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Research Article
Detoxification Assessment of Inorganic Mercury by Bioluminescence of *Vibrio fischeri*

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Abstract

Background and Objective: Heavy metals showed an important environmental problem today. They can be toxic when they are present in excess. Their dangerousness is not only their ubiquitous presence, but also the fact that they exist in several chemical forms. Among the main metal extraction is mercury, its extraction has modified the proportion and the chemical form of this metal in various compartments of ecosystems making it bioavailable and potentially harmful for organisms especially human being. This study evaluated the toxicity of this metal and a bioluminescence method has been optimized. **Methodology:** Bioluminescence method consisted to optimize the intensity and stability of luminescence emitted by bacteria *Vibrio fischeri* (*V. fischeri*) strain NRRL-B-11177 cultivated in a modified LBS medium. Through this method, toxic effects of inorganic mercury ($HgCl_2$) on *V. fischeri* have been determined. All data were analyzed using GraphPad Prism and values were obtained as means±standard errors. **Results:** Results showed aeration (600 mL min^{-1}) and agitation (250 rpm) were essential to *V. fischeri* to emit light. It has found that at concentration 1.4 mg L^{-1}, $HgCl_2$ was capable to inhibit totally the light emitted by *V. fischeri*. **Conclusion:** Thereafter, evaluated the detoxification of $HgCl_2$ in fluidized bed reactor using immobilized and free bacteria *Escherichia coli* strain R100 by the luminescence of *V. fischeri*.

Key words: *Vibrio fischeri*, *Escherichia coli*, mercury, bioluminescence, ecotoxicology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heavy metals constitute an important current environmental problem[1]. Trace metals elements are elements whose concentration in the earth's crust is less than 1 g kg^{-1} or heavy metals (metals which have densities greater than or equal to 4.5 g cm^{-3}) may be toxic when they present in excess in the environment [2,3]. Their dangerousness was not only their ubiquitous presence, but also the fact that they existed in several chemical forms[4]. Each of these forms had unique characteristics which could modify their solubility or give them the power to pass through the biological membranes[5-7]. Environment contamination by heavy metals came from their many uses in anthropogenic activities, including industry. For these activities, metals naturally present in the earth's crust, were extracted in large quantities. This extraction has altered the proportion and chemical form of these metals in various compartments of ecosystems, thus making them bioavailable and potentially harmful to organisms. Toxicity measurement of toxicants such as heavy metals is a most important part to evaluate environmental pollution. In order to assess their toxicities several tools and methodologies were available to ecotoxicologists. Among these tools and methods, tests carried out on living organisms, such as algae, crustaceans, fish and bacteria, generally these methods' aim was to determine the inhibition of an organism[8]. In case of bacteria several types of tests could be considered on isolated bacterial populations. Toxic effects were studied from bacterial growth, bacterial enzymatic activity, adenosine triphosphate content or inhibition of bioluminescence[9-12]. Therefore, to evaluate the pollution, it was preferable to use a simple, rapid, sensitive and cost effective such as bioluminescence inhibition method could indicate specific information on the toxicity of heavy metals[13]. The objective of this study was to find the best conditions of intensity and stability of luminescence of *Vibrio fischeri* (*V. fischeri*) and to assess the detoxification of Inorganic mercury ($HgCl_2$)in fluidized bed reactor.

MATERIALS AND METHODS

This study carried out from March, 2016-April, 2017 at Laboratory of Microbiology, Pharmacology, Biotechnology and Environment, Faculty of Sciences Aïn Chock, University Hassan II.

Bacterial strains: *Vibrio fischeri* (*V. fischeri*) strain NRRL-B-11177 and *Escherichia coli* (*E. coli*) strain R100.

Bacterial growth media: *V. fischeri* strain was grown at 25°C in a modified Luria-Bertani salt (LBS) medium (10 g nutrient broth, 5 g yeast extract, 20 g NaCl, 50 mL 1 M Tris buffer, pH 7.5±0.02, in 1 L of distilled water).

