FABRICATION OF BIOSENSOR FOR TOXICITY EVALUATION OF SOME HEAVY METALS AND BIOCIDES

Ehsan Binaeian[1], Ali Morad Rashidi[2],Mohammad Reza Toosi jamali[1]
[1]Engineering department, Qaemshahr branch, Islamic azad university, Qaemshahr, Iran
[2]Nanotechnology Research Center, Research Institute of Petrochemical Industry (RIPI), Tehran, Iran
(Corresponding author: ehsan.binaeian@yahoo.com), rashidiam@ripi.ir, mrtoosi@gmail.com

ABSTRACT: *In recent years the use of microbial sensors has widely applied for monitoring environmental contamination. In this study, we focus on the effects of biocides such as heavy metals, pesticides and herbicides on bioluminescent bacterium, vibrio fischeri strain DSM 7744 which is used as stable bioindicators. This method makes a correlation between the light of Vibrio fischeri and the concentration of biocides. However, the basic part of this research depends on how to optimize the best condition for maximum bioluminescence. Optimized conditions of Vibrio fischeri were stirring at 120 rpm at a incubation temperature within the range of 23 to 26°C after 24 to 48 h when solid cultures were reserved at 18°C. In this case we use the whole bacteria, Vibrio fischeri which is one of interesting bioluminescence bacteria, coupled with luminometer. In our procedure the LOD for two pesticides, Malathion and Diazinon, and two heavy metals, Mercury and Selenium is about 1ppb.*

Keywords: *Bioluminescence, Vibrio fischeri, Biosensor, Biocide*

INTRODUCTION

The natural phenomenon of bioluminescence is the emission of visible light by living organisms mediated by an enzyme-catalyzed (luciferase) reaction of molecular oxygen with a substrate (luciferin) [1,2].The use of bioluminescent bacteria as bioindicators dates back to the 1950s. There are variety examples of applications range using bioluminescence bacteria in assessment of environmental toxic components [3,4,5]. As bioluminescence bacteria are specially modified to respond to toxic concentrations of heavy metals by increasing an easily detectable signal, for example luminescence, they are very promising tools to detect bioavailable heavy metals such as Cd, As, Sb, Cr, Cu, Hg, Zn and Pb [6]. Conditions for bioluminescence of *Vibrio fischeri* in continuous culture has previously demonstrated[7].Whole organisms are used to measure the potential biological impact (toxicity) of a water or soil sample. These systems are based on the use of luminescent bacteria, *Vibrio fischeri*, to measure toxicity from environmental samples. Bacterial bioluminescence has proved to be a convenient measure of cellular metabolism and consequently, a reliable sensor for measuring the presence of toxic chemicals in aquatic samples [8].The bacterium *V. fischeri* is a well-described marine bacterium, which has a world-wide distribution and can be found preferentially in temperate and sub-tropical waters. It may grow in a free-living planktonic state or in a symbiotic relationship with certain fish and squid [5]. In *V. fischeri*, there are two substrates, luciferin, which is a reduced Flavin Mononucleotide (FMNH2) and a long chain (7-16 carbons) fatty aldehyde (RCHO), which in its natural form is believed to be a tetradecanal. An external reductant acts via flavin mono-oxygenase oxidoreductase to catalyse the reduction of Flavin Mononucleotide (FMN) to FMNH2. The reduced flavin (FMNH2) binds to the enzyme and reacts with O_2 to form a 4a-peroxy-flavin intermediate. This complex oxidizes the aldehyde to form the corresponding acid (RCOOH) and a highly stable luciferase-hydroxyflavin intermediate in its excited state, which decays slowly to its ground state emitting blue-green light with a maximum intensity at about 490 nm[9],[10].

$$FMNH_2 + RCHO + O_2 \xrightarrow{luciferase} FMN + H_2O + RCOOH + h\upsilon(490nm)$$

A biosensor is an analytical device that combines a biological sensing element with a transducer to produce a signal proportional to the analyze concentration [11]. Biosensors have been extensively applied in clinical, food and environmental areas due to the advantages of fast detection speed, high selectivity and sensitivity[12]. In this study, the contribution of bioluminescence and luminometer makes a biosensor for detection of water pollution.

MATERIALS AND METHODS

Organisms

The study was carried out at the molecular and cellular research center, Islamic Azad University, qaemshahr Branch during 2009-2011. *Vibrio fischeri* strain DSM 7744 was kindly provided by Iranian Research Organization for Science and Technology (IROST).

Pollutant

Two organophosphorus pesticides: Diazinon and Malathion, two toxic metals: Mercury (Ag^{2+}) and Selenium (Se^{4+}) are used in this experiment $HgCl_2$ and SeO_2 were prepared from MERK. Diazinon and Malathion were purchased from Parto Nar Company.

