Original Paper

Luminescent Bacteria as Biosensor for Mercury Toxicity

Seema Nair, Ammu Thomas,** Neema Job,** Girija DS,** Unni AKK****

ABSTRACT

Agricultural activities and human industrialization are mainly responsible for the release of heavy metals into the environment, especially the air and the water. The first step towards the effective management of water resources is the assessment of pollution levels. Biosensors for the detection of pollutants in the environment can complement analytical methods by distinguishing bioavailable from inert, unavailable forms of contaminants. A bioassay system for detecting heavy metals in water using bioluminescent bacteria, Vibrio harveyi and Vibrio fischeri has been developed, which offers the advantages of simplicity and rapidity for screening heavy metals in water sources. Bioluminescence was found to be species specific and strain specific. Mercury, zinc and copper showed definite microbial toxicity and inhibition of bioluminescence. The inhibition range for each strain of a species was standardized and its reproducibility verified. The utility of the biosensors to detect heavy metals in tap water was demonstrated with samples supplemented with Hg (II).

Key Words: *bioluminescent bacteria, Vibrio spp, mercury, water analysis*

Introduction

Heavy metal pollution of water resources is a major issue in a developing country such as India.[1] Agricultural activities and human industrialization enhance the release of heavy metals into the environment. Since heavy metals readily accumulate in soil, the concentration in soil is likely to progressively increase over a period of time. Such contamination of soil has deleterious effects on the metabolic processes of the soil microorganisms, which in turn affects microbial activities and the decomposition process of organic matter.[2] The first step towards the effective management of water resources is the assessment of pollution levels. Heavy metals are known to be inhibitory and toxic to the microbial community.[3] There are at present many physical, chemical, and biological methods for the detection of water pollution. Biosensors for the detection of pollutants in the environment can complement these conventional analytical methods by distinguishing bioavailable from inert, unavailable forms of contaminants. As a biological method, luminescent bacteria offer the advantages of a simple test procedure and a rapid response.[4]

Bioluminescence refers to the visible light emission in living organisms that accompanies the oxidation of organic compounds (luciferins) mediated by luciferase.[5] Bioluminescence has been most extensively studied in marine bacteria (V*ibrio harveyi*, *Vibrio fischeri*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*), and to a lesser extent in terrestrial bacteria (*Xenorhabdus luminescens*). It has been found that the light-emitting reactions are quite distinct for different organisms, with the only common component being molecular oxygen. Therefore, significant differences have been found between the structures of the luciferases and the corresponding genes from one luminescent organism to another.[6]

Heavy metals are well known for their toxicity to living organisms. Mercury (Hg) is one such metal, while copper (Cu) is a metal that is an essential nutrient cation at trace levels, which becomes toxic at higher concentrations. The World Health Organization prescribes a

*Assistant Professor (*Corresponding Author*),
**Project Fellow,
***Professor & Head, Biotechnology Laboratory, Research Coordination Division, Amrita Institute of Medical Sciences, Cochin 26, India

maximum limit of 1ppb Hg in potable water. The same limit has been adopted by Indian Standard Specifications since 1983.

Mercury is released into the atmosphere by a variety of natural and anthropogenic sources.[7] Inorganic mercury compounds (such as mercuric chloride) are used in batteries, paper manufacturing, and the chemical industry. Mercury can seep into underground water supplies from industrial and hazardous waste sites. Application of mercury-based pesticides on agricultural lands can wash into nearby surface waters or travel through the soil into underground water supplies.

Toxicity screening for heavy metal ions could use both chemical and biological methods. Currently, the analysis of such low levels of heavy metals is done by time consuming and expensive methods such as Cold Vapor Atomic Absorption Spectrometry and Plasma Mass Spectrometry. This study was aimed at developing a simple and rapid screening system for heavy metals in water sources.

Materials and Methods

The bacterial strains used in this study were isolated from water collected from shrimp ponds in the coastal belt of Cochin of Kerala State (specifically from Chalakadavu located in Chellanam panchayat), and from the intestines of fresh fish *(Rastralliger kanagurta, Lactarius lactarius, Nemipterus japonicus, Leognathus bindhus, Mene maculata* and *Sardinella longiceps)* procured from the fish markets in Cochin. The isolated strains were identified as *Vibrio fisheri* and *Vibrio harveyi* according to Bergey's Manual of Determinative Bacteriology.[8,9] The strains were maintained in Luminescent broth and Luminescent agar (1.5% agar) at 22 + 2^{O}C for 18-24 hours.

The effect of sodium chloride (1%, 2%, 3%, 4%, 5% and 6%) and magnesium sulphate (1%, 0.1%, .01%, .001%, 0.0001%) on growth and luminescence was studied by incorporating them in the growth media and adding 1% innoculum, followed by incubating the tubes at 22 + 2^{O}C for 18-24 hrs. Growth was observed as turbidity, and luminescence measured by luminometer (Turner Designs 20/20 Luminometer).

