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Scientific paper

The Use of Yeast *Saccharomyces Cerevisiae* as a Biorecognition element in the Development of a Model Impedimetric Biosensor for Caffeine Detection

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Abstract

In the present study, an electrochemical-impedimetric biosensor using *Saccharomyces cerevisiae* as an effective biorecognition element was designed to detect caffeine. The presented biosensor consists of a previously developed stainless steel electrochemical cell constructed as a three-electrode system in the RCW side-by-side configuration. The electrochemical stability of the sensing electrode was evaluated by measuring the open circuit potential (OCP), and electrochemical impedance spectroscopy (EIS) was applied to determine the impedimetric response of the biosensor with *Saccharomyces cerevisiae* cells attached to the working electrode (WE) in the absence (0.9% NaCl) and presence (10 mg/mL in 0.9% NaCl) of caffeine. Moreover, the limit of detection (LOD) was determined. In this way, a new approach in biosensor development has been established, which involves assembling a low-cost and disposable electrochemical system to detect alkaloids such as caffeine. The developed biosensor represents a good candidate for detecting caffeine in beverages, foods, and drugs with the merits of time-saving, robustness, low cost, and low detection limit.

Keywords: Impedimetric biosensor, *Saccharomyces cerevisiae*, electrochemical impedance spectroscopy, caffeine

1. Introduction

The demand for biosensors has increased significantly in the recent years due to the need for specific sensors that can provide fast and reliable measurements in various research areas. The development of biosensors is of interest for different applications ranging from biochemical profiling of normal and pathologic cells, over clinical diagnostics and drug discovery to more straightforward analyses such as fermentation, process monitoring, environmental testing, and food and beverages quality control.[1–3] Detection of alkaloids such as caffeine has attracted abundant attention due to their extensive occurrence in beverages and drugs.[4] However, conventional detection methods for caffeine (high-performance liquid chromatography-mass spectrometry (HPLC-MS), thin-layer chromatography (TLC), and immunoassay) have several drawbacks, including expensive equipment as well as complex and laborious sample preparation.[5]

Biosensors, as analytical devices, convert a biological response into an electrical signal and provide us with the information on the concentration of the target analyte.[6,7] Biosensors may present the best candidates for detecting caffeine with the merits of high sensitivity and specificity, convenience, time-saving, low cost, and low detection limit.[8] Biosensors can be based on animal tissues, bacteria, or eukaryotic microorganisms such as yeasts.[9] Although yeasts are highly resistant to adverse environmental conditions, they can sense and respond to a variety of stimuli and, unlike several alternative biological components, do not require sophisticated sterile techniques or complex media.[10,11] Yeast *Saccharomyces cerevisiae* represents a single-cell eukaryotic organism used primarily in the food industry to produce bakery products and alcoholic beverages.[9,12] It is chemoorganotrophic and anaerobic organism classified in the kingdom of Fungi, phylum *Ascomycota*, class *Saccharomycetes*, order S*accharomycetales*, and family *Saccharomycetaceae*.[13] *Saccharomyces cerevisiae* can exist in two different forms, the haploid or the diploid form.[13,14] A yeast cell possesses the typical characteristics of a eukaryotic cell and characteristic organelles such as vacuoles

and lipid droplets.[12] It is usually spherical to slightly spherical and occasionally ellipsoidal to cylindrical.[15]

Caffeine (1,3,7-trimethylxanthine), with the chemical molecular formula of $C_8H_{10}N_4O_2$, has been used for thousands of years and represents one of the most widely consumed food ingredient throughout the world.[1,16] It is found in common beverages such as coffee, tea, and soft drinks, as well as in products containing cocoa or chocolate, and in a variety of medications and dietary supplements.[17,18] Due to the high consumption of caffeinated foods, beverages, and medicines worldwide, caffeine is also considered to be the most representative pharmaceutically active pollutant with regard to its abundance in the environment.[19] Based on the data reviewed, it is concluded that in the healthy adult population, daily caffeine intake at a dose exceeding 400 mg is associated with adverse effects such as general toxicity, cardiovascular effects, effects on bone status and calcium balance, changes in adult behavior, increased cancer incidence and effects on male fertility.[20] The caffeine content in coffee products ranges from 0.27 to 1.85 mg/mL, in tea from 0.11 to 0.23 mg/mL, in energy drinks from 0.30 to 0.37 mg/mL, and in soft drinks such as regular cola from 0.10 to 0.13 mg/mL.[21–23] Given these values, the biosensor may be sufficiently sensitive and robust enough to cover the range of caffeine concentrations present in beverages.

