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P-benzoquinone-mediated amperometric biosensor developed with *Psychrobacter* sp. for toxicity testing of heavy metals

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ABSTRACT

A rapid and reliable *p*-benzoquinone-mediated amperometric biosensor (ToxTell) incorporated with *Psychrobacter* sp. to detect toxicities of heavy metal ions has been developed. This ToxTell biosensor relied on the real-time monitoring of inhibition effect for metabolism by toxicant to provide early detection and assessment of the degree of toxicity to living cells. The effect of growth phase on the sensitivity of *Psychrobacter* sp. biosensor was studied. The results showed that at the middle of the logarithmic phase or transition from logarithmic to stationary phase, the *Psychrobacter* sp. ToxTell biosensor had a higher sensitivity to toxicants. The effects of pH, salinity in respiratory substrates and incubation time on the performance of *Psychrobacter* sp. biosensor were also investigated. EC_{50} values of Cu^{2+}, Cd^{2+}, Zn^{2+}, Cr^{6+}, Hg^{2+} and Pb^{2+} to *Psychrobacter* sp. determined at incubation time 30 min were 2.6 mg/L, 47.3 mg/L, 10.9 mg/L, 14.0 mg/L, 0.8 mg/L and 110.1 mg/L, respectively. The ToxTell microbial biosensor developed in this work demonstrated excellent storage stability for more than 60 days. The biosensor could incorporate different microbial species as biocomponent to reflect the comprehensive values for toxicants in real samples and the results therefore had high degree of validity.

1. Introduction

Heavy metals are extensively used in several industries such as, mining, metallurgical, electronic, electroplating, and metal finishing. The presence of metal ions in industrial effluents is extremely undesirable, as they present a serious threat to human health, even in trace quantities, due to their high non-biodegradability, accumulation in living organisms causing various diseases and genetic disorders, and deleterious ecological effects (Gammoudi et al., 2010). Although many chemical/physical methods are currently employed for the monitoring of heavy metals, the effects of heavy metals in environment, including synergistic and antagonistic effects, can only be inadequately estimated on the basis of the analyzed substance's concentrations (Tzoris et al., 2005). These characteristics can be assessed, however, through bioassay.

Many biological methods for monitoring heavy metals have been developed which monitor the physiological response of living organisms such as fish, daphnia, algae, plant tissue, animal cells, and several bacterial species (Zhu et al., 2011; Nguyen-Ngoc et al., 2009; Xiong et al., 2010; Liu et al., 2007; Wang et al., 2010; Hassan et al., 2010). However, the monitoring of pollutants by higher organisms does not have sufficient sensitivity, the tests are expensive, and they require a long time for detection (Hsieh et al., 2004). Microbial toxicity tests depend on bacteria and other microorganisms that have similar physiological responses as higher microorganisms are viewed as desirable for a number of reasons. These include ethical responsibilities, particularly in comparison to toxicity assays using vertebrates, as well as the relative simplicity, speed, low cost and flexibility of the assays (Catterall et al., 2010).

Many efforts have been initiated to establish cell-based system for toxicity evaluation and detection. Microtox® based on the bioluminescence fading in the presence of toxic materials, is the most widely used method for toxicity assessment (Hsieh et al., 2004; Pasco et al., 2008; Tizzard et al., 2004; Yong et al., 2011). However, some disadvantages and particular characteristics must be mentioned. Luminescence tests depending on optical detection are restricted by cell populations strictly, and are not suitable for samples of high turbidity, which would cause the fluorescence scattering. Furthermore, the luminous bacteria (*Vibrio fischeri*, a marine bacterium) must work in 3% saline solution, which is not always relevant to the environment being monitored, such as freshwater (Liu et al., 2009). Microtox® is also limited by its sensitivity to redox reactions, electrical conductivity, and alkalinity (Tizzard et al., 2004). In order to complement the defects of Microtox® test, microorganisms combined amperometric tests have been developed (Tizzard et al., 2004; Zhao et al., 2007; Shitanda et al., 2009). It uses a working electrode poised at a fixed potential against a reference electrode with current flow being measured, and the analyte detected at the working electrode may be a naturally occurring chemical species in the monitoring environment, for example oxygen, or an introduced chemical, typically a mediator, which can exist in both the oxidized and reduced forms.

