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Heavy metal tolerant halophilic bacteria from Vembanad Lake as possible source for bioremediation of lead and cadmium

M. Sowmya[1], M.P. Rejula[1], P.G. Rejith[1], Mahesh Mohan[1], Makesh Karuppiah[2] and A.A. Mohamed Hatha[3]*

[1]School of Environmental Sciences, Mahatma Gandhi University, Kottayam-686 560, India
[2]Environmental Sciences Division, EnviroApps Inc, Orlando, FL 32835, USA
[3]Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin – 682 016, India
*Corresponding Author E-mail: mohamedhatha@gmail.com

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Abstract

Microorganisms which can resist high concentration of toxic heavy metals are often considered as effective tools of bioremediation from such pollutants. In the present study, sediment samples from Vembanad Lake were screened for the presence of halophilic bacteria that are tolerant to heavy metals. A total of 35 bacterial strains belonging to different genera such as *Alcaligenes, Vibrio, Kurthia, Staphylococcus* and members of the family *Enterobacteriaceae* were isolated from 21 sediment samples during February to April, 2008. The salt tolerance and optimum salt concentrations of the isolates revealed that most of them were moderate halophiles followed by halotolerant and extremely halotolerant groups. The minimum inhibitory concentrations (MICs) against cadmium and lead for each isolate revealed that the isolates showed higher MIC against lead than cadmium. Based on the resistance limit concentration, most of them were more tolerant to lead than cadmium at all the three salt concentrations tested. Heavy metal removal efficiency of selected isolates showed a maximum reduction of 37 and 99% against cadmium and lead respectively. The study reveals the future prospects of halophilic microorganisms in the field of bioremediation.

Key words

Bioremediation, Halophilic bacteria, Heavy metal tolerance, Sediment, Vembanad lake

Introduction

Metal pollution and bioaccumulation/ biomagnification of metals cause serious health and ecological risks. Sediment is the ultimate repository of many chemical compounds including heavy metals from natural and anthropogenic sources. Cadmium generated by mining, industrial activities, sewage sludge and the use of phosphate fertilizer in agriculture - is one of the most toxic heavy metals and ranked number 7 among the top 20 major toxins (Al-Khedhairy *et al.,* 2001; Tang *et al.,* 2006). Lead, a highly useful metal, has been mined for thousands of years. But its wide-spread use in the modern era has resulted in increased metal levels in the human blood, skeletal system and subsequent accumulation in various organs in human body and related health hazards including cancer (Tsalev and Zipranov, 1985; Bruins *et al.,* 2000; Lam *et al.,* 2007). Cadmium can cause developmental abnormalities and increased mortality in fish, renal dysfunction, osteomalacia or severe osteoporosis, bone demineralization and cancer (Khan *et al.,* 1992; Ghoshroy *et al.,* 1998). As a result of increasing population, industrial and agricultural activities as well as larger volume of the sewage treatment plant influent has resulted in an increase in metal concentrations and toxicity of sediments from bay and estuaries (Karuppiah and Gupta, 1998). Lead can affect many organs and cause mental retardation, decrease in IQ levels, especially in children (Rosen, 1995; AAP, 2005). Environmental cadmium and lead exposures at levels currently observed in the United States population increase the risk of hearing loss, the third chronic condition experienced by adults ≥ 65 years of age (Choi *et al.,* 2012). At high blood lead levels (more than 70mg dl^{-1}) nephropathy, behavioral disturbances, learning disabilities, deficit in fine and gross motor development, reduced fertility both in men and women, neuropathy, Alzheimer disease, increased intracranial pressure, seizure and death are common (Mendelsohn *et al.,* 1998; Navas-

Acien *et al.,* 2007; Iqbal *et al.,* 2008; Woodruff *et al.,* 2008).

