Ecotoxicology and Environmental Safety 96 (2013) 61–66

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Bioluminescence enhancement through an added washing protocol enabling a greater sensitivity to carbofuran toxicity

Kun Jia [a], Evgeni Eltzov [b,c], Robert S. Marks [b,c,d,e], Rodica E. Ionescu [a,*]

[a] Laboratoire de Nanotechnologie et d'Instrumentation Optique, Institute Charles Delaunay, Université de technologie de Troyes, UMR-STMR CNRS 6279, 12 rue Marie-Curie CS 42060, 10004 Troyes Cedex, France
[b] Department of Biotechnology Engineering, Ben-Gurion University of the Negev, PO Box 653, 84105 Beer-Sheva, Israel
[c] National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[d] The Ilse Katz Centre for Meso and Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
[e] School of Materials Science and Engineering, Nanyang Technological University, Singapore, 639798

ARTICLE INFO

Article history:
Received 6 December 2012
Received in revised form
6 June 2013
Accepted 15 June 2013
Available online 16 July 2013

Keywords:
Carbofuran detection
Enhancement of bioluminescence
E. coli DPD2794 bacteria
Washing protocol

ABSTRACT

The effects of carbofuran toxicity on a genetically modified bacterial strain *E. coli* DPD2794 were enhanced using a new bioluminescent protocol which consisted of three consecutive steps: incubation, washing and luminescence reading. Specifically, in the first step, several concentrations of carbofuran aqueous solutions were incubated with different bacterial suspensions at recorded optical densities for different lengths of time. Thereafter, the resulting bacterial/toxicant mixtures were centrifuged and the aged cellular supernatant replaced with fresh medium. In the final step, the carbofuran- induced bioluminescence to the exposed *E. coli* DPD2794 bacteria was shown to provide a faster and higher intensity when recorded at a higher temperature at30 °C which is not usually used in the literature. It was found that the incubation time and the replacement of aged cellular medium were essential factors to distinguish different concentrations of carbofuran in the bioluminescent assays. From our results, the optimum incubation time for a "light ON" bioluminescence detection of the effect of carbofuran was 6 h. Thanks to the replacement of the aged cellular medium, a group of additional peaks starting around 30 min were observed and we used the corresponding areas under the curve (AUC) at different contents of carbofuran to produce the calibration curve. Based on the new protocol, a carbofuran concentration of 0.5 pg/mL can be easily determined in a microtiter plate bioluminescent assay, while a non-wash protocol provides an unexplainable order of curve evolutionswhich does not allow the user to determine the concentration.

1. Introduction

Carbofuran, an anticholinesterase carbamate, is commonly used as an insecticide, nematocide, and acaricide in agricultural practice throughout the world under a given trade name of Furadan (Gupta, 1994). It is known that carbofuran is particularly toxic to birds and the fatal dose can be a single pesticide-contaminated grain (Wobeser et al., 2004). Carbofuran is also one of the highest toxic insecticides known to humans. The toxic effects are mainly attributed to its activity as a cholinesterase inhibitor (Bretaud et al., 2000). Carbofuran is a water soluble compound (solubility of 320 mg/L at 25 °C in water) contaminating groundwaters (Goad et al., 2004; Lau et al., 2007; Morales et al., 2012). An example of carbofuran toxicity, is the inhibition of the activity of acetylcholinesterase (AchE) extracted from thebrain of Juvenile goldfish (*Carassius auratus*) after exposure to 50 μg/L (19–28 percent) and 500 μg/L (85–87 percent) carbofuran (Bretaud et al., 2000). Thus, it is crucial to monitor trace concentrations of carbofuran persisting in the environment.

Even though a high specificity and sensitivity can be both readily achieved from conventional chemical analytical methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC), they are limited by some inherent disadvantages like tedious sample pre-treatment, and the requirement of complicated instruments as well as professional technicians. Therefore, promising alternative analytical devices named biosensors have seen increasing applications in the determination of environmental toxicants in the past decades (Melamed et al., 2012; Ionescu et al., 2010; Castillo et al., 2004; Chiriaco et al., 2011).