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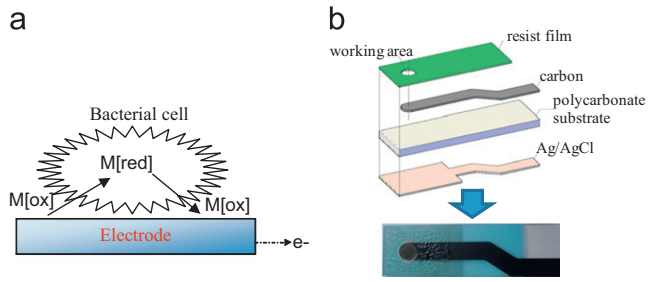

Fig. 1. The electron transfer events of redox mediator between bacterial cell and the surface of biosensor electrode (a): M[ox], oxidized mediator; M[red], and the structure of a screen-printed biosensor electrode (b).

Recently, a mediated amperometric microbial biosensor 'ToxTell' has been developed in our lab. One major advantage of ToxTell bioassay is its ability to utilize different microbial species as the biocomponent, which is unlike the MicroTox® test. As different species of microorganisms had their own metabolic response to toxicant, single microorganism could hardly reflect the true and comprehensive values for the toxicant in real samples. The use of different or multiple bacterial species in the ToxTell test could remedy this defect. In a ToxTell bacterial biosensor, a current can be obtained from bacteria by passively diverting electrons from the electron transport chain (ETC), the cell's central metabolic pathway, which is common to all bacterial species (Yamamori et al., 2012). Redox mediators such as the ferricyanide ion, *p*-benzoquinone, or dimethylferrocene can be used as electron carriers allowing redox events within the cell to be monitored using suitably poised electrodes (Fig. 1a). Mediator reduced (M[red]) by enzymatic events within the cell is reoxidized (M[ox]) at the working electrode surface resulting in a current flow (Roy et al., 1989; Ikeda and Kano, 2001). As injury sustained by a cell in response to a toxicant changes the capacity of some central function of cellular metabolism, compared to unaffected cells. These toxic effects can be easily measured as a deviation away from the respiration signal produced by healthy cells. Work has shown that the current produced in the presence and absence of toxicant is proportional to the level of metabolic activity of the cells, which provide an index of inhibition. This approach has also been subsequently modified to allow the monitoring of the metabolic activity of either pure or mixed cultures of bacterial cells.

The objective of this study is to develop and optimize a mediated ToxTell assay for heavy metals using *Psychrobacter* sp. obtained from an activated sludge plant. Here, *Psychrobacter* sp. is used as a model microorganism for its higher sensitivity to the toxicity of heavy metals (Zhao (2009)). *p*-benzoquinone is employed as a redox mediator to determine the whole effects of chemical toxicity on *Psychrobacter* sp. respiration rather than identifying the chemicals included. Different parameters influencing sensitivity of the ToxTell biosensor are to be optimized. Its toxicity responses and data for a range of heavy metal ions, such as Cd^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+}, are presented.

2. Experimental

2.1. Reagents

A cocktail of respiratory substrates (sodium lactate, sodium succinate and glucose each at 5 mM) in 0.85% saline was used for resuscitating and monitoring the biosensors. *p*-benzoquinone (0.5 mM) was used as mediator. The heavy metals used were ultrapure salts ($CdCl_2$, $PbCl_2$, $HgCl_2$, $ZnSO_4 \cdot 7\ H_2O$ and $CuSO_4 \cdot 5\ H_2O$). All chemicals were of analytical grade. Milipore Mlli-Q nanopure water (resistivity 18.2 MΩcm) was used throughout for the preparation of solutions.

2.2. Growth, maintenance and harvesting of Psychrobacter sp.

The test organism *Psychrobacter* sp. was isolated from a nitrifying activated sludge from a large wastewater treatment plant in Shanghai, China, and maintained on nutrient agar plates at 4 °C. The suitable medium and the incubation temperature were confirmed by guide of China General Microbiological Culture Collection Center (CGMCC).