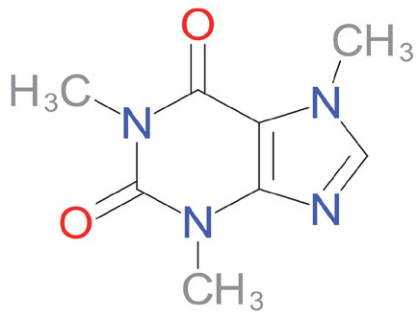

Figure 1: Chemical structure of the caffeine molecule

For the detection of different caffeine concentrations, the electrochemical impedance spectroscopy (EIS) method was applied. EIS is widely used in the production and optimization of biosensors as this method allows for the characterization of the biological component attached to the sensor and of the analyte present in the sample.[24,25] Because biosensors produce a rapid response, they can be applied to monitor molecular events in real-time.[26] The EIS method was used to measure the frequency response of the electric current, which provides data on the adhesion layer of Saccharomyces cerevisiae on the electrode surfaces.

The aim of this study was to develop a model electrochemical impedimetric biosensor for the detection of caffeine using *Saccharomyces cerevisiae* as an effective biorecognition element with many advantageous properties such as cell robustness, ease of maintenance, and cell production rate.

2. Experimental

The developed biosensor consists of a stainless steel electrochemical cell (Figure 2) constructed as a three-electrode system in the RCW-side by side configuration, including the working electrode (WE) with yeast cells on the surface, the reference electrode (RE), and the counter electrode (CE). Such electrochemical cell was previously developed and tested.[10] In assembling the electrochemical cell, stainless steel type SS316 (manufacturer TBJ Industries, Germany) was used. The electrodes were manufactured with a dimension of electrode 20 mm × 5 mm, where the active component was applied to the 5 mm × 5 mm. The electrodes were insulated on the fixation side, and the system was sealed with glass.

Saccharomyces cerevisiae was applied to the working electrode using a technique involving a mold made with a 3D printer, which ensured that the layer thickness (0.10 mm) was similar for all measurements. 3.8 g *Saccharomyces cerevisiae* was mixed with 1 mL 0.9% NaCl to ensure that the mixture was viscous. The mixture was applied on the stainless-steel electrode inserted in the mold. The process of coating the working electrode (WE) was taken at 25 °C and took approximately 30 seconds.

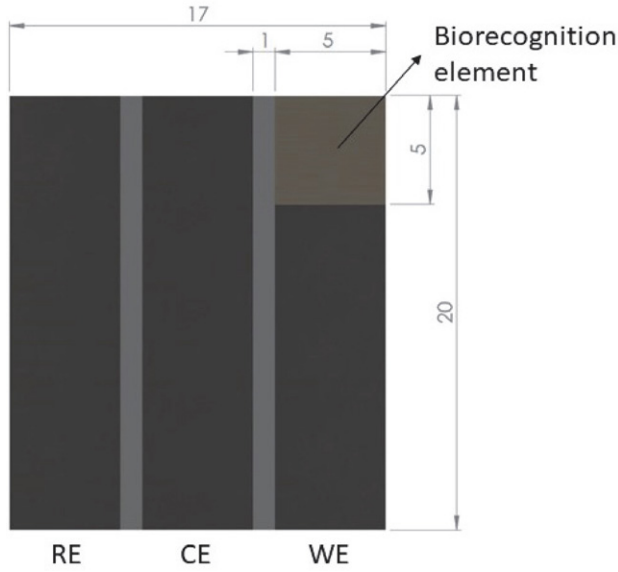

Figure 2: Electrochemical cell in the RCW-side-by-side configuration and the Saccharomyces cerevisiae cells attached to the working electrode (WE).

