Original article:

Isolation of chemically resistant bacterial strains from industrially polluted water body

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Abstract

Bacground: Untreated disposal of chemical waste poses serious environmental hazard. An attempt was made towards presumptive identification of the major genera of microbial contaminants found in a natural pond that receives waste from a pharmaceutical industry of Gonoshasthaya Antibiotic Ltd. during the period of 2008 to 2009. ***Methodology:*** Water sample was collected and physical parameters were determined. Results: A total of 38 bacterial isolates were found from surface water, deep layer water and sediment soil but only five isolates were found to resist up to 0.1 mg/ml of phenol. Microscopic and biochemical test of five isolates presumptively identified them *Staphylococcus sp, Sporosarcina sp, Bacillus sp* and members of family Enterobacteriaceae. All five isolates were resistant to Amoxycillin (30?g), Erythromycin (15?g), and Penicillin-G (10units). Conclusion: Resistance against common therapeutic antibiotics indicates possible epidemiological risk.

Keywords: Chemically resistant bacteria, industrially polluted water.

Introduction

About 80% people in Bangladesh lack clean, safe water because most household and industrial wastes are dumped directly in to the natural water bodies. According to Bangladesh Bureau of Statistics about 37.5% factories lack waste management and industrial waste is dumped into the water bodies without any treatment[1].The oxygen demanding wastes are decomposed by aerobic (oxygen requiring) bacteria. The quantity of oxygen demanding wastes in water can be determined by measuring the biological oxygen demand (BOD), the amount of dissolved Oxygen needed by aerobic decomposer to break down the organic materials in a certain volume of water over a 5-days incubation period at 20^0C (68^0 F)[2].

Microorganisms of polluted environment bear resistant property to the pollutants present. Like other microbes they are identified by morphology, nutritional requirement and growth characteristic. Gonoshasthaya Antibiotic Ltd. (GAL) produces antibiotic (amoxicillin, ampicillin, cephalexin, cloxacillin, flucloxacillin) and has no waster treatment facility. The waste contains of a range of chemical substances including methylene chloride (CH_2Cl_2), isopropyl alcohol (C_3H_8O), pivalic acid ($C_5H_{10}O_2$), triethylamine ($C_6H_{15}N$), small amount of amoxicillin and its degradation products ($C_{16}H_{19}N_3O_5$), ethanol (C_2H_6O) actone (C_3H_6O), cephalexin ($C_{16}H_{17}N_3O_4S$), and its degradation products, ethyl acetate ($C_4H_8O_2$), methyl ketone ($C_6H_{12}O$) and small amount of cloxacillin ($C_{19}H_{18}ClN_3O_5S$), and its degradation products[3, 4]. Effect of pollutant on micro flora of an aquatic environment draws special interest of research to find out possible ways of bioremediation.

Materials and Methods

Collection of sample

A natural pond receiving waste from antibiotic industry (GAL) was chosen and 100 ml water was collected from surface layer, 50 cm deep layer and layer near sediment in sterile Duran Bottles.

Determination of Physical and Chemical Parameters Chemical Oxygen demand (COD) of the polluted water was determined by titrating the water against 0.1 M sodium thiosulfate using 0.25 N $K_2Cr_2O_7$ as the indicator[5, 6].

Dissolved Oxygen (DO) and Biochemical Oxygen Demand (BOD) was determined by the standard 5 day BOD test (BOD_5)[6, 7, 8]. Alkalinity of the waste water was titrated against 0.1 N HCl using methyl orange as the indicator[7]. The temperature and pH were determined respectively by thermometer and Mettler pH meter.

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Isolation of organisms
Each specimen was cultured on nutrient agar plate and incubated at 37^0 C over night. Next day, the types of colonies were observed and recorded. Mac Conkey agar, Eosin Methylene Blue (EMB) Agar, Blood agar, K. F. Streptococcus agar (KFSA) and Mannitol Salt Agar (MSA) were used for cultivation of the suspected organisms in the sample water[9].

