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A rapid method for detection of catalase-positive and catalase-negative bacteria based on monitoring of hydrogen peroxide evolution at a composite peroxidase biosensor

B. Serra [a], J. Zhang [a,b], M.D. Morales [a], A. Guzmán-Vázquez de Prada [a], A.J. Reviejo [a], J.M. Pingarrón [a,*]

[a] *Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, 28040 Madrid, Spain*
[b] *Test Center, Fujian Agriculture and Forestry University, 35002 Fuzhou, China*

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Abstract

The rapid detection of catalase-positive and catalase-negative bacteria in complex culture media has been accomplished by monitoring of hydrogen peroxide consumption or generation with a graphite–Teflon–peroxidase–ferrocene composite electrode. *Escherichia coli* and *Streptococcus pneumoniae* have been used as model catalase-positive and catalase-negative bacteria, respectively. Hydrogen peroxide evolution was amperometrically measured at 0.00 V. Experimental conditions, including the working solution composition, the incubation time and the hydrogen peroxide concentration, were optimized. The reusability of the biosensor was improved by placing a nylon membrane on the bioelectrode surface to prevent fouling caused by the bacterial medium. The developed methodology allowed the detection of *E. coli* and *S. pneumoniae* at concentration levels of approximately 2×10^6 and 2×10^5 cfu/mL, in assays taking 10 and 15 min, respectively, without any pre-concentration step or pre-enrichment procedure.

Keywords: Catalase activity; Hydrogen peroxide; Bacteria detection; Peroxidase biosensor

1. Introduction

Bacterial pathogens detection is particularly relevant in fields like medicine, where the correct treatment is determined by the bacterial species causing an infection, water quality control, food safety, where a close control of the food chain is essential for public health but very difficult to ensure due to its complexity, and biological warfare security. Unfortunately, pathogen detection and identification is an intricate area, as only a few portion of the ubiquitous incredible diversity of bacteria are pathogenic, and the traditional methods for their detection based on specific microbiological and biochemical identifications [1–3] have the mayor limitation of long incubation times (days to weeks depending on the organism) [4] which are inadequate for crucial short time remediation responses.

Therefore, early detection techniques are desperately needed. In this sense, biosensors offer an exciting alternative, allowing rapid "real-time" and multiple analyses [5]. Accordingly, different types of biosensors for bacterial pathogen have been developed [6–16]. Most of the applications are based on the monitoring of pathogen–antibody binding or pathogenic DNA-probe DNA hybridization events. These methods provide great selectivity and very often suitable sensitivity, but they are complex, and therefore difficult to apply to real samples. Moreover, they are targeted to a particular pathogen, which is not very useful when the source of contamination is unknown. Recent developments in the field of pathogen bacteria biosensors, including electrochemical biosensors, have been recently reviewed [17].

The measurement of a metabolic product for bacterial determination is an interesting approach, which provides several

* Corresponding author. Tel.: +34 91 3944315; fax: +34 91 3944331.
E-mail address: pingarro@quim.ucm.es (J.M. Pingarrón).

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advantages: simplicity, low cost, possibility to distinguish viable and non-viable cells, amplification is usually achieved, and detection with no need for the use of a label. For example, bacterial catalase is a sensitive marker of aerobic and facultative anaerobic micro-organism's numbers. The measurement of its activity has been suggested as a method to quantify catalase-positive bacterial content [18], for example in milk [19], sewage treatment plants [20] and soil samples [21]. Catalase can catalyze the dismutation of hydrogen peroxide into water and oxygen. Hydrogen peroxide is a harmful by-product of many metabolic processes and if it is accumulated it can be lethal for bacterial cells. Therefore, catalase is frequently used by cells to rapidly catalyse the decomposition of hydrogen peroxide. Accordingly, monitoring the hydrogen peroxide consumption by bacterial catalase can be used for micro-organisms detection.

On the other hand, many catalase-negative bacteria can generate hydrogen peroxide during their metabolic activity in concentrations up to the millimolar range [22]. Therefore, monitoring its formation seems to be a potential way for catalase-negative bacteria detection.

