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Characterisation of metal and xenobiotic resistance in bacteria isolated from textile effluent

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ABSTRACT

Heavy metals released from various industrial effluents have damaging effects on ecosystem and may become a health hazard to man and animals. Some heavy metals at low concentrations are essential micronutrients for various life forms, but in higher concentrations, they tend to cause metabolic disorders and growth inhibition. Microorganisms have evolved several mechanisms to tolerate the presence of heavy metals by efflux, complexation or reduction of metal ions to non toxic forms. Microorganisms isolated from industrial effluent discharges were found to tolerate high levels of Cadmium (3000 ppm), Lead (600 ppm), Arsenic (1500 ppm) and Mercury (500 ppm). These isolates were seen to have high level of tolerance to various xenobiotic compounds like pesticides and showed multi-drug resistance. The residual heavy metals (Cadmium, Lead, Arsenic and Mercury) after bioaccumulation were analyzed using ICP-AES technique and the isolates showed 70-80% bioaccumulation of heavy metals. The metal uptake property of these isolates show a potential to be applied for the heavy metal removal from industrial effluents thus saving the ecosystem from the disastrous effects due to heavy metal pollution.

Key words: Heavy metal tolerance, antibiotic resistance, Pesticide resistance, Metal Bioaccumulation, Atomic absorption techniques.

Short title: Metal and xenobiotic resistance in bacterial isolates.

INTRODUCTION

Heavy metal releases to the environment are increasing continuously as a result of industrial activities and technological development, posing a significant threat to the environment and public health because of their toxicity, accumulation in the food chain and persistence in nature. It is therefore important to develop new methods for metal removal and recovery from dilute solutions and for the reduction of heavy metal ions to very low concentrations [1, 2] .Many bacterial strains isolated from natural sources have been found to possess unique properties which make them useful for commercial processes and environmental cleanups.

Many metals are essential, e.g. K, Na, Mg, Ca, Mn, Fe, Co, Ni, Cu, Zn, Mo, whereas others have no known essential biological functions, e.g. Al, Ag, Cd, Sn, Au, Sr, Hg, Ti, Pb [3]. Retaining suitable concentrations of essential metals, such as copper and zinc while rejecting toxic metals, such as arsenic, lead, and cadmium was probably one of the toughest challenges of the living cells [4].

Microorganisms play a significant role in bioremediation of xenobiotics (pesticides), heavy metals etc. which contaminated soil and wastewater. These microbes have the ability to produce certain plasmid and chromosome based enzymes that hydrolyse P-O, P-S, P-F and P-C bonds, which are found in a lot of organophosphate pesticides and have evolved several mechanisms to tolerate the presence of heavy metals. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of metal ions inside the cell and reduction of the heavy metal ions to a less toxic state. The presence of certain plasmid based antibiotic resistance genes, confers

resistance to certain antibiotics as well as to certain metals [5, 6, 7, 8]. Coexistence of different metal ions resistance genes and antibiotic/xenobiotic (pesticides) resistance genes on one plasmid is not rare. This has an advantage from evolutionary point of view as the transfer of genes can take place from cell to cell via various mechanisms [9]. Ecological studies have reported that metal and antibiotic/ xenobiotic (pesticides) resistance is becoming a global phenomenon as the frequency of occurrence of plasmid-borne bacteria has become high [10]. The presence of conjugative or mobilizable plasmids in the bacteria indicate that these bacteria have gene transfer capacity with implications for dissemination of heavy metal and antibiotic resistance genes. Anjum *et al* [2011] proposed that plasmids are mainly responsible for the spread of multi-resistant bacteria in the contaminated soils [11].

Considering the importance of these tolerance mechanisms, in the present study microorganisms were isolated from effluent samples obtained from textile industries having the ability to degrade pesticides, antibiotics and heavy metals.

MATERIALS AND METHODS

Isolation of bacteria:

The Effluent sample was procured from Textile dye industry. Enrichment of the effluent was carried out using sterile nutrient broth containing 25ppm of heavy metal salts (Cadmium Sulphate, Lead Acetate, Sodium Arsenate and Mercuric Chloride) and incubated at 28 ± 2ºC for 7 days. Growth was isolated on sterile nutrient agar plates. The colony characters and gram staining was carried out. Biochemical characteristics were performed by KEM Hospital, Pune. The isolates were further identified using 16 S rRNA carried out at NCCS, Pune.