Escherichia coli was grown at 37°C in broth medium (2% w/v nutrient broth in distilled water) and in minimal medium (MM) (1.36 g of potassium hydrogen phosphate (k_2HPO_4), 0.10 g of magnesium sulfate ($MgSO_4$), 0.60 g of ammonium sulfate ($[NH_4]_2SO_4$), 0.02 g of calcium chloride ($CaCl_2$) and 0.50 g of sodium chloride ($NaCl_2$), 220 µL of manganese (II) sulfate ($MnSO_4$), 40 µL zinc sulfate ($ZnSO_4$), 40 µL of copper (II) sulfate ($CuSO_4.5H_2O$) and 28 µL of iron (II) sulfate ($FeSO_4$), in 1 L of distilled water). All products were purchased from Biokar Diagnostics.

Chemicals and samples: 1 mM of $HgCl_2$ suspension was prepared in 2% NaCl (pH7.5±0.02). Serial dilutions (1:1) for $HgCl_2$ were directly prepared in 2% NaCl (pH7.5±0.02). Concentrations of $HgCl_2$ tested were 13.55, 6.78, 3.39, 2, 1.70, 1.4, 1, 0.85, 0.5, 0.42, 0.21 and 0.11 mg L^{-1}. The 2% NaCl (pH7.5±0.02) served as a control and diluent solution for all concentrations.

Luminometer: Luminescence of marine bacterium *V. fischeri* was performed using a 96-well microplate Luminoskan Ascent Luminometer (LAL) Thermo, manipulated by Ascent software version 2.6 ThermoLab systems. The bacteria growth was determined using spectrophotometer (UV-Visible) at the optical density of 600 nm ($OD_{600\ nm}$). The inhibition of luminescence intensity of *V. fischeri* caused by toxicants and was determined as follows:

$$\text{Light inhibition (\%)} = \frac{IT_n}{KF \times IT_0} \times 100\text{-}100$$

$$KF = \frac{IC_n}{IC_0}$$

Where:

- KF = Correction factor
- IC_n = Luminescence intensity of control after contact time (n time) (mV)
- IC_0 = Initial luminescence intensity of control sample (mV)
- IT_n = Luminescence intensity of test sample after contact time (n time) (mV)
- IT_0 = Initial luminescence intensity of the test sample (mV)[14]

Intensity and stability of luminescence of marine bacterium *V. fischeri*

Incubation of *V. fischeri* in LAL: Freeze-dried luminescent bacteria *V. fischeri* were stabilized first at 4°C for 30 min, then inoculated in 5 mL of a modified LBS medium and incubated at 25°C for 24 h. Cultures were harvested when they grew and 0.05 mL were inoculated into a fresh modified LBS medium (5 mL) and incubated at 25°C for 2 h. Prior to transfer bacterial suspension into microplates, 0.1 mL of 2% NaCl (pH7.5±0.02) was pipetted into well in duplicate and acclimated at 25°C for 30 min, which was supplemented with 0.1 mL of bacterial suspension by automatic dispensing in LAL testing chamber. The bioluminescence was recorded during the first 2 sec at 25°C after the microplate was automatically shacked, then incubated at the same temperature for 51 h. Each 60 min the bioluminescence was recorded again after shaking.

Incubation of *V. fischeri* with and without aeration: Luminescent bacteria *V. fischeri* were suspended in a modified LBS medium then aliquots of bacterial suspension (0.1 mL) were transferred into a fresh modified LBS medium (10 mL), incubated at 25°C and stirred (250 rpm). A series of samples were taken during incubations and immediately measured. The bioluminescence was measured by LAL and the bacteria growth by spectrophotometer at $OD_{600\ nm}$. For bioluminescence measurements, 0.1 mL of 2% NaCl (pH 7.5±0.02) was added into well duplicate and acclimated at 25°C for 30 min and then supplemented with 0.1 mL of bacterial suspension manually and measured. Prior each measurement, samples were automatically shaken for 10 sec in LAL. The same procedure was performed with aeration by supplementing air (600 mL min^{-1}).