Two solutions were prepared for the measurements, the 0.9% NaCl solution (Sigma Aldrich, CAS: 7647-14-5, M: 58.44 g/mol) and 10 mg/mL caffeine (Sigma Aldrich,

CAS: 58-08-2, M: 194.19 g/mol) in 0.9% NaCl solution. The electrochemical cell with *Saccharomyces cerevisiae* on the working electrode was connected to the Multi Palmsens4 potentiostat. Initially, 1 mL of the 0.9% NaCl solution was injected into the system, and the open circuit potential (OCP) and EIS measurements were performed. Then, the excess saline (0.9% NaCl) was drained. Afterward, 1 mL of the 10 mg/mL caffeine solution was injected into the system, and OCP and EIS measurements were repeated.

The electrochemical characterization of the working electrode was evaluated by measuring the OCP to assess the stability of the electrode. The duration of the measurement was 600 seconds since living yeast cells were utilized.

The EIS method was applied to evaluate the biosensor with yeast on the stainless-steel surface. Impedance spectra were obtained at a steady open circuit potential (OCP) in the frequency range from 100 kHz to 10 mHz with 10 points per decade and a 20 mV amplitude of the excitation signal. The potential amplitude was chosen at 20 mV since the lower amplitudes have not provided the stable signal due to the yeast layer on the stainless-steel electrode

The EIS measurement's expected duration was 2 minutes and 15 seconds, although this time was often extended up to 3 minutes. EIS was used to obtain data on the processes on the surface of the electrode and the applied layers, and the Bode and Nyquist plots were interpreted as the results.

Moreover, the limit of detection (LOD) was determined based on the impedance drop with the increasing concentration of caffeine in 0.9% NaCl. For the measurement, eight different caffeine concentrations in saline (0.0 mg/mL, 0.05 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, and 5.0 mg/mL) were prepared and 1mL of each sample was injected into the system separately. The blank solution consisted of 0,9% NaCl solution (saline). Three measurements were taken for each concentration, where the mean value (MV), standard deviation (SD), precision, and accuracy of the measurements were calculated. The measurements were taken using identical parameters as in the measurements mentioned before, and the data were obtained at the frequency 125 mHz.

3. Results and Discussion

3. 1. Open Circuit Potential (OCP) Measurements

The electrochemical characterization of the sensing electrode was evaluated by measuring the OCP to assess the stability of the electrode. The OCP provides valuable insight into the thermodynamic stability of the electrode material involved in the electrochemical response.[27]

The results are represented in Figure 1S in the Supplementary information. The data provided from the measurements indicate that the system was thermodynamically stable. When measuring with *Saccharomyces cerevisiae* on the stainless steel surface of the working electrode (WE) with the addition of 0.9% NaCl and the addition of 10 mg/ml caffeine in 0.9% NaCl, a slight difference is observed at OCP in each measurement. The slight change in OCP is due to the living cells on the surface, which react to environmental conditions.

3. 2. Electrochemical Impedance Spectroscopy (EIS)

EIS represents a non-destructive method that can be used to quantify specific parameters and simultaneously monitor multiple electrochemical processes.[28] The measurements are explained with the real (electrical resistance) and imaginary (capacitance) components of the impedance response of an electrochemical system.[10]

In the Nyquist diagram (Figure 3), the decrease in the resistance (real component, x-axis) and the decrease in the capacitance (imaginary component, y-axis) when 10 mg/mL caffeine in 0.9% NaCl was added to the system compared to the blank solution (0.9% NaCl) is seen. The results indicate that the electrode surface was released due to the detachment of yeast cells from the electrode surface.

