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Isolation and Characterization of Heavy Metal Resistant Bacteria from Barak River Contaminated with Pulp Paper Mill Effluent, South Assam

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Abstract A group of 15 heavy metal resistant bacteria were isolated from Barak River contaminated with paper and pulp effluents. These isolates displayed different degrees of chromium tolerance. Four isolates showed 34 %–49 % of growth at a concentration of 4.0 mM of Cr^{6+} and subjected to chromium reduction assay under aerobic condition. The isolate E (4) showed highest reduction (34.38 %) followed by E (3) and K(6)PA6, both showed 28.75 % reduction and then D (2) (27.5 %) after 72 h of incubation. These 4 isolates also showed different degrees of resistance to other heavy metals like Ni, Cu, Co and Cd. Antibiotic sensitivity profile of these selected bacterial strains was determined against 10 different antibiotics. Isolate E (4) appeared to be most susceptible being inhibited by eight antibiotics and resistant to penicillin G and ampicillin. The isolate E (3) was resistant to as many as five antibiotics and showed susceptible responses to the rest of the antibiotics. Both the isolates K(6)PA6 and D (2) were resistant to four antibiotics and showed intermediate to susceptible responses to the rest of the antibiotics.

Keywords Barak River · Metal tolerance · Bioremediation

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Water the most important resource of nature is increasingly becoming a scare resource. Rivers are playing an important role as major water resource in this planet. Unfortunately, rivers are being polluted by indiscriminate disposal of sewage and industrial waste, which also affect physico-chemical properties and microbiological quality (Koshy and Nayar 1999). Most of the rivers in urban areas of the developing world are the end points of effluents discharged from the industries. Paper pulp industries are the sixth largest effluent generating industries of the world (Ugurlu et al. 2007). These effluents have been found to contain approximately 700 organic and inorganic compounds (Tambekar et al. 2008). Some of these substances have been classified as carcinogenic and mutagenic (Karrash et al. 2006).

Microorganisms are considered to be the best indicators of water pollution. In general, they are very sensitive to low concentration of heavy metals but rapidly adapt to the specific habitat conditions. The microorganisms have acquired a variety of mechanisms for adaptation to heavy metals. Among the various adaptation mechanisms, metal sorption, mineralisation, uptake and accumulation, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form and efflux of heavy metals from the cell has been reported (Joshi-Tope and Francis 1995).

Many reports are available on antibiotic and heavy metal resistant bacteria isolated from different polluted environment (Rajbanshi 2008; Ezaka and Anyanwa 2011) but no report is available from Barak River contaminated with paper mill effluent. The present study was an attempt to evaluate the status of heavy metal resistant bacteria

isolated from Barak River water contaminated with paper mill effluent.

Materials and Methods

Water samples were collected from Panchgram site (near Hindustan Paper Corporation) of Barak River during monsoon season into pre-sterilised plastic bottles.

For the isolation and enumeration of bacteria, each water sample was serially diluted in sterile distilled water and plated on nutrient agar and different selective medium. All the media were prepared with the addition of distilled water and autoclaved properly. The plates were prepared 24 h prior to sampling. The bacterial population in different samples was estimated by spread plating method on nutrient agar and selective medium plates with 1 mL of suitable dilutions. Bacterial inoculated plates were incubated at 37°C for 24–48 h and final counts of colonies were noted. All trials were performed in duplicate. Colonies differing in morphology were isolated in pure form and maintained on nutrient agar slants with proper indexing.

Dissolved oxygen was determined by modified Winkler's azide method, pH by digital pH meter, alkalinity and FCO_2 were measured by titrimetric method. All the physico-chemical parameters were analysed following the standard protocols (APHA 1998). The water quality was determined by the standard most probable number (MPN) method using the three tube test with lactose broth was employed. Fermentation tubes were inoculated with 10, 1, and 0.1 mL aliquots of water sample (APHA 1998). The tubes were incubated at 37°C for 24 h. Positive tubes producing acid and gas were used in estimating the presumptive MPN/100 mL. Confirmed test was carried out by transferring a loopful of broth from a positive tube into Brilliant green lactose bile (BGLB) broth, followed by incubation at 37°C for 24–48 h. The tubes were observed for gas formation. Completed test was performed by plating a loopful of broth from a positive BGLB tube on to an Eosine Methylene Blue (EMB) Agar plate. The plates were incubated at 37°C for 24–48 h and observed for dark red colonies with metallic green sheen. Final faecal coliform or *E. coli* count as MPN/100 mL was calculated based on the completed test.