Bioremediation provides not only an economical solution to many pollution problems but is also a safe and effective alternative. The industries which manufacture chemicals such as pesticides, pharmaceuticals and herbicides and oil and gas recovery processes produce waste waters with salinity fluctuating from time to time. Conventional microbiological treatment processes do not function effectively at these salinity variations because of the inadaptability of microbes to varying salt concentrations. Therefore, the use of moderately halophilic or halotolerant bacteria should be considered (Oren *et al.,* 1992; Oren *et al.,* 1993) for bio remediation in saline environments.

Halophiles are microorganisms which are able to survive a wide range of salinity. Using consortia of indigenous microorganisms for the treatment of metal pollution has been proved to be very effective in bioremediation (Vatis Update, 1999; Chowdhury *et al.,* 2011). The present study aims at isolation of halophilic bacteria from Vembanad - Kol wetland – a Ramsar site in Kerala, India and screening them for highly tolerant strains against cadmium and lead and determination of their metal removal efficacy.

Materials and Methods

Study Area : Vembanad - Kol wetland of Kerala (length: 80 km;

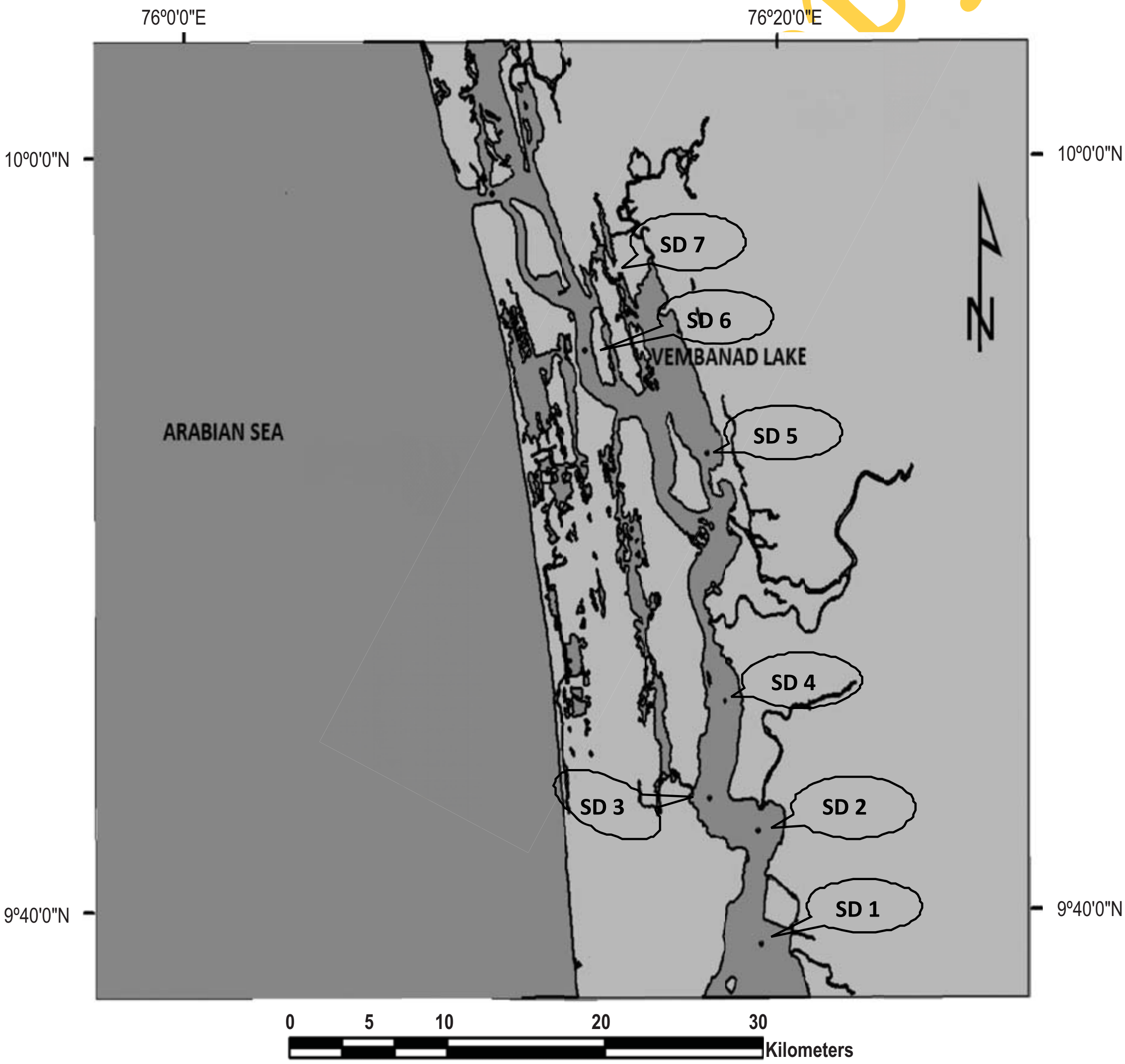

Fig. 1 : Map showing Vembanad lake and sampling sites SD1 – SD7

