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Research Article

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Lead and cadmium resistant bacteria isolated from industrial effluent

***Umamaheshwari K.[1], Poonguzhali P.[1], Sivanandhan S.[2], Khusro A.[3] and Ignacimuthu S.[2]**

[1]*Department of Microbiology, St. Josephs College of Arts and Science, Cuddalore, Tamil Nadu, India*
[2]*Entomology Research Institute, Loyola College, Nungambakkam, Chennai, India*
[3]*Research Department of Plant Biology and Biotechnology, Loyola College, Nungambakkam, Chennai, India*

ABSTRACT

Bacteria play a major role in the biogeochemical cycling of toxic heavy metals. Heavy metal resistant bacteria can be used as bioremediation agents. The aim of this present investigation was to determine the resistance property of bacteria isolated from industrial waste dumped soil samples, to heavy metals (Pb^{2+} and Cd^{2+}) and some antibiotics. Of 29 isolates, only two bacterial strains were found to be highly resistant to lead and cadmium. Further, these two bacterial strains were checked for resistance against heavy metals by culturing them in basal medium in which varying concentrations of heavy metals were incorporated. HML8 and HMC1 isolates showed resistance against Pb^{2+} and Cd^{2+} at all concentrations tested. The morphological and biochemical tests confirmed that the isolates belonged to Bacillus sp. Both the isolates showed similar pattern of growth at different temperature ranging from 25ºC to 42ºC. HMC1 showed better growth in the presence of high NaCl concentration but the same was unable to grow at high pH. On the other hand HML8 showed good growth in the basal medium at high pH. High salt concentration affected the growth of HML8 in the basal medium. Both the isolates also exhibited high tolerance to antibiotics such as Amoxicillin and Penicillin G. The results of the present study clearly suggest that both the strains can be studied and explored further for biotechnological applications especially for lead and cadmium detoxification.

Key words: Antibiotics, Bioremediation, *Bacillus* sp., Heavy metals, Industrial waste

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INTRODUCTION

Environmental contamination is increasing progressively day by day due to an unabated increment in industrialization, population and urbanization [1]. Heavy metal contamination is widespread and it represents a group of metals whose atomic density is greater than 5g/cm3. In recent years, heavy metal contamination has become one of the most serious environmental problems due to its toxicity and detrimental effect to all living organisms. The environmental pollution with toxic heavy metals is spreading throughout the world. Metal contamination, therefore, represents a long-standing and recalcitrant selection pressure with both environmental and clinical importance [2]. Heavy metals such as cadmium, chromium, cobalt, lead, mercury, copper and many more are known to show an adverse effect on humans and other biological systems [3]. Among all the heavy metals, 13000 tons of cadmium are produced yearly worldwide, mainly for nickel – cadmium batteries and it is one of the most toxic pollutants of the surface layer. High exposure to cadmium can lead to obstructive lung cancer, osteomalacia, osteoporosis and increased blood pressure. Lead is another major pollutant that is found in soil, water and air. Exposure to lead can result in a wide range of biological effects in humans depending on the level and duration of exposure.

The remediation of heavy metals is a complex mechanism that is not suitable by physical or chemical methods [4]. At present, bioremediation is an idyllic process for pollution abatement. Bioremediation is an alternative that offers the possibility to degrade heavy metals using natural biological activity. Bioremediation uses biological agents, mainly microorganisms, e.g. yeast, fungi or bacteria to clean up contaminated soil and water [5]. Bioremediation process is mostly affected by temperature, pH, moisture content, hydrogeology, the nutritional state, redox-potential, and microbial diversity of the site [6]. Bacteria possess an adaptive mechanism to deal with heavy metal stress [7] including the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and the reduction of the heavy metal ions to a less toxic state [8]. In view of the wide spread contamination of cadmium and lead in the environment, the present study was carried out to isolate cadmium and lead resistant bacteria from industrial waste dumped soil samples and to study their heavy metal tolerance and antibiotic resistance.

EXPERIMENTAL SECTION

Sample collection
Industrial effluent and industrial waste dumped soil samples were collected from various streets of Ambathur Industrial Estate, Chennai, India. The samples were transported to the laboratory for immediate processing.

Isolation of heavy metals tolerant bacteria
The sample was initially enriched in Muller Hinton broth supplemented with heavy metals ($CdCl_2 \cdot 2H_2O$ and $PbCl_2$) at the concentration of 5 mg/mL. The broth was incubated at 37°C for 48 h in rotary shaker and maintained at 100 rpm. Hundred microliter of the enriched sample was streaked on Nutrient agar plate aseptically and was incubated overnight at 37°C. The selected heavy metal resistant isolates were transferred to Nutrient agar slants aseptically and preserved at 4°C.