The isolates were tested for their resistance to chromate by growth in nutrient broth tubes containing various concentrations of chromium (0.1, 0.5, 2, 4 mM) as K_2CrO_4. These tubes were inoculated with freshly grown culture of the isolates and incubated at 30–37°C for 48 h. The bacterial growth was determined by measuring the optical density using spectrophotometer at 540 nm. Relative growth of the isolates was expressed as the percentage of those obtained in untreated control which was taken as 100 %. Chromate reductase activity of the bacterial isolates was assayed following the standard procedure (Park et al. 2000). For reduction assay, nutrient broth medium containing 100 μm of Cr (Vl) was inoculated with isolates and incubated up to 72 h at 37°C. Samples of inoculated medium were collected during the incubation period after every 24 h interval.

The resistance of the selected isolates against other heavy metals was also tested in the nutrient broth. The other heavy metals that were tested include Cd, Co, Ni and Cu. These metals were used as their chloride salts. The relative resistance of the isolates was determined from the percent inhibition of growth over the control. Growth was also determined by measuring the optical density at 540 nm.

For biochemical characterisation the isolates were tested for catalase activity, indole production, methyl red test, voges-proskauer test, citrate utilisation test, MacConkey agar test and fermentation of eight different sugars. These biochemical tests were done by using the biochemical test kits provided by HiMedia Pvt. Ltd. Identification of the bacterial isolates was carried out according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

To determine the antibiotic sensitivity of the bacterial isolates, antibiotic discs (Hi-media) were placed on freshly prepared lawns of each isolates on nutrient agar plates. The plates were incubated at 30–35°C for 24 h. 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). Discs containing the following antibiotics were used: Tetracycline (30 mcg), Rifampicin (30 mcg), Streptomycin (10 mcg), Vancomycin (30 mcg), Penicillin G (10 units), Ampicillin (10 mcg), Chloramphenicol (30 mcg), Gentamycin (10 mcg), Erythromycin (15 mcg) and Polymyxin B (300 units).

All experiments of physico-chemical parameters were performed in triplicates and statistical analysis was performed according to the standard method (Steel and Torrie 1992).The results are given as mean ± SE values.

Results and Discussion

The physico-chemical parameters and bacteriological load at the site Panchgram of river Barak has been presented in Table 1. The pH value of the water sample was 6.25 ± 0.55, which is slightly acidic. The value of DO, FCO_2 and alkalinity was recorded as 5.36 ± 0.14, 4.3 ± 1.51 and 14.0 ± 2.64 mg/L respectively. The values of total faecal coliform and total viable count (TVC) was recorded as 350 MPN/100 mL and 42 ($\times 10^3$) mL^{-1}.

15 pure cultures of bacterial isolates different in their morphology were isolated in pure form and were subjected to assessment for relative Cr resistance. The chromium concentrations used during screening ranged from 0.1 to

Table 1 Physico-chemical parameters and bacteriological load at the site Panchgram of Barak River

pH	DO (mg/L)	FCO_2 (mg/L)	Alkalinity (mg/L)	Total faecal coliform (MPN/100 mL)	Total viable count ($\times 10^3$)
6.25 ± 0.55	5.36 ± 0.14	4.3 ± 1.51	14.0 ± 2.64	350	42

Table 2 Chromium tolerance of bacteria isolated from river Barak, Assam

Bacterial isolate	Incubation period (h)	Relative growth, %			
		Cr concentration (mM)			
		0.1	0.5	2	4
E (1)	48	97.08	86.40	33	4.85
E (2)	48	88.13	82.20	25.42	19.06
E (3)	48	85.24	80.32	43.85	35.65
E (4)	48	83.14	75.28	73.03	49.55
E (5)	48	88	79.2	34.4	28.4
E (6)	48	93.27	92.85	68.06	16.80
D (1)	48	87.93	78.01	64.22	9.48
D (2)	48	85.71	72.76	56.25	48.01
D (3)	48	91.54	89.43	83.8	15.60
D (4)	48	94.44	91.91	70.7	23.68
D (5)	48	77.68	72.72	34.29	12.80
D (6)	48	97.63	94.88	28.74	27.16
K6PA6	48	87	80.66	55.66	34.66
K5	48	90.38	81.73	10.57	9.61
K4	48	91.74	90.82	7.33	6.42

4.0 mM. Tolerance to these isolates to different concentrations of Cr in broth is shown in Table 2.

It was observed that all the isolates were resistant to Cr showing growth at lowest concentrations. But with the increase of Cr(VI) concentrations, the percent relative growth of the isolates decreased. Based on their growth in different concentrations of Cr^{6+}, isolates D (2), K(6)PA6, E (3) and E (4) have exhibited 34 %–49 % of growth at a concentration of 4.0 mM of Cr^{6+}. The four selected isolates were then subjected to chromium reduction assay.

