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Application of Biosensor for the Detection of Bioluminescent Microbian Hg (II) in Real Samples

Gisele S.Costa[1], Andrea Medeiros Salgado[2*], Paulo R.G. Barrocas[3]

[1]TPQB - Escola de Química - Universidade Federal do Rio de Janeiro
[2] DEB - Escola de Química - Universidade Federal do Rio de Janeiro
Av. Horácio Macedo, 2030 - Bloco E - Sala E-122 - Ilha do Fundão -Rio de Janeiro-Brasil. andrea@eq.ufrj.br
[3]ENSP/FIOCRUZ - Escola Nacional de Saúde Pública Sérgio Arouca

Escola de Química - CT - Universidade Federal do Rio de Janeiro
Mercury presents bioaccumulation and biomagnifications potential by organisms, and it is of big interest to determine its toxic component. This work aims to use the bacteria *Escherichia coli* MC1061 as a detector of bioavailable mercury, due to its capacity to emit luminescence in the presence of Hg, serving as a biosensor component. Because many species of Hg present different behavior, it is necessary to use methods to identify the bioavailable mercury species, once the bioavailability is critical when refers to the toxicity of the metal. Through an experimental plan, it was possible to obtain the variables more relevant for the cultivation of the bacterias´cells (the best environment $M9NO_3$ and time of incubation). Calibration curves were determined for the use of biosensor instrument, once the tested environmental matrices (samples of slurry from different places) were complex, these being defined from the standard additional curves of each local tested. The obtain results showed that the biosensor instrument presents high specificity to Hg(II) and good repeatability. Within the tested samples collected between September and October of 2009, the dump showed the higher fraction of bioavailable Mercury by the tested method, the other two samples (semi-controlled landfill and landfill) presented lower values of bioavailable mercury concentration.

1. Introduction

In the beginning of civilization, the man had a smaller influence on the environment, but when conquered new technologies, he increased the quality of life and also produced many impacts on the environment. The maintenance of environmental quality is an important fact for the present society and for the future generations. According to Lacerda & Malm (2008) the main issue now, approached by the environmental agencies, does not regard only the contamination of the environment but how serious this contamination is.

The mercury methylation is the key step for the Mercury cycle in aquatics systems and it is extremely important in remote and contaminated environments. Bisinoti & Jardim (2004) have demonstrated the importance of studying mercury behavior in the environment for being very complex, therefore, the understanding of its cycle is fundamental as well as the analysis of its toxic effects in the human and biota health.

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The highlight is give to Mercury, because although it occurs naturally in the environment, the indiscriminate use has been causing the increase of Mercury concentration in the environment and damaging the biota. There are interests in the determination of its toxic components, once the study of the chemical species is important for the comprehension of toxicology, therefore, a public health issue, considering the accidents involving this heavy metal in the past.

The traditional methods of detection do not allow distinguishing the pollutants that are available for the biological systems from the ones inert and unavailable, a special issue relating to toxic metals. Thereby, the bioavailability is critical in the determination of the metal toxicity. The biosensors come as an alternative for pollutant detection, presenting itself as new analytical tools to be applied in the diagnosis of environmental conditions (Tecon & Meer, 2008).

These sensors show unique characteristics: selectivity, relatively low cost of construction and storage, potential por miniaturization, ease of automation and construction of simple and portable equipments. The new detection systems, based on microbial sensors, mostly, use the luminescence response to a toxic compound present in the polluted environment (Karube & Nakanishi, 1994). The key of using these organisms genetically modified as biosensors is in the selective and specific response of the contaminated species present. This way, the produced luminescence is proportional to the concentration of the contaminant in the sample.

This work had the general goal of investigating and applying *Escherichia coli* MC1061, the microorganism genetically modified as the biological element of a biosensor (Virta et al., 1995, kindly provided by the research group in Finland) using a luminometer as a transducer for the detection of bioavailable mercury in environmental samples. These samples were tested and monitored regarding the presence of bioavailable mercury. The operating mechanism of *E. coli* MC1061 is in the reporter gene under a promoter control which is induced by intracellular presence of mercury. The slurry real samples used were from three locations that can be defined as: dump semi-controlled landfill and landfill, collected between September and October of 2009.

