Biosensors and Bioelectronics 115 (2018) 1–6

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Development and characterization of a field-effect biosensor for the detection of acetoin

Denise Molinnus[a,b], Lukas Muschallik[a], Laura Osorio Gonzalez[a], Johannes Bongaerts[a], Torsten Wagner[a], Thorsten Selmer[a], Petra Siegert[a], Michael Keusgen[b], Michael J. Schöning[a,c,*]

[a] Institute of Nano- and Biotechnologies (INB), FH Aachen, Campus Jülich, Heinrich-Mußmannstr. 1, 52428 Jülich, Germany
[b] Institute of Pharmaceutical Chemistry, Philipps-University Marburg, Wilhelm-Roser-Str. 2, 35032 Marburg, Germany
[c] Institute of Complex Systems 8 (ICS-8), Research Center Jülich, Wilhelm-Johnen-Str. 6, 52425 Jülich, Germany

ARTICLE INFO

Keywords:
Acetoin biosensor
Acetoin reductase
Butane-2,3-diol dehydrogenase
Field-effect EIS structure
Diluted wine samples

ABSTRACT

A capacitive electrolyte-insulator-semiconductor (EIS) field-effect biosensor for acetoin detection has been presented for the first time. The EIS sensor consists of a layer structure of Al/p-Si/SiO_2/Ta_2O_5/enzyme acetoin reductase. The enzyme, also referred to as butane-2,3-diol dehydrogenase from *B. clausii* DSM 8716^T, has been recently characterized. The enzyme catalyzes the (*R*)-specific reduction of racemic acetoin to (*R,R*)- and *meso*-butane-2,3-diol, respectively. Two different enzyme immobilization strategies (cross-linking by using glutaraldehyde and adsorption) have been studied. Typical biosensor parameters such as optimal pH working range, sensitivity, hysteresis, linear concentration range and long-term stability have been examined by means of constant-capacitance (ConCap) mode measurements. Furthermore, preliminary experiments have been successfully carried out for the detection of acetoin in diluted white wine samples.

1. Introduction

Acetoin and diacetyl are widely distributed in various beverages, and are also used in foods and cosmetics as flavouring and fragrance, as well as in chemical synthesis (Hahn et al., 1987; Lawson et al., 1995; Vasavada and White, 1977; Xiao et al., 2012). These compounds are products of fermentative metabolism in different microorganisms. Acetoin can be formed, e.g., in most bacteria from pyruvate and is thus a product of carbohydrate metabolism, or from diacetyl while NAD(P)H serves as a cofactor of the enzyme acetoin reductase (Cogan et al., 1981; Huang et al., 1999).

During the fermentation process of alcoholic beverages, such as beer or wine, acetoin plays an important role in their quality due to its buttery-like taste, although acetoin is not pungent smelling (Romano and Suzzi, 1996). Typical acetoin concentrations in alcoholic beverages are in the range of 10–50 µM in beer, ~500 µM in white wine and ~150 µM in red wine (Haukeli and Lie, 1975; Reed and Nagodawithana, 1991; Romano and Suzzi, 1996). The detection of acetoin content during the fermentation process could control the quality of alcoholic beverages due to its involvement in the wine bouquet or its influence in the beer flavor. Furthermore, the acetoin concentration during beer storage is used as a parameter to establish the degree of the beer's maturity (Haukeli and Lie, 1975). Precise detection of the acetoin level can be used to avoid unnecessary maturation time (Romano and Suzzi, 1996; Vann and Sheppard, 2005). Hence, its control of concentration change during the fermentation course could help assess the fermentation processes, as well as the maturation process.

Several methods have already been described for the detection of acetoin, mainly colorimetric techniques, like the Voges-Proskauer test, which is the most commonly applied procedure for the detection of acetoin in analytical microbiology or gas chromatography (Levine, 1916; Speck and Freese, 1973). However, none of these techniques provide the advantages that can be achieved by using a biosensor which offers a faster analytical approach and that does not need additional trained staff.

