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Electrochimica Acta

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

Fast determination of viable bacterial cells in milk samples using impedimetric sensor and a novel calibration method

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ARTICLE INFO

Article history:

Received 26 January 2016

Received in revised form 8 March 2016

Accepted 9 March 2016

Available online xxx

Keywords:

IDEA electrodes

Impedance

E. coli detection

calibration method

ABSTRACT

As a first stage toward the development of an impedimetric biosensor to quantify viable bacterial cells, this work defines the relationships between microbial growth and impedance changes associated with acidification of the culture medium. *Escherichia coli* bacterium was used as a model of a common pathogenic bacterium found in a foodstuff. Impedance was measured at a fix frequency of 10 kHz using interdigitated microelectrodes. Impedance changes were found to depend on the number of microorganisms in a sample. However, as the kinetics greatly depends on the bacterial concentration, it was not possible to obtain a single calibration curve in a wide concentration range at a fixed incubation time. To resolve this, a novel calibration method was proposed by measuring the sensor response at 270 and 390 min of incubation and taking a mean value. Obtained calibration plots were used to determine *E. coli* in a spiked milk samples. Performed experiments demonstrated the capacity of the method to detect *E.coli* concentrations in a range between 10^2 and 10^6 cfu mL⁻¹ in milk in only 6 hours with 5–12% error margin.

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1. Introduction

Effective pathogen detection is essential for the prevention and treatment of infectious diseases. Additionally, an accurate differentiation of live bacteria is important in many applications. Biosensors based on different types of transducers may be used for highly sensitive and specific detection of pathogens [1]. However, although those permit to reach good detection limits in most cases they are not able to differentiate between viable and non-viable cells. Conventional and standard methods of viability and quantification in bacteria are traditionally associated with the ability to form colonies on agar growth medium and to proliferate in liquid nutrient broths. However, these traditional culture-based tests are time-consuming and are not providing real-time results or timely information that is needed in applications such as industrial manufacturing.

By metabolic pathways bacteria convert large molecules, such as polysaccharides, lipids, nucleic acids and proteins, into charged, smaller and more mobile metabolites. These may include lactic

acid, acetic acid, carbon dioxide, ammonia, bicarbonate and urea, the presence of which provokes a change in the ionic composition of the growth media [2]. Thus, these changes can be measured and related to bacterial concentration for determination of microbial growth [3]. Since bacterial metabolism may significantly alter electrical conductivity of the growth media, impedance technique can be successfully applied for estimation of microbial biomass [4], detection of microbial metabolism, as well as for determination of the physiological state of bacteria growth [5–7]. Advantages of this approach are high sensitivity, relative simplicity and comparatively low cost of the required experimental equipment [8].

Impedance measurements of microbial growth are commonly related to conductivity changes due to the release of ionic products, however it should be noted that most of metabolic products of bacteria are of acidic nature, so that the microbial growth media can also lead to pH changes of the media [8]. Thus, pH measurements can also be used to control the bacteria growth [9].

Monitoring of acidic products concentration is usually carried out by commercial pH electrodes. Conventional glass pH electrodes have been exploited to detect pH changes in the medium produced by bacteria, for example in fermentation processes [10]. However, glass pH electrodes present several drawbacks, such as high price, large size, fragility, and miniaturization and integration constraints. For these reasons, numerous pH sensor alternatives have

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been explored over the last decades [11,12]. Among them stand out ion-sensitive field effect transistors (ISFETs) [13] and potentiometric ion selective electrodes based on different metal oxides [14]. One of the drawbacks of potentiometric measurements is that the method requires the use of a reference electrode, which makes the whole system too bulky. It is possible to design small size reference electrodes to be integrated within the system, but these have a short working lifetime and require maintenance. Integrated reference electrode–ISFET pair may be used in a small volume samples but the costs are rather high to be implemented in real life applications.

Potentiometric methods in comparison to conductivity measurements are less sensitive and rapid [15]. However, if the initial conductivity of the broth is high, then conductivity changes will be small and pH in this particular case should be a better indicator of bacterial activity.