Percentage resistance of heavy metals
The serially diluted sample from 10^{-5} dilution was directly plated on Nutrient agar medium supplemented with Lead (5mg/ml) and Cadmium (5mg/ml). Nutrient agar medium without supplementing the heavy metals was also prepared for calculating the percentage resistance of bacteria for the samples. Percentage resistance was calculated by the formula:

$$\% \text{ Resistance} = \frac{\text{cfu/ml with heavy metal}}{\text{cfu/ml without heavy metal}} \text{ X } 100$$

Determination of minimum inhibitory concentration (MIC)
The bacterial isolates were inoculated in Nutrient broth and incubated at 37°C for 24 h. Five different concentrations (8 mg/ml, 11 mg/ml, 14 mg/ml, 17 mg/ml and 20 mg/ml) of lead and cadmium were supplemented with Nutrient agar medium and plated onto sterile petridishes. Wells of 6 mm were prepared on the Nutrient agar using cork borer. Ten microliters of the sample were loaded in each well and the plates were incubated overnight at 37°C in order to determine minimum inhibitory concentration of heavy metals.

Identification of bacterial isolates
Heavy metal tolerant bacteria were identified according to Bergey's manual of systemic bacteriology [9]. Morphological and biochemical tests such as Catalase, Oxidase, Voges-Proskauer, Methyl Red, Triple Sugar Iron, Urease, Citrate and Indole were carried out to identify the metal resistant bacteria.

Process optimization for optimum growth of bacteria
The optimum growth conditions such as different pH, temperature and NaCl concentration were tested for the highly tolerant bacterial isolates. Nutrient broth was prepared at different pH ranging from 4 to 9 using 1N HCl and 1N NaOH. The broth was sterilized and the isolates were inoculated. The broth was kept for overnight incubation at 37°C and the results were noted by reading absorbance at 600 nm. The isolates were inoculated in separate broth with optimum pH and incubated at different temperatures ranging from 25°C to 42°C. Absorbance was read at the same wavelength after overnight incubation. The broth with optimum pH was supplemented with different concentrations (3%, 7%, 8% and 9%) of NaCl and the isolates were inoculated aseptically. The broth culture was kept overnight at optimum temperature suitable for the growth of isolates. After overnight incubation, the absorbance was read at 600 nm.

Antibiotic susceptibility test
The antibiotic susceptibility test for the selected isolates was performed using disc diffusion method. The isolates were transferred into Tryptic soy broth and kept for incubation at 37°C for 24 h. After overnight incubation, the cultures were swabbed on Muller Hinton agar plates and antibiotic discs such as Streptomycin, Amoxycillin, Pencillin G, Tetracycline, Gentamycin and Ofloxacin were placed aseptically onto the plates using ethanol dipped

and flamed forceps. The plates were incubated overnight at 37°C and the results were recorded in terms of sensitivity or resistance.

RESULTS

Isolation and percentage resistance of the isolates towards heavy metals

From 9 different samples collected, 29 isolates were taken for further processing (Table 1). The bacterial population obtained with and without heavy metals was determined and percentage resistance of the bacterial isolates towards lead and cadmium for each sample was calculated and tabulated. 53.6% and 57.7% of bacteria obtained from the sample S6 and S8 respectively were found to be resistant to lead. On the other hand 57.1% and 7.7% of the isolates obtained from the sample S6 and S8 respectively were found to be resistant to cadmium (Table 2).

Table 1: Total number of isolates obtained

S. No	Sample	Area/collected site	No. of isolates
1	S1	Ambathur, Industrial estate	Nil
2	S2	Sipcot, Cuddalore	Nil
3	S3	Sipcot, Cuddalore	Nil
4	S4	Sipcot, Cuddalore	9
5	S5	Ambathur, Industrial estate	7
6	S6	Ambathur ,Industrial estate	4
8	S8	Ambathur, Industrial estate	4
9	S9	Ambathur, Industrial estate	5

Table 2: Percentage resistance of isolates towards heavy metals

S. No	Sample No.	cfu/ml ×10⁵			% Resistance to Lead	% Resistance to Cadmium
		Medium with Heavy metal (5mg/ml)		Medium without Heavy metal		
		Lead	Cadmium			
1	S1	0	0	0	0	0
2	S2	0	0	0	0	0
3	S3	0	0	0	0	0
4	S4	38	4	1	32.2	3.4
5	S5	8	3	56	14.3	5.3
6	S6	15	16	8	53.6	57.1
7	S7	17	1	95	17.9	1.5
8	S8	15	2	26	57.7	7.7
9	S9	8	2	16	50	1.5

MIC of isolates towards heavy metals

Of 29 isolates, 37% of the isolates were found to be resistant to lead and 20% of the isolates were tolerant towards cadmium. The isolate HML8 was found to be resistant to lead at all concentrations tested (Table 3). On the other hand HMC1 showed very high resistance to cadmium at all concentrations (Table 4). Some other isolates were also found to be resistant to lead and cadmium but their tolerance level to heavy metals were found to be less when compared to HML8 and HMC1.