The culturing process for *Psychrobacter* sp. was addressed as follows:

(1) Select the bacteria: select a suitable *Psychrobacter* sp. from the plate medium dishes and put it into the liquid culture medium for mixing. Finally, put it in a shaker bath (200 rpm) for 12 h at 37 °C.
(2) A colony culture: taking a colony culture of *Psychrobacter* sp. into a 100 mL fresh liquid medium for culture.
(3) Growth curve: measure the OD_{600} value from the *Psychrobacter* sp. liquid culture medium per hour and finish after 60 h.

Cells were harvested by centrifuging 1 mL aliquots of culture at 10,000 rpm for 2 min, removing the supernatant and rinsing the resulting cell pellets twice in 0.85% (w/v) saline. Finally the cells were resuspended in 150 μL saline for use.

2.3. Biosensor preparation

The structure of the ToxTell biosensor electrode was shown in Fig. 1b. A carbon electrode was fabricated by using a screen-printer (HG, Shanghai). A polycarbonate film (Capton, Toray-Dupont) was used for the substrate. A carbon film was formed on the polycarbonate film with a carbon ink (CH-10, Jujo Ink). The silver/silver chloride (Gwent Ink) was printed on the reverse side of the polycarbonate film as a reference electrode. A resist ink (AC-3G, Jujo Ink) was printed on the carbon film in order to define the surface area of the carbon as working electrode. 8 μL aliquots of above bacterial cell suspension are presented to working electrode (Cell density used here was optimized according to the resultant electrode's characteristics). Once this is dry, a 0.2 μm polycarbonate membrane (Whatman, UK) was used for cell immobilization and secured onto the electrode precoated with a circle of micropore tape.

2.4. Biosensor monitoring

The biosensor electrodes were monitored in stirred vials containing a solution of respiratory substrates in saline solution. The electrodes were set into the vial lid, which also had a sample port for the addition of mediator and toxicants. Up to 8 cell-loaded electrodes were connected to a biosensor interface unit (ToxTell instrument, Shanghai, China), and poised at a constant potential of 500 mV. Operational amplifiers, within the interface, maintained this potential across the reference and working electrodes. Controls were established with either toxicant free additions or exchange of vials. The current was monitored every 4 s and data were displayed in real-time as current against time plots. Fig. 2 illustrated the typical effect of a toxic sample on a ToxTell biosensor and indicated a clear response, detected rapidly after the addition of test sample.

As shown in Fig. 2, the analysis process was as follows: (1) Following a biosensor stabilisation period of 5 min in 0.85% saline, the samples were supplemented with *p*-benzoquinone, by injection via the sample ports, to a final concentration of 5 mM

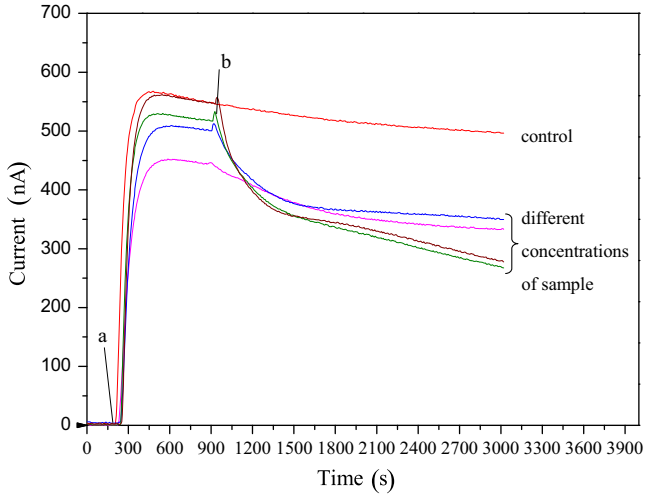

Fig. 2. Typical toxicity analysis current curves of a ToxTell biosensor: (a), (b) are operation starting points.