From presented it follows that the impedance technique to register changes in a growth medium resulting from the metabolic products released by bacterial may be regarded as a most convenient one. Impedance measurements are carried out by applying a small AC signal between a pair of metal electrodes registering the resulting current and a phase shift between the voltage and the current. Electrochemical impedance spectroscopy (EIS) determines the response of an electrochemical cell to a voltage at different frequencies. Thus, impedance spectrum is obtained allowing characterization of the system composed by electrodes and bulk medium. EIS spectra are often analyzed using an equivalent circuit which consists of resistances and capacitances combined in parallel or serially, as required. The most typical equivalent circuit for this type of measurements can be represented by a series combination of a bulk solution resistance and an interfacial capacitance at the electrode–solution interface.

Electrodes for impedance measurements may be arranged as a pair of oppositely placed electrodes. However, it is possible to miniaturize this electrode arrangement by using two in-plain interdigitated microelectrodes. These impedimetric transducers, called interdigitated electrode array (IDEA), has been demonstrated as a promising for monitoring the growth of bacteria [7,16,17], since they present advantages in terms of fast establishment of the steady state signal, increased signal-to-noise ratio and the use of small sample solution volumes.

In this work to miniaturize the system instead of a pH-sensor and reference electrode an impedimetric sensor based on an interdigitated electrode array [18] was used to detect metabolic

activity of *Escherichia coli* bacteria. *Escherichia coli* was chosen as a target because it is an important pathogen responsible for numerous water and food transmitted infections whose growth under aerobic conditions causes the formation of acidic by-products, of which acetate is the most predominant [19,20].

2. Material and Methods

2.1. Sensor Fabrication

The sensor is based on a platinum interdigitated electrode array (IDEA) presented in Fig. 1. The sensor chip with silicon covered with silicon oxide as a substrate has dimensions of 3×3 mm. Parameters of sensors depend on the number of digits, the length and width of each digit and the distance between them. In this work the geometry of the sensor was: width of digits— $3 \mu\text{m}$, separation between electrode digits $20 \mu\text{m}$, number of digits—56, digit length 1.4 mm. Platinum is used as a metal to form the electrodes 120 nm thick embedded into insulating substrate with 1.5 micron thick oxide layer covered with 120 nm silicon nitride formed on silicon. To isolate all the contact lines and leave only the fingers in contact with the ambient, 150 nm thick silicon nitride passivation layer is formed in which a window $1500 \times 1500 \mu\text{m}^2$ is opened over the metal grid. After being cut from the wafer the sensors are glued to a PCB substrate and are wire bonded for electrical connections. Contact pads and wires were encapsulated using epoxy resin.

2.2. Preparation of bacterial suspensions

Escherichia coli ATCC 10536, obtained from the American Type Culture Collection was grown overnight in a 5 mL of Luria-Bertani (LB) broth (Sigma-Aldrich) at 37°C . After growth the culture was centrifuged (9000 G, 5 min) using a OrtoAlresa Biocen20 Eppendorf centrifuge and resuspended in a 5 mL of sterile LB medium for further use. The number of colony forming units per milliliter (cfu mL^{-1}) was counted by plate count after incubation the plates at 37°C overnight. The number of cells usually reached 10^8 cfu mL^{-1} .

The culture was diluted in serial with LB broth (Sigma-Aldrich) and glucose was added to fix its concentration at 80 mM. After this, bacterial suspensions with different concentration were aliquoted in 1 mL tubes as replicates. In the case of *E. coli* detection in milk samples dilutions of initial *E. coli* culture were performed in this

Fig. 1. Layout of the IDEA conductivity sensor.

medium and then 100 μL of each sample was added to 1 mL tubes (replicates) with 900 μL of LB-glucose medium. In both cases, tubes without bacteria inoculation were used as blank.

2.3. Impedance measurements

Impedance of the sensors was measured using an Ivium Compactstat impedance analyzer. Initially, the spectra were recorded in a 100 Hz–1 MHz frequency range with amplitude of the test signal of 25 mV. As the main changes in the impedance module values were observed at 10 kHz, this fixed frequency was chosen to perform measurements in the culture medium. More detailed information on measurement technique and spectra data treatment is presented in “Results and discussion” section. The spectra analysis and fitting were performed using ZView software.