Based on these premises, we have developed a simple amperometric method to detect bacterial pathogens by monitoring the consumption or generation of hydrogen peroxide by different types of bacteria. A graphite–Teflon–peroxidase–ferrocene composite electrode has been employed for the H_2O_2 determination, due to the advantageous analytical characteristics in terms of renewability, sensitivity and robustness exhibited by this amperometric biosensor [23]. To the best of our knowledge, no similar approaches have been described in the literature. However, electrochemical detection and identification of catalase-positive micro-organisms has been reported by combining lateral flow immunoassay with amperometry using a Clark cell at 0.7 V for the detection of hydrogen peroxide [24].

The proposed methodology has been evaluated using *Escherichia coli* and *Streptococcus pneumoniae* as model catalase-positive and catalase-negative bacteria, respectively. In fact, one of the objectives is to check the method usefulness to differentiate catalase-positive from catalase-negative bacteria by hydrogen peroxide evolution in selective culture media.

2. Experimental

2.1. Apparatus and reagents

Amperometric measurements were carried out on a PalmSense Electrochemical Sensor Interface controlled by Hewlet Packard pocket PC software. The electrochemical cell was a BAS VC-2 cell with a BAS RE-5 Ag/AgCl/KCl (3 M) reference electrode, and a Pt wire auxiliary electrode. Graphite–Teflon–peroxidase–ferrocene composite electrodes were used as the working electrode. A Raypa AES-75 steam sterilizer, a P-Selecta Agimatic-N magnetic shaker, a Griffin flask shaker and a P-Selecta Tectron 200 thermostatic bath were also used.

2.2. Reagents and solutions

Wild type *E. coli* were obtained from the Spanish Collection of Type Cultures and *S. pneumoniae R6* were generously provided by Centro de Investigaciones Biológicas, CSIC, in Madrid. Horseradish peroxidase (HRP) (EC 1.11.1.7, type II, activity 180 U mg^{-1} of solid, Sigma), graphite (ultra F purity; Carbon of America, Bay City, USA), Teflon powder (Aldrich) and ferrocene (Fluka, Buchs, Switzerland) were used for the fabrication of the biosensor. Hydrogen peroxide (30% w/w) was supplied by Sigma.

0.1 M hydrogen peroxide stock solutions were prepared daily in 0.05 M phosphate buffer solution of pH 6.5. More dilute standards were prepared by suitable dilution with the same buffer solution. Culture media consisted on Luria broth (LB) for *E. coli* (Scharlau) and brain heart infusion broth (Oxoid) for *S. pneumoniae*, and LB agar (Scharlau) and blood agar plates (Oxoid) were used for plate colony counting for *E. coli* and *S. pneumoniae*, respectively. Culture media and flasks were sterilized by autoclaving at 121 °C during 15 min. All chemicals were of analytical-reagent grade, and the water used was obtained from a Milli-Q purification system (Millipore).

2.3. Fabrication of graphite–Teflon–HRP–ferrocene (GTHF) composite electrode

Composite electrodes were fabricated in the form of cylindrical pellets as described elsewhere [23]. A portion of a 47 mm-diameter, 0.45 μm-pore size nylon membrane (Osmonics) was used for preventing the direct contact between electrode surface and bacterial medium by placing it on the electrode surface by means of a rubber o-ring.

2.4. Bacteria culture and plating method

S. pneumoniae R6 belongs to the micro-organism biosafety level 1 and wild-type *E. coli* to the level 2 group and, consequently, all safety considerations concerning these groups [25] were accomplished in the manipulation of these bacteria. *E. coli* cultures were grown overnight in Luria broth medium at 37 °C with aeration by shaking, which allowed the growing stationary phase to be reached. Then, bacterial cultures were serially diluted (10-fold steps) and 10 μL of samples were applied to LB agar plates and incubated for 24 h at 37 °C for enumeration of colonies. The stationary-phase cultures were properly diluted with 0.05 M phosphate buffer of pH 6.5 in order to obtain 10–10^8 cfu/mL.

S. pneumoniae was grown until mid-log-phase by culture in brain heart infusion (BHI) broth at 30 °C for 6 h under microaerophilic conditions. Bacteria were then centrifuged at 5000 × *g* for 20 min and washed with BHI in order to eliminate hydrogen peroxide from the medium. Then, bacterial cultures were serially diluted (10-fold steps) and 10 μL of samples were applied to blood agar plates and incubated for 24 h at 30 °C for enumeration of colonies. At the same time, the mid-log-phase cultures were diluted to 10–10^8 cfu/mL in BHI.

