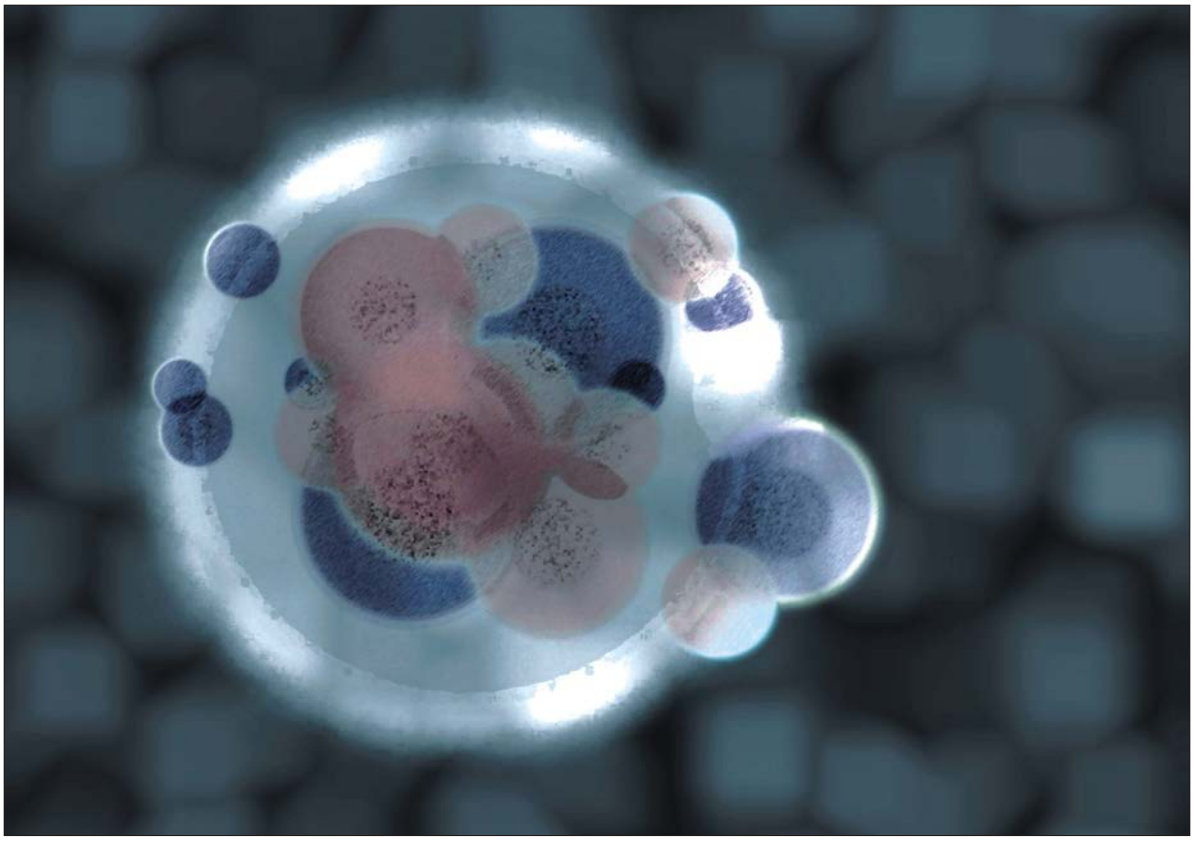

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 8 (5): 935-939, 2008
ISSN 1727-3048

Isolation and Identification of Mercury Resistant Bacteria from Kor River, Iran

N. Mirzaei, F. Kafilzadeh and M. Kargar
Department of Microbiology, Islamic Azad University of Jahrom, Fars Province, Jahrom, Iran