Capacitive EIS sensors are field-effect devices that are used for the detection of surface potential changes, e.g., due to pH alterations (Poghossian et al., 2004). These changes can also be induced by, e.g., enzymatic reactions (Poghossian et al., 2001b; Schöning et al., 2005b; Siqueira et al., 2014; Thust et al., 1996), and binding of charged molecules, such as DNA (Abouzar et al., 2012; Bronder et al., 2015; Poghossian et al., 2001a; Veigas et al., 2015). These sensors can also be applied for the development of enzyme logic gates (Molinnus et al., 2017b; Poghossian et al., 2011, 2015). Furthermore, EIS sensors have many advantages over conventional analytical methods such as small size, low weight and fast response time, and they are easy and cost-

* Corresponding author at: Institute of Nano- and Biotechnologies (INB), FH Aachen, Campus Jülich, Heinrich-Mußmannstr. 1, 52428 Jülich, Germany.
E-mail address: schoening@fh-aachen.de (M.J. Schöning).

https://doi.org/10.1016/j.bios.2018.05.023
Received 6 March 2018; Received in revised form 27 April 2018; Accepted 10 May 2018

effective in fabrication (Poghossian and Schöning, 2014; Schöning et al., 2005a). Furthermore, due to the miniaturized sensor layout, a small sample volume is necessary for the measurement. Additionally, with an array of different modified EIS sensors, several analytes can be detected simultaneously.

In this study, we report on the development of a chip-based biosensor for the acetoin detection for the first time. The sensor is based on a pH-sensitive capacitive EIS field-effect structure consisting of an Al/p-Si/SiO_2/Ta_2O_5 layer set-up. The chip was modified using a recently introduced acetoin reductase from *B. clausii* DSM 8716 ^{T} (Muschallik et al., 2017) accountable for the reduction of acetoin in the presence of NADH as a cofactor (Molinnus et al., 2017a). The local pH shift induced by the enzymatic reaction resulted in the modulation of the flat-band potential of the field-effect biosensor, arising in a shift of the capacitance-voltage (C-V) curve of the EIS structure. Two immobilization strategies, namely adsorptive and cross-linking by forming cross-linkages between the enzyme molecules, have been investigated to attach the acetoin reductase to the pH-sensitive Ta_2O_5 surface of the EIS sensor chip. Characteristic biosensor parameters such as linear concentration range, pH optimum of the field-effect sensor with the immobilized enzyme, sensitivity, lower and upper detection limit, hysteresis and long-term stability will be discussed. In addition, the newly developed acetoin biosensor was applied for measurements in real samples such as white wine.

2. Experimental

2.1. Materials

Acetoin and the cofactor NADH were acquired from Sigma-Aldrich (USA), as well as glutaraldehyde, glycerol and NaCl. TRIS-HCl buffer (0.2 mM) was purchased from Carl Roth (Germany). The respective pH values of the buffer solution were adjusted by addition of 0.1 M NaOH or 0.1 M HCl. The enzyme acetoin reductase from *B. clausii* (~380 U/mL) is produced in our institute as described before (Muschallik et al., 2017).

2.2. Preparation of the sensor structures

The applied capacitive EIS sensor consists of the following layer stack: a p-doped silicon substrate with a thickness of ~400 µm and a specific resistance of ρ = 5–10 Ωcm, a 30 nm thermally grown SiO_2 insulating layer and a 60 nm thick Ta_2O_5 gate insulator layer (for that 30 nm Ta is deposited by electron-beam evaporation, followed by a thermal oxidation step). A rear side contact, consisting of a 300 nm thick aluminum layer is deposited by electron-beam evaporation and annealed afterwards. As a final step, the wafer is separated into 1 cm × 1 cm chips with a diamond saw. Fig. 1 shows the EIS-sensor set-up with the different layers. Detailed information about the sensor's fabrication process is described in Ref. (Schöning et al., 2005a). The Ta_2O_5 layer as gate insulator has been selected because of its well-known excellent pH behavior and high permittivity but also because of its chemical stability (Atanassova and Spassov, 1998; Chaneliere et al., 1998).