2.5. Amperometric measurements of hydrogen peroxide with the composite biosensor

The graphite–Teflon–HRP–ferrocene composite electrode was used to detect hydrogen peroxide. Amperograms were monitored at room temperature, in the electrochemical cell containing 5.0 mL of 0.05 M phosphate buffer which was mechanically stirred at a constant rate. A potential of 0.00 V was applied and the background current was allowed to stabilize. Then the solution containing the appropriate amount of hydrogen peroxide was added and the current was monitored until the steady state was reached.

2.6. Direct detection of E. coli

After the addition of the appropriate amount of H_2O_2 to the electrochemical cell and later current stabilization, different concentrations of *E. coli* were also pipetted into the cell, and the current deflection was monitored.

2.7. Indirect detection of E. coli after a separated consumption step

Indirect detection of *E. coli* consisted of a two separated steps procedure. Firstly, an appropriate amount of hydrogen peroxide was added to an *E. coli* suspension in phosphate buffer, allowing the catalase action on H_2O_2 to take place for a fixed period of time. Secondly, an aliquot of this suspension was added to the electrochemical cell containing 5 mL of 0.05 M phosphate buffer at the appropriate pH and the remaining hydrogen peroxide was measured at 0.00 V. Subsequently, an aliquot from a negative control sample (without *E. coli*) was also added to the cell, and the obtained current was compared to the first one in order to compare hydrogen peroxide consumption by bacteria.

2.8. Indirect detection of S. pneumoniae

Indirect detection of *S. pneumoniae* was carried out by allowing bacterial BHI solution to grow, and, at appropriate periods of time, aliquots of 1 mL of these suspensions were added to the electrochemical cell containing 4 mL of 0.05 M phosphate buffer at the appropriate pH, the generated hydrogen peroxide being measured at 0.00 V.

3. Results and discussion

The determination of hydrogen peroxide, related to bacterial growth, is accomplished in this work by using a composite graphite–Teflon–HRP–ferrocene biosensor developed previously by our research group [23]. This biosensor was selected because of its excellent analytical performance for the determination of hydrogen peroxide. Thus, the amperometric measurements can be carried out at a low potential such as 0.0 V, which minimises possible interferences. Moreover, the biosensor exhibits a fairly good renewability of the electrode surface by polishing as well as a long-term operation and stability.