Assessment of $HgCl_2$ toxicity: $HgCl_2$ suspension of 1 mM was prepared containing 2% NaCl (pH7.5±0.02). Serial dilutions were prepared as previously described and diluents were placed at 25°C for 30 min 2% NaCl (pH7.5 ±0.02) served as a control. Prior to toxicity testing luminescent bacteria *V. fischeri* were reconstituted in a modified LBS medium, aliquots of bacterial suspension (0.1 mL) were transferred into a fresh modified LBS medium (10 mL), incubated at 25°C, stirred (250 rpm) and supplemented air (600 mL min^{-1}) for 22 h 0.1 mL of each concentration was added into different wells in duplicate and was supplemented with 0.1 mL of bacterial suspension by automatic dispensing in the LAL testing chamber, followed by incubation at 25°C for 15 min. The bioluminescence was recorded after microplate was automatically shacked. The signal of the light was measured after 15 min in duplicate.

Volatilization of $HgCl_2$ in fluidized bed reactor using immobilized and free bacteria *E. coli*

Stimulation of the bacteria: Fresh bacteria suspension of 5 mL *E. coli* were transferred in 500 mL of nutrient broth medium containing 1 mM of $HgCl_2$ and incubated at 37°C with agitation (100 rpm) for 72 h, to induce the mercuric-reductase production. This enzyme reduce (Hg^{2+}) to volatile mercury (Hg^0). After 72 h, Cultures were centrifuged at 8600 rpm for 30 min using a centrifuge (SIGMA 2-16PK Heated and Refrigerated Centrifuge).

Fluidized bed reactor set: Two bottles flasks form the fluidized bed reactor, the first one contains 500 mL of minimal medium,10 g of glucose and 1 mM of $HgCl_2$, was incubated at 37°C and inoculated by 0.4 g of *E. coli*. The mercury vapors obtained after mercury reduction by the bacteria were evacuated by current air using an air compressor to a mercury trap flask containing 500 mL of oxidizing solution potassium permanganate ($KMnO_4$, 0.5 mM). The color of $KMnO_4$ changed from purple to orange, this indicate successful detoxification. A series of samples were taken from both bottles during the procedure to evaluate the reduction of $HgCl_2$. At different time samples were taken from bioreactor, centrifuged at 3000 rpm for 15 min and supernatant were measured using luminescence of *V. fischeri* to evaluate the reduction of $HgCl_2$. Samples from the oxidizing solution were measured using Spectrophotometer at 530 nm ($OD_{530\ nm}$) to follow the discoloration of $KMnO_4$. The same procedure was performed using immobilized bacteria *E. coli* until 116 h.

Immobilization of bacteria *E. coli*: The 100 mL sterile sodium alginate solution (2% w/v) was mixed with 0.832 g of bacteria biomass until homogenous. The mixture was extruded through a needle (2 mm i.d.) into 150 mM $CaCl_2$ solution at room temperature for 30 min and rinsed with 50 mM Tris- HCl buffer pH 7.5[15].

Statistical analysis: All data were analyzed using GraphPad Prism (GraphPad Software version 6.0), values are means±standard errors. All experiments were done in triplicate.

RESULTS AND DISCUSSION

The aim of this study was to optimize the intensity and stability of luminescence emitted by bacteria *V. fischeri* cultivated in a modified LBS medium prior to assess the detoxification of $HgCl_2$ which was detoxified by free and immobilized bacteria *E. coli* in fluidized bed reactor. The intensity of luminescence of *V. fischeri* while incubated in a water bath without aeration at 25°C and stirred (250 rpm) was less higher. Therefore, the intensity of luminescence of *V. fischeri* while incubated within LAL instrument was higher. Thereafter, the intensity of luminescence of *V. fischeri* while incubated in a water bath with aeration (600 mL m^{-1}) at 25°C and stirred (250 rpm) was the highest one compared to other two cases. It was found that free bacteria *E. coli* detoxified more rapidly $HgCl_2$ than the immobilized bacteria.