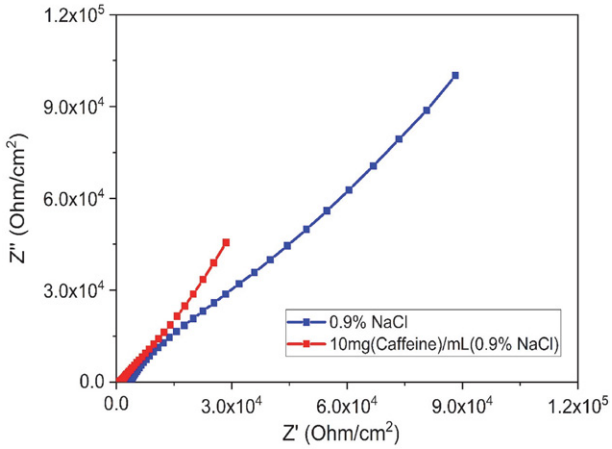

Figure 3: Nyquist diagram of the EIS measurement of the biosensor containing *Saccharomyces cerevisiae* on the WE, with the addition of 0,9% NaCl (blue) and 10 mg Caffeine in 1 mL 0.9% NaCl (red).

Bode plots consist of two spectra simultaneously, the impedance spectrum and the phase spectrum, in which the dependence of impedance (Z) and the dependence of phase angle on the frequency is shown. In the impedance spectrum, the activity at the working electrode is determined from the slopes of the line, and in the phase spectrum, the activity is determined from a phase angle.

Based on the impedance spectrum of the Bode diagram (Figure 4), the solution resistance (R_s) was determined with a slope of approximately 0 (high-frequency range), the capacitance of the electrical double layer (C_{dl}) was deter-

mined with a slope of approximately –0.8, which occurs at the phase boundary between the electrode and the electrolyte (middle-frequency range), the charge transfer resistance was determined with a slope of approximately 0 (R_{ct}) which occurs due to the electrochemical reaction or due to the charge transfer between the electrolyte and the metal (middle- frequency range), as well as the diffusion with a slope approximately –0.5 (low-frequency range) was determined.

In the phase spectrum of the Bode diagram (Figure 5), the resistance is described as the negative phase at approximately 0°, the non-ideal capacitance with the negative phase at approximately 55°, the diffusion at the negative phase at approximately 45°.

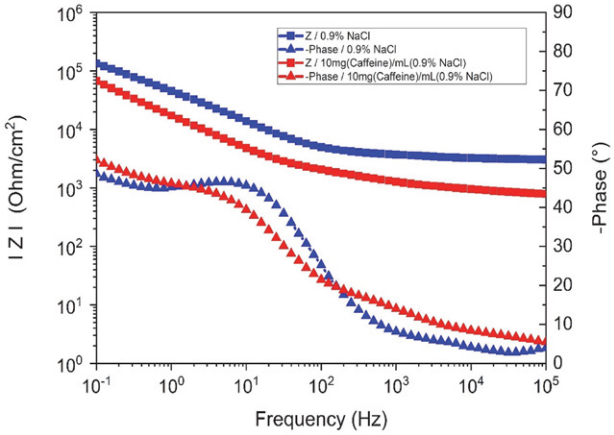

Figure 4: Bode diagram of the EIS measurement of the biosensor containing *Saccharomyces cerevisiae* on the WE, with the addition of 0,9% NaCl (blue) and 10 mg Caffeine in 1 mL 0.9% NaCl (red). The Bode diagram includes an impedance diagram described with squares and a phase diagram described with triangles, where I Z I represents an impedance and -Phase presents a negative phase shift.

The equivalent electrical circuits (EEC) of the stainless steel electrochemical cell without and with the yeast on the working electrode (WE) are shown in Figure 5.

The EEC of the electrochemical cell without yeast cells attached to the stainless steel electrode is depicted in Figure 5A. The equivalent circuit consists of the solution resistance (R_s), the capacitance of the electrical double layer (C_{dl}), the charge transfer resistance (R_{ct}), and the Warburg impedance (W_o).

The EEC of the electrochemical cell with the yeast cells attached to the working electrode is shown in the Figure 5B and consists of the solution resistance (R_s), the yeast layer capacitance (C_y), the yeast layer resistance (R_y), the capacitance of the electrical double layer (C_{dl}), the charge transfer resistance (R_{ct}), and the Warburg open diffusion (W_o).

The comparison of the values of the parameters where the 0.9% NaCl and the 10 mg/mL caffeine in 0.9% NaCl were separately added to the system is provided in Table 1.