In all the cases the methodology was the same. Bacteria samples were placed in the oven at 37 °C. Every hour, three tubes of each concentration (replicates) were extracted from the oven for impedimetric measurements. To eliminate the undesirable effect of the bacteria attachment to the interdigitated electrode surface that might provoke additional changes in the impedance, the process consisted in a single measurement placing a drop of approximately 50 μL of a studied sample on the sensor surface. The impedance measurements were taken in two minutes during which the impedance signal was stable. This means that possible effects of the solution evaporation can be neglected.

After performing the impedance test the samples were discarded to avoid contamination. All the measurements were performed in replicates using four different sensors. After the measurements sensors were washed with ethanol and deionized water.

In order to determine the absolute impedance variation between the bacterial cell sample (Z_{sample}) and the blank control (Z_{ref}), results are present in terms of Normalized Impedance Change (NIC) [4,21], which is defined by the following equation:

$$\text{NIC}(\%) = \frac{Z_{\text{ref}}}{Z_{\text{ref}} + Z_{\text{sample}}} \times 100 \quad (1)$$

The same formula was applied to calculate the Normalized pH Change (NpHC), where instead of the impedance subsequent pH values determined from the sensors calibration curves were used.

3. Results and Discussion

3.1. Impedance detection system

At high growth rates in conditions where an excess of glucose is presented oxidative routes of degradation may be not sufficient thus *E. coli* is able to reorganize the metabolism obtaining also energy by acetic acid formation [22], which may increase the produced pH changes.

Previous experiments performed with a pH metal oxide sensors [12] in the LB medium showed that during *E. coli* growth maximum changes of 2 pH units occur, descending from pH 7 (normal pH of the medium) to pH 5. These values coincide with those published in literature [23]. Earlier studies [24,25] of impedance changes during bacteria growth demonstrated that they are associated with the media conductance effect and are predominant at high frequency.

To detect pH changes in the bacteria growth medium by impedance measurements in the present work an interdigitated electrode array with platinum metal electrodes was used. The IDEA sensor response to changes in the surrounding media or on the electrode surface greatly depends on the sensor geometry, mainly on the electrode digits width and separation between adjacent digits. To reduce the effect of possible electrode surface contamination and its effect on the interfacial capacitance and overall impedance, the electrode width was chosen as small as 3 μm . Penetration of the electric field in the water solution of an IDEA sensor is more or less equal to the distance between two adjacent electrode digits. To reduce possible surface effects and increase the sensitivity to solution conductivity changes the separation between the electrodes was chose to be 20 μm . This results in a signal penetration distance of 23 μm .

Fig. 2 shows the studied IDEA sensor spectrum measured in a 10^{-5} M KCl solution with the conductivity of $2.5 \mu\text{S}\cdot\text{cm}^{-1}$. In the inset, an equivalent electrical circuit for measured spectra fitting is presented that is generally discussed elsewhere [18]. It is formed

Fig. 2. Nyquist plot of the impedance spectrum of the IDEA sensor measured in a 10^{-5} M KCl solution with the conductivity of $2.5 \mu\text{S}\cdot\text{cm}^{-1}$. In the inset the equivalent circuit used for spectra fitting.

by contact resistance, R_{CONT} , representing the resistivity of wire leads and thin film metal electrodes; stray or geometrical capacitance, C_{GEOM} , of the IDEA structure in a water solution; solution resistance, R_{SOL} , between the electrode digits; electrical double layer capacitance at the metal/solution interface, presented as a constant phase element, CPE_{DL} ; and Warburg impedance originating from diffusion in the Nernst layer [26].

The semicircle in Fig. 2 appears due to a parallel combination of the stray capacitance and the solution resistance and its right side intercept with the real impedance axis gives the R_{SOL} value. From spectra measured in solutions with different conductivity it is possible to obtain the calibration plot presented in Fig. 3 which gives the sensitivity of 0.5 cm^{-1} for the studied sensors. It must be noted that in highly conductive solutions the semicircle is not resolved and the sensor sensitivity becomes limited by the contact resistance, R_{CONT} .

Initially the experimental impedance spectra were measured in Luria-Bertani growth medium supplemented with glucose, the medium used for bacteria growth. To emulate pH variation produced by bacteria the test solution pH was changed from 7 to 4 with a one pH increment by adding acetic acid. Fig. 4 presents experimental impedance spectra measured at different pH values. The solution conductivity/resistivity at different pH, presented in Table 1, are in the range of $9.8\text{--}8.4 \text{ mS cm}^{-1}$ and do not vary significantly with the pH changes.