The effect of mercury as mercuric chloride (10ppm, 1ppm, 0.1ppm, 0.01ppm, 0.001ppm) zinc as zinc sulphate (10ppm, 100ppm, 1000ppm, 10000ppm), and copper as copper sulphate (1ppm, 10ppm, 100ppm, 1000ppm, 10000ppm) on luminescence was determined by adding different concentrations in distilled water and tap water, inoculating the culture, and incubating for 30min and then measuring the luminescence after dilution. The percentage of inhibition of luminescence for each *Vibrio* strain against each metal was standardized, and a range was obtained. Tap water samples from five different sources within Cochin city were collected and the percentage of inhibition of luminescence using a standard strain of *Vibrio* was measured.

Vibrio harveyi and *Vibrio fisheri* strains were isolated from the water and fresh fish intestines. The strains were grown in luminescent broth at 22 + 2ºC for 24hrs. Bacterial luminescence is dependent on the growth of the bacteria, pH of the media, oxygen availability, and nutrients. The bioluminescence was found to decrease as the pH decreased and the media became acidic (**Table 1**). Except for the culture grown in media with an initial pH of 9, which exhibited bioluminescence after 72 hours, the other cultures did not show any luminescence at 72hrs as the media becomes increasingly acidic.

Table 1 Effect of pH on Luminescence

pH	Effect on Luminescence		
	After 16 hrs	After 24 hrs	After 72 hrs
9	_	_	+
8	+	+	_
7	+	+	_
6	+	+	_
5	+	_	_

"+" indicates positive luminescence
"-" indicates no luminescence

Effect of Salt and Magnesium on Growth and Bioluminescence

Vibrio spp being halophilic, it was observed that salt concentration of 3-4% was optimum for growth and bioluminescence (**Fig 1**). Luminescence was observed to be directly proportional to the magnesium concentrations (**Fig 2**).

Effect of Heavy Metals on Bioluminescence

The highest concentration of 10^4ppm zinc in the media showed less than 50% inhibition of bioluminescence. Zinc concentrations below 10^4ppm did not show any significant effect on bioluminescence. The sensitivity of this method to detect zinc concentrations below 10^3ppm was

less. Copper showed 100% inhibition at 10^3ppm. The toxicity and effect on luminescence was higher in the case of copper as compared to zinc. Concentrations of copper below 100ppm could be detected with more sensitivity. Mercury showed the highest level of inhibition of luminescence among the heavy metals studied here (**Fig 3**). Different concentrations of mercury below 10ppm could be detected using this method. However below 10^{-3} ppm, mercury could not be detected as the percentage of inhibition goes below 7%. As mercury could be detected adequately by this method, further studies were carried out to standardize the method of detection of mercury from tap water samples.

Standards for Detection of Mercury in Water Samples

The bioluminescence produced by two typical strains belonging to *Vibrio* species in the presence of different mercury concentrations incorporated in distilled water and tap water was read in a luminometer (**Table 2**). The inhibition varied from 100% in 10ppm mercury to 7% in 0.001ppm of mercury. Based on the % inhibition of bioluminescence by the standard strains selected (MMI, HW1 & PMI), a range for each concentration could be identified as below:

100% inhibition of Bioluminescence = contains around 10ppm and above mercury in the sample
95-99% inhibition of Bioluminescence = contains around 1ppm and above mercury in the sample
80-90% inhibition of Bioluminescence = contains around 0.1ppm and above mercury in the sample
50-70% inhibition of Bioluminescence = contains around 0.01ppm and above mercury in the sample
Below 50% inhibition of Bioluminescence = contains less than or equal to 0.001ppm mercury in the sample