The acetoin EIS biosensor was developed by modifying the Ta_2O_5 surface with the enzyme acetoin reductase. Immediately before modification, each sensor was cleaned in acetone, isopropanol and deionized water for 5 min, respectively. Two different immobilization strategies have been investigated. As first immobilization method, the enzyme acetoin reductase is adsorptively bound to the sensor surface. For this, 80 µL of acetoin reductase solution was dropped onto the sensor surface. For the second immobilization procedure, cross-linking is performed by formation of cross-linkages between the enzyme molecules, where the membrane cocktail consisting of 48 µL glutaraldehyde (2 vol%) / glycerol (10 vol%) solution and 32 µL of enzyme solution was mixed. 80 µL of the membrane cocktail was pipetted onto the Ta_2O_5 surface. After drying of the different prepared EIS sensors, they were mounted into a homemade measuring cell, sealed by an O-ring to protect the rear side contact and to define the contact area of the EIS sensor with the analyte solution (~0.5 cm^2). Before measurements, the sensors were stored at 4 °C in the dark.

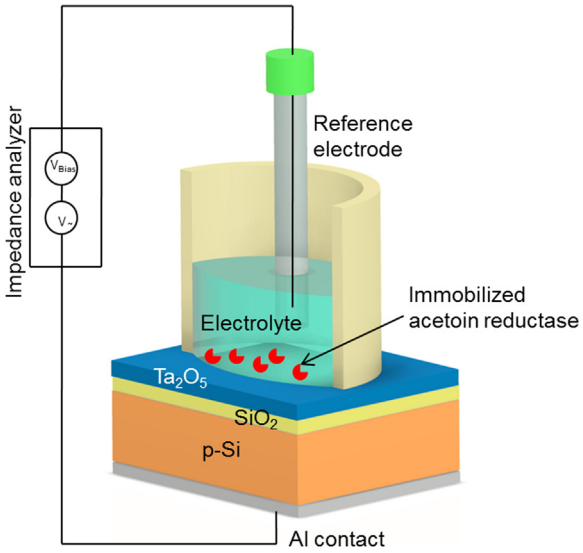

Fig. 1. Schematic of the measurement set-up with the Al-p-Si-SiO_2-Ta_2O_5 EIS sensor modified with the enzyme acetoin reductase for the detection of acetoin.

3. Measurement principle

Fig. 1 illustrates the measurement set-up with the developed acetoin field-effect biosensor. For the electrochemical characterization of the acetoin biosensor chip, C-V (capacitance / voltage) and ConCap (constant capacitance) measurements were performed by connecting the EIS chip with an impedance analyzer IM6 (Zahner Elektrik, Germany). Before performing ConCap measurements, C-V curves of each sensor chip, in a gate voltage range between −2 V and 2 V with steps of 100 mV were recorded to define a fixed capacitance value (in the linear range of the depletion region, ~60% of the maximum capacitance) using a feedback-control circuit. With the help of ConCap measurements, potential and/or charge changes at the Ta_2O_5 surface can be detected in real time. An external liquid-junction Ag/AgCl electrode (Metrohm, Germany) filled with 3 M KCl was applied as the reference electrode. The C-V and ConCap measurements were carried out at a frequency of 120 Hz. A 20 mV ac (alternating current) voltage has been applied between the Ag/AgCl reference electrode and the rear side Al contact, to measure the capacitance.

The measurement principle for the detection of acetoin using the capacitive field-effect sensor is based on the enzymatic reaction as depicted in Eq. (1). (*R*)- and (*S*)-acetoin will be reduced by the R-specific enzyme acetoin reductase to (*R,R*)-2,3-butanediol and *meso*-butanediol, respectively, while NADH serves as a cofactor and will be oxidized to NAD^+.

$$\text{acetoin} + \text{NADH} + H^+ \xrightarrow{\text{acetoin reductase}} 2,3\text{-butanediol} + \text{NAD}^+ \quad (1)$$

As a result of this enzymatic reaction, the hydrogen ion concentration decreases, and this pH change can be detected by the pH-sensitive Ta_2O_5 transducer surface of the field-effect sensor. The resulting change in the flat-band potential of the sensor is recorded and corresponds to the measured acetoin concentration. All measurements were performed in a dark Faraday cage at room temperature. Before starting the measurements, the sensor chip was incubated in 0.2 mM TRIS-HCl buffer solution (pH 7.1) containing 150 mM NaCl for 2 h. All solutions contained 500 µM of the cofactor NADH and all measurements were performed in 1 mL analyte solution containing different acetoin