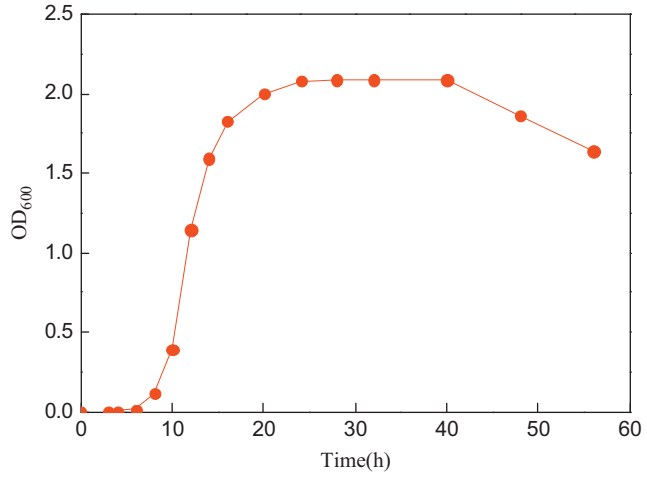

Fig. 3. Growth curves of *Psychrobacter* sp. 37 °C, pH 7.0, LURIA-BERTANI (LB) Medium.

(as shown in Fig. 2, position a); (2) Supplementation of samples with inorganic salts or toxicants were performed in the same way following a second period (5–10 min) of stabilisation (Fig. 2, position b); (3) The biosensors were monitored for at least 30 mins; (4) Then, the effective concentration (EC) values determined under different incubation time of the sample were calculated based on the data of Fig. 2 by the ToxTell software.

3. Results and discussion

3.1. The effect of growth curve phase on cell sensitivity to toxicant

Fig. 3 indicates the growth curve of the *Psychrobacter* sp. Concerning the growth curve of Fig. 3, four culturing points with respect to the transition timing from the lag phase to the logarithmic phase, the middle timing of the logarithmic phase, the transition timing from logarithmic phase to stationary phase and the middle timing of the stationary phase, would be selected as four growth curve phases for realizing the influence of growth phase on the toxicity sensitivity performance of the microbial biosensor. Here, the four culture timings for *Psychrobacter* sp. were set at 10, 15, 24, and 40 h, respectively.

The effect of growth phase of *Psychrobacter* sp. on cell sensitivity to Cu^{2+} was tested. The results of percentage inhibition analysis of Cu^{2+} to *Psychrobacter* sp. with different culturing time were addressed as follows. First, after a culturing time of 10 h for *Psychrobacter* sp., the half inhibitory concentration (EC_{50}) value determined under 30 min incubation for *Psychrobacter* sp. exposed to Cu^{2+} was 3.8 mg/L. Similarly, an EC_{50} value of 2.7 mg/L was produced after a culturing time of 15 h. As for a culturing time of 24 h, an EC_{50} value of 2.6 mg/L was produced. The biosensor with *Psychrobacter* sp. yielded an EC_{50} value of 4.3 mg/L after its culture time of 40 h. As for the effect of culture time of *Psychrobacter* sp. on the performance of biosensor, the *Psychrobacter* sp. at different culture times would represent the different characteristics of growth and further affect its sensitivity to toxicants. Generally speaking, the *Psychrobacter* sp. at the middle of the logarithmic phase or transition from logarithmic to stationary phase would have a higher sensitivity to toxicants because the phase would show a stronger growth rate and a higher metabolism. These observations would be useful to improve the performance of biosensor. From this evidence, a culture time of 24 h would be suggested for *Psychrobacter* sp.

3.2. Influence of pH

The effect of the respiratory substrate pH on the response of the ToxTell microbial biosensor was evaluated between 2.0 and 9.0 by addition of HCl or NaOH solutions. As shown in Fig. 4, there was a distinct degeneration of the current at pH 2.0–5.0. In contrast, the current maintained consistent when pH over 6.0 up to 9.0. This result was mainly due to the occurrence of cell damage in *Psychrobacter* sp. at low pH. Saldaña et al. (2012) reported that microbial inactivation and the cell damage were very dependent on pH. Spetea et al. (1997) also found that low pH accelerated light-induced damage of photosystem II by enhancing the probability of the donor-side mechanism of photoinhibition. These results agreed with those reported for *Nitrosomonas* and *Nitrobacter* organisms (Grunditzm and Dalhammar, 2001), whose optimum activity were at pH values of 8.1 and 7.9, respectively. Hence, for all subsequent sensor measurements the respiratory substrate pH was selected as 7.0.