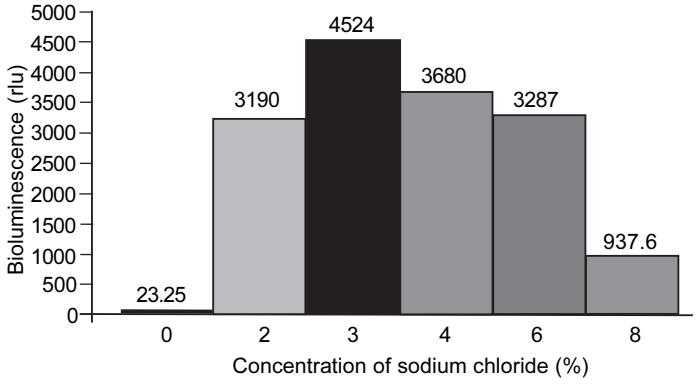

Fig.1 Effect of sodium chloride on Bioluminescence

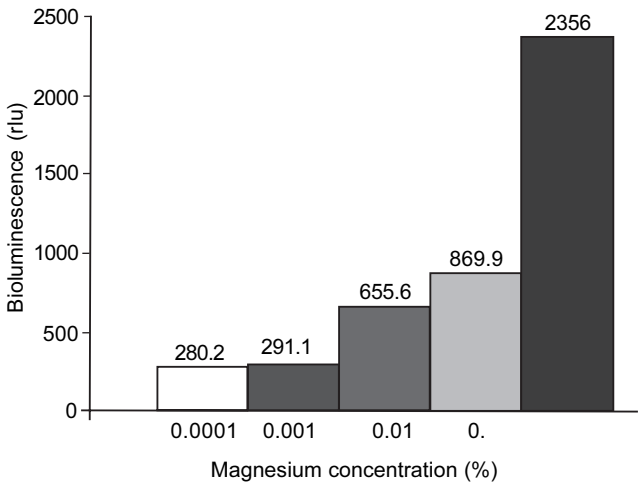

Fig. 2 Effect of magnesium on Bioluminescence

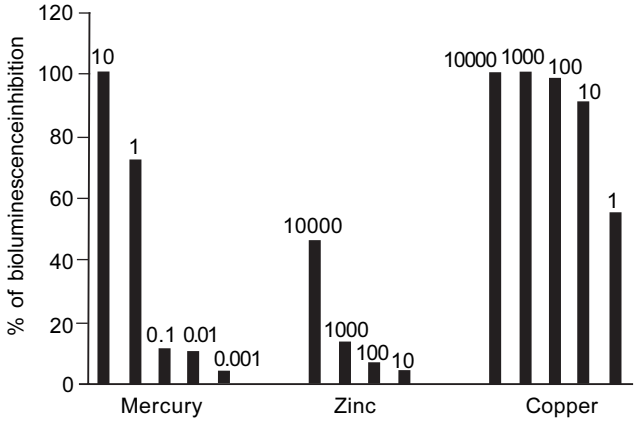

Fig. 3 Effect of heavy metals on Bioluminescence

Table 2 Effect of Mercury on Bioluminescence

Sample	Species	*Percentage inhibition of luminescence at different concentrations of mercury (ppm)				
		10	1	0.1	0.01	0.001
Distilled water	*Vibrio harveyi (MMI)*	100%	98%	82%	68%	52%
	Vibrio fischeri (HW1)	99%	98%	80%	66%	50%
Tap water	*Vibrio harveyi (MMI)*	100%	99%	87%	53%	20%
	Vibrio fischeri (PMI)	100%	99%	82%	10%	<7%

*Values shown are average of three readings

Discussion

Bacterial luminescence is dependent on the growth of the bacteria, ph of the medium, oxygen availability, and nutrients. Bioluminescence was found to decrease as the ph decreases and the medium becomes acidic. *Vibrio spp* is known to have better growth in alkaline media, and hence the growth of the bacteria will directly influence the production of bioluminescence as it is related to cell density. The expression of luminescence in many bacteria has been found to be highly dependent on cell density. That is, bacteria found living free in the ocean do not give off light, whereas luminescence is observed from bacteria that are found at high densities, such as in the confined environments of the light organs of fish or squid. This dependence on cell density can be linked to a small autoregulator, termed the *lux* autoinducer, which provides communication between cells and thus allows them to sense their own population density.[10] bioluminescent bacteria are known to be halophytes with optimum growth and luminescence in halophilic media.[11] salinity affects osmotic balance in the cell; however it is not known to directly affect the luminescence. It has been reported that lowering the salinity from 33 to 27 per thousandth or less resulted in a substantial decrease in re-establishment of bioluminescence, while increasing the salinity to 43 or 48 per thousandth resulted in a small decline.[12] the luminescence reaction is an ATP dependent reaction, which requires magnesium as a cofactor[14] for ATPase activity. However its effect on luciferase is not known.

Biosensors utilizing luminescent bacteria for the detection of pollutants in the environment can complement and supplement analytical methods by distinguishing bioavailable from inert, unavailable forms of contaminants.[15] Bacterial bioluminescence is directly proportional to the rate of metabolism. However it was observed that it was not only the lack of bacterial growth but some other factor also that caused the inhibition of luminescence in the presence of heavy metals. Bacteria are known to concentrate and excrete a variety of metal ions. There are several studies on the effect of heavy metals on the growth of different species of bacteria[16], but there are no such studies on bioluminous bacteria.[17] Mercury, copper, and cadmium have been found to significantly decrease the microbial biomass.[18,19] This agrees with the result of our study, which also reports the high toxicity of mercury and copper, as compared to zinc. The toxic effects on microbial growth were found to correlate with the decrease in luminescence, as it is dependent on cell density. Excessive heavy metal concentration in the soil has been reported to decrease the microbial population.[20] Tap water contains some minerals, which may be responsible for enhancing the luminescence as compared to distilled water, which is totally deionized. Higher percentage of inhibition in distilled water may be due to lack of other compounds in it. The utility of the biosensors in tap water was demonstrated with samples supplemented with Hg (ii). Other reports[21] on the biosensors for detecting bioavailable inorganic mercury (at a nanomolar to micromolar concentration range) in contaminated waters show the validity of such a system.

Mercury is one of the most common metal contaminants in water supply and even minute quantities can be harmful to humans. The results of this study showed good correlation between luminescence inhibition and the concentration of the metal suggesting the effectiveness of the luminescence assay protocol developed. The simplicity and reproducibility of this method makes it efective tool to monitor heavy metal toxicity.

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