As follows from the presented results it is not possible to control pH changes of the studied samples by determining the R_{SOL} values from the high frequency spectra range. However, as shows the Nyquist plot in Fig. 4A, there are significant changes in the spectra shape at lower frequencies. The quality of spectra fitting using the equivalent circuit presented in the inset of Fig. 2 was high and the chi-squared parameter was in the range of $1 \times 10^{-4}\text{--}3 \times 10^{-4}$. The main parameter of the equivalent circuit that changes with pH of the sample is the Warburg diffusion impedance. This is revealed by Fig. 5 where the impedance spectra are plotted as $\log(|Z|)$ versus $(1/\sqrt{\omega})$. The straight line manifests the presence of the Warburg impedance [26].

The Warburg impedance of finite-length diffusion with reflective boundary was used for model fitting. This element has two parameters: W_r and W_c . Impedance of W element, Z_w , is given by the formula:

$$Z_w(\omega) = \frac{W_r}{\sqrt{\omega}}(1-j)\coth[W_c\sqrt{j\omega}] \quad (2)$$

where W_r is equal to Warburg coefficient, inversely proportional to mobile ion concentration and the square root of the diffusion coefficient. $W_c = d/D^{1/2}$ (d —Nernst diffusion layer thickness and D

— diffusion coefficient). W_c values obtained by fitting were of the order 10^{-4} and were not affected by the pH changes.

From the presented we may conclude that changes of pH in the complex medium under study affects the diffusion, hindering it in more acidic solutions. It may be also noted (see Table 1) that the solution conductivity, in contrast to what may be expected, tends to decrease as pH increases.

To perform the measurements the impedance module values were determined at fixed frequency of 10 kHz at which, as it follows from Fig. 4B, the highest response was obtained. $|Z|$ values determined in the growth media solutions at different pH are presented in Table 1.

Calibration curves, obtained on four different IDEA sensors, presented in Fig. 6 confirm the correlation between impedance and pH value of the culture medium at this fixed frequency. In all the cases a linear increase of the impedance module was observed with pH decrease of the growth medium with R^2 of 0.99 which proves that the proposed method of measuring impedance using interdigitated microelectrodes at a fix frequency may be used to control pH changes of the bacteria growth medium. However, the slope of the pH response was slightly different both between the distinct IDEA electrodes ($n=4$) and for the same electrode within different calibrations performed ($n=5$). More concretely, coefficients of intra-electrode variation of $5.1 \pm 0.5\%$ were found, while differences between electrodes (inter-electrode) were higher with variation of $11.4 \pm 0.3\%$. However, it may be noted that inter-electrode variation is of minor importance as individual sensor calibration parameters and not their mean values were used for data analysis.

As it was mentioned in the experimental section, results of the impedance measurements were normalized taking into account the impedance changes in the medium without microorganisms (control), denominated Normalized Impedance Change (NIC) [21] (eq. (1)). Additionally, the growth media pH measurements results were also represented as normalized values (Normalized pH Change, NpHC). This allows us to compare the results obtained with different electrodes and in different bacteria cultures.

3.2. Impedance change caused by bacterial growth

Initially, preliminary experiments were performed to study the effects of bacterial metabolism on the electrical properties of the growth medium and the impedance of the IDEA electrodes. To monitor this the impedance at 10 kHz was measured in samples taken after 6 hours of incubation from the *E.coli* cultures with the bacteria concentration range between 10^2 and 10^6 cfu mL^{-1} .

Fig. 3. The sensor response to the solution resistivity changes.

Fig. 4. Nyquist (A) and Bode (B) plots of the impedance spectra of the sensor measured in the growth culture medium at different pH values adjusted by adding acetic acid to the initial sample.

Table 1

Properties of the test solutions, Warburg coefficient determined from spectra fitting and the impedance module determined at 10 kHz measured in the growth media solutions at different pH.

pH	Cond (mS/cm)	R, Ohm cm	W _r , Ohm s ^{-1/2a}	Z at 10 kHz, Ohm
4.02	8.36	120	12194	5094
5.03	9.68	103	8799	4620
6.01	9.77	102	5760	3599
7.04	9.84	102	4027	2752

^a Mean values of four sensors.