Screening for phenol tolerant organism:
Two sets of test tubes were prepared with phenol containing media. One set had T_1N_1 (Tryptone NaCl) media with 0.1 mg/ml phenol. Another set had 0.85% NaCl solution with 0.1 mg/ml phenol. Both sets were inoculated with the isolates and incubated at 37^0 C for 3 days, the tubes were observed for growth.

Biochemical Test for Presumptive Identification
The phenol-tolerant isolates were taken for Gram staining, spore staining, oxidase test, catalase test, Methyl Red- Voges Proskeur (MRVP) test, Motility Indole Ornithine (MIO), Triple Sugar Iron (TSI) agar test, citrate utilization test, Indole formation, Urease production, carbohydrate fermentation, nitrate reduction, mannitol fermentation and 6.5% NaCl tolerance, hydrolysis of gelatin, starch, casein, DNA and tributyrate tests were done according to bacteriology protocols[9, 10].

Antibiotic Sensitivity Assay of Bacterial Isolates
Bacterial susceptibility to antimicrobial agent was determined in vitro by using the standardized agar disc-diffusion method in Muller-Hinton agar known as the Kirby Bauer method [11]. Imipenem (10 μg), Azithromycin (15μg), Amoxicilin (30 μg), Tetracyclin (30 μg), Nalidixic Acid (30 μg), Erythromycin (15 μg), Vancomycin (30 μg), Cephalexin (30 μg), Amikacin (30 μg), Penicillin-G (10 units) discs were available from Oxoid (USA).

RESULT

DO, COD, Alkalinity, P^H and temperature of the water was determined .The result is recorded in Table I.

Table I: Chemical Parameters of Polluted Water Samples

Source	*DO*	*COD*	*Alkalinity*	*P^H*	*Temperature*
Surface	2.0mg/L	120.0mg/L	0.72mg/L	6.0	27.0^0 C
Deep	3.5mg/L	96.0mg/L	14.9mg/L	7.0	26.0^0 C
Sediment	11.0mg/L	784.0mg/L	5.13mg/L	7.0	25.5^0 C

The water samples were cultured on nutrient agar media and a total of 38 different isolates were found. Among them 11 isolates showed growth on T1N1 supplemented with 1% phenol and 5 isolates could grow in 0.85% NaCl solution. These 5 isolates are chosen for further experiments. The results of microscopic observation are presented in Table II.

Table II: Microscopic Features of Phenol Resistant Isolates

Isolates Code	*Gram Reaction*	*Cell Morphology*	*Spore*
P1	Gram positive (+) ve	Cocci	None
P9	Gram positive (-) ve	Small rod	Not done
B5	Gram positive (+) ve	Cocci	Yes
B9	Gram positive (+) ve	Rod in chain	Yes
B11	Gram positive (+) ve	Large rod	Yes

The growth of the isolates on different cultural media is described in Table III.

Table III: Cultural Characteristics of Isolates

Isolates	MaConkey Agar	Mannitol Salt Agar	Blood Agar	EMB	KFSA
P1	No growth	Yellow	α -haemolytic	No growth	Purple
P9	Pink colony	No growth	α -haemolytic	Shiny colony	White
B5	No growth	White	β -haemolytic	No growth	Bluish
B9	No growth	Off-white	β -haemolytic	No growth	Colorless
B11	No growth	Off-white	γ -haemolytic	No growth	Off white

The result of Biochemical profiling is presented in Table IV.

Table IV: Biochemical Characteristics of Phenol Resistant isolates

olate Label	Oxidase	Catalase	Methyl Red	Voges-Proskeur	Motility	Ornithine ecarboxylation	Glucose Fermentation	Lactose Fermentation	H_2S Production	Citrate Utilization	Urease Production	Indole Production	Fructose Fermentation	6.5% NaCl Tolerance	Nitrate Reduction	Mannitol Fermantation
P1	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+
P6	-	+	+	-	+	+	+	+	-	-	-	+	+	-	-	+
B6	+	+	+	-	+	+	+	-	-	-	-	-	-	+	-	-
B9	-	+	+	-	-	+	+	-	-	-	-	-	-	+	-	-
B11	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+

Further test of hydrolysis was done for the Gram positive isolates, the result of which is presented in Table V.