2. Material and Methods

2.1 Environment and samples of mercury

The environment M9Cl e $M9NO_3$ (a mixture of phosphate salts, prepared in a buffer of pH7) were used both for biosensor growth as luminometric tests (plus growth factors: 0,1M $MgSO_4.7H_2O$; 0,01M Ca $(NO_3)2.4H_2O$; 200 g/L of glucose solution, 100 g/L casamino acids). The difference between the two used environment was the elimination of chloride salts, from the original recepy, and substituting them by nitrate salts and kanamycin 30 mg/L for positive selection of transposon lux. The mercury samples were prepared from a standard solution of mercury (1g/L). The preparation of the cellular suspension used in the tests was made from a new culture (obtained after cellular growth under 150 rpm and 30ºC). The respective cell concentration was calculated from the curve of dry weight. For bioluminescent tests, mercury solutions (10 µL) were add to the wells of luminometric plate, followed by the cellular samples (50 µL), the plates were put in room temperature inside the luminometer chamber for the production of

luciferase, which must be proportional to the mercury incorporated by the bacterial cells. After this step of introducing *E. coli* cells, the measurement process was initiated with the equipment dispensing the luciferin 1mM (100µL) in the well and reading for 12 seconds.

A planning central composite design (DCCR) was used to maximize the variable response, in other words, to obtain the more relevant variables for the cultivation of bacterial cell (the best environment $M9NO_3$ and incubation time). The analysis of the results were made through calculating the estimated effect, standard error and the Student distribution of each control variable under the response variable using the Statistica (version 6.0) program. The independent variables studied were cell concentration and mercury concentration. All experiments were made in a random way with three central points.

In this study, it has worked with complex samples, such as slurry. Therefore, it was necessary the construction of a calibration curve, due to the complexity of the samples, to use with them, with the standard addition where different aliquots of mercury were add in the real analyzed samples themselves, according to different ranges of mercury concentration.

Complementary analysis of pH were made, Chemical Oxygen demand (COD) of the samples by colorimetric method of closed reflux standard (Hach), with $K_2Cr_2O_7$ in acid environment, containing Ag_2SO_4 as catalyst and $HgSO_4$ to eliminate the interference of the chlorets present in the samples (APHA, 1992), and, Oxygen Biochemical Demand (BOD) through incubation method (with dilution), the oxygen was measured by the Analytical Digital Method (APHA, 2005).

The luminometer was used to measure luminescence emissions in the plates where the samples are conditioned, and can operate in low valus of concentration. This equipment was used for all luminometric tests and the unit of light detection is measured in relative light unit (RLU).

3. Results and Discussion

The growth profile of the microorganism *Escherichia coli* MC1061 was observed to obtain the cellular density and determination of the physiological state of cells. This showed in the tested conditions (both means tested M9CL and $M9NO_3$, 150 rpm and 30°C) an exponential phase after about seven hours, andreach the stationary phase were needed around eleven o'clock.

3.1 Experimental Design

By experimental design can determine the variables most relevant to the cell culture and testing luminometric. Only the culture medium $M9NO_3$ showed significant results in time from 20 to 45 minutes. The variable of significance for the model was the concentration of mercury (linear term) in two periods: 20 and 45 minutes. By analyzing the coefficient of determination (R^2) adjusted for $M9NO_3$ in 20 minutes was 51.30% while in this time of 45 minutes was 93.96%, is important to note that the incubation time (20 minutes) may not have been sufficient in the production of luciferase, which does not reflect fully the level of intracellular Hg (II). For this reason, the mean $M9NO_3$

in time of 45 minutes of incubation in the chamber luminometric best fits the model, differently from that of Barrocas (2004).

3.2 Real samples

In the analysis of the contents of bioavailable mercury in real samples of slurry, a complex sample, we took into account the possible matrix effects that could cause these environmental samples, mainly due to the natural ligands present. Thus were the standard addition curves for each leachate, in order to obtain a calibration curve that could be better comparable to the system. After the first trials to determine the calibration curve, the selected track where he obtained a linear region in the data was 1.00 E-7 to 4.00 E-6 mg/L. The values found for the standard deviations indicated that the test was performed with good repeatability. And this occurs even found higher values of coefficient of variation (1.35 and 3.60) in the range of $1x10^{-7}$ to $1x10^{-6}$ mg/ L of mercury, respectively. The best calibration curve for the biosensor instrument, applied to leachate detection in complex matrices has been defined starting from the standard addition curves of each location, directly contaminated with the analyte in question (Figure 1). The curves of standard addition were important for the analysis of quantification of bioavailable mercury in the samples.

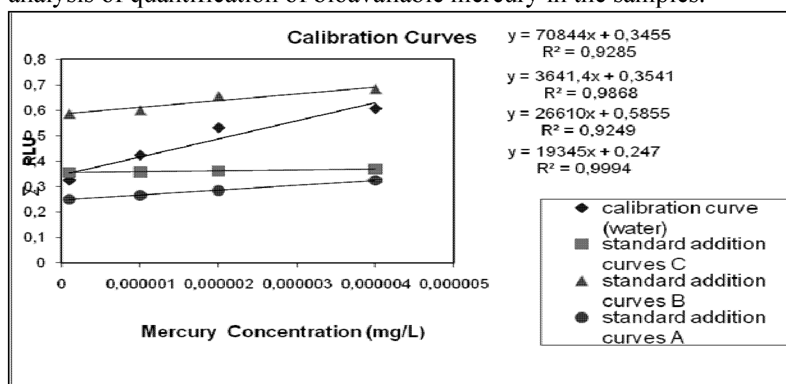

Figure 1: Graph of the calibration curve and standard addition curves for mercury in different concentrations ($1x10^{-7}$ to $1x10^{-6}$ mg/L of mercury).