Bacterial identification

Based upon the heavy metal tolerance result, HML8 and HMC1 were further selected for identification, process optimization and antibiotic susceptibility test. Both the isolates appeared as white colonies on agar plate. The isolates were Gram positive, rod-shaped with motility, indole negative, methyl red positive, oxidase positive, VP negative, citrate negative, triple sugar iron positive, urease negative and catalase positive. Both the isolates were identified as *Bacillus* sp. based upon biochemical tests.

Growth optimization of the isolates

Effect of pH

pH had profound effect on the growth of bacteria. In the present study pH affected the growth of HML8 and HMC1. HMC1 was showing good growth at pH 8. But the isolate was unable to grow at higher pH. The isolate showed slow growth rate at acidic pH. HML8 showed similar pattern of growth as shown by HMC1. High pH favoured the growth of HML8 (Table 5).

Effect of temperature

Temperature is one of the effective factors for the improvement of bacterial growth. In the present context, both the isolates showed similar pattern of growth at different temperatures ranging from 25°C to 42°C. Bacterial growth was enhanced at 42°C (Table 5).

Table 3: MIC of the isolates towards lead

S. No	Isolates	8 mg/ml	11 mg/ml	14 mg/ml	17 mg/ml	20 mg/ml
1	HML1	++++	++++	-	-	-
2	HML2	+++	++	-	-	-
3	HML3	++++	++++	+++	+++	-
4	HML4	++++	+++	+++	+++	+++
5	HML5	-	-	-	-	-
6	HML6	++++	+++	++	++	++
7	HML7	-	-	-	-	-
8	HML8	++++	++++	++++	++++	+++
9	HML9	++++	++++	++++	++++	-
10	HML10	++++	++++	++	++	-
11	HML11	++++	+++	+++	+++	+++
12	HML12	++++	++++	+++	+++	-
13	HML13	+++	+++	++	-	-
14	HML14	++++	++++	+++	++	-
15	HML15	++++	+++	+++	++	-
16	HML16	++++	+++	+++	+++	-
17	HML17	++++	+++	+++	+++	+++
18	HML18	+++	+++	+++	++	-
19	HML19	++++	+++	+++	++	+++
20	HML20	++	++	++	-	-
21	HML21	-	-	-	-	-
22	HML22	++++	+++	+++	+++	++
23	HML23		+++	++	+++	+++
24	HML24	+++	+++	+++	++	++
25	HML25	++++	+++	+++	+++	+++
26	HML26	+++	+++	+++	+++	-
27	HML27	++++	+++	+++	+++	-
28	HML28	+++	+++	+++	+++	+++
29	HML29	+++	+++	+++	+++	+++

Note: +++ = Good growth; ++ = Moderate growth; – = No growth

Table 4: MIC of isolates towards cadmium

S. No	Isolates	8 mg/ml	11 mg/ml	14 mg/ml	17 mg/ml	20 mg/ml
1	HMC1	+++	+++	+++	+++	+++
2	HMC2	++	++	++	++	++
3	HMC3	+++	++	++	++	++
4	HMC4	-	-	-	-	-
5	HMC5	+++	+++	+++	+++	++
6	HMC6	+++	+++	+++	++	++
7	HMC7	+++	+++	++	++	++
8	HMC8	+++	+++	+++	+++	-
9	HMC9	+++	+++	-	-	-
10	HMC10	+++	+++	++	++	++
11	HMC11	+++	+++	++	-	-
12	HMC12	+++	+++	++	++	-
13	HMC13	+++	+++	+++	-	-
14	HMC14	+++	+++	+++	-	-
15	HMC15	+++	++	++	-	-
16	HMC16	+++	+++	+++	-	-
17	HMC17	-	-	-	-	-
18	HMC18	+++	+++	+++	-	-
19	HMC19	+++	+++	+++	-	-
20	HMC20	+++	+++	-	-	-
21	HMC21	+++	+++	-	-	-
22	HMC22	+++	+++	+++	-	-
23	HMC23	+++	+++	-	-	-
24	HMC24	+++	++	-	-	-
25	HMC25	+++	+++	-	-	-
26	HMC26	+++	++	++	++	-
27	HMC27	++	-	-	-	-
28	HMC28	+++	-	-	-	-
29	HMC29	+++	++	-	-	-

