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Fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics

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ABSTRACT

Global urban and industrial growth, with the associated environmental contamination, is promoting the development of rapid and inexpensive general toxicity methods. Current microbial methodologies for general toxicity determination rely on either bioluminescent bacteria and specific medium solution (i.e. Microtox®) or low sensitivity and diffusion limited protocols (i.e. amperometric microbial respirometry). In this work, fast and sensitive optical toxicity bioassay based on dual wavelength analysis of bacterial ferricyanide reduction kinetics is presented, using *Escherichia coli* as a bacterial model. Ferricyanide reduction kinetic analysis (variation of ferricyanide absorption with time), much more sensitive than single absorbance measurements, allowed for direct and fast toxicity determination without pre-incubation steps (assay time = 10 min) and minimizing biomass interference. Dual wavelength analysis at 405 (ferricyanide and biomass) and 550 nm (biomass), allowed for ferricyanide monitoring without interference of biomass scattering. On the other hand, refractive index (RI) matching with saccharose reduced bacterial light scattering around 50%, expanding the analytical linear range in the determination of absorbent molecules. With this method, different toxicants such as metals and organic compounds were analyzed with good sensitivities. Half maximal effective concentrations (EC_{50}) obtained after 10 min bioassay, 2.9, 1.0, 0.7 and 18.3 mg L^{-1} for copper, zinc, acetic acid and 2-phenylethanol respectively, were in agreement with previously reported values for longer bioassays (around 60 min).

This method represents a promising alternative for fast and sensitive water toxicity monitoring, opening the possibility of quick in situ analysis.

1. Introduction

Environmental contamination is becoming a great challenge regarding the global urban and industrial growth. In this context, the development of rapid and inexpensive water toxicity monitoring techniques is highly desirable (Fulladosa et al., 2005). Conventional analytical methods, such as high pressure liquid chromatography (HPLC) or gas chromatography (GC), present high sensitivity and selectivity in the detection of specific chemical pollutants. However, they are not suitable for general toxicity detection in complex matrices, like wastewater or industrial water (Tizzard et al., 2004; Li et al., 2013) Biological assays and biosensors have positioned as one of the most promising alternatives to the traditional methods for general toxicity determination. That is, toxic agents kill or inhibit living organisms in a dose-dependent process, which allows for a quantitative determination of general toxicity of the sample (Tizzard et al., 2004). Many different biological assays using living organisms have been reported until now for the determination of acute or chronic toxicity. Depending on the nature of the living organism, they can be divided into eukaryotic and prokaryotic assays. Eukaryotic-based assays are based on the monitoring of reproduction or lethality of model organisms such as daphnids, fish, and others, during a previously established time period (Levy et al., 2007). Toxicity is expressed, in this case as a percentage of growth inhibition or lethality with the sample concentration. These assays are very useful but rely on long incubation times of the sample with the living organism and demand skilled personnel (Oanh and Bengtsson, 1995; Liu et al., 2009). On the other hand, prokaryotic-based assays are advantageous in terms of simplicity, speed and cost, mostly due to the fast microbial metabolism, versatility (e.g. they can be sensitized to detect specific targets) and easy manipulation of microorganisms (Li et al., 2013; Liu et al., 2009; Tizzard et al., 2004).

Up to now, from all microbial-based toxicity assays developed, Microtox® is, with difference, the most widely used (Oanh and

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Bengtsson, 1995). This method is based on the intrinsic bioluminescence of *Vibrio fisheri* expressing the *lux* genes, which is lost in the presence of toxic agents in the sample. Microtox® presents some limitations such as the requirement of luminescent bacteria or specific medium solution (Liu et al., 2009). In opposition, respirometric microbial assays, where the reduction rate of an electron acceptor is directly linked to the metabolic activity of microorganisms, can operate with a broad microbial spectrum and diverse chemical conditions. Respirometric microbial bioassays present two main issues: (i) the selection of the suitable electron acceptor and (ii) the transduction mechanism. Considering electron acceptor selection, several natural or artificial electron acceptors have been evaluated until now (e.g. oxygen, nitrate, ferricyanide, benzoquinone, among others) (Catterall et al., 2010a, 2010b; Li et al., 2013; Liu et al., 2009; Tizzard et al., 2004; Yip et al., 2014). From all of them, ferricyanide has positioned as the most suitable one due to its low toxicity and an aqueous solubility many orders of magnitude higher than oxygen (Chen et al., 2008; Jordan et al., 2013; Nakamura et al., 2007). Hence, ferricyanide enables the use of highly concentrated bacteria in general toxicity assays, enhancing sensitivity and reducing the time per assay in both general toxicity and biological oxygen demand (BOD) determination (Catterall et al., 2010a; Ertl et al., 2000; Liu et al., 2010; Pasco et al., 2004).