Abstract: Different mercury contaminated areas of the Kor River were surveyed for detection of mercury resistant bacteria. The samples were collected from four stations throughout the Kor River in four seasons. Amount of total mercury in the samples was determined using cold vapor atomic absorption spectrophotometery. The response of bacterial communities to toxic effects of Hg was monitored by enumerating the number of bacteria on agar containing 10 mg L^{-1} $HgCl_2$ and with out Hg (II). Isolation of mercury resistant bacteria was performed using the primary enrichment culture method and directly plating on agar containing 10 mg L^{-1} $HgCl_2$ Hg (II). Total viable counts ranged from 6.5×10^6 to 2.2×10^7 cfu g^{-1} in different stations. Frequencies of mercury resistant bacteria were 42.5% in Pole Khan Station and 3.14% in Droodzan Station. These stations were the most contaminated and uncontaminated areas of the Kor River, respectively. *Pseudomonas* sp., *E. coli*, *Serratia marcescens*, etc. was identified as mercury resistant bacteria. Using the primary enrichment culture method has permitted more effective isolation of Hg resistant bacteria. Present results showed that enhancement of mercury pollution in the environment will increase the probability of the isolation of Hg resistant bacteria.

Key words: Mercury resistant bacteria, enrichment culture method, mercury, *Serratia marcescens*, Kor River

INTRODUCTION

Public health authorities are now becoming greatly concerned about various toxic chemical pollutants such as heavy metals found in waters (Kumar, 1994). Mercury exists naturally in small amounts in the environment. However, its levels have risen due to environmental contamination from human activities (Nascimento and Souza, 2003). Mercury binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions (Wagner Dobler *et al*., 2000b). Even small amounts of mercury are toxic for all organisms. However, some bacterial communities residing in the mercury-contaminated areas can exchange mercury resistance genes between each other, because of continually exposure to the toxic levels of mercury. After the acquisition of resistance genes, these bacteria will be resistant to mercury (Nascimento and Souza, 2003). The mechanism of resistance to mercury in bacteria is mediated by the enzyme mercuric reductase (*merA* gene product). Resistance to mercury is controlled by a set of genes organized in the *mer* operon. *merA* has the key role in the removal of Hg (II). *merA* encodes the enzyme mercuric reductase. This enzyme reduces Hg^{2+} compounds to metallic mercury Hg^0 which is obviously less toxic to them (Deckwer *et al*., 2004; White *et al*., 2005; Kiyono and Pan Hau, 2006).

Moore first reported bacterial resistance to mercury compounds in a clinical isolate of *Staphylococcus aureus* (Barkay *et al*., 2003). After this finding there were several reports of environmental bacteria, which were resistant to mercury compounds (Walker and Colwell, 1974; Olson *et al*., 1979). Masura *et al*. (1999) studied the ability of resistance to Hg in anaerobic bacteria such as Clostridium genus. Mercury resistance ability not only has been reported in bacteria but has also been reported in different archea (Schelert *et al*., 2004; Vetriani *et al*., 2004). Further investigations showed that mercury resistant bacteria have some potential for the treatment of industrial effluents containing Hg (II) (Barkay, 1987; Ray *et al*., 1989). The removal of mercury from wastewaters was first demonstrated by Brunke *et al*. (1993). Moreover, researchers showed that, this process is also applicable to industrial wastewater of the chloralkali industry (Von Canstein *et al*., 1999). Thus, exact identification of mercury resistant bacteria is the first step in the application of biological treatment of mercury containing factory effluents. Therefore, the objectives of this study were to determine mercury contaminated areas of the Kor River to survey of the response of bacterial communities residing in different areas of the Kor River to toxic effects of Hg and to enrich of mercury resistant bacteria and isolating and detecting them in the sediment samples of the Kor River.