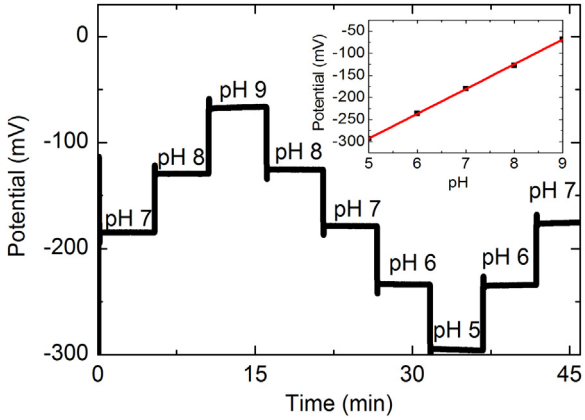

Fig. 2. Typical ConCap measurement for different pH values of Titrisol buffer solution of a bare EIS sensor; inset figure depicts the resulting calibration curve with an average slope of 55.9 mV/pH between pH 5 and pH 9.

concentrations varying from 1 µM to 500 µM. The pH value of the solutions used was additionally controlled by a pH meter (Mettler-Toledo, Germany).

4. Results and discussions

4.1. Electrochemical characterization of the capacitive acetoin biosensor

Before surface modification, the functionality and pH sensitivity of the bare EIS sensor chip was studied. Therefore, the pH sensitivity of each sensor was determined in the ConCap measurement mode with standard pH buffer solutions (Titrisol, Merck, Germany). The result of a typical ConCap measurement of a bare EIS sensor chip is presented in Fig. 2, for the pH values of 7-8-9-8-7-6-5-6-7, respectively. Each pH value has been recorded for 5 min. As the pH buffer solution is changed, an immediate signal step is perceived. This result shows that the EIS sensor is highly reproducible as demonstrated for pH 7.0, which was measured three times within this measurement cycle, yielding a potential at this pH value that is always −179 ± 5 mV. The corresponding calibration curve is shown in the inset figure of Fig. 2 and demonstrates a nearly Nernstian pH sensitivity of 55.9 mV/pH, as described in literature (Schöning et al., 2005a).

Additionally, different acetoin concentrations in the range between 30 µM and 90 µM were tested with the bare sensor chip (i.e., without the immobilized enzyme) (data not shown). The variation of the acetoin concentration only resulted in negligible potential changes of less than 3 mV, which can be related to slight pH variations of the analyte and drift effects of the sensor chip itself. In a further experiment, the EIS sensor chip modified with the enzyme acetoin reductase was also examined with regard to its original pH sensitivity in Titrisol buffer solutions of different pH values (identical procedure as performed in Fig. 2). The sensor modified by the enzyme possesses a similar sensitivity of 55.7 mV/pH as for the bare EIS sensor. Thus, the immobilized enzyme has no influence on the pH response of the pH-sensitive Ta_2O_5 transducer layer.

The immobilization of the enzyme onto the sensor surface is always an essential step in the development of a biosensor. Several immobilization methods such as adsorptive, covalent bonding, entrapment or cross-linking have been discussed in literature (Sassolas et al., 2012). For the immobilization of the enzyme acetoin reductase, two different immobilization methods were investigated, in particular, adsorptive and cross-linking by using glutaraldehyde to form a stable membrane which is drop-coated onto the sensor surface. Biosensor chips were prepared with each immobilization method. The influence of the enzyme immobilization on the sensitivity of the capacitive acetoin EIS biosensor chip was studied at different acetoin