width: 0.5- 4.5 km) being connected to the Arabian Sea at two places, is often exposed to tidal influences that lead to variations in salinity (Fig. 1) (Priju and Narayana, 2007). Sediment samples were collected from seven sites of the Vembanad Lake from Kumarakom to Fortkochi (9°37'47.4"N 76°25'5.2"E - 9°58'08.0"N 76°14'36.1" E) using sediment sampler and transferred to sterile packets and properly labeled. All the samples were kept on ice and immediately transported to the laboratory and examined within 5 hrs of collection. Sampling was done for three months, from February to April, 2008.

Isolation and characterization of salt tolerant bacteria : Isolation of salt tolerant bacterial strains from sediment samples were done as previously described (Amoozegar *et al.,* 2005). The isolates were characterized by Gram staining, spore staining, motility, oxidase test, catalase test and oxidation fermentation (O/F) test. The cultures were then identified as various genera as per the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 2000).

For determination of salt tolerance, all the isolates were aseptically transferred from nutrient agar vials into sterile nutrient broth tubes with 10% (w/v) NaCl and incubated at 37° C for 24 hrs. The exponentially growing broth cultures were subsequently transferred to nutrient broth tubes with different salt concentrations [0%, 3%, 10%, 15% and 25% (w/v) NaCl] with an inoculation loop and incubated at 37° C for 24 hrs. All the broth cultures were visually examined for growth after 24 hr of incubation and then the optical density was taken by Spectronic 20 D spectrophotometer at 550 nm to determine optimum salt

Table 1 : Minimum inhibitory concentration (MIC) of halotolerant bacteria from Vembanad Lake against cadmium and Lead