Corresponding Author: Nima Mirzaei, Shahid Langari Ave, Pasdaran Ave, P.O. Box 1693616719, Tehran, Iran
Tel: +989192546680 Fax: +982122941565

MATERIALS AND METHODS

Study area and sampling: The study area stretched from Droodzan Dam to Lake Bakhtegan, which comprised of four sampling stations of Droodzan (D), Pole Petroshimi (PP) Pole Khan (PKh) and Ghavmishi (G). The entire stretch from Droodzan to Lake Bakhtegan is about 120 km and lies between longitude 52° 25' 32" to 53° 25' 00" E and latitude 29° 51' 00" to 30° 12' 22" N (Fig. 1). Sampling of surface sediments were carried out monthly for a period from summer 2006 to spring 2007 from the sampling stations. The samples were collected with nitric acid pre-rinsed 1 L plastic container for chemical analysis and sterile glass container for microbial culturing. After collection, the samples were placed in cooler boxes with ice bags whilst being transported to the laboratory and kept at about 4°C before chemical and microbial analysis (MOOPAM, 1999).

Sample preparation and determination of mercury levels: The samples were first dehydrated to a constant weight using an oven at 103°C for 2 h and all individual particles were pulverized to a uniform particle size. Then the pulverized samples were digested with a mixture of 6 mL of nitric acid and 2 mL of perchloric acid and heated. The digested samples were filtered with 42 mm Whatman filter paper (MOOPAM, 1999) following the preparation, all samples were analyzed for Hg by cold vapor atomic absorption spectrophotometery using Inductive Coupled Plasma (ICP) Varian model.

Enumeration of viable cell count: All samples were serially diluted in Phosphate-Buffered Saline (PBS) (2.2 g of NaH_2PO_4 per liter, 6 g of Na_2HPO_4 per liter, 5.8 g of NaCl per liter [pH = 7.2]). 0.1 mL of each dilution were spread on Luria Bertani agar (10 g of peptone per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter, 12 g of agar per liter) supplemented with 10 mg of $HgCl_2$ per liter and with out $HgCl_2$. Bacteria grows maximum at 37°C but we monitored the growth of Hg resistant bacteria at 30°C like their natural conditions. The plates were incubated at 30°C for 48 h. After incubation period, the appeared colonies on both LB agar containing Hg (II) and with out Hg (II) were enumerated using total viable plate count method (Prescott and Harley, 2002).

Isolation and identification of mercury resistant bacteria: Isolation of Hg resistant bacteria was performed using the primary enrichment culture method and directly plating on agar containing Hg (II). In directly method after enumerating the number of bacteria the appeared colonies on appropriate plates containing Hg (II) were purified. In the primary enrichment culture method 1 g of each sample was added to 9 mL of Luria Bertani broth medium (10 g of peptone per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter) containing 10 mg of $HgCl_2$ per liter and incubated at 30°C for 48 h. After incubation period, 0.1 mL of enrichment cultures was spread on LB agar and incubated. Then the appeared colonies were purified and identified with gram staining and conventional biochemical tests according to the method of Bergey (Prescott and Harley, 2002).