3.3 Determination of the Concentration of Bioavailable Mercury

Based on the standard addition curves we determined the concentration of bioavailable mercury in samples from three locations, using the equations of the line and the emission of luminescence found in each locality. This is because the calibration curve prepared with deionized water, with the highest values of RLU largest, high angular coefficient of 70,890 (Figure 1) did not show any complexing agent in water to mitigate the emission of luminescence, all bioavailable mercury is seen by the microorganism. Analyzing the curves of standard addition can be seen that the curve of sample B, the landfill, had the highest slope of the line (26 610) when compared to other standard addition curves, indicating that the sample has less matrix effect, inferring that the samples from this location are less complexing agents. While the sample of leachate C, the semi-controlled landfill, it was noticed the flattening of the curve (3641.4) almost no

slope, supporting the idea that this sample has the highest matrix effect. A sample of the leachate A, landfilling was intermediate between the other two curves, standard addition, this curve still showed a better R^2 value (0.9994) and lower residue in the equation of the line (0,247), meaning that the experimental values were more accurate in the test sample A.
However it can be seen that all curves showed a standard addition $R^2 > 0.9$ and, based on these results, we considered the equations of the line curves to accomplish the calculations for determination of mercury in their sample of slurry. Bioavailable mercury concentrations calculated from the equations of the line standard addition curves of the landfill, landfill and semi-controlled landfill are found in Tables 1.

Table 1: Concentrations of bioavailable mercury in the collection sites.

	Average RLU	Concentration of bioavailable mercury (mg/L)
A_1_1	0,315025	172,29 x10^{-7}
A_1_2	0,2452	12,624 x10^{-7}
A_1_3	0,31405	170,66 x10^{-7}
A_1_4	0,24385	9,5257 x10^{-7}
	Average RLU	**Concentration of bioavailable mercury (mg/L)**
B_1_1	1,526825	1768,74 x10^{-7}
B_1_2	0,556125	551,95 x10^{-7}
B_1_3	1,93475	2535,23 x10^{-7}
B_1_4	0,885675	5640,27 x10^{-7}
	Average RLU	**Concentration of bioavailable mercury (mg/L)**
C_1_1	0,44155	120077 x10^{-7}
C_1_2	0,37035	2231,28 x10^{-7}
C_1_3	0,324325	4088,4 x10^{-7}
C_1_4	0,35505	130,44 x10^{-7}

A series of questions can be raised from the values found in these locations (all values were below the values allowed by CONAMA Resolution 357). The sampling sites may have in your array complexing agents, so the organism would not be seeing all mercury bioavailable (sample C showed a greater matrix effect among the three curves of standard addition). Or, these sites could not receive potentially toxic wastes (heavy metals, for example) or are sites that use the separation of possibly toxic waste (which should be done at least in landfills).
And the analysis of BOD and COD have allowed us to infer the geochemical bioavailable fraction of mercury, since it can be considered that mercury has an affinity with organic matter. From these, one can infer that the samples were derived from a leachate which had already gone through the acidic (low pH and high COD). The BOD values found for the places with new landfill leachate are in the range de15.000 - 50,000 mg/L (Segato & Silva, 2000). Thus, one can say that the values found in this study were relatively low and can infer that the influence of organic matter was not very sharp, even to obtain a larger matrix effect in the sample C.

The monitoring of metal levels in these environmental samples is an important tool for environmental management. Aiming to maintain or improve quality of life. The lack of control and treatment of leachate generated in systems of solid waste disposal promotes contamination of soil, air, surface water and groundwater, besides enabling the proliferation of disease vectors, to the detriment of the quality of the environment and public health.

4. Conclusion

The results obtained by biosensor instrument showed high specificity for Hg (II) and good repeatability. Within the sampled locations, the dump was the one which showed higher fraction of bioavailable mercury in the tested method, the other two locations showed lower values of bioavailable mercury concentration. The analysis related to the phisical-chemical characteristics of the samples showed us that referred to places where the landfill had already passed the acid phase, it was old slurry. This way, bioluminescence showed to be a sensitive technique with high potential of application for detection of bioavailable mercury, which is proved in the results of this study.

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