		MIC (mM) of cadmium at different salt concentrations			MIC (mM) of lead at different salt concentrations		
Bacterial genus	**Isolate No**	**5%**	**10%**	**15%**	**5%**	**10%**	**15%**
Alcaligenes	SD6 55	0.1	0.1	0.1	7	5	5
	SD3 64	0	0.1	0.1	0.6	2	7
	SD7 72	0	0.1	0	0	2	0
	SD6 80	0.3	1	4	7	7	7
	SD6 87	4	2	2	7	7	2
Enterobacteriaceae	SD4 51	0.05	0.1	0.1	7	12	12
	SD6 58	0.1	0.1	0.1	7	12	12
	SD2 61	0.1	0.1	0.1	7	12	12
	SD7 69	0.9	0.3	0.1	7	0	0
	SD5 75	0.1	0.1	0.1	7	7	12
	SD2 76	0.1	0.1	0.1	7	7	12
	SD2 77	0.1	0.1	0.1	12	12	12
	SD3 78	0.1	0.1	0.1	7	7	12
	SD4 81	0.1	0.1	0.1	7	7	12
Kurthia	SD5 84	0.1	0.5	1	9	12	12
Staphylococcus	SD4 83	0.5	4	4	7	12	12
Vibrio	SD1 45	0.1	0.1	0.3	7	12	12
	SD3 49	0.1	0.1	0.3	7	12	12
	SD4 52	0.1	0.1	0.3	7	7	2
	SD5 54	0.1	0.1	0.3	7	12	12
	SD6 56	2	0.1	0	12	12	0
	SD1 59	0.1	0.1	0.3	7	12	12
	SD1 60	2	0.1	0	12	12	0
	SD5 66	0.1	0.1	0	12	7	0
	SD4 68	0.1	0.1	0	7	1	0
	SD7 70	2	2	0.3	7	7	0
	SD1 73	0.1	0.05	0	0.6	0	0
	SD1 74	0	0.1	0.1	0	7	12
	SD6 79	0.1	0.6	4	7	7	7
	SD4 82	0.1	0.1	0.1	12	7	5
	SD5 85	2	2	0	7	7	0
	SD6 86	2	2	0	5	5	0
	SD6 88	0.1	0.1	0.6	7	12	12
	SD7 89	0.05	0.1	0	0.1	0.6	0
	SD7 90	4	4	4	7	7	5

concentration for growth.

Determination of minimum inhibitory concentration (MIC) : Minimum inhibitory concentration (MIC) was determined by spot plate method (Amoozegar *et al.,* 2005). In the first step the tolerance range of isolates to cadmium and lead was determined using a wide range of metal concentrations. For this, to each set of nutrient agar, different concentrations of a heavy metal solution ($CdCl_2$ for cadmium and $Pb(NO_3)_2$ for lead) - 0.005 mM, 0.05 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM and 80 mM was added and plated. The same concentrations were used for both the metals. In the second step, for determining the MIC of cadmium, the following concentrations of metal solution were prepared: 0.01 mM, 0.03 mM, 0.1 mM, 0.3 mM, 0.6 mM, 0.9 mM, 2 mM, 4 mM. For determining the MIC of lead, the following concentrations of metal solutions were prepared: 0.6 mM, 0.9 mM, 2 mM, 4 mM, 7 mM, 9 mM, 12 mM, 14 mM. Controls consisted of agar plates without heavy metal and inoculation with the corresponding test microorganisms was carried out in all experiments.

Heavy metal removal/biosorption : Heavy metal removal efficiency of the highly tolerant isolates was determined by biosorption method. The filter sterilized metal solution was added to 25 ml sterilized nutrient broth with varying NaCl concentrations (5, 10, 15%). Uninoculated nutrient broth [with varying NaCl concentrations (5, 10, 15%)] with metal solution was used as control. The concentrations of metal solution were chosen on the basis of highest tolerance shown by the bacterial strains. Initial metal concentration in the broth was read using Voltametric trace metal analyzer (VA Computrace 797, Metrohm). The exponentially growing 24 hr broth cultures of selected isolates were inoculated into the above metal medium, and were incubated at 37°C with shaking for 48 hrs in an orbital shaker. After incubation, the cells were pelletized by centrifugation at 3000 rpm and the supernatant was analyzed for final metal concentration using Voltametric trace metal analyzer. The percentage reduction of metal concentration by the isolate in the broth was determined.

Statistical analysis : Results were analyzed using analyses of variance (ANOVA). The analyses were run at a 5% significant level. Statistix (V. 9.0) was used for statistical analysis (Statistix^R, 2008).