Impedance and pH variation data can be represented as a percentage of the normalized change (Fig. 7) (Eq. (1)) and plotted against the incubation time. Similar trends but in opposite direction were observed in both, impedance and pH changes, as Z vs. pH is a linear function. Variations of 2 and 0.95 units of NIC and NpHC, respectively, were detected after 150 min in cultures with the highest *E. coli* concentration (10^6 cfu mL⁻¹) reaching, after 390 min of incubation, variations of 11.62 (units of NIC) and 5.65 (units of NpHC). These changes became less with the decrease in concentration of the initial bacterial culture. For example, 270 min were necessary to detect NIC variations of 2.93 in cultures with initial concentrations of 10^4 cfu mL⁻¹, while in the case of cultures

with 10^2 cfu mL⁻¹, 390 min were necessary for a similar change (2.88). For all bacteria concentrations within the studied range the impedance increased with time, which according to sensors calibration results reveals acidification of the sample. How it is expected [2,3], these changes are greater and faster the higher the initial inoculum culture is. Typical bacteria growth curves are of a sigmoid shape.

After being introduced into the culture bacteria remain in a “lag phase” in which no growth occur. Durability of this stage depends on different factors, such as the type of a growth medium, temperature or initial concentration of the inoculum. After this the “log phase” starts with exponential growth of bacteria and the release of their metabolic by-products into the medium increases. Finally, microorganisms tend to shift to “stationary phase” of growth and the production of by-products is limited due to the depletion of nutrients necessary for bacterial growth and accumulation of acids [22,27].

In an attempt to determine the incubation time, in which a linear relationship is produced between NIC and bacterial concentration allowing a quantification of the bacteria present in the sample in a widest concentration range, NIC values was represented in relation to *E. coli* concentration to each time (Fig. 8). However, no linear relationship was observed between NIC values and all the concentrations tested at any time. A linear relation was

Fig. 5. Bode graph of the impedance spectra plotted as $\log(|Z|)$ versus $(1/w)^{1/2}$. The straight line manifests the presence of the Warburg impedance.

Fig. 6. Calibration curves, of four different IDEA sensors measured at a fix frequency of 10 kHz, in the growth medium. Conductivity of the test solutions at different pH is also shown.

Fig. 7. Normalized impedance changes (NIC) (solid symbols) and normalized pH changes (NpHC) (open symbols) calculated for different *E. coli* concentration cultures. Standard error bars correspond to the measurements made in three different culture replicate of each *E. coli* concentration measured with 4 different IDEA electrodes.

Fig. 8. Normalized impedance changes (NIC%) measured in the growth media for different initial *E. coli* concentration at different times (from 0 to 390 min). Standard error bars correspond to the measurements made in three different culture replicate of each *E. coli* concentration measured with 4 different IDEA electrodes.

detected after 270 min of incubation in initial culture concentrations between 10^4 and 10^6 cfu mL $^{-1}$, although this time was not enough for lower bacteria concentrations to produce medium changes. In the same way, linearity was detected in the impedance change produced by cultures with concentrations from 10^2 to 10^4 cfu mL $^{-1}$ after 390 min of incubation. Thus, longer incubation times prevent the observation of impedance changes in high *E. coli* concentrations due to the culture reaches the stationary phase.

Concentrations of 10^1 cfu mL $^{-1}$ were also studied but required much longer times to show differences in impedance with respect to the blank. However, these times are out of the time scale of the proposed method of detection and calibration, as at longer times samples with high bacteria concentrations will reach saturation making it impossible to distinguish between different concentrations. That is why we define 10^2 cfu mL $^{-1}$ as the detection limit for the proposed method with working concentration range of 10^2 – 10^6 cfu mL $^{-1}$.

3.3. Development of a calibration curve for *E. coli* concentration determination

Impedance changes were found to depend on the number of microorganisms in the samples in the concentration range 10^6 to 10^2 cfu mL $^{-1}$. However, as the kinetics greatly depends on the bacterial concentration, it was not possible to obtain a calibration curve in a wide concentration range at a fixed incubation time. To resolve this, a novel calibration method was proposed by measuring the sensor response at 270 and 390 min of incubation and taking a mean value.