When caffeine was added to the system, the *Saccharomyces cerevisiae* cells detached from the stainless steel surface, and consequently, the electrode surface was released. Consequently, the resistance of the system dropped, and the capacitance and the impedance of diffusion increased. The χ^2 of the measurement where 0.9% NaCl was added to the system was 2.94×10^{-3}, and for the measurement where the 10 mg/mL caffeine in 0.9% NaCl was added 1.48×10^{-3}.

Table 1: Comparison of the EEC parameters when the 0.9% NaCl and the 10 mg/mL caffeine in 0.9% NaCl were added to the system.

Parameters	0.9% NaCl	10 mg/mL Caffeine
R_s (kOhm/cm^2)	3.07	0.69
R_y (kOhm/cm^2)	55.65	1.08
R_{ct} (kOhm/cm^2)	103.35	78.97
C_y (μF/cm^2)	21.17	2237.20
C_{dl} (μF/cm^2)	77.26	208.72
W_{or} (kOhm/cm^2)	8.42	12.68
W_{oc} (Ohm/cm^2)	0.09	1.21

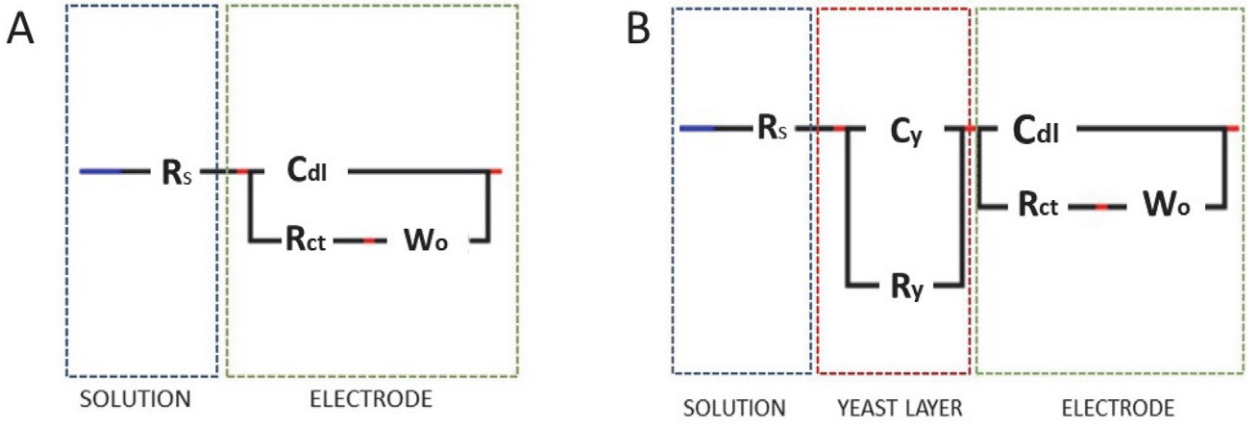

Figure 5: The equivalent electrical circuit (EEC) of the electrochemical cell without the yeast attached to the working electrode (Figure 5A) and with the electrochemical cell with yeast attached to the working electrode (Figure 5B). Figure 5A consists of the solution resistance (R_s), the capacitance of the electrical double layer (C_{dl}), the charge transfer resistance (R_{ct}), and the Warburg impedance (W_o). Figure 5B consists of the solution resistance (R_s), the yeast layer capacitance (C_y), the yeast layer resistance (R_y), the capacitance of the electrical double layer (C_{dl}), the charge transfer resistance (R_{ct}), and the Warburg open diffusion (W_o).

3. 3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

In Table 2, the decrease in impedance (|Z|) with the increasing concentration of caffeine in saline (c) is reported. Compared to the blank solution, the decrease in impedance is observed with the addition of 0.1 mg/mL of caffeine in 0.9% NaCl. The data were obtained at a frequency of 125 mHz. Three measurements were taken for each concentration, where the mean value (MV), standard deviation (SD), precision, and accuracy of the measurements were calculated.