Different biosensor configurations to detect carbofuran pesticide were developed such as fluorescent sensors (Llorent-Martinez et al., 2005; Jin et al., 2004), optical fibers (Andres and Narayanaswamy, 1997), and photothermal biosensors (Pogacnik and Franko, 1999),

* Corresponding author. Fax: +33 3 25 71 84 56.
E-mail address: elena_rodica.ionescu@utt.fr (R.E. Ionescu).

0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.06.013

although the inhibition tests of acetylcholinesterase enzyme extracted from nervous tissue is the most commonly used (Valdes-Ramirez et al., 2008; Liu et al., 2011).

In addition, electrochemical biosensors have also been widely used in the determination of carbofuran, by employing different configurations: classical amperometry (Shulga and Kirchhoff, 2007; Albareda-Sirvent et al., 2001), screen-printed electrodes amperometric electrodes (Palchetti et al., 1997; Cagnini et al., 1995), chronoampometry (Laschi et al., 2007), classical potentiometry and conductometry (Dzyadevych et al., 2005) and potentiometric flow injection-type biosensors (Lee et al., 2001).

Furthermore, genetically modified microorganisms have been also widely used in the screening of environmental toxicants, such as organic pollutants (Hay et al., 2000; Valdman et al., 2004), heavy metals (Jouanneau et al., 2011; Liao et al., 2006), pesticides (Jia et al., 2012), and antibiotics (Eltzov et al., 2008). The main components for these whole-cell biosensors are the promoter gene and the adjacent bioreporter gene (Belkin, 2003); the former one is responsible for the specific recognition of the analyte, while the expression of the latter one leads to the production of bioluminescence via the synthesis of luciferase and its corresponding substrate.

Depending on the toxicants to be detected, a large number of engineered bacteria strains harboring different promoter genes have been constructed, which were summarized in some recent review articles (Robbens et al., 2010; Lei et al., 2006; Girotti et al., 2008; Su et al., 2011; Woutersen et al., 2011). The most used bioreporter *lux* genes responsible for encoding the luciferase enzyme are the *luxCDABE* gene (Abd-EI-Haleem et al., 2002; Hwang et al., 2008; Lopes et al., 2012), while the said operon includes, an independent light generation system, that does not require an additional extraneous substrate (Meighen, 1991).

Even though the engineered bacterial luminescent bioreporters are advantageous in terms of cost-effectiveness, high sensitivity and possible determination of toxicant bioavailability, the generation of bioluminescence is a dynamic process highly dependent of cellular metabolism status (Van Der Meer et al., 2004). Therefore, the bioluminescence can be affected by internal metabolic products which may result in ambiguous positive responses.

In this work, the genetically modified bacterial strain *E. coli* DPD2794, containing a *recA* promoter fused to *luxCDABE* gene originating from *Vibrio fischeri*, was used to evaluate the toxic effects of carbofuran. A new protocol was developed with the purpose of tuning the cellular physiological response of the bioreporter microorganism to increase its sensitivity to the deleterious effect of a target toxicant.

2. Materials and methods

2.1. Materials

Kanamycin sulfate (K1377), LB-broth (L3032) and carbofuran (32056) were purchased from Sigma (Lyon, France). Deionized water was produced by Millipore-Mill-Q water purification system (France) and sterilized prior to preparation of stock analyte solution. Bacteria *E. coli* DPD2794 strain was provided by Prof. Shimshon Belkin from Hebrew University of Jerusalem (Israel). Bioluminescence measurements were performed within 96 wells Costar microtiter-plates (Corning Incorporated, USA).

2.2. Medium preparation

Luria Bertani (LB) medium was prepared by adding 2 mg LB broth powder into 100 mL double distilled water in a 200 mL glass bottle, followed by sterilization in an autoclave-steam sterilizer (2540 ML-Tuttnauer, Netherlands) for 15 min at 121 °C. Fresh sterilized LB medium was used in the preparation of carbofuran dilutions and also in the bacteria washing step after centrifugation.

