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Original Article

Isolation and characterization of heavy metal resistant bacteria and its effect on shoot growth of *Oryza sativa* inoculated in industrial soil

Soumitra Nath[1*], Bibhas Deb[1], Indu Sharma[2], Piyush Pandey[2]
[1]Bioinformatics Centre, Gurucharan College, Silchar, Assam, India
[2]Department of Microbiology, Assam University, Silchar, Assam, India

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Abstract: A total of twenty heavy metal resistant bacteria were isolated from industrial effluents, sewages, garages and petrol pumps of Barak valley region of Assam, India, against copper, zinc, and lead. Decrease in total count and microbial population diversity with increasing metal concentrations were observed. The predominant isolates obtained were *Pseudomonas* sp., *Klebsellia* sp. and *Bacillus* sp. with minimum inhibitory concentration for heavy metals as 60µg/ml (for copper), 180µg/ml (for lead) and 1800µg/ml (for zinc). The present study demonstrated that the isolates having higher tolerance to heavy metals have high resistance pattern towards a group of antibiotics. Pot experimental studies suggest that the isolated bacterial communities live in association with rhizosphere soil and able to withstand high heavy metal concentrations in contaminated soil. Further, it has been observed that a significant increase in shoot length of *Oryza sativa* in contaminated soil when inoculated with heavy metal resistant bacterial strains.

Keywords: Antibiotic, Barak Valley, Heavy Metal, Resistance, Rhizosphere, Tolerance

Introduction

Pollution of the biosphere with trace elements has increased dramatically since the industrial revolution. Primary sources are the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, agro-chemicals and sewage. In addition, natural mineral deposits containing particularly large quantities of heavy metals are also found in many regions. The pollution of the ecosystem by heavy metals is a real threat to the environment because metals cannot be degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain (Igwe *et al*., 2005). Metal toxicity may affect all forms of life including microorganisms, plants and animals, but the degree of toxicity varies for different organisms. Heavy metals may decrease metabolic activity and diversity as well as affect the qualitative and quantitative structure of microbial communities (Giller *et al*., 1998). Traces of these heavy metals are necessary as co-factors of enzymatic reactions, but high levels of them may cause extreme toxicity to living organisms due to inhibition of metabolic reactions. The microorganisms respond to these heavy metals by several processes; including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation reduction reactions (Huang *et al*., 1990; Avery and Tobin, 1993; Brady and Duncan, 1994; Veglio *et al*., 1997).

Naturally occurring bacteria that are capable of metal accumulation have been extensively studied but it is difficult to imagine that a single bacterium could be capable to remove all heavy metals from its polluted site (Clausen, 2000). It is important to study the indigenous microorganisms in heavy metal polluted sites. It may provide new insight into bacterial diversity under unfavorable conditions, new isolates and probably new genetic information on heavy metal resistance, which could be exploited in re-vegetation in future (Fabienne *et al*., 2003). Excessive accumulation of heavy metals in agricultural soils through wastewater irrigation, may not only result in soil contamination, but also lead to elevated heavy metal uptake by crops, and thus affect food quality and safety (Muchuweti *et al*., 2006). Heavy metal accumulation in soils and plants is of increasing concern because of the potential human health risks.

The objectives of this study were to investigate heavy metal stress in bacteria isolated from Barak valley region of Assam, India and to determine whether there is a

***Corresponding Author:**
Soumitra Nath,
Bioinformatics Centre,
Gurucharan College, Silchar,
Assam, India

relationship between heavy metal stress and antibiotic resistance.

Materials and methods

Sample collection:

The present study was conducted in Barak valley region of Assam, India (Latitude-$24^0 8^/$ N to $25^0 8^/$ N, Longitude-$92^0 15^/$ E to $93^0 15^/$ E, Area- 6992 sq km). Soil samples were collected from industrial effluents, sewages, garages and nearby petrol pump in sterile sealed plastic bags and immediately brought to the laboratory. The samples were mixed with 50 ml distilled water and filtered.

Isolation and identification of heavy metal resistant bacteria:

Samples were streaked on Pseudomonas Isolation Agar (PIA), Klebsellia Isolation Agar (KIA) and Starch Agar and incubated at 37^0C for 24 hrs for recovery of potent isolates. Pure cultures were obtained and their biochemical and morphological characters were studied. The predominant bacterial genera isolated were finally characterized and identified by standard identification methods (Holt *et al.,* 1994; Cappuccino and Sherman, 2005).