3.3. Influence of salinity

Effect of NaCl addition on the biosensor performance was also conducted (not shown). The NaCl concentration in the respiratory substrate was gradually increased from 0.5% to 10%. It was found

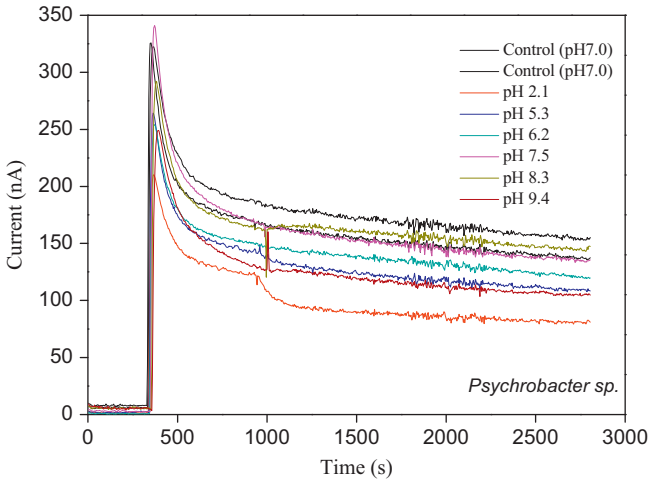

Fig. 4. Effect of buffer pH on the performance of *Psychrobacter* sp. biosensor. pH 7.0; 5 mM substrate cocktail/0.85% saline.

that there was a distinct degeneration of the current at NaCl concentration over 3.0%, and below which, the current maintained consistent. The results showed that the conductivity in the respiratory substrate, which increased with adding NaCl from 0.5% to 3%, did not affect the current. The decrease of current, when NaCl concentration was over 3%, was mainly due to the inhibitory effects of NaCl on the microorganism (Hinton, 1999; Maier, 2009). Dew et al. (1997) also suggested that high concentrations of chloride in solution could damage the membrane of the bacteria. As bacteriological saline (0.85%) provides the optimum osmolarity for *Psychrobacter* sp., the 5 mM substrate cocktail/0.85% saline was employed for toxicity assessment.