Regarding the transduction mechanism, electrochemical methods like amperometry and bulk electrolysis have been used for years in microbial toxicity assays due to their high sensitivity and wide detection range. In amperometric transduction ferrocyanide is oxidized on the electrode surface, obtaining a diffusion limiting current quantitatively related to its concentration (Catterall et al., 2010a; Liu et al., 2009; Tizzard et al., 2004). The use of microelectrodes improved electrochemical detection since the diffusion limiting current could be achieved in the second time-scale with a negligible consumption of the analyte (Morris et al., 2001). However, amperometric detection relies on interfacial mass transport processes, being unable to elicit a direct real-time perspective of ferricyanide reduction kinetics. On the other hand, optical detection techniques can overcome some of this limitations by providing a host of interesting features, such as contactless and non-invasive measurements thus avoiding contamination problems (very important when working with cells), low or null interference with biological processes and allowing for bulk interrogation of the sample (much more sensitive than interface electrochemical methods). Besides, ferricyanide presents an absorption peak at 420 nm (ε_{420}=10,571 cm mol^{-1}) and can be used for optical monitoring of microbial metabolism (Morris et al., 2001). Nevertheless, optical transduction is negatively affected by light scattering (particularly that due to bacterial turbidity), and offers a narrow detection range in comparison to amperometry. Suspended bacteria in aqueous media behave as colloidal particles, acting as light scattering centers causing turbidity and interfering in the spectrophotometric determination of absorbing molecules. Moreover, this interference is not constant over time but change with cell proliferation.

In this study, an optical ferricyanide-based bioassay for direct, fast and sensitive toxicity determination, based on ferricyanide reduction kinetics monitoring, is presented. This protocol exploits the inherent advantages of optical transduction methods overcoming traditional methods limitations (i.e. biomass interference and low analytical range of detection) by using dual wavelength detection and saccharose to enhance the refractive index of the culture medium. Dual wavelength detection allows for biomass interference elimination. That is, due to the additive nature of absorbance, the contribution of biomass in the absorbance magnitude at the wavelength of interest (i.e. 405 nm) can be easily eliminated by considering biomass absorption at a wavelength out of the absorption range of the analyte (i.e. 550 nm). On the other hand, saccharose enhances the refractive index of the medium, reducing biomass dispersion and enlarging the optical detection range. With this protocol, toxicity due to the common toxic agents copper, zinc, acetic acid and 2-phenylethanol is determined and compared with bibliography. This method opens the possibility for fast toxicity assays, which may be performed even in situ.

2. Materials and methods

2.1. Chemicals

Potassium ferricyanide, copper sulfate, zinc sulfate, acetic acid, 2-phenylethanol, potassium dichromate, glucose, saccharose, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate 3-hydrate were purchased from Panreac (Spain) and were of analytical grade, and all solutions were prepared with distilled water, unless otherwise stated.

2.2. Cultivation and preparation of Escherichia coli K-12

E. coli K12 (CGSC 5073) was used. *E. coli* was grown aerobically in 100 mL LB broth flasks for 18 h at 37 °C in a shaker bath (160 rpm). Grown cultures were centrifuged at 10,100*g* for 15 min and re-suspended in 0.1 M phosphate buffer (PB) containing 2% glucose. Optical density of re-suspended bacteria was measured at 600 nm in a Smartspec™ Plus spectrophotometer (Bio-rad, California, US). Bacterial suspensions were diluted in 0.1 M PB to achieve desired cell concentrations. 4′,6-diamino-2-phenilindol (DAPI) staining was used for total cell counts ((Porter and Feig, 1980). Images were acquired with a Zeiss Axio Imager A1 fluorescence microscope (Zeiss, Germany).

2.3. Optical measurements

Optical measurements were performed in 96-well plates using the Thermo Electron Multiskan EX plate reader (VWR International, Pennsylvania, US) and Ascent software (VWR International, Pennsylvania, US). Ferricyanide and toxic agents were freshly prepared and added to bacterial suspensions in PB immediately before each assay. Final mixtures were placed in 96-well plates and monitored by simultaneously recording at 405 and 550 nm at 1 min intervals for the duration of the experiment (60 min). Control samples were always analyzed at the same time.

2.4. RI matching assays

68% (w/v) saccharose solution was freshly prepared by dissolving saccharose in 100 mL distilled water at constant agitation and heating at 60 °C in a thermal magnetic stirrer during 60 min (IKA, RCT basic, Germany) Suitable dilutions were added to bacterial suspensions in PB immediately before each assay. Optical assays were performed as described in Section 2.2. For statistical analysis two-way ANOVA and Bonferroni post-test was carried out.

3. Results and discussion

3.1. Ferricyanide-based optical bioassay optimization

Preliminary experiments were performed to optimize cell and ferricyanide concentrations to be able to determine general toxicity of samples in less than one hour. Biomass should be selected to provide with quick ferricyanide reduction kinetic without interfering ferricyanide determination. For this reason,