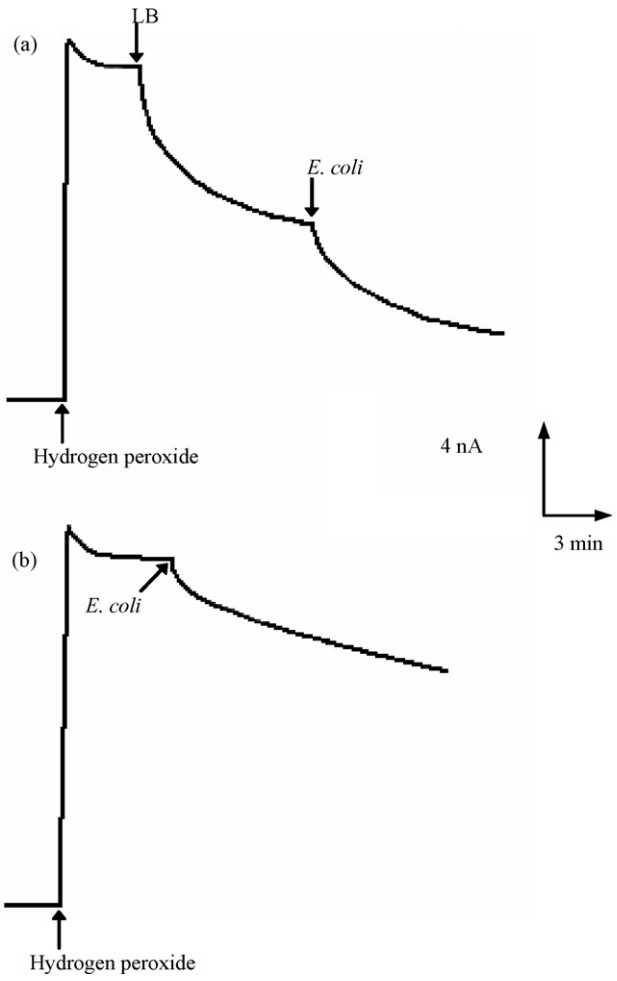

Fig. 1. Current–time curves obtained at a working gold electrode in an electrochemical cell containing 5.0 mL of 0.05 M phosphate buffer (pH 6.5) after the addition of 2.5 mM H_2O_2 and subsequent addition of (a) 100 μL of Luria broth and 100 μL of 10^8 cfu/mL *Escherichia coli* suspension in Luria broth, respectively; (b) 100 μL of 10^8 cfu/mL *E. coli* suspension filtered from Luria broth medium and resuspended in 0.05 M phosphate buffer (pH 6.5). E_{app} = 0.00 V.

3.1. Monitoring of hydrogen peroxide consumption by catalase-positive bacteria using graphite–Teflon–HRP–ferrocene electrode

Although hydrogen peroxide can be amperometrically detected at many electrode surfaces, its direct amperometric detection in a complex medium such as bacteria suspensions can be affected by many interferences. For example, Fig. 1 shows the influence on the amperometric response for H_2O_2 obtained on a gold electrode at 0.00 V when an aliquot of LB medium containing no bacteria (typically used for *E. coli* growing) is added to the amperometric cell and when *E. coli* suspensions in phosphate buffer solution are also added.

It is observed that it was not possible to distinguish between the signal due to bacteria catalase hydrogen peroxide consumption and the current decrease that appeared when an aliquot of the LB medium was injected. On the contrary, if a graphite–Teflon–HRP–ferrocene electrode is used, no influence from the culture medium was observed thus being possible to

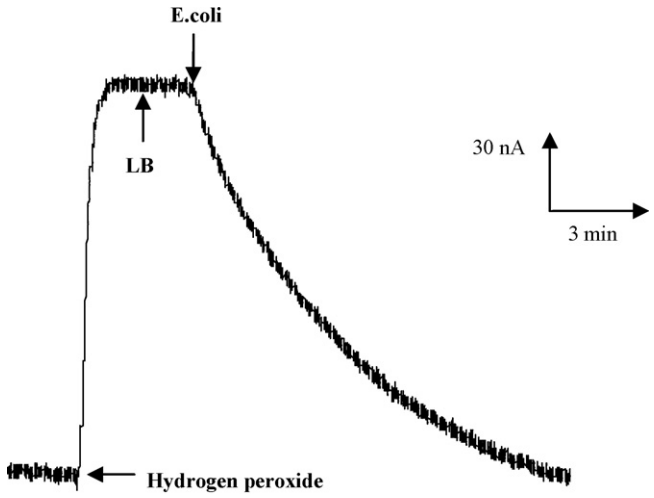

Fig. 2. A current/time response curve for 10^8 cfu/mL *E. coli* LB suspension in 5 mL of 0.05 M phosphate buffer solution (pH 6.5) on a graphite–Teflon–HRP–ferrocene composite electrode. $E_{app} = 0.00$ V; initial H_2O_2 cell concentration, 25 μM. The addition of 100 μL of LB did not affect the response.

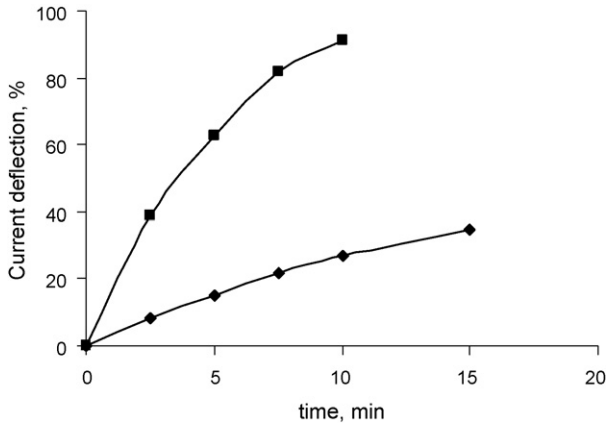

Fig. 3. Effect of assay time on the response of a 0.45-μm nylon membrane–graphite–Teflon–HRP–ferrocene composite electrode to: 1×10^7 cfu/mL *E. coli* (♦) and 1×10^8 cfu/mL *E. coli* (■). Conditions as in Fig. 2.

distinguish the response due to catalase action (Fig. 2). From the figure, it is also observed that the initial amperometric baseline is recovered after 15 min of *E. coli* suspension addition, demonstrating that bacteria consumption of hydrogen peroxide is fast. Therefore, its monitoring can provide a rapid method for the detection of *E. coli*.