Antibiotic and Xenobiotic (Pesticide) resistance:

The Antibiotic resistance was checked using Kirby- Bauer disc diffusion method. The isolates were spread on Sterile Mueller and Hinton agar plates using sterile cotton swabs. The Antibiotic discs (Himedia, India) were placed on the plates aseptically and then incubated at 37 ºC for 24 hours. The zones of inhibition were compared with the standard Kirby Bauer chart (Table 1).

Antibiotic	Symbol	Dosage/ Disc
Penicillin	P	10 Units
Erythromycin	E	15 mcg
Sulphafurazole	SF	300 mcg
Gentamycin	G	10 mcg
Chloramphenicol	C	30 mcg
Vancomycin	VA	10 mcg
Ampicillin	A	10 mcg
Streptomycin	S	30 mcg
Tetracyclin	T	30 mcg
Ciprofloxacin	CF	5 mcg
Mecillinam	MEC	10 mcg
Carbenicillin	CB	100 mcg
Aztreonam	AT	30 mcg
Doxycycline Hydrochloride	DO	30 mcg
Trimethoprim	TR	5 mcg

Table 1: List of Antibiotics used

Sr. No.	Name of the Pesticides	Symbol	Concentration	Solution Made in	Manufactured by
1.	Chlorpyriphos	CP	21.5 %	Distilled water	Godrej
2.	Cypermethrin	CM	36 %	Distilled water	Isagro Asia
3.	Endosulfan	E	35 %	Distilled water	Krushiudyog
4.	Deltamethrin	DM	2.8 %	Distilled water	Godrej
5.	Butachlor	BU	85 %	Distilled water	BSF
6.	Carbofuran	CF	98 %	Methanol	Bayer
7.	Mancozeb	M	50 %	Distilled water	Bayer

Table 2: List of Pesticides (Xenobiotic compounds) Used

Xenobiotic (Pesticide) resistance was checked using spot inoculation method. Sterile Nutrient agar plates containing pesticides of varying concentrations (in ppm) were spot inoculated in grids using a sterile cotton swab. The plates were incubated at 37ºC for 24 hours. The visible growth of the organism indicated that the organism was tolerant to the concentration of pesticide used (Table 2).

Heavy metal tolerance and bioaccumulation:

Metal tolerance was examined on Sterile Nutrient agar plates in which varying concentrations of heavy metal salts- Cadmium sulphate, Lead acetate, Sodium arsenate and Mercuric chloride, - were incorporated. The isolates were spot inoculated using sterile cotton swabs and the plates were incubated at 37º C for 24 hours.

The bioaccumulation activity of the microorganisms was checked using ICP-AES. The organisms were grown in St Nutrient broth containing 500 ppm of the heavy metal salts. After 24 hours the cell pellet was removed by centrifugation and the supernatant was checked for residual heavy metals.

Sr. No.	Biochemical test	Result	Sr. No.	Biochemical test	Result
1.	Ala- Phe- pro- Arylamidase	-	2.	Saccharose/sucrose	-
3.	Adonitol	-	4.	D- tagatose	-
5.	L-Pyrrolydonyl-Arylamidase	-	6.	D- trehalose	-
7.	L-Arabitol	-	8.	Citrate (Sodium)	+
9.	D-Cellobiose	-	10.	Malonate	+
11.	Beta-Galactosidase	-	12.	5-Keto-D- Gluconate	-
13.	H2S Production	-	14.	L –Lactate alkalinisation	+
15.	Beta-N-Acetyl-Glucosaminidase	-	16.	Alpha- Glucosidase	-
17.	Glutamyl Arylamidase pNA	-	18.	Succinate alkalinisation	+
19.	D- Glucose	+	20.	Beta- N-Acetyl- Galactosaminidase	-
21.	Gamma-Glutamyl Transferase	+	22.	Alpha- Galactosidase	-
23.	Fermentation/ Glucose	-	24.	Phosphatase	-
25.	Beta- glucosidase	-	26.	Glycine arylamidase	-
27.	D- maltose	-	28.	Ornithine Decarboxylase	-
29.	D- mannitol	+	30.	Lysine decarboxylase	-
31.	Beta- Xylosidase	+	32.	Decarboxylase base	-
33.	Beta – Alanine Arylamidase pNA	-	34.	L-Histidine assimilation	+
35.	L- Proline arylamidase	+	36.	Courmarate	+
37.	Lipase	+	38.	Beta- glucoronidase	-
39.	Palatinose	-	40.	O/129 Resistnace (Comp.*Vibrio*)	+
41.	Tyrosine arylamidase	+	42.	Glu-Gly-Arg-Arylamidase	-
43.	Urease	-	44.	L-malate assimilation	+
45.	D-sorbitol	-	46.	Ellman	**-s**
47.	L-Lactate assimilation	+			