Intensity and stability for luminescence emission of marine bacterium *V. fischeri*: The best conditions for intensity and stability of luminescence emission and growth of the *V. fischeri* were determined in a modified LBS medium prior to assess $HgCl_2$ toxicity. The modified medium used in this study was different from other LBS medium published[16-22], thus 10 g of bacto tryptone was replaced by 10 g of nutrient broth. This modified medium provides also a high intensity of luminescence and strong growth as well as LBS medium does. During the incubation of *V. fischeri* in LAL instrument at 25°C for 51 h in aerobic conditions, it was found that *V. fischeri* started to emit light after 8 h of growth and the intensity of luminescence continued to rise (Fig. 1). The highest value (2540.2 mV) of the intensity of luminescence was at 34 h, then it decreased. During the incubation of *V. fischeri* in a water bath without aeration at 25°C and stirred (250 rpm). It was found that the intensity of luminescence rose with cell growth until it reached a peak (284.7 mV) at 50 h, then it dropped as the cell went into stationary phase (Fig. 2). During the incubation of *V. fischeri* in a water bath with aeration (600 mL min^{-1}) at 25°C and stirred (250 rpm). It was found that the emission of luminescence started just 7 h of aeration. The intensity of luminescence rose with cell growth until it reached a peak (4372.5 mV) at 25 h, the corresponding cell growth was $OD_{600\,nm}$ (1.8). Then the luminescence dropped as the cell went into stationary phase (Fig. 3). The intensity of luminescence of *V. fischeri* while incubated within LAL instrument was higher. Therefore, the intensity of luminescence of *V. fischeri* while incubated in a water bath without aeration at 25°C and stirred (250 rpm) was less higher. The intensity of luminescence of *V. fischeri* while incubated

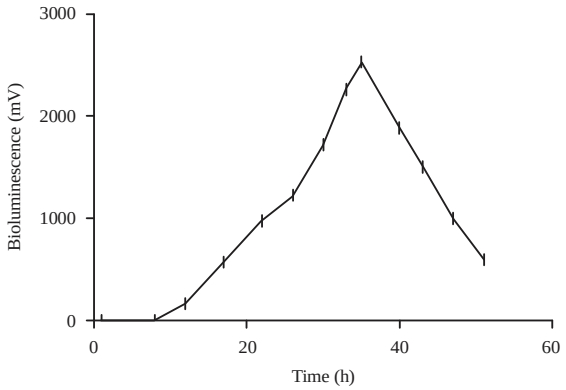

Fig. 1: Luminescence intensity of *V. fischeri* versus time (h) during incubation within 96-well microplate LAL instrument

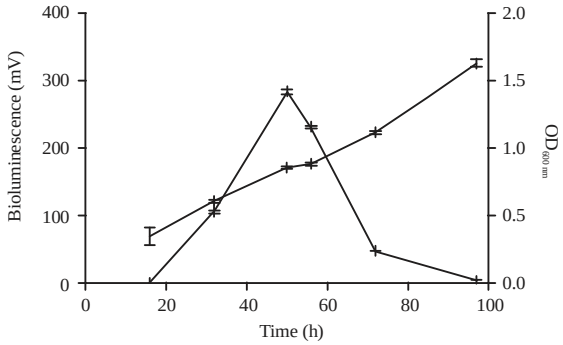

Fig. 2: Growth and luminescence intensity of *V. fischeri* in a modified LBS medium incubated without aeration at 25°C and stirred (250 rpm) for 97 h