3.1.1. Reproducibility of the measurements

Prior to the evaluation of the analytical characteristics of the biosensor for *E. coli* determination, the reproducibility and stability of the device must be studied. It should be remarked that although the composite biosensor exhibited a good analytical performance for hydrogen peroxide determination [23], it is now used in a more complex system, making it necessary to evaluate these aspects again. Therefore, five different measurements were performed in the same conditions as in Fig. 2. The relative standard deviation (R.S.D.) obtained (29.3% for 10^8 cfu/mL *E. coli*) showed a high dispersion of the analytical responses. Therefore, the micro-organisms environment seems to affect the stability of the biosensor, most likely due to surface fouling. Consequently, in order to avoid this surface contamination, the composite electrode was covered with a nylon membrane with a pore size of 0.45 μm to prevent bacteria adhesion on the electrode surface. Five measurements carried out under these conditions yielded an R.S.D. value for *E. coli* responses of 3.1%. These results demonstrate the effective prevention of surface fouling using the nylon membrane. Calibration plots obtained for hydrogen peroxide using a graphite–Teflon–HRP–ferrocene composite electrode non-covered and covered with a nylon membrane are compared. The uncovered electrode showed a range of linearity ($r = 0.998$) between 1.2 and 11.2 μM H_2O_2 with a slope value of 3.52 nA μM^{-1}. In the case of the nylon membrane covered-electrode, the linear range ($r = 0.9990$) was 2.4–22.4 μM H_2O_2 with a slope value of 3.37 nA μM^{-1}. As expected, the response was slightly smaller and the linear range extended as the membrane reduces the diffusion rate of hydrogen peroxide from the solution to the electrode surface.

3.1.2. Optimization of working conditions

3.1.2.1. Effect of pH on the biosensor response to E. coli. In this assay protocol for *E. coli* determination based on the detection of their catalase activity, there are two enzymatic reactions involved: the reduction of hydrogen peroxide catalysed by HRP at the electrode surface and the consumption of H_2O_2 in the bulk of the solution by catalase. With the purpose of studying the effects of pH on both enzyme reactions separately, detection of *E. coli* is carried out using the indirect method described in Section 2.7. Therefore, *E. coli* suspensions at different pH values were allowed to react with hydrogen peroxide for 5 min. Then, an aliquot of these suspensions was added to the electrochemical cell containing phosphate buffer at a pH value of 6.5. In this way, it was possible to observe the optimum pH value for catalase activity in *E. coli*. On the other hand, the response of graphite–Teflon–HRP–ferrocene electrode to H_2O_2 in the electrochemical cell containing working solutions of different pH values was also evaluated in order to determine the optimum value for HRP reaction. No significant changes in current signal for H_2O_2 were observed in the 5.0–7.5 range of pH values in the electrochemical cell. However, a maximum response was observed for the *E. coli* suspensions incubated with H_2O_2 at pH between 5.5 and 6.5. Furthermore, it was found that it took longer to obtain a stable amperometric baseline for lower buffer pHs. Therefore, a pH value of 6.5 was selected for *E. coli* determination with our biosensor system.

3.1.2.2. Optimization of assay time. Fig. 3 shows the relationship between the current deflection and the assay time after *E. coli* addition to the electrochemical cell. Obviously, the longer the assay time the more sensitive the response. Using an *E. coli* concentration of 10^8 cfu/mL, the response increased quickly, and reached 90% of cell hydrogen peroxide consumption after 10 min of assay. For lower *E. coli* concentrations, the response increased slower, but after 10 min the consumption rate also

decreased. Therefore, these results suggested that 10 min of assay could be a good compromise between sensitivity and speed of assay.