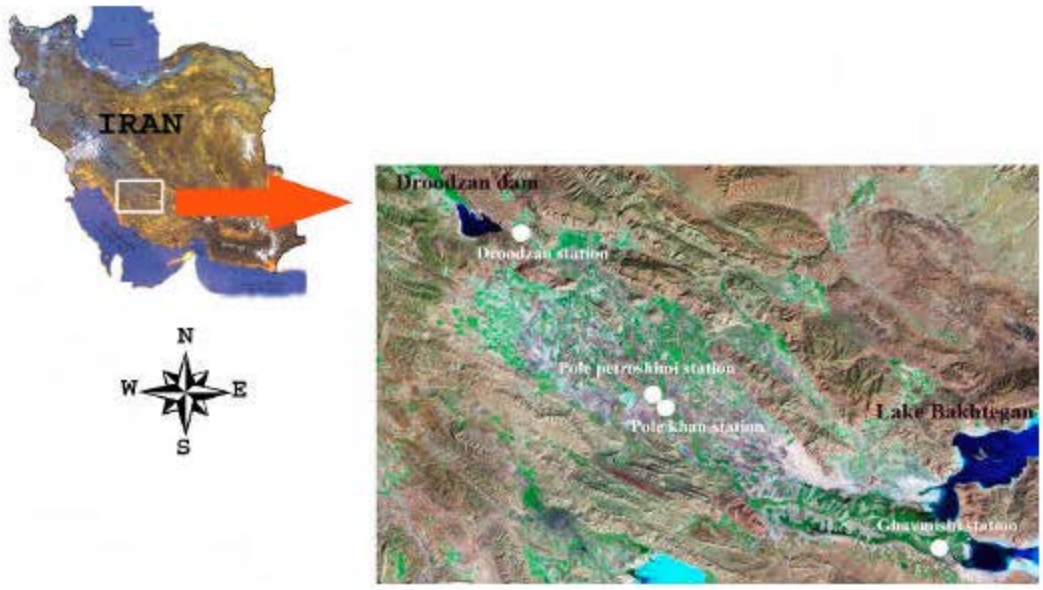

Fig. 1: Study area of the project and the sampling sites

Data analysis: Data analysis was performed by ANOVA and Duncan tests. All statistical analysis was done using SPSS software Ver.12 with significance based on 0.05 in most of the cases.

RESULTS

Amounts of mercury in the samples: The highest level of mercury was 0.758±0.056 ppm. This amount achieved in the samples of Pole Khan Station. The lowest amount of mercury (0.266±0.076 ppm) was obtained in the sediment samples of Droodzan Station (Table 1). There was a significant variation ($p<0.05$) between amounts of mercury in different stations.

Bacterial enumeration: Total viable counts ranged from 6.5×10^6 cfu g^{-1} in Ghavmishi sediment samples to 2.2×10^7 cfu g^{-1} in Pole Petroshimi sediment samples. The frequencies of resistance to Hg varied from 3% in Droodzan Station to 42% in Pole Khan Station (Table 1). There was a significant variation ($p<0.05$) between number of mercury resistant bacteria isolated in different stations.

Mercury resistant bacteria: The 67 environmental isolates were identified to at least genus level by gram staining and subsequent biochemical tests. Thirteen genera were identified as Hg resistant bacteria and 28 isolates to species level. Moreover, 43 isolates were detected by the enrichment culture method and 24 isolates detected with directly plating on LB agar containing 10 mg L^{-1} $HgCl_2$. Species diversity was greatest amongst the Pole Petroshimi and Pole Khan isolates (11 and 10 different species, respectively). Frequencies of gram negative Hg resistant bacteria were higher than gram positives (77% and 33, respectively). The most isolated bacterium was *Serratia marcescens* (88%) and the lowest were *Micrococcus* sp. and *Citrobacter* sp. (6%) (Table 2).

Table 1: Frequencies of Hg resistant bacteria at four sampling station

Sampling station	Total mercury (ppm)	Total bacteria (cfu g^{-1})	Hg resistant bacteria (cfu g^{-1})	Hg resistant bacteria (%)
Droodzan (D)	0.076±0.266	1.1×10^7	3.3×10^5	3.14
Pole Petroshimi (PP)	0.046±0.726	2.1×10^7	4.7×10^6	22.38
Pole Khan (PKh)	0.056±0.758	1.2×10^7	5.1×10^6	42.50
Ghavmishi (G)	0.021±0.307	6.5×10^6	4.1×10^5	6.30

DISCUSSION

The measurement of mercury levels in the samples showed that Pole Khan and Pole Petroshimi were the most contaminated regions of the Kor River. In the pervious studies on mercury pollution of the Kor River, also these regions reported as the most mercury contaminated areas of the Kor River (Kafilzadeh, 2005). Moreover the mean levels of Hg in these stations were higher than the interim standards for aquatic life and domestic use (WHO, 1989). Pole Petroshimi Station was placed in vicinity of a chloralkali plant. Marvdasht urban wastewater and Abbarik industrial town wastewater are directly discharged into the Kor River in Pole Khan Station.