The selected bacterial isolates were capable of reduction of Cr(VI) and the reduction rate was dependent on bacterial species and time of incubation (Table 3). No isolate has shown significant reduction after 24 h of incubation. After 72 h of incubation, the isolate E(4) has shown highest reduction (34.38 %) followed by E(3) and K(6)PA6 (28.75 %) and D(2) (27.5 %).

The promising isolates were tested for their tolerance to other heavy metals. Among the other heavy metals tested, the isolates showed different degrees of resistance to Ni, Cu, Cd and Co at 4 mM. Tolerance to these isolates to different heavy metals in liquid medium is shown in Table 4.

Table 3 Reduction of hexavalent chromium by selected chromium resistant bacteria isolated from river Barak, Assam

Bacterial isolate	Cr(VI) reduction (%)		
	24 h	48 h	72 h
E (3)	8.75	22.5	28.75
E (4)	3.75	27.5	34.38
K(6)PA6	8.75	25.63	28.75
D (2)	5.63	8.75	27.5

Morphological and biochemical characteristics of the selected isolates are shown in Table 5. Based on comparison of these characters with standard descriptions in Bergey's Manual of Determinative Bacteriology and further molecular characterisation, the isolate K(6)PA6 was identified as *Bacillus cereus* (MTCC-JN202315).Comparison of morphological and biochemical characteristics with standard descriptions in Bergey's Manual of Determinative Bacteriology isolates E(3), E(4) and D(2) were identified as bacteria belonging to a group known as "coliform" that exist in intestines of warm blooded animals. However, generic identity of these isolates remained unidentified and needs more detailed studies for confirmation. Data revealed that except K(6)PA6, all the isolates were gram(−)ve. Carbohydrate utilisation of all the isolates showed positive response for glucose and negative response for sucrose, rhamnose and lactose.

The chromium resistant bacterial isolates were tested for their sensitivity to 10 different antibiotics. Isolate E (4) appeared to be most susceptible being inhibited by 8 antibiotics and resistant to penicillin G and ampicillin. The isolate E (3) was resistant to as many as five antibiotics and showed susceptible responses to the rest of the antibiotics. Both the isolates K(6)PA6 and D (2) were resistant to four antibiotics and showed intermediate to susceptible responses to the rest of the antibiotics (Table 6).

The physico-chemical parameters of any particular aquatic ecosystem represent the relation between biotic and abiotic factors. The pH value recorded was within highest desirable limit prescribed by WHO (1998). The value of TVC of water sample was higher than those prescribed by Bureau of Indian Standards (ISI 1991). Dissolved oxygen is a barometer of the ecological health of the river. Normally high dissolved oxygen is encountered in unpolluted areas while at polluted sites a level of

Table 4 Relative growth of bacterial isolates in nutrient broth containing heavy metals

Metals used	Incubation period (h)	Heavy metal concentration (mM)	Relative growth, %			
			Bacterial strains			
			E (4)	E (3)	D (2)	K(6)PA6
Ni	48	0.1	96.42	89.79	95.57	88.88
		0.5	94.64	52.04	92.03	83.70
		2.0	40.17	21.42	73.45	1.48
		4.0	8.03	10.71	50.44	1.48
Cu	48	0.1	90.67	84.45	49.45	83.03
		0.5	85.59	62.18	48.91	70.53
		2.0	6.77	33.61	16.30	32.14
		4.0	5.93	24.36	14.13	16.96
Cd	48	0.1	62.82	83.52	48.77	62.27
		0.5	43.58	75.29	45.90	41.81
		2.0	32.05	42.35	40.16	29.09
		4.0	28.20	40.0	37.70	27.72
Co	48	0.1	99.21	94.69	97.39	85.71
		0.5	97.65	93.93	96.52	74.02
		2.0	7.81	7.57	11.73	5.19
		4.0	7.81	7.57	11.30	4.54

Each value represents average of duplicates

Table 5 Morphological and biochemical characteristics of selected chromium-resistant bacterial isolates

Character	Bacterial isolates			
	E (3)	E (4)	D (2)	K(6)PA6
Gram reaction	Gram (−)ve	Gram (−)ve	Gram (−)ve	Gram (+)ve
Indole test	−	−	−	−
Catalase production	+	+	+	+
Growth on MacConkey	+	+	+	−
MR	−	−	−	−
VP	−	_	−	−
Citrate	+	−	+	−
	Carbohydrate	Utilisation		
Glucose	+	+	+	+
Adonitol	+	−	−	−
Arabinose	+	−	+	−
Lactose	−	−	−	−
Sorbitol	+	+	_	−
Mannitol	+	+	+	−
Rhamnose	−	−	−	−
Sucrose	−	−	−	−

+ positive, − negative

dissolved oxygen was very low. In the present study dissolved oxygen concentration was above 5 mg/L which was well within the WHO permissible limit. FCO_2 value recorded in the present study was not suitable for fish cultivation according to the National Academy of Sciences (Water Quality Criteria 1972). The standard desirable limit of alkalinity in potable water is 120 mg/L and maximum permissible limit is 600 mg/L (WHO 1998). The alkalinity value recorded in the present study was well below the desirable limit.

