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Isolation and Identification of Arsenic Resistant Bacteria from Polluted Water of Ghaziabad Region

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The aim of this study was to isolate and identify the arsenic resistant bacteria. The experiment was conducted through January 2010 to May 2010 at Microbiology Laboratory of Institute of Applied Medicines and Research, Ghaziabad, Uttar Pradesh. The water samples were collected from different polluted water bodies. Twenty one arsenic resistant bacterial strains were isolated form polluted water. Arsenic resistant bacteria were isolated by growing on nutrient agar medium impregnated with high concentration (20 mg/mL) of As_2O_3 and As_2S_3. Four isolates were found more resistant and finally selected for their morphological and biochemical studies in details. All four isolates were able to tolerate very high concentration (> 0.2% or 20 mg/mL). The optimum temperature and pH for growth of all four strains were 30-37 °C and 7.0-8.0 respectively.

Key words: Resistant, Impregnated, Concentration, Biochemical.

Arsenic contamination of groundwater is a natural occurring high concentration of arsenic in deeper levels of groundwater, which became a high-profile problem in recent years due to the use of deep tubewells for water supply in the Ganges Delta, causing serious arsenic poisoning to large numbers of people. A 2007 study found that over 137 million people in more than 70 countries are probably affected by arsenic poisoning of drinking water. Arsenic contamination of ground water is found in many countries throughout the world, including the USA.

Arsenic often is remembered as a poison, used since medieval times to eliminate unsuspecting persons. In those days, arsenic was a perfect murder weapon because the symptoms of death mimicked other causes and, therefore, were hard to trace. Severe poisoning and possibly death can occur from just 100 mg of arsenic. Lower frequent doses like those received from contaminated drinking water build up in the body and produce varying side effects. Cancer, diabetes and cardiovascular problems are linked to arsenic poisoning. (Twarakavi, N. K. C., Kaluarachchi, J. J., 2006)

Arsenic is the 20th most abundant element in the Earth‘s crust 14th in the seawater and 12th in the human body (Woolson, 1975) and is widely distributed throughout nature as a result of weathering, dissolution, fire, volcanic activity and anthropogenic input (Cullen and Reimer, 1989). The last includes the use of arsenic in pesticides, herbicides, wood preservatives and dye stuffs as well as production of arsenic-containing wastes during smelting and mining operations. In arsenic-enriched environments, a major concern is the

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potential for mobilization and transport of this toxic element to ground water and drinking water supplies. Inorganic arsenic consists of arsenic ions combined with chlorine, oxygen and sulfur ions to form compounds known as arsenite (As+3) and arsenate (As+5). Arsenite is considered to be more toxic than arsenate (on average 100 times more toxic) and can be oxidized to arsenate chemically or microbiologically (Ehrlich H.L**.,** 1996, Muller D., 2003).

MATERIAL AND METHODS

Water Sample Collection

The polluted water samples were collected from Ghaziabad, Uttar Pradesh. All the water samples were collected from polluted water bodies. Determination of pH of water was conducted by pH-meter.

Bacterial enumeration

At first, the total number of bacteria was determined in the water samples using the SPC (Standard Plate Count) method. Samples were diluted 10- 1000 fold in normal saline and plated on nutrient agar plates. These plates were incubated for 42 hrs at 37°C and the number of colonies counted. Then morphologically different colonies were randomly picked and isolated after successful purification process on the same medium.

Screening of Arsenic resistant bacteria

For screening of arsenic resistant bacteria, loopfull bacterial culture of different isolates streaked on nutrient agar plates containing 200 µg As (III) per mL. Nutrient agar plates were prepared by dissolving 5 g NaCl, 5 g peptone and 3 g beef extract in 1000 ml distilled water, pH adjusted to 7-7.2 and then 18 g agar was added. The medium was autoclaved at 121°C for 15 min. The growth of the bacterial colonies was observed after 48 h of incubation at 30°C.The population of the resistant bacteria was counted. In the later stage the preservation of the resistant bacteria was performed in slant tubes.

Identification of bacteria

Identification of various resistant bacteria was performed with key biochemical tests and Gram's staining. Gram's staining and cell morphology was investigated under microscope (1000x magnification). Biochemical properties of the isolates were tested according to Bergey's Manual of Systematic Bacteriology (Krieg, 1984). The following properties were determined: indole test, citrate utilization, nitrate reduction, starch hydrolysis, catalase test, oxidase test, oxidation/ fermentation test (O/F), acid production from carbohydrates: glucose, lactose, sucrose.