In the present study we used LB agar and LB broth for detection of mercury resistant bacteria. Mercury resistant bacteria can be isolated in different medium such as Triptic Soy Agar (TSA) (Spangler *et al*., 1973), Tripton Iron Agar (TIA) (Baldi *et al*., 1992), MS agar (Teves, 1994) and Luria Bertani agar (LB) (Von Canstein *et al*., 1999).

The values of Hg resistant bacteria in the samples ranged from 3 to 43% (Table 3). Osborn *et al*. (1993) reported the frequencies of Hg resistant bacteria in the range of 0.05 to 25%. Much of these variations maybe due to differences in mercury levels amongst the sediment samples studied. However, a direct comparison of these

Table 2: Isolated mercury resistant bacteria in different seasons and stations

	Season and station																
	Summer 2006				Autumn 2006				Winter 2007				Spring 2007				
Isolated bacteria	D	PP	PKh	G	D	PP	PKh	G	D	PP	PKh	G	D	PP	PKh	G	(%)
Escherichia coli	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+	69
Serratia marcescens	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	88
Pseudomonas sp.	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-	31
Proteus sp.	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	-	25
Acinetobacter sp.	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	44
Klebsiella sp.	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	+	25
Enterobacter sp.	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	19
Citrobacter sp.	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	6
Alcaligenes sp.	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	19
Salmonella sp.	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	12
Bacillns sp.	-	+	-	+	+	-	-	+	+	+	-	+	+	-	-	+	56
S. aureus	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	19
Micrococcus sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	6

+: Isolated, -: Not isolated

Table 3: Physiological and biochemical characteristics of Hg resistant bacteria

	Isolates												
Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13
Gram	-	-	-	-	-	-	-	-	-	-	+	+	+
O/F	F	F	O	F	O	F	F	F	O	F	F	F	O
Oxidase reaction	-	-	+	-	-	-	-	-	+	-	-	-	-
Yellow pigment on YDC	-	-	-	-	-	-	-	-	-	-	-	-	+
Citrate utilization	-	+	+	-	+	+	+	+	+	-	ND	ND	+
Motility	+	+	+	+	-	-	+	+	+	+	-	-	-
Indole production	+	-	-	+	-	-	-	+	-	-	-	-	-
Voges-Proskaur	-	+	-	-	-	+	+	-	-	-	ND	-	-
Methyl red	+	-	-	+	-	+	-	+	-	+	ND	-	+
H_2S production	-	-	-	+	-	-	-	+	-	+	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	-	-	-	-	+	+	-	+	ND	ND	-
Endospores	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	ND
Urease	-	-	+	+	-	-	-	-	-	-	ND	-	+
D-Nase	-	+	-	-	ND	-	-	-	-	-	ND	-	-
Ornithine decarboxylase	+	+	-	+	ND	-	+	-	ND	+	ND	-	-
Lysine decarboxylase	+	+	-	-	-	ND	ND	-	-	-	ND	-	-
Coagulase	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Phenylalanine deaminase	-	-	-	+	ND	ND	ND	ND	-	-	ND	ND	-
Gelatin liquefaction	-	+	-	+	-	-	-	-	-	-	ND	ND	+

+: Isolated, -: Not isolated, ND: Not Determined

values with those from previous studies is not possible, as variation maybe due to different kinds of media used and to the different $HgCl_2$ concentrations used in media for detection of Hg resistant bacteria. In the present study frequencies of mercury resistant bacteria in the population at Pole Khan and Pole Petroshimi Stations, which are known to be contaminated with mercury, were higher. Frequencies of Hg resistant bacteria in the sediment samples of Droodzan and Ghavmishi Stations, where have the lowest amount of mercury, were low. Previous studies have shown a marked correlation between the number of mercury resistant bacteria and the levels of environmental mercury (Nakamura *et al*., 1990; Petrova *et al*., 2002).