Note: +++ = Good growth; ++ = Moderate growth; – = No growth

Table 5: Growth optimization for resistant bacteria

Isolates	pH				Temperature				NaCl concentration			
	4	5	8	9	25°C	30°C	37°C	42°C	3%	7%	8%	9%
HMC1	++	++	+++	-	++	++	++	+++	++	++	+++	++
HML8	++	++	+++	+	++	++	++	+++	++	++	+++	-

Note: +++ = Good growth; ++ = Moderate growth; + = Limited growth; – = No growth

Effect of NaCl concentration

Different concentrations of NaCl showed profound influence on the bacterial growth. Table 5 clearly indicates that HMC1 has the ability to tolerate high salt concentration when compared to HML8. HML8 was found to be sensitive to high salt concentration. NaCl at the concentration of 8% showed good growth of the bacteria in the broth. The concentration lower than 8% affected the growth of both the isolates.

Antibiotic susceptibility test

In the present finding, Penicillin G and Amoxicillin were found to be resistant towards both the *Bacillus* sp while the other antibiotics such as Tetracycline, Gentamycin, Streptomycin and Ofloxacin were found to be sensitive (Table 6).

Table 6: Antibiotic susceptibility test

Antibiotics	HMC1	HML8
Amoxicillin	R	R
Penicillin G	R	R
Tetracycline	S	S
Gentamycin	S	S
Kanamycin	S	S
Streptomycin	S	S
Ofloxacin	S	S

Note: R= Resistance; S= Sensitive

DISCUSSION

Heavy metals are generally more persistent and stable than pesticides or petroleum by-products and are non-biodegradable [10]. The environmental pollution by heavy metals comes from smelters, mining, fertilizer, sewage sludge and the irresponsible disposal of wastes by various industries. Heavy metals can accumulate in biological systems and ultimately be introduced into food web via different mechanisms [11]. Microbes have developed adaptive mechanisms to tolerate the heavy metals either by the presence of heavy metals through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration [12]. Several bacterial species utilize intra- and extracellular binding mechanisms to avoid toxicity to Pb^{2+}. *Bacillus subtilis* for example, is able to accumulate lead ions in its cell wall and *Bacillus subtilis* MTCC-1427 has been shown to have a high absorptive capacity for Pb^{2+} [13]. In the present investigation, one of the isolates was also found to be highly resistant to lead. This may be due to the lead ions efflux mechanism of the isolate. The finding of the present investigation favours the finding of Khusro *et al.* (14) who demonstrated that *Bacillus subtilis* was able to tolerate lead at the maximum level up to 1000 mg/l. The absorption of Pb^{2+} by *Bacillus subtilis* was considerably influenced by the composition of the growth medium. Removal of lead was also affected by the pH, time and concentration. Cadmium has no beneficial effects on bacterial cells and is toxic even at low concentrations by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity. In the present study we isolated the *Bacillus* strain that was found to be resistant to cadmium even at high concentration. These resistance mechanisms in response to heavy metals may be encoded by chromosomal genes, but more usually loci conferring resistance are located on plasmids.

HML8 was found to grow at high pH. The result of present investigation clearly indicates that the isolate is resistant to alkaline environment. On the other hand HMC1 was able to tolerate high NaCl concentration which indicated the bacterium was saline resistant. The growth of bacteria at 42□C indicates that both the isolates are mesophilic in nature.

In the present study, correlation was found between the resistance of both the isolates to lead and cadmium, and few antibiotics. The bacteria resistant to antibiotics showed correlation between resistance to metal ions and to antibiotics. Similar findings were reported by Rajbanshi [15]. It might be that resistance genes to both antibiotics and heavy metals could be closely located on the same plasmid in bacteria and are thus more likely to be transferred together in the environment [16]. The results of the present study is partially in agreement with the findings of Khusro *et al.* (14) who demonstrated that *Bacillus subtilis* showed tolerance to some of the heavy metals at different concentrations but the strain was susceptible to all the antibiotics tested.

Increased uptake of lead and cadmium could lead to bioaccumulation of these heavy metals within the organisms. Therefore, these bacteria from the environment will help in the bioremediation of these toxic heavy metals thereby decontaminating the environment.

CONCLUSION

Bacteria that resisted high levels of lead and cadmium were isolated from industrial wastes. The isolates have adapted to tolerate the presence of heavy metals as such they can be used for bioaccumulation and bioremediation process. Genome sequencing of the present isolates may provide insights into metabolic pathways in order to identify genes that could be involved in heavy metal tolerance and detoxification.

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