Table 2: The decrease in impedance with increasing concentration of caffeine in 0.9% NaCl

C (mg/mL)	logC (mg/mL)	MV log|Z| (kOhm /cm^2)	SD (kOhm /cm^2)	Precision (%)	Accuracy (%)
0.00	/	237.472	0.539	99.503	99.681
0.01	−2.00	231.935	2.895	97.051	98.318
0.05	−1.30	221.839	2.998	96.827	98.166
0.10	−1.00	106.922	1.929	96.161	97.515
0.25	−0.61	98.402	0.555	98.627	99.310
0.50	−0.30	94.551	0.272	99.312	99.616
1.00	0.00	88.514	0.583	97.949	98.859
5.00	0.70	75.801	0.332	98.175	99.010

The impedance decrease with increasing concentrations is also represented as a box plot (Figure 6). The data were obtained at a frequency of 125 mHz. The box plot shows the mean values as a dot, the upper whiskers represent the maximum, the lower whiskers represent the minimum, and the box represents the interquartile range. The distinct decrease in the impedance is observed at 0.1 mg/mL concentration.

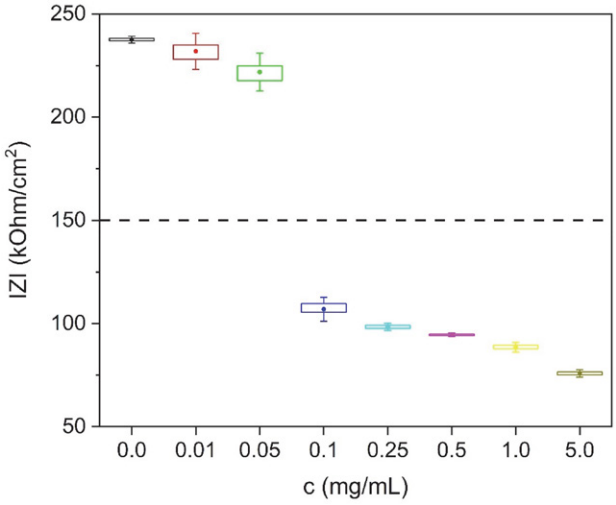

Figure 6: Box plot representing the impedance (log|Z|) decrease with the increasing concentrations (c) of caffeine

The system's linearity was obtained in the concentration range from 0.1 mg/mL to 5 mg/mL with R^2 of 0.997 (Figure 7). The concentrations were calculated to logarithmic values (Table 2) to obtain linear regression since the impedance values read from the Bode plot were logarithmic. Based on the 3-Sigma criteria, the LOD was determined at 0.728 mg/mL, and based on the 10-sigma criteria, the LOQ was determined at a concentration of 0.382 mg/mL. It was observed that the impedance decreased with the increasing concentration of the caffeine in the solution. Thus, it can be concluded that the biosensor can sense the presence of caffeine in the solution.

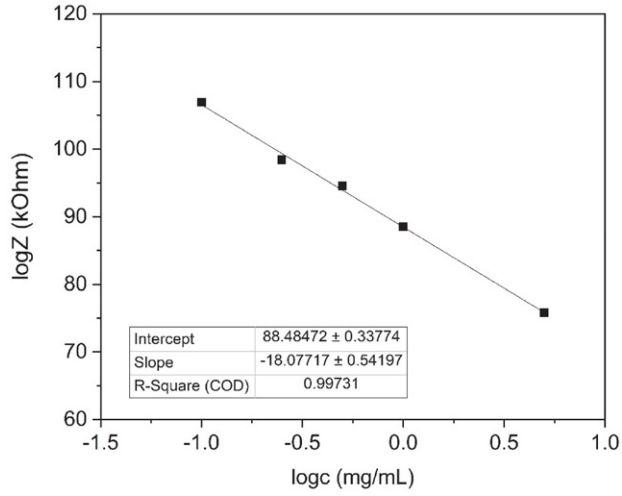

Figure 7: Calibration curve between the impedance (log|Z|) and the caffeine concentrations (logc)

In conclusion, the Nyquist plot and the Bode plot of the investigated biosensor show that adding 10 mg/mL of caffeine in saline decreased the resistance and increased the capacitance, indicating that the electrode surface is released. Due to the *Saccharomyces cerevisiae* detachment from the stainless steel surface, the parameters of the EEC changed: the system's resistance decreased, and the capacitance increased. Consequently, it can be indeed concluded that caffeine can cause the desorption and death of *Saccharomyces cerevisiae* cells.