Mercury resistant bacteria in the present study were isolated by the primary enrichment culture method and directly plating on agar containing Hg (II). The use of direct selection with high levels of $HgCl_2$ with out prior induction may have inhibited the growth of some Hg resistant bacteria (Osborn *et al*., 1993). In most previous studies isolation of mercury resistant bacteria has been performed by plating on agar containing Hg (II) and lower effective isolation of mercury resistant bacteria has been done (Wagner Dobler *et al*., 2000a; Karbasizaed *et al*., 2003).

Different bacterial genera were identified as mercury resistant bacteria (Table 2). Earlier studies have reported *Pseudomonas*, *Micrococcus*, *E. coli*, *Alcaligenes*, *Bacillus* (Pahan *et al*., 1990) and *Acinetobacter* sp. (Petrova *et al*., 2002). Similar results were obtained in this study. But in this report, *Proteus* sp., *Salmonella* sp. and *Citrobacter* sp. were identified as mercury resistant bacteria that earlier studies had not reported.

The results achieved in the present research show that different regions of the Kor River are highly polluted to mercury. Frequencies of Hg resistant bacteria in the contaminated areas were higher than other sites. According to the results of this study it is suggested that mercury resistant bacteria are being isolated with primary enrichment method in the presence of Hg. Mercury resistant bacteria isolated from contaminated environments have high potential to remove Hg from factory effluents. So it is suggested that mercury elimination ability of these bacteria should be evaluated. Moreover we can genetically engineer these isolates to reach better results in removal of Hg.

ACKNOWLEDGMENTS

This study was conducted in the research laboratory of Islamic Azad University of Jahrom. The authors would like to thank research assistant secretary of the Islamic Azad University of Jahrom for financial support of this research with grant No. 86.32 and Mehdi Kargar for excellent technical assistance during this study.

REFERENCES

Baldi, F., A. Boudou and F. Ribeyre, 1992. Response of a fresh water community to mercury contamination in a controlled system. Arch. Environ. Contam. Toxicol., 22: 439-444.

Barkay, T., 1987. Adaptation of aquatic microbial communities to Hg (II) stress. Applied Environ. Microbiol., 53: 2725-2732.

Barkay, T., S.M. Miller and A.O. Summers, 2003. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev., 27: 355-384.

Brunke, M., W.D. Deckwer, A. Frischmuth, J.M. Horn, H. Lundsorf, M. Rhode, M. Rohrocht, K.N. Timmis and P. Weppen, 1993. Microbial retention of mercury from waste streams in a laboratory column containing *merA* gene bacteria. FEMS Microbiol. Rev., 11: 145-152.

Deckwer, W.D., F.U. Becker, S. Ledakowitcz and I. Wagner Dobler, 2004. Microbial removal of ionic mercury in a three phase fluidized bed reactor. Environ. Sci., 38: 1858-1865.

Kafilzadeh, F., 2005. Evaluation of heavy metals pollution of the Kor River; Research project. Department of Biology, IAU Arsanjan Branch.

Karbasizaed, V., N. Badami and G. Emtiazi, 2003. Antimicrobial heavy metal resistance and plasmid profile of coliforms isolated from nosocomial infections in a hospital in Isfahan, Iran. Afr. J. Biotechnol., 2: 379-383.

Kiyono, M. and H. Pan Hau, 2006. Genetic engineering of bacteria for environmental remediation of mercury. J. Health Sci., 52: 199-204.

Kumar, H.D., 1994. General Ecology, Chapter VII, Aquatic Ecology. 1st Edn. Vinkas Publ., New Delhi, India, pp: 497-555.

Masura, N., C. Huang, Y. Takeshi and E. Ginro, 1999. Study on ubiquity of mercury resistance genes and multi heavy metal resistance of Clostridia. Proc. Environ. Eng. Res., 36: 29-37.

MOOPAM., 1999. Manual of oceanographic and observations pollutant analysis methods. ROMPE, Kuwait.

Nakamura, K., M. Sakamoto, H. Uchiyama and O. Yagi, 1990. Organomercury volatilizing bacteria in the mercury polluted sediment of Minamata bay Japan. Applied Environ. Microbiol., 56: 304-305.