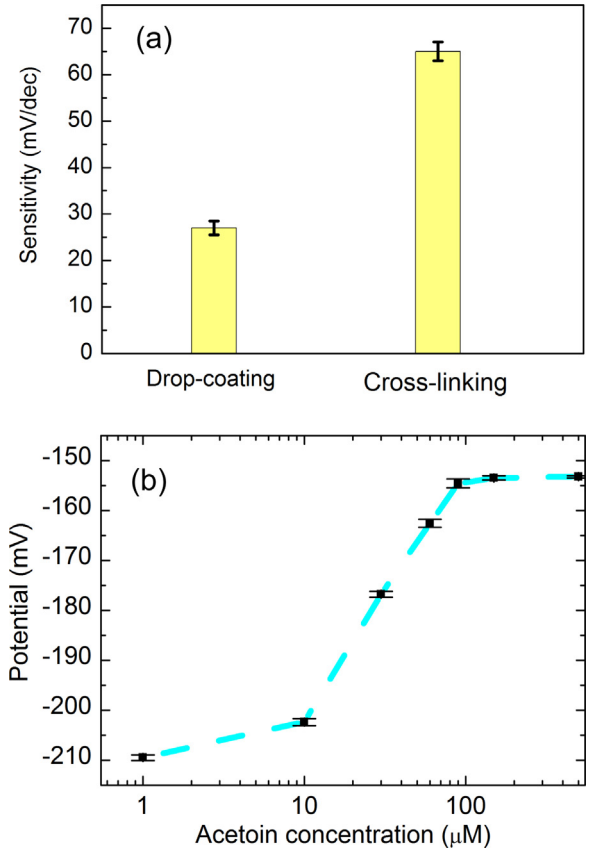

Fig. 3. (a) Mean values of the sensitivity with standard deviation (set of three series of measurements for each immobilization method) for the capacitive acetoin EIS biosensor chip recorded in buffer solution with different acetoin concentrations from 30 µM to 90 µM. (b) Calibration plot with standard deviation obtained with the capacitive, cross-linked acetoin EIS biosensor chip measured in an acetoin concentration range between 1 µM and 500 µM.

concentrations ranging between 30 µM and 90 µM in 0.2 mM TRIS-HCl buffer solution, containing 150 mM NaCl and 500 µM NADH at pH 7.1. Fig. 3(a) shows the results of the mean values of the sensitivities. The two bars depict the mean values of the sensitivities obtained from each three individual sensors modified using adsorptive and cross-linking, respectively. The highest sensitivity has been observed with the sensors modified by means of cross-linking with a mean sensitivity of 65 mV/dec, while the sensors modified using adsorptive immobilization only have a mean sensitivity of 27 mV/dec. These results demonstrate that although the adsorption method causes little to no enzyme inactivation, the enzymes are probably loosely attached to the sensor surface resulting in less amount of enzymes, which are fixed on the surface and hence, a lower acetoin sensitivity was achieved. Therefore, for the subsequent experiments, the sensor was modified by applying the cross-linking method for the immobilization of the enzyme.

To investigate the lower and upper detection limit of the developed biosensor chip towards acetoin, ConCap measurements were furtherly performed in the acetoin concentration range between 1 µM and 500 µM. Fig. 3(b) shows an S-shaped calibration curve, as typically expected for electrochemical biosensors. The sensor signal is plotted versus the logarithmic acetoin concentration. A linear behavior in the acetoin concentration range between 10 µM and 90 µM with a sensitivity of 65 mV/dec is given. A saturation effect is resulting for acetoin concentrations higher than 150 µM. Due to the enzymatic reaction, the pH increase close to the sensor surface might lead to an enzyme inhibition due to the pH dependence of acetoin reductase's activity (see also Fig. 4). Note that a shift in the biosensor signal of about 65 mV for varying acetoin concentrations corresponds to a pH shift from originally