Determination of minimum inhibitory concentration (MIC):

All the twenty isolates were checked for metal tolerance. MIC was determined against respective heavy metals Cu ($CuSO_4.5H_2O$), Pb [$(CH_3COO)_2Pb.3H_2O$] and Zn (Zinc Metal Powder)) by gradually increasing the concentration of the heavy metals on Nutrient Agar (NA) plates until the strains failed to give colonies on the plate. The initial concentration used was 50µg/ml and thereby the concentration was gradually increased by 10-15µg/ml each time on NA plates. The growth of cultures on last concentration was transferred to the higher concentration by streaking on the plate. MIC was recorded when the isolates failed to grow on plates.

Test for antibiotic resistance of bacterial isolates:

The isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method (Bauer *et al*. 1966) to 12 antibiotics. The concentrations of the disc used were Amikacin (30 mcg), Amoxycillin (10 mcg), Ampicillin (25 mcg), Cefalexin (30 mcg), Cefixime (5 mcg), Ceftriaxone (30 mcg), Chloramphenicol (10mcg), Gentamicin (50 mcg), Kanamycin (5mcg), Methicillin (30mcg), Ofloxacin (5mcg), Tetracycline (30mcg). The selected isolates were freshly inoculated on saline water or peptone water, their turbidity was checked by comparing with Mc. Farland solution and inoculated on Mueller-Hinton Agar. The selected antibiotics were placed on the plate and incubated at 37ºC for 24 hours. The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method.

Pot experiment:

Pot experimental studies were performed to determine the bioremediation potential of Heavy metal resistant bacteria (HMRB). Heavy metal contaminated soil collected from paddy field nearby industrial sites was put in five different pots. Pots were coded according to the inoculums added (Ps-1, Ps-4, Ba-6 and K-1) and compared them with a control set (C). The bacteria showing the highest MIC for heavy metals were selected and inoculated in nutrient broth for the formation of bio fertilizers (Table 1). The broth was kept in rotator shaker incubator at 37^0 C for 4-5 days. On the other hand seeds of *Oryza sativa* were soaked in petriplates containing sterile water for 24 hrs and sown on pots. The bacterial broth serving as bio fertilizers and distilled water was added to each pot every day. The shoot length was measured up to 15^{th} day.

Table.1: Pots are marked according to the inoculums used; to each pot broth and distilled water were added on routine basis.

Pot Labelling (codes)	Bacterial strains inoculated	Broth added	Distilled water added	Total volume
C	Control	No	50 ml	50 ml
Ps-1	*Pseudomonas* sp.	5 ml	45 ml	50 ml
Ps-4	*Pseudomonas* sp.	5 ml	45 ml	50 ml
Ba-6	*Bacillus* sp.	5 ml	45 ml	50 ml
Kl-1	*Klebsiella* sp.	5 ml	45 ml	50ml

Results

Isolation and identification of heavy metal resistant bacteria:

Twenty heavy metal resistant bacteria isolated from sewage of industrial effluents, garages and petrol pumps of Cachar district of Assam, India, against copper, zinc, and lead. After performing the biochemical tests

(Table. 2), it was deciphered that the isolated bacterial strains belong to genus *Pseudomonas* sp., *Bacillus* sp. and *Klebsiella* sp.

Table.2: Biochemical tests of all the isolated strains

Bacterial isolates		Biochemical tests											
		Gram	Indole	MR	VP	Citrate	Nitrate Reduction	Starch Hydrolysis	Litmus Milk Test	Urease Test	Catalase Test	Gelatin Hydrolysis	Oxidase
Pseudomonas sp.	Ps-1	Rod -	-	-	-	+	+	-	Rapid peptonisation	-	+	+	+
	Ps-2	Rod -	-	-	-	+	+	-		-	+	+	+
	Ps-3	Rod -	-	-	-	+	+	-		-	+	+	+
	Ps-4	Rod -	-	-	-	+	+	-		-	+	+	+
	Ps-5	Rod -	-	-	-	+	+	-		-	+	+	+
	Ps-6	Rod -	-	-	-	+	+	-		-	+	+	+
	Ps-7	Rod -	-	-	-	+	+	-		-	+	+	+
	Ps-8	Rod -	-	-	-	+	+	-		-	+	+	+
Bacillus sp.	Ba-1	Rod +	-	-	+	-	+	+	Peptonisation	-	+	+	-
	Ba-2	Rod +	-	-	+	-	+	+		-	+	+	-
	Ba-3	Rod +	-	-	+	-	+	+		-	+	+	-
	Ba-4	Rod +	-	-	+	-	+	+		-	+	+	-
	Ba-5	Rod +	-	-	+	-	+	+		-	+	+	-
	Ba-6	Rod +	-	-	+	-	+	+		-	+	+	-
Klebsellia sp.	Kl-1	Rod -	-	-	+	+	+	-	Acid, gas, curd	+	+	-	-
	Kl-2	Rod -	-	-	+	+	+	-		+	+	-	-
	Kl-3	Rod -	-	+	+	+	+	-		+	+	-	-
	Kl-4	Rod -	-	-	+	+	+	-		+	+	-	-
	Kl-5	Rod -	-	-	+	+	+	-		+	+	-	-
	Kl-6	Rod -	-	-	+	+	+	-		+	+	-	-