Microorganisms with the ability to resist heavy metal may be used for detoxification of water contaminated with heavy metals. The removal of heavy metal from aqueous solution by bacteria, fungi, ciliates, algae, mosses, macrophytes and higher plants has been recorded (Pattanapipitpaisal et al. 2002; Rehman et al. 2007). These mechanisms could be utilised for

Table 6 Antibiotic sensitivity profile of chromium resistant bacterial isolates

Antibiotic disc (conc.)	Diameter of inhibition zone (mm)			
	K(6)PA6	D(2)	E(3)	E(4)
Tetracycline (30 mcg)	22 mm (S)	20 mm (I)	30 mm (S)	26 mm (S)
Streptomycin (10 mcg)	NI	14 mm (I)	24 mm (S)	23 mm (S)
Polymyxin B (300 units)	8 mm (R)	9 mm (R)	12 mm (S)	12 mm (S)
Vancomycin (30 mcg)	15 mm (I)	19 mm (S)	NI	16 mm (S)
Penicillin G (10 units)	17 mm (R)	14 mm (R)	NI	16 mm (R)
Ampicillin (10 mcg)	12 mm (I)	9 mm (R)	NI	10 mm (R)
Chloramphenicol (30 mcg)	24 mm (S)	20 mm (I)	18 mm (S)	16 mm (I)
Gentamycin (10 mcg)	13 mm (I)	19 mm (S)	NI	24 mm (S)
Erythromycin (15 mcg)	19 mm (I)	17 mm (I)	21 mm (S)	17 mm (I)
Rifampicin (30 mcg)	NI	12 mm (R)	8 mm (R)	17 mm (I)

Letter in parentheses indicates sensitivity; *R* resistant, *I* intermediate, *S* susceptible

NI no inhibition; diameter of disc = 6 mm

detoxification and removal of heavy metals from polluted environment (Ahmed et al. 2005). Among the various mechanisms the cellular response to the metals such as biosorption by cell biomass, active cell transport, binding by cytosolic molecules, entrapment into cellular capsules, precipitation and oxidation reduction reactions are important ones. Bacteria from polluted environments would represent an appropriate practice to select metal resistant strains that could be used for heavy metal removal and bioremediation purpose (Malik 2004).

Association between resistant to antibiotics and heavy metals has been reported (Ramteke 1997; Verma et al. 2001). Metal tolerance of microorganisms is ecologically important particularly if they are antibiotic resistant. Multiple metal resistances along with antibiotic resistance have been found in native bacteria which may involve genes (Trajanovska et al. 1997). Multiple heavy metal resistance determinants for Cd, Co, Zn, Co, Ni, Cr and Hg have been found in bacterial plasmids (Verma et al. 2001). Large numbers of chromate tolerant bacteria, especially coliforms were capable of multiple antibiotic resistant.

The native aquatic bacterial isolates showed varying levels of Cr(VI) resistance and reduction. The ability of these isolates to reduce Cr(VI) is probably reflecting horizontal genetic transfer (Francisco et al. 2002). Presence of such metal tolerant bacteria in Barak River water may be an indicator that such area is affected by heavy metals. Such an area may foster adaptation and selection for heavy metal resistant organisms (Clausen 2000). Chromium resistant bacteria capable reducing chromate have been reported from chromium polluted environments (Ganguli and Tripathi 2001; Srinath et al. 2001), serpentine soil (Pal and Paul 2004) but no report is available from river water contaminated with paper mill effluent to reduce chromate. In this study, one strain which was identified as *Bacillus cereus* (MTCC-JN202315) showing 28.75 % reduction rate. However, few studies reported the ability of *Bacillus* spp. isolated from chromium polluted environment as well as from serpentine soil (Pal et al. 2005; Sundar et al. 2010).

The presence of high chromium tolerant bacterial strains also revealed that the river water of Barak near Hindustan Paper Corporation may be contaminated with chromium. Reduction of chromium by these bacterial isolates can be considered as an effective tool for bioremediation.

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