Luminosity measurement of bacteria

A Berthold detection system (SIRIUS tube luminometer) made in Germany was used to measure bioluminescence intensity.

Nutrient media

To ensure the best quality of luminescent bacteria with sustainable viability, the bacteria can be inoculated and maintained in culture medium. Although, a variety of media mixtures can be used, the following cultures medium permit maximum luminescence, growth and stability that are useful for the disclosed methods.Three basic growth media were tasted:

1. Bacto Marine Broth (DIFCO 2216) (Table 1)

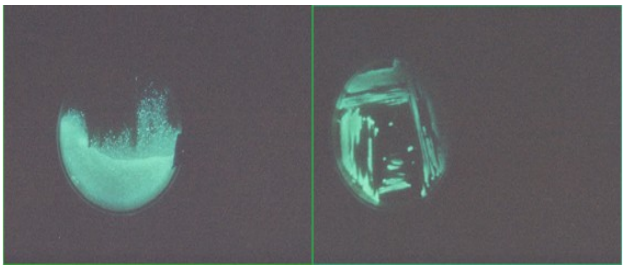

Fig.1 :Vibrio fischeri in sea water agar(Twin pack) media

Table 1: Nutrient media used for reviving bacteria (DSMZ Medium 514:BactoMarine Broth (Difco 2216))

Bacto marine broth(Difco 2216)	
Bacto peptone	5 g
Bacto yeast extract	1 g
Fe (III) citrate	0.1 g
NaCl	19.4 g
$MgCl_2$ (dried)	5.9 g
Na_2SO_4	3.2 g
$CaCl_2$	1.8 g
KCl	0.5 g
Na_2CO_3	0.1 g
$SrCl_2$	34 g
H_3BO_3	22 g
Na-silicate	4 g
NaF	2.4 g
$(NH_4)NO_3$	1.6 g
Na_2HPO_4	8 g
Distilled Water	1000 ml

Table 2 : Nutrient media used for solid cultures. Sea water agar (Twin pack)

Part A	Standard Formula (gL^{-1})
Peptic digest of animal tissue	5
Yeast extract	5
Beef extract	3
agar	15
Part B	Standard Formula (gL^{-1})
Sodium chloride	24
Potassium chloride	0.7
Magnesium chloride	5.3
Magnesium sulphate.$7H_2O$	7
Calcium chloride	0.1

2. Sea water agar (twin pack) (Table 2) (Fig. 1)
3. Sea water agar (Table 3).

The first media was used for reviving; the second one was used for solid cultures and the third one for liquid cultures.
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Optimized condition for bacteria growth

Solid cultures were reserved in incubator at 18°C. After 48 h liquid cultures were incubated at 25°C in an orbital shaker at 120 rpm after inoculation with brightly glowing *V. fischeri* solid culture [13].

Table 3 : Nutrient media used for liquid cultures (DSMZ Medium 246: Sea Water Agar)

Sea water agar	
Beef extract	10 g
peptone	10 g
agar	20 g
Tap water	250 ml
Sea water*	750 ml

	Artificial sea water
Nacl	28.13 g
Kcl	0.77 g
$CaCl_2 \times 2H_2O$	1.6 g
$MgCl_2 \times 6H_2O$	4.8 g
$NaHCO_3$	0.11g
$MgSO_4 \times 7H_2O$	3.5 g
Distilled Water	1000 ml

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Optimized condition for experiments

Decrease of 500 µL bacterial luminescence in effect of 10 µL biocide measured by luminometer after 2 min.

RESULTS

The luminescence of bacteria has long been known to be sensitive to a wide variety of toxic substances (eg., heavy metals, pesticides, etc.). for example the use of Luminescent bacteria has been discussed for the detection of toxins on solid surfaces, such as soil, and in liquid substances, such as in the analysis of waste water [14],[15].The effect of four dangerous biocides (Mercury, Selenium, Malathion and Diazinon) in potable water was tested. Maximum light intensity were reached within 48 h after inoculation.*Vibrio fischeri* liquid cultures prepared from solid cultures and stirred at 120 rpm for 48 h. Bioluminescence of 500 µL *Vibrio fischeri* was measured with luminometer when it was infected with each 10 µL of biocide.

Diazinon

Diazinon 60% at concentration of 100, 20, 10, 1, 0.01, 0.001 ppm were the samples. Bacteria light in effect of Diazinon at 0.001ppm reduced 96% in comparison of maximum bioluminescence.It can be clearly seen that the bacteria light reduced in effect of Diazinon 60% at 100 ppm (about 97%).There is not a great deal of difference between the reduction of light in effect of Diazinon at 100 and 0.001 ppm. But the most important point is an extreme reaction of *Vibrio fischeri* to Diazinon (Table 4).