Table 3a: List of Biochemicals for *Pseudomonas aeruginosa*

Sr. No.	Biochemical test	Result	Sr. No.	Biochemical test	Result
1.	Beta xylosidase	-	2.	D-Mannitol	-
3.	L-Lysine –Arylamidase	-	4.	D-Mannose	-
5.	L-Asparatate Arylamidase	-	6.	D-Melezitose	-
7.	Leucine Arylamidase	+	8.	N-Acetyl-D-Glucosamine	-
9.	Phenylalanine Arylamidase	+	10.	Palatinose	-
11.	L-Proline Arylamidase	-	12.	L-Rhamnose	-
13.	Beta galactosidase	-	14.	Beta-glucosidase	-
15.	L-Pyrrolydonyl –Arylamidase	+	16.	Beta-Mannosidase	-
17.	Alpha galactosidase	-	18.	Phosphoryl choline	-
19.	Alanine arylamidase	+	20.	Pyruvate	-
21.	Tyrosine arylamidase	+	22.	Alpha-glucosidase	-
23.	Beta- N -Acetyl- Glucosaminidase	+	24.	D- tagatose	-
25.	Ala-Phe-Pro-Arylamidase	+	26.	D-trehalose	-
27.	Cyclodextrine	-	28.	Inulin	-
29.	D-galactose	-	30.	D-glucose	-
31.	Glycogene	-	32.	D-ribose	-
33.	Myo-inositol	-	34.	Putrescine (assimilation)	-
35.	Methyl-A-D-Glucopyranoside	-	36.	Growth in 6.5%NaCl	-
37.	Ellman	-	38.	Kanamycin resistance	-
39.	Methyl-D-xyloside	-	40.	Oleandomycine resistance	-
41.	Alpha-Mannosidase	-	42.	Esculin hydrolyse	+
43.	Maltotriose	-	44.	Tetrazolium red	+
45.	Glycine arylamidase	-	46.	Polymixin B resistance	-

Table 3b: List of Biochemicals for *Brevibacillus choshinensis*

RESULTS AND DISCUSSION

The two bacterial isolates were obtained from effluent samples taken from textile industries and they were identified by biochemical tests (KEM Hospital, Pune, India) as *Pseudomonas aeruginosa* and *Brevibacillus choshinensis* (Table 3a &3b). Further identification was confirmed by 16S rRNA sequencing method.

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. *P. aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), pyoverdin (yellow-green and fluorescent), and pyorubin (red-brown). *Brevibacterium* belongs to order Actinomycetales. They are Gram-positive soil organisms. It is the sole genus in the family Brevibacteriaceae.

The Antibiotic and Xenobiotic (Pesticide) tolerance was check for the two isolates using ready to use Antibiotic discs (Hi media) and spot inoculation method on sterile Nutrient agar plates containing varying concentrations of pesticides. (Table 4)

Isolate No.	Antibiotic	P 1 (mm)	P 2 (mm)	P 3 (mm)	Mean ± S.D.	Interpretation
Pseudomonas aeruginosa	P	--	--	--	--	**R**
	E	11	10	10	10.33± 0.57735	**R**
	SF	--	--	--	--	**R**
	G	14	14	15	14.33± 0.57735	**I**
	C	--	--	--	--	**R**
	VA	--	--	--	--	**R**
	A	--	--	--	--	**R**
	S	--	--	--	--	**R**
	T	11	10	11	10.66±0.57735	**R**
	CF	25	24	25	24.66± 0.57735	**S**
	MEC	26	27	26	26.33± 0.57735	**S**
	AT	24	24	23	23.66± 0.57735	**S**
	DO	--	--	--	--	**R**
	TR	10	10	10	10± 0	**R**
	CB	20	20	21	20.33± 0.57735	**S**
Brevibacillus choshinensis	P	--	--	--	--	**R**
	E	21	21	22	21.33± 0.57735	**S**
	SF	--	--	--	--	**R**
	G	14	14	13	13.66± 0.57735	**I**
	C	29	28	29	28.66± 0.57735	**S**
	VA	22	22	21	21.66± 0.57735	**S**
	A	29	30	29	29.33± 0.57735	**S**
	S	--	--	--	--	**R**
	T	18	17	18	17.66± 0.57735	**I**
	CF	23	23	22	22.66± 0.57735	**S**
	MEC	26	26	27	26.33± 0.57735	**S**
	AT	20	20	19	19.66± 0.57735	**I**
	DO	25	24	24	24.33±0.57735	**S**
	TR	20	19	20	19.66± 0.57735	**S**
	CB	29	28	29	28.66± 0.57735	**S**