3.1.2.3. Optimization of H_2O_2 concentration in the electrochemical cell. Hydrogen peroxide concentration in the electrochemical cell will influence the sensitivity of the response to *E. coli*. In order to study this effect, assays were performed using 5, 10 and 25 μM H_2O_2 in the measuring cell. These concentrations lay in the linear range of the calibration plot for H_2O_2 with the graphite–Teflon–HRP–ferrocene biosensor and, therefore, the decrease in current due to bacteria presence could be directly correlated with hydrogen peroxide consumption by bacteria, being possible to assess the speed of bacteria respiration. The results indicated that the lower the concentration of hydrogen peroxide the greater current deflection was observed. So, for 5 μM H_2O_2 a current deflection of 68 ± 4% was obtained whereas 39 ± 1% and 26 ± 4% were obtained for 10 and 25 μM H_2O_2, respectively. Despite this fact, 10 μM hydrogen peroxide was chosen for further studies, considering that the low current obtained for 5 μM gave rise to a lower signal-to-noise ratio than for 10 μM.

3.1.3. Calibration curves for direct E. coli determination

Different *E. coli* concentrations were added to the electrochemical cell containing 10 μM of hydrogen peroxide. The decrease in current was measured after 5 and 10 min of bacteria addition, respectively. Plotting the percentage of hydrogen peroxide consumption versus the logarithm of *E. coli* concentration allowed to obtain the calibration graphs with a non-linear shape. The experimental points can be adjusted to polynomial equations ($y = 7.60x^2 - 78.82x + 210.5$, $r^2 = 0.98$ after 5 min, and $y = 7.70x^2 - 74.88x + 194.0$, $r^2 = 0.94$ after 10 min) which would allow the calculation of bacteria concentration in an unknown sample by interpolating the percentage of hydrogen peroxide consumed into the equation. Therefore, this method allowed the quantification of *E. coli* in water samples with a detection limit of 5.7×10^7 and 3.6×10^6 cfu/mL, for 5 and 10 min assay time, respectively, taking into account the sample dilution after addition to the electrochemical cell. The detection limits were calculated as the bacterial concentration producing a decrease in the hydrogen peroxide signal of 3-fold the background noise.

3.1.4. Indirect E. coli determination

In order to avoid bacteria sample dilution due to the addition of a sample aliquot to the electrochemical cell, methodology described in Section 2.7, where hydrogen peroxide was added to the bacteria sample and was allowed to react for a fixed period of time, was implemented. Then, the remaining H_2O_2 in the *E. coli* sample is measured in the electrochemical cell with the graphite–Teflon–HRP–ferrocene composite biosensor. Prior to establish the analytical characteristics of this method, some experimental variable were optimised.

3.1.4.1. Influence of hydrogen peroxide concentration in consumption step. The hydrogen peroxide concentration in bacteria samples needs to be high enough to allow the measurement of the remaining current in the electrochemical cell. Nevertheless, if cannot be so high that the concentration in the cell is out of the linear range. Furthermore, very high hydrogen peroxide concentrations can be toxic for bacteria, despite the action of catalase. Pericone et al. [22] estimated that the minimum bactericidal H_2O_2 concentration for *E. coli* was of 15 mM. There were no significant differences between the percentages of hydrogen peroxide consumed by *E. coli* for H_2O_2 concentrations between 0.5 and 1.25 mM. Lower or higher concentrations produced smaller responses, and, accordingly, 1.25 mM hydrogen peroxide was selected for further work.

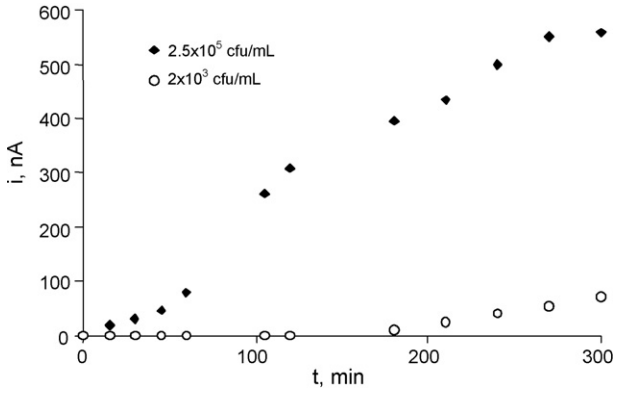

Fig. 4. Amperometric responses obtained with a 0.45-μm-pore size nylon membrane–graphite–Teflon–HRP–ferrocene composite electrode at an applied potential of 0.00 V, when 1 mL of *Streptococcus. pneumoniae* suspension was added to the cell after different incubation times in BHI broth at 30 °C.