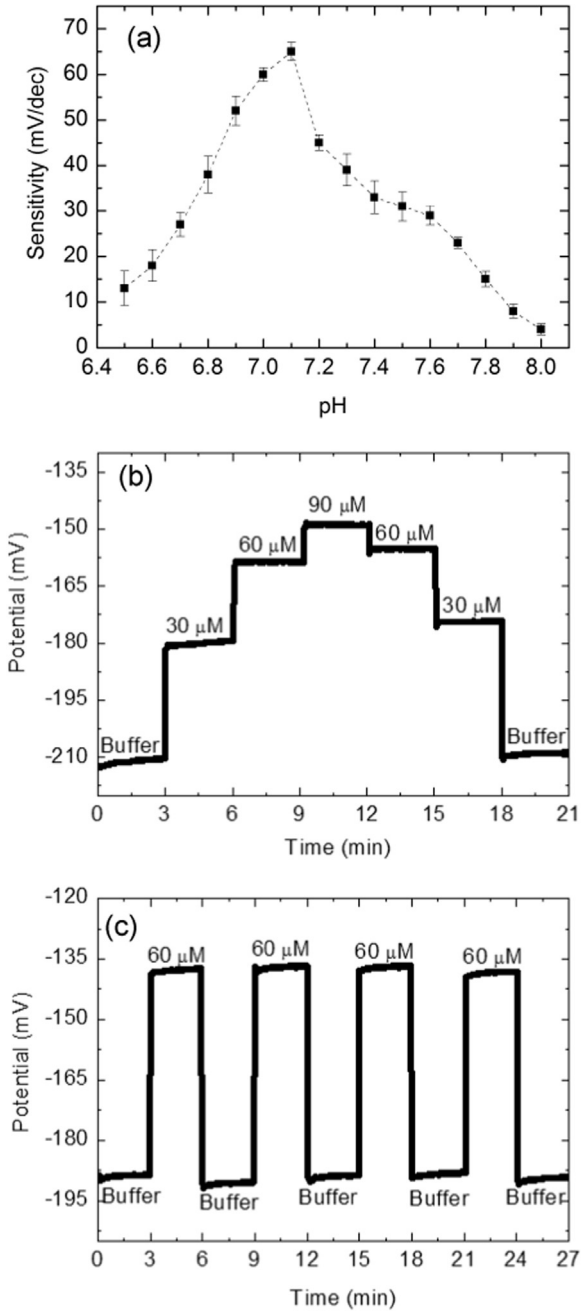

Fig. 4. (a) Mean values of the sensitivity with standard deviation (set of three series of measurements) for three fabricated capacitive acetoin EIS biosensors recorded in TRIS-HCl buffer containing 150 mM NaCl in the pH range between pH 6.5 and pH 8.0 with different acetoin concentrations from 30 µM to 90 µM. (b) Typical ConCap calibration measurement of different acetoin concentrations in the range from 30 µM to 90 µM measured by the acetoin biosensor chip. (c) Reproducibility measurement of buffer solution alternating with 60 µM acetoin.

pH 7.1 to about pH 8.2 at the biosensor surface. This is defined by the pH sensitivity of the Ta_2O_5 layer with immobilized enzyme of about 56 mV/pH as described above. On the other side, the curve starts to get flat at low acetoin concentrations (< 10 µM) that defines the detection limit of the biosensor set-up.

To study the pH influence on the sensitivity of the developed capacitive acetoin EIS biosensor in more detail, measurements in the ConCap mode were performed by variation of the pH of the buffer solution over a range from pH 6.5 to pH 8.0. Different acetoin concentrations ranging from 30 µM to 90 µM (buffer solution) were taken into account for the measurement. Fig. 4(a) summarizes the mean values and their standard deviations of the sensitivity obtained for three individual acetoin biosensors determined at each pH value. Maximum response with a sensitivity of 65 ± 4 mV/dec could be reached at a pH value of ~7.1, which is also consistent with the pH optimum of the used acetoin reductase (~pH 7.0) for the given reaction (Muschallik et al., 2017).

Fig. 4(b) demonstrates an example of a ConCap measurement of the acetoin biosensor with regard to its hysteresis behavior and response time. Acetoin concentrations were varied starting with a concentration of 30 µM up to 90 µM and then decreasing the concentration again down to 30 µM. Each concentration was recorded for 3 min and was varied in steps of 30 µM. A clear dependence of the sensor signal on the acetoin concentration is observed. The sensor signal is increasing with an increase of the acetoin concentration due to the decrease of the H^+-ion concentration as discussed in Eq. (1) affected by the biocatalytic reaction. Furthermore, the sensor output is almost the same in the upward and downward series of the performed measurement with a small hysteresis of less than 5 mV. In contrast to standard measurement techniques for the detection of acetoin (Speck and Freese, 1973), the responds time $t_{90\%}$ is only about 25 s by using the developed acetoin EIS biosensor.

To proof the reproducibility of the developed acetoin biosensor chip, the sensor signal of the measurement performed in TRIS-HCl buffer solution at pH 7.1 was recorded in alternation with an acetoin concentration of 60 µM in the ConCap mode. Fig. 4(c) shows exemplarily the results obtained with one sensor, when this cycle is repeated four (60 µM acetoin) and five (buffer) times, respectively. The sensor signal is increased to a value of −137 ± 1 mV with each acetoin concentration step and again decreased when measured in buffer solution to a value of −189 ± 1 mV. This experiment again underlines that the sensor delivered reproducible and reversible results independent of the titration direction, only with a small hysteresis of ~1 mV.