For this five different overnight cultures with *E. coli* concentrations in the range between $9 \cdot 10^7$ and $9 \cdot 10^8$ cfu mL $^{-1}$ were used to perform the necessary dilutions and obtain different initial concentrations in the test samples. And the measurements were performed with 4 different IDEA sensors. NIC values obtained at 270 and 390 min in each of the culture dilutions and measured with four sensors were calculated and their mean values are presented in Fig. 9. It can be seen that at 270 min incubation time the sensitivity at low bacteria concentration is low, while the 390 min response curve tends to saturation at high concentrations. To obtain a more linear relationship between NIC and the log [*E. coli*] in the whole concentration range a new calibration curve,

presented in Fig. 9 as black dots, was generated using the average values obtained at both times. In this way linear response was achieved with a regression equation: $\text{NIC (\%)} = -2.44 \log [E. coli] + 53.51$, with $R^2 = 0.986$ for the concentration range of 10^2 – 10^6 cfu mL $^{-1}$.

3.4. Quantification of *E. coli* in pure cultures

This approach with our impedimetric biosensor was tested by quantification of *E. coli* in growth culture to demonstrate the capacity and precision of the method to quantify bacteria concentrations in the range between 10^2 and 10^6 cfu mL $^{-1}$. With this aim 5 samples with unknown concentrations within this range were analyzed after 290 and 370 min of incubation. The calculated NIC values at both times and the resulting average are presented in Table 2. Fig. 10 shows the calibration curve and plots NIC average values obtained for each of the blind samples. The extrapolation of the obtained NIC values to the calibration curve allows us to estimate the bacterial concentration present in the sample. However, more precision is obtained by using the equation of the lineal regression model taken from calibration curve (Table 2).

Additionally, the real bacteria concentration in each of the samples was determined by plate count and compared with the *E. coli* concentration calculated by impedance measurements showing a good relation with an error margin of 3–14%. Thus, presented experimental results confirm the capacity of the proposed method to determine bacteria concentration.

3.5. Quantification of *E. coli* in milk samples

In order to assess the performance of the reported method in a more complex matrix the quantification of *E. coli* was performed in milk samples. Milk was chosen as a real matrix due to the necessity of the dairy industry to obtain fast, sensitive and cost effective analytical systems for pathogen detection [28,29].

To minimize the possible matrix effect of the sample on the impedance measurements the electrodes were first calibrated in LB growth medium supplemented with glucose and inoculated with milk demonstrating that no significant interferences affect in impedance measurements with IDEA electrodes in milk samples.

Fig. 9. Normalized impedance change obtained at 270 and 390 minutes of incubation. Average curve is the resulting calibration plot used to determine *E. coli* concentration in samples.

Table 2

NIC values obtained with blind samples (a–d) and the estimates of the initial *E. coli* concentration of each sample using the linear regression obtained from calibration curve ($\text{NIC}\% = -2.44 \cdot x + 53.51$, being (x) the log of *E. coli* concentration. Real bacteria concentration was calculated by plate count. $\Delta\epsilon$ corresponds to coefficient of variation.

		t = 270 min	t = 390 min	Average	Calculated log[E. coli]	Calculated [E. coli] (cfu mL ⁻¹)	Real [E. coli] (cfu mL ⁻¹)	$\Delta\epsilon$ (%)
NIC (%)	a	42.2	38.4	40.3	5.4	2·10⁵	4·10 ⁵	3.7
	b	49.3	42.7	46	3.1	1·10³	4·10 ³	14.6
	c	50	46.2	48	2.2	2·10²	4·10 ²	13.9
	d	46.3	39.6	42.9	4.3	2·10⁴	4·10 ⁴	6.1
	e	40.3	36.9	38.6	6.1	1·10⁶	4·10 ⁶	7.5

Fig. 10. Calibration curve (solid symbol) and NIC value of the blind samples (a–d) (open symbols) obtained by the average of the NIC obtained at 270 and 390 min. Extrapolation of the NIC value of each sample to the calibration curve allows approximating the initial *E. coli* concentration of the sample.

Fig. 11. Calibration curve (solid symbol) and NIC value of the samples (open symbols) with the milk inoculation obtained by the average of the NIC obtained at 270 and 390 min. Extrapolation of the NIC value of each sample to the calibration curve allows approximating the initial *E. coli* concentration of the sample.