MIC determination

The resistance of the bacteria resistant to arsenic has been defined with the agar dilution method. For evaluating the resistance of the bacteria, different media with different concentrations of arsenic were prepared and then the bacteria cultured radially in the plate. The plates were then kept for 2 days in an incubator at 30°C. The absence of bacterial growth indicated its sensitivity, while the presence of bacterial growth in this concentration indicated that bacteria were resistant. The MIC was defined as the minimum concentration of the metal, which inhibits the bacterial growth in the plate (Roane, et al, 1995, Sabry, S. A., 1997, Choundhury, P. & Kumar, R., 1996).

Growth at different temperature

Petri plates of Nutrient Agar were equally inoculated with culture and incubated at 4, 25, 37 and 60 ^{O}C. Growth on nutrient agar was observed after 48 h.

Growth at different pH

Petri plates of Nutrient Agar at different pH (4.0, 5.0, 6.0, 7.0, and 8.0) were prepared in duplicates and after inoculation incubated at 37 ^{O}C. Growth on nutrient agar was observed after 48 h.

RESULTS AND DISCUSSION

Twelve samples of polluted water were collected and stored at 4 ^{o}C for analysis. Total numbers of bacterial isolates were found in polluted water range from 31000- 65000 per mL. Eighty one bacterial isolates were selected according to difference in their colony size, shape and colour. Total 81 isolates were screened for arsenic resistance. Twenty one bacterial isolates were found resistant against arsenic. Finally four isolates were selected that showed the highest arsenic tolerance ability. MIC of the four isolates was also investigated using plate assay presented in Table 1. These four isolated strains were used for biochemical identification according to

Bergey's Manual of Systematic Bacteriology as shown in Table 2. From morphological and biochemical studies indicate that the strain code $S_{6,}$ $D_{15,}$ H_{19} and H_{23} may be *Bacillus polymyxa*, *Micrococcus luteus*, *Lactobacillus plantarum* and *Pseudomonas fluorescens*. Culture characteristics of the selected isolate were shown in table -6.

Organisms were studied in different concentration of arsenic containing culture media e.g., nutrient agar with arsenic at nutrient medium agar plates. The MIC was defined as the minimum concentration of the metal, which inhibit the bacterial growth in the plate. *Bacillus polymyxa* and *Pseudomonas fluorescens* were found to be resistant to As_2O_3 up to a concentration of 0.4 % (40 mg/ mL). *Micrococcus luteus* and *Lactobacillus plantariam* were found to be resistant to As_2O_3 up to a concentration of 0.2% (20 mg/mL). *Bacillus polymyxa* and *Pseudomonas*

Table 1. Screening of Arsenic resistant bacteria

S. No.	Bacterial strain code	Arsenic resistant bacterial strain code
1.	D_1, D_2, D_3, D_4, D_5, D_6, D_7, $D_{8,}$ D_9, D_{10}, D_{11}, D_{12}, D_{13}, $D_{14,}$ D_{15}, D_{16}, D_{17}, D_{18}, D_{19}, D_{20}, D_{21}	$D_{4,}$ $D_{11,}$ $D_{15,}$ D_{19}
2.	H_1, H_2, H_3, H_4, $H_{5,}$ H_6, H_7, H_8, H_9, H_{10}, H_{11}, H_{12} H_{13}, H_{14}, H_{15}, H_{16}, H_{17}, H_{18}, H_{19} , $H_{20,}$ H_{21}, H_{22}, H_{23}, H_{24}, H_{25},$H_{26,}$ H_{27}, $H_{28,}$ H_{29}, $H_{30,}$ H_{31}, H_{32}, H_{33}, H_{34}, H_{35}, H_{36}	H_2, $H_{6,}$ $H_{12,}$ H_{16}, $H_{19,}$ $H_{23,}$ $H_{28,}$ $H_{30,}$ $H_{31,}$ H_{36}
3.	S_1, S_2, S_3, S_4, S_5, S_6, $S_{7,}$ S_8, S_9, S_{10}, S_{11}, S_{12}, S_{13}, S_{14}, S_{15}, S_{16}	S_1, $S_{3,}$ $S_{6,}$ $S_{9,}$ $S_{10,}$ $S_{14,}$ S_{16}