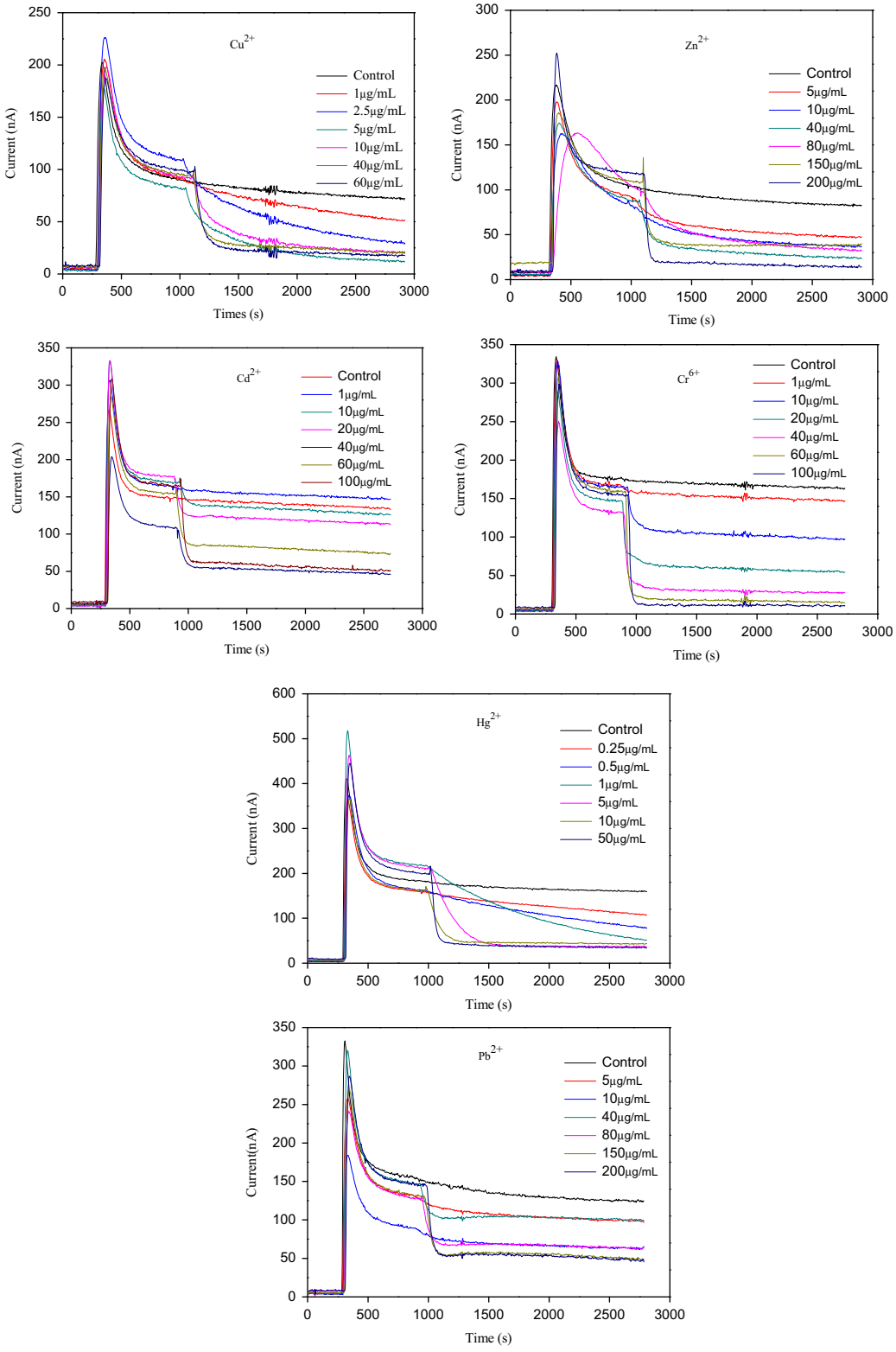

Fig. 5. Response of *Psychrobacter* sp. biosensors to different heavy metal ions. pH 7.0; 5 mM substrate cocktail/0.85% saline.

Table 1
Comparison of EC_{50} values by electrochemical method to other assays.

Toxicity assays	Toxicants, EC_{50} (mg/L)						References
	Cu^{2+}	Cd^{2+}	Zn^{2+}	Cr^{6+}	Hg^{2+}	Pb^{2+}	
ToxTell *Psychrobacter* sp. biosensor, incubation time 30 min	2.6	47.3	10.9	14.0	0.8	110.1	Present study
Amperometry, *E. coli*, incubation time 60 min	60(EC_{20})	–	–	–	40	$EC_{10} > 150$	Liu et al., 2009
FM-TOX, *E. coli,* incubation time 60 min	3.7	7.8	7.5	–	2.0	20.4	Catterall et al., 2010
Nitrification inhibition, nitrifying bacteria, incubation time 2 h	41.5	33.1	22.6	35.6	–	–	Dalzell et al., 2002
Respiration inhibition, activated sludge, incubation time 3 h	40.0	61.1	67.5	36.2	–	–	Dalzell et al., 2002
ATP luminescence, incubation time 30 min	5.5	871.5	no response	37.5	–	–	Dalzell et al., 2002
Microtox *V. fischeri*, incubation time 30 min	0.48	8.5	0.87	22.5	–	–	Dalzell et al., 2002
In vivo L-alanine-aminopeptidase inhibition, incubation time 15 min	10.5	357.1	80.5	228.5	–	–	Dalzell et al., 2002
Amperometry, *E. coli,* incubation time 30 min	–	–	45.0	–	1.6	–	Bentley et al., 2001
Luminescence, incubation time 30 min	–	–	3.5	–	1.5	–	Bentley et al., 2001