Minimum inhibitory concentrations for heavy metals:

Among all the twenty isolated bacteria, eight isolates exhibited high resistance to heavy metals with minimum inhibitory concentration (MIC) for heavy metals ranging from 30µg/ml to 1800µg/ml (Table 3). *Pseudomonas* sp. exhibited high resistance with MIC for heavy metals as 60µg/ml (for copper), 170µg/ml (for lead) and 1800µg/ml (for zinc). *Klebsellia* sp. showed 50µg/ml (for copper), 180µg/ml (for lead) and 1500µg/ml (for zinc) whereas *Bacillus* sp. showed the values similar to *Klebsellia* sp. except lead (150µg/ml). Some isolates also exhibits multiple tolerance to heavy metals (Ps-1, Ps-4, Ba-6 and K-1). Heavy metal tolerance test indicated highest tolerance to zinc by Ps-1 and Ps-4 (1800µg/ml), copper by Ps-4 (60µg/ml) and lead by Kl-1 (180µg/ml).

Table.3: Tolerance of bacterial isolates to heavy metals (Cu, Pb and Zn)

Bacterial Isolates	Strain No.	Minimum Inhibitory Concentration		
		Copper	Lead	Zinc
***Pseudomonas* sp.**	Ps-1	50 µg/ml	170 µg/ml	1800 µg/ml
	Ps-2	60 µg/ml	130 µg/ml	1800 µg/ml
	Ps-3	40 µg/ml	100 µg/ml	1600 µg/ml
	Ps-4	60 µg/ml	160 µg/ml	1800 µg/ml
	Ps-5	50 µg/ml	150 µg/ml	1600 µg/ml
	Ps-6	45 µg/ml	160 µg/ml	1700 µg/ml
	Ps-7	40 µg/ml	160 µg/ml	1500 µg/ml
	Ps-8	35 µg/ml	140 µg/ml	1400 µg/ml
***Bacillus* sp.**	Ba-1	50 µg/ml	150 µg/ml	1500 µg/ml
	Ba-2	30 µg/ml	130 µg/ml	1200 µg/ml
	Ba-3	35 µg/ml	130 µg/ml	1500 µg/ml
	Ba-4	45 µg/ml	150 µg/ml	1300 µg/ml
	Ba-5	50 µg/ml	130 µg/ml	1300 µg/ml
	Ba-6	45 µg/ml	150 µg/ml	1500 µg/ml
***Klebsellia* sp.**	Kl-1	50 µg/ml	180 µg/ml	1400 µg/ml
	Kl-2	35 µg/ml	160 µg/ml	1300 µg/ml
	Kl-3	45 µg/ml	150 µg/ml	1500 µg/ml
	Kl-4	40 µg/ml	130 µg/ml	1100 µg/ml
	Kl-5	50 µg/ml	130 µg/ml	1400 µg/ml
	Kl-6	40 µg/ml	130 µg/ml	1500 µg/ml

Antibiotic sensitivity and resistance pattern of heavy metal resistant isolates:

All the predominant isolates having high MIC values for a set of metals exhibited high resistance pattern towards a group of antibiotics. It was observed that most of the metal tolerant strains (Ps-1, Ps-4, Ba-6 and Kl-1) were resistant to amoxycillin, ampicillin, cefalexin, cefixime, kanamycin, methicillin and tetracycline (Table-4). The present study showed some resemblance with the long back work of Calomiris *et al*., (1984) who found a correlation between the resistance to high level of Cu(II), Pb(II), Zn(II) and antibiotic in the bacterial species found in drinking water.

Effect of HMRB on the Shoot growth of Oryza sativa inoculated in industrial soil:

The effects of HMRB on shoot elongation of *Oryza sativa* in industrial soil, collected from paddy field nearby paper industry is shown in Figure 1. No growth was observed for the first two days, but after 3rd day, the shoots began to develop in some pots. After 15 days of inoculation, it was observed that the pot marked as Ps-1 had a remarkable shoot growth of 34±2.8 cm when compared with control pot with shoot length of 23±1 cm. The lower shoot growth by Ps-4 and Ba-6 reveals the incompetence of the isolates as bio fertilizer.