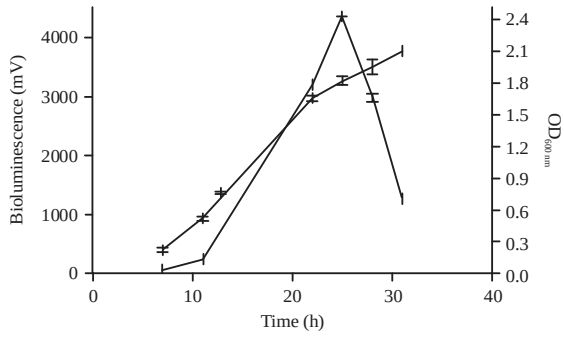

Fig. 3: Growth and luminescence intensity of *V. fischeri* in a modified LBS medium incubated with aeration (600 mL min^{-1}) at 25°C and stirred (250 rpm) for 30 h

in a water bath with aeration (600 mL m^{-1}) at 25°C and stirred (250 rpm) was the highest one compared to other two cases. It was found that the bacteria began to emit luminescence at almost same time with different intensity in two cases. Indeed the maximum intensity of luminescence during aeration was

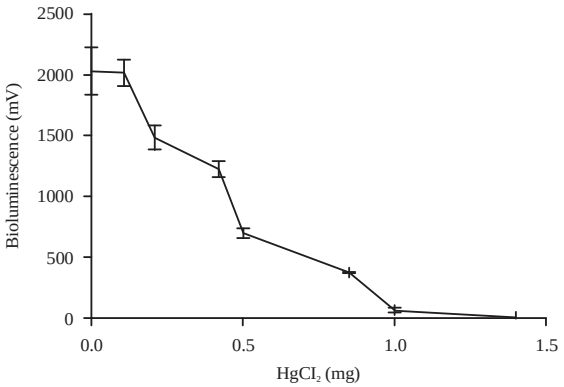

Fig. 4: Measurement of dilutions of 1 mM $HgCl_2$ using luminescence of *V. fischeri* which has grown in a modified LBS medium incubated in a water bath with aeration (600 mL min^{-1}) at 25°C and stirred (250 rpm)
2% NaCl (pH7.5 ±0.02) served as a control and diluent

much higher (4372.5 mV) than that incubated in the instrument (2540.2 mV). In previous researches, the best conditions for intensity and stability of luminescence and growth were obtained when *V. fischeri* were grown in a media at pH (7.2-7.8)[23-25], best temperatures between (20-25°C)[26,27], strong aeration and stirring between (300-400 rpm)[24]. Therefore, in this study *V. fischeri* were cultivated in a modified LBS medium, containing 10 g nutrient broth, 5 g yeast extract, 2% NaCl, 50 mL 1 M Tris buffer, pH 7.5±0.02, temperate at 25°C, aerated (600 mL min^{-1}) and stirred (250 rpm), was in accordance with previous researches. In this best conditions of luminescence emission, the intensity of luminescence increases with cell growth. Thus the best time of toxicity assessment by luminescence of *V. fischeri* is between 16-22 h.

Assessment of $HgCl_2$ toxicity: *V. fischeri* were cultivated in a modified LBS medium and were successfully applied to assess the toxicity of $HgCl_2$. Concentrations of $HgCl_2$ tested were 13.55, 6.78, 3.39, 2, 1.70, 1.4, 1, 0.85, 0.5, 0 .42, 0.21 and 0.11 mg L^{-1}. The 2% NaCl (pH7.5±0.02) served as a control and diluent solution for all concentrations. The control and the concentration (0.11 mg L^{-1}) of $HgCl_2$ were clearly similar which showed that this concentration was not sufficient to inhibit the light emitted by *V. fischeri* consequently it was not toxic to this strain. Therefore, six concentrations (13.55, 6.78, 3.39, 2, 1.70, 1.4 mg L^{-1}) of $HgCl_2$ were extremely toxic, similarly capable to inhibit the luminescence totally. The concentration 0.42 mg L^{-1} presented moderate toxic. Thereafter, inhibition of bioluminescence was closely related to concentrations of $HgCl_2$. Greater concentration induce greater inhibition of luminescence, at the concentration 0.11 mg L^{-1} of $HgCl_2$ there was no inhibition but from this concentration the light began to decrease, at the concentration 1.4 mg L^{-1} of $HgCl_2$ inhibition of the light was almost total. The correlation between bioluminescence and concentrations of $HgCl_2$ was very significant (0.99). Thus, bioluminescence was inversely proportional to the different concentrations of $HgCl_2$ (Fig. 4).