Table 3

NIC values obtained with milk samples (a–e) inoculated with different concentrations of *E. coli* (1–6) and the estimates of this concentration using the linear regression obtained from calibration curve ($\text{NIC}\% = -2.44 \cdot x + 53.51$, being (x) the log of *E. coli* concentration. $\Delta\epsilon$ corresponds to coefficient of variation.

		t = 270 min	t = 390 min	Average	Calculated [E. coli] (cfu mL ⁻¹)	Real [E. coli] (cfu mL ⁻¹)	$\Delta\epsilon$ (%)
NIC (%)	a	50.3	49.2	49.8	3·10²	5·10 ⁵	5.3
	b	50.3	46.2	48.3	1·10³	5·10 ³	11
	c	49.7	42	45.9	1·10⁴	5·10 ²	12.5
	d	46.1	39.3	42.7	3·10⁵	5·10 ⁴	8.8
	e	41.7	38.1	39.9	4·10⁶	5·10 ⁶	6.9

In the experiment milk samples were inoculated with *E. coli* at different concentrations for the subsequent determination of bacterial concentration. After this 100 μL volume of each milk sample was added to the growth culture medium (900 μL) supplemented with glucose, generating a 1/10 dilution. At 270 and 390 min of incubation time impedance measurements were performed. As previously, using the calibration curve bacteria concentration in each of the samples was determined from the obtained NIC average values. These results are presented in Fig. 11 and Table 3.

It is necessary to note that corresponded results were obtained after a 1/10 dilution of the milk sample, therefore the real concentration in the milk is 10 times higher than determined in the test sample.

Comparing the results of *E. coli* concentration obtained by linear regression of calibration curve (Table 3) and those obtained by plate count, it was verified that the developed method permits to determine bacteria concentration with 5–12% error margin. The performed experiments demonstrate the capability of the method to detect *E. coli* concentrations in a range between 10^2 and 10^6 cfu mL^{-1} in milk in only 6 hours. It is possible to find in literature faster detection times reported [28,30], however in these works the detection is referred to total microbial cells present in a sample, not viable cells.

It must be noted that 6 hours incubation for determination of viable bacterial cells at concentrations of 10^2 – 10^6 cfu mL^{-1} can be considered a fast method. Reported techniques using impedance to detect viable cells show detection limits between 10^4 and 10^8 cfu mL^{-1} after 8–5 hours of incubation [31–33] or till 8 cfu mL^{-1} , but after about 15 hours of measurement [34–36].

4. Conclusions

We have reported an impedimetric method applicable for the detection and quantification of *E. coli* bacterium in milk samples in a wide concentration range using an interdigitated electrode array sensor. Impedance measurements performed at a fixed frequency of 10 kHz permit to detect electrical changes in the growth medium originating from pH changes due to bacterial metabolism. In contrast to other publications, where impedance technique is used to detect interaction of bacteria with the electrode surface, the proposed method determines only changes in metabolic products concentration in the sample.

By the analysis of the impedance changes caused by different bacterial concentrations at different incubation times we can conclude that kinetics of impedance change depends on the bacterial concentration. Thus, a unique incubation time could not be established to differentiate within a wide range of bacterial concentrations. To overcome this problem a novel calibration method was developed by registering the sensor response at 270 and 390 min of incubation and taking a mean value of both results. The developed methodology was successfully employed for quantification of *E. coli* concentration in the range between 10^2 and 10^6 cfu mL^{-1} in milk samples with 5–12% error margin.

It is clear that the presented method lacks the specificity as other bacteria, e.g. *Listeria monocytogenes*, which could be present at the same time in test samples may provoke pH changes as well. However, proposed method that defines the relationships between microbial growth and impedance changes associated with acidification of the culture medium should be regarded as a first stage toward the development of an impedimetric biosensor to quantify viable bacterial cells. So, the *Escherichia coli* bacterium was used in this work as a model of a common pathogenic bacterium found in a foodstuff. Currently we are working to convert this method into a specific one for different food pathogens

using biorecognition elements or using specific culture media designed for certain microbial type.

Acknowledgements

Authors acknowledge financial support from Spanish Ministry of Economy and Competitiveness (project CTQ2014-54553-C3-1-R) and European Union (project ENIAC-ED-52 v161210).

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