Table.4: Antibiogram pattern of some selected bacterial isolates.

Antibiotic disc	Diameter of inhibition zone (in mm)			
	Ps-1	Ps-4	Ba-6	Kl-1
Amikacin	16 (I)	13 (I)	14 (I)	13 (I)
Amoxycillin	NI	NI	NI	NI
Ampicillin	NI	NI	5 (R)	NI
Cefalexin	7 (R)	4 (R)	NI	NI
Cefixime	NI	6 (R)	NI	5 (R)
Ceftriaxone	12 (I)	7 (R)	25 (S)	13 (I)
Chloramphenicol	5 (I)	2 (R)	NI	8 (R)
Gentamicin	21 (S)	17 (I)	21 (S)	23 (S)
Kanamycin	NI	NI	NI	NI
Methicillin	NI	NI	NI	7 (R)
Ofloxacin	23 (S)	21 (S)	25 (S)	27 (S)
Tetracycline	NI	4 (R)	NI	NI

NI= no inhibition zone; Diameter of disc=6mm.

Letters in parenthesis indicate sensitivity; R = Resistance; I = Intermediate; S = Susceptible.

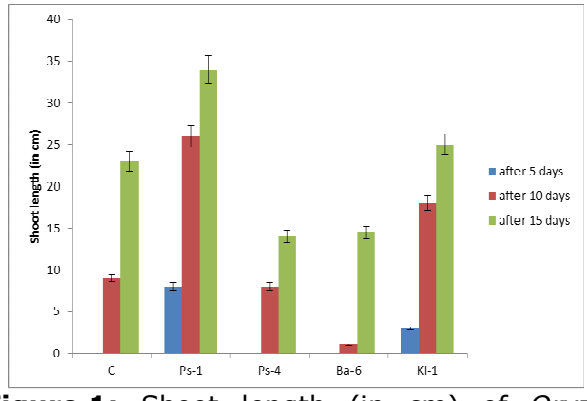

Figure.1: Shoot length (in cm) of *Oryza sativa* seedlings in heavy metal contaminated industrial soil, inoculated with HMRB and control

Discussion

A decrease in growth (cfu/g) of bacterial colonies was observed on increasing the heavy metal concentration on culture plates at any given time interval compared to the control without metal amendment. The lower values of microbial load at higher metal concentrations showed correlation with the study of Anyanwu *et al.,* (2011). Contaminated environments like those in the vicinity of industries or industrial dump grounds accumulate a heavy load of toxic metal ions, organic ions, organic wastes and antibiotics. The present study suggests that the microorganisms resistant to antibiotics and tolerant to metals appear to be the result of exposure to metal contaminated environment, which is fairly consistent with the findings of Ramteke (1997). This fact was also established by other researchers that multiple metal resistant bacterial isolates exhibits high resistance towards a group of antibiotics (Vajiheh *et al*., 2003). Based on the MIC values and antibiogram pattern of the isolated strains and as studied by Bruins *et al.,* (2003), *Pseudomonas* sp. shows resistance to a variety of toxic substances, heavy metals and antibiotics, which have generated a high degree of interest in the area of environmental bioremediation. Pot experimental studies demonstrated that the isolate Ps-1 (*Pseudomonas* sp.) live in association with rhizospheric soil are able to withstand high heavy metal concentrations in contaminated soil. Although a number of studies have demonstrated the importance of bacterial inoculation for plant growth and heavy metal accumulation in heavy metal polluted environments (Abou Shanab *et al*., 2003; Idris *et al*., 2004; Khan, 2005; Sheng and Xia, 2006). It is evident from the present study that the application of HMRB specifically adapted to high concentrations of heavy metals will increase the ability to remediate heavy metal contaminated soils.

Conclusion

Heavy metal contamination of soil by industrial effluents, sewages, garage wastes and petrol pumps can induce serious problems to soil, cropping and vegetation which subsequently hamper human health. The long term effect of pollutants has led to emergence of multi-metal and multi-antibiotic tolerant bacteria in the study area. Pot experimental studies demonstrated a significant increase in shoot length of *Oryza sativa* in contaminated soil when inoculated with heavy metal resistant bacterial strains. Further research will expand the knowledge of the microbial genetics, their application and bio-absorption of heavy metals from contaminated sites. In addition, we need to understand the mechanisms involved in mobilization and transfer of metals into the bacterial cell in order to develop future strategies.

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