Table 4: Antibiotic sensitivity testing

Pseudomonas aeruginosa and *Brevibacillus choshinensis* showed resistance to most of the antibiotic discs used. Xenobiotic (Pesticide) tolerance was checked for various concentrations of the pesticides. It was seen that *Pseudomonas aeruginosa* could tolerate Deltamethrim (10,000ppm), Carbofuran (35,000 ppm), Butachlor (35,000ppm), Mancozeb (35,000ppm), Endosulphan (35,000ppm), Cypermethrin (10,000ppm) and Chlorpyriphos (10,000 ppm), whereas *Brevibacillus choshinensis* could tolerate Deltamethrim (5,000 ppm), Carbofuran (35,000 ppm), Butachlor (35,000ppm), Mancozeb (35,000ppm),Endosulphan (5,000ppm),Cypermethrin (10,000ppm) and Chlorpyriphos (10,000 ppm).

The heavy metal tolerance was checked by subjecting the isolates to varying concentrations of metal salts. The actual heavy metal concentration was calculated and it was seen that *Pseudomonas aeruginosa* could tolerate 294.60 ppm of Mercury and 1596.60 ppm of Arsenic whereas *Brevibacillus choshinensis* could tolerate 58.93 ppm of Mercury and 1011.18 ppm of Arsenic. The concentration of Lead and Cadmium tolerated by both the organisms is same *i.e.* 625.8 ppm for Lead and 3322 ppm for Cadmium. (Fig 1)

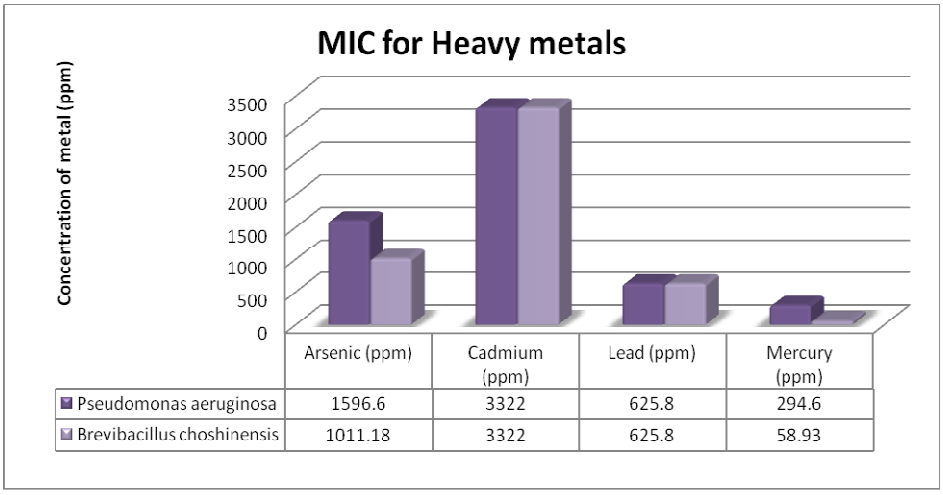

Fig 1: Minimum Inhibitory Concentration

The residual heavy metal concentration was determined by the use of Atomic Absorption Spectrophotometer. It was seen that the metal bioaccumulation capacity was higher for *Brevibacillus choshinensis* than for *Pseudomonas aeruginosa*. (Table 5; Fig 2)

Sr no.	Microorganism	Heavy metal (in ppm)							
		Lead (163.285)		Cadmium (386.704)		Mercury (192.512)		Arsenic (1086.30)	
		A	R	A	R	A	R	A	R
1	***Pseudomonas aeruginosa***	99.372	63.913	107.699	279.005	35.526	156.986	73.50	1012.80
2	***Brevibacillus choshinensis***	136.288	26.997	63.618	323.084	46.083	146.429	105.03	981.27

A: Accumulated; R: Residual.