Results and Discussion

Thirty five isolates of salt tolerant bacteria obtained from the sediment samples of Vembanad lake were characterized up to genus level. The predominant group was Gram–negative rods (94.29%) and Gram-positive bacteria (5.71%) consisted of rods (2.86%) and cocci (2.85%). Generic level characterization revealed genera such as *Alcaligenes* (14.29%), *Enterobacteriaceae* (25.71%), *Kurthia* (2.86%), *Staphylococcus* (2.85%) and *Vibrio* (54.29%). Extreme halophiles were not encountered in the present study. However, 66% of isolates were moderate halophiles. Most halophiles within the domain bacteria were moderate rather than extreme halophiles (Oren, 2002). Halotolerant bacteria and extremely halotolerant (11%) were the other two groups encountered. Study area being a typical tropical estuary subjected to seasonal changes in salinity, turning nearly fresh water during heavy monsoon and considerably saline during the pre-monsoon months (Priju and Narayana, 2007) offers an ideal habitat for bacteria with tolerance to great ranges of salinity.

On the basis of salt range and optimum salt concentration, the isolates (n=35) were grouped into three. Halotolerant (23%) - grows without salt, but tolerates up to 2.5 M (15% (w/v) NaCl), extremely halotolerant (11%) - grows without salt, but tolerates above 2.5 M [15% (w/v) NaCl] and moderate halophiles (66%) - grows optimally between 0.5 M -2.5 M [3 -15% (w/v) NaCl and can tolerate even up to 25%].

The resistance of the isolates to the heavy metals was tested and the minimum inhibitory concentration (MIC) was determined (Table 1). The strain was considered resistant, when its growth was not inhibited by the resistance limit concentration of any metals. Depending on resistance limit concentration, the isolates were grouped to two - metal tolerant and non tolerant. Tolerance to cadmium was found to vary at different salt concentrations. Against cadmium, 40% of *Alcaligenes* were tolerant at 15% salt concentration, while 20% of them tolerated cadmium at 5 and 10% salt concentrations. While *Enterobacteriaceae* and *Kurthia* were found to be intolerant to cadmium at all the salt concentrations *Staphylococcus* tolerated cadmium at 10 and 15% salt concentrations. Most of the strains (31.58%) in genus *Vibrio* showed tolerance to cadmium at 5% salt concentration (Table 2).

Table 2 : Percentage incidence of various genera that are tolerant to cadmium and lead at different salinities

Name of the genera	Total no. of isolates	Cadmium Metal tolerant %			Lead Metal tolerant %		
		5%	10%	15%	5%	10%	15%
Alcaligenes	5	20	20	40	60	100	80
Enterobacteriaceae	9	0	0	0	100	88.89	88.89
Kurthia	1	0	0	0	100	100	100
Staphylococcus	1	0	100	100	100	100	100
Vibrio	19	31.58	21.05	10.53	84.21	84.21	52.63

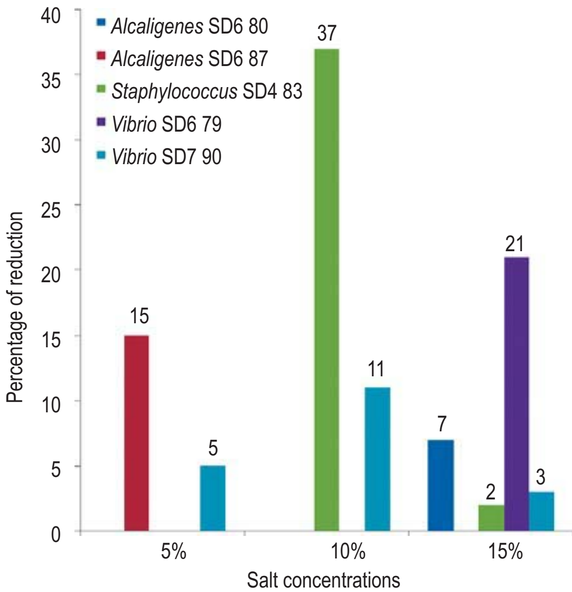

Fig. 2 : Percentage reduction of cadmium by the selected strains of bacteria from Vembanad Lake