The stability of a biosensor often limits its use to a certain number of measurements, and losses of sensitivity are caused due to either the decrease of the applied enzyme activity or the enzyme leakage out from the sensor surface after certain time. Therefore, the biosensor's stability has been studied over a time period of 5 days. At each day, different acetoin concentrations between 30 µM and 90 µM were measured in buffer solution. Three individual biosensor chips were tested. Between the measurements, the sensors were stored under dry conditions in the refrigerator at 4 °C. The results of mean values of the obtained sensitivities with their standard deviations are presented in Fig. 5. Within

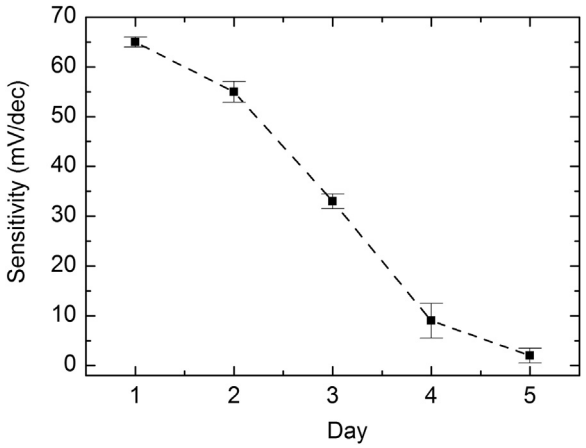

Fig. 5. Long-term stability of the acetoin biosensor. Mean values of the sensitivity with standard deviations of three individual biosensors recorded in buffer solution at different days (day 1 to day 5) with different acetoin concentrations from 30 µM to 90 µM.

the first two days, the biosensors show good sensitivities of about 65 mV/dec and 55 mV/dec, respectively. However, after two performed measurement days, the sensitivity starts to decrease rapidly to a value of 3 mV/dec at day 5. These results are comparable to results obtained, when the pure enzyme activity was investigated in solution without immobilization (Muschallik et al., 2017).

4.2. Application of the acetoin biosensor chip in wine

The aim of the newly developed capacitive acetoin EIS biosensor is to control and monitor fermentation processes to avoid unnecessary storage of beer or wine. Therefore, such biosensor chip should be able to detect acetoin in real fermentation samples.

In this study, preliminary experiments have been performed in diluted white wine samples (alcohol 10 vol%) to verify the ability of the acetoin biosensor. The pH value of white wine was about pH 3.3. After dilution with TRIS-HCl buffer solution (pH 7.1) containing 150 mM NaCl (wine to buffer mixture of 1:50), the pH value was about pH 3.8. Therefore, the pH value was adjusted to pH 7.1, the optimum working pH of the field-effect sensor with the immobilized enzyme, by addition of 0.1 M NaOH. Different acetoin concentrations between 1 µM and 500 µM were spiked to the wine-buffer mixture. As already demonstrated for the buffer solution containing varying acetoin concentrations (see Fig. 3(b)), Fig. 6(a) depicts again a typical S-shaped calibration curve obtained in the wine-buffer mixture with the potential recordings depending on the logarithmic acetoin concentration. Here, a linear behavior was reached in the acetoin concentration range between 10 µM and 90 µM with an average sensitivity of 40 mV/dec. Note