Table 5: Bioaccumulation of heavy metals

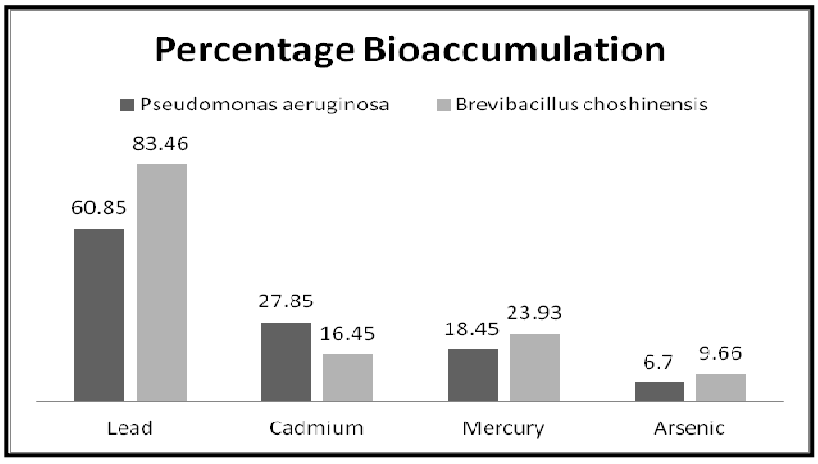

Fig 2: Percentage Bioaccumulation

Shadfiani and Malik [2003] isolated around 64 isolates which have shown tolerance to high levels of pesticides like endosulfan, carbofuran, and malathion. These isolates also showed multi-drug resistance to seven different antibiotics-nalidixic acid, cloxacillin, chloramphenicol, tetracycline, amoxicillin, methicillin and doxycycline [12]. De *et al* (2003) isolated two strains from an area with intense shipping traffic, which grew on seawater nutrient agar solid medium with 75 ppm mercury. In general there is a sharp rise in resistant bacteria capable of tolerating very high concentration of metal mercury in the coastal environment of India and was irrespective of the current levels of pollution [13]. Many earlier studies observed that mercury resistant bacteria are also resistant to many antibiotics and other toxic chemicals [14] by virtue of carrying plasmids and or transposons encoding genetically linked metal and antibiotic resistance. The incidence of multiple resistances either to metal or antibiotics was observed in the Antarctic strains. Similar bacterial resistance to multiple heavy metals was reported from Providence River and the Narragansett Bay [15]. Sabry *et al*. (1997) showed that the response of the isolates to 11 tested antibiotics ranged

from complete resistance to total sensitivity and multiple antibiotic resistance was exhibited by 70.4% of the total isolated population. The highest incidence of metal-antibiotic double resistance existed between lead and all antibiotics (100%), copper and penicillin (95%) and nickel and ampicillin (83.3%) [16]. Singh *et al.* (2010) isolated *Bacillus cereus*, an antibiotic and heavy metal resistant bacterium showing resistance to antibiotics like penicillin, lincomycin, cloxacillin, pefloxacin and heavy metals like arsenic, lead and cesium [17] . Samanta (2012) isolated an organism belonging to the *Bacillus* sp. having the ability to grow in presence of a wide range of metals namely nickel, cadmium, chromium and cobalt in the order Cd^{2+}> Cr^{6+}> Ni^{2+}> Co^{2+}. And also it was observed that the isolate was resistant to a wide range of antibiotics namely Kanamycin (30µg/disc), Ampicillin (25µg/disc) and Methicillin (5µg/disc) [18]. Various yeast and fungal species have shown to have the ability to bioaccumulate heavy metals. Anaemene (2012), isolated yeast and fungal species belonging to the genera Candida, Fusarium and Rhizopus were able to tolerate high levels of Mercury, Zinc, Cadmium, Lead, Iron, Copper, and Chromium. Their results proved that Candida sp biomass was an effective biosorbents for copper, iron and zinc. Candida sp biomass had the ability to biosorped about 80% of the metal ions from the effluent [19].

CONCLUSION

The heavy metal tolerant soil bacteria are a potential indicator of toxicity of heavy metals to other forms of life. The future prospect lies in the application of these microorganisms for purposes like heavy metal remediation and potential use in extracting rare metals from dilute solution or removing toxic metals from industrial effluents. The present studies inform us that the isolated *Pseudomonas aeruginosa* and *Brevibacillus choshinensis* have the properties to resist and accumulate high levels of heavy metals and can resist various antibiotics and pesticides; it may be harmful to human being as well as to the animals. The isolation of these isolates comprise a valuable assemblage for testing for strains degrading pollutants in the presence of high concentrations of mercury, which can be used in bioremediation of mixed wastes.

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