Evaluation of volatilization of $HgCl_2$ in fluidized bed reactor: A series of samples were taken from both bottles during fluidized bed reactor procedure using immobilized and free bacteria *E. coli* to evaluate the degradation of $HgCl_2$. Samples from bottle one are centrifuged at 3000 rpm for 15 min and supernatants were measured using luminescence of *V. fischeri* to evaluate the degradation of $HgCl_2$. Samples from bottle two were measured using a spectrophotometer at $OD_{530\ nm}$ to evaluate the discoloration of $KMnO_4$. The same procedure was performed using immobilized bacteria *E. coli* until 116 h. Free and immobilized bacteria *E. coli* have been used to detoxicate $HgCl_2$, it was found that the bacteria began progressively to degrade $HgCl_2$. In the case of free bacteria, 14 h and 30 min of the degradation, the quantity of $HgCl_2$ remained was not enough to inhibit the luminescence of *V. fischeri*. Thus, *E. coli* reduced the quantity of $HgCl_2$ from 13.55 mg L^{-1} to nearly 0.42 mg L^{-1}. because 0.42 mg L^{-1} was moderately toxic to *V. fischeri*, thus it was not enough to inhibit the luminescence (Fig. 5a). In immobilized case, it was found that after 23 h and 30 min of detoxification, the quantity of $HgCl_2$ remained was moderately toxic to *V. fischeri*, thus the complete detoxification was after 108 h and 30 min (Fig. 5b). The discoloration of $KMnO_4$ was evaluated using spectrophotometer at $OD_{530\ nm}$, it was proportional in both cases. The color changed from purple to orange, this indicate successful detoxification. In the case of free bacteria, after 14 h and 30 min of detoxification $OD_{530\ nm}$ was reduced from 0.78 to nearly 0.70. In the Immobilized case, after 23 h and 30 min of detoxification$OD_{530\ nm}$ was 0.68 (Fig. 6a and b). Therefore, the discoloration was perfectly proportional to the detoxification of $HgCl_2$. Free *E. coli* bacteria is more rapid than immobilized one in detoxification of $HgCl_2$. It was concluded that luminescence of *V. fischeri* which cultivated in a modified LBS medium is simple, rapid, sensitive and cost effective, can be used to evaluate toxicants.

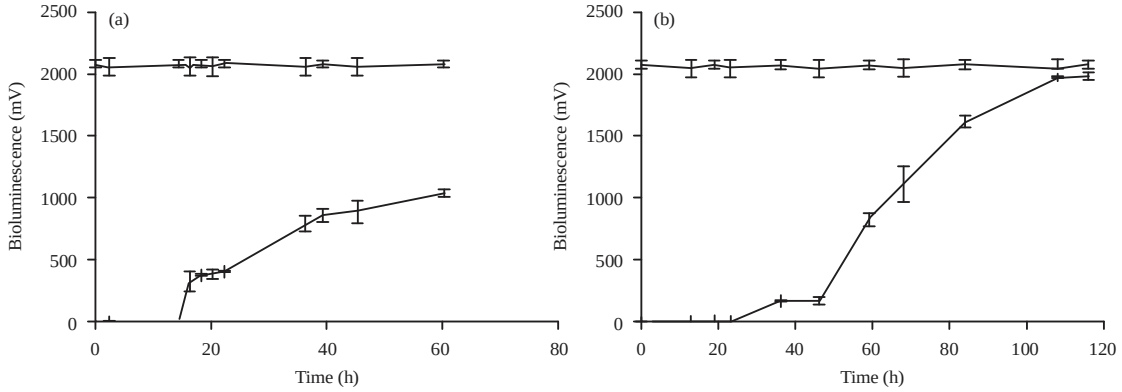

Fig. 5(a-b): Kinetics of luminescence of *V. fischeri* exposed to suspensions of $HgCl_2$ degraded by (a) free and (b) Immobilized bacteria *E. coli*, measured in a 96-well microplate LAL instrument. 2% NaCl served as control