Some other research was done in the field of electrochemical biosensors and caffeine detection. An amperometric biosensor for the determination of caffeine in solutions was developed, where whole cells of *Pseudomonas alcaligenes* were utilized. The biosensor system was able to detect caffeine in solution over a concentration range of 0.1 to 1 mg/mL.[1] In comparison, the linearity range with our biosensor was in the range of 0.1 to 5 mg/mL. A biosensor based on the inhibition of alkaline phosphatase (ALP) enzyme was developed for caffeine determination, where caffeine concentration can be determined accurately between 0.1 and 10 μM and the LOD of the biosensor was 0.08 μM.

This biosensor, compared to ours, had a lower LOD and linearity range as the enzymes were used as a biorecognition element. Also, an electrochemical impedance aptasensor based on a porous organic framework supported silver nanoparticles for ultrasensitively detecting theophylline, with the LOD of 0.191 pg/mL (1.06 pmol/L) in a wide concentration range of 5.0×10^{-4} to 5.0 ng/mL (2.78×10^{-3} to 27.8 nmol/L) was developed.[29]

4. Conclusions

A new approach in biosensor development has been established, which involves assembling a low-cost and disposable electrochemical system for the detection of alkaloids such as caffeine. The caffeine detection with the presented method avoids an excessive use of solvents, requires only a small amount of analyte, and does not require lengthy preparation.

It was observed that the impedance decreased with the increasing concentration of the caffeine in the solution. It can be concluded that the developed biosensor is robust enough to detect the various caffeine concentrations. Based on the linear calibration curve of the impedance decrease with the increasing caffeine concentration, the LOD was determined at 0.728 mg/mL, and the LOQ was determined at 0.382 mg/mL. Therefore, it can be concluded that yeasts, although very resistant to adverse environmental conditions, can sense and respond to caffeine as stimuli.

Biosensors have the potential to represent the best candidate for caffeine detection with the merits of time-saving, robustness, low cost, and low detection limit. In the future, an upgraded impedimetric biosensor with the biorecognition element *Saccharomyces cerevisiae* could be used to detect the caffeine content in various beverages, foods, and medicines. Furthermore, impedimetric biosensors based on the described approach and using a simplified potentiostat/galvanostat, which is transferable and compared to other conventional methods cost less, could also be applied on a large scale for food monitoring, environmental monitoring, or even medical diagnostics.

Acknowledgments

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Povzetek

Za detekcijo kofeina smo razvili elektrokemijski-impedančni biosenzor, ki kot biološko komponento uporablja kvasovke vrste *Saccharomyces cerevisiae*. Predstavljen biosenzor je sestavljen iz predhodno razvite elektrokemijske celice, narejene iz nerjavnega jekla v RCW konfiguraciji. Elektrokemijska stabilnost delovne elektrode je bila ocenjena s potencialom odprtega tokokroga (OCP). Elektrokemijska impedančna spektroskopija (EIS) je bila uporabljena za spremljanje impedimetričnega odziva biosenzorja s celicami *Saccharomyces cerevisiae* na površini delovne elektrode (WE) pri odsotnosti (0.9% NaCl) in prisotnosti (10 mg/mL v 0.9% NaCl) kofeina. Določena je bila tudi meja zaznavnosti (LOD). Razvit je bil nov pristop v razvoju biosenzorjev, ki vključuje sestavo ekonomično dostopnega biosenzorja, namenjenega enkratni uporabi za detekcijo alkaloidov kot je kofein. Razvit biosenzor je dober kandidat za detekcijo kofeina v pijači, hrani ter zdravilih, saj omogoča hitro detekcijo, z nizko mejo zaznavnosti ter z nizko mejo določljivosti, hkrati pa je tudi ekonomičen.