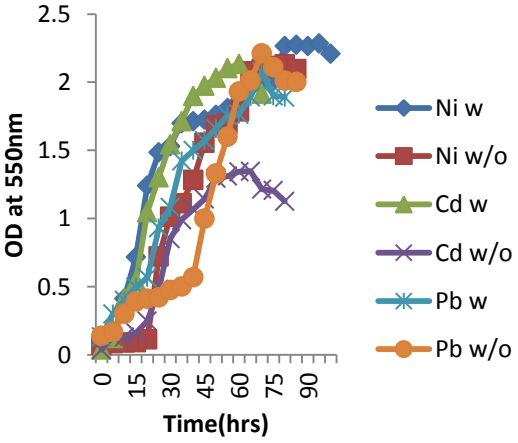

Fig. 6 Growth curve of Culture AV6

International Journal of Novel Research in Life Sciences
Vol. 2, Issue 1, pp: (34-41), Month: January - February 2015, Available at: www.noveltyjournals.com

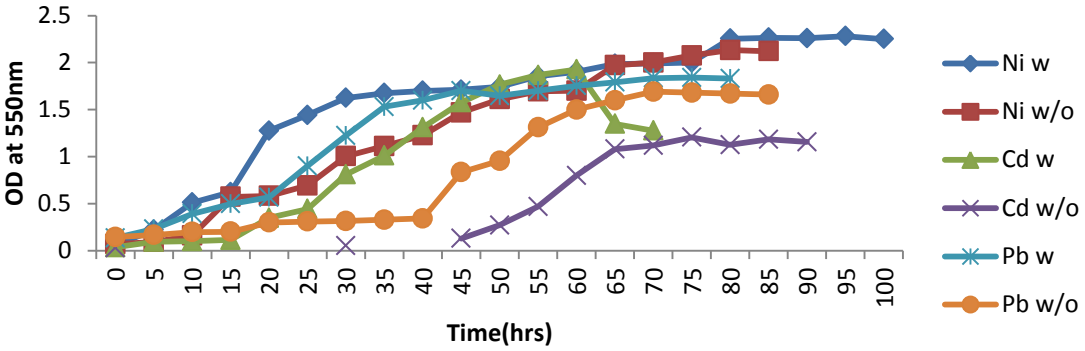

Fig. 7 Growth curve of Culture AV7

Growth kinetics of selected seven bacteria

Note: w- Inoculum developed in the presence of heavy metals; w/o-Inoculum developed without metals

4. DISCUSSION

The above given Fig. 1-7 is showing that in case of culture AV1, AV2, AV6, AV7 it has been observed that the lag phase is reduced in case of all three metals Ni, Cd & Pb when the growth curve was started with inoculum developed in the presence of metal as compared to the growth curves which was started with inoculum developed without metal where the culture had been found to remain in lag phase for longer period of time. So the presented data suggest the involvement of inducible proteins in tolerance mechanism shown by AV1, AV2, AV6, AV7 bacterial isolates. As the longer lag phase observed in growth curve started with inoculums developed in the absence of metal. So the metal acts as inducer which leads to the synthesis of inducible proteins in bacteria which helps in their further growth & tolerance. On the other hand, in case of growth curve for cultures AV1, AV2, AV6, AV7 started with inoculums developed in the presence of metal there is reduced lag phase as the inoculums developed with metal after four subsequent transfers got adapted due to development of an inducible mechanism towards heavy metal tolerance. So due to an already developed inducible mechanism their growth start very soon (resulting in reduced lag phase) after inoculation into the fresh LB medium at the time of start of growth curve.

While on the other hand the cultureAV3 for Ni & Pb, Culture AV4 & AV5 for Cd & Pb have shown the same growth pattern as it was in case of culture AV1, AV2, AV6, AV7 (Lag phase was reduced when the growth curve was started with inoculum developed in the presence of metal and was longer when growth curve was started with inoculums developed in the absence of metal) because of the presence of inducible mechanism.

In case of culture AV3 for Cd and culture AV4 & AV5 for Ni the growth pattern observed was opposite to that of the expected as in above case. For these cultures in case of above mentioned metals, the lag phase has been found to be reduced in case of growth curve started with inoculum developed without metal and was found to be longer in case of growth curve started with inoculum developed in the absence of metal. So the mechanism is not inducible here because in case of growth curve started with inoculum developed in the presence of metal there is long lag phase. Even the growth curve that was started with inoculum developed in the absence of metal have shown a reduced lag phase.

5. CONCLUSIONS

In our work, we isolated 30 bacterial strains from the battery manufactured contaminated soil samples. The selected seven microbial strains were tested on higher concentration of cadmium, lead and nickel (up to 2000ppm) and seven bacterial strains with potential in heavy metal bioremediation were selected. AV1, AV2, AV6 and AV7 bacterial culture are showing more tolerance than culture AV3, AV4 and AV5 as shown in the growth kinetics. So it has been found that the metal act as inducer which leads to the synthesis of inducible proteins and there is a kind of protein inducible mechanism

in the culture AV1, AV2, AV6 and AV7, as a result lag phase is reduced even in the presence of high concentration of three heavy metals.

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Conflict of Interest

The Author(s) here declare(s) that there is no conflict of interest regarding any financial relationships, personal relationships, academic competition and intellectual passion.

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