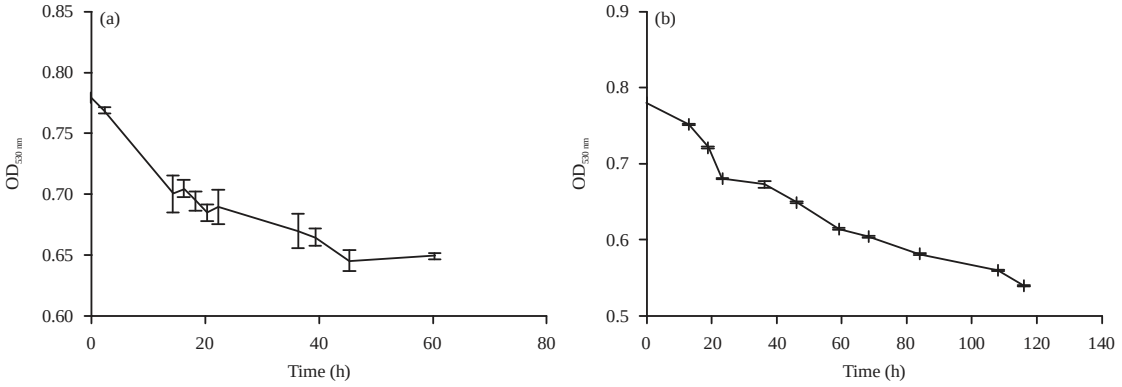

Fig. 6(a-b): Curves (a) Free and (b) Immobilized bacteria *E. coli* represent discoloration of $KMnO_4$ by gaseous monatomic mercury (Hg^0). Measured using spectrophotometer at $OD_{530\ nm}$

CONCLUSION

According to the different results obtained during this study, culture of *V. fischeri* in a modified LBS medium containing 10 g nutrient broth, 5 g yeast extract, 20 g NaCl, 50 mL 1 M Tris buffer, pH 7.5±0.02, temperated at 25°C, aerated (600 mL min^{-1}) and stirred (250 rpm), was the best conditions for intensity and stability of luminescence emission. It was found that, the intensity of luminescence increases with cell growth but the bacteria must be at a certain critical threshold of its growth OD (1-1.8) beyond this threshold the intensity of light decreases. The best time of toxicity assessment by luminescence of *V. fischeri* is between 16-22 h of incubation. $HgCl_2$ is extremely toxic to *V. fischeri*, indeed at a very low concentrations capable to inhibit totally the light emitted by this strain. Thus, inhibition of bioluminescence is closely related to concentrations of $HgCl_2$. Greater concentration induce greater inhibition of light, at the concentration (0.11 mg L^{-1}) of $HgCl_2$ there was no inhibition of light but from this concentration the light began to decrease. The concentration (0.42 mg L^{-1}) of $HgCl_2$ was moderately inhibited the light. In the case of the concentration (1.4 mg L^{-1}) of $HgCl_2$, the inhibition light was total. The correlation between bioluminescence and concentrations of $HgCl_2$ was very significant (0.99). Thus, bioluminescence is inversely proportional to the different concentrations of $HgCl_2$. Thereafter, the discoloration is perfectly proportional to the detoxification of $HgCl_2$. Free *E. coli* bacteria is more rapid than immobilized one in detoxification of $HgCl_2$. It was concluded that luminescence of *V. fischeri* which cultivated in a modified LBS medium is a simple, rapid, sensitive and cost effective, can be used to evaluate toxicants.

SIGNIFICANCE STATEMENTS

This study discovers the possible utilization of bioluminescence of *V. fischeri* to assess the detoxification of $HgCl_2$ that can be beneficial for toxicity evaluation of the metal. This study will help

researchers to uncover the critical areas of toxicology and ecotoxicology. Thus, a new, simple, rapid, sensitive and cost effective method on the assessment of toxicants, may be arrived at.

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