Nascimento, A.M.A. and E.C. Souza, 2003. Operon mer: Bacterial resistance to mercury and potential for bioremediation of contaminated environments. J. Genet. Mol. Res., 2: 92-101.

Olson, B.H., T. Barkay and R.R. Colwell, 1979. Role of plasmids in mercury transformation by bacteria isolated from the aquatic environment. Applied Environ. Microbiol., 38: 478-485.

Osborn, A.M., K.D. Bruce, P. Strike and D.A. Ritchie, 1993. Polymerase chain reaction fragment length polymorphism analysis shows divergence among *mer* determinants from gram negative soil bacteria in distinguishable by DND-DNA hybridization. Applied Environ. Microbiol., 59: 4024-4030.

Pahan, K., S. Ray, R. Gachui, J. Chaudhuri and A. Mandal, 1990. Ecological and biochemical studies on mercury resistance bacteria. Indian J. Environ. Health, 32: 250-261.

Petrova, M.A., S. Mindlin, Z. Gorlenko, E. Kaliaeva, V. Soina and E. Bogdanova, 2002. Mercury resistance bacteria from permafrost sediments and prospects for their use in comparative studies of mercury resistance determinants. Genetika, 38: 1569-1574.

Prescott, L.M. and J.P. Harley, 2002. Laboratory Exercises in Microbiology. 1st Edn. McGraw Hill Publ., New York, USA.

Ray, S., R. Gachhui, K. Pahan, J. Chaudhury and A. Mundal, 1989. Detoxification of mercury and organomercurials by nitrogen fixing soil bacteria. J. Biosci., 14: 173-182.

Schelert, J., V. Dixit, V. Hoang, J. Simbahan, M. Drozda and P. Blum, 2004. Occurrence and characterization of mercury resistance in the hyperthermophilic archaeon *Sulfolobus sulfataricus* by use of gene disruption. J. Bacteriol., 186: 427-437.

Spangler, W.J., J.L. Spigarelli, J.M. Rose, R.S. Flippin and H.H. Miller, 1973. Degradation of methyl mercury by bacteria isolated environmental samples. Applied Microbiol., 25: 488-439.

Teves, F.G., 1994. Mercury resistance bacteria isolated from industrial effluent outlet in Iligan City. Multi. Inf., 9: 13-20.

Vetriani, C., Y.S. Chew, A.M. Miller, J. Yagi, J. Coombs, R.A. Lutz and T. Barkay, 2004. Mercury adaptation among bacteria from a deep sea hydrothermal vent. J. Bacteriol., 13: 1-18.

Von Canstein, H., Y. Li, K.N. Timmis, W.D. Deckwer and I. Wagner Dobler, 1999. Removal of mercury from chloralkali electrolysis wastewater by a mercury resistance *Pseudomonas putida* strain. Applied Environ. Microbiol., 65: 5279-5284.

Wagner Dobler, I., H. Lunsdorf, T. Lubbenhausen, H. Von Canstein and Y. Li, 2000a. Structure and species composition of mercury reducing biofilms. Applied Environ. Microbiol., 66: 4559-4563.

Wagner Dobler, I., H. Von Canstein, K.N. Timmis, Y. Li and W.D. Deckwer, 2000b. Removal of mercury from chemical wastewater by microorganisms in technical scale. Environ. Sci. Tecnhnol., 34: 4628-4634.

Walker, J.D. and R.R. Colwell, 1974. Mercury resistant bacteria and petroleum degradation. Applied Microbiol., 27: 285-287.

White, D.G., M.N. Alkeshun and P.F. Mc Dermott, 2005. Frontiers in Antimicrobial Resistance, Chapter 11, Metal Resistance Loci of Bacterial Plasmid. 1st Edn. ASM Publication, USA., pp: 165-173.

WHO., 1989. Guidelines for drinking water quality. World Health Organization, Geneva, 2.