Table 2. Determination of minimum inhibitory concentration

Strain	Concentration of As_2O_3 in NAM					Concentration of As_2S_3 in NAM				
code	0.1%	0.2%	0.3%	0.4%	0.5%	0.1%	0.2%	0.3%	0.4%	0.5%
S_6	+	+	+	+	-	+	+	+	+	+
D_{15}	+	+	-	-	-	+	+	+	-	-
H_{19}	+	+	-	-	-	+	+	+	-	-
H_{23}	+	+	+	+	-	+	+	+	+	+

Table 3. Biomass evaluation of selected arsenic resistant bacteria in different medium

S. No.	Bacterial Strain code	NAM	Bacterial Growth mg/50mL Medium			
			As_2O_3 (0.2%)	As_2S_3 (0.2%)	Glucose 0.2%	Glucose 0.4%
1.	S_6	371.9	132.0	198.5	855.6	471.7
2.	D_{15}	168.0	128.5	113.1	1081.8	334.5
3.	H_{19}	326.0	112.0	178.4	409.2	5017.5
4.	H_{23}	303.1	118.2	198.8	484.3	724.0

Table 4. Growth pattern at different temperature

Bacterial Strain code	4 °C	25 °C	37 °C	60 °C
S_6	-	+	+++	-
D_{15}	-	+	+++	-
H_{19}	-	+	+++	-
H_{23}	-	+	+++	-

- (no growth), + (mild growth), ++ (moderate growth), +++ (optimal growth)

Table 5. Growth pattern at different pH

Bacterial Strain code	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
S_6	-	+	++	+++	++
D_{15}	-	+	++	+++	++
H_{19}	-	+	++	+++	++
H_{23}	-	+	++	+++	++

- (no growth), + (mild growth), ++ (moderate growth), +++ (optimal growth

Table 6. Biochemical characterization of selected arsenic resistant bacteria

Characters/ Biochemical test	Isolates code Number			
	S_6	D_{15}	H_{19}	H_{23}
Gram Reaction	+ve	+ve	+ve	-ve
Cell Shape	Rod	Round	Rod	Rod
Spore formation	+	_	_	_
Glucose fermentation	+	+	+	_
Lactose fermentation	_	_	+	_
Sucrose fermentation	+	+	+	_
Indole test	_	_	_	_
Citrate utilization	+	_		+
Starch hydrolysis	+	_	_	+
Catalase test	+	+	_	+
Nitrate reductase test	_	_	_	+
Oxidase test	+	+	_	+
Probable identity	*Bacillus polymyxa*	*Micrococcus luteus*	*Lactobacillus fluorescens*	*Pseudomonas plantarum*

fluorescens were found to be resistant to As_2S_3 up to a concentration of 0.5% (500 mg/ mL). *Micrococcus luteus* and *Lactobacillus plantariam* were found to be resistant to As_2S_3 up to a concentration of 0.3% (30 mg/mL). Biomass of arsenic resistant bacteria was also determined in the different media containing As_2O_3, As_2S_3 and glucose shown in Table-3.

Growth responses at different temperature

For the determination of optimum temperature, strains were grown at nutrient agar medium. Plates of nutrient agar were equally inoculated with inoculum and incubated at different temperature 4, 25, 37 and 60ºC. Growth on nutrient agar plates was observed after 48 hrs. Table— indicate that 37º C temperature suitable for optimal bacterial growth.

Growth Responses of different pH

Growth of bacterial strains was observed at nutrient agar medium plates. Growth response of bacterial strains depends upon different pH. Table 4 indicates that 7.0 were suitable for bacterial growth.

CONCLUSION

Isolation of arsenic resistant bacteria from polluted environments would represent an appropriate practice to select arsenic resistant that would be used for arsenic removal and bio remediation purposes. The tolerance of bacteria to arsenic has been proposed as an indicator of potential toxicity of metals to other form of life this is proved in this study that these high arsenic tolerate bacteria confirmed the arsenic contamination in the studied water bodies of Ghaziabad .

Microorganisms with the ability to

tolerate and reduce arsenic can be use for detoxification of environments contaminated with arsenic so these arsenic resistant bacterial strains can be use to remediate the water bodies of Ghaziabad

The bioremediation of arsenic form contaminated sites involves reduction and oxidation of arsenic with the use of arsenic resistant microorganisms. The successful exploitation of these bacterial strains with proper biotechnology for bioremediation of arsenic will be beneficial. Therefore, more advance research is required for a deeper understanding about these bacterial strains to improve arsenic bioremediation process.

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