2.3. Instrumentation

Growth of *E. coli* DPD2794 bacteria strain occured in a water bath (Grant Instruments Type VF, Cambridge Ltd, UK). A DR/2500 spectrophotometer (Odyssey, HACH Company, USA) was used for recording the bacteria optical density (OD), while the mixing between bacteria cultures and different dilutions of toxicant solutions were performed using a Vortex-Genie 2 type G560E. Centrifugation and bioluminescence measurements were conducted by using a centrifuge model type Universal 320R (Andreas Hettich GmbH, Germany) and a luminometer Luminoskan Ascent (Thermo Fisher Scientific, United States), respectively. Luminescence values were presented in relative light units (RLU) and recorded at 30 °C.

2.4. Bacteria growth conditions

Cultivation of *E. coli* DPD2794 strain was performed in 10 mL fresh LB medium supplemented with 10 μL of 50 mg/mL kanamycin aqueous solution to get a final concentration of 50 μg/mL. The cells were grown overnight for 9 h at 37 °C in a thermostated water bath, followed by vigorous vortexing (2000 rpm) of densely formed suspensions of *E. coli* DPD2794 strain cultures. Then, 200 μL of bacterial suspension grown overnight was inoculated in 10 mL fresh LB without antibiotics and kept incubated for another 9 h at 37 °C, the resulting optical density was 0.7 corresponding to a cell concentration of 10×10^8 cfu/mL.

2.5. Bioluminescence measurements

The toxicant stock solution (0.05 mg/mL) was prepared by dissolving 1 mg carbofuran into 20 mL ddH_2O, and then various aliquots of 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} were prepared by diluting stock solutions with fresh LB medium. Taking into account that the limit of detection of carbofuran using an Agilent 6460 triple quadrupole liquid chromatograph/mass spectrometer (LC//MS/MS) with a high energy dynode detector was established at 0.03 ng/mL by a standard laboratory named CARSO (Laboratoire Santé Environnement Hygiène de Lyon, France) the validation of our dilution process down to 10^{-6} corresponded to an experimental concentration of 0.05 ng/mL. Various carbofuran aqueous solutions (one at the time) were directly injected in the HPLC column, followed by the mass spectrometry carbofuran quantitative identification. The HPLC measurements allowed the carbofuran separation from the potential contaminants presented in the aqueous suspension.

For the measurements, 100 μL mixtures of bacterial suspension and toxicant dilution (volume ratio of 9:1) were distributed into the 96-well white microtitre plate, while 100 μL of bacterial suspension alone was used as the control. All the bioluminescent measurements were conducted at 30 °C for 400 min.

The new protocol that enabled higher bioluminescence production contained three main steps (Fig. 1):

Step A: Incubation of combined bacterial suspension and toxicant dilutions. Bacterial suspensions were incubated with various concentrations of toxicants at room temperature for different periods of time (0 without a room temperature incubation step, 2, 4, 6, 9, and 12 h, respectively). See Fig. 3.

Step B: Centrifugation and washing with fresh LB. After the aforementioned incubation periods at room temperature, the bacterial/toxicant mixtures were centrifuged at 856 g for 15 min, where the resulting supernatant was discarded and replaced with fresh LB medium. The washing step was repeated twice. The second set of control experiments were conducted by using “non-washed” bacterial cells under the same experimental conditions.

Step C: Bioluminescence measurements occured at 30 °C. The bacterial/toxicant mixtures (volume ratio of 9:1) were distributed into the 96-well microtiter plates. Bioluminescence measurements were conducted at a favorable physiological higher temperature of 30 °C (unlike in most of the literature), so as to gain a faster bioluminescent response (unpublished results). In each bioluminescence measurement, the resulting signals obtained from the bioreporters that were not exposed to the toxicant (called not incubated samples), were recorded as the third control in each measurement.

2.6. Data processing

In the present work, the whole evolution of bacterial bioluminescence at 30 °C in the presence of toxicant over 400 min was investigated. Since various luminescence peaks were observed during the measurements, the maximum peak intensity was not the only parameter used to evaluate the carbofuran toxicity to cells. Thus, an additional parameter named area under the bioluminescent curve (AUC) was calculated using the build-in algorithm of Origin 8.5 software and the peak luminescent intensities (RLUmax) corresponding to the different peaks for each content of carbofuran measured as summarized in Table S1 (Supplementary material). The variation of the AUC parameter was investigated for different concentrations of toxicant and used in the construction of the calibration curve.