3.4. Incubation time

The results of mean values by duplicate assays for Cu^{2+} inhibition on *Psychrobacter* sp. during 60 min incubation showed that there was an obvious distinction of toxicity on *Psychrobacter* sp. incubated between 15 and 30 min. At 15 min, the inhibitory percentages were below 50% even the concentration of Cu^{2+} reached 10 mg/L. However, the toxicity rapidly increased to 50% inhibition (EC_{50}) when *Psychrobacter* sp. was exposed to 2.6 mg/L Cu^{2+} at 30 min incubation. At 60 min incubation, the EC_{50} only decreased 0.5 mg/L comparing with that of 30 min. This meant that 30 min was enough for determining the toxicity by using the *Psychrobacter* sp. biosensor. A similar phenomenon was also found for other heavy metals, such as Cd^{2+}, Zn^{2+}, Cr^{6+}, Hg^{2+} and Pb^{2+}, respectively. In fact, unlike other ecotoxicological tests, which record the effect of a toxic shock after a single fixed period of time, the ToxTell biosensor provides a near continuous monitoring (such as 4 s intervals) of the microbial activity following toxic shock. Thus from the data generated, inhibition is assessed using a hyperbolic transfer function to relate the biosensor response to the toxicant exposure. This uses the whole of the available data and allows the long-term inhibition to be predicted from the short-term response.

3.5. Toxicities of heavy metals to Psychrobacter sp.

The important heavy metal pollutants, such as Cu^{2+}, Cd^{2+}, Zn^{2+}, Cr^{6+}, Hg^{2+} and Pb^{2+} were investigated under optimum conditions in present study. The responses of *Psychrobacter* sp. based biosensors to the heavy metal ions were shown in Fig. 5. The EC_{50} results obtained were compared with those of published data by other methods (Table 1). According to Table1, the EC_{50} values of ToxTell assay based on *Psychrobacter* sp. were comparable or even better than those of other tests. Our results were in agreement with Catterall et al.'s (2010) report on toxicity order of heavy metals based on ferricyanide-mediated toxicity bioassay with *E. coli*. As for Pb^{2+}, its much lower toxicity than other heavy metals was mainly due to its stronger membrane sequestration (Nies, 1999). Moreover, there was some significant difference in the sensitivities of the various tests and methods, which reflected the fact that each bacterial species and test procedure had its own sensitivity pattern to toxicants.

The complete microbial sensors were stored in sterile circumstances at 4 °C. In order to evaluate the long term stability of this sensor, percentage inhibition analysis of Cu^{2+} to *Psychrobacter* sp. with different interval time was recorded. The EC_{50} values of Cu^{2+} to *Psychrobacter* sp. determined were 2.6 mg/L, 2.9 mg/L, 2.7 mg/L and 3.1 mg/L after 1, 10, 30 and 60 days storage. The ToxTell microbial biosensor developed in this work demonstrated excellent storage stability for more than 60 days.

The reproducibility of the *Psychrobacter* sp. biosensor was evaluated by five replicate assays for Cu^{2+}, Cd^{2+}, Zn^{2+}, Cr^{6+}, Hg^{2+} and Pb^{2+} inhibition on *Psychrobacter* sp. during 30 min incubation. The relative standard deviations (R.S.D.) obtained were 6.5%, 8.2%, 7.0%, 7.3%, 2.5% and 9.1%, respectively. Thus, the sensor showed a good, reproducible behavior and could be used for reproducible measurements. Moreover, with a portable and compact design, the ToxTell microbial bio-sensing system is able to monitor toxicity in real-time into different locations.

4. Conclusion

A rapid and reliable amperometric mediated bacterial biosensor, which adopted *Psychrobacter* sp. as biocomponent and *p*-benzoquinone as mediator, for heavy metal toxicity assessment was developed. Cu^{2+}, Cd^{2+}, Zn^{2+}, Cr^{6+}, Hg^{2+} and Pb^{2+} were examined by using the *Psychrobacter* sp. ToxTell biosensor under optimum conditions, and the EC_{50} values determined were 2.6 mg/L for Cu^{2+}, 47.3 mg/L for Cd^{2+}, 10.9 mg/L for Zn^{2+}, 14.0 mg/L for Cr^{6+}, 0.8 mg/L for Hg^{2+} and 110.1 mg/L for Pb^{2+}, respectively. These results obtained were comparable or even better than those of other toxicity bioassays. The biosensor based on whole cell is a sensitive, rapid and inexpensive alternative with no ethical argument for toxicity screening of chemicals and environmental water monitoring.

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