3.1.4.2. Optimisation of reaction time in H_2O_2 consumption step. The influence of the reaction time on the amount of hydrogen peroxide consumed by *E. coli* in the indirect detection method was evaluated by allowing a 2.5×10^7 cfu/mL *E. coli* suspension to react with 1.25 mM hydrogen peroxide for different periods of time comprised between 2.5 and 15 min. Then, a 40 μL aliquot of this suspension was added to the electrochemical cell containing 5 mL of 0.05 M phosphate buffer solution of pH 6.5, and the current at 0.00 V was compared with that measured after the addition of a negative control solution without *E. coli*. The response increased with reaction time, but the increase was remarkably smaller from 10 to 15 min. A reaction time of 10 min was chosen in order to maintain assay time as short as possible.

3.1.4.3. Calibration curve for E. coli detection with the indirect method. Under the optimised conditions mentioned above, the peroxide consumed was determined by comparison of the measured current with that of a negative control solution. The calibration plot obtained obeyed the equation: hydrogen peroxide consumed %, $y = 20.35x^2 - 247.03x + 760.3$, $r^2 = 0.9455$. The limit of detection obtained in this case, according to the same criterion mentioned above, was 1.8×10^6 cfu/mL of *E. coli*.

3.2. Monitoring of hydrogen peroxide generation by catalase-negative bacteria using the graphite–Teflon–HRP–ferrocene electrode

It has been established that different *S. pneumonia* strains can generate up to 0.7 mM of hydrogen peroxide in their culture media [22]. Monitoring formation of a product instead of its consumption is a much simpler and sensitive approach. Therefore, a sensitive detection of *S. pneumoniae* through its peroxide formation is expected.

In this case, again both direct and indirect detection were considered. Direct detection consisted of the immersion of the electrodes in the bacterial culture from $t = 0$. When initial concentrations were lower than 10^7 cfu/mL, it was not possible to distinguish between the background drift and peroxide signal.

Consequently, in order to achieve an improved sensitivity in short times, indirect measurements were preferred, where aliquots of the culture media were added to the electrochemical cell after certain periods of culture time. Fig. 4 shows the amperometric signal due to hydrogen peroxide presence in the aliquots added to the cell for two different starting *Streptococcus* concentrations. It is observed that it was possible to detect H_2O_2 formation after 15 min of incubation at 30 °C of cultures containing 2.5×10^5 cfu/mL. Moreover, smaller bacteria concentrations, as low as 2×10^3 cfu/mL can be detected after 3 h of growing. Therefore, depending on the initial concentration of bacteria, different incubation times are required, but, in any case, a very sensitive detection is achieved in short periods of time, comparing to other methods.

3.3. Discussion

From the results shown above, it is demonstrated the feasibility to detect both catalase-positive and -negative bacteria in very short time. A standard assay would consist of adding the real sample in the appropriate generic media and allow its growing under aerobic and microaerophilic conditions, and monitor hydrogen peroxide formation or consumption. In this way, it would be possible to distinguish between both types of bacteria in a simple manner, and even more, to detect both of them when they are coexisting, as, under each of the mentioned growing conditions, only one type would grow. Moreover, if more selective information is required, it would be possible to establish a battery of culture in different selective media, or media containing different types of antibiotics, and monitor hydrogen peroxide evolution in all of them sequentially or simultaneously, in order to obtain information that allowed the identification of one species. On the other hand, the developed methodology can also be coupled to a previous selective capture step, such as immunofiltration, for example, in order to achieve specific detection. Future work will involve evaluation of such possibilities.

4. Conclusions

The use of a composite peroxidase biosensor for hydrogen peroxide measurement allowed direct monitoring of catalase-positive and catalase-negative bacteria in complex culture media, as interference effects are minimized at the applied potential value of 0.00 V for the amperometric measurements, in a simple, rapid, sensitive and reproducible way. The proposed methodology under the optimised conditions yielded reproducible measurements and a limit of detection of $\approx 2 \times 10^6$ cfu/mL *E. coli* in 10 min of assay, and of $\approx 2 \times 10^5$ cfu/mL *S. pneumoniae* in 15 min of assay, with no need of any pre-concentration, pre-enrichment or bacteria permeabilization step. Therefore, much lower limits of detection can be achieved easily, for example by sample filtration. The fast and simple detection method can be combined with a selective capture step to obtain rapid and selective analytical procedures for a large number of different bacteria, and can be easily automated for routine application.

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