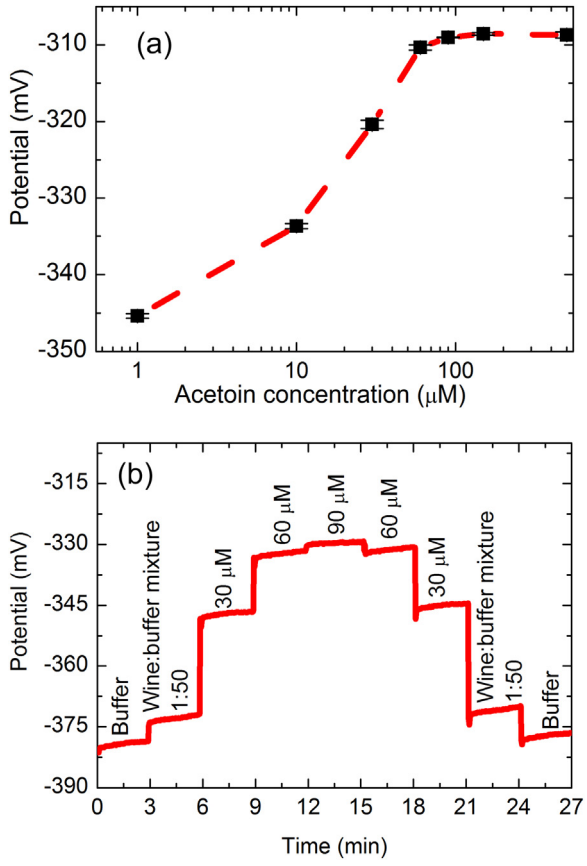

Fig. 6. (a) Calibration plot with standard deviation obtained with the capacitive acetoin EIS biosensor measured in a concentration range between 1 µM and 500 µM in a wine-buffer mixture (1:50). (b) ConCap measurement of different acetoin concentrations in a wine-buffer mixture (1:50) in the range from 30 µM to 90 µM measured by the acetoin biosensor chip.

that the average sensitivity is somewhat lower than for the acetoin measurements in buffer without wine (65 mV/dec). On the other hand, the sensitivity and detection limit of the developed biosensor is sufficient to detect acetoin in the industrially relevant concentration range. For the detection of higher concentrations, the sample might be diluted if necessary.

Fig. 6(b) demonstrates exemplarily a ConCap response of the acetoin biosensor in buffer solution and wine-buffer mixture, both at pH 7.1, without acetoin or spiked with different acetoin concentrations (30–90 µM). The sensor signal obtained in each solution was recorded for 3 min. Again, with increasing acetoin concentrations, the biosensor signal raises, clarifying the clear dependence of the biosensor signal in wine-buffer mixtures with spiked acetoin content. Interestingly, an increase in the biosensor signal was also observed for the pure wine-buffer mixture (without additionally spiked acetoin). The shift in contrast to the sensor signal in buffer might be explained due to the naturally present acetoin content in that sample. These experiments in wine-buffer mixture underline that the developed acetoin biosensor can detect different acetoin concentrations even in real samples.

5. Conclusions

A capacitive EIS field-effect biosensor has been modified by a recently introduced acetoin reductase. Due to the immobilized enzyme, racemic acetoin will be converted to (*R,R*)-2,3-butanediol and *meso*-butanediol, respectively, in a H^+-consuming reaction resulting in a pH change that can be detected by the EIS sensor. The acetoin biosensor has been characterized regarding the immobilization method and the pH optimum. The developed biosensor depicted the highest sensitivity through immobilization of the enzyme via cross-linking by using glutaraldehyde at a pH value of 7.1. Furthermore, reproducible measurement results could be achieved at different acetoin concentrations in the range between 10 µM and 100 µM with an average acetoin sensitivity of 65 mV/dec. The sensor shows a long-term stability of around two days, which should be further improved. Ongoing experiments are dealing with the stabilization of the enzyme's activity. Moreover, first experiments in white wine have been carried out where acetoin concentrations between 10 µM and 90 µM could be successfully detected, giving a hint that the sensor can be applied to real fermentation solutions. In future experiments, possible cross-sensitivity effects towards diacetyl and acetylbutanediol shall be investigated, especially for diacetyl, which is also an important flavor in beer and responsible for the buttery taste (Wainwright, 1973). Additionally, the obtained results in real samples, such as wine or beer, should be compared with already established measurement methods, like gas chromatography.

Thus, both substances (acetoin and diacetyl) can be used as indicator for different fermentation processes and could prove the quality of alcoholic beverages. By applying Boolean operations (such as AND, OR, NAND,…) (Katz et al., 2017; Katz and Minko, 2015) and by defining a threshold voltage of the particular biosensor signal (depending on the fermentation process), the overall sensor read-out could describe the status of the fermentation course. By simultaneously detecting diacetyl and acetoin, the fermentation process could be stopped at the right moment to avoid unnecessary fermentation or maturation time and finally, to accelerate the process. Furthermore, the unpleasant buttery-like flavor, which occurs due to exceeded fermentation/maturation process, can be avoided.

Acknowledgements

The authors thank the Ministry of Innovation, Science and Research of the state of North Rhine-Westphalia for financial support (funding program FH-Struktur 2016). The authors thank A. Poghossian for valuable discussions and H. Iken for the preparation of the EIS sensor chips.

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