Malathion

Malathion 57% at concentration of 100, 20, 10, 1, 0.01, 0.001 ppm was prepared. The reaction of *Vibrio fischeri* light to Malathion provide information that show an increase trend from 0.001 to 100 ppm, however, this increase is not sensible, but it shows a great fall in comparison of maximum bioluminescence. The percentage of light reduction between 0.001 ppm and maximum bioluminescence is about 97% (Table 5).

Selenium

Water spiked with SeO_2 to give the concentration of 100, 20, 10, 1, 0.1, 0.001 ppm Se^{4+}. Bacteria light at 0.001 ppm selenium reduced 95% in comparison of maximum bioluminescence. As an overall look the light have a decrease trend in different concentration of selenium from 0.001 to 100 ppm, but there is an increase from 0.01 to 0.1 ppm that may show the mistake in measurements (Table 6). EPA have set the limit of selenium in drinking water 0.05 ppm.

Mercury

The Environmental Protection Agency (EPA) and Food and Drug Administration (FDA) have set the limit of mercury in drinking water 2ppb.Water spiked with $HgCl_2$ to give the concentration of 100, 20, 10, 1, 0.1, 0.001 ppm Hg^{2+}.Bacteria light at 0.001 ppm mercury reduced 96% in comparison of maximum bioluminescence (Table 7).

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In tables 4 to 7 maximum bioluminescence after 48 hour is 157717 RLU/S .

DISCUSSION

The effect of metal ions and organophosphorus pesticides on luminescence can be investigated. While maximum bioluminescence occurred in 157717 the decrease of light is obvious. In all cases when we move from 100 to 0.001 ppm the bioluminescence is decreased at 0.001 ppm about 95-97% compared with maximum bioluminescence.

The obtained results revealed a dramatic decline in bioluminescence however the amount of biocide is too small and luminometer is very sensitive to measure bioluminescence. The obtained results showed that 2 min is a small time when it compared with Mass Spectroscopy (MS), High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) [15]. Also, our new method is comparable with these old methods because they are complex, time consuming, expensive and require sample pretreatment.Also the results showed that LOD for this method is about 0.001 ppm when it compares with AAS method in detection of Mercury with LOD 0.01 ppm[16] and HG-AAS method in detection of Selenium has shown LOD 0.04 ppm [17]. In addition it was the first time that the effect of biocides on whole bacteria *Vibrio fischeri* tested with luminometer, in previous methods luciferase extracted from *Vibrio fischeri* and then after preparation, the effects of biocides was evaluated. The more future experimental data on other biocides can continue and confirm these data.The data indicates that it's possible to detect the limit of biocides even more than that EPA set.

Table 4 : Effect of Diazinon on bioluminescence of *Vibrio fischeri*

Diazinon(PPM)	100	20	10	1	0.01	0.001
(RLU/S) **Light**	4362	4591	4671	5367	5713	6397.5
SD	84.85281	127.2792	169.7056	233.3452	422.8492	1009.041

SD : Standard deviation , RLU

Table 5 : Effect of Malathion on bioluminescence of *Vibrio fischeri*

Malathion(ppm)	100	20	10	1	0.01	0.001
Light (RLU/S)	3084.667	4735.5	4844	4853.5	4886	4921
SD	35.35534	38.89087	9.899495	3.535534	5.656854	49.49747

: Relative light unit

Table 6 : Effect of Selenium on bioluminescence of *Vibrio fischeri*

Selenium(ppm)	100	20	10	0.1	0.01	0.001
Light (RLU/S)	4832.5	4883	5404	5669	5388.5	7156.5
SD	16.26346	77.7817	152.7351	224.86	430.628	474.4687

Table 7 : Effect of Marcury on bioluminescence of *Vibrio fischeri*

Marcury(PPM)	100	20	10	1	0. 1	0.001
Light (RLU/S)	4617	4960.5	4874	4950	5368	5682.5
SD	4.242641	12.02082	8.485281	50.91169	499.2174	211.4249

CONCLUSIONS

Since the majority of this research depend on optimization of media culture, the media culture was selected which take a short time to prepare in a way to be applicable for a long-term, so the DSMZ MEDIA CULTURE for liquid culture is recommended and for solid culture, Sea Water Agar, is recommended. As it is mentioned before, optimization of media cultures and the temperature of bacteria growth that can be used for biological element of biosensor for detection biocides in water, so 18°C is the temperature that bacteria rows well and has the most bioluminescence light and we can see the light after 24-48 h. The experimental results showed that limit of detection (LOD) in *Vibrio fischeri* for all of the above toxic material is about 1 ppb. Because the traditional methods are not as precise as this method, the present work demonstrated the feasibility of using *Vibrio fischeri* for detecting biocides.

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