Table V : Hydrolysis of Biomolecules by Gram positive isolates

Isolate	Gelatin	Starch	DNA	Tributyrate
P1	-	-	-	+
B5	+	-	-	-
B9	+	+	-	+
B11	+	+	+	+

ical tests, the isolates were identified with reference to Bergey's Manual of Systemic Bacteriology[12] and Bergey's Manual of Determinative Bacteriology[13]. The identity are presented in Table VI.

Discussion

The aim of this study was to assess the microbial flora of industrially polluted water and to find out the tolerance or resistance of the organisms against chemicals. A natural pond with effluent from an antibiotic industry was chosen because this effluent contains antibiotics along with other industrial chemicals[13]. Phenol was chosen because it is a common bacrtericidal agent and there is a correlation between rise of phenol resistance and antibiotic resistance[14]. The bacteria that can survive overnight in 0.1 mg/ml phenol are considered resistant to it. The five phenol-resistant isolates identified on biochemical test were common organisms of tropical water (Table IV). The member of

Table VI : The Presumptive Identification of Phenol Resistant Isolates

Isolate	P1	P9	B5	B9	B11
Genus	*Staphylococcus*	*Escherichia coli*	*Sporosarcina*	*Bacillus*	*Bacillus*

Depending upon the microscopic and the biochem. The resistance of these isolates against antibiotics is listed in Table VII.

Table VII: Antibiotic Resistance of Phenol Resistant Isolates

Antibiotic	*Staphylococcus sp.*	*Escherichia coli*	(*Sporosarcina sp.*	(*Bacillus sp.*	*Bacillus sp.*
A	R	R	R	R	R
P	R	R	R	R	R
Ak	S	S	S	S	R
CFX	S	S	R	S	R
NA	MS	S	S	S	R
E	R	R	R	R	R
ATH	S	S	S	MS	R
Va	S	R	MS	MS	R
Te	S	S	S	MS	MS
Imi	S	S	S	S	S

S=Sensitive R=Resistant MS=Moderately Sensitive
IMI=Imipenem (10 μg), ATH=Azithromycin (15μg), A=Amoxicilin (30 μg), Te=Tetracyclin (30 μg), NA=Nalidixic Acid (30 μg), E=Erythromycin (15 μg), Va=Vancomycin(30 μg), CFX=Cephalexin (30 μg), AK=Amikacin (30 μg), P=Penicillin-G (10 units).

Family Enterobacteriaceae (Table III) is probably *Escherichia coli* because it produces shiny colony on EMB[15]. This isolate is resistant to Penicillin, Amoxycillin, Erythromycin and Vancomycin (Table VII) as expected from Gram negative bacteria[16, 17].

The two *Bacillus* sp. identified were different from each other in microscopic and biochemical feature as well as in cultural characteristic (Table II,III,IV,V). The isolate B9 is of special interest because it is?–haemolytic and resistant to Penicillin and Amoxycillin. Such *Bacillus* sp. are clinically important[18]. Isolate B11 is resistant to all tested antibiotics except Imipenem and Tetracycline (Table VII). This isolate shows the highest degree of drug resistance. Such a pattern of resistance from a natural strain is uncommon since most reported strains are sensitive to Vancomycin and Erythromycin[18]. Probability of adaptation to the antibiotics present in the industrial effluent could be a reason. Detailed study of virulence and molecular characterization of antibiotic resistance factors was not within the scope of this study, but extensive research of pathogenic potential of the *Bacillus* sp. that are antibiotic resistant might generate new information about pollution-induced community tolerance and spread of antibiotic resistance[19, 20].

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