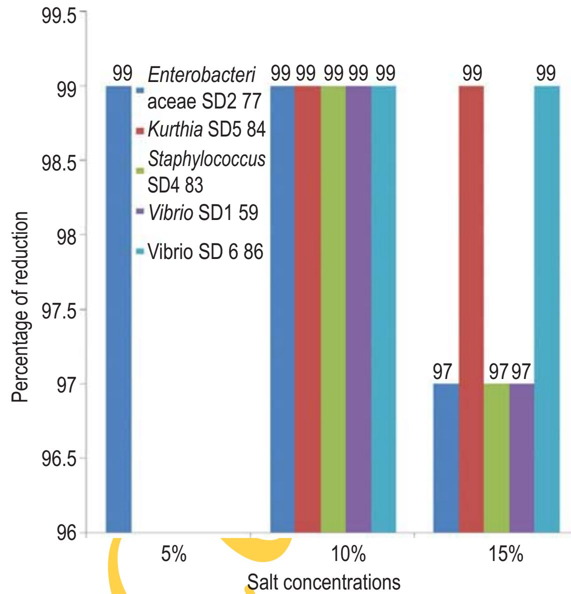

Fig. 3 : Percentage reduction of lead by the selected strains of bacteria from Vembanad Lake

Similarly, tolerance to lead was also found to be dependent on salt concentration in the medium. There was only one strain of *Kurthia* and *Staphylococcus*, which was able to tolerate lead at all the salt concentrations tested. All the strains of the genus *Alcaligenes* and Vibrio showed varying levels of tolerance depending on salt concentration (Table 2). Half of the cadmium tolerant isolates and 57.58% of the lead tolerant isolates showed higher MIC at higher percentage of salt concentrations. The present results differ with some of the previous findings in that the isolates did not show a steady increase in tolerance to both the metals with increasing NaCl concentrations in the medium from 5% to 15% (Amoozegar *et al.,* 2005). Statistical analysis (ANOVA) of the data from Cd and Pb tolerance at different salt concentrations did not seem to be statistically significant.

The isolates which had shown highest MIC for cadmium and lead at each salt concentration were chosen to study heavy metal removal/ reduction when grown in the medium containing these heavy metals. The removal/ reduction in the heavy metal concentration showed variations among different genera of bacteria (Fig. 2). Of all the isolates tested for reduction of cadmium, the genus *Staphylococcus* (SD4 83) showed maximum reduction (37%) at 10% salt concentration. In the case of lead, all the tested isolates showed maximum reduction of 99% at 10% salt concentration. SD2 77, member of the genus *Enterobacteriaceae* had same MIC (12 mM) at all the three salt concentrations, had a reduction efficiency of 99, 99 and 97% at 5, 10 and 15% salt concentrations, respectively (Fig. 3). In case of controls, there was no change between the initial and final metal concentrations.

The metal reduction tests of isolates showed 99% efficiency in removal of lead. In the case of cadmium it was 37%. This may be due to the difference in the toxicity of two metals and the difference in characteristics of the isolates used. Toxicity of metals including Cd and Pb to *Vibrio fischeri*, a bioluminescent, Gram-negative rod bacterium that are natural inhabitants of seawater but can also be found in fresh water, has been extensively used in identification of metal toxicity in Chesapeake Bay tributaries (Karuppiah and Gupta, 1998). Metal removal by bacteria in culture media has been reported by several workers (Iyer *et al.,* 2004; Iyer *et al.,* 2005; Zouboulis *et al.,* 2004). Though cadmium is very toxic to microbes, 70% to nearly 100% removal efficiency of cadmium by sulfate reducing bacteria from acid mine drainage in *in situ* studies has already been reported (Marietta *et al.,* 2008). A bacterium belonging to genus *Halomonas* could uptake more than 90 and 50% of lead and cadmium, respectively (Amoozegar *et al.,* 2012). The study revealed future prospects of halophilic microorganisms in the field of bioremediation of metals.

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